ISSN 0975 - 6299 Vol 2 / Issue 3 / Jul - Sept 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOTECHENOLOGY

STRAIN IMPROVEMENT OF *CURVULARIA LUNATA* TO ENHANCE THE LIPID PRODUCTION BY CHEMICAL MUTAGENESIS

SOWMIYA.E*, JAGANNATHAN.N AND RAJAGOPAL.K

VELS University, Department of Biotechnology, School of Life Sciences, Chennai-600117, Tamilnadu, India

SOWMIYA.E
VELS University, Department of Biotechnology, School of Life Sciences, Chennai-600117, Tamilnadu, India

***Corresponding author**

ABSTRACT

This study demonstrates the effectives of dyes in strain improvement for enhanced lipid production by *Curvularia lunata*. Isolated *Curvularia lunata* were treated with Ethidium bromide and Acridine orange to cause frame shift mutation. Effect of dyes at different time exposure to *Curvularia lunata* survival and growth was studied. The mutants were compared with wild type for lipid production. Ethidium Bromide mutants were EBCV1, EBCV2, EBCV3, EBCV4, and EBCV5. The mutant EBCV3 gave lipid yield, which was 25% more than the wild strain in standard PDA medium. Acridine Orange mutants were AOCV1, AOCV2, AOCV3, AOCV4, and AOCV5. The mutant AOCV4 yields 20% higher than wild strain. Genetic variability of *Curvularia lunata* mutants were studied using RFLP analysis. The results indicated that dyes were effective mutagenic agents for strain improvement to produce high lipids in *Curvularia lunata.*

KEYWORDS

Fungi, *Curvularia*, *lunata,* Mutation, Lipids, Acridine orange, Ethidium bromide

INTRODUCTION

The use of fungi in biotechnological research and industry is significant. Fungi play roles as biocatalysts for the production of food, chemicals and fuels and they are becoming important in the development of solar energy technology, biodegradation and bioremediation. *Curvularia lunata* are known to produce lipids. The production of fungal lipids is receiving great attention, because of its unique properties and wide variety of applications. Fungal lipids have been used as raw material for producing bio fuels. Structural and chemical alteration of cellular nucleic acids occurs relatively throughout an organism's lifetime with differing consequences. Genetic information on DNA sequences in genomes is different not only between species but also between individuals in the same species.

Chemical compounds are most common forms of mutagens. These exert their effects by altering the structure of the DNA strands or the chemical composition of individual nucleotides. A wide range of chemical modifications may consequently be induced, which if not corrected by DNA repair mechanisms may ultimately become mutant. Certain planner aromatic molecules are able to intercalate into double stranded DNA. In this process the intercalating molecule inserts between two neighbouring base pairs in the centre of the helix by hydrophobic and electrostatic interaction. Some intercalates form fluorescing complexes, with DNA which enables the visualization of DNA by irradiation with UV light. However, the intercalation of these molecules changes the form and shape of DNA. The DNA helix is stretched and unwinds in the presence of dyes by 26 per intercalating molecule as an effect of the increasing distance of neighbouring base pair. If the DNA is negatively super coiled, unwinding of the DNA helix results in partial relaxation of the molecule, with an increasing number of intercalating molecules the DNA molecules become fully relaxed and last positively super coiled. The binding of intercalating molecules is proportional to the negative super coil density of the DNA.

Genomic integrity is under constant threat in all species. These threats come in many forms (e.g., agents that damage DNA, spontaneous chemical changes, and errors in DNA metabolism), lead to a variety of alterations in the normal DNA structure and have many direct and indirect effects on cells and organisms (mutations, genetic recombination, the inhibition or alteration of cellular processes, chromosomal aberration, and cell death).

MATERIALS AND METHODS

Organism

The fungi used were *Curvularia lunata* isolated in Department of Biotechnology, School of life sciences, Vels University, Chennai. The culture medium used was the standard PDA medium. The cultures were incubated at 21°C in 1000 ml quantity of the medium for 21 days. The biomass is harvested by centrifugation at 10000 rpm. The dry biomass was used to estimate the lipid content.

Mutagenesis and mutant selection

100 ml culture was harvested by centrifugation at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet was re suspended in 25 ml of phosphate buffer pH7. To 10ml of cell suspension with viability of 10^5/ml, 10 ml of sterile solution of Ethidium bromide, Acridine orange (200 $\mu g/ml^{-1}$) was added separately. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 30, 60, 90, 120, 150 minutes.

Immediately, the samples were resuspended in sterile buffer. The suspended sample was again centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded. Cells were washed thrice with sterile distilled water to remove traces of mutagens. The samples were inoculated on PDA plates. Colonies were inoculated in fresh PDB medium and tested for lipid production.

DNA isolation

Isolated *Curvularia lunata* was grown on PDB medium and incubated at 21°C for two weeks. The fungal biomass was separated by centrifugation at 10000 rpm for 5minutes and pellet was dried under vacuum and used for DNA extraction.0.1 g of biomass is extracted with liquid nitrogen. Extracted solution was mixed with 8ml CTAB buffer and 2ml of 10% SDS incubated at 60°C for 30 minutes. Add equal volume of phenol chloroform (1:1) and centrifuge at 10000 rpm for 5 minutes. Add equal volume of isoamyl alcohol to the supernatant and centrifuge at 12000 rpm for 10 minutes. Add 0ne ml of 70% ethanol to pellet and centrifuge at 12000 rpm for 10 minutes. Dissolve the pellet with Tris buffer. The presence of DNA could confirm by Agarose gel electrophoresis. The extracted DNA was run on 1.2% Agarose gel. The Agarose gel electrophoresis was performed for one hour at 50 volts. The gel viewed under the Trans illuminator (Punekar, et al., 2004).

Estimation of DNA purity and quantification

The DNA isolated from *Curvularia lunata* cells are usually contaminated with protein, RNA, and salts used during the isolation process. The purity of DNA may be estimated by utilizing the property of the heterocyclic rings of the nucleotides of absorbing light strongly in the UV range. DNA absorbs maximum light energy at about 260 nm. An optical density of 1.0 corresponds to approximately 50 mg/mL of double stranded DNA. The ratio of absorbance viz. A260/A28O and A28O/ A260 provides an estimation regarding the purity of DNA. To find out the purity of DNA, make the appropriate dilution with TE buffer, and measure the absorbance at 260 nm and 280 nm. Do not use glass or plastic cuvettes, as lights in the UV range do not pass through these.

Restriction Fragments Length Polymorphism (RFLP) Analysis.

The DNA extracted from mutant strains was restricted with Hind III. To the reaction mixture add 11.8 µl of nuclease free water, 2 µl of 10x assay buffer, 0.2C of 100x BSA, 5 µl of template and 1 µl of restriction enzyme. The mixture was incubated at 37°C for one hour then 65 °C for 10 minutes. 10-15 µl of digested DNA along with dye were loaded on the gel. Electrophoresis was performed for one hour at 50°C and viewed under the Trans illuminator. As a marker 1Mb ladder was used to find out the molecular weight of restricted DNA.

Estimation of Lipids

All *Curvularia lunata* cultures were harvested at 21st day and used for lipid analysis. 50 mg of fungal biomass was homogenized and extracted repeatedly with 30 ml acetone first then with 30 ml hexane. Mix both acetone - hexane extract and concentrated in distillation apparatus with cooling vacuum pump. Dissolve 10 mg of lipid sample in 10 ml of benzene. Fatty acids were analysed by GC (Agilent 1100 model) equipped with FID using Innowax column.

RESULT AND DISCUSSION

Genetic improvement is one of the promising approaches for increased production of lipids by industrially important microfungi. Genetic improvement of the selected fungi was carried out by chemical mutagenesis. Strain improvement can generally be described as the use of any scientific techniques that allow the isolation of cultures exhibiting a desired phenotype. In the present investigation mutation was carried out chemically, by treating the fungi with Ethidium bromide and Acridine orange. Wild strain of *Curvularia lunata* was subjected to chemical mutagenesis. A total 5 mutants were selected

and screened for their lipid production. Five Ethidium bromide mutants EBCV1, EBCV2, EBCV3, EBCV4, and EBCV5 produced 2.12 g/L, 2.84 g/L, 3.45g/L, 2.21g/L, 2.01g/L lipids respectively. The mutant EBCV3 gave maximum lipid production of 3.45 g/L which was 25% more than the wild strain in standard PDA medium. Five Acridine orange mutants AOCV1, AOCV2, AOCV3, AOCV4, and AOCV5 produced 2.21g/L, 2.81g/L, 3.01g/L, 3.79g/L, 1.90g/L lipids respectively. The mutant AOCV4 gave maximum lipid production of 3.79g/L, which was 30% more than the wild strain. More chemical mutation was directly related to immediate deaths of fungi cells. Over the seven -day growth period, the control (0 min) doubled its population for each of the two-day intervals. The 30 min sample showed no growth the first two days but did on the fourth day. Not certain whether it was because of the cells died but grew back or if they did not die at all. On the second day, it grew, signifying that there was DNA repair. The 60 min sample showed decline after the two days, with recovery after two more days. The 60 min sample showed a decline, indicating that mutations occur but the population recovered. This may indicate that the fungal population underwent cell repair. For the 90 min, 120min, and 150 min samples, the populations declined for four days. No fungi cells died from the increased temperature. For the 90min, 120min, and 150 min samples, the populations experienced a downward decline in population, indicating irreversible DNA damage. The relatively small decrease in population during the first two days in these samples suggests that the mutated cells were able to survive and function in interphase.

Isolation of DNA

From the pure cultures, the DNA of all mutant strains were isolated and confirmed by Agarose gel electrophoresis. The DNA bands were clear. The result indicated that all the isolates of five strains samples found to contain DNA with similar molecular weight. Nuclear DNA of Curvularia *lunata* was represented in Fig: 1.

Estimation of DNA

Extracted DNA samples were estimated from all fungal cultures were checked its purity and quantification by using spectrophotometer. About 800-900 ng of DNA is obtained from 1 g of pellet from Ethidium Bromide treated Curvularia sp and 300-400 ng from Acridine Orange treated. The ratio of absorbance at 260 to that at 280 nm (A260/280) of the DNA ranged from 1.8-1.9 for Ethidium Bromide strains and 1.7-1.8 for Acridine Orange strains.

Restriction Fragments Length Polymorphism (RFLP)

The DNA extracted from all mutant strains was restricted with enzyme Hind III. The result shows that the isolates EBCV1 & EBCV2 produced the bands at 1500 kb, 1750 kb and 2000 kb. The isolates EBCV3 & EBCV4 produced the bands at 1250kb and 1500 kb. The isolates EBCV4 & EBCV5 produced the bands at 1200 kb. Only the isolate EBCV4 produced a single band at 2750 kb. The isolate EBCV1, EBCV2, EBCV3 and EBCV4 produced the bands at 1500 kb. The isolate AOCV1, AOCV3 and AOCV5 produced the bands at 1200kb. The isolates AOCV4 and AOCV5 produced bands at 1500kb. The isolates AOCV5 produced a single band at 1750 kb and 2000kb.Purity of the DNA ranged from 1.8-1.9 for Ethidium Bromide mutants and 1.7-1.8 for Acridine orange mutants; both the mutants having same purity but the quantity of DNA were slightly modified. RFLP analysis of Curvularia *lunata* by HIND III digestion was represented in Fig: 2 and 3.

The molecular genotyping of *Curvularia lunata* has proven useful in many epidemiologic situations. One of the most widely used genotyping techniques is RFLP, a relative technically simple and rapid procedure. Although RFLP has been criticized for a lack of reproducibility, this method has been used with the success for *Curvularia lunata* isolates. **Brich and Denning (1998)** used five restriction enzymes to test 14 isolates. **Symones (2000)** tested 43 isolates of environmental and

clinical origins with the Hind III. Among epidemiologically unrelated species, the restriction enzymes were highly discriminatory. In addition, they found RFLP to be useful tool in demonstrating the clonal region of contamination of the environment in a hematology unit.

In the present study there are ten different mutant strains were isolated. Among these mutant strains no differences were found in the microscopic morphology. Similar results were obtained by **Rath (1999).** The RFLP analysis was performed was intended to assess the genetic diversity within the *Curvularia* sp. RFLP fingerprints allow intra specfic genetic diversity studies but are not very good tools for such analysis at the inter specific level. **Cooke (1998).**RFLP analysis of *Curvularia lunata* has proven useful in many epidemiological investigations. **Varga (2000)** applied RFLP and showed a high degree of variability among *Curvularia lunata* even among those indistinguishable based on their internal transcribed spacer sequences. **Rath (1999)** reported nine different genotypes of *Curvularia lunata* by RFLP analysis. Previously, this technique was used successfully in fingerprinting other *Curvularia lunata*. **Brown (1992)** showing a high genetic diversity in *Aspergillus flavus*. Similar results were also found in *Curvularia lunata* in the present study. Therefore, RFLP seems to demonstrate diversity within a species.

Table

1 Lipid production of Curvularia* lunata *by Ethidium bromide mutagenesis

Time (Minutes)	Mutants	Lipid yield (g/L)
0	Control	1.23
30	EBCV1	2.12
60	EBCV2	2.84
90	EBCV3	3.45
120	EBCV4	2.21
150	EBCV5	2.01

Table: 2

Lipid production of Curvularia* lunata *by Acridine orange mutagenesis

Time (Minutes)	Mutant	Lipid yield (g/L)
0	Control	1.23
30	AOCV1	2.22
60	AOCV2	2.81
90	AOCV3	3.01
120	AOCV4	3.79
150	AOCV5	1.90

Table 3

RFLP Profile of Ethidium Bromide mutants with Hind III

S. No	*Curvularia* Mutants	Presence (+) or Absence (-) of Bands at the respective base pairs						
		1000	1200	1250	1500	1750	2000	2750
1	EBCV1	-	-	-	+	+	+	-
2	EBCV2	-	-	-	+	+	+	-
3	EBCV3	+	-	+	+	-	-	-
4	EBCV4	-	+	+	+	-	-	+
5	EBCV5	-	+	-	-	-	-	-

Table 4
RFLP Profile of Acridine Orange mutants with Hind III

S. No	*Curvularia* Mutants	Presence (+) or Absence (-) of Bands at the respective base pairs				
		1200	1250	1500	1750	1800
1	AOCV1	+	-	-	-	-
2	AOCV2	-	+	-	-	-
3	AOCV3	+	-	-	-	-
4	AOCV4	-	-	+	-	-
5	AOCV5	-	+	-	+	+

Graph: 1
Production of Lipids in Curvularia* lunata *by chemical mutagenesis with respect to time.

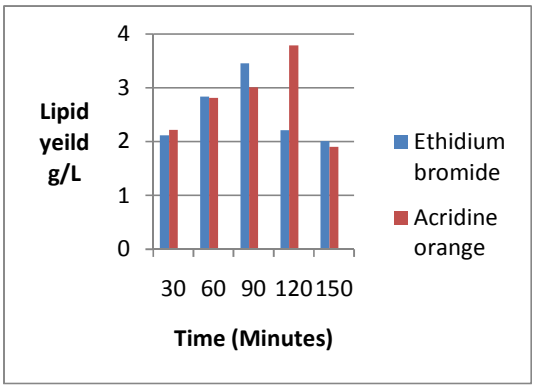
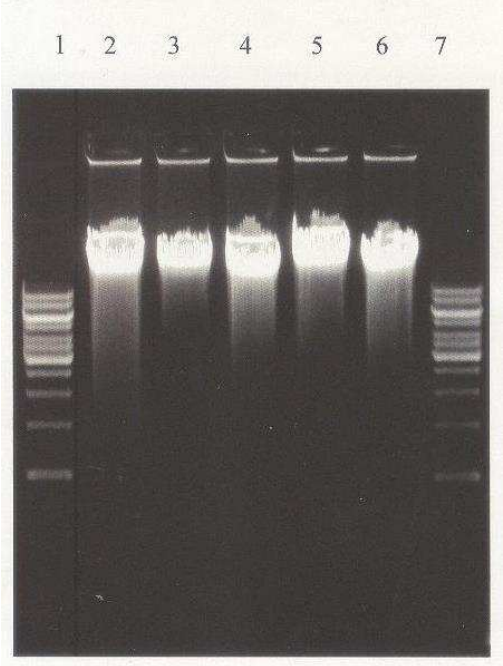
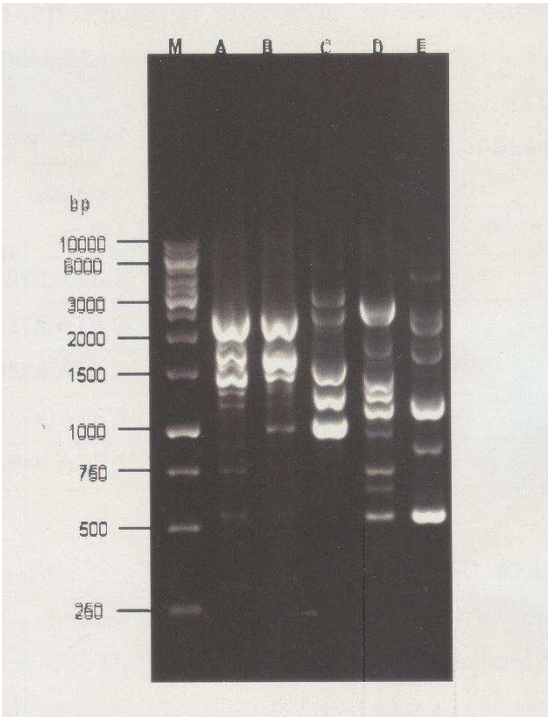


Figure: 1
Genomic DNA of Ethidium Bromide mutant strains



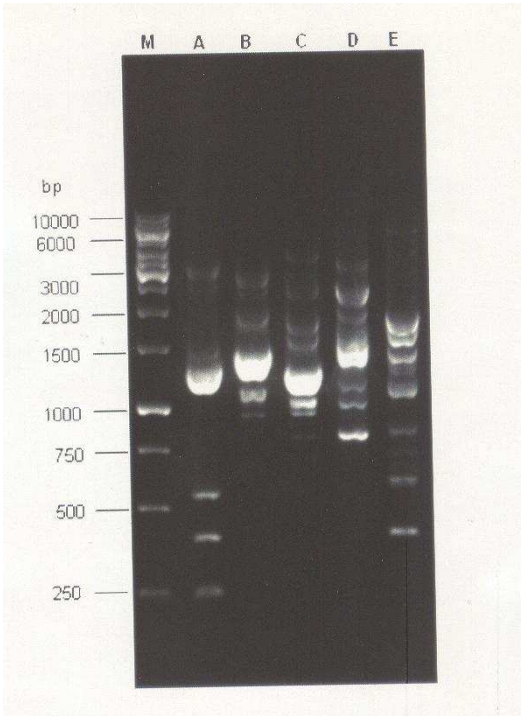
1: 1kb ladder, 2: EBCV1, 3:EBCV2, 4:EBCV3, 5:EBCV4, 6:EBCV5, 7: 1kb ladder

Figure: 2
RFLP of Ethidium Bromide mutant strains with Hind III



M: 10000bP ladder, A: EBCV1, B: EBCV2, C: EBCV3, D: EBCV4, E: EBCV5

Figure: 3
RFLP of Acridine Orange mutant strains with Hind III



M: 10000bP ladder, A: AOCV1, B: AOCV2, C: AOCV3, D: AOCV4, E: AOCV5

CONCLUSION

In the present study it is concluded that in contrast to the phenotypic method, the analysis of RFLP patterns is useful for strain differentiation of *Curvularia lunata.*

REFERENCES

1. Altomare, C., O. Petrini, A. Logrieco. and A. Bottalico, Taxonomic relationships among the taxogenic *species Fusarium acuminatum, Fusarium sporotrichiodes* and *Fusarium tricinctum* by isozyme analysis and RAPDs assay. *Canadian Journal of Botany*, 75: 1674-1684, (1997).
2. Bapiraju K, Sujatha P, Ellaiah P and Ramana T ,Mutation induced enhanced biosynthesis of lipase. Afr. J. Biotechnol. 3: 618- 621, (2004).
3. Berretta, R. E., R. Lecuona, O. Zandomeni. and R. Grau, Genotyping isolates of the entomopathogenic fungus *Beauveria bassiana* by RAPD with fluorescent labels. *Journal of Invertebrate Pathology*, 71: 14-10, (1998).
4. Carson M. L, Assessment of six models of host-parasite pathogen interaction in horizontal pathosystems. *Phytopathology*, 77: 241-246, (1987).
5. Castrillo, L. A. and W. M. Brooks, Differentiation of *Beauveria bassiana* isolates from the darkling beetle, *Alphitobius diaperinus*, using isozyme and RAPD analysis. *Journal of Invertebrate Pathology*, 72: 190-196,(1998).
6. Crowhurst, R. N., F. Y. King, B. T. Hawthorne, F. R. Snderson. and T. Choi-pheng, RAPD characterization of *Fusarium oxysporum* associated with wilt of angsana (*Pterocarpus indicus*) in Singapore. *Mycology Research*, 99:14-18,(1995).
7. Edel-Hermann V., Gautheron N., Alabouvette C., Steinberg C., Fingerprinting methods to approach multitrophic interactions among microflora and microfauna communities in soil. *Biology and Fertility of Soils* **44**: 975-984, (2008).
8. Freese EB , Transitions and transversions induced by depurinating agents. Proc. Natl. Acad. Sci. USA, 47: 540-545, (1961).
9. Haq IU, Ali S, Saleem A, Javed MM , Mutagenesis of *Bacillus licheniformis* through ethyl methanesulfonate for alpha amylase production. Pak. J. Bot. 41: 1489-1498, (2009).
10. Hartmann M., Widmer F., Reliability for detecting composition and changes of microbial communities by T-RFLP genetic profiling. *FEMS Microbiology and Ecology* **63**: 249-260, (2008).
11. Hodgetts J., Ball T., Boonham N., Mumford R., Dickinson M., Use of terminal restriction fragment length polymorphism (T-RFLP) for identification of phytoplasmas in plants. *Plant Pathology* **56**: 357–365, (2007).
12. Hodges, C.F. , Interaction of culture age and temperature on germination and growth of *Curvularia geniculata* and on virulence. *Canadian Journal of Biology*, 50: 2093-2096, (1972).
13. Iftikhar, S., S. Amir, M. Anjum, I. Shazia. and A. Iftikhar., Fungi associated with rice-wheat cropping system in relation to zero and conventional tillage technologies. *Journal of Biological Science*, 3: 1076-1083, (2003).
14. Jeennor S, Laoteng K, Tanticharoen M, Cheevadhanarak S , Evaluation of inoculum performance for enhancing gamma linolenic acid production from *Mucor rouxii*. Letts. Appli. Microbiol., 46: 421-427, (2008).
15. Lamari, L. and C. C. Bernier, Evaluation of wheat lines and cultivars for reaction of tan spot (*Pyrenophora tritici-repentis*) based on lesion types. *Canadian Journal of Plant Pathology*, 11: 49-56, (1989).
16. Liu W.T., Marsh T.L., Cheng H., Forney L.J., Characterization of microbial diversity by determining terminal

restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology,* **63**: 4516-4522, (1997).
17. Ledingham, R. J, Crop rotations and root rot in wheat. *Canadian Journal of Plant Science*, 41: 479-486, (1961).
18. Marsh T.L., Saxman P., Cole J., Tiedje J., Terminal restriction fragment length polymorphism analysis program, a Web-based research tool for microbial community analysis. *Applied and Environmental Microbiology* **66**: 3616-3620, (2000).
19. Meijer, G., B. Megnegneau. and E. G. A. Linders., Variability for isozyme vegetative compatibility and RAPD markers in natural populations of *Phomopsis subordinaria. Mycological Research*, 98: 267-276, (1994).
20. Osborn A.M., Edward R.B.M., Timmis K.N., An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* **2**: 39-50, (2000).
21. Rogers S. O. and A. J. Bendich, Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology*, 5: 69-76, (1985).
22. Sakai M., Matsuka A., Komura T., Kanazawa S., Applications of a new PCR primer for terminal restriction fragment length polymorphism analysis of the bacterial communities in plant roots. *Journal of Microbiological Methods* **59**: 81-89, (2004).
23. Shamoun, S. F., A. K. M. Ekramoddoullah. and R. E. Wall., Isozyme analysis of *Chondrostereum purpureum* used as a mycoherbicide. *Canadian Journal of Plant Pathology*, 13: 285, (1991).
24. Pigliucci, M., "How organisms respond to environmental changes: from phenotypes to molecules (and vice versa)", Trends in Ecology and Evolution,11, pp. 168–173, (1996).
25. Punekar NS., Suresh Kumar SV., Jayashri TN, Anuradha TN., 'Isolation of genomic DNA from acetone-dried *Aspergillus* mycelia", Fung Genet Newsl, 50, pp.15–16, (2004).
26. Waskman, S. A. and E. B. Fred., A tentative outline of the plate method for determining the number of microorganisms in the soil. *Soil Science*, 14: 27-28, (1992).