Effect of cultural conditions on the growth and linamarase production by a local species of *Lactobacillus fermentum* isolated from cassava effluent

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Abstract

Background: This study was designed to investigate the effect of cultural conditions on growth and production of linamarase by a local species of *Lactobacillus fermentum* isolated from cassava effluent. Isolation and identification of bacteria from cassava effluent were carried out using the culture-dependent method and polyphasic taxonomy, respectively, while screening for cyanide degradation, and the effects of cultural conditions on the growth and linamarase activity of *L. fermentum* were investigated based on standard procedures.

Results: A total of twenty-one bacterial isolates were obtained from cassava effluent, and isolate MA 9 had the highest growth of 2.8×10^{10} cfu/ml in minimum medium, confirmed as safe, identified as *Lactobacillus fermentum* and selected for further study. The highest growth of 2.498 OD and linamarase activity of 2.49 U/ml were observed at inoculums volume of 0.10 ml at 48-h incubation period, while optimum growth of 1.926 OD and linamarase activity of 1.66 U/ml occurred at pH 5.5. At 37 °C, the optimum growth of 0.34 OD was recorded with the highest linamarase activity of 0.81 U/ml at 30 °C. However, the incubation period of 48 h stimulated an optimum growth of 3.091 OD with corresponding linamarase activity of 1.81 U/ml, while the substrate concentration of 400 ppm favours a maximum growth of 2.783 OD with linamarase activity of 1.86 U/ml at 48 h of incubation. The supplemented of 10 mM calcium ions stimulated optimum linamarase activity of 2.65 U/ml.

Conclusion: *Lactobacillus fermentum* could be used as starter culture in cassava fermentation for the production cassava-based product with reduced cyanide content.

Keywords: Cultural conditions, Production, Linamarase activity, *Lactobacillus fermentum*, Cassava effluent

Background

Linamarase (EC 3.2.1.21) along with its naturally occurring substrates, linamarin and lotaustralin, is found in a variety of edible plants such as cassava, lima beans and flax. It is a well-studied hydrolytic enzyme that has also been isolated from members of eukaryotes, bacteria, fungi and archaea. It hydrolyses linamarin to produce β-D-glucopyranose and acetone cyanohydrin which are further degraded to acetone and hydrogen cyanide (HCN), respectively (Nartey 1968; Cooke 1978; Rolle 1998; Murugan et al. 2012; Nwokoro 2016). In the production of *garri* (a household West African food material flour), the enzyme plays an important role in the batch-wise detoxification process of cassava fermentation and degradation of cyanogenic compounds to produce HCN which either dissolves readily in water or released into the air in a process known as cyanogenesis (Ikediobi and Onyike 1982; Kostinek et al. 2006). Microorganisms have also been documented to exhibit the ability to

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disintegrate cyanide-containing compounds into acetone cyanohydrins by producing linamarase (Nwokoro 2016). In addition, some phytopathogenic fungi like *Fusarium solani* are capable of degrading cyanide. However, biodegradation by bacteria is preferable because it shows considerable advantage since bacteria are more easily manipulated both at biochemical and at genetic levels (Okafor and Ejiofor 1985; Huertas et al. 2006; Abban et al. 2013; Ahaotu et al. 2013). Cyanide is a toxic chemical compound present in plants seeds, bitter almonds, apricots, peaches and inorganic salts such as sodium and potassium cyanides. In cassava tubers, cyanide is available in the form of cyanogenic glucosides, linamarin and lotaustralin which are major safety concerns among cassava consumers and processor (Dunstan et al. 1996). Cyanide is a widely known inhibitor of cellular energy generation that occurs during oxidative phosphorylation. It binds to a large enzyme of the inner mitochondrial membrane (cytochrome oxidase), thus preventing the transfer of electrons to molecular oxygen and generation of adenosine triphosphate (ATP) (Nelson and Cox 2005; Tefera et al. 2014). Intake of 50 to 100 mg of cyanide has been observed to elicit poisoning which is fatal to humans, while long-time consumption of trace amounts has been implicated in the aetiology of tropical neuropathy, fibrocalculous pancreatic diabetes (FCPD), glucose intolerance coupled with iodine deficiency goitre and cretinism (Mathangi et al. 2000; Cardoso et al. 2005). Hitherto, it had been reported that most locally produced cassava-based products contain cyanide higher than the World Health Organization (WHO)-recommended safety level (Owuamanam et al. 2010). In this context, this research is aimed at investigating the effect of cultural conditions on the growth and linamarase production by a local species of *Lactobacillus fermentum* isolated from cassava effluent.

Methods

Sample collection
Cassava effluent was collected from different cassava processing plants around Agbowo axis in Ibadan, Oyo State, at 10:00am on 26 September 2019. The samples were collected aseptically in clean sterile bottles and transported to the Food Biotechnology Postgraduate laboratory, Department of Microbiology, University of Ibadan, Nigeria, for immediate isolation of bacteria.

Isolation of bacteria from cassava effluent
The sample was serially diluted by transferring 10 ml of cassava effluent into 90 ml of sterile distilled water, and 1 ml from 10^4 dilution test tube was transferred into sterilized Petri dishes. Sterile 20 mls of De Man, Rogosa and Sharpe agar (MRS agar) with pH 5.5 and nutrient

agar (NA) were poured one after the other into the Petri dishes (Harrigan and McCance 1966). The MRS plates were incubated at 37 °C for 24 h in an anaerobic jar, while nutrient agar plates were incubated aerobically. Discrete colonies that grew on the plates were counted and recorded as colony-forming unit per millilitre (cfu/ml). Representative colonies were selected and streaked repeatedly to obtain pure isolates which were maintained on MRS and nutrient agar slants in refrigerator for subsequent use.

Screening for linamarase-producing bacteria
Screening for linamarase-producing bacteria was carried out by inoculating differently a single pure colony of the bacteria isolated from cassava effluent into 10 ml sterile MRS and nutrient broth in different test tubes. Incubation was done anaerobically at 37 °C for 48 h in an anaerobic jar to generate a growing and activated inoculum suspension, while that of nutrient broth was done aerobically. One millilitre of the activated cells suspension was transferred differently into 100 ml of sterile MRS and nutrient broth containing 100 ppm of filter-sterilized potassium cyanide (KCN) as the sole nitrogen source in a 250-ml Erlenmeyer flask placed on rotatory shaker for 48 h at 37 °C. One millilitre of the agitated suspension was pipetted and serially diluted to 10^{-8} dilution, and 1 ml was taken and differently plated on sterile MRS and nutrient agar plates. The MRS was incubated anaerobically at 37 °C for 48 h in an anaerobic jar, while the nutrient media was incubated aerobically at the same time and temperature. Plates were examined for bacterial growth, and the observed colonies were counted physically to measure the growth/survival in cyanide environment. Representative colonies were selected and subcultured severally to obtain pure colonies which were stored on agar slants in McCartney bottles and kept in the refrigerator. The isolate with the highest growth was selected for further studies.

Safety assessment test for bacteria
Gelatinase test
Gelatinase activity was investigated as described by Harrigan and McCance (1966). A 24-h-old culture was streaked on nutrient gelatin agar and inoculated anaerobically for 48 h at 37 °C and flooded with $HgCl_2$ solutions (15% $HgCl_2$ in 20% HCl, v/v). Clear zones surrounding colonies were noted for positive reaction indicating gelatin hydrolysis.

DNase test
The isolate was tested for the ability to produce deoxyribonuclease. This was carried out by streaking the isolate on DNase agar and incubated for 24 h, and the plate was

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flooded with concentrated HCl (Harrigan and McCance 1966).

Haemolytic activity

Haemolytic test was carried out with nutrient agar supplemented with 5% sheep blood. The plates were inoculated aseptically and incubated for 48 h at 37 °C and examined for greenish colouration halos to confirm the production of α-haemolysin which indicates partial haemolysis, while complete clear zone indicated the production of β-haemolysin (Akinjogunla et al. 2014).

Characterization of the isolated bacteria

The isolates were characterized and identified using phenotypic and molecular methods.

Phenotypic characterization

Phenotypic characterization was carried out using criteria such as gram staining, spore staining, indole, starch and gelatin hydrolyses, urease, methyl red, motility, catalase tests, citrate, oxidase tests, growth at 4% and 6% NaCl and sugar fermentation (Sneath 1986; Holt 1994; Cheesbrough 2006).

Molecular characterization (16S rDNA method)

It was done using 16S rDNA gene sequence analysis with a base pair of 1100 bp as shown in Fig. 1. The forward primer used was 27F-AGAGTTTGATCCTGGCTCAG, and the reverse primer was 1492R-GGTTACCTTGTT ACGACTTT (Woo et al. 2002).

DNA extraction

Genomic DNA extraction of cultured bacteria was done with column-based Bacteria DNA Preparation kit, according to the manufacturer's protocol (JENA Bioscience). Bacteria cells were harvested from 500 µl aliquot of bacteria culture using microcentrifuge at 15,000 g for 1 min. The residual pellet was re-suspended in 300 µl of buffer and 2 µl of lysozyme solution. The mixture was homogenized by inverting several times and incubated at 37 °C for 1 h. Re-suspended cells were recovered by centrifugation and lysed by adding 300 µl of lysis buffer and 2 µl RNase and 8 µl proteinase K solution followed by incubation at 60 °C for 10 min. The tube was cooled on ice for 5 min, and 300µ binding buffer was added to the mixture and vortexed briefly. The mixture was cooled on ice for 5 min and centrifuged at 10,000 g for 5 min. The supernatant was transferred directly into the spin column and centrifuged at 10,000 g for 1 min to trap the DNA. The trapped DNA was washed twice with washing buffer and was eluted with 50 µl elution buffer into a clean Eppendorf tube.
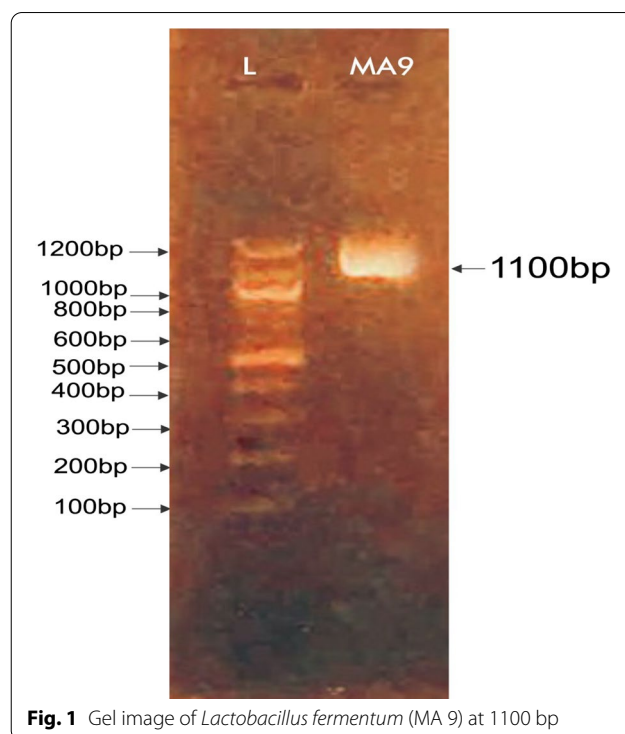

Fig. 1 Gel image of *Lactobacillus fermentum* (MA 9) at 1100 bp

Polymerase chain reaction (PCR) amplification of bacterial 16S rDNA

Each PCR mixture consisted of 5 µl master mix, 1 µl of 10 pmol each of 27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTTACGACTT (Lane 1991), 1 µl DNA template and 17 µl sterile nuclease-free water to make up to a total reaction of 25 µl. PCR amplification was carried out in an Applied Biosystem 2720 thermocycler. The mixture was subjected to an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 60 s, 72 °C for 60 s and a final extension at 72 °C for 10 min. Amplifications were visualized by electrophoresis on 1% agarose gel after staining with ethidium bromide (Hengstmann et al. 1999).

Deoxyribonucleic acid (DNA) sequencing

16S rDNA PCR product was extracted from gel extraction kit QIAquick (Qiagen) and was cloned using TA cloning kit (Invitrogen, San Diego, Calif.) as recommended by the manufacturer. DNA sequencing was conducted using ABI Prism BigDye™ Terminator cycle sequencing Ready Reaction Kit according to instructions of manufacturer (PE Applied Biosystems) ABI PrismTM 377XL DNA Sequencer (PerkinElmer) (Gomaa and Momtaz 2007).

The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). The

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optimal tree with the sum of branch length $= 9.87710978$ is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2017) and are in the units of the number of base substitutions per site. The analysis involved nine nucleotide sequences. Codon positions included were $1st + 2nd + 3rd + noncoding$. All positions containing gaps and missing data were eliminated. There were a total of 1121 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2014).

Standardization of inoculum

Standardization of inoculums was carried out using McFarland standard by preparing normal saline with the addition of 0.085 g of NaCl to 100 ml of sterile distilled water in a 250-ml Erlenmeyer flask. Five millilitres of the normal saline was dispensed severally into test tubes and autoclaved at 121 °C for 15 min. A mixture of 0.05 ml of 1% $BaCl_2$ and 9.95 ml of 1% H_2SO_4 in 100 mls of sterile distilled water served as McFarland standard. A 24-h-old pure culture of the bacteria with the highest growth was inoculated into the normal saline, and the turbidity was compared with that of the McFarland standard in the presence of good lighting. The turbidity of the bacterial suspension with the highest growth was adjusted until it matches that of the McFarland standard.

Linamarase production and assay

The minimal medium used for this assay consists of NaCl 0.3%; $(NH_4)_2SO_4$ 0.1%; KH_2PO_4 0.05%; $MgSO_4$ 0.02%; $CaCl_2$ 0.02%; lactose 3% and KCN 0.15% (Okafor and Ejiofor 1985). One hundred millilitres of the minimal medium in a 250-ml Erlenmeyer flask was sterilized at 121 °C for 15 min and adjusted to pH 5.5 with sterile HCl. Ten millilitres of cells suspension of the bacteria with the highest growth was transferred into the minimal medium and incubated for 24 h on a rotatory incubator 2500 rpm for 30 min at 30 °C. Cells were harvested by centrifugation, and the supernatants obtained were combined and used for enzyme assay.

Assay procedures

Linamarase activity was carried out by determining the HCN liberated from KCN as follows: 0.5 ml of enzyme solution in 0.2 M phosphate buffer (pH 6.5) contained in Eppendorf tubes was added to 0.5 ml of 1 mM buffered solution of KCN and incubated at 32 °C for 20 min. Two millilitres of 2% KOH and 1 ml of sodium alkaline picrate were added into the reaction mixture, and the reaction was stopped by placing the tubes in iced water.

The red colour that developed was read at 540 nm in a spectrophotometer under the above conditions; one unit of activity was defined as the amount of enzyme that released 1 µg HCN in 30 min under assay condition (Nwokoro 2016).

Effect of different cultural conditions on linamarase activity

Determination of the effect of different inoculums volume on growth and linamarase activity

Effect of inoculums volume on growth and linamarase activity was determined by dispensing 250 ml of minimal medium containing NaCl 0.3%; $(NH_4)_2SO_4$ 0.1%; KH_2PO_4 0.05%; $MgSO_4$ 0.02%; $CaCl_2$ 0.02%; lactose 3% and KCN 0.15% separately into four different Erlenmeyer flasks and sterilized at 121 °C for 15 min. One hundred ppm of filter-sterilized KCN was added to the sterilized medium and inoculated with different volumes (cell suspension) of the bacterial cells with the highest growth (0.05, 0.1, 0.15 and 0.2 mls) and incubated on a rotatory shaker at 30 °C for 96 h. Ten millilitres of the medium was taken at 12-h intervals and centrifuged at 2500 rpm for 40 min. The cell growth was measured using spectrophotometer, and the supernatant was used for enzyme assay as described above. The experiment was carried out in triplicate.

Determination of the effect of different pH on growth and linamarase activity

Effect of pH on growth and linamarase activity was investigated by adjusting the pH of sterile MRS medium to pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. One hundred millilitres of MRS medium in a 250-ml Erlenmeyer flask was sterilized, and 100 ppm of filter-sterilized KCN was added to the medium and inoculated with the optimum volume. Incubation was done on a rotatory shaker at 30 °C for 96 h. Ten millilitres of the medium was taken at 12-h intervals and centrifuged at 2500 rpm for 40 min. The cell growth was measured using spectrophotometer, and the supernatant was used for enzyme assay as described above. The experiment was carried out in triplicate (Adeleke et al. 2017).

Determination of the effect of different temperatures on growth and linamarase activity

In order to determine the effect of different temperatures on growth and linamarase activity, 50 mls of sterile MRS medium with pH adjusted to optimum pH was dispensed into sterile screw-capped Erlenmeyer flasks and sterilized at 121 °C for 15 min and 100 ppm of filter-sterilized KCN was added and inoculated with 24-h-old culture of the bacteria with the highest growth using the optimum inoculums volume, pH and incubated separately at 10 °C, 20 °C, 37 °C, 45 °C and

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60 °C for 1 h. Ten millilitres of the medium was taken at 12-h intervals and centrifuged at 2500 rpm for 40 min. The cell growth was measured using spectrophotometer, and the supernatant was used for enzyme assay as described above. The experiment was carried out in triplicate (Adeleke et al. 2017).

Determination of the effect of incubation period on growth and linamarase activity

The effect of incubation period on the growth and linamarase activity was studied by obtaining 100 mls of sterile MRS broth with pH adjusted to optimum pH and dispensed into a 250-ml Erlenmeyer flask and sterilized at 121 °C for 15 min with 100 ppm of filter-sterilized KCN added and inoculated with a 24-h-old culture of the bacteria with the highest growth using optimum inoculums volume, pH and temperature and incubated for 96 h. Ten millilitres of the medium was taken at 12-h intervals and centrifuged at 2500 rpm for 40 min. The cell growth was measured using spectrophotometer, and the supernatant was used for enzyme assay as described above. The experiment was carried out in triplicate.

Effect of substrate concentration on growth and linamarase activity

Different concentrations of filter-sterilized KCN (100 ppm, 150 ppm, 200 ppm, 400 ppm, 500 ppm and 800 ppm) were prepared and added separately to MRS broth adjusted to optimum pH and sterilized at 121 °C for 15 min. The sterilized medium was inoculated with a 24-h-old culture suspension of the bacteria with the highest growth using the optimum inoculums volume, pH and temperature incubation period. Ten millilitres of the medium was taken at 12-h intervals and centrifuged at 2500 rpm for 40 min. The cell growth was measured using spectrophotometer, and the supernatant was used for enzyme assay as described above. The experiment was carried out in triplicate (Adeleke et al. 2017).

Influence of different cations on growth and linamarase activity

Five mM of various metal ions (Hg^{2+}, Urea, NH_4^+, Ca^{2+}, K^+, Na^+, Mg^{2+} and Fe^{3+}) was added to enzyme solution and incubated for 30 min at 37 °C. Linamarase activity was measured by adding KCN, and enzyme assay was carried out at optimum conditions. This was carried out in triplicates. Different concentrations of the best metal ion that stimulated the highest linamarase activity were prepared (5–25%) and added to enzyme solution and incubated for 30 min at 37 °C.

Table 1 Results of microbial growth in minimum medium

MRS isolates	cfu/ml	NA isolates	cfu/ml
MA 2	1.5×10^9	NA 1	1.3×10^9
MA 4	7.0×10^9	NA 4	1.2×10^9
MA 5	2.3×10^9	NA 6	3.7×10^9
MA 7	5.0×10^9	NA 7	7.3×10^9
MA 9	2.8×10^{10}	NA 9	1.6×1.0^{10}
MA 10	1.2×10^9	NA 10	1.9×10^{10}
MA 13	5.2×10^9	NA 11	4.5×10^9
MA 16	1.3×10^9	NA 13	6.3×10^9
MA 19	4.3×10^9	NA 16	2.7×10^9
MA 21	4.1×10^9	NA 18	13×10^9

Table 2 Safety assessment of the bacteria isolate (MA9) with the highest growth

Isolate code	Haemolysis	DNase	Gelatinase tests
MA 9	−	−	−

+, positive; −, negative

Results

Growth of isolated bacteria from cassava effluent in minimal medium

Table 1 shows the result of growth of isolated bacteria from cassava effluent in minimal medium. It was observed that MA9 had the highest growth of 2.8×10^{10} cfu/ml, while the lowest growth was observed with MA 10 with 1.2×10^9 cfu/ml.

Safety assessment of isolate MA 9

Table 2 depicts the safe property of isolate MA9. It was revealed that the isolate tested negatively to all safety assessment and was regarded as safe.

Biochemical and physiological characterizations of Isolate MA9

The result of the phenotypic identification procedure of Isolate MA9 (isolate with the highest growth in the minimal medium) using physiological and biochemical characterizations is shown in Table 3. Isolate MA 9 is a Gram-positive, non-motile and non-spore-forming rod. It showed negative reactions to catalase, VP, methyl red, indole, and starch hydrolysis, oxidase positive but showed growth at 4% and 6% NaCl.

Sugar fermentation pattern of Isolate MA9

The result of sugar fermentation pattern of Isolate MA9 is shown in Table 4. It depicts that that isolate MA9 was able to degrade glucose, maltose, sucrose, fructose,

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Table 3 Biochemical and physiological characterizations of Isolate MA9

Isolate code	Catalase test	Gram stain	Spore stain	Vp	Oxidase	4% NaCl	6% NaCl	Indole	Motility	Probable identity
MA9	−	+	−	−	+	+	+	−	−	*Lactobacillus fermentum*

+, positive; −, negative

lactose, xylose, mannose, ribose, dextrose and raffinose. However, it did not utilize mannitol, galactose, sorbitol and rhamnose.

Identification of Isolate MA9

The combination of results in Tables 2 and 3 with reference to Bergey's Manual of Systematical Bacteriology was used to probably identify Isolate MA9 as *Lactobacillus fermentum*, while the molecular method was done using 16S rDNA gene sequence analysis with a base pair of 1,100 bp as shown in Fig. 1. The forward primer used was 27F-AGAGTTTGATCCTGGCTCAG, and the reverse primer was 1492R-GGTTACCTTGTTACGACTTT.

Identity of the isolate MA 9 was confirmed as *Lactobacillus fermentum* CIP 102960 using 16S rRNA analysis. The strain showed maximum similarity ratio towards *L. fermentum* CIP 102960 (98.02%) accession number NR 104927 using NCBI data base (Fig. 2).

Figure 3 shows the result of the effect of different inoculums volume on the growth and linamarase activity of *L. fermentum*. The highest growth of 2.4980 OD and linamarase activity of 2.49 U/ml were observed at inoculums level of 0.10 ml at 48-h incubation period, while the least growth of 1.263 OD and activity of 1.10 U/ml were observed at inoculums volume of 0.20 ml at 72 h.

Effect of pH on growth and linamarase activity of *L. fermentum*

The effect of pH on growth and linamarase activity of *L. fermentum* is presented in Fig. 4. It was observed that optimum growth of 1.926 OD and linamarase activity of 1.66 U/ml occurred at pH 5.5, while the least growth of 1.254 OD and enzyme activity of 0.66 U/ml were recorded at pH 6.5 and pH 4.0, respectively.

Effect of temperature on the growth and linamarase activity of *L. fermentum*

The effect of temperature on the growth and linamarase activity of *L. fermentum* is shown in Fig. 5. The result revealed that an optimum growth of 0.38 OD and enzyme activity of 1.18 U/ml were observed at 30 °C. However, minimum growth of 0.13 OD and enzyme activity of 0.39 U/ml were recorded at 60 °C.

Effect of incubation period on growth and linamarase activity of *L. fermentum*

The effect of incubation period on growth and linamarase activity of *L. fermentum* is presented in Fig. 6. It revealed that *L. fermentum* showed optimum growth of 3.091 OD and enzyme activity of 1.81 U/ml at 48-h incubation period, while minimum growth of 2.369 OD and enzyme activity 0.97 U/ml occurred at 12 h of incubation.

Effect of different substrate concentrations on the growth and linamarase activity of *Lactobacillus fermentum*

Figure 7 showed the result of the effect of different substrate concentrations on the growth and linamarase activity of *Lactobacillus fermentum*. It can be inferred from the result that *L. fermentum* had the optimum growth of 2.783 OD and linamarase activity of 1.86 U/ml at substrate concentration of 400 ppm, while least growth of 1.472 OD and enzyme activity of 0.49U/ml were recorded at 800 ppm and 100 ppm, respectively.

Effect of different metal ions on the activity of linamarase

The result of the effect of different metal ions on the activity of linamarase is shown in Fig. 8. The highest linamarase activity of 2.65 U/ml was stimulated by the supplementation of calcium ions in the assay medium, while the least activity of 0.43 U/ml was recorded with the supplementation of ammonium ions.

Effect of varying concentrations of calcium ion on the linamarase activity of *L. fermentum*.

Figure 9 shows the result of the effect of different concentrations of calcium ions on the linamarase activity of *L. fermentum*. The maximum activity of 3.42 U/ml was recorded with the supplementation of 10 mM calcium ions, while the least activity of 2.54 U/ml was observed with the use of 25 mM of calcium ions.

Discussion

This present study investigated the effect of cultural medium on the growth and production of linamarase by a local species isolated from cassava effluent identified as *Lactobacillus fermentum*. The isolation of lactic bacteria from effluent reported in this study has earlier been documented by Okafor et al. 1984; Amoa-Awua et al. (1996); Kobawila et al. (2005); Ayansina et al. (2015); Lacerda et al. (2005); and Adeleke et al. (2017). The survival

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Table 4 Sugar fermentation pattern of Isolate MA9

Isolate code	Glucose	Maltose	Sucrose	Galactose	Sorbitol	Fructose	Lactose	Mannitol	Xylose	Mannose	Ribose	Dextrose	Rhamnose	Raffinose	Probable organism
MA 9	+	+	+	−	−	+	+	−	+	+	+	+	−	+	*Lactobacillus fermentum*

+, positive; −, negative

of lactic bacteria in cassava effluent is due to their ability to tolerate the cyanide content present in the effluent and possession of linamarase enzyme capable of hydrolyzing the cyanide to simple end products which can be utilized for growth and metabolism by the bacteria (Offiong et al. 1990; Kostinek et al. 2006). Nwokoro (2016) reported the isolation and identification of a fast growing species of *L. fermentum* in a medium containing 800 mg/l potassium cyanide from fermenting cassava tuber. Equally in this study, the bacterium with the highest growth in minimal medium was identified using both phenotypic and molecular methods as *L. fermentum*; this result is supplementary to the submission of Nwokoro (2016).

The *L. fermentum* tested negative to all the safety assessments (gelatinase, DNase and haemolysis) which confirmed their safety property. There are earlier documented reports on the safety status of lactic acid bacteria which identify them as GRAS (generally regarded as safe) (Narvhus and Axelsson 2003). This unique property accords them acceptability as good starters in food fermentation.

The maximum linamarase activity was observed when the culture medium was inoculated with 0.1 ml of inoculums volume, and further increase led to a decrease in enzyme activity. Similar observation had earlier been reported (Adeleke et al. 2017), and this occurrence might be due to increased competition for carbon and other nutrients. However, the increase in enzyme at lower inoculums volume is related to the presence of decreased number of cells for utilization of the substrate for desired product formation (Kashyap et al. 2002.) Inoculums volume plays a major role in enzyme activity by controlling and reducing the microbial lag phase (Iqbal et al. 2010).

In this present study, optimum linamarase activity was recorded at pH 5.5. This observation is in conformity with earlier findings of Gueguen et al. (1997) and Akinyele et al. (2014), and Maherawati et al. (2017) who reported pH 5.5 for optimum production of β-glucosidase by *Leuconostoc mesenteroides*, cellulase by *Aspergillus niger* NSPRO12 in submerged fermentation and linamarase activity isolated from cassava leaves, respectively. However, Essen (1992) also reported pH 5.8 for optimum production of β-glucosidase, while Yeoh et al. (1995) observed that optimum linamarase activity of *Aspergillus nidulans*, *Penicillium funiculosum* and *Fusarium oxysporum* occurred at pH 5.1, 4.8 and 4.2,

respectively. In addition, Bodade et al. (2010) explained that change in pH might result in morphological changes of the microorganism and decrease in enzyme secretion, thereby affecting enzyme activity. The pH of growth medium plays an important role in enzyme induction and production, and slight change in pH drastically influences microbial growth and enzyme production (Horikoshi 1971; Gurudeeban et al. 2011; Joshi and Yadav 2016). From the results obtained in this study, it can be inferred that *L. fermentum* prefers acidic pH for its optimum linamarase activity and it could be employed for the hydrolysis of cyanide in cassava fermentation which occurs naturally within a pH range of 5.5–5.8 (Nwokoro and Onyebuchi 2011). In addition, Joshi and Yadav (2016) observed that alteration in hydrogen concentration will lead to inhibition of the active site causing a reduction in enzyme activity, while Maherawati et al. (2017) emphasized that alteration in pH will alter the ionization prototropic group in the active site of the enzyme which will ultimately influence enzyme activity.

In this present study, optimum incubation temperature for linamarase activity occurred at 30 °C. This finding corroborates the earlier report of Okafor and Ejiofor (1985) who reported that optimum activity for linamarase activity of yeast species was recorded at 29 ± 2 °C. However, Nwokoro and Onyebuchi (2011) reported optimum linamarase production by *Lactobacillus delbrueckii* at 50 °C which contradicts our findings in this work. *L. fermentum* may be suggested to be a mesophile and prefers a low temperature for its optimum enzyme activity (Maheshwari et al. 2000). However, this phenomenon seems to be strain dependent (Lu et al. 2003; Gautam et al. 2010) and the occurrence of optimum linamarase activity of bacteria at low temperature hinders its application in biotechnological processes (Nwagu and Okolo 2011). It could be suggested that the application of linamarase in the detoxification of cassava should be done at low temperature range. Maherawati et al. (2017) explained that the stability of enzyme is a function of parameters such as temperature, pH, ionic strength, presence of substrate, concentration of enzyme, proteins in the system, incubation time and presence of activators and inhibitor. In addition, an increase in temperature leads to an increase in kinetic energy which causes denaturation and destabilization of thermostable three-dimensional structure of the enzyme at high temperature
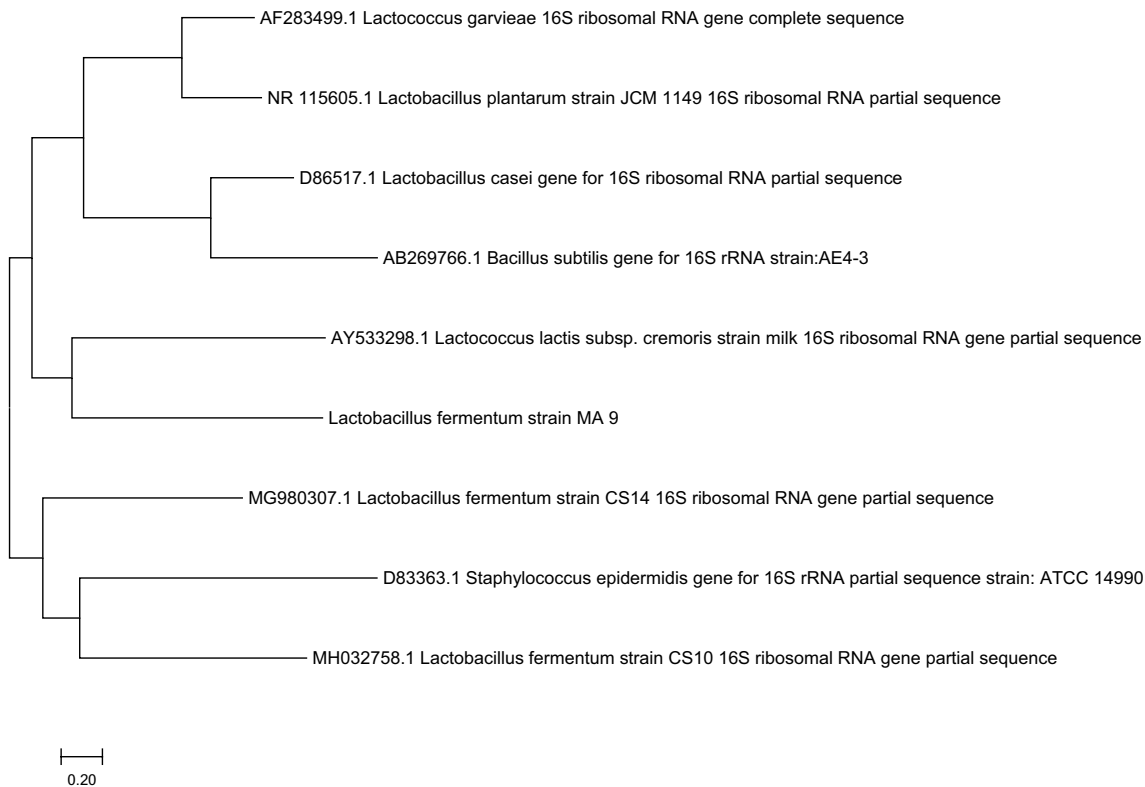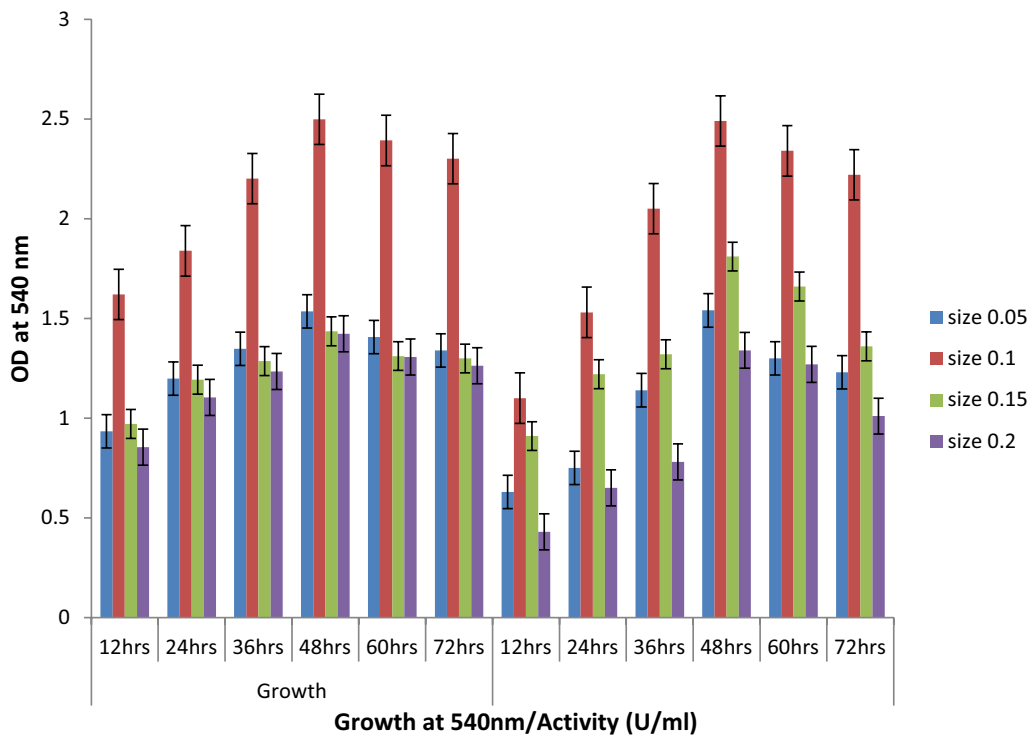
Fig. 2 Phylogenetic tree

Fig. 3 Effect of inoculum volume on growth and enzyme activity of *Lactobacillus fermentum* at different incubation periods
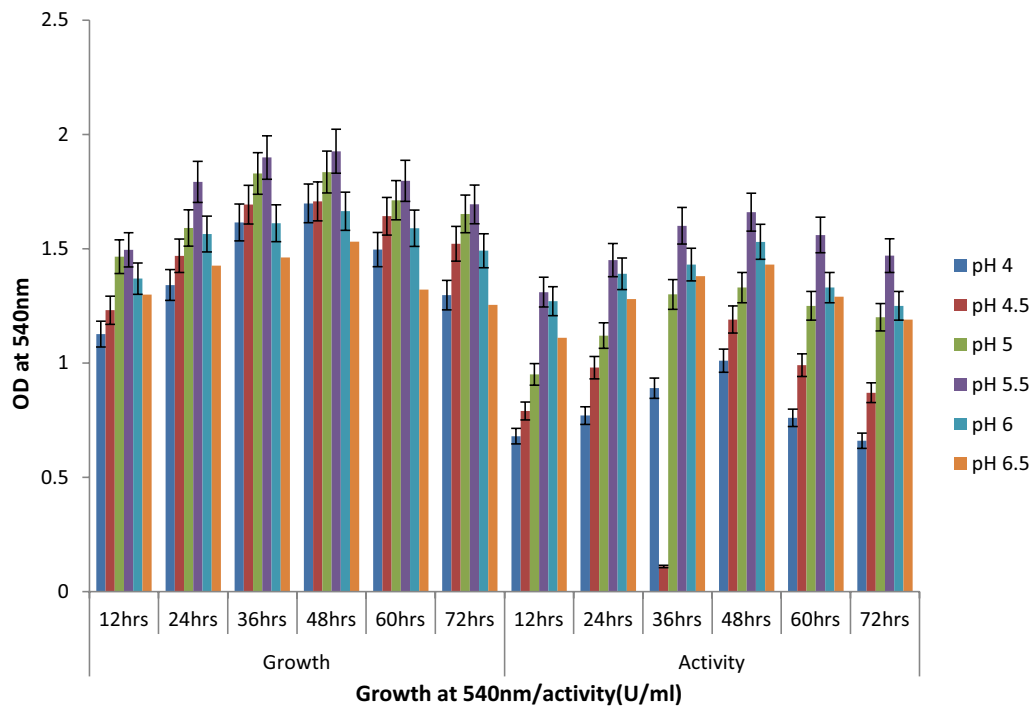
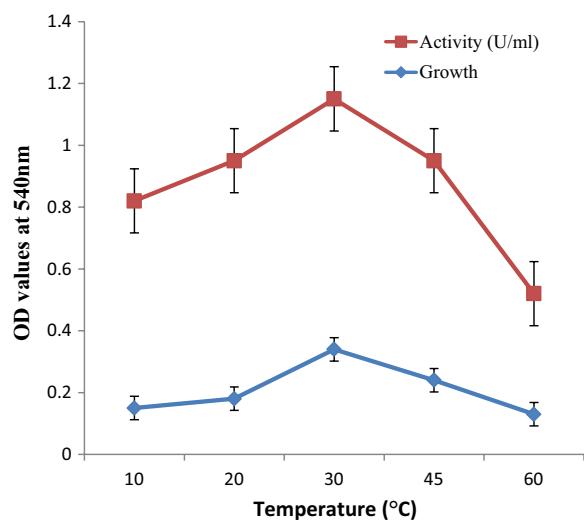**Fig. 4** Effect of pH on growth and linamarase activity of *L. fermentum*

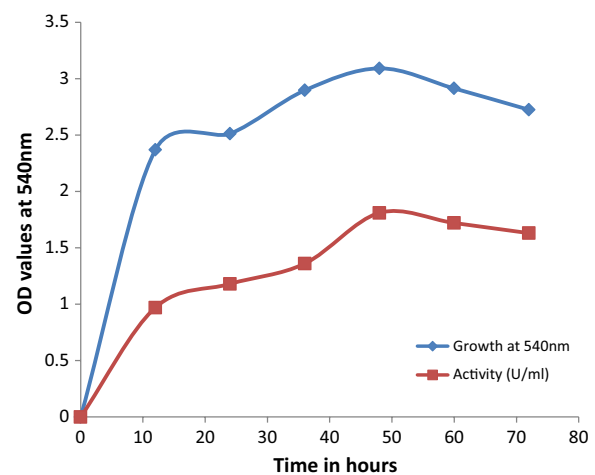**Fig. 5** Effect of temperature on the growth and linamarase activity of *L. fermentum*

Fig. 6 Effect of incubation period on growth and linamarase activity *L. fermentum*

(Bisswanger 2014). However, Petrucciolli et al. (1999) and Nwokoro and Onyebuchi (2011) reported that optimal linamarase activity of *Mucor circinelloides* and *L. delbrueckii* occurred at 40 °C and 50 °C, respectively, which is at variance with our findings in this investigation. It is pertinent to report that each enzyme has its

own optimum temperature for activity which is dependent on the strength of amino acids as monomers of enzymes (Maherawati et al. 2017). Nwagu and Okoli (2011) and Joshi and Yadav (2016) reported that optimal temperature is a paramount factor which affects microbial growth, stability, enzyme induction and production. The effect of incubation period on the activity
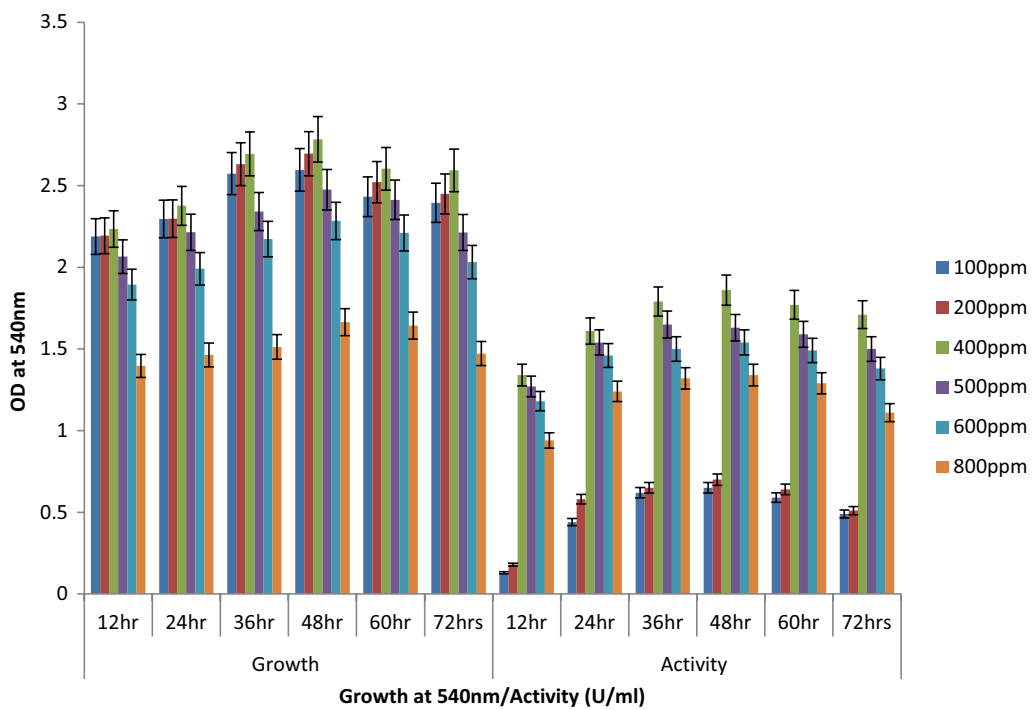
Fig. 7 Effect of substrate concentration on growth and linamarase activity of *L. fermentum*
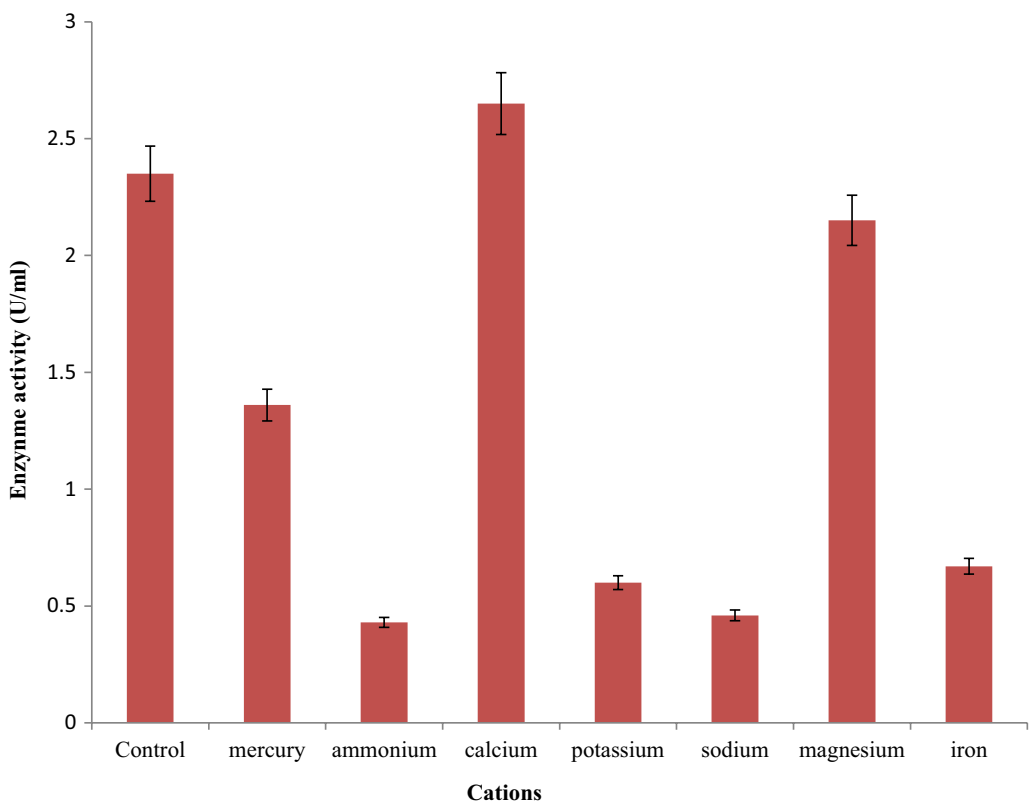

Fig. 8 Effect of different cations on linamarase activity of *L. fermentum*

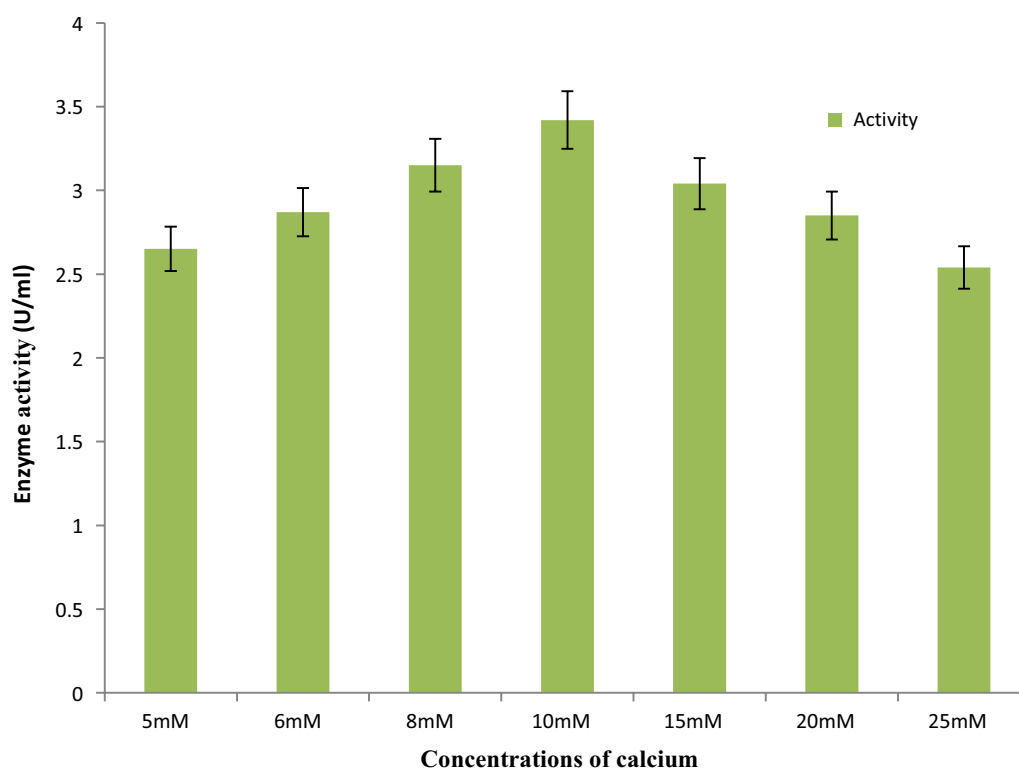Fadahunsi *et al. Bull Natl Res Cent*   (2020) 44:185

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Fig. 9 Effect of different concentrations of calcium on enzyme production

of linamarase by *L. fermentum* showed that linamarase activity increased during the growth phase of the culture, reaching the optimum incubation time at 48 h, and any further increase beyond this period resulted in lower activity. This observation is similar to the findings of Sakthivel et al. (2010) and Rezvani et al. (2016). Reason such as enormous utilization of the accessible part of the substrate which is needed for enzyme production thus leaving the unutilized crystalline portion which cannot be consumed by the bacteria for growth and enzyme production could be adduced to be responsible for this phenomenon (Omojasola et al. 2008). In addition, reduction in enzyme activity after 48 h might be caused by the bacteria transiting to the stationery phase of growth when cells are metabolically inactive. However, Adeleke et al. (2017) reported that optimum linamarase activity of *L. plantarum* was recorded at 22 h of incubation which contradicts our findings in this study. The effect of substrate concentration on the production of linamarase by *L. fermentum* showed that enzyme production increased as substrate concentration increased. Optimum substrate concentration attained a substrate concentration of 400 ppm, whereby enzyme activity ceased to rise regardless of any increase in substrate level. Reasons such as environmental saturation by the substrate occurred,

whereby all enzymes are bound and reacting (Vmax) can be adduced for this occurrence. Similar finding had earlier been reported by Irshad et al. (2013) from an investigation carried out on the production of cellulose. It could be suggested that scaling up of linamarase production by *L. fermentum* could be achieved with 400 ppm substrate concentration (Sakthivel et al. 2010). In addition, Teodoro and Martins (2000) explained that the presence of available substrate has been reported to influence the biosynthesis of many extracellular enzyme via catabolic repression mechanism. In addition, Mamo and Gessesse (1999) explained that the reduction in enzyme activity with an increase in substrate concentration might be caused by end product repression of enzyme production and high viscosity of the medium leading to reduction in oxygen availability (Omidiji et al. 1997). According to Hafiz et al. (2010), substrate concentration is a crucial factor that influences the yield and initial hydrolysis of substrate.

In the present study, Ca^{2+} was observed to stimulate the highest linamarase activity when supplemented in the assay medium. This observation is in conformity with the submissions of Mishra and Maheswari (1996) and Adeleke et al. (2017). According to Lin et al. (1999) and Caiming et al. (2018), Ca^{2+} improves the thermostability

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of enzyme which is in alignment with the documented findings of Pandey et al. (2000) and the reasons for this phenomenon are ambiguous, but it is obvious that metal ions often act as ion bridge between two adjacent amino acids residue, thereby contributing to stimulatory potential of the metal ions on enzyme activity (Lin et al. 1999). The thermostability of enzyme is well documented. According to previous report, Ca^{2+} serves as protection against high temperature in thermostable enzyme (Violet and Meunier 1989; Savehenko et al. 2002). In addition, Ca^{2+} had been reported to stabilize total structure of the enzyme and also maintain structural integrity (Robyt 1984; Krishnan and Chandra 1989; Vihinen and Mantsala 1990; Violet and Meunier 1989; Ptacek et al. 2018) and its omission in the production medium will bring about reduction in enzyme activity (Machius et al. 1995). It can be suggested that linamarase is a metalloenzyme due to the occurrence of Ca^{2+} binding site for its activity and stability (Nwagu and Okolo 2011).

Conclusion

This research showed that linamarase production by *L. fermentum* can be optimized by considering parameters such as inoculums volume of 0.1 ml, pH 5.5, temperature of 30 °C, 48-h incubation period, substrate concentration of 400 ppm and 10 mM of calcium ion. It can be suggested that *L. fermentum* could be used as starter cultures in the fermentation of cassava for the production of cassava-based products with reduced cyanide content.

Abbreviations

OD: Optical density; ATP: Adenosine triphosphate; PCR: Polymerase chain reaction; MRS agar: De Man, Rogosa and Sharpe agar; FCPD: Fibrocalculous pancreatic diabetes; GRAS: Generally regarded as safe.

Authors' contributions

IFF designed, conceptualized and supervised the study. BNK and OSF carried out the experimental protocols and assays. IFF wrote the first draft. The authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

No competing interest exists in the research outcome presented in this article.

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