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Effect of Pullulanase Debranching and Retrogradation on Resistant Starch Yield and Glycemic Index of Garri

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Authors' contributions

This work was carried out in collaboration between both authors. Author FCO designed the study, wrote the protocol, supervised, read and corrected the manuscript. Author NCN managed the analysis of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: This research is aimed at developing a method of processing to increase the quantity of resistant starch in garri and reduce its glycemic index using pullulanase-producing Bacillus subtilis organism.
Place and Duration of Study: Department of applied microbiology and brewing, Nnnamdi Azikiwe University, Awka between January, 2018 and February, 2019.
Methodology: The organism was isolated from different cassava processing sites in Anambra metropolis, Nigeria. It was then identified based on phenotypic, biochemical and molecular characteristics After which the pullulanase assay, the fermentation studies, resistant starch analysis and glycemic index was analysed.
Results: Pullulanase assay result showed Bacillus subtilis as a very good pullulanase producing organism with a pullulanase quantity of. The resistant starch content was found to be higher for the samples fermented with the choice organism and retrograded at 10oC at 14.29%, than the control garri sample fermented without any organism and not retrograded at 4.73%. The glycemic index

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was relatively high in all the garri samples, however, the lowest glycemic index, 62% was observed in the garri sample produced with the choice organism.
Conclusion: This research has been able to show that pullulanase enzyme from Bacillus subtilis is a very useful industrial raw material in production of functional foods with low glycemic index.

Keywords: Pullulanase; resistant starch; bacillus subtilis; glycemic index.

1. INTRODUCTION

Resistant starch (RS) is described as all starch and starch degradation products which resist small intestinal digestion and enter the large bowel in humans [1]. It is measured chemically as the difference between total Starch (TS) obtained from homogenized and chemically treated sample and the sum of RDS and SDS, generated from non-homogenized food samples by enzyme digestion. RS = TS – (RDS + SDS). The starch may not be physically bioaccessible to the digestive enzymes such as in grains, seeds or tubers. Also, the starch granules themselves are structured in a way which prevents the digestive enzymes from breaking them down (e.g. raw potatoes, unripe bananas and high-amylose maize starch) [2]. Resistant starch is naturally found in cereal grains, seeds, heated starch and starch-containing foods such as cassava-based foods, albeit in low quantities [3]. Many methods have been developed for increasing the resistant starch content of foods; these include enzymatic hydrolysis, physical treatments, chemical modifications, exposure to γ-rays, genetic manipulation on amylose levels in plants, and the effects of lipid complexes on the resistant starch content of starches. In the enzymatic hydrolysis method, resistant starch is increased by increasing the apparent levels of amylose *via* debranching amylopectin molecules using pullulanase or isoamylase enzymes or enzyme-producing organisms. The amylose formed is subsequently retrograded to RS by employing heating and cooling cycles [4]. Furthermore, RS has many benefits; it has been hypothesized to reduce colon cancer and to benefit inflammatory bowel disease by the production of a short chain fatty acid, called butyrate. It has also been reported to greatly reduce glycemic index in most starchy foods. These properties of RS makes it an important functional fiber component of food, which can be exploited in the management of diseases such as human type 2 Diabetes Mellitus [5]. The objective of this research study is to isolate a pullulanase producing organism with GRAS status, utilize the organism in the fermentation of cassava to produce garri, assay for the quantity of resistant starch produced by the sample garri and determine the glycemic index of the garri produced.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Bacteria

Soil samples were collected from different cassava processing sites in Anambra metropolis and stored at 4ºC. Isolation of bacteria was performed by preparing a five-fold serial dilution of 1 gram of the sample. Pour plate technique was used for inoculation into nutrient agar plates. The plates were incubated at 37°C for 72h. The bacterial isolates were further sub-cultured in order to obtain pure culture. The bacterial isolates were inoculated into a pullulan –medium which composed of the following in (g/l): pullulan (10), NaCl (2), $MgSO_4.7H_2O$ (0.1), $KH_2PO_4.7H_2O$ (0.12), $NaNO_3$ (0.5) and agar (15); pH: 7.5.The transparent zones of hydrolysis around the colonies after 48 hours of incubation indicated the presence of pullulanase activity.

2.2 Molecular Identification of Isolated Bacteria

2.2.1 DNA extraction, 16srrna amplification and sequencing

DNA was extracted from bacterial colonies harvested after 24 hours of incubation using Zymo® Genomic DNA extraction kit according to the manufacturer's instructions and their A260/A280 and A260/A230 ratios checked to assess their purity. 16S rRNAs were PCR amplified using universal forward 27F (5'-TCCTCCGCTTATTGATATGS -3') and reverse 1535R (5'- GGAAGTAAAAGTCGTAACAAGG -3') primers in a Gene Amp PCR system. Visualization of Amplified fragments was done on safe-view stained 1.5% agarose electrophoresis gels. The size of the amplicon was about 650bp and the DNA ladder from NEB. DNA purification kit was used to purify the amplified 16S rRNA fragments and sequencing was done on both strands using a GeneAmp PCR system 9700 at

the International Institute of Tropical Agriculture Bioscience Laboratory (Ibadan Nigeria). DNA Baser Sequence Assembler was used to edit and assemble contigs from chromatograms. Assembled nucleotide sequences were aligned with those obtained in the GeneBank of NCBI.

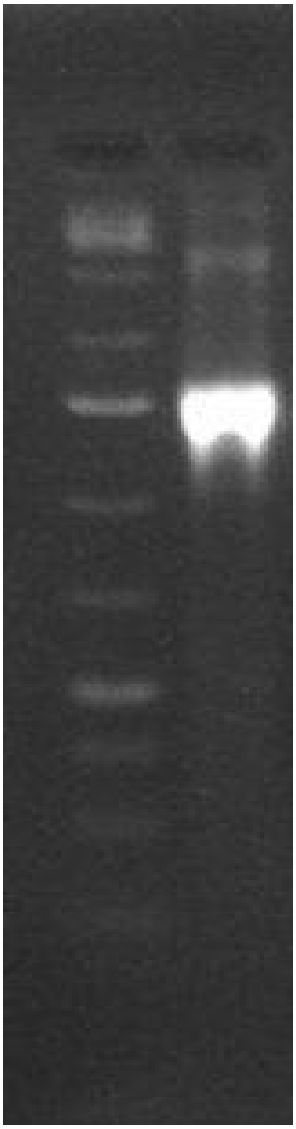

Plate 1. PCR on gel electrophoresis for *B. subtilis*

2.3 Production of Pullulanase Enzyme in Broth

The choice isolate which was identified using molecular methods was inoculated into the production medium, which consisted of: {(g/l) pullulan/cassava flour (10), NaCl (2), $MgSO_4.7H_2O$ (0.1), K_2HPO_4 (0.17), $KH_2PO_4.7H_2O$ (0.12) and $NaNO_3$ (5), pH 7}. The flask was loaded on a rotary shaker incubator at a speed of 200 rpm at 37°C for 48 h after which centrifugation was done at 4,000 rpm for 20 min to separate the cells from the medium. The resulting supernatant was collected and used for the pullulanase assay [6].

2.4 Pullulanase Assay

Pullulanase assay was done as described by [7]. Pullulanase assay was determined by quantifying the reducing sugars released during the activity of the pullulanase enzyme on the substrate. The reaction mixture containing 0.5 ml of crude enzyme and 0.5ml of (1% pullulan in 0.05M potassium phosphate buffer- pH 7.0) including the negative control used for the enzyme assay (0.5ml of 1% pullulan/cassava flour in 0.05M potassium phosphate buffer at pH7 without the enzyme) was incubated at 40°C for 30 min. The reaction was stopped by the addition of 2 ml of 3,5-dinitrosalicylic acid, followed by boiling for 10 min to develop colour. The absorbance of the mixture was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1 µmol glucose per minute under the assay conditions. The selected bacterial isolate which showed very high pullulanase activity was identified using molecular identification methods.

2.5 Fermentation of Garri

This procedure was carried out according to the method described by [7] with slight modifications. Cassava roots which were freshly harvested were peeled using a sterile peeling knife in order to remove the brown outer skin. The peeled roots were thoroughly washed using potable water to remove all sand particles and dirt and reduce microbial contamination. The clean roots were grated to obtain a mash. Grating was done using hand graters, after which 100g of grated cassava mash measured into different sterile containers. A 24-hour old actively growing culture of *Bacillus subtilis* was dissolved in sterile distilled water. A quantity of 30ml of 6.4 x 10^{-7} CFU/ml of the culture was dispensed into each container and mixed thoroughly. The pH of the cassava mash mixture was measured and adjusted to different levels: 4 and 5. A control sample, which was just cassava mash without the addition of any microbial culture or pH adjustment (pH6), was prepared along-side. The mixture was allowed to stand for two hours after which they were loaded

into sacs and dewatered by gently squeezing out excess liquid from the sacs; this was done in order to facilitate a solid state fermentation. It was then left for 3 days to ferment after which the debranching activity and was monitored using different parameters.

2.6 Preparation of Retrograded Garri

Retrograded garri was processed by the adoption of the method described by [8] on the retrogradation of rice, with slight modifications. The cassava mash from experiments above was sieved and roasted (garrified) for 30 minutes at 90^{o}C. It was then cooled at 10^{o}C, for 5 days, after which it was dried using a laboratory oven at 50^{o}C. It was grinded with an electric blender and stored in sacs for the purpose of resistant starch determination.

2.7 Determination of Resistant Starch

Resistant starch was determined using a kit assay (K-RSTAR, Megazyme Bray, Co. Wicklow, Ireland). This kit follows the protocols of the [9] procedures explicated by [10]. Samples (100±0.5 mg) prepared as already described was incubated with pancreatic α-amylase (10 mg/ml) solution containing amyloglucosidase (AMG) for 16 h at 37°C with constant shaking. After hydrolysis, samples were washed thrice with ethanol (99% v/v and 50% ethanol) followed by centrifugation. The separated pellet from supernatant was further digested with 2 M KOH. Digested pellet and supernatant was separately incubated with AMG. Glucose released was measured using a glucose oxidase-peroxidase (GOPOD) reagent kit (K-GLOX, Megazyme Bray, Co. Wicklow, Ireland) by absorbance at 510 nm against the reagent blank. The glucose content of the supernatant and digested pellet was used in calculation of digestible starch (DS) and Resistant Starch (RS) respectively by applying the factor of 0.9.

2.8 Determination of Glycemic Index

Food samples**:** Glucose, test garri samples and control garri sample. The garri samples were prepared by grinding using an electric blender and the flours were packed in polyethylene sacs and stored until used in the next step.

Experimental animals**:** Male albino winstar rats weighing between (100-120g body weights) were housed individually in suspended mesh bottom and front stainless steel hanging cages in a controlled condition, between 20 - 25°C, 12 hours light and dark cycle. After five days of adaptation, these animals were weighed and divided into 4 groups of six animals each, notably, with no statistical differences. Deionized distilled water was offered *ad libitum.* Animals were fasted (overnight fasting) for 15 hours and tested for blood glucose at zero time before given in amount of test food.

Group 1 was fed with sample 1 retrograded garri.

Group 2 was fed with sample 2 retrograded garri

Group 3 was fed with sample non retrodraded garri

Group 4 was fed with glucose

The experimental groups and control group were allowed access to diet for 60 minutes and then the diet was withdrawn. Blood glucose level of all groups was monitored with glucometer at time intervals of 30 minutes, 60 minutes, 90 minutes and 120 minutes after which the diet was withdrawn. The same method was repeated with the control group, by giving 0.15g standard glucose dissolved in distilled water.

Table 1. Pullulanase assay

Carbon Source	Enzyme Quantity
Pullulan	0.69U/ml
Raw Cassava Flour	0.83U/ml

2.9 Measurement of Blood Glucose Response

Blood glucose was determined by using glucose tester Device made by One Touch; Fabricado por, lifeScan Europe, division of Cilag international, Switzerland. Blood samples were taken from Tail tipping. Glycemic Index (GI) for each diet was determined by calculation of Incremental Area Under two hours of blood glucose response or Curve (IAUC) for each diet and compared with the IAUC for glucose solution standard according to the method of [11] which also reported by [12] using the following equation:

$$\mathrm{GI} = \frac{\text{Incremental Area Under 2h blood glucose Curve for food}}{\text{Incremental Area Under 2h blood glucose Curve for glucose}} \mathrm{X}\ 100$$

3. RESULTS AND DISCUSSION

3.1 Molecular Identification of Isolate

The molecular identification confirmed the choice isolate to be *Bacillus subtilis* with Accession number: MT178206.1 This finding is in agreement with the earlier reports that pullulanase enzymes are predominantly extracellular enzymes produced by a variety of bacteria, mainly by *Bacillus* sp. [13].

3.2 Production and Assay of Pullulanase Enzyme in Broth

A higher pullulanase activity was observed (0.83 ±0.01 U/ml) when raw cassava flour was used as carbon source compared with pullulan (0.69 ±0.01 U/ml). Similar findings have been reported by [14], where *Bacillus subtilis* was reported to produce pullulanase enzyme in high quantities.

3.3 Determination of Resistant Starch in Retrograded Garri

The resistant starch quantity of the different garri samples is shown in Table 2, the garri samples which had undergone fermentation using the choice organism *Bacillus subtilis* had much higher quantities of resistant starch (14.29%, 11.06%) than the control garri sample which was not fermented with the choice organism (4.73%). Similar results have been reported by [7] who obtained higher quantities of resistant starch after fermentation with autoclaving and cooling cycles of taro using *Lactobacillus plantarum* and *Leuconostoc mesenteroides.* Resistant starch formation can result from many factors, among which are: fermentation conditions, enzymatic hydrolysis by debranching enzymes, amylose content, autoclaving temperature, molecule chain length and storage time. In this study, the pullulanase enzyme produced by *Bacillus subtilis*, during fermentation of cassava mash led to the debranching of the raw cassava starch which upon retrogradation, led to the ultimate increase of the resistant starch contents. The resistant starch content of the garri samples which were retrograded at 10^{o}C was found to produce much higher resistant starch quantities, (12.56%) than the control garri sample which was kept at room temperature (4.73%). Similar results have been reported by [15], where retrograded banana flour kept at refridgeration temperature yielded a higher amylose content leading to a higher Resistant starch III (RS3) content. Also, [16], retrograded rice at refridgeration temperature and it also yielded a high RS3 content. However, results obtained by [17], showed that lower temperature storage conditions (4°C) did not significantly affect the formation of RS3 in rice. These could be attributed to the formation of several linear amylose fractions during debranching and a greater re-association between the amylose straight chains in the cassava starch as a result of aging and freeze-thaw process which leads to recrystallization of starch at low temperature. This re-crystallization process is also known to reduce the digestibility of starch [18].

3.4 Determination of Glycemic Index in Retrograded Garri Samples

Blood Glucose response of diets containing different samples of garri, for healthy male albino winstar rats using glucose as standard at 30, 60, 90 and 120 minutes respectively are shown in Table 2, while Fig. 1 shows the graph of glycemic response of the four samples of garri at various time intervals. The results for the blood glucose and glycemic index of different samples of retrograded garri with the use of male albino winstar rats and glucose as standard showed that there were major differences in blood glucose responses between test garri samples and the glucose standard. This finding shows that the untreated garri sample had low quantity of resistant starch, resulting to an increased quantity of glycemic index, similar trends have been reported by [19], who observed that higher resistant starch content led to a low glycemic index and an increased glucose concentration in obese dogs. For most starchy foods, a reduction in GI is usually accompanied by a higher content of resistant starch [20]. Also, the mean values of IAUC for garri sample (G1), the untreated garri sample (195 mmol.min/L) was significantly ($P<0.05$) high comparing with the other meals. This could be attributed to a number of reasons. Firstly, since the starch in the control garri samples didn't undergo debranching or retrogradation during processing, the absence of the activity of pullulanase prevented the enzyme hydrolysis of α-1,6-glucosidic bonds of the cassava starch and released linear polymers linked by α -1,4- glucosidic bonds. Secondly, the absence of these linear polymer fragments meant a great availability of digestible starch with high IAUC [21].

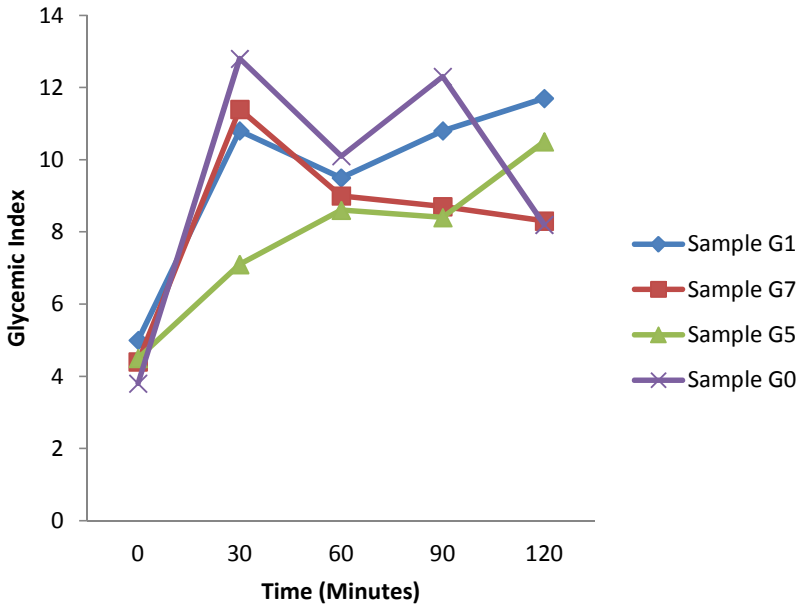

Fig. 1. Blood glucose response and glycemic index for meals of different Garri samples

Key:
Sample G0: Glucose Standard (Glucose D)
SampleG1: Control Sample Garri, fermented for 48 hours, pH6
Sample G5: Garri fermented for 48 hours with B. subtilis, pH 5
Sample G7: Garri fermented for 72 hours with B. subtilis, pH 5

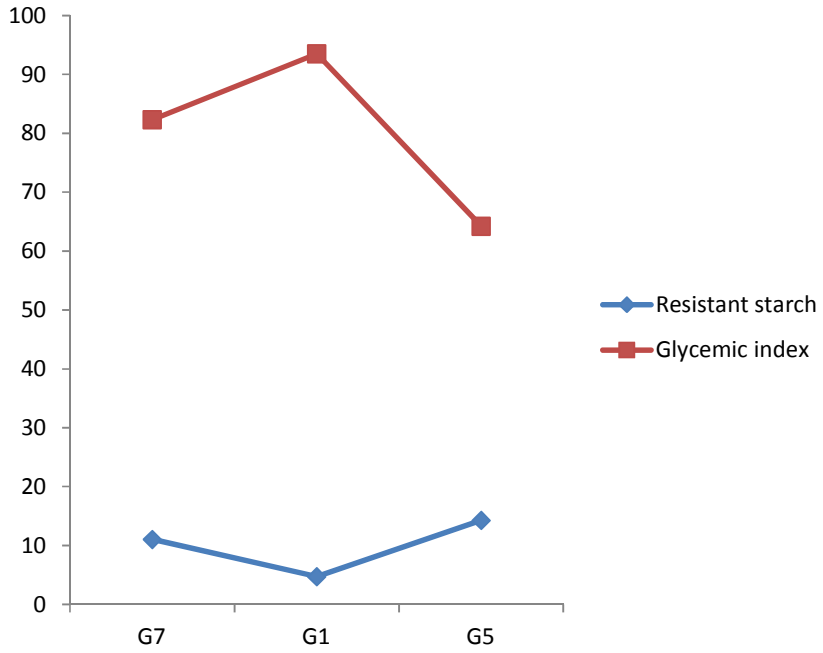

Fig. 2. Relationship between RS and GI of the Garri samples

SampleG1: Control Sample Garri, fermented for 48 hours, pH6
Sample G5: Garri fermented for 48 hours with B. subtilis, pH 5
Sample G7: Garri fermented for 72 hours with B. subtilis, pH 5

Table 2. Resistant starch quantities of retrograded Garri samples

Sample	Resistant starch quantity (%)
G1: Non- Retrograded Control Sample Garri, at 48 hours (fermented at pH6 without choice isolate)	4.73
G2:Garri at 24 hours fermentation with choice isolate, pH 4	7.46
G3:Garri at 24 hrs fermentation with choice isolate, pH 5	12.53
G4:Garri at 48 hrs fermentation with choice isolate, pH 4	6.94
G5:Garri at 48 hours fermentation with choice isolate, pH 5	14.29
G6:Garri at 72 hrs fermentation with choice isolate , pH4	6.10
G7:Garri at 72 hours fermentation with choice isolate, pH 5	11.06

Table 3. Blood glucose responses and glycemic index of Garri samples

Diet	Blood Glucose Response (mmol/l)					IAUC	Glycemic
	Time (minutes)					Mmol/min	Index
	0	30	60	90	120		
Sample G0	3.8±0.71	12.3±0.35	6.7±0.37	12.3±0.61	8.2±0.32	201	100
Sample G1	5.0±0.47	10.8±0.05	9.5±0.08	10.8±0.82	11.7±0.24	187.5	93.5
Sample G7	4.4±0.91	11.4±0.06	9.0±0.22	8.7±0.09	8.3±0.31	163.5	82.3
Sample G5	4.5±0.67	7.1±0.86	8. 6±0.03	8.4±0.21	10.5±0.62	129	64.2

Key:
Sample G0: Glucose Standard (Glucose D)
SampleG1: Control Sample Garri, fermented for 48 hours, pH6
Sample G5: Garri fermented for 48 hours with B. subtilis, pH 5
Sample G7: Garri fermented for 72 hours with B. subtilis, pH 5

4. CONCLUSION

In this study, pullulanase-producin *Bacillus subtilis* isolated from cassava processing sites was used in the fermentation of cassava to produce garri. The microorganism was used to produce garri with high resistant starch, type III yields ranging from 4.73% to 14.25%, the highest resistant starch yields were observed in garri samples which were fermented for 48 hours at pH 5. Also, the lower glycemic index for the garri sample was observed with amongst samples with the higher resistant starch quantities. This study showed that pullulanase from *Bacillus subtilis* is a very useful enzyme in biotechnological applications. However, additional studies are necessary for more detailed characterization and use for other industrial purposes.

ETHICAL APPROVAL

The overall care of the albino rats was maintained in accordance with the provisions of the National Institute of Health Guidelines for Care and Use of Laboratory Animals (PUB No 85-23, revised 1985) under the approval of the University Ethical Committee on the use of laboratory animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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