Poll Res. 40 (4) : 1574-1580 (2021)

ISSN 0257–8050

STATISTICAL OPTIMIZATION OF LACTIC ACID (LA) PRODUCTION BY *Lactiplantibacillus plantarum* (MZ483878) USING WHEY AS SOURCE OF NUTRIENT BY RESPONSE SURFACE METHODOLOGY

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(Received 17 September, 2021; accepted 10 November, 2021)

ABSTRACT

Shake flask experiment was conducted to study the combination of whey water (%), nutrient (%) along with temperature on the production of Lactic Acid using Response Surface Methodology (RSM) with the help of bacterium *Lactiplantibacillus plantarum* so-called *Lactobacillus plantarum* (NCBI - MZ483878) and the produced bioproduct was confirmed as lactic acid by Fourier Transform Infra Red Spectroscopy (FTIR). The optimized whey water (%), nutrient (%) and temperature conditions were 90, 14 and 31 °C respectively to get the maximum lactic acid (20.18 g l^{-1}) from *L. plantarum*. The maximum yield obtained was 20.18 g l^{-1} which was closer to the predicted yield of 20.16 g l^{-1}. Lactic Acid Bacteria isolated from fruit sample (*Malphigia punicifolia*) have greater potential to produce Lactic Acid, under optimized fermentation conditions which are used further for biodegradable plastic production (Poly Lactic Acid).

KEYWORDS : Lactic Acid, *Lactiplantibacillus*, Response surface methodology, Whey

INTRODUCTION

Lactic acid bacteria (LAB) were generally isolated from milk-based products, fruits and vegetables. In nature, surface of fruits and vegetables contain lactobacilli and many studies earlier reported *Lactobacillus* from fruits. Zehra *et al*. (2014) obtained 12 different isolates of *Lactobacillus* spp. from 25 different fruits and vegetables. Recently Frediansyah *et al*. (2019) isolated lactic acid bacteria from Jamaican cherry fruit. In the present investigation, an attempt was made to isolate LAB from Barbados cherry which is the utilization of Lactic acid production.

Acerola/Barbados cherry (*Malphigia punicifolia)* belongs to the family Malpighiaceae, originated from the Caribbean, Central America and Northern South America. It was introduced in tropical and subtropical areas like India.

Whey water contains about 5% (w/v) lactose. Several studies reported lactic acid production using whey (Kim *et al*, 2006). Considering the high content of lactose and proteins present in the wastewater from the milk processing industry, it serves as a better source for the production of LA through the fermentation process. Moreover recycling the whey water as lactic acid will reduce environmental pollution.

Lactic Acid (LA) production has received greater attention due to its application in various fields. Fermentation of LA by microbes plays an imperative role in the process of the final product. Rao *et al.* (2004) studied that the enhancement of LA production is mainly due to controlled factors such as media composition, temperature etc. Different types of components were used in the LA fermentation as a carbon source. One such "waste to worth" material is whey water from the "milk-

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based" industry. Therefore, this investigation was carried to utilize whey (wastewater) into economically important plastic component PLA production by lactic acid bacteria (LAB) isolated from Barbados cherry.

MATERIALS AND METHODS

Sampling and Isolation of LAB

Fully matured and healthy fruits from the small evergreen plant *Malphigia punicifolia* (Barbados Cherry) were collected in and around the campus of Tamil Nadu Agricultural University (TNAU), Coimbatore. Sampling was carried at the time of seasonal fruit production (April, 2020) and collected aseptically and stored in pre-sterilized zip-lock bags and analyzed within 24hr. To analyze LAB present in the fruit sample, 90ml of peptone water was added to 10g of fruits and homogenized. Appropriate dilutions were taken after homogenization and were plated onto MRS agar (De Man *et al*., 1960) and incubated at 30 ^{o}C for 24 to 72 hr. All the experiments were performed in the Department of Agricultural Microbiology, TNAU, Coimbatore.

Individual, identical colonies were picked and purified using streak plate method. Based on the microscopic observation and biochemical assay (acid production), Lactic acid-producing bacteria were screened. Effective LAB isolate was confirmed molecularly using thermo cycler (M/s, Eppendorf Master cycler, Germany). The PCR products were checked using 1.2% w/v agarose gel and it was sent to (M/s. Bioserve Biotechnologies (I) Pvt. Ltd., Hyderabad) for sequencing and identified as *Lactobacillus plantarum* (renamed as *Lactiplantibacillus plantarum*). The gene sequence of the isolate was submitted in gene bank (NCBI) and the accession number is (MZ483878). Glycerol stocks of the same were stored under -20 ^{o}C for future use.

Collection of Whey and Preparation of media

Whey was collected from a local sweetshop (NSR Bakery, Coimbatore, India) and filtered to remove the remnant of denatured protein. Varying concentrations of whey and MRS medium nutrients (peptone 10.0, meat extract 10.0, yeast extract 05.0, d-glucose 20.0, tween-80 01.0, K_2HPO_4 02.0, sodium acetate 05.0, tri-ammonium citrate 02.0, $MgSO_4.7H_2O$ 0.2, $MnSO_4.4H_2O$ 0.05 grams in 1000 ml water) were diluted with distilled water and was made up into 100 ml working volume in 500ml flask. All chemicals and reagents used for the experiments were analytically graded. One per cent of culture *Lactiplantibacillus plantarum* LAB 01 was used as a starter culture for this study.

Fourier Transform Infra Red spectroscopy (FTIR)

The culture filtrate (Lactic acid) sample was extracted from fermented broth and prepared for analysis as recommended by (Helm *et al*., 1991) with minor modifications. Standard LA was also subjected to FTIR analysis, along with the sample to detect the functional groups.

Optimization Study

To determine the optimum combination of whey water and nutrient content at a particular temperature for enhancing acid production, Response Surface Methodology (RSM) was followed. The idea of using RSM in laboratory experiments is to use a sequence of designed experiments to accomplish one or more optimal responses. The experimental design and statistical analyses were generated using Design-Expert Trial Version 13.0.2.0 (Stat-Ease Inc. Minneapolis, USA.) RSM was employed in a central composite design (CCD). The low and high level of variables investigated in the study is mentioned in Table 1. A set of 20 runs and the response from interactions between different factors was specified using a second-order polynomial regression model comprising linear, quadratic and interaction coefficients, as given in equation (1) as follows;

$$Y= \beta_o + \beta_1 + \beta_1 X_1 + \beta_2 X_2 + \ldots\ldots \ \beta_2 X_3 \qquad ..(1)$$

Table 1. High and low level of factors used in central composite design

Symbol	Factors	Low level (-1)	Mid Level	High Level (+1)
A	Whey water	50	65	80
B	Nutrient content	14	10	18
C	Temperature	31	27	35

Where, Y represents LA yield g/l, β_o is the constant term, $\beta_{1,}$ β_2 is the coefficient of linear terms. X_1, X_2, X_3 represents the factors for whey water, nutrient and temperature respectively. For the determination of statistical adequacy of the model test, Analysis of Variance (ANOVA) was performed. By using Fisher's F test and its associated probability, the overall model significance was validated. The quality of the polynomial model equation was judged statistically through the coefficient of determination (R^2) and adjusted R^2. The relationship between the response and experimental value of each independent variable was illustrated using "three-dimensional" surface plots.

RESULTS AND DISCUSSION

Lactic acid (2-hydroxypropanoic acid, CH_3–CH (OH)–COOH) is an organic acid produced by chemical and biological methods with enormous applications in food to household industries and chemical intermediates for a few compounds (Abdel Rahman *et al*., 2011). In microbial fermentation, the biggest challenge is high substrate cost. For the successful production of microbial products; substrate should be cheap and easily available. Whey water is one such cheap available substrate for LA production.

Lactiplantibacillus plantarum (Zheng, 2020) was isolated from the fruit sample and its sequence was submitted in gene bank (NCBI accession number - MZ483878). This experiment was conducted to explore the presence of LAB in fresh fruits and their ability to produce industrially important and interesting products (PLA- Polylactic acid). Tyler *et al.* (2016) reported that plant-associated LAB possess an universal trait of producing maximum amounts of LA and Acetic Acid (AA). Likewise, similar LA producing bacterial strains were isolated from wild flowers and fruits (Ruiz Rodríguez *et al.,* 2019), fresh fruits and vegetables (Trias *et al*., 2008), *Ziziphus mauritiana (*Nyanga *et al*., 2007) have been reported. Strains of *L. plantarum*, *L. fermentum*, *L. lactis*, *L. nageli* and *L. brevis* isolated from fruit industry waste (Barbados Cherry) also have been reported by Garcia *et al*. (2016).

Lactic acid was characterized by Fourier transform infrared spectroscopy (FTIR). Figure 1 depicts the presence of peaks at different wavelengths reveals the functional groups present in the sample. The presence of O- H stretching indicates the acid component (3000 - 3500 cm^{-1}); The presence of peak around the region of 3500 cm^{-1} is related to stretching group of OH is comparable with the results Huy *et al*., (2016) and the peak of C=O group at 1643 cm^{-1} and the result was coherent with the spectrum we obtained at 1600-1700 cm^{-1}. The absorption of the acid group in O-H bond takes place between wavelength at the ranges of 2500 to 3300 cm^{-1} and 3230 to 3550 cm^{-1}. Similar results were reported by (Tripathi *et al*., 2015). The bands

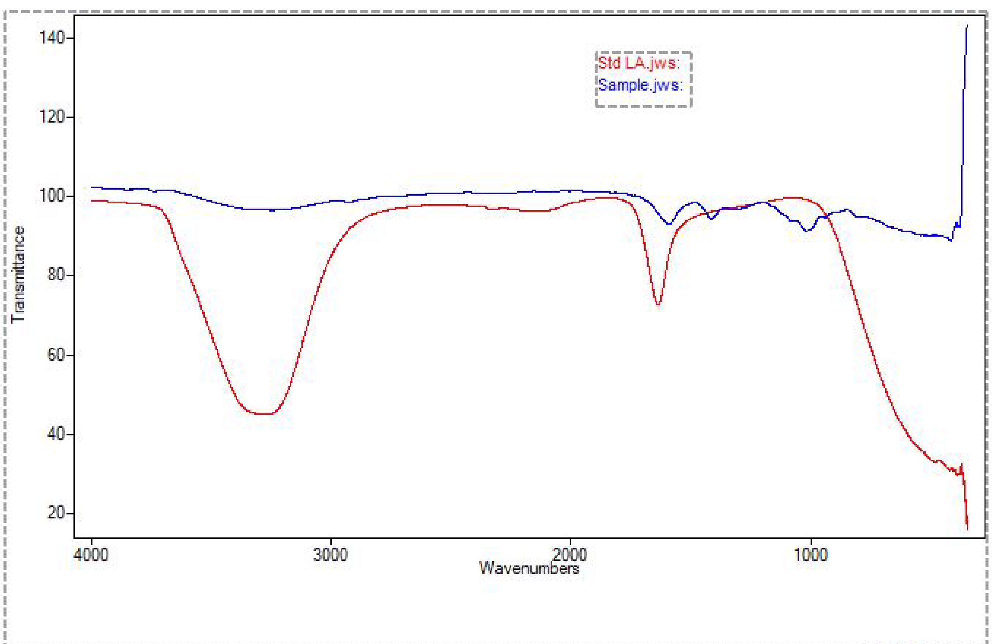

Fig. 1. FTIR spectrum of Lactic Acid produced by *Lactiplantibacillus plantarum*

depicted the stretching mode of C=O (1612 cm^{-1}) and C-O (1080.06 cm^{-1}) represent the structure of lactate. The absorption range at 1730 cm^{-1}indicated C=O which is the characteristic of carboxylic acid groups. Comparable results were obtained by Paucean *et al.* (2017).

As the first step of LA production, Central Composite Design (CCD) was used to optimize the media content and temperature for maximum LA production, a set of 20 experiments were conducted based on twenty different combinations obtained from the simulation and is listed in Table 2. LA production equation is given in equation (2) where factors A, B, C represents whey water, nutrient content and temperature respectively.

Lactic acid production= +10.33874 +0.110842 (A) +0.037269 (B)-0.022733 (C) ……………(2)

The medium with 100 per cent whey had insufficient nutrients for rapid bacterial growth and LA production. The commercial MRS medium for the growth of LAB and production of LA is still expensive. Based on this aspect, the combination of whey water and the MRS nutrient percent at optimum temperature was altered to achieve the best combination for LA production. Whey without supplementation was not an excellent medium for the growth of bacteria and production of lactic acid. Whey is considered as the low-cost substrate for LA fermentation and is effectively used with the combination of other nutrients and product conversion ratio was found high. (Pescuma *et al.*, 2015) in whey added medium. "Likewise" our results also showed higher yield with nutrient supplementation.

Panesar *et al.* (2010) stated that, whey medium supplemented with inorganic phosphate and nutrients resulted in LA production of 33.73 g l^{-1} by *L. caesi* under optimized process parameters was found advantageous for acid production. Our experimental results also more or less related with the yields of previous studies of Sweta *et al.* (2016) with 29.6 g l^{-1} of LA when whey was used as a substrate. Sowmiya and Ramalingam (2019) evaluated the potential of whey water for the production of bio-preservative by *L. plantarum* (NZ7100).

In fermentation process, temperature is considered one among the most influential factors which effects the LA production either positively or negatively. In some cases, the highest lactic acid concentration and yield were obtained at a lower temperature (25 ^{o}C). The optimal temperature for maximum LA production was varying among species. The optimal temperature of *L. casei* was 28°C which was reported by (Nabi *et al.*, 2004). Our experimental results were in close agreement with lactic acid production from glucose present in MRS medium by LAB (Sheeladevi and Ramanathan, 2011).

From the F-value, 44.29 and very low P level < 0.0001 (Table 2) was evident as a highly significant model. The Lack of Fit F-value of 2.47 indicates the Lack of Fit is not significant relative to the pure error. There is a 16.45% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good for the experimental data. The Predicted R^2 of 0.8142 is in logical conformity with the Adjusted $R^2$of 0.8724; i.e. the difference is less. RSM three dimensional graph indicated the maximum LA production (20.18 g/l) which was obtained in the combination of Whey per cent (90.2268), nutrient per cent (14) at the temperature of 31 ^{o}C.

Table 2. Analysis of variance (ANOVA) for response surface quadratic model for lactic acid

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	38.17	3	12.72	44.29	< 0.0001	Significant
A-Whey water	37.75	1	37.75	131.43	< 0.0001	
B-Nutrient content	0.3035	1	0.3035	1.06	0.3193	
C-Temperature	0.1129	1	0.1129	0.3931	0.5395	
Residual	4.60	16	0.2872			
Lack of Fit	3.88	11	0.3528	2.47	0.1645	not significant
Pure Error	0.7148	5	0.1430			
Cor Total	42.76	19				
R squared	0.8925					
Adj R- Squared	0.8724					
Pre R –Squared	0.8124					
Adeq Precision	7.94					

The similarity between predicted and actual analyses of combinations of variables on LA production is shown in Fig.2. The plot displays a close correlation between predicted and actual data as data points were accumulated closer to the line that bisects the plot at an angle of 45°. Based on Table 3 and Fig. 2, it can be deduced that the predicted values attained from the quadratic model have close proximity with the experimental values of Sanusi *et al*. (2021). This RSM tool comprised features for point prediction and interprets graphically through a 3D response surface curve that could be handy in predicting the optimum value of significant factor interaction for maximum LA production. Graphical representation of response surface and contour plots determine the interaction effect. Fig. 3 illustrates the 3D plot for optimum LA production with a combination of significant variables (A, B and C).

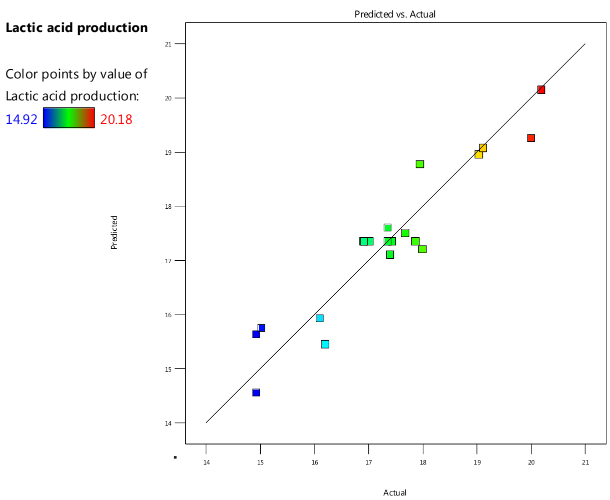

Fig. 2. Similarity plot between predicted and actual values for LA production by *Lactobacillus* strain.

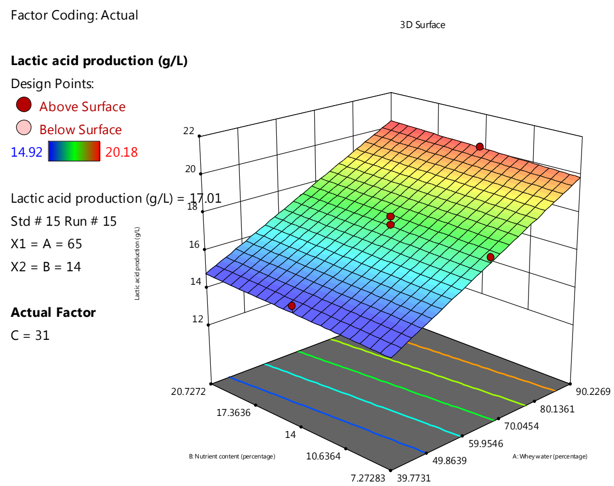

Fig. 3. 3D response surface and contour plots showing maximum LA production by *Lactobacillus*

CONCLUSION

Table 3. Twenty runs of CCD with experimental and predicted values

Run	Factor A Whey water %	Factor B Nutrient Content%	Factor C Temperature °C	Lactic Acid Production(g/L)	
				Experimental	Predicted
1	50	10	35	16.19	15.46
2	65	14	31	17.86	17.36
3	65	14	24.27	17.67	17.51
4	65	7.272	31	17.39	17.11
5	65	14	37.72	17.99	17.21
6	50	18	27	16.09	15.94
7	65	14	31	17.34	17.36
8	50	10	27	14.92	15.64
9	80	18	27	19.99	19.26
10	65	14	31	17.43	17.36
11	80	18	35	19.11	19.08
12	65	20.727	31	17.34	17.61
13	50	18	35	15.01	15.76
14	80	10	35	17.94	18.78
15	65	14	31	17.01	17.36
‘16	90.2268	14	31	20.18	20.16
17	39.773	14	31	14.92	14.56
18	65	14	31	16.89	17.36
19	80	10	27	19.03	18.96
20	65	14	31	16.91	17.36

This study gives a clear insight on the role played by the concentration of whey water and nutrient at optimum temperature (Fermentation conditions) required for maximum LA production, by the native organism *Lactiplantibacillus plantarum*. The presence of encouraging results in the study stated that the LAB strain isolated from cherry fruits could be exploited for industrial purposes. In future, efforts are taken to utilize the industrial whey water for maximum LA production which can be further polymerized to obtain PLA with less expenditure.

ACKNOWLEDGMENT

The authors are grateful to the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore for providing laboratory facilities to conduct this research work.

Conflict of interest: The authors declare that there is no conflict of interest.

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