"GENETIC MANIPULATION OF 'COIRRET' FOR THE APPLICATION ON COIR FOR QUALITY IMPROVEMENT"

AIM

To improve the performance of 'COIRRET' and to formulate a novel eco-friendly microbial consortium for field use on coir fibre/yarn.

INTRODUCTION

Genetic Manipulation is changing the hereditary characteristics of plants and animals by modifying the genetic material. Genetic engineering also called genetic modification, is the direct human manipulation of an organism genome (organism hereditary information) using modern DNA Technology.

Artifical manipulation, modification and recombination of DNA or other nucleic acid molecules inorder to modify an organism or population of organism. The term initially meant any of a wide range of techniques for modifying or manipulating organisms through heredity and reproduction. Now the term denotes the narrower field of recombinant DNA technology, or gene cloning, in which DNA molecules from two or more sources are combined, either within cells or in test tubes, and then inserted into host organisms in which they are able to reproduce.

This technique is used to produce new genetic recombinations that are of value to science, medicine, agriculture or industry. Genetic manipulation has a lot of advantages, but it also comes with a lot of risks.

COIRRET

The 'COIRRET' is a consortium of microbes: ***Mycoplana bullata, Mycoplana dimorpha and Pseudomonas desmolyticum*** belongs to the family actinomycetes.. Coirret is a formulation of phenolic bacteria. They posses the ability of degrading phenolic compounds. Central Coir Research Institute (CCRI) has developed this bacterial consortium 'COIRRET'. (Das A.R,2001)

By applying coirret on mechanically treated fibers we can reduce the retting period from 11 month to 3 month and improve the quality of green husk fiber within 72 hrs. 'COIRRET' is a bacterial cocktail which biosoftens in just 3 days. This process ensure a good quality fiber with less effluent problems.

In the present work, coirret bacterial strains: *Mycoplana bullata (NCIM 2382), Mycoplana dimorpha (NCIM 2383) and Pseudomonas desmolyticum (NCIM 2028)* obtained from National collection of Industrial Micro organism (NCIM), Pune. These bacterial strains were isolated and used for phenol degradation and retting.

OBJECTIVES

Development of an eco friendly biological package for retting coconut husk/coir fibre tanks by the application of biotechnology.

To produce superior quality fibre for the coir industry.

The project target improving the performance of 'coirret' on coconut husk and coir fibre by genetic manipulation.

APPLICATION OF BIOTECHNOLOGY IN COIR INDUSTRY

- It may be well adopted for coir softening and brightening.
- New eco-friendly methods for fibre production having the potential to produce more consistent quality of fibre are of interest.
- Microbial treatment will produce soft, white fibres having better tensile strength and elongation properties. Expected outcome will ensure that the coir fibre achieve the desired softness required for faultless spinning without end breaks and hairiness.
- In coir sector, process efficiency can be achieved by means of using biotechnological tools i.e. by altering the genetic make up of a particular strain for a desired purpose using genetic engineering techniques. All the information process by biotechnology is stored and analysed using bioinformatics softwares.
- Sequence analysis is the use of various bioinformatics methods and tools to determine the biological function and structure of genes and the proteins they code for. Thus we can produce an improved strain having better characteristics. Therefore special techniques have to be formulated to explore the possibilities of enzymatic quality improvement of coir fibre. There by we can minimize the environmental issues related to the coir industry.

EXPERIMENTAL DESIGN

1. To isolate pure cultures of the stains in COIRRET
2. Morphological and biochemical analysis of the bacterial strains.
3. To isolate DNA from 24 hour old broth culture of each of the bacterial strain
4. PCR amplification of DNA with 16s rRNA primer followed by sequencing of the DNA.
5. Sequence analysis by using bioinformatics tools.
6. To perform sequence similarity search by using bioinformatics software to compare the sequence of a particular gene with sequence from other related organisms.
7. Determination of quality and quantity of enzyme production by the bacterial strains.
9. To identify whether the DNA is plasmid or genomic DNA.
10. Physical mutagenesis of the 3 bacterial strains.
11. Determination of quality and quantity of enzyme production by the mutated bacterial strains.
12. Isolation of DNA from the 3 mutated bacterial strains.
13. Designing primers using primer3 bioinformatics software for PCR amplification of the isolated DNA.
14. Sequencing the mutated organism and with the help of Refseq bioinformatics software, check whether the mutation has occurred in the genes coding for enzymes tannase, cellulose, xylanase, pectinase, tannase and laccase etc.
15. Testing of improvement in quality of COIRRET by application on coir fibre (In accordance with the Biosafety rules and regulation guidelines).
16. Testing the quality improvement of coir fibre in brightness, softness and flexural rigidity, tenacity, elongation and breakage force etc and the scanning electron microscope (SEM) analysis and Fourier transform infrared spectroscopy (FTIR) studies.
17. To develop a bio-package for husk retting in the tanks using microbes isolated from the stressed environment.

Flow Chart-

Schematic Representation of "Genetic Manipulation Of 'Coirret' For The Application On Coir For Quality Improvement"

- *COIRRET*

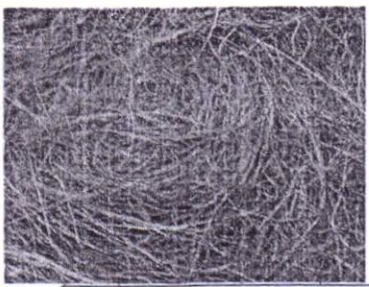
- *Modifying 'COIRRET' by incorporation of new phenol degrading strains.*

- *Study of DNA in all strains*

- *Genetic Manipulation of the DNA in all strains.*

- *Laboratory scale trials on treatment of coir fibre using the modified 'COIRRET'*

- *Assessment of bio brightening / biosoftening of coirfiber by new consortium through tests.*

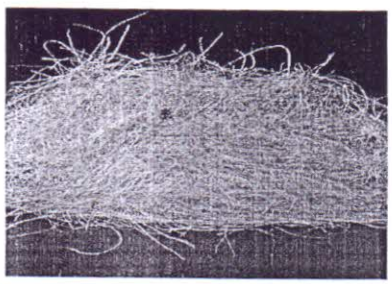


- *Scale up studies on coir fibre*

- *Standardisation of production of modified 'COIRRET' for large scale treatment.*

- *Standardisation of dosage of 'COIRRET' for treatment on coir in large scale.*

- *Demonstrations of treatment of coir entrepreneur through field trials.*



EXPERIMENTAL SELECTION

STUDIES CONDUCTED

1. MUTATION

Mutations are heritable change in the base sequence of DNA. Some mutations can be beneficial to an organism, but most are actually harmful because the mutation will often results in the loss of an important cellular function. It occur naturally in bacteria at a rate $10^{-7} - 10^{-8}$ per base pair during one round of replication. In molecular biology and genetics, mutations are changes in a genomic sequence; the DNA or RNA sequence of a virus. Mutations are caused by radiation, transposons and mutagenic chemicals and viruses as well as errors that occur during meiosis or DNA replication. They can also be induced by the organism; itself by cellular processes such as hyper mutation. Today bacteria are an important tool in the study of genetics and biotechnology, but for 40 years after the rediscovery of Mendel's work and the rebirth of genetics, they were considered too simple to have genes, undergo mutation, or reproduce sexually. Scientist had long observed differences between bacterial colonies, but had never realized that these differences were the results of mutation (Bacterial mutation; Chris Evers, 1943).

Shortwave UV light can kill bacteria and cause a strong sunburn effect on skin. It is harmful to humans and all living things because it has the power to change DNA. The energy in UV light, which is absorbed by DNA, can actually break bonds in DNA. UV light exposed at 254nm for short time periods, such as two or five minutes, was not expected to completely destroy the bacteria. Similarity it was not expected that bacteria exposed to UV light for 1minute would result in almost complete mortality. Surprisingly, very low exposure times, such as 15 and 30 seconds resulted in at least 40% bacteria mortality and bacteria exposed to 254nm for 1minute resulted in at least 95% mortality. In this study the Bacillus sp. and 'COIRRET' cultures are exposed to 5 to 10 minutes UV light for causing mutations for increasing the quality of coir fiber. The short term UV light exposure is for causing only mutation in the bacteria.

MUTATION IN 'COIRRET'

The main aim of this work was to improving the performance of 'COIRRET' on coconut husk and coir fibre by genetic manipulation. To mutating the 'COIRRET' for

checking whether it helps in increasing the quality of coir fiber. The procedure was given below:

1. The culture of 'COIRRET' including *Mycoplana bullata*, *Mycoplana dimorpha* and *Pseudomonas desmolyticum* were obtained from National cultural collection of NCIM, Pune and Five potential phenol degrading bacillus sp. [(B.stratosphericus (S6), (B.licheniforms (S9), (B.safensis(S11), (B.thurengensis (S19)].
2. These culture are exposed to Ultraviolet radiation (254nm) at a time of 5 minutes and 10 minutes for causing mutation in these cultures.
3. After the exposure, these cultures are incubated 37^0C for 24 hours.

BIOSOFTENING OF COIR FIBER

The biotreatment was performed in Ehrlen Mayer flasks (250ml). 20ml distilled water was poured into 18 flask and the flask mouth were plugged with cotton. They were sterilized in an autoclave at 121^0C and 15psi pressure for 20 minutes. After cooling, the flask were divided into 2 set containing 9 flask each, 10g coir fiber was weighed approximately. The fiber was aseptically transferred into the flask containing water with the help of a glass rod. 5ml bacterial inoculums prepared in 1N saline was added to each set excluding the control. The flask were kept at 37^0C for 24 and 48 hr of incubation. Each set flask containing the 5 and 10 minutes mutated bacterial inoculum.

After the treatment, the fiber was carefully taken out of the flask, washed with water and air dried. Softness of the treated fiber was assessed using flexural rigidity tester, Tensile testing and Brightness using spectrophotometer.

2. Study the efficacy of the consortia for quality improvement of coir fibre

In order to identify the suitable formulation for quality improvement in coir fibre, a set of laboratory level experiments will be carried out using different combinations of micro-organisms and monitoring the performance of each with respect to change in fibre properties. After compilation of the results obtained from the performance of each consortium, in terms of its efficacy in enhancing colour, softness and brightness of coir fibre, an ideal formulation will be developed for scale up and field demonstration.

3. Qualitative method for the determination of lignolytic enzyme producing bacteria.

Laccase enzyme involves in the biological degradation of lignin. Bacillus sp. Isolated from coir retting environment was sub cultured in nutrient broth. 1% Lignin extracted from coir fibre was sterilized separately.

Guiacol Screen

Add 0.01% guiacol and 1gm lignin extracted from coir fiber to Nutrient agar medium (100 µl/L) then autoclave and pour plate. The culture microorganisms [(B.stratosphericus(S6), (B.licheniforms(S9), (B.safensis(S11), (B.thurengensis(S19)] and 'COIRRET' consortium (M.bullata, M.dimorpha and P.desmolyticum)] on plates and incubated for 24- 48hrs and observe orange/brown halos around laccase positive colonies.

4. Compatibility of 'COIRRET' with Bacillus sp.

Compatibility of 'COIRRET' with beneficial microorganism bacillus sp. Phenol degrading bacteria isolated from coir retting environment [(B.stratosphericus (S6), (B.licheniforms (S9), (B.safensis(S11), (B.thurengensis (S19)] and 'COIRRET' [(M.bullata, M.dimorpha and P.desmolyticum)] were tested.

Micro organism such as COIRRET and Bacillus sp. was subcultures in Nutrient Agar medium. The subcultured pure colonies were cultured in nutrient broth and incubate for 2 hours. Nutrient agar plates were prepared and 8mm agar well was produced using well cutter. The 2 hours incubated bacillus culture broth was spreaded over the agar plate and incubated at 37^0C for 24 hrs. After 24hrs, the culture broth of bacillus sp. versus COIRRET was using a micro pipette poured in agar wells and kept at room temperature. After 48-72 hrs of incubation, a clear zone of inhibition was seen in Bacillus Sp. versus COIRRET

RESULTS

There was a visible increase in the brightness of the coir fibre by the use of 5 min UV mutated COIRRET consortia, Mb and Md of Trial I treatment than 5min UV mutated Bacillus consortia and S6 of Trial II treatment.

The Flexural Rigidity of the treated sample of Bacillus consortia 5 & 10mins UV (Trial I) treatment is best softer feel to the coir fibre. COIRRET consortia Trial I and II of Mb

Physical test results obtained were given in the table.1

Sample No	Brightness Index		Flexual rigidity (Gf) gcm2		% of softening	
Bacillus Sp.	Trial I (24 hrs)	Trial II (48 hrs)	Trial I (24 hrs)	Trial II (48 hrs)	Trial I (24 hrs)	Trial II (48 hrs)
Control	6.529	6.529	1.1284	1.1284	-	-
S6 UV 5min	8.772	10.637	0.6123	0.6123	45.73	45.73
S6 UV 10min	8.973	9.647	0.6123	0.7639	45.73	32.30
S9 UV 5min	10.331	9.626	0.7639	1.0147	32.30	10.076
S9 UV 10min	10.422	10.006	0.7918	0.9128	29.82	19.106
S11 UV 5min	10.476	9.385	1.0890	1.0689	3.491	3.589
S11 UV 10min	8.643	9.559	0.6599	0.6356	41.51	43.67
S19 UV 5min	8.673	9.707	1.0689	0.8205	3.589	27.28
S19 UV 10min	10.519	9.785	1.0147	0.8205	10.076	27.82
Bacillus consortia 5 min uv	9.184	10.648	0.5460	1.0147	51.612	10.076
Bacillus consortia 10 min uv	8.123	9.405	0.4851	0.9128	57.009	19.106

Sample No	Brightness Index		Flexual rigidity (Gf) gcm2		% of softening	
COIRRET	Trial I (24 hrs)	Trial II (48 hrs)	Trial I (24 hrs)	Trial II (48 hrs)	Trial I (24 hrs)	Trial II (48 hrs)
Control	6.529	6.529	1.1284	1.1284	-	-
Mb UV 5min	16.55	15.90	0.6548	0.5677	52.90	49.689
Mb UV 10min	13.92	12.38	0.6468	0.6055		
Md UV 5min	15.82	14.27	0.8205	0.8490	40.99	38.940
Md UV 10min	11.36	10.39	0.6433	0.6145		
Pd UV 5min	14.53	13.08	0.6849	0.9451	39.303	32.03
Pd UV 10min	12.13	10.19	0.6689	0.9042		
COIRRET Consortia 5 min UV	17.11	15.91	0.6599		41.518	
COIRRET Consortia 10 min UV	14.93	13.55	0.6754			

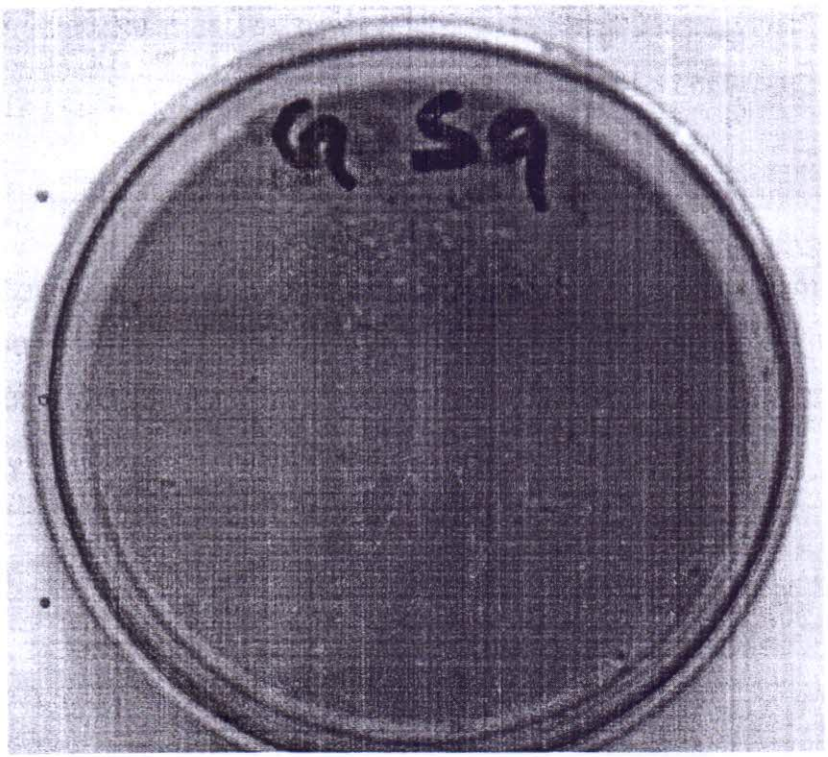
3. Laccase assay

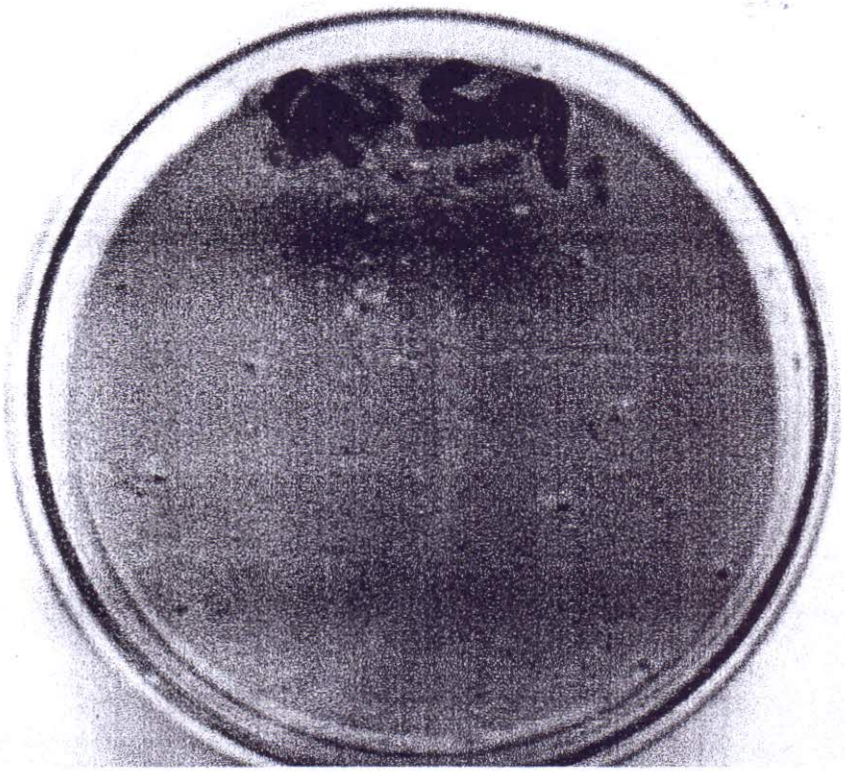
The obtained Bacillus sp. S6- Bacillus stratosphericus, S9- Bacillus licheniforms shows orange halos around laccase positive colonies.

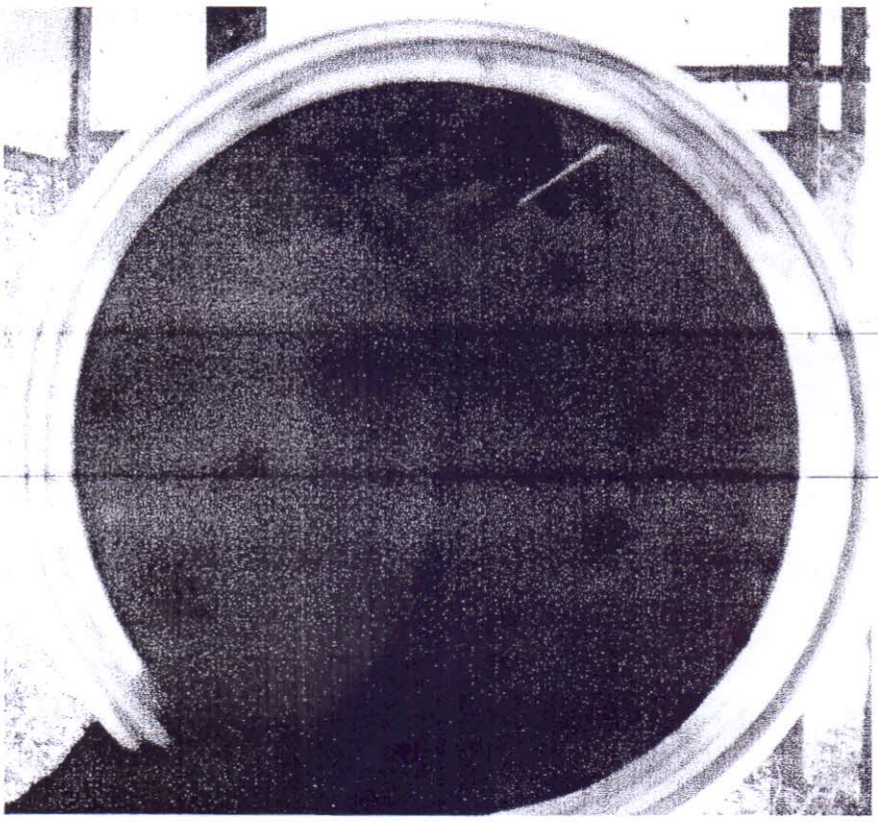
4. Compatibility of 'COIRRET' with Bacillus sp.

ISOLATES Bacillus	'COIRRET'		
	Mb	Md	Pd
S6	2.3 cm	2.5 cm	2.4cm
S9	3cm	-	-
S11	4 cm	3.2 cm	4.5 cm
S19	2.3 cm	-	-

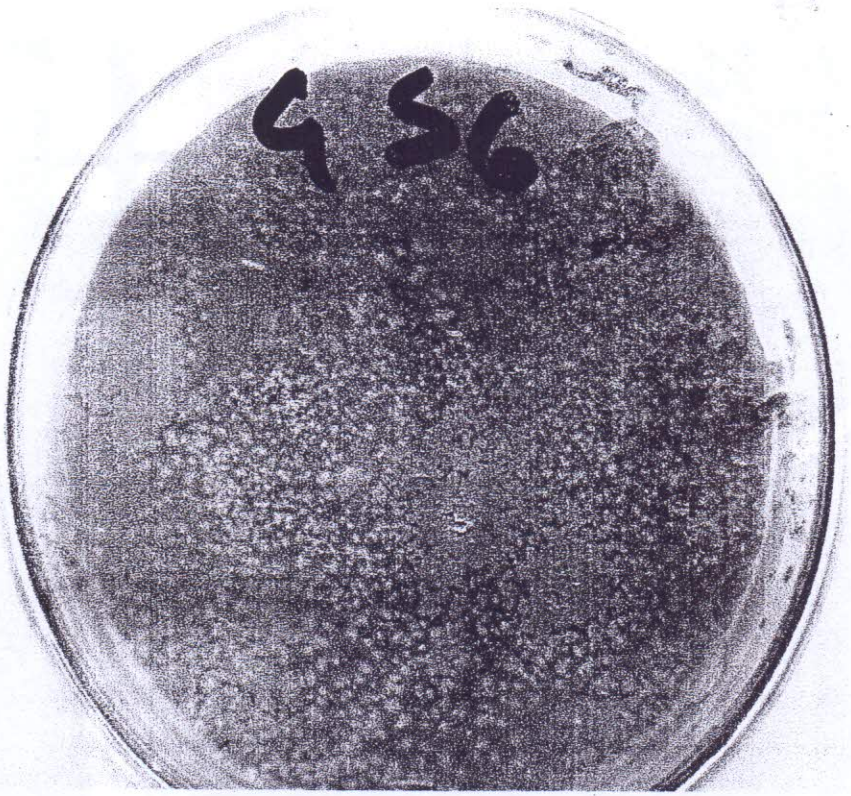
A clear zone of inhibition was found when 'COIRRET' treated against bacillus (S6, S9, S11, and S19). The inoculants were found to be non-compatible with each other and not able to growth simultaneously. 'COIRRET' exhibits a very visible antibiotic property therefore bacillus sp. cannot combine with 'COIRRET'.

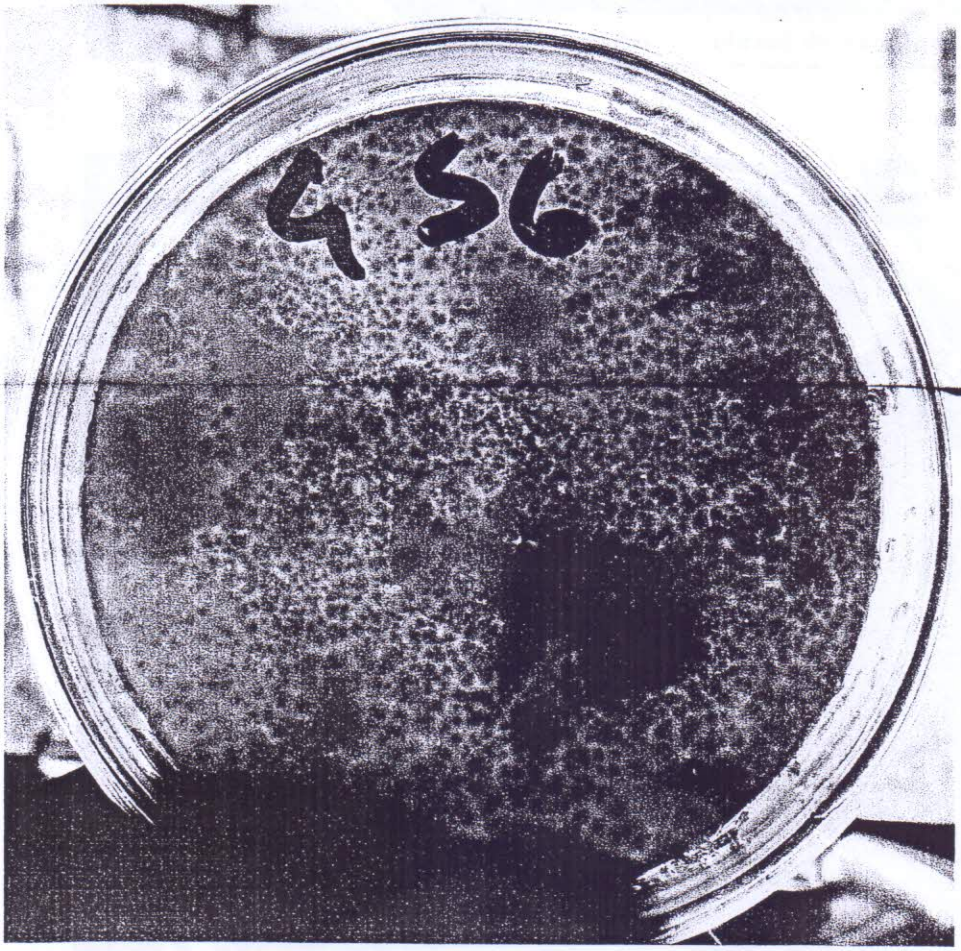




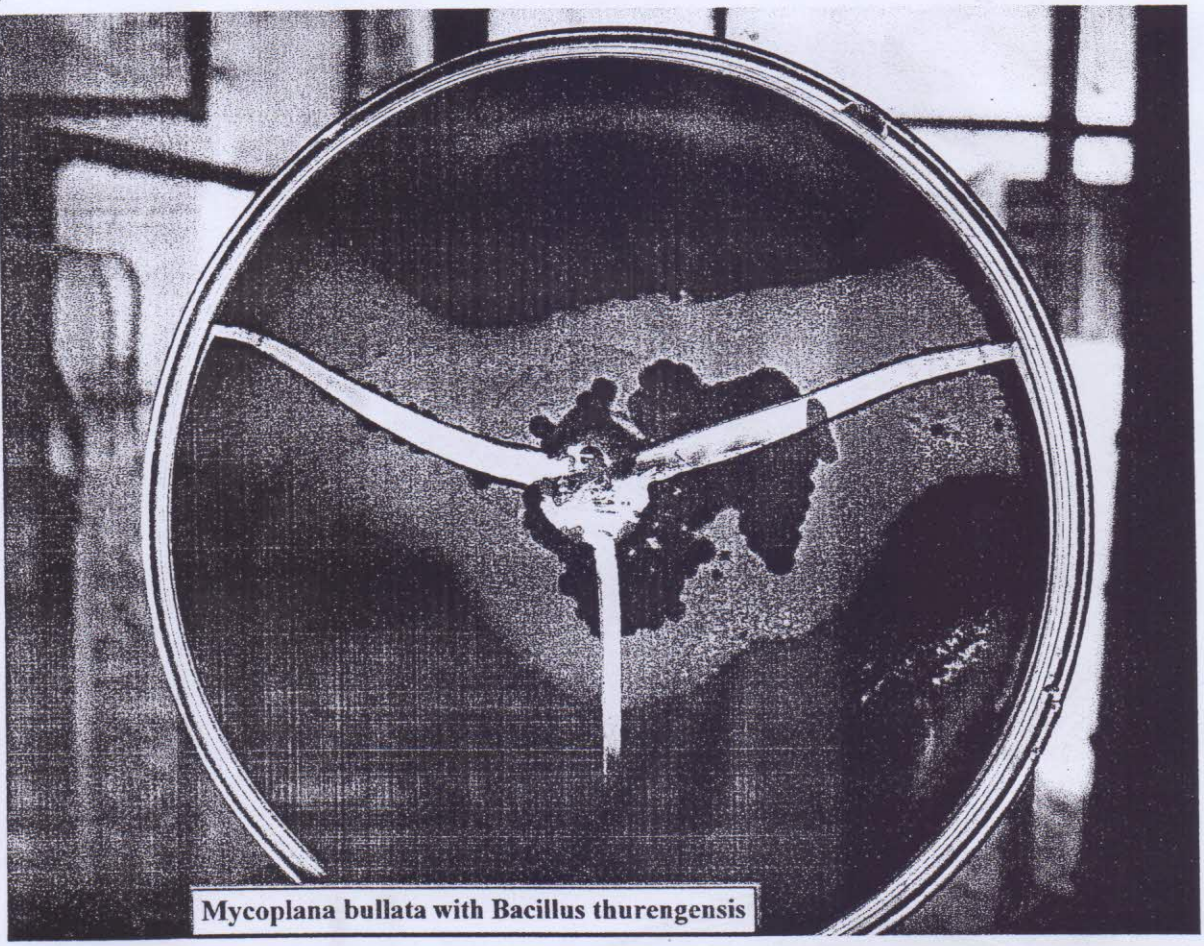


Bacillus licheniforms (S9) shows visible orange halos around laccase positive colonies

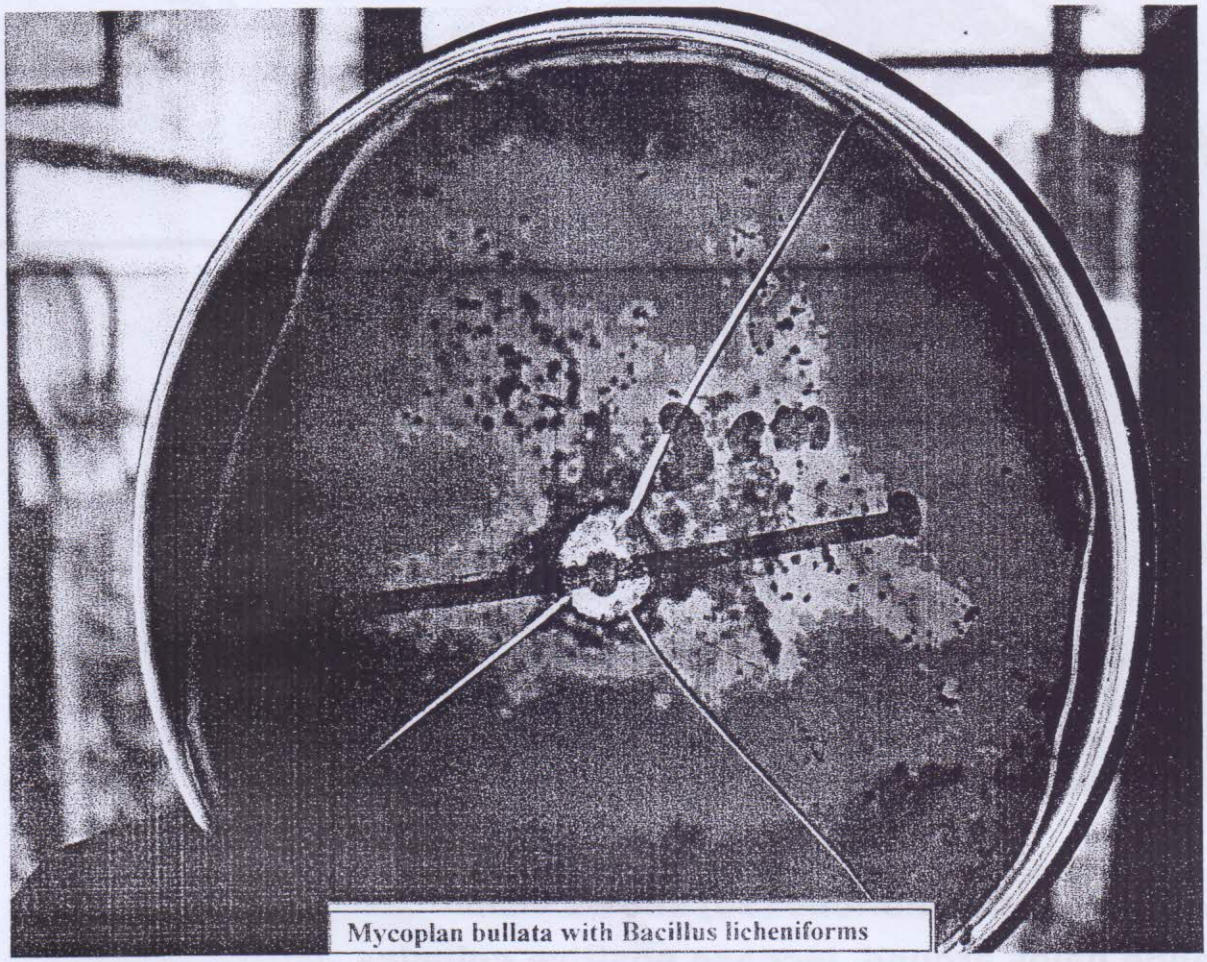




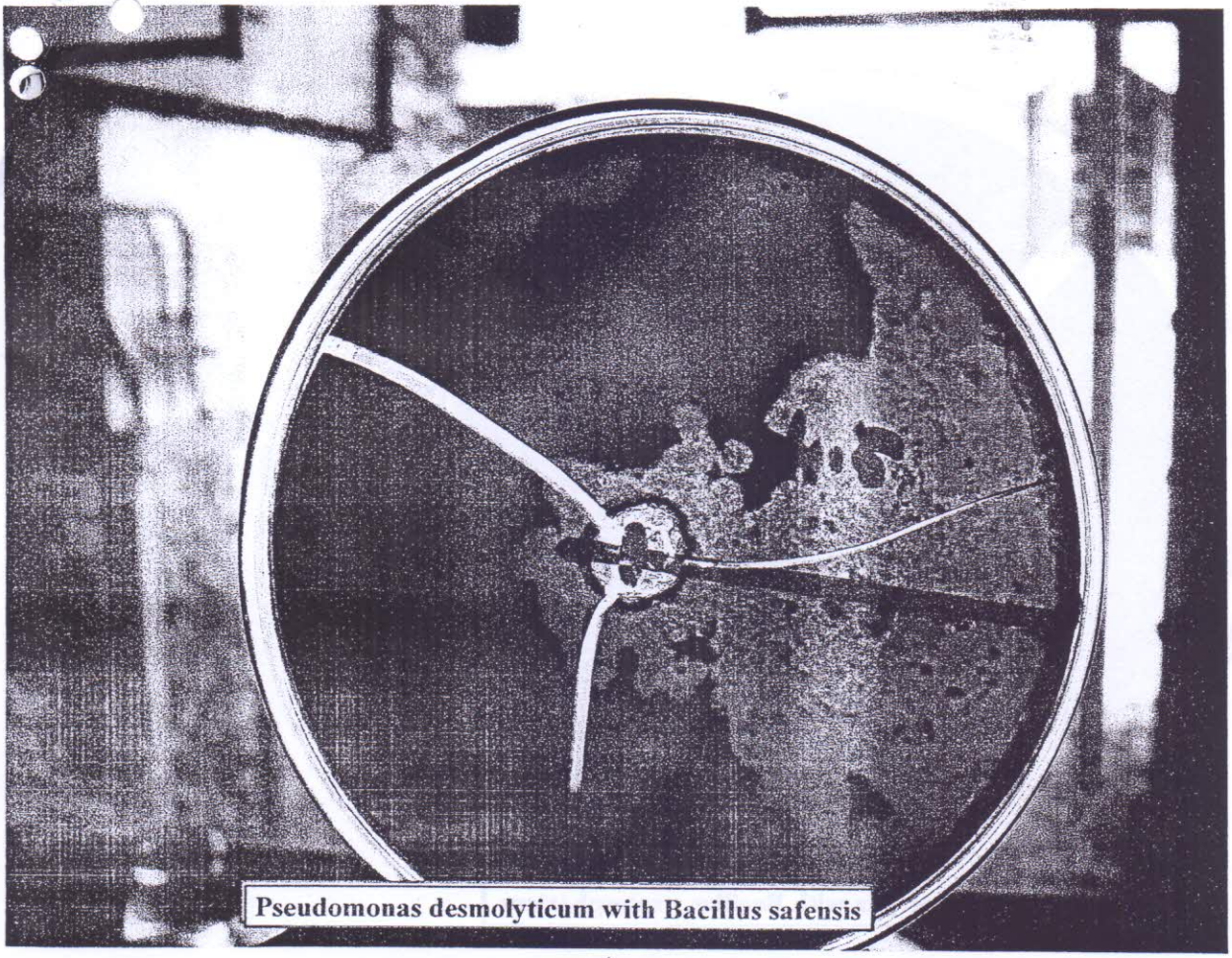
Bacillus stratosphericus (S6) shows visible orange halos around laccase positive colonies



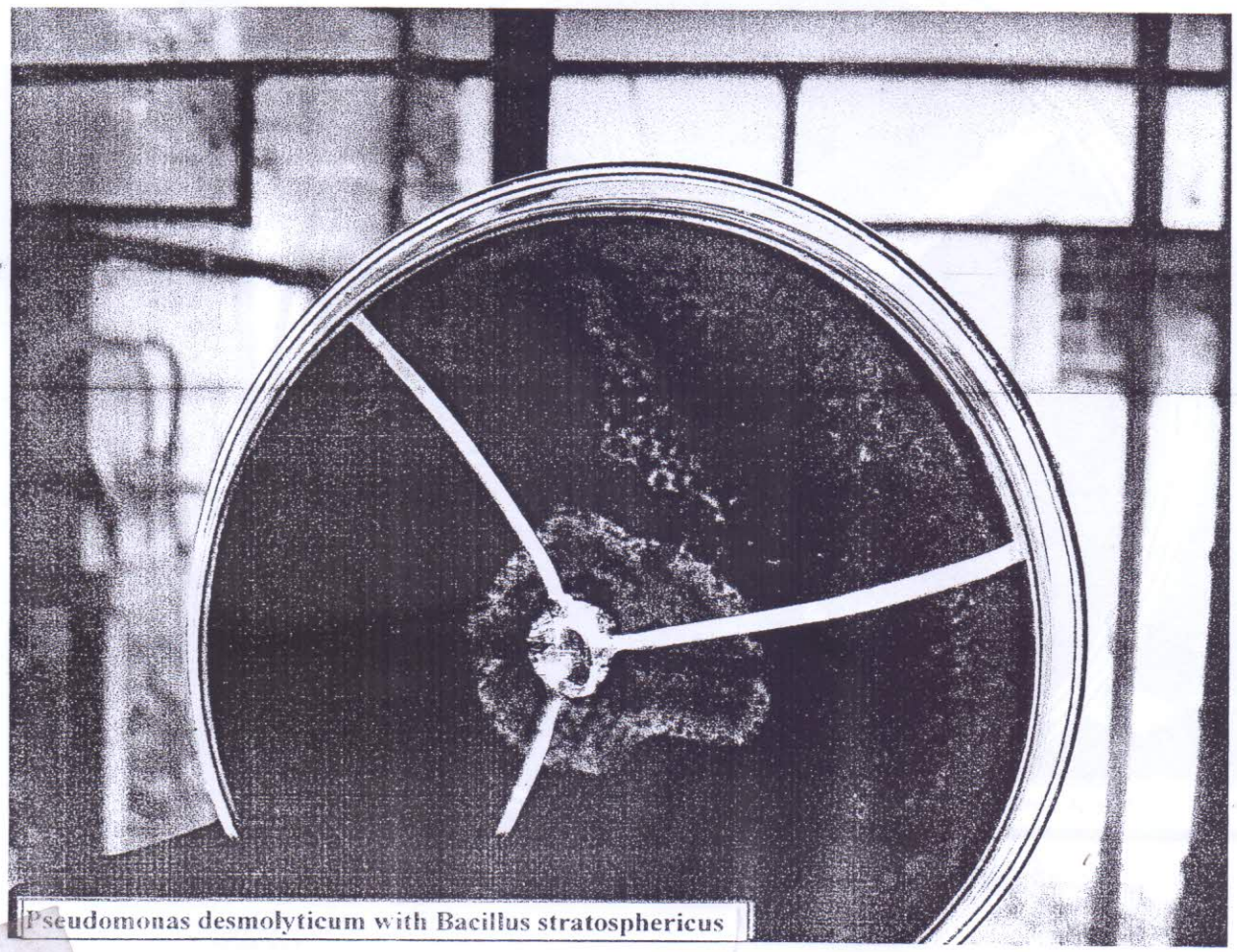
Mycoplana bullata with Bacillus thurengensis



Mycoplan bullata with Bacillus licheniforms

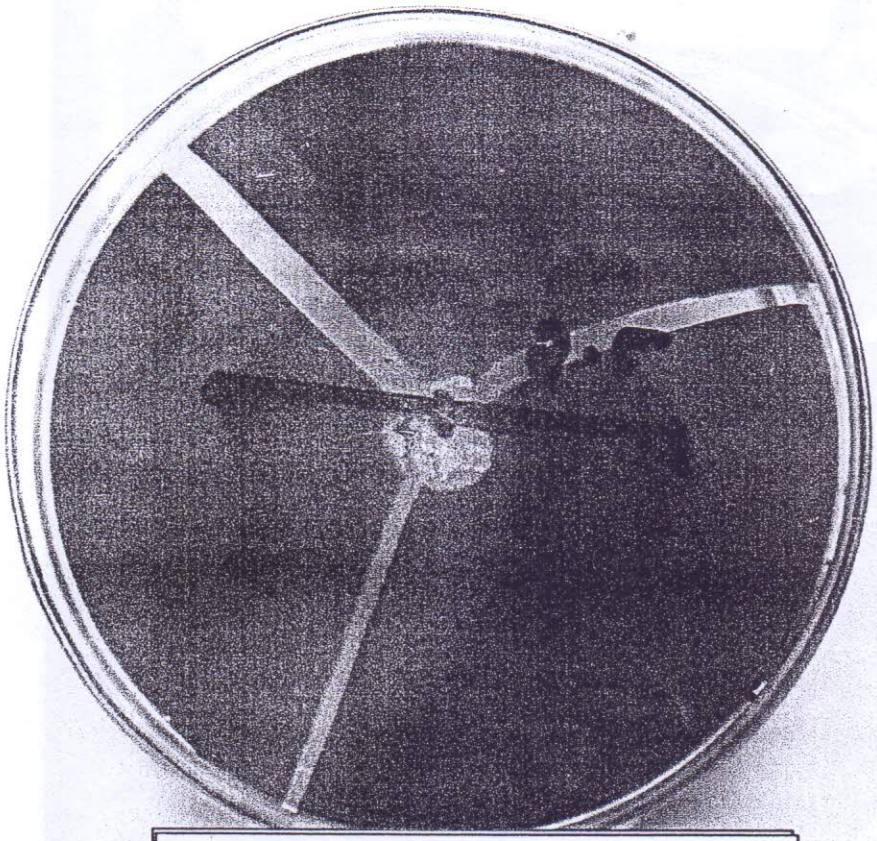


Pseudomonas desmolyticum with Bacillus safensis

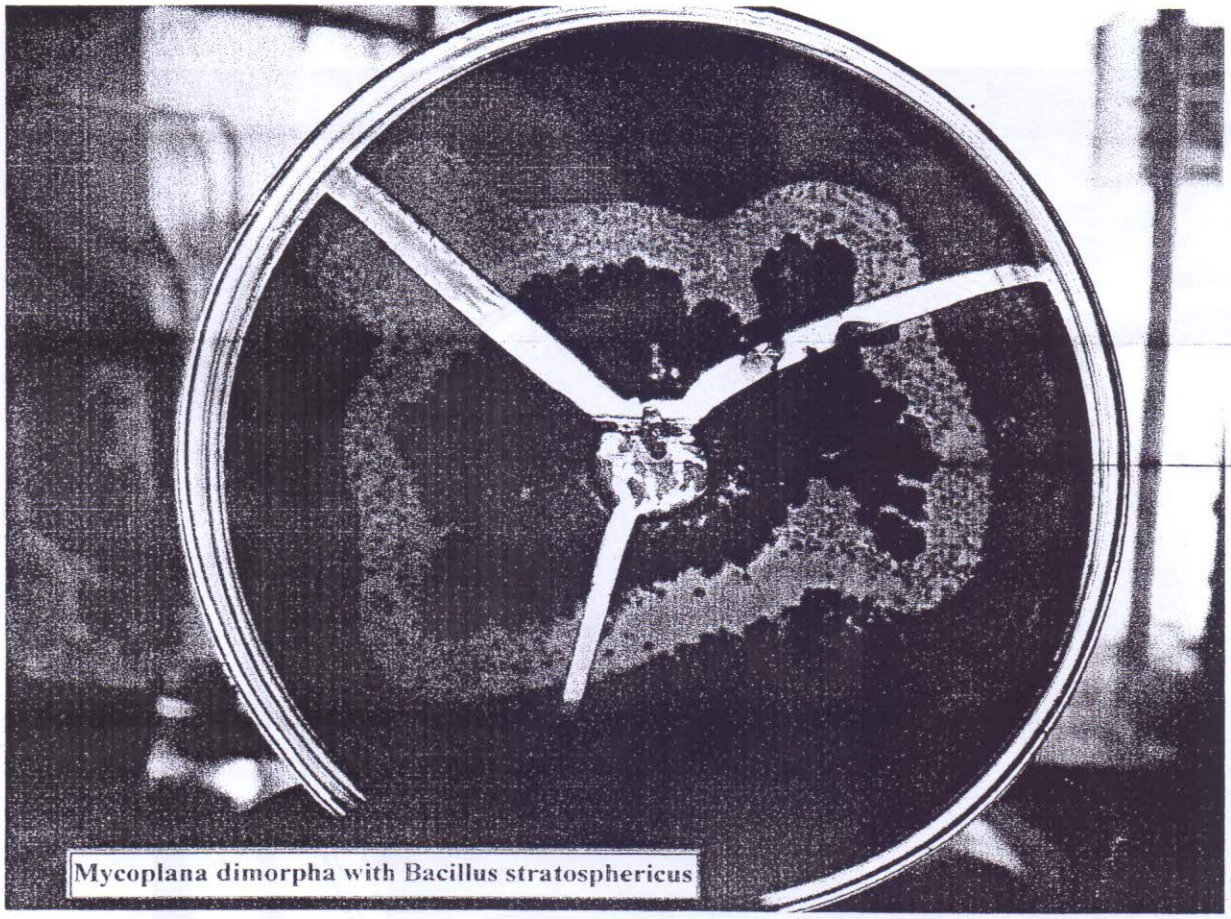


Pseudomonas desmolyticum with Bacillus stratosphericus

~~223~~ 222



Mycoplana dimorpha with Bacillus stratosphericus



Mycoplana dimorpha with Bacillus stratosphericus

CONCLUSION

CCRI has initiated systematic studies on the softening of coir. To improve the physical quality of microbial consortia treated coir fiber and also improving the performance of 'COIRRET' on coir fiber by genetic modification. ASTM lab in CCRI was evaluate the Physical test (Brightness Index and flexural Rigidity) with matured coir fibre to check the efficiency of the strain ('COIRRET' and bacillus sp. phenol degrading bacterial isolates from 'Anjuthengu' the coir retting biotope) in softening. There is an improvement in the brightness properties and softening properties on treatment with a bacterial consortium of selected phenolitic strains. Therefore treatment of coir fiber with UV mutated Bacillus sp. bacterial culture UV 10mins Bacillus consortia and S6 (Bacillus stratosphericus) of Trial II treated recommended for brightness and UV 5 & 10 min Bacillus consortia of Trial I treatment treated recommended for better softeness.

Studies shows that Mb, S6 and S9 gives more brightness and softness to coir fibre. S6 and S9 were found to be Laccase positive. So manipulating these strains will give a genetically modified strain for the application on coir for quality improvement.