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Effect of ultrasound on bioconversion of isoflavones and probiotic properties of parent organisms and subsequent passages of *Lactobacillus*

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A B S T R A C T

This study evaluated the effects of ultrasound (60 W; 3 min) on growth, bioconversion of isoflavones, and probiotic properties of parent organisms and subsequent passages of *Lactobacillus casei* FTDC 2113. Ultrasound significantly enhanced ($P < 0.05$) the cell growth of the parent organisms in mannitol–soymilk fermented at 37 °C over 24 h. Such treatment also enhanced the intracellular and extracellular β-glucosidase activity, leading to increased bioconversion of isoflavones in mannitol–soymilk ($P < 0.05$). In addition, treated cells also exhibited better tolerance toward acidic conditions (pH 2 and pH 3) and intestinal bile salts compared to the control ($P < 0.05$). Ultrasound also promoted cell adhesion ability and antimicrobial activity ($P < 0.05$). All these positive effects were only prevalent in the parent cells without inheritance by first, second and third passage of cells. Our results suggest that ultrasound could enhance the bioactive and probiotic potentials of parent cells of *L. casei* FTDC 2113, and could be used in the production of probiotic foods with enhanced bioactivity.

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1. Introduction

Lactobacilli are known as the most common type of bacteria exhibiting probiotic properties. Probiotics are defined as ‘live microorganisms that when administered in adequate amounts confer health benefits on the host’ (FAO, 2001). These bacteria are claimed to impart health-beneficial effects such as stress alleviation of immune system, modulation of gastrointestinal microbial balance, and prevention of gastrointestinal infection (Liong, 2007). Due to their potential health benefits, there are growing interests in the production of probiotic foods.

Soybeans are well known as an inexpensive protein and carbohydrate source for human consumption. In addition, soy has been reported to exert several health-beneficial effects such as prevention of postmenopausal symptoms, cardiovascular disease, bone health problems, and cancers, which was attributed to the presence of isoflavones (Setchell & Cassidy, 1999). Izumi et al. (2000) have demonstrated that isoflavones aglycones are absorbed faster and in higher amounts by humans than their glucosides. However, approximately 80%–95% of isoflavones in unfermented soy products exist as glucosides which are less bioactive and non-bioavailable. We have previously demonstrated that β-glucosidase-producing lactobacilli and bifidobacteria efficiently biotransform isoflavone glucosides to bioactive aglycones (Yeo & Liong, 2010). In addition, we have also reported that supplementation of prebiotics, especially mannitol, further enhanced the bioconversion of isoflavones in soymilk (Yeo & Liong, 2010). Mannitol has been classified as a prebiotic due to its indigestible properties (Liong & Shah, 2006). These sugar alcohols escape undigested due to their additional hydroxyl group compared to other sugars which makes them less likely to be absorbed by human (Grabitske & Slavin, 2008).

Ultrasound is a type of physical treatment which involves energy generated by high-frequency sound. This physical treatment has been successfully applied on living cells to produce positive effects on growth, enzyme activity of cells, and delivery of therapeutically beneficial macromolecules into cells (Yang, Zhang, & Wang, 2010). Such beneficial effect was predominantly attributed to the reversible pore formations on cell membranes upon treatment. These temporary pores provide a channel for the transport of macromolecules such as proteins, enzymes, DNA, and drugs across the membrane (Yang et al., 2008). Our previous study has demonstrated that ultrasound, especially at 60 W for 3 min, significantly enhanced membrane permeability, which subsequently promoted the growth of lactobacilli and bifidobacteria and bioconversion of isoflavones in mannitol–soymilk (Yeo & Liong, 2011). Past study has demonstrated that bacterial cells expose to stressful condition may possibly exhibit heritable changes and thus the altered characteristics would also be prevalent in subsequent

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passages of cells (Cooley, Carychao, Nguyen, Whitehand, & Mandrell, 2010). However, the inheritance of beneficial effect by subsequent passages of ultrasound-treated cells remains unclear.

Considering the beneficial effect of this physical treatment on lactobacilli and bifidobacteria (Yeo & Liong, 2011), it is also essential that such treatment does not suppress the functional characteristics of these bacteria as a potential probiotic. However, up to date, no attempt has been made to evaluate the effect of ultrasound on their probiotic potentials. In addition, there is also no information available on the probiotic potentials of subsequent passages of treated cell populations.

The objectives of this study were to determine the effect of ultrasound on growth, β-glucosidase activity and bioconversion of isoflavones in soymilk fermented by parent organisms and three subsequent passages of *Lactobacillus casei* FTDC 2113 (previously known as *Lactobacillus* sp. FTDC 2113; Yeo & Liong, 2011). *L. casei* FTDC 2113 was evaluated as we previously demonstrated that this strain showed more prominent increment for bioconversion of isoflavones upon ultrasound compared to other strains (Yeo & Liong, 2011). In addition, the effect of ultrasound on probiotic potentials of parent and subsequent passages of cells were also evaluated. Thus, this study would serve as a theoretical groundwork for practical application of ultrasound on *Lactobacillus* cells.

2. Materials and methods

2.1. Bacterial cultures

Cultures of *L. casei* FTDC 2113 and several pathogens, including *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella typhimurium* and *Bacillus subtilis* were obtained from the Culture Collection Centre of the School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia. Stock cultures of *L. casei* FTDC 2113 and the pathogens were stored in sterile 40 mL/100 mL glycerol at −20 °C. *L. casei* FTDC 2113 cells were propagated in rehydrated de Man Rogosa Sharpe (MRS) broth (Hi media, Mumbai, India) supplemented with 0.15 g/100 mL L-cysteine·HCl (Hi media, Mumbai, India) for three successive times at 37 °C for 24 h prior to use. Strains of pathogens were activated in tryptone soya broth (Hi media, Mumbai, India) at 37 °C for 24 h prior to use.

2.2. Preparation of soymilk

The soymilk was prepared as described previously (Yeo & Liong, 2011). Briefly, whole soybeans were blended and pasteurized at 63 °C for 30 min. Upon cooling to 40 °C, soymilk was then supplemented with 0.15 g/100 mL filter-sterilized L-cysteine·HCl and 1 g/100 mL mannitol (Mannogem, SPI Polyols Inc., New Castle, DE, USA).

2.3. Ultrasound and subsequent passages of cells

The activated culture of *L. casei* FTDC 2113 was washed twice with phosphate buffer (50 mmol/L, pH 6.5) and resuspended into the same buffer. The cell suspension was sonicated at a frequency of 30 kHz (LABSONIC®M, Sartorius Stedim Biotech, Goettingen, Germany). Past studies have demonstrated that ultrasound treatment at 10–40 kHz does not severely compromise the viability of bacteria. Based on these information, we have conducted a preliminary study and found that growth of lactobacilli and bifidobacteria was better upon treatment at 30 kHz compared to other frequencies (10, 20 and 40 kHz; Data not shown). Thus, 30 kHz was selected in our current study. The intensities of treatment were adjusted to 60 W with continuous ultrasound action for 3 min. In all reactions, the tip of the sonotrode (titanium probe 3 mm × 80 mm) was located at 1 cm depth from the surface of the cell suspension (10 mL) in a sample tube (25 mm × 60 mm). All sample tubes were placed in a water bath maintained at 25 °C during sonication.

These treated cells were used as the parent cells. The parent cells were propagated in soymilk (10 mL/100 mL) for 3 successive times (37 °C for 24 h) to produce cell suspension for the first, second and third passage of treated cells. Untreated cell suspension was used as the control. Parent and subsequent passages of control and treated cells (5 mL/100 mL) were added into mannitol–soymilk and fermented at 37 °C over 24 h. Samples were collected every 4 h for evaluation of the following analyses.

2.4. Cell growth

The growth of *Lactobacillus* was determined via pour plate method using MRS agar supplemented with 0.15 g/100 mL L-cysteine·HCl. Plates were incubated anaerobically at 37 °C for 24 h in an anaerobic jar with a gas-generating kit (Merck, Darmstadt, Germany).

2.5. Determination of intracellular and extracellular β-glucosidase activities

Crude enzyme extract for intracellular β-glucosidase activity was prepared and determined as previously described (Yeo & Liong, 2011). Cells were harvested upon fermentation and lysed using sterile glass beads and sonication. β-Glucosidase activity was determined based on the rate of hydrolysis of ρ-nitrophenyl-D-glucopyranoside (ρNPG) which was detected spectrophotometrically at 420 nm. One unit of enzyme activity is defined as the amount of β-glucosidase activity that releases 1 μmol of ρ-nitrophenol from the substrate ρNPG per mL per min under the assay conditions. The protein concentration was quantified using the methods of Bradford (1976). Specific activity was expressed as unit (U) of β-glucosidase activity per mg of protein.

Extracellular β-glucosidase activity of the control and treated cells was determined as previously described (Yeo & Liong, 2011). Briefly, fermented mannitol–soymilk was centrifuged at 14,000× g for 30 min at 4 °C and the supernatant was analyzed for β-glucosidase activity. β-Glucosidase activity was determined as mentioned above.

2.6. Determination of isoflavones

Extractions and determination of isoflavones were performed as previously described (Yeo & Liong, 2011). Concentrations of isoflavones were determined using high-performance liquid chromatography (HPLC) equipped with a UV/Vis detector (Jasco 875-UV, Tokyo, Japan) set at 259 nm. An Inertsil ODS-3 column (150 × 3 mm, 5 μm, GL Sciences, Tokyo, Japan) was maintained at 40 °C. The degassed mobile phase consisted of solvent A (water:phosphoric acid, 1000:1, v/v) and solvent B (water:acetonitrile:phosphoric acid, 200:800:1, v/v/v) used at a flow rate of 1 mL/min. The gradient was as follows: solvent A 100% (2 min) → 65% (29 min) → 50% (31 min) → 100% (45 min) → 100% (50 min). HPLC-grade glucosides and aglycones were used as standards.

2.7. Acid tolerance

Acid tolerance was determined according to Teh, Ahmad, Wan-Abdullah, and Liong (2009). Briefly, control and treated cells from each passage (10 mL/100 mL) were incubated in MRS broths supplemented with 0.3 g/10 mL pepsin (Sigma–Aldrich, St. Louis, MO,

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