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Original Article

Estimation of kinetic parameters using mathematical modeling of beta-galactosidase production under rapid growth of *Bacillus licheniformis*

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Abstract

Background & objectives: β-galactosidase is well known as an enzyme used in the food industry and health domains. Today special attention is paid to its production, therefore determining the appropriate kinetic model can be essential for optimizing its production process.

Material & Methods: The batch fermentation was performed in four different concentrations of lactose (20, 30, 40, and 50 g L^{-1}) as an inducer substrate to model and determine the kinetic parameters of β-galactosidase production by *Bacillus licheniformis*. The kinetic parameters were evaluated by the simultaneous solution of one of the Moser or logistic models with the set of differential equations in MATLAB software.

Results: Lactose (as substrate) has an inhibitory effect on enzyme production in the concentration of 50 g L^{-1}. More than 83% of the final biomass was produced in just the first 8 hours of incubation. Due to the inhibitory effect of substrate on growth, Moser and Logistic models were used to express specific growth rates. Malthus, Luedeking–Piret, and substrate consumption differential equations were used to express the bacterial growth rate, enzyme production, and substrate utilization rate, respectively. The high accuracy of the results (R^2> 0.94) showed that the models have a reliable estimate of the experimental results to describe the kinetic of the β-galactosidase production process.

Conclusion: Scientific analysis of kinetic parameters showed the predictable behavior of *B.licheniformis* in enzyme production from rapid growth to long decelerating phase.

***Keywords*:** β-galactosidase, Lactose, Kinetic parameter, Mathematical model, *Bacillus licheniformis.*

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Introduction

In the fermentation process, hydrolyzing lactose into glucose and galactose by β-galactosidase has received special attention in the production of this enzyme (1). In lactose intolerance disorder, extra gases which are produced during metabolism by microorganisms after defective lactose digestion in the small intestine may cause several abdominal symptoms such as pain, cramping, bloating, or vomiting (2). In such cases, eliminating dairy products from daily diet is an undesirable approach to prevent the disorder because of serious diseases related to calcium deficiency (3). Furthermore, flavor deterioration due to lactose crystallization and non-enzymatic browning are the main problems of storing lactose-containing powders (4). Precipitation problems and the low sweetening power of lactose in the confectionery and ice-cream industries are the other problems in β-galactosidase production (5). As an application of β-galactosidase the recovery of biologically active oligosaccharides from milk, has also been described in the literature (6). Extensive applications of β-galactosidase in the food industry and health domains have persuaded manufacturers to produce lactose hydrolyzed products (7).

Microbial processes are inherently complex; therefore understanding, controlling and optimizing a fermentation process are critical in practical applications. Biological prediction of the dynamic behaviors of a fermentation process has significant benefits in controlling the process and optimizing targeted metabolite production conditions (8). The structured models consider all intracellular metabolic reactions, while the unstructured models describe the variations such as concentrations of biomass, substrate and metabolic products using ordinary differential equations. Various unstructured kinetic models have been recommended for describing organic acids and enzymes production (9-10). However, there are few studies on the kinetic modeling of β-galactosidase production.

In our previous paper, we reported the optimization of fermentation parameters for the β-galactosidase production by a local isolate of *B.licheniformis* using the Taguchi design of experimental (DOE) methodology (11). In this study, experimental data were examined to form the basis of the kinetic model. A series of batch experiments were conducted in shake flasks with different initial substrate (lactose) concentrations in order to identify the model's availability range. Experimental data were used to estimate parameters and validate the models that helped us characterize the behavior of *B.licheniformis* during fermentation. The fermentative characteristics of β-galactosidase production from lactose by *B.licheniformis* were discussed in detail.

Materials and methods

A) Microorganisms: The bacterial strain (*Bacillus licheniformis)* used in this study was obtained from the laboratory of Islamic Azad University of Rasht (12). The isolate was maintained on nutrient agar slant for 4 months at refrigerator temperature and used throughout the study.

B) Fermentation conditions: The culture medium was nutrient broth containing (per liter): 2.0 g yeast extract; 5.0 g peptone; 5.0 g NaCl, and 1.0 g beef extract. After preparation, it was sterilized at 121 ºC for 15 min (13).

Production of β-galactosidase was carried out in 500 ml shake-flasks with 100 ml contained following constituents (g L^{-1}): yeast extract 5.0; peptone 5.0; K_2HPO_4 3.0; KH_2PO_4 1.0, and $(NH_4)_2SO_4$ 3.0. The initial pH value of the medium was regulated to 6.6 before sterilization

(15 min, 121ºC). Lactose was sterilized separately, and added into the medium as a carbon source just before inoculation. To determine the effect of growth limitation and/or inhibition by the substrate (lactose), the initial lactose concentrations in the medium were selected from 20 to 50 g L^{-1}. Cultures were inoculated with the pre-culture and incubated at 32 °C and 150 rpm for 22 hours.

C) Analytical procedures: Periodically, samples were withdrawn every 2 hours, and the concentrations of the cell, residual lactose, and enzyme activity were determined. All experiments were repeated at least three times in order to acquire high accuracy of the results. This procedure gave consistent and reproducible results. The data given here are the average of the measurements. Bacterial growth was monitored using a spectrophotometer at the optical density of 600 nm (OD_{600}); then was converted to dry cell weight (DCW) using a calibration curve. A standard graph was plotted for dry cell weight versus absorbance for further estimation of DCW (13).

The β-galactosidase assay was done spectrophotometrically (λ=415 nm) using O-nitrophenyl β-D-galactopyranoside (ONPG) as previously described (14).

Lactose was estimated in the aqueous supernatants using the modified method of Anthrone reagent as described by Choonia and Lele (15).

D) Kinetic models: At the beginning of batch fermentation, all the essential substances for the growth and maintenance of bacteria must be added to the medium (16). The kinetics of fermentation were modeled using three equations. Malthus equation (Eq. (1)) was used for cell growth rate (r_X), which describes the growth in batch fermentation (17). Luedeking Piret equation (Eq. (2)) was used for β-galactosidase production rate (r_P), as a model that considers both effects of cell growth rate (r_X) and biomass concentration (X) on enzyme production rate (18). Finally, the substrate utilization equation (Eq. (3)), which is a modified form of Luedeking-Piret model, was used for the lactose utilization rate (r_S):

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

$$r_p = \frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (2)$$

$$-r_S = -\frac{dS}{dt} = \frac{1}{Y_{XS}} \frac{dX}{dt} + m_S X + \frac{1}{Y_{PS}} \frac{dP}{dt} \quad (3)$$

where X is the biomass concentration (g L^{-1}), S is the substrate concentration (g L^{-1}), P is the enzyme activity (U mL^{-1}), μ is the specific growth rate (h^{-1}) which gives the characteristic nonlinear behavior of fermentation processes, t is the incubation time (h), α is a growth associated term (U mg $cells^{-1}$), β is a non growth associated term (U mg $cells^{-1}$ h^{-1}), Y_{XS} and Y_{PS} are biomass (g cells g $Substrate^{-1}$) and product (U mg $Substrate^{-1}$) yield coefficients, and m_s is maintenance coefficient (h^{-1}). Yield coefficients Y_{XS} and Y_{PS} are assumed to remain constant during the fermentation.

Kinetic modeling is a preliminary step for a better understanding of the microbial enzyme production process (19). Although there are various kinds of models to describe kinetic microorganism behavior, as explained by Arellano-Plaza *et al*. (8), the most widely used are unstructured models. In this study, the Moser model (Eq. (4)) (20), which describes growth inhibition by high substrate concentration, represents the bacterial growth rate.

Another model that can describe the dynamics of growth, considering the maximum cell concentration and relying on substrate availability, is the logistic equation (16). Eq. (5) shows the

specific growth rate is based on the logistic model (17,21):

$$\mu = \frac{\mu_{max} S^n}{K_s + S^n}, n > 0 \quad (4)$$

$$\mu = \mu_{max}(1 - \frac{X}{X_{max}}) \quad (5)$$

where μ_{max} is the maximum specific growth rate (h^{-1}), X_{max} is the maximum biomass concentration (g L^{-1}), K_S is the substrate saturation constant (g L^{-1}), and n is the exponential term for the Moser model. μ_{max} and K_S are characteristics of the microorganism and may alter by temperature and pH (22).

Results

Since preliminary results showed maximum enzyme production at the incubation time of 22 h, all of the experiments were stopped after this time. The kinetic parameters were estimated by solving the set of differential equations ((Eq. 1-3) and one of the specific growth rate equations (Eq. 4 or 5) using the ode45 solver MATLAB (V 7.11). The results (Table 1) are based on specific growth rate models. These parameters were used then to estimate biomass and substrate concentrations and β-galactosidase activity.

A) Microbial growth: Fig.1 shows the experimental and simulated data obtained from the models in different initial lactose concentrations.

Table 1. Values of kinetic parameters for *β*-galactosidase production. (m_S=0)

Initial lactose concentration	Logistic model						Moser model						
	Biomass		Substrate		Product		Biomass			Substrate		Product	
S_0 (g L^{-1})	μ_{max} (h^{-1})	X_{max} (g L^{-1})	Y_{XS} (g_c g_s^{-1})[a]	Y_{PS} (U mg_s^{-1})	α (U mg_c^{-1})	β (U mg_c^{-1} h^{-1})	μ_{max} (h^{-1})	K_S (g L^{-1})	n	Y_{XS} (g_c g_s^{-1})	Y_{PS} (U mg_s^{-1})	α (U mg_c^{-1})	B (U mg_c^{-1} h^{-1})
20	0.78	6.73	0.325	0.85	0.408	0.037	0.63	9.2	2	0.317	0.644	0.381	0.039
30	1.14	6.94	0.221	0.6	0.306	0.048	0.82	13.12	2	0.216	0.57	0.292	0.05
40	1.18	6.56	0.158	0.42	0.335	0.044	0.85	19	2	0.158	0.5	0.323	0.046
50	0.83	5.97	0.117	0.36	0.414	0.041	0.66	32	2	0.115	0.3	0.392	0.044

[a]g_c, g cell, g_s, g substrate.

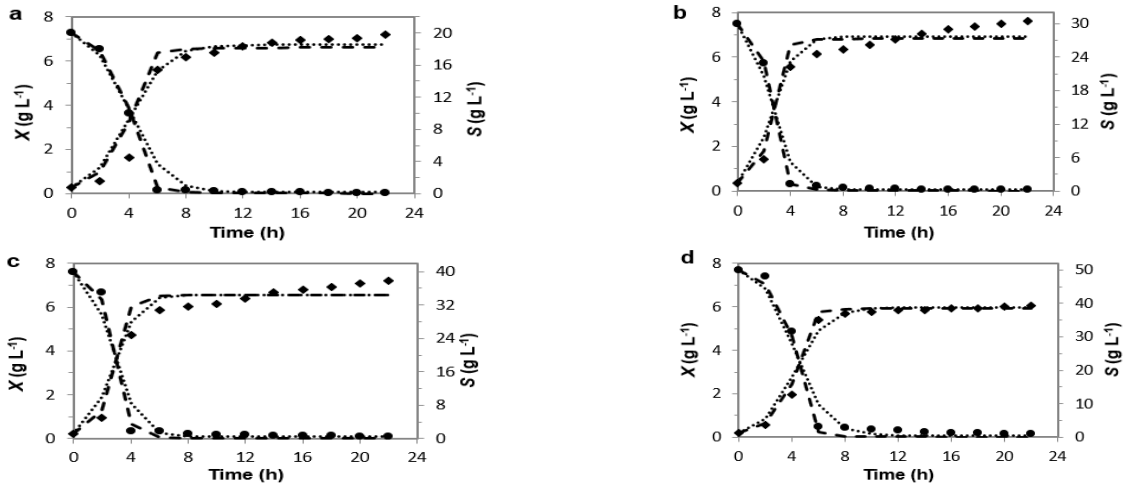

Fig 1. Comparison of experimental concentrations of *X* (diamond) and *S* (circle) and simulated data by Logistic (dotted line) and Moser (dashed line) models at initial substrate concentrations of 20 g L^{-1} (a), 30 g L^{-1} (b), 40 g L^{-1} (c) and 50 g L^{-1} (d).

Bacterial growth started about 1-2 h after inoculation and continued during exponential and decelerating growth phases at initial lactose concentrations of 20, 30, and 40 g L^{-1}. By increasing the initial concentration to 50 g L^{-1}, the lag phase lengthened. Besides, bacterial growth stopped after the exponential growth phase, and the stationary phase continued until the end of fermentation (24h). The long lag phase, which was observed in the initial concentration of 20 g L^{-1} can be due to substrate limitation.

Trends in biomass production and substrate utilization at various initial substrate concentrations were quite similar. However, there was a noticeable reduction in biomass level at the initial concentration of 50 g L^{-1} so that maximum biomass concentrations, which were 7.21, 7.62, and 7.20 g L^{-1} at initial concentrations of 20, 30, and 40 respectively, decreased to 6.04 g L^{-1} in the concentration of 50 g L^{-1}. Additionally, there was an increase in the exponential phase in this concentration compared to the initial concentrations of 30 and 40 g L^{-1} (Fig. 1d). It seems that high osmotic pressure across the cell membrane is responsible for this trend (23).

Models showed close approximations of experimental data and slight deviations of biomass concentration at the decelerated growth phase.

The maximum specific growth rate (μ_{max}) increased with increasing the initial lactose concentration to 40 g L^{-1}, but further increase in the initial lactose concentration resulted in the reduction of μ_{max} in inhibition concentration of 50 g L^{-1} (Table 1). It represents the negative effect of high substrate concentration on the growth of *Bacillus licheniformis*. Achieving a fast growth phase requires a high value of specific growth rate, which means reduction of the fermentation time, and overall productivity improvement (10). Therefore, the high values of μ_{max} obtained from the models explain the rapid growth of biomass in the exponential growth phase. Due to the different structures of the Moser and Logistic models, as was explained before, the values of μ_{max} obtained from these models were different. The higher value of substrate saturation constant (K_S) in the concentration of 50 g L^{-1} was the consequence of high substrate concentration.

B) Substrate utilization: Substrate reduced dramatically (about 95%) during the exponential growth phase, which commonly achieved the highest biomass production. Rapid depletion of lactose substantially reduced the tendency of bacteria to use residual sugar and caused the changes in biomass concentration to be very slow and insignificant during the decelerating growth phase in 20, 30, and 40 g L^{-1} (Fig. 1a–c).

The biomass yield coefficient (Y_{XS}) was a function of the initial lactose concentration (S_0) and decreased with an increase of S_0. The values of the maintenance coefficient were estimated very low (almost zero) for both sets of the models, and at all the initial substrate concentrations. Maintenance is the energy supports metabolism. So, the second term in Eq. (3) ($m_S X$) specifies the amount of substrate used for the living cell population, whether during growth or absence of it (24). The importance of maintenance energy depends on the supply of substrate per unit biomass (25). Jahic *et al*. (26) showed that the most significant requisite for providing high cell mass is a low maintenance requirement. Therefore, estimating very low amounts of maintenance coefficient can be proof for obtaining maximum values for biomass concentration and high values of Y_{XS} and Y_{PS} during the *Bacillus licheniformis* fermentation process.

C) β-galactosidase production: The β-galactosidase activity started simultaneously with the beginning of the exponential growth phase and kept rising in decelerating or stationary phases (Fig 2).

The highest β-galactosidase activity was 7.81 U mL^{-1} at the initial concentration of 30 g L^{-1} during 22 h that is much greater than reports of Siverio *et al*. (27) using *A. uvarum* (1.38 U mL^{-1}, the highest activity among 50 fungi producers of β-galactosidase after 20 days of fermentation). The minimum enzyme activity was 6.46 U mL^{-1} at the initial concentration of 50 g L^{-1} (Fig 2).

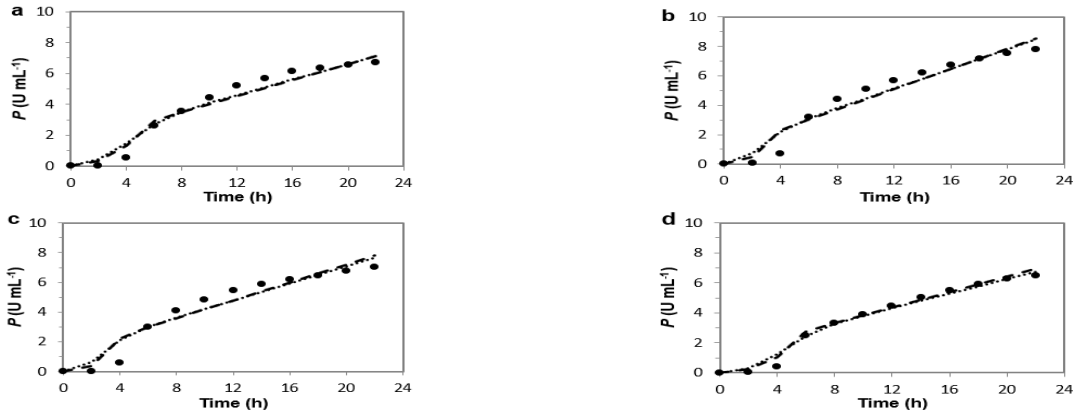

Fig 2. Comparison of experimental concentration of *P*(circle) and simulated data by Logistic (dotted line) and Moser (dashed line) models at initial substrate concentrations of 20 g L^{-1} (a), 30 g L^{-1} (b), 40 g L^{-1} (c) and 50 g L^{-1} (d).

Cell growth rate (r_X) and the β-galactosidase production rate (r_P) changed during the time (Fig 3). At constant temperature and pH during the fermentation process and absence of product inhibition, the growth rate was only influenced by the substrate concentration. The maximum rates of biomass and β-galactosidase production were obtained at 2.07 (g L^{-1} h^{-1}) and 1.25 (U mL^{-1} h^{-1}) respectively, at the initial substrate concentration of 30 g L^{-1}.

The cell growth rate became almost zero after 8 hours of inoculation, but enzyme production continued signifies that β-galactosidase production was a semi-growth-associated process (Fig 3). On the other hand, the peaks of cell growth and β-galactosidase production rates overlapped at the Sixth hour of fermentation in initial concentrations of 20 and 50 g L^{-1} (Fig. 3a, d). It reveals more growth-associated production of β-galactosidase in these concentrations compare with initial concentrations of 30 and 40 g L^{-1}.

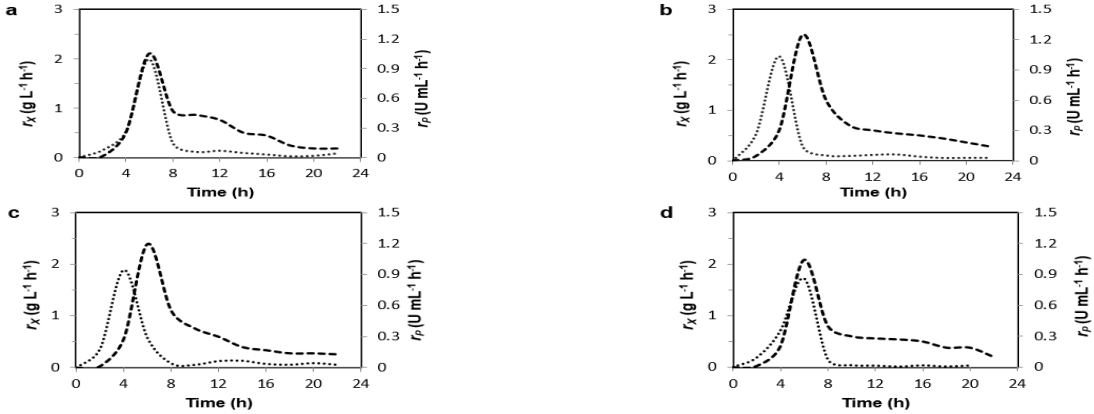

Fig 3. Variation of cell growth rate; r_X (dotted line) and β-galactosidase production rate; r_P (dashed line) of batch fermentations at initial substrate concentrations of 20 g L^{-1} (a), 30 g L^{-1} (b), 40 g L^{-1} (c) and 50 g L^{-1} (d).

As expected, the growth-associated coefficients (α) were estimated greater in initial concentrations of 20 and 50 g L^{-1} than those in 30 and 40 g L^{-1} (Table 1). It shows more dependence of enzyme production to the growth of *B. licheniformis* at concentrations of 20 and 50 g L^{-1} compared to two other concentrations. Subsequently, smaller values of β were obtained in 20 and 50 g L^{-1} than those in 30 and 40 g L^{-1}.

The accuracy of the models was appraised using of the correlation coefficients (R^2) between experimental and predicted data for the biomass, substrate, and product. These values were more than 0.94 for biomass and substrate concentrations and β-galactosidase activity (Table 2). For a better understanding of the performance of the models, the root mean square errors (RMSE) are calculated too.

Table 2. Linear correlations coefficients (R^2) and Root mean square errors (RMSE).

Initial lactose concentration	Logistic model						Moser model					
	Biomass		Substrate		Product		Biomass		Substrate		Product	
S_0 (g L^{-1})	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
20	0.961	0.59	0.983	1	0.974	0.44	0.951	0.62	0.999	0.22	0.973	0.44
30	0.957	0.51	0.981	1.36	0.954	0.61	0.947	0.53	0.999	0.36	0.948	0.64
40	0.96	0.49	0.97	2.43	0.943	0.62	0.945	0.54	0.996	1.01	0.94	0.65
50	0.984	0.3	0.983	2.51	0.987	0.29	0.994	0.2	0.999	1.62	0.99	0.26

There is a good fit between experimental data and model estimations for β-galactosidase activity from the beginning to the stationary phase (Fig 2). The most significant deviations were observed in the initial concentrations of 30 and 40 g L^{-1} simultaneously at the end of the exponential growth phase. The predicted β-galactosidase activities calculated by the two sets of models were very close to each other. It seems reasonable because of quite the same amounts of α or β was obtained from both models.

The higher diffusion rates in submerged cultures lead to faster growth than solid-state cultures (28). Furthermore, the sensitivities of biomass production and specific growth rate are higher to the variation of initial sugar concentration in submerged fermentation than solid-state fermentation because of more free water content and more osmotic pressure (28). Consequently, submerged cultivation not only reduced the fermentation time but also allowed us to study this sensitivity in a smaller range of initial substrate concentrations.

Discussion

In the present era, with the expansion of various programming software, research in mathematical modelling and the determination of kinetic parameters of microbial growth processes under optimal conditions has gained greater attention due to the economic importance of industrial production. Structural models are more commonly used due to their simplicity and fewer synthetic parameters. However, Arijit Nath et al. (2014) first attempted to model the growth of this microorganism using a structural mathematical model in a dual-carbon-source environment (lactose and glucose) by expressing intracellular β-galactosidase in *Bacillus safensis* (JUCHE 1) (29). They developed this structural model and then integrated experimental studies and calculations to predict changes in the biomass concentration, glucose, and lactose in the non-living phase, as well as the intracellular concentrations of lactose, β-galactosidase, and

phospho-β-galactosidase. Most of the model's synthetic parameters were calculated through nonlinear regression with high correlation coefficients. Given that the model showed good agreement with some high-scale experimental data (5-liter bioreactor), they expect this model to be useful for designing large bioreactors for the production of β-galactosidase by *Bacillus safensis* (JUCHE 1) (29).

The production of microbial products in closed processes can generally continue until the stationary phase of microbial growth, which is also correct in the production of enzymes. Therefore, enzyme production is done in two ways, growth-dependent and semi-growth dependent. In the latter case, enzyme production may continue until the stationary phase, after which the production stops or its amount is so low that the continuation of the process is not economically viable. In such processes, the dependence of product production on growth is evaluated with the help of two growth-dependent and non-dependent kinetic parameters. One of the most common problems of working with enzymes is the reduction of enzyme activity in samples due to their instability. Like many proteins, enzymes are subject to denaturation in laboratory conditions (30). This is different in the process of producing enzymes by lengthening the time of keeping in a greenhouse depending on the type of enzyme. If the incubation time for enzyme production lasts longer than the time to reach the maximum activity of the enzyme, the enzyme starts to become inactive and its effectiveness decreases. Therefore, it was necessary to first determine the optimal time for beta-galactosidase production in order to achieve maximum enzyme activity. Because it is not economical to continue the process due to the reduction of enzyme activity.

One of the effective factors in studying the kinetics of microbial growth is the concentration of the growth-limiting substrate. With other factors affecting growth rate such as temperature, pH, and the product not inhibiting growth being constant, the substrate concentration will be the only limiting and inhibiting factor for growth. Growth at a concentration of 50 g/L lactose, after the exponential phase, entered the stationary phase, unlike other initial concentrations. The observation of such changes in the bacterial growth curve at an initial concentration of 50 g/L lactose indicates the inhibitory effect of the substrate on growth at its high concentrations. In general, in microbial fermentation processes, with an increase in the substrate concentration in the medium, the osmotic pressure increases to the point where a large osmotic pressure gradient is created along the cell membrane and prevents substrate transport. The lack of sugar transport will limit the growth rate and lead to cell death (22). The increase in the exponential growth phase at an initial concentration of 20 g/L can be due to substrate limitation. Normally, substrate limitation causes the lag phase to be longer than other concentrations and growth to start with a slight delay. Biomass production depends on substrate metabolism for energy supply. The results showed that biomass production was well correlated with changes in substrate concentration. During the exponential growth phase, the substrate dropped sharply as the yield increased, and after this sudden drop, more than 95% of the initial lactose concentration was consumed, followed by the exponential growth phase of the *B. licheniformis* (which usually produces the highest biomass production) stopping.

Conclusions

Accurate deduction of kinetic parameters leads us to a better understanding of the behavior

of β-galactosidase production process by *B. licheniformis*. Eventually, the estimated parameters were used to predict cell growth, substrate utilization, and β-galactosidase production process. Limitation and inhibition effects of substrate intensified the dependency of β-galactosidase production on the growth of the microorganism. Although our main aim was not to maximize β-galactosidase activity or identify the best β-galactosidase producers, we have achieved good results of β-galactosidase activity produced by *B. licheniformis* in a short period.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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