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Production of Bioethanol Based on *Eichhornia crassipes* Combined with the Pulp of the Ripe Fruit of *Azadirachta indica*

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Abstract:- The management of invasive plants such as *Eichhornia crassipes* and the fruits of *Azadirachta indica* on the banks of the waters of Lake Chad in the Far North of Cameroon remains a challenge to overcome. On the other hand, given the urgency of finding other sources of energy following the scarcity of fossil fuels, it becomes appropriate to turn to renewable energies obtained from lignocellulosic biomasses which constitute an opportunity to be seized as cooking energy. The objective of this study is to determine the ethanoic potential of the biomass of *Eichhornia crassipes* associated with the pulp of *Azadirachta indica*. Thus, the production of bioethanol from the biomass of *Eichhornia crassipes* associated with the pulp of *Azadirachta indica* was done by biochemical route which results in: physical pretreatment of the raw material, thermo-mechanical pre-hydrolysis chemical, alcoholic fermentation with Lactobacillus fermentum yeast in batch mode and distillation. Measuring the pH and volatile fatty acid (VFA) content in different fermented samples made it possible to determine the optimal conditions for better fermentation. During 135 hours of fermentation, with 1281.25±1.09 ml of initial raw material fermented, it was possible to obtain on average 675.47±1.02 ml/g of hydrated ethanol and 640.62 ±5.07 ml/g of CO_2 with an average weight yield of 52.72±3.57%. Thanks to a yeast contribution of 2.5% relative to the dry matter and an L/S ratio = 20, the dosage of the distillate obtained after distillation of the fermented must by the chronometric method made it possible to determine the ethanoic concentration of the solutions studied which is on average 0.94±0.01%V/V of distillate per fermented biomass.

Keywords:- *Bioethanol, Eichhornia crassipes, Azadirachta indica, Fermentation, Cooking Energy.*

I. INTRODUCTION

At the Twenty-eighth Session of the Conference of the Parties to the United Nations Framework Convention on Climate Change (COP28) held from November 30 to December 12, 2023, in Dubai, United Arab Emirates, a global consensus of States to make a transition away from fossil fuels. The IPCC (2022), in its Sixth Assessment Report, estimates that limiting warming to 1.5°C above pre-industrial levels requires that global greenhouse gas emissions be reduced by 43% here 2030 compared to 2019 levels and that a level of net zero global CO_2 emissions is reached by the early 2050s. It is imperative to limit warming to 1.5°C in order to minimize losses and damages linked to climate for people and nature (IUCN, 2023). The increase in pollutant emissions from the combustion of fossil fuels in the atmosphere contributes significantly to global warming with the corollary having a significant impact on ecosystems. This is done via the release of CO_2 mainly through the combustion of fossil fuels (Azad et *al.*, 2015; Dharma et *al.*, 2016.) In addition, the increasingly growing demand for oil and adverse effects such as resulting climate changes (Sabba et *al.*, 2018; Bunthita et *al.*, 2016), have led to the search for alternative energy sources with very little impact on the environment (Siti et *al.*, 2017; Novidzro et *al.*, 2013). Furthermore, ethanol, added to gasoline, improves fuel combustion, thereby reducing exhaust emissions of CO and unburned hydrocarbons (Charles et *al.*, 1996). Compared to gasoline, bioethanol has a 35-40% lower energy content and 35% higher oxygen content, which results in cleaner combustion and lower toxic emissions (Marouf. 2020). Bioethanol helps reduce CO_2 emissions by up to 80% compared to the use of gasoline, thus contributing to a more sustainable environment (Sofien, 2015).

Azadirachta indica is a forest, fodder and ornamental tree which has proven ecological and especially medicinal properties. The annual fruit production of a single tree 8m to 10m high varies from 20.5kg to 55kg in Sahelian countries (Radwanski, 1977) and from 37kg to 100kg (Ketkar, 1976, Saxena et *al.*, 1989) in tropical countries. When mature, *Azadirachta indica* produces an average of 50 kg of sweet fruits annually (Formad, 2013), the pulp of which (48% of the total mass of *Azadirachta indica* fruits) remains unused. As a result, *Azadirachta indica* pulp therefore represents a significant quantity of biomass, which requires energy recovery.

Eichhornia crassipes is an invasive aquatic plant (Adjahatode et *al.*, 2016) with harmful biological consequences on the aquatic ecosystem and human activities threatening fisheries, river transport, tourism, etc. (Fragoso,

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2011). This state of affairs alters the physicochemical and organoleptic quality of the water then reduces fishing stocks (Adjahatode et *al.*, 2016) by reducing the oxygen level in the water making aquatic ecosystems less fertile (Das et *al.*, 2016); It then becomes urgent to find techniques for valorizing *Eichhornia crassipes* that respect the environment in a sustainable manner in all its forms. Furthermore, *Eichhornia crassipes* is a lignocellulosic aquatic plant composed of cellulose, hemicellulose and lignin, with promoter biomass as an energy source due to its rapid growth (Daniel et *al.*, 2020). Other functional characteristics of *Eichhornia crassipes* are its high hemicellulose and cellulose content and low lignin content, which makes it an excellent feedstock for second-generation ethanol production (Zhang et *al.*, 2018) and very effective in several areas such as wastewater purification (Aïna et *al.*, 2012). Bioethanol from water hyacinth is a biofuel which could today be the subject of significant industrial and environmental development given that unlike biogas, bioethanol can be a fuel for engines

Samples of *Eichhornia crassipes* were collected in the waters of Lake Chad in Makari, Logone and Chari in the Far North of Cameroon (12°33′45.98 north latitude and 14°26′51.3 east longitude, 1845 km²) (Figure 1).

The sampling areas for *Azadirachta indica* fruits during the period January-March 2023 are the same as those for *Eichhornia crassipes.*

(Ayissi et *al.*, 2016) and is easily conserved (Ben Chaabane, 2006).

Investigations have shown that *Eichhornia crassipes* has an ethanolic power by alcoholic fermentation (Adjahatode et *al.*, 2016) while the pulp of *Azadirachta indica*, very rich in total sugars, can be transformed into bioethanol by alcoholic fermentation (Sabba et *al.*, 2018). It is in this context that this study was initiated and is essentially oriented towards the experimentation of ethanol production processes (biofuel) from the biomass of *Eichhornia crassipes* associated with the pulp of *Azadirachta indica* more precisely the optimization of the fermentable power of the mixture of these two types of biomass (which are also plant waste) and the determination of its ethanoic degree.

II. MATERIALS AND METHOD

A. Raw Material Acquisition Area and Characterization

Six indices were applied to determine the distribution of individuals of each species in the study area: the average abundance-dominance index and the average recovery rate (MR), the presence index of *Eichhornia Crassipes* (Pi), Shannon-Weaver Index (H'), Evenness Index (R), Frequency of evenness of the species on site (%).

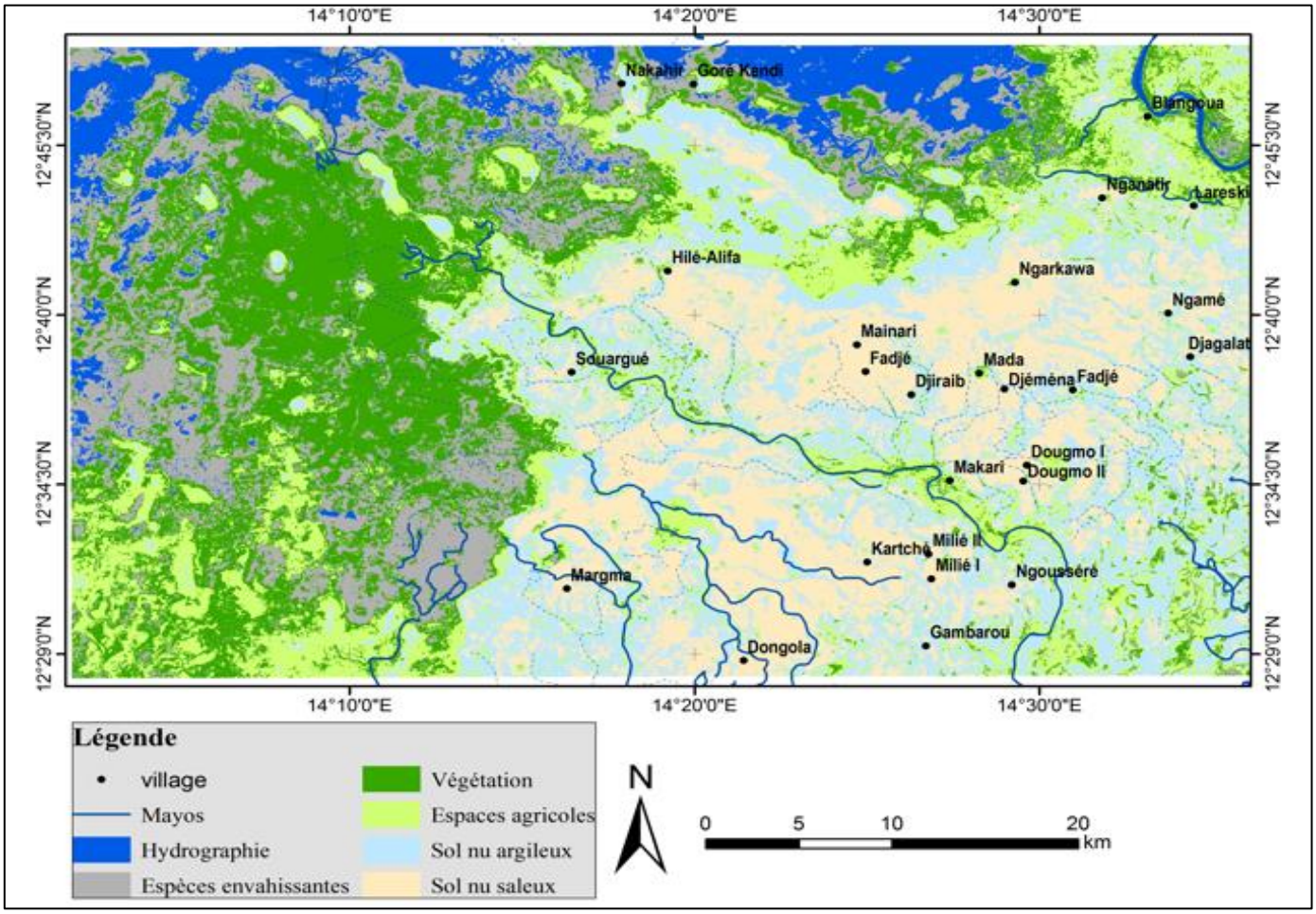

Fig 1: Raw Material Acquisition Area

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B. Pretreatment of the Raw Material and Characterization

After collection, the whole *Eichhornia crassipes* plants were dried in the shade at room temperature for 96 hours, then in the oven at 60°C for 48 hours until a constant weight was obtained. Subsequently, dry *Eichhornia crassipes* were crushed in an electric grinder (Italian Power EG500 2.5 CV) and then sieved through a medium-sized mesh of 850µm. to increase the contact surface area to improve the efficiency of analytical testing. In total, 05 kilograms of *Eichhornia crassipes* powder was obtained.

Azadirachta indica fruits were sorted based on skin color (preferably yellow) and appearance (fresh) to the touch, fallen from the tree between 24 and 48 hours (Sabba et *al.*, 2018). The average weight and proportion of pulp and kernels were determined by weighing batches of 250 fresh and ripe *Azadirachta indica* fruits using a precision balance (0.001 grams). The mass of each element separated from *Azadirachta indica* fruits was determined by weighing after separation of the pulp from the stone in each batch of fresh *Azadirachta indica* fruits. Slow stirring (30 rpm) in a specific container made it possible to separate the seeds from the pulp. This pretreatment of *Azadirachta indica* fruit pulp residues made it possible to obtain 02 liters of pure *Azadirachta indica* pulp juice for 18 kilograms of fruit used. The removed seeds were cleaned in 04 lira. This solution was added to 02 liters of pure *Azadirachta indica* fruit juice collected, which ultimately resulted in 06 liters. This solution was diluted in 30 liters to obtain a final solution concentrated at 1/5 (v/v).

The fiber content of the two raw materials is determined by differential gravimetry of the residues obtained after solubilization of the non-wall constituents (lipids, proteins and water-soluble, etc.), hemicelluloses and lignins. The dry matter was determined on a mass of 5 grams of each raw material, placed in an isothermal oven at 105°C until a constant mass was obtained (AFNOR, 1982). The total ash content was determined by calcination of the test portion used as dry matter, in a high temperature oven at 550 ± 15 °C (AFNOR, 1982).

C. Composition of samples of the raw material to be fermented

➢ *The Test was Conducted Using a Randomized Fisher Block Design Comprising Five (5) Substrates which are:*

- Control1 = 01 liters of Eichhornia crassipes substrate (100g/l);
- Control2 = 01 liters of Azadirachta indica fruit substrate (1/5 (g/l));
- Substrate 1 = 0.33 liters of Eichhornia crassipes substrate (100g/l) + 0.66 liters of Azadirachta indica fruit substrate (1/5 (g/l));
- Substrate 2 = 0.5 liters of Eichhornia crassipes substrate (100g/l) + 0.5 liters of Azadirachta indica fruit substrate (1/5 (g/l));
- Substrate 3 = 0.66 liters of Eichhornia crassipes substrate (100g/l) + 0.33 liters of Azadirachta indica fruit substrate (1/5 (g/l)).

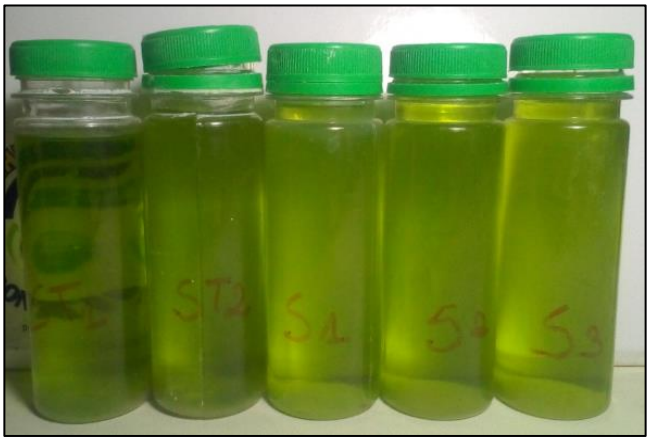

Photo 1: Samples of the Raw Material to be Fermented

Five Bioreactors were prepared with the addition in each of them a volume of water in the respective proportions of 1/3, 1/4, 1/5, 1/6 and 1/7 of the volume of final solution, in order to obtain preliminary dilutions which aim to create environments favorable to bacterial growth during the next inoculation with yeasts.

D. Processing of the Raw Material

The treatment was carried out by subjecting *Eichhornia crassipes* powder to steam explosion which is a very promising technique for lignocellulosic biomass before bioconversion. It is a thermo-mechano-chemical process which destroys lignocellulosic material and partially hydrolyzes it (Cosme et *al.*, 2018). This steam explosion process was developed by W. H. Mason in 1925 for the production of hardboard (Wertz et *al.*, 2016). It is composed of two distinct phases; steam cracking and explosive decompression (Eloutassi et *al.*, 2014). 50g of *Eichhornia crassipes* powder is placed in an Erlenmeyer flask containing 0.5 liters of distilled water. 50 liters of *Eichhornia crassipes* substrate (concentrated at 100 g/l) was obtained. The substrate is pretreated with steam in an autoclave for 1 hour at 270°C. Samples were taken every 10 minutes to assess changes in sugar levels and glucose concentration.

Furthermore, a fraction of 500ml of the 12 liters of *Azadirachta indica* fruit juice collected is brought to the boil at 70°C for 60 minutes with continuous stirring following the method of Chniti et *al.* (2013). Then, the juice of *Azadirachta indica* fruit was filtered using muslin after cooling, then heated to 85°C for 20 minutes to eliminate the bacterial flora and cooled to room temperature following the methods of Diakabana et *al.* (2013) and Massengo et *al.* (2016). Finally, urea (NH_2CONH_2 46% minimum) was added at a rate of 4 g/l to ensure optimal yeast growth and accelerate the fermentation kinetics (Novidzro et *al.*, 2013; Gbohaida et *al.*, 2016).

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E. Alcoholic Fermentation of the Solution of Eichhornia Crassipes Associated with Fresh Fruits and Blackberries of Azadirachta Indica

➢ *Preparation of the Inoculu*

The protocol described by Massengo et *al.* (2016) was used to prepare the inoculum. The yeast is pre-inoculated when introducing 06 g of the dry yeast strain of *Lactobacillus fermentum* into 100 ml of distilled water, containing 44 ml of a 12% (v/v) sucrose solution, under continuous stirring for 90 minutes and at a temperature of 27°C (Gauthier et *al.*, 2005).

➢ *Alcoholic Fermentation*

The prepared inoculum was added to the solution in the ratio Inoculum/Solution=1/500 (v/v) with continuous stirring during fermentation. The simultaneous saccharification and fermentation process used *Saccharomyces cerevisiae* (ATCC 9763), a commercial enzyme at 7 FPU/g dry mass. In each bioreactor, 01 liter of diluted solution after optimized pretreatment with acid was packaged in 50 ml samples coupled to a fermentation device in a pH 4.8 buffer solution. The hydrolysis process was initiated after initial volume gain of the solutions in the fermentation devices then subjected to constant stirring for 120 minutes at 80°C.

The process of enzymatic hydrolysis and separate fermentation which were carried out following conditions identical to the simultaneous hydrolysis and fermentation described by Daniel et *al.*, (2020). The previously prepared enzyme inoculum was used in a buffer solution at pH 4.8. The hydrolysis process was started after measuring the volumes of the solutions in the fermentation devices and constantly stirring using a stirrer (HJ-1S, 1000ml, 0-1200 rpm) at 70°C for 24 hours of hydrolysis at a concentration of 2% of the total volume of the hydrolyzate. The fermentation devices after inoculation were subjected to stirring at 300 rpm at 35ºC for 30 hours in order to similarly determine the quantity of CO_2 produced during fermentation.

From the solutions obtained, 05 alcoholic fermentation tests were carried out following the process described by Sabba et *al.*, (2018): 01 test where the pH of the solution was not adjusted (pH = 5.1) and 04 tests in which the pH of the solutions obtained were adjusted to 4.1, 4.3, 4.5 and 4.7 with a diluted sulfuric acid solution (H_2SO_4, 1.5N)). Once the desired pH is reached for the different adjusted solutions (4.1, 4.3, 4.5, and 4.7) and the unadjusted solutions, the latter are transferred to the 1 liter fermentation devices and carried out anaerobically for 120 hours (Ameyapoh et *al.*, 2006), at a temperature of 30 ± 2°C (Boulal et *al.*, 2013, 2010; Kaïdi and Touzi, 2001).

Fermentation monitoring was carried out by sampling 10 ml using a syringe every 24 hours and for 120 hours (Sabba et *al.*, 2018). The data recorded are: pH, temperature and density of the fermentation must. At the end of alcoholic fermentation, the ethanol contained in the must is extracted by distillation at 78.5°C (Diakabana et *al.*, 2016).

➢ *Alcohol Content of the Bioethanol Obtaine*

Ethanol productivity was determined by direct measurement of the volume of the distillate obtained (after distillation) for each fermentation test (Sabba et *al.*, 2018). The alcoholic degree was determined using the OIML method (1973), after distillation of the solutions following the various fermentation tests.

➢ *Statistical Analysis*

Each test was repeated 03 times for the physicochemical analyzes of the pulp and the physical characterization of the fruits of *Azadirachta indica*. The fermentation tests at different pH were carried out in two stages. The results obtained were expressed in the form: M±σ, with σ = the standard deviation and M the mean. A probability p<5% was considered a non-significant difference in the data analysis.

III. RESULTS

A. Taking Floristic Data on Site

The floristic data of *Eichhornia Crassipes* over 20m² in plots of 01 m² were carried out in Makary: Point 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, a total of 10 points of data collection.

The production data of *Azadirachta indica* over 2500 m² in plots of 25 m² were carried out in Makary: Point 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, a total of 10 data collection points.

From 2022-2023, the harvest of *Eichhornia Crassipes* in Makary in the Lake Chad area allowed us to determine the average abundance-dominance index (3.5±1.3), the average recovery rate (RM =89±0.52%) and the average weight of the biomass (5.20±3.31 kg/day) while the harvest of *Azadirachta indica* fruits in Makary in the Lake Chad area allowed us to obtain the average abundance-dominance index (4.75±0.5), the average recovery rate (RM=84.37±2.85%) and the average biomass weight (0.41±0 .20 kg/day)

Table 1: Estimation of Spatial Heterogeneity of Biomasses

Species studied	Period	Number of relief points	Weight of biomass studied (kg/period)	Number of individuals (Ri)	Average recovery (RM) %	Abundance-dominance indices
Eichhornia Crassipes Plants	June-May	10	467.75±297.83	1004±526.32	89±0.52	3.5±1.3
Azadirachta indica Fruits			37.25±18.34	165±2	84.37±2.85	4.75±0.5

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

➢ *Presence of Biomass in Sampling Site*

The diversity or specific richness of each environment in *Eichhornia Crassipes*, which takes into account both richness and fairness, was determined using the Shannon-Weaver index, the general average of which is 2.37 ±1.09, an average presence index of 0.19±0.14 with an average frequency of 24% and a Regularity Index (R) of 0.67±0.31. *Azadirachta indica* fruits collected on and under *Azadirachta indica* trees made it possible to determine a Shannon-Weaver index of 1.86±0.28 with an average presence index of 0.23±0.05, an average frequency of 9.33±2.31% and a Regularity Index (R) of 0.52±0.08.

Table 2: Quantification of Species Present on the Sites

Species studied	Presence index of Eichhornia Crassipes (Pi)	Shannon-Weaver index (H')	Regularity Index (R)	Frequency of Eichhornia Crassipes (%)
***Eichhornia Crassipes* Plants**	0.19±0.14	2.37±1.09	0.67±0.31	24±12
***Azadirachta indica* Fruits**	0.23±0.05	1.86±0.28	0.52±0.08	9.33±2.31

B. Physical Characterization of the Raw Material

The morphological study of *Azadirachta indica* fruits and *Eichhornia Crassipes* plants allowed us to have their characteristics illustrated in the following Table. The results represent the average of each parameter on 30 batches of 100 fruits of Azadirachta indica and on 30 batches of 10 plants of Eichhornia Crassipes.

Table 3: Average Results of Physical Analyzes

***Azadirachta indica* fruits**		***Eichhornia Crassipes* plants**	
Fruit weight (g)	0.487±0.019	**Plant weight (g)**	0.752±0.001
Pulp weight (g)	0.359±0.004	**Leaf weight (g)**	0.382±0.046
Seed weight (g)	0.123±0.013	**Stem weight (g)**	0.253±2.231
Fruit short axis length (cm)	0.818±0.043	**Leaf length (cm)**	25.932±2.231
Fruit long axis lengths (cm)	1.781±0.067	**Maximum stem lengths (cm)**	32.230±1.654
Pulp/fruit ratio (%)	73.717±1.743	**Leaf/plant ratio (%)**	50.798±7.234
Seed/fruit ratio (%)	25.257±2.214	**Stem/plant ratio (%)**	33.643±6.170
Almond/fruit ratio (%)	10.921±0.039	**Ratio (leaf + stem)/fruit (%)**	84.441±4.894
Color	**Yellowish**	**Color**	**Brown**

The fruit of Azadirachta indica has an average length of approximately 1.781±0.067 cm. The thin skin and bittersweet pulp of the yellowish-white fruit is very fibrous and weighs on average 0.359±0.004g, representing on average 73.717±1.743% of the total fruit weight.

Photo 2: *Azadirachta Indica* Fruit

The *Eichhornia Crassipes* plant has an average maximum stem length of approximately 32,230±1,654 cm while the average leaf length is 25,932±2,231 cm. The *Eichhornia Crassipes* plant weighs approximately 0.752±0.001g, and the aerial part of *Eichhornia Crassipes* (leaf + stem), very fibrous, represents on average 84.441±4.894% of the total weight of the plant. These proportions of material to be used are interesting for use in the fermentation process for the purposes of bioethanol production.

C. Chemical Characterization of the Raw Material

The following table shows that the average rate of raw material is 85.67± 0.42 (g /100g) for *Azadirachta indica* fruits and 79.75±1.19 (g /100g) for *Azadirachta indica* plants.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

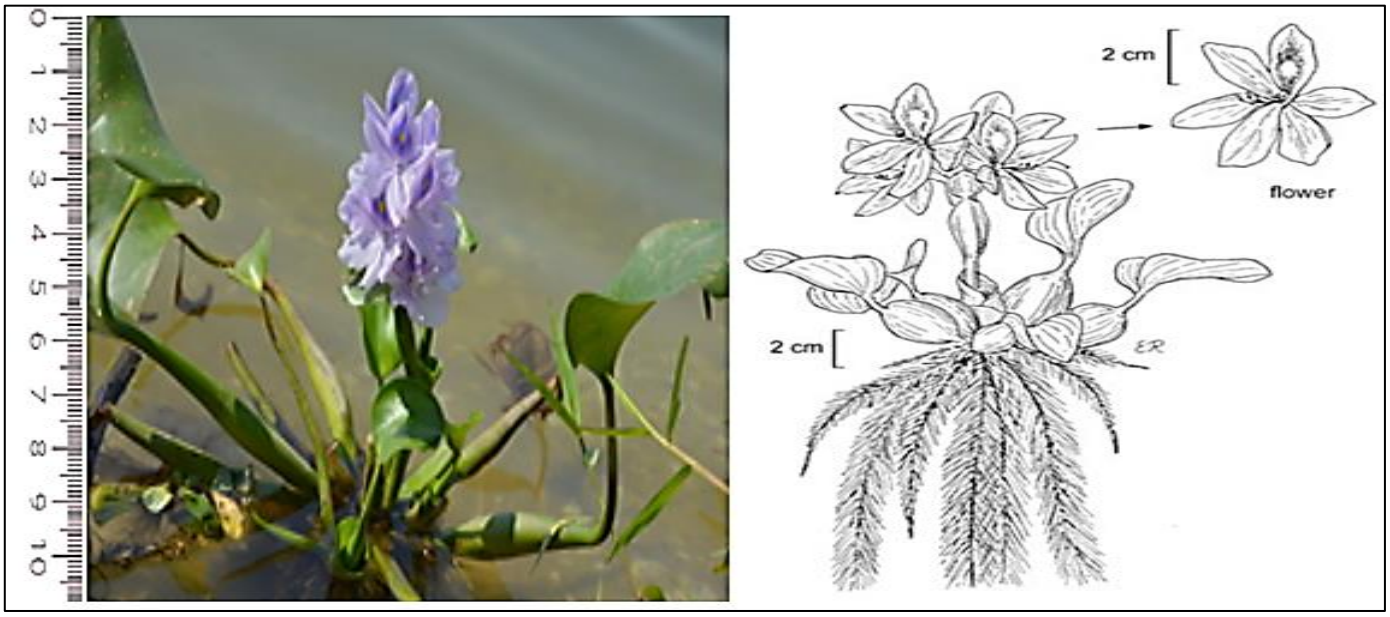

Photo 3: Eichhornia Crassipes Plant

The average pH for Azadirachta indica fruits is 05.12±0.91 and 05.24±0.07 for Eichhornia Crassipes plants. This pH is harmful to the development of bacteria but it is favorable to the proliferation of yeasts and molds, Bocquet (1982) which is a positive point for alcoholic fermentation.

The fruit pulp of Azadirachta indica is woody (13.51±1.65 g/100g DM), with proportions of hemicellulose (17.87±1.21 g/100g DM) and 70.05±2, 32 g /100g DM of cellulose while the Eichhornia Crassipes plant is less woody (08.18±1.85 g /100g DM), with proportions of hemicellulose (27.87±0.23 g /100g DM) and 33.42±0.02 g /100g DM of cellulose.

Table 4: Result of Chemical Analysis of the Raw Material

Chemical parameters	Azadirachta indica fruits	Eichhornia Crassipes plants
pH	05.12±0.91	05.24±0.07
Humidity rate (%)	38.11±3.76	46.07±1.21
Dry matter (g /100g)	85.67± 0.42	79.75±1.19
Cellulose (g /100g DM)	70.05±2.32	33.42±0.02
Hemicellulose (g /100g DM)	17.87±1.21	27.87±0.23
Lignin (g /100g DM)	13.51±1.65	08.18±1.85
Reducing sugar content (g /100kg DM)	74.75±5.07	67.21±2.01
Ether extract (g/100g MS)	05.01±0.02	35.01±1.65
Ash rate (g /100g DM)	07.31±0.29	166.25±0.29

These cellulose, hemicellulose and lignin contents found in this study are similar to the data described by Reales (2013) who proposed the production of ethanol from water hyacinth by pretreatment with sulfuric acid (2% v /v) and describes a considerable fraction of hemicellulose (21.33 g /100g DM) with a low lignin content (4.40 g /100g DM). The lignin presented by water hyacinth contributes to the lower crystallinity and the recalcitrance of the biomass to degrade during fermentation in order to release elements useful for the formation of bio-alcohol. Which directly reflects the better efficiency of the hydrolysis process and conversion of sugars into ethanol (Daniel et *al.*, 2020).

Another interesting aspect is the concentration of carbohydrates (available reducing sugars which constitute the sum of the contents of Cellulose, Hemicellulose, Starch and total soluble sugars) i.e. a total of 74.75±5.07 g /100g DM of carbohydrates for *Azadirachta indica* fruits and 67.21±2.01 g/100g DM for *Eichhornia Crassipes* plants. These data reflect a stoichiometric projection of ethanol; which makes these raw materials an interesting product with high potential for the production of ethanol.

D. Proportionate Mixtures of Solution Based on the Fruits of Azadirachta Indica with the Solution based on Eichhornia Crassipes

The randomized Fisher block device comprising the five (5) substrates which were constituted were arranged as follows:

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Table 5: Proportioned Substrate Mixtures

	Control Solution1 (liter)	Control Solution2 (liter)	Solution1 (liter)	Solution2 (liter)	Solution3 (liter)
***Azadirachta indica* fruit juice diluted with water (1/5 v/v)**	/	01	0.66	0.50	0.33
Fine particles of dry matter from *Eichhornia crassipes* plants diluted with water (100g/l)	01	/	0.33	0.50	0.66

- Control Solution1 = 01 liter of *Eichhornia crassipes* solution (100g/l);
- Control Solution2 = 01 liter of *Azadirachta indica* fruit solution (1/5 v/v);
- Solution 1 = 0.33 liters of *Eichhornia crassipes* solution (100g/l) + 0.66 liters of *Azadirachta indica* fruit solution (1/5 v/v);
- Solution 2 = 0.5 liters of *Eichhornia crassipes* solution (100g/l) + 0.5 liters of *Azadirachta indica* fruit solution (1/5 v/v);
- Solution 3 = 0.66 liters of *Eichhornia crassipes* solution (100g/l) + 0.33 liters of *Azadirachta indica* fruit solution (1/5 v/v).

Each solution, including the control solutions, has an initial volume of 1 liter. Each solution consists of mixing *Azadirachta indica* fruit juice with fine particles of *Eichhornia crassipes* (obtained via the 850µm mesh sieve). The pretreatment of these solutions with steam in an autoclave for 01 hour at 270°C resulted in a first distillation, the results of which were as follows:

Table 6: Initial Property of Solutions

Dilution number	Sugar Content (g /100kg DM)	Density at 20°C	Alcohol Content (%)
Solution T_1	65.21±2.07	1.0370 ± 0.0014	7.5025 ± 0.4231
Solution T_2	69.68±5.11	1.0372 ± 0.0165	7.5056 ± 0.8962
Solution$_1$	72.62±4.82	1.0367 ± 0.01442	6.2637 ± 1.04052
Solution$_2$	71.25±3.76	1.0363 ± 0.0179	6.1787 ± 0.8764
Solution$_3$	71.29±4.65	1.0362 ± 0.0219	6.1218 ± 1.0067

According to the table 6 above, the mixture of solutions allows us to perceive a higher initial alcohol level in solution 1, while it is lower in solution T_2.

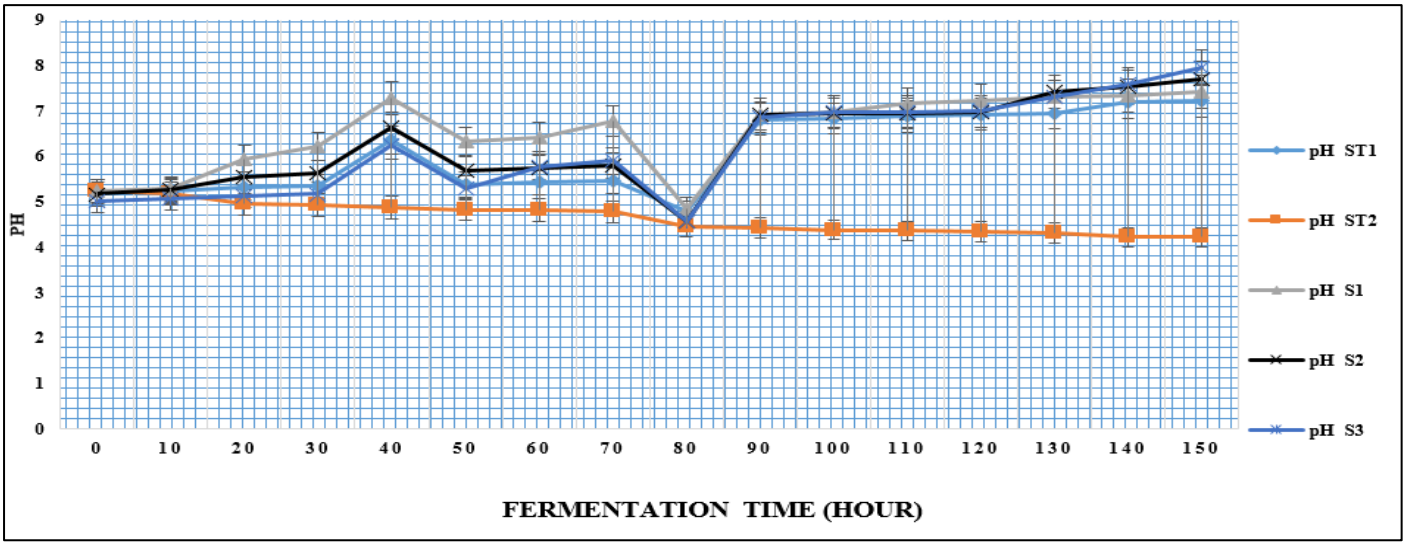
E. Monitoring Physicochemical Parameters during Fermentation

The results of the physicochemical analyzes of the solutions after addition of the inoculum during fermentation are presented as follows:

➢ *The Ph*

Determining pH is an essential step in controlling biological activity. The following graph indicates that the pH decreased from 4.60 to 4.56 during fermentation in control solution 2 while it increased in all other solutions with a jump in pH between 5.45 and 6.85.

As pH is essential for controlling biological activity, the production medium was initially adjusted between 5.01 and 5.23. During alcoholic fermentation, the metabolism of the yeast induces a perpetual change of the environment. Thus the consumption of carbonaceous and nitrogenous substrates is accompanied by the production of metabolites of carboxylic acids or alcohols. The following graph shows a variation in the pH of the solutions during alcoholic fermentation.



Graph 1: Evolution of pH during Fermentation

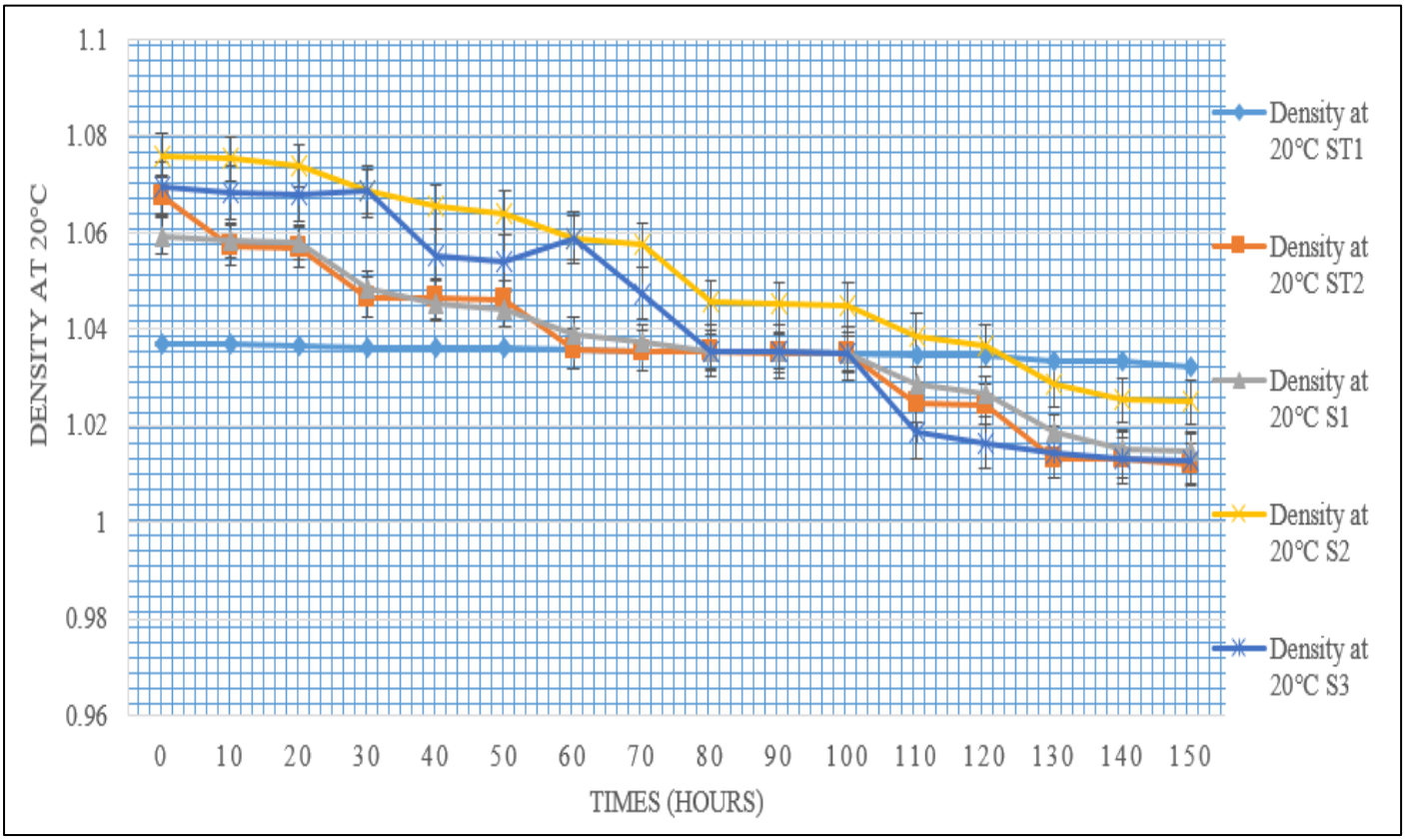
ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

For the solution based on *Azadirachta indica* fruit juice diluted with water (1/5 v/v), this phenomenon is entirely expected because the metabolic activity of the yeasts caused the variation in pH and made the more acidic environment due to the release of organic acids after the degradation of sugars by the action of the yeast used (Mehani et *al.*, 2013; Tadmourt et *al.*, 2020).

However, plant-based solutions of *Eichhornia crassipes* diluted with water (100g/l) have the same tendencies. The evolution of their pH as a function of fermentation duration presents the same oscillation for all solutions. The curve of the sample of control solution 1 (ST_1) (after addition of yeast = 3.5%) results in two peaks; one at 48 hours and the other at 76 hours while the curves of solutions S_1, S_2 and S_3 present peaks between 42 hours and 48 hours but do not present two peaks as well differentiated after 90 hours. The optimal pH range for yeast activity is between 4.6 and 6 (Le Blanc, 2008). Analysis of the results obtained shows that the pH values are between 4.21 and 7.94 during fermentation. These results therefore justify yeast activity in the environment.

➢ *Density*

The following graph represents a remarkable decrease in the density of solutions ST_2, S_1, S_2 and S_3 during fermentation varying from 1.0064 to 1.0147 and corresponding to different pH values between 4.55-7.13, transitional between the acidic environment and the alkali environment.



Graph 2: Evolution of Density during Fermentation

These variations can be explained by the transformation of glucose or fermentable sugars into alcohol and a strong loss of mass in the form of CO_2 (Boulal et *al.*, 2016, 2017; Gaillard et *al.*, 1995).

The Graph 3 below shows after 87 hours of must fermentation. This is due to a significant degradation of the sugar which is intense during the first 36 hours, leading to a decrease in the values between 25.36%, 17.09%, 22.55% and 25.25% respectively for the ST_2, S_1, S_2 and S_3.

However, in the ST_1 solution, there was rather an increase in the level of reducing sugar: which means the yeasts favored the formation of sugars.

➢ *Reducing Sugars*

Observation of the curve during the first 48 h indicates that hydrolysis was very slow in the four hydrolyzed samples (ST_2, S_1, S_2 and S_3). This can be justified by the establishment of the hydrolysis process by enzymes in the media. After 48 hours, some samples (ST_2 and S_2) are hydrolyzed more quickly than others, in particular sample S_2, which gives an interesting concentration (21.29 mg/ml) after 107 hours.

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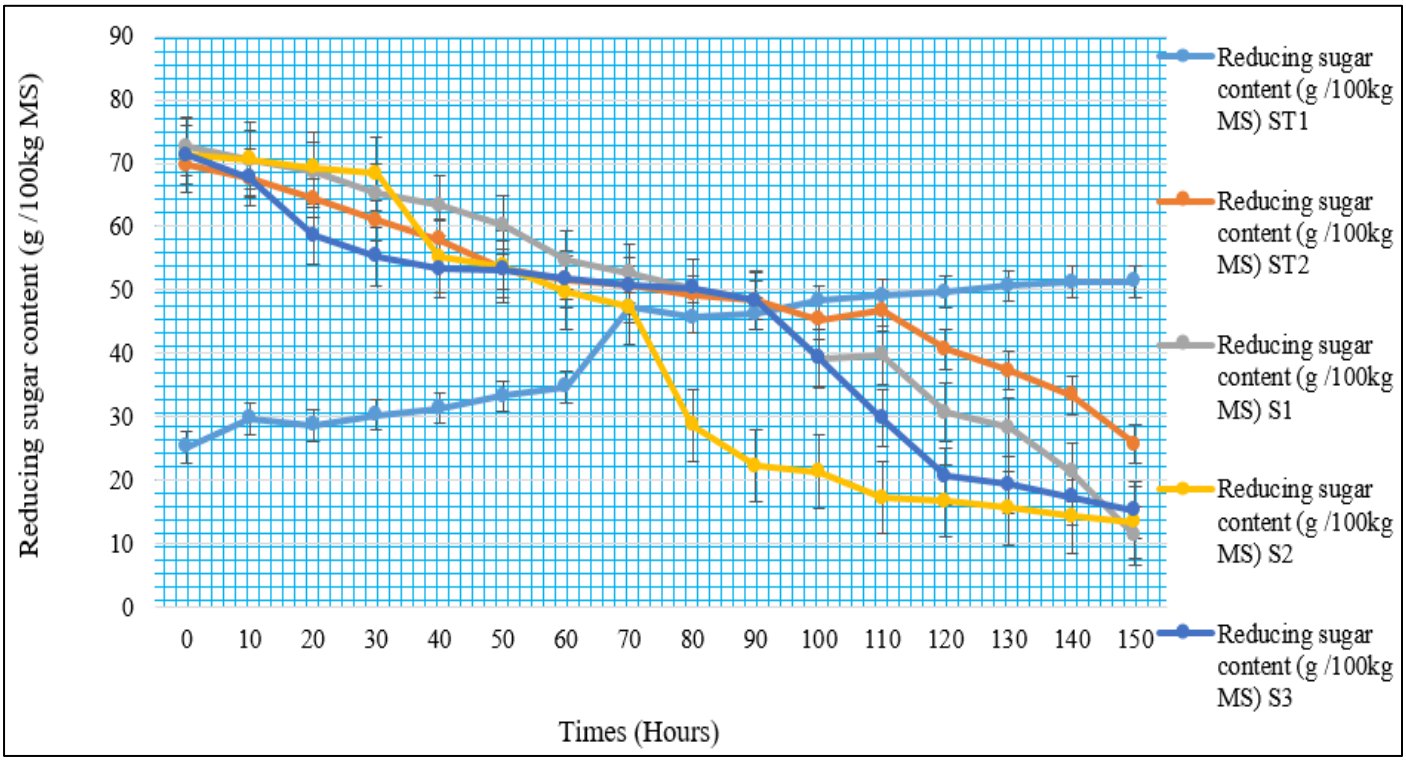


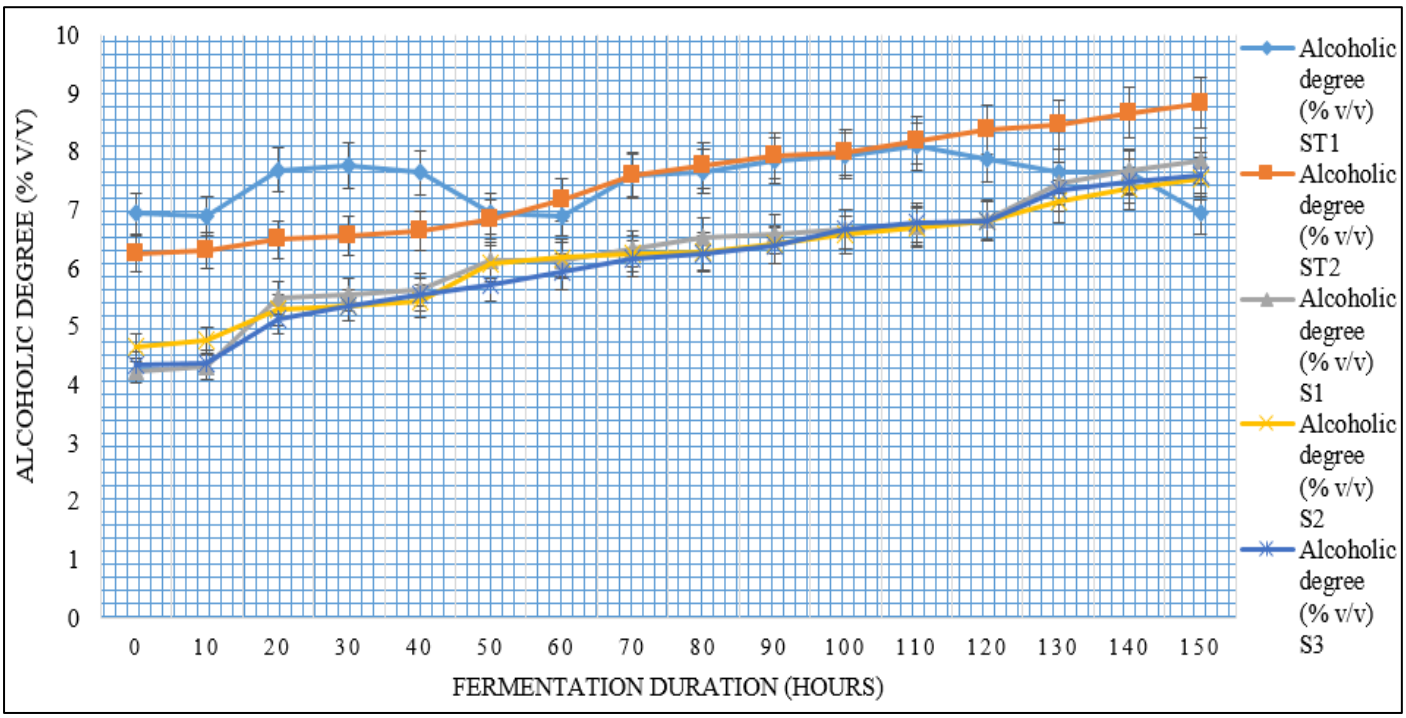
Graph 3: Evolution of the Reducing Sugar Content During Fermentation

The S_1 solution does not release considerable glucose during hydrolysis. This principle is fundamental because complex sugars are broken down into simple sugars by the synergistic action of enzymes which can be defined by good coordination of hydrolysis (Cosme et *al.*, 2018).

The three types of enzymes involved in the process are endo-1,4-glucanase and exo-1,4-glucanase cellulase, which hydrolyze celluloses to cellobiose, and β-glucosidases which hydrolyze cellobioses to glucose (Eloutassi et *al.*, 2014; Kumar et *al.*, 2008).

F. Alcoholic Degree

The following graph clearly shows that the alcohol content increased during fermentation and that the kinetics of alcohol production is related to the sugar content of the substrate in all solutions.



Graph 4: Evolution of the Alcohol Content during Fermentation

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

In the first 48 hours, the alcohol content is constantly increasing for all solutions. The lowest value of the alcoholic degree (4.24%v/v) was observed for the S_1 solution while the highest alcoholic degree (8.84%v/v) was observed in the ST_2 solution. On the other hand, alcohol production increases during the first 48 hours of fermentation. This result is in agreement with that reported by Boulal (2017) and Hadri (2023)

➢ *Analyzes of the Ethanolic Power of the Bioethanol Produced*

- *Volatile Fatty Acid (VFA) Content*

Bioethanol produced from Eichhornia crassipes plants combined with Azadirachta indica fruits is quite volatile, flammable, clear and has a pungent odor.

The results obtained show that the evolution of Volatile Fatty Acid (VFA) contents as a function of fermentation duration also presents two peaks for all samples of all fermented solutions, with similar curves.

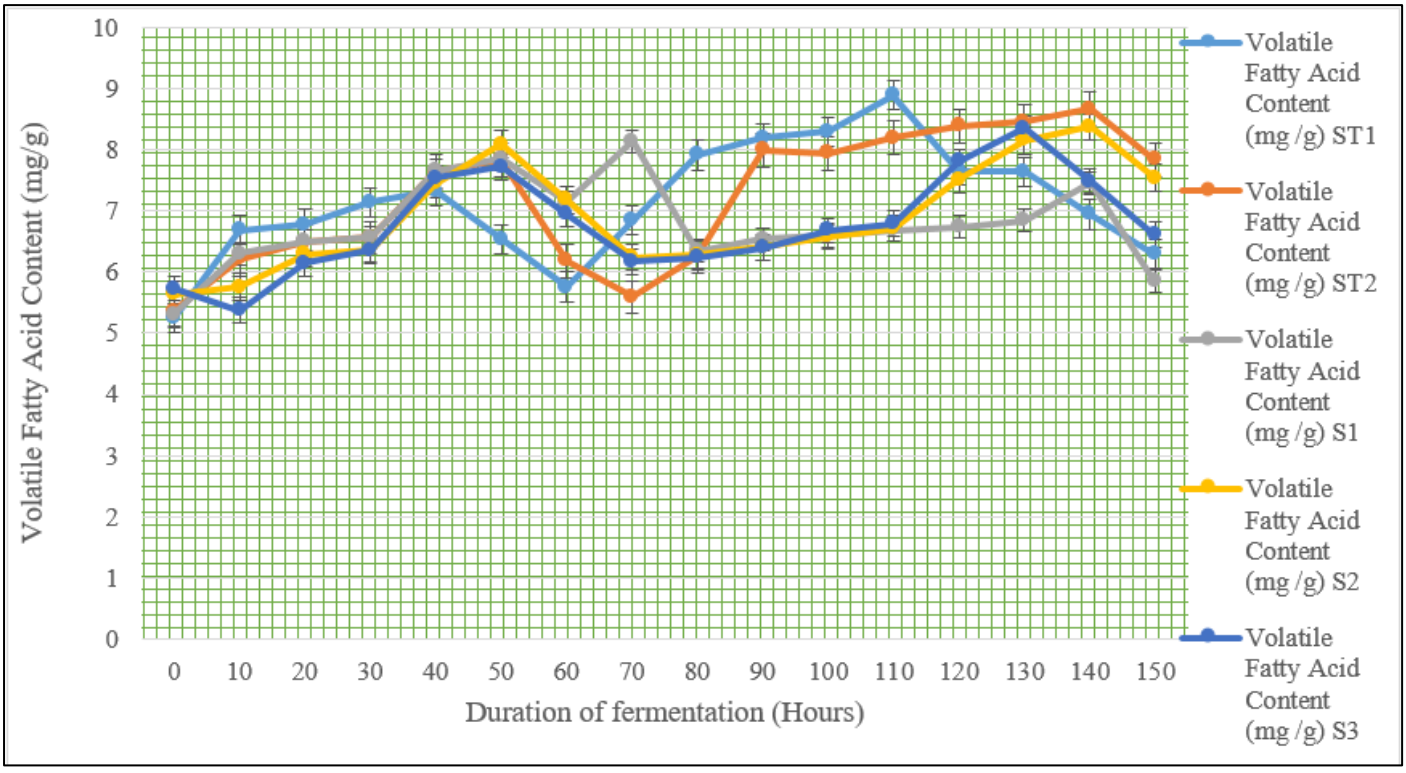
From the start of fermentation, Volatile Fatty Acid (VFA) contents are observed in the medium ranging from 5.24 mg/g (ST_1) to 5.81 mg/g (S_1). This quantity of Volatile Fatty Acid at 0 hour represents more than 50% of the maximum production observed.

During the first phase between 0h and 50h, the highest peak was reached by solution ST_1 is 7.32mg/g) while solution S_1 had only 8.14mg/g, lower than all the other solutions. During this first phase, the production of AGV increases then decreases until around 70 hours of fermentation of all solutions. This first growth could be explained by the principle of the initial decomposition of the ferment in the form of macromolecules (baker's yeast) into microorganisms (*S. cerevisiae*) (Flora et *al.*, 2016).

During the second phase between 130 hours and 150 hours, the second highest peak was reached by solution ST_1 is 8.89mg/g) while solution S_1 had only 7.46mg/g, lower than all other solutions .

For this sample S_1, the ratio between the initial and maximum production of Volatile Fatty Acid is more than 71.37% while for solution ST_1, the ratio between the initial and maximum production of Volatile Fatty Acid is more than 58.94%.

This spontaneous reaction demonstrates that pretreatment accelerates decomposition and facilitates enzymatic activity (Flora et *al.*, 2016).



Graph 5: Volatile Fatty Acid Content of Solutions as a Function of Fermentation Duration

After 74 hours, the resumption of growth results in ascending curves which reach their optimum between 122 hours and 145 hours for all samples. This very pronounced second growth is favored by the fermentation of the substrate by all the microorganisms present in the medium (Flora et *al.*, 2016). Practically, the same results were obtained from the three tests of the experiment. The quantity of VFA produced varies depending on the three parameters studied: the volume of water, the quantity of yeast and the duration of fermentation.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

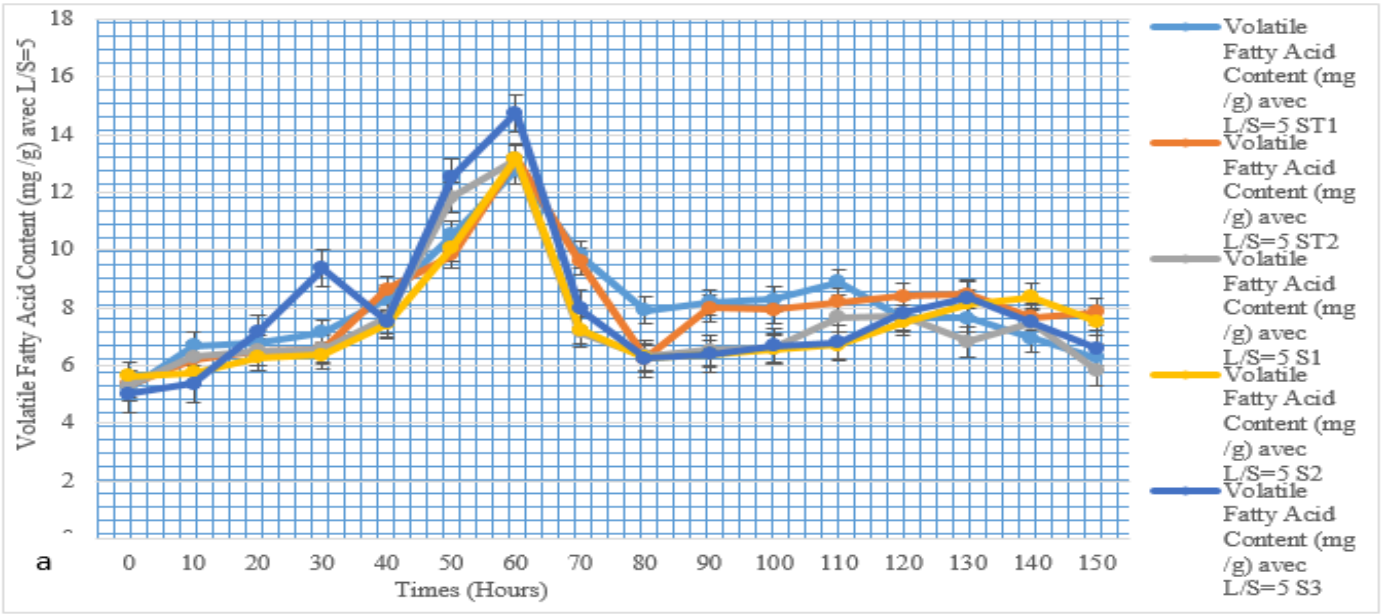
➢ *Influence of the Quantity of Ferment and the Duration of Fermentation*

The yeast used here is *Lactobacillus fermentum*, widely used in the production of dolo, a fermented drink made from the substrate of *Sorghum sp.* The volatile fatty acid (VFA) contents as a function of the fermentation duration of the samples of solutions ST_1, ST_2, S_1, S_2 and S_3 influenced by the addition of 0.5 g (2.5%), 1 g (5 %), 1.5 g (7.5%), 2 g (10%) and 2.5 g (12.5%) of yeast are shown in the following graph 6. The optimal fermentation time is equal to 135 hours. On the other hand, for the hydrolyzed heterogeneous mixture samples S_1, S_2, and S_3, the production of VFA evolves between 0 and 135 h. We will therefore use an optimal fermentation time of 135 hours. The curves have the same appearance.

Furthermore, the samples from batches “b”, “c”, “d” and “e” having had a yeast content greater than 1 g reach their maximum VFA production more quickly than the samples which had no yeast content. This variation follows the principle according to which the provision of enzymatic activity is necessary and makes it possible to accelerate the fermentation of lignocellulosic biomasses (Flora et *al.*, 2016, Almoustapha et *al.*, 2008). Thus, the choice would be a maximum yeast level for good fermentation. But this rate must be limited, or even minimized, for socio-economic reasons: better valorization of biomass at low cost (Flora et *al.*, 2016).

The analysis of the results of the first "a" batch is in line with those obtained by Flora et *al.* (2016) and Fragoso (2011) who found an optimal duration of 120 and 121 hours respectively for the fermentation of water hyacinth. The use of *S. cerevisiae* NBRC 2346 required a fermentation time of 96 hours (Mishima et *al.*, 2008).

We can therefore conclude that the fermentation duration is influenced by the quality and nature of the ferment used (Flora et *al.*, 2016).



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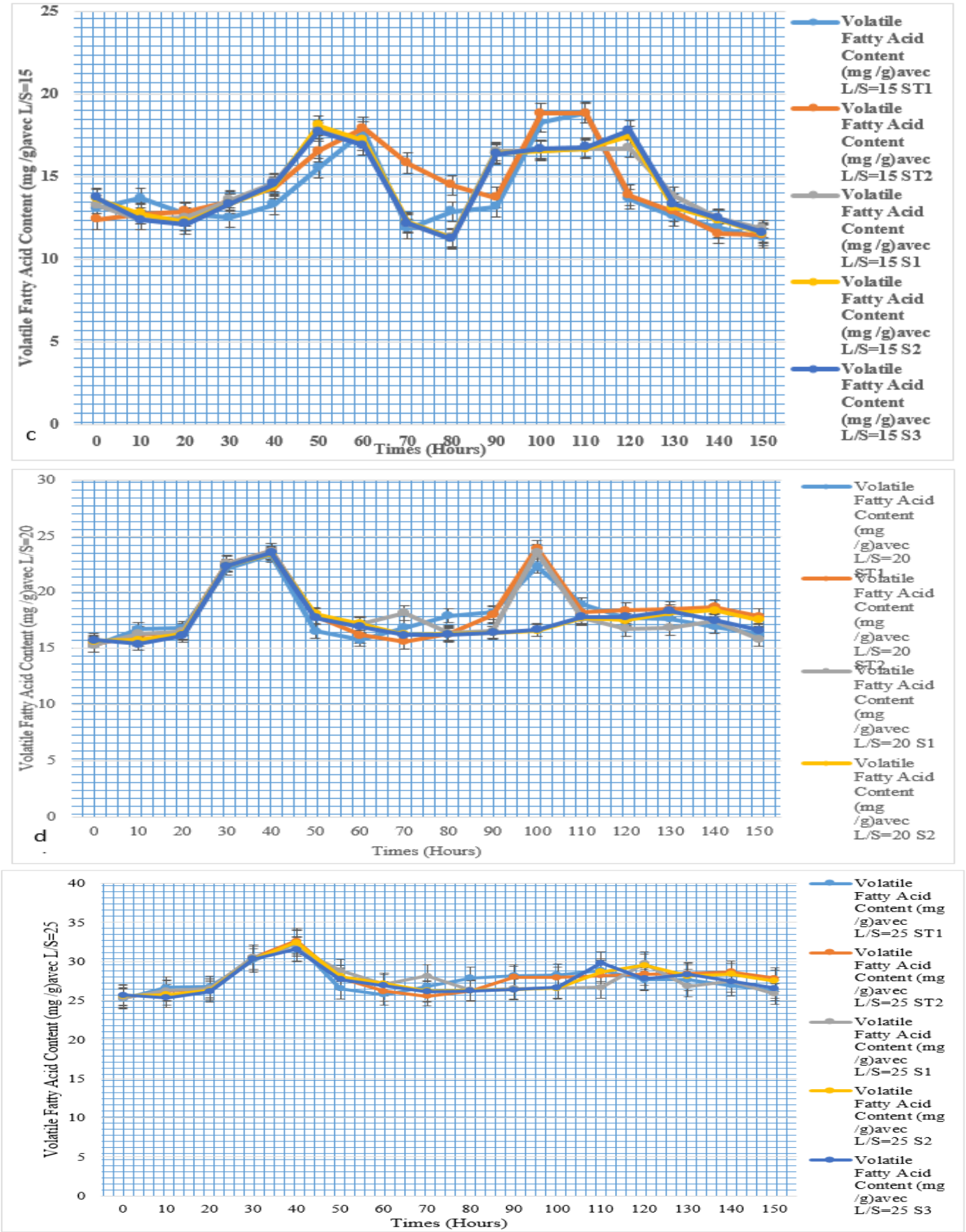


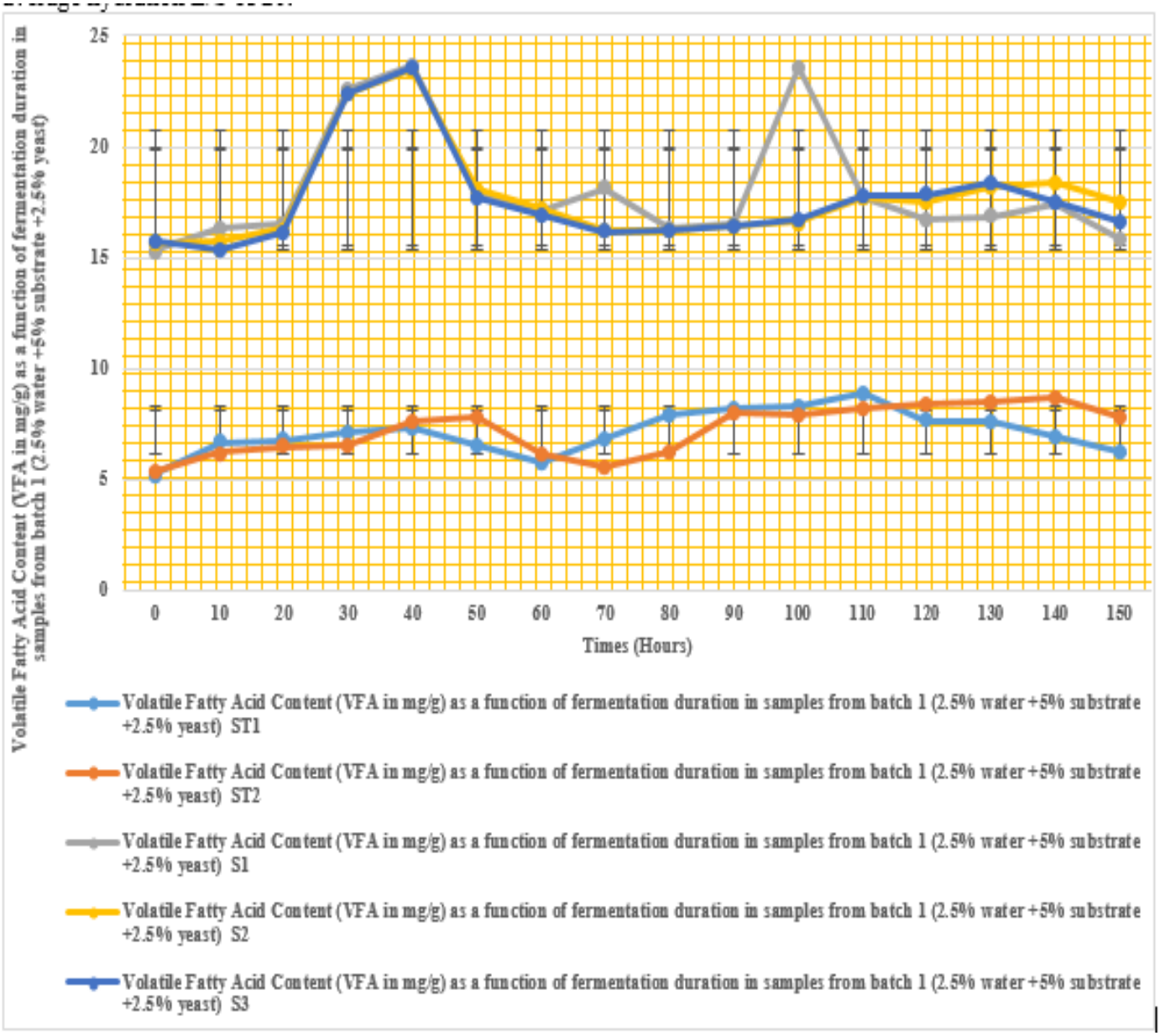
Graph 6: Volatile Fatty Acid (VFA) content for different ferment rates

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

With 2.5% *Lactobacillus fermentum* yeast for 135 hours of fermentation, the non-pre-hydrolyzed substrate based on the fruit of *Azadirachta indica* had a better ethanolic power (7.5056% v/v) than that of the non-pre-hydrolyzed substrate based on *Eichhornia crassipes* plant (7.5025%v/v).

➢ *Influence of the Quantity of Water*

Following the five tests of the experiment, the samples of solutions based on the fruit of *Azadirachta indica* associated with *Eichhornia crassipes* plants (S_1, S_2 and S_3) and the control solutions (ST_1 and ST_2) show that the production of Volatile Fatty Acid (VFA) follows the same principle regardless of the quantity of water added to the different ones whose yeast rate is 2.5% with an average hydration L/S of 20.



Graph 7: Volatile Fatty Acid Content (VFA in mg/g) as a Function of Fermentation Duration in Samples from Batch 1 (2.5% Water +5% Substrate +2.5% Yeast)

It appears from the analysis that the least hydrated sample (2.5% water) has the lowest content of VFA produced and that the production of VFA is not the highest for the sample of control solutions, whether we are in the most hydrated batch or not. On the other hand, the fermentation of media based on the fruit of *Azadirachta indica* associated with *Eichhornia crassipes* plants varies depending on the water content of the medium. This is in line with the results obtained by Flora et *al.*, (2016) which indicates that a high or low hydration rate constitutes a limiting factor for the fermentation of *Eichhornia crassipes*.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24APR135

Graph 8: Volatile Fatty Acid Content (VFA in mg/g) as a Function of Fermentation Duration in Samples from Batch 2 (5% Water + 2.5% Substrate + 2.5% Yeast)

However, for samples S_1, S_2 and S_3, Volatile Fatty Acid is more produced for the first phase of fermentation, over a short period (25th hour and 55th hour), when the added water is at 2.5%. But the production of Volatile Fatty Acid is more intense during the second phase of fermentation and extends over a relatively long period (90th hour and 136th hour) when the water content is 5%. Consequently, hydration at 5 % is favorable for optimal fermentation.

➢ *Weight Yield*

The weight yield is determined by the ratio between the quantity of alcohol produced and the quantity of substrate used. (Boulal et *al.*, 2010). Knowing that 01 liter (100 cl) of pure alcohol weighs 800 grams and that 01 kilogram of glucose gives approximately 500 g of ethanol and 500 g of CO_2, in the context of this study, the weight yield which is the ratio of the mass of bioethanol to the mass of substrate used is calculated and expressed as follows:

$$\text{Weight yield} = \frac{(\text{Quantity of alcohol})}{(\text{Quantity of substrate})}$$

With: Weight yield in %v/v or %g/g; Quantity of alcohol in ml or grams and quantity of substrate in ml or grams.

However, among the solutions based on the fruit of *Azadirachta indica* associated with *Eichhornia crassipes* plants, S_3 has the best production of bioethanol (712.19 ml/g) followed by S_2 (687.87 ml/g) then S_1 (626.37 ml/g). Their average bioethanol production obtained is 675.47 ml/g or 0.5272 g/g of *Azadirachta indica* fruit + *Eichhornia crassipes* fermented with L/S ratios between 20-25.

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Table 7: Weight Yield

Azadirachta indica fruit pulp (Milligram)	Eichhornia crassipes plants (Milligram)	Bioethanol produced (ml/g)	Weight yield (%v/v)
/	1000	750.25	58.56
1000	/	750.56	58.58
330	670	626.37	48.89
500	500	687.87	53.69
670	330	712.19	55.58

Concerning the control solutions, the results are similar to those found by Flora et *al.*, (2016) which indicates that for 0.955%V/V of pure bioethanol obtained, he used 0.08 g/g of *Eichhornia crassipes* fermented with an L/S ratio of 20 while Mishima et *al.* (2008) found a quantity of 0.14 g of pure bioethanol for 01 gram of pretreated *Eichhornia crassipes*.

IV. CONCLUSION

The production of bioethanol based on the fruit of *Azadirachta indica* associated with *Eichhornia crassipes* plants by biochemical means consisted of pre-treatment of the fruits of *Azadirachta indica* and the *Eichhornia crassipes* plants. Pre-hydrolysis via a thermo-mechano-chemical process made it possible to destroy the lignocellulosic material and partially carry out hydrolysis. The addition of urea (NH_2CONH_2) ensured optimal yeast growth and accelerated the fermentation kinetics. Starting the second phase of fermentation by adjusting the pH with a diluted sulfuric acid solution (H_2SO_4, 1.5N) made it possible to obtain varied productions of bioethanol.

The results obtained for the fermentation of samples of the associated and pre-hydrolyzed solutions have an average level of Volatile Fatty Acid of 6.8081±0.7720mg/g while the control solutions give a better average level of Volatile Fatty Acid of 7.174± 1.014 mg/g. The optimal quantities of *Lactobacillus fermentum* yeast used for this purpose are 2.5% per dry matter of *Azadirachta indica* fruit associated with *Eichhornia crassipes* plants and a liquid to solid ratio (L/S) of 20. Furthermore, the distillation of the fermentation must makes it possible to obtain on average 675.476 ml/g of hydrated ethanol and 640.625 ml/g of CO_2 with an average weight yield of 52.72±3.57%.

With a view to optimizing the results obtained, a resumption of the experiment with enzymatic pre-hydrolysis preceded by the characterization of other types of biomass often dumped and not exploited is envisaged, in order to give data useful for understanding the behavior of fermentation enzymes and bioethanol production.

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