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Recycle Batch Fermentation for Acetic Acid Production from Matured Coconut Water by *Acetobacter aceti* Immobilized in Fibrous Bed Bioreactor

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Highlights

- Matured coconut water was used together with muscovado liquid syrup as a medium substrate for the four recycle batches fermentation in the study.
- A natural vinegar or acetic acid was generated from the fabricated fibrous bed bioreactor.
- The vinegar or acetic acid obtained from the recycle batch fermentation with 12^0Bx has 5.2%TA which meet the standard for vinegar set by the Food and Drug Administration

Abstract

Acetobacter aceti was immobilized in a fabricated fibrous bed bioreactor (FBB) for production of acetic acid with matured coconut water mixed with liquid muscovado syrup as substrate. Matured coconut water coming from coconuts was used as raw materials and substrate for acetic acid production using the immobilization of *A. aceti* in FBB.The general objective of the study was to produce acetic acid from coconut water using immobilized cells of *A. aceti* in FBB. Specifically, the potential of the fabricated FBB was assessed based on its production performance. Four (4) recycle batches fermentation with 5^0 Bx, 10^0Bx, 12^0 Bx and15^0 Bx sugar concentrations in the medium, were conducted to determine process performance of the fabricated FBB in terms of productivity, product purity, product yield and final product concentration of acetic acid (%Titrable Acidity). The 12^o Bx recycle batch fermentation produced highest %TA or acetic acid produced equivalent to 5.2%. The lowest product yield of 0.3341 was achieved in 15^0 Bx recycle batch fermentation, but the low sugar concentration of 5^0 Bx recycle batch gave a high yield of acetic acid equal to 0.651g/g. Reactor productivity increased as the sugar concentrations increased from 5^o Bx to 12^o Bx recycle batches fermentation but at 15^0Bx fermentation, it dropped almost four (4) times lower than that of the 12^0Bx recycle batch fermentation due to the low final acetic acid concentration of 1.88g/L. Recycle batch fermentation with 12^oBx has the highest % acetic acid, product purity of 14%. Product purity is affected by the final acetic concentration obtained after the fermentation process and attributed by the low side product formed.

Key Words: Recycle batch fermentation; acetic acid; matured coconut water; reactor productivity; product purity

1. Introduction

In the Philippines, the technology of cells immobilization in fibrous matrix is still on its experimental state. The technology may have started way back in 1990 but there is still a need to establish the basic information on this kind of fermentation strategy or technique. In 1986, the Philippine Coconut Research Foundation and Development Foundation (PCRDF) conducted a research on the state of the art of vinegar making in the Philippines. Based on the study, the most common methods of making vinegar being produced in the Philippines are: (1) slow or "tapayan" process; (2) quick or trickling process and; (3) submerged acetator process. Of these three processes, the "tapayan" method was assessed to be the most practical and convenient method suitable for village farmers for the following reasons: (1) vinegar production can be operated or handled by women in the household and (2) the copra "tapayan" site is a good and immediate source of the coconut water which is normally thrown away.

Using immobilization of cells technique in fermentation is seldom undertaken in research studies here in the Philippines. There are very few or no literature found in immobilization of acetobacter in vinegar production and immobilization technique is used in other fermentation processes.Very few researches were performed using immobilizing cells in matrices like loofah, coconut husk etc for wastewater treatment or other reduction of wastes concentration.

The use of fibrous bed bioreactor in producing acetic acid was studied by Huang et al., (1998), where the fermentation kinetics of acetic acid production from fructose by Clostridium formicoaceticum, a co-culture microorganism at pH 7.6 and 37° C. Immobilized cell fermentation was used,and acetic acid yield from fructose was approximately 1.0 g/g, with a final acetate concentration of approximately 78 g/L and the overall reactor productivity (based on the fibrous bed bioreactor volume) of approximately 0.95 g/(L.h) in the fed-batch fermentation. Fructose is a fermentable sugar commonly found in corn steep liquor and many other food processing wastes.

Fibrous bed bioreactor was also used by You-Hua et al., (2012), in producing bioethanol from fermentation of cassava pulp by a genetically modified microorganism *Thermoanaerobacterium aotearoense*. In the FBB, the fed-batch fermentation, using glucose as the sole carbon source, gave a maximum ethanol production of 38.3 g/L with a yield of 0.364 g/g in 100 h; whereas the fed-batch fermentation, using xylose as the sole carbon source, gave 34.1 g/L ethanol with a yield of 0.342 g/g in 135 h.

Matured coconut water is one resource considered a waste product, which is produced in appreciable quantities in the Philippines, Sri Lanka Thailand and other countries. Coconut water is a good base for vinegar production Its conversion into vinegar therefore presents an attractive option for decreasing wastage and producing a valuable product (Battcock and Ali, 2014).. Thus, there is a need for the Philippines to develop its vinegar manufacturing industry competitive with other markets so that importation will be minimized.

This proposal will introduce a different kind of fermentation technique, wherein only one time inoculation will be made and when cells are already immobilized in the FBB, this will serve as generator of cells without contaminating the system.

The general objective of this study is to examine the potential of the fabricated FBB to produce acetic acid from matured coconut water using immobilized cells of *A. aceti* in the matrix of FBB. The study also determine the process performance of the immobilized recycle batch, in terms of productivity, product purity (in terms of % acidity) and product yield of acetic acid using one substrate i.e.; matured coconut water and the maximum production rates of acetic acid at different substrate concentrations of the recycle batch fermentation mode.

Methods

2.1. MATERIALS

2.1.1. Materials and chemicals

The chemicals used in this project were dextrose (90% glucose technical) isopropyl alcohol, muscovado liquid sugar, hydrogen peroxide and disinfectant liquid.Muscovado liquid syrup was used as part of the medium substrate obtained from Muscovado farmers in Januiay, Iloilo. Matured coconut water was taken from vendors selling matured shredded coconut in Miagao public market.

Other materials used for the project were cotton towel, stainless steel wire, magnetic stirrer, glassware (Erlenmeyer flask, cylinder, beakers, pipettes, test tubes, petri dishes,and slants), paper towel, alcohol lamp, aluminum foil and cotton.

2.2. Microorganism

Acetobacter aceti used in the study was obtained from the Department of Science and Technology, Division of Environmental Biotechnology, Taguig, Metro Manila, placed in a slant inside a test tube. Initially, the microorganism was grown at a range of 25 to 32°C in an Erlenmeyer flask containing medium of glucose, yeast extract, and trypticase (GYET). To maintain viability of the culture, 10% of its prepared culture was transferred to a similar flask and kept in the refrigerator at 10°C. After it was grown on a synthetic medium, the microorganism was transferred and grown in an Erlenmeyer flask containing coconut water to see if the microorganism will grow in coconut water as medium for growth.

2.3. Preparation of Fermentation Medium

The fermentation medium was prepared from matured coconut water. The initial sugar concentration of the coconut water was measured using a refractometer. The sugar concentration of the coconut water was then adjusted to the desired sugar concentration of the fermentation recycle batch.

2.4. Experimental Set-up

2.4.1. Construction of fibrous bed bioreactor (FBB)

The FBB was made of glass with a total effective volume of 800 ml. The fibrous bed of the FBB was made of cotton mounted in a stainless steel and inserted into the bioreactor and was used to immobilize the *A.aceti*.

2.4.2 Preparation, start-up, and operation of FBB

The immobilized sytem was comprised of different components seen in Figure 1. The components were assembled and connected after autoclaving each component at 121^{0}Cand 15 psig for 20 minutes. The FBB was then connected to a 7 liter fabricated sterilized fermentor filled with sterilized medium consisting of matured coconut water with muscovado liquid sugar.The stirred bioreactor or fermentor was filled with 6.5 liters of sterilized medium consisting of coconut water with liquid muscovado sugar. This type of sugar consist of invert sugar (84.38%), total sugars as reducing sugar (15%) and sucrose (66%) (NFA, FDA, 2015).

When the acetic acid concentration of 4% substrate was attained, the fermented broth was removed and new set of medium was used in the next batch. Productivity is measured by the final product concentration obtained divided by the total fermentation time. Product purity is the % titrable acidity (TA) content of the vinegar and its acidity measured by pH. Yield is obtained from the amount of vinegar acid produced divided by the total amount of substrate fed.

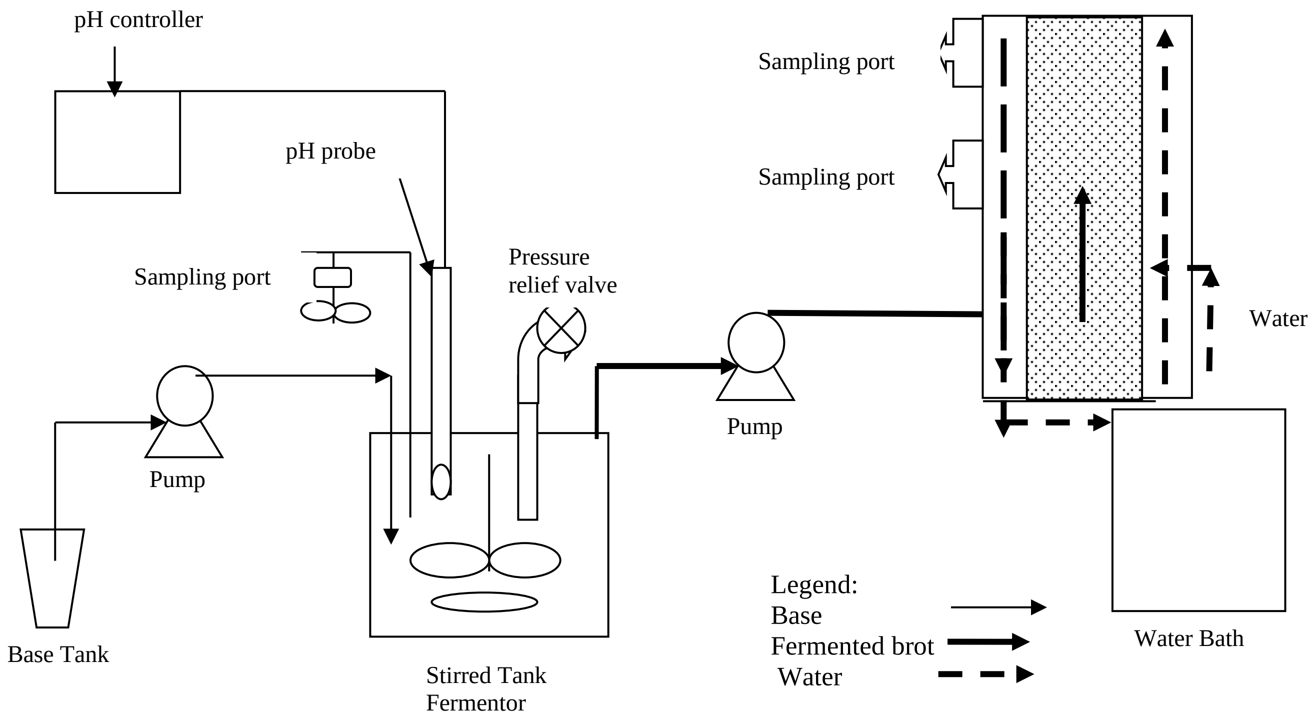

Figure 1. Experimental set-up for acetic acid fermentation using FBB

2.4. Cell Recycle Batch Fermentation

Cell recyle batch fermentation is a process of fermentation where the medium after inoculation was made to return back to the fermentor after the medium has passed the FBB. The medium which continuously flow from the fermentor to FBB is reprocess again from FBB to fermentor and vice versa to form the acetic acid inside the fermentor. Four batches of cell recycle batch fermentation were performed using the experimental set-up in Figure 1, except that in each batch run, the volume of the fermentation medium used was constant at 6.5 liters. Fresh medium was used for each recycle batch run. Each of the batch contained medium of 5%, 10%, 12% and 15% sugar concentration. The pH, %TA, and sugar concentration in terms of Brix, were the parameters measured for each batch run. Process performance parameters such as productivity, product yield and purity were determined. The 6.5 liters medium was made to pass the fibrous bed bioreactor immobilized with *A.aceti* bacteria

2.5 Determination of pH of fermentation broth

pH of the fermentation broth is determined by potentiometric method using a pH meter. The pH meter used was first calibrated by buffer solutions of pH 4 and pH 8 before getting the pH of the broth.

2.6. Determination of Titrable Acidity (TA)

Titrable acidity (TA) was determined using the Potentiometric method for colored samples since the fermented broth turning to vinegar was a colored sample (Nielsen, 2010). Ten milliliters (10ml) was taken from the bioreactor or fermentor and was used to determine the %TA using the following formula below (Nielsen, 2010):

$$\% \text{ acid (wt/vol)} = (N \, x V_1 \, x \, Eq \, wt / \, V_2 \, x \, 1000)100 \qquad (1)$$

$$\text{Or } \% \text{ acid (wt/vol)} = (N \, x V_1 \, x \, Eq \, wt / \, V_2 \, x \, 10)$$

Where:

N = normality of titrant usually NaOH (mEq/ml)

V_1 = volume of titrant (ml)

Eq. wt. = Equivalent weight of predominant acid (mg/mEq)

V_2 = volume of sample (ml)

1000 = factor relating mg to grams (mg/g)

(1/10 =100/1000)

2.7. Determination of sugar concentration

The sugar concentration of the fermentation broth was determined using refractometric method (Nielsen, 2010). A drop of sample taken from the bioreactor or fermentor was placed on the prism of the refractometer and was held against the light to read the brix value equivalent to the sugar concentration of the fermentation broth. For analysis of sugar concentration, the property used was ºBrix. According to the method of the AOAC (2000), ºBrix is the total soluble solids. The concentration of sugars has a linear relationship with ºBrix (Torres Neto et al., 2006), as shown in the Equation below.

$$\text{Sugar (g/L)} = 10.13 * (^{\circ}\text{Brix}) + 1.445 \qquad (2)$$

2.8. Formula used for the calculation of process performance parameters

Product Yield- slope of the linear regression plot of the acid concentration against the glucose concentration and the negative value of the slope of the regression line was the yield. It has been observed frequently that the total amount of acid or cell mass formed is proportional to the amount of substrate utilized (Bailey and Ollis, 1986, Lee, 1992).

Productivity –was calculated based on the definition of Lee (1996), i.e., the amount of acid produced divided by the volume of the reactor and total fermentation time.

Product Purity- is the ratio of the final acetic acid concentration and the sum total of the concentration of acetic acid and alcohol and multiplied by 100 to get % purity.

3.0. Results and discussion

3.1. Cell Recycle Batch Fermentation

Four cell recycle batches fermentation were performed. The cell recycle batches fermentation contained medium with sugar concentration of 5^0Bx, 10^0Bx, 12^0Bx, and 15^0Bx for the first, second, third and fourth batches fermentation, respectively. The medium which contained coconut water and muscovado liquid sugar (or pulot) was used for these experiments. The sugar concentration of matured coconut water was initially measured to be 4.8^0Bx and was adjusted to the desired mentioned sugar concentration. The sugar concentration was expressed in °Bx. The volume of fermentation medium used for all four batches was 6.5 liters and was inoculated with 10% of the volume medium with *A.aceti*.

3.1.1 Sugar concentration of cell recycle batches fermentation

Figure 2 shows the time course of the total soluble solids or the sugar concentrations (Bx) of the four cell recycle batches fermentation. The sugar concentration decreased for all recycle batches fermentation as time progressed. This indicated that the *A. aceti* microorganism was consuming the sugar for its growth and in producing products like acetic acid and alcohol. Acetic acid bacteria have been considered "fastidious" due to their response to growth in culture media (Torija et al. 2010).

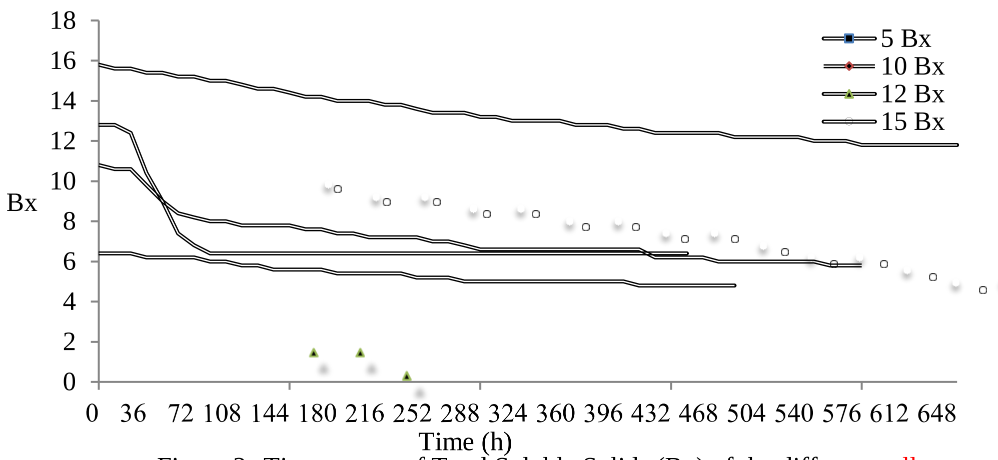

Figure 2. Time course of Total Soluble Solids (Bx) of the different cell recycle batches fermentation

A aceti bacteria are found on substrates containing sugars and/or alcohol, such as fruit juice, wine, cider, beer, and vinegar. On these substrates, the sugars and alcohols are incompletely oxidized, leading to the accumulation of organic acids, such as the production of acetic acid from ethanol or gluconic acid from glucose (Mas et al., 2014). The metabolism of some acetic acid bacteria may include a tricarboxylic acid cycle function, enabling them to completely transform acetic acid to CO2 and water (de Ley J et al. 1984). However, because entry into the acetate cycle is inhibited by the presence of ethanol, it is essential to maintain a low concentration of ethanol in the presence of acetic acid bacteria to prevent this full oxidation. In fact, ethanol concentrations between 0.5 and 1% are regularly maintained in vinegars (Mas et al., 2014). The final concentration of sugar reached 4.5^{0}Bx for the batch with 5^{0}Bx medium concentration, 5.8^{0}Bx for the batch with 10^{0}Bx medium, 6.4^{0}Bx for the batch with 12^{0}Bx medium and 11.2^{0}Bx for the batch with 15^{0}Bx medium. This means that *A. aceti* fermentation process can reached a constant level of sugar concentration, but there was a continued production of vinegar as acetic acid as seen in Figure 2. From Figure 2, it can be seen that the *A. aceti* fermentation was a slow process and as observed by Nanda et al., 2001. It was observed that the decline of sugar concentration was almost half the initial amount of sugar concentration in the medium and fermentation was too slow especially when the sugar concentration was initially high. It was further noted that the sugar concentration for 12^{0}Bx batch fermentation declined abruptly from 12.4^{0}Bx to a lower ^{o} Bx and 24 hours unlike that seen for 5^{0}Bx, 10^{0}Bx and 15^{0}Bx, where the decrease of sugar concentration with time was gradual. Commercial brand vinegar such as Datu Puti (sukang puti) has acetic acid as the final product which has a final sugar concentration of 4.6^{0}Bx.

3.1.2. Vinegar production (%TA) of cell recycle batches fermentation

Titrable acidity is one of the very important physico-chemical parameters of vinegar fermentation process (Saha and Banerjee, 2013). Another term for titrable acidity is total acidity and expressed as acetic acid content (AOAC, 1990). It is possible to assume that total acidity or titrable acidity is a good indicator of acetic acid concentration (Saha and Banerjee, 2013).

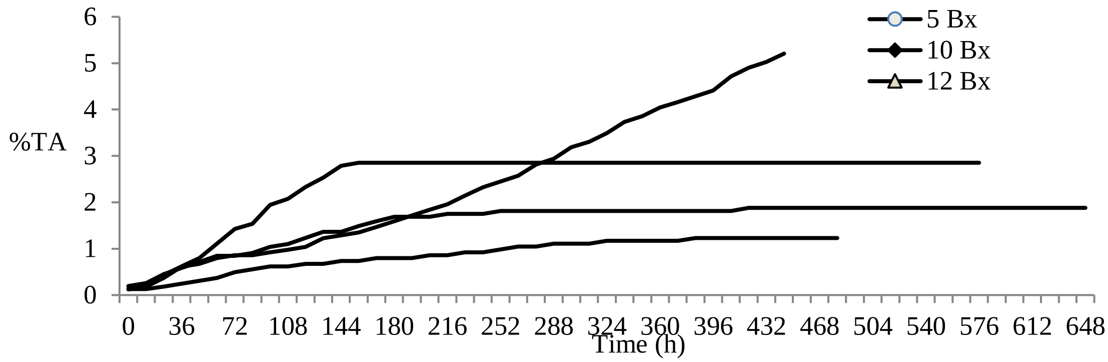

Figure 3. Time course of acetic acid concentration (%) of the different cell recycle batches

Figure 3 shows the time course of acetic acid concentration or %TA of the four different cell recycle batches fermentation. The highest %TA or acetic acid produced was from 12ºBx recycle batch fermentation equivalent to 5.2%. However, for the 5ºBx, 10ºBx and 15ºBx recycle batches, the achieved %TA respectively were 1.3%, 2.86% and 1.88%. The recommended value of the U.S. Food and Drug Administration for %TA is 4% (Punchihewa and Arancon, 1999). The amount of sugar used for each recycle batch has not affected much the production of acetic acid. Results of acetic acid produced decreased as the sugar concentration increased from 12ºBx to 15ºBx. The acetic acid concentration increased started from 5ºBx to 12^{0}Bx. And then the acetic concentration dropped when the sugar concentration of the medium was 15^{0}Bx. At this sugar concentration of the medium, the production of acetic acid was inhibited by the amount of sugar and alcohol fermentation and that of the growth of *A.aceti* (Figure 3).

3.1.3. Process Performance of the Recycle Batches Fermentation

Process performance of the recycle batches fermentation was assessed by the parameters such as product yield, purity, productivity and final product concentration of acetic acid.

- *Product Yield*

Product yield of the cell recycle batches fermentation is shown in Figure 4. The lowest yield of 0.3341 was achieved in 15^{0}Bx cell recycle batch fermentation. This indicated that the amount of acetic acid produced was low and the remaining amount of substrate was high. In the high sugar fermentation, the formation of alcohol can happen and this would proceed to conversion to acetic acid. But if no conversion occurs, there will be less acetic acid produced. The low sugar concentration of 5^{0}Bx cell recycle batch gave a high yield of acetic acid equal to 0.651. This means that there was less amount of side product formed such as alcohol in the fermented broth.

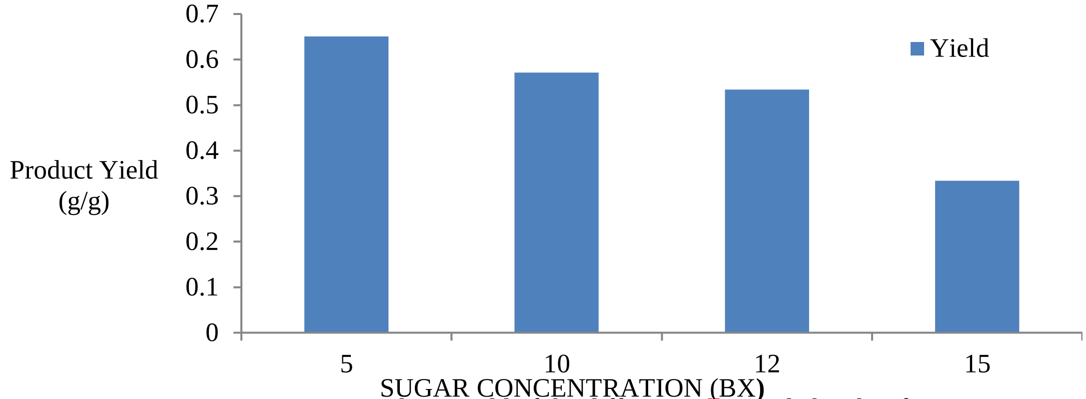

Figure 4. Product Yield of the different cell recycle batches fermentation

- *Productivity*

Reactor productivity was observed to increase as the sugar concentration increased from 5°Bx to 12⁰Bx cell recycles batch fermentation (Figure 5). Sugar concentration has affected the reactor productivity. It was also noted that when the sugar concentration was 15⁰Bx, the productivity dropped almost four (4) times than that of the 12⁰Bx cell recycle batch fermentation. This apparent condition was due to the low final acetic acid concentration equivalent to 1.88g/L (Figure 3). The highest productivity was observed in 12° Bx cell recycle batch equivalent to 0.012 g/L/h.

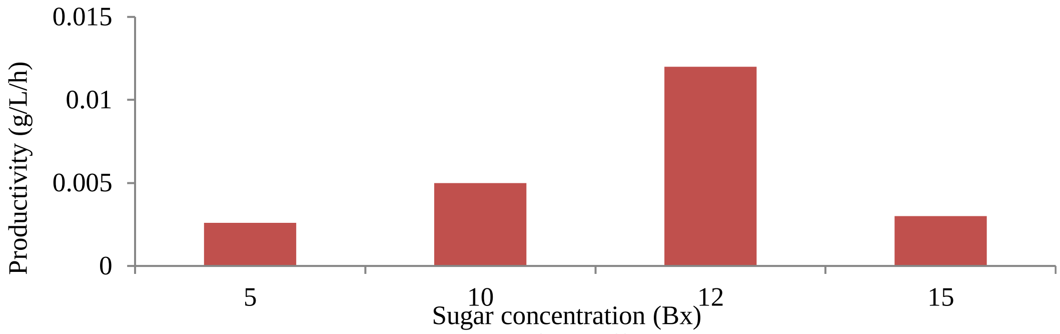

Figure 5. Productivity (g/L/h) of the different cell recycle batches fermentation

- *Product Purity*

The purity of the different cell recycle batches fermentation is given in Figure 6. As seen in the figure, cell recycle batch fermentation with 12°Bx has the highest % product purity of 14% purity of acetic acid. This particular cell recycle batch fermentation has similarly obtained high reactor productivity (Figure 6).Product purity is affected by the final acetic concentration obtained after the fermentation process and attributed by the low side product formed.

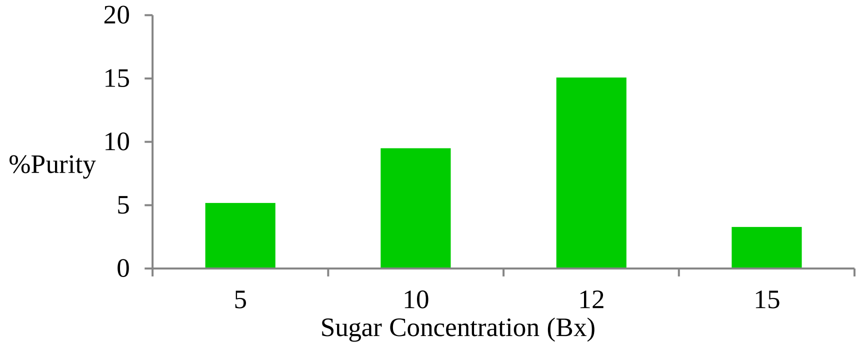

Figure 6 %Purity of the different cell recycle batches fermentation

4. Conclusions

The fabricated bioreactor immobilized with *A.aceti* has shown potential in producing acetic acid commonly known as vinegar from matured coconut water with muscovado sugar as substrate in the medium. The fibrous bed bioreactor served as the continuous supply of *A aceti* to process sugar to acetic acid or vinegar. The acetic acid obtained has met the standard for acetic acid or vinegar by the Food and Drug Administration which is in the range 4.5% to 5%. Cell recycle batch fermentation with 12^{o}Bx showed high purity (15%), and productivity (0.012g/h/L) but the cell recycle batch fermentation with 5^{0}Bx obtained the highest product concentration (0.65g/g).The highest %TA obtained was 5.2% and this occurred in recycle batch fermentation with 12^{0}Bx sugar concentration.

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