EFFECTS OF YEAST EXTRACT AND Ca^{2+}, Fe^{3+} IONS ON THE GROWTH AND SPORULATION OF *BACILLUS COAGULANS*

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SUMMARY

Bacillus coagulans is currently being studied due to its potential applications of its spores in probiotic products. In this study, the effects of yeast extract concentration and Ca^{2+} & Fe^{3+} ions on the growth and sporulation of *B. coagulans* VCIM5914 were investigated. The examination of 12 types of yeast extracts from suppliers revealed a significant dependency of growth and sporulation on the type of yeast extract. An appropriate concentration of yeast extracts effective for cell density increase was ≥ 7.5 g/L, and for spore density, it was ≥ 12.5 g/L. The presence of calcium ions at concentrations ≥ 0.2 g/L increased both cell and spore numbers. Although high concentrations of iron ions did not significantly increase cell density, they enhanced sporulation at concentrations ≥ 0.2 g/L. Fermentation using selected conditions on a fermenter resulted in a cell density of ~5×10^8 CFU/mL with a sporulation rate of 82% after 28 hours cultivation.

Keywords: Bacillus coagulans, calcium Ion, probiotic, sporulation, yeast extract.

INTRODUCTION

Bacillus are applied in many industrial fields to produce fine chemicals, enzymes, probiotic, pharmaceutical ingredients and in agriculture. The *Bacillus* species that have been most extensively studied these are *B. subtilis, B. clausii, B. cereus, B. coagulans* and *B. licheniformis.* The abilities of some *Bacillus* strains to metabolize and transform complex organic compounds are of interest in both pharmaceutical production and agriculture. Thus, characterizing *Bacillus* species growth conditions especially sporulation is interest to optimize the condition to produce endospores (Ben Khedher *et al.,* 2011), (Cao *et al.,* 2020).

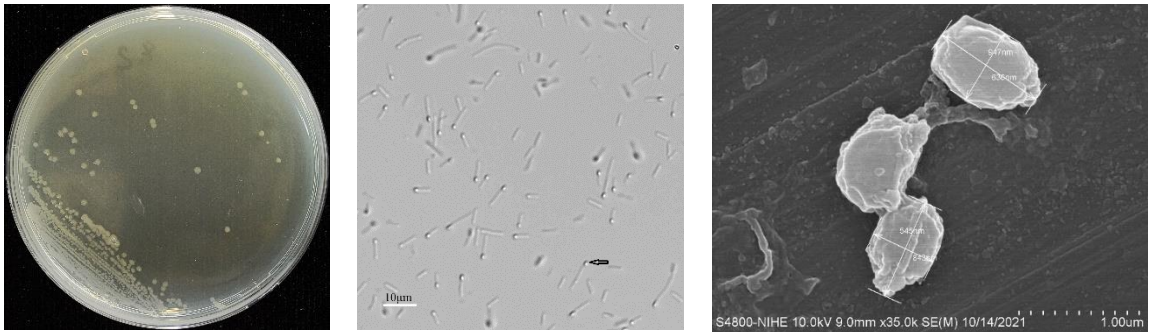
Despite the great diversity of genera, most *Bacillus* species can grow well on conventional media such as nutritional agar or trypticase soy agar, and blood agar. However, some isolates, especially those from poor nutrition environments, may grow poorly on these standard media and therefore require weaker formulations. Most species use glucose and/or other fermentable carbohydrates as sole sources of carbon and energy, but some species do not seem to use carbohydrates (Logan and De Vos 2009). *Bacillus* species can use inorganic and organic nitrogen sources. Little comprehensive information is available on the vitamin requirements of each *Bacillus* species. Many do not require such growth factors, but yeast extract will usually stimulate better growth (Poormontaseri *et al.,* 2017). The nutritional condition from rich to a poor growth medium or population density increases activate the forming of endospore at the end of the exponential growth phase. There are many factors affect endospore formation, including growth temperature, environmental pH, aeration, presence of certain minerals, and carbon, nitrogen and phosphorus sources and their concentrations (Logan and De Vos 2009). Manganese ions are essential for *B. subtilis* sporulation because they are needed for PGA-mutase, an enzyme that necessary for the balance of intracellular metabolites. Calcium and magnesium seem to be effect on spore formula of *B. coagulans* (Sinnelä *et al.,* 2019). De Vries (2004) report the role of calcium in spore germination, Calcium dipicolinate (CaDPA), while Keynan and Halvorson report the mechanism of Calcium ion and dipicolinic acid inter reaction with L-alanine induced germination (Keynan and Halvorson, 1962).

In this study, we focused on investigating the effects of yeast extract, as well as the influence of calcium and iron ions, on the growth and sporulation. Additionally, cultivation in bioreactor was carried out using a 2 L bioreactor to select preliminary conditions for the cultivation of *Bacillus coagulans* VCIM5914, with the aim of maximizing spore production.

MATERIALS AND METHODS

Materials

Bacillus coagulans VCIM5914 was isolated from a commercial probiotic product (BioSpring Jsc., Vietnam) which is identification 99,90% similar with *B. coagulans* strain *S-lac* (GenBank: CP011939) by using rpoB gene, encoding the beta-subunit of RNA polymerase. The morphology of *B. coagulans* VCIM5914 on plate count agar (PCA) medium and spore under microscope and Scanning electron microscope (SEM) as in Fig.1.



A B C

Figure 1. *Bacillus coagulans* morphology: (A) colony on PCA; (B) cells with endospore (arrow); (C) SEM spore image

Yeast extracts are provided Procelys by Lesaffre® (Procelys, Singapore). Twelve yeast extract samples received from Lesaffre Singapore, B.U. from Singapore were used in this study. Procelys kept the composition and recipe for yeast extracts proprietary and under continuous research.

Methods

Cultivation medium: The MRS agar medium (M6411-HiMedia®, India) was used as the medium for activate cell (include for 1 L: 5.0 g yeast extract; 8.0 g meat extract; 10.0 g peptone from casein; 2.0 g K_2HPO_4, 0.1 g $MgSO_4$, 0.05 g $MnSO_4$, 2.0 g $(NH_4)_3$ $C_6H_5O_7$; 5.0 g $CH_3COONa.3H_2O$; 20.0 g glucose, 1 mL Tween 80, and 15 g agar). The cultivation medium modified from sporulation medium described by Cazemier *et al.,* (2001) containing ingredients: 5 g/L Yeast extract, 0.5 g/L K_2HPO_4, 0.25 g.L^{-1} $MgSO_4$, 0.1 g/L KCl, 0.025 g/L $MnSO_4$, adjusted pH to 6.0 and was sterilized at 121°C for 15 minutes.

Effect of yeast extract nutrient: The liquid broth as describe above with different concentration of yeast extract: 0.0, 5.0 7.5, 10.0, 12.5, 15.0, 17.5, and 20 g/L was cultivation total 20 mL in 100 mL flask, shaking 150 rpm at 37°C for 48 hours. Samples were measured turbidity, plate count agar. All experiments were duplicated.

Effect of calcium chloride concentration: Calcium were be add into medium cultivation with range from 0 to 1.0 g/L (0, 0.2, 0.4, 0.6, 0.8, 1.0 g/L) volume in total is 20 mL in 100 mL flask, shaking 150 rpm at 37°C for 48 hours. Samples were measured turbidity, plate counting. All experiments were duplicated.

Temperature: Two different temperature conditions, 37°C and 40°C, were selected to examine cultivation and sporulation capabilities. A volume of 20 mL of medium was incubated with shaking at 150 rpm in a 100 mL Erlenmeyer flask for 48 hours. Samples were measured turbidity, plate count agar. All experiments were duplicated.

Turbidity measure (OD_{600}): The turbidity was measured at wavelength of 600nm in 24-wells microplate (1 mL/well) by BioTek spectrophotometry (BioTek Instruments, Inc.).

Plate counting: For cells counting, one milliliter of each sample was diluted with 9 mL sterile salinity salt to 10^{-5} and 10^{-6} dilution. Amount of 100 µL of 10^{-5} and 10^{-6} solution was spread out using a sterile glass rod. All the plates were incubating at 37°C for 24h then counting the colony forming. For spores, samples were heating up 75°C for 15 minutes to kill vegetative cells before dilution. The colony forming unit (CFU) was calculated by multiplying the number of colony with the dilution per milliliter (CFU/mL).

Bioreactor cultivation: *B. coagulans* VCIM5914 were cultivate in bioreactor 2 L Jupiter Solaris using 1 L medium include 10 g/L Yeast extract, 0.5 g/L K_2HPO_4, 0.25 g/L $MgSO_4$, 0.1 g/L KCl, 0.025 g/L $MnSO_4$, add 0.4 g/L $CaCl_2$, pH adjusted to 6.0, inoculum 10% v/v with preculture. The temperature is 40°C, dissolve oxygen remains 30% control by stirring (150-300 rpm) and air flow (0.2-0.4 lpm), cultivation time for 28h. Samples were measured turbidity and colony counting.

RESULTS AND DISCUSSION

Screening yeast extract for cultivation

Twelve yeast extract test samples received from Procelys by Lesaffre were screened to select the optimal yeast extract source. The composition and recipe for yeast extracts proprietary and under continuous research. However, some samples included crude extracts containing whole yeast cells (YE9, YE10, YE11), making it difficult to observe results using turbidity absorption at a wavelength of 600nm. The results of plate counting as show in Figure 2.

The number of cells ranged from 2×10^8 to 8×10^8 CFU/mL, commonly falling within $2\text{-}3\times10^8$ CFU/mL, while high spore densities only reached $1\text{-}2\times10^8$ CFU/mL. Some yeast extract samples that yielded high cell densities included YE1, YE4, YE2, and YE6. However, it is well-known that bacterial sporulation is often induced under stressful conditions rather than favorable growth conditions. Consequently, samples that produced high spore densities were YE6, YE10, and YE11. Based on these results, YE6, which achieved high spore density while maintaining an average cell count, was selected as the optimal yeast extract for subsequent experiments.

The results demonstrated a variation in the impact of various yeast extract samples on the cells and spores, suggesting that it is one of the primary factors influencing cells and spores yield.

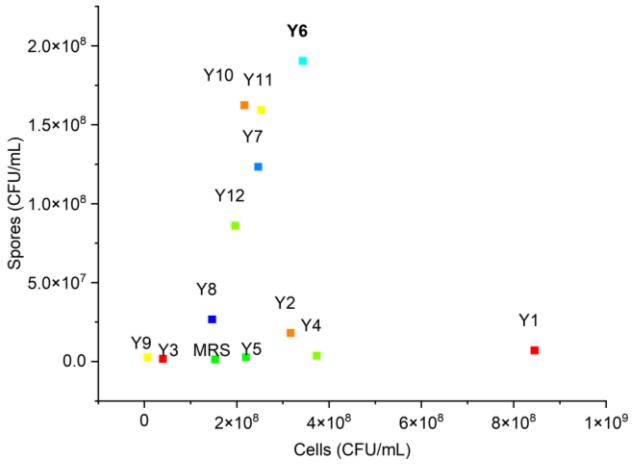


Figure 2. Screening different types of yeast extract nutrient

Yeast extract concentration

The concentration of yeast extract (YE6) was investigated in range from 0 to 20 g/L (0-5-10-15-20 g/L) in a 48 hours cultivation period. The plate counting of cells and spores results are shown in Figure 3. As expect, the increase of YE concentration resulted in increasing of cells and spores.

In the range of yeast extract concentration from 5 to 12.5 g/L, the cell count increase from 5×10^8 CFU/mL to 4.84×10^9 CFU/mL. At 15 g/L, the cell count reached its highest value of 7.7×10^9 CFU/mL, but at 20 g/L it only reached 6.14×10^9 CFU/mL. For spores, in the range of yeast extract from 5 to 10 g/L, the count remained constant at 1×10^8 to 2×10^8 CFU/mL. From 12.5 to 20 g/L, the number of spores increased: at 12.5 g/L, it reached approximately 1×10^9 CFU/mL and reach the highest spore count was observed at 20 g/L with 2.48×10^9 CFU/mL.

Overall, the increase of spores and cells can be observed depending on the increase of yeast extract concentration, however in practical applications, the selection material for production must also consider the sporulation rate and the growth efficiency per unit of raw material. Thus, we selected the amount of 10 g/L yeast extract for further experiment to increase the number of spores in cultivation.

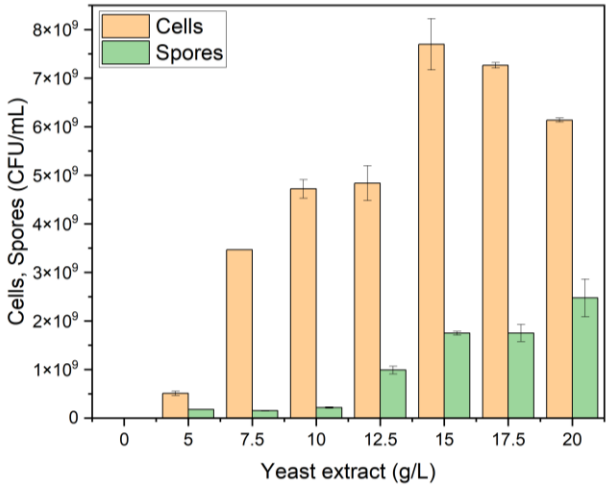


Figure 3. Effect of yeast extract concentration on cells and spores

Effect of calcium and Iron ions

In the present study, the effect of calcium ion concentration on accumulation of cells biomass and spore rate were investigated. The results as shown in Fig. 4.

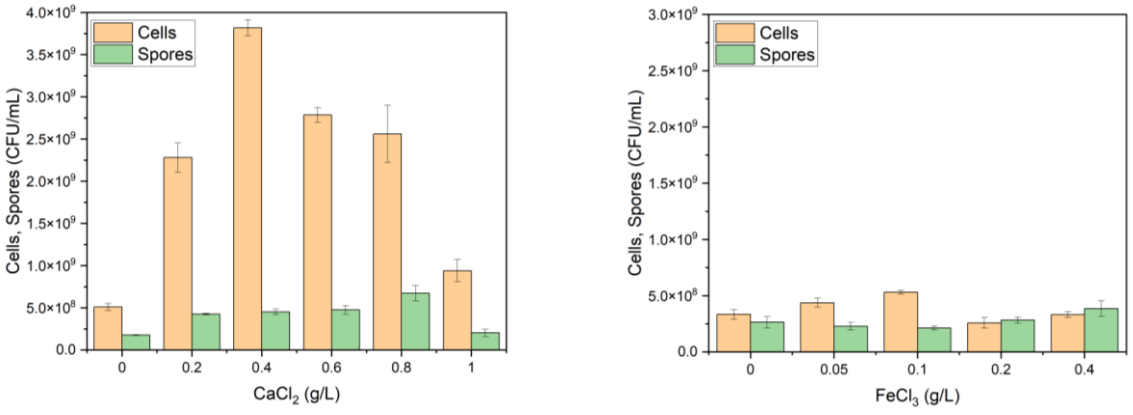


Figure 4. Effect of calcium and iron ion concentration on cells and spores

(A: Calcium chloride, B: Iron (III) chloride)

Supplementing with $CaCl_2$ in concentrations ranging from 0.2 to 0.8 g/L increased cell density from approximately 2.25 $\times 10^9$ to 3.5 $\times 10^9$ CFU/mL and 1×10^9 CFU/mL at the concentration of 1 g/L compared to the control (5×10^8 CFU/mL). This indicated that the addition of $CaCl_2$ positively affects the growth of the strains, enhancing its growth capacity. Spore density also increased from approximately 4×10^8 CFU/mL to 6×10^8 CFU/mL as calcium concentration increased from 0.2 to 0.8 g/L, compared to only 1.7 $\times 10^8$ CFU/mL without supplementation.

For $FeCl_2$, an increasing trend in cell density from 3×10^8 CFU/mL (0 g/L) to 4-5×10^8 CFU/mL also observed, with a maximum cell density achieved at a concentration of 0.1 g/L. However, the change in spore density was not significant. These results indicate that the effect of $FeCl_3$ is minimal compared to the control.

In both experiments, despite the increase in cell density in the samples with $CaCl_2$, there was surprisingly no noticeable increase in spore density, which remained around 3×10^8 CFU/mL. While $CaCl_2$ supplementation increased cell density, it did not affect spore formation capacity, resulting in a spore formation rate of less than 40%.

The sporulation of *Bacillus* was known for the formation of endospore with three main stages: firstly, asymmetric cell division: the mother cell and the forespore; secondly, engulfment: the mother cell covers the forespore with a spore coat, and finally, late sporulation: coat assembly of four layer: (i) inner forespore membrane, (ii) cortex, (iii) outer forespore membrane, and (iv): coat. Then the mother cell lyses to release a mature spore into the environment. During this process, the involvement of Ca^{2+}-dipicolinic acid in replacing water during the late stage of the spore formation process is crucial. The absence of this factor prevents the spores from surviving in dry conditions(Kamat, Lewis, and Pradhan 1985), (McKenney *et al.,* 2013). This may explain the increase in spore density with Ca^{2+} concentration, even when the cell count was not change, as show in the results in Fig. 4A.

Additionally, the homeostatic roles of Fe^{2+} and Mn^{2+} are closely related to each other through the coordination of three metal-regulating protein: the Fe^{2+} sensor Fur, the Mn^{2+} sensor MntR and the oxidative stress response regulator PerR, which functions with either Fe^{2+} or Mn^{2+}. The homeostatic of Fe^{2+} and Mn^{2+} in *Bacillus* is a complex mechanism involving oxidative stress response (Steingard *et al.,* 2023). This explains the high spore ratio observed in high Fe^{2+} concentration in Fig. 4B.

It suggests that an optimum sporulation condition can be archived with separate stages for optimal cell formation and stress-induced sporulation may be more effective than one stage with all components from the start of batch. However, the essential balance during the sporulation stage between manganese, iron, and oxidative stress in *Bacillus* could be resolved through genetic and metabolic analysis.

Temperature

The common cultivation temperature for *Bacillus* in the industry are 30°C, 37°C, and 40°C (Biermann and Beutel 2023). Particularly, studies about the lactic acid producing from *Bacillus coagulans* cultivated at 45°C (Tolieng *et al.,* 2018), (Marshall and Beers 1967). Therefore, two temperatures are 37°C, and 40°C were investigated in this study, the results as showed in Fig.5.

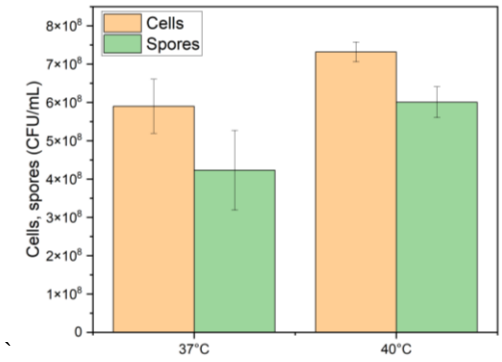


Figure 5. *B. coagulans* cells and spores comparison cultivation at 37°C and 40°C

Comparison of the cultivation at temperatures 37°C and 40°C, it is evident that at 40°C, both cell density and spore ratio were better than at 37°C. Some previous studies have also indicated that the optimal cultivation temperature for this strain is between 40-45°C, although the exact optimal temperature is not clearly reported (Xu *et al.,* 2023). The spore ratio also improved, though not significantly, by about 10%, increasing from 72% to 82% (spore count increased from 4.23 to 6.01×10^8 CFU/mL) (Fig.5). Therefore, a cultivation temperature of 40°C will be used for fermentation conditions in bioreactor.

Cultivation in bioreactor

Aiming to apply in bioreactor scale, cultivation 1L medium was experiment in 2L Jupitor Solaris bioreactor was conducted for scale up. The fermentation parameters controled include temperature: 40°C, dissolve oxygen remain ≥ 30% control by stirring (150-300rpm) and air flow (0.2-0.4 lpm). Yeast extract has used 10 g/L with addition 0.4 g/L $CaCl_2$. The change of pH and oxygen demand were recorded to evaluate the relative with sporulation process.

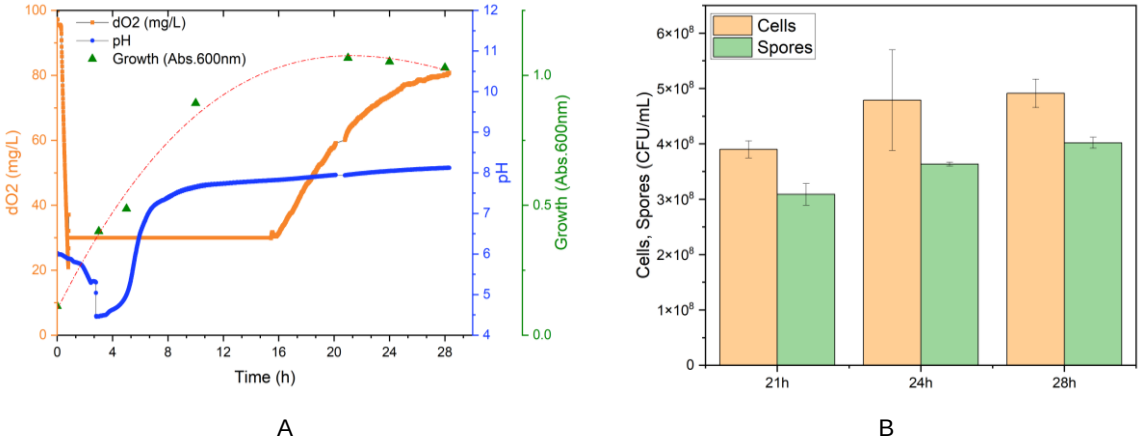


Figure 6. Cultivation in bioreactor (A) pH, oxygen dissolvent, and OD600, (B) Cells and spore at 21, 24, and 28h

The growth rate by OD600nm measurement show the cells growth curve reaches highest after 16-20h cultivation (Fig. 6A). The pH tends to decrease from 6 to 4.5 during 3 first hours then increase from this point to pH 7.5 at about 8h cultivation. From this point pH slightly increases up to 8.0 until the end of fermentation (28h). The most consumption oxygen also occurred during the first 16h. The oxygen drastically falls from 100% to nearly 10% during the first 4th hours cultivation. Then the dissolve oxygen was constantly controlled at value of 30% by stirring speed and air flow 0.02 lpm to 0.04 lpm (the fluctuate of stirring and dissolve oxygen). At 16h time point, the cells growth reaches the highest value then the demand of oxygen slowly down, leading to the increase of dissolve oxygen from 30 up to 80% at the end of cultivation (28h) (Fig. 3C). The oxygen consumption was highest from 2 to 12h cultivation in which is in accordance to report by Li *et al.,* showing the dissolved oxygen was below 20% after 2h cultivation. The spore does not observe at first 10h cultivation (data not show) then quickly reaches 3x10^8 at 21h to 4×10^8 CFU/mL at 28h cultivation, similar with study of Li *et al.,* (2022). The spore rate slightly increases from 79% (at 21h) to 82% (at 24h) and 83% (at 28h) (Fig.6B). The spore yield indicated that under aerated and stirred fermentation conditions, the spore collection time could be more than 24 hours.

Multiple strategies to optimize of endospore were studied, including medium and parameter cultivate condition, in both solid-state and submerge fermentation. The variety of process parameters and media composition reflects

the diversity of the *Bacillus* spp. group (Biermann and Beutel 2023). These results contributed the initial study on optimization to improve cell and spore production, aiming for further application in pilot-scale fermentation in the industry.

CONCLUSION

The composition of medium for *B. coagulans* cultivation was experiment with different yeast extract nutrient and addition of different calcium ion. The result shows the high biomass can reach at 24h but the spore rate seems to be needed more optimization condition. The increase of nutrient and calcium have significant effect higher number of cell and spore rate. In addition, cultivation condition in bioreactor was experiment with control of oxygen, temperature, and stirring. The trend of oxygen demand and pH were observed. The log phase happened during the first 12h cultivation then the stationary phase can be predicted from 12h – 24h, the sporulation phase happened during this phase and extend longer. This study showed the enhance effect of yeast extract nutrient and calcium ion on *B. coagulans* cell and sporulation. Nevertheless, the medium composition and the optimization experiment is necessary for sporulation target, and the optimization media composition for spore/ biomass still is an interesting question to be investigate in further study.

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TÁC ĐỘNG CỦA CAO NẤM MEN VÀ ION Ca^{2+}, Fe^{3+} TỚI SỰ PHÁT TRIỂN VÀ TẠO BÀO TỬ CỦA *BACILLUS COAGULANS*

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TÓM TẮT

Bacillus coagulans đang được quan tâm nghiên cứu do có nhiều tiềm năng ứng dụng bào tử trong các sản phẩm probiotic. Trong nghiên cứu này, ảnh hưởng của nồng độ cao nấm men và ion Ca^{2+}, Fe^{3+} tới khả năng sinh trưởng và tạo bào tử của chủng *B. coagulans* VCIM5914 đã được quan tâm nghiên cứu. Mười hai mẫu cao nấm men từ nhà cung cấp đã được khảo sát cho thấy sự phụ thuộc lớn vào loại cao nấm men đến khả năng sinh trưởng và tạo bào tử. Nồng độ cao nấm men phù hợp có hiệu quả tới tự gia tăng của mật độ tế bào là ≥ 7.5 g/L, mật độ bào tử là ≥ 12.5 g/L. Sự có mặt của ion canxi ≥ 0.2 g/L làm tăng lên về số lượng tế bào và bào tử. Nồng độ ion sắt không làm tăng lên đáng kể về mật độ tế bào nhưng góp phần tăng tỷ lệ tạo bào tử ở nồng độ ≥ 0.2 g/L. Lên men trên thiết bị lên men với các điều kiện lên men đã lựa chọn cho mật độ tế bào ~5×10^8 CFU/mL với tỷ lệ 82% bào tử sau 28h nuôi cấy.

Từ khóa: Bacillus coagulans, ion canxi, lợi khuẩn, tạo bào tử, cao nấm men.

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