OPTIMIZATION OF FERMENTATION CONDITIONS FOR COLLECTING OF *BACILLUS SUBTILIS* NT1 BIOMASS USED IN *CANNA EDULIS*. KER PROCESSING WASTEWATER TREAMENT

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SUMMARY

Canna edulis. Ker processing craft villages in rural area of Vietnam are developing rapidly thus helping local people to create high-income jobs. However, large amount of untreated waste water and solid waste have been causing serious environmental pollution. Strain *Bacillus subtilis* NT1, isolated from wastewater of *Canna edulis*. Ker processing craft village, has proven to be capable of handling wastewater. In order to produce microbial products for wastewater treatment in large scale, fermentation conditions for *B. subtilis* NT1 were optimized by submerged fermentation method. By surveying influenced factors and optimization by the Box-Behnken method, optimal condition for *B. subtilis* NT1 growth was determined at 8.0 (g/l) glucose, 7.0 (g/l) peptone, shaking speed 195 rpm, 30°C, the inoculum rate of 5% (v/v) and initial pH of 6. After 24 hours biomass obtained was 11.98 LogCFU/ml.

Keywords: Bacillus subtilis, biomass, optimization, submerged fermentation, waste water.

I. INTRODUCTION

Starch and vermicelli made from *Canna edulis*. Ker processing are very popular products with mass consumption market. The increase in the number of starch processing villages with untreated wastewater and solid waste has been causing many serious environmental problems, influencing on the health and lives of local people and causing burden to society (Tran Van The et al, 2013).

For example, in Duong Lieu village (Hoai Duc, Hanoi), production of vermicelli has discharged into the environment large amounts of wastewater and solid waste with high organic contents causing serious pollution. In this wastewater there are many exceeded indicators in comparison with (2945:2005) standard such as SS (474 mg/l - exceeding 4.7 times), BOD_5 (5506 mg/l - 110 times), COD (6406 mg/l - 80 times), Coliform ($9x10^5$ CFU/ml - 180 times), N (154.02 - 5 times), P (29.93 - 5 times). The high pressure from environmental pollution has led to the necessary of treatment. Essentially, the pollutants in this wastewater are organic compound that can be decomposed by microorganisms (Cristhiane L. Krueger et al, 2012).

Among bacterial strains capable of metabolizing organic matters in starch processing wastewater, *B.subtilis* has been proven as a potent candidate for treatment (Cristhiane L. Krueger et al, 2012; Nguyen Nhu Ngoc et al, 2016).

The aim of this study is to identify the optimal submerged fermentation conditions for *B. subtilis* NT1 biomass production with the purpose of producing microbial products applied in the treatment of *Canna edulis*. Ker starch processing wastewater.

II. MATERIALS AND METHODS

2.1. Microorganisms and culture media

B. subtilis NT1 strain was isolated from *Canna edulis*. Ker starch processing village, Minh Hong - Ba Vi - Hanoi. Its ability in degrading organic material in the wastewater has been demonstrated in our previous study (Nguyen Nhu Ngoc, Nguyen Van Cach, Nguyen Thi Diep, 2016).

NB medium (10 g/l peptone, 10 g/l NaCl, 3 g/l meat extract) was used for seeding.

Fermentation medium used contained: 0.5 g/l KH_2PO_4, 0.05 g/l $CaCl_2$, 1 g/l K_2HPO_4, 4.4

mg/l ZnSO, 0.1 g/l KCl, 3.3 mg/l $MnSO_4$, 0.5 g/l $MgSO_4$, 0.1 mg/l $CuSO_4$, 0.008 g/l $FeSO_4$, 1 mg/l NaI, 0.5 g/l KH_2PO_4. Initial pH of the tow media was set at 7.0 then the media was autoclaved at 121^{o}C for 20 minutes.

Different carbon sources used 10g/l for each: glucose, saccharose, lactose, starch and CMC.

Different nitrogen sources used 5 g/l for each: $(NH_4)_2SO_4$, $NaNO_3$, yeast extract, peptone.

2.2. Methods

2.2.1. Effects of nutrient feeding on bacterial growth

To find out the suitable carbon and nitrogen sources for strains *B.subtilis* NT1, experiments were carried out followed:

Seeding culture was inoculated in a 250ml flask (containing 100ml of seeding medium) on a shaker (150 rpm) at 37^{o}C for 24 hours.

Effects of carbon sources: experiments were carried out in 250 ml flasks containing 100ml medium supplemented with different carbon sources, the best source was then selected and tested in a concentration range from 5 g/l to 20 g/l.

Effect of nitrogen sources: measuring of bacterial growth using medium with different nitrogen sources (individual and combination), the best source was selected and tested in a concentration range from 1g/l to 9g/l.

These experiments were carried out on a rotator shaker (150 rpm) at 37^{o}C, 3% (v/v) of the inoculum for 24 hours.

2.2.2. Effects of fermentation conditions on bacterial growth

Fermentation conditions have strong impact on the growth of microorganisms. To determine the best fermentation parameters for *B.subtilis* NT1 growth, the experiments were carried out in 250ml flasks (containing100ml of fermentation medium with suitable carbon and nitrogen sources, identified as above (2.2.1) with different seeding ratio (in the ranges of 1%, 3%, 5%, 7%, 9%, 10%…); temperature (from 22^{o}C to 47^{o}C), initial pHs (from 4 to 8) and shaking speed (from 100 to 250 rpm) (Screekumar G, Soundarajan K., 2010).

2.2.3. Determination of bacterial biomass production and spore-forming

Biomass production and spores-forming time were assessed by dilution method via determining the number of colonies (CFU/ml) appeared on agar plates at different times (Afia G., Shahida H., 2009). For spore-forming determination, bacterial cultures were incubated at 80^{o}C for 20 minutes before use. All Experiments were repeated 3 times. In all results, concentration of biomass and spores are represented by log Cfu/ml values.

2.2.4. Design of the optimal conditions for bacterial biomass production

The Box – Behnken Design approach with Design-Expert 7.1.5 software was used to determine the optimum levels of three critical independent variables for increasing the biomass production of *B.subtilis* NT1: glucose concentration (X_1), peptone concentration (X_2) and shaking speed (X_3). The experimental plan consisted of 17 trials with X_1 from 5 – 15g/l, X_2 from 2.5 – 7.5 g/l, X_3 from 150 - 250 rpm. All experiments were carried out in 250ml flasks containing 100ml fermentation medium at seeding ratio of 5% (v/v) at 32^{o}C, initial pH of 6. The generalized polynomial model of three factors was as follows:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2$$

Where: Y - predicted response of fermentation, X_1, X_2 and X_3 are the coded settings for three factors, β_0 - value of fitted response at the center point of the design, β_1, β_2, and β_3 - linear coefficients, β_{12}, β_{13}, and β_{23} - interaction coefficients, β_{11}, β_{22}, and β_{33} - quadratic coefficients.

The analysis of variance (ANOVA) table was generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined.

III. RESULTS AND DICUSSION

3.1. The effect of medium conditions on *B. subtilis* NT1 biomass production

3.1.1. Effects of carbon, nitrogen source and concentration on B.subtilis biomass production

Carbon and nitrogen are important components for microbial growth. The results showed that among various carbon sources tested, glucose was the most suitable carbon source for *B.subtilis* NT1 growth. The highest biomass yield was obtained when using medium containing 10 g/l glucose (figure 1B) and reduced when using other sources of carbon like lactose, starch or CMC (figure 1A).

B.subtilis biomass yield significantly increased when nitrogen was supplemented into fermentation medium (figure 1C) and individual use of peptone at concentration of 5 g/l led to the maximum production biomass, estimated to be 8.51 LogCFU/ml (figure 1D). There are some differences with previous published by Sreekumar G, *B. subtilis* SK09 grew best on the medium containing 8.5 g/l peptone, at pH 6.7, 37^{o}C, shaking speed 160 rpm for 24 hours. The maximum biomass yield was estimated to be 10.05*10^{9} CFU/ml (Screekumar G, Soundarajan K., 2010).

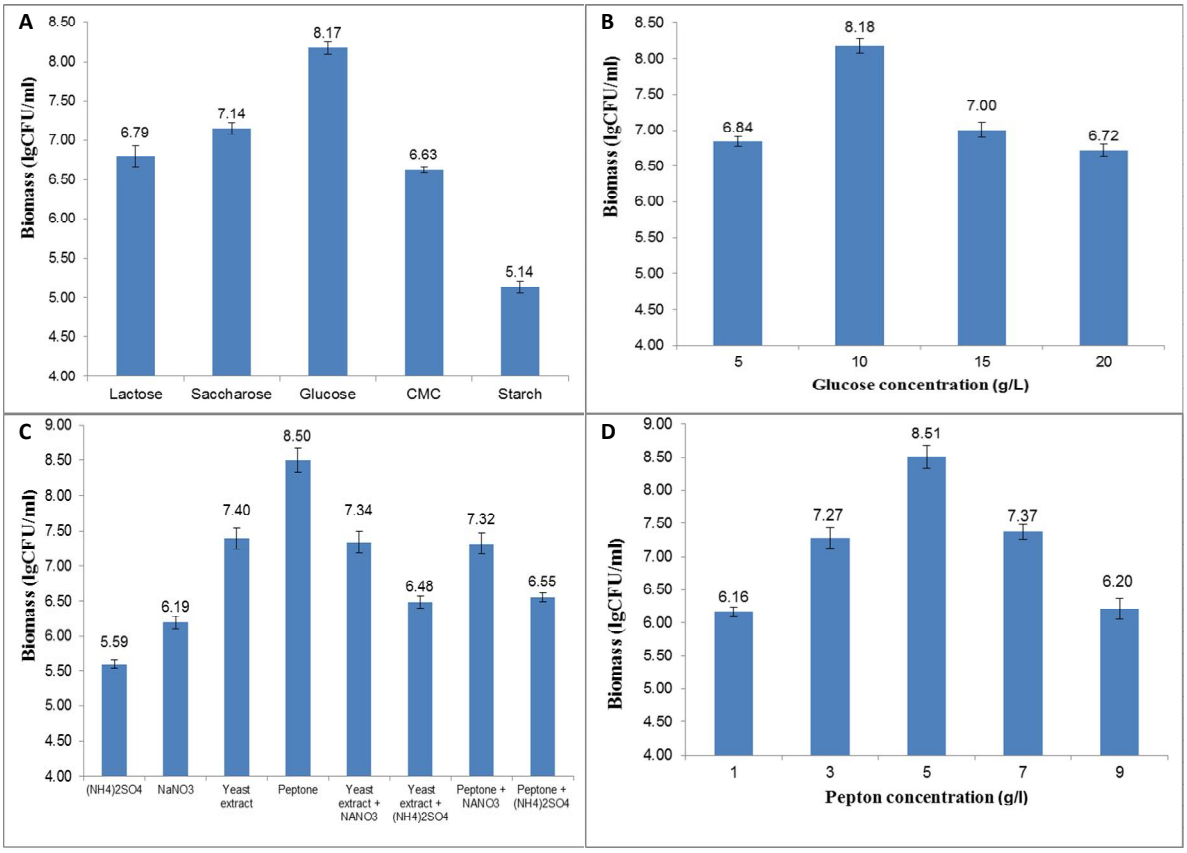

Figure 1. Diagram of the Effect of: carbon sources (A), Glucose concentration(B), nitrogen sources (C) and peptone concentration (D) on biomass production of B.subtilis NT1

3.1.2. Effects of fermentation conditions on B.subtilis NT1 biomass production

Fermentation condition has a major influence on the bacterial biomass production. Figure 2 shows that the highest biomass yield of *B.subtilis* NT1 was obtained when cultivated in these fermentation conditions: inoculum rate of 5% (v/v), initial pH 6, shaking speed 200 rpm, at 30^{o}C, biomass yield was estimated to be 10.98 LogCFU/ml (figure 2). The biomass yield decreased significantly at shaking speed of 250 rpm, this

may be due to the too high speed was not good for cell growth and the best aerobic bacterial growth is limited by the speed at which the microbes can replicate. The result also showed that *B.subtilis* NT1 could grow at a lower temperature and shaking than other *B.subtlis* strains did, at 37°C and 150 rpm shaking speed as reported by Afia and Shahida., 2009. Besides, NT1 strain was also adapted to grow and divide at a wide range of temperature (20°C to 45°C). This may be useful for environmental treatment applications.

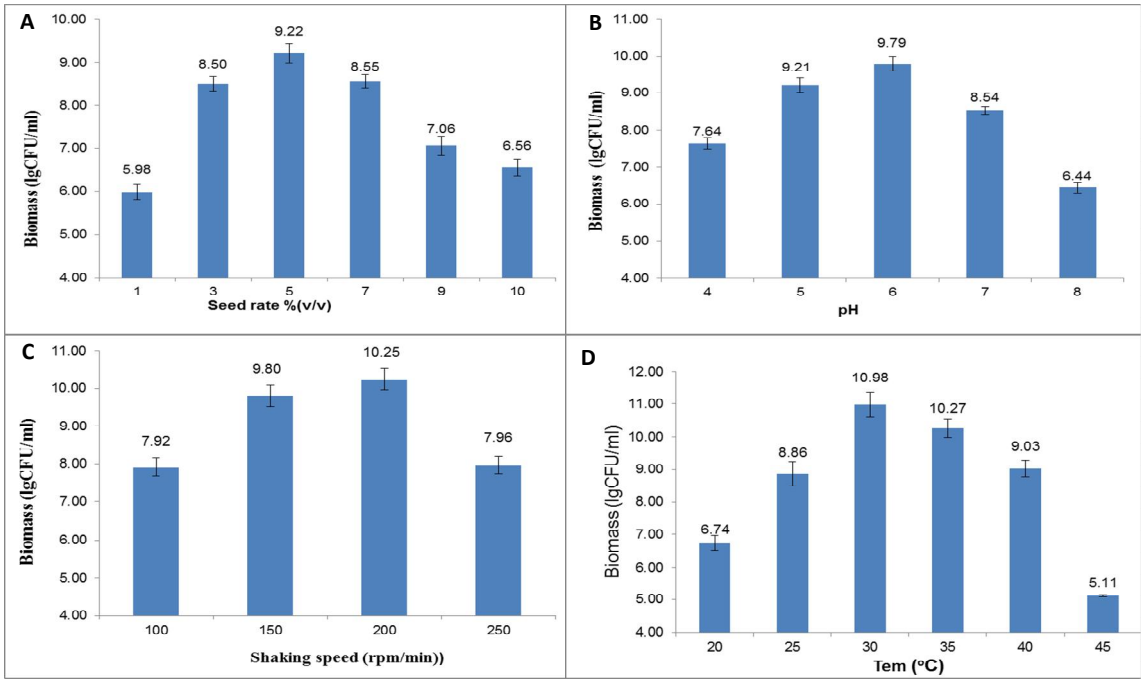

Figure 2. Effect of incubation conditions: The inoculum rate (A), pH (B), shaking speed (C) and temp (D) on biomass production of B.subtilis NT1

3.1.3. The effluence of fermentation time on biomass production

The efficiency and cost of biomass and spores production are greatly dependent on fermentation time. Culturing time for bacterial biomass production and spore-forming was surveyed and in figure 3, the highest biomass yield of *B. subtilis* NT1 was obtained after 24 hours fermentation, estimated to be 10.95 LogCFU/ml and spores-forming was estimated to be 9.22 LogCFU/ml after 72 hours fermentation.

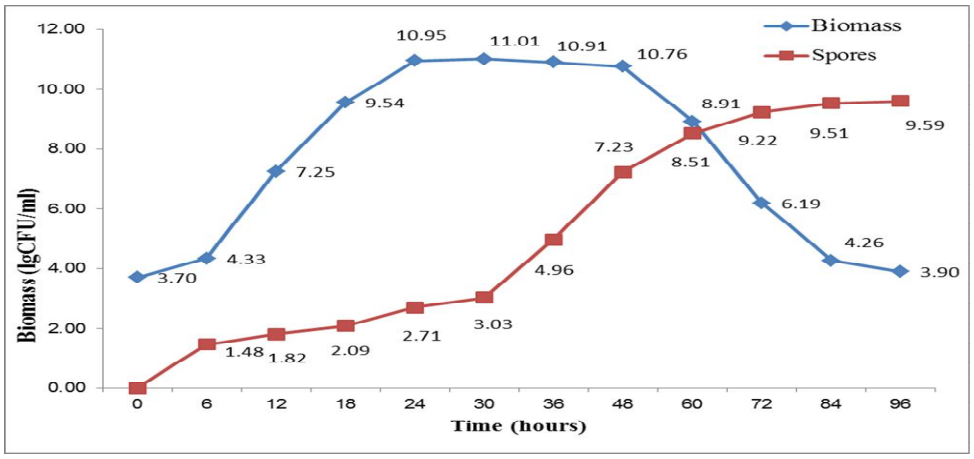

Figure 3. Variation of incubation time on biomass and spores production

3.2. Optimization of the significant variables by applying Response Surface Methodology (RSM)

According to above mentioned result which is about impact of individual factors on bacterial growth, there are three dominant factors on *B.subtilis* NT1 biomass production, including glucose concentration (X_1), peptone concentration (X_2) and shaking speed (X_3). The simultaneous effect of these three factors on *B.subtilis* NT1 biomass production was analyzed by using a Box–Behnken design of the response surface methodology to optimize fermentation conditions for high yield of biomass production (table 1).

Table 1. Experimental design matrix and results of B. subtilis NT1 biomass production

Standard order	X_1	X_2	X_3	Biomass (LogCFU/ml)	Standard order	X_1	X_2	X_3	Biomass (LogCFU/ml)
1	5	2.5	200	6.22	10	10	7.5	150	11.78
2	15	2.5	200	7.15	11	10	2.5	250	5.54
3	5	7.5	200	10.72	12	10	7.5	250	10.37
4	15	7.5	200	11.32	13	10	5.0	200	11.12
5	5	5.0	150	9.03	14	10	5.0	200	10.92
6	15	5.0	150	9.39	15	10	5.0	200	11.04
7	5	5.0	250	6.85	16	10	5.0	200	10.85
8	15	5.0	250	7.82	17	10	5.0	200	11.05
9	10	2.5	150	7.91					

Table 1 show that the highest biomass yield was 11.78 LogCFU/ml at experiment standard order 10 and the lowest was 5.54 Log CFU/ml at standard order 11. Analysis of variance optimization model by DX7.1.5 software was used to test the significance and adequacy of second order polynomial model. The results in table 2 indicate that all three factors: glucose concentration, peptone concentration and shaking speed have strong impact on biomass production of *B.subtilis* NT1. The F-value of 979.38 with $p < 0.0001$ ($p < 0.05$) indicates the model selected was suitable. The p value of "lack of fit" is 0.9263 ($p > 0.05$) which shows that this model is compatible with experimental.

The p value of X_1X_3, $X_2X_3 < 0.05$ shows the strong simultaneous impact of glucose concentration and shaking speed as well as peptone concentration and shaking speed on *B. subtilis* NT1 biomass production.

Table 2. ANOVA of the quadratic model

Source	Sum of squares	F-value	Prob>F	Source	Sum of squares	F-value	Prob>F
Model	66.16	979.38	<0.0001	$X_2.X_3$	0.23	30.69	0.0009
X_1	1.02	136.21	<0.0001	X_1^2	8.08	1076.75	<0.0001
X_2	37.71	5024.31	<0.0001	X_2^2	2.42	322.29	<0.0001
X_3	7.09	944.21	<0.0001	X_3^2	7.54	1004.19	<0.0001
$X_1.X_2$	0.027	3.63	0.986	Lack of fit	5.23E-003	0.15	0.9263
$X_1.X_3$	0.093	12.39	0.0097				

The second-order polynomial model for biomass production is shown in Equation 1:

$$Y = +11.00 + 0.36X_1 + 2.17X_2 - 0.94X_3 - 0.083X_1X_2 + 0.15X_1X_3 + 0.24X_2X_3 - 1.39X_1^2 - 0.76X_2^2 - 1.34X_3^2 \quad (1)$$

A desirability function was used to optimize biomass production that led to 43 experimental plans. According to theoretical calculations, the highest biomass yield could be achieved after 24 hours of culture (11. 996 LogCFU/ml) using 8.09 g/l glucose and 6.96 g/l peptone at shaking speed 193.83 rpm, 30^{o}C, pH 6.

In order to verify RSM suggestion, an experiment was conducted using optimum values of the test variables (8g/l glucose, 7g/l peptone, shaking speed 195 rpm) and the maximum biomass was obtained at 11.97 LogCFU/ml, very close to the predicted value.

IV. CONCLUTION

Optimal conditions for strain *B.subtilis* NT1 produces biomass is: glucose (8 g/l), peptone (7 g/l) shaking 195 rpm, 30^{o}C, pH 6 with 5% v/v inoculum.

REFERENCES

1. Afia G., Shahida H. (2009), Production dynamics of *Bacillus subtilis* strain AG-1 and EAG-2, producing moderately alkaline proteases, *African Journal of Microbiology Research,* **3** (5), pp. 258 - 263.

2. Amran M. (2006), Biomass production and formulation of *Bacillus subtilis* for biological control, *Indonesian Journal of Agricultural Science* , **7** (2), pp. 51 – 56.

3. Cristhiane L. Krueger, et al (2012), Bioconversion of cassava starch by-product into *Bacillus* and related bacteria polyhydroxyalkanoates, *Electronic Journal of Biotechnology*, **15** (3), pp. 1 - 13.

4. Nguyen Nhu Ngoc, Nguyen Van Cach, Nguyen Thi Diep (2016), Isolation and screening of indigenous *Bacillus* which have ability to resolve organic compounds of *Canna edulis, Ker starch processing wastewater,* **11**, pp. 101 - 107.

5. Screekumar G, Soundarajan K. (2010), Enhanced biomass production study on probiotic *Bacillus subtilis* SK09 by medium optimization using response surface methodology, *African Journal of Biotechnology,* **9** (45), pp. 8078 - 8084.

6. Tran Van The, Nguyen Tuan Son, Nguyen Nghia Bien (2013), Evaluation on Economic Losses of Arising Wastes from Food Processing Handicraft Villages in the Red River Delta Region, Vietnam, *J.Sci. & devel*., **11** (8), pp. 1223-1231.

TỐI ƯU ĐIỀU KIỆN LÊN MEN THU SINH KHỐI *BACILLUS SUBTILIS* NT1 ĐỂ TẠO CHẾ PHẨM VI SINH XỬ LÝ NƯỚC THẢI LÀNG NGHỀ CHẾ BIẾN TINH BỘT DONG RIỀNG

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TÓM TẮT

Làng nghề sản xuất tinh bột ở nông thôn Việt Nam đang phát triển mạnh đã giúp cải thiện kinh tế làng nghề. Tuy nhiên, lượng lớn nước thải và chất thải rắn không được xử lý mà thải trực tiếp ra môi trường đang gây ra tình trạng ô nhiễm nghiêm trọng. Để tạo chế phẩm vi sinh xử lý nước thải làng nghề chế biến tinh bột dong riềng ở quy mô lớn, chủng *B. subtilis* NT1 được nghiên cứu điều kiện thu sinh khối theo phương thức lên men chìm. Bằng phương pháp khảo sát ảnh hưởng của đơn yếu tố và tối ưu theo quy hoạch thực nghiệm bậc 2 Box-Behnken. Kết quả nghiên cứu đã xác định được điều kiện tối ưu thu sinh khối cho chủng *B. subtilis* NT1: hàm lượng glucose 8,0 (g/l), pepton 7,0 (g/l), tốc độ lắc 195 vòng/phút, nhiệt độ 30^{o}C, tỷ lệ cấp giống cấp 5% (v/v), pH 6, sau 24 giờ nuôi hàm lượng sinh khối thu được là 11,98 LogCFU/ml.

Từ khóa: Bacillus subtilis, lên men chìm, nước thải, sinh khối, tối ưu.

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