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Non-growth associated production of enzymes in solid state fermentation system: Its mathematical description for two enzymes produced by Bacillus licheniformis M27

M. V. Ramesh, N. C. L. N. Charyulu, Nagin Chand, B. K. Lonsane

Abstract Logistic model, based on more general and realistic assumptions, has been derived to express the production of two non-growth associated enzymes by Bacillus licheniformis M27 in a solid state fermentation system. The model explained the production of alpha-amylase and neutral protease with correlation coefficients ranging between 0.974 and 0.985 in basal and standardized wheat bran media. It is apparent from the values of parameters in the model that the rate constant in standardized medium was lower ($0.15\ l/h^{-1}$) than in the basal medium ($0.32\ l/h^{-1}$), though higher maximum enzyme titres (1.7 times) were observed in the former medium. The data thus indicate dependence of enzyme titres on the maximum biomass formed. The model represents a significant advance in model formulation as it recognizes and takes care of all other products (enzymes etc) formed during fermentation. The model may prove useful in optimizing product synthesis, design of bioreactor and determination of harvest time, especially due to its adequacy and efficiency. Models for predicting product formation in solid state fermentation system are scarce and confined to fungal fermentations. No such model for bacterial solid state fermentation system was available earlier.

1 Introduction

Work on mathematical modelling of the behaviour of microorganisms in solid state fermentation (SSF) system has long been stymied by the general apathy towards SSF processes and consequent concentration of efforts on submerged fermentation(SmF) technique in the West. The mathematical modelling, therefore, has been mostly confined to SmF processes thus far [1–4]. The recent realization of several advantages of SSF over SmF techniques [5, 6] has resulted in a surge of interest in SSF technique throughout the world [7, 8] and its extension to production of those bacterial and fungal metabolites which were earlier produced traditionally or conventionally by employing SmF processes [9–12].

The impasse on the mathematical models applicable to SSF processes [5] was recently overcome by developing a kinetic model for relationship between biomass and substrates as well as the dependence of biomass on temperature [13]. The mathematical formulations were also developed to account for the metabolic heat generation during fermentation and the consequent temperature rise of the fermenting solids [14]. The models for predicting temperature distribution in fermenting solids [15, 16] as well as fungal growth with respect to mass transfer in media solidified by agar, gelatin or other gelling agents [17–20] are also available. These studies have been confined to SSF system involving fungal cultures, while no mathematical model is available on bacterial SSF processes except for a recent publication on biomass formation [21]. The growth characteristics of fungal cultures in SSF system involve the penetration of the solid substrate particles by fungal hyphae [5, 22] in contrast to simple absorption or adherance of the bacterial cells to solid substrate particles in bacterial SSF system [23]. The metabolic and biosynthetic processes in the latter case, therefore, may differ from those in the former system and, consequently, the models developed for fungi may not be applicable to the bacterial SSF system.

This communication describes an attempt to mathematically formulate the production of enzyme in bacterial SSF system. The efficacy of the model, as determined by its testing on the data generated on the production of a major and a minor enzymes by the same culture formed in nutritionally different two media in bacterial SSF system, is reported. Such a system, selected for model testing, has high potentials in industrial exploitation and involves the production of thermostable alpha-amylase by Bacillus licheniformis M27 as a major product in SSF process. The culture also concomitantly produces neutral protease as a minor product [12, 23, 24].

2 Experimental

2.1 Modelling aspects

Classical approach was used in formulating a model to analytically describe the production of enzymes in bacterial SSF system. Simple, tenable assumptions were postulated to

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facilitate the integration of the different classical differential forms, with a view to achieve the model which is amenable to the transformation and afford help in fitting and parameter estimations. The model relationships were linearized by suitable transformation and parameters estimated using the technique of least squares on IBM compatible computer.

2.2
Fermentation aspects

B. licheniformis M27, isolated from the soil sample collected from municipal waste dump area [12], is maintained as described earlier [11]. The basal wheat bran (WB) medium contained 100 g WB moistened with 200 ml of 40 mM phosphate buffer (pH 7.0). Diammonium hydrogen phosphate at 3.3% (w/w) level, based on the weight of commercial WB, was added to basal medium to obtain standardized WB medium. In each case, 15 g moist medium in 250 ml capacity Erlenmeyer flasks was autoclaved at 121°C for 60 min, cooled to about 35°C, inoculated and incubated in inclined position in static condition. The thickness of the layer of the moist WB medium in the flask was less than 3 cm and the flasks were plugged with cotton to facilitate mass transfer. The media were not aerated. Samples were removed every 4 h for the analysis. The seed inoculum was developed in nutrient broth containing 0.3% soluble starch for 16–18 h at 35°C and used at 20% (v/w) level, based on the weight of the commercial WB. The methodology used for extraction of the enzyme from dry bacterial bran (DBB), clarification of the extract, assay technique for alpha-amylase and the definition of alpha-amylase unit were as reported elsewhere [12]. Neutral protease was assayed by modified method of Kunitz [25]. The reaction mixture contained 1 ml of 1% Hammerstein casein, 1 ml of 100 mM phosphate buffer (pH 7.0) and 0.5 ml of the appropriately diluted enzyme extract. It was incubated at 60°C for 10 min and the reaction was stopped by adding 3 ml of 5% (w/v) TCA solution. The precipitated casein was removed by centrifugation and the clear supernatant was taken for estimating aromatic amino acids by Folin–Ciocalteau's reagent [26]. One unit of neutral protease is defined as the enzyme which releases the amino acids equivalent to one micromole of tyrosine per min under the assay conditions.

3
Results and discussion
3.1
Basic concepts in the model

Diverse kinds of logistic, empirical and structural models have been developed by many workers and applied to predict product formation at any given time in the fermentation processes involving the use of media solidified by various gelling agents [17–19]. In the present studies, a kinetic approach with mechanistic scheme was selected, based on the theoretical understanding of the metabolic process in its entirety, to formulate suitable model to describe the production of metabolites under SSF process. The selection of the approach in developing the model has also been influenced by various factors such as simplicity, logistic inferences and imagination, as well as practical applicability to the SSF technique. The kinetic scheme selected in the present studies is based on a general simplified approach that the system as a whole is governed by a single empirical rate constant, which may be considered as the net total effect of all the external and internal rates of related and relevant processes. It, thus, ignores all the phenomena in respect of oxygen, carbon dioxide, substrate and solute components from bulk phase to cell wall as well as through the cell internals per se, but accounts only for the formation of the end product of interest. It is also assumed that no substrate is utilized for cell growth during stationary phase as only negligibly small amounts of the substrate is required for maintaining the cell mass during this phase.

3.2
Model development

Production of alpha-amylase and neutral protease by B. licheniformis M27 in SSF system is non-growth associated, i.e. formed after the log phase of the growth of the culture is over [24]. It is, therefore, logical to express the rate of enzyme produced, $[P]$, as independent of cell growth rate (but not of ultimate biomass formed), by the following equation:

$$\frac{d[P]}{dt} = K'[P][S]. \tag{1}$$

Assuming that the substrate is available for the production of various enzymes and also other metabolites during the stationary phase, then the product of interest, $[P]$, can be given by the material balance as:

$$([S_o] - [S])\, Y_p = [P] - [P_o] + Y_p \sum \frac{P_i - P_{io}}{Y_{ip}}, \tag{2}$$

where Y_p is the yield per unit substrate for $[P]$, and the terms enclosed in the summation sign express all other metabolites and enzymes (P_i), which are being produced concomitantly, with due consideration to their respective yields (Y_{ip}). $[S_o]$ and $[P_o]$ are substrate and product concentrations at the first measurable appearance of the enzyme of interest, (i.e. at time t_o while P_{io} indicates the concentration of other metabolites and enzymes at time t_o. After rearrangement, Eq. (2) becomes:

$$[S] = [S'_o] - \frac{[P]}{Y_p}, \tag{3}$$

where $[S'_o]$ is the total effective substrate concentration for $[P]$ at $t = t_o$:

$$[S'_o] = [S_o] + \frac{[P_o]}{Y_p} - \sum \frac{P_i - P_{io}}{Y_{ip}}. \tag{4}$$

Defining $[P_m]$ as the maximum possible product of interest, and considering it as equal to $[S'_o]$. Y_p:

$$[P_m] = [S_o].\, Y_p + [P_o] - Y_p \sum \frac{P_i - P_{io}}{Y_{ip}}, \tag{5}$$

or:

$$[P_m] = [S'_o].\, Y_p. \tag{6}$$

Incorporating the value of Y_p in Eq. (3), we get:

$$[S] = [S'_o]\left(1 - \frac{[P]}{[P_m]}\right). \tag{7}$$

Substituting for $[S]$ in Eq. (1), one obtains:

$$\frac{dP}{dt} = K[P]\left(1 - \frac{[P]}{[P_m]}\right), \tag{8}$$

where

$$K = K'[S'_o]. \tag{9}$$

On integration with the boundary conditions, $[P] = [P_o]$ at $t = t_o$, the Eq. (5) yields:

$$\ln\left(\frac{[P]}{[P_m]-[P]}\right) = \ln\left(\frac{[P_o]}{[P_m]-[P_o]}\right) + K(t-t_o). \tag{10}$$

Taking $t_o = 0$, and rearranging Eq. (10), one obtains:

$$[P] = \frac{[P_o]\exp(K.t)}{1-([P_o][P_m])[1-\exp(K.t)]}. \tag{11}$$

Equation (11) is the well known logistic equation which is widely reported in literature. However, its derivation, as presented here, is more rigorous and general than traditionally understood. Therefore, the logistic equation, with basis of this nature, can be expected to be much more widely applicable.

If the product of interest is an enzyme, the convenient way of its assay is in terms of its activity in units, which can be related to its concentration by the following:

$$A = \varepsilon.[P], \tag{12}$$

where ε is the activity coefficient per unit concentration of $[P]$. Replacing $[P]$, $[P_o]$ and $[P_m]$ in Eq. (11) by $\varepsilon.[P]$, $\varepsilon.[P_o]$ and $\varepsilon.[P_m]$ one obtains, after incorporating in Eq. (11):

$$A = \frac{A_o.\exp(K.t)}{1-(A_o/A_m)[1-\exp(K.t)]}, \tag{13}$$

The Eq. (13) thus, gives an explicit function for A with time.

The fit of the model to the experimental data on the production of thermostable alpha-amylase in two nutritionally different media and concomitantly produced neutral protease in one medium by B. licheniformis M27 in SSF system was, therefore, attempted.

3.3
Pattern of alpha-amylase production

The experimental values of the production of bacterial thermostable alpha-amylase by B. licheniformis M27 at different fermentation times in basal and standardised WB media in SSF system are presented in Fig. 1. The data show extremely low production of alpha-amylase, which was 1600 and 1800 units/g DBB in basal and standardized media at 36 and 28 h, respectively. The subsequent enzyme production was, however, at a greater pace (rate) until 56 and 72 h in basal and standardized WB media, respectively. The enzyme production remained more or less constant with further incubation up to 65 and 80 h, respectively in the above two cases. The highest enzyme production in basal WB medium was nearly 14,500 units/g DBB at 65 h against that of nearly 24,000 units/g DBB in standardized WB medium, though it occurred 16 h late in the latter case. It is interesting to note that the cell growth reached its maximum at about 28 and 36 h in standardized and basal WB media, respectively, before entering into the stationary phase [21]. As is evident from Fig. 1, the increase in the enzyme production occurring between 16 to 28 h in standardized and 28 to 36 h in basal WB media, i.e., during the period when the cells almost reached their highest growth, was of low magnitudes. The data, thus, showed that the production of alpha-amylase is not

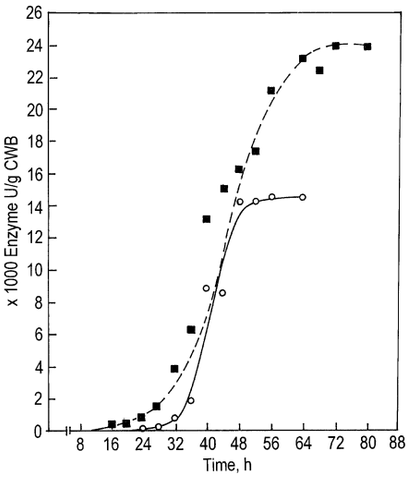

Fig. 1. Pattern of alpha-amylase production in basal and standardized WB media by B. licheniformis M27 in SSF system and the enzyme production curves as predicted by the logistic model formulated. ■: and ○: experimental enzyme production (in 1000 enzyme units/g DBB) in standardized and basal WB media, respectively; - - - - - and ——: predicted enzyme production curve by the model

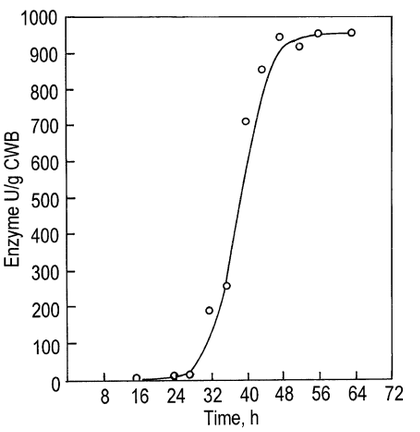

Fig. 2. Concomitant production of neutral protease by B. licheniformis M27 in standardized WB medium under SSF technique. ○: experimental enzyme units/g DBB, ——: predicted enzyme production curve by the model

associated with cell growth and is also of sigmoidal nature. Such a behaviour was also reported for alpha-amylase production in SmF processes by other workers [27, 28].

3.4
Concomitant production of neutral protease

Neutral protease is also produced by B. licheniformis M27 in standardized WB medium in the process for production of alpha-amylase in SSF system. The data on the production of neutral protease, concomitantly produced at different fermentation times, are presented in Fig. 2. Except for the lower production, the pattern of neutral protease formation is similar to that of alpha-amylase. The enzyme formation between 16 to 28 h is of low magnitude, i.e., 18 units/g DBB at 28 h but

increased at a faster rate of attain a peak of 940 units/g DBB at 48 h. In the subsequent fermentation period, it remained nearly constant at the peak value up to 64 h. The production of neutral protease is also of sigmoidal nature and non-growth associated, but it occurred up to 64 h against that of alpha-amylase up to 72 h. The concomitant production of protease by Bacillus cultures in the process for production of alpha-amylase has also been reported by other workers in SmF [29, 30] and SSF [31, 32] systems. The absence of the co-production of protease in case of B. megaterium 16M was, however, also reported [11].

3.5
Parameter estimation

The parameter values were estimated by a least squares technique using the transformed equation:

$$\ln\left(\frac{A}{A_m - A}\right) = \ln\left(\frac{A_o}{A_m - A_o}\right) + K(t - t_o). \qquad (14)$$

The plots of $\ln[A/(A_m - A)]$ vs. time for alpha-amylase production in basal and standardized WB media are presented in Fig. 3. Similar plot for neutral protease production in standardized WB medium is given in Fig. 4. The value of A_o was obtained from the intercept and the slope was equal to K. The estimated values of the parameters (Table 1) are reliable because of the use of integrated expressions, Eq. (14), and an intelligent guess in assigning A_m value from the experimental results. It is worth noting that the inherent errors may be introduced, if differential form, similar to Eq. (8), is used in the analysis of data concerning the product formed per unit time.

3.6
Fit of the model to experimental data

The product A (in so many units/g DBB) at different times in SSF system for alpha-amylase production by B. licheniformis M27 in basal and standardized WB media as well as concomitant production of neutral protease in standardized WB medium were used in estimating values of K and A_o by the method of least squares, using Eq. (14) as explained earlier. The efficacy and adequacy of the model are indicated by a close proximity of the predicted and experimental values for alpha-amylase production in two nutritionally different media (Fig. 1) as well as for another product, i.e., neutral protease produced concomitantly in standardized WB medium (Fig. 2). The results of the regression analysis are given in Table 1. The correlation coefficient for the models fitted for these enzymes ranged between 0.974 to 0.985 ($P \leq 0.0001$). The model basically predicts sigmoidal nature of the pattern of the formation of alpha-amylase and neutral protease, which is also observed experimentally in all these cases and even in nutritionally different media in case of the former enzyme.

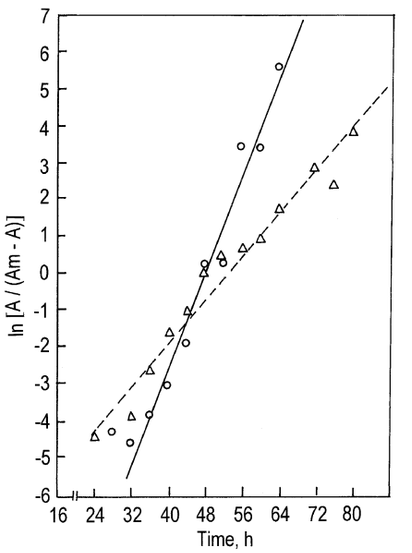

Fig. 3. The plot of $\ln[A/(A_m - A)]$ versus time, which was used in determination of constants in the logistic model developed for predicting alpha-amylase production by B. licheniformis M27 in basal and standardized WB media in SSF system. ○ and △: experimental values in basal and standardized media; ——— and - - - - - -: predicted values in basal and standardized media, respectively

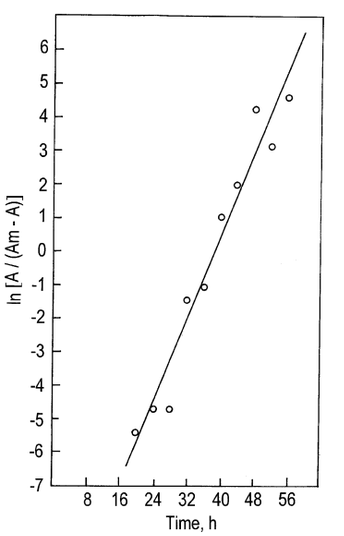

Fig. 4. Concomitant production of neutral protease by B. licheniformis M27 in standardized WB medium: The plot is as described in Figure 3 for determination of model constants. ○ and ———: experimental values and predicted enzyme production curve, respectively

3.7
Quality and importance of the model

The kinetic model, as developed in the present studies, offers a simpler approach where different factors are integrated into a quantifiable mechanistic scheme to explicitly predict the pattern of the production of the enzymes with time in SSF system and also the time at which assayable accumulation of the products (enzymes) occurs. It offers further insights into the general nature of the logistic models and hence reasons for much wider applicability of the same. These facts cannot be expected from or explained by the traditional derivation alone. The model is simple with only three parameters and clear-cut physical significance of each parameter. Its satisfactory fit to the production of alpha-amylase and neutral protease under SSF in a non-growth associated manner in two nutritionally different

Table 1. Estimated parameter values of the kinetic model and the data on the analysis of variance of the predicted and experimental values of the enzymes produced by B. licheniformis M27 in SSF system

Term	Basal WB medium	Standardized WB medium	
	Alpha-amylase	Alpha-amylase	Neutral protease
A_o, units/g DBB	3.21×10^{-2}	28.75	6.03×10^{-3}
A_m, units/g DBB	14,600	24,500	960
K, 1/h	0.3255	0.1500	0.3103
F–ratio	227.30	333.03	147.05
Correlation coefficient	0.985	0.982	0.974
Residual mean sum of squares (df)	3.1320 (7)	3.4107 (12)	6.9148 (8)

media indicates high efficiency. The estimation of parameter values is also relatively simpler. The rate constant and the limiting highest level of the production of these two enzymes give the all important information on the rate and amount of enzymes produced.

The maximum value of alpha-amylase activity was observed in standardized medium, although the rate constant was just half as much (0.15 l/h^{-1}) as compared to that in basal WB medium (0.32 l/h^{-1}). The titres of the enzymes formed are, therefore, probably related to the biomass produced in both the cases. The biomass formed in the former medium was higher (1.1×10^{13} cells/g DBB), while it was only 3.0×10^{11} cells/g DBB in the latter medium [21]. It appears that, the maximum biomass available, before the commencement of the production of enzymes, becomes relevant and should find a place in the model which may be coupled with the rate constant.

The model indicates that four different patterns may arise in the production of the enzymes by B. licheniformis in SSF system. If both K and A_m are low, then too little enzyme will be produced in too long a fermentation time, which represents by far the worst and most undesirable case. Low K and high A_m will take longer time to produce high quantities of the product (alpha-amylase, standardized medium as an example) while high K and low A_m can lead to quick enzyme production which will stagnate soon at lower product value (alpha-amylase, basal medium as an example). The most desirable combination will be a high K as well as a high A_m which will lead to quicker production and formation of highest amount of the product.

The model in the final form is similar to the familiar Pearl's (logistic) model. However, the model described here is more general in nature and closer to realities in terms of dealing with multiple products. The present model, in contrast to the existing models, recognises the production of other metabolites at various stages of the fermentation process. It, therefore, represents a significant improvement in the model formulation and the understanding of the kinetic behaviour. In addition, it provides requisite information for interpretation of observed formation of multiple products (enzymes etc.).

Similar models have been found applicable for diverse products under SmF processes [33–35]. The applicability of the presently derived model to SSF processes, where substrate diffusion is heterogenous and with concentration gradients [17], indicates efficacy and desired adaptability. The model may find its use in maximizing product synthesis under practical conditions (optimization), design of bioreactors and determination of harvest time, which can collectively result in development of efficient and economic technology for industrial exploitation.

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