Acta Alimentaria 51 (2022) 2, 228–240
DOI: 10.1556/066.2021.00255

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Production of protease with *Bacillus megaterium* DSM32: Partial characterisation of the enzyme and modelling of the production

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ORIGINAL RESEARCH PAPER

Received: December 7, 2021 • Accepted: March 28, 2022
Published online: May 10, 2022

ABSTRACT

Proteases hold an important position in today's world commercial enzyme market. Among various microbial producer genera, *Bacillus* is leading the commercial protease production. However, industry is still actively looking for new microbial protease producers with distinctive properties. Therefore, this study was undertaken for the evaluation of protease production by *Bacillus megaterium* DSM 32 strain in terms of its protease productivity, calculation of various production kinetics, partial characterisation of the enzyme, and modelling the protease production process. As results, the highest protease activity, specific cellular protease production rate, and protease productivity were calculated as 255.42 U mL^{-1}, 36.2514 U g^{-1}, and 16.1313 U mL^{-1} h^{-1}, respectively, in shake flask fermentations. Partial characterisation studies showed that the enzyme has 45 °C and pH 8 as optimum working conditions, and its activity increased by 24% with the addition of 5 mM Mn^{+2} to the reaction medium. Additionally, the enzyme showed high stability and kept almost full activity in a cell-free medium for 20 days at 4 °C. Furthermore, modified Gompertz model provided the best fit in describing protease production with the lowest error and high fit values.

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KEYWORDS

Bacillus, protease, enzyme, characterisation, modelling

1. INTRODUCTION

Various human goods are being produced by using microorganisms for thousands of years. Among them, enzymes are one of the major products, which are widely used in various industries such as food, chemical, biotechnology, and detergent industries (Bhunia et al., 2012). Proteases, which take action in breaking down reactions of proteins into their subunits, hold the major portion in food and feed industrial enzyme market, and they are especially used in brewing, meat tenderisation, coagulation of milk, and bread quality improvement processes (Kawamura-Konishi et al., 2013; Wang et al., 2013; Bekhit et al., 2014; Fox et al., 2017; Jadhav et al., 2020). Production of microbial proteases is mainly performed by using *Bacillus* strains due to their several characteristics such as being non-pathogenic, easy to modify via genetic manipulations, low cost of production medium, extracellular production, short production time, and lacking endotoxins (Bhunia et al., 2012; Asker et al., 2013). In one of the example studies from the literature, alkaline protease with 60 °C and pH 10 as optimal conditions was produced by *Bacillus* sp. B001 (Deng et al., 2010). Differently, protease, which was produced by *Bacillus subtilis* DR8806 showed optimum temperature and pH values as 45 °C and 8, respectively. Additionally, it was shown that protease activity increased in the presence of various chemicals such as Ca^{2+}, K^{+}, Mg^{2+}, Fe^{2+}, or dimethyl sulphoxide (DMSO) (Farhadian et al., 2015). In another study, again an alkaline protease was produced by *Bacillus licheniformis* with higher optimum temperature of 70 °C and pH 10 (Hadjidj et al., 2018). In a more recent study, a protease was produced by *Bacillus safensis* RH12 with pH 9 and 60 °C optimum working conditions, retaining its full stability against a commercial laundry detergent (Rekik et al., 2019). Currently, screening and identification studies on protease production by *Bacillus* strains are being actively pursued in the scientific world in order to discover new species capable of producing novel proteases with specific properties, which would meet industrial scale demand. In addition to increasing production, mathematical modelling of the microbial processes is also receiving attention. These models help ensure process optimisation and also provide information on general process kinetics such as yield, cell growth pattern, etc (Sablani et al., 2006). Among others, the Gompertz (Gompertz, 1825), logistic (Pearl and Reed, 1920), and Richards (Richards, 1959) models have been widely used to describe biomass growth and product formation in various microbial processes (Richards, 1959; Zhao et al., 2014; Venkateshkumar et al., 2020; Germec et al., 2020). These three models were further modified to be used for describing biological processes (Zwietering et al., 1990). In the literature, there are only a few studies available on modelling enzyme production by *Bacillus megaterium*. In one of these studies, extracellular proteinase production by *B. megaterium* was studied and a substrate based basic mathematical model was conducted (Votruba et al., 1987). In two further studies, poly-(hydroxybutyrate-co-hydroxyvalerate) (PHA) production by *B. megaterium* was studied, and various kinetics, such as growth-associated product formation, growth-associated rate constant for carbon source consumption, were calculated and processes were described by

mathematical models (Faccin et al., 2012; Porras et al., 2019). However, these studies mostly focused on determination of production kinetics and statistical evaluation of the models was not intensively studied. Currently, there are not many studies available in the literature on modelling and statistical evaluation of protease production by *B. megaterium* DSM 32. Therefore, in this study, protease and biomass production capacity of *B. megaterium* DSM 32 strain was evaluated. Additionally, production kinetics, partial characterisation of the enzyme, and modelling of the production process were addressed in detail.

2. MATERIALS AND METHODS

2.1. Microorganism

B. megaterium DSM32 was kindly donated by Prof. Dr. Gülşad Uslu Şenel from Environmental Engineering Department of Fırat University, Turkey. The strain was cultured in potato dextrose broth (PDB) at 37 °C for 24 h with shaking at 180 r.p.m. Thereafter, the culture was centrifuged at 4 °C and 5,500 r.p.m. (5,250 g) for 15 min. The supernatant was discarded and the cells were suspended in a 50% glycerol solution, transferred into 1 mL sterile tubes, and stored at −86 °C for further studies.

2.2. Inoculum preparation and shake flask productions

Inoculum medium was prepared with the following composition: 1 g glucose, 2 g KH_2PO_4, 3 g meat extract, 10 g peptone, 10 g casein, and 5 g NaCl per litre of distilled water. The pH of the medium was adjusted to 8 with 5 M NaOH solution. The medium was dispensed per 50 ml in Erlenmeyer flasks of 250 mL capacities, then sterilised at 121 °C for 15 min. After cooling down, 1 mL of frozen cells was added, and the flasks were incubated at 37 °C for 24 h at 180 r.p.m to prepare inoculum for the studies. For the enzyme production process, flasks of 1 L volume were filled with 250 mL of sterile medium, then inoculated with 1% v/v inoculum and incubated at 37 °C for 48 h at 180 r.p.m. Samples were taken from the flasks to measure cell concentration and protease activity.

2.3. Analysis

In this study, all samples were taken in triplicate from the production medium, and kinetic and characterisation experiments were also run in triplicate.

2.3.1. Biomass measurement. Microbial growth absorbance values were measured spectrophotometrically at 600 nm using the uninoculated production medium as blank. Thereafter, values were converted to g L^{-1} using the absorbance-dry cell equation (Equation 1) obtained by plotting the graph of absorbance vs dry cell weight.

$$Dry\ cell\ weight\ (g\ L^{-1}) = 0.0023 \times Abs_{600\ nm} + 1.8523 \quad (1)$$

2.3.2. Protease activity measurement. Protease activity was measured as described in the literature with some minor modifications (Vaithanomsat et al., 2008). First, samples were centrifuged at 5,500 r.p.m. for 10 min. Thereafter, 0.5 mL from the supernatant was added to

2.5 mL of phosphate buffer, which contained 0.6% (w/v) casein (50 mM, pH: 8.0). The mixture was incubated in a water bath at 45 °C for 20 min. The reaction was terminated by addition of 2.5 mL of 0.44 M trichloroacetic acid. Then, the mixtures were centrifuged again at 5,500 r.p.m. for 10 min, and 0.25 mL from the upper phase was mixed with 1.25 mL of 0.5 M Na_2CO_3 and 0.25 mL Folin–Ciocalteu phenol liquid. The mixture was incubated at room temperature for 30 min in the dark. Absorbance values were measured at 600 nm using uninoculated production medium as blank. An enzyme unit (U) was defined as the amount of enzyme that would produce 1 µmol of tyrosine per minute at 45 °C.

2.3.3. Calculation of production kinetics. Along with the calculation of biomass and protease activity for each sampling point, various production kinetics, such as microbial specific growth rate (h^{-1}), specific cellular production rate (U g^{-1}), and protease productivity values (U mL^{-1} h^{-1}), were calculated to provide better understanding of overall production dynamics. Calculation of specific growth rate is shown in Equation 2:

$$\text{Specific growth rate } (h^{-1}) = \frac{\ln\left(\frac{\text{Cell concentration at time 2}}{\text{Cell concentration at time 1}}\right)}{\text{Time 2} - \text{Time 1}} \tag{2}$$

2.3.4. Determination of optimal pH and temperature of the protease. The produced enzyme was subjected to enzymatic activity measurement at different temperatures (4–80 °C) and pH (5–10) values to determine optimum working parameters.

2.3.5. Determination of protease stability at different storage temperatures. After production under optimum conditions, the cell-free enzyme solution was transferred to 1 mL sterile centrifuge tubes. Tubes were kept at –20, 4, and 25 °C for 40 days. Samples were taken from each temperature every 5 days and analysed for enzyme stability.

2.3.6. Effect of various chemicals on protease activity. Various chemicals such as EDTA, $CaCl_2$, $MgCl_2$, $FeCl_2$, $MnCl_2$, $ZnCl_2$, and $CoCl_2$ at 1 and 5 mM concentrations were dissolved in the substrate solution and their effects on protease activity were evaluated.

2.3.7. Mathematical models. Since protease is a growth related product, it usually follows a sigmoid pattern similar to cell growth during the production. In this section, modified versions of Gompertz, logistic, and Richards models were implemented and evaluated for their representative capacity of describing protease production. All calculations were performed using Microsoft® Excel® for Microsoft 365 MSO program.

Modified Gompertz model (Zwietering et al., 1990):

$$A_t = A_m \times exp\left[-exp\left\{\frac{Q \times e \times (\lambda - t)}{A_m} + 1\right\}\right] \tag{3}$$

Modified logistic model (Zwietering et al., 1990):

$$A_t = \frac{A_m}{1 + \exp\left(\frac{4 \times Q \times (\lambda - t)}{A_m} + 2\right)} \tag{4}$$

Modified Richards model (Zwietering et al., 1990):

$$A_t = \frac{A_m}{\left\{1 + v \times exp(1+v) \times exp\left(\frac{Q \times (1+v)^{\left(1+\frac{1}{v}\right)} \times (\lambda - t)}{A_m}\right)\right\}^{\left(\frac{1}{v}\right)}} \quad (5)$$

All stated parameters in these models were obtained from experimental productions: A_t is protease activity (U mL^{-1}) at "t" time, A_m is the upper asymptote (255.42 U mL^{-1}), Q is the maximum production (Q_P) (12.19 U mL^{-1} h^{-1}) rate, λ is lag time (3.5 h), e is Euler number (2.718), v is dimensionless shape parameter (0.5), and t is the sampling time (h). A_t, A_m, Q, and λ values were directly obtained from experimental data, however, v was manually assigned to obtain the lowest error values (Germec et al., 2020). Comparison of the models were performed by evaluating values such as root mean square error (RMSE), mean square error (MSE), bias factor (BF), accuracy factor (AF), regression coefficient (R^2), and slope. A mathematical model preferably should have low RMSE and MSE values and AF, BF, R^2, and slope values as 1.

3. RESULTS AND DISCUSSION

3.1. Calculation of production kinetics

In order to calculate production kinetics, biomass concentration and protease activity values were first computed. Their time depended trends are shown in Fig. 1. Since protease is a growth related product, biomass growth and protease production followed similar trends. Cells had approximately 3.5 h of lag phase in the production medium. Thereafter, biomass and protease activity rapidly increased and reached their maximum values around the 30th hour of the process (Fig. 1, Table 1). During stationary phase, the values obtained were calculated as almost zero or negative. Calculation of the negative values is the indication of lower cellular optical density due to probable decrease in vegetative cell numbers because of potential spore formation. Additionally, maximum specific cellular protease production rate value was

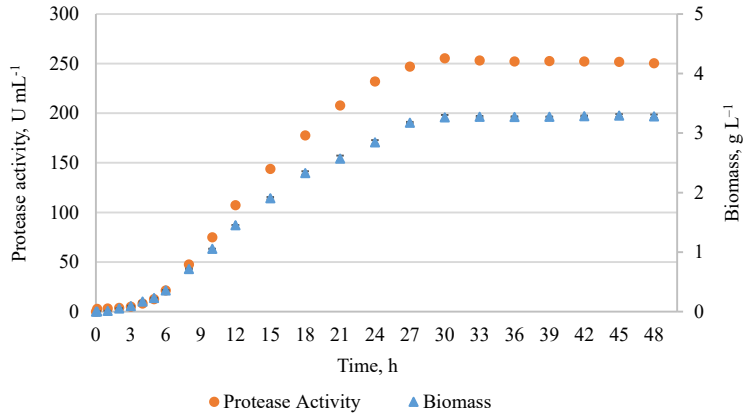

Fig. 1. Biomass formation and protease activity during enzyme production process with *B. megaterium* DSM32

Table 1. Growth and production kinetics calculated for protease production process with *Bacillus megaterium* DSM32

Time, h	Specific growth rate, h^{-1}	Specific cellular production rate, U g^{-1}	Protease productivity, U mL^{-1} h^{-1}
0	–	–	–
0.1	–	–	–
1	–	–	–
2	–	–	–
3	0.5673	11.5223	1.1000
4	0.5739	19.4729	3.3000
5	0.3147	18.9546	4.4000
6	0.4336	24.5719	8.8000
8	0.3479	36.2514	13.0167
10	0.1922	26.0713	13.7500
12	0.1603	22.1998	16.1333
15	0.0904	19.2361	12.2222
18	0.0668	14.4853	11.2444
21	0.0332	11.6864	10.0222
24	0.0335	8.5067	8.0667
27	0.0364	4.7372	5.0111
30	0.0093	2.5842	2.8111
33	0.0010	–0.6721	–0.7333
36	–0.0003	–0.3363	–0.3667
39	0.0003	0.1120	0.1222
42	0.0011	–0.1116	–0.1222
45	0.0008	–0.1114	–0.1222
48	–0.0011	–0.4470	–0.4889

measured as 36.2514 U g^{-1} at the 8th hour of the process and the value decreased continuously until the end of the exponential growth phase. Furthermore, the highest protease productivity value was calculated as 16.1313 U mL^{-1} h^{-1} at the 12th hour of the process, which was quite high compared with the results from the literature. For example, protease productivity values for *Bacillus* species were presented as 5 U mL^{-1} h^{-1} for *Bacillus cereus* FT1 (Bhakthavalsalam and Muthusamy, 2018), 1.48 U mL^{-1} h^{-1} for *B. subtilis* AKAL7 (Hakim et al., 2018), and 1.58 U mL^{-1} h^{-1} for *Bacillus aryabhattai* Ab15-ES (Adetunji and Olaniran, 2020). On the other hand, 20.19 U mL^{-1} h^{-1} alkaline protease productivity value was also published for a *Bacillus* sp. in a response surface methodology optimisation study (Puri et al., 2002). These kinetic values obtained will certainly provide help for future enzyme productions, scale up studies, and modification on the process such as setting up fed-batch and continuous systems.

3.2. Effect of pH and temperature on protease activity

In order to evaluate the effect of pH on enzyme activity, the produced enzyme was mixed with the substrate solution with pH values between 5 and 10. As shown in Fig. 2, optimum pH value for the produced enzyme was determined as 8. Additionally, produced protease could perform 60% of its maximum activity at pH 5 and it showed better performance with only 18% and 27%

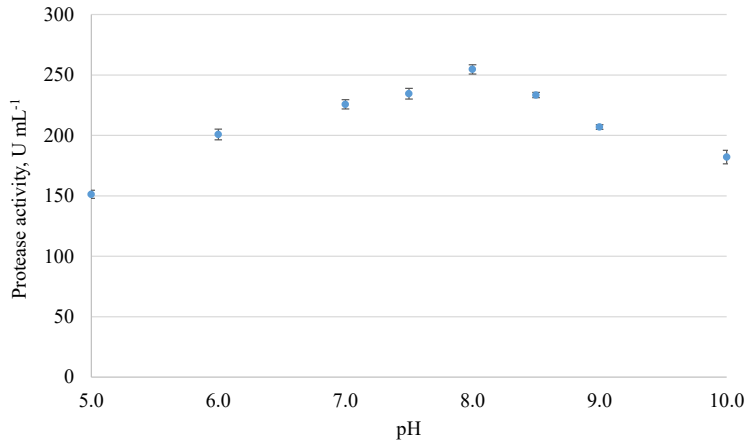

Fig. 2. Protease activity at different pH values

activity loss at pH 9 and 10, respectively. These results clearly show that the produced enzyme is an alkaline protease with a broad pH range. These results were also compared with several studies from the literature. In one of these studies, the optimum pH value of protease produced by *B. subtilis* was stated as 7.4. However, it was also shown that protease activity decreased by 50% and 30% at pH 6.8 and 8, respectively, indicating low stability (Pant et al., 2015). In another study, the optimum pH of the protease produced by another *Bacillus* strain was also determined as 7.4, and only approximately 20% activity loss between pH 7 and 8 was observed. However, only 8% and 10% of the activity could be measured at pH 5.5 and 9, respectively (Asker et al., 2013). Supporting the results of this current study, the optimum pH value for proteases produced by *B. macerans* IKBM-11, *B. licheniformis* IKBL-17, and *B. subtilis* IKBS-10 was reported as 8, but up to 40% decrease in the activity at pH 5 and 10 was presented (Olajuyigbe and Ajele, 2005). On the other hand, optimum pH value was reported as 10 for a protease produced by *B. megaterium* RRM2 (Pearl and Reed, 1920), *B. cereus* strain S8 (Lakshmi et al., 2018), and *B. megaterium* isolated from Thai fish sauce fermentation (Yossan et al., 2006). However, similarly to the studies stated above, wide pH stability of these proteases was low in these studies, too.

The produced protease was also studied under different temperature conditions from 4 to 80 °C. As shown in Fig. 3, the enzyme was able to perform its 60% of maximum activity even at a temperature as low as 4 °C. While the optimum working temperature was recorded as 45 °C, the enzyme was able to maintain its 75% of activity at 60 °C. Similarly, optimum temperature for *B. subtilis* protease was reported between 40 and 50 °C (Pant et al., 2015). As a close value, *B. megaterium* protease optimum temperature was calculated as 50 °C with 80 and 54% activity performance at 60 and 70 °C, respectively (Asker et al., 2013). Protease obtained from *B. megaterium* RRM2 showed a higher optimum temperature of 60 °C, however, this enzyme was able to maintain its 30% activity at 20 °C but was almost totally deactivated at 80 °C (Pearl and Reed 1920). Similarly, optimum temperature for *B. macerans* IKBM-11, *B. licheniformis* IKBL-17, and *B. subtilis* IKBS-10 proteases was reported as 60 °C, however, the enzymes lost approximately 35% of their activity at 65 °C (Olajuyigbe and Ajele, 2005). Higher optimum temperature value was reported for *B. cereus* strain S8 as 70 °C. However, this protease could

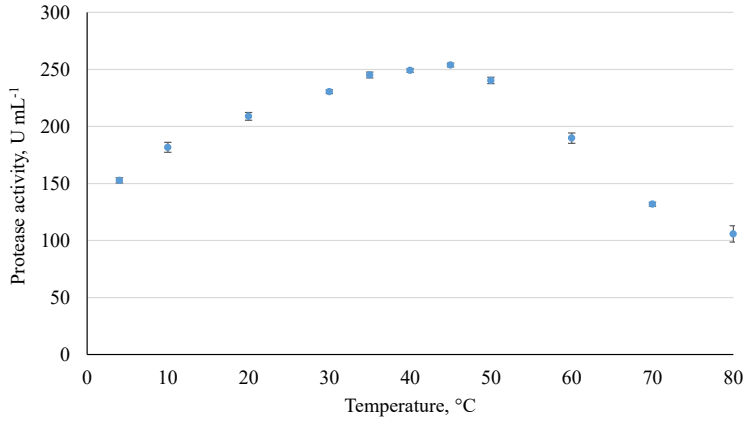

Fig. 3. Protease activity at different temperatures

only perform 40 and 60% of its maximum activity at 30 and 80 °C, respectively (Lakshmi et al., 2018).

3.3. Effect of various chemicals on protease activity

Effects of various chemicals on protease activity are shown in Fig. 4. Among all evaluated chemicals, except for Mn^{+2}, all slightly or significantly decreased enzyme activity. Protease activity increased by 11 and 24% in the presence of 1 and 5 mM Mn^{+2}, respectively. However, the enzyme was able to maintain its 44% of the activity when exposed to 5 mM EDTA. Similarly, addition of Co^{+2} decreased the enzyme activity by 18 and 27% in the presence of 1 and 5 mM concentrations, respectively. On the other hand, addition of 1 or 5 mM of Ca^{+2}, Mg^{+2}, Fe^{+2}, and Zn^{+2} caused a slight (3–15%) decrease in the enzyme activity. In the literature, *B. megaterium* protease activity was presented to increase by 51% in the presence of 5 mM $MnCl_2$ and decrease

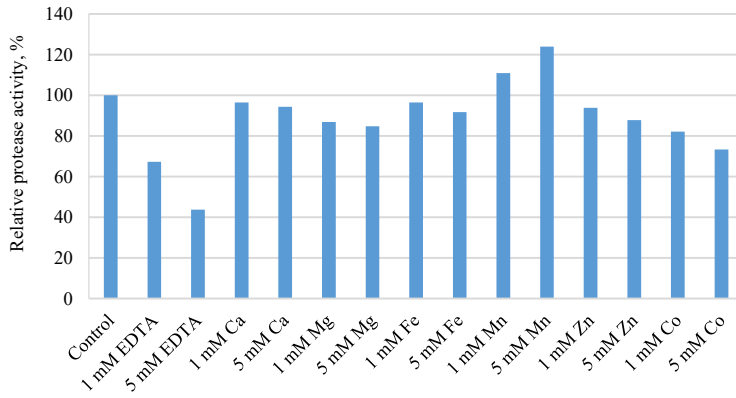

Fig. 4. Effects of various chemicals on protease activity

by 72% and 12% under the effect of 5 mM $ZnCl_2$ and $CuCl_2$, respectively (Yossan et al., 2006). In another study, it was stated that *B. megaterium* RRM2 protease activity increased by 25%, 21%, 19%, and 7% in the presence of 5 mM of Ca^{+2}, Mg^{+2}, K^{+}, and Na^{+}, respectively. However, at the same concentration of Co^{+2}, Fe^{+2}, and Zn^{+2} the maximum activity of the enzyme decreased by 90%, 76%, 79%, respectively (Pearl and Reed, 1920). Opposite to the results of this study, *B. cereus* strain S8 protease activity was not changed by the addition of 5 mM $MnCl_2$, whereas it was increased by more than 20% by the addition of the same amounts of $CuCl_2$, $CaCl_2$, $MgCl_2$, and $MoCl_2$ (Lakshmi et al., 2018).

3.4. Effect of storage temperature on protease activity

The enzyme maintained its activity almost with no loss for 20 days at 4 °C, but the activity decreased by 12% after 40 days (Fig. 5). The enzyme lost 26% of its activity after 40 days at 25 °C. Nevertheless, 46% enzyme activity loss was measured at the first sampling time at –20 °C, but the enzyme activity remained stable until the end of the 40-day-storage. The sharp decrease in enzyme activity may be due to formation of ice crystals in the enzyme solution, negatively affecting the enzyme structure.

3.5. Modelling of protease activity

Production of protease by *B. megaterium* was also modelled by using three modified models. Experimental and predicted values are shown in Fig. 6. At the beginning of the production, protease activity was estimated as 3.53 U mL^{-1} by modified Gompertz, but higher values of 16.57 and 10.47 U mL^{-1} were predicted by modified Logistic and modified Richards models, respectively. All models successfully presented the experimental results with minor differences between the 10th and 15th hour of the production process. After that point, all models underestimated the values until the end of the production period. The maximum protease activity was estimated as 234.06, 243.98, and 240.12 U mL^{-1} by modified Gompertz, modified Logistic, and modified Richards models, respectively, whereas 255.43 U mL^{-1} was measured in the experiment.

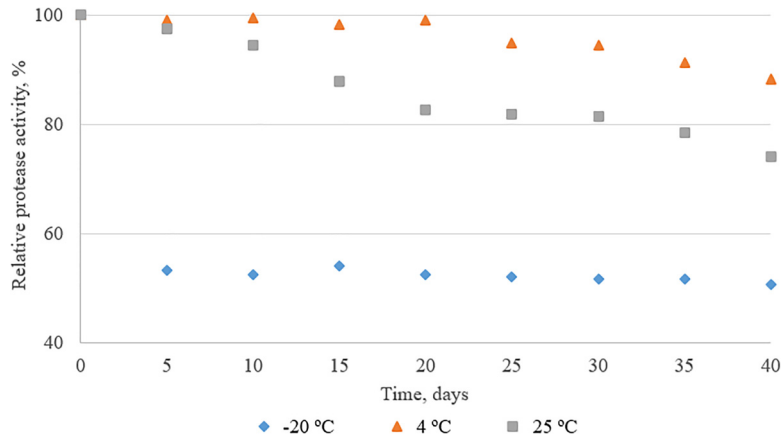

Fig. 5. Effect of storage temperature on protease activity

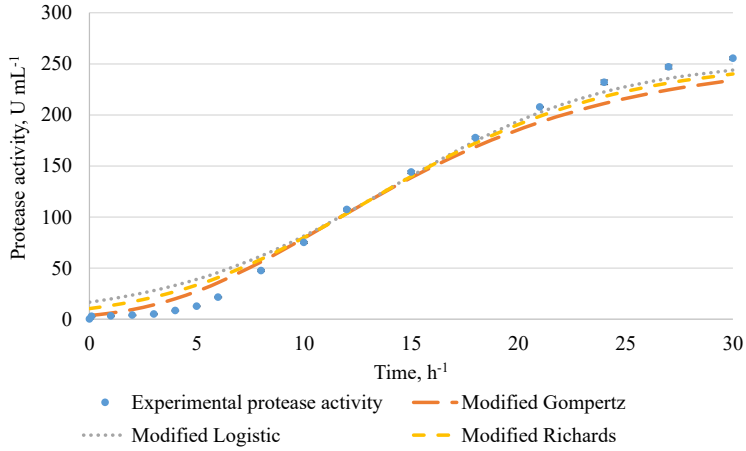

Fig. 6. Protease activity values measured and estimated by different models

Table 2. Analyses of mathematical models used to describe protease activity during its production by *Bacillus megaterium* DSM32

Mathematical models	Protease activity					
	RMSE	MAE	BF	AF	R^2	Slope
Modified Gompertz	12.1595	10.1565	0.5356	1.9745	0.9973	0.8817
Modified Logistic	15.7472	13.7475	0.3479	2.9554	0.9970	0.8667
Modified Richards	12.9922	11.8271	0.4025	2.5816	0.9978	0.8768

Statistical evaluation of protease production by the three models is shown in Table 2. Among them, the lowest RMSE and MAE values were calculated in modified Gompertz model as 12.1595 and 10.1565 U mL^{-1}, respectively. The highest RMSE and MAE values were obtained in modified Logistic model. The main reason for this, although modified Logistic model provided the closest values to experimentally obtained ones, it overestimated the values at the initial points with higher differences than other models. The best values of BF and AF were also obtained for modified Gompertz model. R^2 values for all models were calculated above 0.997, which is an indication of successful estimation. Similarly, slope values were also close to each other for the models, however, the highest value of 0.8817 was obtained for modified Gompertz model again. Therefore, after thorough evaluation, modified Gompertz model was selected as the best model for the estimation of protease production by *B. megaterium* in shake flask fermentation.

4. CONCLUSIONS

In this study, protease production by *B. megaterium* DSM32 was studied in details for the first time. Promising results, such as high production rates, high stability of the enzyme in wide

temperature, pH, and cation concentration ranges, clearly indicate that this enzyme can be a good candidate for industrial scale production. Additionally, calculation of production kinetics and application of mathematical models provided a better understanding of the overall process.

ACKNOWLEDGEMENTS

I would like to thank Ass. Prof. Dr. Hatice Güneş Özhan, who shared her laboratory with me in Izmir Biomedicine and Genome Center. This study was funded by Dokuz Eylul University, Izmir, Turkey, BAP (Project no: 2021.KB.SAG.026).

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