STUDIES ON COCONUT HUSK RETTING AND BIOINOCULANT TREATMENT FOR PROCESS IMPROVEMENT IN A NATURAL SYSTEM

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Microbiology

By

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1999

Dedicated to my beloved Mother

CERTIFICATE

This is to certify that the thesis entitled **Studies on coconut husk retting and bioinoculant treatment for process improvement in a natural system** submitted by Ms. Anita Das Ravindranath for the award of the degree of Doctor of Philosophy in Microbiology is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any other University or Institution.

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STATEMENT

As required under the University ordinance 0.19.9 (xi), I state that the piece of work presented in this thesis entitled **Studies on coconut husk retting and bioinoculant treatment for process improvement in a natural system** is my original contribution and has not been submitted to any other University or Institution for the award of a degree, or diploma or a certificate. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities have been availed of.

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Chapter I

Introduction

Coir is a natural lignocellulosic hard fibre extracted from the outer covering of the coconut "Cocos nucifera" of the family Palmae. It is ecofriendly and therefore is advantageous for environmental operations over synthetics. Its inherent properties (Table 1.1, 1.2) robustness, resistance to biodegradation, low raw material price are suitable for sustainable development (Boben et al., 1999).

India is the largest producer of coconut in the world with a production of 13,968 million nuts against the global production of 53,598 million (Kumar, 1999). Coconut cultivation and production of nuts is prevalent in the different states and union territories of India (Table 1.3). The coconut has to be dehusked to remove the outer covering, which consists of the exocarp and the mesocarp. This outer covering, the husk, is the source of the "coir fibre' which is the raw material for the coir industry.

Table 1.1- Physical Properties of the coir fibre.

1.	Ultimates :		
	Length in mm	:	0.6
	Diameter / Width (microns)	:	16
2.	Single Fibre :		
	Length in inches	:	6-8
	Density (gm/cc)	:	1.40
	Tenacity (gm/tex)	:	10.0
	Breaking Elongation %	:	30
	Moisture Regain at [65% R.H. (%)]	:	10.5
	Swelling in water (dia)	:	5%

Joseph & Sarma, 1997

Table 1.2 - Chemical composition of coir fibre

Water Solubles	:	5.25 %
Pectin and related compounds	:	3.00 %
Hemicellulose	:	0.25 %
Lignin	:	45.84 %
Cellulose	:	43.44 %
Ash	:	2.22 %
		100.00 %

Table 1.3- INDIA: Coconut cultivation and production of nuts.
(1996-97)

State/Union Territory	Area (thousand hectares)	Production (million nuts)
Andhra Pradesh	90	685.9
Assam	19.6	118.4
Goa	24.7	119.0
Karnataka	290	1493.0
Kerala	980	5759.0
Maharashtra	15.1	264.5
Orissa	53	271.5
Tamil Nadu	328	3811.6
Tripura	8.8	5.9
West Bengal	23.7	313.1
Andaman & Nicobar	24.7	86.6
Lakshadweep	2.8	27.5
Pondicherry	2.1	31.5
All India	**1892.5**	**12987.5**

COIR BOARD

1.1. Importance of coir

The coir industry is an export and employment oriented one and the annual global requirement of coir fibre is about 78900 tonnes (Kumar,1999). This export oriented small-scale coir industry fetches a foreign exchange earning of over 200 crores of rupees per annum for India (Table 1.4). It forms a major segment of village & small industries sector in terms of production and employment. It is very important in the national context on account of the employment that it provides in rural areas to the economically weaker sections of the population. The coir sector provides employment to over five lakh households in Kerala alone, the majority being women engaged in the spinning of fibre to yarn.

The fibre is consumed both in the domestic and international market as fibre, yarn, coir products, rope, curled coir and rubberized coir (Table 1.5). Other diversified applications include use of coir netting and matting as geotextile material to prevent soil erosion and

Table 1.4 - Export of coir products from India

ITEM	April 1998- February 1999	
	Quantity in tonnes	Value in lakhs Rupees
Coir Fibre	928	129.73
Coir Yarn	14547	4201.70
Coir Mats	19834	13231.37
Coir Matting	5694	4050.28
Coir Rugs & Carpets	2566	2002.20
Coir Rope	189	50.55
Rubberised Coir	395	305.75
Curled Coir	252	43.91
Coir Geotextiles	927	410.63
Coir Other Sorts	2030	952.04
Coir Pith	2152	264.90
TOTAL	49514	25643.06

COIR BOARD

Table 1.5- Products from Coir

ITEM	USE
Mats, Matting, Mourzouks,Carpets Rugs.	Floor Covering.
Geotextiles	Protection of road, rail & canal Embankments.
Drainage filter material for application as pipe envelopes.	To prevent flooding in grazing meadows, orchards, sport fields & gardens.
Ship Requisites	Fender Rope
Tent Components & Army Requisites	Salitah, Wall bag, Pole & Pin bag Camouflage net, dumping net.
Household Articles	House maids Kneeler, scrubber Shopping bags.
Speciality Articles	Cricket pitch matting, billiard surrounds, golf tee mat, wrestling mat, tablemat, tealeaf bags.

COIR BOARD

in roof surface cooling, as drainage filter material (Sarma, 1999). Needled coir felt also has been observed to possess properties for reinforcement function in cohesionless soils and as filter fabric in cohesive soils (Iyer and Girish, 1999).

Coir, having a greater aspect ratio (length: diameter), can be used successfully in filling or reinforcing thermoplastics with an improvement in flexural properties and toughness and reduction in the cost as compared to the pure thermoplastic. For aesthetic qualities, polypropylene composites with coir and sisal have been stated as the best.

1.2 Production of coir fibre

Coir fibre is classified into "white fibre" (Plate A) and "brown fibre" based on the extraction process. White fibre is obtained by the retting of coconut husks while the brown fibre is extracted by mechanical means.

PLATE A

1.2.1 Retting of husks

Retting is one of the conventional methods of coir extraction, which leads to the production of "white fibre". To obtain the white fibre traditionally by the "retting" process, 11 month mature green coconut husks are arranged neatly into lots ranging between 5000 to 10000 secured with coir nets in a large circular bundle and released into the saline backwaters. The bundles float freely, get drenched, become heavy and gradually sink. The bundles are left undisturbed for 6 to 10 months for completion of retting following which the husks are drawn out, beaten with wooden mallets to yield the "white fibre". This is the ideal fibre for the spinning of coir yarn and weaving into mats, matting and other floor coverings. In India, the abundant backwater facilities available on the southwest coast provide natural retting conditions. The colour of the fibre is not only influenced by the variety of the nut from which it is derived , its maturity, time lapse between dehusking and retting, but also on the nature of the retting process, environmental conditions and duration of retting. The physical appearance and quality of fibre varies widely with respect to colour, length and percentage of impurities. The best quality fibre is bright in natural colour (Grade II in Xenotest), possesses good staple

length (between 15-20 cms) and is comparatively free from pith and impurities.

1.2.2 Mechanical extraction of unretted brown fibre

"Brown fibre" is obtained by subjecting the husks through a power driven crushing, decorticating and combing system. Brown fibre can be classified as two types viz. the green husk fibre and the dry husk fibre.

Eleven-month mature green husks can be subjected to mechanical extraction to yield bright coloured fibre. The time required for the extraction of this fibre is only a few hours and the problem of pollution of backwaters can be eliminated. However the greatest disadvantage of the mechanically extracted fibre is, its inconsistent colour, harsh texture and poor photostability. Photodegradation is one of the disadvantages for production of good coir yarn and products from mechanically extracted fibre. Dry husk brown fibre is brown in

colour, coarse and mainly used for the manufacture of ropes, rubberized coir and in the upholstery industry.

1.3 Present scenario of the coir industry

"White fibre" production by the retting process is confined to Kerala on the south west coast of India, however, a large percentage of the fibre produced in other coconut growing states of India is "brown fibre". Retting of husks is disadvantageous on environmental considerations as the pH of the surrounding waters is lowered from neutral to the acidic range and the BOD levels increase with the progress of retting (Aziz & Nair, 1978), it also involves drudgery with laborious methods for steeping and drawing of retted husks. Retting is also not very economical as the investment towards the cost of husks is blocked for 11 months for retting, after which the fibre is available for use.

It is therefore important that, if the requirement of the coir fibre has to be met with, the environmental implications of "retting" have to be understood and alternate non-polluting technologies need to be developed to harness the husk potential

available in the country and satisfy the demand of the coir industry.

Coir Board has introduced "Coirret" a formulation of three bacterial cultures that can be applied on coconut husks steeped for retting in the backwaters to reduce the retting period to three months. It can also be treated on mechanically extracted green husk fibre to bestow it with the properties of "retted fibre" in 72 hours. "Coirret" is a patented product of Coir Board produced only at the Boards research Institute at Alappuzha in Kerala. Coirret has to be collected from the Pilot Scale Laboratory, CCRI @ Rs.38.00 per kilogram.

Table 1.6 - Statewise coir fibre production & husk utilization.

State	Fibre Production (Tonnes)	Utilization of husk (%)
Kerala	149800	38
Tamil Nadu	91300	23
Karnataka	28900	22
Andhra Pradesh	15000	16
Orissa	3000	15
Others	8000	10
Total	296000	21

COIR BOARD

Conventional monitoring methods for coconut husk retting are subjective, often more of an art than a technology. The lack of experience with coir retting can be a barrier for potential Indian coir producers and processors. Lack of a good retting measurement method even limits laboratory research

aimed at optimizing the retting process. The Flexural Rigdity Tester is an indigenous method developed for comparing the extent of softness imparted to a fibre by a treatment (Mukherjee, 1996).

Global requirement of fibrous materials.

The husk potential utilized in India at present is only 21% and the fibre production is 2,96,000 tonnes (Table 1.6). Most analysts forecast long-term increase in world demand for all types of fibrous materials, at the same time limitations in production capacity is also predicted. New fibre crops, new industrial uses of non-wood fibers, and agricultural diversification in conventional and novel markets, in general,

are therefore subjects of widespread interest for plant fibre . For annually harvested fibre production to be an attractive proposition, the three essential requirements are that the material be produced at a large scale, at a low enough price, with fibre characteristics being adequate for the end use in question. Equally important is that there should be a proven technology available for the processing of the new raw material (Bolton, 1995).The mechanically extracted coir fibre per se is not of the same quality as naturally retted fibre but can be made comparable by biological treatments.

1.4 Aim and scope

The problems associated with the natural retting process, elaborated under 1.3, has been a concern of the environmentalists. Besides, the low availability of the fibre as compared to the demand has been a point of focus of the coir industrialists. An attempt is therefore made in this work to reduce the period of retting of husks for coir extraction, increase the utilization of the husk potential in coconut growing

regions and tackle the environmental problems arising during retting.

An established fact known to cause the delay in the retting of coconut husks is the presence of the high percentage of polyphenols. (Varrier & Moudgil, 1947, Menon & Pandalai 1958, Jayasankar & Bhat 1966). Polyphenols from the coconut husks get constantly leached out into the surrounding steep liquors and such high percentage of polyphenols in the steep liquors appear to significantly influence the retting process, thereby resulting in a delay in extraction of the fibre (Jayasankar & Bhat, 1966). Retting is also a cause of environmental pollution (Aziz and Nair, 1978) as the pH of the environmental waters in a retting zone is lowered from neutral to the acidic range indicating the release of acidic substances and the BOD levels increase considerably leading to the deterioration in the quality of the backwaters which is detrimental to the aquatic life. Recommendations have been

made for adapting fibre pretreatments by improved retting and biobleaching (van Dam, 1999).

It is therefore imperative to develop ecofriendly methods for coir extraction from coconut husks. Alternative measures, like the development of Coirret, have limitations such as insufficient production capacity to meet the requirement of all coconut growing regions and its high cost. Therefore a process which could overcome these shortcomings would be useful for economic utilization of the husk potential in any coconut-growing region.

Bacteria are the most versatile organisms dissimilating an array of aromatic compounds with catechol as the key intermediate involved in the oxidative cleavage of the aromatic ring (Evans, 1974; Sleeper & Stanier, 1950; Simpson and Evans, 1953). Some important degradative bacteria that occur in water and soil environments belong to the following genera

viz. *Pseudomonas, Xanthomonas, Azotobacter, Rhizobium, Agrobacterium, Methylomonas, Methylococcus, Moraxella, Acinetobacter, Alcaligenes, Flavobacterium, Escherichia, Enterobacter, Serratia, Proteus, Aeromonas, and Bacillus* (Cork and Kroeger, 1991). Growth of specific types of microorganisms and their physiological activities are a response to the physicochemical environment. The steeping of coconut husks for retting leads to the establishment of such a unique ecosystem for proliferation of specific microorganisms degrading polyphenols.

The present studies have hence been carried out with a view to explore the possibility of developing a consortium that can survive and proliferate on the leachates from coconut husk which are rich in phenolic compounds. An advantage of such a consortium would be that it can be developed at any site where coconut husk retting needs to be carried out and then develop a method for better utilization of the husk potential in coconut

growing regions. It would lead to increasing the supply of raw material from India and establish coir industry, without high investments in states where natural facilities for retting do not exist. This would generate employment opportunities and increase the economy of the region.

1.5 Objectives of the project

The project undertaken entitled "Studies on coconut husk retting and bioinoculant treatment for process improvement in a natural system " was aimed to study the following objectives:

1. To study the process of retting in a natural system by monitoring the environmental parameters of the retting ecosystem and study the biochemical changes in the husk during the retting process.
2. To develop a consortium growing on husk leachates, isolate and characterize bacteria growing on phenolic compounds from the consortium.

3. To study the effect of seeding of consortium, a mixture of bacterial cultures growing on husk leachate on the retting process.
4. To develop a sustainable system for the coir extraction process using the consortium as bioinoculant.

Chapter II

Literature Survey

Man has been dependent on plant fibres in the form of structural and building materials, of semi-structural materials (packaging, furniture etc), of paper, of textiles and other materials (Table 2.1). The coconut palm, found by Magasthenes the Ambassador of Seleucus Nicator in Sri Lanka as far back as 300 B.C, is one of the most remarkable renewable resources. The early Arabs knew of coir, and the coconut palm has been a source of wealth for centuries (Mukherjee, 1997). The coir is obtained from the husk, which is anatomically, the mesocarp of the coconut fruit. The main tissue of the husk, the ground tissue has a cork like consistency and is white in its fresh living state, darkening to a brown colour as the fruit becomes older and the husk dries out. The ground tissue is transversed longitudinally by many vascular bundles which when extracted are the coir fibres (Heyn, 1951).

In India about 75 % of the coir produced in terms of value is consumed in the Indian domestic market itself, while the remaining 25 % of coir is exported. Countries in Europe and America together consume about 90 % of coir exports from India. Coir is marketed in different forms such as fibre, yarn, mat, matting, rug, carpet, geo-textile, curled coir and rubberized

Table 2.1- Commercially Important Fibre Sources

Material	Name of the plant	World Production (10^3 tonnes)	Source
Wood	(>10,000 sp.)	1,750,000	Stem
Bamboo	(>1,250 sp.)	10,000	Stem
Cotton Lint	Gossypium sp.	18,450	Fruit
Jute	Corchorus sp.	2,300	Stem
Kenaf	Hibiscus cannabinus	970	Stem
Flax	Linum usitatissimum	830	Stem
Sisal	Agave sisilana	378	Leaf
Roselle	Hibiscus sabdariffa	250	Stem
Hemp	Cannabis sativa	214	Stem
Coir	Cocos nucifera	100	Fruit
Ramie	Boehmeria nivea	100	Stem
Abaca	Musa textilis	70	Leaf
Sunn hemp	Crotolaria juncea	70	Stem

Source: Bolton (1994)

coir. The factors that contribute to the increase in export opportunities for coir are the rising cost of synthetic substitutes, a shift in tastes and preferences in favour of natural materials and a greater appreciation of the environmental implications. The 100% biodegradable nature of coir floor coverings has resulted in a steady increase in demand for them as compared with the synthetic materials which result in problems of recycling, fire / health hazards and recalcitrance. A recent development of interest to the coir industry is the growth of the market in Europe and America with consumers demanding materials for use to prevent soil erosion and promote revegetation (Coir Board, 1996).

2.1 Coir fibre

Coir fibre is lignocellulosic in nature; the ultimate fibre is polygonal in section with rounded ends. The cell wall is thin to fairly thick with lens shaped silicified stegmata; delicate reticulate thickenings, few spiral thickenings are also present. Individual fibre cells are 0.3-1.0 mm (average 0.7 mm) long and 0.10-0.024 mm (av.0.020) in diameter; the ratio of length to diameter is 35. The

lumen is medium to large, polygonal-rounded, rounded or elliptic. The vascular bundle is collateral and is surrounded by a thick sclerenchymatous sheath; the cross section is hemiconcentric. Lignins and hemicelluloses, which form the cementing materials of fibre cells, increase with the age of the fibre and pectins decrease. As the lignin content increases, the fibre becomes stiffer and tougher. The physical characteristics of coir fibre such as length, fineness, strength, elongation determine its utility. The fibre has a spiral angle (angle between the direction of fibrils in the cell wall and the fibre axis) of 45^0 and is sufficiently large to be measured by X Ray diagrams. x-ray photographs of fibres isolated from green and dried coconuts are similar and crystallites of cellulose in both types of fibres are oriented at the same fibre angle to the fibre axis. (CSIR, 1960). Coir has a high extensibility (about 37%) and high lignin content of 40% which distinguish it from other cellulosic fibres (Ray and Bandhopadhyay, 1936). The high extensibility of the coir fibre is chiefly because the microfibrils in the cell wall lie in perfect helical spirals, extension of the fibre being related with the changes of the spiral angle, that is, the

angle which a microfibril element makes with the fibre axis. Table 2.2 shows the percentage composition of coconut husk and coir fibre.

Coir is in great demand on account of its natural resilience, durability, resistance to dampness and ecofriendliness. To cater to the demand, coir fibre is presently being extracted from coconut husks by the natural retting process in the coastal backwaters. Many attempts have also been undertaken to obtain the fibre in laboratory conditions using alternate chemical methods in which inferior coloured fibre was obtained (Barker, 1933).

2.2. Natural retting of coconut husk

The natural coconut husk retting process has been studied by various scientists to understand the involvement of microorganisms and the biochemical changes occurring during the process. There are three different methods of retting of coconut husks. "Pit retting " wherein 20,000 to 30,000 husks are buried in basin shaped pits dug on the banks of backwaters. In "net retting", 10,000 husks are placed into nets and transferred to brackish waters

Table 2.2- Percentage composition of coconut husk and coir fibre (dry wt. basis)

Sample	Total water Solubles	Boiling water solubles	Hemi-celluloses	Lignin	Cellulose
Husk from old nuts	26.00	14.25	8.50	29.23	23.87
Husk from young nuts	29.00	14.85	8.15	31.64	19.25
Husk from Very young nuts	38.50	15.25	9.00	20.13	14.39
Fibre from old nuts	5.25	3.00	0.25	45.84	43.44
Fibre from young nuts	16.00	2 .75	0.15	40.52	32.86
Fibre from very young nuts	15.50	4 .00	0.25.	41.02	36.11

CSIR, (1960)

and subjected to tidal action. The retting period ranges from 6 to 9 months. "Stake retting" involves preparation of enclosures in shallow backwaters with stakes and coconut leaves, and putting the husks inside enclosures. Fresh husks treated within a few days after their separation from the nuts give better fibre than dry husks. Husks retted in saline backwater yield stronger and better coloured fibre than those retted in fresh water. Retting is quicker in fresh water but is generally incomplete and the fibre retains a certain amount of pith. When retting is done in stagnant water the fibre obtained is weak and of a dull colour. Retting is quicker in summer months, but the colour of the extracted fibre is brown. Husks soaked in shallow backwaters give better fibre than those retted in deep waters. "Net retting " provides better quality coir fibre than pit retting.

2.3 Microbiological studies

A study on the retting of flax by Winogradsky and Friebes in 1895 led to the isolation of a bacterium, establishing the fact that retting is a microbiological process (Bhat and Nambudiri, 1971). The

main feature of any retting process is the decomposition of pectins that form the chief constituent of the middle lamellas between individual cells. The middle lamellas between the fibre cells are much less easily affected; however, as these lamellas are lignified the technical fibre is not subject to disintegration, but is only liberated from the surrounding tissues. "Soaking" of coconut husks results in decomposition of pectins and hemicelluloses with involvement of bacteria in the decomposition process. The soaking process yields a pure fibre with very little impurities from the surrounding tissues, the retted fibre is lighter in colour and possesses properties like improved lustre and dyeability (Heyn, 1951). The microbial action on the husk leads to steady leaching of compounds such as phenols and pectins into the ret liquor. Bacteria belonging to the genera *Aerobacter, Bacillus, Escherichia, Micrococcus,* and *Pseudomonas* have been isolated from coir rets (Jayasankar, 1966). Fungi such as *Aspergillus, Penicillium, Fusarium, Trichoderma, Mucor* and *Diploidia* (Jayasankar et al., 1961) and yeast strains belonging to *Debaromyces hansenii* have been isolated from

coir rets and retting areas. The ability of the yeast strains to grow on catechol, phenol and some related compounds as sole source of carbon, may also play a role in the retting of the coconut husk (Jayasankar and Bhat, 1966).

The biological retting of coconut husks differs from that of other fibrous materials in that it is not confined to pectin decomposition alone but extends also to the disintegration of the phenolic cement binding the fibres together. The phenols from the husk are constantly leached out into the surrounding steep liquors during the course of retting. The binding matter in the husk which is a complex substrate undergo degradation at a varied rate depending upon the nature of microflora, physicochemical conditions and accessibility of the components to microbial attack. This leads to disproportionate variations in the components such as cellulose, lignin and pectins on retting (Bhat and Nambudiri, 1971). The physical properties of coir fibre treated with fungal cultures were comparable to mature fibre (Basak et al., 1983) and use of specific phenolytic

cultures have been postulated to expedite the retting process and yield fibre of better quality (Nazareth and Mavinkurve, 1987). Retting of coconut husk, under laboratory conditions, could be accomplished within 16 to 20 hours using Hiparol, a mixture of enzymes (Baruah and Baruah, 1944). Enzymes have been applied for retting of jute ribbons (Majumdar, et al., 1990). Field experiments conducted with selected strains of bacterial cultures could reduce the retting period for coconut husks and improve the quality of unretted coir fibre. (Ravindranath, 1991; Ravindranath and Sarma, 1995, 1998). Retting of coconut husk and treatment of unretted fibre can also be carried out in RCC tanks in a reduced span of time (Ravindranath and Bhosle, 1999).

2.4 **Biochemical studies**

Biochemical retting is a process , which depends on the property of the microorganisms causing the decomposition of the pectic and phenolic substances which hold together the fibre in the husk. Within the husks, the pectins exist as water insoluble

protopectins and the polyphenols are catechin like tannins (Jayasankar, 1966). Retting is partly chemical and partly microbiological, the latter action takes place quickly and efficiently only in brackish water and changing water exerts the most beneficial influence in retting (Pandalai et al. 1956). The analysis of coconut husk at different stages showed that the pectin content decreases to traces as retting progresses.

2.5 Environmental studies

The factors affecting retting have been stated to be (a) nature of the husk used, fresh husks give better fibre (b) nature of water, husks retted in saline water give good quality fibre (c) the season, retting is quicker in summer months but the colour of the fibre is brown, (d) means employed for keeping the husks under water, husks soaked in shallow backwaters give better fibre than those retted in deep water, net retting yields better fibre than pit retting (CSIR,1960). The effective organisms reside in the husk and the influx of cold water retards the retting process, indicating that

temperature may play an important part in retting (Fowler and Marsden, 1924). The potentials of retting gas as a new bioenergy source and methane production during retting of coconut husks need to be understood and exploited (Zachariah and Muralidharan, 1993).

Coconut husk retting involves microbial action and in any natural retting process microorganisms are the main agents of leaching. The content of phenols analysed in rets liquor, in a 10 day study, was observed to increase initially and decline after 6 days. This has been attributed to the steady leaching from the husks and a probable enrichment of phenolytic organisms which assimilate these compounds causing the fall of these compounds in ret liquor. Analysis of chemical constituents of husk before and after soaking revealed that the phenolic content decreased within 10 days. Phenols are known to inactivate enzymes and inhibit pectinase and since coconut husk has a high percentage of polyphenolic compounds in the form of lignin, tannins and monomeric phenolic units, they have been stated as potential inhibiting / inactivating agents (Nazareth, 1986). Study of laboratory rets revealed the presence of catechol, benzoic acid, gentisic acid, cinnamic acid and methoxy compounds in ret liquor.

2.6. **Environmental implications of retting.**

Retting of coconut husk is one of the principal sources of aquatic pollution in Kerala where the extensive backwaters that occur between the Arabian Sea and the midlands are sites of coir extraction (Aziz and Nair, 1978). Long years of retting have made the brackish water tracts a unique and complex biotype resulting in the complete failure of fisheries and extermination of the flora and fauna of certain areas. Hydrological studies reveal that the pH of the environmental waters in a retting zone lowers from neutral to the acidic range indicating the release of acidic substances. The BOD levels also increase considerably confirming the fact that retting of coconut husks in the backwaters leads to the deterioration in the quality of the backwaters which is detrimental to the aquatic life. The turbid medium containing tannins and suspended material prevents penetration of light and thus organic production is virtually inhibited. Except for a few tolerant species the polluted area supports no life except during the monsoon period when part of the accumulated byproducts of retting are flushed out of the area to some extent by the monsoon floods.

During this period the region becomes colonized by a variety of organisms including fish from the neighbouring area

The biochemical oxygen demand (BOD) is a vital tool in assessing organic pollution in aquatic biotopes. The concentration of dissolved oxygen in water can be taken as a significant index of its sanitary quality. Dissolved oxygen levels sag followed by its total depletion and this was an important feature of the water in retting zones. This anoxic condition is attributed to the oxidation of the organic matter by bacteria resulting in the utilization of dissolved oxygen (Nandan and Aziz , 1990).

2.7 Alternate methods of retting for extraction of fibre

Considering the extent of impact on the environment, it has been proposed that alternative methods should be derived which should be ecofriendly and cater to the demand of the coir industry. It is possible to

achieve this if a technology is developed based on the use of specific organisms under closed conditions. Such studies on retting of coconut husk have been carried out by inoculating strains of phenol degrading cultures in laboratory conditions which resulted in reducing the period of retting considerably (Ravindranath,1991) and also improving the quality of mechanically extracted coir fibre (Ravindranath and Sarma, 1998). The International Jute Organisation has also recommended the use of special microbial cultures for retting and extraction of jute in a reduced period of time with environment friendly technology. (IJO, 1998). The yield from enzyme retted flax at a semi industrial scale was higher and commercial enzyme preparations have been experimented for retting of jute. (Majumdar et al.1990)

2.8 Techniques for evaluating quality of coir

The physical and chemical properties of the coir viz. fibre length, fineness, strength, extension at break, rigidity were the criteria assessed to determine the quality of coir. Four grades (4F, 3F, 2F, F) were assigned to retted fibre in conformity with the boxed colour standards of

the Indian Standards Institute. The grade 4F represented the best quality fibre having bright natural colour and good staple length, free from pith and impurities (Prabhu, 1959). The Indian Standards Institute specification (IS:898-1985) for retted coir fibre is also classified into four grades based on the colour and maximum permissible impurities as follows :

GRADE	COLOUR	Maximum Impurities (% by mass)
1.	Natural Bright	2.0
2.	Natural light brown and/or light grey	3.0
3.	Natural brown and/ or grey	5.0
4.	Natural dark brown and / or Dark grey	7.0

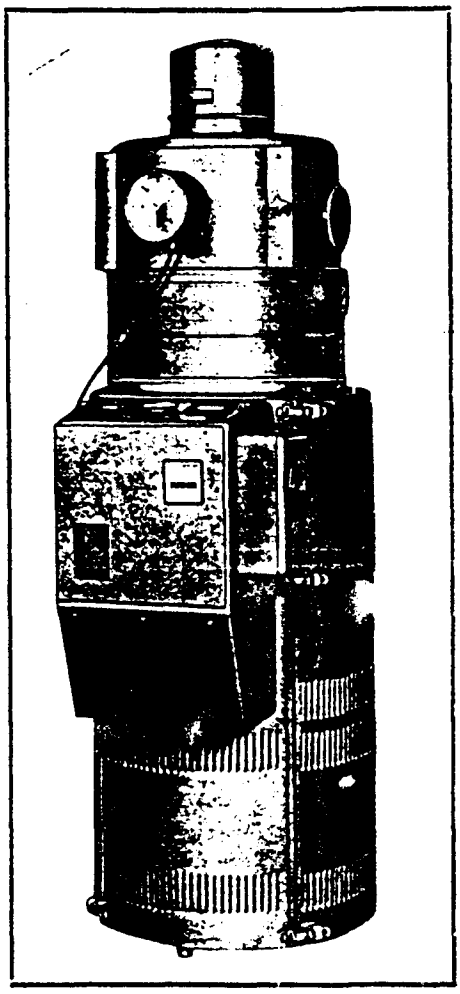
Fibre strength contributes an important part in yarns and products-matting/carpet manufactured from coir. Tensile strength of a fibre is its fundamental ability to resist strain or rupture induced by tension and is a

determining factor in the selection of a fibre for any given purpose. It is expressed as the force per fibre weight per unit length which is termed as tenacity of the fibre and is expressed in gms/denier. The average tenacity of 4F grade retted fibre is 1.3286 gms/denier (Prabhu, 1959).

The quality of coir fibre can also be determined by assessment of lightfastness ratings using the Xenotest (Ravindranath and Sarma, 1993 , Plate B). This is a uniform specification for rating the lightfastness and weatherfastness of materials more quickly than naturally. It has a 1500-watt Xenon arc lamp as a source of radiation; the filtered spectrum of this lamp when used in the Xenotest is the same as sunlight. The samples were subjected to alternate periods of light and dark. This mimics conditions of day and night approximately. A test time of 24 hours in the Xenotest was roughly equivalent to the radiation received over 10 days in the open air averaged throughout the year. Samples fading within 80 min of test exposure were rated as Grade I and those after 80 min as Grade II. The retted fibre exhibits a rating of Grade II whereas untreated mechanically extracted fibre shows a rating of Grade I.

X·ENOTEST® 150

System Cassella ORIGINAL HANAU



Light and

Weather Fastness Tester

with adjustable humidifying and weathering units

PLATE B

The degree of softness imparted to coir by a treatment can be measured by the Flexural Rigidity Tester. This is a device developed by the loop methods to determine the flexural rigidity of the fibre (Mukherjee, 1996). In this method, a ring of radius 'r ' formed by a fibre is allowed to deform with the help of a fixed weight as shown in Fig.2.1.The amount of deformation with a fixed weight depends upon the softness of the fibre.

Flexural rigidity $G_f = 0.0047\ mg\ (2\pi r)^2 \cos\theta/\tan\theta$

where mg = weight of load applied

$\theta = 493/2\pi r$

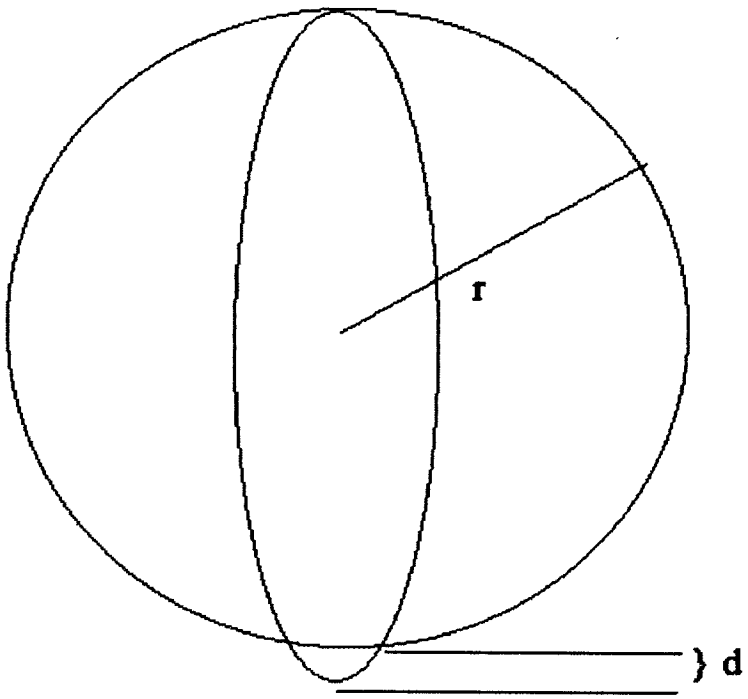
d = deformation of lower end of ring

Fifty samples of the retted fibre were tied up on a PVC rod to attain the form of a ring having a radius of 2.3 cms and allowed to remain for 24 hours. The rings were then tested on a Flexural Rigidity Tester with and without load and the Flexural rigidity G_f calculated.

2.9 Salient features of coconut husk retting.

1. Retting of coconut husk is carried out in natural waters .

Fig.2.1- Flexural Rigidity of coir fibre



r-radius of the coir ring

d- deformation in the lower end of the ring

2. Natural retting process yields superior fibre which is the best in quality.
3. Phenolic compounds are released from coconut husk during retting.
4. Retting of coconut husk involves microbial biodegradation.
5. The environmental implications of retting are alarming and alternate methods are to be developed.
6. Selective strains of phenolytic bacteria could improve the retting process.

2.10 Biodegradation of aromatic compounds

Biodegradation is the catabolism of a compound into molecules that can enter intermediary or central metabolism. Microorganisms as a group display an amazing versatility for recycling elements in nature. The autotrophs may utilize simple inorganic substrates and synthesize complex biomolecules whereas the heterotrophs have the capacity to degrade complex molecules like cellulose , lignin and pectins to simpler compounds for the purpose of utilization.

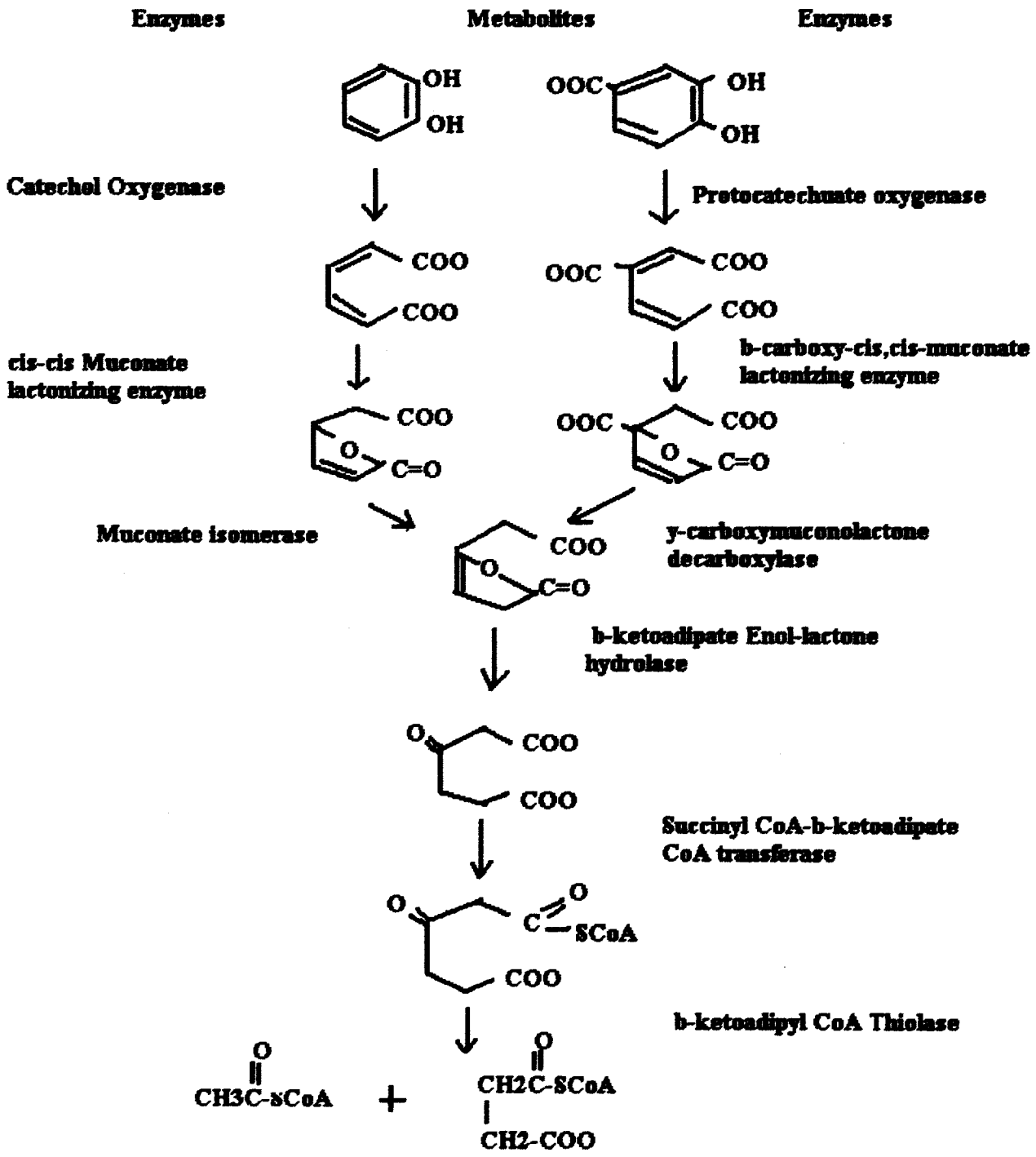


Fig.2.2-Catechol and protocatechuate pathway in P.putida

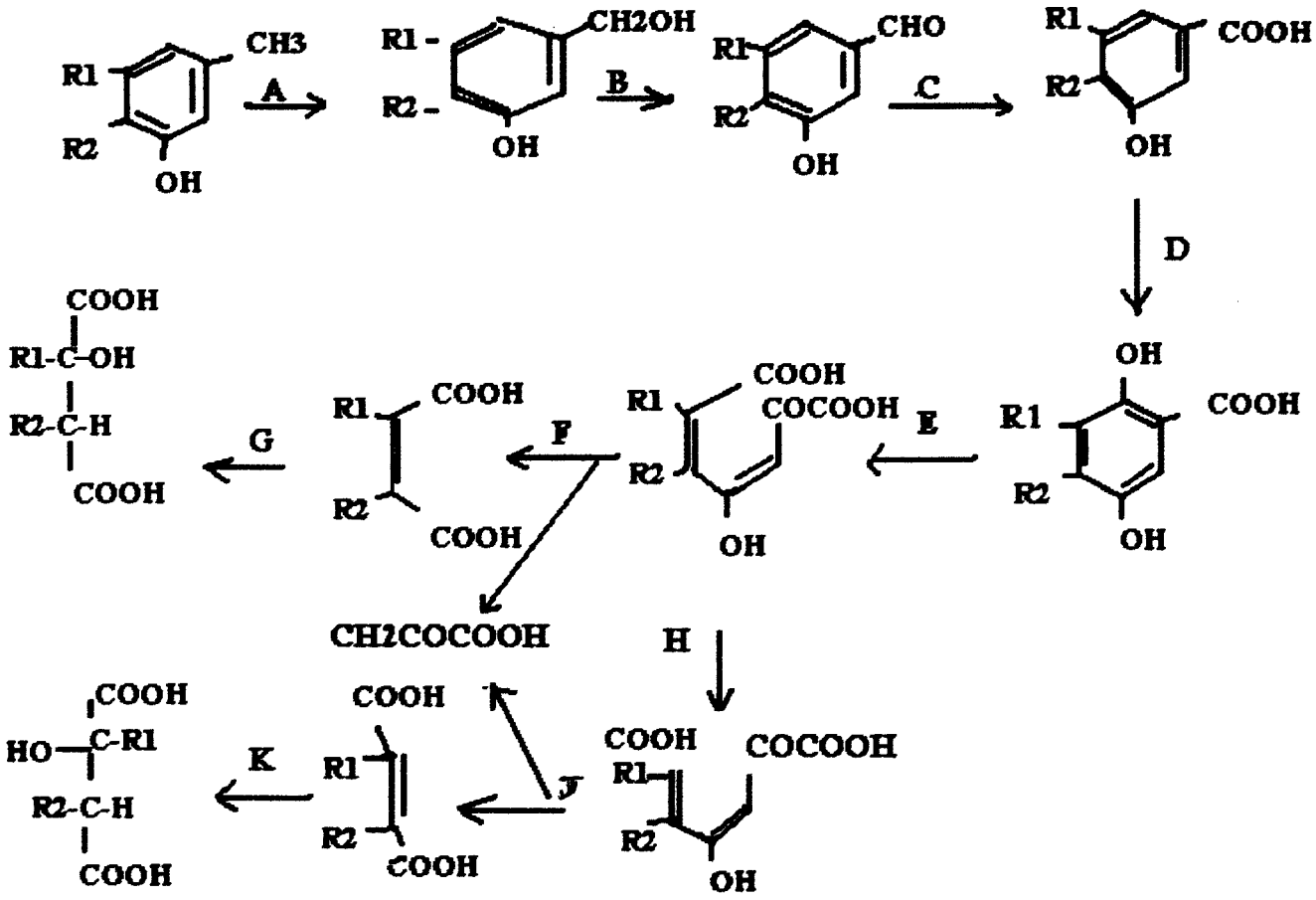


Fig.2.3- The gentisate pathway . A, methyl hydroxylase; B, alcohol dehydrogenase; C, aldehyde dehydrogenase; D, 6-mono-oxygenase; E ,gentisate 1,2-dioxygenase; F, maleylpyruvate hydrolase; G,maleate hydratase; H, isomerase; J , fumaryl pyruvate hydrolase; K, fumarase.

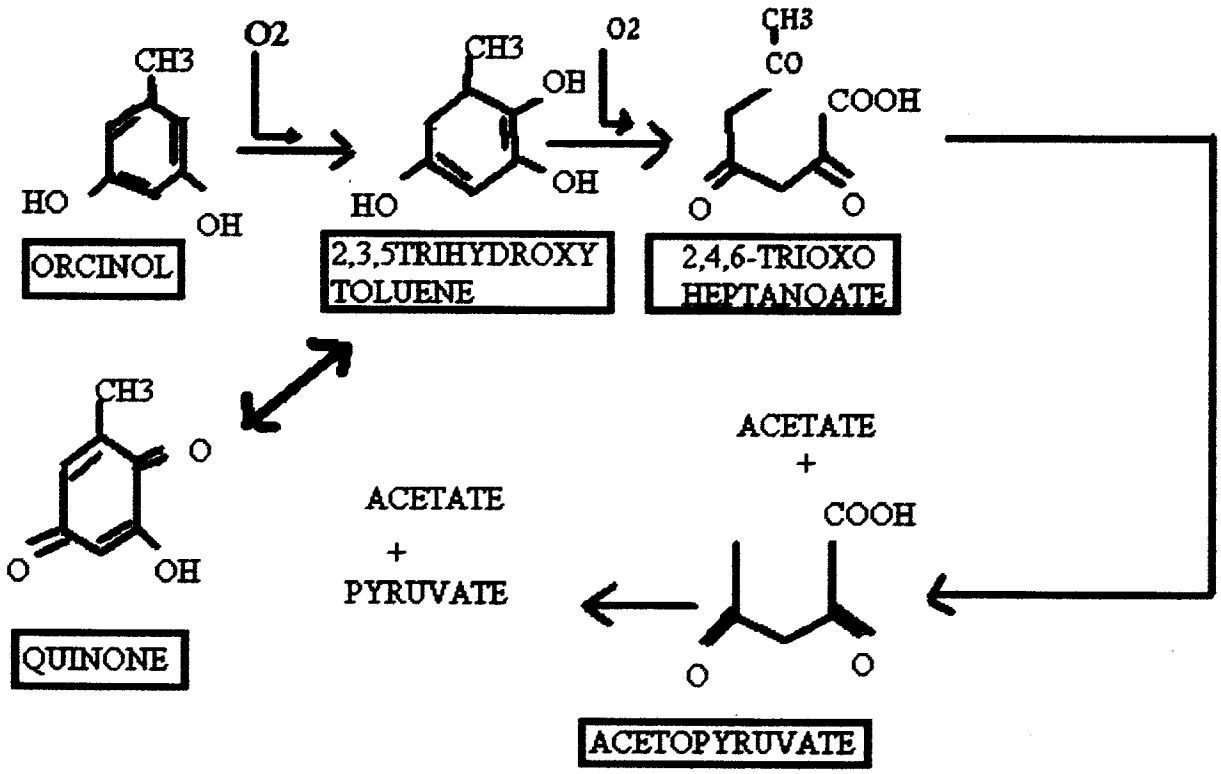
Aromatic compounds can be totally or partially degraded by microorganisms depending on the number of rings and the type of substituent groups present. Biodegradation by a microorganism is initiated with the entry of the organic compound into the cell followed by modification of substituents before ring cleavage. Most of the microorganisms convert benzenoid compounds into catechol, protocatechuate or gentisic acid which are the central metabolites in biodegradation (Fig. 2.2 and 2.3). Mono- and di-oxygenases initiate biodegradation by initial oxidative attack on biochemically inert organic compounds in bacteria. Monooxygenases are involved in hydroxylation of organic molecules using reducing power of NADH or NADPH, as seen in the equation below

$$R\text{-}H + NAD(P)H + H^{+} + H_2O = R\text{-}OH + NAD(P)^{+} + H_2O$$

All monooxygenases are specific for their aromatic substrates. Diooxygenases are responsible for the fixation of oxygen directly into organic compounds. Dioxygenases bring about cleavage of benzene rings by inserting both atoms of molecular oxygen, provided that two hydroxyl groups are placed ortho or para to each other. Thus all aromatic compounds are transformed into ortho or para

dihydroxybenzenes before the action of dioxygenases. In some cases , as in the case of resorcinol, a third hydroxyl group must be introduced before ring cleavage can occur. The formation of hydroxyquinol is followed by ring cleavage to maleylacetate and metabolism via the β-ketoadipate pathway (Chapman and Ribbons, 1976). The biodegradation of resorcinol has been reported in *Pseudomonas putida, Azotobacter vinelandi and Trichosporon cutaneum.* The catabolism of resorcinol in one strain of *Pseudomonas ORC* is via hydroxyquinol and ortho cleavage to give maleyl acetate. Another mutant O1OC was shown to be constitutive for the orcinol pathway (Fig. 2.4). After growth of this strain on resorcinol, two enzymes , hydroxyquinol 1,2,-dioxygenase and maleyl reductase of the resorcinol pathway are induced. Thus hydroxyquinol , formed from resorcinol undergoes both ortho and meta cleavage with formation of both pyruvate and maleyl acetate. The evidence for a third pathway for resorcinol catabolism was seen in *Azotobacter vinelandi* where resorcinol was converted to pyrogallol by resorcinol grown cells. Pyrogallol was the substrate for one of two ring cleavage enzymes induced by growth with resorcinol. Oxalocrotonate, CO_2,

Fig.2.4- Pathway proposed for orcinol catabolism by P.putida O1 & ORC



pyruvate and acetaldehyde have been identified as products of pyrogallol oxidation catalyzed by resorcinol grown cells. The presence of the enzymes pyrogallol 1,2-dioxygenase, oxalocrotonate tautomerase (isomerase), oxalocrotonate decarboxylase and vinylpyruvate hydratase were present in resorcinol grown cells Catechol occurs as an intermediate in the metabolism of both benzoate and phenol by strains of *Pseudomonas putida* and the major strains follow the ortho cleavage pathway (Fiest and Hegeman, 1969). The transformation of various organic compounds are brought about by the microorganisms which results in the formation of products which are amenable for further degradation. The phenomenon where a microorganism may transform a given organic compound to its related alternate products without utilizing it as a carbon or energy source is termed cometabolism and when the reaction is oxidative , it is termed as co-oxidation (Mahtani, 1981).

Cometabolism, cooxidation and fortuitous oxidation are therefore terms used to describe the oxidation and degradation of non growth substrates by microbes. Cometabolism brings about the biological transformation of a compound in the presence of another substrate which acts as a carbon source fortuitous metabolic events, that result from non specific monooxygenase activity. It also seems possible that in some cases of cometabolism, energy derived from the oxidation of the non growth substrate can be utilized to fix carbon from the growth substrate. The capacity for cometabolism and fortuitous activity seems to occur most frequently in hydrocarbon utilizing bacteria (Internet).

In natural environments such cometabolic activities of a group of microorganisms results in the biodegradation of polluting substances, and the specific mixed cultures are enriched in these polluting environment. In the laboratory, such a population can be enriched on target compounds as the only substrate (sole source of carbon and energy) with multiple transfers, permits the population

capable of utilizing the compound to proliferate and metabolize. With each successive transfer the "utilizers" begin to dominate the population and comprise of a mixed culture capable of mineralizing the target compound. Such mixed cultures are also termed as "consortia". After a number of transfers the organisms can be isolated on solid medium and tested for the ability to degrade the target compound (Pelczar and Reid, 1965). The recent attention to bioremediation as a means of alleviating environmental pollution has resulted in a variety of investigations involving the biodegradation of heterogenous mixtures of organic compounds. Since few bacterial strains have the genetic diversity of a heterogenous population, studies involving degradation of mixtures of compounds have involved mixed bacterial cultures that may be undefined, partially defined, or defined (Haigler,et al.,1992). The biodegradation of phenols, benzene, toluene and xylene by mixed cultures, through cometabolic activity have been reported. (Alvarez and Vogel, 1991; Horvath,1972; Schmidt,1987)

In methanogenic environments, complex organic matter (including aromatic compounds) could be converted into a mixture of methane and carbon dioxide by a complex network of various metabolic groups of bacteria. Bacteria in these consortia depend entirely on each other to perform the metabolic conversions and are therefore referred to as syntrophic consortia. In these syntrophic consortia, fermentative bacteria convert complex organic matter into a mixture of acetate and hydrogen or formate , which are substrates for methanogenic bacteria (Kleerebezem et al., 1999).

The adaptation of a mixed aquatic microbial community to phenol was examined in microcosms receiving phenol as sole carbon source . Extended exposure (adaptation) to phenol resulted in adaptation of the microbial community to the structurally related aromatic compounds m-cresol, m-aminophenols and p-chlorophenol. The increased biodegradation potential of the phenol adapted microbial community was accompanied by concurrent increase in the number of organisms able to degrade the three test compounds. Thus adaptation to the three test chemicals was likely as a result of

extended exposure to phenol. Therefore adaptation to a single chemical may increase the assimilative capacity of an aquatic environment for related chemicals even in the absence of adaptation inducing levels of those materials (Shimp and Pfaender, 1987).

A phenol degrading consortia was enriched from anaerobic digester sludge obtained from a municipal plant. The enrichment was maintained for two years with bimonthly transfers of 25 % inocula to fresh revised anaerobic mineral medium. The enrichment was grown at 37 ^{0}C under stationary conditions, and the consortium was immobilized in agar to maintain the syntrophic activity necessary for phenol biodegradation to methane and carbon dioxide (Dwyver et al. 1986).

An anaerobic consortium degrading phenol and p-cresol was isolated and assayed to control and reduce the malodorous compounds in swine waste (Beaudet, et al.,1986). The acclimation culture was carried out with vegetable sediment in anaerobic conditions, activated

sewage sludge, swine waste soil and phenol. The enriched culture was adapted to grow on swine waste by periodic transfer (20 % v/v) in mineral salts medium containing phenol and increasing the concentration of the waste.. The results showed that the degradation of phenolic compounds by bacteria in anaerobic conditions in swine waste is possible (Beaudet, et al.,1986). An anaerobic methanogenic consortium could carboxylate phenol to benzoate (Bisaillon et al., 1991).The carboxylation was carried out by cometabolism in the presence of proteose peptone. The consortium comprised of seven different morphological types of bacterial cultures, one culture was tentatively identified as *Methanotrix* species.

A consortium comprising predominantly of Gram negative rods was also found to degrade phenol under methanogenic conditions. The carboxylation was observed to be accomplished by cometabolism and some degradation products were observed to serve as carbon sources for the growth of the carboxylating bacteria which appear to be present in large numbers in the consortium (Bechard et al., 1990). A

consortium , which in addition to phenol could carboxylate and dehydroxylate ortho-substituted phenolic compounds (Bisaillon et al., 1993). A pathway of benzoate degradation by adapted bacterial consortia has also been postulated (Evans and Fuchs, 1998).

Bacteria co-metabolically degrade a variety of polychlorinated biphenyl (PCB) congeners to the corresponding chlorobenzoic acids through 2,3-dioxygenation. Certain strains degrade even highly chlorinated PCBs through 3,4-dioxygenation. (Furukawa and Kimura, 1995). Studies have identified two distinct biological processes capable of biotransforming polychlorinated biphenyls (PCBs) aerobic oxidative processes and anaerobic reductive processes. It is now known that these two complementary activities are occurring naturally in the environment (Abramowicz, 1995).

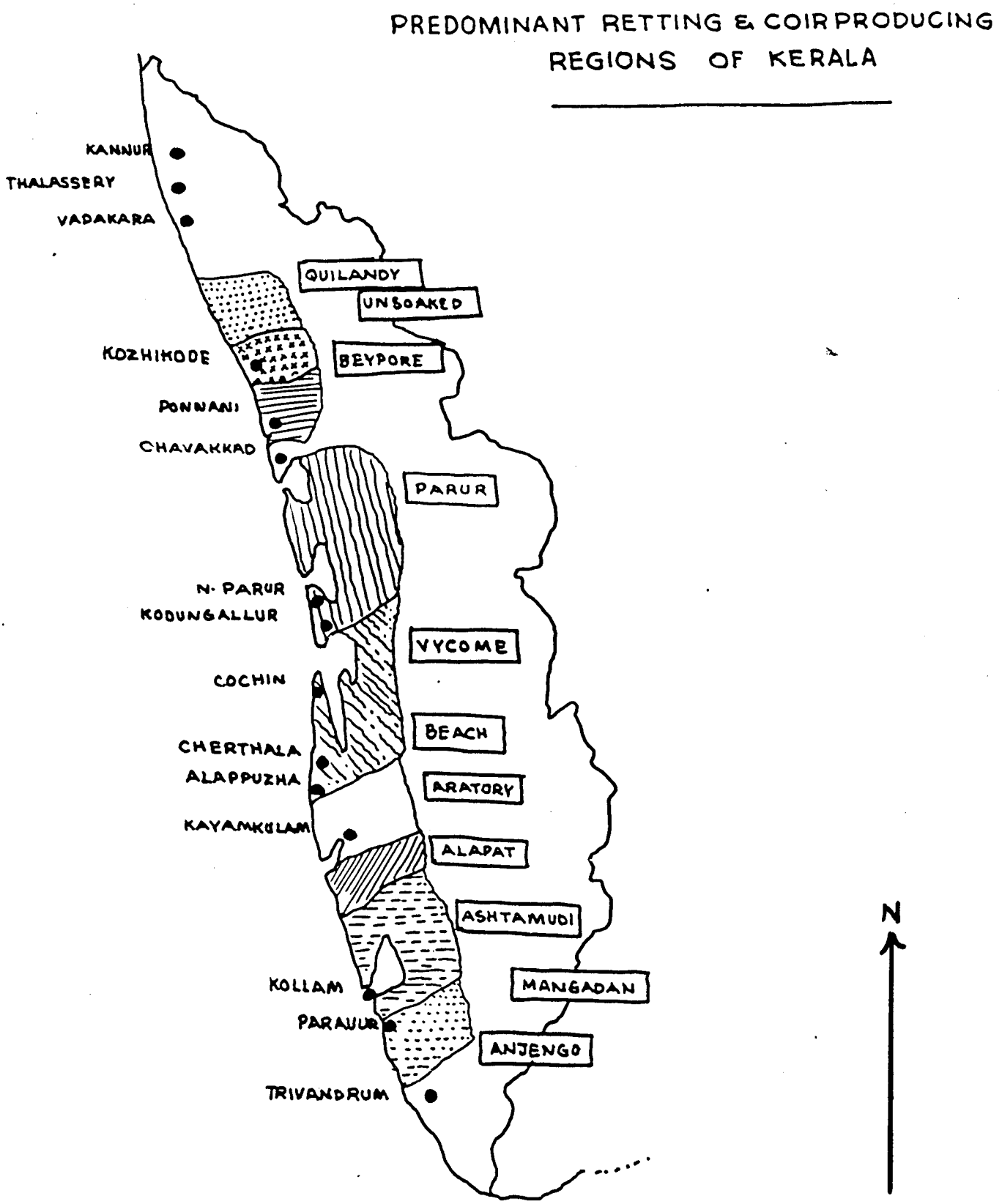
A resorcinol and catechol degrading consortia used the substrates over repeated feedings and the biodegradation was coupled to denitrification (Milligan and Haeggblom, 1998).

Chapter III

Study of conventional retting in a natural system

The retting of coconut husks for the extraction of coir is mainly carried out in Kerala on the southwest coast of India. The major coir retting regions of Kerala have been differentiated on the basis of the quality of coir yarn produced (Fig 3.1). The site selected for this study was at Varanad about 35 kms from Alleppey, which is the main centre for coir. This is a typical retting site producing the Vycom variety of coir from coconut husks steeped for 9 months of retting and has access to the tidal influence of of the Arabian Sea. During the process of retting, the husk is influenced by the pH, temperature and salinity that may have an impact on the quality of the coir fibre and the chemical composition of the husk. During the retting process a number of polyphenols are released which could affect the pH, temperature and salinity of the water around the husk lot. The constituents in the husk would also vary with the leaching out of the polyphenolic compounds and the physical nature of the husk would undergo changes as the retting progresses. The present work was therefore undertaken to study the process of retting under natural conditions and monitoring the variations in the environmental parameters, chemical constituents and the softening of the husk. The evaluation was carried out during a period of nine months.

FIG. 3·1


PREDOMINANT RETTING & COIR PRODUCING REGIONS OF KERALA
KANNUR
THALASSERY
VADAKARA
QUILANDY
UNSOAKED
KOZHIKODE
BEYPORE
PONNANI
CHAVAKKAD
PARUR
N. PARUR
KODUNGALLUR
VYCOME
COCHIN
BEACH
CHERTHALA
ALAPPUZHA
ARATORY
KAYAMKULAM
ALAPAT
ASHTAMUDI
KOLLAM
MANGADAN
PARAVUR
ANJENGO
TRIVANDRUM
N

3.1. Materials and method

3.1.1 Setting up of husks for retting.

A total of 10,000 mature husks were bundled into large nets of coir rope and set to soak for retting anchored and weighted with stones (Plates C and D) in the conventional manner at the selected site (Varanad about 35 kms from Alleppey, Kerala) during the month of May, a premonsoon season.

Samples of husk and water were collected from the site before steeping of the husks and at periodic intervals of thirty days thereafter. The water samples were stored in sterile bottles and transported to the laboratory for determination of the bacterial count on nutrient agar and mineral salts medium with 0.05% resorcinol. Samples of husk were also subjected simultaneously to analyses of the polyphenol and pectin content. The conventional monitoring methods for coconut husk retting involve physical examination (by touch and feel method) of the husk for its ease in removal of the exocarp and softness of the husk, which form the main criteria for evaluating the progress in retting. Samples were drawn out from different points in the lot of steeped husks and assessed for the progress in retting. The study was

PLATE C

PLATE D

continued for nine months at the end of which the husks were drawn out and the fibre extracted.

3.1.2 Analysis of water samples in the natural retting system.

(a) pH

A portable pH meter (Eutech Cybernetics range -1.0 to15.0 , resolution 0.1, accuracy +/-0.1 operating temp 0 to 50 ^{0}C) calibrated with buffer of pH 7 and pH 4 was used for analysis. pH was monitored at four points around the retting pit and the average recorded.

(b) Temperature

Temperature was recorded using a laboratory thermometer at four points around the retting pit and the average recorded.

(c) Salinity

Water samples collected from four points around the pit were pooled and salinity estimated by Mohr Knudsen method as described by Strickland and Parsons (1972).

(d) Extraction and analysis of phenolic compounds

The water samples were extracted thrice in diethyl ether in a separating funnel and the extracts pooled, concentrated and analyzed by TLC.

Thin layer chromatography (TLC): Glass plates (10 X 20 cms) were fitted with adhesive tape applied to upper and lower edges of the plates to provide the required thickness of silica gel layer. Silica gel, made into a slurry with distilled water was then spread evenly on to the plates by means of a glass rod / spreader. The plates were allowed to dry and then activated at 110 degrees for 20 minutes. Chromatography chambers were lined with filter paper soaked with the solvent system to facilitate saturation of the chamber with the solvent vapours. Solvent systems benzene: ethyl acetate: acetic acid (85:15:1, v/v/v) and benzene: dioxan: acetic acid (90:25:4, v/v/v) were tried initially and since the separation was observed to be better in the former solvent system the same was used routinely. Concentrated ether extracts were spotted by means of capillaries, on to activated silica gel plates and developed in the solvent system. Developed chromatograms were visualized by iodine vapours and by freshly prepared phenol specific spray reagent tetrazotized benzidine.

(e) Total bacterial count

The water sample was serially diluted in normal saline from 10^1 to 10^9 and plated on nutrient agar and mineral salts medium with 0.05% resorcinol. The plates were incubated at room temperature under stationary conditions for 48 h. The number of colonies and their cultural characteristics were observed.

3.1.3 Analysis of husk samples for polyphenol and pectin content.

Husk, cut into small size was soaked thrice in 40% acetone (aqueous) in a ratio of 1:2 for extraction of phenols. The extraction was continued till the final wash was colourless and the entire extraction process was completed in 24 hours. Extract was concentrated under vacuum using a flash evaporator to reduce the volume to one third. The polyphenols were precipitated from the concentrated extract by boiling a mixture of the extract, concentrated hydrochloric acid, 40% formaldehyde (aqueous) and water in a ratio of 1:1:1.5:1 for one hour. The precipitate of phenols obtained was collected on G-3 sintered crucible, washed and dried to constant weight. The residual husk after extraction of phenols was rinsed with water, then boiled twice for 2 hours with 0.5% ammonium oxalate

solution in a ratio of 1:2. The extract was concentrated as above and pectin precipitated by addition of 3-4 volumes of distilled 95% ethanol and kept in cold for complete precipitation. The cold solution was filtered under vacuum through G-3 scintered crucible and the precipitate was washed with cold ethanol and dried to constant weight.

3.1.4 Assessment of fibre quality

The husks were drawn out after nine months of retting and the fibre extracted by beating with wooden mallets, and air-dried. Five sets each of fifty rings of the retted fibre were tied up on a PVC rod to attain the form of a ring having a radius of 2.3 cms and allowed to remain at room temperature for 24 hours. The rings were then tested on a Flexural Rigidity Tester with and without load and the deformation in loading determined. Flexural Rigidity (gcms) was calculated using the following formula:

$$\text{Flexural Rigidity} = 0.0047\ mg\ (2\pi r)^2 \cos\theta / \tan\theta$$

where mg = weight of load applied

r = radius of the ring

d = deformation of lower end of ring

$\theta = 493d / 2\pi r$.

The quality of coir fibre was also determined by assessment of lightfastness ratings using the Xenotest which is a uniform specification for rating the lightfastness and weatherfastness of materials more quickly than naturally. It has a 1500 W Xenon arc lamp as a source of radiation; the filtered spectrum of this lamp when used in the Xenotest is the same as sunlight. The samples were subjected to alternate periods of light and dark. This mimics conditions of day and night approximately. A test time of 24 h in the Xenotest was roughly equivalent to the radiation received over 10 days in the open air averaged throughout the year. Samples fading within 80 min of test exposure were rated as Grade I and those after 80 min as Grade II.

3.1.5 Estimation of lignin in coir fibre from different retting regions

The Klason lignin in the fibre was determined by the following method (Klason, 1908). Approximately one gram of finely cut fibre sample from each of the ten retting regions was weighed out in triplicate in weighing bottles (previously tared) and dried at 105 ^{0}C for one hour. The material was weighed thrice till a constant weight (A) was obtained. The material was then carefully transferred to a clean filter paper and rolled to pack the contents. The three packed samples were transferred into the reflux unit of the soxhlet extraction apparatus for the first extraction with ethanol: benzene (2:1v/v) for 4 h. The samples were then allowed to dry and the contents transferred to a 400-mL beaker and refluxed in 200 mL distilled water for 4 h. The cooled samples were then transferred into a 100-mL beaker to which was carefully added 3 mL of 72 % sulphuric acid with the help of a glass rod. Another 22 mL of 72 % H_2SO_4 was added to make the total volume to 25 mL. The sample was then carefully macerated to form a fine paste and kept covered at room temperature for 2 h. The sample was then diluted with distilled water (575 mL) in a one-litre beaker and heated on a water bath for 4 h. After cooling the sample was filtered carefully through a clean tared G-4 sintered Gooch crucible.

The residue was washed till free from acid, dried at 105 degrees and weighed till constant reading obtained (B). The net weight recorded and the % lignin calculated as per the formula

$$\% \text{ Lignin} = B/A \times 100$$

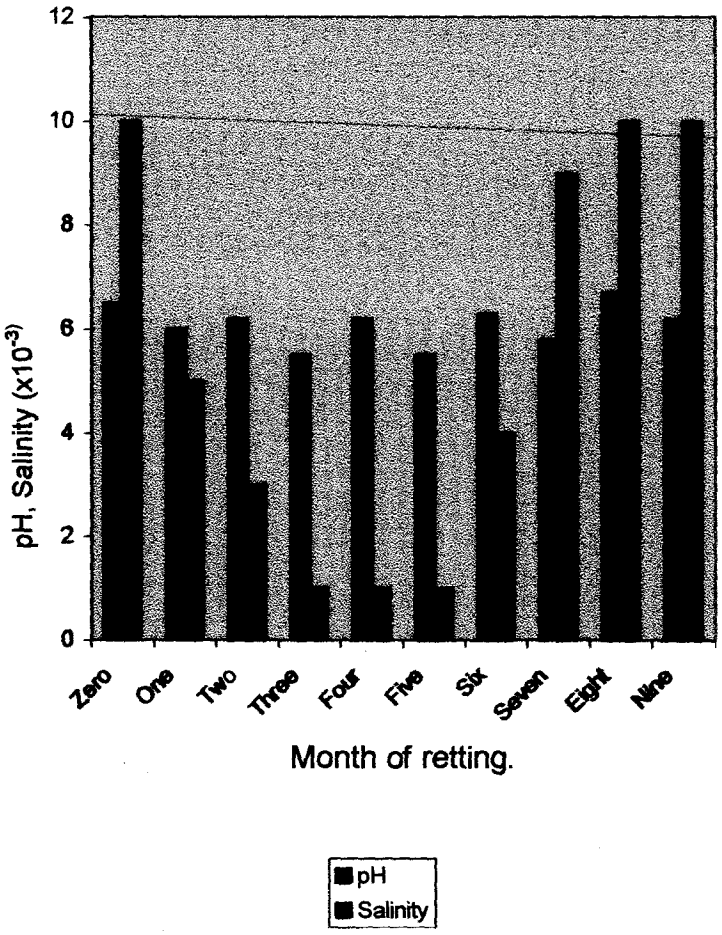
3.2 Results

The water samples collected were analyzed for the pH, temperature and salinity and total bacterial count besides, the phenolic compounds leached out into the water were also extracted and identified in the samples. The mean pH values in the water samples drawn at different stages of retting exhibited a variation (Table 3.1). The maximum pH 6.5 and 6.7 was noted before steeping and in the eighth month of retting. However, the minimum pH of 5.5 was recorded in the sample drawn in the third and the fifth month of retting. (Fig.3.2). The water temperature during the period of study from April to January corresponded to the atmospheric temperature ranging between 24 to 35^0C (Table 3.1).It was interesting to note that salinity varied widely during the period of study, the highest was at 10.0 ($x10^{-3}$) in the month before steeping of husks (May), eighth and

Table 3.1- pH, temperature and salinity in natural retting.

Month of Retting	pH	Temperature ^0C	Salinity ($x10^{-3}$)
0 (May)	6.5	31	10
1 (June)	6.0	24	5
2 (July)	6.2	25	3
3 (August)	5.5	26	1
4 (September)	6.2	25	1
5 (October)	5.5	34	1
6 (November)	6.3	28	4
7 (December)	5.8	27	9
8 (January)	6.7	30	10
9 (February)	6.2	34	10

Fig 3.2- pH & salinity variation in natural retting.



ninth (January and February). A significant decrease in the salinity to 1.0 ($x10^{-3}$) was observed during the third, fourth and fifth months corresponding to August, September and October (Fig.3.2).

Ether extracts of the ten samples drawn at monthly intervals were analyzed by TLC for detection / identification of phenolic compounds leached out. The TLC pattern (Table 3.2) showed the presence of three compounds in the first month , two compounds in the second and seventh month and one each in the third, fourth , fifth and sixth months. No spots were however detected in the zero, eighth and ninth month samples. Interestingly, the spot with the Rf 0.4 remained consistently in the ret liquor for seven months. On comparison with standard phenolic compounds, the Rf values obtained during co-TLC it was confirmed that the spot at 0.4 was corresponding to resorcinol while the other spots were identified as pyrogallic acid, protocatechuic acid (Rf 0.31, 0.95) (Fig.3.3).

Microscopic examination of the ret liquor showed the presence of bacteria and protozoa. It was interesting to note that before steeping

Table 3.2 - Rf Value of spots on TLC of water samples in natural retting.

Month of retting	No. of Spots Detected	Rf Value
Before Steeping (Zero)	-	-
One	3	0.31, 0.40, 0.96
Two	2	0.31, 0.40
Three	1	0.41
Four	1	0.42
Five	1	0.40
Six	1	0.40
Seven	2	0.40, 0.95
Eight	–	–
Nine	–	–

Solvent System: Benzene: Ethyl Acetate: Acetic Acid (85:15:1)

Visualizing agent: - Iodine Vapours, Tetrazotised Benzidine

Rf value of Standard phenolic compounds

Catechol-0.51, Pyrogallic acid-0.31, Resorcinol-0.40 ,Syringic acid -0.53

Tannic acid-0.45, Phenol 0.68, Protocatechuic acid- 0.95

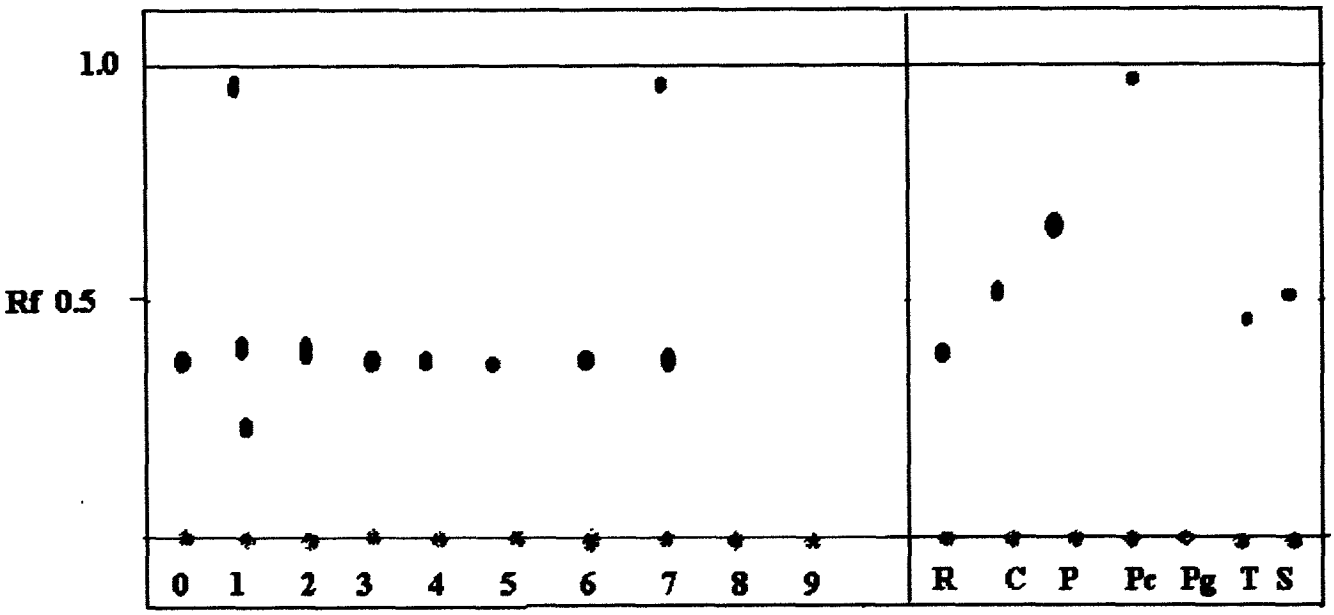


Fig.3.3 TLC of ether extracts of ret liquor samples from natural retting. *0-9* indicate sample drawn before steeping of husks and at monthly intervals for nine months. R, C, P, Pc, Pg, T, S denote authentic standards resorcinol, catechol, phenol, protocatechuic acid, pyrogallic acid, tannic acid and syringic acid. Solvent system *Benzene* : ethyl acetate : acetic acid. Visualising agent- Iodine vapours.

the count on nutrient agar was 148 x 10^4 which showed a sharp decline after three months to 15 x 10^4 increasing rapidly in the third month to a value of 2000 x 10^4 (Table 3.3). The count further remained consistent ranging from 125 to 180 x 10 ^{4}. Significantly a gradual increase in values was also observed on resorcinol medium from 470 to 1660 cells/mL within a period of three months increasing in the fifth month to 2980 cells/mL.

The ratio of total bacterial count to the count on resorcinol medium consistently remained at around 4:1, the difference reduced in the fifth month when the ratio was found to be 3:1. However, no organisms growing on resorcinol medium could be isolated and counted from the sample collected before steeping. Although different types of colonies were observed on nutrient agar only one type of colony was seen on resorcinol medium which was pinnate and transparent showing a bluish sheen on nutrient agar between 16-24 hours of incubation.

Table 3.3- Microbial analysis of water samples from natural retting system.

Month of retting.	Cells/mL ($x10^4$) on Nutrient Agar	Cells/mL on 0.05% Resorcinol Agar
Before Steeping (Zero)	148	–
One	150	470
Two	15	960
Three	2000	1660
Four	125	560
Five	150	2980
Six	150	580
Seven	163	270
Eight	270	340
Nine	180	270

3.2.2 Analysis of husk

Although retting is an age old process, the only scientific criterion reported for determining the completion of retting is the reduction of polyphenols and pectin content in husk (Bhat and Nambudiri, 1971). Besides, the softening of the husk, ease in separation of the exocarp (which peels off without exertion), fibre of length between 15-20 cm and soft texture devoid of extraneous pith material with bright colour have been taken as the main indications of completion of retting.

The husk samples drawn out from the first to fourth month were hard and the exocarp could not be peeled off. The husks drawn out after the sixth month was suppler and the exocarp could be peeled out with some effort. The husk sample drawn out at the end of the ninth month was very soft and the exocarp could be peeled off effortlessly indicating the completion of retting. The polyphenol content in the husk was observed to reduce from over 50% in the sample drawn before steeping (raw) for retting to almost 1% in the seventh month. The pectin content was observed to reduce from 7% in the unretted

husk sample to approximately 1 % in the ninth month sample. (Fig. 3.4).

3.2.3 Evaluation of fibre quality

The fibre (800 kgs) could be extracted after nine months of retting from 10,000 husks. The retted fibre was observed to have a natural bright colour with staple length between 15 –20 cms and less than 2 % impurities. The Flexural Rigidity analysis of five sets (fifty rings each) of the nine month retted fibre sample was found to be 1.47 gcm^2. The lightfastness rating of the fibre was observed to be of Grade II in the test conducted on the Xenotest (Table 3.4). No significant change was observed in the mechanically extracted fibre of green husk, brown fibre (from dry husk) and retted fibre from green husk..

Estimation of the lignin content in coir fibre obtained from different retting regions was observed to range between 34 to 40 % (Table 3.5).

Fig.3.4-Polyphenol & pectin in husk in natural retting.

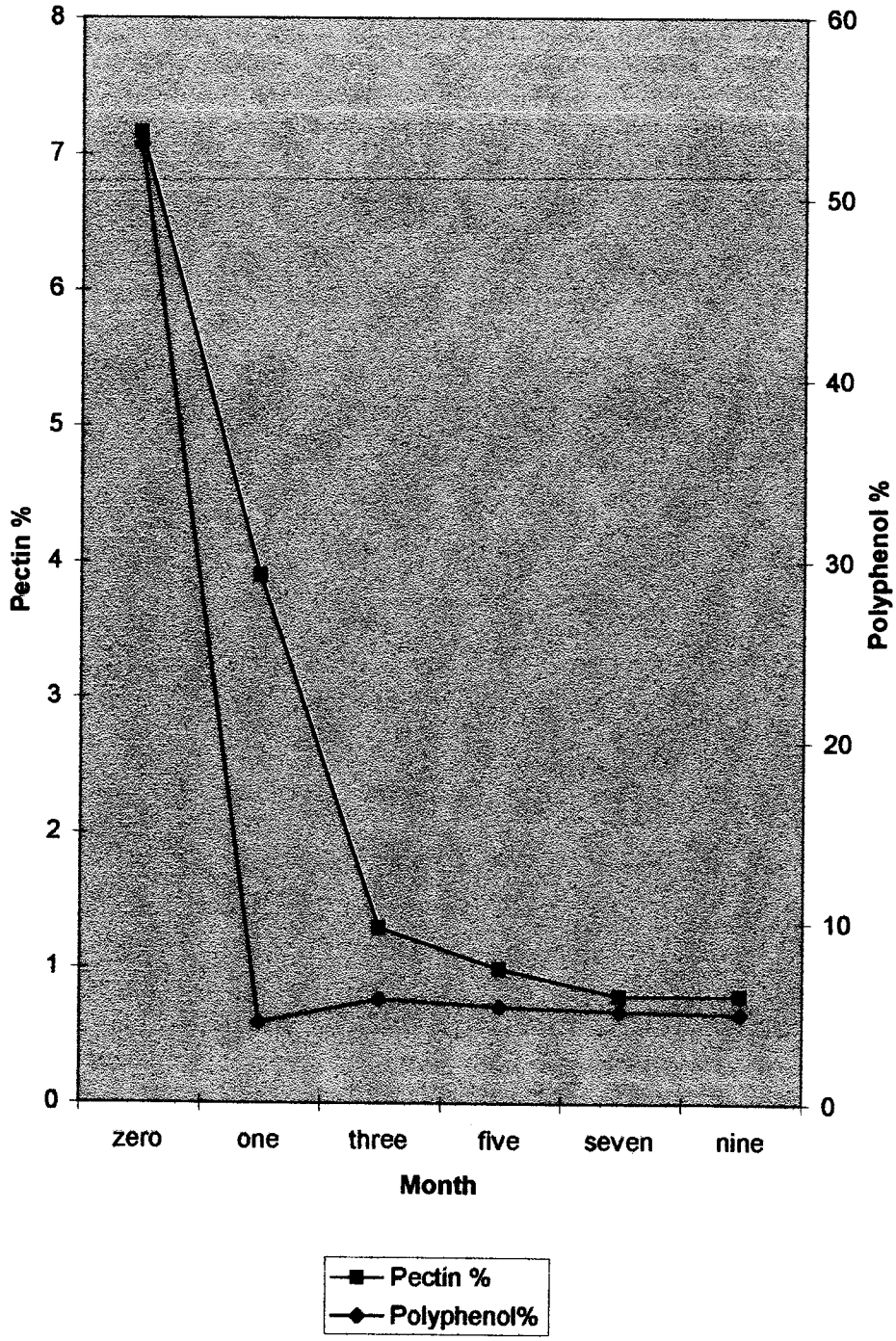


Table 3.4- Lightfastness studies of retted fibre.

Test method	Exposure to xenon arc lamp QUARZLAMPEN GESELLSCHAFT M.B.H., HANAU FRG.
Humidity	70%
Temperature	37 ^{0}C

Sample Description	Grade*
FIBRE FROM UNRETTED HUSK	**1**
FIBRE FROM TRADITIONALLY RETTED HUSK	**11**

*GRADE **1**- Fading within 80 minutes of test exposure
GRADE **11**- Fading within 145 minutes of test exposure

Table 3.5- Lignin content in coir fibre from different retting regions.

SL. No.	Coir Retting Region	Lignin % (Dry weight basis)
1.	Anjengo	39.9
2.	Vycom*	35.27
3.	Quilandy	34.10
4.	Beach	36.70
5.	Alapat	35.30
6.	Unretted Fibre	35.50
7.	Brown Fibre	34.52

*Fibre obtained from the site where the study on natural retting was being conducted.

3.3 Discussion

The main feature of the coconut husk retting process is the decomposition of the binding matter between individual fibre cells without any effect on the chemical constituent of the coir fibre (Heyn, 1951). The lignocellulosic bondage that constitutes the coir fibre is normally resistant to microbial attack and hence the fibre is not disintegrated in the retting process. The lignin content in the retted fibre obtained from different locations showed no significant variation, yet the quality of the fibre obtained form these stations varied with respect to brightness and texture. It is possible that the environmental conditions and the effect of the climatic changes play an important role in defining the quality of the fibre. The Anjengo coir is the most superior fibre and ideal for the manufacture of products.

Study of the environmental conditions prevailing in Varanad has shown that the pH fluctuates during the nine months of retting from 6.5 in the month before steeping of husks (May), to 5.5 in the third month (September) and increasing to 6.7 in January. Besides, the temperature also is found to vary (28-35^{0}C). These changes are attributed to the monsoons

which occur during these months. The effect of monsoons is also reflected on the salinity values which are as low as 1 ($x10^{-3}$) a decline during monsoons, such a drop in salinity due to rainfall has also been reported in the earlier studies (Aziz and Nair, 1978).

During the present study the release of phenolic compounds in the steep liquor in the earlier months of retting has been observed, which may be responsible for lowering of the pH of the retted water. The change in the bacterial count in the third month of retting on nutrient agar and on resorcinol medium confirms the predominance of phenolytic bacteria in these waters. The absence of growth on resorcinol medium in the water samples drawn before steeping of the husks, suggest that the bacteria may originate from the husk or may be enriched in the water samples due to the presence of leached phenolic compounds over the period of three months showing a gradual increase. This is further substantiated by the fact that there is a fall in the polyphenol content and pectin content in the husk samples with the progress in retting. The retting of coconut husk is therefore a unique process and differs from other fibrous materials, as the decomposition of pectins and phenolic cement binding the fibre occurs

simultaneously (Bhat and Nambudiri, 1971). In the present study also polyphenols are reduced from 70% to less than 1% within three months and pectins from 7% to less than 1% within four months. The criterion for determining the end point of retting has been adjudged by the decrease in the polyphenol and pectin content of the husk (Bhat and Nambudiri, 1971).

The fact that retting is a microbiological process was established by Winogradsky and Friebes in 1895 with their studies on flax retting (Bhat and Nambudiri). The degradation of these components is brought about by the microorganisms present in the water. In the present study also the total microbial count is found to increase from 148 x 10^4 to 2000 x 10^4 during the first three months and then remain steady indicating the proliferation of the microorganisms which can breakdown the polyphenols leached out from the husk. The analyses of the retting water samples by TLC have interestingly indicated the presence of resorcinol in the retted waters in considerably high concentrations throughout the retting period while the presence of pyrogallic acid and protocatechuic acid was observed in the first three months. The phenolytic bacteria were therefore quantitatively estimated on resorcinol medium. A significant increase in the phenolytic

bacteria was observed during the third and the fifth month which also reflected in the total count on nutrient agar, although, during these months the influence of monsoons has been noted with respect to temperature and salinity, yet the counts were found to be hundred folds higher than the earlier months. These periods seem to be the most efficient periods for retting as the polyphenols and pectins also were found to reduce in the husk samples.

Although retting is an efficient microbiological process for production of fibre, the retting zone during the course of time turns out to be an ecologically unfavourable environment for aquatic organisms due to the presence of phenolic substances released indiscriminately into the natural water body. Further these backwaters are sites of excellent fishery, providing livelihood for a very large section of the coastal population and serve as a nursery for aquaculture. Therefore development of alternative methods for extraction of coir would lead to elimination of pollution being caused by the retting process.

The presence of bacteria utilising the phenolic compound resorcinol, in the water during retting indicates their involvement in the removal of the phenols. Therefore by allowing the proliferation of a consortium of such phenol degrading bacteria, in the retting environment it was envisaged to hasten the retting process in RCC/ PVC tanks and transfer it to a closed system, leaving the backwaters free for aquaculture and fishery development.

The next chapter deals with the development of such a consortium degrading phenolic compounds.

Chapter IV

Development of a bacterial consortium for retting of coconut husk

Retting of coconut husks involves the biodegradation of mainly polyphenols and pectins which bind the fibre in the husk. (Bhat and Nambudiri , 1971). The efficiency of this process therefore depends on the rate and extent of degradation of these binding components. During the retting process, these polyphenols from the coconut husks leach out into the steep liquors, in significantly high concentrations, resulting in the delay of the retting process due to their toxicity. Inoculation of selected strains of pectin and phenol degrading bacterial cultures resulted in improving "retting" in a poor retting area where dull coloured fibre is produced after ten months of retting. (Ravindranath, 1991).

Most environments support the growth of a wide range of microorganisms having many different metabolic capabilities. Microbial communities have an extremely important role to play in the degradation of simple and complex natural products. It is a commonly accepted observation that often the rate of biodegradation of a particular compound is faster in natural environments than with pure cultures of organisms isolated from that environment. This may be due to the

concerted activity of a community of microorganisms available under natural conditions. Studies on such isolated communities clearly show that mixed populations confer beneficial effects and make the associations more successful than any of the individual populations alone. (Slater and Lovatt , 1984). Such biodegradation processes are advantageous as they can be used in situ unlike other waste processes. Although majority of laboratory based studies on biodegradation have focussed on metabolism of single compounds by pure cultures, in reality, a complex array of compounds are present at a polluted site, thus requiring the presence of a microbial consortium (Ghadi, 1996).

A bacterial consortium degrading phenolic compounds has been isolated and assayed in swine waste (Beaudet et al. 1986). The potential for carboxylation-dehydroxylation of phenolic compounds to benzoate by a methanogenic consortium has also been studied in detail. (Bisaillon et al., 1993). In methanogenic environments, complex organic matter (including aromatic compounds) could be converted into a mixture of methane and carbon dioxide by a syntropic consortia (Kleerebezem et al., 1999). A formulation of microbial consortia has been used for

biodegradation of phenol present in oil refinery effluent. Immobilized cells of the consortia have been observed to degrade 37% of phenol present in the effluent within six hours. (Kapoor et al., 1998). The probable pathways for the degradation of benzoate by a bacterial consortia has been described in detail (Evans and Fuchs, 1988).

Since retting of coconut husk, for extraction of coir, also leads to release of phenolic compounds into the retting environment, it was envisaged that inoculation of microorganisms which can degrade the components in the husk leachate would accelerate the retting process. Studies were undertaken to develop a consortium of indigenous bacteria from coconut husks capable of growing on husk leachates. The effect of this consortium on the retting of husks and fibre has been studied in the laboratory system.

4.1 Materials and method

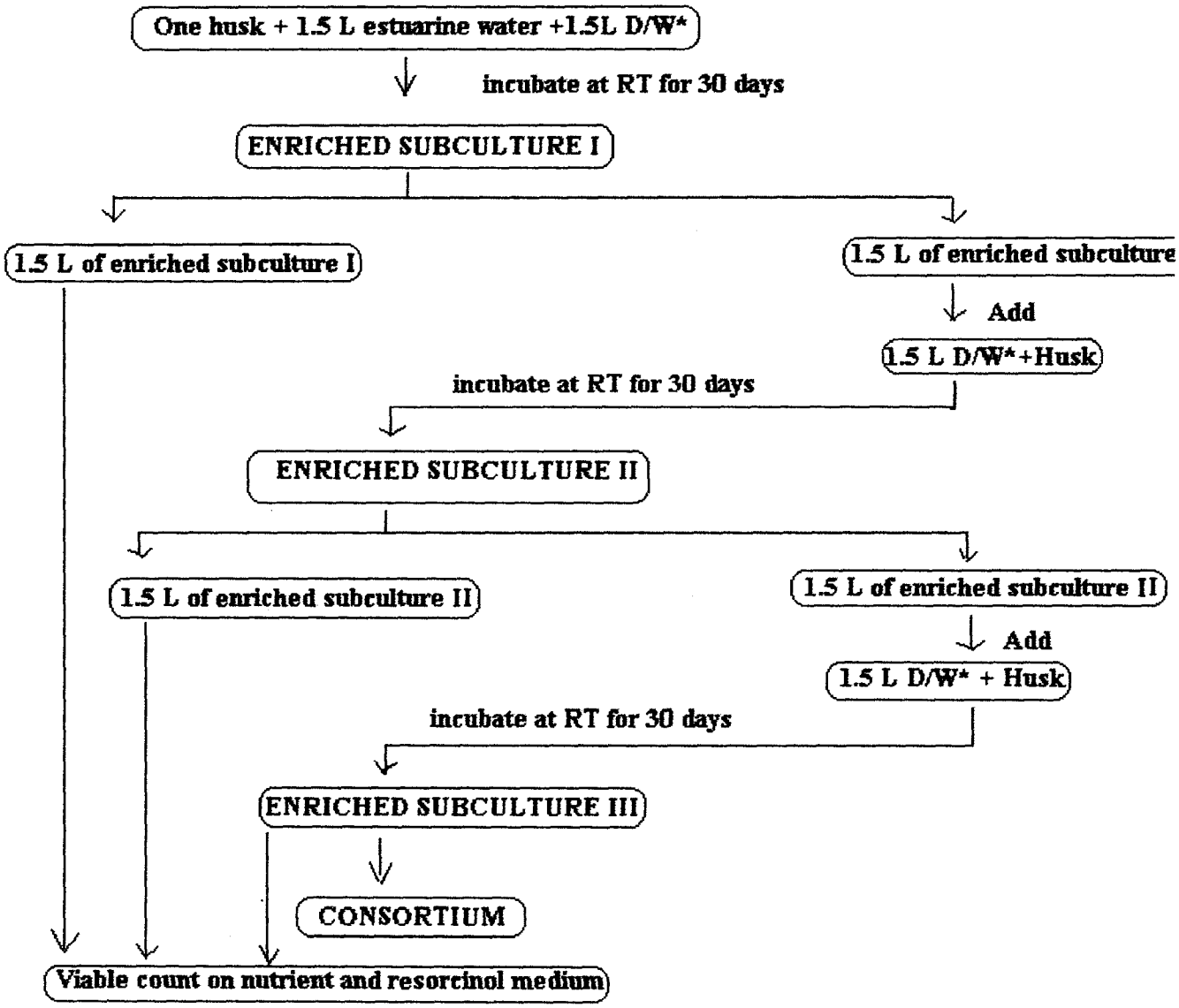
4.1.1 Development of the Consortium

One husk was steeped in 1.5 L of estuarine water of salinity 6 (x 10^{-3}) in a 5 litre beaker. To this 1.5 L of distilled water was added and the salinity adjusted to 6 (x 10^{-3}) with sodium chloride

(FLOWCHART). The mixture was allowed to stand for 30 days, during this period the phenolic compunds leaching out from the husk would act as carbon sources for the indigenous organisms present in the husk. These organisms would get enriched and proliferate utilizing the carbon sources leached out and multiply to give the growth in substantial numbers.

1.5 L of this first enriched culture with the husk was transferred to another beaker and was supplemented with 1.5 L of distilled water with salinity adjusted to 6 (x 10^{-3}) and kept for 30 days to yield the second enriched culture. This second enriched culture was also subjected to the above treatment to give a third enriched culture of the consortium growing on husk leachate. The viable count of the first, second and third enriched cultures were taken on nutrient agar and resorcinol mineral medium (Flowchart). The final consortium was also plated on pectin agar to estimate the viable count of pectin degraders.

FLOWCHART



D/W*- distilled water with salinity adjusted to 6 ppt
One husk was used for the consortium development

4.1.2 Isolation and identification of the bacterial cultures comprising the consortium

The bacterial isolate were purified on nutrient agar and resorcinol mineral salts medium. The cultural and biochemical characteristics were studied using the standard methods. (Bergueys Manual, 1986 and Schmauder, 1997).

4.1.3 Growth of the consortium on nutrient, resorcinol and husk Leachate media.

4.1.3.1. Media

a Nutrient medium

Nutrient broth (Hi Media) was prepared by adding 13 grams powder in one litre distilled water.

b. Resorcinol medium

One litre sterile mineral medium (Appendix) was supplemented with 0.5g of resorcinol. The culture was also inoculated into R medium (Chapman and Ribbons, 1976, Appendix).

c. Husk leachate medium (HLM)

The husk leachate medium (HLM) was prepared by adding one surface sterilized husk into one litre sterile mineral salts medium and allowed to stand for 48 h at room temperature to facilitate leaching out of the polyphenolic compounds from the husk into the medium. The husk was aseptically removed before adding the inoculum.

4.1.3.2 Growth of the consortium

5% of the consortium was inoculated into one litre each of nurient broth (NB), 0.05 % resorcinol mineral medium (RMM) and husk leachate medium (HLM) and incubated at room temperature under stationary conditions. The viable count in each of the three media was determined by drawing out samples at zero and twenty four hours of incubation and plated using spread plate techniques on nutrient agar.

4.1.4 Retting of husks in tanks using consortium

4.1.4.1 Preparation of inoculum.

Surface sterilized husk (50g) was taken in each of the four round bottom flasks (5L capacity). To this 2L of sterile mineral medium was

added and allowed to stand for 48 h. During this period the leachate containing phenolic and other compounds would comprise the nutrient medium for proliferation of the consortium. The medium was decanted aseptically into sterile flasks and was inoculated with 5 % (v/v) of the consortia and incubated at room temperature for 24 h. This was used as inoculum for the laboratory scale retting experiment.

4.1.4.2 Retting of husks in tanks

Mature coconut husks from 11-month-old nuts, which are normally utilized for coir extraction, were used for the laboratory scale study. Three tanks A, B, C were set up with 10 husks immersed in tap water. After 24 h of soaking, tanks A and B were inoculated with the consortium (grown in HLM) in concentrations of 5 and 10 per cent respectively. Tank C was maintained as the untreated control. In all the three sets the final husk : liquor ratio was maintained at 1: 5. A periodic flushing of the water in all the three tanks was carried out by removal of the steep liquor and refilling with tap water at fortnightly intervals. This was done to simulate the flushing action in the environment which gives a brightening effect on the fibre and also

exerts a beneficial influence in retting. In order to supplement the loss of organisms due to flushing , tanks A and B were reinoculated with the consortium in the concentration of 5 and 10 % respectively after one month of the first inoculation.

4.1.4.3 Analysis of ret water in lab-scale retting

The following parameters were studied in the ret water samples drawn from the three tanks A,B and C at monthly/ fortnightly intervals.

a. pH

b. salinity

c. temperature

d. The phenolic compounds leached out from the coconut husks steeped for retting into the ret water in the lab-scale retting tanks were analysed using the High Performance Liquid Chromatograph . The HPLC analysis was performed on Beckman System Gold with a 250 x 4 mm column packed with viosfer C-8 support. The detector was set at 280 nm. The mobile phase consisted

of an isocratic solvent system of acetonitrile / water (20:80) and the flow rate was set at 0.5 mL/min (Semple and Cain,1997).

e. The total bacterial count was enumerated on nutrient agar, 0.05 % resorcinol mineral salts agar. The samples drawn after 60 days were plated on 0.05% pectin mineral salts agar.

4.1.4.4. Analysis of the retted husks

Three husks were drawn out from each of the three tanks every month to monitor the progress in retting. The parameters analyzed were

a. pectin and polyphenol content in the husk samples, analyzed by the method described under Chapter III, 3.1.3.

b. the change in the texture of the husk by physical touch & feel method.

c. the lignin content, lightfastness rating and the degree of softness of the fibre from the husks on completion of three months.

4.1.5 Effect of application of the consortium on mechanically extracted green husk fibre in tanks

10 kilograms of machine extracted coir fibre was soaked in 25 L of tap water in three carbuoys Set A, B and C for 7 hours. The water in all three tanks were drained out to remove the very dark coloured leachates and refilled. Tanks A and B were inoculated with 5% and 10% of the consortium respectively and incubated for 48 hours, Tank C was maintained as uninoculated control. After 48 hours the water in all the three tanks were drained out and the fibre, air dried. The fibre thus obtained from the three sets were subjected to the Xenotest for assessing the lightfastness rating and the degree of softness.

4.2 Results

The consortium was developed on husk leachates after an acclimation period of ninety days with three transfers. An increase in the total count was observed on both nutrient and resorcinol media during the transfers. On nutrient agar the counts changed from 23 x

10^2 to 120 x 10^3 whereas on resorcinol agar it changed from 43 x 10^2 to 141x 10^2 (Table 4.1). Plating of the consortia on nutrient agar showed different types of colonies, the cultural characteristics of which are shown in (Table 4.2). Out of the nine isolates, C1 – C9, obtained on nutrient agar, four were gram positive and five were Gram negative. Most of the organisms were motile showing H_2S production and catalase activity (Table 4.3). On the basis of their biochemical characteristics the organisms were tentatively identified as belonging to the genera of *Azotobacter, Micrococcus, Bacillus, Pseudomonas* and *Actinomycetes.*

Interestingly on plating the consortium on 0.05% resorcinol mineral salts medium and R medium only one type of pinnate colonies (C10) were observed. When streaked on nutrient agar, the colonies were observed to be bigger in size with a translucent blue sheen formed in 24 hours which disappeared on incubation after 24 hours. This culture, which was isolated from both the subcultures and the final consortium was Gram negative coccobacilli, oxidase positive, catalase positive and motile. On the basis of the biochemical tests this isolate was identified as *Pseudomonas* (Table 4.2, 4.3). The

Table 4.1 - Influence of enrichment on total count of enriched cultures.

No. of Subculture	Media	
	Nutrient Agar	Resorcinol Agar
	Cfu / mL $X10^2$	Cfu / mL $X10^2$
1	23	43
2	85	73
Consortium	1200	141

Table 4.2-Colony characteristics of the isolates in the consortium on nutrient agar.

Isolate	Description
C1	Cream coloured opaque colonies 0.1-0.2 mm dia.
C2	Colonies with yellow pigment, and transparent 2mm dia.
C3	White coloured opaque colonies of size 1-3mm dia.
C4	Yellow pigmented, transparent ,pinnate colonies
C5	White transparent colonies 2mm-3m dia.
C6	White colonies showing spreading growth
C7	Cream coloured pinnate , opaque colonies
C8	White coloured opaque colonies of size 0.5-1mm dia.
C9	Cream coloured transparent colonies 0.2mm dia.
C10	White transparent colonies 2mm-3m dia.

Table 4.3-Biochemical tests for identification of isolates in the consortium.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Gram Stain	–	+	–	+	–	–	+	–	+	–
Motility	–	+	+	+	+	+	+	+	+	+
M.R.	+	+	+	+	+	–	–	+	+	+
V.P.	–	–	–	–	–	–	–	–	–	–
Citrate	–	+	+	–	+	–	–	–	–	+
Oxidase	–	–	+	+	+	–	+	+	–	+
Gelatine Hydrolysis	–	–	–	–	–	–	–	–	–	–
Indole	+	+	+	+	+	+	+	+	+	+
H_2S Production	+	+	+	+	+	+	+	+	+	+
Phenylalanine deaminase	–	–	–	–	–	–	–	–	–	–
Starch Hydrolysis	–	–	–	–	–	–	–	–	–	–
Nitrate Reduction	+	–	+	+	+	+	+	–	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Tentative Identity	Azoto-Bacter	Micrococcus	Actinomycetes	N.I.	Pseudomonas	N.I.	Bacillus	N.I.	N.I.	Pseudomonas

N.I.- Not Identified.

isolate C10 appears to be the same as isolate C5 obtained from nutrient agar. The consortium was inoculated (5 % inoculum v/v) into one litre each of nurient broth (NB), 0.05 % resorcinol mineral medium (RMM) and husk leachate medium (HLM) and incubated at room temperature under stationary conditions. The viable count in each of the three media was determined by drawing out samples at zero and twenty four hours of incubation. The viable count on nutrient agar was highest followed by the count on husk leachate medium (Table 4.4)

The inoculation of the tanks with the consortium results in affecting the pH of the steep liquor. The pH decreased in all the three tanks from 6.5 in A and B , 6.8 in C to 5.0, 6.0 and 4.7 respectively in fifteen days (Table 4.5). The tanks A and B on inoculation showed a stable increase in pH in less than 2 months, however in tank C the pH showed a wide variation changing from 6.8 to 4.7 in fifteen days and then increasing back to 6.8 in ninety days. Such a variation was seen in Tank A with the pH changing from 6.5 to 5 in 15 days but reverting to 6.3 in thirty days. Interestingly, in Tank B , where the inoculum

Table 4.4 - Growth pattern of the consortium on different substrates

Incubation period (hours)	Cells/mL ($x10^2$) Media		
	Husk Leachate	Resorcinol	Nutrient
0	48	-	38
24	287	141	300

Table 4.5 -Time bound pH, temperature and salinity profile in coconut husk retting tanks.

Incubation Period Days	PARAMETERS								
	pH			Salinity ($x10^{-3}$)			Temperature ^0C		
	A	B	C	A	B	C	A	B	C
1	6.5	6.5	6.8	1.0	1.0	1.0	31	31	31
15	5.0	6.0	4.7	2.0	2.0	2.0	29	29	29
30	6.3	6.5	4.8	1.0	2.0	3.0	30	30	30
45	6.7	6.7	5.8	1.5	1.5	2.5	31	31	31
60	6.9	7.0	6.5	1.0	1.0	2.5	30	30	30
75	7.0	7.0	6.5	1.0	2.0	4.0	30	30	30
90	7.0	7.0	6.8	1.0	1.0	3.5	30	30	30

was 10% such a variation was not observed with the pH ranging between 6.5 to 7.0 throughout the study (Fig 4.1).

Although the salinity in all the three tanks were observed to increase from 1 (x 10^{-3}) to 2 (x 10^{-3}) within fifteen days in tank C the salinity remained high and increased to 4 (x 10^{-3}) after 75 days. The temperature in all the three tanks however was noted to be same as room temperature (Fig.4.2).

The phenolic compounds leached out from the coconut husks into the ret water during the lab-scale retting were analysed using the HPLC. In the first two months the peaks were found to be similar with the RT ranging from 2.7 to 2.8, 3.5 to 3.4 and 4.3 to 4.8 minutes. The retention time of standards used such as catechol, resorcinol and pyrogallic acid indicated that the first peak represents pyrogallic acid and the third represents resorcinol. The second peak however could not be identified. During the third month , these peaks were not present, however there were other peaks with retention time 0.68, 5.07

Fig.4.1- pH variation in lab-scale retting using consortium.

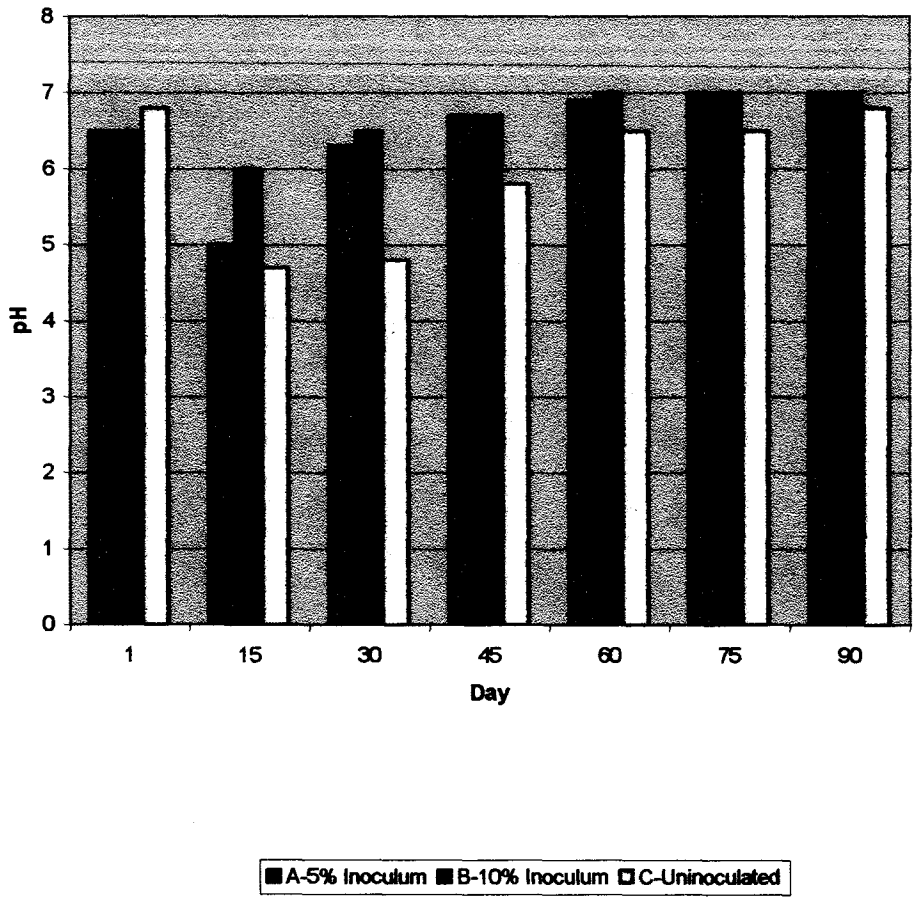


Fig.4.2-Salinity variation in lab-scale retting using consortium.

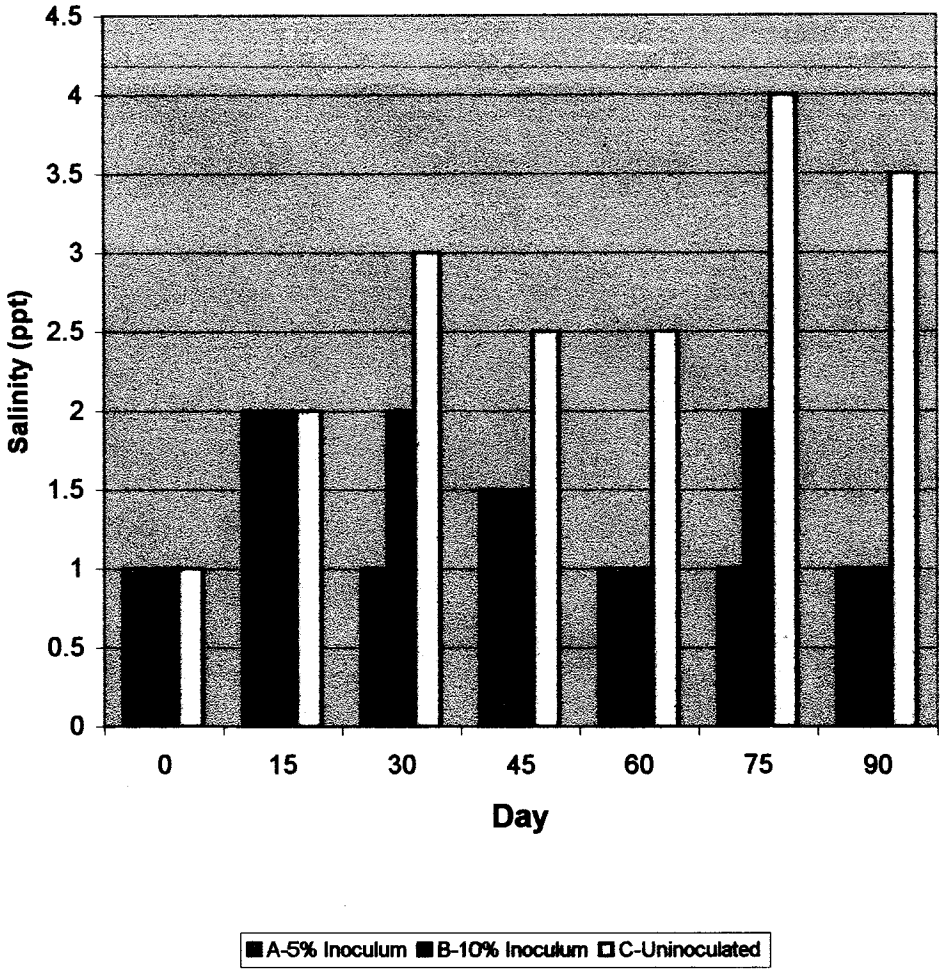


Table 4.6 -HPLC analysis of polyphenols in lab-scale retting.

Sample	Retention Time (minutes)						
	0.5	1.0	2.0	3.0	4.0	5.0	6.0
One Month	-	-	2.750	3.525	4.285	-	-
Two Months	-	-	2.84	3.42	4.80	-	-
Three Months	0.68	-	-	-	-	5.07	6.41
Resorcinol	-	-	-	-	4.49	-	-
Catechol	-	-	-	-	-	5.75	-
Pyrogallic Acid	-	-	2.6	-	-	-	-

HPLC : Beckman System Gold with a 250 x 4 mm column. packed with viosfer C-8 support

Mobile phase: Acetonitrile / water (20:80), flow rate : 0.5 mL/min.

Visualizer: UV-VIS detector, 280 nm.

Table 4.7 Total bacterial count in ret liquor from lab-scale retting with consortium .

Incubation period Days	Bacterial Count / mL								
	Tank								
	A			B			C		
	N.A	R.A	P.A	N.A	R.A	P.A.	N.A	R.A	P.A
0	83×10^5	-	ND	300×10^4	-	ND	196×10^5	-	ND
30	5×10^7	129×10^2	-	21×10^7	67×10^5	-	120×10^4	-	ND
60	53×10^5	143×10^2	43	70×10^5	123×10^3	55	113×10^5	-	-
90	209×10^3	120×10^2	ND	120×10^3	23×10^3	ND	211×10^3	180×10^2	ND

N.A.- Nutrient Agar R.A- Mineral Salt Medium + 0.05% Resorcinol P.A.- Mineral Salt Medium + 0.05% Pectin

and 6.41. The peak at 5.07 corresponded to catechol when used as standard (Table 4.6).

Water samples drawn out from the three retting tanks at intervals of thirty days were plated on nutrient medium and mineral salt medium with 0.05 % resorcinol (Table 4.7). The water sample collected after 60 days of retting was plated on mineral salts medium with 0.05% pectin. The initial count in the tanks inoculated with the consortium was observed to increase from 83×10^5 and 300×10^4 to 5×10^7 and 21×10^5 in tank A and B respectively whereas the count in the control tank was observed to decrease from 196×10^5 to 120×10^4 in a period of thirty days. There was growth on 0.05 % resorcinol mineral medium in samples from the two inoculated tanks in thirty days. Sample from the control tank C during the same period showed no growth and colonies appeared only after ninety days of incubation. Plating of the 60 day water samples on 0.05% pectin mineral medium showed the

emergence of 43 cells/mL and 55 cells/mL from Tanks A and B respectively, however no pectin degraders could be isolated from the control tank. These organisms comprising of four different types of colonies were purified and isolated . The biochemical characteristics were studied and the cultures were identified as *Micrococcus, Alcaligenes*, *Arthrobacter* and *Escherichia* (Table 4.8). The variation in the polyphenol content in the husk samples drawn at monthly intervals is depicted in Fig.4.3. Significant changes were observed in the polyphenol content of the husk which decreased in the inoculated as well as the control tanks. The decrease in the inoculated tanks ranged from 90 % to almost less than 10 % whereas in the control tanks the polyphenol content remained after three months at 30 %. The pectin content was lowered from 7 % to less than 1% in all the three tanks in thirty days and to negligible thereafter (Table 4.9). The husks drawn out from the inoculated tanks were softer and the exocarp could be peeled off easily indicating completion of retting after 90 days. A significant difference was observed with the husks drawn out from the control (untreated tank) which exhibited a hard nature and the exocarp

Table 4.8- Biochemical tests of isolates from Pectin medium.

Test	P1	P2	P3	P4
Gram Stain	–	+	–	+
Motility	+	+	+	–
Methyl Red	–	–	+	–
Vogues Prosker	+	+	–	–
Citrate	+	–	–	+
Oxidase	+	+	–	+
Gelatine	–	–	–	+
Indole	–	–	+	–
H_2S	–	+	–	–
Starch	–	–	–	+
Nitrate Reduction	+	–	+	–
Catalase	+	+	+	+
Urease	–	+	–	+
Tentative Identity	**Alcaligenes**	**Micrococcus**	**Escherichia**	**Arthrobacetr**

Fig. 4.3 Polyphenol variation in husk in lab-scale retting with consortium

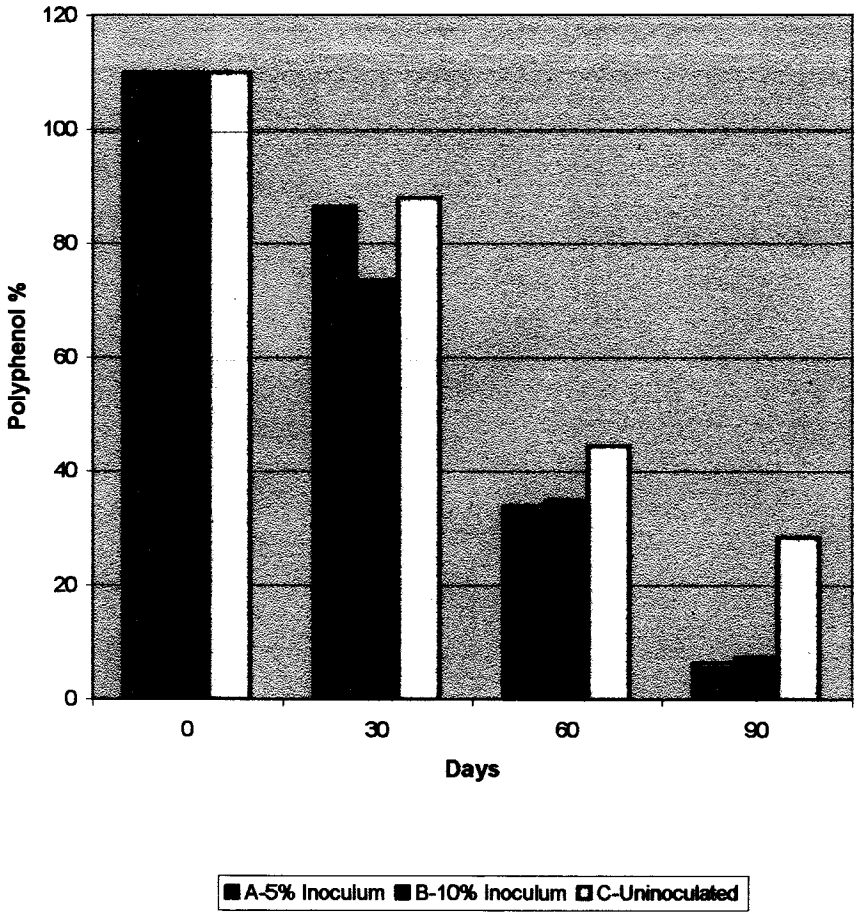


Table 4.9- Polyphenol & pectin content in husk in lab-scale retting with consortium.

Day	Polyphenol %			Pectin %		
	A	B	C	A	B	C
0 (Raw)	52.2	52.2	52.2	7.16	7.16	7.16
30	86.50	73.50	88.00	0.84	0.96	0.72
60	34.0	35.0	44.40	-	-	-
90	6.40	7.04	28.50	-	-	-

would not peel off easily indicating incomplete retting. The fibre could not be extracted from the husk by the conventional beating and hence was extracted by mechanical means.

The fibre extracted from tanks A & B exhibited less pith content as compared to the fibre from Set C which was comparatively inferior having a dull colour (Plates E) The fibre from the three sets were subjected to the Xenotest which rated the fibre from consortia as Grade II whereas the fibre from the untreated husk showed a rating of Grade I. The Flexural rigidity of the fibre extracted from tanks A , B and C were tested for the degree of softness and were found to be 1.19, 1.13 and 2.00 gcm^2 respectively. The lignin content in the fibre from the consortia treated husk was 38 % (Table 4.10).

The mechanically extracted green husk fibre samples treated with 5 and 10 % of the consortium in tanks A, B and C with were air dried after seventy two hours (Plate F). The Xenotest and the degree of softness of mechanically extracted fibre from unretted husk with and without treatment with the consortia was measured. The Xenotest indicated the fibre from the consortia treated tanks to be of

PLATE E

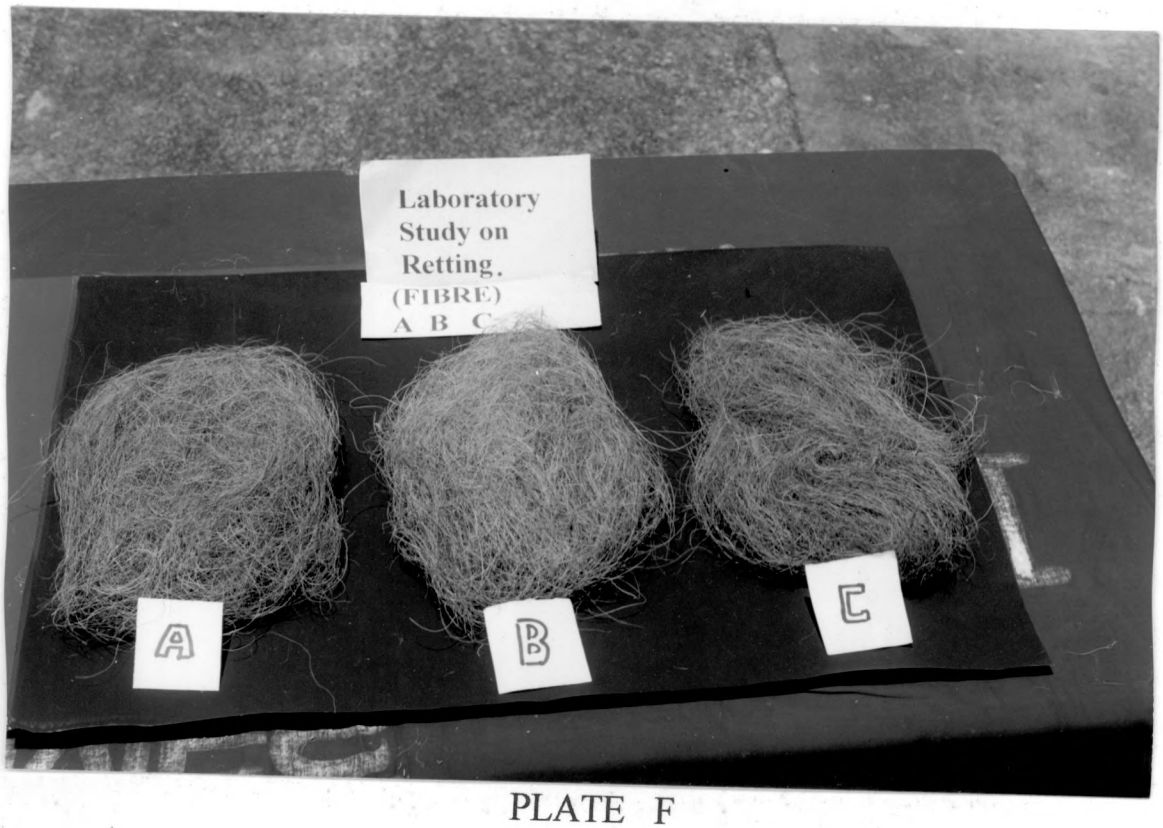


PLATE F

Table 4.10 - Lignin content in coir fibre from consortia treated husk

SL. No.	Sample	Lignin % (Dry weight basis)
1.	Mechanically extract -ed unretted green husk fibre	35.50
2.	Mechanically extracted dry huskbrown bibre	34.52
3.	Fibre from consortia treated husk	38.00
4.	Naturally retted fibre	36.20

Grade I equivalent to that of control fibre from Tank C. However, the Flexural rigidity of the fibre samples from Tanks A, B and C measured 0.69, 0.96 and 2.09 gcm^2 (Table 4.11) respectively indicating a difference in degree of softness of the fibre samples drawn from the three tanks.

4.4 Discussion

Microbes that are the natural components of soil and water environments are potential agents for the biological transformation of aromatic compounds that enter the ecosystem. Mineralisation or complete biodegradation is almost always a consequence of microbial activity (Cork & Krueger, 1991). In nature, microorganisms live in complex communities, often consisting of many different species, which collectively exhibit great physiological diversity but also substantial metabolic redundancy and interdependency. This physiological diversity enables communities to take advantage of a great range of different carbon and energy sources for growth, whereas its metabolic redundancy

Table- 4.11- Flexural Rigdity G_f of fibre samples

Fibre Sample	Average deformation "d" cms	θ	Flexural Rigidity G_f gcm^2
Naturally retted	0.87	22.69	1.47
5% consortium treated husk	1.00	34.13	1.19
10% consortium treated husk	1.03	35.13	1.13
Untreated Control husk	0.71	24.2	2.00
5% consortium treated unretted fibre	1.20	45.05	0.69
10% consortium treated unretted fibre	1.32	38.46	0.96
Untreated Control fibre	0.68	23.21	2.09

endows it with the flexibility to function efficiently under changing environmental conditions. Moreover, microbial communities exhibit a remarkable genetic plasticity and promiscuity, due to the existence of very efficient gene exchange systems, which permit the rapid evolution and spread of new activities in response to new metabolic opportunities or other strong selection pressures. The interactions – metabolic and genetic –between diverse members of microbial communities determine community activities and control the ability of new species to integrate into the community.

The principal change brought about in the plant tissue during retting is the breakdown of pectic substances which form the chief constituent of the middle lamellae between the fibre cells and the cementing material (Bhat and Nambudiri, 1971). The consortium degrading the husk leachates is therefore postulated to contain bacteria belonging to different physiological groups, particularly metabolizers of phenolic compounds and pectin degraders. The growth of colonies on resorcinol and pectin could substantiate the presence of such bacteria which are involved in coconut husk retting. Preliminary evidence of

bacterial growth on resorcinol suggests the presence of bacteria that are able to use the husk leachates as a carbon source,

Consortia development is initiated by enrichment of microorganisms from the source followed by different periods of incubation with periodic transfer of the inocula to fresh medium. The phenol degrading consortium from a municipal plant was maintained for enrichment for two years with bimonthly transfers of 25 % of inocula to fresh mineral medium (Dwyver et al.,1986). To determine the degree of adaptation of a microbial community to p-nitrophenol, the ecocores prepared from sediment and water from the test site and incubated for five days to allow the community to adapt. The ability of communities to adapt varies from site to site. The consortium in the present study has been incubated for thirty days to enable complete leaching of the polyphenols from the husk.

Retting of coconut husks under natural conditions results in the liberation of polyphenols into the surrounding water leading to the lowering of the pH in the environment. This fact could be confirmed by studying the natural system and from the

lab-scale study. A significant finding from the foregoing studies reveals that in the natural system the pH is raised to the neutral range only after the third month, in the lab-scale study the pH has been observed to rise to the neutral range within two months. This can be attributed to the proliferation of the bacteria in the consortium which possess the ability to assimilate the phenolic compounds. There is an increase in the bacterial count on nutrient medium in the water samples drawn during the first month from the two tanks inoculated with the consortium (5 x 10^7 in A & 21 x 10^7 in B) whereas the count in the water sample drawn from the natural system during the same period exhibited a lesser count ($150x10^4$) indicating that the removal of the polyphenols could allow the proliferation of other ret microflora which could play a role in the retting. The salinity under natural conditions has been observed to be influenced by the climatic changes as reflected by the drastic decline in the salinity values during the monsoon months. The salinity values in lab-scale retting are comparatively lower than those in natural retting. The water used for soaking of the husks in the tanks being from a coastal area which has an inherent salinity of 1(x 10^{-3}) which is

reflected in Fig.4.3. Salinity values appear to be a measurement of the halogen ion estimated by the Knudson Mohr salinity measurement method (Strickland and Parson, 1972). It has been reported that all titration methods used for the estimation of salinity reflect the amount of precipitable halogen ions in a sample of water. It is therefore indicative of the halide or halogen ions present in a given sample of water where sea water samples are not used. In the present study, wherein tap water is used for the soaking of the husk, the salinity indicated actually refers to the amount of halogens leached out from the coconut husk and subsequently reduced during the growth of the consortium. The bacteria in the consortium are adapted to the saline conditions maintained during the course of development of consortia. The increase in the salinity is perhaps due to the halides and therefore does not affect the growth and proliferation of the bacteria.

The significant observation that the control tank has a concentration of halogens reaching to almost 4 (x 10^{-3}) after two months could be another limiting factor for inhibiting the activity of naturally occurring microorganisms and hence delaying the retting process. Such an effect appears to be

overcome with the inoculation of specific groups of organisms comprising the consortium wherein the salinity does not go beyond 2 (x 10^{-3}) during the entire incubation period.

There is no significant effect of the inoculum size reflected on the pH values in Tanks A and B. The pH in Tank A falls to 5 within 15 days but the pH in Tank B remains between 6-7 during the three month study and this difference in pH could be attributed to the inoculum size as the pH in Tank C reached a low of 4.7 during the same period. The lowering of the pH is due to the leaching of phenolic compounds. In the inoculated tanks, the phenolytic bacteria reduce the concentration of these components and therefore , the effect on pH is not manifested.

The chemical nature of the phenolic compounds leached out from coconut husks within nine days have been reported to consist of catechol, benzoic , gentisic, vanillic, syringic and p-coumaric acids (Nazareth and Mavinkurve, 1987). The phenolic compounds identified in the present study were catechol, resorcinol and pyrogallic acid which are compounds consisting of single aromatic ring with two hydroxyl groups.

The assimilation of the phenolic leachates by the phenolytic flora in the consortia, allowed the proliferation of the non phenolytic bacteria which was reflected in the viable count on nutrient medium. This fact could be substantiated by the comparison of the viable count on both media from the control Tank C. A reduction in the viable count in the first month which declined further in the third month was observed. The enrichment of phenolytic bacteria was reflected by the growth on resorcinol medium in the second month. The accumulation of the phenolytic leachate could be the cause for reduction in the count on nutrient medium in three months. The initial viable count in the three tanks did not show a wide variation with the increase in incubation period. The counts on resorcinol agar were found to increase in the inoculated tanks with 5 % showing 129 x 10^2 whereas B showing 67x 10^4 . However no colonies could be obtained from the control tank on resorcinol medium. The phenol ytic organisms present in the consortium seem to be proliferating and metabolizing the phenolic compounds which are leached out and hence their total count increases with the incubation period.. In the control tank, however, the indigenous organisms capable

of breaking down phenolic compounds are being enriched and therefore can be counted on resorcinol medium only after 90 days. This fact is also established from the polyphenol content in the husks reducing faster in the inoculated tanks as compared to the control tank. The ultimate effect of these inoculations is seen on the husks from which the fibre could be extracted by light beating. However, the husks in Tank C exhibited incomplete retting even after three months of steeping

A significant difference was observed in the nature of the husks in the inoculated tanks and those in the control tank. The criteria for completion of retting by the touch and feel method were satisfied in the husks from the consortium treated tanks, the polyphenol content in both the inoculated tanks was reduced but the reduction was more pronounced in the inoculated tank with 10 % consortia.

The degree of softness of the fibre in the tank with 10% inoculum, B was slightly greater than A indicating the possibility that the quantity of the consortium inoculated may be important in determining the softness of the fibre.

The fibre extracted from both the tanks inoculated with the consortium were observed to possess characteristics (lightfastness rating and degree of softness) comparable to that of the fibre retted in a natural system for 11 months within a much shorter period of three months.

The study could thus establish the fact that retting of coconut husks could be carried out in tanks by inoculating the consortia to yield fibre in three months. This fibre is comparable in quality with that obtained by natural retting in 9-11 months. The application of the consortium on mechanically extracted unretted coir fibre from green husk resulted in softening the fibre in three days. The potentials of the consortium in biosoftening of the coir could therefore be established.

This method eliminates the environmental pollution caused by retting and also provide an alternate method for tapping the husk potential available in all coconut growing regions for setting up of coir industries.

Chapter V

Transformation of resorcinol & biobleaching and biosoftening of coir

The polyphenols of the husks have been identified as catechin like tannins (Bhat and Nambudiri, 1971) which are complex compounds with built-in catechol and phloroglucinol units (Jayasankar, 1966). The presence of polyphenols in the ret water and phenol enrichments of coir rets have resulted in the isolation of *Pseudomonas desmolytica, Pseudomonas fragi , Pseudomonas dacunhae* which could tolerate 0.05 - 0.1% phenol. Catechol cleaving, and phenol tolerant yeasts have also been isolated from ret liquor (Jayasankar, 1966 ; Ravindranath, 1991). Several phenolic acids viz. benzoic, cinnamic , veratric, p-coumaric and vanillic acids have been resolved in coconut husk ret liquor (Nazareth, 1986).

The degradation of such aromatic compounds in natural environments is brought about by the association of different bacterial strains. The elucidation of catabolic pathways for specific aromatic compounds has been the subject of numerous reports, but the number of chemically permissible solutions for such pathways is not unlimited (Stanier and Ornston, 1973). Studies conducted in Chapter IV have

revealed that the consortium developed on husk leachates could affect the retting of coconut husk by reducing the period of retting to three months and bestowing a greater degree of softness to the mechanically extracted coir fibre. The consortium consisted of different genera of bacteria which possibly by syntrophic activity ret the coconut husk in a lesser period than under natural conditions. Only one isolate in the consortium was observed to grow on a phenolic substrate 0.05 % to 0.2 % resorcinol indicating that the other isolates in the consortium could metabolize the constituents in the husk leachate by cometabolic activities.

Cometabolism, cooxidation and fortuitous oxidation are all used to describe the oxidation and degradation of non growth substrates by microbes. Cometabolism is defined as the biological transformation of a compound, which is unable to support cell replication, but in the requisite presence of another growth substrate it can be metabolized to a simpler compound but not completely

mineralized. This is clearly different from fortuitous metabolic events, which result from non specific monooxygenase activity on an analogous substrate. It also seems possible that in some cases of cometabolism, energy derived from the oxidation of the non growth substrate can be utilised to metabolize the growth substrate. The capacity for cometabolism and fortuitous activity seems to occur most frequently in bacteria metabolizing complex natural and unnatural substrate (Bayly and Barbour, 1984).

Cometabolism in natural ecosystems where a mixed population of organisms are encountered , is of significance in the context of coconut husk retting. An effort to follow the microbiological dissimilation of polyphenols in coconut husk led to the isolation and characterisation of yeast and bacteria such as *Pseudomonas, Alcaligenes, Micrococcus* strains capable of degrading phenolic compounds associated with coconut husks. Enrichments on catechol seeded with samples of ret liquor promoted the development of *Debaromyces hansenii* which could utilize both phenol and catechol. Enrichments on phenol incubated with the same material gave rise to

only bacterial isolates belonging to the genera *Micrococcus* and *Pseudomonas* . The overall mode of attack by *M.varians* on phenol has been postulated in Fig.5.1 (Jayasankar and Bhat,1966).

The degradation of polyphenols in the ret liquor may result in the formation of dihydric phenols such as resorcinol, orcinol or thymol. These dihydric phenols are also known to be catabolized by a number of bacterial cultures. Resorcinol biodegradation has been reported in several microorganisms (Table 5.1*). Pseudomonas putida* ORC catabolizes resorcinol by a metabolic pathway via hydroxyquinol and ortho oxygenative cleavage to give maleylacetate (Fig. 5.2). A mutant strain O1OC was shown to be constitutive for the enzymes of the orcinol pathway (Chapman and Ribbons,1976). *Azotobacter vinelandii* catabolizes resorcinol through a third pathway by converting it first to pyrogallol and then oxidising it to oxalocrotonate, pyruvate, acetaldehyde and carbon dioxide (Fig.5.3, Groseclose and Ribbons, 1981). This suggests that there could exist a number of pathways for the biodegradation of resorcinol and related compounds . The metabolic sequences for resorcinol catabolism in a

Fig.5.1 Mode of attack on phenol by M.varians isolates

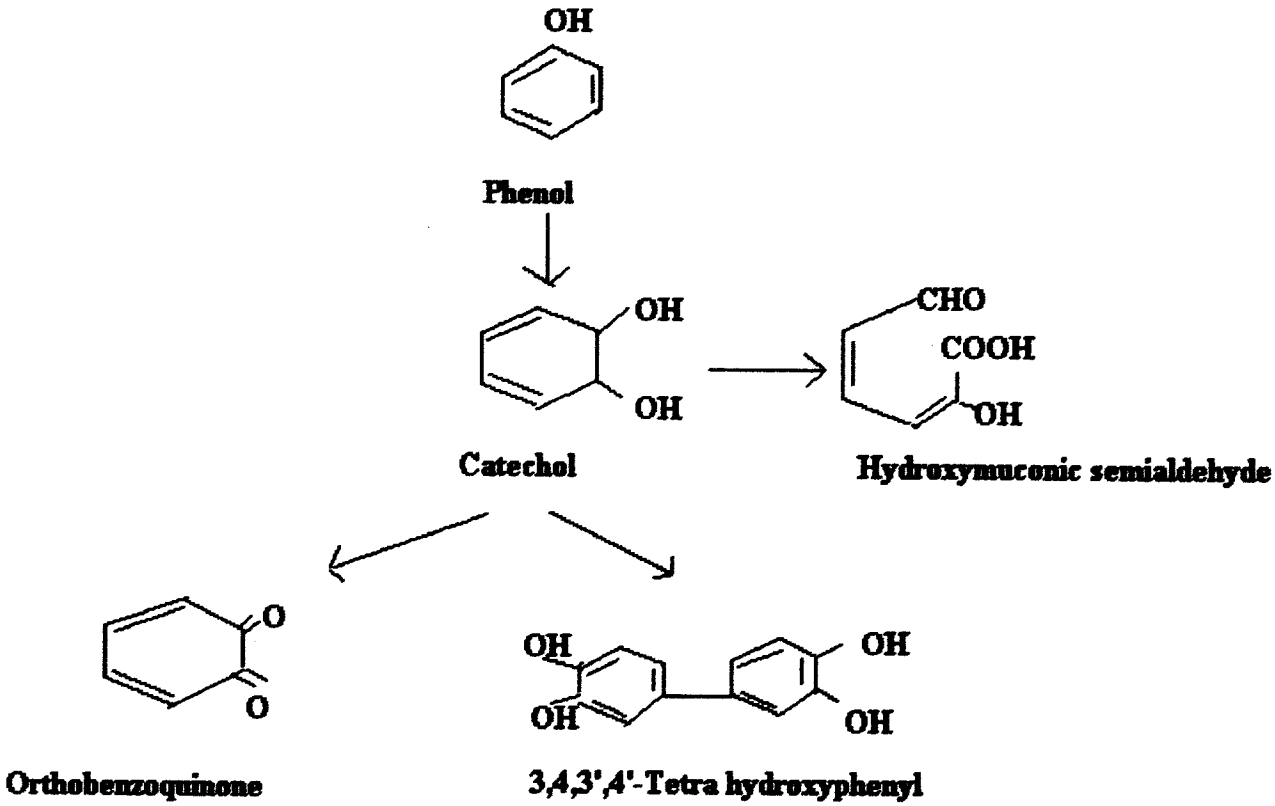


Table 5.1- Microorganisms involved in resorcinol degradation.

Microorganisms	Reference
Pseudomonas putida O1	Chapman and Ribbons, 1976
Pseudomonas putida ORC	Chapman and Ribbons, 1976
Azotobacter vinelandii	Groseclose and Ribbons, 1981
Trichosporon cutaneum	Gaal and Neujahr, 1978
Pseudomonas cepacia AC1100	Ghadi, 1996
Rhodococcus sp.	Straube , 1987
Enrichment cultures	Milligan and Haeggblom, 1998

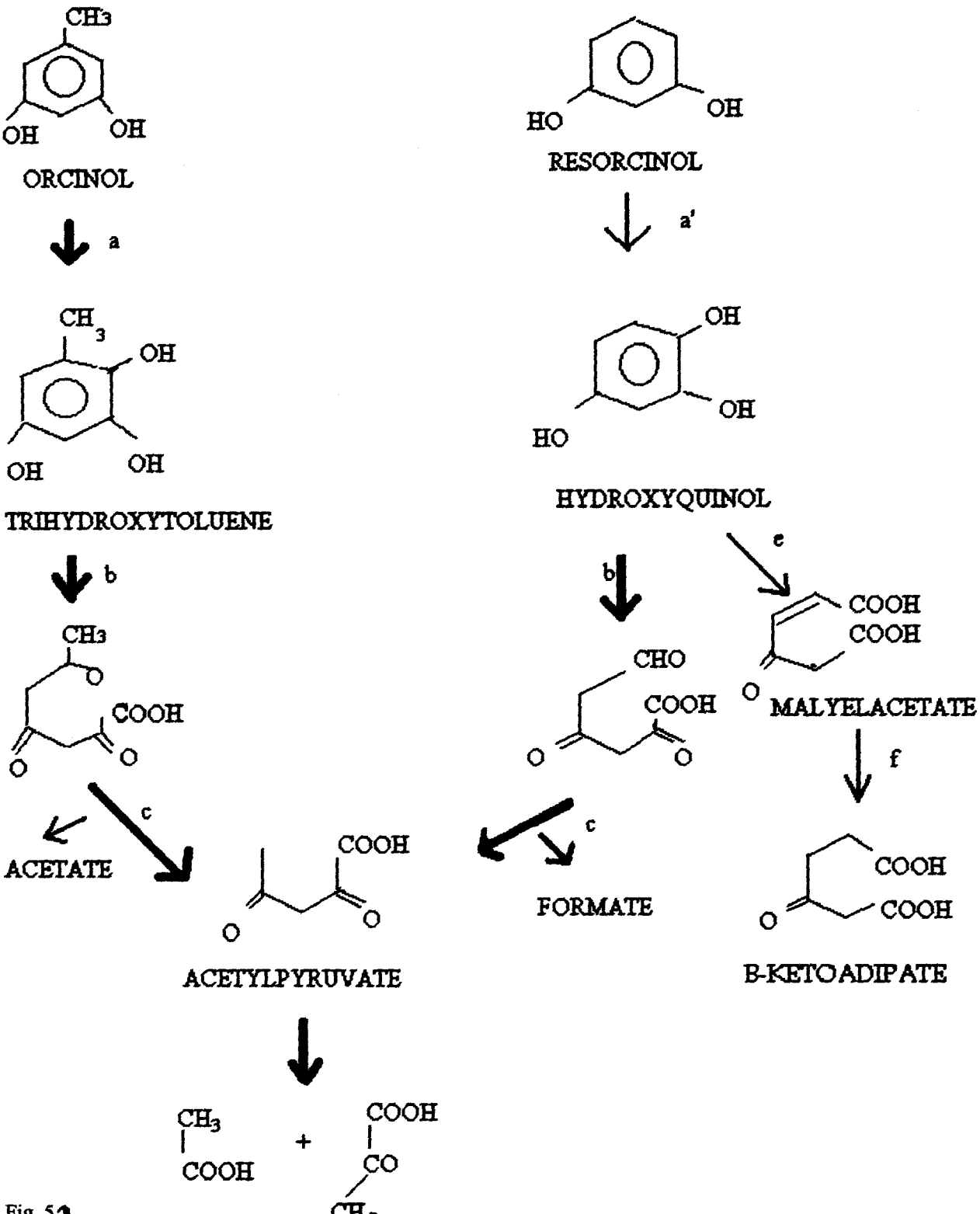
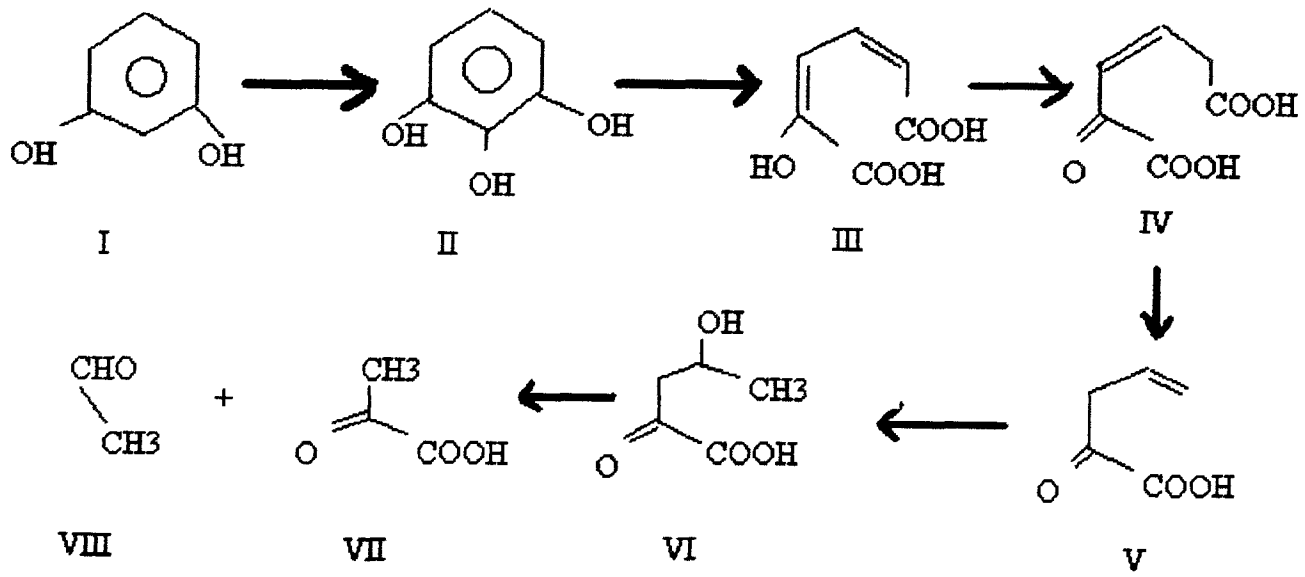


Fig. 5.1 Metabolic sequences for resorcinol catabolism used by P.putida ORC and P.putida OIOC.Light arrows indicate enzymes specifically induced for resorcinol catabolism.Heavy arrows indicate the constitutive enzymes of the orcinol pathway present in P.putidaOIOC, which are also induced in P.putida ORC and O by orcinol and which catalyze pyruvate formation from resorcinol.(a) Orcinol hydroxylase; (a') resorcinol hydroxylase; (b) 2,3,5-trihydroxytoluene 1,2-oxygenase; (c) 2,4,6-trioxoxheptanoate hydrolase; (d)acetylpyruvate hydrolase; (e) hydroxyquinol 1,2- oxygenase; (f) maleylacetate reductase.



Adapted from Groseclose and Ribbons, 1981

Fig.5-3

Proposed pathway for resorcinol catabolism by A.vinelandii: I, resorcinol; II pyrogallol; III and IV, isomers of oxalocrotonate (2-hydroxymuconate); V, vinylpyruvate; VI, 4-hydroxy-2-oxovalerate; VII, pyruvate; VIII acetaldehyde.

yeast *Trichosporon cutaneum* has been observed to be similar to the degradative pathway of *Pseudomonas putida*. Cells grown on resorcinol contain enzymes that are also involved in the degradation of phenol (Gaal and Neujahr, 1979).

The presence of resorcinol degrading bacteria in the consortium perhaps plays an important role in retting of coconut husks along with the other cultures which have cometabolic activity. The studies with culture C10 with respect to growth, utilization of resorcinol and enzyme activity have been compiled in this chapter. Studies were also undertaken to see the effect of the pure culture and consortium on unretted coir fibre from green and dry husk.

5.1 Materials and method

5.1.1 Culture maintenance and its identification

The bacterial culture C10 isolated from the consortium was streaked on resorcinol mineral medium (sterile and non sterile) and on nutrient agar

for its colony and morphological characteristics. The culture was maintained on slants of nutrient agar and mineral salts medium with 0.05 % (w/v) resorcinol and 1.5 % agar and stored at refrigeration temperature. The isolate was subjected to Gram staining and Gram typing. The culture was taken on a slide in one drop of 3 % KOH solution and mixed thoroughly, the presence of slimy threads of nuclear material was observed. The routine biochemical tests (Schmauder, 1997) were carried out and the isolate identified.

5.1.2 Utilisation of other substrates by C10

The culture was inoculated into mineral salts medium with 0.05-0.2% (w/v) of catechol, orcinol, acetate, glucose, benzoate, protocatechuate and pyrogallic acid and incubated under stationary for 24 hours at room temperature. The visual turbidity was observed and the presence of growth confirmed by plating on nutrient agar.

5.1.3 Growth of the culture

Mineral salts medium with 0.05 % (w/v) resorcinol was used for growth of culture and for transformation studies. 48 h Culture grown

on resorcinol medium was inoculated (5% v/v) in mineral salt medium with 0.05 % resorcinol. and incubated under stationary conditions. Aliquots of the culture broth were withdrawn at intervals of one hour and the absorbance was read at 450 nm on a Beckman DU 640 Spectrophotometer to monitor the growth of the isolate C10.

5.1.4 Analysis of the culture broth for transformation of resorcinol.

Each sample was centrifiged at 4000 g and the supernatant was shaken with diethyl ether and the ether extracts were analysed by TLC and paper chromatography. The concentrated extracts were spotted on Whatman chromatography paper and silica gel plates. The paper was developed in the solvent system benzene: ethyl acetate: acetic acid (85:15:1, v/v/v). Developed chromatograms were visualized by iodine vapours and by freshly prepared phenol specific reagent tetrazotized benzidine. The silica gel chromatograms were developed on the solvent system consisting of benzene : methanol : acetic acid (45: 10 :1, v/v/v) and the spots visualised by exposure to iodine vapour and spraying with 2,4- dinitrophenylhydrazine.

5.1.5 Determination of the mode of cleavage of the aromatic ring by Rothera's Test.

The pathway for the degradation of the aromatic ring, via the ortho- or meta- cleavage, was tested by growing the culture stationary in 0.05% sodium benzoate in mineral salts medium for twenty four hours under stationary conditions. Ten mL of the cell suspension was concentrated to 2 mL (≅5000 mg dry weight per litre) by centrifugation at 4000 g. Concentrated cells (0.5 ml) were resuspended in 2 mL of 0.2M Tris (Hydroxymethyl) aminomethane (tris) buffer (pH 8.0), supplemented with 0.5 mL of toluene to solubilize the cell membrane and shaken with 0.2 mL of a 1.0M catechol solution. The appearance of yellow colour within minutes is indicative of "meta" cleavage activity. In the absence of yellow colouration, the reaction mixture was incubated for one to twelve hours. after , 1 g of $(NH_4)_2SO_4$, one drop of ammonia solution (28%) and five drops of freshly prepared 1 % sodium nitroprusside were added. A puple colour indicated "ortho" cleavage (Shih Chien- Chun et al.,1996)

5.1.6. Enzyme assay of 1,2- dioxygenase

The culture was grown on mineral salts medium with0.05 % sodium benzoate as the carbon source for 24 hours. The culture broth was centrifuged , the pellet obtained. The cells were suspended in 20μL of tris buffer and transferred to a 10 mL beaker. The beaker was placed into a100 mL beaker containing ice cubes and the cells were lysed by sonication for 60 seconds in two pulses each of 30 seconds duration, centrifuged at 8000 g at 4^0 C for thirty minutes. The supernatant was used as the enzyme source for the assay.

The assay mixture contained in a final volume of 3.0 mL within a quartz cuvatte with a 1 cm light path, 0.1mM catechol, 1mM EDTA, 33 mM Tris-HCl buffer, pH 8 and from 1-10 milliunits of enzyme activity (lysed cell supernatant). The reaction was initiated by addition of enzyme and the activity monitored spectrophotometrically at 260 nm.

5.1.7 2,4-dinitrophenylhydrazine derivative

The culture broth of cells incubated with resorcinol for twelve hours was centifuged and the supernatant extracted in ether. 2,4- dimitrophenylhydrazine was added to the residue of the ether extract and kept overnight in cold.

5.1.8 Spectrophotometric analysis of resorcinol and orcinol transformation using resting cells of C10.

The culture was grown on nutrient broth/ mineral salts medium with sodium benzoate as the carbon source for 24 hours. The culture broth was centrifuged , the pellet obtained was washed with phosphate buffer and the cells suspended in 0.2M phosphate buffer, pH 7 with 0.05% resorcinol / orcinol. Samples were withdrawn at hourly intervals for 48 hours and scanned on Shimadzu 1601 UV-VIS spectrophotometer, to determine the presence of peaks and absorbance at different wavelengths.

5.1.9 Isolation and detection of plasmid in C10 isolate

Harvesting and lysis of bacteria.

A single bacterial colony of the isolate C10 grown on 0.05% resorcinol was transferred into 2 mL of Luria- Bertani medium and incubated overnight with vigorous shaking. 1.5mL of the culture in a microfuge tube was centrifuged at 12,000 g for 30 seconds at 4 ^{0} C. The medium was removed by aspiration and the pellet dried. The bacterial pellet was resuspended in 100 µL of ice cold Solution I (Appendix) by vigorous vortexing and 200 microlitres of freshly prepared Solution II was added and mixed by rapidly inverting the tube five times. 150 µL of ice cold Solution III was added and gently vortexed in an inverted position to disperse the Solution III through the viscous bacterial lysate. The tube was stored on ice for 3-5 minutes and centrifuged at 12000 g for 5 minutes at 4 ^{0} C in a microfuge tube. The supernatant was transferred to a fresh tube and the DNA precipitated with two volumes of ethanol at room temperature, mixed by vortexing, and allowed to stand for 2 minutes at room temperature. The mixture was centrifuged at 12,000 g for 5

minutes at 4 ^{0} C in a microfuge and the supernatant removed by gentle aspiration and the pellet dried. The pellet of double stranded DNA was rinsed with one mL of 70 % ethanol at 4 ^{0} C and the supernatant removed. The nucleic acid was dried in air for 10 minutes and redissolved in 50 microlitres of Tris EDTA (pH 8.0) containing DNAase free panceatic RNAase (20 µL /mL), vortexed briefly and stored at –20 ^{0} C.

Electrophoretic mobility of the extracted DNA

Preparation of gel slab.

0.7% agarose was prepared in 1 X TAE buffer by heating in a microoven for 2 minutes. The platform for electrophoresis was sealed on the open sides with leucoplast. The comb was adjusted to 1 mm above the gel base and 1.5 cms from one sealed side. To the molten agarose (50 mL) 2 microlitres of ethidium bromide was added from a stock (10 mg/mL) to get a final concentration of approximately 4 µg/mL and poured on to the platform to a thickness of 0.5 cm and allowed to set at room temperature. After setting , the comb and the leucoplast were carefully removed. The gel was placed in the

electrophoretic chamber and the 1 X TAE buffer was added to the chamber till the gel was just below the buffer.

Loading : 10 μL of the sample was mixed with 4 μL of tracking dye bromophenol blue 0.2 %) in the sample slots of the agarose gel using a micropipette. The lid of the chamber was closed. The electrodes were connected to the power supply for flow of power at 80 V and electrophoresis was carried at that constant voltage for two hours. The gel was observed on the UV photodyne transilluminator (Maniatus, et al., 1989).

5.1.10 Biosoftening and biobleaching of mechanically extracted green husk and dry husk fibre

Studies on brightening effect of the consortium and the pure culture C10 on mechanically extracted (unretted) green husk and dry husk were carried out in two sets. Set I comprised of taking two grams of each type of fibre in 400 mL beakers containing mineral salt medium (100 mL). The 48 hour old culture of the consortium and C10 were added in the concentration of 5% and 10% (v/v). The contents were incubated stationary at room temperature. In Set II, two grams of each type of fibre was added to 48 hour old culture suspension (100 ml) of the consortium and C10 separately and the

contents were incubated as above. The experiment was carried out in duplicate and untreated controls were maintained for comparison. In both the sets the incubation period was kept at 15 days at room temperature after which the fibre was removed, washed with water and air dried. The brightness was determined visually and the softness assessed by physical touch and feel method.

5.2 Results

The isolate C10 formed pinnate translucent colonies on resorcinol medium. The growth was faster on mineral salt medium sterilized with 0.05% resorcinol where colonies appeared within 24 hours. The growth was slow and colonies appeared in 48 hours on sterilized mineral salts medium to which resorcinol was added aseptically.

Microscopic examination of the Gram stained culture revealed it to be Gram negative. The Gram typing by the KOH rapid test

according to Gregerson confirmed its Gram negative characteristic. As per Bergey's manual of determinative bacteriology, on the basis of its positive response to the oxidase test, utilisation of glucose, non hydrolysis of gelatin and starch and growth at 4^0 C the isolate was identified as *Pseudomonas putida* (Table 5.2).The isolate was also capable of utilizing different organic compounds such as orcinol, glucose, acetate, and protocatechuate (Table 5.3). The visual turbidity in catechol and pyrogallic acid could not be distinguished due to the dark colour of the substrate and therefore the growth was checked on nutrient agar, however the culture failed to grow on nutrient agar.

The culture showed a purple colour on Rotheras test indicating the ortho cleavage of catechol. As per the the Bergey's manual, it has been indicated that *Pseudomonas putida* has an ortho cleavage pathway, thus confirming the identity of the isolate.

Table 5.2- Biochemical characteristics of isolate C10

	Test	Response of C10
1.	Oxidase reaction	+
2.	Gelatin hydrolysis	_
3.	Starch hydrolysis	_
4.	Utilization of glucose	+
5.	Growth at 4^0C	+
6.	Catechol (ortho cleavage)	+
7.	Cell length μm	3.0
8.	Cell diameter μm	0.8

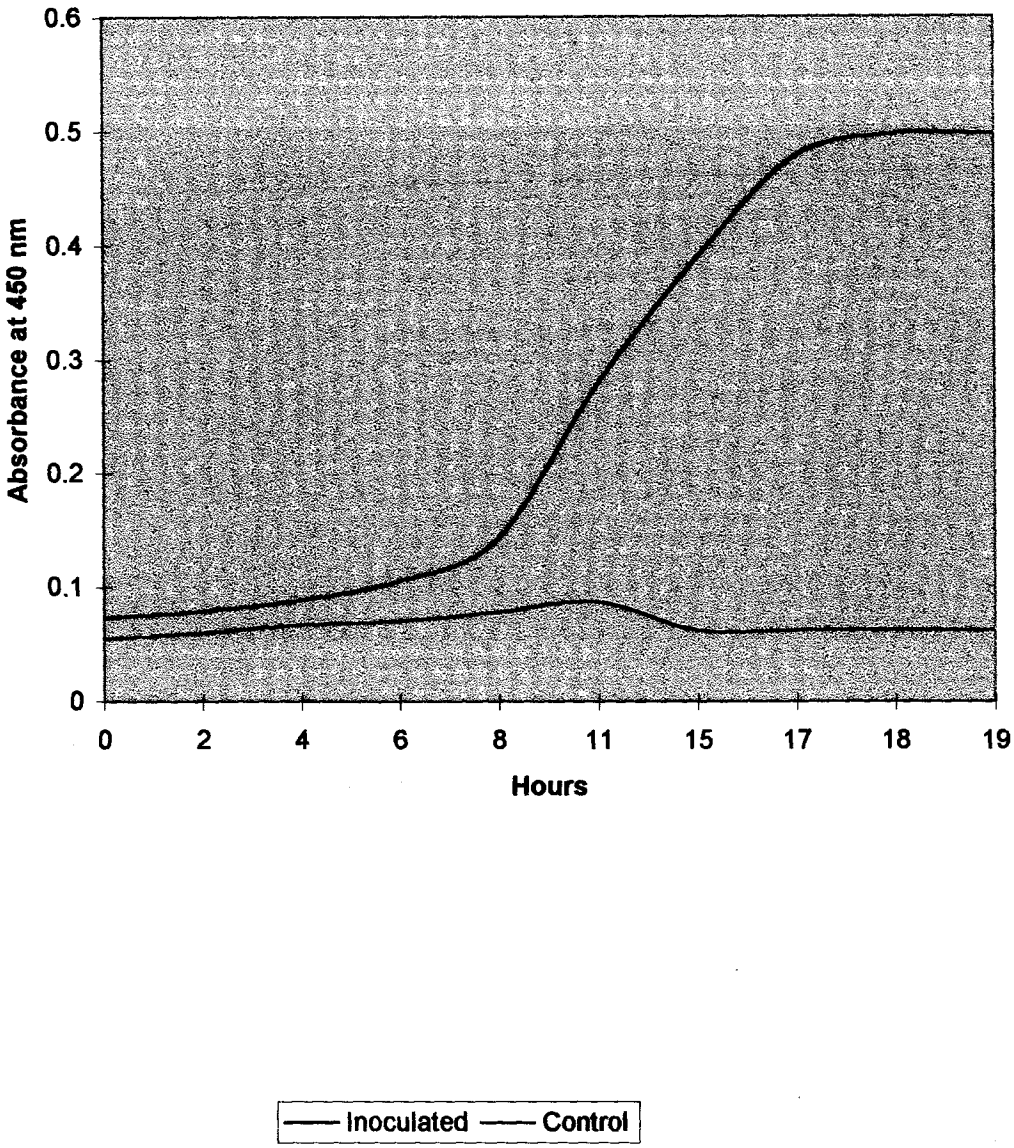
Table 5.3- Utilization of other substrates by C10

Substrate	Incubation period (hours)	Growth
Glucose	24	++++
Acetate	24	++++
Catechol	24	_
Benzoate	24	++++
Orcinol	24	++++
Protocatechuate	24	++++
Pyrogallic acid	24	_

The 1,2 dioxygenase assay using cell lysate of C10 showed the formation of the product muconate , absorbing at 260 nm. The increase was observed from 0.70 to 0.973 in three minutes indicating the activity of 1,2- dioxygenase.

The growth of C10 on mineral salts medium sterilized with 0.05 % resorcinol showed an initial lag of 5 hours the maximum absorbance was seen at 18 hours followed by the stationary phase (Fig 5.4). Further, the ether extracts of the aliquots of the culture broth drawn from inoculated and control flasks with 0.05% resorcinol mineral medium at hourly intervals showed the disappearance of the resorcinol spot from the inoculated flask between 18-20 hours whereas the spot could be detected in the control flask even after 24 hours. This indicates that the culture grows on resorcinol and the growth enters the stationary phase when the resorcinol concentration is reduced and undetectable on the chromatograms. The disappearance of resorcinol from the culture medium after 24 hours could be confirmed by paper chromatography.

Fig.5.4-Growth curve of C10 on resorcinol



The ether extract of the supernatant of C10 cells grown with 0.05% resorcinol showed the appearance of one new spot on the TLC plate having an Rf of 0.4 . The Rf values of resorcinol and catechol spotted as standards were 0.20 and 0.22 respectively. The new spot is attributed to a transformation intermediate during resorcinol catabolism, which is less polar than resorcinol .

Spectrophotometric studies on transformation of resorcinol and orcinol by C10 cells grown on nutrient broth did not show the transformation of these compounds. However, a partial transformation of the resorcinol was observed by cells grown on sodium benzoate. The peaks at different wavelengths showed the presence of hydroxyquinol with sodium benzoate cells indicating the transformation to this compound. (Table 5.4, 5.5). Further, this compound is known to be autooxidised to a quinone which perhaps is seen as a spot on TLC with an Rf of 0.4.

Table 5.4 -Spectral changes during growth of C4 on resorcinol in different media.

Hour	λ nm	Absorbance of cells grown on	
		Nutrient Broth	Sodium benzoate
0	258	-	3.7
	255	4.0	-
1	255	4.0	3.5
	263	-	3.6
2	255	4.0	3.8
	323		1.7
	214	-	3.9
3	272	3.9	-
	263	-	3.7
	257	3.6	-
4	264	3.6	3.9
	272	3.6	-
	230	3.9	-
5	265	3.9	-
	260		4.0
6	273	3.9	-
	204	4.0	-
	263	-	3.9
24	268	3.9	3.0
	323	1.7	-
	214	-	3.3
48	256	1.6	-
	264	-	3.6

Table- 5.5 Study on transformation of orcinol by C4.

Hour	λ nm	Absorbance of C4 grown on	
		Nutrient Broth	Sodium benzoate
0	263	3.7	3.7
1	258	4.0	3.6
	263	3.9	–
2	275	3.9	
	258	3.1	3.6
	214	-	3.9
3	275	3.3	-
	258	2.9	3.7
4	276	3.6	-
	258	2.9	3.9
	212	4.0	-
5	273	3.9	-
	263	-	3.9
24	273	3.9	-
	263	-	3.0
	214		3.3
48	256	1.6-	
	264	-	3.6

The degradation of naphthalene by *Pseudomaonas putida*, phenanthrene and anthracene by *Pseudomaonas putida* has been reported to be plasmid mediated (Menn ,1993). An attempt was made to check the origin of the degradative enzymes of resorcinol. The culture was subjected to phenol alkaline method for isolation of plasmids and the extractes checked on electrophoresis. The electrophoretic pattern however failed to show the presence of any plasmids, in the extracts. It was possible that the resorcinol degradative activity is chromosomal based or perhaps the technique could not separate out the plasmid from the culture. No further attempts were however made to check with alternative methods in view of the objectives of the project.

Biosoftening and biobleaching of coir fibre

The effect of the consortium and the pure culture on the mechanically extracted green husk and dry husk fibre was determined (Table 5.6). The inoculation of the pure culture C10 on green husk fibre in concentrations of 5 and 10 % concentrations was observed to bestow a brightening and softening effect as compared to the control (Plate G). The treatment of the consortium on green husk

Table 5.6-Biobleaching and biosoftening of mechanically extracted green husk/ dry husk coir

Fibre Type	**Inoculum**	**Brightness***	**Softness***
Green Husk	5 % consortium	+++	+++
Green Husk	10 % consortium	+++ +	++++
Green Husk	Control	–	–
Green Husk	48 h consortium	+++	+++
Green Husk	C10- 5 %	++++	++++
Green Husk	C10- 10 %	++++	++++
Dry Husk	5 % consortium	–	+++
Dry Husk	10 % consortium	–	+++
Dry Husk	Control	–	–
Dry Husk	5 % consortium	+	++
Dry Husk	10 % consortium	+	+++
Dry Husk	C10 48 h	+	+

*increasing degree of softness, brightness +, ++, +++, ++++ ,

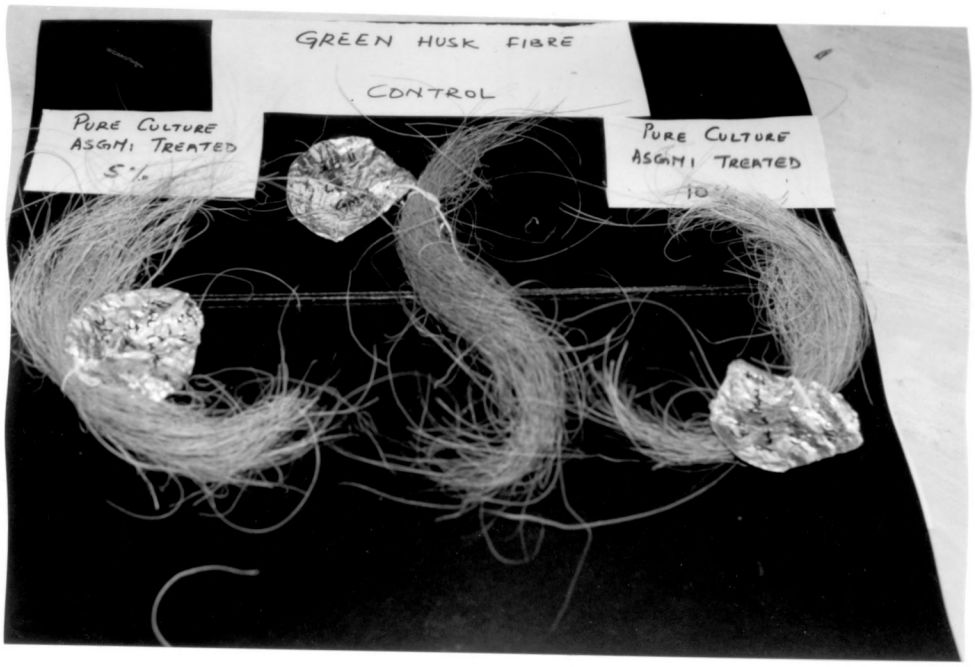


PLATE G

PLATE H

fibre in both 5 and 10 % concentrations also showed a brightening and softening effect on the fibre (Plate H). Although there was no significant improvement in the colour of the dry husk fibre, considerable softening was bestowed by the treatment with the consortium and the pure culture which was comparable to the naturally retted fibre (Plates I, J).

5.4 Discussion

Microorganisms may evolve specific enzymes for the degradtion of non growth substrates or they may be fortuitously be degraded by enzyme systems that have evolved for growth substances. The famous thesis of den Dooren de Jong from Delft listed 80 such compounds that support growth of *Pseudomonas putida* (Jong.,1926) The nature of the substrate determines the mode of its breakdown by bacteria. The metabolic diversity of the aerobic pseudomonads is attributed to the acquisition of nutritional capabilities by the recruitment of enzymes of related metabolic pathways to utilize a non growth substrate. An increasing knowledge of molecular events which lead to adaptation of the microbial

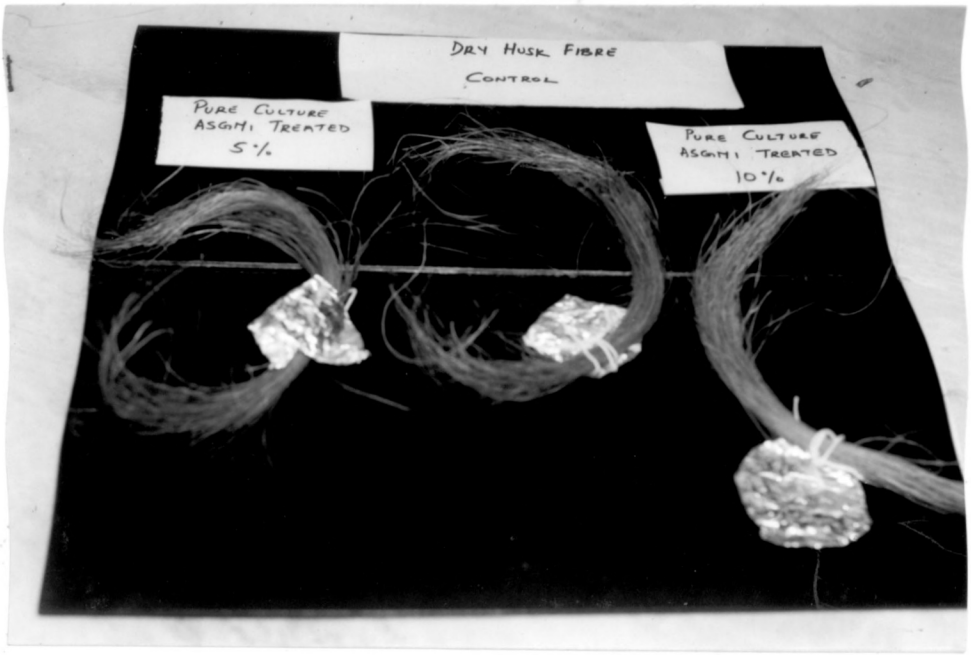


PLATE I

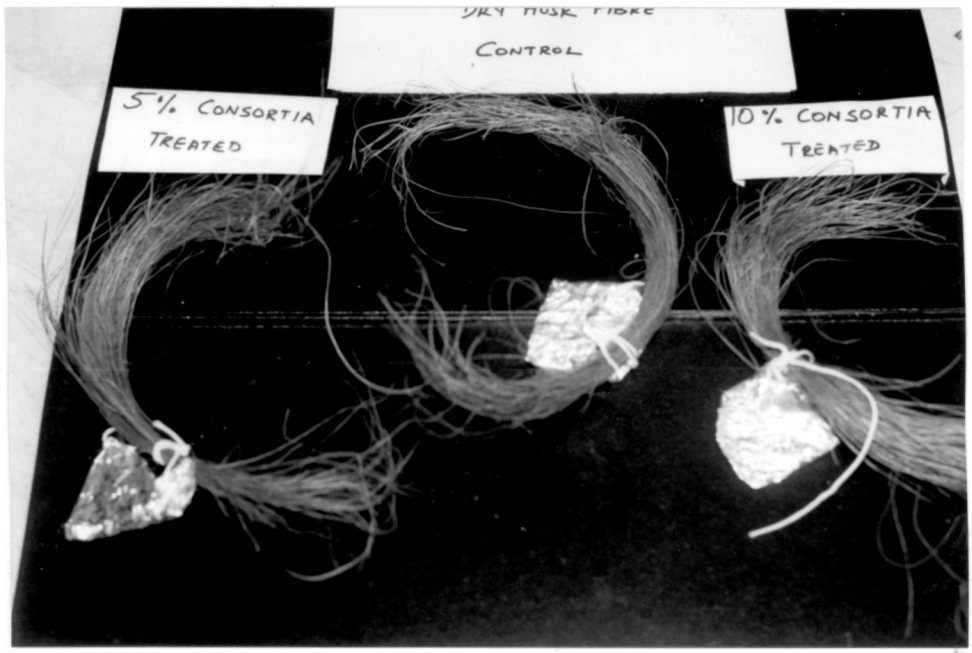


PLATE J

communities for degradation of xenobiotics provide a better insight into the metabolic capacities of microorganisms and may reveal the underlying metabolic diversification in bacteria. Plasmids are extrachromosomal elements carrying genes which, when present in bacteria bestow them with special characteristics. Metabolism of organic compounds is one of the plasmid related properties, besides virulence , exotoxin production, adhesiveness, plant tumor production and biological nitrogen fixation (Internet).

The presence of *Pseudomonas putida* in the consortium and its specific activity on resorcinol has been indicative of its role in degradation of polyphenols in husk leachates. Resorcinol catabolism has been observed in a number of bacteria (Table 5.5) The catabolism of resorcinol by two strains of *P. putida* have been studied in detail (Chapman and Ribbons, 1976). In *Pseudomonas putida*, resorcinol is hydroxylated to give hydroxyquinol (1,2,4-trihydroxybenzene), which is the substrate of ortho and meta ring cleavage enzymes in strain ORC but only for a meta cleavage enzyme in O1. *Pseudomonas*

putida ORC possesses an inducible suite of enzymes for resorcinol caabolism which catalyzes the formation of maleylacetate and its reduction by NADH to β-ketoadipate. In contrast mutants of *P.putida* O1 selected for growth on resorcinol are constitutive for the orcinol pathway enzymes, and pyruvate is formed as one product after meta cleavage of hydroxyquinol. A third pathway for resorcinol catabolism observed in *Azotobacter vinelandii* where it was converted to pyrogallol which formed as a substrate for ring cleavage to form oxalocrotonate, CO_2 , pyruvate and acetaldehyde.

The ability of C10 to use other growth aliphatic and aromatic growth substrates indicates that the enzymes for resorcinol degradation are likely to be induced in the presence of phenolic compounds which is a condition prevaling in the coconut husk retting environment.

Muconates absorb strongly at 260 nm a wave length at which the absorbance of catechol is slight. Therefore the activity of the

oxygenases can be determined by measuring the increment in absorbance at 260 nm in the presence of catechol. Muconate cyclo-isomerase , the enzyme that acts on muconate , requires Mn^{++} and unlike the catechol oxygenases is inhibited by EDTA. Therefore quantitative muconate accumulation from catechol is assured by adding EDTA to the assay mixture. The presence of the enzyme catechol 1,2-dioxygenase system in C10 could be confirmed by its positive response to the Rothera's test. The steady increase in absorbance at 260 nm in the assay mixture comfirmed the presence of an active catechol 1,2 dioxygenase system in the C10 culture.

Studies on the transformation intermediates of resorcinol catabolism by growing C10 on nutrient broth and exposing to resorcinol led to induction of its hydroxylation to hydroxyquinol (Chapman and Ribbons,1976). In the present study with C10 , peaks appear at 272 nm (hydroxyquinol λ_{max} 275nm).Therefore it is envisaged that the transformation of resorcinol by C10 is likely to follow the same pathway of hydroxylation of resorcinol to

hydroxyquinol. The peak at 325 nm observed after 24 hours and 48 hours could be attributed to the product formed during the autooxidation of hydroxyquinol. The autooxidation of hydroxyquinol, which hinders the study of the its degradative pathway, can be inhibited by superoxide dismutase. (Suzuki and Itoh, 1986).

The formation of a precipitate on treatment of the culture supernatant with dinitrophenylhydrazine also indicates the formation of a quinone during growth of C10 on resorcinol.

From the foregoing studies therefore it can be suggested that the C10 isolate in the consortium transforms resorcinol by hydroxylating it to hydroxyquinol followed by ring cleavage and metabolim via the β-ketoadipate pathway. This pathway is a chromosomally encoded convergent pathway for aromatic compound degradation. One branch converts protocatechuate derived from phenolic compounds and numerous lignin monomers to β-ketoadipate. The other branch converts catechol, generated from various aromatic hydrocarbons and

lignin monomers also to β-ketoadipate. The β-ketoadipate pathway (ortho cleavage) is distrbuted widely among taxonomically diverse eubacteria and fungi. It plays a central role in the processing and degradation of naturally occurring aromatic compounds derived from lignin (Harwood and Parales, 1996). Coir extracted mechanically has disadvatages such as poor photostabilty and harsh texture which affect the quality of the coir yarn and products produced. Biological treatments have resulted in improving the quality of the fibre (Ravindranath and Sarma, 1993).

The treatment of the pure culture C10 and the consortium and the on unretted green husk fibre in concentrations of 5 and 10 % was observed to brighten and soften the fibre considerably indicating their potentials in biobleaching and biosoftening. The brightening effect of the consortium and the pure culture on dry husk fibre was comparatively less however the softening effect could be achieved. .

These studies could reveal the properties in bacteria for biodegradation of naturally occurring phenolic compouds which adversely affect the retting process. The potentials of a pure culture of

Pseudomonas putida and a bacterial consortium for application in the coir industry has been elicited. The non requirement of natural retting conditions is advantageous on environmental considerations.

Summary

The coconut husk, an agrowaste yields the "coir fibre" which is of commercial importance owing to its natural origin, physical and chemical properties. The 100% biodegradable nature of coir floor coverings has resulted in a steady increase in demand for them as compared to synthetic materials which result in problems of recycling, fire / health hazards and recalcitrance. Its multiple uses as elegant floor coverings, geotextile material, rubberized coir and in the manufacture of blended products with other natural fibres has increased its global demand. Only 21% of the husk potential in India has been exploited for coir production with export earnings amounting to Rs.200 crores per annum. The coir industry also provides employment to a large sector in the rural coconut growing regions of India.

The extraction of coir fibre from coconut husk is either by natural retting or mechanical extraction. Coir fibre is classified into "white fibre" and "brown fibre" based on the extraction process. White fibre is obtained by the retting of coconut husks while brown fibre is extracted by

mechanical means. Both the processes have their advantages and disadvantages. Retting yields a superior quality coir fibre that is most suitable for the coir industry, however the process is a prolonged one, with drudgery and results in polluting the environment. Mechanical extraction of coconut husk yields coir fibre much faster, however the quality of this fibre is poor with respect to photostability and texture.

A study was undertaken to understand the process of retting under natural conditions and to monitor the variations occurring in the environmental parameters and the chemical constituents of the husk during the period of retting. The pH and salinity were found to fluctuate while the temperature was fairly stable. In the ret liquor resorcinol, pyrogallic acid and protocatechuic acid could be observed. Microbiological analysis of water samples showed a significant count on resorcinol medium within a period of three months with a ratio of total bacterial count to the count on resorcinol medium being 4:1. Different types of colonies were observed on nutrient agar with only one type appearing from resorcinol medium. The husk samples after retting showed a complete reduction in polyphenols and pectin content. The fibre quality at the end of the retting is assessed by flexural

rigidity and the Xenotest showed a Grade II fibre with lignin content of 35.27 %.

An established fact known to cause delay in the retting of coconut husks is the presence of the high percentage of polyphenols. The present studies have hence been carried out with a view to explore the possibility of developing a consortium that can survive and proliferate on the leachates from coconut husk which are rich in phenolic compounds.

Presence of bacteria utilizing the phenolic compound resorcinol, in the water during retting indicates their involvement in the removal of the phenols. Therefore, by allowing the proliferation of a consortium of such phenol degrading bacteria, in the retting environment it was envisaged to accelerate the retting process and transfer it to a closed system, leaving the backwaters free for aquaculture and fishery development.

In this work an attempt has been made to study the natural retting and develop a consortium of bacterial cultures which could reduce the retting

period and also improve the quality of mechanicIly extracted green husk coir fibre.

In natural environments, specific mixed cultures are enriched and their cometabolic activities result in the biodegradation of complex substrates such as tannins, pectins etc. Studies were undertaken to develop a consortium of indigenous bacteria from coconut husks capable of growing on husk leachates. The effect of this consortium on the retting of husks and fibre has been studied in the laboratory system.

The consortium has been developed over a period of ninety days with two subcultures, using coconut husk leachate as substrate. Application of the consortium in 5% and 10% concentrations in PVC tanks under laboratory conditions yielded fibre in three months. The fibre possessed characteristics comparable to the fibre retted in the natural system

Resorcinol was detected as one of the phenolic compounds in the retting environment where the retting process is influenced by presence of these phenolic compounds. One isolate C10 present in the consortium, identified as *Pseudomonas putida* has been observed to utilize resorcinol as sole source of carbon.

This isolate metabolized benzoate via the ortho pathway and degraded resorcinol by hydroxylation to hydroxyquinol, which was autooxidized to quinone and confirmed by DNP. The culture failed to show presence of any plasmid but utilized other phenolic compounds like orcinol, and protocatechuate. The effect of this culture and the consortium on green husk and dry husk coir fibre was assessed.

No significant variation was observed in the environmental parameters in the lab scale study on retting. HPLC analysis of the ret liquor showed the presence of resorcinol for the first two months. The total count

during enrichment showed a significant increase on nutrient agar and resorcinol medium. The viable count in the three tanks did not show a wide variation while the counts on resorcinol agar were found to increase in the inoculated tanks. No colonies were observed from the control tank on resorcinol medium for the first two months.

The bacteria isolated from the consortium were identified as *Bacillus, Micrococcus, Azotobacter, Actinomycetes*. The only isolate obtained on resorcinol media was identified as *Pseudomonas putida*. The potentials of a pure culture of *Pseudomonas putida* and a bacterial consortium for application in the coir industry have been elicited. Retting can therefore be transferred to a closed system without the requirement of natural conditions. The advantage of such a process would be that it can be developed at any site where coconut husk retting needs to be carried out, exploit the husk potential available in that region and reduce environmental pollution.

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APPENDIX

Tetrazotized benzidine

Solution A

Benzidine	:	5g
HCl (conc)	:	14mL

Volume made to 1L with distilled water.

Solution B

Sodium nitrite solution	:	10% (aqueous)

Equal volumes of solution A and solution B mixed before use.

Mineral Salt Medium (Double Strength)

Dipotassium Hydrogen Phosphate	100 mL of stock (12.6%)
Potassium dihydrogen phosphate	20 mL of stock (18.2%)
Ammonium sulphate	20 mL of stock (10%)
Magnesium sulphate	20 mL of stock (1%)
Manganese sulphate	0.2 mL of stock (0.6%)
Sodium molybdate	0.2 mL of stock (0.6%)
Calcium chloride	15 mL of stock (1%)
Ferrous Sulphate*	0.006g
Distilled Water	824.6mL

Add CaCl2 drop by drop by constant stirring.
*Stock solution of Ferrous sulphate was not prepared.

R Medium*

Potassium dihydrogen phosphate	5.4	g/L
Ammonium Sulphate	1.2	g/L
Magnesium sulphate	0.2	g/L
Ferrous Sulphate	0.01	g/L
Sodium Hydroxide	pH 6.8-7.1	

*Chapman and Ribbons. 1976.

Tris (hydroxy methyl) aminomethane HCl
(Tris HCl) Buffer

Solution A- Tris base (0.2M)	:	24.2g
Distilled Water	:	1000 mL
Solution B- HCl	:	4 mL
Distilled Water	:	49 mL

Mix 90 mL of A and 26.8 mL of B to get a buffer of pH8.0

Isolation and detection of plasmid

Solution I

Glucose	:	50mM
Tris-HCl (pH-8)	:	25mM
EDTA (pH 8)	:	10mM

Solution II

NaOH	:	0.2 N
SDS	:	1%

Solution III

Potassium Acetate (5M)	:	60 mL
Glacial Acetic Acid	:	11.5 mL
Distilled Water	:	28.5 mL

Tris Acetate Buffer (pH 8.0)

Tris Base	:	2.42
Glacial Acetic Acid	:	1.00

(Volume made up to 10 mL)

Distilled Water	:	490 mL