RESEARCH PAPER

Standardization and Optimization of Production of Sorbic acid and its salts as Natural preservatives via Lactic acid bacterial isolates

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ABSTRACT
Sorbic acid is an unsaturated fatty acid containing two carbon–carbon double bonds in the trans configuration. It was first isolated in 1859 from the unripe berries of Sorbus aucuparia, from which it derives its name. Sorbic acid and its salts have been widely used in the food industry for many years as important preservatives that inhibit the growth of various bacteria, fungi, and yeasts in acidic media. Their use extends the shelf life of many food products, including baked goods, dairy items, beverages, and processed foods. The ability of sorbic acid to prevent spoilage and contamination supports food safety and quality, making it a valuable additive in numerous applications. The U.S. Food and Drug Administration (FDA) considers sorbic acid safe for regular use, as it has not been linked to cancer or other health issues. In addition to foods, sorbic acid is also used in wines, pharmaceuticals, and cosmetics. Being an acidic preservative, it is most effective at pH values between 5 and 6. In the present study, an attempt was made to standardize and optimize the fermentation process for production of sorbic acid using Lactic acid bacteria (LAB) isolates. The media was optimized in hit and trial studies to understand the behaviour of LAB with pH and temperature optima for production of sorbic acid. The sorbic acid content produced in fermentation was measured by HPLC analysis, using C18 column was used as the stationary phase and 100mM Ammonium acetate and acetonitrile as mobile phase at 25 °C. Further FT-IR studies were conducted to interpret the compound as potassium sorbate..

Keywords: Fermentation process, Lactic acid bacteria (LAB), Sorbic acid, Potassium sorbate, Food Preservative, antimicrobial agent

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1.INTRODUCTION

Food contamination by pathogenic bacteria has become a global concern due to its severe threat to human health (Rather *et al*., 2017; Garvey, 2019). Of particular interest are global outbreaks associated with *Vibrio cholerae* and *Escherichia coli* O157, which have caused a significant number of fatalities worldwide. Between 1998 and 2008, more than 13,000 cases of foodborne outbreaks were documented in the United States (Batz *et al*., 2021). In 2018, the Foodborne Diseases Active Surveillance Network (Food Net) identified 25,606 cases of infections, resulting in 5893 hospitalizations and deaths (Tack *et al*., 2018). In Malaysia, incidences of food poisoning in schools increased by 57%, from 30 cases in 2015 to 45 in 2016 (New *et al*., 2017; Salleh *et al*., 2017). Despite stringent regulations enforced by the Ministry of Health to govern hygienic practices among local food handlers, food poisoning cases remain high. One of the major contributing factors is Malaysia's hot and humid climate, which promotes the growth of pathogenic bacteria. Furthermore, the misuse of antibiotics has accelerated the emergence of drug-resistant bacterial strains, exacerbating the problem. Recent research has explored alternatives to traditional antibiotics, including improved farming practices, natural antimicrobials, nano-antibiotics, lactic acid bacteria (LAB), bacteriocins, cyclopeptides, bacteriophages, synthetic biology, and predatory bacteria (Hashempour Boultrek *et al*., 2019). Among traditional Malaysian fermented foods, *tempoyak*—fermented durian flesh—is a culturally significant condiment typically consumed with local herbs (Juliyarsi *et al*., 2018). It is produced by fermenting durian pulp in

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closed containers for four to seven days, yielding an acidic, strongly flavored semi-solid product with a pH range of 3.96–4.08 (Wasnin *et al*., 2014; Amin *et al*., 2004). Previous studies have confirmed that *tempoyak* harbors diverse LAB microflora, the composition of which varies depending on the quality of the durian and regional production practices (Leisner *et al*., 2002). LAB are known to inhibit the growth of pathogenic bacteria (Chuah *et al*., 2016; Salleh *et al*., 2014) and exhibit probiotic properties with various health benefits (Ahmad *et al*., 2018; Veron *et al*., 2017; Nuraida, 2015; Yuliana and Dizon, 2011). However, there are currently no studies confirming the safety of *tempoyak* consumption for public health.

Lactobacillus species are the predominant LAB in *tempoyak*. In Malaysia, strains such as *Lactobacillus plantarum*, *L. brevis*, *L. mali*, and *L. fermentum* have been isolated (Muhialdin *et al*., 2012; Mohd Adnan *et al*., 2006) whereas *tempoyak* from Indonesia has been reported to contain *L. plantarum*, *L. casei*, *L. corynebacterium*, and *L. casei* (Wirawati *et al*., 2002; Ekowati *et al*., 1998). LAB and their metabolites—generally recognized as safe (GRAS)—produce antimicrobial compounds such as organic acids, hydrogen peroxide, fatty acids, aroma compounds, and other low-molecular-weight substances that inhibit pathogenic bacteria (de Lacereda *et al*., 2016). Moreover, LAB have been shown to modulate immune responses, prevent enteropathogenic contamination, and alleviate diarrhea (Reid, 1999). Therefore, it is crucial to identify novel LAB strains from Malaysian *tempoyak* that produce potent antibacterial compounds. The use of LAB in foods represents a promising, natural alternative to synthetic chemical preservatives. Consequently, the objectives of this study are (1) to isolate and identify LAB from *industrial fermented samples* capable of producing antibacterial compounds, and (2) to evaluate the antibacterial activity of these LAB isolates. It is found that, antibacterial activity of LAB is because of organic acids such as Sorbic acid.

Sorbic acid is an unsaturated fatty acid known chemically as *2,4-hexadienoic acid*, containing two carbon–carbon double bonds in the *trans, trans* configuration. It has the molecular formula $\mathbf{C_6H_8O_2}$ and appears as a colorless to white, free-flowing crystalline powder with a slight characteristic odor. Sorbic acid sublimes readily and is slightly soluble in water but readily soluble in ethanol and glacial acetic acid.

Sorbic acid and its salts—particularly **potassium sorbate** and **calcium sorbate**—are widely used as antimicrobial agents in foods and beverages. They inhibit the growth of molds, yeasts, and fungi, thereby extending the shelf life of perishable products. In its dry and crystalline state, sorbic acid is chemically stable and does not readily degrade when stored at room temperature for long periods.

As an acidic preservative, sorbic acid exhibits maximum antimicrobial activity at **pH values below 6.0.** It is commonly used in various food and pharmaceutical applications, including **cheese, bakery products, wines, meats, shellfish, and other refrigerated foods.** Sorbic acid helps preserve meats due to its natural antibiotic-like properties.

The **U.S. Food and Drug Administration (FDA)** classifies sorbic acid **as Generally Recognized As Safe (GRAS)** for regular use in foods, as it has not been linked to cancer or other adverse health effects.

2.0 Materials and Methods

2.1 Chemicals and Reagent Required

The chemicals and reagent of analytical grade procured were of Ranchem and CDH, India while growth media were procured from Hi Media, India

2.2 Isolation and Identification

The samples of lactic acid fermented broth were collected from fermenters of Prathista Industries Pvt Ltd., Telangana State, India. Samples were placed in sterile screw-cap tubes until further use, kept at 4^0C.

2.3 Enrichment of LAB

Each broth sample (1 mL) was inoculated into 10 mL of sterile reconstituted skim milk (RSM, 12.5% w/v). The inoculated tubes were incubated for 24 hours at three different temperatures:

30°C for mesophilic LAB,
42°C for thermophilic LAB, and
37°C for both groups.

2.4 Selective Culturing

After enrichment, 1 mL of each culture was transferred to 10 mL of selective broth media:

M17 broth — for incubation at 30°C, 37°C, and 42°C
MRS broth — for incubation at 30°C, 37°C, and 42°C
Cultures were incubated for an additional 24 hours to promote LAB growth.

2.5 Isolation and Purification

The enriched cultures were streaked onto M17 agar and MRS agar plates and incubated at 30°C, 37°C, and 42°C for 24–48 hours. Colonies differing in shape, size, and color were randomly selected and sub cultured into corresponding broth media, followed by 24-hour incubation. Purified isolates were streaked on fresh agar plates to confirm purity. Microscopic examination (Gram staining and morphology) was performed, and colonies exhibiting typical lactic acid bacteria characteristics (Gram-positive, catalase-negative rods or cocci) were selected for further characterization.

2.6 Preservation of Isolates

Pure LAB strains were preserved in duplicate at −20°C using sterile reconstituted skim milk (12.5% w/v) supplemented with 15% glycerol as a cryoprotectant (Werpy *et al*., 2004; Xie *et al*., 2006).

2.7 Phenotypic Characterization

2.7.1 Morphological and Physiological Examination

The morphological characteristics of the purified LAB isolates were examined microscopically using an oil immersion lens. Gram staining was performed according to the method of Harigon and McCane (1976). Isolates

exhibiting homogeneous cell morphology were classified as either cocci or rods. Gram-negative isolates or those showing heterogeneous morphology were excluded from further analysis.

2.7.1.1 Catalase Test

The catalase activity of isolates was determined by transferring a drop of actively growing broth culture onto a clean glass slide and adding a drop of 3% (v/v) hydrogen peroxide solution. The appearance of effervescence indicated a positive catalase reaction. Only Gram-positive, catalase-negative isolates were selected and preserved as potential LAB strains. Five isolates were maintained in duplicate at −20°C in 12.5% (w/v) reconstituted skim milk supplemented with 15% glycerol.

2.7.1.2 Carbon Dioxide Production

The ability of isolates to produce carbon dioxide from glucose fermentation was tested as described below. Each purified strain (1 mL) was inoculated into 2 mL of molten MRS agar (for rods) or M17 agar (for cocci) maintained at 40–45°C. After solidification, 2 mL of water agar (1.5%) was added as an agar plug to restrict gas diffusion. Plates were incubated at 37°C for 24–48 h, and gas production was observed as bubble formation or fissures in the agar. Saccharomyces lactis N.C.Y.C. 571, a known CO_2 producer, was used as the positive control.

2.7.1.3 Growth at 45°C

The ability of isolates to grow at elevated temperature was determined by inoculating 1% of each culture into MRS broth (for rods) or M17 broth (for cocci). Tubes were incubated at 45°C for 48 h, and growth was assessed visually by turbidity compared to an uninoculated control.

2.7.1.4 Growth at 10°C

Cold tolerance was evaluated by inoculating 1% of each strain into MRS broth (rods) or M17 broth (cocci), followed by incubation at 10°C for 10 days in a cooling incubator. Growth was assessed by comparison with un-inoculated control tubes incubated under the same conditions.

2.7.1.5 Growth in the Presence of 6.5% NaCl

Salt tolerance, used to differentiate *Lactococcus* and *Enterococcus* species, was assessed by inoculating each strain into M17 broth supplemented with 6.5% (w/v) NaCl. Tubes were incubated for 3 days at the optimal temperature, and growth was evaluated visually by turbidity.

2.7.1.6. Growth at pH 9.6

To further distinguish between *Enterococcus* and *Lactococcus* species, isolates were inoculated into M17 broth adjusted to pH 9.6 and incubated for 48 h at the appropriate temperature. Growth under these conditions indicated tolerance to alkaline pH, characteristic of *Enterococcus* spp., while *Lactococcus* spp. typically failed to grow.

Analysis of fermented broth for sorbic acid production via High performance liquid chromatography

The positive isolates of Lactobacillus was inoculated in MRS broth in provided optimal media and the broth was analyzed for sorbic acid production via HPLC at Roorkee Research and Analytical Laboratory, Roorkee, Uttarakhand, India. The HPLC analysis was performed using a Shimadzu LC- 2030 - HPLC system (Kyoto, Japan), equipped with a Shimadzu LC- 2030C 3D plus PDA detector with a thermo-stated flow cell and a selectable wavelength of 290 nm. The detector signal was recorded on a Shimadzu LC2030C integrator. The column used was C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 250 mm long) with a particle size of 5 µm. Mobile phase used was 100mM Ammonium acetate and acetonitrile at a flow rate of 1.0 ml/minute and at column temperature 30C. Injection volume was 20 µl of the diluted compound sample and detection of the chromatogram was carried in UV range. The complete procedure was performed both for isolated compound and standard. Both the chromatograms were interpreted by comparing the retention time (RT).

Analysis of fermented broth for potassium sorbate production via Fourier Transform Infrared (FTIR) studies

The IR spectrum of the fermentation-based compound was recorded using a computerized FT-IR spectrometer (Perkin Co., Germany) in the range of 2000-400 cm^{-1} by the KBr pellet technique at Roorkee Research and Analytical Laboratory, Roorkee, Uttarakhand, India. The molecular structure and its homology with any reported compound (if any) were determined by probable structural units (PSUs) as determined by IR- spectroscopy.

3.0 Results and Discussion

3.1 Enumeration and Identification of Lactic Acid Bacteria (LAB)

The lactic acid bacteria (LAB) count in the samples varied depending on the type of culture medium used, ranging from 10^4 to 10^6 CFU/g. All isolates obtained from were Gram-positive rods and tested negative for both catalase and oxidase activities. Interestingly, the morphology of the presumptive *Lactobacillus* spp. colonies on MRS agar (MRSA) exhibited irregular or rough margins, which differed from the typical smooth appearance of regular *Lactobacillus* strains. Based on phenotypic and biochemical characterization, a total of twelve LAB isolates were identified. Eight isolates (I4, I5, I7, I8, I12, I15, I16, I18) showed consistent identification results as *Lactobacillus acidophilus* **(Figure 1).** However, ten other isolates showed discrepancies in species identification.

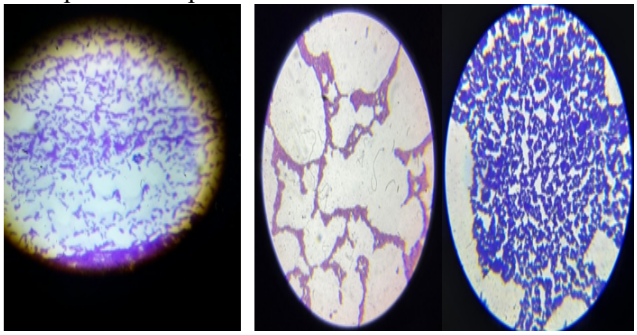

Figure 1: Microscopic images of Lactic acid bacteria

3.2 Production of sorbic acid and its salt during fermentation and its determination by chromatography and spectroscopy

The sorbic acid production occurred after 6 hours of fermentation of the broth with specialized media provided using *Lactobacillus acidophilus*. The sorbic acid production showed the pH at 6.4-6.5 in different replicative fermented sets at 30^0C. Sorbic acid produced via Lactic acid bacteria was tested in the fermented broth. The sorbic acid produced showed retention time at 2.099 minutes in context to standard which showed Retention time at 2.327 minutes **(Figure 2 and Figure 3).** During the fermentation process, there is continuous addition of potash which neutralizes the pH drop during the fermentation process which leads to the preparation of salt of sorbic acid viz potassium sorbate during the fermentation process. Further, after the completion of fermentation process, harvesting process was carried out at 90^0C temperature to kill the microbial cells and purify potassium sorbate produced after concentration and drying. The analysis of potassium sorbate was performed by determination its FT-IR spectra from 2000-400 /cm **(Figure 4).** The PSUs of the FT-IR spectra as interpreted from the library results in interpretation of potassium sorbate **(Table 1).**

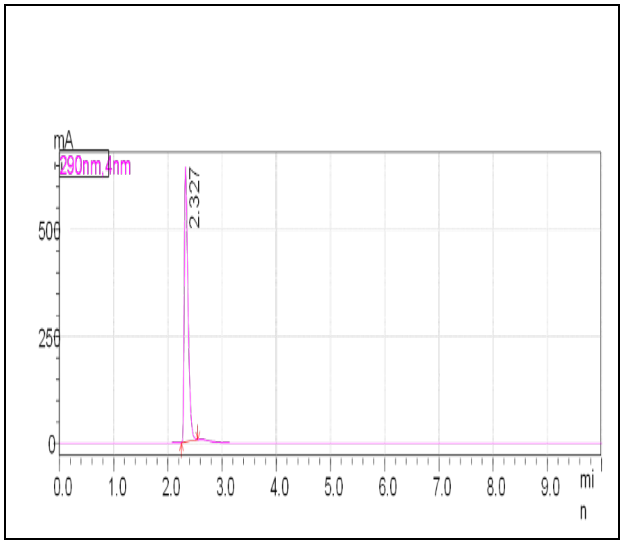

Figure 2: HPLC chromatogram of the standard (sorbic acid)

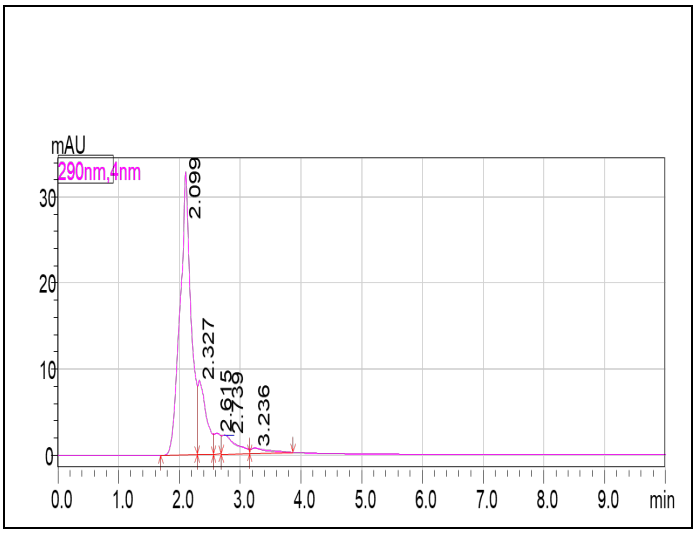

Figure 3: HPLC Chromatogram of sorbic acid produced by Lactobacillus acidophilus

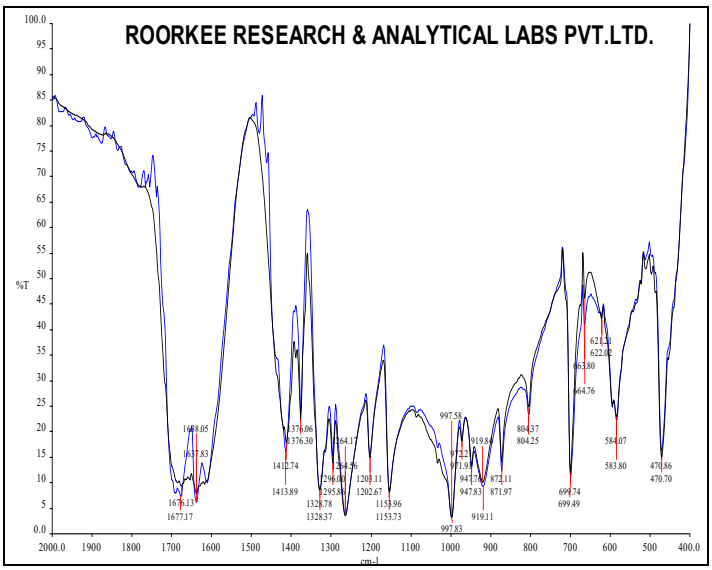

Figure 3: FT-IR – spectra of potassium sorbate produced via fermentation

Table 1: Probable Structure Units (PSUs)/Functional group of potassium sorbate

Wave number (cm-1)	Functional group predicted
584.07	Halogen compound
699.74	Aromatic CH bending
1153.96	Alkyl Amine
1376.06	-CH2 of the saturate
1676.13	Aromatic CH bending

4.0 Discussion and Conclusion

During fermentation, the total nucleic acid content changes in relation to bacterial activity. A small increase occurs during the first 3 hours, followed by a more pronounced rise from the 6th hour onward, reaching about **8-10 mg/100 mL per hour**. By the end of fermentation viz after 10 hours of fermentation cycle, the sorbic acid content was found to be **50-70 mg/100 mL** of sorbic acids. The increase in sorbic acids corresponds with the **growth and proteolytic activity** of lactic acid bacteria, which release free amino acids during fermentation. However, during **storage**, no significant changes in total sorbic acids content were observed

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