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ENZYMATIC HYDROLYSIS OF BREADFRUIT STARCH: CASE STUDY WITH UTILIZATION FOR GLUCONIC ACID PRODUCTION

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ABSTRACT

Enzymatic hydrolysis of breadfruit starch using alpha amylase and glucoamylase were investigated. The possibility of using the hydrolysate obtained to produce gluconic acid compared to synthetic glucose was also studied. The optimum temperature for hydrolysis was established as 60 and 50°C for liquefaction and saccharification steps, respectively. The pH and calcium ion were observed to be factors that increased the rate of hydrolysis. The optimum conditions yielded Dextrose Equivalent (DE) of 20 for the liquefaction step after 40 min and increased to 90 during saccharification step 60 min later. The hydrolysate produced was used to culture *Aspergillus niger* at pH of 5.5 for gluconic acid production. The breadfruit hydrolysate medium yielded 97.20 g/l of gluconic acid from 100 g/l of breadfruit hydrolysate while medium with synthetic glucose as carbon source produced 83.52 g/l of gluconic acid after 72 h of fermentation. About 80% of the breadfruit hydrolysate had been consumed within the first 24 h of cultivation while approximately 90% of the synthetic glucose was consumed within the same period. Maximum biomass concentrations observed were 39.42 and 32.32 g/l for breadfruit hydrolysate and with synthetic glucose media, respectively. These results indicated that breadfruit hydrolysate like other carbon sources can be used to substitute synthetic glucose to produce gluconic acid and this can be scaled-up in a pilot plant.

Keyword: *Breadfruit, enzyme, hydrolysis, Aspergillus niger, gluconic acid*

1. INTRODUCTION

Starch is a major storage product of many economically important crops such as cassava, corn, sorghum, wheat, rice and potato. Beside cellulose, starch is the most abundant carbohydrate in the world [1]. It is used by the plants themselves, by microbes and by higher organisms. The primary industrial use of starch is its hydrolysis to sugar syrups that are employed by the food industry to make sweets, drinks, juices and for fermentation into products like citric acid, ethanol, bakers' yeast, as well as in paper and textile industry [2], [3]. Traditionally, it is hydrolysed by acid catalysis but the method has now largely be replaced by enzymatic processes, as it required the use of corrosion resistant materials, gave rise to high colour and salt ash content (after neutralisation), needed more energy for heating and was relatively difficult to control [4]. The two steps involved in enzymatic process are liquefaction and saccharification [5]. In the first step, the enzyme α-amylase cleaves 1,4-glycosidic bonds to yield shorter chains of soluble dextrins. In the second, the enzyme glucoamylase attacks 1,4 terminal bonds in the degraded molecules to release one glucose unit at time. However, the degradation of starch to glucose is never complete, so the end product is typically a mixture of glucose, maltose and longer-chain-length sugars. The values of optimum pH, temperature and other operating conditions differ between the two steps [6].

Studies of enzymatic hydrolysis of starch from many plants such as cassava [7],[8], corn [9], wheat [10], potato [11] etc. have been widely reported. Solomon et al. [12] reported the use of sorghum malt, combination of sorghum malt and termamyl as well as acid hydrolysis of breadfruit starch. Most reports on breadfruit have been on the nutritive value or physicochemical properties of the fruit. Breadfruit, Artocarpus communis, is a tropical fruit which is grown in Africa [13]. It was introduced to Ifewara, South Western, Nigeria from the Caribbeans and spread to the neighbouring villages. In the South Western Nigeria, it is regarded as poor man's substitute for yam [14]. But elsewhere it is used in a variety of food preparations such as cakes, syrup, jam, cooked into puddings or baked, roasted or fried as chips [15]. The breadfruit has been made into flour and evaluated in bakery products [16],[17]. Adewusi et al. [18],[19] reported the possible utilisation of breadfruit as a component of weaning diets and also as a good source of carbohydrate with complementary protein content. The starch content of unripe mature breadfruit pulp is 77% [14].

The aim of this work was to study the enzymatic hydrolysis of breadfruit starch by examining the effects of temperature, pH and calcium ion on the hydrolysis. This was with a view to establishing the optimum conditions for obtaining high yield of glucose syrup. Prospect of using the breadfruit hydrolysate to produce gluconic acid using *Aspergillus niger* was also investigated.

2. Materials And Methods

2.1 Materials

2.1.1 Breadfruit

Freshly harvested matured but unripe fruits from breadfruit tree were obtained from Ile-Ife, Osun State, Nigeria. The breadfruits were washed in clean water to remove adhering latex and dirt. They were manually peeled and sliced to pieces with a stainless steel knife. The sliced fruits were sun-dried to constant weight and milled to flour.

2.1.2 Enzymes

Alpha-amylase (E.C.3.2.1.1) from bacterium source (*Bacillus licheniformis*) and glucoamylase (E.C.3.2.1.3) from *Aspergillus niger* were both obtained from the Federal Institute of Industrial Research, (FIIRO), Oshodi, Lagos, Nigeria.

2.2 Enzymatic Studies

2.2.1 Starch Hydrolysis with Alpha Amylase

The flour obtained was made into starch slurry by adding appropriate quantity of water. To make 10% slurry, 10 g of flour was weighed into 100 ml distilled water to make slurry. The solution of 40 ppm Ca^{2+} was added for stability of the enzymes. The pH was adjusted to 6.5 with Citrate-phosphate buffer. Gelatinization was done by heating the mixture to 97°C and was held at this temperature for 10 min. The gelatinized starch was cooled to 72°C. Liquefaction was carried out by adding 2% (w/v) of α-amylase for 110 min at this temperature [12]. Samples were withdrawn at regular intervals to follow the kinetics. The enzyme activity was stopped by heating the mixture to 97°C for 15 to 20 min. The procedures described above were done in duplicates.

2.2.2 Studies of Effect of Temperature on Liquefaction

The procedures described above were repeated for the studies on the effect of temperature on liquefaction. The gelatinized starch was liquefied at 50, 60 and 70°C for 40 min. The liquefied starch was buffered to pH of 4.5 and subsequently saccharified with glucoamylase (2 % w/v) at 50°C for 70 min. Samples were withdrawn at regular intervals to follow the kinetics.

2.2.3 Studies of Effect of Temperature on Saccharification

Gelatinization and liquefaction steps were carried as described above. The gelatinized starch was liquefied at 60°C for 40 min. Saccharification of the liquefied starch was carried out at 40, 50 and 60°C for 70 min. Samples were withdrawn at regular intervals to follow the kinetics.

2.2.4 Studies of Effect of pH and Ca2+ on Starch Hydrolysis

The procedures described in Section 2.2.2 were repeated for the studies on the effects of pH and Ca^{2+} on the starch hydrolysis. The gelatinized starch was buffered to pH of 6.5 and then liquefied at 60°C for 40 min. Subsequently, the liquefied starch was buffered to pH of 4.5 and saccharified with glucoamylase (2 % w/v) at 50°C for 70 min. These procedures were repeated with the addition of 40 ppm solution of Ca^{2+} to the starch slurry before carrying out the gelatinization. For the control, the pH values of the gelatinized and liquefied starch were not adjusted and Ca^{2+} solution was not added to starch slurry. Samples were withdrawn at regular intervals to follow the kinetics.

2.3 Gluconic Acid Production

2.3.1 Microorganism

A pure culture of *Aspergillus niger* strain provided by the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria was used throughout this study. The organism was maintained as direct stock culture from which inocula were prepared. Spore formation was carried out on Potato Dextrose Agar (PDA) at 30°C for 6-7 days and then the spores were stored in a 4°C refrigerator with regular sub-culturing.

2.3.2 Medium Composition

The preculture medium was composed of in g/l, glucose, 50; yeast extract, 3; malt extract, 3; polypeptone, 5; calcium carbonate, 20. Production medium was composed (g/l) of carbon source, 100; urea, 1.35; Na_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.15; Calcium carbonate, 60 [20]. The carbon source for production medium was glucose rich syrup obtained as breadfruit hydrolysate or synthetic glucose. All media and flasks were sterilized using an autoclave at 121°C for 25 min.

2.3.3 Inoculum Preparation

Five millilitres of the aseptically harvested spores from the sporulating surface washed with 50 ml sterile water was added to 50 ml of preculture medium in 250-ml Erlenmeyer flask. The inoculated flasks were shaken continuously on an environment-controlled incubator shaker manufactured (New Brunswick Scientific Co., USA) at 200 rpm and 30°C for 18 h before it was used for the fermentation process.

2.3.4 Shake Culture Experiment

For the gluconic acid production, 5 ml of preculture medium was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of production medium. The cultivation was carried out at 30°C at 200 rpm in an environment-controlled incubator shaker for 72 h. Samples were withdrawn at regular intervals for gluconic acid concentration analysis. Other separate samples were also collected, filtered, and the residue was processed for biomass concentration determination. While the clear supernatants were used for the reducing sugar analysis.

2.4 Analytical Methods

2.4.1 Gluconic Acid Concentration

A millilitre of sample from medium was mixed with 1 ml of fresh broth without calcium carbonate. Thereafter, 4 ml of 0.1 M of Ammonium oxalate ($(NH_4)_2C_2O_4.H_2O$) was added with 2 ml of 1/50 N ammonium hydroxide (NH_4OH) to the initial mixture. The mixture was then boiled for 10 minutes. Afterwards, it was cooled to room temperature. The mixture was centrifuged at 8000 g for 5 min and the supernatant was removed. The resulting precipitate was washed with distilled water for three times and dissolved by heating in the presence of 6 ml of 2 N Hydrosulphuric acid (H_2SO_4). The final solution was titrated against N/10 Potassium permanganate, $KMnO_4$ [21].

2.4.2 Reducing Sugar Concentration Assay

The DNS method described by Miller [22] and modified DNS Reagent was used to determine the reducing sugar concentration in this work. The fermented broths were collected and 3 ml of the DNS solution was added in the test tubes and was boiled for 15 min, cooled and diluted appropriately after which their absorbents were measured at a wavelength of 540 nm using the UV-Visible Spectrophotometer.

2.4.3 Biomass Concentration Determination

The filter paper was preweighed before it was used to filter the broth. The filtered mycelia mat was washed with acidified (4 mol l^{-1} hydrochloric acid) double distilled water to convert the insoluble calcium carbonate to soluble calcium chloride. The separated mycelia mat was washed several times with distilled water until pH of washing was neutral (7.0). The residue was oven dried at 80°C for 24 h to constant weight. After which it was allowed to cool and final weight was recorded. The weight of the biomass was determined by subtracting the weight of the filter paper from the weight of the paper plus the cells [23].

3. Results And Discussion

3.1 Enzymatic Hydrolysis of Breadfruit Starch

The results of breadfruit starch hydrolysis are depicted in Figures 1 to 4. When only bacterial alpha-amylase was employed for the hydrolysis at 72°C, maximum dextrose equivalent (DE) obtained was 16 after 40 min. The DE reduced to 9 after 60 min of hydrolysis and this remained constant thereafter (Figure 1). This observation may be attributed to the formation of maltulose (4-α-D-glucopyranosyl-D-fructose), which is resistant to hydrolysis by α-amylases as reported by Chaplin and Bucke [4]. The hydrolysis was repeated without prolong treatment of α-amylase at liquefaction stage at different temperatures (50, 60 and 70°C) followed by saccharification (Figure 2). The maximum DE observed using α-amylase was 16 for liquefaction step at 60°C and immediately glucoamylase was added for saccharification only at 50°C. DE was observed to increase in all three cases considered. The maximum value of 48 was observed in the sample with liquefying temperature of 60°C.

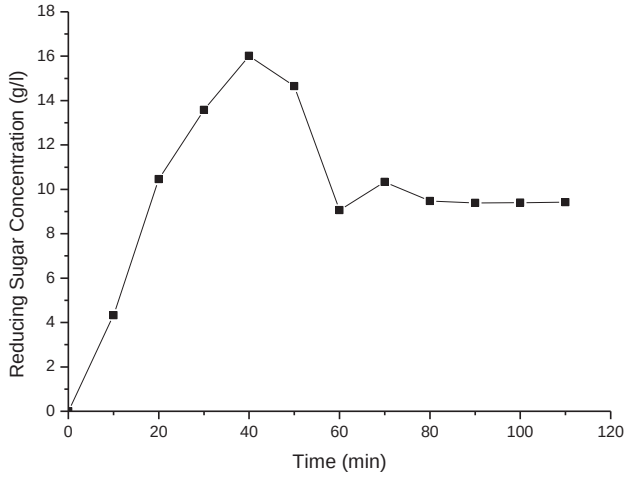

Figure 1: Hydrolysis of breadfruit starch using bacterial alpha amylase only at 72°C

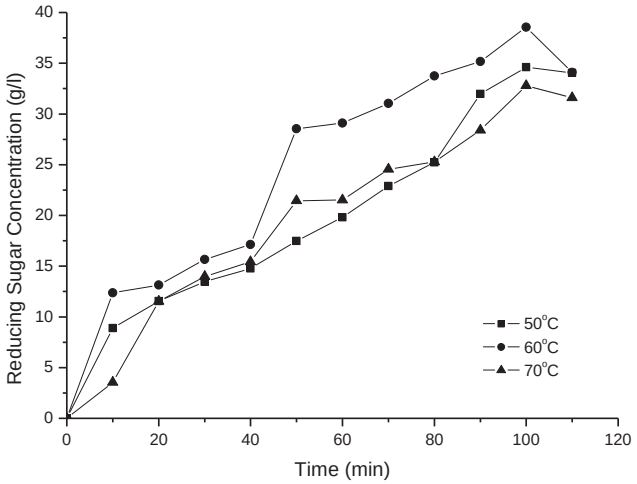

Figure 2: Hydrolysis of breadfruit starch using bacterial alpha amylase at 50, 60 and 70°C for liquefaction step.

Having established the optimum liquefying temperature to be 60°C, the saccharifying temperature was also investigated. The results are displayed in Figure 3. The highest DE of 48 was obtained at 50°C. This observation indicated that glucoamylase operates most effectively at 50°C for the hydrolysis of breadfruit starch. The duration of hydrolysis was comparably lower than that of other starchy materials. Solomon et al. [12] also observed the same period of hydrolysis for breadfruit flour.

Figure 4 showed results of breadfruit starch hydrolysis when the optimal temperatures of 60°C (liquefaction) and 50°C (saccharification) were considered with/without the pH adjustment and with/without Ca^{2+} addition. The control sample yielded maximum DE of 48 while the maximum DE was 75 when pH of both liquefaction and saccharification steps were adjusted. This increased to 90 with the addition of Ca^{2+}. This value doubled the observed DE when pH was not adjusted and the ion was not added. This indicated that pH and the ion had significant effect on the rate of hydrolysis of breadfruit starch. It has been reported that both pH and Ca^{2+} stabilise and activate enzymes (Chaplin and Bucke, 1990).

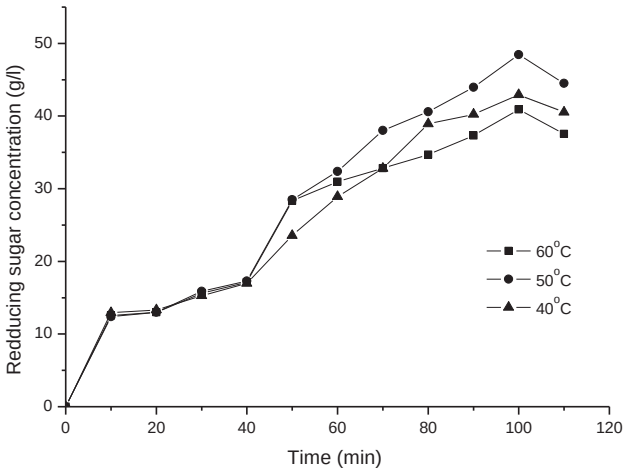

Figure 3: Hydrolysis of breadfruit starch using bacterial alpha amylase at 60°C and glucoamylase at 40, 50 and 60°C for saccharification step.

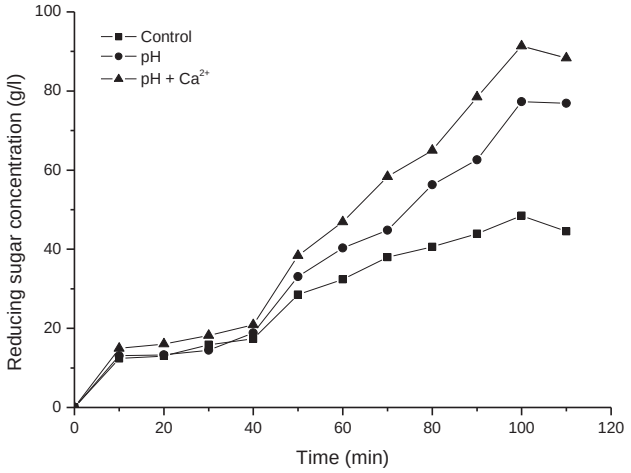

Figure 4: Hydrolysis of breadfruit starch using bacterial alpha amylase at 60°C and glucoamylase at 50°C with/without pH control and calcium ion addition.

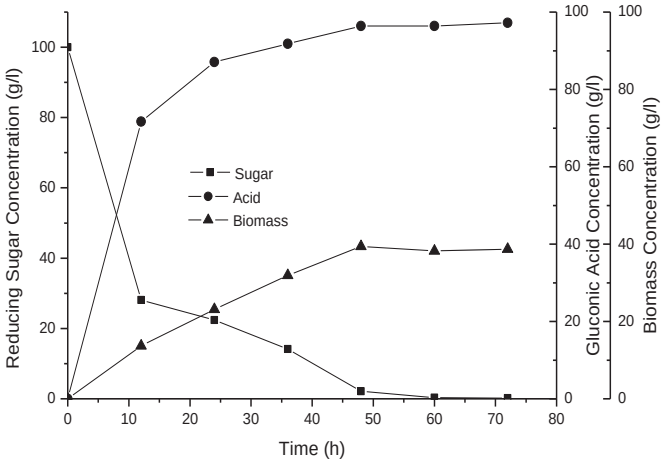

Figure 5: Plot of gluconic acid, reducing sugar and biomass concentrations against Time for the medium with 100 g/l of breadfruit hydrolysate as the sole carbon source at pH of 5.5 under shake culture.

3.2 Gluconic acid Production

The study investigated the prospects of using breadfruit starch hydrolysate as the sole carbon source for the production of gluconic acid using *Aspergillus niger* under shake culture. The mould showed no problem metabolizing the hydrolysate for growth and gluconic acid formation. More than 90 % of the hydrolysate had been consumed 48 h into the cultivation. It was observed that as the hydrolysate was being consumed there was a corresponding increase in the amount of the gluconic acid produced. The results showed that there was a direct relationship between the hydrolysate consumption and the quantity of gluconic acid produced. These results strongly supports the claim that enzymatic hydrolysate does not have any negative effect on gluconic acid production and can be used as an alternative carbon source in the culture of *Aspergillus niger* [24].

The biomass concentration production also increased linearly from the initial phase and reached a maximum after 48 h of cultivation. When synthetic glucose was used, similar profiles of results were obtained. While the breadfruit hydrolysate yielded 96.6% conversion, synthetic glucose gave 84.0% conversion. This observation is corroborated by the report of Ikeda et al. [20]. In their work, they produced 46.0 and 40.4 g/l gluconic acid from 50 g/l of hydrolysed waste paper and synthetic glucose, respectively. The corresponding yields of gluconic acid reported by same authors, based on glucose consumption were 92 and 80%, respectively.

Varieties of carbohydrates have been used for gluconic acid production. It has been suggested that the economics of fermentation of gluconic acid can be improved by using cheap raw materials, provided the producing microorganism can metabolise the particular carbohydrate. Mukhopadhyay et al. [25] and Singh et al. [26] successfully used some agro-food by products (grape must, banana must and sugarcane molasses) for gluconic acid. Vassilev et al. [24] used hydrol (corn starch hydrolysate) as fermentable sugar to produce gluconic acid while Rao and Panda [27] employed Indian cane molasses. The results obtained in this study compared favourably well with these past reports.

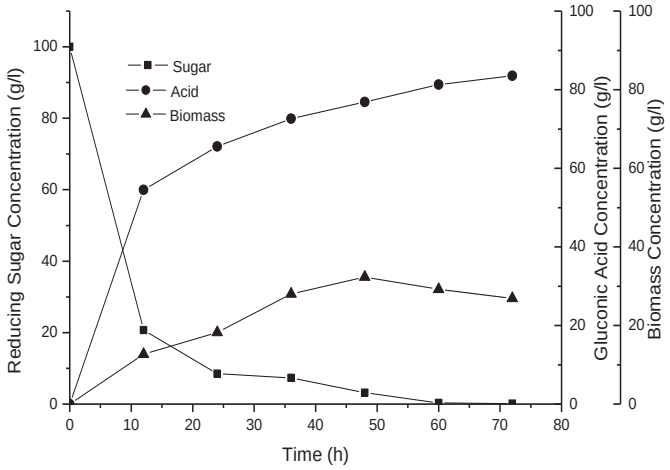

Figure 6: Plot of gluconic acid, reducing sugar and biomass concentrations against time for the medium with 100 g/l of synthetic glucose as the sole carbon source at pH of 5.5 under shake culture.

4. Conclusions

The results obtained in this research showed that 60°C (liquefaction) and 50°C (saccharification) are the optimum temperatures for hydrolysis of breadfruit starch using alpha – amylase and glucoamylase, respectively. It was found that pH and calcium ion can increase the yield of reducing sugar produce in the hydrolysis of breadfruit starch. This work showed that breadfruit hydrolysate can serve as alternative to other carbon sources in production of gluconic acid. The successful use of breadfruit hydrolysate for this purpose is novel and may reduce the cost of production, particularly, the price of the fruit and the fastness of the hydrolysis, unlike starches from many plants such as cassava, corn etc., in which hydrolysis last for 72 h.

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