Korean J. Chem. Eng., 34(1), 118-126 (2017)
DOI: 10.1007/s11814-016-0216-1

pISSN: 0256-1115
eISSN: 1975-7220

Kinetics of growth on dual substrates, production of novel glutaminase-free L-asparaginase and substrates utilization by *Pectobacterium carotovorum* MTCC 1428 in a batch bioreactor

Kumar Sanjay*,, Ashish Prabhu Anand*, Venkata Dasu Veeranki*,†, and Pakshirajan Kannan***

*Biochemical Engineering Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
**Department of Biotechnology, Motilal Nehru National Institute of Technology Allahabad, Allahabad 211004 U.P., India
(*Received 14 March 2016 • accepted 30 July 2016*)

Abstract−Bacterial L-asparaginase has been widely used as a potential therapeutic agent in the treatment of various lymphoblastic leukemia diseases. We studied product and dual substrates utilization kinetics by *P. carotovorum* MTCC 1428 in batch bioreactor. The kinetic study revealed that the maximum growth of *P. carotovorum* MTCC 1428 was achieved at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine, respectively. Different substrate inhibition models were fitted to the growth kinetic data and the additive form of double Luong model was found to best explain the growth kinetics of *P. carotovorum* MTCC 1428. The kinetic parameters of growth studies showed that the predicted maximum inhibition concentration of glucose (S_{mg}) and L-asparagine (S_{ma}) was close to the experimentally observed value 15.0 and 10 g l^{-1}, respectively. Modified form of the Luedeking-Piret model was used to describe the kinetics of L-asparaginase production, and the system seems to be mixed growth associated. Kinetic models of dual substrate growth, L-asparaginase production and substrate(s) utilization by *P. carotovorum* MTCC 1428 well fitted with experimental data with regression coefficients (R^2) value of 0.97, 0.96 and 0.93, respectively.

Keywords: Anti-leukemic Enzyme, L-asparaginase, *Pectobacterium carotovorum*, Growth Kinetics, Multiple-substrate, Production Kinetics

INTRODUCTION

With drastic outbreaks of epidemics worldwide, much focus is on developing novel therapeutic agents for treating chronic diseases. L-Asparaginase (L-asparagine amido hydrolase E.C. 3.5.1.1) is an enzyme of high therapeutic value due to its use in cancer therapies, mainly in acute lymphoblastic leukemia (ALL) [1]. L-Asparaginase acts by cleaving L-asparagine (amino acid essential for lymphoblasts' growth) to ammonia and L-aspartic acid, which results in depletion of L-asparagine in serum and cerebrospinal fluid, and finally leads to destruction of lymphoblasts as they are unable to build their proteins due to the inability of endogenic L-asparagine production [2]. Apart from its therapeutic uses, it is also used in the food industry for the production of acrylamide free food [3], model enzyme for the development of new drug delivery system [4] and L-asparagine biosensor for leukemia [5].

Despite its wide use as an anticancer drug, most of the treatments have been interrupted due to severe side effects and immunological reactions in patients. The therapeutic response of patients rarely occurs without some evidence of toxicity [6]. With a view to characterize enzymes with less toxic side effects, several members of a family of homologous L-asparaginases have been thoroughly investigated [2,7,8]. In most of the bacterial L-asparaginases, the presence of partial glutaminase activity up to 9% was reported [9]. The various side effects of this drug are mainly due to the presence of partial glutaminase activity [10]. Hence, for successful clinical studies glutaminase-free L-asparaginase is highly desirable. Chan et al. [11] recently reported that asparagine synthetase negative leukemic cells were hypersensitive to L-asparaginase treatment without glutaminase activity than with glutaminase activity. The mechanism of action of glutaminase free or associated is summarized in Fig. 1.

Mathematical modeling is necessary to predict the results of industrial fermentations and to choose optimized conditions. Additionally, it helps in understanding the complex mechanisms of growth and product formation of the microorganisms [12]. Kinetic studies allow the prediction of fermentation rate, product yield and the control of the fermentation process. The production of L-asparaginase in batch system by Gram negative bacteria is dependent on many factors and the production system is complex. A complete quantitative description of its kinetic behavior requires mathematical expressions describing the time course of each important variable (substrate utilization, growth and enzyme production). Although the production of L-asparaginase in bioreactor was studied earlier [13,14], the development of kinetic models for dual substrate growth, L-asparaginase production and substrate utilization has not been considered previously.

Our aim was to find the most suitable models to describe the mechanism of dynamic dual substrate (L-asparagine and/or glucose) growth, substrates utilization and L-asparaginase production by *P. carotovorum* MTCC 1428 in batch bioreactors. The effects of the

†To whom correspondence should be addressed.
E-mail: veeranki@iitg.ernet.in

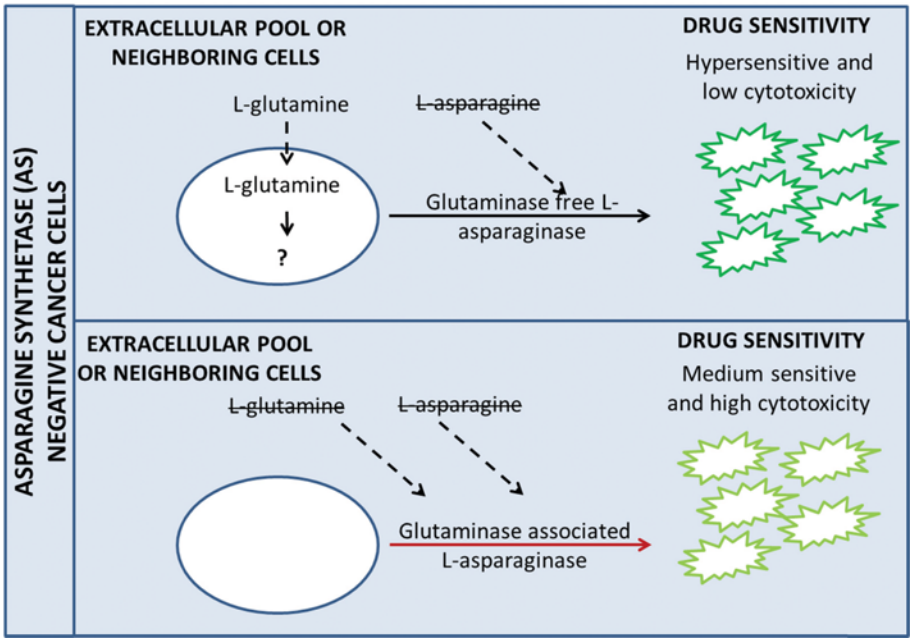

Fig. 1. Mechanism of action of glutaminase free or associated L-asparaginase activity in AS negative cancer cells.

initial L-asparagine and glucose concentration on the growth, its utilization and production of L-asparaginase were studied in pre-optimized physical conditions under shake flasks condition and batch bioreactors [13,14]. Models were developed to describe the kinetic behavior of dual substrate growth, L-asparaginase production and substrate(s) utilization from *P. carotovorum* MTCC 1428 in batch bioreactor. To develop an unstructured dual substrate growth kinetics model, three single substrate models (Double exponential model, Loung model, Yano and Kago 2 model) were adopted and combined in an additive manner. The production of L-asparaginase by *P. carotovorum* MTCC 1428 has also been modeled using a modified form of the Luedeking-Piret model. The substrate utilization kinetics was considered as dual substrate conversion to cell mass, product formation and substrate consumption for maintenance.

MATERIAL AND METHODS

1. Microorganism and Growth Conditions for Experimental Set Up

The bacterium used throughout the study, *P. carotovorum* MTCC 1428, was procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This organism was maintained on the medium containing (g l^{-1}): beef extract 1.0, yeast extract 2.0, NaCl 5.0, peptone 5.0 and agar 15.0 (pH 7.0) at 30 °C. The organism was subcultured every month and maintained at 4±1 °C. The growth assessment, substrate utilization and production of L-asparaginase were studied in batch bioreactor with working volume of 1.5 *l* (Biostat B plus, Sartorius, Germany) of optimized semisynthetic medium using eight different combinations of influent substrate(s) concentration of glucose and/or L-asparagine as shown in Table 1 (g l^{-1}): Glucose, 0-7, L-asparagine, 0-10, $Na_2HPO_4{\cdot}2H_2O$, 6.0; KH_2PO_4, 1.773; NaCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.373; $CaCl_2{\cdot}2H_2O$, 0.015; yeast extract 1.0; peptone, 1.0 and initial pH of 7.0 [13]. The inoculum was prepared by adding a loop full of freshly prepared pure culture of slant into 40 ml of autoclaved above said medium containing glucose as carbon source in a 250 ml shake flask and incubated at 30 °C and 180 rpm in a shaking incubator for 10-12 h (optical density (OD) at 600 nm: 0.6-0.8). A 2.61% of inoculum from the above seed culture was added to 1.5 *l* of the medium in a bioreactor and operated at 20% dissolved oxygen under cascading mode (agitation - 160-600 rpm), aeration rate 1.5 vvm and 30 °C. Samples were withdrawn at regular time intervals and measured for L-asparaginase production, cell growth and substrate utilization. Experiments were conducted in duplicate and enzymatic assay was performed in duplicate for each sample.

Table 1. The search range for kinetic constants of *P. carotovorum* MTCC 1428 in the non-linear regression analysis

Constant	Allowable range
μ_m (h^{-1})	μ_{max}-3μ_{max}
K_{sg} (g L^{-1})	0-20
K_{sa} (g L^{-1})	0-20
K_{1g} (g L^{-1})	0-30
K_{1a} (g L^{-1})	0-30
pH_{op}	8.5-8.8

2. Enzyme Assays and Determination of Protein Concentration

Collected samples were centrifuged at 10,000 *g* for 10 min at 4±1 °C and washed twice with 0.05 M Tris-HCl buffer (pH 8.6). Washed cells were ultrasonicated at 20 MHz, 35% amplitude, four cycles (2 minute per cycles with 1.5 second on and 0.5 second off) and the content was centrifuged at 20,000 *g* for 10 minutes (4±1 °C). The supernatant was analyzed for intracellular L-asparaginase activity. L-asparaginase activity was measured from modified Nessler's

method [14]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μM of ammonia per minute at 37 °C. Specific activity was expressed as U mg^{-1} of protein. Productivity was defined as the number of units of enzyme activity per liter of enzyme solution per hour. Specific activity is the number of units of enzyme activity per milligram of protein.

3. Determination of Protein Concentration

The total protein content of the samples was determined according to the method described by Lowry et al. [15] using bovine serum albumin (Sigma) as standard.

4. Determination of Cell Concentration

The biomass concentration was determined by measuring the OD (Optical Density) of the culture broth at 600 nm using UV-visible spectrophotometer. Dry cell weight (DCW) of the biomass was determined from previously established standard curve between OD at 600 nm *vs* DCW (1 unit OD_{600}=0.272 g l^{-1} dry cell weight).

5. Estimation of Glucose and L-asparagine

Glucose concentration in the media was estimated by DNS method, and L-asparagine concentration was estimated using HPLC method (Varian, USA). Reversed phase C-18 HPLC column (PTH amino acid column, Hypersil gold, 5 μl particle size, 250×4.6 mm, Thermo Ltd. USA) was used with Fluorescence (exc. 263 nm, emm. 313 nm) and UV (263 nm) detection system using 9-fluorenylmethyl chloroformate chloride (FMOC-Cl) derivatisation method [16].

6. Kinetic Model Development in Batch Bioreactor

6-1. Microbial Growth

Three types of multiple-substrate growth models were considered when growth is limited by more than one substrate [17]:

Interactive or multiplicative form:

$$\frac{\mu}{\mu_m} = [\mu(S_1)][\mu(S_2)]\ldots[\mu(S_i)] \quad (1)$$

Additive form:

$$\frac{\mu}{\mu_m} = \frac{\mu(S_1)+\mu(S_2)+\ldots+\mu(S_i)}{i} \quad (2)$$

Non-interactive form:

$$\frac{\mu}{\mu_m} = \mu(S_1) \text{ or } \mu(S_2) \text{ or } \ldots \text{ or } \mu(S_i) \quad (3)$$

Many mathematical models are reported in the literature to correlate the single substrate concentration with microbial growth rate, μ versus S_i [17-19]. To develop multiple-substrate growth kinetics, these individual models can be combined in a manner described by Eqs. (1)-(3), to obtain equations consistent with the experimental data [18]. The specific growth rate in exponential phase is calculated by Eq. (4):

$$\frac{dx}{dt} = \mu X \Rightarrow \ln(X_2 - X_1) = \mu(t_2 - t_1) + C \quad (4)$$

where, X_1 and X_2 are the dry cell weight (DCW) obtained at time t_1 and t_2, respectively. C is an integration constant. We used the following growth kinetics models considered to explain the cell growth kinetics for a single substrate, S_i [17,18,20-22]:

Double exponential model: $$\frac{\mu}{\mu_m} = \mu_m\left[\exp\left(-\frac{S_i}{K_{1i}}\right) - \exp\left(-\frac{S_i}{K_{Si}}\right)\right] \quad (5)$$

Loung model: $$\mu = \mu_m \frac{S_i}{K_{Si}+S_i}\left(1-\frac{S_i}{S_{mi}}\right)^{ni} \quad (6)$$

Yano and Kago 2 model: $$\mu = \mu_m \frac{S_i}{K_{Si}+S_i+\frac{S_i^2}{K_{2i}^2}} \quad (7)$$

where, μ_m is the maximum specific growth rate (h^{-1}), μ is the specific growth rate (h^{-1}), S_i is the limiting substrate concentration (glucose or L-asparagine in this study), K_{si} is the Monod half saturation constant for substrates (glucose or L-asparagine) g l^{-1}, S_{mi} is the maximum inhibitory substrate concentration g l^{-1}, n_i is the constant which accounts the relationship between μ and S_i, and K_{1i}, K_{2i} are inhibition constants, subscript i represents either g or a as glucose and L-asparagine, respectively.

To develop growth models for multiple-substrates, for each combination of L-asparagine and glucose, specific growth rate (μ) was calculated in exponential phase. Different growth models based on single substrate (Eqs. (5)-(7)) were inserted into Eq. (1) or (2), or (3) to find the best additive-substrate model.

7. L-asparaginase Production

L-asparaginase production in batch bioreactor was modeled using a modified form of Luedeking-Piret model [23,24] (Eq. (8)), which includes a term for the effect of the optimum final pH value on L-asparaginase synthesis:

$$\frac{dP_{asp}}{dt} = (\alpha\cdot\mu\cdot X + \beta\cdot X)\left(1-\kappa\left|\frac{pH_{op}-pH_t}{pH_{op}}\right|\right) \quad (8)$$

where P_{asp} is the L-asparaginase concentration (U mL^{-1}), α is a growth-associated constant for L-asparaginase production (U mg^{-1}), which corresponds to the yield product on biomass formed ($Y_{P/X}$) for growth-associated metabolites, and β is the non growth-associated constant (U mg^{-1} h^{-1}), X is the biomass concentration (g of DCW l^{-1}), κ is a constant of pH proportionality, X biomass concentration, pH_{op} is the optimum final pH for maximum L-asparaginase production and pH_t is the pH value in each sampling time.

8. Substrate Utilization

The substrate utilization kinetics was described by Eqs. (9) and (10), which consider substrate conversion to cell mass, to product formation and substrate consumption for maintenance.

Glucose utilization kinetics: $$\frac{dS_g}{dt} = -\frac{1}{Y_{X/Sg}}\frac{dX}{dt} - \frac{1}{Y_{P/Sg}}\frac{dP_{asp}}{dt} - m_g X \quad (9)$$

L-asparagine utilization kinetics:

$$\frac{dS_a}{dt} = -\frac{1}{Y_{X/Sa}}\frac{dX}{dt} - \frac{1}{Y_{P/Sa}}\frac{dP_{asp}}{dt} - m_a X \quad (10)$$

where, S_g, S_a are glucose and L-asparagine concentration (g l^{-1}), $Y_{X/Sg}$, $Y_{X/Sa}$ are yield of biomass on glucose and L-asparagine (g of DCW g^{-1} of substrate), $Y_{P/Sg}$, $Y_{P/Sa}$ are yield of product (L-asparaginase) on glucose and L-asparagine (U of L-asparaginase g^{-1} of substrate), and m_g, m_a are substrates used for maintenance of biomass (g of substrate g^{-1} DCW h^{-1}) respectively.

9. Non-linear Regression Analysis

The kinetic parameters of different models (dual substrate growth, product formation and substrate utilization kinetics) considered were estimated using Microsoft Excel 2007 Solver®, which solves

non-linear regression problems using Newton's method [25]. The following constraint was given to kinetic constants in all models, while estimating the parameters is shown in Table 1. The equations were solved to find values of the kinetic parameters that minimize the objective function, the sum of squares of the differences (SSD) between experimental and theoretical data for specific growth rates, as given by Eq. (11):

$$SSD = \sum_{i=1}^{N} (\mu_{Expt} - \mu_{Pred})^2 \quad (11)$$

To calculate the maintenance and yield factors (Eqs. (9) and (10)), describing the objective function as the SSD between the substrate consumption rates, which were experimentally measured and theoretically estimated, separately for glucose and L-asparagine. The selected growth model equation for growth kinetics was surface fitted by nonlinear surface fit using Origin® Pro 8.0.

10. Best Model for Dual Substrate Growth Kinetics

Using non-linear regression and the selected additive substrate growth kinetics, we calculated the kinetic parameters as described before. The best additive-substrate growth model was selected among different combinations of Eqs. (5)-(7) to give the minimum sum of squares of differences (SSD) and maximum R^2 between the experimental data and model solutions.

Table 2. Production of L-asparaginase and specific growth rate from *P. carotovorum* MTCC 1428 at different concentration of glucose and L-asparagine in batch bioreactor

Batch bioreactor run	Initial substrate concentration		Maximum L-asparaginase activity		Specific growth rate (μ) (h^{-1})
	Glucose (S_g) ($g\ L^{-1}$)	L-asparagine (S_a) ($g\ L^{-1}$)	Enzyme activity ($U\ ml^{-1}$) (h)	Specific activity ($U\ mg^{-1}$)	
1	0	7	12.73±0.37 (14)	26.67±0.64	0.184±0.05
2	7	0	1.95±0.73 (12)	3.35±0.39	0.169±0.02
3	2	5	16.92±0.54 (12)	36.87±0.83	0.344±0.07
4	1	2.5	9.89±0.49 (12)	22.17±0.43	0.292±0.08
5	4	7.5	13.83±0.32 (18)	29.07±0.49	0.321±0.06
6	5	10	10.32±0.65 (22)	21.73±0.61	0.279±0.04
7	2	0	1.23±0.19 (12)	2.73±0.58	0.175±0.07
8	0	5	13.21±0.39 (12)	25.43±0.25	0.199±0.08

Process condition: T_m, 30 °C; pH controlled when reaches <8.5; aeration, 1.5 vvm; agitation, 160-600 rpm (cascading mode); DO level, 20%

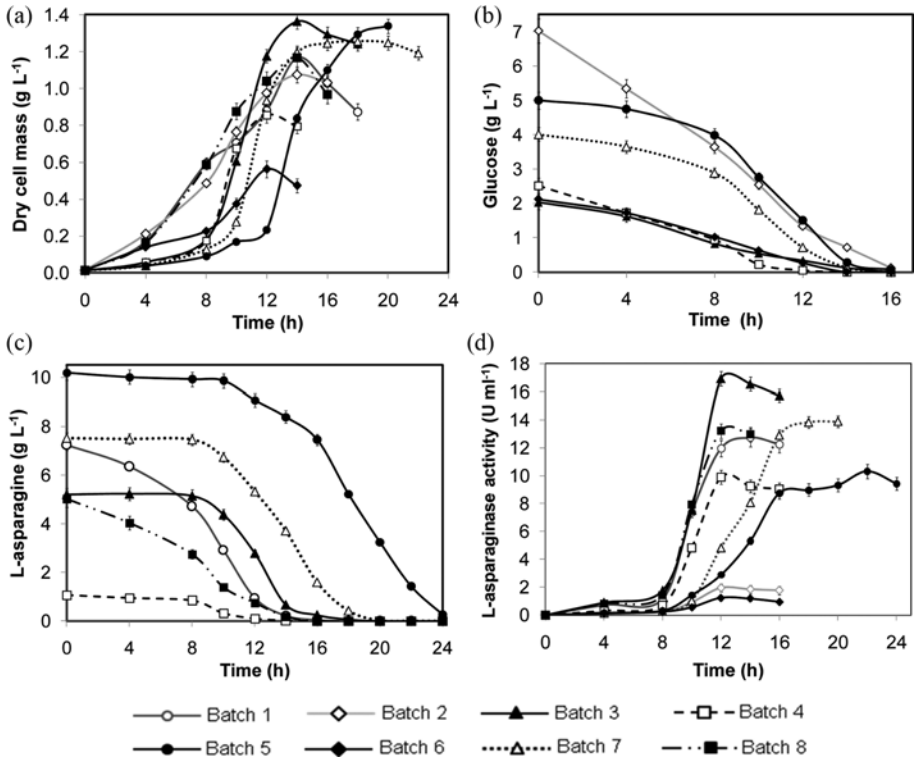

Fig. 2. Profiles of batch bioreactors runs. (a) Biomass production, (b) glucose utilization, (c) L-asparagine utilization, (d) L-asparaginase enzyme production.

RESULTS AND DISCUSSION

Previously, physical parameters (DO level and pH) were optimized to maximize the production of L-asparaginase production in batch bioreactor. It also revealed that the maximum enzyme activities, substrate utilization rate and cell growth were observed when the DO level of the medium was at 20% [26]. Under controlled pH, a sharp decline in enzyme activity was discontinued after 12 h of fermentation in batch bioreactor. Hence, experiments for L-asparaginase fermentation modeling were performed at previously optimized physical condition at different concentration of glucose and/or L-asparagine (Table 2). In previous reports, L-asparaginase production under different cultivation conditions from *P. carotovorum* MTCC 1428 possesses no partial glutaminase activity. This aspect of high specificity toward L-asparagine of glutaminase free L-asparaginase from *P. carotovorum* MTCC 1428 has been reported [14,26,27].

1. Effect of Initial Substrates Concentrations on Production of L-asparaginase and Specific Growth Rate

The cells grew to a maximum concentration of range 0.56-1.36 g l^{-1} at different concentrations of glucose and/or L-asparagine in batch bioreactor (Fig. 2(a)). The maximum DCW was observed at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine concentration, respectively, in batch run 3. Furthermore, *P. carotovorum* MTCC 1428 exhibited shorter lag periods at low substrates concentration, and longer lag was observed at higher concentrations of substrates (Fig. 2(a)). More than 90% of glucose was exponentially assimilated in 14 h fermentation by *P. carotovorum* MTCC 1428 in all conditions (Fig. 2(b)) as preferential substrate than L-asparagine. Initially, 4-8 h of fermentation, L-asparagine was consumed very slowly. Subsequently, L-asparagine was also utilized simultaneously along with glucose, and almost L-asparagine was also exponentially consumed with in 16 h of fermentation in most the cases (Fig. 2(c)). The maximum L-asparaginase production (16.92 U ml^{-1}) was observed at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine, respectively (batch run 3) (Table 2). The production of L-asparaginase was 6-10 times fold higher in the medium containing L-asparagine (batch run 1 and 8) as compared to glucose medium (batch run 2 and 7). The maximum L-asparaginase production was observed at 12 h in all batch conditions except batch run 5 and 6 (Fig. 2(d)).

Specific growth rate (μ) for different combinations of initial glucose and/or L-asparagine concentration (S_g and S_a) were calculated from the plot of ln(X) *vs.* time in logarithmic phase as discussed in Section 2. The slope of the line during the exponential phase gives the specific growth rate varies in each run (Fig. 2(a)). Maximum specific growth rate for only glucose containing basal medium was found to be 0.175 h^{-1} at 2 g l^{-1}, whereas at 7 g l^{-1}, diminishing of specific growth rate was observed (Table 2). Similarly, maximum specific growth rate for only L-asparagine containing basal medium was found to be 0.199 h^{-1} at 5 g l^{-1}. However, a slight decrease in specific growth rate was observed at 7 g l^{-1} of initial L-asparagine concentration. However, the specific growth rate was found to be about 1.5 times higher when both substrates (L-asparagine and glucose) were present in the medium. Specific growth rate increased with increase in initial concentration of glucose and L-asparagine till 2 g l^{-1} and 5 g l^{-1}, respectively. The decrease in specific growth rate with in increasing substrates concentrations suggested that both substrates inhibition occurred simultaneously (Table 2).

2. Modeling of Microbial Growth Kinetics

In addition to the data in Table 2, we also tested the growth-limiting substrates and their interactions. According to these results, the growth rate of *P. carotovorum* MTCC 1428 in the absence of L-asparagine and glucose was very less. A sharp decrease in growth of microorganism, substrate utilization rates and production of L-asparaginase was observed when the concentrations of glucose and L-asparagine exceeded 15 g l^{-1} and 10 g l^{-1}, respectively, in the

Table 3. Estimated kinetic parameters of various growth models with SSD and regression coefficients (R^2)

Model	Equation number for glucose	Equation number for L-asparagine	μ_m	K_{sg}	K_{sa}	K_{1g}	K_{1a}	K_{2g}^2	K_{2a}^2	S_{mg}	n_g	S_{ma}	n_a	SSD×10^{-2}	R^2
1	1	--	0.361	0.348	--	18.380	--	--	--	--	--	--	--	2.348	0.149
2	2	--	0.369	0.234	--	--	--	--	--	7.103	0.144	--	--	1.808	0.959
3	3	--	0.346	0.273	--	--	--	78.24	--	--	--	--	--	2.163	0.221
4	--	1	0.796	--	1.068	--	5.327	--	--	--	--	--	--	0.479	0.795
5	--	2	0.540	--	0.627	--	--	--	--	--	--	11.146	1.048	0.593	0.959
6	--	3	0.581	--	0.960	--	--	--	22.535	--	--	--	--	0.486	0.801
7	1	1	0.491	2.433	2.049	7.094	6.823	--	--	--	--	--	--	0.421	0.955
8	1	2	0.363	1.958	1.391	9.937	--	--	--	--	--	11.957	0.625	0.125	0.963
9	1	3	0.352	0.019	6.142	5.191	--	--	457.914	--	--	--	--	1.864	0.492
10	2	1	0.367	2.436	1.599		8.552			8.328	0.309			0.141	0.936
11	2	2	0.355	1.668	1.464	--	--	--	--	16.190	0.841	11.280	0.476	0.121	0.971
12	2	3	0.371	2.181	2.145	--		--	49.144	13.869	0.843	--	--	0.149	0.959
13	3	1	0.391	2.870	1.699	--	8.076	42.515	--	--	--	--	--	0.191	0.946
14	3	2	0.347	2.060	1.289	--	--	67.213	--	--	--	11.424	0.541	0.139	0.963
15	3	3	0.736	9.180	7.619	--	--	18.749	14.498	--	--	--	--	0.764	0.802

Dual substrate kinetics model 7-15 are additive form of column second and third respectively

medium (data not shown). Hence, growth of *P. carotovorum* MTCC 1428 was represented by a kinetic expression by considering the dual-substrate limitations (glucose and L-asparagine).

Table 3 shows the possible growth models using various combinations of Eqs. (5)-(7), calculated kinetic parameters of various growth models with SSD and regression coefficients (R^2). The minimum SSD were found for model number 11, with R^2 0.971. The model 11, additive combined form of Loung model kinetics. The additive form of double Loung model was best selected model to explain the growth of *P. carotovorum* MTCC 1428 (Eq. (11)).

Double Loung model:

$$\mu = \mu_m \left[\frac{S_g}{K_{Sg} + S_g} \left(1 - \frac{S_g}{S_{mg}} \right)^{ng} + \frac{S_a}{K_{Sa} + S_a} \left(1 - \frac{S_a}{S_{ma}} \right)^{na} \right] \quad (11)$$

where, glucose and L-asparagine represented by subscripts g and a, respectively, in the model equation. For the selected model, calculated kinetic parameters are μ_m=0.355 h^{-1}, K_{sg}=1.668 mg l^{-1}, K_{sa}= 1.464 mg l^{-1}, S_{mg}=16.190 g l^{-1}, n_g=0.841, S_{ma}=11.28 g l^{-1}, n_a=0.476. Fig. 3 shows the experimental specific growth rate fitted over simulated surface using Origin® Pro 8.0 from additive form of double Loung model (Eq. (11)). The high correlation coefficient (R^2=0.971)

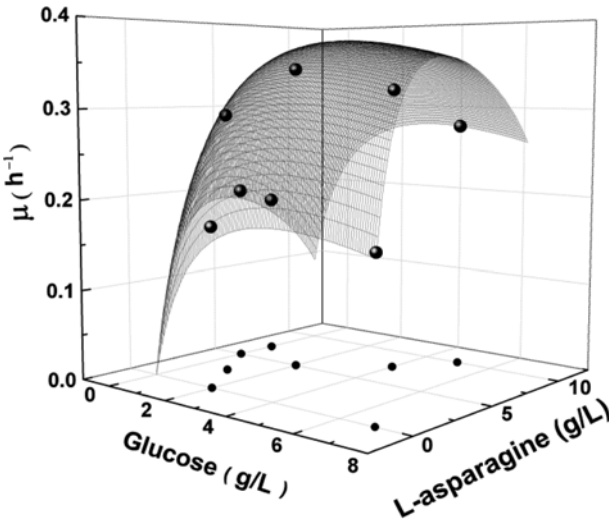

Fig. 3. The specific growth rate from batch bioreactors experiments fitted over simulated surface from dual substrate growth kinetic model (Eq. (11)) (R^2=0.971). Projections of specific growth rates in XY plane.

demonstrates that the growth model accurately represents the growth of *P. carotovorum* MTCC 1428.

So far, no reports are available in the literature on the kinetics of dual substrate growth, production of L-asparaginase and substrates utilization in batch bioreactor. The added value of L-asparaginase isolated from the *P. carotovorum* MTCC 1428 is that it has no glutaminase activity; in addition, enzyme activity is comparable with high level of L-asparaginase producing microorganisms [13,14]. We derived three different equations to describe the detailed kinetic behavior of dual substrate growth, L-asparaginase production and substrate(s) utilization from *P. carotovorum* MTCC 1428. The summarized kinetic parameters evaluated in this study are presented in Table 4.

To have more insights to understand the growth kinetics, selected empirical models in the additive form that describing the substrate inhibition kinetics were employed to fit the kinetic data (Table 3). Initially, the growth data of *P. carotovorum* MTCC 1428 was tested for dual substrate kinetics with various single substrate models (Moser, Tessier, Monod, Contois, Yano and Kago 1 and 2, Andrew, Haldane, Double exponential, and Loung model) in non-interactive, multiple and additive form of these models and only additive form of double exponential, Loung and Yano and Kago 2 model are found to be fitted very well with the experimental data. However, the remaining models did not fit well in any combination (non-interactive, multiple and additive) and showed very less regression coefficient (R^2<0.80) (data not shown). This might be due to the other than selected models and their various combinations not being able to predict decrease in specific growth rate at high concentration of substrates. Therefore, Loung, Double exponential and Yano and Kago 2 models were used in the present study. Loung model was used to explain the inhibition growth kinetics of microorganism for various carbohydrates and nitrogenous sources [21,22]. The values of S_{mg} and S_{ma} predicted by double Luong model (16.19 and 11.28 g l^{-1}) were close to the experimentally observed value (15.0 and 10 g l^{-1}). The constant n_i estimated by Loung model for both substrates suggests that a nonlinear relationship between μ and S exists during inhibition.

3. Modeling of L-asparaginase Production

Previous studies in batch culture show that the glucose is not sufficient to maximize the production of L-asparaginase from *P. carotovorum* MTCC 1428; L-asparagine is essential substrate for higher production of L-asparaginase. Medium pH also plays a vital role for the production of L-asparaginase. When pH of medium

Table 4. Summarized kinetic parameters calculated in this study for dual-substrate growth, L-asparaginase production and substrate utilization models

	Kinetic parameters							R^2
Dual substrate growth model	μ_m	K_{sg}	K_{sa}	S_{mg}	n_g	S_{ma}	n_a	0.971
	0.355	1.668	1.464	16.190	0.841	11.280	0.476	
L-asparaginase production model	α	β	κ					0.962
	3.27	6.421	6.681					
Substrate utilization model	$Y_{X/Sg}$	$Y_{P/Sg}$	m_g	$Y_{X/Sa}$	$Y_{P/Sa}$	m_a		0.935
	0.624	0.808	0.011	0.286	10.910	0.029		

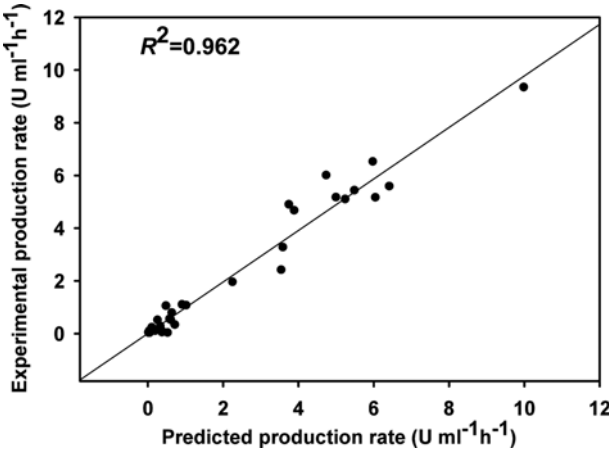

Fig. 4. The experimental batch bioreactor L-asparaginase production rate and predicted from production kinetics model.

reached to <8.5, the maximum production of L-asparaginase was observed. In current study, the maximum production was observed at 12-14 h in a batch bioreactor (Fig. 2(d)) except high substrate concentration of batch run 5 and 6.

With regard to L-asparaginase production in batch cultures from *P. carotovorum* MTCC 1428, the results showed that the L-asparaginase production was clearly mixed growth-associated ($\alpha \neq 0$, $\beta \neq 0$). In addition, the values obtained for pH_{op} oscillated between 8.5 and 8.7 ($P<0.05$) for maximum production of L-asparaginase. The kinetic parameters from the modified form of Luedeking-Piret model for L-asparaginase production are α=3.27 U mg^{-1}, β=6.421 U mg^{-1} h^{-1} and κ=6.681. Fig. 4 shows the relationship between observed production rate from batch bioreactor experiments and predicted value of L-asparaginase production rate obtained from the model, Eq. (3.19). The correlation between the model equation and experimental values obtained from L-asparaginase production was very satisfactory (R^2>0.96). Hence, the production was classified as a pH dependent secondary metabolite.

In general, L-asparaginase production was higher at late exponential phase (24-48 h) and enzyme activity was also a function of cell age [27]. The growth of L-asparaginase producing microorganism is often inhibited not only because of nutrient limitation, but also because of substrate inhibition and the production of ammonia which have toxic and antimicrobial effects, even for the producer cells. In such cases, the classic Monod model would have modified by including terms for accounting the inhibitory effects of substrate inhibition and end product formation [28].

Although L-asparaginase production has been reported by various researchers to be growth-associated [29-31], in some cases, the lack of proportionality between the rate of L-asparaginase production and bacterial growth has been observed [27,32,33]. This was found to be due to the specific influence of several key variables of the culture (like pH or some nutrient limitation) on L-asparaginase synthesis [34-36] and difficult to fit the experimental data with the classical Luedeking-Piret model. Similar type of adoption was also seen for the bacteriocin production from *Lactococcus lactis* subsp. *Lactis* by Guerra et al. [24]. Recent studies of secondary metabolite production like lipid, enzyme from microalgae and fungus used the Luedeking-Piret model to explain the production profile [37,38].

The alkalinization of the medium at low glucose concentration due to complete utilization of this carbohydrate during the initial periods, followed by the release of nitrogen as NH_3 through the degradation of substrates containing amino groups (such as amino acids), as often observed during the fermentation processes [33]. It is known ammonia accumulates in the ionized form (NH_4^+), which further contributes to the acidity of the medium. These results are in good accordance with others showing that NH_4^+ has an inhibitory effect on L-asparaginase production [39,40].

4. Modeling of L-asparagine and Glucose Utilization

The considered models for both substrates (L-asparagine and glucose) were solved using non-linear regression analysis by Microsoft Excel 2007 Solver®. The predicted evolution of the glucose and L-asparagine utilization rate by Eqs. (9) and (10) during the fermentation process together with the experimental data shows the fitting of results was satisfactory (correlation coefficient, R^2>0.93). The values of parameters of the substrate uptake model were calculated as follows for glucose: $Y_{X/Sg}$, $Y_{P/Sg}$, m_g are 0.624 g g^{-1}, 0.808 U g^{-1}, 0.011 g g^{-1} h^{-1} and for L-asparagine $Y_{X/Sa}$, $Y_{P/Sa}$, m_a are 0.286 g g^{-1}, 10.910 U g^{-1}, 0.029 g g^{-1} h^{-1}, respectively.

5. Effect of Initial Glucose and/or L-asparagine Concentration on Substrate Uptake Rate

At different concentration of substrates (L-asparagine and/or glucose) in medium of batch bioreactor studies, a variation in specific substrate uptake rate ($Y_{S/X}$) was observed. This deviation in experimental specific substrate uptake rate from *P. carotovorum* MTCC 1428 at different concentration of glucose and/or L-asparagine in batch bioreactor is presented in Fig. 5. The specific substrate uptake rate ($Y_{Sg/X}$) for glucose varied in range of 0.271-0.551 g of glucose g^{-1} of cells h^{-1}. However, specific substrate uptake rate ($Y_{Sa/X}$) for L-asparagine was very high, ~2 times in all combinations.

CONCLUSIONS

This is the first report on the kinetics of dual substrate growth,

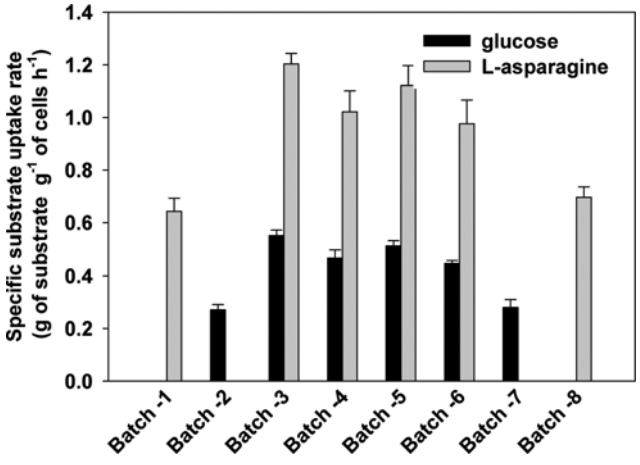

Fig. 5. Experimental specific substrate uptake rate from *P. carotovorum* MTCC 1428 at different concentration of glucose and L-asparagine in a batch bioreactor.

L-asparaginase production and substrates utilization in batch bioreactor using *P. carotovorum* MTCC 1428. The kinetic study revealed the maximum growth of *P. carotovorum* MTCC 1428 at 2 g l^{-1} and 5 g l^{-1} of glucose and L-asparagine, respectively. Additive form of double Loung model was the best dual substrate growth model for explaining the growth kinetics *P. carotovorum* MTCC 1428. The modified form of the Luedeking-Piret model includes a term for the effect of the optimal pH value to explain the L-asparaginase synthesis. The values of kinetic parameters of L-asparaginase production suggested system seems to be mixed growth associated. All kinetic models of L-asparaginase fermentation (dual substrate growth, L-asparaginase production and substrate(s) utilization) by *P. carotovorum* MTCC 1428 fitted well with experimental data. These kinetic models would be used in scale up of the bioprocess of L-asparaginase production.

ABBREVIATION AND NOMENCLATURE

C : integration constant
DCW : dry cell weight [g l^{-1}]
DO : dissolved oxygen [%]
K_{1a} : inhibition constant for L-asparagine [g l^{-1}]
K_{1g} : inhibition constant for glucose [g l^{-1}]
K_{1i} : inhibition constant for substrate i [g l^{-1}]
K_{2a} : inhibition constant in Yano and Kago 2 model for L-asparagine [g l^{-1}]
K_{2g} : inhibition constants in Yano and Kago 2 model for glucose [g l^{-1}]
K_{2i} : inhibition constant in Yano and Kago 2 model for substrate i [g l^{-1}]
K_{sa} : Monod half saturation constant for L-asparagine [g l^{-1}]
K_{sg} : Monod half saturation constant for glucose [g l^{-1}]
K_{si} : Monod half saturation constant for substrate i [g l^{-1}]
m_a : L-asparagine used for maintenance of biomass [g of L-asparagine g^{-1} DCW h^{-1}]
m_g : glucose used for maintenance of biomass [g of glucose g^{-1} DCW h^{-1}]
n_a : constant in Loung model accounts the relationship between μ and L-asparagine
n_g : constant in Loung model accounts the relationship between μ and glucose
n_i : constant in Loung model accounts the relationship between μ and substrate i
P_{asp} : L-asparaginase concentration [U mL^{-1}]
pH_{op} : optimum final pH for maximum L-asparaginase production
pH_t : pH value in each sampling time
R^2 : regression coefficient
S_i : concentration of substrate i [g l^{-1}]
S_a : L-asparagine concentration [g l^{-1}]
S_g : glucose concentration [g l^{-1}]
S_{ma} : maximum inhibitory concentration of L-asparagine [g l^{-1}]
S_{mg} : maximum inhibitory concentration of glucose [g l^{-1}]
S_{mi} : maximum inhibitory concentration of substrate i [g l^{-1}]
SSD : sum of squares of the differences
X : biomass concentration [g of DCW l^{-1}]
X_1 : biomass concentration in batch bioreactor at time t_1 [g of DCW l^{-1}]
X_2 : biomass concentration in batch bioreactor at time t_1 [g of DCW l^{-1}]
$Y_{P/Sa}$: yield of product (L-asparaginase) on L-asparagine [U g^{-1} of L-asparagine]
$Y_{P/Sg}$: yield of product (L-asparaginase) on glucose [U g^{-1} of glucose]
$Y_{Sa/X}$: specific L-asparagine uptake rate [g of L-asparagine g^{-1} of cells h^{-1}]
$Y_{Sg/X}$: specific glucose uptake rate [g of glucose g^{-1} of cells h^{-1}]
$Y_{X/Sa}$: yield coefficient for L-asparagine [g microorganism g^{-1} L-asparagine]
$Y_{X/Sg}$: yield coefficient for glucose [g microorganism g^{-1} glucose]

Greek Letter

μ : specific growth rate [h^{-1}]
μ_{Expt} : experimental specific growth rate [h^{-1}]
μ_m : maximum specific growth rate [h^{-1}]
μ_{Pred} : theoretical specific growth rate [h^{-1}]
A : growth-associated constant for L-asparaginase production [U mg^{-1}]
B : non growth-associated constant [U mg^{-1} h^{-1}]
K : constant of pH proportionality

REFERENCES

1. U. H. Athale and A. K. C. Chan, *Thromb. Res.*, **111**, 199 (2003).
2. G. A. Kotzia and N. E. Labrou, *J. Biotechnol.*, **127**, 657 (2007).
3. F. Pedreschi, K. Kaack and K. Granby, *Food Chem.*, **109**, 386 (2008).
4. E. Teodor, S. C. Litescu, V. Lazar and R. Somoghi, *J. Mater. Sci. Mater. Med.*, **20**, 1307 (2009).
5. N. Verma, K. Kumar, G. Kaur and S. Anand, *Artif. Cells. Blood Substit. Immobil. Biotechnol.*, **35**, 449 (2007).
6. U. K. Narta, S. S. Kanwar and W. Azmi, *Crit. Rev. Oncol. Hematol.*, **61**, 208 (2007).
7. J. C. Wriston and T. O. Yellin, *Adv. Enzymol. Relat. Areas Mol. Biol.*, **39**, 185 (1973).
8. J. Krasotkina, A. A. Borisova, Y. V. Gervaziev and N. N. Sokolov, *Biotechnol. Appl. Biochem.*, **39**, 215 (2004).
9. H. J. Müller and J. Boos, *Crit. Rev. Oncol. Hematol.*, **28**, 97 (1998).
10. J. A. Distasio, A. M. Salazar, M. Nadji and D. L. Durden, *Int. J. Cancer*, **30**, 343 (1982).
11. W. K. Chan, P. L. Lorenzi, A. Anishkin, P. Purwaha, D. M. Rogers, S. Sukharev, S. B. Rempe and J. N. Weinstein, *Blood*, **123**, 3596 (2014).
12. M. Zinn, B. Witholt and T. Egli, *J. Biotechnol.*, **113**, 263 (2004).
13. S. Kumar, K. Pakshirajan and V. V. Dasu, *Appl. Microbiol. Biotechnol.*, **84**, 477 (2009).
14. S. Kumar, V. V. Dasu and K. Pakshirajan, *Process Biochem.*, **45**, 223 (2010).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
16. P. A. Haynes, D. Sheumack, L. G. Greig, J. Kibby and J. W. Redmond, *J. Chromatogr.*, **588**, 107 (1991).
17. "Biochemical engineering fundamentals / James E. Bailey, David F. Ollis. - Version details, *Trove*. (2016).

18. M. L. Shuler, F. Kargı, *Bioprocess engineering: basic concepts*, Prentice Hall (1992).
19. A. Bouguettoucha, B. Balannec, S. Nacef and A. Amrane, *Enzyme Microb. Technol.*, **41**, 377 (2007).
20. T. Yano and S. Koga, *Biotechnol. Bioeng.*, **11**, 139 (1969).
21. J. H. Luong, *Biotechnol. Bioeng.*, **29**, 242 (1987).
22. S. Gokulakrishnan and S. N. Gummadi, *Process Biochem.*, **41**, 1417 (2006).
23. R. Luedeking and E. L. Piret, *J. Biochem. Microbiol. Technol. Eng.*, **1**, 393 (1959).
24. N. P. Guerra, A. T. Agrasar, C. L. Macías, P. F. Bernárdez and L. P. Castro, *J. Food Eng.*, **82**, 103 (2007).
25. L. S. Lasdon, A. D. Waren, A. Jain and M. Ratner, *ACM Trans Math Softw*, **4**, 34 (1978).
26. S. Kumar, A. A. Prabhu, V. V. Dasu and K. Pakshirajana, *Prep. Biochem Biotech.* (2016), DOI:10.1080/10826068.2016.1168841.
27. J. Mukherjee, S. Majumdar and T. Scheper, *Appl. Microbiol. Biotechnol.*, **53**, 180 (2000).
28. R. Callewaert and L. D. Vuyst, *Appl. Environ. Microbiol.*, **66**, 606 (2000).
29. E. Albanese and K. Kafkewitz, *Appl. Environ. Microbiol.*, **36**, 25 (1978).
30. S. Khamna, A. Yokota and S. Lumyong, *Int. J. Integr. Biol.*, **6**, 22 (2009).
31. A. J. Shah, R. V. Karadi and P. P. Parekh, *Asian J. Biotechnol.*, **2**, 169 (2010).
32. F. S. Liu and J. E. Zajic, *Appl. Microbiol.*, **25**, 92 (1973).
33. H. Geckil, S. Gencer and M. Uckun, *Enzyme Microb. Technol.*, **35**, 182 (2004).
34. B. Heinemann and A. J. Howard, *Appl. Microbiol.*, **18**, 550 (1969).
35. Y. R. Abdel-Fattah and Z. A. Olama, *Process Biochem.*, **38**, 115 (2002).
36. R. S. Prakasham, C. S. Rao, R. S. Rao, G. S. Lakshmi and P. N. Sarma, *J. Appl. Microbiol.*, **102**, 1382 (2007).
37. Q. He, N. Li, X. Chen, Q. Ye, J. Bai, J. Xiong and H. Ying, *Korean J. Chem. Eng.*, **28**(2), 544 (2011).
38. D. Surendhiran, M. Vijay, B. Sivaprakash and A. Sirajunnisa, *3Biotech.*, **5**, 663 (2015).
39. T. Tosa, R. Sano, K. Yamamoto, M. Nakamura and K. Ando, *Appl. Microbiol.*, **22**, 387 (1971).
40. D. X. Sun and P. Setlow, *J. Bacteriol.*, **173**, 3831 (1991).