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Short communication

Novel application of κ-carrageenan: As a gelling agent in microbiological media to study biodiversity of extreme alkaliphiles

Sumitra Datta, Kalpana Mody*, Gnanasekaran Gopalsamy, Bhavanath Jha*

Discipline of Marine Biotechnology & Ecology, Central Salt & Marine Chemicals Research Institute, Council of Scientific and Industrial Research (CSIR), G. B. Marg, Bhavnagar 364 021, Gujarat, India

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ABSTRACT

In the present investigation a novel application of κ-carrageenan, as a gelling agent for isolation, purification and identification of extreme alkaliphilic bacteria has been explored. The potassium salt of κ-carrageenan was an appropriate substitute of agar for preparation of solid medium, particularly for isolation of extreme alkaliphiles, as agar de-polymerizes at pH > 12.5. The purpose of formulating a new, stable, highly alkaline, solid medium was to study the diversity of extreme alkaliphilic bacteria by means of conventional cultivation techniques allied with molecular tools. The medium having pH in the range of 8.0–13.5 was used to isolate extreme alkaliphiles in pure form.

1. Introduction

Highly alkaline environments for microorganisms include soda lakes, deserts, soils and industrial-derived waters (Ulukanli & Diğrak, 2002). Diverse prokaryotic communities isolated from such extreme environments are a valuable biotechnological resource (Borsodi et al., 2005; Sorokin, Banciu, Van Loosdrecht, & Kuenen, 2003). Alkaliphilic exoenzymes are used in bioremediation, detergent and other industries (Horikoshi, 1996, 1999; Kanekar, Sarnaik, & Kelkar, 1995; Satyanarayana, Raghukumar, & Shivaji, 2005; Ulukanli & Diğrak, 2002). To employ the right bacterial cultures for their utilization, it is necessary to isolate them in pure form, which is possible only on solid media. To prepare solid media for alkaliphiles, it is necessary to add a gelling agent, which is stable in extreme alkaline condition. In addition, to study the biodiversity of cultivable alkaliphiles, it is necessary to purify them from mixed cultures as individual colonies. To do this they are required to grow on a solid medium so that they grow as individual clones.

For any microbiological solid media, desirable qualities of a gelling agent include (a) stability of the gel over the temperature range for bacterial growth, (b) resistance to digestion by bacteria, (c) lack of syneresis, (d) transparency, (e) and the ability to form a reversible gel. The solid culture medium must be firm enough to facilitate streaking and spreading. In addition, it is desirable that the gelling agent is relatively inexpensive and easily available. Since Koch first introduced agar as a gelling agent in bacteriological media (Koch, 1882), it has become the primary gelling agent throughout the world. However, increased cost and shortage of resources for agar led to discovery of alternatives. Carrageenan extracted from Irish moss and *Eucheuma striatum* has been reportedly used in bacteriological media by various microbiologists (Epifanio, Veroy, Uyenco, Cajipe, & Laserna, 1981; Walker & Day, 1943). Alain (2003) and Taintor (2006) have reported application of κ- or ι-carrageenan and a mixture of agar and ι-carrageenan, respectively, as gelling agents. Parker, Bernsteinas, and Green (1968) had exploited carrageenan based solid medium for isolation of psychrophilic bacteria. Evaluation of different κ-carrageenan samples as substitute for agar, in microbiological media, was carried out by Abbott and Chapman (1981).

All the earlier studies, where κ-carrageenan had been used as gelling agent, were confined to media having neutral or near neutral pHs (Lines, 1977) and none of these reports have indicated the use of κ-carrageenan for preparation of extremely alkaline solid media to be used for isolation of extreme alkaliphiles. Due to the alkali labile property of agar, it was felt necessary to identify an alternative alkali stable gelling agent. Carrageenan is a linear sulphated (anionic) polysaccharide extracted from red seaweeds (Stanley, 1987). It is widely used in the food and pharmaceutical industries as thickening, stabilizing and gelling agents (McHugh, 2003). κ-Carrageenans are predominantly derived from *Eucheuma cottonii* (*Kappaphycus alvarezii*), *Chondrus crispus*, *Gigartina stellata*, *Furcellaria fastigiata*, and *Hypnea* spp. (Rudolph, 2000). It has the

* Corresponding authors. Tel.: +91 278 2567352; fax: +91 278 2570885.
E-mail address: bjha@csmcri.org (B. Jha).

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Table 1
Requirement of super saturated NaOH solution (110 g NaOH/100 ml DW) to achieve desired pH in 100 ml Horikoshi medium.

NaOH solution (μl)	pH achieved
Nil	7.0 ± 0.2
30	8.0 ± 0.1
80	9.0 ± 0.1
130	9.60 ± 0.1
160	10.0 ± 0.1
200	10.4 ± 0.1
250	11.0 ± 0.1
300	11.5 ± 0.1
400	12.0 ± 0.1
500	12.5 ± 0.1
1500	13.0 ± 0.1
3000	13.3 ± 0.1
4000	13.5 ± 0.1

ability to form thermo-reversible gels at room temperature in the presence of monovalent and divalent cations such as potassium and calcium (Campo, Kawano, Braz da Silva, & Carvalho, 2009; Morris, Rees, & Robinson, 1980). Large scale cultivation of carrageenophytes is successfully carried out all over the world including India and hence there is no shortage of resources for carrageenan production. Good cultivation practices and simple extraction procedures makes carrageenan, a cheaper gelling agent, as compared to agar. In this report, we describe a novel application of κ-carrageenan, as the gelling agent in extremely alkaline microbiological media having pH > 12.5, for isolation, purification, identification and to study biodiversity of cultivable extreme alkaliphiles (Gopalsamy et al., 2009).

2. Experimental

2.1. Materials

Analytical grade chemicals from commercial suppliers (Merck, Sigma–Aldrich and Himedia) and refined κ-carrageenan produced at CSMCRI, Bhavnagar were used in the experiments.

2.2. Preparation of isolation media

Liquid Horikoshi I medium [(g/l) glucose 10 g; peptone 5 g; yeast extract 5 g; K_2HPO_4 1.0 g; $MgSO_4{\cdot}7H_2O$ 0.2 g; and distilled water] was used as an alkaline basal medium for the isolation and enrichment of alkaliphilic bacteria. Alkaline pH was obtained by addition of sterilized NaOH solution (Table 1). The best carbon source (glucose, sucrose and starch) for isolation of alkaliphiles was determined.

2.3. Correlation of carrageenan/KCl concentration with gel strength

Concentrations of κ-carrageenan and KCl, required for the formation of solid alkaline media (pH ranging from 10.0 to 13.5) with requisite gel strength, was determined. For this, varying concentrations of refined κ-carrageenan and KCl were added to 100 ml medium. The mixture was boiled to dissolve carrageenan and 50 ml of solution was distributed between two 50 ml beakers. The gel was allowed to form in the beaker at room temperature by natural cooling of solution and then kept in the refrigerator in the inverted position over night at a low temperature of 12–15 °C. Next day the gel was brought to room temperature and gel strength was measured by using Nikkansui type gel tester (Kiya Seisakusho Ltd, Tokyo, Japan). The measurement was performed using a solid cylindrical plunger of 1 cm diameter.

2.4. Isolation and characterization of extreme alkaliphiles

Alkaliphilic bacteria isolated from soda ash effluent and sludge samples (pH ≥ 10.2), collected from West coast of Gujarat (21°50.103′N; 72°11.339′E), India were tested for pH tolerance (10.0–13.5) on solid media with κ-carrageenan as gelling agent at 32 °C for 48 h. Morphological and physiological characteristics of the isolates were studied as recommended by Smibert and Krieg (1994). Antibiotic sensitivity tests (Icosa dics, Himedia) and hydrolytic activity of all the isolates were performed at pH 10 (Carrim, Barbosa, & Vieira, 2006; Sanchez-Porro, Martin, Mellado, & Ventosa, 2002). Molecular identification of the isolates was done using 16S rDNA sequencing. Genomic DNA was extracted using the chloroform–isoamyl alcohol extraction procedure (Wilson, 1999). 16S rRNA genes of these isolates were amplified using the primer set (fD1 and rP2) (Weisburg, Barns, Pelletier, & Lane, 1991). The reaction mixture for PCR amplification contained 10× PCR buffer 5 μl, 200 μmol dNTPs 5 μl, 2.5 U *Taq* DNA polymerase, 20 pmol of each primers (Sigma, India) and 50 ng of template DNA. Amplification was performed in a thermal cycler (Bio-Rad MyCycler, Thermal cycler, CA, USA) for an initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 5 min. Amplification was checked by agarose gel electrophoresis (Sambrook, Fritsch, & Maniatis, 1989). All the amplified PCR products were purified and sequenced for 16S rRNA gene.

2.5. Phylogenetic analysis

Near full-length 16S rRNA gene sequences (accession numbers: GQ280011, GQ280012, GQ280018, GQ280033, GQ280045 and GQ280049) were compared to known sequences in the GenBank (http://www.ncbi.nih.gov) using the BLAST search tool (Altschul et al., 1997). A phylogenetic tree including obtained isolates and their closest relatives was constructed using MEGA software 4 (Tamura, Dudley, Nei, & Kumar, 2007). Pairwise evolutionary distances were computed using the correction method (Jukes & Cantor, 1969) and clustering was performed using the neighbor-joining method (Saitou & Nei, 1987). Bootstrap analysis was performed using 1000 replicates (Felsenstein, 1985).

3. Results and discussion

3.1. Optimization of media

Varying concentrations of super-saturated NaOH solution (110 g NaOH/100 ml DW), ranging from 160 μl to 4.0 ml were added to adjust pH of the medium from 10.0 to 13.5 (Table 1). Bacterial growth was best supported when sucrose was used as carbon source. To understand the roles and structures of microbial communities, sequence data only are not enough, but cultivability of microorganisms is equally important (Borsodi et al., 2005). Pure culture microbiology still represents the best method to study microbial physiology including detailed investigations on the role of genes, proteins and metabolic pathways (Alain & Querellou, 2009).

3.2. Effect of κ-carrageenan and KCl concentration on gel quality and gel strength of the medium

The gel strength of the media prepared using different concentrations of agar and κ-carrageenan was determined using Kiya Seisakusho Ltd, Tokyo, Japan gel tester, with which maximum gel strength of 1100 g/cm^2 could be measured. Due to depolymerization, agar failed to gel at extreme alkaline pH (>12.5) even at 3% concentration. Under this condition, κ-carrageenan in

Table 2
Effect of κ-carrageenan and KCl concentration on gel quality and gel strength of Horikoshi medium at pH 13.0 and 13.5.

Carrageenan and KCl concentration	Gel quality at pH 13.0	Gel strength (g/cm^2) at pH 13.0	Gel quality at pH 13.5	Gel strength (g/cm^2) at pH 13.5
1% Carrageenan:1% KCl	Soft	350	–	–
1.5% Carrageenan:1% KCl	Medium	400	Soft	230
1.5% Carrageenan:1.5% KCl	Hard	515	Soft	310
2% Carrageenan:1.5% KCl	Hard	700	Hard	430
2% Carrageenan:2% KCl	Hard	715	Hard	450
2.5% Carrageenan:1.5% KCl	Hard	770	Hard	700
3% Carrageenan:1.5% KCl	–	–	Hard	810

Table 3
Taxonomic affiliations of the six isolates, determined by phylogenetic analysis of 16S rRNA gene using BLAST tool.

Culture code	GenBank accession number	pH tolerance	Opt pH	Salt tolerance (%)	Opt salt concentration (%)	Nearest match GenBank	Homology	Group
BJ-1	GQ280011	8.0–13.0	11.0	0–5	0.5	*Bacillus* sp. [EU004566]	99%	Firmicutes
BJ-2	GQ280012	7.0–13.0	10.0	0–5	0.5	*Cellulosimicrobium cellulans* [AY114178]	99%	Actinobacteria
BJ-8	GQ280018	8.0–13.0	11.0	0–10	0.5	*Bacillus* sp. [AY347311]	95%	Firmicutes
BJ-23	GQ280033	7.0–13.0	10.0	0–10	3	*Alcaligenes* sp. [EU443097]	98%	β-Proteobacteria
BJ-35	GQ280045	8.0–13.0	11.0	0–5	0.5	*Pseudomonas mendocina* [EU311211]	99%	γ-Proteobacteria
BJ-39	GQ280049	7.0–13.0	11.0	0–10	0.5	*Exiguobacterium* sp. [EU379016]	98%	Firmicutes

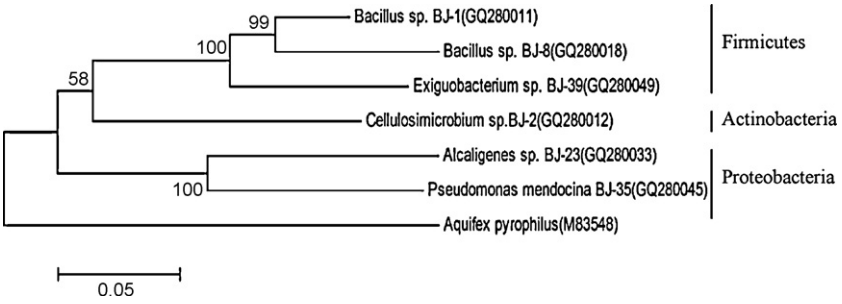

Fig. 1. Unrooted phylogenetic tree based on a comparison of 16S rRNA gene sequences of six alkaliphilic isolates. The tree was created by the NJ method. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 replications. Isolates characterized in the present study are indicated by their respective GenBank accession numbers in brackets. *Aquifex pyrophilus* (accession number M83548) was used as the outgroup taxon. Bar inferred nucleotide substitutions per nucleotides.

the presence of potassium chloride, was used as an alternative gelling agent. Concentrations of κ-carrageenan and KCl, required to obtain the desired gel strength in the medium having pH 13.0 and 13.5, was determined (Table 2). Reasonably hard gels (gel strength ≥ 400 g/cm^2) are required to perform streaking for isolation of bacteria. Streaking was feasible on solid medium of pH 13.0 containing 1.5% κ-carrageenan and 1% KCl (gel strength 400 g/cm^2). At pH 13.5, a gel strength of 435 g/cm^2 was achieved using 2% κ-carrageenan and 1.5% KCl. Likewise, 2.5% κ-carrageenan with 1.5% KCl and 3% κ-carrageenan with 1.5% KCl yielded gels with very high gel strength of 700 and 810 g/cm^2, respectively. However, lower κ-carrageenan concentration (<1.5%) yielded soft gel (gel strength 230–350 g/cm^2) on which streaking was not possible. κ-Carrageenan is used as a gelling agent in food, pharmaceutical, cosmetics, printing and textile industries (Glicksman, 1979; Imeson, 2000) and in biotechnology sectors (De Ruiter & Rudolph, 1997). Though there have been many reports on utilization of carrageenan as substitute of agar for bacteriological culture media (Walker & Day, 1943; Watson & Apirion, 1976), none have used it at extremely high pH value of 13.0 and 13.5.

3.3. Diversity of extreme alkaliphiles

The alkaliphiles showed different colony pigmentation and morphologies on carrageenan plates of pH ≥ 12.5. Colonies of BJ-1, BJ-8 and BJ-23 were cream colored while those of BJ-2 and BJ-35 were yellow and BJ-39 had orange color colonies. BJ-25 and BJ-35 were Gram negative. Only BJ-2 was coccoid in structure. All were motile and catalase positive. Alkaline amylase, caseinase and gelatinase activity was exhibited by most of them. Except BJ-35, all others had multienzyme activity at alkaline pH. BJ-8 exhibited best hydrolytic activity producing all six hydrolases, utilizing twenty different sugars. Alkaline environment is an active microbial habitat where their extra-cellular enzymes play important roles in degrading or recycling the consumable substrates in a nutrient-limited, low energy-flux microbial ecosystem. Isolates BJ-2, BJ-23 and BJ-35 displayed multidrug resistance against six or more antibiotics. Table 3 shows bacterial diversity (Firmicutes, Proteobacteria and Actinobacteria) isolated from environmental samples having high pH. Fig. 1 shows phylogenetic tree constructed using 16S rRNA gene sequence data obtained for the six isolates with Aquifex pyrophilus (accession number M83548) as outgroup.

4. Conclusion

The present study describes, for the first time, a successful application of κ-carrageenan as a gelling agent in microbiological media having pH ≥ 12.5, which is used to study diversity of cultivable extreme alkaliphiles (Gopalsamy et al., 2009). Agar does not form a gel under strongly alkaline conditions. Under neutral condition, 1% κ-carrageenan in the presence of 1% KCl gives strong gel. Under extreme alkaline condition, i.e. at pH 13.0, 1.5% κ-carrageenan and 1% KCl are required to give a firm gel having gel strength of 400 g/cm^2, on which streaking is possible for bacterial isolation. At a pH of pH 13.5, the culture medium required 2% carrageenan with 1.5% KCl, for formation of stable and hard gel with gel strength

of 435 g/cm^2. Isolation and purification of culturable alkaliphilies was achieved on the solid medium of highly alkaline pH, using κ-carrageenan as the gelling agent. The isolated alkaliphiles could be used to study their diversity and can be exploited in future for industrial applications.

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