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# Effects of culture conditions and surfactants on marine lysozyme S-12 production by *Bacillus* sp isolated from East China sea

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**Effects of culture conditions and surfactants on marine lysozyme s-12 production using a statistical approach were studied in this work. Analysis of results were based on statistical calculations carried out with the one factor-at-a-time method and the $L_{16}$-orthogonal array method, using MINITAB 15.0 software. All the fermentation runs were carried out at 30±2°C on a rotary orbital shaker at 200 rpm for 24 h. The individual components selected affected the fermentation process were $CaCl_2$ > glucose >peptone > beef extract >$MgSO_4$. Furthermore, Tween 80 with 0.4 mM could enhance the production of all the extracellular proteins and S-12. The final optimized medium supported S-12 activity of 1971.25 U/mL at the end of 24 h as compared with 1095.00 U/mL before optimization.**

**Key words:** Lysozyme S-12, culture condition, surfactant, optimization, $L_{16}$-orthogonal array.

## INTRODUCTION

Lysozymes are widespread in nature and have been isolated from a variety of organisms (Ibrahim et al., 2001). The most extensively studied enzyme is the chicken-type (c-type) lysozyme, but there is no obvious sequence homology between one family and another (Saedi et al., 1987). Lysozymes have been widely used in food, pharmaceutical and biological applications for many years (Bachali et al., 2002; Hawiger, 1968; Proctor et al., 1988; Yu et al., 2002). Marine-derived lysozyme is a new class recently extracted from marine organisms (Nilsen et al., 1999; Ye et al., 2008). It has been widely recognized that marine organisms need to retain their activity at low temperature, hypoxia, and high-pressure environment, marine-derived lysozyme exhibits special activity compared to vertebrate lysozyme (Faulkner, 2001; Sakai et al., 1995). In our previous studies, a novel lysozyme S-12 from *Bacillus* sp isolated from East China Sea was found (Zhang et al., 2008a). It with pH and temperature stability, strong antibacterial activity and high resistance of va-Gram-positive and Gram- negative bacteria, can be very useful in related applications (Yang et al., 2005; Zhang et al., 2008b). But S-12 fermentation levels in the microbial system are too low to be separated and used for industrial preparations. Therefore, it is necessary to improve fermentation conditions to obtain maximum S-12 levels. In this study we address optimization of lysozyme S-12 production using a statistical approach. The effects of various surfactants added on S-12 production were studied with the aim of obtaining high yields of lysozyme.

## MATERIALS AND METHODS

### Media components

Glucose, lactose, sucrose, maltose, dextrine, soluble starch, peptone, beef extract, yeast extract, sodium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate, ammonium nitrate and urea used in the study were purchased from Shuang - xuan, Beijing,

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China. The surfactants used were: polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monooleate (Tween 80), polyethylene glycoloctylphenol ether (Triton X-100) and sorbitan monooleate (Span 80). All surfactants were purchased from China National Pharmaceutical Group Corporation. All the other chemicals were of analytical grade and commercially available.

**Bacterial strain and medium**

The strain of *Bacillus* sp. used in the present investigation was isolated from sea mud at the bottom of the East China Sea. The basal medium used for lysozyme production consisted of 1.0% tryptone, 0.3% beef extract and 0.5% NaCl (pH 6.5~7.0). Incubation was carried out at 30°C in a rotary shaker, with stirring at 200 rpm.

**Inoculum and fermentation**

A loopful of cells from a slant was transferred to 25 ml of the above mentioned sterile medium in a 250 ml conical flask and incubated at 30°C and 200 rpm for 18 h. This was used as the inoculum. Fermentation was carried out in 500 ml Erlenmeyer flasks, each containing 100 ml of the sterile production medium. The medium was inoculated with 6% (v/v) of 18 h old culture, and the five nutrients (glucose, peptone, beef extract, $CaCl_2$ and $MgSO_4$) were selected for optimizations according to the requirement of experimental design. The inoculated flasks were kept on a rotary shaker at 30°C and 200 rpm.

**Optimization of fermentation medium using one factor-at-a-time method**

***Effects of different carbon sources***

In the basal medium, six different carbon sources were added, viz., glucose, lactose, sucrose, maltose, dextrine and soluble starch. All carbon sources were used at 2% concentration.

***Effects of different nitrogen sources***

To study the effects of different nitrogen sources on lysozyme S-12 production, both beef extract and peptone were replaced with other organic nitrogen sources, such as peptone, beef extract, yeast extract and inorganic nitrogen sources, such as sodium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate, ammonium nitrate and urea at 1.5% concentration.

***Effect of pH***

To study the effect of pH on lysozyme S-12 production, fermentation runs were carried out at initial pHs varying between 6.0 – 9.0.

***Effects of different metal ions***

To study the effect of metal ion on lysozyme S-12 production, NaCl was substituted with other different metal salts, such as $MgSO_4$, $ZnCl_2$, $CaCl_2$, $FeSO_4$, $CuSO_4$, $BaCl_2$ and $KH_2PO_4$. All salts were used at 0.5 % concentration.

***ANOVA***

One-way ANOVA was used to provide evidence that there was significantly different in the dry cell weight (DCW) and enzyme activity among the different culture conditions.

**Optimization of concentrations of the selected medium components using the orthogonal matrix method**

The design for the orthogonal array was developed and analyzed using Minitab(R) 15.0 software. Effects of the C/N ratio, $MgSO_4$ and $CaCl_2$, which were optimized by the $L_{16}$-orthogonal array, were studied.

**Use of surfactants for optimization**

To study the effects of addition of different surfactants on lysozyme S-12 production, Tween 20, Tween 80, Span 80, or Triton X-100 were added at 0.5 mM in the medium, which was optimized by the orthogonal array method. Different concentrations of Tween 80 (0.1-0.8 mM) were added and their effects on lysozyme S-12 production by Bacillus sp. were studied.

**Analytical determinations**

Determination of dry cell weight (DCW) Fermentation broth (25 mL) was withdrawn from the flask at different time intervals, diluted it with an equal volume of distilled water and heated to 80–90°C in a water bath for 10–15 min. The diluted broth was then centrifuged at 8000×g at 4°C for 30 min to separate the cell mass. The detained cell pellet was then dried in hot air oven at 80°C for 6 h (Bajaj et al., 2006).

Enzyme activity and protein concentration assays Lysozyme activity was determined by a diffusion assay done in agar plates with cells of *Micrococcus lysodekticus* (Sigma). The well-diffusion assay, done in Petri plates, consisted of 10 ml of tryptic soy agar (TSA) containing 2.5 × 105 cells of M. lysodekticus. Wells (3 mm in diameter) were cut in the agar and the agar plugs then removed. The wells were filled with 10 ml of the column fractions (Lockey and Ourth, 1996). After 24 h at 30°C, the zone of inhibition was measured in mm using a vernier caliper. One sample repeated 3 times, the data averaged. A standard curve was prepared by serially diluting a 1 mg/ml stock solution of egg white lysozyme. The lowest detectable level for this method was 0.6 mg/ml of egg white lysozyme. The correlation coefficient of standard curve was greater than 0.99 ($p<0.05$), and relative standard differences (RSD) were less than 10%.

The extracellular proteins were measured by the Bradford method with bovine serum albumin as the standard (Lowry et al., 1951).

## RESULTS AND DISCUSSION

### Optimization by one-factor-at-a-time

During microbial fermentation, the carbon source, as an energy source, is a major constituent for the building of cellular materials. Figure 1 shows the effects of different carbon sources on lysozyme S-12 production. It is observed that the carbon source supporting the maximum production of DCW results in the maximum lysozyme S-12 production and vice versa. Glucose supported the maximum production of DCW 0.39 (g/l ) and the maximum activity of lysozyme S-12 (1022.50 U/mL) at the end of 24 h of fermentation.

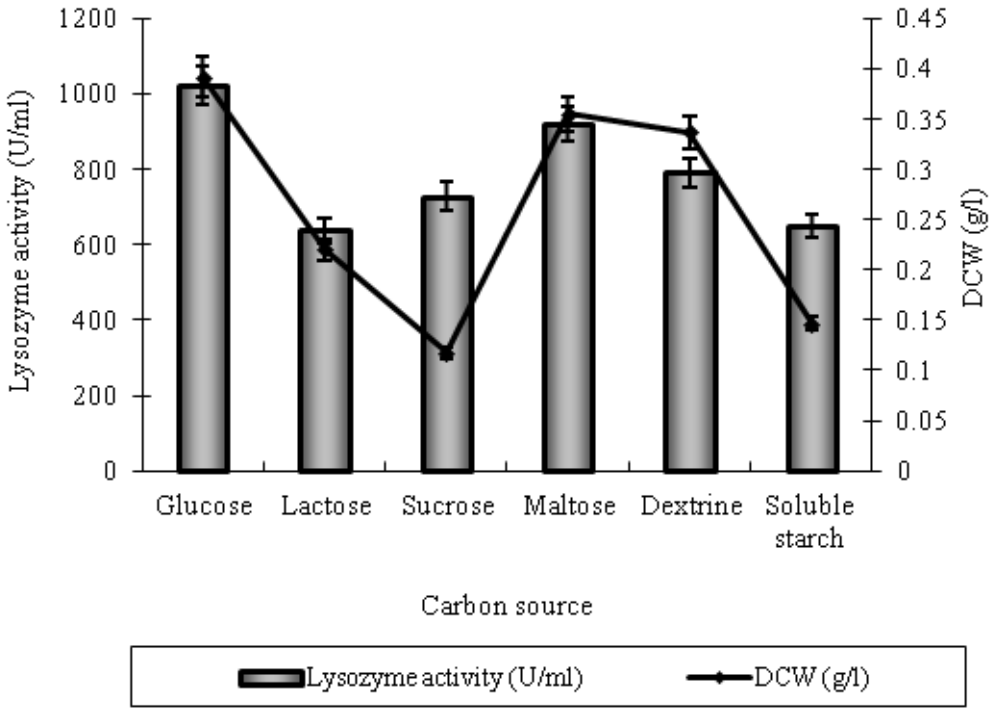


**Figure 1.** Effect of different carbon sources on lysozyme S-12 production by *Bacillus* sp. Incubation was carried out for 24 hour on a rotary shaker at 30°C and 200 rpm. Six different carbon sources were added in the basal medium at 2% concentration. Values are mean ±S.E.M. of triple determinations. Analysis of variance revealed significant differences between the groups ($P < 0.01$).

The nitrogen source is important for metabolite formation, growth, and cell composition, and is also used in the synthesis of enzyme. Figure 2 shows the effects of different organic and inorganic nitrogen sources on lysozyme S-12 production. It was found that Ammonium nitrate gave a higher biomass and the yield of lysozyme S-12 was lower, whereas organic nitrogen sources were able to increase the yield of S-12, but did not support biomass production. As organic nitrogen sources are complex containing amino acids and vitamins, it could be assumed that these constituents in the nitrogen source could be responsible for improving the yield of S-12. Among the various nitrogen sources used, only beef extract and peptone were found to be useful. Peptone supported the maximum activity of (1087.50 U/mL) S-12, whereas beef extract got S-12 with an activity of 1033.75 U/mL at the end of 24 h. Among the inorganic nitrogen sources, ammonium sulphate supported the maximum activity of (753.50 U/mL) lysozyme S-12. Various reports suggest that the utilization of a carbon source depends on the type and concentration of a nitrogen source used in the medium (Ashtaputre and Shah, 1995).

Figure 3 shows the effect of different initial pH values on lysozyme S-12 production. An Initial pH of 8.0 supported the maximum S-12 activity of 1650.00 U/mL but the biomass was lower. It also explained the maximum lysozyme S-12 production at slight-alkaline pH. Figure 4 shows the effects of different metal ions on lysozyme S-12 production. It was found that $ZnCl_2$ gave a higher biomass and the yield of lysozyme S-12 was lower, whereas $CaCl_2$ was able to increase the yield of S-12, but did not support biomass production. Among the metal ions used, only $CaCl_2$ and $MgSO_4$ were found to be useful. They supported the maximum activity of (1042.50 and 1047.50 U/mL) S-12 at the end of 24 h.

### Optimization using $L_{16}$-orthogonal array

Once the best carbon and nitrogen sources were selected, the medium was subjected to final optimization using the $L_{16}$-orthogonal array. The parameters optimized were the concentrations of Glucose, peptone, beef extract, $CaCl_2$ and $MgSO_4$. Medium optimization by the one-factor-at-time method involved changing one variable (example nutrients, pH, and temperature) while fixing the others at a certain arbitrary level. Because most industrial experiments usually involve a significant number of factors, a full factorial design entails a large number of experiments. To reduce the number of experiments to a practical level, only a small set from all the possibilities is selected. Taguchi constructed a special set of general design guidelines for factorial experiments that cover many applications. In this method, a special set of arrays called orthogonal arrays is used which stipulates the way

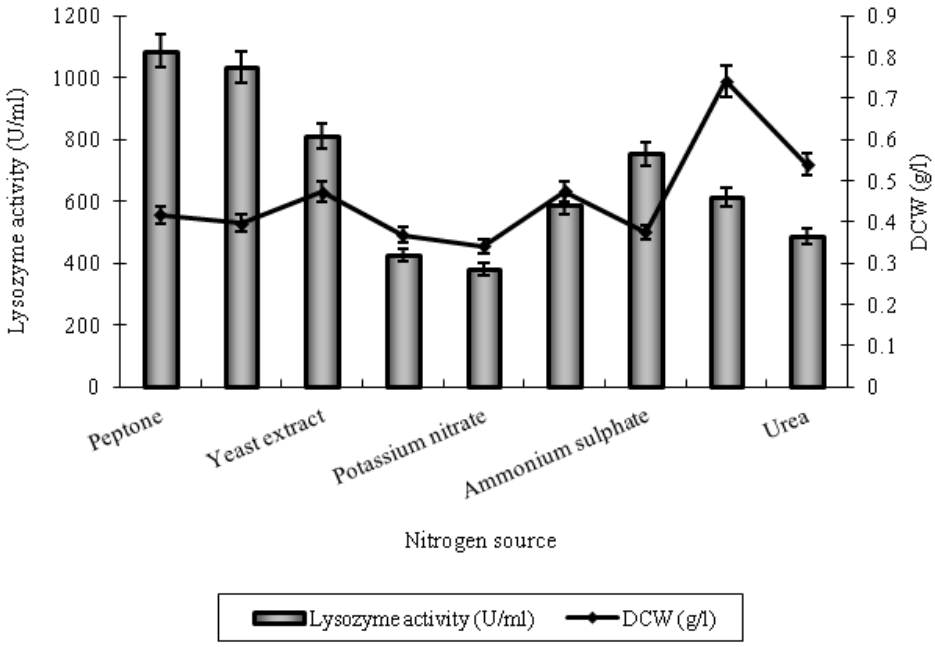


**Figure 2.** Effect of different nitrogen sources on lysozyme S-12 by by *Bacillus* sp. Incubation was carried out for 24 hour on a rotary shaker at 30°C and 200 rpm. Nine different nitrogen sources were added in the basal medium at 1.5% concentration. Values are mean ±S.E.M. of triple determinations. Analysis of variance revealed significant differences between the groups ($P < 0.01$).

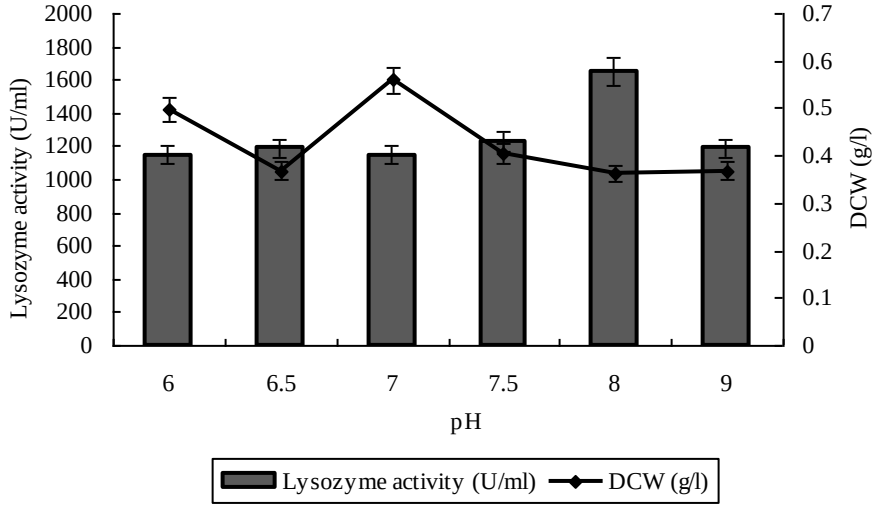


**Figure 3.** Effect of different pH on lysozyme S-12 by by *Bacillus* sp. Incubation was carried out for 24 hour on a rotary shaker at 30°C and 200 rpm. fermentation runs were carried out at initial pHs varying between 6.0 – 9.0. Values are mean ±S.D. of triple determinations. Analysis of variance revealed significant differences between the groups ($P < 0.01$).

of conducting the minimal number of experiments, and provide the complete information of all the factors that affect a performance parameter. While there are many standard orthogonal arrays available, each of the arrays is meant for a specific number of independent design variables and levels. The additive assumption of the Taguchi design implies that the individual or main effects of independent variables on a performance parameter are separable. Under this assumption, the effect of each factor can be linear, quadratic or may have a higher

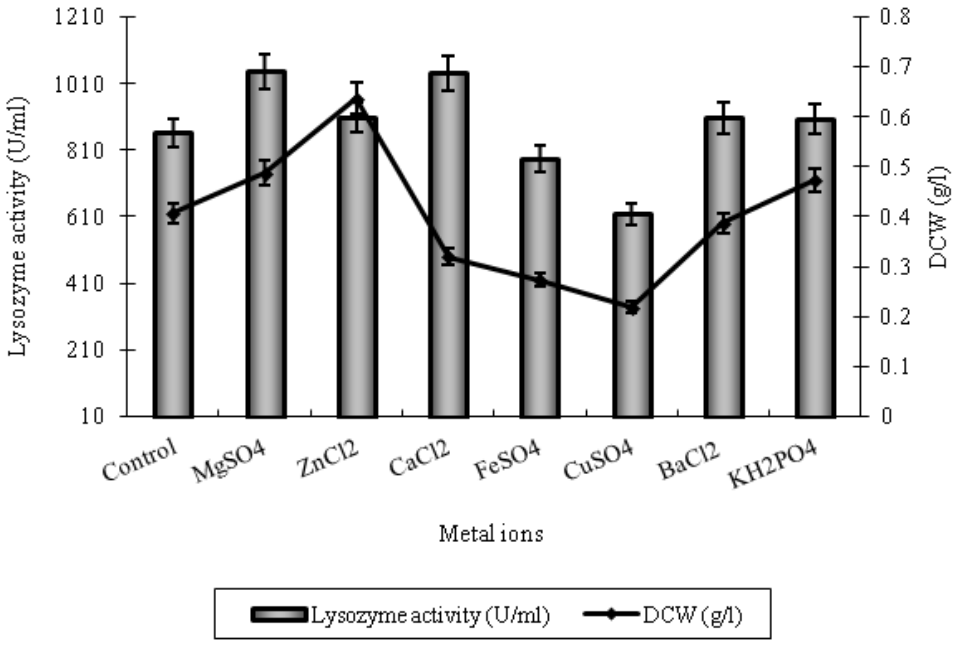


**Figure 4.** Effect of metal ions on lysozyme S-12 by by *Bacillus* sp. Incubation was carried out for 24 hour on a rotary shaker at 30°C and 200 rpm. Eight different metal ions were added in the basal medium at 0.5% concentration. Values are mean ±S.E.M. of triple determinations. Analysis of variance revealed significant differences between the groups ($P < 0.01$).

order, but the model assumes the absence of any cross product effects (interactions) among the individual factors. This means that the effect of independent variable 1 on a performance parameter does not depend on the different level settings of any other independent variables and vice versa. The crux of the orthogonal arrays method lies in choosing the level combinations of input design variables for each experiment (Bajaj et al., 2006; Chen et al., 2002; Xu et al., 2003).

The results of variance analysis are shown in Table 2. Repeated measures and general linear model were used to determine the optimal results, the more accurate regression coefficients and the standard errors were obtained. Table 3 shows the response table for means (larger is better) and for signal-to-noise ratio obtained using the $L_{16}$-orthogonal array. The last two rows in the tables show delta values and ranks for the system. Rank and delta values help in assessing which factors have the greatest effect on the response characteristic of interest. Delta measures the size of the effect by taking the difference between the highest and lowest characteristic average for a factor. A higher delta value indicates a greater effect of that component. Rank orders the factors from the greatest effect (on the basis of the delta values) to the least effect on the response characteristic. The order in which the individual components selected in the present study affected the fermentation process were $CaCl_2$>glucose>peptone>beef extract>$MgSO_4$ suggesting that $CaCl_2$ has a major effect and $MgSO_4$ has least effect on lysozyme S-12 production by *Bacillus* sp (Table 1).

Figure 5 shows the main effect plots for the system, which show how each factor affects the response characteristic. The main effect is present when different levels of a factor affect the characteristic differently. MINITAB generates the main effect plot by plotting the characteristic average for each factor level. These averages are the same as those shown in Table 3. A line connects the points for each factor. When the line is horizontal (parallel to the x-axis), then there is no main effect present. Each level of the factor affects the characteristic similarly and the characteristic average is the same across all factor levels. When the line is not horizontal (parallel to the x-axis), then there is a main effect present. Different levels of the factor affect the characteristic differently. The greater the difference in the vertical position of the plotted points (the more the line is not parallel to the x-axis), the greater the magnitude of the main effect. In the present study it was noted that for each of the five variables at four levels, one level increased the mean compared with the other level. This difference was the main effect that is glucose at level 2, peptone at level 2, beef extract at level 1, $CaCl_2$ at level 2, $MgSO_4$ at level 3 exerted the main effect. These levels also represented the optimal concentrations of the individual components in the medium.

Response tables can also be used to predict the optimal concentration of each component used in the study. To obtain the optimized level or composition of each factor, the intuitive analysis based on statistical calculations is shown in Table 3. To confirm the results

**Table 1.** $L_{16}$ orthogonal array for lysozyme S-12 production.

| Run | Component | | | | | Medium component (%) | | | | | Activity (U/mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | A | B | C | D | E | |
| 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 0.0 | 0.0 | 0.1 | 0.1 | 940.00±10.80 |
| 2 | 1 | 2 | 2 | 2 | 2 | 0.5 | 0.5 | 0.5 | 0.3 | 0.3 | 963.75±28.39 |
| 3 | 1 | 3 | 3 | 3 | 3 | 0.5 | 1.0 | 1.0 | 0.5 | 0.5 | 907.50±21.02 |
| 4 | 1 | 4 | 4 | 4 | 4 | 0.5 | 1.5 | 1.5 | 0.7 | 0.7 | 151.25±8.54 |
| 5 | 2 | 1 | 2 | 3 | 4 | 1.0 | 0.0 | 0.5 | 0.5 | 0.7 | 945.00±30.28 |
| 6 | 2 | 2 | 1 | 4 | 3 | 1.0 | 0.5 | 0.0 | 0.7 | 0.5 | 1146.25±23.94 |
| 7 | 2 | 3 | 4 | 1 | 2 | 1.0 | 1.0 | 1.5 | 0.1 | 0.3 | 920.00±23.80 |
| 8 | 2 | 4 | 3 | 2 | 1 | 1.0 | 1.5 | 1.0 | 0.3 | 0.1 | 1023.75±35.91 |
| 9 | 3 | 1 | 3 | 4 | 2 | 1.5 | 0.0 | 1.0 | 0.7 | 0.3 | 255.00±12.91 |
| 10 | 3 | 2 | 4 | 3 | 1 | 1.5 | 0.5 | 1.5 | 0.5 | 0.1 | 945.00±21.60 |
| 11 | 3 | 3 | 1 | 2 | 4 | 1.5 | 1.0 | 0.0 | 0.3 | 0.7 | 953.75±12.50 |
| 12 | 3 | 4 | 2 | 1 | 3 | 1.5 | 1.5 | 0.5 | 0.1 | 0.5 | 950.00±15.81 |
| 13 | 4 | 1 | 4 | 2 | 3 | 2.0 | 0.0 | 1.5 | 0.3 | 0.5 | 872.50±20.62 |
| 14 | 4 | 2 | 3 | 1 | 4 | 2.0 | 0.5 | 1.0 | 0.1 | 0.7 | 897.50±21.02 |
| 15 | 4 | 3 | 2 | 4 | 1 | 2.0 | 1.0 | 0.5 | 0.7 | 0.1 | 117.50±6.45 |
| 16 | 4 | 4 | 1 | 3 | 2 | 2.0 | 1.5 | 0.0 | 0.5 | 0.3 | 801.25±21.36 |
| Control | | | | | | 0 | 1 | 0.3 | 0.5 | 0.5 | 1095.00±21.73 |

A, Glucose; B, peptone; C, beef extract; D, $CaCl_2$; E, $MgSO_4$. The arrangements of column A, B, C, D and E were decided by orthogonal design for 5 (factor) 16 (run number); every row of run number represents one experimental replicate, every run was replicated 4 times. Values are mean ±S.E.M. of triple determinations.

**Table 2.** Analysis of variance for linear model according to lysozyme activity.

| Source | DF | Seq SS | Adj SS | Adj MS | *F*-value |
|---|---|---|---|---|---|
| A | 3 | 1024247 | 1024247 | 341416 | 760.02** |
| B | 3 | 767078 | 767078 | 255693 | 569.19** |
| C | 3 | 571631 | 571631 | 190544 | 424.17** |
| D | 3 | 3134122 | 3134122 | 1044707 | 2325.61** |
| E | 3 | 618834 | 618834 | 206278 | 459.19** |
| Error | 48 | 21562 | 21562 | 449 | |
| Total | 63 | 6137475 | | | |

DF; Degree of freedom, SS; Sum of squares, MS; Mean Square, Column data marked with different superscripts mean significant difference. *: P<0.05; **: P<0.01.

**Table 3.** Response for means and S/N ratio.

| Level | A | | B | | C | | D | | E | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | S/N | Mean | S/N | Mean | S/N | Mean | S/N | Mean | S/N |
| 1 | 740.6 | 55.46 | 753.1 | 56.47 | 960.3 | 59.57 | 926.9 | 59.33 | 756.6 | 55.13 |
| 2 | 1008.8 | 60.04 | 988.1 | 59.85 | 744.1 | 55.02 | 953.4 | 59.56 | 735.0 | 56.28 |
| 3 | 775.9 | 56.69 | 724.7 | 54.84 | 770.9 | 56.63 | 899.7 | 59.06 | 969.1 | 59.67 |
| 4 | 672.2 | 54.33 | 731.6 | 55.34 | 722.2 | 55.29 | 417.5 | 48.55 | 736.9 | 55.43 |
| Delt | 336.6 | 5.71 | 263.4 | 5.01 | 238.1 | 4.55 | 535.9 | 11.01 | 234.1 | 4.54 |
| Rank | 2 | | 3 | | 4 | | 1 | | 5 | |

A, Glucose; B, peptone; C, beef extract; D, CaCl2; E, MgSO4. The orthogonal array was analyzed using Minitab(R) 15.0 software. The ranks were sorted by main effects for Means and Noise Ratios. The order in which the individual components selected in the present study affected the fermentation process were CaCl2 > glucose >peptone > beef extract >MgSO4.

obtained by orthogonal array method, experiments were carried out using these nutrient concentrations and it was

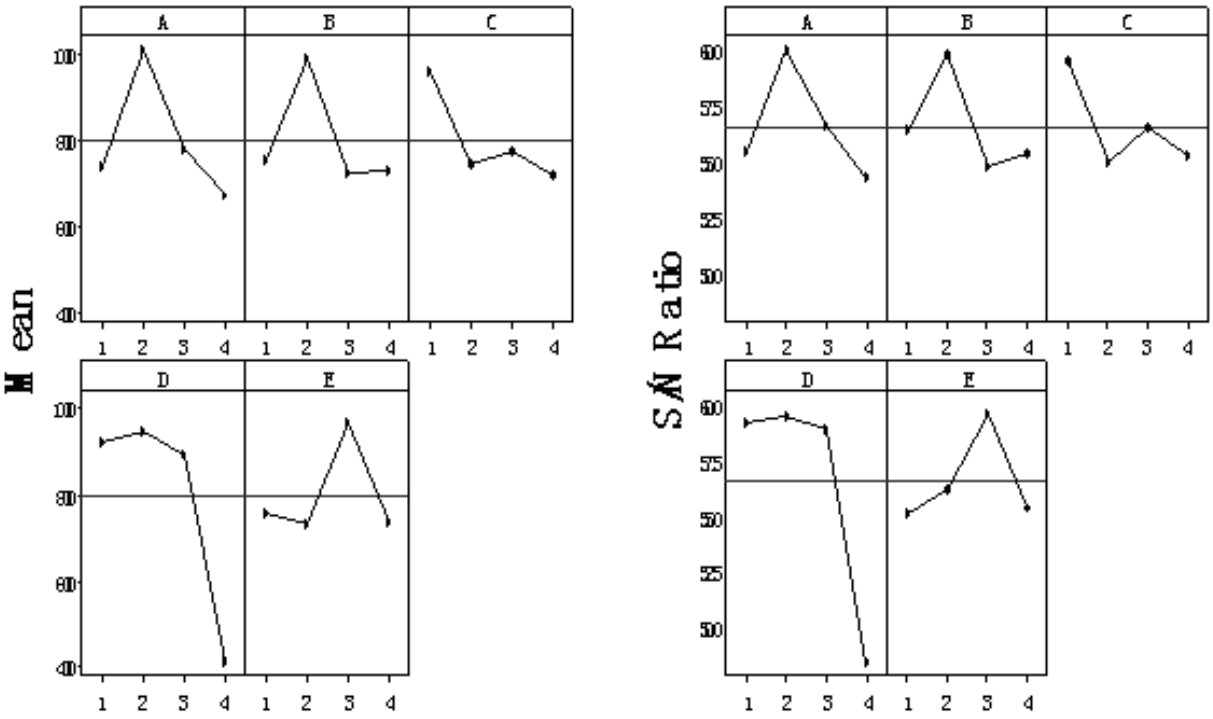


**Figure 5.** Main effect plot for mean and S/N Ratio for different concentrations of glucose (A), peptone (B), beef extract (C), CaCl2 (D) and MgSO4 (E). The main effect was plot by plotting the characteristic average for each factor level. A line connects the points for each factor. These averages are the same as those shown in Table 3. The order of effects was $CaCl_2$ > glucose >peptone > beef extract >$MgSO_4$.

observed that the mean concentration obtained was 1693.77 U/mL as compared with 1682.19 U/mL predicted using Minitab for the same composition. The final optimized medium supported S-12 activity of 1693.77 U/mL at the end of 24 h as compared with 1095.00 U/mL before optimization. This implied that the selected conditions were the most suitable in practice for improving S-12 production.

## Use of surfactants for optimization

The aim of the present study was to analyze the effect of different surfactants on the lysozyme S-12 production of *Bacillus* sp. in culture. All results are the average of three triplicate experiments with a standard error of less than 5%.

Many reports have shown the stimulatory effect of surfactants on extra-cellular enzyme production and release (Kuhad et al., 1994; Oguntimein and Moo-Young, 1991; Okeke and Obi, 1993; Reese et al., 1969; Reese and Maguire, 1969, 1971; Sheppard et al., 1991). It can change the hydrophobicity and zeta potential of the cell surface (Hua et al., 2003; Sheppard et al., 1991), and thus make the release of cell-bound enzyme easily. Accordingly, the current study investigated the effect of a culture broth containing various surfactants on S-12 production.

As shown in Table 4, Tween 80 was the best compound for enhancing the production of all the extracellular proteins and S-12. It caused an increase of 14.30%, respectively on S-12 activity. The extracellular proteins production yield was approximately twofold higher than the control. The effect of Tween 80 on the S-12 production of the strain was studied. Compared to the cell yield of the control (0.64 g/l), the biomass after 24 h incubation showed a trend of increasing with Tween 80 (Figure 6). Cells with a concentration of 0.91 g /l were obtained when 0.4 mM Tween 80 was added. With the addition of Tween 80, the concentration of S-12 went up.

The highest activity of S-12 (1940.12 U/mL) was detected at a Tween 80 concentration of 0.4 mM. The stimulatory effect of Tween 80 on enzyme produc-tion may be a consequence of its action on cell mem-branes causing increased permeability and/or by promo-ting the release of cell-bound enzymes (Reese and Maguire, 1971). The increase in yield with Tween 20, Triton X-100 and Span 80 could be due to an effect similar to Tween 80, as these compounds provoked a rise in extra-cellular proteins in culture supernatants (72%with Tween 20, 126% with Triton X-100 and 58% with Span 80).

Figure 7 shows the profile of lysozyme S-12 production using the optimized medium. The maximum S-12 activity (1971.25 U/mL) was obtained at 24 h, after which the

**Table 4.** Effect of Tween 20, Tween 80, Triton X-100, and Span 80 in the culture medium on cell growth and S-12 lysozyme production in *Bacillus* sp.

| Surfactant | DCW (g/l) | S-12 Activity (U/mL) | Extracellular proteins (μg/mL) |
|---|---|---|---|
| Control | 0.64±0.03 | 1693.77±83.70 | 27.00±1.30 |
| Tween 20 | 0.62±0.02 | 1760.50±88.00 | 46.50±2.30 |
| Tween 80 | 0.87±0.03 | 1935.84±96.70 | 61.00±3.00 |
| Triton X-100 | 0.78±0.01 | 1895.11±94.00 | 65.30±3.20 |
| Span 80 | 0.67±0.02 | 1755.25±87.5 | 42.80±2.10 |

Incubation was carried out for 24 hour on a rotary shaker at 30°C and 200 rpm. Four different surfactants were added in the basal medium at 0.5mM concentration. Values are mean ±S.E.M. of triple determinations. Analysis of variance revealed significant differences between the groups (P < 0.01).

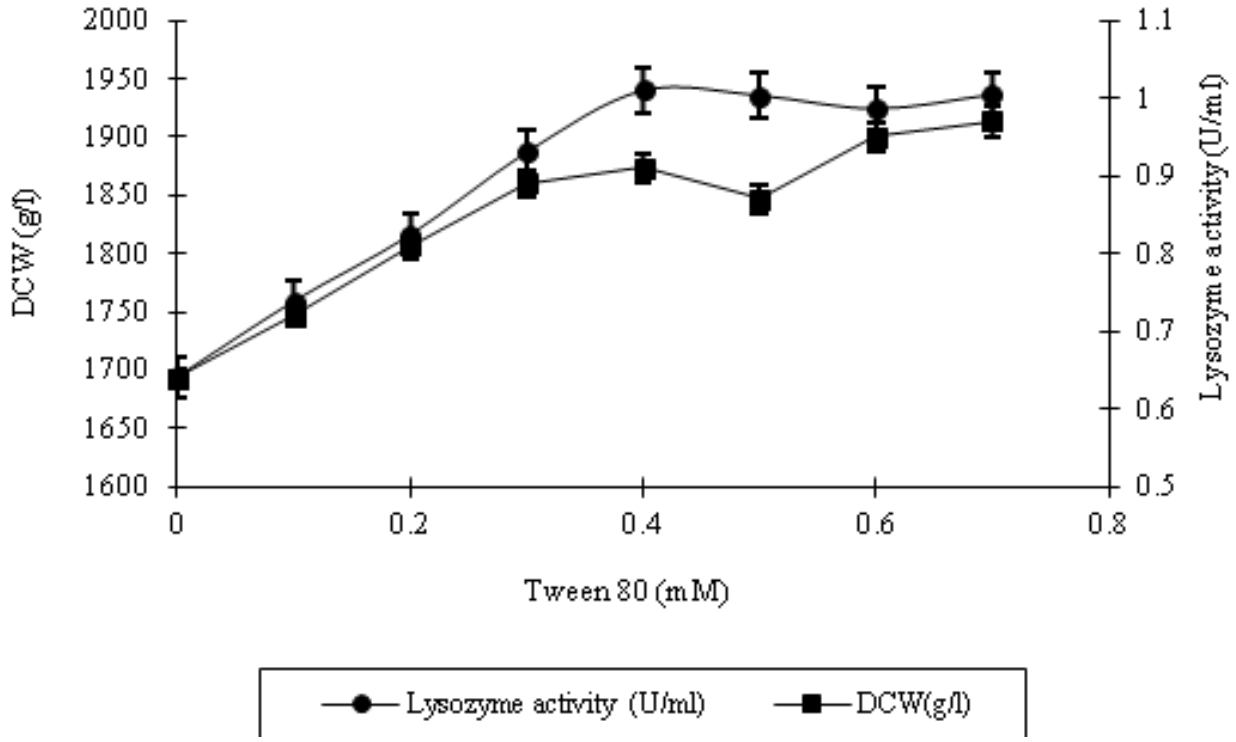


**Figure 6.** Effect of different concentrations of Tween 80 on lysozyme S-12 by by *Bacillus* sp. Incubation was carried out for 24 h on a rotary shaker at 30°C and 200 rpm. Different concentrations of Tween 80 were added in the basal medium at 0-0.7mM concentration. Values are mean ±S.E.M. of triple determinations. Analysis of variance revealed significant differences between the groups (P < 0.01).

yield decreased.

## Conclusions

It is possible to determine optimal operating conditions using the one-factor-at-a-time method and the orthogonal matrix method to obtain the maximum production of lysozyme S-12 by *Bacillus* sp. The supplementation of the fermentation medium with different surfactants, Tween 80 in particular, increased S-12 and extracellular proteins production. However, the exact mechanism for this is not yet clarified. Understanding the exact role of these surfactants in the biosynthesis of lysozyme S-12 could lead to further improvement of the yield of it.

## ACKNOWLEDGMENTS

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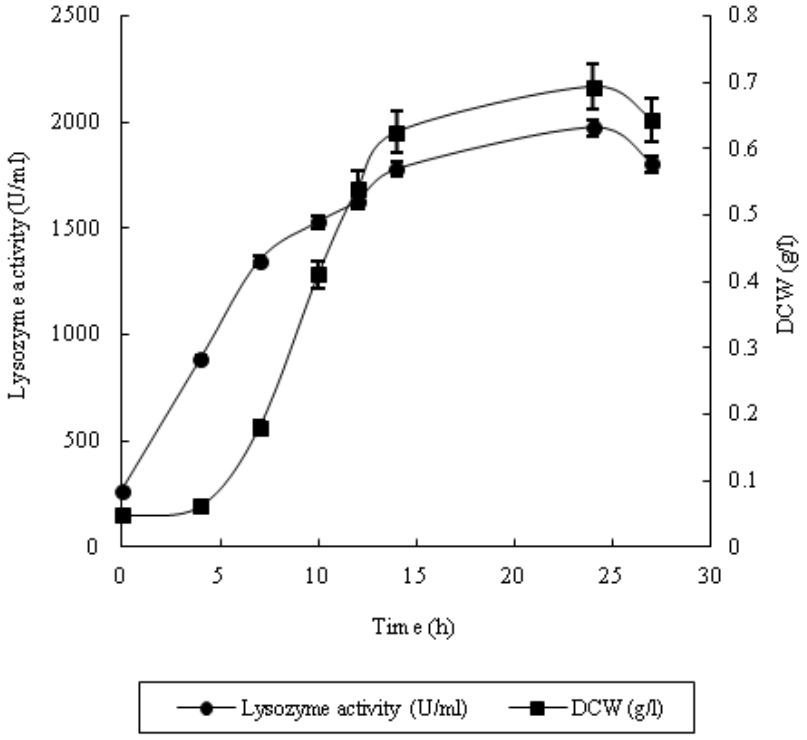


**Figure 7.** Production profile of lysozyme S-12 on the optimized medium. Incubation was carried out for 24 h on a rotary shaker at 30°C and 200 rpm. Values are mean ±S.E.M. of triple determinations. Analysis of variance revealed significant differences between the groups ($P < 0.01$).

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