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ORIGINAL ARTICLE

Enhanced production of 3-phenyllactic acid from novel non-axenic coculture: adaptive evolution and statistical fermentation studies

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Abstract

This research pivots around screening of idoneous lactic acid bacteria (LAB) from cow milk and subjecting them to adaptive evolution experiments to aid superior growth/robustness necessary for 3-phenyllactic acid (3-PLA) production. Conventional and statistical fermentation studies were conducted at batch scale using a non-axenic coculture of three novel LAB strains: *Lactiplantibacillus plantarum* str. nov. plantharim, *Lactobacillus delbrueckki* str. nov. delharim, and *Pediococcus pentasaceous* str. nov. pentharim. Statistically optimized fermentation using Box Behnken technique resulted in 1225 mg/L 3-PLA production using the growth medium: cheese whey—MRS medium mixture (5:2 ratio), phenylalanine (2.69% w/v), and glucose (9.6% w/v). Statistical optimization of fermentation parameters resulted in a substantial increase (17 times higher) compared to the non-optimized fermentation conditions (72 mg/L). Monad growth kinetics of the cow milk whey (CMW) coculture were calculated and estimated as: $\mu_{max} = 0.336\ h^{-1}$, $K_s = 11.64$ mg/mL, $Y_{x/s} = 0.835$ mg/g, $Y_{P/S} = 1.66$ mg/g, $Y_{X/P} = 0.112$ mg/mg. The purified 3-PLA (1.93 mg/mL) showed antimicrobial activity with pathogenic bacteria like *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus*, with a minimum inhibitory concentration of 12 mg/mL.

Keywords Cheese whey · Phenyllactic acid · Lactic acid bacteria · Fermentation · Coculture · Antimicrobial activity

1 Introduction

LAB are resourceful entities which can ferment carbohydrates, fruit-processing residues, vegetable peels (agro-food waste biomass matter) to economically produce lactic acid (LA), and other secondary metabolites like PLA, acetic acid, and bacteriocins [1] [2] [3]. Cheese whey (CW) is the residual fluid generated as a by-product of cheese-manufacturing industries, and can serve as a nutrient-rich growth-matrix which can sustain LAB sustenance for revalorization into useful metabolites [4] [5] [6] [7]. CW hydrolysates augmented with phenyl pyruvic acid or phenylalanine and other nutrients when fermented with *Lactobacillus plantarum* CECT221 could yield LA and PLA [7] [8]. LAB fermentation of yellow mustard and milk whey (as substrates) resulted in the production of DL-3-PLA and LA with considerable antioxidant activity [9]; moreover, CW is an inexpensive nitrogen-rich substrate which can be exploited holistically by LAB [10] [6]. It has been recently reported that LAB-fermented whey could effectively be used as a bio-preservative that could extend the shelf-life of bread due to antimicrobial activities [11].

PLA/2-hydroxy-3-phenyl propionic acid/$C_9H_{10}O_3$/β-phenyllactic acid is a natural organic acid-derivative of phenyl-alanine catabolism which is metabolized by lactate dehydrogenase (during glycolysis) [12, 13], and occurs in probiotic-foods, viz., honey, milk /milk products, cheese, pickles, and sourdough [14] [15]. Chemically PLA occurs in two enantiomeric forms: D-PLA and L-PLA (based upon C_2 position chirality), while the former has better antimicrobial properties with efficacy at operational pH/temperature ranges, better diffusibility and/or water solubility [16] [17]. PLA production from LAB strains isolated from various matrices has been reported: 24 *Lactobacillus* strains were isolated from the pig's caecum and intestines (80–119 mg/L) and pig feces (233.0 mg/L, *L. plantarum* r16) [18], *Geotrichum candidum* (600 ~ 1000 mg/L) strain

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isolated from cheese [19], *Leuconostoc mesenteroides* ITMY30 (0.57 ± 0.04 mM) strain isolated from olive-phylloplane [20], *Lactobacillus plantarum* 21B (56.0 g/L) isolated from sourdough bread [21], and *Pediococcus pentosaceus* SK25 (47.2 mg/L mg/L) isolated from Chinese pickles [22].

Adaptive laboratory evolution (ALE) or metabolically engineering LAB strains could improve their robustness and tolerance to higher temperature, and lactic acid or the substrate (CW) contents; moreover, coculturing two/more LAB strains together could further enhance growth and subsequent PLA yields through inter-dependency without using genetic biotransformation [23], and ALE has also been proven to be inherently restricted to traits benignly associated with cell fitness (like nutrient utilization) [24]. Adapted inoculum effectually promoted lactic acid production while using mixed culture fermentation of food waste [25]. Furthermore, traditional and statistical fermentation steps could be used to control other physicochemical fermentation factors which influence growth and intracellular activity [26]. Optimal PLA production was reported with: *Lactobacillus* sp. SK007 at 30 °C when cultivating in stationary mode [27]; *Lactobacillus paracasei* W2 when cultured at a pH-range of 6.5–7.0 [28]; *Lactobacillus* strains in fed-batch mode with monitored pH, agitation, and supplements (glucose, phenyl pyruvic acid, sodium hydroxide, agitation) [29]; recombinant *Escherichia coli* cultivated under restricted oxygen levels to trigger l-phenylalanine production [30]; *Lactococcus lactis* which produces aminotransferases (converting phenylalanine to phenyl pyruvic acid (PLA-precursor)) [31]; *Lactobacillus plantarum* SK002 which produces lactate dehydrogenases (LDH) triggering bioconversion of phenylalanine substrates at 30–45 °C and 5.5–7.0 [32]; and *Pediococcus pentosaceus* which produces D-LDH using NADH regeneration by *Ogataea parapolymorpha* formate dehydrogenase [22], etc.

Critically reviewing the state-of-art of ongoing researches which adopt impactful/fruitful research strategies for producing 3-PLA using batch stage fermentation studies, herewith we discuss the summative application of a sequential stratagem using adapted non-axenic coculture inoculum for fermenting cheese whey rich growth medium, conventionally/statistically optimizing batch fermentation parameters, and performing Fourier-transform infrared spectroscopy/High-performance liquid chromatography (FTIR/ HPLC) analyses to detect/quantify the produced 3-PLA production levels. A flow chart diagram of the sequential application of the orchestrated strategy is shown in Fig. 1.

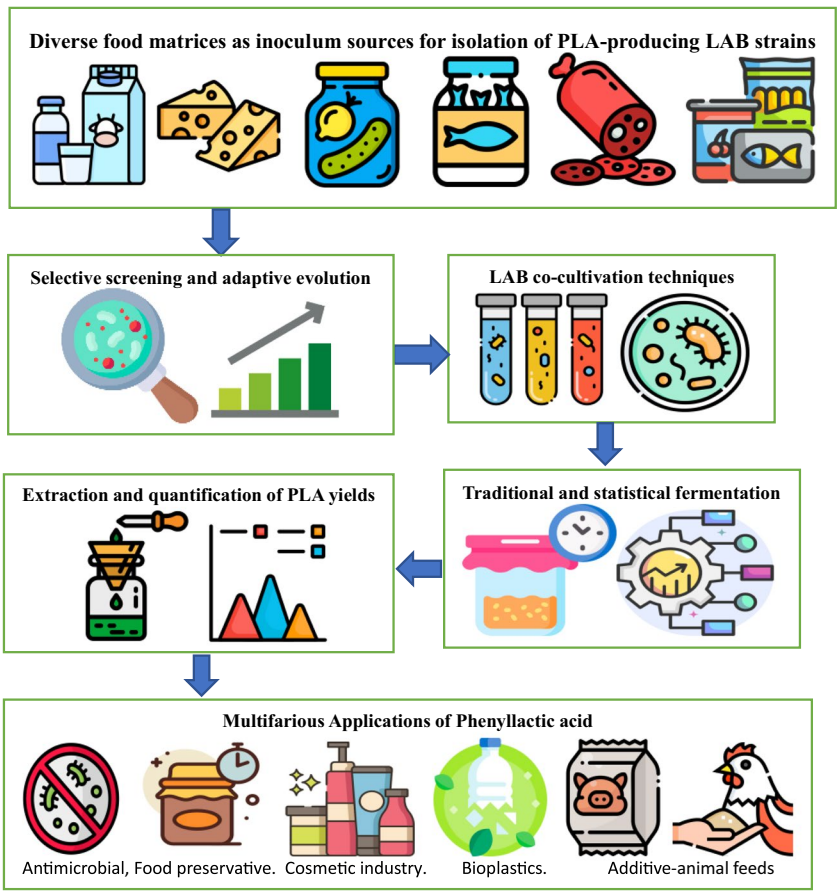


Fig. 1 Schematic showing the sequential strategy for: isolation, selective screening of LAB from diverse food matrices like milk and fermented milk products, etc.; adaptive evolution to improve LAB robustness; LAB cocultivation techniques to promote augmented PLA yields; traditionally and statistically optimizing the fermentation parameters to increase PLA yields; extraction and quantification of the PLA yields; and studying the application prospects of the PLA yielded in suitable industrial sectors as antimicrobial agents finding usage in food preservation, etc.

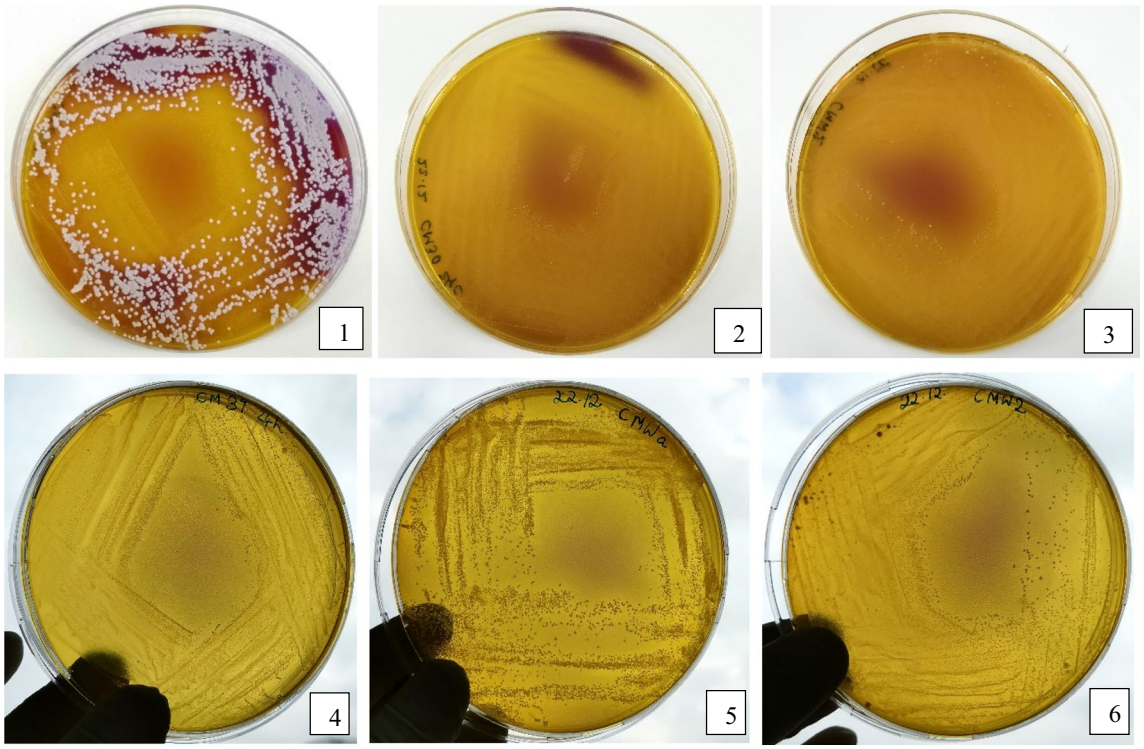


Fig. 2 Pictures of LAB inocula quadrant streaked upon MMRS agar plates. (1) RCW (non-axenic), (2) FCM (non-axenic), (3) CMW (non-axenic), (4) CM30_001 (axenic), (5) CMW_10-3 (axenic), (6) CM30_001 + CMW_10-3 (co-culture). The violet color of the Bromocresol purple dye supplemented within the MMRS agar medium is decolorised into orangish-yellow shades of colors displaying the different metabolisms of medium by the different LAB cultures

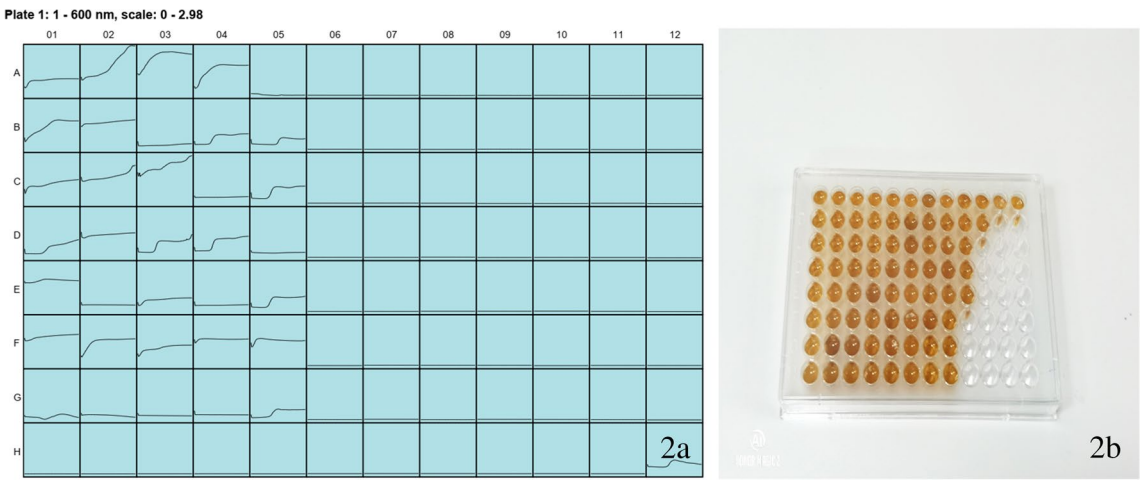
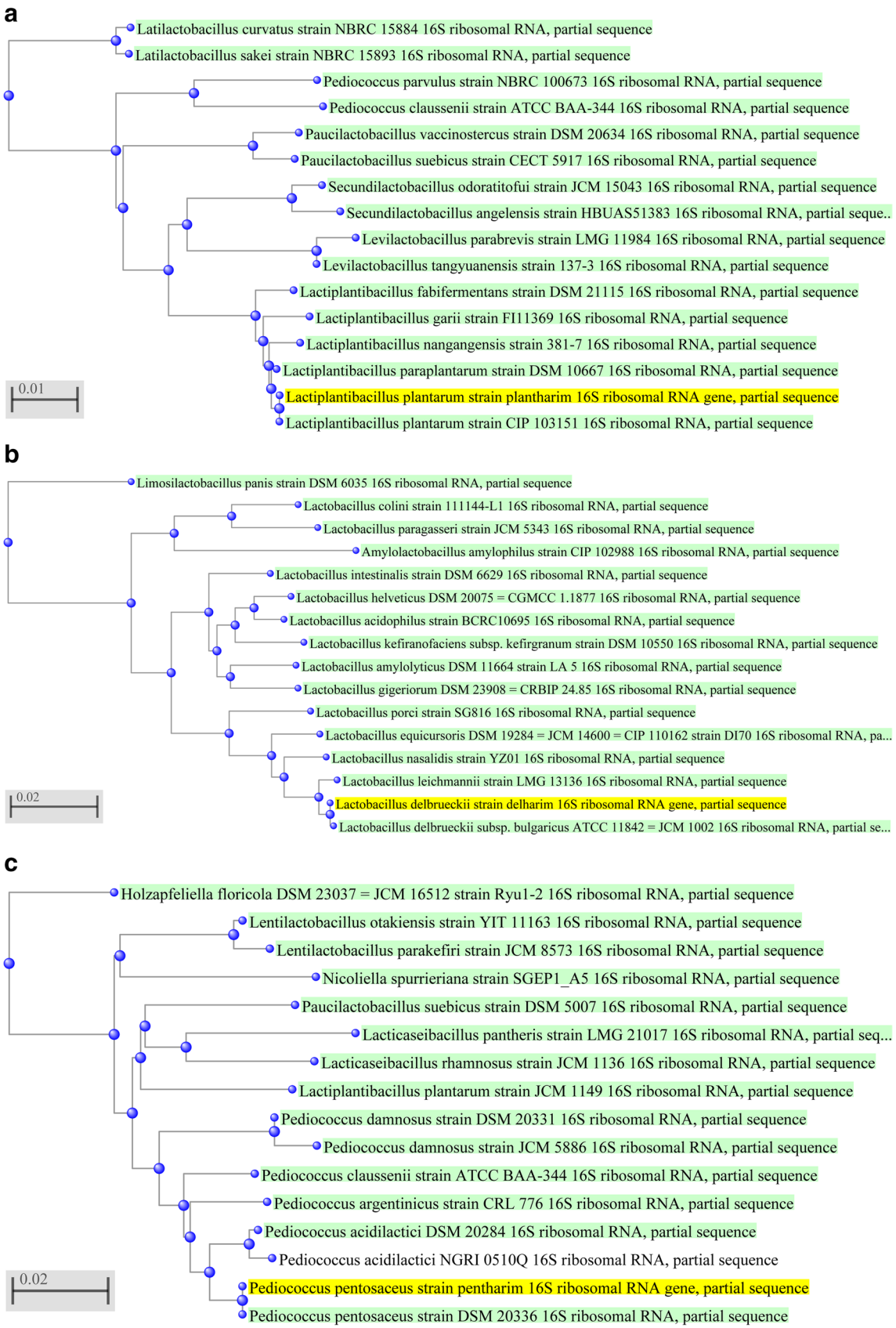


Fig. 3 **a** The best blended growth patterns or curves (left) were recorded for adapted mixed-cultures sourced from RCW (A02), FCM (A03), and CMW (A04), compared to the growth curves of bacterial isolates as single cultures and co-cultures (two/three pure isolates) in various combinations are shown in other wells of 96-well plate (growth absorbance was recorded at 600 nm). Isolate CM30_001 and Isolate CMW_10-3 also showed blended growth as coculture (F02) and as single strains (B01, C02) also. **b** 96-well plate loaded with samples of both non-axenic LAB strains and axenic LAB strains both as single cultures or in various co-culture combinations, incubated for 5 days


a
Latilactobacillus curvatus strain NBRC 15884 16S ribosomal RNA, partial sequence
Latilactobacillus sakei strain NBRC 15893 16S ribosomal RNA, partial sequence
Pediococcus parvulus strain NBRC 100673 16S ribosomal RNA, partial sequence
Pediococcus claussenii strain ATCC BAA-344 16S ribosomal RNA, partial sequence
Paucilactobacillus vaccinostercus strain DSM 20634 16S ribosomal RNA, partial sequence
Paucilactobacillus suebicus strain CECT 5917 16S ribosomal RNA, partial sequence
Secundilactobacillus odoratitofui strain JCM 15043 16S ribosomal RNA, partial sequence
Secundilactobacillus angelensis strain HBUAS51383 16S ribosomal RNA, partial seque..
Levilactobacillus parabrevis strain LMG 11984 16S ribosomal RNA, partial sequence
Levilactobacillus tangyuanensis strain 137-3 16S ribosomal RNA, partial sequence
Lactiplantibacillus fabifermentans strain DSM 21115 16S ribosomal RNA, partial sequence
Lactiplantibacillus garii strain FI11369 16S ribosomal RNA, partial sequence
Lactiplantibacillus nangangensis strain 381-7 16S ribosomal RNA, partial sequence
Lactiplantibacillus paraplantarum strain DSM 10667 16S ribosomal RNA, partial sequence
Lactiplantibacillus plantarum strain plantharim 16S ribosomal RNA gene, partial sequence
Lactiplantibacillus plantarum strain CIP 103151 16S ribosomal RNA, partial sequence
0.01
b
Limosilactobacillus panis strain DSM 6035 16S ribosomal RNA, partial sequence
Lactobacillus colini strain 111144-L1 16S ribosomal RNA, partial sequence
Lactobacillus paragasseri strain JCM 5343 16S ribosomal RNA, partial sequence
Amylolactobacillus amylophilus strain CIP 102988 16S ribosomal RNA, partial sequence
Lactobacillus intestinalis strain DSM 6629 16S ribosomal RNA, partial sequence
Lactobacillus helveticus DSM 20075 = CGMCC 1.1877 16S ribosomal RNA, partial sequence
Lactobacillus acidophilus strain BCRC10695 16S ribosomal RNA, partial sequence
Lactobacillus kefiranofaciens subsp. kefirgranum strain DSM 10550 16S ribosomal RNA, partial sequence
Lactobacillus amylolyticus DSM 11664 strain LA 5 16S ribosomal RNA, partial sequence
Lactobacillus gigeriorum DSM 23908 = CRBIP 24.85 16S ribosomal RNA, partial sequence
Lactobacillus porci strain SG816 16S ribosomal RNA, partial sequence
Lactobacillus equicursoris DSM 19284 = JCM 14600 = CIP 110162 strain DI70 16S ribosomal RNA, pa...
Lactobacillus nasalidis strain YZ01 16S ribosomal RNA, partial sequence
Lactobacillus leichmannii strain LMG 13136 16S ribosomal RNA, partial sequence
Lactobacillus delbrueckii strain delharim 16S ribosomal RNA gene, partial sequence
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 = JCM 1002 16S ribosomal RNA, partial se...
0.02
c
Holzapfeliella floricola DSM 23037 = JCM 16512 strain Ryu1-2 16S ribosomal RNA, partial sequence
Lentilactobacillus otakiensis strain YIT 11163 16S ribosomal RNA, partial sequence
Lentilactobacillus parakefiri strain JCM 8573 16S ribosomal RNA, partial sequence
Nicoliella spurrieriana strain SGEP1_A5 16S ribosomal RNA, partial sequence
Paucilactobacillus suebicus strain DSM 5007 16S ribosomal RNA, partial sequence
Lacticaseibacillus pantheris strain LMG 21017 16S ribosomal RNA, partial seq...
Lacticaseibacillus rhamnosus strain JCM 1136 16S ribosomal RNA, partial sequence
Lactiplantibacillus plantarum strain JCM 1149 16S ribosomal RNA, partial sequence
Pediococcus damnosus strain DSM 20331 16S ribosomal RNA, partial sequence
Pediococcus damnosus strain JCM 5886 16S ribosomal RNA, partial sequence
Pediococcus claussenii strain ATCC BAA-344 16S ribosomal RNA, partial sequence
Pediococcus argentinicus strain CRL 776 16S ribosomal RNA, partial sequence
Pediococcus acidilactici DSM 20284 16S ribosomal RNA, partial sequence
Pediococcus acidilactici NGRI 0510Q 16S ribosomal RNA, partial sequence
Pediococcus pentosaceus strain pentharim 16S ribosomal RNA gene, partial sequence
Pediococcus pentosaceus strain DSM 20336 16S ribosomal RNA, partial sequence
0.02

◂**Fig. 4** Neighbor-joining tree based on almost complete 16S rRNA sequences showing relationships between the novel LAB strains: **a** *Lactiplantibacillus plantarum* plantharim, **b** *Lactobacillus delbrueckki* delharim, and **c** *Pediococcus pentasaceous* pentharim, representatives of their family and related taxa (attached PDF files separately)

2 Materials and methods

2.1 Isolation and adaptive evolution studies

LAB strains isolated from fresh raw cow milk using inoculum samples (non-axenic cultures): raw cow milk, 48 h fermented milk curd at room temperature, and whey separated from fermented milk curd; were fermented in MRS medium supplemented with $CaCO_3$ (5 g/L) and Bromocresol purple (0.12 g/L) for 24 h at 37 °C [33]. Several axenic and non-axenic cultures were detected as LAB strains (strains that discolor bromocresol purple dye to yellow-orange) which can produce 3-PLA confirmed through FTIR analysis. Commercial cheese whey (CW) powder and MRS medium were used as the chief substrates of interest for fermentation studies. The selected LAB strains (non-axenic cocultures and axenic strains) were subjected to adaptive evolution studies by growing in MRS medium supplemented with $CaCO_3$ (5 g/L), at increasing temperatures (up to 37 °C) and higher contents of cheese whey (up to 80%) replacing MRS medium to promote the growth of robust LAB strains [34].

3-PLA production ability was confirmed qualitatively using FTIR—Perkin Elmer FT-IR System, PerkinElmer Spectrum Version 10.5.2. 1% of chosen inoculum (~ 4×10^8 CFU/mL) was inoculated in MRS broth, fermented for 24 h, centrifuged at 4500 × g, 15 min, 4 °C, and the separated supernatant was filtered using 0.45-µm microfilter; this supernatant was used as the 3-PLA extract to be studied in FTIR analysis after vacuum desiccating to remove the water content in it using Labconco Free Zone-4.5 freeze drier. The coculture growth patterns of chosen axenic/non-axenic cultures in desired combinations were studied using 96-well plate method analyzed with Varioskan Flash Instrument version 4.00.52 Microplate reader fitted with SkanIt Software 2.4.3 RE. HPLC analysis was used to detect 3-PLA production quantitatively: a standard graph showing absorbance data (mAU) versus 3-phenyllactic acid (mg/L) was constructed using different contents of pure 3-PLA dissolved in various concentrations of standard MRS broth solution and HPLC analyzed; here the elution was performed with methanol/0.05%TFA(solvent A) and water/0.05% TFA(solvent B) at 1 mL/min and A/B ratios of 10:90, 100:0, 100:0, and 10:90, with run times of 0, 20, 23, and 25 min, respectively [35]. All chemicals used were of analytical grade purchased from Merck Millipore, Germany.

2.2 Optimization studies

The STATISTICA Version 10 software was used to conduct response surface methodology (RSM) studies [36]. Three selective physico-chemical parameters that affect 3-PLA production were optimized using Box Behnken design (adopting 3 variables with 15 runs) where each factor is studied at low, medium, and high points (− 1, 0, + 1) yielding a set of 15 varying experimental design. The application of RSM yielded the below regression equation elucidating the empirical relationship between 3-PLA yield and test variables in coded units.

$$Y = 665 + 276X1 + 27X1X1 + 378X2 + 236X2X2 + 354X3 + 67X3X3 + 136X1X2 - 64X1X3 + 60X2X3;$$

where *Y* is 3-PLA yield; X1, X2, and X3 are coded values of Substrate (CW: MRS) ratio, phenylalanine (or yeast extract), and glucose, respectively. Estimation of regression analysis and analysis of variance (ANOVA) is made to explicate the regression coefficient, and check resemblance between experimental and predicted values. At the defined statistically optimized conditions, batch scale fermentation was conducted using the chosen non-axenic coculture in larger scale. Batch fermentation was conducted in larger scale using the CMW coculture (*Lactiplantibacillus plantarum*, *Lactobacillus delbrueckki*, and *Pediococcus pentasaceous* in 1:1:1 ratio, ~ 4×10^8 CFU/mL) as inoculum in a 5000 mL Duran® laboratory bottle with a screwed cap to maintain an anaerobic cultivation environment. The working volume was 3000 mL sterilized fermentation medium (cheese whey mixed with MRS broth in 5:2

Table 1 ANOVA; R-sqr = .86878; Adj:.63257 (Spreadsheet1) 3 3-level factors, 1 Blocks, 15 Runs; MS Residual = 28,173.03 DV: Var4 (Var4 = 3-PLA yield)

	SS	df	MS	*F*	*p*
(1)Var1 L + Q	155638	2	77818.9	2.762178	0.155574
(2)Var2 L + Q	493408	2	246703.8	8.756735	0.023244
(3)Var3 L + Q	267559	2	133779.5	4.748496	0.069860
1*2	18632	1	18632.2	0.661351	0.453053
1*3	4160	1	4160.2	0.147668	0.716574
2*3	3660	1	3660.2	0.129920	0.733241
Error	140865	5	28173.0		
Total SS	1073474	14			

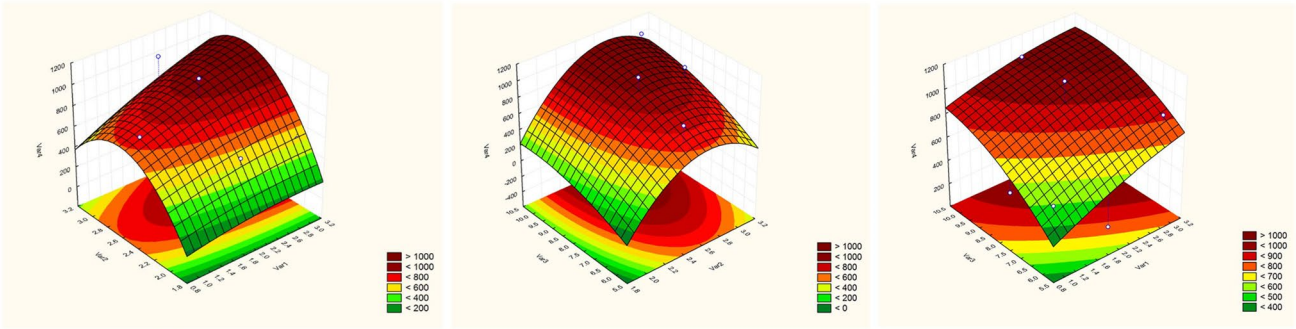


Fig. 5 Response surface plots of the three significant variables (Var1: substrate ratio CW/MRS %v/v, Var2: phenylalanine, Var3: glucose) correlation with the amount of 3-PLA production (Var4) at optimized levels. Contour plots showing 3-PLA production which is highest in the darkest region (darker color depicts increased response or higher 3-PLA production)

ratio, supplemented with 2.69% w/v phenylalanine, 9.6% w/v glucose, 5 g/L $CaCO_3$) and 125 ml was the inoculum/seed content added, the fermentation was done for 3 days maintaining a temperature of 37 °C; the 3-PLA yield (quantitatively measured through HPLC), cell dry weight, and OD at 600 nm were checked every 3 h.

2.3 3-PLA antimicrobial activity

PLA produced in the fermentation-broth of non-axenic CMW coculture after batch fermentation for 24 h was collected and centrifuged at 4500 × g, 15 min, 4 °C; the supernatant is separated and filtered using 0.45-µm microfilter [37] [38]; this supernatant is used as the PLA extract for purification studies. This cell-free supernatant was initially extracted (1:3 v/v) using ethyl acetate (organic solvent), concentrated under vacuum, and evaporated (using rotary evaporator) at a 37 °C. Furthermore, preparative HPLC (Agilent 1200 HPLC) using Agilent PrepHT C18 column (21.2 × 150 mm, 5 µm), elution was performed and read (UV detector-210 nm) using methyl cyanide-water (from 3:97 to 100:0) as elution gradient for half hour at 10 mL/minute with 37 °C column temperature [39], PLA peak fraction was collected and vacuum concentrated. This purified 3-PLA of 1.93 mg/mL concentration/yield and 96% purity is then studied for antimicrobial activity using regular protocol for well diffusion assay [40]. The dried residue of 3-PLA was diluted (by mixing with methanol/water in 1:1 ratio) to prepare different concentrations of purified 3-PLA (15, 12, 10, 5 mg/mL) to check the minimum inhibitory concentration (MIC) on the pathogenic bacteria. 50µL of purified 3-PLA (of different concentrations) was added into welled petriplates containing Mueller Hilton agar seeded with 3 food pathogens (*Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922) and incubated at 37 °C for 24 h [41] [42]. It is expected that 3-PLA added to the wells would seep into the agar to inhibit the growth of the pathogenic bacteria (seeded around) forming a clear zone of clearance commensurate with the content of 3-PLA added inside the well. The MIC would be defined as the minimum amount of purified

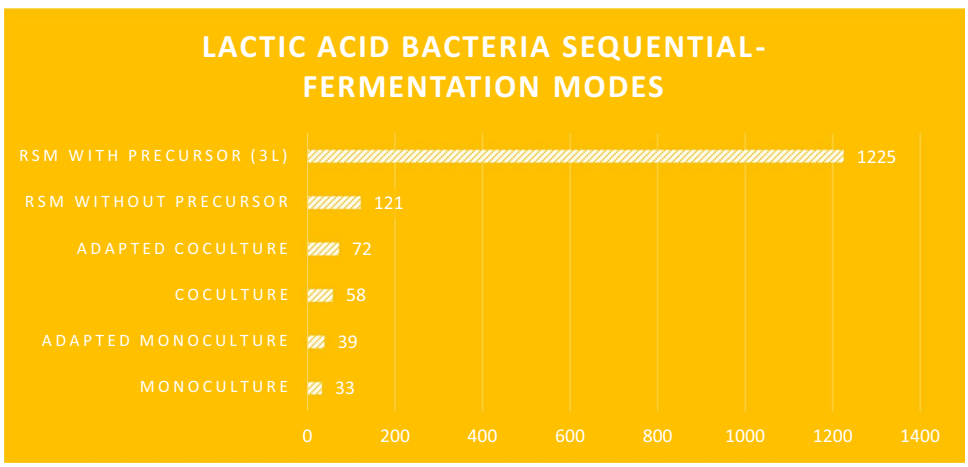


Fig. 6 Graph showing the gradual increase in PLA yield by sequentially adopting various fermentation modes one by one

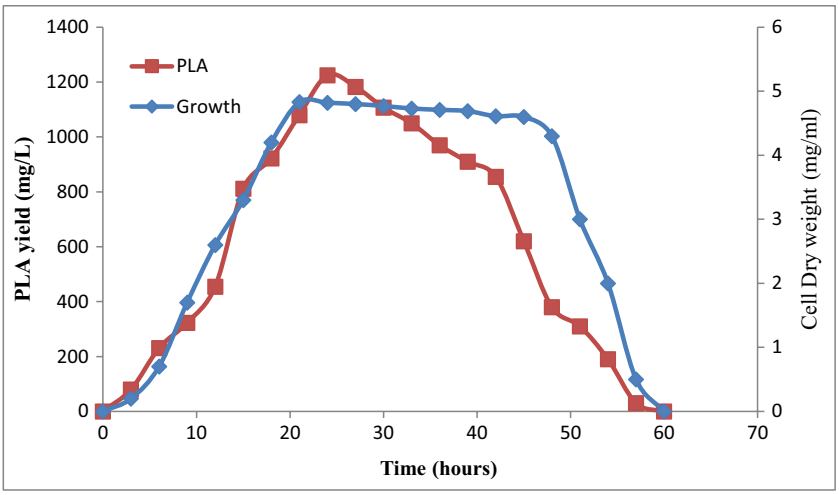


Fig. 7 Growth profile of non-axenic CMW coculture in relation to the 3-PLA production in batch fermentation at optimized conditions. The highest 3-PLA yield (1225 mg/L) was shown at 24th hour of cultivation

3-PLA needed for inhibiting pathogenic bacteria through antimicrobial activity. Good 3-PLA activity would mean production of zones of inhibition comparable to commercial dimethyl sulfoxide which has broad spectrum antimicrobial activity [43].

3 Results and discussion

3.1 Screening of 3-PLA producing LAB, adaptive evolution, identification studies

These adapted inoculum samples (both non-anexic cocultures and anexic/pure cultures) when streaked upon MRS agar plates showed brighter yellowish-orange-colored zones around them due to the hydrolysis of lactate by lactate dehydrogenase (LDH) enzyme, while some LAB cultures showed less/no discoloration around them making the agar to remain violet/purple in color [34] (Fig. 2). Comparatively, non-axenic cocultures RCW, FCM, CMW, and non-axenic cultures CM30_001, CMW_10-3 (axenic), CM30_001 + CMW_10-3, showed better orangish-yellow shades of colors. These chosen LAB strain isolates were subjected to adaptive evolution studies (for 13 weeks) resulting in improved metabolic tolerance to LA (lactic acid), higher CW contents, and higher temperature. Cocultivation studies were conducted to check for the best growth patterns or curves for axenic and non-anexic cocultures in various combinations using a 96 well-plate incubated for 5 days. Results showed that comparatively faster, better, and blended co-cultivation growth was observed with non-anexic cultures (CW, FCM, CMW) (Fig. 3) compared to axenic cultures (CM30_001, CMW_10-3) (previous study results) [44]. Among the various adapted non-axenic cocultures, CMW showed better growth and PLA production yield in the early stationary phase (24th hour). These LAB isolates (of CMW coculture) were confirmed for purity using simple Gram staining test and was morphologically characterized according to Bergey's Manual of Systematic Bacteriology [45]. Through 16srRNA genotypic tests, the CMW coculture cells was identified as novel LAB strains, *Lactiplantibacillus plantarum* plantharim (Genbank no. OR223590), *Lactobacillus delbreuckki* delharim (Genbank no. OR223591), and *Pediococcus pentasaceous* pentharim (Genbank no. OR223592), bearing 98.53%, 98.94%, and 99.42% genotypic similarity with their respective type strains. BLASTN suite of NCBI [46] was used to find the neighbor distance tree results for constructing fast minimum evolution trees (with maximum sequence difference of 0.75) as represented in Fig. 4 (a, b, c).

3.2 Optimized fermentation studies

The non-axenic co-culture CMW (*Lactiplantibacillus plantarum* str. nov. plantharim, *Lactobacillus delbreuckki* str. nov. delharim, *Pediococcus pentasaceous* str. nov. pentharim) was selected as inoculum for further optimization studies. The various physico-chemical fermentation parameters were optimized one by one conventionally using the traditional one-factor-at-a-time (OVAT) methodology followed by statistical techniques [26] in order to promote the highest 3-PLA productivity and yield; the OVAT fermentation parameters were found to be fermentation time of 24 h, 37 °C temperature, 7 pH, $CaCO_3$ (5 g/L), MRS medium (50%), cheese whey (50%), glucose 2%. The finally optimized parameters (critical values) showing best 3-PLA levels were found to be growth medium/substrate ratio 5:2 ratio, CW (71.5%v/v): MRS (28.5%v/v), supplemented with phenylalanine (2.69% w/v), glucose (9.6% w/v), maintaining other parameters constant.

Batch fermentation studies were done at the finally optimized parametric conditions and the maximum yield of

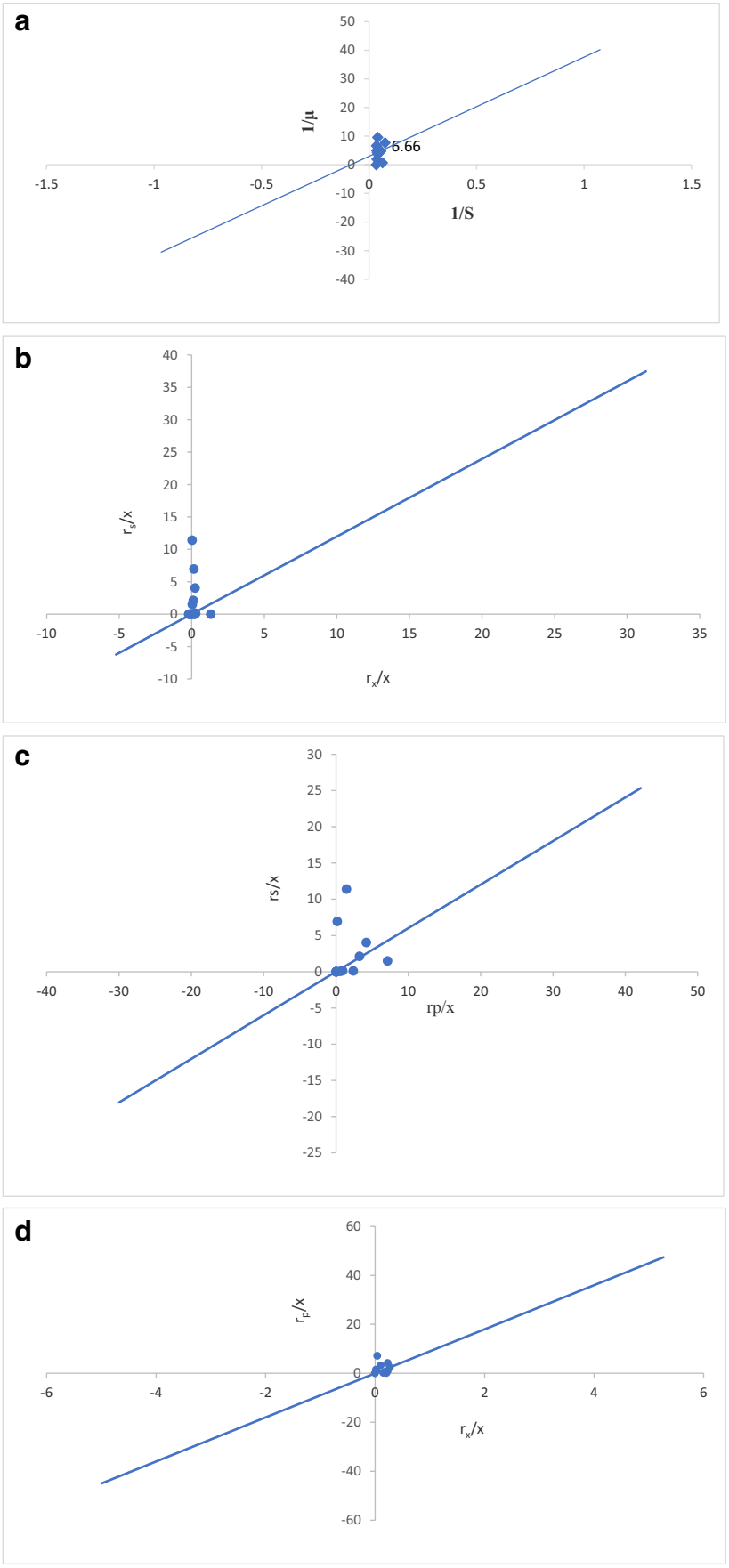


Fig. 8 Growth kinetics of CMW coculture: **a** plot for maximum specific growth rate and limiting nutrient values: $\mu_{max} = 0.336\ h - 1$, $K_s = 11.64$ mg/ml. **b** Plot for yield coefficient of biomass w.r.t. substrate: Yx/s = 0.835 mg/g. **c** Plot for yield coefficient of product w.r.t. substrate: $Y_{P/S} = 1.66$ mg/g. **d** Plot for yield coefficient of biomass w.r.t. product: $Y_{X/P} = 0.112$ mg/mg

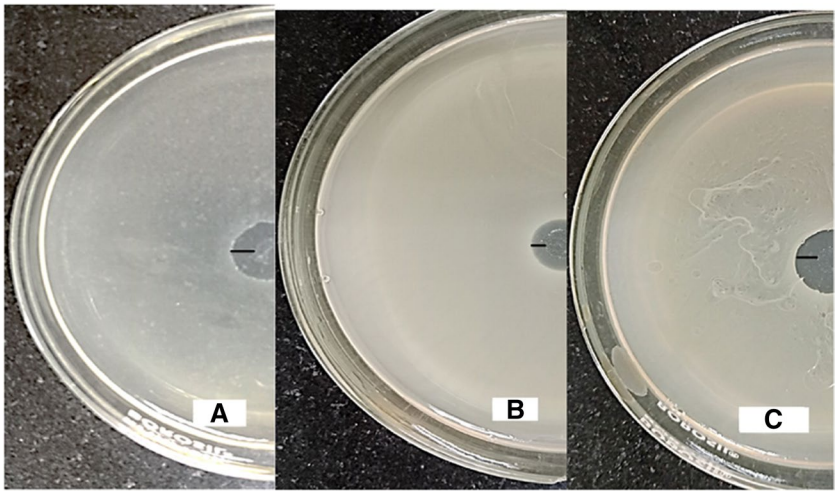


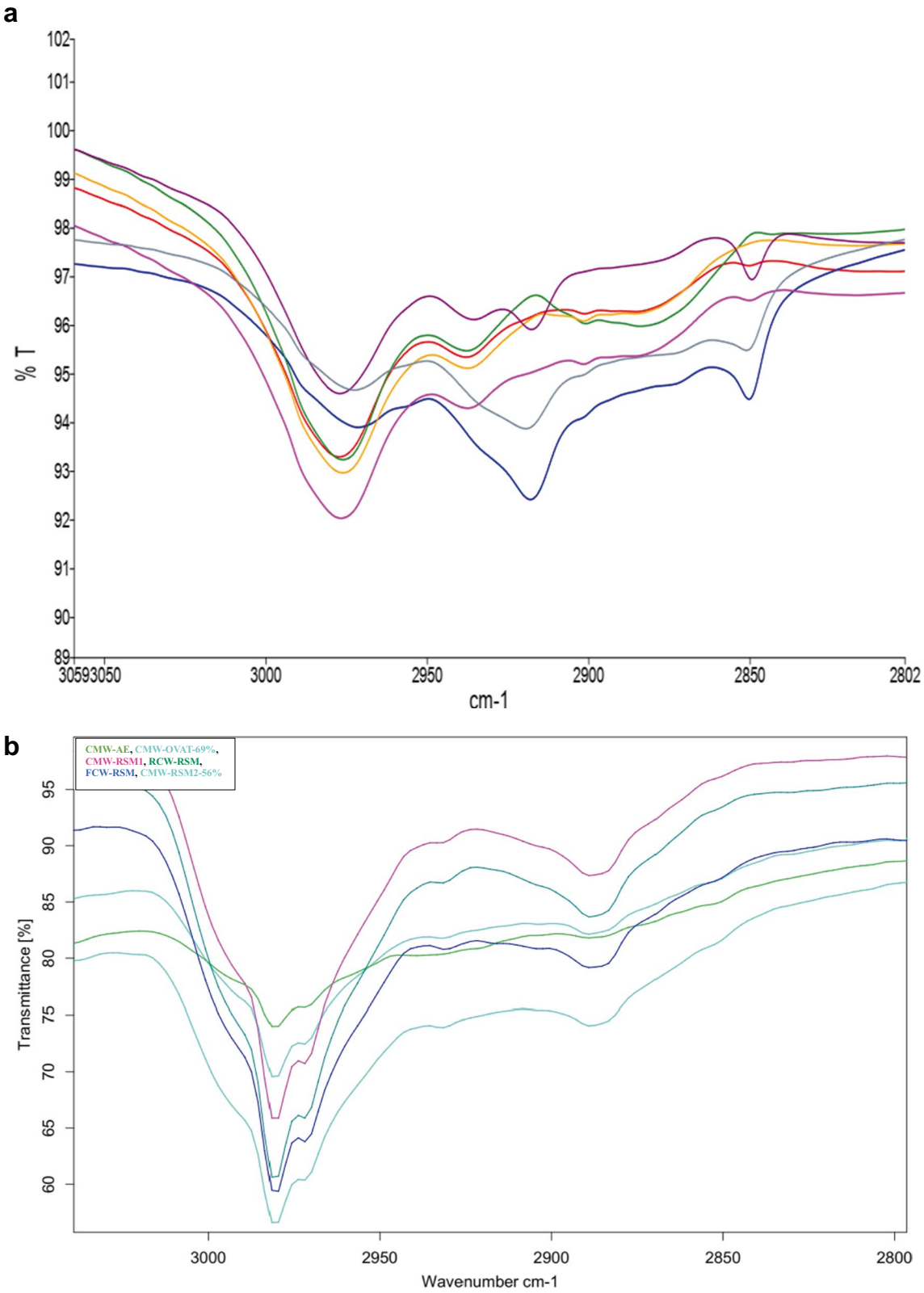
Fig. 9 Pictures of petriplates (sized: 90 mm diameter × 25 mm) with antimicrobial activity showing inhibitory zones (radius). **A** *Pseudomonas aeruginosa* ATCC 27853: 16 mm, **B** *Staphylococcus aureus* ATCC 25923: 9 mm, **C** *Escherichia coli* ATCC 25922: 17 mm

3-PLA production was 1225 mg/L which was similar to the predicted 3-PLA production was 1217 mg/L proving both the validity and effectiveness of the model. Statistical optimization envisaged similarity between predicted and experimental values through the ANOVA results (Table 1) explaining that the model was a good fit as there were few deviations in the experimental values when compared to the predicted values. The response surface graphs using statistical Box-Behnken technique adoption are shown in Fig. 5; presenting the Response surface plots of the three significant variables (Var1: substrate ratio CW/MRS %v/v, Var2: phenylalanine, Var3: glucose) in correlation with the amount of 3-PLA production (Var4) at optimized levels. Contour plots showing 3-PLA production which is highest in the darkest region, because as the color intensity increases response increases showing higher production of Var4 [47]. The benign interactions flanked by independent variables verify significance of each coefficient and the 3-PLA yield can be precisely predicted from the confined surface of the response surface diagrams (Fig. 5). Statistical optimization of fermentation parameters in large scale (3 L) resulted in a substantial increase (17 times higher) compared to the non-optimized fermentation conditions (72 mg/L). Statistical fermentation was conducted in two stages: (i) using yeast extract as supplement (in initial small-scale studies) with low 3-PLA production (121 mg/L) (ii) using phenylalanine as the supplement in large scale producing 1225 mg/L 3-PLA, this increase in production is because phenylalanine acts as the precursor for 3-PLA production [48] [22] (Fig. 6).

This can be interpreted as, PLA being a secondary metabolite it was produced in best quantities in the beginning of the stationary growth-phase, slowly started decreasing until the late-stationary phase, and the PLA yield levels decreased drastically once the stationary phase ended (Fig. 7). To understand the LAB coculture growth in relation with the 3-PLA production, the Monad growth kinetics were calculated and estimated as: specific growth rate $\mu_{max} = 0.336\ h - 1$, limiting nutrient value of $K_s = 11.64$ mg/ml, yield coefficient of biomass w.r.t. substrate Yx/s = 0.835 mg/g, yield coefficient of product w.r.t. substrate $Y_{P/S} = 1.66$ mg/g, yield coefficient of biomass w.r.t. product: $Y_{X/P} = 0.112$ mg/mg (Fig. 8).

3.3 Antimicrobial activity studies of purified PLA

50 µL of purified 3-PLA (15, 12, 10, 5 mg/mL) of 1.9 mg/mL concentration obtained from CMW-coculture was added into welled petriplates containing Mueller Hilton agar seeded with 3 food pathogens (*Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922) and incubated at 37 °C for 24 h. The inhibition activity against the pathogenic bacteria was gradually higher with the increase in 3-PLA concentration, at both 12 mg/mL and 15 mg/mL zone of clearance (pathogen inhibition) was similar, the best MIC for testing antagonistic activity of the purified 3-PLA was confirmed as 12 mg/mL for all the three pathogenic bacteria (Fig. 9). The antimicrobial activity results obtained were comparable to the radial zones of inhibition obtained using dimethyl sulfoxide (DMSO) as control (15 mm), the purified PLA showed greater antagonistic activity against *Pseudomonas aeruginosa* (16 mm) and *Escherichia coli* (17 mm), and lesser zone of inhibition was detected with *Staphylococcus aureus* (9 mm). This is the first report showing antimicrobial activity of 3-PLA from an LAB coculture containing *Lactiplantibacillus plantarum* plantharim, *Lactobacillus delbrueckii* delharim, and *Pediococcus pentosaceus* pentharim. Antimicrobial LAB axenic isolates *Lactobacillus pentosus* BMOBR041 and *Lactobacillus acidophilus* could inhibit standard pathogens showing zones of inhibition: *Pseudomonas aeruginosa* (10 mm, 15 mm), *Escherichia coli* (10 mm, 12.5 mm), and *Staphylococcus aureus* (15 mm, 13 mm), respectively [37].



4 Conclusion

As in, it was confirmed that the supplemented cheese whey medium containing "5 parts of cheese whey added to 3 parts of MRS broth medium supplemented with $CaCO_3$ (5 g/L)," was the most suitable growth medium for production of

◂**Fig. 10** **a** FTIR graph showing the transmittance levels of PLA detected for axenic monocultures of Lactobacillus isolates: **fcm10-2**, **cmw36_10-1**, **cmw36_10-2**, **cm30_001**, **fcm30_10-1**, **cmw10-3**, **fpo37_002** (depiction for comparison: monocultures have lesser 3-PLA yields than cocultures [44]). **b** Graph showing transmittance levels of PLA detected for non-axenic cocultures: after adaptively evolving CMW-coculture (CMW-AE), after optimizing CMW-coculture fermentation parameters using the traditional one-variable-at-a-time (OVAT) method (CMW-OVAT), after statistically optimizing CMW-coculture fermentation parameters without using 3-PLA precursor (CMW-RSM1), after statistically optimizing RCW-coculture fermentation parameters using phenylpyruvate (RCW-RSM), after statistically optimizing FCW-coculture fermentation parameters using 3-PLA precursor (phenylpyruvate) as the supplement (FCW-RSM), after statistically optimizing CMW-coculture fermentation parameters using 3-PLA precursor (phenylpyruvate) as the supplement (CMW-RSM2)

improved 3-PLA using a low-cost substrate like cheese whey which is less expensive compared to MRS medium. Batch fermentation was conducted sequentially adopting various modes of fermentation led to gradual increase in 3-PLA production: firstly using a monoculture (39 mg/L) [44] (previous study), using CMW-coculture (58 mg/L), using adaptively evolved CMW-coculture (72 mg/L), using adaptively evolved CMW-coculture applying statistical optimization without 3-PLA precursor (121 mg/L), using adaptively evolved CMW-coculture applying statistical optimization with 3-PLA precursor-phenylalanine in large-scale (3 L) (1225 mg/L) (Fig. 6). FTIR analysis of the 3-PLA production spectrum was shown to gradually increase step-by-step by fermenting non-axenic cocultures: after adaptively evolving CMW-coculture (74% transmittance), after optimizing fermentation parameters using the traditional one-variable-at-a-time (OVAT) method (69% transmittance), after optimizing statistically without using 3-PLA precursor (phenylpyruvate) (65% transmittance), after optimizing statistically using 3-PLA precursor (phenylpyruvate) as the supplement (56% transmittance). The 3-PLA production levels of monocultures are also shown for comparison purpose within the figure (Fig. 10 a, b). 3-PLA peaks with lower transmittance% represented higher 3-PLA production levels qualitatively [49]. 3-PLA production reported from *Lactobacillus plantarum* YM-4-3y (isolated from chinese fermented soybeans) using MRS medium was 400 mg/L [50], *Lactobacillus delbrueckii* ŁoCK 0987 (isolated from human intestinal tract) grown in MRS broth supplemented with galactosyl polyol was 84.3 mg/L [51], and *Pediococcus pentosaceus* sK25 (sourced from traditional chinese pickles) fermented with MRS broth was 135.6 mg/L [52]; however, this research shows 1225 mg/L 3-PLA production using cheese whey fermented with novel strains *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, and *Pediococcus pentosaceus*. This can be interpreted that high PLA production could be expected using the simple techniques coupled together using adapted non-axenic inoculum (sourced from fermented cow milk) and a cheap substrate like cheese whey. Five of the twelve Green Chemistry Principles are represented in this study: (1) preventing waste generation, (2) designing of benign biochemicals, (3) usage of renewable feedstocks, (4) less hazardous chemical synthesis, and (5) catalysis. Selected mixed microbial cultures (of raw cow milk) when used for fermentation studies under pre-defined fermentation conditions gave iterative results when conducted using raw cow milk samples procured from diverse geographical locations; hence, this sequential experimentation protocol "INTEGRATION VALORIZES SIMPLICITY" can be applied in any part of the globe to produce optimal yields of phenyllactic acid of proven antimicrobial quality and industrially comparable quantity (without using expensive precursors/supplements). This research compilation with orchestration of simple techniques together for production of 3-phenyllactic acid serves as a preliminary/introductory guide to any researcher having basic access to laboratory facilities, yet desirous to do quality research making the best utilization of time, efforts, and resources.

Author contribution Haritha Meruvu: Project Coordinator/ Executive (experienced researcher), conceptualization, experimental design, resources, funding acquisition, writing—original draft, review, editing.

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Data availability Schematic credits for Fig. 1 to FLATICON authors: amonrat rungreangfangsai, Freepik, Smashicons, Superarticons, wanicon, Paul J, photo3idea_studio, Vactor area, SBTS2018, rsetiawan, Darius Dan, Flat-icons-com, Pixelmeetup.

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