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Optimization Study on the Microbial Hydrolysis and Fermentation of *Citrullus lanatus* (L) (Watermelon) Fruit Peels in Bioethanol Production

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Abstract: The aim of this work was to optimize the microbial hydrolysis of watermelon fruit peels for bioethanol production**.** The watermelon fruit peels as an alternative and cost effective feedstock for bioethanol production was also studied. Proximate analysis showed that the dried peels contained 31% Extractives, 16% Lignin, 26% Cellulose and 24% Hemicellulose. The fruit peels were pretreated at 121^{o}C for 1 hour prior to hydrolysis and fermentation. Hydrolysis was carried out using *Aspergillus niger* for 5 days and Baker's yeast *Saccharomyces cerevisiae* was used for the fermentation. The optimization was done using a designed experiment MINI TAB. Analysis of variance ANOVA was used to test for the significance of Designed experiment. The results of the analysis showed that temperature (28^{o}C), time (5 days), and pH (5.0) significantly *($p< 0.05$)* affected the yield of reducing sugar in the hydrolysis of watermelon fruit peels. The result equally showed that temperature, Days, and pH were significant *($p< 0.05$)* factors for hydrolysis. The highest reducing sugar yield was 1.93 ± 0.01 g/L at 28˚C, pH 5.0 and 5 days of hydrolysis. The concentration of the bioethanol produced was determined using the potassium dichromate method. The result therefore revealed the highest bioethanol yield to be 0.16 ± 0.017 mg/L. This Study revealed the optimum conditions for hydrolysis of watermelon peels using *Aspergillus niger* for high bioethanol production and yield are 28˚C, 5 days, and pH of 5.0.
Keywords: Bioethanol, Hydrolysis, Fermentation, Optimization, Percentage Concentration.

INTRODUCTION

Watermelon (*Citrullus lanatus*) of family Cucurbitaceae is a flowering plant. It is mainly originated from southern Africa. Its fruit is called pepo by botanist. It is also known as watermelon. Pepo is a berry like fruit. The exocarp is the thick rind, the outer surface of the fruit and mesocarp and endocarp are the freshly coloured center of the fruit [1].

Watermelon contains a significant amount of citrulline which is measured in the blood plasma. This could be mistaken for citrullinaemia or other urea cycle disorder [2]. The juice or pulp from water melons is used for human consumption, while the rind or peels and seeds are major solid wastes [1]. The peels are usually discarded and applied to feeds or fertilizers, due to their unpleasant taste but they are also and sometimes used as vegetables.

In recent years the world has witnessed a tremendous increase in the search and quest for an alternative energy source to replace the convectional fossil fuels. This was necessitated as a result of the finite nature of crude oil and other fossil fuels [3]. In Nigeria and many developing countries, there is a growing interest in the conversion of the huge biomass of organic wastes generated by the food processing sector and other human endeavors into useful products such as ethanol. A number of studies have been carried out in an attempt to optimize the yield of bioethanol from watermelon fruit peels using different organisms including *Saccharomyces cerevisiae* and *Aspergillus niger* [4].

Bioethanol is an ethanol that is produced from biological materials (biomass). It is water-free alcohol produced from the fermentation of sugar or converted starch [5]. Lignocelluloses are non-edible portion of plants that are more suitable for Bioethanol production. These lignocellulosic plants consist of cellulose, hemicellulose and lignin with sugar monomers that are tightly bound together [6].

MATERIALS AND METHODS

Collection of Samples

The fruit peels were collected in new sterile polythene bags throughout the research from Sokoto central market, Sokoto State. This research work was carried out in the Research laboratory of Department of Microbiology, Usmanu Danfodiyo University, Sokoto.

Determination of Structural Composition of Watermelon Fruit Peels (Extractives, Lignin, Holocellulose, Cellulose and Hemicellulose)

Determination of Extractives

Chemically, extractives in watermelon fruit peels consist of fats, fatty acids, fatty alcohols, phenols, flavonoid, protein, steroids e.t.c. Two (2g) of the watermelon fruit peels powder were extracted with alcohol Acetone (T 204 om-97). The extractives were removed from a 2g watermelon peels by soxhlet apparatus using 250ml a mixture of ethanol-acetone (1:2 v/v) for a total period of 3 hours (flushed was four times per hour). After 3 hours, the excess solvent was removed from the sample and residue was washed 5 times with ethanol and then air-dried to remove the remaining trace of ethanol acetone. The extracted samples were placed in a vacuum oven at 103 ± 2 ^{o}C for 3hours. The following equation was used to calculate the % extractives on a dry weight basis [7].

$$\% \text{ Extractives} = \{(W1-W2/ W1) \text{ X } 100\}$$

Where
W_1 = Weight of feedstock before extraction
W_2 = Weight of feedstock after extraction

Determination of Klason Lignin

The percentage of acid – insoluble lignin, which is defined as the residue, was determined according to TAPPI procedure (T224 om-88). Firstly, 2 g of the watermelon peels residue was put in to 30ml beaker, then, 72% sulfuric acid (15ml) was carefully added for 2 hours. The mixture was stirred frequently during this time. After 2 hours, the sulphuric acid was diluted with distilled water (560ml) to obtain sulphuric acid of 3 % concentration. The solution was then boiled for 4 hour, maintaining constant volume (575mL) by frequent addition of hot distilled water. The solution was then filtered through a medium porosity filtering crucible. The filtered residue (acid insoluble lignin) was washed free of acid with hot distilled water. The crucibles and the contents were dried at 103 ± 2 ^{o}C for 3 hours and weighed [7]. The percentage of acid insoluble lignin in feedstock was calculated using the following equation:

$$\text{Lignin Klason} = \{(W2-W1/ W1) \text{ X } 100\}$$

Where
W_1 = Initial weight of sample
W_2 = Weight of crucible and acid- insoluble lignin after drying in oven
W_3 = Weight of crucible

Determination of Holocellulose

The holocellulose content, which is the combination of hemicellulose and cellulose, was determined in order to find the total amount of cellulose and hemicellulose in watermelon fruit peels. The holocellulose content was determined according to DIN 2403. A flask containing a mixture of 80 mL distilled water, 1 mL acetic acid (98%), 3 g sodium chlorite, and 2 g of feedstock was heated in a water bath at 70^{o}C for one hour. The mixture was stirred every five minutes during this time. Acetic acid (1mL) and sodium chlorite (3 g) were added each hour for the next 3 hours. After 4 hours, the samples were cooled and the holocellulose was filtered. The holocellulose was washed with methanol 3 times then air- dried to remove the remaining trace of methanol and then placed in a vacuum oven at 103 ± 2 ^{o}C for 3 hours [7]. The percentage of holocellulose in feedstock was calculated based on dry weight using the following equation:

$$\% \text{ Holocellulose} = \left[\frac{(W2 - W)}{W1} \text{ X } 100 \right]$$

Where: W =Dry weight of crucible
W_1 = Initial weight of sample
W_2 = Sample weight plus crucible after drying in oven

Determination of α-Cellulose and Hemicellulose

α-Cellulose is the pure cellulose content of the materials which was extracted from holocellulose using alkali solution. The α-Cellulose content of watermelon fruit peels was determined as the residue insoluble in the 17.5 NAOH solutions according to TAPPI 203 om-93 method. Aqueous NAOH solution 17.5 % (25mL) was added to a flask containing sample of holocellulose (1 g) and the mixture was stirred at 20 ^{0}C for 40 minutes, then 25 ml of distilled water was added in to the mixture. After 5 minutes, the residue was filtered with rubber filter, and then 40 ml of 10 % acetic acid aqueous solution was added to the residue, filtered and washed with 1 L of boiling water. The residue (α-Cellulose), was filtered, dried at 103 ± 2 ^{o}C for 3 hours in a vacuum oven and weighed [7].

The percentage of α-Cellulose in the substrate was calculated using the following equation:

$$\% \alpha\text{-Cellulose} = \{(W2/W1 \times \% \text{ holocellulose}/100\%) \times 100\}$$

Where
W_1 = Initial weight of holocellulose
W_2 = Weight of residue (α-Cellulose)

The percentage hemicellulose was calculated by subtracting the percent α-Cellulose from % holocellulose determined [7].

Experimental Design of the Research

In this study, experimental design techniques were used to determine the effects of the pH, hydrolysis time and temperature on the efficiency of reducing sugar yield. A total of 5 experiments were carried out for optimization purpose where the effect of each factor was analyzed by using lower and higher values from optimized conditions.

Table-1: Experimental Design of Microbial Hydrolysis for Reducing Sugar Determination

Sample	Factor 1	Factor 2
	A: pH	B: Temp. (^{o}C)
A	5.0	28
B	5.5	30
C	6.0	32
D	6.5	35
E	7.0	37

Preparation of the Sample

About 5kg of watermelon fruit peels were washed under tap water and chopped into small pieces of about 3-5 cm length using a stainless steel knife. The peels were then dried in hot air oven for 3 days at 60^{o}C to completely remove the water content. Next, the sample was taken out of the drier once they were dry enough to be grind. The cut pieces were milled using a grinder. The maximum particle size was 2 mm. The ground sample was kept at low temperature until the next step of experiment [8].

Isolation and Characterization of Microorganisms

Aspergillus niger was identified by Macro culture method according to the method described by [9]. Floor dust was collected using sterilized spatula and transferred to a sterilized sample container. The soil was serially diluted; a sample suspension was prepared by adding 1.0g of sample to 10ml of distilled water and mixed well for 10 minutes. The suspension was diluted serially 10^{-1}, 10^{-2} and 10^{-3}. 1ml (from the third dilution factor) was measured using a syringe and inoculated into a Potato Dextrose Agar (PDA), a glass spreader. The initial white color of the colonies that later turns black at the top with pale yellow color at the bottom confirm the organism to be *Aspergillus niger*.

Baker's Yeast (*Saccharomyces cerevisiae)* was also reactivated and used in this studies according to the methods described by Alkasrawi [10]. One (1) Gram of Baker's yeast was put in a flask containing a solution of warm distilled water, Glucose broth media and yeast extract. The solution was then subjected to incubation for 24 hours. The reactivated yeast was then inoculated in to Potato Dextrose Agar (PDA and subjected to incubation at 28^{0}C for 5 days. After 5 days, the growth observed was used to form a pure culture. This was done to avoid inhibition of some important inoculums in the yeast.

Pretreatment of the Fruit Peels Powder

Pre-treatment of the watermelon fruit peels powder was carried out by steam expulsion method. In this method, 10 g each of grounded fruit peels were put in to 15 conical flasks of size 250 ml and diluted each with 100 ml of distilled water. The conical flasks were then cover with aluminum foil paper and capped with rubber plugs and subjected to

autoclave at 121^{0}C for 1 hour. After autoclaving, the samples were allowed to cool. The insoluble biomass was made to hydrolyze in the next step which is hydrolysis [8].

Microbial Hydrolysis of the Sample with *Aspergillus niger*

Microbial hydrolysis was carried out according to the method describe by [9]. In this method, the treated watermelon peels were inoculated with 0.5 ml suspension of 96 hours culture of *Aspergillus niger* sub cultured on Saboraud Dextrose Agar plate. Hydrolysis was carried out at room temperature for five days. Samples were taken daily for reducing sugar determination. The samples were then filtered using filter paper No. 1 and the filtrates were used for fermentation.

Determination of Reducing Sugar

The method of Abdul *et al.* [11] was used. One (1) ml of each samples filtrate was taken into test tubes and 1ml of DNS reagent was added in the each of the test tubes. A blank was prepared containing 1ml distilled water and 1ml DNS reagent. The test tubes were boiled in a water bath for 10 minutes and allowed to cool to developed red brown color. The color developed fully after 20 minutes. One (I) ml of 40% sodium potassium tartarate was added to stabilize the colour and the absorbance was read at 540nm wave length using ultraviolet (UV- VIS) spectrophotometer. A standard curve of glucose determination was prepared to calculate the percentage of the reducing sugar.

pH Adjustment

Before addition of yeast to the above prepared samples, the pH of the samples was adjusted to 5.0, otherwise the yeast will be denatured in hyper acidic or basic state. A highly concentrated NAOH solution and concentrated HCL was prepared to adjust the pH and was regularly checked using a digital pH meter [12].

Sterilization

All the hydrolysate samples were sterilized at 121^{o}c for 15 minutes prior to fermentation [13].

Fermentation of the Sample

The hydrolysates were aseptically inoculated with 1ml suspension (6.0×102cfu/ml) of the reactivated yeast. The flasks were then covered with cotton wool and wrapped in with aluminum foil paper. Fermentation was carried out at room temperature for 7 days [14].

Distillation of the Sample

Soxhlet extractor was used for bioethanol extraction at 78^{o}C [15].

Determination of Percentage Concentration of the Bioethanol Produced

Percentage concentration was carried out using acid potassium dichromate reagent according to the methods of [16]. One milliliter (1ml) of standard ethanol was diluted with 99 ml of distilled water to give a concentration of 1 %. Then 0, 2, 4, 6, and 8 ml each of the 1% ethanol was diluted to 10 ml of distilled water to produced 0, 0.2, 0.4, 0.6, and 0.8 ml of the ethanol. To each of the varying ethanol concentrations, 1mls of acid potassium dichromate was added and allowed to stand for an hour for color development. The absorbance of each concentration was measured at 580 nm using UV-VIS spectrophotometer and the reading was used to develop standard ethanol curve [17].

Determination of Quantity (Volume) of Bioethanol Produced

The methods of Amenaghawon *et al.* [18] was used to determine the volume of Bioethanol produced. To determine the quantity of the bioethanol produced, the distillate collected over a soxhlet apparatus heated at 78^{o}C was measure using a measuring cylinder, and express as the quantity of bioethanol produced in g/l by multiplying the volume of the distillate collected at 78^{o}C by the density of bioethanol (0.8033gl/ml).

Statistical Data Analysis

The data obtained was statistically analyzed using Analysis of Variance (ANOVA). The results were expressed as Mean ± Standard deviation. Duncan multiple range tests were used in mean comparison and $p< 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Watermelon fruit peels composed of Extractives, Lignin, Cellulose and Hemicellulose. The extractives which mainly consist of fats, fatty acid and fatty alcohol constitute the highest percentage with 31%, and then holocellulose which is the total amount of cellulose and hemicellulose constitutes 50% with 26% cellulose and 24% hemicellulose respectively. Lignin was found to be least with 16%.

Table-2: Structural Composition of Watermelon Fruit Peels (Extractives, Lignin, Cellulose and Hemicellulose)

PARAMETERS	COMPOSITION
Extractives Content (%)	31
Lignin Content (%)	16
Holocellulose Content (%)	50
Cellulose Content (%)	26
Hemicellulose Content (%)	24

Morphological characterization *of Aspergillus niger* isolated from soil. The organism showed a black mycelium on the agar medium, it had septate hyphae, long and smooth conidiospores, and long unbranched sporangiospores with a large and round head.

Table-3: Morphological Characterization *of Aspergillus niger* Isolated from Soil

Isolate	Colony characterization	Cell shape	Organism
1.	Black, dotted surface as Conidia	Filamentous with septed hyphae	*Aspergillus Niger*

Concentration of Reducing Sugar Produced from Microbial Hydrolysis of Treated Watermelon peels from day One (1) to Five (5). The highest yield of reducing sugar of 1.93 g/l was obtained from microbial hydrolysis after 5 days of hydrolysis at 28^{o}C and pH of 5. But when the pH concentration was increase to 7.0 at 37^{o}C for first day of hydrolysis, a lowest reducing sugar yield of 0.51 g/l was obtained. This means that pH and time have effects on the microbial hydrolysis. However, the reducing sugar concentration of the microbial hydrolysis increases gradually from day One (1) to day five (5). This means that hydrolysis for 5 days is the best for microbial hydrolysis. Statistical Analysis of variance (ANOVA) of the experimental results of the microbial hydrolysis shows that there is significant difference at ($p< 0.05$) in the percentage mean concentration of reducing sugar across the days of hydrolysis and effects of pH.

Table-4: Concentration of Reducing Sugar Produced from Microbial Hydrolysis of Treated Watermelon Peels from Day One (1) to Five (5)

Concentration of reducing sugar produced (mg/l)					
DAYS	A	B	C	D	E
1	$0.62 \pm 0.01^{a(x)}$	$0.45 \pm 0.01^{a(x)}$	$0.61 \pm 0.01^{a(xy)}$	$0.74 \pm 0.01^{a(y)}$	$0.51 \pm 0.01^{a(x)}$
2	$1.67 \pm 0.01^{cd(x)}$	$1.40 \pm 0.01^{c(x)}$	$1.25 \pm 0.02^{b(x)}$	$0.92 \pm 0.02^{b(x)}$	$0.60 \pm 0.01^{a(xy)}$
3	$1.88 \pm 0.01^{cd(z)}$	$1.56 \pm 0.01^{c(x)}$	$1.33 \pm 0.02^{bc(y)}$	$1.18 \pm 0.01^{b(xy)}$	$1.26 \pm 0.04^{b(x)}$
4	$1.89 \pm 0.01^{cd(z)}$	$1.68 \pm 0.01^{cd(x)}$	$1.40 \pm 0.02^{c(x)}$	$1.26 \pm 0.01^{b(x)}$	$1.31 \pm 0.01^{bc(x)}$
5	$1.93 \pm 0.01^{d(z)}$	$1.73 \pm 0.01^{d(x)}$	$1.45 \pm 0.01^{cd(x)}$	$1.31 \pm 0.01^{bc(x)}$	$1.36 \pm 0.03^{c(x)}$

a,b,c,d,e means within a column with different superscripts are significantly different at ($p<0.05$)
x,y,z within a row with different superscripts are significantly different at ($p<0.05$)

Percentage Concentration of Bioethanol produced after hydrolyzed by microbial hydrolysis. The results revealed that the highest bioethanol yield of (0.16 %) at 28^{o}C and pH of 5 after 24 hours of fermentation. However, (0.06 %) was the lowest yield microbial hydrolysis produced at 37^{o}C and pH of 7.

Volume of Bioethanol produced after Hydrolyzed by Microbial hydrolysis. The results revealed that 57 ml was the highest volume produced by sample B. However, the lowest volume of 39 ml was produced by sample A.

Table-5: Percentage Concentration and Volume of Bioethanol Produced after Hydrolyzed by Acidic and Microbial Hydrolysis

Sample (Conc. of Bioethanol produced (mg/l))		Sample (Volume Bioethanol produced (ml))	
A	0.16 ± 0.017	A	39.00 ± 2.5
B	0.14 ± 0.012	B	57.00 ± 2.6
C	0.09 ± 0.058	C	43.00 ± 1.0
D	0.08 ± 0.058	D	40.00 ± 3.2
E	0.06 ± 0.058	E	46.65 ± 2.0

The Structural Composition of watermelon fruit peels (lignin, Cellulose and Hemicellulose) revealed that extractives has the highest percentage of 31% followed by holocellulose which is combination of cellulose and hemicelluloses 50%, which cellulose is having 26% and hemicellolose 24%, respectively. Lignin is the least with 16%. However, Soliman [19] in Egypt reported that whole Gurma watermelon fruits constitute 50% hemicellulose, and 30% cellulose and 8% lignin.

The structural composition of watermelon fruit peels is made up of cellulose, hemicellulose, and lignin. Lignin is the integral part of the secondary cell wall of plants. They are the second most abundant organic polymers that lack a defined structure because of their heterogeneity and help to strengthen xylems in plants. The branches in lignin make it difficult to measure its degree of polymerization. There are three monomers in lignin namely: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [20].

The main feature that differentiates hemicellulose from cellulose is the branches and short lateral chains consisting of different sugars in hemicellulose. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids. The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by - (1, 4) -glycosidic bonds and occasionally - (1, 3) – glycosidic bonds. Also, hemicelluloses can have some degree of acetylation, for example, in heteroxylan. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolysable [21].

Morphological identification of *Aspergillus niger* isolated from soil revealed that *Aspergillus niger* showed a black mycelium on the agar medium, it had septate hyphae, long and smooth conidiospores, and long unbranched sporangiospores with a large and round head. The initial white color of the colonies that later turns black at the top with pale yellow color at the bottom confirm the organism to be *Aspergillus niger*. The results also conform to the work of Oyeleke and Jibrin [16] that reported the isolation of *Aspergillus niger* from soil and stated that the organism can degrade biomass and utilize as their growth factor.

An experimental design of microbial hydrolysis for reducing sugar determination was also studied. The optimum pH used ranged from 5 to 7 whereas temperature ranged from 28^{o}C to 37^{o}C. However, optimum pH and temperature increased the yield of reducing sugar. The best optimum pH and temperature for *Aspergillus niger* was 28 ^{o}C and pH 5 as reported by Adeleke *et al.* [22].

The results of percentage mean concentration of reducing sugar produced by *Aspergillus niger* shows that the highest reducing sugar yield of 1.93 g/l was obtained at 28^{o}C, pH 5 and after five days of hydrolysis of treated watermelon peels with *Aspergillus niger*. This might be because the hydrolyzing microorganisms use the substrate as their source of carbon and at the same time produced enzymes that hydrolyzed the peels into glucose during the process of feeding. The 1.93 g/l reducing sugar obtained is almost in agreement with the 1.82 g/l reducing sugar obtain by [23] and lower than 2.78 g/l from alkali treatment reported by Brooks [24]. The researchers stated that hydrolyzing the substrate with *Aspergillus niger* at 28^{o}C and pH 5 produce more reducing sugar. These studies also reveal that pH and time have effects on the microbial hydrolysis.

However, the reducing sugar concentration of the microbial hydrolysis increases gradually from day One (1) to day five (5). This means that hydrolysis for 5 days is highly recommended for microbial hydrolysis as reported by Oyeleke *et al.* [25]. Statistical analysis using One- way Analysis of variance (ANOVA) reveals that there was significant difference at $p< 0.05$ in the percentage mean concentration of reducing sugar across the days of hydrolysis and effects of pH. But with the effects of temperature only or combination of both temperature and days or pH and days, there was significant difference at $p< 0.05$.

The percentage concentration of bioethanol produced after hydrolysates were fermented using baker's yeast *Saccharomyces cerevisiae* was studied. The highest concentration of Bioethanol obtained was 0.16 % after 24 hours of fermentation. These results revealed a higher production by microbial hydrolysis which may be because *Aspergillus niger* posseses pyruvate decarboxylase as reported by Oyeleke *et al.* [25] which tends to facilitate the breaking down of the watermelon fruit peels. All these might be responsible for high bioethanol produced from the hydrolysates.

The quantified amount of Bioethanol obtained from microbial hydrolysis at 28^{o}C, pH 5 and 5 days of hydrolysis was found to be significantly higher. This was done with reference to the research reported by Zhao *et al.* [26] who stated that further hydrolysis of substrate will produce more bioethanol.

These studies revealed that Bioethanol can be produced from watermelon fruit peels with maximum yield obtained using *Aspergillus niger* for hydrolysis at 28^{o}C, pH 5 and 5 days of hydrolysis.

The results of volume of Bioethanol produced by Microbial hydrolysis revealed that Bioethanol produced by sample B has the highest volume of 57 ml where sample A has the lowest volume of 39 ml bioethanol. This might be as a result of optimum temperature and pH (28^{o}C and pH 5) used for sample B during hydrolysis. Akin-Osanaiye *et al.* [27] used 20kg of the whole watermelon fruits and produced 10 ml concentrated Bioethanol.

CONCLUSION

At the end of this study, it was found that Pretreatment of watermelon fruit peels by steam expulsion method enables the accessibility and ease of hydrolysis of the peels. This study had shown that hydrolysis with *Aspergillus niger* at 28^{o}C, pH 5, and 5 days of hydrolysis produced the highest sugars of (1.93 g/L) from sample A than rest of the samples. This study concluded that bioethanol produced by sample A at 28^{o}C and pH 5 is higher with an average yield of (0.16 %) than sample E at 37^{o}C and pH 7 with an average of (0.06%).

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