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PRODUCTION AND UTILIZATION OF PULLULANASE FROM *BACILLUS CLAUSII* FOR ENHANCED RESISTANT STARCH PRODUCTION IN GARRI

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ABSTRACT

Resistant starch is the portion of starch which escapes digestion in the small intestine and passes into the large intestine where it is fermented by gut microflora. It is considered a functional component of food due to the health benefits it confers following its consumption. The aim of this research was to produce garri with high resistant starch using pullulanase enzyme from *Bacillus clausii*. The organism was isolated from different cassava processing sites in Awka and identified based on phenotypic, biochemical and molecular characteristics. Pullulanase assay was carried out using standard procedures at 540 nm. Optimum conditions for pullulanase production was determined, after which the pullulalanse enzyme was used to modify garri. The test organism with the highest pullulanase activity was identified as *Bacillus clausii*. Optimum pullulalanse activity was observed with an innoculum size of 3.0ml, pH 6, an incubation temperature of 35^{o}C and incubation time of 48 hours. The resistant starch content was found to be higher for enzyme-treated garri samples (13.81%) than for the untreated garri samples with (2.69%). This research shows that pullulanase enzyme from *Bacillus clausii* is a very useful industrial raw material for the increased production of resistant starch in foods.

Keywords: Garri, Pullulanase, *Bacillus clausii*, Resistant starch

INTRODUCTION

In recent times, there is a high demand for functional food which has led to investigations by food industries to produce innovative functional food productions which possess additional health benefits. Due to a recent increased awareness about healthy and nutritious foods, there are concerns with the additional health merits of food **(Huan-Woo *et al.*, 2014)**. Most of the carbohydrate rich foods are considered to be highly glycaemic. This has led to a lot of researches done in order to develop carbohydrate based functional foods with low glycaemic index (GI).

Among carbohydrate based foods, cassava is one of the leading food and feed plants in the world. Although cassava is produced mostly in developing countries, it is ubiquitous as a worldwide tropical staple food because of its ability to grow under harsh climatic conditions, which is common in African countries. Cassava is major source of staple food for majority of Nigerians (**Onyenwoke and Simonyan, 2014**). It has also been estimated that cassava provides food for over 500 million people in the world **(Abu *et al*., 2006)**. It is essentially a carbohydrate food with low protein and fat. It is a very versatile commodity and is consumed in various forms such as: cassava starch, fermented cassava paste, fermented cassava flour, cassava chips, and garri **(Nwafor *et al*., 2015)**. Among these products of cassava, garri is the most popularly consumed in Nigeria and in many West African countries. The food, garri, is creamy-white granular flour made from gelatinized fermented cassava mash. Its wide consumption is attributed to its relatively long shelf life compared to other food products from cassava, as well as its ease of preparation for eating **(Sanni *et al*., 2008)**. However, although garri, as a carbohydrate based food is a major source of energy in the diet of Nigerians; a healthier choice of starchy foods that still provide beneficial functions for sustaining good health is desired. This is because of recent concerns about the side effects of the consumption of garri on the diabetic due to its high glycemic index **(Ogbonna *et al*., 2018)**. Recent studies have shown different attempts to produce functional food products from cassava based foods with properties such as low glycemic index, soluble and insoluble dietary fibre in the gastrointestinal tract **(Ogbo and Okafor, 2015)**. One of such attempts is the production of cassava based foods with high quantities of resistant starch. Resistant starch (RS) is defined as the sum of starch and products of starch degradation not absorbed in the small intestine of a healthy individual **(Sajilata *et al*.,2006)**. RS plays a major role in the health food industry, showing some resistance to human digestive enzymes, leading to a reduced energy intake and hence a low glycemic index. Resistant starch also has the ability to reduce blood cholesterol and triglyceride levels **(Wang *et al*., 2018)**. Different methodologies have been employed to increase resistant starch content in foods such as: chemical treatments using acid hydrolysis, hydrothermal treatments, autoclaving and cooling and enzymatic treatment. Among the enzymatic treatment, the use of pullulanase enzyme has led to great success in increasing the resistant starch content. Pullulanase (pullulan α-glucanohydrolase) is called a de-branching enzymes which is widely used to hydrolyse the α-1, 6 glycosidic linkages in starch, amylopectin, pullulan and related oligosaccharides, to produce maltotriose **(Hii *et al*., 2012)**.

Overall, increasing the RS content in the diet has the potential to provide several significant health benefits and an added value to food products. The aim of this research was to produce garri with high resistant starch using pullulanase enzyme from *Bacillus clausii*. The objectives of this research were specifically to: Isolate and identify pullulanase producing bacteria, study the optimum conditions for producing pullulanase, determine the resistant starch of garri produced with crude pullulanase enzyme from the chosen isolate.

MATERIALS AND METHODS

Isolation of pullulanase producing bacteria

Isolation of pullulanase producing bacteria was done according to the method described by **Shehata *et al.* (2016)**. This was achieved by performing a 6-fold serial dilution of cassava wastes obtained from different cassava processing sites in Awka South Metropolis. Innoculation was done using pullulanase media, which was incubated at 37o C for 48 hours. The pullulan – medium was 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 plates were flooded with 90% ethanol and allowed to sit for 30 minutes, after which the presence of transparent zones of hydrolysis around the colonies was used as an indication of pullulan hydrolysis. Preliminary identification was done using morphological and biochemical identification tests: gram stain, spore stain, catalase, starch hydrolysis, motility, voges proskauer and lecithinase tests. The isolates which were positive for the other listed biochemical tests and negative for lecithinase test were subjected to molecular identification.

Molecular identification of isolated strains

DNA extraction, 16SrRNA amplification, and sequencing

Genomic DNA was extracted from 24 h old bacterial colonies from cassava processing sites 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. Amplified fragments were visualized on safe-view stained 1.5% agarose electrophoresis gels. The size of the amplicon is about 650bp and the DNA ladder from NEB. The amplified 16S rRNA fragments were purified using a DNA purification kit and sequenced on both strands using aGeneAmp 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.

Production of pullulanase enzyme in broth

Enzyme production was done as described by **Shehata *et al*. (2016)** with slight modifications. Fresh 18 hour old culture was inoculated into a culture medium, which consisted of: {(g/l) 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. The cells were removed from the culture medium by centrifugation at 4,000 rpm for 20 min at 10° C. Supernatant was collected and used for the pullulanase assay.

Pullulanase assay

Pullulanase assay was done as described by **Shehata *et al*. (2016)**. Pullulanase assay was determined by measuring the release of reducing sugar from pullulan. The reaction mixture containing 0.5 ml of crude enzyme and 0.5ml of (1% pullulan in 0.05M sodium phosphate buffer- pH 6.8) 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 color. The absorbance of the mixture was measured at 540 nm, and D-glucose is used to create a standard curve, by measuring the absorbance at different concentrations of glucose (0.1-1.0). The OD value of the blank was subtracted from the OD values obtained with all other standard samples to get the relative change in OD (ΔOD). A standard curve was made by plotting a graph of ΔOD as a function of glucose concentration. One unit of enzyme activity is defined as the amount of enzyme releasing reducing sugar equivalent to 1 μmol glucose per minute under the assay condition.

Determination of optimum conditions for pullulanase activity

Effect of optimum incubation temperature for pullulanase activity

The effect of incubation temperature was determined by inoculating the organism into the broth culture and incubating at different temperatures (28, 35, 45 and 55° C) for 48 hours enzyme activity was determined as previously described.

Effect of optimum incubation time on pullulanase activity

The pullulanase production during the incubation period of 96 hours, using the test organism was evaluated. A volume of 100 ml of pullulan medium was dispensed into each 250 ml conical flask. All flasks were autoclaved at 121°C for 15 min. After cooling, the flasks which contained same quantity of media were inoculated with same amount (5.8log CFU/ml) of a 24 hour culture of the organism at 37°C with shaking at 200 rpm and flasks were withdrawn at different time intervals:24,48,72 and 96 hours for assays. The enzyme activity for each flask was determined by centrifugation at 4000 rpm for 20 minutes. The supernatant was used to measure enzyme activity.

Effect of incubation pH on pullulanase activity

The pullulanase activity was studied over a range from pH 3-10, using pullulan as a substrate. The mixture (crude enzyme and pullulan) was incubated for 30 minutes at 40 °C and the activity of the enzyme was measured as previously described.

Effect of inoculum size on pullulanase activity

Pullulan medium was prepared and dispensed into different flasks, with each containing 10 ml of the medium. Each of the flasks was then inoculated with different quantities (1.0, 2.0, 3.0, 4.0 and 5.0ml) of 24 hour culture suspension. They were incubated at 37°C for 48 hours. The cells were precipitated by centrifugation at 4000rpm. Supernatant was assayed for enzyme activity.

Fermentation of cassava and garri production

Freshly harvested cassava roots were peeled using a sterile peeling knife in order to remove the brown outer skin. Peeled roots were washed thoroughly in potable water to remove all sand particles and dirt and reduce microbial contamination. The clean roots were grated to obtain a mash. Grating was carried out by means of hand graters. The pullulanase enzyme was added in a quantity of 50 ml to 50 g of the grated cassava mash and allowed to stand for 4 hours, in order to achieve debranching, after which the resulting mash was loaded into sacs and slowly dewatered by gently squeezing out excess liquid from the sacs. It was then left for 3 days to ferment after which the resulting fermented mash was sieved and roasted through a method of garification. The garri was dried at room temperature, grinded with an electric blender and stored in sacs for the purpose of resistant starch determination.

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 **AOAC. (2003)** procedures explicated by **Niba and Hoffman (2003)**. 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.

Statistical analysis

All the experiments were conducted in triplicate. Statistical difference was analyzed for experiments of optimization by one way ANOVA test using SPSS 21.0. $P < 0.05$ was considered as significant at the 0.05 level of confidence.

RESULTS

Table I Molecular identification of isolates

Isolate Number	Name Of Organism	Max Core	Total Score	Query Cover	E Value	Per Ident	Accession
C1	*Bacillus cereus* strain AJSI 16S ribosomal RNA gene, partial sequence	1234	1234	95%	0.0	97.41	KR706562.1
C2	*Bacillus clausii* strain PJC 14 16S ribosomal RNA gene, partial sequence	1255	1255	97%	0.0	95.01	MK802117.1
C3	*Bacillus cereus* strain ST06 16S ribosomal RNA gene, partial sequence	1618	1618	100%	0.0	99.44	MH475925.1

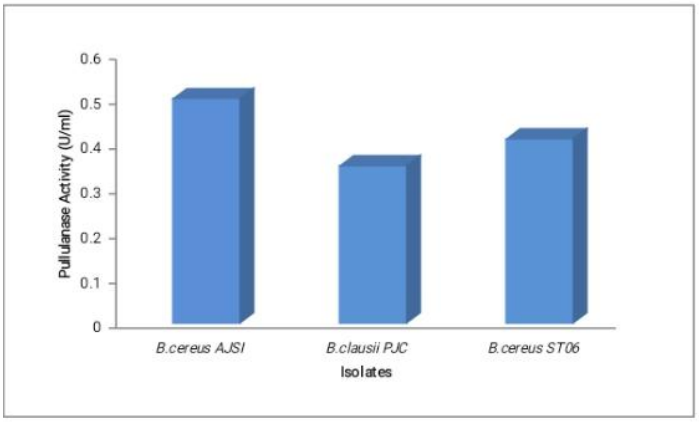

Figure I Production and assay of pullulanase enzyme

The results of the pullulanase production and assay showed different levels of enzyme activity from the different selected isolates are represented in Figure I. The maximum pullulanase activity (0.50 ±0.01 U/ml) was observed with *Bacillus cereus*, while *Bacillus clausii* showed the lowest pululanase activity (0.35±0.005 U/ml). The molecular identification revealed the level of relatedness of the choice isolate to the other *Bacillus* species submitted to the NCBI database as shown in Table II.The isolates were confirmed to be different species of *Bacillus: Bacillus cereus* and *Bacillus clausii.*

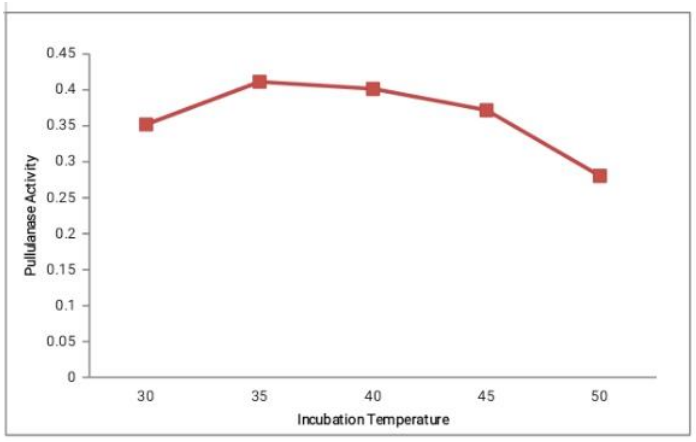

Figure II Effect of incubation temperature on pullulanase production

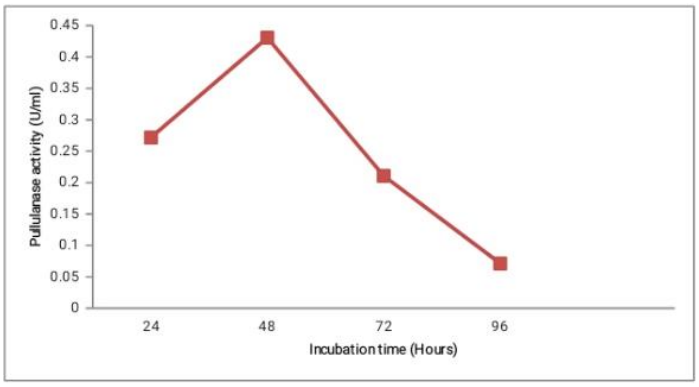

Figure III Effect of incubation time on pullulanase production

Pullulanase production was observed according to figure III, to increase linearly with an increase in period of incubation until the stationary phase of growth was attained. *Bacillus clausii* had its highest enzyme activity at 48 hours (0.51±0.02 U/ml). There was a general decrease in enzyme activity as the period of incubation increased from 72 to 96 hours (0.22±0.01 U/ml and 0.07±0.01 U/ml). The effect of incubation temperature on pullulanase production is shown in Figure II. The enzyme activity from broth cultures containing *Bacillus clausii*, showed a gradual decrease in enzyme activity as temperature increased, with the highest enzyme activity (0.48±0.01 U/ml) at a temperature of 35ºC.

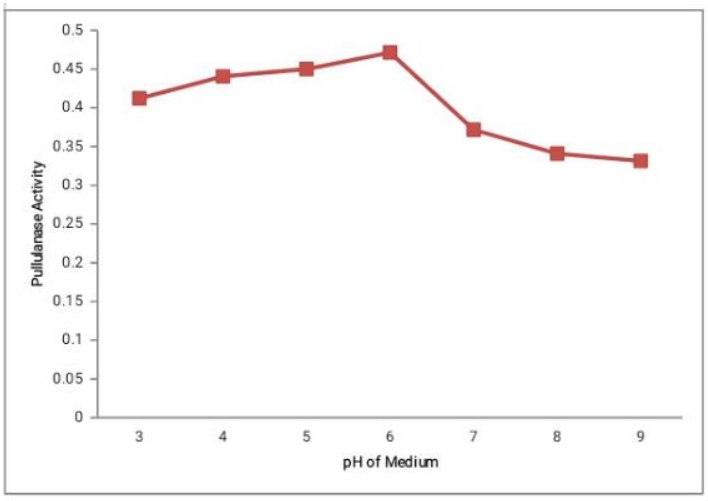

Figure IV Effect of pH on pullulanase production

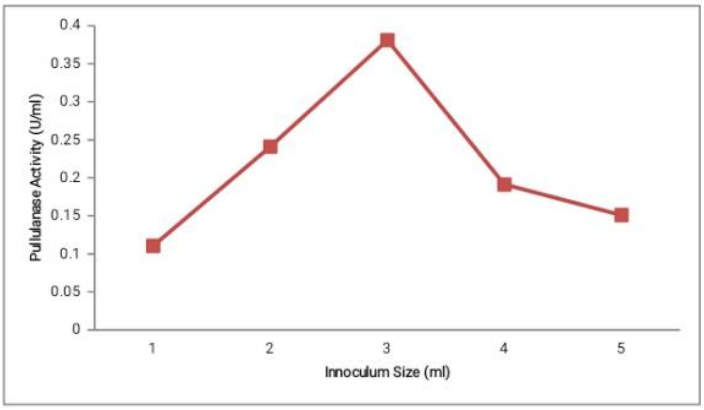

Figure V Effect of innoculum size on pullulanase production

Pullulanase production was monitored using different quantities of the inoculum as shown in Figure V. It was observed that there was a gradual increase in the pullulanase activity as the inoculum size increased from 1 to 3ml (0.11 to 0.43U/ml), however afterwards, there was a decrease in the pullulanase activity as the inoculum size increased from 4 to 5ml (0.28 to 0.15U/ml). The effect of pH on pullulnase activity is shown in Figure IV. Pullulanase from *Bacillus clausii*, showed a highest enzyme activity at pH 6 (0.43±0.02 U/ml), after which there was a consistent decrease in enzyme activity with increase in pH, from pH 7 to 9. The lowest pullulanase activity was observed at pH 9 (0.31±0.01 U/ml).

Table II Resistant starch quantity of garri samples

Sample code	Resistant starch Quantities
Cg0	2.69
Cg1	13.81

Key: Cg0- Control garri sample without pullulanase enzyme, **Cg1**: Test garri sample with pullulanase enzyme

The resistant starch quantity of the different garri samples is shown in Table II. The samples differed in the quantities of their resistant starch according to the different treatments they received. The sample Cg0 (control garri sample with no pullulanase enzyme treatment) had a low quantity of resistant starch (2.69%). However, the resistant starch quantity for the garri sample in which the pullulanase enzyme from *Bacillus clausii* was added, Cg1, had a considerably higher quantity of resistant starch (13.81%).

DISCUSSION

Pullulanase , an important debranching enzyme has been widely utilized to utilize the α- 1,6 glucosidic linkages in the starch/ sugar industry and used predominantly in conjunction with other enzymes to break down starch **(Wei *et al*.,2015)**. Cassava wastes from processing sites, are known to be a repertoire of different microorganisms with industrial advantages. Different *Bacillus* species isolated from different cassava processing sites possessed the ability to produce the microbial pullulanase enzyme. This could be attributed to their ease of adaptation and utilization of the substrates in the environment, which is composed mainly of starch **(Ling *et al*., 2009)**. *Bacillus cereus* had the highest pullulanase activity amongst others (Figure I). Similar results have also been reported by a number of researchers, where *Bacillus cereus* was reported to produce pullulanase enzyme in high quantities **(Waleed *et al*., 2015; Davaeifar *et al*., 2015)**. However, *Bacillus clausii* also showed a good pullulanase activity and was chosen for the enzyme studies due to its GRAS (Generally Regarded as Safe) status.

A substance may be GRAS only if its general recognition of safety is based on the views of experts qualified to evaluate the safety of the substance. GRAS status may either be affirmed by FDA or determined independently by qualified experts. *Bacillus clausii* is an organism with known GRAS status and is effectively used in the food industry as probiotics **(Sewalt *et al*., 2016)**.

Temperature plays a major role in pullulanase production, this is because temperature is very key to microbial growth and metabolite production. There was pullulanase production by the *Bacillus* species at incubation temperatures between 30ºC and 45ºC, (Figure II) however the pullulanase production was highest when incubation temperature was 35ºC with an enzyme activity of (0.48±0.01 U/ml). Similar results were obtained by **Asha (2013)** who reported a stable pullulanse production by *B. halodurans* within a range of 28–65°C with its maximum production at 37°C. Determination of the optimum incubation time for pullulanase production over duration of 96 hours was studied (Figure III). The maximum production of pullulanase was observed at 48 hours of incubation (0.51 ±0.02 U/ml). There was a decrease in the enzyme production, afterwards. This finding is similar with studies carried out by **Prabhu *et al*. (2018)**, who reported same trend during the production of pullulanase in *Klebsilla aerogenes* and **Waleed *et al.* (2015)** who reported the same trend from *Bacillus cereus*. Reduction in pullulanase production after 72 hours (0.07±0.01 U/ml) could be attributed to the decline phase reached by the organism at 72 hours of medium incubation. Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and, in particular enzyme molecules **(Shah and Madamwar, 2005)**. The effect of pH on enzyme activity was studied over a range of pH (Figure IV) and it was observed that the maximum pullulanase activity was achieved at pH 6.0 (0.43±0.02 U/ml). This indicates that pullulanase activity from *B.clausii* has high activity at relatively low pH. Studies from other research have shown that pullulanase has an optimum activity at pH 5.5 **(Chen *et al*., 2014)**. Pullulaanse activity was also observed at higher pH values but at lower levels as reported by **Wei *et al.* (2015)** who observed a similar result where *Bacillus acidopullylyticus* produced a stable but low pullulanase enzyme at alkaline pH.

The resistant starch quantity of the different garri samples is shown in Table II. The cassava mash samples in which the pullulanase enzyme was added produced debranched cassava which after roasting to produce garri, had much higher quantities of resistant starch (13.81%) than the control garri sample whose cassava starch was not debranched (2.69%). Similar results have been reported by other researchers. **Polesi *et al.* (2011)** reported an increase in RS of high amylose rice through pullulanase debranching activity from 16% to 32%. The pullulanase enzyme hydrolyses α-1,6-glucosidic bonds which leads to the release of linear polymers linked by α-1,4-glucosidic bonds. **(Li *et al*., 2011)**.The debranching activity carried out by this enzyme modifies the molecular structure of starch by enabling the chains to aggregate and hence form perfectly crystalline structures, thereby leading to the formation of more Resistant starch.

CONCLUSION

A pullulanase-producing bacterium from soil from cassava waste was identified as *Bacillus clausii.* It was found to be a slightly acidic and mesophilic which can therefore be utilized in starch processing, food industries and other biotechnological applications. This pullulanase, enzyme was also found to be effective in debranching the starch in raw cssava mash, which contributed to a higher resistant starch quantity when it was used in garri production. It is therefore required that further isolation, genetic expression and manipulation be done in order to achieve maximal production for better yield of pullulanase for other industrial applications.

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