Operational Analysis of vertical flow constructed wetland fitted with aerobic-anaerobic chambers for the treatment of Black Liquor

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QUAID-I-AZAM UNIVERSITY
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2022

بسم الله الرحمن الرحيم

Dedicated

TO MY PARENTS

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Maqsood Ahmad Awan

Certificate

This thesis submitted by ***Maqsood Ahmad Awan*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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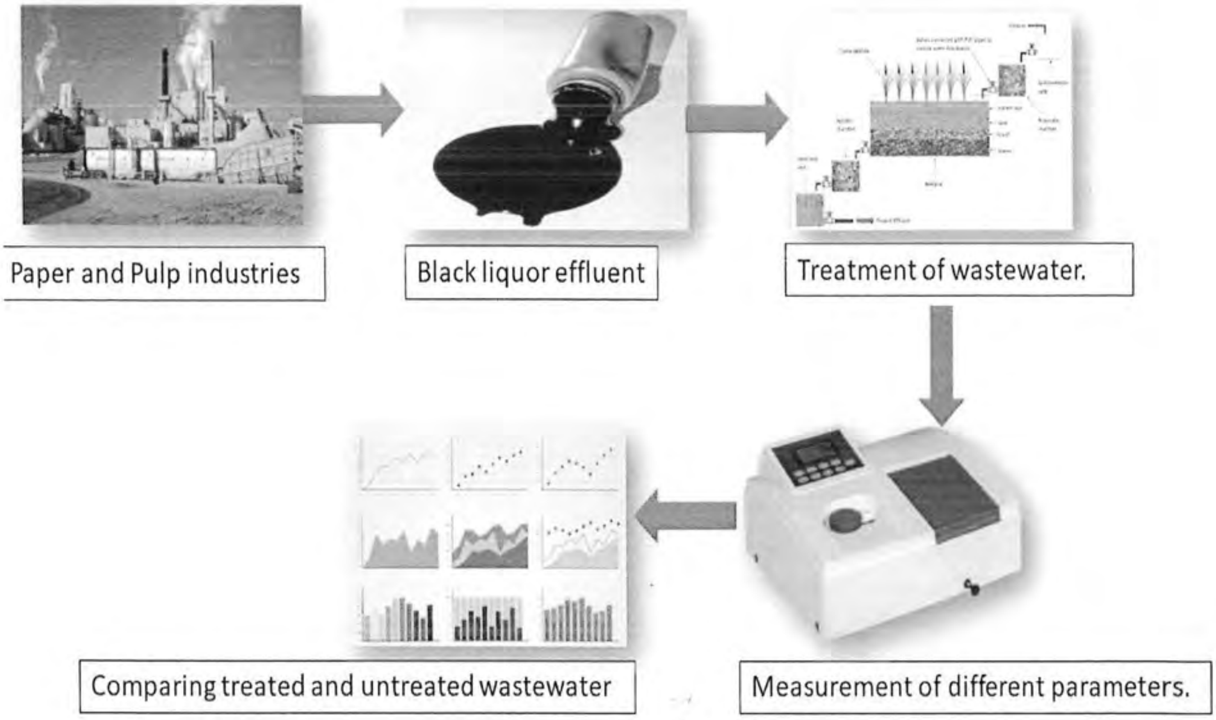
Abstract

Paper manufacturing is among the most energy extensive and wastewater producing industries in our country. The waste water produced from paper industry one of the major environmental threats. Bioremediation has been an important method for the reclamation of environmental pollution caused by pulp and paper industry commonly known as black liquor. In present research, a hybrid wetland was developed for the treatment of black liquor. Hybrid wetland was consisting of primary sedimentation chamber anaerobic chamber, constructed wetland, aerobic chamber and sand bed. Wastewater from industry was treated in hybrid system at flow rate of ten to fifteen ml per min. This process was run for 8 weeks in which the average temperature was a 33°C during day time. The treated wastewater collected and analyzed for various parameters. These parameters include odor, pH, electric conductivity, dissolved Oxygen, BOD, COD, TDS, TSS, sulphates, phosphates, nitrates, CFU and MPN. Our results indicated significant reduction in COD 71%, BOD 71%, EC 72%, TDS 74%, TDS 74.4%, MPN 75%, Nitrate 73%sulphate 72%, phosphate 73%. In next step biofilm was studied that develop in constructed wetland and aerobic chamber. Ten different strains were isolated which were Biochemically tested. These strains belong to genus *Bacillus, Pseudomonas, Rhodococcus and Streptomyces*. In conclusion the hybrid wetland was very efficient to remove major pollutants from the wastewater. The hybrid wetland contains a diversity of microorganism that remove the pollutants. Therefore, in the light of these results the hybrid wetland is very suitable to complex wastewater treatment.


Paper and Pulp industries
Black liquor effluent
Treatment of wastewater.
Measurement of different parameters.
Comparing treated and untreated wastewater

List of Abbreviations

ABR	Anaerobic Baffled Reactor
BOD	Biological oxygen demand
CFU	Colony forming unit
COD	Chemical oxygen demand
Conc.	Concentration
DO	Dissolved oxygen
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
EMB	Eosin metylene blue
EPS	Exopolysaccharide (matrix)
FBR	Fixed biofilm reactor
FC	Final clarifier
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
Hr	Hours
HRT	Hydraulic retention time
IC	Intermediate clarifier
IR	Infrared
KgCOD/m3/day	Kilogram of COD per cubic meter per day
Min	Minute
MPN	Most probable number
NA	Nutrient agar
OD	Optical density
PCA	Pseudomonas citrimide agar
PC	Primary clarifier
pH	Power of Hydrogen ions
Q	Flow of wastewater
RBC	Rotating biological contractor
SOB	Sulphur oxidizing bacteria
Sp	Species

TDS	Total dissolved solids
TSS	Total suspended solids
WHO	World health organization
WWTP	Wastewater treatment plants
'C	Degree Celsius/Centigrade

Contents

List of figures:

List of tables

Introduction

Humans generates approximately 359 billion m^3 of wastewater annually, according to Edward Jones. This amount of water can fill up to 144 million Olympic-sizes swimming pools. Most of time this wastewater is pump to creeks, rivulets and seas. We should save our waterways and water bodies from massive number of pollutants by preventing wastewater from entering into them. We need to keep industrial wastewater out of our waterways and waterbodies reason being their environmental effect is extensive. The paper factories use a lot of clean water and produces a lot of effluent at several phases of making of paper and pulp. The effluent discharged has worst effects on the surroundings and seriously endangers both human and animals living (Gayathiri 2022). Paper industries produce third largest effluent after metal and chemical sectors. Pakistan has more than 100 units that has capacity to produce 650, 000 tons per annum. These industries produce 244.4 million m^3 of wastewater (Sana Akhtar.2013). This wastewater is produced through several different processes. During debarking of wood, chip production, pulp production bleaching and washing and drying. Each procedure is directly corelated with its amount of pulp production and the techniques used during the process (The World Bank Group, 1999). The produced wastewater contains a higher level of BOD and differing quantities of other pollutants depending on the kind of applicable operations. e.g. The effluent from digester has fatty acids AOX, VOX, brown color and a high level of COD and BOD whereas effluent from wood preparation has suspended particles, filth fibers and high level of BOD (Pokhrel and Viraraghavan, 2004).

Resins, lignin tannins and chlorine compounds are the pollutants which are present in effluent that are toxic to marine life (Buzzini and Pires, 2007). The other pollutants that should be eliminated and /or reduced in treatments facility through variety of methods includes adsorbable organic halides (AOX), nitrates nitrites ammonia total suspended solids and chemical oxygen demands. The treatment of wastewater is important because the processed effluent can be reuse. It can also be discharged into the environment if the level of pollutant is reduced to such level that they meet the environmental criteria. Paper and pulp industries also produces greenhouse gases and solid sludge when they treat the wastewater through mechanical or chemical treatment. CO_2 and methane are the three significant greenhouse gases that have been documented to be generated in this process (Ashrafi et al., 2013b).

Mostly wastewater is processed using chemical methods to remove solid and colloidal particles, hazardous substances, floating material and colors. Previously many procedures have been put forward by different people which includes coagulation and flocculation by wong et al., 2006, flotation by Hogenkamp 1999, screening El-Ashtoukhy et ., 2009, sedimentation by Kishimoto et al., 2010, and ozonation electrolysis ultra- filtration by Bhattacharjee et al., 2007. When treating wastewater, either in the primary, secondary or tertiary phases, physiochemical methods are frequently employed. The problem with these methods is that they require either addition of chemical or use of electricity which make them expensive and requires regular maintenance to keep efficacy. According to Thompson et al,. 2001 wastewater might potentially have 80% of its suspended particles removed using sedimentation.

To decompose dissolved pollutant from wastewater the majority of water processing plants employs aerobic and /or anaerobic microbiological techniques. Pulp and paper plants employs aerobic treatments since it is very cheap and effortless and low expenses (Mulligan, 2002). Aerobic methods like 'activated sludge' and 'aerated lagoons' are frequently used (Pokhrel and viraraghavan, 2004). Anaerobic treatments have shown to been effectively remove toxic material at lower pH (Salkinoja-Solonen et al., 1984). Both aerobic and anaerobic treatments have disadvantages because they produce large amount of sludge.

Constructed wetlands were develop in 1950s (Seidel, 1961). Throughout the past two decades the uses of the built wetlands for treating domestic wastewaters have expanded to take in industrial effluents (Vymazal , 2011). Arivoli et al., in 2015 use the constructed wetland with vertical flow to effectively remove the heavy metals up to 80%. But due to presence of very high amount of these metals over time these metals decrease the efficacy of wetland.

To reap the benefits of different treatments in this study we develop hybrid system. First chamber has primary sedimentation to trap the suspended particles. Second chamber has anaerobic chamber that degrades the organic compounds in the effluents. Third chamber was consisting of a constructed wetland that uses both plants and bacteria to degrade the lignin and remove heavy metals from the effluents. Fourth chamber was made up of sand bed that filter out any remaining particles. Both aerobic and anaerobic treatments have disadvantages because they produce large amount of sludge. This set up was built at Soil and water lab using Glass plastic sheets. These sheets were cut down into length width base and top which were glued together using super glue.

Aims and objectives

The aim of the present study was to analyzed vertical flow constructed wetland fitted with aerobic-anaerobic chambers for the treatment of black Liquor

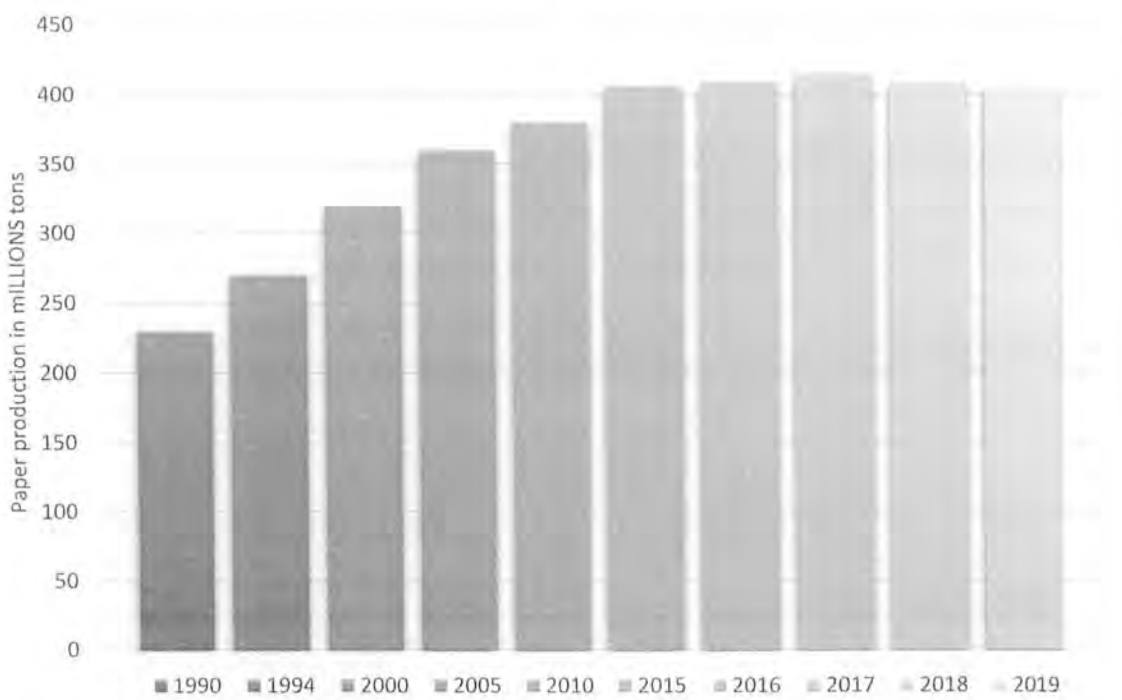
Objectives

- Develop wetland fitted with aerobic and anaerobic chambers.
- Evaluate the performance the bioreactor under varying conditions.
- To study biofilm and associated microorganisms for their role in bioremediation

Literature Review

Paper and pulp industry

Paper and pulp industry is the 3rd biggest industry on the world stage succeeding Petro-chemical and mining. This industry has strong foothold in East Asian Northern Europe and Latin America. In the coming years the Africa and south Asia will become the new hub of this industry. Around the world there are ten thousand paper mills that are making paper every day. These mills employees around millions of people directly and indirectly. Around 647,000 peoples are linked to this industry via 21,000 companies. Total valued of paper market is around 351.53 billion in 2022. Globally the production of the paper and its products were 230 million tons by the year 1990 which rose to 270 million by the year 1994 and 329 million tons by the year 2000. The increasing tread of paper production continue in 2005 and read 360 million tons. During the year of 2010 the production was 380 million tons, in 2015 it was 406 million tons. World's paper production is near 417 million tons and it is estimated to reach to 550 million tons by the year 2025 (Statista 2023)



. **Figure 2. 1 Annual global paper and paperboard production** (Statista 2023)

Paper production in Pakistan

Domestic demands of paper and its products is very high in Pakistan so meeting up to this demands Paper industry has develop into significant size. In Pakistan there are over more than 120 units that are manufacturing paper and these 96.k people are attached to paper industry. These mills produced following types of paper

- text paper
- wrapping paper
- office paper
- color paper
- Brown Paper
- filtering paper

Despite its size in Pakistan, this industry still requires to improve its manufacturing and treatment by implying modern tech and smart innovations. Many companies in Pakistan are investing in developing indigenous technology and procedures to make the technology feasible in Pakistan. But these efforts are disrupted by various factors like ever changing policies of governments. In recent years many governments policies are not in favors of the industrialists compounding this with other factors like unskilled labor force lethargy and low pressure of gas supply are greatly affecting the production of the paper production. Towards the end of the pervious century, the paper and board production were around 149,000 ton which rose to 246,000 tons in 2000s. During the year of 2001 the production fall to 187,000 tons due to economic reasons. From 2002 to 2005 the production of paper gradually and reached to 476,000 tons. In 2006 the production fall again 464,000 tons which by 2010 fall to 330,000 tons. In 2011 the production was 410,000 tons and reached to 680,000 by the year 2014 than again fall to 590,000 tons in 2016. In year 2019 the paper production was around 690,000 tons (Afzal, M.,2008) (CEIC 2023) (Shabbir and Mirzaeian, 2016).

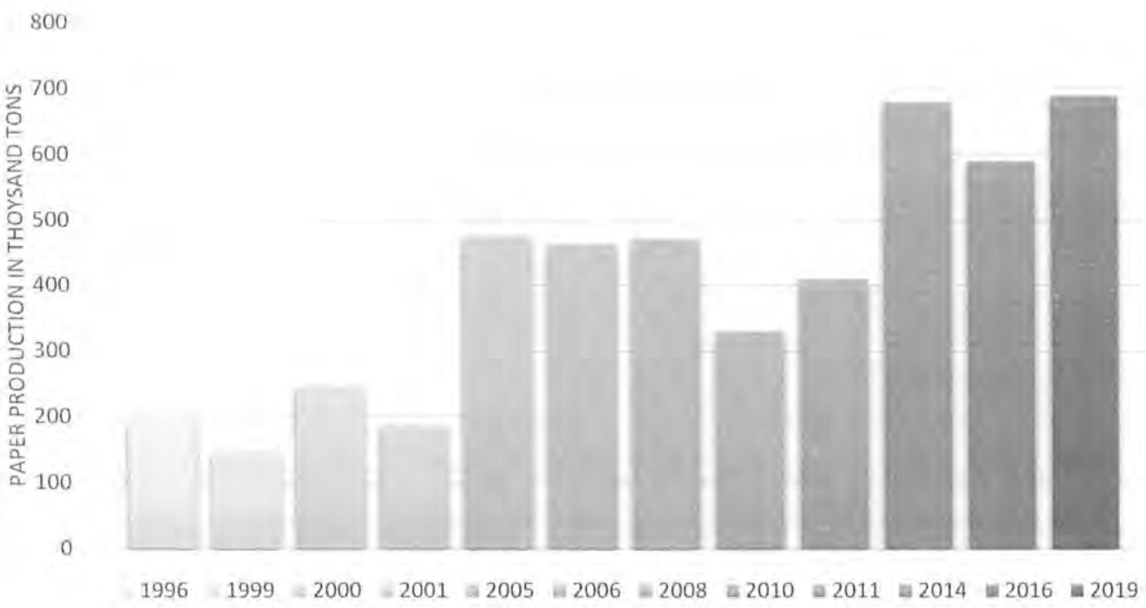


Figure 2. 2 : Paper production in Pakistan by year (CEIC 2023)

Paper making

there are many ways to produce paper on industrial scale but general there are five basic phases. the effluent that is generated from paper industry is a mixed of all wastewaters from different phases. The wastewater composition depends on the procedures implied and the chemicals used

Debarking

Wood from tress commonly known as softwood tree like pine is used in paper making. Wood is demarked and the plant fibers are chopped into small pieces called chips (Moussavi, A., et al. 2023).

Pulping

In the next phase the chips are turned into the pulp. Cellulose rich pulp is obtained by removing hemicellulose and lignin from the wood. There are two treatments i.e., chemical (sulfite pulping. Bajpai, P. 2016 kraft pulping Shrotri, A 2017) and mechanical pulping (Höglund, H. 2009). This process produce wastewater that contains color resin, acids fatty acids, AOX, VOCs, dissolved inorganic compounds, soluble wood.

Bleaching

The color of pulp is brown which is removed by applying bleach. It is done to refine the grade of the paper and bring the final product to desired standards. Oxygen, dioxide chlorine, hydrogen peroxide, chlorine is use in different proportions to remove color. Lignin, phenols, chlorophenols halogenated hydrocarbons AOX, EOX, VOCs are generated as toxins that are generally washout with water.

Washing and drying

Bleaching agents are removed from the pulp obtaining snow white pulp. White pulp is layered into sheets and then pressed to remove extra water. Finally, the sheets are dried on hot roller and folded into rolls. At this stage organic compounds dissolved in water plus dyes and alkali are generated as effluent

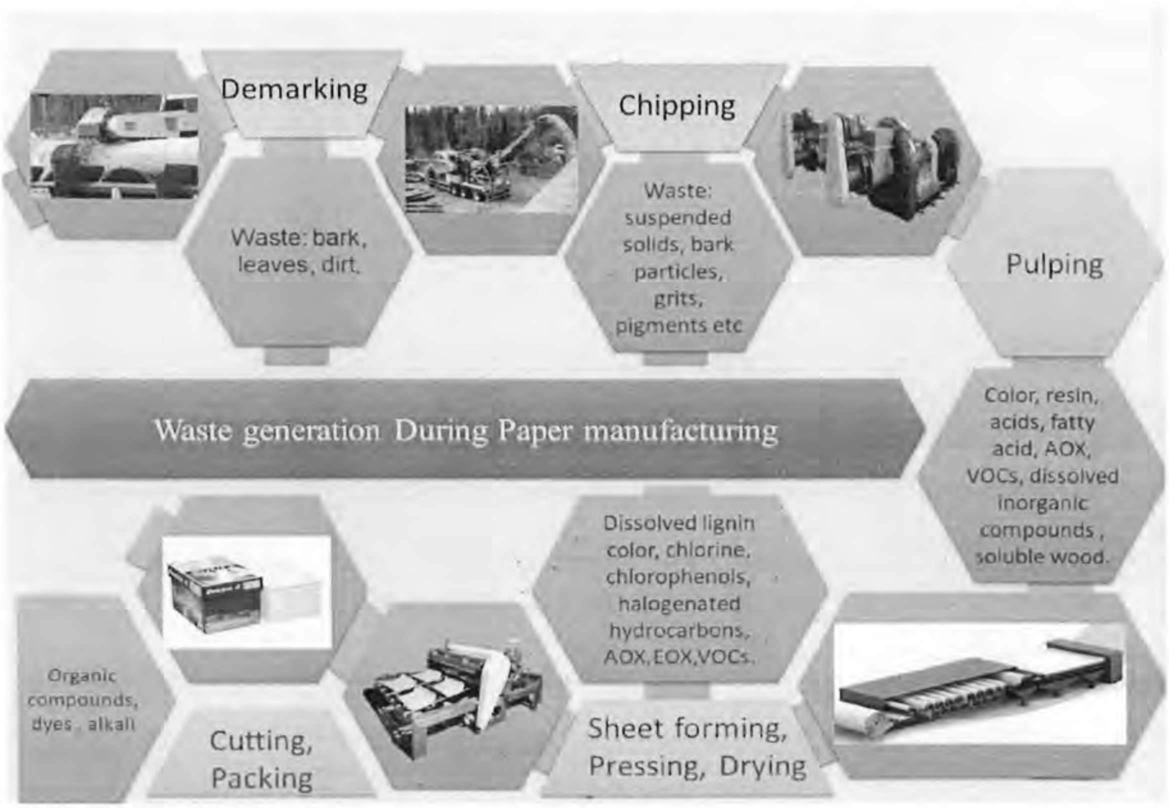


Figure 2. 3 : waste generation During Paper manufacturing at various steps

Black liquor

Due to its benefits over alternative pulping techniques, the Kraft process is currently used in up to 90% of all pulp mills globally to produce pulp (J. Hu et al., 2018). These mostly include getting stronger fibers, how simple it is to apply to various types of wood, and recovering the used chemicals (P. Bajpai, et al 2017). Essentially, there are four steps in the making of Kraft pulp: 1: prepping the wood, 2: 'pulping', 'bleaching', and 'chemical recovery' (A.K. Singh, R et al., 2019). More specifically, bark is removed from the logs and then made into small pieces. These small pieces are then degraded for two to three hours in a watery mixture (called white liquor) containing 15-20% Na_2S and caustic soda at hot temp (155 °C) (A.L. Woiciechowski, 2020). The cellulosic material is segregated from the solubilized chemicals in this process, resulting in a fluid known as weak black liquor.

Numerous variables, including the kind of feedstock utilized, and the operating parameters employed in making pulp, affect the characteristics of Kraft black liquor (M. Cardoso et al 2009). All of these fluids, however, are viscous, have increase pH and carbon content. Both organic (derivatives of phenol, benzene, carboxylate) and inorganic (primarily, caustic soda) components are present in this stream, with dry solids accounting for up to 15% of the total (N.S. Kevlich et al 2017).

The pulp is created, then bleached using oxidation and chlorination procedures to give it the final qualities needed for its further usage, while Kraft black liquid goes through a chemical recovery process. This stage involves first concentrating the black liquid to at least 65% of its solids, then burning it to produce energy and a molten smelt that is high in sodium carbonate and Na2S. (Na2CO3). Following their first dissolution in water (known as "green liquor"), (Reyes, L 2020). these ashes are mixed washing soda and lime with to produce caustic soda, with Na2S remaining inert. The liquid that remains after this reaction is pumped back to the digesting stage after being filtered to eliminate the created lime mud (CaCO3). To regenerate the used CaO, the mud is next cleaned and warmed to an elevated temp. (Chang, M.2017).

The above-mentioned treatment of black liquor is used commercially but it has many environmental drop backs so researcher look for other methods to degrade black liquor, some of these are as follows

Table 2 1 : Physical treatments of Black liquor and their useful products

Physical treatments

Treatments	Parameters	Products
Decantation	20-30% of black liquor, Acidifications, Additives	Tall oil soap (T. Aro et,al 2017).
Filtrations	Micro-membranes, ultra-membranes. Nano-membranes,	Lignin, organic acids and inorganic salts (N.S. Kevlich, et al 2017).
Acidifications	pH 9-2, Acid: Dihydrogen sulfate,	Lignin, acids of nonaromatic hydrocarbons (S.H.F. da Silva et al., 2020).
Fractions	Pre-treatment, Pressure: 0.067-0.173 KPa	Carboxylic acids, lignin (L. Reyes 2020)
Solvent-solvent Extraction	Trioctylphosphine oxide, Alkanes	Carboxylic acids, polycyclic compounds. (D. Núnez, 2022).

Table 2 2 : Chemical treatments of Black liquor and their useful products

Chemical Treatments

Treatments	Parameters	Products
Emulsion	Emulsification agents: 1,2-Dichloroethane, Propionyl Chloride, Formaldehyde. And additives	Bio-carbon (G. Amaral-Labat, E et, al. 2021).
Oxidation	Peroxides: Hydrogen peroxides	Carbon Ash (Z. Al-Kaabi, et,al. 2020).

Table 2 3 : Electrochemical treatments of Black liquor and their useful products

Electrochemical treatments

Treatments	Parameters	Products
Electro-decomposition	Anode/cathode: Nickel/stainless-steel Power: 0.3 V	Lignin H_2 (R.C.P. Oliveira, et al . 2012).
Active carbon filtration using electricity.	Anode/cathode: Tin/Iron Membranes: deionizing membrane	Lignin H_2 Sodium hydroxide Sulphuric acid (J. Heinonen et al. 2020) .

Table 2 4 : Thermochemical treatments of Black liquor and their useful products

Thermochemical Treatments

Treatments	Parameters	Products
Gasified.	High/dry Kraft black liquor Thousand-degree temperature increase atmospheric pressure	Biogas (CO, H2, CH4, mainly) Residue of melted metals (D.T. Pio et al. 2020).
Supercritical H_2O gasified	Weak Kraft black liquor 400-degree temperature increase atmospheric pressure	Biogas Mixture having high carbon content (P. Casademont et al. 2020).
Thermal decomposition	High/dry Kraft black liquor Nine-hundred-degree temperature. No oxygen.	Renewable diesel Biogas Biochar (J. Huang et al. 2020).
Hydrothermal decomposition.	lower Kraft black liquor three-hundred-degree temperature Rise atmospheric pressure No oxygen	Renewable diesel Moist biochar (H. Boucard et al. 2020).
High temperature hydrolysis	lower Kraft black liquor two-hundred-degree temperature increase atmospheric pressure No oxygen	Mixture having high carbon content (L. Pola et al 2019).
Moist decomposition	Lower Kraft black liquor Three-hundred-degree temperature Increase atmospheric pressure	Mixture containing long-chain carbon molecules (L. Pola et al 2021).

There are some drawbacks that are linked with these treatments. first, in order to treat black liquor through these approaches, separate units are to be set up where black liquor is treated. separate units cost a lot of capital to invest. Second, when we set up a separate unit it would requires a set of skill workers and other laborer. third, a lot of these set up are energy intense which make them less profitable and difficult to operate. fourthly products that are recover from these, are not very refined which means they loss their competition to chemical synthesis products. fifthly these systems lost their efficiencies after sometime and increase the bill in terms of repairs and replacement (L.A. Zevallos 2020).

Biochemical treatment/Fermentation

It is important to note at this point that a few publications about the microbial ecology of black liquor have come out, since few microorganisms can survive its harsh environment (C.H. Ko, et al 2011). Actually, *Paenibacillus* was sequestered from Kraft black liquor and utilized to degrade birchwood xylan-rich media, producing xylanase as the primary metabolite (C.H. Ko, et al 2007)

Due to the sugar and lignin concentration of black liquor, biochemical treatments to valorize its components have long been of interest (R. Morya, et al. 2022). For instance, Potvin created citric acid from black liquor by employing the yeast Candida tropicalis (J. Potvin, et al. 1988). However, Kraft black liquid needed to be have concentration of about 10% solids, and fortified with phosphate and Mg salts before it could be used. These results that were obtained using this artificial acetate medium, the 2-hydroxy-1,2,3-propanetricarboxylic acid production was almost one-half of the theoretical peak output. In contrast to the synthetic medium, the black liquor's other organic acids slowed the absorption of acetate and the pace at which products were produced.

Analyzing more recent studies, it was discovered that microorganisms that produce laccases and peroxidases were also used to degrade the lignin in wasewater to create molecules that have high cost of productions (ethanal), while simultaneously lowering black liquor's carbon matter and color intensity (G. Singh, et al., 2019). Paliwal et al. used this method when they concurrently cultivated 'Bacillus megaterium ETLB-1' and '*Pseudomonas plecoglossicida* ETLB-3' at alkaline conditions on Kraft black liquor (R. Paliwal, et al. 2016). They reported after 96 hours that lignin degrading enzymes like Mg^{+} peroxidase had shown notable activity (7-9 U/mL). Most of the original lignin (92%) and chlorophenols (91%) were degraded by these enzymes, resulting in the formation of several commercially valuable chemicals.

It's also noteworthy to note that, in addition to lignin, some Kraft black liquid fractions can undergo biochemical processes after being separated. As a result, some scientists have fermented Kraft hemicellulose to create substances like ethanol and butanol. Hemicellulose has a poor heating value, similar to carboxylic acids, hence it is not advised to use it as a direct source of energy (H.J. Huang et al 2010). In this regard, a residue in semi-solid form was acquired with H_3CO_2 and employed as feedstock by Kudahettige-Nilsson et al. This material is abundant in hemicellulose and phenolics. (R.L. Kudahettige-Nilsson et al 2015).

They remove any enzyme blockers with help of active carbon. Lignin was degraded through the help of Dihydrogen sulphate. Lignin later solidify out of Sulphuric acid and was given to *C. acetobutylicum* as substrate. This bacteria breakdown it into butanol. Although encouraging, the yield was significantly less compared to other medias like $C_5H_{10}O_5$. In order to reduce the development of inhibitors before to fermentation and increase the generation of bioethanol, the authors suggested using additional pre-treatment techniques. (R.L. Kudahettige-Nilsson et al 2015). Faisal et al. tested a later selectively recovered butanol in the solvent with the help of suitable adsorbent by carrying out a similar procedure. The co-adsorption of butanol with this substance was exceedingly selective, according to the results, which is minimized at $pH = 8$ (A.faisal et al. 2018)

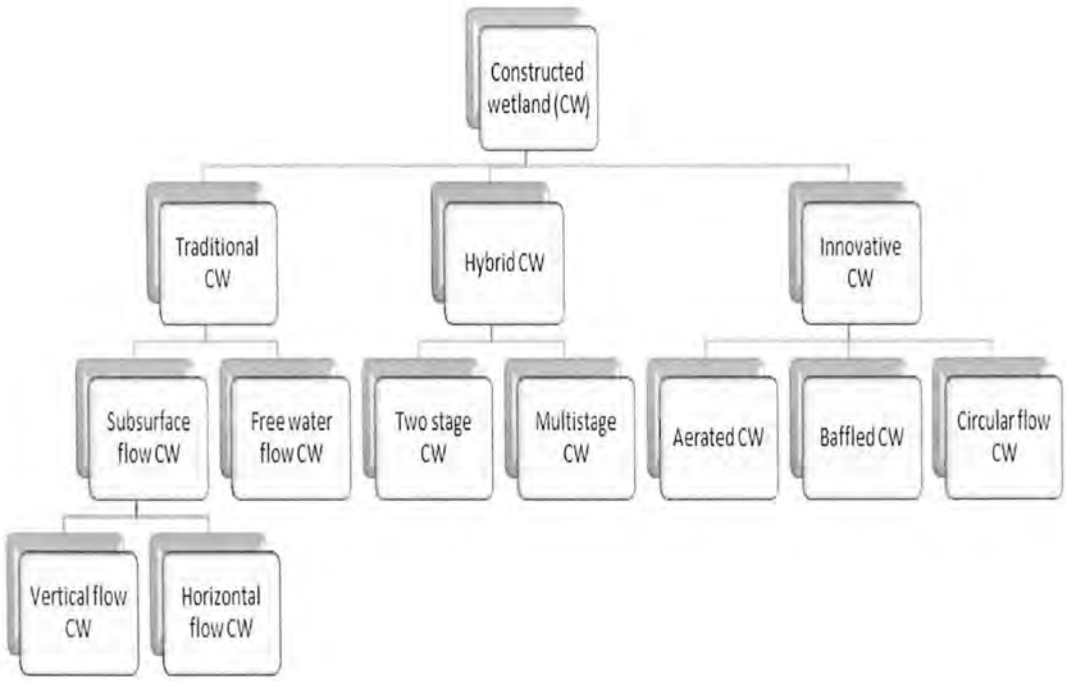
Constructed wetland.

Treatment of wastewater from constructed wetlands was established in 1950s (Seidel et al. 1955) since then different models and designs were put forward by different scientists at different times. Three basic types of constructed wetland on the basics of flow of water are vertical, horizontal, and hybrid. Many cities have central wastewater treatment facilities based on different designs (Maniam, G et al. 2022). One of the problems with central wastewater treatment is that is requires transport of wastewater to the facility through pipes or tanks which can leak out (De Anda et al., 2018). Another problem with these systems is that they require a lot of energy for the mechanical aeration and left behind a huge amount of sludge that needed to be safely disposed of.

Countries are nowadays looking for treatments of the wastewater at the point of its production. These systems are called decentralized wastewater treatment systems (ElZein et al., 2016) These systems usually have one or two anaerobic chambers and adjoining constructed wetlands. These systems also work best in developing countries where operating centralized waste treatment is not cost effective and land is cheap as compared to developed countries (Libralato et al., 2012_). There are some limitations with these systems among them one is substrate clogging which results from high organic content in the wastewater (Hdidou, M.,et al., 2021). These limitations make them ineffective in the long term. The solution to these limitations is the hybrid system which utilized different chambers to maximize the removal of nutrients. Anaerobic reactors have shown quit a efficiency in removal of solid contaminants from the waste water. This led to lower sludge production. Another possibility from anaerobic chambers is the production of methane gas from the sludge produce and use that biogas to generate energy (Álvarez et al., 2008). Mostly the solid organic pollutants are converted into dissolved organic pollutant which are picked up by the roots of plants in the constructed wetland (Brix, H. 2020).

In order to enhance wastewater treatment and get beyond the drawbacks of separate (anaerobic chamber and constructed wetland) units, solutions incorporating both of them have arisen (liu et., 2015). The utilization of the anaerobic reactor and constructed wetland in combination offers a remedy for the drawbacks of anaerobic reactor and constructed wetland when use alone, however research on these combinations is still in its infancy. Only a small portion of was devoted to discussing on hybrid systems and only a handful of their benefits were acknowledged. On the contrary side, a larger range of research, involving septic tanks, bed reactors and blanket reactors, paired with different types of the constructed wetlands were reviewed by Alvarez et al. (2008), however it concentrates mostly on the degradation of organic wastes and TSS and scarcely discuss the reduction of the nutrients.

This combination of constructed wetland with anaerobic reactor have shown high efficacy in different combinations. some of these combinations includes '2-phase parallel flow constructed wetland', 'two stage perpendicular flow constructed wetland,' 'two stage parallel- perpendicular flow constructed wetland' and 'muti-stage constructed wetland'. The conventional system had efficacy of 30% to 66 % while the hybrid systems have shown degradation up to 80% (del Castillo, et al., 2022)



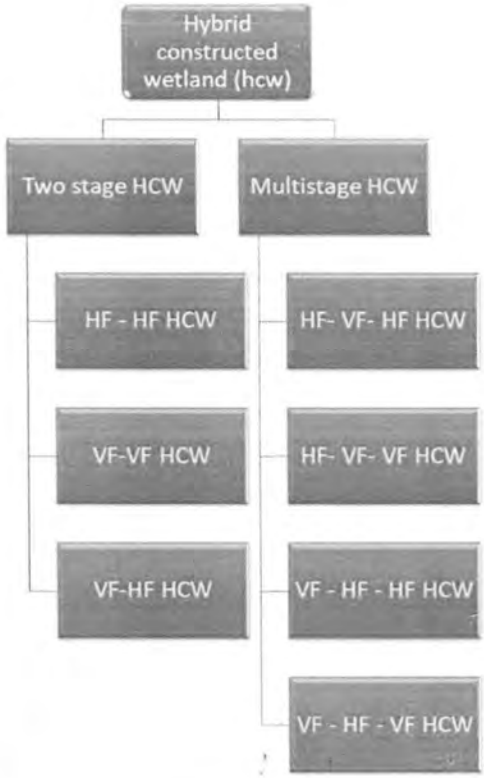


Figure 2. 4 : Different types of Hybrids constructed wetlands

Methodology

3.1. Scheming and Building of Constructed wetland fitted with anerobic aerobic chambers.

A constructed wetland fitted with aerobic and anaerobic chambers was built in Soil and Applied environmental Microbiology Lab. The hydrodynamic flux of this Bench GRIB was 10-15 ml/min (Q = 0.0009 m^3/h), the feed flow rate (Q/A) was 0.02 m^3/day, and the biological loading rate (OLR) was 0.35 g/m^3. It can process roughly 2 liters of black liquor (BL) each day. This hybrid wetland was consisting of four chamber and one constructed wetland. The four chambers had equal length(120mm) width(120mm) and height(220mm). The wetland had length of 406mm width of 304mm and height of 203mm. Wetland was sandwich between four chambers, two before wetland and two after wetland. These chambers were arranged on an iron stand in a step-down arrangement so that wastewater moves down the next chamber through gravitational force. The first compartment served as the main settling tank (PST) which was followed by an anaerobic chamber (ANC), wetland, an aerobic chamber (AC), and one filtration chamber. In PST, ANC and AC stones with a rugged surface area and a volume of roughly 2mm^3 were selected as stuffing materials. Wetland was constructed in layers, first layer was consisting of pebbles with approx. diameter of 2 inches on top of this layer was a layer of gravel, over the layer of gravel was a layer of sand and the uppermost layer was consisting of mud. Each layer in wetland was 2 inches thick. Air pump was used to oxygenate the air in the aerobic chamber whereas paraffin waxes were used to seal the anaerobic chamber. Every chamber in this system had been linked to the next chamber via a PVC pipe system with plastic valves (V1, V2, V3, V4, V5, V6 of internal diameter of 0.1inches and the length of 10 inches). To create biofilms on the surfaces of pebbles, they were maintained in domestic wastewater over 10 days as incubation phase. Figure 1 graphically depicts the hybrid wetland.

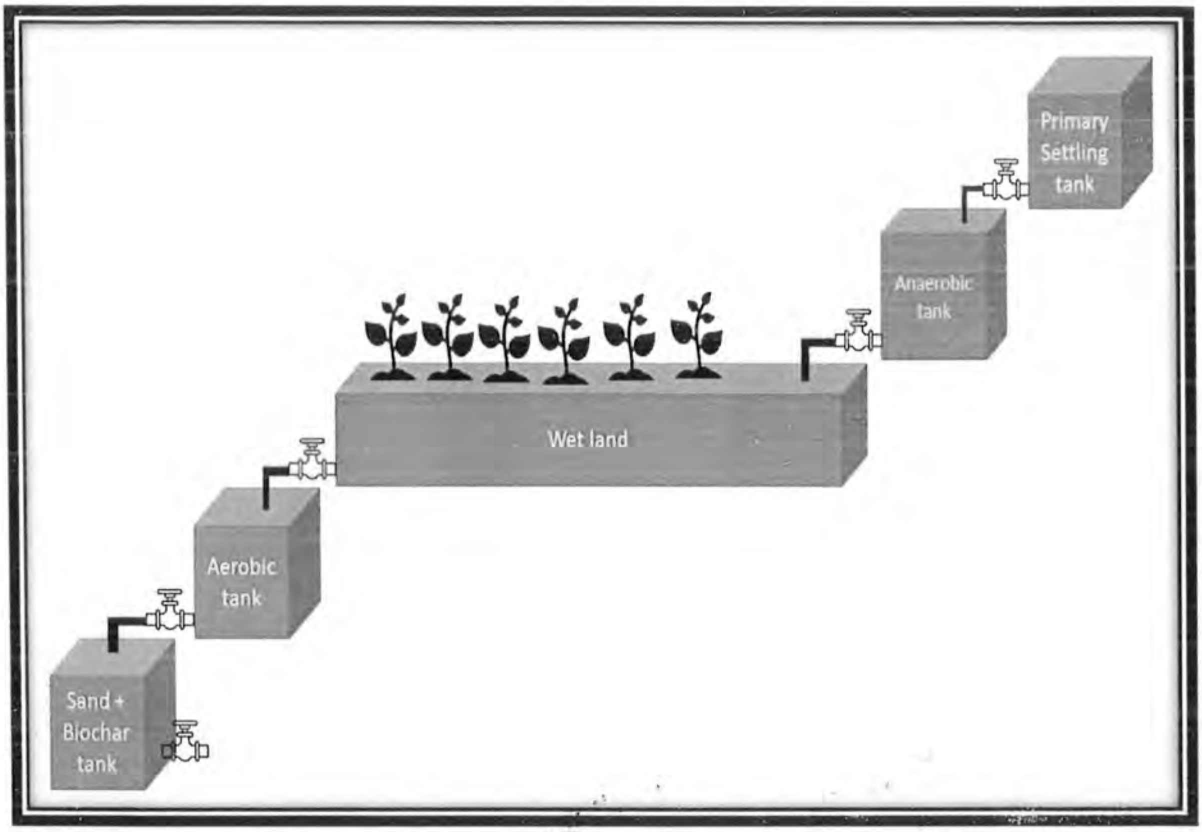


Fig 3. 1 Figure shows the arrangement of different chamber in the system

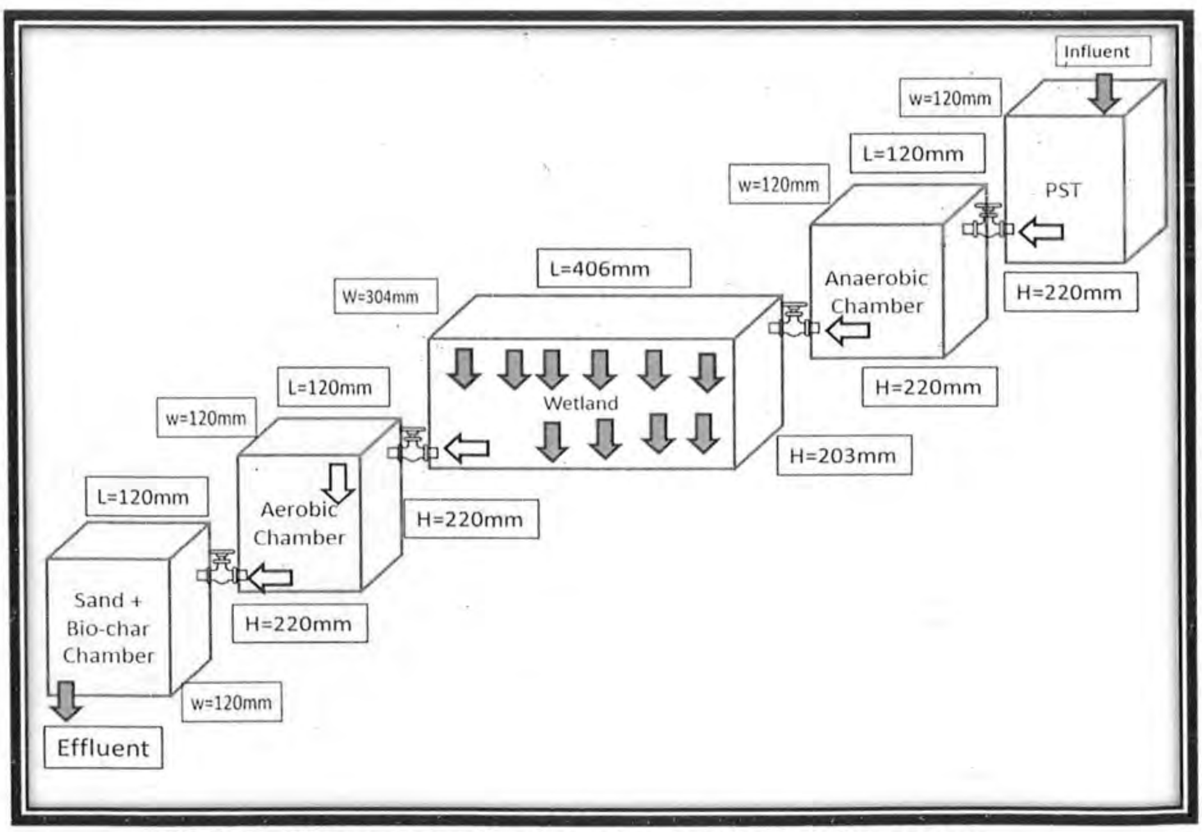


Fig 3. 2 : *F*igure shows the flow of water through the system

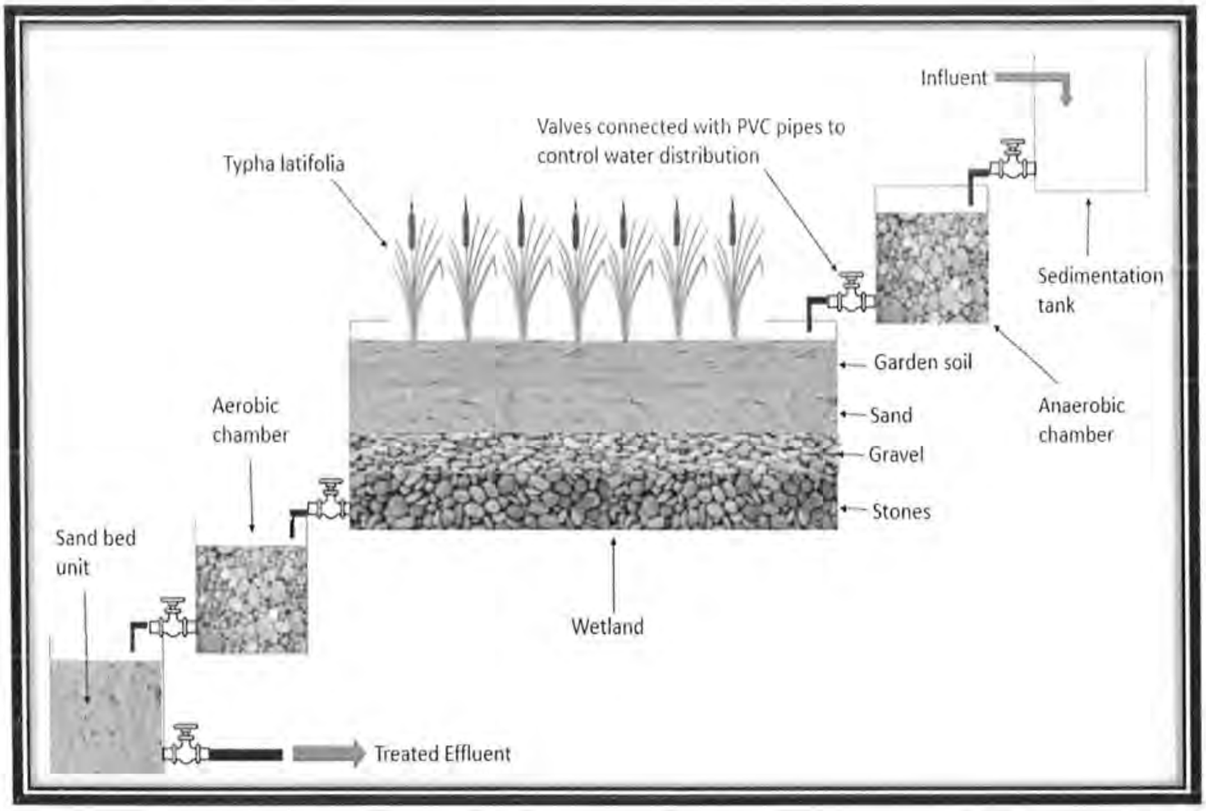


Fig 3. 3 Operational Arrangements of gravity driven interconnected biological reactor

3.2. Operational Scheme of hybrid wetland.

Bulleh Shah Packages Limited, Lahore (Pakistan), provided the effluent (black liquor, BL), which was then received in sterile polyethylene containers and given a holding period of about 24 hours in PST to guarantee sedimentation of colloidal matter and granular debris. After settling, valve V1 was switched on, permitting effluent to pour down to anaerobic chamber. For anoxic degradation, a holding period of around 24 hours was specified. When we open valve V2 the effluent poured down the wetland where waste water was held up for 24 hours. After passing through the wetland the effluent was held up in aerobic chamber for 24 hours via valve V3. The Effluent was then transferred to the filtration chamber made up of sand and biochar via the valve V4.

3.3. Wastewater Sampling and its Physicochemical Analysis

Standards set by the American Public Health Association in 1998 were used to collect the sample before and after treatment to assess them. Samples were collected in sterilized plastic bottles. The Applied Microbiology Lab received samples right away for the prepose of biological analysis. Before storing the sample at 4°C for analysis the dissolved oxygen of samples was determined immediately.

3.3.1. Reduction in Color.

Normally Water has no Flavor, no aroma and no color. Each sample was centrifuge at 10,000 rpm in 4°C for 20 minutes. Standard solution PtCob.0.1214 for Color reduction assay was prepared. Color Reduction was measured by photospectrometry at 465 nm.

3.3.2. pH

The value of pH was measured using pH meter. The Electrode of the pH meter was deionized with distilled water before and after each sample.

3.3.3 Electrical Conductivity.

Using Electrical conductivity meter, the movement of free in ions in water was recorded before and after the treatment.

3.3.4 Dissolved Oxygen.

The Dissolved oxygen of every sample was recorded carefully using digital Dissolved oxygen meter right away. The tip of the meter was cleaned with deionized water before recoding the dissolved oxygen.

3.3.5 Biological Oxygen Demand (BOD)

The concentration of O_2 needed by micro-organism at given temperature and time to decompose the carbon content in water sample is known a biological oxygen Demand. Before and after treating the wastewater, samples were collected and their BOD was recorded according to APHA, 21st Edition. Samples were diluted enough so 1ml of every component was present in one liter of the solution. Aspirator bottle having a volume of 300 ml was taken and 295 ml of it was filled with dilution and 5ml of sample was added in a way that air bubbles were not trapped. Another bottle was kept at 21'C for 120hours after the first BOD was recorded using dissolved oxygen. The last level of dissolved oxygen was recorded after 5 days. The BOD_5 was evaluated using the following formula.

$$BOD_5\ mg/L = \frac{(DO\ i - DO\ f) \times 1000}{used\ dilution\ sample\ volume}$$

Where,

DO_i= Initial Dissolved Oxygen

DO_f=Final Dissolved Oxygen

3.3.6 **Chemical Oxygen Demand (COD)**

The quantity of O_2 needed for the compounds to oxidize is known as chemical oxygen demand. Using the kit having the range of 4 to 1500mg/l, the COD was calculated. For the COD determination, 3ml of sample which was filtered transfer to COD vial, shaken for few minutes and then placed in the digester for 120minutes at 150^{o}C. Spectro quant Pharo 300 was used to record the readings after temperature of kit was lowered.

3.3.7. **Total dissolved & Suspended Solids**

Theses parameters, for every sample, were determined using the digital PCS multi test meter.

3.3.8 **Calculation of Sulfates.**

In order to calculate sulfates, standard method was used, provided by APHA, 21 Edition. A sample of 25ml which was filtered before and placed in a flask of 250ml. A few crystals of Anhydrous BaCl2 were introduced after adding 2.5ml of buffers A and B(Buffer A.: Deionized water was mixed with 30g of Magnesium Chloride Hexahydrate, 1g of NaOAc, and 20 ml of pure ethanoic acid, before being diluted to 1 liter. Buffer solution B. 30g of $MgCL_2$, 20 ml of pure ethanoic acid, 5g of NaoAc, 0.11g of Na_2SO_4, 1g of KNO_3 was added to 1l of water.). The sample was gently blended. The measurements were taken with spectroquant Pharo 300.

3.3.9. **Calculation of Orthophosphates.**

In order to calculate Orthophosphates, standard method was used, provided by APHA, 21 Edition. A 25 ml of sample was brought in a flask that was filtered before. Two or three drops of PH and strong was added to the flask. 25 ml of Stannous chloride solution and 1 ml of $(NH_4)_6Mo_7O_{24}$ was mixed in the flask. 10 minutes were given to the sample to settle down in the mixture, after those steps, measurements were recorded on spectroquant Pharo 300.

3.3.10 **Calculation of Nitrates**

Fertilizers, rotting plant and animal debris, home and commercial effluents, and atmospheric washouts are significant sources of nitrates. Natural water that hasn't been contaminated often only has trace amounts of nitrate. Nitrates are converted to nitrites in newborns' digestive tracts, which might result in methaemoglobinemia or blue baby syndrome, hence excessive concentration in drinking water is regarded as dangerous for them. When nitrate and phosphates are present in high concentrations together, eutrophication results because nitrate is also a necessary ingredient for algal development. When nitrate and phenol disulphonic acid combine, a nitro derivative is created. When this derivative is exposed to alkaline conditions, its structure is altered, giving it a yellow color. When calculated spectrophotometrically at 410 nm, the color of the solution generated is exactly equal to the amount of nitrates present. Each crucible contained 50ml of standard, sample, and blank (distilled water), which were all heated to dryness and then cooled. The leftover material was dissolved in 2 milliliters of phenol disulphonic acid, and the mixture was then diluted to 50 milliliters in a Nessler's tube. To give the solution a yellow tint, 6ml of liquid ammonia was added. The mixture was then vigorously stirred. At 410 nm, the color generated was spectrophotometrically read. Nitrate concentration was recorded (Trivedy and Goel, 1987).

3.11. **Microbiological characterization of wastewater before and after treatment.**

On the regular basis, two analyses were done: overall bacterial colony count by plate count technique and Most Probable Number (MPN) assessment of fecal Coliforms.

3.11.1 **Culture Media and chemicals.**

Media used to culture bacteria from several global firms as Biolife, Chemical Company, Italy; Oxford company, UK were used. All culture medium was formulated in accordance with the company's specifications and requitements. In Order to sterilized, all the materials were autoclaved at 121°C for 15 minutes.

3.11.2. **Determination of CFU**

The plate count technique was performed to calculate the total amount of different bacterial communities such as E. coli. the sample of the wastewater was diluted using the serial dilution method because it has a high concentration of bacterial population. 10 test tubes all holding nine milliliters of saline water and all of them were marked. One milliliter of sample was put in the first test tube and carefully mixed. Following that one milliliter from first test tube was taken and added to the second test tube. Similarly, several dilutions were prepared up to the 10. Following the preparations of the various dilutions, one milliliter of all dilution was distributed on the nutrient agar plates. Serial dilution up to 10^6 were made because the waste water generally contains very high number of bacteria. 0.1ml of all dilutions were taken with pipette and were spread on nutrient agar plates. Each plate was marked according to the dilutions that were put in. The plates were incubated at 37°C for 24 hours. Colonies were counted using Digital colony counter. Using the following formula CFU was recorded.

$$\frac{CFU}{ml} = \frac{Numberof\ Colonies \times Dilution\ fator}{volume\ of\ inculated}$$

3.11.3 **Determination of MPN**

In order to determine the quality of water most probable number was used as qualitative and quantitative essay. This test describes the amount of fecal coliform which ferments lactose and produce gas which was trapped into the Durham's tubes. Nine test tube were stacked in three sets of three and each set was marked differently as 0.1x, 1.0x and 2x respectively. In order to identify methane production, a Durham tube was inverted into every tube to trap gas bubbles. In sets 0.1x and 1.0x lactose broth of 10ml volume, with single -strength was added. In the 2x set double strength of lactose broth of 10ml was poured. All the tubes were autoclaved and then sample was added to tubes and kept for one day at 36.5'C. Trapped bubble indicates the presences of gas producer (+) and no bubble show negative result. The results were recorded according to the standard MPN index.

3.11.4. **Microbial analysis of Biofilm Study**

A thick biofilm-covered stone was taken from the TBF system using a sterile handler, and the biofilm was scraped off with a sterile spatula before being combined with 25mL of autoclaved distilled water. Sample was a 10-minute-old vortex. Following conventional procedures, several dilutions up to 10-5 were created. From each dilution, 0.1 mL was placed on the nutrient agar plate and incubated for one day at 36.5°C. SSA (Salmonella sheigella agar), E. coli/coliform agar, MacConkey agar, Blood agar plates, and eosin methylene blue agar were only a few of the numerous media that were poured over various colonies that were selected from the nutrient agar plates. Afterwards, plates were incubated for one day, and growth was seen.

3.11.4.1: **Colony Morphology**

Each microorganism has a unique morphology and form. Strains of bacteria were classified according to many traits such shape, volume, transparency, coloration, and colony morphology such as raised colonies, scattered colonies, and colony edges.

3.11.4.2: **Gram staining**

Gram+ and Gram- Bacteria may be distinguished using a method developed by Hans Christian Gram. Differential method was used to every bacterial colony. Under a microscope, Gram (+) appear with purple color whereas Gram (-) appear with pink color.

There are four fundamental phases in the Gram staining procedure, including:

1. Drop few drops of crystal violet.
2. Put on few drops of Gram's iodine as a mordant.
3. Wash out with acetone.
4. Drop few drops of safranin

3.11.4.3: **Biochemical test**

Bergey's Manual of Determinative Bacteriology was used to identify isolates obtained colonies (9th Edition). The following tests were conducted:

3.11.4.3.1: **Catalase**

- Catalase-producing organisms are discovered using this assay.
- Add 1-2 ml of H_2O_2 solution on a slide.
- Pick a colony and rub on the slide.
- If bacteria have potential to degrade H_2O_2 if form bubbles.

3.11.4.3.2: **Oxidase**

- Take a piece of filter paper.
- Add the regents on the paper.
- Pick a colony and rub on the paper.
- See for any development of colour

3.11.4.3.3: **Citrate utilization test**

- Pick a small colony.
- Make a slant on the citrate media.
- Keep the test tube at 36.5°C for 8 hours.
- Note any change in color.
- For up to 4-7 days, grow aerobically at 35 to 37C.

3.11.4.3.4: **Indole**

- Take four milliliters of tryptophan broth in a sterile test tube. S
- tore the tube for 24-28 hours at 37 degrees Celsius.
- Kovac's reagent, 0.5 ml, should be added to the broth culture.
- Look out for any ring formation.

3.11.4.3.5: **Motility**

- Pick a colony using syringe loop.
- Pierce through media from middle to down.
- Keep the test tube for 73 hours in 36.4°C.
- If the growth spread out in media, then it has flagella otherwise none.

3.11.4.3.6: **Urease**

- Take all the ingredients in distilled water (one liter) beaker and put 3 milliliters in each test tube.

- Add a strong inoculum in broth
- After inoculation gently wobbles the test tubes and store in 37o c incubator for 1 day and observe the color changed

3.11.4.3.7: **Nitrate**

- Add microbial culture to the nitrate broths.
- Keep the liquid media for 1 day at 36°C
- Look for any N_2 bubbles.
- Drop 7 droplets of other 2 reagents into the test tube.
- Look for any change in colour..
- Put in zn grind and watch for any change in color.

3.11.4.3.8: **Methyl Red**

- Let the broth come to normal temp.
- Pick a small colony from fresh media, add to broth for one day at 37.5°C.
- Take 1 cc of the liquid media to a clean test tube after one day in incubator
- The leftover broth should be re-incubated for a further 24 hours.
- To a test tube, add 1 drops of methyl red indicator.
- Red hue should be quickly seen.

3.11.4.3.9: **Voges Proskauer (VP) Test**

- Let the broth come to normal temp.
- Pick a small colony from fresh media, add to broth for one day at 37.5°C.
- Take 2 cc of the liquid media to a clean test tube after one day in incubator
- Re-incubate leftover media for a further day.
- To aerate, stir in 6 drops of 5% alpha-naphthol.
- To aerate, combine thoroughly and add 2 drops of potassium hydroxide, 40 percent.
- Within 30 minutes, look for a pink-red tint near the surface. During the 30-minute time, aggressively shake the tube.

Results

Physicochemical and microbiological parameters Analysis of treated wastewater from the hybrid wetland.

Black liquor from the Paper making factory was taken to the soil and water lab. We had constructed hybrid wetland where black liquor was poured into primary chamber for sedimentation. After 24 hours the primary chamber was emptied and the wastewater was moved down to Anerobic chamber. More black liquor was poured into the primary chamber. Similarly, the wastewater was moved down to constructed wetland and aerobic chamber after 24 hours. After aerobic treatment the wastewater was passed through sand bed and collected into the sterile plastic bottles and were quickly move to Lab for analysis.

Color reduction of black liquor.

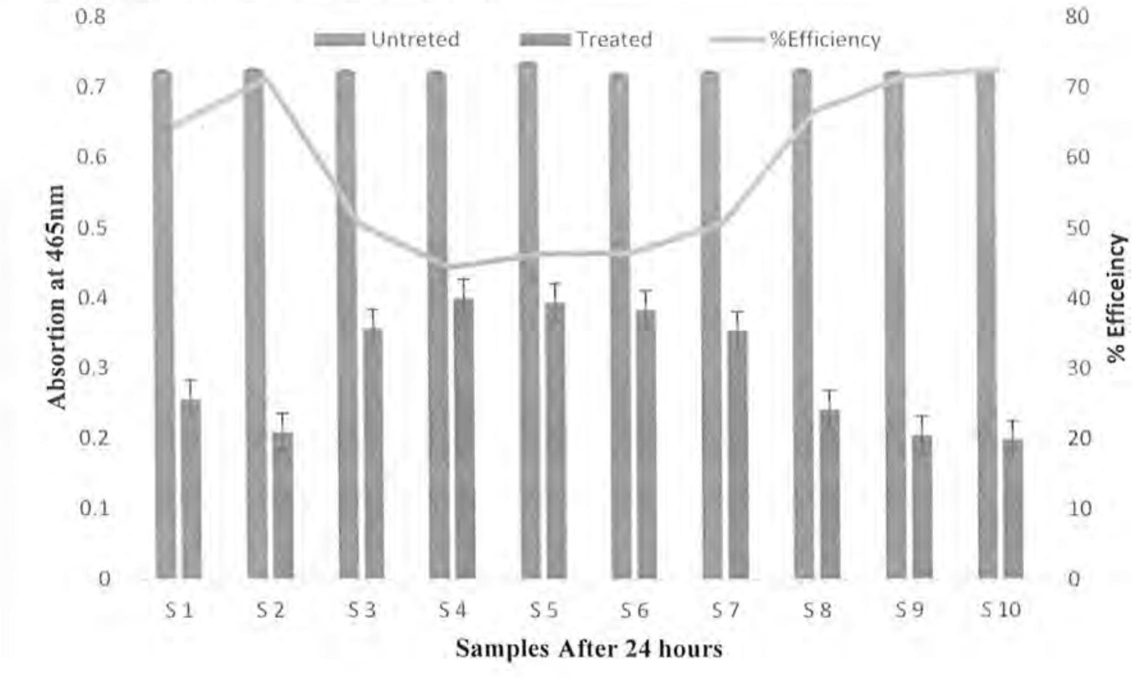


Fig 4. 1 **Effect of wetland treatment on the Color reduction of Black liquor**

The Black liquor has color, reason being of lignin and hemicellulose. The figure represents 10 samples after 24 hours. At first the efficacy was high at 64.51% and then comedown at 46.31% which was saturation point. after that hybrid wetland efficacy increase to 72.5%. after sample 8 there was no significant change in color reduction.

Calculation of pH

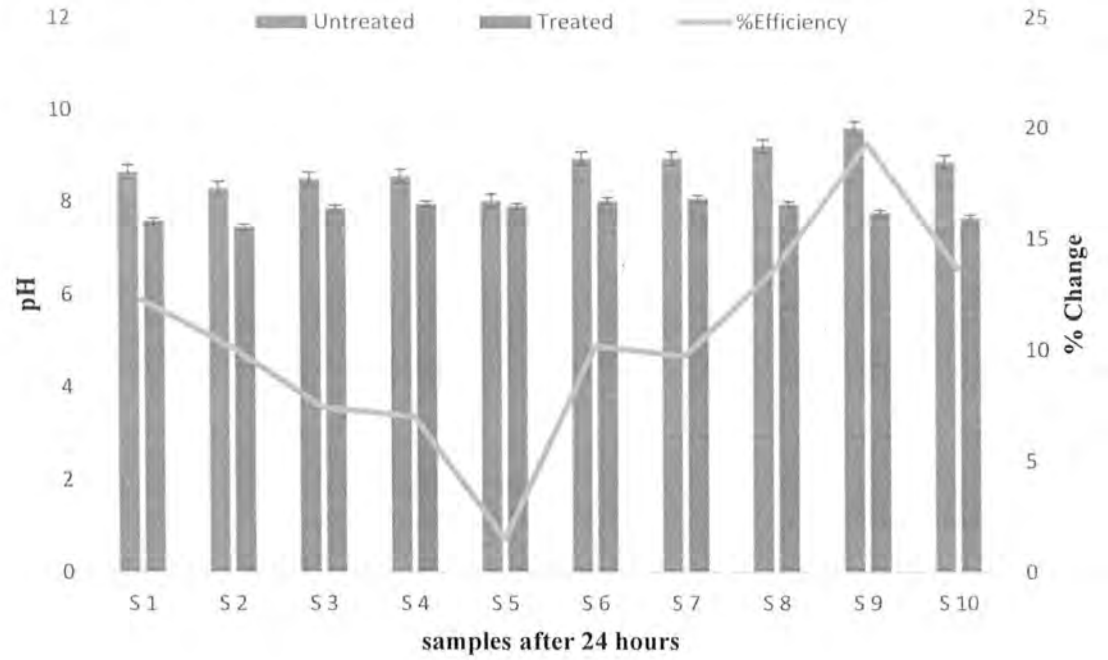


Fig 4. 2: Effect of wetland treatment on the pH of Black liquor.

Usually the pH of black liquor is alkaline. The average value of untreated wastewater was 8.73. The average value of treated wastewater was 7.6 which was near to neutral.

Electric conductivity of samples

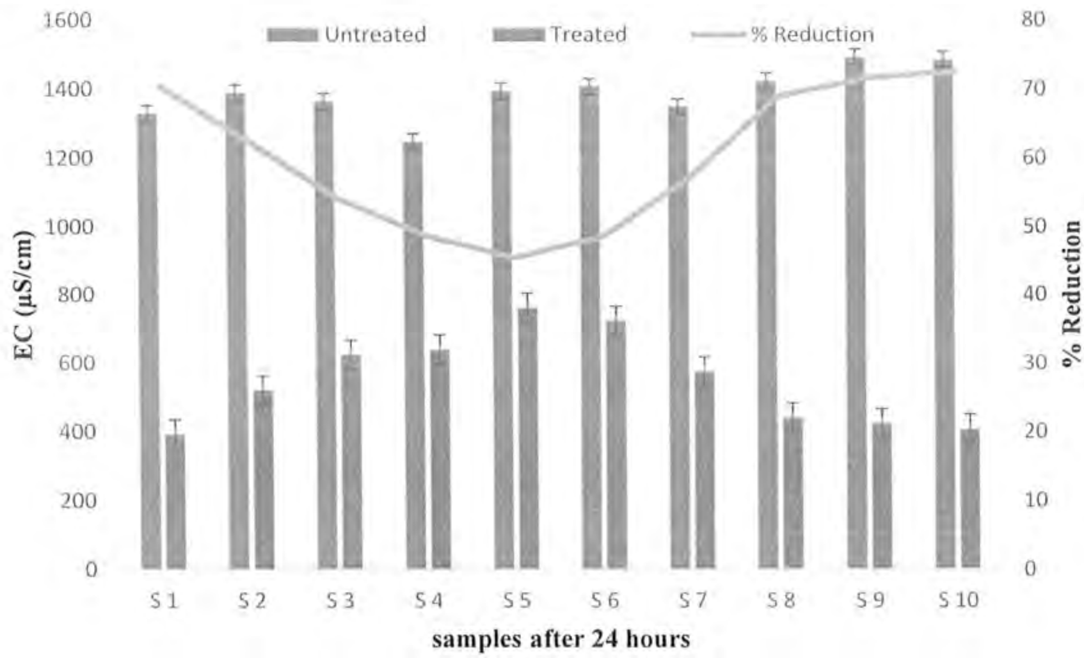


Fig 4. 3: Effect of wetland treatment on the Electric conductivity of black liquor

The organic compound has the ability to carry electrons and free ions that contribute to EC. The EC of untreated wastewater ranges from 1248 to 1493 µS/cm. The maximum EC of treated wastewater was 640 and minimum was 424 µS/cm. The efficacy of wetland was first 70% then reduce to 48% and then rose back to 72% . There was no net change in the EC after sample 8.

Calculation of DO

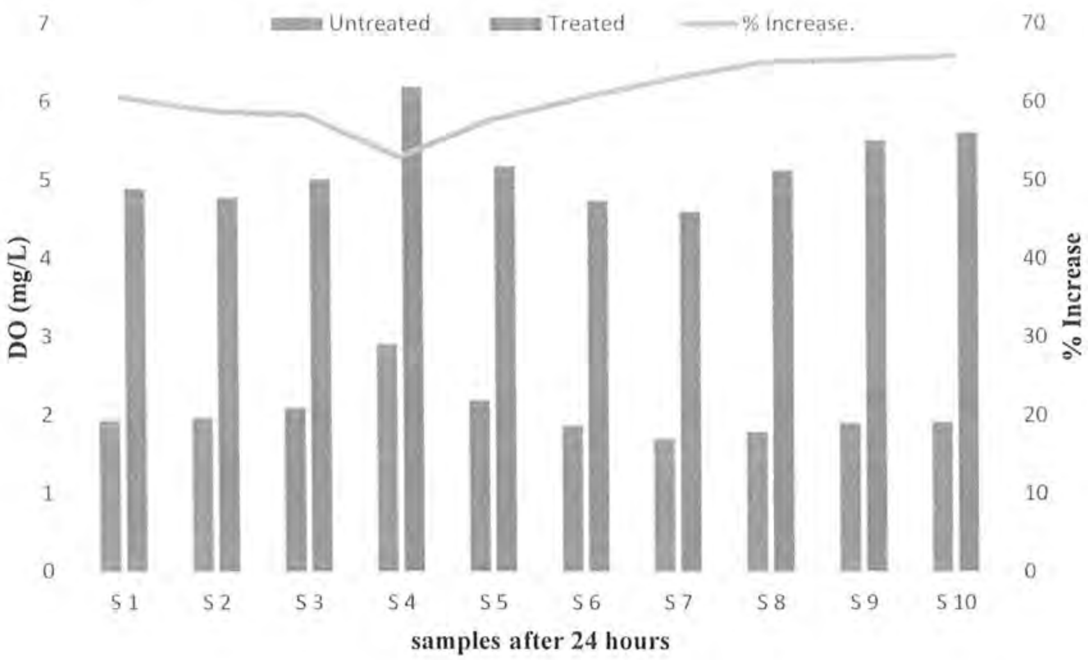


Fig 4. 4: Effect of wetland treatment on the Dissolved Oxygen of black liquor.

Dissolved oxygen in water is ability to carry molecular oxygen in water. The value of dissolved oxygen is low in polluted water and high in clean water. The untreated wastewater had DO value varying between 1.70 to 2.91 mg/L. The highest measurement of DO for treated wastewater was 6.19 mg/l and lowest measurement was 4.6 mg/l. The increase in DO at first was around 60% and then lower to 52%. There was no significant increase in DO after 65%.

BOD of samples

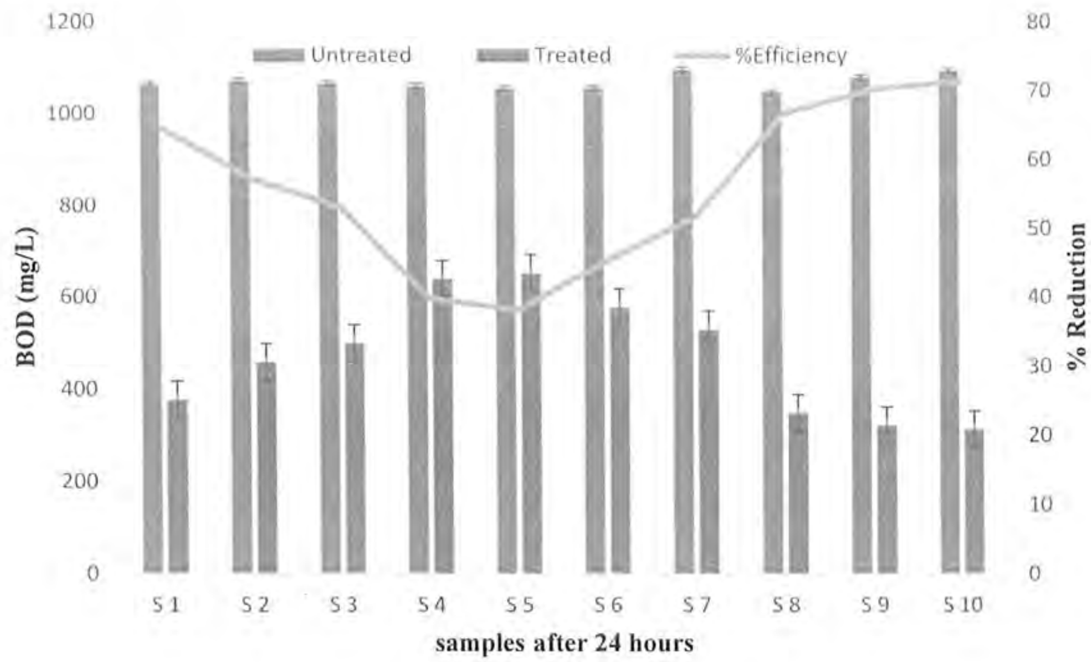


Fig 4. 5: Effect of wetland treatment on the BOD reduction of black liquor

Biological oxygen demand is the concentration of O_2 needed by microorganism to degrade organic compound in polluted wastewater. The averaged measurement of BOD of ten samples of untreated wastewater was 1069.104 ranging from 1047.89 to 1096.854. The maximum valued of BOD for treated wastewater was 653.2mg/l and minimum value of BOD for treated wastewater was 312.8mg/l. The efficiency first was high around 64% and then decrease to around 38% and then increase 71%. After which there was no significant change in BOD reduction.

COD of samples

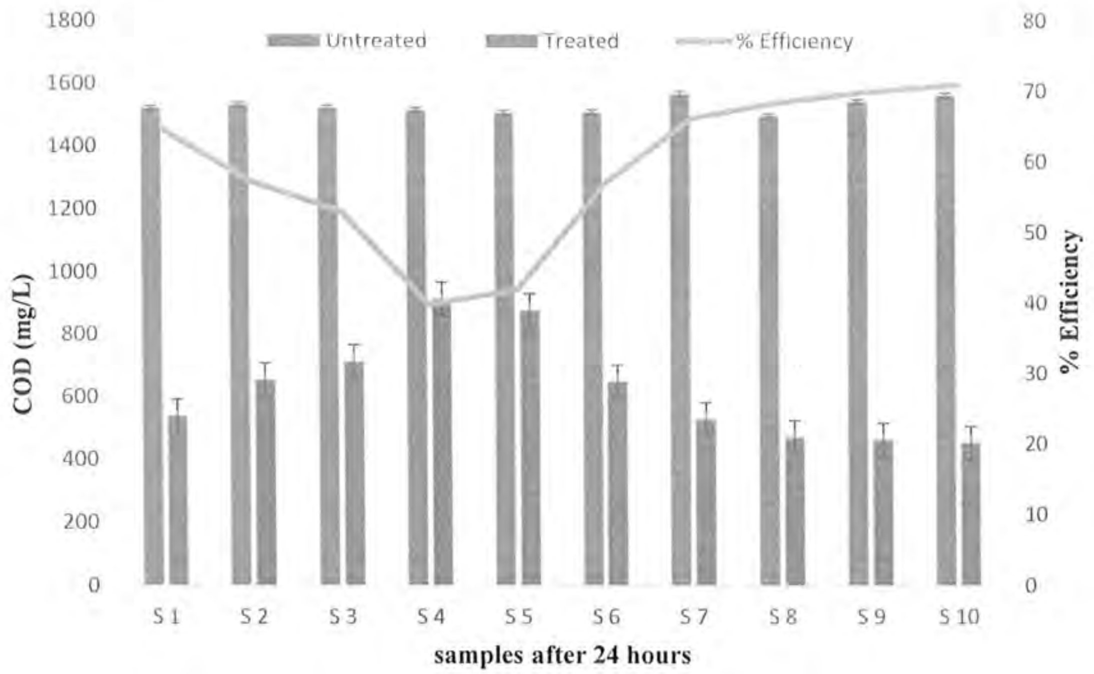


Fig 4. 6: Effect of wetland treatment on the COD level of black liquor.

COD is oxygen needed to oxidized the carbon compounds in wastewater. The untreated wastewater has COD average value of 1527.291mg/l with ranged of 1496.986 to 1566.984. The maximum valued of treated wastewater was 912.977 mg/l for COD. The minimum value of treated wastewater was 454 mg/l. The efficiency at first was around 64.54% then decrease to 39% and rise again to 70%. There was no net change after sample 8 in reduction of COD.

TSS in samples of black liquor

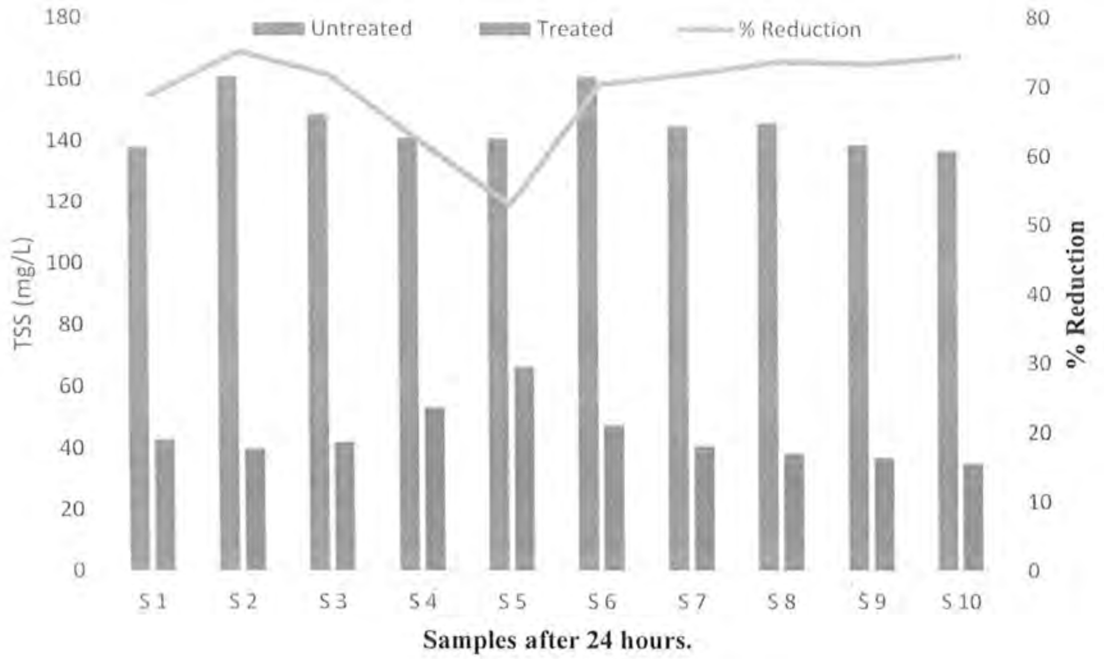


Fig 4. 7: Effect of wetland treatment on the TSS removal of black liquor

Total suspended solid in wastewater are particles that can be seen by naked eye. The average value of untreated wastewater for TSS was 145.8 mg/l with value ranging from 137 to 161 mg/l. The maximum value of TSS in treated wastewater was 66.33mg/l and minimum value was 35 mg/l The reduction in TSS was at first around 68% increase to 75% and then decrease to 52%. After which the efficacy steady increase to 74% and there no net change later on.

TDS in samples of black liquor.

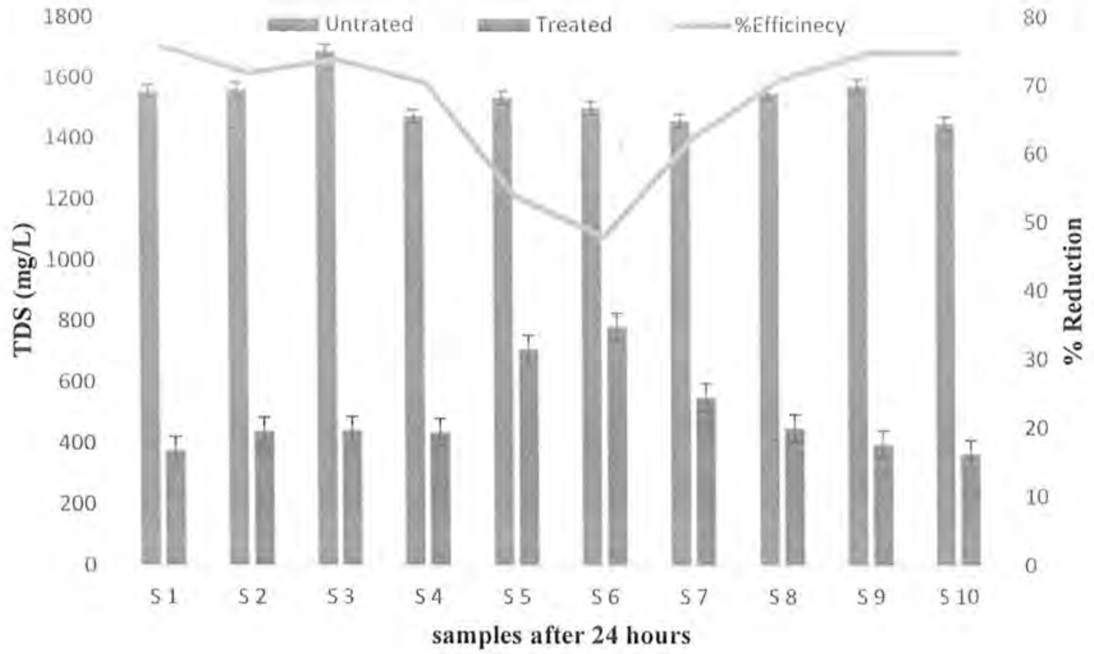


Fig 4. 8: Effect of wetland treatment on the TDS removal of black liquor

Total dissolved solids are the organic compounds that are present in water which cannot be seen by naked eye. The highest measurement of TDS for untreated waste was 1687 mg/L and lowest concentration of TDS was 1450 mg/L wit average value of 1471.5mg/l. the minimum value of TDS for treated wastewater was 365 mg/L and maximum concentration of TDS for treated wastewater was 782 mg/L. The efficiency first rose to 75% and then decrease to 47% and then steady increase to 74.82%. there was no net change in level on TDS removal after sample 8.

Estimation of Sulphates

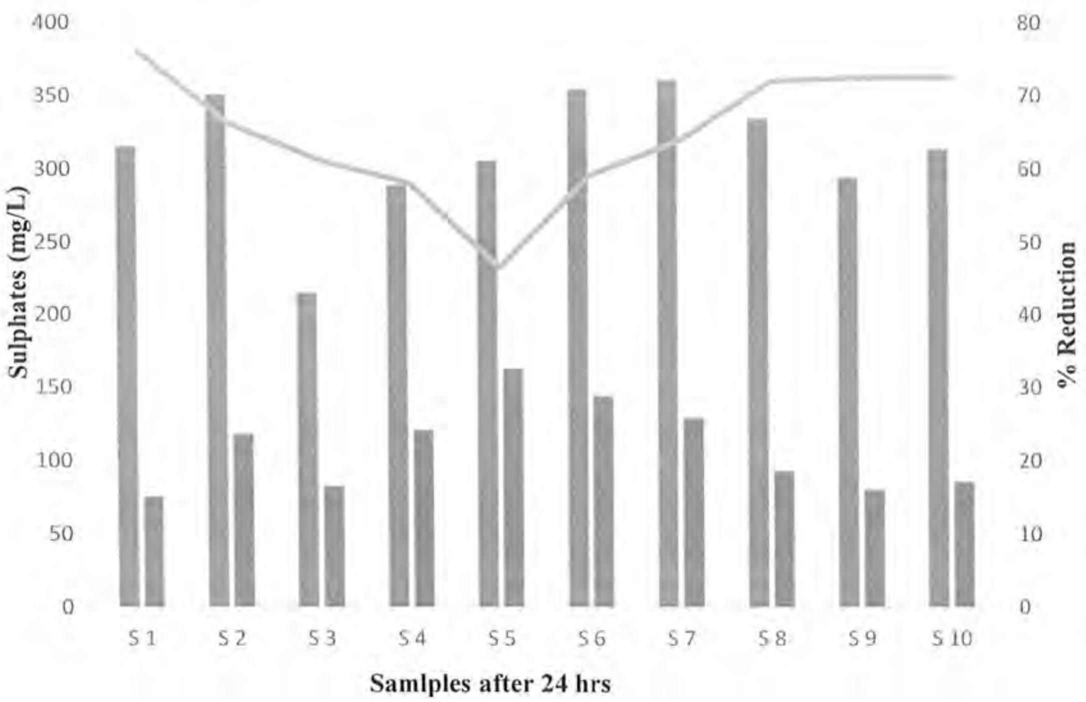


Fig 4. 9: Effect of wetland treatment on the sulphate deduction of black liquor

Sulphates are one of the inorganic compounds that are present in wastewater. The maximum value of sulfates in black liquor was recorded as 361 mg/L and the minimum value was recorded as 215 mg/L.. The minimum value of sulphates in processed wastewater was 76.01 mg/L and maximum amount of sulphates were 163 mg/L in treated wastewater. The reduction in the wastewater started with 75% and the decrease to 46% and then start to increase up to 73%. There was no significant change in the sulphate reduction after sample 8.

Estimation of Phosphates in samples

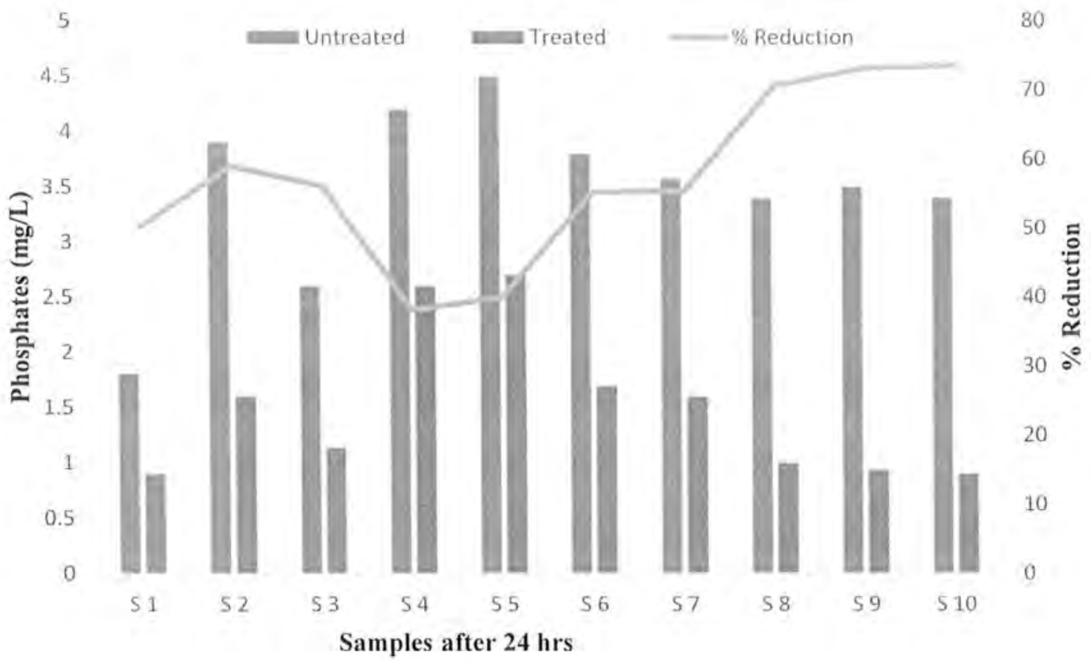


Fig 4. 10: Effect of wetland treatment on the phosphate removal of black liquor.

Phosphates are inorganic compounds that are in wastewater as a consequence of mixing with sewage. The average value of phosphates in untreated wastewater was 3.469 mg/L with variety from 1.81 to 4.5. The maximum levels of phosphates in treated wastewater were 2.7 mg/L and minimum level was 0.9 mg/L. Reduction of phosphate at first around 50% and then lowered to 38%. After this point it start to rise and level up to 73%. After which there was no significant change observed.

Estimation of Nitrates in samples

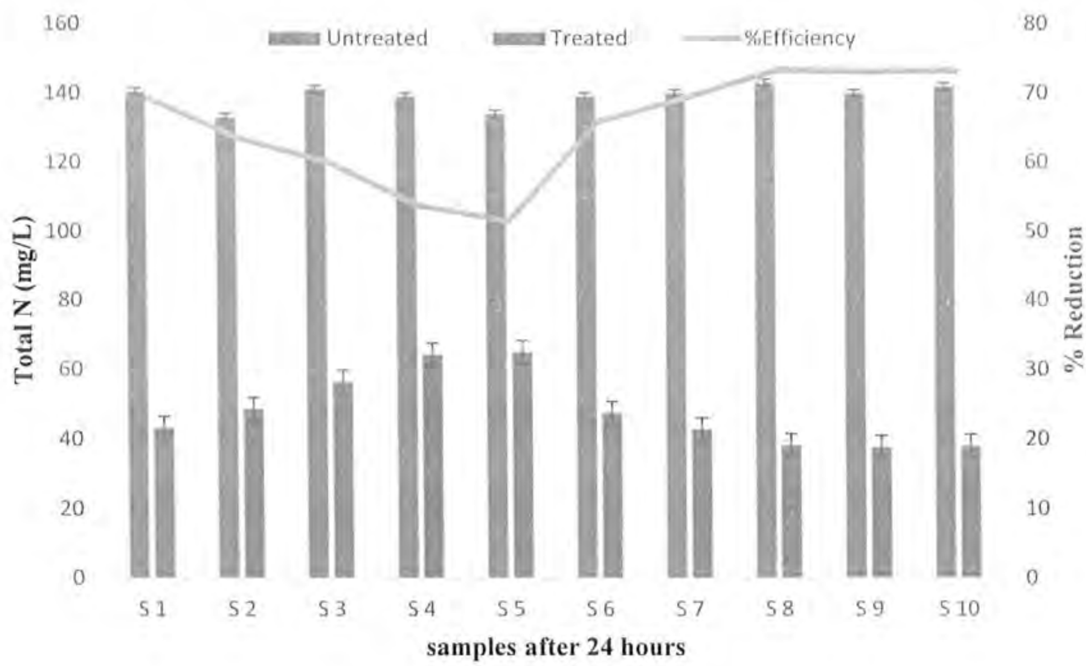


Fig 4. 11: Effect of wetland treatment on the nitrate content of black liquor.

The Nitrates are present in wastewater as pollutants. The average level of nitrates in untreated wastewater was 139.123 mg/L with maximum level 143 mg/L and minimum 133 mg/L. The minimum level of nitrates in treated wastewater were 37.71 mg/L and maximum level as 65 mg/L. The efficiency at start was noted as 69% that decrease to 51% and then rose up and level up to 73%. After that there was no net change in the nitrate content.

Determination of CFU in samples

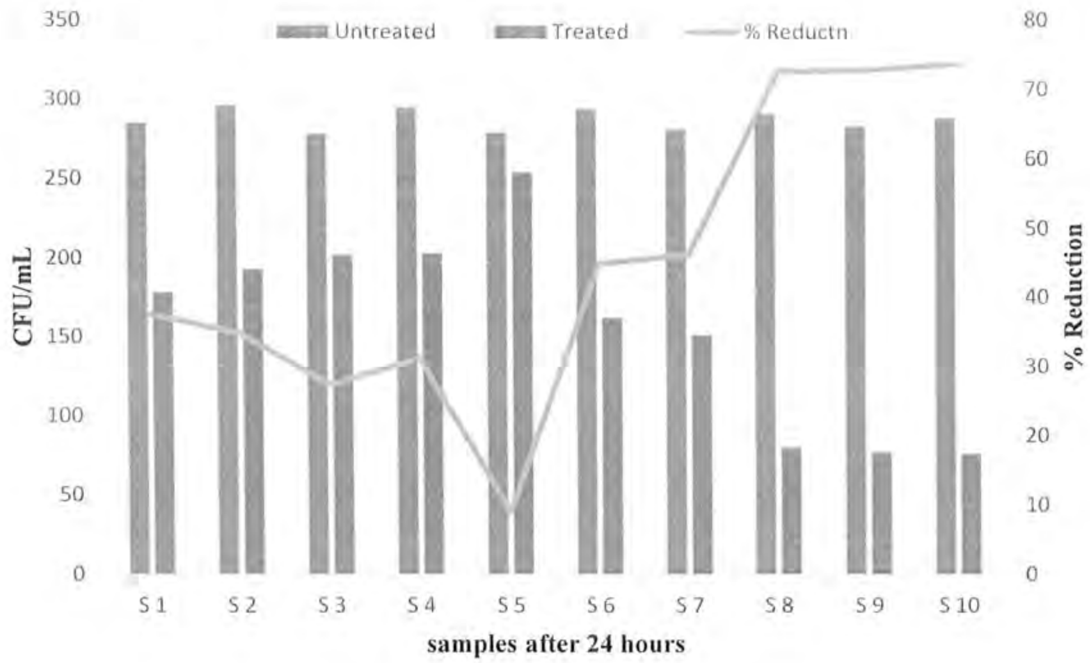


Fig 4. 12: Effect of wetland treatment on the number of bacteria in black liquor. Colony forming unit is one of the methods to measure culturable bacteria in a given sample.

The untreated wastewater had CFU of 287 on average with high end at 296 and low end at 278 the maximum number bacteria in treated wastewater was 254 and minimum was 76. the reduction in cfu started with efficacy of 37% and then steady move up to 73% after which there was no net change.

Determination of MPN in samples.

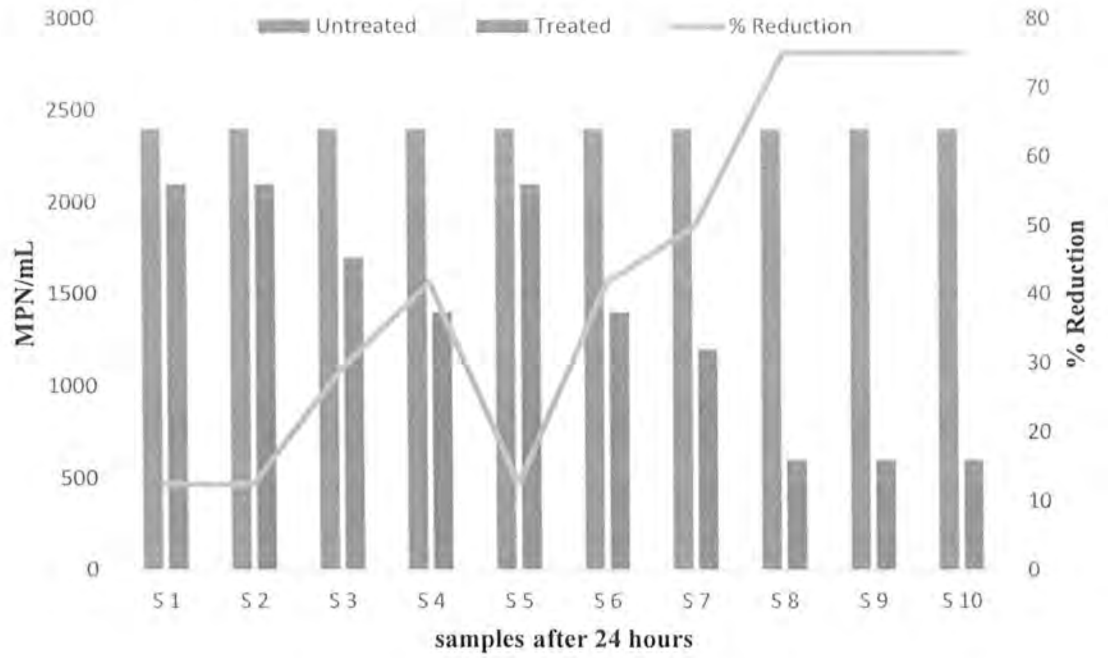


Fig 4. 13: Effect of wetland treatment on the coliform removal in black liquor.

Coliforms bacteria are present in wastewater when it gets mixed in sewage water. Untreated wastewater has 2400 MPN/ml value. The treated wastewater has initial 2100 MPN/ml that decrease to 1400 MPN/ml and then increase again to 2100 MPN/ml and then steady decrease to 600MPN/ml after that there was no significant change

Biofilm study.

Biofilm was developed on the packing material inside the chambers and constructed wetland. This biofilm was developed at the average temperature of 35°C without any inoculum added from outside. The bacteria that were present in the wastewater were allowed to developed biofilm. After completing our treatment with black liquor, the samples for biofilm were taken and isolated strain were obtained and coded. Gram staining was performed on ten strains and results are as follows:

Table 4. 1: Table shows Results of Gram staining on the stains.

Serial number	Code	Gram's Staining	Bacterial Morphology	Pigmentation
1	MAQ16	Gram Positive	Rod	Purple
2	MAQ17	Gram Positive	Rod	Purple
3	MAQ18	Gram Positive	Rod	Purple
4	MAQ20	Gram Positive	Rod	Purple
5	MAQ21	Gram Positive	Rod	Purple
6	MAQ22	Gram Positive	Rod	Purple
7	MAQ23	Gram Positive	Rod	Purple
8	MAQ24	Gram Negative	Rod	Pink
9	MAQ25	Gram Positive	Rod	Purple
10	MAQ26	Gram Negative	Rod	Pink

MAQ24 and MAQ26 were Gram Negative while the rest were Gram Positive.

After Grams staining, we go for biochemical testing on the ten strains whose results as follows:

Table 4. 2: Tables shows the results of Biochemical tests

no.	Code	catalase	oxidase	citrate	indole	motility	urease	nitrate	spore	M R	[illegible]
1	MAQ 16	+	+	+	-	-	+	+	-	+	[illegible]
2	MAQ 17	+	+	-	-	+	+	-	+	-	[illegible]
3	MAQ 18	+	-	+	-	+	-	+	+	+	[illegible]
4	MAQ 20	+	-	-	-	+	-	-	+	-	[illegible]
5	MAQ 21	+	-	+	-	+	-	+	+	-	[illegible]
6	MAQ 22	+	+	+	-	+	-	+	+	-	[illegible]
7	MAQ 23	+	-	-	-	-	+	+	+	+	[illegible]
8	MAQ 24	+	+	+	-	+	-	+	-	-	[illegible]
9	MAQ 25	+	-	+	+	-	+	+	-	+	[illegible]
10	MAQ 26	-	+	-	-	+	-	-	-	-	[illegible]

The results were that obtained were checked against the known bacterial species that can develop biofilm and has ability to degrade Lignin. After comparing we expects that following were the strains we obtained. (confirmation through 16s DNA identification is needed)

Table 4. 3: Tables shows Expected identification of bacterial strains.

Codes	Bacterial species
MAQ16	*Rhodococcus jostii*
MAQ17	*Bacillus ligniniphilus*
MAQ18	*Bacillus atrophaeus*
MAQ20	*Bacillus pumitus*
MAQ21	*Paenibacillus glucanolyticus*
MAQ22	*Bacillus Subtilus.*
MAQ23	*Streptomyces setonii*
MAQ24	*Pseudomonas aeruginosa*
MAQ25	*Rhodococcus opacus*
MAQ26	*Pseudomonas putida*

Discussion

Water is needed for very human activity. With the change in the climate and overuse of water we are facing with water scarcity. Another use in Pakistan is that we were not properly taking care of our wastewater that is usually thrown out into the nearby water body. This leads to health crises and pollute one of the water sources. Industries use a lot of water and produce highly polluted wastewater. Aquatic as well as human can die if they take in effluents from industries. One of such industry is paper industry that produce black liquor rich in carbon contents. One of the key molecules in black liquor is lignin. Lignin cannot be digested by human but it can be degraded by the bacteria. Bacteria produces enzymes like peroxidases that degrade the side chains of the lignin molecule and convert into a useful compound. We develop a system based on our research that bacteria can degrade and clean water. A wetland was deigned that was fitted with anerobic and aerobic chambers to get optimal efficiency of biodegradation.

Any color or smell in the water is a symptom of pollution and is visually awful. Numerous factors, including organic compounds, Sulphur complexes, and the degradation of C=O compounds, contribute to the smell in the water. The elimination of odorous substances from the waste water was one of the goals of current experiment, and longer the pollutant was with the biofilm help to contributed to this goal (Talaiekhozani, A. et aal., 2016). Water was move through different bioreactors in different directions (horizontal and vertical) helped in breakdown the biological compounds and odor elimination (Rodríguez-Hernández, L 2012).

The power of hydrogen ion concentration in every liquid is the pH value. The optimum pH for polluted water should be between 6.5 and 8.5, as per the NEQ Standards. The findings of the current study indicated a slightly alkaline pH of black liquor i.e. 8.73. After treatment with hybrid wetland the pH of the wastewater comes downs to 7.6 which is near neutral. The pH of influent and effluent relies on a number of variables, including nitrification, denitrification, oxidation, and reduction of compounds containing sulphur (Sakuma et al., 2008).

In water or sewage water, loose ions are measured by electrical conductivity. The World Health Organization has established the usual range of electrical conductivity as 400–1200 Siemens/centimeter square. Micro Siemens/centimeter square is the unit for measuring electrical conductivity. Average electrical conductivity in this system was 571 Siemens/centimeter square, which was higher than as WHO's lower limit. In terms of lowering electrical conductivity, the system had an efficiency of 72%. The conversion of nitrate and nitrites to molecular nitrogen (which disperse as gas) was one of the events that contribute to the decline in EC. Similar to how COD, color, suspended/dissolved solids, and turbidity are all related to EC decrease (Chavez et al., 2004). Fluoride ions and the decrease in total suspended solids both significantly contribute to the fall in the EC (Pritchard et al., 2007).

Dissolved oxygen measures the amount of water pollution and the numerous reactions occurring in the water. It is said that water treated with multiple types of flow .i.e. horizontal and vertical flow increase Dissolved oxygen in untreated wastewater . The average DO of untreated waste in the current investigation was 2.04 mg/L, although the average DO of treated water was 5.14 mg/L. Eliminating TSS, TDS, COD, and BOD5 led to an increase in DO (Waqkene, T. 2023). Water treated with this system has a DO that indicates it can provide the oxygen that aquatic life needs.

The microorganisms need oxygen in order to oxidise the organic pollutants in the waste water. Initial BOD levels were generally 1069.104 mg/L on average, with very little DO (2.04 mg/L). The higher number of organic containments in the wastewater was indicated by the low DO value and high BOD value. With a 24-hour retention period, the water treated by hybrid wetland exhibited an 71.4% reduction in BOD. An active catabolism of carbon containing containments in the wastewater was shown by the decrease in BOD5 and increase in DO. Pollutants undergo active catabolism, which produces biomass, carbon dioxide, and water. Microbe oxidation reduces the carbonaceous content, which raises the quantity of dissolved oxygen (US-EPA 2000). Saeed at al.. 2020 reported an 69.2% reduction in BOD of wastewater using hybrid wetland system. While Almuktar, S et al., 2018 reported an 48% reduction in BOD. Similarly, Mustafa 2013 reported an 50% reduction in BOD in treated wastewater by constructed wetland.

Chemical oxygen demand, which is often given in mg/L or g/L, is the quantity of oxygen needed by organic substances to be oxidised into CO_2 and H_2O (Blumenthal et al., 2000). Mukimin, A., & Vistanty, H. (2022) found 70 to 94% reduction in COD with the help of hybrid wetland system. Sim, C. H. 2013 reported 42.2% reduction in COD with constructed wetland and ponds. The COD decrease in the current research was 70.94%. High COD reduction was caused by the formation of an effective biofilm over the stone medium, and a decrease in alkalinity also contributes to the COD reduction.

Total solids, including total suspended solids and total soluble solids, are another crucial indicator of the quality of drinking water and sewage. When treated, TSS and TDS were decreased from 137 and 1450 mg/L in the current study's influent to 15 and 365 mg/L, respectively, with a 24-hour retention period. The lowering of TSS and TDS in the constructed wetland as 68% and 80% was reported by Colares GS et al. 2020. According to Waly, M. M. et al 2023 the constructed wetland reduced TSS by 94% and TDS by 33%.

Sulfates play a crucial role in both drinking water and sewage. Sulfates are naturally found in all forms of water, including home wastewater, industrial effluent, and natural runoff. Salts of Sulphur dissolve in water easily and affect hardness, according to Colleran, E et al. (1995). Current system has showed the ability to reduce 72% of sulfates in wastewater with retentation time of 24 hours. According to Pathirana, K. P. J. J., & Yatawara, M. D. M. D. W. M. K. (2022) constructed wetland had shown sulfates reduction by 61.61%.

Soaps, cleansers, animal and human excrement, leftover fertilizer, and people and livestock faecal matter are the main sources of phosphate in waste water. Because phosphates are taken from the waste water by subcellular microbial buildup, a high amount of phosphate nutrient in the waste water stream promotes eutrophication (Almuktar, S et al 2017). The USEPA specified allowable limits for the phosphate not more than 0.05mg/L but the World Health Organization has not indicated the limitations of phosphate in discharge water. According to the current study, an average 70.96% drop in the levels of organophosphorus was found, which suggested the presence of bacteria that accumulate phosphate. According to Jin et al. (2006), phosphate reduction rate was around 80%. Anders et al 2023 reported 21 to 24% removal of phosphates from wastewater

The formation of a biofilm over time is closely correlated with the rate of nitrogen reduction in a hybrid wetland system. The average decrease in the nitrogenous pollutant was 73.19% with a retention duration of 24 hours and sand filtration in the current investigation, which shows that the rate of nitrogen removal rises with time.

Temperature raises the population of nitrifiers, which has a positive substantial impact on nitrogen removal (Lin, Z.,2022).). Ma, Y. (2020) reported 71.26% removal of total nitrogen from wastewater of iron ore using a constructed wetland. These findings demonstrate unequivocally that connected growth reactors with natural packing media were more effective at removing nitrogen than reactors with manufactured packing media. However, additional phenomena like pH, alkalinity, and temperature are connected to the process of nitrification and nitrogen elimination.

The most likely outcome is to test both qualitatively and quantitatively for the faecal coliforms (Citrobacter, E. coli, Enterobacter, Klebsiella, Salmonella, and Shigella). In the current study, a decrease in MPN was seen as biofilm developed. MPN levels in the treated effluent were within acceptable ranges. Another crucial biological metric for determining and counting the total number of bacteria present in effluent and influent is the colony forming unit (CFU). The slow decline in CFU is likely caused by the retention of microorganisms on the slimy biofilm, followed by their departure owing to natural decay or wanted by other microbes (Nacheva et al., 2008). Chand, N.et al (2021) reported as high as 97.2% reduction in coliform.

This biofilm was developed at average temperature of 34°C, so our goal for current study was to profile the bacteria responsible for the detoxification of waste water and the use of nutrients by investigating biofilm. Under aseptic circumstances, samples of the biofilm were collected and sent right away to the lab for characterization. Pure culture methods were used to characterize the bacteria by spreading and strewing the bacteria on nutritional agar (NA) plates, which were then overnight incubated at 37°C. SSA (Salmonella and Shigella agar), PCA *(Pseudomonas* cetrimide agar), Blood agar, and MaCa Agar were among the many media that were swept on in order to subculture distinct colonies based on differences in morphology. Almost 10 strains were isolated that we were able to further analyze for biochemical testing. These bacteria isolates were *Bacillus subtilus, Bacillus pumitus, Bacillus atrophaeeous, Bacillus ligniniphilus, Paenibacillus glucanolyticus, Pseudomonas aeruginosa, Pseudomonas putida, Rhodococcus opacus, Rhodococcus jostii, Streptomyces setonii.* All these bacteria have shown the ability of lignin degradation previously *as B.subtilus* Riyadi, F et, al. (2022), , *Bacillus pumilus* Zuo, K. et al (2022), *Bacillus atrophaeeous*, Narnoliya LK et, al.(2019) *Bacillus ligniniphilus*, Cui L et al (2022), *Paenibacillus glucanolyticus*, Mathews, S et, al. (2016), *Pseudomonas aeruginosa,* Usman, M., & Dabai, A. I. (2021), *Pseudomonas putida*, Salvachúa, D., et al (2020), *Rhodococcus opacus*, Anthony, W et al, (2019), *Rhodococcus jostii*, Spence, E (2021), *Streptomyces setonii*. Radhika, N., et al., (2022)

Conclusion

Black liquor from Pulp and paper industry can be treated sustainably using Hybrid wetland system. The primary settling chamber removes most of TSS and produces sludge that prevents the clogging of downflow chamber. Anaerobic chamber has biofilm developed which breakdowns most of the TDS and other complex molecules into monomeric forms. Constructed wetland took up the nitrates, phosphates and heavy metals from the wastewater by plants. The bacteria in constructed wetland decompose the lignin into monomeric forms that were taken up by the plants. The Aerobic chamber converts the remaining dissolved organic compounds into CO_2 NO_2 and water. To eliminate any remaining contaminants, the effluent was finally routed through a sand bed. The treated wastewater's characteristics were examined and compared to standards set by international environmental bodies. The findings showed that the contaminants in black liquor were significantly decreased. Biofilm that was develop in different chamber has many potential bacteria that had the ability to degrade lignin into useful compounds. wastewater treatment in hybrid wetlands is more effective and friendly to the environment. It concludes that using artificial wetlands equipped with aerobic and anaerobic chambers is advised for the treatment of black liquor.

Future prospects

Future Prospects

a. Hybrid wetland system works more effectively than simple constructed wetland so effluent from different industries should be treated using them.
b. More designs of Hybrid wetland system should be developed against different environments and effluents.
c. Fungi and other micro-organism should be involved and studied in wetlands and aerobic chambers
d. More Bacterial strains can be isolated from the biofilm.
e. Bacterial strains that are isolated, their potential and ability to degrade lignin should be studied.

Glossary

Black liquor

The inorganic compounds employed in the procedure, leftover lignin, and hemicellulose are all combined in the black liquid to form an aqueous suspension. The black liquid contains 15% by weight of solids, of which two thirds are made up of organic compounds and the rest of inorganic substances.

BOD

BOD is a measurement of the o2 needed to eliminate organic waste compounds from water during aerobic breakdown process by bacteria.

COD

The potential of wastewater to use o2 during the breakdown of organic materials in the water is measured by the term "chemical oxygen demand," or COD.

EC

Water's capacity to carry electricity is measured by its electrical conductivity (EC).

Lignin

The 2nd highest prevalent polymer after cellulose, lignin is a naturally occurring aromatic (phenolic) diverse bio- complex molecule. In addition to being a waste of the paper and lignocellulosic industries, lignin is a component of plant cell walls.

TDS

Particles that are present in water with size less than 2 microns

TSS

Particles that are present in water with size greater than 2 microns

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