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CULTIVATION AND CHARACTERIZATION OF BACTERIAL CONSORTIUM FOR THE TREATMENT OF TANNERY EFFLUENT

A PROJECT REPORT

Submitted by

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in partial fulfillment for the award of the degree

of

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IN

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BONAFIDE CERTIFICATE

Certified that this project report "**CULTIVATION AND CHARACTERIZATION OF BACTERIAL CONSORTIUM FOR THE TREATMENT OF TANNERY EFFLUENT**" is the bonafide work of "**Ms. APARNA K.** AND **Ms. SIVASHANKARI V.**" who carried out the project work under my supervision.

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(APARNA & SIVA SHANKARI)

ABSTRACT

The study revolved around three aspects. First was to identify and characterize the microbial consortium that was isolated from the Upflow Anaerobic Sludge Blanket (UASB) in the environmental technology laboratory, Central Leather Research Institute (CLRI), Chennai. The characterized consortium of six different species was then maintained and grown under microaerophilic conditions and optimized for its efficient growth and activity. Then they were acclimatized to salt levels generally found in the raw tannery effluents discharged from tanneries. Two species were found to be highly salt resistant and showed dominance in the bioreactor study. Finally after optimizing all the required conditions for efficient working of the bioreactor, the consortium was checked for its resistance and degradative capability of phenol, a pollutant in the effluent of leather industries and was found to be marginally effective.

Keywords: *Microaerophiles, Halophiles, Consortium, Dissolved Oxygen, Facultative anaerobes*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BCS	- Basic Chromium Sulphate
BOD	- Biological Oxygen Demand
CLRI	- Central Leather Research Institute
COD	- Chemical Oxygen Demand
Cr (III)	- Chromium
DNS	- Dinitro Salicylic Acid
DO	- Dissolved Oxygen
DS	- Dissolved solids
ETP	- Effluent Treatment Plant
MR/VP	- Methyl Red and Voges Proskauer
MSM	- Minimal Salt Medium
OD	- Optical Density
ORP	- Oxidation Reduction Potential
ppm	- parts per million
RPM	- Revolutions Per Minute
SIM	- Sulfur Indole Motility
SS	- Suspended Solids
TDS	- Total Dissolved Solids
TNPCB	- Tamil Nadu Pollution Control Board
TS	- Total Solids
TSI	- Triple Sugar Iron
UASB	- Upflow Anaerobic Sludge Blanket
UV	- Ultra Violet

INTRODUCTION

CHAPTER 1
INTRODUCTION

The leather products that adorn our living rooms as sofas, that snugly fit us as pricey shoes and those which give us comfortable seating in vehicles, undergo a series of operations before we see it as polished goods. The production processes in a tannery can be split into four main categories:

a) Beam house operations
b) Tan yard operations
c) Post-tanning operations and
d) Finishing operations

During the tanning process many chemicals such as lime, salt, etc. are added per ton of hides. The different steps of a typical tanning process and important aspects concerning the wastewater emergence are delineated in more detail.

1.1 Overview of leather processing methods

The leather processing involves four distinct sets of unit operations. In the first stage referred to as pre-tanning operations, the skins or hides are cleaned free of unwanted materials and made ready for tanning. In tanning, the skins and hides are imparted permanent preservation. In post- tanning and finishing operations, functional and aesthetic properties are imparted to the tanned leather.

1.1.1 Pre- tanning

The pre-tanning or beam-house operations include soaking, liming, deliming, bating, degreasing and pickling. The soaking and liming processes are carried out in pits in many old tanneries especially those processing East India leathers. For hides, it is common now to employ paddles.

1.1.2 Tanning (Tan yard operations):

Principally, there are two possible processes:

- Chrome tanning: After pickling, when the pH is low, chromium (III) salts are added. To fixate the chromium, the pH is slowly increased through addition of a base. The process of chromium tanning is based on the cross-linkage of chromium ions with free carboxyl groups in the collagen. It makes the hide resistant to bacteria and high temperature. The chromium-tanned hide contains about 2-3% by dry weight of Cr (III). Wet blue, i.e. the raw hide after the chrome-tanning process, has about 40% of dry matter.
- Vegetable tanning: Vegetable tanning is usually accomplished in a series of vats (first the rocker-section vats in which the liquor is agitated and second the lay-away vats without agitation) with increasing concentrations of tanning liquor. Vegetable tannins are polyphenolic compounds of two types:

1) Hydrolysable tannins (i.e. chestnut and myrobalan) which are derivatives of pyrogallols and

2) Condensed tannins (i.e. hemlock and wattle) which are derivatives from catechol.

In some cases as much as 50% by weight of tannin is incorporated into the hide.

1.1.3 Wet Finishing (Post-Tanning):

The wet finishing processes are sometimes performed in one single float. Chromium tanned hides or Wet blue are often retanned, during that process the desirable properties of more than one tanning agent are combined and treated with dye and fat to obtain the proper filling, smoothness and color. Before actual drying is allowed to take place, the surplus water is removed to make the hides suitable for splitting and shaving. Splitting and shaving is done to obtain the desired thickness of the hide. The composition of pollutants in the wet finishing effluent is complex due to the presence of dyes, fat liquors and combined tanning agents, but the total amounts generated are smaller than in prior steps and often not considered as significant.

1.1.4 Finishing

The crust that results after retanning and drying is subjected to a number of finishing operations. The purpose of these operations is to make the hide softer and to mask small mistakes. The hide is treated with an organic solvent or water based dye and varnish. The finished end product has between 66 and 85% by weight of dry matter. Environmental aspects are mainly related to the finishing chemicals, which can also reach effluent water.

1.2 Environmental Impacts due to tannery effluent discharge.

The wastewater generated from individual units of leather processing differs widely in quantity and characteristics. The wastewater discharged from leather processing activity in the country has been estimated to be about 30 million cubic meters per year. Most of the constituents present in a tannery effluent are of toxic nature, irrespective of whether they are discharged into river stream, sewer, on land or into ocean.

The characteristics of wastewater vary widely depending on the nature and quantum of input chemicals. The physico chemical characteristics of combined raw effluents prior to the treatment are given in the following table.

Characteristics of composite wastewater

S.No.	Parameter	Value
1.	pH	7.5 – 9.0
2.	Alkalinity as $CaCO_3$	800-2500
3.	Total Solids (TS)	14000-27000
4.	Dissolved Solids (DS)	11000-22000
5.	Suspended Solids (SS)	3000-5000
6.	Chemical Oxygen Demand (COD)	2000-5000
7.	Biological Oxygen Demand (BOD)	800-1600
8.	Chloride as Cl^-	5000-10000
9.	Sulphate as SO_4^{2-}	2500-5000
10.	Sulphide as S^{2-}	75-150
11.	Total Chromium	200-400

Note: all values except pH are expressed in mg/l

1.2.1 Effects on Stream

- The dull brown color of the effluent, which is very difficult to bleach, is imported to the receiving streams and results in unsightly appearance.
- The highly disagreeable foul odor is transmitted to the water.
- The beam house operations (soaking, liming, fleshing, deliming, etc.) generate alkaline streams. The processes from pickling to dyeing generate generally acidic effluents. These extremities results in corrosion and deterioration of concrete, metallic pipes, etc. and the water becomes unsuitable for any use.
- The most damaging constituent sulfide is if present in excess concentration, is toxic to fish and other aquatic life.
- Another detrimental factor is the excess concentration of NaCl, which makes the water unsuitable for irrigation and corrodes structures.
- The dissolved constituents such as proteins and albumin putrefy slowly evolving highly offensive odors.
- The suspended solids, mostly hair, flushing of $CaCO_3$, settle to the bottom affecting the fish spawning grounds and also interfere with the aeration and photosynthetic activities of aquatic plant.
- Chromium in high concentration is toxic to fish and other aquatic life and interferes with the natural purification system.
- The effluent may also contain Anthrax bacilli, which is a pathogen.

1.3 Treatment methods

The need to scale down the level of pollution in the tannery effluent has given birth to a number of chemical and biological treatment methods. Some of the treatment methods are as follows.

1.3.1 Chrome recovery and reuse

The conventional method of chrome tanning, employed in Leather industry, results in very poor exhaustion of chrome, thereby adding to the toxicity of the effluent. One of the cleaner processing options recommended is that the chrome which is let out in the effluent, may be recovered for reuse to ensure not only economy of usage of chrome, but also effective management of this toxic substance. The methodology involves precipitation of chrome with an alkali, whereby the supernatant liquor is separated, allowing the resulting precipitate to settle down. This precipitate is treated with sulfuric acid again to get the Basic Chromium Sulphate (BCS), which can be reused. Commercial polyelectrolytes are used for further facilitation of chrome precipitation.

The technology has been implemented in large number of commercial tanneries. It has been shown that it is possible to recover about 98-99% of chrome. The cost of installation of the plant can be recovered in 12-24 months depending on the scale of operation.

1.3.2 Energy recovery from liquid waste

Biogas and elemental sulfur recovery using UASB Technology with sulfur recovery plant

The Upflow Anaerobic Sludge Blanket (UASB) technology has been well known for treating wastewater primarily because of its low sludge

production and less requirement of energy as well as area. The major advantage of this technology is that it provides a potential possibility to generate energy rather than consuming the same while treating the wastewater.

Despite the aforesaid advantages, there is a major constraint of using this technology for treating tannery wastewater, which is the high sulfate content, that results in generation of methane gas, contaminated with hydrogen sulfide, thereby restricting its use as a source of energy.

An improved system has therefore been developed whereby the sulfate is completely removed as elemental sulfur and the treatment of the tannery wastewater is carried out in conventional UASB reactor, thereby generating energy. This system ensures removal of Chemical Oxygen Demand (COD) and Total Dissolved Solids (TDS) by 60% and 90% respectively, whereby the sulfur can be recovered.

1.3.3 Energy Recovery from solid waste

Biomethanation for solid waste disposal

Solid wastes generated by the leather processing industry are posing a major challenge. Appropriate technology has been developed for the profitable disposal of these solid wastes. Fleshing, which are one of the major solid wastes emanating from the beam house processing in a tannery, are subjected to biomethanation. It is a process whereby the fleshing, effluent sludge and other untanned waste has been digested in anaerobic digesters to produce biogas. The heat value of 3300 Kcal/kg dry matter and high carbon (34% of dry residue) indicate composite waste as a potential source of biogas. The technology has been implemented in Melvisharam,

TamilNadu. The same biomethanation technology can be used for the disposal of other solid wastes as well. The technology has been implemented by CLRI in M/s Alkabeer, Hyderabad and M/s Hind Agro, Aligarh, UP for the disposal of slaughterhouse wastes.

1.3.4 Significance of bacterial consortia in industrial waste treatment

The issue relating to the Total Dissolved Solids in tannery effluents has been of major environmental concern. TDS is generated by the chemicals, which are used in leather processing, but are let out without being fully absorbed in the leather. The effluents having very high TDS affect the ground water adversely and considering this, the Tamil Nadu Pollution Control Board (TNPCB) has set a limit of 2100 ppm in treated effluents. But in practice, the treated effluent is found to contain much more TDS than the prescribed limit. Viable solution for reduction of TDS in tannery effluent is therefore of prime importance.

This technology relates to a series of in plant measures involving a combination of different eco-benign technological options like less salt or salt less curing, enzyme based pre-tanning operations; recycling of pickle liquor, pickle less tanning. Potential for reduction of TDS through these measures has been quantified.

There is a growing demand for information on the fate and effect of toxic chemicals used by the leather industries to have a better understanding on the environmental impact of the products. Many stress factors necessitate the microorganisms to the requirement of fast adaptation to environmental changes or of protection against such influences. The stress factors are the high ion concentration, high temperature, high UV radiation and the reduced solubility of molecular oxygen. Organisms from these extreme environments

(extremophiles) often provide with important tools for processes to make useful chemicals, medicinal and environmental applications. The fact that organisms can develop the capacity to exist and multiply in almost any environment is extremely useful and a potential tool to exploit them. For bioremediation applications, microbial consortia are considered to have several advantages over a pure culture, such as greater stability and increased metabolic capabilities. These characteristics enable the consortium to overcome limitations for the complete biodegradation of toxic compounds.

Microorganisms exist in nature as members of complex, mixed communities. The microbial communities in industrial wastewater bioreactors can be used as model systems to study the evolution of new metabolic pathways in natural ecosystems. The evolution of microbial metabolic capability in these bioreactors is presumably analogous to phenomena that occur in natural ecosystems. The microorganisms in these bioreactors compete for different carbon sources and constantly have to evolve new metabolic capabilities for survival. Thus, industrial bioreactors should be a rich source of novel biocatalysts.

The treatment on chemical pollutants produced from tanning processes were handled through oxidation of these pollutants through high temperature and pressure, ozone or UV radiation to cleave the complicated bonding nature of the aromatic compound. With this view, the present studies were focused on cultivation of robust bacterial consortia in a bioreactor under optimized conditions for biodegradation and remediation of tannery pollutants. In this study, attention was focused on the viability of cultivated bacterial consortia for commercial application, under normal environmental conditions.

REVIEW OF LITERATURE

CHAPTER 2
REVIEW OF LITERATURE

The requirements for treated wastewater are becoming increasingly more stringent, and therefore the improved efficiency of biological treatment processes is indispensable at industrial Effluent Treatment Plants (ETPs). Microorganisms such as bacteria play an important role in the natural cycling of materials and particularly in the decomposition of organic wastes. The knowledge of the interactions among these microbial populations needs to be harnessed for optimum evaluation and functioning of effluent treatment plants. Modern molecular techniques have revolutionized the methods of assessing these microbial populations. The combination of the results of these microbial assessments along with the on-site parameters at ETPs would favor an efficient treatment. (Moharikar *et al.*, 2005). It was thus decided to steer our project in a similar direction with the objective of cultivating and characterizing a facultative bacterial consortium, to be used in secondary treatment plant.

This consortium was isolated from an anaerobic bioreactor, which has been fed with tannery effluent over a period of years in the environmental technology department at the Central Leather Research Institute (CLRI), Chennai. For bioremediation applications, microbial consortia are considered to have several advantages over pure cultures, such as greater stability and increased metabolic capabilities. These characteristics enable the consortium to overcome limitations for the complete biodegradation of toxic compounds (Davison *et al.*, 1994).

The degradative efficacy of a microbial community depends on the stability of the constituent members as well as their ability to degrade or mineralize the target compound. Nevertheless, in numerous degradative studies, uncharacterized biological sludge, microbial consortia have been used instead of defined or characterized consortia. It is important from the perspective of future applications to identify bacteria in the consortium, which are responsible for degradation, as this information could lead to optimization of degradative processes as well as development of monitoring tools. To date, however, there are only a few reports available on the nature of defined microbial consortia capable of degrading toxic organic compounds. (Davison *et al., 1994*). Thus a main objective of the project became identification of the individual bacterial isolates that we were dealing with by biochemical tests.

A microaerophilic condition that is controlled aeration with limited dissolved oxygen of 1- 3 ppm were chosen to cultivate the consortium owing to a number of commercial advantages and success stories in prior works conducted by others. Heterotrophic microaerophilic bacteria are specialized for growth in O_2 limited environments. Microaerophilic bacteria are adapted to maintain essentially constant turnover of primary energy substrates in response to a wide range of physiological O_2. This capacity, oxidative metabolic gearing, allows microaerophilic bacteria to maintain catabolic enzymes, substrates, and cofactors at high steady-state levels. (Ludwig, R.A., 2003). Media and growth conditions were optimized for the micro aerobic cultivation of *Magnetospirillum gryphiswaldense* in flasks and in a fermentor, resulting in significantly increased cell and magnetosome yields, compared with earlier studies. (Heyen, U. and Schuler, D., 2003).

Currently used tests for inhibition of respiration, nitrification and growth of sewage bacteria are normally short-term and deliberately use unacclimatized microorganisms. To investigate the possibility of acclimatization, activated sludge were exposed to six organic chemicals for 3 months. Their toxicity (EC_{50}) was assessed before and after exposure. Results identified only one acclimatized sludge for pentachlorophenate. (Painter, H.A. and King, E.F., 1985). The characteristic feature of the effluent released from primary treatment plants in the tannery effluents, is their high inorganic content. It was thus imperative that in order to survive at the high salt conditions acclimatization of the bacterial consortium to sodium chloride (NaCl) needs to be done.

The halophilic bacteria are capable of tolerating high levels of salt conditions in the environment it dwells in. Complex mechanisms of adjustment of the intracellular environments and the properties of the cytoplasmic membrane enable rapid adaptation to changes in the salt concentration of the environment. Approaches to the study of genetic processes have recently been developed for several moderate halophiles, opening the way toward an understanding of halo adaptation at the molecular level. The new information obtained is also expected to contribute to the development of novel biotechnological uses for these organisms (Ventosa *et al.*, 1998).

According to observations, sodium, if present in a significant concentration, is highly toxic to anaerobic microorganisms and inhibits their activity. Toxicity due to a particular cation is known to be affected by the presence of other cations. A research has already been carried out to

investigate the effect of calcium and potassium on sodium toxicity in anaerobic treatment processes. The results show that the simultaneous use of calcium and potassium is very effective in reducing the toxic effects of sodium and can be used to enhance the efficiency of industrial-scale anaerobic processes (Bashir, B. H. and Matin, A., 2004). Thus the combination of NaCl together with $CaCO_3$, which is one of the major constituents in tannery effluent, was carried out as a part of the acclimatization process in the current study.

A bacterial consortium has been developed in 500 mg/l phenol for four years at 28 ± 3°C which showed a biomass increase. The ability of large membered microbial consortia to maintain its stability with respect to its composition and effectiveness in phenol degradation indicated its suitability for bioremediation applications (Ambujom, S., 2000). Phenolic compounds are generally found in the tannery effluents and thus the bacterial consortium in hand was tested for its degradative capability of phenol as a minuscule part of our study.

All observations of the study were carried out in a continuous bioreactor where fresh nutrient was continually supplied to a well-stirred culture, and products and cells could be continuously with drawn, thereby maintaining growth and product formation for prolonged periods. Continuous culture provides constant environmental conditions for growth and product formation and supplies uniform quality product. Continuous culture is an important tool to determine the response of microorganisms to their environment and to produce the desired products under optimal environmental conditions. (Shuler *et al.*, 2002)

OBJECTIVES

CHAPTER 3

OBJECTIVES

- Acclimatization and development of microaerophilic robust microbial consortia using the existing anaerobic microbial populations present in environmental technology laboratory reactor, CLRI, Chennai, operated with tannery liquid and solid wastes over a period of 20 years.
- Development of chemo-heterotrophic microbes that can grow between pH 6-9 and at the temperature between 20 to 55°C with substrates like glucose and phenol.
- Finding out the tolerance limit of developed microbial consortia for cations and anions like sodium, calcium, and chloride, which are the major pollutants in tannery wastes.
- Conduct a study of the performance and evaluation of acclimatized microbes in bioreactor under varied conditions.

MATERIALS AND METHODS

CHAPTER 4

MATERIALS AND METHODS

4.1 Isolation of cultures:

Isolation of the facultative anaerobic bacterial cultures was made from the Upflow Anaerobic Sludge Blanket (UASB) reactor, which has been fed with tannery liquid and solid wastes over the period of several years in the environmental technology department at the Central Leather Research Institute (CLRI), Chennai. They were further sub-cultured to obtain 6 pure cultures in a reducing medium with a dissolved oxygen concentration not less than 3 mg/l. A step-by-step identification of these slant cultures was performed with the help of a series of tests.

4.2 Biochemical Characterization

4.2.1 Morphology:

The culture was observed through phase contrast microscope to find out the motility and shape of the live bacterial strains.

4.2.2 Gram staining:

Principle

The differential staining divides the bacterial cells into two major groups, Gram positive and Gram negative, which makes it an essential tool for classification and differentiation of microorganism. The Gram positive bacteria will retain the crystal violet and appear deep violet in color. The Gram negative loses the crystal violet on decolourisation and are counterstained by safranin and appear red in color.

Procedure 6

A smear of the culture was made on a clean slide, heat fixed and then:

- The slide was flooded with crystal violet and washed with distilled water after one minute.
- It was then flooded with Gram's iodine and washed off with distilled water after one minute.
- This was followed by washing the slide alternatively with destainer (acetone) and distilled water.
- Finally the secondary stain, safranin, was applied which was again washed after 30 sec, with distilled water.
- The dried slide was observed under an oil immersion objective.

4.2.3 Indole test

Principle

Tryptophan present in peptones of the culture media (SIM agar), is acted upon by the enzyme tryptophanase and converted into Indole, skatol and indole acetic acid. Indole reacts with aldehydes to produce a red colored product. So organisms are grown in tryptophan rich medium and tested for the presence of indole.

SIM agar (pH of the medium is adjusted to 7.3)

Components	Weight in g/l
Peptone	30
Beef extract	3
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.025
Agar	3

Kovacs reagent

Components	Weight in g/l
p-dimethylamino benzaldehyde	5
Amyl alcohol	75 ml
Conc.HCl	25 ml

p-dimethylamino benzaldehyde was dissolved in amyl alcohol and then HCl was added.

Procedure

- Tubes of tryptone broth were inoculated with the organisms and incubated for 24-48 hours at 37^0C.
- 0.2ml of Kovac's reagent was added and shaken well.
- The tubes were allowed to stand for few min and results read.

4.2.4 Methyl red and Voges Proskauer test

Principle

Organisms belonging to *Enterobacteriaceae* ferment glucose via pyruvate and produce mixed acids and other end products. Among them one group produces mixed acids such as acetic, lactic, succinic and formic acids, ethanol, CO_2 and H_2. They do not produce butylene glycol. Because of the abundant acid production, the final pH of the broth drops to less than 4.5, which can be detected by pH indicators. The other group of organisms produces butylene glycol and acetone, which are more neutral in nature, and not much drop in pH noticed. The end products were detected by VP reagent.

MR/VP broth

Components	Weight in g/l
Peptone	7
Dextrose	5
Potassium phosphate	5

Procedure

- The organisms were inoculated into MR/VP broth; incubated at 37^0C for at least 48 hours.
- The broth was divided into two equal halves and to one 0.5 ml of MR reagent was added.
- To the other half, 0.2ml of VP reagent A was added and 0.2ml of VP reagent B was added.
- Each tube was gently mixed and allowed to stand for 15 minutes.

4.2.5 Catalase test

Principle

Some bacteria and macrophages can reduce diatomic oxygen to hydrogen peroxide or super oxide. Both of these molecules are toxic to bacteria. Some bacteria, however, possess a defense mechanism, which can minimize the damage caused by the two compounds. These resistant bacteria use two enzymes to catalyze the conversion of hydrogen peroxide and superoxide back into molecular oxygen and water. One of these enzymes is catalase and its presence can be detected by a simple test. The catalase test involves adding of hydrogen peroxide to a culture sample or agar slant. If the bacterium produces catalase, they will convert the

hydrogen peroxide and oxygen will be evolved. The evolution of gas causes bubble formation, which is indicative of a positive test.

Procedure

- The test organism was inoculated in an agar slant and was incubated for 24 hrs at 37^0C.
- Following incubation, 1ml of 3% hydrogen peroxide was trickled down the slant.
- The tubes were then examined for the evolution of bubbles.

4.2.6 Citrate utilization test

Principle

In the absence of glucose or lactose, some organisms use citrate as the carbon source, which depends on the presence of citrate permease. Citrate is acted upon by the enzyme citrase, which produces oxalo acetic acid and acetate. These are then enzymatically converted to pyruvic acid and CO_2. During this reaction the medium becomes alkaline as the CO_2 combines with sodium and water to form sodium carbonate an alkaline product. The sodium carbonate changes the bromothymol blue from green to deep Prussian blue.

Simmon citrate agar

Components	Weight in g/l
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phospate	1
Sodium citrate	2
Bromothymol blue	0.08
Agar Agar	15

Procedure:

A 4-6 hrs old culture was inoculated into the medium and incubated for 18-24 hrs.

4.2.7 Triple sugar iron (TSI) agar

Principle

Degradation of sugar and acid production is detected by the pH indicator phenol red, which changes its color from red-orange to yellow, on alkalinization it turns deep red. Thiosulfate is reduced to hydrogen sulfide by several species of bacteria, hydrogen sulfide reacts with an iron salt to give black iron sulfide.

TSI agar medium

Components	Weight in g/l
Beef extract	0.3
Yeast extract	0.3
Peptone	2.0
Sodium chloride	0.5
Glucose	0.1
Lactose	1.0
Sucrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	2.5
Phenol red	2.5

The medium was made up to 1000ml with distilled water. The final pH of the medium was 7.3

Procedure

- Slants of TSI agar were inoculated with the test organisms.
- The slant was stabbed down the butt initially and then streaked to the surface the slant.
- The tubes were incubated at 37^0C for 24 hrs.

4.2.8 Carbohydrate utilization

Utilization of different sugars is very important for a lot of microbes. However, despite the fact that the sugar is the primary nutrient used (if the organism uses that particular sugar), when the microbe runs out of it, protein or other nutrients will be attacked causing color changes in the medium with the help of pH indicator added to detect acid production. When proteins are used, alkaline by-products are produced and the medium can change colors. Some of the sugars come in phenol red broth, already with sugar in it lactose, glucose and sucrose.

Phenol red broth

Components	Weight in g/l
Trypticase	10
Sodium chloride	5
Phenol red	0.018
Dextrose or lactose or sucrose	5
Distilled water	1000 ml

Procedure

Phenol red broth:

- The broth can have the specific sugars (glucose, lactose, sucrose) added to each tube has a small upside down tube called a Durham tube that collects CO_2 gas.
- The phenol red broth with the corresponding sugar was inoculated with unknown bacteria.

4.2.9 Lipid hydrolysis

In this procedure, tributyrin agar is used to demonstrate the hydrolytic activities of the extracellular enzyme lipase. The medium is composed of nutrient agar supplemented with triglyeride (tributyrin) as the lipid substrate. Tributyrin forms an emulsion when dispersed in the agar, producing an opaque medium that is necessary for extracellular enzymatic activity. Following inoculation and incubation of the agar plate, organisms excreting lipase show a zone of lipolysis appearing as a clear area surrounding the bacterial growth. This loss of opacity is the result of the hydrolytic reaction yielding soluble glycerol and fatty acids and represents a positive reaction for lipid hydrolysis. In the absence of lipolytic enzymes the medium retains its opacity. This is a negative reaction.

- The tributyrin agar plate was streaked with the bacteria and was incubated at 37^0C for 24 hrs.
- After incubation the plate was viewed and the result was observed.

Tributyrin agar

Components	Weight g/l
Peptone	5
Beef extract	3
Agar	15
Tributyrin	10

4.2.10 Hydrogen sulphide test

To determine the ability of microorganism to produce H_2S from substrates such as the sulphur containing amino acids or inorganic sulphur compounds. There are 2 major fermentative pathways by which some microbes can produce H_2S.

Pathway 1:

H_2S may be produced by the reduction of organic sulphur present in the amino acid cysteine, which is a component of peptones included in the medium. Microbial enzymes to amino acids, including sulphur containing amino acid cysteine, degrade these peptones. This amino acid, in the presence of cysteine d sulphurase loses a sulphur atom and is then reduced by the addition of hydrogen from water to form H_2S gas.

Pathway 2:

Gaseous H_2S may also be produced by the reduction of inorganic sulphur compounds such as thiosulphates or sulphites. The medium contains sodium thiosulphate, which certain microbes are capable of reducing to sulphite with the liberation of H_2S. The sulphur atoms act as hydrogen acceptor during oxidation of the inorganic compound.

Here, Sulphur Indole Motility (SIM) medium contains peptone and sodium thiosulphate as the sulphur substrate, ferrous sulphate serves as indicator. Regardless of which pathway is used, H_2S gas is colorless and therefore not visible. Ferrous ammonium sulphate in the medium serves as an indicator by combining the gas, forming an insoluble ferrous sulphide precipitate that is seen along the line of the stab. Absence of precipitate is indicative of negative reaction. SIM agar can also be used to detect motile organism. Motility is recognized when culture growth of flagellated organisms is not restricted to the line of inoculation.

4.2.11 Starch hydrolysis

Principle

Starch agar is a differential medium that tests the ability of an organism to produce certain extracellular enzymes, including α- amylase and oligo-1, 6-glucosidase, that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria secrete extracellular enzymes to degrade starch into subunits that can then be utilized by the organism.

Starch agar is a simple nutritive medium with starch added. Since no color change occurs in the medium when organisms hydrolyze starch, iodine is added to the plate after incubation. Iodine turns blue, purple, or black (depending on the concentration of iodine) in the presence of starch. A clearing around the bacterial growth indicates that the organism has hydrolyzed starch.

Starch agar

Components	Weight in g/l
Peptone	5
Sodium Chloride	5
Yeast extract	1.5
Beef extract	1.5
Soluble starch	2
Agar	15
pH	7.4 $\pm$ 0.2

Procedure

- The starch agar plate was streaked with the bacteria and was incubated at 37^0C for 24 hrs.
- After incubation the plate was flooded with iodine and the result was observed.

4.3 Bioreactor studies on bacterial consortia with synthetic medium

The living organisms, mainly the heterotrophic ones, require metal ions like K^+, Ca^{2+}, Mg^{2+} and Fe^{2+} in conspicuous quantity in addition to vitamins and other cofactors in micro quantities, which are termed as trace elements, for their normal growth. The following synthetic medium similar to tannery waste constituents was selected for the cultivation of bacterial consortia.

Composition of synthetic medium

Components	Weight in g/l
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.01
NH_4NO_3	3.0
$FeSO_4.7H_2O$	0.01

Trace elements

Components	Weight in µg /l
$MnSO_4.H_2O$	500
$ZnSO_4$	500
$CuSO_4.5H_2O$	5
$CoCl_2.6H_2O$	5

Along with the above metal ions the following components were also added for the carbon and energy source. To maintain the culture medium with the dissolved oxygen of not more than 3 mg/l, reducing agents like casein, cysteine and thioglycollate were also added.

Components	Weight in g/l
Pancreatic digest of casein	15
Yeast extract	5
Glucose	5
Sodium chloride	2.5
Sodium thioglycollate	0.5
L - cysteine	0.25
pH	7.2 ± 0.2 at 25 °C

Procedure:

- The synthetic medium was prepared and autoclaved in the conical flask
- About 10 µl of the consortia was inoculated in 10mL of the medium.
- Growth of the consortia was observed in a spectrophotometer every one hour for six hours and then for 24^{th} hr and 48^{th} hr.
- The absorbance values were recorded at 660nm.
- Growth curve was plotted with absorbance at 660 nm in Y-axis and time in hours in X-axis.

4.4 pH and temperature optimization.

The optimum efficacy of the microorganisms is dependent on their surrounding environment. pH and temperatures are the immediate variants of a medium. It was thus decided to optimize the best working conditions of the microbial consortium. A fairly broad pH range of 6 to 9 was chosen while temperatures between 25 to 55°C was chosen.

Procedure:

- The synthetic medium was prepared and autoclaved.
- The medium's pH values were adjusted to 6, 7, 8, and 9 in four different conical flasks.
- 10 µl of the consortia was inoculated in 10ml of the medium of varying pH.
- Each of these conical flasks was placed in separate incubators adjusted to temperature values of 20°C, 35°C, 45°C, 55°C.
- Growth of the consortia was observed in a spectrophotometer at 24 hrs. The absorbance values were recorded at 660nm.

4.5 Glucose Assay

To find out the utilization rate of substrate, energy consumed or released by the bacteria and to arrive at the feed/mass ratio, glucose estimation was done. By finding out the residual glucose that remains in the culture medium after its exponential phase, the yield coefficient factor may be arrived.

Principle

This assay helps determine the presence of free carbonyl group (C=O). Here oxidation of aldehyde group in glucose and ketone in fructose and reduction of dinitrosalicylic acid (DNS) to 3 amino 5-nitro-salicylic acid in alkaline condition. Sulphite is added to stop oxygen interference with glucose oxidation although it is not required for color reaction. Many side reactions also occur depending on the exact nature of the reducing sugar. Different sugars give different color intensities hence better to calibrate each sugar.

Reagents

DNS reagent 1%

DNS 10g

Phenol 2g

Sodium sulphite 0.5g

Sodium hydroxide 10g

Distilled water 1L

Potassium sodium tartarate solution 40%

Procedure

- 3ml of DNS reagent was added to 2ml of glucose sample in a tightly capped test tube.
- The mixture was heated at 90°C for 5 to 15 minutes to develop reddish brown color.
- 1ml of 40% potassium sodium tartarate solution was added to stabilize the color.
- The solution was cooled to room temperature in a cold water bath and the absorbance was recorded at 575nm.

4.6 Salts tolerance efficiency

Salts like sodium chloride and calcium chloride/carbonate are the major pollutants contributing to dissolved solids in water bodies, which is one of the major problems in tannery industry. Hence the microbes isolated from UASB reactor were already acclimatized to resist these salts. To find out salts tolerance capacity in the individual isolates and as a consortium the following experiment was performed.

Procedure

- The synthetic medium was prepared and autoclaved.
- Autoclaved NaCl solution was added to the above medium to make it to a final concentration of 10% NaCl.
- Autoclaved $CaCl_2$ was added to the above medium to make it to a final concentration of 0.1% $CaCl_2$
- Enumeration of the total bacterial population in the media was done by using plate count.
- Growth of the consortia was observed in a spectrophotometer at 24 and 48 hours.

4.7 Resistance to phenol

Phenol is one of the toxic compounds found in the tannery effluent. To be able to use phenol as a carbon source and grow would be a microbe's feat and an environmentalist's boon.

Procedure

- MSM was prepared with phenol as the sole carbon source in concentrations of 50 mg and 500 mg respectively.
- The size of inoculum taken was 10µl for 10 ml of the medium.
- Enumeration of the total bacterial population in the media was done by using plate count.
- Growth of the consortia was observed in a spectrophotometer at 24, 48 and 72 hrs.

4.8 Serial dilution - agar plate technique

To determine the viable cells in a bacterial culture, the serial dilution - agar plate technique was used.

This method involves serial dilution of a bacterial suspension in sterile water blanks, which serves as a diluent of known volume, once diluted, the suspensions are placed on suitable nutrient media. The pour-plate technique is the procedure usually employed. Molten agar, cooled to 45°C, is poured into a Petri dish containing a specified amount of the diluted sample following of the molten and cooled agar, the cover is replaced and the plate is gently rotated in a circular motion to achieve uniform distribution of microorganism. This procedure is repeated for all dilution to be plated. Dilutions should be plated in duplicate for greater accuracy, incubated overnight and counted on Quebec colony counter either by hand or by an electronically modified version of this instrument.

Plates suitable for counting must contain not fewer than 30 or more than 3000 colonies. The total count of the suspension is obtained by multiplying the number of cells/plate by the dilution.

The total bacterial population in the media was estimated by the serial dilution technique followed by a plating technique. The total number of bacteria present in the sample per ml was estimated by multiplying the number of colonies with the dilution factor.

Reagents

Preparation of dilution water (500 ml):

Phosphate solution:

Potassium dihydrogen phosphate	0.34 g
Distilled water	5 ml

The pH was adjusted to 7.2 with 1N NaOH. Rest of the distilled water was added to make up to 10 ml.

Magnesium sulphate solution:

Magnesium sulphate	0.5 g
Distilled water	10 ml

Dilution water:

Phosphate solution (1)	0.625 ml
Magnesium sulphate solution (2)	2.5 ml
Distilled water	500 ml

Procedure

- 9 ml of dilution water was taken in 7 test tubes plugged with cotton and were autoclaved for 30 minutes at 120°C.
- 1 ml of the sample was added using micropipette and transferred to the first test tube, which forms a dilution of 10^{-1} and then mixed well.
- From the first test tube 1ml was taken using another micro syringe and transferred to second test tube resulting in a dilution of 10^{-2}.
- This procedure was carried out for a dilution up to 10^{-8}.
- 1 ml of the sample was taken from the test tubes and poured into the Petri plate close to the flame.
- Once the agar has come to bearable warmth, it is poured into the Petri plate, followed by clockwise and anticlockwise rotation.
- The plate is then kept in an inverted position in the incubator and then observed for colonies after 24 hours at 37°C.

4.9 Performance of bacterial consortia in a Bioreactor

The bioreactor present in CLRI Environmental technology Department is stirred tank bioreactor (Reactors with internal mechanical agitation) bought from LARK Innovative Company. The robust microbial consortium was developed and maintained in this indigenous 3-litre bioreactor employing the synthetic culture medium with sensors for maintaining temperature, pH, Oxidation Reduction Potential (ORP) and dissolved oxygen conditions by automatic controlled and measurement systems.

It is one of the continuous cultivation devices called as chemostat. The name chemostat refers to constant chemical environment. Gas under

pressure is supplied to the spargers with single discharge point, as the media is with high levels of solids hence they are more resistant to plugging.

The impellers provide sufficiently rapid agitation to disperse bubbles throughout the tank, to increase the residence time within the liquid, and to shear larger bubbles into small bubbles. The bioreactor in hand employed has flat blade turbine impeller.

The vessel itself is made of glass. It is built with a height to diameter ratio of 2 to 3. It uses internal stainless steel coils for heat removal. Internal coils provide advantages over jacketed vessel in terms of efficiency of heat removal, owing to the larger surface area for heat transfer.

Problem of foaming is controlled by the addition of surface-active chemical agents. The stirred tank reactor is provided with headspace for the gas to disengage from the liquid. The working volume in the reactor is about 75% of the total volume.

Procedure

- The bioreactor was completely cleaned and autoclaved together with synthetic medium.
- The bioreactor was maintained in a continuous operation at the optimum pH of 7 and at temperature of 32°C.
- The working volume in the bioreactor was maintained at 2 liters to provide a sufficient headspace to prevent air clogging in the funnel membranes provided.
- Online parameters were recorded daily for RPM, temperature, pH, and dissolved oxygen.

RESULTS AND DISCUSSION

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Biochemical characterization of isolates

The biochemical tests were performed for the six bacterial isolates that are going to use in bioreactor studies as consortia and the results were recorded as follows.

Table 5.1: Biochemical characterization

No.	Tests	A	B	C	D	E	F
1.	Gram Reaction	-ve	-ve	-ve	-ve	-ve	-ve
2.	Cell Shape	Straight rods	Short Rods	Rods	Long rods	Curved rod	Very short rods
3.	Chemoorgano-trophic growth						
	a. Lactose	+ve	+ve	+ve	+ve	+ve	++ve
	b. Dextrose	-ve	+ve	-ve	+ve	+ve	-ve
	c. Sucrose	+ve	-ve	-ve	+ve	-ve	+ve
4.	Hydrolysis of						
	a. Starch	-ve	-ve	-ve	-ve	-ve	+ve
	b. Lipid	Good	Poor	Poor	Good	Poor	Poor
5.	Catalase production	+ve	+ve	+ve	+ve	+ve	+ve
6.	Indole production	-ve	-ve	+ve	+ve	-ve	-ve

7.	Methyl Red Reaction	+ve	+ve	+ve	+ve	+ve	+ve
8.	Voges Proskauer Reaction	-ve	-ve	+ve	-ve	+ve	+ve
9.	Citrate utilization	-ve	+ve	-ve	-ve	+ve	-ve
10.	H_2S production	-ve	-ve	-ve	-ve	-ve	-ve
11.	Growth on TSI agar	+ve	-ve	+ve	-ve	+ve	+ve
12.	Cell aggregation	In chains	single	single	single	single	single

The above characterization infers that (Staley *et al.,* 1989) the isolates fall under *Enterobacteriacea* family, and the identification of the six species were as follows:

A – *Klebsiella*

B – *Citrobacter*

C – *Serratia*

D – *Kluyvera*

E – *Cedecae*

F – *Enterobacter*

5.2 pH and temperature optimization

Before the isolates were fed into the bioreactor, optimization studies were conducted in 250 ml Erlenmeyer flasks to find out their maximum growth. The experiment was performed to record the optimum pH and temperature. Four different pH and temperatures were set and the observations were as follows.

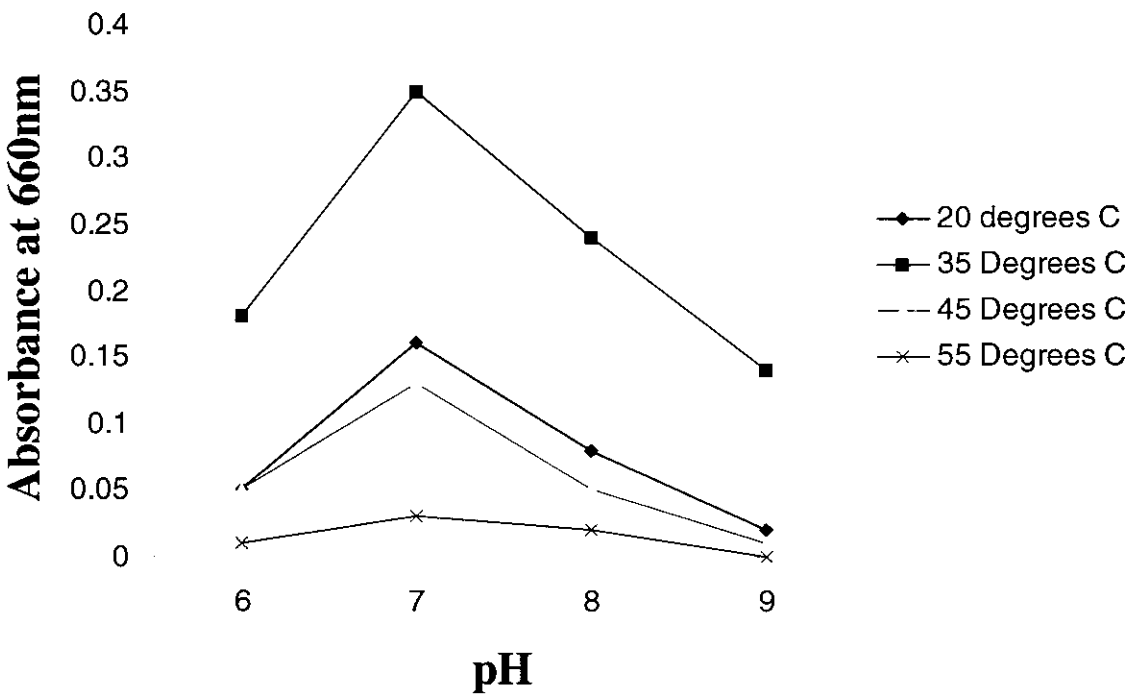

Figure 5.1. pH and Temperature optimisation

From the above graph, the optimum pH that can be used for the cultivation of the bacterial consortium is found to be 7.00, and the temperature is 35°C.

5.3 Tolerance level of the bacterial isolates for NaCl and $CaCl_2$

The major pollutants present in the tannery effluent were sodium calcium and chloride. To find out the resistance capacity of the consortia that is going to be fed into the bioreactor, each of the individual bacterial isolates was checked for their resistance to 10% NaCl and 0.1% $CaCl_2$ under the optimum conditions of pH and temperature.

Table 5.2: Salt resistance of the bacterial isolates

Microbe	24 hours	48 hours
Klebsiella	0.165	0.090
Citrobacter	0.108	0.380
Serratia	0.118	0.210
Kluyvera	0.121	0.086
Cedecae	0.106	0.100
Enterobacter	0.159	0.500

From the above tabulation, it is evident that the *Citrobacter* and the *Enterobacter* species have good resistance to salts. The *Klebsiella*, *Kluyvera* and *Cedecae* species are susceptible to the salts. *Serratia* species show moderate resistance to the salts.

5.4 Growth potential of the bacterial consortia.

The following figure shows a pattern of growth of the bacterial consortia under normal conditions, in the presence of 10% NaCl and 0.1% $CaCl_2$ and in the presence of phenol.

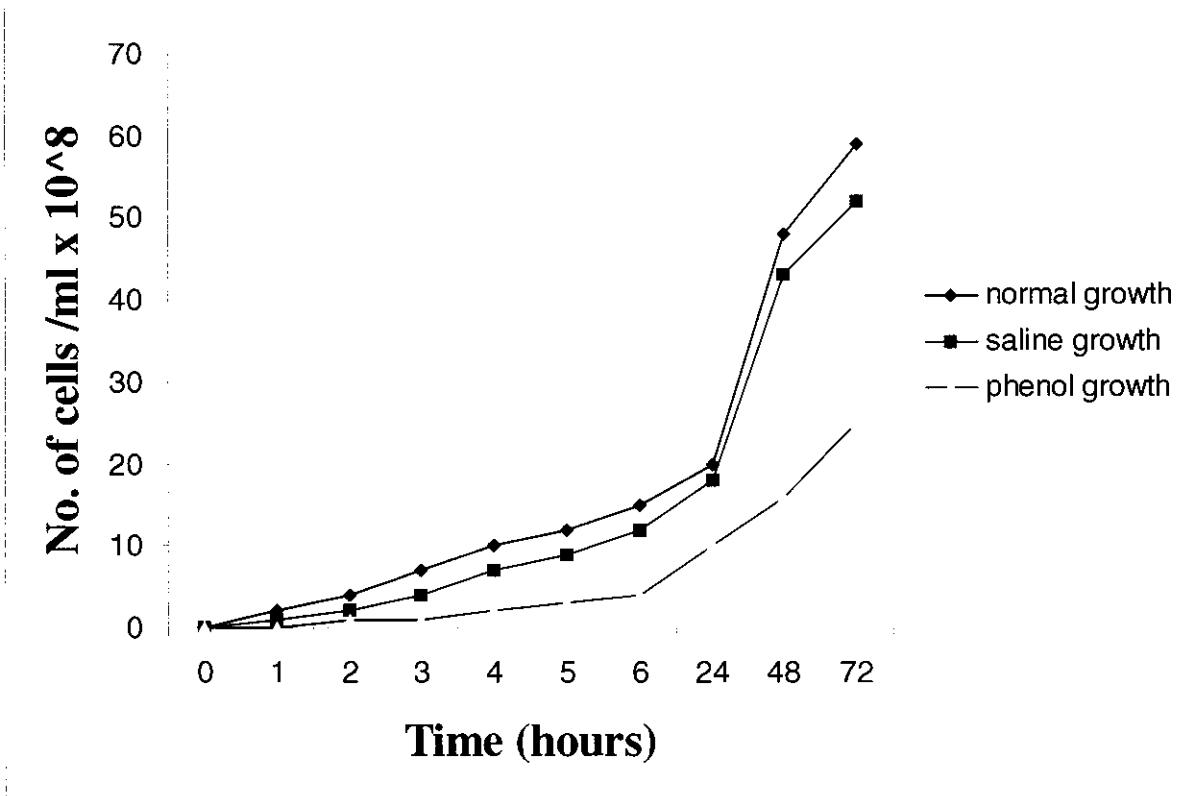

Figure 5.2. Growth curve of bacterial consortium

Generation time: Generation time is the time interval required for a cell to double, which is represented as n given by the following formula.

$$n = \frac{\log N - \log No}{\log 2}$$

Doubling rate: Doubling rate is the number of cells grown per time interval. It is calculated from the slope of the logarithmic phase of the growth curve.

Growth Rate constant is arrived from the following formula.

$$k = \frac{\ln 2}{\text{Doubling time}}$$

From the above growth curve, it is observed that the average doubling rate for the bacterial consortia under normal growth conditions is 1.167×10^8 cells/ hour and the number of generations is 1.26.

The consortium's doubling rate however drops to 1.042×10^8 cells/ hour under saline conditions with a generation time of 1.25.

The growth rate constant under normal conditions is 0.550 and under saline growth conditions it is 0.5545.

It is clear that the bacterial consortium grows very slowly in the phenol medium of concentration 50mg/l. However, with the sudden shock load of 500mg/l of phenol, the bacteria could not sustain itself in the medium and grow. It is thus required for a slow and gradual feeding of phenol of increasing concentrations to support the growth of the bacterial consortium.

5.5 Glucose consumption rate

To arrive at the food/microbe ratio (glucose), substrate utilization rate and energy yield coefficient were studied in conical flask for the consortia and the results were tabulated as follows.

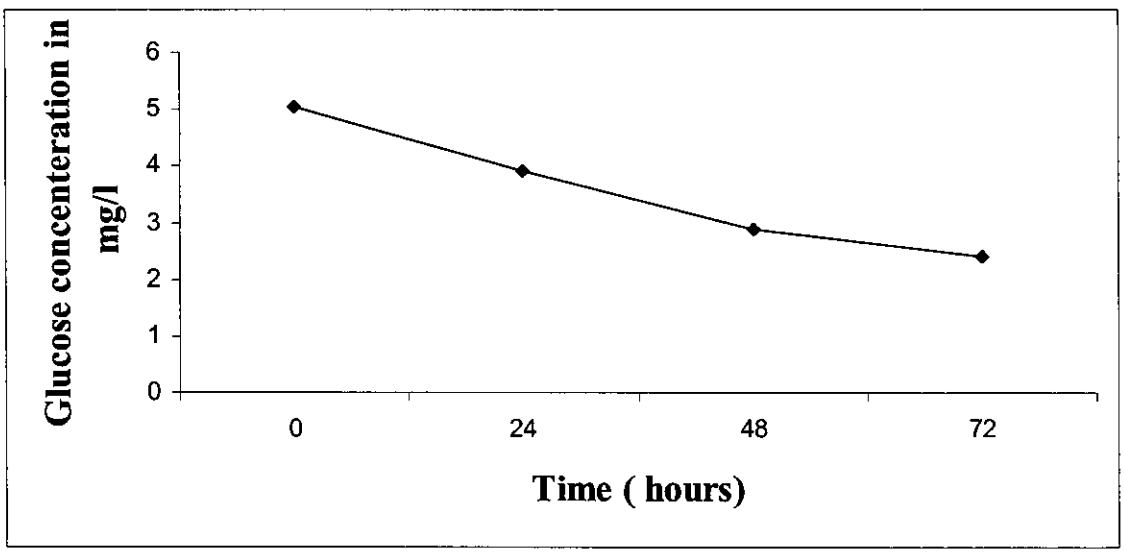

Figure 5.3: Residual glucose concentration

It is evident from the graph that glucose consumption was high in the first 48 hours with corresponding growth of the microbial consortium. Glucose consumption has dropped after 48 hours confirming the onset of stationary phase.

The graph plotted above between the substrate (glucose) taken up in a certain period of time against the number of cells, gives the specific rate of substrate uptake for cellular maintenance. This is given by

$$m = - \frac{[dS/dT]}{X}$$

It is thus evident that the maintenance coefficient is – 0.014 g ml /cell / hr and that the process of substrate uptake is exothermic.

5.6 Bioreactor studies

A synthetic medium of 2 liters was prepared and operated as batch reactor with an inoculum size of 0.1% in suspension, initially with synthetic medium consisting of necessary metal ions, carbon and nitrogen for food and energy, together with reducing agents to maintain dissolved oxygen level of not more than 3 mg/l. Once the consortia reached stability in its growth under normal nutrient rich synthetic medium, pollutants were added one by one to test its tolerance efficiency. By observing the growth of the consortia, the stability time taken for normal and pollutants was studied. The results are given in the following tables 5.3, 5.4, 5.5 and Fig.5.4.

Table 5.3: Bioreactor studies with inoculum size of 0.1 % consortia

Date	pH	Temp (°C)	RPM	DO mg/ml	OD at 660nm	Plate count x 10^8	Cell dry weight g/100ml
Day1	7.0	32.0	110	3.0	0 .304	45	0.0165
Day2	7.0	32.0	115	3.5	0.630	58	0.0238
Day3	7.0	32.1	110	3.7	0.749	94	0.0349
Day4	7.02	32.4	120	2.8	0.788	98	0.0400
Day5	6.83	32.2	112	3.1	0.760	88	0.0360

Table 5.4: Bioreactor studies of synthetic medium enriched with 10% NaCl and 0.1% $CaCl_2$

Date	pH	Temp. (°C)	RPM	DO mg/ml	O.D. at 660nm	Plate count x 10^8	Cell dry weight g/100ml
Day6	7.0	31.7	110	3.0	0.780	95	0.0390
Day7	7.0	32.0	115	3.0	0.797	104	0.0430
Day8	7.0	32.1	110	3.7	0.834	146	0.0498
Day9	7.02	32.0	120	2.9	0.856	154	0.0521
Day10	6.83	32.2	112	3.2	0.829	142	0.0501

Table 5.5: Bioreactor studies of medium enriched with slow feeding of phenol (maximum concentration of 50mg)

Date	pH	Temp. (°C)	RPM	DO mg/ml	O.D. at 660nm	Plate count x 10^8	Cell dry weight g/100ml
Day11	7.0	32.0	110	3.0	0.838	147	0.0512
Day12	7.0	32.4	115	3.5	0.878	140	0.0563
Day13	7.0	32.6	110	3.7	0.900	132	0.0587
Day14	7.02	32.4	120	2.7	0.956	128	0.0613
Day15	6.9	32.5	112	3.2	0.976	120	0.0623

Note: The data given in the above tables for pH, temperature, DO and RPM were the average of whole through the day.

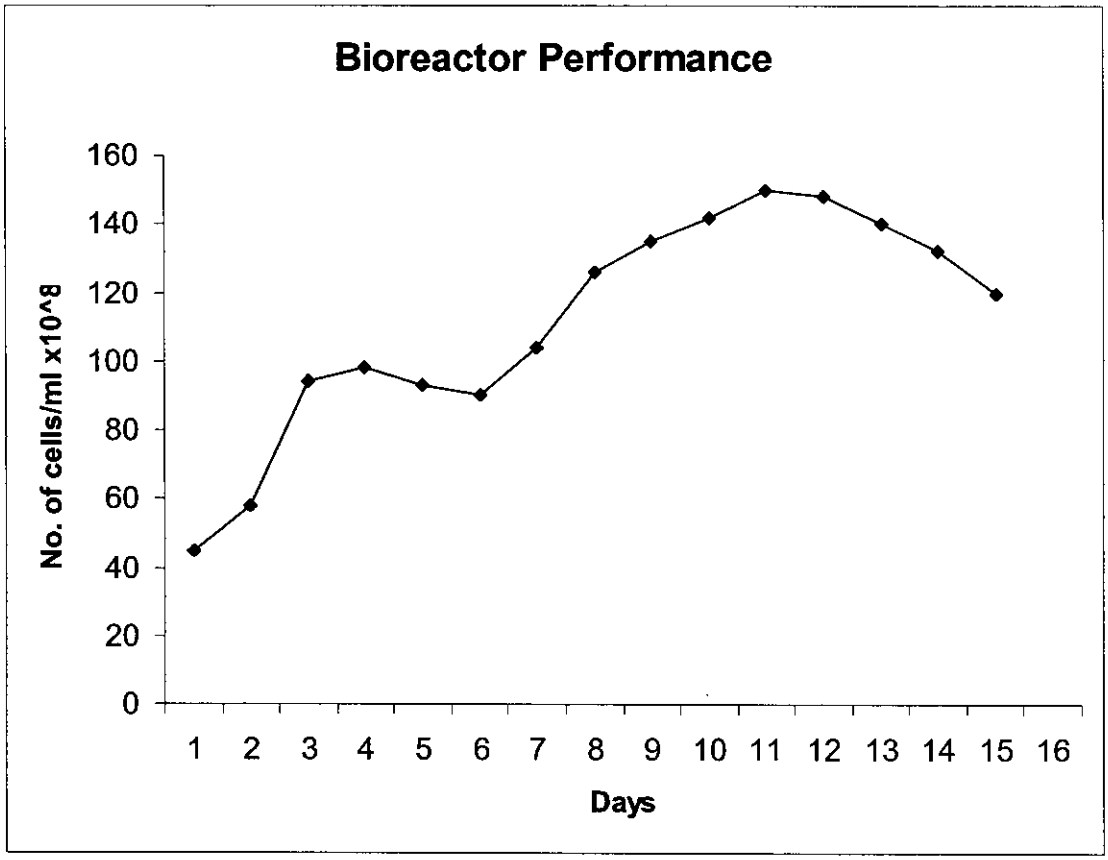

Figure 5.4: Bioreactor studies over a period of 15 days

By observing the biomass developed in the bioreactor under phase contrast microscope at the end of the 15 days study, the dominant species found were *Citrobacter* and *Enterobacter* species revealing their robustness out of the six isolates present in the consortia. Though the other four also resist, their growth rate is reduced compare to their normal doubling rate.

CONCLUSION

CHAPTER 6
CONCLUSION

The selected area of investigation was about cultivation and development of robust microbial consortia and application of these microaerophilic microorganisms for degradation processes on toxic chemicals. In many industrial wastes, pre-treatment of these effluents is compulsory before the biological treatment is applied. So by screening out robust microorganisms which can grow in wide pH / temperature ranges, and high concentration of cations and anions like sodium, calcium, and chloride the consortium may be applied directly without prior pre-treatment.

Further studies may be made to immobilize the microbial consortium on natural material, check its tolerance and finally be screened for its ability to decolorize dyes, fungicides, biocides, solvents and surfactants that are used in tannery operations.

Once it is capable enough to resist and also degrade the pollutant, when they utilize it as carbon or energy source, metabolic pathway and molecular sequencing will be done to identify the bacterial populations of the robust microbial consortia.

ANNEXURES

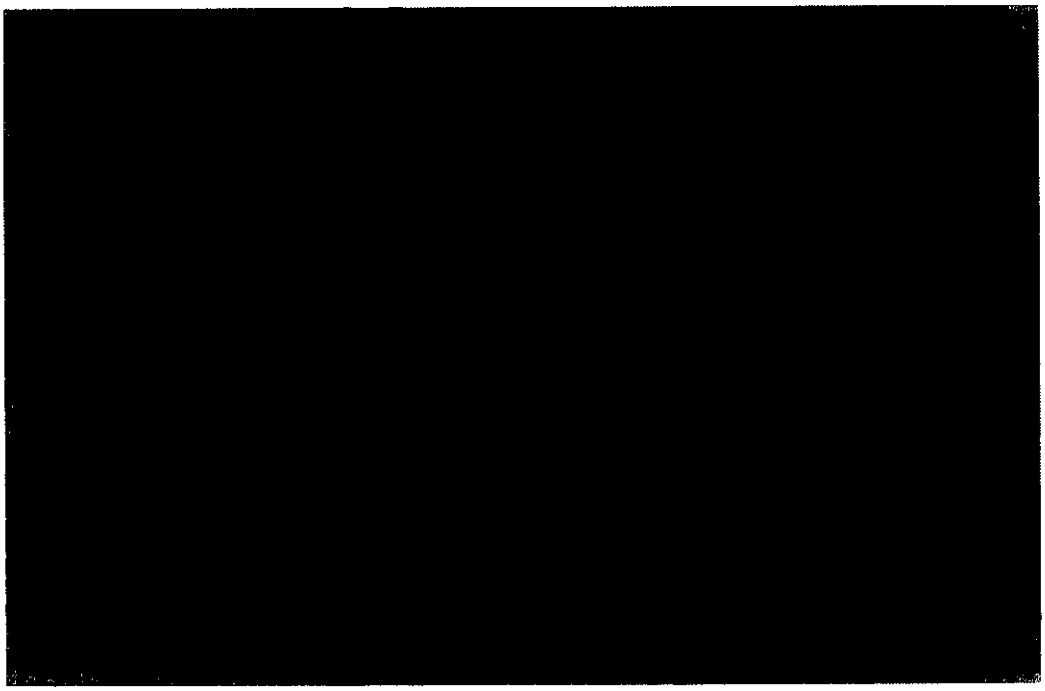

Figure 1. Gram stained view of *Klebsiella*

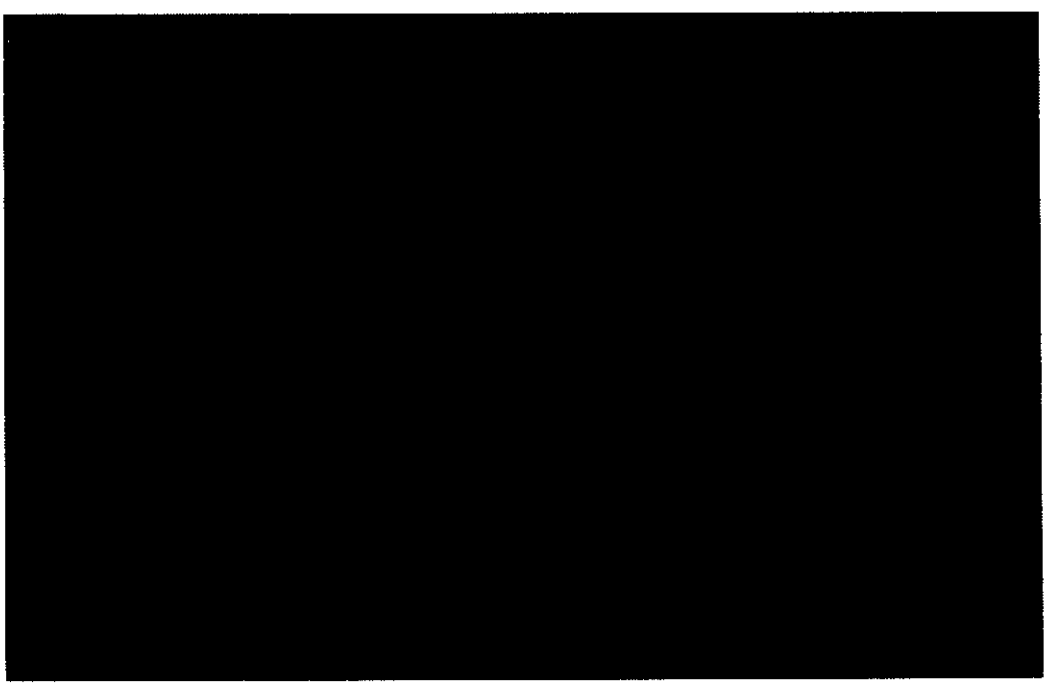

Figure 2. Gram stained view of *Citrobacter*

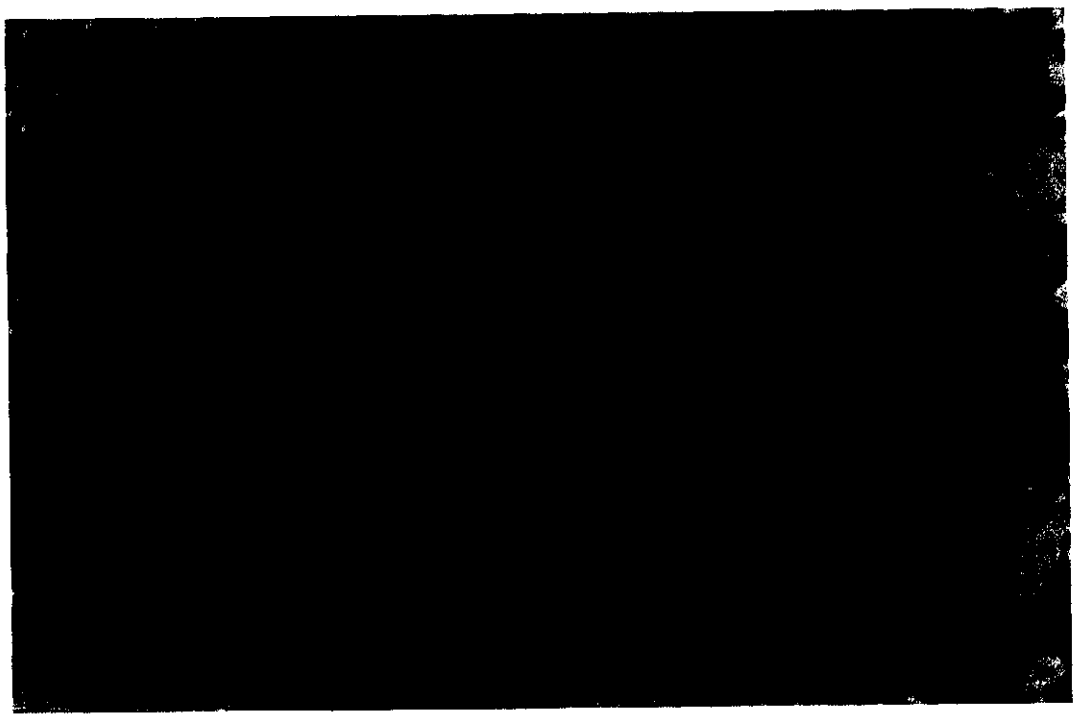

Figure 3. Gram stained view of *Serratia*

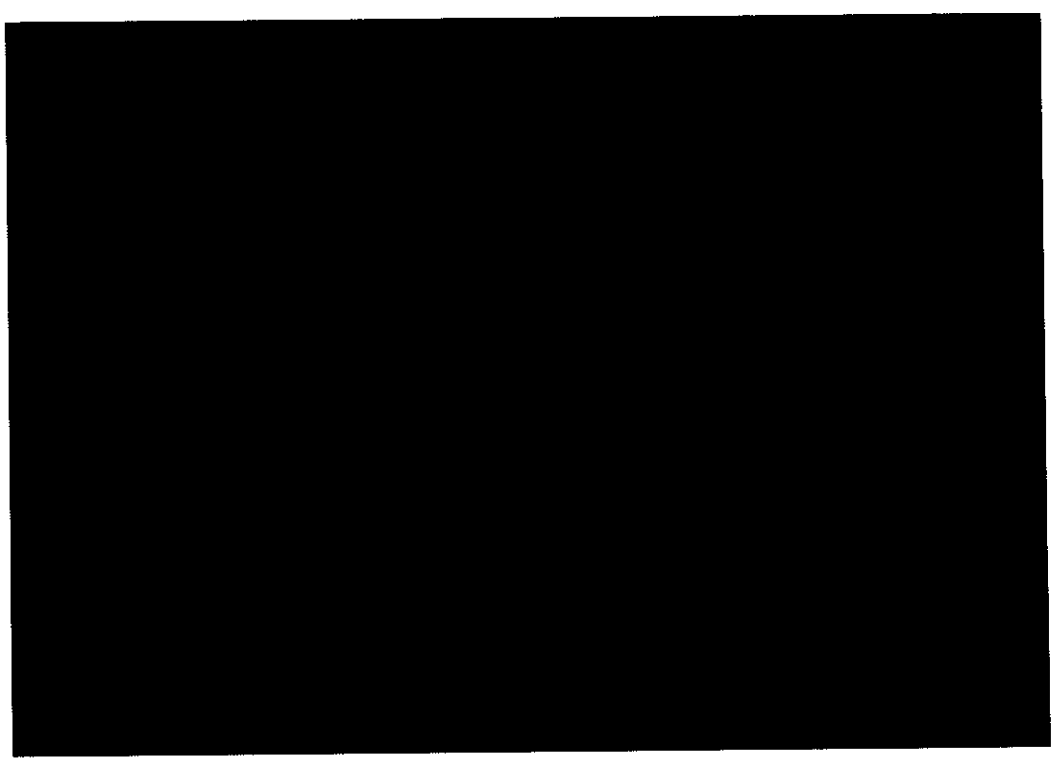

Figure 4. Gram stained view of *Kluyvera*

Figure 5. Gram stained view of *Cedecae*

Figure 6. Gram stained view of *Enterobacter*

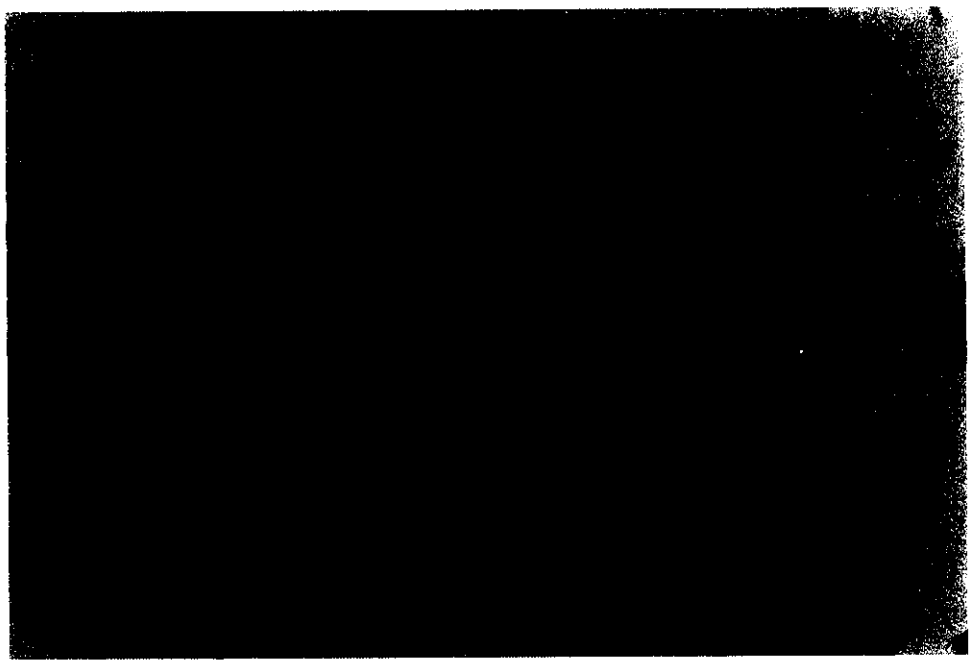

Figure 7. Gram stained view of bacterial consortium

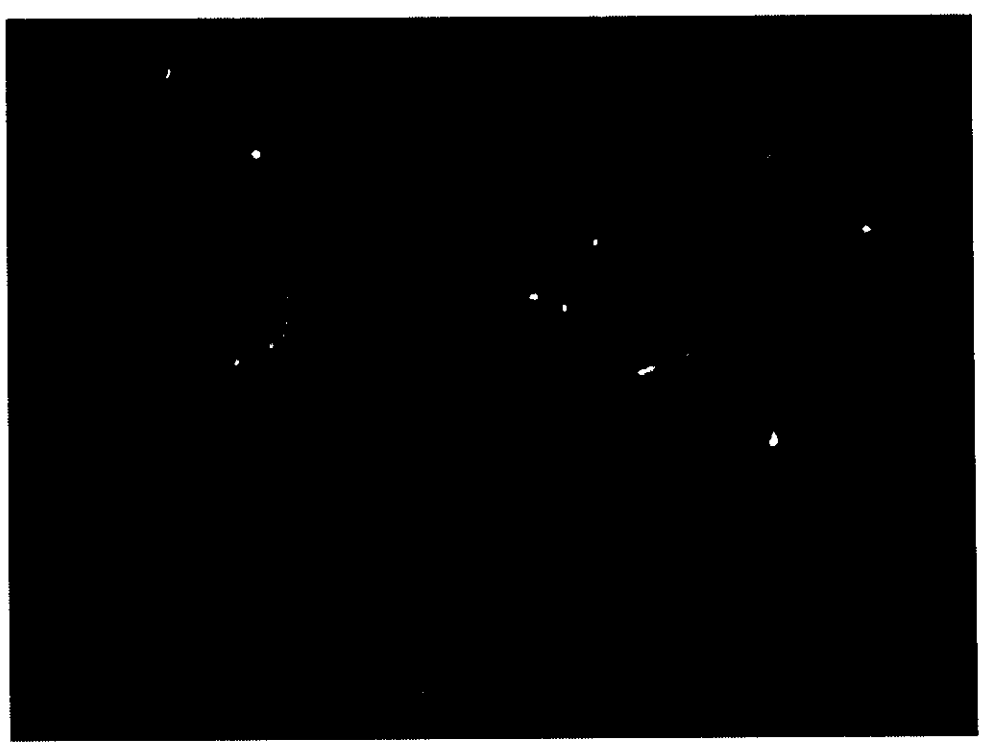

Figure 8. Live consortium

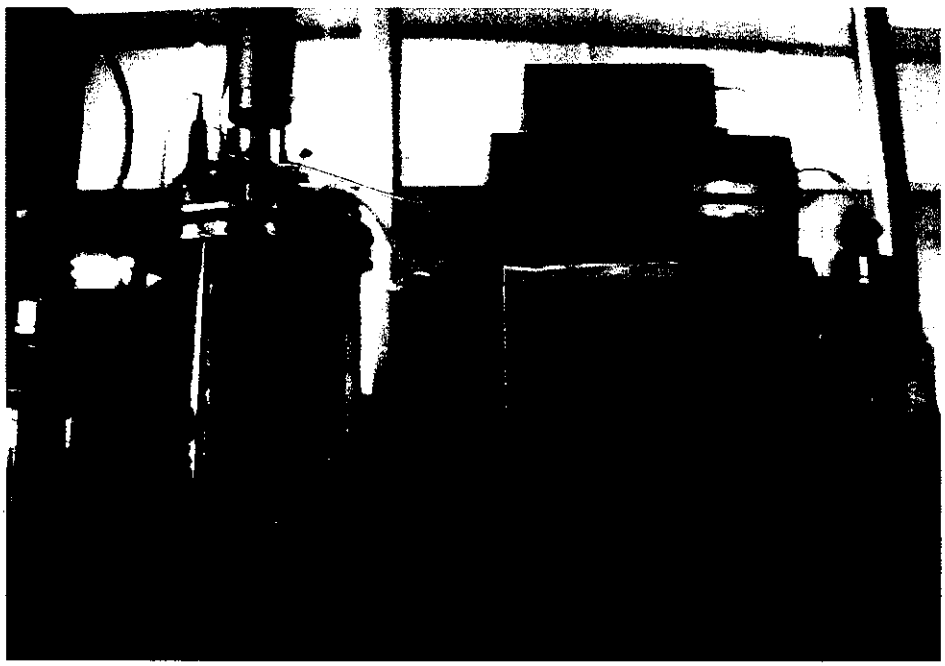

Figure 9. Bioreactor vessel (3 liters)

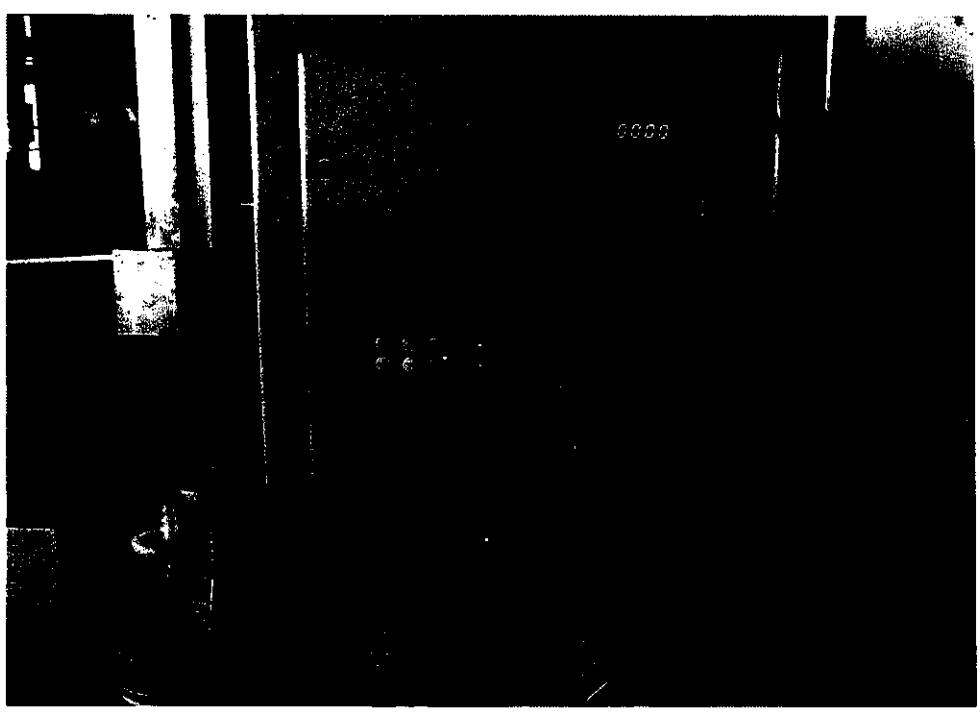

Figure 10. Bioreactor control

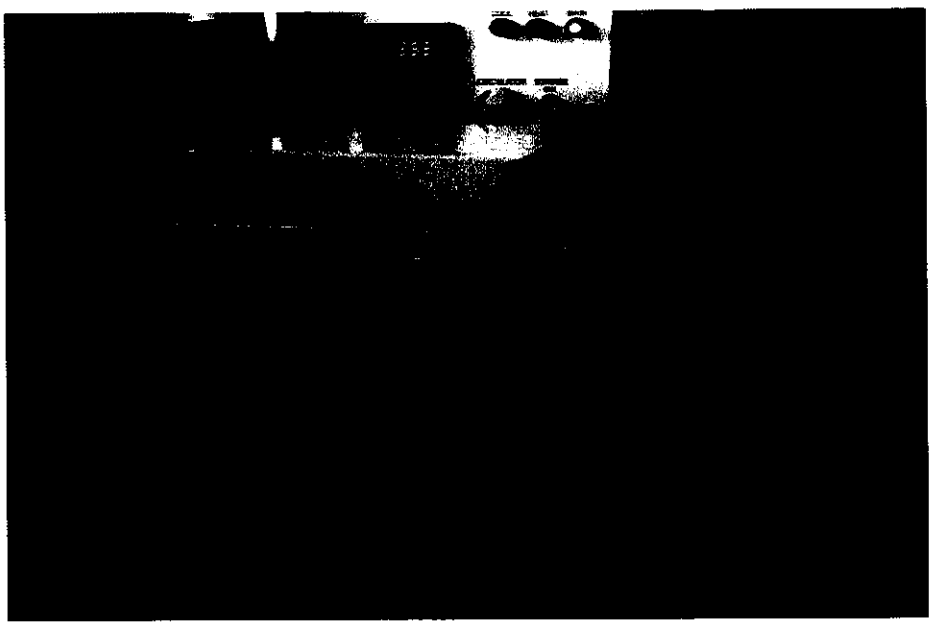

Figure 11. Bioreactor circulating bath for temperature control

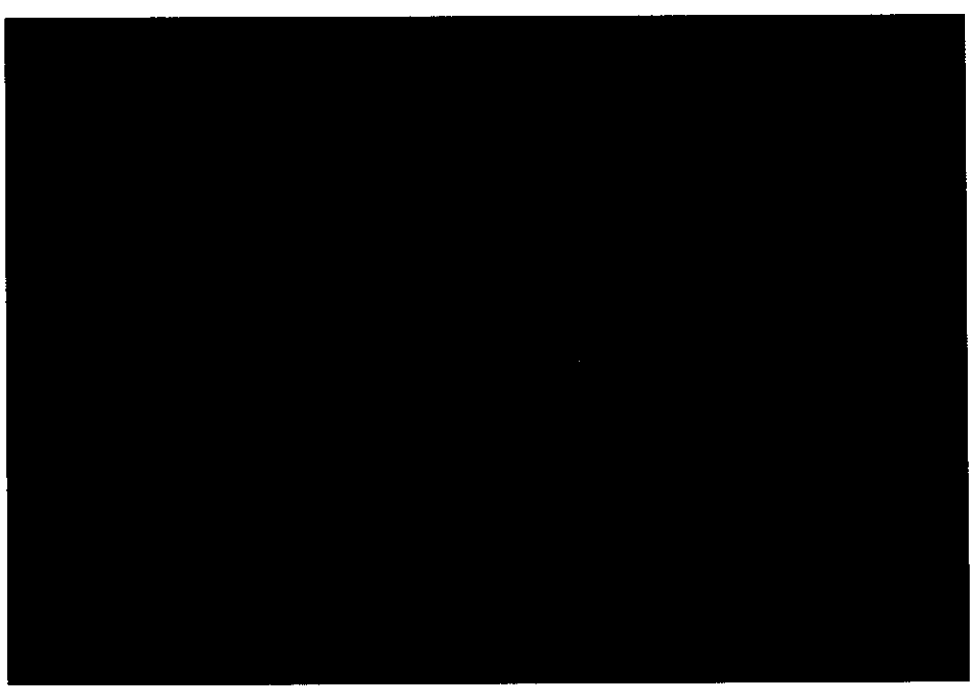

Figure 12. Plate count of consortium using colony counter

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