Production of bioethanol from liquid waste from cassava dough during gari processing

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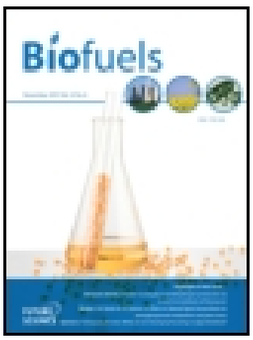

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Production of bioethanol from liquid waste from cassava dough during gari processing

Francis Kotoka, Samuel Kofi Tulashie and Daniel Dodzi Setsoafia

University of Cape Coast, College of Agriculture and Natural Sciences, School of Physical Sciences, Department of Chemistry, Industrial Chemistry Section, Cape Coast, Ghana

ABSTRACT

Starch is a polysaccharide that can be hydrolyzed by enzymes and fermented into bioethanol. It is commonly found in Ghana as the effluent during cassava-gari processing. The copious amount and its related stench in the environment as effluent have necessitated its conversion into ethanol. This present study investigated bioethanol production by enzyme catalysis using effluent from cassava dough during gari processing. The starch was converted to dextrins using α-amylase enzymes extracted from paddy rice after which glucoamylase extracted from *Aspergillus niger* was used to hydrolyze the dextrins to glucose. The glucose was subsequently fermented into ethanol using yeast, and the ethanol was recovered by fractional distillation. The optimum temperature and pH of the enzymes applied were investigated. Hence, the optimum pH and temperature for the α-amylase during the starch liquefaction was 4 and 60 °C, respectively, while that of the glucoamylase for the starch saccharification was 3.5 and 60 °C, respectively. The average ethanol yield was 26.5 $\pm$ 0.15% v/v, and the distillate purity was determined as 65.33 $\pm$ 0.58%. This suggests that bioethanol production from the starch effluent of cassava dough during gari processing by enzyme catalysis may be employed industrially in addition to the conventional acid enzyme catalysis if the process is fully optimized.

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Gari (granulated-cassava powder); starch; bioethanol; glucoamylase; *Aspergillus niger*; α-amylase

Introduction

Ethanol production is a vital global industrial product due to the excellent fuel properties exhibited by ethanol [1] and its significant effects on the reduction of greenhouse emissions when used as fuel [2]. The need for alternative or renewable fuels has been a major concern in many countries due to the global energy crisis [3]. Enzymatic hydrolysis is preferred over acid hydrolysis as a result of energy consumption, yield, operating conditions, and other factors [4,5]. For example, fungal amylases such as α-amylase and glucoamylase have been reported in other studies to have merits such as cost effectiveness, consistency, ability to modify and optimize process easily, and less production time and space [6]. Several agricultural materials are also gaining attention as they are used as feedstocks for bioethanol production. These raw materials are classified into three main groups: sugars, starches, and cellulose [7]. Among the starch groups, cassava starch was reported to be one of the best agricultural materials for first generation biofuels such as bioethanol fermentation and production [8–10]. Fresh cassava was reported to have significant starch content up to about 30% [7]. Bioethanol produced from cassava starch, involving hydrolysis of the starch into sugars followed by fermentation, has been reported in several literatures. For instance, 185 to 200 L of anhydrous bioethanol was produced from 1 ton of starch in fresh cassava root through dual enzyme activities [11]. Conversely, 0.35 g/100 mL and 3.60 g/100 mL bioethanol were produced through dual enzyme catalysis, respectively, from 1% and 8% cassava starch substrates [12]. Again, 53.0% bioethanol distillate was obtained from cassava starch when amylolytic enzymes were used during saccharification and liquefaction of the cassava starch [13]. However, these methods have their associated drawbacks. One major drawback is the slower enzymatic degradation of fresh cassava starch or ungelatinized starch unlike that of gelatinized starch [14]. Another drawback is the toxic cyanide content in fresh cassava starch or ungelatinized starch [15]. Gelatinized starch is needed to enhance enzymatic degradation during the liquefaction and saccharization stage, thereby reducing the amount of enzymes required for the liquefaction stage. Additionally, gelatinization has the advantage of largely liberating the cyanide content through heat. In view of these, production of bioethanol from cassava starch by means of gelatinization, liquefaction, saccharification, and fermentation will be considered in this study.

The cost effectiveness of the ethanol production process is a key issue, therefore, the use of low-cost

CONTACT Samuel Kofi Tulashie stulashie@ucc.edu.gh

raw materials is a key factor to efficient bioethanol production [16,17]. Cassava is economically abundant in many countries [18], therefore using it as a raw material can be a better approach to reducing production costs. Africa produces the largest quantity of inexpensive cassava, and uses it as a major source of low-cost carbohydrate. An important characteristic of cassava is its ability to produce significant yield on infertile lands where other crops cannot be cultivated [19]. In tropical parts of the world, sugarcane and palm tree are the most common agricultural products used as raw materials for bioethanol production. However, these two major materials have associated significant limitations. For instance, copious amounts of water are required for sugarcane production, and the availability of suitable lands for sugarcane cultivation is also limited while suitable sites for cassava cultivation are available in the tropics [20]. Traditionally, in Ghana, cassava tubers are processed and utilized in various ways according to local preferences. However, the use of cassava also has its related drawbacks: bigger starch particles which require more enzymes to break down [18] and higher energy input needed for starch gelatinization [14]. Despite these difficulties, the disposal of large amounts of the effluents from gari processing activities is becoming a major concern due to its associated stench and its role as breeding grounds for mosquitoes and maggots. It may also result in loss of aquatic lives if the toxic cyanide content ends up in water bodies.

Therefore, this present study investigates the production of bioethanol by enzyme catalysis using effluent from cassava dough during gari processing. The optimum temperature and pH of the enzymes applied will be presented and discussed.

Materials and methods

Germination of paddy rice

Germinated cereals were chosen because they had been reported to exhibit a significant increase in α-amylase activity [21]. Paddy rice was selected because of its abundance and its ability to produce high α-amylase from 72–120 h sprouting [22]. The procedures used were adopted from Machaiah et al. on the germination of paddy rice and extraction of α-amylase [23]. In order for the rice to germinate, 20 g of the paddy rice was washed with 1% NaOCl and rinsed with distilled water to remove any adhering material or dirt. The rice seeds were steeped in distilled water and imbibed for 16 h. The seeds were then moistened with penicillin and incubated in the dark at 25 °C for five days to germinate.

Extraction of α-amylase

The vegetative parts of the germinated seeds were broken off, and the endosperm tissues homogenized at 3 °C in 0.01 M sodium acetate buffer containing 0.03 M $CaCl_2$ at a pH of 5.6. The extract as shown in Figure 1 was then centrifuged at 5000 g for 10 min, after which the supernatant was adjusted to pH 8 using 1.0 N $NaOH_{(aq)}$. β-amylase in the resulting mixture was deactivated by warming the mixture to 70 °C and then cooling to 4 °C. After β-amylase deactivation, the solution was centrifuged and the supernatant (crude α-amylase) was cooled and stored below 4 °C.

Partial purification of the crude α-amylase

The crude α-amylase was partially purified by ammonium sulphate precipitation method [24]. Sodium

Figure 1. The left beaker contains the homogenized endosperm tissues while the right beaker contains the crude extract of α-amylase and β-amylase.

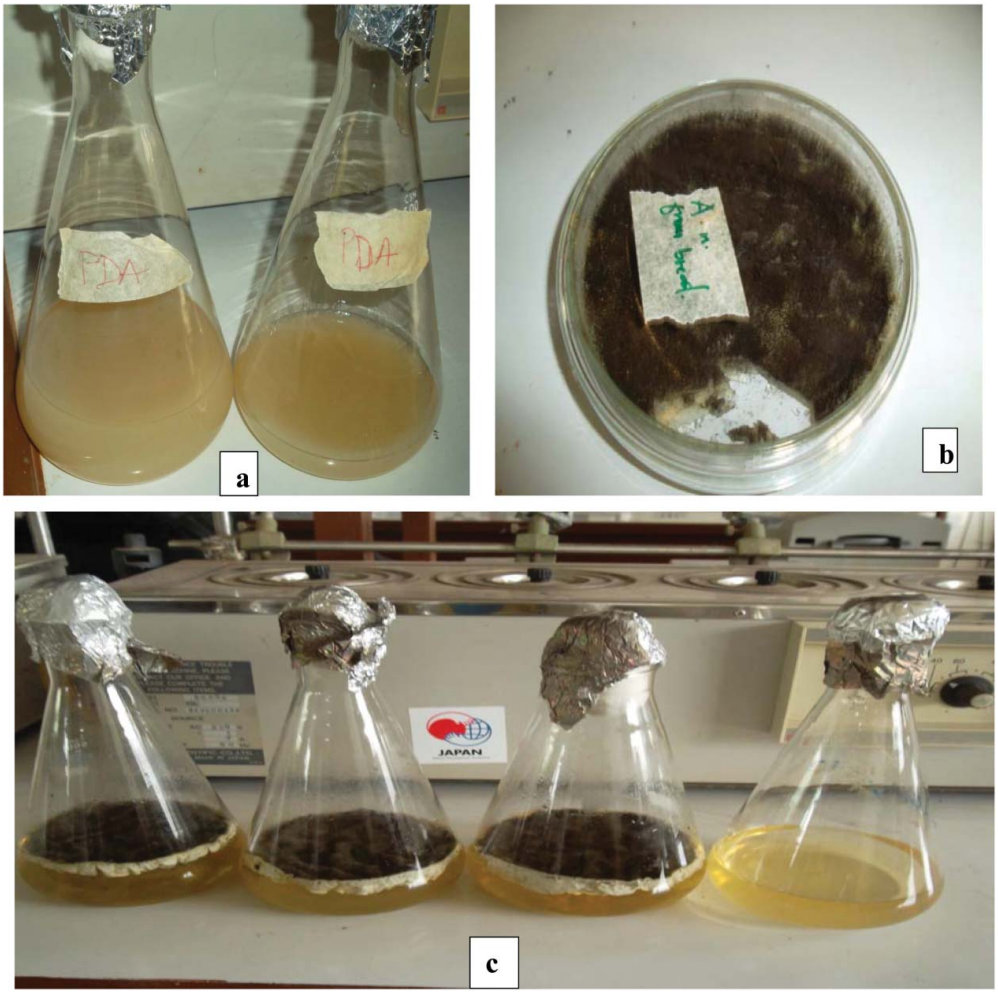

Figure 2. (a) Potato dextrose agar. (b) *Aspergillus niger* culture. (c) *Aspergillus niger* subculture.

acetate buffer was used to dissolve the precipitated enzyme [23]. The selection of ammonium sulphate as the precipitant was based on its high solubility, and its ability to stabilize proteins by preferential solvation [25]. Acetate buffer was also chosen because it does not have the potential for precipitating calcium ions required for full fungal amylases like phosphate buffer can do to disturb the enzyme activity [26].

The crude α-amylase was 45% saturated with ammonium sulphate salt and kept overnight at 4 °C to precipitate the enzyme. The suspension was centrifuged at 1200 g and the precipitate recovered by filtration. The precipitate was dissolved in 0.01 M sodium acetate buffer containing 0.03 M $CaCl_2$ at pH 5.6 and the resulting solution was used as α-amylase.

Glucoamylase production

Three main processes, namely growth, culturing, and submerged fermentation, were involved. Submerged fermentation method was applied due to the ease of handling, better control of temperature and pH, and it is a commercially well-known method for amylase production [14].

Growth of Aspergillus niger

Aspergillus niger had been reported to be very prolific in glucoamylase production [27], thermostable and abundantly available [28]. They can be found in industrial waste potatoes [27], soils [29], and contaminated foods. A piece of bread was moistened and kept at room temperature in the dark for four days to grow *Aspergillus niger*.

Culturing Aspergillus niger

The black mold on the bread was inoculated on potato dextrose agar (PDA) which had initially been poured on three autoclaved Petri dishes and allowed to harden. The PDA contained: 100 mL of boiled potato extract; 20 g agar powder; 20 g dextrose. The Petri dishes were then covered and kept at room temperature in the dark for three days to produce a subculture of *Aspergillus niger* into PDA. Figure 2 shows PDA, culture, and subculture of *Aspergillus niger*.

Glucoamylase production by submerged fermentation

A total of 100 mL of the glucoamylase production medium was taken and submerge-fermented in an

Figure 3. (a) Crude glucoamylase after filtration. (b) Supernatant of centrifuged glucoamylase.

Erlenmeyer flask [30]. The fermentation media contained: 100 mL potato dextrose broth (25 g dextrose and 100 mL potato extract); 0.03 M $CaCl_2$; 0.2 M NH_4NO_3. The pH of the medium was controlled at 6, and incubated on a rotary shaker with 120 rpm for six days at 30 °C [31]. The fermented medium was suction filtered to remove the foam present. The filtrate was centrifuged at 5000 g at 4 °C and the supernatant collected as crude glucoamylase as shown in Figure 3. Partial purification was carried out by the same method used for the α-amylase described above.

Investigating the optimum conditions for α-amylase and glucoamylase

Several factors affect the activity and structure of enzymes. Therefore, the optimum conditions such as pH and temperature are very important to enzymes performance, effectiveness, and efficiency in a medium. In this study, the optimum pH and temperature for α-amylase and glucoamylase were also investigated prior to the ethanol production process.

Preparation of starch/iodine standard curve

Serial dilutions of the starch were employed: 1.0 mL of iodine was added to test tubes containing stock standard starch. The solutions were shaken to mix thoroughly and topped up with 1 mL phosphate buffer at pH 7.4. Using UV-vis at 590 nm wavelength, a standard curve of absorbance against concentration of starch–iodine mixture was prepared.

Investigating the optimum temperature for α-amylase

Starch–iodine method developed by Fuwa was adopted using cassava starch as the substrate to ascertain the α-amylase activity [32]. This method was chosen because of its broad use and its ability to detect a greater percentage of the enzyme activity even in the presence of maltose [33]. To identify the optimum temperature, a temperature range of 30–90 °C in increments of 10 °C was applied. Seven test tubes were separately placed in seven beakers (in a water bath) each with 500 mL capacity containing hot water. The temperature of the water in the beakers was adjusted so that they ranged from 30–90 °C. To each of the test tubes, 5.0 mL (5 mg/L) of the cassava starch solution was added, followed by 1.0 mL phosphate buffer solution. The solutions were topped up with 1 mL of the α-amylase to initiate the reaction. The temperature of each of the reaction mixtures was allowed to attain equilibrium with that of the water in their corresponding beakers. The reaction was stopped after 5 min using 1 M HCl solution after which two drops of acidified iodine solution were added to each of the test tubes and mixed thoroughly to test for the presence and quantity of starch remained. One unit of α-amylase activity was defined as the quantity of enzyme that converted 1 mg/mL of starch into dextrins per minute.

Investigating the optimum pH for α-amylase

A total of 1.0 mL phosphate buffer solutions at pH range 2.0–8.0 were separately added into another set of seven test tubes followed by the addition of 5 mL of the cassava starch solution and 1 mL of α-amylase. The temperature of the test tubes was maintained at 60 °C since it was the optimum temperature previously determined. The reaction mixtures were allowed to stand for 5 min and the reaction was terminated with 1.0 M HCl solution. The test tubes were tested for the presence and quantity of starch remaining as stated earlier.

Investigating the optimum temperature for glucoamylase

Glucose oxidase per oxidase method was used to determine the amount of glucose produced from the dextrins by the glucoamylase enzyme. The choice of this method was based on the report that it combines specificity with great simplicity [34]. The glucoamylase activity was investigated within the temperature range 30-90°C. Following starch hydrolysis by α-amylase as described above (without terminating the reaction), 1 mL of glucoamylase was added to each of the test tubes containing the reaction mixtures. The mixtures were allowed to stand for 5 min and tested for the presence and quantity of glucose produced. One unit of glucoamylase activity was defined as the quantity of enzyme that produced 1 mg/mL of glucose per minute.

Investigating the optimum pH for glucoamylase

The glucoamylase activity was investigated at pH range 2.5-8.5 in increment of 0.5 interval. Following hydrolysis of the starch by α-amylase, the pH of the mixtures in each of the test tubes was adjusted by gradual addition of 1.0 M NaOH solution so that the range was 2.5–8.5. The temperature of the mixtures was maintained at 60 °C since it was the optimum temperature for glucoamylase activity as determined above. The amount of glucose produced by the glucoamylase at the various pH was then determined by the glucose oxidase per oxidase method.

Production of bioethanol from cassava starch using α-amylase and glucoamylase

The process employed five main steps as illustrated in Figure 4: gelatinization, liquefaction, saccharification [35], fermentation [36], and distillation. Gelatinized starch is more readily degraded by enzymes than non-gelatinized starch [14]. Enzymes and yeast were used to convert starch to sugar, and then ferment sugar to ethanol [37]. The effectiveness and efficiency of the enzymes depend on the pH, temperature, enzyme concentration, substrate concentration, and mixing rate [16].

In order to gelatinize the starch, 300 mL of the effluent starch was measured and diluted with distilled water to 940 mL. The diluted solution was then heated, followed by the addition of 50 mL of α-amylase extracted. This step was done for 6 h at 60 °C and pH 4. The liquefied solution was saccharized for 8 h at 60 °C and pH 3.5 using 50 mL glucoamylase. The resulting glucose solution was then cooled to 35 °C [36] and seeded with 50 g of baker's yeast to initiate the fermentation process. The fermenting mixture was allowed to stand for 11 days after which the fermented solution was distilled by means of fractional distillation. The ethanol was then recovered at 75 °C. All statistical analysis, such as average, standard deviations, tables and graphs were done using Microsoft excel 2010.

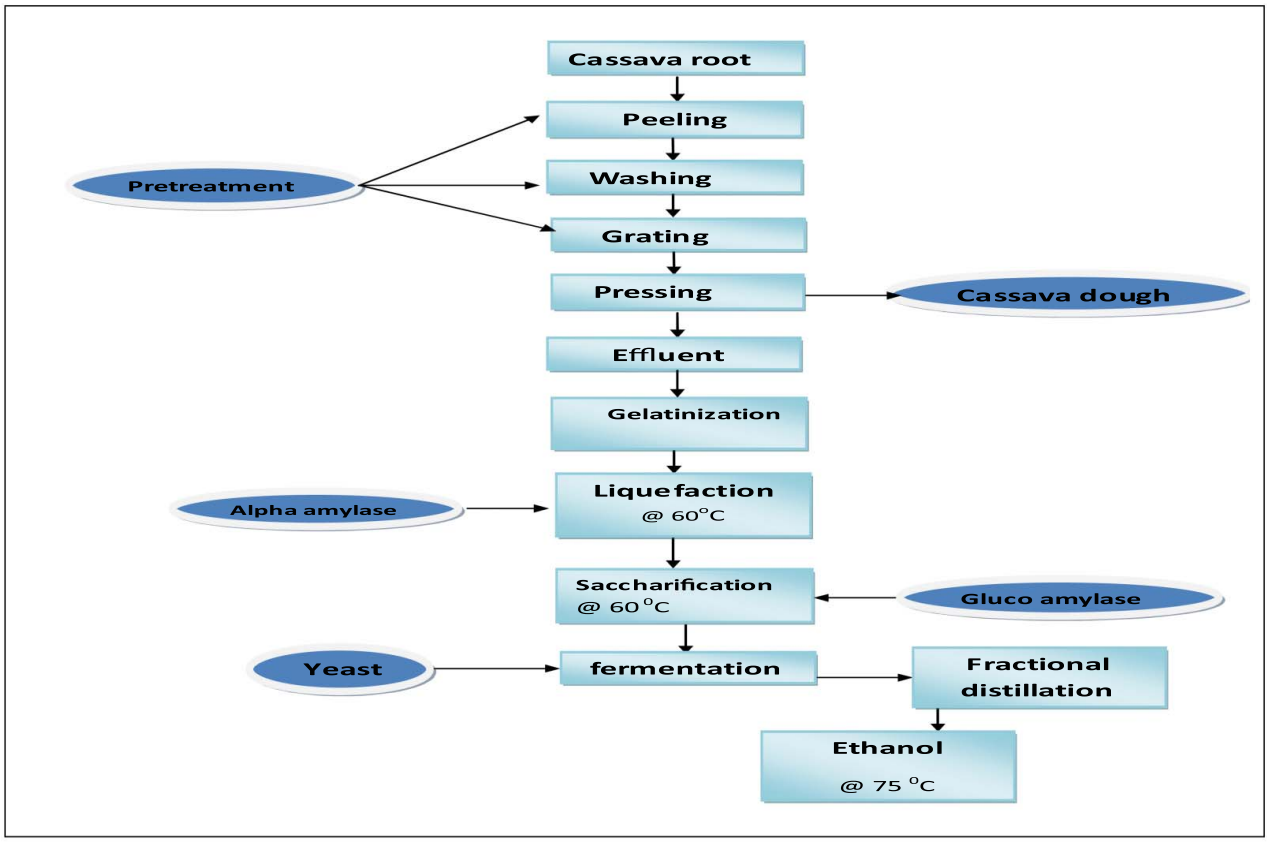

Figure 4. Schematic diagram of bioethanol production from effluent of cassava dough during gari processing.

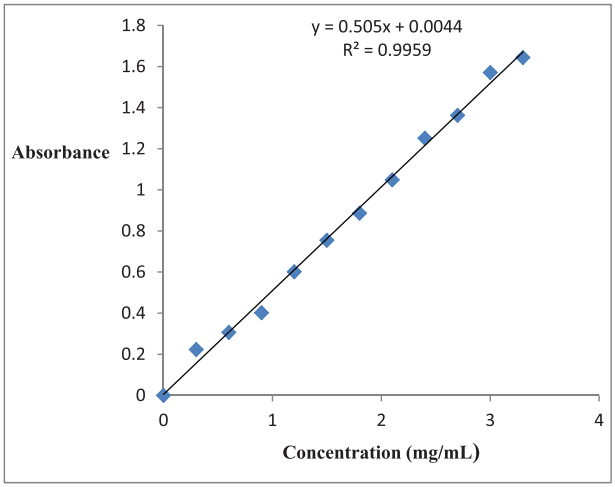

Figure 5. The standard working curve for determining the final concentration of starch hydrolyzed at various temperatures and pH.

Results and discussion

Figure 5 shows the working curve for the starch–iodine mixture used for determining the final concentration of starch hydrolyzed at various temperatures and pH values. Serial dilutions of the starch concentrations ranging from 0.3 mg/mL to 3.00 mg/mL were prepared from 5 mg/mL stock standard solution using the relation $C_1V_1 = C_2V_2$. The absorbance increased with increasing starch concentration, and corresponded with the intensity of the blue-black formation of the starch upon addition of iodine.

Investigating the optimum conditions for α-amylase activity

Enzymes are very sensitive biological organisms, hence a change in their optimum conditions such as temperature and pH may drastically affect their activities in a medium. Thus, enzymes perform best in the optimum conditions. As shown in Table 1, the highest absorbance reading was observed at a temperature of 80 °C with the least activity value of 0.647 mg/mL/min. It may be implied that the starch hydrolysis was inefficient at that temperature. The optimum temperature was found to be 60 °C. Thus, the lowest absorbance (0.380) was recorded at this temperature value with the highest activity value of 0.891 mg/mL/min as shown in Figure 6. This was seen in the test tube that had the faintest blue-black coloration after a test for starch. Therefore, the optimum temperature within which the enzymes could effectively and efficiently hydrolyze the starch into dextrins was 60 °C.

Table 1. Effect of temperature on enzyme activity for α-amylase.

Test tube	I	II	III	IV	V	VI
Temperature (°C)	30	40	50	60	70	80
Absorbance	0.709	0.687	0.629	0.380	0.594	0.781
Concentration (mg/mL)	1.600	1.551	1.4199	0.8578	1.3409	1.7630
Activity (mg/mL/min)	0.680	0.689	0.716	0.891	0.730	0.647

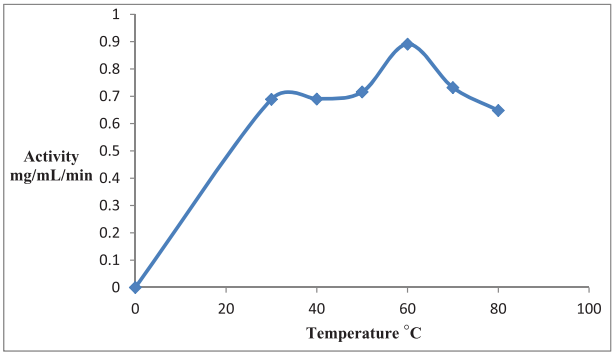

Figure 6. α-amylase activities with varying temperature.

A similar study reported that α-amylase of *Aspergillus niger* strain isolated from soil had pH optima range 4–6 and temperature optima range 30–40 °C. [29] The optimum temperature in this study varied from the reported optimum temperature because it is obvious that the optimum temperature of enzymes vary depending on the method of extraction and the source from which the enzyme was extracted. As seen in Table 2, varying the pH also varied the activity of the enzyme. The highest enzyme activity value (0.833 mg/mL/min) was recorded at pH 4. Unlike the optimum temperature, the optimum pH of the α-amylase was within the range of that reported in literature [29]. The pH of the medium to which an enzyme is subjected also affects the enzyme activity. At higher pH values, enzymes may be denatured, drastically affecting the enzyme activities. As shown in Figure 7, this phenomenon was clearly observed at pH 8, which had the lowest enzyme activity value (0.647 mg/mL/min). The pH of the enzyme catalyzed reaction must, therefore, be controlled to enhance enzyme activities.

Table 2. Optimum pH of α-amylase enzyme.

pH	2.0	3.0	4.0	5.0	6.0	7.0	8.0
Absorbance	0.513	0.633	0.469	0.568	0.735	0.6903	0.745
Concentration (mg/mL)	1.158	1.429	0.833	1.282	1.659	1.558	1.682
Activity (mg/mL/min)	0.714	0.768	0.858	0.744	0.688	0.668	0.664

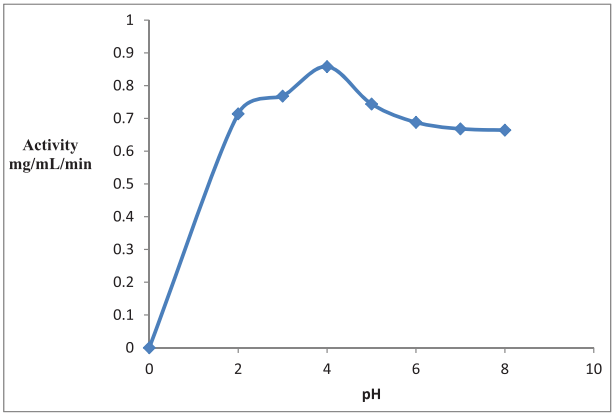

Figure 7. α-amylase activities with varying pH.

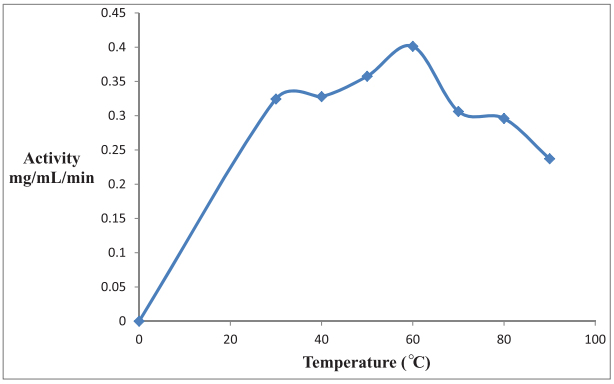

Figure 8. Glucoamylase activities with temperature changes.

Similar studies showed that pH and temperature optima for α-amylase activity were 5.0 and 70 °C [38,39] and 7.5 and 70 °C [40]. The enzymes were isolated from different sources such as *Sorghum bicolor* malt, millet malt, and thermophilic *Bacillus* sp. The variation in the optimum pH and temperature may be as a result of the method of extraction and the sources of the enzymes.

Investigating the optimum conditions for glucoamylase extracted from Aspergillus niger

As shown in Figure 8, enzyme activity value varied with varying temperature. The lowest activity reading was 0.237 mg/mL/min at a temperature of 90 °C. This implies that the conversion of the dextrins to glucose was very low at this temperature. The highest enzyme activity was 0.401 mg/mL/min and was recorded at 60 °C. Thus, the optimum temperature was found to be 60 °C. This temperature was equal to that of the α-amylase, but with a different activity value. As shown in Figure 9, varying the pH had different effects on enzyme activity. The lowest enzyme activity was observed at pH 8.5 with the activity value of 0.655 mg/mL/min. The highest enzyme activity (0.829 mg/mL/min) value was recorded at pH 3.5. This indicates that the optimum pH of the enzyme glucoamylase was 3.5, and it was lower than the optimum pH of α-amylase.

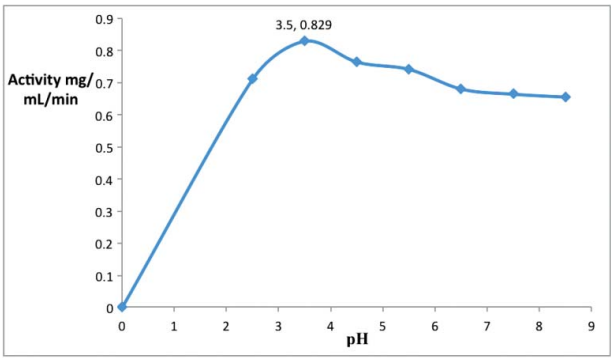

Figure 9. Glucoamylase activities with varying pH.

Table 3. Percent yield and purity of distillate.

	run1	run2	run3	Average
Fermented vol. (mL)	300	300	300	300
Distillate vol. (mL)	79.2	79.5	80.1	79.60
% yield v/v	26.4	26.5	26.7	26.53 ± 0.15
% purity v/v	65	65	66	65.33 ± 0.58

This result is similar to other studies conducted on glucoamylase optima. The optimum pH and temperature of glucoamylase of *Aspergillus awamori* were 3.5–4.5 and 55 °C respectively [41]. The pH and temperature optima for glucoamylase from *Aspergillus oryzae* were 5.0 and 50 °C respectively [42]. Table 3 shows the percentage yield and purity of the distillate obtained by fractional distillation. The average percentage yield and purity of the bioethanol distillate were 26.53 ± 0.15% v/v and 65.33 ± 0.58% v/v respectively. Indeed, if the distillate was redistilled at least twice, higher purity would be achieved.

Although the yield was smaller than expected, it was higher than those reported in some studies. When α-amylase and glucoamylase from *Aspergillus awamori* were used to directly ferment raw starch to ethanol, 10.3% v/v ethanol was yielded [43]. Also, 17.5−18.5% v/v of ethanol was yielded from raw ground corn using glucoamylase from *Saccharomyces* sp. HO [44]. Crude glucoamylase from *Rhizoctonia solani* was also used to saccharify potato and cooked starch which yielded 5.89% v/v ethanol when yeast strain was used for the fermentation process [45].

Conclusions

This study presents the production of bioethanol by enzyme (α-amylase and glucoamylase) catalysis using effluent from cassava dough during gari processing. The results of this study showed that the average ethanol yield and purity using α-amylase, glucoamylase, and baker's yeast as converting enzymes were 26.53 ± 0.15% v/v and 65.33± 0.58% v/v, respectively.

This suggests that ethanol production from the starch effluent of cassava dough during gari processing by enzyme catalysis may be employed industrially in addition to the conventional acid enzyme catalysis if the process is fully optimized. The abundance of the effluent and the environmental friendliness of the ethanol production process also indicate that it may be beneficial for gari producers to convert the effluent to ethanol rather than allowing it to go to waste with its associated environmental problems.

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Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Francis Kotoka http://orcid.org/0000-0002-3568-4030
Samuel Kofi Tulashie http://orcid.org/0000-0002-6744-2917

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