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Statistical Optimization of the Production of NaCl-Tolerant Proteases by a Moderate Halophile, *Virgibacillus* sp. SK37

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Summary

The objectives of this study are to optimize the conditions for providing high yield of NaCl-tolerant extracellular protease from *Virgibacillus* sp. SK37 based on a fish-based medium and to investigate the effects of the key factors (mass per volume ratios of dried anchovy, yeast extract and NaCl, and initial pH of the medium) on the secretion pattern of proteases. Based on the predicted response model, the optimized medium contained 1.81 % of dried anchovy, 0.33 % of yeast extract and 1.25 % of NaCl at pH=7.8. Under these conditions, a 5.3-fold increase in protease production was achieved, compared with the broth containing only 1.2 % of dried anchovy (5 % of NaCl at pH=7). The cubic regression adequately described the protease production. Protease activity was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on the synthetic substrate (Suc-Ala-Ala-Pro-Phe-AMC). Proteases of molecular masses of 19, 34, 35 and 44 kDa were secreted in the presence of NaCl, whereas those of 22 and 42 kDa were the main proteases detected in the absence of NaCl. In addition, no secreted proteases were detected when initial pH of the medium was pH=6. The peptide mass fingerprint of the medium cultured with 10 % NaCl showed a higher abundance of peptides with lower mass of 500–1000 *m*/*z* compared with the medium containing 0 % NaCl, indicating the higher proteolytic activity of the high-salt medium. The *Virgibacillus* sp. SK37 proteases showed a marked preference towards Lys, Arg and Tyr in the presence of NaCl and towards Lys and Arg in the absence of NaCl.

Key words: response surface methodology, protease, *Virgibacillus* sp., moderate halophile, fish sauce

Introduction

Bacterial protease production is greatly influenced by the medium composition and physical factors. Therefore, to achieve efficient protease production, the optimization of these parameters is needed. The Plackett-Burman design (PBD) can be applied to reduce a large number of variables for determining the key factors affecting the re-

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sponse (*1*). Subsequently, the identified key factors are optimized using the response surface methodology (RSM). RSM is a useful statistical technique for the optimization using a minimum number of experiments. This method has been successfully applied for the optimized production of cold-active protease from *Colwellia* sp. NJ341 (*2*), nattokinase from *Bacillus subtilis* (*3*) and organic solvent--tolerant protease from *Geomicrobium* sp. EMB2 (*4*). The optimization of NaCl-tolerant protease production from moderately halophilic bacteria has never been reported.

Moderately halophilic bacteria are able to grow over a wide range of salt mass fractions, from 3 to 15 % (*5*). They have been used for diverse applications, including as a source of hydrolytic enzymes and compatible solutes, for degrading toxic industrial residues and forming biopolymers for oil recovery (*6*). The genus *Virgibacillus*, which is classified as a moderate halophile, was proposed by Heyndrickx *et al.* (*7*), and there are 30 species that have been identified. *Virgibacillus* species have been isolated from different samples, such as saline environments (*8*) and traditional fermented products (*9*). Recently, the protease-producing bacterium, *Virgibacillus* sp. SK37, which was isolated from fermented fish sauce, showed great promise for use as a starter culture (*10*). The strain was isolated from one-month-old fish sauce mash and showed highest extracellular protease activity among 165 studied isolates (*11*). It was identified as *Virgibacillus* sp. SK37 according to 16S rRNA gene sequence with the GenBank/NCBI accession number of DQ910840. *Virgibacillus* sp. SK37 is a Gram-positive or -variable, nonmotile long rod (0.6 to 0.7×3.0 to 6.6 μm) bacterium, containing terminal or subterminal ellipsoidal spores. It can grow at a wide pH range from 4 to 11 and temperature from 20 to 45 °C. The bacterium produced cell-bound and extracellular proteases which exhibited high activities at high salt concentrations when the broth containing yeast extract as a main nitrogen source was used (*11,12*). However, the use of yeast extract as a culture broth for large-scale enzyme production in fish sauce industry is not economically viable due to its high cost. The optimum medium based on inexpensive nitrogen source is, thus, required.

Fish sauce is a popular condiment consumed in Southeast Asia. It is produced through a natural fermentation by mixing uneviscerated anchovy and solar salt at a ratio of 3 to 1, which gives a final product with approx. 27–30 % NaCl. The complete fermentation takes at least one year due to the extremely high salt content and prolonged protein hydrolysis. The application of NaCl-tolerant proteases with high activity would be a viable approach to accelerate proteolysis during fish sauce production (*10*). Efficient protease production based on the complex medium that contains ingredients compatible with fish sauce is extremely critical. Anchovy could be a potential raw material for the medium development since it is the essential component of fish sauce. However, the complex medium like anchovy broth has been reported to suppress protease production by *Virgibacillus* sp. during fish sauce fermentation (*12*). The optimum anchovy-based medium that can promote the protease production by *Virgibacillus* sp. SK37 must be developed and optimized so that the strain can be efficiently applied as a starter culture in fish sauce production.

The proteases produced by *Virgibacillus* sp. SK37 showed different activities on anchovy proteins at various NaCl concentrations (*10*). The composition of culture broth can affect not only protease activity but also the type of secreted protease. Different proteases might exhibit various substrate specificities, leading to various species of produced peptides. However, the effect of culture broth composition on the type of protease secreted by *Virgibacillus* sp. SK37 has not been systematically elucidated. The aim of this study is to optimize the critical parameters affecting the protease production by *Virgibacillus* sp. SK37 on the anchovy-based medium using a rotatable central composite design (RCCD). In addition, the pattern of protease secretion and the resulting peptide fragments derived from *Virgibacillus* sp. SK37 will be elucidated.

Materials and Methods

Inoculum preparation and cultivation conditions

Virgibacillus sp. SK37 was obtained from the Culture Collection Center at Suranaree University of Technology (Nakhon Ratchasima, Thailand). A single colony of the strain grown on tryptic soy agar (Merck KGaA, Darmstadt, Germany) containing 2.5 % NaCl at 40 °C for two days was transferred into 30 mL of yeast extract broth (1 % yeast extract, 0.3 % trisodium citrate, 0.2 % potassium chloride, 2.5 % $MgSO_4 \cdot 7H_2O$) containing 2.5 % NaCl (*12*) and incubated at 40 °C on a rotary shaker at 100 rpm for 1 day. The culture medium was diluted to obtain a final absorbance of 0.25 absorbance unit (AU) at 600 nm ($A_{600\ nm}$) with sterile 0.85 % NaCl. The diluted samples were subsequently used as an inoculum.

The inoculum (2 %) was transferred into the experimental medium as described below. The samples inoculated with approx. 10^6 CFU/mL were incubated at a rotary shaker at 150 rpm for 2 days. After incubation, the total viable cells were counted using a drop plate technique (*13*) on tryptic soy agar containing 2.5 % NaCl. The cell--free supernatant was collected by centrifugation at 15 000×*g* and 4 °C for 15 min (Sorvall™ Legend™ Micro 21 Microcentrifuge, Thermo Fisher Scientific, Bremen, Germany), and the extracellular protease activity was determined as described below.

Plackett-Burman design

The effects of independent factors, namely dried anchovy, yeast extract, $MgSO_4 \cdot 7H_2O$, glucose and NaCl mass per volume ratios, initial pH, and incubation temperature were evaluated for protease production. Two levels, *i.e.* low (–1) and high (+1), of all of the factors were investigated. The –1 and +1 values were set to 0.2 and 2 % dried anchovy, 0.1 and 0.5 % yeast extract, 0.1 and 1 % $MgSO_4 \cdot 7H_2O$, 0.1 and 1 % glucose, 0.1 and 1 % NaCl, initial pH values of 7 and 9, and incubation temperatures of 30 and 40 °C. Eleven independent variables, including four dummy variables, were screened in 12 experiments according to the experimental design proposed by Plackett and Burman (*14*). The statistical package (SPSS Statistics for Windows, v. 17.0, IBM Corporation, Chicago, IL, USA) was used to analyze the experimental design. Dried anchovy powder was prepared by drying anchovy in an

air oven set at 70 °C for 35 h, grinding with an IKA M20 universal laboratory mill (IKA-Werke GmbH & Co., Staufen, Germany), and sieving through 140 mesh (Fritsch GmbH, Idar-Oberstein, Germany).

Response surface methodology

Four factors, including dried anchovy (A), yeast extract (B) and NaCl (C) mass per volume ratios, and initial pH (D), were fed to a RCCD for optimization. Five levels (–2, –1, 0, +1 and +2) taken for each component were set to 0, 0.5, 1, 1.5 and 2 % dried anchovy and yeast extract each; 0, 2.5, 5, 7.5 and 10 % NaCl, and initial pH values of 6, 7, 8, 9 and 10. In total, 32 experiments with eight central points were performed. The experimental media were incubated at 40 °C, and the protease production was measured as the response. The experiment was performed in three replicates. The response data were analyzed by Design Expert® v. 9.0.3 (Stat-Ease, Inc., Minneapolis, MN, USA). To validate the model, experiments selected within the design space were conducted.

Protease assay

The protease activity was assayed following the method proposed by Sinsuwan *et al.* (*15*) with minor modifications. The reaction mixture (1 mL) contained 50 μL of the crude extracellular proteases, 1 μM of Suc-Ala-Ala-Pro--Phe-7-AMC, 200 mM of Tris-HCl (pH=8.0) and 30 mM of $CaCl_2$, and was incubated at 65 °C for 5 min. The activity of proteases was terminated by the addition of 1.5 mL of the mixture containing 30 % butanol, 35 % methanol and 35 % deionized water (by volume). The fluorescence intensity was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (RF-1501 spectrofluorophotometer, Shimadzu Co., Kyoto, Japan). One unit of protease activity was expressed as the release of amino-4-methylcoumarin (AMC) in micromoles per minute.

Zymographic analysis

The effect of the medium composition on extracellular protease secretion was evaluated using a fluorogenic peptide zymogram (*16*,*17*). A volume of 2 mL of the inoculum, prepared as described above, was transferred into 18 mL of various media and incubated at 40 °C and a shaking speed of 150 rpm for two days. The following media were used: (*i*) anchovy broth with 5 % NaCl, pH=8, containing dried anchovy at mass per volume ratio of 0.5, 1.25 and 2 %; (*ii*) yeast extract broth with 5 % NaCl, pH=8, containing 0.5, 1.25 and 2 % yeast extract; (*iii*) dried anchovy 0.5 % and yeast extract 0.5 % broth with 0, 5 and 10 % NaCl at pH=8; and (*iv*) dried anchovy 0.5 % and yeast extract 0.5 % broth with 5 % NaCl at initial pH values of 6, 7.5 and 9. The cell-free supernatants were collected by centrifugation at 15 000×*g* and 4 °C for 15 min (Sorvall™ Legend™ Micro 21 Microcentrifuge, Thermo Fisher Scientific). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 4 and 12.5 % acrylamide stacking and separating gels, respectively. The separating gel was prepared by copolymerization with 500 μM of Suc-Ala-Ala-Pro-Phe-AMC in the dark at 4 °C for 1 h. The crude enzymes were mixed with a treatment buffer (4 % SDS, 10 % 2-mercaptoethanol (β-ME), 20 % glycerol, 125 mM Tris-HCl (pH=6.8) and 0.1 % Bromophenol Blue) at a ratio of 1:1. A volume of 10 μL of the mixtures was loaded into the gel. The electrophoretic separation was carried out in the dark at 4 °C and a constant current of 10 mA. Subsequently, the gel was washed with cold 2.5 % Triton X-100 at 4 °C for 5 min and then twice with cold deionized water. The proteases were activated by incubating the gel with 200 mM Tris-HCl (pH=8.0) and 30 mM $CaCl_2$ at 65 °C for 5 min. The fluorescent bands indicating the existence of proteases were detected immediately using a Gel Doc™ XR system (Bio-Rad Laboratories, Hercules, CA, USA). Prestained SDS-PAGE standard including myosin (209 kDa), β-galactosidase (124 kDa), serum albumin (80 kDa), ovalbumin (49.1 kDa), carbonic anhydrase (34.8 kDa), trypsin inhibitor (28.9 kDa) and lysozyme (20.6 kDa) (Bio-Rad Laboratories) was used for the molecular mass estimation.

Mass spectrometry

The anchovy and yeast extract broth containing 0.5 % dried anchovy, 0.5 % yeast extract, pH=8, and 0 and 10 % NaCl was selected for mass spectrometry studies. The cell-free supernatant was collected by centrifugation at 15 000×*g* and 4 °C for 15 min. A fraction containing peptides smaller than 10 kDa was collected by Nanosep® centrifugal devices with an Omega™ membrane with a molecular mass cut-off of 10 000 Da (Pall Corporation, Ann Arbor, MI, USA) and then subjected to the desalting spin column (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The desalted samples were dried by vacuum centrifugation and dissolved in a mixture of 0.1 % acetonitrile and 0.1 % trifluoroacetic acid (1:2). The peptide samples were mixed with the matrix mixture containing 2,5-dihydroxybenzoic acid and applied onto a sample holder. The peptide mass fingerprint of the samples was determined by matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometer (Ultraflex III TOF/TOF, Bruker Daltonik GmbH, Bremen, Germany). The MALDI-TOF spectrum was obtained at the positive ion mode, an acceleration voltage of 20 kV and an extraction delay of 400 ns.

The *de novo* peptide sequencing was performed using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with the Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Co., Waltham, MA, USA). The peptides were separated using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Coventry, UK) coupled to an UltiMate 3000 LC System (Dionex Ltd., Surrey, UK) equipped with a nanocolumn (PepSwift monolithic column 50 mm×100 μm i.d.). Eluent A was 0.1 % formic acid, and eluent B was 80 % acetonitrile containing 0.1 % formic acid. Peptide separation was performed with a linear gradient from 10 to 70 % B for 13 min at a flow rate of 300 nL/min, including a regeneration step at 90 % B and an equilibration step at 10 % B. The *de novo* peptide sequencing was achieved using the Pepnovo program without the assistance of a sequence database (*18*).

Results and Discussion

Selection of physical and chemical factors

The protease production by *Virgibacillus* sp. SK37 significantly increased with an increase in the mass per volume ratios of yeast extract and $MgSO_4 \cdot 7H_2O$, and initial pH, but was suppressed by glucose ($p<0.05$, Table 1). The effect of dried anchovy and NaCl mass per volume ratios and incubation temperature on the protease production was not significant ($p>0.05$). Yeast extract is a rich source of free amino acids, vitamins, minerals and other growth factors essential for protein biosynthesis; therefore, it appeared to be a critical component for protease production. Although the growth of the strain was significantly increased by glucose ($p<0.05$), this substrate appeared to suppress protease production by *Virgibacillus* sp. SK37 due to carbon catabolite repression. Carbon catabolite repression in Gram-positive bacteria involves the metabolite-activated HPr(ser)-kinase, the phosphocarrier protein HPr and the transcription factor CcpA (*19*). These key proteins involved in the repression are also found in the whole-genome sequences of *Virgibacillus* sp. SK37 (*20*). Elevations in the levels of the glycolytic intermediate fructose-1,6-bisphosphate resulted in the activation of HPr(ser)-kinase and the phosphorylation of HPr protein (HPr~P). Subsequently, HPr~P formed a complex with CcpA protein, which acted as a repressor of the synthesis of many proteins (*19*). This regulation is, therefore, most likely to repress the synthesis of *Virgibacillus* sp. SK37 proteases. An increase in the mass per volume ratio of $MgSO_4 \cdot 7H_2O$ in the medium positively increased the protease production ($p<0.05$), despite the fact that $MgSO_4 \cdot 7H_2O$ had no effect on the growth ($p>0.05$). The secretion of α-amylase from *Bacillus subtilis* decreased markedly when the cells grew in the medium containing EDTA, even though the bacterial growth was not affected by the presence of EDTA (*21*). Mg^{2+} can neutralize the negative charges on the cell wall of Gram-positive bacteria (*22*). The major secreted proteases from *Virgibacillus* sp. SK37 showed an acidic pI, implying the presence of numerous negative charges in their structures (*23*). The repulsive forces between the negative charges of secretory proteases and the cell wall may be minimized during protein translocation by the presence of Mg^{2+}, resulting in an improvement in protease secretion. NaCl at mass per volume ratios up to 10 % was considered to be an insignificant factor for protease production ($p>0.05$). However, the halophililic characteristic of the protease production of this strain has been reported (*24*). For this reason, NaCl was still selected for further optimization. Although dried anchovy had no effect on protease production at the studied level ranging from 0.2–2 %, it greatly affected growth ($p<0.05$, Table 1). Thus, dried anchovy was selected for further optimization.

Optimization of culture conditions and validation of models

Protease production and growth under various conditions within the RCCD are shown in Table 2. The analysis of variance (ANOVA) suggested that the cubic regression model for the response was satisfactory (Table 3). The F-values and the significant p-values implied that the obtained experimental data fit well with the model (Table 3). The R^2 value indicated that the cubic regression model could explain 90.1 % of the total variations in protease production ability. The value of the adjusted regression coefficient (Adj R^2) was correlated to R^2, advocating the high significance of the models. A slightly high value of the coefficient of variation (CV) for the protease production model was obtained. The lack of fit was insignificant (Table 3), indicating that the model may significantly describe the variation of the response. The adequate precision value is an index of the signal-to-noise ratio. The adequate precision value of the model was higher than 4, suggesting that the model was satisfactorily used to navigate the design space. The regression equation coefficients were determined as shown in Eq. 1 for protease production:

$$\begin{aligned}\text{Protease production} = {} & 0.38 - 0.116A + 0.053B - \\ & 0.002C - 0.046D - 0.047AB + 0.040AC + 0.033AD - \\ & 0.003BC - 0.051BD + 0.047CD - 0.023A^2 - 0.052B^2 - \\ & 0.083C^2 - 0.064D^2 - 0.005ABC - 0.017ABD - \\ & 0.017ACD + 0.024BCD - 0.107A^2B - 0.077A^2C + \\ & 0.012A^2D + 0.131AB^2 / (\text{mU/mL})\end{aligned} \quad /1/$$

where A, B, C and D represent dried anchovy, yeast extract and NaCl mass per volume ratios, and initial pH, respectively.

To confirm the validity of the cubic regression model, some conditions within the design space were tested. The predicted values were comparable with the experimental

Table 1. Linear regression analysis of the Plackett-Burman experiment

Variables	Bacterial growth[1]			Protease production[2]		
	Regression coefficient	*t*-value	p-value[3]	Regression coefficient	*t*-value	p-value[3]
Dried anchovy	0.286	5.099	0.000	0.021	0.609	0.551
Yeast extract	0.154	2.747	0.014	0.259	7.496	0.000
$MgSO_4 \cdot 7H_2O$	0.048	0.852	0.407	0.143	4.145	0.001
Glucose	0.175	3.118	0.007	–0.290	–8.389	0.000
NaCl	–0.003	–0.052	0.959	–0.059	–1.702	0.108
Initial pH	0.015	0.273	0.788	0.140	4.051	0.001
Incubation temperature	–0.197	–3.516	0.003	0.020	0.575	0.573

[1]R^2=0.883; [2]R^2=0.954; [3]Significant differences at $p<0.05$

Table 2. Experimental design and results of a rotatable central composite design

Standard run	Coded value				Experimental value	
	$\frac{m(\text{dried anchovy})}{V(\text{solvent})}$ / %	$\frac{m(\text{yeast extract})}{V(\text{solvent})}$ / %	$\frac{m(\text{NaCl})}{V(\text{solvent})}$ / %	Initial pH	Bacterial growth / log CFU/mL	Protease production / mU/mL
1	–1	–1	–1	–1	7.83	0.338
2	1	–1	–1	–1	8.69	0.259
3	–1	1	–1	–1	7.35	0.457
4	1	1	–1	–1	8.22	0.234
5	–1	–1	1	–1	8.36	0.039
6	1	–1	1	–1	8.63	0.164
7	–1	1	1	–1	9.15	0.029
8	1	1	1	–1	9.47	0.053
9	–1	–1	–1	1	8.36	0.212
10	1	–1	–1	1	9.11	0.360
11	–1	1	–1	1	9.92	0.060
12	1	1	–1	1	9.44	0.010
13	–1	–1	1	1	7.66	0.030
14	1	–1	1	1	9.72	0.330
15	–1	1	1	1	8.90	0.021
16	1	1	1	1	8.11	0.005
17	–2	0	0	0	7.95	0.508
18	2	0	0	0	8.60	0.042
19	0	–2	0	0	8.43	0.051
20	0	2	0	0	8.81	0.261
21	0	0	–2	0	7.77	0.037
22	0	0	2	0	8.39	0.030
23	0	0	0	–2	9.15	0.201
24	0	0	0	2	0.00	0.018
25	0	0	0	0	8.50	0.420
26	0	0	0	0	8.14	0.462
27	0	0	0	0	8.03	0.376
28	0	0	0	0	8.64	0.417
29	0	0	0	0	7.77	0.252
30	0	0	0	0	8.57	0.336
31	0	0	0	0	8.23	0.517
32	0	0	0	0	8.09	0.244

Table 3. Analysis of variance (ANOVA) of the cubic regression models generated from the experiments for the protease production by *Virgibacillus* sp. SK37

F-value	17
p-value	<0.0001
R^2	0.9012
Adjusted R^2	0.8482
Coefficient of variance (CV)	31.75 %
p-value of lack-of-fit	0.4630
Adequate precision	13.08

ones (Table 4), suggesting the validity of this model. This result suggests that response surface methodology (RSM) can be applied to optimize the protease production by halophilic bacteria with satisfactory reliability. The significances of each coefficient were determined. The linear effects of dried anchovy (A) and yeast extract (B) mass per volume ratios, and initial pH (D) on protease production were significant. The interactions, namely AB, AC, AD, BD, CD, A^2B, A^2C and AB^2, were also significant. The protease production gradually increased with the increase of initial pH and reached a maximum at pH=7.5–8 (Fig. 1a). This result was similar to that reported by Lapsongphon

Table 4. Validation of the cubic models for protease production within the design space

Validated levels				Protease production/(mU/mL)	
$\frac{m(\text{dried anchovy})}{V(\text{solvent})}$ / %	$\frac{m(\text{yeast extract})}{V(\text{solvent})}$ / %	$\frac{m(\text{NaCl})}{V(\text{solvent})}$ / %	Initial pH	Experimental	Predicted[1]
0.9	1	5	7.9	0.564	0.407
1	0.25	8.75	8	0.029	0.000
1	0.9	4.9	8	0.358	0.367
1	1.75	0.125	8	0.039	0.040
1	1.75	8.75	8	0.140	0.146
1.75	1	5	6.5	0.030	0.000
1.75	1	5	9.5	0.031	0.056

[1]The prediction value was calculated according to Eq. 1

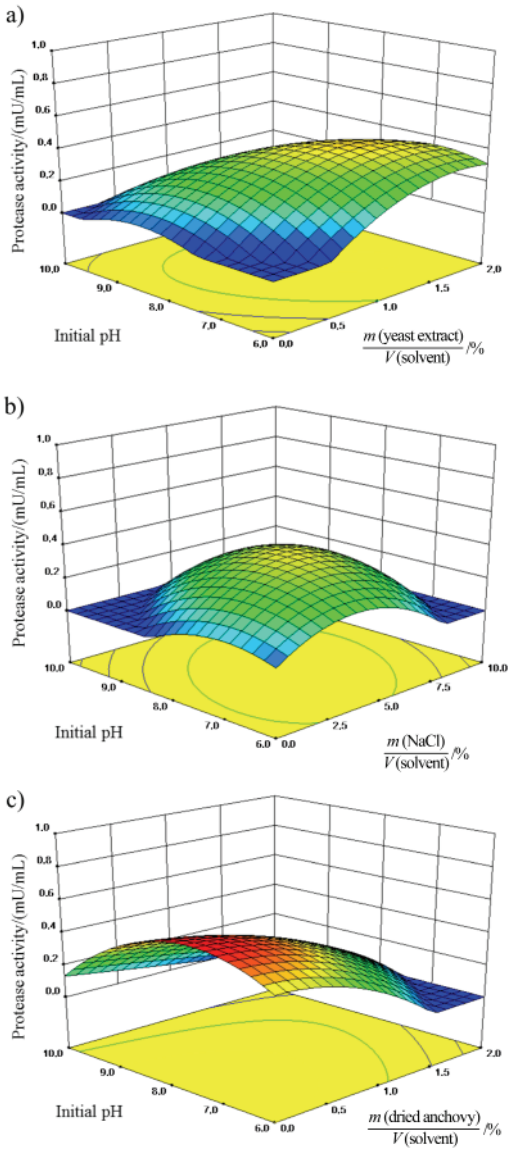

Fig. 1. Three-dimensional (3D) surface plot for protease production as functions of: a) yeast extract mass per volume ratio and initial pH, b) NaCl mass per volume ratio and initial pH, and c) dried anchovy mass per volume ratio and initial pH of *Virgibacillus* sp. SK37 when the other factors were held at zero level

et al. (*24*). In addition, higher mass fraction of yeast extract favoured an increase in the protease production by *Virgibacillus* sp. SK37 (Fig. 1a). The strain required NaCl for protease production, but higher NaCl mass fraction (>7.5 %) reduced the production (Fig. 1b). When the medium contained 1 % yeast extract, the addition of dried anchovy (>0.5 %) reduced the protease production (Fig. 1c). Nitrogen catabolite repression in *Virgibacillus* sp. has been previously reported (*12*). The excessive amount of available nitrogen supply results in the inhibition of protease production. Based on the model and the response surface plots, the optimal levels to attain high protease production can be predicted: 1.81 % dried anchovy, 0.33 % yeast extract and 1.25 % NaCl at pH=7.8. These conditions required 67 % less yeast extract than the yeast extract broth used to induce protease production by *Virgibacillus* sp. (*12*). When compared to the broth containing only anchovy (1.2 % dried anchovy and 5 % NaCl at pH=7), a 5.3-fold increase in protease production was obtained. The activity obtained under the optimized conditions was approx. eight times higher than that previously reported in neopeptone broth (0.5 % neopeptone, 1 % $MgSO_4 \cdot 7H_2O$, 0.2 % KNO_3, 0.0005 % ferric citrate and 1 % glycerol) containing 10 % NaCl (pH=7.0) (*11*). The protease production by *Bacillus* obtained in the optimized medium determined by RSM increased 1.5- to 12.9-fold compared with that obtained in the unoptimized media (*25,26*).

Detection of secreted proteases by zymogram

The proteases secreted from *Virgibacillus* sp. SK37 exhibited activity only towards Suc-Ala-Ala-Pro-Phe-AMC among other synthetic substrates tested, indicating subtilisin-like character (*11*). Therefore, Suc-Ala-Ala-Pro-Phe-AMC was used for substrate zymography. The dominant proteases had molecular masses (*M*) of 19, 34, 35 and 44 kDa, and these were essentially secreted at all of the studied mass per volume ratios of dried anchovy (Fig. 2a). Minor proteases were those of larger molecular mass (Fig. 2a). This was in agreement with the results reported by Phrommao *et al.* (*23*), who reported that the major proteases of *Virgibacillus* sp. SK37 had molecular masses of 19, 34 and 44 kDa. The band intensity appeared to increase with the addition of dried anchovy to the medium and corresponded to the growth of *Virgibacillus* sp. SK37 (Fig. 2a). Similar results were also observed with an increase in

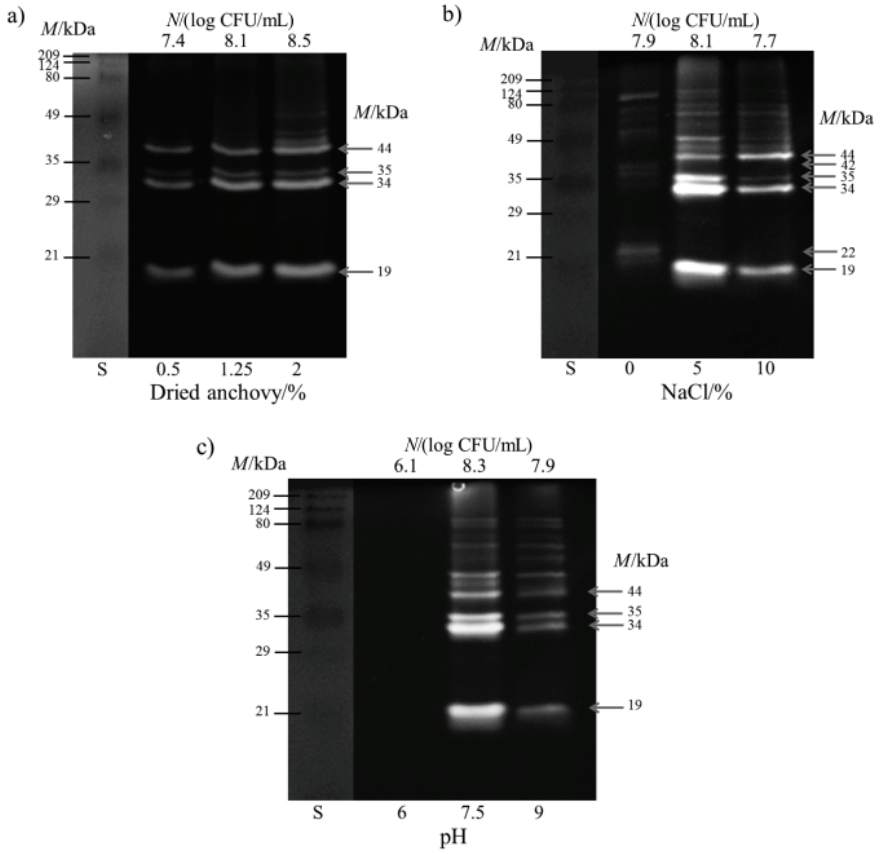

Fig. 2. Zymogram (12.5 % T) of *Virgibacillus* sp. SK37 using the synthetic Suc-Ala-Ala-Pro-Phe-AMC substrate. The strain was cultivated in various media with various mass per volume ratios of: a) dried anchovy, b) NaCl and c) different initial pH values. S=molecular mass standard. The total viable cell count was measured by a drop plate technique and expressed as log CFU/mL

the yeast extract mass per volume ratio (data not shown). Hence, the production of proteases by *Virgibacillus* sp. SK37 in the medium containing dried anchovy or yeast extract varied proportionally with the changes in biomass.

NaCl strongly affected the secretion pattern (Fig. 2b). In the absence of NaCl, proteases with *M* of 22 and 42 kDa were detected, whereas proteases with *M* of 19, 34, 35 and 44 kDa were predominant in the presence of 5 and 10 % NaCl. Our results indicated that NaCl mass per volume ratio does not only affect protease production by *Virgibacillus* sp. SK37 (Fig. 1b), but also the type of protease produced (Fig. 2b). The DegS-DegU two-component system controlled the expression of degradative enzymes involved in the response of *B. subtilis* to NaCl stress (*27*). Two major component genes, namely *degS* (encoding membrane-associated histidine kinase) and *degU* (encoding cytoplasmic response regulator), were also found in the whole genome of *Virgibacillus* sp. SK37 (*20*). Phosphorylated DegU (DegU~P) recruited RNA polymerase at the promoter region of the genes to stimulate or inhibit a transcriptional process (*28*). At high NaCl mass per volume ratio, the expression of the alkaline protease *aprE* was repressed, but levansucrase, encoded by *sacB*, was stimulated by DegU~P (*27*). It may be postulated that DegU~P might regulate the level of expression of *Virgibacillus* sp. SK37 protease genes in a medium with high NaCl mass per volume ratio.

The highest activity was observed at an initial pH=7.5 (Fig. 2c). The same activity pattern obtained at initial pH values of 7.5 and 9 indicated no specific inducers on protease secretion (Fig. 2c). However, activity bands at initial pH=6 were not detected, despite an apparent bacterial growth of 6.1 log CFU/mL. The expression of glutamate dehydrogenase (GDH) was attributed to an efficiency of secretory enzyme production in *B. subtilis* (*29*). The production of α-amylase by *B. subtilis* was greatly decreased in a medium with acidic pH because of the lower expression of GDH (*29*). It is speculated that GDH expression in *Virgibacillus* sp. SK37 may be down-regulated at pH=6, inhibiting the transcriptional process of protease genes. Our findings clearly demonstrated that the medium composition affected not only the yield of protease production but also the type of the secreted protease.

Peptide patterns and sequences

Since secretion of proteases appeared to vary with different mass per volume ratios of NaCl, peptides resulting from proteolysis are expected to be different. The peptide mass fingerprint (PMF) was, therefore, evaluated (Fig. 3). The non-inoculated samples showed different PMF patterns in the absence or presence of NaCl (Figs. 3a and c). This difference could be due to the different protein extractability at various NaCl mass per volume ratios. The inoculated sample at 10 % NaCl apparently ex-

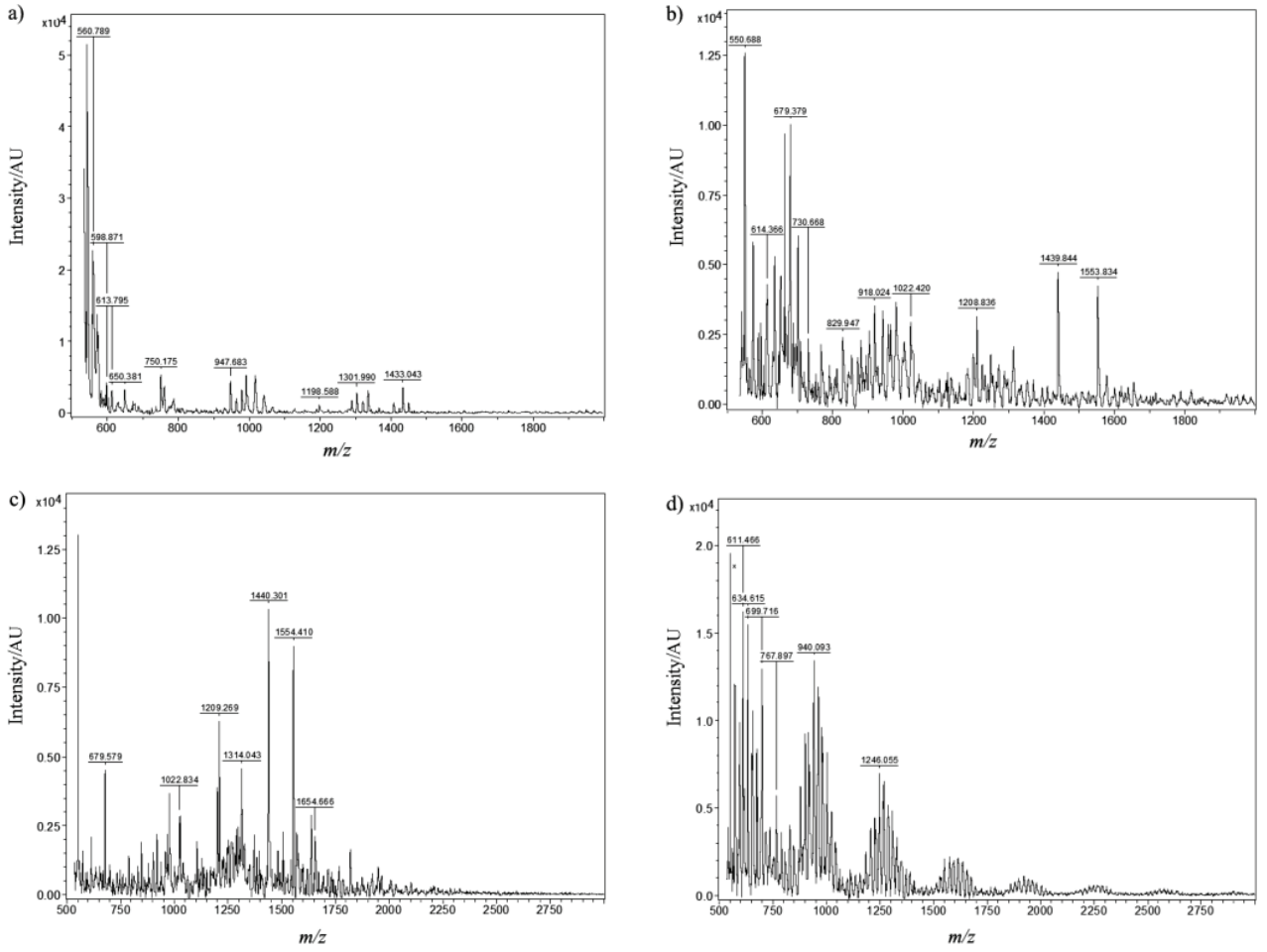

Fig. 3. Peptide mass fingerprint (PMF) of *Virgibacillus* sp. SK37 cultivated in media (0.5 % dried anchovy, 0.5 % yeast extract, initial pH=8) in the absence (a, b) or presence (c, d) of 10 % NaCl. Non-inoculated samples (a, c) served as a control

hibited higher mass intensities and peptides with smaller mass-to-charge ratios (*m*/*z*) of approx. 500–1000 (Fig. 3d), indicating that a larger extent of proteolysis occurred at 10 % NaCl. In contrast, a high abundance of peptides of approx. 1439 and 1553 *m*/*z* was detected in the inoculated medium without NaCl (Fig. 3b). In addition, *de novo* peptide sequencing revealed different major peptides at 0 and 10 % NaCl (Table 5). The different PMFs and peptides suggested that the active proteases in the absence of NaCl and in the presence of 10 % NaCl are likely different. This finding was concomitant with the zymogram results, which showed different dominant proteases at various salt mass per volume ratios (Fig. 2b). The proteases in the medium without NaCl preferably hydrolyzed Lys and Arg, whereas those in the high-salt medium preferred the cleavage sites at Lys, Arg and Tyr (Table 5).

The detection of trypsin cleavage sites in the medium without NaCl may lead to the assumption that proteases with apparent *M* of 22 and 42 kDa, as detected by the specific substrate for subtilisin (Fig. 2b), possessed broad substrate specificity. It could also be possible that trypsin-like protease(s) was (were) present. These results demonstrated that NaCl greatly governed the type of proteases secreted by *Virgibacillus* sp. SK37.

Table 5. The *de novo* sequencing of the peptides in the culture media generated by the halophile *Virgibacillus* sp. SK37 in the absence or presence of NaCl

0 % NaCl[1]	10 % NaCl[1]
ALVPK	GAVAFSK*
EEGEFLR*	GSLVLAH
ELGSQFLQQK*	HALLAR
ELLFR	LLPKYDR*
ELTETRR*	QDLLAH
ELVEEER*	SGVVGPY*
ELVHAKP	SHLFR
ENQGYGR	YLLGQDLLLLTK*
FNEKAR	
GFLQAER*	
HAVNLCR*	
LDQAWHR*	
LEGNEQFLNAAK*	
LEQAHVPK	
MLNYR	
SFAK	
SHVEEER	

[1]*De novo* peptide sequences were not observed in the non-inoculated samples

*Abundant peptide fragments based on an intensity greater than 900 AU

Conclusions

Four key variables, including dried anchovy, yeast extract and NaCl mass per volume ratios, and initial pH value, were found to be important for protease production and secretion pattern by *Virgibacillus* sp. SK37. A 5.3--fold increase in protease production was obtained in the medium containing 1.81 % dried anchovy, 0.33 % yeast extract and 1.25 % NaCl at pH=7.8. The acidic medium at pH=6 inhibited the secretion of proteases. Proteases with *M* of 22 and 42 kDa were detected in the medium without NaCl, whereas those with *M* of 19, 34, 35 and 44 kDa were predominant in the NaCl-containing medium. The resulting peptides greatly varied with the salt content of the medium. This study provides the first report of the optimized anchovy-based medium for protease production by moderate halophile, *Virgibacillus* sp., which is a potential starter culture for fish sauce fermentation.

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References

1. Rispoli F, Shah VA. A new efficient mixture screening design for optimization of media. Biotechnol Prog. 2009;25:980–5. http://dx.doi.org/10.1002/btpr.225
2. Wang Q, Hou Y, Xu Z, Miao J, Li G. Optimization of cold-active protease production by the psychrophilic bacterium Colwellia sp. NJ341 with response surface methodology. Bioresour Tech. 2008;99:1926–31. http://dx.doi.org/10.1016/j.biortech.2007.03.028
3. Deepak V, Kalishwaralal K, Ramkumarpandian S, Babu SV, Senthilkumar SR, Sangiliyandi G. Optimization of media composition for nattokinase production by Bacillus subtilis using response surface methodology. Bioresour Tech. 2008; 99:8170–4. http://dx.doi.org/10.1016/j.biortech.2008.03.018
4. Karan R, Singh SP, Kapoor S, Khare SK. A novel organic solvent tolerant protease from a newly isolated Geomicrobium sp. EMB2 (MTCC 10310): Production optimization by response surface methodology. New Biotechnol. 2011;28:136–44. http://dx.doi.org/10.1016/j.nbt.2010.10.007
5. Ventosa A, Nieto JJ, Oren A. Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev. 1998;62:504–44.
6. Ventosa A, Nieto JJ. Biotechnological applications and potentialities of halophilic microorganisms. World J Microbiol Biotechnol. 1995;11:85–94. http://dx.doi.org/10.1007/BF00339138
7. Heyndrickx M, Lebbe L, Kersters K, de Vos P, Forsyth G, Logan NA. Virgibacillus: a new genus to accommodate Bacillus pantothenticus (Proom and Knight 1950). Emended description of Virgibacillus pantothenticus. Int J Syst Bacteriol. 1998; 78:99–106. http://dx.doi.org/10.1099/00207713-48-1-99
8. Yoon JH, Kang SJ, Jung YT, Lee KC, Oh HW, Oh TK. Virgibacillus byunsanensis sp. nov., isolated from a marine solar saltern. Int J Syst Evol Microbiol. 2010;60:291–5. http://dx.doi.org/10.1099/ijs.0.009837-0
9. Tanasupawat S, Chamroensaksri N, Kudo T, Itoh T. Identification of moderately halophilic bacteria from Thai fermented fish (pla-ra) and proposal of Virgibacillus siamensis sp. nov. J Gen Appl Microbiol. 2010;56:369–79. http://dx.doi.org/10.2323/jgam.56.369
10. Sinsuwan S, Rodtong S, Yongsawatdigul J. Hydrolytic activity of Virgibacillus sp. SK37, a starter culture of fish sauce fermentation. World J Microbiol Biotechnol. 2012;25:2651–9. http://dx.doi.org/10.1007/s11274-012-1075-5
11. Sinsuwan S, Rodtong S, Yongsawatdigul J. NaCl-activated extracellular proteinase from Virgibacillus sp. SK37 isolated from fish sauce fermentation. J Food Sci. 2007;72:C264–9. http://dx.doi.org/10.1111/j.1750-3841.2007.00375.x
12. Sinsuwan S, Rodtong S, Yongsawatdigul J. Production and characterization of NaCl-activated proteinase from Virgibacillus sp. SK33 isolated from fish sauce fermentation. Process Biochem. 2008;43:185–92. http://dx.doi.org/10.1016/j.procbio.2007.11.015
13. Hoben HJ, Somasegaran P. Comparison of the pour, spread, and drop plate methods for enumeration of Rhizobium spp. in inoculants made from presterilized peat. Appl Environ Microbiol. 1982;44:1246–7.
14. Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1946;33:305–25.
15. Sinsuwan S, Rodtong S, Yongsawatdigul J. Purification of characterization of a salt-activated and organic solvent-stable heterotrimer proteinase from Virgibacillus sp. SK33 isolated from Thai fish sauce. J Agric Food Chem. 2010;58:248–56. http://dx.doi.org/10.1021/jf902479k
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227: 680–5. http://dx.doi.org/10.1038/227680a0
17. Yasothornsrikul S, Hook VY. Detection of proteolytic activity by fluorescent zymogram in-gel assays. Biotechniques. 2000; 28:1166–73.
18. Frank A, Pevzner P. PepNovo: De novo peptide sequencing via probabilistic network modeling. Anal Chem. 2005;77:964–73. http://dx.doi.org/10.1021/ac048788h
19. Saier MH, Chauvaux S, Deutscher J, Reizer J, Ye JJ. Protein phosphorylation and regulation of carbon metabolism in Gram-negative versus Gram-positive bacteria. Trends Biochem Sci. 1995;20:267–71. http://dx.doi.org/10.1016/S0968-0004(00)89041-6
20. Phrommao E. Characterization of proteinases, proteinase genes, and genome of Virgibacillus sp. SK37 isolated from Thai fish sauce [PhD thesis]. Nakhon Ratchasima, Thailand: Suranaree University of Technology; 2010.
21. Leloup L, Haddaoui EA, Chambert R, Petit-Glatron MF. Characterization of the rate-limiting step of the secretion of Bacillus subtilis α-amylase overproduced during the exponential phase of growth. Microbiology. 1997;143:3295–303. http://dx.doi.org/10.1099/00221287-143-10-3295
22. Beveridge TJ, Murray RGE. Sites of metal deposition in the cell wall of Bacillus subtilis. J Bacteriol. 1980;141:876–87.
23. Phrommao E, Rodtong S, Yongsawatdigul J. Identification of novel halotolerant bacillopeptidase F-like proteinases from a moderately halophilic bacterium, Virgibacillus sp. SK37. J Appl Microbiol. 2010;110:191–201. http://dx.doi.org/10.1111/j.1365-2672.2010.04871.x
24. Lapsongphon N, Rodtong S, Yongsawatdigul J. Spent brewery yeast sludge as a single nitrogen source for fibrinolytic enzyme production of Virgibacillus sp. SK37. Food Sci Biotechnol. 2013;22:71–8. http://dx.doi.org/10.1007/s10068-013-0010-3
25. Chauhan B, Gupta R. Application of statistical experimental design for optimization of alkaline proteinase production

from Bacillus sp. RGR-14. Process Biochem. 2004;39:2115–22. http://dx.doi.org/10.1016/j.procbio.2003.11.002

26. Saran S, Isar J, Saxena RK. Statistical optimization of conditions for protease production from Bacillus sp. and its scale-up in a bioreactor. Appl Biochem Biotechnol. 2007;141:229–39. http://dx.doi.org/10.1007/BF02729064
27. Kunst F, Rapoport G. Salt stress is an environmental signal affecting degradative enzyme synthesis in Bacillus subtilis. J Bacteriol. 1995;177:2403–7.
28. Murray EJ, Kiley TB, Stanley-Wall NR. A pivotal role for the response regulator DegU in controlling multicellular behavior. Microbiology. 2009;155:1–8. http://dx.doi.org/10.1099/mic.0.023903-0
29. Manabe K, Kageyama Y, Tohata M, Ara K, Ozaki K, Ogasawara N. High external pH enables more efficient secretion of alkaline α-amylase AmyK38 by Bacillus subtilis. Microb Cell Fact. 2012;11:74. http://dx.doi.org/10.1186/1475-2859-11-74