BACTERIAL DEGRADATION OF BLACK LIQUOR – A PULP MILL EFFLUENT

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **BACTERIAL DEGRADATION OF BLACK LIQUOR-A PULP MILL EFFLUENT** in fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** and submitted in the **DEPARTMENT OF CHEMISTRY** of the University is an authentic record of my own work carried out during a period from **DECEMBER 28, 1991** to **APRIL, 1995** under the supervision of **Prof. S.K. SRIVASTAVA** and **Prof. A.K. SHRIVASTAVA**.

The matter presented in this thesis has not been submitted by me for the award of any other degree.

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ABSTRACT

With increasing industrialization and urbanization, the problems of pollution have manifested into disastrous proportions all over the world. In India, with the promulgation of Water and Air acts, it has become mandatory on the part of industries to treat their wastes to effluent standards before disposal.

The pulp and paper industry in recent years have registered a boom, specially with the introduction of agricultural wastes as raw material. Water discharged from these industries is one of the major problems of river pollution in India. The most toxic waste emanating from these mills is "black liquor" generated from digesters. It has characteristically high BOD, COD, suspended solids, dissolved solids and colour apart from slowly biodegradable lignin compounds and its derivatives.

The present day technology for the treatment of black liquor comprises of chemical precipitation or biological treatment in anaerobic lagoon/digesters or in activated sludge process when mixed with the other waste of the paper mill. In the first method large quantities of sludge generated further pose disposal problems, whereas the digesters require skilled operation and maintenance and lagoons are land intensive, requiring a detention period of 25 to 30 days. In case of activated sludge process, the problems of frothing and high power costs are common. As such a need for techno-economically feasible solution to the treatment of black liquor is of paramount importance

A few species of fungi and bacteria are found to degrade lignin fast and easily. However, not much work has, so far, been reported on the degradation of black liquor by these microorganisms and more investigations in this direction are called for.

Investigations presented in this dissertation are centered on the degradation of black liquor by three bacterial strains viz. *Pseudomonas putida*, *Aeromonas*

formicans and *Acinetobacter calcoaceticus* obtained from the National Chemical Laboratory, Pune, India. Black liquor was collected from the effluent channels (at first extraction stage) of an agricultural residue based small pulp and paper mill. This plant is located in Himachal Pradesh, India and employs soda process for pulping. Sterilized black liquor was used throughout the study.

In preliminary experiments conducted for research, the growth pattern for all the three strains were studied in basal medium and generation time was calculated for each strain. Effect of physico-chemical parameters such as pH and aeration on the growth of selected bacteria was investigated. Growth was measured by bacterial enumeration (7 days growth) method. The growth pattern of bacteria indicate a well defined log period which extends upto the 10th day and then rounds off to a stationary phase. Generation time varied between 0.67 and 0.98 days.

Various physico-chemical parameters e.g. black liquor concentration, pH, amount of glucose, ammonium chloride and phosphate as extra carbon, nitrogen and phosphorus sources respectively were optimized for the best growth of bacterial strains in black liquor using bacterial cells suspension in distilled water (10^2 -10^4 cells per ml) as inocula. The degradation of black liquor was monitored as percent removal efficiency of COD, colour and lignin.

Batch studies were conducted to degrade black liquor under optimized conditions for each strain and the reaction mixture was analysed per day for pH, viable cell count, COD, colour and lignin up to 20 days of incubation. The results of batch studies show that the efficiency of removal of COD, colour and lignin ranged between 70 to 90 percent for each strain and most of the removal occurred in first 8 to 10 days of the experiment. There is a gradual fall in pH in initial stages and this becomes constant in last few days, while the bacterial cell count increased exponentially initially and reached to a constant level in last stage of experiment. The decrease in pH was suspected as a result of acid formation after degradation of lignin present in black liquor.

The uninoculated incubated black liquor used as a control and samples obtained after 20 days of degradation by individual bacterial strain in batch studies were analysed on gas chromatography to identify the products of lignin degradation. Ethyl acetate extractives were prepared from the supernatants obtained after filtration of black liquor in each case and analysed on gas chromatograph as trimethyl silyl derivatives. Various peaks were obtained due to unknown compounds present in the sample and some of these compounds were identified as phenolic acids by matching their retention time with known standards. The identified compounds formed as a result of interaction of *P.putida* with lignin are p-hydroxy phenyl acetic acid (PHPA), p-hydroxy benzoic acid (PBA), vanillic acid (VA), protocatechuic acid (PCA), syringic acid (SA), p-coumaric acid (CA) and ferulic acid (FA). The identified products of lignin degradation by *A. formicans* include-PHB, VA, PCA, SA and FA and the same in the case of *A. calcoaceticus* are PHPA, PHB, VA and FA. These compounds are lignin fragments can be reasonably justified on the basis of their structures. Phenolic acids were formed due to oxidative degradation of lignin by different bacterial strains.

Continuous degradation of black liquor was carried out at laboratory scale and for this purpose completely mixed, continuous flow aerated reactors/lagoons were used. The reactors were fabricated using plexiglass having a capacity of six litre with an outlet at four litre. Black liquor was taken at optimized conditions and inoculated by tested strain of bacteria. These lagoons were kept in batch for 8 days to mature and removal efficiencies of COD, colour and lignin were measured every day. On the 8th day, 500ml of black liquor was fed to the reactor from the reservoir at a rate of 2.88 ml per minute to give a detention time of around 8 days. The effluent from the reactor was tested regularly for COD, colour and lignin removal as parameters of treatment. The values of these parameters were in the range of about 70 to 90 percent for each strain of bacteria. Experiments were continued for 27 days after continuous fedding of black liquor in the reactor.

Various kinetic parameters were also calculated from the above results and these can be utilized in the design of reactors for COD, colour and lignin removal from black liquor by selected bacterial strains. The detention time can be found out with the help of these parameters for achieving the desired efficiency of removal.

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First I bow down at the lotus feet of **MAA SARASVATI** and pray, "Let noble thoughts come to me from the universe and by the eternal blessings of Omniscient, Almighty as to enable persist always in me the strong belief of devotion and determination that anything incomprehensible on this holy earth is comprehensible through team-work with the spirit of cooperation, goodwill and hardwork".

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LIST OF PUBLICATIONS

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4. Degradation of Black Liquor, A Pulp Mill Effluent by Bacterial Strain *Pseudomonas putida*.
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CHAPTER -1

INTRODUCTION

1.1 GENERAL

There is a sense of desperation in our society that modern technologies have introduced a bewildering array of potential hazards to human health and to our environment. There is an accompanying sense of frustration that our prodigious basic research capabilities and our technological ingenuity have not yielded practical ways to control many pollutants and waste streams or-better still-to convert them into useful products.

To translate it into practice it is necessary, that biological scientists and engineers join forces with both traditional and newly emerging techniques to make substantial progress in controlling environmental pollution. There is a need for the development of efficient technology for the removal of toxic, persistent chemicals from the environment.

The waste and toxic substances in the environment can be more effectively and safely managed by microbial-technology. More over some microbes are capable of degrading many such pollutants into their basic elements, which can be recycled and help to maintain C, N and S balances in the environment. Thus the biotechnology dealing with the interaction between bacterial population and environmental pollutants becomes important for pollution abatement. The purpose is to identify and assess strategies for effective and safe management of wastes and toxic substances in the environment, through use of genetically engineered microorganisms.

Water is of vital importance to all life on earth. But the consequence of

manifold usage is the generation of waste water and concombitant increase in the level of pollution. Water pollution from pulp and paper industries is one of the biggest environmental problems. Many treatment technologies are available to treat this waste water, but very few of them are cost effective. As such there is a need to develop a clear and economical treatment technology.

The present research project deals with the application of biotechnology for the treatment of black liquor-a pulp mill effluent, by conducting studies on specified bacterial strains that can utilize lignin present in black liquor, as a sole carbon and energy source and degrade it into its derivatives. These strains are found to be highly effective in removing large quantities of lignin and its derivatives from waste water, and as a consequence reduce its BOD, COD, colour etc.

1.2 PULP AND PAPER INDUSTRY IN INDIA

Paper pervades all walks of human activity and use of paper and its products is highly linked with cultural and economic development of a country. In fact the quantum of paper used is an index of prosperity and development of any nation.

Machine made paper industry in India made its debut in 1867 with the establishment of first paper mill at Bali in Bengal. Since then a slow and steady growth of paper mills is recorded in various parts of the country. During the last 40 years there has been a tremendous growth of these mills, and as on January 01, 1989, there were 305 paper and paper board mills in India [1,2], with an installed capacity of 3.0 million tons per annum (TPA).

Large paper mills (LPM) with capacities greater than 55 tons per day (TPD) and numbering 34, account for 51 percent of total installed capacity. A number of small paper mills (SPM) with 10-30 TPD capacity and using agricultural residues and waste paper, have been set up all over the country since 1975. The SPM numbering 271 account for 49 percent of the total installed capacity [1,2].

1.3 RAW MATERIAL REQUIREMENTS

The fibrous raw material is the most important raw material with which the paper industry is concerned. Indian paper industry was originally based on the use of bamboo as main cellulosic raw material. However with the increase in paper demand and decreased availability of bamboo, other secondary sources such as hard wood e.g. Eucalyptus, Salai, agricultural residues and waste papers are exploited. The non-wood pulp materials like rice and wheat straw, bagasse, gunny and jute cuttings, Kenaf (*Hibiscus cannabinus*), Sarkanda grass and few other annual crops and grasses are generally the main raw materials now used in small paper mills.

1.4 WATER REQUIREMENTS

As a raw material for use in manufacture of pulp and paper, water is the second in importance only to the fiber itself. Water requirement varies from 250-400 cubic meter per ton of paper made in LPM, while speciality paper requirement is 370-1220 cubic meter per ton of paper produced [3]. It is estimated that for an agricultural residue based paper mill (SPM), its requirement is 200-300 cubic meter per ton of paper made, and corresponding range for waste paper based mills is 100-150 cubic meter per ton of product [3,4].

1.5 PROCESS OF MANUFACTURING

The two basic operations involved in pulp and paper mills are :

(i) Pulp making or pulping

(ii) Paper making

1.5.1 PULP MAKING

Pulp preparation is the initial phase of paper manufacturing. It consists of cooking the raw material i.e. cellulosic and non-cellulosic materials in suitable chemicals in a digester under controlled conditions of temperature, pressure and

time. Main pulping process that are normally employed in paper industry include;

(i) Chemical pulping

(ii) Mechanical pulping

(iii) Chemo-mechanical pulping

(i) Chemical pulping

Chemical pulping may be defined as cooking of raw material by sulfate (Kraft), soda or sulfite process to a point where the fibres can be easily separated from each other. The above chemical processes differ from one another only in the chemicals used to digest the raw materials. The range of pH for reaction is from 2 to 13. The rate of lignin removal, extent of cellulose degradation, extent of hemicellulose removal, etc. are pH dependent.

(ii) Mechanical pulping

For waste paper and recycled paper, hydropulping is adopted. This process involves the reduction of raw material to the fibrous state by mechanical means-generally by grinding raw materials to pulp against a large grindstone. The yield of pulp is high by this process (about 90%), but the pulp is of low purity and there is considerable fibre damage.

(iii) Chemo-mechanical pulping

As the name suggests, the process involves features of both chemical and mechanical process in series. The raw material is first given a chemical treatment and then subjected to drastic mechanical treatment to separate fibres. This process enables more of the lignin and hemicellulose constituents of raw material to be retained in pulp than that in chemical pulping process. So the pulp obtained by this process is named "High Yield Pulp".

The other phase of pulp making involves processing followed by bleaching.

After digestion, chemically digested pulp is discharged on to a blow pit or on a perforated floor where part of the black liquor drains out. The drained pulp is then washed and passed through screens, cleaners and refiners to remove knots and other nondisintegrated matter. A cylindrical screen, called a decker, revolving across the path of the pulp partially dewaters it, after which it passes to bleach tanks, (if bleached pulp is required) where it is mixed in a warm dilute solution of calcium hypochlorite or hydrogen peroxide. The semi-dried, bleached pulp is then ready for making the paper.

1.5.2 Paper Making

The paper making operations consist of :

(i) Stock preparation through blending and conditioning i.e treating the pulp to the required degree of fitness; and

(ii) Paper making, where the treated pulp is passed on continuous moulds/wires to form sheets.

Blending provides the required pulp to water ratio before sending it to the paper machine. The pulp mixture is disintegrated and mixed in a beater to which various fillers and dyes are added to improve the quality of end product and sizing to fill the pores of the paper. This pulp is sent to a paper machine of a moving wire mesh and rotary driers. Steam is used in driers to drive away moisture from the sheet of paper formed on the wire mesh and picked up by the driers. The finished product is cut to the required size and is ready for marketing.

A generalized flow sheet [5] of the processes adopted for making paper in SPM is depicted in figure 1.1.

1.6 WASTE WATER GENERATION

Large quantity of water is used during pulping and paper manufacturing and practically the entire quantity reappears as effluent. The chief sources of waste water generation are :

(i) Digester house

Black liquor is generated in this section. In LPM, although chemical recovery of black liquor is done, leaks and spills of black liquor and gland cooling water appear as waste water. In SPM, there is no chemical recovery and all the black liquor appears as pulping mill wastewater.

(ii) Pulp washing section

The main component is the pulp wash water from the pouchers, often referred to as brown stock wash or unbleached decker wash.

(iii) Pulp bleaching section

Chlorination stage waste water contains chlorolignins and caustic extraction waste water contains chlorolignins and hypochlorite waste water.

(iv) Paper machine

Waste water contains fibres and sizing chemicals.

The different types of wastes generated [5] from different section of pulp and paper mill (SPM) are shown in figure 1.1.

1.7 CHARACTERISTICS OF WASTE WATER

The characteristics of waste water vary appreciably and depend on the raw material used, process employed, the efficiency of black liquor recovery and other factors. The waste water obtained from different sections of an agricultural residue based paper mill (SPM) have different characteristics.

The liquid waste generated from digester i.e. black liquor consists of organic as well as inorganic dissolved solids and excess of chemicals. The suspended matter contains fibres, silt and sand. It has dark brown colour and hence the name-black liquor. Black liquor in sulphate and soda process is highly alkaline with pH in the

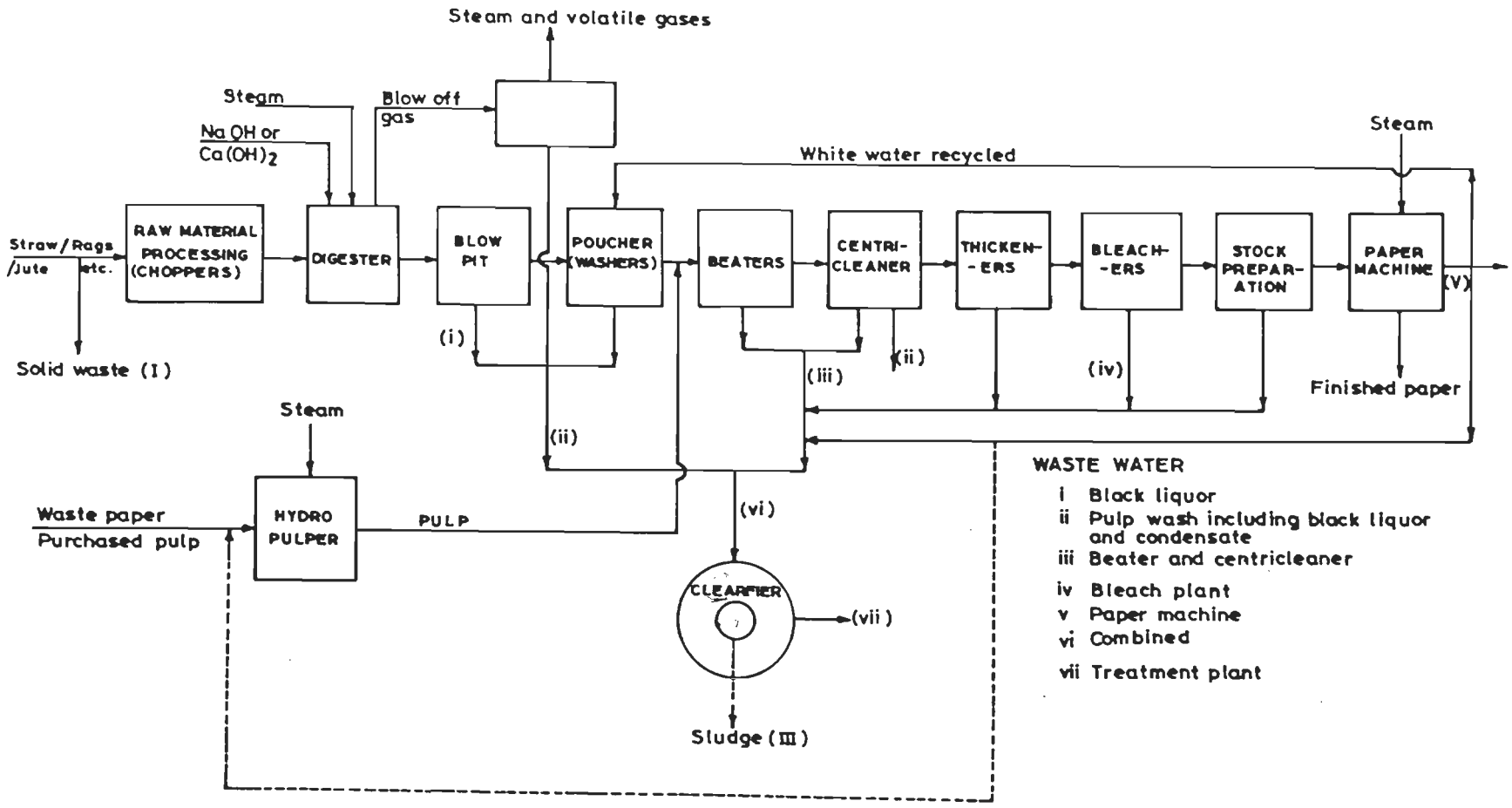


FIG. 1.1 GENERALISED PROCESS FLOW SHEET OF SMALL KRAFT PAPER MILLS AND SOURCES OF WASTE WATER

range of 10-12, but the spent sulfite liquor is highly acidic with pH varying between 3 and 4. The total solids may be varying between 50,000 to 2,00,000 mgl^{-1}. The BOD values may be in the range of 10,000 to 60,000 mgl^{-1} . Whereas COD may be varying between 50,000 to 2,00,000 mgl^{-1} . The COD to BOD ratio may be high ranging between 2.5 and 4.5 and is mainly due to lignin and fibres which are not easily biodegradable. The lignin contents may vary in the range 7,000 to 90,000 mgl^{-1} .

Waste water generated from pulp washing section contributes nearly 80 percent of the total pollution load. The characteristics of waste water from pulp washing and bleaching operations depend upon the process employed and may have pH in the range 6-8, suspended solids in the range of 400 to 800 mgl^{-1}. BOD between 200 to 900 mgl^{-1}, COD between 400 to 1,500 mgl^{-1} and lignin about 200 mgl^{-1} . The paper machine effluent is least polluting among all the sections. The paper machine effluent has pH between 6 and 8. Suspended solids range from 1,000 to 1,500 mgl^{-1}, COD between 500 to 1,000 mgl^{-1}, BOD between 200 to 500 mgl^{-1}, but the lignin content may be very small (10-15 mgl^{-1}).

1.8 BLACK LIQUOR AND ENVIRONMENTAL POLLUTION

Black liquor from agricultural residue based paper mills (SPM) constitutes the most polluting waste.On an average, about 10 cubic meter of black liquor is generated per ton of pulp made. Pulp washing section also contributes its share of black liquor and this section accounts for 20-25% of total waste water and contributes 70-80% of pollution load in SPM using agricultural residue as raw material. These wastes are discharged intermittently and alter the composition of combined waste water appreciably [1].

1.8.1 CHEMICAL COMPOSITION OF BLACK LIQUOR

Lignin of wood, during pulping, dissolves in cooker liquor as sodium phenate. A variety of degraded and condensed lignin fragments of different molecular

weights remain present in the black liquor. In kraft black liquor, a mixture of alkali and thiolignins are present, while only alkali lignin is formed in soda black liquor. Sulfite black liquor contains lignosulphonic acid.

Some hemicelluloses and celluloses are also degraded to give rise to isosaccharinic acid and low molecular weight carbohydrates [6]. Other minor wood components like resin and fatty acids are saponified and are also present in black liquor. Silica present in bamboo, bagasse and straw ends up in black liquor as sodium silicate.

1.8.2 TOXIC EFFECTS OF BLACK LIQUOR

The pollution potential of pulp and paper industries is based on their location. Mostly these are located on the banks of small rivers and streams. These rivers and streams receiving the pulp mill effluent are generally characterized by a black colour and frothy surface and this type of waste water is very much toxic to flora and fauna of aquatic ecosystem. High BOD and COD of this effluent have adverse effect on fishes and other test species. Lignin and its derivatives, besides imparting colour, cause aesthetic pollution for long stretches of river as these are not readily biodegradable. The problem of colour is enhanced in regions where waters are to be used for industrial or recreational purposes. The colour of effluent is meeting increased criticism on various grounds [7,8] as detailed below :

(i) The dark brown colour of effluent inhibits photosynthetic production of oxygen by decreasing light penetration.

(ii) The colour component may be toxic to some of the lower scale of organisms in the aquatic ecosystem.

(iii) At some down-stream point, the coloured water may become part of a municipal water supply source where the colour removal might be troublesome.

(iv) Regulatory bodies are becoming less tolerant of any organic material in an effluent.

(v) Chlorination of the compounds responsible for colour are found to be responsible for mutagenic effect i.e. a change in genetic code of living cells.

(vi) Aesthetically, strong colour is inconsistent with present public attitudes.

(vii) These colour pollutants may be chelate metal ions, thus increasing the cost of water treatment and there is a likelihood of contamination by dissolving heavy metals.

Thus, discharge of untreated paper mill effluents into water courses damages the water quality and the colour in water persists for long distances. As paper industry is scattered all over the country, and only a few industries are providing treatment facilities, it may be said that no river is spared from pollution due to discharge of these waste waters. The pulp and paper industry is, therefore, numbered among those industries, which are the most damaging to the environment.

1.8.3 BLACK LIQUOR AND TREATMENT STRATEGIES

The black liquor is generated both in SPM and LPM except the mills using waste paper as raw material and the one that are not undertaking any pulping activity and utilizing purchased pulp [9]. Various treatment methods of black liquor depend on raw material used, size of paper mill, availability of land and the effluent standards desired. The strategy behind treating black liquor is to reduce its pollutional parameters first and then mixing it with total mill effluent for final treatment.

1.8.3.1 Treatment of Black Liquor in LPM

Black liquor is normally separated out for chemical recovery in LPM and pollution load is reduced considerably in addition to saving in chemicals. In some mills (LPM) black liquor is treated partially in anaerobic lagoons removing 50-70% BOD load. It is then mixed with other process waste water and treated in activated sludge process or other aerobic processes.

1.8.3.2 Treatment of Black Liquor in SPM

Chemical recovery from black liquor in SPM is considered uneconomical due to abnormally high cost of installing chemical recovery plant. Thus, the pollution load from a small paper mill with no chemical recovery is nearly 3.5 times that of a corresponding mill with chemical recovery [10] and is generally treated as combined waste water or may be first treated to reduce its BOD and COD load before subjecting it to combined treatment [11,12].

Being small and intermittent discharge, no segregation of black liquor is practised in SPM. It is allowed to join other streams of water from various further operations after equalization and pumping at constant rate. It is then treated like normal municipal waste water with pre treatment, primary treatment and secondary biological treatment and in some cases a separate treatment for colour.

1.9 MINIMAL NATIONAL STANDARDS

The Minimal National Standards (MINAS) for pulp and paper mill effluent have been evolved after looking into practical difficulties, limitations, techno-economic feasibility and economic impact on the industry.

The basic considerations taken into the development of the MINAS are as follows :

(i) Characteristics of effluent from small pulp and paper mills without chemical recovery system.

(ii) Achievability and techno-economic feasibility of various waste water treatment alternatives.

(iii) Maintain ratio of annualised cost to the turn over of the industry. Based on the above aspects, the recommended tolerance limits for effluent from small pulp and paper industry [5] are tabulated below :

MINAS For Small Pulp And Paper Industry

Parameters	Values
pH	6.0-9.0
Suspended solids	100 mgl^{-1}
BOD	50 mgl^{-1}

Permissible limits for COD and lignin are not prescribed as no suitable economical technology is presently available. COD limits shall be introduced in MINAS as and when the suitable economic treatment system for removal of COD/ lignin would be available.

1.10 RELEVANCE AND OBJECTIVES OF PRESENT STUDY

The problem of treatment of black liquor from pulp mill is still persisting. In large paper mills chemical recovery processes have been quite successful but in small paper mills, recovery plants are found uneconomical. Existing methods of treatment include precipitation and removal of lignin through physico-chemical methods, or biological treatment in anaerobic lagoons or in activated sludge process (ASP) mixed with paper machine effluent. In precipitation methods, the cost of chemicals becomes prohibitive and enormous amount of sludge produced, poses a disposal problem. Anaerobic lagoons with around 25-30 days detention provides 50-60% efficiency only and require large land area. In case of ASP or aerated lagoons, the problems of frothing and high power costs are common. Hence a need for techno-economically feasible solution for the treatment of black liquor is of paramount importance. The methodology should have low power and land costs, giving amicable efficiency of treatment.

Some species of aerobic/facultative bacterial strains are reported in literature to degrade lignin and there is a need to investigate and identify the various bacterial

strains, which can be preferentially used for this purpose. To accomplish this, objective, investigations were conducted using black liquor from SPM to investigate the possibilities of colour, COD and lignin removal by different aerobic bacterial strains chosen for this purpose.

The objectives of the present study are to :

(i) Study the growth pattern of selected bacterial strains in basal medium and effect of aeration in the same medium.

(ii) Optimize the culture parameters like black liquor concentration, pH, extra carbon source, additional nitrogen and phosphorus concentration for maximum removal of COD, colour and lignin.

(iii) Study percent removal of COD, colour and lignin from black liquor by each selected strain at optimized conditions and to observe change in pH and viable cells count during batch experiment.

(iv) Identify the phenolic acids formed during biodegradation of black liquor in batch study by gas chromatography; and

(v) Study the continuous aerobic lagoon treatment of black liquor by all the three selected strains of bacteria on laboratory scale.

CHAPTER -2

LITERATURE REVIEW

2.1 PULP AND PAPER MILL EFFLUENT TREATMENT

2.1.1 GENERAL

The pulp and paper industries have treatment and disposal problems [13-15]. The offensive colour in the effluent of paper mill waste is due to lignin and its derivatives. Black liquor is most polluting among the wastes generated by all sections of pulp and paper mills. Various options are available for the effective treatment of pulp and paper mill waste water [13]. The literature cited below has broadly been divided into four treatment groups viz. Physico-chemical, anaerobic, anaerobic-aerobic and aerobic treatment methods and the data and conclusions arrived by various investigators are reported in this dissertation.

2.1.2 PHYSICO-CHEMICAL TREATMENT

The current physical and chemical processes, best suited for pulp and paper mill effluent treatment, include such methods as ultrafiltration, ion exchange, chemical coagulation and precipitation, adsorption, soil percolation and oxidation.

2.1.2.1 Ultrafiltration

Mishra and Bhattacharya [16] studied the feasibility of electrolytic treatment of alkaline black liquor in a three compartment electrodialyzer under batch conditions. Influence of current density (5-25 mA cm^{-2}), pH, residence time (15-18 min) and percent soda recovery with membrane arrangements were studied. In terms of percent caustic recovery and specific power consumption, the cation

exchange-cellophane membrane arrangement gave better results than the cellophane-cellophane arrangement. With the cation exchange-cellophane arrangement, it is possible to recover more than 95% of caustic in batch electrodialysis.

Bodzek et al. [17] used acetyl cellulose membrane in the ultrafiltration of waste water from washing of sulphate and sulphite pulp.

Simpson and Groves [18] discussed the treatment of bleach effluent from pulp and paper manufacturing plant by reverse osmosis. It was observed that membrane fouling was significant and membrane rejection of both inorganic and organic components were more than 90% for total solids concentration of 5-30 gml^{-1}.

2.1.2.2 Coagulation and Precipitation

E.W. Lang [19] investigated the effectiveness of alum coagulation on total mill effluent. He reported that alum consistently removed 81% to 93% of colour, 50% to 62% of total organic carbon, 20% to 25% of BOD and gave a product almost free of suspended solids and turbidity at an optimum pH of 5.5.

Sheela and Dastidar [12] used a method of precipitating the lignin from black liquor of small paper mills with the application of gypsum in presence of CO_2. Almost 63% of the dissolved organic solids were removed from the black liquor out of a total of 95% before treatment. Maximum reduction was observed after 4 hours of reaction time and removal of the organic fraction from the reacted liquor was supported by changes in pH, colour and COD values. Ye Binglin [20] precipitated the lignin from soda black liquor only in presence of CO_2 and highest yield of precipitation of lignin was obtained under conditions of 25% concentration of total dissolved solids, at 80^0C, a relatively low pH, short storage time and no oxidation of liquor.

2.1.2.3 Adsorption

Lang et al. [21] confirmed the use of activated carbon for treating caustic bleaching effluents as technically sound and achieved 94% removal of colour and 84% of total organic carbon (TOC). Carpenter et al. [22] and Dove [23] used powdered activated carbon in activated sludge treatment of pulp mill effluent, at a dose of 450-1000 mgl^{-1}, for the removal of additional colour attained in conventional process but total solids were found to be more.

Moss et al. [24] studied the treatment of bleach plant effluent from the pulp and paper industry by means of the anaerobic biological granular activated carbon process. It was found that over 50% of the COD and colour could be successfully removed from this effluent. The adsorptive capacity of the activated carbon was extended as a result of microbial activity inside the anaerobic reactor. The results of these investigations suggest that the anaerobic biological granular activated carbon process could be used to alleviate the pollution problems experienced by the pulp and paper industry.

2.1.2.4 Electrolysis

Cloutier et al. [25] utilized a technically feasible method of electrolytic treatment of weak black liquor in a continuous 90 hour experiment with a laboratory scale system. The process was shown to be able to reduce the organic load to the recovery furnace by precipitating and removing upto 75% of lignin, while converting a major fraction of the sodium salts to high quality NaOH solution.

2.1.2.5 Oxidation

Sun et al. [26] effectively removed total organic chlorine and colour of the high molecular-weight chlorolignin from spent alkali extraction stage effluent by an oxygen oxidation process. Under the best conditions, about 70-80% of the total

organic chlorine with an initial value of 2200 mgl^{-1} and 60-70% of the colour with an initial value of 220,000 Co-Pt units were removed in less than one hour. The high molecular weight chlorolignins were degraded to a lower molecular weight ones.

Gonzalez et al. [27] described the possibility of utilizing kraft black liquor generated in a paper mill, using *Eucalyptus globulus* as raw material, for the production of a nitro-humic soil conditioner. Two processes were considered : (i) oxidation of the kraft black liquor with nitric acid and (ii) precipitation of the lignin contained in the kraft black liquor with CO_2 and further oxidation of this lignin by nitric acid. The second process was more efficient with regards to the product yield and safety of operation.

2.1.3 ANAEROBIC TREATMENT

There are various anaerobic treatment methods which are normally employed for pulp and paper mill effluent treatment. These methods include anaerobic digestion, anaerobic lagoon, anaerobic fixed bed and upflow anaerobic sludge blanket (UASB) reactors. The main principle of anaerobic treatment in all the above mentioned methods is the biological decomposition of organic matter in absence of oxygen. This takes place primarily in two sets of reaction in anaerobic digestion, the first one is known as acid fermentation and the second as methane fermentation.

2.1.3.1 Anaerobic Digestion

Khanolkar and Pudumjee [28] treated the black liquor by two stage digesters in Pudumjee Pulp and Paper Mills Ltd. They have reported, that the BOD and COD reduction efficiencies are slightly above 70% and 50% respectively. The active biomass builds up slowly and generally takes a couple of months to reach a concentration of 10 mgl^{-1}. The specific gas production was reported to range between 0.42-0.5 $m^3\ kg^{-1}$ COD destroyed.

Bremmon et al. [29] studied the production of methane from desugared spent sulfite liquor (SSL). Ozonated SSL was fed continuously to three anaerobic fermenters for three months. Methane production from ozone treated SSL was about 17 mgl^{-1} of volatile solids fed. It was concluded that ozonation increased methane fermentation.

Nitchals et al. [30] studied the anaerobic treatment of mixture of two waste streams from sulfite pulping, caustic extraction stage bleaching waste and sulfite evaporator condensate. This process produced energy and reduced the waste loads on aerobic treatment systems. Treatability was evaluated in batch test and in a continuous flow reactor. Mixtures with pH as low as 4.6 were treatable, with 70 to 80% BOD removal and 42 to 51% TOC and COD removal.

Latola [31] treated paper mill waste water anaerobically having COD 1800 mgl^{-1} in a multistage reactor with COD loading 5-6 kgm^{-3} and retention time of 4-6 hours. The reduction in BOD was reported to be 60-75 percent.

Saerner [32] treated a mixture of condensate and extraction liquor from a dissolving pulp mill, anaerobically in a pilot plant. The result showed that caustic extraction liquor could be used for pH control and no chemicals were needed for the purpose. No toxic effects were seen, if the flow of caustic extraction was kept in the range of 25-30% of the total flow. Average COD reduction was about 50% and the corresponding reduction of BOD was 70-80%.

Anderson et al. [33] treated waste water from chemi-thermo-mechanical pulp bleaching with metal salts for detoxification followed by a double stage anaerobic treatment. The anaerobic effluent was treated in a trickling filter. A methane yield of 0.25 m^3kg^{-1} COD was obtained.

2.1.3.2 Anaerobic Lagoon

Deshmukh [34] treated mixture of prehydrolysate liquor and waste water from washings, anaerobically after supplementation of nitrogen. Daily

requirements of nitrogen and phosphorus for the waste water flow of 1800 m^3 per day were reported to be 2.14 and 0.39 ton respectively, giving BOD:N:P of 100:2:0.4 for efficient anaerobic digestion.

Kroiss et al. [35] conducted laboratory scale and semi technical scale experiments for anaerobic treatment of condensate and effluent from two mills. Neutralization, solid retention and sulfur dioxide toxicity has been discussed. It is reported that the start up of the process presented great difficulty.

Webb [36] studied anaerobic treatment of waste water from the production of peroxide bleached chemi-thermo-mechanical pulp. Treatment of waste water in pilot plant at low loading rate gave a COD reduction of 50-60% and biogas yield of 0.3 m^3kg^{-1} COD removed.

Hall and Carnacchio [37] described anaerobic treatability of 43 waste water samples from pulp and paper mills in Canada. Average soluble COD removal efficiency of 90% was achieved in the treatment of recovery plant waste water. Methane production accounted for about 90% of the COD removal, observed for the wood room and chip wash and non sulphur pulping waste waters. Bleaching plant waste water was very toxic to unacclimatized anaerobic microorganism.

2.1.3.3 Anaerobic Fixed Bed Reactors

Pichon et al. [38] described studies conducted on bleached chemi-thermo-mechanical pulp effluent in anaerobic fixed bed reactors with upflow or downflow feed modes. Upflow reactors were more efficient than downflow reactors with a maximum loading rate of 3.5 kg COD m^{-3} per day. A COD loading rate of 4.7 kg m^{-3} per day could be maintained with a residence time of 2 days and a pollution load removal efficiency of 45% for COD with complementary oxidation.

Frostell et al. [39] constructed a full scale anaerobic contact reactor system (ANAMET). The plant treated waste water for thermo-mechanical pulp production with evaporate condensate and aerobic excess sludge. After 5 months of

operation, a COD reduction of 60% and BOD reduction of 70% were obtained. Methane gas production was 0.14-0.15 m^3 per kg COD loaded.

Aivasidis [40] treated evaporate condensate from sulfite pulping in an anaerobic loop reactor, containing a porous glass sinter as microorganism support. At a residence time of 11 hours and loading of 100 kg COD m^{-3} per day, the COD removal obtained was 84%.

Pichon et al. [41] treated chemi-thermo-mechanical pulping effluent in upflow anaerobic packed bed filter reactor. COD and BOD removal efficiency were 60 and 80 percent respectively. A loading rate of 20 kg COD m^{-3} per day was achieved with 12 hours retention time and methane production was 3.5 m^3kg^{-1} of COD per day.

2.1.3.4 Upflow Anaerobic Sludge Blanket Reactors

Russo and Dold [42] studied the treatment of paper waste from a paper industry by upflow anerobic sludge blanket reactors (UASB). Effluent from a paper plant (4700 mgl^{-1} of COD and 2500 mgl^{-1} of SO_4) was treated in 9 litre laboratory scale UASB reactors operated at 25 ^{0}C and 33^{0}C. COD removal of 85% were attained in both the reactors from inlet to outlet.

Rintala et al. [43] studied the anerobic treatment of sulphate rich (COD/SO_4 ratio 1.4-2.1) clarified white water from a thermo-mechanical pulping (TMP) in three laboratory scale UASB reactors at 55^{0}C and in batch digesters at 55^{0}C and 65^{0}C. Different seed materials were used in the UASB reactors.

The highest COD removal efficiency was obtained in the UASB reactors. About 55% COD removal efficiency was obtained at a loading rate of about 41 kg COD m^{-3} per day in the UASB reactors seeded with thermophilic sludge cultivated with volatile fatty acids. Sulphate reduction was almost complete in the batch digesters whereas the value for the same in UASB reactors was 24-64%.

2.1.4 ANAEROBIC-AEROBIC TREATMENT

Priest [44] presented an anaerobic-aerobic treatment system for paper mill waste water. COD was reduced from 175-187 mgl^{-1} to 46-48 mgl^{-1}.

Rintala and Vuoriranta [45] discussed anaerobic-aerobic treatment of clarified white water from an integrated thermo-mechanical pulp producing plant. At the anaerobic stage 60-70% of the soluble COD was removed at loading rates of 5-8 kg COD m^{-3}. After aerobic treatment, COD reductions were 80-85%. A similar COD reduction was achieved in a single stage activated sludge treatment. Methane production of the anaerobic stage was 0.22-0.33 m^3 kg^{-1} COD removed. Aerobic post treatment changed the colour of waste water.

Qui et al. [46] conducted sequential anaerobic and aerobic treatment with combination of kraft evaporator condensate and caustic extraction stage bleaching effluent. A large fraction of the BOD in the combined waste could be removed at a loading of 15 kg COD m^{-3} per day. Removal efficiencies were higher for the low molecular weight fractions. One third of the influent sulfur was removed as hydrogen sulfide in the anaerobic stage while the remaining amount gets oxidized to sulfate in the aerobic stage with nearly complete elimination of odour.

Cocci et al. [47] investigated a bench scale treatability of neutral sulfite semi-chemical pulp mill effluent by anaerobic-aerobic treatment. COD, BOD and suspended solids removal of 48.6, 70.8 and 65.0 percent respectively were reported in the anaerobic filter. Removals were 60.6, 92.7 and 66.5 percent respectively in the overall system. The average loading rate in the anaerobic reactor was 1.3 kg COD m^{-3} per day and the operating temperature was 35^0C. The average specific methane generation rate was found to be 0.23 m^3 kg^{-1} COD removed.

2.1.5 AEROBIC TREATMENT

Lot of research has been conducted to isolate and identify bacterial and fungal strains, which are able to degrade lignin and its compounds faster, thus

helping more efficient black liquor treatment. Although numerous bacteria can decompose monomeric lignin substructure models, only a few strains are able to attack lignin derivatives obtained from different pulping process or can decay modified lignins isolated from lignocelluloses. A selective literature survey of lignin degradation present in pulp mill effluents by different bacterial and fungal strains is described as here under :

2.1.5.1 Fungal Degradation of Lignin

Marton et al. [48] reported that a strain of white rot fungus *Polyporus versicolor* significantly reduced the colour of the diluted pine kraft black liquor. Lignin was not sufficient as sole carbon source to support cell propagation. Cell growth required an easily metabolizable sugar and other nutrients. The intensity of cell growth and, simultaneously, the degree of decolorization were higher under aerobic conditions.

Eaton et al. [49] demonstrated that *Phanerochaete chrysosporium*, a white rot fungus can decolor the highly coloured first extraction stage stream originating in kraft bleach plant. The culture conditions favouring fungal growth were quite different from those favouring fungal decolorization. About 60% of colour reduction was achieved in 2-4 days with pH 4.5 at 39 ^{0}C and with 80% agitation. Fungal biomass could be recycled for at least 60 days. Addition of a co-substrate such as glucose or cellulose was necessary, while addition of a nitrogen source was not necessary.

Eaton et al. [50] showed that ligninolytic fungi *P.chrysosporium* can decolorize the first alkali extraction stage effluent from kraft bleach plant. BOD and COD were reduced by about 40 and 60 percent respectively. Colour reduction by this method had been scaled up using 2.5 litre rotating biological contactor. The primary sludge discharged by the pulp and paper mill was cited as co-substrate having 3.5 times more cellulose than required for complete decolorization.

Golovleva et al. [51] isolated 24 fungal cultures from various natural sources and from accumulative cultures. These cultures were capable of growing on media containing native lignin. Out of these cultures, the cultures of *Sporotrichum pulverulentum* and *Trichosporium capitatum* were having the capacity of breaking down 60-70% of lignin, within 15 days. Effect of different co-substrates as glycerol, cellulose and glucose was also studied.

Reid et al. [52] studied synthetic lignin to CO_2 by *P.chrysosporium* on a gyratory shaker. They observed that agitated cultures also degraded the lignin in aspen wood to CO_2 and water-soluble products as well as static cultures. These results were in contradiction to the results of Kirk et al. [53] which show that agitation inhibited the culture growth and heavy mycelial pellets of fungus were observed in stationary cultures.

Hakulinen [54] described the use of enzymes for the treatment of pulp and paper mill waste. He discussed that ligninase, cellulase and peroxidase are most potential enzymes, specially peroxidase, which can be used for colour removal in bleaching effluents. It is also possible to mix enzymes together with special microbes, which normally do not have high enzyme activity and remove recalcitrant and harmless compounds from waste water.

Conwell et al. [55] studied the degradation of a synthetic 14C-beta labelled guiacyl lignin by an immobilized culture of *Phanerochaete chrysosporium*. The results of degradation by immobilized cultures were similar to non-immobilized agitated cultures of *P.chrysosporium*. Immobilization of the fungus greatly facilitated periodic replacement of the extracellular medium and the porous supports were reusable after removal of the spent mycelium.

Livernoche et al. [56] studied the decolorization of combined effluent of a kraft mill with fungus *Coriolus versicolor* immobilized by entrapment in calcium alginate beads. The colour removal efficiency achieved within three days was 80% in presence of sucrose (10 mM) as an extra carbon source for fungal growth.

Archibald et al. [57] demonstrated that the white rot basidiomycete *Coriolus versicolor* decolorize the stable high molecular weight chromophores released by kraft mill bleacheries. Simple carbohydrates were shown to be essential for effective decolorization, and a medium composed of inexpensive industrial byproducts provided excellent growth and decolorization.

Bergbauver et al. [58] studied the degradation of chlorinated lignin compounds in a bleach plant effluent by the white rot fungus *Trametes versicolor*. With glucose as co-substrate, about 90% colour reduction was achieved within 3 days. Simultaneously, the concentration of chloro-organic compounds measured as adsorbable organic halogens decreased by about 45%. The residue obtained after degradation was extremely recalcitrant.

Esposito et al. [59] screened 51 ligninolytic strains of fungi to examine their ability to decolorize kraft mill bleach effluent with no additional carbon source. The selection showed that *Lentinus edodes* strain removed 73% of colour with a COD reduction efficiency of 60% in five days.

Arora and Garg [60] carried out degradation of wheat straw and pine wood saw dust by fungal cultures of *Coriolus hirsutus, Daedalea flavida, Polystictus sanguineus, Daldinia concentrica* and *Postia placenta* in an attempt to find an efficient lignin decomposer. From each substrate *Coriolus hirsutus* and *Daedalea flavida* were found to be the best decomposer in terms of total weight loss as well as lignin loss in wheat straw and pine wood saw dust respectively. On both the substrates *Polystictus sanguineus* was the best laccase producer.

2.1.5.2 Bacterial Degradation of Lignin

Woodard et al. [61] isolated three bacterial species from activated sludge which were used to treat the black liquor from a pulp mill waste water. These species were *Leptothrix ochracea, Pseudomonas multistreata* and *Flavobacterium* species. This activated sludge system was able to degrade lignin by 90% with a colour removal efficiency of 98%. The COD was reduced by 80%.

Mobius [62] biologically treated paper mill waste water with BOD/COD quotient of 0.57. It was reported that a lowering of BOD sludge loading and increase of residence time diminished the quotient for a given waste water.

Deschams et al. [63] isolated 19 strains of bacteria from enrichment cultures of decaying bark and decaying wood from pine and oak. Out of these strains, *Aeromonas* sp. was shown to grow on industrial kraft lignin as sole carbon source, degrading 98% of the lignin after 5 days of incubation.

Kawakami et al. [64-67] studied the biodegradation of kraft and alkali lignins, lignin sulfonates and milled wood lignin by *Pseudomonas ovalis*. Alkali lignin was found to be more readily degradable than kraft and lignin sulfonates. Further pine kraft lignin was decomposed more readily than bleached kraft lignin.

Crawford [68] used *Streptomyces viridosporus* for bioconversion of lignin to useful chemicals after degradation. It was observed that the grass and corn lignocellulose were degraded much more extensively than the spruce and maple lignocelluloses.

Larrea et al. [69] carried out diffused aeration experiments at 21^0C in batch reactors containing a diluted kraft black liquor in presence of an acclimatized seed of activated sludge. The experiments were performed by varying aeration intensity and filtrable COD, lignin and colour variations with time were also determined in the course of experiments. It was observed that filtrable lignin and COD decreased rapidly during the first 2 days. The magnitudes of the final reduction of both parameters were linearly related and increased with aeration intensity. Filtrable colour reductions were found to be lower than those expected on the basis of filtrable lignin reductions obtained by the polycondensation-adsorption mechanism.

Pekarovicova et al. [70] isolated eight bacterial strains from the enrichment of a sulfate pulp mill. These strains were utilizing glucoisosaccharinic acid (GISA), one of the main components of sulfate black liquor hydroxyacids, as

carbon source. The best GISA-utilizing bacterium was isolated from soil and it was identified as *Micrococcus lylae*. The growth of this strain on GISA in the pH range 5.0-9.0 was studied. Optimum growth and utilization of GISA were observed at pH 8.0. *M.lylae* also utilized D,L-lactic acid and black liquor separated from the lignin.

Bhatt et al. [71] showed that a remarkable increase in the biodegradation of pretreated gamma irradiated lignocellulosic materials, by *Flavobacterium* sp. isolated from soil, was observed. The percent increase in overall degradation of pretreated materials ranged between 137% (*Robinia* bark) and 456% (*Pinus wallichiana* wood). The needle of *Cedrus deodara* were more susceptible to the degradative action of *Flavobacterium* sp. followed by barks and wood materials.

Bharti et al. [72] investigated the role of two cyanobacteria in the removal of lignin from two pulp and paper mills waste water. It was observed that the maximum amount of lignin was removed on the 5th day and the level dropped from 93.0 mgl^{-1} to 25.0 mgl^{-1} by *Chroococcus mintus* and to 25.5 mgl^{-1} by *Phormidium ambiguum* in Mysore Paper Mill (MPM) waste water. In West Coast Paper Mill (WCPM) waste water, lignin level dropped from 11.0 mgl^{-1} to 8.8 mgl^{-1} by *C.mintus* and to 6.5 mgl^{-1} by *P. ambiguum*. At the closure of the experiment on the 30th day, the lignin level was 6.6 mgl^{-1} and 3.0 mgl^{-1} in case of *C.mintus* and 10.0 mgl^{-1} and 4.0 mgl^{-1} in case of *P.ambiguum* in MPM and WCPM waste waters respectively.

Vasudevan and Mahadevan [73] observed that *Acinetobacter* sp. utilized non-phenolic ß-0-4 model lignin compounds, 2-methoxy-4 formyl-phenoxyacetic acid and veratrylglycerol-ß-guiacyl ether as sole carbon source. vanillin, vanillic acid, protocatechuic acid and catechol were detected in 2-methoxy-4 formyl-phenoxyacetic acid amended culture. Veratryl alcohol, veratraldehyde, veratric acid,, vanillic acid, protocatechuic acid, catechol and guiacol were identified from veratryl glycerol-ß-guiacyl ether culture. *Acinetobacter* sp. produced

catechol 1,2 dioxygenase and protocatechuic 3,4 dioxygenase that cleaved catechol and protocatechuic acid respectively.

2.2 FACTORS LIMITING THE RATE OF BIODEGRADATION

For efficient treatment of black liquor and effective degradation of lignin present in black liquor by bacteria, certain factors like nutrient requirements, pH, temperature, oxygen and solid retention time are considered important and so are described in subsequent paragraphs.

2.2.1 NUTRIENT REQUIREMENTS

Nutrients are necessary to be taken into the microorganism from the environment in order to satisfy the requirements of organism for biosynthetic raw material and energy. The inorganic nutrients, necessary for growth and required in the highest concentrations, are nitrogen and phosphorus. Black liquor from a small pulp mill contains a very low amount of these nutrients. As such there is a need to supply these nutrients from outside. The required amounts of nutrients that should be added for growth can be determined from the chemical composition of the cell. Using an average cell composition $C_5H_7NO_2$, the nitrogen content required is about 14% of the dry weight of the cell, while phosphorus is about one fifth of that for nitrogen or about 3% of the dry weight of the cell [74]. If the production of solid is 1 gm per gm COD, the nitrogen and phosphorus requirements would be 0.14 and 0.03 gm per gm of COD respectively. For aerobic stabilization, a BOD:N:P ratio of 90:5:1 was reported to be optimum [75].

2.2.2 pH

Maintenance of system pH in proper range is necessary for efficient growth of microorganism in waste water. Most bacteria have pH optima near neutrality and minimum and maximum pH values for growth near 5.0 and 9.0 respectively

[74]. Many metabolic activities of microorganisms result in the formation of acidic or alkaline products. Organisms growing in an aerobic environment produce acids. It may produce CO_2 to lower the pH values significantly, if the aqueous environment is not sufficiently buffered. As such the growth of microorganisms and rate of biodegradation are affected due to lowering of pH. Accumulation of these acids should be controlled by addition of alkaline materials such as lime or sodium hydroxide.

2.2.3 TEMPERATURE

Maintenance of constant and uniform temperature is imperative for the system. Microorganisms possess no means of controlling internal temperature and the temperature within the cell is therefore determined by the external temperature. Most microorganisms are eurythermal that is, these are capable of growing in a range of 30^0C to 40^0C [74]. Growth of the bacteria are most rapid at optimum temperature. The optimum temperature for most microorganism is much closer to the maximum than to the minimum temperature.

2.2.4 OXYGEN

The availability of molecular oxygen is considered to be an important factor of lignin degradation by microbes as it is largely an oxidative process and requires oxygen for two purposes [74]. The major requirement is as a terminal electron acceptor for electron transport system necessary for the generation of energy and a very small amount of oxygen is required in certain enzymatic reactions like lignin biodegradation. Oxygen is a limiting factor for the aerobic treatment of black liquor as the dissolved oxygen (DO) of black liquor is almost nil. For effective aerobic treatment, 3-8 mgl^{-1} DO should be maintained in waste water.

2.2.5 SOLID RETENTION TIME

Retention time is a key factor for bacterial growth. Microorganism must be in sufficient quantity and concentration and must have adequate retention time for metabolism. Biologically solid retention time (SRT) is operationally defined as mass of solids contained in reactor divided by mass of solids discharged or washed from system per day [76]. SRT should be greater than regeneration time of the slowest growing bacteria. A long SRT can be achieved by keeping substrate and organisms in reactor for a longer period than the minimum regeneration time.

2.3 RECALCITRANT MATERIAL-THE LIGNIN COMPONENT

Lignin and its derivatives are the most recalcitrant materials present on the earth and becoming a major concern to ecologists and environmentalists because of the inability of microorganisms to efficiently degrade these materials [3].

2.3.1 STRUCTURE OF LIGNIN

Lignin is extremely complex, difficult to characterize, non-carbohydrate fraction of extractive free wood. It comprises 20-40% of wood by weight and does not occur alone in nature. Lignin is basically an aromatic polymer comprised of a heterogeneous, branched network system with no evidence of simple repeating unit. It is evident that the lignin sample is in the form of spherical particles of varying size. The molecular weight of isolated lignins are in the range of 1000 to 12000, depending on the extent of chemical degradation or condensation during isolation.

The structural back bone of lignin is composed of phenylpropanoid units linked together in many ways [77]. (A) arylglycerol-ß-aryl ethers account for half the linkages (B) diphenyl; and (C) phenyl coumarin, each accounting for about 10% of the linkages. Each type of linkage is shown in Fig.2.1. However there is no evidence of the pattern in the sequence of linkages.

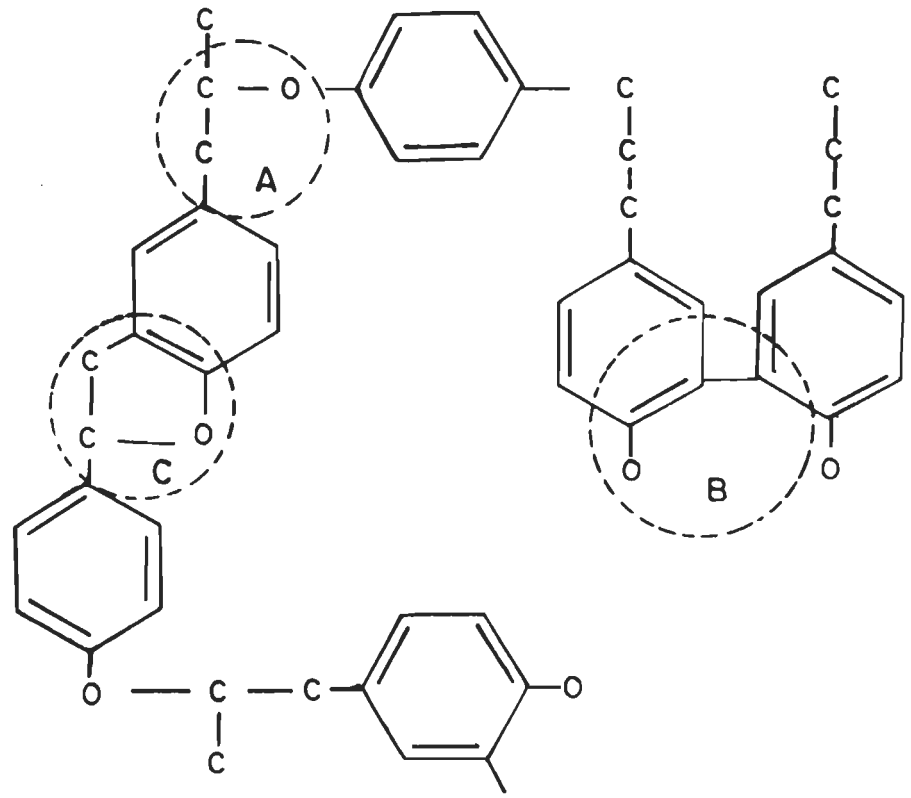
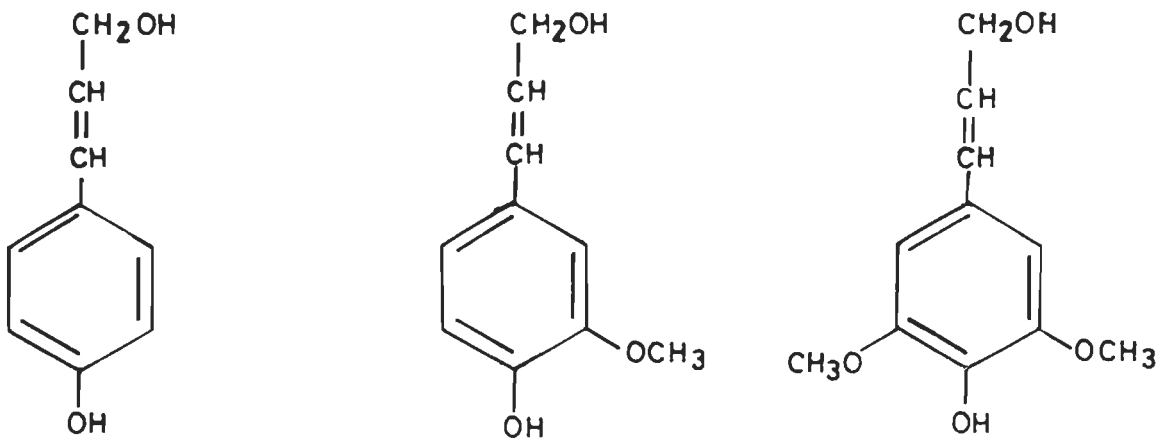


FIG. 2.1 A SMALL FRAGMENT OF LIGNIN TO ILLUSTRATE THE THREE MOST COMMON TYPES OF LINKAGES
(A) Arylglycerol-β-aryl ether (B) Diphenyl (C)
(C) Phenyl coumarin



(A) P-Coumary alcohol (B) Coniferyl alcohol (C) Sinapyl alcohol

FIG. 2.2 STRUCTURES OF THREE PRECURSORS OF LIGNIN SYNTHESIS

Lignin is a natural polymeric product arising from enzyme initiated dehydrogenative polymerization of three primary Precursors viz. (i) p-coumaryl (ii) coniferyl; and (iii) sinapyl alcohol. Structure of these compounds are shown in Fig. 2.2.

Gymnosperms lignin is formed from coniferyl alcohol, while the angiosperms lignin is a mixture of coniferyl and sinapyl alcohols and the grass lignin is composed of a mixture of coniferyl, sinapyl and coumaryl alcohols.

2.4 MECHANISM OF LIGNIN DEGRADATION

Lignin breakdown involves multiple biochemical reactions that take place more or less simultaneously viz. cleavage of intermonomeric linkages, demethylations, hydroxylations, side chain modifications and aromatic ring fission followed by dissimilation of the aliphatic produced. Insolubility of lignin and lack of its stereoregularity contribute to making it a substrate that is difficult for the microflora to degrade [78].

There have been two prominent theories as to how microorganisms degrade the complex aromatic macro-molecular substance, lignin. These hypotheses centre on either the mediation of lignin decay by classical microbial enzymes [79] or by active oxygen species [80] such as hydroxyl radical, superoxide anion singlet oxygen and hydrogen peroxide.

The white rot fungus *Phanerochaete chrysosporium* contains a family of extracellular enzymes collectively called as ligninases, which are peroxidases and act through a mechanism involving free radical formation [81]. *P.chrysosporium* have at least two peroxidases which are haem containing and are responsible for the cleavage of most, if not all, of the inter phenol linkages in lignin [77].

Bacteria of several genera, including *Pseudomonas, Alcaligens, Arthrobacter, Nocardia* and *Streptomyces*, readily degrade single ring aromatic compounds that build up the lignin macromolecules [78]. However, the extent to which bacteria are able to bring about the decay of the lignin polymer itself has not been assessed.

The ability, of bacteria to grow on low molecular weight lignin oligomers as the sole source of carbon and energy, indicates that bacteria produces enzymes catalysing cleavage of intermonomeric linkages. The involvement of specific enzymes in lignin degradation by *Streptomyces viridosporus* has been confirmed. Evidence has been presented to show that *S. viridosporus* produces a ligninolytic enzyme complex involved in demethylation of lignin aromatic rings and in the oxidation of lignin side chains and cleavage of ß-ether linkages within the polymer [82]. The combined activities of these enzymes generate water soluble polymeric modified lignin fragments, which are then slowly degraded further by *S. viridosporus*. The ß-ether cleaving enzyme complex is probably membrane associated, but it is not extracellular.

CHAPTER -3

OPTIMIZATION OF CULTURE PARAMETERS

3.1 GENERAL

The toxic waste produced by pulp and paper mills contains large amount of modified lignin and its derivatives. The rivers and streams, receiving pulp and paper mill waste, are reported to lose their original colour and acquire a blackish or coffee colour with considerable foaming on the surface. However fungi belonging to basidiomycetes, ascomycetes and a few species of bacteria are have been claimed to show degradation activity towards lignin [83]. Biodegradation of lignin, present in black liquor, is influenced by physical, chemical and biological factors. As such optimization of growth conditions of bacterial strains employed for the biodegradation of black liquor is quite essential. This chapter presents the characteristics, optimization of conditions and growth pattern of bacterial strains selected for investigations.

3.2 CHARACTERISTICS OF BACTERIAL STRAINS

Three bacterial strains viz. *Pseudomonas putida, Aeromonas formicans* and *Acinetobacter calcoaceticus* were chosen for the degradation of lignin present in black liquor. These strains were obtained from the National Collection of Industrial Micro-organisms, National Chemical Laboratory, Pune-411008, India. These were checked for their purity according to Bergey's manual of systematic bacteriology [84]. The major characteristics of the above mentioned bacterial strains are as under :

***Pseudomonas putida* (NCIM 2174, DSM 549)**

These are gram negative, straight or slightly curved rods, but not helical, 0.5-1.0μm in diameter by 1.5-5.0μm in length, motile by polar flagella and chemoautotrophs. These are aerobic having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor and are unable to grow under acidic conditions (pH below 5.0). Optimum temperature required for growth is 25-34^{0}C and these can be isolated from soil and water after enrichment in mineral media with various carbon sources like phenol, butanol and cresol.

***Aeromonas formicans* (NCIM 2319, CDC-RH-1)**

These are gram negative, straight, rod shaped with rounded ends to coccoid, 0.3-1.0μm in diameter by 1.0-3.5μm in length, motile by a single polar flagellum and chemoautotrophs using a variety of sugar and organic acids as carbon sources. These are facultative anaerobes and metabolize glucose in both respiration and fermentation. These are oxidase and catalase positive and show growth in the optimum pH range of 5.8-8.0 at 22-28^{0}C. These can be isolated from fresh water and sewage.

***Acinetobacter calcoaceticus* (NCIM 2886, DSM 590, ATCC 11171)**

These are gram negative, rod shaped, 0.9-1.6μm in diameter, 1.5-2.5μm in length, non motile, with polar fimbrae present and are chemoautotrophic. These are aerobic having a strictly respiratory type of metabolism with oxygen as terminal electron acceptor. These require slightly acidic pH (4.8-6.3) for growth and grow in temperature range of 20-30^{0}C with optimum temperature range of 33-35^{0}C. These are used to breakdown the aromatic rings and can metabolize phenol as a carbon source. These can be isolated from soil and water in enrichment media after vigorous shaking at 30^{0}C.

3.3 GROWTH OPTIMIZATION IN BASAL MEDIUM

The growth patterns of all selected strains were observed by growing these in basal medium and then some physico-chemical studies like pH and aeration in the same medium were conducted to optimize the growth parameters. Bacterial enumeration was done by serial dilution method [85]. The composition of basal medium was as follows:

Basal Medium

$(NH_4)_2\ SO_4$	0.26%
K_2HPO_4	0.10%
KH_2PO_4	0.01%
$MgSO_4$	0.02%
$CaCl_2$	0.001%
$FeSO_4$	0.0001%
Yeast extract	0.01%
Glucose	0.20%

pH was adjusted to 7.2 with phosphate buffer before autoclaving the medium.

3.3.1 EXPERIMENTAL METHODOLOGY

3.3.1.1 Growth and Purification of Bacterial Strains

Bacterial strains were developed on nutrient agar plates by streak plate method [85] at 32^0C. Purification was performed on the same medium plates by single colony isolation method. These colonies were picked up with the help of inoculation loop and used for further growth. Composition of nutrient agar was as under :

Nutrient Agar (per litre)

Medium was prepared as per Standard Methods [85].

Beef extract	3.0 gm
Peptone	5.0 gm
Agar	15.0 gm

pH was adjusted to 7.2-7.4 before autoclaving the medium.

3.3.1.2 Maintenance of Bacterial Strains

All bacterial strains were maintained on the slants of nutrient agar at 4-8^{0}C and transferred periodically.

3.3.1.3 Growth Patterns of Bacterial Strains

The growth patterns of bacteria were studied by growing these in 150 ml of basal medium taken in 500 ml of erlenmeyer flasks. These flasks were kept at 32 ^{0}C in a rotary shaker and bacterial enumeration was done by serial dilution of these neat cultures to obtain single colonies of bacteria [85]. The serial dilutions were done in the tubes containing 0.9 ml of saline (0.9% w/v).

0.1 ml of neat culture was added to the first tube and shaken well to get 10^{-1} dilution. 0.1 ml of this diluted culture was added to the next tube and shaken well to get 10^{-2} dilution and the process was continued to make further dilutions till the desired dilution was attained.

0.05 ml of this diluted culture was used to plate on the nutrient agar plates and these plates were incubated at 32^{0}C. After 48 hours, the colonies were counted and the average of each set of three readings was taken.

3.3.1.4 Physico-Chemical Study

Effect of physico-chemical parameters such as pH and aeration in basal medium on the growth of all the three bacterial strains was investigated. Growth

was measured by bacterial enumeration method (7 days growth). The period was so chosen as to have microbes in log phase. The effect of the variable parameter was observed by keeping other parameters constant.

3.3.2 RESULTS AND DISCUSSION

The growth pattern of the three bacterial strains are shown in Fig. 3.1. The curves indicate that log phase is well defined for all strains and it extends up to the 10th day and then rounds off to a stationary phase. Although within the framework of the experimental protocol, a distinct stationary phase was not deciphered except for *A. formicans*.

The growth pattern showed increase in number of bacterial cells as a function of time. Maximum growth was observed on the 16th day of incubation except in the case of *P. putida*, where the same is recorded on the 14th day.

Experimental data corresponding to Fig.3.1 has been utilized for the analysis of growth pattern of all the bacterial strains, in order to obtain the generation time and a mathematical expression for log phase of growth.

(i) Generation time

It is defined as the time required for the population to become double. The generation time 'g' can be determined from the number of generations 'n' that occur in a particular time interval 't'. It can be expressed by the following formula :

$$g \frac{t}{n} = \frac{t}{3.3\ (\log_{10} N - \log_{10} N_0)}$$

where,

N_0 = Initial number of bacterial cells

N = Number of bacterial cells after time interval 't'

The generation time varies between 0.67-0.98 days for various bacterial

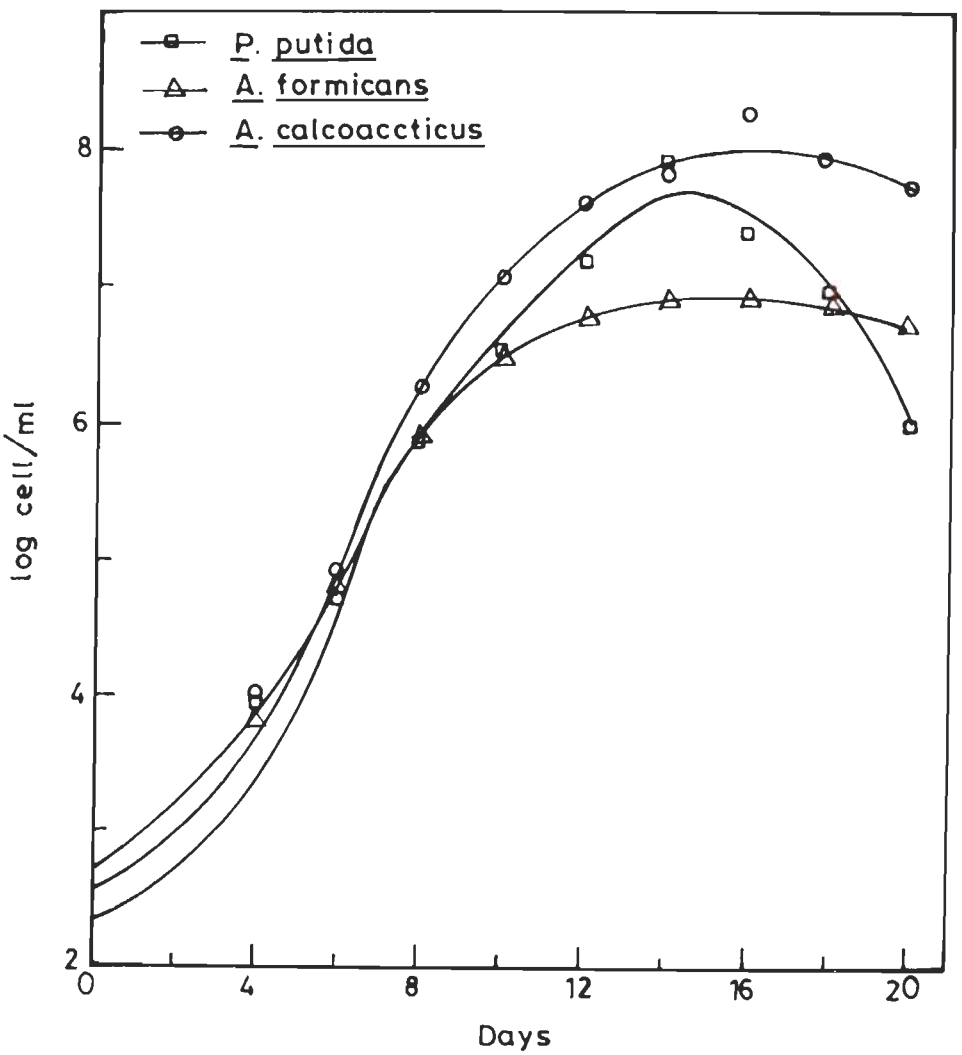


FIG. 3.1 GROWTH CURVES OF BACTERIA IN BASAL MEDIUM

strains as tabulated below:

Bacterial Strains	Generation Time
P. putida	0.77 days
A. formicans	0.98 days
A. calcoaceticus	0.67 days

From the above it is clear that the generation time of *A. calcoaceticus* is less than *P. putida* which is less than *A. formicans*.

(ii) The following mathematical expression for growth curves has been tried for the analysis of data of log phase :

$$Y_{log} = b\ t_{days} + C$$

where,

Y_{log} = log bacterial cells per ml at time t

b = Slope of line i.e. growth rate

t_{days} = Time in days

C = Constant or intercept on y- axis

The results of the above correlation and regression analysis are presented in Table 3.1.

Table - 3.1

LINEAR REGRESSION OF GROWTH CURVES

Bacterial Strains	Regression Line	Coefficient of Correlation (r)
P. putida	$Y_{log} = 0.41\ t_{daya} + 2.38$	0.992
A. formicans	$Y_{log} = 0.37\ t_{daya} + 2.59$	0.973
A. calcoaceticus	$Y_{log} = 0.46\ t_{daya} + 2.28$	0.991

It is apparent that the growth rate of *A. calcoaceticus* is fastest and that of *A. formicans* is slowest, while *P. putida* falls in between the two.

Effect of varying pH on the yield of bacterial cells is shown in Fig.3.2. It is evident that *P. putida* and *A. formicans* have maximum cell yield around neutral pH, while *A. calcoaceticus* prefers to grow in slightly acidic environment. Accordingly, pH is an important environmental factor in controlling the growth rate of microbes. The optimum pH range i.e. the pH at which maximum number of bacterial cells are observed is tabulated as under :

OPTIMUM pH RANGE FOR BACTRIAL STRAINS

Bacterial Strains	Optimum pH Range
P. putida	7.0-8.0
A. formicans	7.0-8.0
A. calcoaceticus	5.0-6.0

Effect of agitation on the bacterial cell yield was also observed and the results are reported in Table 3.2. It is observed that the effect of agitation, although not very much significant, is quite perceptible. *A. formicans*, a facultative anaerobe is not much affected by agitation as it can grow with or without oxygen. The other two bacterial strains are strict aerobes and give better yield only in presence of oxygen. The increase in cell yield, due to agitation, is probably due to the reason that oxygen is required by the bacteria in some metabolic processes necessary for the generation of energy.

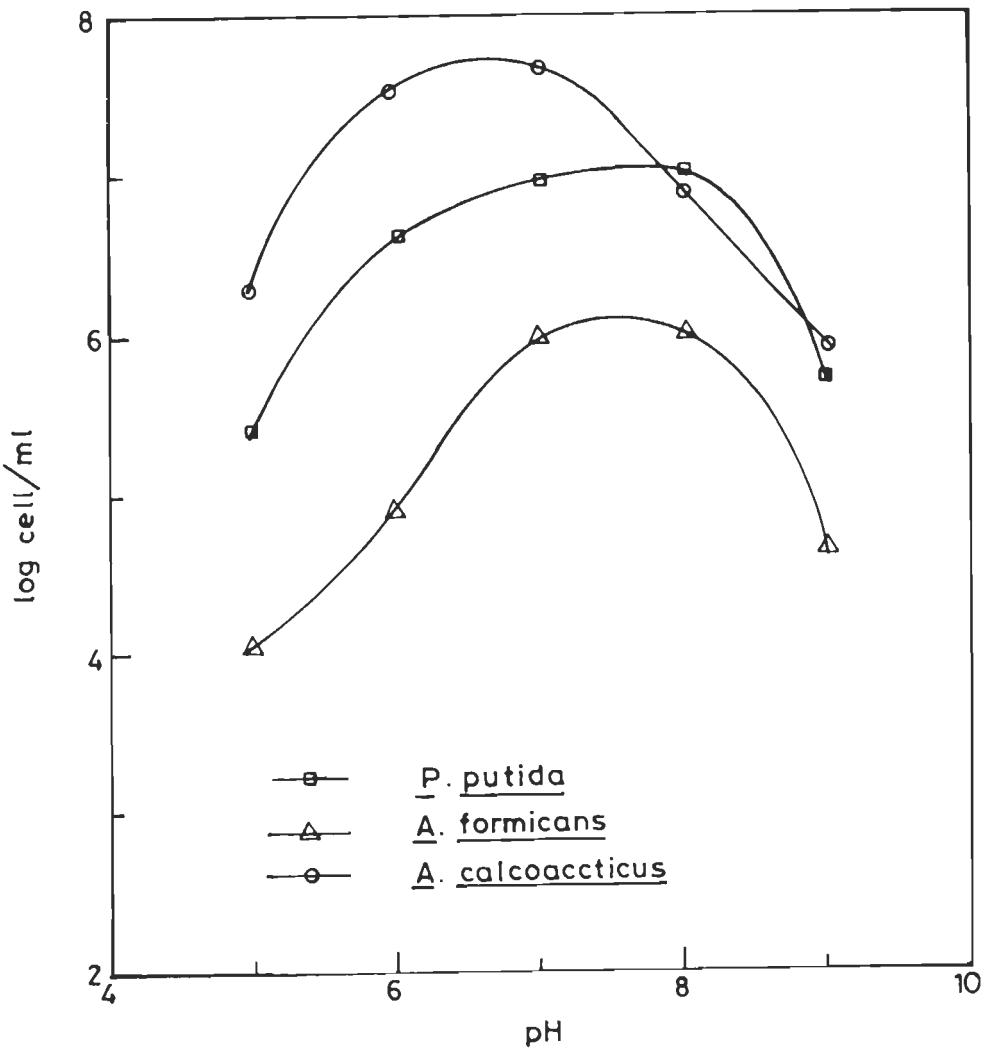


FIG. 3.2 EFFECT OF pH ON BACTERIAL GROWTH IN BASAL MEDIUM

Table - 3.2

EFFECT OF AERATION ON THE CELL YIELD OF BACTERIA

Bacterial Strains	On Agitation (cells per ml)	Stationary (cells per ml)
P. putida	1.81×10^6	4.63×10^5
A. formicans	9.76×10^5	9.18×10^5
A. calcoaceticus	2.12×10^7	7.94×10^6

3.4 GROWTH OPTIMIZATION IN BLACK LIQUOR

For the growth of bacterial strains in black liquor, parameter optimization was attempted to have better efficiency for the degradation of lignin and removal of COD and colour. The details of sampling and optimization are presented below :

3.4.1 EXPERIMENTAL METHODOLOGY

3.4.1.1 Sampling

The black liquor was collected from the effluent channels (at first extraction stage) of an agricultural residue based small paper mill employing soda process. The mill is located in Himachal Pradesh, India. Samples were collected in a clean ten litre polyethylene can by grab technique. The collected samples were transported to laboratory in an ice box, stored at 4 ^{0}C and were used within three months.

3.4.1.2 Effluent Characteristics

The dark coloured and turbid effluent was filtered through 0.45µm membrane filter and analyzed for various parameters as per Standard

Methods [86]. Sterilized black liquor was used throughout this study. The main characteristics of black liquor are given in Table 3.3.

3.4.1.3 Preparation of Inocula

Initially bacterial strains were grown in 500ml flasks containing 150ml of basal medium. Medium were inoculated with one specific bacterial strain to be studied and incubated on a rotary shaker at 30^0C for 72 hours. The optimum pH of basal medium for each strain was adjusted with phosphate buffer. The bacteria were collected by centrifugation on a R 24 REMI research centrifuge at 2000 rpm for 15 minutes, washed with sterile water and resuspended in 150ml of sterile water to give a cell suspension of 10^2 -10^4 cells per ml. In all experiments, the above homogenates arrived in this manner served as the inoculum for the degradation of black liquor.

3.4.1.4 Determination of Colour Units

The pH of the sample was measured and adjusted to 7.6 with 2M NaOH. Colour units were determined as cobalt-platinum (Co-Pt) units on spectronic-100 (Bausch and Lomb) at a wavelength of 465nm as given in Standard Methods [86]. According to Eaton et al. [50], one colour unit is the amount of coloured material present in 1.0ml giving an absorbance of 1.0 at 465nm and pH 7.6 by a path length of 1.0cm.

3.4.1.5 Determination of COD

The chemical oxygen demand (COD) was determined by the well known dichromate method, described in Standard Methods [86].

3.4.1.6 Determination of Lignin

To calculate the amount of lignins present in black liquor, lignins were precipitated using hydrochloric acid [87] and coagulated by warming. It was

Table - 3.3

AVERAGE CHARACTERISTICS OF BLACK LIQUOR

Parameters	Values
pH	11.3
Total solids	17465.0
Dissolved solids	15360.0
Suspended solids	2105.0
COD	10578.0
Colour (Co- Pt Units)	15300.0
BOD at 20^0C	4906.0
Lignin	4360.0
Sodium	650.0
Magnesium	1600.0
Calcium	1800.0
Total nitrogen	6.3
Phosphorus	1.2
Glucose	Nil

(All values are in mgl^{-1} except pH)

allowed to stand overnight, filtered and washed to make it free from acids and dried at 55^{0}C. The dried lignin (5 gm) was dissolved in pyridine: acetic acid : water solution (9:1:4) with stirring. The solution, after complete dissolution of lignin, was extracted with chloroform (250 ml) and the organic extract was reduced to about 100ml under reduced pressure and poured slowly with stirring into 1.25 litre of anhydrous diethyl ether. The ether insoluble lignin was filtered and again washed three times with 250ml of ether and separated to form an aqueous suspension and dried.

A standard solution of the above purified lignin was prepared in 1 M NaOH and used to determine extinction coefficient [88,89]. Thereafter, estimation of lignin from the sample was done with the help of the following expression :

$$B = \frac{A}{19.4}.D$$

where,

A = Absorbance at 280 nm

B = Lignin content in gml^{-1}

D = Dilution factor (if the absorbance of the sample is higher than 0.7, the sample is diluted) expressed as V_D/V_0, where V_D is the volume of the diluted sample and V_0 the volume of the original sample taken and 19.4 lgm^{-1} cm^{-1} is the extinction coefficient or absorptivity calculated from standard solution of lignin.

3.4.1.7 Physico-Chemical Study

Preliminary studies on the degradation of lignin reveal that the addition of extra carbon, nitrogen and phosphorus source is necessary and if one of these is lacking, the efficiency of removal of COD, colour and lignin is reduced due to subdued growth of bacterial strains. Although a weak decolorization is observed even without adding a co-substrate (glucose) and this may be due to endogenous storage material derived from preculture in nutrient media [63]. As

such various physico-chemical parameters e.g. the concentration of black liquor, pH and the amount of ammonium chloride, glucose and phosphate (as nitrogen, extra carbon and phosphorus source respectively) necessary for the optimal growth of bacterial strains in black liquor were optimized. Black liquor treatment was monitored by observing the percent removal efficiency of COD, colour and lignin.

For the optimization of above parameters for each bacterial strain 10ml portions of cell suspension, having 10^2-10^4 cells per ml, were used to inoculate 100 ml of sterile black liquor supplemented with glucose, nitrogen and phosphorus, taken in 250ml of erlenmeyer flasks. These flasks were kept in a rotary shaker at 30^0C. Each experiment was done in triplicate. The effect of the variable parameter was observed by keeping other parameters constant and a series of blanks were also run in each experiment with and without seeding and nutrients. After five days of incubation, the black liquor was membrane filtered (0.45 μm) and analyzed for COD, colour and lignin.

3.4.2 RESULTS AND DISCUSSION

Initial assessment of biodegradability of black liquor was accomplished in the laboratory. However, a substance that was degraded in laboratory may be recalcitrant in the natural environment because of physical factors such as oxygen, temperature, pH or other nutritional factors. The effect of various physico-chemical factors on the efficiency of the removal of COD, colour and lignin from black liquor, as obtained in batch studies, are described below :

3.4.2.1 Effect of Black Liquor Concentration

The effect of the concentration of black liquor diluted with sterile tap water on removal efficiency of COD, colour and lignin for each strain is shown in Figs. 3.3, 3.4 and 3.5. It is apparent that the removal efficiency of various parameters

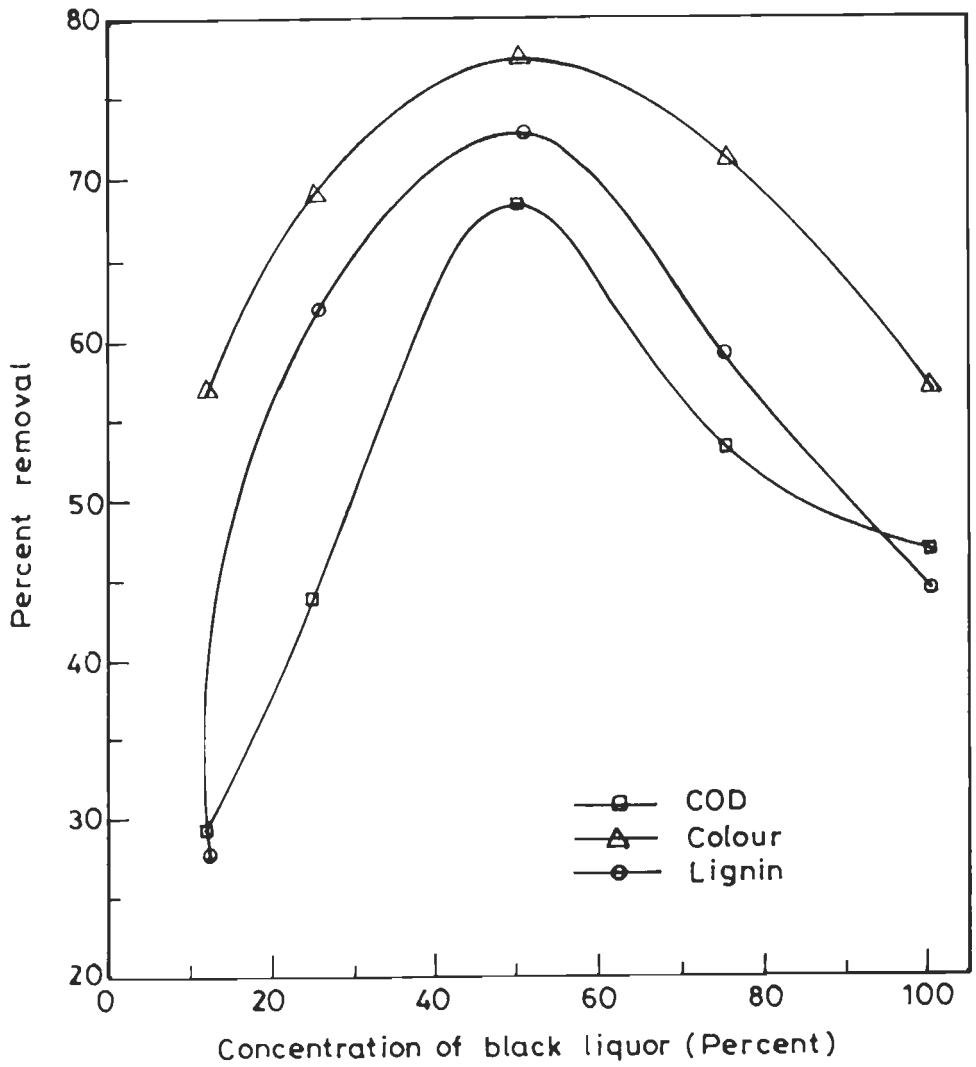


FIG. 3.3 EFFECT OF BLACK LIQUOR CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN BY P. putida

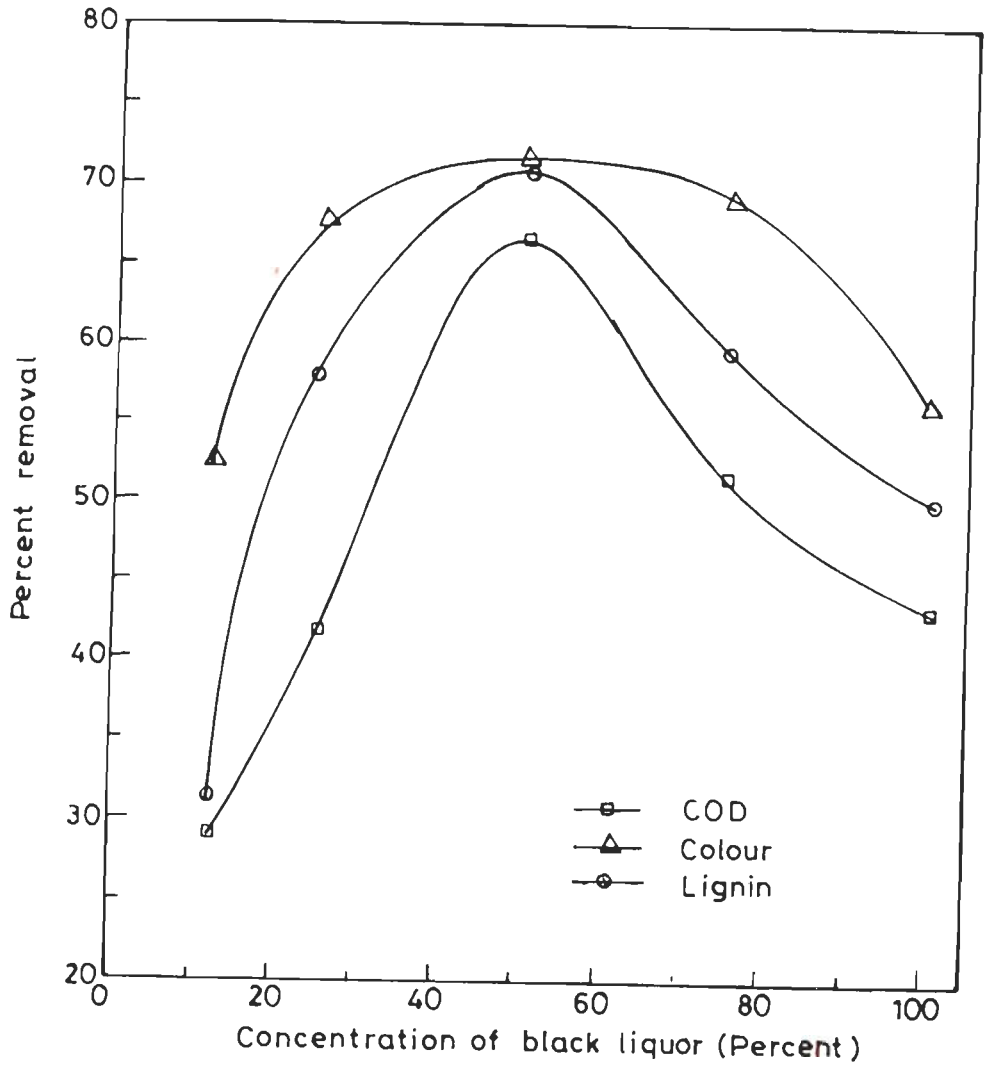


FIG. 3.4 EFFECT OF BLACK LIQUOR CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN BY A. formicans

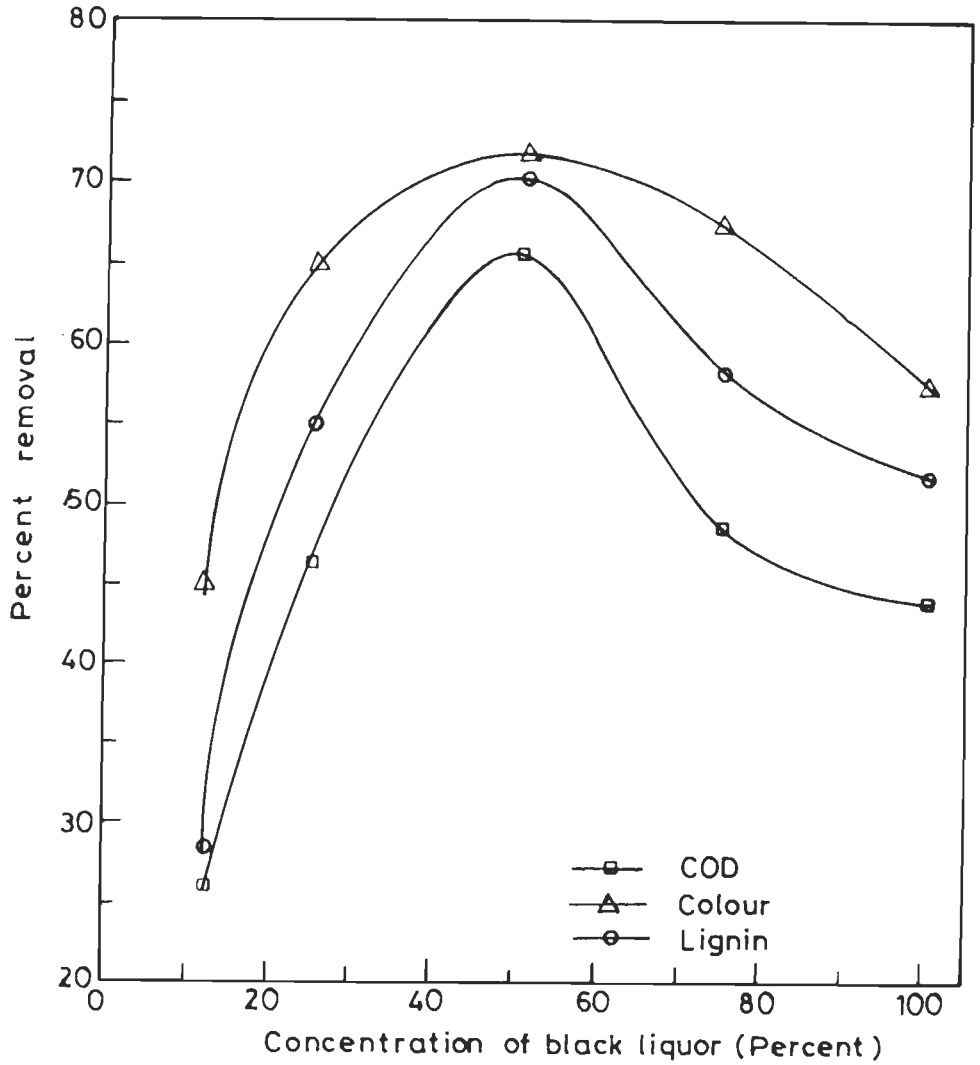


FIG. 3. 5 EFFECT OF BLACK LIQUOR CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN BY A. calcoaceticus

increases with increasing concentration of black liquor (12.5 to 50%) and it is maximum at 50% concentration. At higher concentrations (75% and 100%) a fall in the efficiency of removal is observed. As such it is concluded that a higher concentration of black liquor is inhibitory to the growth of all the strains of bacteria due to its toxic effects and 12.5-50% of black liquor is promotory for all bacterial strains. Hence 50% of black liquor was taken as optimum concentration for subsequent studies.

3.4.2.2 Effect of pH

Batch study data on the efficiency of removal of COD, colour and lignin with varying pH (4.0 to 9.0) is presented in Figs. 3.6, 3.7 and 3.8 for *P. putida*, *A. formicans* and *A. calcoaceticus* respectively.

Figs. 3.6 and 3.7 indicate a marginal increase in the removal of COD, colour and lignin between pH 4.0 to 7.0 and a marked increase is observed at pH 8.0. On further increasing the pH a marked decrease in all the parameters is observed. Thus a pH of 8.0 is the optimum working hydrogen ion concentration for the process under investigation. In case of *A. calcoaceticus* (Fig. 3.8) the optimum functional pH is 6.0. Beyond this pH value, a decrease in the efficiency of removal is observed with these microbes.

These observations lead to the following conclusions :

(i) All the strains show maximum efficiency between pH 7.0 and 8.0 except *A. calcoaceticus* which functions best in the range of pH 5.0 to 6.0. These results are in accordance with Bergey's manual of systematic bacteriology [84].

(ii) Optimum pH in black liquor for the growth of all strains is same as observed in basal medium (Fig. 3.2). It is assumed that the bacterial activity is maximum at optimum pH due to the release of some enzymes of lignin degrading systems from bacterial cells and these start degrading

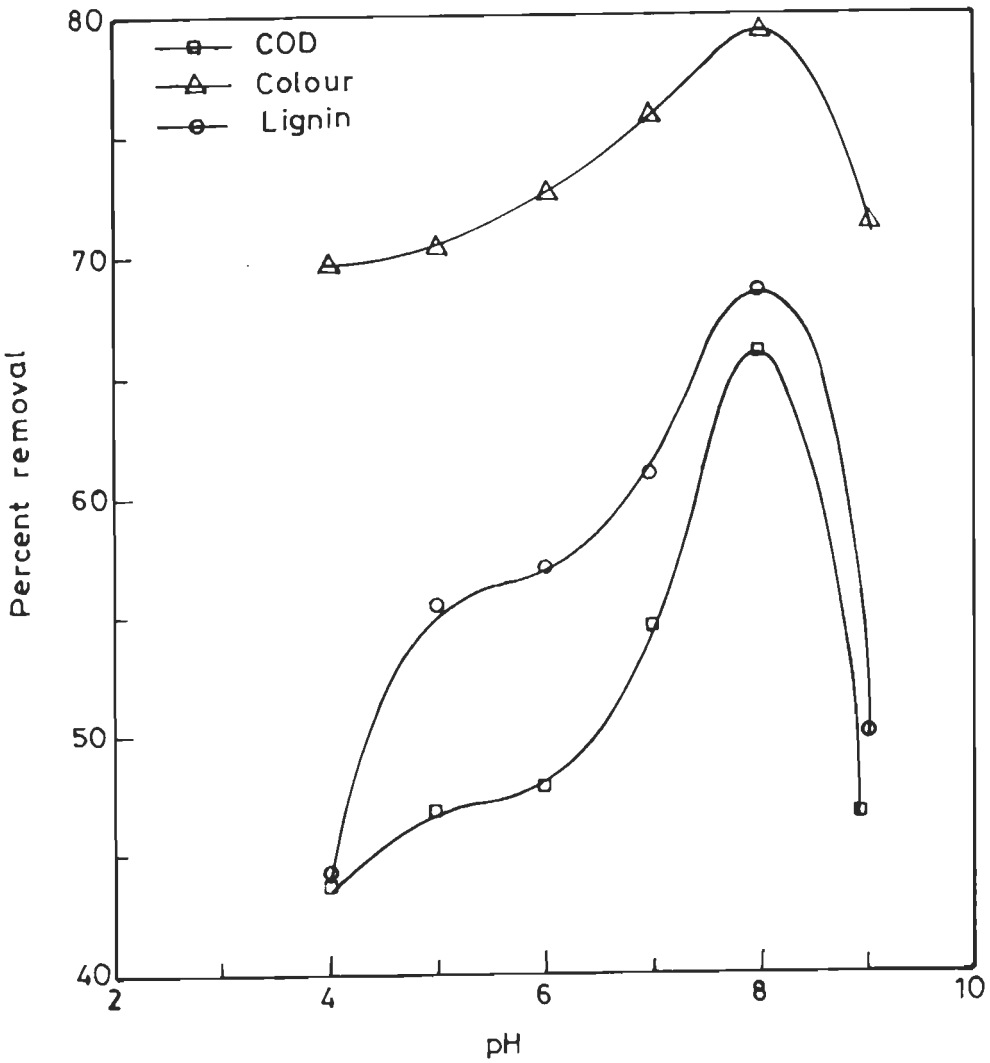


FIG. 3.6 EFFECT OF pH ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY *P. putida*

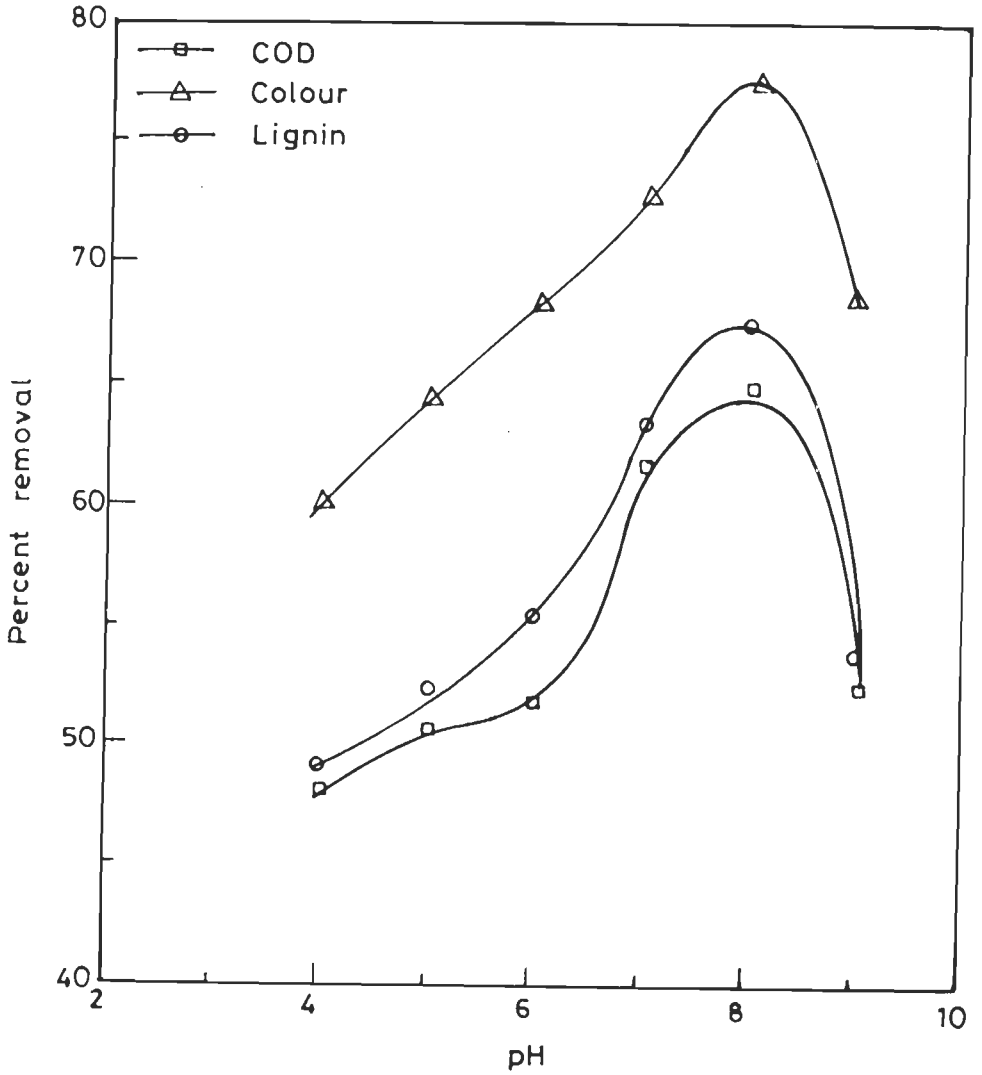


FIG. 3.7 EFFECT OF pH ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. formicans

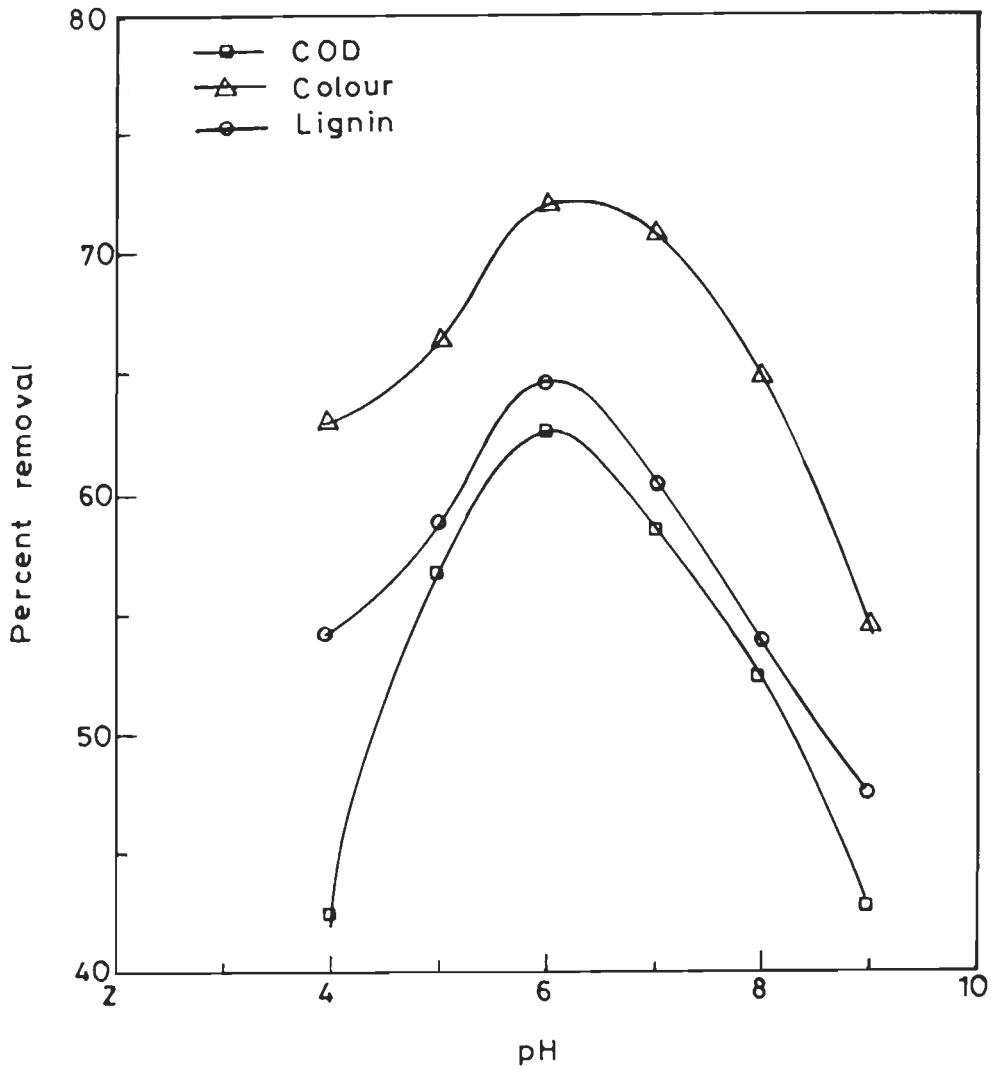


FIG. 3.8 EFFECT OF pH ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. calcoaceticus

lignin into colourless aromatic compounds or low molecular weight lignin compounds.

(iii) Bacterial activity is minimum in the range of pH 4.0 to 7.0 except in case of *A.calcoaceticus* and the observed removal of COD, colour and lignin can be attributed to some chemical reactions presumably due to acidic precipitation of lignin accompained by slower bacterial activity at lower pH values.

This fact was verified by keeping a series of blanks having uninoculated black liquor at different pH (4.0-9.0), containing same constant nutrients as in inoculated black liquor. The removal of colour, COD and lignin was found to decrease after five days when pH varies from 4.0 to 6.0 and very little change was observed at pH 7.0 No change in COD, colour and lignin removal efficiencies was observed at higher pH. It shows that, at lower pH, the precipitation of lignin takes place and this is responsible for the removal of colour and COD in black liquor.

A marked decrease in the efficiency of removal at pH higher than 8.0 is due to decay and/or slower activity of bacterial cells in highly alkaline medium.

3.4.2.3 Effect of Glucose Concentration

Since carbon is an essential element in all organic compounds and comprises 50% of the dry weight of the cell, the presence of an utilizable carbon source is a major determining factor in the ability of any microbial species to flourish in a given habitat. Some prototrophs, such as some species of the genus *Pseudomonas*, are capable of utilizing any one of approximately 100 different type of organic molecules as sole carbon soruce, whereas others are quite restricted in their carbon source utilization [74].

The effect of increasing glucose concentration as an extra carbon source (0.2-1.0% w/v) on the efficiency of removal of colour, COD and lignin are presented in Figs 3.9, 3.10 and 3.11.

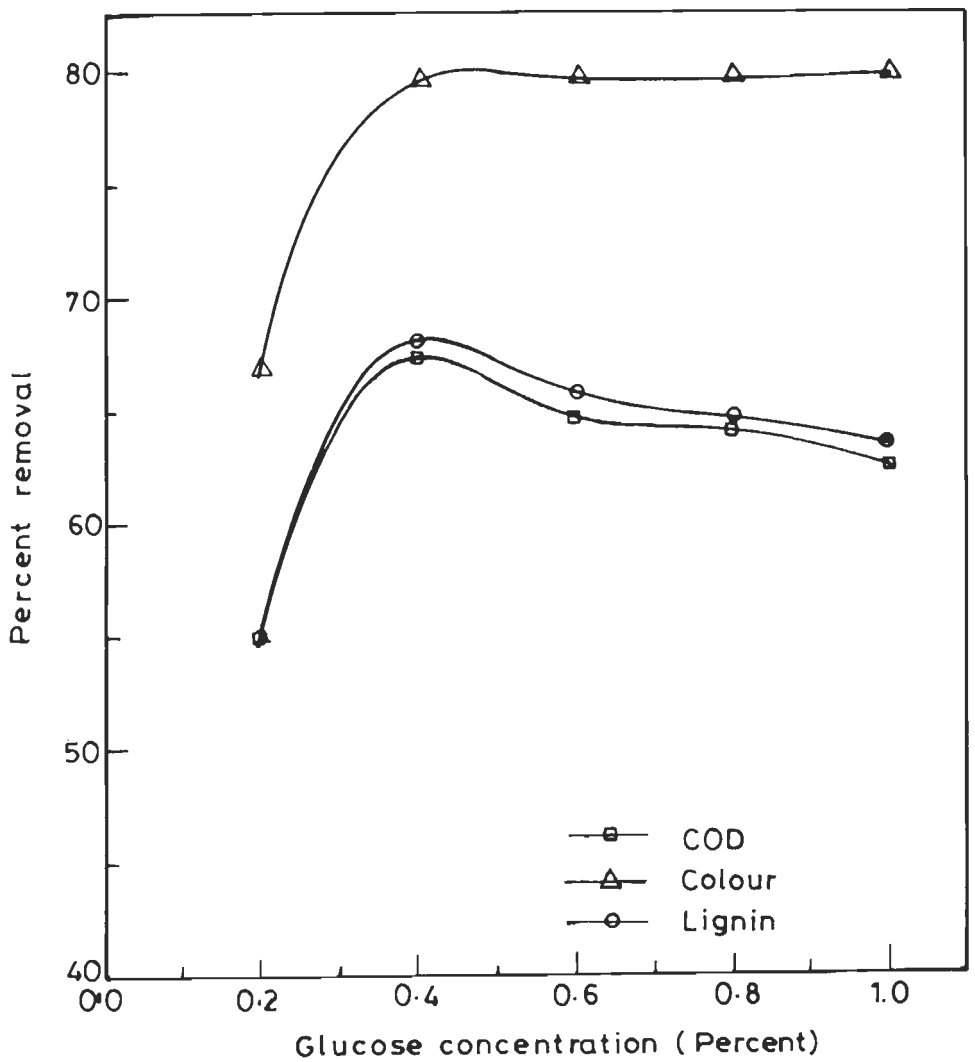


FIG. 3.9 EFFECT OF GLUCOSE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY *P. putida*

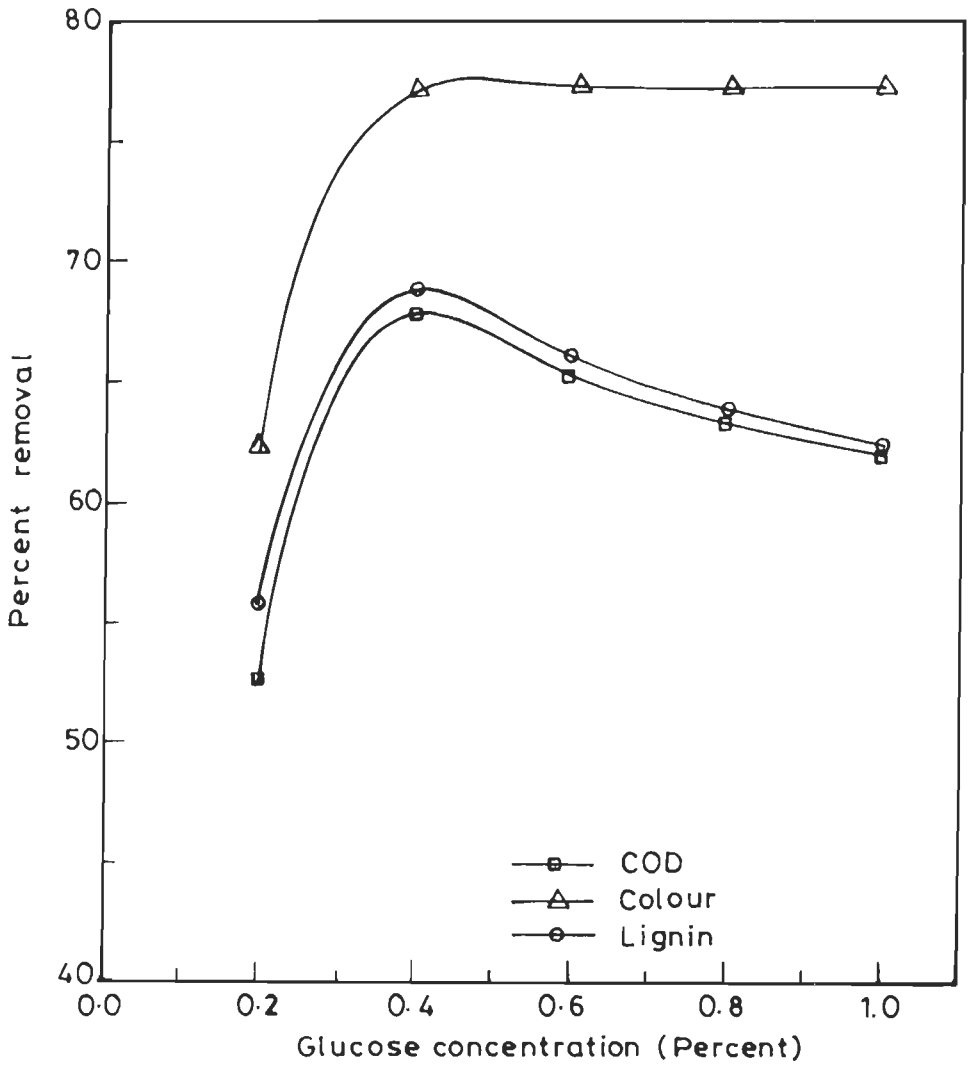


FIG. 3.10 EFFECT OF GLUCOSE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. formicans

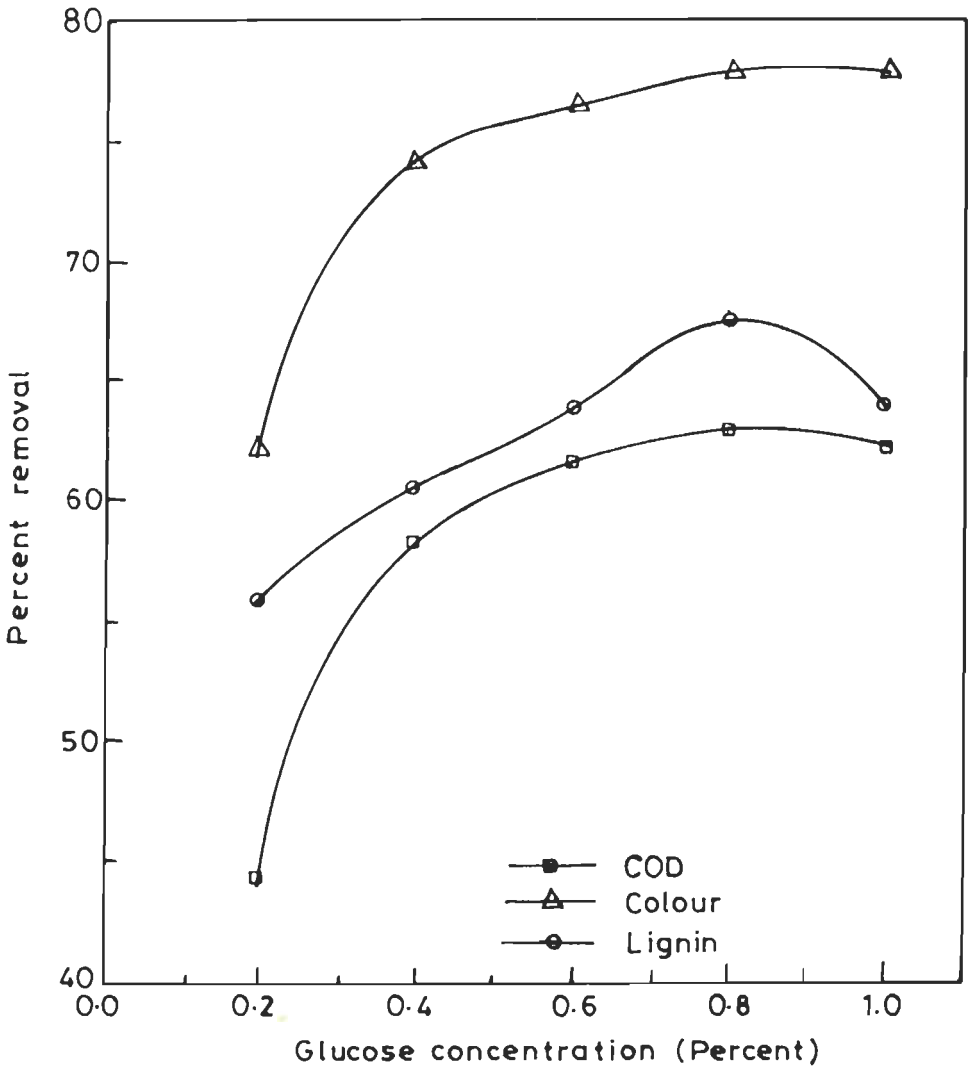


FIG. 3.11 EFFECT OF GLUCOSE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. calcoaceticus

A perusal of Figs. 3.9 and 3.10 reveals that COD, colour and lignin removal is maximum at 0.4% of glucose concentration. On further increasing the glucose concentration (0.6% to 1.0%) the percentage of colour removed remains fairly constant, but a slight fall in lignin degradation as well as COD removal efficiency is observed. As such 0.4% of glucose can be considered as optimum extra carbon source for the growth of *P.putida* and *A.formicans* in black liquor.

Fig. 3.11 indicates that the efficiency of strain *A. calcoaceticus* is maximum at 0.8% of glucose concentration and beyond this the colour removal efficiency remains constant, but a fall is observed in the removal of COD and lignin. Consequently, 0.8% of glucose concentration is the optimum value for the degradation of black liquor by *A.calcoaceticus*.

A fall in COD removal efficiency at higher glucose concentrations, may probably be attributed to undegraded lignin which is not utilized by the bacteria. This is also corroborated to a slight decrease in the efficiency of lignin removal observed at higher glucose concentrations.

The requirement of a growth substrate (glucose) is surprising as lignin potentially is one of the most abundant source of carbon and energy for microbes. Although the basis of this requirement is not known, but the following speculations are suggested :

(i) The energy recovered in the metabolism of lignin degradation is simply too little to support the growth of bacteria.

(ii) The level of the ligninolytic activity is too low to support growth.

3.4.2.4 Effect of NH_4Cl Concentration

Nitrogen constitutes about 14% of the dry weight of microbial cell and hence this element is required for growth as a macro-nutrient. In growth medium nitrogen may be supplemented in organic or inorganic form. The most common

inorganic forms are NH_3, NO_2^-, NO_3^- and N_2, out of which ammonia (NH_3) has been found to be most readily utilized.

To elucidate the effect of additional nitrogen, (total nitrogen in filtered black liquor was about 0.3mg per 100ml), varying concentration of ammonium chloride (30-150 mg per 100ml) were added as a source of nitrogen and its effect on removal efficiency of COD, colour and lignin were observed. The result of these experimental studies are depicted in Figs. 3.12, 3.13 and 3.14 for *P. putida*, *A. formicans* and *A. calcoaceticus* respectively.

It is apparent (Figs. 3.12, 3.13 and 3.14) that in each strain the COD and lignin removal efficiency increases with increasing NH_4Cl concentration and a maxima is observed at 120mg per 100ml concentration of NH_4Cl. Any further increase in NH_4Cl (150mg per 100ml), however, casts a marked decrease in the removal efficiency of COD as well as lignin. Thus 120mg per 100ml of NH_4Cl concentration can be considered as the optimum concentration of nitrogen for the process.

The decrease in the efficiency of removal of COD and lignin, at higher NH_4Cl concentration, clearly indicates its adverse effect on the growth of microorganisms in black liquor and inhibition to lignin degradation as well as COD removal. Reasons of this adverse effect of high nitrogen concentrations on lignin metabolism is not known but three possibilities have been suggested by Kirk et al. [53] :

(i) High nitrogen promotes rapid depletion of the growth substrate known to be necessary for lignin metabolism.

(ii) Nitrogen metabolism competes with lignin metabolism through requirements for the same cofactor(s).

(iii) Nitrogen regulates the synthesis of one or more components of lignin degrading system. Bu'lock has observed [90] that bikaverin synthetase system in *Giberella fujikuroi* is not synthesized until nutrient nitrogen

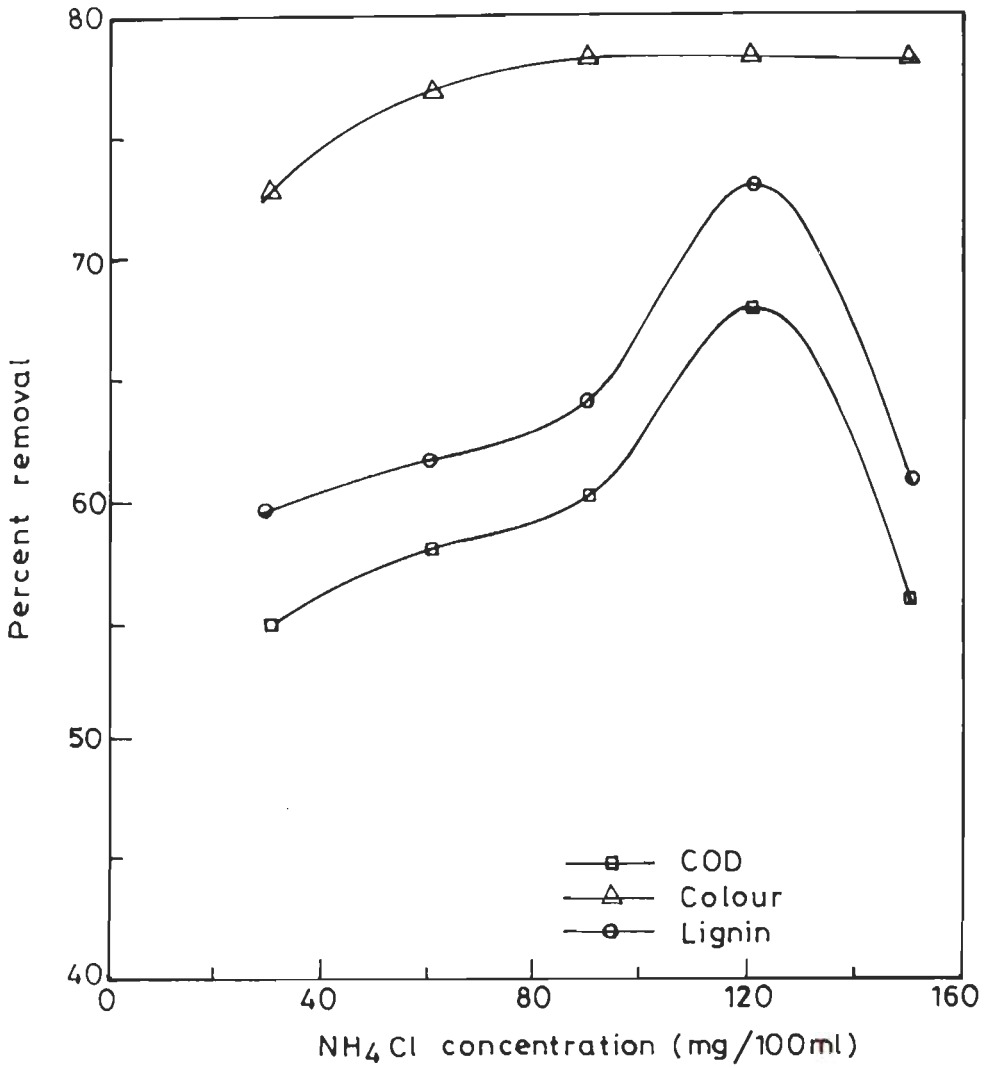


FIG. 3.12 EFFECT OF AMMONIUM CHLORIDE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY *P. putida*

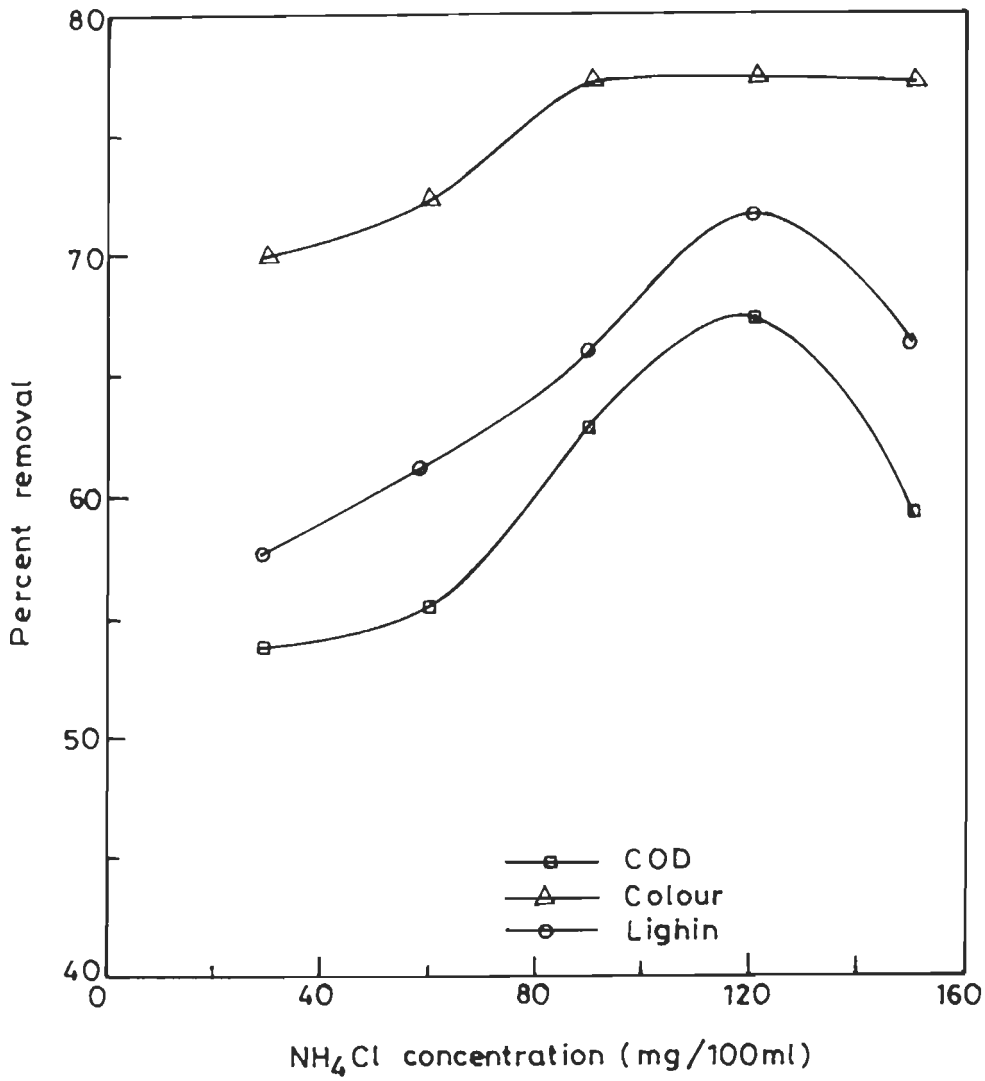


FIG. 3.13 EFFECT OF AMMONIUM CHLORIDE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. formicans

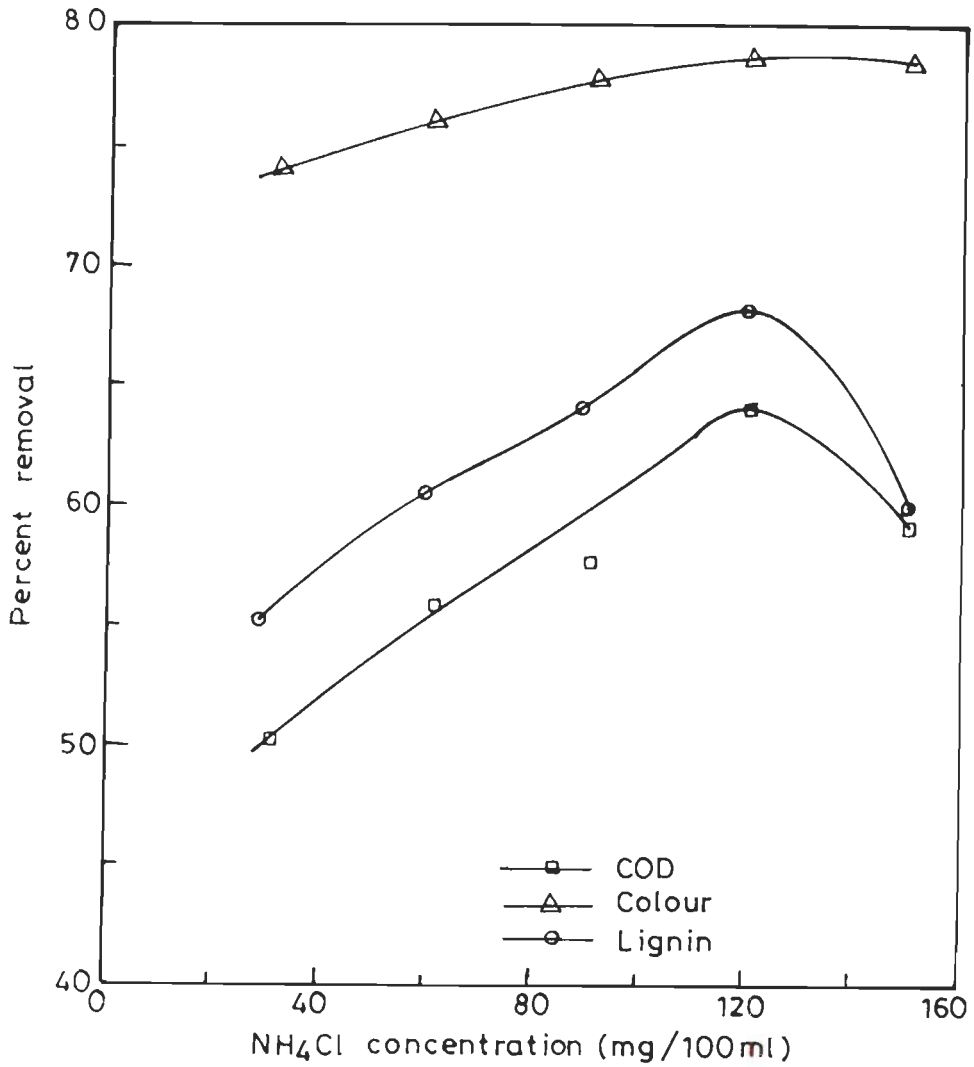


FIG. 3.14 EFFECT OF AMMONIUM CHLORIDE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. calcoaceticus

becomes limiting and thus provides an example of the third possibility for the nitrogen effect.

The variation in colour removal efficiency with varying ammoniom chloride concentration presents a different trend. For each strain, the colour removal efficiency increases with increasing NH_4Cl concentration in the range of 30 to 90 mg per 100ml and thereafter it almost becomes constant at higher concentrations i.e. at 120 and 150 mg per 100ml. This clearly indicates that the colour is attributed by only a part of lignin and some fraction of lignin molecules are colourless, which account for the COD and are biodegraded at optimum NH_4Cl concentration (120 mg per 100ml). But at higher concentration (150 mg per 100ml) this colourless fraction of lignin is not degraded and is responsible for the decrease in COD removal efficiency.

3.4.2.5 Effect of Phosphate Concentration

Phosphorus constitutes about 3% of the dry weight of a bacterial cell. This nutrient is also utilized by the microorganisms in the form of phosphate, which is an essential component for the synthesis of genetic materials-DNA and RNA. Thus phosphate in various concentrations (16-32 mg per 100ml) was added in black liquor, in order to observe its effect on the efficiency of removal of different parameters viz. COD, colour and lignin and the results are presented in Figs. 3.15, 3.16 ad 3.17 for *P. putida*, *A. formicans* and *A. calcoaceticus* respectively.

It is evident from the above referred Figs. that COD and lignin removal efficiency increases slowly with increasing phosphate concentration and a maxima is observed at 28 mg per 100ml of phosphate concentration. A slight fall in removal of COD with no further enhancement in lignin degradation is observed at higher phosphate concentration.

Colour removal efficiency for each strain depicts a very small increase with increasing phosphate ion concentration in the range of 16 to 24 mg per

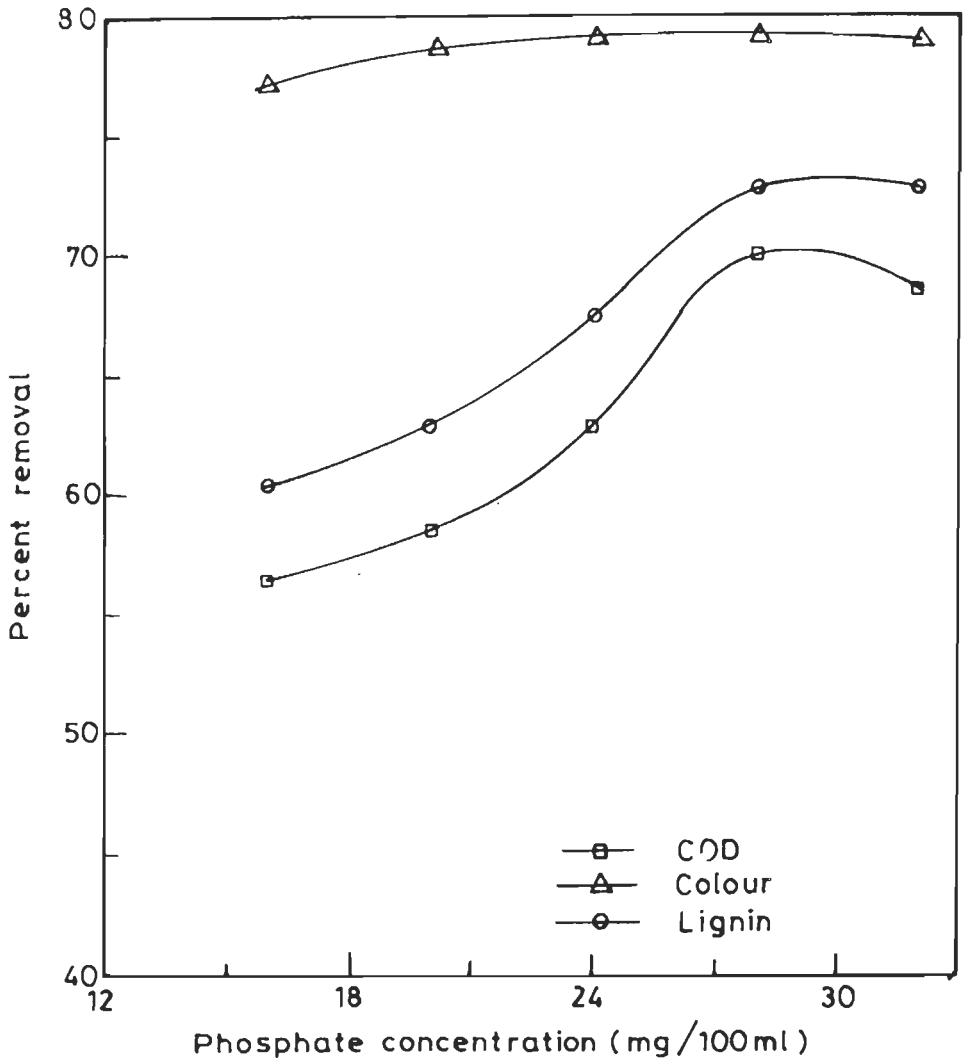


FIG. 3.15 EFFECT OF PHOSPHATE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY P. putida

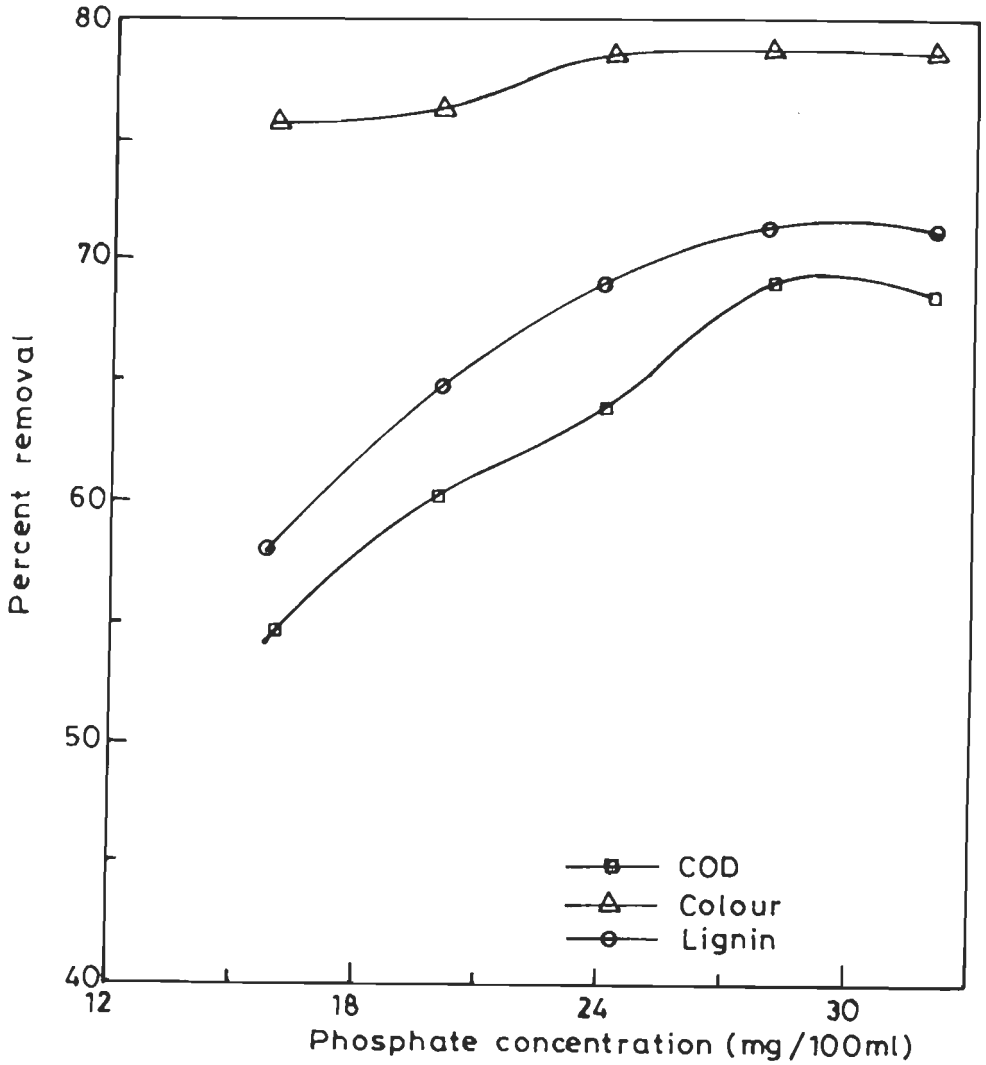


FIG. 3.16 EFFECT OF PHOSPHATE CONCENTRATION ON THE REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. formicans

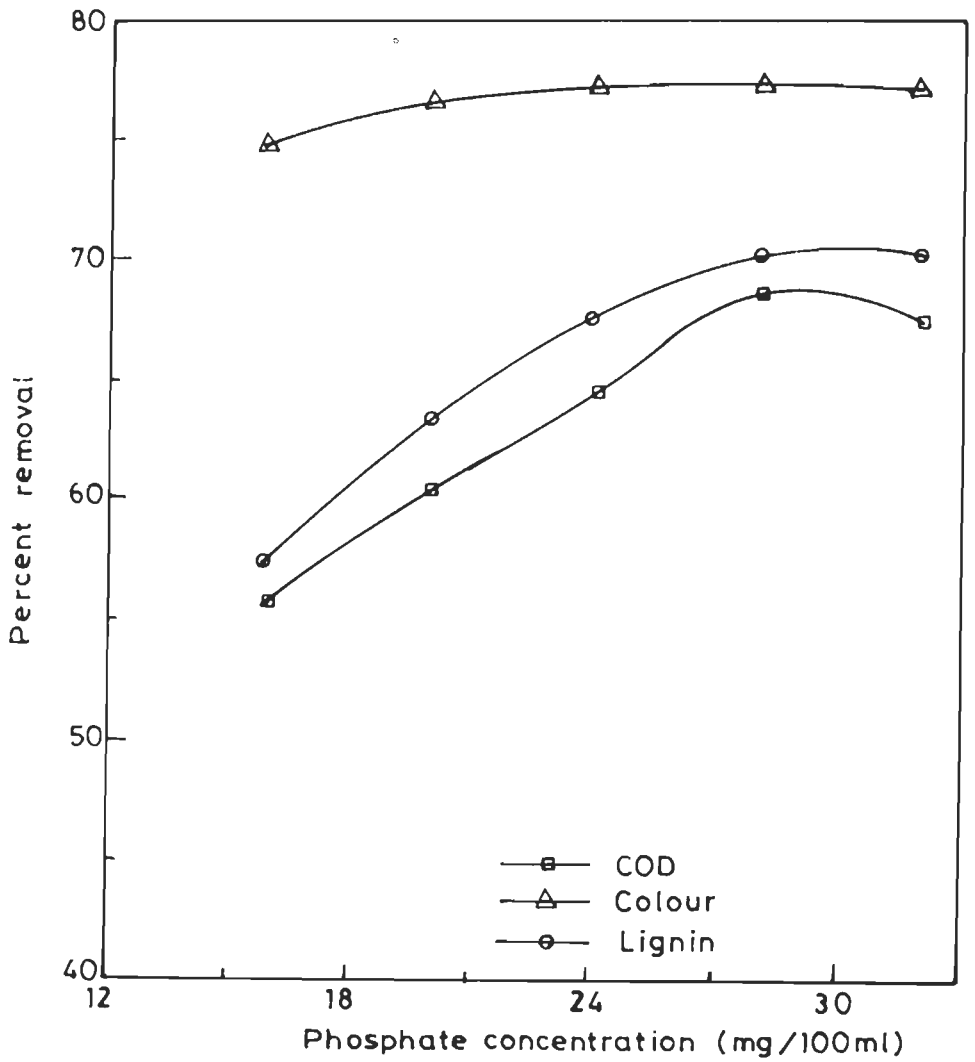


FIG. 3.17 EFFECT OF PHOSPHATE CONCENTRATION ON THE REMOVAL OF COD COLOUR AND LIGNIN FROM BLACK LIQUOR BY A. calcoaceticus

100ml, and beyond this concentration no change in this property of black liquor is observed. Thus a concentration of 28mg per 100ml of phosphate can be considered to be the optimum dose for the removal of COD, colour and lignin from black liquor.

These results clearly indicate that at low concentration of phosphate, no marked enhancement in colour removal efficiency is observed, but a marked increase is observed in COD and lignin removal efficiency. A possible explanation given for these results is :

(i) It seems that the removal of colour does not depend on the additional phosphate concentration, whereas the same is necessary for the effective decrease in COD and lignin from black liquor.

(ii) Further it is also possible that only colour providing lignins are degraded at low phosphate concentration.

At higher phosphate concentrations (>28mg per 100 ml) again no change is observed in colour and lignin removal, while the removal of COD decreases slightly. The reasons of these observations are not very clear.

Thus the optimum conditions for the degradation of black liquor are (Table 3.4) as follows:

Table - 3.4

OPTIMUM CONDITIONS FOR THE DEGRADATION OF BLACK LIQUOR BY DIFFERENT BACTERIAL STRAINS

Bacterial Strains	Optimum Concentration of Black Liquor(%)	Aeration Effect	Optimum pH	Glucose as Carbon Source (%)	NH_4Cl as Nitrogen Source (mg per 100ml)	Phosphate as phosphorus Source(mg per 100ml)
P. Putida	50	+	8.0	0.4	120	28
A. formicans	50	+	8.0	0.4	120	28
A. calcoaceticus	50	+	6.0	0.8	120	28

CHAPTER -4

DEGRADATION OF BLACK LIQUOR - BATCH STUDIES

4.1 GENERAL

This chapter deals with batch studies conducted on the applicability of selected bacterial strains for the treatment of black liquor. Measurement of bacterial growth by viable cell count and that of treatment efficiency by observing the COD, colour and lignin removal, are taken as the parameters in these experiments. The methodology and the results obtained are described as under :

4.2 EXPERIMENTAL METHODOLOGY

Batch experiments were conducted in 500ml erlenmeyer flasks, each containing 200ml sterilized black liquor (optimum concentration). These flasks were supplemented with nutrients like extra carbon, nitrogen and phosphorus at optimum concentration and pH (3.4.1.7) for each strain. Inocula was also prepared by the method described earlier (3.4.1.3). 10ml portions of cell suspension containing 10^2 to 10^4 cells per ml were used to inoculate 100ml of the black liquor. All flasks were kept in water bath at 30 ^{0}C. Uninoculated flasks containing effluent supplemented with nutrients and also without nutrients were kept as control. Each set was run in duplicate. The level of dissolved oxygen (DO) was maintained at 1-1.5mgl^{-1} in each flask throughout the experiment by introducing compressed air by air diffuser pipes with nozzles and DO was measured by DO probe. The contents of each flask were analyzed for pH, viable cell count, COD, colour and lignin etc. at the start of the experiment and also after 1 to 6, 8, 10, 15 and 20 days of incubation. Viable cell count was done by plate count method [85].

4.3 RESULTS AND DISCUSSION

The result of batch studies of black liquor treatment by selected strains of bacteria are summarized below :

4.3.1 GROWTH PATTERN OF BACTERIAL STRAINS

Growth patterns i.e. the increase in the number of bacterial cells as a function of time, of all the three strains in black liquor are shown in Fig. 4.1. The plots indicate an exponential increase in bacterial cells after a short lag phase. Thereafter, it reaches a peak value and comes to a stationary phase around the 10th day. The stationary phase is not very distinct for each strain and even at the end of 20th day, the bacterial growth continues at a smaller rate. Mathematical analysis of growth pattern of all the bacterial strains in black liquor was done to calculate the generation time and growth kinetic constants as detailed in chapter 3 (3.2.2). The generation time of selected strains in black liquor are :

Bacterial Strains	Generation Time
P. putida	0.55 days
A. formicans	0.63 days
A. calcoaceticus	0.49 days

Thus *A. calcoaceticus* is the fastest growing of the three strains, while *A. formicans* is the slowest growing bacteria having maximum generation time. The growth curves have also been subjected to correlation and regression analysis by fitting the data in the following equation :

$$Y_{log} = b\ t_{days} + C$$

Where,

Y_{log} = log of bacterial cells per ml at time 't'

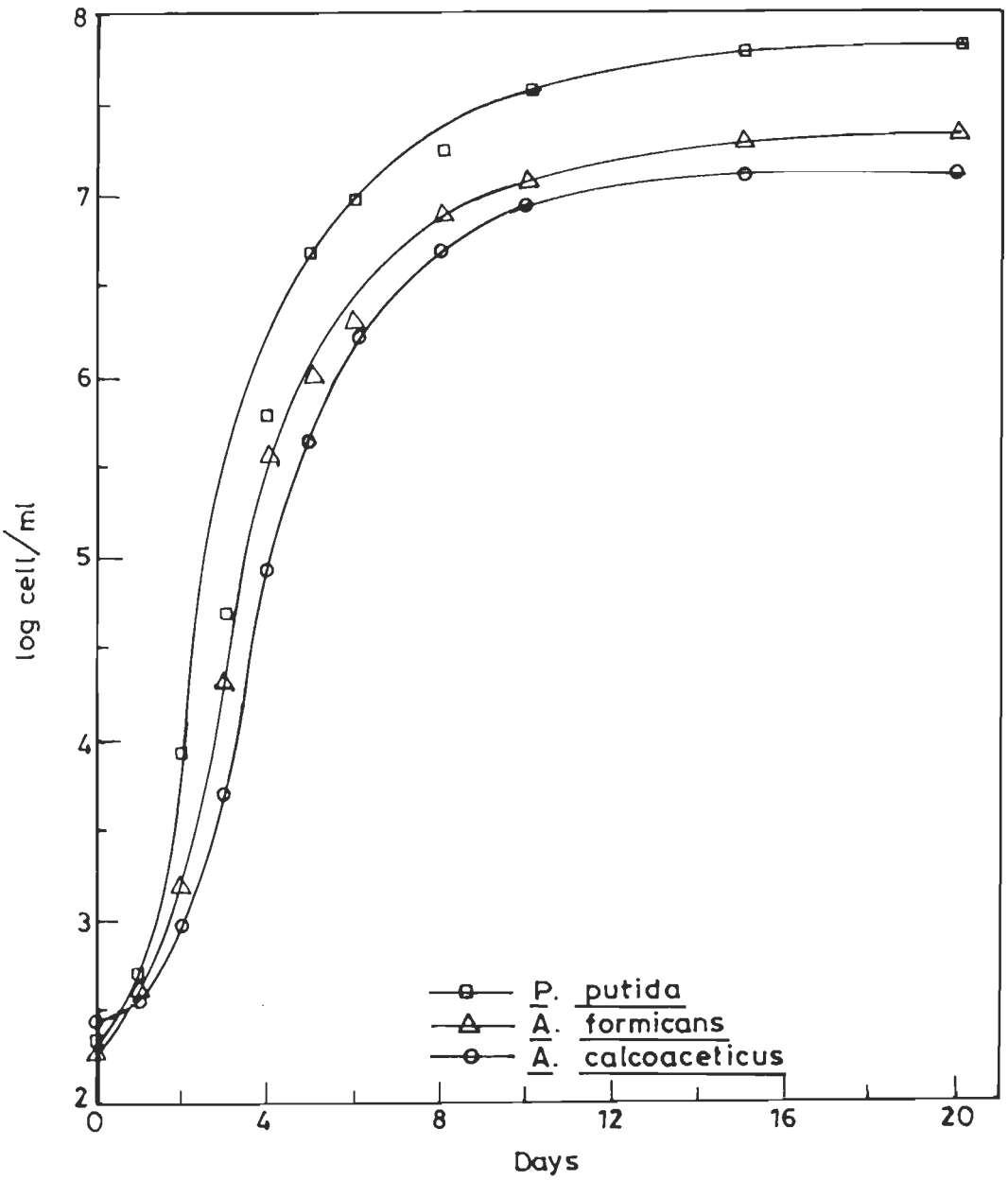


FIG. 4.1 GROWTH CURVES OF BACTERIA IN BLACK LIQUOR

b = Slope of line i.e rate of growth

t_{days} = Time in days

C = Constant or intercept on Y-axis

Results of the above mentioned analysis are presented in the Table 4.1:

Table - 4.1

LINEAR REGRESSION OF GROWTH CURVES

Bacterial Strains	Regression Line	Coefficient of Correlation (r)
P. putida	$Y_{log} = 0.57\ t_{days} + 3.19$	0.936
A. formicans	$Y_{log} = 0.45\ t_{days} + 3.16$	0.911
A. calcoaceticus	$Y_{log} = 0.64\ t_{days} + 2.03$	0.958

An appraisal of the data (Table 4.1) reveals that the growth rate of *A. calcoaceticus* is highest and that of *A. formicans* is smallest, while *P. putida* is having an intermediate value. A significant increase in cell yield as a function of time corresponds to high values of coefficient of correlation.

4.3.2 CHANGE IN pH

During the experiments conducted in batch studies, it was noticed that the pH of black liquor does not remain constant, but shows variations. The change in pH, during bacterial degradation of black liquor, as a function of time are depicted in Fig. 4.2.

A perusal of Fig. 4.2 reveals a sharp initial fall in pH of all the three strains. The decrease continues up to the 10th day of the experiment and thereafter a slight

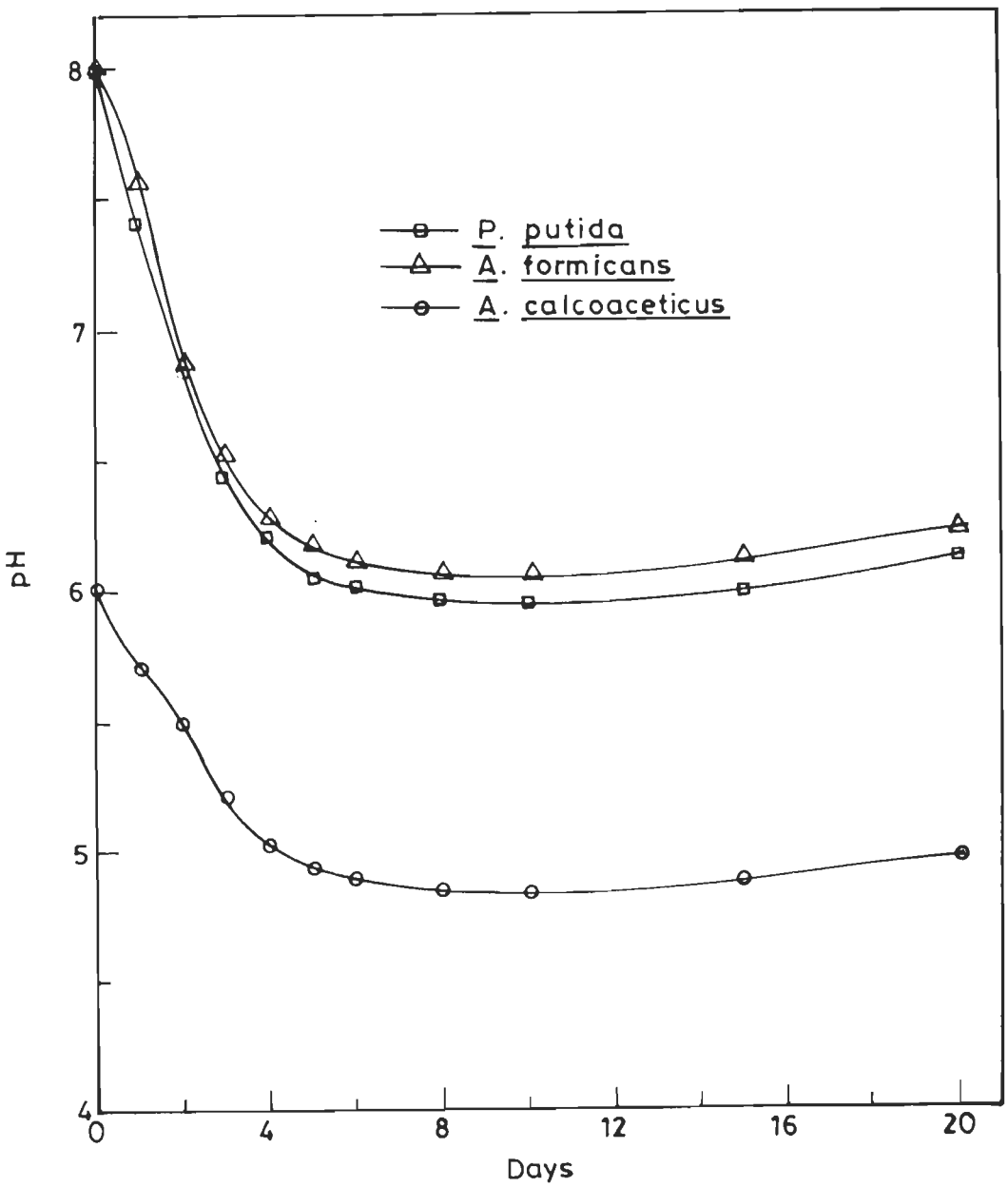


FIG. 4.2 CHANGE IN pH OF BLACK LIQUOR AS A FUNCTION OF TIME DURING BACTERIAL DEGRADATION

increase is observed. The pH values recorded on 20th day of the experiment in case of *P. putida* and *A. formicans* are 6.12 and 6.22 respectively, while the same for *A. calcoaceticus* is 4.96. The reason for the concombitant decrease in pH can be attributed to the formation of some acidic products due to the degradation of lignin and its derivative compounds present in black liquor. These acidic products may be phenolic acids, hydroxy acids and other organic compounds. Some of these have been identified by gas chromatography and are reported in chapter 5.

Similar results were also noticed by Livernoche et al. [56] who recorded a drop in pH during decolorization of a kraft mill effluent with fungal mycelium immobilized in calcium alginate gel. Reason given for the decrease in pH was the biosynthesis of organic acids by the immobilized fungus.

4.3.3 EFFICIENCY OF COD, COLOUR AND LIGNIN REMOVAL

The efficiency of removal of COD, colour and lignin by *P. putida*, *A. formicans* and *A. calcoaceticus*, determined as a function of time in batch studies is depicted in Figs. 4.3, 4.4 and 4.5 respectively.

The efficiency of removal of COD, colour and lignin, by various bacterial strains, increases very fast with time from 0 to 10 days and most of the removal takes place in first six days. Beyond this time (ten days), the plots level off and become fairly constant with negligible increase. The maximum removal of COD, colour and lignin by *P. putida* is 72.49, 86.31 and 77.02 percent respectively (Fig. 4.3). The same parameters in case of *A. formicans* are 69.64, 83.03 and 75.12 percent (Fig. 4.4) and 69.08, 84.51 and 74.36 percent respectively for *A. calcoaceticus* (Fig. 4.5). The data pertains to the 8th day of the experiment in each case.

Bacterial strains used in these investigations show continued activity until about the end of the 20th day of the experiment. Maximum efficiency of removal of COD, colour and lignin as observed up to the 20th day of the experiment, for all the three strains, are shown in Figs. 4.6, 4.7 and 4.8 respectively. These figures

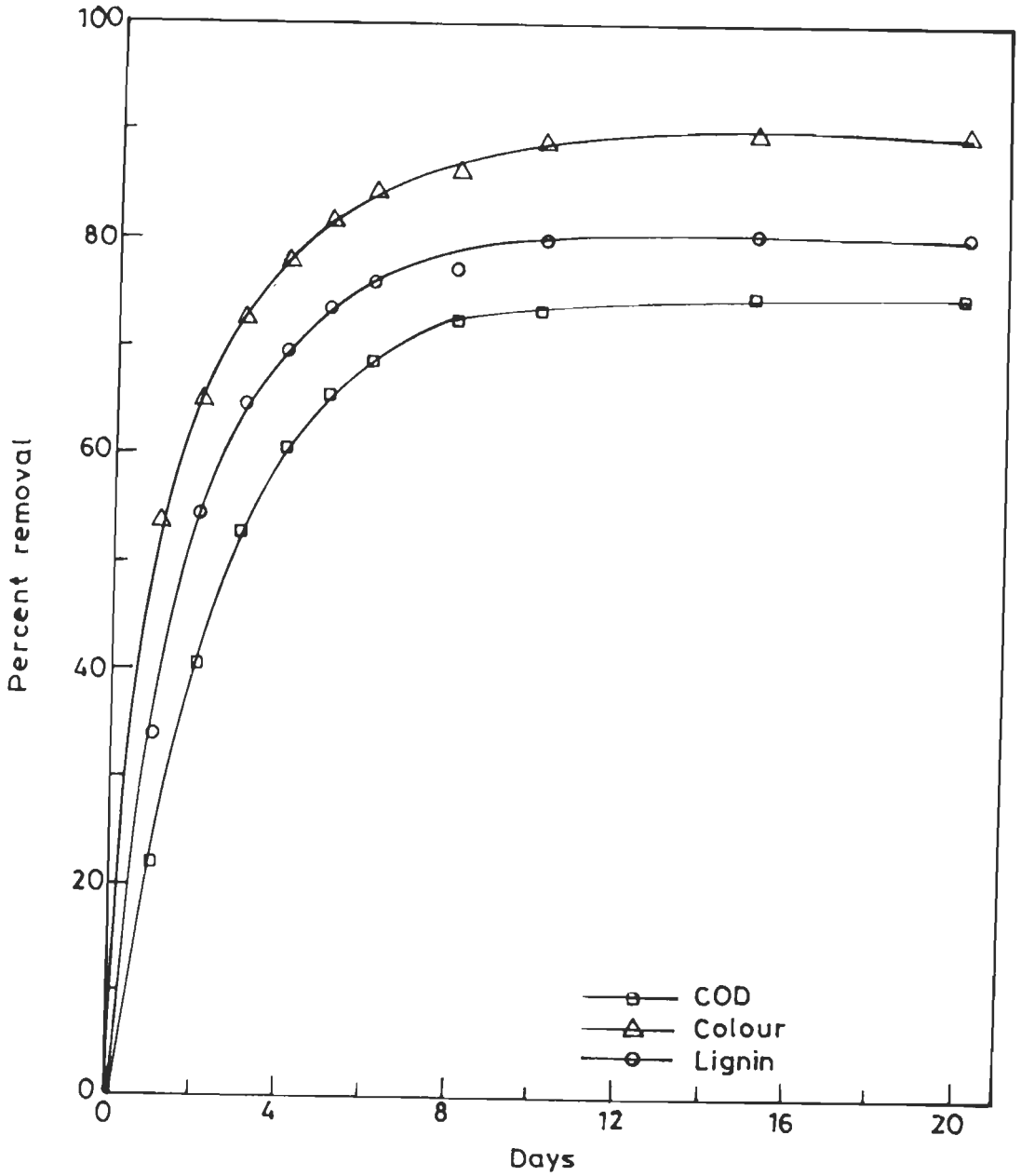


FIG. 4.3 REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR AS A FUNCTION OF TIME DURING DEGRADATION BY *P. putida*

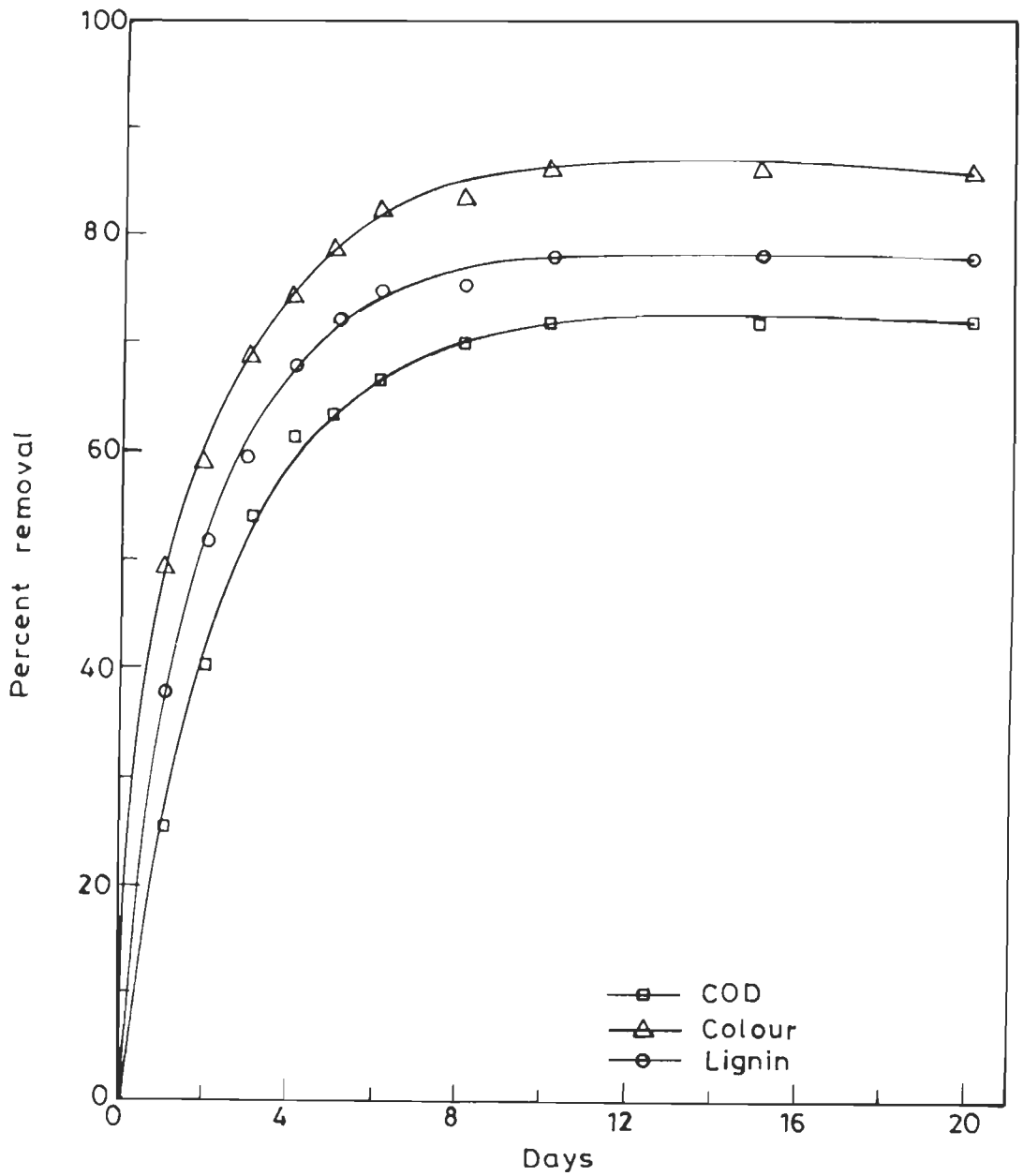


FIG. 4.4 REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR AS A FUNCTION OF TIME DURING DEGRADATION BY A. formicans

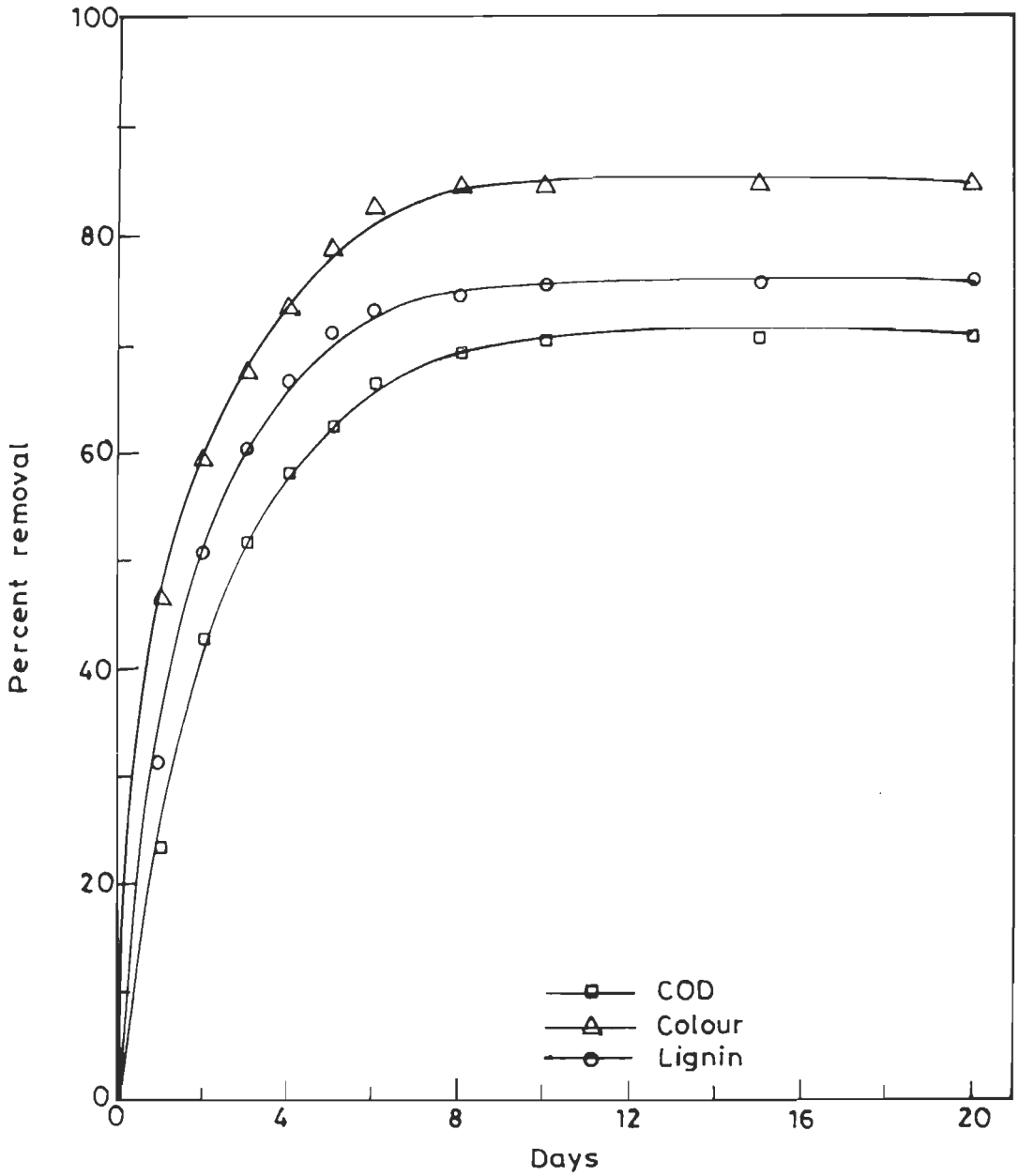


FIG. 4.5 REMOVAL OF COD, COLOUR AND LIGNIN FROM BLACK LIQUOR AS A FUNCTION OF TIME DURING DEGRADATION BY A. calcoaceticus

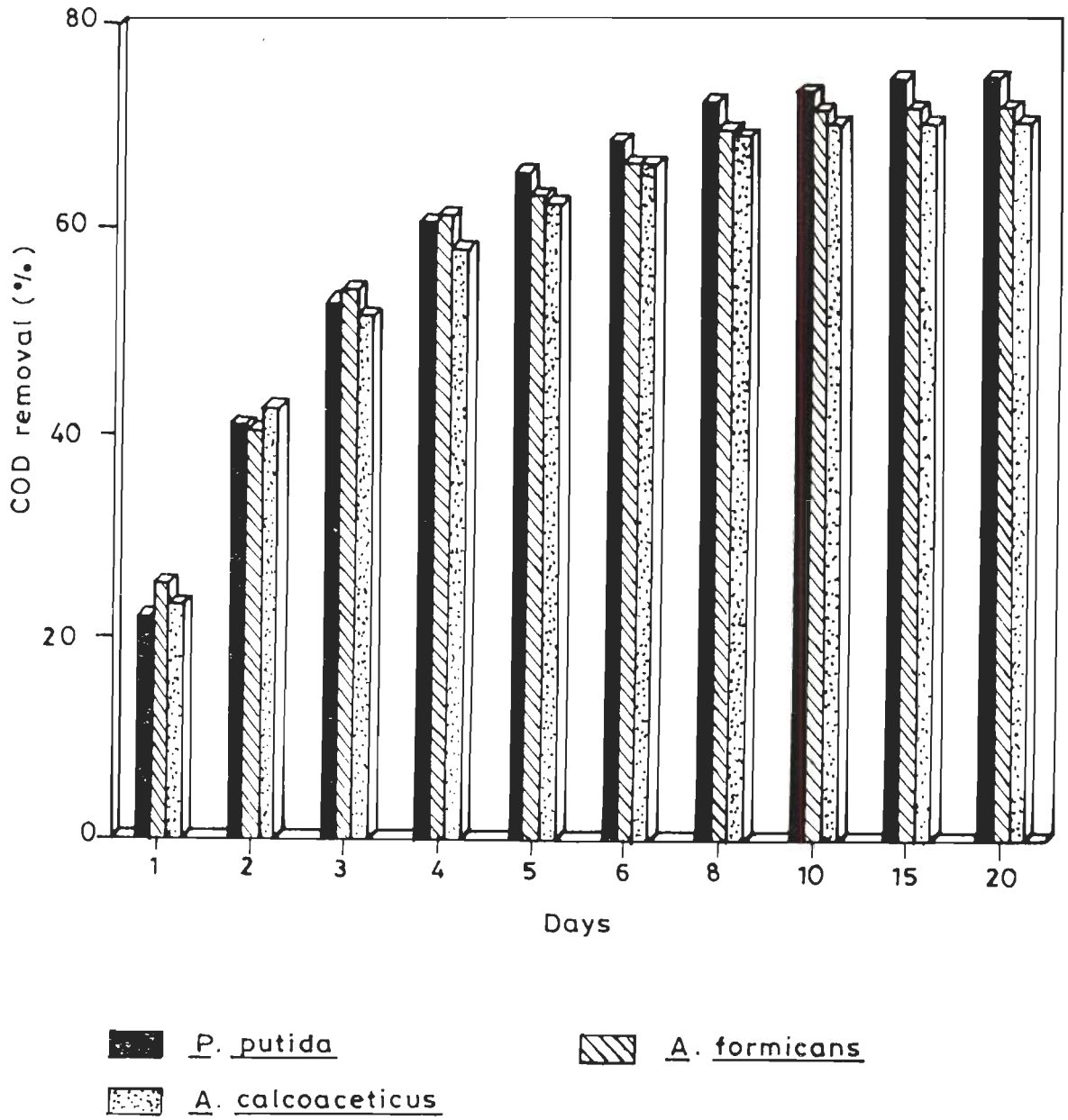


FIG. 4. 6 COD REMOVAL IN BLACK LIQUOR AS A FUNCTION OF TIME DURING DEGRADATION BY THREE STRAINS OF BACTERIA

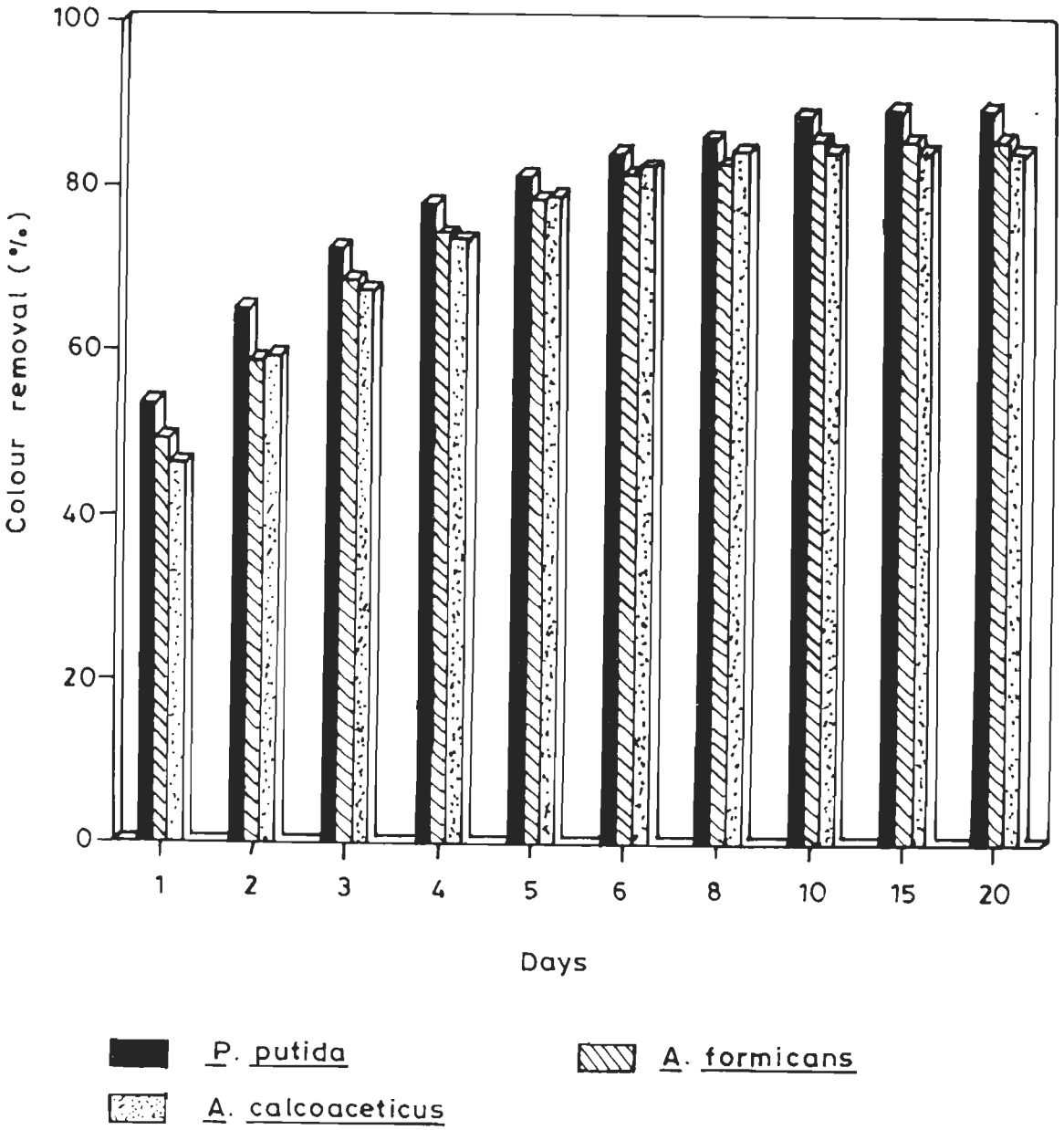


FIG. 4.7 COLOUR REMOVAL IN BLACK LIQUOR AS A FUNCTION OF TIME DURING DEGRADATION BY THREE STRAINS OF BACTERIA

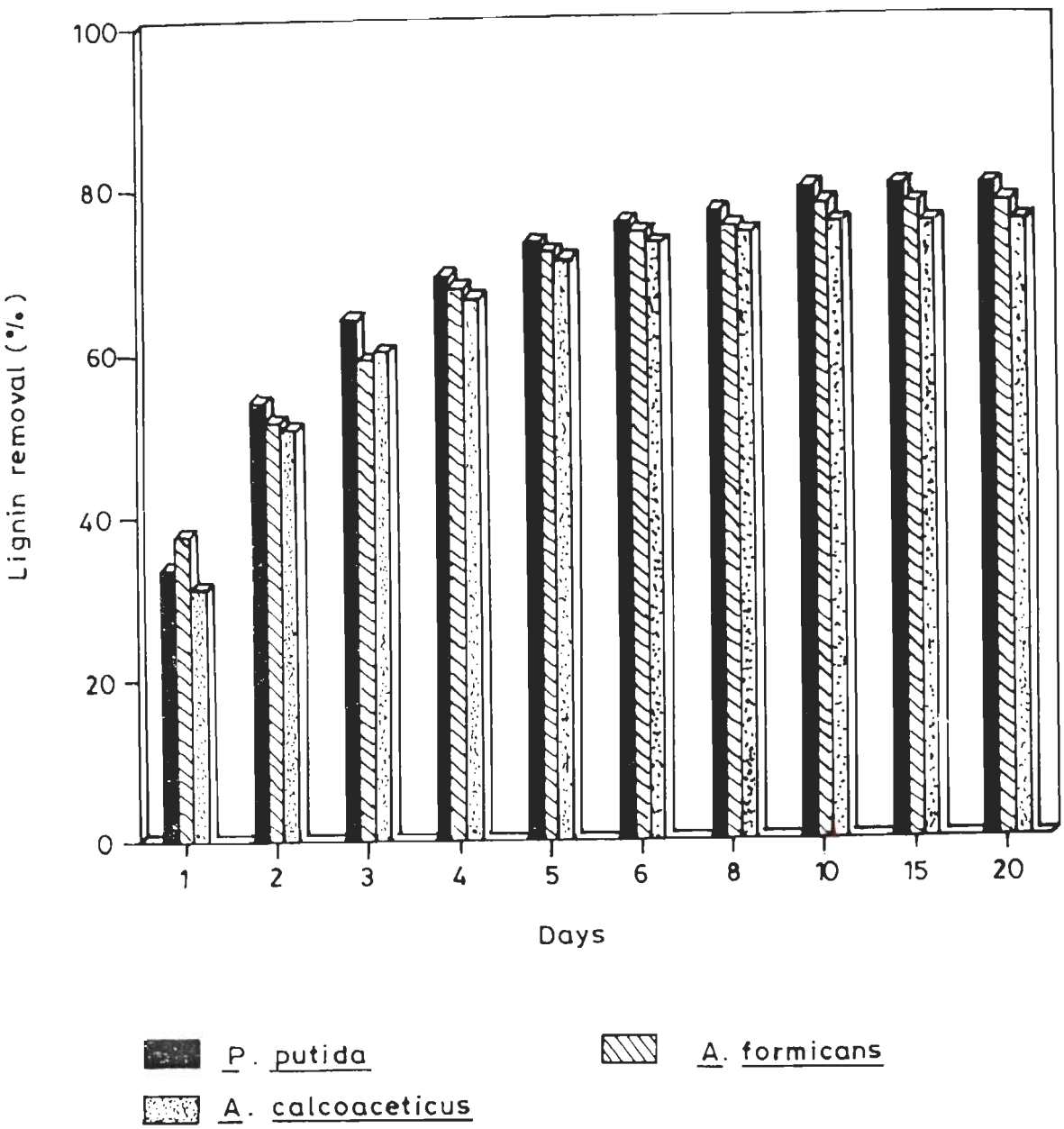


FIG. 4.8 LIGNIN REMOVAL IN BLACK LIQUOR AS A FUNCTION OF TIME DURING DEGRADATION BY THREE STRAINS OF BACTERIA

indicate that *P. putida* exhibits highest removal efficiency, while the lowest efficiency is recorded in the case of *A. calcoaceticus*. The efficiency of removal by *A. formicans* is in between these two strains. However, the difference in the efficiency of removal, by these strains, is not much i.e. the activity of all the strains seems to be almost equal.

The spectra of black liquor, as such, and the one treated with bacterial strains, have also been recorded between 200-350 nm (Fig. 4.9). The peak at 280 nm and its intensity indicates the presence of lignin and also provides a rough estimate of its amount. This peak is considerably reduced in the sample obtained after the treatment with bacterial strains. The extent to which the absorption maxima (corresponding to lignin) is affected in treated effluent, depends on the comparative tendency of various strains to utilize lignin as an organic carbon source, ultimately resulting in its removal.

No perceptible changes in COD, colour and lignin content of black liquor were observed in controls without nutrients. In controls having nutrients, the change in COD and colour were negligibly small (almost ~ 10%) in eight days time. This may be attributed to chemical oxidation of lignin due to aeration [91]. Chemical oxidation affects some functional groups of kraft lignin, but does not modify the absorbance at 280nm which is normally used to evaluate its concentration.

4.3.4 KINETICS OF THE COD, COLOUR AND LIGNIN REMOVAL

A cursory survey of the data (Figs. 4.3, 4.4 and 4.5) exhibits a first order kinetics of the removal of each parameter. As such the values of rate constants have been evaluated in each case with the help of the following expression :

$$\frac{d\eta_t}{dt} = -K\eta_t$$

Integrating and solving the above expression

$$\eta_t = \eta_{max}\left(1 - e^{-Kt}\right)$$

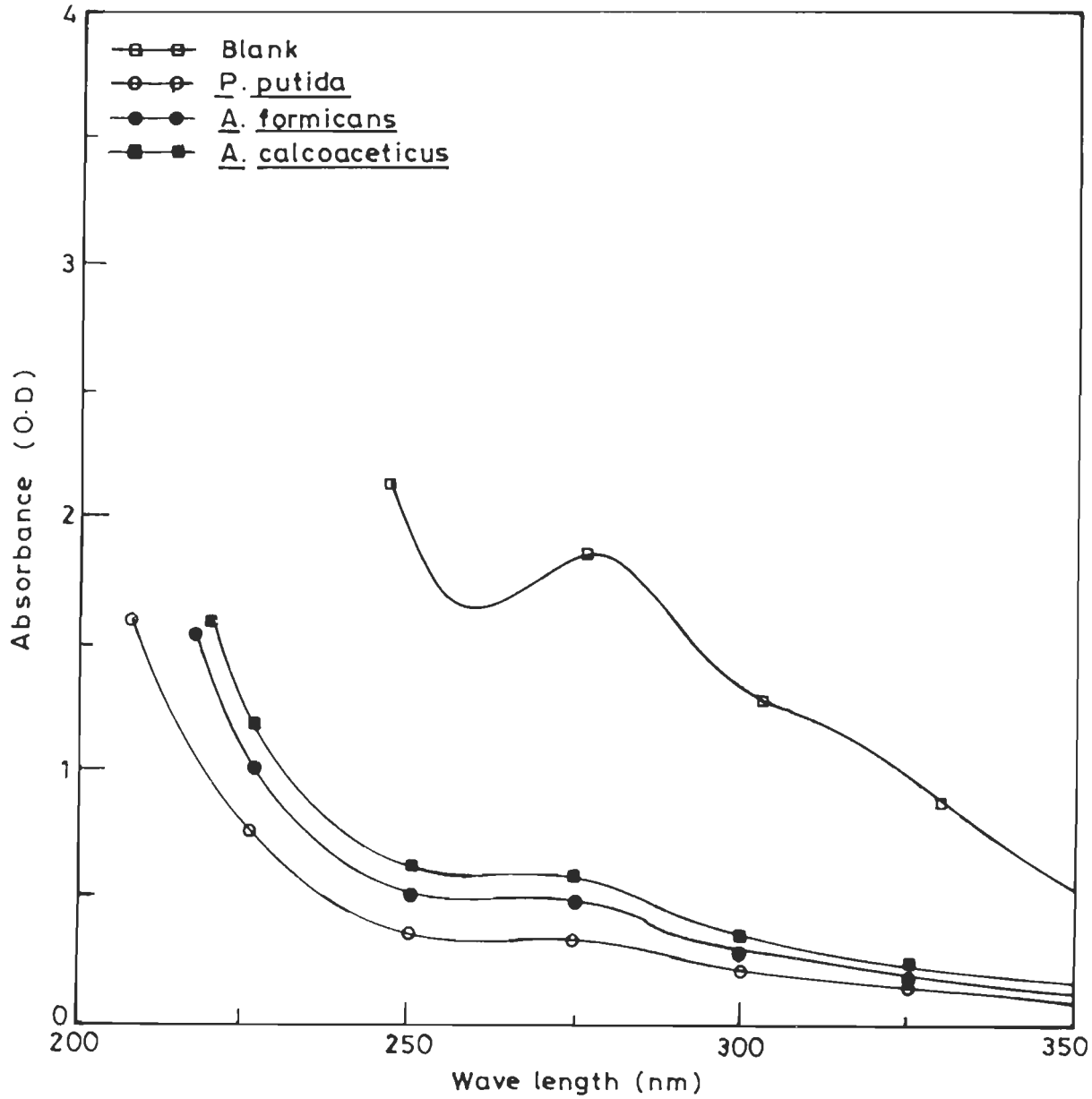


FIG. 4.9 ABSORPTION SPECTRA OF BLANK AND DEGRADED BLACK LIQUOR BY THREE STRAINS OF BACTERIA

where,

η_t = Percent removal of COD, colour or lignin at time t

η_{max} = Maximum attainable percent removal of COD, colour or lignin

K = Kinetic coefficient (rate constant)

t = Time in days.

Using a computer programme the experimental data shown in each figure was used to fit in the above equation and values of parameters η_{max} and K were calculated. The curve fitting was carried out by minimizing the error estimate CAPE (Cumulative Absolute Proportionate Error) as suggested by Srivastava [92]. Where CAPE is defined as :

$$CAPE = \sum \left| \frac{\eta_{cal} - \eta_{obsr}}{\eta_{cal}} \right|$$

where,

η_{cal} = Calculated value

η_{obsr} = Observed value

The calculated values of K and η_{max} for COD, colour and lignin removal by *P. putida*, *A. formicans* and *A. calcoaceticus*, are given in the Table 4.2.

Table - 4.2

VALUES OF KINETIC CONSTANTS FOR COD, COLOUR AND LIGNIN REMOVAL

Bacterial Strains	COD Removal		Colour Removal		Lignin Removal	
	η_{max}	K	η_{max}	K	η_{max}	K
P. putida	74.91	0.39	86.64	0.69	79.65	0.55
A. formicans	71.84	0.43	84.11	0.60	77.42	0.55
A. calcoaceticus	70.77	0.43	84.78	0.60	75.57	0.53

The above values of rate constants (K) can be utilized to determine the detention time necessary to design lagoons for the removal of COD, colour and lignin from black liquor for a desired efficiency.

4.3.5 INTERRELATIONSHIP BETWEEN pH, CELL CONCENTRATION AND PERCENT REMOVAL EFFICIENCIES

The interrelationship between the change in pH, cell concentration and percent decrease in COD, colour and lignin, for each strain, are depicted in Figs. 4.10, 4.11 and 4.12 respectively.

A perusal of plots show that an increase in the cell number of bacteria is responsible for the degradation of black liquor i.e. removal of COD, colour and lignin and associated decrease in pH. 73.42, 88.90 and 79.81 percent of COD, colour and lignin removal are observed at the end of the 10th day of incubation for *P. putida* accompained by a viable cell counts of 3.63×10^7 cells per ml and a reduction in pH from 8.0 to 5.96. The reduction in COD, colour and lignin on the 10th day by *A. formicans* are 71.49, 85.89 and 77.81 percent respectively. A cell concentration of 1.17×10^7 cells per ml is also observed along with a change in pH from 8.0 to 6.06. *A. calcoaceticus* however, exhibits the smallest removal efficiency of COD, colour and lignin i.e 70.17, 84.63 and 75.41 percent respectively, at a pH of 4.83 with cell concentration of 8.91×10^6 cells per ml. All the three strains of bacteria show a drop in pH and an increase in cell count (range 8.19×10^6 to 3.6×10^7 cell per ml).

Analysis of the above data reveals that with ten fold increase in cell number, there is a drop in pH of 0.17 to 0.26 units. Consequently a fall in pH is directly associated with the cell growth. This can be attributed to the utilization of lignin as a carbon source by the bacteria to have growth and oxidizing the lignin to some organic acid which are responsible for the drop in pH.

Attempts have been made to find a mathematical expression between cell

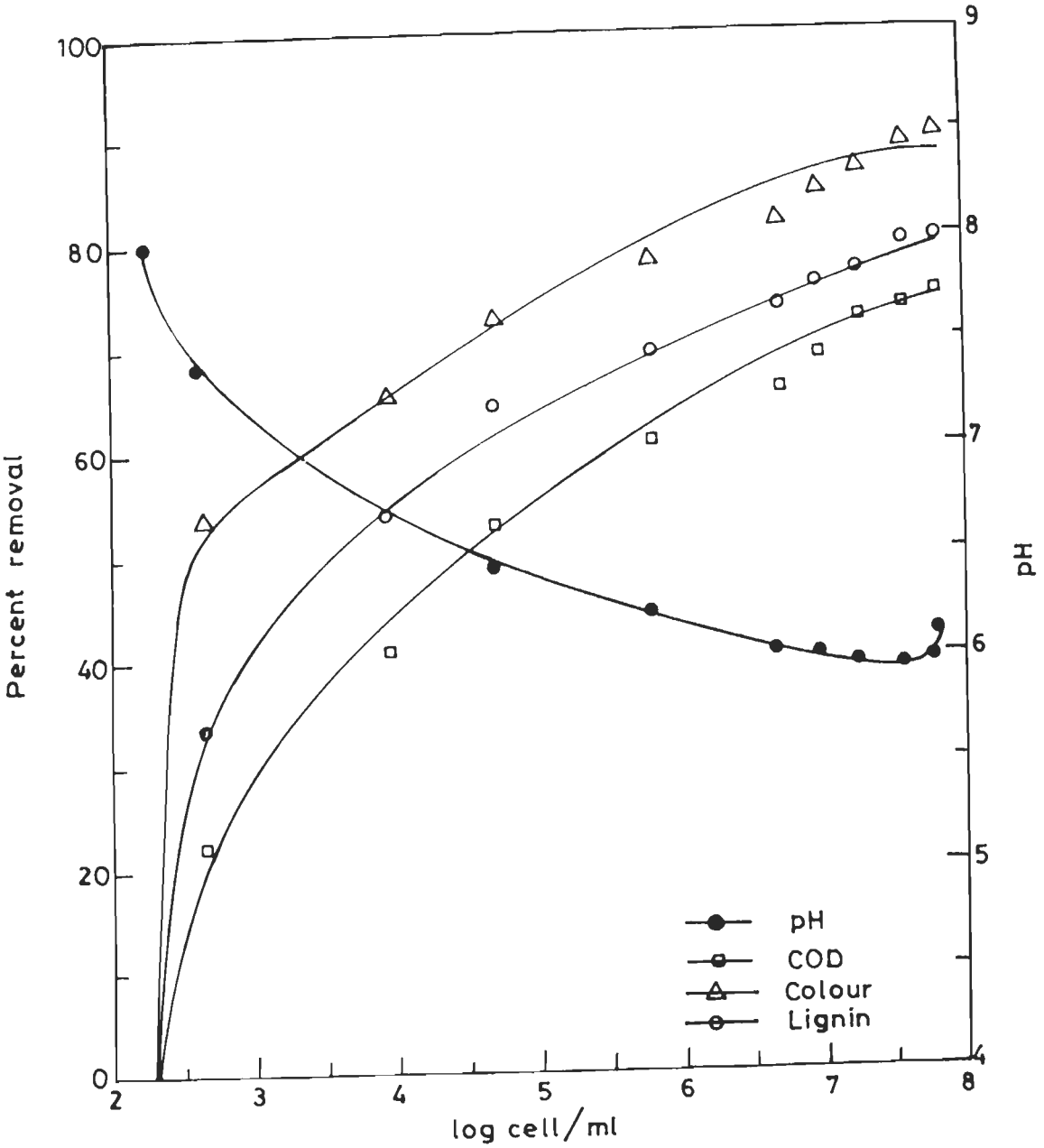


FIG. 4.10 RELATIONSHIP BETWEEN PERCENT REMOVAL OF COD, COLOUR, LIGNIN, pH AND CELL YIELD OF P. putida DURING DEGRADATION OF BLACK LIQUOR

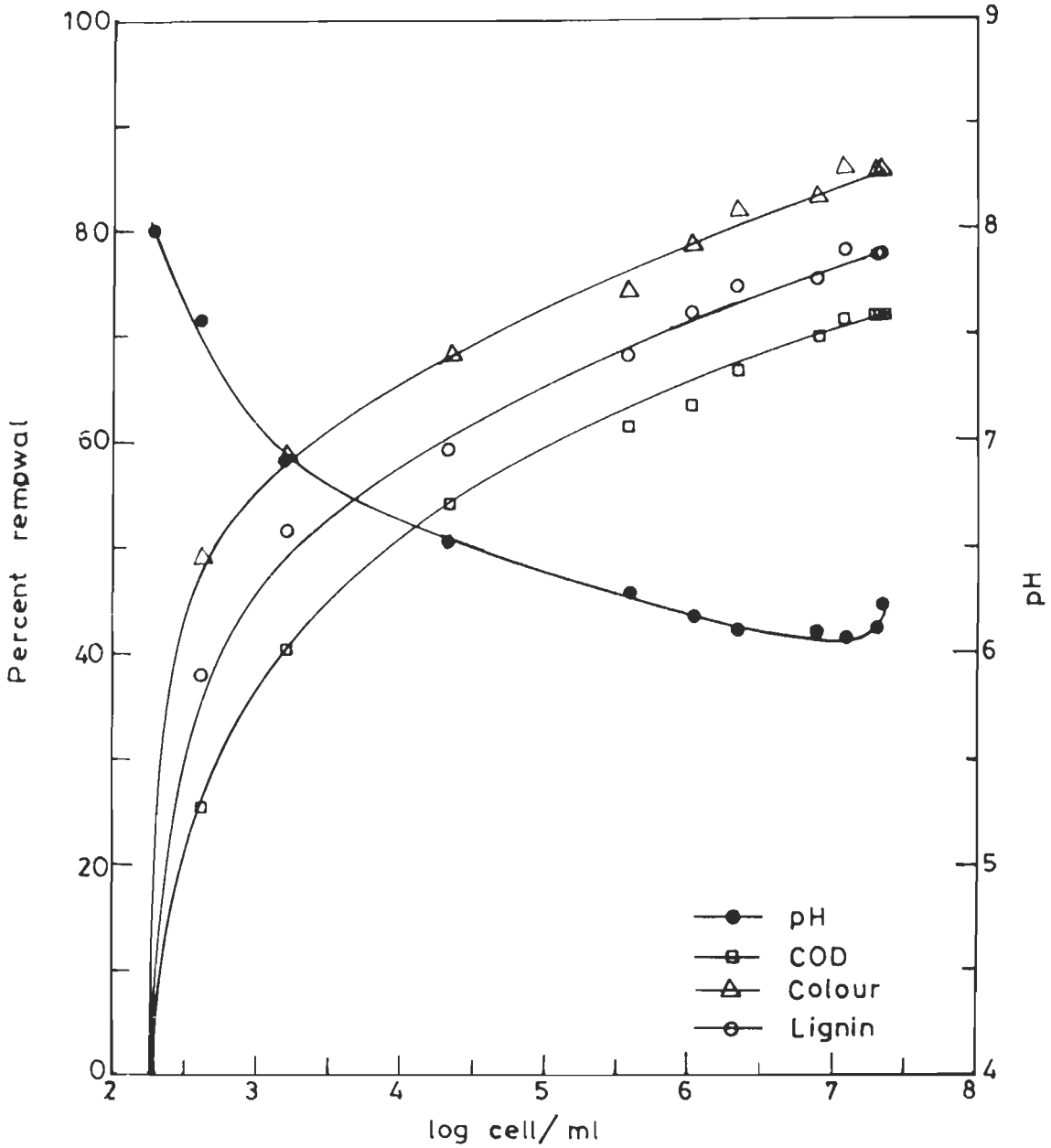


FIG. 4.11 RELATIONSHIP BETWEEN PERCENT COD, COLOUR, LIGNIN, pH AND CELL YIELD OF A. formicans DURING DEGRADATION OF BLACK LIQUOR

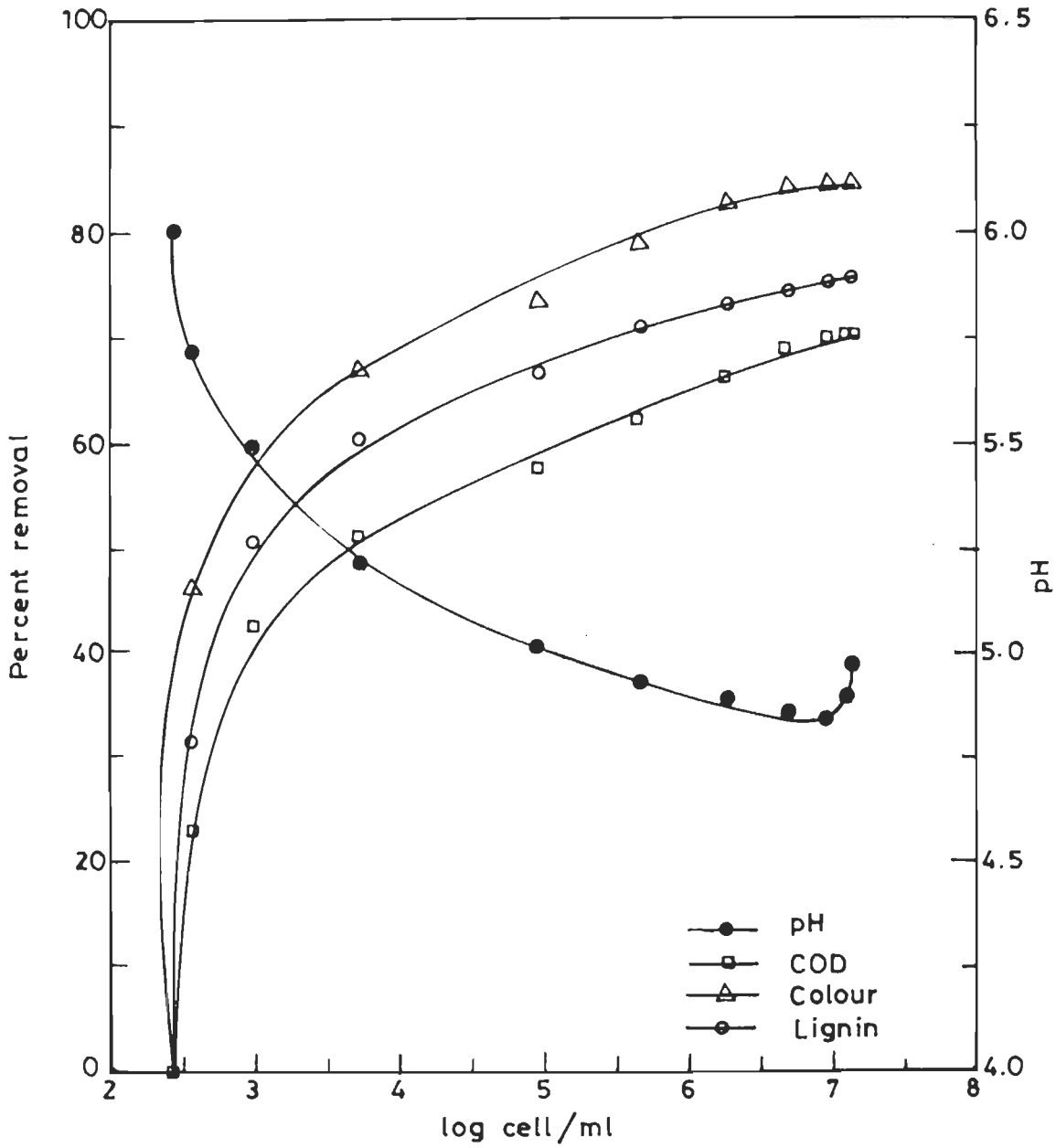


FIG. 4.12 RELATIONSHIP BETWEEN PERCENT REMOVAL OF COD, COLOUR, LIGNIN, pH AND CELL YIELD OF A.calcoaceticus DURING DEGRADATION OF BLACK LIQUOR

concentration (log phase) and the removal efficiency of COD, colour and lignin for each selected strain of bacteria. There seems to be a straight line relationship between these parameters and as such the data is also subjected to regression analysis by fitting the following equation :

$$Y_r = b_r X_{log} + C$$

Where,

Y_r = Percent removal of COD, colour or lignin

b_r = Slope of line i.e. rate of removal of COD, colour or lignin

X_{log} = log bacterial cells per ml

C = Constant or intercept on Y - axis.

The results of the correlation and regression analysis i.e. the equation for COD, colour and lignin removal (Y_{COD}, Y_{col}, Y_{lig}) and values of coefficient of correlation (r) are shown in Table 4.3.

Table - 4.3

LINEAR REGRESSION OF PERCENT COD, COLOUR AND LIGNIN REMOVAL VERSUS CELL CONCENTRATION

Bacterial Strains	Regression Line	Coefficient of Correlation (r)
P. putida	$Y_{COD} = 8.25 X_{log} + 11.24$	0.988
	$Y_{col} = 6.18 X_{log} + 42.19$	0.990
	$Y_{lig} = 6.10 X_{log} + 32.15$	0.985
A. formicans	$Y_{COD} = 7.28 X_{log} + 19.61$	0.986
	$Y_{col} = 6.52 X_{log} + 38.91$	0.993
	$Y_{lig} = 6.48 X_{log} + 31.59$	0.990
A. calcoaceticus	$Y_{COD} = 6.43 X_{log} + 25.60$	0.992
	$Y_{col} = 5.98 X_{log} + 43.72$	0.991
	$Y_{lig} = 5.90 X_{log} + 37.81$	0.973

It is obvious that in case of *P. putida*, COD removal rate is faster (8.25) in comparison to the other two strains. It is also evident that the colour and lignin removal rate in all the three cases are approximately same. Regression lines also show that in all the three strains COD removal rate is higher as compared to lignin and colour removal rate. The regression analysis further shows a very significant relationship in the cell concentration of different strains vis a vis the removal of COD, colour and lignin. As borne out by high values of correlation coefficients there is a distinct enhancement in removal efficiency of various bacterial strains with increasing cell concentration

Based on the above results, attempts have also been made to analyse the data of log phase for all the three strains together for the removal efficiency of COD, colour and lignin versus cell concentration.

Figs. 4.13, 4.14 and 4.15 show the plots for COD, colour and lignin removal versus cell concentration along with the regression line and value of coefficient of correlation (r). It is apparent that for every ten fold increase in the number of bacterial cells, COD removal is about 7 percent and the values for colour and lignin removal are 6.14 and 5.94 percent respectively. COD removal rate is highest as compared to the removal of colour and lignin which are almost equal. High values of correlation coefficient (r) also exhibit a significant relationship in all the three strains for the removal of COD, colour and lignin versus cell concentration.

4.3.6 RELATIONSHIP BETWEEN COD, COLOUR AND LIGNIN REDUCTION

It is observed that the removal of COD and colour depends upon lignin removal for all the three bacterial strains. As such a correlation and regression analysis of the experimental points (COD and colour removal efficiency versus lignin removal efficiency) is carried out with the help of following expression :

$$Y_r = b_r X_{lig} + C$$

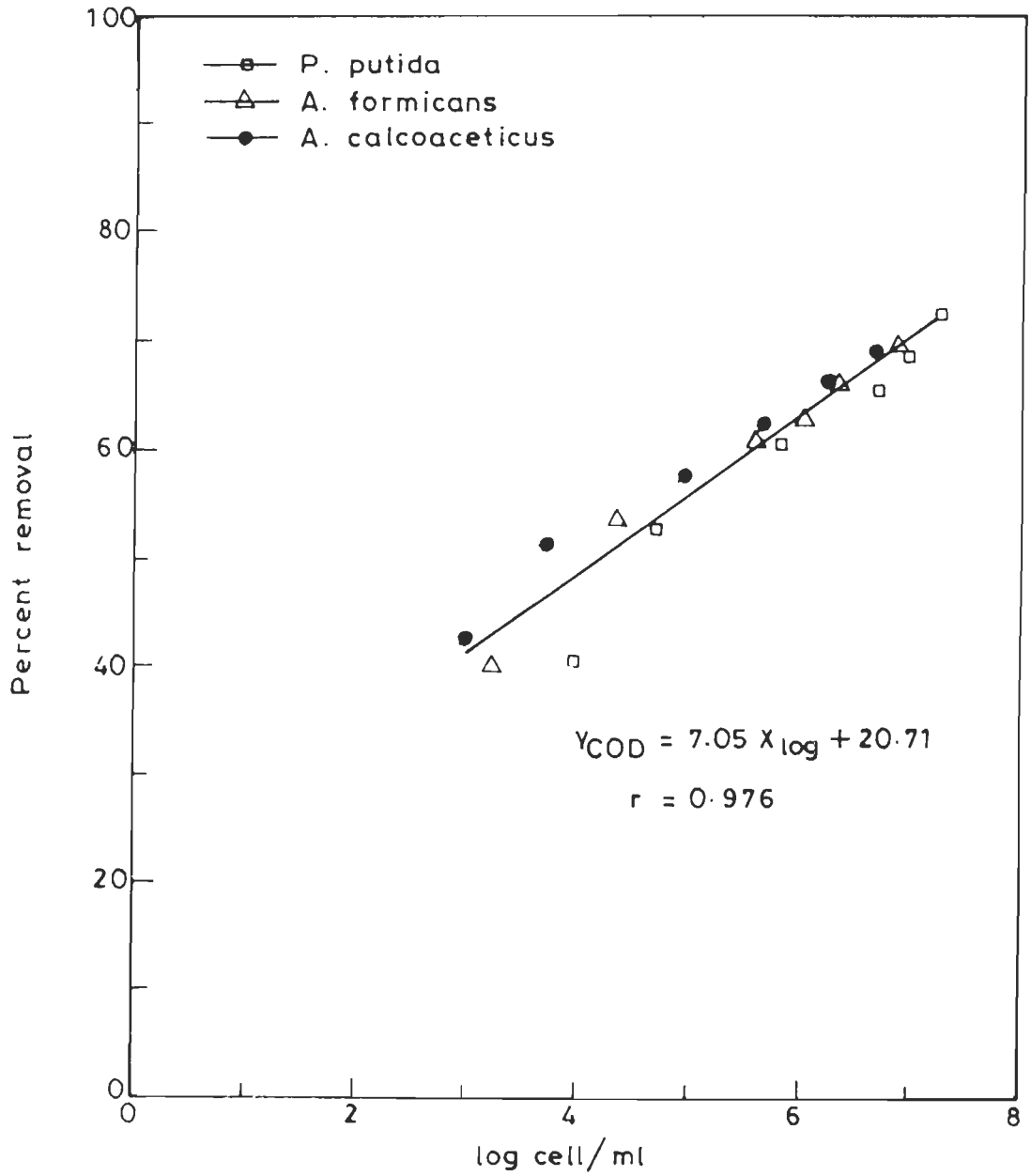


FIG. 4.13 RELATIONSHIP BETWEEN PERCENT COD REMOVAL AND CELL YIELD OF BACTERIA FOR DEGRADATION OF BLACK LIQUOR

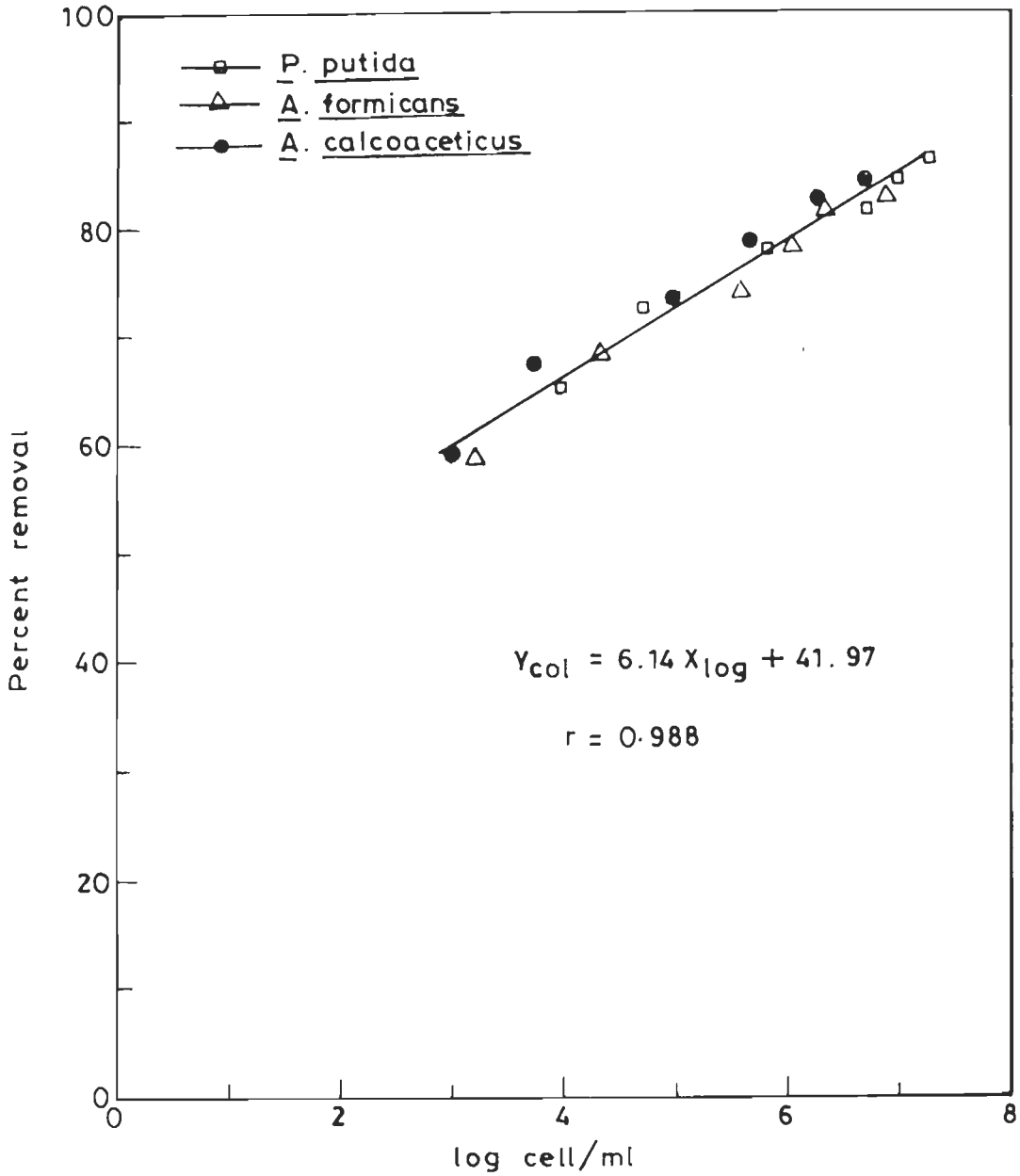


FIG. 4.14 RELATIONSHIP BETWEEN PERCENT COLOUR REMOVAL AND CELL YIELD OF BACTERIA FOR DEGRADATION OF BLACK LIQUOR

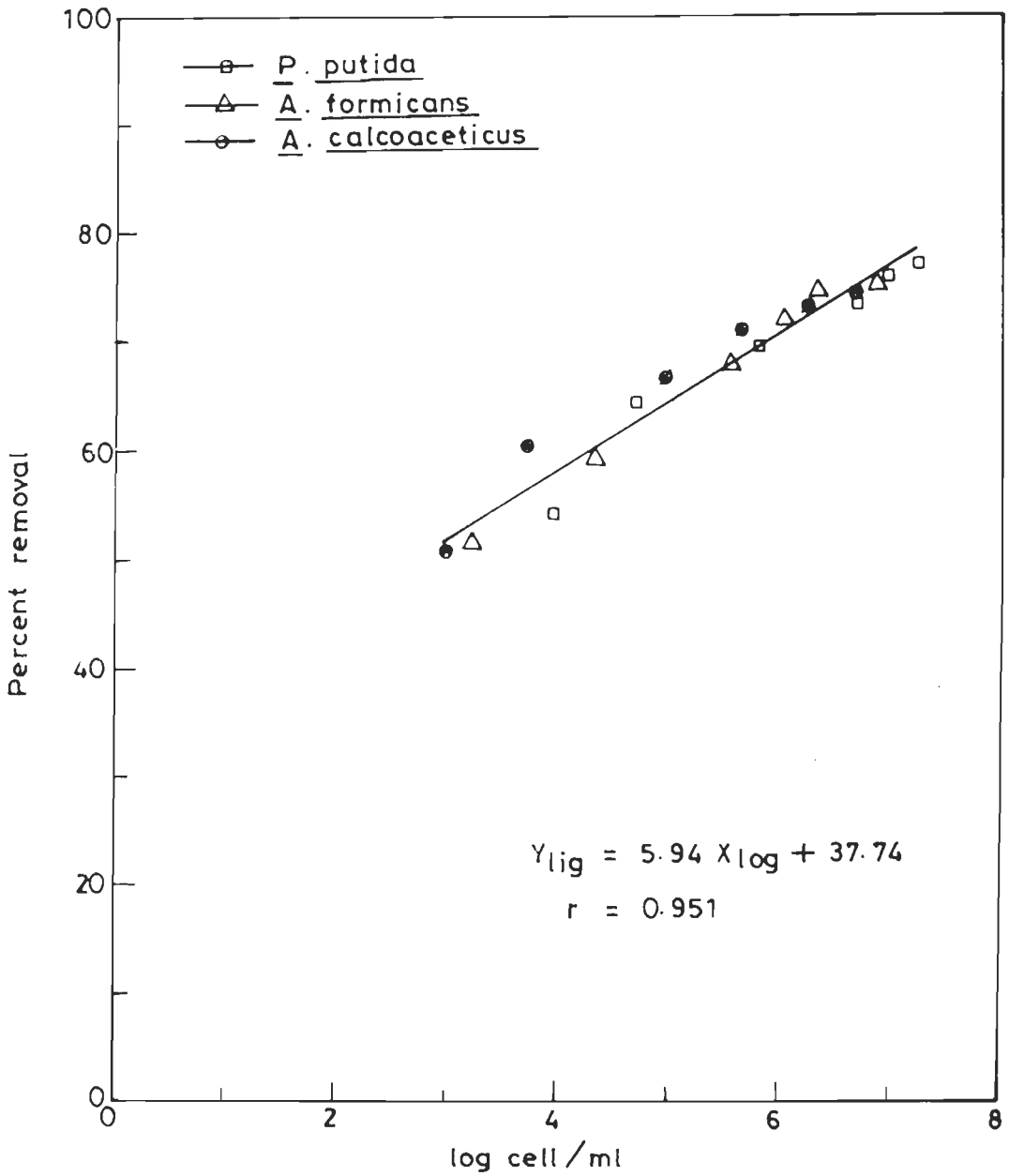


FIG. 4.15 RELATIONSHIP BETWEEN PERCENT LIGNIN REMOVAL AND CELL YIELD OF BACTERIA FOR DEGRADATION OF BLACK LIQUOR

Where,

Y_r = Percent removal of COD or colour

b_r = Slope of line i.e. rate of removal of COD or colour

X_{lig} = Percent removal of lignin

C = Constant or intercept on Y - axis.

Results of the analysis are shown in Table 4.4 and the plots are presented in Figs. 4.16 and 4.17. These figures represent the points that relate COD and colour reductions along with lignin reduction, obtained in all batch experiments, for each selected strain.

Table - 4.4

LINEAR REGRESSION OF RELATIONSHIP BETWEEN COD AND COLOUR REMOVAL VERSUS LIGNIN REMOVAL

Bacterial Strains	Regression Line	Coefficient of Correlation (r)
P. putida	$Y_{COD} = 1.32\ X_{lig} - 31.56$	0.997
	$Y_{col} = 0.97\ X_{lig} + 11.15$	0.995
A. formicans	$Y_{COD} = 1.11\ X_{lig} - 15.17$	0.987
	$Y_{col} = 1.00\ X_{lig} + 7.52$	0.995
A. calcoaceticus	$Y_{COD} = 1.18\ X_{lig} - 18.17$	0.955
	$Y_{col} = 1.06\ X_{lig} + 4.05$	0.992

The linear relationship between COD and colour reduction (Y_{COD}, Y_{col}) with respect to lignin reduction (Y_{lig}) is justified by high values of coefficient of correlation. It further shows that the rate of COD removal (Fig. 4.16), in comparison to colour (Fig. 4.17), is high and this may be ascribed to the fact that COD removal does not entirely depend on lignin removal alone but also depends on other oxidizable compounds present in black liquor.

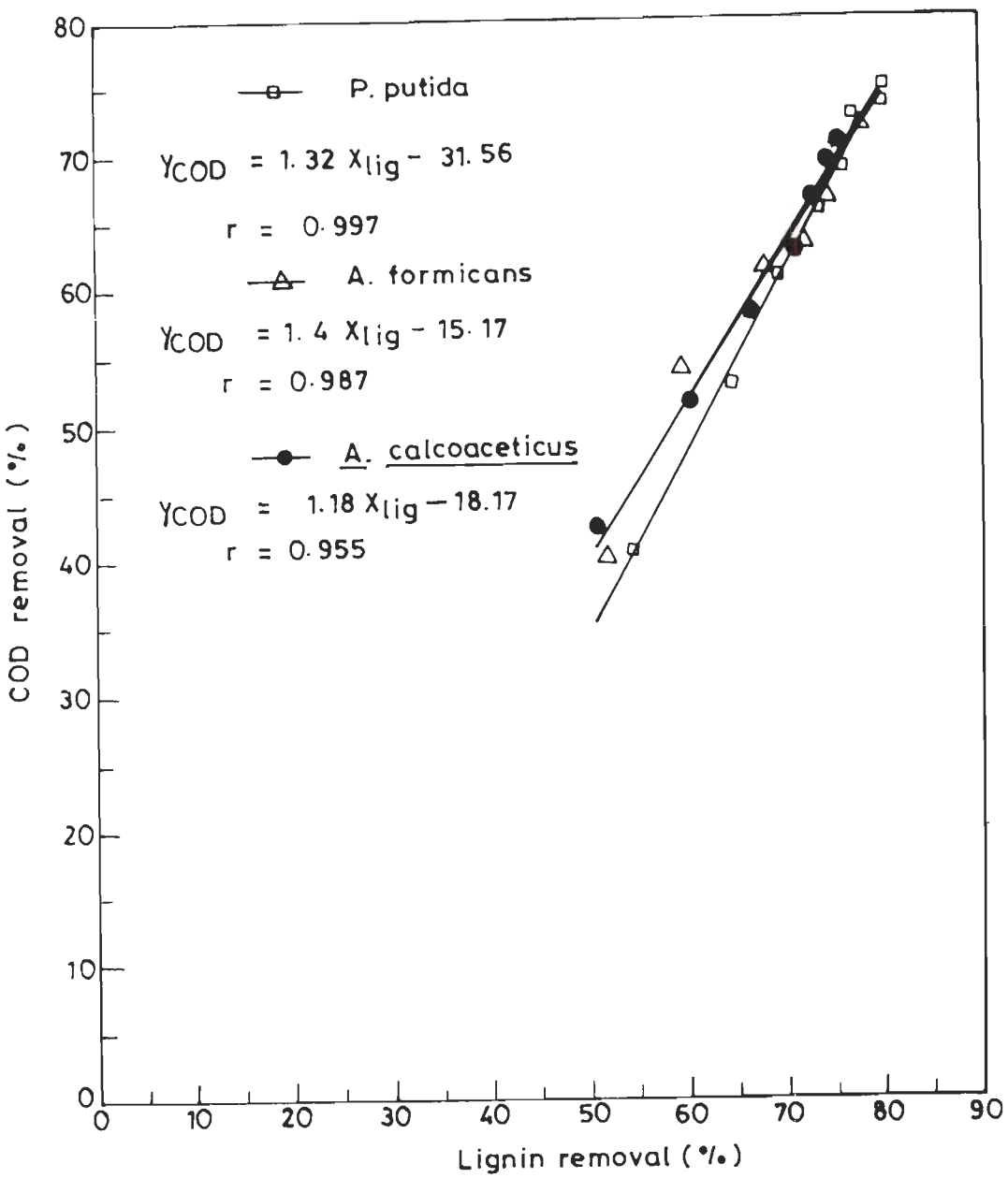


FIG. 4.16 RELATIONSHIP BETWEEN PERCENT COD REMOVAL AND LIGNIN REMOVAL FROM BLACK LIQUOR

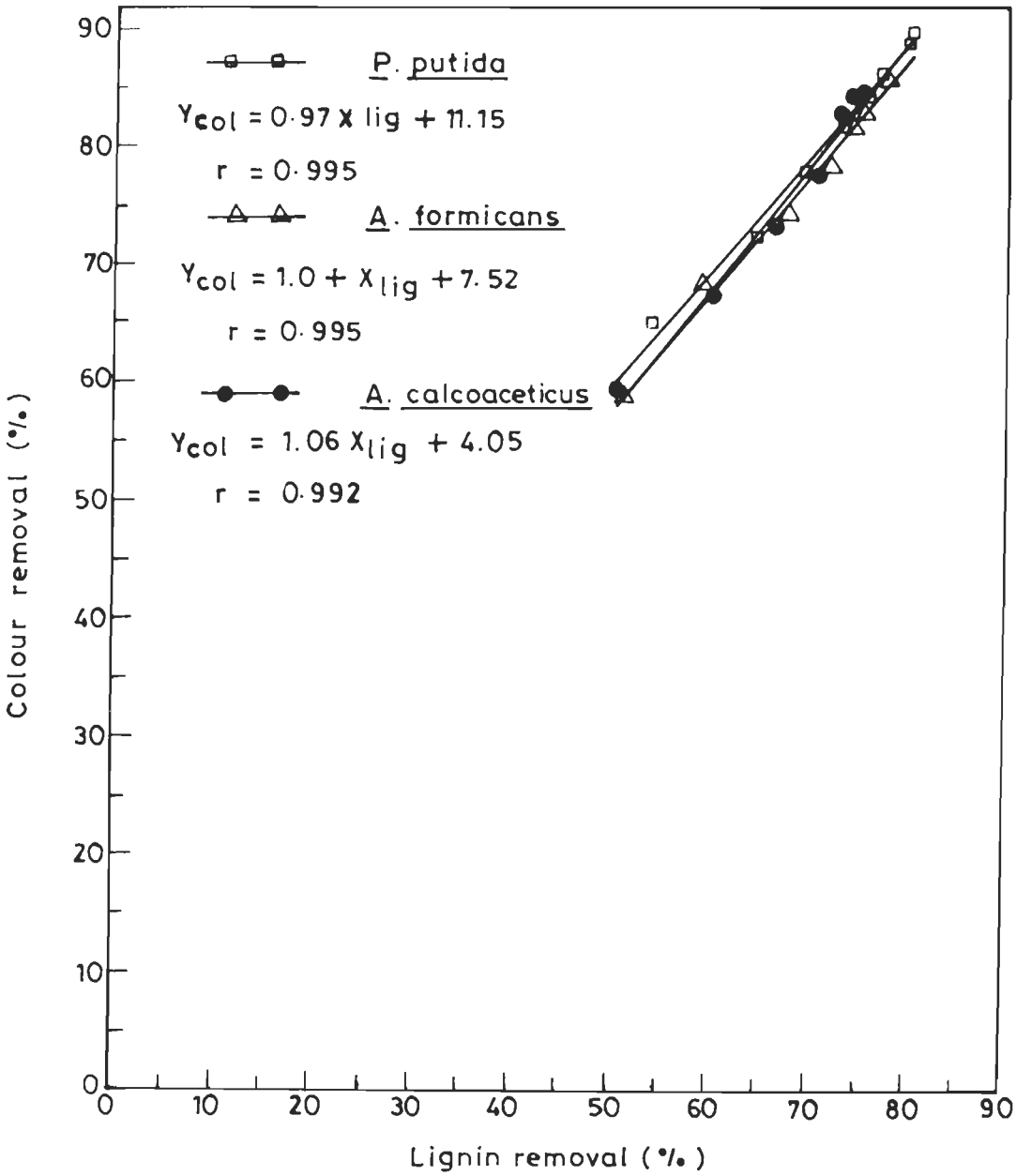


FIG. 4.17 RELATIONSHIP BETWEEN PERCENT COLOUR REMOVAL AND LIGNIN REMOVAL FROM BLACK LIQUOR

Similar results were also obtained by Larrea et al. (91) during aeration and precipitation experiments of kraft mill waste water which showed a linear relationship between COD and colour reduction versus lignin reduction in both cases of aeration and precipitation.

Various mechanisms [93] have been suggested to explain the activity of bacterial strains involved in the removal of COD, colour and lignin from black liquor.

In one case, the removal of organics (lignin) is explained on the basis of coagulation of colloidal portion when waste water comes in contact with a culture of microorganisms. The coagulated mass is adsorbed onto the microbial mass surface. At the same time a large portion of the soluble organic matter is rapidly absorbed or diffuses into the cells. Later on the adsorbed portion gets hydrolysed and released back into the solution and this diffuses back inside the cell for metabolism.

In the other mechanism, it is believed that organic matter is transported across a bacterial cell wall by a specific set of enzymes called permeases. Once inside the cell, enzymatic reactions in metabolic pathways are completed for the synthesis of new protoplasm and production of new cells. It is also proposed that extracellular hydrolase enzymes are secreted to hydrolyse the long chain, high molecular weight substances into smaller units, so that these may be transported into the cell by permeases. A schematic representation of the process is shown in Fig.4.18. Several lignin degrading extracellular enzymes collectively called as ligninases have been identified earlier [81] in the white rot fungus *P. chrysosporium*. These enzymes act through a mechanism involving free radical formation. Evidence has been presented to show that *Streptomyces viridosporus* produces a ligninolytic enzyme complex [82] involved in demethylation of lignin aromatic rings, and cleavage of ß-ether linkages within the polymer. These evidence give support to the second mechanism of lignin removal or its degradation in black liquor.

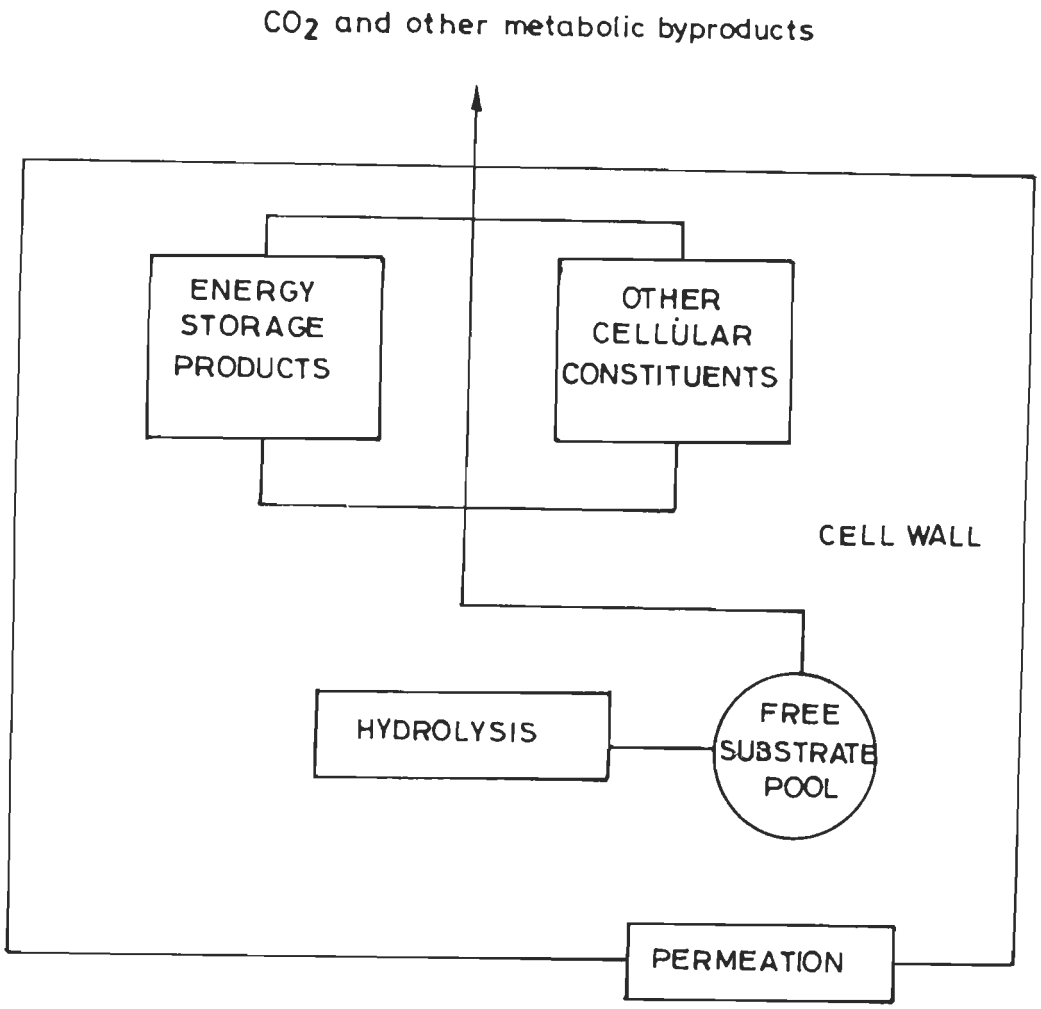


FIG. 4.18 SCHEMATIC REPRESENTATION OF ENZYMATIC THEORY

CHAPTER -5

ANALYSIS OF LIGNIN DEGRADATION PRODUCTS BY GAS CHROMATOGRAPHY

5.1 GENERAL

This chapter includes the gas chromatography of black liquor (supernatants) obtained after degradation, at the end of batch studies by all the three tested bacterial strains. For the identification of lignin degradation products, control i.e. uninoculated incubated black liquor was also subjected to gas chromatography. The methodology used for the sample preparation, conditions of experiment and results obtained are described in this section.

5.2 EXPERIMENTAL METHODOLOGY

5.2.1 EXTRACTION AND RECOVERY OF AROMATIC COMPOUNDS

Extraction and recovery of aromatic compounds from the samples were carried out as described by Crawford [68]. To recover the aromatic compounds present in the supernatant of reaction mixture at the end of batch experiment on the 20th day, all the three inoculated and control samples (50ml) were filtered across a millipore filter (0.45 μm) to separate the sludge. The resulting solutions were then acidified to pH 1.5 with concentrated HCl and thoroughly extracted with ethyl acetate to recover low molecular weight aromatic compounds present in supernatants. Thereafter sodium sulfate was added to dewater ethyl acetate. The yield of ethyl acetate extractives were determined by evaporating away ethyl

acetate in pre-weighed (±0.1mg) beakers and then reweighing the beakers. The schematic representation of the total process is given in Fig.5.1. These aromatic extractives were subjected to gas chromatography after the preparation of trimethylsilyl derivatives.

5.2.2 PREPARATION OF TRIMETHYLSILYL DERIVATIVES

Aromatic compounds present in ethyl acetate extractives of inoculated culture supernatants and controls were characterized by gas chromatography as trimethylsilyl (TMS) derivatives. Unknown compounds present in extractives were identified by matching their retention times with known standards, also chromatographed as TMS-derivatives. For the preparation of derivatives [94] about 3 mg of extractive containing unknown compounds was dissolved in 100 µl dioxane and 10 µl pyridine and to this 50 µl of N,O-bis (trimethyl silyl) acetamide (BSA) (Pierce Chem. Co., Rockford III) was added. The process of derivatization was same for all the extractives obtained from inoculated culture supernatant and control. Derivatives of known standards were also prepared with dioxane, chloroform and dimethyl formamide as solvent. The process of silylation was done at room temperature and reaction mixtures were analyzed by gas chromatography after 5 minutes of silylation.

5.2.3 GAS CHROMATOGRAPHY

Gas chromatographic analysis was performed at Indian Institute of Petroleum (IIP), Dehradun, India. It was accomplished on a Varian 5700 gas chromatograph and the detailed conditions of the analysis and instrument are as under :

Column	:	Stainless steel 6' x 1/8"
Column support	:	Chromosorb-G, acid washed, 80/100 mesh, treated with dimethyl dichlorosilane

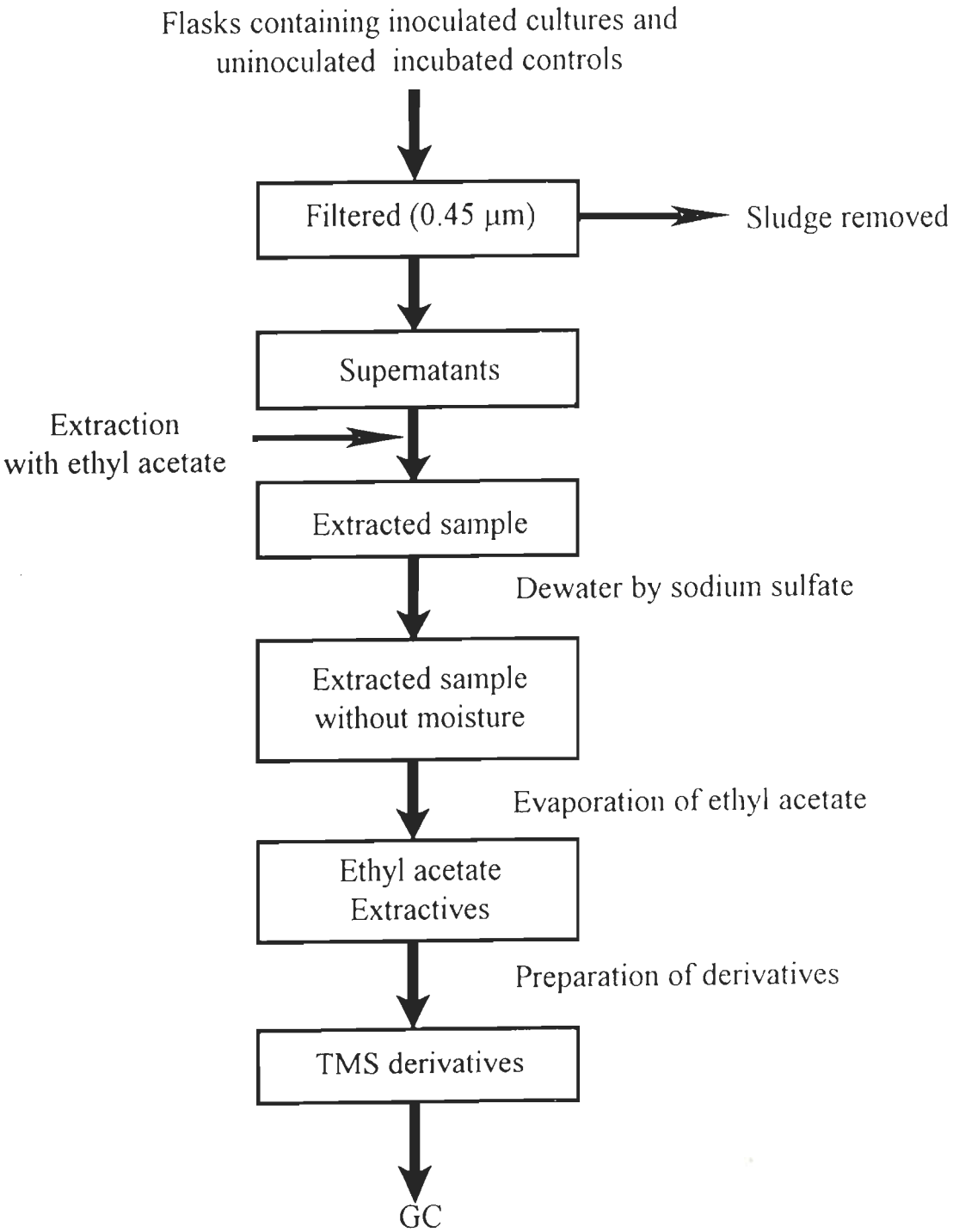


FIG. 5.1 SCHEMATIC REPRESENTATION OF THE PROCESS ADOPTED FOR PREPARATION OF SAMPLES FOR GC ANALYSIS.

Stationary phase	:	OV-17, 3% by weight of solid support (Applied Science Co., Switzerland)
Detector	:	Flame ionization detector (FID)
Carrier gas	:	Nitrogen, 30 ml per min
Injection temperature	:	240°C
Detector temperature	:	280°C
Column temperature	:	230°C

5.3 RESULTS AND DISCUSSION

The yields of ethyl acetate extractives in inoculated culture supernatants and control on the 20th day are shown in Table 5.1 and TMS derivatives of these extractives were subjected to analysis. The results are shown in Tables 5.2, 5.3 and 5.4 for *P.putida*, *A.formicans* and *A.calcoaceticus*, respectively. These Tables present the retention time of peaks formed due to the compounds present in extractives of inoculated culture supernatants as well as control. Figs. 5.2, 5.3, 5.4 and 5.5 depict the peaks of compounds present in the extractives of control and inoculated culture supernatants respectively.

Table 5.1 shows that the recoveries from inoculated culture extractives of ethyl acetate are lower than that of control. Low molecular weight aromatics are released from lignins in control experiments as a result of leaching during the extended incubation period. The inoculated cultures, have some additional compounds which are released from lignin by microbial action. The mircobes, presumably metabolize many of the aromatic fragments and this may be the primary reason for lower recoveries from inoculated culture supernatant in comparison to control.

Table - 5.1

RECOVERIES OF ETHYL ACETATE EXTRACTIVES FROM ACIDIFIED INOCULATED CULTURE SUPERNATANTS AND CONTROL

Bacterial Strains	Ethyl Acetate Extractives (mg)
P. putida	3.1
A. formicans	3.7
A. calcoaceticus	4.2
Control	9.7

Tables 5.2, 5.3 and 5.4 depict the retention time of peaks formed due to compounds present in extractives of inoculated culture supernatants and control along with the identified compounds confirmed by matching their retention time with known standards. The identified compounds formed as a result of interaction of *P.putida* with lignin (Table 5.2) are p-hydroxy phenyl acetic acid (PHPA), p-hydroxy benzoic acid (PBA), vanillic acid (VA), protocatechuic acid (PCA), syringic acid (SA), p-coumaric acid (CA) and ferulic acid (FA). The identified products of lignin degradation by *A. formicans* (Table 5.3) include-PHB, VA, PCA, SA and FA and the same in the case of *A. calcoaceticus* (Table 5.4) are PHPA, PHB, VA and FA. These compounds are reasonably justified to be lignin fragments on the basis of their structures [68]. None of these are detected in incubated controls. Numerous other extractives recovered from supernatants of control and inoculated cultures could, however, not be identified.

The accumulation pattern for the principal substituted benzoic acid in supernatants that would be expected to arise from microbial attack on coumaryl,

Table - 5.2

LIGNIN FRAGMENT ACCUMULATION PATTERNS IN CULTURE SUPERNATANT OF CONTROL AND *P. putida* INOCULATED BLACK LIQUOR AFTER 20 DAYS OF INCUBATION

Peak Retention Time (min)	Present in		Identified Compounds*
	Control	Inoculated	
0.73	+	+	
0.91	-	+	
1.14	+	+	
1.33	-	+	
1.66	-	+	PHPA
1.72	-	+	PHB
1.99	+	+	
2.10	+	-	
2.42	-	+	VA
2.93	-	+	PCA
3.30	+	-	
3.57	-	+	SA
3.98	+	-	
4.10	-	+	CA
5.05	+	+	
5.78	-	+	
6.31	-	+	FA

* Confirmed by matching the retention time with known standards: PHPA, p-hydroxy phenyl acetic acid-TMS; PHB, p-hydroxy benzoic acid-TMS; VA, Vanillic acid-TMS; PCA, Protocatechuic acid-TMS; SA, Syringic acid-TMS; CA, p-coumaric acid-TMS; FA, Ferulic acid-TMS.

Table - 5.3

LIGNIN FRAGMENT ACCUMULATION PATTERNS IN CULTURE SUPERNATANT OF CONTROL AND *A. formicans* INOCULATED BLACK LIQUOR AFTER 20 DAYS OF INCUBATION

Peak Retention Time (min)	Present in		Identified Compounds*
	Control	Inoculated	
0.73	+	+	
0.91	-	+	
1.14	+	-	
1.33	-	+	
1.72	-	+	PHB
1.99	+	+	
2.10	+	+	
2.42	-	+	VA
2.93	-	+	PCA
3.30	+	-	
3.57	-	+	SA
3.98	+	-	
5.05	+	+	
5.38	-	+	
6.31	-	+	FA

* Confirmed by matching the retention time with known standards: PHB, p-hydroxy benzoic acid-TMS; VA, Vanillic acid-TMS; PCA, Protocatechuic acid-TMS; SA, Syringic acid-TMS; FA, Ferulic acid-TMS.

Table - 5.4

LIGNIN FRAGMENT ACCUMULATION PATTERNS IN CULTURE SUPERNATANT OF CONTROL AND *A. calcoaceticus* INOCULATED BLACK LIQUOR AFTER 20 DAYS OF INCUBATION

Peak Retention Time (min)	Present in		Identified Compounds*
	Control	Inoculated	
0.73	+	+	
1.14	+	+	
1.33	-	+	
1.66	-	+	PHPA
1.72	-	+	PHB
1.99	+	-	
2.10	+	-	
2.42	-	+	VA
3.30	+	+	
3.98	+	+	
5.05	+	+	
6.31	-	+	FA

* Confirmed by matching the retention time with known standards: PHPA, p-hydroxy phenyl acetic acid-TMS; PHB, p-hydroxy benzoic acid-TMS; VA, Vanillic acid-TMS; FA, Ferulic acid-TMS.

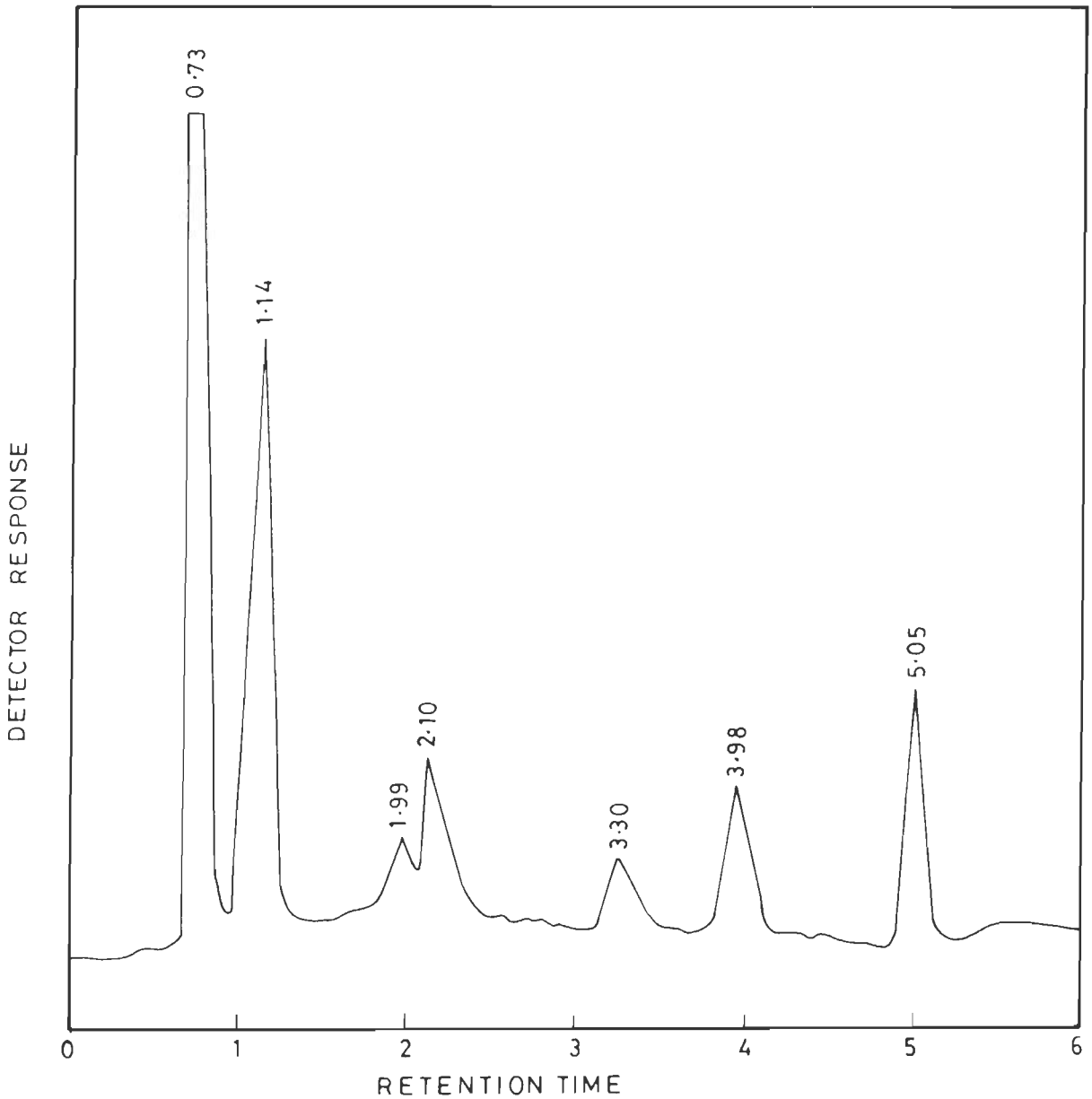


FIG. 5·2 GAS CHROMATOGRAPHIC ANALYSIS OF UNINOCULATED INCUBATED BLACK LIQUOR (CONTROL)

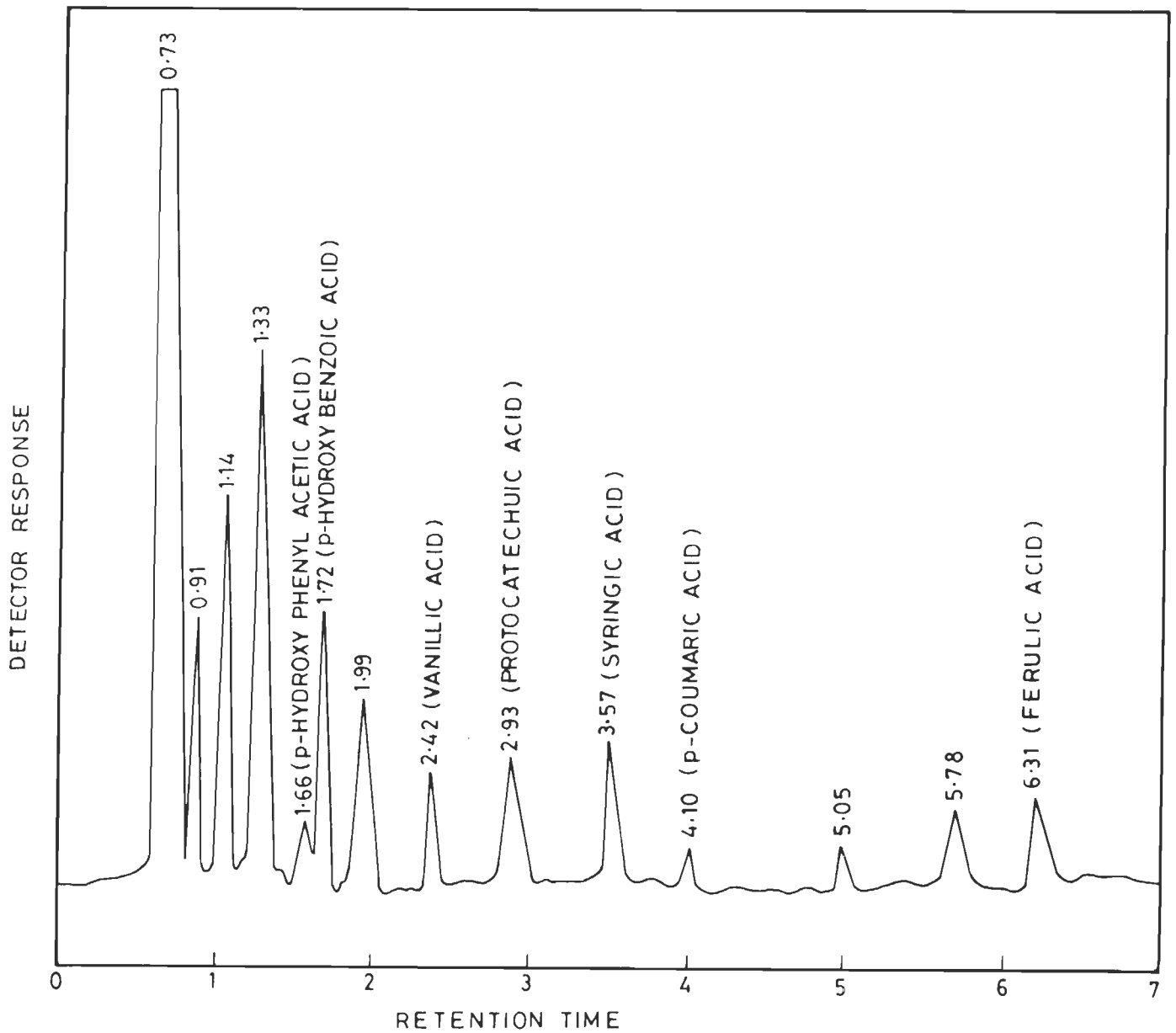


FIG. 5·3 GAS CHROMATOGRAPHIC ANALYSIS OF BLACK LIQUOR AFTER DEGRADATION BY P. putida, SHOWING RETENTION TIME OF PEAKS AND NAMES OF IDENTIFIED COMPOUNDS

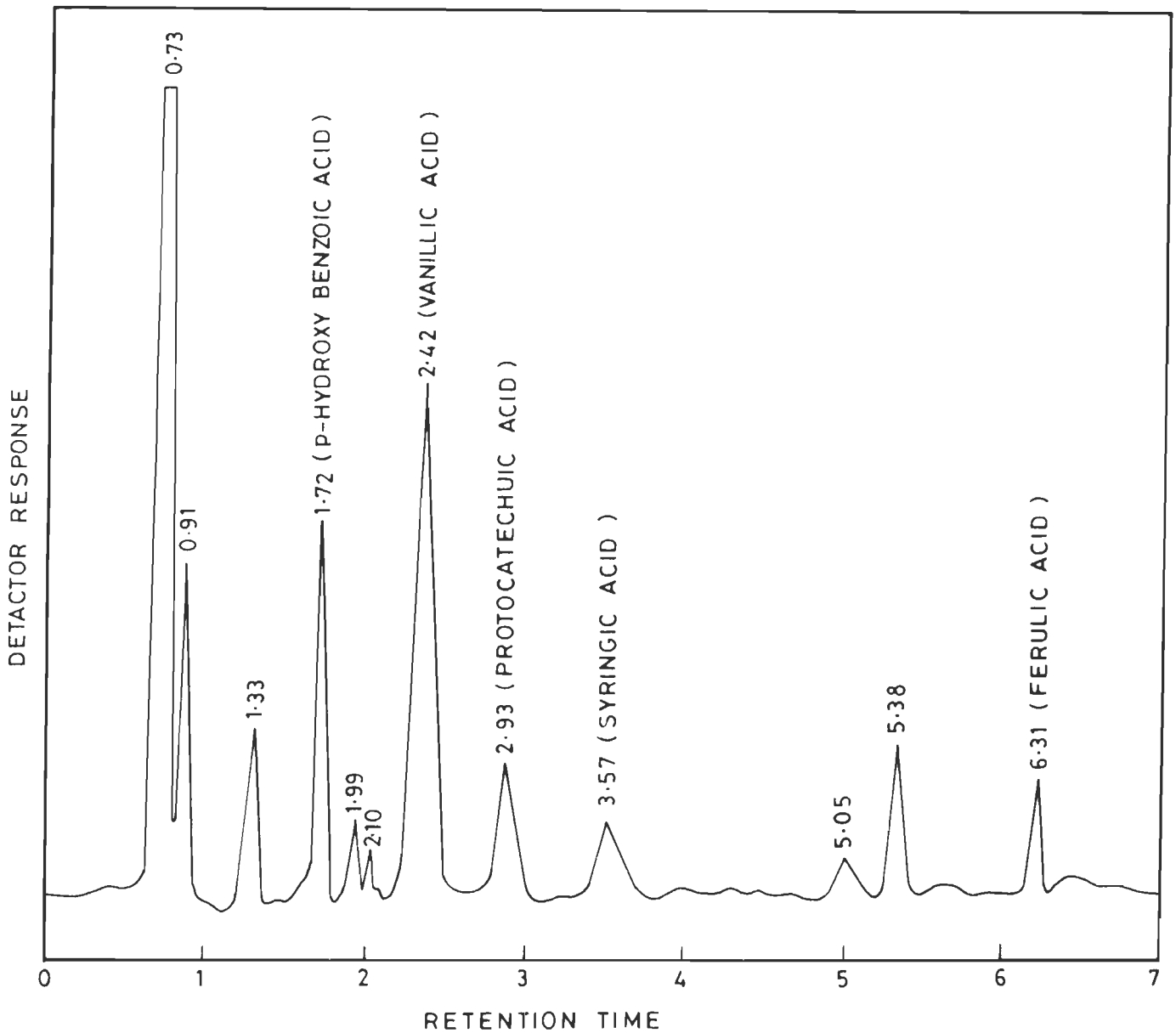


FIG. 5·4 GAS CHROMATOGRAPHIC ANALYSIS OF BLACK LIQUOR AFTER DEGRADATION BY A. formicaus SHOWING RETENTION TIME OF PEAKS AND NAMES OF IDENTIFIED COMPOUNDS

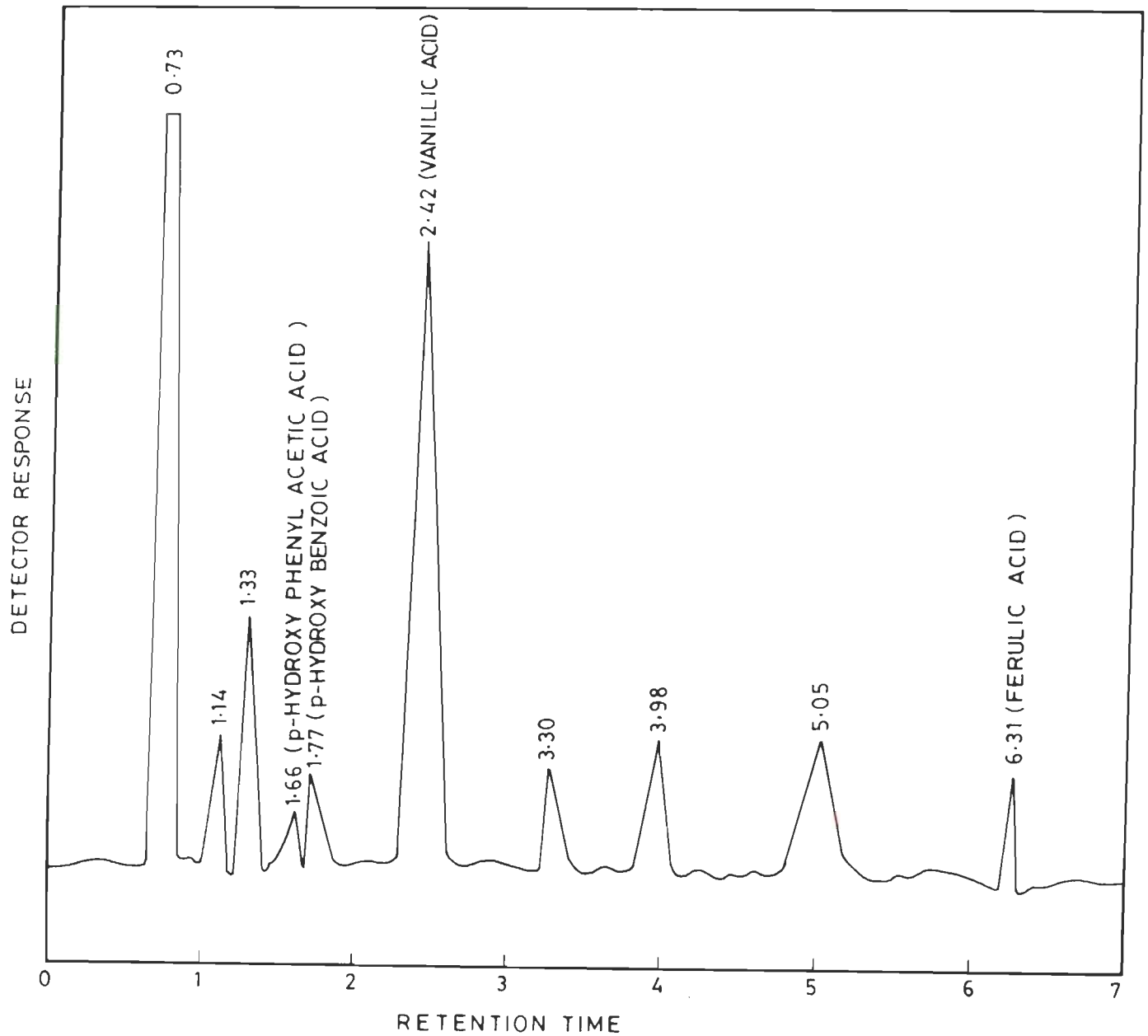


FIG. 5·5 GAS CHROMATOGRAPHIC ANALYSIS OF BLACK LIQUOR AFTER DEGRADATION BY A. calcoaceticus, SHOWING RETENTION TIME OF PEAKS AND NAMES OF IDENTIFIED COMPOUNDS

guiacyl or syringyl units in lignin, include PHB, VA and SA respectively. Each of these aromatic acids has earlier been reported as a lignin degradation intermediate in case of fungi [95]. These acids would probably be released from lignin due to the oxidative attack on terminal phenyl propane units, in particular [96]. It is observed (Tables 5.2, 5.3 and 5.4) that PHB is recovered, as a fragment, in all the three inoculated culture supernatant extractives. As PHB is released in inoculated cultures the appearance of protocatechuic acid takes place, as a degradative intermediate in the case of *P.putida* and *A.formicans* [97]. The release of PHB in cultures may be due to the oxidative degradation of coumaryl units in the lignin, especially peripheral units covalently linked to the main polymer via benzyl-aryl ether bonds [97].

Vanillic acid (VA) is also present in all the three inoculated culture supernatants. It may be a very significant catabolic intermediate because of the significant content of guiacyl units in lignin polymer. However, if guiacyl structures were demethylated and/or their aromatic rings cleaved within a largely intact polymer, VA would not necessarily be released as a catabolic intermediate [68]. For example, if demethylation occurs prior to fragment release, protocatechuic acid (PCA) would be released. PCA is readily metabolized by microbes and results in the formation of vanillic acid. No recovery of PCA in case of *A. calcoaceticus* (Table 5.4) shows that all the PCA has been metabolized to VA. Alternatively, ring cleavage reactions within an intact polymer, as reported for white rot fungus [96], would release neither PCA nor VA as lignin degrading intermediate.

p-Coumaric acid (CA) and syringic acid (SA) are detected in the supernatants of extractives of the reaction mixture inoculated by *P.putida*. Syringic acid is also detected in the extractive of *A.formicans* inoculated culture supernatant. Since lignins present in black liquor also contain coumaryl and syringyl units, besides the guiacyl units, the release of syringic acid or p-coumaric acid is not unexpected.

Figs. 5.2, 5.3, 5.4 and 5.5 have several other peaks indicating the presence of some unidentified aromatic compounds.

CHAPTER -6

DEGRADATION OF BLACK LIQUOR-CONTINUOUS PROCESS

6.1 GENERAL

Based on the results of batch studies on treatment of black liquor, by the three selected strains of bacteria viz. *P.putida*, *A.formicans* and *A.calcoaceticus*, continuous treatment studies on laboratory scale were also conducted. Investigations reported in this chapter describe the continuous degradation of black liquor, on laboratory scale, for the removal of COD, colour and lignin, using above mentioned three strains of bacteria.

6.2 EXPERIMENTAL METHODOLOGY

To study the continuous treatment of black liquor on laboratory scale, completely mixed, continuous flow aerated reactors were used. The reactors were fabricated by using plexiglass of six litre capacity and the working volume was 4 litre. Compressed air was introduced by air diffuser pipes with nozzles and the concentration of dissolved oxygen was measured by DO probe and the same was never allowed to drop below 3 mgl^{-1} in order to maintain the aerobic conditions. A thorough mixing of the black liquor in the reactor was maintained by stirrer. The substrate feed was allowed to flow under gravity by a constant head arrangement from a reservoir (capacity 8 litre) by rubber tubings. All experiments were conducted at room temperature (25-30^{0}C). A schematic representation of the experimental setup is shown in Fig.6.1.

To start the experiment, lab models of reactors having 4 litre of black liquor

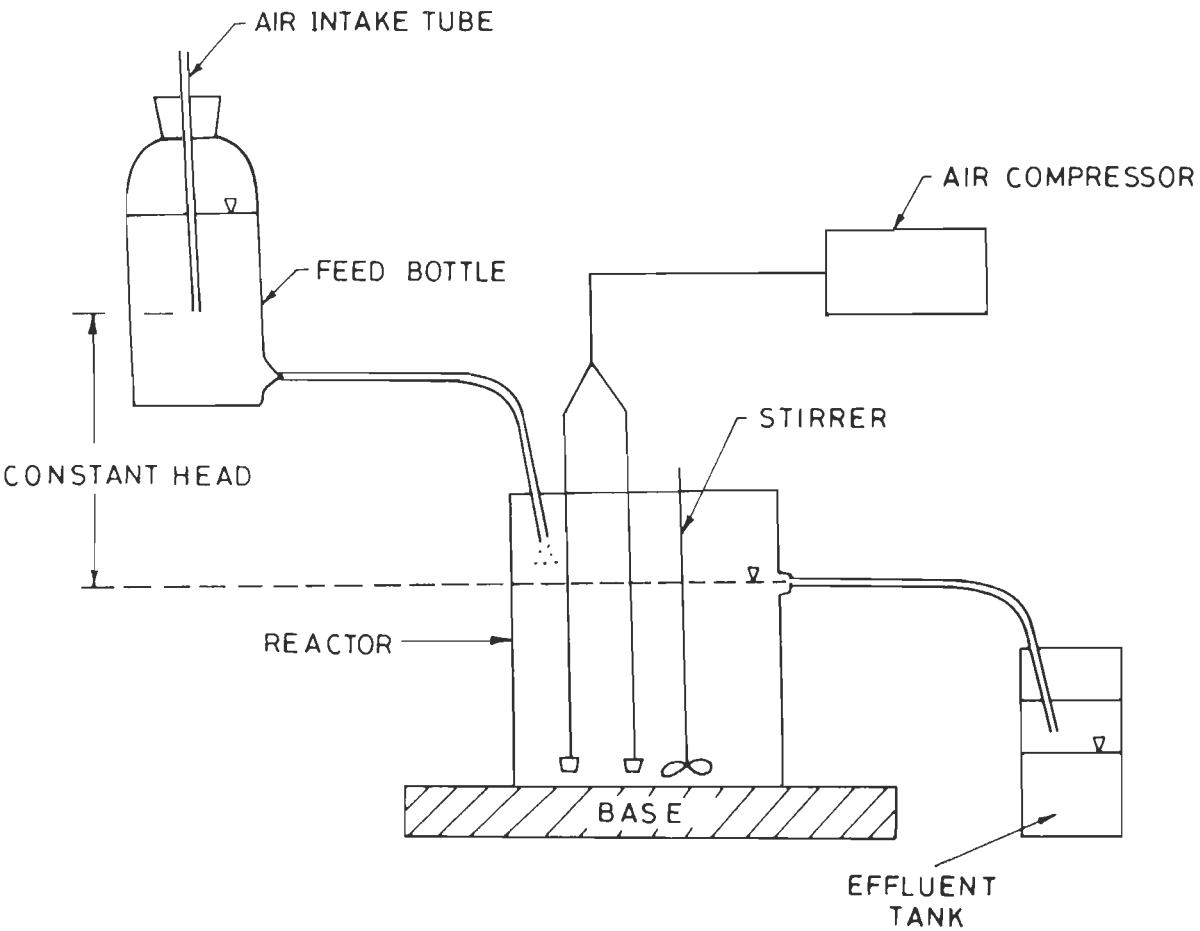


FIG. 6·1 CONTINUOUS REACTOR EXPERIMENTAL SET-UP

were inoculated with tested microbes separately. Inocula was prepared as described in chapter 3 (3.4.1.3) and other conditions of the experiment like black liquor concentration, pH and amount of extra nutrients added (C,N,P) were kept at optimum values for each strain as used in batch studies. These reactors were kept in batch for 8 days to mature and the efficiency of removal of COD, colour and lignin was measured after 1-6 and 8 days of incubation. A time period of 8 days was fixed up on the basis that the bacteria remains in log phase for some time (8 to 10 days) and most of the removal of COD, colour and lignin takes place in this period, as observed in batch studies.

Thereafter 500 ml of black liquor was fed everyday (starting from the 8th day) drop by drop continuously from the reservoir, at a rate of 2.88 ml per minute to give a detention time of around 8 days. The influent which had a constant concentration and pH, was supplemented with nitrogen and phosphorus sources as before. Further addition of glucose as extra carbon source was not required. The sludge coming out with the reractor's effluent was not recycled and thrown away. The effluent from the reactor was tested regularly for COD, colour and lignin removal as parameters of treatment.

6.3 RESULTS AND DISCUSSION

The results of the continuous treatment of black liquor on laboratory scale are presented in Figs.6.2, 6.3 and 6.4 for the bacterial strains *P.putida*, *A.formicans* and *A.calcoaceticus* respectively.

A perusal of figures indicate that in case of each strain the removal efficiency of COD, colour and lignin increases with time from 0th to 8th day of incubation during batch phase.It is further observed that a 70 to 80 percent removal, in all the parameters, is achieved with each strain and these values are almost equal to the one obtained in batch studies.

Further analysis of curves (Figs. 6.2, 6.3 and 6.4) reveal that when black

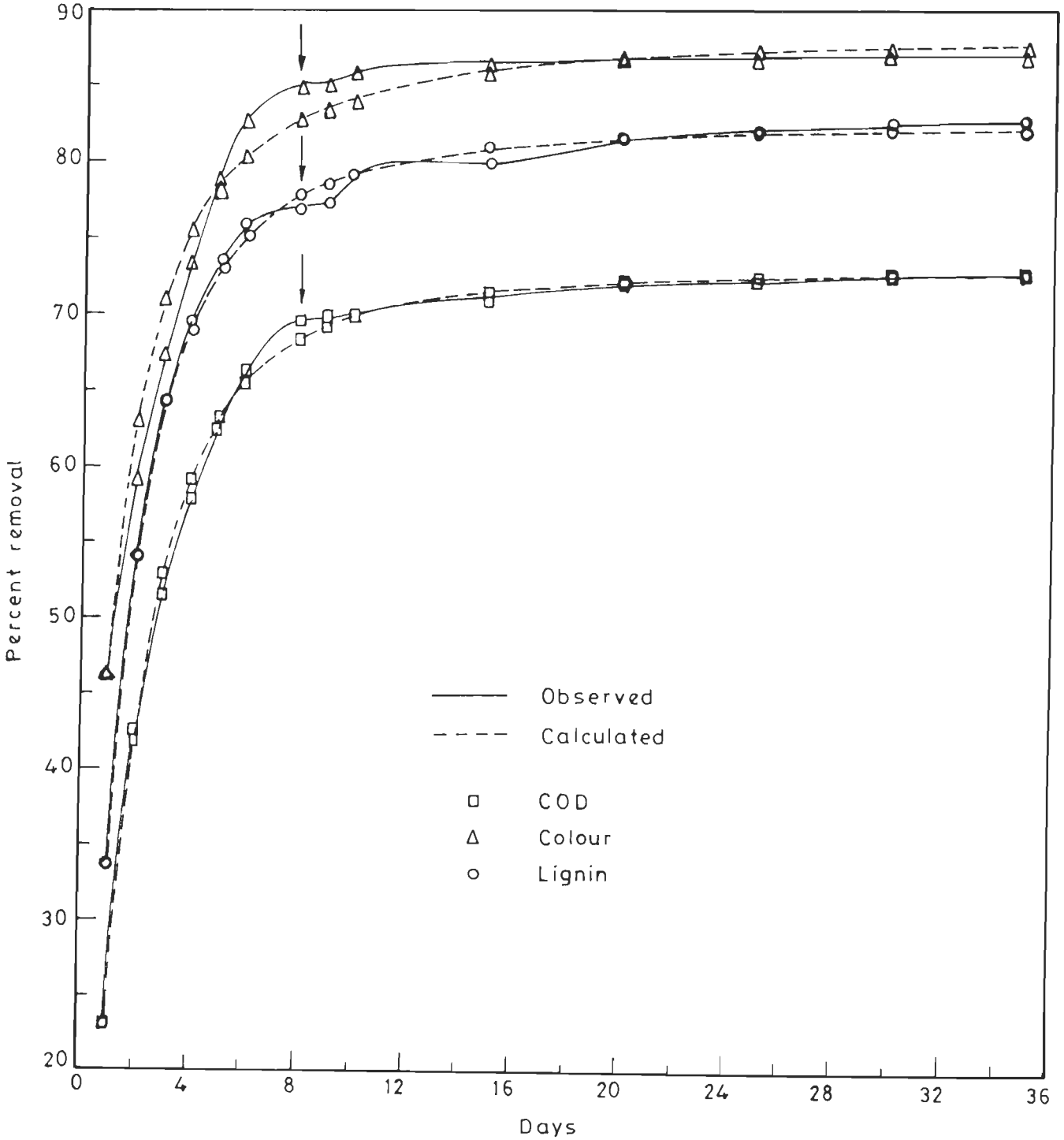


FIG. 6.2 CONTINUOUS REMOVAL OF COD, COLOUR AND LIGNIN DURING BLACK LIQUOR DEGRADATION BY P. putida THE ARROW (⟶) INDICATING THE START OF THE CONTINUOUS FEEDING OF BLACK LIQUOR

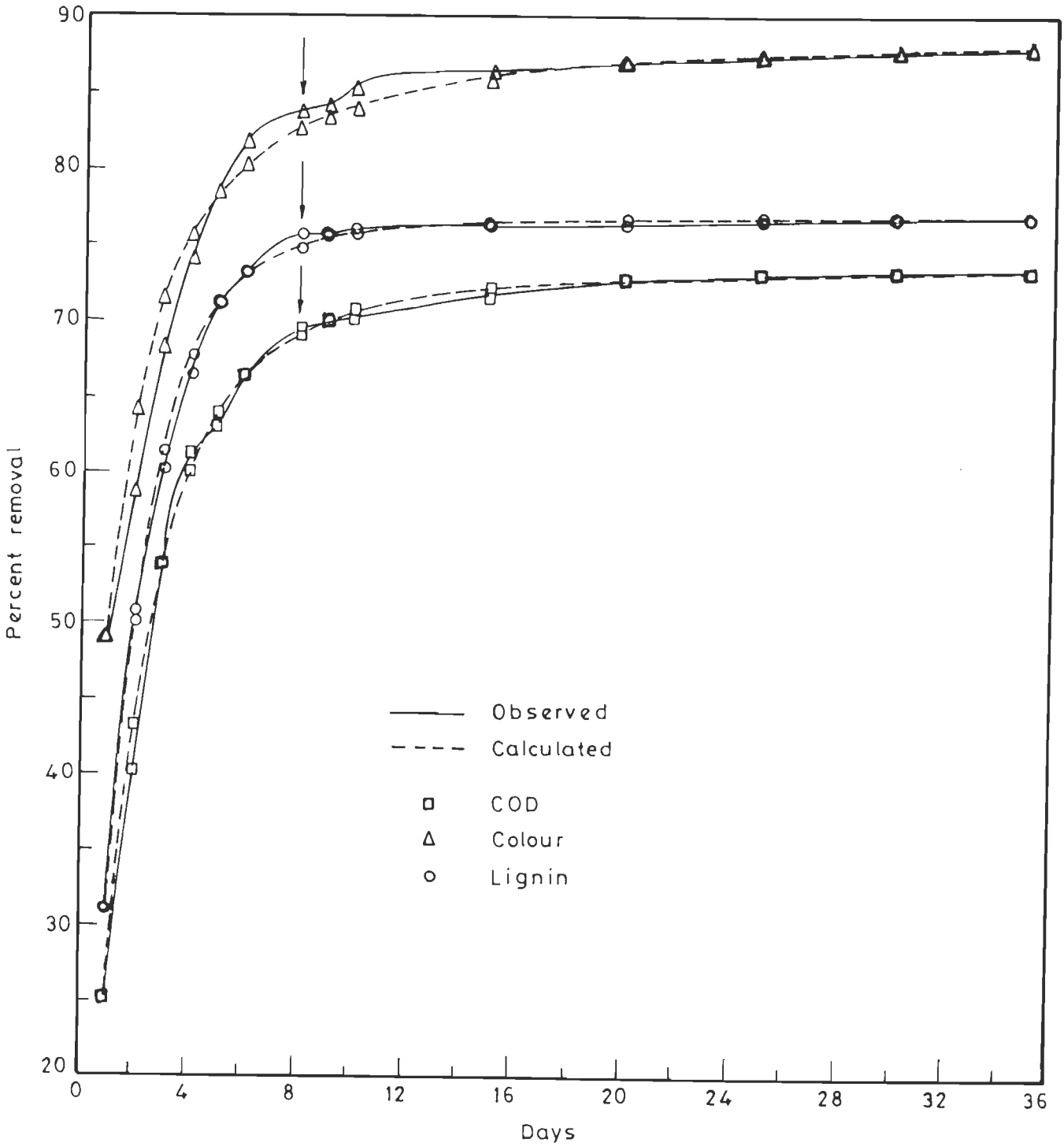


FIG. 6.3 CONTINUOUS REMOVAL OF COD, COLOUR AND LIGNIN DURING BLACK LIQUOR DEGRADATION BY A. formicans THE ARROW (⟶) INDICATING THE START OF THE CONTINUOUS FEEDING OF BLACK LIQUOR

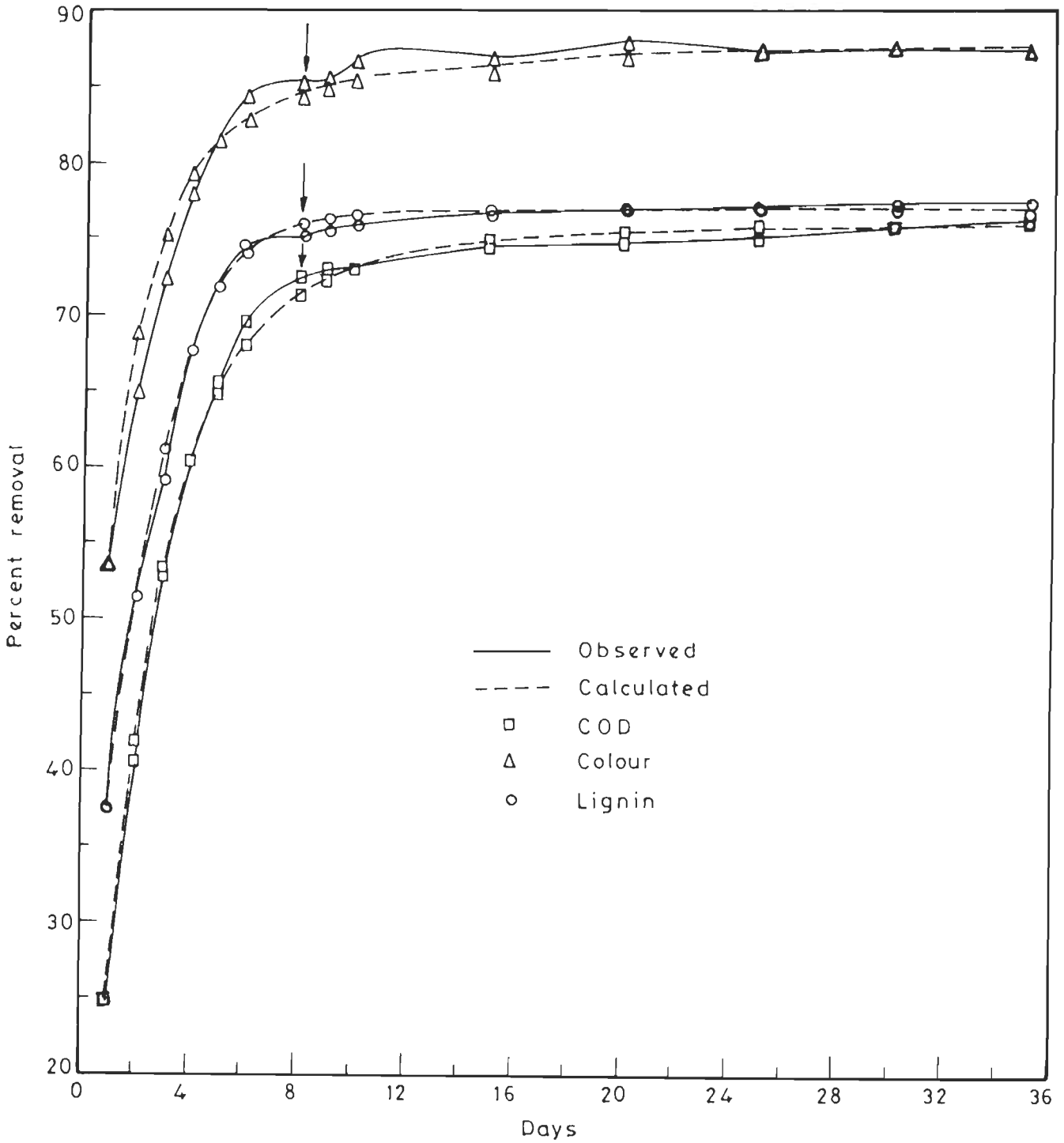


FIG. 6.4 CONTINUOUS REMOVAL OF COD, COLOUR AND LIGNIN DURING BLACK LIQUOR DEGRADATION BY A. calcoaceticus THE ARROW (⟶) INDICATING THE START OF THE CONTINUOUS FEEDING OF BLACK LIQUOR

liquor is continuously fed in the reactor from the 8th day, a negligible increase in efficiency is obtained for the first two days. But after two days time, a slow enhancement in the removal efficiency of COD, colour and lignin is observed up to the 20th day and becomes fairly constant, after this period, for each strain. The negligible increase in the first two days was due to shock loading of the reactor.

The maximum efficiency of removal at the end of experiment (35th day) for *P.putida* (Fig.6.2) are found to be 72.68, 86.97 and 82.69 percent for COD, colour and lignin respectively. The same values for the other two strains i.e. *A.formicans* (Fig.6.3) and *A.calcoaceticus* (Fig. 6.4) are 73.21, 82.72 and 77.44 percent and are 76.23, 87.43 and 77.45 percent for COD, colour and lignin respectively.

An equation of the following type has been proposed by Srivastva [92] for BOD exertion.

$$\eta_t = \eta_{max} \frac{(t/t_*)^m}{\left[1+(t/t_*)^{m/n}\right]^n}$$

where,

η_t = Percent removal of COD, colour or lignin at time t

t = Time in days

η_{max} = Maximum attainable percent removal of COD, colour or lignin

t_* = Apparent ultimate time in days

m = Removal rate exponent

n = Transition exponent

Attempts have been made to propose similar equations for the COD, colour and lignin removal in black liquor by the three selected strains of bacteria. A computer programme was written for fitting the experimental data for each strain, in the above equation, to evaluate the corresponding parameters. The fitting of the curves was carried out by minimizing the error estimate CAPE [92].

The calculated values for COD, colour and lignin removal (percent) by all the three strains are shown plotted (dotted lines) in Figs. 6.2, 6.3 and 6.4 along with the observed values. The values of different parameters viz. m, n, t_* and η_{max} are shown in Table 6.1 for COD, colour and lignin removal efficiency by each strain of bacteria.

It is apparent (Table 6.1) that the rate of removal (m) of COD, colour and lignin by *P.putida* is maximum in comparison to the other two strains. *A.calcoaceticus* have lowest rate of removal and intermediate values are recorded for *A.formicans* for all the three parameters of black liquor treatment.

To calculate the percent COD, colour and lignin removal efficiencies at any time for each selected strain, the following relationships are obtained from the parameters shown in Table 6.1:

P.putida

(i) COD

$$\eta_{t,COD} = 72.93 \frac{(t/2.40)^{1.18}}{\left[1+(t/2.40)^{1.18/0.64}\right]^{0.64}}$$

(ii) Colour

$$\eta_{t,col} = 88.55 \frac{(t/1.51)^{0.85}}{\left[1+(t/1.51)^{0.85/0.65}\right]^{0.65}}$$

(iii) Lignin

$$\eta_{t,lig} = 82.61 \frac{(t/1.60)^{1.25}}{\left[1+(t/1.60)^{1.25/0.79}\right]^{0.79}}$$

A.formicans

(i) COD

$$\eta_{t,COD} = 73.43 \frac{(t/2.62)^{1.02}}{\left[1+(t/2.62)^{1.02/0.54}\right]^{0.54}}$$

Table - 6.1
VALUES OF KINETIC PARAMETERS FOR EACH BACTERIAL STRAIN

Bacterial Strains	COD Removal				Colour Removal				Lignin Removal			
	m	n	t_* (days)	η_{max} (%)	m	n	t_* (days)	η_{max} (%)	m	n	t_* (days)	η_{max} (%)
P. putida	1.18	0.64	2.40	72.93	0.85	0.65	1.51	88.55	1.25	0.79	1.60	82.61
A. formicans	1.02	0.54	2.62	73.43	0.74	0.61	1.51	89.09	0.80	0.31	3.03	76.79
A. calcoaceticus	0.87	0.41	3.49	76.16	0.68	0.47	1.53	88.11	0.46	0.11	4.77	76.97

(ii) Colour

$$\eta_{t,\,col} = 89.09\,\frac{(t/1.51)^{0.74}}{\left[1+(t/1.51)^{0.74/0.61}\right]^{0.61}}$$

(iii) Lignin

$$\eta_{t,\,lig} = 76.79\,\frac{(t/3.03)^{0.80}}{\left[1+(t/3.03)^{0.80/0.31}\right]^{0.31}}$$

A.calcoaceticus

(i) COD

$$\eta_{t,\,COD} = 76.16\,\frac{(t/3.49)^{0.87}}{\left[1+(t/3.49)^{0.87/0.41}\right]^{0.41}}$$

(ii) Colour

$$\eta_{t,\,col} = 88.11\,\frac{(t/1.53)^{0.68}}{\left[1+(t/1.53)^{0.68/0.47}\right]^{0.47}}$$

(iii) Lignin

$$\eta_{t,\,lig} = 76.97\,\frac{(t/4.77)^{0.46}}{\left[1+(t/4.77)^{0.46/0.11}\right]^{0.11}}$$

These equations can be utilized in the design of reactors for COD, colour and lignin removal from black liquor by specific strain, where the detention time t (hydraulic retention time) can be found out for achieving the desired efficiency η_t.

CONCLUSIONS

The preceding chapters of this dissertation cover a study on the degradation of black liquor by three selected strains of bacteria. Optimization of growth conditions for each bacterial strain was done in black liquor and degradation efficiency was monitored in terms of percent COD, colour and lignin removal. Further a detailed study was done on degradation of black liquor in batch and continuous systems. Samples obtained after the batch treatment of black liquor were analyzed on gas chromatography to identify the lignin degradation products. These investigations have led to the following conclusions :

1. Three bacterial strains viz. *Pseudomonas putida*, *Aeromonas formicans* and *Acinetobacter calcoaceticus* were chosen for the degradation of black liquor. The growth pattern of these strains in basal medium were similar to the exponential growth of microbes with not so defined lag phase. A distinct stationary phase was also not deciphered except for A.formicans.

2. Effect of varying pH, of basal medium, on the yield of bacterial cells show that *P.putida* and *A.formicans* have maximum cell yield around neutral pH. While A.calcoaceticus prefer to grow in slightly acidic environment.

3. All the three strains exhibit positive response to agitation, i.e. the bacterial cell yield increases on agitation during growth in basal medium.

4. Black liquor concentration around 50% was found to be most significant for the optimum removal of COD, colour and lignin by all the three strains.

5. The two strains *P. putida* and *A. formicans* show maximum efficiency of

COD, colour and lignin removal between pH 7.0 and 8.0 whereas the strain *A. calcoaceticus* functions best in the range of pH 5.0 to 6.0.

6. Optimum concentration of glucose as extra carbon source is found to be 0.4% for *P. putida* and *A. formicans*, and the same is 0.8% for *A. calcoaceticus* for best results.

7. 120 mg of ammonium chloride and 28 mg of phosphate per 100 ml of black liquor are required as optimum nitrogen and phosphorus sources to achieve maximum efficiencies of COD, colour and lignin removal from black liquor.

8. pH of the black liquor decreases during the treatment by batch process and the removal efficiency of COD, colour and lignin were in the range of 70 to 90 percent in 8 to 10 days of incubation for all the three strains of bacteria. *P. putida* exhibits highest removal efficiency, while the lowest efficiency is recorded in the case of *A. calcoaceticus*. However, the difference in the efficiency of removal, by these strains is not much.

9. Some phenolic acids are identified as lignin degradation products by gas chromatographic analysis of samples obtained in batch studies. The compounds formed as a result of the interaction of *P. putida* with lignin are identified as p-hydroxy phenyl acetic acid (PHPA), p-hydroxy benzoic acid (PHB), vanillic acid (VA), protocatechuic acid (PCA), syringic acid (SA), p-coumaric acid (CA), and ferulic acid (FA). The compounds obtained due to lignin degradation by *A. formicans* included-PHB, VA, PCA, SA and FA and the same in the case of *A. calcoaceticus* are PHPA, PHB, VA and FA.

10. Degradation of black liquor by all the three strains of bacteria in a continuous

reactor system show that the removal efficiency of COD, colour and lignin are almost same as observed in batch studies after a detention period of 8 days. All the strains show a continuous activity until the end of the experiment (35 days).

11. It is concluded that the treatment of black liquor by the three selected strains of bacteria is possible and economically feasible as the hydraulic retention time is drastically reduced with these microbes from 25 to 30 days (anaerobic lagoon treatment) to 8 days (aerated reactor system).

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