Biological and Applied Sciences

Vol.60: e17160215, January-December 2017
35http://dx.doi.org/10.1590/1678-4324-2017160215
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

A N I N T E R N A T I O N A L J O U R N A L

Characterization of Alkaliphilic Bacteria Isolated from Bauxite Residue in the Southern Region of Minas Gerais, Brazil

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ABSTRACT

The aim of this study was to isolate and characterize bacterial strains from bauxite residue in the southern region of Minas Gerais, Brazil, by 16S rRNA gene sequencing and biochemical assays. Bacillus cohnii, Bacillus pseudofirmus, *and* Bacillus clarkii *were identified among the isolates. The isolates were able to use a wide range of carbon sources and to grow at NaCl concentrations of up to 10%, temperatures from 10 to 40 °C, and pH from 7 to 10.5, producing a wide variety of organic acids. This is the first report on microbial composition of bauxite residue in Brazil.*

Key words: Bauxite residue, 16S ribosomal RNA gene, Alkaliphilic bacteria.

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Brazil is one of the three biggest bauxite producers, with an annual output of ~30 megatons [1]. Alumina extraction from bauxite using concentrated sodium hydroxide in the Bayer process generates a slurry and an extremely alkaline (pH of 9 to 13) byproduct known as bauxite residue or red mud. For each ton of alumina extracted from bauxite, approximately 1.5–2.0 tons of bauxite residue is generated [1,2]. Disposal of such a byproduct is a serious problem at alumina plants because of environmental risks and financial costs.

The use of alkaliphilic microorganisms is considered an attractive alternative method for treatment of industrial alkaline residues, owing to their ability to metabolically reduce alkalinity and their tolerance of high concentrations of ions and metals and low availability of organic carbon and nutrients [1-8]. Few research groups have studied alkaliphilic bacteria from bauxite residues [5-7], and there are no reports addressing the microbial composition in such an environment in Brazil. Because climatic, geological, and other environmental conditions as well as variations in alumina extraction methods are strong determinants of biological characteristics of bauxite residue deposits [1,2], it is important to perform microbial analysis of bauxite residues from unexplored geographic locations. The aim of this study was to isolate and characterize bacterial strains in bauxite residue from the region of Poços de Caldas, Brazil.

Samples were collected in a pond located in Poços de Caldas, Minas Gerais, Brazil (21°51′14.53″S 46°34′53.24″W), at an approximate altitude of 1200 m. The region is characterized by dry winter and rainy summer, with a pluviometric mean of 1300–1700 mm per year and mean temperature of 19.9 °C. Samples were collected in December (rainy period), at the depth of 0.25, 0.5, and 1 m and stored in sterile plastic bags at 4 °C until analysis. Chemical characterization of the samples involved an X-ray fluorescence (XRF) method according to Leroux and Mahmud [9].

Bauxite residue samples from each depth were separately cultured in an enrichment medium using oxalate (one of the most likely carbon sources in red mud) as described by Agnew et al. [10]. Five successive 4-day cultures were carried out at 30 °C under aerobic conditions for bacterial enrichment. Bacteria were isolated from the enriched cultures by the method of agar plate streaking. Colonies were picked and maintained in a liquid medium, i.e., a simpler alkaline medium described by Horikoshi [11] (Horikoshi medium) (g/L): 10.0 glucose, 1.0 yeast extract, 1.0 $(NH_4)_2SO_4$, 0.136 KH_2PO_4, 0.024 $Mg_2SO_4{\cdot}7H_2O$, and 10.6 Na_2CO_3. Five isolates were chosen for genotyping. DNA was extracted from the isolates using the Wizard Genomic DNA Purification Kit (Promega®). The 16S rRNA gene was amplified by PCR using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1401R (5′-CGGTGTGTACAAGGCCCGGGAACG-3′). The reaction mixture consisted of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer, and 0.04 U of Taq DNA polymerase (Invitrogen, USA) in a final volume of 50 μl. The amplification program was as follows: 94 ºC for 4 min; 30 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72ºC for 1 min; and the final extension step at 72 ºC for 7 min. The amplicons were sequenced on an ABI 3730 DNA Analyzer. The 16S rRNA gene sequences from isolates BRA.1, BRA.2, BRA.3, BRA.4, and BRA.5 were deposited in the GenBank database (accession numbers: KT592279, KT592280, KT592281, KT592282, and KT592283, respectively). A phylogenetic tree was constructed by the neighbor-joining method by means of the Ribosomal Database Project tool.

Catalase, oxidase, nitrate reduction, and amylase assays were performed as described by Logan and De Vos [12], with adapted media containing 10.6 g/L Na_2CO_3 to ensure alkaline pH.

Growth assays were all conducted in triplicate, with 150-rpm shaking and the Horikoshi medium [11], at the initial pH of 10.5 (except for pH assays) at 30 ºC (except for temperature assays), and bacterial growth was evaluated by measurement of absorbance at 600 nm. For pH growth testing, pH 7.0, 8.0, 9.0, 10.0, 11.0, or 12.0 was established using the carbonate/bicarbonate (0.1 M) buffering system, with addition of NaOH for adjustment of pH to 11.0 and 12.0. Samples were collected at intervals of 4 h for 28 h. Growth at different temperatures (10, 20, 30, 40, or 50 ºC) was tested after 1, 2, 3, and 15 d of cultivation. Tolerance of NaCl was evaluated in cultures containing 0%, 5%, 10%, and 17% of NaCl, and growth was evaluated after 1, 2, and 7 d. For testing of carbon sources, the medium was supplemented with 50 mM of one of the following: glucose, mannitol, fructose, sucrose, xylose, or lactose; growth was quantified after 16 and 24 h. Samples of the culture supernatant of 24-h cultures were also collected for pH measurement and organic-acid detection by the methods of Penteado et al. [13].

Table 1 shows percentages of the oxides detected in bauxite residue. As expected, aluminum (18.53–31.77%), iron (21.99–34.65%), and silicon (16.53–22.21%) oxides, typically the most abundant elements in bauxite residue [2], were predominant. Aluminum content was particularly high, reaching values near the maximal levels ever registered for bauxite residues [2]. Sodium was also found at significantly high concentrations (3.94–8.27%). The pH of bauxite residue samples varied from 10.53 to 10.73, and alkalinity was greater than 90%.

Table 1. Chemical characterization of bauxite residue samples collected at different depths.

	Concentration, %		
Component	0.25 m	0.5 m	1 m
Al_2O_3	31.77	18.51	28.1
CaO	2.22	2.96	2.78
Cr_2O_3	0.01	0.02	0.02
Fe_2O_3	21.99	34.65	25.85
K_2O	2.22	1.26	1.56
MgO	0.13	0.11	0.09
MnO	0.40	0.93	0.91
Na_2O	8.27	3.94	5.86
P_2O_5	0.50	0.41	0.36
SiO_2	22.21	16.53	17.36
TiO_2	4.54	9.58	5.85
V_2O_5	0.07	0.10	0.08
ZnO	0.02	0.07	0.05
ZrO_2	0.66	1.82	0.80
Others	5.00	5.00	5.00

Sequences of the 16S rRNA gene (length of approximately 1.4 kb) from the isolates were compared to sequences from the GenBank database. Five isolates were found to belong to the alkaliphilic *Bacillus* genus, forming three distinct phylogenetic groups (Fig.1) In general, similarity of more than 99% between bacterial 16S rRNA gene sequences of ~1.3 kb is considered a reliable threshold for their classification in the same species [14,15]. The five isolates were >99% similar to alkaliphilic *Bacillus* strains. BRA.1 and BRA.2 were respectively 99.7% and 99.8% similar to *Bacillus cohnii.* BRA.3 and BRA.4 were 99.7% similar to *Bacillus pseudofirmus*; BRA.5 was 99.7% similar to both *Bacillus clarkii* and *Bacillus polygoni* (Table 2). 16S rRNA gene sequences of these last two species were reported to be more than 99% similar, but they have well-distinguishable physiological characteristics [16].

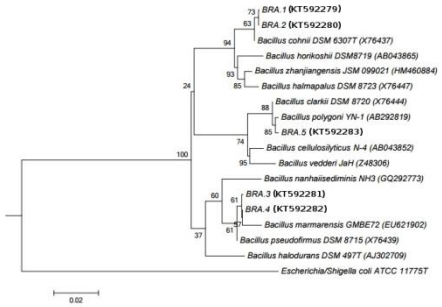

Figure 1. Phylogenetic tree constructed based on a comparison of 16S rRNA gene sequences of bauxite residue isolates with the some of the closest relatives retrieved from GenBank database. The tree was created using the neighbor-joining method. Bar represents 2 substitutions per 100 nucleotides. Numbers in each branch indicate bootstrap values from 1000 replicatesf.

Table 2. Phylogenetic affiliations of the bauxite residue isolates according to comparison of 16S rRNA gene sequences.

Isolate (GenBank accession No.)	Closest relative in GenBank database (accession No.)	% similarity
BRA.1 (KT592279)	*Bacillus cohnii* DSM 6307T (X76437)	99.7
BRA.2 (KT592280)	*Bacillus cohnii* DSM 6307T (X76437)	99.8
BRA.3 (KT592281)	*Bacillus pseudofirmus* DSM 8715 (X76439)	99.7
BRA.4 (KT592282)	*Bacillus pseudofirmus* DSM 8715 (X76439)	99.7
BRA.5 (KT592283)	*Bacillus polygoni* YN-1(AB292819); *Bacillus clarkii* DSM 8720 (X76444)	99.7

Due to the high similarity in 16S rRNA sequences among the isolates within the same group (Fig. 1), only one representative strain from each group (BRA.1, BRA.3, and BRA.5) was chosen for the phenotypic analyses. The three strains were catalase positive. BRA.1 and BRA.5 were oxidase positive and reduced nitrate, whereas BRA.3 tested negative for both characteristics (Table 3). Amylase activity was detected in both BRA.1 and BRA.3 but not in BRA.5. The three isolates can grow at pH from 8.0 to 10.5 and temperatures from 20 and 40 ºC. BRA.1 and BRA.3 also grow at neutral pH, and BRA.3 is the only strain that grows at 10 ºC. BRA.3 and BRA.5 were found to be dependent on the presence of NaCl, and all three strains tolerate high concentrations of NaCl (up to 10%). All three strains can grow on glucose, fructose, sucrose, mannitol, or yeast extract, but none was found to grow on xylose or lactose. These characteristics are consistent with those described in the literature on the respective closest strains from genetic analyses (Table 3) [17-19], confirming BRA.1 as a *B. cohnii* strain, BRA.3 as *B. pseudofirmus*, and BRA.5 to be phenotypically closer to *B. clarkii* than *B. polygoni*.

Table 3. Biochemical characteristics of the isolates and their respective closest strains retrieved from GenBank.

	BRA.1	B. ***cohnii***[2]	BRA.3	B. ***Pseudofirmus***[3]	BRA.5	B. ***clarkii***[4]	***B. polygoni***[5]
Gram stain	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	-	-	+	+	-
Amylase	+	+	+	+	-	-	-
Nitrate reduction	+	+	-	-	+	+	-
pH							
6.7	-	-	-	-	-	-	-
7.2	+	v	+	v	-	-	-
8	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+
11	-	v	-	v	-	v	+

12	-	v	-	-	-	-	+
Temperature							
10 ºC	-	+	+	+	-	-	+
20 ºC	+	+	+	+	+	+	+
30 ºC	+	+	+	+	+	+	+
40 ºC	+	+	+	+	+	+	+
50 ºC	-	-	-	-	-	-	-
NaCl (%)							
0	+	+	-	-	-	v	-
5	+	+	+	+	+	+	+
10	+	v	+	+	+	+	+
17	-	-	-	v	-	+	-
Carbohydrates							
Glucose	+	+	+	+	+	+	+
Fructose	+		+	+	+		
Sucrose	+		+	+	+		
Mannitol	+		+	nd	+		
Xylose	-		-	v	-		
Lactose	-		-	-	-		

nd: not detected
v: variable (variations among strains classified as the same species)
[1]Results of the present study
[2] (18, 19)
[3] (15, 20)
[4] (16, 21)
[5] (16)

Firmicutes has been described as one of the most representative bacterial phyla in bauxite residue, and the *Bacillus* genus as one of the most frequent [5-7]. The species *B. cohnii*, *B. pseudofirmus*, and *B. clarkii* isolated in the present work have been found in a variety of environments, such as soil and animal feces [17-19]. Nonetheless, to our knowledge, this is the first formal report on the detection of these species in bauxite residue, with successful isolation.

As preliminary evaluation of the isolates' ability to reduce pH of a strongly alkaline (pH 10.5) environment, the pH of the culture supernatants was measured at the end of 24-h cultivation. The best performance among the three strains was obtained with glucose as a carbon source, and the maximal decrease in pH was observed in the cultures of *B. clarkii* isolate BRA.5 (Table 4). Other research groups reported the same magnitude of a pH decrease for other alkaliphilic bacteria [6,22,23]. Organic-acid production is associated with the pH decrease in the culture medium by alkaliphilic bacteria [7,22,23]. As shown in Table 5, organic acid production profiles of strains BRA.1, BRA.3, and BRA.5 differed depending on the carbon source, but in general, considerable amounts of acetic, isobutyric, and isovaleric acids were produced by the three strains.

Analysis of the performance of the isolates in a microcosm study on bauxite residue treatment is the next goal in order to better evaluate these bacteria for their potential utility in bioremediation methods.

Table 4. Analysis of growth and pH in cultures of the isolates, with different carbohydrates as a carbon source.

	B. cohnii			*B. pseudofirmus*			*B. clarkii*		
Carbohydrate	Max. growth [1]	Max. pH decrease [2]	Min. pH [3]	Max. growth	Max. pH decrease	Min. pH	Max. growth	Max. pH decrease	Min. pH
Glucose	1.54	1.26	9.2	1.65	0.96	9.51	1.2	1.43	9.04

Fructose	0.525	0.5	10.1	0.53	0.45	10.14	0.4	0.4	10.18
Sucrose	0.461	0.52	10.05	0.53	0.44	10.13	0.4	0.44	10.14
Mannitol	0.714	0.85	9.7	0.75	0.45	10.06	0.56	0.53	9.97
Lactate	0.09	0.32	10.26	0.09	0.46	10.12	0.15	0.39	10.18
Xylose	0.09	0.35	10.2	0.09	0.43	10.11	0.22	0.41	10.13
Yeast extract	0.12	0.24	10.34	0.12	0.37	10.22	0.25	0.39	10.2

[1]Maximal optical density observed during 24 h of cultivation
[2]Minimal pH observed during 24 h of cultivation
[3]The pH decrease calculated from the variation between the initial pH (10.5) and the minimal pH value during the culture

Table 5. Concentrations of acids (mg/L) produced in cultures with different carbohydrates as a carbon source.

	B. cohnii				*B. pseudofirmus*				*B. clarkii*			
Carbohydrate	A*	iB	iV	T	A	iB	iV	T	A	iB	iV	T
Glucose	365	251	279	1036	173	248	94	779	401	415	271	1246
Fructose	87	207	100	524	104	71	45	918	64	223	0.5	762
Sucrose	129	89	38	447	97	18	44	558	68	203	14	446
Mannitol	295	250	210	1410	73	118	62	644	87	207	100	524

*A = acetic acid; iB = isobutyric acid; iV = isovaleric acid; T = total organic acids.

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Received: February 03, 2016;
Accepted: July 14, 2016