International Journal of Engineering

Journal Homepage: www.ije.ir

RESAERCH NOTE

Batch Kinetics and Modeling of Alkaline Protease Production by Isolated *Bacillus* sp.

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PAPER INFO

Paper history:
Received 27February 2015
Received in revised form 04 April 2015
Accepted 30 April 2015

Keywords:
Alkaline Protease
Fish Waste Hydrolysate
Modeling
Bacillus

ABSTRACT

The aim of this study was the use of fish waste hydrolysate (FWH) as a substrate for alkaline protease production and the enzyme production kinetics using isolated *Bacillus* sp. in a batch system. The results show that with the addition of FWH to the fermentation medium with a final concentration of 4% (optimal concentration), alkaline protease value reached a maximum value (89 U/ml), which is 63% higher than that of the control medium. Several kinetic models were evaluated; the combination of the Moser and Boulton kinetic model gave the best prediction. The results show that a good agreement between the model solutions and the experimental data for cell, substrate concentrations and enzyme activity was obtained, and this nonlinear mathematical model performed satisfactorily on biomass, substrate, and enzyme predictions. Also, the quadratic correlation coefficients obtained were higher than 0.98.Therefore, the kinetic model then may be used to simulate the fermentation variables.

doi: *10.5829/idosi.ije.2015.28.06c.03*

NOMENCLATURE

S_0	Initial substrate concentration (g/l)	K_i	Substrate concentration at which the substrate inhibition factor (g/l)
S	Substrate concentration (g/l)	$Y_{X/S}$	Yield coefficient for cell on substrate (g/g)
K_s	Initial substrate concentration at half the maximum specific cell growth rate	dX/dt	Cell growth rate (g/l h)
X	Cell concentration (g/l)	dS/dt	Substrate utilization rate (g/l h)
μ	Specific cell growth rate (1/h)	**Greek Symbols**	
μ_m	Maximum specific growth rate (1/h)	α	Growth-associated product formation constant
K_d	Specific cell death rate (1/h)	β	Non-growth associated product formation constant
m_s	Maintenance energy coefficient (1/h)		

1. INTRODUCTION

Proteases constitute one of the commercially important groups of extra-cellular microbial enzymes and are widely used in several industrial sectors, particularly in the detergent, food, pharmaceutical, chemical, leather and silk, as well as waste treatment. Among all proteases, alkaline proteases are robust in nature and mainly used as detergent additives [1].

For economic production of protease, microbes can be the best choice. They can be cultured in large quantities in a relatively short time by established methods of fermentation, and they also produce an abundant, regular supply of the desired product [2]. Microbial proteases can be produced from bacteria, fungi and yeast. Bacteria of the genus *Bacillus* are active producers of extracellular alkaline proteases [3]. Currently, large portions of commercially available alkaline proteases are derived from *Bacillus* strains [4].

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Please cite this article as: G. Khayati, M. Zamani, Batch Kinetics and Modeling of Alkaline Protease Production by Isolated *Bacillus* sp., International Journal of Engineering (IJE), TRANSACTIONS C: Aspects Vol. 28, No. 6, (June 2015) 841-847

Use of waste biomaterials for biotechnological products especially enzyme has been getting attention in the recent years [1, 5]. Fish waste hydrolysate, known as fibrous protein, is widely produced in the world. Increasing concern about pollution occurring from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. Furthermore, other fibrous proteins like feather, nail and hair are available as waste.

There are several reports describing use of waste material for the production of alkaline protease, e.g. proteinaceous solid waste from tanneries by *Synergistes*sp. [6], shrimp shell wastes by *Bacillussubtilis*TKU007 [7]**.** However, Fish waste hydrolysate (FWH) has never been reported before for the production of industrially important protease enzyme.

Microbial processes are inherently complex, and it is the critical step in practical applications, such as understanding, controlling, and optimizing fermentation process. Kinetic modeling of a fermentation process presents some advantages such as process control [8] and determination of an optimal operation condition for the production of a target metabolite [9]. A good model must, however, take into account the effects of substrate limitation, substrate inhibition and product inhibition as well as maintenance energy and cell death on the cell growth and metabolism.

The objective of this study was to use FWH in a fermentation medium for the production of alkaline protease using a local isolate of *B.sphaericus*in batch fermentation. Also, experimental data from batch fermentations of alkaline protease were examined in order to form the basis of kinetic model of the process. However, little kinetic study on the fermentative production of alkaline protease has been performed.

2. MATERRIALS AND METHODS

2. 1. Microorganism Bacterial strain used in this study was isolated from alkali soils collected at different locations in northern Iran. The Isolated bacterium was identified as *Bacillus sphaericus* [10]. The strain was maintained on nutrient agar and used throughout the study.

2. 2. Hydrolysis of Fish Waste Hydrolyses were prepared by the method of Gao [11]. In this process, firstly the wastes were minced by a grinder. Secondly the minced wastes were mixed with water to make fish wastes (wet weight)/water a ratio of 1:1. Thirdly the initial pH of the waste slurry was set at 1 by the addition of 6 M HCl. And lastly the slurry was hydrolyzed at 121°C for 20 min and was further centrifuged at 4000 rpm for 20 min. The supernatant was used as a nutrient source for the production of alkaline protease after neutralized.

2. 3. Culture Conditions and Fermentation Process The experimental medium contained 1% glucose, 0.1% casein, 0.1% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$. In order to determine the effects of FWH on the alkaline protease production, 0 (control medium, CM), 1–6% v/v FWH (FWHM medium) were added to the production medium, respectively.

The experiments were conducted at different FWH concentrations (0–6% v/v). Batch fermentations were carried out in a 2-L fermentor with a working volume of 1 L. The airflow rate was adjusted at 0.7 v/v/m. The culture temperature and agitation rate was automatically maintained at 34ºC and 200 rpm, respectively.

The biomass was determined following centrifugation at 8000×g for 20 min in 4ºC, drying the cell mass at 80ºC overnight and weighting the resulting dry cell biomass. The supernatant was used for the determination of the residual sugars and protease activity.

2. 4. Analytical Methods Samples for the determination of the enzyme activity, residual sugar and cell dry weight were withdrawn at interval of 2h. Bacterial growth was assessed measuring the optical density with a spectral photometer (Biosience, Ultrospec 2100 pro (UV/Vis) photometer).

The concentration of residual sugar in the medium was determined calorimetrically using 3, 6-dinitrosalicylic acid (DNS) [12]. For this purpose, 1 ml of diluted supernatant, obtained after removal of the biomass, was mixed with 1 ml of DNS and heated to 100°C for 5 min. The reaction was stopped by incubation on ice and the optical density at 540nm was read. The concentration of residual sugar was determined against a glucose standard graph.

Proteolytic activity was measured by hydrolysis of casein. The supernatant (0.5ml) was mixed with 1.0ml of 0.5% casein solution in glycine–NaOH buffer (0.05M, pH 9.0), preincubated at 34°C for 30min. The mixture was incubated at 34°C for 30min., and 2ml of 10% TCA (trichloroacetic acid) solution was added to the mixture immediately to stop the reaction. The reaction mixture was centrifuged at 10000rpm and 4°C for 10min. Tyrosine content in the supernatant was determined by colorimetry at 650nm using Folin–phenol reagent [13]. The enzyme activity was defied as the amount of the enzyme that liberated 1mg of tyrosine per minute under the conditions used in this study.All experiments were replicated twice and averaged values are presented in this study.

3. RESULTS AND DISCUSSION

3. 1. Fermentation Experiments In this study, FWH was considered as a supplement in the fermentation medium because of its amino acid content and fibrous protein. It is clear that alkaline protease production could be increased when FWH was employed as a supplement in the fermentation medium. First, we have investigated the effects of different initial FWH concentration (0-6%) on the biomass and alkaline protease production. These results demonstrated that up to 4% FWH addition could result in promoted *B.sphaericus* growth and increased alkaline protease production (see Table 1).

Obviously, the concentration of 4% FWH had a significant effect on the biomass and enzyme production. As seen in Table 1, the highest biomass (6.7 g/l) and alkaline protease (87 U/ml) were obtained from 4% FWH. Thus, this study showed that the optimal concentration of FWH for production of alkaline protease was 4%. It was found that applications higher than 4% had an inhibitory effect. For example, the lowest biomass (1.32 g/l) and alkaline protease (18 U/ml) yields were obtained when 6 % FWH was applied. This is probably due to the high concentrations of salts after neutralization that may cause critical problems during fermentation as they inhibit the growth of *B.sphaericus*. Gao, et al. [11] also reported the same results in lactic acid production. These results suggested that the increase in FWH had a negative effect on the cell growth rate, and the increase in alkaline protease production rate was due to the increase in the cell growth rate. Therefore, we used 4% FWH for subsequent studies.

The effects of incubation time on the alkaline protease activity, biomass yield and residual sugar of the cultures are shown in Figure 1. During these fermentations, no product inhibitions were observed.

Notably, the use of FWH in the fermentation medium has a significant effect on the alkaline proteaseactivity and sugar consumption. The results of FWHM (4% FWH+CM) (Figure 1a) are higher than that of CM (Figure 1B).

The highest alkaline proteaseactivity in the FWHM was observed at 26 h. This value was 89 U/ml, and the rate of sugar consumption was approximately 95%. However, the alkaline proteaseactivity in the CM for the same incubation time was measured as 48 U/ml, and the rate of sugar consumption for this medium was about 84%.The highest alkaline proteaseactivity in the CM was observed at 30 h. This value was 55 U/ml; the rate of sugar consumption was approximately 100%. Nevertheless, this value is lower than that of FWHM. At 30 h incubation time, the content of alkaline protease in the FWHM is 63% higher than that of CM.

TABLE 1. Biomass and alkaline protease activity of *B.sphaericus* after 26 h at different FWH concentration

Media	Biomass yield (g/l)	Enzyme activity (U/ml)
1% FWH[a]+CM [b]	4.73	56
2% FWH+CM	5.48	66
3% FWH+CM	6.11	78
4% FWH+CM	6.70	87
5% FWH+CM	3.17	34
6% FWH+CM	1.32	18
CM	3.48	36

a. fish waste hydrolysate (FWH)
b. control medium (CM)

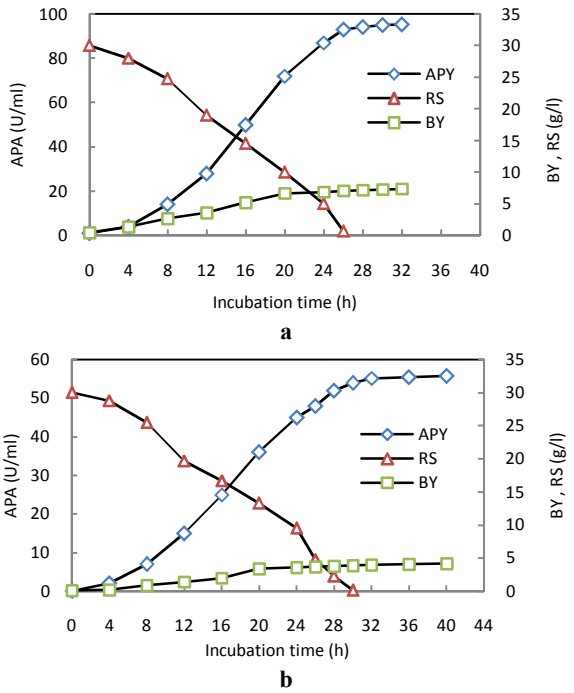

Figure 1. Residual sugar (RS), biomass yield (BY) and alkaline proteaseactivity (APA) on FWHM (A) and CM (B) at various incubation times

The CM gave the maximum enzyme production after a long time (30 h), while the FWHM gave it after a short time (26 h). Therefore, utilizing the FWHM for protease production may be more economical. It resulted in an improvement in both fermentation time and yield.

3. 2. Development of Kinetic Models The unstructured kinetics of fermentation can be normally described by set of three coupled differential equations for cell growth rate (r_X), product formation rate (r_P), and substrate utilization rate (r_S). These equations are:

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

$$r_s = \frac{dS}{dt} = -\left[Y_{X/S}^{-1} + Y_{P/S}^{-1}\right]\mu X - m_s X \quad (2)$$

$$r_p = \frac{dP}{dt} \alpha Y_{p/s} \mu X + \beta X \quad (3)$$

where X is the biomass concentration, S the substrate concentration , P product concentration (protease activity), Yx/s and Yp/s specific yield coefficients, ms maintenance coefficient, α a growth associated term, β a non growth associated term, and μ the specific growth rate, which gives the characteristic nonlinear behavior of fermentation processes. Several approaches that describe the kinetic microorganism behavior (kinetic models) are shown in Table 2.Using each kinetic model given by (4-8) alone with the set of differential Equations (1-3) cannot describe satisfactorily the enzyme and biomass production and sugar consumption in the fermentation process as is shown in Figure 2.

However, combinations of these kinetic models may improve the model prediction. Also, Table 2 contains different combinations of kinetic models (e.g. Equation (9) uses the kinetic models of Haldane [14] and Boulton [15], Equation (10) uses the kinetic models of Haldane and Levenspiel [16], and so on). Figure 3 shows the performance of the different kinetic models (9-14) on the prediction of protease production. Each model (11-16) was evaluated by means of a linear regression between the experimental data and the predicted data for the biomass, substrate, and product. The correlation coefficient (R^2) of each set of fermentations was averaged and their values are shown in Table 3.

Excellent correlation coefficients were obtained for models of Haldane – Boulton (9), Moser [17] – Boulton (12), and Moser – Luong [18] (14). However, the model with the best correlation coefficients is the one with the combination of Moser and Boulton. The simulated data was obtained by integrating differential Equations (1-3) along with the specific growth rate Equation (12) and the parameter values shown in Table 4, using the Runge-Kutta method ode45 with MATLAB. In the model, with respect to the magnitude of α parameter (23.1 g g-1) (Table 4), it appears that the biosynthesis of alkaline protease can be attributed to a growth-associated type. It can be seen that protease formation is strongly linearly related to cell growth. The *B.sphaericus* started enzyme production when the cells entered the exponential phase, and as a result, cell growth and alkaline protease production took place simultaneously.

The biomass (X), substrate (S), and enzyme (P), profiles obtained with the unstructured kinetic model are shown in Figure 4. The results show that the Moser-Boulton model is more accurate in predicting utilization of substrate during microbial growth and protease production. Also, good agreement between the results of the prediction model and the experimental data for cell, substrate concentrations and enzyme activity was obtained. A linear regression for the predicted biomass, the substrate, and the enzyme with the experimental data was produced. The results for 30 g/L initial substrate concentrations are shown in Figure 5. The quadratic correlation coefficients obtained were higher than 0.98, so the kinetic model can be used for fermentation process optimization and scale up this bioprocess.

TABLE 2. Kinetic models

Simple kinetic models:		
Boulton, 1980	$\mu = \frac{\mu_m K_p}{K_p + P}$	(4)
Haldane, 1930	$\mu = \frac{\mu_m S}{K_s + S + S^2 K_i^{-1}}$	(5)
Levenspiel, 1980	$\mu = \mu_m (1 - \frac{P}{K_p})^m$	(6)
Luong, 1985	$\mu = \mu_m (1 - (\frac{P}{K_p})^m)$	(7)
Moser, 1958	$\mu = \frac{\mu_m S^n}{K_s + S^n}, n > 0$	(8)
Combinations of kinetic models:		
Haldane – Boulton	$\mu = \frac{\mu_m S}{K_s + S + S^2 K_i^{-1}} \frac{K_p}{K_p + P}$	(9)
Haldane – Levespiel	$\mu = \frac{\mu_m S}{K_s + S + S^2 K_i^{-1}} (1 - \frac{P}{K_p})^m$	(10)
Haldane – Luong	$\mu = \frac{\mu_m S}{K_s + S + S^2 K_i^{-1}} (1 - (\frac{P}{K_p})^m)$	(11)
Moser – Boulton	$\mu = \frac{\mu_m S^n}{K_s + S^n} \frac{K_p}{K_p + P}$	(12)
Moser – Levespiel	$\mu = \frac{\mu_m S^n}{K_s + S^n} (1 - \frac{P}{K_p})^m$	(13)
Moser – Luong	$\mu = \frac{\mu_m S^n}{K_s + S^n} (1 - (\frac{P}{K_p})^m)$	(14)

TABLE 3. Linear correlations for kinetics models

Kinetics Model	Mean Value among the correlation coefficient (R^2)
Haldane – Boulton	0.9816
Haldane Levespiel	0.9690
Haldane – Luong	0.9783
Moser – Boulton	0.9881
Moser – Levespiel	0.8503
Moser – Luong	0.9810

TABLE 4. Kinetic parameters used in the process fermentation of protease production using Moser - Boulton kinetic model

Parameter	Value
μ_m= maximum specific growth rate (1/h)	0.528
K_s= Substrate affinity (g/L)	35
K_p = Inhibition term (g/L)	100
m_s= maintenance coefficient (g/L)	0.056
Y_{xs}= yield coefficient	0.74
Y_{ps}= yield coefficient	0.52
α = growth associated term	23.1
β = *non* growth associated term	0.07
n = exponential term for Moser model	1
m= exponential term for Boulton model	9

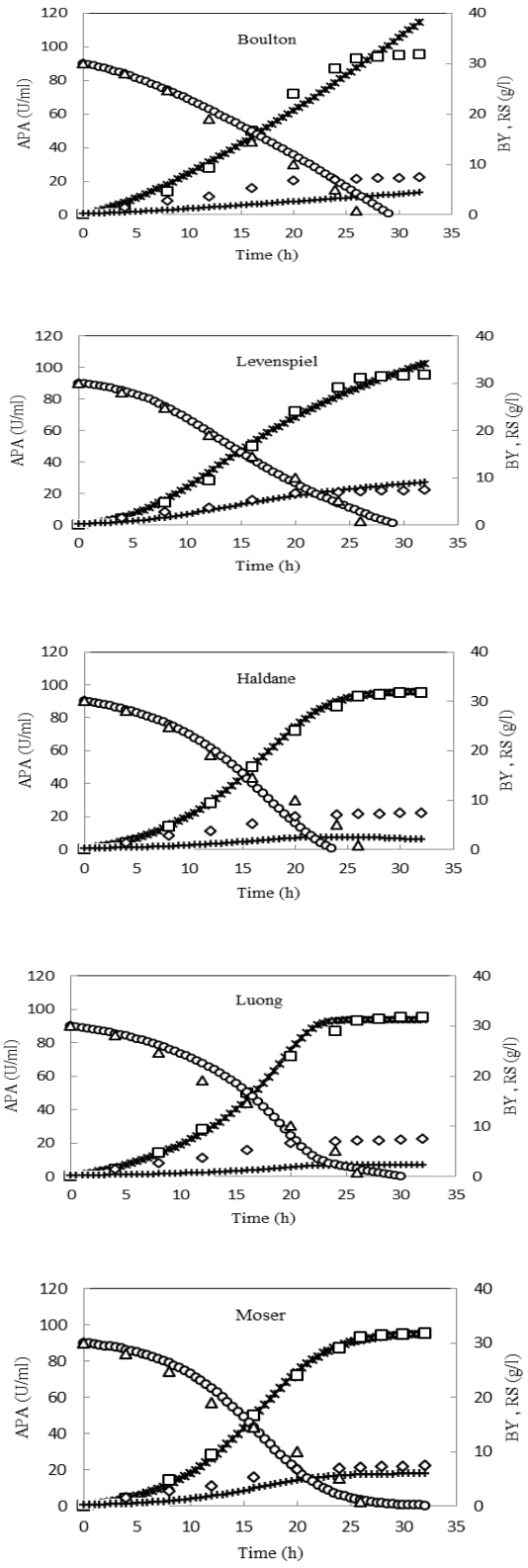

Figure 2. Process prediction using the simple kinetic models from Table 2. Experimental points for biomass (◊), substrate (Δ), enzyme ( ); modeled data for biomass (+++), substrate (ooo) and enzyme (×××).

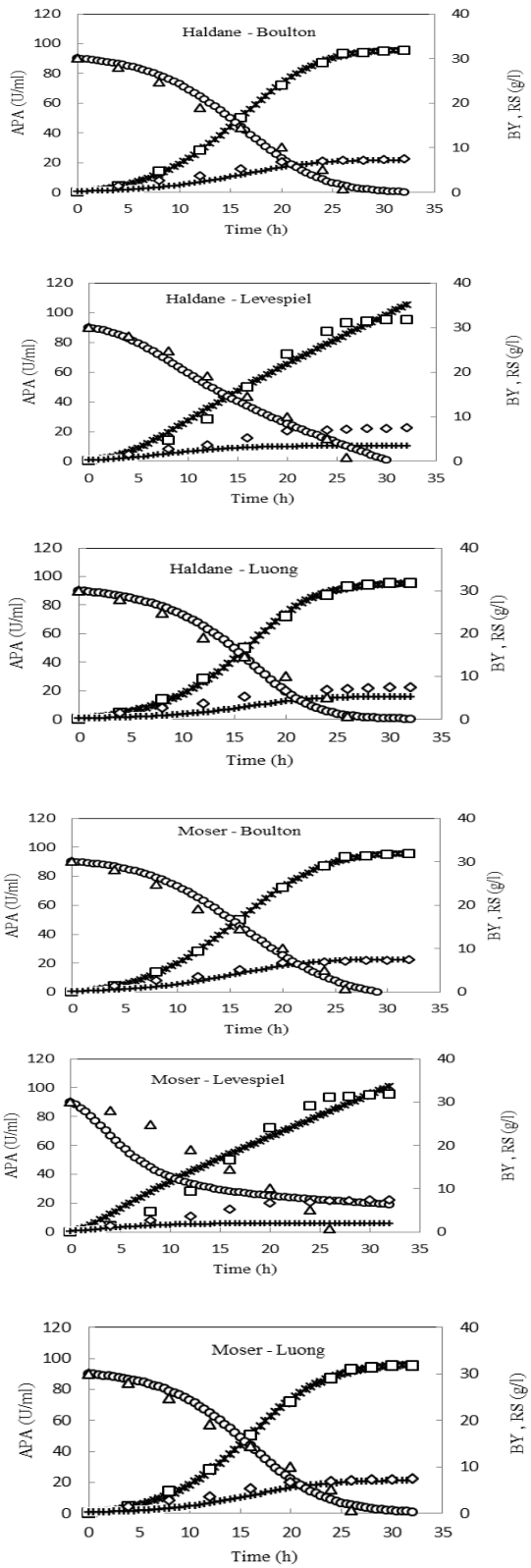

Figure 3. Process prediction using the combinations of kinetic models from Table 2. Experimental points for biomass (◊), substrate (Δ), enzyme ( ); modelled data for biomass (+++), substrate (ooo) and enzyme (×××).

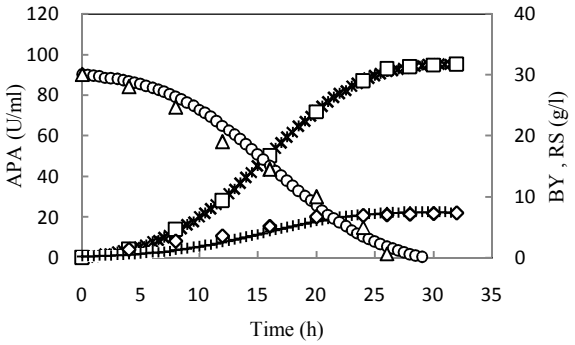

Figure 4. Comparison of the experimental and predicted kinetics of enzyme production based on Moser - Boulton kinetic model. Experimental biomass (◊), substrate (Δ), enzyme ( ), Predicted biomass (+++), substrate (ooo) and enzyme (×××).

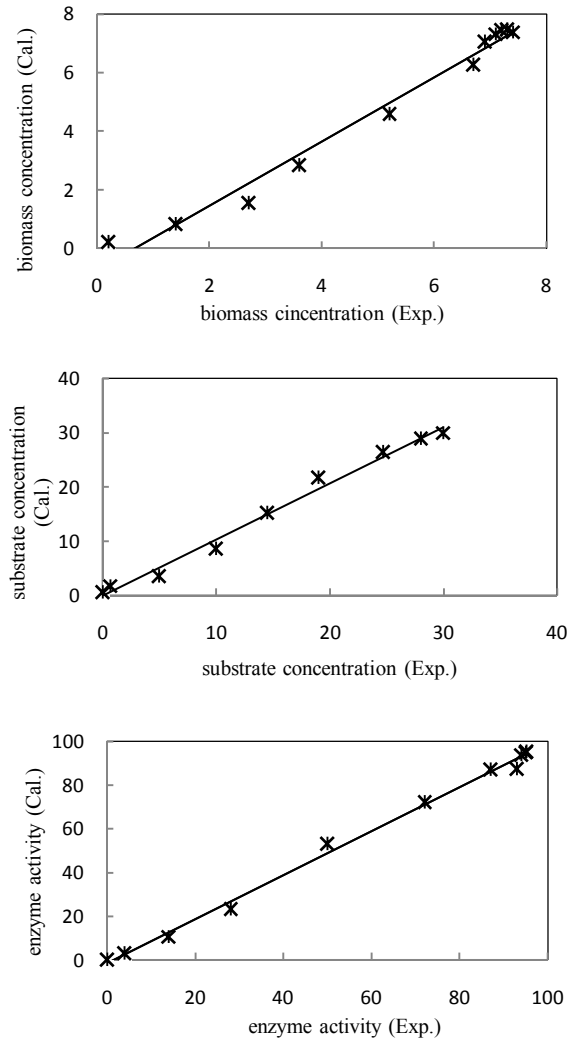

Figure 5. Linear regressions for experimental and predicted data based on Moser - Boulton kinetic model. For biomass R^2 = 0.981, substrate R^2 = 0.988 and enzyme activity R^2 = 0.995.

Although some works has been done on modeling enzyme fermentation processes [19, 20], currently there are no previous results reported on mathematical modeling of the FWH for enzyme production.

4. CONCLUSIONS

During the recent years, efforts have been directed to explore the means to reduce the enzyme production costs through improving yield, and e of either cost-free or low-cost waste materials for protease production. Our finding showed that FWH is a suitable substrate for alkaline protease production, and FWH may be a valuable supplement in biotechnology. Therefore, due to its excellent positive influence on fermentation time and enzyme activity, we decided to develop a mathematical model based on this experimental result. After examining several models, the Moser-Boulton model was capable of successfully explaining cell growth, enzyme production and substrate utilization. Therefore, the use of this model may lead to the development of better strategies for the optimization of the fermentation process to ensure its economic viability.

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RESAERCH NOTE

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P A P E R I N F O

Paper history:
Received 27 February 2015
Received in revised form 04 April 2015
Accepted 30 April 2015

Keywords:
Alkaline Protease
Fish Waste Hydrolysate
Modeling
Bacillus

چکیده

هدف از این مطالعه استفاده از هیدرولیز ضایعات ماهی (FWH) به عنوان یک سوبسترا برای تولید پروتئاز قلیایی و بررسی سینتیک تولید آنزیم با استفاده از جدایه باسیلوس در سیستم بسته بود. نتایج نشان داد که با با افزودن ٤درصدFWH به محیط تخمیر حداکثر میزان تولید پروتئاز قلیایی به ۸۹U/ml رسید که ٦٣٪ بالاتر از محیط کنترل بود. پس از بررسی چندین مدل سینتیکی، مدل ترکیبی موزر و بولتون به عنوان بهترین مدل انتخاب شد. نتایج نشان داد که سازگاری خوبی بین حل مدل و داده‌های تجربی برای غلظت سلول، سوبسترا و فعالیت آنزیم وجود دارد و این مدل ریاضی غیرخطی مقدار زیست توده، غلظت سوبسترا و فعالیت آنزیم را به‌خوبی پیشگوئی می کند. همچنین، ضریب همبستگی حاصله بالاتر از ۰/۹۸ بود.بنابراین، مدل سینتیکی می‌تواند برای شبیه‌سازی متغیرهای تخمیر مورد استفاده قرار گیرد.

***doi**: 10.5829/idosi.ije.2015.28.06c.03*