Egypt. J. Chem. **Vol. 68**, No. 7 pp. 17 - 31 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Sustainable Production of Lactic Acid by *Enterococcus Gallinarum* from Agricultural Wastes

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Abstract

Recently, there has been a great deal of interest among researchers in the production of lactic acid and its conversion to polylactic acid. Environmental issues such as global warming and plastic pollution are forcing society to look for alternatives to traditional plastics. Polylactic acid (PLA), made from lactic acid (LA) and one of the most famous environmentally friendly biodegradable bio-based polyesters, has been extensively studied for its applications and is a promising candidate for petroleum-based plastics. It is considered a good alternative. The goal of this research was to produce lactic acid from agricultural wastes as a low-cost, renewable substrate and to produce the most commercially successful bioplastic from agricultural wastes. Eighteen bacterial strains were isolated from agricultural wastes (cotton waste, banana waste, potato waste, and Nile Flower). Chemical hydrolysis of agricultural wastes is carried out using hydrochloric acid, sulfuric acid, and sodium hydroxide. Cotton and banana waste produced the highest concentrations of total reducing sugars of 6568.99 and 7460.37mg/L respectively, through chemical hydrolysis. Biological hydrolysis of these wastes was also achieved. Optimal conditions for lactic acid production have been promoted as temperature of 30◦C, pH 7, inoculum size of 10% (v/v), and agitation rate at 150 rpm under the stationary growth incubation of 24 h. The most promising bacterial isolate for lactic acid production was identified by 16S rRNA as *Enterococcus Gallinarum* with similarity of 99%. The isolate *Enterococcus Gallinarum*, recommended for using in lactic acid production, showed the highest lactic acid concentrations of 4.516 g/L after 24 h, as qualitatively determined by high performance liquid chromatogram (HPLC) analysis.

Keywords: Agricultural Wastes; Hydrolysis; Fermentation; Lactic Acid production; Optimization; Polymerization; Polylactic Acid.

1. Introduction

Since lactic acid and polylactic acid have different applications in all fields, researchers have been increasing the production of lactic acid from different sources every year. Currently, biodegradable plastics are gradually replacing traditional petroleum-based plastics, which are expensive to produce, pollute the environment, and are harmful to human health[1].

In recent decades, the development of petroleum-based synthetic plastics has led to economic development and brought great benefits to human life. Global plastic production is predicted to reach 1.8 billion tons per year by 2050 [2]. Approximately 70 million tons of the 90 million tons of plastic produced by humans accumulate in the environment and end up breaking down into microplastics, making serious health risks [3].Plastic trash on beaches and sea surfaces can be removed, but debris and microplastics floating in the ocean and on the seabed are difficult to remove [3].

Global warming is another problem that is caused by CO_2 as a greenhouse gases excessive emission. Concerns about natural resource conservation and climate change are believed to be motivating academic and industrial researchers around the world to reduce consumption and dependence on fossil fuels [4]. Due to environmental issues such as global warming and plastic pollution mentioned above, demand for alternative materials is increasing. This is one of the causes of improper waste disposal [5].

Many researchers have focused on converting wastes to value-added products in order to reduce wastes and environmental problems [6], [7], [8]. Society is trending towards introducing natural-based biodegradable polymers that are environmentally friendly to convert and consume. Therefore, biodegradable biobased polymers such as Polylactic acid (PLA) and itssynthetic raw material (Lactic Acid) have been attracting a great attention in recent years. Renewable biomaterials and biological substrates other than fuels are considered as potential replacements for petroleum-based products [9].

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Received date 02 October 2024; Revised date 30 December 2024; Accepted date 10 March 2025
DOI: 10.21608/EJCHEM.2025.323555.10566

Starchy and Lignocellulosic biomass such as agricultural and solid wastes, woody plants, and several industrial wastes are dry solids consists of two types of carbohydrate polymer, Cellulose and Hemicellulose, and lignin which is considered an aromatic-rich polymer; these carbohydrate-containing polymers contain varies sugar monomers (six and five carbon sugars) [10].

The above-mentioned substrates are used to produce sugars which could microbially be fermented into LA and fuels, acetate, ethanol, propionate, butyrate, lactate, hydrogen and so on, making that substances a good alternative production way for these economical products [11], [12],[13][14], [15]. Lactic acid fermentation, like any other fermentation process, depends on factors such as the raw materials used, the nutrients present in the culture medium, and the microorganisms used. Four different fermentation methods are practiced: batch fermentation, fed-batch fermentation, continuous fermentation, and repeated fermentation for LA production[16][17].

Biological production of lactic acid has important advantages over chemical routes. The advantage is that production costs can be reduced by using inexpensive raw materials rich in fermentable sugars [18], and reducing the usage of petrochemicals [19]. Chemical pathways involve the production of intermediate products derived from petrochemical feedstocks, which have negative effects on the environment [20]. Racemic D/L(±)-lactic acid is also obtained by chemical routes, whereas L(+)- or D(-)-lactic acid is obtained with high optical purity by microbial fermentation [21].

LA is produced by a variety of bacterial species, including Lactic acid bacteria (LAB) that are known as the major bacterial sources of LA production due to its high acid tolerance, high yield productivity, low fermentation temperature, and low contamination risk. Such LAB as *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Lactobacillus, Oenococcus*, *Leuconostoc*[22], [23], [24].*Bacillus* sp., *E. Coli*,*Corynebacterium glutamicum,* and *Weizmannia coagulans*can also produce LA [25][26].

LA, needed to reach USD 9 billion of annually growth in 2025 [13], has also been used as a good raw material for thepolylactic acid formation in recent years (materials industries)[27][28]. polylactic acid, also known as polylactide, has paid attention among researchers as the most commercially excellent biocompatible bio-plastics[29]. Briefly, has advantages such as environmentally friendly, and less to without carcinogenic effects [30]. As PLA consume less energy (25-55%) to produce than petroleum-based polymers [30], it is reported the most useful and biodegradable polymer in the industrial fields[31]. Polylactic acid (PLA) has different applications in all fields such as in agriculture, chemistry, and medicine[32], [33].

This article details the production of lactic acid as a valuable product from agricultural wastes by batch fermentation under aerobic conditions. We discuss parameters that increase lactic acid yield such as temperature (°C), agitation speed (rpm), pH, inoculum size, and incubation time. Furthermore, the chemical, biological, and physical pretreatments of the substrate were discussed. Additionally, the extraction, purification, and characterization of lactic acid produced from the fermentation medium were studied.

2. Material and Methods

2.1. Collection of agricultural wastes for lactic acid fermentation

There are four types of wastes were collected (Cotton whole plant, Banana whole plant without the fruit, Potato peel, Nile Flower whole plant) from an agricultural area at Fayoum governate, Egypt. The wastes were dried in an oven at temperature of 50 °C for 48 h and then milled, sieved and stored util used in the experimental sequences.

2.2. Isolation and purification of microorganisms

LB medium (g/L, Sigma-Aldrich, USA) was prepared with ingredients of 10 g tryptone, 5 g yeast extract and 10 g NaCl; per liter of distilled water. The pH was adjusted to 6.9±0.2. The medium was then autoclaved at 121 °C for 20 min. One gram of each substrate was added separately to 100 ml of sterile LB broth medium. Then, one ml of each solution was cultured in LB agar plates. Plates were put in an incubator for 24 h at 30°C under aerobic conditions. The growing colonies were selected and streaked onto sterile LB agar plates in order for purification. Eighteen pure bacterial isolates were purified on sterile LB agar for further testing.

2.3. Preparation of hydrolysates for fermentation

Generally, feedstock pretreatment and minerals need [34], [35] are the most important stages for reducing the particle size [36] to obtain definite sugars [37], and to get LA at high rate of fermentation. There are many pretreatment methods for lignocellulosic materials, termed as Physical, Chemical and Biological[27].

2.3.1. Physical hydrolysis

Physical pretreatment methods usually contain mechanical and thermal pretreatment. Physical pretreatment is done mechanically by cutting the all four substrates to decrease the size and to increase the surface area to make it easier contact of microorganisms with the substrate. The substrates are also dried in an oven at 50^{o}C [36], [38].

2.3.2. Chemical hydrolysis

In this study, 10% (w/v) of Cotton, Banana, Potato, and Nile Flower wastes were used, each was treated at various concentrations of 0.5g%, 1g%, 2g%, 3g%, 5g% (v/v) NaOH, 1-5% (v/v) H_2SO_4, and 1-5% (v/v) HCL for hydrolysis. Each type of substrate was autoclaved at 121^{o}C for 20 min [39], [40]. After hydrolysis, filtration is done to remove solid materials and the supernatant was neutralized at pH 7 and then the 3, 5-dinitro salicylic acid (DNS) method is used to calculate the total reducing sugars (TRS) using UV spectrophotometer at 540 nm wavelength [41]. Here the standard is glucose. Hydrolysates were then refrigerated at 5–7 ^{0}C until used for lactic acid release.

2.3.3. Biological hydrolysis

Biological pretreatment by eighteen bacterial isolates was achieved. About 10g of banana and cotton waste were moistened with 20 ml of distilled water and autoclaved for 20 min at 121 °C. Then, each of these substrates were inoculated with 5 ml of eighteen bacterial isolates, placed in a shaking incubator at 37 °C/150 rpm for 48 h, filtered, and the reducing sugars produced from each substrate were calculated.

2.4. Reducing Sugars Quantification Using DNS (3,5-Dinitrosalicylic Acid) Assay

The experiments were done according to Miller [41]. 1 ml of hydrolysate is added to 1 ml of DNS (3,5-dinitrosalicylic acid) reagent. Then, the mixture was heated at 100 °C for 10 min in water bath, and cooled before being mixed with 4 ml of deionized water. The residual reducing sugar content of the fermentation broth was calculated by UV spectrophotometer at 540 nm.

2.5. Screening the best concentration (alkali or acid), substrate, and isolate producing the highest TRS for further LA processes.

Depending upon the study of El-Sheshtawy *et al*. 2022, it was reported that the higher the reducing sugar, the better thelactic acid content. In the present study, the different types of concentrations (alkali or acid) and substrates were put in comparison to investigate the highest TRS yield. After that, the hydrolysate containing the highest TRS Conc. was injected by all bacterial isolates to select the best isolate for LA production using the best substrate with the best concentration.

2.6. Identification of the most potent bacterial strain using 16S rRNA sequencing

The 16S rRNA by Sigma Scientific Services Co., Egypt has been used to identify the *Enterococcus Gallinarum*. The bacterial cells were collected in the enriched medium up to 2 x 10^9 cells, then the DNA was extracted through the protocol of the Gene Jet genomic DNA purification Kit (Thermo) (Sigma Scientific Services Co., Egypt). For amplification of the 16S rDNA genes, a polymerase chain reaction (PCR) was used by (50-AGAGTTTGATCCTGGCTCAG-30) (50-GGTTACCTTACGACTT-30) as forward and reverse primer respectively. The PCR product was purified by the Gene-JET™ PCR Purification Kit.

Then, 45 µL binding buffer was added in the PCR mixture. This mixture was transferred to the Gene- JET™ purification column and centrifuged for 30–60 s at >12000×g, and then the flow were removed. After that, a 100 µL wash buffer was added to the GeneJET™ purification column, centrifuged for 30–60 s to remove the flow, and placed the purification column back into the collection tube. The mixture was centrifuged again for 1 min at an empty GeneJET™ purification column to get rid of any further wash buffer. The purification column was sent to a clean 1.5 mL microcentrifuge tube with adding 25 µL of elution buffer, centrifuged for 1 min. Finally, discarding the column and the purified DNA was stored at -20 ^{o}C. After the PCR products' purification, the positive clone's DNA sequence was subjected to a matched search called BLAST on the website of NCBI (http://www.ncbi.nlm.nih.gov) and stored in the GenBank. Several 16S rRNA gene sequences with validate published names were taken to be references from the Gen- Bank.

2.7. Lactic acid quantification using HPLC

HPLC Thermo Scientific Dionex Ultimate 3000 Series equipped with photo diode array detector is used for quantification of the LA produced at 250 nm wavelength. The column C8 (250 mm x 4.6 mm, Egyptian Petroleum Research Institute, Egypt) was equilibrated in 50:50mixture of methanol and water (mobile phase) with a flow rate 1 mL/min at 25 ^{o}C, and an injection volume of 0.02 ml [42] .

2.8. Optimum conditions for lactic acid production by the most promising bacterial isolate

Five parameters were investigated to maximize LA production. Each parameter was examined at four levels. pH was 5, 6, 7, and 8; temperatures were 25, 30, 35, and 40 °C; inoculum sizes were 2.5 % v/v (1.25 x 10^6 CFU/ml), 5 % v/v (2.5 x 10^6 CFU/ml), 7.5 % v/v (3.75 x 10^6 CFU/ml), and 10 % v/v (5.0 x 10^6 CFU/ml); incubation periods were 24, 48, 72, and 96 hours; and the agitation speeds were 100, 150, 175, and 200 rpm.

2.8.1. Influence of different pH values on lactic acid Production

The pH, as well as temperature, are important factors in lactic acid fermentation[27]. Four Screw capped bottles (100 ml) containing 50 ml of fermentation medium obtained from 3% (v/v) H_2SO_4 hydrolysis of banana waste after autoclaving at 121 °C for 20 min are used. The pH was adjusted to 5.0, 6.0, 7.0, and 8.0 using 5% (w/v) $Ca(OH)_2$. Bottles were inoculated with 10% *Enterococcus Gallinarum* and incubated at a temperature of 30 °C under agitation speed of 150 rpm. Measurements of the produced lactic acid were recorded after 24 h of incubation using HPLC analysis.

2.8.2. Influence of temperature on (LA) production

The variability in temperature should be taken in consideration for enhancement of the microbial growth and lactic acid concentration [43]. The above optimum pH was done here at 5 various temperatures (25, 30, 35, and 40°C) with 150 rpm and the lactic acid produced was estimated after 24 h of incubation and 10% of inoculation with the *Enterococcus Gallinarum*.

2.8.3. Influence of different inoculum size on (LA) production

Many studies reported inoculum sizes between 1-10% (v /v) [36]. Here we studied the different inoculum sizes of the bacterial strain (2.5%, 5%, 7.5%, and 10% (v/v)) under the above recorded optimum pH and temp. The lactic acid production was measured after 24 h incubation on 150 rpm.

2.8.4. Influence of different incubation periods on (LA) production

To find out the optimum incubation periods for maximal lactic acid generation, the hydrolysate was incubated at 24, 48, 72, and 95 h respectively under the above optimized conditions of (pH, temp, and inoculum size). At the end of each incubation period, lactic acid released was estimated via HPLC technique.

2.8.5. Influence of rpm on lactic acid Production

To monitor the effect of agitation rate on the lactic acid fermentation, different rpm at (100, 150, 175, 200) were chosen under the above optimized conditions and the lactic acid yield was estimated after 24 h using (HPLC).

2.9. Lactic acid production by fermentation process

Lactic acid production on Fermentor-scale was completed after LA optimization using the selected most potent bacterial strain (*Enterococcus Gallinarum*). 600 g of banana waste was hydrolyzed with 6 L of 3% H_2SO_4 (v/v). The flask containing wet banana was autoclaved at 121°C for 20 min. After hydrolysis, the solid wastes were removed from the flask by filtration, and the supernatant is neutralized (pH of the Fermentation is 7.0) with an effective neutralizing agent such as $Ca(OH)_2$ to generate calcium lactate and water [44], [45]. Sterilization of the banana waste hydrolysate was proceeded in the autoclave system. After the sterilization step, the fermented broth was cooled, then inoculated with 10% (v/v) of the bacterial isolate. The batch fermentation was incubated under aerobic conditions at 30 °C and 150 rpm for 24 h. The produced lactic acid was reported using HPLC analysis and the highest concentration of lactic acid was obtained.

2.10. Downstream processing (Extraction, Purification, and Quantification of Lactic Acid)

To dispose cellular biomass and contaminants such as production microorganisms, proteins, polysaccharides, inorganic ions, and organic acid byproducts, the fermentation medium was centrifuged at 8000 rpm for 10 min. The supernatant was filtered, the activated carbon (5% w/v) was added to the filtrate to remove colored impurities, and the mixture was gently stirred for 1 h and centrifuged for activated carbon removal. Finally, this cell-free supernatant was used for purification of LA.

In this study, solvent extraction method was usedfor LA downstream processing[46]. The best lactic acid recovery was done in a pH 2.5 medium using concentrated HCL. The supernatant containing lactic acid (10 ml) was placed in a flask. Ammonium sulfate (5g) was added and completely dissolved. Additionally, 30 mL of n-butanol was added for proper mixing, followed by shaking at 30 °C for 2 h. The mixture was then transferred to a separatory funnel and allowed to stand until the aqueous and organic phases are separated. After phase separation, the upper organic layer was evaporated on a rotary evaporator at 50 °C. The lactic acid obtained in the dry organic layer was dissolved in 5 mL distilled water, centrifuged at 10,000 rpm for 10 min, and the clear supernatant was used for lactic acid measurement by HPLC.

2.11. Fourier transformation infrared (FT-IR) spectral analysis

The FTIR spectrum of the lactic acid produced after further processing is compared with a standard lactic acid sample to identify the functional groups of the purified lactic acid. The spectra were collected using Nicolet IS-10 spectroscopy, German, within the range of wavelength between 4000 and 400 cm^{-1}. This powder was mixed with KBr powder and pressed in a vacuum to obtain 0.5 mm thickness of a homogeneous disk. The concentration of powder in the sample, 2% , was calculated based on KBr [47].

2.12. Flowchart for the production of lactic acid

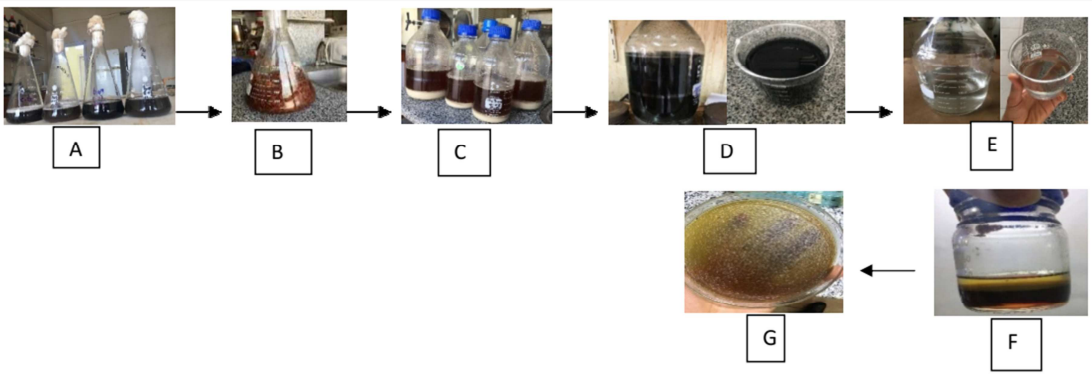

Figure 1: Flowchart for lactic acid production. (A) Shows the chemical acid hydrolysis of Banana and Cotton wastes with 1% HCL and 3% H_2SO_4. (B) Shows 3% H_2SO_4 of Banana Wastes. (C) The hydrolysate of the Banana wastes in Acid Medium. (D) The hydrolysate of the Banana wastes after adjustment to the optimum pH (7.0). (E) The Purified Hydrolysate after addition of the Activated Carbon. (F)The mixture of the aqueous phase and the upper organic phase containing lactic acid. (G) The purified Lactic Acid as the final product.

3. Results and Discussion

The raw materials used in the production of lactic acid are currently considered one of the most common challenges in biological production routes and represent a major issue for production prices. Many papers have focused on the possibility of producing lactic acid from inexpensive materials such as food, industrial, and agricultural wastes[48], [49], [13]. Therefore, the solution proposed in this study is to produce lactic acid from agricultural waste.

3.1. Isolation and purification of bacterial isolates

In this study, eighteen bacterial isolates were isolated from banana, cotton, Nile flower, and potato wastes using LB media. Among several isolates, these strains showed the highest growth rates in culture and were purified for further studies. Many researchers have studied and isolated several microorganisms from different kinds of wastes for lactic acid production [50-52].

3.2. Feedstock Pretreatment

After calculation the total reducing sugar (TRS) by DNS method, the amount of (TRS) produced by chemical pretreatment is higher than those in other types of pretreatments, especially after physical pretreatment that provides small molecule waste products. The TRS concentrations obtained from hydrolysis by 3% H_2SO_4 and 1% HCL are higher than those produced by NaOH (Fig. 1). In addition, it is considered that the TRS concentrations in banana and cotton wastes were higher than those in potato and Nile flower wastes, as shown in (Figures 3-6). Finally, it was figured out that the highest total reducing sugar concentration of 7460.37 mg/L in banana waste was obtained by 3% H_2SO_4 using DNA assay. The highest amount of TRS produced can vary according to not only the concentrations of the alkali or the base, but also the type of substrate. Many studies proved that the TRS produced from acid hydrolysis is higher than that is produced form alkali hydrolysis [50], [53].

Biological pretreatment of banana and cotton waste with eighteen bacterial strains resulted in low TRS concentrations, so there are no further processes involved in this type of hydrolysis, as seen in (Fig. 7). Also, Asoaka et al., 2011; Kosarik et al., 2011 Reported that fermentable sugars produced through chemical hydrolysis is higher than those from the enzymatic process because of its ability to breakdown the cellulose is better than enzymatic breakdown [54].

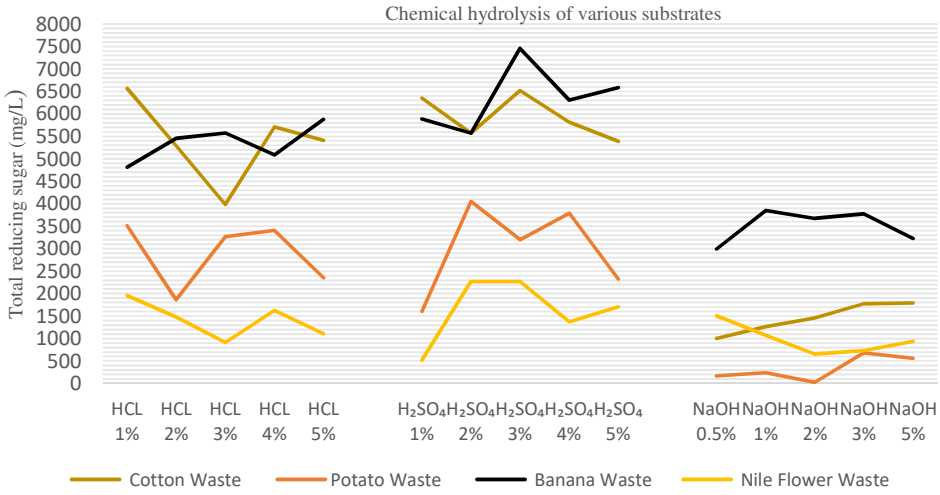

Figure 2: Comparison between the amount of total reducing sugar (mg/dL) produced by various substrates after chemical hydrolysis.

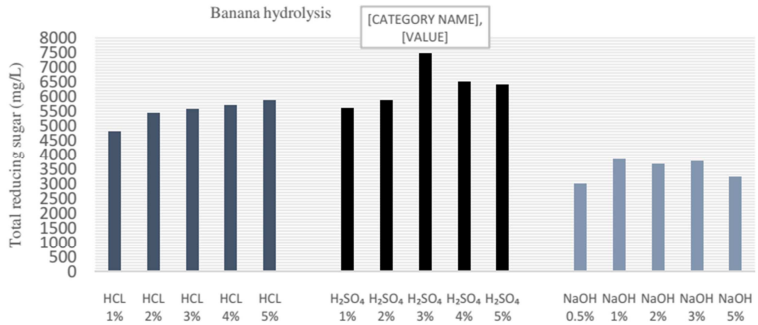

Figure 3: Total reduced sugar produced by HCl, H_2SO_4, and NaOH hydrolysis of banana wastes.

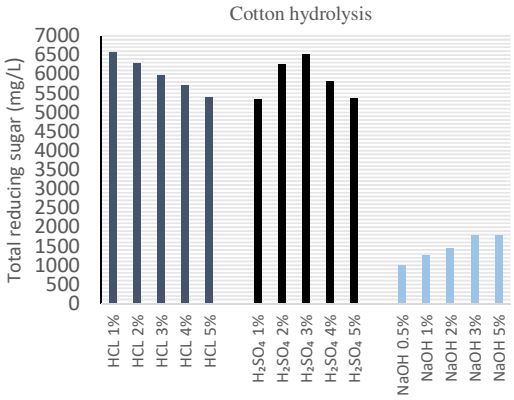

Figure 4: Total reduced sugar produced by HCl, H_2SO_4, and NaOH hydrolysis of cotton wastes.

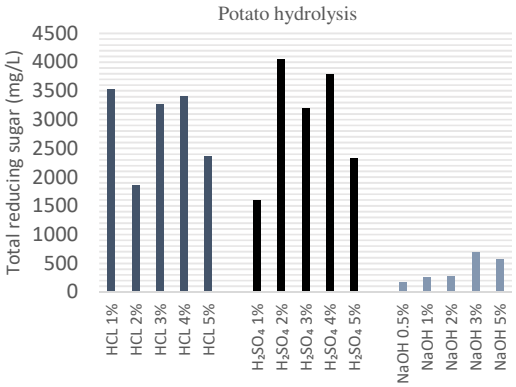

Figure 5: Total reduced sugar produced by HCl, H_2SO_4, and NaOH hydrolysis of potato wastes.

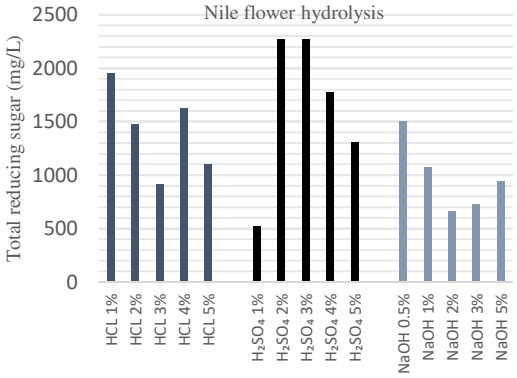

Figure 6: Total reduced sugar produced by HCl, H_2SO_4, and NaOH hydrolysis of Nile Flower wastes.

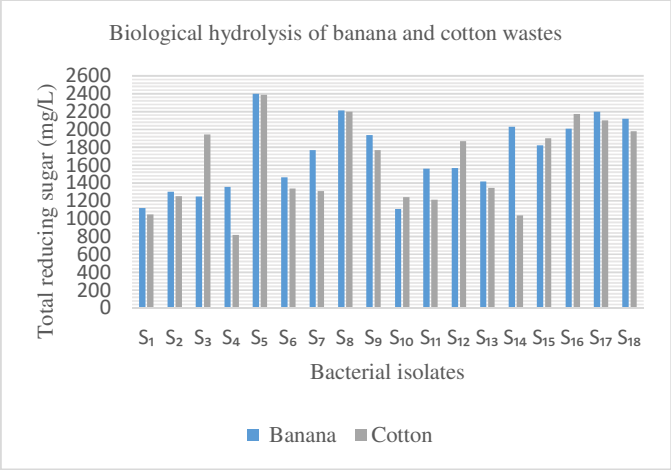

Figure 7: Total reduced sugar produced by biological hydrolysis of banana and cotton wastes by all 18 isolates.

3.3. Testing the ability of bacterial isolates for producing lactic acid

Eighteen bacterial isolates showed different abilities for total reducing sugar (TRS) production. As concluded that the hydrolysate of banana and cotton wastes produced by 3% H_2SO_4 and 1% HCL hydrolysis gave the highest conc. of TRS, their hydrolysates were injected by these isolates to produce lactic acid. Finally, using HPLC Analysis bacterial strain (S_5) showed the highest LA concentration at 4.516 g/L within 24h after 3% H_2SO_4 Banana hydrolysis as shown in Fig. 8. And then it was selected for upcoming studies.

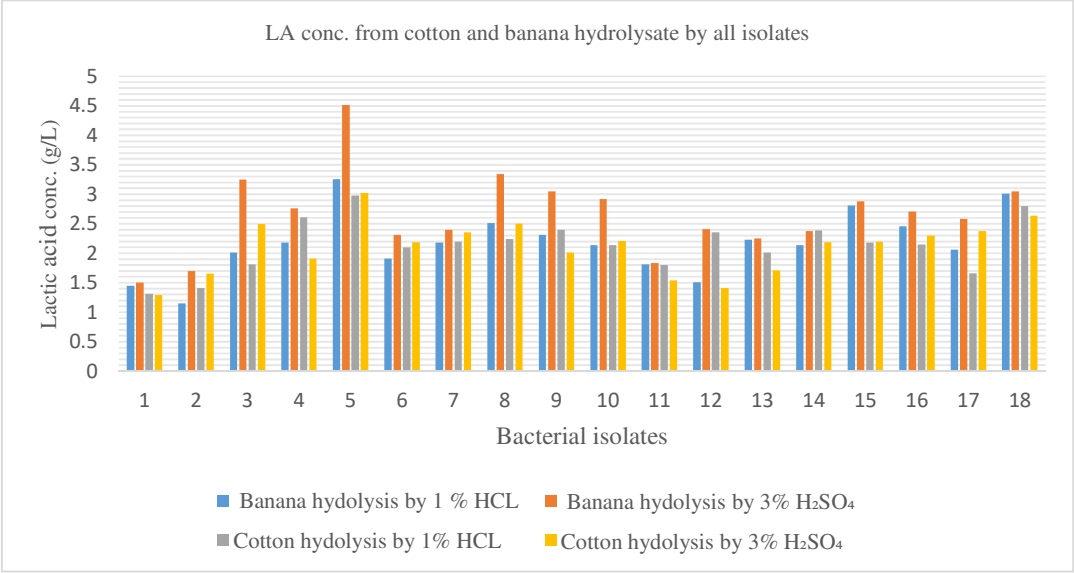

Figure 8: Lactic acid production by acid hydrolysis (3% H_2SO_4, 1% HCL) of banana and cotton wastes using all 18 bacterial isolates.

3.4. Identification of the most potent bacterial strain using 16S rRNA sequencing

Morphological characterization of the selected bacterial strain after 24 h incubation at 30 °C in LB solid medium is performed as *Enterococcus Gallinarum* that is constituted by Gram-positive, catalase-negative, non-spore-forming cocci, mesophilic (can grow in wide range of temperature between 10-45 °C), facultative anaerobic lactic acid bacteria [55].The most potent lactic acid-producing bacterial isolate (S_5) was identified by 16S rRNA. The NCBI blast identification procedures showing sequence identity as compared to established sequences in the NCBI database by the 16s rRNA are found in Table 1. The sequence of the isolate confirmed that it is 99 % similar to *Enterococcus Gallinarum* Fig 9.

Table 1: NCBI blast results showing sequence identity with the 16s rRNA

Scientific Name	*Enterococcus Gallinarum* Strain LMG 13129
Max Score	1585
Total Score	1585
Query Cover	100 %
E Value	0.0
% Identification	99.88 %
Accession Number	NR 104559.2

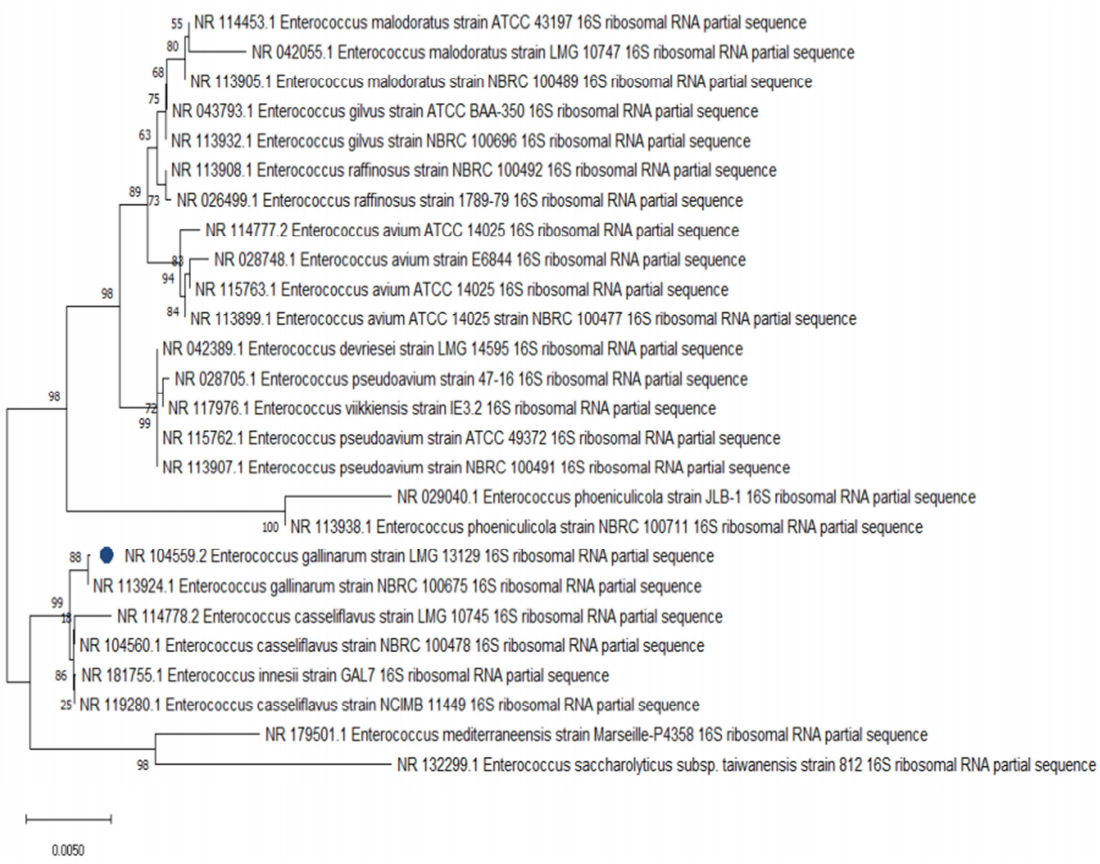

Figure 9: A Phylogenetic tree showing the phylogenetic position of *Enterococcus Gallinarum* sp. strain based on 16S rRNA gene sequences of closely related reference bacterial strains retrieved from the NCBI 's GenBank database. Digits are shown with nodes at bootstrap values (1000 replicates).

3.5. Optimum conditions of *Enterococcus Gallinarum* for producing lactic acid

To achieve high yields of lactic acid, optimal conditions of *Enterococcus Gallinarum* (pH, temperature, inoculum size, incubation time, agitation speed) have been prescribed [56], [57].

3.5.1. Effect of different pH values on (LA) production

For optimization, fermentation medium was adjusted to different pH values (5.0, 6.0, 7.0, and 8.0) and stored in a shaking incubator at 30 °C with rotating speed 150rpm. Lactic acid yield was recorded after 24 h of 10% (v/v) inoculation with the bacterial isolate. The results of lactic acid production at different pH values were showed Fig. 10. From pH 5.0 to 6.0, lactic

acid amount increases dramatically, but after an optimum pH of 7.0, lactic acid production decreases until pH 8.0 is reached. In this section, the best value of lactic acid concentration was achieved at pH 7.0 (3.716 g/L).

Let's prove our results. However, it is unclear what the optimal pH is, the pH for LA production has been reported to range from 3.2 to 11 [58], [59]. There was no significant difference between the amount of LA at optimal pH 8.0 (29.99 g/L) and the amount of LA at pH 7.5 (29.36 g/L) [57]. Similarly, 95 % lactose conversion (w/v) corresponding to lactic acid production was suitable at pH 6.5 [60]. Also El-Sheshtawy *et al*. reported that the Maximum LA was achieved at optimum pH (7.0) [50]. Finally, the higher value of lactic acid production using cafeteria waste was obtained at a pH of 7.29 [61]. All of the above results suggest that pH 7.0 is optimal for maximizing lactic acid production from hydrolysate of Banana wastes by *Enterococcus Gallinarum*.

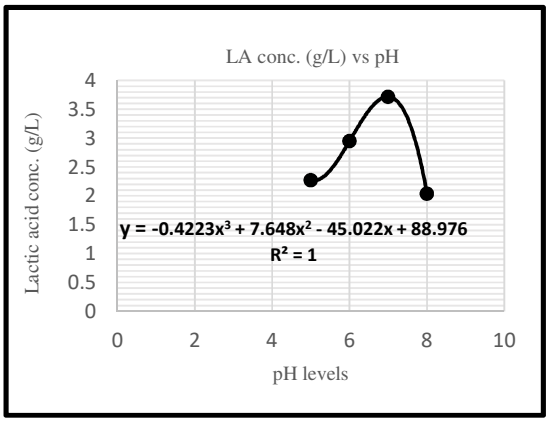

Figure 10: Effect of different pH values on the lactic acid production by *Enterococcus Gallinarum*

3.5.2. Effect of temperature on lactic acid production

To find the optimum temperature for lactic acid production, the experiment was performed at temperatures of 25 °C, 30 °C, 35 °C, and 40 °C, and a rotation speed of 150 rpm at the above optimum pH (7.0) of fermentation medium. The lactic acid production was estimated after 24 h of 10%(v/v) inoculation with *Enterococcus Gallinarum*. The results showed that the lactic acid amount increased sharply when the temperature increased from 25°C up to 30°C; and maximum production was found to be (3.346 g/L) at 30°C. Then, there was a decrease at 35 °C, and 40 °C (Fig. 11).

The optimal temperature for lactic acid fermentation differs according to the substrates and inoculum size used. The highest lactic acid titer and productivity are achieved between 30 and 43 °C [62]. *Lactobacillus amylophilus* released LA at the highest yield and productivity under 35 °C, and *Escherichia coli mundtii* produced the highest lactic acid titre and yield within 30-43 °C [63]. *Rhizopus oryzae* showed an enhanced lactic acid production when the temperature increased from 22 to 30 °C during hydrolysis of substrate and 30–40 °C during fermentation [64]. Finally, El-Sheshtawy *et al*. and Xavier, J.R., et al. reported that the maximum LA was achieved temperature 30^{o}C [50], [52] respectively.

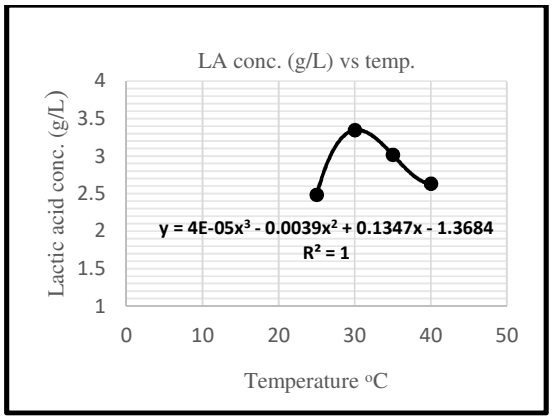

Figure 11: Effect of different temperatures on the lactic acid production by *Enterococcus Gallinarum*.

3.5.3 Effect of different Inoculum Sizes on (LA) production

Generally, an inoculum of 5–10% (v/v) is used for optimal LA production. Here, different inoculum sizes (2.5, 5, 7.5, and 10.0 % v/v) were used to optimize the inoculum concentration for lactic acid production. The Lactic acid production gradually increased as the inoculum concentration increased up to 7.5% (v/v). Maximum lactic acid production of 3.562 g/L was observed after addition of 7.5% (v/v) inoculum with *Enterococcus Gallinarum*. After that, the production decreased despite increasing the inoculum concentration to 10% (v/v) **Fig. 12**.

Small inoculum size may result in insufficient biomass, whereas large inoculum size may result in nutrient depletion or mass transfer limitation [65]. A maximum lactic acid production was observed with the addition of 7% (v/v) inoculum of sugarcane molasses by isolated *Lactobacillus bacteria*[57]. *Lactobacillus casei* was studied at 3–8% (v/v) inoculum size for rice straw fermentation, and the optimal inoculum size was 6% (v/v) [66]. The above studies showed that the 7.5 % (v/v) would be the optimum inoculum size used for LA production by *Enterococcus Gallinarum*.

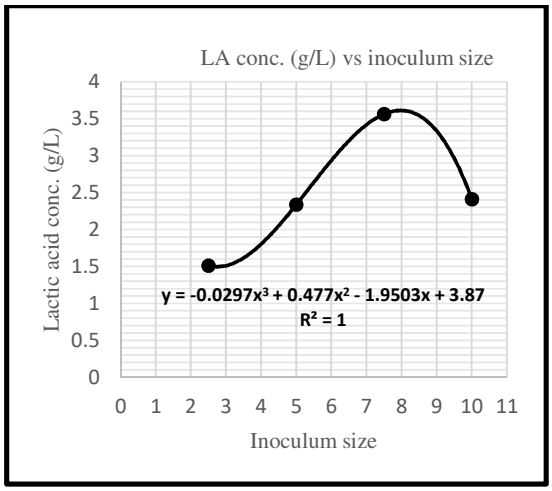

Figure 12: Effect of different inoculum sizes % (v/v) on the lactic acid production by *Enterococcus Gallinarum.*

3.5.4. Effect of different incubation periods on (LA) production

To find out the optimum incubation period for the maximal lactic acid production, the fermentative medium inoculated with bacterial isolate (7.5% v/v) was incubated for 24 h, 48h, 72h, and 96 hrs respectively on 150 rpm under the found above optimized conditions (pH 7.0 and Temp. 30^{o}C). At the end of each incubation period, lactic acid produced was estimated by HPLC. The results showed that highest level of lactic acid production was obtained within 24h (3.891 g/L), followed by a decrease in lactic acid production after 96h of incubation (Fig. 13). Therefore, in our study, a 24h incubation period was considered optimal for lactic acid production.

The optimal incubation period for LA production depends on several factors, including the Substrates and the isolates used in the process. The incubations time of 24h was considered the optimum period of production of lactic acid (from cow-milk whey by *Lactobacillus delbruckii*[67]. Another study proved that the incubation time of 120 h was the optimal for lactic acid production [68]. Many researchers mentioned that incubation period of 48 h has been generally used for lactic acid production using various lactobacilli cultures [69], [70]. The 144h is considered the optimum Incubation time for lactic acid production from cane sugar by *Lactobacillus spp.*[57].

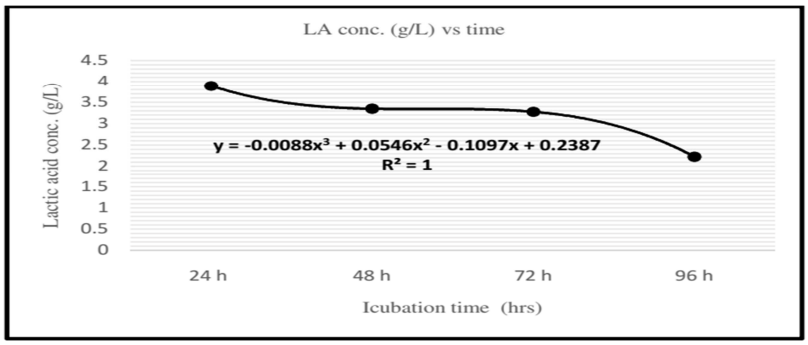

Figure 13: Effect of different incubation periods on the lactic acid production by *Enterococcus Gallinarum.*

3.5.5. Effect of different agitation speed (rpm) on lactic acid production

To investigate the effect of stirring speed on maximum lactic acid production, different speed values of (100, 150, 175, and 200) were set sequentially to the fermentation system respectively under the found above optimized conditions and the lactic acid production was measured after 24 h. The LA curve increased till reaching 150 rpm, and at lower (rpm) levels, a decrease in the productivity of (LA) was investigated.

In this study, the data in Fig. (14) showed that the highest production of lactic acid reached 4.516 g/L at 150 rpm which is matched with the study. Many studies have proved that the optimum (rpm) for LA production from whey as a substrate by using isolated *Lactobacillus* from curd sample is 150 rpm [68]. Also the maximum lactic acid production was obtained at 150 rpm form wheat wastewater by *Rhizopusoryzae*[71].

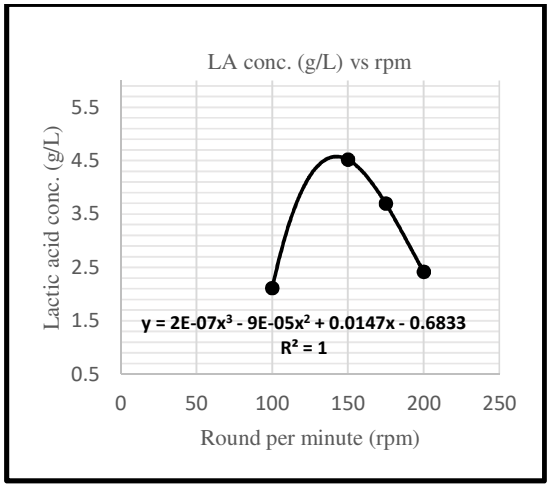

Figure 14: Effect of different agitation rate on the lactic acid production by *Enterococcus Gallinarum*.

3.6. Lactic acid production by the fermentation process

Six liters of the banana waste hydrolysate from (3%H_2SO_4) hydrolysis were sterilized under the above-mentioned optimum conditions of pH 7, temperature at 30◦C, 150 rpm and 10% (v/v) inoculum size of *Enterococcus Gallinarum* under 24h of incubation time. The yield of LA measured by HPLC would be higher when chemical hydrolysis was followed by enzymatic hydrolysis [72-74]. Generally, that process of pH adjustment typically produces gypsum (calcium sulfate which must be disposed of as solid waste with limited use) as a byproduct from the reaction of calcium lactate with sulfuric acid.

In this study, it was concluded that the maximum lactic acid production achieved in the stationary growth phase of the strain in batch fermentation was 4.516 g/L. Fig. (15) showed HPLC chromatogram sample of lactic acid during the fermentation process at pH 7.0 after 24 hours of incubation. The retention time of lactic acid appeared to be approximately 3.070 min. Those results can be slightly similar to the lactic acid as a maximum concentration (6.21 g/L) produced from agro-wastes using *Lactobacillus plantarum*[51].

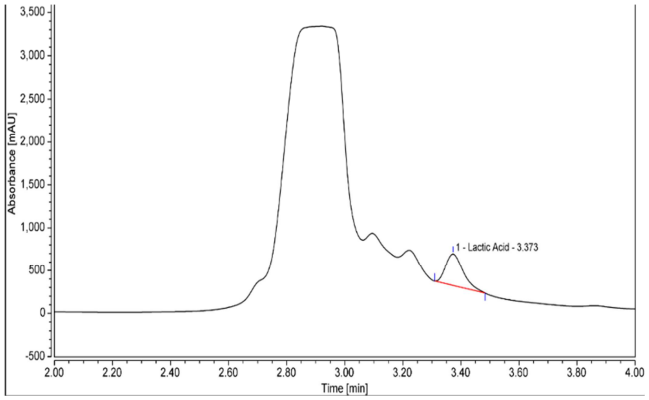

Figure 15: An HPLC chromatogram of lactic acid production during the fermentation process.

3.7. Downstream processing

3.7.1. Lactic acid (LA) extraction from culture fermentation

Generally, downstream lactic acid extraction techniques have been carried out using a variety of methods, each with advantages and disadvantages. It is important to select high-efficient, and low cost-effective technologies that improve the yield efficiency of LA [45].

In this study, the solvent extraction method, as one of the most commonly used methods for lactic acid removal, is performed for lactic acid extraction from the fermentation medium [75]. The fermentation medium (solution) containing calcium lactate needs to be acidified with HCL (optimized to pH 2.5) to release the lactic acid from the salt for further recovery. Using ammonium sulfate can't affect the LA yield, it is contributed to better dispersion as the aqueous phase dissolves in butanol without ammonium sulfate. Additionally, the used butanol can be recovered during rotary evaporation and used again. Finally, the produced LA was yellow to colorless crystals, ready for its characterization by FTIR technique.

3.7.2. Spectrophotometry analysis by Fourier-transform infrared spectroscopy (FTIR) (Characterization of lactic acid)

FTIR spectra were also recorded to characterize the purified lactic acid. To characterize the purified lactic acid, FTIR spectroscopy was used to determine the wavenumbers of the functional groups of purified lactic acid. FTIR spectra pattern of standard lactic acid from Sigma and the purified was similar (Fig. 16, 17). The FTIR spectra of lactic acid sample purified from broth shows stretching frequencies for (-OH), (-CH), (-CH3), (C=O), (-CO) was 3370 cm^{-1}, 2985 cm^{-1}, 2883 cm^{-1}, 1742 cm^{-1}, and 1127 cm^{-1} respectively. Also, Zakariyah *et al.; and* EL-Sheshtawy *et al.* showed FTIR characterization of lactic acid that are slightly similar to the above-mentioned results [76], [50]. In one study, frequency stretching in the FTIR spectrum of purified LA was observed, demonstrating the effectiveness of FTIR spectroscopy as a means of detecting lactic acid in fermentation media [47].

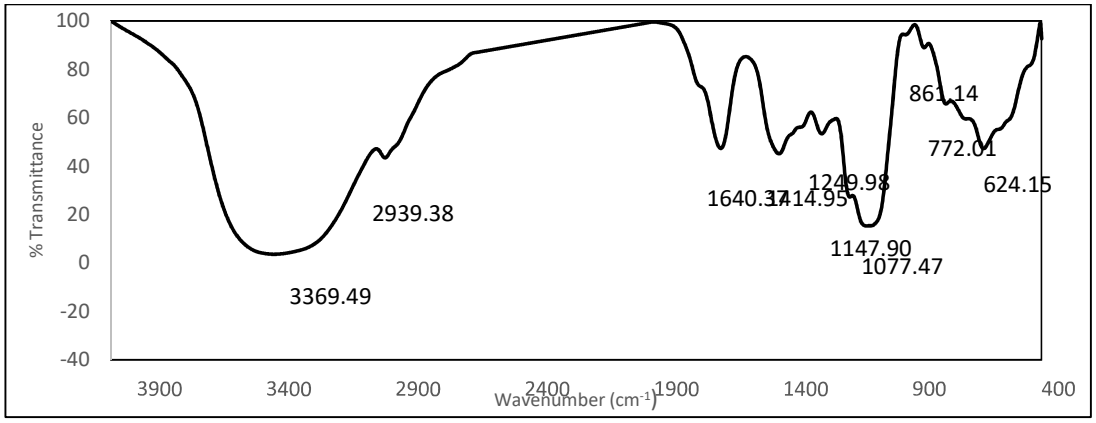

Figure 16: FTIR Spectrum of the Standard lactic acid.

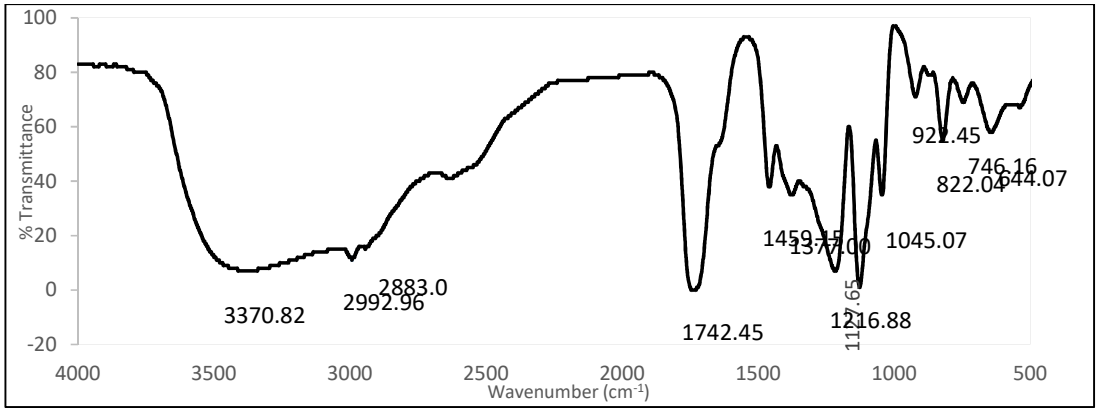

Figure 17: FTIR spectrum of the purified lactic acid lactic acid

Conclusions

In this study, the hydrolysate of banana wastes as an agricultural waste was selected as the best carbon source for lactic acid production among other substrates (Cotton, Nile Flower, Potato) by the *Enterococcus Gallinarum* strain at laboratory scale compared with other strains. The treatment of the banana waste by different concentrations of acid or base provided that the 3% H_2SO_4 hydrolysis has shown the highest yield of reducing sugars and Lactic Acid. The effect of some factors such as temperature, pH, incubation period, inoculum size. and agitation rate on the production of lactic acid from banana waste by that strain explained that the maximum lactic acid production was obtained at 4.516 g/L as a qualitative determined with the optimum conditions of temperature of 30◦C, pH 7, inoculum size of 10% (v/v), and agitation rate at 150 rpm under the stationary growth incubation of 24 h. The LA produced was extracted by solvent extraction method as a high-efficient, and low cost-effective technique.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the research reported in this article.

Acknowledgments

The authors would like to thank the Egyptian Academy of Scientific Research and Technology (ASRT) for financial support through his SNG grant and the Egyptian Petroleum Research Institute (EPRI) for the experimental facilities. Cairo, Egypt.

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