THE ANTIFUNGAL ACTIVITY OF PATHOGENIC FUNGI ISOLATED FROM COTTAGE CHEESE

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ABSTRACT: The present study evaluated the pathogenic fungi present in cottage cheeses. All the 8 type of cheese were collected form Puducherry, India cottage cheese whole sale area. All the samples were serially diluted and get into the plate for the fungal growth. The isolated fungal colonies were confirmed using Lacto phenol cotton blue staining method. The isolated fungal strains were properly maintained for further studies. The isolated funguses are confirmed using DNA isolation method and BLAST sequence editing. The isolated 4 fungal stains are treated against the commonly available antifungal agent namely Clotrimazole, Miconazole, Econazole, Ternbinofine, Fluconazole, ketoconazole. In those antifungal agents Clotrimazole, Miconazole shows the greater zone of inhibition on isolated 4 fungi. Aim of this study is to reveal about some unhealthy cottage cheeses.

Keywords: antifungal agent, cottage cheese, DNA sequences, fungal culture.

CHAPTER 1:
INTRODUCTION

The familiar “Grana Padano” and “Parmigiano Reggiano" is collectively called as common cheese in cottage industries. In compliance with PDO, grana cheeses have an extended ripening period; from 9 months for normal, up to many years for premium goods. Cheese production relies on the action of bacteria, yeasts and filamentous fungi (ff) which convert the processed milk into cheese, contributing to its final characteristics like consistency, taste, and flavour. There are two types of starter culture have been used for making of cheese production, i.e.

1. The primary microbiota is mainly composed of starter lactic acid bacteria;
2. The secondary microbiota includes salt-tolerant bacteria, yeasts, and filamentous fungi which perform degradation of proteins, sugars and lipids.

In this type of cheese, the development of filamentous fungi usually occurs during ripening and it is only limited to the crust like the most of cheeses [1].

In recent years, there is an ever-increasing interest among consumers for food products that contain less total fat, saturated fat, cholesterol and calories. Cheese analogues are able to meet special dietary needs and can act as a vehicle for a health supplement, e.g. cholesterol-free and enriched with vitamin, mineral, fibre, [2].

Filled cheese is a type of cheese in which milk fat is partly or fully replaced by vegetable oils, which in turn could be partially hydrogenated to impart eating profile similar to that of milk fat. Filled cheeses are often made to possess required quantum of fat through the use of healthful vegetable oils. A directly acidified, low-cholesterol filled-Mozzarella cheese has been made up of skimmed milk emulsified with sunflower-seed oil[3].

CHAPTER 2:

ISOLATION METHODS FOR PATHOGENIC FUNGI

Morphological Identification- preparation of lacto phenol cotton blue

1. Cotton blue (Aniline blue) -0.05g
2. Phenol crystals -20g
3. Glycerol -40ml
4. Lactic acid -20ml
5. Distilled water -20ml

Procedure

1. Dissolve cotton blue in distilled water and leave overnight (to eliminate insoluble dye).
2. Next day, wear hand gloves add the phenol crystals to the lactic acid in glass beaker and stirrer well.
3. Add glycerol slowly to the beaker.
4. Filter the cotton blue, phenol, glycerol, lactic acid mixture.
5. Mix well and store the room temperature.

NOTE:

Phenol is used to kill the fungi and the lactic acid preserves the fungal structure.

Staining Method

1. Place a drop of lacto phenol Cotton Blue Stain in the center of a clean slide.
2. Remove a fragment of the fungus colony 2-3mm from the colony edge using an inoculating or teasing needle.
3. Place the fragment in the drop of stain and tease gently.
4. Apply a coverslip. Do not push down or tap the cover slip as this may dislodge the conidia from the conidiophores.

5. Examine the preparation under low and high, dry magnification for the presence of characteristic mycelia and fruiting structures.

DNA Isolation (modified by[3]**)**

1. The cells were grown overnight in Sabouraud dextrose broth.
2. 1.5ml of culture was transferred to a tube and centrifuged at 10,000 rpm for 2 minutes.
3. The pellets were collected and repeated the centrifugation with another 1.5ml of culture containing cells.
4. Drained the tubes on a paper towel briefly.
5. 10µl of 10% SDS were added.
6. Incubate at 55˚c for 2 hours.
7. After incubation it was chilled on ice for 10 minutes.
8. 250µl of 6M Nacl was added.
9. Again it was kept on freezer for 5 minutes.
10. After freezing the sample was spinned at 8000 rpm for 15minutes.
11. 500µl of supernatant was taken and transferred into a new 1.5ml tube.
12. 1ml of 100% ice cold ethanol was added and inverted several times.
13. Again the sample was spinned at 10,000 rpm for 15 minutes.
14. The supernatant was removed and rinse with 500µl of 70% ethanol.
15. The sample was spinned at 10,000rpm for 5 minutes.
16. The supernatant was removed and dry the pellet at room temperature.
17. 100µl of 1X TE buffer was added to the pellet.
18. 5µl of DNA sample was added to the 0.8% agarose gel.
19. Visualized under the UV Transilluminator.

Agarose Gel Electrophoresis

1. 0.24g of agarose in 30ml of TAE buffer was mixed.
2. The agarose solution was boiled till get a clear solution.
3. 1.5µl of EtBr was added the solution gets completely cooled..
4. The clear solution was poured in a gel casting plate with already adjusted gel comb.
5. The casting tray was cooled at room temperature for 30 minutes for solidification.
6. After solidified, 5µl of DNA sample with 2µl of loading buffer were mixed and load in the well.
7. Run the gel 50V for about 20 minutes.
8. Observed the bands in UV light.

Polymerase Chain Reaction (modified by[4]**)**

Primer Mix

1. ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′) -25 pmol
2. ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3) -25 pmol

Master Mix Components (modified by[5]**)**

1.	Distelled water	-	16µl
2.	10X Assay buffer	-	2.5µl
3.	Primer mix	-	0.5µl
4.	dNTPs mix	-	2µl
5.	Mgcl (30mM)	-	3.0µl
6.	Taq polymerase	-	0.5µl
7.	Template DNA	-	1µl

Pcr Programme For 18S rRNA (modified by[3]**)**

Polymerase chain reactions for ITS1 gene can be performed by following the temperature and timing condition programmed in a thermal cycler.

1. Initial denaturation at 95˚c for 5 minutes.
2. Number of cycles 30.
3. Denaturation at 94˚c for 1 minute.
4. Annealing at 45˚c for 45 seconds.
5. Extension at 72˚c for 1 minute.
6. Final extension at 72˚c for 10 minutes.
7. Check the amplified products in 1.5% Agarose gel electrophoresis and the molecular weight was assessed using molecular weight marker (100bp ladder).

ANTIMICROBIAL ACTIVITY (modified by[5]

1. The broth culture was prepared of test samples and fungal pathogens. The broth was incubated at overnight.
2. The Sabouraud dextrose agar plates were prepared and named properly. The standard antifungal agent plates were prepared and named as duplicate plates.
3. The plated were allowed to solidify.
4. After solidification the fungal pathogens were inoculated by using cotton swab method.
5. Standard antibiotic discs were prepared with 80µl concentration.
6. Place the plates in incubator for 24 hours.

7. Observed the plates and note the zone formation.

Sequencing Editing

The obtained sequences were edited based on the electropherogram peak clarities. Sequences with noisy peaks were excluded from the analysis. The sequences were assessed to check the insertion or deletions and codons in MEGA 5.0 software.

Sequencing Characterization

Multiple sequence alignment and pairwise sequence alignment were performed using Clustal W program implemented in MEGA 5.0 in all the sequences. Nucleotide differences were carefully monitored and the differences were observed and edit manually. Sequences were translated into amino acid sequences using vertebrate mitochondrial codon pattern in the MEGA 5.0 for checking the pseudo-gene status. All the sequences were correctly translated into amino acid sequences with their respective starting primers without any internal stop codon.

Blast Search

The amplified sequences were confirmed by similarity index built in the NCBI's BLAST program. Based on the percentage similarity and query coverage against the reference species, the species were confirmed.

CHAPTER 3:
RESULTS

Table 2: Staining results

ORGANISM NAMES	LACTOPHENOL COTTON BLUE
Aspergillus flavus	Postitive
Candida albicans	Postitive
Fusarium oxysporum	Postitive
Stachybotrys chartarum	Postitive

Table 3: Antibacterial activity test

Organism name	Clotrimazole	Miconazole	Econazole	Ternbinofine	Fluconazole	ketoconazole
Fusarium oxysporum	Positive	Positive	Low positive[1]	Low positive[1]	Positive	Low positive[1]
Candida albicans	Positive	Positive	Comparatively positive[2]	Low positive[1]	Comparatively positive[2]	Negative
Aspergillus flavus	Positive	Positive	Negative	Negative	Negative	Negative
Stachybotrys chartarum	Positive	Positive	Positive	Negative	Positive	Negative

Low positive[1:] antifungal activity is low. Comparatively positive[2]: antifungal activity is very low.

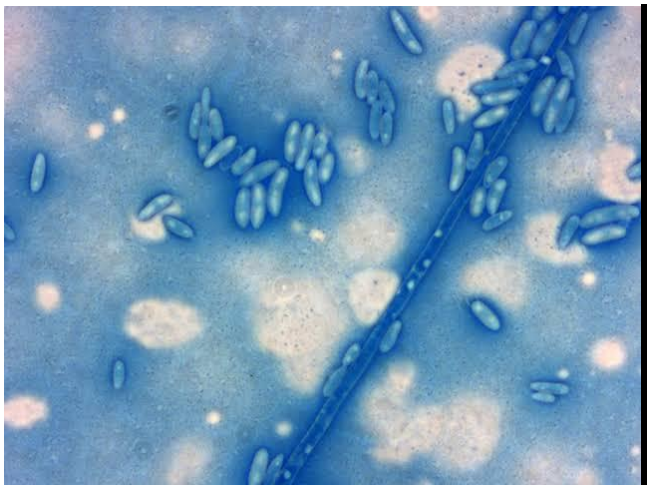

Figure 1a: Fusarium oxysporum

Figure 1b: Fusarium oxysporum plate

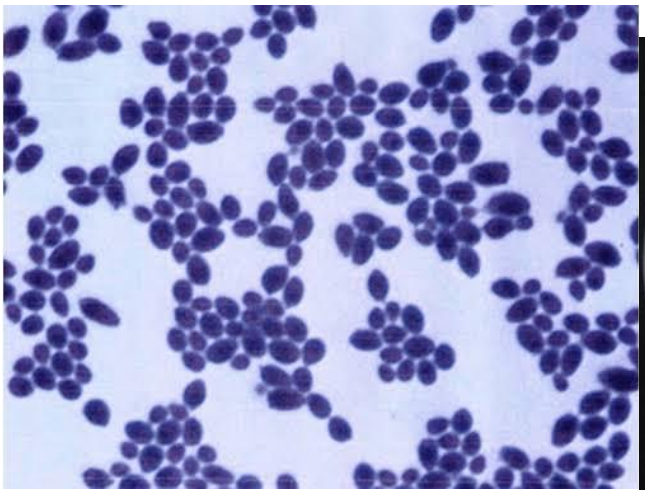

Figure 2a: Candida albicans

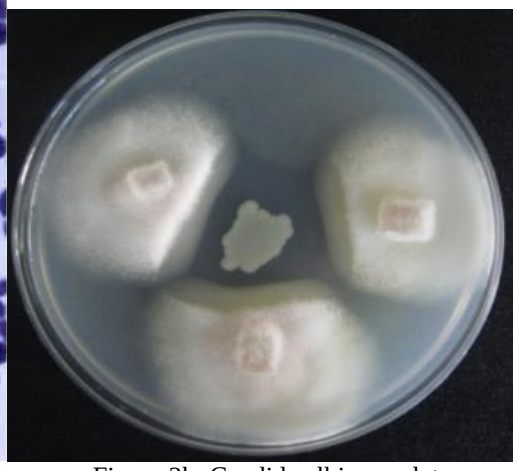

Figure 2b: Candida albicans plates

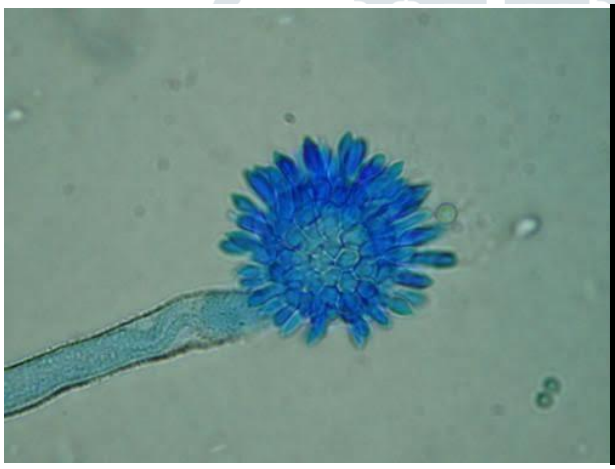

Figure 3a: Aspergillus flavus

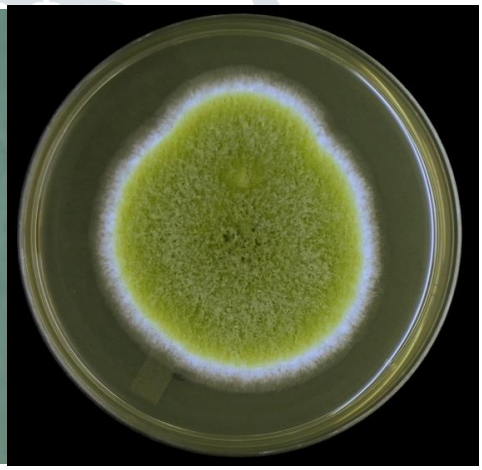

Figure 3b: Aspergillus flavus colony

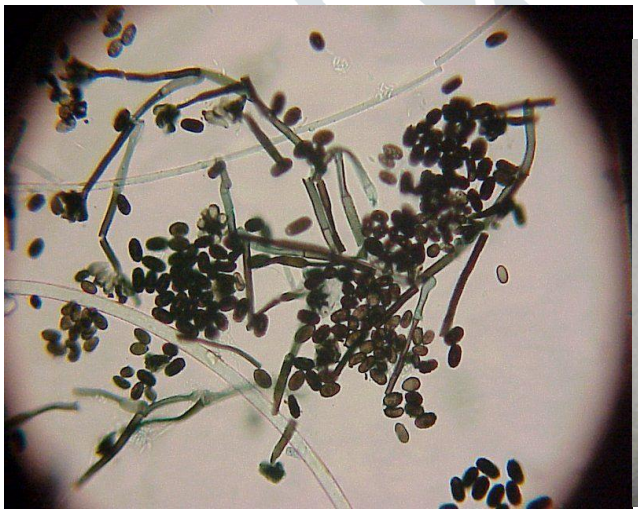

Figure 4a: Stachybotrys chartarum

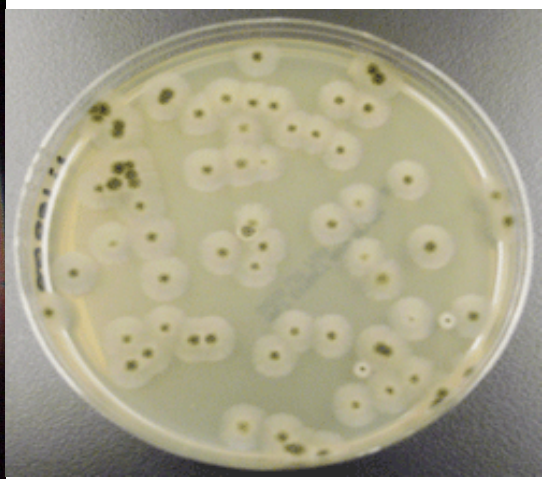

Figure 4b: Stachybotrys chartarum colony

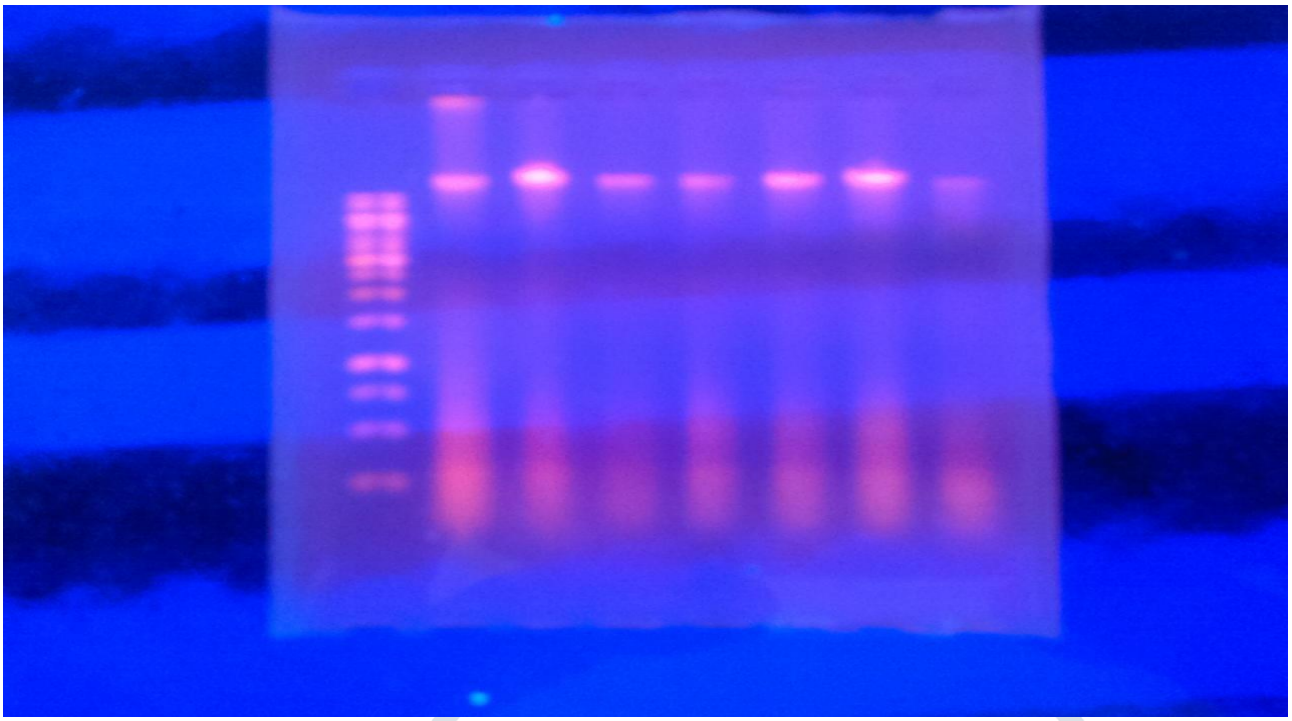

Figure 5: DNA isolation lane 1,2,3,4

Figure 6: PCR lane 1,2,3,4.

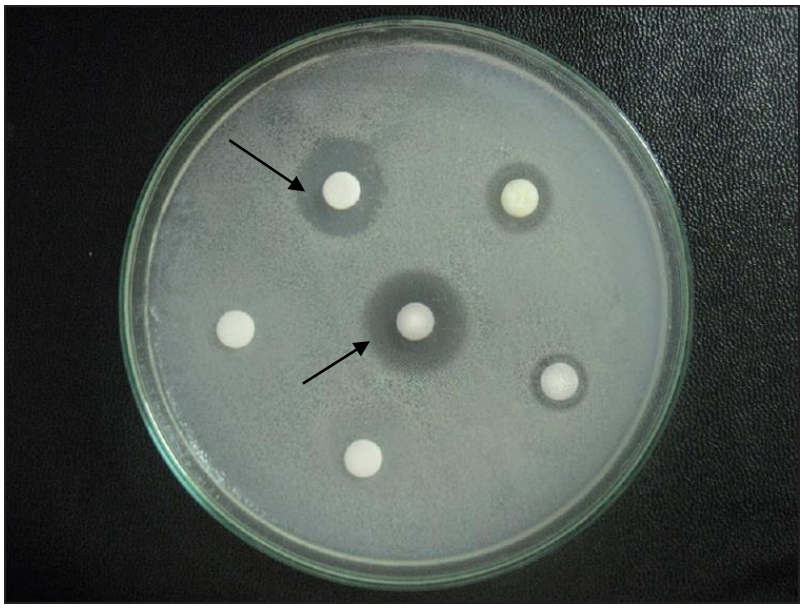

Figure 7: Aspergillus flavus against Clotrimazole and Miconazole

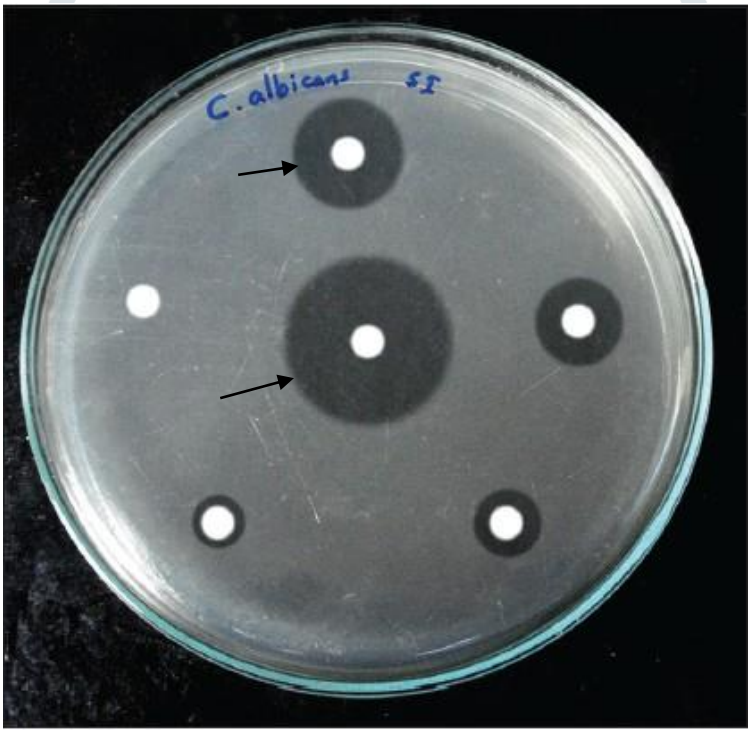

Figure 8: Candida albicans against Clotrimazole, Miconazole.

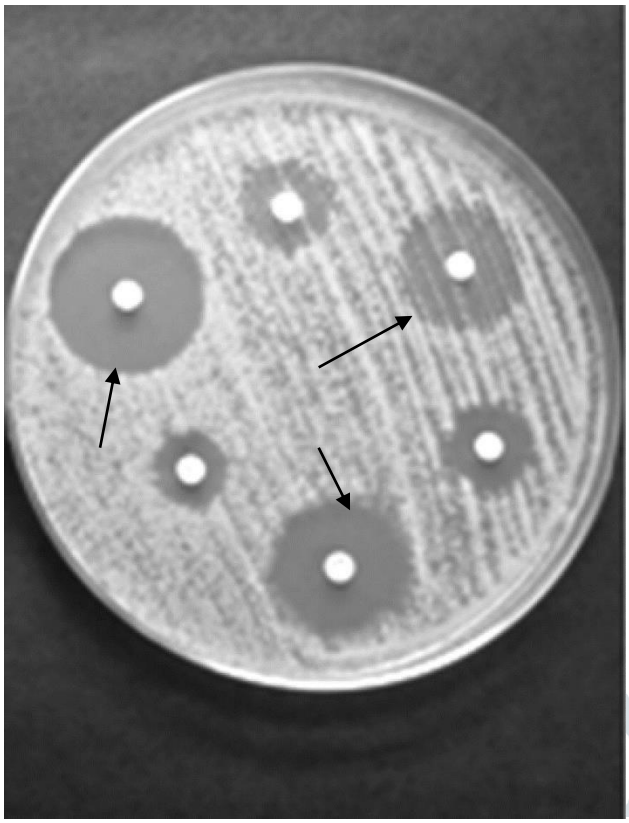

Figure 9: Fusarium oxysporum against Clotrimazole, Miconazole and Fluconazole.

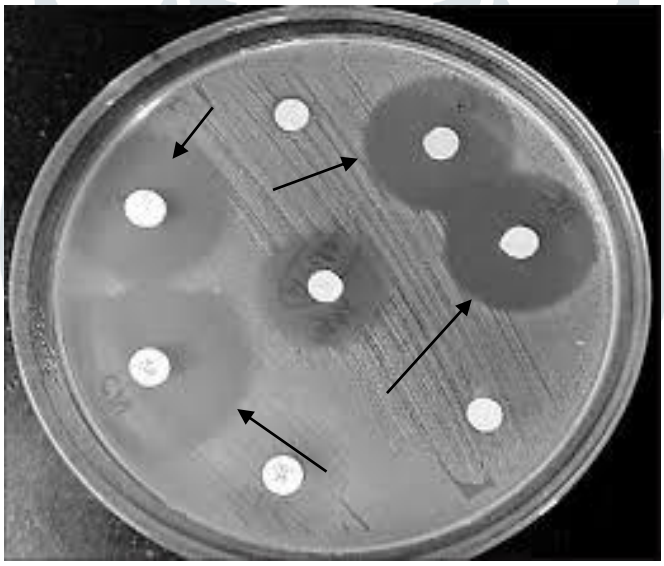

Figure 10: Stachybotrys chartarum against Clotrimazole, Miconazole, Econazole and Fluconazole.

Note: We did not include low positive antifungal agent (see table 2).
All the unmarked standards were shows that the antifungal agents as controls.

Fusarium oxysporum 18S ribosomal RNA gene, partial sequence
TCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGT
GAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCC
CTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTC
GAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAAC
TTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCA

Candida albicans 18S ribosomal RNA gene, partial sequence

TATCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGC
AATTTATACAGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACTT
GGATAACCGTGGTAATTCTAGAGCTAATACATGCTTAAAATCCCGACTGTTTGGAAGGGATGTATTTATT
AGATAAAAAATCAATGCCTTCGGGCTCTTTGATGATTCATAATAACTTTTCGAATCGCATGGCCTTGTGC
TGGCGATGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTTTCA
ACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG
GCAGCAGGCGCGCAAATTACCCAATCCCGATTCAGGGGAGGTAGTGACAATAAATAACGATACAGGGCCC
TTTTGGGTCTTGTAATTGGAATGAGTACAATGTAAATACCTTAACGAGGAACAATTGGAGGGCAAGTCTG
GTGCCAGCAGCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAG
TTGAACTTTGGGCTTGGCTGGCCGGTCCATCTTTTTCGATGCGTACTGGACCAGCCGAGCCTTTCCTTCT
GGTAGCCATTTATGGCGAACCAGGACTTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTC
GAATATATTAGCATGGAATAATAGAATAGGACGTTATGGTTCTATTGTGTTGGTTTCTAGGACCATCGTA
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CTCACCAGGTCCAGACACAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATTTTGTGGGTGGTGGTGC
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AATAGTGCTGCTAGCATTTGCTGGTATAGTCACTTCTTAGAGGGACTATCGACTCCAAGTCGATGGAAGT
TTGAGGCAATAACAGGTCTGTGATGCCCTTAGACGTTCTGGGCCGCACGCGCGCTACACTGACGGAGCCA
GCGAGTATAAGCCTTGGCCGAGAGGTCTGGGAAATCTTGTGAAACTCCGTCGTGCTGGGGATAGAGCATT
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CCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGTTTAGGA
AAGGGGGCAACTCCATTCTGGAACCGAGAAGCTGGTCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTA
ACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTA

Aspergillus flavus 18S ribosomal RNA gene, partial sequence

TCCCTAACTCGTTAGCTCTCACAGGCGTGCTTTAAGGAAAGATTAAAAAAAGTAAAAGGAACTCGGCAAA
CACGAACCCCGCCTGTTTACCAAAAACATCACCTCTAGCATAACAAGTATTAGAGGCATTGCCTGCCCAG
TGACTAAAGTTAAACGGCCGCGGTATCCTGACCGTGCAAAGGTAGCATAATCACTTGTTCCTTAATTAGG
GACTAGAATGAATGGCTAAACGAGGGTTCAACTGTCTCTTACTTTCAATCAGTGAAATTGACCTTCCAGT
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AAAAACAACCTAATGGGCTAAAACAAAATAAATATGAACTAAAAAATTTCGGTTGGGGTGACCTCGGAGA
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TTAGGGTTTACGACCTCGATGTTGGATCAGGACATCCCAATGGTGCAGAAGCTATTAATGGTTCGTTTGT
TCAACGATTAAAGTCCTACGTGATCTGAGTTCAGACCGGAGCAATCCAGGTCGGTTTCTATCTATTTACA
ATTTCTCCCAGTACGAAAGGACAAGAGAAATGGAGCCTCCTTACCATAAGTGCTCCCAACCAATTTATG

Stachybotrys chartarum 18S ribosomal RNA gene, partial sequence

ATTGCCTTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCCCCAGGCCCGAGTTGTA
ATTTGCAGAGGATGCTTTTGGCGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAG
CCCCGTCTGGTTGGATACCAAGCCTTTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTC
TAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGATC

GAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAACAGCACGTGAAATTGTTAAAAGGGAAGCGTTTATGA

CCAGACTTGGGCCGGTTAATCATCCAGCGTTCTCGCTGGTGCACTTTGCCGGTCCAGGCCAGCATCAGTT

CGCTGCGGGGGATAAAGGCGTCGGGAATGTGGCTCCTCTGGAGTGTTATAGCCCTTCGCGCAATACCCTG

CGGTGGACTGAGGTTCGCGCATCTGCAAGGATGCTGGCGTAATGGTCATCAACGACCCGTCTTGAAACAC

GGACCAAGGAGTCGTCTTCGTATGCGAGTGTTCGGGTGTAAAACCCCTACGCGTAATGAAAGTGAACGCA

GGTGAGAGCTTCGGCGCATCATCGACCGATCCTGATGTTCTCGGATGGATTTGAGTAAGAGCATACGGGG

CCGGACCCGAAAGAAGGTGAACTATGCCTGTATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGCA

GCGGTTCTGACGTGCAAATCGATCGTCAAATATGGGCATGGGGGCGAAAGACTAAT

CHAPTER 4:

SUMMARY AND CONCLUSION

Market of cheese in India is 1250 crores in 2013 and forecasted to grow at rate of 20% annually (articles.economicsimes.indiatimes.com). The total consumption of cheese in India is about 7,500 tons. The per capita consumption of cheese in India is 2.5 kg per annum as against 16.0 kg per annum in US. Anand Milk Union Limited (AMUL), one of the pioneers in introducing Mozzarella cheese commercially in India has a large contribution to the cheese market in India. Amul's annual sale of Mozzarella cheese was about 800 tons in 2001. Amul has captured about 55.0 per cent share in the Rs. 70 crores industry cheese markets. Fungal growth on cheese is a common problem for the cheese manufacture during ripening and curing as well as for the retailer and consumer during refrigeration storage. Species of *Penicillium* and *Aspergillus* are common contaminants of cheese. By the searching in the medical references, it was observed that, most of these fungi had the ability to human and animal pathogenicity or produced toxins. The growth of toxigenic fungi during ripening of cheese must be considered as a problem of safety for human consumption. During the ripening of cheese, non-toxigenic strains of fungi should be avoid, moreover, fungi growth on the cheese surface causes economic losses and quality problems. In this study concluded that how the cottage cheeses are contaminated by pathogenic fungi and mold.

ABBREVIATION

PDO - Protected Designation of Origin certification
PPI - Peanut Protein Isolate
AMF - Anhydrous Milk Fat
XG - Xanthan Gum
LBG - Locust Bean Gum
EMC - Enzyme Modified Cheese

CHAPTER 5:

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