Apr 22, 2022

Isolation and identification of potential probiotic bacteria from the soil of Saint Martin's island Bangladesh

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dx.doi.org/10.17504/protocols.io.ewov1nzokgr2/v1

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Soil bacteria of Saint Martin's Island modulates gut microbiota and prevents *Enterococcus faecalis* infection in tilapia (*Oreochromis niloticus*)

DOI

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Md. Mahbubur Rahman, Sulav Indra Paul 2022. Isolation and identification of potential probiotic bacteria from the soil of Saint Martin's island Bangladesh. **protocols.io** https://dx.doi.org/10.17504/protocols.io.ewov1nzokgr2/v1

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Collection of soil sample.

1 the marine soil samples were collected from the shore of Saint Martin's Island (20°37'40.3"N' 92°19'22.3"E) of the Bay of Bengal, Bangladesh

2 the samples were collected from 6-15 inch depth using an auger

3 immediately transferred to a 50 mL sterile falcon tube

Isolation and maintenance of bacteria.

4 1 g of each soil sample was suspended in 9 mL of autoclaved seawater in an individual test tube

5 100 µL suspension from each diluted stock (10^{-2} and 10^{-3}) was aseptically inoculated on individual Starch Casein Agar (SCA)

6 After incubation, different colonies were picked up based on their colony characteristics

Phenotypic identification of bacterial isolates.

7 Individual colonies grown on SCA plates were carefully observed and colony characteristics *viz,* colony size, shape, color, type and elevation were recorded

8 Biochemical tests such as oxidase, catalase, motility, oxidative-fermentative (O-F) tests were accomplished

Screening of antagonistic activity of marine soil isolates.

9 Bacteria were grown in Marine Broth (MB) 2216 (Merck, USA) for 7 days at 28°C

10 The broth culture was centrifuged at 10,000 ×g for 15 min and the culture supernatant was passed through a 0.22 µm millipore membrane filter

11 The inhibitory activity of the culture supernatant was determined by agar well diffusion assay

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Molecular identification of marine soil isolates.

12 Genomic DNA of the selected isolates was extracted by using a commercial GenJET genomic DNA purification kit (Thermo Fisher Scientific, USA) #K0721

13 DNA was amplified by using universal primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGATACCTTGTTACGACTT-3').

14 The PCR amplification condition was done by an initial denaturation at 94 °C for 5 min; 35 cycles of a denaturation at 94 °C for 1 min, an annealing at 57 °C for 40 sec and an extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min.

15 Then the PCR amplicons were purified by using a commercial kit (Thermo Fisher Scientific, USA)

16 16S rRNA gene sequencing was done by Sanger Sequencing

Evaluation of digestive enzymes activity of the marine soil bacteria.

17 Enzymatic activity such as, protease, lipase, amylase, and cellulose activity of the *B. haynesii* strain CD223 and *A. mimigardefordensis* strain SM421were assessed to evaluate their probiotic effects.

Evaluation of the viability of bacteria in different pH and bile esculin.

18 The pH of SCA broth was adjusted from pH 3–9. Then, *B. haynesii* strain CD223 and *A. mimigardefordensis* strain SM421 were inoculated in this broth and kept for 24 h incubation at 28°C.

19 The viability of the cells was confirmed by inoculating them onto SCA agar plates (pH 7) by the spread plate method

Preparation of bacterial extracellular products (ECPs).

20 Bacteria were enriched in MB at 28^{O}C for 10 days

21 The ECPs were harvested and mixed with an equal volume of ethyl acetate in a separatory funnel

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22 The air-dried extract was weighted and dissolved in methanol for further use

Minimum inhibition concentration (MIC).

23 MIC was measured with different concentrations of ECPs extracts (1000, 500, 250, 125, 62.5, and 31.25 µg mL^{-1})

Measurement of Hematological Parameters

24 Fish from each treatment were anesthetized with the clove oil (0.05 mL per 500 mL of water) for hematological analysis

25 Blood was collected from fish using a 3 cc syringe containing 10% blood anti-coagulant (EDTA) inserted into the caudal peduncle region to drag out blood.

26 The blood was transferred to a test tube coated with EDTA, and stored at -30 oC until use.

27 Red blood cells (RBCs) and white blood cells (WBCs) were counted using an improved Neubauer hemocytometer (MarienFeld Company Germany) under the light microscope (DM 100; Leica, Wetzlar, Germany)

28 To measure hemoglobin, fresh blood was collected from fish from each treatment and was poured in the edge of a strip of hemoglobin meter before the coagulation of blood.

29 Estimation of immunoglobulin (IgM) was carried out by using Humalyzer-3000 analyzer.

Metagenomics study

30 Gut microbiome DNA was extracted using a commercial kit

V3 and V4 primer were used for sequencing

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