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Nano-Enzymatic Hydrolysis and Fermentation of Waste Starch Sources for Bioethanol Production: An Optimization Study

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Abstract

With the inevitable depletion of the world's energy supply and the rising pollution issues, there has been an increasing worldwide interest in alternative energy sources. One of the best options to beat this energy crisis is biofuel. Bio-ethanol is the best biofuel that can be produced by simply converting the sugar content of any starchy material into alcohol with the evolution of CO_2 under controlled environmental conditions. More quantitative ethanol production can be carried out using a hydrolyzed starchy source. The hydrolysis of the starch is achieved through various methods, viz. acid hydrolysis, heat treatment, and enzymatic treatment, out of which the enzymatic method of hydrolysis shows prevalence. In the present study, the hydrolysis of starch sources is carried out by a nano-enzyme bio-conjugate. The enzyme used is α-amylase in association with silver nanoparticles. Previous studies indicate the efficacy of nano-enzymatic bio-conjugate, i.e., silver nanoparticles in association with á-amylase, showed a 2-fold increase in its efficacy in reaction mixtures over converting the substrates to products. Thus the usage of the catalys, silver nanoparticles-α-amylase bio-conjugate in the reaction mixture enhances the reaction rate in hydrolyzing the starch sources, thereby, more breakdowns of the sources be enabled in lesser time. The waste starch sources used in the current study are corn waste, rice husk, and potato peels which can reduce the economy of biofuel production. In this study, pretreatment of waste starch sources for hydrolysis was carried out using nano-enzyme bio-conjugate. Further to this, the efficacy of the hydrolyzed starch source in producing bioethanol was assessed in comparison with the non-hydrolyzed starch source when subjected to fermentation of hydrolyzed and non-hydrolyzed sources using baker's yeast for 16hrs. The percentage of ethanol produced from hydrolyzed and non-hydrolyzed sources is estimated by gas chromatography. The factors affecting the bioethanol production are estimated by optimizing various ethanol process parameters, viz. time, pH, temperature, concentrate of starch source, and biomass concentration by the yield of bio-ethanol produced. The maximum percentage of bioethanol produced using hydrolyzed starch sources using nano-enzyme catalyst under the optimized condition is 63% in comparison with non-hydrolyzed sources, which was 13%.

Keywords: *Bioethanol, biofuel, α-amylase, silver nanoparticles, optimization, cost estimation*

1.0 Introduction

Global warming and an ever-growing need for liquid fuels in recent years have made bioethanol a possible gasoline substitute[1-4]. First-generation bioethanol produced from edible crops has sparked debate about various social concerns, including food security and rivalry with food sources[4]. The majority of the carbohydrates used as a source to produce bioethanol come from plants that contain more amount of sugar or starch, viz. maize, sugarcane, sweet

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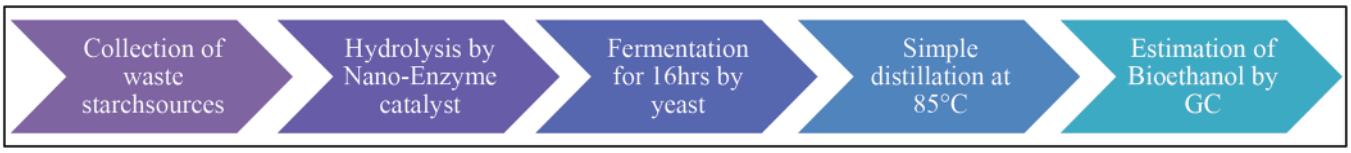

Figure 1: Process flow diagram of Bioethanol production

sorghum, or lignocellulose biomass. These crops, which include corn, wheat, and other grains, as well as waste straw, willow and other common trees, sawdust, reed canary grass, cord grasses, miscanthus, and sorghum plants, are planted explicitly for energy usage[1]. Ethanol (C_2H_5OH) is an inert liquid that is biodegradable, has a low degree of toxicity, and only slightly pollutes the environment. When C_2H_5OH burns, water and carbon dioxide are produced. Lead has been replaced with ethanol or ethyl alcohol as the preferred octane booster in gasoline. By blending ethanol with gasoline, which oxygenates the fuel combination and helps it burn thoroughly, emissions can be reduced substantially.

The present study focuses on ethanol production from waste sources such as potato (Solanum tuberosum) peel, rice (Oryza sativa) husk, and American sweet corn (Zea mays) waste. Literature survey shows an immense study of using hydrolyzed sources for a better yield of bioethanol in a shorter time. The chosen waste sources in the present study for bioethanol production are hydrolyzed using the nano-enzyme bio-conjugate method with enzyme α-amylase and silver nanoparticles. In our previous study, substantial evidence of increased stability and activity of the enzyme α-amylase when combined with silver nanoparticles is proved[4]. The enzyme kinetics and the behavior pattern under different parameters during starch hydrolysis reactions were studied[6]. The sources selected for the current study are based on the starch content and possibly the waste materials used.

The hydrolysis of the starch sources in the current study was carried out by the enzyme nano-enzyme conjugate, i.e., α-amylase with silver nanoparticles, followed by fermentation. For attaining maximum hydrolysis and high yield of bioethanol production during fermentation, the parameters viz. the effects of substrate concentration, pH, and temperature on the enzymatic hydrolysis of waste starch sources were studied.

2.0 Material and Methods

2.1 Collection of source

The waste potato (Solanum tuberosum) peels, rice (Oryza sativa) husks, and American sweet corn (Zea mays) waste from the local vendors were collected. The wastes were weighed (50g) and then washed under running tap water to remove any surface contaminants and soaked in water for 30 min. 100mL of water was added to the samples and blended in a mixer to make a homogenized solution of 50g/100mL concentration.

2.2 Starch estimation

The starch content present in the chosen samples were estimated by iodine test. The standard starch (AR grade, Sigma Aldrich) solution of 100mg/100mL was prepared, from which aliquots of different concentrations were prepared, and a standard graph was plotted. From the standard graph, the concentrations of starch present in the samples were estimated.

2.3 Nano-enzyme catalyst for hydrolysis

The enzyme used for the starch hydrolysis process for the selected sources was 1% a-amylase (procured from HIMEDIA). The silver nanoparticles used were green synthesized from the plant extract Jatropha curcas under optimized conditions[6]. The green synthesized silver nanoparticles were characterized using SEM and EDX for structural analysis. The silver nanoparticles of concentration 5µg/mL were incubated at 37°C in an incubator with 1% of a-amylase for 30 min to make a nano-enzyme catalyst of concentration 5µg of silver nanoparticles/10 mg of a-amylase/mL. This nano-enzyme bio-conjugate (silver nanoparticles with a-amylase) was used to hydrolyze the starch substrate.

2.4 Yeast culture

The baker's yeast (Saccharomyces cerevisiae) was purchased from the local supermarket and activated in lukewarm water for 5 min. 5g of yeast cells were weighed and diluted in 50 mL of water to make a concentration of 5g/50mL. The concentration of yeast used for the fermentation process was 0.1g/mL.

2.5 Hydrolysis

The hydrolysis of the selected starch sources by the nano-enzyme catalyst were carried out as an intermediate step before fermentation. The starch sources were pre-treated with nano-enzyme catalysts in order to decrease the size of the feedstock and open up the plant structure in order to obtain sugars from the selected starch sources thereby enabling more quantitative production of bioethanol in lesser amount

of time. Enzymes or weak acids are used to hydrolyze the starch and hemicellulose parts to produce glucose/maltose sugar, which is fermented to produce ethanol[3].

In the present study, 100 mL of the homogenized starch sources were hydrolyzed with 10 mL of nano-enzyme catalyst (1% α-amylase and 5µg/mL of silver nanoparticles) under room temperature for 2hrs.

2.6 Fermentation

The fermentation was carried out in an Erlenmeyer flask with a stopper under anaerobic conditions with hydrolyzed starch sources (potato peel, rice husk, and corn waste) and 10 mL of the yeast (with a concentration of 0.1g/mL). A reference flask containing 100mL of non-hydrolyzed starch sources (potato peel, rice husk, and corn waste) was also fermented using yeast (with a concentration of 0.1g/mL). The flasks were incubated in a shaker incubator (130 rpm) at 30°C for 48 hrs.

2.7 Distillation

The fermented flasks of hydrolyzed and non-hydrolyzed starch sources were subjected to simple distillation at 85°C to collect the bioethanol formed. The volume of distillate collected during simple distillation is noted, and the concentration of the bioethanol present in distillate was estimated using gas chromatography (GC).

2.8 Estimation of bioethanol

Gas chromatography (GC) was used to measure the amounts of bioethanol using a Mayura Analytical GC Model 1100 fitted with a flame ionization detector. The capillary column of Internal Diameter (I.D.) of 0.2 cm and has a length of 2.0m. The working temperature 225°C was maintained at the injection port and detector. The oven's initial temperature was set to 100°C for 2 minutes, and then raised to 225°C at a rate of 10°C per minute and maintained for 9.5 minutes. A sample injection of 200 µL was made for each GC analysis. The carrier gas was helium, and the internal standard was 2-pentanone at a concentration of 0.5 per cent (v/v).

The percentage of bioethanol produced in each sample was determined using the area under the peak.

2.9 Optimization of bioethanol production conditions

The yield of bioethanol production was optimized under various reaction conditions, viz. time, pH, temperature, substrate concentration, and biomass concentration.

2.9.1. Effect of time

To study the suitable time required to produce a substantial quantity of bioethanol, hydrolysis time required to hydrolyze the reaction mixture containing the starch source (1.5% w/v) and nano-enzyme catalyst of 10mL was exposed to different time intervals at room temperature and neutral pH as 2 hrs, 3hrs, 4hrs, 5hrs, and 6 hrs. The flasks containing non-hydrolyzed starch source flasks were maintained at the same conditions as a reference for comparison. Further to the hydrolysis at different time intervals, the flasks of hydrolyzed and non-hydrolyzed sources were fermented with yeast for 48 hrs.

2.9.2 Effect of pH

To study the suitable pH required for bioethanol production, the hydrolysis of the reaction mixture containing the starch sources (1.5% w/v) and the nano-enzyme catalyst (10mL) was exposed to different pH of 4, 6, 8, 10, and 12 for 3 hrs at room temperature as. The pH of the reaction mixtures was maintained using 0.1 N HCl and 0.1 N NaOH solutions. The flasks of the non-hydrolyzed sources were also maintained at the same pH without the nano-enzyme catalyst. Furthermore, the flasks of hydrolyzed sources and non-hydrolyzed substrates were fermented with yeast for 48 hrs.

2.9.3 Effect of Temperature

To study the suitable temperature for quantitative yield of bioethanol, the hydrolysis of the reaction mixture containing the starch substrate (1.5% w/v) using nano-enzyme (10mL) catalyst was exposed to different temperatures as 30°C, 40°C, 50°C, and 60°C at neutral pH for 3hrs. The flasks of the non-hydrolyzed sources were also maintained at the same temperatures without the nano-enzyme catalyst. The flasks of hydrolyzed sources and non-hydrolyzed sources were fermented with yeast for 48 hrs.

2.9.4 Effect of Substrate concentration

To study the suitable substrate (starch source) concentration required to produce substantial yield of bioethanol, the hydrolysis of the reaction mixture containing the starch substrate with different concentrations (0.5%, 1.0%, 1.5%, 2%, and 2.5% w/v) and nano-enzyme catalyst (10mL) was exposed to neutral pH for 3hrs at 30°C. The same substrate concentrations were maintained for non-hydrolyzed substrates without a nano-enzyme catalyst, and both sets of flasks were fermented with yeast for 48 hrs.

2.9.5 Effect of Biomass concentration

To study the suitable concentration of biomass required to ferment and yield bioethanol, various concentrations of the biomass were used. The hydrolysis of the reaction mixture containing the starch sources of constant concentration (1.5% w/v) with nano-enzyme catalyst (10mL) was exposed to neutral pH for 3hrs at 30°C. The non-hydrolyzed flasks were

also maintained at similar conditions without nano-enzyme conjugate. During fermentation, the biomass concentration was varied for the hydrolyzed and non-hydrolyzed flasks as 0.05, 0.1, 0.5, and 1 g/mL.

2.9.6 Estimation of glucose concentration

1mL of the sample was collected periodically from the flasks subjected to hydrolysis maintained at different time intervals, pH, temperature, substrate concentration, and biomass concentrations. The glucose content in the hydrolyzed substrate was estimated by the Ortho Toluidine method. Standard glucose of 100mg/mL (AR grade, Sigma Aldrich) was diluted and aliquot into different test tubes, making a concentration range from 0 to 100 mg/mL. Ortho Toluidine reagent was added and heated in a water bath for 20 min. The standard graph was prepared using the known concentrations. The samples drawn from the hydrolyzed flasks were subjected to the Ortho Toluidine test. The concentration of the glucose present in the hydrolyzed flasks was estimated from the standard graph.

3.0 Result and Discussion

3.1 Green synthesized nanoparticles

The green synthesized silver nanoparticles were characterized using SEM (Figure 2) for their size determination and structural morphology. The size of the silver nanoparticles was estimated as 41nm and spherical in shape. The EDX profile gives the elemental analysis in which the peak of the silver ions was observed around 3KeV and found to be 16% of the total composition.

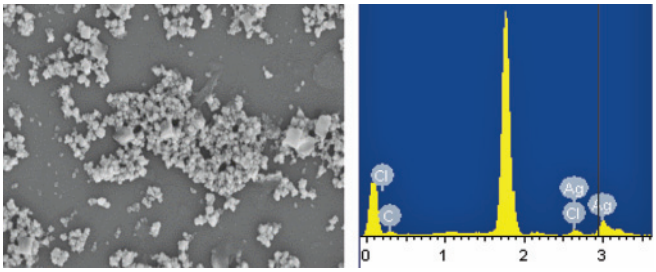

Figure 2: SEM and EDX image of silver nanoparticles

3.2 Starch estimation

The starch content present in the selected homogenized starch sources is shown in Table 1.

From the result, it is seen that the corn waste has the maximum starch content of 0.98 mg/mL, potato peel of 0.93 mg/mL and Rice husk of 0.8mg/mL.

As shown in Table 2, the corn waste produced a maximum percentage yield of bioethanol compared to the other sources. Corn waste is considered a significant raw material in bioethanol production in countries like the USA, where more usage of bioethanol as an alternative fuel has been reported. For further study on optimization of process parameters to increase the yield of bioethanol, only corn waste is chosen as it contains more starch content as well as more quantitative bioethanol production capacity[2].

3.3 Optimization of Bioethanol Production

The effects of the parameters selected for the optimization study were performed on corn waste as a starch source. The percentage production of the bioethanol was estimated using GC. The following are results obtained on optimizing various parameters for bioethanol production.

3.3.1 Effect of time

The effect of time on hydrolysis for bioethanol production was studied as in Figure 3. As time increases, the bioethanol produced increases and subsequently the glucose concentration increases until it reaches a saturation point. The bioethanol produced in hydrolyzed starch source at 3 hrs is 61%, whereas beyond 3hrs there is a very gradual increase

Table 1: Starch content present in selected starch sources

Starch source	Concentration of starch
Corn waste	0.98 mg/mL
Potato peel	0.93 mg/mL
Rice husk	0.8 mg/mL

Table 2: Bioethanol yield and volume collected from selected starch sources

Starch Source	Bioethanol yield (%)		Bioethanol Volume collected (mL)	
	Hydrolyzed	Non-Hydrolyzed	Hydrolyzed	Non-Hydrolyzed
Corn waste	63	14.8	52.3	22.3
Potato peel	58	12	42	28
Rice husk	42	10.2	35	20

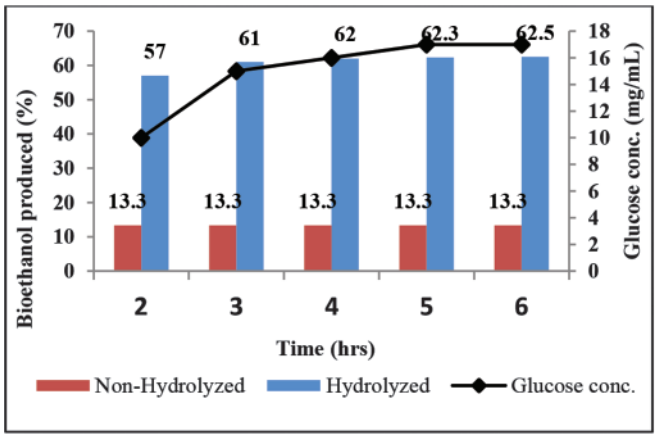

Figure 3: Effect of time on Bioethanol production

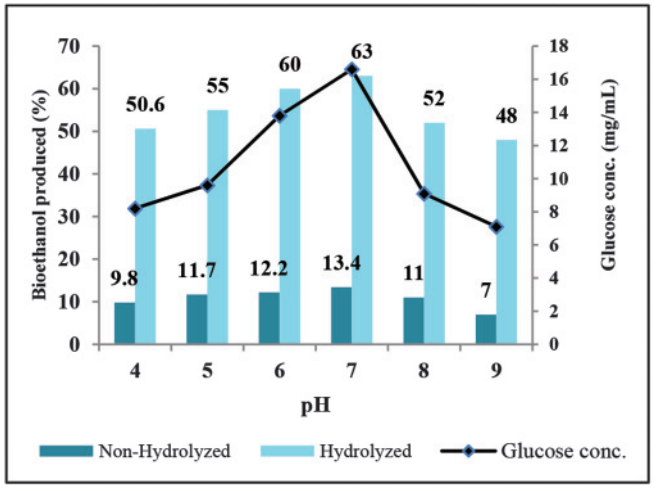

Figure 4: Effect of pH on Bioethanol production

indicating that the hydrolysis reaction has come to saturation. The bio-conjugate catalyst have acted on the corn source, and the active sites present on the surface of α-amylase would have gotten saturated as all the substrates got attached to the active sites making the reaction to attain saturation in 3 hrs. The presence of nano-enzyme catalyst has enhanced the activity of the amylase and resulting in production of more bioethanol in hydrolyzed condition. The glucose concentration produced in hydrolyzed is found to be 16 mg/mL, which is also consistent after 3 hrs confirming there is saturation in reaction and no hydrolysis beyond 3 hrs of time. The non-hydrolyzed source starch source produced an ethanol yield of 13.2%.

3.3.2 Effect of pH

The results of the experiment in Figure 4 show how pH affects the hydrolysis of corn waste collected. It is observed that at neutral pH 7 maximum yield of bioethanol is 63%. The non-hydrolyzed source produced bioethanol of 13% at neutral pH. The glucose concentration improved from 5mg/mL to 17mg/mL when the pH of the medium was raised from 4 to 7. This observation is in line with[8], who discovered that the ideal glucose concentration was at a pH of 7. However, the glucose production significantly decreased when the pH rose above 7; as a result the bioethanol production also decreased. The finding that pH has an impact on glucose production can be explained in the manner as follows. The enzyme's activity increased, and more starch biomass was hydrolyzed when the pH was raised from 4 to the pH 7 that amylase prefers. The enzyme was most active and produced the highest amount of glucose at pH 7. The enzyme's activity then gradually decreased when pH was raised above its optimal level. The electrostatic interaction between the enzyme and substrate during the hydrolysis process is impacted by pH value changes[9]. As a result, when an enzyme's charges are disturbed, the enzyme's 3D shape changes, rendering the active site unsuitable for catalyzing hydrolysis processes[10]. From pH 7 to 9, the fall in glucose level in hydrolyzed samples from 17 mg/mL to 5mg/mL was observed. The bioethanol produced proportionally decreased. The presence of nano-enzyme catalyst has increased the production of bioethanol with 63% a maximum at pH7.

3.3.3 Effect of Temperature

Another factor that significantly affects enzyme activity is temperature. Temperatures between 30°C and 70°C were used to hydrolyze solid corn wastes using nano-enzyme bio-conjugate. After 3 hours of hydrolysis, it was found that glucose yeild first increased to 16 mg/mL with a temperature rise from 30°C to 50°C and subsequently decreased to 6 mg/mL with a further temperature increase to 70°C, as shown in Figure 5. The highest glucose production of 17mg/mL was obtained at a temperature of 60°C, which was the ideal nano-enzymatic hydrolysis temperature. The ideal temperature for the amylase activity is usually between 40°C to 50°C; the presence of nanoparticles with the amylase in nano-enzyme conjugate would have increased the thermal resistance of the enzyme and its maximum activity at 60°C. Additionally, it has been found that this temperature works well for the enzymatic hydrolysis of several starch biomass sources[9,10]. Cold glucose yields were seen at a temperature below 50°C, which may have happened because the enzyme's activity is inhibited by low temperatures. Once the temperature was elevated to 70°C the glucose content was substantially lower than the yields at lower temperatures. The kinetic energy of enzymatic processes was impacted by temperature changes, which raises the likelihood of a source colliding with an enzyme's active sites. Thus, heat agitation may lead to the denaturation of enzymes, which would reduce the number of active sites[10].

Figure 5 shows the effect of temperature on the production

of bioethanol. It is observed that at 60°C maximum amount of bioethanol produced is 63% from the hydrolyzed substrate. The non-hydrolyzed source produced the maximum bioethanol at 12% at 50°C; further it is seen decreasing. It is seen that adding nanoparticles to the enzyme has increased the optimum temperature of amylase activity from 50°C to 60°C, increasing the thermal stability of the enzyme.

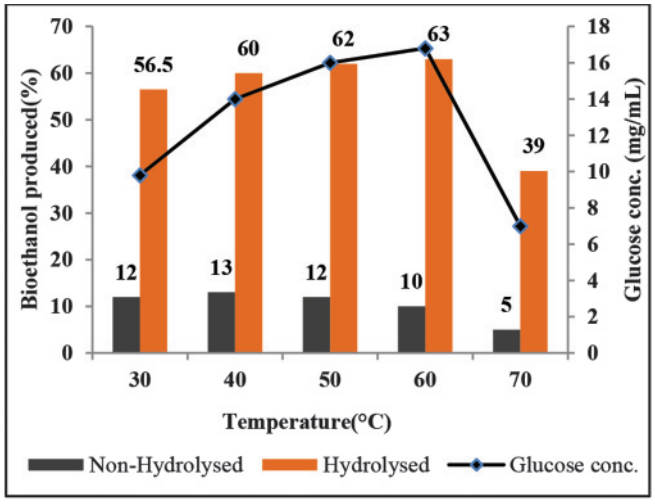

Figure 5: Effect of Temperature on Bioethanol production

3.3.4. Effect of substrate concentration

The concentration of the starch source (corn waste) for the study impacts the bioethanol production. The optimum substrate concentration required for the enzyme activity was studied and depicted in Figure 6. It is seen that there was an increase in bioethanol production as the substrate concentration increases

The hydrolysis efficiency of the catalyst (nano-enzyme bio-conjugate) also increases till it reaches the saturation point. Further to the increase in the substrate, there is no more increase in the production of bioethanol indicating that the enzyme has reached its saturation point where no more active sites are available for the enzyme to act. Proportionally the glucose increase was observed to change from 7 mg/mL to 17.2 mg/mL. This shows that the increase in substrate concentration increases the production from 55% to 67% when the substrate concentration is increased from 0.5% to 2%, further to this, there is no more increase in bioethanol percentage. The glucose production significantly increased when substrate concentration was raised from 0.5% (w/v) to 2% (w/v). Other studies have also reported finding the optimal substrate concentration[8,9]. A low glucose yield results from end-product inhibition and mass transfer limits within the reaction mixture brought on by the high viscosity of the slurry due to the high concentration of the corn waste, which

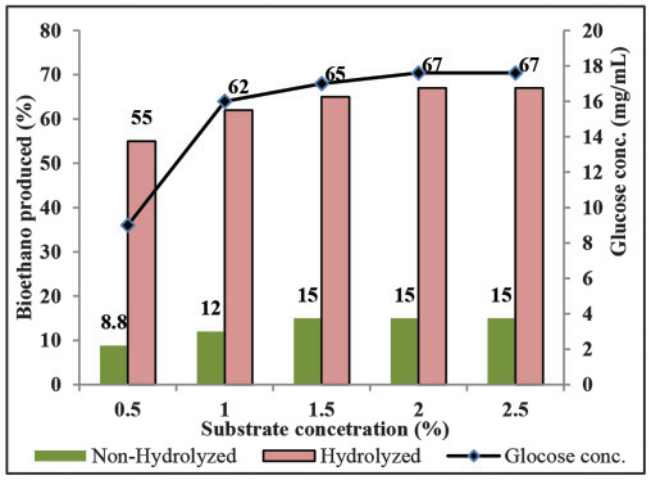

Figure 6: Effect of substrate concentration on Bioethanol production

is why this optimal concentration is required. This study shows us that the ideal concentration of substrate used for the conversion can be 1.5%

3.2.5 Effect of Biomass concentration

The optimum biomass concentration for fermentation impacts bioethanol production. The glucose concentration increased from 6 mg/mL to 17 mg/mL when the biomass was increased from 0.025 g/mL to 1 g/mL. Proportionally the production of bioethanol increased from 40 % to 65%. Thus indicating the increase in bioethanol production with an increase in biomass. The maximum bioethanol production of bioethanol was observed at a biomass concentration of 0.5 g/mL with an ethanol concentration of 63%. Beyond a particular concentration of biomass, the bioethanol production has reached saturation as there was no more substrate available for the biomass to convert it into

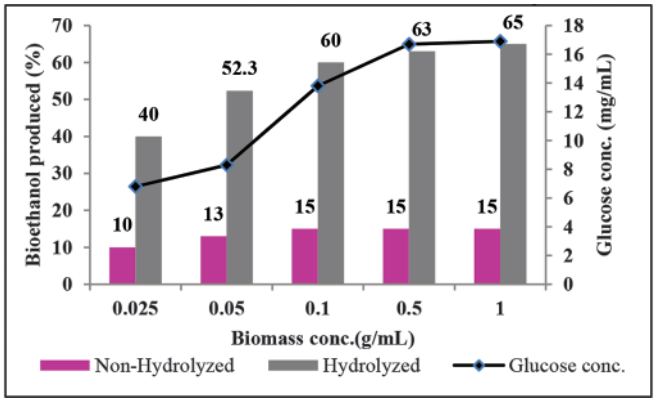

Figure 7: Effect of biomass concentration on bioethanol production

bioethanol. The same study repeated for the non-hydrolyzed source shows that there was no more increase in bioethanol percentage even when the biomass concentration increases as the starch source was in complex form for the biomass to convert to bioethanol. This shows that in non-hydrolyzed flasks the biomass concentration did not have an impact on bioethanol yeild which produced a maximum of 15%. This study was conducted to find the optimum concentration of biomass that can be used for fermentation of hydrolyzed sources can be 0.5 g/mL.

4.0 Conclusions

The present study clearly shows us the efficiency of the nano-enzyme bio-conjugate in hydrolyzed corn waste for the production of bioethanol. Due to the high cost of many enzymes as well as the inhibitory properties of certain compounds on enzyme, it can lower the efficiency of glucose production, the enzymatic hydrolysis process is currently still a difficult path to the efficient production of bioethanol. Using α-amylase-Silver nanoparticles bio-conjugate as a catalyst for enzymatic hydrolysis can be a low-cost method that boosts the value of the bioethanol production is safe for use. The hydrolyzed substrate using the nano-enzyme catalyst showed an enormous increase in bioethanol production compared to non-hydrolyzed waste starch source. On optimizing the reaction conditions, more quantitative bioethanol production can be enabled. It is seen a maximum production yield of 63% of bio-ethanol is obtained from the hydrolyzed source under optimized conditions. In comparison the non-hydrolyzed starch source showed a maximum bioethanol production of 13%. As nations all over the world have started their journey aggressively to pursue the use of ethanol as an alternative fuel, the future of ethanol fuels appears to be more on demand. Since its production can lessen the adverse effects of greenhouse gas emissions from fossil fuels and thus limit global climate change, bioethanol can significantly decarbonize our future energy needs. When compared to other fuels, commercializing ethanol production will make it a more affordable and green fuel. In future studies, scale up of waste starch sources can be done for bioethanol conversion, which is a waste-to-wealth technology, and cost estimation per liter of bioethanol can be done.

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