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Production of Lactic Acid from Banana Waste Peel by batch Fermentation using Lactic Acid Bacteria (LAB) isolated from Milk Products

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Abstract

Lactic acid is a chemical compound that plays a role in various biochemical processes and also widely used in the food, cosmetic, pharmaceutical, and chemical industries. The aim of this study was to establish a process of lactic acid (LA) production from organic banana peel waste using lactic acid bacteria isolated and growth in MRS media at 37°C for 24 h from milk product. The results indicate that lactic acid production using lactic acid bacteria are identified as Gram-positive, non-spore forming rods, catalase-negative, usually non-motile. There was significant pH difference from 5.64 to 3.92 in the fermentation production medium containing banana waste peel + inoculum and 5.64 to 3.89 in the medium containing banana waste peel extract + inoculum + malt extract during the course of time from 0 hours to 96 hours when compared to control containing only banana waste peel without inoculum. There was dramatic increase in the percentage of crude lactic acid production was similar along with change in pH from ranging from 6.14 to 6.71 before decolorization without activated charcoal and 7.31 to 7.53 after decolorization with activated charcoal for fermentation production medium during the course of 24 to 96 hours. Similarly, the percentage of crude lactic acid production increases ranging from 6.73 to 6.98 before decolorization without activated charcoal and 7.79 to 7.96 after decolorization with activated charcoal for fermentation production medium containing banana waste peel + Malt extract and inoculum from 0 to 96 hours. This may due to the high rate of consumption of starch compounds to lactic acid with the help of lactic acid bacteria.

Key words: Banana peel, Lactic acid, MRS Medium, Lactic acid bacteria, Batch Fermentation.

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Introduction

Back ground of the study :

Lactic acid, also called α -hydroxypropanoic acid or 2-hydroxypropanoic acid, has a wide range of application in different fields and in general in preservation of human food stuffs (Davison et al., 1995). Discovered by Scheele in 1780 and was considered as a milk component and in 1789 was named as "acide lactique" by Lavoisier. Later in 1857, Louis Pasteur discovered that it was a fermentation metabolite released by certain microorganism rather than being a milk component (Benninga, 1990).

The production of lactic acid can be performed in two ways: by chemical synthesis or by fermentation (Abdel-Rahman et al, 2011). Chemical synthesis is based on petrochemical resources and has the disadvantage of giving rise to a racemic mixture of DL-lactic acid . As for the second lane of production, it corresponds to lactic fermentation based on a biomass carbon source; this path is more advantageous for its low energy consumption and its pure LA production by selecting the appropriate lactic acid bacteria (LAB) strain. (Hofvendahl and Hahn-Hägerdal, 2000).

The organic waste arising from banana peel approaches 30 - 40% of the gross weight and indicates that banana peel is not effectively used after the fruits are consumed.

Banana peel waste contains cellulose, hemicellulose, starch, pectin and polysaccharides and can be used as a supplementary source for the production of industrial enzymes provides renewable energy resources (Rehman et al., 2014). Lactic acid production is not typically a highly complex procedure. However, for unutilized biomass to be used as a carbon source, it must be saccharified and/or pretreated because most lactic acid bacteria cannot utilize it directly. Thus, the types of biomass and hydrolytic enzymes as well as lactic acid producing microorganisms can affect the efficiency of lactic acid production (Luo et al., 1997).

The present study investigated the optimization of L-lactic acid production from banana peel as an unutilized biomass substrate by anaerobic fermentation process at laboratory level by using the LAB pure cultures isolated from yogurt (Ergo).

Statement of problem :

Lactic acid can be produced by bacteria, yeasts, and molds including genetically modified forms of these organisms using carbohydrate substrates. Banana peels are suitable substrates for some species of lactic acid bacteria. This project covers lactic acid fermentation processes, product recovery methods, and commercial applications of lactic acid, including foods, cosmetics, medical products, and biodegradable polymers.

General objective :

- Production and synthesis of lactic acid in laboratory scale from starch (obtained from waste banana peel) via fermentation using lactic acid bacteria (LAB) from milk products

Specific objectives :

- Isolation of Lactic Acid Bacteria (LAB) from fermented Milk products.
- To synthesize Lactic acid from waste banana peel via fermentation using Lactic acid bacteria isolated from milk product.
- To determine the crude and purified Lactic acid from waste banana peel by titratable method and variable incubation period and pH on the lactic acid yield.
- To determine antimicrobial potential of Lactic acid against other bacterial isolates.

Scope and limitation of the study :

- This work involves investigation of lactic acid production starting from isolation of potential lactic acid bacteria (LAB), media formulation and fermentation processes. The ultimate objective of the whole project report is to improve value of the agricultural product for the efficient production of lactic acid by fermentation processes. Lactic acid fermentation was performed using waste banana peel starch as the main substrate.

Materials and Methods

Materials:

- The study area was in Arba Minch located in the Gamo Gofa zone of the southern nations, nationality, and people region the most common vegetation type grow in Arba Minch are mango

tree, banana and acacia plants.

- **Study Design:** The study area was in Arba Minch located in the Gamo Gofa zone of the southern nations, nationality, and people region. Observational study was conducted. The study was on fermented milk products found in Arba Minch town between October 2017 and October 2018. The laboratory used for study were chemistry and microbial biotechnology laboratory, Arba Minch University, Arba Minch.

Sample Collection :

The samples were collected in different houses or purchased from local market of Arba Minch. Two types of samples were collected. The first sample was curd (irgo) and the second was fermented milk product (cheese). The samples were transported to AMU microbial biotechnology laboratory and placed in refrigerator at 4°C.

Media and Reagents :

The following solutions will be used : de Man-Rogosa-Sharpe (MRS) broth, MRS-A (MRS + 1.5% of bacterial agar), 0.85% saline water, methylene blue, crystal violet, safranin, Gram's iodine, acetone (95%), 3% hydrogen peroxide solution, NaCl(2, 4 and 6.5%), tryptone (1.0%), yeast extract (1.0%), beef

extract (1.0%), NaCl (1.0%), glucose (1.0%), 1 M NaOH and 1 M HCl.

Test (indicator) strains :

Salmonella thyphi (Clinical isolate), *Shigella flexineri* (Clinical isolate), *Staphylococcus aureus* (ATCC-25923) and *Escherichia coli* (ATCC-25922) were obtained from Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia were used as test microorganisms.

Isolation of lactic acid bacteria (LAB) :

For isolation of LAB, serial dilution agar technique was used. Fermented milk was used directly for isolation of lactic acid bacterial strains, while cheese was serially diluted in saline solution (P.K. Nagalakshmi et al., 2013). Ten gram of each sample (except curd taken 10 ml) was dissolved into 90 ml of MRS broth. After dissolving into MRS broth shake homogeneously and incubated at 37°C for 24 hrs in an aerobic condition. In the serial dilution agar plate technique, 10ml of a stock solution was added to 90 ml water blanks to form a microbial suspension. Serial dilution of 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ by pipetting 10ml into 90ml water blanks. 10 ml of each dilution was inoculated to MRS agar plates and incubated at 37°C for 24 hrs for bacterial growth.

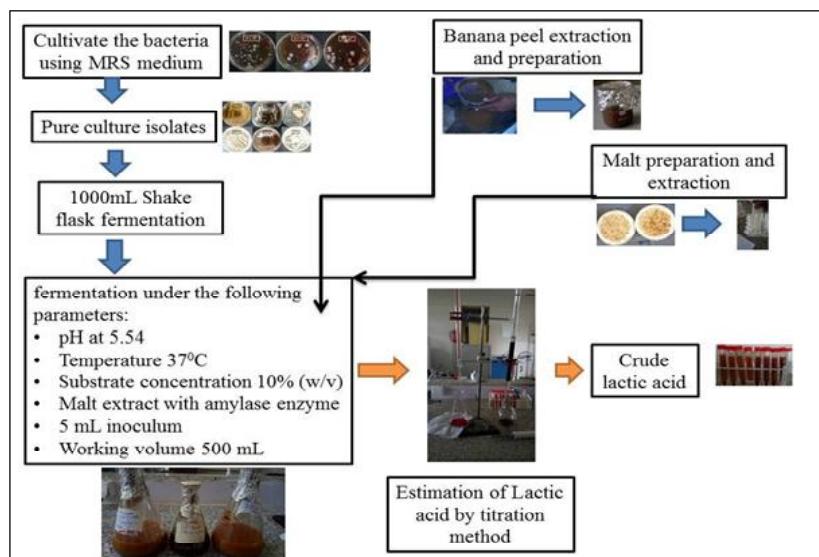


Figure 1: Schematic flow Chart diagram for the production of lactic acid from lactic acid bacteria isolated from milk product.

The plates for appearance of colonies observed. Purified bacteria by streak plate method on MRS agar and incubated at 37°C for 24hrs and transferred to MRS agar slants and placed in refrigerator at 4°C (Ram kumar *et al.*, 2013).

Isolation, screening and identification of lactic acid producing LAB :

Inoculate the isolated LAB strains in 5ml MRS broth and incubated under anaerobic condition at 30°C for 18-24 hr. Obtain cell-free supernatant (CFS) by centrifugation of this culture using Biofuge fresco centrifuge at 10.000 x g for 10 min at 4°C. To clarify whether the antimicrobial activity detected derived from an organic acid or hydrogen peroxide (H_2O_2), adjust the CSF to PH 7.0 by adding 1N NaOH to eliminate the inhibitory effect of organic acids and add 3000 U/ml of catalase to eliminate the potential inhibitory effect of hydrogen peroxide produced by the isolates. Filter the so treated CFS through 0.45 μ m filter and used as crude lactic acid bacteria solution.

Biochemical characterization of LAB isolates :

Biochemical characterization of isolates on the basis of catalase, Methyl Red (MR) test, Triple Sugar Iron Agar test (TSI test), Citrate utilization test and Voges Proskauer (VP) Test were studied.

Preparation of Inoculum :

A single bacterial colony from potential isolate were inoculated in to 1 ml of MRS broth under aseptic condition and incubated at 37°C for 24 h. After 1 day of incubation, growth (turbidity) was appeared in inoculated broth and this 1ml bacterial suspension transferred to 10ml of MRS broth under aseptic condition and incubated at 37°C for 24 h. After 1 day of incubation, growth (turbidity) was appeared in inoculated broth which preparation was directly used as a source of inoculums.

The Process of Fermentation :

The fermentation was carried out by taking

three 1000-mL Erlenmeyer flask with 500 mL production medium were optimized by changing their concentration of MRS broth with banana peel waste as substrate, inoculum and malt extract having set 1 batch fermentation (only MRS broth and banana peel extract) as control, set 2 batch fermentation (MRS broth, banana peel and inoculum) and set 3 batch fermentation (MRS broth, banana peel, inoculum and malt extract with enzyme) for getting the optimum production of crude lactic acid. All equipment, including the medium, was sterilized at 15 psi and 121°C for 15 min containing reaction mixture with substrate and MRS broth. Autoclaved with medium substrate was leave for some time to cool down at 37°C temperature; initial quantity of 5mL inoculum and 10% substrate was poured in reaction mixture in Laminar flow in order to avoid contamination with other microbes and kept back inside incubator further for fermentation for production of lactic acid with frequent hand shaking the fermentation flasks. During fermentation, the temperature was kept at 37°C, and the pH adjusted at 5.5 to 6.0 for all three fermentation flasks by adding 0.1N NH_4OH where necessary.

Production and Extraction of Crude Lactic Acid :

Crude lactic acid was produced from 3 different fermentation broths viz. MRS broth, inoculated with the bacterial culture in three different conical flasks under incubation via frequent shaking with hand from 24 hours to 90 hours. After the desired incubation the fermentation broths were taken and centrifuged in a high speed cooling centrifuge at 10,000 rpm for 10 minutes and the supernatant (crude lactic acid) was collected and stored for further use.

Decolourisation of crude lactic acid :

These operations were carried out in glass columns filled with the renewable granulated active charcoal Purolite AC 20 was chosen and used for decolourisation. The flow rate of the cell-free fermentation broth after centrifugation through the column was 1 bed volume per hour for decolourisation

was performed.

Titrimetric Assay (Titratable Acidity) of Lactic Acid:

This method takes into account the concentration of disassociated hydrogen molecules and un-disassociated hydrogen ions. Acidity is related with hydrogen in the solution so TA to measure the total acidity is a better indication of lactic acid levels. In this process the sample is titrated against 0.1N NaOH with the addition of 2-3 drops of Phenolphthalein indicator until the colour of the sample turns light pink. The percentage of purity of lactic acid is calculated using the given formula.

$$R = \frac{V(\text{titr}) \times C(\text{titr}) \times MW \times F \times 100}{1000 \times W(\text{sample})}$$

Where, R= % of Lactic Acid

V (titr) = Total volume of titrant needed to reach the end point in ml.

C (titr) = Concentration of titrant.

M W = Molecular weight of lactic acid = 90.08

F = Dilution Factor

W (Sample) = Sample amount in either gram or ml.
100 is multiplied to obtain the percentage of lactic acid present.

Antimicrobial Activity and Bioassay By Agar Well – Diffusion Assay :

Petri plates were prepared by pouring 20ml of respective sterile molten media for test microorganisms and allowed it to solidify. Spreading the agar plates with 100 µl of each standardized tested microorganisms. Dry the plates and make two wells (each 7mm in diameter) into agar plates with sterile borer and load wells with 25 µl of crude lactic acid fermentation broth filtrate (supernatant after centrifugation), after decoloration with activated charcoal samples and 25 µl sterile broth. Then incubated the plates at 37°C for 24 hrs for test bacteria (Lihua et al., 2013). Diameter of zone of inhibition was Measured (Kumar et al., 2013). Lactic acid activities

were determined by measuring the diameter (mm) of inhibition zone around the discs (Tatsikou et al., 2017).

Replications and Statistical Analysis :

All experiments were repeated three times on different days. The average values ±SE are presented for few observations only. Statistical analysis of the data was done using the software Sigma plot (version 12.0) and Microsoft excel 2010.

Results and Discussion

Lactic acid has a long history of uses for fermentation and preservation of human food stuffs and can be produced by either microbial fermentation or chemical synthesis. Due to present environmental concerns and the limited nature of petrochemical feedstock, lactic acid can be commercially produced by microbial fermentation. In recent years, lactic acid consumption has increased dramatically because of its role as a monomer in the production of biodegradable Poly lactic acid (PLA), which is well-known as a sustainable bio plastic material. However, the global consumption of lactic acid is expected to increase rapidly in the near future. Lactic acid bacteria can be classified into two groups: homo-fermentative and hetero-fermentative. While the homo-fermentative LAB convert glucose almost exclusively into lactic acid, the hetero fermentative LAB catabolize glucose into ethanol and CO as well as lactic acid (Hofvendahl and Hahn-Hagerdal², 2000). Only the homo-fermentative LAB has the industrial importance and used as commercial production of lactic acid (Yun et al., 2003). The homo-fermentative Lactic acid bacteria were from the genera *Lactobacillus*, *Sreptococcus* and *Pediococcus* (Stainer et al., 1976). The results of the present study in the Lactic acid production using Lactic acid bacteria are identified as according to (Bergey's manual, 1986) is Gram-positive, non-spore forming rods, catalase-negative, usually non-motile, that do not reduce nitrate, indole is not formed and that utilize glucose (Figure 4 and 5).

According to the morphological and biochemical characteristics of the six LAB isolates were identified respectively based on the Bergey's manual of systematic Bacteriology (Table 1, Figure 5).

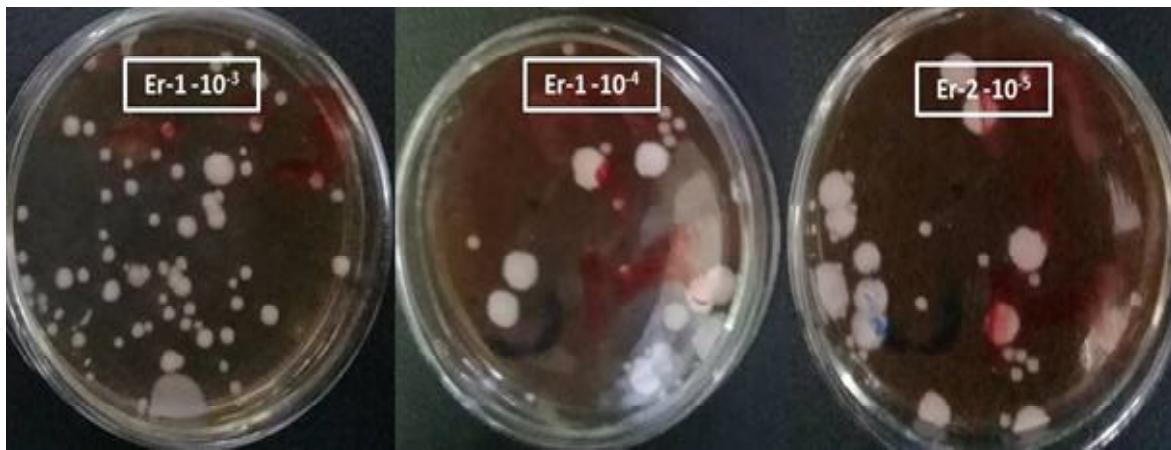


Figure 2: Growth of mixed culture of lactic Acid Bacteria on MRS Agar.

These colonies were carefully picked up and inoculated in MRS broth and consequently in MRS agar media to obtain pure culture of lactic acid bacteria (Figure 5).

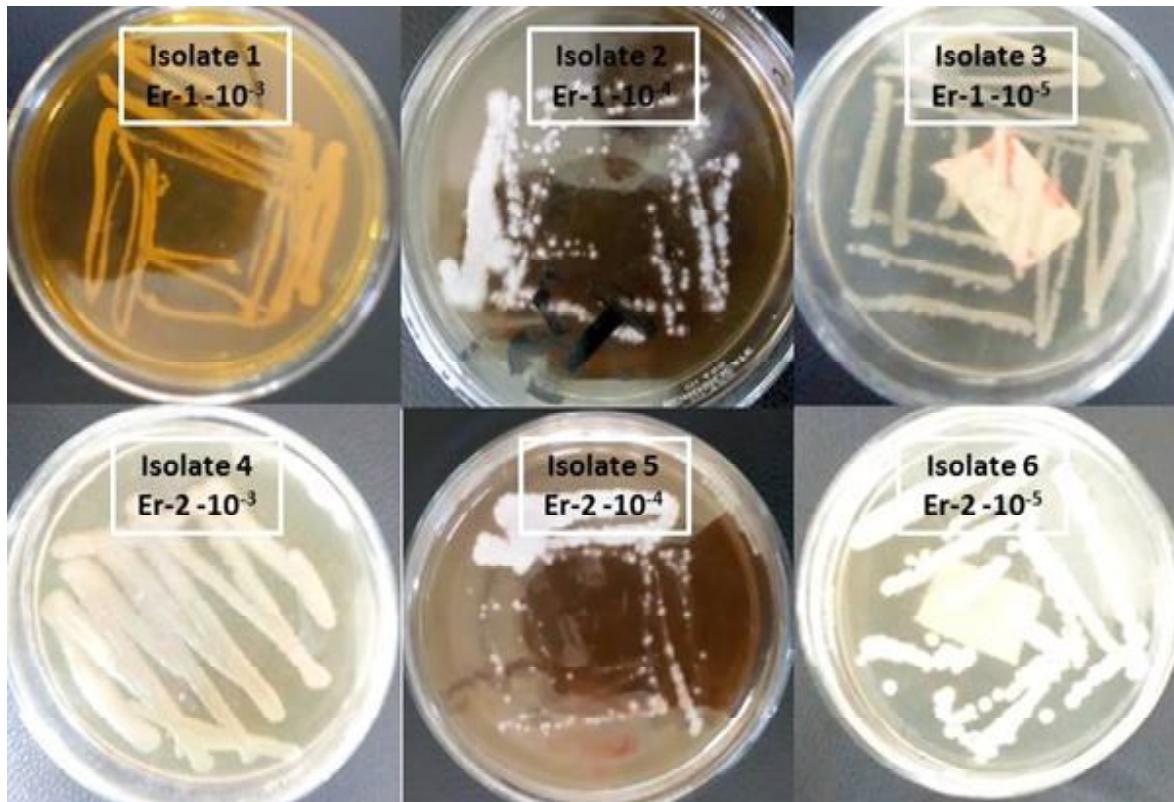


Figure 3: Pure culture of Lactic Acid Bacteria isolated from Ergo

Table 1: Morphological, cultural and biochemical characteristics of lactic acid producing isolates of lactic acid bacteria isolated from ergo.

Characteristics	Lactic acid producing lactic acid bacteria isolates					
	Er-1 ⁽⁻³⁾	Er-1 ⁽⁻⁴⁾	Er-1 ⁽⁻⁵⁾	Er-2 ⁽⁻³⁾	Er-2 ⁽⁻⁴⁾	Er-2 ⁽⁻⁵⁾
Colony Morphology	Ft	Cir	Cir	Cir	Ft	Irg
Cell Morphology	Cocci	Cocci	Rod	Cocci	Cocci	Rod
Gram Staining	+ve	+ve	+ve	+ve	+ve	+ve
Catalase Reaction	-ve	-ve	-ve	-ve	-ve	-ve
Methyl Red (MR) Test	+ve	+ve	+ve	+ve	+ve	+ve
Triple Sugar Iron Agar Test (TSI Test)	+ve	+ve	+ve	+ve	+ve	+ve
Citrate Utilization Test	+ve	+ve	+ve	+ve	+ve	+ve
Voges Proskauer (Vp) Test	ND	ND	ND	ND	ND	ND

Legend: Ergo (Er) Positive reaction (+ve), Negative reaction (-ve), Not Determined (ND), Flat (Ft), Raised (Ra), Circular (Cir), Irregular (Irg).

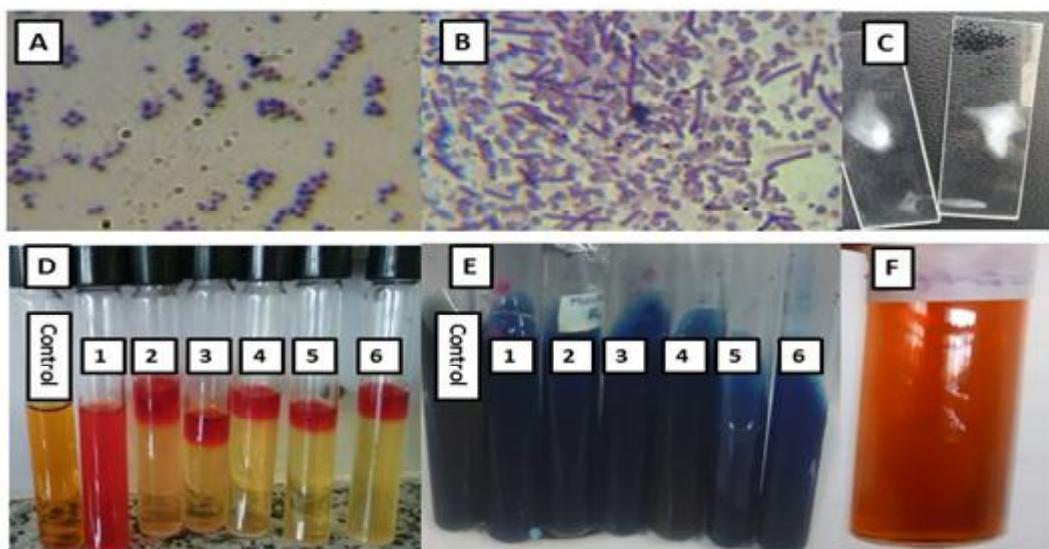


Figure 4: Morphological and biochemical test of Lactic Acid Bacteria ([A] Gram +ve cocci shaped bacteria [B] Gram +ve rod shaped bacteria [C] Catalase test reaction [D] Methyl red test [E] Citrate utilization test [F] Bacterial inoculum).

The fermentation was conducted based on the production of lactic acid banana waste peel hydrolyzate addition in medium (MRS Broth) was set at 10% (w/v) with and without malt extract obtained. The pH was recorded before autoclaving the different fermentation medium and set the pH to 5.5 to 6.0 at 0 hours and started the fermentation. The results indicate that there is significant pH difference from

5.64 to 3.92 in the fermentation production medium containing banana waste peel + inoculum and 5.64 to 3.89 in the medium containing banana waste peel hydrolyzate + inoculum + malt extract during the course of time from 0 hours to 96 hours when compared to control containing only banana waste peel without inoculum (Table 2).

Table 2: pH at different time intervals before and after autoclaving of different fermentation production medium.

Different fermentation Production medium	Fermentation pH at different time intervals					
	Before autoclaving	Set at 0 hours	24 hours	48 hours	72 hours	96 hours
Flask 1	6.46	5.64	5.54	5.39	5.32	5.21
Flask 2	6.46	5.65	4.95	4.15	4.05	3.92
Flask 3	6.49	5.64	4.92	4.13	4.03	3.89

Flask 1: MRS broth + 10% banana peel extract

Flask 2: MRS broth + 10% banana peel extract + 5 mL inoculum

Flask 3: MRS broth + 10% banana peel extract + 5 mL inoculum + 50 mL of Malt extract

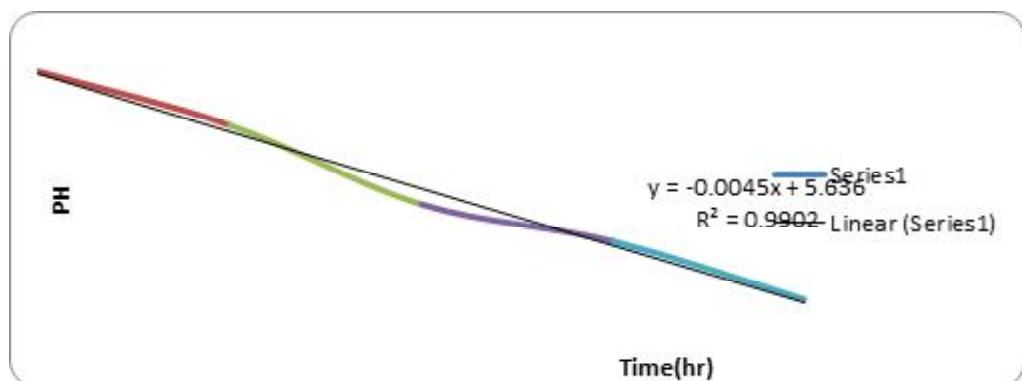


Figure 5: graph of pH versus time of fermentation for flask₁

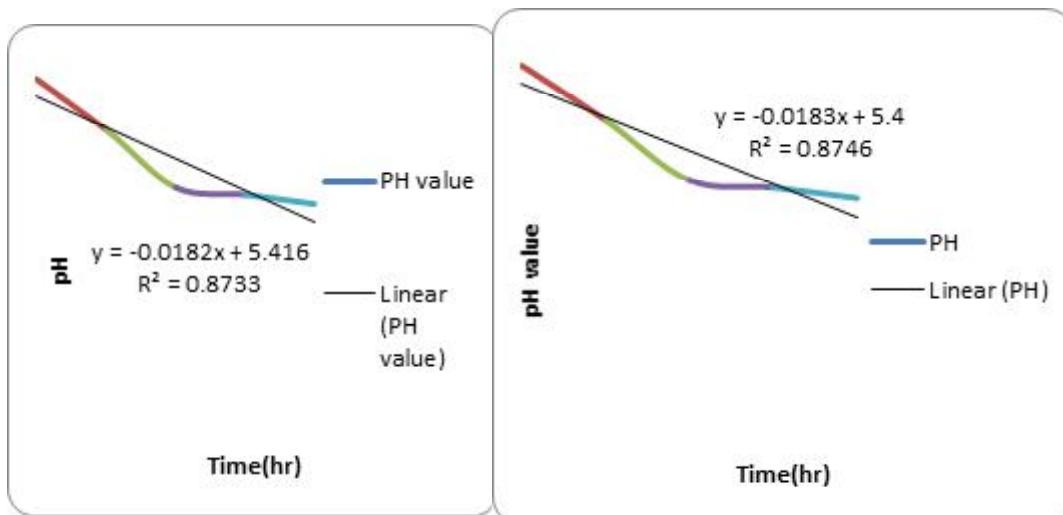


Figure 6: graph of PH versus Time
of fermentation for flask₂

Figure 7: graph of pH value versus Time
of Fermentation for flask₃

Accordingly, the percentage of crude lactic acid production was similar along with change in pH ranging from 6.14 to 6.71 before decolorization without activated charcoal and 7.31 to 7.53 after decolorization with activated charcoal for fermentation production medium containing banana waste peel + inoculum during the course of 24 to 96 hours (Table 4 and 5). Similarly, the percentage of crude lactic acid production was ranging from 6.73 to 6.98 before decolorization without activated charcoal and 7.79 to 7.96 after decolorization with activated charcoal for fermentation production medium containing banana waste peel + Malt extract and inoculum (Table 4 and 5). This may be due to high cellulosic content of (46-72 %) (Li et al., 2010) banana waste was considered as a potential

source for production of fermentable sugars. Maximum enzymatic hydrolysis with the help of malt extract containing amylase was obtained by decreasing the crystallinity of the cellulose and hemicellulose present in the banana peel biomass. The percentage of purity of lactic acid is calculated using the given formula.

$$R = \frac{V (\text{titr}) \times C (\text{titr}) \times MW \times F \times 100}{1000 \times W (\text{sample})}$$

Where, R= % of Lactic Acid

V (titr) = Total volume of titrant needed to reach the end point in ml.

C (titr) = Concentration of titrant.

Table 3: Estimation of crude lactic acid from different fermentation production medium without activated charcoal by titration method.

Different fermentation Production medium	Percentage of LA/100 mL sample at different time intervals of fermentation			
	24 hr	48 hr	72 hr	96 hr
Flask 1	2.02±0.21	2.23±0.19	2.37±0.17	2.33±0.16
Flask 2	6.14±0.37	6.54±0.39	6.68±0.35	6.71±0.35
Flask 3	6.73±0.41	6.91±0.47	6.97±0.43	6.98±0.43

Flask 1: MRS broth + 10% banana peel extract

Flask 2: MRS broth + 10% banana peel extract + 5 mL inoculum

Flask 3: MRS broth + 10% banana peel extract + 5 mL inoculum + 50 mL of Malt extract

* The titration was carried out with two dilutions for the sample (1:1 i.e., 5 mL sample and 5 mL distilled water and 1: 9 i.e., 1 mL sample and 9 mL distilled water).

Table 4: Estimation of crude lactic acid from different fermentation production medium after decolorization with activated charcoal by titration method.

Different fermentation Production medium	Percentage of LA/100 mL sample at different time intervals of fermentation			
	24 hr	48 hr	72 hr	96 hr
Flask 1	2.16±0.17	2.21±0.15	2.31±0.19	2.29±0.16
Flask 2	7.31±0.35	7.49±0.37	7.54±0.31	7.53±0.34
Flask 3	7.79±0.40	7.97±0.42	7.99±0.44	7.96±0.41

Flask 1: MRS broth + 10% banana peel extract

Flask 2: MRS broth + 10% banana peel extract + 5 mL inoculum

Flask 3: MRS broth + 10% banana peel extract + 5 mL inoculum + 50 mL of Malt extract

* The titration was carried out with two dilutions for the sample (1:1 i.e., 5 mL sample and 5 mL distilled water and 1:9 i.e., 1 mL sample and 9 mL distilled water).

During fermentation, the rate of lactic acid concentration increase dramatically with addition of malt extract which indicates that the rate of glucose consumption is highest in case of production medium compared to the control with and without inoculum. Similar results were obtained by Balmakki *et al.*, (2016) showed that the enzymatic hydrolysis yield was significantly improved.

Conclusion

The required bacterial colonies were isolated from milk sample (Ergo) by serial dilution method by gram staining, endospore staining and biochemical tests. The culture was inoculated into three different mediums which include control without inoculum for 0 to 96 hours respectively. Percentage of crude lactic acid was increased dramatically in malt extract containing fermentation medium compared to with and without malt extract. Decolorization by activated charcoal was performed to increase percentage of lactic acid yield.

Significance of the study :

The significance of this study can be seen from five different angles.

- The first one is accompanying with economy. If poly lactic acid (PLA) will be produced in Ethiopia, then the cost will be incurred for importing PP, LDPE, HDPE, PVC, PU, PS and other will be greatly reduced. That means production of lactic acid from available renewable resource material in Ethiopia can be seen as huge import substitution.
- The second significance of this study is comprised on implementation of new technology in the country. As we know technology is transferring from developed countries to developing countries in transition of globalization. etc

Recommendation:

- Further, purification of lactic acid through column chromatography may give better production and yield.
- Addition of synthetic enzymes in fermentation production medium may help the further production and yield in the large scale synthesis.
- We performed the fermentation production in batch

culture, but in future, if we use continuous fermentation production method, we may get more production and yield at the industrial level.

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