Journal of Scientific & Industrial Research
Vol. 67, July 2008, pp. 531-537

Direct conversion of raw starch to lactic acid by *Lactobacillus plantarum* MTCC 1407 in semi- solid fermentation using sweet potato (*Ipomoea batatas* L.) flour

S H Panda[1] and R C Ray[1]*

[1]Central Tuber Crops Research Institute (Regional Centre), Dumduma PO, Bhubaneswar 751 019

Received 03 September 2007; revised 08 May 2008

Lactobacillus plantarum MTCC 1407 was used for direct fermentation of raw starch in sweet potato (*Ipomoea batatas* L.) flour (SPF) to lactic acid (LA) under semi- solid fermentation using Mann Rogassa Sharpe medium containing (3%) SPF in lieu of glucose (2%) as carbon source. Response Surface Methodology (RSM) was used to evaluate effect of incubation period, temperature and pH on LA production, using a full factorial Central Composite Design (CCD). At optimum parameters (incubation period, 120 h; temperature, 35°C and pH, 6.5), maximum starch conversion by *Lb. plantarum* MTCC 1407 to LA was 56.4%. The organism produced (L+) LA (23.86 g) from starch (55 g) present in SPF (100 g); LA production yield (mass LA produced mass starch present in SPF^{-1} x 100) was 43.4%.

Keywords: *Lb. plantarum* MTCC 1407, Raw starch, Lactic acid, Response surface methodology, Semi- solid fermentation, Sweet potato flour

Introduction

Lactic acid (LA) is used as food additive in food and non-food industries[1-3]. It can be obtained on industrial scale either by microbial fermentations or by chemical synthesis[2,4]. In recent years, fermentation approach has become more successful because of increasing market demand for naturally produced LA[5,6] with an estimated worldwide demand[2] of 130, 000-150, 000 t/y. Utilization of cheap agricultural residues in bioprocess provides an alternative way to replace costly raw materials (glucose, lactose, etc) and bulk use of such materials will solve environmental hazards[2,4,7]. Chemical synthesis only produces racemic mixture of L (+) and D (–) enantiomers but fermentation has added advantage of producing biologically active L (+) or D (–) or DL form of LA[3,8]. Use of agricultural starchy wastes involves a two-step process of saccharification followed by *Lactobacillus* fermentation, which is often expensive[9]. Amylase- producing *Lactobacillus* spp. (*Lb. amylophilus* and *Lb. amylovorus*) are often used for direct fermentation of starchy materials into LA, which can lead to significant reduction in operating cost[2,10].

*Author for correspondence
E-mail: rc_ray@rediffmail.com

Response Surface Methodology (RSM) has been successfully applied for optimization of media and culture conditions in many cultivation processes for production of amino acids, ethanol and enzymes[11,12]. Several LA- based fermented food products have been produced from sweet potato (SP) (*Ipomoea batatas* L.) such as lacto- pickle[13], lacto- juice[14] and curd[15, 16] using *Lb. plantarum* MTCC 1407 as the starter culture. *Lb. plantarum* was chosen as starter culture because it is homofermentative [produces only (L+) LA but not ethyl alcohol], and is a probiotic that confers various health beneficial effects to consumers[17], and is frequently encountered in human gastrointestinal tracts[18]. Further, assuming that strain *Lb. plantarum* MTCC 1407 might be possessing significant amylase activity by virtue of which it could convert efficiently SP starch into glucose and finally to LA in these food and beverage products, different process parameters were optimized for amylase production.

SP is world's seventh most important food crop, of which over 80% is grown in Asian countries like China and India[19]. Tubers are consumed fresh or utilized commercially for extraction of starch and flour[20]. Sweet potato flour (SPF), a cheap source of carbohydrate and other nutrients, can be used as a fermentation substrate for production of ethanol, organic acids, etc[21].

Present study investigates direct conversion of SPF to LA by *Lb. plantarum* MTCC 1407 in semi- solid fermentation and optimization of important fermentation parameters (incubation period, temperature and pH) by applying RSM.

Materials and Methods

Culture

Lb. plantarum MTCC 1407 was procured from Institute of Microbial Technology, Chandigarh, India and maintained on Mann Rogassa Sharpe (MRS) [22] agar slants at 4°C.

Effect of Shake and Still Flask Cultures on LA Production

Inoculum was prepared in 250 ml Erlenmeyer flasks containing 100 ml of modified MRS liquid medium (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; and $CaCO_3$, 4.0 g l^{-1}; Tween 80, 0.1 ml and pH 6.8) by transferring a loop full of organism (*Lb. plantarum*) from a stock culture and incubated at 35°C and 120 rpm for 48 h in an orbital incubator- cum -shaker (Remi, India, Pvt. Ltd., Bombay, India). Inoculum contained 1×10^7 CFU ml^{-1}.

For LA production, modified MRS semi-solid medium containing SPF (3%) in lieu of glucose (2%) as carbon source was used. SPF (starch, 55; total sugar, 12.5; crude fibre, 10.5, moisture, 11.5; total nitrogen, 2.3 and ash, 3.1 % dry wt basis) was obtained by crushing dried SP (var. ST- 10) chips in a laboratory mill (Pelican Equipments, Chennai, India) and was stored in an air- tight container. Erlenmeyer flasks (250 ml) containing 100 ml of sterile (autoclaved at 120°C for 15 min) MRS semi-solid medium with SPF (3%) was divided into two sets and both sets were inoculated with 2% 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 sets were kept at 35°C for 120 h. After incubation, 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 clear cell free supernatants were used for LA estimation.

Effect of Different Concentrations of SPF on LA Production

Effect of different concentrations of SPF on LA production by *Lb. plantarum* was investigated by varying SPF concentrations (1 - 11%) in MRS semi-solid medium and samples (n=3) were incubated for 120 h at 35°C under still conditions. After incubation, cell free supernatants were used for LA estimation.

Effect of $CaCO_3$ Concentration, Inoculum volume and Surfactant on LA Production

Optimum concentration of $CaCO_3$ for LA production was evaluated by varying $CaCO_3$ concentration [0.1 - 0.5 % (w/v)] in MRS semi-solid medium. Different surfactants (0.1 %) like Tween 40, 60, 80 and sodium dodecyl sulphate (SDS) were used to standardize fermentation conditions for maximum LA production. Five levels of inoculum size [1-5% (v/v)] were optimized for LA production with 3% SPF in MRS semi-solid medium and all samples were incubated at 35°C for 120 h under still conditions. After 120 h of incubation period, cell free supernatants were used for LA estimation.

Optimization of Incubation period, Temperature and pH by Applying RSM

A central composite design (CCD) was used to pick factors that influence LA production significantly. Experimental design, which consisted of three variables at five levels, required 20 formula combinations. All variables were taken at a central coded value considered as zero. Minimum and maximum ranges of variables were used and full experimental plan was listed in coded form. The results quoted were mean of LA production and were taken as independent variables or response (Table 1).

Statistical Analysis and Modeling

The data obtained from RSM of LA production was subjected to analysis of variance (ANOVA) for significant sequential models in developing regression equation. The results obtained from RSM were used to fit a second order polynomial equation as

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

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. Statistical significant was determined by 'F' test and multiple coefficient of determination R squared (R^2) value. Design expert

Table 1— Range of values for RSM

Independent variables	Levels				
	-α	-1	0	+1	+α
Incubation period, h	39.27	72	120	168	200.73
Temperature, °C	1.36	15	35	55	68.64
pH	4.8	5.5	6.5	7.5	8.9

RSM- response surface methodology

(Ver, 7.1, STATEASE INC; Minneapolis, MN, USA) was used in this investigation.

Effects of pH (5.5-7.5), temperature (15-55°C) and incubation period (72-168 h) on LA production by *Lb. plantarum* in MRS semi-solid medium consisting of 3% SPF were investigated. pH was measured with a Systronics– make pH meter (Model 351, Pvt. Ltd, Ahmadabad, India) using glass electrode. pH (5.5-6.0) was maintained with acetate buffer (0.2 M) and pH (6.5-7.5) with phosphate buffer (0.1 M).

Biochemical Analysis

Starch, total sugar, crude fibre, moisture, total nitrogen, ash and LA contents were estimated[23]. Starch, total sugar and LA were estimated by using a UV – VIS spectrophotometer (Cecil Instruments, UK) and expressed as g LA $100g^{-1}$ SPF. Moisture was determined by vacuum oven, total nitrogen and ash were determined by the Kjeldahl and muffle furnace methods, respectively. Crude fibre was determined by enzymatic–gravimetric method using fibre tech instrument (Pelican Equipments, Chennai, India).

Results and Discussion

Effect of Shake and Still Flask Cultures on LA Production

Using shake and still flask cultures, LA production by *Lb. plantarum* was higher in case of still flask (23.84 g LA $100g^{-1}$ SPF) in comparison to shake flask cultures (19.65 g LA $100g^{-1}$ SPF) at the end of 120 h incubation period. So, further experiments were conducted only with still flask cultures.

Effect of Different Concentrations of SPF on LA Production

LA production increased from 19.8g $100g^{-1}$ SPF to 23.98g $100g^{-1}$ SPF as concentration of SPF in MRS semi-solid medium was increased from 1 to 3%, respectively; after which, there was gradual decrease (4.8-17.8%) in LA production. LA production at 11% SPF was lowest (10.0g $100g^{-1}$ SPF). This might be due to increasing viscosity of culture medium beyond 3% SPF 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[5,24]. Naveena *et al*[4] reported a similar decrease in LA production with 10% wheat bran using a strain of *Lb amylophilus* GV6 as an inoculant.

Effect of $CaCO_3$ Concentration, Inoculum volume and Surfactant on LA Production

LA production was highest (23.85 g $100g^{-1}$ SPF) at 0.4% concentration of $CaCO_3$. Further, there was 13.5% decrease in LA production at 0.5% $CaCO_3$ concentration. A decrease in the production of LA or utilization of starch above m/v (mass per volume) ratio of 0.4% $CaCO_3$, might be due to inhibition of enzyme activity and ultimately growth of microorganism responsible for biosynthesis of LA[25].

Out of five levels of inoculum volume, 2% was found to be best for LA production (23.86 g $100g^{-1}$ SPF) and inoculum levels higher than 2% had adverse effect (11.1-13.9 % decrease on LA production). In case of semi-solid fermentation, inoculum level varies according to the initial sugar or starch content used in liquid mediumt:[4]. In general practice, a low level sugar medium needs 2-3% inoculum volume and high sugar level needs 5-10% inoculum volume[10].

Among surfactants tested, *Lb. plantarum* produced highest amount of LA (23.87 g $100g^{-1}$ SPF) with 0.1% Tween 80 in comparison to other surfactants (Tween 40, 60 and SDS). Chen & Yanagida[26] reported that broth containing surfactants such as Tween 20 and 80, *Lb animalis* exhibited high growth and production of bacteriocins-like inhibitory substance but imparted low production without surfactants.

Table 2 — Experimental design and result of CCD of RSM

Std	A: Incubation Period, h	B: Temperature °C	C: pH	LA production (g LA 100 g^{-1} SPF) Predicted	Experimental
1	-1	-1	-1	14.45	15.54
2	-1	-1	-1	17.31	17.66
3	-1	-1	-1	17.61	17.71
4	-1	-1	-1	20.41	19.83
5	-1	-1	1	15.22	16.10
6	-1	-1	1	18.11	18.30
7	-1	1	1	18.45	18.40
8	1	1	1	21.49	20.50
9	-α	0	0	20.87	12.80
10	+α	0	0	21.24	19.30
11	0	-α	0	22.42	12.30
12	0	+α	0	20.98	19.90
13	0	0	-α	19.89	20.72
14	0	0	+α	21.75	22.55
15	0	0	0	22.57	23.85
16	0	0	0	22.57	22.40
17	0	0	0	22.57	22.14
18	0	0	0	22.57	21.83
19	0	0	0	22.57	22.45
20	0	0	0	22.57	22.84

LA, lactic acid; RSM- response surface methodology; CCD, central composite design; SPF, sweet potato flour

Table 3 — ANOVA for LA production in submerged fermentation with 3% SPF

Source	Sum of squares	Degree of freedom	Mean square	p-value
Model	2.70	9	0.30	0.0001
Pure error	0.027	5	0.005	
Total	2.87	19		
R^2	=	0.9425		
Adjusted R^2	=	0.8908		
Predicted R^2	=	0.6218		
Adequate Precision	=	11.786		
Lack of Fit F- value	= 5.07			

LA- lactic acid; SPF- sweet potato flour; ANOVA- analysis of variance

Optimization of Incubation period, Temperature and pH by Applying RSM

Effect of three independent variables (incubation period, temperature and pH) for LA production by *Lb. plantarum* MTCC 1407 with 3% SPF are presented along with predicted and observed responses (Table 2). Regression model for LA production was highly significant ($P<0.01$) with a satisfactory value of determination co-efficient ($R^2 = 0.9425$) indicating that 94.25% of the variability in the response could be explained by second order polynomial equation (Table 3).

$$Y = 4.75+0.17 \times A +0.19 \times B + 0.046 \times C - 0.26 \times A^2 - 0.26 \times B^2 - 0.022 \times C^2 - 5.34 \times AB -1.90 \times AC + 1.24 \times BC.$$

where Y is enzyme production, A is incubation period (h), B is temperature (°C) and C is pH. An adequate precision of 11.786 for LA production was recorded. Predicted R^2 (0.6218) is in agreement with adjusted R^2 (0.8908). Hence, ANOVA result showed that this

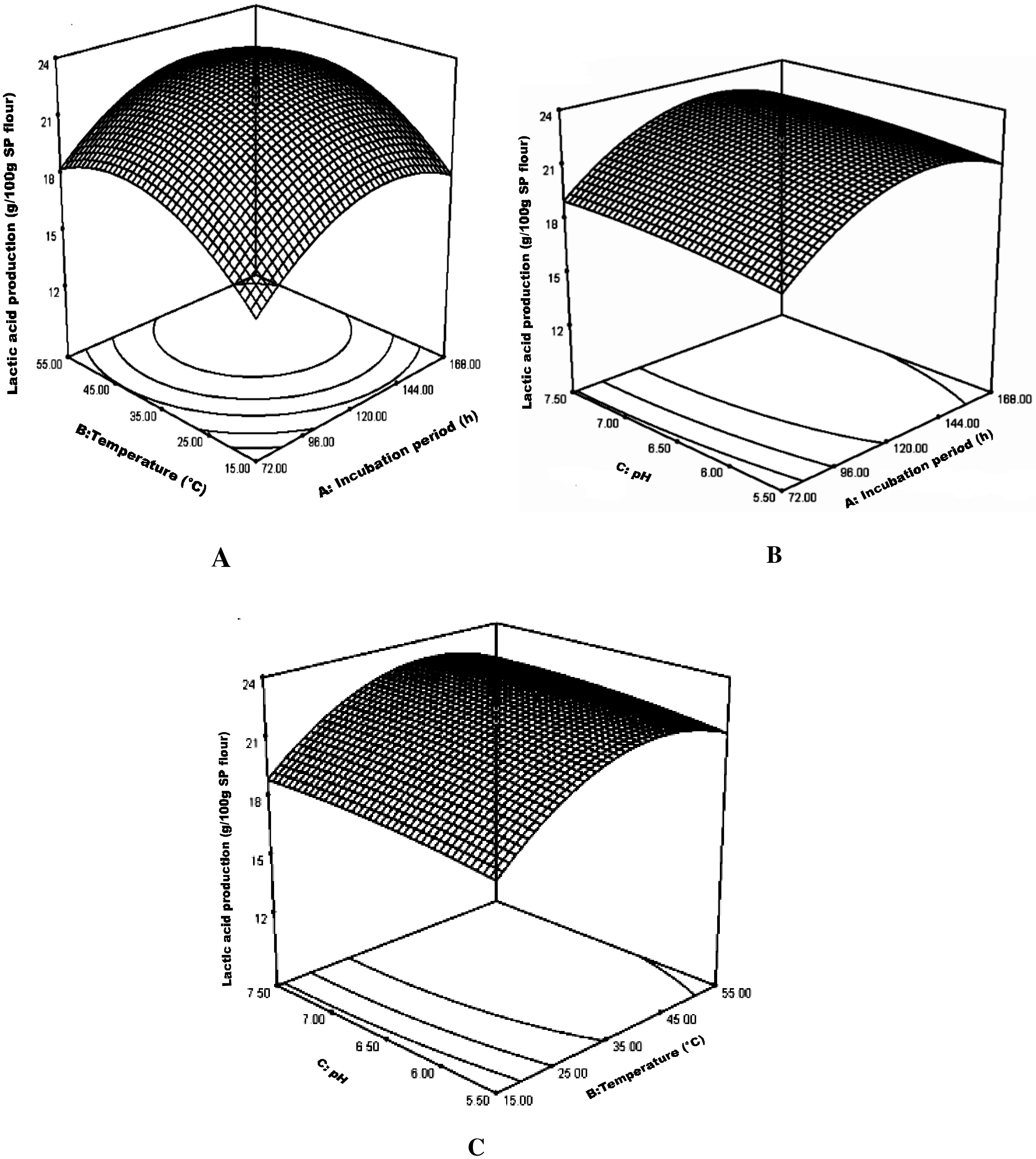

Fig. 1— Statistical optimization of enzyme production using RSM: A) temperature and incubation period; B) pH and incubation period; and C) pH and temperature

model is appropriate and a good agreement between experimental and predicted value for LA production was observed.

The model F- value of 18.21 and values of Prob > F (< 0.05) indicated that model terms are significant. For LA production, A, B, A^2 and B^2 are significant model terms. "Lack of fit F-Value" at 5.07 implied that "lack of fit" is significant.

Maximum LA production (23.8 g LA 100 g^{-1} SPF) was observed when incubation period and temperature were increased upto 120 h and 35°C, respectively and thereafter, it declined (Fig. 1A). The response between incubation period and pH (keeping temperature at 0 level) indicated that a pH of 6.5 was optimum with 120 h incubation period for LA production (Fig. 1B). An interaction between temperature and pH suggested a little difference with the earlier response (Fig. 1C). Thus, optimum level of incubation period (120 h), pH (6.5) and temperature (35°C) were chosen to achieve maximum yield of LA (23.86 g LA 100 g^{-1} SPF). The decrease in LA production beyond and above optimum conditions might be due to inhibition of growth and enzyme activities of *Lb. plantarum* MTCC 1407 that were responsible for biosynthesis of LA. The results in present work further proved its use in the optimization.

Validation of Model

Validation was carried out in still flasks under conditions predicted by the model. A high similarity was observed between predicted and experimental results, which reflected accuracy and applicability of RSM to optimize the process for LA production in semi-solid fermentation. Predicted response for LA production was 22.57 g LA 100g^{-1} SPF, while actual (experimental) response was 23.86 g LA 100g^{-1} SPF, thus proving the validity.

Lb. plantarum MTCC 1407 is amylolytic and it could convert directly raw starch present in SPF to LA in a single step fermentation process. The organism produced 23.86 g of LA (43.4% yield) from 55 g of starch present in 100 g of SPF showing 56 % conversion after 120 h of incubation. A yield of 41 g LA l^{-1} in mineral liquid medium was reported by Fu & Mathews[25] using a strain of *Lb. plantarum* ATCC 21028. In general, yield of LA (22-65g l^{-1}) were reported for *Lactobacillus* spp. using *Lb. bulgaricus*[27], *Lb. pentosus*[28], *Lb. amylophilus* GV6[10], *Lb. amylovorus* ATCC 33622[3] and *Lb. helveticus* ATCC 15059[29]. In conclusion, expensive pure starch or glucose could be replaced with SPF which is cheaper, underutilized and abundantly available in countries like China and India[19] and will reduce substrate cost of several folds to make whole LA production process more economical. Though LA yield (43%) using SPF may be lower than that obtained from pure glucose or lactose (70-80%)[25,30] but considering the very low cost of substrate (US$ 0.1-0.15/kg SPF), the process appears to be economically sound.

Conclusions

SPF, a low cost and easily available material, could provide an economic advantage as a solid substrate as well as carbon source for production of LA by *Lb. plantarum*. Experimental results showed optimum incubation period, temperature and pH at 120 h, 35°C and 6.5, respectively. Maximum starch conversion by *Lb. plantarum* MTCC 1407 to LA was 56.4%. Organism produced 23.86 g of (L+) LA from 55g of starch present in 100 g of SPF.

Acknowledgement

Authors thank Director, Central Tuber Crops Research Institute, Thiruvanathapuram, for facilities.

References

1 Calderon - Santoyo M, Loiseau G & Guyot J P, Fermentation by *Lactobacillus fermentum* of carbohydrates occurring naturally in cereals: consequences on growth energetics and amylase production, *Int J Food Microbiol*, **80** (2003) 161-169.

2 Wee Y J, Kim J N & Ryu H W, Biotechnological production of lactic acid and its recent applications, *Food Technol Biotechnol*, **44** (2) (2006) 163-172.

3 John R P, Nampoothiri M K & Pandey A, Solid state fermentation for L (+) lactic acid production from agro - wastes using *Lactobacillus delbrueckii*, *Process Biochem*, **41** (2006) 759-763.

4 John R P, Nampoothiri M K & Pandey A, Production of lactic acid from biomass: an overview on process developments and future perspectives, *Appl Microbiol Biotechnol*, **74** (3) (2007) 524-534.

5 Naveena B J, Altaf Md, Bhadrayya K, Madhavendra S S & Reddy G, Production of L (+) lactic acid by *Lactobacillus amylophilus* GV6 in semi-solid state fermentation using wheat bran, *Food Technol Biotechnol*, **42** (2004) 147-152.

6 Naveena B J, Altaf Md, Bhadrayya K, Madhavendra S S & Reddy G, Direct fermentation of starch to L (+) lactic acid in SSF by *Lactobacillus amylophilus* GV6 using wheat bran as support and substrate medium optimization using RSM, *Process Biochem*, **40** (2005) 681-690.

7 Oh H, Wee Y J, Yun J S, Han S H, Jung S & Ryu H W, Lactic acid production from agricultural resources as cheap raw materials, *Biores Technol*, **96** (2005) 1492-1498.

8 John R P, Nampoothiri K M, Nair A S & Pandey A, L (+) lactic acid production using *Lactobacillus casei* in solid state fermentation, *Biotechnol Lett*, **27** (2005) 1685-1688.

9 Linko Y Y & Javanainen P, Simultaneous liquefaction, saccharification and lactic acid fermentation on barley starch, *Enz Microb Technol*, **19** (1996) 118-123.

10 Vishnu C, Seenayya G & Reddy G, Direct fermentation of various pure and crude starchy substrates to L(+)-lactic acid using *Lactobacillus amylophilus* GV6, *World J Microbiol Biotechnol*, **18** (2002) 429-433.

11 Liew S L, Ariff A B, Raha A R & Ho Y W, Optimization of medium composition for the production of a probiotic microorganism, *Lactobacillus rhamnosus* using response surface methodology, *Int J Food Microbiol*, **102** (2005) 137-142.

12 Boyaci I H, A new approach of determination of enzyme kinetic constants using response surface methodology, *J Biochem Eng*, **25** (2005) 55-62.

13 Panda S H, Parmanick M & Ray R C, Lactic acid fermentation of sweet potato (*Ipomoea batatas* L.) into pickles, *J Food Process Preserv*, **31** (2007) 83-101.

14 Panda S H & Ray R C, Lactic acid fermentation of ²- carotene rich sweet potato (*Ipomoea batatas* L.) into lacto- juice, *Plant Food Hum Nutr*, **62** (2007) 65-70.

15 Panda S H, Naskar S K & Ray R C, Production, proximate and nutritional evaluation of sweet potato curd, *J Food Agric Environ*, **4** (2005) 124-127.

16 Mohapatra S, Panda S H, Sahoo S K & Ray R C, Beta- carotene-rich sweet potato curd: production, nutritional and proximate composition, *Int J Food Sci Technol*, **42** (2007) 1305-1314.

17 Montet D, Loiseau G & Zakhia-Rozis N, Microbial technology of fermented vegetables, in *Microbial Biotechnology in Horticulture*, **vol I**, edited by R C Ray & O P Ward (Science Publishers, Inc., Enfield, New Hampshire, USA) 2006, 309- 343.

18 Kleerebezem M, Boekhorst J, Kranenburg R, Molenaar D, Kuipers O P, Leer R, Tarchini R, Peters S A, Sandbrink H M, Fiers M W E J, Stiekema W, Lankhorst R M K, Bron P A, Hoffer S M, Groot M N N, Kerkhoven R, Vries M, Ursing B, deVos W M &, Siezen R J, Complete genome sequence of *Lactobacillus plantarum* WCFS1, *Proc Natl Acad Sci*, **100** (2003) 1990-1995.

19 Ray R C & Ravi V, Post harvest spoilage of sweet potato in tropics and control measures, *Crit Rev Food Sci Nutr*, **45** (2005) 623-644.

20 Van Hal M, Quality of sweet potato flour during processing and storage, *Food Rev Int*, **16** (2000) 1-37.

21 Ray R C & Ward O P, Post harvest microbial biotechnology of tropical root and tuber crops, in *Microbial Biotechnology in Horticulture*, **vol I**, edited by R C Ray & O P Ward (Science Publishers Inc., Enfield, New Hampshire, USA) 2006, 345-395.

22 Sharpe M & Elisabeth Pyer T F, Identification of the lactic acid bacteria, in *Identification Method for Microbiologists*, **part A**, edited by B M Gibbs & F A Skinner (Academic Press, London and New York) 1996, 65-69.

23 Amerine M A, Ough C S, *Methods for Analysis of Musts and Wines*, (Wiley – Inter Science Publication, John Wiley and Sons, New York) 1984, 447.

24 Giraud E, Gosselin B, Harin B, Parida L & Raimbault M, Purification and characterization of an extracellular amylase activity from *Lactobacillus plantarum* strain A6, *J Appl Bacteriol*, **75** (1993) 276-82.

25 Fu W & Mathews A P, Lactic acid production from lactose by *Lactobacillus plantarum*: kinetic model and effects of pH, substrate and oxygen, *J Biochem Eng*, **3** (1999) 163-170.

26 Chen Y S & Yanagida F, Characteristics and effects of temperature and surfactants on bacteriocin-like inhibitory substance production from soil isolated *Lactobacillus anomalis* C060203, *Curr Microbiol*, **53** (2006) 384-387.

27 Burgos-Rubio C N, Okos M R & Wankat P C, Kinetic study of the conversion of different substrates to lactic acid using *Lactobacillus bulgaricus*, *Biotechnol Progr*, **16** (2000) 305-314.

28 Bustos G, Moldes A B, Cruz J M & Domínguez J M, Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by *Lactobacillus pentosus*, *J Food Sci Agric*, **84** (2004) 2105-2112.

29 Schepers A W, Thibault J & Lacroix C, *Lactobacillus helveticus* growth and lactic acid production during pH–controlled batch cultures in whey permeate/yeast extract medium. II: kinetic modeling and model validation. *Enz Microb Technol*, **30** (2002) 187-194.

30 Berry A R, Franco C M M, Zhang W & Middelberg A P J, Growth and lactic acid production in batch culture of *Lactobacillus rhamnosus* in a defined medium, *Biotechnol Lett*, **21** (1999) 163-167.