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Enhanced production of protease by *Pseudoalteromonas arctica* PAMC 21717 via statistical optimization of mineral components and fed-batch fermentation

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ABSTRACT

The objective of this study was to statistically optimize the mineral components of the nutritional medium required for enhancing the production of a cold-active extracellular serine-type protease, W-Pro21717, by the Antarctic bacterium *Pseudoalteromonas arctica* PAMC 21717. Skim milk was identified as the major efficient inducer. Among the 12 components included in the unoptimized medium, skim milk, NaCl, Na_2SO_4, $Fe(C_6H_5O_7)$ (ferric citrate), and KCl were determined, by the Plackett–Burman and Box–Behnken design, to have a major effect on W-Pro21717 production. Fed-batch fermentation (5 L scale) using the mineral-optimized medium supplemented with concentrated skim milk (critical medium component) resulted in a W-Pro21717 activity of 53.4 U/L, a 15-fold increment in production over that obtained using unoptimized flask culture conditions. These findings could be applied to scale up the production of cold-active protease.

KEYWORDS

Fermentation; *Pseudoalteromonas arctica* PAMC 21717; psychrophile; statistical mineral optimization; W-Pro21717

Introduction

Extracellular enzymes play decisive roles in the circulation of organic matter in polar environments.[1,2] These cold-active enzymes display high catalytic competency at low temperatures, and can be applied in the agricultural, energy, food, medical, structural material, and textile industries.[3,4] Among the various industrial enzymes, proteases constitute a major portion of the enzyme market.[4–6] Many types of proteases have been identified, including the serine (EC 3.4.21.), sulfhydryl (EC 3.4.22), aspartic (EC 3.4.23), and metallo (EC 3.4.24) proteases.[7] Alkaline proteases (EC 3.4.21–24, -99) are active in a neutral to alkaline pH range. Alkaline serine proteases, specifically, have a wide range of applications in molecular biology, detergent preparation, and the leather and food industries.[8–10] Recent studies have demonstrated the increased interest in cold-active proteases, and various protease-producing psychrotrophic microorganisms have been identified.[5] The optimal growth conditions of these microorganisms must be determined in order to ensure a good yield for industrial applications. The production of extracellular proteases is influenced by nutritional factors, such as the carbon and nitrogen sources, and inorganic salt content; casein, citrate sodium, and Tween-80 were selected as optimal media constituents for the growth of *Colwellia* sp. NJ341, and Zn^{2+} and Cr^{2+} enhanced the protease production in *Curtobacterium luteum*.[11,12] As all psychrotrophic microorganisms grow in specific optimized media, it is necessary to investigate the optimal conditions for the production of the cold-active protease of interest.

An extracellular cold-active serine-type protease, W-Pro21717, was isolated and purified in our laboratory from the Antarctic bacterium *Pseudoalteromonas arctica* PAMC 21717.[13] This study attempted to investigate the effects of medium composition on the production of wild-type W-Pro21717, and to develop the optimal conditions for enhanced protease production in this bacterium. The optimization of medium using the classical method, varying one independent factor at a time while keeping the other variables constant, is slow and ignores the interactions among the various factors. Statistical design, such as the response surface methodology and the two-level factorial design strategy, has proven to be efficient for culture medium optimization.[14–18] Therefore, we conducted a statistical experimental design in order to identify the best mineral components in the medium. In this study, we have described a statistical approach and batch and fed-batch fermentations to increase the productivity of cold-active W-Pro21717.

Materials and methods

Strain and media

The bacterium *P. arctica* PAMC 21717, which produces the protease W-Pro21717, was isolated from a soil sample collected from the Barton Peninsula (S 62°13′, W 58°47′) on King George Island, Antarctica. The seed culture was obtained by inoculating cells in a flask containing Zobell's medium (peptone, 5 g/L; yeast extract, 1 g/L; $FePO_4 \cdot 4H_2O$, 0.01 g/L; seawater 750 mL/L; pH adjusted to 7.0–7.2; prepared from a glycerol stock), and incubating these for 24 hr at 15°C.[19] The primary culture medium was inoculated with the seed culture (10%) in order to facilitate cell growth and production of cold-active protease. The unoptimized Marine Broth basal medium was composed of $Fe(C_6H_5O_7)$ (ferric citrate)

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(0.1 g/L), NaCl (19.45 g/L), $MgCl_2$ (5.9 g/L), Na_2SO_4 (3.24 g/L), $CaCl_2$ (1.8 g/L), KCl (0.55 g/L), $NaHCO_3$ (0.16 g/L), KBr (0.08 g/L), and a trace metal solution (1 mL/L composed of $FePO_4$, 10.0 g/L; $SrCl_2$, 34.0 g/L; H_3BO_3, 22.0 g/L; Na_2HPO_4, 8.0 g/L; Na_2SiO_3, 4.0 g/L; NaF, 2.4 g/L; and NH_4NO_3, 1.6 g/L).[20] The medium was optimized using powdered skim milk (Becton, Dickinson and Company, Franklin Lakes, NJ), various carbon sources, and complex nitrogen sources. The cultures were prepared in 250-mL Erlenmeyer flasks with 50 mL culture medium, unless otherwise stated. Growth of *P. arctica* PAMC 21717 was evaluated by measurement of the optical density at 600 nm (OD_{600}) of the culture, using a spectrophotometer (S-3100; Scinco, Seoul, Korea).

Selection of suitable carbon source

The carbon source effects on liquid cultures of *P. arctica* PAMC 21717 were investigated using 10 g/L each of glucose, galactose, fructose, lactose, sucrose, maltose, glycerol, starch, and cellulose, in the presence or absence of 10 g/L of skim milk as an induction agent for protease production.[21,22] The cells were grown in medium containing 10 g/L each of the different carbon sources. The cell density and W-21717 activity were measured after 3 days of cultivation at 15°C.

Selection of suitable complex nitrogen source

The identification of the suitable complex nitrogen source was performed by growing the cells in unoptimized medium supplemented with 0.6 g/L each of peptone (enzymatic digest of animal protein), yeast extract (extract of autolysed yeast cells), tryptone (pancreatic digest of casein), soy peptone (enzymatic digest of soybean flour), and tryptic soy broth (soybean–casein digest broth), in the presence of skim milk.[22,23] These cells were incubated for 3 days at 15°C, following which the cell density and W-Pro21717 activity were measured.

Identification of significant mineral components

The medium components that wielded considerable influence on the production of W-Pro21717 from *P. arctica* PAMC 21717 were determined by Plackett–Burman design.[24] Based on this design, 12 components of the unoptimized Marine Broth, and the selected carbon and nitrogen sources and skim milk, were considered at two levels (Table 1): low (−) and high (+) concentrations. This resulted in a first-order model, $\mathbf{Y} = \beta_0 + \Sigma\beta_i\mathbf{X}_i$, where $\mathbf{Y}$ is the predicted response (W-Pro21717 production), β_0 is the model intercept, β_i is the linear coefficient, and $\mathbf{X}_i$ is the value of the independent factor. The various interactions among the factors (medium components) were not described in this model, as it was only used to screen the significant variables influencing the response.

Determination of the concentration of selected mineral components

The concentrations of $Fe(C_6H_5O_7)$, NaCl, Na_2SO_4, and KCl, which were previously chosen using the Plackett–Burman design, were optimized using a Box–Behnken design (Table 2).[25] Each component was quantified into three levels: (−), (0), and (+), for low, intermediate, and high concentrations, respectively. The optimal mineral component concentrations were predicted using a second-order equation, which depicted the relationship between the independent factors (mineral components) and the response: $\mathbf{Y} = \beta_0 + \Sigma\beta_i\mathbf{X}_i + \Sigma\beta_{ij}\mathbf{X}_i\mathbf{X}_j + \Sigma\beta_{ii}\mathbf{X}_i^2$, where $\mathbf{Y}$ is the predicted response (W-Pro21717 production), β_0, β_i, β_{ij}, and β_{ii} are the constant and regression coefficients of the model, and $\mathbf{X}_i$ and $\mathbf{X}_j$ are the values of independent variables. Minitab (ver. 14; Minitab Inc., State College, PA) was used for the design.

Batch and fed-batch fermentations

The batch and fed-batch cultures were prepared using mineral-optimized medium in a 5-L jar fermenter (Minifors, Infors AG, Bottmingen, Switzerland) at 15°C. The batch cultures were performed using different media conditions in each step, in order to identify the enhancing step. All fermentation steps were carried out using 2 L of the optimized medium (initial pH, 6.8), with shaking at 100–200 rpm.

The cells in the fed-batch culture were initially grown for 18 hr in the batch mode. Subsequently, a dissolved oxygen (DO)-stat fed-batch culture was prepared by the addition of skim milk (100 g/L) and tryptone (6 g/L). Samples were obtained every 12 or 24 hr, until the fed-batch fermentation was stopped (after 144 hr of culture). The fed-batch fermentation using the DO-stat was performed according to the

Table 1. Statistical analysis of medium components using the initial Plackett–Burman experiment.

Variable	Mineral component	−Value (g/L)	+Value (g/L)	Effect[a]	*t* statistic[b]	*p* value[c]
X_1	$Fe(C_6H_5O_7)$	0.01	0.1	1.632	2.13	0.037
X_2	NaCl	1.945	19.45	3.520	4.59	0.000
X_3	$MgCl_2$	0.59	5.9	−1.142	−1.49	0.142
X_4	Na_2SO_4	0.324	3.24	2.352	3.06	0.003
X_5	$CaCl_2$	0.18	1.8	0.841	1.10	0.277
X_6	KCl	0.055	0.55	1.406	1.83	0.072
X_7	$NaHCO_3$	0.016	0.16	0.687	0.90	0.374
X_8	KBr	0.008	0.08	0.803	1.05	0.299
X_9	Trace elements	0.0082	0.082	−2.718	−3.54	0.001
X_{10}	Skim milk	1.0	10	3.994	5.20	0.000
X_{11}	Fructose	1.0	10	−2.952	−3.85	0.000
X_{12}	Tryptone	0.06	0.6	0.091	0.12	0.906

[a,b]The "effect" and "*t* statistics" indicate whether or not the difference between the averages of two groups is large enough to have practical meaning (whether or not it is statistically significant).

[c]The medium components having a p value < 0.1 were considered to be factors significantly affecting W-Pro21717 production.

Table 2. Box–Behnken optimization of selected significant mineral components.

Variable	Mineral component	−Value (g/L)	0 Value (g/L)	+Value (g/L)
X_1	$Fe(C_6H_5O_7)$	0.02	0.11	0.2
X_2	NaCl	3.89	21.395	38.9
X_3	Na_2SO_4	0.648	3.564	6.48
X_4	KCl	0.11	0.605	1.1

following standard: When the DO values dropped below the set point of 30% air saturation, the feeding was stopped until the recovery of DO to more than 30% air saturation.

Analytical methods

The W-Pro21717 activity in the broth was measured using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA, Sigma-Aldrich, Inc., St. Louis, MO) as a substrate. The cultured broth was centrifuged (at 12,000 × g for 5 min) and the supernatant was used as the enzyme (W-Pro21717) solution. At least 3 different volumes of the W-Pro21717 solution (10–100 μL) were diluted up to 1 mL using the standard buffer containing the substrate (final volume: 10 m*M* of sodium phosphate buffer [pH 7.5], 1 m*M* of substrate). These reaction mixtures were incubated at 37°C for 10 min. The amount of *p*-nitroaniline produced as a result of enzyme activity was calculated by measurement of the OD_{410}, and applying a molar extinction coefficient of 8,800 M^{-1} cm^{-1}. One unit of enzyme activity per liter (1 U/L) was defined as the amount of enzyme required to hydrolyze 1 μmol substrate to *p*-nitroaniline per minute per culture volume.

Results

Selection of the suitable carbon source

The carbon source suitable for the production of W-Pro21717 by flask-cultivated *P. arctica* PAMC 21717 was selected by investigating nine different carbon sources (10 g/L). Most carbon sources expressed a positive effect on the cell concentration, whereas cellulose negatively affected cell concentration in the absence of skim milk (Figure 1A). Glucose, galactose, fructose, lactose, and maltose positively influenced the production of W-Pro21717, whereas sucrose, glycerol, starch, and cellulose appeared to inhibit protein production. Only fructose and lactose positively affected W-Pro21717 production in the presence of 10 g/L of skim milk (Figure 1B). W-Pro21717 activities in culture media using fructose as the carbon source were 3.4 and 8.0 U/L in the absence and presence of skim milk, respectively. This indicated an approximately 2.4-fold higher enzyme activity in the presence of skim milk. Therefore, fructose was chosen as the carbon source for all subsequent experimentations.

Selection of the suitable complex nitrogen source

The serine protease, W-Pro21717, was produced in a basal medium supplemented with skim milk (10 g/L; protease inducer) and a variety of complex nitrogen sources, including peptone, tryptone, yeast extract, soy peptone, and tryptic soy broth. The addition of tryptone in the presence of skim milk resulted in an increase in W-Pro21717 activity from 4.4 to 7.5 U/L (approximately 1.7-fold higher enzyme activity; Figure 2). Therefore, tryptone was selected as the complex nitrogen source for W-Pro21717production in flask medium.

Determination of significant components in the medium

In order to exclude the medium components that do not supplement W-Pro21717 production, all 12 different compounds used in the preparation of the marine broth basal medium were investigated. The results of the tested values, effects, *t*-statistics, and *p* values for all medium components are listed in Table 1. The medium components with a *p* value <0.1 were accepted as factors significantly affecting W-Pro21717 production. $MgCl_2$, $CaCl_2$, $NaHCO_3$, KBr, and tryptone did not significantly affect protease production ($p > 0.1$). Two medium components, trace elements and fructose, were excluded from further analyses owing to their negative effects on the production of W-Pro21717. However, $Fe(C_6H_5O_7)$, NaCl, Na_2SO_4, KCl, and skim milk positively affected the production of W-Pro21717 ($p < 0.1$). Skim milk was determined to be an essential source of carbon and nitrogen and an inducing agent for the production of W-Pro21717; consequently, $Fe(C_6H_5O_7)$, NaCl, Na_2SO_4, and KCl were chosen as the mineral sources significantly affecting W-Pro21717 production (Table 1).

Production of W-pro21717 by mineral component optimization

The mineral components significant for W-Pro21717 production were further optimized by Box–Behnken design. Table 2 shows the tested levels of mineral components for Box-Behnken design, calculated using the Minitab software (ver. 14; Minitab Inc., State College, PA).

The effects and interactions of the significant mineral components that influence W-Pro21717 production are displayed in three-dimensional plots (Figure 3). Eventually the following second-order equation was obtained:

$$\begin{aligned} Y = &-1.65 + 47.98X_1 + 0.46X_2 + 1.75X_3 + 3.86X_1 - 91.53{X_1}^2 \\ &- 0.01{X_2}^2 - 0.16{X_3}^2 - 3.96{X_4}^2 - 1.23X_1X_2 - 2.04X_1X_3 \\ &+ 13.79X_1X_4 - 0.00X_2X_3 - 0.07X_2X_4 - 0.31X_3X_4 \end{aligned}$$

Here, Y is the predicted response (W-Pro21717 production), and X_1, X_2, X_3, and X_4 are the concentrations of $Fe(C_6H_5O_7)$, NaCl, Na_2SO_4, and KCl, respectively. The predicted quantity of W-Pro21717 produced was 11.7 U/L when the concentrations of $Fe(C_6H_5O_7)$, NaCl, Na_2SO_4, and KCl were 0.1, 24.8, 4.4, and 0.7 g/L, respectively.

Bioreactor operations using mineral optimized medium

In order to confirm our hypothesis, the production of W-Pro21717 from *P. arctica* PAMC 21717 was performed in a jar fermenter. The cells were cultured at 15°C with different

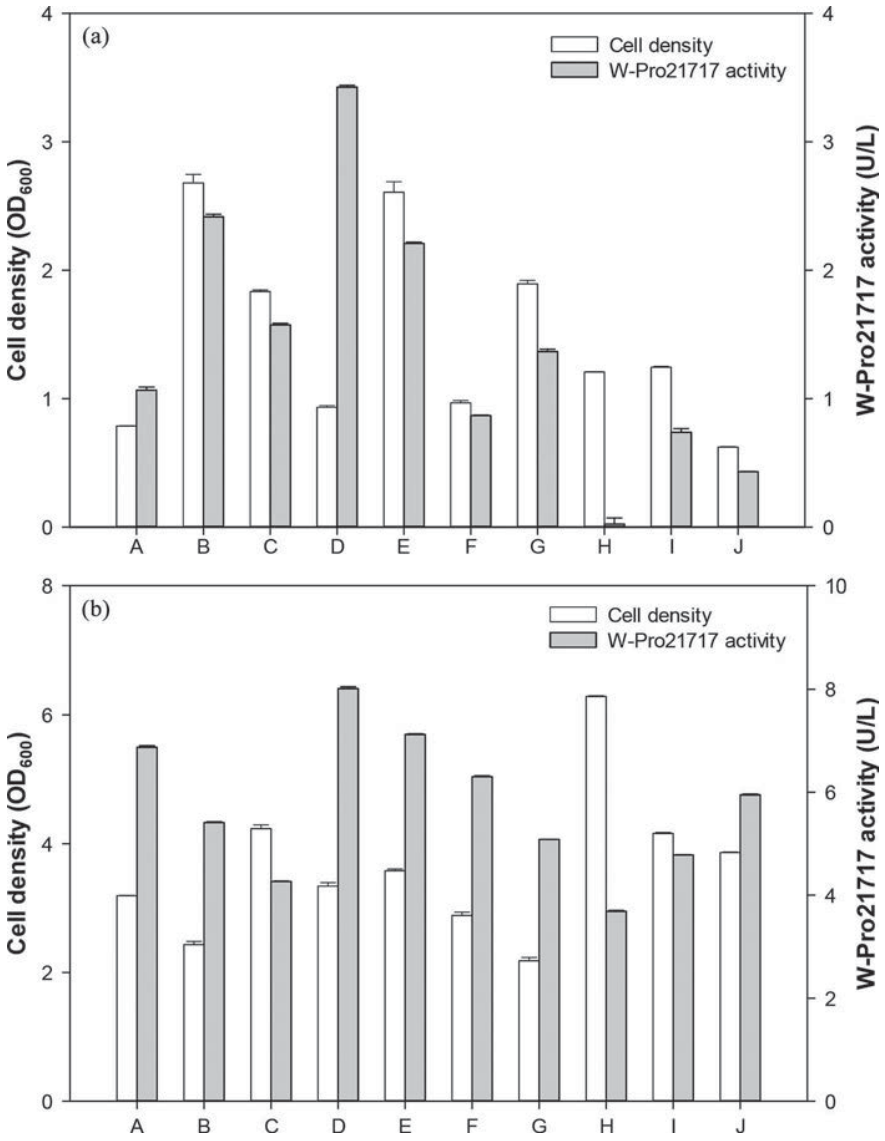

Figure 1. Effect of the carbon source on cell growth and W-Pro21717 production (a) without skim milk and (b) with skim milk. Cells were grown in medium containing 10 g/L each of various carbon sources in Erlenmeyer flasks: A, control; B, glucose; C, galactose; D, fructose; E, lactose; F, sucrose; G, maltose; H, glycerol; I, starch; and J, cellulose. After 3 days of cultivation, the cell density (white bar) and W-Pro21717 activity (gray bar) were measured. Error bars represent the standard deviation of three runs.

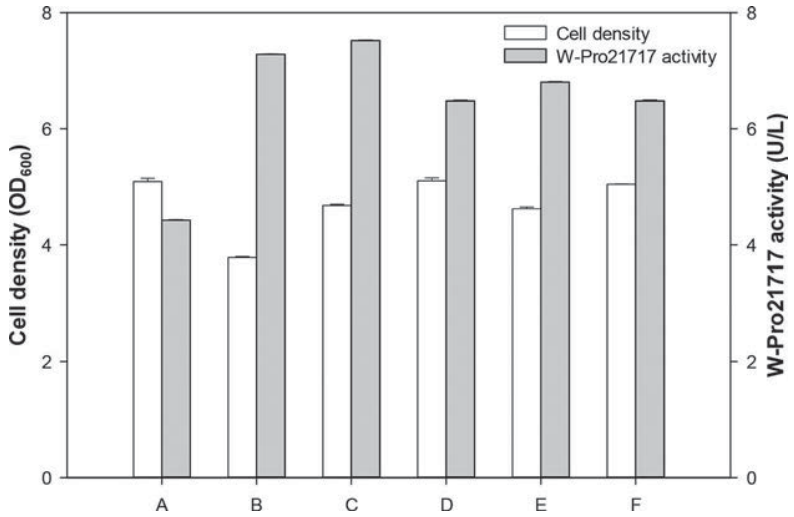

Figure 2. Effect of the nitrogen source on cell growth and W-Pro21717 production in the presence of skim milk. Cells were cultivated in medium containing 0.6 g/L each of the different nitrogen sources in Erlenmeyer flasks: A, control; B, peptone; C, tryptone; D, yeast extract; E, soy peptone; and F, tryptic soy broth. After 3 days of cultivation, the cell density (white bar) and W-Pro21717 activity (gray bar) were measured. Error bars represent the standard deviation of three runs.

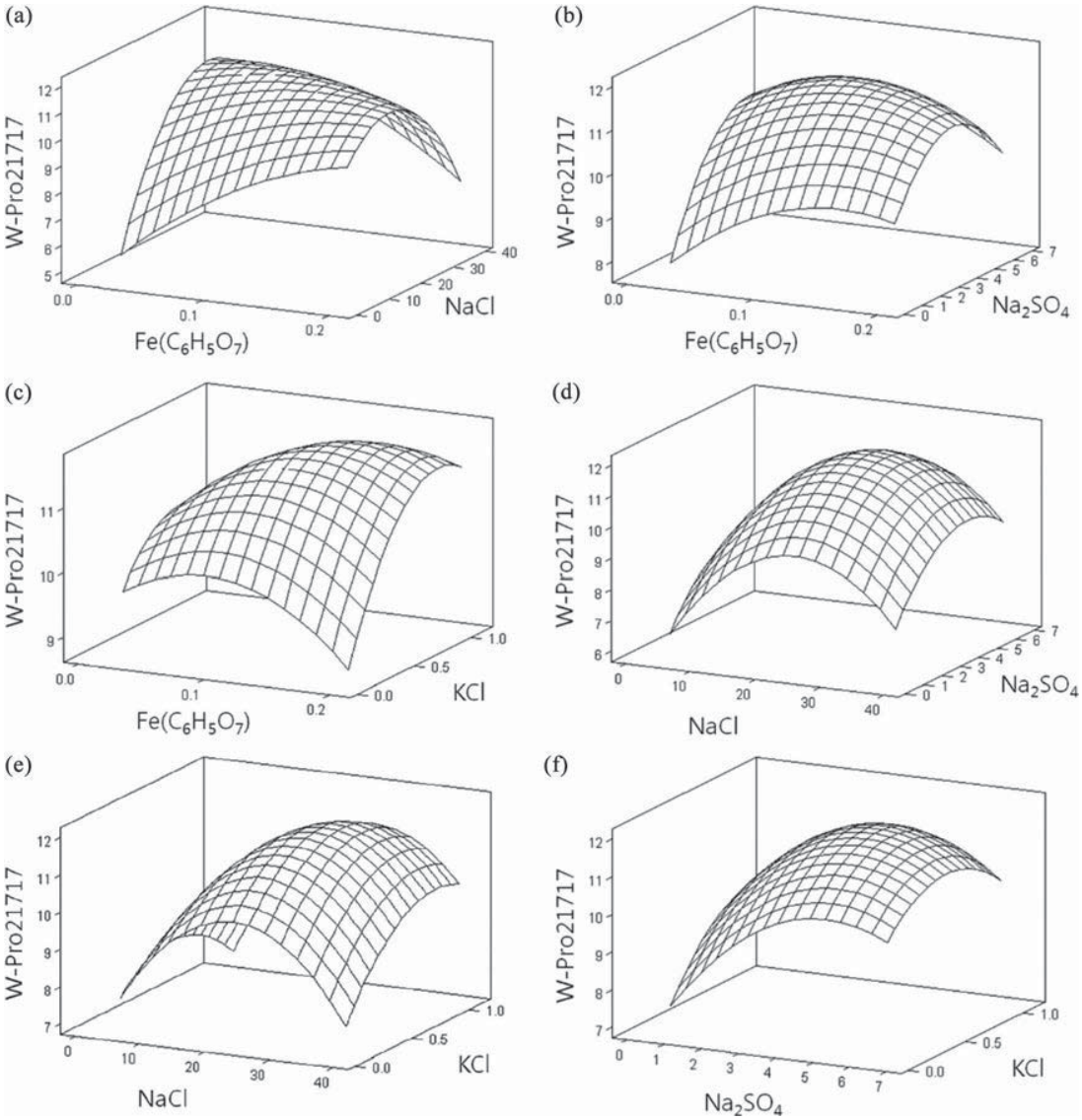

Figure 3. Three-dimensional response plot displaying the effect of (a) $Fe(C_6H_5O_7)$ and NaCl; (b) $Fe(C_6H_5O_7)$ and Na_2SO_4; (c) $Fe(C_6H_5O_7)$ and KCl; (d) NaCl and Na_2SO_4; (e) NaCl and KCl; and (f) Na_2SO_4 and KCl, on the production of W-Pro21717.

types of media: Marine Broth basal medium without skim milk, Marine Broth basal medium with skim milk, and mineral component-optimized medium with skim milk. Figure 4A displays the changes in cell growth and W-Pro21717 production over time using different media but identical operation conditions. Maximal W-Pro21717 activities of 5.5, 7.4, and 12.9 U/L were attained in basal medium without skim milk, basal medium with skim milk, and mineral component-optimized medium, respectively. These results indicated that the mineral component optimization and use of skim milk led to an increase in W-Pro21717 productivity. In order to increase the production of W-Pro21717 without effecting catabolic repression, the DO-stat fed-batch fermentation was conducted in mineral component-optimized medium. With the addition of skim milk and tryptone, the maximal W-Pro21717 activity drastically increased to 53.4 U/L, 4-fold higher than that obtained by batch fermentation (Figure 4B).

Discussion

In this study, the optimized mineral component conditions required to facilitate an increase in the productivity of cold-active protease W-Pro21717 in the Antarctic microorganism *P. arctica* PAMC 21717 were investigated.

Skim milk exhibited a major positive impact on W-Pro21717 production. This is consistent with the observations of previous studies, where some proteases were secreted in the presence of skim milk.[12,26-28] A statistical approach was found to be a very efficient tool for optimizing the mineral components in a controllable number of experimental runs.[22,23] The final mineral-optimized medium composition is shown in Table 3. Recent research and development have increasingly focused on the use of statistical methods in this regard. Previous analyses using statistical experimental design have been performed on the *Bacillus* spp.,[29–33] *Teredinibacter* sp.,[34] *Microbacterium* sp.,[35]

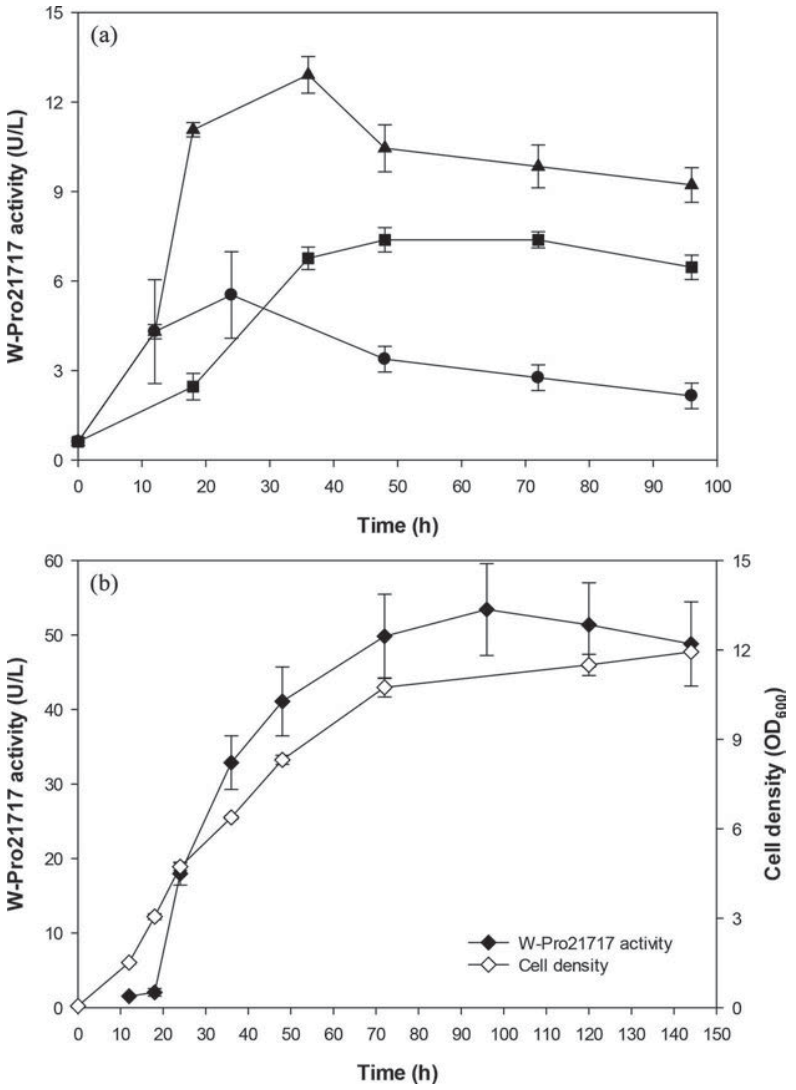

Figure 4. Time profile of W-Pro21717 production in (a) batch fermentation (●, basal medium without skim milk; ■, basal medium with skim milk; ▲, mineral source-optimized medium) and (b) fed-batch fermentation (—◇—, cell density; —◆—, W-Pro21717 activity). Cells were grown in a 5-L jar fermenter, and samples were taken periodically in order to analyze the W-Pro21717 activity. Error bars represent the standard deviation of at least three samples taken from a single run.

Aspergillus niger,[36] and *Colwellia* sp.[11] Overall, our findings demonstrated that the mineral component optimization caused a 1.7- to 7.3-fold increase in *P. arctica* PAMC 21717 protease production. To date, the effect of mineral component optimization of growth media has been investigated for only a few psychrophilic proteases; the 3.4-fold increase in protease production as a result of mineral component optimization observed in our study was comparable with the 3-fold increment in protease production observed using psychrophilic *Colwellia* sp.[11]

Table 3. Summary of the quantity of medium components required and the W-21717 activity for each step.

		Unoptimized medium	Optimized medium (Box-Behnken design)	Fed-batch fermentation
Medium component (g/L or mL/L)	$Fe(C_6H_5O_7)$	0.1	0.1	Optimized medium +feeding solution (100 g/L of skim milk and 6 g/L of tryptone)
	NaCl	19.45	24.8	
	$MgCl_2$	5.9	5.9	
	Na_2SO_4	3.24	4.4	
	$CaCl_2$	1.8	1.8	
	KCl	0.55	0.7	
	$NaHCO_3$	0.16	0.16	
	KBr	0.08	0.08	
	Trace elements	1 mL	—	
	Skim milk	—	10	
	Fructose	10	—	
	Tryptone	—	0.6	
Predicted value of W-21717 activity		—	11.7	—
Experimental value of W-21717 activity		3.4	12.9	53.4

The fed-batch fermentation process was performed in a bioreactor, in order to obtain a high protease yield.[37–40] The overall protease yields displayed a 10-fold improvement upon application of the fed-batch process.[37] In this study, the fed-batch culture of *P. arctica* PAMC 21717 in mineral-optimized media resulted in maximal W-Pro21717 production (53.4 U/L), approximately 4-fold higher than that obtained using batch fermentation. A comparison of the W-Pro21717 activity with the previously reported protease activities would be arduous because of the differences in their units and the substrates used.[7] Overall, we observed a 15-fold enhancement in W-Pro21717 production by statistical optimization of the mineral components and fed-batch culture, compared to the unoptimized basal Marine Broth conditions.

Conclusions

To the best of our knowledge, medium optimization for the production of cold-active protease by *Pseudoalteromonas* species has not been feasible. This study identifies the suitable conditions for enhancing protease productivity of *P. arctica* PAMC 21717. The optimal composition of mineral components determined in this study would be useful for large-scale production of W-Pro21717.

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