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Isolation and Molecular (RAPD) Identification of Alkaline Protease Producing Fungi from Dissimilar Soil Areas of Andhra Pradesh

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ABSTRACT

Three soil samples were collected in depth of 5-6 cm from soil. Alkaline pH was observed across the sampling sites and a total of 3 samples were found with plenty of microflora occurrence. The total microorganism counts of the soils were estimated by standard dilution plate technique. The isolated microbes were identified by their cultural and morphological characteristics. Total 74 microorganism forms (includes bacteria (4), fungi (69) and actenomycetes (1)) were obtained from the positive three soil samples. These are 10 fungal forms from Kakinada, 9 from Vishakhapatnam and 40 from Tenali black field soils. Alkaline proteases fungi were isolated using milk agar plate assay consists of 0.5% casein from different soils collected from Kakinada, Vishakhapatnam beach soil and Tenali black soil fields of Andhra Pradesh. Three soil fungal isolates were examined for protease producing microorganism. Isolation and RAPD-identification of the highest alkaline protease producer under submerged fermentation, using czepekdox media.

KEYWORDS : Enzyme production and optimization, fungal protease, tyrosine production.

Introduction

Soil is the natural medium for the growth of both microbial and biological activities (Griffin, 1972). Prescott *et al.* (1993) Microbial fauna was always depends on different influencing factors such as nutrients, moisture and aeration rate pH, temperature etc. Environmental factors play vital role in the conversion of complex organic compound into reusable compounds in the nature. Plant and animal origin complex matter conversion of bioavailability of nitrates, sulfates, phosphates etc., and by different biochemical processes produce industrially useful primary and secondary metabolites such as enzymes, amino acids and vitamins and antibiotics, alcohol and organic acids (Bridge and Spooner, 2001).

High depth of soil samples having much biomass (fungi) than bacteria, and also where the soil nutrients were more concentrated than surface area, which also plays crucial role (Ainsworth and Bisby, 1995). Fungi are fundamental microorganism in soil ecosystem and execute useful ecological processes to maintain the quality of human life, which facilitate economic benefits. According to the Hawks worth and Ross man, in the year (1997) and (2001) predicted that 70,000 fungi were identified and used as the industrial source out of 1.5 million fungal species on earth.

Atlas and Bartha, studied in the year 1998 and describes that the count of fungi for gram soil was up to 5×10^3 to 9.0×10^6 and were accomplished to grow and survive at broad range of pH. But fungi were destructive at acid, neutral and alkaline pH. Fungi were able to uptake and utilize high concentration of substrates at favorable conditions with adequate moisture, aeration (Miyanoto *et al.,* 2002). Waksman (1922) and Warcup (1950) isolated fungi from soil by plate count method. Manivannam and Kathiresan (2007) isolated fungi from Rhizosphere soils and Charles *et al.,* (2008) isolated fungi named as Aspergillus nidulans from poultry farm soils. Nehra *et al.,* (2002) Kalpana Devi *et al.,* (2008) isolated proteolytic fungi from diverse sources such as garden soil and alkaline soil. Al- Falih, (2001), isolated yeast from sandy soil and soil samples from butcheries (Usama, 2008) etc.

Proteases have wide applications in many industries such as textiles, detergents, food processing, meat tenderizing, animal nutrition, pharmaceuticals, paper industry and food industry and these proteases account for 60% of industrial enzymes in the market demand (Negi and Banerjee, 2006). The detergent industry is the major users of hydrolytic enzymes, working on alkaline pH and now more than a quarter of the global enzyme production. The huge amounts of alkaline protease used commercially and industrially. Alkaline proteases are used as the index of some horrible diseases such as cancer and AIDS. Hence clinical importance was increased and the demand for the highest alkaline protease producers was increased day by day (Rath *et al.,* 2002). The present study aim to isolate and molecular identification of the alkaline protease producing fungi from different soils, such as Kakinada and Vishakhapatnam beach soil and Tenali black soils from fields of Andhra Pradesh.

MATERIAL AND METHODS

Identification of the culture

The fungal isolate was subjected to some gross morphological and biochemical studies viz., gelatin liquefaction, casein hydrolysis, tyrosine utilization and carbohydrate utilization. Gelatin was added at 15% level to sterile nutrient agar plates, inoculated with the fungal isolate and incubated at 30°C for 96 h. After incubation, the plates were kept at a temperature of 2-3° for 1h and tested for liquefaction if any. PDA, supplemented with 20% skimmed milk, was used for casein hydrolysis and incubated at 30°C for 96 h for the organism.

Culture and Identification

A morphological identification was performed using culture media, potato dextrose agar media for the primary identification of isolated molds (Moghim *et al.,* 2012). The fungal strain was then confirmed by molecular identification using restriction fragment length polymorphism (RFLP) followed by random amplified polymorphic DNA (RAPD) for the epidemiological linkage among clinical and environmental sources.

DNA extraction

Aspergillus mycelial mass was harvested from the 12 to 24 hr fungal liquid cultures, filtered and purified. The genomic DNA was extracted by glass beads and phenol-chloroform method, (a solution of 1mM EDTA, 1% SDS, 100mM NaCl, 10 mM Tris-HCl and 2% Triton X-100, in distilled water, pH 8.0 was used as Lysis Buffer). The extracted DNA was checked by using 1.5% agarose gel electrophoresis (Rath *et al.,* 2002).

PCR for identification

The PCR assay was performed using 5 µl of the DNA template in a total reaction volume of 50 µl (consisting of PCR buffer [20 mMTris-HCl at pH 8.0], 50 mM KCl, 0.1 mM each of forward [ITS: 5-TCC GTA GGT

GAA CCT GCG G-3] and reverse [ITS-4 5-TCC TCC GCT TAT TGA TAT GC-3] primers for ITS regions of rDNA [purchased from Mirhendi Molecular Biology Lab, TUMS], and 1.5 U of Taq DNA polymerase). We used universal primers for the amplification of Aspergillus ITS regions (forward primer: 5′-TCC GTA GGT GAA CCT GCG G - 3′, reverse primer: 5′-TCC TCC GCT TAT TGAT TAT GC-3′) (Loudon *et al.,* 1993).The reactions were performed in a thermo cycler (XP Cycler, BIOER, China). Thermal program included an initial DNA denaturation at 95°C for 5 min that followed by 30 cycles, consisting of the stages; denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min following the last cycle. The DNA fragments were length separated by electrophoresis through 1.5% agarose gels in Tris Borate EDTA (TBE) buffer and 0.50 mg of ethidium bromide per ml. Results were documented by using a UV tarns illuminator (SynGene SYDR4/680X, UK) (Mirhendi *et al.,* 2007).

Digestion of PCR products using RFLP method:

Digestion of amplified ITS fragments by using a novel restriction enzyme, MwoI at 37°C enabled us to differentiate most of Aspergillus isolates in level of species. The method was performed for both clinical and environmental isolates. For the restriction enzyme digestion, 13 µl of each PCR product was directly digested by 5 U (0.5 µl) of the restriction enzyme MwoI, 1.5 µl of the enzyme buffer, and then incubated at 37°C for 180 min (Mirhendi *et al.,* 2007). Digested PCR products were subjected to a 2% agarose gel electrophoresis and visualized intrans illuminator (Gel Doc system). Aspergillus isolates were identified comparing the electrophoretic DNA patterns with standard measures (Table 1).

Random amplification of polymorphic DNA (RAPD)

For the RAPD-PCR, six single primers which successfully tested before were used as random primers on *Aspergillus* genomic DNA. The primers: P1 (Rp4): 5´-CAGATGCTTC-3´, P2 (Rp1): 5´-TAGGATCGGA-3´, P3 (SOY): 5´-AGGTCACTGA-3´, P4 (RP2): 5´-AAGGATCAGA-3´, P5 (R108): 5-GATTTGCCCT-3´, P6 (UBC90): 5´-GGGGGTTAGG3´were randomly selected from many available primers (Purchased from Molecular Biology Lab, TUMS). The primers were run into a PCR master mix containing 3 mM $MgCl_2$, 200 pmol of each primer, and 5 ng of DNA, in final volume of 100 µl. The thermal protocol used in thermo cycle system included 5 min at 95°C ,45 cycles of 94°C for 45 sec, 35°C for 1 min, 72°C for 1.5 min and a final 72°C for 5 min (Mirhendi *et al.,* 2009). Agarose gel electrophoresis of the PCR products followed by an ethidium bromide staining showed various patterns making DNA bands which analyzed by using Image Master software (Gene Snap Tool, SynGene, version: 4.01.0, UK). Reproducibility of DNA patterns was demonstrated by the analysis of two to three Aspergillus subcultures. The RAPD-PCR patterns were compared between the clinical and environmental *Aspergillus* isolates for each case and the similarity of RAPD patterns was analyzed.

RESULTS AND DISCUSSION

Isolation and morphological Identification of fungi from collected soil samples:

Totally four samples were collected from various areas of Andhra Pradesh includes Kakinada beach soil, Vishakhapatnam beach soil and Tenali black soil from fields. The soil samples were serially diluted and counted for no of microbial organisms. Which includes bacteria, fungi and actenomycetes etc. Total 74 strains were identified by morphological identification and microscopic identification (Visagie *et al.,* 2014). Further screened for alkaline protease screening media with the 0.5% of casein agar media and the results revealed that 20 fungi were able to produce zone of clearance on gelatin agar and milk agar media that indicates that fungi could able to degrade the protein. Among the 20 fungi five has shown highest protein degradation, and further conformed by RAPD characterization method and named as which includes *Pencilium oxalicum* KRSS-S-FP10 produced highest alkaline protease activity (25.64 ±0.02 U/mL) comparison with known sources has shown that alkaline proteases excreted effectively by *penicillin* species among the best alkaline producers (*Aspergillus terreus* KRSS-S-FBR1 (04.9 ±0.02), *Aspergillus niger* KRSS-S-FG8 (16.24 ±0.02), *Talaromyces radicus* KRSS-S-FYG7 (17.48 ±0.05) and *Aspergillus flavus* KRSS-S-FBL3 (18.56 ±0.05)).

Isolation and Screening for protease producing organisms:

A total of 74 and 69 fungi from beach soils and black soils respectively were obtained by employing different techniques of isolations (Table 1). Among these, 10 from Kakinada beach soil and 9 from Vishakhapatnam beach soil and 40 fungi from Tenali black soil. Among the isolated and screened fungi 5 were selected as highest protease producers at 30°C. A total of 74 fungi were isolated from the soil samples collected from beach soils and black soil (Visagie *et al.,* 2014). Single isolated colonies were selected and inoculated on casein agar plate for protease activity. The diameters of hydrolyzed zones around the colonies were calculated as a measure of caseinolytic activity of isolates. Isolates were initially screened based on zone of clearance on agar plates. Among the 74 isolates FCS1, FCS3, FCS7, FCS8 and FCS10 were found to have maximum protease activity and was selected for further studies (Maria Papagianni (2014).

Table 1- screening for alkaline protease enzyme activity

species code	Zone of clearance (mm)	Enzyme activity U/mL
FCS1	3.5	4.6
FCS2	0.3	1.0
FCS3	1.5	18
FCS4	1.8	3.8
FCS5	0.8	1.8
FCS6	0.5	1.4
FCS7	4.8	16
FCS8	2.4	14
FCS9	0.5	3.2
FCS10	5.6	22.4
FCS11	0.48	2.0
FCS12	0.32	1.4
FCS13	0.38	1.8
FCS14	0.30	1.4
FCS15	0.24	0.8
FCS16	0.28	1.2
FCS17	0.2	2.0
FCS18	0.30	0.4
FCS19	0.24	1.4
FCS2O	0.2	0.14

Molecular identification of isolated fungal strains by RAPD

For making an exact correlation among the environmental isolates, the findings of RAPD-PCR were analyzed. The random primers, P1-P6 made different electrophoretic DNA patterns for most cases. For example, application of P1 resulted in different DNA patterns for the pairs (clinical and environmental isolates) 16, 36 and 37 from 65 cases of *Aspergillosis*. Just in one case (pair 31) same DNA patterns were observed (Fig: 1c).

Use of primer P2 resulted in identical patterns for three pairs:

16, 31 and 37 as shown in (Fig: 1 d). At the same way, the other primers created common patterns not more than one. Comparison of environmental pairs with RAPD-PCR using six random primers revealed similar electrophoretic DNA patterns for environmental *Aspergillus* isolates (Fig: 1 a, b) just in two of 28 pairs including 32 and 45. *A. niger* and *A. flavus* were included in the pairs 32 and 45 respectively. The *Aspergillus* isolate of bronco alveolar lavage of case 32 was completely similar to that of relevant air conditioner used in the private room. Also, DNA pattern of *A. flavus* isolated from sinus discharge of case 45 was similar to the isolate of wall swabs. Other *Aspergillus* pairs showed no similarity (Vaishali Choudhary and Jain (2012).

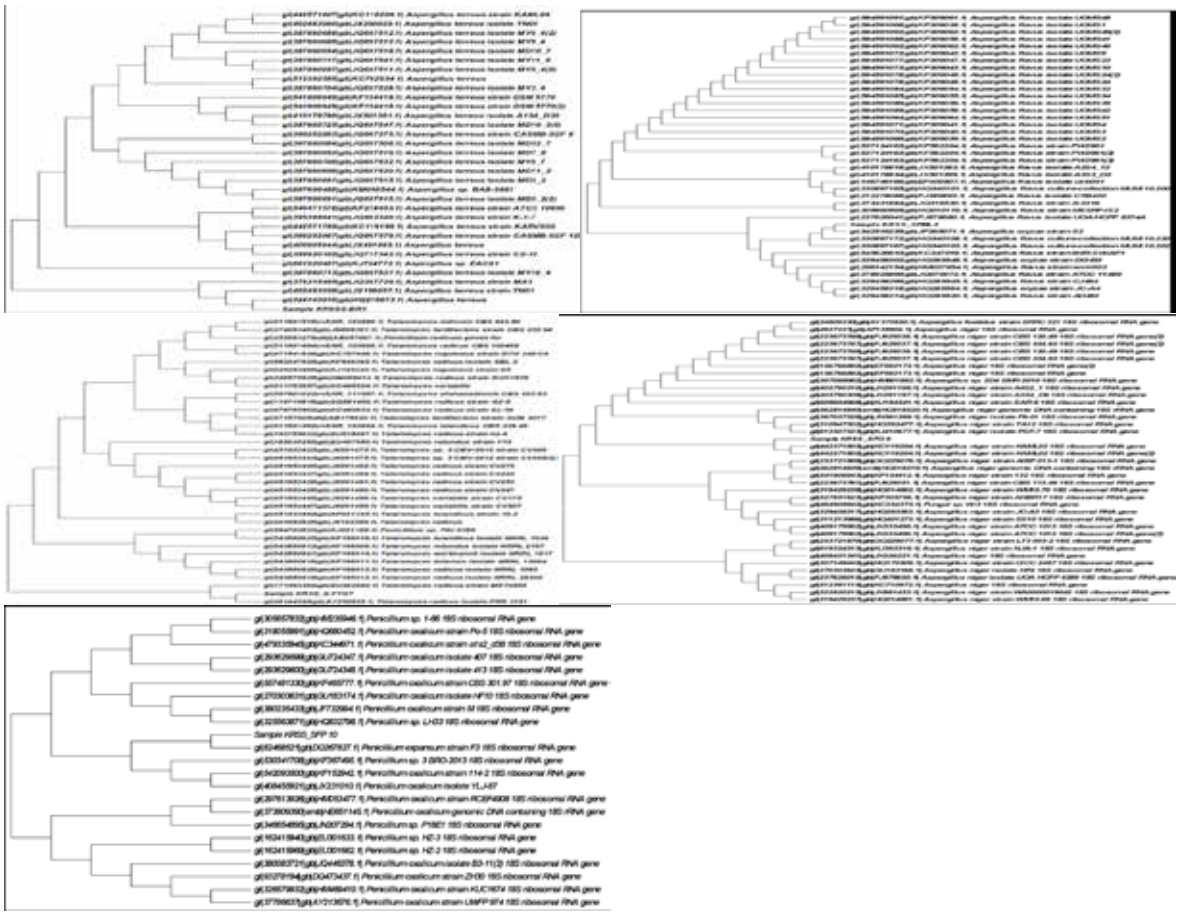

Fig No: 1- Dendrogram of isolated fungi

Talaromyces radicus BankIt1756133 *Talaromyces* KM486548 KRSS-S-FYG7
Aspergillus terreus BankIt1756143 *Aspergillus* KM486549 KRSS-S-FBR1
Pencillium oxalicum BankIt1756444 *Pencillium* KM486550 KRSS-S-FP10
Aspergillus flavus BankIt1756449 *Aspergillus* KM486551 KRSS-S-FBL3
Aspergillus niger BankIt1756450 *Aspergillus* KM486552 KRSS-S-FG8

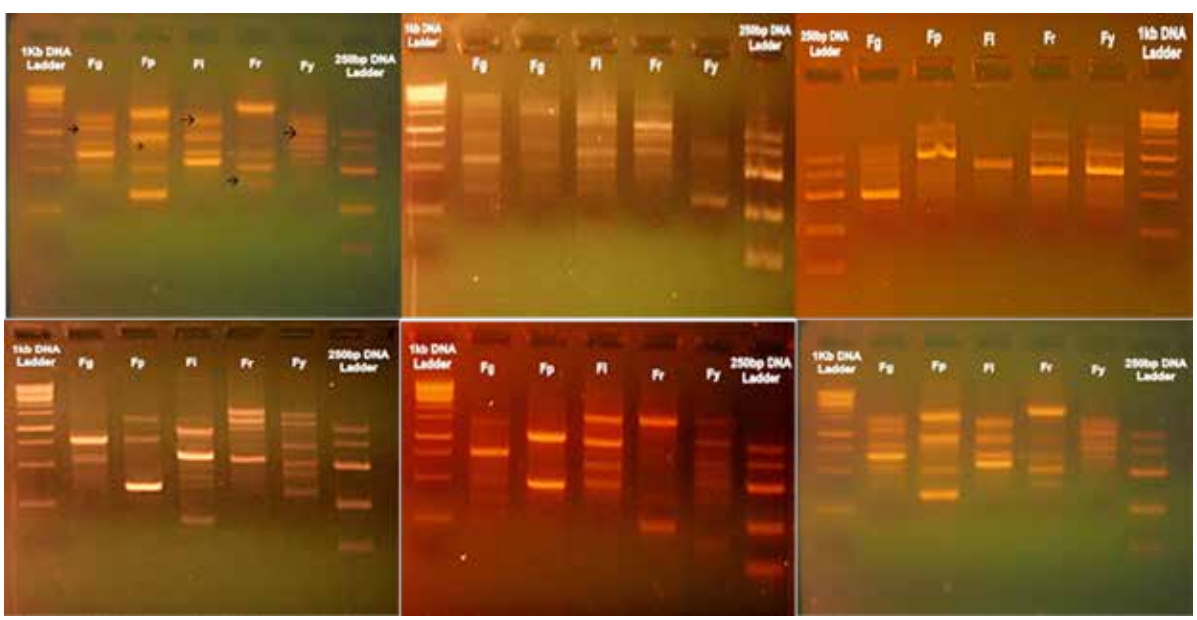

Fig 2. Banding pattern of Random amplified polymorphic DNA (RAPD)
Banding pattern of Random amplified polymorphic DNA (RAPD) obtained from 4 isolates of *Aspergillus* **and one belongs to** *Pencillium* **(Lane 1 to 28) M=1kbp ladder with random primers.**

In the present investigation, RAPD-PCR based tools could be used to characterize the fungal species or molecular identification of micro-organism. Before subjecting with RAPD-PCR analysis assessed the differences in the DNA configurations can be produced so that the data useful for fingerprinting. *A. fumigatus, A. flavus*, *A. niger* and *A. terreus (*Kambiz Diba *et al.,* 2014*)*.

In the RAPD analysis, random primers were used to screen among the data present in the gene bank *Aspergillus* species, *Penicillin* and *Teleromyces* species (Novak *et al.,* 2004). In fact, we considered a 100% similarity of RAPD patterns of *Aspergillus* isolates, *Penicillin* isolates and *Teleromyces* species. In these cases, *A. niger, Aspergillus terrius* and *A.flavus* were included as the *Aspergillus* species. Present results of *Aspergillus* species identification of collected samples is confirmed by other studies, so that in a similar study, *A. fumigatus* and *A. flavus* were the most frequently isolated *Aspergillus spp*. PCR based methods for detection of fungi have been described and there is a problem of finding the real source of opportunistic fungi such as *Aspergillus* spp..

The previous data verify that RAPD analysis is useful for fingerprinting *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* (Raclavsky *et al.,* 2006).

Conclusion

As a conclusion, RAPD-PCR can be applied as a simple, rapid, and useful method, but it plays a trivial role in finding the hospital sources of *Aspergillus* clinical isolates. It should be noted, however, that selecting the random primers is an important point to find the highest level of molecular similarity.

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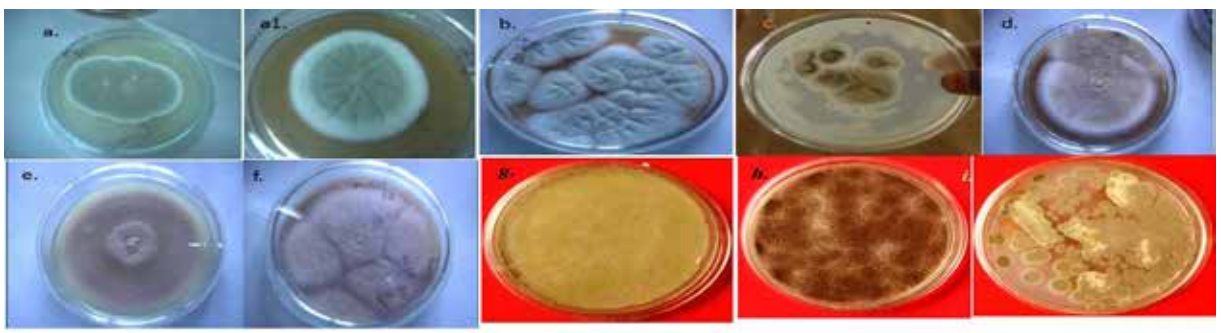

Figure 3: a.,a1.,b. KRSS-S-FP10-*Penicillium oxalicum at pH8,10;c.* KRSS-S-FP10- *Penicillium oxalicum with clearance zone;d,e.* KRSS-S-FBR1-*Aspergillus terrus at pH 11;f,g.* KRSS-S-FBL3-*Aspergillus flavus at pH 11; h.* KRSS-S-FG8 *Aspergillus niger;i.* KRSS-S-FYG7 *Talaromyces radicus*

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