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3 **Effect of Dilute Acid and Alkaline Pretreatment of *Typha australis* (Typha grass) for**
4 **Bioethanol Production**

5
6 **ABSTRACT**

7 *Typha australis* (Typha grass) obtained from Kware Lake was used in this research to produce
8 bioethanol. Different pretreatment methods including dilute acid (0.2M H₂SO₄), dilute alkaline
9 (0.2M NaOH) and liquid hot water pretreatments were used to pretreat the Typha grass sample
10 before enzymatic saccharification for 7 days using *Aspergillus niger* isolated from soil sediment
11 and the hydrolysate was seeded with *Saccharomyces cerevisiae* isolated from palm wine to
12 produce bioethanol. HPLC was used to analyze bioethanol product. The result showed that
13 pretreatment with 0.2M H₂SO₄ removed more hemicelluloses (7.0%) when compared with other
14 pretreatment methods used, but pretreatment with 0.2M NaOH and liquid hot water removed
15 more lignin (14.29%) than dilute acid pretreatment. The highest percentage reducing sugar
16 concentration of 0.58% was obtained from lower part of the sample pretreated with liquid hot
17 water while Typha grass pretreated with 0.2M H₂SO₄ and 0.2M NaOH produced the highest
18 percentage reducing sugar concentration of 0.32% each from the upper part of the sample. Also,
19 the highest Bioethanol concentration of 2.07% was obtained at day 6 of fermentation from the
20 Typha grass pretreated with liquid hot water while Typha grass pretreated with 0.2M H₂SO₄ and
21 0.2M NaOH produced highest Bioethanol concentration of 0.43% and 0.54% respectively. The
22 results indicate that Typha grass can be harnessed for bioethanol production thereby reducing
23 their negative impact on Lakes.

24 **Keywords:** Typha grass, Pretreatment, Bioethanol, HPLC,

25 **Introduction**

26 Consumption of energy has increased steadily over the last few decades as the population of the
27 world has grown, and more countries have become industrialized. Crude oils have been the
28 major natural resource to meet the growing demand for energy. Along with this, the usage of the

29 fuels direct to global warming, environmental pollution, and other related hazards [1].
30 Production of liquid biofuels from lignocellulosic biomass will significantly reduce the
31 dependence on petroleum-based fuels and therefore it has become a research area of great
32 interest to many research scientists and government agencies [2]. Potential source intended for
33 low-cost ethanol production is the utilization of lignocellulosic biomass such as grasses,
34 agricultural residues, wood chips, and sawdust [3].

35 *Typha* grass has been identified as a particularly suitable biomass crop for wetland, because of
36 their superiority, productivity, pest resistance, adaptability and chemical composition [4].

37 Kware lake serves a lot of functions to the nearby communities including a source of water for
38 domestic activities such as washing and bathing. *Typha* grass has prolific vigorously, nearly
39 impenetrable stand, getting to close the view of the water and take over the lake. This has raised
40 concern that it may take over the lake and prevent a lot of activities from the lake. The present
41 research is aimed at pretreating and hydrolyzing *Typha grass* for bioethanol production thereby
42 adding more value to the grass, increase conservation and sustainability of the plant and lake,
43 contributing towards alternative energy supply and also create profit and jobs opportunity.

44 **Methodology**

45 **Collection and Preparation of Samples**

46 The *Typha* grass sample was collected from Kware Lake in Kware local government area,
47 Sokoto State, Nigeria. The sample was washed under tap water and then cut into two separating
48 the upper part that grows above the water level and the lower part that grow inside water. The
49 two portions were shade dried separately for 14 days. The dried samples were then grounded into
50 powder using and stored at room temperature for further analysis.

51 **Isolation and Identification of *Aspergillus niger* and *Saccharomyces cerevisiae***

52 *Aspergillus niger* was isolated from soil sediment collected from kware lake and identified base
53 on microscopic (morphological) and macroscopic characteristics (colour, texture appearance and
54 diameter of colonies) according to Sourza *et al.* [5]. The soil was serially diluted; a sample
55 suspension was prepared by adding 1.0g of sample to 10ml of distilled water and mixed well for
56 10 minutes. The suspension was diluted serially 10^{-1} , 10^{-2} and 10^{-3} . 1ml (from the third dilution
57 factor) was measured using a syringe and inoculated into a Sabraud Dextrose Agar (SDA) plate

58 and incubated at 37 °C for five days. The initial white color of the colonies that later turns black
59 at the top with pale yellow color at the bottom confirm the organism to be *Aspergillus niger*.
60 and *Saccharomyces cerevisiae* was isolated from palm wine sample collected from Giginya
61 Barrack market, Sokoto and identified by the standard morphological and physiological test and
62 identification keys described by Barnett *et al.* [6]. The palm wine sample was serially diluted; a
63 sample suspension was prepared by adding 1.0 ml of sample to 10ml of distilled water and
64 mixed. The suspension was diluted serially 10⁻¹, 10⁻² and 10⁻³. 1ml (from the third dilution
65 factor) was measured using a syringe and inoculated into a Sabraud Dextrose Agar (SDA) plate
66 and incubated at 28 °C for five days. Capability of the organism to hydrolyze starch and form
67 bud under microscope **was used** to confirm the organism as *Sacchromyces cerevisiae*.

68 **Determination of Structural Composition of Typha Grass**

69 The percentage of acid – insoluble lignin, which is defined as the residue, was determined
70 according to TAPPI procedure (T224 om-88). The holocellulose content, which is the
71 combination of hemicellulose and cellulose, was determined in order to find the total amount of
72 cellulose and hemicellulose in Typha grass. The holocellulose content was determined according
73 to DIN 2403. α-Cellulose is the pure cellulose content of the materials which was extracted from
74 holocellulose using alkali solution. The α-Cellulose content of Typha grass was determined as
75 the residue insoluble in the 17.5 % NaOH solutions according to TAPPI 203 om-93 method.

76 **Dilute Acid Pretreatment**

77 2 g of *Typha* grass sample was mixed with 0.2M H₂SO₄ solution in a 250 mL flask with a stopple
78 and then allowed to suck for 24hrs. The mixture was subsequently autoclave at 121 °C for
79 15mins and then cooled and filtered through a Whatman filter paper no. 1 to separate the solid
80 residue. The residue was washed with distilled water until neutral pH. The sample was air dried
81 and stored in tightly sealed plastic bag in a refrigerator for further use [7].

82 **Dilute Alkaline Pretreatment**

83 Approximately 2 g of *Typha* grass sample was soaked in 0.2M NaOH solution in a 250 mL flask
84 for 24hrs and then **autoclaved** at 121 °C, for 15 minutes. The solid residue was separated from
85 the mixture by filtration with Whatman filter paper no. 1 and thoroughly washed with distilled
86 water to neutralize its pH. Finally, the filtrate was dried and stored as above [7].

87 **Liquid Hot Water Pretreatment (LHW)**

88 In liquid hot water pretreatment of the *Typha* grass sample, the grounded powdered of the plant
89 was slurried with distilled water using a solid to liquid ratio of 10% (w/w) and autoclaved at 121
90 °C for 15 minutes. After autoclaving, the sample was filtered using **Whatman** filter paper no. 1
91 and the solid residue was air dried and stored for further use [8].

92 **Enzymatic Saccharification of Pretreated Typha Grass**

93 The enzymatic saccharification of *Typha* grass was carried out using **Aspergillus niger** isolated
94 from soil sediment as described by Gupta [9]. In this method, the pretreated *Typha* grass samples
95 were inoculated with 0.5 ml suspension of 96 hours culture of *Aspergillus niger*. Hydrolysis was
96 carried out at room temperature for 7 days. Samples were taken daily for reducing sugar
97 determination using 1,4-dinitro salicylic acid (DNS) method to find out the net yield of
98 fermentable sugars. The samples were then filtered using Whatman filter paper No. 1 and the
99 filtrates were used for fermentation.

100 **Fermentation of the Hydrolysate and Bioethanol Production**

101 The fermentation studies were carried out using *Saccharomyces cerevisiae* isolated from palm
102 wine. The hydrolysates were autoclaved at 121 °C for 15 min and the flasks were then cooled to
103 room temperature. The pH of the fermentation medium was adjusted to 6.5 and then 1ml of
104 prepared suspension of yeast isolated was added in the hydrolysate [10]. The fermentation was
105 allowed for 7 days and samples from the medium were withdrawn periodically at 24 hrs interval
106 from the flasks to determine ethanol quantity using UV-visible spectrophotometer at 540 nm.

107 **Distillation**

108 The fermented broth was filtered using **Whatman** filter paper no. 1. Each sample was weighed
109 into Microkjeldahl flasks and then heated at 78 °C on the Microkjeldahl apparatus until the
110 solution turned colourless. The presence of bioethanol was determined using high performance
111 liquid chromatography (HPLC) [11].

112 **Statistical data analysis**

113 All the experiment were carried out in triplicate and their mean **was expressed** as the result of the
114 experiment. Analysis of variance (ANOVA) was used for statistical analysis at $p < 0.05$

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116

117

118 **Results and Discussion**

119 **Structural Composition of Typha Grass Obtained from Kware Lake Before Pretreatment**

120 The structural composition of both the upper and lower part of Typha grass before pretreatment
121 show that, hemicelluloses has the highest composition of 28.70% and 30.00% while Lignin has a
122 composition of 28.15% and 24.93% respectively, and α cellulose has least composition of
123 12.30% and 10.00% respectively in the Typha grass collected from Kware Lake. The result of
124 structural composition of hemicelluloses and lignin is in conformity with the report of Bajpai
125 [12] who reported that hemicelluloses compose between 35-50% and lignin compose 10-30% of
126 grasses. However, this current study disagrees with his report that α -celluloses compose of 25-
127 40%. This research shows that the upper part of Typha grass has higher structural composition
128 than the lower part of the plant (Table 1).

129 **Table 1:** Structural Composition of Typha Grass Obtained from Kware Lake Before
130 Pretreatment

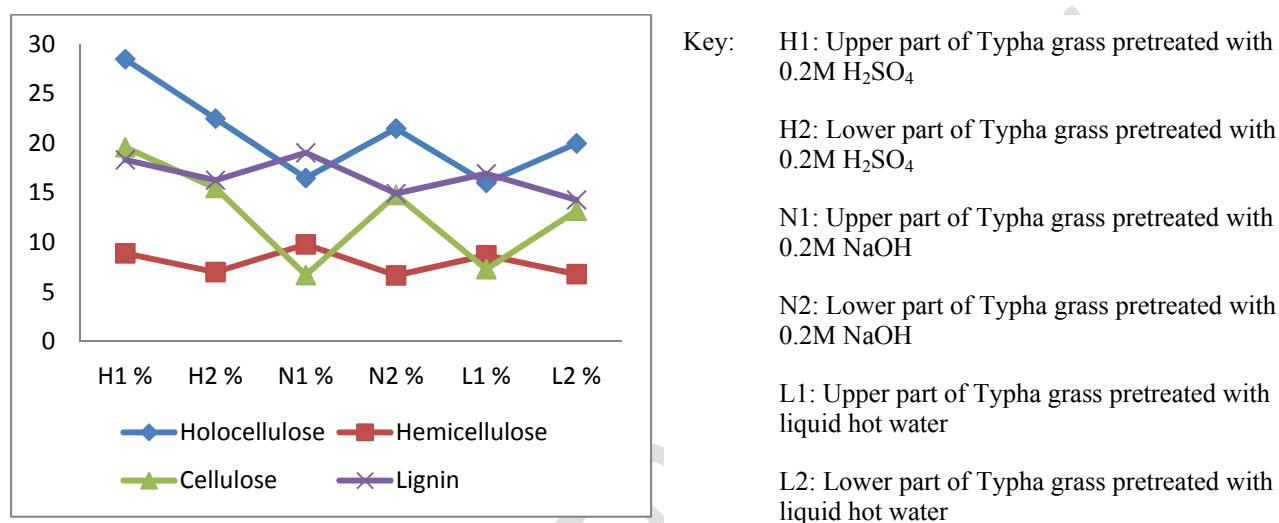
Contents	Sample A (%)	Sample B (%)	Key: Sample A: Upper part of Typha grass that Grows above water level Sample B: Lower part of Typha grass that grow inside water
Holocellulose	41.00	40.00	
Hemicelluloses	28.70	30.00	
α cellulose	12.30	10.00	
Lignin	28.15	24.93	

131

132 **Structural Composition of Typha grass Obtained from Kware Lake after Pretreatment**

133 Pretreatment of Typha grass with 0.2M H_2SO_4 produced lowest lignin content of 16.28%
134 cellulose content of 15.5% and hemicelluloses content of 7.0%. Pretreatment with 0.2M NaOH
135 produced lowest lignin content of 14.94% cellulose content of 13.8% and hemicelluloses content
136 of 7.67%. Typha grass sample pretreated with liquid hot water has lowest lignin content of
137 14.29% cellulose content of 12.2% and hemicelluloses content of 7.8%. The result indicates that
138 0.2M H_2SO_4 remove more hemicelluloses content than the other pretreatment process (figure 1).
139 This is in agreement with the result of many other researches such as [4] and [14]. Mosier *et al.*,

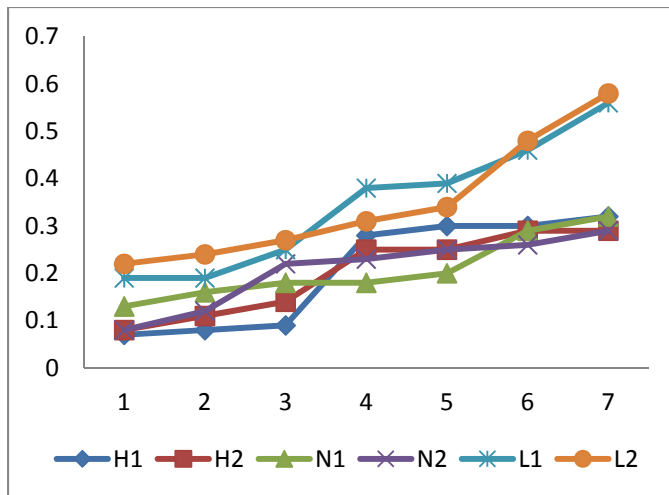
140 [13 also reported that hemicelluloses are removed when dilute H₂SO₄ is added and this enhances
 141 digestibility of cellulose in the residual solid. Also, dilute acid pretreatment was not good for
 142 lignin removal when compared with dilute alkaline and liquid hot water pretreatment. Chang *et*
 143 *al.*, [15] reported that Alkaline pretreatment removes amorphous substances such as lignin,
 144 which increases the crystallinity index of lignocellulosic materials.



145
 146 Figure 1: Structural Composition of Typha Grass after Pretreatment with 0.2M H₂SO₄, 0.2M
 147 NaOH, and Liquid Hot Water

148 Reducing Sugar Concentration of Typha Grass

149 Pretreatment with 0.2M H₂SO₄, 0.2M NaOH and liquid hot water produces highest reducing
 150 sugar concentration of 0.58% at day 7 when liquid hot water was used to pretreat 2g of the
 151 sample (figure 2). Chemicals used in this research produce small quantity of reducing sugar. This
 152 agrees with the work of Arumugan and Manikandan, [8] who said significant sugar production
 153 was not recorded from pretreatment with dilute chemicals, but the current studies disagree with
 154 their findings that said more reducing sugar from dilute acid pretreatment was produce than the
 155 liquid hot water pretreatment. Also, the production of low reducing sugar in the present study
 156 from dilute chemicals can be attributed to the washing of the chemical after pretreatment in order
 157 to neutralize their pH. It can also be as result of the solubilization of the carbohydrate by the
 158 chemical during pretreatment [14]. According to Ogier *et al.*, [16] and Laser *et al.*, [17], liquid
 159 hot water pretreatment can be a promising pretreatment method that presented elevated recovery
 160 rates of sugars which does not generates inhibitors.



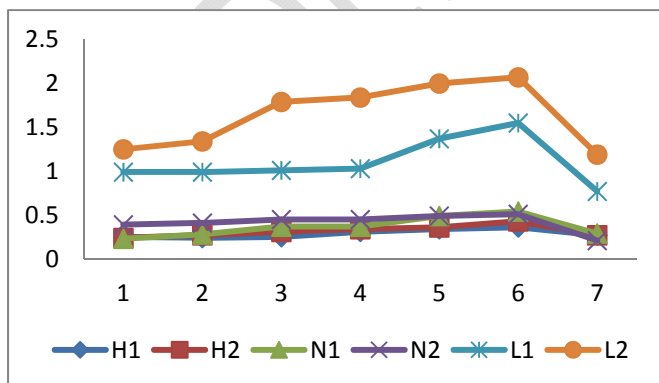
Key: H1: Upper part of Typha grass pretreated with 0.2M H₂SO₄
 H2: Lower part of Typha grass pretreated with 0.2M H₂SO₄
 N1: Upper part of Typha grass pretreated with 0.2M NaOH
 N2: Lower part of Typha grass pretreated with 0.2M NaOH
 L1: Upper part of Typha grass pretreated with liquid hot water
 L2: Lower part of Typha grass pretreated with liquid hot water

161

162 Figure 2: Results of Reducing Sugar Produce after Pretreatment of 2g of Typha Grass with
 163 Liquid Hot Water Pretreatment, 0.2M H₂SO₄ and 0.2M NaOH

164 **Bioethanol concentration of Typha grass**

165 **The** highest bioethanol concentration of 2.07% was produced from the sample that was
 166 pretreated with liquid hot water and sample pretreated with dilute NaOH and H₂SO₄ produces
 167 highest bioethanol concentration of 0.54% and 0.43% respectively (figure 3). The result of
 168 ethanol yield from dilute NaOH and H₂SO₄ pretreated sample is almost the same with 0.5%
 169 reported by Fish *et al.*, [18]. Also, Grous *et al.*, [19] reported that 90% efficiency of enzymatic
 170 hydrolysis was achieved in 24 h for poplar chips pretreated by liquid hot water, compared to only
 171 15% hydrolysis of untreated chips.



Key: H1: Upper part of Typha grass pretreated with 0.2M H₂SO₄
 H2: Lower part of Typha grass pretreated with 0.2M H₂SO₄
 N1: Upper part of Typha grass pretreated with 0.2M NaOH
 N2: Lower part of Typha grass pretreated with 0.2M NaOH
 L1: Upper part of Typha grass pretreated with liquid hot water
 L2: Lower part of Typha grass pretreated with liquid hot water

172

173 Figure 3: Percentage Concentration of Bioethanol Produce from the Sample after Pretreatment
 174 with Liquid Hot Water, 0.2M H₂SO₄ and 0.2M NaOH

175 **Conclusion**

176 From the result of this research, it has been find out that pretreatment using dilute acid and
177 alkaline were very effective in removing lignin and hemicelluloses from typha grass.
178 Pretreatment with dilute acid removed hemicelluloses by 77% which proved to be better than
179 dilute alkaline which removed hemicelluloses by 75%. Dilute alkaline removed more lignin in
180 typha grass by 60% than dilute acid pretreatment which removed lignin by 35%. However,
181 cellulose content of sample pretreated with dilute H₂SO₄ increased by 59% which is higher than
182 50% increase by sample pretreated with dilute NaOH. Also, sample pretreated with dilute NaOH
183 produce highest bioethanol concentration of 0.54% than H₂SO₄ which produce 0.43%.
184 Determination of bioethanol by HPLC conclude that bioethanol can be produced from Typha
185 grass and pretreatment of the sample can increase accessibility to cellulose and increase reducing
186 sugar production thereby increasing bioethanol production.

187 **Ethical approval and consent are not applicable.**

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