

Improvement of *Bacillus clausii* HH1 cell density via culture medium optimization and pH-stat fed batch fermentation

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Abstract. In this study, the culture medium and fermentation modes were studied aiming to improve the cell density of *Bacillus clausii* HH1. Firstly, the factorial design method using Minimum Run Resolution IV design was used to evaluate the relative importance of culture medium components to the growth of *Bacillus clausii*. The results showed that three components peptone, yeast extract and malt extract were the components significantly affecting the bacterial biomass. Then, the optimization of these three ingredient concentrations using a response surface methodology with the Box-Behnken design resulted that the maximal biomass had been achieved using the medium containing 7.64 g/L peptone, 10 g/L yeast extract and 6.36 g/L malt extract. Finally, the pH-stat fed batch fermentation was conducted in a 2-liter bioreactor where the 9X concentrated optimal culture medium was feed into the bioreactor based on the pH signal. As a result, the microbial cell density increased by 2.9-fold compared to that achieved through batch fermentation.

Keywords: *Bacillus clausii*, culture medium optimization, pH-stat fed batch fermentation.

Classification numbers: 1.3.2.

1. INTRODUCTION

The genus *Bacillus* are the main micro-organisms that currently receive much interest as probiotics with numerous advantages including the production of interest extracellular enzymes and the ability of heat-stable spores formation which facilitates its processing, storage, and use [1]. Among the genus, *Bacillus clausii* is known to be able to produce antibacterial substances against pathogen gram-positive bacteria such as *Enterococcus faecium*, *Clostridium difficile*, to stimulate the synthesis of immunomodulatory substances such as NOS II, IFN-gamma [2]. The bacterium indeed is known to resist to several antibiotics and suggested to be used with the antibiotic treatment with safety [3]. Furthermore, it has been shown to be effective and safe in

the treatment of acute diarrhea and prevention of recurrent respiratory tract infections in children [4]. In addition, the ability of *B. clausii* spores to germinate in experimental conditions mimicking the gastrointestinal tract is consistent with the beneficial health reported for this spore forming bacterium [5]. Therefore, *B. clausii* spores have been used in commercial probiotic products.

One of challenge in production of probiotics *B. clausii* is to attain a high cell density in the fermentation. *B. clausii* is known as a fast-growing, alkaliphilic aerobic microbe so a suitable medium is required for a better biomass production [6]. Statistical experimental designs are generally used to determine the main medium components [7], then to optimize the media for micro-organisms to growth and produce the products [8, 9].

Controlling nutrient concentration and biological state of the bacteria during the fermentation is another approach to improve the biomass in the culture. Fed batch fermentation techniques have been applied extensively in industrial fermentations to improve the cell density. The major advantage of such techniques is the possibility to adjust the medium component concentration in the culture broth to values favorable for cells to prolong the desired development phase of the bacterial culture [9, 10]. The pH-stat fed batch fermentation is a simple feedback control scheme that controls nutrient feeding via measurement of pH of the culture [11]. It can be used singly or in combination with other fed-batch techniques to enhance the microbial biomass in the bioreactor [12, 13].

In this study, a statistic experimental using Minimum Run Resolution IV design was used to investigate the main culture components significantly affecting *B. clausii* growth. Then, the response surface methodology (RSM) using Box-Behnken design was carried out to investigate the relationships between the main independent variables and to optimize the culture medium. Finally, the pH-stat fed batch fermentation to improve the *B. clausii* cell density in bioreactor was examined and discussed.

2. MATERIALS AND METHOD

2.1. Materials

Bacillus clausii HH1 provided by the School of Biotechnology and Food Technology (SBFT – HUST) was used in this study. This strain was stored at -20 °C, activated in nutrient agar plate before use.

2.2. Methods

Bacterial cultivation

A seed culture was grown in 250 mL baffled flask containing 50 mL of seed medium (peptone 5 g/L; yeast extract 5 g/L; meat extract 2 g/L; K₂HPO₄·3H₂O 1.9 g/L and NaCl 1 g/L) at 37 °C, 120 rpm for 12 h.

Flask culture: 50 mL culture medium (peptone 10 g/L; yeast extract 10 g/L; meat extract 3 g/L) in 250 mL baffled flask was inoculated with the seed culture to achieve the OD_{600nm} 0.1. The flask was cultivated at 37 °C, shaking 120 rpm. The optical density at 600 nm was measured for monitoring the bacterial growth at various intervals.

Screening of culture medium components

Minimum-Run resolution IV screening design was used to evaluate the relative importance of various media components including glucose, peptone, yeast extract, meat extract and malt extract at three levels (+1; 0 and -1) for *B. clausii* growth. Total 16 experiments in 250 mL baffled flask were carried out following the condition indicated in the Table 1. The microbial growth was evaluated at 12 h by measurement the OD_{600nm}.

Optimization of the culture medium

The central composite matrix experiment was designed to investigate the effect of the main components of the culture medium to *B. clausii* biomass. In this study, an RSM combined with Box-Behnken design was used. This design was composed of three levels (low, medium, and high, being coded as -1, 0 and +1) and a total of 15 runs in 250 mL baffled flask were carried out to optimize the level of three independent variables: peptone concentration (A) yeast extract concentration (B) and malt extract concentration (C) (Table 2). The *B. clausii* growth was evaluated by OD_{600nm} measurement.

Batch-fermentation

Batch fermentation in 2 L bioreactor: 1 L optimal culture medium was filled in 2 L bioreactor equipped with DO (dissolve oxygen), pH, temperature, and antifoam probes. After sterilization at 110 °C for 30 mins, the temperature was maintained at 37 °C and pH was automatic adjusted to 8.5 by adding 2 N HCl or 2 N NaOH. The inoculum was pumped to the bioreactor to achieve OD_{600nm} 0.1. The pO₂ was maintained at 20 % by automatically changing the stirring speed from 400 to 750 rpm, then manually changing the aeration rate from 1.8 to 2.4 vvm when stirring speed achieved maximum value but pO₂ was below 20 %.

pH-stat fed batch fermentation

The pH-stat fed batch was initiated as a batch fermentation for 6 h in 2 L bioreactor containing 800 mL optimal culture medium. The 2 N HCl bottle then was replaced by the feeding bottle. The 9X concentrated culture medium (7.64 g/L, yeast extract 10 g/L and malt extract 6.36 g/L) was used for feeding into the bioreactor. The feeding medium was automatically pumped into the bioreactor whenever the pH was higher than 8.5 through acid pump. The optical density at 600 nm was measured for monitoring the bacterial growth. The standard plate count on LB medium was used to determine the bacterial numbers.

3. RESULT AND DISCUSSION

3.1. Screening of culture medium components for *B. clausii* growth

The composition of the nutrient medium has a great influence on the microbial growth. Various components usually used for culture of *Bacillus* [14, 15], among them, naturally derived nutrient such as glucose, malt extract, peptone, meat extract and yeast extract were the most components used as carbon and nitrogen sources. However, the effect of each ingredient on microbial growth are still controversial. Glucose was used to optimize the *B. subtilis* growth, and maximum cell density achieved at 33.4 g/L in the study of Mosquera [16]. Contrarily, using similar species, Koim-Puchowska *et al.* showed that glucose was not as good as saccharose or trehalose for microbial growth. According to these authors, the effect of nitrogen was in the order of yeast extract ~ peptone > malt extract > beef extract [15]. In the research of Ojha *et al.*,

that order was changed to was yeast extract > tryptone > beef extract > malt extract > peptone [17].

Table 1. Minimum run resolution IV design for evaluation of medium component.

Run	Glucose (g/l)	Peptone (g/l)	YE (g/l)	Meat extract (g/l)	Malt extract (g/l)	OD 600 nm
1	1 (10)	-1 (1)	-1 (1)	-1 (0)	1 (5)	2.02
2	-1 (0)	-1 (1)	-1 (1)	-1 (0)	-1 (0)	3.45
3	-1 (0)	1 (10)	-1 (1)	-1 (0)	-1 (0)	5.51
4	-1 (0)	1 (10)	1 (10)	1 (5)	-1 (0)	12.05
5	-1 (0)	-1 (1)	-1 (1)	1 (5)	-1 (0)	3.96
6	-1 (0)	1 (10)	-1 (1)	-1 (0)	1 (5)	9.31
7	0 (5)	0 (5.5)	0 (5.5)	0 (2.5)	0 (2.5)	11.74
8	-1 (0)	-1 (1)	1 (10)	-1 (0)	1 (5)	13.19
9	1 (10)	1 (10)	1 (10)	1 (5)	1 (5)	10.03
10	1 (10)	1 (10)	-1 (1)	1 (5)	-1 (0)	6.49
11	1 (10)	-1 (1)	1 (10)	1 (5)	-1 (0)	6.61
12	0 (5)	0 (5.5)	0 (5.5)	0 (2.5)	0 (2.5)	11.48
13	1 (10)	1 (10)	1 (10)	-1 (0)	1 (5)	9.75
14	1 (10)	-1 (1)	1 (10)	0 (5)	1 (5)	8.09
15	-1 (0)	1 (10)	-1 (1)	0 (5)	1 (5)	12.85
16	1 (10)	-1 (1)	1 (10)	-1 (0)	-1 (0)	5.39

(In parentheses are the actual concentration of each component)

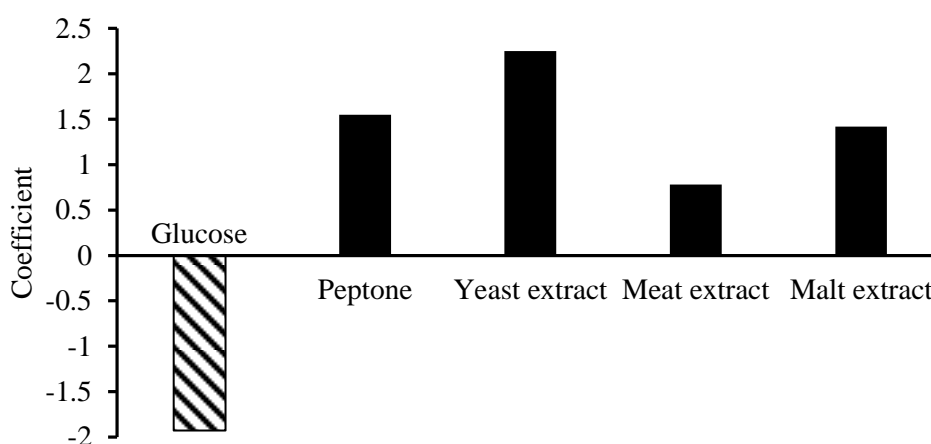


Figure 1. The effective coefficient of each medium components to *B. clausii* growth.

Bacillus clausii is well-known species and is used in many commercial probiotic products, however, the optimal medium culture for its growth has not been mentioned. In this paper, to evaluate the importance of nutrients on the growth of *B. clausii*, total 16 experiences were carried out following the Minimum Run Resolution IV design (Table 1).

The ANOVA analysis using Design Expert 11 software showed that this design was significant at the 95 % level ($p = 0.008 < 0.05$). Glucose had a negative effective coefficient while the other four variables showed positive effective coefficients (Figure 1). The use of glucose causes a decrease in pH of the fermentation medium [18, 19], which could also inhibit alkaline strains such as *B. clausii*, especially when the experiments were not adjusted to pH 8.5 during fermentation process. Therefore, the addition of glucose could be avoided in further experiments. The four variables showed efficiency coefficients as following order: yeast extract > peptone > malt extract > meat extract (Figure 1). Among them, meat extract showed the least positive coefficient to *B. clausii* growth, so it can be excluded in further experiments.

3.2. Optimization of culture medium using response surface methodology

The study of individual and interactive effects of three independent and positive effective variables (peptone, yeast extract and malt extract) to the growth of *B. clausii* was carried out using the statistic design approach of RSM. Total 15 experiments were carried out following the Box-Behnken design (Table 2).

Table 2. Central composite matrix for optimization of culture medium using Box-Behnken design.

Run	Peptone (g/L)	Yeast extract (g/L)	Malt extract (g/L)	A	B	C	OD _{600nm}
1	5.5	10	10	0	1	1	13.28
2	5.5	1	10	0	-1	1	7.67
3	10	10	5.5	1	1	0	13.36
4	5.5	10	1	0	1	-1	12.58
5	5.5	1	1	0	-1	-1	3.25
6	1	5.5	1	-1	0	-1	2.24
7	10	5.5	1	1	0	-1	10.13
8	1	1	5.5	-1	-1	0	3.32
9	10	5.5	10	1	0	1	9.72
10	10	1	5.5	1	-1	0	7.70
11	1	10	5.5	-1	1	0	10.33
12	1	5.5	10	-1	0	1	7.98
13	5.5	5.5	5.5	0	0	0	12.23
14	5.5	5.5	5.5	0	0	0	9.20
15	5.5	5.5	5.5	0	0	0	11.43

$$\text{Biomass (OD}_{600\text{nm}}) = 10.95 + 2.14A + 3.46B + 1.31C - 1.54AC - 1.99A^2 - 1.45C^2 \quad (1)$$

here A: peptone; B: yeast extract; C: malt extract.

A quadratic model was suggested to fit the experiment data. The ANOVA analysis showed that P-value of this model was 0.0033 and the lack of Fit was not significant (P-value 0.9136). The quadratic model therefore can be used to fit the experimental data (Equation 1). The dependence of *B. clausii* on peptone, yeast extract and malt extract were expressed as in the

following equation (insignificant coefficients were removed). The interaction between peptone-malt extract or peptone-yeast extract to the bacterial growth were shown in the Figure 2.

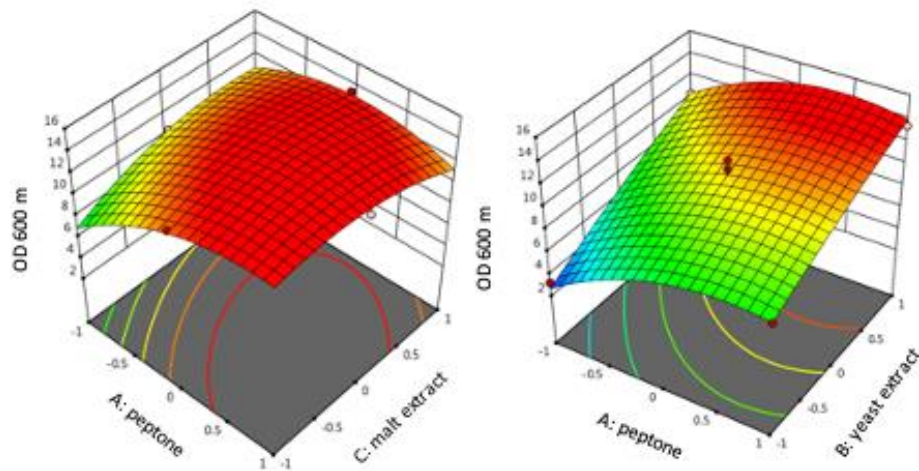


Figure 2. Response surface plots showing the effect of malt extract –peptone and peptone-yeast extract to *B. clausii* growth.

The optimization of culture medium for *B. clausii* growth was performed using the Design Expert 11 software. It predicted that the highest *B. clausii* cell density could be attained (up to OD₆₀₀ 14.85) in the medium containing peptone 7.64 g/L, yeast extract 10 g/L and malt extract 6.36 g/L. The experimental data at the optimal culture medium gave the maximum of OD₆₀₀ of 14.42, compatible with the predicted value by the software. The growth of *B. clausii* in optimal medium was increased 1.3-fold comparing to that in the initial culture medium.

3.3. Batch fermentation

B. clausii was cultivated in 2 L bioreactor following the batch mode using optimal culture medium. The fermentation kinetic was showed in the Figure 3. The pO₂ was approximately maintained at 100 % at the first three hours corresponding to the microbial lag phase. Then the pO₂ decreased sharply and reached the minimum after 5 h of fermentation, corresponding to the log phase of the bacteria. To maintain the pO₂, the agitation was automatically increased from 400 rpm to 740 rpm.

The maximal OD_{600nm} achieved at 8 h of fermentation, then *B. clausii* entered in the stationary phase; the agitation was then slowed down to maintain the pO₂ 20 % corresponding to the decrease of microbial oxygen demand. The specific growth rate of *B. clausii* achieved 0.73 (1/h), and maximal microbial density was 20.04 at OD_{600nm}.

Bacillus clausii is an alkaline species [6, 18], so the culture medium is usually brought to alkaline pH for its optimal growth. Tabandeh *et al.*, launched the *B. clausii* at initial pH 10.5 without pH control [18]. These authors observed that the pH of the culture medium decreased from 10.5 to 8.5 at the end of the exponential phase, then it gradually increased to 9.5 during the stationary phase. Based on the literature, in this study, the pH was adjusted to 8.5. As mentioned in Fig. 3, to maintain the pH 8.5, 2 N NaOH was automatically pumped into bioreactor until the 7.2 h; after that 2 N HCl was fed to the bioreactor that coincided with the transition time between the logarithm phase and the stationary phase of *B. clausii* (Fig. 3). Suzuki *et al.* [20]

discussed phenomenological change of pH during fermentation, at stationary phases using organic carbon–nitrogen sources such as peptone, cells consume a small amount of nutrients for energy to maintain their basic metabolism, probably from decomposition of pre-accumulated substances inside the cells or from partial lysis of the cells. As a result of oxidation of carbon compounds for maintenance energy formation, the cells excrete excess nitrogen as NH_4^+ which causes the pH rise, suggesting that substrates can be fed automatically in a link with pH rise. In the next experiments, fed batch fermentation based on pH (pH-stat) will be performed.

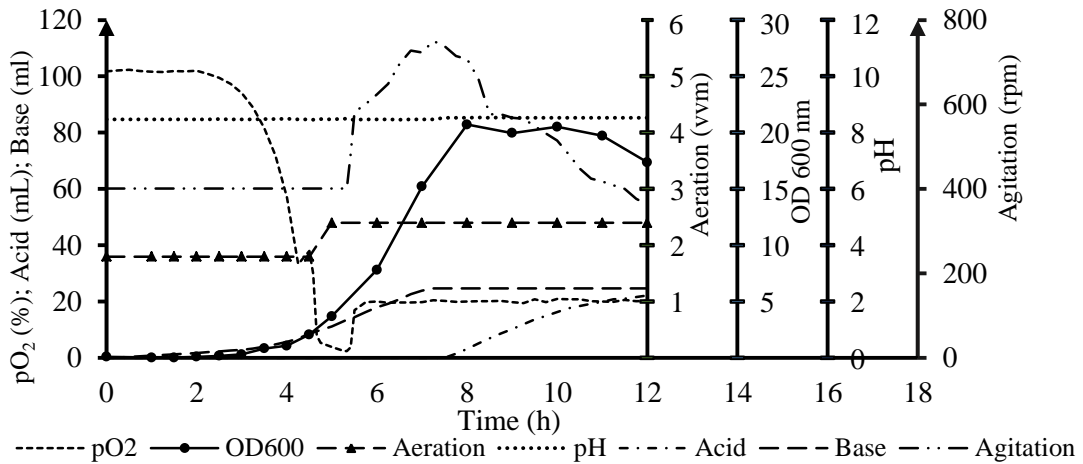


Figure 3. Kinetic of the batch fermentation of *B. clausii*.

3.4. pH-stat fed batch fermentation

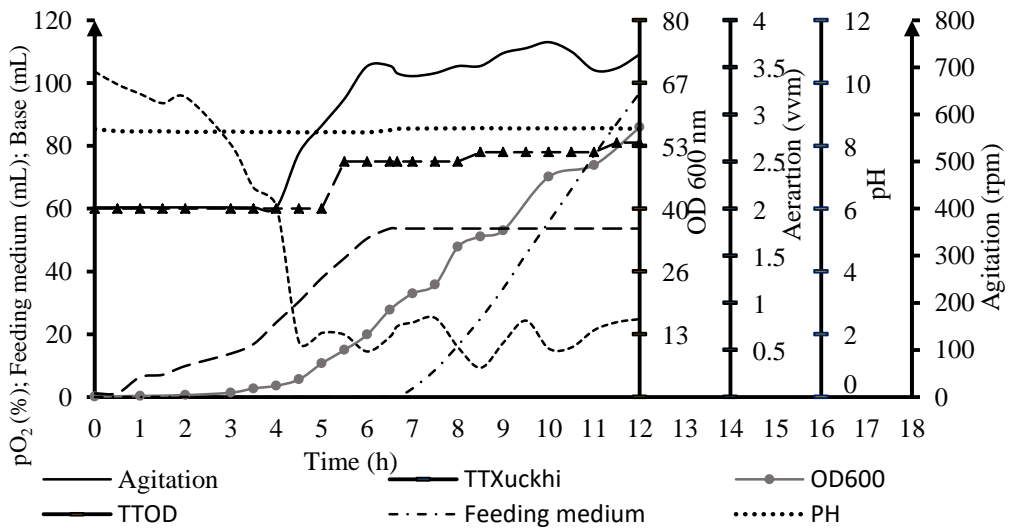


Figure 4. Kinetic of pH-stat fed batch fermentation of the *B. clausii*.

To enhance bacteria growth, it is necessary to extend the log phase in the fermentation process. The fed-batch fermentation techniques can be used to prolong the log phase and resulting in higher biomass [21]. In this study, pH-stat fed batch fermentation was carried out to

extend the log phase of *B. clausii* in the bioreactor aiming to improve *B. clausii* growth (Figure 4). A concentrated medium should be used for feeding to the culture to avoid diluting the culture, therefore the 9X optimal culture medium for biomass was used for feeding into bioreactor in pH-stat fed-batch fermentation.

At the first 6.6 hours of fermentation, 2 N NaOH was used for maintaining the pH 8.5 in the bioreactor. Then, from 6.6 h to 12 h of the fermentation, the feeding medium was automatically added via the acid pump. *B. clausii* log phase was initiated at 4 h of fermentation and last until 12 h, it was 4 h extended comparing with that in the batch fermentation. The maximal OD_{600nm} achieved of 57.3; 2.9-folds higher than that of the batch fermentation. The bacterial density was 1.48×10^{10} CFU/mL after 12 h of fermentation.

4. CONCLUSION

Using screening the components by Mini Run IV design and optimization of the main components via the response surface methodology with Box-Behnken design, the optimum culture medium for *B. clausii* HH1 growth was established. In the optimal culture medium, the microbial growth was enhanced 1.3-fold comparing the initial medium. The pH-stat fed batch fermentation applied could extend the microbial log phase for 4 h resulting in the improvement of the cell density of *B. clausii* 2.9-folds higher than in the batch fermentation mode. This result is very promising and is subject to a further scaling up trial.

Credit authorship contribution statement. Pham Tuan Anh: Methodology, Writing the manuscript. Nguyen Thi Phuong: experiment, analysis. To Kim Anh: Editing, Supervision.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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