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Antibacterial polyketides from *Bacillus amyloliquefaciens* associated with edible red seaweed *Laurenciae papillosa*

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ABSTRACT

Heterotrophic *Bacillus amyloliquefaciens* associated with edible red seaweed, *Laurenciae papillosa* was used to isolate antibacterial polyketide compounds. Antibacterial activity studies integrated with the outcome obtained by polyketide synthetase (*pks*) coding genes established that seaweed-affiliated bacterial flora had a wide-ranging antibacterial activities and potential natural product diversity, which proved that the bacterium is valuable reservoir of novel bioactive metabolites. Bioactivity-guided isolation of 3-(octahydro-9-isopropyl-2*H*-benzo[h]chromen-4-yl)-2-methylpropyl benzoate and methyl 8-(2-(benzoyloxy)-ethyl)-hexahydro-4-((*E*)-pent-2-enyl)-2*H*-chromene-6-carboxylate of polyketide origin, with activity against human opportunistic food pathogenic microbes, have been isolated from the ethyl acetate extract of *B. amyloliquefaciens*. Structure-activity relationship analysis revealed that hydrophobic descriptor of the polyketide compounds significantly contribute towards its antibacterial activity. Seaweed-associated microorganisms were shown to represent a potential source of antimicrobial compounds for food and health benefits. The antibacterial polyketide compounds described in the present study may find potential applications in the food industry to reduce food-borne pathogens.

1. Introduction

The surfaces of seaweeds are rich with symbiotic bacteria having antimicrobial properties that can provide the host with bacterial-mediated defense properties. These seaweed-associated bacterial communities are potential sources of biotechnological significance as they produce biologically active compounds of greatly diverse characteristics (Chakraborty, Thilakan, & Raola, 2014; Gomez, Soria-Mercado, Rivas, & Ayala-Sánchez, 2010). The recovery of seaweed-associated bacterial strains possessing activities against pathogenic food microbes led to the belief that the seaweed might form an ideal ecological niche that harbors valuable microbial varieties. This warrants carrying out research into the bioactive metabolites from seaweed associated bacterial flora for food and health benefits (Kanagasabhapathy, Hideaki, & Shinichi, 2008; Penesyan, Marshall-Jones, Holmstrom, Kjelleberg, & Egan, 2009). Microbial symbionts from various marine eukaryotic hosts, such as sponge and marine algae, were reported as the real architect to biosynthesize valuable secondary metabolites of food and medicinal significance (Kubanek et al., 2003; Zhang, Zhang Li, Miao, Meng, & Zang, 2009). Previous studies demonstrated that the seaweed-associated microbial populaces are prolific sources of bioactive metabolites, which were of great demand in health related fields, such as food supplements and antimicrobial agents (Chakraborty et al., 2014; Singh, Kumari, & Reddy, 2015).

The greater attention to control the food borne pathogens, and search for newer metabolites of marine origin has been expanded during the recent decades, which resulted in the exploration of the bacteria-seaweed relationships and isolation of antimicrobials (Ben Ali et al., 2012; Goecke, Labes, Wiese, & Imhoff, 2010). Seaweed-associated bacteria were found to be rich in bacterial strains with polyketide synthase (*pks*) gene (Thilakan, Chakraborty, & Chakraborty, 2016; Zhao, Yang, & Zeng, 2008), which was demonstrated as the functional gene determining the active bacterial metabolites. It was additionally reported that the seaweed derived polyketides actually belong to the seaweed-associated bacterial metabolites (Chakraborty et al., 2014; Kubanek et al., 2003). Inadequate works on these natural compounds and their derivatives related to the defeating mechanisms against various food borne pathogens, lack of antibiotics and novel antibacterial agents has created a diminishing reservoir of alternative solution to control the emerging food pathogens. Biopreservation of Brine Shrimp (*Pandalus borealis*) by bacteriocins from lactic acid bacteria was reported in a previous literature (Einarsson & Lauzon, 1995). With this background, we developed a program to

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isolate and characterize the antibacterial lead molecules from edible red seaweed-associated bacteria collected from marine habitats as novel antimicrobial agents against food pathogens. The present study acquired a culture-dependent method to isolate the heterotrophic bacteria, *Bacillus amyloliquefaciens* associated with the red seaweed *Laurenciae papillosa* harvested from the southeast coast of India. The two new polyketides of presumed activity against human pathogenic bacteria have been isolated from the ethyl acetate extract of *B. amyloliquefaciens*. Structure-activity relationship analyses were used to analogize various molecular descriptor variables, which appreciably contributed towards the antibacterial activities of polyketides. The present work further investigated the chances of improvisation of natural food preservatives and its synthetic derivatives using these isolated metabolites, which can cease the growths of food pathogenic bacteria.

2. Materials and methods

2.1. General

Liquid chromatography-mass (LCMS), Fourier Transform Infra-Red (FTIR), and ultra violet-visible spectra, of the purified compounds were acquired by Applied Biosystems QTrap 2000 (Applied Biosystems) connected to Agilent 1100 HPLC (Agilent), Thermo Nicolet, Avatar 370 (using KBr), and Varian Cary 50 Conc UV–vis spectrometer, respectively (Chakraborty et al., 2014). The encompassing nuclear magnetic resonance (NMR) spectral experiments were recorded on a Bruker NMR spectrometer (Bruker, AVANCE III 500 MHz) by dissolving the pure compounds in an aprotic solvent, $CDCl_3$ (Chakraborty et al., 2014; Raola & Chakraborty, 2016). The Microbial Type Culture Collection Centre (MTCC, Institute of Microbial Technology, India) supplied the microbial strains, whereas food pathogen *Vibrio parahemolyticus* (ATCC® 17802™) was procured from American Type Culture Collection (ATCC, Manassas, VA 20110). The reagents for assays and solvents for extractions, spectral studies, and chromatography were purchased from E-Merck (Germany), and were of analytical, chromatographic, or spectroscopic grades.

2.2. Isolation of bacterial strains

The seaweed *L. papillosa* was collected from the Gulf of Mannar located in the southeast (SE) coast of India (9°17′ N; 79°7′ E), and the samples were processed as described previously (Chakraborty et al., 2014). The cleaned seaweeds were used to isolate and subsequent culture of the bacterial strains that were tested against food pathogens for anti-microbial potentials (Chakraborty et al., 2014). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) staining of the living cells visualized the inhibition regions around the cultured isolates. The biochemical methods along with fatty acid fingerprinting identified the microbes with antagonistic properties, supported by 16S rRNA gene sequence (Chakraborty et al., 2014) using the primers AGAGTTTGATCCTGGCTCAG (forward) and ACGGCTACCTTGTTACGACTT (reverse) (Weisburg, Barns, Pelletier, & Lane, 1991). Seaweed associated *B. amyloliquefaciens* (deposited as MTCC 10456) was further screened for its potential metabolites having polyketide synthetase (*pks*-I) in their natural biosynthetic pathway using the primers, RTRGAYCCNCAGCAICG (forward) and VGTNCCNGTGCCRTG (reverse) (Chakraborty et al., 2014).

2.3. Purification and characterization of antibacterial metabolites from seaweed-associated B. amyloliquefaciens

The secondary metabolites were prepared and separated by surface-culturing technique on nutrient agar (Chakraborty et al., 2014). The bacterial secondary metabolites accumulated on the surface of nutrient agar plates were extracted with ethyl acetate (EtOAc) by refluxing before being evaporated (Heidolph, Schwabach, Germany) to afford the EtOAc extract (6.3 g) from whole culture volume (4 L). The crude extract was fractionated through vacuum liquid chromatography using adsorbent silica gel (180–230 mesh), with a stepwise gradient of *n*-hexane to ethyl acetate as solvent system (EtOAc: *n*-hexane 1–19 to 4–1, v/v) to yield B_4E_1–B_4E_{14} as fourteen fractions (50 ml each). The fraction B_4E_3 (773 mg) was found to have antibacterial activity of 24 mm, but a mixture of compounds, so which was fractionated by flash chromatography (Biotage SP1, 230–400 mesh, 12 g) on a Biotage No. 25+M 0489-1 column (silica gel 230–400 mesh, Biotage, Sweden) at collection UV wavelength at 246 nm (CH_2Cl_2-MeOH; 0–100% MeOH) to furnish 125 fractions (9 ml). The similar fractions were mixed together to afford seven pooled fractions (B_4E_{15}–B_4E_{21}) after TLC methods (9% *n*-hexane-EtOAc, v/v). The next sub-fraction B_4E_{16} (159 mg) as eluted at CH_2Cl_2-MeOH (19:1, v/v) was again separated by chromatography on silica gel coated on a preparatory thin layer plate using a stepwise gradient solvent system of 0–0.5% MeOH-CH_2Cl_2 yielding 3-(octahydro-9-isopropyl-2*H*-benzo-[h]-chromen-4-yl)-2-methylpropyl benzoate (**1**, inhibition zone diameter of 16 mm against *Aeromonas hydrophilla*, 25 mcg per disk). The active fraction B_4E_{23} (72 mg) separated at 0.3% MeOH/CH_2Cl_2 was further chromatographed over preparatory TLC using MeOH-CH_2Cl_2 (0.5–95.5, v/v) to afford methyl 8-(2-(benzoyloxy)-ethyl)-hexahydro-4-((*E*)-pent-2-enyl)-2*H*-chromene-6-carboxylate (**2**, ~99% purity, 6.2 mg, inhibition zone diameter of 14 mm against *A. hydrophilla*, 25 mcg per disk) and TLC over pre-coated silica gel (Si gel GF_{254}) using *n*-hexane/EtOAc (4:1, v/v) validated the purity.

2.3.1. 3-(Octahydro-9-isopropyl-2H-benzo [h] chromen-4-yl)-2-methylpropyl benzoate (**1**)

Yellowish oil; UV (MeOH) λ_{max} (log ε): 362 nm (3.11); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 1:9, v/v) R_f: 0.8; R_t (GC): 25.18 min.; IR ν_{max} (KBr) cm^{-1} (δ_{OOP} = out of plane bending, ν = stretching, δ = bending, ρ = rocking vibrations): 726.24 (C—H ρ), 1000.14 (aromatic C—H δ), 1352.24 (C—H ρ), 1526.21(C=C aromatic ν), 1654.40 (C=C ν), 1692.21 (C—CO—O ν), 1722.86 (C=O ν), 2923.22 (C—H ν), 2955.04 cm^{-1} (C—H ν), 3067.12 (C—H aromatic ν); ^1H NMR (500 MHz, $CDCl_3$ δ in ppm), ^{13}C NMR (125 MHz, $CDCl_3$ δ in ppm), ^1H—^1H—COSY, and HMBC data, refer Table 1; HRMS (ESI) *m/z* calcd for $C_{27}H_{37}O_3$ 409.2743, found 409.2756 $[M + H]^+$.

2.3.2. Methyl 8-(2-(benzoyloxy) ethyl)–hexahydro-4-((E)–pent-2-enyl)-2H-chromene-6-carboxylate (**2**)

Colorless oil; UV (MeOH) λ_{max} (log ε): 254 nm (3.62); TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 1:9, v/v) R_f: 0.6; R_t (GC): 37.68 min.; IR ν_{max} (KBr) cm^{-1}: 838.17 (C—H ρ), 968.1 (HC = CH δ), 1454.38 (C—C ν), 1578.21(C—C aromatic ν), 1642 (C=C ν), 1742 (C=O ν), 2923.22 (C—H ν), 3010.04 (C—H ν); ^1H NMR (500 MHz, $CDCl_3$ δ in ppm), ^{13}C NMR (125 MHz, $CDCl_3$ δ in ppm), ^1H—^1H—COSY, and HMBC data, see Table 1; HRMS (ESI) *m/z* calcd. for $C_{25}H_{33}O_5$ 413.2329; found 413.2364 $[M + H]^+$.

2.4. Determination of antibacterial activities

The title compounds were subjected to antibacterial assays by disk diffusion method on Mueller Hinton agar dressed with food pathogenic bacterial suspensions (~10^5 CFU/ml) as reported previously (Chakraborty et al., 2014), and the results were compared with antibiotic disk for gram negatives (HiMedia, India).