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PRODUCTION AND OPTIMIZATION OF LACTIC ACID USING *Chlorella vulgaris* AS A SOURCE OF FERMENTABLE SUGAR

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ABSTRACT

The use of plant waste product for maximum sustainability has led to the search for bio based raw material such as Chlorella vulgaris which can make a tremendous contribution on the ecological and economical production of different organic acids like lactic acid. This study aimed to evaluate the enzymatic and or acidic hydrolysis of Chlorella vulgaris cell wall Intercellular starch as a source of fermentable sugar for lactic acid production. Lactic acid was produced through acid hydrolysis, enzyme hydrolysis and a combination of acid and enzyme hydrolysis of four different substrates. Each of the substrate was inoculated with a lactic acid bacterium (Lactobacillus plantarum) and a fungus (Rhizopus oryzae) while acid/enzyme hydrolysis was inoculated with mixed culture of the organism within a retention period of ten days. Results showed that the physico-chemical properties of C. vulgaris had a total carbohydrate of 17.4% and a reducing sugar of 24.0µg/ml. According to treatment, C. vulgaris had the highest reducing sugars (6.4 ± 0.2) and (4.4 ± 0.3) with Lactobacillus Enzyme Hydrolysis (LEH) and Rhizopus Enzyme Hydrolysis (REH) treatments, respectively indicating that C. vulgaris was the most hydrolyzed sample. Results of the optimization of various responses showed that using Corn Steep Liquor (CSL), REH day 2 treatment for fermentation at 37^0C for 48h were best combinations that yielded the optimal of Y1=0.249, Y2= 5.461 and Y3=33.704, respectively with a desirability of 0.619. The results indicated Chlorella vulgaris produced the highest lactic acid of about 20% signifying that its biomass contains high source of carbohydrate accessible for lactic acid production.

Keyword: *Chlorella vulgaris*, hydrolysis, Lactic acid bacteria, Simultaneous Saccharification and Fermentation treatments.

INTRODUCTION

Lignocellulosic biomass is complex biological materials that are relatively low cost feedstocks with suitable source of carbohydrates for sustainable industrial biotechnological production. They include domestic wastes, office wastes, industrial wastes, forestry waste products and agricultural residues especially from legumes, cereals, pulses, forage and silage crops (Wang *et al*., 2011; Dong, *et al*., 2016; Kim, 2018). These waste materials are normally preferred because of their relative availability, non-seasonal and abundance (Zhang *et al*., 2016). They comprise mainly of plant cell wall materials which are composed mainly of cellulose - β-1, 4 and β-1, 6-glucans; hemicellulose in form of polysaccharides (Fukuda *et al*. 2009; Hendriks and Zeeman, 2009). Through application of pretreatment processes of acid, alkali and enzyme hydrolysis, complex sugars of lignocellulosic materials are broken down into smaller

macromolecules with specific functions for biosynthesis production (Keshwani and Cheng, 2009; Sanchez 2009). Lactic acid is an organic acid known as 2-hydroxypropanoic acid, a versatile product known and produced worldwide with diverse application in food, manufacturing, pharmaceutical, plastics, cosmetics and the chemical industries (Lima *et al*., 2009, Pinaki *et al*., 2019). It can be produced through chemical synthesis and microbial biosynthesis using the lactic acid bacteria which allows the biologically active L(+) form of the acid to be obtained with little or no side effects (Dan *et al*., 2013). Lactic acid bacteria are traditionally fastidious microorganisms and have complex nutrient requirements due to their limited ability to synthesize B-vitamins and amino acids (Ali *et al*., 2009).

Chlorella vulgaris is a photoautotrophic green microalga capable of producing large biomass in the presence of light and carbon dioxide. The cells are rich in chlorophyll, protein, vitamin, minerals, nucleic acid and carbohydrate which can be used to produce lipid for biodiesel production (Ho *et al*., 2013). The carbohydrate and protein content of the cell which form part of its biomass normally serves as a waste product and the reducing sugar obtained from it can be used for the production of vast biological products (Brennan and Owende, 2010). Rodrigues and Bon (2011) evaluated the use of *Chlorella* sp. as source of fermentable sugar for the production of a second generation biological product because of its cell wall component, intracellular spaces and carbohydrate content. Zhou *et al*. (2011) studied the methods of C*hlorella vulgaris* hydrolysis in the presence of some acids. Albarico *et al*. (2017) evaluated the potential of *Chlorella vulgaris* for the production of reducing sugar via acid hydrolysis. Phwan *et al.* (2018) revealed the technologies for microalgae pretreatment and fermentation processes of bioethanol and Phwan *et al.* (2019) studied the reducing sugar concentrations of *Chlorella* sp. biomass by pretreating with dilute concentrations of acids. The commercial viability of microbial lactic acid production relies on the utilization of an inexpensive carbon substrates derived from waste resources (Eiteman, and Ramalingam, 2015). Production of lactic acid by simultaneous saccharification fermentation is possible using waste material such as defatted rice bran (Tanaka *et al*., 2006); cassava bagasse starch hydrolysate (John and Nampoothiri, 2008); corn stover hydrolysate (Cui *et al*., 2011). Certain parameters such as temperature, pH, agitation speed and dissolved oxygen level affects their production rate during fermentation due to the metabolic processes involved (Abdulkarim *et al*., 2006). A number of industrial by-products or wastes have been evaluated as substrates for lactic acid production with the aim of decreasing the cost of the process, such as sugarcane, molasses and whey as carbon sources and CSL as a nitrogen source (Komesu *et al*., 2017). The statistical tool Response Surface Methodology (RSM) normally used in the production of biochemicals and biotechnology processes was adopted in the study to experimentally model and optimize the production of lactic acid. This investigation was carried out to assess the possibility of producing lactic acid using *Chlorella vulgaris* biomass as a source of potential feedstock and its optimization.

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MATERIALS AND METHODS

Sample Collection and Processing

A total of four samples namely, Corn cob, Corn Steep Liquor, Corn germ and *Chlorella vulgaris* were used for the analysis.

Corn Cob: The dried corn cob was milled into a fine powder and preserved until when needed (Umeh and Agwa, 2001). Corn Germ was steeped in hot water for 2-3 days, wet milled, sieved and corn starch extracted. The germs were washed using a sieve material to remove the starchy material for making pap (Ogi). The dewatered and clean germs were sundried for 4 days (Bai *et al*., 2008). Corn Steep Liquor (CSL) was steeped in hot water for 2-3 days, the broth separated and used as part of the fermentation substrate (Zhaopeng *et al.*, 2006).

Isolation and characterization of Microorganism

The microorganisms were obtained from the Department of Microbiology University of Port Harcourt, Rivers State, Nigeria. The fungus, *Rhizopus oryzae* was cultured on PDA for 2-5 days at room temperature, while *Lactobacillus plantarum* was cultured on Mann Rogosa Sharpe (MRS) agar and incubated anaerobically in a gas jar for 24h. After 24h, colonies observed were milky white colonies with rough edge (Zhou *et al*., 2006; Dan *et al*., 2013). The microalga (*Chlorella vulgaris*) used in the study was bloomed with a freshly sterilized aqueous poultry manure digestate using the technique of Agwa *et al.* (2014). These organisms were further sub cultured on slants and stored for further use.

Inoculum Development for Lactic Acid Production

One milliliter (1 m1) of *Lactobacillus plantarum* was added to 9 ml distilled water, inoculated in MRS broth and incubated for 48 h at 37^0C. Thereafter, 5 ml of the microbial suspension was transferred into the fermentation broth for lactic acid production. About 1ml of the inoculum suspended in MRS broth was transferred into 9 ml of sterile distilled water and was observed for turbidity. *Rhizopus oryzae* of 4 - 6 days old spores was added to a mixture of 10 ml sterile distilled water and 2 drops of 0.1% Tween 80 was poured into PDA slant and was aseptically harvested. One milliliter of the *Rhizopus* suspension was transferred into each of the sterilized fermentation broth, respectively (Zhou *et al*., 2003).

Screening for Amylase Production

Lactobacillus plantarum and Rhizopus oryzae were screened for amylase production on nutrient agar plates supplemented with starch solution. These isolates were spot inoculated on the nutrient agar plate containing 1% starch solution, incubated at 37^0C for 24 h, clear zones of hydrolysis around the colonies were noted after staining the isolates with iodine solution. The isolate that showed highest zone of clearance on screening for amylase production was selected and stored as amylolytic producing fungus (Ashiwini *et al*., 2011).

Substrate Pre-treatment for lactic acid fermentation

Three types of pre-treatment were administered with the four substrates used in this research.

Acid Hydrolysis Pretreatment: Fifty grams of each substrate was separately soaked in 100 ml of dilute hydrochloric acid and autoclaved at 121^0C for 15 min (Parviz *et al*., 2011).

Enzyme Hydrolysis pretreatment: Fifty grams of each biomass was soaked into the fermentation media and autoclaved accordingly. A suspension of *Lactobacillus plantarum* and *Rhizopus oryzae* were inoculated and incubated for 3-4 days at 37^0C in their respective condition (Rodrigues and Bon, 2011).

Enzyme and Acid Hydrolysis pretreatment: Twenty-five grams of raw substrates and 25 g of pretreated acid substrates were added into the fermentation media, autoclaved and allowed to cool. A suspension of *Lactobacillus plantarum* and *Rhizopus oryzae* were inoculated and incubated for 3-4 days at 37^0C in their respective condition according to Idris *et al.* (2009).

Physico-chemical Analysis

Physico-chemical Analysis of the substrates was determined following the method of Ali *et al.* (2009). They include Fat Extraction; total carbohydrate, Moisture content determination; Trace metal determination and Reducing sugar Estimation (DNS Method using 10 M glucose as a standard).

Simultaneous Saccharification and Fermentation (SSF)

The modified method of Coehlo *et al.* (2011) was adopted for the study. To produce sugar hydrolyzate for fermentation, the samples were subjected to acid hydrolysis, Enzyme hydrolysis and combination of both acid and enzyme hydrolysis. Three fermentation set ups were monitored in triplicates: the first part of the samples used were all hydrolyzed with amylase enzyme; while the second part were hydrolyzed with 7% w/v Hydrochloric acid (HCl) and the third, a combination of the amylase enzyme and HCl. The hydrolysates were inoculated with the isolated microorganisms; subsequently, fermentation was carried out at 28 ± 2^0C, respectively with regular agitation for ten days in a media (g/l) containing: *Rhizopus oryzae* (KH_2PO_4 – 5.0; $MgSO_4.7H_2O$ - 0.1; NH_4NO_3 - 2.0); *Lactobacillus plantarum* (glucose-20; KH_2PO_4- 5; $MgSO_4.7H_2O$ – 0.1; yeast extract – 5.0; peptone – 10.0; ammonium citrate – 2.0) and a combination of the two organisms with the required nutrient. The pH of the respective media was adjusted to 6.5 with 5% $CaCO_3$. They were sterilized by autoclaving at 121^0C at 15psi for 15min and, allowed to cool before inoculating. During the fermentation period, samples were taken periodically for various analyses - pH, temperature, titrable acidity and reducing sugar. Part of the filtrate was centrifuged at 4000 rpm for 15 min and the supernatant filtered. The concentration of lactic acid produced from the hydrolysates was compared with that produced from *Chlorella vulgaris*.

At the end of the fermentation period, the lactic acid produced was recovered by using a modified method of Pal *et al*. (2009). The fermentation medium was filtered to remove cell debris and mycelia and centrifuged at 4000 rpm for 15 min. After centrifugation, calcium hydroxide was added, mixed and allowed to settle; the clear calcium lactate was decanted and mixed with the filtrates from the slurry. The combined mixture was treated with sodium sulphide, decolourized by adsorption with activated charcoal and subsequently

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filtered. The calcium lactate liquor was then dried using hot air oven.

Statistical Analysis: The Analysis of variance (ANOVA) was used to compare the mean acidity, % hydrolysis and lactic acid yield from the different sources used. The Dunn's Post's test was used to compare difference in lactic acid yield and glucose hydrolysis between *Chlorella vulgaris* and other sources. All tests were done with the Graph pad Prism V6 software at a 95% confidence interval and a p value of < 0.05 was considered significant.

RESULTS

A total of four fungi colonies were picked from beans and soil samples; out of which only one of the isolates obtained from bean sample had amylase producing potential. The cultural and physiological properties of the colonies appeared as white cottonous mycelia which covered the entire surface of the petri dish with non-septate hyphae, brown spores on sporangia. The strain was identified from the colonial and microscopic features as *Rhizopus oryzae*. A total of six isolates were encountered, two each from corn steep liquor, yoghurt and soil samples and only one isolate was identified from the colonial and microscopic features as *Lactobacillus Plantarum* - a milky white colonies with rough edges, showing a wide zone of clearance on screening for amylase production, stored as amylolytic lactic acid producing bacteria.

The Physiochemical characteristics of the substrates used in the production of lactic acid are presented in Table 1. The result showed that Corncob had the highest carbohydrate (149.2%) and the least *Chlorella vulgaris* (17.4%).

Table1: Physiochemical characteristics of the substrates used in the production of lactic aci

Substrates	Fat (%)	Total Carbohydrate (%)	Reducing sugar (µg/l)	Moisture (%)	Trace metal (ppm)	Salt (%)
Corn cob	-58.4	149.2	21.0	8.3	K=7.2 Zn=0.18 Cu=2.80 Mg=35.0	1.9
Corn germ	8.0	69.9	25.0	21.3	K=84.0 Zn=0.06 Cu=0.44 Mg=0.82	1.4
Corn Steep Liquor (CSL)	4.0	36.3	34.0	56.6	Mg=0.26 Cu=0.27 Zn=0.18 K=5.8	3.6
Chlorella vulgaris	55.8	17.4	24.0	23.4	Mg=0.17 Cu=3.51 Zn=0.0 K=9.6	3.7

Note
K=Potassium
Zn =Zinc
Mg=Magnesium
Cu=Copper

The differences in the rate of reducing sugar according to the type of treatment administered are represented in Table 2. *Chlorella vulgaris* was used in comparison with Corn Cob, Corn Steep Liquor (CSL) and Corn Germ. In the Rhizopus Acid Hydrolysis (RAH): Corn Cob (3.5); CSL (3.6); *Chlorella* sp. (4.4) and Corn Germ (4.1). This shows that *Chlorella vulgaris* had the highest reducing sugar in the RAH treatment. From the Lactobacillus Enzyme Hydrolysis

(LEH): Corn cob (5.8); CSL (3.8), Corn Germ (5.3) and *Chlorella vulgaris* (6.4). In other words, the percentage of reducing sugar from *Chlorella vulgaris* was also the highest in LEH treatment. Only LAH and EAH are significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris.* But the treatments of REH with corn germ, RAH and LEH with corn cob and corn germ and LAH with CSL are not significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris.*

Table 2: Differences in Reducing Sugar according to treatment

Treatment	Corn Cob	Corn Steep Liquor	Corn Germ	*Chlorella vulgaris*	ANOVA
REH	3.5±0.1[a]	3.6±0.5[a]	4.1±0.1[b]	4.4±0.3	0.0387*
RAH	5.2±0.2[b]	6.4±0.4[b]	5.1±0.1[b]	5.7±0.6	0.0267*
LEH	5.8±0.8[b]	3.8±0.3[a]	5.3±0.3[b]	6.4±0.2	0.0016*
LAH	5.5±0.4[a]	3.7±0.4[b]	5.3±0.1[a]	3.8±0.4	0.0008*
EAH	5.3±0.1[a]	4.1±0.02[a]	4.1±0.1[a]	4.4±0.1	0.0001*

All figures are presented in mean ±SD.

*Difference across the groups is statistically significant ($p < 0.05$)

[a]Difference is statistically significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris*

[b]Difference is not statistically significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*

REH= *Rhizopus* Enzyme Hydrolysis

RAH= *Rhizopus* Acid Hydrolysis

LAH= *Lactobacillus* Acid Hydrolysis

EAH= Enzyme and Acid Hydrolysis

LEH= *Lactobacillus* Acid Hydrolysis

The initial reducing sugar content of the lactic acid produced was monitored during the fermentation period (Fig. 1). The investigation revealed REH as the highest treatments (corncob 27µg/l; CSL 25µg/l; *Chlorella vulgaris* 24µg/l and corn germ 23µg/l), followed by EAH (corncob 17µg/l; CSL 16µg/l; *Chlorella vulgaris* 24µg/l and CSL 23µg/l).

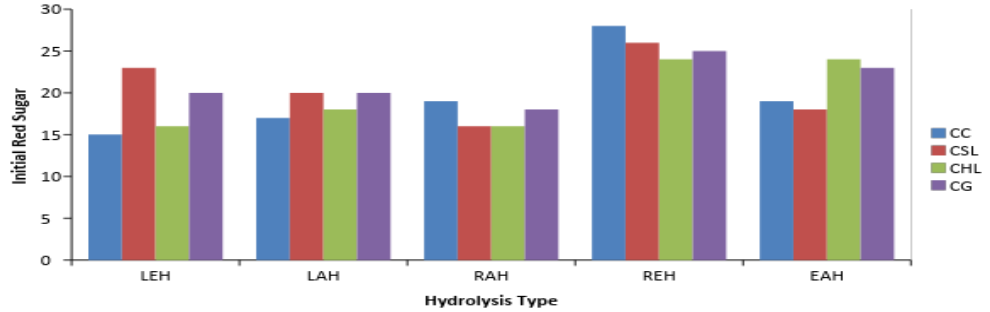

Fig 1 Histogram of initial Reducing Sugar (µg/l)

REH= *Rhizopus* Enzyme Hydrolysis CC=Corn cob
RAH=*Rhizopus* Acid Hydrolysis CSL=Corn Steep Liquor
LEH=*Lactobacillus* Enzyme Hydrolysis CHL= *Chlorella*
LAH=*Lactobacillus* Acid Hydrolysis CG=Corn Germ
EAH=Enzyme and Acid Hydrolysis

The final reducing sugar content of the lactic acid produced was monitored during the fermentation period (Fig. 2). The sugar content was properly hydrolyzed by LAH treatments

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with *Chlorella vulgaris* 40µg/l and CSL 37µg/l followed by LEH with CSL 34µg/l and the least hydrolyzed treatment LAH with corn cob and corn germ samples both had 15µg/l each)

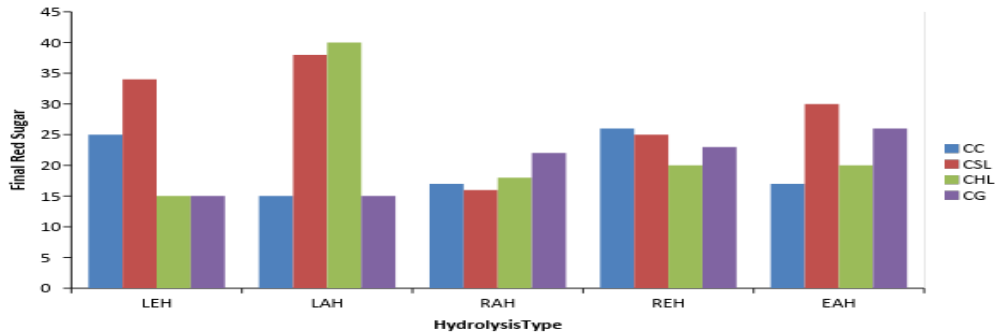

Fig 2 Histogram of Final Reducing Sugar (µg/l)

REH= *Rhizopus* Enzyme Hydrolysis
RAH=*Rhizopus* Acid Hydrolysis
LEH=*Lactobacillus* Enzyme Hydrolysis
LAH=*Lactobacillus* Acid Hydrolysis
EAH=Enzyme and Acid Hydrolysis
CC=Corn cob
CSL=Corn Steep Liquor
CHL = *Chlorella*
CG=Corn Germ

The differences in % hydrolysis according to treatment during fermentation are illustrated in Table 3. Corn cob, CSL and Corn Germ was statistically significant ($p<0.05$) compared to treatment on *Chlorella vulgaris* implying that the % hydrolysis of *Chlorella vulgaris* was higher than that of the other substrates. But in the treatment of RAH with CSL, LEH with corn cob and EAH with corn germ are not significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*

Table 3: Differences in % Hydrolysis according to treatment

Treatment	Corn Cob	Corn Steep Liquor	Corn Gem	*Chlorella vulgaris*	ANOVA
REH	3.5±0.1[a]	3.7±0.2[a]	4.1±0.1[a]	4.3±0.2	0.0001*
RAH	5.2±0.1[a]	6.6±0.3[b]	5.5±0.4[a]	6.3±0.1	0.0013*
LEH	6.5±0.1[b]	4.2±0.1[a]	5.2±0.1[a]	6.6±0.1	0.0001*
LAH	5.3±0.2[a]	6.8±0.2[a]	5.3±0.1[a]	6.4±0.1	0.0001*
EAH	5.5±0.4[a]	5.3±0.2[a]	4.5±0.1[b]	4.1±0.1	0.0020*

All figures are presented in mean ±SD.
*Difference across the groups is statistically significant ($p < 0.05$)
[a]Difference is statistically significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris*
[b]Difference is not statistically significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*
REH= *Rhizopus* Enzyme Hydrolysis
RAH=*Rhizopus*Acid Hydrolysis
LEH=*Lactobacillus* Enzyme Hydrolysis
LAH= *Lactobacillus* Acid Hydrolysis
EAH=Enzyme and Acid Hydrolysis

The differences in % acidity according to all the treatments are significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris* except RAH which is not significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris.*

Table 4: Differences in %Acidity according to treatment

Treatment	Corn Cob	Corn Steep Liquor	Corn Gem	*Chlorella vulgaris*	ANOVA
REH	0.02±0.001[a]	0.01±0.002[b]	0.01±0.001[a]	0.01±0.001	0.0017*
RAH	0.02±0.002[b]	0.03±0.002[b]	0.12±0.16[b]	0.02±0.002	0.4102**
LEH	0.02±0.001[b]	0.017±0.001[a]	0.02±0.001[b]	0.022±0.001	0.0003*
LAH	0.02±0.001[a]	0.025±0.001[b]	0.023±0.001[b]	0.02±0.001	0.0001*
EAH	0.02±0.002[a]	0.023±0.001[a]	0.016±0.002[b]	0.01±0.004	0.0093*

All figures are presented in mean ±SD.
*Difference across the groups is statistically significant ($p < 0.05$)
**Difference across the groups is not statistically significant ($p > 0.05$)
[a]Difference is statistically significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris*
[b]Difference is not statistically significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*
REH= *Rhizopus* Enzyme Hydrolysis
RAH=*Rhizopus* Acid Hydrolysis
LEH=*Lactobacillus* Enzyme Hydrolysis
LAH=*Lactobacillus* Acid Hydrolysis
EAH=Enzyme and Acid Hydrolysis

The difference in the yield of lactic acid and glucose produced from the different sources revealed that corn cob gave the highest yield in lactic acid and *Chlorella vulgaris* produced the highest glucose yield. The differences across the different sources is significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris* (Table5).

Table 5: Differences in Lactic Acid and Glucose yield from different sources

Yield	Corn Cob	Corn Steep Liquor	Corn Gem	*Chlorella vulgaris*	ANOVA
LacticAcid Yield	0.0053±0.001[a]	0.0046±0.001[b]	0.004±0.001[b]	0.003±0.002	0.0084*
Glucose Yield	4.15±0.1[b]	4.19±0.2[b]	3.68±0.2[a]	4.26±0.3	0.0040*

Glucose unit = (µg/l)
Lactic acid unit = (Mol/l)
All figures are presented in mean ±SD.
*Difference across the groups is statistically significant ($p < 0.05$)
[a]Difference is statistically significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris*
[b]Difference is not statistically significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*

The differences in the efficiency of the lactic acid produced within the different treatments can be seen in table 6. All the substrates used within the treatments were significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris* except LEH with corncob and LAH with CSL which were not significant ($p > 0.05$) compared to treatment on *Chlorella vulgari*

Table 6: Differences in Lactic Acid Efficiency of Different Treatments

Treatment	Corn Cob	Corn Steep Liquor	Corn Gem	*Chlorella vulgaris*	ANOVA
REH	0.006±0.0001[a]	0.0050±0.0002[a]	0.0042±0.0001[a]	0.0033±0.0001	<0.0001
RAH	0.0055±00001[a]	0.0045±0.0001[a]	0.0062±0.0001[a]	0.0034±0.0002	<0.0001
LEH	0.0031±0.0001[b]	0.0043±0.0001[a]	0.0047±0.0001[a]	0.0033±0.0001	0.0011
LAH	0.006±0.0003[a]	0.0036±0.001[b]	0.0043±0.0001[a]	0.0035±0.0001	<0.0001
EAH	0.005±0.0001[a]	0.0038±0.000001[a`]	0.0035±0.0001[a]	0.0044±0.0002	<0.0001

Lactic acid unit = (Mol/l)
All figures are presented in mean ±SD.
*Difference across the groups is statistically significant ($p < 0.05$)
**Difference across the groups is not statistically significant ($p > 0.05$)
[a]Difference is statistically significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris*
[b]Difference is not statistically significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*

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Graphical Designs of Response Surface Methodology: **Response of % Acidity to Substrates, Days and Treatments**

The optimal line chart
The All Factor Line Graph

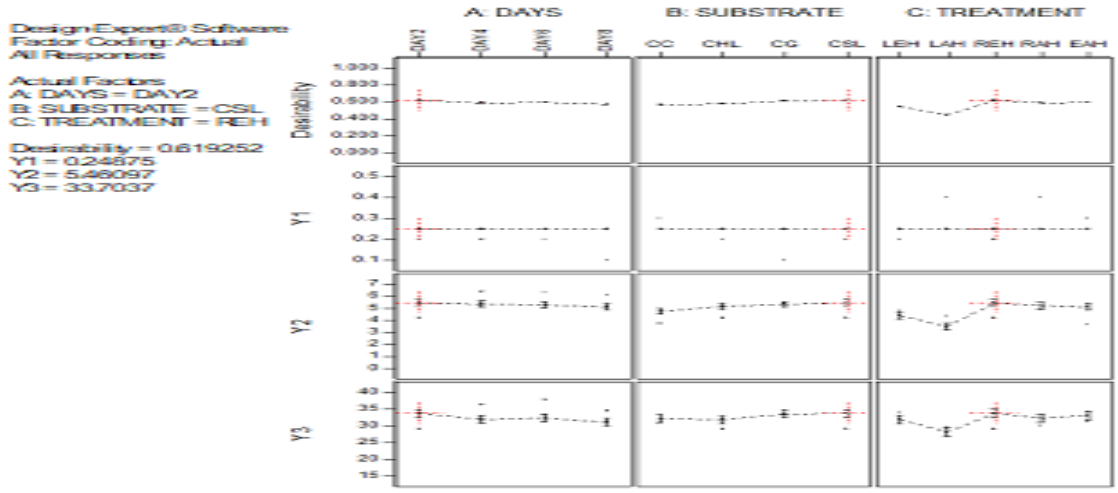

Fig. 3: RSM Optimization stage graph for all factors

The results from the analysis of variance shows that the factorial model is not significant, since p – value (0.1825) > 5% significant level. Hence, the factors (days, substrate and treatments) do not significantly have effect on response Y1 (Fig. 3). The result from the analysis shows that the factorial model is significant, since p – value (0.0001) < 5% significant level. The result further shows that factor A (days) was insignificant to the model but factor B (substrate) and C (treatments) significantly had effect on response Y2 with a p – value (0.033 and 0.0001) < 5% significant level respectively (Fig. 4 a and b). Likewise, the result from the analysis of variance shows that the factorial model is significant, since p – value (0.0018) < 5% significant level indicating that factor A (days) and factor B (substrate) were significant to the model with p – value (0.16 and 0.25) > 5% significant level respectively. But factor C (treatments) significantly had effect on response Y3 with a p - value (0.0005) < 5% significant level (Fig. 5a and b). Applying the various models in the optimization of the various responses, results shows that Day 2, substrate CSL and treatment REH are best combinations that will yield the optimal of Y1=0.249, Y2= 5.461 and Y3=33.704 respectively with a desirability of 0.619 (Fig. 6 a and b).

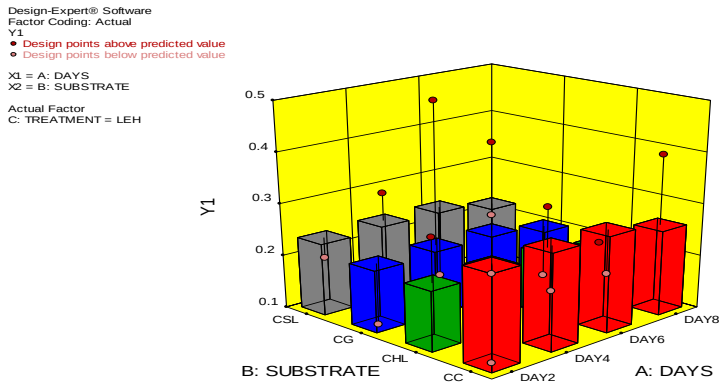

Fig. 4a: RSM Model for Response Y1 LEH

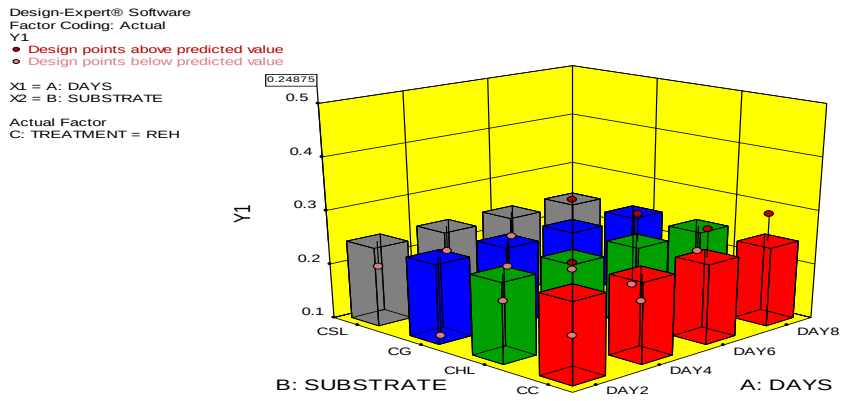

Fig. 4b: RSM Model for Response Y1 REH

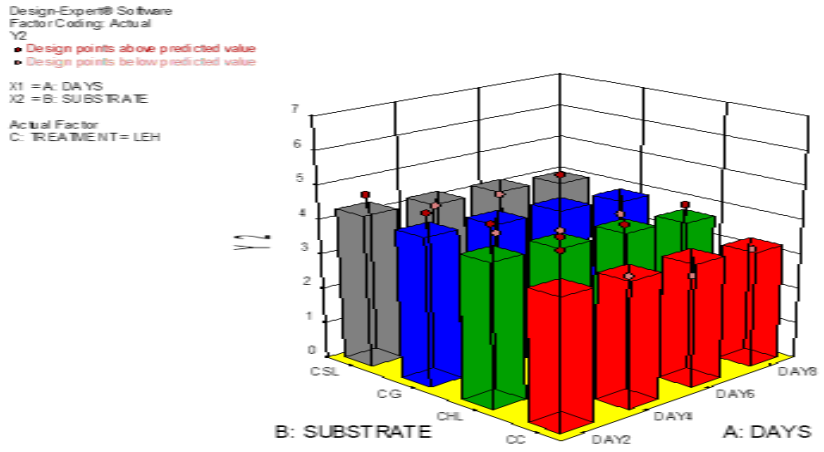

Fig. 5a: RSM Model for Response Y2 LEH

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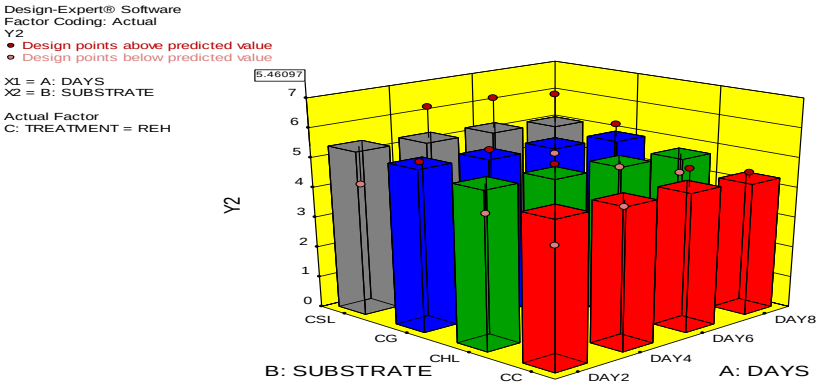

Fig. 5b: RSM Model for Response Y2

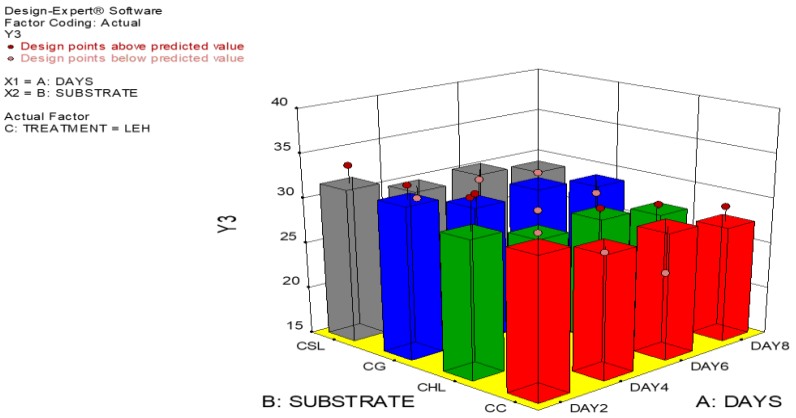

Fig. 6a: RSM Model for Response Y3 LEH

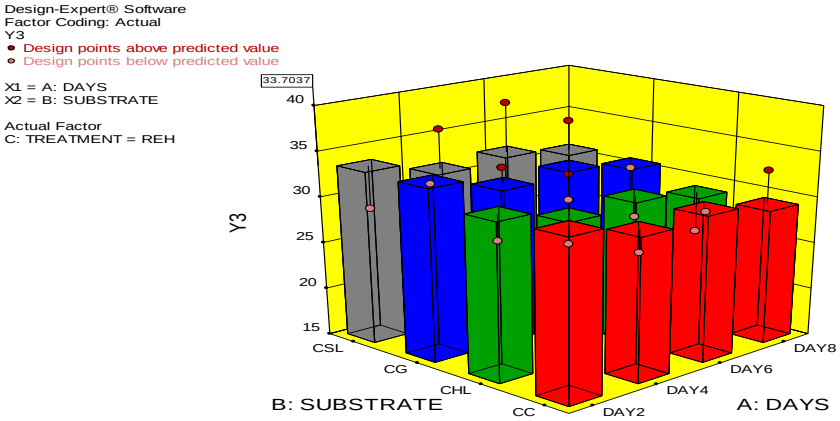

Fig. 6b: RSM Model for Response Y3 REH

Table 7 Response Surface Optimization

		Constraints				
Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:DAYS	is in range	DAY2	DAY8	1	1	3
B:SUBSTRATE	is in range	CC	CSL	1	1	3
C:TREATMENT	is in range	LEH	EAH	1	1	3
Y1	maximize	0.1	0.5	1	1	3
Y2	maximize	0.891	6.361	1	1	3
Y3	maximize	17.5	38.7	1	1	3

Table 8 Solutions for 80 combinations of categoric factor levels

Number	DAYS	SUBSTRATE	TREATMENT	Y1	Y2	Y3	Desirability	
1	DAY2	CSL	REH	0.249	5.461	33.704	0.619	Selected
2	DAY2	CG	REH	0.249	5.331	33.544	0.611	
3	DAY2	CSL	EAH	0.249	5.117	33.029	0.595	
4	DAY6	CSL	REH	0.249	5.282	32.314	0.593	
5	DAY2	CSL	RAH	0.249	5.212	32.360	0.590	
6	DAY4	CSL	REH	0.249	5.339	31.904	0.590	
7	DAY2	CG	EAH	0.249	4.987	32.869	0.587	
8	DAY6	CG	REH	0.249	5.152	32.154	0.585	
9	DAY2	CG	RAH	0.249	5.081	32.200	0.582	
10	DAY4	CG	REH	0.249	5.209	31.744	0.582	
11	DAY2	CHL	REH	0.249	5.145	31.859	0.581	
12	DAY8	CSL	REH	0.249	5.117	31.144	0.570	
13	DAY6	CSL	EAH	0.249	4.939	31.639	0.568	
14	DAY2	CC	REH	0.249	4.760	32.119	0.566	
15	DAY4	CSL	EAH	0.249	4.996	31.229	0.565	
16	DAY6	CSL	RAH	0.249	5.033	30.970	0.563	
17	DAY8	CG	REH	0.249	4.986	30.984	0.562	
18	DAY4	CSL	RAH	0.249	5.090	30.560	0.560	
19	DAY6	CG	EAH	0.249	4.808	31.479	0.560	
20	DAY4	CG	EAH	0.249	4.865	31.069	0.557	
21	DAY2	CHL	EAH	0.249	4.802	31.184	0.556	
22	DAY6	CG	RAH	0.249	4.902	30.810	0.555	
23	DAY6	CHL	REH	0.249	4.966	30.469	0.553	
24	DAY4	CG	RAH	0.249	4.960	30.400	0.552	
25	DAY2	CHL	RAH	0.249	4.896	30.515	0.551	
26	DAY4	CHL	REH	0.249	5.024	30.059	0.550	
27	DAY2	CSL	LEH	0.249	4.408	31.935	0.546	
28	DAY8	CSL	EAH	0.249	4.773	30.469	0.545	
29	DAY2	CC	EAH	0.249	4.416	31.444	0.540	
30	DAY8	CSL	RAH	0.249	4.867	29.800	0.539	
31	DAY6	CC	REH	0.249	4.581	30.729	0.539	
32	DAY2	CG	LEH	0.249	4.278	31.775	0.537	
33	DAY8	CG	EAH	0.249	4.643	30.309	0.536	

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34	DAY2	CC	RAH	0.249	4.510	30.775	0.536
35	DAY4	CC	REH	0.249	4.638	30.319	0.536
36	DAY8	CG	RAH	0.249	4.737	29.640	0.531
37	DAY8	CHL	REH	0.249	4.801	29.299	0.529
38	DAY6	CHL	EAH	0.249	4.623	29.794	0.528
39	DAY4	CHL	EAH	0.249	4.680	29.384	0.525
40	DAY6	CHL	RAH	0.249	4.717	29.125	0.522
41	DAY6	CSL	LEH	0.249	4.230	30.545	0.519
42	DAY4	CHL	RAH	0.249	4.774	28.715	0.519
43	DAY4	CSL	LEH	0.249	4.287	30.135	0.516
44	DAY8	CC	REH	0.249	4.416	29.559	0.515
45	DAY6	CC	EAH	0.249	4.237	30.054	0.513
46	DAY6	CG	LEH	0.249	4.099	30.385	0.510
47	DAY4	CC	EAH	0.249	4.295	29.644	0.510
48	DAY6	CC	RAH	0.249	4.332	29.385	0.508
49	DAY4	CG	LEH	0.249	4.157	29.975	0.507
50	DAY2	CHL	LEH	0.249	4.093	30.090	0.506
51	DAY4	CC	RAH	0.249	4.389	28.975	0.505
52	DAY8	CHL	EAH	0.249	4.457	28.624	0.503
53	DAY8	CHL	RAH	0.249	4.552	27.955	0.497
54	DAY8	CSL	LEH	0.249	4.064	29.375	0.494
55	DAY8	CC	EAH	0.249	4.072	28.884	0.488
56	DAY2	CC	LEH	0.249	3.707	30.350	0.488
57	DAY8	CG	LEH	0.249	3.934	29.215	0.485
58	DAY8	CC	RAH	0.249	4.166	28.215	0.483
59	DAY6	CHL	LEH	0.249	3.914	28.700	0.477
60	DAY4	CHL	LEH	0.249	3.971	28.290	0.474
61	DAY6	CC	LEH	0.249	3.528	28.960	0.459
62	DAY4	CC	LEH	0.249	3.586	28.550	0.457
63	DAY8	CHL	LEH	0.249	3.748	27.530	0.451
64	DAY2	CSL	LAH	0.249	3.460	28.135	0.444
65	DAY2	CG	LAH	0.249	3.330	27.975	0.434
66	DAY8	CC	LEH	0.249	3.363	27.790	0.434
67	DAY6	CSL	LAH	0.249	3.281	26.745	0.414
68	DAY4	CSL	LAH	0.249	3.339	26.335	0.411
69	DAY6	CG	LAH	0.249	3.151	26.585	0.404
70	DAY4	CG	LAH	0.249	3.208	26.175	0.401
71	DAY2	CHL	LAH	0.249	3.145	26.290	0.399
72	DAY8	CSL	LAH	0.249	3.116	25.575	0.386
73	DAY2	CC	LAH	0.249	2.759	26.550	0.378
74	DAY8	CG	LAH	0.249	2.986	25.415	0.376
75	DAY6	CHL	LAH	0.249	2.966	24.900	0.367
76	DAY4	CHL	LAH	0.249	3.023	24.490	0.363
77	DAY6	CC	LAH	0.249	2.580	25.160	0.346
78	DAY4	CC	LAH	0.249	2.638	24.750	0.344
79	DAY8	CHL	LAH	0.249	2.800	23.730	0.337
80	DAY8	CC	LAH	0.249	2.415	23.990	0.317

DISCUSSION

An agricultural waste with high content of lignocellulose is one of the most abundant renewable feedstocks that have been extensively utilized as a substrate for the production of various biochemicals. Lactic acid is one of the mostly utilized biochemical in the manufacturing sector because of its numerous potentials. The microalgae (*Chlorella vulgaris*) were used in comparison with various corn waste products (corn cob, corn germ and corn steep liquor (CSL)). The physico-chemical properties revealed the total carbohydrate for the four untreated substrates were as follows: *Chlorella* (17.4%); CSL (36.3%); Corn germ (69.93%) and Corn cob (149.2%). This result was synonymous with the reports of Albarico *et al.* (2017) who obtained a total carbohydrate of 20.41% from lipid extracted waste biomass of *Chlorella vulgaris.* High biomass and high carbohydrate content were necessary for production of lactic acid from C*hlorella vulgaris*. C*hlorella vulgaris* had high proton efficiency which enables it to convert starch into biochemical products (Kumar *et al.*, 2016). Lignocellulose feedstock had certain experimental issues which affects the metabolic process and hinders production. Therefore, pretreatment, hydrolysis and fermentation of the feedstock was eminent for effective production of metabolites (Agwa *et al*., 2018). The process of pretreatments makes the substrate easily accessible to the fermentation microorganisms and increases the digestibility of the feedstock without any alterations in their chemical and structural properties (Silverstein *et al*., 2007; Zhu *et al*., 2006). Pretreatments reduced the crystalline nature of the cellulose, removed lignin, and liberated the sugars (xylose, glucose, arabinose, galactose, mannose and other soluble oligomers) for fermentation, increasing their porosity and surface areas (Galbe and Zacchi, 2007; Sun and Cheng, 2005; Wyman *et al*., 2005; Sorensen *et al*., 2008, Shrestha *et al*., 2008; Hendriks and Zeeman, 2009). Oktaviani *et al*. (2019) stated that the process of pretreatment tends to release the organic acids from the hydrolysate. Hydrolysis of agricultural wastes was normally carried out using acid hydrolysis into fermentable sugars but when treated with strong acid under high temperature results in the production of by-products (Gupta *et al*., 2009; Keshwani and Cheng, 2009; Tasic *et al*., 2009). Microorganisms isolated from the environment are normally used to carry out enzymatic hydrolysis. The process of enzymatic hydrolysis is not rapid due to the release of certain substances by microorganisms that hindered the process (Shallom and Shoham, 2003; Chang, 2007). These setbacks from the hydrolysis spurred the combination of acid and enzyme hydrolysis in this research to eliminate any defaults that might occur in the process. Because of the presence of hydronium ions, acid hydrolysis increased the yield of reducing sugar that was available in the medium initiating cleavage of glycosidic bonds releasing more sugars in the process (Albarico *et al*., 2017). Combination of acid and enzyme hydrolysis made use of synergistic action and breakdown complexes into forms easily accessible for simultaneous saccharification and fermentation process which reduces end

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product inhibition and loss of fermentable sugars (Shapouri, 2007; Chang *et al*., 2017). To obtain high yield and improve hydrolysis, pretreatment was normally carried out especially on agricultural wastes such as maize (Sun and Cheng, 2002; Silverstein *et al*., 2007). Rodrigues and Bon (2011) opined that high yield of fermentable sugar was obtained from the microalgal biomass by enzymatic hydrolysis. The results from the various treatments according to reducing sugar content showed that acid hydrolysis gave the highest yield with CSL and corncob; enzyme hydrolysis was synonymous with *Chlorella vulgaris* and the combination of corncob followed by *Chlorella vulgaris* (Harun and Danquah, 2011a; Ho *et al*., 2013; Hernandez *et al*., 2015). CSL was a fermentation product from the corn mill industry which is an inexpensive nutrient source for microorganisms with very high content of amino acids, peptides and polypeptide with considerable amounts of B-complex vitamins. It was one of the dominating nutrients for the production of lactic acid and has been proven as a suitable substitute for expensive substrates (Lima *et al*., 2010). In the percentage hydrolysis treatments; acid hydrolysis CSL was the highest hydrolyzed, enzyme hydrolysis *Chlorella vulgaris* and the co-combination corn cob was the highest hydrolyzed. This investigation indicated that *Chlorella vulgaris* showed a significant increase in reducing sugar and high percentage hydrolyzed making them potential feedstock for lactic acid production (Rodrigues and Bon, 2011; Albarico *et al*., 2017). *Chlorella vulgaris* can grow anywhere, have small life span and possess high fermentable sugars with little or no cellulose (Nguyen *et al*., 2012; Albarico *et al*., 2017). This result was similar to the investigations of Idler *et al*. (2015) who reported that although acid pretreatment facilitated the hydrolysis of cellulosic material, it lead to the release of toxic residues. Palmarola and Adrados *et al*. (2005) stated that after acid treatment, some degradation by-products like furfural and 5-hydroxy furfural were produced. But the findings of both Rodrigues and Bon (2011) and Agwa *et al*. (2018) further revealed that enzyme hydrolysis treatment was more considerable than acid treatment for *chlorella vulgaris.*

The differences in % acidity in all the treatments was significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris* except RAH which was not significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris.* The difference in the yield of lactic acid and glucose produced from the different sources revealed that corn cob gave the highest yield 0.0055 ± 00001^{a} in lactic acid and *Chlorella vulgaris* produced the highest glucose yield (6.4 ± 0.2) and (4.4 ± 0.3) with *Lactobacillus* Enzyme Hydrolysis (LEH) and *Rhizopus* Enzyme Hydrolysis (REH) treatments, The differences across the different sources were significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris*. All the substrates used within the differences in the efficiency of the lactic acid produced within the treatments were significant i.e ($p < 0.05$) compared to treatment on *Chlorella vulgaris* except LEH with corncob and LAH with CSL which were not significant ($p > 0.05$) compared to treatment on *Chlorella vulgaris*. Similar results was obtained by Guo *et al.* (2010) who determined efficient production of lactic acid from sucrose and

corncob hydrolysate by a newly isolated *Rhizopus oryzae* GY18. Corncob hydrolysates obtained by dilute acid hydrolysis and enzymatic hydrolysis of the cellulose- enriched residue were used for lactic acid production by *R. oryzae* GY18. A yield of 355 g lactic acid per kg corncobs was obtained after 72 h incubation. Lactic acid Production rely on the fermentation condition, inoculum size, metabolic route and substrate involved with little or no residue. The best simultaneous saccharification and fermentation (SSF) process conditions were recorded at a temperature of 38^0C for 48 h and pH 6.5. The temperature was in line with Abdel-Rahman *et al.* (2011) who stated that temperature range of between 30 - 43^0C was suitable for lactic acid production. Silveira (2009) opined that pH of 6.5 is ideal for production of lactic acid. The final concentration of lactic acid yield for corn cob was 5.2 g/l and *Chlorella* was 3.4 g/l. In this study, initial inoculum size played an important role in *Chlorella vulgaris hydrolysis* where the maximum reducing sugar yield was registered at 4% inoculum size, above this level, the reducing sugar yield decreased. This could also be explained that inoculum size higher than the optimum value may produce a high amount of biomass which could rapidly deplete the nutrients necessary for growth and product synthesis. On the other hand, lower inoculum size may give insufficient biomass and allow the growth of undesirable organisms in the hydrolysis production medium and introduce some toxic substances into the fermentation medium thereby limiting the production. Kunasundari *et al.* (2017) studied the effect of different parameters on the production of lactic acid using *Geobacillus stearothermophilus* andtheir investigations revealed that 5.65 ± 0.07 g/L lactic acid was produced at pH 5.5, an agitation speed of 200 rpm with an operating condition of 60°C for 48 h.

The results from the analysis of variance showed that the factorial model was not significant, since P– value (0.1825) > 5% significant level. Hence, the factors (days, substrate and treatments) do not significantly have effect on response Y1. The result from the analysis shows that the factorial model was significant, since p – value (0.0001) < 5% significant level. The result further shows that factor A (days) was insignificant to the model but factor B (substrate) and C (treatments) significantly had effect on response Y2 with a p – value (0.033 and 0.0001) < 5% significant level respectively. Likewise, the result from the analysis of variance shows that the factorial model is significant, since p – value (0.0018) < 5% significant level indicating that factor A (days) and factor B (substrate) were significant to the model with p – value (0.16 and 0.25) > 5% significant level respectively. But factor C (treatments) significantly had effect on response Y3 with a p- value (0.0005) < 5% significant level. Applying the various models in the optimization of the various responses, results shows that Day 2, substrate CSL and treatment REH are best combinations that will yield the optimal of Y1=0.249, Y2= 5.461 and Y3=33.704 respectively with a desirability of 0.619. The result from the Response Surface methodology in table 7 indicated that response Y1 is was not significance to the factors, response Y2 show that factor A (days) was insignificant to the model but factor B (substrate) and C

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(treatments) were factors that, significantly has effect on the response with a p-value (0.033 and 0.0001) < 5% significant level respectively but response Y3 at p – value (0.0018) < 5% significant level significantly had effect on all the factors A and B with a p – value (0.16 and 0.25) > 5% significant level while factor C had effect on response Y3 with a p- value (0.0001) < 5% significant level respectively. Applying the various models in the optimization of the various responses, results shows that Day 2, substrate CSL and treatment REH are best combinations that will yield the optimal of Y1 = 0.249, Y2 = 5.461 and Y3 = 33.704 respectively with a desirability of 0.619, This implies that during SSF fermentation, CSL under optimized conditions of temperature, pH, and acidity, yield the best concentration of lactic acid. Similar result was recorded by Lima *et al.* (2010), who carried out L (+) lactic acid production by *Lactobacillus* sp. at an optimum temperature of 39.6 °C and pH 5.9. Saelee and Sriroth (2014) used oil palm trunk juice as substrate for the optimization of nutrient during the fermentation of lactic acid. Their result revealed an optimum production of lactic acid at 40^0C, pH 7.0 at 48 h cultivation time. Patel and Patel (2016) observed similar result with cheese whey medium, about 50% of lactic acid was produced using *Lactobacillus casei* with CSL and other mineral salts were identified to be significant at 37^0C for 48 h. Mufida *et al*. (2017) obtained similar result with banana peel as substrate at 37^0C, pH 6.3 for 48 h. But, Sridevi *et al*. (2015) report was in contrast with our investigation, maximum production of lactic acid using *Lactobacillus plantarum* at 36.39^0C for 96 h and pH 6.43. *Chlorella vulgaris* was also used in comparison with Corn cob, Corn Steep Liquor and Corn germ in terms of their lactic acid efficiency, in the *Rhizopus*Acid Hydrolysis (RAH) treatment, Corn Cob lactic acid efficiency was 0.0055±0001, CSL was 0.0045±0.0001, Corn Germ was 0.0062±0.0001 and *Chlorella* was 0.0034±0.0002 in RAH. This implies that these substrates were all statistically significant ($p < 0.05$) compared to treatment on *Chlorella vulgaris* and significant differences with ANOVA result were showed between the groups. This study provides an encouraging means of producing lactic acid which is one of the top potential building block chemicals from lignocellulosic resource such as the low-cost C*hlorella vulgaris*. Therefore, the search for cheap raw materials is an objective to reduce the production costs and use of agricultural waste by-products sterns the trend in sustainable resource management which is a key tool in biotechnology.

CONCLUSION

This study aimed to evaluate the enzymatic and/or acidic hydrolysis of *Chlorella vulgaris* cell wall intercellular starch as a source of fermentable sugar for lactic acid production. This study also provides an encouraging means of producing lactic acid which is one of the top potential building block chemicals from lignocellulosic resource using a low-cost carbon source such as C*hlorella vulgaris* The use of plant waste product for maximum sustainability has led to the search for bio based raw material such as *Chlorella vulgaris* which can make a tremendous contribution on the ecological and economical production of different organic acids like lactic acid.

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