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Enzymatic degradation and fermentation of Corn Bran for Bioethanol production by *Pseudomonas aeruginosa* AU4738 and *Saccharomyces cerevisiae* using Co-culture technique

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Abstract

Ethanol is one of the bioenergy sources with low environmental and high efficiency impact. The aim of this study was to screen for the bacterial isolate capable of degrading starch, investigate the enzymatic hydrolysis and fermentation of corn bran through submerged fermentation using co-culture technique for bioethanol production. The isolate was identified using 16S rRNA sequence technique as *Pseudomonas aeruginosa* AU4738. Corn bran was used as substrates with and without garlic powder (*Allium sativum* L.) as activator and subsequently optimized for production of bioethanol. Reducing sugar from the hydrolysate and ethanol concentration of the distillate were analyzed using spectrophotometry and gas chromatography mass spectrometry techniques respectively. There was an increase in glucose concentration (23.8% and 17.8%) in the culture medium with and without activator at 48 h respectively but steadily decreased from 72 h to 168 h. Maximum ethanol concentration obtained in substrate culture with activator was 35% higher compared with that without activator at 120 h fermentation time. Thus a cheap, renewable and readily available agricultural waste has been effectively utilized as substrate for bioethanol production and incorporation of activator also had significant effect on the viability of fermenting organisms thus subjugating the intolerance of alcohol concentration.

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1. Introduction

Sources of biofuel have become more salient as economical substitute to declining and much exorbitant fossil fuels [1]. Bioethanol is the only liquid fuels that do not contribute to the greenhouse gas effect [2]. Due to fluctuating prices and dwindling oil reserves at global market, fermentation processes have attracted great demand in comparison to conventional production of bioethanol [3]. Thus high cost has resulted to energy catastrophe in african countries that are oil contingent. It has been delineated that in many part of the world biofuel remains censorious energy development target if petroleum prices be a cut above US $ 60

per barrel [4]. Brazil is the world prime biofuel producer and Nigeria has joined the confederation of biofuel users from sugarcane and cassava sources [5]. Cassava, yam, and sweet potato are main starches that serve as staple foods for people throughout the world's humid and hot regions [6]. However, potatoes are high starchy value crops which do not require complex pretreatment. Waste byproducts from sweet potato cultivation could be utilized for bioethanol production [7, 8]. The use of these staple starchy crops poses threat to food surveillance in the face of growing ethanol fuel demand. Imaginably, the divergence of food resource to bio-fuel production may to a large extent cause food crises worldwide [9]. As a result it becomes exigent that spotlight be turned to the use of non-food starchy piece for bioethanol production.

Saccharomyces cerevisiae (baker's yeast) is one of the most significant microbes in the fermentation of sugar to bioethanol due to its high tolerance to ethanol concentration, high fermentation rate, high ethanol yield, high selectivity, good tolerance to substrate concentrations, low accumulation of by-products, and lower pH value [10, 11]. Several researchers, Abouzied and Reddy, [12], Oyeleke *et al*., [13] Duhan *et al*., [1] George *et al.,* [14] combined *Saccharomyces cerevisiae* with other group of saccharifying fungi such as *Aspergillus* sp., *Kluyveromyces* sp., *Zymomonas*

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mobilis, Gloeophyllum sepiarium, Trichoderma sp. and *Pleurotus ostreatus* to improve bioethanol production. This research was aimed at assessing the appropriateness of using the corn bran through two-step processes: saccharification and fermentation using co-culture method for bioethanol production by *Pseudomonas aeruginosa* AU4738 and *Saccharomyces cerevisiae*. Evaluation of the substrate pre-treatment processes for transformation of starch into fermentable sugar and successive use of the spice from garlic as activator was conducted to alleviate product inhibition potency of microorganism in the production of ethanol.

2. Materials and Methods

2.1. Materials

The bacterial isolate was obtained from Culture Collection Centre, Department of Biological Sciences, McPherson University, Seriki Sotayo, Nigeria and was characterized using 16S rDNA sequencing technique while the baker's yeast was purchased from Apongbo Market, Lagos Island Local Government, Lagos. Corn bran, soya beans and garlic were purchased from Awolowo Market, Sagamu, Remo North Local Government, Ogun State, Nigeria. Nutrient agar (NA) (Accumix, Diagnostic, Ltd., India), Yeast Extract Agar (YEA) (LOBA Chemie Pvt. Ltd. India). Iodine, 3,5-Dinitosalicyclic acid (DNS) (Baker Inc., USA), sodium potassium tartrate (Klincent Laboratories, Mumbai, India) and Tetraoxosulphate (VI) acid were of analar grade.

2.2 Methods

Screening of starch hydrolyzing bacteria

Pure culture of the bacterial isolate was inoculated into nutrient agar containing 1 g soluble starch and stay for 5 min and visualized for hydrolytic activity. Clear zones which appeared around growing bacteria indicate hydrolysis of starch [15].

Determination of hydrolysis rate of the isolate

Ability of the bacterial isolates to degrade starch was described by the starch degrading index (SDI). The isolate was re-plated on starch agar and their halo diameter (Z) and colony diameter (C) was determined after 24 h incubation at 35 ºC. The formula $Z - C / C \times 100$ was employed to calculate the percentage hydrolysis efficiency according to the method of Sreedevi and Reddy [16].

Molecular identification of gene sequences of the bacterial isolate

The total genomic DNA extraction, Polymerase Chain Reaction (PCR) and DNA sequencing according to the standard protocols were carried out. The isolate which demonstrated starch degrading ability was subjected to extraction of total genomic DNA according to the procedures of Zymo Research Bacterial DNA MiniPrep™ instruction manual and kit.

In determining the phylogenetic grouping of sample genomic DNA, this was amplified using standard PCR. The genomic DNA extract was amplified using PCR reaction and completed within 36 cycles under conditions of initial denaturation (94 ºC for 5 min); denaturation (94 ºC for 30 s); annealing (56 ºC for 30 s); extension (72 ºC for 45 s); final extension step (72 ºC for 7 min); and indefinitely final holding at 10 ºC. Thus, this protocol helped to amplify the 16S rRNA gene of interest.

A 5 µl sample of PCR product reaction mixture was analyzed by 1.5% agarose gel electrophoresis in 1 x Tris Acetic EDTA buffer. It was run at 80 V and 107 mA for 45 min. A staining medium ethidium bromide was applied on the gel and visualization of bands under UV illumination was evidence. The 16S rRNA sequences were determined by fluorescently labeled 16s RNA products analysis generated by PCR cocktail mix on a DNA sequencer AB 373a Stretch (short gun). Primers (27F:AGAGTTTGATCMTGGCTCAG and 1525R: AAGGAGGTGWTCCARCCGCA) were used in all sequencing reactions. The 16S rRNA sequences obtained were aligned with the non-reductant nucleotide database at Genbank using the BLAST program (http://www.ncbi.nlm.nch.gov). A phylogenetic tree was constructed by the neighbour-joining method using MEGA 7 package [17].

Preparation and pretreatment of substrates

Corn bran and soya bean were blended into powdery form using the electric grinder (Marlex, Electroline) and stored in an air tight container prior to use. Garlic was peeled, dried at 40 ºC, blended and stored in an air tight container for subsequent use [18].

Preparation of inocula

The bacterial inoculum was prepared in 50 ml broth containing nutrient broth (0.65 g), corn bran (1 g) and soya bean (0.5 g). The medium was adjusted to pH 6.5 solutions autoclaved and cooled at room temperature. It was inoculated with 100 µl *Pseudomonas aeruginosa* AU4738 (2.3×10^4 cfu) and incubated for 24 h at 35 ºC.

Saccharomyces cerevisiae inoculum was prepared in 100 ml yeast extract broth (0.75 g), adjusted to pH 6.5 autoclaved, cooled at room temperature and inoculated with baker's yeast (1 g). It was then incubated at 120 rpm for 24 h (30 °C) [19].

Preparation of production medium and substrate treatments

Pretreatment slurry of substrate was prepared by adding 20 g corn bran powder and 2 g soya bean powder to 250 ml distilled water (w/v) with and without 1 g garlic as activator respectively [20]. Production media were prepared in duplicate and adjusted to pH 6.5 and thereafter autoclaved at 121 ˚C for 30 min. Co-culture technique involved simultaneous addition of both starch hydrolytic and fermentation microorganisms. Optimized protocol with very little modification was adopted [20]. The adjustment includes fermentation conditions such as ethanol production parameters (pH 6.5; temperature 37 °C; incubation period 72 h). They were inoculated with *P. aeruginosa* AU4738 (5 ml) and Baker's yeast (12.5 mL) at the same time and incubated at 37 ºC for 144 h. All the treatments were manually mixed at 24 h interval to promote uniform utilization of substrate. pH was checked and also recorded each day.

Generation of glucose standard curve

Standard glucose stock solution was prepared by dissolving 0.25 g glucose in 100 mL distilled water. Working standard solution was also prepared by adding up 10 ml stock solution to the 100 mL.

Glucose standard solution ranging from 0.1 – 1.0 µmol/mL was transferred into clean, dry test tubes, 1 mL DNS reagent was added to each tube and cotton plugged. A blank was prepared with 1 mL DNS added, the test tubes were boiled in water bath for 5 min cooled at room temperature and 9 mL distilled water was added. Absorbance at 540 nm using a spectrophotometer (GS-UV11, General Scientific) was read against the blank.

Determination of reducing sugar from the fermented broth

Fermented broth (10 mL) was centrifuge at 3000 rpm for 15 min to obtain supernatant for each sample with duplicates. One (1) mL supernatant was dispensed into a test tube, followed by another 1 ml prepared DNS reagent. The resulting mixture was boiled at 100 °C for 5 min, cooled and 10 mL distilled water was added and absorbance reading was taken at 540 nm. Thus, concentration values were interpolated from the glucose standard curve [18].

Distillation process

Distillation was carried out using distillation apparatus after fermentation process. Top fermented broth (15 mL) was transferred into round-bottom flask with an enclosed distillation apparatus of a running tap water flask in a heating mantle and fixed to the other end of distillation column for the collection of distillate at 78 °C (standard temperature for ethanol production).

Determination of Ethanol concentration

The ethanol concentration was determined by spectrophotometric method [22]. Distillate (0.5 mL) was measured into a conical flask containing 15 mL distilled water, and 12.5 mL K_2CrO_7 solution was added. The resulting mixture (20 mL) was transferred into a test tube and incubated at 60 ˚C for 20 min in a water bath and then cooled at room temperature. Five (5) mL was taken and diluted with 5 mL distilled water and absorbance was determined at 600 nm using spectrophotometer. The ethanol concentration was calculated from absolute ethanol standard curve, while the ethanol yield was determined using Yoswathana and Phuriphipat [23] procedure as shown below:

$$\text{Ethanol Yield} = \frac{\text{Ethanol measure in Sample}}{\text{Amount of initial sugar content x 0.5}}$$

Gas chromatography mass spectrometry (gcms) analysis of bioethanol

Gas Chromatography Mass Spectrometer (Shimadzu QP 2010 Ultra, Japan) equipped with Mass Spectrometer Detection 5975C (VLMSD) and injector (Auto) 7683B series) was used for the analysis. Absolute ethanol GC grade was used as internal standard in Gas Chromatography measurements. An aliquot (1 µl) reaction medium was measured and diluted in absolute ethanol (GC grade). The column temperature was kept at 40 °C, held for 1 min, raised to 290 °C at the rate of 3 °C /min, and then maintained at this temperature for 1.65 min. The final run time was 54.2 min. The detector and injector temperatures were set at 240 and 230 °C respectively. GC measurements were taken in triplicate.

3. Results and Discussion

Screening of microorganisms capable of hydrolyzing starch and hydrolysis efficiency

Screening of bacterial isolates for ability to hydrolyze starch depends on clear zone exhibition around their colonies. Zone of inhibition obtained from the screened bacterium showed appreciable differences in ability to hydrolyze starch (Figure 1). Hennessy *et al.* [24] described series of methods for the isolation, screening, and selection of glycoalkaloids (GA)-degrading bacteria. The screening of bacterial crude extracts for the ability to hydrolyze GAs was performed using a combination of thin layer chromatography (TLC), high performance liquid

chromatography (HPLC), and liquid chromatography mass spectrometry (LC-MS). These revealed the principal monomer of sugar constituents available for microorganisms' consumption for hydrolysis.

However, the bacterium in question was selected based on exhibition of halo zone diameter (19 mm) with calculated hydrolysis efficiency of 63.3%. Occurrence of isolate producing such large diameter of zone of inhibition was an indication that this substrate could serves as potential amylase producing bacterial medium [16, 20]. The starch hydrolysis was otherwise described by Gudeta (25) as starch degrading index (SDI).

Though the procedures carried out by the extracellularly secreted commercial α-amylase from bacteria could be very expensive for large scale production. The zone of inhibition exhibited by the bacterial isolate was an indication that it is more efficient on starch hydrolysis for monomeric sugars production. Furthermore, starch molecules are too large to enter bacterial cells, hence their transportation after hydrolysis into the cell and are thereafter used for metabolism reactions. These exoenzymes vis α - amylase and oligo-1,6-glucosidase are able to hydrolyze starch (amylase test) into dextrin, maltose, or glucose subunits using the starch agar as differential nutritive medium [26].

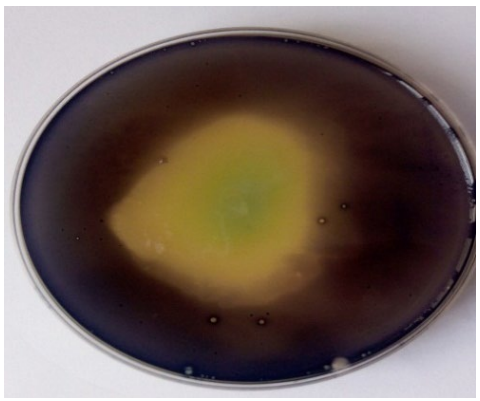

Figure 1. *Pseudomonas aeruginosa* showing Zone of Clearance on Starch Agar plate

Identification of the PCR amplified 16S rRNA

The isolate with NCBI Accession number HQ1481651.1 was identified as *Pseudomonas aeruginosa* AU4738. The maximum percentage identity was 93 % as shown in Figure 2.

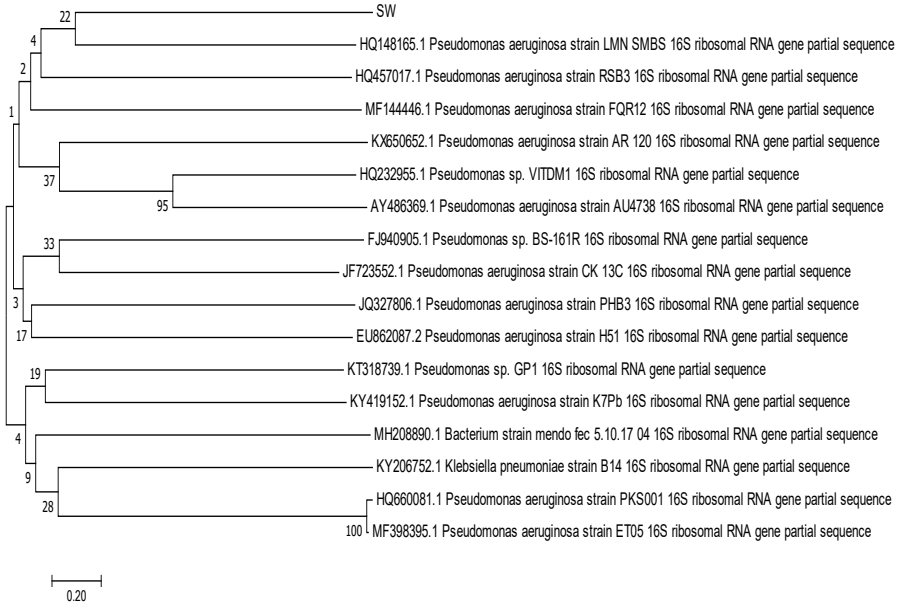

Figure 2. Phylogenetic Tree based on relationship of *Pseudomonas aeruginosa* strain AU4738

Optimization of the Glucose and Bio-Ethanol from the Corn bran substrate

There was an increase in activity of the starch degrading enzyme for glucose production by 23.8 and 17.8% at 48 h in culture with and without activator respectively and a steady decrease at 72 h to 168 h (Figure 3). This revealed fast starch-degrading enzyme produced by *P. aeruginosa* AU4738. According to Zakpa *et al.,* [27] the reducing sugar concentration retention may be directly proportional to the initial starch concentration available in corn bran. Incomplete hydrolysis of starch at a given saccharification time possibly resulted in reduction of reducing sugar that could have been converted even after addition of baker's yeast. This was in conformity with the results of Bekele *et al.* [28] when they produced bioethanol from potato waste peels.

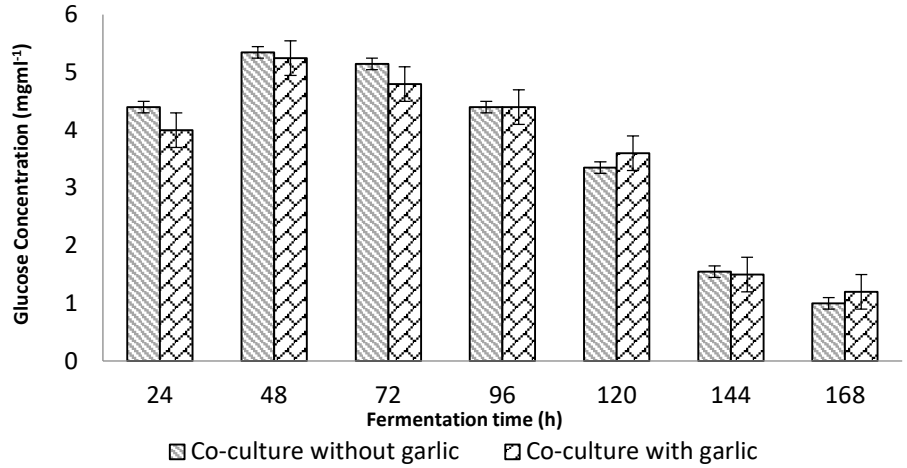

Figure 3. Effect of Culture Techniques on Glucose Production during the Fermentation of Corn Bran with or without Activator

In studying the effect of garlic powder on bioethanol synthesis, activated corn bran has maximum ethanol concentration of 18.25% v/v which was 35% higher than its counterpart without activator that recorded 11.86 % v/v at 120 h fermentation time (Figure 4). Subsequent fermentation process time drastically reduced the concentration. The procedure could be assumed as an alternative cost-efficiency in the trailing of fuel ethanol production protocol. Moreover, the incorporation of activator in the medium had significant effect on the bioethanol yield. Consequently the intolerance alcohol concentration posed to the viability of fermenting microorganisms was subdued.

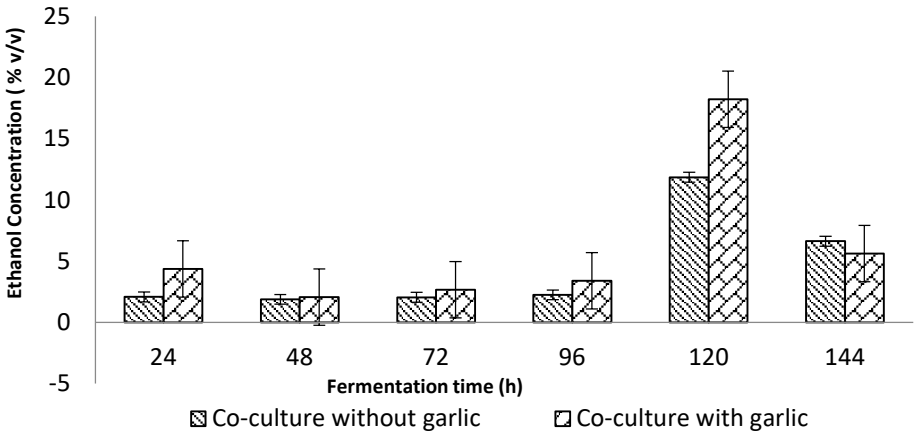

Figure 4. Effect of Culture Technique on Ethanol Production during the Fermentation of Corn bran with or without Activator

Garlic extract has been reportedly used to prevent acetaldehyde synthesis from ethanol by inhibiting the alcohol dehydrogenase [29]. Teixeira *et al.* [30] noted that the discrimination of alcohol concentration greater than 10% (v/v) will pose difficulty on the adequacy of fermenting microbes if it has potential to produce ethanol concentrations ≥ 17% (v/v). There was a report on reduction in the levels of acetaldehyde and acetate, after the introduction of garlic to investigation animals with significant increase in ethanol concentration [31]. In a work conducted by Abouzied and Reddy, [12] when *Aspergillus niger* and *Saccharomyces cerevisae* were co-cultured for bioethanol production, the barrier of the intolerance of the yeast to alcohol concentration was supposedly a concern. The activator circumvented this obstacle which also might be due to its phenolic

constituent. Garlic has been contemplated as one of the richest vegetable origin for total phenolic compounds [32]. As a result, most researchers now look plant origin for phytochemicals that could specifically target and prevent enzyme synthesize of these fermentation inhibitors distinctively acetate.

Pseudomonas aerugenosa AU4738 and baker's yeast concomitantly employed in this study enhanced the bioethanol synthesis. This was also in agreement with Igbokwe *et al.,* [33] that reported a keen increase in the percentage ethanol yield from 120 to 216 h incubation. Moreover, from glucose and lignocellulosic biomass hydrolysate substrates according to Joshi *et al.* [34], ethanol was efficiently and effectively produced by a coalescence of *S. cerevisiae* CDBT2 and *W. anomalous* CDBT7 yeast strains and indicated almost complete utilization of reducing sugars. Contrariwise, there was no improvement in ethanol production obtained after inoculation with *S. stipitis* in the sequential co-culture of *S. cerevisiae* and *S. stipitis* but lower ethanol was recovered with simultaneous co-culture in the consumption of xylose and glucose substrates. Moreover, *S. cerevisiae* fermentation of Kraft pulp hydrolysate in fermenter resulted in a slightly lower ethanol productivity and yield [35].

It has been told of that final ethanol concentration acquired differ either in the type of substrate concentration, pre-treatment given to substrate, mode of operation, substrate detoxification procedure, temperature, or fermentation strain [36]. Consequently ethanol from amylolytic fungus hydrolysates was lower than ethanol from acid hydrolysate of various substrates [37].

Identification test for Bio-Ethanol

A conventional method for determination of reducing sugars and total alcohols in raw fermentation broths has been developed and widely employed. The fermented broth culture is often pretreated to remove polysaccharides, proteins, glycerol and organic acids. The colorimetric change from total alcohols and reducing sugars were measured by potassium permanganate oxidation and determined by DNS test and subtracted. The remaining portion of colorimetric change was then used to calculate the total alcohol concentration in the sample. However, ethanol concentration can also be determined using ethanol oxidase or ethanol dehydrogenase, but the results are easily disturbed by the presence of various enzymes in the fermentation broth [38]. In this study, the GC analysis revealed 5.73% w/v and 3.6% w/v total component of ethanol from corn bran with and without activator using co-culture respectively as presented in Figures 5 and 6 respectively. However, the ethanol concentration determination by spectrophotometric method was estimated by percentage whereas the GC analysis revealed the component base on proportion, hence the disparity in quantification. Though there are some disadvantages in using this method. Potassium permanganate being unstable as it react with water at low pH and complicate the test results. To mitigate this setback, potassium permanganate solution was prepared right before it is used and kept in dark.

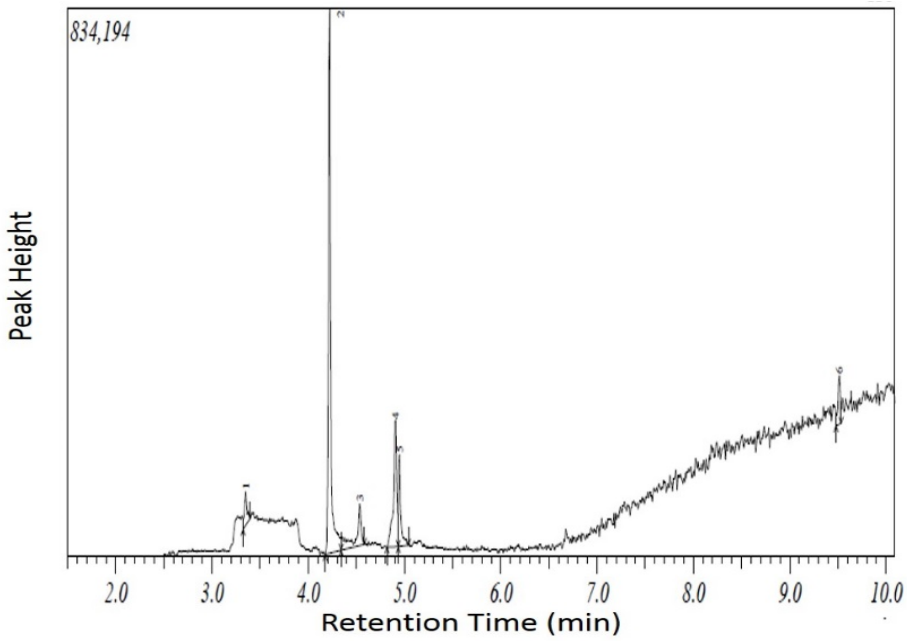

Figure 5. Gas Chromatogram of Ethanol concentration from Corn bran with activator

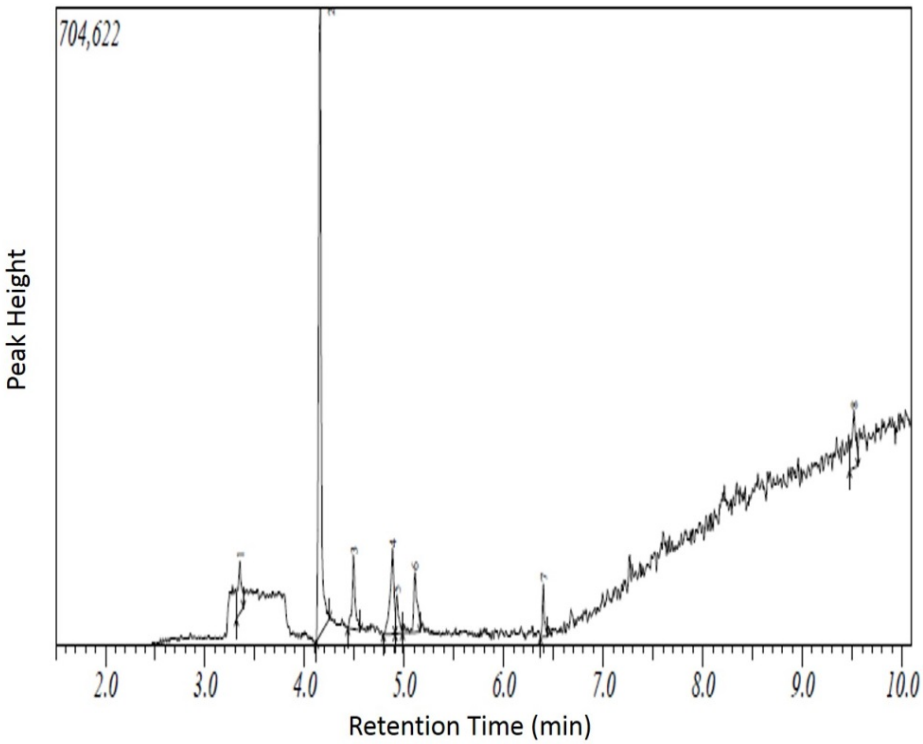

Figure 6. Gas Chromatogram of Ethanol Concentration from Corn Bran without Activator

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Conflicts of interest

The author simply declares there is no conflict of interest.

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