Induction of *Clostridioides difficile* L-form

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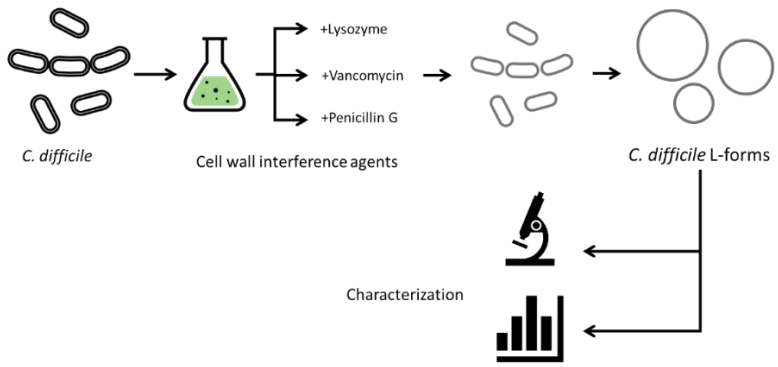
Abstract: L-forms are cell wall deficient variants of conventional bacteria that are capable to proliferate. Although L-forms were discovered for several decades, little is known about their general properties, mainly due to the lack of reliable methods for generating such bacteria. This work aims to create and characterize L-forms of *Clostridioides difficile* using cell wall interfering agents, including penicillin G, vancomycin, and lysozyme at various concentrations on osmoprotective brain heart infusion agar. The results revealed that *C. difficile* L-forms could be induced by 100 µg/mL penicillin G and 250 µg/mL vancomycin. The induced L-forms showed distinguished water drop-like colony morphology on agar plate. In addition, the abnormal shape or pleomorphic cells in induced condition was observed for L-forms by phase contrast microscopy. Muramic acid assay showed the significantly decrease of peptidoglycan abundance in L-form under the treatment of penicillin G. This study provides new investigation and characterization of the *C. difficile* L-form, which would be initiate a valuable research tool for gene delivery and biotechnology in *Clostridioides spp.*

Graphical abstract:



Keywords: L-forms; wall-less proliferation; *Clostridioides difficile*

1. Introduction

In the mid-1900s, the novel type of cell wall deficient bacteria (CWDBs) was discovered in serum from a patient with antibiotic treatment. The bacteria were named L-forms in honor of the Lister institute where they were discovered [1, 2]. L-forms are bacterial variants that lack a cell wall, however, they can still maintain the ability to grow and proliferate in osmoprotective conditions [2]. The CWDBs referred to pleomorphic bacteria with damaged or absent peptidoglycan cell walls. They are distinguished into 2 types, including spheroplast and protoplast, which are induced from Gram-negative and Gram-positive bacteria, respectively [3]. Basically, CWDBs fail to proliferate, rendering the

limitation for further applications. However, the discovery of L-form bacteria initiates the idea to break the limitation. Thus, L-forms induction and characterization are challenging topics for many researchers [4, 5].

In normal cells, cell wall precursor synthetic pathway and cytoskeletal protein are required for cell division [4, 6]. In contrast, the mechanism of cell division in L-form occurred through membrane dynamics utilization such as vesiculation, tubulation, and blebbing [4]. Crucially, due to the lack of the cell wall, L-form bacteria require extra osmoprotective conditions to facilitate growth and survival. Many works used L-form bacteria as a model to study the primitive cell division [4, 7]. The generation of L-forms of many bacteria, such as *Bacillus subtilis, Escherichia coli, Streptobacillus moniformis, Streptomyces viridifaciens, Clostridium thermocellum*, and *Bacteroides* strains were successfully generated [5, 8-11]. The methods to induce an L-form state often involve the utilization of cell wall synthesis inhibitors [1, 5, 8, 9]. Moreover, many reports have suggested that the L-form induction can also be completed by cultivation in selective growth media [1, 5, 8, 9]. The induced bacterial L-forms displayed remarkable application potential in biotechnology. Cell wall deficient phenotypes can be advantageous for the production of recombinant protein as well as being a heterologous recipient for large foreign DNA such as phage genomic DNA.

Presently, *Clostridioides difficile* becomes a great concern as it has become one of the leading causes of healthcare-associated infections worldwide [12, 13]. Complication of the treatment of *C. difficile* infection is continuously raising due to the emergence of antibiotic resistant strains. An alternative way for *C. difficile* treatment is therefore mandatory [12]. One alternative therapeutic approach so-called "phage therapy" is known as a method to exploit bacteriophage – a group of viruses that is able to infect bacteria, in treatment of drug resistant bacterial infection [14, 15]. To further develop phages suitable for therapeutic purposes, the genome of bacteriophage of interest needs to be modified and amplified in a suitable host [12, 13]. In Gram-negative bacteria, many of these organisms have been reported to be an efficient recipient for DNA transformation [8, 16]. Nevertheless, there are only a few Gram-positive bacteria able to incorporate genetic material because of the thick cell wall. Therefore, the L-form bacteria become candidate recipients for transformation. L-forms are not only the suitable recipient for accepting large genetic material but also capable to replicate, transcribe, and translate the introduced DNA [5, 9]. Nevertheless, reports of L-forms induction are still lacking in *Clostridioides* species. Therefore, this study aims to introduce an effective protocol for L-form production in *C. difficile*. The optimized induction protocol for *C. difficile* L-forms presented in this work may be of great interest to be used as an alternative tool for genetic engineering of *Clostridioides* species.

2. Materials and Methods

2.1. Bacterial culture

C. difficile strain NIH21 was cultured and routinely maintained in BHI (brain heart infusion) medium at 37°C under anaerobic condition. A single colony from agar plate was inoculated into 3 mL of BHI broth using aseptic technique and incubated overnight before the experiment. For L-form cultivation, the bacteria were maintained in BHI broth supplemented with 5% (w/v) glucose as an osmoprotectant.

2.2 Agar well diffusion

Agar well diffusion technique was used for screening of the suitable cell wall targeting agents for the L-form induction. The log-phase culture of *C. difficile* (OD_{600} = 0.5) was prepared, then 150 µL of the culture were spread into the L-form supporting agar plate (BHI agar containing 10% glucose) and let it dried. After that, the agar plates were cut on the central area using a sterile 6 mm cork borer. Each well was then filled with 20

μL filter sterilized solutions of either 10 mg/mL vancomycin, 10 mg/mL penicillin G, or 50 mg/mL lysozyme. Sterile water was used as negative control. The appearance of inhibition zones were recorded by a stereo microscope after overnight incubation at 37 °C. The L-form-like cells at the edges of each zone were examined using phase contrast microscopy. The agent that yielded the best indication of L-form induction of *C. difficile* was selected for subsequent experiments.

2.3 Optimization of L-form induction and osmotic pressure

Inducer concentration optimization step was carried out by cultivation of *C. difficile* NIH21 in BHI broth supplemented with 5% (w/v) glucose for 16 h at 37°C under anaerobic condition. The culture was then adjusted to an OD_{600} of 0.5 with sterile medium. 300 μL of the *C. difficile* NIH21 culture were then added in 96-well plate containing various concentrations of the selected agents. Plate was incubated overnight and cells were harvested by centrifugation at 6000xg for 2 min for morphological observation.

Osmotic pressure optimization was performed by cultivation of *C. difficile* NIH21 in BHI broth containing either 100 μg/mL penicillin G or 250 μg/mL vancomycin with the addition of glucose at 1, 2.5, 5, 7.5, and 10% w/v. Then, 100 μL of the culture were harvested and centrifuged at 6000xg for 2 min. Cell pellet was collected and washed twice with 100 μL of 1X PBS. Cell morphology was observed using live cell fluorescence imaging system, IX-83ZDC, DIC mode at 60X magnification. The microscopic experiment was supported by Center of Nanoimaging (CNI), Faculty of Science, Mahidol University. Images were captured and analyzed in quantitative data using ImageJ program.

2.5 Peptidoglycan measurement

Muramic acid assay is a simple colorimetric method to determine the amount of muramic acid in bacterial peptidoglycan structure. 100 μL of bacterial culture from 100 μg/mL penicillin G and 250 μg/mL vancomycin treatments were harvested and incubated with 50 μL of 1 M NaOH at 37 °C for 30 min. Then, 1 mL of 18 M H_2SO_4 was added and incubated with samples in a boiling water bath for 3 min. After incubation, the samples were rapidly cooled down on ice for 1 min. Then, samples were mixed with 10 μL of 0.16 M $CuSO_4.5H_2O$ and 20 μL of 0.09 M p-hydroxy biphenyl (PHD), followed by incubation at 30 °C 30 min. After incubation, the absorbance was measured at 570 nm in a spectrophotometer Shimadzu UV-1280 and calculated into percentage. Statistical significance was determined using a one-way ANOVA test in Microsoft Excel with a P-value threshold of ≤0.05.

3. Results

3.1. Screening of cell wall interference agents

The candidate agents used in this experiment and their mechanism of actions are listed in Table 1. The screening of cell wall disturbing solutions was done by agar well diffusion. The inhibition zones were observed in the conditions of penicillin G and vancomycin treatments (Figure 1c-d). Whereas, no inhibition zone was observed around the well of control and lysozyme treatment conditions (Figure 1a and b). Nevertheless, in the lysozyme treatment, a few colonies appeared on the plate compared with those of control (Figure 1b). The colony morphologies from penicillin G and vancomycin were different from the control condition. *C. difficile* exhibited various colony sizes with rough surfaces, cloudy colors and swarming growth, as shown in the control condition (Figure 1e). Colony morphology had changed into water drop-like appearance after penicillin G

and vancomycin treatments. Moreover, the colonies were smaller with a smooth edge, and no signs of swarming growth (Figure 1g and h). For lysozyme condition, colony morphology of *C. difficile* was similar to that of the control (Figure 1f). As the alteration of colony morphology suggesting the effect of agents on bacterial cell wall, therefore, these two antibiotics were chosen as candidates for the L-form induction.

Table 1. List of cell wall interference agents used in this study and their action mechanism

Solution	Action mechanism
Penicillin G	Inhibit transpeptidase, enzyme related to cell wall synthesis [17, 18]
Lysozyme	Hydrolyze linkage of NAG-NAM in peptidoglycan [19]
Vancomycin	Prevent cross-link of peptide chain by binding with D-Ala-D-Ala [20]

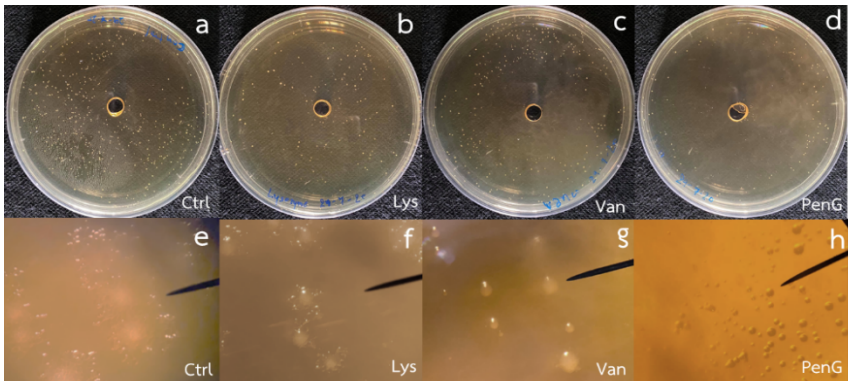


Figure 1. *C. difficile* colony pattern (a-d) and morphology (e-h) from agar well diffusion under stereo microscope. (a, e) the normal colony pattern and morphology of control condition. (b, f) The colony pattern and morphology of lysozyme condition. (c, g) The abnormal colony patterns and morphologies of vancomycin and (d, h) penicillin G conditions.

3.2. Cell morphology of L-form

Colonies were selected from each agar well diffusion and observed for cell morphology under a light microscope using DIC mode. The cells were counted and compared in terms of morphology (Figure 2). For the lysozyme condition, the population was likely to be a mix of endospores and bacillus-shaped vegetative cells. Whereas, abnormal shapes or pleomorphic cells were observed in vancomycin and penicillin G treatments. Because all treatments presented a mixed population of abnormal and normal cells, therefore, the proportion of L-forms was analyzed to compare between each condition.

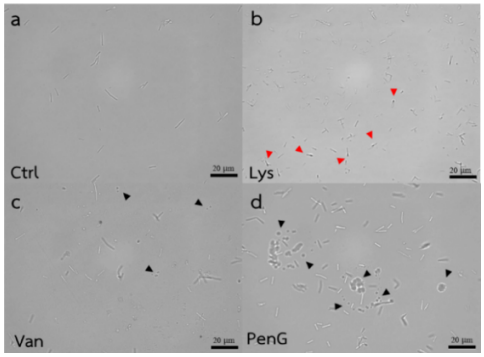


Figure 2. Cell morphology after cell wall interference agent treatments including (a) control, (b) lysozyme, (c) vancomycin, and (d) penicillin G. The black arrows indicate pleomorphic cells, and red arrows indicate spore-like particles, scale bar =20 μm.

Vancomycin and penicillin G conditions presented the highest percentage of pleomorphic cells (Figure 3). The results suggested that these two agents could be candidates for the L-form induction in *C. difficile*. The optimal concentration of vancomycin and penicillin G would therefore be optimized for efficiency of the L-form induction.

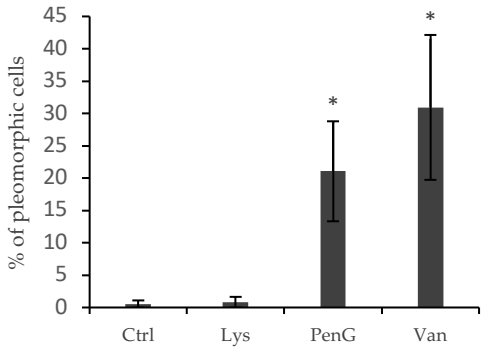


Figure 3. The percentage of pleomorphic cell observed under microscope in each tested condition. Statical analysis by ANOVA (p-value<0.05).

3.3. Optimization of the inducer concentration and osmotic pressure

To achieve the effective method for L-form induction, *C. difficile* was cultured and induced in BHI broth with penicillin G or vancomycin at the concentration ranging from 0-1000 μg/mL. For penicillin G, the highest percentage of pleomorphic cells was achieved at 100 μg/mL, which was calculated to be 65% (Figure 4a). Whereas, the percentage of pleomorphic cells was dramatically dropped at the concentration of penicillin G higher than 100 μg/mL. The result of 100 μg/mL penicillin G treatment on cell morphology was shown in Figure 4a. No positive effect on L-form induction was observed when 5% glucose was added along with penicillin G as an osmoprotectant.

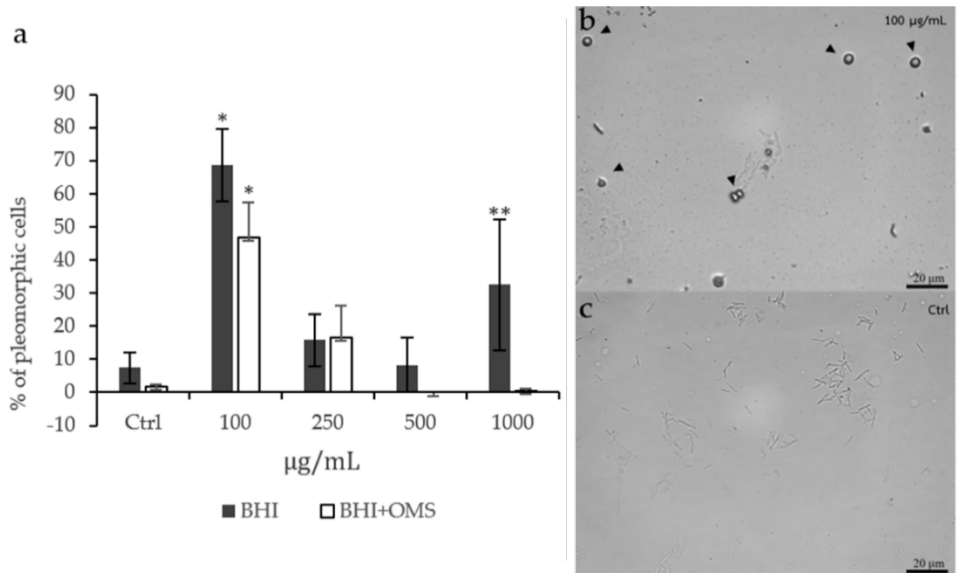


Figure 4. (a) The pleomorphic cell percentage at various concentration of penicillin G with and without addition of 5% glucose as osmoprotectant. Statistical analysis was done using ANOVA (p-value<0.05). (b) Cell morphology of *C. difficile* from 100 μg/mL penicillin G treatment and (c) control conditions. The arrows indicate pleomorphic cells, scale bar =20 μm. OMS; media with 5% (w/v) glucose.

For vancomycin treatment, the results showed significantly different pleomorphic cell percentage among the samples (Figure 5a). The highest percentage of pleomorphic cells was achieved at 250 μg/mL with the addition of 5% glucose as an osmoprotectant. In contrast to treatment with penicillin G, addition of 5% glucose tended to improve the yield of pleomorphic cells under the treatment with vancomycin.

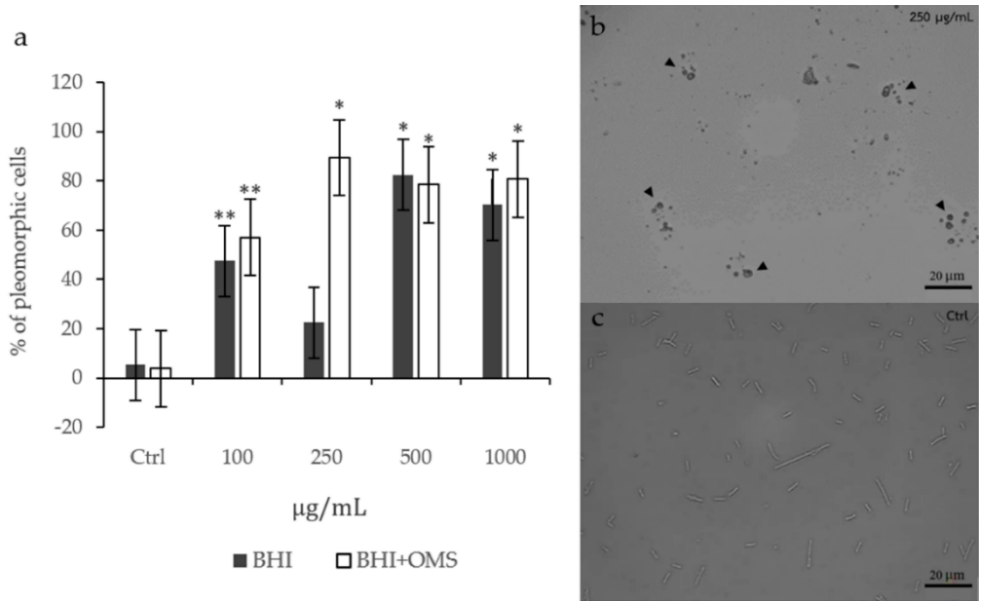


Figure 5. (a) The comparison of pleomorphic cell percentage at various concentration of vancomycin with and without 5% glucose as osmoprotectant. ANOVA (p-value<0.05) was used for statistical analysis. (b) Cell morphology of *C. difficile* treated with 250 μg/mL vancomycin and (c) control. The arrows indicate pleomorphic cells, scale bar =20 μm. OMS; media with 5% (w/v) glucose.

Furthermore, the glucose concentration was varied to determine the effect of osmotic pressure toward the L-form induction. As shown in Figure 6, the addition of glucose increased pleomorphic cells for both penicillin G and vancomycin. The highest yield of pleomorphic cells was obtained in the induction condition of 100 μg/mL penicillin G containing 2.5% (w/v) glucose. When the glucose concentration was increased beyond 2.5%, percentage of pleomorphic cells was not significantly different among each the antibiotic-inducing sample.

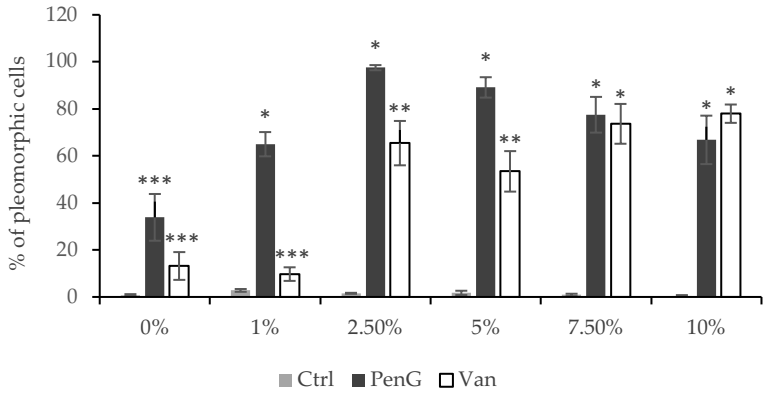


Figure 6. The pleomorphic cell percentage at various concentration of glucose in penicillin G-, vancomycin inducing condition. ANOVA was done for statistical analysis (p-value<0.05).

3.4. Muramic acid measurement

The primary characterization of L-forms was performed by determining the abundance of peptidoglycan. As shown in Figure 7, the lowest A_{570} which reflecting peptidoglycan abundance are significantly decreased to 17% and 28% in penicillin G and vancomycin treatments, respectively.

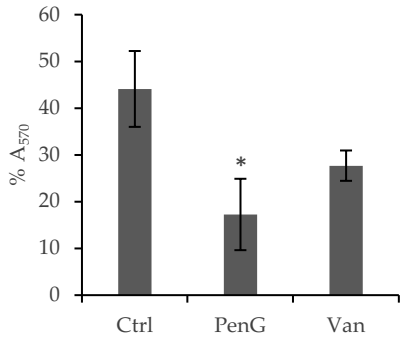


Figure 7. Muramic assay of *C. difficile* induced with 100 μg/mL penicillin G and 250 μg/mL vancomycin. ANOVA was used for statistical analysis (p-value<0.05).

4. Discussion

In previous work, various methods have been useful for inducing L-form in various bacteria. The induction has often been achieved by routine treatment with cell wall interfering agents [1, 21]. The cell wall targeting agents including enzymes and antibiotics, such as lysozyme and penicillin G, were shown to be effective for L-form induction in *Streptomyces hygroscopicus*, *Bacillus subtilis*, and *Streptomyces viridifaciens* [2, 4]. A more generic drug against *C. difficile* infection, vancomycin, was also effective inducer in Gram-positive bacteria such as *Staphylococcus aureus* [20, 22].

In this work, we screened various cell wall interrupting agents to establish a protocol for inducing L-form formation in *C. difficile*. Notably, the results revealed that penicillin G and vancomycin were relatively effective in inducing the production of *C. difficile* L-forms, which is agreeable to the previous reports. On the other hand, the addition of lysozyme did not show inhibition zone in the agar well diffusion assay. The result could be potentially due to the fact that certain *C. difficile* may exhibit lysozyme resistant capability [23, 24]. In many bacterial strains, the colony of L-form bacteria was often described as a "fried-egg colony" [1, 2, 25]. The fried-egg phenotype was reported to be derived from a unique vesiculation process of the L-forms proliferation [26]. However, our results clearly showed that vancomycin and penicillin G induced L-form colony of *C. difficile* adopted water drop-like colony morphology. At this point, it has still yet to determine what mechanism creates this unique water drop-like colony. Further characterization to elucidate this particular colony pattern may shed light on the mechanism of L-form formation of *C. difficile* and related groups of anaerobic firmicutes.

Due to its deficiency in the cell wall structure, the L-form bacteria often have pleomorphic cell morphology [1, 2]. The examination of bacterial cell shapes using regular light microscope was shown to be sufficient to distinguish pleomorphic cells from normal rod-shaped cell [1, 8]. This type of characterization could be helpful for estimation of induction rate [8]. We used the same microscopic technique to carefully scrutinize the effects of different agents in the induction of L-forms. In our optimization, 100 μg/mL of penicillin G and 250 μg/mL of vancomycin could induce high percentage of pleomorphic cells,

presumably representing the cell wall deficient *C. difficile* or the L-forms. This concentration is markedly correlated well with the previously reported concentration of drugs around 200 μg/mL used for induction of L-forms in other bacteria [1, 2, 27]. In any case, lysozyme treatment did not show positive results in inducing the formation of L-forms. Further microscopic investigation revealed the mixed population of *C. difficile* endospores and normal bacillus-shaped cells, suggesting that the stress caused by the lysozyme induced cell wall damage instead have triggered the formation of endospores rather than the L-forms. This ability to switch into spore state has been reported in various bacteria with the resistant phenotype [23, 24]. It is worthy to further investigate the underlying principle behind switching between endospore state and L-forms formation.

Furthermore, L-forms can be classified into 2 types: stable and unstable L-forms [1]. The unstable L-forms are the cell wall deficient cells that temporarily form the L-forms state. This type of L-forms can revert into normal cells once the stress inducing agent is removed. Due to their intrinsically dynamic nature, this type of L-form may not be particularly well suited for future characterization or biotechnological application [1, 2, 4]. In our hand, mixed population of pleomorphic cells, normal cells, and spores were found in all treatments. Several methods have been successfully exploited to isolate stable L-forms such as filtration, and osmotic isolation [27, 28]. In our work, the optimal glucose concentration to maintain the L-forms was determined. Similar to prior experiments, our result showed that the glucose concentration suitable for maintaining the L-forms is in the range of 2.5%-10% (w/v) [1, 21, 28, 29]. Nevertheless, it should be noted that addition of too high concentration of glucose could cause a lethal effect to the cells while providing insufficient amount of glucose may provide no osmoprotective effect to maintain the L-form structure [30]. Due to that, we therefore, chose 5% (w/v) of glucose as the optimal condition to maintain and isolate L-forms.

To verify the abundance of induced *C. difficile* L-form in each condition, we chose to detect of presence of peptidoglycan using muramic acid assay, a calorimetric detection of muramic acid in peptidoglycan structure [31]. It is anticipated that the amount of peptidoglycan in a successful induction of L-forms structure would be dramatically lower than that of normal untreated *C. difficile* with rod-shaped cells or even completely depleted. Despite the fact that the L-form bacteria were seen in various vancomycin treatment. After isolation with 5% (w/v) of glucose, the quantification of muramic acid content in the sample did not show a marked decrease in the peptidoglycan content, suggesting that there might still be a significant number of *C. difficile* cells contaminated in isolation. It is also possible that the addition of 5% (w/v) of glucose might not be enough to preserve the induced L-forms state from reverting to regular vegetative cell with intact cell wall. An investigation to optimize the maintenance and isolation of the L-forms should be further scrutinized to achieve better isolation of L-form bacteria for future application.

5. Conclusions

In conclusion, this work presented the induction protocol for *C. difficile* L-forms production could be completed by routinely treatment with 100 μg/mL penicillin G and 250 μg/mL vancomycin. Moreover, we suggested that 5% (w/v) of glucose can be used as osmolarity medium in L-forms production. The characterization of pleomorphic cells showed significantly decrease of muramic acid in peptidoglycan structure in penicillin G treatment. The decreasing of peptidoglycan components suggested that pleomorphic cells could be L-forms in *C. difficile*. However, pleomorphic cells from this protocol were mixed with normal cells. Then, the protocol is needed to develop more for L-forms isolation and characterization.

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Conflicts of Interest: The authors declare no conflict of interest.

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