

Experimental design for optimization of D-lactic acid production using a UV-light selected strain

Abstract

Lactic acid is a molecule with several applications in different industries. Many applications require high isomeric purity, in order to attain the need properties of the final product. L-lactic acid production has already been extensively studied, however, D-lactic acid production remains a challenge since the majority of the strains used in lactic acid production are L producers. Bearing all this in mind, this study presents UV-light as an alternative tool to enhance D-lactic acid production. Different UV-light selected strains from *S. porolactobacillus nakayamae* subsp. *nakayamae* were studied in order to obtain high D-lactic acid concentration. Preliminary tests were performed using different carbon and nitrogen sources. Further, an experimental design was used to optimize the medium composition. As result, the selected strain presented a better D-lactic acid production compared to the natural strain, with 97% of isomeric purity. The maximal production of D-lactic acid reached 74.46g/L from clarified sugarcane juice and yeast extract, with a productivity of 3.10g/Lh.

Keywords: D-lactic acid, UV-light, *S. porolactobacillus nakayamae*, experimental design, optimization

Volume 5 Issue 4 - 2018

Regiane Alves de Oliveira,¹ Luciana Fontes Coelho,² Natalia Sozza Bernardi,³ Jonas Contiero²

¹Department of Processes and Products Development, University of Campinas (Unicamp), Brazil

²Department of Biochemistry and Microbiology, Universidade Estadual Paulista "Julio de Mesquita Filho" (UNESP), Brazil

³Department of Pharmaceutical Technology, University of Coimbra, Portugal

Correspondence: Jonas Contiero, Department of Biochemistry and Microbiology, Universidade Estadual Paulista (UNESP), Avenida 24A, 1515, Bela Vista, 13506-900, Rio Claro-SP, Brazil, Tel +55 19 35264180, Email jconti@rc.unesp.br

Received: July 18, 2018 | **Published:** August 22, 2018

Introduction

Lactic acid (LA) is an organic acid with a hydroxyl and an acid functional group.^{1,2} It can be produced by humans, plants, animals and microorganisms.² It has an asymmetric carbon and it naturally occurs as two optical isomers, D- and L-LA, due to the existence of a chiral centre.^{1,3} It was discovered in 1780 by the experimental chemist Carl Wilhelm Scheele, isolated from sour milk. Currently is known as 2-hydroxypropanoic acid.⁴

LA chemical production leads to racemic mixtures of D and L isomers.⁵ This circumstance makes impossible to control the chemical and physical properties of the final product. Besides that, the racemic mixture is not suitable to be used in food, pharmaceutical, and medical industries. The proportion of each isomer confers different physical properties to the final product. It makes the enantiomeric purity a crucial factor for LA industrial production, as in the manufacture of biodegradable plastics and polymers as poly-LA.⁶

The polymerization of a racemic mixture of L- and D-LA generally leads to the synthesis of poly-DL-LA (PDLLA), which is amorphous. The crystallinity and many other important physical properties such as rate of degradation,³ melting and boiling point are controlled by the ratio of enantiomers D and L used.^{3,7} Due to the chiral nature of the LA, several different types of polymers can be produced, for example, poly-L-LA (PLLA) is the product resulting from the polymerization of L-LA, which has high crystallinity and high melting point.^{4,8} The melting temperature of the PLLA may be increased to 50°C by the physical mixture with the polymer PDLA (poly-D-LA) which can form a highly regular stereo-complex with increased crystallinity and thermal stability.^{9,10}

Due to the necessity of enantiomeric purity in the industries for some specific applications, the LA production by fermentation has been more attractive instead of chemical production. Currently, almost all LA in the world is produced by fermentation,¹¹ however, the majority of the microorganisms used and most of the studies in the

literature are focused on the L-LA biosynthesis. So far, cost-effective production of L-LA, used for PLLA production, is well established compared to that of D-LA, used for PDLA production, production process.^{12,13}

This finding has led to increased interest in the production of optically pure D-LA because the commercial lactic acid market historically has been dominated by L-LA.^{10,14}

Several microorganisms have been reported to be natural D-LA producers.¹⁵⁻¹⁷ Also, genetically engineered microorganisms have been reported to be pure D-LA producers.^{10,12,14,18-21} However, to the best of our knowledge, it is the first time that UV-light is used as a tool to enhance D-LA production.

Bearing all this in mind, the main objective of this study is to improve the D-LA production with high isomeric purity, using an isolated *S. porolactobacillus nakayamae* subsp. *nakayamae* submitted to UV-light mutation. It was also evaluated the effects of different media composition, such as different carbon and nitrogen sources on D-LA production.

Methods

Microorganism

The microorganism *S. porolactobacillus nakayamae* subsp. *nakayamae* Vini6 is gram-positive, rod-shaped, spore-forming, and a D-LA producer. It was isolated from *Desmodium adscendens* root.²² The strain was stored in GYP (Glucose-Yeast-Peptone) medium 23 with 20% glycerol (v/v) at -20°C.

The microorganism was cultivated in GYP culture medium and incubated at 35°C for 24hours. Afterwards, the bacterial suspension was transferred to an Erlenmeyer flask at the same conditions and used as the inoculum. 10% of the workload was inoculated in all fermentation tests.

UV light mutation

S. nakayamae subsp. *nakayamae* strain was cultivated in GYP media for 24hours at 35°C, thereafter it was transferred to solid GYP and incubated for 48hours at 35°C. The bacterial colonies were exposed to UV-light (254nm) for 20minutes. After that, NaCl 0.85% was added and the colonies were mixed. The aliquot was used in serial dilutions in solid GYP with CaCO₃ 1% and incubated at 35°C for 48hours. Selection of colonies was based on rapid growth, as well as on the largest acid production halo. The selected colonies were mixed and incubated to grow and again exposed to UV irradiation for another 20minutes. Three cycles of exposure to UV-light were performed.

Fermentation

All fermentation tests were performed in 40mL Erlenmeyer flasks containing 20mL of medium, at 35°C and 150rpm. Initial pH was adjusted to 7.0 and 60g/L of CaCO₃ was added before the inoculum, in order to control the pH during the fermentation time. All tests were performed in triplicate. Samples were stored at -20°C to further analysis after the centrifugation to remove cells and CaCO₃.

For each test, LA production (g/L), productivity (Equation 1) and remaining total reducing sugar (RTRS) (Equation 2) were calculated to evaluate the process.

$$P_{(g\ L^{-1}\ h^{-1})} = \frac{lactic\ acid_{(g/L)}}{time_{(h)}} \quad (1)$$

$$R_{TRS} \left(\frac{g}{L} \right) = TRS_{initial} \left(\frac{g}{L} \right) - TRS_{final} \left(\frac{g}{L} \right) \quad (2)$$

Carbon source determination

The fermentation media was composed of 5g/L of sodium acetate, salts solution of GYP media (4% MgSO₄; 0.2% MnSO₄; 0.2% FeSO₄; and 0.2% NaCl), 30g/L of yeast extract and 100g/L of TRS (total reducing sugars) from four different carbon sources: sugarcane juice; clarified sugarcane juice; commercial glucose; commercial sucrose.

Clarified sugarcane juice was prepared using Ca(OH)₂, as the method called simple defecation with lime and heating 24: sugarcane juice was limed with Ca(OH)₂ to pH 8.0 and placed in the water bath 105°C for 1hour and afterwards filtered, in order to remove the solids.

Nitrogen source determination

The microorganism was incubated in culture media with 5 g/L of sodium acetate, salts solution of GYP media, and 100g/L of TRS (total reducing sugars) from the best carbon source previous determined (item 2.4). Four different nitrogen sources were tested, as shown in Table 1: yeast extract, yeast autolyzed, corn steep liquor (CSL), and pro-floo (cottonseed protein). The statistical analysis was performed using Statistica 7.0 (Statsoft Inc). The results were submitted to F-test with the average results compared by Tukey's test.

Table 1 Nitrogen source concentration

Nitrogen source	Concentration A	Concentration B
Yeast extract (g/L)	15	30
Yeast autolyzed (g/L)	12.75	25.5
CSL (mL/L)	36.75	73.5
Pro-floo (g/L)	15.47	30.94

The amount of each nitrogen source corresponds to the same amount of nitrogen contained in 15g/L and 30g/L of yeast extract. Concentration A corresponds to 1.725g of nitrogen/L; Concentration B corresponds to 3.45g of nitrogen/L.

Experimental design

In order to evaluate the influence of TRS, nitrogen source, sodium acetate and GYP salts solution for D-LA production, it was performed a factorial central composite design with four replicates at the central point, totalizing 28 experiments. Table 2 shows concentrations and levels of the 4 independent variables analysed. The Statistica 7.0 (Statsoft Inc.) was used to analyse the experimental design. The confidence level for the regression test was fixed at 95%. The results were submitted to the F-test and the average results compared by Tukey's test.

Table 2 Real values of the coded independent variables

Variables	Codes	Levels				
		-2	-1	0	1	2
TRS (g/L)	X ₁	0	50	100	150	200
Yeast extract (g/L)	X ₂	0	15	30	45	60
Sodium acetate (g/L)	X ₃	0	5	10	15	20
Salts of GYP (mL/L)	X ₄	0	2.5	5	7.5	10

The association between the factors and response was modeled using polynomial equation given by Equation 3, in which: X₁, X₂, X₃ and X₄ are the independent coded variables, β₀, β₁, β₂, β₃, β₄, β₁₂, β₁₃, β₁₄, β₂₃, β₂₄ and β₃₄ are the regression coefficients, and Y is the response function.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (3)$$

Analytical methodology

For LA and sugars quantification it was used a high-performance liquid chromatography (HPLC) (Shimadzu, Prominence series) equipped with an ultra-violet detector at 210nm and refractive index detector. The column used was Rezex ROA (300 x 7.8mm) from Phenomenex, eluted with 5mM of H₂SO₄ as the mobile phase. The flow was 0.6mL/min and the injection volume was 5 μL. The temperature was set at 65°C.

In order to determine the concentration and the isomeric purity of LA, HPLC was equipped with a Chirex 3126 column (150 x 4.6mm) from Phenomenex and a UV detector. The flow was adjusted to 1mL/min using 1mM of CuSO₄ as the mobile phase and the temperature was fixed at 26°C.

Results

The microorganism *S. nakayamae* subsp. *nakayamae* Vini6 is a natural producer of D-LA with a yield up to 50%. The natural strain was able to produce 12.77g/L of LA from 20g/L glucose, with 92.01% of D-LA purity.

The natural strain was exposed to UV-light for 20minutes. After the first UV-light exposure originated the selected strain called Vini6 2x (second generation). This new strain was exposed to UV-

light and generated the strain Vini6 3x (third generation). The UV-light cycle was repeated one more time and generated Vini6 4x (fourth generation), Vini6 4x2 (fourth generation colony 2), Vini6 4x3 (fourth generation colony 3), and Vini6 4x4 (fourth generation colony 4). In total, 6 strains were selected from UV-light exposure, which are depicted in Table 3. All selected strains presented a better D-LA production when compared to the natural strain. The highest LA production obtained from 100g/L of glucose was 88.98 g/L using the selected strain *S. nakayamae* Vini6 2x, which was chosen as the strain to be used in the next tests.

Table 3 D(-) acid lactic production by 6 strains submitted to UV mutation from 100g/L of glucose

Strain	D(-) lactic acid (g/L)
<i>S. nakayamae</i> Vini6 natural strain	60.64
<i>S. nakayamae</i> Vini6 2x	88.98
<i>S. nakayamae</i> Vini6 3x	76.9
<i>S. nakayamae</i> Vini6 4x	59.85
<i>S. nakayamae</i> Vini6 4x2	81.95
<i>S. nakayamae</i> Vini6 4x3	79
<i>S. nakayamae</i> Vini6 4x4	79.68

After the strain selection, D-LA production was tested using different carbon sources: sugarcane juice, clarified sugarcane juice, commercial glucose, and commercial sucrose. The highest D-LA production occurred in the presence of commercial sucrose as a carbon source, both after 24 and 48hours (56.61 and 58.98g/L, respectively). However, commercial sucrose has high market prices, which justifies its replacement by a more economically viable source of carbon. Therefore, it is noted the clarified sugarcane juice has a slightly higher production of D-LA (53.80g/L) when compared to sugarcane juice (48.18g/L). The clarification was performed in order to precipitate the largest possible amount of impurities, resulting in an improvement of the microorganism performance. The process did not affect the sucrose content, but pectin, albumin, mucilage, starch, fibres, waxes, proteins, pigments, and other components precipitated and were removed with a simple filtration.²⁴ Front of the results clarified sugarcane juice was chosen as the carbon source to D-LA production.

The next step was to test different nitrogen sources to be used for D-LA production: yeast extract, yeast autolyzed, CSL, and pro-floo. The results of the nitrogen source influence in production, productivity and yield of D-LA are shown in Table 4. The highest D-LA production was 67.89g/L using yeast extract on the concentration B, after 24hours of fermentation. Remaining TRS also presented the best result using yeast extract (6.15g/L) on the concentration B. Only when LA yield is considered, corn steep liquor presented the best result, reaching 0.75g/g after 48hours of fermentation, also on concentration B. However, yeast extract also presented a good yield (0.58g/g), being chosen as the nitrogen source to be used for D-LA production.

An average value followed by different letters in vertical (lowercase) and horizontal (uppercase) differ at 5% according to Tukey's test.

Based on the preliminary tests, clarified sugarcane juice and yeast extract were used in the central composite design in order to optimize the media composition for D-LA production. Table 5 shows the variables and the real concentrations of each component: TRS

from clarified sugarcane juice (X_1), yeast extract (X_2), sodium acetate (X_3), and salts solution from GYP (X_4), as well as the average results obtained from a triplicate of D-LA production, productivity and remaining TRS after 24hours of fermentation.

Table 4 D(-) lactic acid production (g/L) after 24 and 48 hours of fermentation, remaining TRS (g/L), and yield (g/g), according to the nitrogen source used

Nitrogen source	Concentration A	Concentration B
Production - 24 hours		
Yeast extract	50.82 Ba	67.89 Aa
Yeast autolyzed	3.83 Ac	5.02 Ac
Corn steep liquor	37.60 Ab	39.13 Ab
Pro-floo	0.37 Ac	0.48 Ac
Production - 48 hours		
Yeast extract	50.92 Aa	54.83 Aa
Yeast autolyzed	1.24 Ab	1.82 Ab
Corn steep liquor	45.58 Ba	58.67 Aa
Pro-floo	0.13 Ab	0.47 Ab
Remaining TRS - 48 hours		
Yeast extract	12.61 Ab	6.15 Ab
Yeast autolyzed	55.89 Aa	62.00 Aa
Corn steep liquor	17.56 Ab	19.70 Ab
Pro-floo	57.55 Ba	68.99 Aa
Yield - 48 hours		
Yeast extract	0.58 Aa	0.58 Ab
Yeast autolyzed	0.03 Ab	0.05 Ac
Corn steep liquor	0.55 Ba	0.75 Aa
Pro-floo	0.00 Ab	0.02 Ac

The highest D-LA production was obtained in test 14, with 74.46g/L of LA produced from 150g/L of TRS, 45g/L of yeast extract, 5g/L of sodium acetate, and 7.5mL/L of GYP salts solution, as shown in Table 5. On these conditions, productivity and remaining TRS were 3.10g/Lh and 31.06g/L. D-LA isomer purity presented an average of 97%±2% between the 28 tests, showing that this microorganism may be suitable for an industrial process for D-LA production.

The application of multiple regression analysis for each response from Table 5 resulted in the regression Equations 4, 5 and 6. Equation 4 represents the regression model for LA production (LA), Equation 5 for productivity (P), and Equation 6 for remaining TRS (RTRS). X_1 , X_2 , X_3 and X_4 are the coded values of the test variables TRS, yeast extract, sodium acetate and GYP salts, respectively.

$$LA = 57.632 + 18.017X_1 + 15.283X_2 - 3.651X_3 + 0.218X_4 - 8.796X_1^2 + 9.032X_1X_2 - 7.568X_1X_3 + 2.989X_1X_4 - 7.974X_2^2 + 3.942X_2X_3 - 3.923X_2X_4 - 1.394X_3^2 - 4.486X_3X_4 - 4.618X_4^2 \tag{4}$$

$$P = 2.640 + 0.527X_1 + 0.651X_2 - 0.160X_3 + 0.002X_4 - 0.499X_1^2 + 0.424X_1X_2 - 0.198X_1X_3 + 0.068X_1X_4 - 0.495X_2^2 - 0.036X_2X_3 - 0.077X_2X_4 - 0.275X_3^2 - 0.224X_3X_4 - 0.117X_4^2 \tag{5}$$

$$R_{TRS} = 3.352 + 16.203X_1 - 23.479X_2 + 0.841X_3 - 3.684X_4 + 6.946X_1^2 - 15.789X_1X_2 + 5.253X_1X_3 - 4.196X_1X_4 + 19.503X_2^2 - 5.534X_2X_3 + 3.494X_2X_4 + 3.921X_3^2 + 7.242X_3X_4 + 4.065X_4^2 \tag{6}$$

Table 5 Central composite design (real values), and the results after 24 of fermentation

Test	Concentration				Production (g/L)	Productivity (g/Lh)	Remaining TRS (g/L)
	TRS	Yeast extract	Acetate	GYP salts			
1	50	15	5	2.5	1.37	0.06	46.58
2	50	15	5	7.5	1.77	0.07	48.51
3	50	15	15	2.5	7.48	0.31	36.97
4	50	15	15	7.5	6.98	0.29	33.65
5	50	45	5	2.5	4.92	0.2	33.94
6	50	45	5	7.5	25.88	1.08	5.4
7	50	45	15	2.5	36.53	1.52	2.83
8	50	45	15	7.5	6.34	0.26	30.58
9	150	15	5	2.5	3.36	0.14	138.58
10	150	15	5	7.5	27.89	1.16	96.12
11	150	15	15	2.5	8.92	0.37	132.14
12	150	15	15	7.5	3.21	0.13	132.34
13	150	45	5	2.5	73.49	3.06	28.91
14	150	45	5	7.5	74.46	3.1	31.06
15	150	45	15	2.5	58.51	2.44	45.79
16	150	45	15	7.5	55.75	2.32	54.55
17	0	30	10	5	3.93	0.16	0
18	200	30	10	5	48.48	2.02	97.91
19	100	0	10	5	1.4	0.06	100
20	100	60	10	5	51.67	2.15	32.34
21	100	30	0	5	63.39	2.64	3.16
22	100	30	20	5	31.89	1.33	26.55
23	100	30	10	0	64.27	2.68	4.92
24	100	30	10	10	61.12	2.55	3.64
25	100	30	10	5	66.58	2.77	4.15
26	100	30	10	5	64.27	2.68	3.7
27	100	30	10	5	61.05	2.54	3.93
28	100	30	10	5	61.57	2.57	3.58

The identification of significant parameters was performed through hypothesis test using t-Student statistic method. The probability was used as a tool to verify the significance of each coefficient. The maximum probability of error in the test was 5%, thus the parameters with the value $p < 0.05$ were considered significant.

The main variables affecting production, productivity and remaining TRS are X_1 (TRS) and X_2 (yeast extract). Based on these results, it was possible to imply that acetate (X_3) and salts solution from GYP (X_4) were not essential for D-LA production using *S. nakayamae* Vini6 2x.

Fisher-test was used to analyse the statistical significance of the Equations 4, 5 and 6 through ANOVA, as shown in Table 6.

For Equation 4, $F_{14,13}$ calculated (5.32) was higher than $F_{14,13}$ tabulated (2.55) with 95% of confidence level, showing that the model is significant and adequately explains experimental data variation for LA production. The good fit of the model was verified by the determination coefficient (R^2) and multiple correlation co-efficient (R). In this case, the value of R^2 indicates that 85% of the variability in the data was explained by the mathematical model. The high R-value (0.92) shows a high degree of agreement between the experimental data and predicted values (Table 6). Based on the mathematical model it was constructed the response surface for LA production in function of TRS (X_1) and yeast extract (X_2). To achieve high lactic acid concentrations, Figure 1A shows that the optimum concentration of TRS should be between 100 and 200g/L and between 30 and 60g/L for yeast extract.

Table 6 ANOVA table for the responses with 95 % of confidence level

Production					
Source of variation	Sum of Squares	Degrees of Freedom	Mean Square	F _{calculated}	P
Regression	19782.06	14	1.41	5.32	0.00232
Residues	3453.05	13	265.62		
Lack of fit	3432.77	10	343.27		
Pure error	20.28	3	6.76		
Total	23235.11	27			
R ² = 0.85; R = 0.92					
Productivity					
Regression	3.18	14	2.27	7.82	0.000333
Residues	378.25	13	0.29		
Lack of fit	374.91	10	0.37		
Pure error	0.03	3	0.01		
Total	3.56	27			
R ² = 0.89; R = 0.94					
Remaining TRS					
Regression	35426.3	14	2.53	4.94	0.00329
Residues	6657.28	13	512.1		
Lack of fit	6657.11	10	665.71		
Pure error	0.18	3	0.06		
Total	42083.58	27			
R ² = 0.84; R = 0.92					

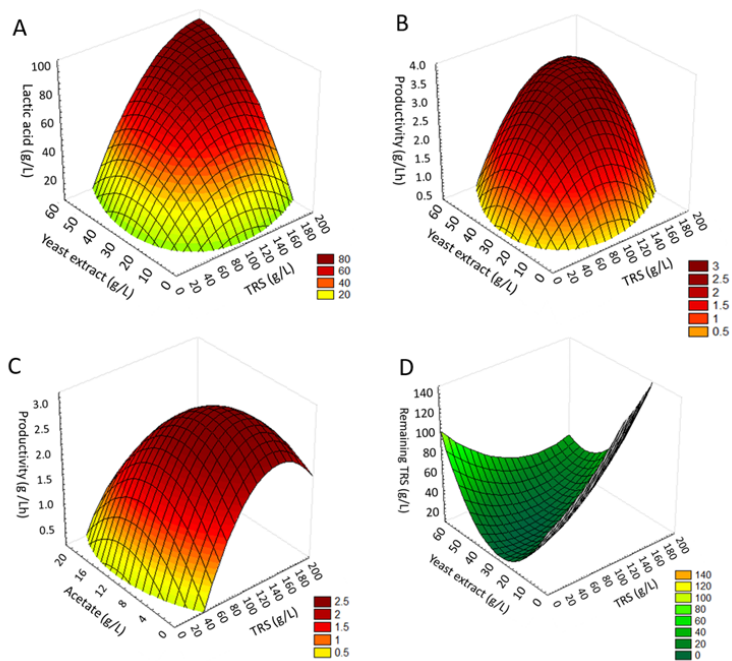


Figure 1 Response surface of (A) lactic acid production in function of TRS (X₁) and yeast extract (X₂); (B) lactic acid productivity in function of TRS (X₁) and yeast extract (X₂); (C) lactic acid productivity in function of TRS (X₁) and acetate (X₃); and (D) remaining TRS in function of TRS (X₁) and yeast extract (X₂).

Considering the productivity (Equation 5) Fisher-test also confirmed that the mathematical model was predictive. $F_{14,13}$ calculated (7.82) was higher than $F_{14,13}$ tabulated (2.55) with 95% of confidence level, showing that the model is significant. In this case, R^2 indicated that 89% of the variability in the data was explained by the model. The high R-value (0.94) shows a high degree of agreement between the experimental data and predicted values (Table 6). Thus, it was possible to construct the response surface for LA productivity in function of TRS (X_1) and yeast extract (X_2) as shown in Figure 1B; and in function of TRS (X_1) and acetate (X_3) as shown in Figure 1C. According to Figures 1B and 1C, better productivities can be achieved when the concentrations of TRS are between 120 and 180g/L of TRS, yeast extract is between 35 and 55g/L, and sodium acetate is between 4 and 10g/L.

According to Equation 6, $F_{14,13}$ calculated (4.94) was higher than $F_{14,13}$ tabulated (2.55) with 95% of confidence level, showing that the mathematical model was significant and adequately explained the experimental data variation for LA yield. In this case, the value of R^2 indicated that 84% of the variability in the data was explained by the model. The high R-value (0.92) also shows a high degree of agreement between the experimental data and predicted values (Table 6). Based on these results, Figure 1D shows that in order to obtain low amounts of remaining TRS, initial TRS concentrations should be around 100g/L and yeast extract should be 30g/L.

In all cases, residual distribution responses were random around zero with no tendency. The observed values were close to those predicted, leading to a steady trend and a normal distribution.

In general, it was observed that high TRS concentration (up to 150g/L) leads to a decrease in the productivity and a higher amount of remaining TRS. In this context, the best media composition for *S. nakayamae* Vini6 2x should be composed by 150g/L of TRS from clarified sugarcane juice and 35g/L of yeast extract. 4g/L of sodium acetate could also be used as a complementary nutrient in order to enhance the productivity.

Discussion

Several microorganisms have been reported to be D-LA producers, such as *Lactobacillus delbrueckii*¹⁵ *Lactobacillus coryniformis*¹⁷ and *Lactobacillus plantarum*.^{10,14,18,19} Several *S. porolactobacillus* sp. strains have also been reported to be D-LA producers.^{16,22,25–33} Even though, D-LA production is still a challenge for the industry,^{12,13} once the majority of good LA producers have key-enzymes to L-LA production.

In this sense, many studies report the use of metabolic engineering tools to obtain higher D-LA production.^{10,12,14,18–21,29,33,34} Instead of using metabolic engineering tools, this study presents a UV-light mutation approach to improve the D-LA production by *S. nakayamae* *nakayamae*. This kind of approach is widely used for mutation and selection of microorganisms in order to improve their capacity of producing biologically active substances.^{35,36} The use of UV-selection mechanism is an effective mutagenesis tool towards improving lactic acid production in different strains, as demonstrated for *Lactobacillus lactis* BME5-18 with a productivity 46% higher than the parent strain 37 and *Rhizopus oryzae* R1021 with an improvement of 52% of LA production.³⁶

Clarified sugarcane juice and yeast extract were the best carbon

and nitrogen sources between the ones evaluated for D-LA production by *S. nakayamae* Vini6 2x.

Yeast extract had been chosen once yeast extract makes it possible to have a faster D-LA production. A study using *S. inulinus* detected that the free amino acids are the limiting factor for the lactic acid production.³⁰ It turned out that this is the reason why yeast extract is a good nitrogen source for this microorganism. Furthermore, Brazil has a high potential of using the remaining yeast from the ethanol industry to produce yeast extract with low costs. As highlighted by Pleissner et al.,³⁸ the costs of biotechnological inputs are region dependent. For instance, in the case presented here, yeast extract can be an important input if the LA production could be done close to an ethanol and yeast production plant, in a biorefinery concept. Ethanol fermentation as it is done in Brazilian distilleries generates an excess of 20kg of dried yeast per 1000L of produced ethanol³⁹ that can be converted to a low-cost yeast extract. This process is already cost-effective in Brazilian scenario.⁴⁰ As an example, a Sugarcane 1st Generation Biorefinery would process sugarcane to sucrose, ethanol, yeast products and LA. The remaining bagasse can be still used to provide steam and electricity to sustain processes and the excess of electricity available can be even exported to the grid.

Regarding the optimization process, the main components affecting D-LA production by *S. nakayamae* Vini6 2x are clarified sugarcane juice and yeast extract. Higher D-LA concentrations can be obtained using 150 g/L of TRS from clarified sugarcane juice and 35g/L of yeast extract. 4g/L of sodium acetate can also be used as a complementary nutrient in order to enhance the productivity. Zheng et al.³² proved that the presence of sodium ions in the early growth phase of *S. inulinus* improve the productivity of D-LA by the key-enzyme phosphofructokinase. Besides, sodium acetate is also known as an efficient medium component to prevent the contamination of the process by fungi.⁴¹

Conclusion

S. nakayamae Vini6 2x is a useful strain to produce D-LA with high purity ($\approx 97\%$). UV-light is a suitable tool for increasing the LA production, as well as keeping a high isomeric purity. The use of central composite design was a useful tool to optimize the production process of D-LA. Further, the obtained results are very promising for scaling-up the fermentation process. As next steps, it is intended to scale-up the fermentation process in order to obtain higher D-LA production and productivity.

Acknowledgements

None.

Funding details

Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (2012/21679-9)

Conflict of interest

Author declares that there is no conflict of interest.

References

1. Auras R, Lim LT, Selke SEM, et al. *Poly (Lactic Acid): Synthesis, Structures, Properties, Processing, and Applications*. In: Grossman RF, Nwabunma D, editors. New Jersey: John Wiley & Sons, Inc.; 2010.

2. Vijayakumar J, Aravindan R, Viruthagiri T. Recent trends in the production, purification and application of lactic acid. *Chem Biochem Eng Q*. 2008;22(2):245–264.
3. Zhou S, Causey TB, Hasona A, et al. Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110. *Appl Environ Microbiol*. 2003;69(1):399–407.
4. Ghaffar T, Irshad M, Anwar Z, et al. Recent trends in lactic acid biotechnology: A brief review on production to purification. *Journal of Radiation Research and Applied Sciences*. 2014;7(2):222–229.
5. Juodeikiene G, Vidmantienė D, Basinskiene L, et al. Green metrics for sustainability of biobased lactic acid from starchy biomass vs chemical synthesis. *Catalysis Today*. 2015;239:11–16.
6. Farah S, Anderson DG, Langer R. Physical and mechanical properties of PLA, and their functions in widespread applications—A comprehensive review. *Advanced Drug Delivery Reviews*. 2016;107:367–392.
7. Södergård A, Stolt M. Properties of lactic acid based polymers and their correlation with composition. *Progress in Polymer Science*. 2002;27(6):1123–1163.
8. Oh H, Wee YJ, Yun JS, et al. Lactic acid production from agricultural resources as cheap raw materials. *Bioresour Technol*. 2005;96(13):1492–1498.
9. Ikada Y, Jamshidi K, Tsuji H, et al. Stereocomplex formation between enantiomeric poly (lactides). *Macromolecules*. 1987;20(4):904–906.
10. Hama S, Mizuno S, Kihara M, et al. Production of D-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*. *Bioresour Technol*. 2015;187:167–172.
11. Alves de Oliveira R, Komesu A, Vaz Rossell CE, et al. Challenges and opportunities in lactic acid bioprocess design—From economic to production aspects. *Biochemical Engineering Journal*. 2018;133:219–239.
12. Yamada R, Wakita K, Mitsui R, et al. Enhanced D-lactic acid production by recombinant *Saccharomyces cerevisiae* following optimization of the global metabolic pathway. *Biotechnol Bioeng*. 2017;114(9):2075–2084.
13. Klotz S, Kaufmann N, Kuenz A, et al. Biotechnological production of enantiomerically pure D-lactic acid. *Appl Microbiol Biotechnol*. 2016;100(22):9423–9437.
14. Zhang Y, Vadlani PV, Kumar A, et al. Enhanced D-lactic acid production from renewable resources using engineered *Lactobacillus plantarum*. *Appl Microbiol Biotechnol*. 2016;100(1):279–288.
15. Zhang Y, Vadlani PV. D-Lactic acid biosynthesis from biomass-derived sugars via *Lactobacillus delbrueckii* fermentation. *Bioprocess Biosyst Eng*. 2013;36(12):1897–1904.
16. Wang L, Zhao B, Li F, et al. Highly efficient production of D-lactate by *S_poro*lactobacillus sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. *Appl Microbiol Biotechnol*. 2011;89(4):1009–1017.
17. Yáñez R, Moldes AB, Alonso JL, et al. Production of D(–)-lactic acid from cellulose by simultaneous saccharification and fermentation using *Lactobacillus coryniformis* subsp. *torquens*. *Biotechnol Lett*. 2003;25(14):1161–1164.
18. Zhang Y, Kumar A, Hardwidge PR, et al. D-lactic acid production from renewable lignocellulosic biomass via genetically modified *Lactobacillus plantarum*. *Biotechnol Prog*. 2016;32(2):271–278.
19. Yoshida S, Okano K, Tanaka T, et al. Homo-D-lactic acid production from mixed sugars using xylose-assimilating operon-integrated *Lactobacillus plantarum*. *Appl Microbiol Biotechnol*. 2011;92(1):67–76.
20. Liu Y, Gao W, Zhao X, et al. Pilot scale demonstration of D-lactic acid fermentation facilitated by Ca(OH)₂ using a metabolically engineered *Escherichia coli*. *Bioresour Technol*. 2014;169:559–565.
21. Wang Y, Tian T, Zhao J, et al. Homofermentative production of D-lactic acid from sucrose by a metabolically engineered *Escherichia coli*. *Biotechnol Lett*. 2012;34(11):2069–2075.
22. Beitel SM, Sass DC, Coelho LF, et al. High D(–) lactic acid levels production by *S_poro*lactobacillus *nakayamae* and an efficient purification. *Annals of Microbiology*. 2016;66(4):1367–1376.
23. Kozaki M, Uchimura T, Okada S. *Experimental Manual of Lactic Acid Bacteria*. Tokio: Asakura Syoten; 1992.
24. Prati P, Moretti RH. Study of clarification process of sugar cane juice for consumption. *Ciência e Tecnol Aliment*. 2010;30(3):776–783.
25. Fukushima K, Sogo K, Miura S, et al. Production of D-lactic acid by bacterial fermentation of rice starch. *Macromol Biosci*. 2004;4(11):1021–1027.
26. Zhao B, Wang L, Li F, et al. Kinetics of D-lactic acid production by *S_poro*lactobacillus sp. strain CASD using repeated batch fermentation. *Bioresour Technol*. 2010;101(16):6499–6505.
27. Bai Z, Gao Z, Sun J, et al. D-lactic acid production by *S_poro*lactobacillus *inulinus* YBS1–5 with simultaneous utilization of cottonseed meal and corncob residue. *Bioresour Technol*. 2016;207:346–352.
28. Zhao T, Liu D, Ren H, et al. D-lactic acid production by *S_poro*lactobacillus *inulinus* Y2–8 immobilized in fibrous bed bioreactor using corn flour hydrolyzate. *J Microbiol Biotechnol*. 2014;24(12):1664–1672.
29. Sun J, Wang Y, Wu B, et al. Enhanced production of D-lactic acid by *S_poro*lactobacillus sp. Y2–8 mutant generated by atmospheric and room temperature plasma. *Biotechnol Appl Biochem*. 2015;62(2):287–292.
30. Klotz S, Kuenz A, Prübe U. Nutritional requirements and the impact of yeast extract on the D-lactic acid production by *S_poro*lactobacillus *inulinus*. *Green Chem*. 2017;19(19):4633–4641.
31. Bai Z, Gao Z, He B, et al. Effect of lignocellulose-derived inhibitors on the growth and D-lactic acid production of *S_poro*lactobacillus *inulinus* YBS1–5. *Bioprocess Biosyst Eng*. 2015;38(10):1993–2001.
32. Zheng L, Liu M, Sun J, et al. Sodium ions activated phosphofructokinase leading to enhanced D-lactic acid production by *S_poro*lactobacillus *inulinus* using sodium hydroxide as a neutralizing agent. *Appl Microbiol Biotechnol*. 2017;101(9):3677–3687.
33. Zheng H, Gong J, Chen T, et al. Strain improvement of *S_poro*lactobacillus *inulinus* ATCC 15538 for acid tolerance and production of D-lactic acid by genome shuffling. *Appl Microbiol Biotechnol*. 2010;85(5):1541–1549.
34. Ou MS, Awasthi D, Nieves I, et al. Sweet sorghum juice and bagasse as feedstocks for the production of optically pure lactic acid by native and engineered *Bacillus coagulans* strains. *BioEnergy Research*. 2016;9(1):123–131.
35. Goodarzi A. UV – Induced mutagenesis in lactic acid bacteria. *Int J Genet Genomics*. 2016;4(1):1–4.
36. Bai DM, Zhao XM, Li XG, et al. Strain improvement of *Rhizopus oryzae* for over-production of L(+)-lactic acid and metabolic flux analysis of mutants. *Biochemical Engineering Journal*. 2004;18(1):41–48.
37. Bai DM1, Zhao XM, Li XG, et al. Strain improvement and metabolic flux analysis in the wild-type and a mutant *Lactobacillus lactis* strain for L(+)-lactic acid production. *Biotechnol Bioeng*. 2004 Dec 20;88(6):681–689.
38. Pleissner D, Dietz D, van Duuren JB, et al. Biotechnological production of organic acids from renewable resources. *Advances in Biochemical Engineering/Biotechnology*. Springer, Berlin, Heidelberg; 2017. p. 1–38.

39. Sgarbieri VC, Alvim ID, Vilela ESD, et al. Pilot plant production of yeast (*Saccharomyces* sp.) derivatives for use as ingredients in food formulations. *Brazilian J Food Technol.* 1999;2:119–125.
40. Alves de Oliveira R, Vaz Rossell CE, Lunelli BH, et al. Different strategies to improve lactic acid productivity based on microorganism physiology and optimum operating conditions. *Ind Eng Chem Res.* 2018;57(31):10118–10125.
41. Cortés-Zavaleta O, López-Malo A, Hernández-Mendoza A, et al. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *Int J Food Microbiol.* 2014;173:30–35.