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Studies on Optimization of Sustainable Lactic Acid Production by *Bacillus amyloliquefaciens* from Sugarcane Molasses through Microbial Fermentation

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Abstract: Lactic acid is the meekest hydroxyl carboxylic acid (2-hydroxy propionic acid) which is a colorless, odorless, hygroscopic, organic compound with no toxic effect, a very inevitable and versatile chemical used in the Food, cosmetics, textile, and pharmaceutical industries for very long years. Lactic acid was produced as non-racemic when specific microbial strains were used; therefore, microbial fermentation gained more attention. Albeit the substratum used for the microbial fermentation price is much exorbitant. Wherefore, identifying the best and cheap substrates is a bottleneck for the scientific community. Sugarcane molasses is the best source of components for microbial growth and cheap raw material for Lactic acid fermentation. This study produced sustainable lactic acid from sugarcane molasses by the *Bacillus amyloliquefaciens* J2V2AA strain with a higher production of 178 gm/L/24 h. The produced lactic acid was characterized and analyzed by UV-Visible Spectrum, FTIR Spectrum, TLC, and HPLC.

Keywords: optimization; microbial fermentation; lactic acid production; sugarcane molasses; *Bacillus amyloliquefaciens*

Citation: Vignesh Kumar, B.; Muthumari, B.; Kavitha, M.; John Praveen Kumar, J.K.; Thavamurugan, S.; Arun, A.; Jothi Basu, M. Studies on Optimization of Sustainable Lactic Acid Production by *Bacillus amyloliquefaciens* from Sugarcane Molasses through Microbial Fermentation. *Sustainability* **2022**, *14*, 7400. https://doi.org/10.3390/su14127400

Academic Editor: Sergi Maicas

Received: 16 May 2022
Accepted: 14 June 2022
Published: 16 June 2022

1. Introduction

Lactic acid is a colorless, odorless, hygroscopic, organic compound with no toxic effects, which is a very inevitable and versatile chemical used in the Food, cosmetics, textile, and pharmaceutical industries for very long years [1–4]. Lactic acid (LA) is the meekest hydroxycarboxylic acid (2-hydroxy propionic acid) expressed in two different forms, such as levorotatory (L) and dextrorotatory (D) forms [5]. The reactive hydroxyl group (OH) and Carboxylic group (COOH) can be modified chemically, thus making it a versatile chemical [6] applied in various forms in the manufacturing of ointments, lotions, parenteral solutions in the medical field [7]. This creates more demands for lactic acid and thus makes Lactic acid the most desired biopolymer of the decade [8]. In other terms, as of 2018, the Polymer industries, Food and beverages industries, Chemical industries, Cosmetics and pharmaceutical industries wise demands lactic acid at 31%, 24%, 18%, 13%, and 11%, respectively [7]. Though the demand for lactic acid is at high alert, it can only be produced by two modes, viz., microbial fermentation and chemical synthesis. When comparing environmental sustainability and quality of the lactic acid produced by two modes, lactic acid produced by microbial fermentation is heave, whence microbial fermentation gained more attention [7]. Albeit the substratum used for microbial fermentation is much

exorbitant in price [6,7]. Wherefore, identifying the best and cheap substratum is again a bottleneck for the scientific community [9,10]. Various researchers have employed many cheap raw bio-refinery materials such as molasses, rye, corn, whey, and potato starch to produce lactic acid [11,12]. Sugarcane molasses is a cheap raw material and the best source for lactic acid production when bearing in mind the productivity of Sugarcane in India. From the first decade of the 21st century, India is one of the largest countries to produce more than 20 million tons of cane sugars from sugarcane. Hence the wastes produced from these are higher. These waste resources have also been a reservoir for the lactic acid producing bacteria, especially milk industry effluents. Lactic acid producing bacteria utilize milk effluent as the chief source of the substratum. From the literature, Lactic acid has been produced through various raw and cheap agricultural and non-agricultural wastes, primarily through sugarcane molasses [1,11,13–17] utilizing this waste in the lactic acid production will be bi-functional reduces the waste and produces the lactic acid. Therefore, this study's objective is to produce sustainable lactic acid through microbial fermentation using SM a cheap agricultural resource [18].

2. Materials and Methods

2.1. Isolation, Screening of Lactic Acid Producing Bacteria

Milk effluent samples were collected from: 1. Aavin Milk Co., Sivagangai District Cooperative Milk Producers' Union Ltd., Karaikudi, Tamil Nadu, India; 2. Aavin Milk Co., Madurai, Tamil Nadu, India; and 3. SPS Milk Industry, Dindigul, Tamil Nadu India (Figure 1A,B). Samples were subjected to serial dilution and spread plate technique on MRS media (pH 7.2) supplemented with 0.12 gm/L of L-cysteine hydrochloride [19,20] as an oxidizing agent to suppress other non-lactic acid producing bacteria and to enhance the growth of the desired lactic acid producing bacteria (LAB) (Table S1). Morphologically different microbial colonies were isolated and continuously sub-cultured on MRS media (Table S2) and they are preserved in (50%) glycerol for long-term storage. Isolates were tested for the Lactic acid production through the Bromocresol Purple MRS Agar Medium (BCP-MRS) plating technique as described by [21–23] (Figure 2).

(**A**) (**B**)

Figure 1. (**A**) Milk effluent sample collected at Karaikudi Aavin Milk Co-operative Society; (**B**) Milk effluent sample collected at Madurai Aavin Milk Co-operative Society.

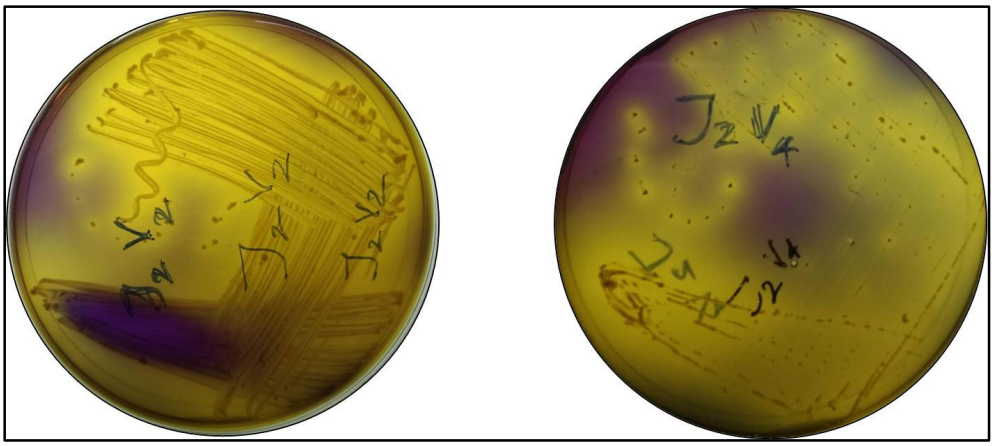


Figure 2. BCP-MRS Based Identification of LA Producing Bacteria.

2.2. Screening for Best LAB Isolates

16 Lactic acid producing bacteria were subjected to fermentation for 72 h in the MRS fermentation broth media with an addition of 20 gm of Glucose (10% inoculum), 150 rpm at 30 ± 2 °C. The fermented broth further had undergone downstream processing to separate the produced lactic acid from fermentation broth media as suggested by [24]. The microbe produced lactic acids were estimated through the spectrophotometric method [25] and the Acidity test [26]. Two Best LA Producing bacteria were identified from the fermentation experiment.

2.3. Pilot Scale Test of LA Production Using Sugarcane Molasses as Carbon Source

The sugarcane molasses sample were collected from District Co-Operative Sugarcane Industry, Madurai, Tamil Nadu (Figure 3) To check the suitability of the SM as a carbon source for the LA production Pilot-scale experiment was carried out with minimal SM (3.75 mL and 7.5 mL) concentration. The Best 2 LA producing bacteria were used in the pilot-scale (50 mL, 10% inoculum) to test LA production using Sugarcane molasses as a carbon source. Glucose availability in sugarcane molasses is estimated through the Phenol-sulfuric acid method. Modified MRS (mMRS) media was prepared with the Sugarcane molasses with an equal glucose concentration of 5 gm/L and 10 gm/L subjected to fermentation for 72 h, 150 rpm at 30 ± 2 °C.

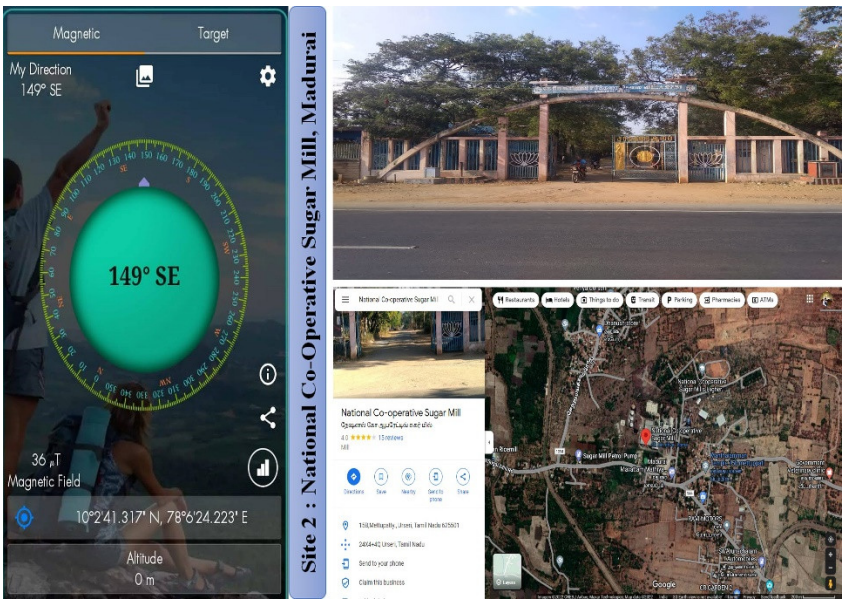


Figure 3. Location for Sugarcane Molasses sample collection.

2.4. Effect of Sugarcane Molasses as Carbon Sources on LA Production

From the Previous Pilot Scale RSM experiment, it has been observed that the J2V2AA isolate has shown promising LA production results. Therefore, optimization was carried out to explore the best possible combination of the Sugarcane molasses for the maximized LA production. In this case, mMRS media used in this experiment is by replacing glucose with Sugarcane molasses with several concentrations (5%, 10%, 15%, 20%, 25% and 30%) in the fermentation for 24 h, 150 rpm at 30 ± 2 °C. A 250 mL Erlenmeyer flask with 100 mL of media supplemented with SM was inoculated with 10% inoculum. All the experiments were carried out in triplicates.

2.5. Molecular Identification and Phylogenetic Tree Construction of LAB

24 h grown bacterial broth was centrifuged at 8500 rpm for 15 min and pellets were collected. The bacterial pellets were subjected to DNA isolation based on CTAB, the Liquid-liquid extraction method. 12.5 µL PCR reaction performed with 2 µL of isolated DNA and 1 µL of each 16 s RNA forward and reverse primers (27F 5′-AGAGTTTGATCCTGGCTCAG-, 3′ 1492R 5′-AGAGTTTGATCCTGGCTCAG-3′), 6 µL of Ampliqon Red Master Mix (1.5×) with 2.5 µL of Molecular grade water. The PCR programme is as follows: Initial Denaturation at 94 °C for 5 min, Denaturation at 94 °C for 60 s, Annealing at 54 °C for 30 s, Extension at 72 °C for 120 s, and Final Extension at 72 °C for 10 min for 30 cycles. Sequenced result aligned and BLASTed with nucleotides available in the NCBI Genbank databases. The distance matrix was generated, and the phylogenetic tree was constructed using MEGA 11 software.

2.6. Downstream Processing of LA

The microbial fermented Lactic acid was separated from the fermentation broth media through downstream processing, i.e., phase separation. In short, 10 mL of cell-free fermented broth were brought down to a pH of Less than 2.5 for high precision LA separation. 5 gm of Ammonium sulphate and 10 mL n-Butanol were added to the broth vortex for 2 h. The organic phase was separated and evaporated using Rotary Evaporation. LA was dissolved with 5 mL Milli Q water [24].

2.7. Characterization of LA

Fourier transforms infrared spectroscopy (FTIR):

100 µL of the down-streamed Liquid LA sample and standard LA were directly placed on the FTIR spectrophotometer (Bruker, Alpha II Model Advanced, Berlin, Germany), and spectral ranges from 400–4000 cm^{-1} were recorded with a scan of 24 per sample. The normal smoothening, baseline correction, atmospheric compensation, and peak picking were performed as per the instrument manual protocol. The FTIR spectrum is used for functional group identification [24].

2.7.1. UV-Vis Spectrophotometer Analysis of LA

The separated LA and standard LA were scanned in the range of 200–400 nm in a UV-vis Spectrophotometer) against blank and the spectrum was analyzed for a similar peak. Additionally, the determination of lactic acid in the fermentation broth was analyzed as described by [25]. In short, fresh 0.3% $FeCl_3$ solutions were prepared and 50 µL of samples were added to 2 mL of the $FeCl_3$ solution and stirred vigorously. The absorbance was measured at 390 nm [27]. A stock of standard LA dilutions was prepared, and the standard curve was plotted.

2.7.2. Thin Layer Chromatographic for the Identification of LA

The experiment was carried out in the TLC rectangular chamber. The stationary phases of TLC plates were made using Silica Gel 60 or Pre-coated Silico gel purchased from HiMedia. The mobile phase of the system is prepared as a mixture stated by [28] acetone: water: chloroform: ethanol: ammonium hydroxide (60:2:6:10:22). The chromatogram

developed by spraying indicator solution contains 0.25 gm of methyl red and 0.25 gm of bromophenol blue in 100 mL of 70% ethanol. Plates were drained in a hot air oven for 10 min to overnight.

2.7.3. Analysis of LA Using HPLC

The separated LA were quantified using HPLC (High-Performance Liquid Chromatography) equipped with a Photodiode detector operated in the UV Range (195 nm to 400 nm). Fractions and quantifications were performed on the C18 column/Waters Column. The 20 µL sample injected into the HPLC instrument ran for 30 min with PDA 280.0 nm. The concentration of the produced lactic acid was estimated using the Lactic acid standard curve. The standard curve was plotted using a different working concentration of Standard LA dilutions.

2.8. Statistical Analysis

All the data generated during the experiments were processed and analyzed using IBM SPSS statistics 25 Version and Excel 2010 with XLSTAT 2021. The graphs were generated using Excel 2010 and Origin Pro 8.5.

3. Result and Discussion

3.1. Isolation, Screening of Lactic Acid Producing Bacteria

Totally 16 bacterial isolates were isolated from all four sample sites through the BCP-MRS plating method. BCP-MRS plates with purple at a pH of 7.2, when bacterial isolates use glucose to convert into lactic acid, the color of the media changes into yellow (less than 4.0 pH) due to the production of acid which in turn brings pH down. 16 bacterial isolates plates showed yellow coloration preliminary confirms the production of LA.

3.2. Screening for Best LAB Isolates

16 isolates were subjected to fermentation and 6 isolates among them showed promising LA production. In the 6 isolates, 2 isolates denoted as J2V2AA and J2V4AA showed a significant reduction in the level of pH with more acidity due to LA production. J2V2AA showed maximum LA production (147.8 mg/mL) in the short span of 24 h. In the overall experiment, on the completion of the experiment, i.e., 72 h, Maximum LA was produced by two isolates J2V2AA (155 mg/mL) and J2V4AA (133 mg/mL) (Figure 4A,B, Table S3). All the isolates showed a decline in the LA production after 24 h of the experiment, May due to the feedback mechanisms, i.e., reutilization of LA thus reduces the LA production [11,29]. In both isolates, there are no significant differences were found after the 24 h of the fermentation, it has also been an advantage that both isolates produce LA in a short span of fermentation.

3.3. Pilot Scale Test of LA Production Using Sugarcane Molasses as Carbon Sources

The two best LA producing bacteria were primarily tested for the production of LA using Sugarcane Molasses as a carbon source. Both strains (J2V2AA and J2V4AA) utilized the Sugarcane Molasses and produced Lactic acid viz., 28 mg/mL/24 h and 24 mg/mL/24 h, respectively. Similar to the screening test, after 24 h of fermentation, no significant differences were found in terms of LA production, which confirms that the isolates are capable of producing LA in a short duration, which is of paramount importance for large scale productions. The bacterial isolate J2V2AA was chosen for further experimental assays based on the pH analysis and LA production (Figure 5, Table S4).

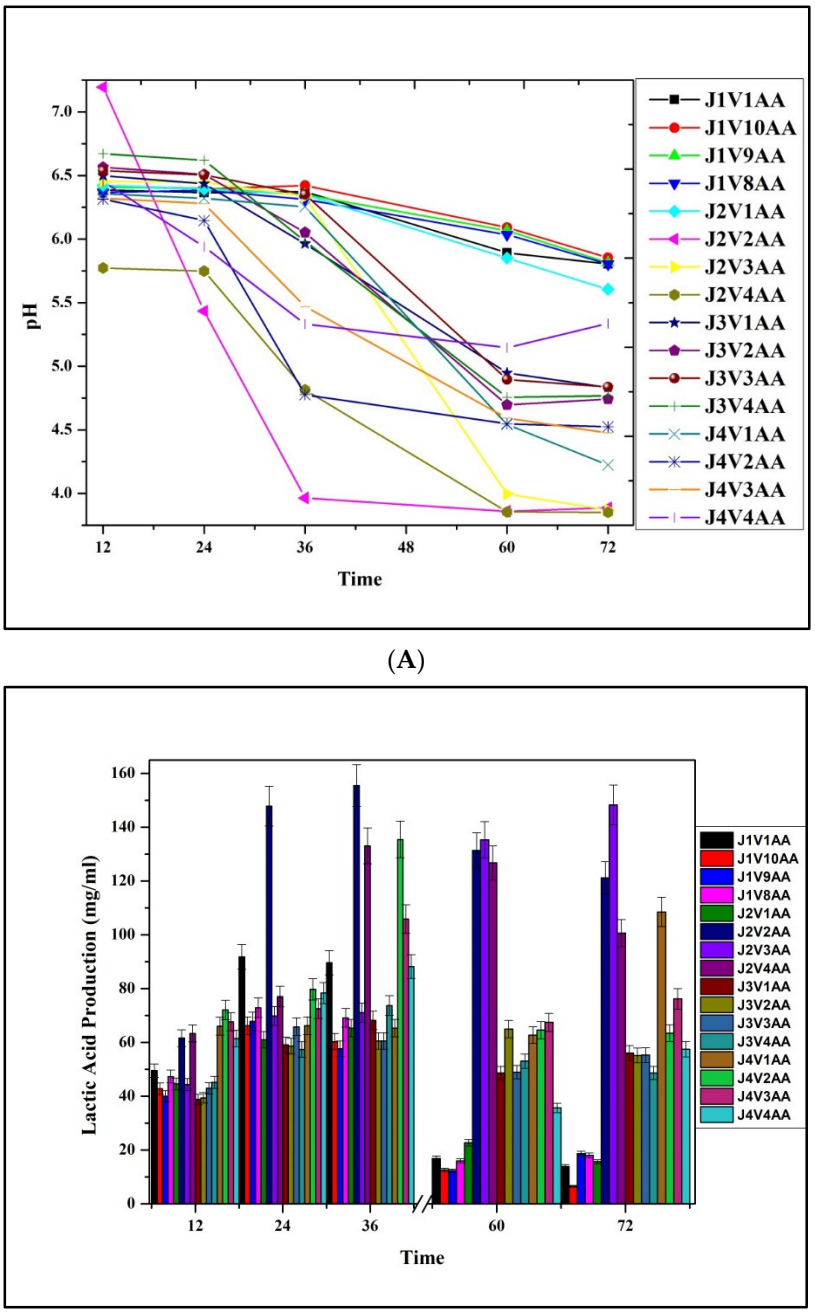


Figure 4. (**A**) Changes in the pH during Production of LA by 16 bacterial isolates; (**B**) Production of LA by 16 bacterial isolates.

3.4. Molecular Identification and Phylogenetic Tree Construction of LAB

The J2V2AA was identified as Non-fastidious *Bacillus amyloliquefaciens* molecularly using 16S rRNA Sanger Sequencing. The best-producing isolate was sequenced against 16SrRNA Sequencing and BLASTed. Based on the E Value and Percentage of Match the isolate was confirmed as *Bacillus amyloliquefaciens* and the phylogenetic tree was constructed (Figure 6A,B). The sequence of the isolates was submitted to GenBank with an accession numbers (OM698825 and OM698824).

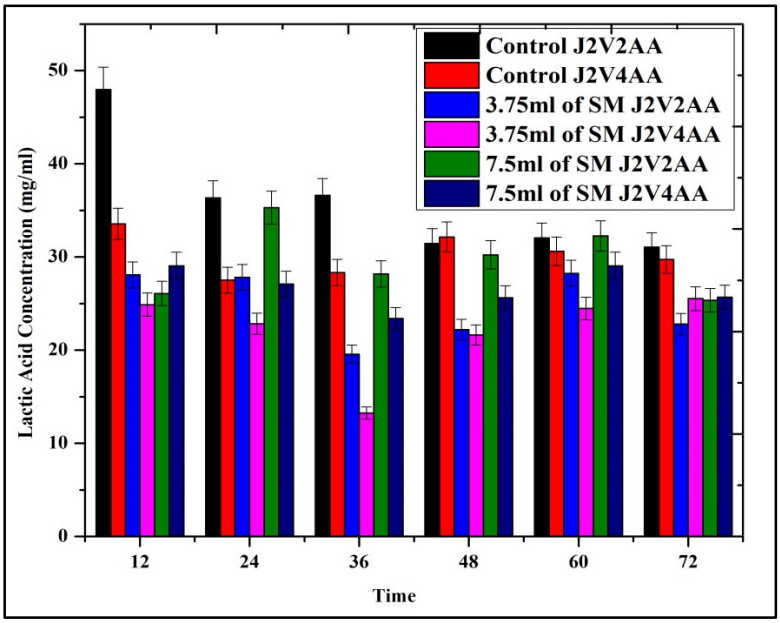
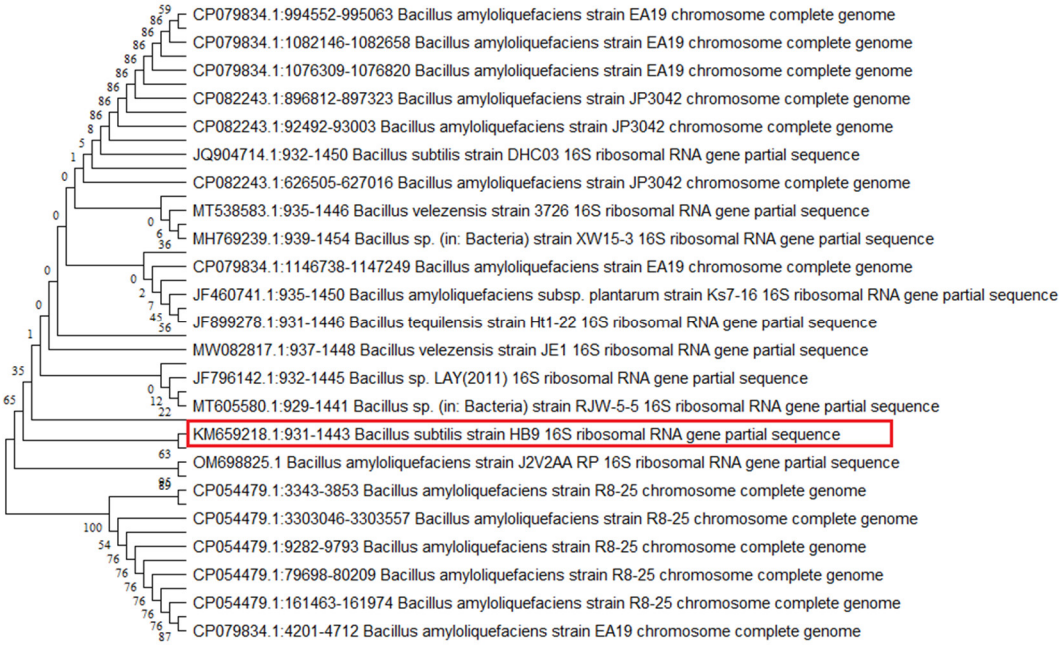
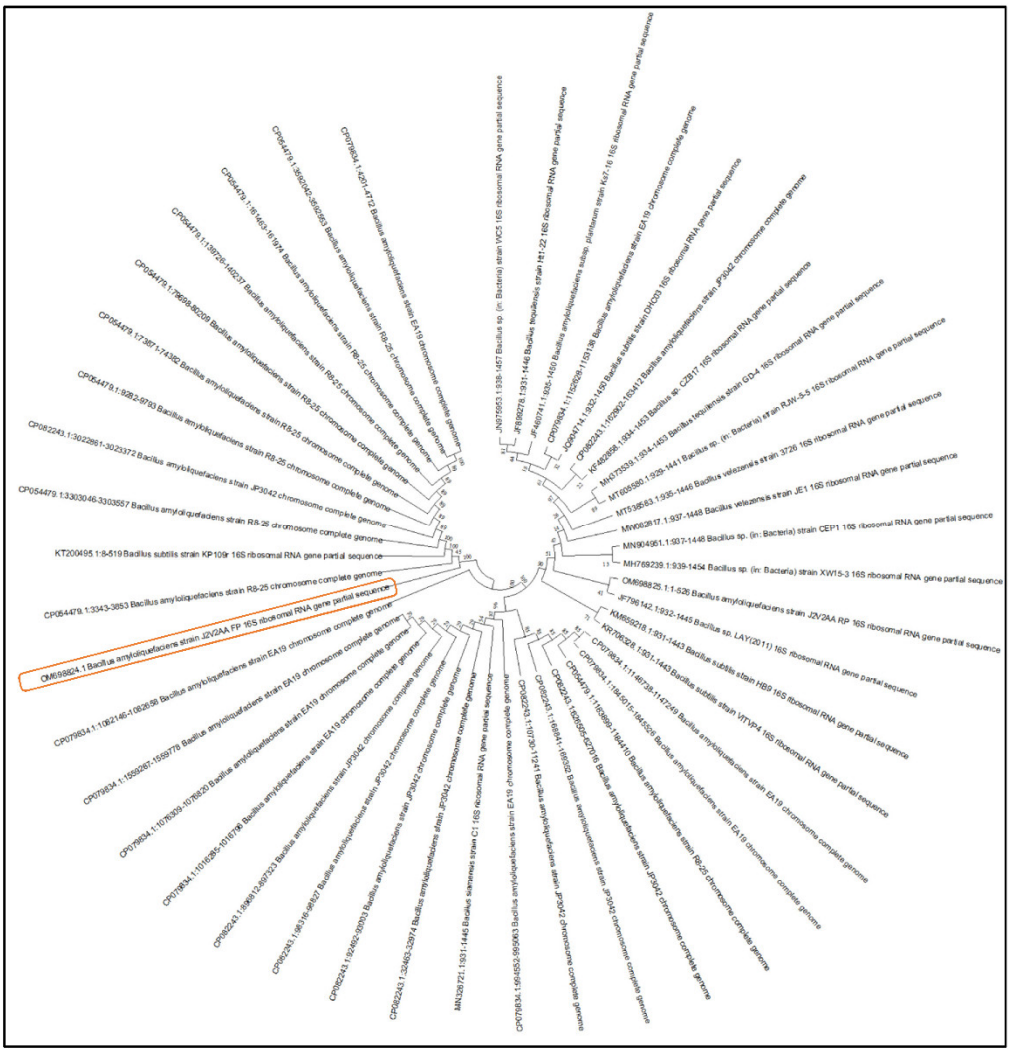


Figure 5. Pilot Scale Production of LA using Sugarcane Molasses as Carbon sources.



(**A**)

Figure 6. *Cont*.



(**B**)

Figure 6. (**A**) Phylogenetic Tree neighboring-distance relation of LA producing bacteria; (**B**) Circular Phylogenetic Tree neighboring-distance relation of LA producing bacteria.

3.4.1. UV-Vis Spectrophotometer Analysis of LA

The microbial fermented lactic acid was further characterized by a UV-visible spectrophotometer compared with the standard lactic acid. A peak between 200–250 nm and ~225 nm represents the presence of lactic acid in the sample (Figure 7).

3.4.2. Thin Layer Chromatographic for the Identification of LA

The chromatogram showed two red spots which represent the presence of Lactic acid. The Rf value of the Lactic acid upper spot and lower spot were 0.50 and 0.33, respectively, comparable to the previous reports [28]. (Figure S1).

3.5. Effect of Sugarcane Molasses Concentration on LA Production

Different concentrations of SM were supplemented to optimize the best combination for the highest production of LA as the isolates are capable of producing LA by utilizing SM to replace Glucose. *Bacillus amyloliquefaciens* strain J2V2AA showed the maximum LA production of 178 mg/mL/24 h in mMRS supplemented with 30% Sugarcane Molasses (Figure 8C). pH analysis resulted in a decrease in the level of pH from 6.5 to 4.6 (Figure 8A). The obtained results are comparable with other literature data [30], up to 30% of Sugarcane molasses showed improved production of LA which is similar to that of the result obtained

by [29,31]. It is suggested that more than 40% of SM showed the deprivation of LA [11] due to maybe the presence of entrained hazardous substances and high sugar concentration [29].

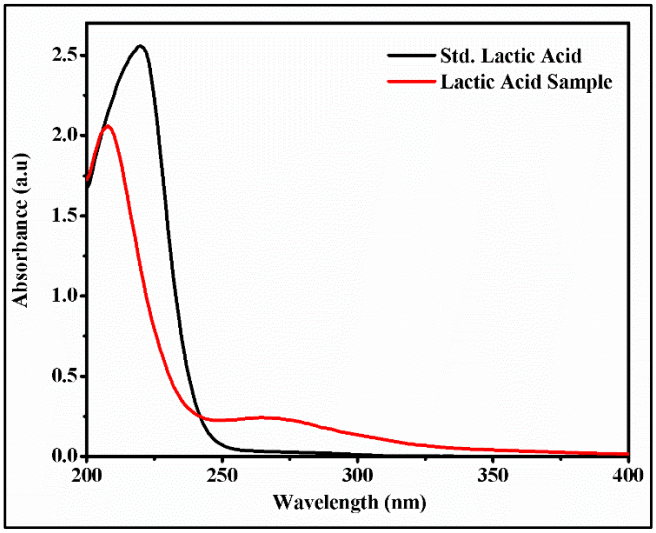
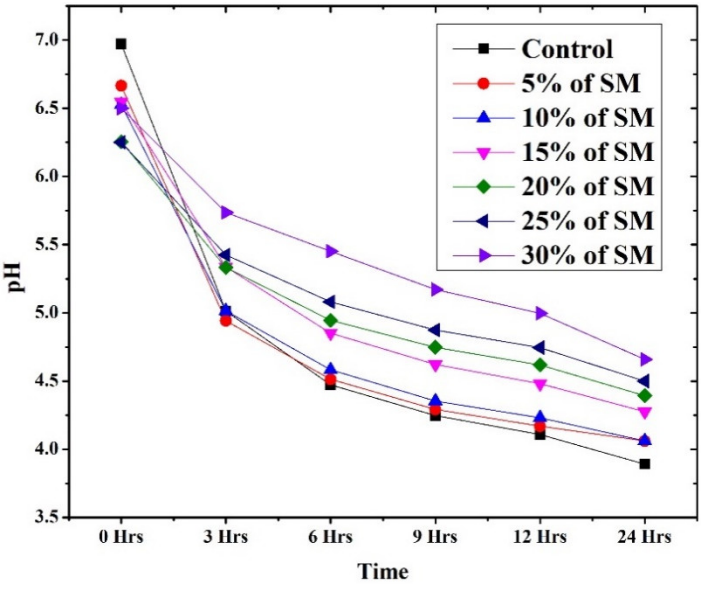
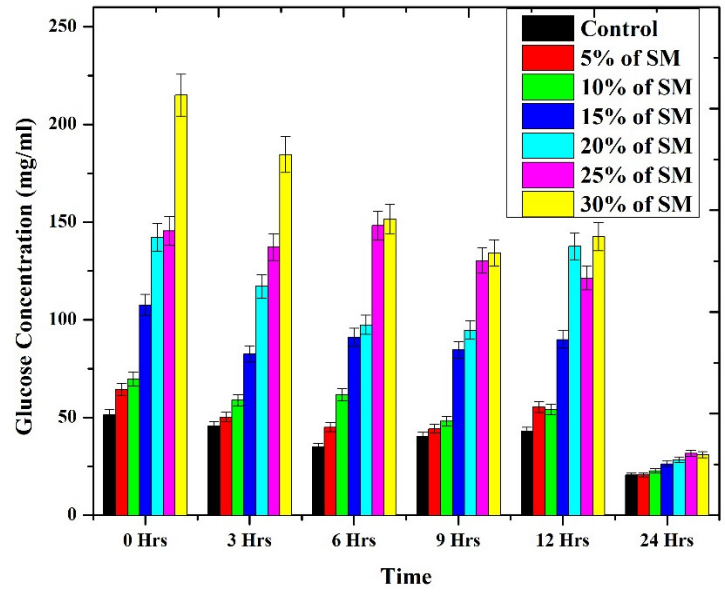


Figure 7. UV Spectrum of Standard and Microbial Produced Lactic Acid.



(**A**)

Figure 8. *Cont.*



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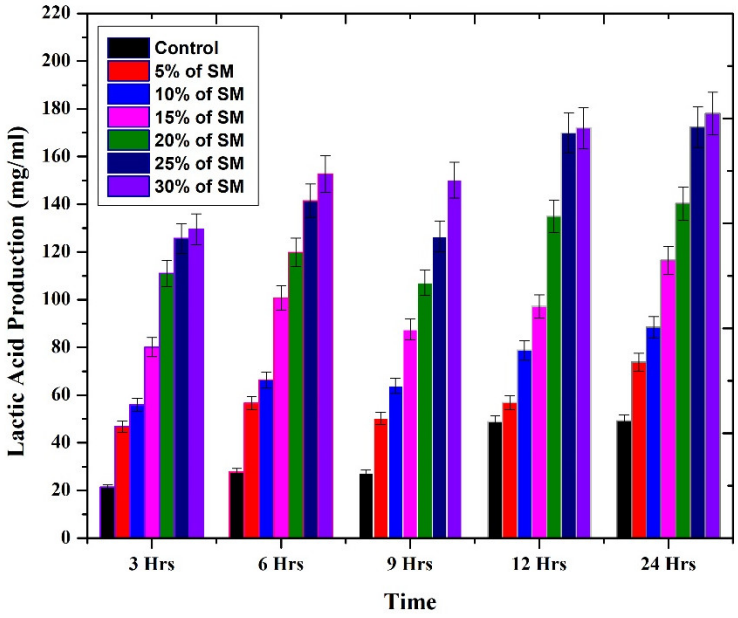


(**C**)

Figure 8. (**A**) Effect of Sugarcane Molasses Concentration on LA Production indicated by decrease in the pH Level; (**B**) Effect of Sugarcane Molasses Concentration on LA Production indicated by decrease in the Glucose Concentration; (**C**) Effect of Sugarcane Molasses Concentration on LA Production.

During the initiation of the fermentation, the pH of the media was around 6.5 to 7, which gradually reduced to 4.75 to 5.75 after 3 h of fermentation denoting the production of LA. At the 24 h of fermentation, the pH of all concentrations was brought down to less than 4.5. Simultaneously the glucose concentrations were also gradually converted to LA. At the initial stage of the fermentation, the glucose concentration of 30% SM was around 225 mg/mL which gradually reduced to around 25 mg/mL at 24 h of fermentation (Figure 8B, Table S5). All the glucose concentration of SM was brought down at the end of the fermentation. The control glucose was also reduced and converted into LA, but the conversion rate was slow in the control and the rate of conversion of SM into LA was higher which could be distinguishably observed in the inter-conversion plate. Initially, at 0 h of the fermentation, no LA has been produced or negligible which is indicated in the plate. Then, inter-conversion was observed in the 3, 6, 9 and12 h in the plate and at the

24th hour of fermentation, the maximum amount of glucose was converted into LA (Figure 9). The conversion into LA was higher in the SM as it contains a high glucose concentration in it. The productions of LA were compared with the other kinds of literature (Table 1), which describes that the LA produced through this study was higher.

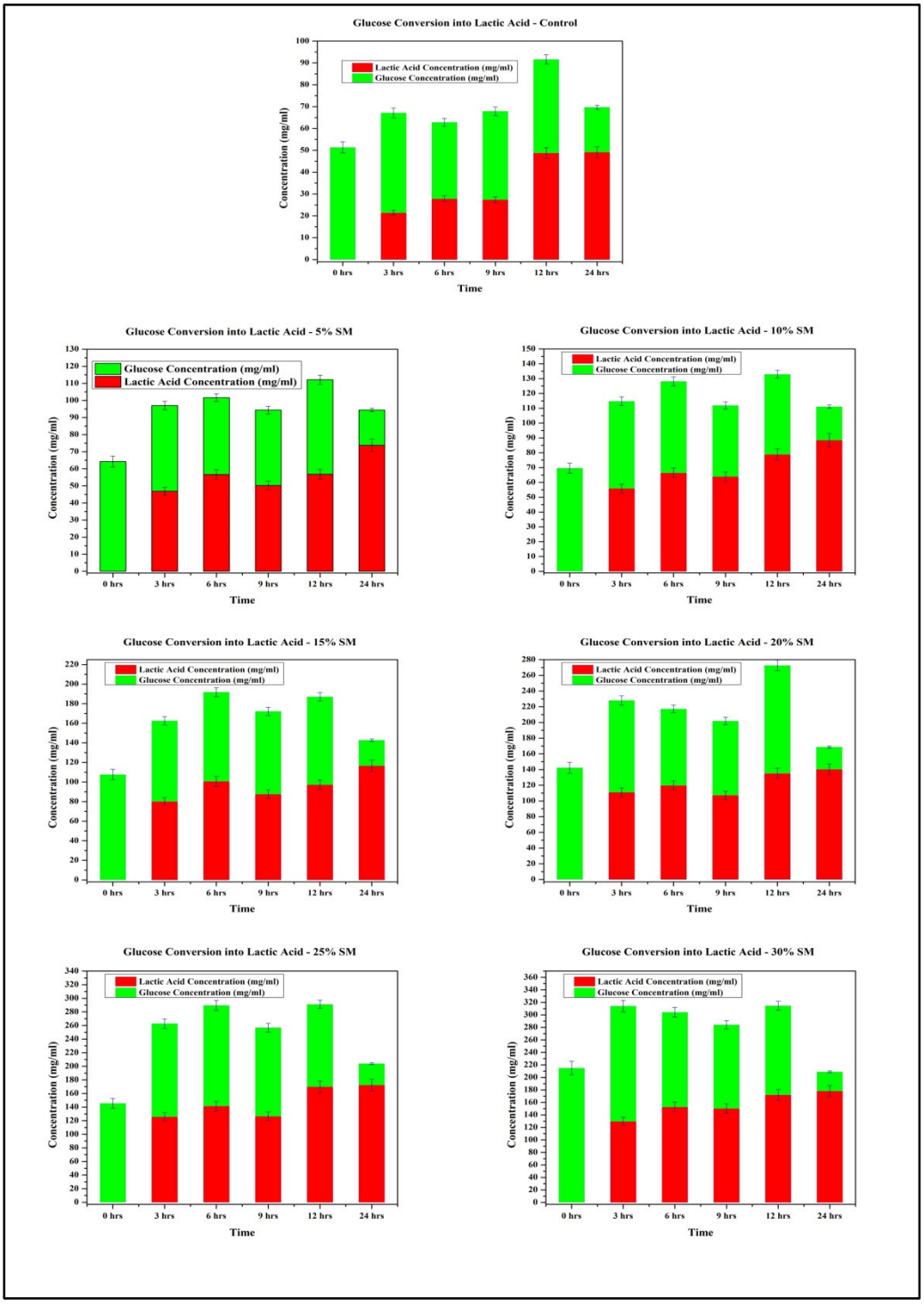


Figure 9. Diagrammatic representation of Inter-Conversion of Glucose into Lactic Acid.

Table 1. List of Lactic acid production through different substratum and their efficacy.

Sl. No	Fermentation Media/Substratum	Microorganisms	Lactic Acid Production
1.	Corn-Steep Liquor with acid-hydrolysate Soybean meal	*Lactobacillus casei* LA-04-1	162.5 g/L [32,33]
2.	Bakery waste hydrolysates and lucerne green juice	*Bacillus coagulans*	62.2 g/L [34]
3.	Diluted sugarcane molasses	Bacterial isolate 25	8.5 g/L [35]
4.	Cane molasses	*Lactobacillus delbrueckii* NCIM 2025	84.50 g/L [36]
5.	Sugar and yeast autolysate	*L. mesenteroides* B512	116.9 and 44.25 g/L [37]
6.	Sugarcane molasses	*Lactobacillus casei* M-15	38.33 g/L [38]
7.	Sugarcane molasses, sugarcane juice and sugar beet juice	*Lactobacillus delbrueckii*	107 g/L, 120 g/L and 84 g/L, respectively [39]
8.	Cassava bagasse	*B. coagulans*	110 g/L [40]
9.	Cassava bagasse	*L. rhamnosus* and *B.coagulans*	112.5 g/L [40]
10.	Date pulp	Indigenous Microbiota	21.66 g/L [41]
11.	Corn steep liquor	*Lactobacillus casei*	180 g/L [42]
12.	Agro-industrial wastes	*Lactobacillus delbrueckii*	112.3 g/L [43]
13.	*Sophora flavescens* residues	*Rhizopus oryzae*	46.78 g/L [44]
14.	Liquefied cassava starch	*Rhizopus microsporus*	84.3 g/L (Batch) and 105–119 g/L (Fed Batch) [45]
15.	Yam tuber reducing sugar, wheat bran hydrolysate and persimmon juice	*Lactobacillus rhamnosus* HG09F5-27	157.22 g/L [46]
16.	Fermentation Broth	*Actinobacillus succinogenes* 130Z	183.4 g/L [47]
17.	Fermentation Broth	*Rhizopus oryzae* As3.819	80.2 g/L [48]
18.	Fermentation Broth	*Bacillus amyloliquefaciens* J2V2AA	49.17 g/L (This Study)
19.	Sugarcane Molasses	*Bacillus amyloliquefaciens* J2V2AA	178 g/L (This Study)

3.5.1. Fourier Transforms Infrared Spectroscopy (FTIR) Analysis of Lactic Acid

Lactic is a Bi-functional molecule with one Carboxylic acid with a hydroxyl group. The microbial fermented lactic acid was separated from the MRS media through downstream processing. The LA were analyzed and compared with standard lactic acid through FT-IR Spectrum (Figure 10), the –OH bond stretching was observed at 3396 cm^{-1} in standard LA, and 3333 cm^{-1} in Microbial fermented LA. A band at 1719 cm^{-1} and 1632 cm^{-1} represents the C = O stretches, 1121 cm^{-1} represents C–O stretches [24].

3.5.2. HPLC Analysis of Lactic Acid

The standard and the microbial produced in the LA were separated and analyzed through the HPLC system (Figure 11A,B). A single peak at 3.370 RT was obtained in the Standard LA, similar Peaks were observed in the Microbial produced LA. The standard curves were plotted with appropriate dilutions and LA productions were calculated.

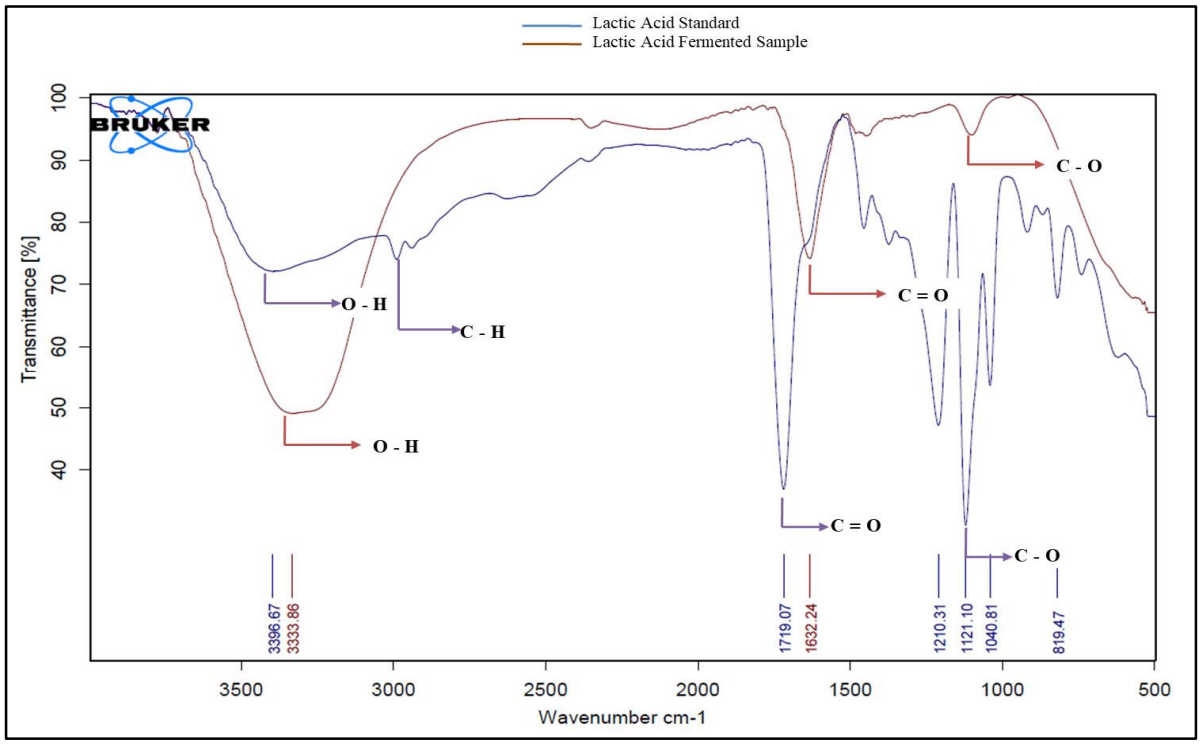
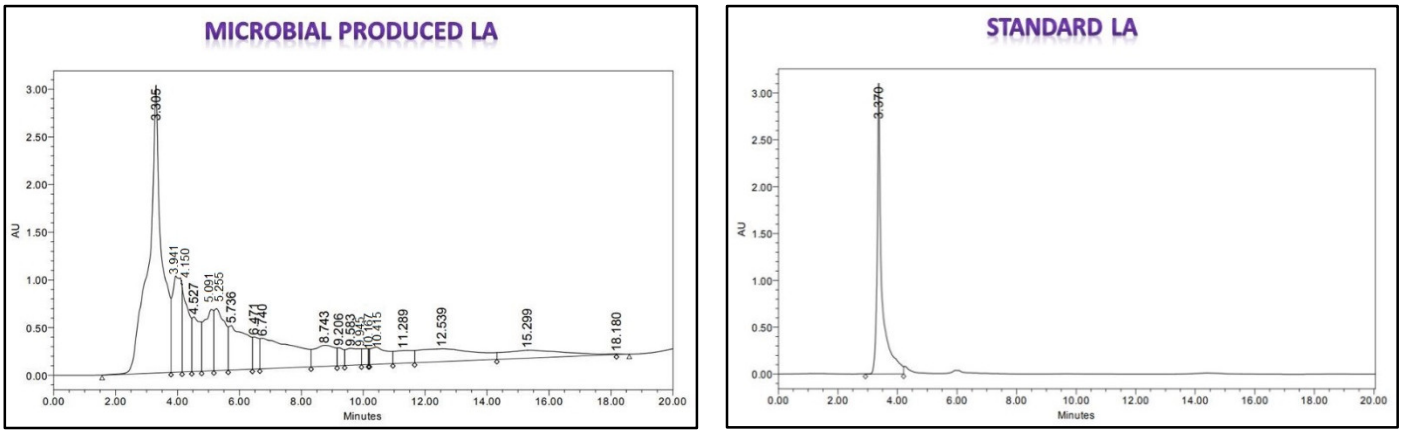


Figure 10. FTIR Spectrum comparison of Standard and Microbial Produced Lactic Acid.



(**A**) (**B**)

Figure 11. (**A**) HPLC analysis of Microbial Produced Lactic Acid; (**B**) HPLC analysis of Standard Lactic Acid.

4. Conclusions

In this study, lactic acid has been produced by microbial fermentation through bioconversion of sugarcane molasses. The Lactic acid producing bacteria isolated from Milk industry effluent, further screened by BCP-MRS assay and identified by 16s rRNA sequencing. The produced lactic acid was identified and analyzed by UV-Visible Spectrum, FTIR Spectrum, TLC and HPLC. Optimization of Lactic acid production using *Bacillus amyloliquefaciens* strain J2V2AA showed efficient highest production of Lactic acid 178 mg/mL/24 h against mMRS Media supplemented with 30% Sugarcane Molasses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14127400/s1, Figure S1: TLC of Standard LA and Microbial LA; Table S1: List of bacterial isolates isolated from their respective sites; Table S2: MRS Composition; Table S3: Production of LA by 16 bacterial isolates (pH); Table S4: Production of LA by 16 bacterial isolates (LA); Table S5: Pilot Scale Production of LA using Sugarcane Molasses as Carbon sources; Table S6: Effect of Sugarcane Molasses Concentration on LA Production (pH); Table S7: Effect of Sugarcane Molasses Concentration on LA Production (LA); Table S8: Effect of Sugarcane Molasses Concentration on LA Production (Glucose Concentration).

Author Contributions: B.V.K.: conceptualization; data curation; formal analysis; methodology; writing—original draft; B.M.: formal analysis; methodology; M.K.: formal analysis; methodology; J.K.J.P.K.: formal analysis; methodology; S.T.: formal analysis; methodology; A.A.: formal analysis; methodology; data curation; investigation; supervision; M.J.B.: conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable for studies not involving humans or animals.

Informed Consent Statement: Not applicable for studies not involving humans.

Data Availability Statement: E-supplementary data of this work can be found in the online version of the paper.

Acknowledgments: All the authors' thanks to DST-PURSE, Department of Botany (Phase II—3rd Installment), and RUSA 2.0 (Letter No. F. 24-51/2014-U, Policy (TN-Multi-Gen), Dept. of Edn. Govt. of India, Dt. 09.10.2018), Alagappa University for the financial support of this research work, and also Research Instrumentation facilities of Department of Botany, Department of Animal Health and Management, Department of Bioinformatics, Department of Microbiology, Department of Biotechnology, Department of Industrial Chemistry and University Science Instrument Centre (USIC), Alagappa University significantly acknowledged and Instrument facilities, Department of Botany, Bharathidasan University also significantly acknowledged. Special thanks to PU. Mahalingam, G. Rajagopal, D. Subashri, M. Sudhakar, M. Sethupathi, and C. Raj for their academic support.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

LA	Lactic Acid
SM	Sugarcane Molasses
BCP	Bromocresol Purple
MRS	de Man, Rogosa & Sharpe
mMRS	Modified MRS
FM	Fermentation Media
LAB	Lactic acid producing bacteria

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