

**NEUTRALIZATION OF ALKALINE WASTE WATER FROM PULP  
AND PAPER INDUSTRY BY ALKALIPHILES**

A Dissertation

Submitted in the partial fulfilment of the requirements for the award of the  
degree of

**Master of Technology**

in

**Environment Science and Technology**

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Under the guidance of

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**DEPARTMENT OF BIOTECHNOLOGY AND ENVIRONMENT SCIENCES**

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**PATIALA-147004**

## **Declaration**

I, the undersigned, hereby declare that the research work presented in the M.Tech project entitled “**Neutralization of alkaline wastewater from pulp and paper industry using Alkaliphiles**” has been carried out by me under the supervision and guidance of Dr. Anita Rajor, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala.

Further, I declare that no part of this Dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

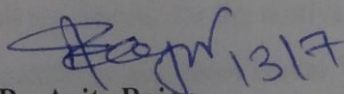
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## CERTIFICATE

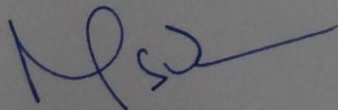
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To the best of our knowledge, the matter embodied in this thesis has not been submitted to any other university/institute for award of any degree/diploma.

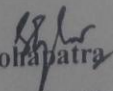
  
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## Abstract

Two alkaliphilic bacterial strains T1 and T2 capable of lowering pH and alkalinity of alkaline wastewater from pulp and paper industry were isolated in the present study. Bacterial strain T1 reduces the pH from 12 to 8.1 and alkalinity from 1040 mg/L to 480 mg/L whereas strain T2 reduces the pH from 12 to 7.95 and alkalinity from 1080 to 460 mg/L. Both alkaliphilic bacterial strains were gram positive and spore forming. Strain T1 has regular margin, glistening, convex and oval round in shape whereas strain T2 has regular margin, flat and irregular in shape. Both the strains can grow at wide range of Temperatures (10 - 44°C), pH (6 - 12) and NaCl concentrations (0 – 20 %). Strains T1 and T2 can utilize all the different types of Carbohydrates. Strain T1 can hydrolyse starch and show positive result for Catalase Screening whereas strain T2 can utilize citrate, hydrolyse casein and show positive results for Lipase Screening, pectinase screening and gelatin liquification test. Neutralization of highly alkaline wastewater without addition of any external carbon source highlights the potential use of the isolate as an alternative to the conventional acid neutralization method for treatment of alkaline wastewater.

**Keywords:** Alkalinity, Alkaliphiles, Neutralization, pH, wastewater

## CONTENTS

	PAGE No.
DECLARATION	ii
CERTIFICATE	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xi
<b>1 INTRODUCTION</b>	<b>1-10</b>
1.1 Modern paper production typically involves the following steps	1
1.2 The Pulp and Paper Industry in India	1
1.3 Recycled fibre (waste paper) sources	3
1.4 Energy benchmarking of Indian mills	3
1.5 Present Scenario (2000-2010) of Indian pulp and paper industry	4
1.6 Pollution problems of Pulp and Paper Industries	5
1.7 Microorganisms used for degradation of pulp and paper industry effluent	7
1.8 Alkaliphiles	7
1.9 Physico-chemical ways for neutralization of wastewater	8
1.9.1 Sulfuric Acid in Alkaline Wastewater Neutralization	8
1.9.2 Hydrochloric Acid in Alkaline Wastewater Neutralization	8
1.9.3 Neutralization with carbonic acid	9
1.9.4 Neutralization with flue gas	9
1.10 Alkalinity hazards on Plants and Crops	10
<b>2 LITERATURE REVIEW</b>	<b>11- 25</b>
2.1 Pulp and Paper Process	11
2.1.1 Wood Preparation	11
2.1.2 Pulping	11
2.1.2.1 Mechanical Pulping	11
2.1.2.2 Chemical Pulping	11
2.1.2.3 Kraft Pulping Process	12
2.1.2.4 BCTMP Process	12

2.1.2.5 Other Processes	12
2.1.3 Bleaching	13
2.1.4 Chemical Recovery	13
2.1.5 Papermaking	13
2.2 Bleaching of pulp generates the highly alkaline wastewater	14
2.3 Alkaliphiles: ‘basic’ molecular problems of pH tolerance and bioenergetics	14
2.4 Cytoplasmic pH Measurement and Homeostasis in Bacteria and Archaea	15
2.5 Type of Alkaliphiles	15
2.5.1 Geoalkalibacter Ferrihydriticus	15
2.5.2 Bacillus Okhensis	16
2.5.3 Alkalibacterium Iburiense	17
2.5.4 Amphibacillus Xylanus	18
2.5.5 Thermococcus Alkaliphilus	18
2.5.6 Thermococcus Acidaminovorans	18
2.6 Industrial Application of Alkaliphiles	19
2.6.1 Alkaline cellulases of alkaliphilic <i>Bacillus</i> strains	19
2.6.2 Lipases	20
2.6.3 Xylanases	21
2.6.4 Pectinases	21
2.6.5 Chitinases	22
2.7 Neutralization of alkaline industrial wastewaters using <i>Exiguobacterium sp.</i>	23
2.8 Biotechnological process for neutralizing alkaline beverage industrial wastewater	23
2.9 Biological neutralization of highly alkaline textile industrial wastewater	24
2.10 Biological neutralization of chlor-alkali industry wastewater	24
<b>3 MATERIALS AND METHODS</b>	<b>26- 38</b>
3.1 Collection of Sample	26
3.1.1 Effluent Samples	26
3.1.2 Reagents and instruments	26
3.1.3 Physiological characterization	26
3.2 Isolation of Bacteria	26
3.3 M9 medium	27

3.3.1 Composition of M9 media	27
3.4 Characteristics of Effluent	28
3.4.1 Biological Oxygen Demand	28
3.4.2 Chemical Oxygen Demand	28
3.4.3 Hardness Test	28
3.4.4 Chloride Test	28
3.4.5 Alkalinity Test	28
3.4.6 Phosphate Test	29
3.4.7 TSS, TDS and Total Solids	29
3.4.8 Carbohydrate Test	29
3.5 Morphological Studies	29
3.5.1 Gram Staining	29
3.5.2 Spore Staining	30
3.6 Identification of selected bacterial isolates	30
3.6.1 a) Morphological and biochemical characterization	30
3.6.1 b) Biochemical Tests	30
3.6.1.1 Starch Hydrolysis	30
3.6.1.2 Gelatin Agar medium	31
3.6.1.3 H <sub>2</sub> S Production	31
3.6.1.4 Lipase Screening	32
3.6.1.5 Methyl Red-Voges Proskaver test (MR-VP broth)	32
3.6.1.6 Carbohydrate Utilization	33
3.6.1.7 Xylanase Screening	34
3.6.1.8 Pectinase Screening	34
3.6.1.9 Citrate Utilization	35
3.6.1.10 Cellulase Screening	35
3.6.1.11 Catalase Screening	36
3.7 Physiological Testing	36
3.7.1 Effect of Incubation Temperatures on growth	36
3.7.2 Effect of pH on growth	37
3.7.3 Effect of salt concentrations on growth	37
3.8 pH and alkalinity degradation by bacterial strains	38

<b>4</b>	<b>RESULTS AND DISCUSSION</b>	<b>39-57</b>
4.1	Characteristics of Pulp and Paper Industry	39
4.2	Isolation and selection of Bacteria	40
4.2.1	Alkalinity Reduction Test	41
4.2.2	Identification of selected bacterial isolates	41
4.2.2.1	Morphological and biochemical characterization	41
4.3	Biochemical Tests	41
4.3.1	Biochemical activity of microorganisms	41
4.3.2	Intracellular enzyme	42
4.3.3	Pectinase Screening	42
4.3.4	Lipase Screening	42
4.3.5	Xylanase Screening	43
4.3.6	Starch Hydrolysis	43
4.3.7	Cellulose Activity	43
4.3.8	Gelatin Liquefaction	44
4.3.9	H <sub>2</sub> S Production	44
4.3.10	Citrate Utilization	45
4.3.11	Methyl Red Test	45
4.3.12	Voges Proskaver Test	45
4.3.13	Catalase Test	45
4.3.14	Carbohydrate Utilization Test	46
4.4	Physiological Test	47
4.4.1	Effect of temperature on bacterial growth	47
4.4.2	Effect of pH on growth of bacterial strains	48
4.4.3	Effect of salt concentrations on growth of bacterial strains	50
4.5	pH and Alkalinity degradation by bacterial strains	52
	<b>CONCLUSION</b>	<b>55</b>
	<b>REFERENCES</b>	<b>56-62</b>

## LIST OF TABLES

<b>Table No.</b>	<b>Title</b>	<b>Page</b>
1.1	Energy Consumption	4
3.1	M9 media Composition	27
3.2	Composition of Starch Hydrolysis	30
3.3	Composition of Gelatin Agar Medium	31
3.4	Composition for test of H <sub>2</sub> S Production	31
3.5	Composition for Lipase Screening Test	32
3.6	Composition for MR-VP Test	32
3.7	Composition of Carbohydrate Utilization Test	33
3.8	Composition for Xylanase Screening Test	34
3.9	Composition for Pectinase Screening Test	34
3.10	Composition for Citrate Utilization Test	35
3.11	Composition for Cellulase Screening Test	35
3.12	Composition of Catalase test	36
4.1	The characteristics of pulp and paper industry effluent	39
4.2	Morphological characteristics of bacterial isolates	41
4.3	Biochemical characterization of selected bacterial isolates	43
4.4	Biochemical characterization of selected bacterial isolates	44
4.5	Carbohydrate utilization test of the bacterial isolates	46

## LIST OF FIGURES

<b>Figure No.</b>	<b>Title</b>	<b>Page</b>
4.1	Growth of bacterial strains in M9 medium at different temperatures	47
4.2	Growth of bacterial strains in Nutrient broth at different temperatures	48
4.3	Growth of bacterial strains in M9 medium at different pH	49
4.4	Growth of bacterial strains in Nutrient broth at different pH	49
4.5	Growth of bacterial strains in M9 medium at different salt concentrations	50
4.6	Growth of bacterial strains in Nutrient broth at different salt concentrations	51
4.7	pH degradation by bacterial strain T1	52
4.8	Alkalinity degradation by bacterial strain T1	53
4.9	pH degradation by bacterial strain T2	53
4.10	Alkalinity degradation by bacterial strain T2	54

# CHAPTER 1

## INTRODUCTION

The pulp and paper industry wastewater is characterized by dark color, foul odour, high organic content and extreme quantities of COD, BOD and pH. The dark color in wastewater is a major environmental concern as the discharge of dark colored wastewater to water bodies inhibits the photosynthetic activity of aquatic biota by reducing sunlight, besides exhibiting the toxic effects on biota. (Swamy et al., 2011)

Pulp and paper mills generate varieties of pollutants depending upon the type of the pulping process. This paper is the state of the art review of treatability of the pulp and paper mill wastewater and performance of available treatment processes. A comparison of all treatment processes is presented. Combinations of anaerobic and aerobic treatment processes are found to be efficient in the removal of soluble biodegradable organic pollutants. Color can be removed effectively by fungal treatment, coagulation, chemical oxidation, and ozonation. Chlorinated phenolic compounds and adsorbable organic halides (AOX) can be efficiently reduced by adsorption, ozonation and membrane filtration techniques. (Pokhrel et al., 2004)

### **1.1 Modern paper production typically involves the following steps:**

- Producing and acquiring fiber (for example, from trees, used paper or agricultural residues)
- Chemically or mechanically processing the fiber into pulp
- Running the pulp through a paper machine to create large rolls of paper
- Converting the paper into products such as boxes, office paper or paper towels

### **1.2 The Pulp & Paper Industry in India**

The first paper mill in India was set up at Sreerampur, West Bengal, in the year 1812. However, large scale mechanized technology of papermaking was introduced in India in early 1905. Since then the raw material for the paper industry underwent a number of changes and over a period of

time, besides wood and bamboo, other non-conventional raw materials have been developed for use in the papermaking. The paper industry is categorized as forestbased and agro-based and others (waste paper, secondary fibre, bast fibers and market pulp). Currently, the Pulp and Paper industry in India is the 15th largest paper industries in the world. The paper industries in India have been categorized into large-scale and small-scale. Those paper industries, which have capacity above 24,000 tonnes per annum, were designated as large-scale paper industries. Indian paper industry has been de-licensed under the Industries (Development & Regulation Act, 1951) with effect from 17th July, 1997. Foreign Direct Investment (FDI) up to 100% is allowed on automatic route on all activities except those requiring industrial licenses where prior governmental approval is required.

Growth of paper industry in India has been constrained due to high cost of production caused by inadequate availability and high cost of raw materials, power cost and concentration of mills in a particular area. Government has taken several policy measures to remove the bottlenecks of availability of raw materials and infrastructure development. For instance, to overcome short supply of raw materials, duty on pulp and waste paper and wood logs/chips has been reduced. As of 2007-08, the Indian paper industry has a total turnover of more than Rs 10,000 crore and provides direct employment to 200,000 people and indirectly to another 100,000 persons. Despite low per capita (4 kg) consumption of paper and paper boards, the industry has made a steady progress in the last five decades. At present, about 60.8 per cent of the total production is based on non-wood raw material and 39.2 per cent on wood. The capacity utilisation of the industry is low at 60 per cent as about 194 paper mills particularly small mills are sick/or lying closed. Import of paper and paper products have been growing over the years. The imports during 2000-01 were to the tune of 0.152 million metric tons and are estimated to be 0.165 million metric tons in 2001-02. About 0.14 mmt of paper was exported in 2000-01. The domestic demand for newsprint is met partly from indigenous production and partly by import. Free imports and low customs duty have made the newsprint market competitive.

The demand of paper and paper products in India has continuously been increasing over the time. However, per capita paper consumption in India is about 5.5 kg in the year 2003 as against of world average of 50 kg (TERI, 2006). There are about 525 pulp and paper mills with an installed capacity of 6.5 million tonne. The installed capacities of Indian mills vary over a wide range of 5

tpd to 600 tpd. Indian paper mills are categorized into (1) large mills with installed capacity of more than 100 tonne per day, and (2) small mills with capacity less than 100 tonne per day. The small units account for more than 50% production capacity, and characterized by poor energy efficiency. About 80–85% of energy is used for process heating while the share of electricity accounts for 15–20%. More than 80% of electricity used in large wood-based mills is met by cogeneration units (Narayanan et al., 2010).

### **1.3 Recycled fibre (waste paper) sources**

Waste paper based paper mills account for about 35% of Indian paper mill production capacity. Recovery of waste paper has increased from 650,000 tonnes in 1995 to 850,000 tonnes in 2000 but due to alternative uses the recovery rate for paper industry is still about 20 % as against China's 33% & Germany's 71 % (IL&FS Ecosmart limited, 2010).

To sum up, future fibre supply in India depends very much on the following factors:

- **Increase in availability of domestic wood:** Hardwood pulp production is expected to increase from 0.90 MT in 2000 to 1.5 MT by 2015 & 1.8 MT by 2020, depending on further development of plantations. However most of softwood pulp and shortfall in hardwood pulp may still have to be imported. The cost of wood to Indian players is \$50 per tonne compared to around \$30 internationally.
- **Growth in non-wood pulp production** which is expected to increase from 1.3 MT in 2000 to 3.2 MT by 2020. Even though the non-wood fibres required are expected to be available in India, the actual utilisation may be lower due to technical/environmental/logistical reasons.
- **Improvement in waste paper recovery rate in India** which is expected to increase to 38% by 2020 (IL&FS Ecosmart limited, 2010).

### **1.4 Energy benchmarking of Indian mills**

- Indian forest based mills consume a lot of steam and electricity, but produce usually all from wood material without any major oil or coal usage or purchased power.

- Indian agro based mills consume considerably less energy but have to purchase about half of the electricity consumption. (Jaakko Poyry Consulting, 2002)
- European waste based mills use more energy because deinking process is better and final product cleaner.

**Table 1.1: Energy Consumption**

Energy Consumption by integrated mill type	Heat	Power	External supply	
	GJ/t	kWh/t	GJ/t	kWh/t
Indian Agro based mills	15	1000	0	500
Indian Forest based mills	25	1800	0	0
European Forest based mills	15	1200	0	700
Indian Waste based mills	6	850	5	700
European Waste based mills	8	900	7	900

External supply means based on oil, coal or purchased power.

[Source: Jaakko Poyry Consulting, 2002]

### **1.5 Present Scenario (2000-2010) of Indian Pulp and paper industry:**

The Indian paper and paperboards industry is on the growth path. The Indian paper and paperboards industry grew by nearly 7.8 percent during the period 2000-2006. This is substantially higher than the Asian average of 5.1 percent. India's paper manufacturing capacity is expected to grow at a Compounded Annual Growth Rate (CAGR) of 7.4 percent from 8.4 million MT per annum to 11.2 million MT per annum between 2008 and 2010. The Indian per capita paper consumption is among the lowest at 7.0 kg, while Asian and global averages are

11.0 kg and 49.0 kg respectively. But the demand for paper is increasing given the rising disposable incomes particularly of the expanding middle income group.

The literacy level in India which is on the increase is further set to improve the demand for paper in the future. The Government of India's increased budget allocation for education sector is expected to further improve the literacy rates in both urban and rural areas, resulting in increased demand for writing paper. The Indian Pulp and paper industry is expected to grow at 7.4 % CAGR over the period 2008 – 10. With Indian economy in one of its best ever growth mode, the Indian paper industry continues to be a major beneficiary. There are more than 600 paper mills in India in 2010, and installed capacity is about 9 million tonnes / annum. The current per capita consumption of paper in India is about 8 Kg. In the present scenario, apart from capacity augmentation, there is an immense need to improve the Energy Efficiency of the individual units. Many of the Indian Paper mills are also working actively in the areas of water and environmental management not only to better the statutory norms but also in a proactively move closer to cleaner production. The Indian Pulp and Paper industry has to become World class in operations, energy consumption and environmental impact. The Paper Industry is sub divided into three groups namely Wood, Agro and Recycled fibre group.

**2010:** In the present scenario, apart from capacity augmentation, there is an immense need to improve the Energy Efficiency of the individual units. Many of the Indian Paper mills are also working actively in the areas of water and environmental management not only to better the statutory norms but also in a proactively move closer to cleaner production. With the liberalization of the Indian economy leading to global competition as well as the growing emphasis on the environment, it is imperative for the Indian Pulp and Paper industry to become World class in operations, energy consumption and environmental impact. (<http://www.slideshare.net/Harijan29/indian-paper-industry19902010>)

## **1.6 Pollution problems of Pulp and Paper Industries**

Pulp and paper mills are a major source of industrial pollution worldwide. The pulping and bleaching steps generate most of the liquid, solid, and gaseous wastes. Pulping is a process in

which the raw material is treated mechanically or chemically to remove lignin in order to facilitate cellulose and hemicelluloses fiber separation and to improve the papermaking properties of fibers. Bleaching is a multistage process to whiten and brighten the pulp through removal of residual lignin. Pulping and bleaching operations are energy intensive and typically consume huge volumes of fresh water and large quantities of chemicals such as sodium hydroxide, sodium carbonate, sodium sulfide, bisulfites, elemental chlorine or chlorine dioxide, calcium oxide, hydrochloric acid, and so on. A partial list of the various types of compounds found in spent liquors generated from pulping and bleaching steps is shown in. The effluents generated by the mills are associated with the following major problems:

- Dark brown colouration of the receiving water bodies result in reduced penetration of light, thereby affecting benthic growth and habitat. The colour responsible for causing aesthetic problems is attributable to lignin and its degradation products.
- High content of organic matter, which contributes to the biological oxygen demand (BOD) and depletion of dissolved oxygen in the receiving ecosystems.
- Presence of persistent, bio-accumulative, and toxic pollutants.
- Contribution to absorbable organic halide (AOX) loads in the receiving ecosystems.
- Measurable long-distance transport (>100km) of organic halides (such as chloroguaiacols), thereby contaminating remote parts of seas and lakes.
- Cross-media pollutant transfer through volatilization of compounds and absorption of chlorinated organics to wastewater particulates and sludge.

Significant solid wastes from pulp and paper mills include bark, reject fibers, wastewater treatment plant sludge, scrubber sludge, lime mud, green liquor dregs, boiler and furnace ash. The bulk of the solid wastes are generated during wastewater treatment. Sludge disposal is a serious environmental problem due to the partitioning of chlorinated organics from effluents to solids. The major air emissions are fine and coarse particulates from recovery furnaces and burners, sulfur oxides (SO<sub>x</sub>) from sulfite mills, reduced sulfur gases and associated odour problems from Kraft pulping and chemical recovery operations, volatile organic compounds (VOC) from wood chip digestion, spent liquor evaporation and bleaching, nitrogen oxides (NO<sub>x</sub>) and SO<sub>x</sub> from combustion processes. Volatile organics include carbon disulfide, methanol, methyl ethyl ketone, phenols, terpenes, acetone, alcohols, chloroform, chloromethane, and trichloroethane. The extent of pollution and toxicity depends upon the raw material used, pulping

method, and pulp bleaching process adapted by the pulp and paper mills. For example, the pollution load from hardwood is lower than softwood. On the other hand, the spent liquor generated from pulping of nonwood fiber has high silica content. Volumes of wastewater discharged may vary from near zero to 400 m<sup>3</sup> per ton of pulp depending on the raw material used, manufacturing process, and size of the mill. Thus, the variability of effluent characteristics and volume from one mill to another emphasizes the requirement for a variety of pollution prevention and treatment technologies, tailored for a specific industry.

### **1.7 Microorganisms used for degradation of pulp and paper industry effluent**

Paper and pulp mill is a source of major pollution generating industry leaving huge amount of intensely coloured effluent to the receiving end. Rapid increase of population and the increased demand for industrial establishments to meet human needs have created problems such as over exploitation of available resources, increased pollution taking place on land, air and water environment. The intention of this research paper is to identify predominant bacteria and fungi in paper and pulp mill effluent in addition to evaluate the degradation efficiency of individual isolates and combination of isolates. Treatment efficiency of individual isolates and combination of isolates are evaluated by shake flask method. Combination of *Pseudomonas Alkaligenes*, *Bacillus subtilis* along with *Trichoderma reesei* shows higher BOD, COD reduction of 99% and 85% respectively. As individual isolates *Pseudomonas Alkaligenes* show 92% BOD reduction and 77% COD reduction over other bacterial isolates and *Trichoderma reesei* removed 99% BOD and 80% COD respectively (Saraswathi et al., 2010)

### **1.8 Alkaliphiles**

The isolation and characterization of microorganisms that are able to thrive in extreme environments have received a great deal of attention because of their potential biotechnological applications. The alkaliphiles are unique microorganisms, with great potential for microbiology and biotechnological exploitation for the environmental applications. The aspects that have received the most attention in recent years include (i) extra cellular enzymes and their genetic analysis, (ii) mechanisms of membrane transport and pH regulation, and (iii) the taxonomy of alkaliphilic microorganisms. Alkaline enzymes should find additional uses in various fields of

industry, such as chiral-molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents. Furthermore, alkaliphiles may be very good general genetic resources for such applications as production of signal peptides for secretion and promoters for hyper production of enzymes.

The term “alkaliphile” is used for microorganisms that grow optimally or very well at pH values above 9.0. The first paper concerning an alkaline enzyme of alkaliphilic microorganisms was published in 1971 (Horikoshi 1971). Over the past two decades, our studies have focused on the enzymology, physiology, ecology, taxonomy, molecular biology and genetics of alkaliphilic microorganisms. Industrial applications of these microorganisms have also been investigated extensively and some enzymes, such as alkaline proteases, alkaline amylases and alkaline cellulases, have been put to use on an industrial scale.

In present study, a biological technology for neutralization was developed to treat the alkaline industrial waste water. This is a striking example of applied research wherein the use of existing knowledge of extremophilic bacteria was manipulated. This technology offers a cutting-edge biotechnological tool to treat the alkaline waste water in an efficient, advanced and economic way. This technology excludes the use of chemicals as used in the conventional treatment of waste water (Kumar et al., 2008).

## **1.9 Physico-chemical ways for neutralization of wastewater**

### **1.9.1 Sulfuric Acid in Alkaline Wastewater Neutralization**

Neutralization with sulfuric acid ( $H_2SO_4$ ) is typically the cheapest alternative. Reaction times of 15-30 minutes are needed. The formation of calcium sulfate sludges is possible when calcium-containing wastewaters are neutralized (Eckenfelder et al., 1989).

### **1.9.2 Hydrochloric Acid in Alkaline Wastewater Neutralization**

Hydrochloric acid (HCl) is more expensive than sulfuric acid. Reaction times of 5-20 minutes are needed. No sludges are typically formed. However, the acid forms a very corrosive mist during the reaction (Eckenfelder et al., 1989).

### **1.9.3 Neutralization with carbonic acid (CO<sub>2</sub>)**

Carbonic acid is the most inexpensive and environmentally friendly neutralization agent for alkaline waste waters. It is often produced in breweries as excess fermentation product.

Carbonic acid is a harmless product; there is no risk of cauterization or waste water over acidification. The use of hydrochloride acid is not necessary and an increased chloride concentration is avoided (Choi et al., 1999).

Carbon dioxide is weak acid (unlike mineral acids) occurs naturally in water. This means that carbon dioxide can be safely applied as a substitute for mineral acids to neutralize alkaline wastewater. Whereas the use of strong acids can lead to sudden changes in pH values, the neutralization curve is considerably smoother with carbon dioxide, meaning that pH values can be more easily set and better controlled. Using carbon dioxide also increases the buffering capacity of treated effluent.

Reaction time is typically short (1.5-2 minutes). This approach is typically costly unless flue gases are readily available. Calcium carbonate precipitates can be formed when calcium-containing wastewaters are neutralized.

### **1.9.4 Neutralization with flue gas**

Flue gas also contains a considerable amount of carbonic acid (7.5 to 12 %), which can be used for neutralizing alkaline waste waters. It is produced in heating plants and power stations and is therefore available free of charge ([www.frings.com](http://www.frings.com)).

Neutralization with flue gas is of particular interest for companies equipped with their own heating plants or power stations, e.g. in dairies or textile plants. The FRINGS immersible aerator is installed in a waste water collection basin. As neutralization with flue gas requires a large amount of flue gas, this gas is not injected into the air suction pipe, but distributed over the pipe cross-section via a branch line. This means that only flue gas or only air can be injected.

The injection is controlled by a multi-way valve. If the pH value exceeds the legally admissible discharge limits, a pH control and measuring device activates the multi-way valve and the flue gas injection starts.

## 1.10 Alkalinity hazards on Plants and Crops

The most important criteria for evaluating salinity hazards is the total concentration of salts. The quantity of salts dissolved in water is usually expressed in terms of electrical conductivity (EC),  $\text{mg l}^{-1}$  (ppm). Mostly cations like  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and anions like  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  are the major constituents contained in saline waters. Plant growth is adversely affected with saline water, primarily through the effects of excessive salts on osmotic pressures of the soil solution resulting in reduced availability of water. Under the field situations, the first reaction of plants to application of saline water is reduction in germination. A general conclusion can be that the detrimental effect of salinity include, reduced initial growth resulting in smaller plants (lower leaf area index). Experimental evidences indicate that the inter-play of factors like nature and contents of salts, soil type, rainfall, water table conditions and nature of crop and water management practices followed determine the resultant salinity build up vis a vis crop performance from long term use of saline water.

Some waters, when used for irrigation of crops, have a tendency to produce alkalinity hazards depending upon the absolute and relative concentrations of specific cations and anions. The alkalinity is generally measured in terms of sodium adsorption ratio (SAR), residual sodium carbonate (RSC) and adjusted SAR (Adj SAR). Irrigation with sodic waters contaminated from  $\text{Na}^+$  relative to  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and high carbonate ( $\text{CO}_3^{2-}$  and  $\text{HCO}_3^-$ ) leads to an increase in alkalinity and sodium saturation in soils. The increase in exchangeable sodium percentage (ESP) adversely affects soil physical properties including infiltration and aeration. In the early stages of sodic irrigation, large amounts of divalent cations are released into soil solution from exchange sites. Under monsoonal climate alternating irrigation with alkaline water and rainwater induces cycles of precipitation and dissolution of salts. Several field observations have shown that though steady state conditions are not reached in monsoonal climate, but quasi-stable salt balance is reached within 4-5 years of sustained sodic irrigation and further rise in pH and ESP is very low (Minhas et al., 1998).

# CHAPTER 2

## REVIEW LITERATURE

### 2.1 Pulp and Paper Process

The five principal steps in pulp and paper production are wood preparation, pulping, bleaching, chemical recovery, and papermaking. The following step by step description is adapted from the World Energy Council, 1995.

#### 2.1.1 Wood Preparation

Wood preparation involves breaking wood down into small pieces suitable for subsequent pulping operations. Major wood preparation processes include debarking and chipping. This process requires little energy. (Schumacher et al., 1997).

#### 2.1.2 Pulping

The pulping process can be divided into three categories: chemical pulping, mechanical pulping, and semi-chemical pulping (combination of two above categories).

##### 2.1.2.1 Mechanical Pulping

Mechanical pulp is produced by grinding or shredding the wood or non-wood materials to free the fibers. In addition heat and pressure may be applied to assist the process. Mechanical pulping provides low grade pulps with high colour and short fibers, but with a high yield converting 95% of the wood into pulp and produces minimal on-site air pollution and relatively water loads (Anonymous, 1981).

##### 2.1.2.2 Chemical pulping

Chemical pulping is done by digesting to free fibers from the wood chips, nonwood materials such as bamboo, straw, grass, cotton, in chemical solutions that help to dissolve the lignin binding material. Pulp yield is normally in the range of 35 to 57% and about 95% of the lignin is

removed in pulping (McCubbin, 1984). Chemical pulps may be subdivided into Kraft (sulphate), sulphite, semi-chemical and soda.

### **2.1.2.3 Kraft Pulping Process**

Kraft pulping, first used in 1879, is a modification of the caustic soda process in that sodium sulphite ( $\text{Na}_2\text{S}$ ) is added to the cooking liquor. The presence of caustic soda in the cooking liquor is suitable for use of practically all wood species. Sodium sulphate is on duty of buffering, that digestion can be implemented at a lower OH- ion concentration. Thus damage to the fibers is reduced and high strength pulps is produced (UNEP, 1977).

### **2.1.2.4 BCTMP Process**

Bleached Chemi-Thermo-Mechanical Pulping (BCTMP) is a chemi-mechanical pulping process. These high-yield pulps differ from chemical pulps, such as sulfite and kraft, in that the lignin is retained. Chemical pulps have most of their lignin dissolved away by using chemical processes, but this results in a yield around half of that achieved for BCTMP. High-yield pulps may replace some of hardwood kraft pulp in “free sheet” papers.

A variety of hardwoods and softwoods can be used to make BCTMP. Woods typically used are maple, aspen, birch, spruce, and pine. Softwood BCTMP has an ISO brightness range of 80 - 83 and a typical pulp yield of 90%. Hardwood BCTMP, on the other hand, has an ISO brightness range of 85 - 87 and a typical pulp yield of 88%. In particular, aspen has a typical pulp yield of 87%. The use of aspen is of particular importance because its economic rotation length is 25 years, as opposed to the longer rotation lengths of other hardwoods. Ease of regenerating hardwoods is another advantage to using hardwood pulps. (Johnson et al., 1991).

### **2.1.2.5 Other Processes**

According to Palmer (1983) recognizing the difficulties of using established processes on a small scale, a number of alternative processes are being developed which are claimed to be specially suitable for small-scale operation either because they are non-polluting (in at least one case it is claimed that digestion liquor can be used as fertilizer) or because the recovery process is relatively simple. Examples of these processes are:

- Ammonia-based pulping.

- Oxygen (air) and alkali processes.
- Universal process (acid based)

### **2.1.3 Bleaching**

Bleaching whitens pulps for the manufacture of writing, printing, and decorative papers. The process alters or removes the lignin attached to the wood fiber. Chemical pulps are bleached through the use of alternating treatments of oxidizing agents and alkali solutions. The Kraft process produces a darker pulp which requires more bleaching. Mechanical pulps are treated with hydrogen peroxide or sodium hydrosulfite to reduce the light absorption of the lignin rather than remove it. (Schumacher et al., 1997)

This is done in two stages. Firstly the pulp is treated with NaOH in the presence of O<sub>2</sub>. The NaOH removes hydrogen ions from the lignin and then the O<sub>2</sub> breaks down the polymer. Then, the pulp is treated with ClO<sub>2</sub> then a mixture of NaOH, O<sub>2</sub> and peroxide and finally with ClO<sub>2</sub> again to remove the remaining lignin.

### **2.1.4 Chemical Recovery**

Chemical recovery regenerates the spent chemicals used in Kraft chemical pulping. Chemical pulping produces a waste stream of inorganic chemicals and wood residues known as black liquor. The black liquor is concentrated in evaporators and then incinerated in recovery furnaces, many of which are connected to steam turbine cogeneration systems. The wood residues provide the fuel and the chemicals are separated as smelt which is then treated to produce sodium hydroxide. Sodium sulfide is also recovered. (Schumacher et al., 1997).

### **2.1.5 Papermaking**

Papermaking consists of preparation, forming, pressing and drying; preparation and drying are the most energy intensive processes. During preparation, the pulp is made more flexible through beating, a mechanical pounding and squeezing process. Pigments, dyes, filler materials, and sizing materials are added at this stage. Forming involves spreading the pulp on a screen. The water is removed by pressing and the paper is left to dry. In one of the most common papermaking processes, the paper is pressed, drained and dried in a continuous process. In

another, a pulp matt is formed in layers with water removal and treating occurring between deposits. (Schumacher et al., 1997)

Various ancilliary processes result in the recovery of CaO, NaOH and Na<sub>2</sub>S, the major chemicals used in the process. Various utilities ensure that such conditions as sufficient reaction times and adequate mixing are met.

On site processing removes the lignin from the liquid wastes, and solid wastes are generally taken to a landfill. Efforts continue to be made to reduce water consumption by recycling, as smaller volumes are easier to process. The most obvious environmental problem continues to be the sulfurous emissions that give Kraft pulping plants their characteristic smell. These are decreased by gas incineration, but are not able to be wholly eliminated.

## **2.2 Bleaching of pulp generates the highly alkaline wastewater**

The pH of the raw effluent is very high as the incoming wastewater is highly alkaline in nature. The bleaching agents used in the process are reasons for high alkaline wastewater. After washing, if a white product is desired, the pulp must be bleached to remove color associated with remaining residual lignin. Typically in the bleaching process, the bleaching chemicals are injected into the pulp, and the resulting mixture is washed with water. This process occurs several times and generates a large volume of liquid waste. Depending on the bleaching chemicals used, the waste stream from the bleaching process may contain chlorine compounds and organics. The mixture of chemicals may result in the formation of a number of toxic chemicals such as dioxins, furans and chlorinated organics (USEPA, 1997).

## **2.3 ALKALIPHILES: ‘basic’ molecular problems of pH tolerance and bioenergetics**

Alkaliphilic *Bacillus* species provide experimental opportunities for examination of physiological processes under conditions in which the stress of the extreme environment brings issues of general biological importance into special focus. The alkaliphile, like many other cells, uses Na<sup>+</sup>/H<sup>+</sup> antiporters in pH regulation, but its array of these porters, and other ion-flux pathways that energize and support their activity, result in an extraordinary capacity for pH

homeostasis; this process nonetheless becomes the factor that limits growth at the upper edge of the pH range. Above pH 9.5, aerobic alkaliphiles maintain a cytoplasmic pH that is two or more units below the external pH. This chemiosmotically adverse  $\Delta\text{pH}$  is bypassed by use of an electrochemical gradient of  $\text{Na}^+$  rather than of protons to energize solute uptake and motility. By contrast, ATP synthesis occurs via completely proton-coupled oxidative phosphorylation that proceeds just as well, or better, at pH10 and above as it does in the same bacteria growing at lower pH, without the adverse pH gradient. (Krulwich, T.A., 1995)

## **2.4 Cytoplasmic pH Measurement and Homeostasis in Bacteria and Archaea**

Of all the molecular determinants for growth, the hydronium and hydroxide ions are found naturally in the widest concentration range, from acid mine drainage below pH 0 to soda lakes above pH 13. Most bacteria and archaea have mechanisms that maintain their internal, cytoplasmic pH within a narrower range than the pH outside the cell, termed “pH homeostasis.” Some mechanisms of pH homeostasis are specific to particular species or groups of microorganisms while some common principles apply across the pH spectrum. The measurement of internal pH of microbes presents challenges, which are addressed by a range of techniques under varying growth conditions. This review compares and contrasts cytoplasmic pH homeostasis in acidophilic, neutralophilic, and alkaliphilic bacteria and archaea under conditions of growth, non-growth survival, and biofilms. We present diverse mechanisms of pH homeostasis including cell buffering, adaptations of membrane structure, active ion transport, and metabolic consumption of acids and bases. (Slonczewski et al., 2009)

## **2.5 Type of Alkaliphiles**

### **2.5.1 Geoalkalibacter Ferrihydriticus**

Most iron reducers are mesophilic and neutrophilic organisms, adapted to the conditions prevailing on the Earth’s surface (Straub et al., 2001). However, acidophilic iron reducers are also known; they have been isolated primarily from drainage waters of coal and sulfide ore mines (Johnson et al., 2003). It can be seen from our brief review that the capacity for iron

reduction is widespread in the microbial world and that the habitats of the currently known iron reducers include virtually all possible ecological niches.

However, under alkaline conditions, exemplified by soda lakes, only one representative of dissimilatory iron reducers has been found, namely *Alkaliphilus metalliredigens*, isolated from an alkaline pond formed at a site of boron extraction in the United States (Ye et al., 2004). In addition, the ability to reduce ferric iron in an NTA–Fe(III) complex has been revealed for cell suspensions of *Bacillus arsenicoselenatis* (Blum et al., 1998). The thermoalkaliphilic bacterium *Anaerobranca californiensis* should also be mentioned, which reduces iron in the presence of peptides. The possibility of reduction of amorphous and weakly crystalline iron oxides in alkaline environments has been subject to doubt due to the low mobility of Fe(III) under these conditions.

### **2.5.2 Bacillus Okhensis**

Haloalkaliphilic and alkalitolerant microbes occur naturally in alkaline and saline environments, and during the past decade, studies on the ecology, physiology and taxonomy of these organisms have revealed an impressive diversity. Haloalkaliphiles possess special adaptation mechanisms for survival under these two extremities. The members of the alkaliphilic and alkalitolerant *Bacillus* species form the sixth rRNA group (Nielsen et al., 1994) and have attracted much attention as they secrete extracellular enzymes that are both active and stable at high salt concentrations and high pH. These properties make them an interesting tool for fundamental research and for biotechnological exploration (Demirjian et al., 2001). The vast majority of haloalkaliphiles and alkalitolerant species have been isolated from athalassohaline environments (Duckworth et al., 1996; Jones et al., 1998; Pikuta et al., 2003; Rees et al., 2004) while thalassohaline environments have remained relatively less explored (Munoz et al., 2001; Amoozegar et al., 2003).

Cellular characterization and sporulation tests performed as described previously (Patel et al., 2003) showed that the cells of strain Kh10-101<sub>T</sub> were rod-shaped and actively motile. Electron microscopy of negatively stained cells showed the presence of up to three subpolar flagella. The cells stained Gram-positive and thin sections revealed the presence of a typical thick Gram-

positive cell wall. Microscopic examination did not reveal the presence of inclusion bodies or spores.

### **2.5.3 Alkalibacterium Iburiense**

Alkaliphilic bacteria have been isolated in order to investigate their diversity in terms of environments, taxonomy, physiological adaptation to high pH and the industrial application of their enzymes (Duckworth et al., 1996; Thongaram et al., 2003). Most of the strains used for investigation and industrial utilization belong to the genus *Bacillus*. Such alkaliphilic bacteria have been isolated mostly from soil samples and other environments, e.g. fermentation process and water environments. Alkaliphilic bacteria are distributed not only in soil but also in sea water, fresh water, the intestines of insects, alkaline soda lakes and deep-sea and artificial environments (Nielsen et al., 1995; Thongaram et al., 2003; Yumoto et al., 2004). In artificial environments, alkaliphilic bacteria belonging to genera other than *Bacillus* also exist.

The traditional method for producing indigo blue dye had been based on fermentation by alkaliphilic bacteria existing in ubiquitous environments in Japan. The harvested indigo leaves are air-dried and then appropriately wetted before being further processed using moisture-controlled bacterial oxidation. The product obtained is further processed by microbial reduction under alkaline conditions (pH values above 10), at which point the original insoluble oxidized form of indigo is converted to a soluble reduced form. The fermentation method for producing indigo dye by using alkaliphilic bacteria had declined by around 1960 because of the difficulty in managing the fermentation process. Then, a chemical reducing reagent was introduced to replace the bacterial reduction. If one can understand the characteristics of these bacteria in relation to fermentation, it might be easier to manage the fermentation process. A return to the traditional method might diminish the use of the chemical reagent. Indigo-reducing bacteria have been isolated by and have been identified as a *Bacillus* species and *Clostridium isatidis*, respectively. In addition to strains M3T, 41A and 41C, we have also isolated *Alkalibacterium psychrotolerans*, which is an indigo-reducing bacterium that has recently been described taxonomically (Yumoto et al., 2004).

#### **2.5.4 Amphibacillus Xylanus**

Three strains of gram-positive, facultatively anaerobic, sporeforming, rod-shaped bacteria were isolated from composts of manure with grass and rice straw. These organisms grew well in an alkaline medium and digested xylan both in strictly anaerobic cultures when titanium(III) citrate was used as a reducing agent and in aerobic cultures with shaking. The cells contained meso-diaminopimelic acid, and their cellular fatty acids consisted of iso- and anteiso-branched acids and considerable amounts of straight-chain acids. The DNA base composition of these strains ranged from 36 to 38 mol% guanine plus cytosine. Cytochromes, isoprenoid quinones, and catalase activity were not detected. DNA-DNA homology determinations did not show relatedness to strains of representative species of the genera *Bacillus*, *Clostridium*, and *Sporolactobacillus*. Considering the uniqueness of the characteristics, the sequence of the 5S rRNA, and the unique metabolic pathways, we propose *Amphibacillus xylanus* gen. nov., sp. nov., for these strains. The type strain is strain EpOI (= JCM 7361) (Niimura et al., 1990).

#### **2.5.5 Thermococcus Alcaliphilus**

A novel coccoid-shaped, hyperthermophilic, heterotrophic member of the archaea was isolated from a shallow marine hydrothermal system at Vulcano Island, Italy. The isolate grew between 56 and 90°C with an optimum around 85° C. The pH range for growth was 6.5 to 10.5, with an optimum around 9.0. Polysulfide and elemental sulfur were reduced to H<sub>2</sub>S. Sulfur stimulated the growth rate. The isolate fermented yeast extract, peptone, meat extract, tryptone, and casein. Isovalerate, isobutyrate, propionate, acetate, CO<sub>2</sub>, NH<sub>3</sub>, and H<sub>2</sub>S (in the presence of S<sup>0</sup>) were detected as end products. Growth was not inhibited by H<sub>2</sub>. Based on DNA-DNA hybridization and 16S rRNA partial sequences, the new isolate represents a new species of *Thermococcus*, which we named *Thermococcus alcaliphilus*. The type strain is isolate AEDII12 (DSM 10322) (Keller et al., 1995).

#### **2.5.6 Thermococcus Acidaminovorans**

From a shallow marine hydrothermal system at Vulcano (Italy), a new hyperthermophilic member of the Archaea was isolated. The cells are coccoid-shaped and possess up to five flagella. They grow between 56 deg. C and 93 deg. C (optimum 85 deg. C). The organism is strictly anaerobic and grows heterotrophically on a defined amino acids and complex organic

substrates such as casamino acids, yeast extract, peptone, meat extract, tryptone and casein. Polysulfide sulphur is reduced to H<sub>2</sub>S. In the absence of polysulfide or elemental sulphur, the isolate grows at a significantly reduced rate. Growth is not influenced by the presence of H<sub>2</sub>. (Dirmeier et al., 1998).

## 2.6 Industrial Applications of Alkaliphiles

### 2.6.1 Alkaline cellulases of alkaliphilic *Bacillus* strains

Commercially available cellulases display optimum activity over a pH range from 4 to 6. No enzyme with an alkaline optimum pH for activity (pH 10 or higher) had been reported before the rediscovery of alkaliphiles. *Horikoshi and co-workers* found bacterial isolates (*Bacillus* sp. strains N4 and 1139) producing extracellular alkaline CMCase. One of these, alkaliphilic *Bacillus* sp. strain N-4 (ATCC 21833), produced multiple CMCases that were active over a broad pH range (pH 5 to 10). *Sashihara* cloned the cellulase genes of *Bacillus* sp. strain N-4 in *E. coli* HB101. Several cellulase-producing clones with different DNA sequences were obtained. Another bacterium, *Bacillus* sp. strain 1139, produced one CMCase, which was purified and shown to have optimum pH for activity at pH 9.0. The enzyme was stable over the pH range from 6 to 11 (for 24 h at 4°C and 10 min at up to 40°C). The CMCase gene of *Bacillus* sp. strain 1139 was also cloned in *E. coli* (Fukumori et al., 1986; Fukumori et al., 1987). *Beppu's group* many chimeric cellulases from *B. subtilis* and *Bacillus* sp. strain N-4 enzyme genes in an effort to understand the alkaliphily of N-4 enzymes. Although the genes have high homology, the pH activity profiles of the two enzymes are quite different; the *B. subtilis* enzyme (BSC) has its optimum pH at 6 to 6.5, whereas the *Bacillus* sp. strain N-4 enzyme (NK1) is active over a broad pH range from 6 to 10.5. The chimeric cellulases showed various chromatographic behaviors, reflecting the origins of their C-terminal regions. The pH activity profiles of the chimeric enzymes in the alkaline range could be classified into either the BSC or NK1 type, depending mainly on the origins of the fifth C-terminal regions.

*Chaetomium* sp. was found to grow in a wide range of pH (between 4 to 12). This alkaline tolerant fungi was further tested for the production of alkaline cellulases ( $\beta$  - endoglucanase,  $\beta$  - exoglucanase,  $\beta$  - glucosidase), using agricultural and industrial wastes as substrates in the submerged (SMF) and solid state fermentation (SSF) processes (Ravindran et al., 2010). The

results revealed that cellulase enzymes produced under alkaline conditions had high stability and activity than the non alkaline conditions used at pH 5, 7, 12 & at 50 °C.

### 2.6.2 Lipases

Although the initial motivation for studying alkaline lipase was its application to detergents, many alkaline lipases were significantly inhibited in the presence of either alkylbenzene sulfate or dodecyl benzene sulfonate.

Scientists conducted an extensive screening for alkaline lipase-producing microorganisms from soil and water samples. Two bacterial strains were selected as potent producers of alkaline lipase. These were identified as *Pseudomonas nitroreducens* nov. subsp. *thermotolerans* and *P. fragi*. The optimum pH of the two lipases was 9.5. Both enzymes were inhibited by bile salts such as sodium cholate, sodium deoxycholate, and sodium taurocholate at 0.25%. However, further work has not been reported (Watanabe et al., 1977).

A thermophilic lipase-producing bacterium was isolated from a hot-spring area of Yellowstone National Park (Wang et al., 1995). The organism, characterized as a *Bacillus* sp., grew optimally at 60 to 65°C and in the pH range from 6 to 9. The partially purified lipase preparation had an optimum temperature of 60°C and an optimum pH of 9.5. It retained 100% of the original activity after being heated at 75°C for 30 min.

Optimal lipase activity was achieved in medium using combination of sunflower oil and Tween 80 (1% v/v each) as carbon sources. Simple sugars such as glucose and fructose, however, did not promote the production of lipase. Peptone (from meat) at 0.33% (w/v) was the most suitable nitrogen source for lipase production by this Gram-negative bacterium. The presence of  $\text{Ca}^{2+}$  in the cultivation medium possessed significant effect on lipase production while  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  exhibited inhibitory effect towards the enzyme production. Initial culture pH in the range of 5 to 11 were found suitable for lipase production, with the maximum level of lipase activity recorded in the medium with initial culture pH of 9.0 (Lau et al., 2011).

The isolated fungus *Aspergillus fumigatus* MTCC 9657 confirmed the production of alkaline lipase in media having pH in the range of 8.5 to 10.5 along with tributyrin substrate which is used for screening. *A. fumigatus* isolated from aged rice bran oil (Rajan et al., 2011).

### 2.6.3 Xylanases

The first paper describing the isolation of a xylanase from alkaliphilic bacteria was published in 1973 by *Horikoshi and Atsukawa*. The purified enzyme of *Bacillus* sp. strain C-59-2 exhibited a broad optimal pH ranging from 6.0 to 8.0. In the culture broth of *Bacillus halodurans* C-125, two xylanases were found (Honda et al., 1986). Xylanase A had a molecular weight of 43,000, and xylanase N had a molecular weight of 16,000. Xylanase N was most active at pH 6 to 7, and xylanase A was most active pH 6 to 10 and had some activity at pH 12. The xylanase A gene was cloned, sequenced, and expressed in *E. coli* (Honda et al., 1985; Honda et al. 1986).

Since the demonstration that alkali-treated wood pulp could be biologically bleached by xylanases instead of by the usual environmentally damaging chemical process involving chlorine, the search for thermostable alkaline xylanases has been extensive. (Dey et al., 1992) isolated an alkaliphilic thermophilic *Bacillus* strain (NCIM 59) that produced two types of cellulase-free xylanase at pH 10 and 50°C. (Khasin et al., 1993) reported that alkaliphilic *B. stearothermophilus* T-6 produced an extracellular xylanase that optimally bleached pulp at pH 9 and 65°C. (Nakamura et al. 1991) also reported that an alkaliphilic *Bacillus* strain, 41M-1, isolated from soil, produced multiple xylanases extracellularly. One of the enzymes, xylanase J, was most active at pH 9.0. The optimum temperature for the activity at pH 9.0 was around 50°C. Then, an alkaliphilic and thermophilic *Bacillus* strain, TAR-1, was isolated from soil. The xylanase was most active over a pH range of 5.0 to 9.5 at 50°C. Optimum temperatures of the crude xylanase preparation were 75°C at pH 7.0 and 70°C at pH 9.0. These xylanases did not act on cellulose, indicating a possible application of the enzyme in biological debleaching processes.

*Bacillus sp 99* was isolated from sugarcane molasses is thermoalkalophilic. it grows in wide range of different temperature and pH. It produced extracellular cellulose-free xylanase produced was purified and characterized. The Optimum temperature and pH of xylanase were 45°C and 10.0, respectively (Kumar et al., 2011).

### 2.6.4 Pectinases

The first paper on alkaline *endopolygalacturonase* produced by (Horikoshi et al., 1972) alkaliphilic *Bacillus* sp. strain P-4-N was published in 1972. The optimum pH for enzyme action was 10.0 for pectic acid. *Fogarty and co-workers* then reported that *Bacillus* sp. strain RK9

produced endopolygalacturonate lyase. The optimum pH for the enzyme activity toward acid-soluble pectic acid was 10.0. The application of alkaline pectinase-producing bacteria in the retting of Mitsumata bast was reported by *Yoshihara and Kobayashi*. The pectic lyase (pH optimum 9.5) produced by the alkaliphilic *Bacillus* sp. strain GIR 277 has been used in improving the production of a type of Japanese paper. A new retting process produced a high-quality, nonwoody paper that was stronger than the paper produced by the conventional method. (Tanabe et al., 1960) tried to develop a new waste treatment by using the alkaliphilic *Bacillus* sp. strain GIR 621-7. Cao et al. isolated four alkaliphilic bacteria, NT-2, NT-6, NT-33, and NT-82, producing pectinase and xylanase. One strain, NT-33, had an excellent capacity for degumming ramie fibers.

During growth on citrus pectin, *Geobacillus thermoglucosidasius* grew optimally at 60°C and pH 8.5, the strain produced pectinolytic lyases, which were excreted into the medium and the enzymes from *G. thermoglucosidasius* PB94A converted pectin isolated from hemp fibres (Juárez et al., 2009).

### **2.6.5 Chitinases**

(Tsuji et al., 1992) isolated chitinases from the alkaliphilic *Nocardiopsis albus* subsp. *prasina* OPC-131. The isolate produced two types of chitinases. The optimum pH of chitinase A was pH 5.0, and that of chitinase B was pH 7.0. Recently, *Bhushan and Hoondal* isolated the alkaliphilic, chitinase-producing *Bacillus* sp. strain BG-11. The purified chitinase exhibited broad pH and temperature optima of 7.5 to 9.0 and 45 to 55°C, respectively. The chitinase was stable between pH 6.0 and 9.0 at 50°C for more than 2 h. Ag<sup>+</sup>, Hg<sup>2+</sup>, dithiothreitol, β-mercaptoethanol, glutathione, iodoacetic acid, and iodoacetamide inhibited the activity up to 50%. No further studies have been reported.

A chitinase producing marine fungus was isolated as the marine environment serves as the most abundant source of chitin in nature. In addition to the inducer, the media for solid state fermentation was also supplemented with a salt solution containing essential components for fungal growth as well as chitinase production. The chitinase production was assessed in terms of catalytic activity of the enzyme extracted from the solid state media after four days

of incubation. Out of these four solid state fermentation media, the dried potato skin with colloidal chitin was found to be the best combination for chitinase production. In spite of high level of chitinase production in the medium containing dried potato skin and fish scale, concomitant production of protease in the same medium reduced its activity (Das et al., 2011).

## **2.7 Neutralization of alkaline industrial wastewaters using *Exiguobacterium* sp**

A facultatively alkaliphilic bacterium capable of lowering the pH of highly alkaline wastewater from 12.0 to 7.5 within just 2.0 h was isolated from industrial wastewater drained sludge of a local beverage industry. The metabolic product which the bacterium produces in order to neutralize alkaline wastewater was found to be carboxylic acid in nature by Fourier Transform- Infrared (FT-IR) spectroscopy. The isolate belongs to genus *Exiguobacterium* as determined by 16S rRNA gene sequencing and biochemical characterization. It was found to be gram-positive, nonspore-forming, nonmotile, rod shaped, growing aerobically and anaerobically at a wide range of temperatures (5–40 °C), pH (6.5–12.0) and NaCl concentrations(0–9.5%). The strain was able to hydrolyze starch and was catalase positive but lacked oxidase or aminopeptidase activity. Acid was produced from glycerol, cellobiose, D-mannose, mannitol, methyl  $\alpha$ -D-glucoside, amygladin and arbutin. From the 16S rRNA gene sequence analysis, the strain appeared to be most similar (99%) to the sequence from *Exiguobacterium aurantiacum* strain DSM 6208. Neutralization of highly alkaline wastewater without addition of any external carbon source highlights the potential use of the isolate as an alternative to the conventional acid neutralization method for treatment of alkaline wastewater (Kulshrestha et al., 2010).

## **2.8 Biotechnological process for neutralizing alkaline beverage industrial waste water**

A biologically pure culture of the strain *Exiguobacterium* sp., identification number MTCC 5183, deposited at International Depository at IMTECH, Sector 39A, Chandigarh, India, said strain being capable of growth in a medium with a pH in the range of 10-12.00 and being capable of lowering a pH of 12.0 to 11.5 of beverage industrial wastewater to a

neutral pH of 7.5 to 7.00 within a period of 1-1.5 hours, said strain being Gram positive, non motile, rod shaped and oxidase negative, and being capable of hydrolyzing starch and producing acids from glycerol cellobiose, D-mannose, mannitol, methyl .alpha.-D-glucoside, amygdalin and arbutin (Kumar et al., 2008).

## **2.9 Biological neutralization of highly alkaline textile industrial wastewater**

Bacterium, *Kurthia sp.*, is aerobic in nature, is gram positive, shows optimal growth at a temperature in the range of 25 - 42° C., is capable of growth in a high pH environment (pH 12.00), is capable of hydrolyzing starch, is motile, catalase positive and reduces nitrate. In a neutralization experiment, textile industrial wastewater was taken from a local textile industry. The bacterium *Kurthia sp.*, as screened above, was inoculated in 200 - 220 ml basal carbonate medium. The culture was incubated at 35 - 37° C for 8 hours under shaking conditions (100 - 120 rpm). After observing the heavy bacterial growth (Optical Density (O.D)=1.5), the culture was centrifuged at 5000 - 7,000 rpm at about 1 - 4° C. The culture pellet was dissolved in about 20 ml phosphate buffer according to the size of pellet. This pellet was added in a flask containing 200 ml textile industrial waste water (pH 12.0 - 11.5). The flask was kept at shaking conditions (100 - 120 rpm). Decrease in pH was observed after 1.5 - 2 hours. This bacterium, *Kurthia sp.*, (MTCC 5181) has been capable to bring down the pH from 12 - 11.5 to 7.5 - 7 within a short period of 1.5 - 2 hours pH was monitored by a pH meter (Kumar et al., 2007).

## **2.10 Biological Neutralization of chlor-alkali industry wastewater**

The present work reports biological neutralization of chlor-alkali industrial effluent by an alkaliphilic bacterium, isolated from the Gujarat coast, which was identified as *Enterococcus faecium* strain R-5 on the basis of morphological, biochemical and partial 16S rRNA gene sequencing. The isolate was capable of bringing down the pH of waste water from 12.0 to 7.0 within 3 h in the presence of carbon and nitrogen sources, with simultaneous reduction in total dissolved solutes (TDS) up to 19–22%. This bacterium produced carboxylic acid, as revealed by FT-IR analysis, which facilitated neutralization of alkaline effluent. The presence of unconventional raw materials viz. *Madhuca indica* flowers or sugar cane bagasse as carbon and

nitrogen sources could effectively neutralize alkaline effluent and thus making the bioremediation process economically viable. The time required for neutralization varied with size of inoculum. To the best of our knowledge, this is the first report on biological neutralization of a chlor-alkali industrial effluent (Jain et al., 2011).

# CHAPTER 3

## MATERIALS AND METHODS

This chapter discusses the materials used and methodology adopted during the study.

All the chemicals used and reagents employed were of analytical grade with sufficient purity. The calibration curves were prepared prior to estimation of unknown concentration and used throughout the study.

### 3.1 Collection of Sample

#### 3.1.1 Effluent Samples

The combined effluent of bleaching and pulping stages was collected from Satia paper mill and it was stored in refrigerator at 4°C until further investigation was carried out. Satia Paper Mills Ltd. (SPML) is a medium sized paper mill with located in Rupana, Distt. Muktsar, Punjab, India. SPML is well established in the production of Pulp & Paper (paper products) principally from agro waste & recycled paper.

#### 3.1.2 Reagents and instruments

The chemicals used in treatment were of analytical grade and were obtained from various furnishers. The pH, optical density and Fe(III) concentrations were analyzed using instruments pH meter, UV-Visible Spectrometer.

#### 3.1.3 Physicochemical characterization

The characteristics of water samples such as pH, color, COD, TSS and TDS were determined using standard methods of APHA (American Public Health Association).

### 3.2 Isolation of Bacteria

1g sludge of black liquor from Star Paper mill, Saharanpur was added in the nutrient broth and incubated for 2 days at 37°C on rotary shaker at 150°C. After 24 hours, dilutions were made  $10^{-1}$

–  $10^{-6}$  and surface spread on nutrient agar plates. This process repeated 2-3 times. After that the isolated strains were maintained in M9 medium and M9 media slants. Out of these the best grown isolates on M9 media were selected on the basis of their growth.

### 3.3 M9 medium

It is a minimal growth medium used for bacterial cultures. It has the advantage of being cheap. The Medium prepared according to the table 1 and pH was adjusted according to the requirement as given below:

#### 3.3.1 Composition of M9 media

**Table 3.1: M9 media Composition**

Component	Concentration (g/l)
Sucrose	10.0
K <sub>2</sub> HPO <sub>4</sub>	2.50
KH <sub>2</sub> PO <sub>4</sub>	2.50
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	1.00
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.20
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.01
MnSO <sub>4</sub> .7H <sub>2</sub> O	0.007
Distilled water	1000ml
Agar (if solid M9 is required)	15

Separately autoclave the Media and KCl/NaOH buffer (KCl- 4.473g/300 ml H<sub>2</sub>O and NaOH 6.336g/792ml H<sub>2</sub>O, mix both) at 121<sup>0</sup>C for 15 mins. Added 32.9 ml KCl/NaOH buffer with 70 ml of medium to get the required pH.

### **3.4 Characteristics of Effluent**

Characterization of effluent was done according to the method given in APHA (1989).

#### **3.4.1 Biological Oxygen Demand**

Biochemical Oxygen Demand (BOD) refers to the amount of oxygen that would be consumed if all the organics in one liter of water were oxidized by bacteria and protozoa.

It was estimated by the method no. 5210

#### **3.4.2 Chemical Oxygen Demand**

The Chemical Oxygen Demand (COD) is used as a measure of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant.

It was estimated by the method no. 5220 C closed refluxed titric method.

#### **3.4.3 Hardness Test**

An excellent way to determine water hardness is to perform a complexometric titration using a standard ethylenediaminetetraacetic acid (EDTA) solution. Due to steric hindrances, EDTA will complex with calcium and magnesium in a one-to-one molar ratio. The endpoint in this experiment will be determined using a EBT indicator. Hardness is estimated according to the method no. 2340 C.

#### **3.4.4 Chloride Test**

In a neutral or slightly alkaline solution Potassium Chromate can indicate the end point of the Silver Nitrate titration of Chloride. The method no. used was 4500-Cl<sup>-</sup> B.

#### **3.4.5 Alkalinity Test**

Alkalinity of water is its acid neutralizing capacity. It is the sum of all the titrable base. The measured value may vary significantly with the end point pH used according to the method no. 2320 A.

### **3.4.6 Phosphate test**

Phosphorus occurs in natural waters and in waste waters almost solely as phosphates. For the estimation of phosphorus, digestion procedure was used 4500-P B Nitric acid and Perchloric acid digestion and for Colour development by the method 4500-P D stannous chloride method.

### **3.4.7 TSS, TDS and Total Solids**

Solids were dried at 105°C till constant weight according to the method no. given 2540 B, 2540 C, 2540 D respectively for TS, TDS and TSS.

### **3.4.8 Carbohydrate test**

#### **Total Carbohydrate with anthrone test**

#### **Procedure:**

Take 0.1 ml of sample in test tube. Add 5ml of 2.5N HCl. Keep it in boiling water bath for 3 hours (90-100°C). Cool at room temp. and neutralize it with solid Na<sub>2</sub>CO<sub>3</sub> till the effervescence stops. Make volume 100 ml with distilled water. Take suitable amount of digested sample. Prepare standard by taking 0, 0.2, 0.4, 0.6, 0.8, 1.0 ml from working standard solution. Make volume upto 1ml with distilled water. Add 4 ml of Anthrone reagent in each test tube. Boil test tubes in water bath for 8-10 minutes (cover test tubes with aluminium foil). Cool rapidly and take O.D. of green- dark green colour at 630nm. Determine the concentration of carbohydrate in sample by standard curve.

## **3.5 Morphological studies**

### **3.5.1 Gram staining:**

Thin smear of the cultures was made on separate glass slides. Smear was air dried and heat fixed. Covered the smear with crystal violet for 30 seconds then washed with distilled water. After that covered the smear with gram's iodine solution for 60 seconds and washed with decolorizer and after that with distilled water. Applied safranin for 30 seconds and washed with distilled water. Stained slides were air dried and examined microscopically.

### 3.5.2 Spore staining:

Smears were made on separate clean slides. Smears were air dried and heat fixed. Flooded the smears with malachite green and heated the slides to steaming and steamed for 5 minutes, more stain to the smear was added from time to time. Washed the slides with distilled water and counterstained with safranin for 30 seconds. Washed smear with distilled water and examined microscopically.

## 3.6 Identification of selected bacterial isolates

### 3.6.1 a) Morphological and biochemical characterization

Selected isolates were characterized by colony morphology and gram staining and morphological characteristics according to Cappuccino and Sherman (2010). Additional biochemical test were performed according to Aneja (2008) for taxonomic characterization which included gelatine liquefaction, lipase screening, casein hydrolysis, H<sub>2</sub>S production, lipase test, xylanase test, pectinase screening, methyl red-voges proskauer (MR-VP), citrate utilization, carbohydrate utilization and amino acids utilization.

### 3.6.1 b) Biochemical Tests

#### 3.6.1.1 Starch Hydrolysis

**Table 3.2: Composition of Starch Hydrolysis Test**

<b>Composition</b>	<b>Concentration(g/l)</b>
Starch	20
Peptone	5
Beef extract	3
Agar	15
Distilled Water	1000ml

**Procedure:**

Plates of starch agar were inoculated with the bacteria's and were incubated at 37°C for 24-48 hours. The plates were flooded with iodine solution, a clear zone was observed around the colonies.

**3.6.1.2 Gelatin Agar Medium****Table 3.3: Composition of Gelatin Agar Medium**

Composition	Concentration(g/l)
Gelatin	40
Tryptic Soy broth	30
Distilled water	1000ml

**Procedure:**

Stab a small inoculum of the bacterium about  $\frac{3}{4}$  of the way to the bottom of a tube of “deep” agar with the inoculating needle. Repeated with separate stab tubes for each of bacteria and incubated at 37°C for 24-48 hours. Place the incubated inoculated stab and the uninoculated control into the refrigerator for approximately 30 minutes. Compare the inoculated stab with the control by tapping the tubes gently.

**3.6.1.3 H<sub>2</sub>S Production****Table 3.4: Composition for test of H<sub>2</sub>S Production**

Composition	g/l
Peptone	30
Beef extract	3
FAS	0.2
Sodium thiosulphate	0.0210
Agar	15
Distilled Water	1000 ml

**Procedure:**

An inoculating needle was used to stab a small inoculum of each of the bacteria approximately  $\frac{3}{4}$  of the way to the bottom into separate SIM agar tubes. Incubate the deep stabs at 37°C for 24-48 hours. The inoculated deep stab was compared with the control.

**3.6.1.4 Lipase Screening****Table 3.5: Composition for Lipase Screening Test**

<b>Composition:</b>	<b>Concentration(g/l)</b>
Nutrient Broth	13
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.1
Tween 80	10 ml
Agar	20
Distilled water	1000 ml

**Procedure:**

Medium was sterilized by autoclaving at 121°C for 15 mins. Tween 80 was autoclaved separately and added to medium. Incubate inoculated plates at 37°C in an inverted position for 24-48 hours. Observed the plates for the formation of a opaque zone around the growth.

**3.6.1.5 Methyl Red-Voges Proskaver test (MR-VP broth)****Table 3.6: Composition for MR-VP Test**

<b>Composition</b>	<b>Concentration(g/l)</b>
Peptone	7
Glucose	5
Potassium Phosphate	5
Distilled Water	1000 ml

**Procedure:**

Inoculated separate tubes of MR-VP broth with the bacteria's and incubated the tubes at 37°C for 24-48 hours. After incubation, transferred approximately 1/3rd of each culture into empty glass test tubes and set aside for VP test. Five drops of the methyl red indicator was added to the remaining broths in the original tubes and colour change was observed. Added 12 drops of VP reagent 1 (5% naphthol solution in absolute ethanol) to each of the broths that were set aside for the VP test. Each culture was shaken to mix the reagent with the rest of the broth. Immediately add 2-3 drops of VP reagent 2 (40% KOH in water) and shake the cultures again. Shake the cultures again every 3-4 minutes until approximately 15 minutes had passed. Results were compared with the control.

**3.6.1.6 Carbohydrate Utilization****Table 3.7: Composition of Carbohydrate Utilization Test**

<b>Composition</b>	<b>Concentration (g/l)</b>
Peptone	10
NaCl	15
Sugar *	5
Distilled Water	1000 ml

Sugars used are Glucose, Fructose, Sucrose, Mannitol, Maltose.

**Procedure:**

Transferred 0.1 ml of inoculum of each of the bacteria's into broths (glucose, sucrose, fructose, xylose, mannitol, matlose) and incubated the inoculated broths at 37°C for 24-48 hours in a rotary shaker at 150 rpm. Results were observed for each broth and compared to the uninoculated controls.

### 3.6.1.7 Xylanase Screening

**Table 3.8: Composition for Xylanase Screening Test**

Composition	Concentration(g/l)
Yeast extract	1
Xylan powder	5
Agar	20
Distilled water	1000 ml

[1% Congo red, 100 ml and 1M NaCl, 250 ml]

#### **Procedure:**

Inoculated the plates with the bacteria and incubated at 37<sup>0</sup>C in an inverted position for 24-48 hours. After growth occur, flooded the plates with congo red solution (1% solution) for 30 minutes, then washed with NaCl. Observe the plates for the formation of a zone around the growth.

### 3.6.1.8 Pectinase Screening

**Table 3.9: Composition for Pectinase Screening Test**

Composition	Concentration (g/l)
Ammonium Sulphate	1
K <sub>2</sub> HPO <sub>4</sub>	6
KH <sub>2</sub> PO <sub>4</sub>	3
Pectin	5
Yeast extract	1
Distilled Water	1000MI

#### **Procedure:**

The autoclaved medium, cooled to 45-50 °C was poured into sterile petri plates and allowed to solidify. Aseptically inoculated the plates and incubated at 37 °C for 24-48 hours. After growth

flooded with CTAB buffer (1% solution) for 10 min and clear zone was observed around the growth on the media.

### 3.6.1.9 Citrate Utilization

**Table 3.10: Composition for Citrate Utilization Test**

Composition	Concentration (g/l)
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.1
K <sub>2</sub> HPO <sub>4</sub>	1
NaCl	5
Sodium Citrate	2
MgSO <sub>4</sub>	0.2
Bromothymol blue	0.08
Agar	2
Distilled Water	1000 ml

#### Procedure:

The surface of slants of Citrate agar was inoculated with the bacteria by using a small amount of inoculum and incubated at 37°C for 48 hours. The inoculated slant was compared with the control.

### 3.6.1.10 Cellulase Screening

**Table 3.11: Composition for Cellulase Screening Test**

Composition	g/l
Yeast extract	1
Carboxymethylcellulose sodium salt	5
Agar	20
Distilled water	1000 ml

**Procedure:**

Incubated the inoculated plates at 37<sup>0</sup>C in an inverted position for 24-48 hours and flooded the plates with congo red solution (1% solution) after the growth occurred. Observe the plates for the formation of a zone around the growth.

**3.6.1.11 Catalase Screening****Table 3.12: Composition for Catalase test**

<b>Composition</b>	<b>g/l</b>
Trypticase	15
Phytone	5
Sodium chloride	5
Agar	15

**Procedure:**

Inoculated the trypticase soy agar slants and kept an uninoculated trypticase soy agar slant as control. Incubate the cultures at 37<sup>0</sup>C for 24-48 hours. Observe each culture for the appearance or absence of gas bubbles.

**3.7 Physiological Testing**

The optimal growth temperature, pH and salinity concentration for the two strains were determined.

**3.7.1 Effect of Incubation Temperatures on growth**

Temperature is one of the most important physical factors affecting microorganisms. Bacteria may be divide into three major groups w.r.t their temperature requirements: i) Psychrophiles, those with optimum temperature b/w 0<sup>o</sup> to 20<sup>o</sup> C; ii) Mesophiles, those with optimum temperature b/w 20<sup>o</sup> to 40<sup>o</sup> C; and iii) Thermophiles, with optimum temperature b/w 40<sup>o</sup> to 80<sup>o</sup>C.

**Procedure:**

Inoculate the flasks containing M9 media and Nutrient Broth with the bacterial cultures. Incubate the inoculated flasks and the control flasks at different temperatures (10°C, 20°C, 28°C, 35°C and 44°C) for 24 to 48 hours. Examine the cultures after 2 days of incubation for presence or absence of growth and the degree of growth, spectrophotometrically at 600 nm.

**3.7.2 Effect of pH on growth**

Each species has the ability to grow within a specific pH range, that may be broad or limited, with the most rapid growth occurring within a narrow optimum range.

**Procedure:**

Inoculate each of a series of the tubes with bacterial cultures by adding 0.1 ml of the culture to M9 and nutrient broth. Incubate the bacterial inoculated tubes for 24-48 hours at 37°C at different pH (6-12) in a rotary shaker at 150 rpm. Examine the growth after 2 days by using spectrophotometer at 600 nm.

**3.7.3 Effect of salt concentrations on growth**

Bacteria can be divide into four groups depending on their ability to grow at various sodium chloride concentration; non halophilic (grow at less than 2% sodium chloride concentration), halophilic (grow above 2% sodium chloride concentration). Halophilic may further be of three types: slightly halophilic (grow between 2 and 5% NaCl), moderately halophilic (grow upto 10%) and extremely halophilic(grow at more than 20% NaCl).

**Procedure:**

Different salt concentrations (4%, 8%, 12%, 16% and 20% of NaCl) were added to the M9 medium tubes and Nutrient broth tubes. Inoculated each of a series of the tubes with bacterial cultures by adding 0.1 ml of the culture to each tube and incubated the bacterial inoculated tubes for 24-48 hours at 37°C in a rotary shaker at 150 rpm. Growth was examined after 2 days with mass spectrophotometer at maximum wavelength of 600 nm.

### **3.8 pH and alkalinity degradation by bacterial strains**

Two selected bacterial isolates (T1 and T2) were evaluated for their ability to decrease alkalinity and pH. Inoculums were prepared in M9 medium and nutrient broth by growing the cells at 37°C overnight and used to inoculate the wastewater effluent (100 ml) containing 5% sucrose were incubated at 37°C at 150 rpm in shaking incubator.

After each interval the degraded pH and alkalinity of the effluent is recorded.

## CHAPTER 4

### RESULT AND DISCUSSION

This chapter presents the results of the study conducted and a discussion on the results obtained. The study period was from January-June 2012.

#### 4.1 Characteristics of Pulp and Paper Industry

**Table 4.1:** The characteristics of pulp and paper industry effluent are:-

Parameter	Value
pH	9.8
COD	2960 mg/L
BOD	1700 mg/L
TDS	4460 mg/L
TS	5220 mg/L
Chloride	2000 mg/L
Alkalinity	860 mg/L on CaCO <sub>3</sub> scale
Hardness	200 mg/L on CaCO <sub>3</sub> scale
Carbohydrate	822 mg/L
Total Phosphate	4 mg/L

The effluent from pulp and paper industry was collected from satia paper mill, Muktsar which has the **pH** value around 10. The raw waste water from the industry is very polluted. The amount of oxygen needed to consume the organic and inorganic materials is called the **Chemical**

**Oxygen Demand (COD).** It has been found that the Chemical oxygen demand is around 2960 mg/L. **Biochemical Oxygen Demand (BOD)** refers to the amount of oxygen that would be consumed if all the organics in one liter of water were oxidized by microorganisms. The BOD value is calculated and its value comes 1700 mg/L. **Total solids, TS,** is a measure of all the suspended, colloidal, and dissolved solids in a sample of water and its value in the effluent is estimated as 5220 mg/L. **Total Dissolved Solids, TDS** is a measure of the combined content of all inorganic and organic substances contained in a liquid in: molecular, ionized or micro-granular suspended form. **Chloride concentration** in this waste water effluent is found to be 2000 mg/L. **Phenolphthalein alkalinity** is present only when free carbon dioxide (CO<sub>2</sub>) is absent and therefore exists only when the pH exceeds 8.3 and is expressed in mg/L CaCO<sub>3</sub>. The alkalinity value is observed as 860 mg/L on CaCO<sub>3</sub> scale. **Water hardness** is an expression for the sum of the calcium and magnesium cation concentration in a water sample. These cations form insoluble salts with soap, decreasing soaps cleaning effectiveness. They also form hard water deposits in hot water heaters. Hardness value of waste water effluent is 200 mg/L CaCO<sub>3</sub>. **Carbohydrate** test is used to identify the concentration of carbohydrates in the effluent. By using standard curve of carbohydrate, at 630 nm O.D the concentration in the effluent is measured as 822 mg/L. The **phosphate** concentration in the effluent is observed by making a standard curve for phosphate and then by comparing and analyzing by the standard curve, the phosphate concentration in wastewater effluent is found to be 4 mg/L

## 4.2 Isolation and selection of Bacteria

The bacterial strains are isolated from sludge generated near the black liquor drainage at Star Paper Mill, Saharanpur.

Bacterial isolates were isolated on nutrient broth. 1g sludge was added in 100 ml of NB and incubated for 24 hours at 150 rpm on rotary shaker. The broth was serially diluted ( $10^{-1} - 10^{-7}$ ) and then spread on nutrient agar plates. Six different types of colonies which observed on agar plates were transferred on minimal medium (M9). Four colonies were grown on M9 medium, out of which two strains namely T1 and T2 showed good growth as well as alkalinity reduction. Thus these two strains T1 & T2 were selected for further study.

### 4.2.1 Alkalinity Reduction Test

The pH of the minimal M9 medium was adjusted to 12 with KCl/NaOH buffer. Before pouring the plate added 2-3 drops of phenolphthalein indicator per 100 ml of medium.

- i. Pour the plates and let the medium solidify.
- ii. Surface spread all the four strains on the plate. The medium in T1 and T2 plates become colourless which indicates production of acid. Other strains unable to change the colour.

### 4.2.2 Identification of selected bacterial isolates

#### 4.2.2.1 Morphological and biochemical characterization

Out of the 6 bacterial strains the two bacterial isolates T1 and T2 were analyzed on the basis of pH and alkalinity reduction of the effluent. The colony morphology of T1 has regular margins, glistening, convex and oval round in shape. T2 also has regular margin, matt, flat and irregular in shape. Both the isolates were gram positive and all were rod shape. Spore staining revealed the presence of spores in T1 and T2.

**Table 4.2: Morphological characteristics of bacterial isolates**

STRAIN	GRAM STRAIN	SHAPE	SPORE STAIN	PLATE COUNT
T1	+	Regular, convex, Oval-round	+	1.47 X 10 <sup>7</sup> /ml
T2	+	Round, irregular	+	1.02 X 10 <sup>7</sup> /ml

The Plate spread technique was used to count the number of viable cells in a sample. The isolated colonies grown on M9 medium were used for Biochemical and Physiological test.

## 4.3 Biochemical Tests

### 4.3.1 Biochemical activity of microorganism:-

Micro organism must be selected, separated and identified for wide variety of reasons such as:

- a) Determination of pathogens

b) Selection and isolation of strain of fermentative microorganism necessary for industry to produce vitamins, organic acid, antibiotics and industrial enzyme

c) Comparison of biochemical activities for taxonomic purpose.

The chemical reaction which takes place in the cell is defined as cellular metabolism and biochemical transformation that occur both inside and outside the cell. Most high molecular weight substances are not able to pass through cell membrane therefore material food stuff such as polysaccharide, lipid and protein must be degraded to low molecular weight material nutrients before they can be transported in to cell these test are starch, casein, lipid and gelatin hydrolysis.

#### **4.3.2 Intracellular enzyme:-**

These enzymes function inside the cell and are mainly responsible for synthesis of new protoplasmic requirement and production of cellular energy from assimilated material test are carbohydrate fermentation, H<sub>2</sub>S production, Nitrate and catalase reaction, Indole, Methyl red, Voges Proskaver and Citrate utilization.

#### **4.3.3 Pectinase Screening**

Pectinase is the collective term for a row of enzymes that are able to break down or to transform pectins. Some fruits form pectinase during natural gestation. A commercial pectinase might typically be activated at 45 to 55 °C and work well at a pH of 4.5 to 5.5. CTAB buffer (1% solution) was flooded on the petriplates for 10 min and clear zone was observed around the growth on the media. The isolated strain T1 shows negative result and strain T2 shows positive result for pectinase production.

#### **4.3.4 Lipase Activity:**

Lipase and high molecular weight compounds possesses large amount of energy. The degradation of lipids such as triglycerides is accomplish by hydrolyzing enzymes called lipases that cleave the ester bond in the molecule by the adding of water to form a building blocks glycol and fatty acid. Isolate T1 shows negative Lipase activity whereas isolate T2 shows positive Lipase activity.

#### 4.3.5 Xylanase Screening

Xylanases are genetically single chain glycoproteins, ranging from 6–80 kDa, active between pH=4.5–6.5 and at temperature between 40 and 60 °C. Xylanases from different sources differ in their requirements for temperature, pH, *etc.* for optimum functioning. Xylanase production was determined by flooded the plates with congo red solution (1% solution) for 30 minutes, then washed with NaCl, a zone around the growth was observed. The isolated strains T1 and T2 shows negative result for xylanase production.

#### 4.3.6 Starch Hydrolysis

The starch hydrolytic activities include degradation of macro molecules which require co-enzyme. The starch serves as the polysaccharides substrate. The detection of hydrolytic activity of starch, the starch test was performed to determine the presence and absence of starch in solid medium. Starch in the presence of iodine will impart blue color to medium and clear zone around the colony indicating presence of starch hydrolyzing bacteria. The isolate T1 showed positive result and isolate T2 showed negative result.

**Table 4.3: Biochemical characterization of selected bacterial isolates**

Strain	Pectinase Screening	Lipase Screening	Xylanase Screening	Starch Hydrolysis	Cellulase Activity	Gelatin Liquefaction
<b>T1</b>	--	--	--	+	--	--
<b>T2</b>	+	+	--	--	--	+

#### 4.3.7 Cellulase activity

In cellulase activity, the enzyme complex breaks down cellulose to beta-glucose. Cellulase action is considered to be synergistic. Cellulase activity can be determined by flooded the plates with congo red solution (1% solution) for 30 minutes, then washed with NaCl, a zone around the growth was observed. The isolated strains T1 and T2 shows negative result for cellulase activity.

#### 4.3.8 Gelatin Liquefaction test:

Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue. Below temperature 25<sup>0</sup>C gelatin will maintain its gel properties, and exist as solid, temperature above 25<sup>0</sup>C gelatin is liquid. Liquefaction is accomplished by some micro organisms capable of producing a photolytic extra cellular enzyme called gelatinase which act to hydrolyze this protein to amino acid. Once degradation occur even very low temperature of 4<sup>0</sup>C will not restore the gel characteristics. The isolate T2 shows the positive results when the culture was placed in a refrigerator at 4<sup>0</sup>C for 30 minutes. Culture remain liquefied produce gelatinase and called as rapid hydrolyser, whereas isolate T1 showed gelatinase negative results.

**Table 4.4: Biochemical characterization of selected bacterial isolates**

Strain	H <sub>2</sub> S Production	Citrate Utilization	Methyl Red test	Voges Proskaver	Catalase Screening
<b>T1</b>	--	--	--	--	+
<b>T2</b>	--	+	--	--	--

#### 4.3.9 Hydrogen sulphide production:-

There are two fermentative pathways by which some microbes are able to produce H<sub>2</sub>S.

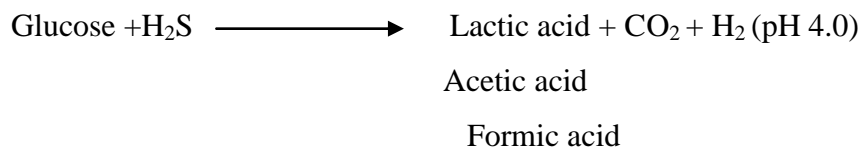
1. H<sub>2</sub>S may be produced by the reduction of organic sulfur present in the amino acid which is a component of peptones contained in the medium which can be degraded enzymatically.
2. By the reduction of inorganic sulfur compound such as thio-sulphate, sulfate and sulphides. The SIM medium contain peptone and sodium thio-sulphate as the sulfur substrate and produce the H<sub>2</sub>S colourless gas. The ferrous ammonium sulfate in the medium serve as a indicator by combining with the gas forming black colour ferrous sulfide precipitate that was seen along the line of stab inoculation the isolate T1 and T2 did not produce any gas so both the culture showed negative results for H<sub>2</sub>S production.

#### 4.3.10 Citrate utilization process:-

In absence of fermentable glucose or lactose some microorganism are capable of using citrate as a carbon source for their energy this ability depend on the presence of citrate. During the growth of bacteria reaction take place and medium become alkaline  $O_2$  that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of sodium carbonate change the bromo-thymol blue indicator incorporated into the medium from green to deep Persian blue. The strain T1 showed citrate negative whereas T2 showed citrate positive result.

#### 4.3.11 Methyl red:-

The hexose monosaccharide glucose is the major substrate oxidized by bacteria for energy production. In this test methyl red indicator was used which give red colour. Both the isolates T1 and T2 showed negative results.



End point: Methyl red indicator gives red colour.

#### 4.3.12 Voges-proskour test:-

This test determine the capability of some organism to produce non acidic or neutral end product such as acetyl methyl carbonyl from the organic acid that result from glucose metabolism this reaction occur in the presence of the alpha naphthol of catalyst and a guanidine group that is present in peptones of the MR-VP medium as a pink complex is formed. Isolate T1 and T2 showed –ve result.

#### 4.3.13 Catalase production:-

During aerobic respiration microorganism produce hydrogen peroxide and in some case an extremely toxic super oxide was produced. Accumulation of these substances will results in death of organism unless they can be enzymatically degraded under anaerobic condition,

carbohydrate degraded for energy production organisms capable of producing catalase rapidly degraded hydrogen peroxide as:-



Catalase production can be determined by adding the substrate  $\text{H}_2\text{O}_2$  on agar plate. If catalase is present the chemical reaction mentioned is indicated by bubbles of free oxygen gas this shows a positive catalase test and the absence of bubble formation shows negative catalase test. Isolate T1 showed positive result and isolate T2 showed negative results which show non toxicity in the process.

**Table 4.5: Carbohydrate utilization test of the bacterial isolates**

Carbohydrate » Strain ↓	Sucrose	Glucose	Lactose	Mannitol	Xylose	Maltose
<b>T1</b>	+	+	+	+	+	+
<b>T2</b>	+	+	+	+	+	+

#### 4.3.14 Carbohydrate utilization Test:-

Most organism obtained energy through a series of orderly and integrated enzymatic reaction leading to bio-oxidation of a substrate some organism are capable of fermenting sugar such as glucose aerobically and anaerobically. In Aerobic bio-oxidation, in which molecular  $\text{O}_2$  can serve as final electron acceptor where as in anaerobic bio-oxidation, in which inorganic ions other than  $\text{O}_2$  such as  $\text{NO}_3$  and  $\text{SO}_4$  can serve as final electron acceptor.

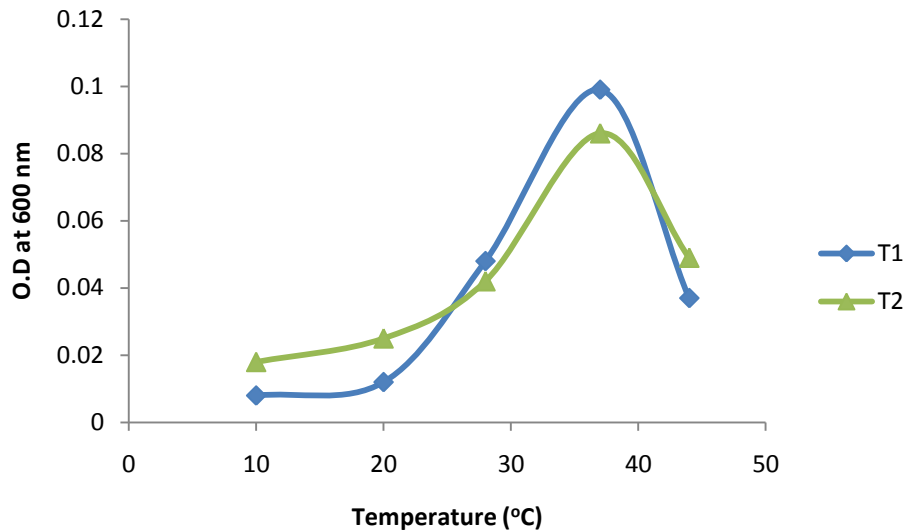
The ability of a microorganism to utilize carbohydrates can be key to its identification. Different carbohydrates (0.5% concentration) with peptone and NaCl are used in testing of utilization for each strain. Both the strains T1 and T2 utilizes all the 6 types carbohydrates (glucose, sucrose, lactose, mannitol, xylose & maltose) and gives the positive result.

## 4.4 Physiological Test

### 4.4.1 Effect of temperature on bacterial growth

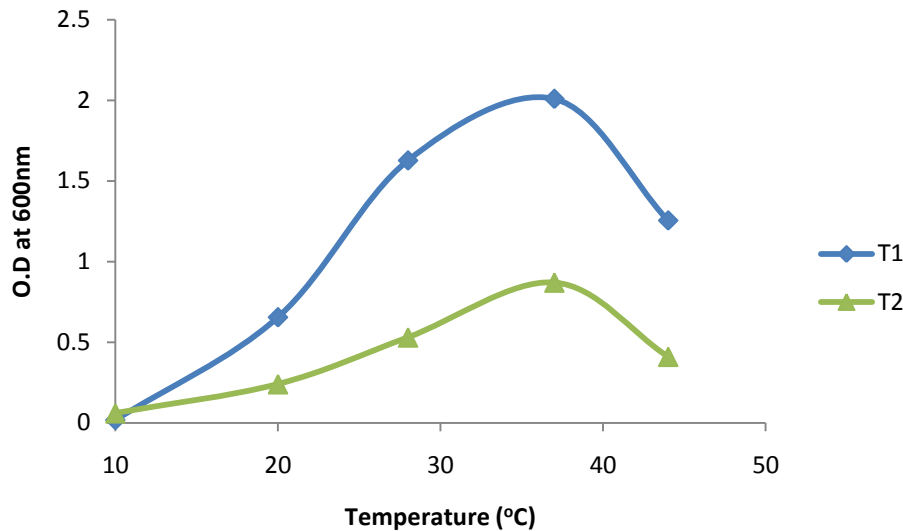
Temperature is one of the most important physical factors affecting microorganisms. Bacteria may be divided into three major groups. a) Psychrophiles which grows in between 0°C - 20°C. b) Mesophiles having optimum temp between 20°C – 40°C. c) Thermophiles, with optimum temperature between 40°C to 80°C.

The growth of selected microorganisms was compared in two medium Nutrient broth (enriched medium) and minimal medium (M9) which is having all inorganic sources.



**Figure 4.1: Growth of bacterial strains in M9 medium at different temperatures**

The bacterial strains were tested at different temperatures (10°C, 20°C, 28°C, 37°C & 44°C) over a period of 24 hours in M9 medium and Nutrient Broth. At 37°C, optimum temperature at which the both the bacterial strains (T1, T2) have their highest growth comparatively to other temperatures in both media. Both bacterial strains T1 and T2 are mesophilic in nature.



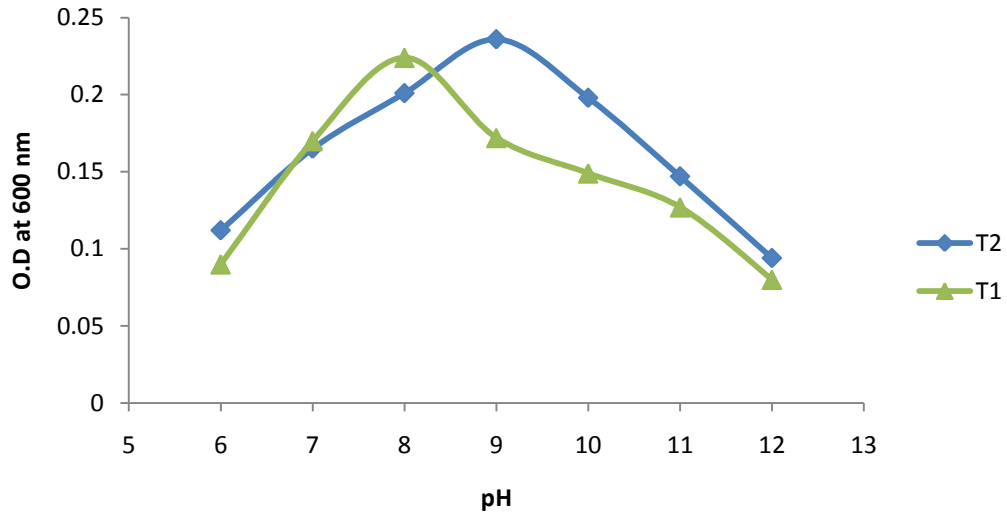
**Figure 4.2: Growth of bacterial strains in Nutrient broth at different temperatures**

From the graph it was observed that both the strain T1 and T2 grows in a different fashion on M9 medium. The growth between 10°C to 20°C was almost linear, but after 20°C the growth increased and maximum growth was observed at 37°C fig. 4.1 whereas in Nutrient broth as it is an enriched medium it shows different growth pattern. Though both having the maximum O.D at 37°C but strain T1 had maximum O.D (2) whereas T2 has only (0.8) O.D in NB which was 0.1 and 0.08 respectively in M9 medium.

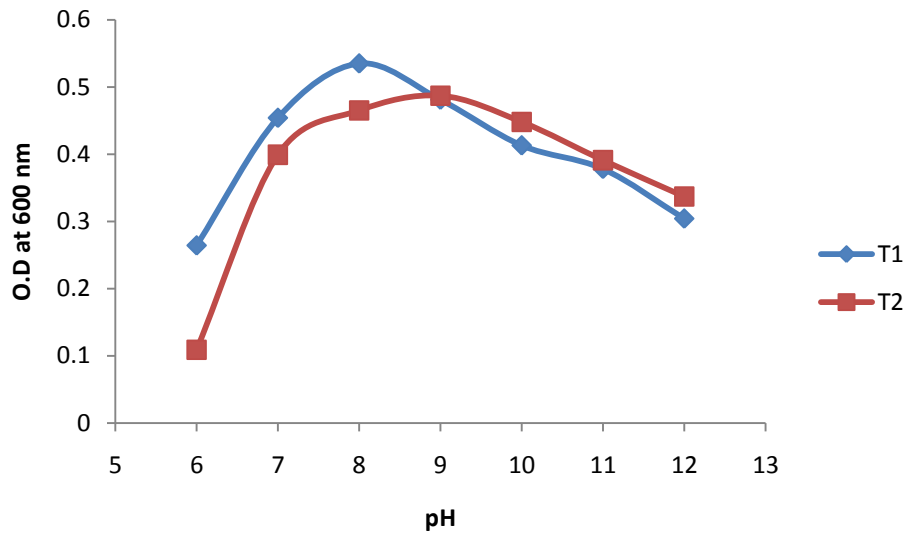
#### 4.4.2 Effect of pH on growth of bacterial strains

Water is polluted mainly by traditional organic waste generated through industrial processes; now a days neutralization of such alkaline wastewaters is not economically feasible (Prisciandaro et al., 2005). So other alternative for neutralization is biological treatment. As attempt has been made to isolate some alkaliphilic bacteria from different sources some studies has been performed to check their effect on pH and salinity other than temperature also.

Each organism has a pH range within which growth is possible. Most organisms have pH optima in 5-9 range. Very few species can grow at pH values below 2 or above 10. Organisms capable of living at low pHs are called acidophiles. Those capable of living at very high pHs are called alkaliphiles.



**Figure 4.3: Growth of bacterial strains in M9 medium at different pH**



**Figure 4.4: Growth of bacterial strains in Nutrient broth at different pH**

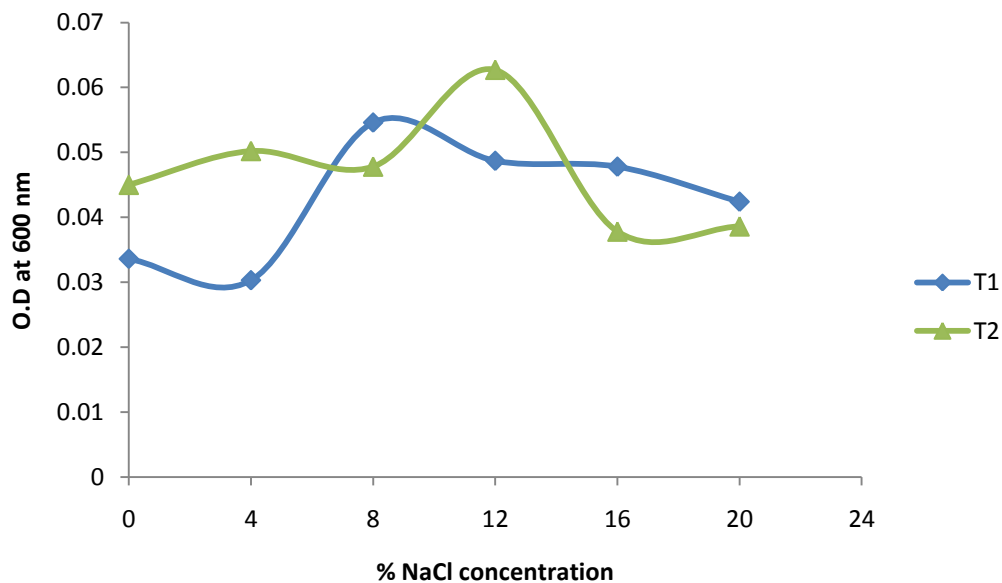
The bacterial strains (T1, T2) are tested at different pH's (6-12) in M9 medium and Nutrient broth. Optimum pH for Strain T1 is found to be pH 8 whereas for Strain T2 the optimum pH is 9, in both M9 medium and NB. Both the bacterial strains showed growth at pH more than 7, hence these strains are alkaliphilic in nature. The growth was maximum at pH 9 in T2 where the optical density is 0.24 whereas in T1 maximum growth is at pH 8 and O.D found to be 0.22, which is just half of the values obtained in Nutrient broth where the highest O.D at pH 9 is 0.55 in T1

strain whereas it was 0.49 in T2 strain, so it was concluded from the result that the Nutrient broth was found to be good in comparison to M9 medium in case of growth at different pH.

Kumar et al., 2011 has also used two medium A and B and found that bacteria *Bacillus alkalophilus* grow very well uptill pH 9 at medium A and B both. In medium B it grows uptill 11.0 pH but growth is comparatively poorer than the growth obtained at pH 9.0. *Bacillus sp.* ATCC no. 27647 also gives good growth with medium A at pH 9.0 and moderate growth in medium B. Medium A does not promote the growth of this bacterial culture after pH 9.0.

The change in pH and alkalinity of the waste water attributed to the production of organic acids in the wastewater by the strain T1 and T2. Paavilainen et al., (1994) also observed production of some metabolic compounds by some alkaliphilic bacteria eg. *Bacillus* cause the reduction in pH and acid production by the *microbial consortium* due to the carbohydrate metabolism.

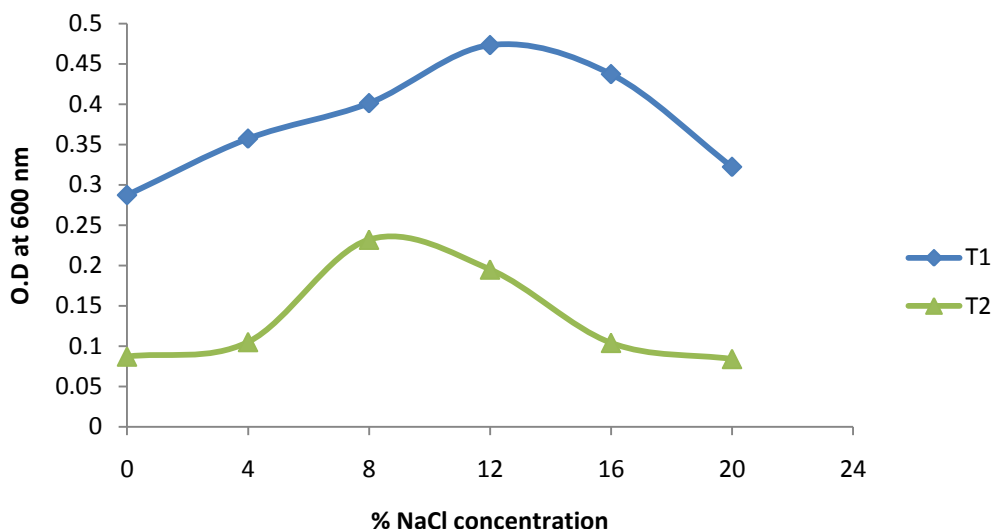
#### 4.4.3 Effect of Salt concentrations on growth of bacterial strains



**Figure 4.5: Growth of bacterial strains in M9 medium at different salt concentrations**

Bacteria can be divide into four groups depending on their ability to grow at various sodium chloride concentration; non halophilic (grow at less than 2% sodium chloride concentration), halophilic (grow above 2% sodium chloride concentration). Halophilic may further be of three

types: slightly halophilic (grow between 2 and 5% NaCl), moderately halophilic (grow upto 10%) and extremely halophilic (grow at more than 20% NaCl).



**Figure 4.6: Growth of bacterial strains in Nutrient broth at different salt concentrations**

All the three strains showed growth in varied salt concentrations (0% - 20%) in both M9 medium and NB.

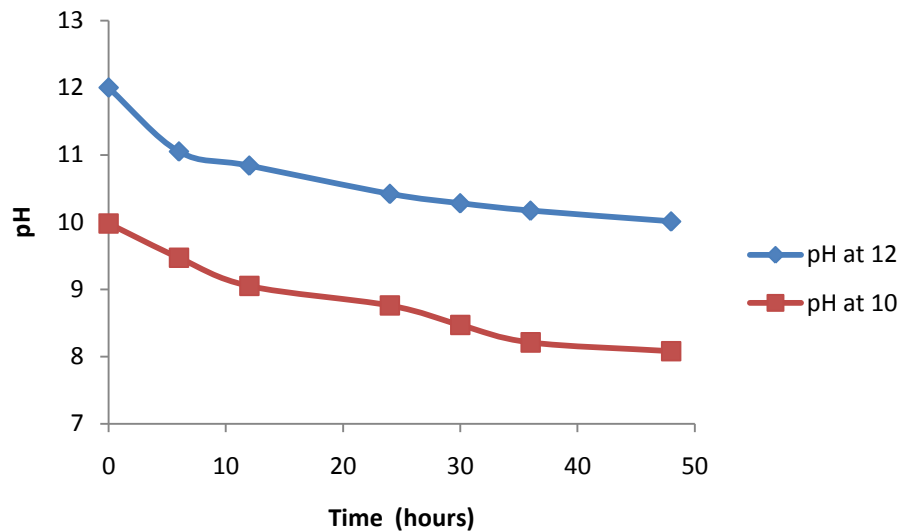
Bacterial strain T1 shows maximum growth at 12% NaCl concentration whereas T2 shows maximum growth at 8% NaCl concentration in both M9 medium and NB. Both the bacterial strains are moderately halophilic in nature. If salinity will increase the growth in both the medium get affected.

The growth of the selected microorganism gave different efficiency in both the medium. As NB is an enriched medium the growth observed better. The maximum growth (on the basis of O.D) was found to 0.46 in T1 and 0.24 in T2 whereas in M9 medium maximum O.D was 0.066 in T1 and 0.05 in T2. So in case of salt concentration studies also it was concluded that NB proved to be better than M9 medium.

## 4.5 pH and Alkalinity degradation by bacterial strains

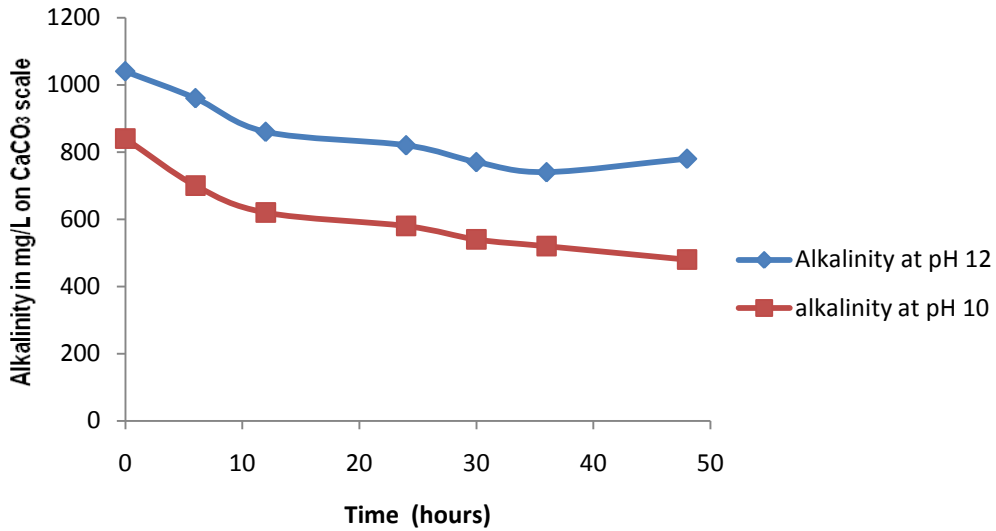
The pretreatment of alkaline wastewaters generated from different industries is now a days a problem to the nation. The neutralization of such alkaline wastewaters chemically is not cost effective. So the biological tool is better remedial process which is adapted in this work

The actual pH of the wastewater effluent from pulp and paper industry was around 9.7-9.8 but to check the degradation efficiency of these bacterial strains, pH was maintained at 10 and 12. As shown in biochemical results the growth of pH was optimum at pH 8 and 9.0, so the pH was adjusted to 10 and 12 to check the efficiency of bacterial strain T1 and T2.



**Figure 4.7: pH degradation by bacterial strain T1**

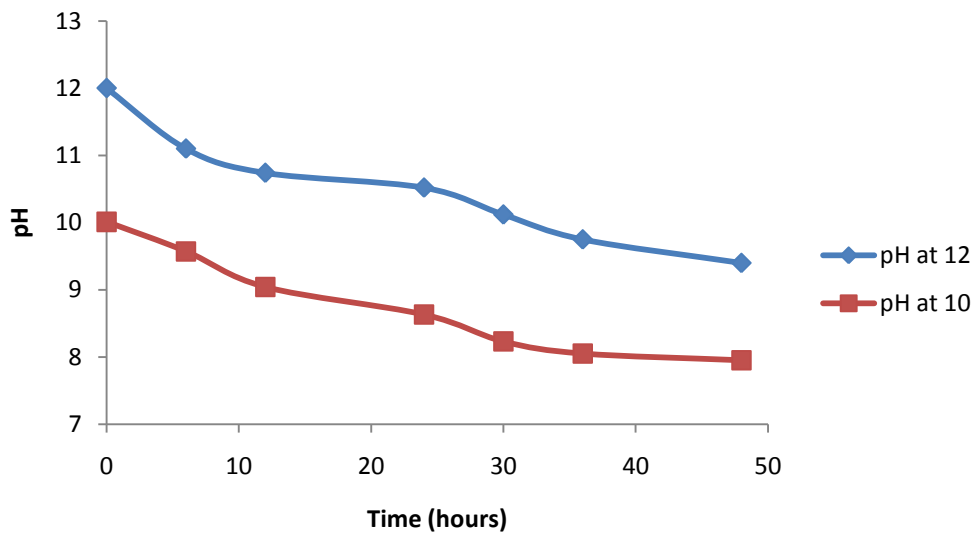
The pH and alkalinity were reduced accordingly in T1 strain. It was observed from fig. 4.7 the pH reduced from 10 to 8.1 and pH 12 to 10 in M9 medium. Fig. 4.8 shows that alkalinity in T1 is reduced from 840 mg/L to 480 mg/L when pH was 10 on CaCO<sub>3</sub> scale and when pH was 12 it reduces from initial alkalinity 1040 mg/L to 780 mg/L. These results indicate that T1 strain is very good and can be utilized for industrial application where reduction of pH or alkalinity is required.



**Figure 4.8: Alkalinity degradation by bacterial strain T1**

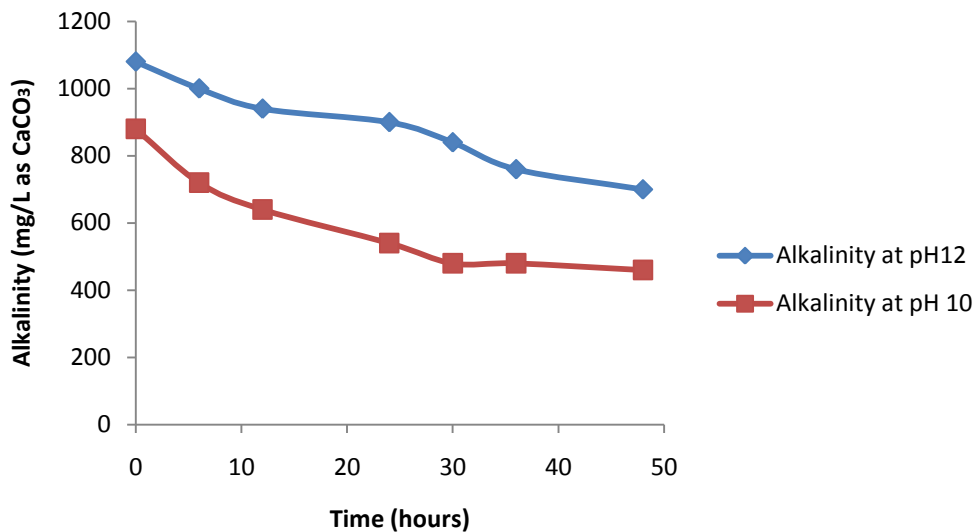
Kulshreshtha et al., (2010) isolated *Exiguobacterium sp.* which has reduced pH from 12 to 7.5 within just 2 hours.

Paavilainen et al., (1994) showed that maximum pH decrease occurring per unit time in alkaliphilic bacteria is 0.13 unit per hour which is quite low as compared to more than two unit decreased during initial one hour of incubation reported in his study.



**Figure 4.9: pH degradation by bacterial strain T2**

The similar trend was observed in pH and alkalinity with strain T2. The pH 10 reduced to 7.95 within 48 hours and pH 12 to 9.4 as showed in Fig. 4.9. In the case of alkalinity, at pH 10 alkalinity was 880 mg/L which reduced to 460 mg/L, which is 48.72% decrease within 48 hours. But when alkalinity was 1080 mg/L at pH 12 it reduces to 700 mg/L which is 36.19% decrease in 48 hours.



**Figure 4.10: Alkalinity degradation by bacterial strain T2**

From fig. 4.10 it was observed that in 48 hours, the pH is reduced from 10 to 7.95 & 12 to 9.4. The alkalinity get reduced from 880 mg/L to 460 mg/L on CaCO<sub>3</sub> scale at pH 10 & from 1080 mg/L to 700 mg/L on CaCO<sub>3</sub> scale at pH 12.

Kumar et al., (2011) isolated several microorganisms out of them two grow well at pH higher than pH 10 and reduced the alkalinity also.

The use of bacteria for neutralization will reduce the final volume of the wastewater to be discharged after treatment as large volumes of mineral acids are no longer required to be added for neutralization.

## CONCLUSION

- Present study concluded that alkaliphilic bacterial isolates T1 and T2 are able to neutralize highly alkaline wastewater from pulp and paper industry.
- Strain T1 reduces the pH from 12 to 8.1 and alkalinity from 1040 mg/L to 480 mg/L whereas strain T2 reduces the pH from 12 to 7.95 and alkalinity from 1080 to 460 mg/L.
- Alkaliphiles can neutralize the alkaline wastewater within 48 hours which is economical.
- Thus we can conclude that isolated alkaliphiles can be used for neutralization of alkaline wastewater from pulp and paper industry and other industries generating alkaline wastewater.

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