Rubber Seed Shell as a Low-cost Medium to Produce Lactic Acid Using *Lactobacillus plantarum* LB2 and Fabrication of a Polylactic Acid-Chitosan Composite for Fish Fillet Packing

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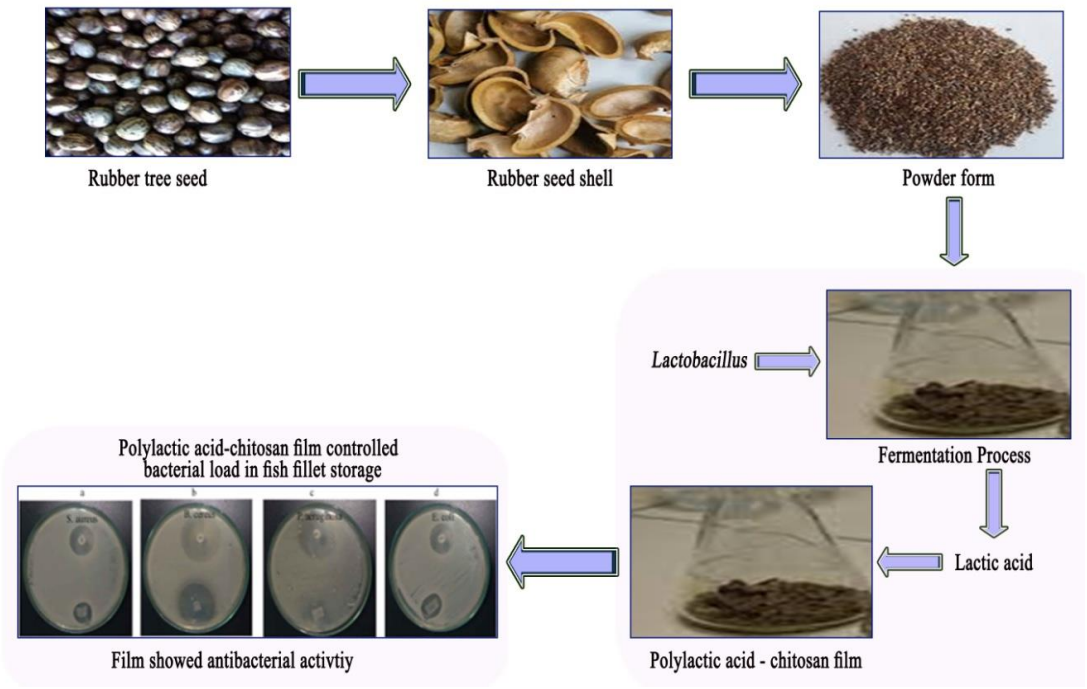
GRAPHICAL ABSTRACT



Rubber Seed Shell as Low-cost Medium to Produce Lactic Acid Using *Lactobacillus plantarum* LB2 and Fabrication of a Polylactic Acid-Chitosan Composite for Fish Fillet Packing

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Rubber seed shell (*Hevea brasiliensis*) was used as a low-cost substrate to produce lactic acid *via Lactobacillus plantarum* LB2. The medium components were initially screened by two-level full factorial design. Three variables (pH, moisture, and polyoxyethylene sorbitan monooleate (PSM)) were used in the central composite design and response surface methodology. The amount of PSM was found to be a significant variable in lactic acid production. Lactic acid was purified and used for the chemical fabrication of a polylactic acid-chitosan composite film. Compared with the polylactic acid, the composite film improved the tensile strength, elongation strength, and tearing strength. The film prepared with 1% chitosan-polylactic acid exhibited the maximum antibacterial activity against *Bacillus cereus* (21 ± 1 mm) and the lowest activity against *Escherichia coli* (10 ± 1 mm). The polylactic acid-chitosan film prepared with 1% chitosan was used as a packing material to store the fish fillets and presented reduced mesophilic (4.3 ± 0.1 Log CFU/g) and psychrotropic (3.2 ± 0.2 Log CFU/g) bacterial populations compared with those of the control (4.9 ± 0.2 Log CFU/g and 3.7 Log CFU/g). Rubber seed shells can be used as an alternative culture medium for lactic acid production, to reduce the production cost of polylactic acid.

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Keywords: Rubber seed shell; Agro-wastes; Biomass; Polymer; Polylactic acid; Chitosan; Packing material

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INTRODUCTION

Polylactic acid has good commercial value and excellent applications in the food processing industry, such as food packing, in the pharmaceutical industry, and in the production of fabrics for industrial processes (Fortunati *et al*. 2012). Its wide range of use is due in part to its good tensile strength, which makes it an ideal material for various processing methods such as injection moulding, melt-extrusion moulding, foam moulding, blown film moulding, and vacuum-forming. It is used for the preparation of nondismantling surgical sutures and for the preparation of drug packaging agents. Polylactic acid shows good properties and improved transparency, gloss, heat resistance,

and hand feel (Vasir and Labhasetwar 2007). Plant biomass, such as sago, potato peel, corn, and other biomass from food processing industries, can be used for the biosynthesis of lactic acid. These biomasses are primarily starch-based substances that are subjected to lactic acid production. Lactic acid is further used to produce polylactic acid (PLA), a degradable biopolymer. Microbial lactic acid is purified and serves as a monomer. Lactic acid is purified *via* downstream processing. Lactic acid has several uses in the textile, chemical, food, pharmaceutical, and other industries. Recently, the demand for lactic acid has increased as lactic acid can be applied as a monomer for the synthesis of polylactic acid (PLA), a degradable bioplastic that is an effective alternative to synthetic polymers (Zhang *et al.* 2016).

Lactic acid has received attention in recent years, has been identified as a major building block chemical. It is applied in the food processing industry as an acidulant, emulsifying agent, preservation agent, and buffering substance. It has deinking properties and antibacterial characteristics. Owing to these properties, it is widely used in the paper and detergent industries. Recently, it has been used as a monomer to produce biodegradable polylactic acid or as a precursor to acrylic acid (Djukić-Vuković *et al.* 2019). Approximately 90% of lactic acid is produced by microbial fermentation (anaerobic fermentation) (Leroy and De Vuyst 2004). In the microbial fermentation process, sucrose and glucose are considered the major starting biomasses. The other feedstocks include disaccharides or polysaccharides from beet extracts, molasses, corn syrups, starches, and whey (Dusselier *et al.* 2013). The use of agro-industrial wastes and agri-food waste represents an important alternative to costly chemical supplements, and bulk materials can be used to produce products and solve various hazardous pollutants (Girotto *et al.* 2015). Examples of substrates include molasses, cassava bagasse (Pandey *et al.* 2000), sugar beet pulp (Xue *et al.* 1992), sugarcane-bagasse (Pandey *et al.* 2000), apple pomace (Antier *et al.* 1993), coffee husk and pulp (Antier *et al.* 1993), rice bran and wheat bran (Peng and Chen 2008), and oil cakes (Ramachandran *et al.* 2007).

Polylactic acid is prepared in three steps, microbial fermentation, purification, and polymerization. Polylactic acid is commonly synthesized by ring opening polymerization of lactide, and this process involves various catalytic reactions involving catalysts such as tin, zinc, aluminium, and lead, as well as solvents such as toluene, chloroform, and diphenyl ether. The molecular weight of the polylactic acid produced by ring opening polymerization can be influenced by the catalyst type, residence time, and temperature. The ratio and sequence of D-lactic acid and L-lactic acid units in the polymer are also controlled (Hamad *et al*. 2015). In addition, polylactic acid can be fabricated *via* various methods including, film casting, melt extrusion, fiber spinning, and blow molding because of its thermal stability compared with other reported biomaterials. The direct polycondensation method is frequently employed for the preparation of polylactic acid in bulk by distillation of condensation water without or with the presence of a chemical catalyst, while the temperature is increased under controlled conditions. Inorganic, organic, homogenous or heterogeneous catalysts have been used in the polycondensations of lactic acid (Balla *et al*. 2021).

Microorganisms produce lactic acid, including lactic acid bacteria (LAB), *Escherichia coli*, *Bacillus* strains, and *Corynebacterium glutamicum* (Abdel-Rahman *et al.* 2013). LABs are commonly used for the production of lactic acid on an industrial scale. Among LAB, *Lactobacillus* strains are safe for industrial processes and have several health benefits. They have long been used for the preparation of fermented foods. LAB strains, such as *L. casei*, *L. delbrueckii* spp. *bulgaricus*, and *L. helveticus*, are widely used because

of their potential to convert mono- and disaccharides and increase tolerance to wide pH and temperature ranges (Mazzoli *et al.* 2014). These LABs produce D-lactic acid or L-lactic acid *via* metabolic pathways (homo or heteroacid fermentation) (Van De Guchte *et al.* 2002). Agro-industrial residues are largely considered ideal substrates for producing LAB, and natural biomass increases the proliferation of LAB. Polyoxyethylene sorbitan monooleate (PSM) is a nonionic surfactant and it is used as an additive in pharmaceutical and packed food preparations, and as a dispersant, stabilizer and emulsifier (Neta *et al.* 2015). PSM has also been used to improve the intake of nutrients into cells; hence, these nutrients are supplemented in the culture medium to improve bacterial growth. In addition, PSM has proven useful in the growth of *Lactobacillus* and lactic acid production (Qi *et al*. 2009).

Hevea brasiliensis is commonly called the rubber tree. The native growing region of this species is the Amazon rainforest, whereas the species is now distributed in almost all Southeast Asian countries and contributes approximately 95% of the world latex market (Singh *et al.* 2017). Latex is considered an economic product, and seeds are considered waste material. Approximately 25% of seeds are estimated to be collected from the field, and the remaining seeds are not utilized or considered waste material, leading to the loss of valuable biomass (Onoji *et al.*2017). The production of rubber seeds has reached more than 2000 kg/ha/year, and the major contributors are Thailand, Indonesia, and Malaysia (Reshad *et al.* 2018). The seeds are available almost all year and are considered one of the major feedstocks for the preparation of bioenergy to manage waste. The kernel contains crude protein and nonedible vegetable oil (Oluodo *et al.* 2018). Rubber seed shells contain a low ash content (0.1 to 0.3%), similar to that of wood, and are considered as a biofuel (Hassan *et al.* 2014). The conversion of rubber seed waste into valuable products reduces waste but also adds additional value thus minimizing environmental problems. Waste valorization has become a sustainable and green technology for the conversion of waste into industrial products and effectively fosters economic opportunities (Putra *et al*. 2023). In this study, rubber seed shells were used to produce lactic acid *via* a solid-state fermentation process. Optimization of the process parameters was performed *via* central composite design and response surface methodology. Lactic acid was purified, used as a monomer for polylactic acid production, chitosan-polylactic acid, and tested for its biological properties.

EXPERIMENTAL

Isolation of Lactic Acid Bacteria from Cattle Dung

Cattle dung was used as the sample to isolate the lactic acid bacteria (LAB). Cattle dung was collected from the farmhouse between January 2022 and December 2022. The dung sample was collected in a sterile container and transported to the laboratory for microbial analyses. The sample was serially diluted and spread on Petri dishes with deMan-Rogosa-Sharp (MRS) (Himedia, India) agar plates supplemented with an antimicrobial agent (2% nystatin). The plates were incubated under anaerobic conditions for 48 to 72 h. The morphology, color, and shape of the colonies were observed and only 10 LAB strains were selected for lactic acid production.

Screening of LAB for Lactic Acid Production

The isolated bacterial strains were cultured individually in 50-mL of MRS broth medium in 250-mL Erlenmeyer flasks. The mixture was incubated for 24 h at 28 °C and the cell free extract was obtained after centrifugation (5000 ×g, 10 min). The synthesis of lactic acid was assayed by titrating 5 mL of the cell-free extract against 0.25 M sodium hydroxide using 1 mL of phenolphthalein indicator. The percentage of lactic acid production was assayed by analyzing the titratable acidity (Fortina *et al.* 1993). Lactic acid production was further assayed by high performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a Hi-Plex H column (7.7 mm × 300 mm), with an injection volume of 3 µL, employing isocratic elution at 0.5 mL/min, which consisted of 2.25 mM H_2SO_4 in water at 60 °C. Reference lactic acid was used as internal standard.

Storage of Bacterial Strains

A bacterial strain that showed maximum lactic acid production was selected for storage. The selected strain LB2 was inoculated in MRS broth and incubated for 24 h at 28 °C. The culture was mixed with glycerol (50%) in a sterile vial and stored at -20 °C. The stored bacterial culture was activated and cultured in MRS broth before the experiments were performed.

Molecular Characterization of LAB Strain Isolated from Cattle Manure

The isolated LAB were cultured in MRS broth and incubated for 18 h at 28 °C. The genomic DNA was extracted *via* the Higher Purity™ Bacterial Genomic DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of the DNA was tested using a nanodrop instrument. It was used as a template DNA, and a polymerase chain reaction was performed using a Thermal Cycler machine (Applied Biosystems, CA, USA) to sequence the amplified product. Universal oligonucleotide forward and reverse primers were used for this study. The final sequences were evaluated using the BLAST algorithm, and the sequences were compared with the gene bank databases. The sequences were submitted to gene bank databases, and accession numbers were assigned.

Substrate and Analysis

Raw rubber seeds were collected from the rubber estate and the rubber seed shells were dried and separated. The material was powdered mechanically and was used as a substrate for the production of lactic acid. The moisture, volatile matter, fixed carbon, and ash contents were determined using a macrothermogravimetric analyzer (LECO TGA801, LECO Corporation, 3000 St. Joseph, MI, USA). The total protein content was determined by Folin phenol reagent method and the carbohydrate content was determined by anthrone method (Yanez *et al.* 2017). The percentages composition of carbon, nitrogen, carbon, oxygen and sulphur were assayed using CHNS Elemental Analyzer.

Inoculum

The *L. plantarum* LB2 strain was inoculated on MRS agar plates and incubated for 24 h at 28 °C. Then, a loopful culture was inoculated into MRS broth medium (100 mL) in an Erlenmeyer flask and incubated overnight at 28 °C. The bacterial growth was measured using a spectrophotometer (AQUASpec™, ELICO Limited, Chennai, Tamilnadu, India) which was used as an inoculum.

Solid State Fermentation

Lactic acid fermentation of rubber seed shells was performed by the selected *L. plantarum* LB2 in solid state fermentation. Briefly, 5 g of solid substrate was added to an Erlenmeyer flask and the substrate was moistened with buffer at pH 6.0. It was sterilized and cooled. The sterile solid medium was inoculated with *L. plantarum* LB2 cells (18 h old cells), mixed and incubated for 48 h. After 48 h, double distilled water was added and the mixture was shaken for 30 min at 150 rpm. Then, the mixture was centrifuged for 10 min at 5000×g and a clear supernatant was obtained. The amount of lactic acid produced was measured using a high-performance liquid chromatography (Agilent Technologies, 1260 Infinity II LC System, Santa Clara, CA, USA).

Optimization of Lactic Acid Production by Response Surface Methodology

To select the variables that influence lactic acid production, a two-level full factorial design was selected. The values of the selected five variables were fixed within a two level full factorial design, on the basis of the nutrient requirements of the candidate bacterium. The selected variables were moisture (60 to 80%), pH (6.0 to 8.0), Polyoxyethylene sorbitan monooleate (PSM, Tween-80) (0.1 to 1.0%), glucose (0.1 to 1%), and yeast extract (0.2 to 2%). These five parameters were selected on the basis of preliminary experiments. The two level full factorial design was performed in 32 experimental runs and five parameters were tested. Table 1 shows the variables and levels (low (–) and high (+)) of the five variables.

Table 1. Two-Level Full Factorial Design for the Screening of Significant Variables in Solid State Fermentation

Factor	Name	Units	Low Actual	High Actual	Mean
A	Moisture	%	60	80	70
B	pH		6	8	7
C	Tween-80 (PSM)	%	0.2	2	1.1
D	Glucose	%	0.1	1	0.55
E	Yeast extract	%	0.1	1	0.55

A central composite design, with six central points was used, and a total of 20 experimental runs were carried out (Table 2). The three most significant factors (moisture, pH, and PSM amount) were selected on the basis of the p value and positive coefficient estimate in the screening experiments. Table 2 shows the variables and levels of the three variables. The lactic acid yield (g/kg substrate) was calculated after the extraction of lactic acid from the fermentation medium. Central composite design optimization of solid waste was performed by selecting three variables at five different levels for the improved production of lactic acid. The experimental results obtained from the designed central composite design use the following second-order quadratic Eq. 1,

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i j_i \quad (1)$$

where Y_i is the predicted lactic acid yield, $x_i x_j$ is the independent variables, β_0 is the offset term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

The experiments were performed in triplicate, and the mean values were used for analysis. Design Expert software (version 8) was used for the analysis of data to predict

the optimum response. An F-test was carried out to analyze the significance of the fitted quadratic polynomial model. Analysis of variance was used to evaluate the statistical significance ($p<0.05$) of the quadratic model using a design expert software. The multiple correlation coefficient (R) and the determination coefficient (R^2) were used to analyze the performance of the regression equation and the optimum levels of the selected factors. The optimum concentrations of the factors were obtained by analyzing the 3D response surface plots.

Table 2. Variables and Levels in Central Composite Design

Factor	Name	Units	Low Actual	High Actual	Mean
A	pH		5.5	7	6.25
B	Moisture	(%)	60	80	70
C	Tween-80 (PSM)	%	0.2	2	1.1

Purification of Lactic Acid

The fermented medium was mixed with 100 mL of double distilled water and placed on an orbital shaker for 30 min at 125 rpm. The mixture was filtered, and a clear supernatant was obtained. The mixture was evaporated, and the product was concentrated under vacuum evaporator and stored at −4 °C before being used it for the purification process. Then, n-butanol (50 mL) was added, the mixture was heated to 155 °C, and the mixture was rotated frequently. The lower phase was extracted, and it was filtered to remove butyl lactate and solid precipitates. The hydrolyzing vessel was heated continuously at 110 °C. Then, the water phase of the column was refluxed, and lactic acid was purified from the hydrolyzing vessel (Li *et al.* 2021).

Polymerization of Lactic Acid and preparation of Polylactic Acid-Chitosan Composite Materials

A total of 50 g of lactic acid was dissolved in 200 mL of p-xylene. The chemical reaction was catalyzed using tin (II) dichloride (0.5 g) and the reaction was performed under heating. The reaction was continued until a clear distillate appeared in the receiver tube. Then, a glass tube was packed with 40 to 50 g of molecular sieves and mounted onto the unit and the reaction was performed at 138 °C for 4 h. The white powder was washed with methanol and dried for 3 h at 70 °C. Chitosan was dissolved in a 1% acetic acid solution and the final concentration of chitosan was 3 mg/mL. A sodium tripolyphosphate solution was prepared at 1 mg/mL concentration and mixed at a 3:1 ratio (chitosan: sodium tripolyphosphate). The pH of the solution was adjusted to 6.0, and the solution was mixed for 30 min at room temperature (30±1 °C). The mixture was subsequently centrifuged at 5000 rpm for 30 min at 4 °C and freeze-dried at −80 °C for 2 h. Polylactic acid (5 g) was mixed with chloroform (100 mL) and stirred for 1 h at room temperature. Then chitosan nanoparticles (compatibilizer) were added at 1%, 1.5%, and 2% with chloroform containing polylactic acid. The mechanical properties were analyzed to ensure the quality of the composite material.

Analysis of Properties of Film

The physical and mechanical properties of the prepared films were determined. To determine the water vapour permeability, the test tube containing silica gel (anhydrous) was sealed with the prepared film and maintained at 21 °C with relative humidity. The weight was calculated, and the water vapour transmission rate was determined. The moisture content of the film was calculated (AOAC 1999). Briefly, a film sample (100 mg) was dried at 105 °C to achieve a constant weight. The solubility of the film was determined. Briefly, 0.1 g film was immersed in double distilled water for 24 h, after which the film solubility was determined (ASTME96–E80 1989). The tensile strength of the film was determined as described previously (Jeong *et al.* 2020). The tensile strength of the film and elongation at break values were determined from the stress-strain curves. The tear strength of the film calculated according to ASTM D1938 (ASTM 2002).

Antibacterial Activity of Polylactic Acid-Chitosan Composite Materials

Bacterial species, such as *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, were selected for antibacterial studies. These bacterial species were cultured in Mueller Hinton broth (Himedia, India) and incubated for 24 h at 37 °C. Then, 50 µL broth was spread on Mueller Hinton agar medium and the nanocomposite sheet prepared with 1% chitosan (0.7 mm square) was placed on the plates. After 24-h incubation, the zone of inhibition (mm) was calculated (Wu *et al.* 2020; Al-Ansari *et al.* 2021; Valan Arasu *et al.* 2023).

Application of Polylactic Acid-Chitosan Nanocomposite Film in Fish Fillets

Yellow fin tuna fish were collected from the fishing harbour and cut into small fillets (100 g each). The prepared test packing material was used to pack the fish fillet. The nanocomposite material was not applied to the control fish fillet. It was stored at 20 °C and 4 °C. The fish samples were taken periodically and subjected to microbial analysis. The aerobic mesophilic count was performed by plating the samples on nutrient agar plates at 7 °C and the psychrotrophic count was also performed.

RESULTS AND DISCUSSION

Lactic Acid Isolates from the Cattle Manure

Cattle manure is one of the major sources of LAB and is considered a microbial niche because of the increased concentration of fibers, carbohydrates, and acidic pH value, thus maintaining an optimum environment for the proliferation and survival of LAB. The LAB isolated from the cattle manure were phenotypically identical. The experimental results demonstrated that cattle manure is a well-suited natural habitat for LAB. Lactic acid bacteria have been isolated from cattle manure (Asano *et al.* 2010), cow feces (Lin *et al.* 2020), rumen fluid and dairy cow feces (Guo *et al.* 2020), and the rumen and feces (Han *et al.* 2014).

Characteristics of Lactic Acid Bacteria Isolated from Cattle Manure

The culture characteristics were determined by analyzing the characteristics of the LAB strains on MRS medium. The selected bacterial strain did not produce CO_2 in the liquid medium, which indicated that the isolate utilized homolactic fermentation, and that the supplemented glucose was converted into lactic acid. The selection of LAB was based on the potential to produce lactic acid by homolactic fermentation.

Proximate Analysis of Rubber Seed Shell

An analysis of nutrients was performed on the rubber seed shell. The fixed carbon content was 17.5%, and the total volatile content was 73.9%. The ash content of the rubber seed shell was 0.19%, and the moisture content was 14.0%. The crude protein content of the rubber seed kernels was 22.1%, which was comparable to the values reported in previous studies such as 21.9% (Oyewusi *et al*. 2007) and 11.4% (Eka *et al*. 2010). The carbohydrate content was 13.4% in the rubber seed kernels, which was similar to the results of previous studies (Narahari and Kothandaraman 1984). The rubber seed kernels contained 65.31% carbon, 7.45% hydrogen, 4.56% nitrogen, and 0.29% sulphur. The amount of rubber seed kernels was similar to that of rubber seed kernels from Malaysia. The percentage carbon and nitrogen contents of the kernels analyzed in this study were greater and the percentages of hydrogen and sulphur were lower than those reported previously (Hassan *et al*. 2014).

Lactic Acid Production by LAB Strains

Strain LB2 produced a significant amount of lactic acid (2.04 ± 0.03 g lactic acid/100 mL of culture). The strain LB09 produced the lowest amount of lactic acid (1.09 ± 0.03 g/100 mL culture) (Fig. 1).

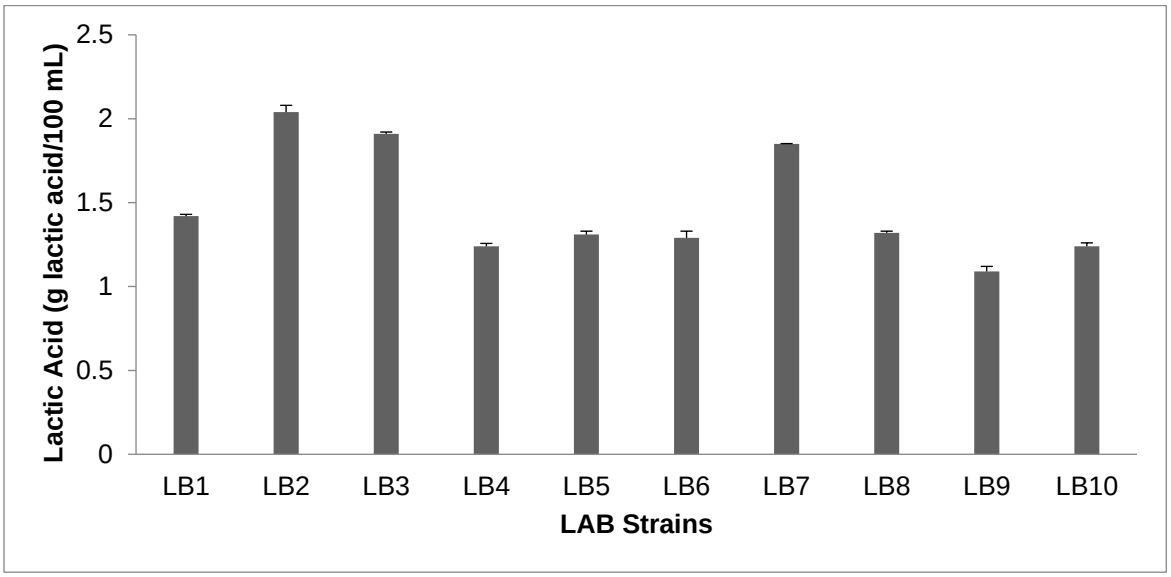

Fig. 1. Production of lactic acid by lactic acid bacteria isolated from cattle manure

Lactic acid is safe organic acid and is used as a decontaminant, fermentation agent, and antioxidant substance. Microorganisms use various natural biomasses, especially carbohydrates and produce organic acids. Lactic acid production varies with pH, available nutrients, temperature, and LAB isolate type (Wang *et al.* 2021). Acid tolerance and high productivity are reported among *Lactobacillus* strains which are widely used in the food industry (Kylä-Nikkilä *et al.* 2000). The lactic acid available in the medium affects the growth and proliferation of LAB and the growth is generally affected below a pH of 4.0 (Wang *et al.* 2021).

Molecular Characterization of LAB

A potent lactic acid producing LAB strain was selected for characterization studies. 16S rDNA gene sequencing is a widely used method for the determination of bacterial strains. The identified LAB strain LB2 was characterized as *Lactobacillus plantarum* LB2. The lactic acid bacteria, such as *Lactobacillus*, *Pediococcus*, *Lactobacillus brevis*, *Pediococcus acidilactici*, *Pediococcus parvulus*, and *Lactobacillus brevis*, have been isolated from various sources (Robert *et al.* 2009). Probiotic, such as *Enterococcus faecium*, was isolated to produce probiotic molecules, including organic acids (Tan *et al.* 2013).

Statistical Optimization of Lactic Acid Production in the Solid State Fermentation

Lactobacillus plantarum LB2 utilizes rubber seed shells and produced lactic acid in solid state fermentation. Lactic acid production ranged between 2.3 g/kg substrate and 109.2 g/kg substrate. Table 3 shows the designed matrix and lactic acid level in the fermented rubber seed shell during solid state fermentation. The analysis of variance (ANOVA) results represent a statistically significant full factorial model (Table 4). The model F-value was 35.15 and the p-value was 0.0001. The individual variables tested, such as moisture, pH, PSM, glucose, and yeast extract were significant ($p<0.01$). The coefficient estimate was positive for moisture, pH, PSM, and glucose, whereas yeast extract showed negative coefficient estimate (Table 5). There is only a 0.01% chance that a "Model F-Value" that is of that size could occur due to noise. In this case A, B, C, D, E, AB, AC, AD, AE, BD, BE, CD, DE, ABD, ACD, ACE, ADE, BCD, CDE, ACDE, BCDE were significant model terms. The R^2 of the model was 0.99 and the adjusted R^2 value was 0.96. The signal to noise ratio was greater than 4 (26.590), which indicated adequate signals.

Optimization of Culture Conditions by Central Composite Design

The experiments with combinations of PSM, different moisture, and pH level of the medium influencing lactic acid production were assayed (Table 6). The levels of the selected independent variables used were obtained from central composite design. The results were analyzed using ANOVA and the designed model was found to be appropriate (Table 7). The R^2value of the model was 0.9337 and the value >0.75 shows fitness for the CCD model. An R^2 value can be generally within 1.0, and a value very close to 1.0 indicates fitness of the model. The adjusted R^2 value of the model was 0.8740, which showed that the analyzed quadratic model fit the data. The "F-value" of the model was 15.6 and the "p-value" was < 0.0001, which showed that the model was adequate and statistically significant. The values, A, A^2, B^2, and C^2 were significant. The first order effects revealed that the PSM (C) concentration had a significant impact on lactic acid production.

Table 3. Factorial Design for the Screening of the Highly Significant Factors upon Solid State Fermentation

Runs	Moisture	pH	PSM	Glucose	Yeast Extract	Lactic Acid (g/kg)
1	80	8	2	0.1	1	34.93
2	60	8	2	1	0.1	25.09
3	60	6	0.2	1	0.1	39.5
4	80	8	0.2	0.1	1	29.05
5	80	8	2	0.1	0.1	20.1
6	80	6	2	0.1	0.1	50.3
7	80	6	2	1	1	38.04
8	60	6	2	0.1	1	10.02
9	80	6	2	1	0.1	25.35
10	60	8	0.2	1	1	20.41
11	60	6	0.2	0.1	1	5.01
12	80	8	2	1	0.1	80.3
13	80	6	0.2	1	1	15.2
14	80	8	0.2	1	1	53.49
15	60	8	0.2	0.1	1	40.2
16	60	8	0.2	0.1	0.1	43.5
17	80	8	0.2	0.1	0.1	57.4
18	60	8	2	0.1	0.1	20.2
19	80	6	0.2	0.1	0.1	20.1
20	80	6	0.2	0.1	1	4.89
21	60	8	2	0.1	1	20.35
22	60	6	2	0.1	0.1	50.7
23	80	6	0.2	1	0.1	19.98
24	60	6	2	1	1	7.51
25	60	6	0.2	1	1	5.19
26	80	6	2	0.1	1	19.94
27	80	8	2	1	1	109.2
28	80	8	0.2	1	0.1	30.2
29	60	6	2	1	0.1	10.2
30	60	8	2	1	1	31.2
31	60	8	0.2	1	0.1	10.1
32	60	6	0.2	0.1	0.1	2.3

Table 4. ANOVA of the Selected Five Variables in Solid State Fermentation

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	16571.8	25	662.8	35.1	0.0001
A-Moisture	2227.6	1	2227.6	118.1	< 0.0001
B-pH	2840.5	1	2840.5	150.6	< 0.0001
C-Tween-80 (PSM)	769.3	1	769.3	40.8	0.0007
D-Glucose	264.3	1	264.3	14.0	0.0096
E-Yeast extract	115.1	1	115.1	6.1	0.0484

Table 5. Analysis of Coefficient Estimate in a Full Factorial Experiment

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	29.68594	1	0.76757869	27.8077402	31.56413
A-Moisture	8.343438	1	0.76757869	6.46524016	10.22163
B-pH	9.421563	1	0.76757869	7.54336516	11.29976
C-Tween-80(PSM)	4.903438	1	0.76757869	3.02524016	6.781635
D-Glucose	2.874063	1	0.76757869	0.99586516	4.75226
E-Yeast extract	-1.89656	1	0.76757869	-3.7747598	-0.01837

The other physical parameters (pH and moisture) were shown to have less impact on lactic acid production ($p>0.05$). Therefore, PSM acts as a limiting factor and supplementation with PSM may improve lactic acid production, whereas the other factors have minimal impacts on lactic acid production. The 2D graph shows the interactive effects between moisture and the pH of the medium (Fig. 2a), pH and PSM (Fig. 2b), and moisture and PSM (Fig. 2c). Biomass, such as household food waste (Anagnostopoulou *et al.* 2022), corn steep liquor (Yu *et al.* 2008), dairy wastewater (Moradi *et al.* 2023), khat ("Catha edulis") waste (Tefara *et al.* 2022), and beet molasses (Alrefaey *et al.* 2021) is frequently used to produce lactic acid. The selection of suitable environmental conditions for lactic acid production is very critical.

Table 6. Central Composite Design for Analysis of Optimum Response upon Solid State Fermentation

Runs	pH	Moisture	Tween-80 (PSM)	Lactic Acid (g/kg)
1	5.5	60	2	70.5
2	5.5	60	0.2	6.21
3	6.25	70	1.1	119.5
4	5.5	80	2	35.06
5	6.25	53.1820717	1.1	77.05
6	6.25	70	-0.4136135	34.09
7	7	80	0.2	24.06
8	6.25	70	1.1	135.9
9	6.25	70	2.61361355	149.2
10	4.988655	70	1.1	1.4
11	6.25	86.8179283	1.1	55.03
12	7	60	2	43.8
13	6.25	70	1.1	148.2
14	6.25	70	1.1	152.04
15	6.25	70	1.1	147.4
16	7	80	2	118.5
17	7	60	0.2	35.06
18	6.25	70	1.1	142.05
19	5.5	80	0.2	15.04
20	7.511345	70	1.1	3.05

Factors such as pH, nutrients, and medium pH, have been reported to influence lactic acid production. The pH is associated mainly with cellular metabolism, which influences the growth of bacteria, accumulation of substrate, and lactic acid biosynthesis (Ojo and de Smidt 2023). Lactic acid production increased with increasing in PSM concentration. Figure 3 shows the interactive effect of moisture and pH (Fig. 3a). The interactive effect was positive, but these interactions were not significant for lactic acid production. Figure 3b shows the interactive effect of pH and PSM. The response surface graph shows that the amount of PSM in the solid substrate improved lactic acid production exponentially, however, pH had less of an effect. Figure 3c shows the interactive effect of moisture and PSM on lactic acid production. This result reveals a significant improvement in lactic acid yield by PSM than moisture content of the solid substrate. PSM is one of the inducers of lactic acid production in LAB and has been previously reported in the literature. Nagarjun *et al.* (2005) used PSM and improved production of lactic acid was reported.

Table 7. ANOVA of the Selected Variables in Central Composite Design

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F
Model	57781.0	9	6420.1	15.64	< 0.0001
A-pH	694.4	1	694.4	1.69	0.2225
B-Moisture	0.00023	1	0.000237	5.77E-07	0.9994
C-Tween-80 (PSM)	10633.7	1	10633.6	25.9	0.0005
AB	1019.487	1	1019.48701	2.48364549	0.1461
AC	44.50961	1	44.5096125	0.10843306	0.7487
BC	214.5556	1	214.555613	0.52269433	0.4863
A^2	35960.21	1	35960.208	87.605244	< 0.0001
B^2	10812.13	1	10812.1325	26.340212	0.0004
C^2	4846.28	1	4846.27987	11.8063703	0.0064
Residual	4104.801	10	410.480085		
Lack of Fit	3400.895	5	680.178953	4.83146097	0.06
Pure Error	703.9061	5	140.781217		
Cor Total	61885.82	19			

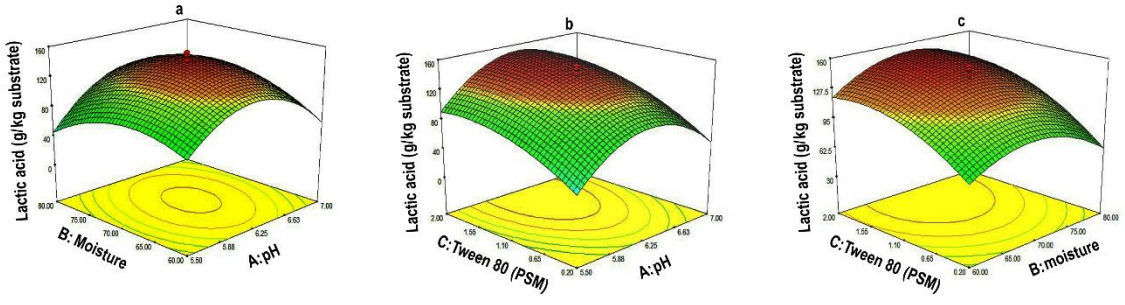

Fig. 2. 2D contour plot of lactic acid production by *L. plantarum* LB2 showing interaction between (a) Moisture and pH, (b) pH and PSM, and (c) Moisture and PSM

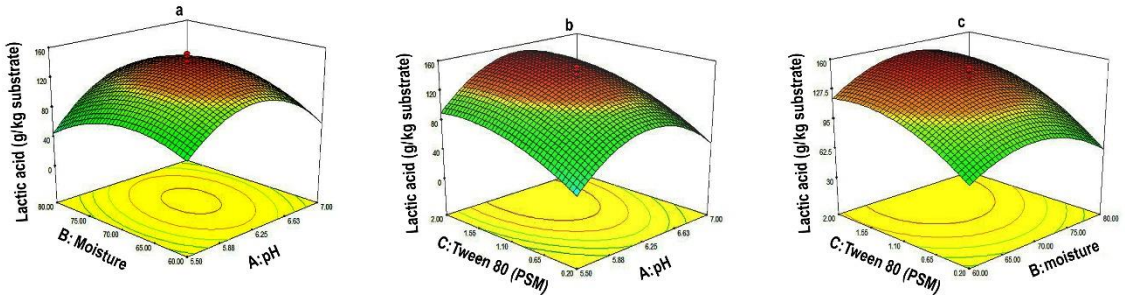

Fig. 3. 3D response surface plot of lactic acid production by *L. plantarum* LB2 showing interaction between (a) Moisture and pH, (b) pH and PSM, and (c) Moisture and PSM

Properties of Film

The appearance of the film prepared with 1% chitosan is shown in Fig. 4. The PLA-composite film was brownish in colour. The colour of the film is due mainly to the fabrication of chitosan. The physical and chemical properties of the chitosan-polylactic acid composite material and polylactic acid are described in Table 8. In addition, the tearing strength of the composite film prepared using 1% chitosan was greater than that of polylactic acid. These findings revealed that this film is suitable for food packaging. The incorporation of chitosan improved the tensile, and elongation strengths, and improved the tearing strength. The supplementation of chitosan may affect continuities in polylactic acid, thus affecting tensile strength (Bonilla *et al*. 2013). The supplemented chitosan particles improved the tearing strength at a 1% chitosan concentration. The present findings revealed that the optimum amount of chitosan improved the tearing strength. The moisture content and solubility (%) were affected at higher chitosan concentrations in the composite material. This can be attributed mainly to the hydrophilic nature of the chitosan particles (Wang *et al*. 2019). These findings revealed that this film is suitable for food packaging. The physical and mechanical properties of polylactic acid/chitosan-modified composite material were determined previously by Wu and Wu (2006) and Kasirajan *et al.* (2019). In this study, the increasing concentration of chitosan increased the water vapour transmission rate, with 2% chitosan-polylactic acid film resulting in a high water vapour transmission rate (0.91 ± 0.12 g mm/m^2 day kPa). The increased vapour transmission rate at higher chitosan concentrations in the film may be due to the hydrophilic property of chitosan (Wang *et al*. 2019).

Fig. 4. Appearance of polylactic acid-chitosan. The prepared polylactic acid was mixed with chitosan and physical and chemical properties were improved for packing.

Table 8. Physical and Chemical Properties of Polylactic Acid-Chitosan Film

Characters	1% CH-PLA	1.5% CH-PLA	2% CH-PLA	PLA
Tensile strength (Kgf/cm^2)	198 ± 2.3^a	173 ± 2.3^b	148 ± 5.8^c	338 ± 3.1^d
Elongation at break (%)	312 ± 2.9^a	281 ± 2.2^b	214 ± 3.5^c	379 ± 1.9^d
Tearing strength (gf)	84 ± 1.5^a	82 ± 1.1^a	75 ± 1.2^b	42 ± 0.9^d
Solubility (%)	1.85 ± 0.21^a	0.58 ± 0.13^b	0.16 ± 0.02^c	0.93 ± 0.08^d
Moisture (%)	2.72 ± 0.04^a	2.89 ± 0.08^b	2.96 ± 0.09^c	0.19 ± 0.03^d
Water vapor permeability (g mm/m^2 day kPa)	0.72 ± 0.04^a	0.89 ± 0.13^b	0.91 ± 0.12^b	0.49 ± 0.03^c

Data are given as mean ± SD (n=5). Different superscript lowercase letters in the same row indicates significant difference ($p<0.05$).

Antibacterial Activity of Polylactic Acid-Chitosan Composite Materials

The antibacterial activity of the polylactic acid-chitosan composite prepared with 1% chitosan was analyzed, and the results are depicted in Fig. 5. Compared with the other selected bacterial strains, the nanocomposite material was highly active against *B. cereus* (21 ± 1 mm). In addition, the polylactic acid-composite material was moderately active against *S. aureus* and *E. coli* (Fig. 6), and the zone of inhibition was <15 mm diameter.

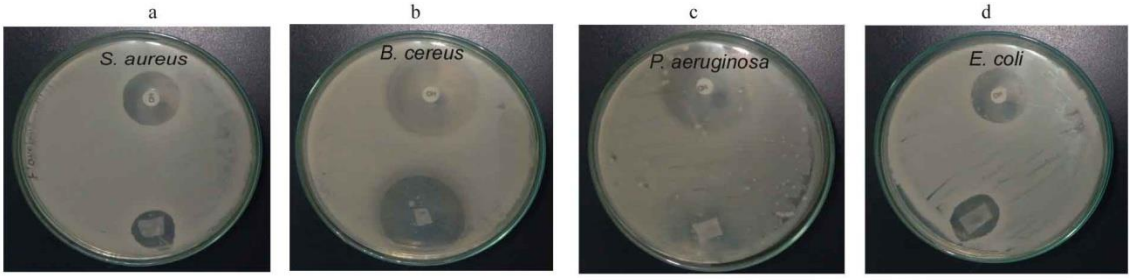

Fig. 5. Antibacterial activity of the composite material against human pathogens by disc diffusion method. PLA-chitosan sheet prepared using 1% chitosan was cut and placed on Mueller Hinton agar plates and incubated for 24 h. A clear zone was observed around the sample.

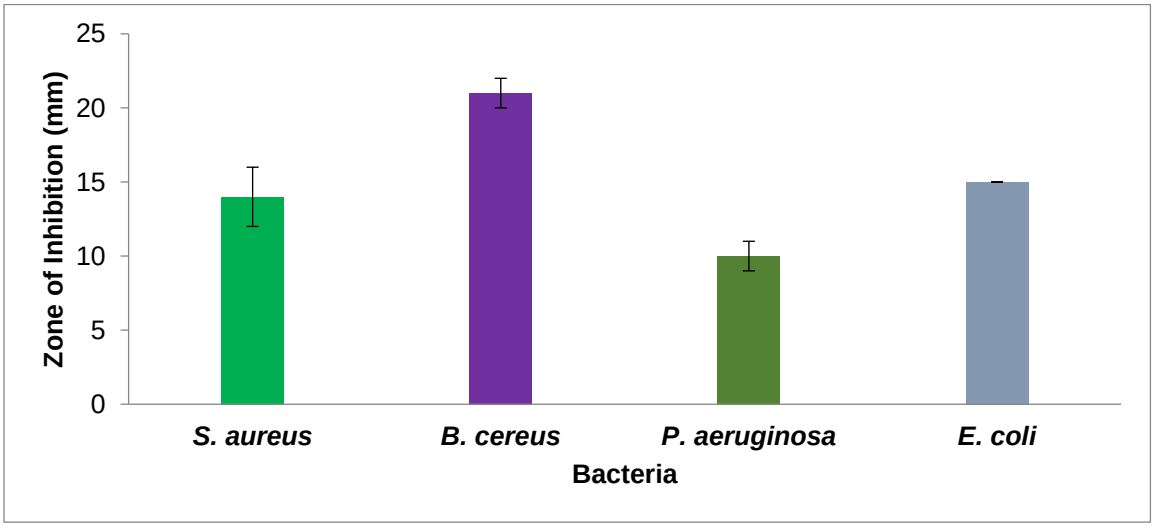

Fig. 6. Antibacterial activity of polylactic acid-chitosan against human bacterial pathogens; the error bars indicate the standard deviation.

The results obtained in the present study were similar with the antimicrobial properties of silver doped polylactic acid/chitosan nanofibers (Goh *et al.* 2016), which are active against *E. coli*. Chang *et al.* (2021) fabricated chitosan–PLA plastic films and 0.5% supplemented chitosan showed antibacterial efficacy against *Pseudomonas fluorescens*, *Escherichia coli*, and *Staphylococcus aureus*. The antibacterial activity obtained in this study was lower than that of the PLA-silver-chitosan nanocomposite materials. Demchenko *et al.* (2022) fabricated a silver-containing polylactic acid–chitosan material and the presence of silver ions in the composite resulted in significant activity against *E. coli*, and *S. aureus*, with zones of inhibition of 25 mm, and 25.8 mm, respectively.

Application of Polylactic Acid-Chitosan Film in Fish Fillet Storage

Foodborne disease outbreaks are difficult to assess and are rarely reported. In the United States, muscle or meat significantly contributes to foodborne outbreaks. The number of outbreaks ranges from 500 to 1500 every year (Dehnad *et al*. 2014). In India, more than 30 foodborne outbreaks have been reported and most of these outbreaks are due to bacterial etiological agents such as *Salmonella* spp., *Staphylococcus aureus* and *Vibrio* spp. (Rao *et al*. 2012).

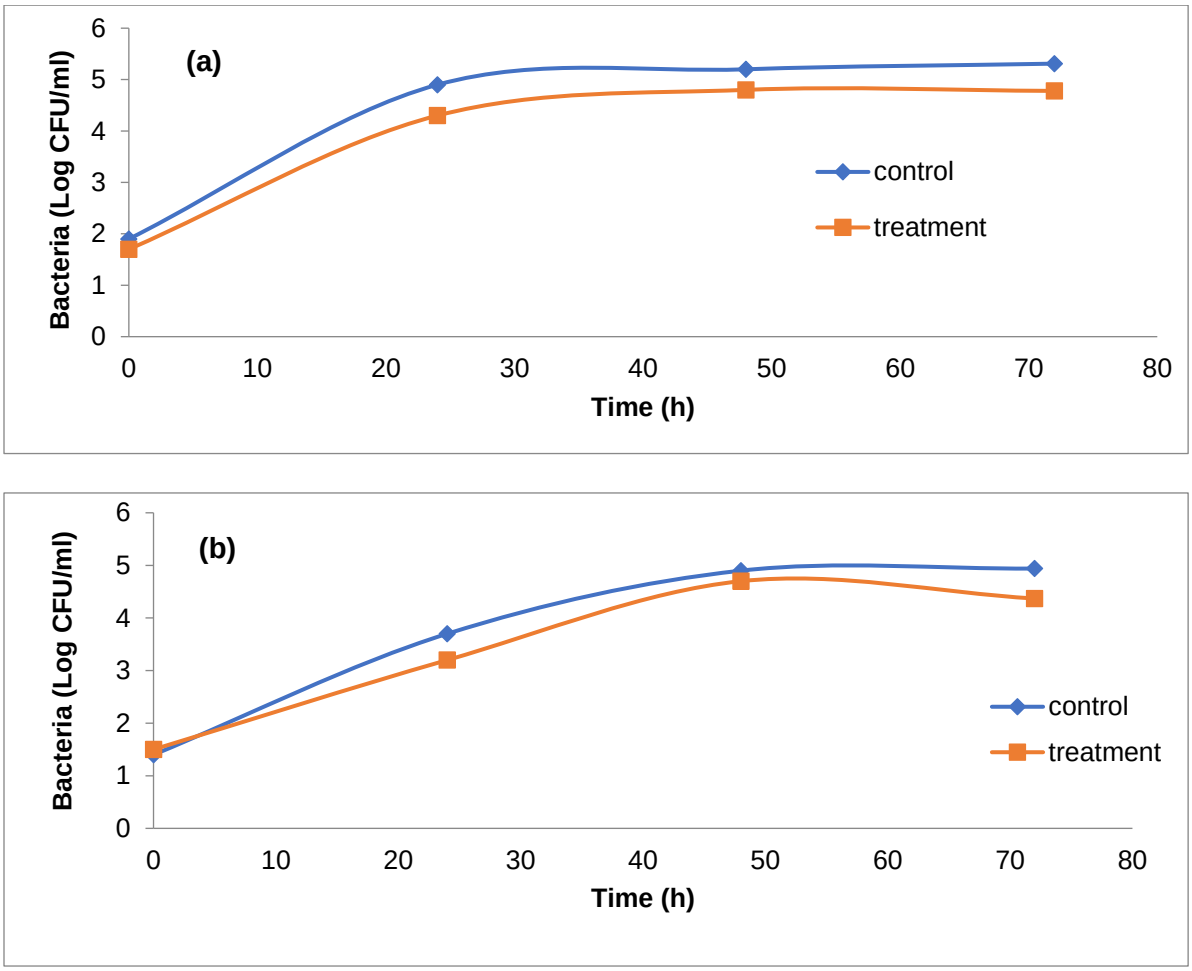

Fig. 7. Total mesophilic (a) and psychrotrophic bacterial population of fish fillet packed with film (experiment) and without film (control)

Fishes are highly perishable food items; surface contaminants enter the inner layers, and at this stage it is very complicated destroy or inactivate pathogens from the food items. The addition of antimicrobial agents to packing materials has become a highly popular alternative to synthetic food preservatives. In this line of natural product research, the fabricated chitosan-based materials (chitosan coatings containing ε-polylysine and rosmarinic acid) have been used to preserve fillets (Li *et al*. 2019). Chitosan is a preservative that is frequently used in the preparation of packing materials. The present study revealed that the prepared polylactic acid-chitosan film protected against food spoiling bacterial strains during storage at various temperatures. Variations in the microbial population under mesophilic conditions in the control and film wrapped fish fillets were observed. The mesophilic bacterial population was 4.3 ± 0.1 Log CFU/g in the film wrapped fillet and it was high in the control fish fillet (4.9 ± 0.2 Log CFU/g) (Fig. 7a). In the film wrapped fish fillets, the psychrotrophic count was 3.2 ± 0.2 Log CFU/g after 24 h and in the control increased bacterial count was observed after 24 h of treatment (3.7 Log CFU/g) (Fig. 7b). The polylactic acid chitosan-based composite materials reduced bacterial load during storage. Due to its biodegradability and renewability, it is used in the preparation of non-toxic packing materials to store perishable foods (Scaffaro *et al.* 2018). Polylactic acid-based composite materials were used to improve the shelf life of fish (Khanjani *et al.* 2023), and PLA-chitosan-essential oil coated packing improved the quality of chicken (Fiore *et al.* 2021).

CONCLUSIONS

1. Rubber seed shells are abundant in East Asian and Asian countries and pose a threat to the environment. The utilization of rubber seed shell substrate helps to alleviate environmental pollution because more than 50% of rubber seeds are not utilized for commercial use.
2. The results of the present study revealed that the screened *Lactobacillus plantarum* LB2 was the most effective bacteria for lactic acid production.
3. The lactic acid was purified and utilised in the synthesis of polylactic acid-chitosan films.
4. The film prepared with polylactic acid and 1% chitosan exhibited antibacterial activity and was effective against Gram-positive and Gram-negative bacteria.
5. This film was effective at storing fish fillets and reducing the bacterial population.

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