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Original Research

Isolation and production of organic solvent tolerant protease from bacterial burn infection, *Staphylococcus aureus* KP091274

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Abstract: Organic solvent-resistant proteases are used to synthesize valuable pharmaceutical and industrial compounds. Using an available and inexpensive source can be very effective in producing this enzyme. For this purpose, *Staphylococcus aureus* KP091274 was isolated from burn infection and a medium optimization procedure in the presence of organic solvents was considered for four factors of incubation time, the concentration of Mg^{2+}, glycerol and sorbitol using the response surface methodology. The results of this statistical method showed that incubation time has the most effect and glycerol concentration has the least positive effect on enzyme secretion. As a result of applying the optimized conditions in the bacterial culture medium (3mM of Mg^{2+}, 1.5% W/V of glycerol, 0.4% W/V of sorbitol and 72 hours of incubation), the enzyme secretion reaches its maximum.

Key words: Protease; Organic solvent; Nosocomial infection; Optimization.

Introduction

Among the accidents that endanger human health and life, burn accident is one of the most severe. When the skin is damaged, pathogens invade the body and burn wounds become infected by resistant microorganisms shortly after injury, which is the leading cause of death for patients following burns (1). But these microorganisms can have both positive and negative effects (2-5). Microorganisms are the most important and major source of enzyme production. In industry, plant and animal resources produce 8% and 4% of the total enzymes, respectively, and other enzymes have a microbial origin (6, 7). Most commercial microbial enzymes are found in a small number of fungal and bacterial genera, of which Bacillus, Pseudomonas, and Staphylococcus are the best known as bacterial species (6, 8).

Bacteria are the main producers of most commercial proteases (9). However, the discovery of enzymes with specific properties has always been an interest to scientists, prompting them to search for new protease sources with a variety of properties and functions. Proteases have many applications in many industries, including peptide synthesis, protein processing, food, pharmaceuticals, and detergents. These enzymes hydrolyze the peptide bond in an aqueous medium and the absence of water, form a peptide bond (9, 10).

In general, the advantages of using enzymes in the presence of organic solvents include increasing the solubility of nonpolar substrates, changing the direction of thermodynamic equilibrium towards synthesis instead of hydrolysis, limiting reactions water-dependent side, substrate change and selectivity of chirality required in chemical and pharmaceutical industries, increase of temperature stability, removal of microbial contamination. Also, these enzymes can be used directly in the next or new chemical process. Despite these advantages, organic solvents break down the three-dimensional structure and denature the protein by separating the protein water molecules, and their use in the organic solvent medium is always associated with this great challenge (11, 12). Therefore, many efforts have been made to stabilize proteases in the presence of organic solvents, including optimizing the environmental conditions of enzymatic activity and optimizing the stability of the enzyme itself (13). Thus, finding organic solvent-resistant protease-producing bacteria is one of the interesting topics that have many capabilities in industrial and pharmaceutical biotechnology (12).

Because burn infections are an available and inexpensive source of bacterial enzyme production, many researchers use these nosocomial bacteria to isolate and produce enzymes (14). *Staphylococcus aureus* is one of these bacteria. *Staphylococcus aureus* is gram-positive cocci and optional anaerobic that appear in clusters under a microscope and its colony is yellow. This bacterium produces the enzyme catalase. The enzyme catalase breaks down hydrogen peroxide (H_2O_2) into water and oxygen. The catalase test can also be used to distinguish strap from staph. In addition to the enzyme catalase, other enzymes such as lipase, amidase and protease are produced by this bacterium for industrial and pharmaceutical uses (15-17).

In this study, our attempt was focused on the Isolation of organic solvent tolerant protease from *Staphylococcus aureus* KP091274 as a bacterial nosocomial in-

fection and optimization of effective factors on a culture medium to achieve a suitable medium for producing this enzyme in the presence of organic solvents.

Materials and Methods

Chemical compounds

Tris, tryptone, yeast extract and casein were purchased from Liofilchem (Italy). Materials required for PCR such as 10X PCR buffer, dNTP (10mM), $MgCl_2$ (20 mM) and Taq DNA polymerase were purchased from Sigma-Aldrich and other required chemicals from Merck (Germany). The required primers were also synthesized by Bioneer (Korea). The organic solvents were purchased from Merck.

Screening of organic solvent-resistant protease-producing bacteria

After the preparation of the bacteria from the bacteriological laboratory, they were cultured in a nutrient broth medium containing 30% cyclohexane and toluene for 48 hours at 37 °C (to prevent evaporation of organic solvent). After this period, 5ml of this medium was removed and inoculated into a fresh medium containing 30% cyclohexane and toluene (18). After 24 hours of growth at 37°C, 100 µl of this medium was removed and cultured on SKM-specific solid medium. The bacterial species that showed the highest halo after 48 hours of incubation was selected as the optimal strain for producing organic solvent-resistant proteases.

Strain identification

16S rDNA molecular method was used to identify the species (19). The bacteria were first cultured on a nutrient agar medium and exposed to 37°C for 24 hours.

The grown bacteria were collected from the surface of the culture medium using a loop and a suspension was prepared in sterile distilled water. The resulting suspension was centrifuged at 4°C for 10 min at 8000rpm. Genomic DNA isolation was performed using the Sigma-Aldrich kit. In order to estimate the quality and quantity of the extracted genomic DNA, the adsorption of the diluted DNA solution (at a dilution of 100) was measured at 260 and 280 nm. Using relation DNA (µg/ml) = 50.d.A_{260} Conc. and A_{260}/A_{280} ratio were estimated for DNA concentration and purity, respectively (20). The primers were designed in accordance with general primers to amplify the 16S rDNA gene as follows (21):

Forward primer: 5′-GTTAGCGGCGGACGGGTGA-3′

Reverse primer: 5′-GGTTACTCGACCGACTTCGG-3′

The PCR reaction was performed according to the following program: 1) initial temperature of 94°C, for 5 minutes; 2) 30 cycles, each includes: 94°C, 45 seconds and 54°C, 45 seconds and 72°C, 90 seconds; 3) For final replication at 72°C, for 8 minutes. The PCR product was purified after 1% agarose gel electrophoresis using a DNA extraction kit. The DNA sequence was then determined using a DNA sequencer by Bioneer (Korea).

Enzyme production and partial purification

The pre-culture medium contains the following compounds:

Nutrient broth (8 g/L), soy meal (17 g/L), peptone (10 g/L), tryptone (10 g/L), NaCl (5 g/L)

The pH of these media was increased to 6.8 before autoclaving. Enzyme culture medium included: Yeast extract (17 g/l), tryptone (10 g/L), NaCl (5 g/L).

48 hours after inoculation of the production medium with the pretreatment medium, the culture medium containing the amplified bacteria was centrifuged for 4 minutes at 4°C at 5000rpm and transferred to the supernatant, which was transferred to another vessel, MS PMSF solution (Phenylmethyl sulfonyl fluoride) was added; So that its final concentration in the culture medium is 1mM. Ammonium sulfate was added to the stirring supernatant to 65% saturation. Then it was placed at 4°C for five hours and after this time it was centrifuged at 4°C at 12000rpm for 15 minutes and the resulting precipitate was dissolved in a minimum buffer volume of 20mM Tris with pH 7.8. A concentrated protein solution containing protease activity was dialyzed at least three times at 4°C in the presence of this buffer and used for enzymatic studies.

Measurement of protease activity

Protease activity was studied by casein substrate as a natural substrate. Casein hydrolysis was measured by enzymatic solution with measurement of adsorption at 280nm with endpoint method. The 500µl reaction solution consisted of 250µl of 50mM Tris buffer with pH of 8, 200µl of 2% casein solution and 50µl of enzyme solution. This solution was incubated for 10 minutes at 50°C. Then 500µl of 10% trichloroacetic acid solution (TCA) was added and precipitated at 12000rpm for 10 minutes. The supernatant contains hydrolyzed amino acids of the casein substrate which adsorption was measured at 280nm (22). In all reactions, a control solution was used, which was added to the TCA solution before adding the enzyme. The experiments were repeated five times and their standard deviation was calculated.

Evaluation of enzyme stability in the presence of organic solvents

To evaluate the stability of the enzyme in organic solvents at room temperature, concentration (V/V) of 40% of organic solvents (dimethylformamide, methanol, isopropanol, chloroform and cyclohexane) was prepared in 50 mM Tris buffer with the desired pH. Equal amounts of this solvent and enzyme were then mixed in a micro-tube. The final solvent concentration was 20% at incubation (V/V). The micro-tubes were then incubated at 25°C for 3 hours. After this period, the amount of residual enzyme activity was evaluated (23). The final solvent concentration (V/V) was two percent.

Experimental design and optimization

The response surface methodology was done in order to find the optimal medium for enzyme production in the presence of a different organic solvent. The response surface methodology consists of a set of experiments that evaluate the relationship between the factors and measure the resulting response (24). Studies have shown that a variety of factors can affect bacterial growth and enzyme secretion, including the presence of inducers such as surfactants, long-chain substrates, and metal ions (25). Previously, the amount and effec-

tive concentration of various factors for *Staphylococcus aureus* KP091274 were measured by the univariate method. Among these various factors, incubation time, the concentration of Mg^{2+}, glycerol and sorbitol were more effective on enzyme secretion than others. In the next step, the Behnken-Box method was used to study the effect of four effective components on the culture medium. The experiments were performed in 100ml Erlenmeyer flasks containing 20ml of culture medium with 2ml of the organic solvents (2% V/V of dimethylformamide, methanol, isopropanol, chloroform and cyclohexane) at 30°C and aerated at 180rpm. The 3-level-4-factor Box-Behnken factorial design with 5 replications at the center point was used to determine the quadratic response surface. Regression analysis was performed to evaluate the accuracy of the results. For this purpose, enzyme activity is considered as a dependent variable or response (Y). Based on the results of this method, a quadratic polynomial model was considered to predict the response. The proposed model for each response (Y) was as equation 1:

$Y = \beta0 + \beta1X1 + \beta2X2 + \beta3X3 + \beta4X4 + \beta22X22 + \beta33X32 + \beta44X42 + \beta12X1X2 + \beta13X1X3 + \beta14X1X4 + \beta23X2X3 + \beta24X2X4 + \beta34X3Xs$ (Equation 1)

Where Y is the predicted response, β_0 is the model constant, X_1, X_2, X_3 and X_4 are the independent variables, β_3, β_2, β_1 and β_4 are linear coefficients and β_{11}, β_{22}, β_{33} and β_{44} are quadratic coefficients.

Design-Expert Software (V. 8.0.4 Trail, 2010) was used for regression analysis and plotting. The optimal amount and concentration of the above variables have been determined with the help of contour diagrams. Statistical analysis of the model is shown in the form of data analysis of variance (ANOVA).

Results and discussion

Measurement of protease activity

In the first stage, 91 samples of *Staphylococcus aureus* were isolated from burn infections, among which 48 samples showed protease activity. Finally, a sample that showed better protease activity was selected.

Identification of *Staphylococcus aureus* strain KP091274

According to bacteriological methods (26), this strain belongs to the genus Staphylococcus. Ribosomal RNA molecules play an important role in the production and synthesis of bacterial proteins and are conserved in all living organisms, and their sequences are well used for the phylogenetic classification of bacteria. For this purpose, the 16S rDNA fragment of the bacterium was amplified and its sequence was determined. The length of this amplified fragment is 1374bp. 16S rDNA sequences for the genus Staphylococcus were provided by NCBI Website (http://www.ncbi.nlm.nih.gov) and they were compared by ClustalW (version 1.82). The phylogenetic tree was drawn by the nearest neighbor method. Multiple comparisons of the sequences and the phylogenetic tree showed that the strain KP091274 belongs to the genus Staphylococcus and it is 99% similar in sequence to the 16S rDNA of *Staphylococcus aureus* (Figure 1).

Fitting the model

Preliminary results in this study showed that incubation time, the concentration of Mg^{2+}, glycerol and sorbitol increase enzyme secretion. As shown in Table 1, the values of these factors are considered as three codes -1, 0 and 1 for low, medium and high values, respectively. To optimize the culture medium, 29 experiments or Runs are designed that includes the effect of all four factors at different levels (Table 2).

According to Table 2, the Box-Behnken method designs a set of 29 experiments with 5 replications at the center point. The resulting model is shown by Equation 2, in which the activity of Protease (Y) is a function of the amount of independent variables.

Activity (U/ ml) = +9.76+3.58 X1 -1.68X2 +2.93X3+ 6.92 X4 -1.88X1X2 -0.22X1X3 +0.40 X1X4 +0.65X2X3 -4.55X2X4 +1.15X3X4 +1.17X12 +0.18X22 +0.98X32 +3.74X42 (Equation 2)

As shown in Equation 2, the coefficient of the three factors (Mg^{2+}, sorbitol, and incubation time) is positive, while the coefficient of the glycerol factor is negative. The negative effect of glycerol on the response function

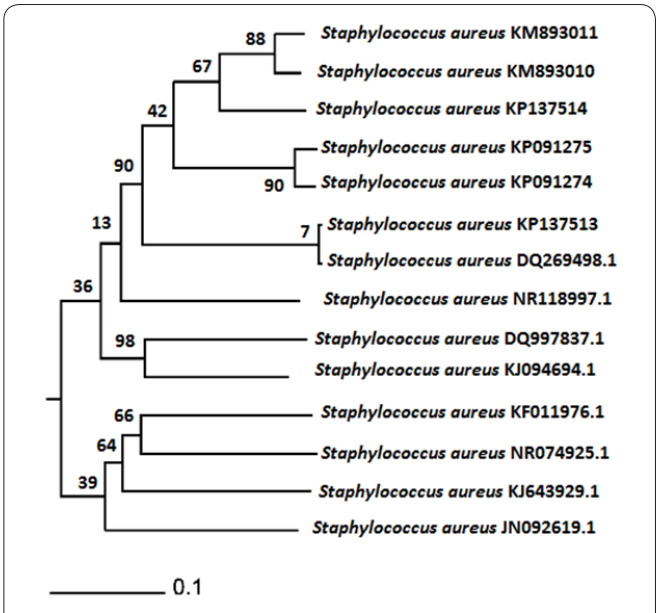

Figure 1. Drawing a phylogenetic tree of 16S rDNA ribosomal sequence of Staphylococcus using ClustalW software.

Table 1. Selected levels and codes for Mg^{2+}, glycerol, sorbitol, and incubation time used to Box-Behnken experiment design.

Independent factors	Factor	Unit	Coded levels		
			-1	0	1
Mg^{2+}	X1	mM	1.5	2.25	3
Glycerol	X2	%W/V	1.25	1.87	2.5
Sorbitol	X3	%W/V	0.2	0.3	0.4
Incubation Time	X4	h	24	48	72

Table 2. Box-Behnken design table with measured and predicted answers. Enzyme activity is considered as the response.

Run	Factor X1	Factor X2	Factor X3	Factor X4	Measured answer A(U/ml)	Predicted answer A(U/ml)
1	-1	0	0	1	17.6	17.6
2	0	0	0	0	10.1	9.7
3	0	0	0	0	10	9.7
4	1	1	0	0	11.5	11.1
5	1	0	0	1	25.6	25.5
6	1	1	1	0	10.2	9.7
7	1	0	-1	0	12.1	12.7
8	-1	0	-1	0	5.2	5.1
9	1	0	1	0	17.3	18.1
10	1	-1	0	0	19.2	18.2
11	0	-1	1	0	14.7	14.8
12	0	-1	0	-1	3.7	3.8
13	0	-1	0	1	26.1	26.8
14	1	0	0	-1	11.2	10.9
15	0	1	0	-1	9.5	9.6
16	0	1	0	1	13.7	14.3
17	0	0	1	1	26.3	25.4
18	0	1	1	0	13.2	12.8
19	-1	0	1	0	11.3	11.4
20	0	0	0	0	7.4	9.7
21	1	-1	0	0	7.4	7.7
22	0	0	-1	1	17.9	17.3
23	0	0	-1	-1	5.6	5.7
24	0	-1	-1	0	10.2	10
25	-1	0	0	-1	4.8	4.5
26	0	0	0	0	8.4	9.7
27	0	0	1	-1	9.4	9.3
28	0	1	-1	0	6.1	5.6
29	1	-1	0	0	7.6	7.3

indicates that higher values of this parameter lead to lower protease production. Among these three parameters, the incubation time has the most effect, which its linear coefficient indicates this fact. Then the concentration of Mg^{2+} (with a coefficient of 3.58) and sorbitol (with a coefficient of 2.93) have the greatest effect on enzyme production and secretion, respectively. Rahman et al. (27) previously reported that incubation time is the most effective factor in protease secretion. Statistical evaluation of Equation 2 was performed for quadratic response surface methodology by F-test and analysis of variance (ANOVA) and the result is given in Table 3. ANOVA data confirm the accuracy of this quadratic model. Parameter F is a measure of the deviation of data from the mean value. In general, for a model that successfully predicts test results, the value of F is usually very high. The value of the Prob> F parameter less than 0.0500 also means that the model is significant. The F-value for this model was 95.214, which indicates that the model is quite significant. In this case, there will be only 0.01% error probability for the model. In this equation, linear parameters include Mg^{2+} concentration (X_1), glycerol concentration (X_2), sorbitol concentration (X_3) and incubation time (X_4), and quadratic parameters X_1^2, X_3^2 and X_4^2 along with the interaction parameters of Mg^{2+} and glycerol concentrations (X_1X_2). The concentration of glycerol and incubation time (X_2X_4) and the concentration of sorbitol and incubation time (X_3X_4) are the index parameters of the model. The results showed that all four independent factors have significant effects on enzyme activity. The mismatch calculation can be a good reason for the accuracy of the model data. In fact, the non-significance of this parameter is desirable and it means that this model can predict the activity of the enzyme in different conditions of the combination for 4 independent factors. The value of this parameter is not significant for the regression of Equation 2 (P= 0.6077). In examining the model, the question arises as to what extent can a change in response be justified? To answer this question, the value of R-squared must be measured. The value of this parameter for protease production is 0.9931, which indicates the accuracy of the model. The closer R-squared value to 1, better relationship is calculated between the laboratory and the results. Finally, adequate precision measures the signal-to-noise ratio. A ratio greater than 4 is acceptable. For this model, the signal-to-noise ratio was 43.719, which indicates a high signal-to-noise ratio.

Analysis of response surface methodology

The effects of four factors on protease activity are shown in Figure 2 in the form of graphic designs of

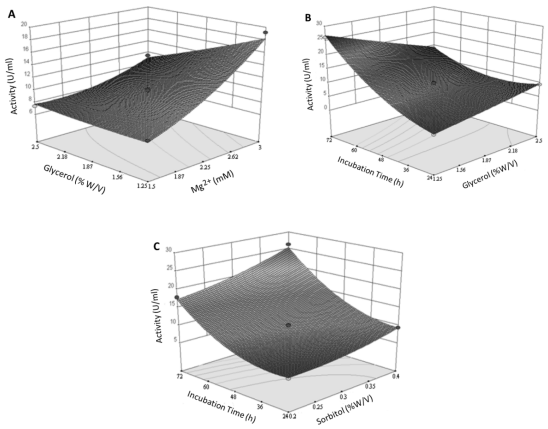

Figure 2. (A) Effect of Mg^{2+} and glycerol concentration on protease activity. Sorbitol concentration and incubation time are considered constant. (B) Effect of Incubation time and glycerol concentration on protease activity. Sorbitol and Mg^{2+} concentrations are kept constant. (C) Effect of Incubation time and sorbitol concentration on enzyme activity. Mg^{2+} and glycerol concentration are kept constant.

response surface and contour diagram. In each of these images, the response surface diagrams are plotted as a function of two parameters and the other two parameters are considered constant. By examining the shape of the response surface diagrams (elliptical or circular), it can be determined whether the relationship between the parameters is significant or not (28). The contour diagram between glycerol and Mg^{2+} and between incubation time and sorbitol is elliptical, in other words, the interaction of these parameters can have significant effects on enzyme activity. The effect of the interaction between glycerol and Mg^{2+} concentrations on enzyme activity is shown in Figure 2A. According to this figure, increasing the concentration of Mg^{2+} increased the production of the enzyme, while increasing the concentration of glycerol not only did not have much effect on the activity of the enzyme but even had an inhibitory effect on the activity of the enzyme at higher concentrations. This result can also be seen in Figure 2B. In this diagram, increasing the concentration of glycerol does not have a positive effect on enzyme activity and incubation time at low concentrations of glycerol is more effective on enzyme activity. Thus, to produce the maximum amount of protease, the concentration of glycerol must be kept at its minimum (1.5% W/V). Also, the results showed that at constant concentrations of glycerol and Mg^{2+}, both incubation time and sorbitol factors had a positive effect on enzyme production (Figure 2C). Figures 2B and 2C emphasize the key role of incubation time in enzyme secretion. This result is consistent with Equation 2.

Experimental approval of the model

To confirm the above results, *Staphylococcus aureus* KP091274 were cultured under the optimal conditions proposed by the model (3mM of Mg^{2+}, 1.5% W/V of glycerol, 0.4% W/V of sorbitol and 72 hours of incubation) and the resulting enzyme activity was measured (35.7 U/ml), which is very close to the predicted value (38.04 U/ml). In this way, the model can well predict the results of experiments and optimize the culture medium of the bacterium in the presence of organic solvents.

Organic solvent-resistant bacteria have received special attention due to their enzymes with high resistance and efficiency in the presence of organic solvents. Solvent-resistant proteases can overcome the challenge of inactivating proteases in organic solvents without the use of costly and time-consuming methods of protein engineering. On the other hand, using burn infection as an inexpensive source of bacteria can make the production of this enzyme very cost-effective. The results of this study showed that *Staphylococcus aureus* KP091274, as a nosocomial infection, is a protease generator that has significant stability in the presence of organic solvents. Therefore, this enzyme can be used in industry to synthesize valuable pharmaceutical and industrial compounds in the presence of organic solvents. Also, this study provided a model that predicted the results of experiments and optimized the culture medium for the bacterium in the presence of organic solvents.

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