Original Research Article

BIOREMEDIATION OF PAPER MILL EFFLUENT USING *Bacillus subtilis AND Escherichia coli*

ABSTRACT

Effluent from onward paper mill limited Nigeria was collected and analyzed. *Bacillus subtilis*, *Escherichia coli* and mixed cultures of both organisms were used to bioremediate the effluent collected. The setups were inoculated with *Bacillus subtilis, Escherichia coli* and mixed cultures of these two organisms, respectively. Setup C was not inoculated and used as a control. Physico-chemical and microbiological analyses were carried out for 12weeks to check the effectiveness of the organisms in carrying out the bioremediation process. The nitrate, phosphate and sulphate levels showed a reduction throughout this experiment. The pH ranged from 6.4 to 7.6. The BOD levels decreased in all setups, indicating microbial activities from 120 mg /me to 92 mg /me in setup A. The mean population density of all the microorganisms for culture development also increased. It increased from 2.3 X 104 to 3.5 X 10 for Bacillus subtilis. This shows microbial growth and multiplication. Also, the dissolved oxygen level increased from 3.8mg /me to 4.7mg /me in setup D. The experiment indicates that the inoculated organisms carried out a certain degree of bioremediation.

Keywords: *Bacillus subtilis,* Bioremediation, *Escherichia coli,* Microorganisms, Papermill effluent.

1.1 INTRODUCTION

Industrialization increases the economic value of a nation, but it also leads to the degradation of the environment [1, 2]. The Paper industry has earned a reputation as one of the world's most important industrial segments due to its economic benefits; however, the energy efficiency mechanisms and the management of consequential pollutants are issues facing paper mills [3]. Paper mills are considered the sixth-largest polluter (after oil, cement, leather, leather, textile, and steel industries), discharging a variety of gaseous, liquid, and solid wastes into the atmosphere. Besides the high-water consumption, the environmental problems of the pulp and paper industry are not limited to the environmental problems [4]. Paper industries produce large quantities of wastewater that need to be treated before being discharged into the environment, which is a significant environmental and

economic problem. One major issue is the persistent dark brown colour caused by lignin and its derivatives, such as chlorolignin, in the released effluent discharged from the pulp bleaching processes [5]. When this effluent is discharged untreated, they do much harm to drinking water due to its high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and chlorinated compounds measured as absorbable organic halides (AOX), suspended solids, mainly fibers, fatty acids, tannins, resin acids, lignin and its derivatives, sulfur and sulfur compounds, etc. [5].

Date back to the present, many physical-chemical treatment techniques have been used to remove the colour and toxicity of these effluents by precipitation, coagulation, adhesion, and membrane filtration, but none of which is commercially viable due to some significant drawbacks [6].

However, the most widely used biological treatment system is the activated sludge process.

Bioremediation uses living microorganisms or microbial processes to detoxify and degrade environmental pollutants to revive the environment [7]. This biotechnological process helps to facilitate the biodegradation of contaminants or toxic materials, causing environmental pollution and health hazards [7]. Biodegradation of pollutants in the effluent is becoming an increasingly important wastewater treatment method [8], thus restoring the original surroundings [9]. Pollutants are degraded naturally using active microbial strains. The merits of these options include that they appear to be the most environmentally friendly method for removing pollutants since other methods, such as chemicals, introduce more toxic compounds to the environment [9]. Biological treatment is advantageous for removing pollutants, mainly when the wastewater contains considerable amounts of organic solvent found in paint effluent. However, paint effluent is co-contaminated with organic and metal pollutants. Metallic powders used as additives have a biostatic and biocidal effect on microorganisms [10]. Hence, this study aimed at paper mill effluent bioremediation using *Bacillus subtilis* and *Escherichia coli.*

2.0 MATERIALS AND METHODS

2.1 Sources of Samples and Microorganisms

Paper effluent was collected from Onward Paper mill limited Mobolaji Johnson Avenue Ikeja, Lagos, Nigeria. The pure cultures of *Escherichia coli* and *Bacillus subtilis* were obtained from the Nigerian Institute of Medical Research (NIMR). The pure cultures were inoculated into four nutrient agar slants – 2 slants for each microorganism and taken to the laboratory.

2.2 MEDIA

Approximately 28g of nutrient agar, 39g of potato dextrose agar, and 25g of nutrient broth were weighed individually, put into four 1000mls flasks containing distilled water, and placed into a water bath to melt and dissolve completely. The conical flask containing the various media was sealed with a stopper, and the flask was placed inside the autoclave for sterilization. After the sterilization, the culture medium was removed and cooled at 45^{o}C in a pre-set water bath.

2.3 SUBCULTURING OF MICROORGANISMS

The organisms were subculture on nutrient agar plants from the agar slants containing them using the streak plate technique. The pure cultures were obtained by transferring the organisms from the nutrient agar plates back to agar slants and inoculated on a fresh nutrient agar plant. The plates and slants were incubated at 37^{o}C for 24hours.

2.4 INOCULATION OF THE ORGANISMS INTO NUTRIENT BROTH

Aliquot of 2ml of the prepared broth was poured into each plate containing the colonies of the microorganisms' under-aseptic conditions. A sterilized inoculating loop was used to scrape the growth of the microorganism and poured into the respective broth containing the labeled organisms.

The flasks were incubated at 37^{o}C after inoculation. From the broth, 1ml was taken to carryout serial dilutions; it was plated out, the plates were incubated, and colonies were counted after 24 hours.

2.5 GROWTH MEASUREMENT

The microbial growth was monitored using a colorimeter at a wavelength of 620nm, and standard plate count determined their population densities on nutrient agar plates. The turbidity reading was taken twice daily at 12 hours to determine microbial mass. For the standard plate, the growth was measured by counting the number of present colonies. An aliquot of 0.1ml of inoculated broth was transferred unto a nutrient agar plate. A sterilized hockey stick spread the inoculum evenly around the nutrient agar plate. This was done aseptically to avoid contamination. The plates were incubated at 37^{o}C for 24hours, and the colonies present after this inoculation was counted.

2.6 INOCULATION OF EFFLUENT WITH NUTRIENT BROTH

Aliquots of 250mls of *Escherichia coli* and *Bacillus subtilis* were prepared in two 500mls conical flasks of nutrient broth. 150mls of distilled water was prepared in a third conical flask. Into setup 1 150mls of *Bacillus subtilis* was poured, and into setup 2 15omls of *Escherichia coli* was poured. Into setup 3, 75mls of *Escherichia coli* and 75mls of *Bacillus subtilis* were poured. Into the control, 150mls of distilled water was poured. Four clean plastic bowels were labeled A - *Bacillus subtilis*, B - *Escherichia coli*, C-control containing only the effluent and D - *Bacillus subtilis* and *Escherichia coli*. Two litres of the effluent were poured into each bowl, and the inoculated broth containing the organisms was poured into the bowl as directed by the label. The setup was exposed, placed in the laboratory and stirred at the interval.

2.7 SERIAL DILUTION TECHNIQUE

The serial dilution technique used was 10-fold serial dilutions. The distilled water was sterilized in the autoclave at 121^{o}C for 15minutes and allowed to cool. Ten test tubes containing 9mls of sterile

diluents were placed in a test tube rack and labeled 1-10. 1ml was taken from the sample using a syringe of 2mls calibration and put in test tube 1 to give 10^{-1} dilution. This was done until the test tube 10 was given 10^{-10}. The diluents inoculated into nutrient agar plates were 10^{-3}, 10^{-6} and 10^{-9}. The plates were incubated aerobically for 24 hours at 37^{o}C.

2.8 MICROBIOLOGICAL ANALYSIS

The media were potato dextrose agar for isolation of fungi and Nutrient agar for isolation of bacteria.

2.8.1 Total Bacterial Count

Aliquots (0.1ml) of appropriate dilutions were put on the surface of the agar plate by spread plate technique to obtain the total bacterial counts of the sample. The plates were incubated aerobically at 37^{o}C for 24hours, and the total bacteria counts of the sample were estimated. This was based on the colony counted and multiplied by the reciprocal of the dilution factors and reported as colony forming units per gram.

2.8.2 Estimation of Fungal Population

This was done by introducing 0.1ml of dilution (10^{-2}) on potato dextrose agar using the spread plate technique. The fungal population were estimated and reported as colonies/g. Streptomycin was added to prevent bacteria growth.

2.9 DETERMINATION OF PHYSICO-CHEMICAL PARAMETERS

2.9.1 Hydrogen Ion Concentration (pH)

The effluent pH was obtained using a glass electrode pH meter. Using 7.0 buffers, the pH was standardized to pH 4.0. The electrode was dipped into the sample until it covered the bulb. The reading was taken when the Value of the pH was stable.

2.9.2 Temperature

The temperature of the effluent was obtained by using the mercury bulb thermometer. The thermometer was dipped into the effluent, and the reading was recorded.

2.9.3 Electrical Conductivity

A conductivity meter was used. The conductivity meter was standardized with 342ppm sodium chloride solution. The conductivity value was obtained and recorded.

2.9.4 Titratable Acidity

This was determined by the use of methyl orange method. Two (2) drops of methyl orange indicator were added to 50cm^3 of the sample in a conical flask placed over a white surface. The solution was then titrated with standard 0.02 NaOH until the colour changed to a pale orange colour.

$$\text{Acidity mg/L} = \frac{\text{A} \times \text{M} \times 50{,}000 \times \text{D}}{\text{Volume of Sample}}$$

A = Volume of NaOH used in the titration

M = Normality of NaOH

D = Dilution Factor

2.10 DETERMINATION OF THE NUTRIENT CONTENTS SULPHATE (SO_4^{2-}), NITRATE (NO_3), PHOSPHATE (PO_4^{3-})

The inorganic nutrient content was determined using the HACH DR2000 direct reading spectrophotometer, which was calibrated internally. For each nutrient parameter, HACH reagents in pillows were used and added to 25ml of the water sample. The concentration was obtained by reading the spectrophotometer after zeroing with the blank.

The Total Bicarbonate (HCO_3) was determined by titrating 50cm^3 of the effluent sample with 0.1 of H_2SO_4 in a conical flask. Few drops of phenolphthalein indicator were already added until a colourless solution was observed. This was designated (V_1). After the titration was done, two (2)

drops of methyl red were added to the same conical flask, and the titration was continued till the solution gave a yellow to rose-red colour. This was designated (V_2)

$$\%HCO_3 = \frac{61 \times (V2 - V1) \times 0.1}{100}$$

V_1 = Volume of 0.1m H_2SO_4 used in phenolphthalein titration

V_2 = Volume of 0.1m H_2SO_4 used in methyl-red titration

2.11 DISSOLVED OXYGEN (DO)

The Value of dissolved oxygen was determined using a dissolved oxygen meter (Model: Hanna H19142) by inserting the probe into the effluent sample.

2.12 BIOCHEMICAL OXYGEN DEMAND (BOD)

For BOD determination, bottles containing 250g of sample were incubated at 20^oC for 5days. On day 0 the initial dissolved oxygen value was obtained, and this was repeatedly obtained each day for 5 days. The difference in the value of the dissolved oxygen (DO) at day zero and the value of DO on day 5 was multiplied by the dilution factor to give the BOD Value.

3.0 RESULT

3.1 Result of Physico-chemical Analysis

The results of the Physico-chemical analysis of the effluent setups and the parameters used are shown in table 1. The pH of the whole setup remained considerably stable, and the pH ranged between 6.4 and 7.6 throughout the experiment. In table 1, the BOD levels, the phosphate, nitrate and sulphate levels decreased over the period allocated to the experiment. The Dissolved Oxygen (DO) levels increased. For nitrate levels, there was a noticeable decrease at weeks 9 and 12. The Bicarbonate levels remained somewhat constant. The nitrate levels, phosphate and sulphate levels were reduced in the setup containing the organisms' mixed cultures. The total acidity levels were also reduced during the experiment.

Table 1: Result of Physico-chemical Analysis

Wk	No	pH	Conductivity (μscm^{-1})	Total acidity	BOD (mg/l)	DO (mg/l)	NO_3^- (mg/l)	PO_4^{3-} (mg/l)	SO_4^{2-} (mg/l)	HCO_3^- (mg/l)
0	A	6.5	38000	166	120	3.8	43.0	10.0	51.0	0.25
	B	6.7	4200	150	122	3.6	46.0	8.2	50.0	0.30
	C	6.5	6000	148	110	3.9	48.0	6.0	48.0	0.20
	D	6.6	5600	106	240	3.8	49.5	5.3	60.0	0.40
3	A	6.8	5600	145	109	4.0	38.3	8.6	49.21	0.20
	B	7.0	8600	138	115	4.2	43.0	6.8	48.0	0.20
	C	6.7	9000	128	106	3.4	42.0	5.2	42.0	0.15
	D	6.8	7700	94	205	4.2	40.0	4.0	50.0	0.20
6	A	7.1	8000	127	98	4.3	35.7	7.3	37.45	0.10
	B	7.5	11000	120	110	4.4	40.2	5.2	46.2	0.10
	C	6.6	12000	102	97	3.1	41.3	4.8	41.0	0.10
	D	6.8	18000	86	190	4.2	35.0	3.6	42.0	0.10
9	A	6.5	21000	104	96	4.4	29.2	5.6	33.0	0.10
	B	7.2	15800	114	97	4.5	36.0	4.0	42.0	0.15
	C	6.9	18000	99	87	3.6	38.0	4.5	38.0	0.10
	D	7.0	22000	70	158	4.4	32.0	4.0	36.0	0.10
12	A	6.4	24500	87	92	4.7	23.5	4.2	26.4	0.10
	B	7.6	20400	108	94	4.6	32.0	3.6	39.0	0.10

	C	6.8	21000	96	81	3.2	36.0	1.3	36.0	0.10
	D	7.1	22500	62	126	4.7	30.0	2.9	32.0	0.10

Note: A = Effluent + *Bacillus subtilis*,**B** = Effluent + *Escherichia coli*, **C** = Control, **D**= Effluent + Mixed Culture.

3.2. Resultof the Mean Population Density

Table 2 shows the mean population density of *Bacillus subtilis* and *Escherichia coli* during the culture development period. For *Bacillus subtilis*, the population density increased from 2.3 x 10^4 cfu/ml at time 0 hour to 3.5 x 10^6 at 48 hours. The population density of *Escherichia coli* increased from 1.8 x 10^4 cfu/ml at 0 hour to 2.6 x 10^6 at 48 hours. In table 2, the increase in mean population density of mixed culture in the nutrient broth is more pronounced than in the other three (3). The control has lower values of mean population density.

Table 2: Mean Population Density

Time (hours)	***Bacillus subtilis***	***Escherichia coli***	**Control**	**Mixed**
0	2.3 x 10^4	1.8 x 10^4	1.2 x 10^4	2.4 x 10^4
12	7.4 x 10^4	8.0 x 10^4	6.7 x 10^4	8.2 x 10^4
24	3.5 x 10^5	3.3 x 10^5	3.0 x 10^5	3.7 x 10^5
36	8.7 x 10^5	7.5 x 10^5	7.7 x 10^5	8.0 x 10^5
48	3.5 x 10^6	2.6 x 10^6	2.2 x 10^6	3.6 x 10^6

4.0 DISCUSSION AND CONCLUSION

According to research, bioremediation is safe and cost-effective in some cleanup situations. The physic-chemical properties of effluent samples influence the responses of organisms to toxicants [5, 11].

From table 1 above, the Physico-chemical analysis shows a decrease in inorganic nutrient content levels. The decrease is due to the nutrient utilization by the selected microorganisms because the microorganisms require these nutrients sources for growth [12]. If any of the required inorganic nutrients become limiting or lacking, the bio-remediating organisms will not grow. For example, the consistent decrease in the microorganisms for sugar phosphorylation, as a component of cell membranes and nucleic acids [13].Hence, from the results of this study, in the bioremediation of paper wastes using microorganisms, it may be necessary to monitor the levels of available inorganic nutrient sources and adjust appropriately to enhance microbial growth.

According to the results, BOD was reduced overtime. This can be seen in the difference between the level of BOD in week 0 and the BOD level in week 12; this is due to the breakdown of organic matter by the selected microorganisms utilizing nutrients [14]. The pH was relatively stable. This may be attributed to the fact that in all cases, the pH of paper mill effluent is adjusted to the range of 6-8 prior to biological treatment and discharge. The pH range of 6-8 is commonly accepted as necessary to sustain the survival of most aquatic life [12]. The reduction in BOD and nutrient levels indicates the effective ability of the organisms as bioremediation agents. It was observed that the setup containing mixed cultures of the selected organism used up more than one nutrient compared to other setups. This is because of the presence of two organisms inoculated into using up the nutrient. It can be deducted that more than one organism as a bioremediating agent is more effective than using only one organism. This experiment has shown that many inorganic nutrients must be present to facilitate bioremediation, which is essential for cell growth.

Table 2 shows that the mean population density during culture development increased. This is due to cell growth and multiplication of the organisms using the available nutrients.

In conclusion, paper mill effluents pollute water, air and soil, causing a significant environmental threat. The best method for paper waste treatment depends on the efficiency of BOD_5 removal nutrient demand, sensitivity to climatic conditions etc. Bioremediation can bring contaminated groundwater to certain drinking standards within a certain period. A knowledge of effluent components must be achieved to know what best bioremediation method to use. Based on the studies carried out, it is evident that the effluent has undergone some form of treatment due to the results of the Physico-chemical analysis. One factor that limits the widespread application of bioremediation is that its "success" has been difficult to prove in the field, but as scientists learn more about its capabilities, it is likely to become one of the best technologies used for cleanup and protection of our environment.

Ethical approval and consent to participate

Not applicable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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