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Research Article

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Differentiation of *Leuconostoc mesenteroides* media modified with different sugars

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ABSTRACT

Leuconostoc mesenteoides is commonly used in fermented food. The study on developing molecularly targeted drugs to achieve a higher grade of drug delivery system that is one of the indispensable issues in pharmaceutical fields. This paper reported the effects of carbon sources including in glucose, maltose, lactose, saccharose at the different concentrations as 0, 5, 10, 20, 30 g/L on the cell differentiation of Leuconostoc mesenteroides VTCC-B-871. As results, L. mesenteoides VTCC-B-871 formed minicells with highly significant number of 4.6 ± 0.3 (%) starting cells in modified MRS broth with 20 % glucose. The minicells were collected and checked for the less than 400 nm in size and round shape under scanning electron microscope. Therefore, minicells could be used as a nanoparticle in pharmaceutical science.

Keywords*: Leuconostoc mesenteroides*, differentiation, minicells, scanning electron microscopy.

INTRODUCTION

It is undeniable that there were several existences such as drug resistance, dose-limiting toxicity, toxic side effects and difficulties of targeted delivery, which cause damages to normal cells as kidney and liver cells. These problems are daunting challenges in terms of medical treatment. Therefore, the combination of pharmaceutical science with advances in cell biotechnology, chemical science and bioinformatics is required to limit the obstacles on the drug development.

The development of nanotechnology in recent years [1] is applied as nanoscale drug delivery vehicles have shown the advantage for directing the drugs to specific target by attaching specific ligands on to their surface, improved stability and therapeutics index and reduce side effects, but increasing the circulation time and bioavailability by manipulating the particle size and surface characteristics of nanoparticles.

Nanoparticles are particles sized from 10 to 1000 nm [2] that can be made using a variety of materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes), magnetic, even inorganic or metallic compounds (silica, iron) and bacteria (bacterially derived nanoparticles or “minicells”) [3- 5].

However, there were several important limitations that have been highlighted and identified in the development of drug delivery system. The ineffective distribution in cells and tissues, limited oral availability, and retention in by passing organs and by macrophages of the reticuloendothelial system after systemic administration [6] are typical examples. Beside the enhanced efficacy demonstrated by many targeted nanoparticles, they also face main restrictions as a major dose loss due to receptor-mediated endocytosis and subsequent lysosomal digestion, immunogenicity and non- specificity of the target ligand resulting in accelerated blood clearance, and further impaired cell penetration such as tumor cells compared to the non-targeted nanoparticles [7].

The minicell producing strains have been isolated from *Escherichia coli* [8], *Bacillus subtilis*, *Salmonella typhimurium*, *Haemophilus influenza* and the other Gram-positive bacteria (*Listeria monocytogenes*) and Gram-negative bacteria (*Shigella flexneri* and *Pseudomonas aeruginosa*) [3-4]. LAB improves nutritional value of food, control of intestinal infections; improve digestion of lactose, control of some types of cancer. Scientists developed natural antimicrobial products for bio-control of pathogens and have exploited LAB for the competitive exclusion of pathogens and delivery of vaccines and bioactive compounds [9]. LAB also plays an important role in the treatment of people suffering with tumors and immune compromised subjects [10-11]. The evidence that LAB effects on human health is remarkable and fascinating for effective utilization. They seem to have relatively low toxicity compared to other treatments [12]. However, exploiting cocci bacteria belonging to lactic acid bacteria (LAB) in nanoscale wasn't much studied. The cocci bacterium used commonly in food is *Leuconostoc mesenteroides*. *Leuconostoc mesenteroides* is spherical, but often lenticular coccoid cells in pairs and chains. *Leuconostoc mesenteroides* size is about 0.5-0.7 micrometers by 0.7-1.2 micrometers.

Based on these benefit properties of *Leuconostoc mesenteroides*, the main aim of this study was to open the unknown differentiation of *Leuconostoc mesenteroides* in order to develop a new nanoparticle.

EXPERIMENTAL SECTION

Bacterial strains and media

Leuconostoc mesenteroides VTCC-B-871 obtained from Vietnam Type Collection Culture (Academic Institute, Hanoi, Vietnam). *Leuconostoc mesenteroides* was grown in *Lactobacilli* MRS broth [13].

Design conditions for differentiation in *Leuconostoc*

With the aim of studying on the impact of various carbon sources on the minicell formation, this study implemented the experiments on difference kinds of sugar with different concentration in the bacterial culture medium. The bacterium was inoculated into the modified *Lactobacilli* MRS broth which containing each kind of sugar separately (glucose, sucrose, maltose), in altered concentration (0%, 5%, 10%, 20%, 30%) for 48hrs in order to cause differentiation.

Subsequently, the modified *Lactobacilli* MRS media that provide the high yield of minicells.

Minicell isolation

The cultured bacteria with minicells were subjected to the minicell isolation for removal of the parent bacterial cells and cellular debris. Firstly, to separate partially the large parent cells, the culture was centrifuged at 3500 g for 20 minutes. Then, the supernatant was collected to undergo further the first and second filtration through the 0.45 µm filter membrane to separate completely minicells. Finally, the filtered supernatant was centrifuged at 14000 rpm for 20 minutes in order to collect the minicells.

Microscopic studies for morphological characterization of minicells

The isolated minicells were observed by microscopic for the morphological alterations and counted using cell counting under a light microscopy with a total magnification of 100X using a Neubauer hemocytometer. The minicell amount was obtained by counting in five small squares (the four 1/25 sq. mm corners plus the middle square) in the central area into focus at low power.

The isolated minicells were examined at scanning electron microscopy laboratory, Vietnam Academy of Science and Technology, 01 Mac Dinh Chi Street, District 1, Ho Chi Minh City to observe the morphology and size of minicells by using the Scanning Electron Microscope (SEM, S-4800, Hitachi Japan) at 10 kV.

Microbial inhibition assay

The agar diffusion method was applied in order to test the antimicrobial effects of minicells (6.10^5) on *Pseudomonas aeruginosa (P. aeruginosa)* ATCC 27853. These samples were dropped on the sterilized paper discs (5 mm in diameter) placed on the petri plates. The plates were incubated at suitable temperature for 18- 24 hrs. Subsequently, the inhibition zone diameter was measured. Phosphate buffer saline (PBS) solution was used as references. The potency of minicell in antimicrobial activity equaling to $AgNO_3$ was calculated following the formulation:

Data Analysis

The results of triple replicates for all experiments were expressed as mean± standard deviation and then analyzed using one-way ANOVA and post-hoc Tukey's test for paired comparisons of means. The statistically significant differences were considered with $p < 0.05$.

RESULTS AND DISCUSSION

Morphological differentiation

Leuconostoc has a normal size less than 1 μm (Figure 1), however, this bacterium is spherical, oval or sometimes rod in chain. Therefore, to apply this bacterium as an ideal nanoparticle, the separately homogenized round shape should be required. The study tried to find out the effect of sugar on the cell division. *Leuconostoc* was let to grow in different carbon sources (glucose, sucrose and maltose) with the optimal concentrations (0%, 5%, 10%, 20%, and 30%). The morphological differentiation of *Leuconostoc* was check under light microscope (Figure 1).

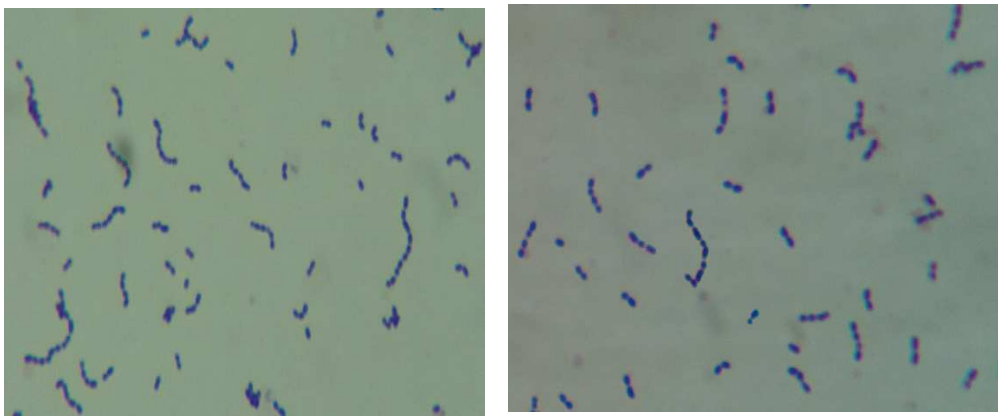

Figure 1: Photomirograph of *Leuconostoc* and its minicells (100X): (A) The morphology of *Leuconostoc* in basic MRS culture medium; (B) The formation of minicells (arrow) in the modified MRS medium with glucose 20% w/v

The differentiation occurred in the modified media with sugars because the sugar affected on the cell division inhibition proteins such as FtsZ, a GTPase which well interacted with glucose; or MinD, an ATPase improved the cell division inhibition process when supplied with sugars [14-16]. Also, to understand well the reasons of differentiation, more studies should be done in future.

Minicell production evaluation by *Leuconostoc* in different sugars

The abnormal small cells of the bacterium were called minicells (Alder *et al*., 1966). The minicells produced in different sugar containing media were collected, concentrated by filtration, centrifugation. The minicells were checked the size by using SEM (Figure 2). The SEM images of minicells with their diameter ranged 400 nm. As a consequence, minicells were generated successfully as nano-size cells. The nanoparticles were utilized with their size up to 1000 nm (Shim and Turos, 2007). Therefore, these minicells were considered continuously in pharmaceutical science. Besides, the isolated minicells were demonstrated the absence of bacterial colonies by inoculating on *Lactobacilli* MRS agar discs and incubating at 37^{o}C for 18- 24hrs. After checking the size of minicells, minicells collected from different sugar conditions were quantitated (Table 1) and analyzed.

Table 1: The proportions of produced minicells by *Leuconostoc* in modified MRS medium containing each kind of carbon sources (selected sugars) in different final concentrations

Sugars	The percentage of minicell production (%)				
	The sugar concentrations in the culture medium				
	0%	5%	10%	20%	30%
Glucose	0.7 ± 0.2	2.2 ± 0.2	4.1 ± 0.3	4.6 ± 0.3	3.4 ± 0.3
Sucrose	0.7 ± 0.1	1.5 ± 0.3	2.6 ± 0.2	3.4 ± 0.2	1.9 ± 0.3
Maltose	0.7 ± 0.1	1.4 ± 0.2	2.3 ± 0.2	2.9 ± 0.1	1.5 ± 0.1

The analyzed data was means ±SD

The minicells were collected, concentrated by filtration, centrifugation and then calculated. The analyzed data which was shown in Table 1 identified that carbon sources affected significantly on the minicell generation and the minicells were produced as a consequence of the sugar concentration ($p<0.05$). Table 1 showed clearly that minicell production changed very low, closed to zero (0.7%) when *Leuconostoc* was cultured in medium without sugar. At all levels of sugar concentrations (from 5% to 30%) that were presented data, the maximum number of minicells was obtained when using glucose as a carbon source. It was followed by the amount of minicells which were produced in glucose MRS medium. The lowest percentage levels of minicell generation were similar in sucrose and maltose MRS medium by 1.9 and 1.5 percent.

As a result from Table 1, the amount of obtained minicells was increased considerably when *Leuconostoc* was cultured in the modified Lactobacilli MRS media for all tested sugars with final sugar concentrations from 5% to 20%. The obtained minicells were the highest amount for each of kind of sugar at level of factor treatment of 20% sugar concentration. In the modified Lactobacilli MRS medium including glucose, sucrose and maltose, the percentage of generated minicells was 4.6 ± 0.3%, 3.4 ± 0.2% and 2.9 ± 0.1%, respectively. The quantity of produced minicells decreased at very high sugar concentration (30%) by falling to 3.4 ± 0.3%, 1.9 ± 0.3% and 1.5 ± 0.1% respectively. Hence, this glucose medium (20% glucose w/v) was utilized as appropriate condition in order to produce minicell.

Minicell production in temperature conditions

In different temperature conditions (room temperature, 37^{o}C, 40^{o}C, and 45^{o}C) with the selected sugar (glucose with 20% in medium) for minicell generation, minicell proportion was showed in table 2. The minicells were isolated, and concentrated by filtration, centrifugation and then calculated. The analyzed data which was presented in table 2 identified that the temperature effected significantly on the minicell generation ($p<0.05$). Table 2 shown evidently that minicell production was increased dramatically from 3.28% at room temperature to 12.78% at 40^{o}C and then dropped slightly to 12.02% at 45^{o}C when the modified Lactobacilli MRS medium (20% glucose). At all levels of temperature conditions which were presented data, the maximum amount of minicells was obtained when incubating the culture at 40^{o}C by 12.78 ± 2.13%. It was followed by the percentage of minicells which were produced in 45^{o}C (12.02±1.67%). The lowest percentage levels of minicell generation were 3.28 ± 0.84% for inoculating in the room temperature condition.

The collected data were analyzed by the utilization of Post Hoc tests (SPSS 20, SPSS Inc., Chicago, USA) for multiple comparisons of means between tested conditions. The good condition of temperature (40^{o}C) which provided the highest proportion of minicell was significant different with the 37^{o}C and 45^{o}C ($p<0.05$). However, the percentage of produced minicells at the 40^{o}C condition was different insignificantly in comparisons with room temperature condition ($p>0.05$). In conclusion, 40^{o}C could be used to produce minicells with high purity in the medium containing 20% glucose.

Table 2: The proportions of produced minicells by *Leuconostoc* in modified MRS medium in different temperature

Temperature	25^{o}C	37^{o}C	40^{o}C	45^{o}C
Percentage of minicells	3.28 ± 0.84	7.33 ± 0.80	12.78 ± 2.13	12.02 ± 1.67

Antimicrobial activity of minicells

To test the ability of *Leuconostoc* minicells, they were used to test for antimicrobial activity. By using agar diffusion test, minicells ($6x10^5$) gave the inhibition zone diameter by 14.3±1.2 mm, equaled to about 0.85 µg $AgNO_3$ corresponding to the inhibition zone diameter of 24.5±0.5mm. Consequently, minicells could be used as nanoparticles for antimicrobial activities without toxicity as silver nitrate.

CONCLUSION

This is the study on minicell production by *Leuconostoc*. The study optimized four kinds of sugars (glucose, sucrose, and maltose) in different concentration (0%, 5%, 10%, 20%, and 30%). The results suggested the method to produce minicells (400 nm) that could be a source of nanoparticle in the inhibition on *Pseudomonas aeruginosa*. Therefore, these minicells could be also used in drug delivery applied in pharmaceutical field.

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