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Lactic acid production from cassava fibrous residue using *Lactobacillus plantarum* MTCC 1407

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Abstract: *During extraction of starch from cassava, fibrous residue is a major waste released into the environment. Owing to the high starch content (60- 65% on dry weight basis) and organic matter of cassava fibrous residue (CFR), an attempt has been made to utilize it for the production of lactic acid (LA) in semi- solid state fermentation using Mann Rogassa Sharpe medium containing [5 % (wv^{-1})] CFR in lieu of glucose [2 % (wv^{-1})] as the carbon source. Response Surface Methodology (RSM) was used to evaluate the effect of main variables, i.e. incubation period, temperature and pH on LA production. The experimental results showed that the optimum incubation period, temperature and pH were 120 hr, 35^{0}C and 6.5, respectively. Maximum starch conversion by Lactobacillus plantarum MTCC 1407 to LA was 63.3%. The organism produced 29.86 g of (L+) LA from 60 g of starch present in 100 g of CFR. The LA production yield (i.e. mass LA produced mass starch present in CFR^{-1} x 100) was 49.76 %.*

Key words**:** *Cassava fibrous residue, Lactobacillus plantarum MTCC 1407, Lactic acid, Response surface methodology, Semi- solid fermentation*
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Introduction

Cassava (*Manihot esculenta* Crantz, Family: *Euphorbiaceae*), is a starchy crop having 20-30 % extractable starch depending on the varieties and climatic conditions. In India, more than 1500 cottage and small scale industries crush over 5000 tonnes of cassava per day during harvest season (Edison *et al*., 2006). In extraction of starch from cassava the major waste product is cassava fibrous residue (CFR) that constitutes about 15- 20% by weight of the cassava chips/tubers processed. CFR is stocked near factory sites causing pollution of the environment (Ray, 2004). CFR contains about 55- 65% starch and organic matter (10- 15%) on dry weight basis (Sriroth *et al*., 2000; Jyothi *et al*., 2005) by virtue of which it can serve as an excellent substrate for production of various bioproducts like microbial enzymes and organic acids in fermentation processes (Pandey *et al*., 2001).

Lactic acid (LA) 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, flavour enhancer and preservative nature (Ray *et al*., 2006; Wee *et al*., 2006). LA is classified as GRAS (Generally Recognized as Safe) for use as a general purpose food additive by Food and Drug Administration (FDA) in US. It can be obtained on an industrial scale either by microbial fermentations or by chemical synthesis. It can be obtained in its pure form by choosing a strain of lactic acid bacteria (LAB), whereas chemical synthesis results in a racemic mixture of LA (Ryu *et al*., 2003; Vishnu *et al*., 2006).

In India annual production of LA is 6000 tonnes, but globally LA market is expected to grow by 8.6% annually (Pandey *et al*., 2001). One of the main obstructions in the large scale production of LA is the cost or raw material. Application of cheap agricultural substrates and agri- waste residues in bio- process provides an alternative way to solve many environmental hazards (Oh *et al*., 2005; Wee *et al*., 2006). Therefore, LA is commercially produced from renewable cheaper substrates available in form of agricultural wastes such as rice kernels, corn cobs, alfalfa fibre, potato waste, wheat bran, cassava bagasse and sugarcane bagasse (Sreenath *et al*., 2001; Naveena *et al*., 2005). Hence the development of strains for single step production of stereo-specific L (+) LA from starchy substrates through fermentation can lead to significant reduction in cost of operations (Linko and Javanainen, 1996).

Response Surface Methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions of factors for desirable responses (Liew *et al*., 2005). Statistical optimization not only allows quick screening of large experimental domain, but also reflects the role of each of the components. RSM has already been successfully applied for optimization of the media and culture conditions in many cultivation processes for the production of primary and secondary metabolites (Sivaramakrishan *et al*., 2006; Swain and Ray, 2007).

Lactobacillus plantarum MTCC 1407, a LAB that produces only L(+) LA was previously used in our laboratory for production of fermented foods (lacto- pickle and lacto- juice) (Panda *et al*., 2007, 2009). This strain also possesses α- amylase activity and molecular characterization of the enzyme has been studied (Panda *et al*., 2008).

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In the present study, the bioconversion of residual starch in CFR to LA has been studied employing this amylolytic LAB strain (*Lb. plantarum* MTCC 1407) in semi- solid fermentation. Further, RSM has been applied to optimize the three most important fermentation parameters (incubation period, temperature and pH) for LA production.

Materials and Methods

***Lactobacillus plantarum* MTCC 1407 culture:** *Lb. plantarum* MTCC 1407 culture was procured from the Institute of Microbial Technology, Chandigarh, India. The bacterial culture was maintained on Mann Rogassa Sharpe (MRS) (Sharpe and Elisabeth Pyer, 1996) agar slants at 4°C.

Cassava fibrous residue: CFR collected during starch extraction from cassava was de-watered and sun dried for 6-8 days to prevent microbial deterioration. The dried CFR having composition : [(g 100 g^{-1} dry weight); moisture, 11.2; starch, 60.0; total sugar, 3.0; crude fibre, 10.8; crude protein, 0.88 and total ash, 1.2] (Ray *et al*., 2008) was stored in an air- tight container until required.

The inoculum was prepared in 250 ml Erlenmeyer flasks containing 100 ml of MRS liquid medium [(gl^{-1}): peptone, 10.0; beef-extract, 10.0; yeast extract, 5.0; glucose, 20.0; Na_2HPO_4, 2.0; sodium acetate, 5.0; triammonium citrate, 2.0; $MgSO_4$, 0.2; $MnSO_4$, 0.2; $CaCO_3$, 4.0; Tween 80, 0.1ml and pH adjusted to 6.8] by transferring a loop full of organism (*Lb. plantarum*) from a stock culture and incubated at 35 ^{0}C and 120 rpm for 48 hr in an orbital incubator- cum -shaker (Remi, India, Pvt. Ltd., Bombay, India). The inoculum contained 1×10^7 c.f.u. ml^{-1}.

Modified MRS semi-solid medium containing [5% (wv^{-1})] CFR in lieu of glucose [2% (wv^{-1})] as the carbon source was used for LA production. Erlenmeyer flasks (250 ml) containing 100 ml of sterile (autoclaved at 120°C for 15 min) MRS medium containing CFR [5 % (wv^{-1})] was divided into two sets and both the sets were inoculated with 2% (vv^{-1}) freshly prepared inoculum. One set of flasks (n=3) was kept in still condition and the other set (n=3) was agitated at 120 rpm in an incubator - cum - shaker. Both the sets were kept at temperature of 35°C for 120 hr. After the incubation period, culture broth from individual flasks was taken out and centrifuged at 8000 g in a refrigerated centrifuge (Model C -24, Remi Pvt. Ltd., Bombay, India) for 20 min. The supernatant was used for estimation of LA.

The effect of different concentrations of CFR on LA production by *Lb. plantarum* was investigated by varying the concentrations of CFR in the MRS medium from 1-11% (wv^{-1}) and the samples (n=3) were incubated for 120 hr at 35°C under still conditions. At the end of incubation period, all cell free supernatants were used for LA estimation.

Effect of $CaCO_3$ concentration, inoculum volume and surfactant on LA production: The optimum concentration of $CaCO_3$ for LA production was evaluated by varying the concentration of $CaCO_3$ in MRS semi-solid medium from 0.1-0.5 % (wv^{-1}). Different surfactants (0.1%) like Tween 40, 60, 80 sodium dodecyl sulphate (SDS) and TritonX100 were used to standardize the fermentation conditions for maximum LA production. Five levels of inoculum size [*i.e.* 1- 5% (vv^{-1})] were optimized for LA production with 5% (wv^{-1}) CFR in MRS medium and all the samples were incubated at 35°C for 120 hr under still conditions. At the end of 120 hr incubation period, cell free supernatants were used for LA estimation.

Optimization of incubation period, temperature and pH by applying RSM: RSM was used to optimize the above factors with reference to LA production. The experimental design was a central composite experimental plan (CCD) with three factors: incubation period, temperature and pH of the medium at five levels of $-\alpha$, -1, 0, +1, $+\alpha$. All variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used and the full experimental plan was listed in coded form.

Statistical analysis and modeling: The data obtained from RSM of LA production was subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second order polynomial equation (1) as it represents the behaviour of such a system more appropriately.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1 \beta_1 A^2 + \beta_2 \beta_2 \beta^2 + \beta_3 \beta_3 C^2 + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC \quad (1)$$

Where Y is response variable, β_0 is intercept, β_1, β_2 and β_3 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$ and $\beta_{2,3}$ are interaction coefficient and A,B,C, A^2, B^2, C^2, AB, AC and BC are level of independent variables. The statistical significance was determined by 'F' test and the multiple coefficient of determination R squared (R^2) value. Design expert (Ver, 7.1, STATEASE INC; Minneapolis, MN, USA) was used in this investigation.

The effects of pH, temperature and incubation period on LA production by *Lb. plantarum* in MRS medium consisting of 5% (wv^{-1}) CFR were investigated at various pH (4.5-8.5), temperature (15-55°C) and incubation period (72-168 hr). The pH measurements were carried out with a Systronics– make pH meter (Model 351, Pvt. Ltd., Ahmadabad, India) using glass electrode. The pHs of 4.5-6.0 were maintained with acetate buffer (0.2 M) while pHs 6.5-8.5 were achieved with phosphate buffer (0.1 M).

LA estimation: LA content was estimated by the method given by Amerine and Ough (1984) using a UV- VIS spectrophotometer (Cecil Instruments, UK) and expressed as g LA 100 g^{-1}CFR.

Results and Discussion

Effect of shake and still flask cultures on LA production: Using shake and still flask cultures, it was ascertained that LA production by *Lb. plantarum* was higher in case of still flasks (29.86 g LA100g^{-1}CFR) in comparison to shake flask cultures

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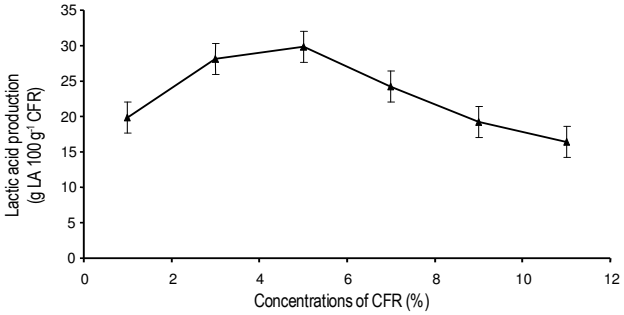

Fig. 1: Lactic acid production by *Lb. plantarum* MTCC 1407 from different concentration of CFR

(20.11 g LA100 g^{-1} CFR) at the end of 120 hr incubation period (Table 1). Although shaking microbial culture increases the rate of substrate utilization and consequently, the rate of production of bio-molecules, there are exceptions. For example, Ray *et al.* (1990) reported rhodanese production was higher in still flask culture than shake flask culture. Hence, further experiments were conducted only with still flask cultures.

Effect of different concentrations of CFR on LA production: LA production was found to increase as the concentration of CFR in MRS medium was increased from 1 to 5 % (wv^{-1}); beyond that there was a gradual decline (Fig. 1). At 11% (wv^{-1}) concentration it was the lowest. This might be due to increasing viscosity of the culture medium beyond 5% (wv^{-1}) CFR level, which led to decreased water activity as the process might have shifted from semi-solid to solid state. Generally bacteria grow at higher water activity (Naveena *et al*., 2004). Naveena *et al*. (2005) reported a similar decrease in LA production with 10% (wv^{-1}) wheat bran using a strain of *Lacto bacillus amylophilus* GV6 as an inoculant. Similarly, MRS medium containing more than 3% sweet potato flour resulted in decreased LA production by *Lb. plantarum* (Panda and Ray, 2008). The reason explained that higher wheat bran or sweet potato flour percentage apparently decreased the utilization of starch beyond 9% (wv^{-1}) substrate, which might be due to increase in osmotic effects or due to hydrolysis of starch to reducing sugars or the organism was incapable of hydrolyzing the starch present in wheat bran at 10% (wv^{-1}) or above.

Effect of $CaCO_3$ concentration, inoculum volume and surfactant on LA production: LA production was found to be optimum at 0.4% (wv^{-1}) concentration of $CaCO_3$ (Fig. 2a). Further it was observed that there was 32.6 % decrease in LA production at 0.5 % (wv^{-1}) $CaCO_3$ concentration. Buffering agent like $CaCO_3$ has significant effect on LA production (Naveena *et al*., 2004). The mass $volume^{-1}$ (mv^{-1}) ratio of $CaCO_3$ of 0.4% was found to be optimum for LA production beyond that there was a decline, which might be due to decrease in enzyme activity or increase in pH of the medium that ultimately inhibited the growth of microorganism responsible for biosynthesis of LA (Fu and Mathews, 1999).

Out of five levels of inoculum volume, 2% (vv^{-1}) was found to be best for LA production and inoculum levels higher than 2% had adverse effect (Fig. 2b). In case of semi-solid fermentation, the inoculum level varies according to the initial sugar or starch content used in the fermentation (John *et al*., 2007a,b). Because, growth of the microorganisms in SSF depends on the substrate: moisture ratio in correlation with environmental factors like pH and temperature. John *et al.* (2006) reported a similar inoculum volume level in LA production from agro-wastes using *Lb. delbrueckii* as an inoculant.

Table - 1: Experimental design and result of CCD of response surface methodology

A: Incubation period (hr)	B: Temperature (°C)	C: pH (H+)	LA production (g LA 100 g^{-1} CFR)	
			Predicted	Experimental
-1	-1	-1	18.38	18.48
1	-1	-1	20.62	21.89
-1	1	-1	17.87	17.91
1	1	-1	20.12	21.33
-1	-1	1	18.77	18.36
1	-1	1	21.04	21.78
-1	1	1	18.25	17.8
1	1	1	20.54	21.21
$-\alpha$	0	0	21.75	17.89
$+\alpha$	0	0	19.28	18.99
0	$-\alpha$	0	22.14	19.99
0	$+\alpha$	0	19.98	19.26
0	0	$-\alpha$	20.26	19.05
0	0	$+\alpha$	21.94	20.99
0	0	0	28.02	29.85
0	0	0	28.02	28.62
0	0	0	28.02	26.98
0	0	0	28.02	28.22
0	0	0	28.02	25.45
0	0	0	28.02	26.32

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Table - 2: ANOVA for α- amylase production in submerged fermentation

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	3.42	9	0.38	19.70	0.0001
Pure error	0.062	5	0.01		
Total	3.62	19			

R^2 = 0.9466, Adjusted R^2 = 0.8985, Predicted R^2 = 0.7003, Adequate Precision = 11.763, Lack of Fit F- value = 7.59

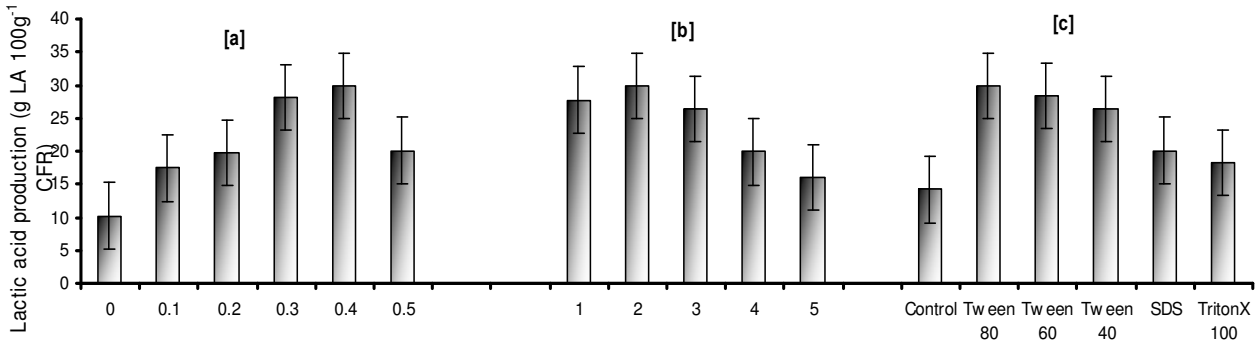

Fig. 2: Effect of different concentrations of $CaCO_3$ (a), inoculum volume (b) and surfactants (c) on lactic acid production

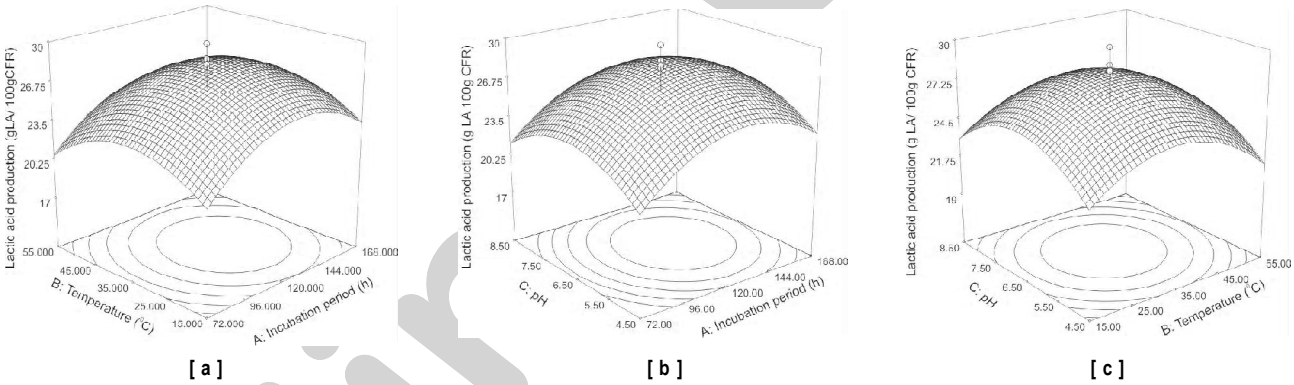

Fig. 3: Statistical optimization of lactic acid production using RSM, (a) temperature and incubation period; (b) pH and incubation period and (c) pH and temperature

Figure 2c shows the production of LA with different surfactants. In this study, tween 80 [0.1 % (vv^{-1})] was found to have maximum additive effect on LA production in comparison to other surfactants (Tween 40, 60, Triton X 100 and SDS). Chen and Yanagida (2006) reported that in broth containing surfactants such as Tween 20 and 80, *Lactobacillus animalis* exhibited high growth and production of bacteriocins-like inhibitory substance but imparted low production in absence of these surfactants.

Optimization of incubation period, temperature and pH by applying RSM: Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second order equation was checked by an F-test (ANOVA) and the data are shown in Table 2. The regression model for LA production was highly significant ($P<0.01$) with a satisfactory value of determination co-efficient (R^2 = 0.9466) indicating that 94.66 % variability in the response could be explained by second order polynomial equation.

$Y = 5.29 + 0.13 \times A - 0.03 \times B + 0.02 \times C - 0.33 \times A^2 - 0.28 \times B^2 - 0.27 \times C^2 + 1.37 \times AB + 2.77 \times AC - 3.45 \times BC$.

Where Y is LA production, A is incubation period (h), B is temperature (°C) and C is pH (H^+). The R^2 value is always between 0 and 1. The closer the R^2 value to 1.0, the stronger the model and better it predicts the response (Swain and Ray, 2007; Panda and Ray, 2008b). An adequate precision of 11.763 for LA production was recorded. The predicted R^2 of 0.7003 is in reasonable agreement with the adjusted R^2 of 0.8985. Hence, the ANOVA

result showed that this model is appropriate and a good agreement between the experimental and predicted value for LA production was observed. RSM used in this investigation suggested the importance of various fermentation parameters at different levels. The methodology employed has successfully been used in the optimization of factors for LA production (Naveena *et al*., 2005; John *et al*., 2006). In this study, an incubation period (120 hr), temperature (35°C) and pH (6.5) were the major factors that influenced the enzyme titre.

The model F- value of 19.70 and values of Prob > F (< 0.05) indicated that model terms are significant. For LA production, A, A^2, B^2 and C^2 are significant model terms. The "lack of fit F-value" of 7.59 implied that "lack of fit" is significant.

The optimum conditions have been selected using surface graphs, contour plots, steepest ascent techniques and canonical analysis (Sogi *et al*., 2003). However, in the current study surface graphs and contour plots were employed by plotting the effect of independent variables (incubation period, temperature and pH) versus the response (LA production). Out of three variables, one was fixed at zero level while the other two were varied. Fig. 3a depicts three dimensional diagram and a contour plot of calculated response surface from the interaction between incubation period and temperature while keeping the other variable (pH) at '0' level. The result demonstrated that the maximum LA production was observed when incubation period and temperature were increased upto 120 hr and 35 ^{0}C, respectively and thereafter, it declined. The response between incubation period and pH (keeping the temperature at '0' level) indicated that a pH of 6.5 was optimum with 120 hr incubation period for LA production (Fig. 3b). An interaction between the remaining two parameters (temperature and pH) suggested a little difference with the earlier response (Fig. 3c). Thus the optimum level of incubation period (120 hr), pH (6.5) and temperature (35 ^{0}C) were chosen to achieve the maximum yield of LA (29.86 g LA100 g^{-1}CFR). Cultural conditions have been found to have a profound influence on LA production. The decrease in LA production beyond and above these optimum conditions [incubation period (120 hr), temperature (35 ^{0}C) and pH (6.5)] might be due to inhibition of growth and enzyme activities of *Lb. plantarum* MTCC 1407 that were responsible for the biosynthesis of LA. The results obtained in the present work further proved its usage in the optimization.

Validation of model: Validation was carried out in still flasks under conditions predicted by the model. The experimental values (29.86 g LA100 g^{-1} CFR) were found to be very close to the predicted values (28.02 g LA100 g^{-1} CFR) hence, the model was successfully validated.

CFR, a low cost agri-residue can provide an economic advantage as carbon source for production of LA by *Lb. plantarum*. The organism produced 29.86 g of LA from 60 g of starch present in 100 g of CFR showing 63.3% conversion after 120 h of incubation. The organism delivered 49.76% LA yield (*i.e.* mass LA produced mass starch in CFR^{-1} x 100). Similar results of LA yield (22 - 42 g 100 g^{-1}) on starchy substrates were reported for *Lactobacillus casei* (John *et al*., 2007b), *Lb. amylophilus* GV6 (Naveena *et al*., 2004) and *Enterococcus faecalis* (Oh *et al*., 2005). Further studies are being carried out in our laboratory on genomic characterization of this strain for LA production.

Practical applications: LA has wide applications in food, beverage, bioplastic, pharmaceuticals and chemical industries, and microbial fermentation of refined substrates like starch and sugar is the major route for its production. Utilization of starchy agro-wastes like CFR for LA production by amylolytic LAB (*Lb. plantarum*) has dual advantages: cheaper production cost as compared with refined substrate and an appropriate technology for value addition of agro-wastes and environmental waste management.

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