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Biological Treatment of Alkaline Cement Kiln Dust by using Alkalitolerant Bacteria

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The present study was aimed at assessing the acid producing ability of alkalitolerant bacterial isolate to reduce the alkalinity of cement industry waste (cement kiln dust). A Gram positive, alkalitolerant bacterium, designated strain KG4, was isolated from rhizospheric soil capable of growth at high pH (~12) and screened on the basis of its potential to reduce the pH of the alkaline medium. The strain exhibited phenotypic properties consistent with its classification in the genus *Bacillus*. Growth was observed at pH 6-12, at temperature 28-50°C, and at NaCl concentration of 0-16%. The highest level of 16S rRNA gene sequence similarity was with *Bacillus sonorensis* NRRL B-23154 (94.7%). On the basis of the phenotypic characteristics and genotypic distinctiveness of strain KG4 from other phylogenetic neighbours of alkaliphilic *Bacillus* species, the name *Bacillus* sp. KG4 is proposed with GenBank accession number JN969986 (=NCIM 5440). This strain was then utilized in reducing the alkalinity (79%) and chloride (82%) of cement kiln dust which represents its economical and industrial application in treatment of alkaline industrial wastes.

Key words: alkaliphiles, alkalinity reduction, *Bacillus* sp., Cement kiln dust.

The most diverse occurrences of organisms are generally observed in moderate environments. Certain environmental conditions such as pH, temperature and salinity conditions are extremely high or low on earth which was thought to prevent the existence of life, and such environments are regarded as extreme environments. Extreme environments are populated by certain groups of organisms adapted to these conditions which are usually referred to as alkaliphiles, halophiles, acidophiles and thermophiles, reflecting the particular type of extreme environment which they inhabit. Microorganisms which inhabit such extreme environments are also termed as "extremophiles". Extremophiles are gaining a lot of research interest because of their remarkable conversion potential and their capability to abate environmental pollution under stressful conditions [1, 2, 3].

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In recent years, there has been increasing interest in alkaliphilic bacteria due to their potential industrial application that require enzymes (proteases, xylanases, cellulases, etc.) that are stable at high pH values (pH > 9.5) and at temperature above 50°C [4, 5, 6]. Alkaliphilic bacteria have been studied for their adaptation to high pH, isolated from variety of environments and most of the isolates belong to genus *Bacillus*. Studies on alkaliphilic *Bacillus* strains have shown that the characteristics of these bacteria differ depending on the strain[7]. The genus *Bacillus* currently includes 19 species characterized as alkaliphilic and alkalitolerant, and many of them have attracted much attention with regard to industrial, basic research and biotechnological applications[8, 9]. Effluent discharge from textile industries also have high pH or alkaline as the dyeing process of fabric involves different chemicals and dyes such as basic dyes. So, the environment around the discharge outlet also alkaline in nature and may acts as the habitat for alkaliphiles.

As the pH or alkalinity is the important parameter of the waste generated by industries that needs to be controlled, several environment authorities such as EPA (Environmental Protection Agency in US) and CPCB (Central Pollution Control Board in India) have set guidelines and directs that the pH of the effluent or waste discharged by the industries should be in the range of 6.5-8.5. Various chemicals such as hydrochloric acid[2], sulphuric acid[10], phosphoric acid[11] and carbon dioxide[12, 13] are available to neutralize the high alkaline industrial waste by-products and waste water. However, these chemicals are difficult to handle as they are hazardous and corrosive, and to neutralize the alkaline waste by-products and waste water, large quantities of acid would required which is economically not feasible and have dangerous effect on the health of the workers as well as on the industrial processes.

Cement kiln dust (CKD), is such an alkaline waste by-product of cement industry generated during the manufacturing of cement clinker from electrostatic precipitators and baghouses. The chemical composition of CKD may vary with the type of raw material and the cement manufacturing process. During the clinker production in kiln the volatile sulfates, alkalis (K_2O and Na_2O) and chlorides are preferentially drawn towards the CKD. In 2011, Indian cement industry generates approximately 37-50 million metric tons of cement kiln dust[14] and major part of it is dumped on open land as landfill material as due to high alkalinity, CKD is not reused in the cement kiln. Mohamed and El-Gamal[15] and Gebhardt[16] treated the alkaline CKD with carbon dioxide gas to remove the alkalinity, but these methods are highly expensive and laborious. The better alternative to the use of chemical process is the biological treatment of alkaline wastes using a class of alkaliphiles which grow well at high pH. Kulshreshtha et al.[17] and Kumar et al.[18] conducted an experiment to neutralize the alkaline industrial waste water by using *Exiguobacterium* sp. and blend of *Bacillus* sp. respectively, but these isolates are not available for research work as these are patented. Thus, in the present study, we undertook the task of isolating and identifying new alkalitolerant bacterium, designated strain KG4 belonging to genus *Bacillus*, which grow well at very high pH (~ 11-12), from soil and exploited beneficially for the alkalinity removal of cement kiln dust for proper disposal and reuse.

EXPERIMENTAL

Isolation of alkaliphilic bacteria

The alkaliphilic bacterial strain investigated in this study was isolated from the rhizospheric soil of citrus plant. For isolation, an enrichment medium was used which consisting of 10 g glucose, 10 g peptone, 5 g yeast extract, 1 g K_2HPO_4 and 15 g agar per 1000 mL of the water. The pH of the enrichment medium was adjusted to 10.5 with 1 N NaOH. Glucose was autoclaved separately and added to the medium aseptically. Soil sample was diluted serially and 100 µL of the aliquot was then spread on the agar plates. The agar plates were incubated for 2 days at 35 ± 2ºC. On the basis of colony morphology and color microbial isolates were selected. The single isolated colonies were then picked and re-streaked on same agar medium plates till pure colonies were obtained. The pure cultures were then stored at 4ºC for further use.

After the isolation, pure isolates were then screened for their tolerance to pH 11 and 12 and this was performed on a minimal medium (M9) instead of enrichment medium as the components of the enrichment medium gets precipitated at such high pH. The composition of minimal media used was sucrose (10 g), K_2HPO_4 (2.5 g), KH_2PO_4 (2.5 g), $(NH_4)_2HPO_4$ (1 g), $MgSO_4.7H_2O$ (2 g), $FeSO_4.7H_2O$ (0.01 g), and $MnSO_4.4\ H_2O$ (0.007 g) per 1000 mL of the medium. Medium of such high pH was prepared using appropriate biological KCl-NaOH buffer instead of NaOH due to precipitation or turbidity of the medium with NaOH. The cultures isolated on enrichment medium were then subcultured on M9 medium having pH-11 and then finally acclimatized on M9 medium having pH-12 by subculturing the isolates a number of times on same medium.

Screening of the isolates for reducing the alkalinity of cement kiln dust

The isolates were then screened on the basis of their potential to reduce the pH of the alkaline medium. For this, the production of acid by alkaliphilic microorganism was observed in liquid minimal medium as well as in enrichment medium having pH~12. The acid production by

microbial isolates was observed by monitoring the pH of the media with the help of pH meter (Cyberscan pH 510) and decrease in pH was monitored for up to 5 days at regular intervals in three replications of the treatment.

Alternatively, the reduction of the pH in the minimal medium (pH~12) by isolates was also observed by adding phenolphthalein pH indicator to the medium. At high pH (>10), the indicator gives bright pink color to the alkaline solution while at low pH (i.e. pH below 8) it is colorless. Both the medium were inoculated with culture isolate which had an OD value of 1.0. Change in color from pink to colorless was the indicator of the acid production by the microbial isolates. After every sampling at regular intervals from the medium the broth was centrifuged at 10,000 rpm for 5 min to palletize the cells so that cells might not interfere in analyzing the optical density of the supernatant with spectrophotometer at 554 nm wavelength.

The potential isolate was then picked and utilized in the treatment of cement kiln dust. Cement kiln dust (CKD) was finely powdered and grey black in color. CKD was collected from the electrostatic precipitators of the cement industry. The chemical composition of the CKD was analyzed by energy dispersive X-ray spectrometry (JEOL JSM-6510 LV, USA) and shown in Table 1. The cement kiln dust was mixed with different OD values of isolated bacterial strain KG4 ranging from 0.6-1.0 in the ratio CKD to culture (4:1). The culture showed OD value of 1.0 had approximately 10^8 cells. The treatment mixture was incubated at 35°C for 20 days in three replications and moisture was maintained in the form of water for the strain. The addition of water to CKD was such that water provides only moisture to CKD, i.e., CKD should be in the form of moist powder not the slurry or in the liquid mixture. After 5 days of incubation, sucrose solution (10%) was added only once during treatment to provide carbon source for the growth of bacterial strain. As no work has been done on such treatment so sucrose solution was used only once during the treatment which is economically feasible. Aeration in the treatment was provided by manual mixing of the sample at regular intervals during the treatment. A control CKD without bacteria was also incubated at similar conditions as for bacterial treated samples. At the onset of 20 days, samples from different treatments were removed and mixed with water (1:10) in conical flask with shaking (@ 130 rpm for 1 h) to generated the leachate which then analyzed for the alkalinity and chloride along with control treatment[19]. To confirm the decrease in alkalinity, bacterial treated CKD was analyzed with energy dispersive X-ray spectrometry (EDX) for change in chemical composition and with X-ray diffraction (XRD) for change in alkali phases (such as arcanite) in CKD. All CKD samples were air dried and grounded to make fine powder before EDX and XRD analysis.

The samples analyzed with EDX (JEOL JSM-6510 LV, USA) were mounted on brass stubs using carbon tape and then determined for the change in chemical composition. The XRD analysis was carried out by using PANalytical X'pert Pro (Netherlands) system. The random powder mount specimens were scanned from 10 to 60° 2θ. Interpretation of the X-ray patterns was carried out by usual methods, involving matching of the peaks with the reported literature and by using standard JCPDS cards, a database of X-ray powder diffraction patterns maintained by the International Center for Diffraction Data (ICDD).

Characterization and Identification of the strain

Characterization of the strain was performed using standard biochemical test[20] and 16S rRNA based phylogenetic analysis. Cell morphology was examined by compound microscope (Nikon E100). The Gram character was determined by using Gram staining as per method given by Cappuccino and Sherman[21]. Several biochemical tests such as starch hydrolysis, gelatin hydrolysis, H_2S production, methyl red – Voges Proskauer test, citrate utilization, catalase test and carbohydrate utilization test was performed as per the method given by Aneja[20]. All the tests were inoculated with fresh culture and then incubated at 37°C. Growth pH and medium used for biochemical characterization was maintained as per the given methods[20]. The observations were recorded at 48 h of incubation period. Growth experiments at different pH (5-12), at various NaCl concentrations (0-20%), and at various temperatures (20-55°C) was investigated in both enrichment broth medium and minimal broth medium. The cultures were incubated at 37°C for 48 h at 120 rpm. The efficient acid producing alkaliphilic bacterial strain were then presumptively identified as per Sneath[22], Cowman and Steel's

manual[23] and study conducted by Yoon *et al*.[24].

The 16S rRNA gene sequencing study was performed by Xcelris Labs Limited, Ahmedabad (India). DNA was isolated from bacterial strain KG4 by using QIAamp DNA Purification Kit (Qiagen). The 16S rRNA gene was amplified by using PCR method with universal bacterial primers 8F (AGA GTT TGA TCC TGG CTC AG) and 1492R (ACG GCT ACC TTG TTA CGA CTT). Amplified PCR product was purified using Qiagen Mini elute Gel extraction kit according to the manufacturer's protocol. Sequencing of the purified 16S rRNA gene was performed using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) as recommended by manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically using ABI 3730xl Genetic Analyzer (Applied Biosystem, USA). The 16S rRNA gene sequence of the strain KG4 was processed manually, analyzed at NCBI (National Centre for Biotechnology Information) server (http://www.ncbi.nlm.nih.gov) using BLAST tool and compared to the corresponding neighbour sequences from the GenBank-NCBI database. Multiple alignment of the strain KG4 was performed with related *Bacillus* species (from GenBank-NCBI database) using Multalin program[25] and phylogenetic tree was constructed by the neighbor joining method[26]. Evolutionary distance matrices for the neighbor joining method were calculated using the algorithm of Kimura's two-parameter model[27]. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 replicates. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of the strain KG4 and related *Bacillus* species are shown in Fig. 5.

Statistical Analysis

Statistical analysis (t-test and analysis of variance) was calculated for the data by using Graphpad Prism (version 4) software. The significant results were represented at $P<0.05$ significant level.

RESULTS AND DISCUSSION

Alkaliphiles have been isolated mainly from neutral environments, sometimes even from acidic soil samples[28]. The frequency of alkaliphiles in the environment especially in the ordinary neutral soil sample has been shown to be 10^2-10^5 per g of soil, which corresponds to 1/10 – 1/100 of the population of the neutrophiles. In the media used to isolate alkaliphilic microorganisms, the sample could be enriched with different substrates such as peptones, glucose, bile salts, casamino acids and caseine[29]. An alkaline media has to be used by adjusting the pH of the medium to around 10 with sodium carbonate (Na_2CO_3) and/or borax-NaOH, Na_2HPO_4/NaOH, KCl/NaOH buffer systems (buffering capacity over the range of pH 9-12 in various media). Out of a number of microorganisms isolated from soil on agar media plates, based on colony morphology, color and screening for acid production, a bacterial strain KG4 was isolated and showed good growth in culture broth at higher pH (11 and 12). The selected isolate KG4 was isolated from the rhizospheric soil as such soil is the habitat of abundant different types of microorganisms and the cultures isolated were able to tolerate and grew at higher pH values by adjusting their cell metabolism essential for survival[18, 30]. Initially, the strain was isolated on enrichment medium having pH 10.5 then sub cultured number of times at particular pH 11 and then 12 in solid minimal medium for acclimatization of the culture at such high pH values. Few studies have been reported on the use of buffer systems such as phosphate buffer[31, 32] and carbonate buffer[32, 18] to increase the pH of the growth medium but no such work has been reported on the use of KCl-NaOH buffer as buffer system for increasing pH of the medium. The pH of the KCl-NaOH buffer system is around 13 so can be used for preparing medium of high pH value such as 12.

Acid production by the strain KG4 was observed in both minimal medium and enrichment medium having high pH (Fig. 1) and demonstrated highly significant ($p<0.0001$) reduction in pH. In minimal medium, the overall change in pH of 3.89 units, after an incubation period of 3 days. At 3 days of incubation the pH was significantly lowered by strain KG4 whereas at 4 and 5 days of incubation change in 0.1-0.18 pH units was observed which seems to be negligible when compared with change in pH up to 3 days of incubation. Similar trend was also observed in enrichment medium. Decrease in pH was mainly observed at 3 days of incubation. The results

showed that at 3 days of incubation the maximum reduction in pH was there which corresponds to the acid production by the microbial isolates. In minimal medium sucrose was the sole carbon source; microbial isolates efficiently utilized the carbon source and produced acids which eventually decrease the pH of the medium[28].

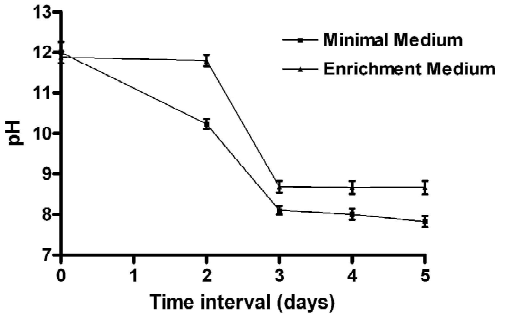

Fig. 1. Reduction of pH of the alkaline growth medium by *Bacillus* sp. strain KG4

An alternative method was also performed to screen the acid production potential of the strain KG4 by using phenolphthalein indicator. Decrease in the optical density of the minimal medium with respect to change in color of phenolphthalein also indicated acid production by the isolate (Fig. 2). Initially at high pH (~12) the M9 medium found bright pink in color and after inoculating the strain the decrease in pH was indicated by fading of the color from pink to colorless with growth. Fig. 2 shows the significant ($p = 0.028$; α value = 0.05) decrease in the optical density of the colored medium with time and observed that strain KG4 decreased the pH of the medium (fading of the color of the medium) within 60 h of inoculation. The change in pH possibly be attributed to the production of organic acids in the medium by enzymes and these results were in agreement with the observation of Paavilainen *et al.*[33]. By comparing these two media compositions for microbial growth, minimal media was observed better media compared to enrichment media as decrease in pH in minimal medium was observed within 2 days of incubation while no such reduction in pH was observed in enrichment medium. Also, the minimal medium is an economical and defined media compared to enrichment medium; so, minimal media was used for the sub culturing process of the isolate for this study.

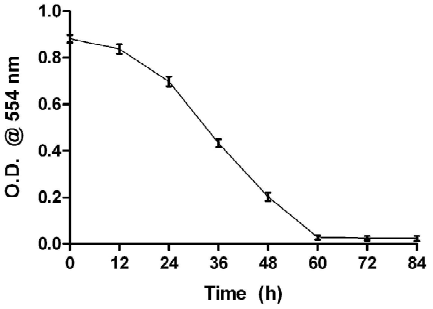

Fig. 2. Screening of the acid production by *Bacillus* sp. strain KG4 in minimal medium at different intervals of time. Optical density of the medium was measured at 554 nm based on the color fading of the medium containing phenolphthalein

Several environmental agencies have set guidelines to check the pH or alkalinity of industrial wastes before discharging to environment as this is the important parameter to check surface and ground water pollution. The removal of the alkalinity of CKD by using alkaliphilic bacterial strain was the challenging task for this study as till date no study was performed in treating the alkaline solid industrial wastes with biological organisms. Fig. 3 represents the alkalinity reduction of the cement kiln dust after treatment with strain KG4 having different cell density (OD 0.6 to 1.0). The

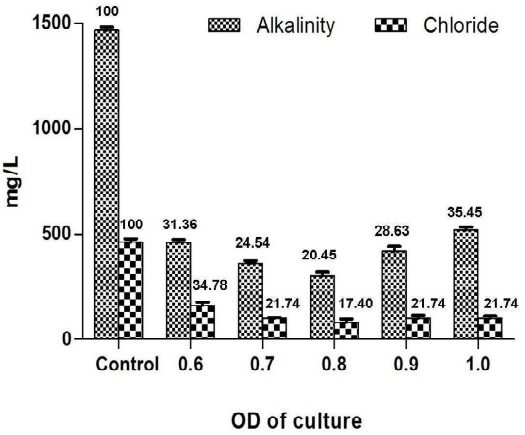

Fig. 3. Alkalinity and chloride reduction of cement kiln dust (CKD) treated with different concentration (in terms of optical density) of *Bacillus* sp. strain KG4. The numbers on the bars represents the percent alkalinity and chloride, respectively, in different treated CKD samples compared to untreated CKD (control). Statistical analysis shows $p<0.05$ using t-test at 95% confidence limits

alkalinity of the control CKD in leachate was 1467 mg/L whereas after treatment with strain KG4, 79.55 % alkalinity was reduced with culture having OD of 0.8. The results exhibited significant ($p<0.05$; t-test at 95% confidence limits) reduction in the alkalinity of the CKD in leachate compare to control.

The chloride content in the control CKD leachate was 460 mg/L which is higher than the maximum permissible limits (250 mg/L) given by EPA and WHO standards for drinking water. CKD treated with strain KG4 having 0.8 optical density showed significant reduction (82%; $p<0.05$; t-test at 95% confidence limits) in chloride content in leachate compared to control. As CKD contains only inorganic mineral salts mainly in the form of oxides (CaO, SiO_2, Al_2O_3, Na_2O, K_2O etc.), so, for the growth of isolates in CKD, carbon source should be added and the whole environment itself acts as the growth medium for the isolate. Preliminary studies in this work conducted to check the growth of isolate in CKD leachate containing 2% glucose solution as carbon source. A cell density (OD) of 0.27 was observed compared to control medium (without inoculation) which showed that the above said medium supports the growth of isolate to some extent. The maximum reduction in alkalinity in CKD + culture (OD 0.8) was due to the proper concentration of cell for the treatment. Low concentration of cells affects the efficiency of the reaction whereas high concentration generates the unfavourable conditions for growth as there was limited nutrition for large number of cells. Thus, we can say that the treatment of CKD with cell density (OD) of 0.8 bacterial culture gives the promising results.

The CKD sample treated with bacterial culture (OD 0.8) was then analyzed with EDX and XRD to confirm the decrease in the alkalinity. The EDX spectrum of CKD revealed the presence of calcite ($CaCO_3$), and quartz (SiO_2) as the major components along with peaks for Al, Mg, K, S and O. Analysis of CKD showed absence of Na and Cl while small amount of Fe, Cu and Zn was detected (Table 1). Thus, in the sample CKD the alkalinity was mainly due to presence of K (K_2O) and S (SO_3). The compound % of K_2O and SO_3 in control CKD was 1.12 and 1.13, respectively. On treatment with bacterial strain KG4, K_2O content was reduced to 0.92 which corresponds to 17.86% reduction compared to control CKD (no bacterial treatment). After treatment SO_3 content was not observed in the treated CKD analyzed with EDX. This confirmed significant ($p<0.0001$; two way ANOVA) reduction in alkalinity of the CKD after treatment with bacterial strain KG4.

Table 1. Chemical composition of cement kiln dust without and with bacterial treatment. The values in brackets denote the percent of compound increased or decreased compared to control. Data is statistical significant at $p<0.0001$ using two way ANOVA

Chemical Composition (compound %)	CKD control	CKD treated with Isolate KG4
$CaCO_3$	21.56 (100)	28.95 (+34.28)
MgO	0.69 (100)	0.54 (-21.74)
Al_2O_3	2.38 (100)	1.89 (-20.59)
SiO_2	13.17 (100)	10.86 (-17.54)
SO_3	1.13 (100)	Not detected
K_2O	1.12 (100)	0.92 (-17.86)
CaO	55.78 (100)	53.66 (-3.80)
Fe_2O_3	2.62 (100)	2.62 (0.0)
CuO	0.89 (100)	1.13 (+26.97)
ZnO	0.66 (100)	0.99 (+50.00)

The XRD results (Fig. 4) indicate that the CKD mainly consists of calcite ($CaCO_3$), anhydrite ($CaSO_4$), dolomite ($CaMg(CO_3)_2$) and free lime (CaO). Traces of crystalline alkali sulfate phases, such as arcanites (K_2SO_4), and the hydration product phases such as syngenite ($K_2(CaSO4)_2$), ettringite and calcium hydroxide ($Ca(OH)_2$) were also observed. Absence of arcanite phase peak (at 31Ú 2è) in bacterial treated CKD also confirmed alkalinity reduction compared to control (Fig. 4). EDX and XRD analysis supported the results in terms of absence of sulfate and crystalline alkaline sulfate phases in treated powdered CKD compared to control. The free sulfates and K_2O content in the control CKD on treatment converted into bound form such as anhydrites or ettringite (calcium aluminium sulfate phase) or syngenite (potassium calcium sulfate phase) which reduced the free sulfate content in CKD and thus, reduced the alkalinity. These results can be supported by the fact that microorganisms utilize carbohydrates usually by fermentation and respiration process; and acid production may have been due to the carbohydrate metabolism. Sucrose used in the

treatment as carbon source, first converted into glucose and fructose by strain KG4 which then metabolized oxidatively to organic acids, results in lowering alkalinity of CKD. Bacterial isolates are highly diverse in the metabolism and channel the diversity of materials into different central pathways (glycolysis, Kreb's cycle, etc.) of cell metabolism for complete oxidation, thereby producing an array of organic acids[18].

Preliminary investigations in this work showed that the direct addition of organic and inorganic acids in neutralization of cement kiln dust is not feasible as large amount of these acids would be needed which is not economical. Use of inorganic acids such as hydrochloric acid, sulfuric acid, etc. in neutralization process can be

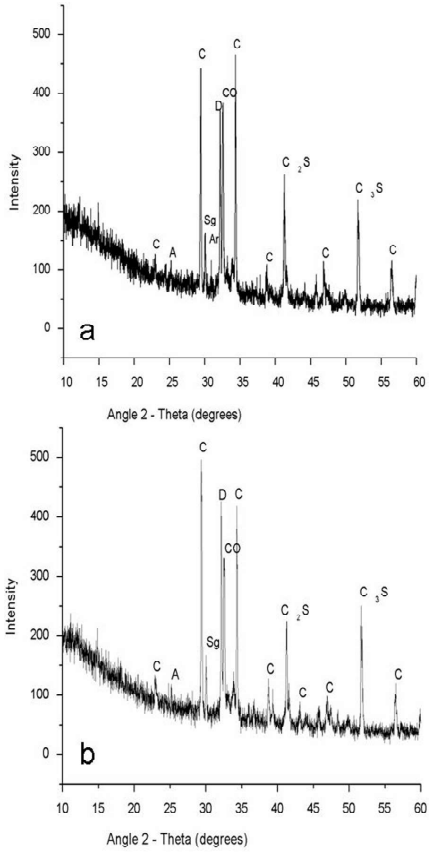

Fig. 4. Diffraction pattern including the crystalline phases in the control CKD (a) and bacterial treated CKD (b). C-calcite ($CaCO_3$), D-dolomite ($CaMg(CO_3)_2$), A-anhydrite ($CaSO_4$), CO-calcium oxide (CaO), Sg-syngenite ($K_2Ca(SO_4)_2$), Ar-arcanite (K_2SO_4) C2S-dicalcium silicate, C3S-tricalcium silicate

hazardous and corrosive and may cause health problems to the workers. Chloride also increases the alkalinity in the CKD. Reduction in chloride content also marks an important parameter to study as its high concentration in ground water through leachate affects the taste of the ground water and increases its alkalinity also. In this study approximately 82% chloride was reduced which showed that CKD on bacterial treatment can safely be reused and dispose off to landfill. Thus, use of biological tool for the neutralization of alkaline solid waste acts as a better alternative to conventional chemical methods. Growth of bacterium on minimal medium also shows its economic effectiveness and acts as cheap growth medium compared to ready to prepare enriched growth medium such as nutrient broth, etc.

Characterization and identification of the bacterial strain

The phenotypic characteristics (morphological and biochemical) of the strain KG4 are shown in detail in Table 2. The isolates KG4 was Gram positive, rod shaped and growing aerobically. The isolate was able to hydrolyze starch and gelatin and exhibited the catalase and citrate test. Strain KG4 showed characteristic utilization of the carbohydrate substrates glucose, sucrose, fructose, maltose, xylose, mannitol and lactose. Grows at pH 6-12. Growth rate and intensity at pH 8-10 are better than those at acidic pH. Grows in 0-16% NaCl concentration (enrichment medium) and 0-8% NaCl concentration (minimal medium). The growth temperature range is 28 - 50°C and optimum growth temperature is 33 - 37°C.

The 16S rRNA gene sequence (1466 bases) of strain KG4 was analyzed in order to determine the phylogenetic position of the isolate. It was compared, in terms of sequence similarity, with sequences of previously reported strains, and a phylogenetic tree was constructed using closely related taxa retrieved from the GenBank database by neighbour joining method (Fig. 5).

The results of morphological, biochemical and phylogenetic analysis suggested that strain KG4 is a member of group six[34] (alkaliphile) of the genus *Bacillus* and formed a clade with *B. aerius* 24K (AJ831843) with a bootstrap value of 87%. Pair wise sequence analysis revealed that the highest sequence similarity was with *B. sonorensis* NRRL B-23154

Table 2. Characteristics of *Bacillus* sp. KG4 and some related*Bacillus* species

Characteristics	*Bacillus* sp. KG4	*Bacillus sonorensis* NRRL B 23154[a]	*Bacillus aerius* 24K[b]	*Bacillus mojavensis*[a]	*Bacillus atrophaeus*[a]	*Bacillus aquimaris* TF-12[c]	*Bacillus aerophilus* 28K[b]
Gram stain	+	+	+	+	+	v	+
Cell shape	Rod	Rod	Rod	ND	ND	Rod	Rod
Colony color	White shiny	Yellowish	White	ND	ND	Pale orange yellow	White
Colony shape	Rhizoid	Irregular	Irregular	ND	ND	ND	Irregular
Growth at:							
45°C	+	+	-	+	+	-	-
50°C	+	+	-	+	+	-	-
pH 5	-	ND	-	ND	ND	ND	-
pH 10	+	-	+	ND	ND	-	+
pH 12	+	-	ND	ND	ND	-	ND
Optimum pH range	8-10	5.6	6-10	5.6	5.6	6.0-7.0	6-10
Tolerance of NaCl							
5%	+	-	+	+	+	+	+
7%	+	-	+	+	+	+	+
10%	+	-	+	+	+	+	+
12%	+	ND	-	ND	ND	ND	ND
16%	+	ND	-	ND	ND	+	ND
20%	-	ND	-	ND	ND	ND	ND
Carbon source utilization							
Glucose	+	+	+	+	+	+	+
Sucrose	+	ND	+	ND	ND	+	+
Fructose	+	ND	+	ND	ND	+	+
Maltose	+	ND	+	ND	ND	+	+
Mannitol	W	+	+	+	+	-	+
Xylose	+	+	+	+	+	-	+
Lactose	+	ND	+	-	-	-	+
Citric acid	-	ND	+	ND	ND	ND	+
Methyl red	-	ND	-	ND	ND	ND	-
-Voges-Proskauer	+	ND	+	ND	ND	ND	+
Catalase	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	ND	+
Starch hydrolysis	+	+	+	+	+	+	+
Gelatin hydrolysis	+	ND	+	ND	ND	ND	+

Characteristics are scored as: +, positive reaction; -, negative reaction; w, weak reaction; v, variable; ND, not determined. For all growth assays: +, growth; -, no growth; w, weak growth. Superscript after the taxa denotes the reference from where the data is obtained. a, Palmisano *et al*.[36]; b, Shivaji *et al*.[37]; c, Yoon *et al.*[38].

(94.7%) followed by *B. aerius* 24K (94.1%) and the remaining species with validly published names showed less than 94% similarity. Strain KG4 can be differentiated from other phylogenetic neighbors of previously reported alkaliphilic *Bacillus* species on the basis of morphological, biochemical and physiological characters given in Table 2. The generally recommended and accepted criteria for depicting bacterial species state that strains with a 16S rRNA gene sequence dissimilarity greater than 3% or with a DNA-DNA hybridization relatedness of less than 70% are considered to belong separate species[35]. It is recommended that bacterial strains with difference in 16S rRNA gene sequence of less than 3% cannot be allocated as new species without the support of DNA-DNA relatedness studies. In our study, the bacterial strain KG4 showed difference in 16S rRNA gene sequence of 5.3% with the closely related *Bacillus* species (94.7% sequence similarity with *B. sonorensis* NRRL B-

23154). Thus, on the basis of morphological, biochemical, physiological and phylogenetic results, it is proposed that strain KG4 be classified as the type strain of the novel species, *Bacillus* sp. KG4 nov. The 16S rRNA sequence of strain KG4 was submitted to GenBank-NCBI and assigned accession number JN969986. The culture is deposited at NCIM (National Collection of Industrial Microorganisms), NCL (National Chemical Laboratory) Pune (India) and assigned deposition number NCIM 5440.

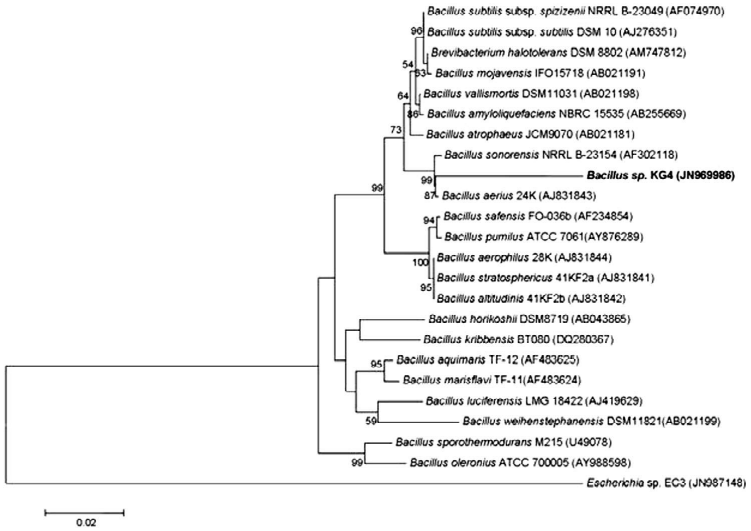

Fig. 5. Phylogenetic tree showing the relationships of the identified bacterial strains and the type strains of closely related Bacillus species, constructed using the neighbour-joining method based on 16S rRNA gene sequences. GenBank accession numbers are given in parentheses. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branch points. Bar, 0.02 nucleotide substitutions per site

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