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Bacteriocin-producing lactic acid bacteria isolated from mangrove forests in southern Thailand as potential bio-control agents in food: Isolation, screening and optimization

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ABSTRACT

A total of 386 isolates of lactic acid bacteria isolated from mangrove forests (soil, water, leaf, twig and fruit) in southern Thailand were screened for bacteriocin production. Only 4 strains that produced bacteriocin-like inhibitory substance (BLIS) in MRS broth, named KT2W2G, KT2W2L, TS9S17 and TS9S19 showed an inhibition zone against *Lactobacillus sakei* subsp. *sakei* JCM 1157, *Listeria monocytogenes* DMST 17303 and *Brochothrix thermosphacta* DSM 20171 as indicators by using agar well diffusion assay. None of the inhibitory activities were related to the production of either organic acids or hydrogen peroxide. The BLISs produced by these strains were not affected by heating but were sensitive to proteolytic enzymes. The isolate KT2W2L was identified as *Lactococcus lactis* subsp. *lactis* while the isolates KT2W2G, TS9S17 and TS9S19 were identified as *Enterococcus faecalis*. These BLISs showed a wide range of antibacterial activity against similar bacterial strains, food-spoilage and food-borne pathogens, but were inactive against the Gram-negative bacteria tested. Statistical experimental designs, based on the Plackett–Burman protocol, were applied to optimize the bacteriocin production by *Ent faecalis* KT2W2G in flask cultures. By using Plackett–Burman protocol, lactose and temperature were found to be the most important factors for bacteriocin production. The effects of the two main factors on bacteriocin activity were further investigated using a central composite design (CCD) and the optimum composition was found to be lactose 14.85 g/l and temperature 25.59 °C. Optimum conditions were validated by experiment in which bacteriocin activity (Arbitrary Unit/ml) was increased 8-fold (640 AU/ml) in 18 h fermentation.

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1. Introduction

Mangroves are coastal wetland forests mainly found at the inter-tidal zones of estuaries, backwater, deltas, creeks, lagoons, marshes and mudflats of tropical and subtropical latitudes. Mangrove forests are large ecosystems distributed in 112 countries, and territories comprising a total area of about 181,000 km^2 are over a quarter of the total coastline of the world's tropical and subtropical coastlines (Holguin, Vazquez, & Bashan, 2001). Mangrove ecosystem is a bridge between terrestrial and marine ecosystem and harbors unique microbial diversity. Mangroves are present in the coastal areas of tropical countries and support abundant life through a food chain that starts with the trees and the microbiota (Smith, Boto, Frusher, & Giddins, 1991). Root exudates of higher plants serve as a food source for the microorganisms living in this ecosystem. Very little information is available about the microbial diversity, mechanisms and their interactions in the mangrove ecosystem. Mangrove litter decomposition and release of nutrients are important aspects of the function of adjacent coastal ecosystems (Wider & Lang, 1982). By participating in various steps of the decomposition and mineralization of litter fall, sediment microorganisms play crucial roles in the mangrove ecosystem and make an essential contribution to the productivity of the mangrove ecosystem (Alongi, 1996; Holguin et al., 2001).

It has been reported that mangroves are unique inter-tidal ecosystems of the tropics, which support genetically diverse groups of aquatic and terrestrial organisms. This ecosystem is ideally situated at the inter-phase between the terrestrial and marine environment and supports a rich and diverse group of microorganisms such as bacteria, actinomycetes and fungi. These microorganisms perform various activities in the mangrove ecosystem like photosynthesis, nitrogen fixation, methanogenesis,

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agarolysis, production of antibiotics and enzymes (Das, Lyla, & Ajmal Khan, 2006). Analysis of microbial biodiversity from these ecosystems will help in isolating and identifying new and potential microorganisms for various applications (Holguin et al., 2001) such as bacteriocin-like inhibitory substances (BLISs) producing lactic acid bacteria (LAB).

LAB are Gram-positive, non-spore forming cocci, coccobacilli or rods. They generally have anaerobic respiration and lack catalase (Makarova et al., 2006; Medina, Katz, & González, 2004; Pringsulaka et al., 2012; Slover & Danziger, 2008). Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative. During fermentation these bacteria do not produce only lactic acid but they are also known to produce and excrete compounds with antimicrobial activity, including bacteriocins (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013; Dalié, Deschamps, & Richard-Forget, 2010; De Martinis & Freitas, 2003; Jiang et al., 2012; Pal, Jamuna, & Jeevaratnam, 2005)

Bacteriocins are antimicrobial substances that are ribosomally synthesized, releasing bioactive peptides or peptide complexes with bactericidal or bacteriostatic effects (Klaenhammer, 1993; Nes et al., 1996). Bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: (i) are generally recognized as safe (GRAS) substances, (ii) are inactive and nontoxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria, (vi) show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Cleveland, Montville, Nes, & Chikindas, 2001; Gálvez, Abriouel, López, & Ben Omar, 2007; Schillinger, Geisen, & Holzapfel, 1996). BLIS producing LAB have been successfully isolated and screened from many sources such as swine (Mélançon & Grenier, 2003), nasopharynx (Walls, Power, & Tagg, 2003), soil in Japan (Chen & Yanagida, 2006), Mexican traditional sausage (Alvarez-Cisneros et al., 2010), traditional Chinese fermented cabbage (Gao, Jia, Gao, & Tan, 2010), boza—a cereal-based fermented beverage from Bulgaria (Todorov, 2010) and kurut, a traditional naturally-fermented yak milk from Qinghaie Tibet plateau (Luo et al., 2011), but there are no reports of BLIS producing bacteria isolated from mangrove forests.

The objectives of this study were to isolate, screen and optimize bacteriocin-producing LAB from mangrove forest in southern Thailand, which should be used as potential bio-control agents in refrigerated shrimp in further research.

2. Materials and methods

2.1. Sample collection

Samples (soil, water, leaf, twig and fruit) from mangrove forests in southern Thailand, namely Krabi, Nakhon Si Thammarat, Phang-Nga, Satun, Songkhla and Trang (Fig. 1 and Table 1) were collected and used to isolate LAB. Temperature, percent of NaCl and pH of water were measured and used in LAB cultivation conditions.

2.2. Indicator bacterial strains

The indicator organisms (*Enterococcus faecalis* LPS04, *Lactobacillus plantarum* D6SM3, *Lactococcus sakei* subsp. *sakei* JCM 1157, *Leuconostoc mesenteroides*, *Streptococcus salivarius* LD219, *Bacillus cereus* DMST 5040, *Brochothrix thermosphacta* DSM 20171, *Escherichia coli* DMST 4212, *E. coli* TISTR 780, *Listeria monocytogenes* (hospital strain), *L. monocytogenes* DMST 17303, *Salmonella* spp., *Salmonella enteritidis* DMST 15676, *Salm. typhimurium* DMST 16809, *Staphylococcus aureus* DMST8840, *Staph. aureus* TISTR 118 and *Vibrio parahaemolyticus*) obtained from Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Thailand, were used in BLIS screening procedures.

Fig. 1. Map of the area, indicating the mangrove forest sampling sites in southern Thailand.

2.3. Isolation of LAB from mangrove forests

Twenty-five grams of sample was aseptically transferred to a sterile stomacher bag and 225 ml of 0.85% normal saline was added to obtain a 1:10 dilution. The sample was blended for 1 min using a stomacher. Appropriate decimal dilutions were prepared in 0.85% normal saline and poured into sterile Petri dishes on de Man, Rogosa and Sharpe agar (MRS, Labscan Asia Co., Ltd., Thailand) containing 2% (w/v) NaCl, 0.02% (w/v) bromocresol purple and 0.7% cycloheximide (v/v) (Difco, Detroit, USA) (Maragkoudakis et al., 2006). This was subjected to incubation at 37 °C for 24–48 h. The bacterial colonies which changed color of agar from purple to yellow were picked and streaked on MRS agar containing 0.02% (w/v) bromocresol purple. This procedure was repeated in order to purify the isolates.

2.4. Screening of BLIS producing LAB

2.4.1. Preparation of cell free supernatants (CFS)

The isolates (from section 2.3) were grown in MRS broth for 24 h at 37 °C. CFS were obtained by centrifugation (9500 *g* for 10 min at 4 °C) and were adjusted to pH 7.0 by means of 6 N NaOH to exclude the antimicrobial effect of organic acids. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 1 mg/ml catalase (Sigma, St. Louis, USA). Samples were heated at 100 °C for 10 min to inhibit enzyme activity (Schillinger & Lucke, 1989).

2.4.2. Determination of BLIS producing strain by agar well diffusion assay

MRS soft agar (1% agar) was seeded with 10^6 colonies forming units (CFU) per ml of *Lb. sakei* subsp. *sakei* JCM 1157. BHI (Hi Media Laboratory Pvt Ltd. India) soft agar (1% agar) was seeded with 10^6 CFU/ml *L. monocytogenes* or *Br. thermosphacta* DSM 20171. Each mixture was poured into sterile Petri dishes, wells of 5 mm in

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