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Study on Minicell Generation of *Lactobacillus acidophilus* VTCC-B-871 for Drug Delivery

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ABSTRACT

The practice of developing molecularly targeted drugs to achieve a higher degree of cancer therapy and antibiotic resistance is indispensable. Recently, bacterially derived 400nm sized minicells was reported on the ability of encapsulation with chemotherapeutic drugs. *Lactobacillus acidophilus* strain participated in the anti-cancer effects and performed the high-level specificity for cancer cell lines. Here we discuss minicells generation of *Lactobacillus acidophilus* VTCC-B-871 for drug delivery. The present work was the first report on that *L. acidophilus* VTCC-B-871 formed minicells with highly significant ratio (25 %) in modified MRS broth with fructose 10 g/l carbon source concentration. The minicells were packaged with Paclitaxel (10 µg/ml) and Cephalosporin (10 µg/ml) at different times of incubation (10, 15, 24 hours) at 37°C. As a result, minicells could completely absorbed in 10 hours by detecting the extraction after drug packaged minicells on antimicrobial activities on *S. aureus* ATCC 25923, *E. coli* ATCC 9637, *Salmonella typhi* ATCC 19430, *C. albicans* ATCC 14053, *P . aeruginosa* ATCC 27853.

INTRODUCTION

The effective cancer therapy continues to be a daunting challenge due mainly to considerable tumor cell heterogeneity, drug resistance of cancer cells, dose-limiting toxicity of chemotherapeutics, and difficulties of targeted delivery to tumors (MacDiarmid *et al.*, 2011). Consequently over the past decade a significant global effort has focused on the discovery and development of molecularly targeted drug delivery systems (DDSs) (MacDiarmid *et al.*, 2009). Molecularly targeted drugs such as cell cycle inhibitors are being developed to achieve a higher degree of tumor cell specificity and reduce toxic side effects. A potential strategy to limit toxicity is to encapsulate the drug and target it directly to cancer cells. However, current strategies that used liposomes (Medina *et al.*, 2004), nanoparticles (Brannon *et al.*, 2001; Ferrari *et al.*, 2005) or polymer therapeutics (Duncan, 2003) are hampered by shortcomings such as drug leakage lack of versatility in terms of packaging a diverse range of different drugs, thereby reducing drug potency, and difficulties in production scale-up, particularly for nanoparticles (MacDiarmid *et al.*, 2007a). Recently, a promising new technology for targeted and intracellular delivery of chemotherapeutic drugs relies on using bacterially derived nano sized (100-400nm in diameter) particles (termed as minicells) to package a range of different chemotherapeutic drugs. This technique has been experimented successfully for both Gram-positive (*Listeria monocytogenes* strain) and Gram-negative bacteria *Salmonella typhi (S. typhimurium), Escherichia coli (E. coli), Shigella flexneri, Pseudomonas aeruginosa (P. aeruginosa)*; drug-packaged nano-sized particles effect apoptosis of tumor cells both *in vitro* and *in vivo*; and they also targeted to cancer cells *in vivo* with high specificity and, thus, delivered in high concentration *in vivo* without toxicity (MacDiarmid *et al.*, 2007b).

Besides, antibiotic resistance is increasing due to the target for treatment. A large number of lactic acid bacteria (LAB) have been proposed to be used commonly as probiotic strains, live microorganisms, in order to apply in foods, pharmaceuticals and animal husbandry. The benefits of probiotics were found protection against gastrointestinal pathogens, enhancement of the immune system, reduction of lactose intolerance, reduction of serum cholesterol level and blood pressure, anti-carcinogenic activity, improved utilization of nutrients and improved nutritional value of food (Philippe, 2001).

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LAB are also usually used to study the antibacterial and anti-cancer discovery besides being effect on human immune system (Choi *et al.,* 2006). Among the tested LAB strains, *Lactobacillus acidophilus (L. acidophilus)* strain participated in the anti-cancer effects and performed the high-level specificity for cancer cell lines (Choi *et al.,* 2006). Thanks to their benefits and the biocells application (MacDiarmid *et al.,* 2009) in drug delivery, it is necessary to develop new molecularly targeted drug delivery systems from *L. acidophilus*. Recently, there were studies on detection of minC (Nguyen *et al.,* 2012) and minD from *L. acidophilus* for minicell study (Nguyen *et al.,* 2013a; Nguyen *et al.,* 2013b). Therefore, this paper presents the study on minicells generation of *Lactobacillus acidophilus* VTCC-B-871 for drug delivery and the investigation if *L. acidophilus* strain can package with chemotherapeutic drugs; in order to detectable a robust and versatile system for *in vitro* drug delivery using minicells, a bacterially-derived lactic acid bacteria carrier.

MATERIAL AND METHODS

Bacterial strains and growths conditions

A strain *Lactobacillus acidophilus* VTCC-B-871 obtained from stock cultures maintained by the Vietnamese Type Culture Collection (Hanoi, Vietnam), was used in all experiments of this study. *L. acidophilus* VTCC-B-871 was grown in Lactobacilli MRS broth (Difco, Detroit, Mich., U.S.A) at 37°C in aerobic condition.

The preparation of inoculums started with transferring the stock cultures of *L. acidophilus* VTCC-B-871 into liquid MRS medium and incubated overnight at 37°C in aerobic condition. After the growth of culture, the microorganisms were transferred to the plate of solid MRS medium. The plate incubated at 37°C for 48h in order to allow sufficient growth of colonies. All plates were then stored at 4°C. Prior to each experiment, the *Lactobacillus* inoculums were individually prepared by inoculating a single colony of them into 10mL broth media which was then incubated overnight at 37 °C.

Design conditions for minicell production in *Lactobacillus* strains

According to biochemical reactions of *Lactobacillus* strains with sugar and to study the effects of culturing, *L. acidophilus* VTCC-B-871 were inoculated into modified *Lactobacilli* MRS broth which containing each of carbon sources separately: glucose, lactose, sucrose, maltose, fructose, in different concentration (0 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) and incubated at 37 ºC for 30 h in order to produce minicells. Microscopic cell morphologies were observed under a light microscope with a total magnification of 100X.

Minicell isolation

Optimized culture of minicell-producing bacteria was subjected of the minicell isolation techniques described in various combinations to obtain a preparation of desired isolation to homogeneity and free of contaminating parent bacterial cells, cellular debris. Initial differential centrifugation of minicell producing bacterial cell culture was performed at 2000 g for 20 minutes, removes most parent bacterial cells. This step was thus incorporated after the initial 0.45 µm cross-flow filtration steps and the resulting filtrate was subjected to 0.2 µm cross-flow filtration to remove small contaminants like bacterial membrane debris. As a result, the final minicell preparation was sterile as confirmed by plating the entire preparation on growth agar plates and incubating it overnight at 37ºC to demonstrate the absence of bacterial colonies.

Packaging of chemotherapeutic drugs into minicells

To determine if minicells can be packaged with chemotherapeutic drugs and the kinetics of minicell in response to a concentration of drug in varying times of incubation. Preparations of purified minicells derived from *L. acidophilus* VTCC-B-871 will be separately incubated with the chemotherapeutic drug, paclitaxel (Sigma, U.S.A) (10 µg/ml) and cephalosporin (Sigma, U.S.A) at different times of incubation (10, 15, 24 hours) at 37°C with rotation. The minicells were harvested by centrifugation at 13000 rpm for 5 min and resuspended in sterile buffered saline gelatin (BSG). Minicells were then washed 10 times with 10 ml of BSG per wash solution.

Drugs were extracted from packaged minicells to prepare for antimicrobial activity. The extract was centrifuged for 5 minutes at 13000 rpm to pellet debris and the clear supernatant was sterilized by filtration (0.45 µm), thus yielding cell-free filtrates.

Antimicrobial activity tests

Antimicrobial effects were tested on *S. aureus* ATCC 25923, *E. coli* ATCC 9637, *Salmonella typhi*ATCC 19430, *C. albicans* ATCC 14053, *P . aeruginosa* ATCC 27853 by the agar diffusion method. The tested microorganisms were propagated twice and then grown for 18 to 24 h in 10 ml of appropriate growth media. Turbidity of the culture broth was compared with McFarland tubes to give an estimate of bacterial population (1×10^6 CFU/ml). Steriled paper discs (5 mm of diameter) were then prepared and dropped on using 20 µl of cell-free filtrate. The inoculated plates were incubated for 18 to 24 h at appropriate temperatures, and the diameter of the inhibition zone was measured in millimeters with calipers. The measurements recorded were from the edge of the zone to the edge of the wall.

RESULTS & DISCUSSION

Generation of minicells in *Lactobacillus* strains

Based on the biochemical tests on sugar of *Lactobacillus acidophilus*, the different sugars were used to study the effect on morphological changes in *Lactobacillus acidophilus*. The result was shown in Table 1 and Figure 1. As presented in Table 1, the ratios of minicells in the total cells were lowest (about 2.5 %) without tested sugar condition. The minicells were increasing from

5 g/L to 10 g/L for all tested sugars and decreasing after this conditions. However, the formed minicells formed by fructose at the concentration of 10 g/L were highest (Table 1).

Table. 1: Percentage of Minicells produced from *Lactobacillus acidophilus* VTCC-B-871 in total *L. acidophilus* bacterial cells.

	0 g/l	5 g/l	10 g/l	15 g/l	20 g/l	30 g/l	40 g/l	50 g/l
Glucose	2.3	15	18	10	14.4	11.2	10	9
Lactose	2.4	16	20	11	15.3	12.9	11.7	10
Fructose	2.6	20	***25***	13.8	18	15	14.6	13.8
Maltose	2.4	18	23.3	12.1	16.2	13.9	13	12
Saccharose	2.5	13	15	8	6.2	5.9	5	4.9

Data are expressed as percentage. Data were the mean ± SD from experiments. Statistical analysis was performed using one-way ANOVA and the Turkey-Kramer *post- hoc* test.

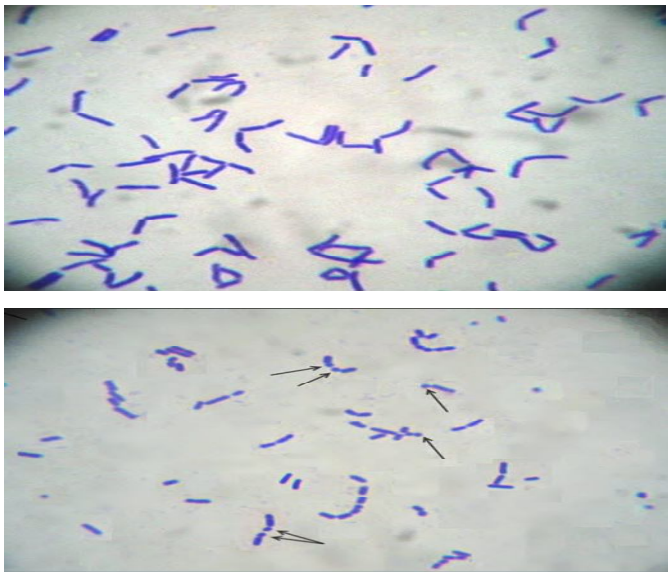

Fig. 1: Photomicrograph of *Lactobacillus acidophilus* (upper) and its minicells (lower). The arrows showed the formation of minicells.

Fructose could affect on the sugar tolerance of *L. acidophilus*. Saccharose effect on the minicell phenotype of the bacteria was the least. In fact, during *minD* expression, saccharose led the filamentation in cells (Nguyen *et al.*, 2013a). Glucose and lactose, even maltose also caused the minicells (18-23.3%), but not higher than fructose (25%). It might be the reason that why the normal medium for lactic acid bacteria did not contain fructose. This result revealed that carbon source actually affected on the minicell production and cell division of *L. acidophilus* VTCC-B-871 strain. The Figure 1B showed the minicell generation fructose condition. The morphology changed clearly while the wild type of *L. acidophilus* was absolutely rod (Figure 1A).

Minicell isolation for drug pakaging

Minicells was achieved by using a procedure to homogenize and eliminate contaminants such as parent bacterial cells, cellular debris (Figure 2). By filtering through the filter (0.2 μm), the generated minicells had the size less than 200 nm. Therefore, this study could produce minicells that were used as nano-cells. The study tried to test the storage in sterile buffered saline gelatin (BSG) and check whether the shape recovered into the rod. Remarkably, the minicells still keep their own shape. The final minicell preparation was suggested to demonstrate the absence of bacterial colonies by plating the entire preparation on MRS agar plates and incubating it overnight at 37ºC. The result meant that the buffered saline gelatin could be used as the best medium for storage the minicell for drug delivery.

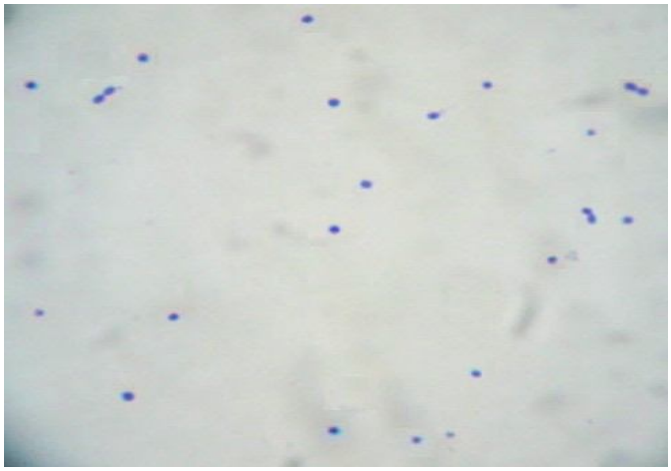

Fig. 2: Photomicrograph of *Lactobacillus acidophilus* minicells after isolation.

Antimicrobial activity tests

To test the ability and potency of drug deliver of our minicells, the minicells were packaged with two different drugs incubated with different time. The extracted solution from drug-packaged minicells was centrifuged and used to test the activities on the above mentioned pathogens. The results showed the activities on both of gram negative and positive bacteria (Table 2, Figure 3). Presented in Figure 4 was the inhibition of the growth of *S. aureus* ATCC 25923, *E. coli* ATCC 9637, *S. typhi* ATCC 19430, *C. albicans* ATCC 14053, *P. aeruginosa* ATCC 27853 by the action of the supernatants. The inhibition zone diameters were 17 to 19 mm for cephalosporin and paclitaxel, respectively and showed that there were significantly different packaging at different incubation time (10, 15, 24 hours) at 37ºC with rotation ($P > 0.05$). The zone inhibition showed the highest activity at 10 hour incubation with the tested drugs. Therefore, the generated minicells could package with many drugs. To test how the mincells could package with the drugs, more studies should be done so far.

Table. 2: Antimicrobial spectrum of cell free supernatant extracted from drug-packaged minicells of *Lactobacillus acidophilus* VTCC-B-871 against Gram-positive and Gram-negative bacteria.

Test organism	Inhibition [1]
Gram-positive	
Staphylococcus aureus ATCC 25923	(+)
Gram-negative	
Escherichia coli ATCC 9637	(+)
Salmonella typhi ATCC 19430	(+)
Pseudomonas aeruginosa ATCC 27853	(+)
Yeast	
Candida albicans ATCC 14053	(+)

[1](+) Inhibited by cell-free supernatant, (-) not inhibited.

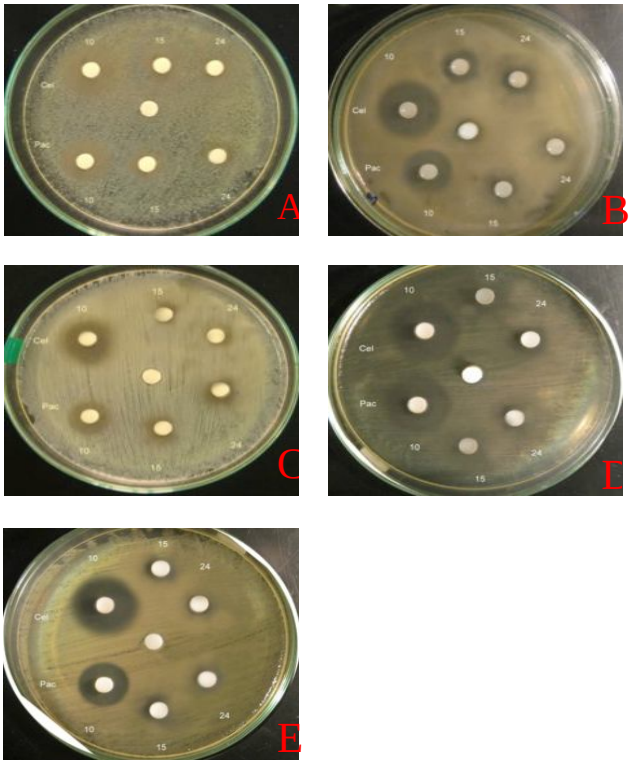

Fig. 3: Antimicrobial activity tests on bacteria to check the drug packaging ability. The activities are showing in (A) *S. aureus* ATCC 25923, (B) *E. coli* ATCC 9637, (C) *Salmonella typhi* ATCC 19430, (D) *P . aeruginosa* ATCC 27853, (E) *C. albicans* ATCC 14053.

CONCLUSION

The study was the first report that *L. acidophilus* in the modified MRS broth with carbon source fructose at 10 g/L gave the minicell quantity in significant number. The study also detected the ability of drug deliver of minicells by testing the antimicrobial activities. This result revealed that carbon source actually affected on the minicell production or cell division of *L. acidophilus* VTCC-B-871 strain. Frequently, the following minicell production is due to the *min* deletion. The practice of modifying carbon source to produce minicell has the potential role in drug delivery. This really helped significantly to the combination of probiotics which contain useful bacteria (including *L. acidophilus* strain), with different kinds of antibiotics and some chemotherapeutic drugs. From those points, the drug absorption by human body may also be better. More studies should be done so far for the mechanisms.

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