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Pediocin production by *Pediococcus acidilactici* in fed batch fermentation using meat processing waste

Barnali Mandal* and Aditia Chakraborty

Department of Chemical Engineering, University of Calcutta, 92, Acharya Prafulla Chandra Road, Kolkata-700 009, India

E-mail: bmandal20@yahoo.co.in

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Pediocin production by *Pediococcus acidilactici* was studied in a bio-reactor under two fed batch fermentations (fed batch operation I and fed batch operation II) at constant pH condition using meat processing waste. In fed batch operation II, higher pediocin activity of 4572 AU/ml was achieved comparing to fed batch operation I (pediocin activity of 3657 AU/ml). The kinetics of cell growth was described by two substrates (glucose and protein) model. The correlation between two products (pediocin and lactic acid) and cell growth was described by Luedeking and Piret model. The comparison of simulated data with experimental results obtained from the study was well matched and the validated mathematical model was developed for fed batch processes.

Keywords: *Pediococcus acidilactici*, Pediocin, meat processing waste, fed batch fermentation, Luedeking and Piret model.

Introduction

Bio-preservatives are gaining increase attention in food preservation due to detrimental effect of chemical additives. Nowadays bacteriocins have received considerable priority as bio-preservatives to enhance the shelf life and quality of food with natural flavor and texture[1,2]. Two types of bacteriocin such as nisin and pediocin produced by different genera of *lactobacillus* bacteria are getting interest as a bio-preservative in food industries because of their wide spectrum of inhibitory activity against Gram-positive food spoiler and pathogenic bacteria[3,4].

Use of protein waste obtained from food processing industries for the production of bacteriocin was followed an economic route. Several research works have been reported for the utilization of protein waste in pediocin production[5,6].

The production of pediocin as well as cell growth may be decreased significantly due to nutrient depletion[7] or pH reduction[8] in batch fermentation process. In order to enhance the pediocin production, fed-batch fermentation is a suitable technique comparing to batch process. The periodical substrate feeding and re-alkalizations are successively maintained in the culture medium during fed-batch operation[8].

Under the present study, meat processing waste was used as the protein source for the growth of *Pediococcus acidilactici* to produce pediocin. The performance of two fed batch operations of bioreactors was compared for the production of pediocin by *P. acidilactici*. Fed batch processes adopting intermittent feeding by (a) glucose concentrate and (b) glucose and protein concentrates with simultaneous re-alkalization was studied. Mathematical model has an important role for scaling up of lab scale reactors to larger ones. An attempt has been made to simulate the performance of bioreactor for both fed batch fermentation processes by developing a suitable mathematical model.

Materials and methods

A. Microorganism and inoculum:

Pediococcus acidilactici NCIM 2292 procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (Pune, India) was used as producer strain of pediocin and *Listeria monocytogenes* MTCC 839 procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (Chandigarh, India) was used as indicator strain. Stock cultures of both microorganisms were maintained at –4°C as slants using nutrient agar (Himedia, India).

B. Analytical determinations:

B.1. Determination of cell concentration:

A calibration curve was prepared against absorbance versus cell dry weight (CDW) for the determination of cell concentration. A UV-Visible spectra-photometer at wavelength of 600 nm was used to measure absorbance of microbial growth. CDW was obtained by centrifugation (1000 rpm for 20 min) of microbial growth. Cell concentration of any sample was determined using standard curve.

B.2. Determination of pediocin activity:

Pediocin screening was performed by agar well diffusion method[9] using cell free supernatant (CFS) obtained by centrifugation of microbial growth. Sterilized nutrient agar (1.5% w/v) was inoculated with indicator strain (*Listeria monocytogenes*) culture and was placed into petri dishes for solidification. The wells of 5 mm diameter were made by a cork borer on the solid agar surface and 70 μL CFS solution with different extent of dilution was poured into each well. The petri dishes were incubated for one night at 30°C. A clear inhibition zone was recorded as positive. Pediocin activity was defined as reciprocal of the maximum serial dilution of CFS giving inhibition zone and was expressed as arbitrary units per milliliter (AU/ml). The titre of pediocin solution was calculated by the formula as follows[10]:

$$\text{AU/ml} = \frac{1000}{d} \times D$$

where, d is dose, i.e. the volume of CFS containing pediocin added on each well (μL), D is highest dilution factor that allowed no growth of indicator strain.

B.3. Determination of lactic acid:

Lactic acid concentration in Cell free supernatant (CFS) was measured using *p*-hydroxydiphenyl[11]. Calibration curve was prepared using known concentrations of lactic acid (10–500 μg/ml) in aqueous solution. Absorbance of the colored solution was measured by spectrophotometer, at 560 nm.

B.4. Estimation of glucose by dinitrosalicylic acid reagent:

Glucose concentration of CFS was estimated using dinitrosalicylic (DNS) acid[12]. A standard curve was prepared against absorbance of CFS versus known concentration of glucose. The absorbance was measured by a UV-Spectrophotometer at wavelength of 575 nm.

B.5. Determination of protein concentration:

The protein concentration of CFS was determined by folin phenol reagent[13]. Bovine serum albumin (BSA) was used as a standard protein for calibration curve. A standard plot was prepared by graphing absorbance against known protein concentrations (BSA). Absorbance of the colored solution was measured by spectrophotometer, at 550 nm.

C. Culture media and mode of fermentation conditions:

Goat meat processing waste, enzymatically hydrolyzed with papain, and supplemented with 2% glucose was used as growth media of microorganism as described in previous work[14]. Protein concentration in the meat waste hydrolysate, obtained under optimum condition was 12.67g/L[14]. The initial pH of media was adjusted to 6.5. The media was sterilized at 121°C for 15 min.

In fed batch fermentation, nutrient feeding was repeated every 2 h with 20 ml volume and pH was controlled automatically at 6.5 by pH controller. The feeding was done in the bioreactor with 150 g/L glucose solution for fed batch operation I and; glucose (150 g/L) supplemented with concentrate meat waste hydrolysate with protein concentration of 21.86 g/L for fed batch operation II. Volume of 20 ml samples were also withdrawn at the time of feeding to keep the reactor volume constant. The same strategies of sampling, re-alkalization and feeding were repeated throughout the fed batch fermentation.

D. Experimental equipment:

In all fermentation experiments, a 5 L stirred bioreactor (Eyela, Tokyo Rikakikai Co. Ltd., Japan) with working volume of 3 L was used. The agitation was kept at 50 rpm to maintain spatial homogeneity throughout the fermenter. The fermenter was equipped with automatic temperature and pH controllers and two peristaltic pumps. One peristaltic pump was used for feeding of alkali for adjustment of pH and the other was used for intermittent feeding of nutrients. Temperature was maintained at 30°C by recirculation of water from constant temperature bath. For each run (both fed batch operation I and fed batch operation II), samples were withdrawn from the reactor at every 2 h to analyze the concentration of biomass, pediocin, lactic acid glucose and protein. The average concentration of biomass, products (pediocin and lactic acid) and substrates (glucose and protein) has been considered during interval of any consecutive sampling period.

E. Mathematical modeling:

It has been observed from experimental study and literature review[5] (Guerra *et al*. 2008) that cell growth of *P. acidilactici* is highly dependent on glucose and protein. As observed by previous researchers[5,15], pediocin and lactic acid production are partly related to cell growth and partly independent of growth. Thus Luedeking-Piret model[16] is applicable for the rates of these two products. The differential mass balance equations for biomass, two substrates (glucose and protein), pediocin and lactic acid around the bioreactor are as follows:

Biomass,

$$\frac{dX}{dt} = \frac{(X_{n+1} - X_n)(1 - V_s/V)}{t_{n+1} - t_n} = \mu_n X_n \quad (1)$$

where,

$$\mu_n = \frac{\mu_m S_{1n}}{K_{S_1} + S_{1n}} \frac{S_{2n}}{K_{S_2} + S_{2n}} \quad (2)$$

Glucose,

$$\frac{dS_1}{dt} = \frac{(S_{1_{n+1}} - S_{1_n})(1 - V_s/V) + (V_f/V)S_{2f}}{t_{n+1} - t_n} = \frac{\mu_n}{Y_{X/S_1}} X_n \quad (3)$$

Protein,

$$\frac{dS_2}{dt} = \frac{(S_{2_{n+1}} - S_{2_n})(1 - V_s/V) + (V_f/V)S_{2f}}{t_{n+1} - t_n} = \frac{\mu_n}{Y_{X/S_2}} X_n \quad (4)$$

Lactic acid,

$$\frac{dLA}{dt} = \frac{(LA_{n+1} - LA_n)(1 - V_s/V)}{t_{n+1} - t_n} = (\alpha_1 \mu_n + \beta_1) X_n \quad (5)$$

Pediocin,

$$\frac{dP}{dt} = \frac{(P_{n+1} - P_n)(1 - V_s/V)}{t_{n+1} - t_n} = (\alpha_2 \mu_n + \beta_2) X_n \quad (6)$$

Since the volume of culture broth in the bio-reactor remains constant with time, therefore,

$$\frac{dV}{dt} = 0 \quad (7)$$

where, $\mu \left(= \frac{1}{X} \frac{dX}{dt} \right)$ is specific growth rate (h^{-1}), μ_m is the maximum specific growth rate (h^{-1}), t is time (h), X is biomass concentration (g/L), V is working volume of fermenter (L), V_s is sampling volume (L), V_f is feeding volume (L).S_1 is glucose concentration (g/L) and S_2 is protein concentration (g/L).S_{1_f} is glucose concentration (g/L) and S_{2_f} is protein concentration (g/L) in feed solution. K_{S_1} is saturation constant for glucose (g-glucose/L) and K_{S_2} is protein saturation constant (g-protein/L). Y_{X/S_1} (g-biomass g-glucose^{-1}) and Y_{X/S_2} (g-biomass g-protein^{-1}) are yield coefficients for two substrates (glucose and protein) respectively. LA is lactic acid concentration (g/L). α_1 is growth-associated constant (g-lactic acid g-biomass^{-1}) and β_1 is non growth-associated constant (g-lactic acid g-biomass^{-1} h^{-1}) for lactic acid. Where, P is pediocin activity (AU/ml), α_2 is growth-associated constant (AU mg-biomass^{-1}) and β_2 is non growth-associated constant (AU mg-biomass^{-1} h^{-1}) for pediocin production. n and (n+1) are denoting n-th and (n+1)-th feeding positions respectively.

The model parameters, namely, μ_m, K_{S_1}, K_{S_2}, Y_{X/S_1}, Y_{X/S_2}, α_1, β_1, α_2 and β_2 have been determined by nonlinear regression through the minimization of sum of squared errors of difference between the predicted and experimental value of the variables, namely, biomass, pediocin, lactic acid, residual protein and residual glucose as obtained from experimental runs using eqs. (1-6)[17].

The accuracy of the model fits is evaluated by the mean squared error (MS_E) Criterion

$$MS_E = \frac{\sum_{i=1}^{n} (y_{obs_i} - y_{pre_i})^2}{q - p} \quad (8)$$

where, i is any value, q is sample size, p is no. of variables involved in model equations, y_{obs}, is observed value of variable, y_{pre}, is predicted value of variables.

Results and discussion

Both experimental and simulated data [eqs. (1), (3), (4), (5) and (6)] of the biomass, pediocin, lactic acid, glucose and protein concentration were plotted with time as shown in Figs. 1(a), 1(b), 1(c), 1(d) and 1(e) for fed batch operation I. Cell growth with production of pediocin and lactic acid has

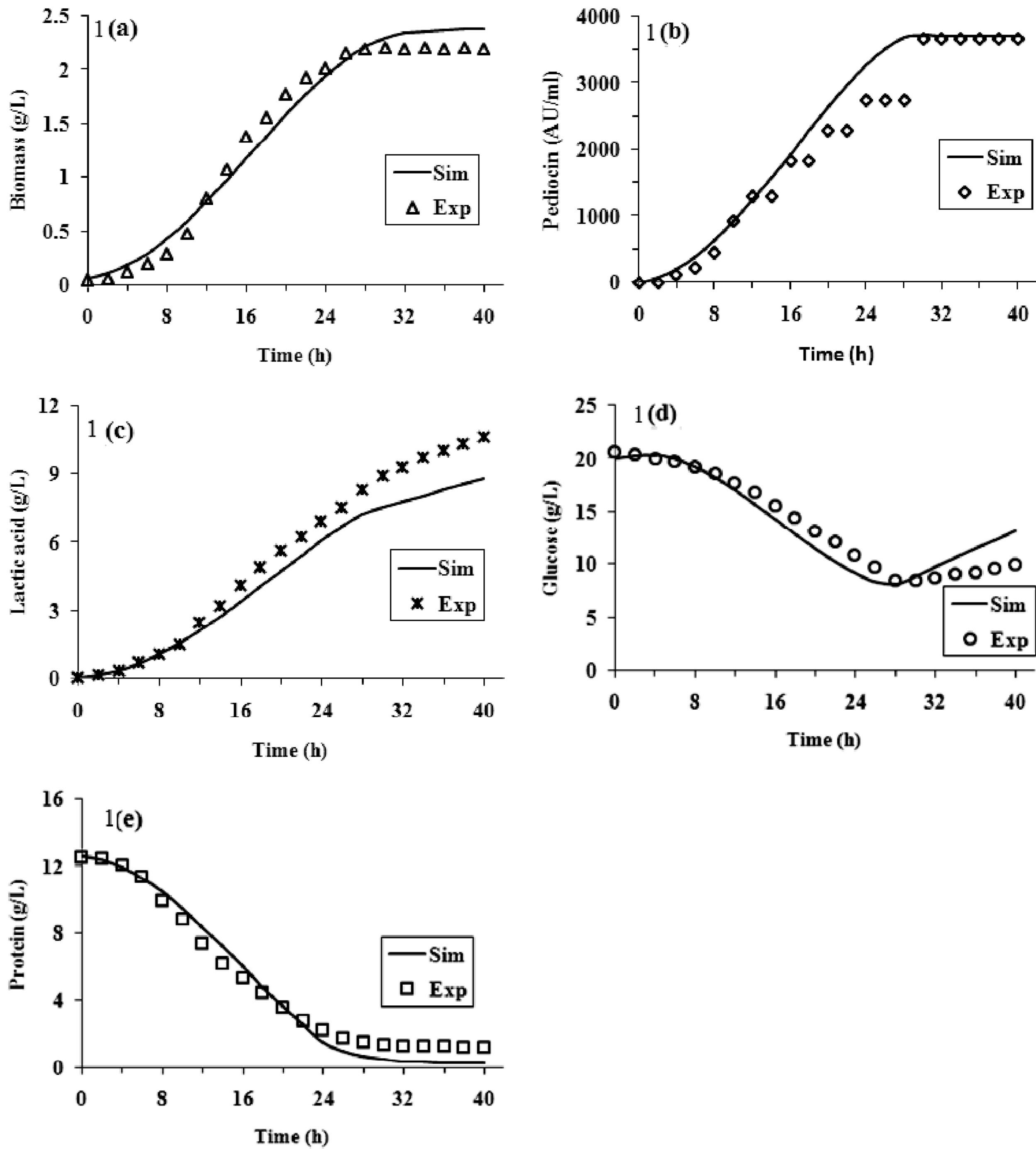

Fig. 1. Fed-batch operation I: fed meat processing wastes with concentrated glucose (150 g/L): under controlled pH condition in bioreactor. (a) Biomass (g/L), (b) pediocin (AU/ml), (c) lactic acid (g/L), (d) glucose (g/L) and (e) protein (g/L). Continuous lines representing simulated data and points indicating experimental results.

been observed with simultaneous glucose and protein consumption as shown in Figure. From the Figs. 1(a) and 1(b) it is clear that the cell concentration and pediocin activity reached the saturation after 30 h operation. The highest pediocin activity, biomass and lactic acid concentration were 3657 AU/ml, 2.2 g/L and 10.62 g/L respectively at the end of

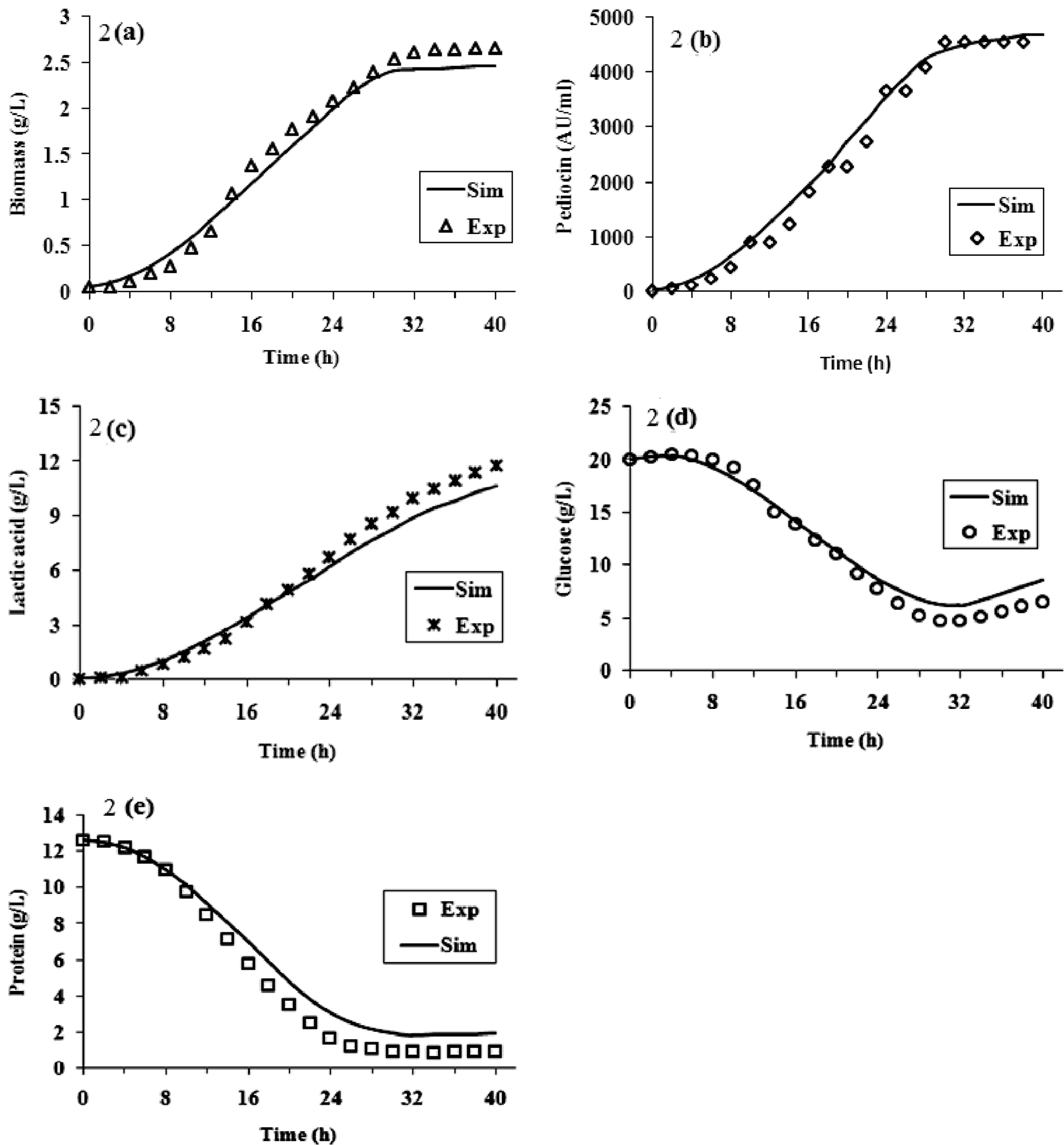

Fig. 2. Fed batch operation II: feeding with concentrated goat meat processing wastes and glucose (150 g/L): in bioreactor under controlled pH condition. (a) Biomass (g/L), (b) pediocin (AU/ml), (c) lactic acid (g/L), (d) glucose (g/L) and (e) protein (g/L). Continuous lines represented simulated values corresponding to the experimental results (points).

the fermentation. The Fig. 1(a) and 1(b) reveal that the concentration of cell and pediocin are saturated after 30 h operation. As shown in Fig. 1(a), the pediocin production is strongly dependent on cell growth, but the Fig. 1(c) indicate that the production of lactic acid is related with both growth and non-growth of cell. The Figs. 1(d) and 1(e) indicate the simultaneous consumption of both glucose and protein with cell growth. The average consumption rate of two substrates

such as glucose and protein are 0.362 $gL^{-1} h^{-1}$ and 0.286 $gL^{-1} h^{-1}$ respectively during the fermentation period.

For fed batch operation II, the concentration of biomass, pediocin, lactic acid, glucose and protein were plotted with time and the simulated data [eqs. (1), (3), (4), (5) and (6)] have been compared with experimental ones shown in Fig. 2(a), 2(b), 2(c), 2(d) and 2(e). The highest concentration of biomass, lactic acid and pediocin activity were 2.7 g/L, 11.76 g/L and 4572 AU/ml at saturation level after 32 h as shown in Figs. 2(a), 2(b) and 2(c). In fed batch operation II, biomass, pediocin and lactic acid concentration were respectively 1.23, 1.25 and 1.1 higher comparing to fed batch operation II. The improvement of production is probably due to addition of enough protein in feeding that indicates the importance of two nutrient such as protein and glucose on the cell growth as well as pediocin production. On the other hand, from Fig. 2(c), it is clear that concentration of lactic acid never reaches saturation. This is due to predominantly mixed, i.e. association with growth and non-growth, nature of production of lactic acid. Figs. 2(d) and 2(e) clearly indicate the importance of two substrates, namely glucose and protein on the cell growth and pediocin production. The average consumption rates of glucose and protein were 0.811 $gL^{-1} h^{-1}$ and 0.726 $gL^{-1} h^{-1}$ respectively. In both the cases (fed batch operation I and fed batch operation II), the agreement between experimental and simulated results is satisfactory (Figs. 1 and 2). From Fig. 2(d), it is evident that the time trajectory of glucose concentration follows a decreasing trend up to 32 h beyond which an increasing pattern is observed while the decreasing trend may be explained by dominance of utilization rate of substrate over the feeding rate trend. The increasing pattern is due to accumulation of substrate due to attainment of saturation in biomass concentration. Due to sampling and nutrient feeding, the fluctuation of biomass, products (pediocin and lactic acid) and substrates (glucose and protein) concentration in the bioreactor, has been considered in mathematical model as shown in eqs. [(1)-(6)]. On the contrary, the time course of protein concentration always follows decreasing trend with ultimate saturation at 32 h. Since the concentration of protein in feed solution is low compared to glucose, there is no increasing trend in its time trajectory. The decreasing pattern up to 32 h is clearly explained by its utilization by *Pediococcus acidilasctici* NCIM 2292. In this case, the agreement between experimental and simulated results is satisfactory.

A. Model parameters:

The model eqs. [(1), (3), (4), (5) and (6)] were solved by A MATLAB 7.0 program using 4-th order Runge-Kutta method. The simulated results were well matched with experimental ones for the concentration of biomass, pediocin, lactic acid, glucose and protein during both fed batch fermentation processes. The values of correlation coefficient (R^2) were in the range of 0.94 to 0.99 respectively that proved the validity of proposed model for the prediction of experimental results with high accuracy. The values of model parameters are given in Table 1. The value of non-growth associated constant (β_2) for pediocin is very small (0.004), which strongly supports the growth-associated production of pediocin. On the other hand, the values of α_1 and β_1 indicates both growth and non-growth associated production of lactic acid. Similar observations have also been reported by previous researchers[5,6].

Table 1. Estimated kinetic parameters

Kinetic parameter	Value	R^2
K_{S_2} (g-protein/L)	0.77	0.996
K_{S_1} (g-glucose/L)	1.34	0.98
μm (h^{-1})	0.45	0.987
Y_{X/S_1} (g-biomass g-glucose^{-1})	0.089	0.99
Y_{X/S_2} (g-biomass g-protein^{-1})	0.169	0.99
α_1 (g-lactic acid g-biomass^{-1})	2.661	0.947
β_1 (g-lactic acid g-biomass^{-1} h^{-1})	0.36	0.947
α_2 (AU mg-biomass^{-1})	1732	0.988
β_2 (AU mg-biomass^{-1} h^{-1})	0.004	0.988

Conclusions

Under the present investigation, *Pediococcus acidilactici* NCIM 2292 strain was used for the production of pediocin on fed batch fermentation mode using the waste from meat processing industries. It is expected that the use of meat processing waste will able to reduce the production cost of pediocin as well as healthy and green environment will be gifted by diminishing pollutants. A Mathematical model has been developed satisfactorily for fed batch operation. The experimental results were well matched with simulated data obtained from the model. In future, the validated model will be helpful for the design of bioreactor for large scale commercial production of pediocin by *P. acidilactici*.

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