Research Article

One-factor-at-a-time and response surface statistical designs for improved lactic acid production from beet molasses by *Enterococcus hirae* ds10

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Abstract
In this study, the production of lactic acid (LA) from beet molasses, a by-product of the beet sugar industry was investigated using newly isolated potential lactic acid bacteria. Isolate ds10 was selected amongst 138 bacterial isolates obtained from natural sources. This isolate was identified as *Enterococcus hirae* ds10 based on morphological, biochemical and molecular characteristics using 16S rRNA sequence. Direct utilization of molasses achieved low LA production at 2.01 g L^{-1}. Different molasses' pretreatment methods were investigated. Molasses treated with EDTA were considered as the best substrate achieving effective LA production at 11.39 ± 2.07 g L^{-1}. Furthermore, medium constituent was optimized, where supplementation of 0.5% (*w/v*) ammonium chloride and 0.05% (*w/v*) yeast extract exhibited the best fermentation medium. Further optimization of fermentation factors was performed by using one-factor-at-a-time (OFAT) and response surface Minitab 18 software approaches. OFAT technique achieved the maximum LA production of 25.4 ± 0.42 g L^{-1} after 24 h at sugar molasses conc., 4% (*w/v*); inoculum size, 10% (*v/v*); pH, 8.0; and temperature, 40 °C. Whereas, response surface Minitab 18 software approach resulted in a 60% increase in LA production achieving 40.69 g L^{-1} at 60 g L^{-1} sugar concentration, 0.625 g L^{-1} yeast extract, 40 °C, pH 8 and 9.5% inoculum size. The optimization strategy in this study could achieve a 20-fold increase in LA production as compared to initial production.

Keywords Lactic acid · Beet molasses · *Enterococcus hirae* ds10 · Response surface optimization · One-factor-at-a-time

1 Introduction

Lactic acid (LA) is an organic acid that has vital roles in many industrial applications including food, textile, chemical, cosmetics, and pharmaceutical industries. It has an increasing demand as a feedstock for the manufacturing of biodegradable polylactic acid (PLA) materials as alternatives to petroleum-derived synthetic plastic [1, 2]. Global LA market is forecasted to grow at an estimated CAGR of 19.43% between 2019—2025 generating around USD 10.06 billion by 2025 (https://www.zionmarketresearch.com/report/lactic-acid-market). LA can be produced by chemical synthesis or biological processes using microbial fermentation. For fermentation processes

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to be competitive, they should meet several characteristics including low-cost substrate/ process, substrate availability, low medium-cost, with little or no by-product formation [3, 4].

Substrates are the cornerstone factor affecting LA production costs. Therefore, waste materials are preferred as substrate not only for its cost but also for solving environmental problems related to its disposal. Among several agro-industrial wastes, beet and sugarcane molasses are produced from sugar industries, readily available, cheap raw materials contacting sugars and therefore can be utilized for LA production [5]. Beet molasses constitutes about 5.5% (*w/w*) of the beet weight used for sugar production. In Egypt, there is about 15,000 tons of beet molasses as a by-product produced annually from three beet sugar factories [6]. Sucrose is the main sugar in molasses that accounts for around 48−50% (*w/v*). These materials also contain limited nitrogen content. On the other hand, it includes some substances such as 5-hydroxymethylfurfural and excessive metallic ions that considered to be microbial inhibitory limiting their efficient utilization [7].

The cost of the fermentation medium is also one of the challenges limiting effective LA production because lactic acid bacteria are fastidious that require a complex nutritional requirement such as peptone, beef extract, and yeast extract. Yeast extract alone has accounted for 38% of fermentation costs. Some researchers used various low-cost nutrients as nitrogen sources like dry yeast cells, shrimp waste, fish waste hydrolysate or silkworm larvae as an alternative to the high-cost yeast extract [8]. Therefore, the utilization of inexpensive waste materials that contain high nutritional values should add additional saving parameters for LA production [9, 10].

Optimization of fermentation factors is critical to maximizing the yield of a specific product before large scale production. Several optimization approaches can be used including classical "one-factor-at-a-time" to recent statistical and mathematical approach, viz. genetic algorithm (GA), artificial neural network (ANN), etc. Each technique has advantages and disadvantages. The combination of various optimization approaches can provide a desirable result [11].

The most common method for improving fermentation medium components and conditions is the "one-factor-at-a-time" approach. it is based on fixing all parameters except one independent variable with individual effect on medium components and fermentation conditions. This approach is easy and simple. On the other hand, it is time-consuming, do not investigate the interaction between variable, and expensive especially for conducting a large number of experiments [12]. However, statistical and mathematical approaches are effective and might overcome these limitations. These methods depend on changing more than one factor at a time [13]. They are more effective than "one-factor-at-a-time" approach in screening interactions between different variables and describing the role of the interactions of each component in the process. Of these methods, Response Surface Methodology (RSM) is effective and a suitable design model that explains the combined effect and study several factors affecting fermentation responses by varying them in a limited number of experiments [14].

Therefore, this study aimed to achieve cost-effective LA production from waste materials (beet molasses) using thermotolerant lactic acid bacteria. Establishing cheap fermentation media and optimization of LA production using traditional and statistical methods for enhanced LA production were evaluated.

2 Material and methods

2.1 Media and bacterial isolation

de Man, Rogosa and Sharpe (MRS) medium is consisted of (g L^{-1}): glucose, 20; yeast extract, 5; peptone, 10; beef extract, 8; K_2HPO_4, 2; $MgSO_4$, 0.1; $MnSO_4$, 0.05; sodium acetate, 5; ammonium citrate, 2 and tween 80, 1 ml [15]. GYP (glucose-yeast extract-peptone) medium is consisted of (g L^{-1}): glucose, 20; yeast extract, 5; peptone, 5. Solid media were supplemented with 1.5% (*w/v*) agar, and 1% (*w/v*) $CaCO_3$. Modified MRS (mMRS) and modified GYP (mGYP) media were composed of the same components as MRS or GYP, respectively but glucose is substituted by molasses at concentrations mentioned in each experiment. pH was adjusted at 7.0 using 1 N NaOH/1 N HCl. Sterilization was done by autoclaving at 121 °C for 20 min. Sugar solutions were sterilized separately at 110 °C for 10 min.

Forty-three natural sources were used for the isolation of LA bacteria. They include milk, rotten fruits, cheese, jam, beet molasses, can molasses, compost, cattle manure, decomposed plant and soil samples that were collected from different localities in Gharbia, Beheira, Cairo and Kafr El-Sheikh Governorates, Egypt. The samples were taken aseptically and packaged into clean bags, then stored at 4 °C.

Bacterial isolation was carried out by two methods. Firstly, either 1 g or 1 mL of sample was mixed with 0.75% (*w/v*) NaCl solution and then serially diluted at ten-fold. Each dilution was spread directly onto mMRS and mGYP agar plates and incubated at 40 °C for 72 h. In the second method, 1 g of each sample was inoculated into 50 ml of MRS and GYP broth media and then incubated at 40 °C for 72 h. The broth was then diluted and spread onto the same agar media containing 1% $CaCO_3$ and incubated at 40 °C

for 72 h. Colonies showing a clear zone were individually picked and purified. Purity was checked up using simple and Gram's stains. Gram-positive isolates were assayed for catalase activity.

2.2 LA production from different substrates

138 bacterial colonies were tested for the productivity of LA in de Man, Rogosa and Sharpe (MRS) broth medium at 40 °C for 36 h. After incubation, LA concentration was determined. mMRS containing sucrose, fructose (Sigma Aldrich), or beet molasses as sole carbon source separately were used at 20 g L^{-1} for the most potent isolates. Beet molasse was obtained from the Hamool beet sugar factory (Delta Sugar Company), Kafr El-Sheikh Governorate, Egypt. The sugar content of beet molasses used in this study was about 48–51% of sucrose, 1% glucose and fructose, and about 1% raffinose. Total nitrogen content in the raw molasses is ranged from 0.8–1.2%.

2.3 Characterization and identification of the most potent isolate

Morphological characteristics including shape, arrangement and Gram reaction of strain ds10 were conducted using Gram's stain [16]**.** Catalase activity using 3% hydrogen peroxide was assayed as previously described [17]. Biochemical identification using VITEK 2 test was performed in Theodor Bilharz Research Institute, Giza, Egypt. Partial 16S rRNA sequence was analyzed using universal primers 27f, 5′- AGAGTTTGATCCTGGCTCAG-3′; and 1492r, 5- GGTTACCTTGTTACGACTT -3′. Total genomic DNA was extracted from ds10 cells using Gene Jet genomic DNA purification Kit (Fermentas) and used as a template for PCR. PCR product was sequenced in GATC Biotech Company using ABI 3730xl DNA sequencer. A similarity index was performed at the GenBank database using the BLAST algorithm.

2.4 Pretreatment of beet molasses

For sulphuric acid (SA) treatment, molasses solution (diluted molasses contain 10% sugar conc.) was adjusted to pH 3.0 using with 1 N H_2SO_4, incubated for 24 h, and then centrifuged for 15 min at 5000 × g. With 5 N NaOH, the pH of the supernatant was readjusted to 7.0 [18]; For activated carbon (AC) treatment, 3% (*w/v*) of AC was mixed to the molasses for 1 h, centrifuged at 5000 × *g* for 15 min. The process was repeated for supernatants and then pH was adjusted to 7.0 [19]; For tricalcium phosphate (TCP) treatment, molasses was mixed with 2% (*w/v*) TCP and autoclaved at 105 °C for 5 min, cooled, and centrifuged at 5000 × g for 15 min. The supernatant's pH was adjusted to 7.0.[19]; for TCP/SA treatment, TCP pretreated supernatants were acidified with concentrated H_2SO_4 by mixing for 24 h at pH 3.0. The mixture was then centrifuged at 5000 × *g* for 15 min, pH was adjusted to 7.0 [19]; for SA/AC treatment, SA-treated liquors obtained as explained above were subjected to 3% (*w/v*) AC as described above [19]; for TCP/SA/AC treatment, molasses solutions pretreated with TCP followed by acidification with SA were subjected to 3% (*w/v*) AC treatment as explained above [19]; For potassium ferrocyanide and EDTA treatment, molasses solution was adjusted to pH 5.5 using 5 N HCl and heated at 100 °C for 15 min. 100 ppm EDTA or potassium ferrocyanide were added to molasses while hot (90 °C), then, mixtures were allowed to settle for 24 h at room temperature and then centrifuged at 5000 × *g* for 20 min. The supernatant was then used for fermentation [18]. All experiments were carried in triplicates.

2.5 Lactic acid production from treated molasses

For LA production, treated molasses was used at 2% (*w/v*) sugar concentration in mMRS media with initial pH 7.0. The inoculum was prepared by transferring 1 mL of bacteria in glycerol stock culture to test tube containing 9 ml medium for refreshment and incubated for 18 h at 40 °C before inoculation in Erlenmeyer flask containing 100 ml of liquid MRS medium for pre-culture. Then, the culture in 500 mL-Erlenmeyer flasks containing 200 mL of production medium was inoculated with 5% inoculum (pre-culture with 0.40 ± 0.03 OD_{600}) and incubated 40 °C for 36 h. All experiments were carried in triplicates.

2.6 Optimization of fermentation medium components:

2.6.1 Replacement of medium component with organic/inorganic nitrogen sources

This experiment was performed in 500 ml-Erlenmeyer flasks with 200 mL working volume. Molasses solution treated with EDTA was adjusted at 2% (*w/v*) initial sugar and used for fermentation with/without other components as follows. Supplementation/exclusion of mMRS media components, or addition of 0.5% (*w/v*) of yeast extract only was firstly investigated. Also, supplementation of 0.5% (*w/v*) of ammonium sulfate, ammonium molybdate, ammonium acetate, ammonium chloride, sodium nitrate, urea, peptone, and beef extract was investigated separately on the base of 0.5% nitrogen source addition equivalent to optimal yeast extract concentration. The media were inoculated with 5% (*v/v*) inoculum and then incubated at 40 °C for 36 h.

2.6.2 Effect mixed nitrogen sources

Molasses solution containing 20 g L^{-1} initial sugar and 5 g L^{-1} ammonium chloride was supplied with different concentrations of yeast extract (0.5, 1, 2, 3, and 5 g L^{-1}), dried yeast cells (0.5, 1, 2, and 3 g L^{-1}), or dried fish wastes (0.5, 1, 2, and 3 g L^{-1}). Fish wastes were collected from local markets in Egypt, dried in the oven at 70 °C until constant weight. Then grounded to be used for experiments. Also, dried yeast cells or dried fish wastes were used at 5 g L^{-1} without additional ammonium chloride. pH was adjusted to 7.0 with 1 N HCl/1 N NaOH and sterilized at 121 °C for 15 min. The experiment was carried out in 500-ml Erlenmeyer flasks containing 200-ml of fermentation medium and incubated for at 40 °C for 36 h.

2.7 Design of experiment for optimization of LA production

2.7.1 One-factor-at-a-time (OFAT)

All experiments were carried out in 500-ml Erlenmeyer flasks containing 200-ml of fermentation medium and incubated for 36 h. Treated molasses solution with EDTA was used as substrate supplemented with 0.05% (*w/v*) yeast extract and 0.5% (*w/v*) ammonium chloride. LA production, pH, optical density and consumed sugar was checked every 12 h.

To study the effect of sugar concentration, treated molasses were used at different concentrations viz., 2, 4, 6, 8 and 10% (*w/v)* initial sugar. The media were adjusted at pH 7.0 (initial pH value), inoculated with 5% of the inoculum and incubated at 40 °C for 36 h. This experiment was conducted at the same condition but with the addition of $CaCO_3$ as a buffering agent at a half concentration of sugar tested. To study the effect of temperature, media with an initial sugar 4% (*w/v*) were adjusted to pH 7 and inoculated at 5% (*v/v*). Then incubated at different temperature viz., 30, 35, 40, 45 and 50 °C. To investigate the influence of culture pH, fermentation media with an initial sugar 4% (*w/v*) were adjusted to different pH values (viz., 5.0, 6.0, 7.0, 8.0 and 9.0), inoculated with 5% (*v/v*) inoculum size, and incubated at 40 °C for 36 h. To investigate the optimal inoculum size, cells from stock cultures at wavelength 600 nm (0.40 ± 0.03) were transferred to molasses media at different inoculum levels (1, 3, 5, 7, 10 and 15%, *v/v*). The fermentation medium (pH 8) was then incubated at 40 °C, for 36 h.

2.7.2 Response surface optimization methods

Response surface methodology (RSM), and MINITAB 18 statistical package for data analysis. Statistical optimization of media was done using five factors at five levels for the optimization process viz., yeast extract, molasses concentration, temperature, pH and inoculum size. The experimental data obtained from the design were analyzed by the response surface regression procedure using the following second-order quadratic equation: $Y = B0 + \sum BiXi + \sum BiiX^2_i + \sum BijXiXj$.

Where, Y was the predicted response which is a dependent variable, i.e. LA production; B0 is an offset term (constant); Bi is linear effect; Bij is quadratic effect when i = j and interaction effect when i < j; Bii is a squared term; Xi is the variable, which is called as independent variables.

Thirty-two experiments were conducted using a central composite statistical design. The role of each variable, statistical analysis, and their interactions to obtain predicted yield of LA is explained by applying the second-order polynomial method. The results obtained are analyzed statistically and response surface contour plots were constructed. The optimum levels of the variables were determined by running experiments using the optimum values for variables given by response optimization for confirmation of predicted value and the LA production was confirmed.

2.8 Analytical methods and analyses

The amount of LA was determined by a colorimetric method according to [20]**.** Total sugar concentrations were determined by the phenol–sulphuric method [21]**.** Cells were centrifuged, washed and readjusted with water. The growth was measured spectrophotometry at 600 nm and described as OD_{600}. The yield of LA-based on the consumed sugar is defined as the ratio of LA produced (g L^{-1}) to the amount of consumed sugar (g L^{-1}). LA productivity (g L^{-1} h^{-1}) was calculated as the ratio of LA concentration to the fermentation time at which the maximum LA concentration was obtained. Maximum LA productivity (g L^{-1} h^{-1}) was calculated between each of the sampling periods.

3 Results

3.1 Isolation and screening of LA producing bacteria

Isolation of microbes from natural sources is the most powerful mean for obtaining genetically-stable strains for industrial products. A basal mMRS and mGYP medium fortified with non-soluble $CaCO_3$ as pH indicator (pH 7) were used for the isolation of lactic acid bacteria. Acid production would lower the pH value and change $CaCO_3$ solubility leading to the formation of a clear zone around the colony. Using 43 natural sources, one hundred and

thirty-eight acid-producing bacterial isolates were obtained. Of these, forty-three isolates were Gram-positive and catalase-negative and selected for further studies. LA production in mMRS broth medium containing glucose (20 g L^{-1}) was investigated as shown in Table S1 (see supplementary data). Amongst all, isolate ds10 exhibited the highest LA production at 14.0 g L^{-1} after 36 h with LA yield at 0.9 g $g^{-1}_{\text{-consumed glucose}}$. Further screening for the utilization of molasses and molasses-derived sugars (Fig. 1) was investigated. Isolate ds10, could efficiently utilize all pure sugars (sucrose, glucose, and fructose) with high titer (ranged 14—18 g L^{-1}) at high yields (0.66—0.75 g $g^{-1}_{\text{-consumed sugar}}$) and productivities (0.272—0.389 g L^{-1} h^{-1}). In contrast, this strain could hardly have utilized crude molasses directly with the production of low titer (2.01 g L^{-1}), yield (0.522 g $g^{-1}_{\text{-consumed sugar}}$) and productivity (0.055 g L^{-1} h^{-1}). As a result, isolate ds10 was selected as the most potent isolate for further studies aiming to improve LA production from beet molasses. This strain was isolated from soil samples collected from the Kafr-Elsheikh governorate, Egypt.

3.2 Characterization and identification of isolate ds10

Morphological and physiological properties of isolate ds10 were investigated. This isolate is Gram-positive, KOH negative, cocci, catalase-negative, and none spore former. This isolate was also identified using VITEK 2 that assesses various metabolic activities such as alkalization, acidification, enzyme hydrolysis, and growth in the presence of inhibitory compounds. As shown in Table S2 (see supplementary data), isolate ds10 showed 93% similarly to *Enterococcus faecium*.

Molecular identification based on 16S rRNA sequence analysis showed 99% identity to that of reference strain *Enterococcus hirae* strain LMG 6399 (accession number NR-114783.2) available in the NCBI. The phylogenetic analysis of isolate is shown in Fig. 2. Accordingly, we concluded that isolate ds10 was identified as *Enterococcus hirae* ds10. The 16S rRNA sequence of this isolate was deposited in GenBank under the accession number MK910108.

3.3 Pretreatment of molasses for effective LA production

The production of LA by strain ds10 from pretreated beet molasses using different chemicals is shown in Fig. 3. Beet molasses treated with H_2SO_4, $Ca_3(PO_4)_2$, activated carbon and $K_4Fe(CN)_6$ separately or in mixture showed decreased fermentation efficiency in terms of produced LA. On the other hand, treatment with EDTA exhibited better results regarding LA concentration, yield, and productivity compared with untreated molasses and other treatments. At which, the highest value of LA at 11.3 ± 2.07 g L^{-1} with LA yield of 0.74 (g $g^{-1}_{\text{-consumed sugar}}$) and productivity of 0.32 g L^{-1} h^{-1} was obtained.

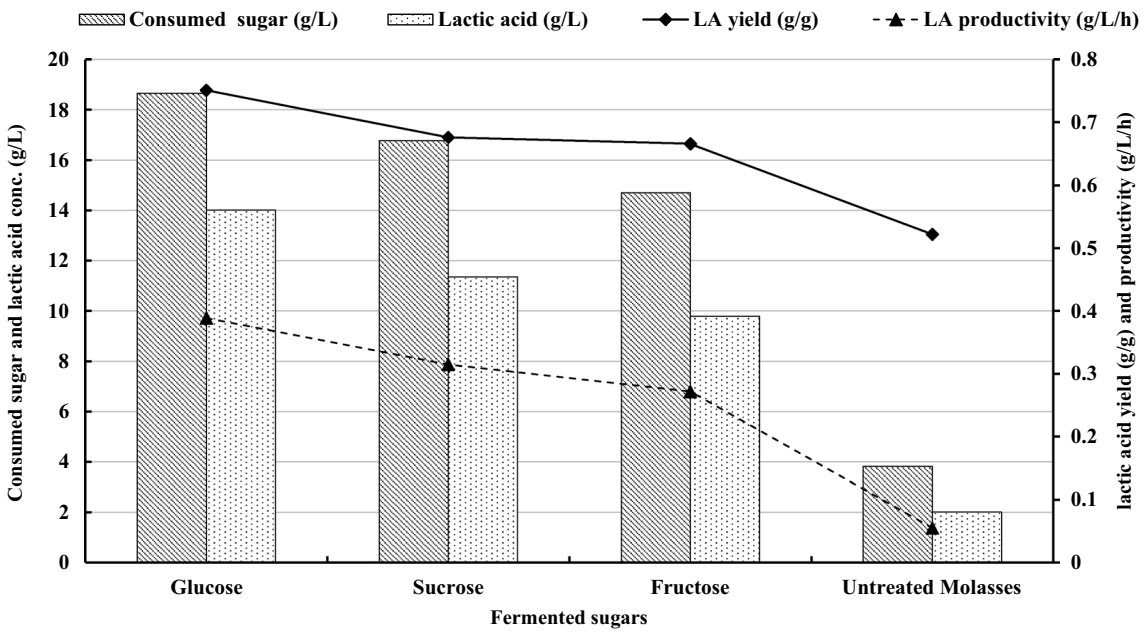

Fig. 1 Utilization of molasses and molasses–derived sugars by bacterial isolate ds10

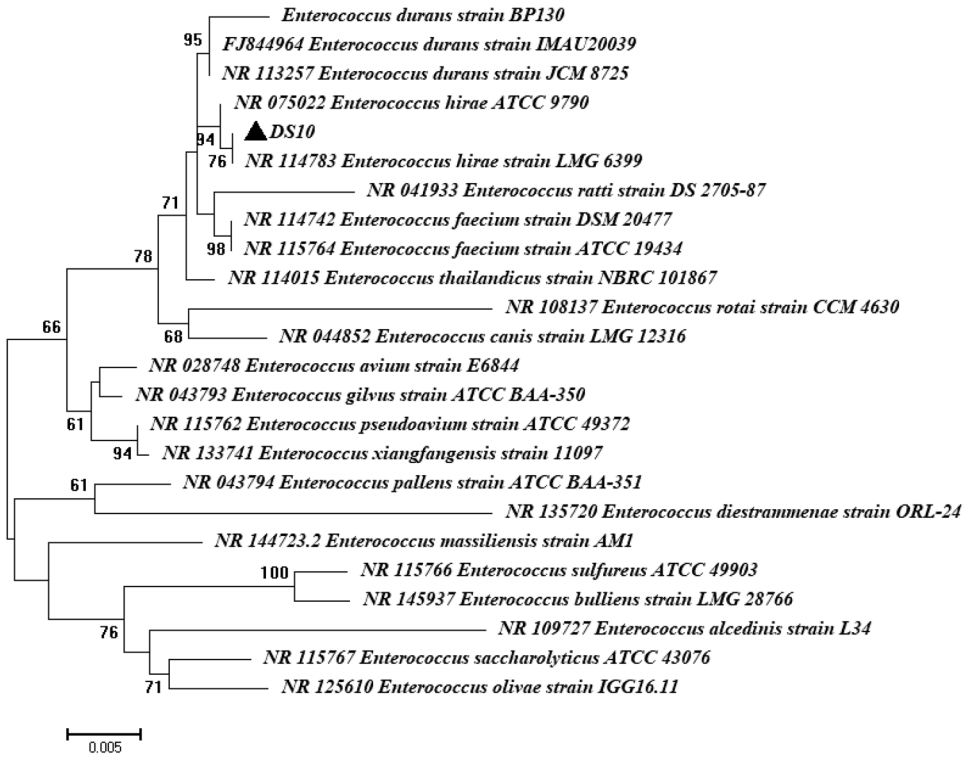

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura two-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Black triangle indicate the bacterial isolate ds10 in this study. GenBank accession numbers of reference sequences are indicated. Bar 0.02 nucleotide substitutions per position

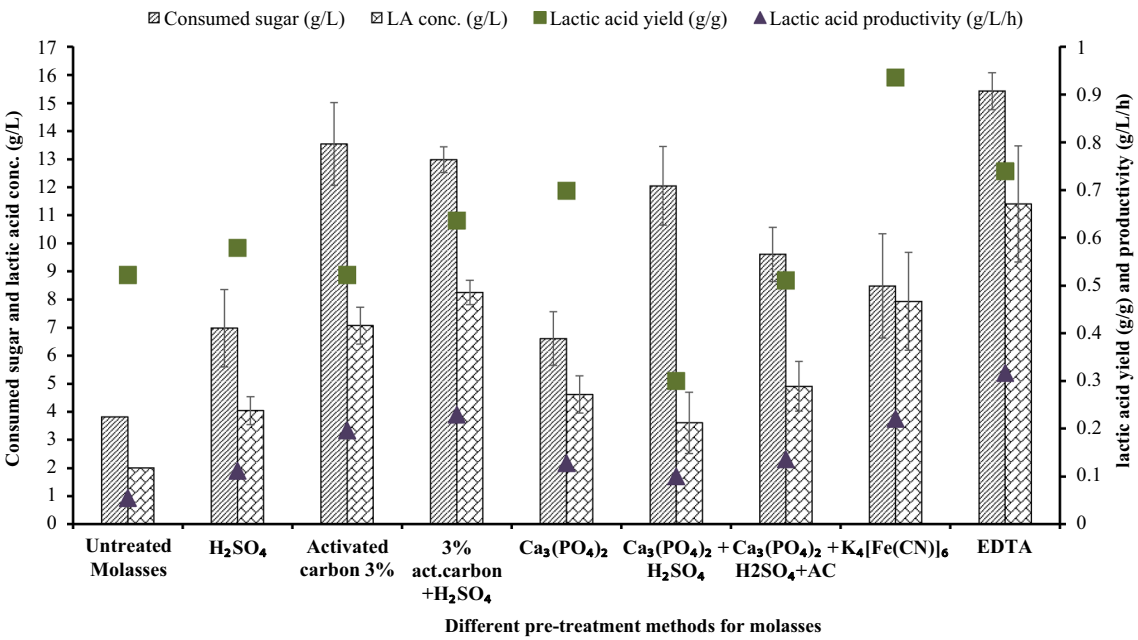

Fig. 3 Effect of treatment of beet molasses on lactic acid fermentation by *Enterococcus hirae* ds10

3.4 Optimization of production medium

3.4.1 Replacement of medium component with different nitrogen sources

LA fermentations using EDTA-treated molasses (2%, initial sugar *w/v*) supplemented with 0.5% (*w/v*) nitrogen source were compared with that obtained without the addition of yeast extract and that supplemented with MRS medium components. Fermentations were conducted at

40 °C, pH 7, for 36 h with 5% (*v/v*) inoculum. As shown in Table 1, the supplementation of MRS medium components resulted in LA production at 11.3 g L^{-1}. On the other hand, supplementation of yeast extract only exhibited LA concentrations at 7.35 ± 0.08 g L^{-1} with yield 0.63 g g^{-1} consumed sugar. Ammonium chloride was the next best nitrogen source where it showed LA concentration at 4.04 ± 0.02 g L^{-1} with yield 0.96 g $g^{-1}_{\text{-consumed sugar}}$. Although dried yeast cells achieved LA production of 4.18 g L^{-1}, it achieved a lower yield at 0.81 g/g. Other nitrogen sources exhibited lower LA production ranged 1.29−3.6 g L^{-1}. Exclusion of medium components results in only 0.86 g L^{-1} LA with very low LA yield at 0.340 g $g^{-1}_{\text{-consumed sugar}}$.

3.4.2 Effect of mixed nitrogen source

Due to the high cost of yeast extract, this experiment was conducted to reduce the cost of fermentation medium components. Ammonium chloride was used as the main nitrogen source while yeast extract, dried yeast cells, or dried fish wastes were used at different concentrations as supplementary vitamin sources for LAB growth and LA fermentation (Table 2). During fermentation, it was observed that the highest LA was produced with the lowest concentration of yeast extract 0.5 g L^{-1} achieving 14.1 ± 0.24 g L^{-1} at LA yield of 0.84 g $g^{-1}_{\text{-consumed sugar}}$ and productivity of 0.39 g $L^{-1}.h^{-1}$ and decreased gradually when yeast extract was increased.

On the other hand, treated molasses supplemented with dried yeast (2 g L^{-1}) gave the highest LA concentration of 7.68 ± 0.35 g L^{-1} compared with the other concentrations. But almost half the amount of LA was produced compared with yeast extract. Besides, supplementation of dried fish wastes as vitamin source was unsuccessful, resulting in very low concentrations of LA ranged 0.29−0.86 g L^{-1}. Also, it was difficult to measure bacterial growth. Therefore, yeast extract 0.5 g L^{-1} of yeast extract with 5 g L^{-1} ammonium chloride was selected as the best component for LA production by strain ds10.

3.5 Different designs for optimization of LA production

3.5.1 One-factor-at-a-time (OFAT) method

To investigate the optimal conditions for utilization of molasses by *Enterococcus hirae* ds10, different initial sugar concentrations (2%, 4%, 6%, 8% and 10%, *w/v*) were investigated for LA production. As shown in Table 3, LA concentration was increased with the decrease of sugar concentration and LA production was observed to be in a limited range of 17−21 g L^{-1}. The highest LA concentration (20.92 ± 0.53 g L^{-1}) was obtained at the sugar concentration of 20 g L^{-1} with an LA yield of approximately 1.0 g $g^{-1}_{\text{-consumed sugar}}$. The result implies that *Enterococcus hirae* ds10 could metabolize molasses sugars homofermentatively through the EMP pathway. To remove such LA limitations, $CaCO_3$ was added to the medium. But almost

Table 1 Effect of medium components on LA production from EDTA-treated molasses by *Enterococcus hirae* ds10

Medium components	$OD_{600\ nm}$ [a]	Final pH	Consumed sugars (g L^{-1})	LA conc. (g L^{-1})[b]	Y_{LA}(g.g^{-1})[c]	P_{LA} (g $L^{-1}.h^{-1}$)[d]
MRS	0.53 ± 0.04	5.36 ± 0.40	15.4 ± 1.65	11.39 ± 2.07	0.74	0.32
Molasses only	0.20 ± 0.03	5.03 ± 0.05	2.48 ± 0.37	0.86 ± 0.35	0.34	0.02
Yeast extract (5 g L^{-1})	1.05 ± 0.04	4.56 ± 0.05	11.6 ± 0.53	7.35 ± 0.08	0.63	0.20
Amm. molybdate (5 g L^{-1})	0.34 ± 0.32	5.80 ± 0.0	5.29 ± 0.86	2.02 ± 0.02	0.38	0.05
Amm. Chloride (5 g L^{-1})	0.20 ± 0.02	5.10 ± 0.0	4.17 ± 0.21	4.04 ± 0.02	0.96	0.11
Amm. acetate (5 g L^{-1})	0.12 ± 0.02	5.60 ± 0.1	3.04 ± 0.29	1.29 ± 0.22	0.42	0.03
Urea (5 g L^{-1})	0.09 ± 0.01	5.35 ± 0.07	3.42 ± 0.85	2.45 ± 0.33	0.71	0.06
$NaNO_3$ (5 g L^{-1})	0.23 ± 0.15	5.00 ± 0.1	3.42 ± 0.53	1.58 ± 0.88	0.46	0.04
Peptone (5 g L^{-1})	0.35 ± 0.00	4.80 ± 0.0	3.83 ± 0.70	3.60 ± 0.40	0.94	0.10
Beef extract (5 g L^{-1})	0.21 ± 0.02	4.90 ± 0.0	3.42 ± 0.85	2.02 ± 0.13	0.59	0.05
Dried yeast cells (5 g L^{-1})	0.94 ± 0.03	4.80 ± 0.0	5.11 ± 0.47	4.18 ± 0.53	0.81	0.11
Dried fish wastes (5 g L^{-1})	NA	5.30 ± 0.1	11.1 ± 0.06	1.29 ± 0.35	0.11	0.03

NA, not analyzed

[a]OD, optical density

[b]Lactic acid concentration after 36 h

[c]Lactic acid yield

[d]Lactic acid productivity at the end of fermentation time

Table 2 The effect of mixing ammonium chloride (5 g L^{-1}) with different concentration of yeast extract on LA production from molasses by *Enterococcus hirae ds10*

Amm. chloride (5 g L^{-1}) supplemented with:		$OD_{600\,nm}$[a]	Final pH	Consumed sugars (g L^{-1})	LA conc. (g L^{-1})[b]	Y_{LA}(g g^{-1})[c]	P_{LA} (g L^{-1} h^{-1})[d]
Supplementation	Conc. (g L^{-1})						
Yeast extract	0.5	0.71 ± 0.09	4.46 ± 0.05	16.7 ± 1.06	14.0 ± 0.24	0.84	0.39
	1.0	0.86 ± 0.04	4.50 ± 0.0	8.5 ± 1.77	8.37 ± 0.40	0.98	0.23
	2.0	0.9 ± 0.04	4.50 ± 0	11.9 ± 1.83	7.22 ± 0.24	0.61	0.20
	3.0	0.95 ± 0.03	4.50 ± 0	9.73 ± 2.95	5.77 ± 0.20	0.59	0.16
Dried yeast cells	0.5	0.95 ± 0.03	4.90 ± 0	9.67 ± 2.29	5.77 ± 0.53	0.59	0.16
	1.0	0.95 ± 0.03	4.80 ± 0	10.2 ± 0.53	6.68 ± 1.42	0.64	0.18
	2.0	0.94 ± 0.03	4.80 ± 0	11.1 ± 2.26	7.68 ± 0.35	0.69	0.21
	3.0	0.94 ± 0.03	4.80 ± 0	6.11 ± 1.47	4.18 ± 0.53	0.68	0.12
	5.0	0.94 ± 0.03	4.80 ± 0	3.86 ± 1.91	2.59 ± 1.54	0.67	0.07
Dried fish wastes	0.5	NA	5.10 ± 0.1	7.16 ± 0.95	0.72 ± 0.40	0.1	0.02
	1.0	NA	5.00 ± 0.17	8.92 ± 1.85	0.86 ± 0.35	0.09	0.02
	2.0	NA	5.15 ± 0.21	9.67 ± 0.45	0.58 ± 0.81	0.06	0.02
	3.0	NA	5.15 ± 0.21	9.67 ± 0.45	0.58 ± 0.81	0.06	0.02
	5.0	NA	5.30 ± 0.1	12.1 ± 1.06	0.29 ± 0.35	0.02	0.01

NA, not analyzed AC

[a]OD, optical density

[b]Lactic acid concentration after 36 h

[c]Lactic acid yield

[d]Lactic acid productivity at the end of fermentation time

all fermentation parameters were decreased with the addition of $CaCO_3$ (Data not shown). As all sugars were almost consumed when 2% sugar concentration was used, further improvement OFAT experiments were conducted at 4% (*w/v*).

The effect of different incubation temperatures was studied at the initial sugar concentration of 4% (*w/v*) and pH 7. The data indicated that the optimal temperature for LA fermentation by strain ds10 was 40 °C, where LA was 14.5 ± 0.20 g L^{-1} (24 h) at a yield of 92.0 g $g^{-1}_{\text{-consumed sugar}}$. Above and below this value, all fermentation parameters were decreased achieving LA concentration ranged 4.04−11.5 g L^{-1} (Table 3).

For optimizing the pH, fermentation media was adjusted to different pH (5.0, 6.0, 7.0, 8.0 and 9.0) at 40 °C. The maximum LA production (21.0 ± 0.2 g L^{-1}) with a yield of 0.93 g $g^{-1}_{\text{-consumed sugar}}$ was achieved at pH 8.0 after 36 h. However, at higher and lower pH levels, decrease in all parameters were obtained (Table 3).

To find the effect of inoculum size on LA production, fermentation medium (pH 8) was inoculated with different inoculum sizes (1, 3, 5, 10 and 15%, *v/v*), separately and incubated at 40 °C for 36 h. An increase in sugar molasses utilization and LA production and resulted in a decrease in fermentation time was observed when bacterial inoculum size increased (Table 3). The maximum LA production of 25.4 ± 0.42 g L^{-1} (24 h) was obtained with 10% (*v/v*) inoculum size.

3.5.2 Statistical optimization design

To maximize LA production, Response Surface Analysis of the Minitab 18 software (version 18) was used. 5 variables of yeast extract, sugar molasses concentration, temperature, pH and inoculum size that was found to be significant by classical OFAT design were investigated. Run design leads to a set of 32 experimental runs as shown in Table 4. Each independent variable was tested at two levels, high and low, which are denoted by (+ 1) and (− 1), respectively. Based on the data, yeast extract concentrations were (+) 0.25 and (−)1 g L^{-1}, molasses concentrations were (+) 40 and (−) 80 g L^{-1}, temperature was (+) 35 and (−) 45 °C, pH were (+) 7 and (−) 9, and the inoculum size were (+) 7 and (−) 12%. LA production was taken as the response for each variable. The interactions between variables and statistical analysis indicated the possibility of enhancement in LA production. The model showed that the highest LA production achieved in the verification experiment was 40.6 g L^{-1} (as seen in run 30).

Results indicated that the maximum LA production achieved by *Enterococcus hirae* ds10 was recorded with high levels (positive effect) of yeast extract, sugar molasses

Table 3 Variables used in OFAT technique for optimization of lactic acid production from *Enterococcus hirae* ds10 using molasses as substrate

Variable	Conc./Value	$OD_{600\ nm}$[a]	Final pH	Consumed sugars (g L^{-1})	LA conc. (g L^{-1}) at indicated time	Y_{LA} (g g^{-1})[b]	P_{LA} (g $L^{-1}.h^{-1}$)[c]	Max. P_{LA} (g $L^{-1}.h^{-1}$) (0–12 h)
Sugar concentration (%, *w/v*)	2	0.78 ± 0.01	4.7 ± 0.0	20.9 ± 0.27	20.9 ± 0.53 (12 h)	0.99	1.74	1.69
	4	0.90 ± 0.01	4.83 ± 0.09	16.8 ± 0.95	14.5 ± 0.20 (24 h)	0.86	0.61	0.96
	6	0.91 ± 0.01	5.00 ± 0.08	21.4 ± 0.70	18.6 ± 1.54 (36 h)	0.86	0.52	1.40
	8	0.96 ± 0.00	5.10 ± 0.08	26.8 ± 0.06	18.6 ± 0.35 (36 h)	0.69	0.52	0.94
	10	1.14 ± 0.00	5.16 ± 0.04	26.8 ± 0.30	17.1 ± 0.20 (36 h)	0.64	0.48	0.80
Temperature (°C)	30	0.33 ± 0.01	5.73 ± 0.12	4.97 ± 0.46	4.04 ± 0.20 (36 h)	0.81	0.11	0.28
	35	0.67 ± 0.01	4.80 ± 0.08	9.48 ± 0.79	7.07 ± 0.41 (36 h)	0.75	0.19	0.41
	40	0.87 ± 0.02	4.86 ± 0.04	15.8 ± 0.95	14.5 ± 0.20 (24 h)	0.92	0.61	0.98
	45	0.50 ± 90.01	4.80 ± 0.08	14.7 ± 2.53	11.5 ± 1.81 (36 h)	0.78	0.32	0.60
	50	0.39 ± 0.01	5.10 ± 0.00	5.16 ± 1.41	4.33 ± 0.35 (24 h)	0.84	0.18	0.28
pH	5	0.23 ± 0.01	5.00 ± 0.0	10.4 ± 0.26	6.78 ± 0.20 (36 h)	0.65	0.18	0.47
	6	0.38 ± 0.00	4.93 ± 0.05	12.6 ± 0.26	8.37 ± 0.88 (24 h)	0.66	0.34	0.64
	7	0.29 ± 0.00	5.06 ± 0.04	12.8 ± 0.46	11.2 ± 0.35 (36 h)	0.87	0.31	0.78
	8	0.42 ± 0.01	5.00 ± 0. 0	22.6 ± 0.53	21.0 ± 0.20 (24 h)	0.93	0.87	1.44
	9	0.23 ± 0.01	5.36 ± 0.04	14.5 ± 0.46	13.7 ± 0.53 (24 h)	0.94	0.57	1.08
Inoculum size (%, *v/v*)	1	0.52 ± 0.03	4.83 ± 0.04	13.6 ± 0.95	10.2 ± 0.53 (24 h)	0.75	0.42	0.67
	3	0.75 ± 0.04	4.70 ± 0.1	15.4 ± 0.26	10.9 ± 0.41 (36 h)	0.71	0.30	0.78
	5	0.42 ± 0.01	4.73 ± 0.04	22.6 ± 0.53	21.0 ± 0.20 (24 h)	0.93	0.87	1.44
	7	0.69 ± 0.02	4.70 ± 0. 01	25.6 ± 0.70	22.6 ± 0.20 (36 h)	0.88	0.62	1.47
	10	0.67 ± 0.00	4.66 ± 0.07	30.1 ± 0.95	25.4 ± 0.42 (24 h)	0.84	1.05	2.07
	15	0.54 ± 0.01	4.66 ± .03	22.0 ± 0.26	18.3 ± 0.73 (24 h)	0.83	0.76	1.48

[a]OD, optical density

[b]Lactic acid yield

[c]Lactic acid productivity at the end of fermentation time

concentration, temperature, pH and inoculum size. Whereas, the LA produced by studied strain ranged from 19.4 to 40.6 g L^{-1} compared by maximum LA produced 25.4 ± 0.42 g L^{-1} (24 h) was observed by classical OFAT.

4 Discussion

Commercial LA production requires the use of robust biocatalysts utilizing a low-cost substrate with a minimal amount of nitrogenous source to meet global needs [22]. Besides, tolerance to high temperature, production of negligible amounts of byproducts is highly recommended to avoid contamination risk and decrease the down streaming processes cost [23]. In this study, isolation and identification of the thermotolerant bacterium for the production of high amounts of LA using cheap agro-industrial wastes and low-cost nitrogen sources were investigated.

A basal mMRS and mGYP media supplemented with $CaCO_3$ were used for the isolation of LAB from various natural sources. Acid production would change pH and solubility $CaCO_3$ forming a clearing zone around microbial growth. One hundred thirty-eight bacterial isolates were obtained, of these, 43 isolates are considered as LAB based on preliminary characteristics of LA production, Gram-positive, spore formation, and catalase-negativity. Based on screening data for utilization of molasses, sucrose, glucose, and fructose, isolate ds10 was selected as the most potent strain for this study. The selected isolate was characterized by a bacterial identification kit of VITEK 2 system using BCL colorimetric card and 16S rRNA sequencing. The phylogenetic analysis of strain ds10 using its 16S rRNA sequence data provide additional evidence that is related to genus *Enterococcus*. Strain ds10 had the highest homology (99% identity) to that of reference strain *Enterococcus hirae* strain LMG 6399 16S ribosomal RNA. Accordingly, we concluded that the strain ds10 was identified as *Enterococcus hirae* ds10.

Many reported studies for LA production had used pure substrates such as lactose or glucose that is costly and competitive with food and feed. In this study, we aimed to reduce the cost by using waste products such as beet molasses as inexpensive industrial by-products containing fermentable sugars [2, 10, 24]**.** It contains approximately

Table 4 Results for optimization of five variables for production of lactic acid by *Enterococcus hirae* ds10

Run Order	Yeast extract (g L^{-1})	Sugar conc. (%, *w v*$^{-1}$)	Temp. (°C)	pH	Inoculum size (%, *v v*$^{-1}$)	Lactic acid (g L^{-1})	FITS1	RESI1
1	1	4	45	8.5	7	22.07792	22.5223	−0.44438
2	0.625	6	40	8	4.5	27.27273	25.89696	1.375771
3	0.625	6	40	8	9.5	36.79654	38.0608	−1.26427
4	0.625	6	40	9	9.5	29.87013	32.96766	−3.09753
5	0.625	6	40	8	9.5	38.52814	38.0608	0.467336
6	0.625	6	40	8	14.5	25.54113	28.85511	−3.31398
7	0.25	4	35	7.5	12	24.67532	22.77483	1.900498
8	1.375	6	40	8	9.5	36.36364	34.91572	1.447921
9	0.25	8	35	7.5	7	26.83983	26.59878	0.241047
10	1	8	35	8.5	7	24.67532	24.57858	0.096747
11	1	8	45	7.5	7	26.83983	28.83543	−1.99561
12	1	4	45	7.5	12	22.94372	23.27988	−0.33615
13	0.625	6	40	7	9.5	34.19913	33.03981	1.15932
14	1	8	45	8.5	12	30.30303	30.06198	0.241047
15	0.25	4	45	7.5	7	24.24242	24.5425	−0.30008
16	0.25	8	45	8.5	7	26.83983	26.5627	0.277122
17	0.625	2	40	8	9.5	19.48052	21.42365	−1.94313
18	1	8	35	7.5	12	29.87013	29.66516	0.204972
19	0.25	4	45	8.5	12	22.07792	20.14135	1.936574
20	0.25	8	35	8.5	12	36.36364	33.88594	2.477699
21	0.625	6	40	8	9.5	38.96104	38.0608	0.900236
22	1	4	35	7.5	7	26.40693	26.88738	−0.48045
23	0.625	6	30	8	9.5	22.07792	25.10331	−3.02538
24	0.125	6	40	8	9.5	30.30303	33.68916	−3.38613
25	0.25	4	35	8.5	7	27.27273	25.48045	1.792273
26	0.625	6	40	8	9.5	37.22944	38.0608	−0.83137
27	0.625	10	40	8	9.5	30.30303	30.29811	0.004919
28	0.625	6	50	8	9.5	22.94372	21.85655	1.08717
29	1	4	35	8.5	12	30.30303	28.54683	1.756198
30	0.625	6	40	8	9.5	40.69264	38.0608	2.631838
31	0.625	6	40	8	9.5	38.09524	38.0608	0.034435
32	0.25	8	45	7.5	12	29.87013	29.48478	0.385347

Multiple Response Prediction for *Enterococcus hirae ds10*

Variable	Setting
Yeast extract g L^{-1}	0.632576
Different sugar conc.	6.84848
Different temp.	39.4949
Different pH	8.07071
Different inoculum size	10.2576

Response	Fit	SE Fit	95% CI	95% PI
LA	38.66	1.10	(36.24, 41.09)	(32.03, 45.29)

50% of sugars (sucrose, fructose, glucose, and raffinose), nitrogen compounds, amino acids, organic acids, heavy metals, etc.[25].

Unfortunately, the direct utilization of molasses exhibited low LA production compared with the utilization of pure sugars (sucrose, glucose, and fructose). This might be attributed to the presence of metal ions/inhibitory compounds that retard microbial growth. Abdel Aziz et al. [48] reported that beet-molasses contain high concentration of Na^+ (986.8 mg/100 g)

and K^+ (201 mg/100 g). Besides this, it also contains high concentrations of Fe^{2+} (7.8 mg/100 g), and Zn^{2+} (12.6 mg/100 g). Metal ions caused a critical problem during fermentation as it inhibited microbial growth, and inactivated the enzymatic activities associated with final product production [26]. Therefore, pretreatment steps for molasses are pending necessary to enhance the fermentation processes.

Amongst various chemical pretreatments methods, molasses treated by EDTA and $K_4[Fe(CN)_6$ achieved higher LA concentrations compared with other methods. EDTA was chosen considering its low cost and it resulted in LA production at 11.39 ± 2.07 g L^{-1} as compared to 2.01 ± 0.02 g L^{-1} that obtained from untreated molasses. This might be attributed to the characteristics of these chemical agents as EDTA is a metal complexing agent and ferrocyanide ions might precipitate out the iron and zinc ions from the medium, and consequently decreased their toxic effect on the microbial strain and enhanced LA production [16, 25]. Tiwaki et al. [27] found that treatment of malt extract with EDTA has increased LA yield by *Lactobacillus delbrueckii* up to 1−2%. It was found that the treatment of molasses with EDTA exhibited the highest LA production while treatment with sulphuric acid, tricalcium phosphate, and cation exchange resin showed an adverse effect on LA production by *Lactobacillus delbrueckii* NCIMB 8130 [25]. Also, the treatment of sweet potato starch hydrolysate with EDTA has enhanced citric acid production by *Aspergillus niger* IIB-A6 [28].

LAB requires complex nutrients as they regarded as fastidious microorganisms that have limited ability to biosynthesize amino acid/vitamins [29]. Interestingly, molasses contains nitrogen and vitamin substrates in its composition that might save the nitrogen source cost [30]. For fermentation cost reduction, LA-fermentation of pre-treated molasses with/without external nutrient supplementation was investigated. We notice that there is no big difference between fermentation conducted with or without MRS fortified with yeast extract only. LA fermentation by *E. faecalis* was significantly affected by yeast extract concentration [26].

As yeast extract is one of the expensive component [38% of total medium-cost [31, 32]. Therefore, alternative and cheaper nitrogen sources are particularly recommended. For that, we studied the effects of substitution yeast extract with other lower-cost nitrogen sources. However, the highest LA production was obtained using yeast extract. But the substitution of yeast extract with ammonium chloride has achieved good results. Ammonium sulfate [33] and malt combing nuts [34] were reported as good alternatives to yeast extract for LA production. Ammonium chloride was used as a nitrogen source and yeast extract as a growth promoter for LA fermentation from sugarcane and cassava bagasse by *Lactobacillus delbrueckii* [35].

Further investigation was conducted to minimize medium cost. We used ammonium chloride as nitrogen source with yeast extract, dried yeast cells or dried fish wastes as vitamin source at different concentrations. Interestingly, the best result was obtained with the lowest concentration of yeast extract at 0.5 g L^{-1}. Therefore, utilization of molasses (substrate) supplemented with 5 g L^{-1} ammonium chloride (nitrogen source) and 0.5 g L^{-1} yeast extract (vitamin source) would achieve better competitive cost for industrial production of LA by *Enterococcus hirae* ds10. It was also reported that increased nitrogen concentration adversely affects fermentation kinetics [36]. Our results indicated that supplementation of dried yeast cells and fish wastes did not improve LA production by strain ds 10. Besides this, the mixed ammonium chloride with fish wastes exhibited very low concentration of lactic acid (0.29—0.86 g L^{-1}), lower than that obtained on molasses with only ammonium chloride (4.04 g L^{-1}). This might be attributed to some inhibitory components in fish waste materials. Similarly, Shi et al. [37] reported that the efficiency of LA production from glucose was greatly reduced when the unhydrolyzed fish manure wastes (Nile tilapia and channel catfish) were used instead of yeast extract using *Lactobacillus pentosus*. On the other hand, Gao et al. [38] have reported that acid hydrolyzed fish wastes showed high efficiency for LA production and could be used as a substitute for YE.

To find the optimum conditions for LA production by strain ds10, we studied factors affecting LA production using a one-factor-at-a-time (OFAT) approach and a statistical approach [4, 29]. The highest LA concentration was obtained with an initial sugar concentration of 20 g L^{-1}. But as expected, lower growth rate, LA concentration, and high residual sugars were obtained at higher sugar concentrations indicating substrate inhibition. It was reported that LA production by *Lactobacillus delbrueckii* NCIMB 8130 was increased with an increase of initial beet molasses sugar concentration up to 100 g L^{-1} [25].

pH is one of the factors influencing the metabolic and enzymatic activities of the microorganisms [29]. During the LA fermentation process, pH was decreased that affected the fermentation process. Therefore, we speculated that the addition of neutralizing agents would partially overcome such inhibition. Unexpected, addition of neutralizing agent (calcium carbonate/sugar conc., 2:1) exhibited an adverse effect on the LA fermentation process. It was found that a high concentration of $CaCO_3$ than 7%, (*w/v*) exhibited a negative effect on LA production [25]. On contrast, $CaCO_3$ (10%, *w/v*) in the production medium exhibited a good effect on LA production by *Lactobacillus LMI8* sp. [36]. Therefore, we might attribute such inhibition

depending on special reactions with the used substrate or due to strain specificity. Overall, our study would result in LA production with no environmental wastes (i.e. gypsum) due to no use of $CaCO_3$ in fermentation processes.

To find the influence of the initial pH of the medium, the pH of the fermentation medium was adjusted to varying pH. The maximum LA production by *Enterococcus hirae* ds10 (11.26 ± 0.35 g L^{-1}) was observed at pH 8.0. However, at higher and lower pH levels, a decrease in all parameters were obtained. It was reported that the optimal pH for LA production by most LAB varies between 5.0−7.0, being dependent on the microbial species [40]. pH 6.5 was the optimal for LA production by *Enterococcus faecium* No. 78 [41].

Temperature is one of the critical factors affecting growth rate and metabolite production [42, 43]. Our results showed that LA production was markedly influenced by the initial temperature whereas optimal fermentation temperature for *Enterococcus hirae* ds10 was 40 °C. At lower or higher temperature, the strain showed lower biomass and LA production, but interestingly, this strain can tolerate higher temperature until 50 °C. LA production by *Enterococcus faecium* S.156 was almost steady from 32 −40 °C [39]. Tan et al. (2017) reported that optimal LA production by *Enterococcus faecium* QU 50 was obtained at 50 °C.

The effect of inoculum size on LA production was also studied. The maximum LA production by *Enterococcus hirae* ds10 of 25.40 ± 0.42 g L^{-1} (24 h) was obtained using a 10% (*v/v*) inoculum size. According to [4], the inoculum size is usually 5−10% (*v/v*) of the working volume. The inoculum size 10% (*v/v*) was used for the production of LA from *Bacillus* sp. [44], and *Lactobacillus amylophilus* GV6 [45].

Response surface Minitab 18 software was used to optimize the composition of the medium. This method is very economical and efficient as it studies the relationships between various independent variables [46]. According to the regression model, the maximum LA concentration (40.69 g L^{-1}) was reached at 0.625 g L^{-1} yeast extract, 60 g L^{-1} sugar concentration, 40 °C, pH 8.0 and 9.5% inoculum size. The optimal temperature, and pH defined by the model were as the same as earlier experiments, however, the inoculum size and yeast extract were slightly differ with 9.5% and 0.625 g L^{-1} from 10% and 0.5 gL^{-1}, respectively. With this parameter, the carbon source was increased from 40 g L^{-1} to 60 g L^{-1} and exhibited maximum LA concentration at 40.69 g L^{-1} that is almost double the amount compared by maximum LA produced [25.4 ± 0.42 g L^{-1} (24 h)] by classical OFAT. Altaf et al. [47] used response optimization with MINITAB-13 and found that the possible LA output to be 4.5 g from the highest output of LA production observed was 3.8 g/6.0 g starch present in 10 g of wheat bran. As have been noted from both optimization methods, high residual sugar concentration has limited the fermentation. This might be attributed to the high LA concentrations that lead to decreased pH value affecting the enzymatic activities for further sugar utilization. The increased free LA might also inhibit bacterial growth and retard fermentation. Although utilization of $CaCO_3$ as a neutralizing agent did not overcome this problem, therefore, further investigations utilizing various neutralizing agents would be necessary for studies.

5 Conclusion

Our results conclude the possibility of using molasses as an economical source for LA production after pretreatment using EDTA. Ammonium chloride is a good alternative nitrogen source that replaced the traditional costly yeast extract in the optimized medium by using yeast extract as a vitamin source at very low concentration (0.05%, *w/v*). Homolactic acid fermentation by *Enterococcus hirae* ds10 was optimized by a statistical method that increased about two-folds than by traditional method on low-cost medium contained beet molasses, ammonium chloride, and yeast extract. Further enhancement was obtained using a statistical method that evaluated the most efficient factors affected LA production achieving 40.69 g L^{-1} in low-cost production medium.

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Data Availability The data used to support the findings of this study are available from the corresponding author upon request.

Compliance with ethical standards

Conflict of interest Authors declare that there are no conflicts of interest.

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