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VISUAL EXPERIMENT

Extraction, isolation and purification of exopolysaccharide from lactic acid bacteria using ethanol precipitation method

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ABSTRACT

Lactic acid bacteria are classified 'Generally Recognized As Safe' (GRAS) with most effective potential to divert significant amount of fermentable sugars towards the biosynthesis of functional exopolysaccharide. Exopolysaccharides from lactic acid bacteria are receiving a renewed interest due to the claims of human health benefits, such as modulation of immune response system and more importantly in food and pharma industries as a texturizer, viscosifer, emulsifier and syneresis-lowering agent. Its purification methodology involves: a) Extraction of cell-free supernatant from lactic acid bacteria; b) Denature of protein using trichloroacetic acid; c) Ethanol precipitation; d) Dialysis; and e) Freeze drying. However, depending on nature of research, compounds can be further purified using scanning electron microscopy (SEM), infrared spectrum (IR); and nuclear magnetic resonance (NMR) spectral analyses.

INTRODUCTION

Synthesis of exopolysaccharides by lactic acid bacteria is well known phenomenon which exists as a cell-bound exopolysaccharides, adhering closely to the bacterial surface, and released exopolysaccharide that releases into the surrounding medium (Wang et al., 2011; Tallon et al., 2003). The exopolysaccharides are associated with microbial cells protection against the adverse environments including desiccation, toxic materials and osmotic stress (Wang et al., 2011). The exopolysaccharides are thought to play a significant role in the colonization of lactic acid bacteria to various ecosystems by facilitating the colonization of bacteria to intestinal mucosa, thus enhance the immunity of host (Gorska et al., 2010). Now-a-days the exopolysaccharides are used as bio-thickeners due to their stabilizing, emulsifying or gelling properties especially in the food industry (Wang et al., 2011). In addition, some of the exopolysaccharides produced by lactic acid bacteria may confer health benefits such as immunomodulatory, anti-tumor, anti-biofilm and antioxidant activities (Ramchandran and Shah, 2009; Gorska et al., 2010). Therefore, the objective of this study was to provide visual demonstration of step-by-step extraction, isolation and purification of functional exopolysaccharide from lactic acid bacteria. However, the production cost of these antimicrobials is expensive due to optimized media composition and laborious purification process in addition to low yield in food-grade medium. Therefore, cost-effective medium and purification processes are of great need.

MATERIALS AND EQUIPMENTS

1. Bacteria growth media
2. Centrifuge tubes (depending on volume)
3. Trichloroacetic acid

4. Absolute ethanol
5. Dialysis membrane ((Mw cut-off 12,000–14,000 Da) and tubing closure
6. Falcon™ 50 mL conical centrifuge tubes
7. Aluminum foil
8. Refrigerated centrifuge
9. Deionized water
10. Magnetic stirrer
11. Freeze dryer

PREPARATION OF REAGENTS

Bacteria growth media: Dissolve 56 g of MRS media (for lactic acid bacteria) in 1 liter of distilled water supplemented with 10 g glucose/liter, stirrer until it gets properly dissolved and autoclave it.

Preparation of 80% stock of trichloroacetic acid: Dissolve 80 g trichloroacetic acid in 1 liter of sterile distilled water.

Absolute ethanol: Purchased from Sigma (USA)

VIDEO CLIPS

Part 1: 7 min 42 sec

Part 2: 3 min 36 sec

METHOD

Extraction, isolation and purification of exopolysaccharide

1. Lactic acid bacterium was cultured at 37°C for 18~24 hours in MRS modified medium supplemented with 10% glucose.
2. After centrifugation (8,000 ×*g* for 20 min at 4°C) of culture, the supernatant was collected and added with a final concentration of 14% trichloroacetic acid to denature the protein content.
3. The culture was further left for homogenization in a shaker (90 rpm) for 30-40 min followed by centrifugation at 8,000 ×*g* for 20 min at 4°C.
4. The supernatant was then added to cold absolute ethanol (two-fold volume of supernatant) at 4°C for 24 hours, followed by centrifugation at 8000 ×*g* at 4°C for 20 min
5. These steps resulted in the isolation of crude precipitate.
6. Finally, the precipitate was dissolved in deionized water and dialyzed using Spectra/Por molecularporous tubular dialysis membrane for 24~48 hours.
7. The precipitate was then lyophilized in an IIShin freeze dryer (Korea).
8. The freeze-dried lyophilized powder of lactic acid bacterium was considered to be purified exopolysaccharides.
9. The purified exopolysaccharide was stored at -80°C for further analysis.

PROTOCOL

1. Grow the exopolysaccharide producing bacterium in 1 liter of MRS broth followed by incubation at 37 °C for 18~24 hours.
2. Add 14% final volume concentration of trichloroacetic acid to denature the protein.
3. Incubate the culture broth at 37°C for 30-40 min in a shaking incubator at 90 rpm.
4. Centrifuge the culture at 8,000 ×*g* for 20 min to pellet down the cells.

5. Collect the supernatant in a sterilize container.
6. Mix cold absolute ethanol in ratio of 1:2 (Sample supernatant: absolute ethanol).
7. Incubate the reaction mixture at 4°C for 24~48 hours for precipitation.
8. Discard the supernatant and collect the precipitate (Repeat the process if any leftover).
9. Divide the precipitate into 50 mL falcon tubes equally depending on the volume
10. Centrifuge the precipitate at 8,000 ×*g* for 20 min to remove traces of media component.
11. Mix equal volume of deionized water to each tube containing the precipitate.
12. Pore the precipitate in dialysis membrane bags according to size and volume needed.
13. Perform dialysis of precipitate at 4°C for 24~48 hours to remove traces of protein (change the distilled water of container 1-2 times during dialysis).
14. Collect the precipitate in a 50 mL falcon tube and keep the tube in a refrigerator at -80°C for few hours.
15. Lyophilized the precipitate using a freeze drier which is a purified exopolysaccharide (Figure 1).
16. Depending on nature of work, further analysis such as SEM, IR or NMR analyses can be performed to identify specific exopolysaccharide.

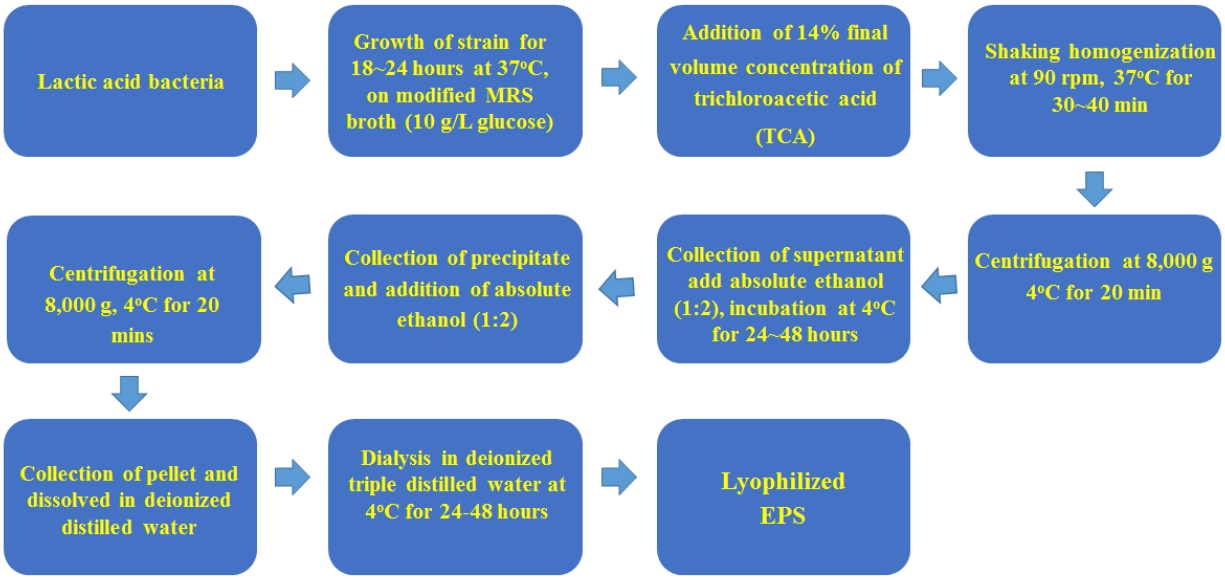

Figure 1: Flow chart of partial purification of functional exopolysaccharide

DISCUSSION

It is well known that *Lactobacilli* are useful microorganisms in dairy technology, along with documented history of use in foods. To date, a number of lactic acid bacteria strains have been screened for their ability to produce exopolysaccharides as well as their health beneficial effects. Moreover, production of exopolysaccharides is dependent on specific growth phase of bacteria and media components. Hence, favorable media composition especially carbon sources (glucose and/or fructose) and bacterial growth (early stationary phase) should be optimized in order to get better exopolysaccharides yield from lactic acid bacteria. Several concerns have been raised that during late stationary phase production of glycohydrolases may catalyze degradation of polysaccharides, resulting in decreased exopolysaccharides yield (Cerning et al., 1994). Degradation of exopolysaccharide production on prolonged incubation has been reported previously for other lactic acid bacteria strains (Degeest et al., 2001). Since regulation of the exopolysaccharides biosynthetic pathway in lactic acid bacteria is dependent on the carbohydrate/carbon sources added to the growth medium (Grobben et al., 1995), supplementation of these sources may result in the variations of exopolysaccharides recovery rate from different lactic acid bacteria strains. One of the

major drawbacks of exopolysaccharides purification is cost effectiveness and prolong-time consumption. However lactic acid bacteria producing exopolysaccharides have received considerable interest over the past couple of years because of their use in food and pharma industries as food preservatives as well as bio-thickener and viscosifer agents.

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PRECAUTION

Before adding sample to dialysis membrane, it is highly recommended to wash the membrane with ultrapure water or buffer for at least 30 min to remove contaminants such as sulfur, heavy metals and glycerol to avoid any possible effects of these components on sample.

In addition, the rate of dialysis also depends on surface area of the membrane as well as the concentration of molecule. Therefore, it is recommended using bigger flasks to carry out dialysis on magnetic stir with regular distilled water and/or suitable buffer exchange.

It is important to repeat sample dialysis process 2-3 times in order to get rid of undesirable protein from exopolysaccharides sample. More removal of protein content from sample during dialysis will provide more purified exopolysaccharides with diverse functional ability.

Also it is advisable to repeat ethanol precipitation process 2-3 times to get release of maximum amount of exopolysaccharides from cell-free bacterial supernatant.

Lyophilisation of exopolysaccharides is crucial stage, hence, precautions are needed while drying it completely so a further desired analysis could be performed in an easy way.

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