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A Kinetic Study of the Enzymatic Hydrolysis of Cassava Starch

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Abstract- This paper presents the kinetics of the hydrolysis of cassava starch by amyloglucosidase. Dried cassava flour was hydrolyzed using amyloglucosidase at varying temperature, pH and substrate concentration and their effects carefully studied over time to determine suitable kinetic parameters for the production of glucose syrup. The result obtained showed the optimum temperature for the activity of amyloglucosidase in the production of glucose syrup to be 60^0C, with pH of 5.5 and optimum substrate concentration was obtained 3wt%. The Michaelis-Menten constant, K_m was found to be 0.167M and the maximum velocity of hydrolysis, V_{max} was found to be 0.08M/min. This work explains how the kinetic parameters were obtained and the path through which the optimum conditions were achived.

Keywords- *Chemical Kinetic; Enzymatic Hydrolysis; Cassava Starch.*

I. INTRODUCTION

Cassava, known botanically as Manihot species is a dicotyledonous perennial plant belonging to the family Euphorbiaceae. It is a starchy root crop that is grown almost entirely in the low land tropics. The total amount of cyanogenic glycosides in cassava roots is often used to place the numerous cassava cultivars into two major groups: the bitter varieties, in which the cyanogenic glycosides are distributed throughout the tubers and are at a high level and the sweet varieties, in which the cyanogenic glycosides are confined mainly to the peel and are at a low level. The cassava root is covered on the outer part by a peel which consists of an inner and outer layer. The outer layer is mainly cork, which is darkish brown in colour, and can be removed by brushing in water, or in some cases can be removed by slicing it away. The inner part of the peel is in two parts, and this separates the peel from the food portions of the root. Both peel and flesh of tuber contain certain significant amounts of two major cyanogenic glycosides; linamarin and lotaustralin. The enzyme linamarise in cassava can hydrolyze the two glycosides to produce hydrocyanic acid is reduced by heating. The cassava tuber consists of about 15% peel, and 85% flesh. The fresh tuber flesh consists of approximately 62% water, 20 - 25% starch, I - 2% protein, I - 2% fibre, and traces of fats and minerals [1].

Cassava can be profitably planted between the beginning of the rainy season and about six weeks before the end of the rainy season. The short growth periods for is usually 8-10 months and result in a poor yields. However, when left for a longer time (15 to 20 months), the roots develops and yield increases. It is essentially a tropical crop. It does best where mean temperatures of 25 - 29°C, and a rainfall of 1000 - 1500mm per annum. Cassava roots deteriorate rapidly after harvesting and therefore they should be consumed or processed into more durable products such as cassava flour, flour or dry or fried chips. Several methods have been used to extend the shelf life of fresh cassava roots. These methods consist of coating roots with paraffin wax, storage in earth pits lined with wet straw, blanching in boiling water for five minutes, immersion in water, coating roots mud or wet ash or by removing stems and leaving roots in the ground. The shelf life of cassava was extended by two or ten days by these methods. However, these methods are tedious, costly and of little practical benefit to consumers and commercial processes. Fresh peeled cassava is eaten as a vegetable after boiling or roasting. In some West African countries, cassava is boiled, and pounded with boiled plantain to form an elastic dough called "fufu". Peeled cassava roots are also often sliced, dried and ground into flour. The main form in which cassava is eaten in West Africa is a roasted, granular product prepared from peeled, grated and fermented cassava roots called "gari". Pellets and chips processed from cassava are a source of energy in animal feeds.

Apart from its use as direct food and feed, cassava is used in the food industry, principally in the form of starch. The low amylose, high amylopectin content of cassava starch gives it the unusual viscosity characteristics and great dimensional strength. Cassava can be hydrolyzed to produce glucose. It can also be converted to alcohol. Dextrins made from cassava flour can be used to make adhesives that are superior to starch-based adhesives in a number of characteristic. Cassava stalks have been used to make particle boards. Starch belongs to one of the three groups of energy given food called carbohydrate. The others are fat and proteins. Carbohydrates are classified as simple or complex. Complex carbohydrates (polysaccharides) include starch, cellulose and glycogen. Polysaccharides can be broken down by hydrolysis into simple carbohydrates [2].

A. *Manufacture of Starch from Cassava*

Local Process

The cassava are washed and peeled by hand. Any dirt remaining on the smooth surfaces of the peeled root is washed off. The roots are then turned into a pulp, in which most of the starch granules are known as rasping. This is achieved by the use of a hand or foot driven rasper. The amount of starch set free with one rasping varies between 70-90% of the starch present in the root. Good quality water is then added to the pulp to separate the free starch from the pulp. The mixture is vigorously stirred with a wooden stick, and the slurry passed through a screen. This process is called screening. The two operations may be combined in a process called wet screening. This is done by raising the pulp mass on a screen in an excess of water which is added continuously from a tap above the screen, while the pulp is stirred using both hands. The screening is completed when the water running out of the cloth screen is clean. The remaining pulp, which still contains some unrefined starch, is pressed by hand and dried in a well-ventilated area on mats, or in the sun. It is mixed into feed for animals. Settling is done in tanks. Holes provided with stoppers are fitted into the walls at different heights or levels to let off the water left above the settled cake of starch. After the tanks are filled, settling takes at least six hours. The stoppers are then removed from the holes, to let the water out, beginning with the upper ones. The remaining moist flour is stirred with water and left to settle again. The starch obtained is dried and sifted to obtain dried starch powder [3].

Mechanized Process

The outer corky layer of skin on the cassava root is cleaned off with any adhering dirt during washing. However, the thick inner layer of skin, which also contain starch is not usually peeled off. Washing is achieved using brush washers. The cleaned cassava roots are grated into pulp using raspers driven by engines. For industrial screening, mechanized rotating screens are used. Water is sprayed into the pulp as it passes through the screening equipment. The starch water is caught in a cement basin below the whole screen length, from where it runs along channels into sedimentation tanks or starch tables. The washed out pulp is dried and pulverized into an off grade flour. Settling is done m settling tanks. Chemicals such as alum (aluminium sulphate) are added to aid sedimentation. The moisture content is reduced to about 35-40% with centrifuges, with final drying done by evaporators. The starch flour is then packed [3].

B. *Hydrolysis of Starch*

The hydrolysis of starch can be achieved either by Acid, Acid-Enzyme or Enzyme-Enzyme hydrolysis. The acid hydrolysis is performed by Saccharification of starch in an aqueous suspension at 100-150°C with the addition of hydrochloric acid of usually 2-3g of gaseous hydrogen chloride per 1kg of dry starch in periodic or continuous saccharifiers. Recently, acid hydrolysis has been replaced by enzymatic hydrolysis because: it required the use of corrosion resistant materials; gave rise to high colour and salt ash content; requires more energy for heating; difficult to control. In the Acid-Enzyme Hydrolysis, Starch is first liquefied and hydrolyzed to specific dextrose equivalents with hydrochloric acid. By choosing two or more types of enzymes (a-amylases, a-amylases or glucoamylases) and adjusting the initial acid hydrolysis, syrups with different ratios of dextrose, maltose and higher saccharides can be obtained.

The Enzyme - Enzyme Hydrolysis is used for the production of various types of starch syrups and allied technologies. This is the most advanced industrial enzyme application in the food industry, and with the exception of the production of biodetergents, it is the most successful enzyme technology of all. When compared with acid hydrolysis, the enzyme technology is characterized by higher yields, wider range of products, higher product quality and energy economy. It is achieved by the heating of the starch slurry above the gelatinization temperature so that starch granules swell and burst. Liquefaction is then carried out by starch thinning and degradation of the original polysaccharide molecules into oligosaccharides by the use of a-amylase. For the purpose of this work, this process will be used to obtain glucose syrup from starch.

II. Chemical Kinetic of Enzyme Catalyzed Reactions

The mechanism of Enzyme Catalyzed reaction is the sequence of individual chemical steps whose overall result produces the observed reaction. By mechanism is meant all the individual collision or elementary process involving molecules that takes place simultaneously or consecutively in producing the overall rate [4]. When enzymatic catalyzed reaction takes place, the following event takes place: A, at constant temperature, elementary reaction rates vary with reactants concentration in a simpler manner. B, at moderate concentration of substrate and very low concentration of enzymes the reaction velocity reaches a maximum, independent of the further increase in concentration. These observations suggest that at higher substrate concentration, all the enzymes sites contain bound substrate, hence further increase in substrate concentration do not lead to an increase in reaction rate. Experiment demonstrating this saturation phenomenon was done by Michelis and Menteen in 1913 [5]. The rate enhancement is attributed to a lowering of the activation energy of the reaction by the enzymes. At a low fixed enzyme concentration, the reaction velocity increases hyperbolically with increasing substrate concentration reaching a concentration independent of maximal velocity at high substrate concentration.

The activation energy for the breakdown of the reactant alone starting of a simple enzyme substrate interaction is giving by:

$$E + S \leftrightarrow ES \rightarrow E + P \quad (1)$$

Where, E is the enzyme, S is the substrate, ES is the enzyme-substrate complex, P is the product of the enzyme-catalyzed reaction,

$$V = k_2[ES] \quad (2)$$

Where, k_1 is the rate constant of the forward reaction of E+S and V is the reaction velocity.

Michealis constant, K_m relate to the enzyme-substrate complex as:

$$[ES] = \frac{[E][S]}{K_M} \quad (3)$$

Since in most cases, the enzyme concentration is very small, the concentration of the uncombined S is almost equal to the total concentration of S. The concentration of uncombined E is equal to the total enzyme concentration [E_o] minus the concentration of the complex [ES]:

$$[E] = [E_0] - [ES] \quad (4)$$

Therefore, equation (3) becomes

$$[ES] = [E_0]\frac{[S]}{[S]+K_M} \quad (5)$$

And equation (2) becomes:

$$V = k_2[E_0]\frac{[S]}{[S]+K_M} \quad (6)$$

The maximum reaction velocity, V_{max}, is reached when all enzyme are saturated with the substrate. At maximum reaction velocity, equation (6) becomes:

$$V_{max} = k_2[E_0] \quad (7)$$

Equation (6) becomes the Michealis-Mentis equation [5]:

$$V = V_{max}\frac{[S]}{[S]+K_M} \quad (8)$$

Re-arranging the Michealis-Mentis equation (8), we obtain the Lineweaver-Burke equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (9)$$

When $\frac{1}{V}$ is plotted against $\frac{1}{[S]}$, we obtain a straight line with a y-intercept = $\frac{1}{V_{max}}$ and a slope =$\frac{K_m}{V_{max}}$. This plot is called a Lineweaver-Burke plot. The Lineweaver-Burk plot is a double reciprocal method that will be basically used in this work to determine the kinetic constants, K_m and V_{max}. To predict the performance of an enzyme - utilizing system, we need to determine V_{max} and K_m for the enzyme of interest. Also, the knowledge of V_{max} and K_m is useful for a number of biochemical purposes for estimation of intracellular reaction rates, comparison of iso-enzymes from different tissues or organism, quantitative comparison of alternative substrates and for definition of potency of inhibitors or activators. This can be achieved by measuring the rate of reaction at a range of reactant concentrations as shown in the figure below. It can be shown algebraically that a rate equal to half V_{max} is obtained when the reactant concentration equals K_m.

III. METHODOLOGY

A. *Materials, Chemicals and Apparatus*

Amyloglucosidase enzyme is use in the saccharification of the hydrolyzed maltose to obtain glucose unit molecules. The Amyloglucosidase was gotten from the enzymology unit of the Biotechnology department of Federal Institute of Industrial research, Oshodi (FIIRO). The enzyme was preserved in the refrigerator at 4^0C until they were used. Cassava starch (food grade) was also obtained from the cassava processing plant of FIIRO and stored in a plastic container until used.

The Chemicals and Reagents used include: (1) 3.5 dinitrosalicylic acid reagent (DNS), consisting of Sodium Potassium Tartrate (30g in 50ml of distilled water); 3.5 dinitrosalicyclic acid (dissolved 1g of this reagent in 20ml of 2N NaOH); DNS is prepared by mixing a solution of the above two and making up to 100ml with distilled water. (2) 0.1N of Sodium Hydroxide (NaOH). (3) 0.1N of Hydrochloric Acid (HCl) and (4) Distilled water. The apparatus used include Hydrolysis Reactor (250ml Erlenmeyer flask), Water Bath, colorimeter, digital weighing balance, stop watch, water bottle, glass stirrer, thermometer, pipette, heater, 50ml conical flask and a pH meter.

B. *Preparation of experimental solutions*

Preparation of solution for standard curves for glucose concentration

Different concentrations of glucose ranging from 0.2 to 2.0 were weighed. 1ml of distilled water and 1ml of DNS reagent was then added. It was then boiled in water bath at 100^0C for color development for 5 mins [6]. The absorbance of the resulting coloration was then read at 540nm against a blank made by substituting the sugar extract with distilled water. The various absorbances for the different concentration will be plotted with the absorbance on the Y-axis and concentration on the X-axis. The slope will be determined from this curve.

Miller Method

3ml of the reaction mixture was taken from the flask and introduced into a boiling tube and 1ml of DNS was added to it. The test tube was covered and placed in a boiling water bath at 100^0C for a color development for 5minutes after which the tube was allowed to cool to ambient temperature. The mixture was filtered and the absorbance of the resulting coloration was read at 540nm against a blank. A blank coloration was made up of 1ml of DNS reagent and 3ml of distilled water. One unit of the amyloglucosidase activity was defined as the amount of enzyme that will liberate 1.0 mole of D-glucose from starch in a 10µ reaction under assay condition. The concentration of glucose produced following the activity of the enzymes at varying temperatures was determined by

making reference to the standard curve of known glucose concentration.

C. *Experimental Procedures*

Three different procedures were used, each for the effect of temperature, pH and Substrate Concentration.

Temperature

Two grams of starch was accurately weighed into a 250ml Erlenmeyer flask. 200ml of water was then added and then placed into the water bath. 3ml of amyloglucosidase was then added and the activities of the enzymes were observed for temperatures ranging from 30-80^{0}C at an intervals of 10^{0}C. The Miller method was used at each interval to determine the activity of the enzymes.

pH

3g of starch was accurately weighed and dissolved in 200mls of distilled water in a 250ml beaker. 5ml of amyloglucosidase was added and thereafter dispensed as 30ml in a 50ml beaker and their pH values adjusted from 3.0 to 6 at intervals of 0.5 using 0.1N of NaOH and 0.1N of HCl. This was then placed in a water bath at 60^{0}C and held for 2hrs. The glucose produced was then assayed using the method of Miller [6].

Substrate Concentration

Varying concentrations of starch was prepared at 1% - 10%w/v. 2ml of amyloglucosidase was then added to each reaction mixture and incubated at 60^{0}C for 2hrs. The glucose produced was then assayed using the Miller method. The concentration of glucose was known by making reference to the glucose standard curve of known concentration of glucose.

After the above procedures, values were plotted to achieve the Lineweaver-Burk plot, from which the Michaelis-Menten constant and maximum reaction velocity are calculated.

IV. RESULTS AND DISCUSSION

Figures 1, 2 and 3 shows the effect of temperature, pH and substrate concentration respectively, on yield of sugars when Amyloglucosidase was used for the hydrolysis of cassava flour.

A. *Effect of temperature on the concentration of glucose produced*

The yield of sugars increases linearly as the temperature increases from 30^{0}C through 60^{0}C as shown in Figure 1. However, the yield began to decline beyond 60°C, revealing an optimum temperature for the hydrolysis of cassava starch. The effects of enzyme-catalyzed reaction passed through a maximum concentration with increased temperature. This was due to the rate of enzyme-catalyzed reaction increases with increase in temperature as does the rate of their chemical reaction, and enzymes being protein are denatured and hence inactivated at high temperature. Thus increasing the temperature past the optimum temperature tends to reduce reaction velocity by reducing enzyme activity. Finally, at optimum temperature the two effects are balanced to the best advantage. In this study, the temperature was maintained at 60^{0}C so as to optimize the pH and substrate concentration.

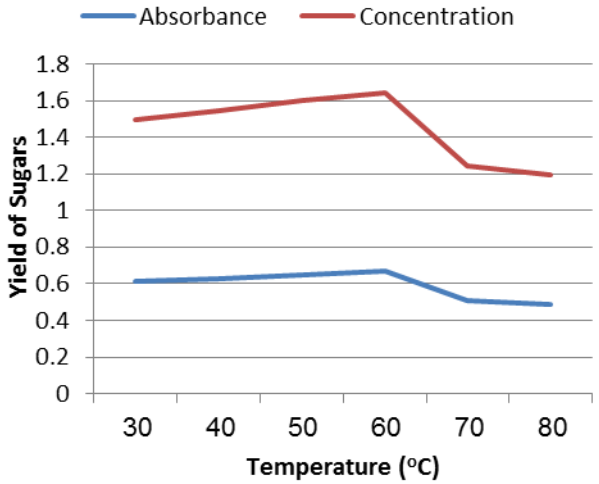

Figure 1. The effect of temperature on concentration

B. *Effect of pH on the concentration of glucose produced*

Sugars yield first decreases from a pH of 3 to 4. Then increases linearly until it reaches its peak at a pH of 5.5 as shown in Figure 2. This phenomenon agrees with Brumm [7], who reported that the AMG has a broad pH optimum between 4.0 and 5.5 [7]. However, the rate of decrease in the yield of sugars seemed to be faster beyond 5.5 than it did take to rise to that point, while yield of sugars increased rapidly with increasing time. AMG showed a very good stability at optimum conditions (pH 5.5 and 55°C), however stability is greatly affected at 60°C or higher [8]. Near optimum value, the effects of changing pH are usually reversible. This means the maximum activity can be restored by bringing the pH back to the optimum value. If the pH is taken too far to the acid or alkaline side, the changes may become irreversible because large changes in pH denature the enzyme protein since most active sites contain acidic or basic groups.

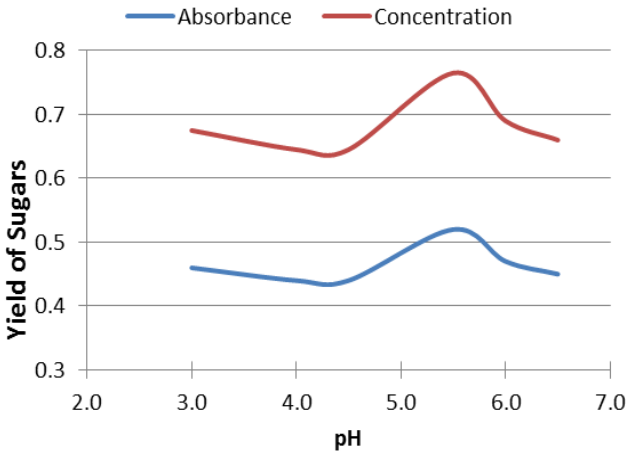

Figure 2. The effect of pH on concentration

C. *Effect of substrate on the concentration of glucose produced*

The yield of sugars increases in a curvilinear manner as substrate concentration increased. The optimum yield seems to have occurred at 6% w/v, further increase in substrate concentration (at very high concentration) do not lead to an increase in reaction rate. The possible explanation for these significant effects of the pH, temperature and substrate concentration conditions for the enzymatic reactions on the end product profiles of starch hydrolysis might be due to various mechanisms such as a shift in the route of substrate hydrolysis, the alteration in the enzyme-substrate affinity constraints and the rate of disintegration of the enzyme-substrate complexes [9].

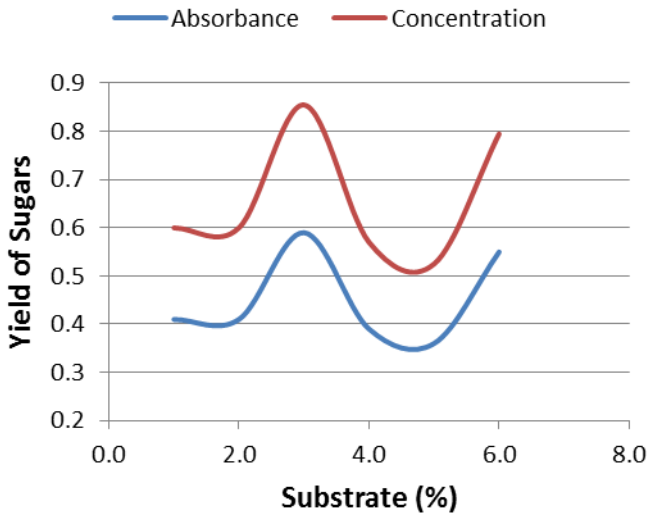

Figure 3. The effect of substrate on concentration

D. *Michaelis Menten and Lineweaver-Burk equations*

The Lineweaver-Burk equation is used to describe the kinetics of amyloglucosidase. Figure 4 shows the variation of glucose produced with time at different substrate concentrations. The slopes of these lines where found, inverted (1/V) and plotted against the inverse of substrate concentration (1/S) to give the Lineweaver-burk plot shown in Figure 5. The Lineweaver-burk plot is a straight line with a y-intercept = $\frac{1}{V_{max}}$ and a slope = $\frac{K_m}{V_{max}}$. The Lineweaver-Burk plot is a double reciprocal method that will be basically used in this work to determine the kinetic constants, K_m and V_{max}. From the intercept and slope values, the Michaelis-Menten constant was found to be 0.167M and the maximum reaction velocity is 0.08M/min. In taking reciprocals the most significance is placed on the rates obtained at low reactant concentration which will be to the greatest experimental error. This may account for any large deviation of experimental values from theoretical value. Sugar syrups produced by Amyloglucosidase in combination with other enzymes contain glucose mainly as well as other sugars. The sugar distribution in any one type of syrup is, of course, dependent upon its method of manufacturing or production and the type and quantity of enzyme used [10]. Rice malt has been said to be rich in many enzymes especially "-amylase, amyloglucosidase and dextrinases [11]. The "-amylase and dextrinases might be responsible for the production of maltose. The quantity of glucose present is a function of the enzyme type being used and the conditions at which it is produced.

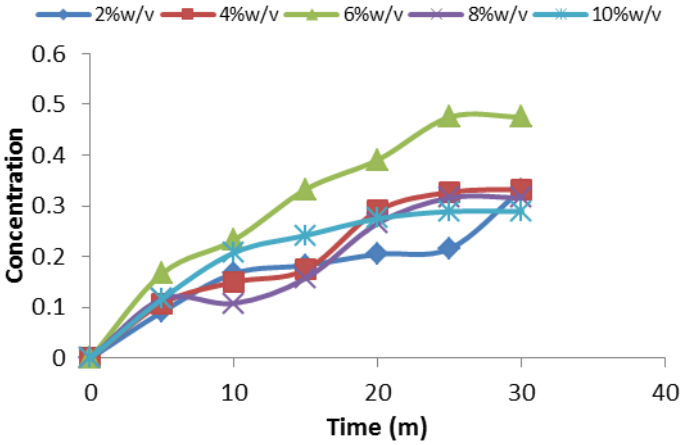

Figure 4. Concentration against time

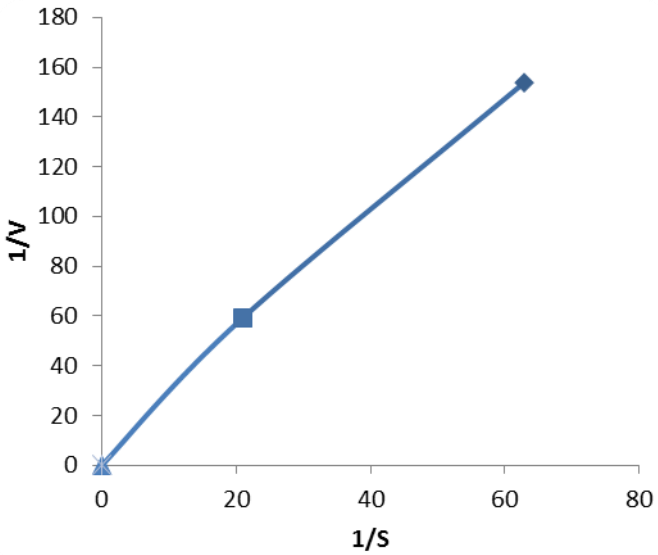

Figure 5. Lineweaver – Burk Plot of 1/V against 1/S

V. CONCLUSION

From the experiments performed, it was observed that amyloglucosidase hydrolyzes cassava starch to yield glucose and this reaction path is predicted by the Lineweaver-Burk plot. The Michaelis-Mentene constant, K_m was found to be 0.167M and the maximum rate of hydrolysis, $V_{max,}$ was found to be 0.08M/min. Hence, the design of a reaction path for the production of glucose syrup from cassava starch with amyloglucosidase as a major enzyme at a pH of 5.5, temperature of 60^0C and substrate concentration of 6%w/v was established.

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