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Research Article

OPTIMISATION OF *Bacillus amyloliquefaciens* FE-K1 EXTRACELLULAR PEPTIDASE PRODUCTION BY RESPONSE SURFACE METHODOLOGY

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Abstract: In this study, it was aimed to optimise the extracellular peptidase production of *Bacillus amyloliquefaciens* FE-K1, previously isolated from ropy wholemeal bread, by using response surface methodology (RSM) based on central composite design (CCD). The temperature (20-45°C), initial pH of the enzyme production medium (pH 5-9) and inoculation level (1-5%, v/v) were used as the factors for RSM, and the fermentation time was determined for each trial separately. Results showed that the optimum peptidase production occurred at 33.4°C, pH 6.62 and 2.3% inoculation. It was determined that the fermentation time was only 7h, the crude enzyme had a peptidase activity of 49.17U/mL and a specific activity of 504.77U/mg under the optimised conditions.

Key words: *Bacillus*, central composite design, peptidase, response surface methodology.

Özet: Bu çalışmada, daha önce sünmüş ekmekten izole edilmiş olan *Bacillus amyloliquefaciens* FE-K1'in ekstraselüler peptidaz üretiminin, merkezi kompozit tasarıma (MKT) dayalı yanıt yüzey yöntemi (YYY) ile optimize edilmesi amaçlanmıştır. Yanıt yüzey yönteminde sıcaklık (20-45°C), enzim üretim ortamının başlangıç pH değeri (pH 5-9) ve inokülasyon seviyesi (%1-5, v/v) faktör olarak kullanılmış, fermentasyon süresi her deneme için ayrı ayrı belirlenmiştir. Sonuçlar optimum peptidaz üretiminin 33,4°C, pH 6,62 ve %2,3 inokülasyon seviyesinde elde edildiğini göstermiştir. Optimum koşullar altında fermentasyon süresinin sadece 7 saat, ham enzimin aktivitesinin 49,17U/mL, spesifik aktivitesinin ise 504,77U/mg olduğu tespit edilmiştir.

Introduction

The enzyme group that converts proteins or large polypeptides into smaller peptides or free amino acids by hydrolysing peptide bonds are peptidases. Although typically known as proteases, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends using the terms 'peptidase' or 'peptide hydrolase' for any enzyme that hydrolyses peptide bonds (Rawlings *et al.* 2007). Therefore, the term 'peptidase' has been used throughout this paper.

Bacillus (Cohn) species have been the most commonly used microorganisms in microbial fermentation applications. The ability to produce and secrete large quantities (20–25g/L) of extracellular enzymes into the culture medium, a high adaptability to environmental changes, high growth rates that lead to short fermentation cycles, GRAS (generally recognised as safe) status, and the vast amount of information about these species have placed them among the most important enzyme producers (Schallmey *et al.* 2004).

Bacillus species are widely used for peptidase production and most of the commercially used neutral and alkaline peptidases are produced by these species (Outtrup *et al.* 1990, Vetter *et al.* 1995, Sandhya *et al.* 2005, Leong 2006, Kolkman *et al.* 2016). They secrete extracellular peptidases principally at the end of the exponential growth phase (Simonen & Palva 1993), in the early stationary phase or during the stationary growth phase (Mabrouk *et al.* 1999), but peptidase production at the beginning of the exponential phase and death phase has also been reported (Beheshti Maal *et al.* 2011). Peptidase secretion by *Bacillus* species may be associated with nutrient depletion (particularly, nitrogen and carbon sources) during the stationary phase, as well as to the sporulation (Hanlon & Hodges 1981, O'hara & Hageman 1990). Thus, strategies of prolonging the stationary phase can be useful to increase the peptidase activity (Gupta *et*

al. 2002). According to Hamoen *et al*. (2003) if the nutrients are limited in the stationary growth phase, *Bacillus subtilis* (Ehrenberg) Cohn secrete degradative enzymes, such as peptidases, to liberate nutrients from alternative sources that are usually difficult to access. Prolonged nutritional stress results in the development of competence and, ultimately, in sporulation of the bacterial population.

Members of the genus *Bacillus* secrete the proteins across their single membrane system directly into the culture medium at high concentrations. *B. subtilis* produce two groups of extracellular peptidases, known as "quality control" and "feeding" peptidases. While quality control peptidases have major roles in the removal of potentially detrimental molecules, such as cleaved signal peptides, incompletely synthesised proteins and misfolded proteins, feeding peptidases provide nutrients to the cells by degrading the proteins or peptides in the environment (Harwood & Cranenburgh 2008, van Dijl & Hecker 2013).

In some of the studies related to production of *Bacillus* peptidases, strains have been isolated, particularly, from soil (Gupta *et al*. 1999, Kumar *et al*. 1999, Singh *et al*. 2001, Beg & Gupta 2003, Shafee *et al*. 2005, Chu 2007, Nadeem *et al*. 2008, Reddy *et al*. 2008, Kim *et al*. 2016, Hussain *et al*. 2017), soil mixed seawater (Patel *et al*. 2006), wastewater near a milk processing plant (Chu 2007), slaughterhouse waste water (Hammami *et al*. 2018), tannery waste (Anandharaj *et al*. 2016), marine sediment (Uttatree & Charoenpanich 2016, Uttatree *et al*. 2017), raw milk (Matta & Punj 1998), sugarcane molasses (Johnvesly & Naik 2001), leather by-products (Genckal & Tari 2006, Tari *et al*. 2006), compost (Denizci *et al*. 2004), industrial waste discharge (Gerze *et al*. 2005, Orhan *et al*. 2005), leather, soil and horse faeces (Akbalik *et al*. 2004), cheese (Molva *et al*. 2009) or obtained directly from culture collections (Yang *et al*. 2000, Beg & Gupta 2003).

Response surface methodology (RSM) is an experimental strategy applied to evaluate the interactions among different parameters and to find the best combination parameters for multivariable systems. RSM is widely used for the optimisation of microbial fermentation parameters such as medium composition, pH, temperature, inoculation level, agitation rate etc. The major advantage of RSM is to minimize the number of experiments and time (Lakshmi & Hemalatha 2016, Ahsan *et al*. 2017).

The *Bacillus* strain used for peptidase production in this study (*Bacillus amyloliquefaciens* FE-K1) has previously been isolated from ropy wholemeal bread (Erem *et al*. 2009). Rope is a type of bread disease seen in regions where the climate is warm and moist. The causative agent is commonly *B. subtilis*, and some other *Bacillus* species, such as *B. licheniformis* (Weigmann), *B. megaterium* (Bary), *B. pumilus* (Meyer & Gottheil) and *B. cereus* (Frankland & Frankland), which may contaminate bread through the raw materials and bakery equipment used (Kirschner & von Holy 1989, Collins *et al*. 1991, Bailey & von Holy 1993). Although most of the vegetative forms of these bacteria are killed during the baking process, the spores can survive the baking process and germinate during storage of bread, causing the disease (Volavsek *et al*. 1992, Thompson *et al*. 1993). The liquefaction that occurs in the centre of the bread results from the degradation of starch and protein molecules by the amylases and peptidases secreted from the germinated spores (Kirschner & von Holy 1989, Voysey 1989, Rosenkvist & Hansen 1995, Ellis *et al*. 1997, Thompson *et al*. 1998, Pepe *et al*. 2003, Erem *et al*. 2009). Hence, this study was performed to optimise the extracellular peptidase production of *B. amyloliquefaciens* FE-K1, considering the probability that *Bacillus* species isolated from ropy bread can be a good source for peptidase production.

Materials and Methods

Materials

Bacillus amyloliquefaciens FE-K1 has previously been isolated from ropy wholemeal bread (Erem *et al*. 2009), and its 16S rRNA gene sequence was determined by Gene Research and Biotechnology, Ankara, Turkey (REFGEN). The strain was also investigated for haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) production and verified as an HBL- and NHE-negative strain (unpublished data). Nucleotide sequence data for *B. amyloliquefaciens* FE-K1 are available in the GenBank databases under the accession number MH045777.

Preparation of stock culture and pre-culture

A single colony of *B. amyloliquefaciens* FE-K1, grown overnight on nutrient agar, was inoculated into 50mL of nutrient broth (in 250-mL baffled flask) and incubated at 37°C and 250rpm for 18h. Then, the culture was mixed with 50% glycerol (1:1, v/v) and 1-mL aliquots were distributed into 2-mL sterile cryo-tubes and maintained at -80°C.

For pre-culture preparation, the whole contents of one tube stock of culture were inoculated into a 250-mL baffled flask, containing 50mL of growth media and incubated at 37°C, with shaking (250rpm), until reaching the mid-log phase (7.5h). The growth medium contained 2.0g/L glucose, 10.0g/L yeast extract, 1.0g/L KH_2PO_4, 3.0g/L $K_2HPO_4.3H_2O$, 2.0g/L Na_2SO_4 and 0.1g/L $MgSO_4.7H_2O$.

Some of the parameters used in this study for the production of *B. amyloliquefaciens* FE-K1 peptidase have previously been determined with one factor at a time approach (Erem & Certel 2018). Carbon (glucose) and nitrogen (yeast extract) sources, which provide the maximum peptidase activity, were selected among different sources and they used in the growth medium. Furthermore, a carbon/nitrogen ratio of 1:5 and agitation rate of 250rpm were determined as the best values in terms of obtaining higher peptidase activity.

Measurement of optical density (OD)

Samples taken from the fermentation medium were diluted with sterile distilled water, and the absorbance was measured at 600nm by using the sterile fermentation medium as the blank.

Determination of the growth curve

In order to determine the growth curve of *B. amyloliquefaciens* FE-K1, the pre-culture was inoculated into 50mL of growth media by setting the initial OD value at 0.1 and the OD was measured at 1-h intervals during 24h of fermentation. The growth curve was obtained by plotting the OD values versus fermentation time.

Experimental design

Temperature (20-45°C), initial pH of the growth medium (pH 5–9) and inoculation level (1-5%, v/v) were chosen as factors for RSM. A central composite design (CCD), which was generated with Minitab statistical software (version 17, Minitab Inc. USA), was implemented to identify the optimum values of these factors. The minimum and maximum values for the factor levels, which represent the axial points in the CCD, were determined according to preliminary experiments. Central Composite Inscribed (CCI) design which is one of the three types of CCD was used to limit the factor levels within the values given above in the text. A total of 40 fermentation trials were conducted by using duplicate samples and triplicate analyses. Duplicate samples were evaluated as blocks in the CCD, and six centre points were used per block. Table 1 shows the experimental design.

Peptidase activity values obtained were evaluated with the analysis of variance (ANOVA), and the regression coefficients (R^2) and equations were determined. Factors with a p value of $p<0.05$ were evaluated as statistically significant. The polynomial regression equation (Eq. 1) depending on the factor levels was then fitted to the data by the software:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad \text{(Eq. 1)}$$

where Y, predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, squared coefficients; β_{12}, β_{13}, β_{23}, interaction coefficients; A, B, C, factors.

Response surface graphs were then generated by the Minitab statistical software using this model.

Peptidase production

The pH of the growth medium (50mL) was adjusted with 6N HCl and 6N NaOH, according to the pH values in Table 1, before sterilisation. The sterilised medium was inoculated with the determined level of pre-culture, as seen in Table 1, for each trial. Fermentation experiments were conducted in 250-mL baffled glass flasks, at 250rpm, using a shaking incubator (Sartorius Certomat IS, Germany) until the OD of the supernatant reached its maximum value. Then, the culture broth was centrifuged at 20,000*g* and 4°C for 15min (Biochrom Libra, Cambridge, England) to obtain the cell-free crude enzyme solution. Peptidase activity, protein content and glucose determination were assayed in the cell-free supernatants obtained at 1-h intervals during the fermentation.

Peptidase assay

Peptidase assay was performed as described by Cupp-Enyard (2008), with a slight modification, in that the transparent solutions were obtained by centrifugation (10000*g* and 4°C for 10min) instead of using syringe filters and the volume of samples and all solutions used in the analysis were decreased with a ratio of 5:1. The peptidase activity of the cell-free clear supernatant was assayed at 37°C in potassium phosphate buffer (50mM, pH 7.5) by using 0.65% (w/v) casein as the substrate. One unit of peptidase was equivalent to the amount of enzyme required to release 1mg of tyrosine/mL/min under standard assay conditions.

Protein content

The total protein content of the enzyme solution was determined by the Bradford method (Bradford 1976), using a Coomassie Plus (Bradford) assay kit (Thermo Scientific, Pierce Biotechnology, USA) with bovine serum albumin as the reference standard. Accordingly, 100µL of crude enzyme solution was mixed with 3mL of Bradford reagent. The mixture was incubated at 25°C for 10min, and the absorbance was measured at 595nm. Protein analysis was done immediately after obtaining the enzyme solution.

Glucose assay

Glucose was determined by modifying the method of Miller (1959). Briefly, 600µL of cell-free supernatant was mixed with 600µL of 3,5-dinitrosalicylic acid reagent. The mixture was kept at 90°C for 15min and, then, 200µL of 40% potassium sodium tartrate was added. Next, the mixture was cooled in an ice bath for 5min, and the absorbance was measured at 575nm. A glucose calibration curve was established.

Table 1. Central composite design used for the optimisation of *Bacillus amyloliquefaciens* FE-K1 peptidase.

	Experimental values				
Variables	-α (-1.682)	-1	0	+1	+α (1.682)
Temperature (°C)	20.00	25.07	32.50	39.93	45.00
pH	5.00	5.81	7.00	8.19	9.00
Inoculation level (%)	1.00	1.81	3.00	4.19	5.00

Results and Discussion

Growth curve of B. amyloliquefaciens FE-K1 and variation in its peptidase production

The growth curve and peptidase production of *B. amyloliquefaciens* FE-K1 (Fig. 1) revealed that the strain grew rapidly after the lag phase and reached a high OD level of 8.0. Then, a prompt decrease in the OD value occurred, and there was no observable stationary phase. It is known that metabolites produced towards the end of the log phase could prevent the growth of the bacteria (Tunail 2009). Therefore, the pH of the fermentation medium was measured to check whether the strain produced acid that could hinder its proliferation. However, the measured pH of the sterile medium increased from pH 6.86 to neutral levels during the growth of *B. amyloliquefaciens* FE-K1. It was also thought that due to the rapid growth, the sugar could be insufficient to sustain the cells. However, although the glucose level decreased, there was still some sugar present that the cells could utilise. Therefore, it was decided that either the bacteria needed a specific nutrient or due to the high OD level there was a competition for the nutrients between the living cells. Consequently, the cells which could not access the nutrients were lysed, and the OD quickly decreased. In order to verify cell lysis, gram staining was employed (not shown). The results showed that after the highest OD level, cells began to seem pink which is the result of cell wall degradation. This was probably due to the intracellular peptidoglycan hyrolases released from the lysed cells. Nevertheless, with the intention to obtain a satisfactory peptidase activity over a short fermentation time, the study was continued, and the reason for the drop in the OD level was considered to be the subject of another study.

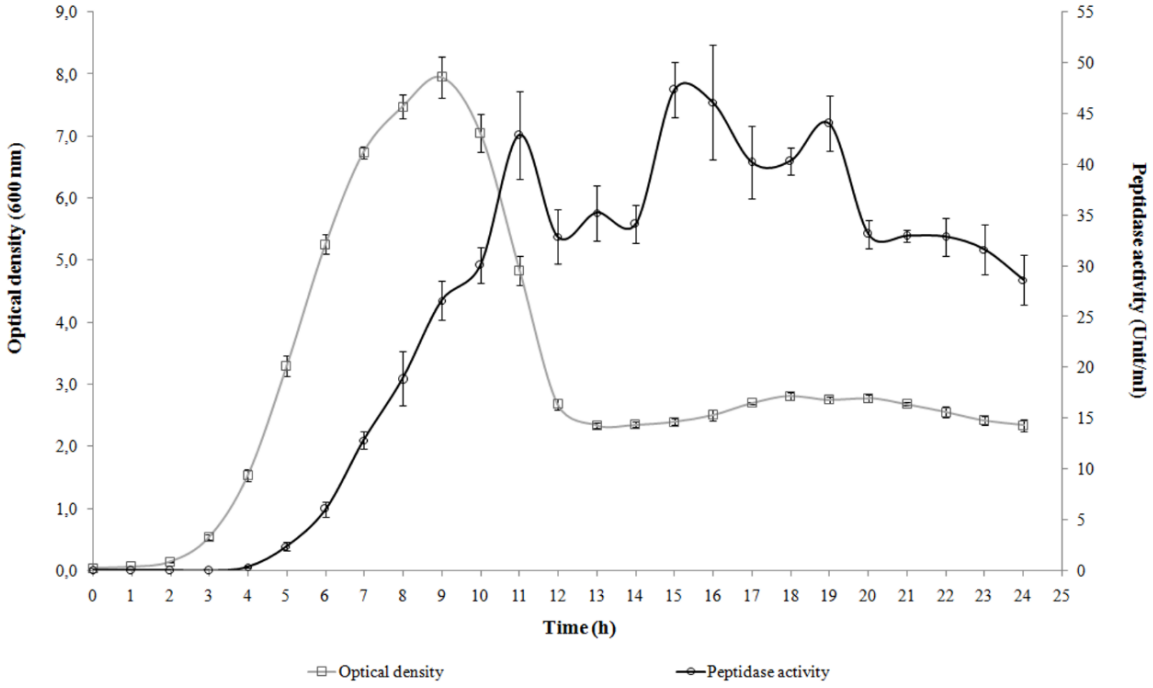

Fig. 1. Growth curve and peptidase production of *Bacillus amyloliquefaciens* FE-K1 at 30°C.

Optimisation of peptidase production by RSM

As seen in Fig. 1, even though the OD decreased, the peptidase activity kept increasing. This behaviour was most probably because of the intracellular peptidases secreted during cell lysis. However, although the actual reason for the cell lysis was not known, the main interest of this study was to optimise the extracellular peptidases of *B. amyloliquefaciens* FE-K1. Therefore, in RSM, the maximum peptidase activity reached before cell lysis, that is, the peptidase activity obtained at maximum OD level was used as the response, to exclude intracellular peptidases. Nonetheless, the time necessary to reach the maximum OD level was variable, depending on the factor levels. For accurate determination of the incubation time of each fermentation experiment, it was necessary to measure the OD and peptidase activity at short intervals. Thus, after inoculating the fermentation media with pre-culture, samples were withdrawn from the fermentation medium at 1-h intervals to perform the glucose and protein content analysis in addition to the OD and peptidase activity measurements. The glucose and protein contents were determined to obtain information about the nutrient content during fermentation, particularly to observe the nutrient content change at the time the OD began to decrease.

In contrast to the results of Gençkal & Tari (2006) and Razak *et al.* (1997), the OD and peptidase activity values obtained under various conditions (Fig. 2) showed that peptidase production is closely linked to the growth of *B. amyloliquefaciens* FE-K1. Even at different temperatures, pH values and inoculation levels, the strain started to produce peptidase consistently shortly after the beginning

of the log phase, and the peptidase activity increased with the growth of the strain. However, due to the sudden cell death observed after reaching a high cell density, the association between the growth of the strain and its extracellular peptidase production could not be determined after the log phase.

The glucose contents measured under various fermentation conditions demonstrated that glucose was not entirely consumed at the point where the fermentation was terminated. Sugar consumption by the bacteria was relatively low, particularly at lower temperatures, such as 20.00 and 25.07°C. Sugar consumption differed when the fermentation was performed at the same temperature but with different pH values. For instance, at 25.07°C, the bacteria consumed more glucose when the initial pH value was higher. When the fermentation media had an initial pH of 5.81, the glucose consumption rate at maximum OD level was 50 and 47% for the inoculation levels of 1.81 and 4.19%, respectively. However, the glucose consumption rate was 84 and 77% under the same conditions, when the fermentation started at pH 8.19. It can be understood from the results that at a given pH, the inoculation level had low influence on glucose consumption. For fermentation at 32.5°C and 3% inoculum, the glucose consumption rates were 56, 76 and 89%, at pH 5, 7 and 9, respectively. Pantamas *et al*. (2003) also found that *B. licheniformis* and *B. coagulans* (Hammer) consumed more glucose at higher alkali conditions.

For all fermentation trials, protein content and its rate of increase were quite low, until the OD reached its maximum value. It is considered that at this stage, the protein content was based on the peptidases secreted by the bacteria into the fermentation medium because no protein was detected in the sterile enzyme production medium (it contains low molecular weight of peptides and amino acids) and the protein content could be determined just after the peptidase activity analysis started to give positive results. Typically, a sudden increase was observed in the protein content under all fermentation conditions after the maximum OD level had been reached. This tendency was also probably due to the intracellular proteins secreted by the bacteria depending on the cell lysis. While living cells kept secreting extracellular proteins, principally peptidases, intracellular proteins were also secreted into the medium because of the lysed cells, increasing the protein content.

The specific activity values at maximum OD and 1h after maximum OD levels were reached (Table 2) indicated that the specific activity of the peptidase started to decrease, generally more than 50%, only at 1h after the maximum OD level, most probably due to secretion of the intracellular proteins during cell lysis. Peptidase activity also increased after cell lysis but the decrease in the specific activity suggested that proteins other than the peptidases were responsible. Therefore, peptidases which have a relatively high specific activity will be obtained by using the peptidase activity level corresponding to the maximum OD level as the response in RSM.

Table 2. Specific activity of peptidase at different fermentation conditions.

Factor levels	Specific activity (U/mg)[a]	Specific activity (U/mg)[b]
25.07°C, pH 5.81, 1.81%	1056.57	474.15
39.93°C, pH 5.81, 1.81%	1318.22	550.62
25.07°C, pH 8.19, 1.81%	760.96	392.21
39.93°C, pH 8.19, 1.81%	663.40	701.63
25.07°C, pH 5.81, 4.19%	795.53	823.70
39.93°C, pH 5.81, 4.19%	1466.99	644.38
25.07°C, pH 8.19, 4.19%	477.69	266.48
39.93°C, pH 8.19, 4.19%	1423.25	579.85
20.00°C, pH 7.00, 3.00%	1508.35	761.18
45.00°C, pH 7.00, 3.00%	1447.31	320.27
32.50°C, pH 5.00, 3.00%	754.18	489.61
32.50°C, pH 9.00, 3.00%	782.15	148.63
32.50°C, pH 7.00, 1.00%	563.02	268.09
32.50°C, pH 7.00, 5.00%	859.87	440.24
32.50°C, pH 7.00, 3.00%	921.70	419.36

[a] Spesific activity at maximum OD level; [b] Spesific activity at 1 hour after the maximum OD level.

Peptidase activity values obtained at maximum OD level were evaluated with analysis of variance (ANOVA), by using Minitab statistical software (version 17, Minitab Inc. USA), and the regression coefficients (R^2) and equations were determined. The polynomial regression equation (Eq 2), depending on the factor levels of temperature (t), initial pH of fermentation medium (p) and inoculation level (i) was as follows:

$$\text{Peptidase activity} = -283.5 + 6.79t + 63p + 1.12i - 0.0853t\text{^}2 - 4.219p\text{^}2 - 1.366i\text{^}2 - 0.214\, t \times p + 0.148\, t \times i + 0.007p \times \text{i} \quad \text{(Eq 2)}$$

The results of the CCD experiments, for the effects of temperature, initial pH of fermentation medium and inoculation level on peptidase production, are presented in Table 3. The predicted values were determined by the statistical software program according to the regression equation.

The ANOVA results in Table 4 verify that according to the p-value, the effect of "block" on peptidase activity is not statistically significant. In this study, the "blocks" represent the duplicate samples (fermentations carried out under the same conditions but at different times). These fermentations were performed to determine the effects of the possible differences that may arise during the preparation of the fermentation medium at various times, although, the same boxes of the enzyme production medium components were used throughout the study. Based on the ANOVA results, however, there were no differences in peptidase activity between the fermentations done under the same conditions but at different times.

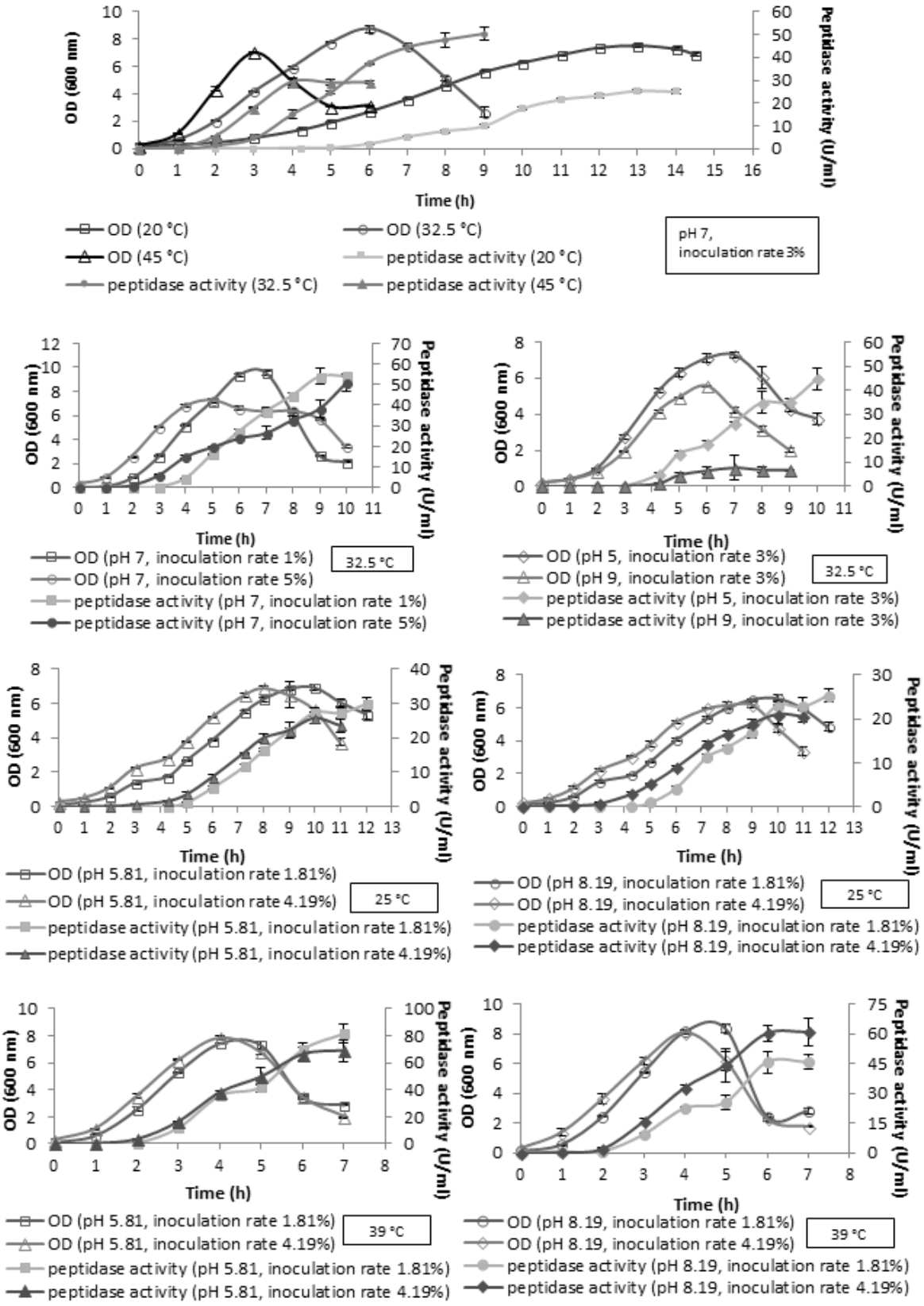

Fig. 2. Changes in optical density (OD) and peptidase activity values in different fermentation trials.

Table 3. Observed and predicted peptidase activities of *B. amyloliquefaciens* FE-K1 depending on the factor levels obtained with CCD.

Run order	Block	Factors (actual levels)			Time (h)[a]	Peptidase activity (U/mL)	
		Temperature (°C)	pH	Inoculation level (%)		Predicted response	Observed response
1	1	25.07	5.81	1.81	10	29.64	27.61 ± 1.25
2	1	39.93	5.81	1.81	4	33.66	35.84 ± 1.65
3	1	25.07	8.19	1.81	10	26.17	23.00 ± 0.87
4	1	39.93	8.19	1.81	5	22.62	25.29 ± 3.61
5	1	25.07	5.81	4.19	8	21.74	19.97 ± 1.26
6	1	39.93	5.81	4.19	4	31.00	38.05 ± 1.13
7	1	25.07	8.19	4.19	9	18.32	18.97 ± 0.94
8	1	39.93	8.19	4.19	4	20.01	32.91 ± 0.94
9	1	20.00	7.00	3.00	13	22.28	25.30 ± 0.38
10	1	45.00	7.00	3.00	3	27.08	17.79 ± 0.25
11	1	32.50	5.00	3.00	7	27.21	26.40 ± 0.66
12	1	32.50	9.00	3.00	6	15.05	6.36 ± 1.46
13	1	32.50	7.00	1.00	7	36.96	36.95 ± 1.96
14	1	32.50	7.00	5.00	5	28.12	19.62 ± 0.61
15	1	32.50	7.00	3.00	6	38.01	37.49 ± 0.08
16	1	32.50	7.00	3.00	6	38.01	37.40 ± 4.85
17	1	32.50	7.00	3.00	6	38.01	44.43 ± 4.92
18	1	32.50	7.00	3.00	6	38.01	43.53 ± 4.71
19	1	32.50	7.00	3.00	6	38.01	39.86 ± 0.11
20	1	32.50	7.00	3.00	6	38.01	38.12 ± 2.90
21	2	25.07	5.81	1.81	8	29.64	26.48 ± 0.72
22	2	39.93	5.81	1.81	4	33.66	35.44 ± 0.52
23	2	25.07	8.19	1.81	9	26.17	27.05 ± 1.05
24	2	39.93	8.19	1.81	5	22.62	30.37 ± 1.77
25	2	25.07	5.81	4.19	7	21.74	22.23 ± 0.24
26	2	39.93	5.81	4.19	4	31.00	35.38 ± 2.44
27	2	25.07	8.19	4.19	8	18.32	22.84 ± 0.33
28	2	39.93	8.19	4.19	4	20.01	21.43 ± 1.51
29	2	20.00	7.00	3.00	12	22.28	25.80 ± 0.26
30	2	45.00	7.00	3.00	3	27.08	16.91 ± 1.45
31	2	32.50	5.00	3.00	6	27.21	27.14 ± 0.65
32	2	32.50	9.00	3.00	7	15.05	11.71 ± 0.18
33	2	32.50	7.00	1.00	7	36.96	37.27 ± 4.03
34	2	32.50	7.00	5.00	6	28.12	23.41 ± 2.84
35	2	32.50	7.00	3.00	7	38.01	35.51 ± 0.37
36	2	32.50	7.00	3.00	7	38.01	35.83 ± 0.34
37	2	32.50	7.00	3.00	7	38.01	38.67 ± 0.99
38	2	32.50	7.00	3.00	7	38.01	34.35 ± 1.42
39	2	32.50	7.00	3.00	7	38.01	35.81 ± 1.87
40	2	32.50	7.00	3.00	7	38.01	37.29 ± 2.36

[a] Fermentation time= time necessary to reach maximum OD level.

Table 4. The results of ANOVA analysis of peptidase activity.

Source	Total Degrees of Freedom	Adjusted Sums of Squares	Adjusted Mean Squares	F-value	P-value
Model	10	2238.35	223.84	7.58	<0.001
Block	1	4.87	4.87	0.16	0.688
Linear	3	601.43	200.48	6.79	0.001
Temperature (*t*)	1	55.62	55.62	1.88	0.180
pH (*p*)	1	357.16	357.16	12.10	0.002
Inoculation level (*i*)	1	188.65	188.65	6.39	0.017
Square	3	1547.28	515.76	17.48	<0.001
*t***t*	1	639.86	639.86	21,68	<0.001
*p***p*	1	1025.85	1025.85	34.76	<0.001
*i***i*	1	107.58	107.58	3.65	0.066
2-way interaction	3	84.78	28.26	0.96	0.426
*t***p*	1	57.31	57.31	1.94	0.174
*t***i*	1	27.47	27.47	0.93	0.343
*p***i*	1	0.00	0.00	0.00	0.994
Error	29	855.85	29.51		
Lack-of-fit	19	795.88	41.89	6.99	0.002
Pure error	10	59.96	6.00		
Total	39	3094.20			
Model Summary	**Standard deviation**	**R^2**	**R^2 (adjusted)**		**R^2 (predicted)**
	5.43249	**72.34%**	**62.80%**		**38.85%**

While the effects of initial pH and inoculation level on peptidase activity were significant ($p<0.05$), the temperature had no effect on peptidase activity (Table 4). Furthermore, *t***t* and *p***p* interactions were also significant ($p<0.05$), suggesting that there is a quadratic relationship between the peptidase activity and these factors.

The ANOVA results indicated a relatively low satisfactory adjustment of the model to the experimental data, due to the R^2 value of 72.34%. The generated model could only explain 72.34% of the variability in the peptidase activity. For a good adjustment of the model, the R^2 value should be higher than 75% (Puri *et al*. 2002). Moreover, the lack-of-fit value was significant ($p<0.05$) which was also an indicator of the inadequacy of the model. Determining the temperature as statistically insignificant factor was probably the main reason of obtaining a relatively incompatible model. Several studies have reported that fermentation temperature is a major factor regarding peptidase activity (Puri *et al*. 2002, Chauhan & Gupta 2004, Gorlach-Lira *et al*. 2010). Finding temperature as an insignificant factor in the present study was probably because of using the peptidase activity values at maximum OD level as the response in RSM, which caused different fermentation times under different fermentation conditions. Fig. 2 illustrates how the temperature had a greater effect on the fermentation time than the pH and inoculation level. For example, the time necessary to reach maximum OD at 32.5°C and pH 7 was 7 and 5h for inoculation levels of 1 and 5%, respectively. In other words, when the other factors were constant, a 5-fold increase in the inoculation level resulted in a 2h decrease in the fermentation time. Conversely, at a fixed pH 7 and 3% inoculation level, the OD reached its maximum value after 13 and 3h at 20 and 45°C, respectively. Therefore, the temperature was an important factor regarding the growth of the bacteria which was also closely associated with its peptidase production. As a result of selecting the peptidase activity corresponding to maximum OD level as the response, the peptidase activities obtained at 20 and 45°C were quite similar to each other despite the different fermentation times. Thus, the temperature was determined as an insignificant factor in the model. A constant fermentation time for all fermentation conditions would solve the problem. However, it would be impossible to determine the extracellular peptidase activity of *B. amyloliquefaciens* FE-K1 in such conditions due to the reasons explained

above. Another possible reason for the model incompatibility is considered to be the composite structure of the crude enzyme solution. It may contain several different enzymes and also various types of peptidases. A peptidase that is active under one fermentation condition may lose or decrease its activity under another condition. If this is the case, it is probable to obtain a variation in the measured response values (peptidase activity), compared to the predicted values.

The contour and surface plots of peptidase production are presented in Figs. 3 and 4, respectively. Given the plots can show the interaction of two factors, the third factor was fixed at the centre points. The circular and elliptical shapes of the contour plots indicated that peptidase activity had reached its maximum level. The same result could be seen in the surface plots (Fig. 4). When the temperature was close to the centre point (approximately 30°C), maximum peptidase activity could have been obtained and a decrease was noted in the activity at lower and higher temperatures. Gençkal and Tari (2006) determined that 30 or 37°C were the optimum temperatures both for the growth of bacteria and enzyme production, and the decrease in enzyme activity, particularly at 45°C or higher, was associated to the protein denaturation and degradation caused by the proteolytic activity of the peptidase produced. Previous studies have documented the optimum temperature for peptidase production as 25°C (Jaswal *et al*. 2008), 34.58°C (Rao *et al*. 2007), 37°C (Chauhan & Gupta 2004, Ali *et al*. 2016) and 40°C (Anadharaj *et al*. 2016).

It is well-known that the culture pH strongly affects many enzymatic processes (Bhunia *et al*. 2012). The surface plots (Fig. 4) confirmed that maximum peptidase activity occurred when the initial pH was around 6.5-7.0, and a substantial decrease in the activity appeared at higher initial pH values.

Bacillus amyloliquefaciens FE-K1 is a bacterial strain isolated from bread and, therefore, it was not expected to grow at high pH values. However, considering that the

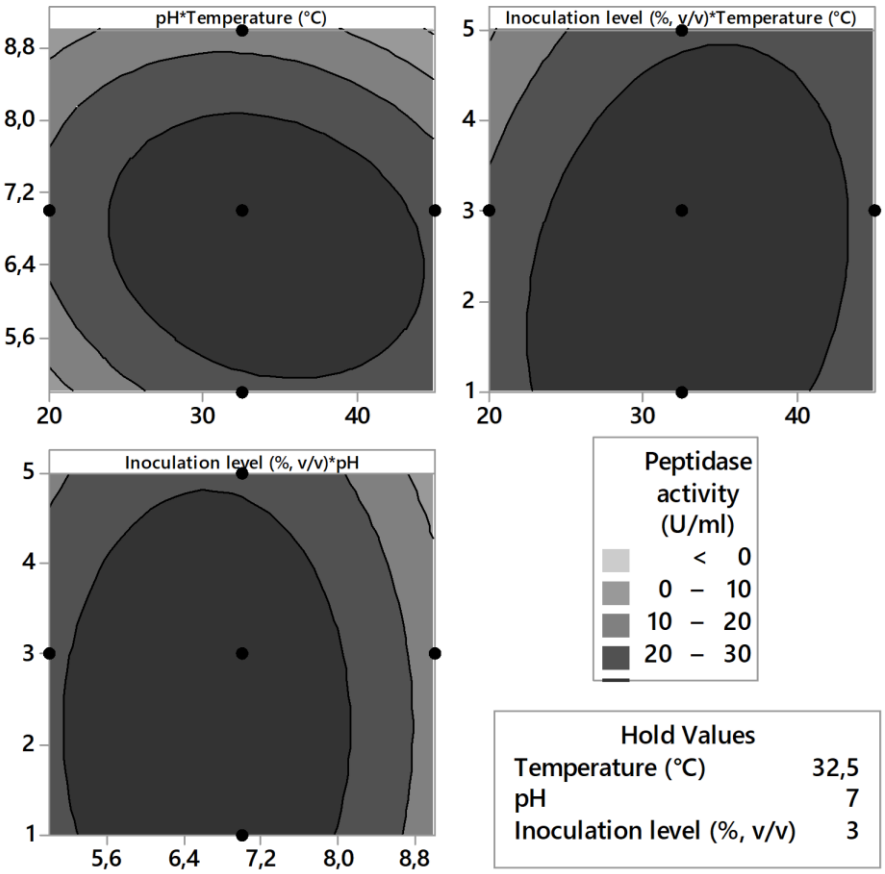

Fig. 3. Contour plots of peptidase activity (U/mL).

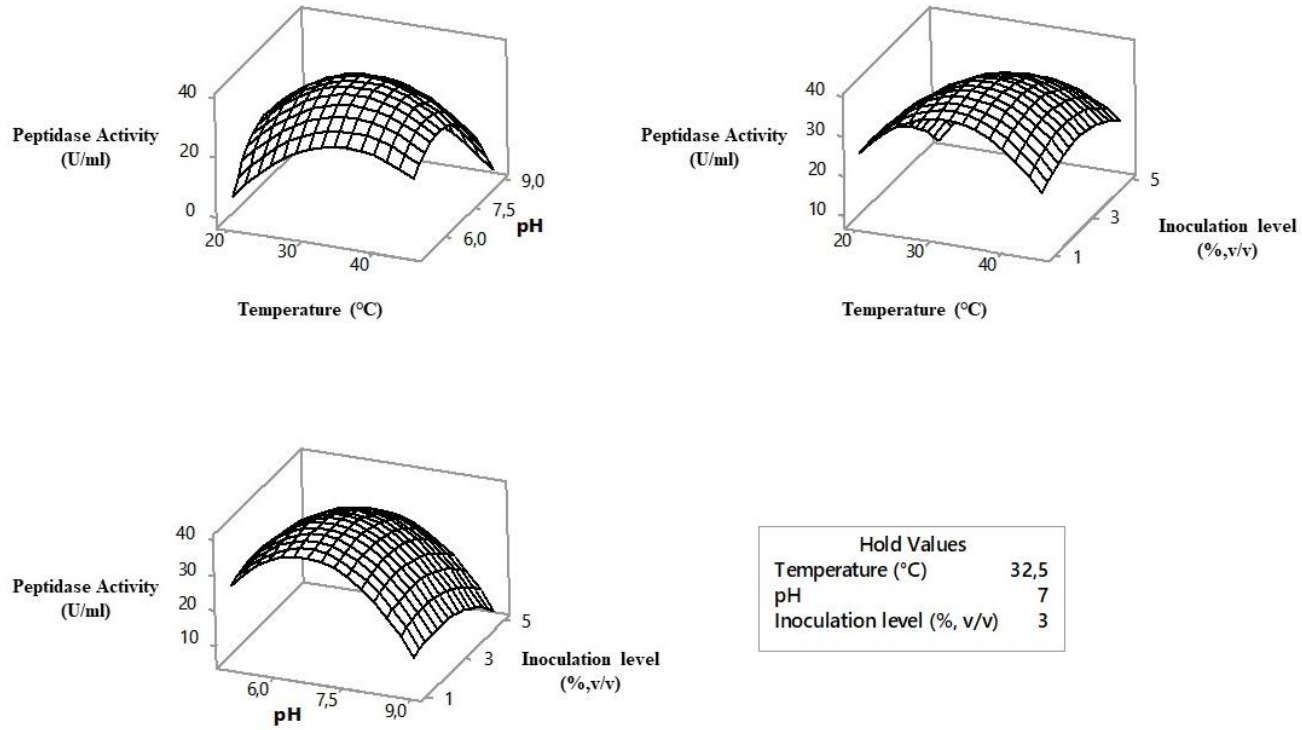

Fig. 4. Surface plots of peptidase activity (U/mL).

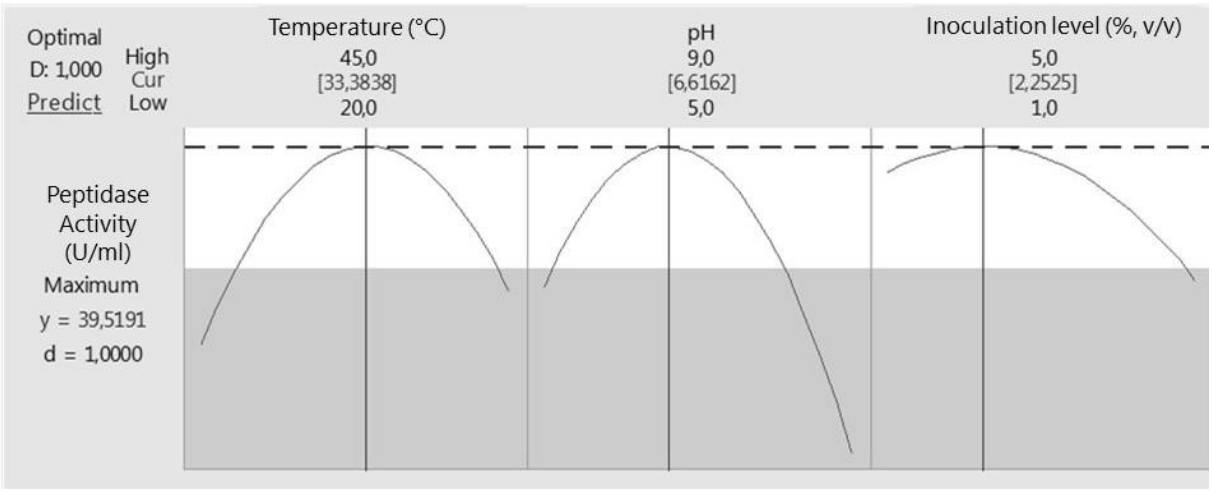

Fig. 5. Optimisation plot for peptidase activity of *Bacillus amyloliquefaciens* FE-K1.

correlation between growth and peptidase production of this strain was not known at the beginning of the study, high pH values (i.e., pH >7) were also added to the CCD. The pH was measured at the end of each fermentation. Irrespective of whether higher or lower initial pH values were established, the pH of the medium was always close to neutral levels at the end of all fermentations. The final pH was between 6.7 and 8.0, depending on the initial pH. This finding indicates that the bacteria have created neutral conditions by producing acidic or alkali metabolites to sustain its growth. Qadar *et al*. (2009) noticed an increase in the pH value of the fermentation medium and proposed that the increase was due to metabolite accumulation. Several studies determined the optimum pH of the culture as 10.5 (Jaswal *et al*. 2008), 10.0 (Ali *et al*. 2016), 7.0 (Qadar *et al*. 2009) and 4.8 (Gorlach-Lira *et al*. 2010).

The optimum inoculation level was approximately 2.5% (Figure 3 and 4). Prakasham *et al*. (2006) applied an inoculum value between 1–5% and found that the optimum level for peptidase production was 3%. Puri *et al*. (2002) selected 1–3% inoculum for *Bacillus* sp. and noted that the inoculation level did not affect the peptidase production. Suganthi *et al*. (2013) documented an optimum inoculation of 1%, for *B. licheniformis* peptidase. The authors reported that lower inoculum doses (<1%) caused a reduction in enzyme production due to the insufficient number of bacteria while higher inoculum levels (>1%) led to the same reduction effect because of

reduced dissolved oxygen and increased competition towards nutrients.

The dashed line in the optimisation plot, generated by the Minitab 17 software (Fig. 5), represents the maximum peptidase activity and the vertical lines show the necessary factor levels to reach this maximum value. This plot indicates that temperature, pH and inoculation level should be 33.4°C, 6.62 and 2.3%, respectively, to provide the maximum peptidase activity of 39.519U/mL. Furthermore, the *d*-value of 1.0 shows that the optimum factor levels have been reached.

Validation of optimisation

In order to validate the optimisation results, triplicate fermentations trials were undertaken at 33.4°C and pH 6.62 with 2.3% inoculum. The change in OD, peptidase activity, and glucose and total protein contents during the fermentations (Fig. 6) demonstrated that the maximum OD was reached at 7h and corresponded to 49.172±1.014U/mL peptidase activity. There were almost 10U difference between the measured and predicted (39.519U/mL) optimum peptidase activity values. The most important reason for this difference is the inadequacy of the model. However, it is possible to evaluate the results from another perspective. Given the fermentation time is not a factor in the CCD or the time is not constant for all the fermentations, it is not possible for the software to determine the fermentation time, namely, the time necessary to reach maximum OD for the optimised conditions. Therefore, the conditions in CCD, which were very close to the optimum factor levels (33.4°C, pH 6.62, 2.3% inoculum level), should be considered. In any case, the software determines the optimum activity level according to the values in the CCD. Fermentation conditions closest to the factor levels that gave the optimum peptidase activity were located at the centre point (32.5°C, pH 7, 3% inoculum level) in the CCD (Table 3). When the fermentations were done at the centre points, the fermentation time was determined as 6 and 7h, respectively, in the different blocks. Therefore, it is possible to accept a 6 or 7h fermentation time for the optimum factor levels. If the time is selected as 6h, it can be seen from Fig. 6 that the corresponding peptidase activity is 40.566U/mL, and this is within proximity to the predicted optimum value (39.519U/mL).

When the optimum peptidase activity of 49.172U/mL was compared to the optimum activity values of similar studies (Table 5), it was determined that a relatively high value was obtained even though the fermentation time was short. In this study, one unit of peptidase was equivalent to the amount of enzyme required to release 1mg of tyrosine/mL/min under standard assay conditions. On the other hand, in most of the other studies related to peptidase production of *Bacillus* species, one unit of peptidase was defined as the amount of enzyme required to release 1µg of tyrosine/mL/min under standard assay conditions. One of the highest activity values encountered for crude enzyme solution was 8320U/mL for *B. circulans* (Jordan) peptidase (Rao *et al*. 2007). When one unit of peptidase activity was evaluated in terms of 1µg tyrosine/mL/min released, the activity value for *B. amyloliquefaciens* FE-K1 peptidase was calculated as 45607U/mL which is almost 5.5-fold higher than the activity value of *B. circulans*. Furthermore, Rao *et al*. (2007) reached the mentioned value after 24 hour fermentation which is almost 4 times longer than the fermentation time of *B. amyloliquefaciens* FE-K1. Peptidase activity value of *B. amyloliquefaciens* FE-K1 in terms of 1µmol tyrosine/mL/min released is equal to 0.272U/mL which is lower than the activity value of 808.68U/mL determined by Jaswal *et al*. (2008) who used relatively longer fermentation time.

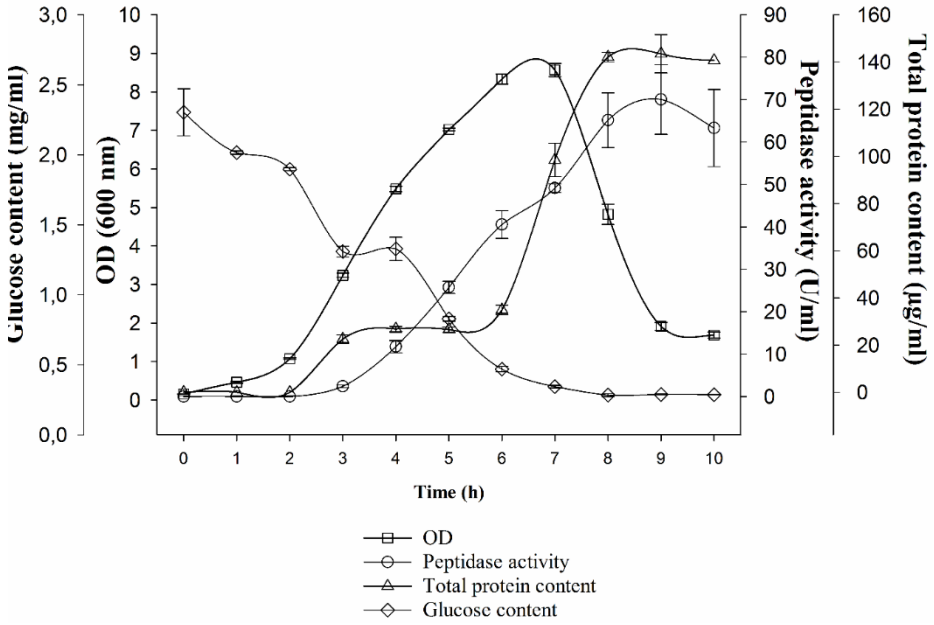

Fig. 6. OD, peptidase activity, total protein content and total glucose content variation with respect to the optimised factor levels.

Table 5. A comparison of results of some studies related to peptidase production by *Bacillus* species

Bacillus strain	Type of peptidase	Enzyme activity*	Fermentation time (h)	References**
Bacillus sp.	Alkaline	1939U/mL[a]	96	Puri *et al*. (2002)
Bacillus sp. RGR-14	Alkaline	3996U/mL[b]	96	Chauhan & Gupta (2004)
Bacillus sp.	Alkaline	222U/mL[a]	96	Gençkal & Tari (2006)
Bacillus sp.	Alkaline	2560U/mL[a]	48	Chu (2007)
Bacillus sp. HS08	Neutral	6804U/mL[a]	18	Guangrong *et al*. (2008)
Bacillus sp. RKY3	Alkaline	939U/mL[a]	24	Reddy *et al*. (2008)
Bacillus sp. PCSIR EA-3	Neutral	~0.7 U/mL[c]	48	Qadar *et al*. (2009)
Bacillus cereus SV1	Metallo-peptidase	5700U/mL[a]	48	Manni *et al*. (2008)
Bacillus circulans	Alkaline	8320U/mL[a]	24	Rao *et al*. (2007)
Bacillus circulans	Alkaline	808.68U/mL[c]	101	Jaswal *et al*. (2008)
Bacillus clausii GMBAE 42	Alkaline	45U/mL[a]	65	Denizci *et al*. (2004)
Bacillus licheniformis N-2	Alkaline	991U/mL[a]	24	Nadeem *et al*. (2008)
Bacillus licheniformis	Alkaline	185.40U/mL[a]	72	Lakshmi & Hemalatha (2016)
Bacillus megaterium	Alkaline	6.57U/mL[a]	15	Uttatree *et al*. (2017)
Bacillus mojavensis	Alkaline	2389U/mL[a]	12	Beg *et al*. 2003
Bacillus mojavensis SA	Alkaline	1467.27U/mL[a]	24	Hammami *et al*. (2018)
Bacillus subtilis FBL-1	Neutral	578.55U/mL[a]	36	Kim *et al*. (2016)

One unit of peptidase was defined as the amount of enzyme required to release a: 1µg of tyrosine/mL/min, b: 1mg of tyrosine/mL/min, c: 1 µmol of tyrosine/mL/min under standard assay conditions

* The given activity values are the maximum peptidase activity reached in crude enzyme solution.

**The studies were selected irrespective of whether they were carried out by using shaking incubator or bioreactor.

Conclusion

This study showed that *B. amyloliquefaciens* FE-K1 grew rapidly to a high OD level and then underwent cell lysis, most probably due to nutrient competition. Therefore, maximum peptidase activity before cell lysis was selected as the response for RSM, to eliminate intracellular peptidases. It was concluded that owing to the different and relatively short fermentation times (3-13h) encountered in this study, depending on the fermentation trials in the CCD, the present experimental design (selecting the peptidase activity of maximum OD level as response) was not an effective method to optimise the extracellular peptidase production of *B. amyloliquefaciens* FE-K1. Particularly, although a significant effect of temperature was evident from the raw data, determining the temperature as an insignificant factor for the peptidase production in the model, due to the different fermentation times, resulted in an incompatible model (R^2 = 72.34%) with a significant lack-of-fit value. However, despite the incompatible model, maximum peptidase activity (49.172U/mL) was obtained compared to the factor levels used in the CCD by applying the optimised factor levels determined by Minitab software according to RSM. This activity was relatively high compared to the literature data, even though the fermentation time was brief. If the cell lysis can be prevented, for instance, by conducting the fermentation in a fermenter, it is possible to improve the extracellular peptidase activity by increasing the fermentation time.

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