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Production of L(+) Lactic Acid by *Lactobacillus amylophilus* GV6 in Semi-Solid State Fermentation Using Wheat Bran

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Summary

Lactobacillus amylophilus GV6 was used for direct fermentation of raw starch in wheat bran to L(+) lactic acid in semi-solid state fermentation. At *m*/*V* ratio of wheat bran 9 % (9 g of wheat bran in 100 mL of medium) with *m*/*V* ratio of $CaCO_3$ 0.375 %, 2.5 g of L(+) lactic acid was produced. To maximize the production of lactic acid, process variables like the volume of inoculum and incubation period were optimized keeping *m*/*V* ratio of wheat bran and $CaCO_3$ at 9 and 0.375 %, respectively, and constant pH=6.5, using response surface method (RSM). The organism produced 3.5 g of L(+) lactic acid from 3.96 g of starch present in 9 g of wheat bran. Maximum starch conversion to lactic acid was observed at process conditions of wheat bran *m*/*V* ratio 9 % at 37 °C, pH=6.5, inoculum volume 3.5 mL and incubation period of 130 h.

Key words: *L. amylophilus*, wheat bran, response surface method, lactic acid, amylolytic strain, direct fermentation

Introduction

Lactic acid is one of the oldest microbial metabolites known in fermented foods. It has wide applications in food, beverage, pharmaceutical and chemical industries, primarily as an acidulant, flavor enhancer and preservative (*1*,*2*). Lactic acid is classified as GRAS (generally recognized as safe) for use as a general-purpose food additive by FDA in US and other regulatory agencies (*3*). L(+) isomer, one of the racemic forms of lactic acid, is preferred in dairy and food products, due to the presence of L-lactate dehydrogenase in human beings, which can metabolize only L(+) lactic acid. D(–) lactic acid is detrimental to human metabolism causing acidurea and/or decalcification. Hence, only L(+) lactic acid is used in human consumables to avoid health problems. Major use of lactic acid (accounts to 85 % of demand) is still in food and food related applications (*3*). Lactic acid is added to margarine, butter, yoghurts *etc*. for its pleasant taste, and calcium lactate is added to milk and other sports and diet drinks as mineral supplement. It is used as pickling agent for sauerkraut, olives, and pickled vegetables, and as gelling agent for jams and jellies. Lactic acid and its salts can increase the shelf life of food products like sausages, hams, poultry, fish, *etc*. by 30 to 50 % by inhibiting the growth of food spoilage organisms (*1*). A large mass fraction of (>50 %) L(+) fermentation grade lactic acid is used to produce emulsifying agents such as sodium and calcium stearoyl lactates in bakery goods. Calcium salt is a good dough conditioner and sodium salt is both conditioner and emulsifier for yeast-leavened bakery products. Polylactide polymers are biocompatible, biodegradable and resorbable materials used in medical materials (*4*). It is reported that annual produc-

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tion capacity of lactic acid is 6000 t in India and there is an estimated gap of 2300 t in supply by the year 2015 (assuming that the present production is not increased) as given by Technology Information Forecasting and Assessment Council (TIFAC) in India (*5*). Globally lactic acid market is expected to grow by 8.6 % annually and in the USA alone, its demand is expected to be 49 600 Mt (*6*).

Depending on the specific microbial strain, desired stereo isomers, L(+), D(–) or a racemic mixture (DL) are produced (*2*,*7*). The production of lactic acid is focused on using whey (*8*) and synthetic medium containing refined sugars in submerged fermentation, which are expensive (*2*,*3*). Lactic acid is also produced from renewable cheaper substrates available in the form of agricultural wastes such as rice kernels, cassava fibrous residue, pearled barley, pearled wheat, wheat bran and rice bran (*9*). The use of such starchy raw materials involves a 2--step process of saccharification followed by *Lactobacillus* fermentation, which is expensive (*7*). Hence, the isolation and development of strains for single step production of stereo specific L(+) lactic acid from starchy substrates through fermentation can lead to significant reduction in the cost of operations. Keeping this as criteria, we have isolated and reported a strain of *Lactobacillus amylophilus* GV6, which can ferment starch directly to L(+) lactic acid in submerged fermentation (*10*).

In semi-solid state fermentations the insoluble solid substrate is a solid porous matrix, which absorbs water with a relatively high water activity and also contains available carbohydrates, nitrogen sources and mineral nutrients. The attraction towards this type of culturing comes from its similarity to the natural way of life for many microorganisms (*11*) and usage of starchy agricultural wastes makes the whole process more economical. As it would be attractive to use organisms growing well on raw starch, the present report describes the use of *Lactobacillus amylophilus* GV6, an amylolytic organism, for the production of L(+) lactic acid from starch using wheat bran as an insoluble solid substrate. Thus, mathematical modelling was proposed to determine the most suitable concentrations of certain selected parameters that would contribute to maximum lactic acid production. Its emphasis is on the optimization of fermentation conditions applying a statistical method by which large improvement in the production of lactic acid was observed. According to the available literature there are no reports on the production of L(+) lactic acid through semi-solid state fermentation.

Materials and Methods

Microorganism, its culture conditions and fermentation experiments

The *Lactobacillus amylophilus* GV6, an amylolytic lactic acid producing organism, was isolated from cornstarch processing industrial wastes using MRS medium. The organism was maintained in prereduced modified MRS medium and subcultured every 30 days. Inoculum of about 10^9 cells per mL was obtained by growing the culture anaerobically in modified MRS broth (pH=6.5) at 37 °C for 24 h containing 1 % of soluble starch (*10*).

Prior to this study, preliminary experiments were carried out to screen the best solid support and substrate using various inexpensive natural, unprocessed crude substrates. Out of all the solid substrates tested wheat bran was found to be the best solid support and substrate in solid state fermentation (*12*). Wheat bran was obtained from local mills and sieved through a mesh with a pore size of 0.5 mm and the coarse bran obtained was used as substrate. All the experiments were carried out in 120-mL serum vials containing 20 mL of medium. The medium with insoluble substrate (wheat bran) was sterilized by autoclaving at 121 °C for 15 min. Wheat bran m/V ratio ranging from 1–10 % (Table 1) was used as substrate in prereduced MRS broth containing (g/L): peptone 10, yeast extract 5.0, sodium acetate 5.0, triammonium citrate 2.0, $NaH_2PO_4 \cdot 2H_2O$ 2.0, $MgSO_4 \cdot 7H_2O$ 0.1, $MnSO_4 \cdot 4H_2O$ 0.05, resazurin 0.002, and 1 mL of Tween 80, pH=6.5 with nitrogen atmosphere, the volume of inoculum of 2 mL and incubation period of 5 days. In further experiments for improving the production of lactic acid, mathematical model was employed where

Table 1. Effect of substrate concentration on L(+) lactic acid production by *L. amylophilus* GV6 using insoluble substrate (wheat bran)

$\frac{m(\text{wheat bran})}{V(\text{substrate})}$ / %	$\frac{m(\text{starch})}{V(\text{substrate})}$ / %	w(moisture) / %	$\frac{m(\text{lactic acid})}{V(\text{substrate})}$ / %
1	0.44	99.00	0.3275
2	0.88	98.03	0.6100
3	1.32	97.08	0.7725
4	1.76	96.15	0.9205
5	2.20	95.23	1.0215
6	2.64	94.33	1.3201
7	3.08	93.45	1.6225
8	3.52	92.50	1.9010
9	3.96	91.70	2.0090
10	4.44	90.90	1.3380

Fermentation time = 5 days, inoculum volume = 2 mL, incubation temperature = 37 °C.
Experiment was conducted without the addition of buffering agent ($CaCO_3$).
The values given above are the average of 3 experiments, each in triplicate conducted on different occasions

vials with 20 mL of MRS broth containing m/V ratio of wheat bran 9 % and m/V ratio of $CaCO_3$ 0.375 % (buffer) were inoculated with actively growing culture (with different inoculum volume) and incubated at 37 °C for different time intervals (incubation period) as for experimental design (Table 2). Lactic acid produced was extracted and estimated.

Estimations

Lactic acid produced after the fermentation was extracted into the 20-mL medium, in which wheat bran was dispensed, by squeezing the fermented bran using cheesecloth. The extract was centrifuged (8000 rpm for 20 min) and the supernatant was taken to estimate lactic acid (*13*). Total starch content in bran before and after the fermentation was determined by standard acid hydrolysis method (*14*).

Experimental design and statistical analysis

The statistical analysis of the data was performed using the Minitab package. Details of response surface method can be found in Neter *et al.* (*15*). The levels of factors used in the experimental design are displayed in Table 2. These factors were chosen for statistical experimental design after a series of experiments conducted to standardize the percentage of wheat bran (solid substrate and support) and $CaCO_3$ (buffer), as shown in Tables 1 and 2. Insoluble substrate and $CaCO_3$ were kept constant while running the statistical experimental design. The experimental region extended from –2 to 2 in terms of the coded independent variables having experimental levels: –2, –1, 0, 1 and 2. The actual level of each variable was calculated by the following equation (*15*):

$$\text{coded value} = \frac{\text{actual value} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2} \qquad /1/$$

The coding facilitated the computation for regression analysis and optimum search. The experiment in the central point was replicated 5 times to provide sufficient degrees of freedom for estimating the purely experimental uncertainty variance. According to this design, 9 experiments were performed. The matrix for this design along with the experimental and predicted results is shown in Table 2. The most commonly used empirical model, polynomial response surface, was fitted to measure the response variable. When two factors are considered, the model fitted to response gives an equation of the term

$$Y = B_0 + \sum B_i X_i + \sum B_{ii} X_i^2 + \sum B_{ij} X_i X_j \qquad /2/$$

where B_0 is constant, B_i is a linear effect, B_{ii} is a squared term, B_{ij} is an interaction effect, and X_i is the i^{th} variable, called independent variable, and X_i and X_j are the algebraic representations of interaction effects between the independent variables.

Results

Effect of wheat bran m/V ratio

Total starch content present in the wheat bran was 44.4 %. An increase in the lactic acid production was observed up to m/V ratio of wheat bran 9 % (Table 1). Further increase in the wheat bran m/V ratio did not show any significant increase in the production of lactic acid. At m/V ratio of substrate of 10 % and above, the fermentation process was no more in semi-solid state but in solid-state fermentation. As the wheat bran content increased (from 1 to 10 %), as shown in Table 1, there was a decrease in moisture content, and moisture content of 91.7 % was found to be optimum. It was observed that the initial pH of the medium adjusted to 6.5 before the sterilization changed after the sterilization. At

Table 2. Central composite design for optimization of two process variables (each at 5 levels) for production of L(+) lactic acid by *L. amylophilus* GV6 using insoluble substrate (wheat bran)

Run	*V* (inoculum)	*t* (incubation)	*m*(lactic acid)/wheat bran 1.8 g	
	mL	h	experimental values	predicted values
1	4	72	0.1939	0.1472
2	6	72	0.2184	0.2184
3	4	120	0.4300	0.4336
4	6	120	0.2080	0.3036
5	3.6	96	0.1825	0.2218
6	6.4	96	0.3020	0.2123
7	5	62	0.2300	0.2417
8	5	130	0.5320	0.4729
9	5	96	0.1920	0.2037
10	5	96	0.2160	0.2037
11	5	96	0.2044	0.2037
12	5	96	0.1940	0.2037
13	5	96	0.2120	0.2037

m/V ratio of insoluble substrate (wheat bran)=9 % and $CaCO_3$=3.06 %, pH=6.5, t=37 °C.
Note: higher and lower inoculum volume and incubation period are 6 and 4 mL, 72 and 120 h, respectively.
The values given above are the average of 3 experiments, each in triplicate conducted on different occasions

m/V ratio of wheat bran of 10 %, the observed change in pH was higher than in substrates containing 1–9 % of wheat bran.

Optimization of lactic acid production

Encouraging results were observed in lactic acid production during preliminary experimentations in solid-state fermentation when the moisture content was increased (*12*). This led to the investigations of semi-solid fermentations. Wheat bran *m/V* ratio of 1–9 % (insoluble substrate) gave semi-solid fermentation, as shown in Table 1. $CaCO_3$ was found to be suitable buffer (*16*). Preliminary experiments were carried out for the optimization of the buffer concentration ($CaCO_3$) and 0.375 % of $CaCO_3$ was observed to be the optimum (data not shown). Substrate *m/V* ratio of 9 % and initial pH=6.5 were found to give maximum mass ratio (lactic acid, starch in wheat bran 2.5/3.96) with 0.375 % of $CaCO_3$ concentration (data not shown). Therefore, these parameters were kept constant, further optimizations were done for the inoculum volume and incubation period (at 5 levels) and their interactions on lactic acid production were determined. It is seen that the highest observed output is 0.5320 g of lactic acid at run 8 (Table 2). The student's t-distribution and the corresponding values, along with the estimated parameters, are given in Table 3. By applying the multiple regression analysis on the experimental data, a second order polynomial model could explain the role of each variable and their second order interaction in the production of lactic acid. The coefficient of determination (R^2) for the production of lactic acid is 0.801. The value of R^2 is a measure of total observed variations of the mean values explained by the fitted model. The coefficient of correlation (R) for the production of lactic acid is 0.900 (Table 3). As the maximum of R-value can be 1.000, this observed R-value of 0.900 shows a good agreement between experimental observations and predicted values. The observed variation in the production of lactic acid can be explained by the fitted model equation /2/. The P-values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of P-value the more significant the correlation with the corresponding coefficient is. The results show that among the independent variables, incubation period had a significant effect. According to the analysis of variance of the regression, linear effects and output are quite significant for the production of lactic acid (Table 4).

The graphic representation of the response surface, shown in Fig. 1, helps us visualize the effect of inoculum volume and incubation period on the production of lactic acid. The overlaid contour plot and surface plot of yield indicated that there are regions where the output could be more than 0.53 g (Fig. 1). Hence, the response optimization was done with Minitab 13, which indicated possible output to be 0.612 g. Therefore, the parameters given by response optimization (inoculum volume of 3.5 mL, incubation period of 130 h) were used for the confirmation of the predicted value of 0.612 g of lactic acid output. Maximum lactic acid production of 0.630 g was obtained under optimized conditions.

Table 3. Regression coefficients and t-values for production of L(+) lactic acid by *L. amylophilus* GV6 using insoluble substrate (wheat bran)

Term	Coefficients	Standard error of coefficients	t-value	Probability
Constant	0.20368	0.02788	7.306	0.000
Inoculum	–0.00339	0.02204	–0.154	0.882
Incubation	0.08160	0.02204	3.702	0.008
Inoculum × inoculum	0.00683	0.02364	0.289	0.781
Incubation × incubation	0.07646	0.02364	3.235	0.014
Inoculum × incubation	–0.06163	0.03117	–1.977	0.089

S = 0.06234, R – Sq = 80.1 %, R – Sq (adj) = 65.8 %, R = 0.900

Table 4. Analysis of variance for production of L(+) lactic acid by *L. amylophilus* GV6 using insoluble substrate (wheat bran)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.109288	0.109288	0.021858	5.62	0.021
Linear	2	0.053359	0.053359	0.026679	6.86	0.022
Square	2	0.040738	0.040738	0.020369	5.24	0.041
Interaction	1	0.015191	0.015191	0.015191	3.91	0.089
Residual error	7	0.027204	0.027204	0.003886		
Lack of fit	3	0.026753	0.026753	0.008918	78.98	0.001
Pure error	4	0.000452	0.000452	0.000113		
Total	12	0.136492				

DF – degrees of freedom, Seq SS – sum of squares, Adj SS – adjusted sum of squares, Adj MS – adjusted mean sum of squares, F – variance ratio, P – probability

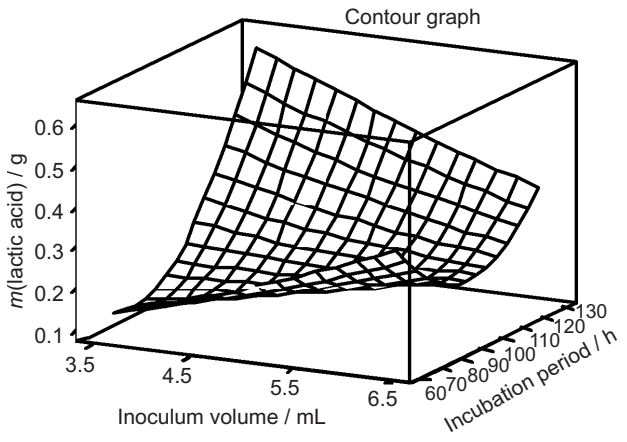

Fig. 1. Response surface showing the effect of inoculum volume (mL) and incubation period (h) on L(+) lactic acid production by *L. amylophilus* GV6 using insoluble substrate (wheat bran)

Discussion

The available amylolytic bacterial wild strains reported so far have 90 % yield efficiency (*17,18*) for direct conversion of starch to lactic acid compared to the wild strain *Lactobacillus amylophilus* GV6, which has about 98 % yield efficiency (*i.e.* mass (lactic acid produced)/mass (starch utilized)). In the present study *Lactobacillus amylophilus* GV6 has shown greater efficiency in the utilization of crude starch present in the substrate (wheat bran) compared to the wild bacterial amylolytic strains. Lactic acid production was observed to increase as the concentration of substrate increased from *m/V* ratio 1–9 %. There was a decrease in the utilization of starch beyond *m/V* ratio of substrate of 9 %, which may be due to the increase in the osmotic effects or the organism is incapable of hydrolyzing the starch present in *m/V* ratio of wheat bran at 10 % or above. At *m/V* ratio of wheat bran of 10 %, the change (decrease) in pH after sterilization was higher, which did not favour the organism's growth. This may be due to the presence of different minerals in wheat bran (*19*) or to the hydrolysis of starch to reducing sugars (*20*) or to decreased water activity as the process slightly shifts to solid-state. Generally, bacteria grow at higher water activity values. Buffering agent $CaCO_3$ has significant effect on the production of lactic acid (*16,21*). The *m/V* ratio of $CaCO_3$ of 0.375 was found to be optimum. There was a decrease in the production of lactic acid or utilization of starch above *m/V* ratio of $CaCO_3$ 0.375 %, which may be due to the inhibition of enzyme activities that are responsible for the biosynthesis of lactic acid. High concentrations of $CaCO_3$ inhibit the growth of microorganisms (*21*). Experiments without $CaCO_3$ did not show maximum lactic acid production. $CaCO_3$ helped to neutralize a small amount of lactic acid produced by the organism during the development of its biomass and starch hydrolysis for the first 2 days by maintaining the pH at 6.5. Later the organism actively utilized the hydrolyzed starch to produce lactic acid, which caused the pH to drop down to 3.5 at the end of fermentation.

There are various reports on the usage of wheat bran hydrolysate and extracts of wheat bran as nitrogen source and refined sugars as carbon source for the production of lactic acid (*21*). The present study involves the use of wheat bran as carbon source (crude starch), which is directly fermented to L(+) lactic acid by *Lactobacillus amylophilus* GV6. RSM was used, which is an efficient experimental strategic tool for optimizing operating conditions and media ingredients in a number of bioprocesses (*21*). The results obtained in the present work further proved its usage in the optimization. The results showed that, in spite of the inoculum volume of 3.5 mL, the production of lactic acid was very much influenced by fermentation time (130 h). This was due to higher starch content bound to wheat bran and not available freely as in submerged fermentation with soluble substrates.

Almost all the sugars produced from starch are utilized for the production of L(+) lactic acid. The organism produced 35 g of L(+) lactic acid from 44.4 g of starch present in 100 g of fermented wheat bran. *Lactobacillus amylophilus* GV6 showed higher amylolytic efficiency than previously reported strains of *Lactobacillus amylophilus*. A detailed study was made to find out the lactic acid yield (*i.e.* mass (lactic acid produced)/mass (starch utilized)). The organism gave 98 % lactic acid yield. Lactic acid production yield (*i.e.* mass (lactic acid produced)/mass (starch in wheat bran)) is 78.8 %. The expensive pure starch could be replaced with wheat bran, cheaper underutilized abundantly available byproduct. The replacement of pure starch with wheat bran will reduce the cost of substrate several folds and will make the whole process more economical in terms of substrate costs. Hence *L. amylophilus* GV6 has a considerable potential in direct fermentation of crude starch (wheat bran) to L(+) lactic acid. Such strains can prove to be beneficial in reaching the demand for lactic acid due to its widest applications in food industries.

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Proizvodnja L(+) mliječne kiseline s pomoću *Lactobacillus amylophilus* GV6 fermentacijom na polučvrstoj podlozi od pšeničnih posija

Sažetak

Bakterija *Lactobacillus amylophilus* upotrijebljena je za izravnu fermentaciju sirovoga škroba na polučvrstoj podlozi od pšeničnih posija za proizvodnju L(+) mliječne kiseline. Korištenjem 9 % posija (9 g pšeničnih posija u 100 mL podloge) uz 0,375 % $CaCO_3$, dobiveno je 2,5 g L(+) mliječne kiseline. Za maksimalnu proizvodnju mliječne kiseline, metodom odzivnih površina (response surface method – RSM), optimirani su volumen inokuluma i vrijeme inkubacije, a pritom su udjel posija (9 %), $CaCO_3$ (0,375 %) i pH=6,5 održavani konstantnima. *L. amylophilus* GV6 proizveo je 3,5 g L(+) mliječne kiseline iz 3,96 g prisutnoga škroba u 9 g pšeničnih posija. Od 9 % pšeničnih posija pri 37 °C i pH=6,5, te u volumenu inokuluma od 3,5 mL i vremenu inkubacije od 130 h postignuta je maksimalna pretvorba škroba u mliječnu kiselinu.