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Abstract. A study was conducted to screen parameters affecting the production of chitinase by *Trichoderma virens* on solid state fermentation of shrimp waste using the application of 2-level factorial design. The variables having most significant effect on chitinase production of shrimp waste were identified using a 2-level factorial design. The variable that were included in the screening experiment and their settings are incubation time, temperature, moisture substrate, pH, inoculum size and concentration ammonium sulphate. Half factorial experimental design for six variables was selected with six replicates at center point, leading to a total 38 experiments. All data obtained were then used as input to the Design Expert software for further analysis, according to steps outline. ANOVA analysis showed that the models were significant ($p < 0.0001$) for chitinase production. Incubation, temperature and moisture substrate were identified as significant factor affecting chitinase production on solid state fermentation. Statistical analysis revealed that the linear model is significant with R^2 value of 0.9448 for chitinase production. Chitinase production was found to be high at the optimal condition were: moisture substrate 50%, temperature 25^0C and incubation 5 days. The obtained optimal process parameters predicted, $Y = 0.265$ U/g and verified by confirmation experiments, $Y = 0.261$ U/g. The actual experimental results were in agreement with the prediction. This statistical design proved to locate the optimum levels of the most significant parameters for chitinase production, with minimum effort and time

INTRODUCTION

Chitin a homopolymer of N-acetyl-D glucosamine (GlcNAc) residues linked by β-1,4 bonds, and its derivatives, the second most abundant polysaccharide in nature. It not only forms a major structural component of skin the arthropod, crustacean and insect cuticle but also contributes to the formation of several fungal cell walls [1]. In general, the main contribution of chitin to nature is to marine invertebrates. In this case, it was reported that the annual chitin recovery from marine invertebrate processing was approximately 3.7 x 10 4 metric tons per year [2].

Chitinase is enzyme responsible for the chitin biological conversion [1]. Chitinase can be produced by fungi, yeast, bacteria, plants, and insects [3]. This enzyme has great potential in major applications in agriculture, medicine, biotechnology and waste management. Recently, several reports illustrate that chitinase plays a key role in mosquito control and plant defense systems against chitin-containing pathogens [4].

Shrimp waste is considered an important source of chitin which produces up to 14-30% chitin from its total dry weight [5]. Since the shrimp are processed, the total weight of shrimp was reported to be in the range of 10-50% are produced as waste [6]. Due to the increased production and consumption of the world's shrimp, the seafood industry needs to find the right way to reuse the waste [7]. In fact, effective utilization will not only reduce

the environmental impact of shrimp waste but also provide considerable economic benefits [6]. Rachmawaty and Madihah [8] studied the potential of shrimp waste as a substrate for chitinase production.

Solid State Fermentation (SSF) is a low-cost technology fermentation process, particularly suitable for the needs of developing countries. The commonly used substrates forSSF are plant products. Shrimp waste can also be used as substrates for SSF [8]. Utilizing shrimp waste as solid substrateswould revolutionize industrial biotechnology, and solve theproblem of solid waste disposal.

There are many factors that have the influence upon the chitinase activity and fermentation rate in solid state fermentation (SSF) process, such as temperature, pH of the medium, moisture substrate, inoculum size, incubation time etc. Study on the optimization of chitinase has been reported earlier with effects of different factors on its production, however, using a one-factor-at-a-time approach. This has not led to an understanding of factors that can exert an interactive effect on chitinase production [9].

Numerous variables may affect the response of the system studied, and it is practically impossible to identify and control the small contributions from each one. Therefore, it is necessary to select those variables with major effects. Screening designs should be carried out to determine which of the several experimental variables and their interactions present more significant effects. Full or fractional two-level factorial designs may be used for this objective principally because they are efficient and economical [10].

In the search for physical factors that may influence the chitinase production of pretreatment shrimp waste by T. virens using SSF, the first order model 2- level factorial design was applied. First order model is used in an attempt to detect factors that exhibit large main effect and to discard from further study any factors with no noticeable effects [11]. The 2-level factorial design is the most popular first order design owing to their simplicity and relatively low cost. The factorial design is also able to reduce the number of experiments, time, overall process cost and to obtain better response [12]. A major use of fractional factorials is in screening experiments. These are experiments in which many factors are considered for the purpose of identifying those factors that have large effects. Screening experiments are usually performed in the early stages of a project when it is likely that many of the factors initially considered having little or no effect on the response. The factors that are identified as important are then investigated more thoroughly in a subsequent experiment [13]. Many authors have applied factorial design in the preliminary studies or in initial optimization steps for the production of chitinase. The design allows free interaction with data, the ability to make comparisons, seeking similarities, differences, and trends. It can also be used to determine simple response surfaces that are linear with respect to all of the investigated factors [1].

In this study aims at screening physical factors that affect chitinase production by *Trichoderma virens* using shrimp waste on Solid State fermentation based on two-level factorial design.

MATERIAL AND METHODS

Preparation of Substrate and *Pretreatment*

Shrimp waste was used as a substrate in the production of chitinase by *T. virens*. Shrimp waste was obtained from a market in Makassar, South of Sulawesi. This material was dried in the microwave for ten minutes until it is weight constant and the water content is 10% (w/v). Dry shrimp waste was ground and stored in a dry place at room temperature (30^0C), was used as a solid substrate for SSF.

Substrate Preparation for Solid State Fermentation (SSF)

Five grams of dry shrimp shell was supplemented with appropriate volume of basal medium containing 0.17% (w/v) $(NH_4)_2SO_4$; 0.025% (w/v) $MgSO_4.7H_2O$; 0.028% (w/v) KH_2PO_4; 0.007% (w/v) $CaCl_2.2H_2O$ [14]. The initial pH substrate was adjusted to 5.5. The thoroughly mixed substrate was autoclaved at 121ºC, 1.03 bar for 20 minutes, and cooled to room temperature before inoculation.

Production of chitinase in SSF systems

The sterilized solid substrates medium was inoculated with spore suspension. Spore suspension was transferred into solid substrate medium, mixed thoroughly and the flasks were placed in an incubator at the desired temperature and for the desired time interval according to experimental design. Different conditions were tested according to 2-level factorial design. The variables considered for the design are listed in **Table** 1. All the sets were prepared in duplicates.

Preparation of Microorganisms and Inoculums

T. virens was grown on potato dextrose agar (PDA) plates at 30^0C for 7 days. Spores were harvested and suspended in 1% (v/v) of Tween 80. Spore suspension was then centrifuged at 4000 rpm for 20 minutes. The spore was suspended in distilled water to prepare spore count suspension as an inoculum. The inoculum size of each experiment was according to the experimental design and ranged between 6.0 spore/substrate to 8.0 spore/substrate with the midpoint was 7.0 spore/ substrate.

TABLE 1. Coded and actual values of variables used in 2-level factorial design

Variables	Unit	Level		
		-1	0	1
Incubation time (A)	Day	1	3	5
Temperature (B)	^{0}C	25	30	35
Moisture substrate (C)	%	50	60	70
pH (D)	-	3.5	5	6.5
Inoculum size (E)	Spore / substrate	6	7	8
Concentration Ammonium sulphate (F)	%	0.1	0.25	0.4

Sampling

Sampling was carried out at daily intervals. During the fermentation process, 1 gram of sample was taken every 24 hours for 10 days for assay. The 25 ml of citrate phosphate buffer (pH 4.0) was added into the sample. The sample was centrifuged at 4^0C for 20 minutes at 4000 rpm. The supernatant were used for determination of the protein content and chitinase activity.

Enzyme Extraction

Fermented samples were collected every 24 h. In each sampling, 1 g of the sample was drawn out and mixed with 25 mL of citrate phosphate buffer (pH 4.0). The mixture was then placed on a vortex for 1 minute to ensure that enzyme or sugars on the surface were well-mixed with the buffer. Next, the suspension was centrifuged at 4000 rpm at 4˚C for 20 minutes to separate the solid and liquid phase. The supernatant was used as crude enzyme for various assays.

Analytical Procedures

The activity of chitinase was determined by the method of dinitrosalycylic acid (DNS) [15]. Chitinase activity was measured by incubating 1 mL of enzyme solution with 0.5 g (w/w) of colloidal chitin in 1 mL of 0.15 M citrate phosphate buffer (pH 4.0) at 50°C for 1 h. The reaction was terminated by placing the tubes in a boiling water bath for 5 min and the undigested materials were removed by centrifugation at 4000 rpm for 5 min. The reduced sugar produced was measured colorimetrically using the dinitrosalicylic acid (DNS) reagent with N-acetyl-D glucosamine as the standard. Colloidal chitin was prepared by following the method of Azaliza *et al*. [3]. One unit (U) of chitinase activity is defined as the amount of enzymes that is required to release 1 µmol of N-acetyl-β-D-glucosamine per minute under assay conditions. Protein assay was carried out accordingly to Lowry's method [16]

RESULT AND DISCUSSION

Six variables, which were expected to have an effect on chitinase production, were identified by preliminary experiments. the variables having most significant effect on chitinase prouction of shrimp waste were identified using a 2-level factorial design. The variable that were included in the screening experiment and their settings are given in Table 1. Each independent variable was investigated at a high (+1) and a low (-1) level. A 2^6 half factorial experimental design for six independent variables [13] was selected with six replicates at center point, leading to a total of 38 experiments. Table 1 shows the design matrix covering six variables to evaluate their effect on chitinase production, it also gives the response evaluated as chitinase activity (U/g). the runs were randomized for statistical reasons. The variables having major effects on chitinase production were identified on the basis of confidence levels above 94% ($P < 0.05$).

Screening design (2^6) was used to detect the factors or independent variables that had higher impact on the response variable the chitinase activity. Table 2 shows the predicted levels of chitinase activity from enzymatic degradation of shrimp waste along with experimental data. Interpretation of result was analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used. The results are included in Table 3

Interaction of variables, incubation (A), temperature (B), moisture substrate (C), pH (D), inoculum size (E), concentration ammonium sulphate (F) on chitinase production in solid state fermentation are suumarized in the regression equation below

Chitinase activity (U/g) = 0.18 + 0.018 A + 0.013 B – 0.024 C + 5.075E-003 D – 5.759E-003 E + 3.890E-003 F – 0.033 AB – 5.619E-003 AC – 6.070E-003 AD – 8.462E-004 AE – 5.673E-003 AF – 6.305E-003 BC + 1.585E-003 CE + 7.611E-004 DF + 6.4888E-003 ABC + 8.494E-003 ACE – 6.461E-003 ADF

To determine the significant factors affecting the response, the half normal plot can be utilized. The half normal plot showed the symbol of factors which are far away from linear line are the most significant factors toward chitinase production. In Figure 1, the effect for interaction between incubation and temperature (factor

AB) obviously falls furthest for away from the line, so that it represents a strong signal. This result correlates with the P value < 0.0001. The next significant factors are moisture substrate (factor C), incubation (factor A), temperature (factor B) and interaction between incubation, temperature and moisture substrate (factor ABC).

The factor moisture substrate was found as a significant effect in chitinase production. The moisture content in SSF is most important parameter [17]. Fungi are well known to favor moist environments for their growth. An optimum moisture level however has to be maintained since, lower moisture tends to reduce nutrition diffusions, microbial growth, enzyme stability and substrate swelling [18].

Incubation time was one of the most important variables. Its statistical significance being one of the highest with P value < 0.0001. Incubation time of fermentation strongly influences the rate of growth and synthesis of enzyme or metabolites. It allowed enzyme to react with substrate in a time which the reaction favor to produce their product effectively [19].

Temperature is next significant factor identified in this statistical analysis with P value of 0.0002. The significance of temperature in development of biological process is such that it could determine the effects of protein denaturation, enzyme inhibition, promotion or suppression of the production of a particular metabolite, cell viability and death [20]

The interaction between incubation time and temperature (factor AB) was highly significant (P < 0.0001), the lowest response was observed when incubation and temperature were highest (Table 2, experiment 8, 12, 16, 20, 24 and 28). Increased amount of incubation time will increase production of chitinase at low temperature (Table 2, experiments 2, 6, 10, 14, 18, 22, 26 and 30).

The effect of each factor was statistically significant. The main effects A (incubation time), B (temperature), C (moisture substrate), interaction AB (incubation time with temperature), AD (incubation time with pH), BC (temperature with moisture substrate), ABC (incubation time with temperature and moisture substrate), ACE (incubation time with moisture substrate and inoculum size) and ADF (incubation time with pH and ammonium sulfate concentration) are of higher statistical significance for chitinase production. Effect D (pH), E (inoculum size), F (ammonium sulfate concentration) and interaction AC, AE, AF, CE and DF which possess larger P value of more than 0.05 were statistically in significant factors has positive effect on the response. Howefer, moisture substrate. showed a greater degree of positive effect on the response compared to the other factors (Figure 1)

The fit model was examined by the coefficient of determination $R^2 = 0.9448$, which implies that the sample variation with more than 94.5 % is associated with the variable. Only 5.50% of the total variance cannot be explained by the model. The adjusted coefficient of determination (Adj $R^2 = 0.8954$) is also satisfactory to confirm the significance of the model. In addition, this model has an "adequate precision value" of 14.95, indicating that the model can be used to navigate the design space. The optimum variables tested (incubation time (A), temperature (B), substrate moisture (C), pH (D), number of inoculum (E), and ammonium sulfate concentration (F)) were obtained from ANOVA. The optimal level for variables is as follows: incubation 5 days , temperature 25^0C , moisture substrate 50.03% , pH 3.5, number of inoculum 1 x 106 spores/substrate and 0.38% ammonium sulfate concentration with Y = 0.265 U/ g . To validate this model, these predicted parameters were tested in the laboratory and samples were taken at certain intervals during the fermentation time for chitinase production and other analyzes. The final chitinase production obtained was 0.261 U / g, which almost reached the predictive value under the same conditions. These results reinforce the validity and effectiveness of this model. Figure 2 shows the optimum level estimated from six independent variables with the desirability of 91.8 %.

TABLE 2. 2-level factorial design of variables (in coded levels) with chitinase activity (U/g) as the responses

Bil.	Incubation time (day) A	Temperature (°C) B	Moisture of substrate (%) C	pH D	Inoculum size (spore/substrate) E	Conc.ammonium sulphate (%) F	Chitinase activity (U/g) Y	
							Actual Value Y (U/g)	Predicted value Y' (U/g)
1	-1	-1	-1	-1	-1	-1	0.100	0.10
2	1	-1	-1	-1	-1	1	0.26	0.27
3	-1	1	-1	-1	-1	1	0.22	0.22
4	1	1	-1	-1	-1	-1	0.22	0.22
5	-1	-1	1	-1	-1	1	0.100	0.11
6	1	-1	1	-1	-1	-1	0.19	0.18
7	-1	1	1	-1	-1	-1	0.18	0.17
8	1	1	1	-1	-1	1	0.15	0.14
9	-1	-1	-1	1	-1	1	0.15	0.14

continued

10	1	-1	-1	1	-1	-1	0.28	0.27
11	-1	1	-1	1	-1	-1	0.22	0.23
12	1	1	-1	1	-1	1	0.20	0.21
13	-1	-1	1	1	-1	-1	0.12	0.11
14	1	-1	1	1	-1	1	0.16	0.17
15	-1	1	1	1	-1	1	0.20	0.21
16	1	1	1	1	-1	-1	0.14	0.15
17	-1	-1	-1	-1	1	1	0.10	0.11
18	1	-1	-1	-1	1	-1	0.21	0.22
19	-1	1	-1	-1	1	-1	0.21	0.22
20	1	1	-1	-1	1	1	0.19	0.19
21	-1	-1	1	-1	1	-1	0.087	0.080
22	1	-1	1	-1	1	1	0.19	0.19
23	-1	1	1	-1	1	1	0.17	0.15
24	1	1	1	-1	1	-1	0.13	0.14
25	-1	-1	-1	1	1	-1	0.12	0.11
26	1	-1	-1	1	1	1	0.23	0.22
27	-1	1	-1	1	1	1	0.28	0.27
28	1	1	-1	1	1	-1	0.20	0.19
29	-1	-1	1	1	1	1	0.11	0.12
30	1	-1	1	1	1	-1	0.19	0.19
31	-1	1	1	1	1	-1	0.14	0.15
32	1	1	1	1	1	1	0.15	0.14
33	0	0	0	0	0	0	0.14	0.15
34	0	0	0	0	0	0	0.14	0.15
35	0	0	0	0	0	0	0.15	0.15
36	0	0	0	0	0	0	0.18	0.15
37	0	0	0	0	0	0	0.14	0.15
38	0	0	0	0	0	0	0.13	0.15

TABLE 3. Regression analysis (ANOVA) for the chitinase activity using 2-level factorial design

Source	Sum of squares	DF	Mean Squares	F-value	P > F	R^2
Model	0.082	17	4.811E-003	19.41[a]	<0.0001[b]	0.9448
A	0.010	1	0.010	41.32	<0.0001	
B	5.306E-003	1	5.306E-003	21.11	0.0002	
C	0.019	1	0.019	75.46	<0.0001	
D	8.243E-004	1	8.243E-004	3.28	0.0860	
E	1.061E-003	1	1.061E-003	4.22	0.0539	
F	4.841E-004	1	4.841E-004	1.93	0.1813	
AB	0.035	1	0.035	139.81	<0.0001	
AC	1.010E-003	1	1.010E-003	4.02	0.0595	
AD	1.179E-003	1	1.179E-003	4.69	0.0433	
AE	2.291E-005	1	2.291E-005	0.091	0.7665	
AF	1.030E-003	1	1.030E-003	4.10	0.0573	
BC	1.272E-003	1	1.272E-003	5.06	0.0365	
CE	8.035E-005	1	8.035E-005	0.32	0.5785	
DF	1.854E-005	1	1.854E-005	0.074	0.7889	
ABC	1.347E-003	1	1.347E-003	5.36	0.0320	
ACE	2.309E-003	1	2.309E-003	9.18	0.0069	
ADF	1.336E-003	1	1.336E-003	5.31	0.0326	
Curvature	3.758E-003	1	3.758E-003	14.95	0.0010[c]	significant
Residual	4.777E-003	19	2.514E-004			
Lack of Fit	3.007E-003	14	2.148E-004	0.61	0.7882[d]	Not significant
Pure Error	1.770E-003	5	3.540E-004			
Cor Total	0.090	37				

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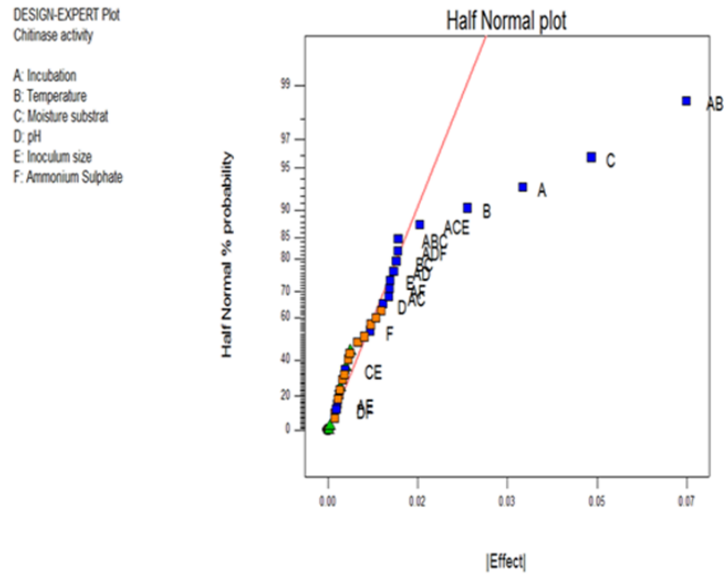
FIGURE 1. Half-normal probability plot the effect of incubation (A), tempeature (B), moisture substrate (C), pH (D), Inoculum size (E) and ammonium sulphate concentration (F)
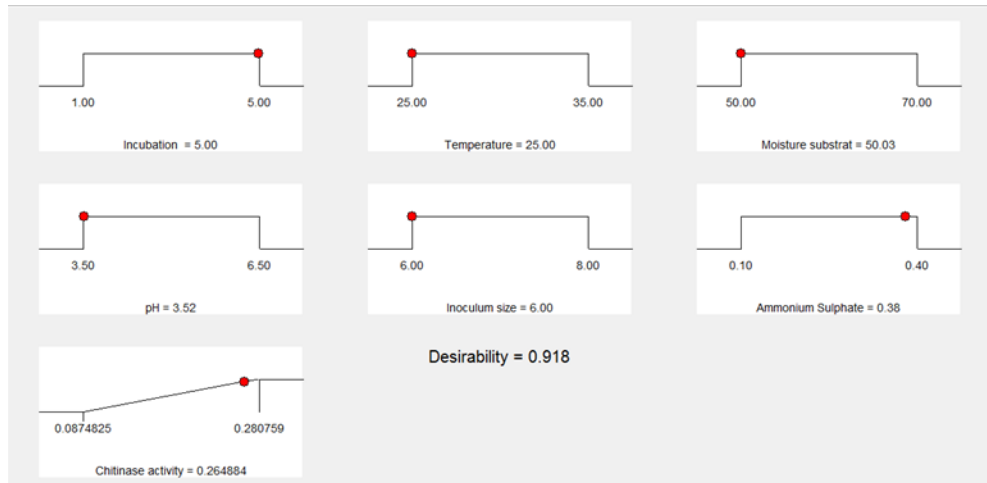
FIGURE 2. Predicted optimum levels of the six independent variables indicating the highest production of chitinase

CONCLUSION

2-Level Factorial Design is useful for filtering influence six variables that affect chitinase production. The experimental results indicated that the proposed mathematical model could adequately describe the performance of the factors being investigated with determination coefficient 0.9448. The three variables tested were found to be significant factors that could affect the chitinase production of *T. virens* with P-value of less than 0.05. Thus, incubation time, temperature and moisture substrate This design suggests that the optimal value for each variable is; incubation time 5 days , temperature 25^0C , moisture substrate 50.03% , pH 3.5, number of inoculum 1×10^6 spores/substrate and 0.38% ammonium sulfate concentration with predicted chitinase production 0.265 U/g . This prediction value was performed in the laboratory and obtained the production of chitinase 0.261 U/g. The actual

experimental results match the prediction. The statistical design proved to be able to find the optimum level of the most significant parameters for chitinase production, with a minimum of effort and time**.**

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