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The Use of Sugar Cane Waste (Molasses) in the Production of Lactic Acid by *L. paracasei* CAU 9836 and its Identification by Infrared Spectrum (FT.IR)

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Abstract. A total of twelve bacterial isolates from different samples of yoghurt and milk purchased from local markets in Diwaniyah Governorate. The preliminary screening was carried out all bacterial isolates by gram staining. The six isolates were selected based on the intensity of colour variation in the fermentation medium and were identified according to phenotypic, microscopic, and biochemical examinations, which included twelve isolates of *Lactobacillus spp*. Vitek II Compact instrument was used with all isolates to confirm the initial identification. The secondary screening was performed for isolates of *Lactobacillus spp*.species. Given the amount of the produced lactic acid, the L.casei strain 1859 had the highest lactic acid production, which was 64.32 g/L. The 16S rRNA test allowed the identification of this isolate. The results showed that the best conditions for acid production were 62.76 mg/L and the L.casei strain 9836 grown on the alternative medium containing 50 % whey, 7% molasses, and pH 6.9, incubated on an orbital shaker at 38°C for 24 hours, and agitated at 250 rpm. FTIR techniques were used to identify the nucleus of the hydrogen atom (proton) ^{1}H and the carbon atoms ^{13}C in the presence of standard lactic acid for comparison. The results revealed the peaks and bands of the produced acid were some similar to that of the standard acid.

Keywords. CAU 9836, FTIR techniques, Molasses.

1. Introduction

Lactic acid is one of the most important organic acids found in nature and is used in many fields as a result of its distinct physical and structural properties and the fact that it is mainly produced from safe and harmless bacteria, which are lactic acid bacteria through microbial fermentation, whether it is homogeneous or heterogeneous according to the type and strain of the microbe used in the fermentation process, Creating the ideal fermentation conditions for bacteria through the ideal temperature, pH, incubation period, the concentration of the ideal production medium and the frequency of the vibrating incubator in order to achieve the best production under these conditions[1] .

As a result of the importance of lactic acid in the industrial, medical, food and commercial fields, the demand for it has increased significantly, which prompted researchers to intensify efforts and studies in developing production and extraction methods at low cost and from cheap and constantly available sources [2].

As agricultural waste and food factory waste, such as molasses, whey, date juice, molasses, decomposed corn and meat extract, were used as carbon and nitrogen sources as alternatives to the production medium for their low cost and high productivity of lactic acid in good quantities. The lactic

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acid produced from the fermentation process is diagnosed using several highly efficient and ideal techniques such as infrared spectroscopy FT-IR and high-performance liquid chromatography HPLC, Infrared spectroscopy FT-IR is one of the highly efficient methods for diagnosing lactic acid through effective groups in the structural structure of the acid such as carboxyl COOH, hydroxyl OH, methylene CH_2 and methyl groups CH_3[2,3].
Fourier transform spectroscopy is a less intuitive way to obtain the same information. Rather than shining a monochromatic beam of light (a beam composed of only a single wavelength) at the sample, this technique shines a beam containing many frequencies of light at once and measures how much of that beam is absorbed by the sample. Next, the beam is modified to contain a different combination of frequencies, giving a second data point. This process is rapidly repeated many times over a short time span. Afterwards, a computer takes all this data and works backward to infer what the absorption is at each wavelength[3,4].

2. Materials and Methods

2.1. Prepare of Samples

- Prepare 4 samples from sources of dairy products, raw milk, local yoghurt, dry milk and laboratory milk with appropriate decimal dilutions in order to grow lactic acid bacteria and extract lactic acid from these sources and you are the best bacterial isolate in acid production .
- Preparation of alternative production medium (molasses) fortified with whey and other nutrients, As well as preparing the standard medium for the fermentation process MRS Broth .

2.2. Isolation and Selection of LAB Strains

Colonies with a different appearance (based on color, shape and size) were extracted from the MRS Broth and purified by streaking on a fresh MRS Broth plate. The purification process was repeated until single colonies with distinct appearance were obtained. The pure isolates were tested for Gram and catalase reactions. Cell morphology was observed under the microscope. The isolates that were Gram positive and catalase negative were taken as presumptive LAB. The LAB isolates were stored at -20 °C in MRS broth containing 20% (v/v) glycerol until required for further tests [5].

2.3. Identification of LAB by Sequencing 16S rRNA

The 16S rRNA target region was amplified using DreamTaqTM DNA polymerase (Thermo ScientificTM, Waltham, MA, USA) and the primers 16S-27F (sequence 5′-AGAGTTTGATCMTGGCTCAG-3′) and 16S-1492R (sequence 5′-CGGTTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) products were gel extracted (Zymo Research, ZymocleanTM Gel DNA Recovery kit), and sequenced in the forward and reverse directions on the ABI PRISMTM 3500 x l Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-upTM kit) were analysed using CLC Main Workbench 7 followed by a BLAST search on the database of the JAPAN National Center for Biotechnology Information.11 Of the 16 LAB strains initially identified from emahewu using the API 50 CH kit, 2 were identified using the 16S rRNA method. I took out the dough Allbeckrat container and wash well, to offset the center distributed the dough on Filaskat (250 ml) and (250 g) of the beaker and one blamed these decanters with all its material, As *L.paracasei strain* CAU 9836 was diagnosed genetically by the previous method, and it was the best bacterial isolate in terms of lactic acid production from molasses medium and standard medium[6,7].

2.4. Determination of the Amount of Lactic Acid Produced from Alternative and Standard Medium

The amount of lactic acid produced from the alternative and standard medium was estimated using the most efficient lactic acid bacteria by using the standard curve of lactic acid, then the absorbance of the samples was measured by using Spectrophotometer UV with a wavelength of 560 nm through lactic acid constriction that standard and produced[8].
After the end of the fermentation period of the standard production medium and molasses, a centrifugation is done at a speed of 4000 revolutions / min for 15 minutes for the obtained solution with the addition of some chemicals as a modification of some previous studies to make the standard

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curve of lactic acid in order to measure and estimate the lactic acid in the alternative medium (molase) as It is shown as table (1).

Table 1. Show the values of standard lactic acid and distilled wate

Sample	Lactic acid volum (ml)	Water volum (ml)	Final Volum (ml)	Lactic acid Const (ml\l)
1	0,0	1.0	1.0	0.0
2	0.05	0.95	1.0	5.0
3	0.1	0.90	1.0	10.0
4	0.15	0.85	1.0	15.0
5	0.20	0.80	1.0	20.0
6	0.25	0.75	1.0	25.0
7	0.30	0.70	1.0	30.0

2.5. Steps of the Method for Estimating Lactic Acid

Add 100 µl of NaOH (1 N) to 200 µl of each concentration of the concentrations prepared in the table above and placed in sealed tubes, then the sealed tubes are incubated at a temperature of 38 C for 25-30 minutes, then add 2 ml of concentrated H_2SO_4 At a concentration of 70% for each tube separately and the tubes are sealed tightly, then the tubes are placed in a boiling water bath at 100 ° C for 5-7 minutes, then the tubes are cooled in an ice water bath and 20 microliters of $CuSO_4.5H_2O$ (aqueous copper sulfate) are added) With good and continuous mixing, then add 40 microliters of bromophenol reagent (2%) to each tube separately and close the tubes tightly, the tubes are placed in a vibrating incubator at a speed of 150 rpm for 20-30 minutes, After that, a Spectrophotometer (UV) is used at a wavelength of 650 nm to read the absorption for each concentration fig(1) (the process is repeated for three repetitions) [9,10] .

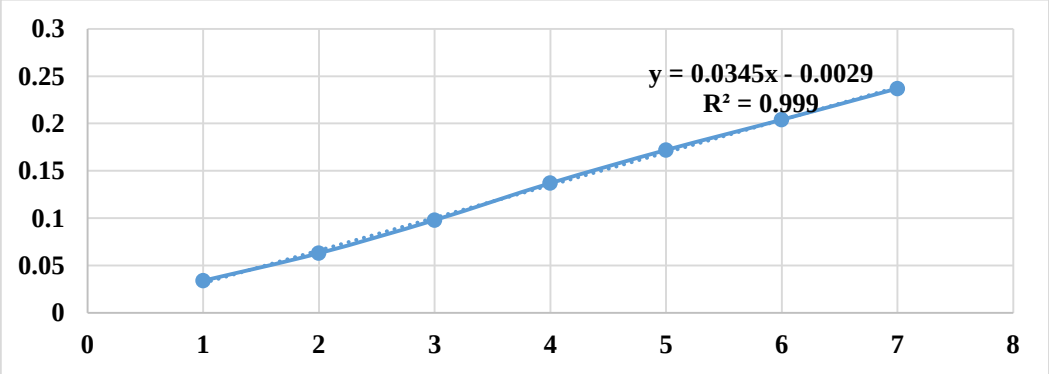

Figure 1. Showing the concentration of lactic acid by UV.

2.6. Identification of Lactic Acid using Infrared Spectrum(FT-IR)

This technique is very effective in identifying the active and active groups in the chemical compounds under study, and their presence is evidence of a good diagnosis and detection of the compound to be diagnosed, and in order to determine the active groups of lactic acid produced from the alternative medium by the bacteria L.paracasei strain CAU 9836 and the active groups of lactic acid The standard Fourier Transform Infrared Spectroscopy (FTIR) Shimadzu FT-IR Prestige-21 device located in the central laboratory of the College of Pharmacy / University of Kufa was used[11], and the examination was carried out by following the following steps:

- Weight of 185 mg of anhydrous potassium bromide KBr.
- Adding 50 microliters of each of the standard lactic acid samples and the lactic acid produced from the standard medium and the substitute to anhydrous potassium bromide separately.
- Mix the mixture well to make it solid.
- Grind the mixture well until it becomes a fine powder.
- The final amount of powder is placed in a double iron disc, and the disc is pressed and pressed well.

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- The sample is placed in the device for the purpose of reading the spectrum of the active aggregates at a wavelength of (400-4000) cm^{-1}.
- The readings are recorded for each of the three acid samples separately.

3. Results and Discussion

3.1. Isolation and Identification of LAB Isolated from Local Dairy Product

The results of the isolation of lactic acid bacteria showed that 12 isolates belonging to the genus Bacillus of lactic acid bacteria were obtained, 3 isolates of curd from the products of the Qadisiyah Dairy Factory, 3 isolates of raw milk from the Diwaniya cow station, and 3 isolates of local curd from the Diwaniyah markets. And 3 isolates of al-Shanina milk for the Taj al-Nahrain laboratory in Diwaniyah, as 6 bacterial isolates were isolated and diagnosed during the study, and they were classified and given the following symbols (L1, L2, L3, L4, L5, L6). For colonies growing on the culture medium MRS Broth of lactic acid bacteria, and then it was found that the use of culture medium MRS Broth led to the inhibition of the growth of spherical bacteria and the growth of lactic acid bacteria due to the decomposition of the compound Disodium B-Glycerophosphate Which works to lower the pH to less than 5.2, and as a result of the decomposition of this compound, the medium is suitable for the growth of lactic acid bacteria, as the pH is between (5.7-7.8) with very little growth observed for spherical bacteria that are resistant to conditions The middle[12].

3.2. Primary Screening of Bacteria Isolates

The primary screening of the 12 isolates obtained from the isolation processes and from different sources was carried out on the MRS Broth culture medium in order to diagnose these isolates. Used as starters for the manufacture of dairy milk products Based on the following examinations:

3.2.1. Morphological Tests

The phenotypic examination of the growing colonies showed that they are white to a creamy glossy color, and they are cylindrical, rod-like, rod-like, or spherical. There may be a little spherical, and they may be single or clustered in the form of chains, some of which have smooth convex edges, varying in size. Small and large ones, as shown in figure (2) as these characteristics were identical to the studies[1315].

Figure 2. Lactobacillus colonies on the culture medium MRS Broth.

3.2.2. Microscopic Tests

The results of the microscopic examinations revealed that all bacterial cells were positive for the Gram stain when stained with the Gram stain, as they appeared in violet color, in the form of long or short

cylindrical or rosary chains as shown in the figure (3), and that they are immobile, and these results were consistent with the study[16,17].

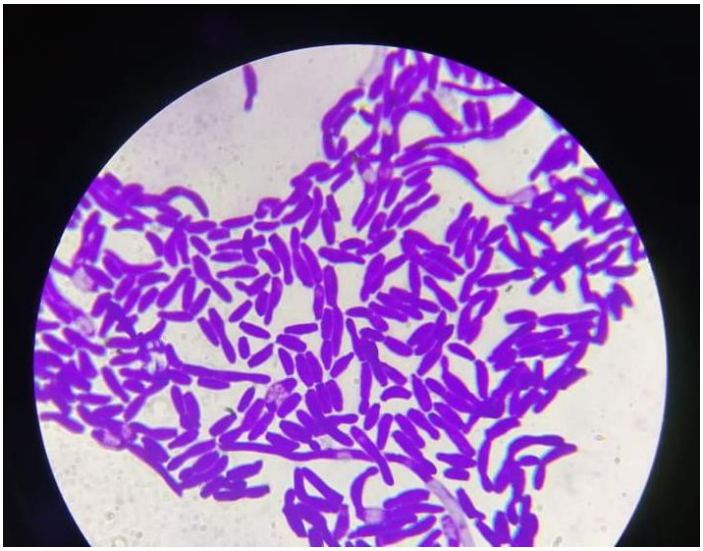

Figure 3. Gram-positive Lactobacillus cells under a light microscope.

3.2.3. Biochemical Tests

The results of the biochemical tests, as shown in Tables ( ) and ( ), revealed that all 6 bacterial isolates do not produce catalase and oxidase enzyme, that is, they are negative for the catalase and oxidase test, and the reason for this is that LAB does not have those enzymes that analyze hydrogen peroxide H2O2 To water and oxygen gas, which is the main cause of the appearance of bubbles when tested[18,19]. The results also showed that the aforementioned bacterial isolates were unable to consume citrate and produce indole, while the isolates were positive for the fermentation of carbohydrates as a carbon source through the production of gas, as they were able to ferment glucose, lactose and galactose, while they were unable to ferment xylose and arabinose and were positive in the whey hydrolysis test[20]. Which is a nitrogen source in the study, by the appearance of a transparent halo around the bacterial colonies, and it had the ability to grow in anaerobic conditions, and it was able to grow at temperatures (35-42) C, and it was able to grow at a pH number (5.4-8.2)[21.22]. And it was unable to grow in high salt concentrations (6-8)% and was able to grow in concentrations (2-5)%, and all of these results were consistent with[23].Table(2,3).

Table 2. Biochemistry of bacterial isolates obtained from different sources.

Symbol of isolation	Shape	Sugar Fermentation	Catalase	Oxidase	Citrate	Indol	Starch analysis	Whey analysis	Salinity tolerance	Gram stain
L1	Bacilli	+	-	-	-	-	+	+	+	+
L2	Bacilli	+	-	-	-	-	+	+	+	+
L3	Bacilli	+	-	-	-	-	+	+	+	+
L4	Bacilli	+	-	-	-	-	+	+	+	+
L5	Bacilli	+	-	-	-	-	+	+	+	+
L6	Bacilli	+	-	-	-	-	+	+	+	+

Table 3. Results of carbohydrate fermentation of bacterial isolates obtained from different sources.

Carbohydrates	*L. rhamonsus*	*L.paracasei strain CAU 9836*	*L. delbrueckii subsp.lactis*	*L. helveticus*	*L. reuteri*	*L. plantarum*
	L1	**L2**	**L3**	**L4**	**L5**	**L6**
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Xylose	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-

3.3. Molecular Assays for the 16S rRNA Test to Select the Bacterial Isolate with the Highest Lactic Acid Production

Figure (4) , The diagnosis was confirmed by using an examination on the 16S rRNA gene to diagnose the highest isolate in terms of lactic acid production, as the polymerase chain reaction (PCR) test was performed and the DNA extracted for the selected isolate, and then the concentration and purity of the DNA were measured and the results of amplification were shown The primers indicate the presence of the 16S rRNA gene by 100% in size (1500 bp), and the results were consistent and identical to the study[24.25]. Bacterial strains isolated from dairy products during the emergence of bundles at (1500 bp) belonging to *Lactobacillus spp.* , and that *Lactobacillus* spp.(ABST) is completely identical to *Lactobacillus paracasei strain*.CAU 9836 registered earlier in the International Genbank, and through the sequence of nitrogenous bases in the 16S rRNA gene amplification test and data analysis in the International Gene Bank NCBI, the ABST code was assigned to the *Lactobacillus bacterium spp*. It is a strain officially registered in the Japanese International Genome Bank[26].

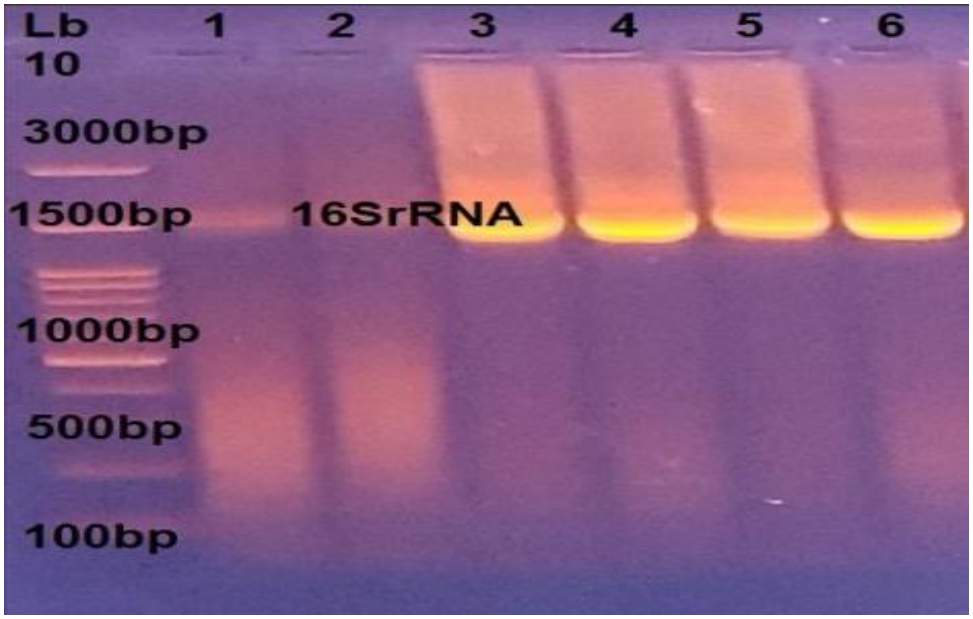

Figure 4. Electrophoresis on 1.5% acarose gel, voltage 100, voltage difference 80 ampere, for an hour and a half for investigation, Determination of the 16S rRNA gene by PCR of isolates of Lactobacillus spp. isolated from dairy products And Lb means Marker ladder 3000-100bp for lactobacilli and isolates (1-6) isolates of positive bacteria.

3.4. Media Production Effect

Two types of culture media were used in the production of lactic acid in order to make a comparison between them in terms of production, as the standard culture medium MRS Broth liquid was used first, and secondly the alternative medium (sugar honey) supplemented with whey was used as a nitrogen source in addition to calcium carbonate (CaCO3) by using isolation The genetically diagnosed bacteria (*L. paracasei strain*.CAU 9836) and the obtained results were to obtain the highest production of

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lactic acid by using the alternative medium (molasses) supplemented with whey and calcium carbonate, and the concentration of the acid was (62.51) g / liter, while the concentration of the acid in the medium was Standard MRS Broth Liquid (54.63)g/L, The reason for the difference and discrepancy in the results obtained is that the molasses medium contains reducing sugars by 58.24% and protein 2.6%, in addition to minerals and vitamins, and these substances are essential sources for the growth of bacteria and acid production in ideal proportions[27], as well as among those reasons is the concentrations in relation to the carbon and nitrogen sources, in addition to the presence of some compounds that stimulate and support metabolic products such as mineral ions, which leads to an effect on the concentration of lactic acid produced (Chen et al, 2012). A study[28], confirmed that sugar cane contains reducing sugars such as glucose, fructose, sucrose, and lactose, as well as some of the B12 group of vitamins, namely vitamin B1 (Thiamin), vitamin B2 (riboflavin), and vitamin B3 (Niacin), Vitamin B5 (Pantothenic acid)), Vitamin B6 (Pyridoxine), in addition to Vitamin A And vitamin C, it also contains antioxidants and alpha hydroxy acid, which are used in the pharmaceutical and cosmetic industries, in addition to containing calcium, potassium, phosphorus, zinc and magnesium, and it is free of fat and cholesterol.

3.5. Diagnosis Lactic Acid Through Active Groups Using Infrared Spectroscopy Technique (FT-IR)

Figures (65) and (6)and (7)show that the results of the diagnosis of lactic acid produced from the alternative medium, the standard medium, and the standard lactic acid using the bacterial isolate *L. paracasei strain*.CAU 9836 using infrared spectroscopy (FT-IR) analysis, and the presence of differences in the positions of the peaks The effective totals of lactic acid produced from the alternative medium and the standard medium compared with the standard lactic acid, as a result of the presence of some impurities and other compounds, in the acid produced from the alternative medium and the standard medium, as for the standard lactic acid, it is more pure and free from impurities, The emergence of bands that amounted to 3408.22, 3375.43, and 3363.86 cm-1 for each of the lactic acid produced from the alternative medium, the standard, and the standard lactic acid, respectively, is due to the expansion of the O-H bond of the hydroxyl group included in the lactic acid formula that contains lactate. (Lactate), while the bands that appeared for the lactic acid produced from the alternative medium, the standard, and the standard lactic acid at the limits of 2985.81, 2933.73, and 2995.45 cm-1, respectively, are due to the expansion of the C-H bond belonging to the methyl group (CH3), which is one of the types alkyl, while the bands in the range of 2347.37, 2349.30, and 2357.01 cm-1 each Of the lactic acid produced from the alternative medium, the standard medium, and the standard lactic acid, respectively, due to the expansion of the double bond, C=O, belonging to the carboxyl group (COOH), one of the structural bonds of lactic acid. The standard 1120.64, 1055.06 and 1124.50 cm-1, respectively, are due to the expansion of the C-O single bond belonging to the alcohol group[27]. The table (4) shows the active groups in each of the lactic acid produced from the alternative medium, the standard medium, and the standard lactic acid[29,30].

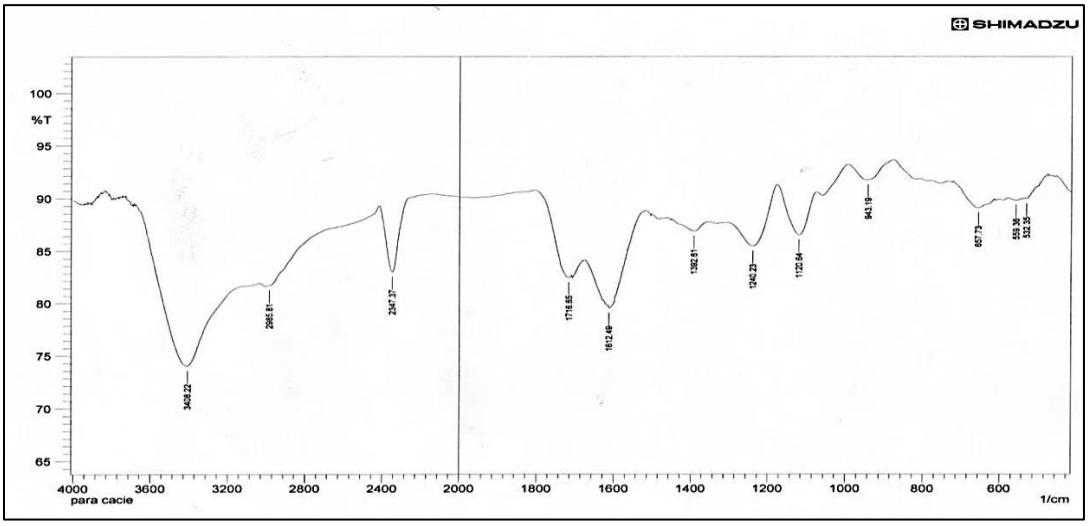

Figure 5. Diagnosis of lactic acid produced from the alternative medium using FT-IR technique.

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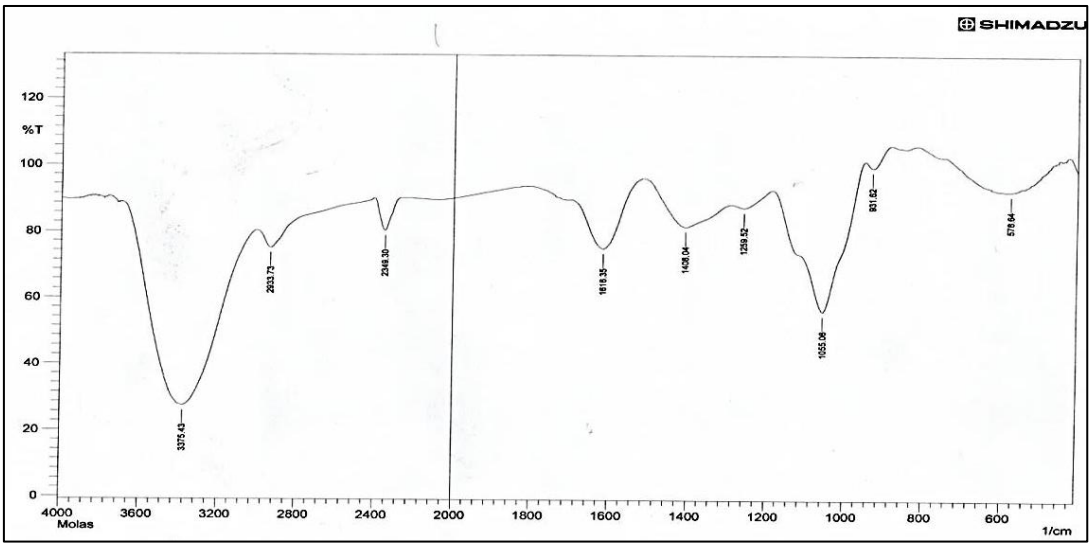

Figure 6. Diagnosis of lactic acid produced from standard medium using FT-IR technique.

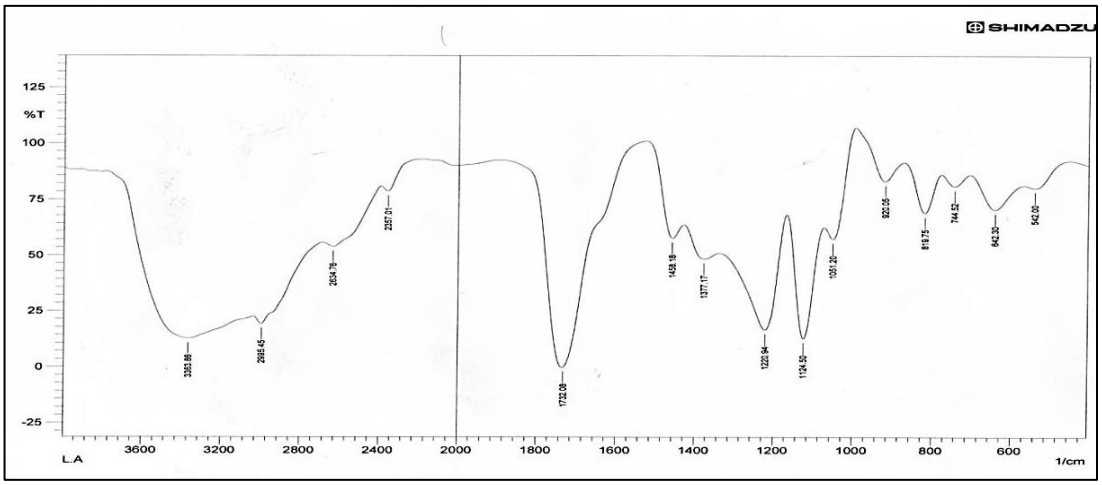

Figure 7. Diagnosis of standard lactic acidosis using FT-IR technique.

Table 4. Active totals of standard lactic acid and product from alternate and standard media.

Sample	Hydroxy group O-H	Methylene C-H	Carboxyl C=O	C- O stretch
Lactic acid by molases	3408,22 cm^{-1}	2985,81 cm^{-1}	2347,37cm^{-1}	1120,64cm^{-1}
Lactic acid by MRS Broth	3375,43 cm^{-1}	2933,73 cm^{-1}	2349,30 cm^{-1}	1055,06cm^{-1}
Standard lactic acid	3363,86 cm^{-1}	2995,45 cm^{-1}	2357,01cm^{-1}	1124,50cm^{-1}

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