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Production of High Cell Mass of *Bacillus firmus* Using Statistical Medium Optimization

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Growing concern over agricultural losses due to nematodes infection had drawn huge interest in using biological control of *Bacillus firmus* as an agent to reduce the damages and increase crop yield. *Bacillus firmus* strains are well known for their ability to terminate nematode *Meloidogyne incognita* activity. They are also able to secrete beneficial enzyme for use in food, animal feed and industrial sector. Therefore, production of high cell mass for this microbeis required. Extensive study for the development of a suitable medium composition and cultivation strategy is needed. The aim of this study is to increase the biomass production of *Bacillus firmus* through optimization of medium composition using statistical approach. Different production media from previous literatures were screened to supports high cells growth. The best medium was found to yield the highest cell mass of about 6.27 g L^{-1}. The composition of the medium was screened for significant factors and further being optimized using statistical method which employed Box Behnken design. The optimized mediumwas found to efficiently increased the cell mass of about 10.22 g L^{-1}. This value is 49.85% higher compared to the unoptimized medium.

Keywords: *Bacillus firmus*, Statistical method, High cell mass cultivation, Factorial design, Box-Behnken design

Introduction

Over the years, the agricultural sector is attacked by plant parasites nematode which caused severe economic losses. Plant parasitic nematodes are worms that travel through soil and they infest the plant root for feed which cause plant damage[1]. Over 1400 species of plant parasitic nematode reported to be the major constraint towards world food security[2]. It was reported that the estimated capitalized damaged was about $US 118 billion per year which is about 11% of the total crop value[3]. *Bacillus firmus* as bio-control agent is introduced into the agricultural sector for crop protection and may serve as an active agent in the new generation of biopesticide[4]. Based on their high capacity for wide range of metabolites production, *Bacillus sp.* have been widely used for the production of many industrial enzymes and bioactive compounds [5,6,7,8,9]. A well-design screening and optimization process of an industrial medium are required to produce high cell mass in cost efficient manner. Several studies conducted by previous researchers proved that the development of optimized industrial media are important to enhance the production of high cell mass[10,11,12]. This study focuses on the development of a novel culture medium for high cell mass production of *B. firmus*.

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Materials and Methods

Microorganism

The strain of *Bacillus firmus* MPB04 was used in this study. The strain was obtained from Malaysian Pepper Board (MPB), Malaysia. To produce master cell culture, cells were cultivated in Nutrient Agar with formulation consist of (in g L^{-1}): agar, 15.0; meat extract, 1.0; peptone, 5.0; sodium chloride, 5.0; and yeast extract, 2.0. The plates were incubated for 24 hours in the shaking incubator at temperature 28°C. The cells were then suspended in sterile glycerol of 50% v/v aqueous solution and transferred into 2 ml Nalgene Cryogenic Vials. The cryobox was stored in Ultra-Low Temperature Freezer at -80°C. Sub-culturing was continued using the master cell

culture to produce working cell culture under the exact conditions.

Inoculum preparation

The strain was activated by sub culturing of cells from the working cell culture on nutrient agar plates and incubated for 24 hours at 28 °C. The grown cells were then suspended in 5 ml of sterile saline solution for inoculum preparation. Inoculum in form of 1 OD (at 600 nm) was transferred into 250 ml Erlenmeyer flask containing 50 ml of sterile nutrient broth consists of (g L^{-1}): meat peptone, 5.0 and meat extract; 3.0. The inoculated flasks were incubated on the rotary shaker at 200 rpm and 28°C. After 24 hours, the grown cells were used to inoculate the production medium.

Production medium and cultivation conditions

During medium screening, five different media were used to determine the most suitable medium composition for further optimization (Table 1). The pH of each medium was adjusted to 7.2 before sterilization and the carbon sources were sterilized separately and added to cultivation medium before inoculation. Cultivation media were then inoculated with 5 ml of vegetative cells and were incubated in rotary shakerat 200 rpm and 28 °C. All experiments were carried out in triplicate.

Statistical medium optimization

Medium optimization using statistical method was conducted to study the interaction effects between each medium component on *B. firmus* cell growth. Plackett Burman design was used to identify the significant components for the production of *B. firmus*. A two level, five factors full factorial design were used to study the significant effect of glucose, peptone, yeast extract, phosphate salt and magnesium sulphate heptahydrate on cell growth of *B. firmus*. The medium components were screened at two levels where (-1) for a low level and (+1) for a high level. A total number of 36 experiments was design using Minitab 17 software. Data received in full factorial design was subjected to response surface methodology using Box Behnken design experiment (2^{nd} order polynomial) to find the optimum concentration of each significant component for high cell mass cultivation of *B. firmus*. A total number of 45 experiments was design using the same software and data was subjected to ANOVA analysis.

Optical density and cell dry weight determination

Prior to optical density assay, cell dry weight of *B. firmus* was determined by using gravimetric method. 5 ml of the sample was measured into the pre-weight falcon tube and centrifuged at 9000 rpm for 10 min to precipitate the cells. After centrifugation, the cells were separated from thesupernatant. The supernatant was used for glucose analysis. Samples were then diluted accordingly and the optical densities of the samples were recorded using UV-VIS single beam spectrophotometer (HACH DR/2500, Germany) at wavelength of 600 nm. Samples dilutions were

Table 1 — Different media compositions used for *B. firmus* MPB04 cultivationMedium Number

	1	2	3	4	5
Component	Composition g L^{-1}				
Glucose	10.0	10.0	-	20.0	-
Casein	5.0	-	-	-	-
yeast extract	5.0	5.0	-	2.0	-
KH_2PO_4	2.0	-	-	5.0	-
K_2HPO_4	2.0	-	0.5	-	1.5
$MgSO_4.7H_2O$	1.0	0.2	0.5	2.0	1.5
Peptone	-	5.0	-	5.0	20.0
Na_2HPO_4	-	1.0	-	-	-
Na_2CO_3	-	5.0	-	-	-
Glycerol	-	-	80.0	-	10.0
MSG	-	-	20.0	-	-
Citric acid	-	-	12.0	-	-
NH_4Cl	-	-	7.0	-	-
$CaCl_2.2H_2O$	-	-	0.15	-	-
$MnSO_4.H_2O$	-	-	0.15	-	-
Trace element solution ($FeCl_3.6H_2O$, 0.04 g L^{-1})	-	-	10 mL	-	-

preceded accordingly. Falcon tubes with cells in pellet form were dried in oven at 90ºC for 48 hours and re-weighed until constant weight achieved[13].

Glucose determination

Residual glucose concentration was determined using DNS method. This method was conducted by observing the color changes of the samples whereby redox reaction occurs between reducing sugar and DNS reagent (3,5-Dinitrosalisylic acid) in citrate buffer solution. 0.1 ml of the samples was added into the test tube containing 0.7 ml of citrate buffer together with 1.2 ml of dinitrosalicylic acid (DNS). After mixing using vortex, test tubes were then heated in water bath at 95°C for 5 minutes. Test tubes were then cooled immediately in ice bath for 3 minutes to stop the reaction. The optical density of the solution was read using spectrophotometer at wavelength of 540 nm.

Result and Discussion

Cultivation in different media

In this experiment, five production media were selected from previous literatures and screened for suitable media forhigh cell mass cultivation of *B. firmus*. Figure 1 shows the cell dry weight (g L^{-1}) achieved using the five production media. The highest cell mass of *B. firmus* was achieved using medium 4 with cell mass of about 6.27 g L^{-1} and final pH. Medium 4 contains glucose, peptone, yeast extract, KH_2PO_4 and $MgSO_4.7H_2O$ as tabulated in Table 1. Medium without nitrogen source shows low cell mass production. Previous researchers reported that nitrogen component was found as favourable source

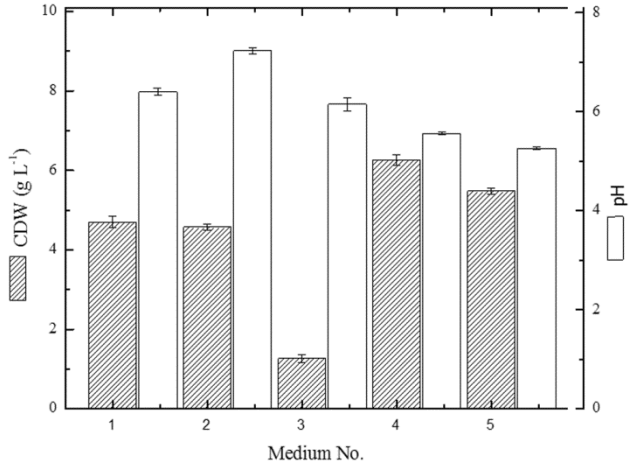

Fig. 1 — Cell dry weight and final pH of *B. firmus* cultivation in shake flask using different media.

for the growth of *B. firmus* cells[14,15]. Medium no. 4 was selectedas it provides the highest cell biomass production and thus was used for further optimization. After this stage, the effect of different carbon sources on cell growth was studied. Results showed that maltose supported better biomass production than glucose (data not shown). Therefore, glucose was replaced by maltose in medium optimization studies.

Plackett burman Design

Five factors, two levels Placket Burman experiment was designed using Minitab 17 software to observe the factors that have the most significant effect on cell mass production. Among the five factors tested, three components passed the significant line that was maltose, peptone and $MgSO_4.7H_2O$. Yeast extract and KH_2PO_4 did not pass the significant line and hence were not considered for further medium optimization.

Box-behnken design

The Box-Behnken design is an application of response surface methodology which does not contain fractional factorial design but allowed points to fall within the combination range of low, middle and high factor levels[16]. Box-Behnken design provides modelling that takes an account on the concentration of each component within the boundaries and eliminates the possibilities of outside boundaries called corner points. This design used second order polynomial regression model with a view to find interaction between components for production of high cell mass. Medium components such as maltose, peptone and $MgSO_4.7H_2O$ were subjected for further optimization using Box-Behnken design to determine the optimum concentration required to produce high cell mass.The interaction between the coded unit and components can be observed clearly under the surface plots. Figure 2 (a), 2 (b) and 2 (c) shows an increase in cell mass where maltose and peptone have high effect on the cell mass production of *B. firmus.* Based on the curve of surface plot, if it is linear, these indicate that the interaction between the components is not significant whereby if the shape are curvature, it shows there is good interaction between the components. The interactions shown between maltose and peptone in surface plot of Figure 2 (a) are more significant followed by interaction between peptone and $MgSO_4.7H_2O$. Table 2 shows the estimated regression coefficients for production of *B. firmus* cell mass using Box-Behnken in coded, un coded units and analysis of variance (ANOVA) respectively. The

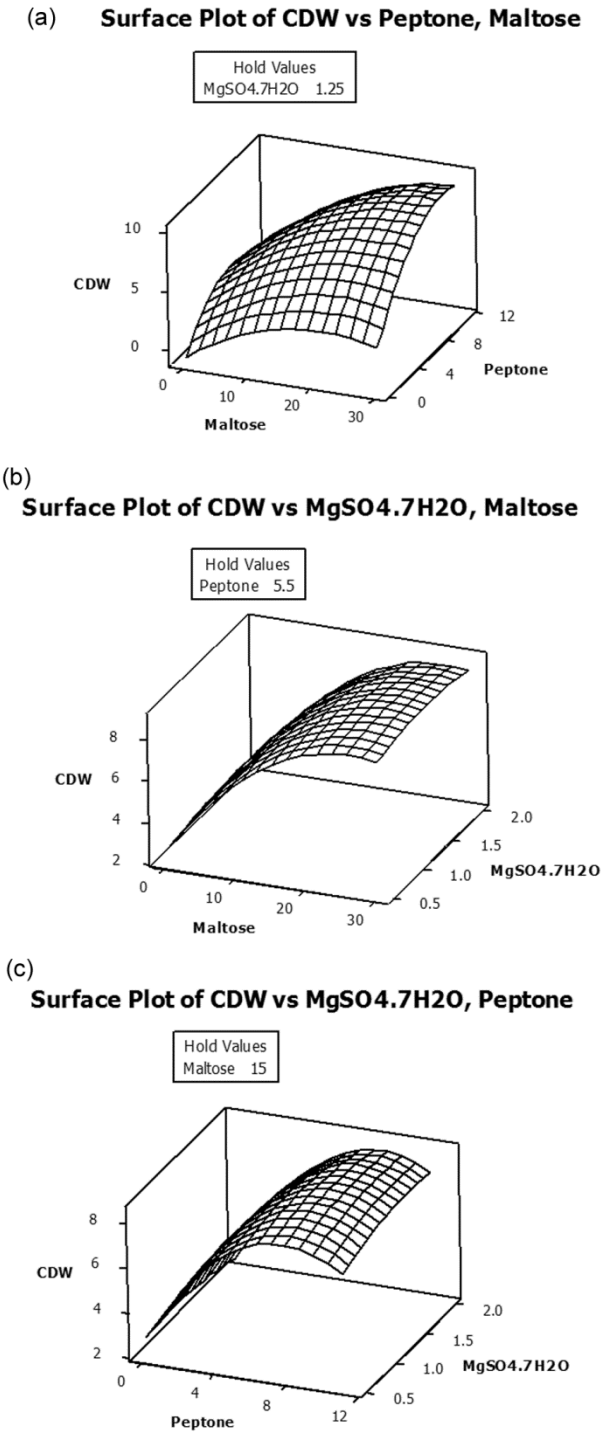

Fig. 2 — (a) Surface plot of peptone and maltose effect on cell mass production (b) Surface plot of $MgSO_4.7H_2O$ and maltose effect on cell mass production (c) Surface plot of $MgSO_4.7H_2O$ and peptone effect on cell mass production

result of Box-Behnken experiment design could be explained in following second order polynomial equation: Cell mass, X (g L^{-1}) = -1.33 + 0.3715A + 1.073B + 1.21C- 0.00882A^2 - 0.0858B^2 - 0.631C^2 + 0.01509AB + 0.0047AC + 0.0628BC.Based on the regression model from Box-Behnken experimental design, an optimization plots indicating optimum concentration for three components were generated in Minitab17 software. The optimum concentration for maltose, peptone and $MgSO_4.7H_2O$ is (in g L^{-1}) 29.70, 9.44 and 1.55 respectively. The analysis of variance, ANOVA for Box-Behnken design is tabulated in Table 2. Lower p-value of 0.010 indicates the significant of each component as well the interaction between each independent component. The regression model lack-lack-of-fit did not show any significance.In addition to that, the Fisher's F-testshowed high significance of regression ($P<0.05$). The results fit the model of coefficient of determination (R^2) which is 0.9023 indicating higher level of significant with 90.23% of the variability of the response could be explained by the model. However, by comparingthe adjusted R^2 (0.877) and predicted R^2 of 0.828, it showed high significant which indicate that the regression model can be applied for component analysis[17].

Growth kinetic comparison

The growth kinetic of *B. firmus* in un optimized and statistically optimized medium in shake flask culture was carried out. Cultivation using these two media were conducted in shaking incubatorat 28 ºC and 200 rpm agitation for 48 hours. Figure 3 illustrates the growth kinetic of *B. firmus* in un optimized and statistically optimized medium cultivated in shake flask culture. The cell mass production achieved in un optimized and statistically optimized medium was6.82 g $L^{-1}$and 10.22g L^{-1}, respectively. Cultivation in optimized medium provides an increase of about 49.85 % compared to the un optimized medium. The lag phase for both media was observed during the first 3 hours of cultivation. The cells grew exponentially from hour 3 to hour 18 in bothcultivations with depletion of glucose and maltose concentration. Specific growth rate, μ of *B. firmus* for un optimized and optimized medium was recorded as 0.15 $h^{-1}$and 0.16 h^{-1}.The cell yield using glucose, $Y_{x/s}$ in un optimized medium is 0.66 gg^{-1} and cell yield produced using maltose, $Y_{x/s}$ in optimized medium was 0.50 gg^{-1}.Table 3 summarizes the growth kinetic of *B. firmus* cultivated in un-optimized and optimized medium.

Table 2 — The ANOVA for cell mass production using Box-Behnken design.

Source	DF	Adj SS	Adj MS	F	P
Regression	9	473.135	52.571	35.91	0.000
Linear	3	343.620	114.540	78.23	0.000
Square	3	110.089	36.696	25.06	0.000
Interaction	3		6.475	4.42	0.010
Residue Error	35	51.244	1.464		
Lack-of-Fit	3	47.575	15.858	138.32	0.000
Pure Error	32	3.669	0.115		
Total	44	524.379			

Table 3 — Growth kinetic of *B. firmus* cultivated in un-optimized and optimized medium.

Parameters	Unoptimized medium	Optimized medium
Cell mass, X(g L^{-1})	6.82	10.22
Maximum cell mass per hour, $X_{max}h^{-1}$ (g $L^{-1}h^{-1}$)	0.23	0.28
Specific growth rate, μ (h^{-1})	0.15	0.16
Carbon source consumption rate, Q_S (g $L^{-1}h^{-1}$)	0.61	0.96
Yield, $Y_{X/S}$ (g g^{-1})	0.66	0.50

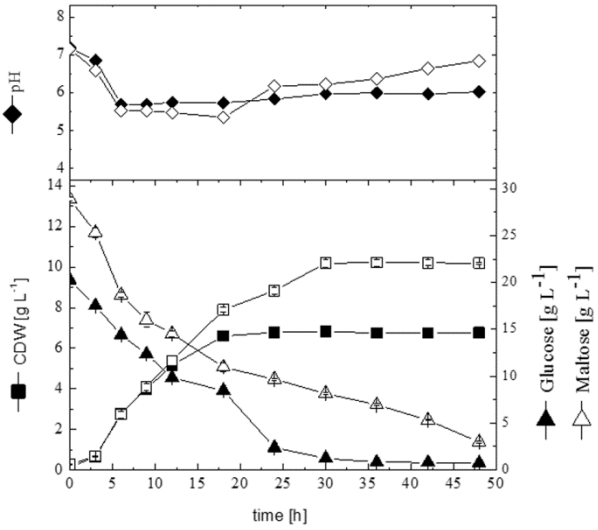

Fig. 3 — Time course of cell dry weight, glucose consumption and pH changes in shake flask cultures where open and closed symbol for optimized and unoptimized medium, respectively

Conclusion

In conclusion, the results obtained revealed that optimization of medium components using statistical approach enhanced high cell mass production of *B. firmus*. The new medium formulation leads to a significant increase in biomass by about 49.85 %. The new medium formulation has high potential use for industrial scale production of this bacteria for economic production of this important biocontrol agent.

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