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Optimization of fermentation medium compositions from dewatered wastewater sludge of beer manufactory for *Bacilus thuringiensis* delta endotoxin production

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Abstract: Optimization of medium compositions from dewatered wastewater sludge of beer manufactory for delta endotoxin production by *Bacillus thuringiensis* was investigated in flask fermentation. Its composition consisted of wastewater sludge hydrolysis broth with several agricultural byproducts (rice bran, soybean meal, corn flour) and mineral salts ($MgSO_4.7H_2O$; K_2HPO_4; KH_2PO_4; $MnSO_4$; $CaCl_2$; NaCl). Rice bran and $MgSO_4.7H_2O$, $CaCl_2$ were all found to have a significant influence on delta endotoxin production. The optimal concentration of these three factors were then sequentially investigated using the response surface methodology with a central composite design. The resulting optimal medium components for delta endotoxin production were determined as follows: WWS (20 g/l, dry weight), $CaCl_2$ (0.3 g/l), $MgSO_4.7H_2O$ (0.32 g/l), and rice bran (5.8 g/l). Using this optimized fermentation media, the yield of delta endotoxin was increased by 34% to 565 mg/l compared with unoptimal medium. Viable cell and spore counts obtained in optimum fermented broth were $1.25x10^9$ CFU/ml, $1.07x10^9$ CFU/ml, respectively. The LC50 value of *Bacillus thuringiensis* serovar *israelensis* against *Culex quinquefasciatus* was 0.056 mg/l and there was no significant difference between LC50 value of bacterium grown in the medium.

Keywords: *Bacillus thuringiensis*, Delta endotoxin, Dewatered Wastewater Sludge, Bio-Pesticide

1. Introduction

Last century, *Bacillus thuringiensis* (Bt) was the most studied in biological agents for control of pest insects. Moreover in last four decades *Bacillus thuringiensis* was used to forming bio-pesticide to control pest insects in agriculture, forestry and mosquito that is vector causes many diseases such as: malaria, yellow fever, Japanese encephalitis, and dengue fever. *Bacillus thuringiesis* – based bio-pesticides have utmost importance and occupy more than 90% of the world bio-pesticide market and not harmful for people, animals and environment [4]. Today, bio-pesticides are world wide applied generously to protect plant in the world. However, using bio-pesticides still limited as chemical pesticides so high cost. Stanbury et al. (1995) estimated cost of materials for producing bio-pesticides about 35 – 59%. So finding cheap and popular materials to reduce product cost is target of productions [18].

Today, in the world there are a lot of experiments to reuse industry, agriculture waste to produce bio-pesticide. In Canada, there are a lot of studies about treating, reusing slaughterhouse, starch industry wastewater sludge as material for production of bio-pesticides to provide for agriculture. Reusing wastewater sludge can reduce about 30% cost as TSB [1, 11]. Besides that, there are also studies reusing other organic waste as material for *Bacillus thuringiensis* fermentation: experiment of Subbiab Poopathi and Archana shown that use chicken feather from poultry farm to culture *B. thuringiensis* subsp. *israelensis* maybe reduce 49 times cost of product as TSB [19]. Recently, Songqing Wu et al (2014) studied to reuse spent mushroom

substrate to culture *Bacillus thuringiensis* by the solid – state fermentation method. The toxicity of this product against fourth instars larvae of *Culex quinquefasciatus* was 1.487IU/mg and cost about US$ 0.075/kg [17]. In this paper we show about optimization of medium composition for delta – endotoxin production by *Bacillus thuringiensis* used dewatered wastewater sludge from beer manufactory.

2. Material and Methods

2.1. Bacterial Strain and Media

- *Bacillus thuringiensis* serovar *israelensis,* H14 (Bti) used in this study provide by Department of Microbial Genetics, Institute of Biotechnology, Vietnam Academy Science and Technology, Hanoi Vietnam.
- *Reference medium* ─ Tryptic Soya broth (TSB) was used as a control media (g/l): soybean meal 5.0; glucose 5.0; starch 5.0; K_2HPO_4 1.0; KH_2PO_4 1.0; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$ 0.02; $ZnSO_4.7H_2O$ 0.02; $CaCO_3$ 1.0
- *Inoculum preparation*: The inoculum was prepared follow reported by Lachhab et al [13]. The Bti was subcultured on tryptic soya agar (TSA) tube, incubated for 48 h at 30 ± 0.1°C and then preserved at 4 ± 0.1°C for future use.
- Media used for inoculum preparation were adjusted to pH 7.0 ± 0.1 before autoclaving. A loopful of Bti grown on TSA tube was used to inoculate a 200 ml Erlenmeyer flask containing 50 ml of sterilized tryptic soya broth (TSB) medium. The flask was incubated in a rotary shaker at 200 revolutions per min (rpm) and at 30 ± 0.1°C for 8 – 12 h. A 2% (v/v) inoculum from this flask was then used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of sterilized medium [12].

2.2. Sludge

The dewatered wastewater sludge (DWS) of Saigon Hanoi brewing factory (Hanoi, Vietnam) was used as a raw material for Bti growth. DWS was sampled and stored at 4 ± 0.1°C to minimize microbial degradation.

2.3. Sludge Pre-Treatments

The dewatered wastewater sludge was pre-treatment by thermal alkaline hydrolysis [21]. The total solid of DWS was approximately 20%, was diluted to 2% and raised to pH 10±1 and treated at 121°C for 30 minutes. The chemical composition of DWS hydrolysis broth was showed at Table 1. In which, the chemical composition was determined by standard methods [2]. The DWS hydrolysis broth was adjusted to pH7±0.1 before using for studies.

2.4. Fermentation

Erlenmeyer flasks (500 ml) containing 100 ml of DWS hydrolysis broth were inoculated with 2% (v/v) inoculum and incubated in a shaker at 200 rpm and 30 ± 0.1°C for 48 hrs.

2.5. Estimation of Total Cell and Spore Count

The total cell count and spore count were performed by counting colonies grown on TSA plates [11]. The samples were serially diluted with sterile saline solution (0.85% NaCl) to determine total cell count and spore count, 0.1 ml appropriately diluted samples were plated on TSA plates. All samples were incubated at 30°C for 24 hrs to form fully developed colonies. To determine spore count, appropriately dilute samples were heated in a silicon bath at 80°C for 10 min. and then chilled on ice for 5 min. [20]. Counting colonies grown on nutrient medium to estimate total cell and spore count. Each dilution was repeated three times and took the average of three replicate.

Table 1. *Characteristics of DWS hydrolysis broth*

Characteristics	Concentration (mg/l)
Total organic carbon (TOC)	306
Total nitrogen (TN)	16.7
Total phosphorous (TP)	1.94
Al	21.8
Ca	38.9
Cd	0.0029
Cr	0.0171
Cu	0.242
K	27.8
Mn	0.155
Ni	1.070
Pb	0.021
Fe	20.80
Mg	8.760
Zn	6.3
Na	172

2.6. Estimation of Delta-Endotoxin

Delta-endotoxin concentration was estimated according to Khanh Dang vu et al 2008 based on the solubility of insecticidal crystal proteins in alkaline condition[12, 22-24]: 1 ml of sample was centrifuged at 10,000g for 10 min. at 4°C. The pellet containing a mixture of insecticidal crystal proteins, spores, cell debris and residual suspended solids was used to determine the concentration of alkali soluble insecticidal crystal proteins (delta-endotoxin). These pellets were washed with 1 ml of 0.14 M NaCl - 0.0 1% Triton X-100 solutions three times to eliminate the soluble proteins and proteases that might be added on to the pellet and could affect the integrity of the crystal protein. The delta endotoxin in the pellet was dissolved with 0.05 N Na0H (pH 12.5) at 30°C for 3 h with stirring. The suspension was centrifuged at 10,000g for 10 min at 4°C and discard the pellet which containing spores, cell debris and residual suspended solids. The supernatant was used to determine the delta-endotoxin concentration by Bradford method using bovine serum albumin as standard protein [6].

2.7. Medium Optimization Using One-Factor-at-a-Time

The production of delta endotoxin was optimized base on

adding nutrients to the hydrolysis sludge broth. Three organic compounds: rice bran, corn bran, soybean meal were added at a concentration of 5 g/l each; Mineral salt: $CaCl_2$, $MgSO_47H_2O$, NaCl, K_2HPO_4, KH_2PO_4 was added at a concentration of 0.1 g/l and 0.5 g/l.

2.8. Central Composite Design and Response Surface Methodology

A central composite design was adopted to optimize the effect of the major variables (rice brand, $CaCl_2$, and $MgSO_4.7H_2O$) on delta endotoxin production. The effect of each variable was studied at five different levels, namely -α, 1, 0, +1, +α, and the set of 20 experiments was performed in triplicate (table 3). The central coded value for all the variables was set at zero. The data obtained from the RSM on delta endotoxin production were subjected to analysis of variance (ANOVA). The results of the RSM were used to fit a second-order polynomial, Eq. (1), to represent the behavior of the system:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_1b_1X_1^2 + b_2b_2X_2^2 + b_3b_3X_3^2 + b_1b_2X_1X_2 + b_1b_3X_1X_3 + b_2b_3X_2X_3 \quad (1)$$

Where Y is the response variable representing delta endotoxin concentration, b_0 is the intercept, b_1, b_2, b_3, are the linear coefficients, b_1b_1, b_2b_2, b_3b_3 are the squared coefficients, b_1b_2, b_2b_3, b_1b_3 are the interaction coefficients, and X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, X_2X_3, X_1X_3 are the levels of the independent variables. The data analysis and generation of the response surface graphs were conducted using the statistical software Design Expert (Design-Expert 9.0.2).

2.9. Validation of the Model

The validation experiment was carried out in triplicate tests under the optimized condition to verify the predicted results.

2.10. Insect Bioassays

The larvae Culex quinquefasciatus (Diptera: Culicidae) received from National institute of Malariology Parasitology and Entomology, Hanoi, Vietnam.

The bioassays were carried out as described previously [3]. Preparing a stock solution of delta endotoxin (100 mg/l) and serial dilution were made (0.003 – 0.1 mg/l). Bioassays were carried out in disposable cups, containing 100 ml of water with appropriate dosage. 10 third instar larvae were taken into the paper cups separately. Bioassays were carried out at room temperature (28 – 30^{o}C) and larvae mortality was monitored in 24 hrs. The LC50 value was estimated by probit analysis calculations [3]. The experiments were carried out simultaneously with control samples and also observed in 24 hrs.

3. Results

3.1. Effects of Mineral and Organic Matter Added to the DWSB Thermal Alkaline Hydrolysis Broth on Delta Endotoxin Production

The effects of mineral salts and organic matter were investigated by adding $MgSO_4.7H_2O$, $CaCl_2$, NaCl, KH_2PO_4, K_2HPO_4, $MnCl_2$, rice brand, soya meal to the DWS hydrolysis broth on delta endotoxin production was shown on Table 2.

Table 2. *Cell, spore counts and delta endotoxin concentration of fermented broth when adding minerals and organic matter*

Culture medium	Cell count (x10^{8}CFU/ml)	Spore count (x10^{8}CFU/ml)	Con. of delta endotoxin (mg/l)
DWSHB	1.5	1.3	420
Mineral salts			
DWSHB + $MgSO_4.7H_2O$ (0.5g/l)	3.1	2.7	510
DWSHB + $CaCl_2$(0.5g/l)	3.2	2.5	505
DWSHB + NaCl(0.5g/l)	2.5	2.0	448
DWSHB + KH_2PO_4 (0.5g/l)	2.2	2.1	442
DWSHB BTSH + K_2HPO_4 (0.5g/l)	2.5	2.4	438
DWSHB + $MnCl_2$ (0.01g/l)	0.02	0.001	-
Agro. byproduct			
DWSHB + rice bran (5g/l)	5.1	4.8	509
DWSHB + corn flour (5g/l)	3.8	3.3	452
DWSHB + soya meal (5g/l)	5.5	3.9	504

As shown in Table 2, Out of six inorganic salts examined, $MgSO_4.7H_2O$ and $CaCl_2$ were found to have a significant influence on delta endotoxin production. Viable cell counts and spore counts increased by two times, the concentration of delta endotoxin also increased by 20% compared to other media. Meanwhile, NaCl, KH_2PO_4, K_2HPO_4 had no obvious effect and $MnCl_2$ even had a negative effect on growth potential or delta endotoxin production of Bt.

All of organic matters were significant influence on delta endotoxin. There was no significant difference between delta endotoxin concentration obtained from DSW + soya meal and DSWHB + rice bran fermented broth. A maximum delta endotoxin was observed in the medium DSWHB + rice bran (509 mg/l) increased about 25%, viable cell counts and spore counts also increased by 3 times compared to DSWHB media (Table 2). However, rice bran is an agro-industrial, cheap, popularity so rice bran was chosen for further studies. Interestingly, it was observed that the medium containing all of $MgSO_47H_2O$, $CaCl_2$, rice bran and DWS yielded the highest delta

endotoxin production. Thus, $MgSO_4$, $CaCl_2$, rice bran were chosen to optimize the medium for delta endotoxin.

3.2. Optimization Using Central Composite Design

Table 3. *Experimental design of the model*

No.	X_1: $CaCl_2$ (g/l)	X_2: $MgSO_4.7H_2O$ (g/l)	X_3: rice brand (g/l)
1	1	0.6	0
2	0.5	0.3	13.40896
3	0.5	0.3	0
4	1	0	10
5	0.5	0.804538	5
6	0	0.3	5
7	1	0	0
8	0.5	0.3	5
9	1	0.6	10
10	1.340896	0.3	5
11	0	0	0
12	0.5	0.3	5
13	0.5	0	5
14	0	0.6	10
15	0.5	0.3	5
16	0.5	0.3	5
17	0	0.6	0
18	0	0	10
19	0.5	0.3	5
20	0.5	0.3	5

The effect of each variable on delta endotoxin was characterized at five different levels, namely -1.68, -1, 0, +1, +1.68. Thus, a set of three factors with five levels and a total of 20 treatments were performed (Table 3). The experiments were repeated three times. The significance of each term in the model is presented in Table 4. The ANOVA for the selected quadratic model showed that the model was significant with a Model F = 32.92 and P > F-value < 0.0001. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, X_3, X_1X_3, X_1^2, X_2^2, X_3^2 are significant model terms.

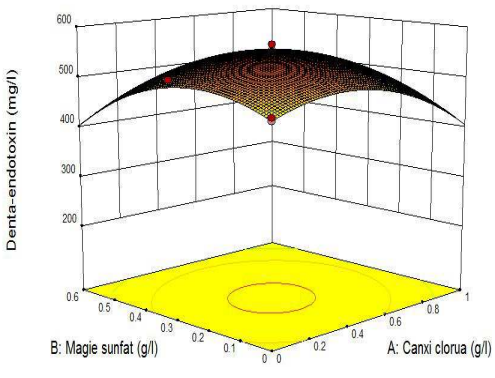
The F-value and P-value of 'lack of fit' were 2.6604 and 0.1367, respectively, which indicated that 'lack of fit' was non significant. A non-significant value of lack of fit indicates that model was significant [5, 14]. It was also suggested that the quadratic and linear terms of rice bran, $MgSO_4.7H_2O$ and $CaCl_2$ of the model primarily determined endotoxin production by Bti. Thus, the response of delta endotoxin production (Y) by *Bacillus thurringiensis* serovar *israelensis,* H14 could be expressed in terms of the following regression equation 2:

Table 4. *Analysis of variance (ANOVA) for the model regression representing delta endotoxin production*

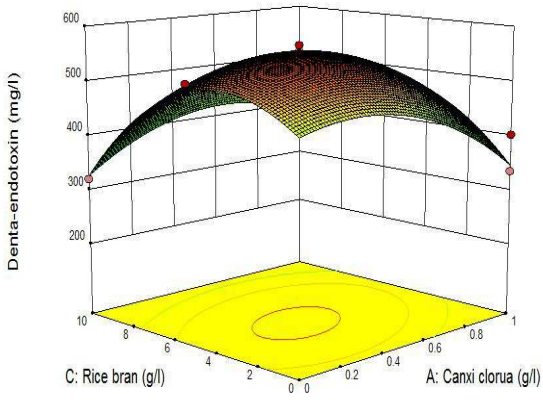
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	188417.1	9	20935.24	304.255	5.39E-11
X_1-Canxi clorua	19391.3	1	19391.3	281.8167	1.18E-08
X_2-Magie sulfate	113.995	1	113.995	1.656707	0.227042
X_3-Rice bran	180.1918	1	180.1918	2.618755	0.136677
X_1X_2	162	1	162	2.354371	0.155941
X_1X_3	24.5	1	24.5	0.356062	0.563965
X_2X_3	1300.5	1	1300.5	18.90037	0.001449
X_1^2	81250.14	1	81250.14	1180.821	1.03E-11
X_2^2	13011.62	1	13011.62	189.0999	8.04E-08
X_3^2	13125.63	1	13125.63	190.7568	7.71E-08
Residual	688.0819	10	68.80819		
Lack of Fit	512.7486	5	102.5497	2.924422	0.131945
Pure Error	175.3333	5	35.06667		
Cor Total	189105.2	19			

$$\text{Delta endotoxin (Y)} = 409.00502 + 84.7006*X_1 + 4407.36222*X_2 + 36.38446*X_3 + 142.79838*X_1*X_2 + 17.68827*X_1*X_3 - 17.20373*X_2*X_3 - 245.53815*X_1^2 - 740.09354*X_2^2 - 4.49948*X_3^2 \quad (2)$$

Where, X1 is concentration of $CaCl_2$, X2 is concentration of $MgSO_4$, and X3 is concentration of rice bran.



(a)



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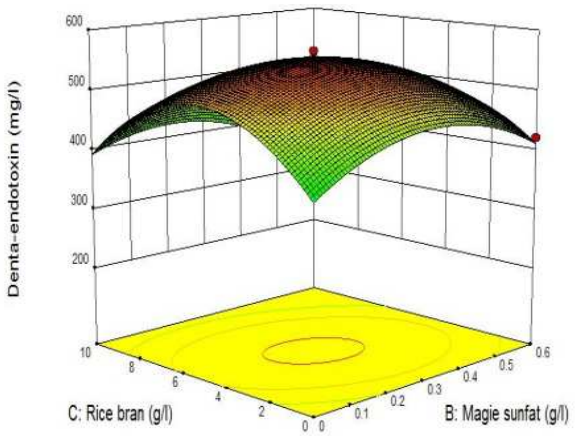

(c)

Figure 1. *Three-dimensional response surface plots of delta endotoxin production by Bti under conditions optimized using RSM*

(a) Effect of interaction of Magie sulfat and canxi clorua; (b) effect of interaction of rice bran and canxi clorua; (c) effect of interaction of rice bran and magie sulfat

A 3D response surface was drawn based on the model equation to investigate the interaction among the variables and determine the optimum concentration of each factor for maximum delta endotoxin production by Bti (Fig.1).

The model predicted that the maximum delta endotoxin production was up to 559 mg/l U/ml when the medium was DWSHB + $CaCl_2$ 0.3 g/l, $MgSO_4 7H_2O$ 0.32 g/l and rice bran 5.8 g/l.

3.3. Validation of the Model

On the basis of medium optimization, the quadratic model predicted that the maximum production of delta endotoxin by *Bacillus thuringiensis* serovar *israelensis*, H14 was 559 mg/l, when the DWS hydrolysis broth was added $CaCl_2$ 0.3 g/l, $MgSO_4$ 0.32 g/l and rice bran 5.8 g/l (Table 5). To confirm the accuracy of the model, validation experiment was performed in triplicate tests. The results presented in table 5 shown that the difference between concentration of delta endotoxin predicted and experimental was insignificant, therefore, the model was validation. Thus, the optimal medium was DWS hydrolysis broth + $CaCl_2$ 0.3 g/l, $MgSO_4$ 0.32 g/l and rice bran 5.8 g/l. When using this medium, delta endotoxin concentration of fermented broth increased by 34% compared to DWSHB media at the end of fermentation (Table 5).

Table 5. *Experimental verification of combined effect of optimized medium on delta endotoxin production*

Medium	Delta endotoxin (mg/l)		Cell count (CFU/ml)	Spore count (CFU/ml)
	Predicted	observed		
DWSHB+ rice bran 5.8 g/l + $MgSO_4.7H_2O$ 0.32 g/l + $CaCl_2$ 0.3 g/l	559	565	$1{,}25x10^9$	$1{,}07x10^9$
DWSHB		422	$1.8x10^8$	$1.3x10^8$

3.4. Toxicity of Bt Product on Culex Quinquefasciatus

The toxicity of Bti produced from two culture media (TSB, DWSHB + rice bran + $CaCl_2$ + $MgSO_4.7H_2O$) was tested with larvae Cx *quinquefasciatus* larvae. The results showed that, there was no significant difference between LC50 value of Bti grown in the TSB media or optimum DWS media. The LC50 value of Bti against Cx *quinquefasciatus* larvae was 0.056mg/l

4. Discussion

Microorganisms require nutrients and minerals for their growth and metabolic product formation. Nutrient has a strong effect on delta endotoxin synthesis. According Zouari N (1999) when changing the nutrient composition of the fermentation medium will alter the ability to synthesize delta endotoxin of Bt. In this study, concentration of delta endotoxin increase by 20 – 30% when adding some minerals and organic matters to DWS hydrolysis broth. Içgen et al. (2002) and Braun (2000) showed that deficiency of Mg or Mn reduced growth and sporulation, but the most noteworthy effect was a decrease in toxin biosynthesis [7, 9]. Magnesium acts as an enzymatic regulator, by activating some enzymes involved in spore formation [1, 9]. Ozkan et al. (2003) indicated that Mg increased toxin biosynthesis when provided at $8x10^{-3}$M concentrations [16]. In addition, Içgen et al. (2002) shown that growth, sporulation and delta endotoxin synthesis were all very effective when Mg was provided at $8x10^{-5}$ M to $4x10^{-3}$M. In our study, delta endotoxin concentration of fermented broth achieved highest when Mg was provided at $1.3x10^{-3}$M, the lower or higher concentrations both reduced delta endotoxin production. Thus, $MgSO_4.7H_2O$ $1.66x10^{-3}$M (original Mg concentration of DWS hydrolysis broth 0.33^{-3}M, table 1) was best effect on delta endotoxin production.

In Ozkan study, canxi also plays an important role in the composition of the Bt fermentation medium at concentrations of 5.5 $x10^{-4}$M. However, our study results shown that Mg of $3{,}67x10^{-3}$M had a best effect on delta endotoxin production. Subbial Poopathi et al. (2012) shown production of Bti from chicken feather medium was increased significantly by adding $MnCl_2$ at concentration of $3.9x10^{-2}$M. Mn^{2+} concentration of DWS hydrolysis broth was $2.8x10^{-6}$M (Table 1) when adding $MnCl_2$ at concentration of $7.9x10^{-5}$M (0.01 g/l) had a negative effect on Bti production [19]. In agreement with this result, Ozkan et al. (2003) also found that Mn^{+2} were the most critical element for the biosynthesis of toxins at 10^{-6} M concentration [16].

Traditional methods to optimize fermentation medium composition change one independent variable, while the variables are keeping fixed at a certain level. However, the single-dimensional search not only is laborious and

time-consuming but also incapable of reaching a true optimum owing to the interactions among the variables [8]. The response surface method was first described by Box and Wilson in 1951 [5]. This is an effective strategy for seeking the optimum conditions for a multivariable system. Jaekoo Lee et al. (2013) shown that galactosidase activity of *Bacillus* sp. LX-1 increased by 6.3 times compared to that value previously obtained under basal conditions [10]. Gao et al. (2013) found that using response surface method to optimize the culture medium for laccase production by *Trichoderma harziamum* ZF-2, the yield of laccase was increased 59.68 times compare with the laccase production with an unopitimized medium [8]. Linna Du et al. (2012) also shown that the biomass, adenosine, polysaccharide and cordyceps acid yields were enhanced by 8.200, 3.580, 23.170 and 31.510%, respectively, when optimizing culture medium by response surface method [15]. In this study, optimization of culture medium for delta endotoxin production by Bti using response surface method, the yield of delta endotoxin production was increased by 34% compare with the delta endotoxin productional obtained from unoptimum medium.

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