Enhancement of Cellulase (CMCase) production from marine actinomycetes \textit{Streptomyces} sp. Bse 7-9: Optimization of fermentation medium by Response Surface Methodology

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Abstract. Optimization of fermentation conditions is important in the development of bioprocesses. Here, we used the Taguchi experimental design and Response Surface Methodology to optimize fermentation medium for enhancing carboxymethyl cellulase (CMCase) production by marine actinomycetes \textit{Streptomyces} sp. Bse 7-9. Optimization using the Taguchi experimental design we found that bagasse has a significant effect on other factors. The Central Composite Design was used to determine the interaction between the factors selected in the fermentation medium to obtain the optimal concentration of ingredients. The experimental results demonstrated that bagasse as a carbon source plays as the key component for the production of CMCase by marine actinomycetes \textit{Streptomyces} sp. Bse 7-9. In conclusion, the optimal fermentation medium for maximum cellulase production by marine actinomycete \textit{Streptomyces} sp. Bse 7-9 was as follows: bagasse concentration (1.84 \% w/v), yeast extract (0.5 \% w/v) and CaSO$_4$ (0.02 \% w/v). The maximum activity of CMCase at this optimum medium was 4.496 U mL$^{-1}$.

1. Introduction

Cellulase has become an important biocatalyst for widespread applications in the fields of industry, such as in the process of conversion of lignocellulosic biomass. To convert lignocellulose and produce fermentable sugar effectively, microorganisms such as certain cellulolytic bacteria can produce hydrolytic enzymes capable of degrading lignocellulosic substrates. The complete hydrolysis of lignocellulose requires three types of enzymes, i.e., endoglucanases, celllobiohydrolases, and β-glucosidases, which work synergistically to hydrolyzed lignocellulose [1]. Huge of the bacteria are known for the production of hydrolytic enzymes, most of them originating from the fungal cellulases and only a few have been studied from the source of actinomycetes [2].

Actinomycetes are Gram-positive bacteria and living widespread, commonly found in soil, leaf litter, the sediment of freshwater and marine ecosystems [3]. Actinomycetes form an important part of the microbial communities that are responsible for the degradation of cellulose [4], known as one of the cellulose producers. Actinomycetes play a vital role in the degradation of lignocellulose from plant cell walls, thus attracting the interest of research for their potential to generate fermentable sugars from cellulose that can be useful for their growth and also for many industrial applications [5; 6].

Actinomycetes growth to form branching hyphae that are useful to penetrate in lignocellulose degradation. Cellulase of actinomycete is induced extracellular enzymes [7], which can be generated
during their growth on a variety of cellulosic materials. A search for new microorganisms that can degrade cellulose is increasing, and a variety of new species have been isolated from a variety of natural habitats. Actinomycetes are rarely explored from the marine habitats, and only limited literature is available on their occurrence, diversity, and enzymatic potential. Therefore, studies on the marine actinomycetes would be of great significance. The marine actinomycetes are largely unexplored for the novel primary and secondary metabolites. In recent years, the possibility of obtaining high yields of useful cellulases from marine origin actinomycetes has attracted the attention of researchers. In this paper, we use marine actinomycetes _Streptomyces _sp. BSE 7-9 of Biotechnology Culture Collection (BTCC). The strains isolated from marine sediments of the seashore on Kuta beach, Bali, Indonesia, for the study of isolation and actinomycetes diversity of coastal environments in Indonesia. The strain can produce cellulase enzyme and examine for the possibility of degradation the cellulose.

Optimization of fermentation conditions is an essential issue in the development of bioprocesses. The interaction between the combinations of components medium with the desired compound production and optimal processes can be developed using procedure experimental design [8; 9]. Effective bioconversion of cellulosic material depends on the properties of the cellulose, the source of cellulolytic enzymes and conditions optimal for the catalytic activity and production of enzymes. The optimization of fermentation media ingredients and environmental factors for the optimum enzymes production is more effective strategies [10].

Statistical experimental designs have been used in various stages of optimization strategies, such as for preliminary screening to find the optimal conditions for a targeted response. One popular choice in applying statistical design for the bioprocessing is Taguchi Experimental design. A Statistical tool of Design of Experiments is the latest trend in the development of the bioprocess and process optimization using response surface methodology (RSM). This tool is searching for the optimum condition through a collection of mathematical and statistical techniques to design an experiment of the factors for the desired response, and evaluate the relative importance of several factors that influence in the presence of complex interactions [11]. RSM is used to determine the various variables under control and optimal process conditions for the production of enzymes, followed by a polynomial equation generation bioprocess models, to estimate the relationship between the variables under control and observe the results [12].

The objective of this research was to study and optimize enzymatic carboxymethyl cellulase (CMCase) production from marine actinomycete _Streptomyces _Bse 7-9 using agricultural waste sugarcane bagasse to provide a high yield of cellulase enzymes.

2. Material and Methods

2.1 Microorganism and culture media

_ Streptomyces _sp. Bse 7-9 used in this study was isolated from the sea sediment in seashore Kuta, Bali Island. Indonesia (E: 115°10‘7.8” S: 8°43‘5.5”). Approximately 1 g sample was diluted in a serial dilution with artificial seawater (Nihon Pharmaceutical), and 0.2 ml of each dilution was spread on plates of NBRC medium 802 [0.2% (w/v) polypeptone, 0.04% (w/v) yeast extract, 0.02% (w/v) MgSO₄ .7H₂O and 1.5% (w/v) agar; pH7.0]. The medium was supplemented with 5.0% (w/v) NaCl, 0.002% (w/v) nalidixic acid and 0.005% (w/v) cycloheximide to avoid unwanted growth of Gram-negative bacteria and fungi. After cultivation at 30 °C for one week and single colony isolation twice, strain Bse 7-9 was obtained. The stock culture was maintained on glycerol stock. The medium composition for maintenance comprises of: NBRC medium 802 supplemented with 2.0% (w/v) NaCl, and, 1.5% (w/v) agar; pH 7.0.

2.2 Detection for CMCase producing actinomycete strain

A preliminary analysis for cellulolytic activity was conducted using Congo red dye. The clear zone methodology with Congo red was used to detect the CMCase activity of the strain. _Streptomyces _Bse 7-9 was grown on ISP2 broth without glucose, substituted with 1% bagasse as a carbon source. The
medium consists of: bagasse, 1.0% (w/v); yeast extract 0.4% (w/v) and malt extract 0.2% (w/v). The medium was incubated at 30 °C for seven days under a shaking condition at 120 rpm. At the end of the incubation, the medium was centrifuged at 10,000 rpm for 10 minutes. Free cells supernatant were used as the crude CMCase enzyme. Crude enzyme was then concentrated until 40-fold using Amicon. Three µl of the free cell supernatant and 1 µL of 40-fold concentrated free cells supernatant, were spotted onto substrate agar containing 0.5% carboxymethyl cellulose (CMC), and 1.7% agar, diluted in 50 mM Phosphate buffer pH 6.8. The CMC plate was incubated at 30 °C for three days to allow hydrolyzed the CMC substrate. Plates were then flooded with 0.25 % Congo red for 15-20 min, then washed with 1 M NaCl for 15 min. The clear zone formation was indicated hydrolysis of cellulose degradation [13].

2.2.1 SDS-PAGE and zymogram analysis.
The samples supernatant were mixed with the sample buffer containing 1% β-mercaptoethanol. The sample was then heating at 70 °C for 20 minutes and subsequently loaded on 5% stacking gel. For zymogram analysis, CMC with a final concentration of 0.1% (w/v) was incorporated into 12% separating gel as substrate. After electrophoresis was run, SDS gel then soaked in 0.1 M phosphate buffer, pH 6.8 for 15 min with gentle shaking, and then was immersed in 0.1% Triton X-100 for 30 min with gentle shaking. The gel was incubated in the 0.1 M phosphate buffer for 1 h at 35 °C with gently shaking to allow the proteins to refold. The gel was then stained in 0.25% Congo red for 1 h and destained with 1 M NaCl until the clear zone appeared against a red background. Another duplicated SDS gel containing the same loading pattern was stained with Coomassie brilliant blue-R250 and destained with the destaining solution. The positions of the cellulase enzyme on the both SDS gels were compared to a determined molecular mass of the active bands.

2.2.2 Fermentation for cellulase production.
Production of cellulase (CMCase) from marine actinomycete Streptomyces Bse 7-9 was analyzed in several media as described in Table 1. The isolate was grown in CYP [14] broth for 24 h under shaking 120 rpm and then inoculated (2%, v/v) into 25 mL fresh of each medium broth

Table 1. Several media for CMCase production by marine actinomycete Streptomyces sp Bse 7-9

| Medium 1          | Medium 2          | Medium 3          | Medium 4          |
|-------------------|-------------------|-------------------|-------------------|
| CMC 10 g/L        | KH₂PO₄ 1 g/L      | Yeast extract 1 g/L | Glucose 0.1 g/L  |
| Yeast extract 1 g/L | MgSO₄ .7H₂O 0.5 g/L | NaCl 0.5 g/L      | K₂HPO₄ 0.1 g/L   |
| Peptone 1 g/L     | NaCl 1 g/L        | Peptone 1 g/L     | Yeast extract 0.05 g/L |
| NaCl 2 g/L        | FeSO₄ .7H₂O 0.01 g/L | Starch 10 g/L    | Tryptone 0.5 g/L |
|                   | MnSO₄ .H₂O 0.01 g/L |                   |                   |
|                   | NH₄Cl 1 g/L       |                   |                   |
|                   | CMC 10 g/L        |                   |                   |
|                   | Yeast extract 2 g/L |                   |                   |

Fermentation was conducted at 30 °C under shaking (120 rpm) for four days of incubation. Samples were centrifuged (Tomy Refrigerated Centrifuge MX-307) at 10,000 rpm for 10 min. The cell-free supernatants were considered as crude extracellular enzymes and used as the source of crude cellulase enzymes for assaying cellulase activity.

2.2.3 Enzyme Assay
CMCase activity was determined by monitoring the release of reducing sugars. CMCase activity was measured following the dinitro salicylic acid (DNS) method of Miller [15]. A reaction mixture composed of 0.5 ml of an appropriately diluted enzyme to 0.5 ml of 1 % (w/v) of carboxymethyl cellulose (CMC) in 50 mM Phosphate buffer, pH 6.8 and incubating at 50 °C for 15 min. The amount of reducing sugars released during the reaction was measured using the DNS method, and D-glucose was used as the standard. One unit (U) of carboxymethyl cellulase (CMCase) activity was defined as
the amount of enzyme that liberated 1 μmol of glucose equivalent under the given assay conditions [15].

2.3 Experimental Design and Statistical Analysis
Screening of ingredients components for medium optimization was done by Taguchi experimental design. Experimental design for optimization can make easier to evaluate the effect of several factors in achieving optimum conditions to produces a desirable response, an experiment that uses a small number of the possible [16]. A standard experimental array L9 (3^4) was used to study the effects of carbon and nitrogen sources, metal ions, and salinity on CMCase production. The levels of the factors investigated and the layout of the L9 Taguchi’s experimental array are shown in Tables 1 and 2. The L and the subscript (9) represent the Latin square and the number of trial runs, respectively. Results obtained from the experiments were then analyzed to determine independent factors that influence the main effect; analysis of variance technique was then applied to determine which factors were statistically significant. The controlling factors are further identified, with the qualified effects and a statistically significant effect. Thus, the optimal conditions are determined by combining the level of the factors that have the highest value of the main effect.

2.4 Optimization of a concentration of the selected medium components using response surface methodology.
Response surface methodology (RSM) was applied to optimize the selected three significant nutrient components: bagasse as a carbon source, yeast extract as a nitrogen source and CaSO4 as a selected metal ion in various concentrations in the fermentation medium. A factorial, central composite design (CCD) for three independent variables were studied at three different levels of the independent variables at the center point, and star points were used in this investigation and shown in Table 5. A centered design consisting of 20 experimental runs (Table 6), including six replicates at the center point was employed. The variables were bagasse (X1), yeast extract (X2), and CaSO4 (X3). The response variable was the CMCase activity (Unit mL^-1). The experimental data were regressed according to Equation (1) as a second-order polynomial equation: The quadratic model equation for predicting the optimal point is expressed by Eq. (1) as followed:

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \epsilon \quad \ldots (1)
\]

where Y is the predicted response (CMCase activity, U.mL^-1), \(\beta_0\) is constant coefficient, \(\beta_i, \beta_{ii}\) and \(\beta_{ij}\) are coefficient for the linear, quadratic and interaction effects, \(X_i\) and \(X_j\) are factors (independent variables) while \(\epsilon\) is the error [17].

A regression model was established, and the experimental verification of the model was validated. Design Expert statistical software (version 9.0.6.2., Stat-Ease Inc., USA) was used for experimental data regression analysis and to plot the response surface.

3. Results
3.1 CMCase producing actinomycetes
Marine actinomycetes strain to produce cellulase has been screened in this study for their potential to hydrolyzed CMC substrate by using the Congo red test. Cellulose in CMC agar was used as the sole carbon source. In a preliminary detection test, marine actinomycete Streptomyces sp. Bse 7-9 displayed considerable cellulase-producing ability on carboxymethyl cellulose (CMC) agar plates. This isolate was able to degrade CMC substrate, as indicated by the formation of a clear zone around the dropped crude enzyme (Figure 1a; 1b), measured as the ratio of the size of CMC hydrolysis on dropped enzyme diameter after three days of incubation at 30 °C. The clear zone diameter produced by Streptomyces Bse 7-9 was 25 mm for free cells supernatant and 37 mm for 40-fold concentrated free cells supernatant. This result was strong evidence that cellulase was produced in the supernatant of Streptomyces Bse 7-9.
The SDS-PAGE of supernatant *Streptomyces* Bse 7-9 revealed a few protein bands after staining (Fig. 1c(i)). Corresponding to the zymogram analysis, supernatant and concentrated free cells supernatant of *Streptomyces* Bse 7-9 showed a few clear bands revealing cellulolytic activity in-gel zymography (Fig. 1c(ii)). Based on the results of this initial detection test, strain Bse 7-9 which showed a substantial cellulase activity and representing from marine ecological habitat was selected for further study.

The denatured of biological mass CMCase was separated by using SDS-PAGE revealed the cellulolytic activity, and shown a molecular weight around 24 kDa (Fig. 1c(i)). In-gel zymography activity contained CMC as substrate suggesting that the enzyme was refolded correctly and had cellulolytic activity after SDS-gel electrophoresis and the following incubation step. The molecular weight of the enzyme estimated from SDS-PAGE was similar to the molecular weight of the bacteria, yeast, beetle, and termites, which are in the range of 29-52 kDa [18; 19; 20; 22; 23; 24].

![Figure 1](image)

Figure 1. (a) Clear zone by *Streptomyces* sp Bse 7-9 supernatant stained with 0.25% Congo Red, (b) with an addition 5% acetic acid glacial, (c) SDS-PAGE and zymogram analysis of CMCase enzyme from *Streptomyces* sp Bse 7-9. (i) Stained gel with Coomassie brilliant blue R-250 and ((ii) in-gel activity staining or zymography. Lane M: Marker, Lane 1: Crude sample (free cells supernatant), Lane 2: Concentrated 40-fold free cells supernatant

3.2 Medium selection for CMCase production

![Figure 2](image)

Figure 2: CMCase production by marine actinomycete *Streptomyces* sp Bse 7-9 using several media, fermentation were conducted at 30 °C under shaking 120 rpm for 4 days incubation.

The strain *Streptomyces* sp Bse-7-9 was found to be capable of hydrolyzing cellulose. To optimize culture medium conditions, a set of experiments was performed under different carbon source,
nitrogen source, and metal ions. The strain *Streptomyces* sp Bse 7-9 exhibited high cellulase activity. Determination of the CMCase activity of the strain was calculated by estimating the amount of reducing sugar (U mL$^{-1}$) released by the strain. It was investigated that the highest amount of reducing sugar released due to CMCase activity by the strain *Streptomyces* sp Bse 7-9 was in Medium 2 as presented in Figure 2.

3.3 Selection of significant variables by Taguchi experimental design

Optimization is performed to improve the yield of bioactive substances such as CMCase produced by actinomycetes strain. From the clear zone of hydrolysis, it is observed that strain *Streptomyces* sp Bse 7-9 gives a good hydrolytic activity. Therefore, an attempt has been made to improve the yields of *Streptomyces* sp Bse 7-9 by optimizing biophysical parameters such as salt, metal ions and nutrient concentrations considering at three levels of experiments. Taguchi’s experimental design has been applied to find the optimum combination of these four factors for maximum yield. Each factor is taken at three levels in an L$^4$ experimental array. The variation reflected the importance of the optimization studies for higher cellulase production. The medium was formulated as per the design, and the flask culture experiment was performed. The response was calculated at the rate of enzyme production and expressed as U mL$^{-1}$. All analyses were performed in duplicate. The average of the rate of enzyme production was considered as the response. The matrix design for the screening of significant variables that have the most profound influence on cellulose production and the corresponding responses are presented in Table 2 and Table 3.

### Table 2: The Taguchi experimental design for the screening of medium variables for production of CMCase from marine actinomycete *Streptomyces* Bse 7-9

| Factors              | Level 1      | Level 2      | Level 3       |
|----------------------|--------------|--------------|---------------|
| Carbon source (1%)   | Glucose      | CMC          | Bagasse       |
| Nitrogen source (0.5%) | Peptone      | Yeast extract | (NH$_4$)$_2$HPO$_4$ |
| Metal ion (0.02%)    | Ca$_2$SO$_4$ | Fe$_2$SO$_4$ | MnSO$_4$      |
| Salinity (%)         | 0            | 1            | 2             |

### Table 3: L9($3^4$) experimental array of Taguchi experimental design and corresponding CMCase, production by marine actinomycete *Streptomyces* Bse 7-9

| Run | A: Carbon (1%) | B: Nitrogen (0.5%) | C: Metal ion (0.02%) | Salinity (%) | CMCase (U mL$^{-1}$) |
|-----|----------------|---------------------|----------------------|--------------|----------------------|
| 1   | 2              | 1                   | 2                    | 3            | 1.97                 |
| 2   | 1              | 2                   | 2                    | 2            | 0.48                 |
| 3   | 2              | 3                   | 1                    | 2            | 0.82                 |
| 4   | 3              | 1                   | 3                    | 2            | 3.06                 |
| 5   | 1              | 1                   | 1                    | 1            | 0.55                 |
| 6   | 1              | 3                   | 3                    | 3            | 0.83                 |
| 7   | 3              | 3                   | 2                    | 1            | 2.64                 |
| 8   | 2              | 2                   | 3                    | 1            | 1.60                 |
| 9   | 3              | 2                   | 1                    | 3            | 3.36                 |

Carbon: 1=Glucose, 2=CMC, 3=Bagasse; Nitrogen source: 1=Peptone, 2=Yeast extract, 3= (NH$_4$)$_2$HPO$_4$; Metal ion: 1=Ca$_2$SO$_4$, 2=Fe$_2$SO$_4$, 3=MnSO$_4$; Salinity: 1=0%, 2=1%, 3=2%.

Among the four ingredients of cultural variables examined based on Taguchi experimental-designed experiments (carbon source, nitrogen source, metal ions, and salinity), one variable Carbon source, A (p-value=0.0011), have maximum influence on the CMCase production (Table 4). Factors showing statistical p-values of less than 0.05 were considered to have a significant effect on the response that is enzyme activity. The model F-value of 26.15 indicates that the model is significant. Only 0.79% chance exists that this large model F-value could be due to noise. The statistical values of
R2=0.9886, Adj R2=0.9581, Pred R2=0.8170, and Adeq Precision = 17.102 indicate the significance of the model and the reliability of the experiments. One of the advantages of the Taguchi experimental design is that it helps to rank the effect of different variables on the measured response independent of its nature either nutritional or physical factor [25].

Table 4: Results of ANOVA obtained for CMCase production by Streptomyces sp Bse 7-9 for Taguchi experimental design

| Source            | Sum of squares | DF | Mean squares | F value | Prob>F  |
|-------------------|---------------|----|--------------|---------|---------|
| Model             | 2.22          | 2  | 1.11         | 26.15   | 0.0011  |
| A-Carbon source   | 2.22          | 2  | 1.11         | 26.15   | 0.0011  |
| Residual          | 0.26          | 6  | 0.043        |         |         |
| Cor Total         | 2.48          | 8  |              |         |         |

The results shown in Table 4 suggest that only carbon source (bagasse) is the positive factor in the response, while other variables such as nitrogen source, metal ions, and salinity were found to be relatively unimportant and non-significant variables. Even though marine actinomycetes can grow without salt, they usually showed the lowest growth rate. Na⁺ ions are still important for their membrane transport system to maintain high intracellular potassium concentration for the functioning of enzymes [26].

3.4 Optimization of CMCase production by Response Surface Methodology

The three variables for cellulase production identified based on Taguchi experimental-designed experiments were further optimized using RSM, keeping other variables at constant levels. The effective variables were analyzed using the Design-Expert software.

Table 5: Independent variables and their coded levels for the central composite design used for cellulase production by Streptomyces sp Bse 7-9

| Variables       | Code | Variable Levels |
|-----------------|------|-----------------|
| Bagasse (%)     | A    | 0.5 1 1.5       |
| Yeast extract (%)| B    | 0.25 0.5 0.75  |
| CaSO₄ (%)       | C    | 0.01 0.02 0.03 |

The experimental values were fed into the response column as presented in Table 6.

Table 6: Central composite design for cellulose production by Streptomyces sp. Bse 7-9

| Run | Factor A: Bagasse (%) | Factor B: Yeast extract (%) | Factor C: CaSO₄ (%) | Response 1: CMCase (U mL⁻¹) actual | CMCase (U mL⁻¹) predicted |
|-----|------------------------|-----------------------------|---------------------|-------------------------------------|---------------------------|
| 1   | 1.00                   | 0.50                        | 0.02                | 3.486                               | 3.49                      |
| 2   | 0.50                   | 0.25                        | 0.01                | 2.390                               | 2.09                      |
| 3   | 1.50                   | 0.75                        | 0.01                | 4.026                               | 4.07                      |
| 4   | 1.00                   | 0.50                        | 0.02                | 3.357                               | 3.49                      |
| 5   | 1.00                   | 0.50                        | 0.037               | 2.674                               | 2.91                      |
| 6   | 0.16                   | 0.50                        | 0.02                | 0.631                               | 1.09                      |
| 7   | 1.00                   | 0.50                        | 0.02                | 3.203                               | 3.49                      |
| 8   | 1.50                   | 0.75                        | 0.03                | 4.094                               | 4.11                      |
The run-13 showed the highest CMCase activity up to 4.496 U mL$^{-1}$ when the experimental culture medium conditions were set to bagasse concentration, 1.84%; yeast extract, 0.5% and CaSO$_4$, 0.02%, respectively. It was noticed that when the experiment was conducted in conditions at temperature, 28°C; with shaking at 120 rpm, and 4 days incubation, the lowest CMCase activity was attained i.e. 1.09 U mL$^{-1}$.

### Table 7. ANOVA for CMCase production

| Source       | Sum of Squares | df | Mean Square | F     | p-value |
|--------------|----------------|----|-------------|-------|---------|
| Model        | 15.84          | 9  | 1.76        | 15.07 | 0.0001  |
| A - Baggase  | 0.74           | 1  | 0.74        | 6.33  | 0.0306  |
| B - Yeast Extract | 0.050        | 1  | 0.050       | 0.43  | 0.5274  |
| C - CaSO$_4$ | 0.051          | 1  | 0.051       | 0.43  | 0.5247  |
| AB           | 0.055          | 1  | 0.055       | 0.47  | 0.5083  |
| AC           | 0.056          | 1  | 0.056       | 0.48  | 0.5033  |
| BC           | 0.0051         | 1  | 0.0051      | 0.044 | 0.8385  |
| A$^2$        | 0.93           | 1  | 0.93        | 7.98  | 0.0180  |
| B$^2$        | 0.11           | 1  | 0.11        | 0.91  | 0.3616  |
| C$^2$        | 0.89           | 1  | 0.89        | 7.59  | 0.0203  |
| Residual     | 1.17           | 10 | 0.12        |       |         |
| Lack of Fit  | 0.73           | 5  | 0.15        | 1.69  | 0.2893  |
| Pure Error   | 0.43           | 5  | 0.087       |       |         |
| Cor Total    | 17.01          | 19 |             |       |         |

ANOVA was performed using the experimental design implemented. The Model F-value of 15.07 implies the model is significant (Table 7). There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.050 indicate model terms are significant. In this case, A, A$^2$, and C$^2$ are significant model terms. A regression equation for the quadratic model was suggested as:

\[
\text{CMCase activity} = -60.91 - 81.56A + 1.43B - 1.43C + 0.83AB - 0.84AC + 0.025BC - 25.43A^2
\]

In which Y is a function of the variables of bagasse concentration (A), yeast extract (B) and CaSO$_4$ (C).
The values for lack of fit F (1.69) and lack of fit P>F (0.2893) implies that the lack of fit effects is not significant to the pure error. There is a 28.93% chance that a "Lack of Fit F-value" this large could occur due to noise. The fit of the model was also expressed by the coefficient of regression (R²) = 0.9313, indicating that 93.13 % of the confidence level of the model to predict the response. Adj R²=0.8695, Pred R²=0.6367, Adeq Precision=13.928. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 13.928 indicates an adequate signal indicator for correlation between the observed and predicted values and their liability of the experiments

Figure 3. The interaction between (A) Bagasse and Yeast extract concentrations (%); (B) Bagasse and CaSO₄ concentration (%); and (C) Yeast extract and CaSO₄ concentration (%) on the activity of the cellulase enzyme (U mL⁻¹).

Figure 3 (A) demonstrates the 3D response surface plot for the interaction effects of bagasse versus yeast extract concentration and keeping CaSO₄ constant at 1%. By optimizing with a coded factor, the maximum CMCase production observed by the model was 4.496 U mL⁻¹ as could be attained when bagasse concentration at 1.84% and yeast extract at the concentration of 0.5%, respectively. The plot revealed that the CMCase production from marine actinomycete *Streptomyces* sp Bse 7-9 was high
and increasing bagasse concentration as carbon source resulted in an increasing CMCase production whereas increasing yeast extract concentration resulted in less production of cellulase. The CMCase activity was increased when the bagasse concentration was raised from 0.5 to 1.84%.

Figure 3 (B) shows the interaction of bagasse (0.5 – 1.84%) and CaSO₄ (0.01 – 0.03%) with the fixed coded value of yeast extract concentration (0.5%). An increasing both bagasse and CaSO₄ concentration have initially increased the production of CMCase until they reach optimal CMCase production. Figure 3 (C) reveals the interaction between yeast extract and CaSO₄ concentration on CMCase production. When bagasse concentration was fixed at 1.84%, yeast extract concentration and CaSO₄ were set to 0.5 – 0.75% and 0.01 – 0.03%. An initial increase is in CaSO₄ concentration resulted in increased production of CMCase until reached a point for the optimal production of CMCase, however on increasing in yeast extract concentration; there has no affected in the CMCase production.

By numerical optimization, the quadratic model predicted that the maximum CMCase activity is 4.45 U.mL⁻¹ when the optimal values of the factors in the code unit tests that bagasse 1.84%, yeast extract 0.5%, and CaSO₄ 0.02% (Table 6). Validation of the results statistically using optimized media was carried out in duplicate. Maximum CMCase activity obtained experimentally found to be 4.496 U mL⁻¹, (Table 6) are in close agreement with the predicted values (4.45 U mL⁻¹). Therefore, this model is considered reliable and accurate to predict the CMCase production by *Streptomyces* sp. Bse 7-9.

### 4. Discussion

Actinomycetes, particularly *Streptomyces* species are known as the source of thousands of bioactive compounds. These microorganisms have a system of secondary metabolic pathways to produce various compounds that show various impressive and diverse biological activities. Enzymes are one of the important products produced in the metabolic pathway of actinomycetes. However, in comparison with the terrestrial microorganisms, only limited groups of actinomycetes from the aquatic ecosystems are known for the cellulose degradation [6].

Actinomycetes have been found in many unique marine environments, such as marine organic aggregates as the major microbial community [27], seashores and estuaries [28], mangrove soils, sediments and plants [29]. Seashores are highly dynamic ecosystems these ecosystems are home to diverse flora and fauna of marine, freshwater and terrestrial species [30]. Marine microorganisms are encouraged to develop an enzyme system adapted to the complex environment, because of the competition between microorganisms for space and nutrients in the marine environment is one of a significant selective force that can lead to evolution. The constant changes in environmental factors such as tidal and salinity gradients in the mangrove environment can be considered as the driving force to adapt to their metabolic pathway. This situation can lead to the production of unique and unusual metabolites. Therefore, currently has been increased exploitation of microorganisms from the mangrove environment [31]. Microbial fermentation is a process in which the product concentration varies under different conditions. Components of the media play a significant role in metabolism for CMCase production. Various factors of the medium were screened by Taguchi experimental design and then the interaction of the selected factors was studied using CCD.

Taguchi experimental design is the study to identify the effect of individual factors in the system provided by a set of independent variables over a certain level of interest, studying the relationships among variables and also a performance at optimum levels. By studying the major effects of each factor, the trend of influencing factor on the process will be predictable and controlled to provide the desired results. Furthermore, the level of a factor to produce the best results can be predicted [32]. Any individual factor may interact with the other factors creating the possibility of the presence of interactions. From the results of Taguchi experimental design, nitrogen source and metal ions are relatively insignificant coefficients, whereas carbon sources were found significant with positive coefficients.

In this study, to evaluate the accuracy of Taguchi experimental design, a medium of the following composition: temperature 30°C; incubation time 4 days; inoculum size 2% (v/v); agitation speed 120
rpm; bagasse 1%; yeast extract 0.5%, and CaSO\(_4\) 0.02 %, which expected to be optimum gives CMCase activity of 3.36 U mL\(^{-1}\) which is higher than result obtained from the basal medium (medium no 2) before applying Taguchi experimental design by more than one and half times (2.20 U mL\(^{-1}\)).

Bagasse mainly consists of cellulose and hemicellulose, utilized widely by different microorganisms as a carbon and energy source for cellulase production. Several Streptomyces strains were isolated from Brazilian soils and grown on sugarcane bagasse in varying concentrations to produce cellulase with the highest production of CMCase (1.11 U mL\(^{-1}\)), after 48 hours fermentation [33]. Streptomyces sp.LX was shown to secrete a carboxymethyl cellulose (CMC)-liquefying enzyme that cleaves the CMC chains, which has a molecular mass of 9.8 kDa [34]. Jaradat et al. [35] also were reported that Streptomyces strain (J2) was observed as the highest crude cellulase enzyme activity as 0.432 U mL\(^{-1}\) in a medium that was supplemented with 0.5% glucose.

Sugarcane bagasse has a softer structure compare to others that could be easy to break down [36]. In sugarcane bagasse, the cellulose content was reported as high as 32-44% [37]. Indonesia is the large sugar producer in South East Asia region with the production of sugar cane as much as 2.6 million ton; there would be produced solid waste bagasse as much as 13 million ton [38]. Thus, the availability of abundant as agricultural waste from sugar production process can be utilized for the production of CMCase.

In this study, a response surface methodology (RSM) was used to optimize the conditions for CMCase production by Streptomyces sp. Bse 7-9. Using RSM based on CCD model, the optimum medium ingredients for CMCase production by Streptomyces sp. Bse 7-9 were: bagasse of 1.84% (w / v), yeast extract of 0.5% (w / v), CaSO\(_4\) of 0.02% (w / v) and incubation time of 4 days. The maximum CMCase production observed by the models by optimizing medium was 4.496 U mL\(^{-1}\), more than two-fold higher than CMCase activity in basal medium. RSM has been widely employed for the optimization of variables for maximum cellulase as reported in production from Cellulomonas uda NCIM 2353 [39] Bacillus VITRKHB [40], Trichoderma reesei WX-112 [8].

The central composite design revealed the optimum ratio of respective critical media components for CMCase production, while the Taguchi experimental design showed the utmost contribution towards CMCase production to have come from bagasse as carbon sources are known to be the most relevant factors for microbial growth. Response surface methodology (RSM), has successfully been used in the optimization of bioprocesses in evaluating the effects of the concentration of medium ingredients and searching for the optimum conditions of CMCase production for marine actinomycetes Streptomyces sp. Bse 7-9.

5. Conclusion

Biodiversity of marine ecosystems provides an abundant natural source of biocatalyst and useful substances. Streptomyces sp. Bse 7-9 can efficiently utilize agro waste bagasse from sugar production as a carbon source for growth. CMCase production can be increased by 2-fold by using statistics-based optimization Taguchi experimental design and response surface methodology.

This study focused on the increased production of the CMCase by Streptomyces sp. Bse 7-9 at various concentration levels of the media production. The results showed that the optimal level of ingredients in the media for the production of CMCase is a combination of 1.84% bagasse, 0.5% yeast extract 0.02% CaSO\(_4\) within four days of incubation. Taguchi experimental design and RSM found to be highly useful in selecting and optimizing medium components in a manageable number of experimental trials. The production improves doubled in CMCase activity. The optimal culture media obtained in this study will be useful for further research to claim the potential of Streptomyces sp. Bse 7-9 in the large-scale fermentation for the efficient production of the CMCase for commercial applications.

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7. References

[1] Lynd L R, Weimer P J, Zyl W H V and Pretorius I S 2002 Microbiol Mol Biol Rev 66 5577
[2] Chellapandi P and Jani H M 2008 Braz J Microbiol 39 122-127
[3] Crawford D L 1986 FEMS Symposium pp 34-728
[4] Vinogradova S P and Kushnir S N 2003 Appl Biochem Microbiol 39 573-575
[5] Jang H D and Chen K S 2003 World J Microbiol Biotechnol 19 263–268
[6] Arunachalam R, Wesely E G, George J and Annadurai G 2010 Curr Res Bacteriol 3 15-26
[7] Ibrahim S A, El-Diwany A I 2007 J Basic Appl. Sci 1 473-478
[8] Hao X C, Yu X B and Yan Z L 2006 Food Technol Biotechnol 44 (1): 89–94
[9] El-Sersy N A, Ibrahim H A H, Abou-Elela, G M 2010 Curr Res Bacteriol 3 1-14
[10] Van Zyl W H 1985 Biotechnol Bioeng 27(9) 1367-1373
[11] Bezerra M A, Santelli R E, Oliveira E P, Villar L S, Escaleira L A 2008 Talanta 76 965-977
[12] Zambare V 2011 J Food Agric 23 37–47
[13] Ariffin H, Abdullah N, Umi Kalsom MS, Shirai Y, and Hassan M A 2006 Int J Eng Technol 3 47-53
[14] Gupta M, Sharma M, Singh S, Gupta P, and Bajaj BK 2015 Energy Technol 3 216-224
[15] Miller G L 1959 Anal Chem 31 426–428
[16] Dong J, Mandenius C F, Lűbberstedt M, Urbaniak T, Nűssler A K N, Knobeloch D, Gerlach J C, Zeilinger K 2008 Cytotechnology 57 251–261
[17] Lai L W, Sarenah S, Yahya M, Nor N M, Sulongo M R 2016 MJAS 20(1) 21 – 30
[18] Mawadza C, Hatti-Kaul R, Zvauya R and Mattiasson B 2000 J Biotechnol 83 177-187
[19] Lee S J, Kim S R, Yoon H J, Kim I and Lee K S 2004 Biochem. Physiol B 139 107-116
[20] Inoue T, Moriya S, Ohkuma M and Kudo T 2005 Gene 349 67-75
[21] Thongekkaew J, Ikeda H, Masaki K and Iefuji H 2008 Protein Expr Purif 60 140-146
[22] Wang C Y, Hsieh Y R, Ng C C, Chen H, Lin H T, Tzeng W S, Shyu Y T 2009 Enzyme Microb Techno 144 373–379
[23] Harshvardhan K, Mishra A and Jha B 2013 J Mol Catal B Enzymatic 9: 51-56
[24] Annamalai N, Rajeswari M V, Elayaraja S and Balasubramanian T 2013 Carbohydr Polym 94 409-415
[25] Youseff G A and Barekaa M M 2009 Biotechnology 8(2) 212-219.
[26] Black J G, and Lewis LM 2005 Microbiology: principles and explorations (7th ed.) (New Jersey : John Wiley & Sons) p 156
[27] Lam K S 2006 Curr Opinion Microbiol 9 (3): 245–251
[28] Dhanasekaran D, Selvamani S, Paneeserselvam A, Thajuddin N 2009 Afr J Biotechnol 8(17) 4159-4162
[29] Hong K, Gao A., Xie Q 2009 Marine Drugs 7 24-44
[30] Jennerjahn T C.and Ittekkot V 2002 Naturwissenschaten 89(1) 23–30
[31] Lee L H, Zainal N, Azman A S, Eng S K, Goh B H, Yin W F, AbMutalib N S, and Chan K G 2014 The Scientific World Journal Article ID 698178 14
[32] Chang M Y, Tsai G. and Houting Y Y 2006 Enzy Microbial Technol 38 407-414
[33] Macedo E P, Cerqueira C L O, Souza DAJ, Bispo A S R, Coelho R R R and Nascimento R P 2013 Braz J Chem Eng 30(4) 729 – 735
[34] Li X and Gao P 1997 J Appl Microbiol 82 73-80
[35] Jaradat Z, Dawagreh A, Ababneh Q, Saadoun I 2008 Jordan J Biol Sci (4) 141-146
[36] Maryana R, Ma'rifatun D, Wheni A, Satriyo K W, Rizal W A 2014 Procedia 47 250 – 254
[37] Supranto S, Tawfiequrrahman A, and Yunanto D 2014 Someche & Rsce 1 (013)
[38] Badan Pusat Statistik Indonesia (June 12, 2013) http://www.bps.go.id/tab_sub/view.php?kat=3&tabel=1&daftar=1&id_subyek=54&notab
[39] Kumar D V R R, Chakri S, Sowjanya M, Yugandhar N M and Reddy D S R 2010 *IJBSE* 1(1) 119-127
[40] Singh K, Richa K, Bose H, Karthik L, Kumar G, Venkata K, Rao B 2014 *3 Biotech* 4 591–598