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Statistical Optimization of α-Amylase Production by Probiotic *Lactobacillus plantarum* MTCC 1407 in Submerged Fermentation

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Abstract

Production and purification of α-amylase by probiotic *Lactobacillus plantarum* MTCC 1407 has been investigated under submerged fermentation using Mann Rogassa Sharpe medium containing (1%) soluble starch in lieu of glucose (2%) as carbon source. Response Surface Methodology was used to evaluate the effect of main variables, *i.e.* incubation period, pH and temperature on enzyme production. A full factorial Central Composite Design was applied to study these main factors that affected α-amylase production. The experimental results showed that the optimum incubation period, pH and temperature were 36 h, 7.0 and 35°C, respectively. The purified enzyme (by ammonium sulphate precipitation) had a molecular mass of 75 450 Da in SDS-PAGE.

Key words: α-amylase, optimization, response surface methodology, submerged fermentation

Introduction

Lactic acid bacteria (LAB) are commonly considered as powerful probiotics that exert many beneficial effects in gastrointestinal (GI) tract and enhance health (Nowroozi *et al*., 2004). The probiotic *Lactobacillus* spp. include *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, *L. gasseri* and *L. rhamnosus*. Lactobacilli have been periodically associated with anticarcinogenic, antimutagenic and antitumourigenic activities; therefore, the consumption of LAB fermented foods may elicit the above effects (Ray and Panda, 2007). Further, probiotic lactobacilli are of considerable significance in fermentation-based industries for production of variety of fermented foods ranging from fermented vegetables such as cabbage (*sauerkraut* and *kimchi*) and cucumbers, curd and yoghurt, lacto-pickles, kefir and fermented milk (Steinkraus, 2002) to the production of lactic acid (LA) as food additive (Vishnu *et al*., 2006). Some species of lactobacilli such as *L. acidophilus* and *L. plantarum* are commonly used as "starter cultures" in vegetable and fruit fermentation (Panda *et al*., 2007).

Some LAB possess amylase activity (Vishnu *et al*., 2006). *Lactobacillus* amylase exhibits significant role in GI tract of chicken and mammals like pig, rabbit, horse and human beings including infants (Nowroozi *et al*., 2004). They degrade the starch present in food to LA and fermentable monosaccharides that can be easily assimilated in the body, thus improving utilization of dietary starch and enhancing digestion. LA produced in GI tract lowers the pH of the environment, thus inhibiting the growth of pathogenic bacteria like *Salmonella*, *Staphylococcus* and *Escherichia coli* (Rincker *et al*., 2000). Amylolytic LAB are now implicated in preparing high energy density cereal-based foods for improving utilization of dietary starch in infants and children (Nguyen *et al*., 2007). Collington *et al*. (1990) reported inclusion of a probiotic (a mixture of multiple strains of *Lactobacillus* spp. and *Streptococcus faecium*) resulting in significantly higher carbohydrate enzyme activities in small intestine of piglets. Similar results were also found in case of rats, broiler chickens and rabbits (Rincker *et al*., 2000). Due to low pancreatic α-amylase content in young pigs, *Lactobacillus* 123 was evaluated as a source of amylase for weanling in pigs to improve digestibility of starch in small intestine (Rincker *et al*., 2000).

Response Surface Methodology (RSM) can be employed as a tool to overcome a number of process optimization problems. In addition, this technique can be

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used to determine optimal conditions of a particular procedure (He *et al*., 2004). 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 the culture conditions in cultivation processes for the production of primary and secondary metabolites, *i.e.* amino acid, ethanol and enzymes (He *et al*., 2004).

In our previous studies in developing lactic-fermented food products from sweet potato, *i.e.* curd (Mohapatra *et al*., 2007), lacto-pickles (Panda *et al*., 2007) and lacto-juice (Panda and Ray, 2007), *L. plantarum* MTCC 1407 was used as the starter culture. Since, sweet potato is a starchy crop, it was assumed that this strain may be possessing sufficient amount of amylase activity by virtue of which it could convert the sweet potato starch into glucose and finally to LA. To confirm this hypothesis, the present study was carried out to investigate the α-amylase production by *L. plantarum* MTCC 1407 in submerged fermentation and optimization of the fermentation parameters (incubation period, temperature and pH) by applying RSM.

Experimental

Materials and Methods

***Lactobacillus plantarum* MTCC 1407 culture.** *L. plantarum* MTCC 1407 culture was previously used in our laboratory for fermentation studies (Mohapatra *et al*., 2007). The bacterial culture was maintained on Mann Rogassa Sharpe (MRS) (Sharpe and Elisabeth Pyer, 1996) agar slants at 4°C.

Optimization of incubation period, temperature and pH by applying RSM. The characterization of different factors for α-amylase production was optimized by applying RSM. The statistical model was obtained using Central Composite Design (CCD) with three independent variables [incubation period (A), temperature (B) and pH (C)]. Each factor in this design was studied at five different levels (Table I). A set of 20 experiments was performed. All variables were taken at a central coded value considered as zero. Upon completion of the experiments, the average of α-amylase production was taken as the dependent variable or response.

Table I
Range of the values for the response surface methodology

Independent variables	Levels				
	–α	–1	0	+1	+α
Incubation period (h)	–4.363	12	36	60	76.363
Temperature (°C)	1.364	15	35	55	68.636
pH (H+)	3.636	5	7	9	10.364

Statistical analysis. The data obtained from RSM on α-amylase 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.

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

Where Y is response variable, β_0 is intercept, $\beta_1\beta_2$ and β_3 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are squared coefficient, $\beta_{1,2}$, $\beta_{1,3}$ and $\beta_{2,3}$, are interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC and BC are level of independent variables. Statistical significance of the model equation was determined by Fisher's test value, and the production of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) value. Design expert (Ver, 7.0; STATEASE INC; Minneapolis, MN, USA) was used in this investigation.

Effect of incubation period. The inoculum was prepared in 250 ml conical flasks containing 100 ml of MRS liquid medium (g/l: 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; distilled H_2O, 1l; pH adjusted to 6.8) by transferring a loop full of organism (*L. plantarum*) from stock culture and incubated at 35°C and 120 rpm for 48 h in an orbital incubator shaker (Remi, India, Pvt. Ltd., Bombay, India). The inoculum contained 1×10^7 CFU/ml.

The amylase production was carried in MRS liquid medium containing soluble starch (1%) in lieu of glucose (2%) as the carbon source. Fifty ml of sterile MRS liquid medium taken in 250 ml Erlenmeyer flasks were inoculated with 2% inoculum and, agitated at 120 rpm for 48 h at 35°C in an incubator shaker. Triplicate flasks were maintained for each treatment. At interval of 12 h, 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 at 4°C. The clear cell free supernatant was used for amylase assay.

Effect of temperature. The culture of *L. plantarum* in MRS liquid medium containing 1% soluble starch was incubated at 15–55°C and 120 rpm for 36 h in an incubator shaker. At the end of incubation, cell free supernatants were prepared and analyzed for amylase activity.

Effect of pH. The effect of pH on amylase production by *L. plantarum* was investigated by varying the pH of the medium from 5.0–9.0 and the samples were incubated for 36 h at 35°C. The pH measurements were carried out with a Systronics-make pH meter (Model 351, Pvt. Ltd, Ahmadabad, India) using glass electrode. The pHs of 5.0–6.0 were maintained

with acetate buffer (0.2 M) while pHs 7.0–9.0 were achieved with phosphate buffer (0.1 M).

Purification of the enzyme. Amylase was purified by ammonium sulphate fractionation followed by dialysis and DEAE cellulose column chromatography (Swain *et al*., 2006). A total of 100 ml of culture filtrate was centrifuged at 8000 × g for 20 min at 4°C to remove the cells. The supernatant was brought to 50% ammonium sulphate saturation at 4°C for 12 h in an ice bath. The precipitated protein was collected by centrifugation at 8000 × g at 4°C and dissolved in a minimum volume of phosphate buffer (0.1 M, pH 6.8). The enzyme solution was dialyzed at 4°C against the same buffer for 24 h at 4°C with continuous stirring and three changes of the same buffer. The DEAE cellulose-ion exchange column was pre-equilibrated with the same buffer. The dialysate was applied to the DEAE cellulose column and the flow rate was maintained at of 0.6 ml/min with 50 ml linear NaCl (0.1 to 0.5 M) gradient. Fractions of 10 ml were collected and each fraction was analyzed for protein concentration and α-amylase activity. The active fractions were pooled and concentrated through a Rotary Evaporator (Model – 102/202, Strike, Italy). The final concentrated enzyme solution was taken for sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). The optimal production of the partially purified enzyme at various pHs (5.0–9.0), incubation periods (12–60 h) and temperatures (15–55°C) were also studied. The flow-chart for purification of the enzyme is given in Fig. 1.

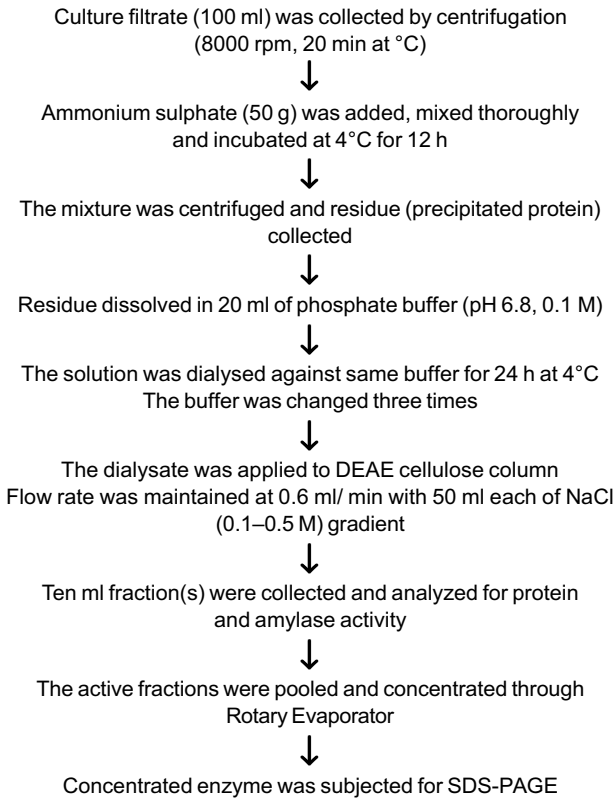

Fig. 1. Flow-chart for purification of the enzyme.

Electrophoresis and molecular mass determination. SDS-PAGE was performed with 12% polyacrylamide gel using a Mini-GEL electrophoresis system (Model No. 0502, Bangalore Genei Pvt. Ltd., Bangalore, India) as described by Laemmli (1970). The bacterial proteins were stained with 0.2% Coomassie Brilliant Blue. The molecular mass of the partially purified amylase was estimated using a standard "protein marker" (PMW-M) of known molecular mass (14 300–97 400 Da) (Bangalore Genei Pvt. Ltd., Bangalore, India).

Thin layer chromatography (TLC). The end products liberated by the action of amylase on starch were identified by spotting the starch digest and standard sugars (glucose and maltose) on a silica gel plate activated at 80°C for 30 min. The plates were developed in butanol: ethanol: water (50: 30: 20) and dried overnight at room temperature (32±2°C). The individual sugars were visualized by spraying with acetone-silver nitrate solution (0.1 ml saturated solution of $AgNO_3$ in 20 ml of acetone).

Amylase assay. The amylase assay was based on the reduction in blue colour intensity resulting from enzymatic hydrolysis of starch and formation of starch-iodine complex (Swain *et al*., 2006). The reaction mixture consisted of 0.2 ml enzyme (cell free supernatant), 0.25 ml of 0.1% starch solution and 0.5 ml of phosphate buffer (0.1 M, pH 6.8) incubated at 35°C for 10 min. The reaction was stopped by adding 0.25 ml of 0.1 N HCl and the colour was developed by adding 0.25 ml of I/KI solution (2% KI in 0.2% I). The optical density of the blue colour solution was determined using a UV-VIS spectrophotometer (Model No CE 7250, Cecil Instrument, UK) at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that causes 0.01% reduction of blue colour intensity of starch iodine solution at 35°C in one minute per ml (Swain *et al*., 2006).

LA production by *L. plantarum*. A 3% (w/v) suspension of sweet potato flour (starch, 550 mg/g flour; total sugar, 12 mg/g flour) in 100 ml water was incubated with 2 ml of *L. plantarum* crude enzyme at 35°C in an incubator shaker. The rate of LA production was evaluated at 12 h interval up to 144 h and LA was estimated by spectrophotometric method (Amerine and Ough, 1984).

Results

TLC. Using TLC analysis, it was ascertained that the amylase produced by the *L. plantarum* was α-amylase owing to the presence of glucose as the end product during starch hydrolysis (Fig. 2).

Optimization of incubation period, temperature and pH by applying RSM. The effects of three

Table II
Experimental design and result of CCD of response surface methodology

Std	A: Incubation period (h)	B: Temperature (°C)	C: pH (H+)	Enzyme production (U/min/ml)	
				Predicted	Experimental
1	–1	–1	–1	1940	2065
2	1	–1	–1	2169	2143
3	–1	1	–1	2012.82	1932
4	1	1	–1	2232.71	2010
5	–1	–1	1	2134.39	2163
6	1	–1	1	2355.27	2242
7	–1	1	1	2217.18	2050
8	1	1	1	2428.07	2109
9	–α	0	0	3672.19	3050
10	+α	0	0	3034.11	3770
11	0	–α	0	2568.12	1350
12	0	+α	0	2469.59	1950
13	0	0	–α	2452.16	1930
14	0	0	+α	2969.78	2475
15	0	0	0	3887.35	4022
16	0	0	0	3887.35	3910
17	0	0	0	3887.35	3825
18	0	0	0	3887.35	3950
19	0	0	0	3887.35	3850
20	0	0	0	3887.35	3720

independent variables (*i.e.* incubation period, temperature and pH) on α-amylase production by *L. plantarum* MTCC 1407 are presented along with the predicted and observed responses in Table II. The statistical significance of the second order equation was checked by F-test (ANOVA) and the data are shown in Table III. The regression model for amylase production was highly significant ($p<0.01$) with a satisfactory value of determination co-efficient ($R^2=0.9609$) indicating that 96.09% variability in the response could be explained by second order polynomial equation given below.

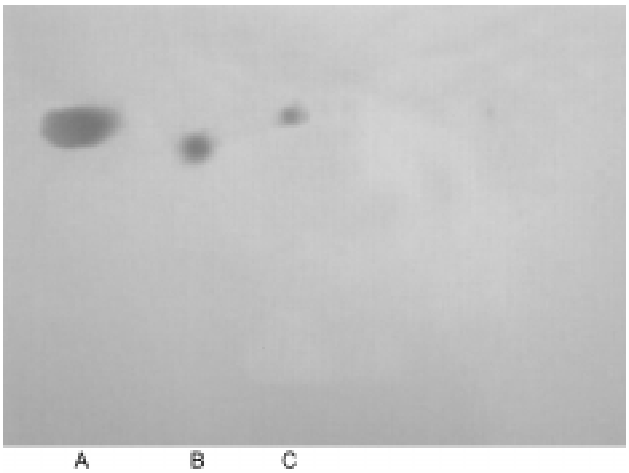

Fig. 2. Hydrolysis products of starch with amylase by thin layer chromatography.
1. Glucose (standard), 2. Maltose (standard) and 3. Hydrolysate of amylase from *L. plantarum*.

Table III
ANOVA for α-amylase production in submerged fermentation

Source	Sum of quares	Degree of freedom	Mean Square	F-Value	p-value
Model	1366.35	9	151.82	27.27	0.0001
Pure Error	3.59	5	0.72		
Total	1422.02	19			

$R^2 = 0.9609$; Adjusted $R^2 = 0.9256$; Predicted $R^2 = 0.7186$;
Adequate Precision = 14.309; Lack of Fit F-value = 14.53

$$Y = 62.35 + 1.00 \times A + 0.50 \times B + 1.05 \times C - - 1.83 \times A^2 - 8.14 \times B^2 - 5.88 \times C^2 - 0.021 \times \times AB - 0.030 \times AC + 0.036 \times BC$$

Where Y is enzyme 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 is to 1.0, the stronger the model and the better it predicts the response (Rao and Satyanarayana, 2003). An adequate precision of 14.309 for α-amylase production was recorded. The predicted R^2 of 0.7186 is in reasonable agreement with the adjusted R^2 of 0.9256. This indicated a good agreement between the experimental and predicted value for α-amylase production. The model F-value of 27.27 and values of prob >F (<0.05) indicated that the model terms are significant. For α-amylase production A^2, B^2 and C^2 are significant model. The "lack of fit F – value" of 14.53 implied that the "lack of fit" is significant.

Fig. 3A depicts three dimensional diagram and a contour plot of calculated response surface from the

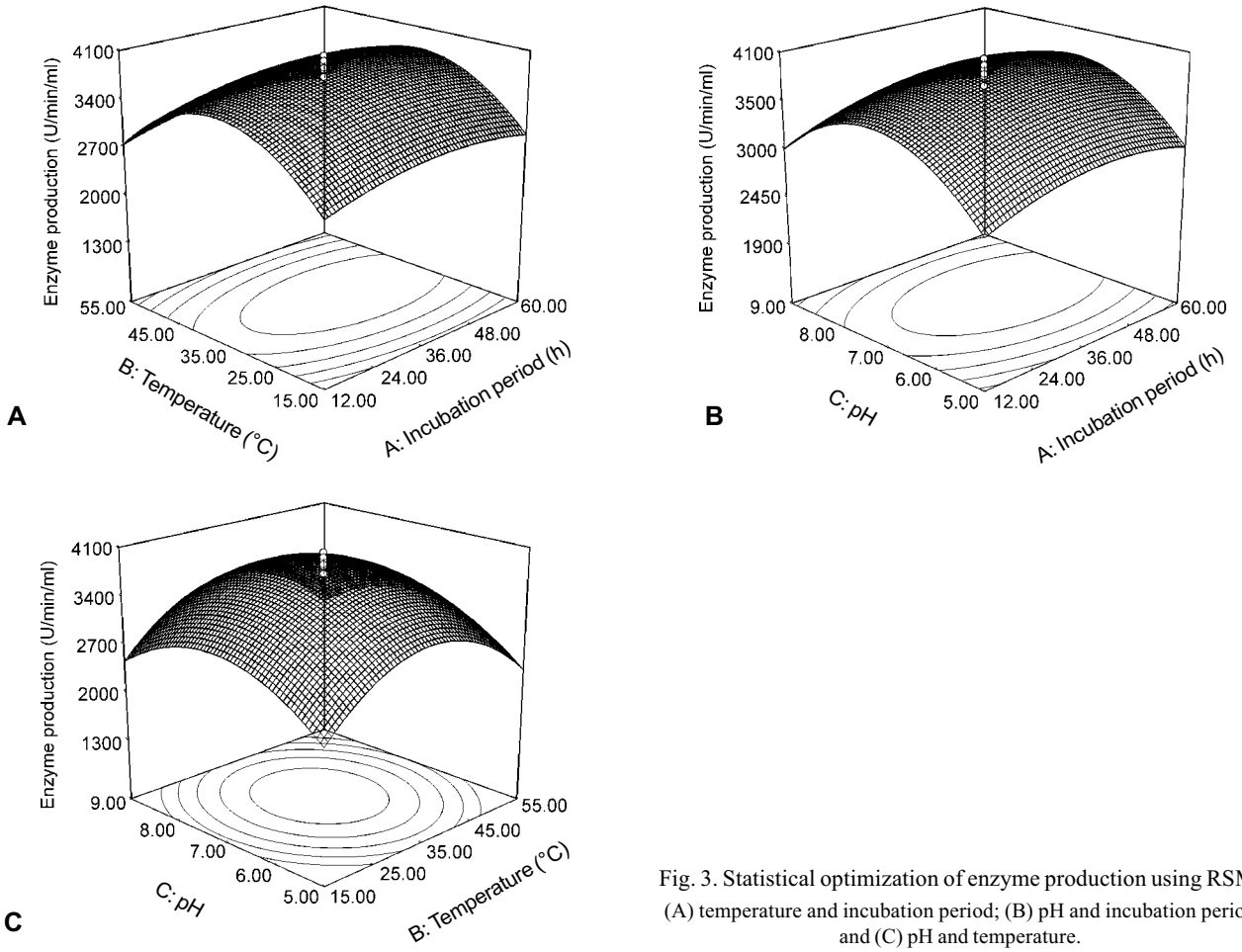

Fig. 3. Statistical optimization of enzyme production using RSM. (A) temperature and incubation period; (B) pH and incubation period and (C) pH and temperature.

interaction between incubation period and temperature while keeping the other variable (pH) at '0' level. A linear increase in α-amylase production was observed when incubation period was increased up to 36 h, and thereafter, it declined. Similarly, when the level of temperature was increased from 15°C to 55°C, a linear increase in α-amylase production was recorded up to 35°C and thereafter declined. At the '0' level of temperature, the response between incubation period and pH indicated that a pH of 7.0 was optimum with 36 h incubation period for α-amylase production (Fig. 3B). An interaction between the remaining two parameters (temperature and pH) (Fig. 3C) suggested a little difference with the earlier responses.

Validation of model. Validation was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The predicted response for α-amylase production was 3887.35 U/min/ml, while the actual (experimental) response was 4022 U/min/ml, thus providing the validity.

Partial purification and molecular mass determination. α-Amylase was partially purified with ammonium sulphate precipitation. The crude extract contained 2.97 mg/protein ml and showed a specific activity of 1411.05 U/mg/protein. After partial purification, the specific activity increased to 3126.91 U/mg/protein with a yield of 23.3% and 2.2-fold purification. The details of purification steps are given in Table IV. Electrophoresis studies showed that the molecular mass of partially purified α-amylase was approximately 75 450 Da (Fig. 4).

Table IV
Partial purification of α-amylase from *L. plantarum*

Purification Steps	Total Volume (ml)	Total Enzyme Activity (Units)	Total Protein (mg)	Yield (%)	Specific Activity (Umg^{-1} Protein)	Fold of Purification
Culture filtrate (crude)	100	418 800	296.8	100	1411.05	0
Ammonium sulphate precipitation	20	77 420	58.35	18.5	1327.31	0.9
After dialysis	25	97 528	31.19	23.3	3126.91	2.2

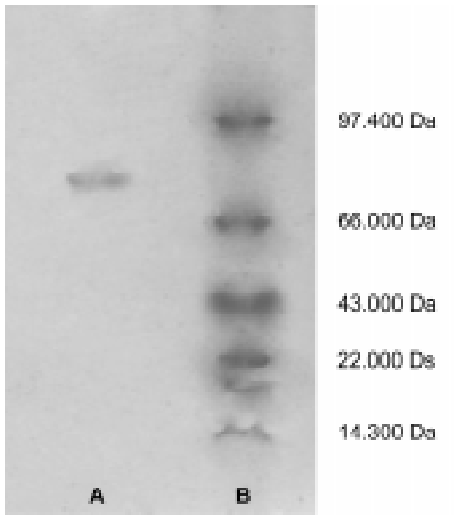

Fig. 4. Determination of molecular mass by SDS-PAGE.
A. Standard protein markers (PMW-B);
B. α-amylase by *L. plantarum* MTCC 1407.

LA production from sweet potato flour by *Lactobacillus plantarum*. The rate of LA production from sweet potato flour by *L. plantarum* MTCC 1407 is shown in Fig. 5. There was a gradual increase in LA with increase in incubation period from 12 to 120 h and thereafter it remained more or less constant. With application of 2 ml crude enzyme (≈8044 Units), 56% conversion of starch in sweet potato flour to LA was achieved.

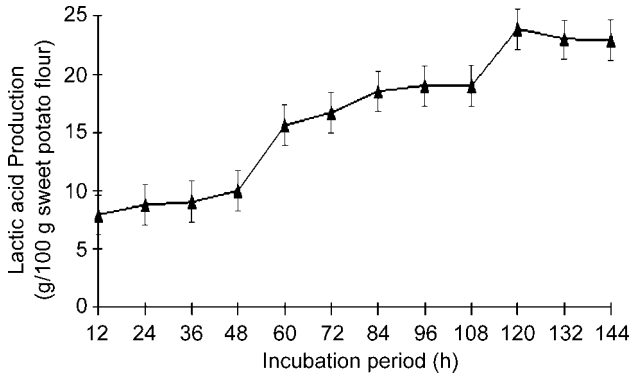

Fig. 5. Lactic acid production from sweet potato flour by addition of crude enzyme (2 ml) from *L. plantarum* MTCC 1407.

Discussion

L. plantarum, a versatile lactic acid bacterium, is one of the best characterized organisms in Gram-positive bacteria. It is safe, stable and widely used in industrial fermentation process and probiotic preparations for humans and animals as it is a homolactic fermentation and produces only (L+) lactic acid (Rincker *et al*., 2000; Ray and Panda, 2007). It has proven ability to survive gastric transits and can colonize the intestinal tract of birds, humans and other mammals to maintain a healthy microflora (Ray and Panda, 2007). Along with other amylolytic LAB (*i.e. L. acidophilus*, *L. bifidus*, *etc*) colonizing the GI tract (Rincker *et al*., 2000), they increase the intestinal amylase activity that improves dietary starch utilization in weaning animals including human infants (Nguyen *et al*., 2007). Formulations of probiotics containing enzymes like amylase, pectinase and protease producing lactobacilli, vitamins and minerals are being developed to specially aid digestion of all food groups, prevention against gas formation, constipation and mal-absorption of nutrients, and to provide support for GI tract by creating an environment that is conducive to the colonization of the probiotic organisms (Collington *et al*., 1990). Some of the probiotic enzymes marketed are under the trade name "Polyenzyme plus", "Probiotic Bioplus 2b, C", F-biotic, *etc* (Naidu *et al*., 1999).

RSM used in this investigation suggested the importance of various fermentation parameters at different levels. The methodology employed will be successful to any process, where an analysis of the effects and interaction of many experimental factors are required. CCD maximizes the amount of information that can be obtained, while limiting the numbers of individual experimental (Rao and Satyanarayana, 2003). Thus, smaller and less time consuming designs could generally suffice for the optimization of many fermentation processes. The result of this study endorses this view point. In this study, a high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for enzyme production in submerged fermentation. MRS medium has been standardized since 15 years for growth of lactobacilli (Coeuret *et al*., 2003); hence, the composition was not further optimized or altered except incorporation of starch (1%) instead of glucose (2%) for α-amylase production. Preliminary studies have shown that concentration of starch (>1%) make the medium thick which is unsuitable for growth of *L. plantarum* MTCC 1407.

The optimum temperature, pH and incubation period for α-amylase productions by *L. plantarum* MTCC 1407 were 35°C, 7.0 and 36 h, respectively, which were the major factors that influenced the enzyme titre. The decrease in enzyme production after the optimum incubation period (36 h) may be due to denaturation or decomposition of α-amylase because of interaction with other components in the culture medium (Gangadharan *et al*., 2006). The purified enzyme had a molecular mass of 75 450 Da using the above process parameters. There were wide variations in molecular mass of different *Lactobacillus* spp., *i.e. L. amylophilus* GV6 strain (90 000 Da and 140 000 Da) (Pompeyo *et al*., 1993), *L. amylovorus* ATCC 33 621 (47 000 Da), *L. manihotivorans* (135 000 Da) (Aguilar *et al*., 2000).

Vishnu *et al*., (2006) reported amylopullunase production from raw starch at pH 6.5, temperature 37°C and incubation period of 48 h by *L. amylovorous* GV6. The strain *L. plantarum* MTCC 1407 used in our study is amylolytic by virtue of possessing α-amylase that converts starch to fermentable sugars and further to LA. When the application of the *L. plantarum* amylase in liquefying sweet potato flour to LA was studied at its optimum temperature 35°C, pH 6.5 the crude enzyme could converted 56% starch present in the flour to LA after 120 h of incubation. To conclude, the present study revealed that *L. plantarum* MTCC 1407 is a potential strain for production of extracellular α-amylase in submerged fermentation. Because of the amylolytic activity of *L. plantarum* MTCC 1407, it can be utilized in production of starch based fermented foods, feed and pharmaceuticals to improve the physiology of GI tracts of humans and animals.

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