Journal of Microbiological Methods 129 (2016) 44–54

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A selective medium for recovery and enumeration of endolithic bacteria

Kaushik Bhattacharjee, S.R. Joshi *

Microbiology Laboratory, Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong 793022, India

ARTICLE INFO

Article history:
Received 9 May 2016
Received in revised form 29 July 2016
Accepted 29 July 2016
Available online 30 July 2016

Keywords:
Endolithic bacteria
Colony forming unit
Plackett-Burman
Box-Behnken

ABSTRACT

The study of lithic microbial communities, inhabiting rock substrates has been gathering momentum due to a growing attention of their wide importance as model systems in ecological studies and for their community structure. It is generally accepted that the success of cultivation-based technique is primarily based on suitable culture medium for isolation. The media available for enumeration and recovery of endolithic bacteria are mainly specific to particular type of rock which may not be suitable to isolate endolithic bacterial community from diverse lithobiontic niches. In this study, a new unoptimized medium was formulated, designated LM10 (unoptimized) for enumeration and recovery of endolithic bacteria by addition and/or omission of media components to the basal medium R2G, which was selected after experimental evaluation of five different existing media. The endolithic bacterial count in LM10 medium (unoptimized) was significantly higher than the R2G medium ($t = -12.57$, $p < 0.0001$). The culture and nutritional parameters associated with unoptimized LM10 medium were optimized using statistical approach to maximize the recovery and enumeration of endolithic bacteria. The first phase of the study comprised of a Plackett-Burman (PB) design experiment conducted to screen thirteen medium components and two culture parameters as variables with effect on bacterial enumeration and recovery. Out of these, Yeast extract, Casein hydrolysate, Glucose, Starch and Sodium thiosulphate were found to be significantly affecting the bacterial count ($p < 0.05$) based on PB design. On keeping rest of the media components and culture conditions at fixed value as per the PB design analyses ($p > 0.05$ and coefficients), further optimization was carried out for significant factors using Box-Behnken design (BBD) of response surface methodology (RSM). Optimized media components obtained by BBD were Yeast extract, Casein hydrolysate, Glucose and Starch in 0.05 g/l each and Sodium thiosulphate in 0.047 g/l concentrations. The composition of optimized LM10 medium formulated (per litre) is 0.05 g Yeast extract, 0.05 g Casein hydrolysate, 0.05 g Glucose, 0.05 g Starch, 0.01 g K_2HPO_4, 0.02 g Sodium pyruvate, 0.2 g $MgSO_4$, 0.001 g $FeSO_4 \cdot 7H_2O$, 0.285 g NH_4Cl, 0.039 g $CaCl_2 \cdot 2H_2O$, 0.047 g $Na_2S_2O_3 \cdot 5H_2O$, 0.002 g $NaHCO_3$ and 11 g Gellan gum (pH = 7.4). Validation of optimized LM10 medium using nine different rock samples from Meghalaya clearly indicated that optimized LM10 medium was better suited for higher recovery and enumeration of endolithic bacteria under both aerobic and anaerobic conditions.

1. Introduction

The study of lithic microbial communities, inhabiting rock substrates has been gathering momentum due to a growing attention of their wide importance as model systems in ecological studies and for their community structure (Warren-Rhodes et al., 2007). They are also relevant to astrobiologists due to their potential as analogs for possible life on Mars (da la Torre et al., 2003). Lithic microbial communities also play a vital role in microbe-mineral interactions (Blackhurst et al., 2004). The rock inhabiting microorganisms were first observed and described about 100 years ago (Horath and Bachofen, 2009). Subsequently several studies were reported of the presence of lithic microorganisms in sandstone (Bell, 1993), limestone (Gerrath et al., 2000), gypsum (Hughes and Lawley, 2003; Stivaletta et al., 2010; Rhind et al., 2014), dolomite (Sigler et al., 2003; Horath and Bachofen, 2009), carbonates (Hoppert et al., 2004), ignimbrite (Wierzchosa et al., 2013) and granite (Li et al., 2013). Among the different lithobiontic habitats, the microorganisms which are present in interior of rock are termed as endoliths (Olsson-Francis et al., 2010). They are usually the dominant form of life in extreme environment and bacteria represent the main component of that niche (McNamara et al., 2006; Olsson-Francis et al., 2010).

It is generally accepted that the success of cultivation-based technique is primarily based on suitable culture medium to isolate them. It is an axiom of microbiology that most culture media are selective for subsets of the total bacterial community; therefore, advancement of a variety of culture media is required to maximize the recovery of diverse microorganisms from different niches (Stevens, 1995). To isolate the endolithic bacteria, researchers have experimented various media

* Corresponding author at: Department of Biotechnology & Bioinformatics, North-Eastern Hill University, Mawlai, Shillong 793022, Meghalaya, India.
E-mail address: srjoshi2006@yahoo.co.in (S.R. Joshi).

http://dx.doi.org/10.1016/j.mimet.2016.07.026
0167-7012/

composition suitable to isolate them (Fike et al., 2002; Hirsch et al., 2004; Fajardo-Cavazos and Nicholson, 2006; Nunes da Rocha et al., 2015). Each of the media is composed of different components which can influence the growth of endolithic bacterial community specific to a niche. But these media are specific to a particular type of rock which may not be used to isolate endolithic bacterial community from diverse lithobiontic niches. At this point, the necessity of new culture media that would facilitate increased isolation of endolithic bacteria from different types of rocks is essential.

The classical method used for medium formulation is time-consuming and expensive. To overcome this difficulty, formulation of medium and optimizing the parameters by statistical method can be used as suitable alternative. Several statistical methods are existing for optimization experiments (Montgomery, 2002). Previously, statistical experimental design has been used in many areas including isolation of bacteria, media optimization for various purposes like secondary metabolites production and biodegradation process (Stevens, 1995; Yao et al., 2009; Grahovac et al., 2014). Plackett-Burman (PB) design is one of such approaches for initial screening of medium components among a large number of variables (Plackett and Burman, 1946). It is a factorial design which allows the analysis of '4n-1' variables in '4n' number of experiments (Plackett and Burman, 1946). In the experimental design, each row signifies an experiment and each column denotes an independent variable. The main drawback of this design is that it considers first-order effects and ignores the interactions between the variables (Zheng et al., 2014). Hence to find the interactions between the variables and to optimize the selected highly influencing factor, response surface methodology (RSM) by Box-Behnken Design (BBD) experiments is widely used statistical technique (Dean and Voss, 2006; Ghanem et al., 2010; Kanmani et al., 2013; Zheng et al., 2014). RSM includes factorial design and regression analyses for optimization experiments considering interaction between variables (Ghanem et al., 2010; Zheng et al., 2014).

In the present study, emphasis has been given to the formulation of a novel improved culture medium that forwarded the higher growth and recovery of endolithic bacteria. In the process of different permutation and combination of medium components, a novel medium LM10 has been formulated for higher recovery of endolithic bacteria.

2. Materials and methods

2.1. Collection of rock samples

Natural outcrops of rocks were collected from nine localities of Meghalaya (25°5′N and 26°10′N latitude and 89°47′E and 92°47′E longitudes), India comprising representatives from sedimentary, igneous and metamorphic rocks (Table 1). The region is an uplifted Precambrian crystalline complex forming the north-eastern extension of the Indian peninsular shield. A sterile knife and chisel blade was used to remove the small superficial outcrop fragments when needed during collection of rocks. The collected rock samples were brought to laboratory and were washed in running water to remove other debris and soil particles. They were then surface sterilized with 70% alcohol for 30 s followed by washing with sterile water several times. The surface sterilized rock samples were then aseptically crushed and grinded with hammer by wrapping in triple layer of pre-sterilized aluminium foil followed by sterile mortar and pestle. These crushed and grinded rock samples were suspended in Ringer's solution supplemented with 0.001% Tween 80 (ratio 1:10 w/v) and shaken vigorously for 1 h (Abdulla, 2009). Suspensions were then diluted 10- to 1000-fold and spread on sterilized medium on Petri plates.

2.2. Selection of basal medium and LM10 medium

A total of five already reported media, R2A agar (Nunes da Rocha et al., 2015), R2G agar (Nunes da Rocha et al., 2015), Tryptic Soy Agar (TSA), M9 media, Mineral salt thiosulphate yeast extract (MSTY) agar (Panda et al., 2009) with 10 fold dilutions of each were evaluated for the recovery of endolithic bacteria from nine diverse collected rock samples. Aliquots of 0.1 ml of each rock suspensions were spread onto plates of each of the test media and the plates were incubated at 30 °C for 15 days for visible growth, and the numbers of colony forming units per gram of rock (CFU/g) were counted. All experiments were performed in triplicates. The best medium with highest bacterial count was selected as basal medium for the formulation of a new medium.

The R2G agar was used as the basal medium in this study, and contained (per litre): 0.5 g Yeast extract (HiMedia, India), 0.5 g Casein hydrolysate (HiMedia, India), 0.5 g Glucose (HiMedia, India), 0.5 g Starch (HiMedia, India), 0.03 g K_2HPO_4 (Sigma-Aldrich, USA), 0.03 g Sodium pyruvate (Sigma-Aldrich, USA), 0.5 g $MgSO_4$ (Sigma-Aldrich, USA), 0.5 g Proteose peptone (HiMedia, India), 4.5 g NaCl (Sigma-Aldrich, USA) and 12 g Gellan gum (Sigma-Aldrich, USA). From this basal medium, proteose peptone and NaCl were omitted and ingredients like 0.01 g $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich, USA), 0.52 g NH_4Cl (Sigma-Aldrich, USA), 0.07 g $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, USA), 0.5 g $Na_2S_2O_3 \cdot 5H_2O$ (Sigma-Aldrich, USA) and 0.2 g $NaHCO_3$ (Sigma-Aldrich, USA) were added to the composition list on basis of their importance to support the growth of lithic bacteria to formulate the new medium that was designated as LM10 (unoptimized). The pH of the medium was adjusted to 7.8 prior to sterilization (autoclaving for 15 min at 121 °C and 15 lb./in.^{2}) with KOH or HCl as required.

A few of these media components are standard ingredients in microbiological media for the isolation and enumeration of microscopic organisms from lithobiontic corner. The nutrient supplement proteose peptone is an acid hydrolyzed product of casein and a source of amino acids, nitrogen (N), sulphur (S) and phosphorus (P) which can be partially achieved by addition of casein hydrolysate. The extra addition of NaCl provides a Na^+ source for halophiles but with an additional inhibitory effect to non-halophiles. This can be balanced by the casein hydrolysate which also contains some amounts of NaCl (<3.5%). The source for iron (Fe) supplemented was $FeSO_4 \cdot 7H_2O$ which formed a key micronutrient for the isolation of Fe-oxidizing bacteria (FeOB) which comprised a part of the lithic bacteria. The inorganic source of nitrogen (N) supplemented was NH_4Cl, $Na_2S_2O_3 \cdot 5H_2O$ as inorganic energy source and $NaHCO_3$ as inorganic carbon (C) source. This LM10 (unoptimized) medium was used for enumeration and recovery of lithic

Table 1
Rock samples collected with geo-locations of sampling sites.

Sample	Source	Code	Latitude	Longitude	Elevation (m)
Quartz pebble (Sedimentary)	Riverbed	Sn1	25°11.352′ N	92°01.079′ E	79.35
Lateritic sandstone (sedimentary)	Rocky outcrop	Sn2	25°14′46.59″N	90°49′11.60″E	353.1482
Friable sandstone (sedimentary)	Rocky outcrop	Sn3	25°11′51.99″N	90°38′39.88″E	30.2959
Basalt (igneous)	Rocky outcrop	Ig1	25°11.352′ N	92°01.079′ E	81.21
Decomposed granite (igneous)	Rocky outcrop	Ig2	25°36′9.96″N	91°33′29.03″E	1688.99
Granitic pebble (igneous)	Riverbed	Ig3	25°12.309′ N	91°53.869′ E	495.46
Quartzite (metamorphic)	Rocky outcrop	Me1	25°21.604′ N	91°52.915′ E	1556.46
Quartzite (metamorphic)	Rocky outcrop	Me2	25°21′16.00″ N	92°31′0.23″ E	1171.46
Phyllite (metamorphic)	Rocky outcrop	Me3	25°21.604′ N	91°52.915′ E	1556.46