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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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**

**Inoculation 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 % MgSO4·7H2O) 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_{600nm}) 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 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, MgSO4·7H2O, 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 % MgSO4·7H2O, 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

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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 CaCl₂, and was incubated at 65 °C for 5 min. The activity of proteases was terminated by the addition of 1.5 mL of 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 CaCl₂ 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 (Fall 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-di-hydroxybenzoic acid and applied onto a sample holder. The peptide mass fingerprint of the samples was determined by matrix-assisted laser desorption/ionization tandem mass spectrometer (MALDI-TOF/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 (PepSwi Legend™ Micro 21 Microcentrifuge, Thermo Fisher Scientific). Sodium dodecyl sulfate-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-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 CaCl₂ 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.
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\(_4\cdot7\)H\(_2\)O, 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 metabolism of 

\[ \text{acidic pI, implying the presence of numerous negative proteins involved in the repression are also found in the HPr and the transcription factor CcpA (}} \]

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\[ \text{fructose-1,6-bisphosphate resulted in the activation of HPr(ser)-kinase, the phosphohydrolysis of HPr protein (HPr-P). Subsequently, HPr-P formed a complex with CcpA protein, which acted as a repressor of the synthesis of many proteins (}} \]

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\[ \text{These key proteins involved in the repression are also found in the whole-genome sequences of Virgibacillus sp. SK37. An increase in the mass per volume ratio of MgSO\(_4\cdot7\)H\(_2\)O in the medium positively increased the protease production (p<0.05), despite the fact that MgSO\(_4\cdot7\)H\(_2\)O had no effect on the growth (p>0.05). The secretion of \alpha\)-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 (}} \]

\[ \text{Mg\(^{2+}\) can neutralize the negative charges on the cell wall of Gram-positive bacteria (}} \]

\[ \text{The major secreted proteases from Virgibacillus sp. SK37 showed an acidic pl, implying the presence of numerous negative charges in their structures (}} \]

\[ \text{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 halophilic characteristic of the protease production of this strain has been reported (}} \]

\[ \text{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:

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

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Bacterial growth(^1)</th>
<th>Protease production(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression coefficient</td>
<td>t-value</td>
</tr>
<tr>
<td>Dried anchovy</td>
<td>0.286</td>
<td>5.099</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.154</td>
<td>2.747</td>
</tr>
<tr>
<td>MgSO(_4\cdot7)H(_2)O</td>
<td>0.048</td>
<td>0.852</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.175</td>
<td>3.118</td>
</tr>
<tr>
<td>NaCl</td>
<td>-0.003</td>
<td>-0.052</td>
</tr>
<tr>
<td>Initial pH</td>
<td>0.015</td>
<td>0.273</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>-0.197</td>
<td>-3.516</td>
</tr>
</tbody>
</table>

\(^1\) R\(^2\)=0.883; \(^2\) R\(^2\)=0.954; Significant differences at p<0.05
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 Lapsongphhon
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·7H2O, 0.2 % KNO3, 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 and initial pH of Virgibacillus sp. SK37 when the other factors were held at zero level.

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

<table>
<thead>
<tr>
<th>m(dried anchovy)</th>
<th>m(yeast extract)</th>
<th>m(NaCl)</th>
<th>Initial pH</th>
<th>Experimental</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>V(solvent) %</td>
<td>V(solvent) %</td>
<td>V(solvent) %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9 1</td>
<td>5</td>
<td>7.9</td>
<td>0.564</td>
<td>0.407</td>
<td></td>
</tr>
<tr>
<td>1 0.25</td>
<td>8.75</td>
<td>8</td>
<td>0.029</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>1 0.9</td>
<td>4.9</td>
<td>8</td>
<td>0.358</td>
<td>0.367</td>
<td></td>
</tr>
<tr>
<td>1 1.75</td>
<td>0.125</td>
<td>8</td>
<td>0.039</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>1 1.75</td>
<td>8.75</td>
<td>8</td>
<td>0.140</td>
<td>0.146</td>
<td></td>
</tr>
<tr>
<td>1.75 1</td>
<td>5</td>
<td>6.5</td>
<td>0.030</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>1.75 1</td>
<td>5</td>
<td>9.5</td>
<td>0.031</td>
<td>0.056</td>
<td></td>
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</tbody>
</table>

The prediction value was calculated according to Eq. 1.
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 levsansucrase, 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-
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

<table>
<thead>
<tr>
<th></th>
<th>0% NaCl</th>
<th>10% NaCl</th>
</tr>
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<tbody>
<tr>
<td>ALVPK</td>
<td>GAVAFSK*</td>
<td></td>
</tr>
<tr>
<td>EEEFLR*</td>
<td>GSLVLH</td>
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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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