Monitoring the Production Process of Friendly Bacteria Using Hplc

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***Abstract*- With the development of microbiological and nutritional sciences in the late 19th century came the technology necessary to produce cultured dairy products on an industrial or commercial basis. However ten Lactic acid bacterial (LAB) strains were isolated from milk and its products. They were identified as *Lactobacillus plantarum, Lactobacillus fermentum, Lactococcus lactis subspecie cremoris, Lactobacillus acidophilus, Lactobacillus brevis and Streptococcus thermophillus*. *Lactobacillus plantarum* produces the highest quantity of lactic acid at 72 hours of incubation (2.89g/l) while *Lactobacillus fermentum* produce the lowest (1.11g/l). *Lactococcus lactis subspecies cremoris had* the highest quantity of diacetyl (1.72g/l) while *Streptococcus thermophillus* and *Lactobacillillus fermentum* had the lowest quantity of 0.52g/l and 0.47g/l respectively. At 72 hours of incubation *Lactobacillus acidophilus* produced the highest quantity of hydrogen peroxide (1.91g/l) while *Streptococcus thermophillus* produced the lowest. The quantity of hydrogen peroxide increased up to 48hours before declination sets in. Results of the high performance liquid chromatography (HPLC) used to quantify organic acid revealed that, *Lactobacillus plantarum* produced the highest yield of Lactic acid (280.16 mg/ml) closely followed by *Lactobacillus fermentum* (215.9 mg/ml). Whereas the lowest yield (48.253mg/ml) was produced by *Lactococcus lactis* subsp *cremoris* with the retention time of 3.231. Thus considering the easiness and conciseness of sample preparation, high performance liquid chromatography could be considered as an efficient, accurate and rapid method of organic acid determination in fermented products.**

***Key word*: Lactic acid bacteria, High Performance liquid chromatography, Mam ragosa sharpe, Analytical profile index.**

I. INTRODUCTION

Lactic acid bacteria refer to a large group of beneficial bacteria that have similar properties and all produce lactic acid as an end product of the fermentation process. Lactic acid bacteria are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits [1]. Species used for food fermentations belong to the genera *Lactococcus, Streptococcus Pediococcus, Leuconostoc, Lactobacillus,* and the newly recognized *Carnobacterium.*

These organisms have been isolated from grains, green plants, dairy and meat products, fermenting vegetables, and the mucosal surfaces of animals [2].

Lactic acid is an organic acid that is produced as a result of fermentation metabolism by the lactic acid bacteria. The fermentative production of lactic acid is advantageous over the synthetic production in that by choosing a stream of lactic acid an optically pure product can be obtained. Lactic acid is also used as acidulate, flavoring, pH buffering agent or inhibitor of bacterial spoilage in many processed food. The esters of lactic acid are used in baking foods, as emulsifying agents [3] [4].

The production of Lactic acid can be done in two ways, which are chemical synthesis and carbohydrate fermentation [4]. The method of carbohydrate fermentation is relatively cheaper and so is preferable. However, the titrimetry methods are generally not selective nor sufficiently sensitive or precise to detect a small acid content [5]. To avoid these problems, gas chromatography (HPLC) methods have gained importance in organic acid analysis. Because of the speed, selectivity, sensitivity, reliability and simple sample preparation methods involved, in HPLC. These are the most widely used techniques. Analytes are detected in HPLC with the aid of refractive index detector [6].Today the reversed phase HPLC methods are very popular in food analysis.

II. MATERIALS AND METHODS

Sample Collection

Milk and its products, used for this study, were purchased locally. These samples (Yorghurt, goat milk and cow milk) were collected with a sterile McCartney bottle and transported to Microbiology laboratory of University of Ibadan under hygienic conditions. The clinical indicator strains were collected from University College Hospital, Microbiology and parasitological laboratory, while indicator strains from the environment were collected from the Environmental microbiology laboratory, University of Ibadan, Oyo state Nigeria.

Culture Media

MRS (de Mann Ragossa Sharpe) broth and agar medium, Nutrient agar, and Peptone water were prepared and homogenized according to the manufacurer's instructions. Sterilization of medium was carried out at 121°C for 15min, autoclaved, and cooled to a temperature of 45 ^{o}C before use.

Isolation of Microorganisms

Serial dilutions were made from the samples by mixing 10 ml sample to 90 ml of sterile peptone water various dilutions to give 1:9 dilutions. Dilutions were made up to 10^{-8}, using a sterile pipette. One ml of appropriate dilutions was

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plated out using different culture media [7]. The plates were left for solidifying, inoculated in duplicates, and inverted.

MRS plates were incubated at 37°C for 48hours under aerobic conditions; Nutrient agar was used to resuscitate the indicator organisms, which were incubated aerobically at 30 to 37 °C for 24hours. After incubation period, representative colonies of the various isolates were selected at random and sub-cultured repeatedly to obtain a pure culture.

Culture Preservation

Pure cultures of LAB isolates were sub- cultured into MRS broth /agar and incubated at 30°C until growth becomes visible, and overlaid then with 12% (v/v) glycerol for maintenance of medium. The stock cultures were stored at 4 °C for subsequent use and sub-cultured at 4 weeks interval.

Characterization

Characterization of isolates was carried out by employing macroscopic, microscopic and biochemical tests using API50CHL strip. One milliliter of broth cultures of each isolate was used for all the tests except where otherwise stated.

Macroscopic Examination

The isolates were examined for growth type, shape, elevation, size, pigmentation and consistency.

Sugar Fermentation Test

API 50CHL strip is a standardized system associating 50 biochemical test which is used for the study of the carbohydrate metabolism of microorganism. The fermentation tests are inoculated with API 50CHL medium which rehydrates the substrates, during incubation, fermentation is revealed by a color change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the chosen medium. The first tube which does not contain any active ingredient is used as a negative.

Quantification of Organic Acids (HPLC)

Lactic and acetic acids were quantified by high pressure liquid chromatography (HPLC) of 12000 series. The Analysis of both Lactic and Acetic acids were performed according to the methods described by the manufacturer (OACD.M) at ambient temperature of 20 to 25°C.

Quantification was carried out via an ion exchange column using the following separation techniques: The flow rate of 1ml/min of methanol: 25mMKH2PO4(20:80)% mobile phase, injection volume of 1.25/ 2.5, 5.0/ 7.5 and 10mg/ml respectively, UV detection at 60^{0}C, Retention /Elution time of 3 to 4mins and Column of supelcosil c18 (250×4.6)mm,5µm.

III. RESULTS AND DISCUSSION

The API 50CHL kit used in this work was able to differentiate the isolate to strain level. The entire LAB fermented glucose, fructose, lactose, maltose and galactose. However there is variation in fermentation of other sugars.



Plate 1: API Kit + Medium+ LAB before incubation

Plate2: API Kit + Medium + LAB after incubation.

Plate.1API 50CHL strip for the identification of LAB isolate.

The quantity of Antimicrobial compound produced by LAB was investigated. It was observed that, *Lactobacillus plantarum* produced the highest quantity of antimicrobials at 72hours of incubation (2.89g/l) while *Lactobacillus fermentum* produced the lowest Figure1.

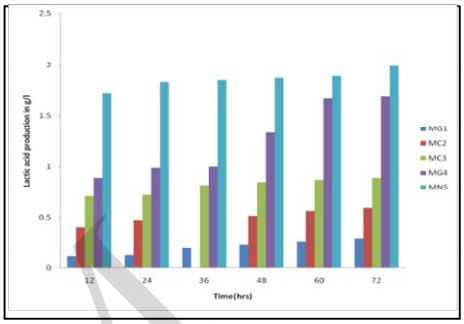


Fig. 1: Quantitative estimation of Lactic acid production by LAB isolates
KEY: MG1-Lactobacillus plantarum, MC2-Streptococcus thermophillus, MC3- Lactococcus lactis sub. cremoris, *MG4- Lactobacillus acidophilus* MN5- *Lactobacillus fermentum*

However for the quantitative estimation of Lactic Acid Bacteria, it was observed that concentration of LAB increased by increasing the incubation period. as the incubation period increases Lactic acid concentration increased. As incubation period increases, the quantity of diacetyl produced increases.

Lactococcus lactis sub. cremoris had the highest quantity of diacetyl at 72 hours of incubation (1.72g/l) while *Streptococcus thermophilus Lactococcus lactis sub. cremoris* and *Lactobacillus fermentum* had the lowest quantity of 0.086g/l at the 12hours of incubation (Figure II).

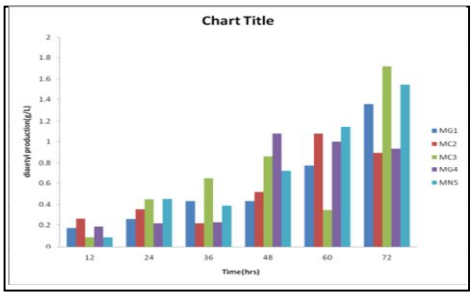
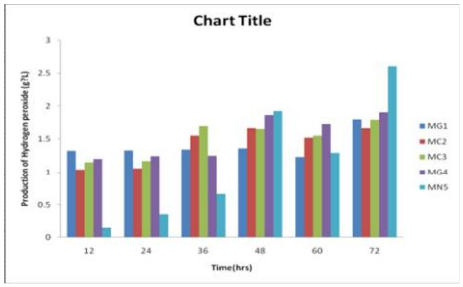


Fig. 2: Quantitative estimation of diacetyl production by LAB isolates

As incubation period increases, the quantity of diacetyl produced increases. *Lactococcus lactis sub. cremoris* had the highest quantity of diacetyl at 72 hours of incubation (1.72g/l) while *Streptococcus thermophilus Lactococcus lactis sub. cremoris* and *Lactobacillus fermentum* had the lowest quantity of 0.086g/l at the 12hours of incubation (Figure II). The quantity of hydrogen peroxide produced by the test isolate varies from one species of organism to the other. Hence it was observed that as the incubation period increased the quantity of hydrogen peroxide increased up to 48hours before declination sets in. However at 72 hours of incubation *Lactobacillus acidophilus* produced the highest concentration of hydrogen peroxide (1.91g/l) while *Streptococcus thermophillus* produced the lowest.Fig.III



a)

Fig. 3: Quantitative estimation of hydrogen peroxide production by LAB Isolates

The separation of the two organic acid in the standard mixture was given. The uptimization of the chromatograph conditions, i.e., the pH and flow rate of the mobile phase, the adsorption wavelength, and column temperature, all affect the separation degree and peak shape of the organic acid, as they were obtained. The organic acid production ability of different LAB species varies, and this is a critical feature in characterization of some LAB species. In the analysis for the four species of LAB used, it was clear that their production of Lactic acid and Acetic acid varied considerably as shown by the chromatograms in Fig. and VI.

In the HPLC analysis and quantification as indicated in Fig IV. *Lactobacillus plantarum* had the highest quantity of Lactic acid (280.16mg/ml) followed closely by *Lactobacillus fermentum* (215.9mg/ml) while *Lactococcus lactis* sub. *cremoris* produced the l (48.253mg/ml). In the case of Acetic acid. *Lactobacillus plantarum* has the highest production of Acetic acid with a value of 200mg/ml followed closely by *Lactobacillus fermentum* (16.3mg/ml). (52mg/ml).

Lactobacillus acidophilus had the least value of 35.5mg/ml. In general, it was observed that *Lactobacillus fermentum* and *Lactobacillus plantarum* were the best producers of Lactic acid when compared with *Lactococcus lactis subspecies cremoris* and *Lactobacillus acidophilus.* The quantity and concentration of Lactic acid and Acetic acid produced by LAB were converted to percentage as shown in Table 1. However, changes in the value of organic acids based on their difference in quantity (mg/ml) were also determined. It was observed that, *L. plantarum* had the highest percentage change in Lactic and acetic values (71.7%) showing a high variability in organic acid production. *L. acidophillus* had a percentage change in organic acid values of 37.7%, while *Lactococcus lactis sub. cremoris* and *Lactobacillus plantarum* had 9.44% and 7.92%, respectively , showing their variability status with regards to organic acid production.

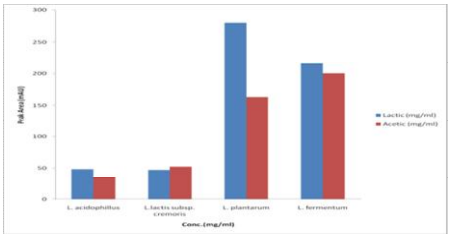


Fig. 4: Quantification of organic acids produced by Lactic and Acetic acid (HPLC)

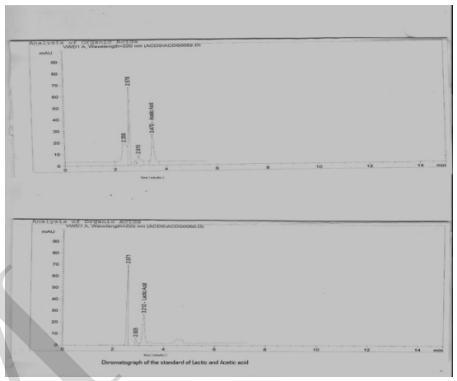


Fig. 5: Chromatogram of the standard of Lactic and Acetic acid showing the Peak area, Retention time and Elution of *Lactobacillus fermentum* and *Lactococcus lactis subsp.cremoris*

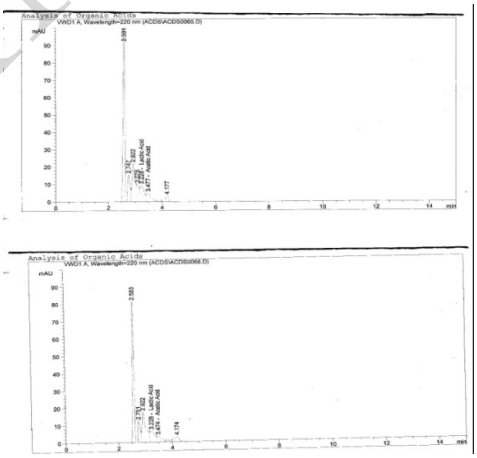


Fig. 6: Chromatogram of Lactic and Acetic acid showing the Peak area, Retention time and Elution of *Lactobacillus plantarum* and *Lactobacillus acidophilus*.

Table.1

Sample ID	**Quantity of Lactic and Acetic acid produces by LAB isolates in mg/ml and their percentage change** Sample Concentration:1in25		% change
Sample ID	Lactic mg/ml	Acetic mg/ml	% change
L. acidophilus	48.2533392	35.55191372	35.72642
L.l subs. cremoris	47.15250257	52.07231973	9.448047
L. plantarum	280.1629238	163.0894481	71.78483
L. fermentum	215.9474534	200.0951575	7.922379

IV. CONCLUSION

With the development of microbiological and nutritional sciences in the late 19th century came the technology necessary to produce cultured dairy products on an industrial or commercial basis. Considering the easiness and conciseness of sample preparation, high performance liquid chromatography could be considered as an efficient, accurate and rapid method of organic acid determination in fermented products.

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