ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 16, Issue 11, 2023

Online - 2455-3891
Print - 0974-2441

Research Article

CONTINUOUS PEDIOCIN PRODUCTION BY *PEDIOCOCCUS ACIDILACTICI* USING DAIRY WASTE

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Received: 17 May 2023, Revised and Accepted: 03 July 2023

ABSTRACT

Objective: The production of pediocin by *Pediococcus acidilactici* has been conducted in continuous operation using dairy waste, namely whey.

Methods: Whey supplemented with yeast extract was used as growth media of *P. acidilactici*. A series of batch fermentation have been conducted in whey with varying concentrations (0–40 g/L) of yeast extract. A continuous mode of fermentation was performed at the dilution rate of 0.05–0.25/h. The volumetric productivity of pediocin in continuous run has also been compared with batch process. A mathematical model has been developed to explain the reactor performance for pediocin production in both batch and continuous processes.

Results: The highest specific pediocin production rate (36.8 AU mg/cell dry mass/h) and growth rate (0.41/h) were observed in batch runs using whey broth supplemented with 20 g/L yeast extract. Maximum cell growth and pediocin activity were obtained at 1.36 g/L and 2286 AU/mL in batch experiments. The highest pediocin productivity (514125 AU/h) was found in continuous mode for a dilution rate of 0.0625/h. The model was validated through well matching of simulated data with experimental ones.

Conclusion: The study should be suitable for large-scale implementation of pediocin production.

Keywords: Pediocin, *Pediococcus acidilactici*, Whey, Continuous fermentation, Mathematical model.

 DOI: http://dx.doi.org/10.22159/ajpcr.2023v16i11.48352. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Because of harmful effects on human health, chemical preservatives are restricted to be used in processed food. The bio-preservatives are gaining increased interest for the improvement of the quality and shelf life of food, maintaining its natural flavor and texture. Bacteriocins are usually produced by lactobacillus, which shows inhibitory activity toward a variety of microorganisms [1,2]. Based on antimicrobial properties, bacteriocins were extensively studied to be used as potential bio-preservative [3,4]. Pediocin is a well-known bacteriocin approved as an effective food preservative and generally recognized as safe according to the Food and Drug Administration [5]. It is non-toxic in nature and easily degradable by intestinal proteases [6].

The cost of pediocin production can follow the economic route through the utilization of waste from food processing industries. Whey is a liquid dairy effluent carrying high values of biochemical oxygen demand and chemical oxygen demand, which poses a pollution threat to water bodies [7]. The reuse of whey as cheap growth media of producer strain is an innovative effort along with the reduction of water pollution. A few research works were reported on the use of whey in the production of lactic acid [8,9], ethanol [10], bacteriocin [11-13], etc. Some nitrogen sources such as peptones, beef, or yeast extract, are needed to mix up with whey for the enhancement of cell growth and its production [11].

Bacteriocin productivity is usually low in conventional batch fermentation mostly studied under well-controlled culture conditions (nutrients, temperature, and pH) [14,15]. Sometimes cell growth is inhibited by the accumulation of metabolic products such as lactic acid [16]. The limitations can be overcome through continuous mode of operation. The basic advantages of the continuous process are high volumetric productivity, nutritional availability, long-duration operational stability with the least contamination, and large-scale application. Product inhibition can be avoided through continuous replacement by fresh media. One limitation of a continuous system is cell washout at dilution rates higher than the maximum specific growth rate of the producer strain [12,13].

Under the present work, dairy waste, namely whey, has been used as growth media for pediocin production by *Pediococcus acidilactici*. To test the optimal condition of pediocin production, whey was supplemented with varying concentrations of yeast extract in batch fermentation. A set of continuous processes has been conducted by consecutive dilution rates in the bioreactor. The volumetric pediocin productivity of continuous mode has been compared with batch one. A mathematical model has been attempted for both batch and continuous pediocin production.

METHODS

Pre-treatment of whey and media preparation

Whey containing 40.5±0.5 g/L lactose, 4.8±0.5 g/L protein, and 0.6 g/L lactic acid, was collected from a local sweet processing shop. Whey was mixed up with yeast extract. It contained milk protein and supplemented yeast extract together was denoted as total protein of whey broth. The pH of the solution was adjusted to 6.8. The whey broth was sterilized at 121°C for 20 min and was used as a culture medium.

Microorganism and growth medium

P. acidilactici national collection of industrial microorganisms (NCIM) 2292 (pediocin producer strain) and *Listeria monocytogenes* NCIM 1143 (indicator organism for pediocin assay) were collected from NCIM, National Chemical Laboratory (Pune, India). Stock cultures of both microorganisms were maintained as nutrient agar slants at 4°C. Nutrient broth was used for the growth of *L. monocytogenes* and *P. acidilactici* was propagated in whey broth.

Effect of yeast extract on pediocin production

To study the effects of yeast extract on cell growth and pediocin production by *P. acidilactici*, a series of batch fermentations have been

conducted in 250 mL conical flasks containing 100 mL of whey broth. Yeast extract was added to liquid whey at varying initial concentrations (0–40 g/L). Each batch run was carried out with an initial pH of 6.8 at a temperature of 30°C for 24 h incubation period.

Pediocin production by batch fermentation

Batch fermentation of *P. acidilactici* has been carried out in a 5 L bioreactor of 3 L working volume using whey medium under controlled temperature and pH conditions. The temperature of the bioreactor was maintained at 30°C by automatic temperature system with attached water circular bath and pH was maintained at 6.8 by adding 1 N sodium hydroxide solution into the reactor. The reactor was equipped with two peristaltic pumps. One pump was used for pH adjustment and another pump was used for adding sterile fresh media into the reactor. The whey broth was inoculated with a 2% (v/v) of working culture. Samples from the reactor have been withdrawn aseptically at regular intervals for the determination of cell growth, pediocin, lactic acid, lactose, and protein concentration.

Pediocin production by continuous fermentation

After achieving mature cell growth, batch fermentation was switched to a continuous process in the bioreactor by feeding the same sterile whey medium at the same condition of pH (6.8) and temperature (30°C). The continuous production of pediocin by *P. acidilactici* was studied in the bioreactor at dilution rates from 0.05/h to 0.25/h. The process was monitored by periodically withdrawing samples. The schematic diagram of pediocin production in continuous mode is shown in Fig. 1.

Analytical methods

The concentration of lactose, protein, and lactic acid in whey broth has been measured by the colorimetric methods using dinitrosalicylic acid [17], folin phenol reagent [18], and p-hydroxydiphenyl [19], respectively.

The collected samples in both batch and continuous fermentation processes were divided into two aliquots. The optical density (OD) of the first aliquot was measured using a spectrophotometer (Varian, India) at 600 nm. A standard curve was drawn by correlating cell dry mass (CDM) with OD at 600 nm. The CDM (cell growth) concentration of each sample was determined from the standard plot.

The second aliquot was centrifuged (12,000 g for 15 min) at 4°C. The collected supernatant has been passed through 0.22 μm membrane (Cellulose Nitrate Membrane Filters, Whatman) for the removal of the cells. The cell free supernatant was used to determine pediocin activity [20], lactose [17], protein [18], and lactic acid [19]. The determination procedure of pediocin activity, lactose, protein, and lactic acid has been described in previous studies [21]. The unit of pediocin titer was denoted in arbitrary units (AU/mL). One arbitrary unit was defined as the reciprocal of the highest two-fold dilution showing a clear zone of growth inhibition by indicator organisms [22]. Pediocin activity in AU/mL was calculated as $(\frac{1000}{d})D$, where D is the dilution factor and d is the dose.

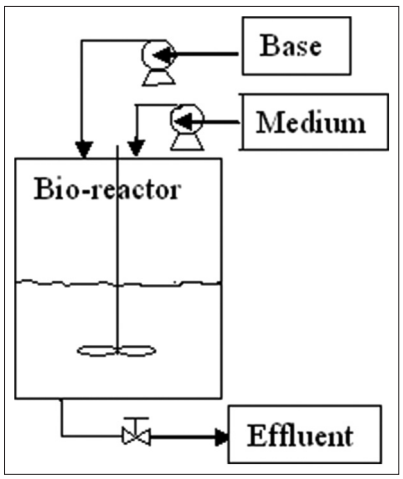

Fig. 1: Schematic of continuous pediocin production by *Pediococcus acidilactici* in the bioreactor

Model development

Batch fermentation

The mathematical model equations have been developed for cell growth, lactose, and protein consumption as well as for pediocin and lactic acid production in batch reactor systems [23,24]. Lactose and protein are two important nutrient sources for cell synthesis. For dual growth-limiting substrates, a modified Monod model has been introduced to describe cell growth. For batch fermentation, cell growth is expressed by the following equation:

$$\mu = \mu_m \frac{S_1}{S_1 + K_{S_1}} \frac{S_2}{S_2 + K_{S_2}} \quad (1)$$

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (2)$$

where μ is the specific growth rate (h^{-1}); μ_m is the maximum specific growth rate (h^{-1}); X is the concentration of cell growth (g/L); t is time (h); S_1 is lactose concentration (g/L); S_2 is protein concentration (g/L); K_{S1} (g-lactose/L) and K_{S2} (g-protein/L) are the corresponding saturation constants for lactose and protein, respectively;

Substrate (lactose and protein) consumption is described by the following equations:

$$q_1 = -\frac{1}{Y_{X/S_1}}\mu \quad (3)$$

$$q_2 = -\frac{1}{Y_{X/S_2}}\mu \quad (4)$$

where the yield coefficient of biomass for lactose is $Y_{X/S1}$ (g-CDM g/lactose) and for protein is $Y_{X/S2}$ (g-CDM g/protein); Specific consumption rate of lactose and protein are q_1 (g-lactose g/CDM/h) and q_2 (g- protein g/CDM/h), respectively.

Pediocin and lactic acid production are related to cell growth [14]. Hence, the Luedeking–Piret model [25] is applicable for the two products formation.

For pediocin generation,

$$q_P = K_P\mu + K_N \quad (5)$$

$$q_P = \frac{1}{X}\frac{dP}{dt} \quad (6)$$

where the specific pediocin production rate is q_p (AU g/CDM/h), P is the pediocin activity (AU/mL), K_p is the growth-associated constant (AU g/CDM) and K_N is the non-growth-associated constant (AU g/CDM/h) for pediocin.

For lactic acid production,

$$q_{LA} = \alpha\mu + \beta \quad (7)$$

$$q_{LA} = \frac{1}{X}\frac{dLA}{dt} \quad (8)$$

where the specific lactic acid formation rate is q_{LA} (g lactic acid g/CDM/h), α is the growth-associated constant (g-lactic acid g/CDM) and β is the non-growth-associated constant (g-lactic acid g/CDM/h) for lactic acid, LA is the lactic acid concentration (g/L).

Continuous fermentation process

In a continuous bioreactor system, overall mass balance for cell growth, substrate (lactose and protein) consumption, pediocin, and lactic production can be expressed as follows:

Accumulation = Inlet + Production _ outlet _ consumption (9)

Cell growth

$$V\frac{dX}{dt} = QX_i + \mu XV - K_d XV - QX_e \quad (10)$$

where V is the working volume of the reactor (L), Q is the effluent flow rate (L/h), microbial cell concentration is X_i (g/L) in influent, and X_e (g/L) in the effluent stream. K_d is the endogenous microbial decay coefficient (h^{-1}). At steady state condition, $\frac{dX}{dt}=0$, Eq. (10) can be written as

$$(\mu - K_d)X = \frac{Q}{V}(X_e - X_i) \quad (11)$$

Eq. (11) can be written as follows:

$$\mu - K_d = D \quad (12)$$

where (μ-K_d) is the net specific growth rate (h^{-1}). Since any bacterial cells are not introduced in the influence of the bioreactor, *Xi=0*. Hence, the cell concentration will be the same in the system, and in the effluent, *X=Xe*, *D* is the dilution rate ($\frac{Q}{V}$).

Substrate consumption

$$QS_i - QS_e - qXV = V\frac{dS}{dt} \quad (13)$$

At steady state condition, $\frac{dS}{dt}=0$. Eq. (13) can be written as follows:

$$qX = D(S_i - S_e) \quad (14)$$

where S_i is the substrate concentration (g/L) in influent, S_e is the substrate concentration (g/L) in effluent; and q is the specific substrate utilization rate.

For lactose consumption

$$q_1 X = D(S_{1i} - S_{1e}) \quad (15)$$

where, S_{1i} and S_{1e} are denoted as influent and effluent lactose concentration, respectively.

For protein consumption

$$q_2 X = D(S_{2i} - S_{2e}) \quad (16)$$

where, S_{2i} and S_{2e} are denoted as influent and effluent protein concentration, respectively.

Pediocin production

$$V\frac{dP}{dt} = QP_i + q_P XV - QP_e \quad (17)$$

where, P_i and P_e are inlet and outlet pediocin activity, respectively. At steady state condition, P_e=P, $\frac{dP}{dt}=0$, and P_i=0. Eq. (17) can be expressed as

$$q_P X = DP \quad (18)$$

Lactic acid production

$$V\frac{dLA}{dt} = QLA_i + q_{LA} XV - QLA_e \quad (19)$$

where, LA_i and LA_e are influent and effluent lactic acid concentrations, respectively.

At steady state condition LA_e=LA, $\frac{dLA}{dt}=0$. The value of LA_i is not zero, because whey contains 0.6 g/L lactic acid. Hence, Eq. (19) can be written as

$$q_{LA} X = D(LA - LA_i) \quad (20)$$

Determination of kinetic parameters

The model parameters (μ_m, Ks_1, Ks_2, $Y_{X/S1}$, $Y_{X/S2}$, K_P, K_N, α, β, and K_d) have been evaluated by non-linear regression. The values of μ_m, Ks_1, Ks_2, $Y_{X/S1}$, $Y_{X/S2}$, K_P, K_N, α, and β were determined from simulation of batch fermentation using equations (1), (3), (4), (5), and (7). The value of K_d was determined from continuous fermentation using equation (12). The variables, namely cell growth (X), pediocin (P), lactic acid (LA), lactose (S_1), and protein (S_2) concentration were obtained from experimental runs. The sum of squared differences between the predicted and experimental values was minimized by varying the values of the model parameters. The accuracy of the model fits is evaluated by the mean squared error (MS_E) criterion.

$$MS_E = \frac{\sum_{i=1}^{n}(y_{obs_i} - y_{p_i})^2}{n-m} \quad (21)$$

where i is any value; n is the sample size; m is the number of variables involved in model equations; y_{obsi} is the observed value of the variable; y_{pi} is the predicted value of variables. The values of model parameters are presented in Table 1. The model equations (1, 3, 4, 5, and 7) and (12, 15, 16, 18, and 20) were solved by MATLAB 7.0 using the 4th order Runge–Kutta method for batch and continuous fermentation, respectively

RESULTS AND DISCUSSION

Effect of yeast extract

In Fig. 1, the specific growth rate of *P. acidilactici* and the specific pediocin production rate obtained from batch fermentation results in conical flasks have been plotted against varying concentrations of yeast extract used in the experiments. The figure indicates that cell growth and pediocin production are maximized for yeast extract concentration of 20 g/L. The value of the highest specific pediocin production rate and maximum growth rate were 36.8 AU mg-CDM/h and 0.41/h, respectively. Based on experimental results, the whey was supplemented with 20 g/L yeast extract and was used further as growth media of *P. acidilactici* for pediocin production in both batch and continuous fermentation process.

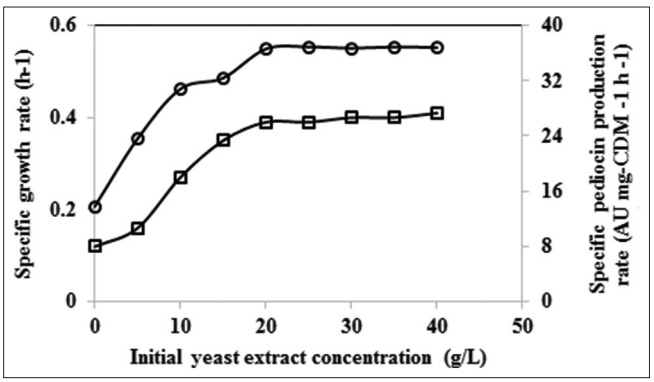

Fig. 2: Batch fermentation of *Pediococcus acidilactici* with initial yeast extracts concentration (0–40) g/L: Specific growth rate (□); specific pediocin production rate (○)

Batch fermentation

In (Fig. 3a-e), the concentration of cell growth, pediocin, lactic acid, lactose, and protein obtained from batch experimental and simulated results using equations (1), (3), (4), (5), and (7) have been plotted against time. Batch fermentation of *P. acidilactici* culture has been performed using yeast extract (20 g/L) supplemented whey media for 24 h in the bioreactor at 30°C temperature and pH of 6.8. The highest cell growth and pediocin activity were obtained at 1.36 g/L and 2286 AU/mL, respectively, after 20 h of fermentation as shown in (Fig. 3a and b). The figures indicate the production of pediocin is increased with cell growth at the exponential growth phase and reaches a maximum value at the stationary phase. In (Fig. 3c), the highest concentration of lactic acid was observed at 10.63 g/L. It validates the concept that the production of pediocin is growth-associated, where lactic acid formation is related to both growth and non-growth-associated as shown in (Fig. 3a-c). It has already been observed by previous researchers that bacteriocin is a growth-associated product [14,26]. The (Fig. 3d and e) depict that most of the lactose and protein were consumed by the *P. acidilactici* strain. The average consumption rate of lactose and protein are 1.54/gL/h and 0.97/gL/h, respectively. The production of pediocin by *P. acidilactici* in batch cultures occurred during the active growth phase. This indicated that pediocin productivity is strictly dependent on cell growth. Hence, high cell densities provide optimal pediocin production. The cell growth is usually influenced by the variables (pH, temperature, and composition of nutrients). The cell growth could be restricted at a higher or lower limit of pH and temperature values. Due to having a suitable amount of lactose, minerals, and vitamins, whey is a valuable medium for cell growth and pediocin production. The cell growth may be inhibited by the production of lactic acid. The continuous feed of fresh media provides nutrients as well as removes lactic acid.

Continuous fermentation

The pediocin production in the continuous fermentation process has been monitored in the bioreactor at dilution rates of 0.05–0.25/h. Both experimental and simulated data of cell growth, pediocin, lactic acid, lactose, and protein concentration were plotted against dilution rate using equations (12), (15), (16), (18), and (20) as shown in (Fig. 4a-e). The highest and almost the same concentration of cell growth, pediocin, and lactic acid were observed at the dilution rates of 0.05/h and 0.0625/h as shown in (Fig. 4a-c). The cell growth and pediocin were

Table 1: Results of batch and continuous fermentation

Dilution rate (h^{-1})	Continuous fermentation					Batch fermentation
	0.05	0.0625	0.083	0.125	0.25	
X_{max} (g/L)	1.45	1.46	1.31	1.01	0.56	1.36
P_{max} (AU/mL)	2742	2742	1828	914	229	2286
dP/dt (AU mL/h)	137	172	152	114	57	112
q_p (AU mg-CDM/h)	93	118	115	113	102	84
VdP/dt (AUh^{-1})	411300	514125	455172	342750	171750	336000

X_{max}: Maximum concentration of cell growth; P_{max}: Maximum pediocin activity; dP/dt: Pediocin productivity; VdP/dt: Volumetric pediocin productivity; q_p: Specific pediocin production rate

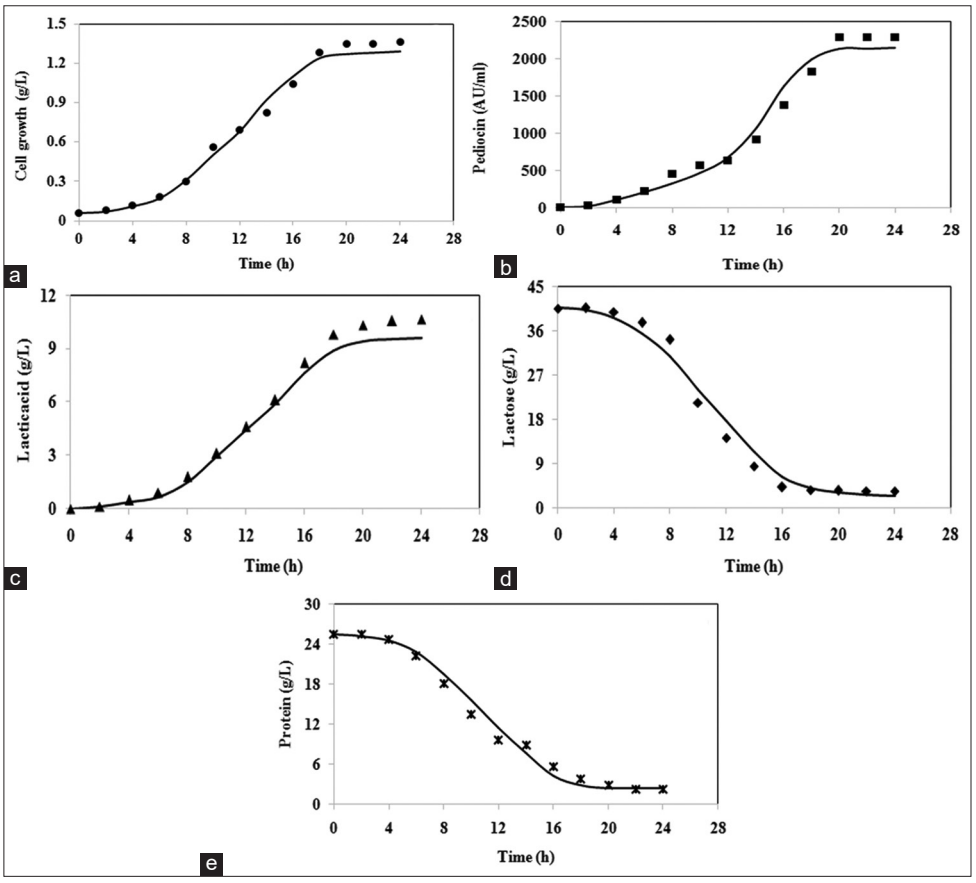

Fig. 3: Experimental (points) and simulated (lines) data of batch fermentation of *Pediococcus acidilactici* on whey supplemented with yeast extract of 20 g/L in the bioreactor: (a) cell growth (●), (b) pediocin (■), (c) lactic acid (▲), (d) lactose (◆), and protein (*)

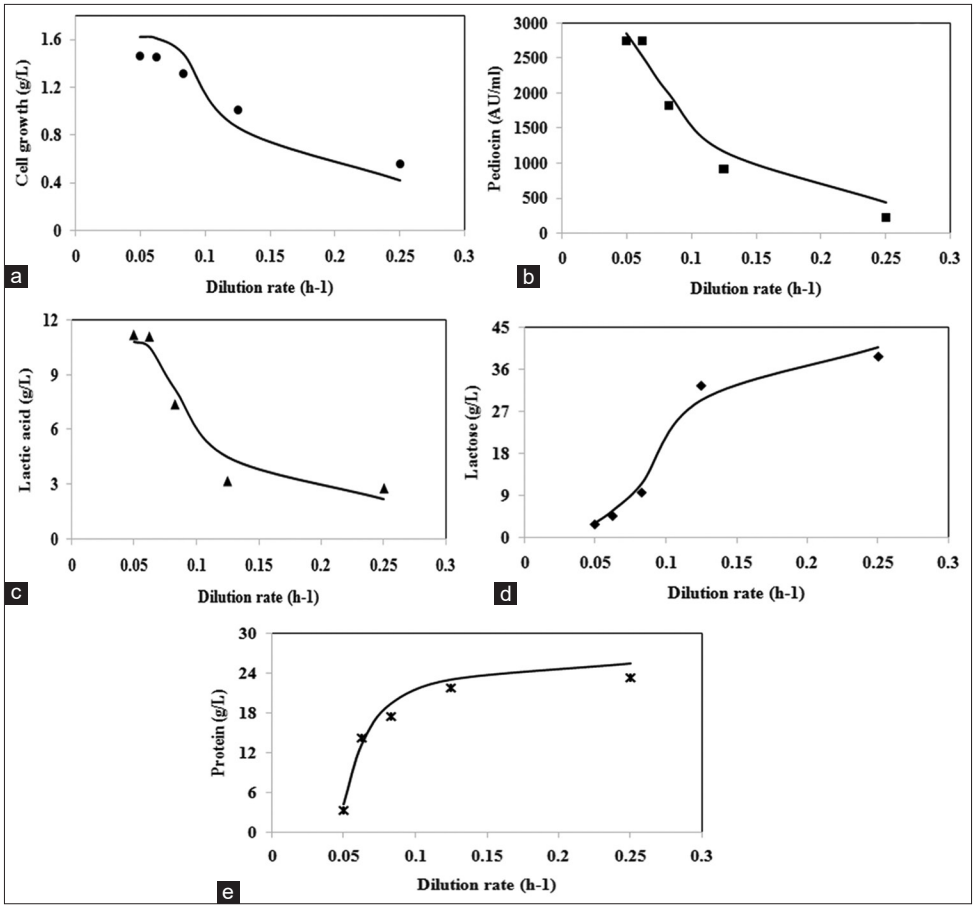

Fig. 4: Experimental (points) and simulated (lines) data of continuous fermentation of *Pediococcus acidilactici* on whey broth in bioreactor: (a) Cell growth (●), (b) pediocin (■), (c) lactic acid (▲), (d) lactose (◆), and (e) protein (∗)

Table 2: Estimated kinetic parameters for cell growth, lactose and protein consumption, lactic acid, and pediocin production

Parameter	Value	Correlation coefficient
μ_m	0.41 (h^{-1})	0.97
K_{S1}	1.46 (g-lactose/L)	0.98
K_{S2}	0.82 (g-protein/L)	0.98
$Y_{X/S1}$	0.082 (g-CDM g-lactose^{-1})	0.99
$Y_{X/S2}$	0.16 (g-CDM g-protein^{-1})	0.99
K_P	1.5 (AU mg/CDM)	0.98
K_N	0.006 (g-protein/L)	0.98
α	2.56(g-lactic acid g-CDM)	0.99
β	0.34 (g-lactic acid g/CDM/h)	0.99
K_d	0.31/h	0.97

1.45 g/L and 2742 AU/mL, respectively, at a dilution rate of 0.0625/h. The maximum lactose and protein consumption were observed for both dilution rates of 0.05/h and 0.0625/h shown in (Fig. 4d and e). The values of volumetric pediocin productivity (pediocin activity × dilution rate × working reactor volume) and specific pediocin production rate for both batch and continuous fermentations are given in Table 1. The highest volumetric pediocin productivity and specific pediocin production rate were 514125AU/h and 118 AU mg-CDM/h, respectively, for the dilution rate of 0.0625/h shown in Table 1. The specific pediocin production increased with increasing dilution rate (Table 1).

The values of kinetic parameters estimated from the experimental results of the batch process are given in Table 2. The predicted results of cell growth (X), pediocin (P), lactic acid (L), residual lactose (S_1), and residual protein (S_2) concentrations were satisfactorily matched with the experimental data. The value of K_N (0.006 g/protein/L) is negligible compared to the value of K_p (1.5 AU mg/CDM), indicating that pediocin is a growth-associated product. On the other hand, the value of α (2.56 g-lactic acid g/CDM) and β (0.34 g-lactic acid g/CDM/h) indicates that lactic acid is both a growth and non-growth-associated product as shown in Table 2. The values of R^2 are ranging from 0.97 to 0.99 shown in Table 2.

CONCLUSION

The present investigation focuses on the usage of dairy waste for the cost-effective production of pediocin by *P. acidilactici* in a continuous fermentation process followed by batch experiment under well-controlled culture conditions. Pediocin is a highly growth-associated product. The maximum volumetric productivity of pediocin (514125 AU/h) has been obtained in a continuous process for a dilution rate of 0.0625/h. A mathematical model has been developed successfully to describe the bioreactor performance. The study will be helpful for the industrial production of pediocin as an innovative bio-preservative in the future.

AUTHOR CONTRIBUTIONS

This is author's sole research work and not contributed by other ones.

CONFLICT OF INTEREST STATEMENT

Declared none.

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