Effects of Temperature and pH on *Trichoderma reseei* Cellulase Activity in Glucose Production from Water Melon Peel

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Abstract- Optimal enzyme activity occurs within a limited set of conditions such as temperature and pH which must thus be carefully controlled to promote high reaction rates. Therefore, watermelon peel was collected, processed, pretreated and subjected to hydrolysis in a series of batch reactors. *Trichoderma reseei* was isolated from decayed wood and the enzymes released by *Trichoderma reseei* were used for the hydrolysis for glucose production from watermelon dry peel. The effects of temperature at selected temperatures of 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C and 45 ^{o}C; as well as effects of pH on enzyme activity at selected pH ranges of 3.0 to 5.5 were studied. From the study, the results obtained within the limit of the experimental conditions show an optimal temperature of 30 ^{o}C, having a corresponding glucose concentration of 0.091728 g/l, optimal pH of 4.5 with a corresponding glucose concentration 0.6436 g/l, which implies that the enzymes from *T. reseei*gives high yield of glucose when controlled at these conditions. Also, the watermelon dry sample used in the study was highly deliginified, leading to high glucose yield.

Index Terms- Waste, Fruits, Hydrolysis, Biomass, Concentration, Environment

I. INTRODUCTION

Waste is defined as any unavoidable material resulting from domestic activity or industrial operation for which there is no economic demand and which must be disposed off (Sridhar and Hammed, 2014). In developing countries, where there is high degree of poverty and hunger; ignorance and negligence are major factors contributing to the contamination of the environment through indiscriminate disposal of wastes. As the world's population grows, production of waste materials also increases proportionately (Ososanya, 2012). In Nigeria, Agricultural Post-Harvest waste, vegetables and fruit peels form a major part of these wastes. This is because; fruits like banana, cucumber, watermelon, pineapple and the rest are not eaten with the peels. Often a times, these peels from the aforementioned fruits and certain parts of the vegetable which are common in Nigeria are regarded as waste with no value and are often thrown away littering the environment or may be fed to animals. If well harnessed, these peels which are hitherto regarded as waste can be enzymatically hydrolyzed to produce bio-energy (Oladeyo, 2010). One of the most pre-dominant fruit and the most common fruit in Nigeria, particularly in the North east is watermelon. Watermelon comes from the cucurbit family; a warm season crop which is usually cultivated in the warmer parts of the world as Africa and Asia. The fruit constitutes 68% flesh, the rind 30% and the seed 2% of the total weight (Kumar *et al.*, 2012). Studies conducted by Abdulla and Masudal (2015), revealed that dry watermelon peel contains 73.8 % carbohydrate from which bio-energy can be produced.

Trichoderma reseei, a haploid filamentous fungus has the ability to produce Cellulase enzymes which can be used for conversion of plant biomass into glucose. Enzyme performance is typically tested or assayed to determine their optimal activity based on variation of environmental factors affects enzymes physical and chemical properties such as temperature and pH. Optimal enzyme activity occurs within a limited set of conditions and must thus be carefully controlled to promote high reaction rates (Obnamia, 2014).

The objective of this work is to investigate the effects of temperature and pH on *T. reseei* Cellulase activity and to determine the appropriate conditions (pH and temperature) for optimal enzyme activity for achieving high glucose yield in glucose production from watermelon peel.

II. METHODOLOGY

Watermelon fruit used was procured from Gomboru market, Custom area of Maiduguri, Borno State, Nigeria for the study. The peels were removed and transferred to the laboratory in a sterilized polyethylene bag, where they were dried in an oven at 50^{0}C for 1 hourand stored in an air-tight plastic container. The equipment used in this work include: Electronic precision balance (Model Tl-5000), Water bath (Techmel and Techmel TT420), pH meter (Jensway 3150), Incubator (Gallenkamp 1EF097 XX2.5), Light microscope (Olympus Venox-T Model), Micro pipette, pasture pipette, Conical flask (pyrex), Measuring cylinder (pyrex), Petri dishes, 4 roll Cotton wool, one role Aluminium foil, Slide and cover slip, (1pack each), Autoclave (New life medical instrument England, Model 280-A), Glucose lab kit (Chemelex), Hotbox oven (BFL 300-749-I Borel Laboratory), Muslin clothe, Spectrophotometer.

The following samples were collected for isolation of the fungi: decaying wood, termite housing soil (THS), maize grain (MG), sorghum grain (SG), millet grain (MLG), cowpea seed and deteriorated groundnut seed were procured from Custom Market in Maiduguri, Borno State, Nigeria. The samples were carefully collected in sterilized container with a cover, labeled and transferred to the laboratory for analysis.

Figure 1: Fresh Watermelon Peels

A. Media Preparation

Trichoderma reseei mycelia that were used through out the study were cultured in Pathology Laboratory of the Department of Crop Production, University of Maiduguri. Potato Dextrose Agar (PDA) was used as the media for isolating the organism. The PDA used is composed of potatoes, infusion from 4.0g (200g of potatoes extract is equivalent to 4.0g of potatoes infusion), D (+) glucose 20.0g, Agar-agar 15.0g. Demineralized water was prepared by boiling water in a current autoclave at 121 ^{0}C for 15 minute, after 39g of the PDA, measured on an electric precision balance was suspended in 1 liter demineralized water in a 1000 ml flask. The orifice of the flask was covered with cotton, wrapped with aluminium foil. Petri dishes, wrapped with aluminium foil were sterilized by autoclaving for 20 minute at the pressure of 0.14-0.16 Mpa and temperature of 121 ^{0}C. The flask containing the media was heated to obtain completely dissolved media. The content of the media was allowed to cool to 45 ^{0}C as given in Figure 2.

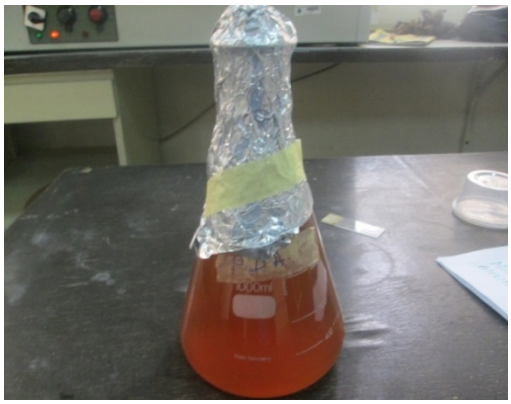

Figure 2: Potato Dextrose Agar (PDA)

B. Plate Preparation and Pouring

Petri dishes with cover were washed in a10% commercial bleach for 5 minute after which they were washed with detergent and rinsed under running tap. The Petri dishes were then placed on a table, sterilized by swabbing with ethanol to avoid contaminations. The petri dishes were then sterilized in a hot oven at 150 ^{0}C for 90 minutes.The mouth of the conical flask containing the medium in Figure 2 was flame-sterilized by using a spirit lamp to destroy surface micro-organisms. Two drops of lactic acid were poured into each of the petri-dishes to inhibit the growth of bacteria and then the medium was poured and covered, and left to solidify as given in Figure 3.

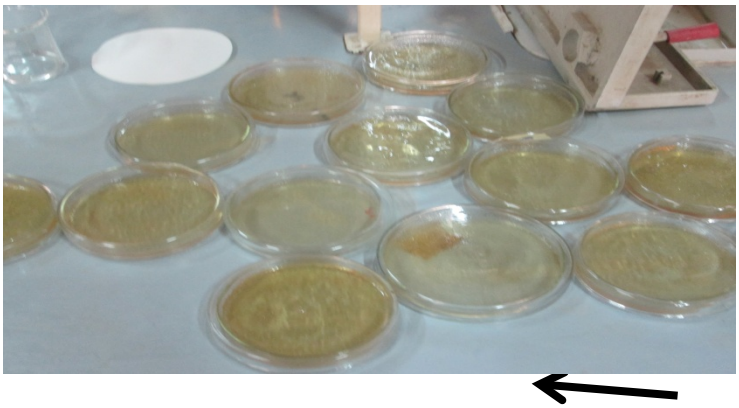

Figure 3: Sets of Petri dishes Containing PDA

C. Isolation of Organism

Sample collected were divided into two batches, with equal number and same sets of samples (i.e. each batch consisting of six samples, namely: decayed wood, termite housing soil, maize grain, sorghum grain, decayed groundnut seed and cowpea seed). The first batch was surfaced sterilized in 10% commercial bleach in a petri dish to destroy secondary pathogens and rinsed with distilled water while samples in the second batch was left surfaced unsterilized. Flame sterilized forceps was used to pick five pieces per sample from the two batches of samples and transferred to the PDA medium aseptically by swabbing the work bench with alcohol. The petri dishes were then incubated ta a temperature between 28^{0}C-30^{0}C for 72 hours, and the growth of micro-organism was monitored.

D. Slide Preparation

Drop of Lactophenol cotton blue was placed on a clean glass slide and a small portion of the fungalmycelium was gently taken from the culture and placed in the drop of Lactophenol using a flame sterilized teasing needle;the mycelium was carefully spread on the slide with the aid of the inoculating needle. A cover slip was then gently applied with little pressure to avoid the trapping of air bubbles in the stain.The slide was covered with a slip and heated slightly to remove air bubbles and the slide was then mounted and examined under a light microscope (x10 and x40), and the colony morphological characteristics of *T. reseei* were identified.

E. Sub-Culturing of T. reseei

Inoculating needle was sterilized by flaming in the blue portion of spirit lamp until it is red and dipped in alcohol to cool, and was used to carefully transfer a tuft of the isolated *Trichoderma reseei*on to a sterile PDA plates by point inoculation. Pure cultures were maintained by sub-culturing onto freshly prepared PDA routinely, and kept at 28^{o}C in an incubator as a stock culture.

F. Mechanical Pretreatment

The dried watermelon peel (100g) was milled or grinded using electric grinding machine to powder after which it was sieved using 100µm sieve size to obtain fine powder so as to increase the area/volume ratio. The peel powder was then collected and kept in a sterilized container with a cover as given in Figure 4.

G. Chemical Pretreatment

Fifty gram (50g) of the milled watermelon rind was mixed with 20 ml of 10% sulfuric acid (H_2SO_4) at room temperature in a 1 liter beaker with constant stirring for 2 minute to delignify the substrate. The sulfuric acid was then separated from the substrate by filtering through a muslin clothe. The substrate was then washed three times with distilled water to remove the residual acid from the watermelon peel. It was then oven dried to a constant weight at 50 ^oC.

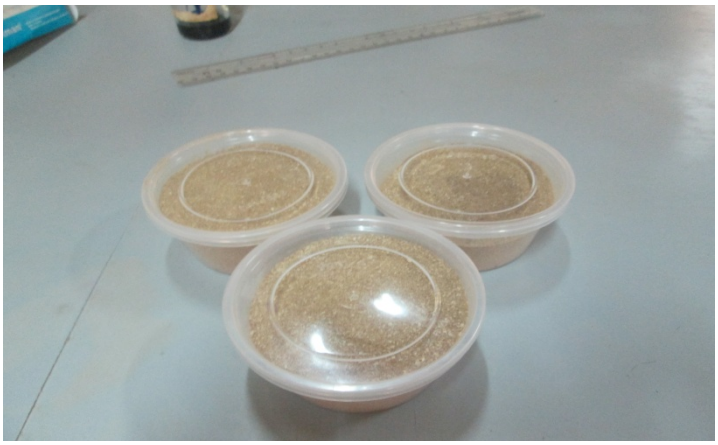

Figure 4: Watermelon peel powder

H. Preparation of Mineral salt Media

Peeled Irish Potato (200g) was chopped into small pieces and added into 1 liter of sterilized distilled water and boiled gently at 60 ^oC after which it was filtered through muslin clothe into a 1 liter conical flask. 3g of sodium acetate and 0.5g of magnesium sulfate were added to the peeled potato extract in the 1 liter conical flask forming a mineral medium. Then 0.5g and 27g of sodium chloride and glucose also were added respectively to the medium. Again 0.5g each of calcium chloride and potassium sulfate were then added. The mineral salt medium formed is given in Figure 5.

Figure 5: Mineral salt media

I. Acetate Buffer Preparation

Solution A (sodium acetate trihydrate) was prepared by adding 13.60 g of sodium acetate trihydrate ($CH_3COONa.3H_2O$) into 1000 ml of deionized water and solution B (acetic acid) was prepared by adding 5.80 ml of acetic acid(CH_3COOH)into 100 ml of deionized water and then the solution was brought to 1000 ml. Each of the solution is of 0.1 M concentration. The various desired pH values were obtained by adjusting with either 0.1 M of solution A or B.

J. Assays for Glucose Concentration

The reducing sugar (glucose) concentration analysis in all the experimental runs was carried out using a glucose oxidase-peroxidase labkit (GOD-PAP). A micro-pipette was used to collect 1000 µl of the sample and was mixed with 1 ml of the reagent. The solutions were then placed on a cycling vibrator for 5 minutes to homogenize the mixture after which it was incubated for 25 minute at temperature of 20 ^oC. Measurement of absorbance of the standard ($A_{standard}$) and sample (A_{sample}) was done against the reagent blank within 60 minute at a wave length of 546 nm.

K. Effect of Temperature and pH on T. reseei Cellulase Activity

To obtain an improved rate of hydrolysis, watermelon peel powder was pretreated with sulfuric acid solution to expose the cellulose to the enzymes to attack.In a typical run, the temperature of the water bath was set at selected temperatures of 20 ^oC, 25 ^oC.30 ^oC, 35 ^oC, 40 ^oC and 45 ^oC. Batch reactors made of plastic containers were used, where 50ml of mineral salt, 1g *T. reseei* mycelium and 1.5g of pretreated watermelon peel were added into each of the reactors. Other reaction conditions such as pH and culture age were kept constant at 4.5 (Sodium Acetate, Trihydrate Buffer) and 5days respectively. For the effect of pH on *T. reesie* Cellulase activity, selected pH ranges from 3.0 to 5.5 were used while other conditions such as temperature, cell loading, substrate concentration and culture age were held constant at 30 ^oC, 1g, 20g/l and 5 days respectively. Optimal temperature and pH for the hydrolysis were evaluated by analyzing the concentration of glucose produced at different temperature and pH levels. The glucose produced was determined at 24 hours interval, using glucose oxidase-peroxidase lab kit (GOD-PAP) and a Spectrophotometer. Each run was repeated two times and the mean value was reported.

III. RESULTS AND DISCUSSIONS

The microorganism *T. reseei* was identified and the morphological features of the organism. To obtain an improved rate of hydrolysis, watermelon peel powder was pretreated with sulfuric acid solution. Figure 3 shows the effect of the chemical pretreatment carried out on watermelon peel powder in glucose production. From the Figure, it can be inferred that high delignification was obtained on the treated sample and likewise the hemicelluloses were well broken which led to the exposure of the cellulose to the enzymes to act upon, yielding to higher glucose production of 0.89347 g/l in the treated sample and 0.19715 g/l in the untreated sample after 168 hours.

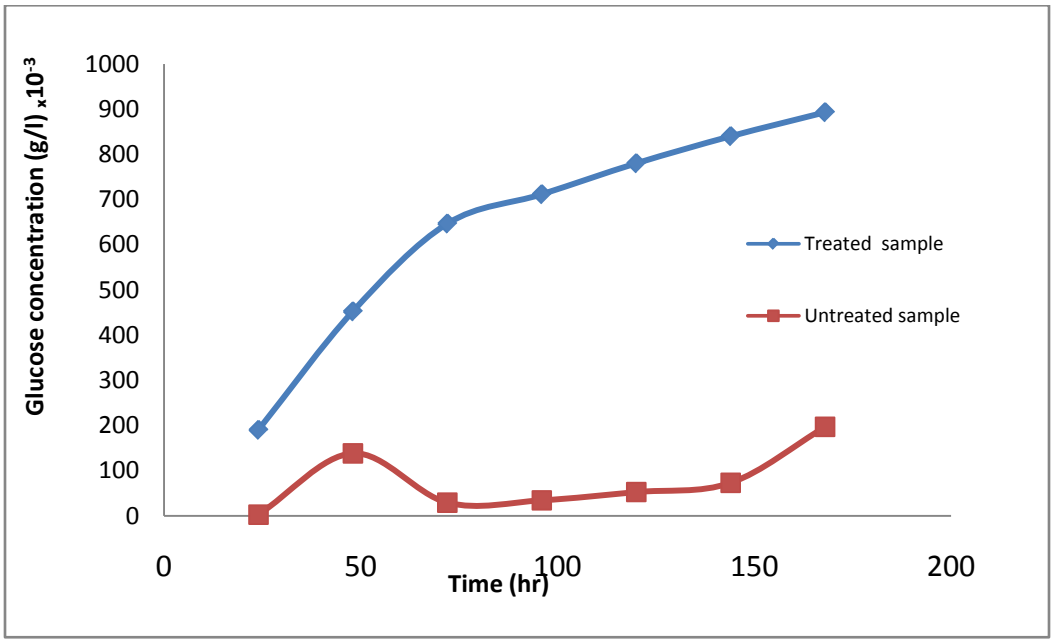

Figure 3: Effect of Pretreatment on Watermelon Peel Hydrolysis

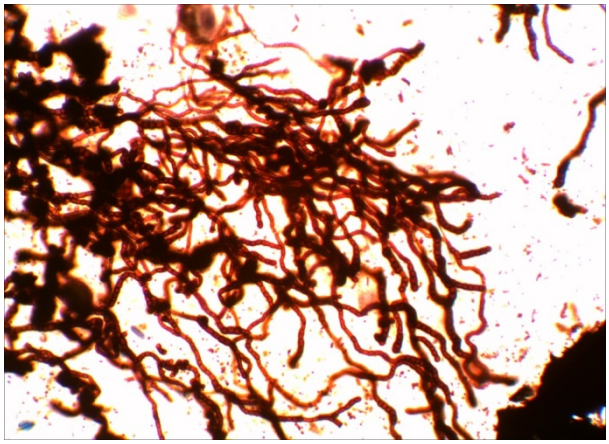

Figure 4: Micrograph of *T. reseei* (x400)

Keeping temperature, substrate concentration and mycelium cell loading constant, the effect of pH on the enzyme activity from *Trichoderma reseei* at different pH values is depicted in Figure 5. Enzyme activity increases as pH increases from an acidic environment, reaches a maximum (optimal pH) of 4.5 with a corresponding glucose concentration of 0.6436 g/l and decreases towards a basic environment as the pH of the batch reactor rises to 5.5, where enzyme activity became deactivated as described by Obnamia (2014). Similar result was obtained by Ososanya (2012).

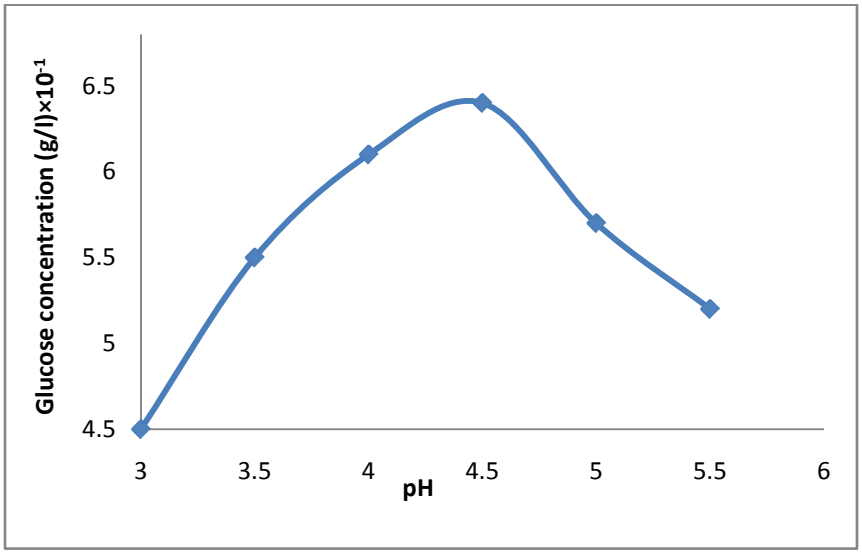

Figure 5: Effect of pH on Glucose Production from Watermelon peel

The effect of temperature on the activity of enzymes from *T. reseei* was studied at variousof temperatures: 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C and 45^{o}C. This was done to determine the optimum temperature for glucose production. Enzymes activity increases between 20 ^{o}C to 35 ^{o}C with an optimum activity at 30 ^{o}C. This was followed by a corresponding decline in the enzyme activity as the temperature rises above 30 ^{0}C. This was because; at high temperature the enzyme was denatured which lead to lost of structural integrity. Similar report was given by Samuel et al., (2002) and Hui (2013), where they reported that majority of *Trichoderma* cultures grow rapidly between the temperature of 25 ^{o}C to 35 ^{o}C and not growing at all at 35 ^{o}C, yet some species grow at 35^{o}C.

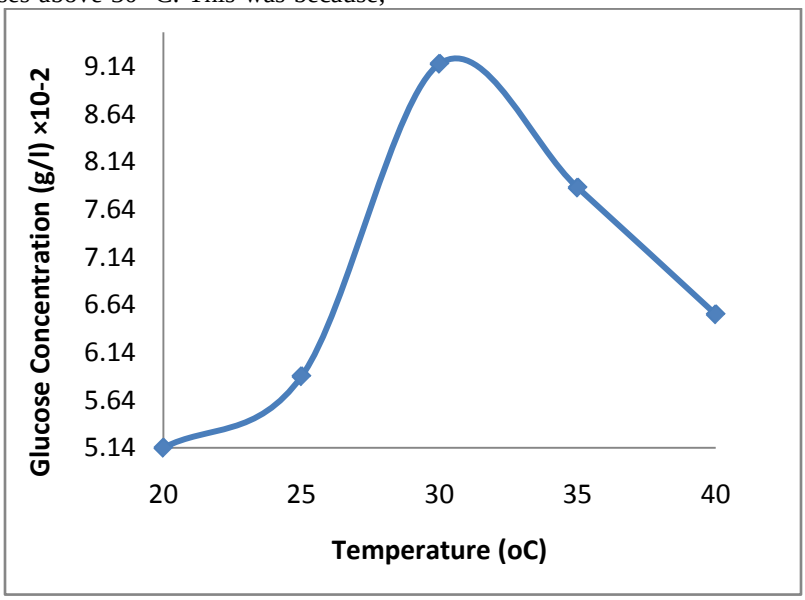

Figure 6: Effect Temperature on Glucose Production from Watermelon peel

Figure 7 and 8 shows the composite effect of temperature and pH on *T. reseei* Cellulase activity in glucose production from watermelon peel.

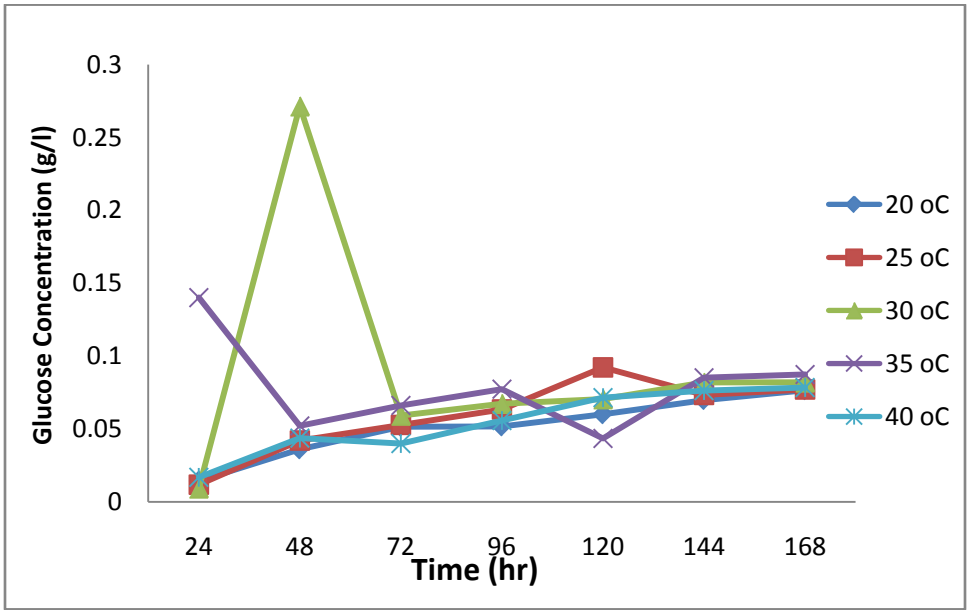

Figure 7: Composite Effect of Temperature on Glucose production from watermelon peel

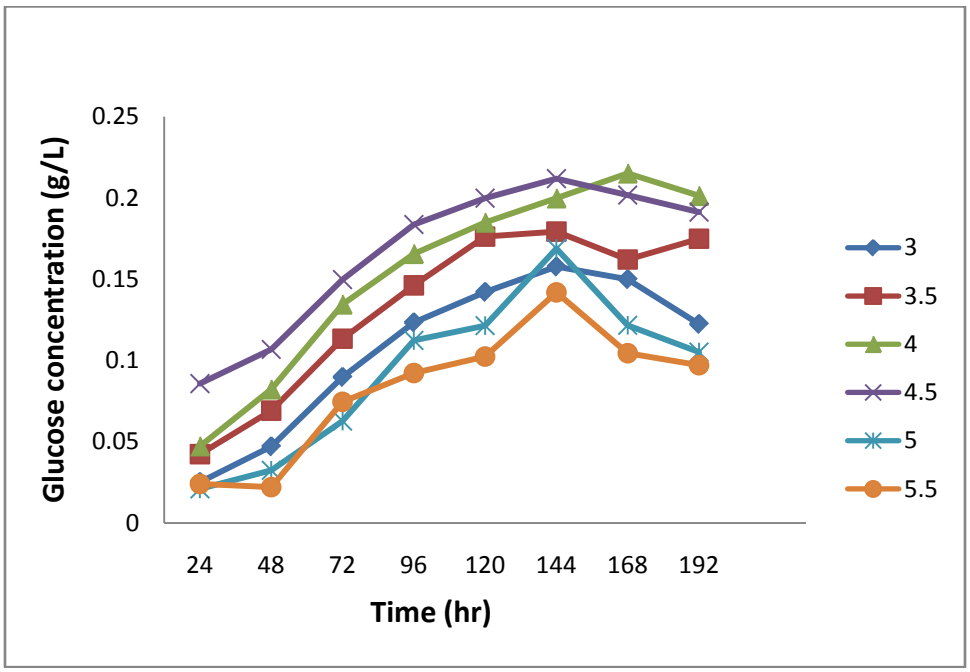

Figure 8: Composite Effect of pH on Glucose Production from Watermelon Peel

From figure 7, it can be inferred that glucose concentration was at the maximum of 0.1402 g/l in the 24th hour at 35 ^{o}C and gradually decreases to 0.04353 g/l in the 120th at the same temperature. Also, high glucose concentration of 0.27145 g/l was produce in the 48th hour at 30 ^{o}C which gradually deceases to 0.04005 g/l in the 72 hour at same temperature. High enzyme activity was observed between the temperatures of 20 ^{o}C to 35 ^{o}C. From Figure 8, it can be seen that high glucose concentration was found in the reactor with pH of 4.5, followed by the rector with pH of 4.0. Maximum enzyme activity was observed in the 120th hour at pH of 4.5. Enzyme activity generally decreases as time increases because substrate concentration decreases.

IV. CONCLUSION

The effect of pretreatment, temperature and pH in glucose production using *T. reseei*fungus was investigated. It was shown that high deliginification was achieved by pretreating the sample and as result the hemicellulose were well broken which led to the exposure of the cellulose for the enzymes to act upon, yielding higher glucose production from watermelon dry peel. The effects of temperature at 20, 25, 30, 35, 40 and 45 ^{0}C as well as the effects of pH on enzyme activity at pH of 30 ^{0}C with corresponding glucose concentration of 0.6436 g/l.

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