DEVELOPMENT OF MICROBIAL TREATMENT OF RET LIQUOR GENERATED IN A COIR RETTING BIOREACTOR

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By

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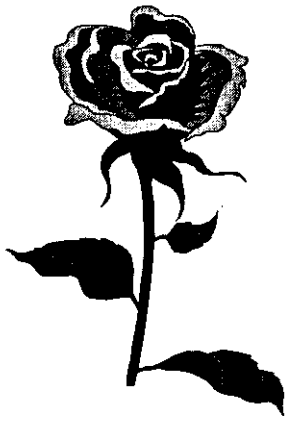

DEDICATED TO THE WELFARE OF

COIR INDUSTRY AND FOR

THE PROTECTION OF

THE ENVIRONMENT

CERTIFICATE

This is to certify that the research work presented in the thesis entitled **"Development of microbial treatment of ret liquor generated in a coir retting bioreactor "** is based on the original work done by Mr.Puthumana Illathu Narayanan under my guidance, in the School of Environmental Studies, Cochin University of Science and Technology, Cochin -682016, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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April 1999

DECLARATION

I hereby do declare that the work presented in this thesis entitled **"Development of microbial treatment of ret liquor generated in a coir retting bioreactor "** is based on the original work done, by me under the guidance of Dr. I.S. Bright Singh, Reader in Microbiology, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682016 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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GENERAL INTRODUCTION

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GENERAL INTRODUCTION

Retting coconut husk in brackish water system is an age old traditional practice carried out for the extraction of coir fibre (Bhat & Nambudiri, 1971)for which the husk is steeped in water for 6-12 months(Menon&Pandalai,1958).To facilitate this Kerala has long stretches of backwaters and estuaries spread along the coast line from north to south. The total area of these ecosystems is estimated to be around 2,42, 600 sq. km(Anon, 1987). They form important areas of fisheries, transportation and other human uses and have played significant role in the socio-economic and cultural history of the state. Cochin backwaters the largest estuarine system in the west coast of India is known for its high productivity, and support rich and diverse aquatic life.

The term retting is a technical form of the word 'rotting' and designate the process of decomposition of tissues surrounding the vegetable fibres. As a result of retting large quantities of organic substances including pectin, pentosan, fat, tannins, and also toxic phenols are liberated into water. This practice not only reduce the total area available as shrimp and fish nursery grounds, but also considerably alter the physical factors like tidal flow, water circulation and currents. All these processes contribute to pollution of water

and air and mercaptan like smell pervade the yards and nearby areas. This is reported to have devastating effect on the flora and fauna.

Most of the retting areas are concentrated in the back waters of Trivandrum, Quilon, Alleppey, Ernakulam, Trichur and Kozhikode districts which include nearly 30,000 retters. Majority of the retting areas are located in the Alleppey district assuming 45% of the total. The number of units in Kerala engaged in retting is estimated to be 69456(Anon, 1985) and in the state about 233.9 lakhs of coconut husks are retted annually.

Chemically, coir is composed of cellulose, cellulosan, lignin, pectin and hemicellulose, the percentage of which vary very much depending upon the age of the nut, from which the coir is derived. The biological retting of coconut husk is not one of pectin decomposition as in the case of flax or jute, but of disintegration of a phenolic cement binding fibre with refuse.

Traditional coir retting is practiced by soaking the husk in saline waters or the husk are steeped in pits dug out with in the reach of tidal action. Retting is also carried out in canals which have influence of water from river and free connection to the sea. In such areas pits of four or five meters deep are dug out and the husk are dumped and covered with mud. In estuaries where there is tidal influence, husks are bundled together called

'mallis' and dumped in water. Rocky shores are also under use for retting where the cavities of rocks or space between adjacent rocks or on the seashore are used. Another method practiced is by arranging the husk in coir rets in to bundles and floating them freely in the backwaters until they get soaked, becomes heavy and gradually sink to the bottom. Often the bundles are weighed down by piling on their top with mud collected from the bottom of the retting yard.

Unlike other plant fibres, which get released with in a few days of retting coir fibre takes a long time to get separated from pith, the binding material; the retting time varying from 4-12 months depending on the area and the variety of yarns required to be produced.

Properly retted husk are removed from rets, washed free of adhering slime, mud and sand and exocarp is then easily peeled of with hand. The husks are beaten there after on granite stones with wooden mallets in order to separate the fibres from cork and pith. A further cleaning by hand, rewashing in water and light beating again are also restored with a view of getting rid of the pith adhering to the fibres. The fibres are then spread out in the sun to dry (Fowler and Marsden, 1925).

It is generally agreed upon that stagnant water is unsuitable and that a periodical change is necessary which takes place during the rise and fall of

the tide amounting to about $1^1/_2$ feet in the backwaters and channels extending along the whole coast. Experiments in stagnant water have shown that the action soon ceases when the husk becomes dark in color.

When the husk is soaked in water, for the first few days, it gets tinged distinctly brown due to the organic liberated from it, but the colouring principle is not entirely removed until retting is completed and danger of discolourisation is always present until the retted fibres have been well washed in water. After a few days the water becomes somewhat milky and this increases until after about thirty days a scum may form on the surface, a distinct smell of sulphurated hydrogen becomes noticeable and there is a marked increase of temperature in the mass of husk, bubbles of gas frequently rising from it. The turbidity, gas formation, smell and rise of temperature increases from the third to the fifth month, but after six months the water becomes clearer, the smell and evolution of gas appearing to diminish (Fowler and Marsden, 1925).

Polyphenols and pectins are major constituents representing as much as 75 to 76 and 16 to 17gm per Kg of husk material (Jayasankar. 1966 and Bhat 1969), the polyphenols getting constantly leached out. A positive correlation has also been found to exist between the progress of the extent of retting and the rate of disappearance of these compounds. The pectinolytic

activity has been attributed to bacteria such as *Micrococcus*, *Aerobacter, Bacillus, Arthrobacter, Escherichia, Paracolobacterium* and by the yeast *Rhodotorula* and *Cryptococcus* (Jayasankar, 1966). Associated with the leaching out of polyphenols are *Pseudomonas* sp and *Micrococcus* sp(Jayasankar and Bhat,1966). Further oxidation of phenol produces a diffusable melanin like pigment which is also released in to the medium during coir fermentation. The association of unusual bacteria and yeasts during the decomposition of pectin and phenolics respectively bestows on the microflora of the coir rets as distinctive character hitherto unknown to other retting material. Markedly offensive odours resembling those of hydrogen sulphide emerate from the retting zones during the decomposition of of pectin. A rise in turbidity, gas formation, foul smell, and depletion of oxygen are few of the many noticeable features associated with retting (Azis and Nair, 1978).

All these foreign constituents have contributed to convert this highly productive aquatic biotope in to a barren noxious zone. These conditions are likely to deteriorate further consequent to expansion of the coir industry. These have resulted of late in the complete failure of fisheries and the extermination of the flora and fauna of certain areas. An ecosystem with microaerobic and anaerobic properties has developed in these backwaters.

An inverse relationship is discernible between the fluctuations of hydrogen sulphide and oxygen (Azis and Nair, 1978).

Ajithkumar and Alagarswami,(1986) on studying the effect of pollution due to coconut husk retting on reproductive potential of green mussel *Perna viridis* observed localized pollution in an intertidal mussel bed off Elathur, near Calicut along the Malabar coast due to coconut retting and was found to affect the reproductive potential of the population of the above animal population . The environmental changes brought about by retting at the site were an offensive odour resembling that of hydrogen sulphide, relatively higher turbidity and temperature, lower levels of dissolved oxygen and reduction in phytoplankton productivity. Growth of mussels at the site was very much negatively affected. The population was composed entirely of males over the observation period of an year. Spawning was fractional and incomplete in individual males while it was normal in the stocks in the unaffected areas of the mussel bed.

As described above, coir retting, even though traditional and age old, is not an environment friendly practice primarily due to the fact that it is performed in backwaters which support rich and diverse life forms and provide crucial nurseries for shrimps and fishes as well and serve as the

habitat, for oysters, clams and mussels affecting adversely the productivity of the backwaters.

During the initial phase of retting enormous polyphenols are liberated in to water which cause acute toxicity to the biological community. During the retting process lignolytic, pectinolytic, cellulolytic and hemicellulolytic organisms altogether degrade the fibre binding material of the husk liberating large quantities of organic matter into the environment which include pectin, pentosans, polyphenols etc. This leads to heavy biological demand and enormous production of hydrogen sulphide. Obviously community diversity of plankton decreases (Remani *et al* 1989) and ecosystem with microaerobic and anaerobic properties emerge in retting zones which subsequently exterminate the flora and fauna (Azis and Nair 1978). Survey of fishing wealth showed that the fishing in the retting zones was adversely affected in terms of community diversity as well as zoomass productivity. Only tolerant species like *Arius* sp., *Etroplus* sp. etc., were found in the retting grounds. Fish biomass in the retting zones was three times less than that in non retting zones. Similarly the abundance of mollusks and crustaceans was also much greater towards the non retting zone compared to the retting yard (Azis and Nair,1978).

For any industry to achieve sustainability the raw material should be with consistently good quality. This requirement is not always met with coir industry where the quality of fibre, the raw material, is left to the vagaries of nature, as the quality varies from season to season and place to place. Retting yards with very good tidal influence generate comparatively good quality fibre because aeration leads to faster degradation of the products of fermentation which under normal conditions tend to accumulate and adversely effective the quality (Bhatt and Nambudiri, 1971). A better control over the quality of this raw material has to be acquired for the smooth running of industry.

May be because of its traditional nature, the people who are involved in this industry are the financially weaker sections of the society and this is especially true with those who work in the retting yards. With the argumentation of literacy programmes the newer generation becomes increasingly reluctant to work in such environments and all of them aspire for 'decent jobs'. This sociological transition shall adversely affect the industry in large in the next century. The longer duration required for the completion of retting makes one to wait longer for getting the returns from the money invested. Further it is complained that the entire lot of the husks dumped are not able to be retrieved always which result in substantial loss.

Painfully enough those who work continuously in the unhygienic retting yards do suffer from skin diseases too(Anon, 1998).

All these indicated that an alternative process for the production of retted fibre has to be evolved which would still perpetuate the pristine glory of the golden fibre of Kerala known world ever. Such an alternative method should be environment friendly and economically viable too.

In this context a Coir Retting Bioreactor was conceptualized and developed (Anon, 1998) which consisted of three major components.

a) Polyphenol stripping device

b) Retting reactor, and

c) Ret liquor treatment system

The present work is concerned with the development of the third component, the Ret Liquor Treatment System.

In this coir retting bioreactor ret liquor of two category are generated. In the first instance that is during the pre retting operation copious polyphenolic compounds are liberated which now as per the existing configuration of the reactor, can be removed by reverse osmosis. But, at the same time if the polyphenols are not commercially used at any time there must be sufficient proversion also to degrade the compound biologically. Secondly during the microbial retting process the remaining polyphenols

along with the once generated by the hydrolysis of the lignin also get liberated in to water. The treatment system envisaged should be capable enough to manage polyphenols of these two categories so that the recirculatory configuration is attained. The ret liquor generated where 20ppt seawater is used in the reactor contained 0.358 $\pm$ 0.24 $mg.ml^{-1}$ polyphenols, 5.67 $mg.ml^{-1}$ sugars 19.47 $\pm$ 5.7 $mg.ml^{-1}$ proteins and 16.11 $\pm$ 6.28 $mg.ml^{-1}$ lipids and 50 $\pm$ 25 BOD (Anon,1998). This is the upper limit which one can expect in the reactor and in the real life situation the concentrations are likely to be very low. The treatment system developed should be capable enough to bring down the highest concentration of the above components drastically so that the effluent generated is fit to be reused in the retting reactor.

For the development of such a system the followings were required:

a. Appropriate groups of microorganisms which can uptake polyphenols , proteins sugars and lipids as sole source of carbon and energy.

b. Appropriate design of the treatment system

c. Fabrication of the system and standardization

The following chapters deals with the above vital components of the ret liquor treatment system.

CHAPTER - 2

DEVELOPMENT OF MICROBIAL CONSORTIA FOR RET LIQUOR TREATMENT

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DEVELOPMENT OF MICROBIAL CONSORTIA FOR RET LIQUOR TREATMENT

2.1. INTRODUCTION

For the development of ret liquor treatment systems an assemblage of microorganisms capable enough to degrade the polyphenols, proteins, lipids, carbohydrates, cellulose, hemicellulose and pectins in ret liquor, generated during the retting process, is required. It was quite appropriate to make a search for such organisms in the retting ground itself as due to years together of the retting ~~of the retting~~ there could have happened segregation, colonisation, and even mutation of the type of organisms which can cleave the fairly resistant polyphenols. Before isolating such organisms the samples were subjected for selective enrichment in ret liquor to eliminate the used organisms. This chapter deals with the procedure for the isolation of such organisms followed and the description of organisms obtained.

2.2 MATERIALS AND METHODS

For generating appropriate microbial consortia towards the envisaged ret liquor treatment system three types of enrichment procedures were executed as follows:

2.2.1. Successive enrichment in husk infusion

2.2.1a Preparation of husk infusion

Husk infusion was prepared by boiling 500g freshly split husk in 20ppt seawater for 30 minutes. The supernatant was decanted off and the salinity determined and corrected to 20ppt by adding distilled water. For preparing the enrichment medium 225ml husk infusion was supplemented with NH_4Cl (2gm) , $NH_4 NO_3$ (0.5gms) and yeast autolysate (250mg) and altogether autoclaved at 15 lbs for 15min. The system was completed by inoculating the medium with 25ml sample prepared out of sediment obtained from a retting ground at Chellanum north. The sediment sample (20gm) from the above retting ground was suspended in 20ppt sterile seawater (100ml) and agitated vigorously for 5 minutes. After allowing the sediment to settle for a brief period 25ml supernatant was inoculated in 225ml husk infusion medium. The preparation after adjusting the pH to 7 was

incubated over magnetic stirrer and monitored for the factors such as colour , pH, and total polyphenols, once in every 24hours.

2.2.1.b. Determination of colour

Colour was determined following APHA (1989) by visual comparison of the sample culture with known concentration of coloured solution. A series of known concentrations of chloroplatinum standard was made in distilled water and the sample was compared visually to determine the colour which was expressed as colour units.

2.2.1.c. Determination of pH

Change in pH of the culture was determined by using narrow range pH paper (E-Merck) which was already calibrated using a pH meter (Elico model). Bits of pH paper was placed on ceramic tiles and a drop of culture was aseptically removed and placed on it. The change in colour was noted after one minute and within two to three minutes.

2.2.1.d. Determination of total polyphenols

Total polyphenol content in the enrichment system was determined colourimetrically following APHA(1989). In this method tungstophosphoric acid and molybdophosphoric acids were reduced by the aromatic hydroxylated groups of tannins and lignins. In this method to 50ml portions of the sample 1ml Folin phenol reagent and 10ml carbonate tartarate reagent were added and the blue colour developed after 30 minutes was read at 700nm. Tannic acid was run as the standard along with reagent blank which was run in distilled water.

2.2.1.e. Successive enrichment

On attaining sufficient disappearance of polyphenols from the culture broth, 25ml of the culture was passaged to another broth of 225ml having the same composition and incubated for the same period along with monitoring of the same parameters. The process was repeated three more times and at every stage of transference heterotrophic bacteria were isolated as described below.

2.2.2. Isolation of heterotrophic bacteria from the enrichment systems

The cultures developed in the husk infusion was serially diluted at every stage of subculturing / passage with 20ppt aged seawater as diluent and inoculated by pour plate method on to nutrient agar prepared in 20ppt aged sea water, husk infusion prepared with 20ppt aged seawater, and also in Sabouraud dextrose prepared in husk infusion.

Nutrient Agar (NA)

Peptone	:	0.5g
Beef extract	:	0.5g
Yeast extract	:	0.1g
20 ppt aged seawater	:	100ml
Agar	:	2.0g
PH	:	7.5 $\pm$ 0.1

Husk Infusion Nutrient agar (HINA)

Peptone	:	0.5g
Beef extract	:	0.5g
Yeast extract	:	0.1g
Husk infusion prepared in 20ppt sea water	:	100ml

Agar	:	2.0g
pH	:	7.5 $\pm$0.1

Husk Infusion Sabouraud Dextrose Agar

Dextrose	:	2.0g
Peptone	:	1.0g
Husk infusion in 20ppt sea water	:	100ml
Agar	:	2.0g
pH	:	4.8

pH was adjusted by adding 4ml tartaric acid after autoclaving the medium at 10 lbs for 10 min.

The plates were incubated at 28$\pm$0.4^{0}C for 7 days and 20 bacterial colonies from each sample and medium were isolated onto respective medium slants .Altogether 180 colonies were isolated which were subsequently purified and stocked in media vials overlaid with sterile liquid paraffin for further study.(Fungi were not found to have developed on husk infusion Sabouraud dextrose agar plates.)

2.2.3. Capability of the isolated heterotrophic bacteria to utilize tannic acid, pyrocatechol and pyrogallol.

A mineral base medium having the following composition was used.

NH_4CL	:	5g
NH_4NO_3	:	1g
Na_2SO_4	:	2g
K_2HPO_4	:	3g
KH_2PO_4	:	1g
NaCl	:	10g
Yeast extract	:	10mg
Thiamine	:	0.1mg
Trace metal mixture	:	5ml
Distilled water	:	1000ml

Trace metal mixture

$Fecl_3$	:	80mg
$Mncl_2$	:	90mg
$Co(NO_3)$	:	65mg
$MgSO_4$	:	12.5mg
$ZnSO_4$	:	20mg
$CuSO_4$	:	0.5mg
$CaCl_2$	:	675mg
Distilled water	:	500ml

While preparing the medium yeast extract and thiamine were filter sterilized and added to the autoclaved medium.

The above medium was aseptically divided into three lots and to one lot filter sterilized tannic acid to the second and third lots pyrocatechol and pyrogallol respectively were incorporated separately after autoclaving at 10 lbs for 10 min. to a final concentration of 0.5% (w/v).The three sets of media thus prepared were dispensed into tubes at 3ml aliquots each .The control set of tubes included one set of medium without any carbon source.

Inoculation and incubation

All tubes were inoculated with nutrient agar grown slant cultures and incubated at 28± 0.4^{0}C in a BOD incubator for 7days. Turbidity as a result of growth was considered the capability of the organisms to use the organics as carbon and energy source. Altogether 220 bacterial isolates were subjected for the above test.

2.2.4 Determination of the capability of the isolated bacterial cultures to degrade the polyphenolics present in ret liquor

The isolated cultures were subjected for tests to evaluate their capability to degrade polyphenolics present in ret liquor. Ret liquor was prepared by steeping freshly split husk in 20ppt seawater for 72 hours. It

was filtered and stored at 20^0C till used. Hundred ml aliquots were autoclaved in 250ml conical flasks at 10 lbs 10 min. The flasks were inoculated from a nutrient agar slant grown culture and incubated at 28 ± 0.4^0C for 72 hours as static cultures. Controls included uninoculated husk infusion incubated under the same conditions. Initial and final polyphenol content was estimated following the methods described under section 2.2.1.d

2.2.5 Determination of the percentage consumption /degradation of polyphenols by the selected cultures.

Tannic acid was used as the substrate in this determination. A 0.5% tannic acid solution was prepared in the mineral base medium (for composition see section 2.2.3) and divided into aliquots of 20ml each in 100ml conical flasks. They were autoclaved at 10 lbs for 10 min. and inoculated from a bacterial suspension of having 0.5 O.D prepared in mineral base medium. In all cases one loopfull of the culture was inoculated uniformly after determining the initial polyphenol content. The preparation was incubated at room temperature over rotary shaker at 100 rpm. At 24^{th}, 48^{th}, 72^{nd},120^{th}, 40^{th}, 168^{th} and 192^{nd} hours of incubation the available

polyphenol content in each flask was determined from which the percentage consumption of the polyphenol was worked out. Polyphenol content was determined following the colourimetric method as described under section 2.2.1.d. Uninoculated controls were run in parallel.

2.2.6 Determination of percentage consumption of tannic acid as sole source of carbon and energy by the segregated cultures.

The segregated bacterial cultures which were found to have the capability to utilize the tannic acid as sole source of carbon and energy were again subjected for a study oriented towards assessing the percentage consumption of tannic acid in a mineral base medium. For this tannic acid was incorporated in to the mineral base medium having the composition as described under section 2.2.3. and dispensed in to 250ml conical flask at 100ml aliquots and autoclaved at 10 lbs for 10min. The cultures were grown in nutrient agar slants and harvested in the mineral base medium and O.D adjusted to 0.5 at 600nm. From this cell suspension 0.1ml inoculum was transferred in to the above media and incubated at room temperature($28\pm0.5^{0}C$) for 5 days. The control flask included uninoculated ones incubated under the same conditions.

Initial and final concentration of the tannic acid was determined and the percentage consumption was worked out over a period of 5 days.

2.2.7 Hydrolytic properties

2.2.7a Lignolysis

Lignolytic property of the segregated cultures were tested in Crawford's Agar having the following compositions per litre.

Glucose	:	1g
Yeast extract	:	1.5g
Na_2HPO_4	:	4.5g
KH_2PO_4	:	1g
$MgSO_4$	:	0.12g
NaCl	:	0.2g
$CaCl_2$	:	0.5g
Tannic acid	:	5g
Agar	:	20g
pH	:	7.5
Distilled water	:	1000mL

The medium was autoclaved at 10lbs for 10 min. and poured in to plates. The cultures were spot inoculated and incubated at 28 ± 0.5^0C for seven days. The colonies were examined for brownish diffusing zone around them indicating the production of polyphenol oxidase characteristics of the presence of lignin degradation system(LDS) with the cultures. Besides, the

plates were examined for halozones around the colonies indicating the total utilization of tannic acid as carbon and energy source.

2.2.7.b Cellulolysis

Cellulolytic potential of the segregated cultures were tested in mineral base medium supplemented with filter papers. The mineral base medium having the composition as detailed under section 2.2.3. was dispensed in test tubes at 10ml aliquots and weighed strips of filter papers, one set of Whatman No.1 and another set of ordinary filter paper, were introduced in such a way that 0.5cm of filter paper was projecting out of the medium. The tubes were autoclaved at 10 lbs for 10min and inoculated from a nutrient agar grown young culture (24 hour growth). The inoculated tubes were incubated at $28\pm0.4^{0}C$ for 10 days. Whenever evaporation and subsequent lowering of the level of medium was observed the loss of water was compensated by adding distilled water. Uninoculated controls were also run along with the test.

On completion of 10 days incubation the tubes were observed for visible degradation of the filter paper, loss of weight and reducing sugar

output in each case. A culture was designated positive when it caused any of the above tested changes.

2.2.7.c. Hemicellulolysis

For checking the potential of the cultures to assimilate hemicellulose (Xylan) the above basal medium was used supplemented with 0.2% Xylan(Sigma Chemical Company, USA) and dispensed at 2ml aliquots in to culture tubes and autoclaved at 10lbs for 10 min. The tubes were inoculated from a freshly grown culture and incubated at $28\pm0.4^{0}C$ for 7 days. Development of turbidity was considered as growth achieved due to the utilization of hemicellulose as sole source of carbon and energy.

2.2.7.d. Proteolysis

Proteolytic property of the cultures were examined by determining their gelatinolytic potential in nutrient gelatin.

Nutrient gelatin:

Peptone	:	0.5%
Beef extract	:	0.5%
Yeast extract	:	0.1%
NaCl	:	2%
pH	:	7.5
Distilled water	:	100ml

The above nutrient medium supplemented with 15% gelatin was autoclaved, poured in to plates. The cultures were spot inoculated and incubated at $28\pm0.4^{0}C$ for 72 hours. Gelatinase production was tested by flooding the plates with Mercuric chloride solution of the following composition.

Mercuric chloride solution

HgCl2	:	15g
HCl	:	20ml
Distilled water	:	100ml

Cultures with a clearing zone around was designated as gelatinolytic and accordingly proteolytic.

2.2.7.e. Lipolysis

Tween compounds are generally used as the substrate for detecting the hydrolysis of fats . In addition to tween, the medium must contain a soluble calcium salt so that the released fatty acid is then turned to insoluble calcium salt around the colony. The medium contained the following ingredients.

Peptone	:	10g
$CaCl_2$	:	0.1g
Tween 80	:	10g
NaCl	:	20g
Agar	:	20g
Distilled water	:	1000ml
pH	:	7.2 ± 0.2

The medium was autoclaved and poured into plates and the organisms are spot inoculated and incubated at 28 ± 0.4^0C for 7days. Opaque zone surrounding microbial growth consisted of calcium salts of free fatty acid and are usually taken as being indicative of a positive lipolytic activity.

2.2.7.f . Amylolysis

Amylase production was checked in nutrient agar supplemented with 2% starch. The medium was autoclaved and poured into plates and spot inoculated and incubated for 72 hours.

The plates were then flooded with Gram's iodine

Grams iodine

Iodine	:	1.0g
KI	:	2.0g
Distilled water	:	100ml

Amylase producing organisms showed clearing zone around them and the colour of the zones depended on the degree of the hydrolysis of starch. The zones with reddish brown indicated that the starch was hydrolysed to dextrin and when the hydrolysis further continued the zone became glassy. Amylolysis was ascribed to both these reactions.

2.2.7.g. Pectinolysis

Ability of microorganisms to grow in a defined medium containing pectin or pectic acid as the sole source carbon is not a reliable criterion of their ability to degrade pectic substances. This is largely because most commercially available pectins are chemically complex and can undergo further changes during sterilization (Jayasankar and Graham,1970). Therefore to determine whether the organisms are pectinolytic in addition to testing their ability to utilize it as the sole source of carbon and energy an

alternative method described by Jayasankar and Graham (1970) was as followed.

In the first category assays mineral base medium prepared as per the composition described under section 2.2.3.was supplemented with 0.2% pectin (BDH)(apple pectin dimethylated at pH 8.6)dispensed into culture tubes at 3ml aliquots autoclaved at 10 lbs for 10min.The tubes were inoculated from freshly grown culture in nutrient agar supplemented with 2% NaCl and incubated for 7 days at $28\pm0.4^{0}C$ .The resultant turbidity was considered the capability of the organisms to utilize pectin as the sole source of carbon and energy.

The medium employed for examining pectinolytic property through the alternative method has the following composition:

Pectin (BDH) (Apple pectin ,demethylated at pH 8.6)	:	5.0g
K_2HPO_4	:	0.5g
$MgSO_4$ $7H_2O$	:	0.1g
NaCl	:	20.0g
$CaCl_3$ $2H_2O$	:	0.2g
FeCl3.6H2O	:	0.01g
Yeast extract	:	1g
Agar	:	20g
pH	:	7.5
Distilled water	:	1000mL

The medium was autoclaved at 10lbs for 10 min. and poured in to plates. The plates were spot inoculated and incubated at 28±0.4^{0}C for 72 hours and flooded with 1% aqueous solution of cetavlon (Cetyl trimethyl ammonium bromide) and allowed to stand for 20 – 30 min. Cetavlon is a polysaccharide precipitant and thus microorganisms which decompose pectic acid are surrounded by a clear zone in on otherwise white and opaque medium. Such cultures were designated pectinolytic.

2.2.8. Selective enrichment and isolation of polyphenol degrading microorganisms through ret liquor as the enrichment medium

In this method ret liquor was used as the enrichment system where the polyphenol contained was regulated to 100ppm.

2.2.8.a Samples

The samples consisted water, sediment and partly retted husk from 18 stations which were active retting zones. The samples were collected in fresh polythene bags and transported to the laboratory by maintaining at 4^{0}C in a thermocool box.

2.2.8.b Enrichment medium

Freshly split coconut husk was steeped in 20ppt sea water, for 3days and the ret liquor generated was filtered through cotton and stored at -20^0C till used. At the time of use it was diluted using 20ppt seawater to attain a final concentration of 100ppm polyphenol and was supplemented with

NH_4Cl	:	1%
$NH_4 NO_3$	:	0.2%
Yeast autolysate	:	0. +001%
pH	:	7.0$\pm$ 0.5

The medium was autoclaved at 10 lbs for 10min.

2.2.8.c Inoculation and incubation

Water samples were used as such, sediment samples were diluted using 20ppt sterile seawater by mixing 10g sediment with 100ml sea water. Partly retted husk pieces (10gms) were crushed and agitated with sterile 20ppt seawater. Inoculations were made to final volume of 10%(v/v) and incubated at room temperature (28$\pm$1^{0}C) on a rotary shaker for 7 days.

2.2.8.d. Isolation of organisms

Two types of isolations were made, one for bacteria and the other for fungi. For the isolation of bacteria the ret liquor based medium mentioned above at pH 7.5 was solidified with 2% agar autoclaved at 10 lbs 10 min. and converted into plates. For the isolation of fungi pH of the medium was lowered to 4.5 by using tartaric acid and solidified with 2% agar and autoclaved in the same pattern and converted into plates. The plates were dried at 37^0C overnight before use.

The enrichment cultures were diluted to 10^{-6} using seawater at 20ppt as the diluent and were swabbed on to the medium plates, and incubated at 28 ± 0.4^0C for 7 days and observed for the development of bacterial and fungal colonies. From each sample 20 bacterial cultures were isolated on to nutrient agar and Sabouraud dextrose agar and 20 fungal colonies prepared in ret liquor.

Nutrient agar in ret liquor

Peptone	:	0.5g
Beef extract	:	0.1g
Yeast extract	:	0.1g
Ret liquor (20 ppt)	:	100ml
Agar	:	2.0g
pH	:	7.5

Sabouraud dextrose agar in ret liquor

Dextrose	:	2.0g
Peptone	:	0.1g
Ret liquor (20 ppt)	:	100ml
Agar	:	2.0g
pH	:	4.8

(pH was adjusted with tartaric acid after autoclaving.)

2.2.8.e. Segregation of potent strains.

The isolated bacterial and fungal cultures were purified by streaking on the corresponding media plates and were isolated.They were tested for their potency to utilize polyphenols as the carbon source.

Primary screening :

To test the capability of the isolated cultures to consume polyphenols, ret liquor prepared with 20ppt seawater supplemented with

NH_4Cl	:	1%
NH_4NO_3	:	0.2%
Yeast autolysate	:	0.001%
pH	:	7.5

was used. The above test medium was taken at 10 ml aliquots in test tubes and autoclaved at 10 lbs for 10min. The tubes were inoculated from the bacterial and fungal slant cultures after determining the initial polyphenol content following the colourimetric method as described under section 2.2.1.d. Freshly grown bacterial cultures were then harvested in 20ppt seawater and inoculated individually to obtain a final OD of 0.05 at 600nm. In the case of fungi, the slants were incubated till sporulation took place and harvested in 20ppt seawater and inoculated to obtain a final spore count of $10^2.ml^{-1}$. Controls included uninoculated set of tubes.All tubes were uniformly incubated at 28 ± 0.4^0C for 10days and the final polyphenol content was estimated for every tube following the above mentioned method. From the values the percent consumption of polyphenol in every tube was worked out. The (>25%) were segregated and identified to genera following Buchanan and Gibbons(1974)and Oliver(1982). This formed the primary screening of polyphenol consuming organisms.

Secondary screening

The above segregated cultures were subjected for secondary screening, using ret liquor without any supplemented nutrients. Ret liquor prepared as described above was transferred in 10ml aliquots into test tubes

after adjusting pH to7.5±0.2 and autoclaved at 10 lbs for 10 min. Inoculations were made as described above and incubated and the percentage consumption of polyphenols was worked out for every culture. The cultures which exhibited fairly higher percentage (25%) polyphenol consumption were subjected for characterization.

The above segregated bacterial and fungal cultures were tested for their hydrolytic properties such as lignolysis, cellulolsis proteolysis, lipolysis, amylolsis and pectinolysis following the methods described under section 2.2.7.a. to 2.2.7.g

2.2.9. Selective enrichment and isolation of polyphenol degrading microorganisms through a packed bed treatment system

This is the third category of enrichment and isolation of microorganisms capable to degrade polyphenols in ret liquor. Speciality of this method is that an enrichment was made in a sand based packed bed reactor.

2.2.9.a. The enrichment system.

The pre-enrichment system was set up in a 30L capacity plastic basin in which a submerged sand based filter bed was set up in the pattern of biological filter. Water in the filter bed was circulated using an air lift pump as depicted in Fig .1. The basin was filled with ret liquor with the polyphenols regulated to 1mg. ml^{-1} .Water and sediment samples from various retting grounds were collected and introduced in to the to the final system in 10ml and 1g aliquots respectively. Nutrients such as NH_4Cl concentration of 1% NH_4NO_3 to the final concentration of 0.2% were supplimented, pH adjusted to 7.5$\pm$ 0.2 with 1N NaOH and the air lift pump in the packed bed reactor was operated. The system was monitored for the drop in pH and polyphenol content.

As the pH dropped it was adjusted to neutrality with 1N NaOH and as the polyphenol content was brought down to less than 0.1ppm a part of the ret liquor was drained off and replaced with fresh ret liquor and the process continued until the system got conditioned. On conditioning the system removal of polyphenol from the ret liquor was attained at the rate of 1mg.ml^{-1} per day. Two such systems were developed and maintained for the isolation of bacteria and fungi for the envisaged ret liquor treatment system.

2.2.9.b. Isolation and segregation

From the treated ret liquor and the filtrant grains of the above enrichment system bacteria and fungi were isolated through ret liquor solidified with agar medium.

Ret liquor prepared in 20ppt seawater was supplemented with 1% NH_4Cl, 0.2% NH_4NO_3, 0.001% Yeast autolysate and for the isolation of bacteria the pH was adjusted to 7.5 and for that of fungi the pH was brought down to 4.8 using 1N NaOH and tartaric acid respectively. The medium was solidified with 2% agar and the polyphenol in the medium was around 1ppm.The plates made out of these media were swabbed with diluted samples of treated ret liquor and filtrant grains. Dilution of the samples were made in 20ppt seawater aseptically. Plates were incubated at $28\pm0.4^{0}C$ for 7days.

Isolation

From each sample bacterial and fungal culture were isolated into nutrient agar and Sabouraud dextrose agar slants prepared in ret liquor base as described under section 2.2.8.d

Segregation of potent strains

As described under section 2.2.8.e. the cultures were subjected for primary screening in ret liquor supplemented with NH_4Cl,NH_4NO_3 and Yeast autolysate identified to genera and subjected to secondary screening in ret liquor without any addition of nutrients. Those which exhibited fairly higher percentage of polyphenol consumption were segregated and subjected for further characterization as described under the section 2.2.7.a to2.2.7.g

Maintenance of cultures

The segregated bacterial and fungal cultures were maintained in nutrient agar and Sabouraud dextrose agar vials prepared in ret liquor (20ppt) and one set was overlaid with sterile liquid paraffin and the other sealed with paraffin wax.

2.3. RESULTS AND DISCUSSION

2.3.1. Successive enrichment and isolation of polyphenol degrading microorganisms

An enrichment broth in husk infusion was developed for the successive enrichment of polyphenol degrading microorganisms Table 2.1 summarises the parameters which were used for the monitoring of the system as it progressed from one system of flask to another. pH throughout the study remained almost steady. Colour reduction in all the first three systems was substantial and comparatively lower in the fourth system. Meanwhile polyphenol consumption over a period of 192 days was above 90% in all the first three systems (stages) and was very low in the fourth stage. Though Husk Infusion Nutrient agar, Husk Infusion Sabouraud Dextrose agar and Nutrient agar 180 bacterial cultures could be isolated and subjected for further study.

From the isolates thus made 8 bacterial cultures could be segregated based on their capability to utilize tannic acid, pyrocatechol and pyrogallol and to degrade or consume polyphenol from ret liquor (Table 2.2). All these cultures were found to be capable to utilize any one of the polyphenol, (either tannic acid, pyrocatechol and pyrogallol) as the sole carbon and

energy sources and their percentage consumption of polyphenols in ret liquor was between 40 to 50 over a period of 5 days.

From this, four cultures were further selected and tested for their capability to degrade/consume tannic acid over a period of 7days, and the results are summarised in Table 2.3.

The selected four cultures were further segregated for tests to reveal their hydrolytic potential (Table 2.4). Accordingly all cultures were found to be lignolytic, hemicellulolytic, and pectinolytic. Cellulolytic, proteolytic, lipolytic, and amylolytic potentials were absent except in one culture which was amylolytic.

2.3.2. Selective enrichment and isolation of polyphenol degrading microorganisms through ret liquor as the enrichment medium.

Enrichment of samples from 18 stations was made in ret liquor (Table 2.5), and subsequently 191 bacterial cultures were isolated and screened for their potential to degrade /consume. Accordingly 52 cultures could be segregated based on their per cent consumption of polyphenols. They were identified to genera and were found to belong to *Planococcus*,

Pseudomonas, Acinetobacter, Micrococcus, Bacillus, Enterobacteriacea, *Aeromonas,* Coryneform group .The segregated cultures were further subjected for a secondary screening procedure and accordingly 47 bacterial cultures were further segregated as summarised in Table 2.6. On examining the hydrolytic potential of the segregated cultures it was observed that all the tested hydrolytic potentials were distributed among the cultures, in the descending order of prominence as proteolytic, pectinolytic, amylolytic, lipolytic, lignolytic, hemicellulolytic, and cellulolytic.

In the same pattern through the enrichment and isolation procedures followed 161 fungal cultures could be isolated which on further screening based on the potential to degrade/consume polyphenols in ret liquor 31 cultures could be further segregated. Majority of them belonged to *Aspergillus* and one culture was *Fusarium*, and two of them belonged to *Penicillium* and three cultures were unidentified Phycomycetes(Table 2-7). Through the secondary srceening procedures followed 13 cultures were further segregated based on their potential to consume polyphenols(Table 2-8). On examining the hydrolytic potential it was observed that the proteolytic potential and the lipolytic potentials were not represented in the collection. At the same time except one culture of *Aspergillus* all others were lignolytic and weakly cellulolytic. Only two cultures were

hemicellulolytic, only one amylolytic and at the same time several of them were pectinolytic.

2.3.3. Selective enrichment and isolation of polyphenol degrading microorganisms through a packed bed treatment system.

From both the packed bed treatment systems which were the pre-enrichment systems eight samples as shown in Table 2-9 were used for the isolation of bacteria through ret liquor solidified with agar. Accordingly 183 bacterial cultures could be segregated altogether. From them 31 cultures could be segregated based on their capability to consume polyphenols in ret liquor supplemented with nutrients. This assemblage was composed of *Pseudomonas, Alteromoas, Acinetobacter, Micrococcus, Bacillus, Moraxella* and coryneform group. These cultures could be confirmed of being capable to degrade polyphenols by growing in ret liquor not supplimented with nutrients(Table 2-10). Among the segregated lot the tested hydrolytic potentials were distributed in the following decreasing order of dominance as pectinolytic, lignolytic, proteolytic, amylolytic, lipolytic, cellulolytic, and hemicellulolytic.

In a similar manner from 7 samples collected from the pre–enrichment treatment system 68 fungal cultures could be obtained (Table 2.11) from which 23 could be segregated based on their capabilty to degrade /consume polyphenols. Majority of them were *Aspergillus* species and a very few were unidentified Ascomycetes. On subjecting them for further screening 13 cultures could be segregated(Table 2.12)which were able to consume polyphenols in ret liquor without being supplemented with nutrients. All of them were pectinolytic and the lignolytic potential was seen. In all cultures except in two strains of the *Aspergillus* sp. Most of them were weakly cellulolytic except two cultures which were with very good hydrolytic potential. Meanwhile two were not exhibiting any such property. Only one culture was hemicellulolytic and none of them were proteolytic, lipolytic, and amylolytic.

Subsequently from all the three enrichment systems together 33 bacterial cultures were segregated, as summarized in Table 2.13, based on their capability to consume /degrade polyphenol and to produce the hydrolytic enzymes. This assemblage was consisted of Enterobacteriaciae, *Aeromonas, Planococcus, and Alteromonas*.

In a similar manner from the fungal cultures segregated from the above described two enrichment systems 22 fungal cultures were finally selected based on their capability to degrade/consume polyphenol and their hydrolytic potentials.(Table 2.14). Majority of them were belonging to *Aspergillus*, two were unidentified Ascomycetes and one culture was *Fusarium* sp.

This collection of bacteria and fungi formed the source of organisms for the development of different ret liquor treatment systems.

The main objective of this investigation was to develop appropriate culture collection for the ret liquor treatment systems envisaged. To make sure that all possible sources of the organisms are being explored a variety of samples were used for the isolation which included water and sediment from around 35 stations and retted husk from areas where from good quality fibre is generated. In the same way to make sure that the right organisms are isolated these different types of enrichment systems were used where ret liquor was used as enrichment medium and the removal /disappearance of polyphenol was used as the criterion for the detection of polyphenol degrading microorganisms. The isolation protocols included only the ret liquor solidified with agar and the organisms which could grow in such

media were segregated. These procedures had the advantage that the weed organisms were totally avoided and the bacteria and fungi which could live in the organics present in ret liquor alone were allowed to grow. From among such organisms the one which could consume /degrade polyphenols were alone segregated. This protocol confirmed the isolation of the most potential strains for the treament system.

Eventhough such vigorous enrichment and isolation procedures were followed surprisingly a variety of bacterial genera could be isolated which were capable to degrade polyphenols. This indicated that in the natural environment polyphenol degradation potential was not limited to certain genera such as *Pseudomonas* and *Bacillus* alone. The involvement of several bacterial genera for a single purpose indicates the effectiveness of the process in the retting ground. In addition they are with the potentials to hydrolyze lignin , pectin, cellulose, hemicellulose, protein, lipid and carbohydrate even though not in all the strains but distributed among several genera. These findings lead to the conclusion that polyphenol degradation in natural environment is a result of concerted action of several groups of organisms and when a treatment process is developed same sort of assemblage of organisms has to be built up. According to Evans (1963) the capability to degrade aromatic compounds is distributed among the

families such *Micrococcaciae, Mycobacteriacea, Pseudomonadaciae, Spirillaciae, Bacteriaciae, Bacillaciae.* Jayasankar and Bhatt,(1966) could isolate several species of *Pseudómonas* and *Micrococcus* capable to degrade phenols from coir rets. Earlier studies have pointed out that *Vibrio* sp,(Evans,1947) *Azotobacter* sp,(Tchan ,1946) and *Pseudomonas* and Corynebacterium (wieringa,1946) could convert phenolic compounds to more complex coloured compounds. However, among the fungi isolated *Aspergillus* species dominated. Among the 22 strains of fungi finally consolidated most of them were lignolytic, pectinolytic, and weakly cellulolytic all of them were capable to use tannic acid as the sole carbon and energy sources. But none of them were proteolytic, lipolytic, and amylolytic. According to Evans (1963) the yeast *Oospora, Candida, Debaryomyces, Pichia* and *Saccharomyces* grow in media with catechol as sole carbon source. Certain higher fungi eg. *Aspergillus, Penicillium,*and *Neurospora* attack aromatics and a variety of soil and wood rotting fungi dissimilate the aromatic polymer lignin as well as other plant phenolics. The capability of *Aspergillus* sp.for the biodegradation of polyphenol has been studied by Maria and Amalia (1998). On studying the biodegradation of polyphenols by *Aspergillus carbonarius* ACTC 92 in carob solid state fermentation.

Precisely, the collection of bacteria and fungi obtained from the retting ground as the organisms for the development of ret liquor treatment system is satisfactory and a consortium of organism can be built up from this assemblage as and when required.

Table 2.1. **Monitoring an enrichment system (successive) for isolating polyphenol degrading microorganisms.**

Stage	Parameters		Hours of Incubation								Total Bacterial Cultures isolated.		
			0	24	48	72	120	148	168	192	HINA	HISDA	NA
1	PH	T	5.8	6.4	7.0	7.5	7.5	7.5	7.5	7.0	20	20	NG
		C	5.9	6.5	7.0	7.5	7.0	6.0	6.5	7.0			
	Colour (CU)	T	17500	25000	12500	7500	2000	1600	1400	1200			
		C	17500	26000	15000	12000	10000	9000	9000	8500			
	% Consumption	T	0	6.53	76.56	86.35	94.36	96.62	96.62	96.62			
	of Polyphenols	C	0	4.50	45.70	50.00	70.90	75.00	75.00	75.00			
2	PH	T	6.5	6.5	6.8	7.0	6.8	6.8	6.8	6.9	20	NG	20
		C	6.8	6.8	6.8	7.0	6.8	6.8	6.8	6.8			
	Colour	T	17500	18000	8000	5000	3000	2750	2500	2350			
	(CU)	C	17500	22500	10000	9000	5400	4000	4000	20			
	% Consumption	T	0	30.49	74.80	79.27	86.99	88.21	90.26	96.22			
	of Poly phenol	C	0	6.77	48.21	52.19	70.92	75.29	76.30	76.30			
3	PH	T	5.5	5.5	6.0	6.0	6.5	6.5	6.5	7.0	20	20	20
		C	5.5	5.5	6.0	6.0	6.0	7.0	7.0	7.5			
	Colour	T	12000	11000	10000	8500	2700	2000	2000	2000			
		C	12500	12000	12000	11000	10000	10000	9000	9000			
	% Consumption	T	0	17.99	23.74	32.37	82.20	88.49	89.50	90.00			
	of Polyphenol	C	0	12.42	13.50	14.60	20.00	59.62	58.00	58.00			
4	PH	T	5.5	5.5	6.0	6.5	6.5	6.5	6.5	6.5	20	NG	20
		C	5.5	5.0	6.0	6.0	6.0	6.0	6.5	6.5			
	Colour	T	4800	4800	4500	4500	4000	4000	4000	3500			
		C	4800	4800	4500	4500	4500	4500	4500	4500			
	% Consumption	T	0	36.36	49.49	59.59	59.59	53.03	67.67	67.67			
	of Poly phenol	C	0	2.30	5.50	7.80	11.06	25.81	48.39	50.00			

HINA : Nutrient agar in Husk infusion
HISDA : Sabouraud Dextrose agar in husk infusion
NA : Nutrient agar in 20 ppt sea water.
NG : No growth on plate
T : Test
C : Control

Table 2.2 **Segregation of polyphenol utilizing /degrading bacteria**

Stage	Culture No.	General Family	Utilization as sole source of Carbon / energy			Degradation capability of the segregated strains	
			Tannic acid	Pyro catechol	Pyro gallol	Final polyphenol content (Mg./ml)	% Consumption of Poly phenols
I	CRPD 1	Enterobacteriacae	+	-	-	0.460	47.13
	CRPD 3	Enterobacteriacae	+	+	-	0.390	55.17
	CRPD 4	Enterobacteriacae	+	+	-	0.38	50.57
	CRPD 5	Enterobacteriacae	+	-	-	0.36	50.50
	CRPD 7	Enterobacteriacae	+	+	-	0.39	55.17
	CRPD 8	Enterobacteriacae	+	+	-	0.47	45.98
	CRPD 9	Enterobacteriacae	+	+	-	0.41	40.00
II	CRPD 42	*Actinomycetes*	+	-	-	0.38	40.00
		Uninoculated Control				0.870	10.25

Table 2.3. Percent Consumption/degradation of tannic acid by the selected cultures

Culture No	T/C	Hours of Incubation							
		0Hr	24 Hrs	48 Hrs	72 Hrs	120 Hrs	148 Hrs	168 Hrs	192 Hrs
CRPD 4	T	0	6.53	76.56	86.35	94.36	96.62	96.62	96.62
	C	0	6.55	30.48	35.48	35.40	35.50	35.50	35.50
CRPD 5	T	0	30.48	74.79	79.26	86.99	88.21	89.20	92.30
	C	0	6.77	30.40	35.45	35.45	35.50	35.50	35.50
CRPD 7	T	0	17.99	23.74	32.37	82.01	88.48	95.0	98.0
	C	0	6.77	30.45	35.45	35.45	35.50	35.50	35.60
CRPD 8	T	0	36.36	48.50	60.0	65.0	75.0	80.0	85.28
	C	0	6.77	30.45	35.0	35.25	35.50	35.50	35.60

T : Test
C : Control

Table 2.4 Capability of the selected cultures to degrade / consume Polyphenols and to hydrolyse other organics.

Culture No.	Family/Genus	Percent consumption of Polyphenol	Percent consumption of tannic acid	Hydrolytic Properties							
				Ligno Lytic	Cellulo lytic	Hemicellulolytic	Proteo lytic	Lipo lytic	Amylo lytic	Pectinolytic	
										Tube test	Plate test
CRPD 4	Enterobacteriacae	50.57	61.12	+	-	+	-	-	+	+	+
CRPD 5	Enterobacteriacae	50.50	56.80	+	-	+	-	-	-	+	+
CRPD 7	Enterobacteriacae	55.17	62.40	+	-	+	-	-	-	+	+
CRPD 8	Enterobacteriacae	45.98	49.68	+	-	+	-	-	-	+	+

Table 2.5 **Enrichment, isolation, primary screening and segregation of bacteria from retting ground capable to degrade polyphenols in ret liquor**

Sl. No.	Sample-Station (Retting ground)	Total Cultures isolated and screened	Segregated cultures based on performance	Percent consumption of polyphenols	Genera/ family
1.	Water - Stn. 1.	10	CRPD 226	50.27	*Aeromonas*
			CRPD 260	51.46	*Bacillus*
			CRPD 261	49.64	Coryneform group
2.	Water Stn. 2.	9	CRPD 252	60.92	Coryneform group
			CRPD 253	56.99	*Bacillus*
3.	Water Stn. 3.	10	CRPD 291 a	52.41	*Pseudomonas*
4.	Retted Husk - Stn.5	5	CRPD 337 b	28.21	*Pseudomonas*
			CRPD 339	73.88	*Micrococcus*
5.	Retted Husk - Stn. 5	5	CRPD 451	39.40	Coryneform group
			CRPD 452	65.11	*Acinetobacter*
			CRPD 456	73.0	*Acinetobacter*
6.	Water - Stn 6	11	-	-	-
7.	Water-Stn 7	22	CRPD 458	51.59	Coryneform group
			CRPD 459	49.33	*Bacillus*
			CRPD 465 b	71.60	*Acinetobacter*
8.	Water - Stn. 8	14	CRPD 542	61.16	*Micrococcus*
9.	Sediment - Stn 9	8	CRPD 603	50.00	Coryneform group
			CRPD 607	53.06	*Pseudomonas*
			CRPD 608	52.74	*Enterobacteriacae*
			CRPD 614 a	54.58	*Enterobacteriacae*
			CRPD 614 b	53.19	Coryneform group
			CRPD 678 a	57.57	*Aeromonas*
			CRPD 678 c	49.21	*Aeromonas*
10.	Water Stn. 10	2	-	-	-
11.	Sediment Stn. 11	8	CRPD 668	61.62	*Bacillus*
			CRPD 669	77.31	Coryneform group
			CRPD 672	73.27	*Bacillus*
			CRPD 679	49.51	*Aeromonas*
			CRPD 680	55.94	*Micrococcus*

Table 2.5. continued

Sl. No.	Sample-Station (Retting ground)	Total Cultures isolated and screened	Segregated cultures based on performance	Percent consumption of polyphenols	General family
12.	Water - Stn. 1.	19	CRPD 701	56.60	*Bacillus*
			CRPD 710	52.88	*Pseudomonas*
			CRPD 728	52.79	*Pseudomonas*
13.	Sediment - Stn.13	11	CRPD 759 b	64.36	*Acinetobacter*
			CRPD 750	26.26	*Micrococus*
			CRPD 751	66.6	*Micrococcus*
14.	Water Stn. 14	25	CRPD 780	67.54	*Bacillus*
			CRPD 785	53.52	*Pseudomonas*
			CRPD 787	61.74	*Pseudomonas*
			CRPD 805 b	50.88	*Micrococcus*
			CRPD 810 c	69.96	*Pseudomonas*
15.	Sediment - Stn 15	15	CRPD 867	79.60	*Acinetobacter*
			CRPD 869	54.50	Coryneform group
			CRPD 810 x_2	57.45	*Aeromonas*
			CRPD 864	52.24	*Bacillus*
			CRPD 864 c	55.61	*Pseudomonas*
			CRPD 877 a	28.49	*Planococcus*
			CRPD 880	57.08	*Micrococcus*
			CRPD 890	55.57	*Aeromonas*
16.	Water - Stn. 16	10	CRPD 927	54.92	*Bacillus*
			CRPD 938	63.57	*Planococcus*
17.	Sediment - Stn. 17	-	-	-	-
18.	Water - Stn. 18	7	CRPD 1011	66.87	Coryneform group
			CRPD 1017	51.88	*Pseudomonas*
			CRPD 1024 a	52.84	*Pseudomonas*
			CRPD 1024 b	51.76	*Acinetobacter*

Table 2.6. **Secondary screening of the segregated cultures isolated from retting ground and further characterisation to select the most suitable ones.**

Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties							
			L	C	He	Pr	Li	Am	Pectinolytic	
									Tube test	Plate test
CRPD 226	*Aeromonas*	62.35	+	-	-	+	-	-	-	+
CRPD 260	*Bacillus*	55.71	-	+	+	+	-	+	+	+
CRPD 261	Coryneform group	40.59	-	+	-	+	+	+	-	+
CRPD 252	Coryneform group	42.61	-	-	-	+	+	+	-	-
CRPD 253	*Bacillus*	25.23	-	-	-	+	-	+	-	-
CRPD 291 a	*Pseudomonas*	29.78	-	-	-	-	-	-	-	+
CRPD 337 b	*Pseudomonas*	22.0	-	-	-	-	-	-	-	+
CRPD 339	*Micrococcus*	42.61	-	-	-	-	-	+	+	+
CRPD 451	Coryneform group	41.12	-	-	-	+	+	+	-	+
CRPD 452	*Acinetobacter*	0.50	-	-	-	-	-	+	-	-
CRPD 456	*Acinetobacter*	29.54	+	-	-	+	+	+	-	+
CRPD 458	Coryneform group	26.98	-	-	-	+	+	+	-	-
CRPD 459	*Bacillus*	17.05	-	-	-	+	-	-	-	-
CRPD 465 b	*Acinetobacter*	9.307	+	-	-	-	-	-	-	-
CRPD 542	*Micrococcus*	1.280	-	-	-	-	-	-	-	-
CRPD 603	Coryneform Group	0.65	-	-	-	-	-	-	-	-
CRPD 607	*Pseudomonas*	61.09	+	-	-	-	-	-	-	+
CRPD 608	Enterobacteriacae	63.36	+	-	-	-	-	-	+	+
CRPD 614 a	Enterobacteriacae	77.23	-	-	+	+	-	-	+	+
CRPD 614 b	Coryneform group	47.15	-	-	-	+	+	-	-	+
CRPD 678 a	*Aeromonas*	50.22	+	-	-	-	-	+	-	+
CRPD 678 c	*Aeromonas*	62.24	+	-	+	+	+	-	-	+
CRPD 668	*Bacillus*	63.34	-	-	-	+	+	-	+	+
CRPD 669	Coryneform group	38.21	-	-	-	+	-	+	-	-
CRPD 672	*Bacillus*	49.53	-	-	-	+	-	-	-	+
CRPD 679	*Aeromonas*	65.98	-	-	-	+	-	-	-	+
CRPD 680	*Micrococcus*	49.82	-	-	-	+	-	-	-	+
CRPD 701	*Bacillus*	28.13	-	-	-	+	+	-	-	-
CRPD 710	*Pseudomonas*	65.78	+	-	-	+	-	-	-	-
CRPD 728	*Pseudomonas*	72.82	-	-	-	+	-	+	-	+

Table 2.6. continued

Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties							
			L	C	He	Pr	Li	Am	Pectinolytic	
									Tube test	Plate test
CRPD 759 b	*Acinetobacter*	66.15	-	-	+	+	+	+	-	+
CRPD 750	*Micrococcus*	15.84	-	-	-	-	+	-	-	-
CRPD 751	*Micrococus*	28.71	-	-	-	+	-	+	-	+
CRPD 780	*Bacillus*	45.14	-	-	-	+	+	-	+	-
CRPD 785	*Pseudomonas*	20.92	+	-	-	-	-	+	+	+
CRPD 787	*Pseudomonas*	34.58	-	-	-	+	+	-	+	+
CRPD 805 b	*Micrococcus*	59.80	+	-	-	+	+	-	-	+
CRPD 810 c	*Pseudomonas*	65.83	+	-	-	+	-	+	-	-
CRPD 867	*Acinetobacter*	46.32	-	-	-	-	-	-	-	-
CRPD 869	Coryneform group	45.85	-	-	-	+	-	-	-	-
CRPD 810 x_2	*Aeromonas*	65.17	-	-	-	+	+	-	-	+
CRPD 864	*Bacillus*	47.14	-	-	-	+	-	+	-	-
CRPD 864 c	*Pseudomonas*	1.74	-	-	-	+	-	-	-	-
CRPD 877 a	*Planococcus*	38.39	-	-	-	+	-	-	-	+
CRPD 880	*Micrococcus*	21.46	-	-	-	+	-	-	+	+
CRPD 890	*Aeromonas*	54.11	-	-	-	+	-	-	-	-
CRPD 927	*Bacillus*	3.56	-	-	-	+	-	-	-	-
CRPD 938	*Planococcus*	52.57	-	-	+	-	-	-	+	+
CRPD 1011	Coryneform group	41.35	-	-	-	-	-	+	-	+
CRPD 1017	*Pseudomonas*	49.32	-	-	-	-	-	-	-	-
CRPD 1024 a	*Pseudomonas*	59.81	-	-	-	-	-	+	+	-
CRPD 1024 b	*Acinetobacter*	51.48	+	-	-	-	-	-	-	-

Table 2.7 **Enrichment, isolation, primary screening and segregation of fungi from retting ground capable to degrade polyphenols in ret liquor.**

Sl.No.	Sample - station on Retting ground	Total cultures isolated and screened	Segregated cultures based on performance	Percent consumption of Polyphenols	Genera/ Family
1.	Water - Stn. 1	8	CRPD 222	46.04	*Aspergillus*
2.	Water - Stn. 2	8			
3.	Water - Stn. 3	13	CRPD 280	48.57	*Aspergillus*
			CRPD 282	51.60	*Aspergillus*
4.	Retted Husk - Stn.4	20	CRPD 333	57.60	*Aspergillus*
			CRPD 336	50.50	*Aspergillus*
			CRPD 340	57.80	Ascomycetes (Unidentified)
			CRPD 342	45.00	Ascomycetes (Unidentified)
			CRPD 433	54.71	*Aspergillus*
			CRPD 435	51.66	*Aspergillus*
5.	Retted Husk- Stn. - 5	13	CRPD 443	50.66	*Aspergillus*
			CRPD 444	50.85	*Aspergillus*
			CRPD 447	52.20	*Fusarium*
			CRPD 473	47.10	*Aspergillus*
6.	Water - Stn. 6	-	-	-	-
7	Water - Stn. 7	-	-	-	-
8	Water - Stn. 8	5	CRPD 546	45.30	*Aspergillus*
			CRPD 549	41.50	*Aspergillus*
9.	Sediment - Stn. 9	10	CRPD 607	37.86	*Aspergillus*
10.	Water - Stn. 10	13	CRPD 633	70.72	*Aspergillus*
			CRPD 639	53.02	*Aspergillus*
			CRPD 640	54.52	*Aspergillus*
			CRPD 648	48.57	*Aspergillus*
11.	Sediment - Stn. 11	8	CRPD 674	50.70	Penicillium
			CRPD 675	45.60	Unidentified Phycomycetes
			CRPD 700	43.43	*Aspergillus*
12.	Water - Stn 12	24	CRPD 709	53.30	*Aspergillus*
			CRPD 714	53.60	*Aspergillus*
			CRPD 725	40.41	*Penicillium*
13.	Sediment - Stn. 13	-	-	-	-
14	Water - Stn. 14	26	CRPD 792	57.04	*Aspergillus*
			CRPD 799	35.43	*Aspergillus*
15.	Sediment - Stn. 15	10	CRPD 875	24.57	*Aspergillus*
16.	Sediment - Stn. 17	2	CRPD 973	68.95	*Aspergillus*
17.	Water - Stn. 18	1	CRPD 1010	70.65	*Aspergillus*

Table 2.8 **Secondary Screening of the segregated fungal cultures and their further characterization to select the most suitable ones.**

Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties							
			L	C	He	Pr	Li	Am	Pectinolytic	
									Tube test	Plate test
CRPD 282	*Aspergillus*	59.66	+	W+	W+	-	-	-	+	+
CRPD 333	*Aspergillus*	45.95	+	W+	W+	-	-	+	+	+
CRPD 336	*Aspergillus*	57.69	+	W+	-	-	-	-	-	-
CRPD 340	Ascomycetes	4.55	+	W+	-	-	-	-	-	+
CRPD 433	*Aspergillus*	37.43	+	W+	-	-	-	-	-	+
CRPD 435	*Aspergillus*	59.24	+	W+	-	-	-	-	+	-
CRPD 443	*Aspergillus*	59.35	+	W+	-	-	-	-	+	+
CRPD 447	*Fusarium*	49.50	+	+	-	-	-	-	+	+
CRPD 549	*Aspergillus*	62.61	+	W+	-	-	-	-	+	+
CRPD 714	*Aspergillus*	62.64	-	W+	-	-	-	-	-	+
CRPD 792	*Aspergillus*	41.99	+	W+	-	-	-	-	+	+
CRPD 973	*Aspergillus*	5.53	+	W+	-	-	-	-	+	+
CRPD 1010	*Aspergillus*	26.05	+	W+	-	-	-	-	+	+

Table 2.9. **Enrichment, isolation, primary screening and segregation of bacteria from a packed bed treatment system set up in the laboratory**

Sl.No.	Sample (Treatment System)	Total cultures isolated and screened	Segregated cultures based on performance	Percent consumption of Polyphenols	Genera/ Family
1.	Treated ret liquor from the system I	16	TSE 12	38.06	*Alteromonas*
			TSE 14	35.56	*Alteromonas*
			TSE 15	46.35	*Alteromonas.*
2.	Treated ret liquor from the System II	6	TSE 40	33.16	*Pseudomonas*
			TSE 41	43.89	Coryneform Group
3.	Filtrant grains from the system I	29	TSE 104	38.26	*Acinetobacter*
			TSE 109	43.65	*Pseudomonas*
4.	Treated ret liquor from the System II	20	TSE 116	49.38	*Pseudomonas*
			TSE 119	40.67	*Micrococcus*
5.	Filtrant Grains from the system II	38	TSE 185	69.57	*Bacillus*
			TSE 190	56.54	*Alteromonas*
6.	Treated ret liquor from the System I	18	TS 191	38.57	*Acinetobacter*
			TS 104	61.60	*Acinetobacter*
7.	Filtrant grains from the system I	30	TS 140	10.79	*Moraxella*
			TS 145	45.06	*Moraxella*
			TS 151	46.32	*Pseudomonas*
			TS 154	43.92	*Moraxella*
			TS 156	37.68	*Pseudomonas*
			TS 157	31.07	*Pseudomonas*
			TS 158	44.38	*Pseudomonas*
			TS 160	45.36	*Bacillus*
8.	Filtrant Grains from the System II	26	TS 297	52.90	*Alteronomonas*
			TS 301	17.20	*Pseudomonas*
			TS 307	58.63	*Pseudomonas*
			TS 308	18.51	*Pseudomonas*
			TS 309	22.49	*Pseudomonas*
			TS 310	58.67	*Pseudomonas*
			TS 313	58.63	*Pseudomonas*
			TS 317	53.26	*Pseudomonas*
			TS 320 a	59.69	*Alteromonas*
			TS 320 b	22.25	*Pseudomonas*

Table 2.10 **Secondary screening of segregated bacterial cultures from the packed bed treatment and their further characterization to select the most suitable ones.**

Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties							
			L	C	He	Pr	Li	Am	Pectinolytic	
									Tube test	Plate test
TSE 12	*Alteromonas*	38.06	+	-	-	-	-	-	-	-
TSE 14	*Alteromonas*	35.56	+	-	-	-	-	-	-	-
TSE 15	*Alteromonas*	46.35	+	-	-	+	+	-	+	+
TSE 40	*Pseudomonas*	33.16	+	-	-	+	+	-	-	+
TSE 41	Conyneform group	43.89	+	-	-	+	-	+	-	+
TSE 104	*Acinetobacter*	38.26	+	-	-	+	-	-	-	+
TSE 109	*Pseudomonas*	43.65	+	-	-	+	+	+	-	+
TSE 116	*Pseudomonas*	49.38	-	-	-	-	-	-	-	+
TSE 119	*Acinetobacter*	40.67	-	-	-	-	-	-	+	+
TSE 185	Conyneform group	69.57	-	-	-	+	-	-	-	-
TSE 190	*Alteromonas*	56.54	+	-	-	+	+	+	-	+
TS 191	*Acinetobacter*	38.57	-	-	-	+	-	-	-	-
TS 104	*Acinetobacter*	61.60	-	-	-	-	-	-	-	-
TS 140	*Moraxella*	10.79	+	-	-	-	-	-	-	+
TS 145	*Moraxella*	45.06	-	-	-	-	-	-	-	-
TS 151	*Pseudomonas*	46.32	-	-	-	-	-	-	-	+
TS 154	*Moraxella*	43.92	-	-	-	-	-	+	-	+
TS 156	*Pseudomonas*	37.68	-	-	-	-	-	-	-	+
TS 157	*Pseudomonas*	31.07	-	-	+	-	-	+	+	+
TS 158	*Pseudomonas*	44.38	-	-	+	-	-	+	+	+
TS 160	*Bacillus*	45.36	-	-	-	+	-	+	-	-
TS 297	*Alteromonas*	52.90	+	-	-	-	-	-	-	+
TS 301	*Pseudomonas*	17.20	-	-	-	-	-	-	-	-
TS 307	*Pseudomonas*	58.63	-	-	-	-	-	-	-	+
TS 308	*Pseudomonas*	18.56	+	-	-	-	-		-	+
TS 309	*Pseudomonas*	22.49	-	-	-	-		-	+	+
TS 310	*Pseudomonas*	58.69	-	-	-	-	-	-	+	+
TS 313	*Pseudomonas*	59.04	-	-	-	-	-	-	+	+
TS 317	*Pseudomonas*	53.26	-	-	-	-	-	-	+	+
TS 320 a	*Alteromonas*	59.69	-	+	-	-	-	+	+	+
TS 320 b	*Pseudomonas*	22.25	+	+	-	-	-	-	+	+

Table 2.11. **Enrichment, isolation, primary screening and segregation of fungi from a packed bed treatment system**

Sl.No.	Sample - station on Retting ground	Total cultures isolated and screened	Segregated cultures based on performance	Percent consumption of Polyphenols	Genera/ Family
1.	Treated ret liquor from the system I	17	TSE 38	53.9	*Aspergillus*
			TSE 42	56.5	*Aspergillus*
			TSE 43	54.85	*Aspergillus*
			TSE 44	53.83	*Aspergillus*
2.	Treated ret liquor from the System II	26	TSE 55	50.61	*Aspergillus*
			TSE 62	53.55	*Aspergillus*
			TSE 71	49.22	*Aspergillus*
			TSE 73	54.15	*Aspergillus*
			TSE 80	54.95	*Aspergillus*
			TSE 84	63.60	*Aspergillus*
			TSE 85	59.17	Ascomycetus (Unidentified)
3.	Filtrant grains from the system I	10	TSE 143	51.51	*Aspergillus*
			TSE 145	43.65	*Aspergillus*
			TSE 146	55.76	Ascomycetus (Unidentified)
			TSE 147	54.04	Ascomycites (Unidentified)
4.	Filtrant Grains from the system II	4	TSE 156	56.47	*Aspergillus*
			TSE 157	46.52	*Aspergillus*
			TSE 159	51.36	*Aspergillus*
5.	Treated ret liquor from the System I	2	TS 101	43.56	*Aspergillus*
6.	Filtrant grains from the system I	4	TS 126	47.26	*Aspergillus*
			TS 147	16.90	*Aspergillus*
7.	Filtrant grains from the system II	5	TS 292	38.34	*Aspergillus*
			TS 293	33.32	*Aspergillus*

Table 2.12 **Secondary Screening of the Segregated fungal cultures and further characterization to select the most suitable ones.**

Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties							
			L	C	He	Pr	Li	Am	Pectinolytic	
									Tube test	Plate test
TSE 42	*Aspergillus*	5.53	+	-	-	-	-	-	+	+
TSE 43	*Aspergillus*	42.68	+	W+	-	-	-	-	+	+
TSE 69	Unidentified Ascomycetes	44.69	+	W+	-	-	-	-	+	+
TSE 71	*Aspergillus*	41.78	+	W+	-	-	-	-	+	+
TSE 80	*Aspergillus*	51.32	+	W+	-	-	-	-	+	+
TSE 85	*Aspergillus*	47.51	+	-	-	-	-	-	+	+
TSE 92	*Aspergillus*	56.69	+	W+	-	-	-	-	+	+
TSE 234	*Aspergillus*	59.24	+	W+	-	-	-	-	+	+
TS 101	*Aspergillus*	43.56	-	+	-	-	-	-	+	+
TS 126	*Aspergillus*	47.26	+	W+	-	-	-	-	+	+
TS 147	*Aspergillus*	16.90	-	W+	-	-	-	-	+	+
TS 292	*Aspergillus*	38.34	+	+	+	-	-	-	+	+
TS 293	*Aspergillus*	33.32	+	W+	-	-	-	-	+	+

Table 2.13. **Bacterial Cultures finally selected for developing the treatment system**

Sl. No.	Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties							
				L	C	He	Pr	Li	Am	Pectinolytic	
										Plate Test	Tube Test
1	CRPD 4	Enterobacteriace	50.57	+	-	+	-	-	+	+	+
2	CRPD 5	Enterobacteriace	50.50	+	-	+	-	-	-	+	+
3	CRPD 7	Enterobacteriace	55.17	+	-	+	-	-	-	+	+
4	CRPD 8	Enterobacteriace	45.98	+	-	+	-	-	-	+	+
5	CRPD 226	Aeromonas	62.35	+	-	-	+	-	-	-	+
6	CRPD 260	Bacillus	55.71	-	+	+	+	-	+	+	+
7	CRPD 261	Coryneform group	40.59	-	+	-	+	+	+	+	-
8	CRPD 607	Pseudomonas	61.09	+	-	-	-	-	-	-	+
9	CRPD 608	Enterobactericae	63.36	+	-	-	-	-	-	+	+
10	CRPD 614 a	Enterobactericae	77.23	-	-	+	+	-	-	+	+
11	CRPD 614 b	Coryneform group	47.15	-	-	-	+	+	-	-	+
12	CRPD 672	Bacillus	49.53	-	-	-	+	-	-	-	+
13	CRPD 668	Bacillus	63.34	-	-	-	+	+	-	+	+
14	CRPD 678 a	Aeromonas	50.22	+	-	-	-	-	+	-	+
15	CRPD 678 c	Aeromonas	62.24	+	-	+	+	+	-	-	+
16	CRPD 679	Aeromonas	65.98	-	-	-	+	-	-	-	+
17	CRPD 680	Micrococcus	49.82	-	-	-	+	-	-	-	+
18	CRPD 710	Pseudomonas	65.78	+	-	-	+	-	-	-	-
19	CRPD 728	Pseudomonas	72.82	-	-	-	+	-	+	-	+
20	CRPD 759 b	Acinetobacter	66.15	-	-	+	+	+	+	-	+
21	CRPD 780	Bacillus	45.14	-	-	-	+	+	-	+	-
22	CRPD 805 b	Micrococcus	59.80	+	-	-	+	+	-	-	+
23	CRPD 810 c	Pseudomonas	65.83	+	-	-	+	-	+	-	-
24	CRPD 810 x_2	Aeromonas	65.17	-	-	-	+	+	-	-	+
25	CRPD 867	Acinetobacter	46.32	-	-	-	-	-	-	-	-
26	CRPD 869	Coryneform group	45.85	-	-	-	+	-	-	-	-
27	CRPD 890	Aeromonas	54.11	-	-	-	+	-	-	-	-
28	CRPD 938	Planococcus	52.57	-	-	+	-	-	-	+	+
29	CRPD 1024 a	Pseudomonas	59.81	-	-	-	-	-	+	+	-
30	CRPD 1024 b	Acinetobacter	51.48	+	-	-	-	-	-	-	-
31	TS 151	Pseudomonas	46.32	-	-	-	-	-	-	+	-
32	TS 158	Pseudomonas	44.38	+	-	-	+	+	-	+	-
33	TS 190	Alteromonas	56.54	+	-	-	+	+	+	+	-

Table 2.14. **Fungal Cultures finally for developing the treatment system**

Sl. No.	Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties								
				L	C	He	Pr	Li	Am	Pectinolytic		Tannic acid as sole carbon
										Plate Test	Tube Test	
1	CRPD 282	*Aspergillus*	59.66	+	W+	W+	-	-	-	+	+	+
2	CRPD 333	*Aspergillus*	43.95	+	W+	W+	-	-	-	+	+	+
3	CRPD 336	*Aspergillus*	57.67	+	W+	-	-	-	-	-	-	+
4	CRPD 340	Ascomycetes (Unidentified)	4.55	+	W+	-	-	-	-	-	+	+
5	CRPD 433	*Aspergillus*	37.43	+	W+	-	-	-	-	-	+	+
6	CRPD 435	*Aspergillus*	59.25	+	W+	-	-	-	-	+	-	+
7	CRPD 443	*Aspergillus*	59.35	+	W+	-	-	-	-	+	+	+
8	CRPD 447	*Fusarium*	49.50	+	+	-	-	-	-	+	+	+
9	CRPD 549	*Aspergillus*	62.61	+	W+	-	-	-	-	+	+	+
10	CRPD 792	*Aspergillus*	41.99	+	W+	-	-	-	-	+	+	+
11	CRPD 973	*Aspergillus*	5.53	+	W+	-	-	-	-	+	+	+
12	CRPD 1010	*Aspergillus*	26.05	+++	-	-	-	-	-	+	+	+
13	TSE 42	*Aspergillus*	5.53	+	W+	-	-	-	-	+	-	+
14	TSE 43	*Aspergillus*	42.68	+	W+	-	-	-	-	+	+	+
15	TSE 19	Ascomycetes (Unidentified)	44.69	+	-	-	-	-	-	+	+	+
16	TSE 71	*Aspergillus*	41.78	+	W+	-	-	-	-	+	+	+
17	TSE 80	*Aspergillus*	51.32	+	W+	-	-	-	-	+	+	+
18	TSE 85	*Aspergillus*	47.51	+	-	-	-	-	-	+	+	+
19	TSE 234	*Aspergillus*	59.24	+	W+	-	-	-	-	+	+	+
20	TSE 126	*Aspergillus*	47.26	+	W+	-	-	-	-	+	+	+
21	TSE 292	*Aspergillus*	38.34	+	+	-	-	-	-	+	+	+
22	TSE 293	*Aspergillus*	33.32	+	W+	-	-	-	-	+	+	+

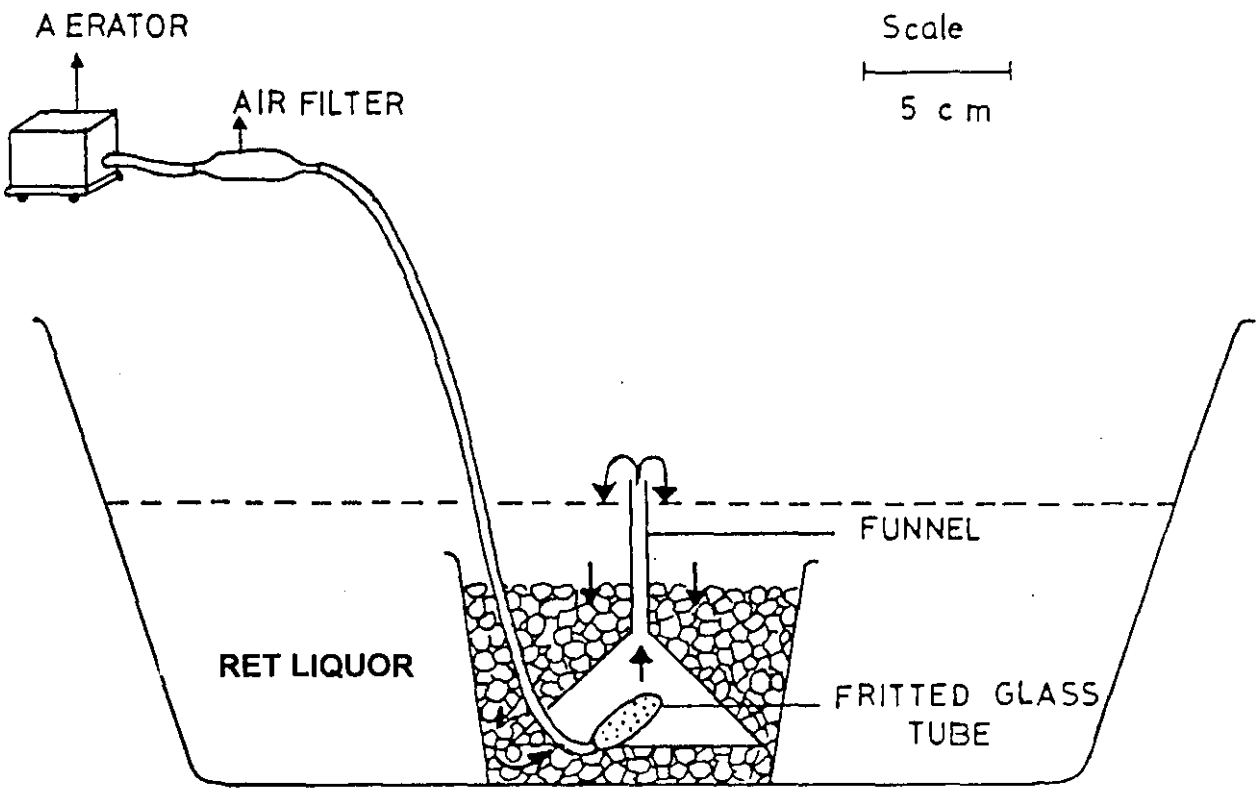


Fig.2.1 Selective enrichment in a packed bed treatment system

CHAPTER - 3

DEVELOPMENT OF ACTIVATED SLUDGE SYSTEM

CHAPTER 3

DEVELOPMENT OF ACTIVATED SLUDGE SYSTEM

3.1 INTRODUCTION

The activated sludge process, the most efficient biological waste water treatment system was conceptualized way back in 1900 by two English men (Junkins *et al.*, 1983). This process consists of living organisms plus organic matter in a controlled liquid- oxygen- rich (aerobic) environment. The wastewater containing the organic is mixed with microorganisms in the presence of dissolved oxygen for a sufficient period of time and allowed the microorganisms to breakdown the complex organic as a food source to produce more microorganisms which are eventually settled out; carbon dioxide which is dispersed into the atmosphere; water which leaves as part of the final effluent and energy which the microorganisms utilize to maintain their life system .(Junkins *et al.*,1983). Such a system was thought to be most appropriate for the ret liquor generated from the retting bioreactor as it contained organics such as polyphenol, proteins, lipids, carbohydrates, cellulose, hemicellulose, and residues of lignin.For the total mineralization of these compounds hydrolytic and degradative potential of several organisms have to be put into operation. An assemblage of such organisms

have already been developed and as the next step an activated sludge system was required to be integrated in to the coir retting bioreactor.

3.2.MATERIALS AND METHODS

3.2.1. Development of activated sludge

From the segregated 33 bacterial cultures 15 were again selected for the development of activated sludge system representing all genera and also in such a way as to incorporate all the hydrolytic potentials in the system.(Table 3.1). The activated sludge was developed in a plastic container of 2 ft height and 1 ft diameter. Ret liquor containing 100 ppm polyphenol prepared in 20 ppt seawater was used as the medium. pH of the ret liquor was adjusted to 7.5 and nutrients such as NH_4^+-N and PO_4^--P were added in the form of commercial grade Ammonium phosphate to a final concentration of 1%.The above bacterial cultures were grown in nutrient agar slants (one slant per culture) and harvested in to the medium. The system was agitated with a heavy duty motor from top and was also aerated (1 litre per minute) from a laboratory aerator. The system was monitored for drop in pH, polyphenol content, and the contents of NH_4^+-N and PO_4^-- P.

Once in 24 hours the pH was adjusted to 6.2± 0.80. When 90% of the polyphenol and the nutrients were consumed 1/3rd of the ret liquor was replaced with fresh ret liquor and nutrients for the total volume were added. The system was monitored for the formation of flocs and their settling. The process was continued for three months till the activated sludge got matured and at this stage the system was capable enough to consume polyphenol rapidly within 30 minutes. It was maintained by continuous aeration, adjustment of pH, addition of polyphenols and nutrients

3.2.2 Characteristics of the developed activated sludge system

The following characteristics of the activated sludge system developed was recorded on its maturation. pH was measured using narrow range pH paper(E-Merck India pvt.ltd.)and a pH meter (Elico). Conductivity was measured by using a conductivity meter. (Chemitto 130 autoranging conductivity meter),Dissolved oxygen by winkler's titrimetric methed COD and BOD following APHA, 1989,NH_4^+-N and PO_4^--P following Dutch states mines(DSM).

The sludge volume was determined by allowing 100ml in a measuring cylinder to settle for 30 minutes.

MLSS

Prepared a pad with asbestos slurry in the Gooch crucible and heated it at 103-105^{0}c for an hour. Stored it in desiccator till it was cooled completely and weighed before use (B mg). Taken 10ml of the well mixed sample and filtered through the previously weighed crucible. Dried the crucible with the residue for atleast an hour in an oven at 103-105^{0}c, cooled in a desiccator. Weighed and repeated the he procedure till a constant weight is obtained(A mg).

$$\text{MLSS content mg /L} = \frac{(A-B) \times 1000}{10}$$

NH_4^+- N

A known volume of the sample was taken (10ml or Diluted if NH_4^+-N was more) added 2 drops of Potassium sodium tartarate, Mixed well added 1ml of Nesseler's reagent and made upto 100ml waited for 10 min, and the absorbency was measured at 420nm.

P_2O_5

About 25ml of filtered sample was taken in 250 ml beaker. Added 100ml of distilled water , 25ml citric molybdate. The beaker was placed in

a hot plate and kept boiling gently for 3minutes, Added 10ml quinoline hydrochloride drop wise while stirring the sample. A whatman filter paper no.1 in the crucible and prepared an asbestos pad over that. Applying suction and filtered the beaker containing quinoline phosphomolybdate precipitate, washed the ppt with neutral water until on adding 2 drops of methyl orange indicator, the wash water passing through the stem of the funnel showed no acidity. About 100ml water was taken out in the beaker, and the crucible , was placed in it as such and, added few drops of mixed indicator along with add 10ml of 0.1N Sodium hydroxide and dissolved the precipitate completely. Titrated the excess sodium hydroxide with 0.1N hydrochloric acid($v_1$ml).As blank 10ml of 0.1N NaOH was titrated with 0.1N hydrochloric acid.

P_2O_5 was calculated using the equation

$$P_2O_5(ppm) = \frac{V_0 - V_1 \times N \times 2.732 \times 1000}{\text{Volume of sample}}$$

3.2.3 Uptake of polyphenols by the activated sludge.

To determine the polyphenol uptake potential of the activated sludge developed 1ml sludge (120mg dry weight) was inoculated ret liquor

containing 100 ppm polyphenol with a pH of 6.5± 0.5. The preparation in 250ml conical flask was maintained on rotary shaker at 100rpm and the polyphenol content was measured once in 24 hours following(APHA,(1989), pH when dropped was adjusted using 1N NaOH. As control one set of flask without the inoculum was maintained in the same fashion. The experiment was run for 16 days and repeated four times.

3.2.4.Relationship between polyphenol uptake and oxygen consumption

The relationship between polyphenol uptake and oxygen consumption was determined in oxygen bottles. The experiment was designed for 5days,and one set of oxygen bottles for the test and another set for the control (12nos) were sterilised by immersing in boiling water for 30 minutes. Ret liquor containing 100ppm polyphenol was supplemented with commercial grade ammonium phosphate to the final concentration of 1% and sterilized by autoclaving at 10 lbs for 10min. The preparation was agitated for 15 minutes manually to aerate adequately and was poured into the oxygen bottles aseptically. Subsequently 6 bottles were seeded with 1ml activated sludge (116.5mg dry weight)and sealed immediately . Control set of bottles were maintained without inoculation. Starting from the zero hour for 6 consecutive days content of oxygen and polyphenol were maintained

following the methods described earlier. Before the addition of the reagents the bottles were streaked on nutrient agar prepared in 20ppt seawater from the values obtained percent consumption of oxygen and polyphenol once in every 24 hours in both the set of bottles were worked for 5 days. Subsequently the simple correlation coefficient between the rate of uptake of polyphenol and oxygen by the activated sludge was determined following Snedecor and Cochran(1967) where the correlation coefficient,

$$\Upsilon = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{\sqrt{[N\Sigma x^2 - (\Sigma x)^2][(N\Sigma y)^2 - (\Sigma y)^2]}}$$

The computer programme `Excel' was used to compute the results.

3.2.5 Fabrication of the system

To be integrated into the bench scale retting reactor an aeration basin and a clarifier were designed and fabricated in perspex.

Aeration basin

The aeration basin has 50 cm height and 31cm^2 base. An inlet pipe having 1.5 cm inner diameter is connected to the tank at 36cms height from the bottom which bends and reaches the bottom of the tank. The pipe is

connected to the retting reactor through a flexible hose. On the opposite side an out let pipe of the same diameter is fixed at the same height from the bottom. For receiving the return sludge from the clarifier an inlet pipe is fixed to one of the sides at 36.5 cm from the bottom. An aeration point is given in this pipe so that it would work as an air lift pump to draw return sludge from the clarifier. For agitating the effluent a heavy duty motor fixed with baffles is used. Besides, an aeration point also is given in the tank.

Clarifier

The clarifier is also made of perspex having 40 cm height, 30 cm width and 31 cm length, fitted with sludge arrestors. Sludge arrestors are dismantlable sheets of perspex with baffles on the inner side. To receive the treated effluent from the aeration basin, there is an inlet pipe of 1.5cm inner diameter fixed at 36 cm height which bends and reaches the bottom of the tank. From the point at which the treated effluent from the aeration tank is discharged, an out let pipe of 1.5 cm inner diameter connects to the air lift pump fixed at the side of the aeration basin through a flexible hose. Opposite to the inlet pipe of the clarifier an out let pipe of the same dimension is fixed at the same height through which the clear fluid can go out.

3.3 RESULTS AND DISCUSSION

For the development of activated sludge system the consortium of bacteria used consisted of 15 cultures belonging to *Pseudomonas, Alteromonas, Acinetobacter, Aeromonas,* Enterobacteriaceae, *Micrococcus*, *Bacillus*, and Coryneform group. All these cultures were the ones which exhibited comparatively higher percentage consumption of polyphenol in ret liquor and all cultures put together the consortium as a whole has all the required hydrolytic potentials expected in an activated sludge system.(Table 3.1). Such a diverse flora with the potential to hydrolyse variety of compounds was essential for an activated sludge system for the treatment of ret liquor as it contained several organics in varying concentrations. In general in an activated sludge system bacterial genera which most frequently occur are *Pseudomonas, Flavobacterium, Achromobacter, Chromobacterium, Azotobacter, Micrococcus,Bacillus, Alcaligens, Arthrobacter, Acinetobacter, Mycobacterium, Nocardia, Leptomonas*,(Hawks, 1963., Mc Kirney ,1962.,Gils, 1964., Harkners,1966., Lighthart and Oglesby,1969., Benedict and Carlson, 1971., Pike and Curds, 1971., Pike and Carrington, 1972.,).Along with bacteria molds and Yeasts also can be seen in lesser amounts(Hawks, 1963.,Mc Kinney ,1962., Pike

and Curds, 1971.,Pike and Carrington 1972., Cooke,1963., Cooke and pipes,1969).Higher organisms such as Protozoa, Rotifers,worms , and others are regularly present in activated sludge system (Hawks, 1962., Mc Kinney I962, Pike and Curds 1971.)But the present activated sludge system is the one developed from pure bacterial cultures, Filamentous fungi and protozoans and rotifers were not seen.

Floc formation was rapid and with is three months the sludge could et matured enough for the rapid removal of polyphenols, BOD, and COD. Sludge at this stage was characterized and compared with that of 20ppt seawater and the ret liquor as influent (Table 3.2). A COD of 2000, BOD of 10, Sludge Volume of 30± 5.0ml per litre and MLSS of 1123.±56.0 were the noteworthy features of the activated sludge in the aeration basin. Generally an effluent in the aeration basin of an activated sludge system have COD of 250 mgL^{-1} and BOD of 30 mgL^{-1} the sludge volume should be with in the range of 100-200 SVI and MLSS should be 2000-3000 μgL^{-1}. Accordingly the activated sludge generated could be said to be fairly satisfactory. Good flocculation resulting in good sedimentation of sludge flocs are one of the most valuable properties of the natural mixed cultures (Pike and Curds, 1971).

Kinetics of the sludge was determined and on beginning with 100ppm polyphenol, around 90% of them got consumed with in 16 hours(fig. 1). Between flasks wide fluctuations in the per cent consumption of polyphenol could be found, but towards the completion the deviations became much narrower suggesting that the sludge flora got stabilized with in a period of 14 - 16 hours. In the control sets of flasks also there was disappearance of polyphenol, but only to a maximum of 20%.

The relationship between polyphenol uptake and oxygen consumption was determined in oxygen bottles and is represented in figs 3.2 and 3.3. The process of polyphenol uptake was found to be very much an oxidative process and the polyphenol uptake stopped when 45% of the available oxygen was consumed with in 72 hours. Even after 24 hours of incubation the rate of polyphenol uptake was halved even when 25% of oxygen consumption had been consumed. The simple correlation coefficient between polyphenol uptake and oxygen consumption was worked out to be 0.7769(Fig 3.2).On the other hand in uninoculated controls disappearance of polyphenol was very little, and within 48 hours around 60% of the oxygen had been consumed. The correlation coefficient between the oxygen consumption and polyphenol uptake in the control sets of bottles was found to be 0.3117.

On comparing the oxygen consumption of the activated sludge with that occurred in a control set of flasks without any inoculum at 48th hour of incubation it was found that 60% of the oxygen was consumed by the bacterial flora in sea water which might not have taken part in the consumption of polyphenols. Meanwhile at 24th hour of incubation oxygen consumption in both sets of bottles was around 38% . In the control sets of flasks the oxygen consumption was very meagre, 65 to 70% during 72 to 120 hours and at the same time in bottles inoculated with the activated sludge the oxygen consumption during the same period of incubation was from 92 to100% .

The data clearly indicate that bulk of the oxygen is consumed by weed organisms / contaminants which thrived on the components of ret liquor. And, therefore just like any other activated sludge system the one developed here also requires to be aerated adequately to maintain rapid rate of polyphenol uptake.

Thus there existed a positive correlation between the polyphenol uptake and oxygen consumption by the activated sludge which was found to be statistically significant. The wider standard deviation obtained in the case of polyphenols uptake and the situation of not having such deviations in the case of oxygen consumption in the corresponding

bottles did not appear to have affected the correlation between the two processes. Observation made in the control set of bottle indicate that oxygen uptake was not due to polyphenol uptake alone, but may be due to hydrolysis of other components such as proteins, lipids and carbohydrates in the ret liquor as evidenced by bacterial growth in the control set of bottles. It is concluded that polyphenols uptake is an oxygen dependent process, however the oxygen depletion need not be necessarily due to polyphenol uptake alone. It again emphasis the fact that sufficient quantity of oxygen has to be pumped continuously for the active progressive uptake of polyphenols.

To carry out the activated sludge an aeration basin and a clarifier were designed and fabricated in perspex.

The aeration basin has 50cms height and 31cm^2 base . An inlet pipe having 1.5cms inner diameter is connected to the tank at 36cms height from the bottom which bend and reaches the bottom of the tank. The pipe is connected to the retting reactor through a flexible hose. On the opposite side an outlet pipe of the same diameter is fixed at the same height from the bottom. For agitating the effluent a heavy duty motor fixed with baffles is used . For receiving the return sludge from the clarifier an inlet pipe is fixed to one of the sides of the aeration basin at 36.5cm from the bottom.

When air is passed through the pipe it functions as an airlift pump to draw sludge from the clarifier (Fig. 3.4).

The clarifier has 40cms height, 30cms width, 31 cms length filled with sludge arrestors, all made of perspex . The sludge arrestors are slanted perspex sheets with baffles facing downward. To receive the treated effluent from the aeration basin there fitted an inlet pipe of 1.5cms inner diameter fixed at 36cms height from the bottom. This pipe bends and reaches the bottom of the tank. For the clarified effluent to flow out an out let pipe is fixed at the same height on the opposite side.

Ret liquor from the retting reactor is directed to the aeration basin and a retention time of 24 hours is given for the effluent to flow out into the clarifier. Since it is a continuous system the same period is given there also for the settling of sludge. There is a continuous movement of the sludge to the aeration basin through the air lift pump which serves as the inoculum. The clarified fluid flows to the packed bed reactor.

Activated sludge process is one of the major biological waste water treatment techniques. The bioflocculated microbial aggregates, known as flocs, are the essential components of the system. The primary objective of the process is the transformation of organic matter into carbondioxide and cell materials, and incorporation of colloidal matter into settlable solids are

ultimately implemented by individual flocs (Li and Ganczarczyk (1990). On the other hand many major operating problems in the process, such as those which occur in solid - liquid operation ,can also be attributed to the properties of the flocks. The activated sludge developed here for the treatment of ret liquor are quite larger particles easily settable. This sludge could remove polyphenol at the rate of 4.37 ppm per hour considering the 20% removal observed in the control flasks. Moreover, this is an oxygen dependent process and should be adequately aerated to maintain an oxygen concentration not less than 3.5mg. L^{-1}. One of the advantages of the present activated sludge system is that the sludge has been generated from the pure cultures of bacteria assuring its reproduction at any moment. This considerably enhance the viability of the process and makes it user friendly.

The bench scale aeration basin and clarifier can be easily integrated into the retting reactor. The retting reactor works in a batch mode and as such when the retting gets completed the ret liquor is directed to the treatment system(Anon , 1998). In the pilot plant which is proposed (Anon,1998) for the 20 retting reactors together one activated sludge system would be sufficient so as to treat the ret liquor from one of the retting reactors emptied.

In this mode the aeration basin and the clarifier have 120cm height and 160 cm diameter which will serve the requirement of a plant having the retting capacity of 250 husk per day. Because during the retting process the reactor can be isolated and no exchange of water becomes necessary. Retting gets completed with in 45 days and only after removal of the retted husk, the ret liquor requires treatment and if there are 20 retting reactors surrounding one ret liquor treatment system one reactor each can be loaded and charged once in two days and thus when the last reactor gets filled up the first one will be ready enough to be emptied. Consequently the ret liquor treatment system remains always functional. The total cost of the activated sludge system to be integrated in to the pilot plant is estimated to be 28500/-.

For fine polishing of the effluent a packed bed reactor is used, subsequent to the activated sludge system..

Table 3.1. **Bacterial Cultures finally selected for developing the treatment system**

Sl. No.	Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties								
				L	C	He	Pr	Li	Am	Pectinolytic		Tannic acid as sole carbon
										Plate Test	Tube Test	
1	CRPD 668	*Bacillus*	63.34	-	-	-	+	+	-	+	+	-
2	TS 151	*Pseudomonas*	46.32	-	-	-	-	-	-	+	-	-
3	TS 158	*Pseudomonas*	44.38	+	-	-	+	+	-	+	-	-
4	CRPD 226	*Aeromonas*	62.35	+	-	-	+	-	-	+	-	-
5	CRPD 260	*Bacillus*	55.71	-	+	+	+	-	+	+	+	+
6	CRPD 261	Coryneform group	40.59	-	+	-	+	+	+	+	-	-
7	CRPD 607	*Pseudomonas*	61.09	+	-	-	+	-	-	+	-	-
8	CRPD 608	Enterobacteriacae	63.36	+	-	-	-	-	-	+	+	-
9	CRPD 678 c	*Aeromonas*	62.24	+	-	+	+	+	-	+	-	-
10	CRPD 710	*Pseudomonas*	65.75	+	-	-	+	-	-	-	-	-
11	CRPD 805 b	*Micrococcus*	59.80	+	-	-	+	+	-	+	-	-
12	CRPD 810 c	*Pseudomonas*	65.83	+	-	-	+	-	+	-	-	-
13	CRPD 810 x_2	*Aeromonas*	65.17	-	-	-	+	+	-	+	-	-
14	CRPD 1024 b^2	*Acinetobacter*	51.48	+	-	-	-	-	-	-	-	-
15	TSE 190	*Alteromonas*	56.54	+	-	-	+	+	+	+	-	-

Table 3.2 Characteristics of the activated sludge system developed (n=4)

SAMPLE S	PARAMETERS								
	pH	Conducti -vity	DO	COD	BOD	NH_4^+-N $mg.L^{-1}$	P_2O_5 $mg.L^{-1}$	Sludge volume $ml.L^{-1}$	MLSS $mg.L^{-1}$
20ppt sea water as blank	7.9±0.25	0.92±0.3	4.0±0.2	240	3	8.5±0.5	3.5±0.5	Nil	Nil
Activated sludge	6.2±0.75	7.5±0.50	3.5±0.5	2300	10	186±50	687±45.5	30±5.0	1123±56
Influent	7.3±0.5	0.72±0.5	4.2±0.3	4000	50	58±12	30.20±10.3	Nil	Nil

Fig.3.1 Rate of uptake of polyphenols by the developed activated sludge (n=4)

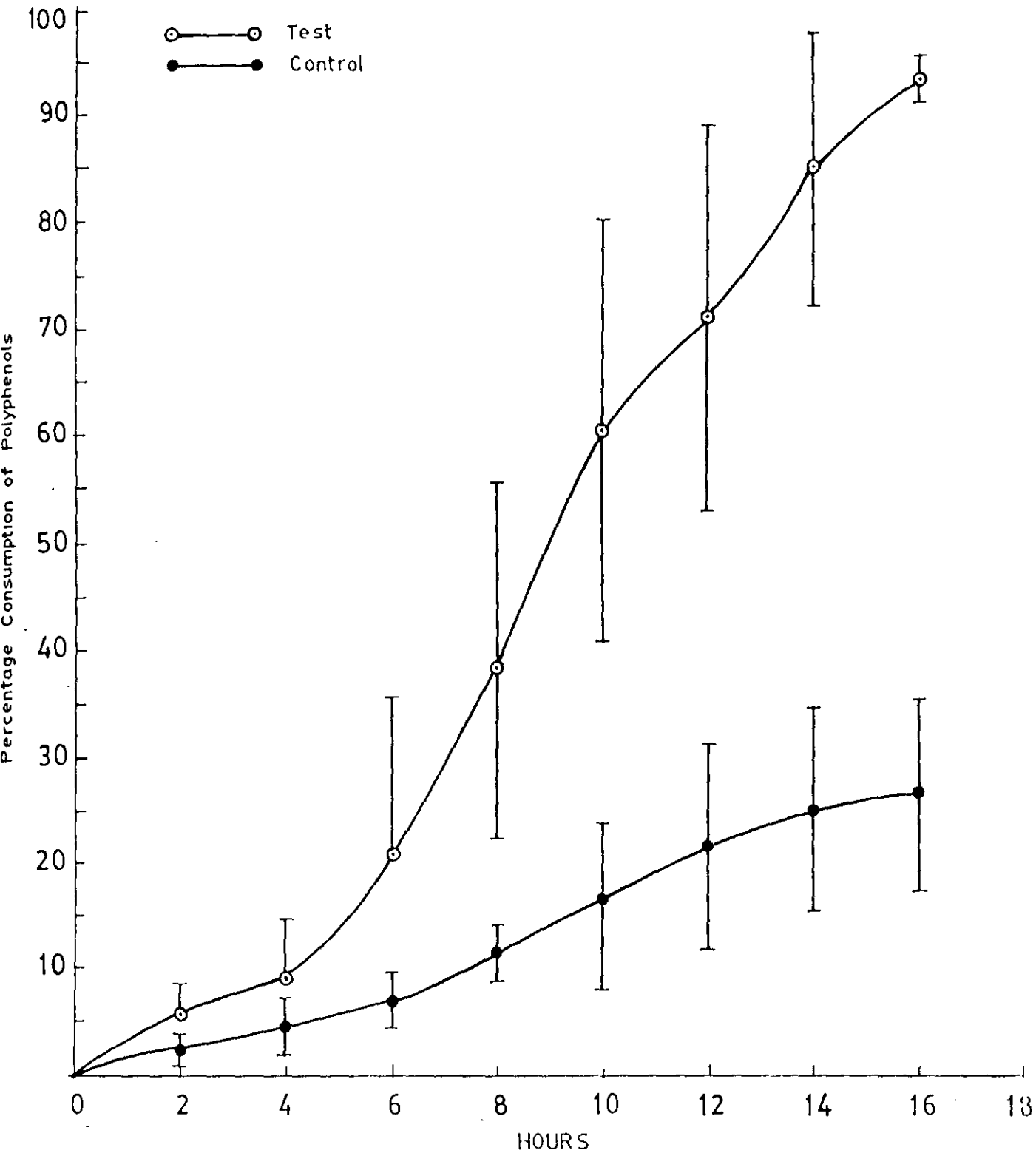


Fig.3.2 Relationship between polyphenol uptake and oxygen consumption by the activated sludge (**Test**; n=4)

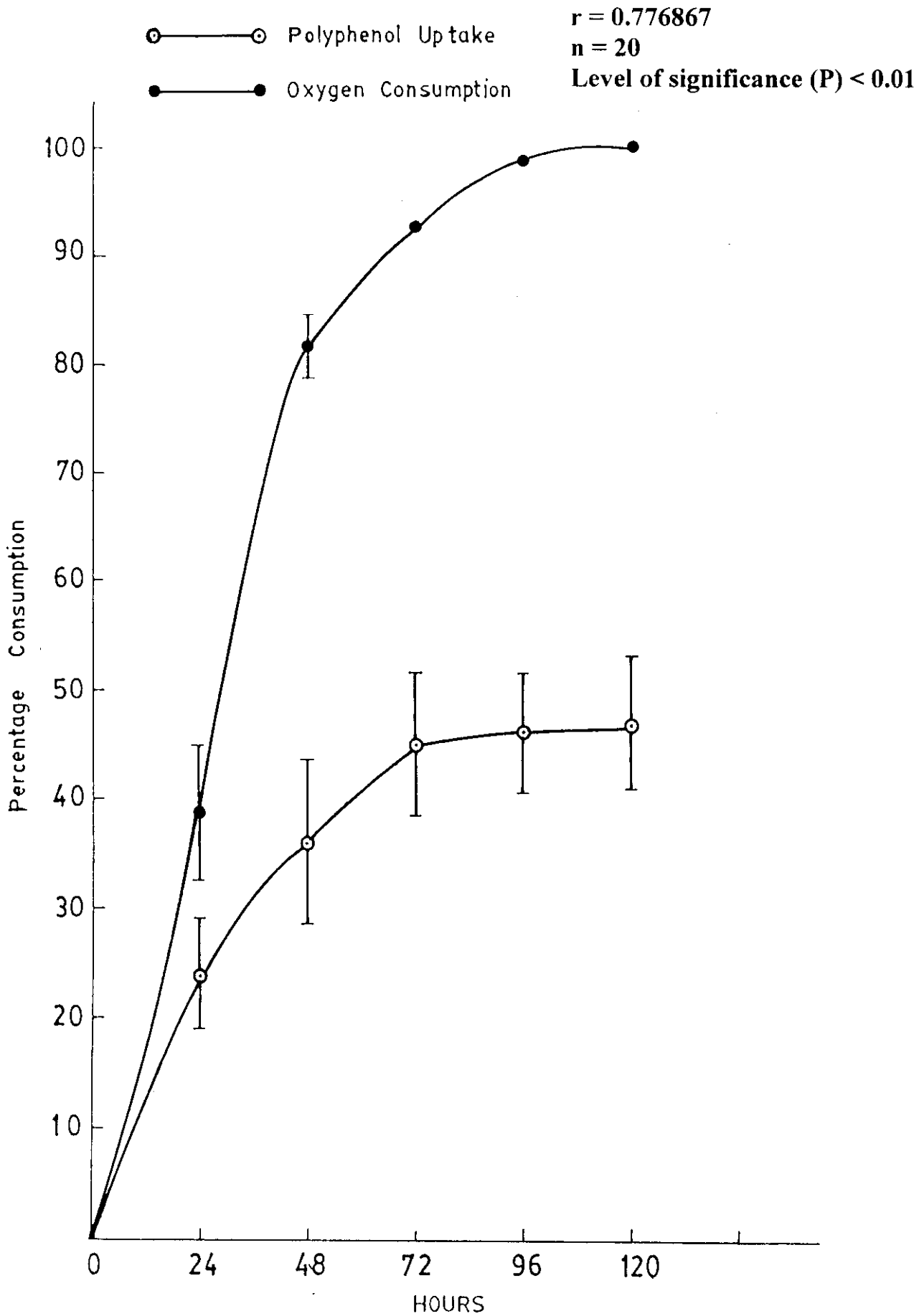
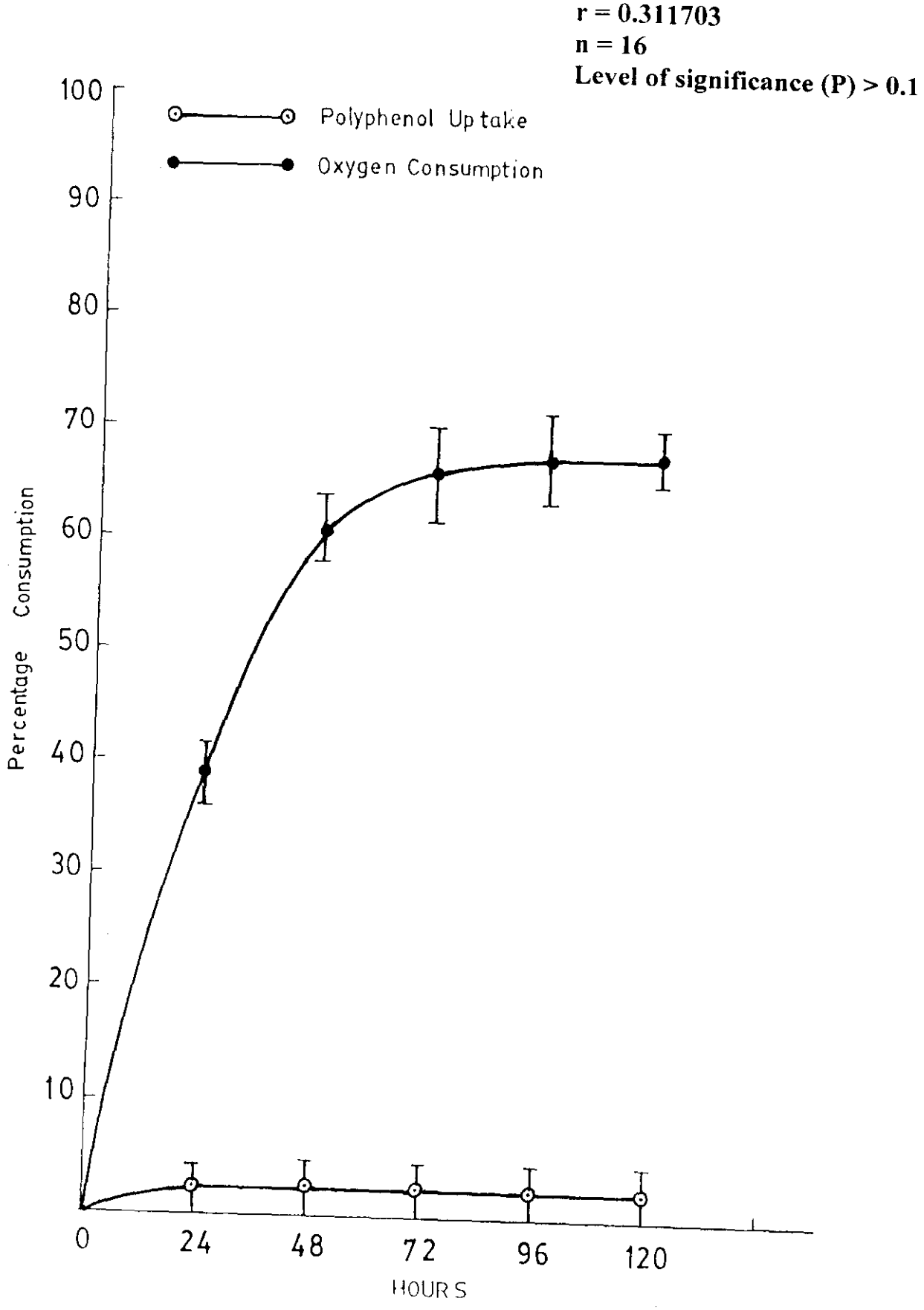


Fig.3.3 Relationship between polyphenol uptake and oxygen consumption by the activated sludge (**Control**; n=4)



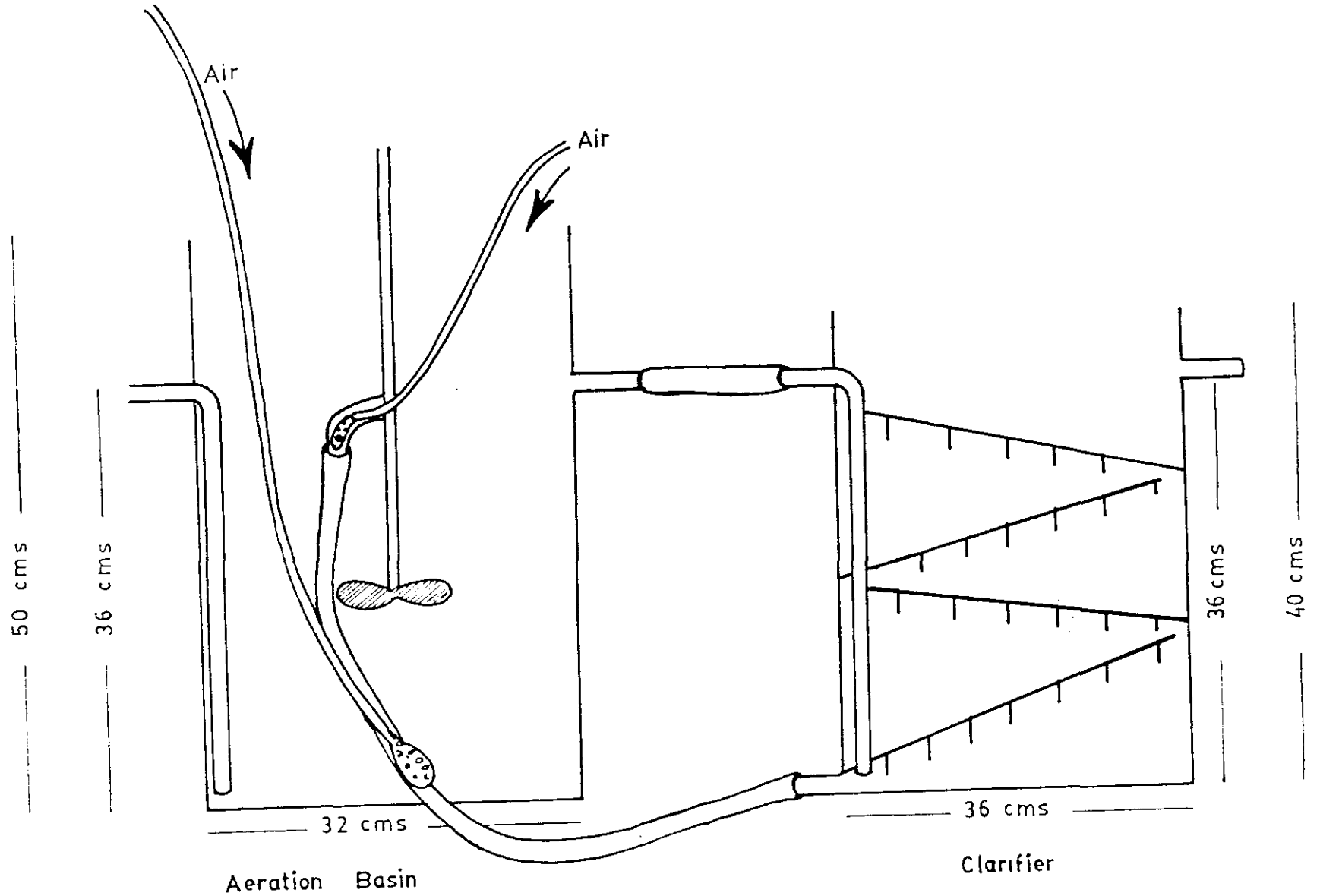


Fig.3.4 Activated sludge system

CHAPTER - 4

PACKED BED REACTOR

CHAPTER 4

PACKED BED REACTOR

4.1 INTRODUCTION

For the final polishing of the ret liquor (effluent) after the treatment in the activated sludge system a packed bed reactor also has been developed. This has been developed with the idea to remove any more polyphenol left in the ret liquor even after passing through the activated sludge system. While the influent passes through the activated sludge system there happens the reduction in pH due to the fermentation of sugars. To counter act this change the reactor is made with crushed oyster shells as the support material. The design of the reactor is made in such a way that the effluent which enters it percolate down and again lifted up through an air lift pump before it goes out after the treatment.

4.2. MATERIALS AND METHODS

4.2.1 Designing and fabrication

The bench scale packed bed reactor designed has 30 cms width and 30 cms breadth and 40 cms overall height. It is made of perspex and at a

point 36 cm from the base an inlet pipe is fixed. At the center of a perforated platform positioned at the base of the tank, 5 cm above the bottom is a 31 cm tall P V C pipe with 1.5 cm inner diametre. An out let pipe fitted at the base of the tank takes a 'U' turn and rises 36cm and discharges the effluent to a collection tank.

The collection tank is made of fibre glass and has dimension of 40 cm^3. It receives the effluent from the packed bed reactor through an inlet pipe of 1.5 cm diameter. An overflow pipe is fixed on the opposite side at the same height limiting the storage capacity to 46.65 litres.

When the storage tank is filled up through an 1.5cm diameter pipe the collected treated effluent is pumped to an overhead tank by a magnetic couple pump.

The reactor is filled with crushed oyster or clam shells as the support materials for the bacteria (Fig . 4.1).

4.2.2. Organisms for the packed bed reactor

The bacterial cultures used for the activated sludge system were used for the packed bed reactor also. Along with them one culture of *Aspergillus* sp chosen from the segregated lot of fungi which exhibited higher level of

polyphenol uptake as exhibited by the halozone in the Crawford's agar was also incorporated (Table 4.1).

Before inoculating the packed bed reactor efficacy of the consortium of bacteria and the fungus in consuming the polyphenol in ret liquor was determined. The bacteria were grown in nutrient agar slants and the fungus in Sabouraud dextrose agar. The bacteria were harvested in 20ppt seawater and absorbency recorded in a UV-visible spectrophotometer at 600nm and diluted to attain an absorbency of 0.05 O.D using 20ppt seawater. Similarly when the fungus generated spores they were also harvested in 20ppt seawater and the spores were enumerated microscopically and diluted to attain 10^2 spores per ml. From the above suspensions of bacteria and fungi 0.1 ml aliquot each were pipetted out in to a series of flask containing 100ml autoclaved ret liquor containing 100ppm polyphenol not supplemented with any nutrient, having the pH adjusted to 7.5 with 1N NaOH or HCL . Appropriate uninoculated controls were also maintained. The flasks were incubated at room temperature on a rotary shaker and once in 24hours for 144 hrs the total polyphenol content was estimated following the method described under the section 2.2.2.d

4.2.3. Inoculating the packed bed with the consortium and testing the efficacy.

The consortium of bacteria and the fungus was prepared as described above and used for charging the packed bed reactor. Ret liquor prepared in 20ppt seawater containing 100ppm polyphenol was introduced into the reactor, pH adjusted to 7.5 and nutrients such as ammonium phosphate was added to a final concentration of 1% . The final inoculum composed of 15 bacterial cultures and one culture of *Aspergillus* sp. was inoculated to a final concentration of 1% with the culture suspension having 0.05 O.D absorbency. It contained fungal spores of 10^{2} ml $^{-1}$. The air lift pump was operated and the effluent was circulated continuously. Total polyphenol content, pH, Nutrients, (NH_4^+-N and PO_4^--P)and formation of flocs were monitored daily. When there was 90% consumption of polyphenols the entire fluid was drained off and fresh equalized ret liquor was added and the process continued.

4.3 RESULTS AND DISCUSSION

The designing of the packed bed reactor was in such a way that when the air lift pump was operated the effluent which entered the reactor flew

down ward percolating through the crushed oyster/clam shells to be lifted up through the central pipe. This type of circulation takes several times before the effluent goes out to be collected in the collecting chamber for recirculation. By adjusting the flow rate of water from the overhead tank to the retting reactor, the rate of flow through the packed bed reactor also can be regulated. Turnover rate through the airlift pump in the packed bed reactor also can be regulated by regulating the quantity of air passed through the air lift pump which may vary from 1 to 7 litres per minute.

The organisms employed in the packed bed reactor have all the potentials to remove the organics present in the ret liquor. Efficacy of the above consortium in removing polyphenols from the ret liquor was tested and it was found that within an incubation period of 144 hours around 97% of the polyphenols could be consumed (Fig. 4.2). Interestingly with in this period the effluent became very clear with the formation of rapidly settling sludge.(Fig. 4.3). These clearly indicated that the consortium developed was suitable for ret liquor treatment in general and for using in the packed bed reactor in particular.

On applying the consortium in the packed bed reactor and circulating through, it was observed that 87.2% of the polyphenols could be removed with in 48 hours. On completion of one cycle the entire fluid was added and

the process repeated without any addition of the inoculum. The process was repeated several times and in all such occasions the rate at which the polyphenol got removed was increasing indicating that the organisms inoculated have already got immobilized on the shell surface. This again indicate that once charged it would be sufficient to continue the process without any more addition of the inoculum (Fig. 4.4).

This packed bed reactor precisely function with the immobilized cells on the support material, the shells of clams or the mussles. The success of any immobilization relies on the proper choice of the carrier. Properties of any carrier can be reviewed in relation to the following criteria (Mosbach, 1976; Royer *et al* 1976)

1. Strength
2. Form
3. Stability
4. Hydrophobicity
5. Porosity
6. Reactivity
7. Reusability
8. Economy

Of all these properties the bivalve shell pieces used here matches with all the specifications except the fifth one, porosity, where in, since the bacteria are immobilized on bivalve shells by adsorption porosity of the support material does not form an important requirement here. The immobilization achieved here is through adsorption primarily through either

ionic or hydrophobic interactions. One of the specialities of the support material used in this reactor is that it impart a favourable pH for the reactor. Since the ret liquor contains variety of sugars fermentation of acid and subsequent lowering of pH. The bivalve shells, which are nothing but calcium carbonates, bring back the pH to neutrality. Further the shells are freely available minimising the cost of the reactor .

The initial interaction between a particle viz. a cell in suspension and a solid surface referred to as adsorption here in is controlled by well defined physical – chemical properties of the cell, solid surface and the suspending liquid (Adamson, 1982 ,Hieminz1986, Neumann *et al.*,1974). Adsorption, here, is defined as the adhesion of the particles on to the surface of a support that has not been specifically altered for covalent or receptor–ligand type bonding. In such a system one must consider the surface tension of the interacting components, pH, ionic strength of the liquid, the zeta potential of the cell surface and the support as well as the concentration of particles in the bulk fluid because these particles have shown to influence adsorption and the subsequent retention of the cells on the support (Di cosmo *et al.*, 1989)

In the packed bed reactor developed here the condition created in the reactor as mentioned above are very much congenial to attain ready

adsorption of cell on to the shell surface and to form an irreversible biofilm. This sort of artificial cellular immobilization actually mimics a natural process. Microorganisms which are involved in the biofilm formation produce a variety of extracellular polysacchride or polypeptide capsule polymers often referred to as the glycocalyx (Costerton *et al.*, 1981 and 1987). When growing on surfaces, biofilm or sessile bacteria are physiologically quite different from their planktonic counter parts(Marshell, 1985.,Gresey and White, 1990.,Van Loosdrecht *et al.*, 1990) These biofilms can be defined as encapsulated communities of cells (Costerton *et al* 1987) This mode of growth enables bacteria to demonstrate remarkable resistance to physical disruption (Reid *et al* 1990) inspite of liquid shear forces (Mc lian *et al.*,1980) which is a persistent force acting upon the biofilm community as the effluent flows down through the packed bed and goes up through the air lift pump.

The biofilm formation resulted here is due to non- specific adhesion of bacteria on to the shell pieces. The role of surface chemistry of the attachment site is of paramount importance as several investigations have shown that hydrophobic surfaces are more prone to colonisation than the hydrophilic ones(Mc Eldowrey and Fletcher, 1986., Pringle and Fletcher, 1986., Humphries *et al*, 1987., Van Loosdrecht *et al*,

1987.,Stenstonm,1989.,)In this case adhesion can be diminished by the addition of detergents(Marshall et al,1989., Cowan *et al*,1987.,Bryant et al, 1984.,)or enhanced by the addition of cations (Fletcher,1988.,)such as Na^{3+}, Ca^{3+}, La^{3+} or Fe^{3+}. In the developed packed bed reactor no detergent stream is coming in to and there is abundant availability of Na^{3+} , Ca^{3+} and Fe^{3+} in the system as they are present in seawater(20ppt) used and especially $Ca^{3+}$is available with the support material as $CaCO_3$.

Adhesion and the consequent biofilm formation is not a simple out come of hydrophobic interaction. Cowan *et al*(1987)found that while hydrophobic interactions are evident, electrostatic bonding was even more significant. It is likely that the bonding chemistry consists of a mixture of hydrophobic, electrostatic, hydrogen, Vanderwaals and possibly even co-valent bonds. The importance of each type of bond is influenced by the chemical environment of the surface and surroundings fluids, and the physiological status of the organisms (Mc Lean *et al*,1994). This has to be further investigated in this reactor.

Aquatic microorganisms tend to colonize surfaces more readily in streams than in a pond (Mills and Manbery, 1981). Presumably the higher water flow of the stream carried more organisms part these rocks and also created a stress factor in which these organisms were obliged to adhere in

order to persist in this environment. This principle very true with regard to the mode of immobilization of cells on the bivalve shell surfaces. When the fluid is flown through the packed bed the stress factor created might be forcing the organisms to attach on to the support material supported by other favourable conditions as described above.

Precisely, the packed bed reactor developed here is compact, easy to set up, maintain and is less expensive and efficient to treat the effluent which flows out of the activated sludge system and it forms the component of the ret liquor treatment system.

Table 4.1 **Bacterial and fungal Culture finally selected for developing the treatment system**

Sl. No.	Culture No.	Family / Genus	Percent Consumption of Polyphenols	Hydrolytic Properties								
				L	C	He	Pr	Li	Am	Pectinolytic		Tannic acid as sole carbon
										Plate Test	Tube Test	
1	CRPD 668	*Bacillus*	63.34	-	-	-	+	+	-	+	+	-
2	TS 151	*Pseudomonas*	46.32	-	-	-	-	-	-	+	-	-
3	TS 158	*Pseudomonas*	44.38	+	-	-	+	+	-	+	-	-
4	CRPD 226	*Aeromonas*	62.35	+	-	-	+	-	-	+	-	-
5	CRPD 260	*Bacillus*	55.71	-	+	+	+	-	+	+	+	+
6	CRPD 261	Coryneform group	40.59	-	+	-	+	+	+	+	-	-
7	CRPD 607	*Pseudomonas*	61.09	+	-	-	+	-	-	+	-	-
8	CRPD 608	Enterobacteriaceae	63.36	+	-	-	-	-	-	+	+	-
9	CRPD 678 c	*Aeromonas*	62.24	+	-	+	+	+	-	+	-	-
10	CRPD 710	*Pseudomonas*	65.75	+	-	-	+	-	-	-	-	-
11	CRPD 805 b	*Micrococcus*	59.80	+	-	-	+	+	-	+	-	-
12	CRPD 810 c	*Pseudomonas*	65.83	+	-	-	+	-	+	-	-	-
13	CRPD 810 x_2	*Aeromonas*	65.17	-	-	-	+	+	-	+	-	-
14	CRPD 1024 b^2	*Acinetobacter*	51.48	+	-	-	-	-	-	-	-	-
15	TSE 190	*Alteromonas*	56.54	+	-	-	+	+	+	+	-	-
16	CRPD 1010	*Aspergillus*	26.05	+	-	+	-	-	-	+	+	+

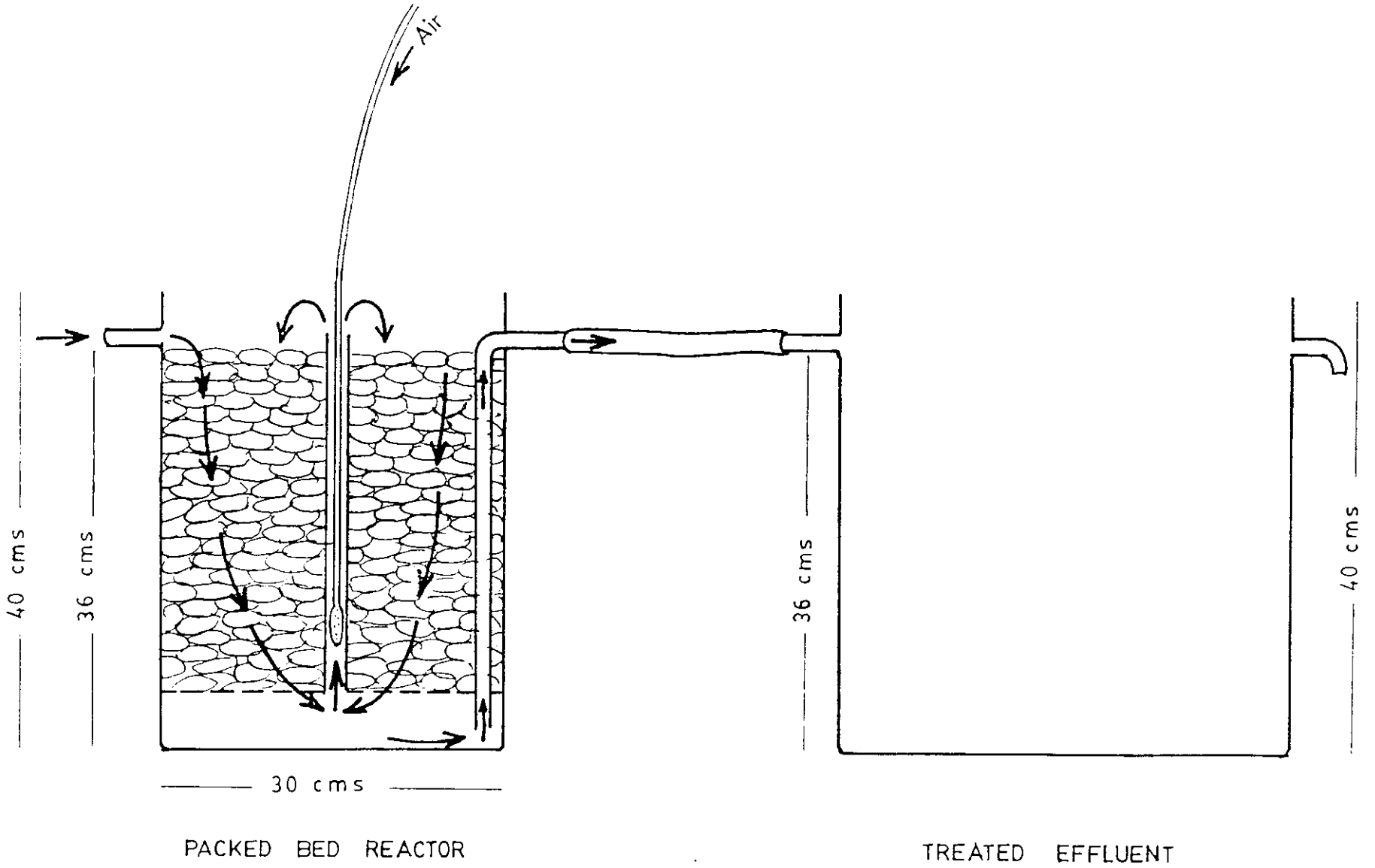
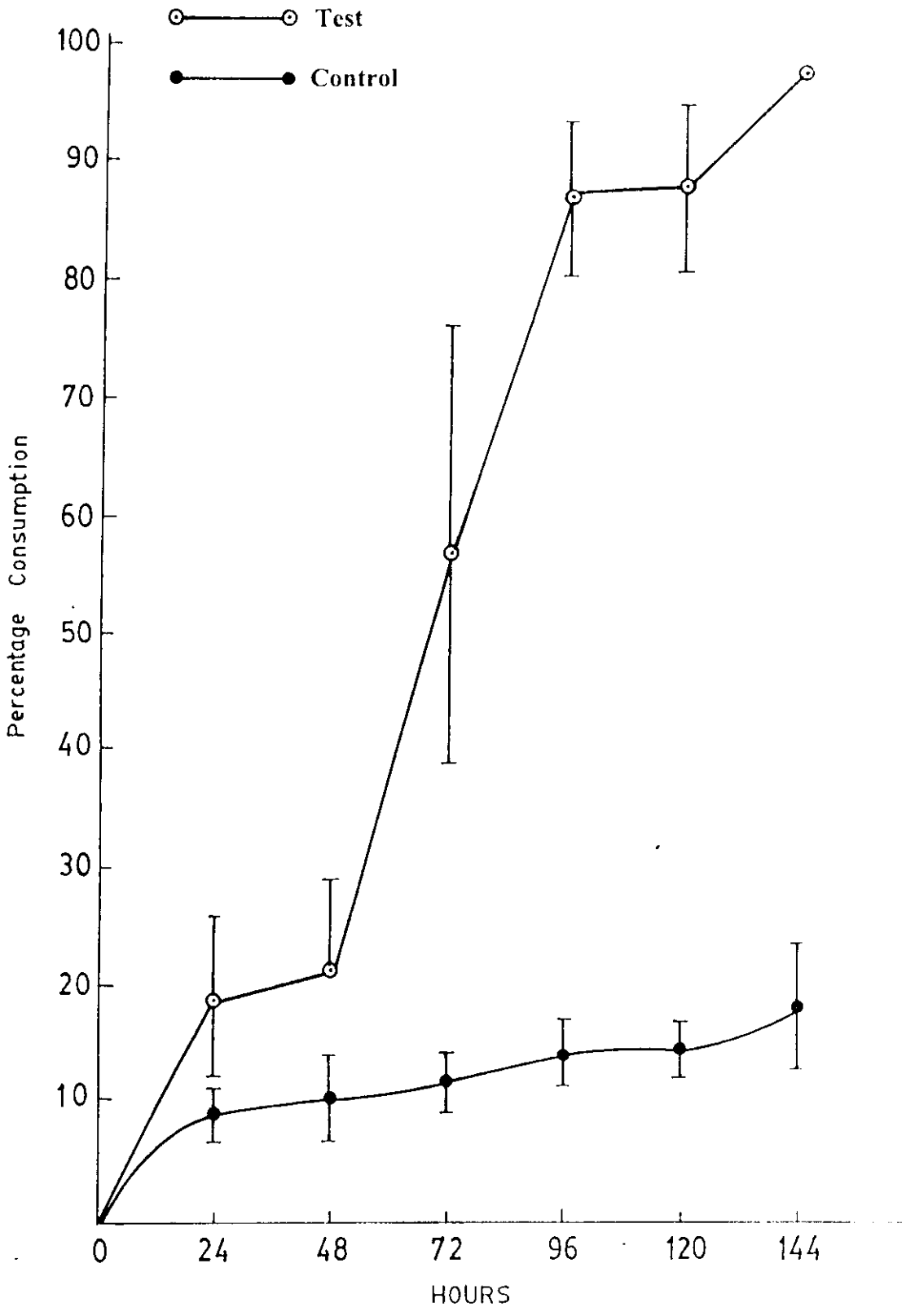


Fig.4.1 Packed bed reactor

Fig.4.2 Efficacy of consortium in removing polyphenols from the ret liquor *in vitro*



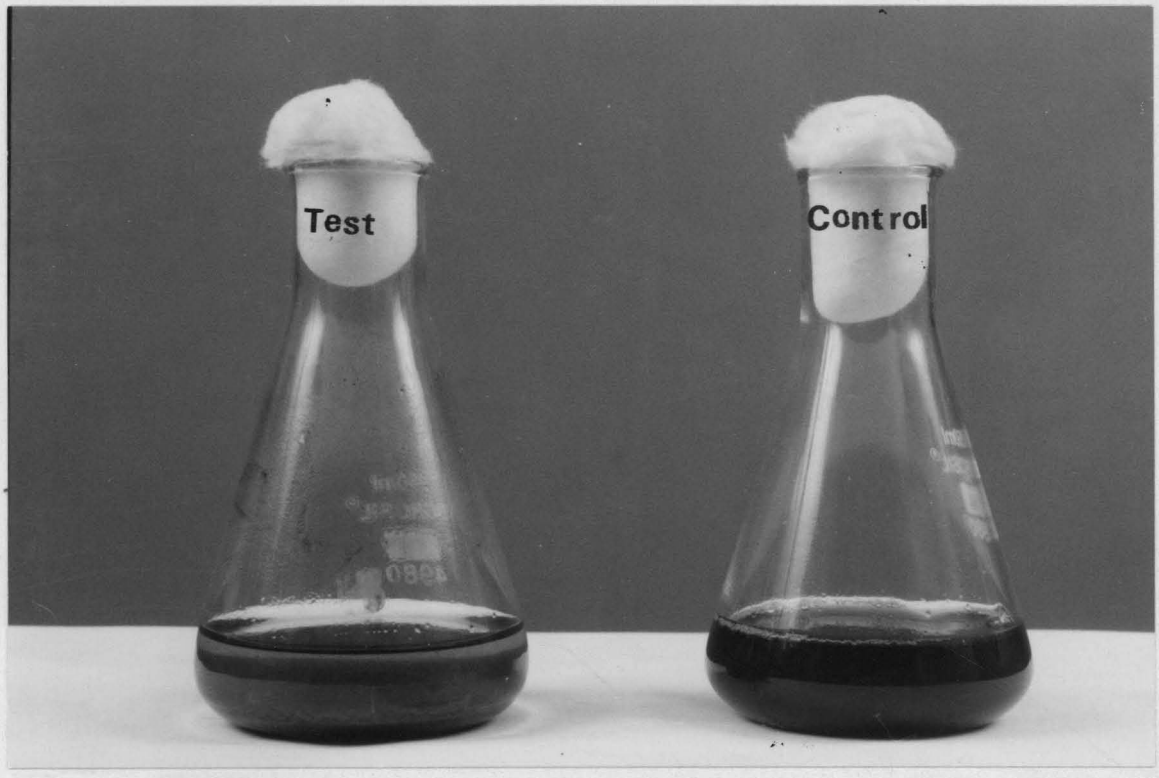
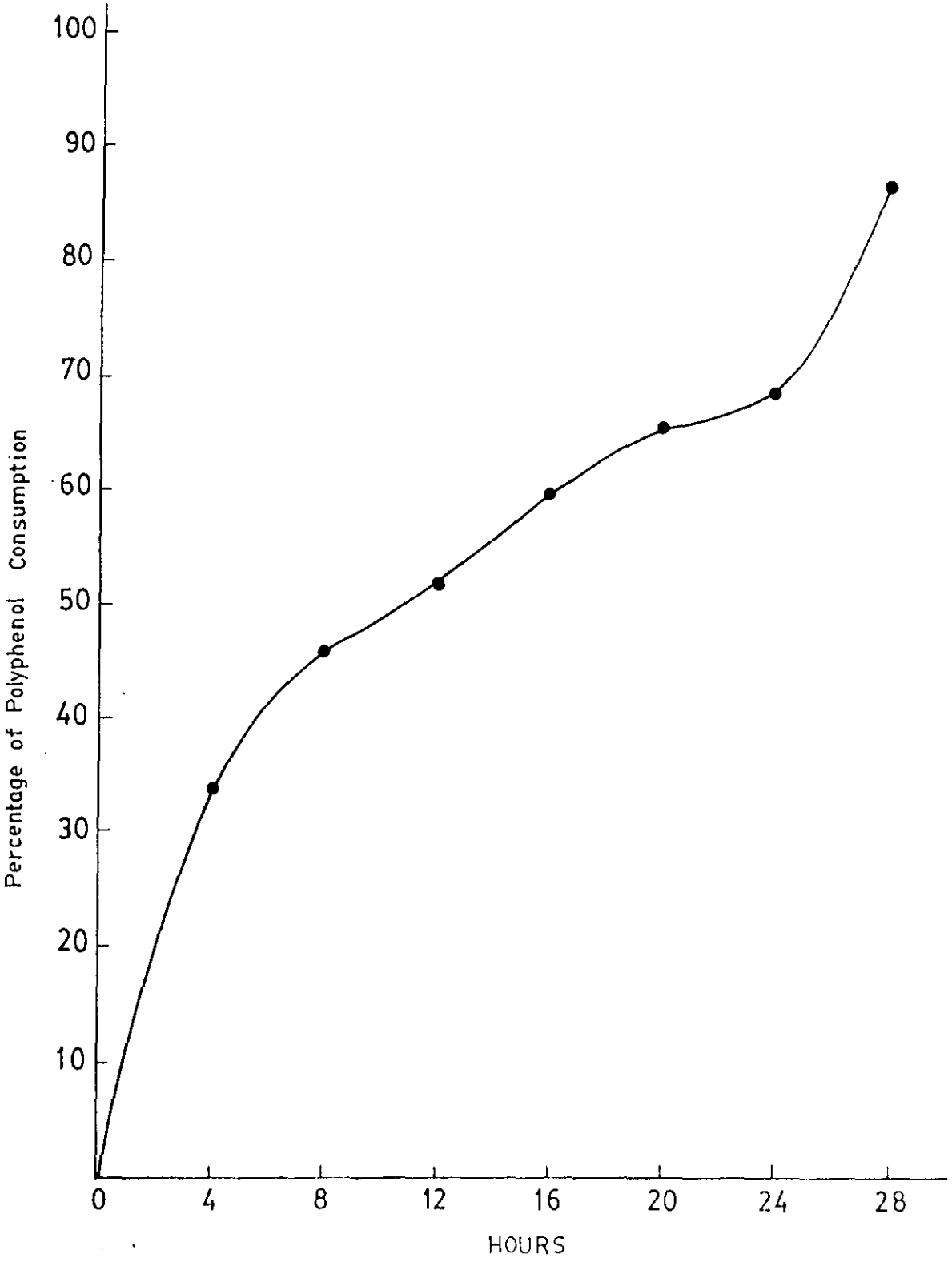


Fig.4.3 Photograph showing efficacy of consortium in removing polyphenols

Fig.4.4 Polyphenol consumption in the packed bed reactor



CHAPTER - 5

CONCLUSION

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CONCLUSION

Retting coconut husk for the extraction of coir fibre is a traditional age old practice which forms backbone of the Coir industry. For achieving retting the green husk is steeped in water for periods ranging from 6 to 12 months. This leads to immense pollution of aquatic system and much of the fauna and flora are lost from the retting ground. The only way to abate pollution and to get a better control over the process is to lift the whole process from open waters and to perform it in a confined system. With this as the objective a bioreactor has been developed which consist of three components

a. **Polyphenol stripping system (pre-retting operation).**

b. **Retting reactor.**

c. **Ret liquor treatment system.**

The present work focuses attention on the third component, the ret liquor treatment system and this chapter summarizes how such a system was developed and standardized.

- For obtaining the right microbial cultures for the ret liquor treatment system three types of enrichment systems were built up .

- The first of its category was successive enrichment system made in husk infusion which was completed by inoculating the medium with 25 ml sample prepared out of sediment obtained from a retting ground. The system was monitored for color, pH, total polyphenol and on attaining sufficient disappearance of polyphenols from the culture broth, 25ml of the culture was passaged to another broth of 225 ml having the same composition and incubated for the same period along with monitoring of the same parameters.
- The process was repeated three more times and at every stage of transference heterotrophic bacteria were isolated on Nutrient agar, Husk Infusion Nutrient agar and Husk Infusion Sabouraud dextrose agar, and accordingly altogether 180 bacterial isolates could be obtained.
- The second category of enrichment system comprised ret liquor as the medium with 100 ppm polyphenol and the samples consisted of water, sediment and partly retted husk from 18 stations which were active retting zones.
- Employing Nutrient agar and Sabouraud dextrose agar prepared in ret liquor 191 bacterial cultures and 161 fungal cultures could be isolated.
- The third category is the selective enrichment and isolation of polyphenol degrading microorganisms through a packed bed treatment system which

was inoculated with water and sediment samples from various retting grounds. The system was monitored for the drop in pH and polyphenol content. From the treated ret liquor and the filtrant grains of the above enrichment system, bacteria and fungi were isolated through ret liquor solidified with agar, supplemented with 1% NH_4Cl and 0.2% NH_4NO_3 and 0.001% yeast autolysate and pH adjusted to 7.5 for bacteria and 4.8 for fungi . Accordingly 183 bacterial cultures and 68 fungal cultures could be obtained from the system.

- The above isolated cultures were screened for their capability to utilize tannic acid , pyrogallol and pyrocatechol as sole source of carbon and energy their capability to degrade the polyphenols present in ret liquor, percentage consumption / degradation of polyphenols, and their hydrolytic properties such as lignolysis, cellulolysis, hemicellulolysis, proteolysis, lipolysis , amylolysis and pectinolysis.
- Accordingly from all the three enrichment systems altogether 33 bacterial cultures were segregated based on their capability to consume / degrade polyphenols and to produce hydrolytic enzymes. This assemblage was consisted of Enterobacteriaceae, *Aeromonas* , *Bacillus,* Coryneform group, *Pseudomonas, Micrococcus, Acinetobacter, Planococcus,* and *Alteromonas.*

- In a similar manner from the above enrichment systems 22 fungal cultures were selected based on their capability to degrade / consume polyphenols and their hydrolytic potential, majority of them belonging to *Aspergillus,* two were unidentified Ascomycetes and one culture was *Fusarium* sp. This collection of bacteria and fungi formed the source of organisms for the development of different ret liquor treatment systems.
- The study indicated that even though rigorous enrichment and isolation procedures were followed surprisingly a variety of bacterial genera could be isolated which were capable to degrade polyphenols. This indicated that in the natural environment polyphenol degradation potential was not limited to certain genera such as *Pseudomonas* and *Bacillus* alone. This further indicate the effectiveness of the process in the retting ground which leads to the conclusion that polyphenol degradation in natural environment is a result of concerted action of several groups of organisms and when a treatment process is developed same sort of assemblage of organisms has to be built up.
- Among the fungi isolated *Aspergillus* species dominated which are capable to use tannic acid as the sole source of carbon and energy.

- From the segregated 33 bacterial cultures 15 were again selected for the development of activated sludge which represent all genera in such a way to incorporate all the hydrolytic potentials in the system.
- Using the consortia of 15 bacterial cultures an activated sludge was developed within a period of 3months.
- The developed sludge was characterized by good floc formation and rapid settling within 30 minutes.
- At this stage in the aeration basin a COD of 2300mg.L^{-1}, BOD of 10mg.L^{-1}, Sludge volume of 30 $\pm$ 5.0 ml. L^{-1} and MLSS of 1100 $\pm$ 56.0 $\mu g.L^{-1}$ was constantly noted which was found to be satisfactory.
- The activated sludge thus developed was found to be capable enough to consume 4.33ppm polyphenol per hour considering the 20% removal observed in the control flask.
- The sludge on introducing into ret liquor was found to get stabilized within a period of 14-16 hours.
- The relationship between polyphenol uptake and oxygen consumption was determined in oxygen bottles and the process of polyphenol uptake was found to be very much an oxidative process and the polyphenol uptake stopped when 45% of the available oxygen was consumed within 72 hours.

- There existed a positive correlation between the polyphenol uptake and oxygen consumption by the activated sludge which was found to be statistically significant. The wider standard deviation obtained in the case of polyphenol uptake and the situation of not having such deviations in the case of oxygen consumption in the corresponding bottles did not appear to have affected the correlation between the two processes.
- In the control set of bottles also oxygen consumption was noticed but without any polyphenol uptake, indicating that oxygen uptake was not due to polyphenol uptake, but may be due to hydrolysis of other components such as proteins, lipids, and carbohydrates in the ret liquor as evidenced by bacterial growth in the control set of bottles.
- It could be concluded that polyphenol uptake is an oxygen dependent process, but the oxygen depletion need not necessarily be due to polyphenol uptake alone.
- It again emphasis the fact that sufficient quantity of oxygen has to be pumped continuously for the active and progressive uptake of polyphenols.

- To carry out the ret liquor treatment by the activated sludge an aeration basin and a clarifier were designed and fabricated in perspex .
- Ret liquor from the retting reactor is directed to the aeration basin and a retention time of 24hours is given for the effluent to flow out in to the clarifier. Being it a continuous system same period is given there also for the settling of sludge. There is a continuous movement of the activated sludge to the aeration basin through the air lift pump. The clarified fluid flows to the packed bed reactor.
- The packed bed reactor has been developed with the idea to remove any more polyphenol left in the ret liquor which comes out of the activated sludge system.
- In this reactor oyster / clam shells are used as the support material in order to bring back the pH of the effluent to neutrality as the effluent which enters the reactor from the activated sludge system would be slightly acidic due to the fermentation of sugars.
- The designing of the packed bed reactor is in such a way that when the air lift pump is operated the effluent which enters the reactor flows downward percolating through the crushed oyster shells to be lifted up through the central pipe.

- This type of circulation takes several times before the effluent goes out to be collected in the collecting chamber for recirculation.
- From the collecting chamber the effluent is pumped to the overhead tank using a magnetic coupled pump.
- By adjusting the flow rate of effluent from the overhead tank to the retting reactor, the rate of flow through the packed bed reactor can be regulated .
- Turn over rate through the air lift pump in the packed bed reactor also can be regulated by regulating the quantity of air passed through the air lift pump which may vary from 1-7 litres per minute.
- The bacterial cultures used for the activated sludge system were used for the packed bed reactor also along with which one culture of *Aspergillus* sp. which exhibited higher level of polyphenol uptake was also incorporated.
- Before employing in the reactor the efficacy of the above consortium in removing the polyphenol from the ret liquor was tested *in vitro* and it was found that with in an incubation period of 144 hours, starting from 100ppm, around 97% of the polyphenols could be consumed. Within this period the effluent became very clear with the formation of rapidly settling sludge.

- On applying the consortium in the packed bed reactor it was observed that 87.2% of the polyphenolics could be removed, starting from 100ppm, with in 48 hours.
- On completing one cycle, the entire fluid was drained off and fresh liquor was added and the process was repeated several times without any change in the rate of removal of polyphenols indicating that the organisms have already immobilized on the substratum. This again indicate that once charged it would be sufficient to continue the process without any more addition of the inoculum.
- The activated sludge system and the packed bed reactor could be easily integrated to the retting reactor (Fig. 5.1).
- The ret liquor treatment technology developed here is the first of its kind even thought of to be integrated with the coir industry.
- By combining the activated sludge system with the packed bed reactor it is possible to get the required quality of the effluent (ret liquor) for recirculation.
- Apart from integrating within the coir retting bioreactor, the technology can find application in treating the effluent generated by bio bleaching of unretted fibre now widely been carried out in Kerala and other neighbouring states.

LEGEND

1. Magnetic coupled pump
2. Retting reactor
3. Perforated platform
4. Air lift pump
5. Air from compressor
6. Ret liquor outlet to reverse osmosis
7. Opening to the activated sludge treatment system
8. Activated sludge treatment system
9. Agitator with baffles
10. Aerator
11. Return sludge port
12. Clarifier
13. Sludge arresters
14. Return sludge valve
15. Waste sludge outlet
16. Packed bed reactor
17. Clam shells
18. Outlet from the bottom
19. Perforated platform
20. Air from compressor
21. Treated ret liquor collecting tank
22. Magnetic coupled pump
23. Overhead tank

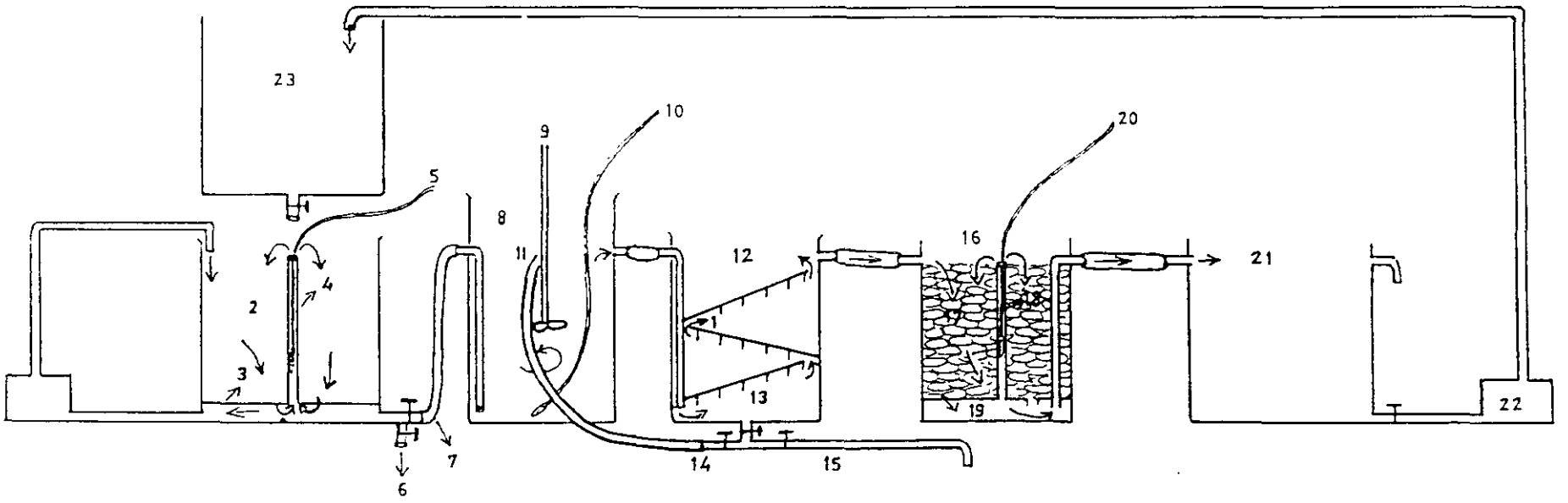

Fig.5.1 Integration of the activated sludge system and packed bed reactor with the coir retting bioreactor

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