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Optimization of Lactic Acid Production in Starch-Based Substrates by Lactic Acid Bacteria

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ABSTRACT

This study investigated lactic acid production using starch-based substrates by lactic acid bacteria. Starchy substrates; yam, cassava, corn, potatoes and rice were used for lactic acid fermentation. Isolation of microorganisms was done using de Man, Rogosa, and Sharpe (MRS) medium. Ten pure colonies were identified and classified presumptively using standard morphological and biochemical identification processes. Optimization of culture conditions was done by varying temperature (25ºC, 30ºC, 35ºC and 40ºC), pH (6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0), and fermentation Time (24, 48, 96 and 122hrs). Optimal culture conditions were obtained at 35ºC, pH 6.0 and 122hrs. At optimal conditions, *Lactiplantibacillus pentosus* strain BSR3 (SL2) produced the highest concentration of lactic acid (0.43 ± 0.10 g/L) in yam broth. *Lactiplantibacillus plantarum* strain Z2 (SL1) produced the highest concentration of lactic acid (0.55 ± 0.12 g/L) in cassava broth. In corn broth, the highest quantity of lactic acid (0.55 ± 0.14 g/L) was produced by *Lactiplantibacillus plantarum* strain Z2 (SL1). *Lactiplantibacillus plantarum* strain Z2 (SL1) produced the highest concentration of lactic acid (0.56 ± 0.35g/L) in potato broth. In rice broth, the highest concentration of lactic acid (0.46 ± 0.40 g/L) was produced by *Lactiplantibacillus pentosus* strain BSR3 (C3P). Overall highest concentration of lactic acid (0.56 ± 0.35g/L) was produced by *Lactiplantibacillus plantarum* strain Z2 (SL1) in potato broth. This study shows that fermentation of potatoes, a low-cost substrate by *Lactiplantibacillus plantarum* strain Z2 (SL1) has a high-rate lactic acid production. This bacterium and substrate can be used in large-scale production of lactic acid for many industrial uses.

Keywords: Fermentation, Starchy Substrates, Lactic Acid Bacteria, *Lactiplantibacillus plantarum*, Optimization,.

Introduction

Lactic acid is a versatile organic acid with broad applications across multiple industries, including food, pharmaceuticals, cosmetics, textiles, chemicals, and leather production (Sreenath *et al.*, 2001; Naveena *et al*., 2004; Mariano, 2015; Zhang, 2008; Gao *et al*., 2011). Global production, estimated at 1.5 million metric tons, is projected to grow at a compounded annual rate of 8.2% by 2030 (Ojo and de Smidt, 2023). This increasing demand is largely driven by its use as a food preservative (Aguirre-Garcia *et al*., 2024), its antibacterial and detergent properties in pharmaceuticals and personal care products (Ruiz-Ruiz *et al*., 2017), and its role in producing polylactic acid (PLA), a biodegradable thermoplastic (Ahmad *et al*., 2024).

The efficiency of lactic acid fermentation depends on various factors affecting microbial growth and metabolism. Chemical parameters (pH, nutrient content), physical conditions (temperature, mixing, fermentation time), and biological aspects (biomass) play crucial roles in optimizing production (Comparetti *et al*., 2013).

Additionally, operational factors such as substrate size, inoculum selection, sugar and acid concentrations, and inhibitors in biomass hydrolysates influence yield and efficiency. Among these, temperature and pH are particularly significant, as they impact microbial metabolism, substrate utilization, and overall lactic acid production (Eiteman and Ramalingam, 2015; Rawoof *et al*., 2020).

Fermentation occurs across different temperature ranges—ambient, mesophilic, thermophilic, extreme thermophilic, and hyperthermophilic (Peinemann and Pleissner, 2020). Bacteria exhibit distinct growth characteristics based on temperature classification: psychrophiles thrive between 0°C and 20°C, mesophiles between 15°C and 45°C (e.g., *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus lactis*, and some *Streptococcus* strains) (Sakai *et al.*, 2012; Bernardo *et al.*, 2016; Kurbanoglu and Kurbanoglu, 2003; Pleissner *et al*., 2017), while thermophiles grow optimally between 50°C and 85°C (Todar, 2012).

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Another critical factor in lactic acid fermentation is pH, typically maintained between 4 and 6. As lactic acid accumulates, it lowers the medium's pH, inhibiting microbial growth by affecting cell wall integrity, enzyme activity, and proton motive force (Hofvendahl and Hahn-Hagerdal, 2000; Wee *et al*., 2006). The undissociated form of lactic acid permeates microbial membranes, leading to cytoplasmic acidification, energy depletion, and eventual cell death. Enzymatic activity is also compromised as changes in amino acid ionization destabilize enzyme structures (Puzanov, 1999). To mitigate these effects, *in situ* acid removal during fermentation is necessary to sustain microbial viability and optimize production conditions (Wee *et al*., 2006; Singh *et al.*, 2011).

Fermentation time significantly influences lactic acid yield, as glucose metabolism and bacterial growth occur in distinct phases. During the logarithmic phase, bacterial populations expand rapidly, synthesizing cellular components at varying rates depending on the strain. The efficiency of glucose utilization during fermentation reflects microbial amylase activity and carbohydrate metabolism, making time optimization crucial for maximizing lactic acid production.

The aim of this study is to access the effect various temperatures, pH, and fermentation Time on lactic acid production using starch-based substrates such as yam, cassava, corn, potatoes and rice by lactic acid bacteria. As to determine the appropriate temperature, pH, and fermentation Time for the optimal production of lactic acid.

Materials and Methods

Microbial Sample Collection

Microbial samples were obtained from soils of six cassava processing plants located in Ohuhu, Umuahia North Local Government Area, Abia State, Nigeria. To ensure consistency, only cassava processing plants that had been operational for at least five years were selected.

Soil samples were specifically collected from areas that exhibited the highest spillage of grated cassava and from a depth of 10–12 cm using a sterile trowel, following the removal of surface debris. Triplicate soil samples were obtained from different locations in each cassava processing plant.

These samples were placed in sterile containers and transported to the laboratory within two hours for further analysis. Serial dilutions were prepared, and 1.0 mL of the fourth dilution (10^{-4}) was used for microbial inoculation onto de Man, Rogosa, and Sharpe (MRS) medium to facilitate lactic acid bacteria (LAB) isolation.

Isolation and Characterization of Lactic Acid Bacteria (LAB)

LAB isolation was performed following the method described by Abd *et al.* (2010). The fourth dilution (10^{-4}) of the microbial soil samples was spread onto MRS agar plates and incubated under anaerobic conditions at 37°C for 48 hours. After incubation, distinct colonies were randomly selected and subcultured through repeated streaking on fresh MRS agar plates to obtain pure isolates. These pure LAB isolates were maintained on MRS agar slants and in broth cultures (in duplicates) at 4°C until further analysis.

Preliminary characterization of the isolates involved cell morphology, Gram staining, catalase, coagulase, citrate, and motility tests, along with sugar fermentation and growth tolerance in 4% and 6.5% NaCl. Only Gram-positive, non-motile, catalase-negative, coagulase-negative, and citrate-negative isolates were selected for further analysis, as per Karnwal *et al.* (2016). The isolates' sugar fermentation capability was assessed using Durham tubes containing sugar broth.

Molecular identification of the isolates was conducted by sequencing the 16S rRNA gene and performing BLAST searches to compare sequences with reference strains.

Substrate Collection, Treatment and Processing

Five starch-based raw substrates—yam, cassava, corn, potatoes, and rice—were purchased from Nkwoegwu Market in Ohuhu, Umuahia North Local Government Area, Abia State, Nigeria.

The treatment and processing of starch-based substrates were carried out following the methods described by Vishnu *et al*. (2002), Wakil and Ajayi (2013), and Odunfa and Adeyele (1985). Cereal grains were manually sorted to remove stones, debris, and defective seeds. The grains were then ground using a blender, sieved through a 150 µm mesh sieve, and used in fermentation experiments. For root-based substrates (cassava, yam, and potatoes), the samples were peeled, washed with distilled water, and cut into smaller portions.

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These pieces were blended and placed in sterile muslin cloths, securely tied, and left to drain in a funnel. These raw materials were considered as unprocessed or crude starch substrates. Starch solutions were prepared by dissolving 10 g of each substrate in 100 mL of distilled water.

Growth and Lactic Acid Production by LAB Isolates

The synthetic medium, starch-based solutions, culture media, and inoculum were prepared following the methods of Mudaliyar and Kulkarni (2011), Mudaliyar *et al*. (2012), and Cheng *et al.* (1991). Each lactic acid bacterium isolate was inoculated into the culture medium using a 5% inoculum, as described by Karnwal *et al*. (2016). This procedure was repeated for each LAB isolate and starch solution.

Molecular Identification of Lactic Acid Bacteria

DNA Extraction

Genomic DNA was extracted from LAB isolates using Zymo-Spin™ kits, following the manufacturer's instructions. The extracted DNA was separated by electrophoresis on a 1% agarose gel.

PCR Analysis

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1429R (5′-TACGGCTACCTTGTTACGAC-3′). PCR was conducted in a thermal cycler (T Gradient model, Biometra, Germany) under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 1 min, with a final extension at 68°C for 10 min. PCR amplicons were maintained at 4°C and visualized using EZvision® Bluelight DNA Dye on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd).

Sequencing and BLAST Analysis

PCR products were purified using the ExoSAP protocol and sequenced with the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Catalogue No. D4053) using an ABI 3500XL Genetic Analyzer at Inqaba Biotec, South Africa. The 16S rRNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI platform to determine similarity with known type strains.

Phylogenetic Analysis

Sequences were aligned using ClustalW, and phylogenetic relationships were inferred using the Neighbor-Joining method. Evolutionary distances were computed using the Maximum Composite Likelihood method, with ambiguous positions removed through pairwise deletion. Phylogenetic analyses were conducted using MEGA X software (Saitou & Nei, 1987; Kumar *et al*., 2018).

Effect of Temperature on Lactic Acid Production

A sterilized culture medium was inoculated with LAB isolates and incubated at pH 6.0 for 24 hours. The cultures were then incubated at varying temperatures (25°C, 30°C, 35°C, and 40°C). After 24 hours, supernatants were collected following the methods of Karnwal *et al*. (2016) and Vishnu *et al*. (2000), and lactic acid production was quantified using the total titratable acidity (TTA) method.

Effect of pH on Lactic Acid Production

To evaluate the influence of pH on lactic acid production, the culture medium was adjusted to pH values of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 using sterile 1 M NaOH. The medium was then inoculated and incubated at 35°C for 24 hours. Lactic acid yield was estimated following the protocol of Karnwal *et al*. (2016) and Vishnu *et al*. (2000).

Effect of Fermentation Time on Lactic Acid Production

LAB isolates were incubated at pH 6.0 and 35°C for different time durations (24, 48, 96, and 122 hours) to determine the effect of fermentation time on lactic acid production. The amount of lactic acid produced was quantified following Karnwal *et al.* (2016) and Vishnu *et al.* (2000).

Lactic Acid Estimation

Lactic acid production was quantified using the TTA method as described by A.O.A.C. (1990) and Parimala and Muthusamy (2017). In this method, 50 mL of fermented broth was centrifuged at 10,000 rpm for 5 minutes to pellet bacterial cells. A 25 mL aliquot of the supernatant (crude lactic acid) was transferred into a 100 mL flask, and three drops of phenolphthalein indicator were added. The solution was titrated with 0.1 M NaOH from a burette until the first appearance of a pink colour. Each mL of 0.1 M NaOH corresponded to 90.08 mg of lactic acid.

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The final lactic acid concentration was calculated using the standard formula for total titratable acidity (TTA).

Total titratable acidity of lactic acid (g/L) =

$$\frac{\text{ml NaOH} \times N\text{NaOH} \times \text{MW Lactic acid} \times 100}{\text{Volume of sample used} \times 1000}$$

where: N = Molarity of NaOH
ml = Volume of NaOH (titrant).
MW = Molecular weight of lactic acid (90.08g/mol).

The lactic acid was calculated and expressed as g of lactic acid L^{-1} of culture medium (gL^{-1}).

Statistical Analysis

All experiments were conducted in triplicate, and data were analyzed using IBM SPSS Statistics 25. Descriptive statistics, including mean and standard deviation, were used to summarize the data. One-way analysis of variance (ANOVA) was performed to compare the means of different parameters. Duncan's Multiple Range Test was applied as a post hoc analysis to determine significant differences between groups. Statistical significance was established at $p < 0.05$, with values presented as means ± standard deviation. Means within the same column that bore different superscripts were considered significantly different at $p < 0.05$.

Results

The morphological, biochemical, and sugar fermentation tests, as presented in Table 1, confirmed the presence of seven (*Lactobacillus* spp.), one (*Lactococcus* sp.), one (*Leuconostoc* sp.), and one (*Streptococcus* sp.) among the isolates.

The effect of temperature on lactic acid production is summarized in Table 2. Statistical analysis indicated that variations in temperature did not result in significant differences ($p > 0.05$) in lactic acid production among the tested lactic acid bacteria. The tested temperature range fell within the optimal incubation range for mesophilic lactic acid bacteria. Based on these findings, a temperature of 35°C was selected for further studies, as it aligns with previous research on lactic acid production by *Lactobacillus* spp. and other lactic acid bacteria.

The effect of pH variation on lactic acid production by lactic acid bacteria is presented in Table 3. When fermentation was conducted at pH 6.0, the highest lactic acid concentration (0.78 ± 0.13 g/L) was produced by *Lactobacillus* sp. (SL1). At pH 6.5, the highest lactic acid concentration (0.75 ± 0.20 g/L) was observed in *Lactobacillus* sp. (SL2). When the pH was adjusted to 7.0, *Lactobacillus* sp. (SL5) exhibited the highest lactic acid production (0.71 ± 0.20 g/L).

At pH 7.5, the highest lactic acid concentration (0.69 ± 0.14 g/L) was produced by *Leuconostoc* sp. (SL7). When the pH was increased to 8.0, *Lactobacillus* sp. (C3P) demonstrated the highest lactic acid yield (0.60 ± 0.02 g/L). At pH 8.5, *Lactobacillus* sp. (SL5) again recorded the highest production (0.54 ± 0.24 g/L). At pH 9.0, *Lactobacillus* sp. (SL6) produced the highest lactic acid concentration (0.69 ± 0.15 g/L).

Statistical analysis revealed that the effect of pH on lactic acid production was significant ($p < 0.05$) at pH 6.0, whereas variations in lactic acid production at other pH values were not statistically significant. Consequently, pH 6.0 was determined to be the optimal pH and was maintained in subsequent experiments.

The impact of fermentation duration on lactic acid production is presented in Table 4, indicating a progressive increase in lactic acid concentration over time. At 24 hours, all *lactic acid bacteria* exhibited varying fermentation capacities, with *Lactobacillus* sp. (C3P) producing the highest lactic acid concentration (3.41 ± 0.37 g/L). By 48 hours, lactic acid production increased in all bacterial strains except *Lactobacillus* sp. (SL1). The highest lactic acid concentration at this time point was recorded in *Lactobacillus* sp. (SL5) (4.24 ± 0.40 g/L).

At 96 hours, all strains demonstrated further increases in lactic acid production, with *Lactobacillus* sp. (SL5) yielding the highest concentration (6.31 ± 3.48 g/L). By 122 hours, peak lactic acid concentrations were attained by most of the strains. Since the highest lactic acid concentration was observed at 122 hours, this fermentation time was determined to be optimal and was selected for further investigations.

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Table 1: Cultural, morphological, biochemical characteristics and probable identity of lactic acid bacteria isolated from cassava processing plant soil.

Cultural Morphology			Microscopy		Biochemical							Sugar Fermentation							Probable Organism
LAB Isolate Code	**Color**	**Cell Occurrence**	**Gram Reaction**	**Cell Shape**	**Catalase**	**Motility**	**Coagulase**	**Citrate**	**Gas Production**	**Growth at 4% NaCl**	**Growth at 6.5% NaCl**	**Glucose**	**Mannitol**	**Fructose**	**Galactose**	**Lactose**	**Maltose**	**Mannose**	
SL1		Single	+	Rounded ends	-	-	-	-	-	+	+	+	+	+	+	+	-	+	*Lactobacillus* sp.
SL2	Milky	Single, pair.	+	Rounded ends	-	-	-	-	-	+	-	+	+	-	+	+	+	+	*Lactobacillus* sp.
SL3	Creamy	Mucoid	+		-	-	-	-	-	+	-	+	+	-	+	+	+	+	*Lactobacillus* sp.
SL4	Creamy		+	circular	-	-	-	-	-	-	+	+	+	+	+	+	+	+	*Lactococcus* sp.
SL5		Pair, short chain.	+	Straight, rounded	-	-	-	-	-	+	-	+	+	+	+	+	+	+	*Lactobacillus* sp.
SL6	Creamy	Short-chain.	+	circular, smooth.	-	-	-	-	-	+	+	+	+	+	+	+	+	+	*Lactobacillus* sp.
SL7	milky	slimy	+	circular	-	-	-	-	-	+	+	+	+	+	+	+	+	+	*Leuconostoc* sp.
SL8	milky	slimy	+	circular	-	-	-	-	-	+	-	+	+	-	+	+	+	+	*Streptococcus* sp.
C3P		Single, pair.	+	rounded	-	-	-	-	-	+	-	+	+	+	+	+	+	+	*Lactobacillus* sp.
C2Y		Short-chains.	+	Straight, rounded ends.	-	-	-	-	-	+	-	+	+	+	+	+	+	+	*Lactobacillus* sp.

Key: LAB = Lactic acid bacteria

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Table 2: Effect of temperature on lactic acid production (g/mL) by lactic acid bacteria (LAB) isolates

LAB Isolate Code	Temperature (ºC) of lactic acid production (g/mL)			
	25ºC	30ºC	35ºC	40ºC
SL1	0.33 ± 0.27^{a}	0.34 ± 0.12^{b}	0.44 ± 0.28^{ab}	0.37 ± 0.02^{a}
SL2	0.53 ± 0.26^{a}	0.47 ± 0.20^{ab}	0.29 ± 0.10^{b}	0.65 ± 0.16^{a}
SL3	0.38 ± 0.17^{a}	0.48 ± 0.16^{ab}	0.24 ± 0.06^{b}	0.60 ± 0.27^{a}
SL4	0.43 ± 0.19^{a}	0.42 ± 0.06^{ab}	0.21 ± 0.10^{b}	0.48 ± 0.33^{a}
SL5	0.39 ± 0.22^{a}	0.45 ± 0.14^{ab}	0.75 ± 0.13^{a}	0.50 ± 0.37^{a}
SL6	0.27 ± 0.03^{a}	0.70 ± 0.12^{a}	0.37 ± 0.19^{ab}	0.31 ± 0.25^{a}
SL7	0.25 ± 0.23^{a}	0.45 ± 0.16^{ab}	0.37 ± 0.09^{ab}	0.50 ± 0.33^{a}
SL8	0.46 ± 0.33^{a}	0.60 ± 0.21^{ab}	0.39 ± 0.26^{ab}	0.32 ± 0.19^{a}
C3P	0.39 ± 0.27^{a}	0.28 ± 0.08^{b}	0.39 ± 0.32^{ab}	0.55 ± 0.36^{a}
C2Y	0.30 ± 0.34^{a}	0.40 ± 0.31^{ab}	0.48 ± 0.32^{ab}	0.47 ± 0.30^{a}

Values presented are means of triplicates ± standard deviation. Means with same superscript across a column are non-significant ($p < 0.05$). NS = Not significant, S = Significant.

Table 3: Effect of pH on lactic acid production (g/L) by the lactic acid bacteria (LAB) isolates

LAB Isolate Code	pH of lactic acid production (g/mL)						
	6.0	6.5	7.0	7.5	8.0	8.5	9.0
SL1	0.78 ± 0.13^{a}	0.59 ± 007^{ab}	0.42 ± 0.10^{ab}	0.50 ± 0.11^{ab}	0.40 ± 0.17^{a}	0.21 ± 0.08^{b}	0.40 ± 0.10^{ab}
SL2	0.39 ± 0.17^{bcde}	0.75 ± 0.20^{a}	0.52 ± 0.19^{ab}	0.32 ± 0.15^{b}	0.34 ± 0.09^{a}	0.37 ± 0.20^{ab}	0.56 ± 0.22^{ab}
SL3	0.22 ± 0.03^{e}	0.47 ± 0.16^{ab}	0.55 ± 0.17^{ab}	0.36 ± 0.08^{b}	0.38 ± 0.16^{a}	0.40 ± 0.18^{ab}	0.37 ± 0.15^{ab}
SL4	0.37 ± 0.15^{cde}	0.34 ± 0.15^{b}	0.30 ± 0.17^{b}	0.23 ± 0.15^{b}	0.57 ± 0.29^{a}	0.28 ± 0.20^{ab}	0.50 ± 0.36^{ab}
SL5	0.23 ± 0.07^{de}	0.36 ± 0.13^{b}	0.71 ± 0.20^{a}	0.50 ± 0.17^{ab}	0.52 ± 0.20^{a}	0.54 ± 0.24^{a}	0.30 ± 0.01^{b}
SL6	0.31 ± 0.08^{cde}	0.61 ± 0.26^{ab}	0.54 ± 0.33^{ab}	0.39 ± 0.04^{b}	0.34 ± 0.12^{a}	0.37 ± 0.05^{ab}	0.69 ± 0.15^{a}
SL7	0.49 ± 0.22^{bcd}	0.46 ± 0.30^{ab}	0.43 ± 0.28^{ab}	0.69 ± 0.14^{a}	0.38 ± 0.05^{a}	0.28 ± 0.08^{ab}	0.64 ± 0.19^{ab}
SL8	0.65 ± 0.13^{ab}	0.59 ± 0.08^{ab}	0.44 ± 0.06^{ab}	0.34 ± 0.16^{b}	0.30 ± 0.18^{a}	0.51 ± 0.22^{ab}	0.54 ± 0.12^{ab}
C3P	0.51 ± 0.19^{bc}	0.34 ± 0.16^{b}	0.29 ± 0.16^{b}	0.46 ± 0.32^{ab}	0.60 ± 0.02^{a}	0.34 ± 0.11^{ab}	0.40 ± 0.29^{ab}
CYY	$0.77 \pm .11^{a}$	0.74 ± 0.09^{a}	0.54 ± 0.03^{ab}	0.47 ± 0.06^{ab}	0.51 ± 0.04^{a}	0.47 ± 0.06^{ab}	0.47 ± 0.07^{ab}

Values presented are means of triplicates ± standard deviation. Means with different superscript across a column are significantly different ($p < 0.05$). S = Significant, NS= Not significant.

Table 4: Effect of fermentation Time on lactic acid production (g/L) by bacterial isolates

LAB Isolate Code	Fermentation Time (hr) of lactic acid production (g/mL)			
	24 hrs	48 hrs	96 hrs	122 hrs
SL1	3.05 ± 0.23^{ab}	2.82 ± 0.31^{b}	4.68 ± 1.17^{a}	6.05 ± 1.48^{ab}
SL2	2.06 ± 0.58^{ab}	3.18 ± 0.63^{ab}	4.53 ± 1.66^{a}	5.36 ± 1.54^{ab}
SL3	3.05 ± 1.11^{ab}	2.91 ± 0.66^{b}	4.68 ± 0.59^{a}	7.11 ± 1.35^{ab}
SL4	2.14 ± 0.11^{ab}	2.96 ± 0.76^{ab}	4.54 ± 0.55^{a}	7.36 ± 2.75^{ab}
SL5	2.92 ± 0.14^{ab}	4.24 ± 0.40^{a}	6.31 ± 3.48^{a}	8.01 ± 1.80^{a}
SL6	3.12 ± 0.74^{ab}	3.04 ± 0.82^{ab}	5.45 ± 3.02^{a}	4.42 ± 0.51^{b}
SL7	2.09 ± 0.42^{b}	3.64 ± 0.27^{ab}	5.46 ± 1.60^{a}	5.63 ± 1.74^{ab}
SL8	2.79 ± 0.61^{ab}	3.13 ± 0.97^{ab}	4.79 ± 0.66^{a}	6.12 ± 2.72^{ab}
C3Y	3.41 ± 0.37^{a}	3.70 ± 0.67^{ab}	6.25 ± 2.03^{a}	5.17 ± 0.91^{ab}
C2Y	2.73 ± 0.29^{ab}	2.91 ± 0.84^{b}	6.13 ± 2.02^{a}	7.27 ± 1.09^{ab}

Values presented are means of triplicates ± standard deviation. Means with different superscript across a column are significantly different ($p < 0.05$). NS = Not significant.

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Plate 1 shows the result of the Molecular analysis of the four (4) lactic acid bacteria isolates (SL1, SL2, C3P and C2Y) were selected for further studies haven showed best production of lactic acid under the three-factor growth parameters for the optimization processes. Molecular characterization of the lactic acid bacteria species was done based on genotypic characteristics (16S rRNA gene sequences similarity with the type strains) during BLAST searches and identified as *Lactiplantibacillus plantarum* strain FM02 (C2Y), *Lactiplantibacillus pentosus* strain BSR3 (C3P), *Lactiplantibacillus plantarum* strain Z2 (SL1) and *Lactiplantibacillus pentosus* strain BSR3 (SL2).

The Phylogenetic tree showing the evolutionary distance between the bacterial isolates is presented in Figure 1.

The Lactic acid bacteria isolates and geneBank relatives showing % relatedness and accession numbers are presented in Table 5.

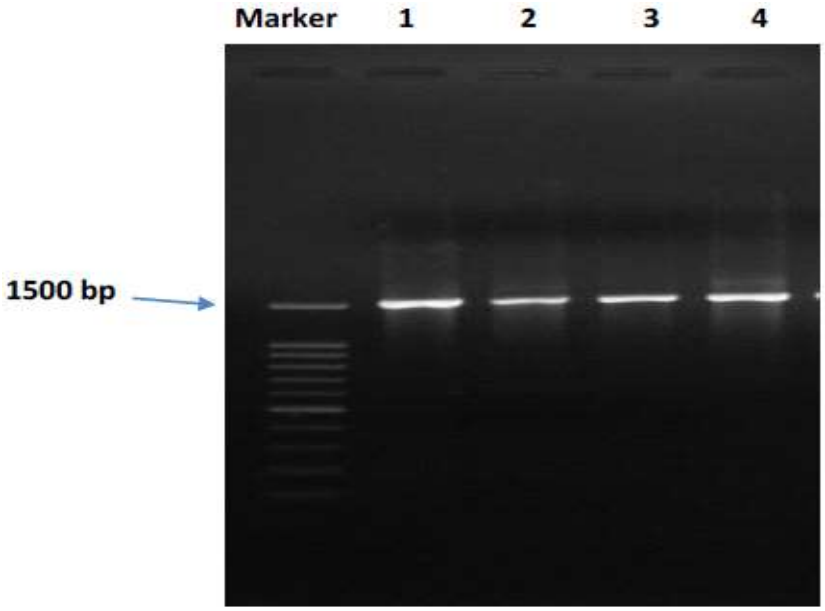

Plate 1: Gel electrophoresis of the Marker and 16S rRNA of the bacteria isolates showing bands at 1500bp
Lane 1= *Lactiplantibacillus plantarum* strain FM02 (C2Y), Lane 2= *Lactiplantibacillus pentosus* strain BSR3 (C3P), Lane 3= *Lactiplantibacillus plantarum* strain Z2 (SL1) and Lane 4= *Lactiplantibacillus pentosus* strain BSR3 (SL2).

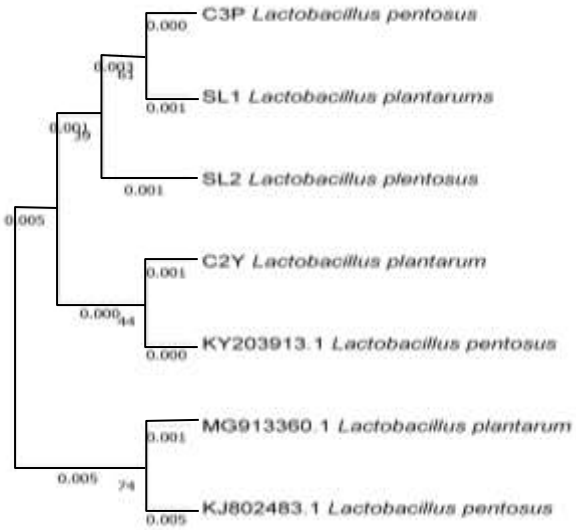

Figure 1: Phylogenetic tree showing the evolutionary distance between the bacterial isolates

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Table 5: Lactic acid Bacteria Isolates and GeneBank Relatives Showing % Relatedness and Accession Numbers

S/N	Seq ID	GeneBank Relative	% Relatedness	Accession number
1	C2Y	*Lactiplantibacillus plantarum* strain FM02	97.54%	MG913360.1
2	C3P	*Lactiplantibacillus pentosus* strain BSR3	100.00%	KY203913.1
3	SL1	*Lactiplantibacillus plantarum* strain Z2	99.86%	ON063304.1
4	SL2	*Lactiplantibacillus pentosus* strain BSR3	99.86%	KY203913.1

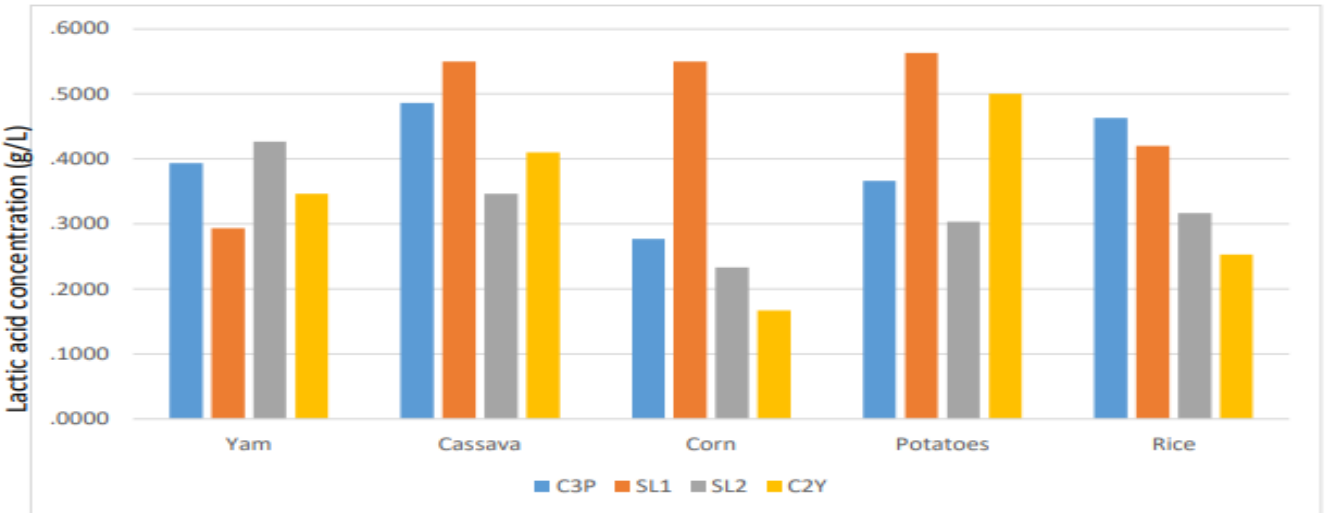

Figure 2: Lactic acid production (g/L) by the identified lactic acid bacteria
C3P = *Lactiplantibacillus pentosus* strain BSR3; SL1 = *Lactiplantibacillus plantarum* strain Z2;
SL2 = *Lactiplantibacillus pentosus* strain BSR3**;** C2Y = *Lactiplantibacillus plantarum* strain FM02.

The lactic acid concentrations produced by *Lactiplantibacillus* strains from different starch-based substrates are presented in Figure 3. From yam broth, the highest lactic acid concentration (0.43 ± 0.10 g/L) was produced by *Lactiplantibacillus pentosus* strain BSR3 (SL2), while *Lactiplantibacillus plantarum* strain Z2 produced the lowest concentration (0.29 ± 0.25 g/L).

In cassava broth, *Lactiplantibacillus plantarum* strain Z2 produced the highest lactic acid concentration (0.55 ± 0.12 g/L), whereas *Lactiplantibacillus pentosus* strain BSR3 (SL2) had the lowest (0.35 ± 0.24 g/L).

For corn broth, *Lactiplantibacillus plantarum* strain Z2 produced the highest concentration (0.55 ± 0.14 g/L), while *Lactiplantibacillus plantarum* strain FM02 recorded the lowest (0.17 ± 0.06 g/L).

In potato broth, the highest lactic acid concentration (0.56 ± 0.35 g/L) was produced by *Lactiplantibacillus plantarum* strain Z2, with the lowest (0.30 ± 0.11 g/L) produced by *Lactiplantibacillus pentosus* strain BSR3 (SL2).

Using rice broth, *Lactiplantibacillus pentosus* strain BSR3 (C3P) produced the highest concentration (0.46 ± 0.40 g/L), while *Lactiplantibacillus plantarum* strain FM02 had the lowest (0.25 ± 0.21 g/L). Overall, the highest lactic acid concentration (0.56 ± 0.35 g/L) was produced by *Lactiplantibacillus plantarum* strain Z2 from potato broth.

Statistical analysis revealed that only corn broth fermentations were statistically significant ($p = 0.010$, $p < 0.05$). Fermentations using yam, cassava, potato, and rice broths yielded p-values greater than 0.05, indicating that results from these substrates were statistically non-significant.

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Discussion

This study successfully isolated, characterized, and screened lactic acid bacteria (LAB) from starch-based substrates following standard procedures, consistent with methods reported in previous investigations on lactic acid production (Karnwal *et al*., 2016; Vishnu *et al*., 2000; Wakil and Ajayi, 2013; Ray *et al*., 2009; Maheshwaran and Palaniswamy, 2017; Mudaliyar and Kulkarni, 2011). The optimization of growth parameters for enhanced lactic acid production closely aligned with the findings of these researchers, further validating the experimental protocols employed.

The optimized incubation temperature for the selected LAB strains agreed with previously reported temperature ranges for optimal lactic acid production by mesophilic bacteria (Ray *et al*., 2009; Karnwal *et al*., 2016; Pleissner *et al*., 2017; Liang *et al*., 2014). Temperature plays a critical role in microbial metabolism, and the selected temperature was suitable for the growth and enzymatic activity of the LAB strains evaluated.

The role of pH in lactic acid fermentation is fundamental, as it directly affects microbial growth, metabolism, and product yield. The pH value selected for optimal lactic acid production in this study fell within the acidic range, consistent with values reported by other researchers (Bernardo *et al*., 2016; Karnwal *et al*., 2016; Maheshwaran and Palaniswamy, 2017; Ray *et al*., 2009; Yang *et al*., 2006). The agreement between these results and prior studies underscores the importance of maintaining appropriate pH during fermentation for enhanced lactic acid production.

Lactic acid production gradually increased over time, with the highest yield recorded at 122 hours of fermentation. This result is consistent with the findings of Wakil and Ajayi (2013), Ray *et al*. (2009), Karnwal *et al*. (2016), and Mudaliyar and Kulkarni (2011), who reported optimal fermentation durations ranging from 4 to 7 days. The results emphasize the importance of prolonged incubation for maximizing acid production from starch-based substrates.

The lactic four LAB strains characterized in this study—*Lactiplantibacillus pentosus* strain BSR3 (C3P, SL2), *Lactiplantibacillus plantarum* strain Z2 (SL1), and *Lactiplantibacillus plantarum* strain FM02 (C2Y)—were identified based on morphological, physiological, biochemical, and genotypic characteristics, including 16S rRNA gene sequence analysis (Plate 1, Figure 1 and Table 5). The identification of *Lactiplantibacillus plantarum* strains is consistent with findings by Ray *et al*. (2009), Coelho *et al*. (2011), and Saavedra *et al*. (2021). Additionally, the production of lactic acid by *Lactiplantibacillus pentosus* strains aligns with reports from Garde *et al*. (2002), Tabacof *et al*. (2023), and Gonzalez-Leos *et al*. (2019).

The lactic acid production performance of the LAB strains varied across the different starch-based substrates. From yam broth, the highest lactic acid concentration (0.43 ± 0.10 g/L) was produced by *Lactiplantibacillus pentosus* strain BSR3 (SL2), while the highest yield (2.06 g/g) was achieved by *Lactiplantibacillus plantarum* strain FM02 (C2Y). During cassava broth fermentation, *Lactiplantibacillus plantarum* strain Z2 (SL1) produced the highest concentration (0.55 ± 0.12 g/L), whereas the highest yield (1.11 g/g) was recorded by *Lactiplantibacillus plantarum* strain FM02 (C2Y). For corn broth fermentation, *Lactiplantibacillus plantarum* strain Z2 (SL1) produced both the highest lactic acid concentration (0.55 ± 0.14 g/L) and the highest yield (0.85 g/g). During potato broth fermentation, *Lactiplantibacillus plantarum* strain Z2 (SL1) produced the highest lactic acid concentration (0.56 ± 0.35 g/L) and yield (1.56 g/g). In rice broth, *Lactiplantibacillus pentosus* strain BSR3 (C3P) produced both the highest lactic acid concentration (0.46 ± 0.40 g/L) and the highest yield (1.05 g/g).

Among all the substrates, the highest overall lactic acid concentration (0.56 ± 0.35 g/L) was produced by *Lactiplantibacillus plantarum* strain Z2 (SL1) from potato broth fermentation, while the highest yield (2.06 g/g) was obtained from yam broth fermentation by *Lactiplantibacillus plantarum* strain FM02 (C2Y). These results highlight the strain-specific and substrate-dependent nature of lactic acid production.

Conclusion

This study demonstrates the efficiency of different *Lactiplantibacillus* strains in lactic acid production from various starch-based substrates, with *Lactiplantibacillus plantarum* strain Z2 (SL1) and *Lactiplantibacillus plantarum* strain FM02 (C2Y) exhibiting superior production performance.

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The findings align with prior research, reinforcing the significance of strain selection and substrate type in optimizing lactic acid production. The study provides valuable insights into the potential of starch-based substrates for sustainable lactic acid production in industrial applications.

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