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Lactic acid yield by mono/mixed *Lactobacilli* starter culture during cassava fermentation: a comparative study

Kubrat Abiola Oyinlola[1*] and Anthony Abiodun Onilude[2]
[1]Department of Biological Sciences, Faculty of Applied Sciences, KolaDaisi University, Ibadan, Nigeria
[2]Department of Microbiology, Faculty of Science, University of Ibadan, Nigeria
kubrat.oyinlola@koladaisiuniversity.edu.ng

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Abstract

Lactic acid, generally regarded as safe, is important for numerous industrial applications. Projected global increase (15.5%) in demand between 2014-2020 puts expected yield to 1960.1 kilo tons. High production cost hinders large scale application. Although, increase in yield was linked to temperature, fermentation time, and substrate, selection of suitable microbial strains for fermentation is also essential. This study compares lactate yield by utilizing single and mixed Lactobacilli starter cultures. Lactobacillus plantarum F2C, L. plantarum U2A and L. plantarum U2C were used as starter cultures (singly and randomly-combined) to ferment cassava for 72hours. High Performance Liquid Chromatography technique was used to quantify lactic acid. Data were subjected to statistical analysis at 5% level of probability. Fermentation with single and combined cultures had lactate quantities ranging between 4.08mg/mL-5.59mg/mL and 5.50mg/mL-6.91mg/mL respectively. Spontaneously fermented batch had overall least lactic acid (3.98mg/mL) and the highest (6.91g/mL) was produced by consortium of the three starters. Quantities produced were significantly unequal at 5% probability level. Mixed cultures produced more lactic acid than single starter cultures. Furthermore, the spontaneous fermentation produced the least quantity. Therefore, consortium of starter cultures could be employed to improve the yield of lactic acid during fermentation.

Keywords: Lactate, starter culture, fermentation metabolite, *Lactobacillus*, Co- culture.

Introduction

Fermentation, as a means of producing lactic acid in the industry, dated back to1880[1], having two isomers, the L (+) and the D (-) form. L (+) is the most important to humans since we have L (+) lactate dehydrogenase to metabolise it[2]. It has a diverse range of potential use in food (as additives and preservatives), cosmetics, pharmaceuticals, leather and textile production[1,2]. There was a projected increase in its global consumption rapidly over the years[3]. In 2012, worldwide demand was between 130 000 to 150 000 tons yearly[4], even though, a forecast of over 1 million ton annual production by 2020 was presented in 2010[5]. Moreso, a more recent projected 15.5% increase in demand between 2014 and 2020 puts the expected yield at 1960.1 kilo tons.

Chemical synthesis or microbial fermentation are the two methods by which lactate can be produced. However, 95% of the lactate produced in the industries was through microbial fermentation[6], since it was proven to be advantageous than chemically synthesized lactic acid which produces a raceme mixture[4]. Therefore, its occurrence in fermented products could be said to be due to hydrolytic breakdown of molecules, microbial metabolic activities.

The most commonly used organisms in fermentation processes are the Lactic Acid Bacteria (LAB). They produce significant amount of lactate from carbohydrates even though, certain fungi such as *Rhizopus* sp. (*Rhizopus arrhizus* and *R. oryzae)* are widely accepted lactic acid producers[5,7]. However, the former produced higher quantities. Strains of *Escherichia, Bacillus*, *Kluyveromyces* and *Saccharomyces have also been reported*[8]. One of the most desirable effects of LAB in fermentation is acidification, which is usually as a result of carbohydrate and pH reduction when lactate is produced in the fermentation medium[9]. Quantification of organic acids have therefore, been continuously used as an index of microbial metabolic activities and classification of fermented foods[10-12]. Quantification of lactate in fermented foods profer insight into chemical and nutritional properties, sensory factors and microbial population in such food product. Some of the methods employed include enzymatic assay[13], High Performance Liquid Chromatography[14] and biosensor[15] analyses.

Lactate production from probiotic bacteria[9], its preservative/ antimicrobial property[16], its importance as additives/ flavour agents[17], production optimization[18] were among the numerous studies conducted on lactic acid but due to its growing demand (5%-8% estimated yearly growth[19]) for a wide range of applications, high production rate which had been attributed to temperature, fermentation time and substrate level[18] is essential, however, the selection of suitable microbial strains in the process of fermentation was also emphasized[20]. The advantages of mixed culture system reported[21] and more recently

reviewed[22,23] indicated its high potential for industrial applications since most processes still depend on the mono culture system. This study therefore aimed at comparing lactic acid yield using mono and mixed *Lactobacilli* strains.

Materials and methods

Starter culture: *Lactobacillus plantarum F2C* (Accerssion number KJ778117), *L. plantarum* U2A (Accerssion number KJ778118) and *L. plantarum* U2C (KJ778119) isolated from a cassava fermentation process and identified genotypically were used as starter cultures.

Starter cultivation: The LABs were cultivated on sterile de Man Rogosa and Sharpe (MRS) broth (LABM, UK) and incubated at 30^{o}C for 48 hours. 1mL of broth cultures were introduced into a fresh broth and incubated for 24hours. The broth culture was centrifuged (Himac CR21GII, Japan) at 5000rpm for 10 minutes. The pellet was washed with sterile distilled water after the supernatant was decanted. This was re-centrifuged before being introduced in sterile normal saline. The pellet was diluted with sterile normal saline to McFarland standard (No 4) to give 0.669 optical density at 600nm (Spectrophotometer, Cecil CE 1011, Cambridge, England), resulting in approximate cell density of 1.2×10^{9} cfu/mL. 5mL of the resultant diluents were used as inoculum both singly and randomly combined.

Fermentation with starters: Cassava tubers were cut into small pieces of about 3-5cm. 200g was weighed, washed in sterile distilled water and surface sterilized using 1% Sodium hypochloride for one minute, then in 70% alcohol[24]. The cassava pieces were rinsed again with sterile distilled water. 5mL of each starter was used as inoculum in all cases either singly or mixed to inoculate 200g of the cassava soaked in 2L sterile distilled water and fermentation was allowed for 72hours.

Quantification of lactic acid produced: Samples for analysis were taken after 72-hour fermentation. Centrifugation was done at 10 000rpm for 10minutes at 4°C. Resulting supernatant was diluted using 50mM KH_2PO_4 buffer with 5% acetonitrile and 37% hydrochloric acid in the following ratio (sample volume: buffer: HCl = 0.1:0.8:0.1mL). This was then filtered through a 0.2μm syringe filter.

High performance liquid chromatography (HPLC) method[25], with slight modifications was used. Lactic acid quantities were measured by HPLC (CECIL CE4200, Cambridge England) machine, equipped with a C18 column (Ultra Aqueous, 5μm, 150mm x 4.6 mm, Restek). Mobile phase used was 50mM KH_2PO_4 buffer with 5% acetonitrile (pH 2.5 adjusted by 37% HCl) at a flow rate 1.0mL/minute. 20μL sample was introducedfor analysis with UV detection at 210nm. The analysis was externally calibrated using lactic acid standard solution (Sigma Aldrich, Germany) as prepared for the samples. Lactate peak regions were identified on the chromatograms (Figure-2-7) and evaluated by comparison to lactate standard curve (Figure-1) and the quantity of lactic acid produced was calculated.

Data analysis: Data obtained were subjected to statistical analysis (SPSS) at 5% level of significance and presented as average of replicates ±SD.

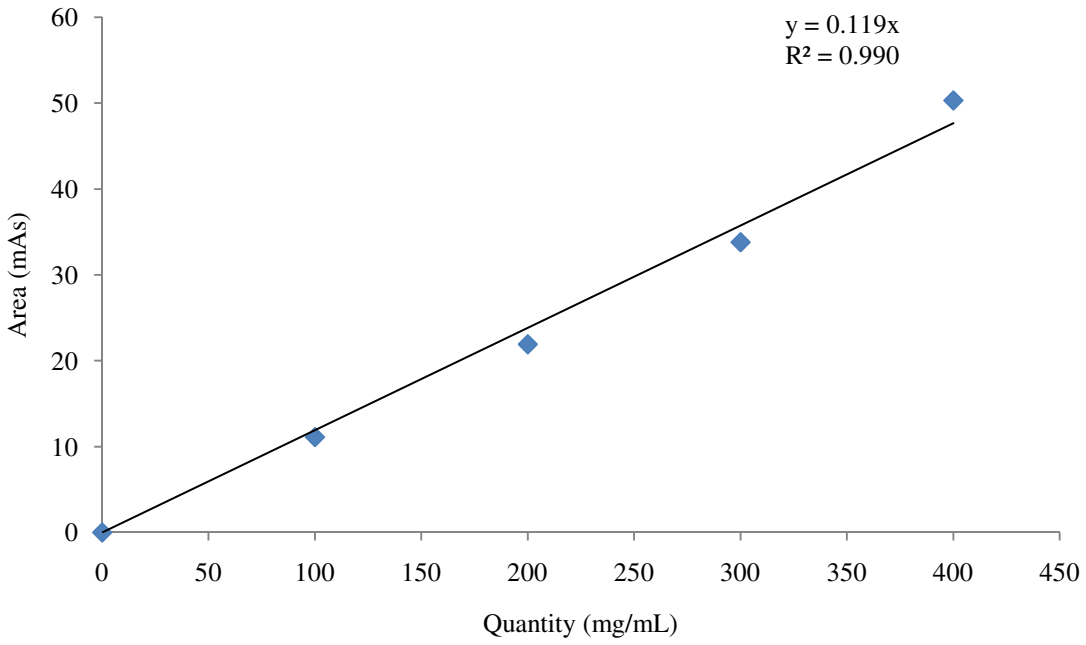

Figure-1: Lactic acid calibration curve (retention time 02:06 mins:sec).

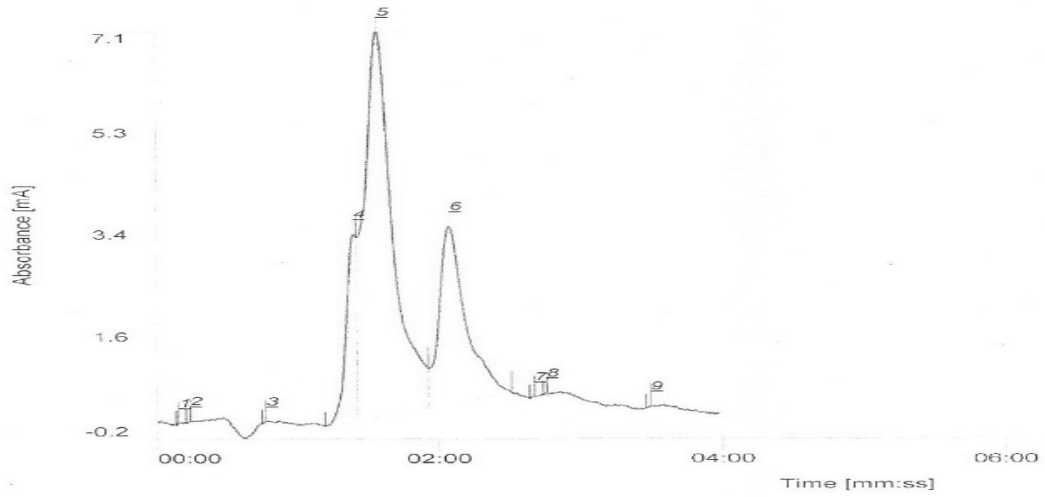

Figure-2: Chromatogram showing lactic acid peak produced by *Lactobacillus plantarum F2C*.

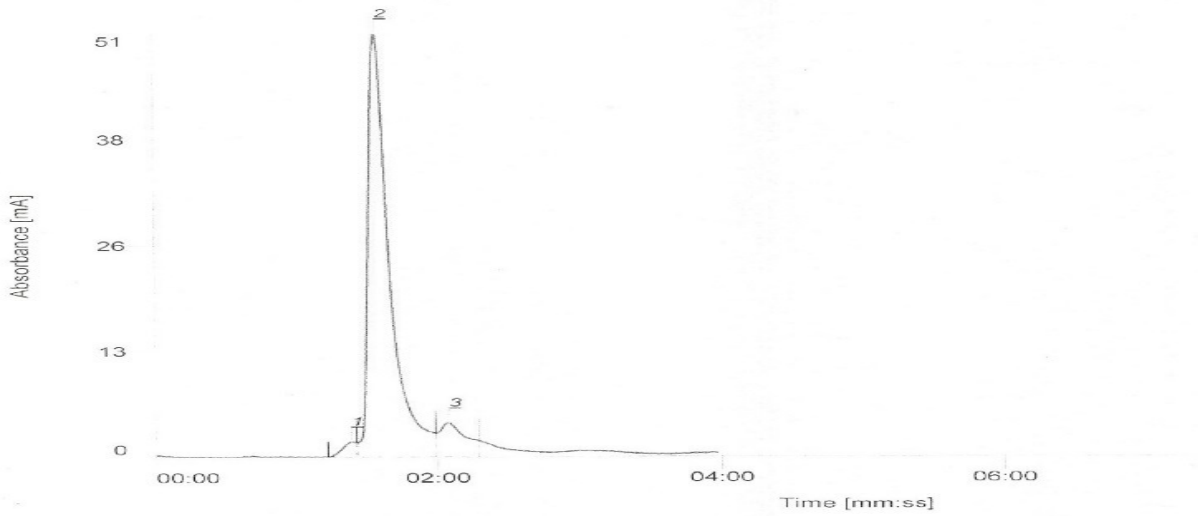

Figure-3: Chromatogram showing lactic acid peak produced by *Lactobacillus plantarum U2A*.

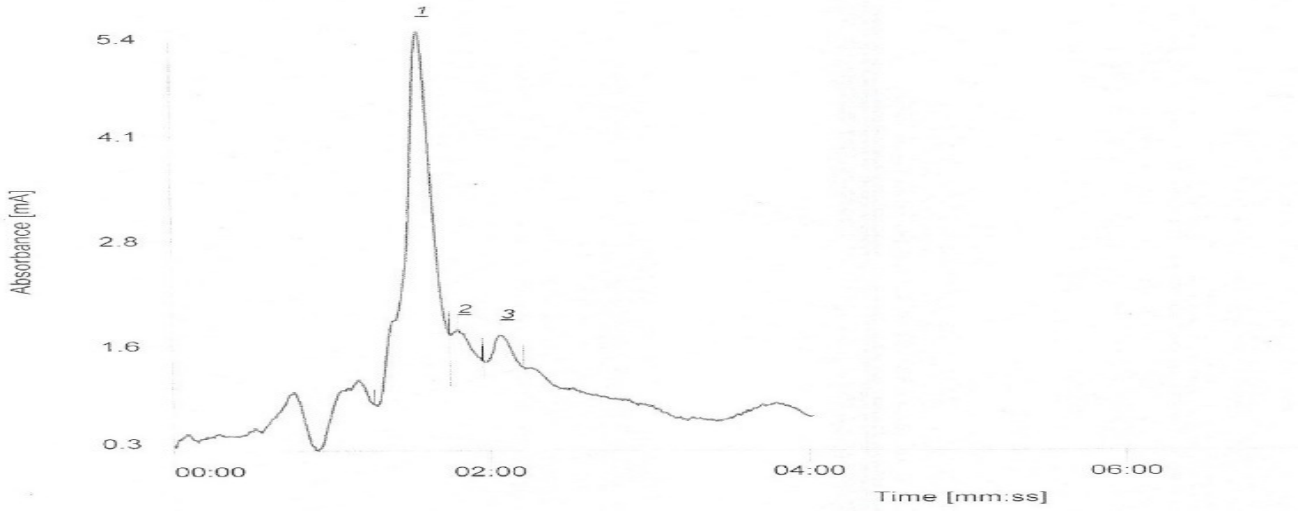

Figure-4: Chromatogram showing lactic acid peak produced by *Lactobacillus plantarum U2C*.

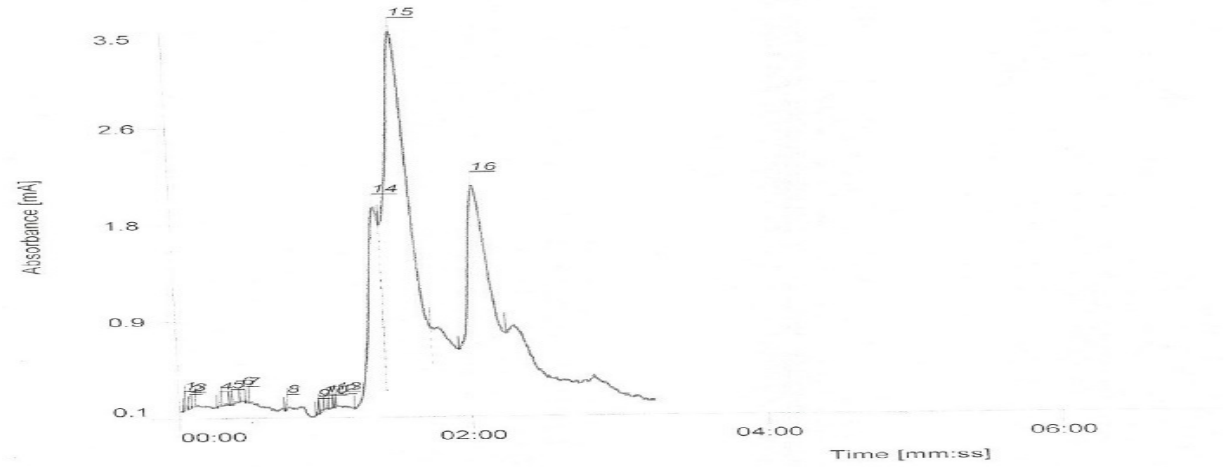

Figure-5: Chromatogram showing lactic acid peak produced by *Lactobacillus plantarum F2C* and *Lactobacillus plantarum U2A*.

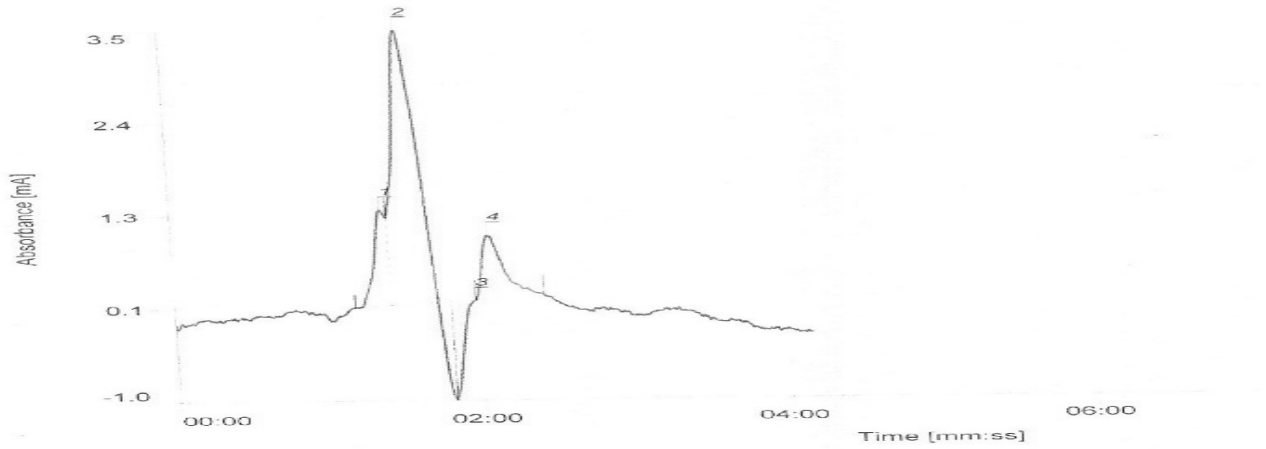

Figure-6: Chromatogram showing lactic acid peak produced by *Lactobacillus plantarum F2C*, *Lactobacillus plantarum U2A* and *Lactobacillus plantarum U2C*.

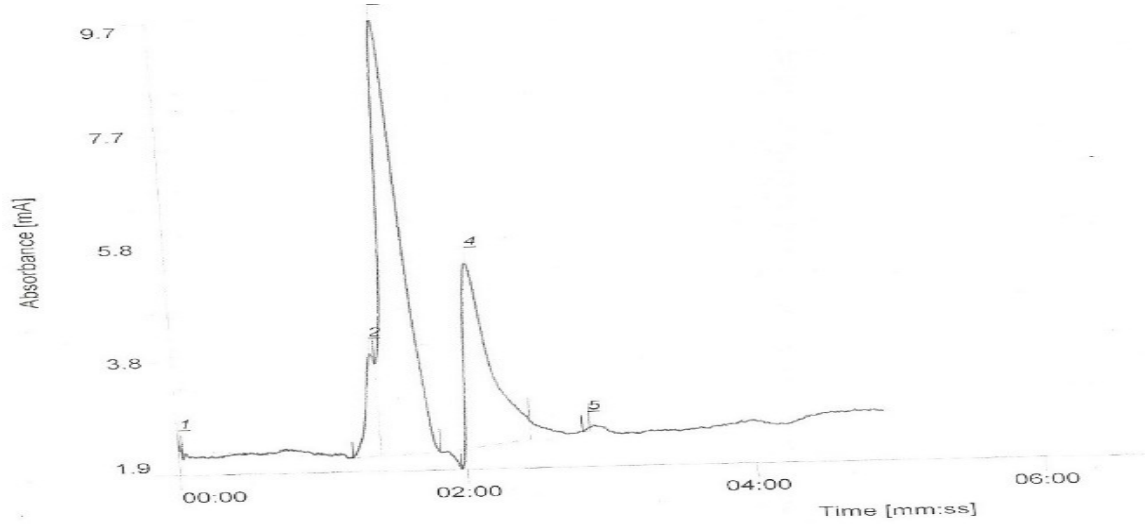

Figure-7: Chromatogram showing lactic acid peak produced by *Lactobacillus plantarum U2A* and *Lactobacillus plantarum U2C*.

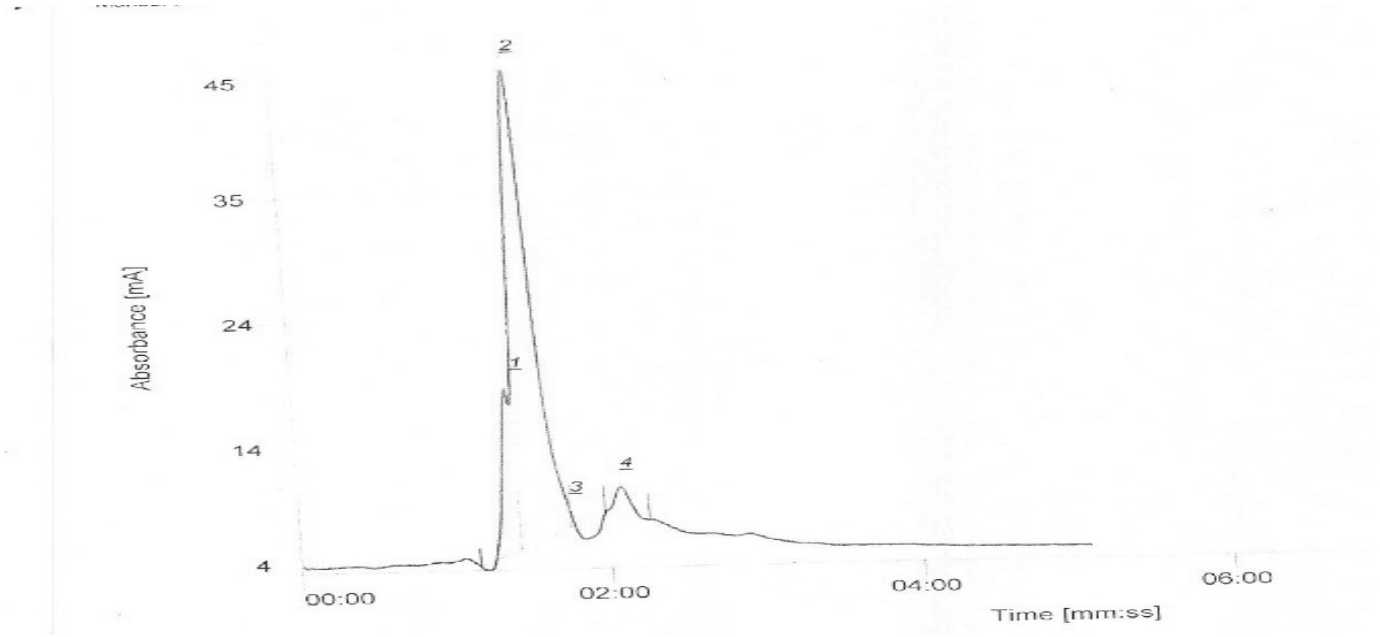

Figure-8: Chromatogram showing lactic acid peak produced during spontaneous fermentation.

Results and discussion

Table-1 shows the quantities of lactate produced by the starters. The differences in the quantities were significant at 5% level of probability. It was observed that single inoculum produced lower quantities than the mixed cultures and this could be as a result of the combined metabolic action of the latter as previously reported [26] that the enhancement of production of metabolites in co-culture may be taken to be indicative of metabolic interactions. However, it was suggested that utilising mixed culture tends to result in difficulty in optimisation as each strain has varied optimal growth conditions[27]. In this study, the highest lactic acid quantity (6.91g/mL) was produced by combination of the three isolates, whereas, the least (3.98mg/mL) was obtained from the spontaneously fermented batch. This confirms the rapid acidification rate effect of starter cultures through the production of acid metabolites as against a spontaneous process. Fermentation with each of the starter had lactic acid quantities ranging between 4.08mg/mL and 5.59mg/mL while those with the combined starter ranged between 5.50mg/mL and 6.91mg/mL. It was however observed that the un-inoculated batch had the overall least lactic acid (3.98mg/mL) produced.

Previously, there were reports that temperature, fermentation time and substrate affects the production of lactate[28] but several authors have however reported varied ranges of lactic acid produced by different microorganisms as well during fermentation. Utilising single cultures of *Lactobacillus bulgaricus* PTCC 1332, *L. plantarum* PTCC 1058, *L. delbruekii* subsp. *delbruekii* PTCC 1333 and *L. casei* subsp. *casei* PTCC 1608 produced 0.024mg/L, 0.029mg/L, 0.015mg/L and 0.019mg/L lactic acid, respectively[29]. Other Scientists also experimented with four different *Lactobacilli* strains for lactic acid production. The optimal fermentation time was 48 hours and overall highest quantities produced were 5.2g/L, 2.6gL, 4.0g/L and 2.4g/L by *Lactobacillus acidophilus, L. plantarum, L. delbruekii* and *L. casei*, respectively[30].

Table-1: Lactic acid (mg/mL) produced after 72hours fermentation using single and mixed *Lactobacillus plantarum* strains.

Starter combination	Lactic acid (mg/mL)
Lactobacillus plantarum F2C	$4.76\pm2.3^{e*}$
L. plantarum U2A	4.08 ± 3.1^{f}
L. plantarum U2C	5.59 ± 2.4^{c}
Lactobacillus plantarum F2C + *L. Plantarum* U2A	6.02 ± 0.5^{b}
L. plantarum U2A +*L. plantarum* U2C	5.50 ± 1.1^{d}
Lactobacillus plantarum F2C + *L. Plantarum* U2A +*L. plantarum* U2C	6.91 ± 0.4^{a}
Un-inoculated	3.98 ± 1.1^{g}

Values are mean± standard deviation, the means reported with the same superscript (*) in each column indicated no significant difference ($p\leq0.05$).

Fahkravar *et al.*[3] while investigating the effect oflactose concentration in lactate production from cheese whey using *Lactobacillus bulgaricus*, obtained maximum lactic acid quantity of 0.025mg/L while 0.034mg/L was from fermentation with *Lactobacillus casei*[32].

However, utilizing co-culture of *L. amylovorus* and *L. casei* to produce lactic acid (0.036mg/L) using barley flour as substrate[33]

gave quantities lower than those in this study. Better performance of co-culture utilisation for lactic acid production using inexpensive carbon source (whey) was also discovered[34]. Researchers obtained maximum production 50.12g/L with co-culture of *Lactobacillus delbruekii* NCIM2025 and *L. pentosus* NCIM2912 as against its individual constituent strains and other singly utilised *Lactobacilli* strains which had a range between 6.60g/L and 46.1g/L lactic acid. These were in accordance with the study that ascertained the fact that production of lactic acid by LAB can be optimised by utilising cultures of single- and mixed-strains of *Lactobacillus sp.* and *Pediococcus acidilactici*.[35].

From this study, it could be observed that higher lactic acid quantity was produced by co-cultures than the singly utilised strains. This could be inferred to be due to the joint action of the individual organism in the mixed culture, being able to overcome the nutritional limitations of utilised substrate[35].

Conclusion

In conclusion, it was observed that *Lactobacilli* co-cultures produced higher quantities of lactic acid than singly utilised starter cultures. Furthermore, the spontaneous fermentation produced the least lactic acid quantity. Therefore, the combination of multiple starter cultures could be employed to improve yield of lactate during fermentation.

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