

Full Length Research Paper

Detoxification of cyanides in cassava flour by linamarase of *Bacillus subtilis* KM05 isolated from cassava peel

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Accepted 27 October, 2011

Defensive cyanogenic glucoside linamarin accounts for 80% cyanide content of cassava and is known to cause severe diseases upon continual consumption. Detoxification of this cyanide would enhance the nutritive quality and hence market value of cassava flour. We isolated cyanogenic glucoside utilizing indigenous bacteria from cyanide rich cassava peel waste and exploited their potential for detoxification. Among the isolates, *Bacillus subtilis* KM05 utilized cyanogenic glycoside through assimilatory degradation with the release of hydrogen cyanide and ammonia. The partially purified linamarase (53 KDa) enzyme from this organism showed considerable activity (9.6 U/ml) and effected rapid cyanide reduction in cassava flour. The results indicate scope for enzymatic detoxification of cassava cyanide without compromising nutrients in sago industries.

Key words: Cyanogenic glucoside, enzymatic treatment, linamarin, linamarase.

INTRODUCTION

Cassava, the vital food in the tropical areas of Africa, Asia and Latin America is the third most important source of calories in the tropics, after rice and corn. The enlarged root of the cassava plant contains a highly digestible starch of important nutritional value. Products derived from cassava are the principal food source of 500 million to 1 billion people in tropical countries (Sornyotha et al., 2010). Over 160 million tons of cassava is produced globally per annum, ranking it as the 4th crop in worldwide production after rice, wheat and maize (Ugwuanyi et al., 2007). This major staple food has two major deficiencies, that is, high content of the poisonous cyanogenic glucoside linamarin (and to a lesser extent,

lotaustralin), and low content of protein and free amino acid (Cooke and Coursey, 1981).

The perishable tuber is normally stored as cassava flour, a substitute for wheat and rice. Cassava flour is produced primarily by the wet milling of fresh cassava roots which includes the following five main stages: preparation (peeling and washing), rasping/pulping/grating, purification (starch washing), dewatering and drying, and finishing (milling and packaging). Upon tissue disruption, the cyanogenic glucosides are brought in contact with glycosidase and hydroxynitrile lyases and are degraded into cyanohydrins, hydrogen cyanide and ketones (Conn, 1980). When cassava products are used as a primary staple food, careful processing to remove these toxic constituents is required to avoid chronic cyanide intoxication (Onabolu et al., 2002). These cyanogens upon intake may cause cyanide poisoning with symptoms of vomiting, nausea, dizziness, stomach

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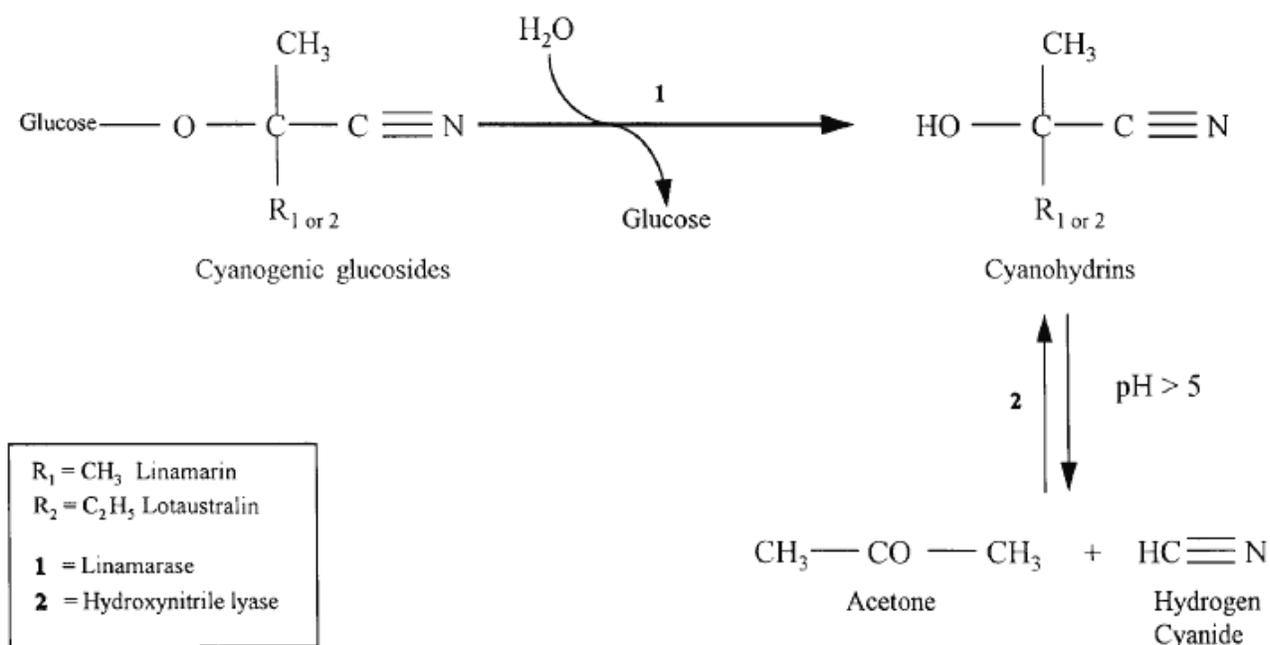


Figure 1. Enzyme catalyzed degradation of cyanogenic glycosides.

pains, weakness, headache and diarrhea and occasionally death (Akintonwa et al., 1994; Mlingi et al., 1995). While incomplete processing result in high cyanide exposure and associated severe diseases like tropical ataxic neuropathy, konzo, etc., the careful processing generally results in loss of proteins, vitamins and minerals. Hence, a well-organized method for detoxifying cassava without nutrient loss is desired. Though a number of processes aiming at the degradation and thereby reduction in cyanide content were developed, all of them either failed to yield the expected reduction in cyanide or resulted in the reduction of the nutrient composition.

Silano et al. (1982) reported that about 50% of the nitrogen content of cassava is in the form of free amino acids and they may be lost during processing. Separation of the starch granules from the tuber in pure form is essential in the manufacture of cassava flour. The linamarin is a β -glucoside of acetone cyanohydrin and ethyl-methyl-ketone-cyanohydrin whose Linamarin β -linkage can only be broken under high pressure, high temperature and use of mineral acids, while its enzymatic break occurs easily (Cereda and Mattos, 1996). Hence removal or conversion of these antinutrient cyanogenic glycosides would be possible through target specific enzymatic conversion if the methods of application are standardized. Linamarase or β -D-glucosidase (EC 3.2.1.21), an enzyme found in many plants including cassava and in microorganisms converts the cyanide containing compounds into acetone cyanohydrins, which spontaneously decomposes to hydrogen cyanide (HCN)

(Rolle, 1998). The HCN then either dissolves readily in water or is released into the air (Figure 1).

When the roots are completely disrupted, all linamarin will come out in contact with its hydrolytic enzyme (linamarase), resulting in hydrolysis and subsequent removal of the breakdown products during washing. Then, addition of exogenous linamarase directly into completely disrupted root is sufficient to hydrolyze all the free linamarin. Several investigators have explored linamarase to facilitate the process of cassava cyanide detoxification (Ikediobi and Onyike, 1982; Petruccioli et al., 1999; Yeoh and Sun, 2001). The objective of this study was to ameliorate cassava starch quality through bioprocesses, towards reduction of any remaining linamarin in the mash without any nutritional compromise and if possible simultaneously increase the protein content using linamarase isolated from indigenous bacteria degrading linamarin rich cassava peel wastes.

MATERIALS AND METHODS

Isolation of cyanide degrading bacteria

Composite soil and partially degraded cassava peel were collected from waste dumping sites at the cassava processing unit, SPAC Tapioca products and Chellapa sago factory located in Poonachi, Erode district of Tamilnadu, India. The cyanide-utilizing microorganisms were isolated from the cassava peel soil waste by inoculating 1 g of sample into an isolation medium containing $\text{K}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; CaCl_2 , 0.01 g; NaCl , 0.01 g; MnSO_4 , 0.2 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 mg; ZnSO_4 , 0.2 mg; glucose, 2.0 g; tryptone, 1.0 g (Watanabe et al., 1998). The enriched culture

was screened for linamarin utilization and linamarase production by spreading appropriately diluted growth on sterile nutrient agar plates having 2 ml of 1% p-nitrophenyl- β -D glucoside (PNPG). A control plate contained PNPG but not the organism. All plates were incubated at 30°C and examined after 24 h. Since linamarase is a β -glucosidase, PNPG broke down into citron-yellow coloured nitrophenol which diffuses into the medium around the colony indicating linamarase activity. Positive isolates were graded in the same way as for linamarin assay, based on the strength and spread of the yellow coloration. Isolates that grew on the plate but without yellow coloration beyond the colony margin were considered negative (Ugwuanyi et al., 2007).

The glycogenic cyanide utilization by the isolates was confirmed using a medium containing bacteriological peptone, 0.5 g; meat extract powder, 0.2 g; Tween 80, 0.1 ml; mineral solution (ZnSO₄·7H₂O, 1 g; CuSO₄·5H₂O, 0.5 g / 100 ml), 0.5 ml; agar, 1 g; sodium phosphate buffer, 0.1 M; and 0.01% linamarin [previously isolated from young leaves of cassava and partially purified as described by King and Bradbury (1995)]. Inoculated agar plates were then incubated at 37°C for 48 to 72 h. The breakdown of linamarin during growth was detected by means of placing aseptically a filter paper impregnated with alkaline picric acid into the headspace of agar slant. Discoloration of filter paper caused by HCN was taken as indication of linamarase activity (Okafor and Ejiofor, 1986). Controls were setup by incubating uninoculated medium. The cyanide degrading bacterial isolate obtained was characterized and identified on the basis of cell and colony morphology, Gram staining, physiological and biochemical reactions according to Bergey's manual (Holt et al., 1994).

Cyanide utilization pattern of the isolate

Determination of hydrogen cyanide production (Lorck, 1948)

A modified nutrient agar medium having peptone, 10 g; NaCl, 5 g with 4.4 g glycine/lit was prepared and sterilized. Cyanide degrading bacteria were streaked and Whatman filter paper No 1 soaked in sodium carbonate (2%) in 0.5% picric acid solution was placed on the top of the plate. Plates were sealed with parafilm and incubated at 37°C for four days. After incubation, colour change from yellow to brown was observed.

Determination and quantification of ammonia release (Ahmad et al., 2002)

Cyanide utilizing bacteria were inoculated into 10 ml of peptone water in each tube and incubated for 48 h at 37°C. After incubation, 0.5 ml of Nessler's reagent was added into tubes and the colour change was observed (pale yellow to dark yellow). The released ammonia was assayed by mixing 0.5 ml samples with equal volume of a 1:3 dilution of commercially available Nessler's reagent and measuring the absorbance at 420 nm using SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Richmond, USA).

Enzyme extraction

Cell-free extracts were prepared from cells grown in M9 minimal medium (without ammonium and citrate) adjusted to pH 9.5 on a rotary shaker (Thermoscientific, USA) at 230 rpm and 30°C in the presence of 2 mM linamarin solution as nitrogen source and 50 mM acetate as carbon source (Luque-Almagro et al., 2005). Cells were harvested at the latest stage of the exponential growth and resuspended in 50 mM Tris/HCl buffer (pH 8.5). Cells were broken

by cavitations and three pulses of 5 s at 90 W. After centrifugation (Sigma Laborzentrifugen GmbH, Germany) at 19000 g, supernatants were collected and used as a source of enzymes. The cell-free extract was subjected to ammonium sulphate precipitation (30 to 60%) and the precipitate obtained after centrifugation, was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer. Protein concentrations were determined as described (Bradford, 1976) using bovine serum albumin as a standard. The extent of the purity of preparation and the molecular mass of the purified enzyme preparation were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The test sample solution was prepared by dilution (1:1) in sample solubilizing buffer, which were placed for 10 min in a boiling (100°C) water bath. After cooling to room temperature, the samples were spun for 1 min. The samples containing equal amount of proteins were loaded into the wells of polyacrylamide gels. The medium range molecular weight markers were used and electrophoresis was carried out at constant voltage of 75 V for 2 h. The gels were stained with 0.2% Coomassie brilliant blue solution.

Enzyme assay

The linamarase activity of the isolate was quantified as described (Ugwuanyi et al., 2007). Quantification of linamarase was based on the degradation of linamarin analogue PNPG and determination of the released p-nitrophenol (Ikediobi et al., 1980). The assay medium included 0.5 ml of enzyme extract, and 1.0 ml of 5 mM PNPG in 0.01 M phosphate buffer with pH 6.8. The mixture was incubated for 15 min at 65°C, and the reaction was terminated by the addition of 2 ml of 0.2 M borate buffer with pH 9.8. Colour of the released p-nitrophenol was measured at 425 nm in Genesys 20 Spectrophotometer against enzyme blank. Temperature inactivated enzyme was used as blank. One unit of linamarase activity was expressed as the amount that caused a change in absorbance of 0.01 units against an enzyme blank under the defined assay conditions (Ugwuanyi et al., 2007).

Effect of enzymatic treatment on cassava flour

An aliquot of 500 mg of cassava flour sample was put in a plastic vial, and a small filter paper impregnated with pH 6 buffer was placed in the vial. Linamarase, followed by 2.5 ml of water and a yellow picrate paper were added to the vial and the cap was immediately closed and left at 30°C overnight. The next day, the yellow-brown picrate paper was separated from the plastic backing strip and placed in 25 ml of water. The absorbance of the solution was measured at 510 nm and the total cyanide content in ppm was calculated by multiplying by 396 (Bradbury et al., 1999). To determine and compare the effect of enzyme treatment on cassava flour, two sets each containing 5 g of flour were taken in a beaker. One batch was well mixed with 6.25 ml water, the other with 5 ml water and 1.5 ml enzymatic extract. The beakers were placed in an incubator at 30°C for 5 h, after which the cyanide analysis was carried out as described (Cumbana et al., 2007). The changes in moisture (923.03), crude protein (Kjeldhal method, N \times 6.25), fat (Soxhlet method) and ash (923.10) were also determined as specified by the Association of Official Analytical Chemists (AOAC, 1995).

RESULTS

15 isolates with different colony morphology that showed

Table 1. Taxonomic characteristics of *B. subtilis* KM05.

S/N	Name of test	Result
1	Gram staining	Gram positive, straight, large, uniform multiseptate
2	Endospore	Positive
3	Acid fast staining	Negative
4	Oxidase	Negative
5	Catalase	Positive
6	Vogus- Proskaur	Negative
7	Methyl red	Negative
8	Indole	Negative
9	Citrate	Positive
10	Esculin hydrolysis	Positive
11	Casein hydrolysis	Positive
12	Gelatin hydrolysis	Positive
13	Growth at 6.5% NaCl	Negative
14	Growth above 55°C	Negative

utilization of cyanide on screening medium were obtained and all the isolates were screened for their ability to utilize cyanogenic glycoside as their substrate. Positive isolates were graded based on the strength and spread of the yellow coloration in the PNPG medium. Out of 15 bacterial isolates, only one was selected based on the cyanogenic glycoside utilization pattern and discoloration of the picrate paper color from yellow to brown. Based on the cultural and biochemical tests, the cyanogenic glycoside utilizing bacteria were identified as *Bacillus subtilis*. The cultural and biochemical characteristics of the *B. subtilis* KM05 are presented in Table 1. They formed flat, round rough reticulate colonies having diameter of 5 to 7 mm on nutrient agar plates. It was observed that *B. subtilis* produced hydrogen cyanide (HCN) in addition to ammonia during cyanogenic glycoside utilization.

Results obtained for the partial purification of *B. subtilis* KM05 cyanide degrading enzyme by conventional ammonium sulphate fractionation are shown in Figure 2. The protein precipitate formed within the saturation range of 30 to 60% was found to retain the enzyme activity. Electrophoretic analysis of crude extract and ammonium sulphate precipitate showed the presence of single band with a molecular mass of 53 KDa (Figure 2). The protein concentration in the enzyme preparation was found to be 0.23 mg/ml and the linamarase activity was found to be 9.6 U/ml. It was also inferred that the enzyme has the ability to degrade cyanogenic glycosides effectively.

The results presented in Table 2 revealed that the untreated cassava flour had higher cyanide content and a significant reduction in both wet and enzyme treated flour. Before inoculation, the cyanide concentration of the flour was found to be 210 ppm/kg. The cell free extract on treatment of cassava flour extract over night led to rapid

reduction in cyanide concentration of 8 ppm/kg, whereas in the untreated control, the cyanide concentration remained as 35 ppm/kg (Table 2). It was also observed that there was no significant change in the nutrient level except the small increase in protein. Results indicate that *B. subtilis* KM05 enzyme effectively detoxified the cassava cyanogenic glycosides.

DISCUSSION

The inadequate processing methods of cassava often result in linamarin and its hydrolytic product cyanohydrin, as a residue in the product. Hence, there is a need to improve the methods of processing cassava to foods that are popular in different communities in the tropics (Oluwole et al., 2002). The new processing method suggested to remove cyanogens from cassava flour involves mixing dry flour with water and leaving the wet flour in a thin layer in the shade for 5 h or for 2 h in the sun to allow the catalyzed breakdown of linamarin to hydrogen cyanide, facilitating a three to six fold reduction in total cyanide content of cassava flour. The wet flour obtained can be used for cooking on the same day (Bradbury and Denton, 2010). Even though a considerable level of cyanide content is reduced, many times, the residual cyanide level exceeds FAO limit of 10 ppm and also it depends exclusively on endogenous enzyme level which shows considerable variation.

Several microorganisms including *Bacillus* sp. (Amoa-Awua and Jakobsen, 1995), lactic acid bacteria (Cohen, 1994), Lactobacilli, *Leuconostoc*, Streptococci and yeasts (Obilie et al., 2004), *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma* strains (Yeoh et al., 1995) are known for their detoxification activities on native cassava

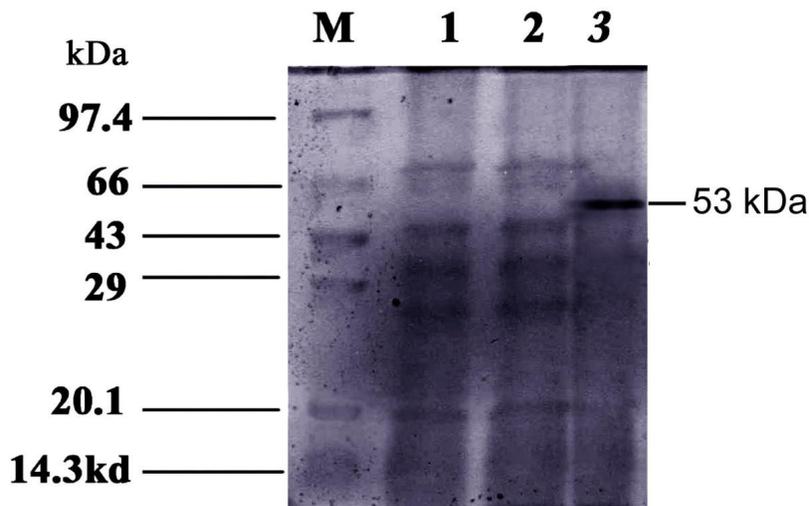


Figure 2. SDS-PAGE of *B. subtilis* KM05 linamarase showing lane 1 (medium range protein markers), lanes 2 and 3 (crude extracts) and lane 4 (ammonium sulphate precipitated sample).

Table 2. Changes in the nutrient and cyanide level in the treated and raw cassava flour.

S/N	Type of treatment	Nutritional parameter				
		Moisture	Crude protein	Fat	Ash	Cyanide
1	Raw flour	14±0.4	1.36±0.2	0.16±0.01	0.96±0.01	210±7
2	Enzyme treated flour	13±0.5	1.46±0.4	0.17±0.02	0.96±0.02	8±2
3	Wet treated flour	13±0.6	1.31±0.2	0.15±0.01	0.96±0.01	35±5

fermentation. To the authors' knowledge, this study reports for the first time the potential of cassava peel waste degrading organisms in cassava detoxification meant for human usage.

Species of *Bacillus*, *Pseudomonas* and *Klebsiella oxytoca* have been reported to biodegrade cyanide to non-toxic end-products using cyanide as the sole nitrogen source under aerobic and/or anaerobic environment (Kaewkannetra et al., 2009). Some previous studies reported *Bacillus* species involvement in detoxification associated with cassava fermentation. However, their role varies as determined by the type of fermentation (Amoa-Awua and Jakobsen, 1995; Oyewole and Odunfa, 1988). Oyewole (1992) noted that in the submerged fermentation process for 'fufu' production, *Bacillus* species which appear at the beginning of the fermentation became extinct towards the end of the process, while Amoia-Awua and Jakobsen (1995) reported the high occurrence and persistence of *Bacillus* species throughout the dough fermentation during the solid state fermentation (SSF) of cassava to 'agbelima' as done in Ghana. Hence instead of applying the whole cell

fermentation, the application of its enzyme would achieve the desired result.

A cyanotrophic microorganism requires an assimilatory pathway that is able to convert cyanide into ammonium (Luque-Almagro et al., 2005). The formation of HCN and release of ammonia as an end product evidenced the assimilatory degradation of linamarin by *B. subtilis* KM05. It is also evident that this bacterium is able to grow, utilizing linamarin as the sole N source and acetate as the C source. Therefore, this strain offers new perspectives in the detoxification of cassava cyanide. The increase in the protein content of the cassava products could be attributed to the enzyme extract addition. It has been observed earlier that the SSF, employing various fungi improves the protein content and also reduces the antinutrients but separation of the fermentative organisms from the mass is practically difficult and limits their application. Hence, this study on nutrient enrichment and detoxification of cassava flour using enzyme from easily growing cheap, non-pathogenic bacteria *B. subtilis* KM05 would increase the productivity, efficiency and quality output in cassava based industrial processing operations

in many developing countries.

ACKNOWLEDGEMENT

The authors acknowledge the Rector, KSU and the Deanship of Scientific Research, College of Science Research Center, King Saud University for their support of this project

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