

# Unstructured Kinetic Modeling of Glutathione Production by *Saccharomyces cerevisiae* NCIM 3345

Abhinandan Dhavale, Atul Vhanmarathi, Shrinivas Deshmukh  
and Seema Dabeer

## 1 Introduction

Glutathione (GSH) is a biologically active tri-peptide consisting of L-glutamate, L-cysteine, and glycine. It is an abundant and ubiquitous low-molecular-mass thiol in living tissues. GSH is an important component in the cellular mechanisms that protect against UV (Sollod et al. 1992), heavy metals (Perego et al. 1997), and many exogenous organic substances (Goto et al. 1995). GSH also plays a very critical role in sacrificial defensive mechanism against oxidative damage in organisms (Berhane et al. 1994). It is now widely used as a medicine, in health foods to prevent hepatotoxicity induced by acetaminophen, vinyl ethers or bromobenzene in animals and in the cosmetic industry. Glutathione majorly functions as an antioxidant, an immunity booster, and as a detoxifier (Pastore et al. 2003).

Recently several studies have described GSH producing yeast strains, which are commonly used for commercial production such as *Saccharomyces cerevisiae* and *Candida utilis* (Sakato and Tanaka 1992; Wei et al. 2003a, b). Many studies have tried to improve the GSH production by supplementing certain materials, such as glucose, minerals, ATP, and phosphorus to the culture medium (Li et al. 1998). The studies have also been reported for investigating the different concentration of amino acids and related compounds such as cysteine on glutathione production (Alfafara et al. 1992). Finally, optimization of fermentation process parameters and conditions were achieved so as to enhance the GSH production using *S. cerevisiae* (Cha et al. 2004).

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A. Dhavale (✉) · A. Vhanmarathi · S. Deshmukh · S. Dabeer  
K.I.T.'s College of Engineering, Kolhapur 416006, Maharashtra, India  
e-mail: abhibiocpd@gmail.com

As mentioned above, many kinds of literature provide information about the optimization of the fermentation process for GSH production using *S. cerevisiae*, but very few references in the literature provide information about kinetic modeling of the production processes. It is essential to have an in-depth understanding of the fermentation process so as to establish better controls at commercial scale production to achieve a consistent and quality product. The already known and well-established kinetics models help develop these strategies (Ghaly et al. 2005). In the present study GSH, production was carried out under the optimal condition and various renowned unstructured kinetic models were used to understand the kinetics of GSH production.

## 2 Materials and Methods

### 2.1 Microorganism

*Saccharomyces cerevisiae* NCIM 3345 was procured from NCL Pune, India. While all the media components (LR grade) were bought from Hi-media Ltd, Mumbai, India.

### 2.2 Culture Maintenance

*Saccharomyces cerevisiae* NCIM 3345 stock culture was maintained using nutrient media containing; Peptone 10 (g/L), Beef extract 3 (g/L), NaCl 5 (g/L). This culture was used to inoculate seed medium with working volume 100 mL containing Glucose 1 g, Peptone 0.5 g, Yeast extract 0.3 g and Malt extract 0.3 g. Seed flask i.e. inoculum was incubated at 30 °C for 24 h.

### 2.3 Fermentation

The GSH formation using *Saccharomyces cerevisiae* NCIM 3345 was achieved in the same 100 mL production medium in 250 mL Erlenmeyer flask containing these components in (g/L): Glucose 30, Yeast extract 30,  $\text{KH}_2\text{PO}_4$  0.6 and L-cysteine 0.6. The pH of media was set to 5.5 and further subjected to sterilization using autoclave at 121 °C for 30 min. Post sterilization, the flasks were inoculated with 8 % (v/v) of matured seed obtained from seed flask. To achieve desired growth and product formation, production flasks were incubated in an orbital incubator at 30 °C with a speed of 150 rpm for 16 h. Sampling was performed as per the protocol for estimation of biomass growth, glutathione production, and glucose utilization. All the analysis was carried out in triplicate.

## **2.4 Biomass Determination**

The concentration of cell biomass was obtained by calculating dry cell weight (DCW) of the cells. Eight mL broth was taken in pre-weighted centrifuge tubes and was subjected to centrifugation at 5000 rpm for 10 min at room temperature. Supernatant was discarded and the pellet was washed using distilled water (twice). The tubes containing pellets were further kept in hot air oven at 100 °C for drying. The weight of each tube was checked after 24 h of drying, ensuring all moisture had evaporated.

## **2.5 Glucose Determination**

To calculate the residual substrate concentration i.e. glucose in the production flask, the 3,5-Dinitrosalicylic acid method was used (Miller 1959).

## **2.6 Glutathione (GSH) Determination**

After 16 h of fermentation, broth was subjected to centrifugation to achieve biomass pellets, which were further suspended in 0.2 M phosphate buffer (pH 7.2) and disrupted by sonication. The glutathione concentration in the supernatant achieved after centrifugation was measured using published methods (Cohn and Lyle 1966), such as measuring the absorbance maxima ( $\lambda_{\text{max}}$ ) at 412 nm using a UV spectrophotometer (Thermo-Scientific CHEMITO 215D). A standard curve was generated by preparing samples of different known concentrations of GSH, which was used to determine specimen concentrations.

## **2.7 Fermentation Description Using Unstructured Kinetics Models**

### **2.7.1 Biomass Growth**

Various unstructured models had characterized the cell culture growth patterns in fermentation kinetics. In an unstructured model, the total biomass concentration (whole quantity) is considered as a single component in representations (Chandrasekhar et al. 1999). Many mathematical equations and theories are available in literature which can explain the sigmoidal relationship between the specific growth rate of cells and key limiting substrate used for biomass growth. In current

study, the most suitable kinetic model tested for describing cell growth was logistic equation.

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

where  $\mu_0$  are initial specific growth rate ( $\text{h}^{-1}$ ) and  $X_{\max}$  maximum attainable biomass concentration (g/L), which on integration, with the initial condition that at  $t = 0$ ,  $X = X_0$ , yields

$$\ln \frac{X}{(X - X_{\max})} = \mu_{\max} t + \ln \frac{X_0}{(X_{\max} - X_0)} \quad (2)$$

On rearrangement, and explicit function for biomass is obtained as:

$$X_t = \frac{X_0 e^{(\mu_0 t)}}{1 - \frac{X_0}{X_{\max}} (1 - e^{(\mu_0 t)})} \quad (3)$$

### 2.7.2 Glutathione Production Kinetics

The GSH production kinetics was analyzed according to the Luedeking-Piret equation. This model illustrates that the rate of product formation is mainly dependent on; (1) the desired cell mass for product formation ( $X$ ) and (2) the rate at which biomass is increasing with respect to time ( $dX/dt$ ) in a linear fashion.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (4)$$

In Eq. (4),  $\alpha$  and  $\beta$  are the constants determined experimentally, which provides the basis for classification of microbial metabolites into growth associated ( $\beta = 0$ ), non-growth associated ( $\alpha = 0$ ), and mixed ( $\alpha \neq 0$  and  $\beta \neq 0$ ). Integration of Eq. (4) using Eq. (3) and initial conditions, ( $X_0$ ,  $P_0$ ) yields,

$$P_t = P_0 + \alpha A(t) + \beta B(t) \quad (5)$$

In Eq. (5)  $A(t)$  and  $B(t)$  as follows,

$$A(t) = \left[ \frac{X_0 e^{\mu_0 t}}{1 - \left( \frac{X_0}{X_{\max}} \right) (1 - e^{\mu_0 t})} - 1 \right] \quad \text{and} \quad B(t) = \frac{X_{\max}}{\mu_0} \ln \left( 1 - \frac{X_0}{X_{\max}} (1 - e^{\mu_0 t}) \right)$$

Here, Eq. (5) can be used to calculate approx. GSH concentration produced at any given time ( $t$ ) in fermentation.

### 2.7.3 Glucose Consumption Kinetics

The modified Luedeking-Piret equation is used to describe the glucose consumption kinetics in yeast culture. This model consider that, the substrate utilized by cells mainly functions for biomass and product formation and maintenance activities in cell (Weiss and Ollis 1980).

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt} - \frac{1}{Y_{P/S}} \frac{dP}{dt} - K_e X \quad (6)$$

Substituting  $r_{fp} = -Y_{p/s} r_{fs}$  values in Eq. (6) and rearranging,

$$\frac{dS}{dt} = -\gamma \frac{dX}{dt} - \eta X \quad (7)$$

where  $r_{fp}$  is the rate of product formation,  $r_{fs}$  is the rate substrate utilization

$$\text{And } \gamma(gS/gX) = \frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}} \quad \eta(gS/gX.h) = \frac{\beta}{Y_{P/S}} + K_e$$

Equation (7) is the modified Luedeking-Piret equation for substrate utilization kinetics. Rearranging Eq. (7)

$$-\frac{dS}{dt} = \gamma + \frac{\eta}{\mu} \quad (8)$$

Integrating Eq. (8) with initial conditions  $X = X_0$  ( $t = 0$ ) and  $S = S_0$  ( $t = 0$ ) give

$$S_t = S_0 - \gamma m(t) - \eta n(t) \quad (9)$$

where

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

and

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

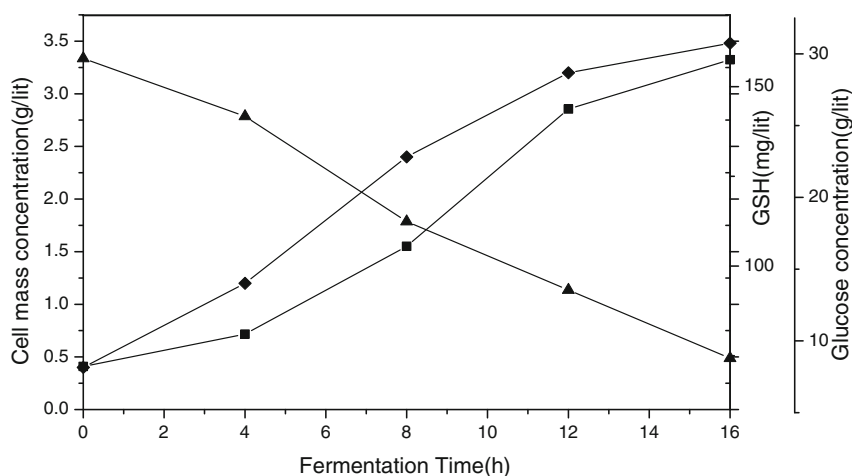
This Eq. (9) can be used to obtain the residual substrate concentration in the production media.

### 3 Results and Discussion

#### 3.1 Glutathione Production (Product Formation Kinetics)

The GSH production kinetics using *S. cerevisiae* NCIM 3345 was studied with the optimized medium and process parameters; temp at 30 °C, media pH 5.5 and mixing at 150 rpm. Figure 1, illustrates the kinetic study profiles of biomass formation, GSH production, and substrate consumption with respect to time using the optimized media and process parameters.

The rate of GSH production was found to be significantly increasing along with the exponential growth phase of micro-organisms proving that the GSH is being the growth associated product. The highest GSH concentration obtained was 157.5 mg/L in 16 h of the fermentation period. After 16th h, production reduces slowly. This might be due to the production of ethanol. GSH production and accumulation in yeast cells are favorable in lower ethanol concentration, while higher ethanol concentration inhibits the glutathione production. The maximum biomass concentration achieved was 3.48 g/L in 16 h duration as stationary phase hadn't contributed towards the increase in biomass concentration. First 4 h duration was an adaptation phase of cells to operating conditions and optimized production media. Next 6 h duration in fermentation was characterized as an exponential growth phase of the microorganism. The rate of product formation and substrate consumption were observed to be highest in this period. Yeast cells had consumed almost 92 % of the glucose till the fermentation ends and maximum GSH production was obtained at this stage correspondingly. The mathematical expression of obtained data from experimentation concluded that, unstructured kinetic models have the ability to explain and deliver process understating of the fermentation process for Glutathione production.



**Fig. 1** Fermentation profile of cell mass concentration (filled diamond), glucose concentration (filled triangle) and GSH production (filled square)

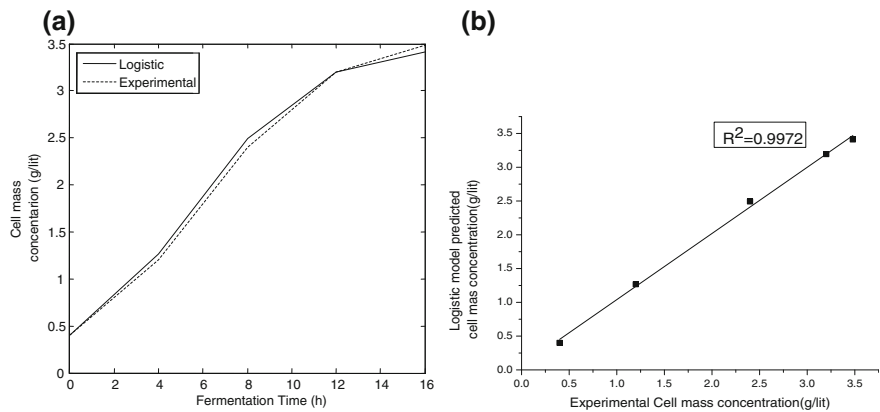
3.2 Unstructured Kinetic Models

The models used in this study had provided a good estimation of predictive capability despite having very limited cellular mechanism information. Following Table 1, gives the calculated values for the estimated kinetic parameters used in unstructured models.

The relationship between actual versus predicted values is explained briefly using statistical tools such as regression coefficient ( $R^2$ ) values for biomass growth, GSH production and glucose utilization profiles observed in fermentation. The regression coefficient measures the strength of linear relationship between the experimental (Actual) and predicted values obtained using the kinetic models. The linear relationship modeled by the straight line illustrates the steady increase or decrease. In Fig. 2a unbroken line shows estimated the response of Logistic model and broken line experimental response. Figure 2b gives a comparison of actual

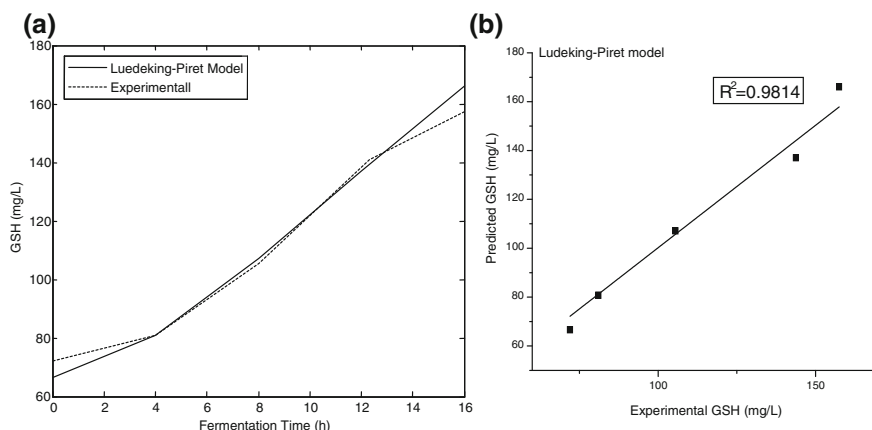
**Table 1** Estimated kinetic model parameters for GSH production

Parameter	Value
$\mu_0$ (1/h)	0.37
$X_{\max}$ (g/L)	5.60
$X_0$ (g/L)	0.88
$\alpha$ (gP/gX)	8.95
$\beta$ (gP/gX.h)	2.04
$\gamma$ (gS/gX)	4.10
$\eta$ (gS/gX.h)	0.52
$Y_{x/s}$ (g/g)	0.15
$Y_{p/s}$ (mg/g)	4.09
$Y_{p/x}$ (mg/g)	27.76
$K_e$ (g/g.h)	0.018

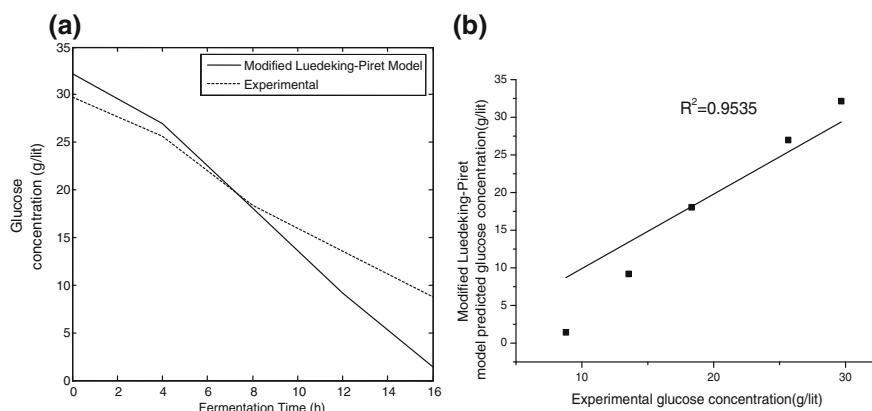


**Fig. 2** a Experimental (dashed line) and model prediction (dotted line) biomass concentration. b Comparison of actual versus predicted biomass concentration by Logistic mode

versus predicted values obtained for biomass concentration with ( $R^2 = 0.9972$ ). Figure 3a show response of Luedeking-Piret model for GSH production and Fig. 3b show comparison between experimental and model predicted GSH production with ( $R^2 = 0.9814$ ). In Fig. 4a response of Modified Luedeking-Piret for glucose consumption and comparison of experimental and model predicted consumption shown in Fig. 4b. ( $R^2 = 0.9535$ ). As observed in figures, product formation rate was maximum in the exponential growth phase of the cells i.e. ( $\alpha' \gg$  than  $\beta'$ ) thus, the product glutathione was considered as primary metabolite due to its growth associated production during the fermentation process.



**Fig. 3** a Experimental (dashed line) and model prediction (dotted line) of GSH. b Comparison of experimental and predicted GSH by Luedeking-Piret model



**Fig. 4** a Experimental (dashed line) and model prediction (dotted line) Glucose consumption. b Comparison of experimental and predicted glucose consumption by modified Luedeking-Piret model



## 4 Conclusion

The mathematical modeling and expression of unstructured kinetics models were found to be relatively simple and easy. The greater prediction capability of the models had made them highly flexible for applying in any fermentation processes. In the present study, the models like Logistic equations for biomass growth, Luedeking-Piret model for GSH production and modified Luedeking-Piret model for glucose consumption i.e. substrate utilization were found best suitable to describe the glutathione production process using *Saccharomyces cerevisiae* NCIM 3345. All the models tested were able to predict profiles with higher  $R^2$  values. The parameter of Luedeking-Piret model clearly indicated that GSH production is strictly growth associated.

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