Scanning and Transmission Electron Microscopy Evaluation Of The Effects And Efficiency Of Formulated L. lactis Cell Suspended In Skim Milk In The Presence Of Starch And Gellan Gum As Excipients

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Abstract: A cost-effective method towards improving shelf life of L. lactis cells was developed. In this study, L. lactis cells suspended in skim milk in the presence of starch and gellan gum as excipient were prepared via freeze drying technique. The effects and efficiency of starch and gellan gum to enhance and protected high numbers of cells were investigated. The viability of the cell were high in the presence of both starch and gellan gum. Scanning electron micrograph confirmed that the freeze drying process did not cause noticeable damage or morphological changes to the L. lactis cell. From the transmission electron microscopic (TEM) result, it showed that the size of the cell wall decrease due to compatibility of the starch and gellan gum that covered and protect the L. lactis cell wall where the interaction formed between the L. lactis cell and the excipients in solution may be attributed from the force exerted upon cell-excipients contact aided by hydrogen bonds, dispersion forces and hydrophobic or electrostatic interactions to form intermacromolecular complexes.

Key words: L. lactis cells, excipient, freeze drying technique and intermacromolecular complexes.

INTRODUCTION

Viability of bacteria and their effectiveness is important for various areas of science and technology study such as microbiology and biotechnology because of their great significance to both theory and practice as well as could lead to more rapid developments in basic scientific research and in practical applications. The decrease in viability can be reduced by several of the method including cell immobilization (entrapping, covalent attachment and ionic attachment), spray drying, extrusion, emulsion, phase separation and microencapsulating (extrusion and emulsion) the bacteria (Chandramouli, V., et al., 2004). Nevertheless, all of these method must meet various criteria such as, it must be simple, gentle, long-live, have a high activity and cheap (Bucke, C., 1983). A progressive research effort had lead towards improving the shelf life of viable bacteria with cost and time saving effect. The substance used as excipients and support material should be gentle and non-toxic as these can influence the viability of the cell. There were more than a few of biopolymer materials used as excipients such as calcium alginate, K-carrageenan (Gardin, H., A.Pauss, 2001), gellan gum (Moslemy, P., et al., 2002; Oliveira, J.T., et al., 2010) and gelatin. However, each of these polymers has its drawback such as poor mechanical strength, durability and not economical.

The use of starch and gellan gum as a support material is one of the promising way in biotechnology application as it has ability to improve viable of bacteria (Sultana, K., et al., 2000) and also can respond to surrounding conditions such as pH and temperature. Starch and gellan gum is a natural material from polysaccharides as well as abundant biomass resources and could be considered as inexpensive. Due to its distinctive biological and physicochemical characteristics with proven safety properties as do not induce a toxic reaction, biocompatible and biodegradable, starch and gellan gum has a wide potential, effectively and have been progressively to be used in industrial applications such as in delivery transportation for food product, controlled drug release (Chen, L., et al., 2007; Vieira, A.P., et al., 2007; Balmayor, E.R., et al., 2009) and manufacture of various adhesives and bioplastics (Singh, N., et al., 2003; Gaspár, M., et al., 2005). In this paper, we report an approach to improved viability of L. lactis cell suspended in skim milk and supported with starch and gellan gum via freeze drying technique. Starch and gellan gum was chosen due to the simplicity of the preparation under mild conditions and easy to work with where the process does not utilize organic solvents which are toxic to microbial cells as well as to its promising properties (mechanical and thermal stability) (Teramoto, N., et al., 2003; Araújo, M.A., A.M. Cunha, 2004; Mali, S., et al., 2005; Pareta, R., M.J. Edirisinghe, 2006; French, D., 1973). The current technique implemented in this work is much simpler and involve fewer steps compared to those previously reported (O’Riordan, K., et al., 2001) which is required high temperature in the process which may not be suitable for encapsulating bacterial cultures and expensive.
The aim of this study was to devise a cost-effective method towards improving shelf life of \textit{L. lactis} cells via freeze drying technique. Furthermore, the effect and the compatibility of starch and gellan gum as support material/excipients for maintenance of cell viability were investigated. Systematic investigations on the interactions between the excipients and cells as well as physicochemical properties of the resultant biomaterial were performed using scanning electron microscopy, SEM and transmission electron microscopy, TEM.

**MATERIALS AND METHODS**

\textbf{Bacterial Culturing:}
\textit{L. lactis} pNZ9000 strains, was furnished by Laboratory of Marine Biotechnology (MARSLAB), Institute of Bioscience, Universiti Putra Malaysia, which was previously stored at -80°C in glycerol stock prior to use and was cultured in the laboratory and checked routinely for purity based on their morphology and biochemical characteristics during this investigation. 10 µl of \textit{L. lactis} were cultured in a 10 ml Luria-Bertani broth (Merck, Damstadt, Germany) seeded with 5% glucose and chloramphenicol. Incubation was allowed for growth at 30°C for 48 hours. A loop whole of 24 hours culture broth were then streaked on GM17 agar for single colony. The agar plates were then incubated invertedly for 48 hours. A single colony of the culture was then picked for gram staining. The 48 hours culture broth were then diluted 1:10 in LB broth seeded with 5% glucose and chloramphenicol. Incubation was allowed for growth under the previous same conditions. All culture broth was then preceded to GM17 agar plate culture for colony count. Approximately 1 g of bacteria (cfu/ml) was added to 1 ml of skimmed milk solution in order to provide greater protection against loss of viability of the bacteria and shrinking of the cell (Cody, W.L., et al., 2008).

\textbf{Samples Preparation:}
Starch from rice: In sterile conditions, 1.0 % wt/vol of starch (Sigma) was dissolved by heating at 60°C± 5°C in distilled water. Heating process was continued until the solution became concentrated. After cooling to 50-55°C ± 5°C, 1 ml of cell suspension were added wisely and mixed vigorously to give a cells-to-biopolymer ratio of 1 : 10 (on a dry weight basis) before freeze-drying process for 12 hours using Freeze Dry System Freezezone 12 (LABCONCO, Japan).

Phytagel (gellan gum): 1 g of phytagel (Sigma) was dissolved by heating at 100°C in 90 ml of distilled water and sterilized by autoclaving (121°C for 20 min). After cooling to 55°C, 10 ml of cell suspension (1 g dry weight/10 ml) were added and mixed. Scheme 1 (Figure 1) shows the diagram of the procedure towards improving shelf life of \textit{L. lactis} cells suspended in skim milk using starch and gellan gum as excipient by freeze drying technique.

![Scheme 1: Diagram of procedure](image)

**Fig. 1:** Schematic diagram showing the formation step towards improving shelf life of \textit{L. lactis} cells suspended in skim milk using starch and gellan gum as excipient by freeze drying technique.
Characterization Of The Resultant Samples:
The resultant biomaterial size and polydispersity index (PDI) were determined using Zeta sizer (Malvern instruments). The zeta potential of *L. lactis* pNZ9000 was carried out after dilution of the formulations in sterile water (warm) and the size was measured in triplicate. Each sample was measured 3 cycles (10 times each).

For the iodine-starch test, 1.0 g of the resultant samples was added to 100 mL of deionized water and the mixture was stirred until suspension was formed. The iodine-KI reagent was dropped into the suspension, and a blue black or red brown colour (blue for amylose and red brown for amylopectin) was observed if starch existed.

For cell viability a cell was suspended and dispersed in 10 ml of phosphate buffer (PBS, pH 6.8). A serial dilution of this suspension was made until a suitable cell density was obtained. The cell suspension was then spread onto the pre-dried MRS agar (Merck, Darmstadt, Germany) plates. The plates were then incubated at 30°C overnight. This plating procedure was carried out in triplicates.

Morphology:

**Scanning Electron Microscope (SEM):**
The morphology and structure of the samples were determined using a Scanning Electron Microscope (SEM) model JEOL JSM 6400. The sample was cut into a number of 1cm³ slice and put into separate vials and fix in fixative (4 % Glutaraldehyde) for 2 days at 4°C. The sample was then washed with 0.1 M Sodium Cacodylate Buffer for 3 changes of 30 minute each and post fixed in 1 % Osmium Tetroxide for 2 hours at 4°C in the same buffer for 3 changes of 30 minutes each before dehydration. Dehydration was done in a graded acetone series (30 – 100 %). After dehydration process, sample was transfer into specimen basket and put into critical dryer for about ½ hour and then sticks onto the stub using double sided tape or colloidal silver before gold coating in sputter coater and views the samples.

**Transmission Electron Microscope (TEM):**
The interaction between starch and bacteria and distribution of the bacteria cell in the starch was investigated by using a HITACHI H-7100, Transmission Electron Microscope (TEM). The sample was cut into a number of 1 mm³ slice and put into separate vials and fix in fixative (4 % Glutaraldehyde) for 2 days at 4°C. The sample was then washed with 0.1M Sodium Cacodylate Buffer for 3 changes of 30 minute each and post fixed in 1 % Osmium Tetroxide for 2 hours at 4°C in the same buffer for 3 changes of 30 minutes each before dehydration. Dehydration was done in a graded acetone series (30–100%) and infiltration the sample with acetone and resin mixture. After infiltration process, the samples were embedded into beam capsules and fill up with resin before polymerize in oven at 60°C for 24-48 hours and sectioning. Thin sections were mounted on grids, covered with collodion film, and post-stained with 2 % uranyl acetate for 15 minutes and wash with doubled distilled before viewing the samples.

RESULT AND DISCUSSION

**Characterisation Of The Samples:**
In order to observe the effect of starch and gellan gum as support material on the particle size of *L. lactis* cell, the size was measured in triplicate using Zeta sizer (Malvern instruments) and the particle size of a representative of freeze dried cell of *L. lactis* suspended in skim milk supported with starch and gellan gum are presented in Table 1. It was observed that, when the *L. lactis* cell was supported by gellan gum, the volume-based mean diameters were slightly smaller than supported by starch. This could be due to slightly thick layer of starch which surrounded and contact with the cell wall compared to gellan gum thus conducted to the increment of the size of the particles and this result will be supported and discussed by TEM measurement in the next section. Consecutively to investigate the efficacy of this technique in the presence of starch and gellan gum as effective protective agents, plate counting technique was vital to examined whether the cells were alive or dead. Colonies of bacteria were counted and converted to log CFU (colony forming units) and was shown in Table 1. The survival of *L. lactis* cells reported as percentage viability was calculated with reported values are averages of the three replicates. Numbers of viable bacteria were high in the presence of both starch and gellan gum therefore indicated the advantages the used of starch and gellan gum to enhanced and protected high numbers of cells. Incorporation of starch and gellan gum improved the viability of the cell (Vidhyalakshmi, R., *et al.*, 2009) and as compared to when the bacteria were support without starch and gellan gum. The protective action of both biopolymer may be attributed to formation of microcolonies within the the matrix surrounding the bacteria and serves as a potential barrier against detrimental environmental factors thus aid the viability of the cell (Moslemy, P., *et al.*, 2004).
Table 1: Values of size, polydispersity index and bacteria counts for _L. lactis_ suspended in skim milk supported with starch and gellan gum prepared via freeze drying technique.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Size (µm)</th>
<th>Poly Dispersity Index</th>
<th>Before capsulation (cfu/ml)</th>
<th>After capsulation (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>2.04 ±0.6</td>
<td>0.67</td>
<td>2.27x10^4 ± 7.18x10^8</td>
<td>2.11x10^4 ±5.04x10^8</td>
</tr>
<tr>
<td>Phyta Gel</td>
<td>1.80 ± 60.2</td>
<td>0.68</td>
<td>1.99x10^4 ± 7.5x10^8</td>
<td>2.53x10^4 ±4.74x10^8</td>
</tr>
</tbody>
</table>

*Polydispersity index closer to 1 indicate different size of particles were present.

3.2 Scanning Electron Microscopy:

Freeze drying technique have been choose due to a number of advantages such as convenient method for the protection and long term storage of a extensive assortment of microbes. Figure 2a present SEM micrographs for the freeze dried cell of _L. lactis_ suspended in skim milk. It appeared with _coccip_ form so as to group in pairs and short chains. This indicated that the freeze drying process did not produce any noticeable damage or morphological changes to the _L. lactis_ cell. Besides, from the technique used the risk damaging the microbial have been reduced because of the short contact time in the dryer. SEM micrographs in Figure 2b and Figure 2c demonstrated the morphology of starch and gellan gum surface containing _L. lactis_ cells suspended in skim milk after freeze dried. Differences in surface appearances of starch and gellan gum were seen, as evidenced by Figure 2b and Figure 2c. The rod-shaped with smooth and even bacterial cells were observed to attach on the particle surface of the starch. To further confirm the presence of starch, chemical analysis method (the starch-iodine test) was employed to confirm the starch component. The red brown was very evident after the addition of the iodine-KI reagent in the mortar suspension. It indicates that starch is still present (Yang, F., B. Zhang, Q. Ma, 2010). However, it could be observed from the images that there was the freeze-dried cells were clumped together on the gellan gum sample. As gellan gum is used as gelling agent and work as cation-induced gelation, the warm of concentrated water solution of gellan gum (Patil, J.S., _et al._, 2010) was transformed from solution to gel at lower temperature (temperature-dependent) and easily harden by addition of water due to complexation with cations and hydrogen bonding with water thus conducted to the bacteria clumping thus covering the cells to provide protection. From the result obtained, it shows that the technique applied was complementary for protecting cell since freezing slow the chemical reactions and occurs under vacuum and in the absence of oxygen which make it impossible for oxidative reaction to occur.

![Fig. 2](image_url)

Fig. 2: Scanning electron micrograph of _L. lactis_ cell (a) untreated, (b) after treated with starch, and (c) after treated with gellan gum.
3.3 Transmission Electron Microscopy:

Understanding the interactions between the excipients and cells and also the physicochemical properties of the resultant biomaterial could lead to more rapid developments in basic scientific research and in practical applications. Transmission electron microscopy (TEM) is generally used to investigate the internal structure of microbial cells and to monitor the contacts between the excipients and cells. Transmission electron micrographs of freeze dried cell of *L. lactis* suspended in skim milk presented a continuous thin smooth cell wall (Figure 3a). It is believed that skim milk solution used has provided greater protection against loss of viability of the bacteria. All *L. lactis* were in ovoid shape and had intact smooth cell wall which surrounded regions of cytoplasm with an unchanged physical appearance of cells upon suspension. It was observed that the size of the cell wall decrease as the starch and gellan gum covered the *L. lactis* cell as shown in Figure 3a-3c. The sizes of *L. lactis* cell wall are about 684 nm (Figure 3a), while 627 nm and 606 nm for *L. lactis* cell in the presence of starch (Figure 3b) and gellan gum (Figure 3c) as excipients respectively. This is due to cell wall have been compressed by the excipients hence led to the decreasing in the cell wall size of the *L. lactis*. It is believed that these results were affected by the physical and molecular changes due to compatibility of the starch and gellan gum that covered and protect the cell wall. These results suggest that the interaction formed between the *L. lactis* cell and the excipients (biopolymer) in solution may be attributed from the force exerted upon cell-excipients contact aided by hydrogen bonds, dispersion forces and hydrophobic or electrostatic interactions to form intermacromolecular complexes (Henke, A., et al., 2005).

![Fig. 3](image-url)
Conclusion:
In conclusion, the technique implemented in this work towards improving shelf life of *L. lactis* cells suspended in skim milk in the presence of starch and gellan gum as excipient were successfully prepared via freeze drying technique. The survival of *L. lactis* cells in the presence of starch and gellan gum were high indicated the efficiency of both starch and gellan gum to enhanced and protected high numbers of cells. SEM result indicated that the freeze drying process did not produce any noticeable damage or morphological changes to the *L. lactis* cell. From the TEM result, we noticed that the the size of the cell wall decrease due to compatibility of the starch and gellan gum that covered and protect the *L. lactis* cell wall. It was shown that the interaction formed between the *L. lactis* cell and the excipients in solution may be attributed from the force exerted upon cell-excipients contact aided by hydrogen bonds, dispersion forces and hydrophobic or electrostatic interactions to form intermacromolecular complexes.

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