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THE OPTIMIZATION OF GROWTH CONDITION OF THE BACTERIA PRODUCING COLD-ACTIVE PROTEOLYTIC ENZYME FROM THE ANTARCTIC REGION

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ABSTRACT: The growth conditions of bacteria producing cold-active protease isolated from an Antarctic sample were screened using one-factor-at-time (OFAT). Then, crude protease of the strain was extracted during the late logarithmic phase for enzymatic assay. A strain that showed the highest enzyme activity was selected for optimization via response surface method (RSM). The parameters studied were incubation temperature (4 – 36 °C), pH media (4 – 10) and NaCl concentration (0 – 8%). Based on the OFAT results, all eight strains showed the highest growth rate at 20 °C, pH 7 and 4% (w/v) NaCl. The assay showed that the crude enzyme extracted from strain SC8 exhibited significantly higher activity (0.20 U and 0.37 U) than the positive control (0.11 U and 0.31 U) at -20 °C and 20 °C. RSM suggested that the optimized setting for growth of SC8 were at 20.5 °C, pH 6.83 and 2.05% (w/v) of NaCl with the results of the bacterial growth rate value was $3.70 \pm 0.06 \times 10^6$ cells/hr. Optimal growth conditions of SC8 from this study are useful for the large-scale production of cold-active protease in future.

ABSTRAK: Keadaan pertumbuhan bakteria yang menghasilkan enzim protease aktif sejuk daripada sampel Antartika disaring menggunakan satu faktor pada masa (OFAT). Kemudian, enzim protease ini diekstrak pada lewat fasa logaritma untuk ujian enzimatik. Strain yang menunjukkan aktiviti enzim tertinggi telah dipilih untuk tujuan pengoptimuman melalui kaedah permukaan tindak balas (RSM). Parameter yang dikaji ialah suhu pengeraman (4 – 36 °C), pH media (4 – 10) dan kepekatan NaCl (0 – 8%). Berdasarkan OFAT, kesemua lapan bakteria menunjukkan kadar pertumbuhan tertinggi pada 20 °C, pH 7 dan 4% NaCl. Hasil ujian enzimatik menunjukkan bahawa enzim protease yang diekstrak daripada SC8 mempamerkan aktiviti yang jauh lebih tinggi (0.20 U dan 0.37 U) daripada kawalan positif (0.11 U dan 0.31 U) pada -20 °C dan 20 °C.

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RSM mencadangkan tetapan optimum untuk pertumbuhan SC8 adalah pada 20.5 °C, pH 6.83 dan 2.05% NaCl dengan keputusan kadar pertumbuhan bakteria ialah 3.70 ± 0.06 x 10^6 sel/jam. Keadaan pertumbuhan optimum SC8 daripada kajian ini bermanfaat untuk menghasilkan produk protease aktif sejuk secara besar-besaran pada masa hadapan.

KEYWORDS: *cold-active protease; Antarctica; one factor at time; response surface method*

1. INTRODUCTION

Antarctica is located at the South Pole of the earth and acknowledged as one of the world's seven continents. This pristine place has recorded the lowest temperature on earth [1]. Compared to the Arctic, this continent encompasses solid land and is mostly covered with a thick snow layer. Roughly, the environment of the Antarctic is extremely cold and exposed to strong ultraviolet radiation [2]. Its sediment and soil contain a low level of carbon and nitrogen sources, contaminated with toxic metal compounds, fluctuating salt concentration, and exposure to low oxygen levels [3-5]. Although it looks like deserted terrain, a diverse microbial community has been discovered from its snows, soil, and sediment [6,7]. Recently, several novel environmental bacteria species have been identified such as *Paenisporosarcina antarctica* [8], *Labilibaculum antarcticum* [9] and *Pseudomonas fildesensis* [10].

Throughout history, many conventional methods used catalysts to speed up the process, either chemically or biologically. Nowadays, bio-catalyst-like enzymes have been accepted by communities. It has been widely used in daily life and industry. The discovery of novel enzymes has replaced the former catalyst and provides more convenient, eco-friendly and cost-effective processing. Several enzymes have been successfully extracted from microorganisms originated from the Antarctica regions [11-13]. One of the important enzymes is a protease. Generally, this enzyme breaks down proteins or polypeptides into smaller subunits or single amino acids. Microbial proteases have been widely used in many different industries including poultry, detergent, food and feed, leather, chemicals, waste management, medical, and research [14-16].

Cold-active protease is an extremozyme that enables a system to run at lower temperature where the majority of the commercial or industrial enzymes require higher temperatures for efficient catalytic activity and denaturation process. Consequently, introducing higher temperatures will always initiate an undesirable chemical reaction and loss of volatile compounds. Thus, cold-active protease has potential as an alternative to overcome these limitations. However, isolating and growing microorganisms in the laboratory directly from a sample is very challenging, especially samples from an extreme environment. A lot of factors must be considered, and the best idea is to mimic the sample environment to prepare the media conditions. In short, optimization of several parameters is important to provide ideal growing conditions for the isolated bacteria. The basic parameters used in this study was incubation temperature, pH media, and salt concentration. Based on previous studies, bacteria from the Antarctic region at 4 - 36 °C, pH 4 - 10 and 0 - 8% NaCl concentration was isolated [17]. Experimental design for optimization was conducted through response surface method (RSM). Initially, RSM was introduced by Box & Wilson [18] as a design of experiment for chemical processing and nowadays, the method has been widely applied in many areas. This system is supported by its regression analysis and allows us to investigate the effect and interaction of multiple variables on one or more responses [19]. In this study, we analyzed the growth conditions of isolated bacteria producing cold-active proteolytic enzymes from the Antarctic region.

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2. MATERIALS AND METHODS

2.1 Determination of Bacteria Growth Phase

Previously, several bacteria that showed positive activity of cold-active extracellular protease on skim milk agar were isolated and stored in glycerol [20]. Samples BB and ROB were collected from moss communities, sample ROS was moist soil covered with alga and cyanobacterial mats, and sample SC was composed at an abandoned skua nest. The parameters to be characterized for bacteria growth rate were incubation temperature, pH media, and sodium chloride concentration. For every experiment, the bacteria were cultured in 10 mL of preferred LB broth with the initial OD_{600} set at 0.1 in a sterile 50 mL tube [21]. The culture was incubated for 24 hours with an orbital shake at 200 rpm. A preliminary experiment disclosed that the strain reached the late logarithmic phase after a 24-hour incubation period. Later, bacteria were harvested during this phase to be incubated in skim milk media for enzymatic assay. After 24 hours incubation period, the final OD_{600} of the culture bacteria was measured and the growth rate (cells x 10^6 /hr) was calculated based on the Eq. (1):

$$\text{Growth rate} = \frac{\text{OD (final)} - \text{OD(initial)}}{24 \text{ hours}} \text{ x } 5{,}85 \text{ x } 10^7 \text{ cells} \quad (1)$$

2.1.1 Effect of Temperature

The first parameter to be characterized for bacteria growth rate was temperature while fixing the other parameters. The bacteria-producing cold-active proteolytic enzyme was incubated in LB broth at three different temperatures, 4 °C, 20 °C and 36 °C. The NaCl concentration and pH of the media were maintained at 1% (w/v) and 7, respectively.

2.1.2 Effect of pH

Different pH, i.e. 4, 7, and 10 of LB broth were prepared and autoclaved. The pH of the media was adjusted using HCl and KOH. Then, these media were used to characterize the growth rate of isolated strains. Based on temperature optimization results, these bacteria were incubated at 20 °C and the NaCl concentration of the media was kept constant at 1%.

2.1.3 Effect of NaCl Concentration

The last parameter to be characterized for bacteria growth rate was NaCl concentration. LB broth media with 0%, 4%, and 8% of NaCl concentration were prepared and autoclaved. The pH of this media was maintained at 7 and the bacteria culture was incubated at 20 °C.

2.2 Proteolytic Assay

Azo-casein (Sigma Aldrich) was used as a substrate to quantify the protease activity enzymatic assay as decribed by García-Cano et al. [22]. For the enzymatic assay, 250 µL of 1% azocasein, 250 µL of 0.5 M Tris and 250 µL of the crude enzyme from culture supernatant were added into 1.5 mL sterile tube and incubated at –20 °C, 20 °C, and 60 °C. Before adding the crude enzyme, the assay was pre-incubated at mentioned temperatures for 10 minutes. For positive control, 0.2 units/mL of protease from the bovine pancreas (optimum temperature at 37 °C) was used. After 20 minutes, 500 µL of 25% of trichloroacetic acid (TCA) was added to stop the reaction and centrifuged at 12,000 rpm for 30 minutes. This supernatant was transferred into a 1.5 mL cuvette and the absorbance was read at 340 nm. For blanks, only 250 mL of 1% azocasein and 250 µL of 0.5 M Tris were incubated before adding 500 µL of 25% TCA. Then, 5 % of skim milk

media were added and centrifuged at 12,000 rpm for 30 minutes. The supernatant was transferred into a new cuvette and labelled as blank. The data represented as enzyme unit mean ± standard deviation (STDEV). Statistical analysis was calculated using one-way analysis of variance (ANOVA), SPSS 26.0. The results were considered significant differences if the p-value was < 0.05.

2.3 Response Surface Method (RSM)

The bacteria strain that showed the fastest shifting of clear skim milk media and highest protease activity were selected and preceded with an optimization process using response surface method (RSM) [23]. The experiment was designed based on three-level parameters of face centered central composite design (FCCCD) as in Table 1. The response variable was the growth rate of the bacteria. The initial and final OD_{600} of bacteria cultures were recorded and one unit of OD_{600} corresponded to 5.85 x 10^7 cells. The culture was incubated for 24 hours with an orbital shake at 200 rpm. A total of 20 experimental runs were generated by the software Design-Expert® Version 7.0.0 (State-Ease Inc., Minneapolis, MN) with six runs at center points included.

Table 1: Independent variables and their corresponding levels for FCCCD in a response to bacteria growth rate

Factor	Variables	Level		
		-1	0	1
A	Temperature [°C]	4	20	36
B	pH	4	7	10
C	Sodium chloride concentration [%, w/v]	0	4	8
Response	Growth Rate [A/hr] [cells x 10^6 cells/hr]			

3. RESULTS

From the previous study, the total amount of bacteria producing extracellular protease isolated from Antarctica's soils and sediments were 35 strains [20]. These strains were further tested on their capability to produce extracellular protease within 24 hours through halo zone formation around the colony on skim milk agar. Therefore, 8 bacteria strains consisting of BB1, DI25, ROB8, ROS7, ROS8, SC8, S10, and SC11 were selected for further analysis for their growth conditions. Experimental designs to analyze the growth rate conditions for each strain were carried out in a conventional one-factor-at-a-time (OFAT) approach. This approach was performed by altering one parameter at a time and maintaining the other parameters, so that the impact of the altering parameters can be accessed.

3.1 Effect of Temperature on Bacteria-producing Cold-active Proteolytic Enzyme

From the graph in Fig. 1, the optimum temperature for growth rates for all bacteria was 20 °C. There was a significant difference observed in all isolated strains at different temperatures. All bacteria grew faster at 20 °C compared to 4 °C and 36 °C.

3.2 Effect of pH on Bacteria-producing Cold-active Proteolytic Enzyme

The second factor, pH has been found as the important factor affecting the bacteria growth rate. Based on the graph in Fig. 2, all strains showed the highest growth rate at neutral pH 7. Besides, several strains can tolerate lower pH or acidic conditions such as BB1, ROB1, ROS7, ROS8, SC8, SC10 and SC11.

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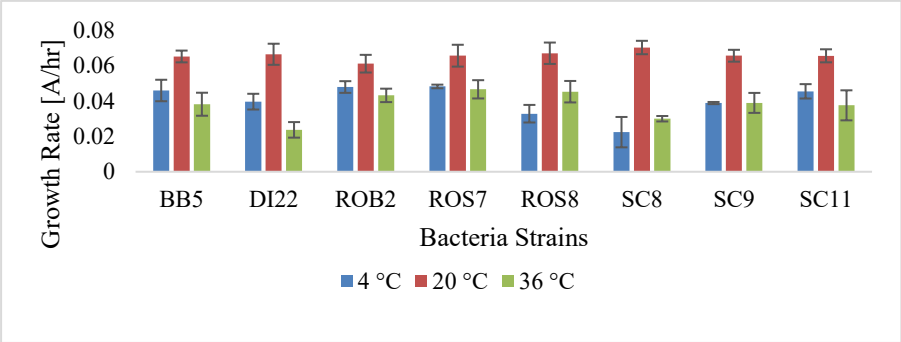

Fig. 1: Effect of different temperature levels to bacteria growth rate. The pH of the media and NaCl concentration were maintained at 7 and 1%, respectively.

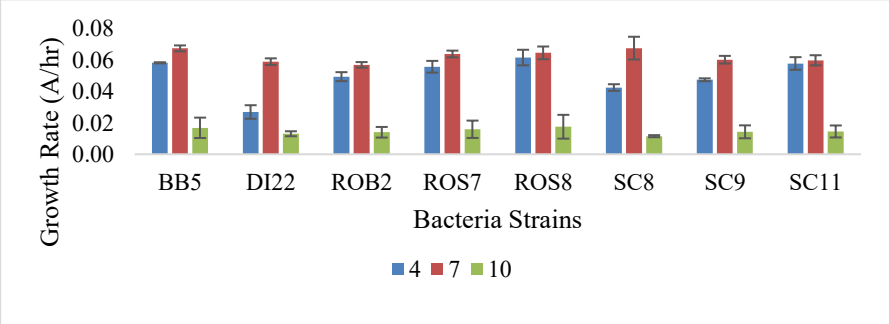

Fig. 2: Effect of different level of pH in a response to the bacteria growth rate. The incubation temperature and NaCl concentration were maintained at 20 °C and 1%, respectively.

3.3 Effect of NaCl Concentration on Bacteria-producing Cold-active Proteolytic Enzyme

The concentration of sodium chloride (NaCl) was considered an important factor as the coastal environment influenced the sample. All bacteria strains showed an optimum growth rate at 4% NaCl concentration as in Fig. 3. In the absence of NaCl, the slower growth rate of these bacteria compared to 4% NaCl was observed. However, with an increase in the concentration of NaCl from 4% to 8%, the growth rate of the bacteria was significantly reduced.

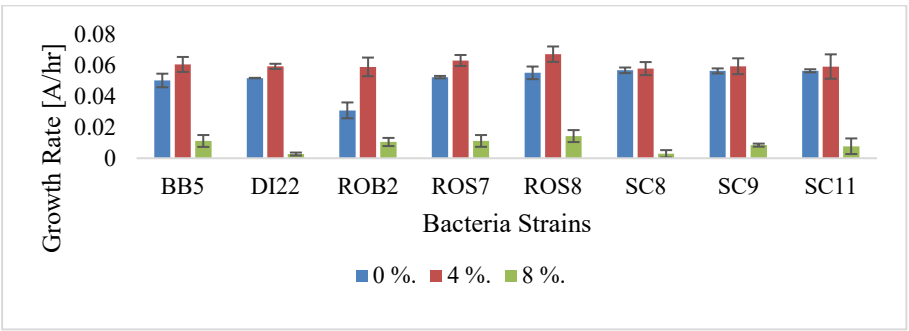

Fig. 3: Effect of NaCl at different levels to the bacteria growth rate. The incubation temperature and pH of the media were maintained at 20 °C and 7, respectively.

3.4 Proteolytic Assay

Change of white skim milk media to clear indicated that the bacteria produced extracellular protease to degrade the casein of milk. From the graph in Fig. 4, the crude enzyme extracted from strains SC8 and ROS8 showed higher proteolytic activity compared to other isolated bacteria. This indicates that the amount of protease produced was higher from both strains. In addition, both crude enzymes showed a significantly higher activity at -20 °C compared to the positive control.

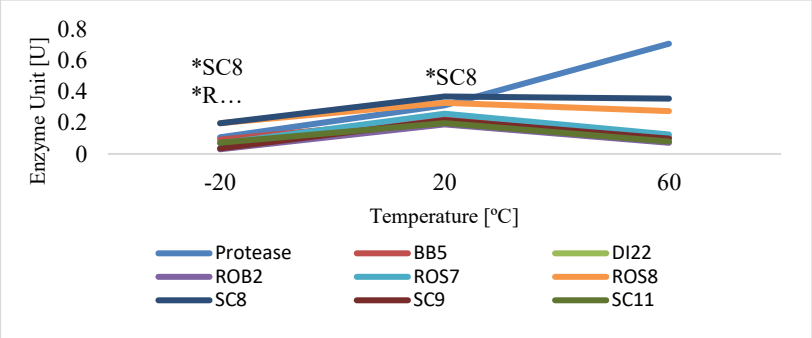

Fig. 4: Results of crude protease assay at three different temperatures. SC8 showed the highest proteolytic activity at the lowest temperature. * $p < 0.05$

3.5 Optimization of Growth Conditions of Bacteria Producing Cold-active Proteolytic Enzyme by Response Surface Methodology (RSM)

The growth rate of isolated bacteria SC8 was selected to be further optimized using Response Surface Method (RSM). This strain showed the fastest shifting of clear skim milk media and the highest proteolytic activity at the lowest temperature. The result of RSM is shown in Table 2. From the results, the bacteria growth rate was varied from 0.014 x 10^6 to 3.93 x 10^6 cells/hr.

Table 2: Experimental design and results of face centered central composite design (FCCCD). The experimental growth rate data represented as mean ± standard deviation (STDEV).

Treatment Number	NaCl [%, w/v]	pH	Temp. [°C]	Response Growth Rate [A/hr]	Bacterial Growth Rate [cells x 10^6/hr]
1	0	4	4	0.0469 ± 0.0005	2.74 ± 0.03
2	8	4	4	0.0242 ± 0.0204	1.42 ± 1.19
3	0	10	4	0.0385 ± 0.0002	2.25 ± 0.01
4	8	10	4	0.0195 ± 0.0009	1.14 ± 0.05
5	0	4	36	0.0408 ± 0.0003	2.39 ± 0.02
6	8	4	36	0.0305 ± 0.0009	1.78 ± 0.05
7	0	10	36	0.0425 ± 0.0007	2.49 ± 0.04
8	8	10	36	0.0275 ± 0.0018	1.61 ± 0.12
9	0	7	20	0.0597 ± 0.0100	3.49 ± 0.59
10	8	7	20	0.0506 ± 0.0211	2.96 ± 1.23
11	4	4	20	0.0525 ± 0.0008	3.07 ± 0.05
12	4	10	20	0.0563 ± 0.0008	3.29 ± 0.05
13	4	7	4	0.0476 ± 0.0046	2.78 ± 0.27
14	4	7	36	0.0541 ± 0.0198	3.16 ± 1.16
15	4	7	20	0.0671 ± 0.0082	3.93 ± 0.48
16	4	7	20	0.0604 ± 0.0077	3.53 ± 0.46
17	4	7	20	0.0576 ± 0.0073	3.37 ± 0.43
18	4	7	20	0.0661 ± 0.0070	3.87 ± 0.41
19	4	7	20	0.0563 ± 0.0079	3.29 ± 0.46
20	4	7	20	0.0632 ± 0.0069	3.70 ± 0.40

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Preliminary analysis showed that the highest growth rate was at the center of the design. Based on the fit summary, the model suggested was quadratic because the sequential model sum of squares (Type I) was significant and the lack of fit tests was insignificant. An insignificant lack of fit was good because the model should be fitted. The value of Prob > F less than 0.05 indicated that the model terms were significant. From the analysis of variance (ANOVA) in Table 3, for the response surface quadratic model, the model F-value of 45.87 implied the model was significant. In this experiment, the concentration of NaCl (A), temperature (C) and algebraic contribution (A2, B2, C2) were significant model terms. The multiple correlation coefficient of the model or R2 of 0.9574 meant that the regression prediction was capable of estimating 95 % fit to the actual data points. Besides, the predicted R-squared of 0.9191 was in reasonable agreement with the adjusted R-squared 0.8051 because the difference was less than 0.2. The adequate precision greater than 4 (14.948) indicated an adequate signal. The final equation in terms of coded factors was generated as Eq. (2):

$$\begin{aligned}\text{Growth rate} = {} & 0{,}0622 - 0{,}0076\,\text{A} - 0{,}0011\,\text{B} + 0{,}0019\,\text{C} - 0{,}0001\,\text{AB} \\ & + 0{,}0021\,\text{AC} + 0{,}0015\,\text{BC} - 0{,}0077\,\text{A2} - 0{,}008487\,\text{B2} \\ & - 0{,}0120\,\text{C2}\end{aligned} \tag{2}$$

Table 3: Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of Squares	Degree of Freedom	F-Value	p-value
Model	2.096E-003	9	34.23	< 0.0001
A-NaCl	1.568E-004	1	23.05	0.0020
B-pH	6.300E-005	1	9.26	0.0188
C-Temp	2.704E-006	1	0.40	0.5484
AB	1.378E-005	1	2.03	0.1977
AC	1.513E-007	1	0.022	0.8857
BC	8.001E-005	1	11.76	0.0110
A^2	3.110E-005	1	4.57	0.0698
B^2	3.335E-004	1	49.02	0.0002
C^2	2.791E-004	1	41.02	0.0004
Residual	4.763E-005	7		
Lack of Fit	3.570E-005	5	1.20	0.5135
Pure Error	1.193E-005	2		
Cor Total	2.144E-003	16		

The graphs were constructed by plotting the response against any two independent variables while maintaining the other variable at the optimal level. For this experiment, as in Fig. 5, contour and the 3D surface graph showed the bacterial growth rate as a response was plotted at the z-axis, meanwhile, the x- and y-axis were assigned for two different variables. Higher growth rate was observed around 2% NaCl at pH 7 (Fig. 5A), 2% NaCl at 20 °C (Fig. 5B) and at pH 7 and 20 °C (Fig. 5C).

For optimization, the criteria were selected to maximize the bacterial growth rate while keeping all the variables in the range. The solution suggested by the design expert software to optimize the bacterial culture conditions were concentration of sodium chloride at 2.05% (w/v), pH of the solution at 6.83, and the incubation temperature at 20.5 °C. New experiments were conducted as post-analysis to validate these statistical models and regression equations. The experiments were run in triplicate under optimized variables as suggested previously. The predicted bacterial growth rate calculated by the software under these optimized variables was 3.75 x 10^6 cells/hr and the average observed experimental value was 3.70 ± 0.06 x 10^6 cells/hr. From these validation results, it

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confirmed that the model was good enough because the error between predicted and actual value was only 1.2 %.

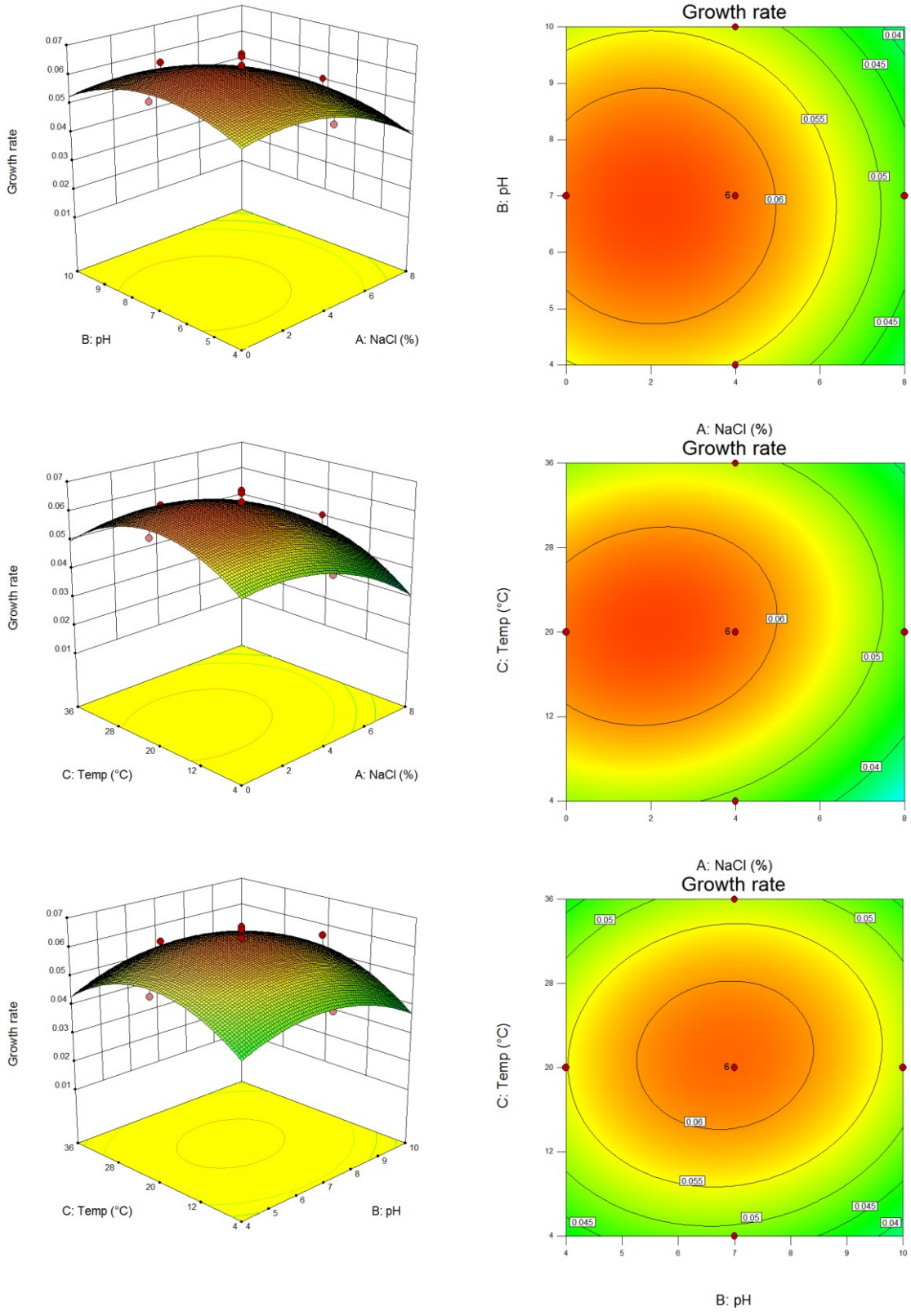

Fig. 5: 3D surface and contour graph showed the interaction of three independent variables with the response to the bacteria growth rate.

4. DISCUSSION

Generally, psychrophiles are microorganisms that can live under very low temperatures. The majority of these extremophilic organisms inhabit a permanently ice-covered environment on Earth. The word 'living' means that they can grow and reproduce under extremely low temperatures rather than hibernate. Some researchers characterized these psychrophiles into two groups: obligate psychrophiles and psychrotolerant organisms. Obligate psychrophiles referred to organisms with an optimum growth temperature of 15 ºC that cannot withstand higher temperatures of more than 20 ºC [24]. Psychrotolerants are organisms that can withstand a broader range of temperatures between 0 °C to approximately 36 °C [25]. Some researchers called them facultative psychrophiles or psychrotrophs and their maximal or optimal growth temperature was above 20 ºC [26,27]. Based on the OFAT, all isolated strains showed the fastest growth rate at 20 °C. These bacteria can be classified as psychrotolerant because they can live in a wide range of temperatures (4 – 36 °C). Besides, these bacteria can tolerate up to 4 % NaCl concentration indicating that their living environment was influenced by the coastal environment. On average, seawater contains a salt concentration of about 35 parts per thousand or about 3.5 % and the major sources of the salt are sodium and chloride [28]. Wind effect brought seawater onto the ice and during the summer season, the ice melted and the salt was concentrated in the soils and sediments [29]. Furthermore, the majority of the isolated strains can endure acidic conditions. This is possible because these strains were influenced by the surrounding environment at the collection point. Researchers have reported that lower pH on Antarctic soils and sediments from moss communities and wetlands were due to the accumulation of organic matter [30]. Besides, birds' nests and excrement created in situ unfavorable chemical reactions that led to acidic conditions of the soil and sediment [31].

An interesting aspect of these psychrophiles is their survivability is supported by the cold-adapted enzyme. They produce biocatalysts that are capable of working at very low temperatures. There were several theories on this unique characteristic of enzymes and the majority of the researchers claimed that flexibility of the protein structure is the main contributor [32]. Comparison studies between psychrophilic and mesophilic enzymes showed that structural modification and adaptation remain obscure although both active sites are preserved [30]. Some researchers claim that the amino acid composition affects the bonding between residues and the structural rigidity of the protein [22]. Besides, loop modification vicinity to active site matched to the induced fit model for enzymatic reaction [24]. These strategies were designated to consume less energy during enzymatic reactions when a smaller amount of heat is available at low temperatures. In this case, crude enzymes extracted from SC8 and ROS8 strains showed significantly higher protease activity than positive control at a lower temperature but gradually decreased at the higher temperature. This showed that increasing the flexibility of the enzyme adversely affected its thermostability [35]. Cold active proteases have been extracted from various species of psychrophiles and the majority showed optimal activity at a temperature range between 30–40 ºC [36-39]. A thorough search on cold-active protease yielded the lowest optimal temperature at only 15 ºC [40,41].

In this experiment, strain SC8 showed the capability to produce extracellular protease faster and higher compared to the other strains. It has a high potential for bioprospecting in future. Thus, we focused on this strain for growth rate optimization. Initially, the term optimization for the OFAT method was not accurate according to some scholars [42,43]. This is because changing one variable at a time could not estimate the interaction between

variables and might miss the optimal setting for each variable [44]. Frequently, the OFAT technique was used in the experiment objectively to identify the maximal or minimal effect [45]. Maximal effect is the highest or greatest result's magnitude whereas, the optimal effect is the most desirable condition for the model. Yet, results from OFAT could be applied as a screening method to determine significant high and low levels for each variable [46,47]. Although the factorial method is best used as screening design in Design-Expert software before optimization using the response surface method, this OFAT technique is lower in cost and easier to conduct. In this experiment, based on the RSM result in Table 2, the fastest growth rate, 3.93 x 10^6 cells/hr, was treatment number 15 with variables set at 4% NaCl, pH 7, and 20 ºC. However, the optimized growth rate-setting suggested was slightly different from the fastest growth rate setting. Clarification on these results was RSM calculated the optimal setting for each variable or resource in the design to achieve maximized results. Based on the validation results, the optimum bacterial growth rate at 2.05% of NaCl, pH 6.83 and 20.5 °C was 3.70 x 10^6 cells/hr and it was only 5% to achieve the fastest result, 3.93 x 10^6 cells/hr. Therefore, this model could achieve a higher response and save the usage of NaCl by using the optimal setting recommended by RSM.

5. CONCLUSION

In conclusion, the isolated strains of bacteria-producing cold-active protease from the Antarctica region in this experiment could be categorized as psychrotolerants. Based on the crude enzymatic assay, the SC8 strain showed potential for bioprospecting as its protease activity was significantly higher than positive control and other strains at a lower temperature. Optimal growth conditions of SC8 strain from this study will be useful for the development of large-scale production of cold-active protease in future. In the food industry, this cold active protease may act as the commercial meat tenderizers (papain and bromelain) as it is applied to the meat during cold storage and export journey.

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