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INDUCTION ENDOSPORE FORMATION OF *Bacillus subtilis* KM16 AS A PROBIOTIC CANDIDATE BY OPTIMIZATION OF THE GROWTH CONDITIONS

Stella Magdalena[1]*, Adelaide Geraldine[2], Yogiara[3]
[1]Department of Food Technology, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, BSD Campus, Banten, Indonesia, 15345
[2]Department of Biotechnology, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, BSD Campus, Banten, Indonesia, 15345
[3]Department of Magister Biotechnology, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Semanggi Campus, South Jakarta, Indonesia, 12930
Corresponding author: stella.magdalena@atmajaya.ac.id

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ABSTRACT

Probiotics are living microorganisms that have beneficial effects. One of the benefits is enhancing immunity. B. subtilis KM16, as an endospore-producing bacteria, has been known to increase probiotic resistance to confront the unstable conditions of the digestive tract. Spore formation by Bacillus is supported by the optimal conditions of the growth medium and incubation periods. The sufficient availability of carbon, nitrogen, and mineral sources in the growth medium and environmental conditions can support the formation of optimal endospores. This research aimed to determine the suitable sugar composition of a medium and the optimum incubation time suitable for the endospore formation of B. subtilis KM16. Glucose and maltose were utilized as a carbon source in several concentrations as a growth medium for B. subtilis KM 16. The incubation time was between 48 and 72 hours. The viable number of vegetative cells was not significantly different for the type of sugar and incubation periods. Based on the glucose concentration, 0.2% glucose exhibited the highest number of cells, while 1% glucose concentration caused a decrease in vegetative cells. In this study, 0.2% glucose with a 48-hour incubation period showed the highest percentage of sporulation frequencies, up to 48%, but it was not significantly different from the other glucose concentrations. The medium that contains 0.2% glucose with a 48-hour incubation period is the best medium for the utilization of B. subtilis KM16 cells and spores as probiotic candidates.

INTRODUCTION

Probiotics are non-pathogenic living microorganisms that are beneficial to enhance digestibility and increase immunity, but they must be consumed in sufficient numbers to work effectively. Lactic acid bacteria are mainly known as probiotics, although a few bacterial species, such as *Escherichia coli*, *Streptococcus*, *Lactococcus lactis*, some *Enterococcus* species, *Streptomyces,* and *Bacillus,* can be probiotic candidates. However, some of these probiotic strains still do not have heat-resistant traits or other resistances, such as UV radiation resistance, the supplementation of chemical compounds during the production process, and resistance to pancreatin, pH conditions, and bile salt in the digestive tract. Therefore, bacteria that form endospores are more preferable for the production of probiotics.

Bacillus sp. is a spore-producing bacteria resistant to extreme environmental conditions such as heat, radiation, chemical components, and limited nutrition. Additionally, the spores of *Bacillus* sp. that have been manufactured into probiotics can be stored at room temperature. Several species of *Bacillus* sp. whose spores have been produced commercially are *B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans,* and *B. licheniformis* (Patel et al., 2019). In this experiment, *Bacillus subtilis* KM16, isolated from Crater Lake in Indonesia, is used as a probiotic candidate because the endospores have good heat resistance, antimicrobial attributes, and resistance to bile salts, while the vegetative cells are motile and can build biofilms (Sanjaya, 2020). The endospores produced can be affected by the accessibility of optimum nutrients in the media to boost the development of vegetative and spore cells.

Sufficient carbon, nitrogen, and mineral sources are the capital components for the conformation of optimal endospores as probiotic candidates. In this study, glucose and maltose were used as carbon sources in various concentrations. Glucose and maltose are optimal carbon sources to produce vegetative and spore cells of *Bacillus* sp. (Khardziani et al., 2017; Sukumaran et al., 2019). The incubation period is implemented at 48 and 72 hours to form endospores. The aim of this study is to identify the optimum incubation period and suitable sugar composition of a medium for endospore formation of *B. subtilis* KM16 as identified by the number of sporulation frequencies.

MATERIALS AND METHODS

Two types of sugars were used as a carbon source for *B. subtilis* KM16 endospore formation, which were glucose and maltose. The incubation time was between 48 and 72 hours. In the first stage, the cultivation of cells was done in basal medium with the addition of glucose or maltose. Sporulation frequencies were calculated to determine the optimum medium condition by the spread agar method. After determining the optimum sugar and incubation period, the second stage of cell cultivation was done to determine the best concentration of sugar for endospore formation.

Culture preparation

The *B.subtilis* KM16 culture was isolated earlier from Crater Lake in Indonesia (Magdalena et al., 2021). Isolate was grown in Luria Agar (LA) (Oxoid) and incubated at 37 °C for 24 hours. Each isolate was kept at 4 °C for stocks and -80 °C for cryopreservation.

Seed culture preparation

For seed culture preparation, one loop of isolate was inoculated into a 250-mL Erlenmeyer flask containing 50 mL of sterile LB medium of the following composition (g/L): tryptone 10.0 (Oxoid), yeast extract 5.0 (Oxoid), and sodium chloride (Oxoid) 10.0. The inoculated flasks were incubated in a water bath shaker at 37 °C for 19 hours and 120 rpm under aerobic conditions until total colonies reached 10^8 CFU/mL (OD~1).

Cultivation condition

This assay was performed as described by Khardziani et al. (2017) with medium modification. One mililiters of seed culture (2% v/v) in the form of OD = 1 at 600 nm was transferred into 250 mL Erlenmeyer flasks containing 50 mL of sterile basal medium (g/L): KH_2PO_4(Merck) 1.0, $MgSO_4$ (Merck) 0.5, yeast extract (Oxoid) 3.0, peptone (Oxoid) 3.0, and glucose (Oxoid) 1.0. Glucose and maltose in concentrations of 5 g/L were used as carbon sources. Four Erlenmeyer flasks of cultivation medium were incubated in a water bath shaker at 37 °C and 120 rpm under aerobic conditions (Table 1).

Table 1. Different media composition and incubation time used for Bacillus subtilis KM16 endospores production

Flasks	Medium Composition	Incubation time
1	Basal medium + 0.5% glucose	48 h
2	Basal medium + 0.5% maltose	48 h
3	Basal medium + 0.5% glucose	72 h
4	Basal medium + 0.5% maltose	72 h

(Khardziani et al., 2017).

The optimum incubation time and carbon source in the first stage of cell cultivation were used for cell cultivation in the second stage. In the second stage of endospore production, various variations in sugar concentration were employed, ranging from 0.2%, 0.5%, 0.7%, and 1.0%, as shown in **Table 2**. Four Erlenmeyer flasks of cultivation medium were incubated in a water bath shaker for 48 hours at 37 °C and 120 rpm under aerobic conditions.

Table 2. Different media concentration used for Bacillus subtilis KM16 endospores production

Flasks	**Medium Composition**
1	Basal medium + 0.2% glucose
2	Basal medium + 0.5% glucose
3	Basal medium + 0.7% glucose
4	Basal medium + 1.0% glucose

(Khardziani et al., 2017).

Determination total number of vegetative cells and spore

This assay was performed with dilution and spread volume modifications. After cultivation, one milliliter of each culture was taken from the flasks and suspended in 9 mL of sterile saline water, and a serial dilution was prepared until 10–6. After serial dilution, dilutions from 10^{-4} to 10^{-6} were spread into Luria Agar plates. The LA plates were incubated at 37 °C for 14–16 h and counted as colony-forming units (CFU/mL). Each dilution of each medium experiment was done in three replicates.

For determining the spore yields, Erlenmeyer flasks were heated at 80 °C for 20 min as a heat shock treatment. Then, a serial dilution was prepared in sterile saline water until 10–6. After serial dilution, dilutions from 10^{-4} to 10^{-6} were spread into LA plates. The LA plates were incubated at 37 °C for 14–16 hours and counted as spores per milliliter. Each dilution of each medium experiment was done in three replicates. Sporulation frequencies were calculated as equation (1) (Khardziani et al., 2017):

$$\text{Sporulation frequency} = \frac{\text{spore}}{\text{vegetative cells} + \text{spore}} x\ 100\% \quad (1)$$

RESULTS AND DISCUSSION

Bacillus sp. is one of the gram-positive bacteria in the form of bacilli. Based on its oxygen requirement, it is classified as an aerobic bacteria. However, several species can live in facultative anaerobic conditions. Bacillus can grow in a wide range of temperatures, from 7 °C to 55 °C, but its growth is optimum at 37 °C. These microorganisms can also grow in conditions of pH 4 to 6, with salinity levels ranging from 5 to 15% (Kurniatuhadi et al., 2023). The specialty of *Bacillus* is that its vegetative cells can form endospores in environmental conditions that are less supportive for their growth. When the environmental conditions return to their optimum, the spores formed can develop into vegetative cells again. This process of spore formation is referred to as sporulation (Riley et al., 2021). Limited availability of nutrients, dehydration, UV radiation, and exposure to chemical compounds such as hydrogen peroxide and lysozyme enzymes can trigger the formation of endospores (Moeller et al., 2014).

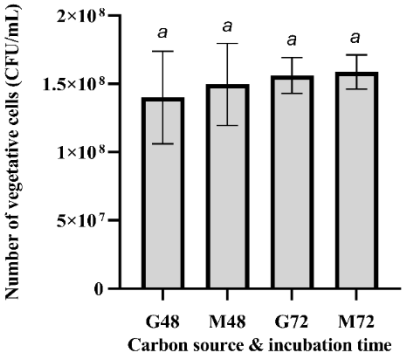

Figure 1. Total number of Bacillus subtilis KM16 based on carbon source and incubation time (G48 = glucose 48h, M48 = maltose 48h, G72 =glucose 72h, M72 = maltose 72h)

According to the result shown in Figure 1, the vegetative cells ranged from 1.40×10^8 to 1.59×10^8 CFU/mL. Basal medium with glucose as a carbon source and incubated at 48 h shows the lowest vegetative cell. Overall, there was an increase in vegetative cells from the seed starter, which amounted to 10^8 CFU/mL, and it was sufficient to be categorized as probiotics. Food that could be classified as probiotics must

contain at least 10^6 CFU/g of viable bacteria (FAO/WHO, 2002). The numbers of colonies between all four treatments were not significantly different ($P > 0.05$).

B. subtilis could utilize both types of sugar, namely glucose and maltose, as a carbon source. Glucose transport and phosphorylation in B. subtilis were regulated by the Phospho Transferase System (PTS) (Chen et al., 2023). B. subtilis could utilize maltose because it produces the α-glucosidase enzyme, which is encoded by the malL gene (Saburi et al., 2015). Maltose would enter the cell via a specific transporter protein for maltose, namely EIICB, that was encoded by MalP. Inside the cell, cytoplasmic maltose would be converted from MalA into maltose 6-phosphate and glycolytic intermediates (Heravi et al., 2019).

Based on Figure 2, glucose at 72 hours of incubation time shows the highest sporulation frequencies (49%), followed by maltose at 72 hours (44%), glucose at 48 hours (40%), and maltose at 48 hours (16%). Only maltose incubated for 48 hours was significantly different from the other treatments.

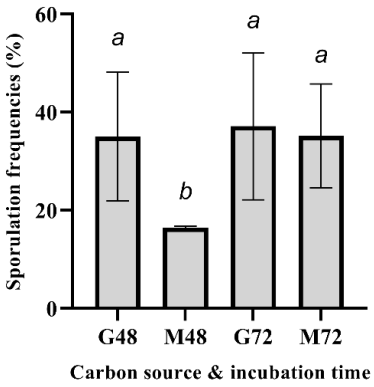

Figure 2. Sporulation frequencies of *Bacillus subtilis* KM16 based on carbon source and incubation time (G48 = glucose, 48h, M48 = maltose, 48h, G72 = glucose, 72h, M72 = maltose, 72h)

The incubation between 48 and 72 hours did not show a significant difference in the number of vegetative cells. Figures 1 and 2 show that the longer the incubation time, the higher the number of vegetative cells and the ability of *Bacillus* to form spores. The incubation time of 48 hours was the optimal time for the growth of *Bacillus* spp. During that time, the organism produces the highest metabolite compounds, such as antibiotics and enzymes (Henshaw and Deputy, 2019). The two incubation times used in this study were still in the cell growth phase, so the number of vegetative cells was still sufficient. If the incubation time exceeds 72 hours, it has started to enter the cell death phase due to

a decrease in the availability of nutrients and the denaturation of enzymes. Time efficiency is very important in this study; therefore, the fastest incubation time was chosen because it did not show a significant difference in the number of vegetative cells at both incubation times (Figure 1).

The vegetative cells for the four treatments were not significantly different. Compared to other treatments, the ability of *B. subtilis* KM16 to form spores when using medium supplemented with maltose during 48 hours of incubation was very low (Figure 2). Based on previous research, cell and spore growth had been produced on a medium containing a mixture of glucose and other carbon sources such as raffinose, sucrose, and fructose. The number of vegetative cells was not significantly different. Meanwhile, the number of spores was lower on a medium containing a mixture of glucose and other carbon sources. It was because these carbon sources did not initiate the formation of sporogenesis directly (Elisashvili et al., 2019). However, the ability to form spores in the basal medium with maltose at 72 hours was quite high. If the carbon source only comes from glucose, glucose has been used entirely by cells, so the spore rate appears earlier than the combination of glucose and maltose sugars. Meanwhile, the carbon source, with the addition of maltose, causes the growth rate of vegetative cells to be slower. The longer the incubation time, the higher the cell number. The availability of nutrients in the media decreases, which could cause an increase in the number of spores.

In the glucose treatment between 48 hours and 72 hours of incubation time (Figure 2), the number of sporulation frequencies was slightly different because the spore formation phase was at a stationary stage. Meanwhile, with maltose sugar, the sporulation frequencies are very different between both incubation times because the spore formation phase is in the log phase. The formation of spores using monosaccharide sugars could be done at a faster incubation time than with disaccharide sugars. The monosaccharide and disaccharide sugars for spore formation could affect the growth rate and spore rate (Bressuire-Isoard et al., 2018). As the incubation time increases, the growth rate also continues to increase due to the cannibalism mechanism in *B. subtilis*. The mechanism of cannibalism is to postpone sporulation for as long as possible. Sporulation killing factor (SKF) and sporulation delaying protein (SDP) toxins would secrete cells that were not resistant to these two toxins, so the dead cells could be used as nutrients for cannibal cells (Höfler et al., 2016).

The number of vegetative cells ranged from 8.47×10^7 to 1.59×10^8 CFU/mL with glucose as the carbon source. Glucose concentrations of 0.2%, 0.5%, and 0.7% indicated an increase in the number of vegetative cells from the seed starter, which was 10^8 CFU/mL. However, the number of vegetative cells at 1% glucose showed a decrease at 8.47×10^7 CFU/mL. The number of colonies between glucose 0.5% and 0.7% was not significantly different, but it was significantly different from glucose 0.2% and 1.0% (Figure 3).

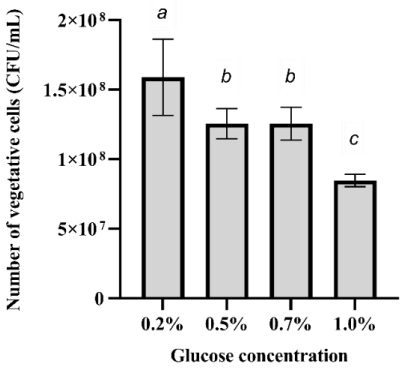

Figure 3. Total number of Bacillus subtilis KM16 based on glucose concentration

Based on Figure 4, glucose 0.2% shows the highest sporulation frequencies (48%), followed by glucose 0.7% (47%), glucose 0.5% (40%), and glucose 1.0% (30%). The sporulation frequencies between all four treatments were not significantly different ($P > 0.05$).

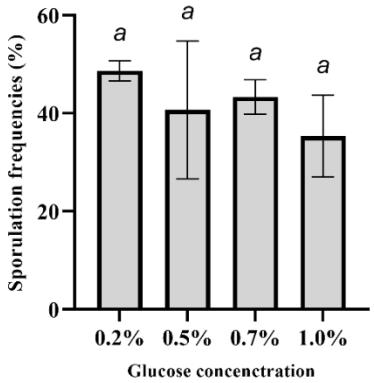

Figure 4. Sporulation frequencies of Bacillus subtilis KM16 based on glucose concentration

Sporulation frequencies in basal medium with the addition of 0.2% glucose (2 g/L) showed the best results, which were 48% (Figure 4). In previous studies, the addition of glucose concentrations up to 5 g/L could produce the largest number of vegetative cells

and spores. However, the addition of glucose exceeding 5 g/L could inhibit the sporulation process (Monteiro et al., 2005). Therefore, the amount of carbon given must meet the needs of cells because each cell has different nutritional needs. The larger the cell, the more carbon sources were consumed. An excess amount of carbon in the cell could cause catabolite repression. For example, in *B. subtilis*, excessive amounts of glucose could cause catabolite repression and inhibit the action of the pyruvate kinase enzyme, which plays a role in the glycolysis pathway (Sousa et al., 2019).

Catabolite repression in *B. subtilis* could be reduced by limiting the entry of glucose into cells. For example, the transcriptional regulation of GlcT and CggR suppresses the expression of genes encoding glycolytic enzymes so that glucose does not continuously enter cells and prevents the process of gluconeogenesis (Buffing et al., 2018). Gluconeogenesis is a metabolic pathway for the formation of glucose, so if this regulation occurs, it can inhibit cell growth (Chung et al., 2015). This could be seen in the 1% glucose treatment, where the number of vegetative cells decreased to 10^7 CFU/mL from the starter culture, which amounts to 10^8 CFU/mL (Figure 3). The bacterial response to a given carbon source not only causes catabolite repression but also affects gene expression to determine the preferred carbon for the microorganism (Buffing et al., 2018).

Spores are resistant to heat because they are covered by a layer of cortex and coat. The function of the spore coat was to filter molecules that enter cells, such as enzymes and nutrients, as well as chemical compounds that can accelerate cell death. The second layer of spores was composed of peptidoglycan, which was responsible for the spore structure forming, followed by the cortex and germ cell wall in the third layer. The core of the spore consists of genetic material in the form of DNA, ribosomes, dipicolinic acid (DPA), and SASPs, which function to protect DNA (Setlow, 2014). Meanwhile, DPA would form a complex with a cation, namely calcium (Checinska et al., 2015). The DPA-calcium complex determines the dehydration conditions and mineralization in the spore core, which protect the spores from wet heat (Jamroskovic et al., 2016). The source of nutrients would affect the formation of a protective layer of spores such as the outer coat, inner coat, and cortex, whose thickness could be observed using an electron microscope (Abhyankar et al., 2016).

Proteins that were bound together by disulfide bonds became the backbone of the *Bacillus* spore coat. One of the most abundant amino acids found in the spore coat was

tyrosine (Ursem et al., 2021). Tyrosine is composed of a carboxyl group and an amine group. Therefore, the source of nutrients in the form of carbon and nitrogen greatly determines the formation of the spore coat to protect the genetic components of *Bacillus* against exposure to an unfavorable environment. In this study, glucose, maltose, yeast extract, and peptone determined the formation of the amino acid tyrosine. Tyrosine could be formed from fumarate in the TCA cycle metabolic pathway. Acetyl Co-A was involved in the TCA cycle obtained from pyruvate, the product of glycolysis. Pyruvate could be obtained through the glycolysis step, which comes from the breakdown of glucose molecules. Therefore, carbon sources play an important role in the formation of tyrosine, which is the main backbone of the spore coat.

Overall, the number of vegetative cells showed a slight increase from the starter culture, and sporulation frequencies were low (Figures 1–4), which could be due to the production of *B. subtilis* KM16 cells and spores under uncontrolled pH conditions. After 48 hours of fermentation, the medium pH was slightly acidic, which could reduce the amount of sporulation efficiency. Meanwhile, alkaline pH (around 8 or 9) could improve sporulation efficiency (Monteiro et al., 2005).

In the application of *Bacillus* as probiotics, vegetative cells and spores could be used, but each had its advantages and disadvantages. Vegetative cells that survived during production and the gastrointestinal environment must be processed using bioengineering first, for example, using protective compounds, encapsulation, and cell immobilization (Nguyen et al., 2016). Meanwhile, spores did not need a bioengineering process. However, spores may not be able to fully germinate into vegetative cells when they reach the digestive tract (Bernardeau et al., 2017). Spores would germinate into vegetative cells if the surrounding environmental conditions, such as nutrients and oxygen supply, were sufficient (Wu and Chang, 2021). In addition, other factors that could influence spore germination are the genetic characteristics of the host and the location of the germinating spores in the digestive tract (Wells-Bennik et al., 2016).

CONCLUSION

Glucose and maltose could be used by *Bacillus subtilis* KM16 to produce endospores. The utilization of vegetative cells based on incubation time did not show any

difference. So, the most effective and efficient incubation time was 48 hours. Basal medium with the addition of glucose and an incubation time of 72 hours showed the highest number of sporulation frequencies, which is equal to 49%. Based on carbon concentration, glucose 0.2% shows the highest yield of vegetative cells. Hence, glucose 1% shows a decrease in the number of vegetative cells in the starter culture. Glucose with a 0.2% concentration shows the highest number of sporulation frequencies. Both vegetative cells and spores could be used as probiotics.

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