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Effect of culture conditions and kinetic studies on extracellular tannase production by *Lactobacillus plantarum* MTCC 1407

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Production of tannase and microbial biomass by *Lactobacillus plantarum* MTCC 1407 was studied in submerged batch fermentation. The tannase production was performed in different media compositions maintaining the fermentation conditions constant at 30°C, initial *p*H 5.5 and agitation speed at 120 rpm. Maximum tannase activity of 5.22 U mL^{-1} was obtained at 24 h in M3 medium containing the following composition (g L^{-1}): tannic acid, 10; glucose, 1; NH_4Cl, 3; $MgSO_4.7H_2O$, 2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; and $CaCl_2$, 1. The medium optimization studies show that the tannic acid (1% w/v) as inducer along with glucose (0.1% w/v) as carbon source gave maximum tannase activity. Unstructured kinetic models, namely, logistic model for cell growth and Luedeking-Piret model for tannase production, were used to predict the fermentation kinetics. The estimated values of the kinetic model parameters, α and β for tannase production indicated that the tannase production by *L. plantarum* was growth associated.

Keywords: Kinetics, optimization, tannase, unstructured modeling

Introduction

Tannase (tannin acyl hydrolase, E.C.3.1.1.20) is an extracellular hydrolase enzyme that catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins or gallic acid esters, liberating glucose and gallic acid (GA) as a final product[1]. Tannase cleaves the ester linkages between galloyl groups present in various compounds, such as, epigallocatechin and epigallocatechin gallate that are present in green tea leaves[1,2]. Tannase finds wide application in food, beverage, brewing, cosmetic and chemical industries[1], and is mainly used in the production of gallic acid, instant tea, acron wine, coffee flavoured soft drinks and high grade leather tannin. Tannase is also used as a clarifying agent in clarification of beer, fruit juice and various food stuffs and as a hydrolyzing agent in cleaning up the highly polluting tannin (polyphenols) from the effluent of leather industry[1,3]. The produced gallic acid (3,4,5 trihydroxy benzoic acid) has several applications in chemical and pharmaceutical industries as a precursor in the production of propyl gallate (antioxidant), pyrogallol, trimethoprim (antibacterial drug) and semiconductor resin[4].

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Tannase production was studied in numerous organisms ranging from prokaryotes to eukaryotes like fungus, higher plants and animals (ruminants, insects). Tannin rich parts of the plants, such as, fruits, leaves, branches and barks, possess considerable amount of tannase. Plants like penduculate oak (*Quercus rubra*), myrobolano (*Terminalia chebula*) and babul (*Acacia arabica*) are rich in tannase[5,6]. Tannase can be extracted from bovine intestine and ruminal mucous[1]. The enzyme produced from microbial sources find immense application in various industries due to its higher stability and availability[1]. Among the various microbial sources for tannase production, filamentous fungi like *Ascochyta, Aspergillus, Chaetomium, Mucor, Myrothecium, Neurospora, Rhizopus, Trichothecium, Fusarium, Trichoderma* and *Penicillium* strains were studied extensively[1,7]. Tannase producing yeasts have also been isolated but they were not extensively studied[8]. Bacterial sources, such as, *Bacillus, Corynebacterium, Klebsiella*[9], *Streptococcus bovis*[10] and *Selenomonas ruminantium*[11], have been studied for tannase production. Lactic acid bacteria play a vital role in hydrolyzing tannins present in food and intestines. High tannase activity was reported in lactic acid bacteria, *Lactobacillus plantarum*[12-15].

The present study reports the effect of various medium compositions on tannase production by *L. plantarum* MTCC 1407 and for the first time the kinetic modeling for tannase fermentation was attempted using unstructured models.

Materials and Methods

Microorganism Maintenance and Inoculum Preparation

L. plantarum MTCC 1407 was obtained from the Microbial Type Culture Collection (MTCC) and Gene Bank Centre, Institute of Microbial Technology, Chandigarh, India. The *L. plantarum* MTCC 1407 stock culture was maintained in agar slants containing (g L^{-1}): beef extract, 10.0; glucose, 20.0; yeast extract, 5.0; peptone, 10.0; Na_2HPO_4, 2.0; sodium acetate, 5.0; triammonium citrate, 2.0; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.2; agar, 2.5; and Tween 80, 1 (mL L^{-1}). Inoculum was prepared by growing the organism in 100 mL sterile seed medium (composition same as maintenance medium excluding agar) in 250 mL Erlenmeyer flask for 24 h on a temperature controlled rotary shaker at 35°C and 120 rpm. The medium components and chemicals used in this study were procured from Himedia Ltd, Mumbai, India.

Batch Fermentation

The tannase production by *L. plantarum* MTCC 1407 was conducted in 250 mL Erlenmeyer flask with 100 mL of the production medium. The production medium was adjusted to the initial *p*H of 5.5 using 1 *M* NaOH or 1 *N* HCl and sterilized (121°C for 20 min). Growth profile of the organism in the seed medium was shown in Fig. 1. The production medium was inoculated with 5% (v/v) of seed culture in the mid exponential phase at 24 h (Fig. 1). The flasks were incubated in an orbital shaker at 120 rpm and 30°C for the fermentation period of 60 h. Aliquot of sample from the fermentation broth was withdrawn at 6 h interval without much change in the culture volume to maintain constant oxygen transfer. The cells were separated from the medium by centrifugation at 10,000 rpm for 15 min. The clarified supernatant was used for the analysis of tannase activity, protease activity, total soluble protein and glucose. Various production media M1, M2, M3, M4 and M5 were tested for tannase production by *L. plantarum* MTCC 1407 as shown in Table 1.

Tannase Activity Assay

Tannase activity was determined spectrophotometrically by the method of Libuchi *et al*[16]. 0.5 mL of culture supernatant (crude enzyme) was added to 2.0 mL of 0.35% (w/v) tannic acid solution in 0.05 *M* citrate buffer (*p*H 5.5) in a test tube. 20 µL of the reaction mixture was taken out and 2.0 mL of 95% ethanol was added to the reaction mixture to stop the enzyme reaction. The absorbance at 310 nm was noted immediately (t_1). The test tube was then incubated in a water bath at 37°C for 10 min (t_2), after which ethanol was added to the reaction mixture (20 µL) to stop the enzyme reaction. The absorbance was measured at 310 nm. One unit (U) of tannase activity is defined as the amount of enzyme required to hydrolyze 1 µ mol of ester in 1 min and is expressed as U mL^{-1} min^{-1}.

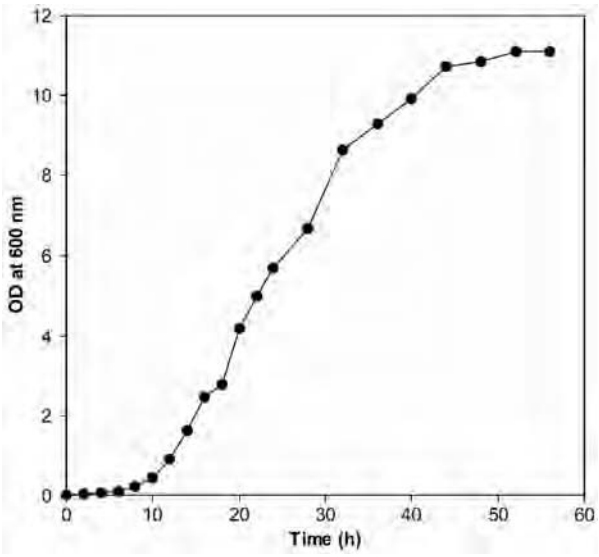

Fig. 1—Growth profile of *L. plantarum* MTCC 1407 in the seed medium (g L^{-1}): Beef extract, 10.0; glucose, 20.0; yeast extract, 5.0; peptone, 10.0; Na_2HPO_4, 2.0; sodium acetate, 5.0; tri ammonium citrate, 2.0; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.2; and Tween 80, 1 (mL L^{-1}).

Table 1—Various medium composition used for the production of tannase by *L. plantarum*

Medium	Composition (g L^{-1})
Medium 1 (M1)	Tannic acid, 7; glucose, 2 ; ($NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.4; KH_2PO_4, 7; $FeSO_4.7H_2O$, 0.02; casein hydrolysate broth, 2.
Medium 2 (M2)	Galactose, 2; tri sodium citrate dihydrate, 0.5; DL maleic acid, 5; casein hydrolysate broth, 4.
Medium 3 (M3)	Tannic acid, 10; glucose, 1; NH_4Cl, 3; $MgSO_4.7H_2O$, 2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $CaCl_2$, l.
Medium 4 (M4)	Tannic acid, 10; NH_4Cl, 3; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 0.5.
Medium 5 (M5)	Lactose, 40; peptone, 10; yeast extract, 5; NH_4Cl, 0.75; $MgSO_4.7H_2O$, 0.1; K_2HPO_4, 2; Tween 80, 2 mL L^{-1}

Protease Activity Assay

The protease activity was assayed by modified Anson method[17] using casein as the substrate. 2 mL of 1% (w/v) casein solution was mixed with 0.5 mL of enzyme solution and incubated at 37°C for 30 min. 2.5 mL of 0.4 *M* trichloroacetic acid was added to arrest the reaction. The solution with precipitate was filtered and to the 1 mL of filtrate, 5 mL of 0.4 *M* Na_2CO_3 and 0.5 mL of Folin's reagent were added. After 10 min of incubation, the colour density developed was determined at 660 nm. One unit (U) of protease activity was defined as 1 µg of tyrosine liberated per minute by 1 mL of enzyme.

Biomass, Glucose and Protein Determination

The bacterial cell growth was determined by measuring the optical density at wavelength of 600 nm (Double beam UV Visible spectrophotometer, Elico India Limited, India). The biomass concentration was determined with a calibration curve made from the relationship between optical density at 600 nm and dry cell wt. The glucose concentration in the fermentation broth was determined by dinitrosalicylic acid method[18]. The total soluble protein in the medium was determined by Lowry's method[19].

Unstructured Model Development for Fermentation Kinetics

The exponential growth phase of the microorganism can be characterized by the following first order equation which states that the rate of increase in cell mass is proportional to the quantity of viable cell mass at any instant time,

$$\frac{dX}{dt} = \mu X \qquad \ldots (1)$$

where dX/dt is the growth rate (g L^{-1} h^{-1}); X is the concentration of biomass (g L^{l}); µ is the specific cell growth rate (h^{-1}). The growth of cell is governed by a hyperbolic relationship and there is a limit to the maximum attainable cell mass concentration. Such growth kinetics is described by logistic equation[20] as,

$$\frac{dX}{dt} = \mu_0\left(1-\frac{X}{X_{max}}\right)X \qquad \ldots (2)$$

where μ_0 is the initial specific growth rate (h^{-1}) and X_{max} is the maximum cell mass concentration (g L^{-1}). Equation (2) on integration using $X_0 = X$ (t = 0) gives a sigmoidal variation X(t) that may empirically represent both an exponential and a stationary phase.

$$X(t) = \frac{X_0 e^{\mu_0 t}}{1-\left(\frac{X_0}{X_{max}}\right)\left(1-e^{\mu_0 t}\right)} \qquad \ldots (3)$$

The kinetic parameter, μ_0 in this equation is determined by rearranging equation (3) as,

$$\mu_0 t = \ln\left[\frac{X_{max}}{X_0}-1\right]+\ln\left[\frac{\overline{X}}{1-\overline{X}}\right] \qquad \ldots (4)$$

where $\overline{X} = \frac{X}{X_{max}}$, if the logistic equation describes the data suitably, then a plot of $\ln\left[\frac{\overline{X}}{1-\overline{X}}\right]$ *vs* t should give a straight line of slope 'μ_0' and intercept $-\ln\left[\left(\frac{X_{max}}{X_0}-1\right)\right]$

The kinetics of tannase production was described by Luedeking-Piret equation[21] which states that the product formation rate depends upon both the instantaneous biomass concentration (X) and growth rate (dX/dt) in a linear fashion.

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad \ldots (5)$$

where α and β are empirical constants that may vary with fermentation conditions. Integrating equation (5) using equation (2),

$$P_t = P_0 + \alpha A(t) + \beta B(t) \qquad \ldots (6)$$

where P_0 and P_t are the product concentrations at initial time and at any time't', respectively and,

$$A(t) = X_0\left[\frac{e^{\mu_0 t}}{1-\left(\frac{X_0}{X_{max}}\right)\left(1-e^{\mu_0 t}\right)}-1\right] \qquad \ldots (7)$$

$$B(t) = \frac{X_{max}}{\mu_0}\ln\left[1-\frac{X_0}{X_{max}}\left(1-e^{\mu_0 t}\right)\right] \qquad \ldots (8)$$

The parameters α and β in equation (6) are determined by plotting $[P_t–P_0]/B(t)$ *vs* A(t)/B(t) which is a straight line with slope 'α' and intercept 'β'.

Results and Discussion

The objective of the present study is to optimize the medium composition for tannase production by

L. plantarum MTCC 1407 and to predict the fermentation profile using unstructured kinetic models. Various reports on microbial production of tannase revealed that the fermentation parameters, namely, culture condition, culture composition and substrate concentration, had significant effect on production of tannase and biomass[22]. Five different media compositions, namely, M1, M2, M3, M4, and M5, were used in this study as shown in Table 1, which were selected based on literature on previous studies. The kinetics of tannase activity, total soluble protein, protease activity, cell mass and *p*H was observed for all the production media studied.

Comparative analysis of cell mass, protease and tannase production by *L. plantarum* grown in different media was illustrated in Fig. 2. The cell mass production was high in the order of M5>M1>M2>M3>M4 as shown in Fig. 3. Maximum cell mass was obtained in the media M5, which contains lactose as the major carbon source. Presence of lactose in the growth media enhanced the growth rate of the cells than any other carbon source, such as, glucose, galactose and tannic acid. Very low cell mass was obtained in medium M4 when tannic acid was used as the sole carbon source.

Maximum cell mass concentration of 0.87 g L^{-1} was obtained in medium M1 which contains tannic acid and glucose at the concentration of 0.7% w/v and 0.2% w/v, respectively. In medium M3, the maximum cell mass concentration of 0.13 g L^{-1} was obtained which contains 1% w/v of tannic acid and 0.1% w/v of glucose. The presence of glucose in the medium induces the cell growth and reduces the lag phase in the growth of the microorganism. In a medium with both tannic acid and glucose, the cell mass yield differed with the proportions in which they were added. This might be the reason for the low cell mass yield in M3. In media M2, the cell mass yield was high compared to M3 and M4 media as the carbon source used was galactose that acts similar to glucose. The cell mass yield depends greatly on the carbon source provided, especially the tannic acid and glucose concentration in the medium. In the presence of higher levels of tannic acid the production of cell mass was less compared to the cell mass produced in a medium with higher amount of glucose[12]. However, Bradoo *et al*[23] reported that the addition of carbon sources, such as, glucose, fructose, sucrose, maltose, and arabinose, to the culture medium at initial concentrations from 10 to 30 g L^{-1} improved the tannase production by *A. niger*.

The increasing order of tannase production by *L. plantarum* MTCC 1407 strain in various media used was M3>M2>M1>M4>M5 (Fig. 4). Maximum tannase production was obtained in medium M3, which contained tannic acid (1% w/v) and glucose

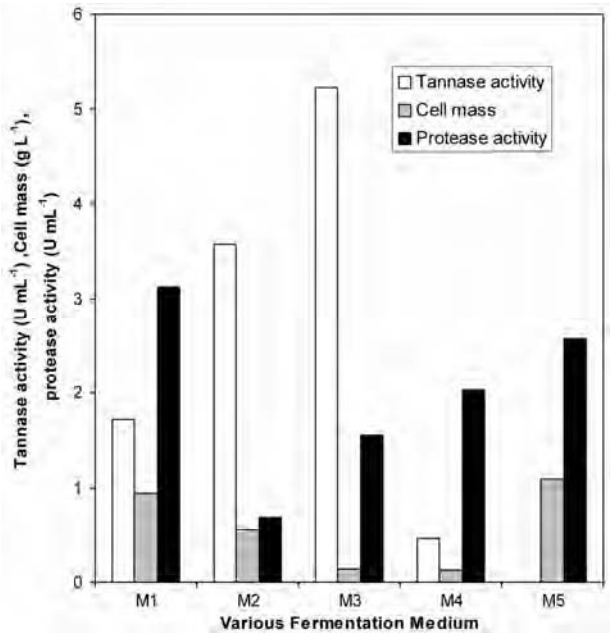

Fig. 2—Comparison of tannase activity, cell mass and protease activity in various production medium

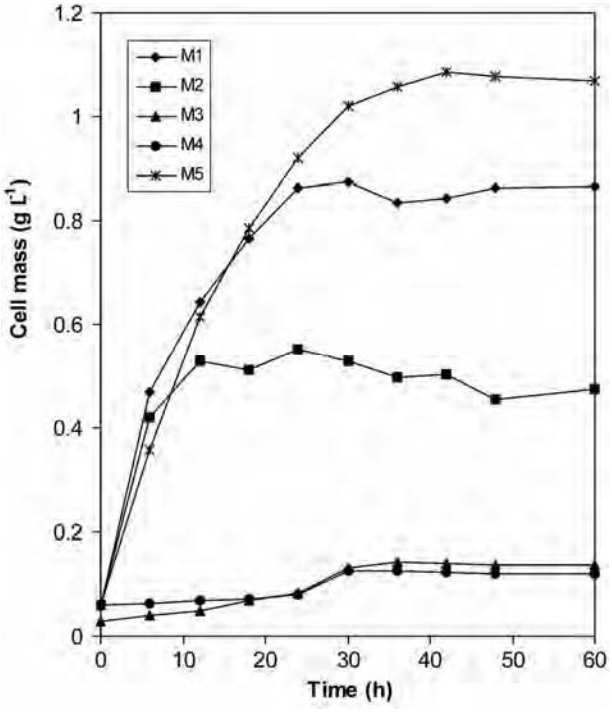

Fig. 3—Time course of cell mass in various medium compositions in tannase fermentation

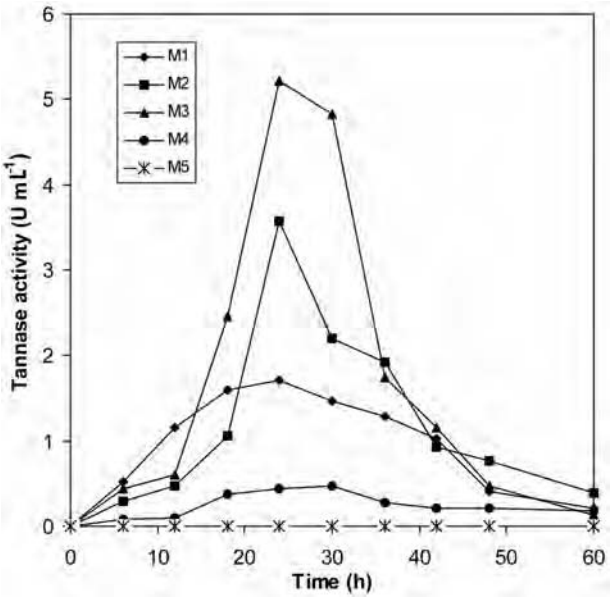

Fig. 4—Time course of tannase activity in various medium compositions in tannase fermentation

(0.1% w/v) during the mid exponential phase of 24 h (Fig. 4). Deschamps *et al*[9] observed maximum tannase production by the bacteria at their active phase of growth. Presence of higher concentration of glucose decreased the tannase production in *A. niger* Aa-20 under solid state fermentation[24]. In submerged fermentation, the increase in glucose concentration considerably increased the enzyme production[24]. Very high concentration of glucose repressed the enzyme production, which is the result of catabolic repression exerted by glucose on metabolite production. Ayed and Hamdi[12] reported that the presence of glucose and tannic acid could directly affect the tannase production in *L. plantarum* species isolated from olive wastes and the optimal concentration of glucose and tannic acid was found to be 2 g L^{-1} and 7 g L^{-1}, respectively. In medium M2, the tannase production was comparatively higher than M1, M4 and M5 media. Even though tannic acid was completely absent in the M2 medium, the presence of galactose, sodium citrate, maleic acid, casein hydrolysate (rich source of protein digest) gave maximal tannase activity of 3.57 U mL^{-1} at 24 h next to M3 media with 5.22 U mL^{-1} tannase activity. *L. plantarum* CECT 748^T (ATCC 14917) produced considerable amount of tannase in the tannic acid free medium[25]. In M1 medium with glucose concentration of 0.2% w/v and tannic acid concentration of 0.7% w/v, the maximum tannase activity of 1.46 U mL^{-1} was observed at 24 h. In the medium M4, where tannic acid was used as the sole carbon source along with basal salts in the absence of glucose, maximum tannase of 0.47 U mL^{-1} was produced at 30 h. Medium M5, which was devoid of tannic acid, showed no tannase activity. Presence of lactose in medium M5 activated the cellular growth and did not show any role in the production of tannase. Production of tannase by *A. niger* HA37 on a synthetic culture medium containing tannic acid at different concentrations was studied by Aissam *et al*[26]. They found that tannase activity increased proportionally to the initial concentration of tannic acid; 0.6, 0.9 and 1.5 U mL^{-1} tannase activity in the presence of 0.2, 0.5 and 1% of tannic acid, respectively. From these results, it can be concluded that the tannic acid acts as an important carbon source and inducer for the tannase production.

The tannase production by *L. plantarum* MTCC 1407 was found to be growth associated in all the fermentation runs conducted, as the tannase production increased with the cell mass, except M5 where tannic acid and glucose were absent. Mondal and Pati[22] also reported that the tannase production was directly proportional to the growth of *B. licheniformis* KBR6 and the extracellular enzyme accumulation increased with the number of cells. In tannase fermentations using fungal cultures, tannase is mostly extracellular when produced by solid state fermentation and it can be easily extracted with water or a buffer, whereas tannase location during its production by submerged fermentation depends on the cultivation time[27]. Tannase is mainly intracellular at the beginning of the culture, and it is further secreted to the culture medium. However, up to 80% of tannase remained bound to the mycelium in fungal fermentations when the maximum overall tannase titter is attained. Cell-bound tannase can be extracted after cell-wall hydrolysis with digestive enzymes such as chitinase[28]. In case of bacterial fermentations, the tannase is mostly extracellular[29].

In the present study, the protease activity was determined in all the fermentation runs to find the effect of protease on tannase production. The protease production was maximal in the medium M1 and it was in the order of M1>M5>M4>M3>M2 (Fig. 2). The decrease in tannase production during the late phase of fermentation might be due to the action of protease. Similar results have been obtained by Aguilar *et al*[24] in *A. niger* Aa-20.

Kinetics of Tannase Production

The kinetics of tannase fermentation using *L. plantarum* was studied using various culture

medium at 30°C, initial *p*H of 5.5 and agitation speed at 120 rpm. The maximum tannase production was observed in the medium M3, which contained the following composition (g L^{-1}): tannic acid, 10; glucose, 1; NH_4Cl, 3; $MgSO_4.7H_2O$, 2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; and $CaCl_2$, 1. The kinetic profile of tannase activity, protease activity, cell mass concentration, substrate utilization, *p*H and total soluble protein concentration in the culture medium M3 is given in Fig. 5.

The tannase production was found to be increased gradually from 8 h of the fermentation period when the growth of the microorganism reached the early exponential phase, which shows that the tannase production is growth associated. The maximum tannase activity was found in the mid exponential phase and early stationary growth phase of *L. plantarum* as reported by Ayed and Hamdi[12]. The maximum tannase activity of 5.22 U mL^{-1} was obtained at the 24 h of fermentation and the activity reduced significantly after 24 h. The decrease in tannase activity might be due to the increased protease production during the stationary phase of the microorganism and also due to the unavailability of the substrate. The protease activity reached a maximum value of 1.853 U mL^{-1} at 24 h at the late exponential phase and early stationary phase. Maximum cell mass concentration of 0.144 g L^{-1} was observed at 36 h during the stationary phase and there was no further increase in the cell mass concentration. The *p*H of the fermentation medium was found to be decreased from initial *p*H of 5.5 to 3.2 at 12 h and then increased to *p*H 3.78 at 24 h and remained constant thereafter for the entire fermentation period. The rate of glucose utilization by the microorganism increased rapidly after 8 h of the fermentation when the microorganism reached the exponential phase and 90% of the glucose was consumed at 60 h of fermentation.

Unstructured kinetic models, namely, logistic model for cell growth and logistic incorporated Luedeking-Piret model for tannase production, were used to predict the kinetic profile. Logistic model predicted the cell growth kinetics of *L. plantarum* with high R^2 (coefficient of determination) value of 0.955 but the R^2 value was 0.60 for tannase activity prediction by logistic incorporated Luedeking-Piret model. The product formation kinetic model was able to predict the kinetics of tannase production during the exponential phase of the microorganism accurately. But during the stationary phase,

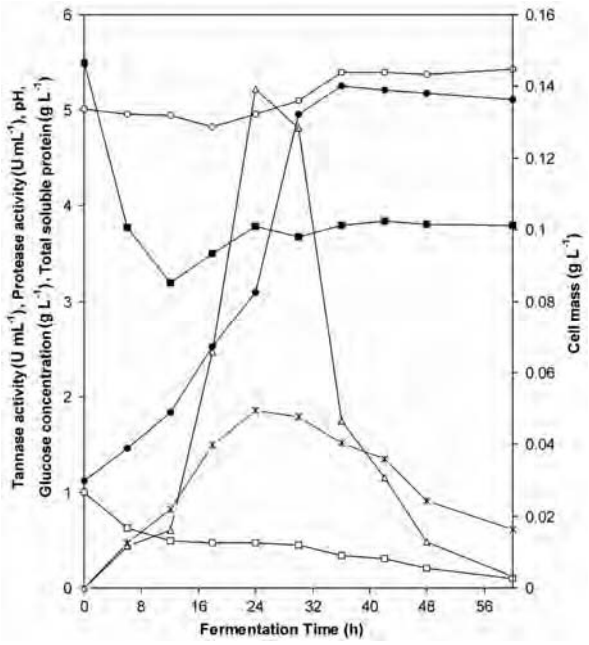

Fig. 5—Profile of tannase activity (△), protease activity (∗), *p*H (■), cell mass concentration (●), glucose concentration (□) and total soluble protein (O) in batch tannase fermentation by *L. plantarum*.

Luedeking-Piret model was not able to predict the tannase activity precisely since the unstructured models did not hold a term for the action of protease enzyme during the later stages of the fermentation. Fig. 6 shows the experimental and model predictions of cell growth by logistic model and tannase activity by logistic incorporated Luedeking-Piret model at the optimized conditions. The estimated kinetic model parameters for batch tannase production by *L. plantarum* are given in Table 2. The value of μ_0, α and β is 0.13 (h^{-1}), 5.5 and 0.685, respectively. The values of α and β signifies that the tannase production by *L. plantarum* is growth associated since the magnitude of the growth associated parameter 'α' is much greater than the magnitude of non-growth associated parameter 'β' in Luedeking-Piret model.

Conclusion

The effect of various medium compositions on extracellular tannase production by *L. plantarum* MTCC 1407 was studied. The *L. plantarum* MTCC 1407 has the ability to produce maximum tannase within a short period of cultivation. Kinetic profile of cell growth and tannase production was predicted using unstructured kinetic models. The use of tannase on a large scale remains dubious due to increased production cost and insufficient knowledge about the enzyme mechanism and its regulation. Therefore, it is

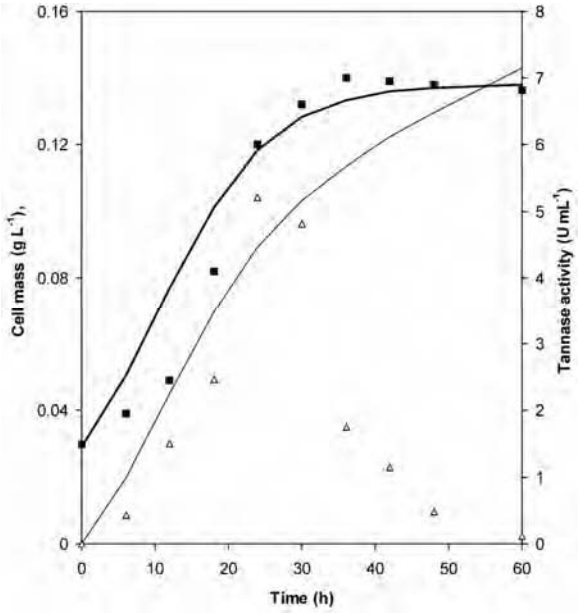

Fig. 6—Experimental and model predictions of cell growth (■) by logistic model (——) and tannase activity (▲) by logistic incorporated Luedeking-Piret model (—) for tannase fermentation by *L. plantarum* at the optimized medium composition (M3).

Table 2—Unstructured kinetic model parameters evaluated using batch data for tannase production by *L. plantarum*

Kinetic model parameters	
μ_o (h^{-1})	0.13
X_{max} (g L^{-1})	0.14
X_0 (g L^{-1})	0.029
α (U gX^{-1})	5.5
β (U gX^{-1} h^{-1})	0.685

necessary to understand the regulation, catalytic capacity and other aspects of optimization in order to increase its production at industrial scale. Further attempts will be made in the future to optimize the process parameters using statistical methods for tannase production in a modular fermentor.

Nomenclature

μ_o [h^{-1}]	Initial specific growth rate
X_{max} [g L^{-1}]	Maximum cell mass concentration
X_0 [g L^{-1}]	Initial cell mass concentration
α [U gX^{-1}]	Growth associated rate constant for product formation
β [U gX^{-1} h^{-1}]	Non growth associated rate constant for product formation

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