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Kinetic Studies on Microbial Production of Tannase Using Redgram Husk

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Abstract**—**Tannase (tannin acyl hydrolase, E.C.3.1.1.20) is an important hydrolysable enzyme with innumerable applications and industrial potential. In the present study, a kinetic model has been developed for the batch fermentation used for the production of tannase by *A.flavus* MTCC 3783. Maximum tannase activity of 143.30 U/ml was obtained at 96 hours under optimum operating conditions at 35^{o}C, an initial pH of 5.5 and with an inducer tannic acid concentration of 3% (w/v) for a fermentation period of 120 hours. The biomass concentration reaches a maximum of 6.62 g/l at 96 hours and further there was no increase in biomass concentration till the end of the fermentation. Various unstructured kinetic models were analyzed to simulate the experimental values of microbial growth, tannase activity and substrate concentration. The Logistic model for microbial growth , Luedeking - Piret model for production of tannase and Substrate utilization kinetic model for utilization of substrate were capable of predicting the fermentation profile with high coefficient of determination (R^2) values of 0.980, 0.942 and 0.983 respectively. The results indicated that the unstructured models were able to describe the fermentation kinetics more effectively.

Keywords**—***Aspergillus flavus,* Batch fermentation, Kinetic model, Tannase, Unstructured models.

I. Introduction

TANNASE or tannin acyl hydrolase (E.C.3.1.1.20) is an inducible enzyme produced by variety of microorganisms such as fungi, bacteria and yeast [1]. It catalyses the hydrolysis of hydrolysable tannin such as tannic acid, releasing glucose and gallic acid. Gallic acid has significance in food and pharmaceutical industries [12]. Tannase enzyme has been used in the preparation of instant tea, wine, beer, and coffee flavored soft drinks and also as additive for detanniffication of food [6]. In addition, it is also used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid ester [5].

Tannase can be obtained from plant, animal and microbial sources. From plant sources, the enzyme is present in tannin rich vegetables mainly in their fruits, leaves, branches and barks of trees like konnam, mirobolano and badul [7]. Many fungal species have been proved for its ability to produce tannase that have higher enzyme activity compared to bacterial and yeast tannase. The most important source to obtain the enzyme is by microbial way, because the produced enzymes are more stable than similar ones obtained from other sources [6]. In this study, the kinetics of tannase production using *A. flavus* with redgram husk was studied and various unstructured kinetic models were used to characterize the fermentation process.

Mathematical models of culture condition can be used as tools to improve enzyme production. Adequate models allow the estimation of kinetic parameters for both process optimization with enzyme production and substrate consumption and simultaneously cell growth prediction [14]. The use of models may lead to development of better strategies for the optimization of the fermentation process and thus ensures its economic viability [3].

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II. Materials and Methods

A. Chemicals

Chemicals used in the experiments were purchased from Hi-Media, Mumbai and were of the highest purity.

B. Microorganism and Culture Maintenance

The fungal organism *Aspergillus flavus* (MTCC 3783) for tannase production obtained from IMTECH, Chandigarh, India was maintained in Modified Czapek Dox agar media with a composition of sucrose 30 g/l, sodium nitrate 2 g/l, magnesium glycero phosphate 0.50 g/l, potassium chloride 0.50 g/l, dipotassium sulphate 0.35 g/l, ferrous sulphate 0.01 g/l and agar 12 g/l at 30ºC for 7 days.

C. Inoculum Preparation

The inoculum required for submerged fermentation is prepared using Modified Czapek Dox medium harvesting the spores from 96 hours old culture (grown at 35ºC). The spore suspension was collected in sterile falcon tube and stored at 4ºC until the actual study.

D Batch Fermentation

A modular fermentor (New Brunswick, USA) of 3L capacity was used to study the kinetics of tannase enzyme production. The Modified Czapek Dox Medium of 1L and 3% (w/v) of substrate redgram husk was sterilized for 15 minutes at 121°C and was cooled after the sterilization. 50 ml of the seed culture of *A. flavus* was used to inoculate the sterile medium. The production of tannase was carried out under optimum operating conditions at 35^{o}C, an initial pH of 5.5 and with an inducer tannic acid concentration of 3% (w/v) for a fermentation period of 120 hours. The samples were drawn at

regular time intervals and analyzed for tannase activity, biomass concentration and residual substrate concentration.

E. Assay of Tannase

0.05 ml of enzyme solution was incubated with 0.3 ml of 1.0 % (w/v) tannic acid and 0.2 M acetate buffer (pH 5.0) at 40°C for 10 min and then the enzyme production was stopped by cooling to 0°C by the addition of 3 ml Bovine Serum Albumin (1 mg/ml), which precipitates the remaining tannic acid. Simultaneously, a control without the enzyme was incubated and the samples were analyzed. The tubes were then centrifuged (5000 x g, 10 min) and the precipitate was dissolved in 3 ml of Sodium Dodecyl Sulphate - triethanolamine (1% (w/v) SDS in 5 % (v/v) triethanolamine) solution and the absorbency was measured at 530 nm after addition of 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 N HCl) .

One Unit of Tannase activity is defined as the amount of enzyme required to hydrolyze one micro mole of ester linkage of tannic acid in 1 min at specific condition [8].

F. Biomass Estimation

A known volume of fermented broth was taken and centrifuged. Then the supernatant separated out from the fermented broth for analyzing the activity and the settled cell mass was dried and weighed. The difference between initial and final weight of centrifuge tube will be the weight of the cell mass [11].

G.Estimation of Tannin

The estimation of tannin content was done the protein precipitation method of [4]. Dried substrates were ground finely in methanol and kept overnight at 4°C. One ml of extract was taken in a tube and 3 ml of Bovine Serum Albumin solution was added and kept for 15 min at room temperature. The tubes were centrifuged at 5000 x g for 10 min, supernatant was discarded and pellet was dissolved in 3ml of Sodium Dodecyl Sulphate - triethanolamine solution. One ml of $FeCl_3$ solution was added and tubes were kept for 15 min at room temperature for color stabilization. The color was read at 510 nm against the blank using UV Spectrophotometer.

H.Kinetic Model for Fungal Growth

Fermentation models consist of two classes: structured models, which consider intracellular metabolic pathway, and unstructured models, which assume biomass as one variable. Unstructured models are much easier to use and therefore, it is used in this research.

Unstructured Model

Unstructured kinetic models are the most frequently employed for modeling microbial systems based on simplicity and technical robustness. However, the formulation of an unstructured model was based on Monod model and logistic model [2], [13]. Unstructured models are simpler than the structured models. Thus, it is necessary to measure smaller numbers of important components. So, the model may describe at least the evolution of biomass, carbon sources, products and often, another substrate, mainly nitrogen [10]. In a typical fermentation process, a number of these rate processes are important, viz, the rate of change of the amount of biomass and its different components, the rate of consumption of nutrients, and the rate of production of products and metabolites [15]. In general, the biomass growth is modeled using Monod's equation or logistics equation for the specific growth of cells. The coefficient of the microbial decay for the microbial growth is also included in the differential equation [10].

I. Kinetic Models for Growth Kinetics

The most widely used unstructured models to describe cell growth are the Monod kinetic model and Logistic growth model.

Monod Model

Monod model is the first model used to represent the growth kinetics of microorganisms. Monod model [9] relates the specific growth rate and the concentration of the limiting substrate and is described by (1). The relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics.

$$\mu = \frac{\mu_m S}{K_s + S} \tag{1}$$

Monod model relates the growth rate to the concentration of a single growth limiting substrate [$\mu = f(s)$] via two parameters, the maximum specific growth rate (μ_m) and the saturation constant for substrate (K_S). Since growth is a result of catabolic and anabolic enzymatic activities, these processes, i.e., substrate utilization or growth associated product formation can also be quantitatively described on the basis of growth models.

Logistic Growth Model

The logistic growth model is a substrate independent model. The logistic model states that the rate of growth of the cell is proportional to the cell mass concentration present at any time. When the cell mass reaches the stationary phase there is no growth and hence the rate becomes zero. The growth rate thus depends on how far the cell mass concentration is away from the stationary phase.

$$\frac{dX}{dt} = kX \tag{2}$$

Equation (2) implies that the growth rate increases with an increase in cell mass concentration and is independent of the substrate concentration. In reality, the growth of the cell is governed by a hyperbolic relationship and the logistic equation is given by,

$$\frac{dX}{dt} = k(1 - X / X_s)X \tag{3}$$

Let $\beta = 1/ X_s$, then (3) becomes

$$\frac{dX}{dt} = k(1 - \beta X\ )X \tag{4}$$

where X is the biomass concentration (g L^{-1}), k is the rate constant (h^{-1}) and β is the logistic constant. The logistic curve is sigmoidal and leads to a stationary population of size X_S = 1/ β. Integrating (4) with the initial conditions, X = X_0 at t = 0 gives a sigmoidal variation of X(t) that may empirically represent both the exponential and stationary phase is given by,

$$X = \frac{X_o e^{kt}}{\left[1 - \frac{X_o}{X_s}\left(1 - e^{kt}\right)\right]} \tag{5}$$

where X_0 is the initial biomass concentration (g L^{-1}) and t is time (hr). Monod and the other models predict the growth stops when the limiting substrate is exhausted. In reality due to the accumulation of toxic metabolites or due to the inhibition, the growth may stop even when substrate is present. These conditions are taken care of by the logistic model. This model neglects the effect of substrate concentration on the growth rate. The advantage of using this model is sigmoidal curve of X as a function of t that can represent growth both in the exponential and stationary phase.

J. Product Formation Kinetics Model

Luedeking-Piret Model

This two parameter kinetic expression, often termed Luedeking-Piret kinetics has proved extremely useful and versatile in fitting product formation data from different fermentation. According to this model, the product formation rate depends upon both the instantaneous cell mass concentration (X) and growth rate (dX/dt) in a linear manner.

$$\frac{dp}{dt} = \alpha\ \frac{dX}{dt} + \beta X \tag{6}$$

where p is the product concentration (g L^{-1}), α is the growth associated product formation constant and β is the non-growth associated product formation constant, (h^{-1})

Substrate Utilization Kinetics Model

The substrate utilization kinetics is given by the equation (7), which considers substrate conversion to cell mass, to product formation and substrate consumption for cell maintenance [16]

$$\frac{dS}{dt} = \frac{1}{Y'_{X/S}}\frac{dX}{dt} - \frac{1}{Y'_{P/S}}\frac{dp}{dt} - kX \tag{7}$$

where $Y'_{X/S}$ is the biomass yield coefficient and $Y'_{P/S}$ is the product yield coefficient.

III. Results and Discussions

A. Kinetics of Tannase Production

The production of tannase was carried out under optimum operating conditions at 35°C, an initial pH of 5.5 and with an inducer tannic acid concentration of 3% (w/v) for a fermentation period of 120 hours. The samples were drawn at regular time intervals and analyzed for tannase activity, biomass concentration and residual substrate concentration and the results are shown in Fig. 1. The tannase activity was found to increase with respect to fermentation time and reaches a maximum of 143.30 U/ml at the end of 96 hours of fermentation and then decreases gradually till the end of fermentation. The biomass concentration reaches a maximum of 6.62 g/l at 96 hours and further there was no increase in biomass concentration till the end of the fermentation.

The lag phase of 12 hours and an exponential phase of 84 hours were observed for the growth of fungi *A. flavus* and tannase enzyme production was very significant during the exponential phase of the microorganism. The rate of substrate utilization was found to increase rapidly till 96 hours in an exponential manner and it was found to be very low in the stationary phase upto 120 hours. The biomass yield coefficient ($Y_{X/S}$) was found to be 0.23 g of biomass/g of substrate and the product yield coefficient ($Y_{P/S}$) was found to be 18.03 U/g of substrate. Table I also showed that the kinetics of tannase enzyme production using *A. flavus* with redgram husk as substrate.

TABLE I
KINETICS OF TANNASE ENZYME PRODUCTION USING *A. FLAVUS* WITH RGH

Run No	Time (Hr)	Biomass Concentration (X), G/L	Tannic Acid Concentration (S), G/L	Tannase Activity (P), U/Ml
1	0	0.52	30.88	0.00
2	12	1.10	28.14	10.14
3	24	1.87	25.11	24.59
4	36	2.75	21.22	37.14
5	48	3.92	16.09	61.36
6	60	5.10	10.95	82.12
7	72	5.98	8.25	98.12
8	84	6.23	6.12	120.14
9	96	6.62	5.14	143.30
10	108	6.60	4.62	141.30
11	120	6.48	4.21	136.21

The unstructured models provide a good approximation of the fermentation profile even though the complete mechanism of microbial growth is not considered in the models. The Monod model (R^2 = 0.35) used to find maximum specific growth rate (0.0158 h^{-1}) and Monod constant (9.65 g L^{-1}), Logistic model (R^2 = 0.98) used to estimate specific growth rate (0.061 h^{-1}). Among the two empirical kinetic models tested the logistic model for microbial growth was suited. Substrate utilization kinetic model (R^2 = 0.983) was used to find the yield coefficient (0.377) Luedeking - Piret model (R^2 = 0.942) was used to find growth associated parameter 'α' (18.02) and non-growth associated parameter 'β' (0.041). The

R^2 of the experimental and the predicted values were analyzed to find the best-suited model for tannase enzyme production.

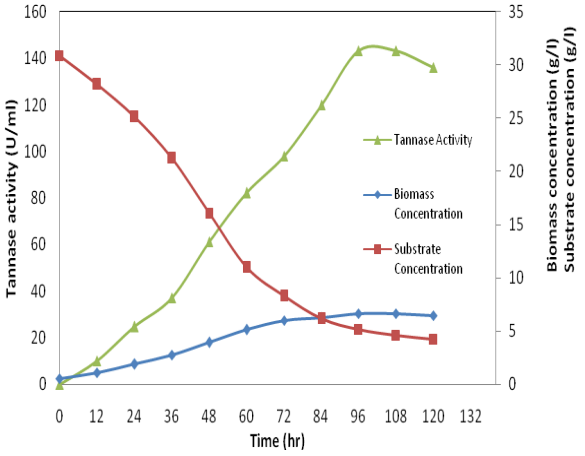

Fig. 1 Tannase enzyme Production using *A. flavus* with RGH as substrate under optimum conditions

IV. Conclusion

The statistical analysis of the experimental data on kinetic studies showed that the unstructured models could satisfactorily explain the batch kinetics of the tannase enzyme production using *A.flavus* MTCC 3783 with redgram husk using modular fermenter. The logistic model for microbial growth, Luedeking - Piret model for tannase production and substrate utilization kinetic model for tannic acid utilization kinetics were found as best models in predicting the formation profile of tannase production more accurately.

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