IJMAAS
International Journal of Microbiology and Applied Sciences
ISSN 1115-4004 *Volume 3 issue 3 Sept, 2024* Research Article

Effect of Varying Temperature, pH and Incubation Time on Amylase Production by Amylolytic Lactic Acid Bacteria Isolated from Fermented Starchy Foods

Ejike, E. N. and Onyeanula, E. O.
Department of Microbiology,
Michael Okpara University of Agriculture,
Umudike, Abia State, Nigeria.
***Corresponding Author:** *emmaejiken@yahoo.com*

ABSTRACT

Amylases are among the most important enzymes in present-day biotechnology. The present study aimed at varying temperature, pH and incubation time to optimize the activities of amylolytic lactic acid bacteria isolated from fermented "Ogi" and retted cassava. Amylase positive bacterial strains were identified based on the clear zone formation around the bacterial growth. Amylase producing bacteria were grown on the starch production media in submerged fermentation method. Fermentation conditions were optimized by varying the following parameters, pH (3-9), temperature (20-70ºC), and incubation time (12-72 h). The most prominent amylolytic index (AI) recorded in isolate AK1 (Ogi), CA5 (cassava) and CA2 (cassava) were 2.63±0.06, 2.06±0.03 and 2.55±0.05 respectively. The highest enzyme activity (0.85±0.05U/ml) was exhibited by AK1. The three strains were identified as *Lactobacillus plantarum*FMO2(AK1), *Lactobacillus plantarum*Z2(CA5) and *Lactobacillus pentosus*BRS3(CA2) by 16S rRNA sequencing. The extracellular amylase activity produced by strain FMO2 coincided with the cell growth. The optimum pH range and temperature for the activity of amylase were found to be pH 6 and 45-50^{0}C respectively. The amylase from *Lactobacillus plantarum*FMO2 was purified 2.63 fold with a specific activity of 0.10U/mg. The enzyme was stable at 50^{0}C and 60^{0}C up to 180 min. The residual enzyme activity of 85.1% was retained after 60mins of incubation at temperature of 70^{0}C and affected by different metal ions in a dose dependent manner, although activated by $CaCl_2$ ion. The results showed that Ogi is a good substrate for amylase production. These bacteria strains may be developed into starter cultures to facilitate production of enzymes that can be used for industrial purposes since the enzyme they produced are thermostable.

.

Keywords: Amylase, retted cassava, *Lactobacillus plantarum*, amyloytic index, fermentation, thermostable.

Introduction

Amylolytic lactic acid bacteria account for a substantial portion in different types of foods and are widespread among the non-dairy food environments and different geographical areas. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The industrial sources are maize, tapioca, potato, and wheat, but limitations such as low shear resistance, thermal resistance, thermal decomposition and high tendency towards retrogradation in some industrial food applications are major restraints (Goyal *et al*., 2005). Therefore, it is very important to obtain a thermostable enzyme, an enzyme that can work over a wide temperature range by isolating directly from nature that lives in these conditions (Annamala *et al*., 2011).

Bacterial amylases are generally preferred to fungal amylases due to several characteristics and advantages they offer (Pandey *et al.*, 2000).

Bacteria have become an important source for producing thermostable α-amylase with better properties than fungi (Prakash and Jaiswal, 2010). Among the bacteria, *Bacillus* sp. is widely used to produce thermostable α-amylase which is required in industry. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known as good α-amylase producers and these have been widely used for commercial enzyme production in various applications (Sivaramakrishnan *et al*., 2006).

Though they can originate from plants, animals and microorganisms, fungal and bacterial amylases are mainly used for the industrial production, because of higher production rate, thermostability, less time and space for production and easier process modification and optimization (Suman and Ramesh, 2010).

The ability of lactic acid bacteria to regulate their cytoplasmic or intracellular pH is one of the most important physiological requirements of the cells.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Cells unable to maintain a near neutral intracellular pH during growth or storage at low extracellular pH may lose viability and cellular activity. Thus, optimization of nutritional and physicochemical parameters is of utmost importance to perform the industrial process cost-effective and economically viable (Singh *et al*., 2011). The production of bacterial thermostable α-amylases has been thoroughly investigated in submerged fermentation (SmF) and solid state fermentation (SSF). However, because of the greater control of environmental factors and ease of handling, SmF is more common and is affected by a variety of physicochemical parameters such as composition of growth medium, inoculum age, pH, temperature, nitrogen source and carbon source (Souza, 2010). Hence, the aim of this work is to optimize the physical parameters (Temperature, pH and Incubation Time) for efficient activities of amylolytic lactic acid bacteria from starchy foods.

Material and Methods

Sample Collection

Samples of freshly fermented maize gruel (Ogi) and retted cassava (Fufu) were obtained from local producers located at Umungasi in Aba North Local Government area of Abia State, Nigeria. The fermented products were transferred into universal sterile bottles. These samples were packaged inside a cooler containing ice cubes and quickly brought to the laboratory for immediate analysis.

Isolation and Characterisation of Lactic Acid Bacteria

Isolation of LAB was carried out using the method as described by Bhattacharya and Das (2010). For all samples, 10 g will be added to 90 ml of sterile diluent's containing 0.1% peptone water and homogenized for 30s and 0.1ml aliquots of appropriate dilutions (10^{-3}, 10^{-6}, and 10^{-9}) of the samples was inoculated into de Man Rogosa Sharpe (MRS) agar by streaking method fortified with 50mg of nystatin and incubated in anaerobic condition at 30°C for 48 h for the colonies to develop. Following incubation, different colonies for each sample was randomly selected from the MRS agar plates. The colonies subcultured and purified by repeated streaking on MRS agar to obtain pure cultures. Pure isolates was then cultured on MRS agar slants and broths (in duplicates) and stored at 4°C until used.
Cell morphology, Gram staining, Oxidase and Catalase tests was performed as a preliminary screening for Lactic acid bacteria.

Gram positive, non-spore forming and Catalase negative strains were selected for further studies as described by Cheesbrough (2000). The isolated strains were inoculated into sugar broth tubes containing Durham's tube to find out the fermentation capability of the organisms in different sugars.

The isolates were also identified on the basis of genotypic characteristics (16S rRNA gene sequences similarity with the type strains) during BLAST searches

Screening for Amylase Production

Screening of amylase producing isolates

One millilitre of the dilution factors 10^{-1} - 10^{-6} was introduced into Petri dishes in triplicates. Pour plate method was used. The medium containing 1% starch and 2% agar was used as described by Hattingh *et al*. (2015). Plates were incubated at 37^0C for 72 h. Only the amylase producing bacteria were able to utilize the starch as sole carbon source and were able to grow on this medium. After the isolation of amylase producing bacteria, they were streaked subsequently on starch agar plate and Nutrient agar until pure isolated colonies were obtained. Bacteria isolates were flooded with iodine on starch agar plates and zone of clearance was determined after 60 minutes. The amylolytic index (AI) was calculated as the ratio R/r, where R was the diameter of the entire clear zone, and r was the diameter of the agar well with the LAB colony (Chen *et al*., 2018). The starch utilization was monitored by the disappearance of the blue colour of the medium based on the intensity of amylase production.

Growth and amylase production by lactic acid bacteria isolates

The method of Ekka and Namdeo (2018) was adopted. A loopful of each amylase producing bacterial cultures were transferred to 150ml of the amylase production medium in a 250ml Erlenmeyer flask, incubated at 37^0C with shaking at 150rpm. The initial pH of the basal medium was adjusted to 6.5 with phosphate buffer. About 5ml of the culture media were withdrawn after 48hours and the cell density determined by recording the OD (optical density) at 660 nm in UV-VIS spectrophotometer (Varian, Cary® 50 Bio, Australia) up to 72hours. After the removal of cells by centrifugation (8000xg, 30 min, 4°C), the supernatant was considered as crude enzyme solution.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Amylase activity assay

Amylase enzyme activity was determined using 1% starch dissolved in distilled water as substrate. The reaction mixture containing 1.0mL substrate and 2mL enzyme solution was incubated at 40°C for 15 minutes. And the reaction was stopped by adding 3 mL DNS (3,5-dinitrosalicylic acid) reagent.

All enzyme samples were assayed in triplicates with boiled enzyme blanks for each determination. The enzyme activity was expressed as U/min/mL which corresponded to µ-mole of glucose equivalent released per minute under the assay conditions as described by Ekka and Namdeo (2018).

Then, 0.2 mL of the reaction mixture pipetted into a 96-well plate and absorbance at 600 nm wavelength was measured using microplate reader. One amylase unit was defined as the enzymatic activity that liberates one microgram of maltose per minute per millilitre CFS.

Molecular identification of lactic acid bacteria strains

DNA extraction

DNA extraction was conducted using the facilities of the Center for Molecular Biology and Biotechnology (CMBB), Michael Okpara University of Agriculture, Umudike (MOUAU), Nigeria. Genomic DNA from the isolated LAB was extracted with Zymo-Spin™ kits according to the manufacturer's instructions and the extracted DNA was separated on a 1% agarose gel electrophoresis.

PCR analysis

The 16S rRNA coding gene was amplified through polymerase chain reaction (PCR) using universal primers, 27F (5‟-AGAGTTTGATCMTGGCTCAG-3‟) and 1429R (5‟- TACGGCTACCTTGTTACGAC -3‟). PCR amplifications were carried out in a thermal cycler (T Gradient model, Biometera, Germany) using the following steps: one cycle of denaturation for 5 min at 94°C followed by thirty-five cycles of 94°C for 30 s. Annealing was performed at 50°C for 30 s, extension at 68°C for 1 min and final extension was done at 68°C for 10 min. The PCR products were kept at 4°C and the integrity of the PCR amplicons were separated on a 1% agarose gel electrophoresis (CSL-AG500, Cleaver Scientific Ltd) and visualized by staining with EZ-vision® Bluelight DNA Dye.

Sequencing

PCR products were purified using ExoSAP Protocol and sequencing was done with the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Catalogue No. D4053) using ABI 3500XL Genetic Analyser by Inqaba Biotec, South Africa.

BLAST analysis

The resulting 16S rRNA gene sequences were analyzed in NCBI website (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) Software in the GenBank nonredundant/nucleotide collection (nr/nt) to compare sequences and identification was performed on the basis of 16S rRNA sequence percentage similarity with the type strains.

Phylogenetic analysis

The sequences were aligned using ClustalW and the evolutionary history was inferred using the Neighbor-Joining method whereas the evolutionary distances were computed using the Maximum Composite Likelihood method. All ambiguous positions were removed for each sequence pair (pairwise deletion option) and the evolutionary analyses were conducted in MEGA X (Saitou and Nei, 1987; Kumar *et al*., 2018).

Effect of temperature on the growth and amylase production

250-mL Erlenmayer flask containing 50 mL of amylase production medium was inoculated with 2% v/v inoculum from 24 h-old seed culture and flasks were incubated in a shaking incubator at different temperatures ranging from 20–70°C at intervals of 10^0C for 24 h. 5ml of the culture medium was withdrawn and the cell density determined. The initial pH was adjusted to 7.0. Amylase activity was determined from culture broth obtained after centrifugation (11,300× g for 10 min at 10 °C) at 600nm.

Effect of pH on the growth and amylase production

The effect of pH on enzyme production was investigated by adjusting the pH range of 3.0-9.0, using 50mM of three buffer solutions: Tris-HCl (pH 3.0), Citrate phosphate (pH 4.0 – 6.0), and Glycine-NaOH (pH 7.0-10.0). The media was inoculated by the isolate and incubated at 37^0C for 24 hours. Samples were withdrawn and the enzyme activity determined as described previously.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Effect of Incubation time on the growth and amylase production

The incubation time on the growth of amylase producing bacterial culture was determined by transferring a loopful to 150ml of the amylase production medium in a 250ml Erlenmeyer flask, incubated at 37^0C with shaking at 150rpm. The initial pH of the basal medium was adjusted to 6.5 with phosphate buffer. 5ml of the culture medium was withdrawn and the cell density determined at 600nm. at regular intervals of 12hours and the cell density determined by recording the OD (optical density) at 660 nm in UV-VIS spectrophotometer Varian, Cary® 50 Bio, Australia) up to 72hours. After the removal of cells by centrifugation (8000xg, 30 min, 4°C), the supernatant was considered as crude enzyme solution.

Protein Estimation

In order to determine the specific activity and purification fold, Protein content was measured by the method of Bradford and bovine serum albumin (BSA) is used as a standard protein measured at a concentration of 0.1-1.0 mg / mL protein Bradford (1976). The specific activity of crude amylase was calculated by dividing unit activity with protein content.

Amylase purification

The crude enzyme preparation obtained from *Lactobacillus plantarum* FM02 was purified by ammonium sulphate fractionation followed by dialysis and DEAE cellulose column chromatography as described by Suman and Ramesh (2010). A total of 100ml of the culture supernatant was centrifuged at 8000rpm for 20mins at 4^0C to remove the cells. The supernatant was brought to 80% ammonium sulphate saturation at 4^0C for 12hours in an ice bath. The precipitated protein was collected and dissolved in a minimum volume of phosphate buffer (0.1M, pH 6.8). The enzyme solution was dialysed at 4^0C against the same buffer 24hours with mechanical stirring at 4°C. The DEAE cellulose column chromatography (Merck, Bangalore, India) was pre-equlibrated with the same buffer. The dialysate was applied to the DEAE cellulose column and the flow rate was maintained at 0.6ml/min with 50ml linear NaCl (0.1 to 0.5M) gradient. Fractions of 10ml were collected and each fraction was analysed for protein concentration and amylase activity.

Thermal Stability of the amylase enzyme

The temperature stability was determined by incubating the purified enzyme solution in water bath for temperature range of 50-90°C at intervals of 10^0C for 0, 30, 60, 90, 120, 150 and 180mins and then cooled with tap water. The remaining α-amylase activity was measured and expressed as the percentage of the activity of untreated control taken as 100%.

Statistical analysis

All the experiments were done in triplicates and data were represented as means ± standard deviation of mean. The study was subjected to one–way analysis of variance (ANOVA) using SPSS 23.0 to determine the significant variations between the tests. Mean analysis was carried out using Duncan Multiple Range test at 95% confidence level.

Results

The phenotypic and carbohydrate fermentation of the isolates as seen in Table 1 show the presence of *Lactobacillus* sp. The result of amylolytic ability and of the amylase activity of the lactic acid bacteria isolates is presented in Table 2. The diameter of the clearance zone is shown in the first column while the amylolytic index (AI), which is the ratio of the diameter of clear zone produced as a result of starch hydrolysis to the diameter of the agar well with the lactic acid bacteria colony.

The resulting clear zone ranged from 6.63mm to 13.17mm while the amylolytic index ranged from 1.32 to 2.63. The highest clear zones were recorded in the lactic acid bacteria isolate *Lactobacillus plantarum*FMO2: AK1 (13.17±0.29), *Lactobacillus pentosus* BRS3:CA2 (10.32±0.16) and *Lactobacillus plantarum*Z2:CA5 (12.77±0.25).

Result of the amylase activity of the lactic acid bacteria isolates showed that some potential isolates produce the highest amylase activity. These include AK1 (0.85±0.05 U/ml), AK4 (0.61±0.02 U/ml) derived from Ogi and CA2 (0.70±0.10 U/ml), CA5 (0.75±0.05 U/ml) derived from retted cassava. The isolate that had the highest amylase activity was AK1 (*Lactobacillus plantarum*FMO2) from ogi with enzyme activity of 0.85±0.05U/ml while the lowest amylase activity in AK5 isolates is 0.16±0.04U/ml (Table 2).

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Table 3: Cultural, morphological, biochemical characteristics and probable identity of Lactic acid bacteria (LAB) isolated from fermented starchy foods

Isolate code	Cell shape	Margin	Elevation	Motility	Endospore	Gram rxn	Catalase	Indole	Methyl red	VP Test	Citrate	Oxidase	Arginine	Mannitol	Fructose	Galactose	Raffinose	Glucose	Sorbitol	Mannose	Arabinose	Rhamnose	Lactose	Salicin	Gas from TSI	Probable organisms
AK1	Cocci	S/E	Raised	–	–	+	–	–	–	–	–	–	–	+	+	+	+	+	+	+	–	–	+	+	–	*Lactobacillus* sp
AK2	Short rods	S/E	Flat	+	+	+	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	*Lactobacillus* sp
AK3	Rods	R/I	Flat	–	–	+	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	_	*Lactobacillus* sp
AK4	Rods	S/E	Flat	+	+	+	–	–	–	–	–	–	–	+	–	+	+	+	+	+	+	+	+	+	–	*Lactobacillus* sp
AK5	Cocci	S/E	Raised	–	–	+	–	–	–	–	–	–	–	+	+	+	+	+	+	+	_	_	+	+	_	*Lactobacillus* sp
CA1	Short rods	R/I	Flat	–	–	+	–	–	–	–	–	–	+	+	+	+	–	+	+	+	–	–	+	+	–	*Lactobacillus* sp
CA2	Rods	R/I	Flat	–	–	+	–	–	–	–	–	–	–	–	–	+	+	+	–	+	+	–	+	+	+	*Lactobacillus* sp
CA3	Short rods	R/I	Flat	–	–	+	–	–	–	–	–	–	+	+	+	+	+	+	–	+	+	–	+	–	–	*Lactobacillus* sp
CA4	Rods	R/I	Flat	+	–	+	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	–	+	–	–	*Lactobacillus* sp
CA5	Short rods	R/I	Flat	+	–	+	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+	–	+	+	–	*Lactobacillus* sp

Key: +ve = positive; -ve = negative; AK=Ogi; CA= retted cassava (fufu) S/E=Smooth and entire; R/I=Rough and irregular

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Table 2: Screening of Amylolytic Lactic Acid Bacteria and Amylase activity of lactic acid bacteria isolates

Lactic Acid Bacteria Isolates	Diameter of Clearance Zone (mm)	Amylolytic index	Amylase activity (U/ml)
AK1	13.17±0.29^{f}	2.63±0.06^{f}	0.85±0.05^{f}
AK2	7.27±0.25^{b}	1.45±0.05^{b}	0.42±0.03^{c}
AK3	8.23±0.25^{c}	1.65±0.05^{c}	0.34±0.05^{bc}
AK4	9.33±0.15^{d}	1.87±0.03^{d}	0.61±0.02^{d}
AK5	9.70±0.17^{d}	1.94±0.03^{d}	0.16±0.04^{a}
CA1	8.27±0.21^{c}	1.65±0.04^{c}	0.28±0.03^{b}
CA2	10.32±0.16^{e}	2.06±0.03^{e}	0.70±0.10^{e}
CA3	7.43±0.06^{b}	1.49±0.01^{b}	0.32±0.03^{b}
CA4	6.63±0.55^{a}	1.32±0.11^{a}	0.34±0.03^{bc}
CA5	12.77±0.25^{f}	2.55±0.05^{f}	0.75±0.05^{e}

Values presented are means of triplicates ± standard deviation. Means with different superscript across a column are significantly different ($p < 0.05$)

These isolates were identified on the basis of genotypic characteristics (16S rRNA gene sequences similarity with the type strains) during BLAST searches as *Lactobacillus plantarum* strain FMO2, *Lactobacillus pentsus* strain BSR3 and *Lactobacillus plantarum* strain Z2 (Figure 1 and 2)with percentage identity and accession number as seen in summary of the blast (Table3).

The effect of temperature on the growth and amylase production was investigated. Hence amylase production by the strains, *Lactobacillus plantarum*FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus*BSR3 were tightly linked to cell growth and temperature changes. The relationship between pattern of cell growth and amylase production was observed (Table 4) in all the 3 strains.

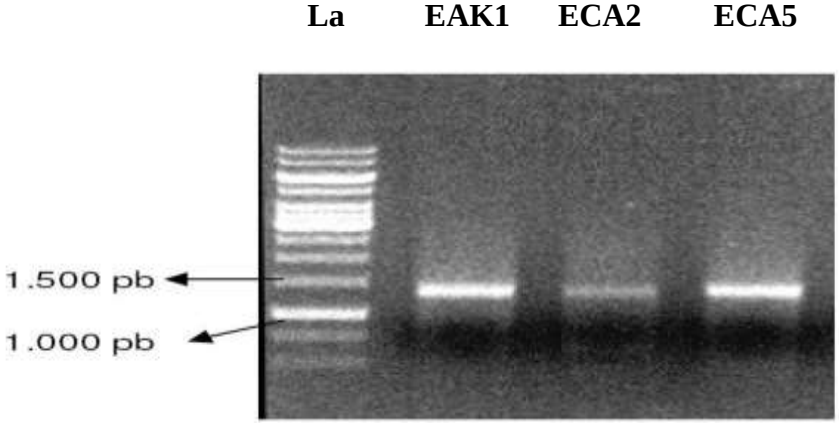

Figure 1: Agarose gel electrophoresis of the 16S rRNA gene of the *Lactobacillus plantarum* strain FMO2 (AK1), *Lactobacillus pentosus* strain BSR3 (CA2) and *Lactobacillus plantarum* strain Z2 (CA5). Lane La represents the molecular ladder.

Table 3: Summary of Blast Prediction

S/N	Seq ID	Matched organism	% Identity	Accession number
1	ECA2	***Lactiplantibacillus pentosus* BSR3**	99.86%	KY203913.1
2	ECA5	***Lactiplantibacillus plantarum* Z2**	100%	ON063304.1
3	EAK1	***Lactiplantibacillus plantarum* FM02**	100%	MG913360.1

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

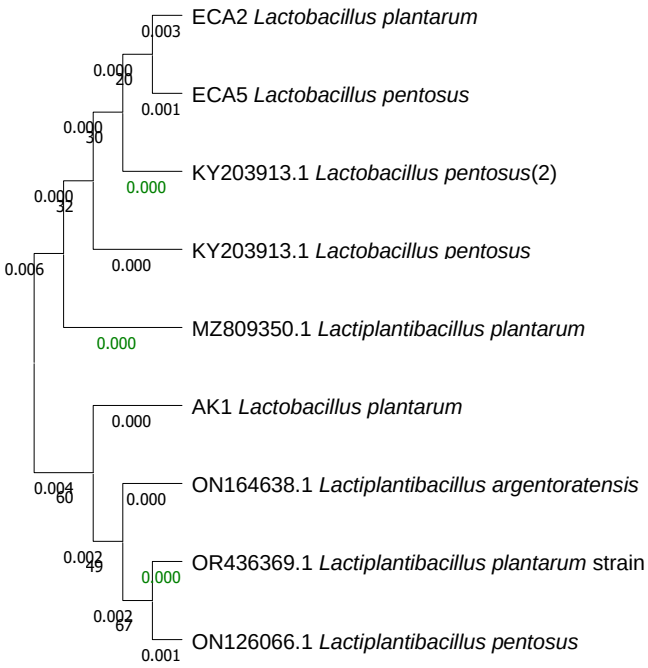

Figure 2: Phylogenetic tree showing the evolutionary distance between the bacterial isolates

Table 4: Effect of temperature on the growth and amylase production

Lactic Acid Bacteria Strains	Temperature (^{0}C)	Cell density (Log CFU/ml)	Amylase Activity(U/ml)	Relative Amylase Produced (%)
Lactobacillus plantarum FMO2	30	1.23±0.25^b	0.12±0.06^a	16.7
	35	1.23±0.04^b	0.22±0.06^a	30.6
	40	1.30±0.02^b	0.36±0.05^a	50
	45	1.42±0.03^a	0.41±0.06^a	56.9
	50	1.82±0.03^c	0.72±0.06^a	100
	55	1.21±0.03^a	0.20±0.05^a	27.8
	60	1.06±0.04^a	0.15±0.06^a	20.8
Lactobacillus plantarum Z2				
	30	1.28±0.16^c	0.09±0.31^a	18
	35	1.31±0.04^c	0.10±0.23^a	20
	40	1.42±0.03^c	0.13±0.07^b	26
	45	1.52±0.03^b	0.50±0.26^b	100
	50	1.42±0.02^b	0.22±0.06^b	44
	55	1.44±0.05^c	0.12±0.04^b	24
	60	1.40±0.02^b	0.08±0.19^b	16
Lactobacillus pentosus BSR3				
	30	1.05±0.06^a	0.11±0.24^a	22
	35	1.11±0.01^a	0.50±0.18^a	100
	40	1.21±0.02^a	0.28±0.19^a	56
	45	1.42±0.02^a	0.20±0.34^a	40
	50	1.31±0.006^a	0.19±0.07^a	38
	55	1.30±0.006^b	0.12±0.12^a	24
	60	1.09±0.006^a	0.09±0.10^a	18

Values represented are means of triplicates ± standard deviation. Means with different superscript across a column are significantly different ($p < 0.05$). *****Relative enzyme production is expressed in comparison to the maximum enzyme produced which is taken as 100%.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Result of the pH of the 3 lactic acid bacteria strains is presented in Table 5 and which showed that pH 5.0-6.0 was the most favourable for α-amylase production and bacterial growth. *Lactobacillus plantarum*FMO2

recorded the highest microbial growth at pH 5.0 and 6.0(1.80±0.16 and1.91±0.09) with a corresponding increase in amylase production of 0.82±0.063 and 0.92±0.06 higher than *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus*BSR3 as in Table 5.

Result of the effect of incubation time on the growth and amylase production is presented in Table 6.

The Lactic acid bacteria strains showed an increase in cell density and amylase production with time ($P<0.05$) (Table 6). The decline of cell growth and amylase activity occurred after 48 h of incubation.

Table 7 and Figure 3 shows the protein content of the cell free supernatant of the strain by Bradford method. Owing to the high activity observed in all the lactic acid bacteria strains, *Lactobacillus plantarum*FMO2 showed maximal activity. The bovine serum albumin of different concentrations (0.1 to 0.5) was used as standard and absorbance measured at 600nm.

Table 5: Effect of pH on the growth and amylase production

Lactic Acid Bacteria Strains	pH	Cell density (log CFU/ml)	Amylase activity(U/ml)	Relative amylase produced (%)
Lactobacillus plantarum FMO2	**3**	1.14±0.25^{a}	0.70±0.06^{a}	82.35
	4	1.27±0.18^{a}	0.75±0.06^{a}	91.46
	5	1.61±0.05^{a}	0.80±0.05^{a}	97.56
	6	1.80±0.16^{b}	0.82±0.06^{a}	100
	7	1.31±0.09^{ab}	0.52±0.06^{a}	63.41
	8	1.20±0.09^{a}	0.48±0.05^{a}	58.54
	9	1.02±0.29^{c}	0.20±0.06^{a}	24.39
Lactobacillus plantarum Z2	**3**	1.28±0.16^{a}	0.10±0.31^{a}	16.39
	4	1.31±0.09^{a}	0.12±0.23^{a}	19.67
	5	1.40±0.09^{ab}	0.36±0.07^{b}	59.01
	6	1.50±0.29^{ab}	0.61±0.26^{b}	100
	7	1.59±0.30^{a}	0.29±0.06^{b}	47.54
	8	1.43±0.11^{a}	0.20±0.04^{b}	32.79
	9	1.19±0.23^{a}	0.15±0.19^{b}	24.59
Lactobacillus pentosus BSR3	3	1.05±0.23^{a}	0.11±0.24^{a}	22.00
	4	1.10±0.59^{a}	0.28±0.18^{a}	56.00
	5	1.23±0.72^{a}	0.35±0.19^{a}	70.00
	6	1.34±0.63^{c}	0.50±0.34^{a}	100
	7	1.41±0.66^{b}	0.29±0.07^{a}	58.00
	8	1.29±0.20^{b}	0.21±0.12^{a}	42.00
	9	1.09±0.39^{a}	0.09±0.10^{a}	18.00

Values represented are means of triplicates ± standard deviation. Means with different superscript across a column are significantly different ($p < 0.05$). *****Relative enzyme production is expressed in comparison to the maximum enzyme produced which is taken as 100%

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Table 6: Effect of incubation time on growth and amylase production

Lactic Acid Bacteria Strains	Incubation Time (hours)	Cell Density (Log CFU/ml)	Amylase Activity (U/ml)	Relative Amylase Produced (%)
Lactobacillus plantarum FMO2	**12**	1.14±0.25[a]	0.45±0.06[a]	75.6
	24	1.28±0.18[a]	0.50±0.06[a]	80.7
	36	1.31±0.05[a]	0.53±0.05[a]	85.5
	48	1.80±0.16[b]	0.62±0.06[a]	100
	60	1.31±0.09[a]	0.22±0.06[a]	35.5
	72	1.20±0.09[a]	0.12±0.05[a]	19.4
Lactobacillus plantarum Z2	**12**	1.28±0.16[a]	0.10±0.31[a]	25.0
	24	1.31±0.09[a]	0.12±0.23[a]	30.0
	36	1.35±0.09[ab]	0.26±0.07[b]	65.0
	48	1.60±0.29[ab]	0.40±0.20[b]	100.0
	60	1.29±0.10[a]	0.21±0.06[b]	52.5
	72	1.13±0.11[a]	0.20±0.04[b]	50.0
Lactobacillus pentosus BSR3				
	12	1.05±0.23[a]	0.11±0.24[a]	22.0
	24	1.10±0.59[a]	0.18±0.18[a]	36.0
	36	1.63±0.72[a]	0.25±0.19[a]	50.0
	48	1.74±0.63[c]	0.50±0.34[a]	100
	60	1.31±0.66[b]	0.29±0.07[a]	58.0
	72	1.29±0.20[b]	0.21±0.12[a]	42.0

Values represented are means of triplicates ± standard deviation. Means with different superscript across a column are significantly different ($p < 0.05$)*****Relative enzyme production is expressed in comparison to the maximum enzyme produced which is taken as 100%.

Table 7: Concentration of Bovine Serum Albumin(BSA) at 600nm ODAbsorbance @600nm

Conc of BSA	R1	R2	R3	Mean	Total Protein
0.1	0.172	0.196	0.148	0.172±0.02	
0.3	0.3	0.356	0.319	0.325±0.03	
0.5	0.594	0.597	0.555	0.582±0.02	
0.7	0.776	0.711	0.725	0.737±0.03	
0.9	0.923	0.953	0.91	0.927±0.02	
Cell free supernatant of *Lactobacillus plantarum*FMO2	0.753	0.867	0.854	0.825±0.06	22.59524

Key: R1 = absorbance from 1st replicate; R2 = absorbance from 2nd replicate; R3 = absorbance from 3rd replicate

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

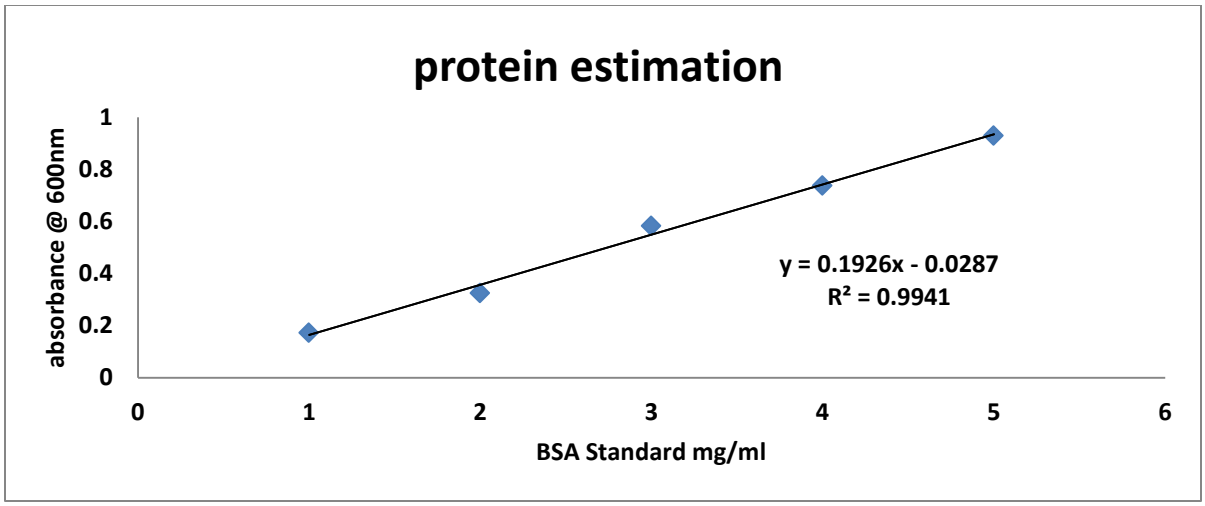

Figure 3: Protein estimation of cell free supernatant using Bradford method

Key: 0.192= slope; 0.028=intercept; Total protein= {OD(cell supernatant)-slope}/intercept

Result of enzyme purification protocol (characteristics of purified enzyme) is presented in Table 8. The α-amylase activity was assayed at different temperatures ranging from 50˚C to 90˚C with time at optimal pH 7 under standard assay conditions (Figure 4).

The enzyme showed stability over a temperature range of 50˚C to 70˚C. Activity generally decreased when temperature and heating time were increased. The effect was more profound at 80°C and 90°C for longer holding times.

Table 8: Characteristics of Purified Enzyme

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
1. Culture supernatant	0.85	22.60	0.038	1.00	100
2. Ammonium sulphate precipitate (80%) and dialysis	0.55	11.23	0.049	1.29	64.7
3. DEAE Cellulose chromatography	0.37	3.62	0.10	2.63	43.5

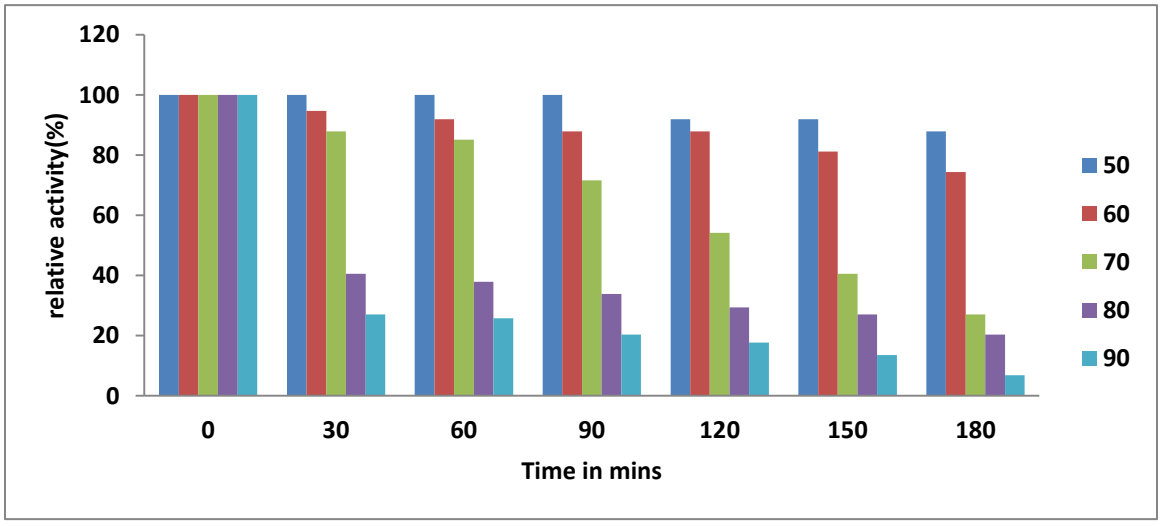

Figure 4: Thermal stability on purified amylase

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Discussion

Amylase-producing LAB represent a valuable resource in food technology, agriculture, and health. Table I shows clearly the phenotypic and carbohydrate fermentation of the lactic acid bacteria isolates from both Ogi and retted cassava. All of this micro organisms have been reported in previous microbiological studies done on spontaneously fermenting millet 'ogi' (Olasupo *et al*.,1997), fermenting maize 'ogi'(Wakil and Daodu, 2011), fermenting sorghum 'ogi'(Odunfa and Adeleye, 1985) and fermenting cassava (Oyewole and Odunfa, 1990). Table 2 shows the resulting clear zone ranging from 6.63mm to 13.17mm while the amylolytic index ranged from 1.32 to 2.63. The absence of a clear zone around the colony indicates a reaction, between iodine reagents and non-hydrolyzed starches in starch-containing mediums (Cappuccino and Sherman, 2002). Gana *et al.* (2014) reported that the ability or the power to produce the enzyme amylase is characterized by the formation of clear zones in the medium containing starch. Ten isolates of microbes that have the ability to produce the enzyme amylase were tested for their ability based on the amylolytic index (AI). Based on the amylolytic index, various AI values were obtained. A total of 1 isolate from Ogi sample and 2 from retted cassava samples were potential or capable of producing AI ≥ 2.00 or moderate to high category in producing amylase compared to other isolates, namely isolates AK1 (*Lactobacillus plantarum*FMO2), CA2 (*Lactobacillus pentosus*BSR3) and CA5 (*Lactobacillus plantarum*Z2) with AI values of 2.63±0.06, 2.06±0.03 and 2.55±0.05 respectively. This is in accordance with the research of Ouedraogo *et al.* (2014), who succeeded in isolating yeast and screening potential amylase-producing yeasts from potatoes with an AI value of 2.35. Based on the measurements of amylase activity (Table 1), some isolates that produce the highest amylase activity include *Lactobacillus plantarum*FMO2 (0.85±0.05), *Lactobacillus plantarum*Z2 (0.75±0.05) and *Lactobacillus pentosus*BSR3(0.70±0.10).

There is a relationship between amylolytic index and enzyme activity. The isolate that has the highest enzyme activity is the *Lactobacillus plantarum*FMO2 with enzyme activity of 0.85 ± 0.05 U/mL with a fairly high or moderate category.

Some studies that are able to produce enzyme activity are quite high, including, Ouédraogo *et al.* (2014), successfully measured the activity of amylase enzymes in the potential yeast of amylase-producing results from potatoes of 0.774 U/mL. Oliveira *et al.* (2015) successfully measured the activity of amylase enzyme in yeast *Saccharomyces cerevisiae* enzyme activity of 0.734 U/mL. The LAB isolates were characterized and identified on the basis of morphological, physiological, biochemical and genotypic characteristics (16S rRNA gene sequences similarity with the type strains) (Figure 1and 2).

The effect of varying incubation temperature on growth and amylase production was studied to determine the optimum temperature for enzyme production. A maximum amylase production was observed at 50°C with about 20% enzyme loss at 60°C (Table 4). Similar results on maximum amylase production at 50°C were achieved by *Bacillus* spp and *Lactobacillus plantarum* MTCC (Panda *et al*., 2008). The obtained results are close with that obtained by Deb *et al*., (2013) who reported that the temperature was considered an important environmental factor which controls the growth and production metabolic substances by microorganisms and this is usually varied from one organism to another. Saleem and Ebrahim, (2014) reported that the decrease in enzyme activity was observed at higher temperature because of change in membrane composition which cause protein catabolism.

In contrast, optimal amylase was produced at 50°C by *Bacillus licheniformis* (Gupta *et al.*, 2018). The ability of *L. plantarum* FMO2 to produce maximum amylase at 50°C suggests the organisms to be a thermophile. Thus, the gradual temperature increase reduced the amount of amylase (Table 4), probably due to distorted systematic physiological activities leading to uncoordinated stress response by the organism.

The result in this study (Table 5) showed that pH 5.0-6.0 was the most favourable for α-amylase production and bacterial growth. The fermentation medium pH is one of the important physical parameters which play an important role in enzyme production. The pH range observed during the growth of microorganisms also affects the product stability. pH is known to affect the synthesis and secretion of amylase.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

*Lactobacillus plantarum*FMO2 recorded the highest microbial growth at pH 5.0 and 6.0 (1.91±0.09 and 1.80±0.16) with a corresponding increase in amylase production of 0.80±0.05 and 0.82±0.06 respectively. The result obtained in table 3 was similar to the findings of Nyarko *et al*.,(2019) where maximum amounts of amylases were achieved in a fermentation medium of pH of 6.5. In contrast to this result, pH of 7 was reported to be the most effective for maximum amylase production by *L. plantarum* MTCC 1407 (Panda *et al*., 2008). Further increase in pH beyond 6 led to a gradual decrease in amylase yield (Table 5). However, up to (82.35% and 24.39%),(16.39% and 24.59%) and (22% and 18%) relative amylase yields for *Lactobacillus plantarum*FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BSR3 respectively were recovererd at pH 3 and 9 which are within acidic and alkaline range, although the highest was observed with *Lactobacillus plantarum*FMO2. This gave an indication that pH changes did not have tremendous effects on the amylase yield. This can be supported by Giraud *et al*. (1991) report, which also indicated that fermentation pH which affected the growth of *L. plantarum* did not have much effect on its production of lactic acid. However, maximum amylase production at pH6 by *L. plantarum*FMO2 could be explained from the fact that the organism mostly preferred semi- neutral environment to others as Ruby *et al*. (2012) also reported optimum growth condition at 6.0.

The time course study on fermentation for optimum amylase revealed 48h (Table 6) as the most effective period and this result is in line with reports from Gupta *et al.* (2018) where highest amylase yields were also obtained within 48h. However, the findings of Divakaran *et al.* (2011) had 72h fermentation period as best for *L. plantarum* amylase yield. The ability to obtain maximum amylase yield at 48h fermentation period indicated the producer to be a fast-grower with adequate potential for amylase production. It could also be due to optimized condition which facilitated both the growth of the organism and its metabolic synthesis. It is interesting to note that the *Lactobacillus plantarum* FMO2 was able to produce amylase with a relative activity of 80.7% within 24h. This positions *L. plantarum*FMO2 as a good industrial organism. The amylase production reached maxima value of 0.62±0.06U/ml at 48 hours when the cell population also at its maximum 1.80±0.16 log CFU/ml.

Hence amylase production by the 3 lactobacillus strains (*Lactobacillus plantarum*FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BRS3 were tightly linked to cell growth. The decline of cell growth and amylase activity occurred after 48 h of incubation and could be attributed to the raise of acidity due to lactic acid production in fermented broth (Singh *et al.*, 2006).

Commercial use of amylase does not require purification process but application of amylase in pharmaceutical and clinical purpose require high purity of enzyme. *Lactobacillus plantarum*FMO2 was selected for the purification process. Different concentrations (0.1-0.9)(Table 7) of Bovine Serum Albumin (BSA) was prepared and compared with the unknown cell free supernatant of *Lactobacillus plantarum*FMO2. The absorbance measured at 600nm(Figure 3) The results of the purification of α-amylase produced by the strain *Lactobacillus plantarum*FMO2 is summarized in (Table 8) which shows a high increase in enzyme specific activity for each purification step, especially the results of the DEAE column chromatography. The first step in purification was conventional $(NH_4)_2SO_4$ fractionation. The fraction (80%) saturation of ammonium sulfate revealed maximum enzyme specific activity (4.56U/mg). It was further purified by the DEAE-cellulose column chromatography exhibited 34.3% of the total initial activity and there was a 2.15 fold increase in specific activity (7.04U/mg) when compared with the crude culture filtrate (3.28U/mg).

This high increase in specific activity indicates that the enzyme purification stage carried out was quite good. The total enzyme activity for each purification stage experienced a high decrease. This high decrease in total activity is probably due to the enzyme loss of activity during the process, especially when deposited with ammonium sulfate, which is because the solution used is very dilute.

The maximum purification level achieved was about 2-fold and the recovery yield was approximately 35%. Recently, Xie *et al.* (2014) have indicated 13.1 purification fold and 7.0% yield, Abdel-Fattah *et al.* (2012) have pointed out 59.3 purification fold and 12.6 % yield, and Shukla and Singh (2015) have reported 37 purification fold and 16.8% yield using chromatography methods.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Thermal stability of the purified enzyme produced by *Lactobacillus plantarumFMO2* was examined by incubating the enzyme at different temperatures (50^0C-90^0C) for 30-180 min. The result (Figure 4) showed that purified enzyme was steady at temperature of 50 °C and 60 °C up to 180 min. The 87.8% of residual enzyme activity was retained after 30 min of incubation at the temperature of 70 °C, the enzyme activity decreased up to 27%. Similarly at 80^0C, loss of activity was 20.3% of its activity. The enzyme was comparatively stable at 50^0C and 60^0C even after 180mins and lost only 25.7% of its activity even after 180mins incubation, but lost almost all (93.2%) of its activity for 180mins (Figure 4) The stability of the enzyme could be due to the genetic adaptability to carry out its biological activities at higher temperatures (Mathew and Gunathilaka, 2015). According to the results obtained from thermal stability of purified α-amylase, the enzyme can be applied in brewing and food processing industries.

In conclusion, this study has shown that, the selected fermented food products (Ogi and Fufu) contain several strains of Lactobacilli, which are capable of producing amylase. Among the isolated LAB from Ogi and Fufu, *Lactobacillus plantarum*FMO2, *Lactobacillus plantarum*Z2 and *Lactobacillus pentosus* BSR3 exhibit good thermal stability and thus may find use in different industries where high temperature is required during commercial process.

References

Abdel-Fattah, Y.R., Soliman, N.A., El-Toukhy, N.M., El-Gendi, H. & Ahmed, R.S. (2012). Production, Purification and Characterization of Thermostable a-amylase Produced by *Bacillus licheniformis* Isolate AI20. *Journal of Chemistry***,** *1*, 1-11.

Annamalai, N., Thavasi, R., Vijayalakshmi, S. & Balasubramanian, T. (2011). Extraction , purification and characterization of thermostable alkaline tolerant α- amylase from *Bacillus cereus*. *Indian Journal of Microbiology***,** *51(4)***,** 424

Bhattacharya, S. & Das, A. (2010). Study of physical and cultural parameters on the bacteriocins produced by lactic acid bacteria isolated from traditional Indian fermented foods. *American Journal of Food Technology***,** *5***,**111-120.

Bradford, M.M.(1976). A rapid and sensitivemethod for the quantitation of microgram quantities of protein utilizingthe principle of protein-dye binding. *Annals of Biochemistry*, *72*, 248 – 254.

Cappuccino, J.G. & Sherman, N. (2002). Microbiology: A laboratory manual, *9th ed Addison-Wilsey* California.

Chen, P.H., Chen, R.Y. & Chou J.Y. (2018). Screening and evaluation of yeast antagonists for biological control of *Botrytis cinerea* on strawberry fruits. *Mycobiology*,*46*,33–46.

Chessbrough, M.(2000). District laboratory practice in tropical countries. (part 2). UK: Cambridge University Press.

Deb, P., Talukdar, S. A. Mohsina, K. ,Sarker, P. K. & Abu Sayem, S.M. (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *Springerplus*, *2* (*154*), 1-12.

Divakaran, D., Chandra, A. & Chandran R., (2011). Comparative study on production of α– amylase from *Bacillus licheniform* strain. *Brazilian Journal of Microbiology*, *42 (4)*, 1397 – 1404.

Ekka, A. & Namdeo, N. (2018). Screening, isolation and characterization of amylase producing bacteria and optimization for production of amylase. *Journal of Biotechnology and Biochemistry*, 4 (2), 50 – 56.

Gana, N.H.T., Mendoza, B. C.& Monsalud, R. G. (2014). Isolation, screening, and characterization of yeasts with amylolytic, lipolytic, and proteolytic activities from the surface of Philippine bananas (*Musa* spp.) *Philipine Journal of Sciences*, 143, 81–87.

Giraud, E., Brauman, A., Keleke, S., Lelong, B. & Raimbault, M. (1991). Isolation and physiological study of an amylolytic strain of *Lactobacillus plantarum*. *Applied Microbiology and Biotechnology, 36 (3)*, 379-383.

Goyal, N., Gupta, J. K. & Soni, S. K. (2005). A novel raw starch digesting thermostable a amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme Microbial Technology*, 37: 723–734.

Gupta, R. Hiteshi, K. & Didwal, G. (2018). Production, optimization of α–amylase from *Bacillus licheniformis*. *Journal of Advaned Research in Biology and Pharmacy Research*, *1 (5)*, 1 –15.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Hattingh, M., Alexander, A., Meijering, I., Van Reenen, C.A. & Dicks, L.M.T. (2015). Amylolytic strains of *Lactobacillus plantarum* isolated from barley. *African Journal of Biotechnology, 14 (4),* 310-310.

Kumar, S., Stecher, G., Li, M., Knyaz. C. & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution, 35(6*), 1547-1549.

Mathew, C. & Gunathilaka, R. (2015). Production, purification and characterization of a thermostable alkaline serine protease from *Bacillus lichniformis* NMS-1. *International Journal of Biotechnology and Molecular Biology Research, 6,* 19-27.

Nyarko, C., Mills, J., Afortude, J., Kizzie, N., Agbale, C. M. & Nyarko, S. B. (2019). Effect of pH stability on alpha amylase extracted from *Aspergillus niger* on starch from local rice in Ghana. *Asian Journal of Microbiology and Biotechnology*, *4 (1),* 24-34.

Odunfa, S. A. & Adeyele, S. (1985). Microbiological changes during the traditional production of *ogi-baba*, a West African fermented sorghum gruel. *Journal of Cereal Science*, *3*, 173-180.

Olasupo, N. A., Olukoya, D. K. &Odunfa, S. A. (1997). Assessment of a bacteriocin-producing *Lactobacillus* strain in the control of spoilage of a cereal-based African fermented food. *Folia Microbiology. 42(1),* 31–34.

Oliveira, A.P.A., De Silvestre, M. A., Alves-prado, H. F.,Rodrigues, A., Fossa, M., Fonseca, G. G. & Leite, R. S. R. (2015). Bioprospecting of yeasts for amylase production in solid state fermentation and evaluation of the catalytic properties of enzymatic extracts *African Journal of Biotechnology, 14(14),* 1215–1223

Ouédraogo, N., Savadogo, A., Zongo, C., Somda, K. & Traoré, A. (2014). High performance amylolytic yeast strains isolation and identification for valorization of potatoes waste available in Burkina Faso *International Food Research Journal,* 19**(**4), 1463–1469.

Oyewole, O. B. & Odunfa, S. A. (1990). Characterisation and distribution of lactic acid bacteria in cassava fermentation during fufu production. *Journal of Applied Bacteriology*, 68,145-152.

Panda, S., Swain, M., Kar, S., Ray, R. & Montet, D. (2008). Statistical optimization of α-amylase production by probiotic *Lactobacillus plantarum* MTCC 1407 in submerged fermentation. *Polish Journal of Microbiology*, 57 (2), 149-155.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V. T., Singh, D. & Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry, 31*, 135-152.

Prakash, O. & Jaiswal, N.(2010). Alpa amylase: an ideal representative of thermostable enzymes. *Applied Biochemistry and Biotechnology, 160 (8)***,** 2401-2414.

Ruby, U., Serrano, A. & Traifalgar, R. (2012). Effect of pH on amylase, cellulase and protease of the Angelwing clam, *Pholas orientalis*. *European Journal of Experimental Biology, 2 (6),* 2280-2285.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4(4),* 406-425.

Saleem, A. & Ebrahim, M. K. H. (2014). Production of amylase by fungi isolated from legume seeds collectedin Almadinah Almunawwarah, Saudi Arabia. *Journal of Taibah University for Science*, *8*, 90–97.

Shukla, R.J. & Singh, S.P. (2015).Production optimization, purification and characterization of a-amylase from thermophilic *Bacillus licheniformis* TSI-14. *Starch/Stärke, 67,* 629-639.

Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K. & Garg, S. K. (2011). Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic *Pseudomonas putida* SKG-1 isolate. *Microbial Cell Factory, 10*, 114-127

Singh, S.K., Ahmed, S.U. & Pandey, A. (2006). Metabolic engineering approaches for lactic acid production. *Process Biochemistry, 41*, 991-1000.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R. & Pandey, A. (2006). α-amylase from microbial sources- An overview on recent developments *Food Technology and Biotechnology, 44(2),* 173-184

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.

Souza, P. M. D. (2010). Application of microbial a-amylase in industry- a review. *Brazilian Journal Microbiology, 41*, 850-861.

Suman, S. & Ramesh, K. (2010). Production of a thermostable extracellular amylase from thermophilic *Bacillus* species. *Journal of Pharmaceutical Sciences and Research*, *2*, 149–154.

Wakil, S.M. & Daodu, A.A. (2011). Physiological properties of a microbial community in spontaneous fermentation of maize (*Zea mays*) for *ogi. International Journal of Microbiology, 2(3)*, 109-115.

Xie, F., Quan, S., Liu, D., Ma, H., Li, F., Zhou, F. & Chen, G. (2014).Purification and characterization of a novel a-amylase from a newly isolated *Bacillus methylotrophicus* strain P11-2. *Process Biochemistry, 49*, 47-53.

Citation: Ejike and Onyeanula. (2024). Effect of varying temperature, pH and incubation time on amylase production by amylolytic lactic acid bacteria isolated from fermented starchy foods. *International Journal of Microbiology and Applied Sciences*. *3(3)*: 22 – 36.