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Mathematical modeling for L-methionine fermentation by a mutant strain of *Corynebacterium glutamicum* X300

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ABSTRACT

In this present investigation, a mathematical model has been developed for shake-flask fermentation of L-methionine by a multiple analogue resistant strain *Corynebacterium glutamicum* X300. Using computer simulation, different parameters of kinetic model were evaluated. The kinetic model developed in this present investigation revealed better assumptions for bacterial growth, L-methionine accumulation and substrate utilization compared to the assumptions as predicted in the available literature.Sensitivity analysis revealed that the predicted model has heighest impact on L-methionine fermentation. In this present study, non-growth associated product formation coefficient had maximum inhibitory effect on L-methionine fermentation.

Introduction

L-methionine is a sulfur-containing essential amino acid required in human diet.Plant and animal proteins are deficient in L-methionine content and consequently fail to meet the nutritional requirement for L-methionine. Deficiency of L-methionine leads to hair loss, depression, rheumatic paralysis, toxemia etc [1].The market for this amino acid increases constantly. L-methionine can be produced by chemical synthesis, protein hydrolysis and fermentation.Chemical synthesis produces racemic mixture of DL -methionine from which separation of L-methionine is difficult and protein hydrolysis is a very complex process [2].Thus, now a days, maximum concern is focused on fermentatine production of this amino acid. A high-yielding strain of *Corynebacterium glutamicum* has been already developed in our laboratory [3].In this present study, a kinetic model has been developed for modeling the shake-flask fermentationof L-methionine by a multiple analogue resistant strain of *Corynebacterium glutamicum* X300.

Materials and methods

Selection of microorganism: Previously, a regulatory mutant *Corynebacterium glutamicum* X1 (accumulated only 0.6 mg /ml L-methionine) developed in our laboratory by induced mutation using UV irradiations from its parent strain *Corynebacterium glutamicum*(basically a L-glutamic acid producing bacterium which does not accumulate L-methionine) that was isolated from North Bengal soil was used for mutational study.*Corynebacterium glutamicum* X1 was then subjected for mutational studies as described below:

Chemical and Physical mutagensis: To develop a high L-methionine yielding strain, the above mentioned regulatory strain was subjected to mutational treatments using Ethyl Methane Sulfonate (EMS) and UV irradiations as Chemical and Physical mutagens respectively as f **Exposure to EMS:** 1 ml cell suspension (containing $3x10^8$ cells) was added to 9ml EMS solution of different concentrations (221.8 mmol /ml, 186.3 mmol /ml 76.9 mmol /ml respectively) and was incubated (10,20,30,40 and 60 minutes respectively). From each sample, 1 ml cell suspension was then plated on CD agar medium and kept at 30^0C for 48 h.

Treatment with UV irradiation: 2 ml cell suspension (containing $3x10^8$ cells/ml) was taken in a petridish (5 cm diameter) and expose it to UV irradiation, using Hanovia germicidal lamp (15 Watt) from a distance of 12 cm for different periods of time (1-9 minutes). The UV treated cells were plated in similar ways as mentioned above.

Development of multiple L-methionine resistant strain: Multiple L-methionine analogue-resistant strain was develop by adding different L-methionine analogue (20-100 mg/ml) to the growth medium [namely: α-Methyl methionine , DL-ethionine , D-methionine sulphate and DL-norleucine] [3].

Composition of basal salt medium for L-methionine production: L methionine production was carried out using the following basal salt medium (per litre): glucose, 60 g; $(NH_4)_2SO_4$, 1.5 g ; K_2HPO_4, 1.4 g; $MgSO_4{\cdot}7H_2O$, 0.9 g; $FeSO_4{\cdot}7H_2O$, 0.01 g ;biotin, 60µg (Ganguly *et al*. 2014).

Optimum cultural conditions: Volume of medium ,25 ml; initial pH ,7.0; shaker's speed ,150 rpm; age of inoculum ,48 h; optimum cell density ,$4.0X10^8$ cells/ml; temperature28^0C and period of incubation ,72 h [4].

Composition of synthetic medium(per Liter): glucose, 100 g; $(NH_4)_2SO_4$, 8.0 g (in terms of nitrogen) ; K_2HPO_4 , 2.2 g; $MgSO_4.7\ H_2O$, 1.5 g ; $FeSO_4.7H_2O$, 0.03 g; KH_2PO_4,2.0 g ; $ZnSO_4.7H_2O$, 1.6 mg ; $CaCO_3$, 1.5 g; $Na_2MoO_4.2H_2O$, 5.0 mg; $MnSO_4.4H_2O$, 2.5 mg; biotin ,80 mg and thiamine- HCl, 70 µg [5].

Analysis of L-methionine: Descending paper chromatography was employed for detection of L-methionine in culture broth and was run for 18 hours on Whatman No.1 Chromatographic paper. Solvent system used includes n-butanol: acetic acid: water (2:1:1). The spot was visualized by spraying with a solution of 0.2 % ninhydrin in acetone and quantitative estimation of L-methionine in the suspension was done using colorimetric method [6]. All the chemicals used in this study were analytical grade (AR) grade and obtained from E mark .Borosil glass goods and triple distilled water used throughout the study.

Estimation of Dry Cell Weight (DCW): The cell paste was obtained from the fermentation broth by centrifugation and dried in a dried at 100^0C until constant cell weight was obtained [7] available in literature:

$$\frac{d}{dt}[B] = \mu_{max}\frac{[S]}{[S]+K_i(1+K_i)} \text{----------(1)}^{[8]}$$

[Where, μ_{max}= Maximum specific growth rate (h^{-1}); [S] = Substrate concentration ($mg.ml^{-1}$); Kg = Monod growth Constant for substrate ($mg.ml^{-1}$); Ki= Inhibition Constant for growth by product ($mg.L^{-1}$)]

$$\frac{d}{dt}[S] = -\frac{1}{Y_b}\frac{dB}{dt} - \frac{1}{Y_P}\frac{dP}{dt} \text{----------(2)}^{[9]}$$

[Where, $_{Yb}$ = Yield Cofficient biomass from substrate ($mg.mg^{-1}$); $_{Yp}$ = Yield Cofficient product from substrate ($mg.mg^{-1}$); P= Product Concentration ($mg.mg^{-1}$)]

$$\frac{d[P]}{dt} = X_{Pmax}.\frac{[S]}{K_p + \frac{[S]}{(1+\frac{[S]}{K_X})}}.B \text{----------(3)}^{[10]}$$

[Where, = X_{Pmax} = Maximum specific production rate; Kp= Monod product constant for the substract; Kx= Monod growth constant for the specific biotin Concentrstion ($\mu g.L^{-1}$)]

Using the logistic model, we can write:

$$\frac{dB}{dt} = \mu_{max}[S]\left(1-\frac{[B]}{[Bm]}\right) \text{----------(4)}^{[11]}$$

(Where, $[B_m]$ = Maximum Biomass Concentration)

This equation is substrate-independent. The cellular growth can be predicted from the following equation:

$$B = \frac{[Bo][Bm]^{*\mu_{max}}}{[Bm]-[B0]+[Bm]^{*\mu_{max-1}}} \text{----------(5)}^{[12]}$$

[Where, B_o= Initial Biomass Concentration ($mg.mg^{-1}$)]

This equation is applicable when t= 0 and B_o= B_m, thus, it shows the relationship between cellular growth and fermentation time. μ_m and B_m can be estimated from non-linear regression. According to Luedeking- Piret model, the rate of L-methionine production is linearly related to both bacterial cell mass (B) and growth rate (dB/dt) as:

$$\frac{dP}{dt} = C.\frac{dB}{dt} + \gamma B \text{----------(6)}^{[13]}$$

[Where, C= Growth associated product formation Coefficient ($mg.mg^{-1}$)]

L-methionine production is associated with bacterial growth rate only when C≠0 and γ+0.As the substrate utilized for cellular growth, maintenance of the cells as product formation, thus we can write:

$$-\frac{d[S]}{dt} = \frac{1}{Y_b}.\frac{dB}{dt} + 1Y_p.\frac{d[P]}{dt}.\ m_c.B \text{----------(7)}^{[14]}$$

[Where, Y_p= Yield Coefficient product from substrate ($mg.mg^{-1}$); [P] = Concentrationof product ($mg.mg^{-1}$) ; m_c = Maintenance Coefficient ($mg.mg^{-1}$).]

This equation describes the utilization of substrate for L-methionine production, bacterial growth and cellular maintenance.

Product formation can be calculated from the Bona and Moser's formula as follows:

$$P = C[B-B_o] + \frac{Bm\gamma}{\mu max}.\ln\frac{Bm-Bo}{Bm-B} \text{----------(8)}^{[15]}$$

[Where, B = Biomass Concentration at a given time ($mg.mg^{-1}$); B_m = Maximum Biomass ($mg.mg^{-1}$); B_o= Initial Biomass (($mg.mg^{-1}$)].

The Sum Squares of Weighed Residues for estimation of kinetic parameters were examined using the following formula:

$$SSWR = \sum_{X=0}^{a}\sum_{Y=0}^{b} D^2_{XY}/W^2_Y \text{----------(9)}^{[16]}$$

[Where, a= Number of experimental data; b=Number of process variable; D_{XY}= Difference between the model and experimental data of X^{th} variable in Y^{th} experimental point; WY=Maximum weight of variable($mg.mg^{-1}$)]

The Standard Error of Mean (SEM) of the variable was calculated using the following formula:

$$SEM(\Delta) = \frac{1}{a}\sum_{X=1}^{a}\Delta XY \text{----------(10)}^{[17]}$$

The Residual Variance of the error (R) was calculated as follows:

$$R = \frac{1}{a-1}\sum_{X=1}^{a}\left(\partial_{XY}-\Delta\right)^2 \text{----------(11)}^{[18]}$$

The statistical adequeacy for the acceptance of this model can be calculated as follows:

The definition of statistic 'λ' can be presented as follows:

$$\lambda = \frac{(a-b)a}{(a-1)b} = \sum_{Y=1}^{b}\frac{D_b^2}{R} \text{----------(12)}^{[19]}$$

The End Point Deviation (EPD) in L-methionine fermentation was estimated as follows:

$$EPD = \frac{[P]-[P]_C}{[P]_C} \times 100 \text{----------(13)}^{[20]}$$

[Where, [P] =Product Concentration in Experiment (mg.mg-1); $[P]_c$= Product Concentration in control (mg.mg-1)]

The mathematical problem regarding this modeling was solved using Microsoft EXCEL.

Results and discussion

In this present study, three basic aspects namely: bacterial growth (B), product formation (P) and substrate uptake [S] were considered as presented in Table1.

Table 1: Comparison of kinetic parameters between present model and Bona and Moser's model

Kinetic parameters		Present model	Bona and Moser's model
1.Bacterial growth	μ_{max}	0.468	0.20
	B_m	12.610	0.30
2.Substrate utilization	B_o	0.096	0.70
	Y_b	0.968	0.40
	Y_p	0.638	0.40
3.L-methionine production	m_c	0.121	0.10
	C	0.011	200
	Γ	0.126	0.166
4. Error values	Sswr	0.160	0.664
	Λ	0.093	28.66

L-methionine production was obtained in stationary phase .The calculated value of B_0 was lower than experimental value probably due to the viability of cells. This model depicts better resolution than Bona and Moser's model [(20)].L-methionine production is associated with bacterial growth in a very complex manner.By fitting the data of the present investigation in equation 7, we get Y_b=0.968 $mg.mg^{-1}$, Y_p=0.683 $mg.mg^{-1}$ and m_c=0.121.Thus, in our present study, it is found that the resolution is much better than Bona and Moser's experiment. The percentage changes in the estimation of L-methionine were depicted in Table 2 as follows:

Table 2: ED is response to ±5% and ±10% changes for each kinetic parameters in the present model

Kinetic parameters	$ED_{5\%}$		$ED_{10\%}$	
B_o	-	-	-	-
B_m	-0.013	0.013	-0.030	0.030
μ_{max}	-0.011	0.011	-0.010	0.010
Y_b	-	-	-	-
Y_p	-	-	-	-
m_c	-	-	-	-
C	-0.018	0.018	-0.009	0.009
Γ	-2.613	2.613	-3.618	3.618

Increase in γ has negative effect on ED, leading to inhibition of L-methionine fermentation. Here, 5% and 10% increase in γ lead to a 2.613% and 3.618 % decrease in ED respectively. A 5% and 10% elevation of B_m led to 0.013% and o.030% reduction in ED and thus, inhibition of L-methionine production. μ_{max} and C have quantitative negative impactson ED and thus on L-methionine fermentation. But other kinetic parameters had no effect on L-methionine production in this present study. Thus from the present study, it can tentatively concluded that, elevation of non-growth associated L-methionine fermentation coefficient caused maximum detrimental effect on L-methionine fermentation.

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