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SHORT COMMUNICATION

An alkaliphilic and xylanolytic strain of actinomycetes *Kocuria* sp. RM1 isolated from extremely alkaline bauxite residue sites

Pankaj Krishna · Amita Arora · M. Sudhakara Reddy

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Abstract We have isolated and characterized a xylanolytic actinomycete strain (RM1) from the extremely alkaline bauxite residue obtained from National Aluminum Company Ltd., Damanjodi, India. The phenotypic features and complete sequence of 16S rRNA revealed that this strain belong the genus *Kocuria* and showed 98% sequence similarity with *Kocuria aegyptia*. The RM1 strain was able to grow at pH 10.5 in buffered and unbuffered media and utilize 40 different carbon substrates. The RM1 strain under optimal conditions produced extracellular xylanase at 311 U/ml. The xylanase produced by RM1 showed a wide range of temperature (30–85°C) and pH (4.5–9) tolerance by retaining 90% of its activity. This is the first report of isolation of actinomycetes, *Kocuria* sp., which produces high amount of xylanase, from bauxite residue and offers a new source of xylanase-producing strains.

Keywords Actinomycetes · *Kocuria* sp. · 16S rDNA · Xylanase · CMCase · Bauxite residue

Introduction

Xylan is the major structural component of plant cell walls and the most renewable hemicellulose, composed of 1, 4-linked β-D-xylopyranosyl residues. A great deal of attention has been received due to the potential application of xylanases in the food, feed, pulp and paper industries (Wong and Saddler 1992; Niehaus et al. 1999; Beg et al. 2001; Kapoor et al. 2001). Xylanases are hydrolytic enzymes that facilitate the complete hydrolysis of xylan. Many studies have been performed on xylanases from bacteria and fungi, but actinomycetes have been explored to a lesser extent, especially regarding xylanase production (Kansoh and Nagieb 2004), although they are traditionally considered as a rich source of primary and secondary metabolites. Actinomycetes can be an important group of microorganisms involved in lignocellulose degradation in general and xylan in particular (McCarthy 1987).

Red mud (bauxite residue) is waste generated during aluminum extraction from bauxite ore with concentrated sodium hydroxide at elevated temperature in the Bayer process (Evans 1993). The use of NaOH in the Bayer process results in the bauxite residue being extremely saline, sodic and alkaline (Wong and Ho 1991). Reports describing the characterization of xylanase-producing alkalophilic bacteria from an artificial alkaline environment like red mud (bauxite residue) have not been reported in the literature. This is the first report in which we have isolated and characterized a strain of *Kocuria* sp. producing a large amount of xylanase.

Material and methods

Sample collection and isolation

Red mud samples were collected randomly from the red mud pond impoundment of National Aluminium Company Limited (NALCO), Damanjodi, Orissa (India) which is one of the largest alumina producers in Asia. Samples were collected by using sterile equipment and in sterile

P. Krishna · A. Arora · M. S. Reddy
Department of Biotechnology and Environmental Sciences, Thapar University, Patiala 147004, Punjab, India

M. S. Reddy (✉)
Department of Biotechnology, Thapar University, Bhadson Road, Patiala 147004, Punjab, India
e-mail: vasu70@yahoo.com

containers. Samples were kept at 4°C until the experiment was carried out. Red mud was directly plated on nutrient media (pH 10.0), tryptic soya media (pH 10.0) and Horikoshi media by serial dilution method. To determine the xylanolytic behavior of the isolate, it was placed on the nutrient media supplemented with 1.0% oat spelt xylan. The xylan-degrading bacteria were identified on the basis of the halo zone in the agar plate. The plates were then stained with Congo red solution [0.5% (w/v)] for 15 min and destained with 1 M NaCl (Teather and Wood 1982).

Phenotypic characterization

Pure cultures obtained as above were subjected to microscopic examination of the shape and size. Each isolate was characterized by determining its carbon substrate utilization pattern with Biolog GN/GP plate according to the manufacturer's instructions (Biolog Inc., Hayward, USA). The Biolog GN/GP plates were inoculated with 150 µl of cell suspension that had been adjusted to a density of $\sim 3.0 \times 10^6$ cells/ml by comparison with the turbidity standard supplied by the manufacturer. These plates were incubated at 37°C for 24 h. The color development in the micro plate wells was interpreted as positive, negative and borderline (when it was not possible to differentiate positive from negative), using the Biolog microlog GN/GP release 14.01/b database.

A total of 35-carbohydrate fermentation tests were performed according to the manufacturer's direction (Himedia Lab., Bombay, India). Gram stain, catalase, oxidase, citrate utilization, lysine, ornithine, TDA, nitrate reduction, acid phosphatase, urease and H_2S production tests were performed by standard methods. To determine the growth of the bacterial cells in alkaline condition, different buffers were used to adjust the pH of media. All buffer solutions used were prepared and sterilized separately and added to the media to a final concentration of 100 mM. Phosphate buffer, Tris buffer and carbonate–bicarbonate buffer were used to adjust the pH at 7, 8 and 9. Carbonate–bicarbonate buffer was used to adjust the pH 10.5. Different sodium chloride concentrations (0, 2, 5, 7, 10, 12 and 15%) were amended in the alkaline nutrient broth (pH 10) to determine the survival of the bacterial isolates in saline conditions. Growth of bacterial isolates was recorded by measuring the absorbance at 600 nm of 24 h-grown cultures. Four temperatures (25, 30, 37 and 45°C) were chosen to test the growth pattern of bacterial isolates.

16S rRNA gene amplification and sequence analysis

For DNA extraction, overnight-grown bacterial cells from alkaline nutrient broth were harvested and suspended in 800 µl of saline EDTA (0.15 M NaCl and 0.1 M EDTA of pH 8.0) incubated at 37°C for 1 h after addition of crystalline lysozyme ($\sim$100 µg). SDS was added to final concentration of 2.0% and incubated at 65°C for 15 min. Phenol:chloroform:isoamyl alcohol (25:24:1) extraction was performed once and the DNA in aqueous phase was precipitated by isopropanol. The DNA was dissolved in 50 µl of TE buffer (10 mM Tris of pH 8; 1 mM EDTA of pH 8.0) and was checked on a 0.7% agarose gel in 1× TAE buffer.

For amplification of 16S rRNA gene the following primers were used: Forward primer 5′-AGAGTTTGATCCTGG CTCAG-3′ and reverse primer 5′-ACGGGCGGTGTGTTC-3′ (Weisberg et al. 1991). DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA). Reaction mixture for the PCR contained 1× PCR buffer (InVitrogen, USA), each deoxynucleotide triphosphate at a concentation of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µM and 2.5U of Taq DNA polymerase (InVitrogen, USA) in a final volume of 100 µl. PCR conditions were as follows: Preheating at 92°C for 120 s, 36 cycles of 92°C for 60 s, 48°C 30 s and 72°C for 120 s and final extension 72°C for 370 s. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5 µl) on a 1.0% agarose gel in 1× TAE buffer. 16S rDNA amplicon was gel eluted using QIAquick columns (Quiagen Inc., USA) and was ligated into the pGEM-T easy vector as per manufacturer's instruction (Promega Inc., USA). Ligated plasmid was used to transform chemically competent *E. coli* DH5α by heat shock method. After screening of the right clone, the sequence was generated by chain termination method using an Applied Biosystem automated sequencer (Delhi University South Campus, Delhi, India). The 16S rDNA gene sequence was compared with Ribosomal Database Project-II (RDP-II) (Cole et al. 2003) and with those from GenBank using the BLASTN program (Altschul et al. 1997). The sequences were aligned using the CLUSTALW program (Thompson et al. 1997). The evolutionary distance was calculated by Kimura 2 parameter, a phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analysis was based on 1,500 resamplings. The MEGA 4.0 package (Tamura et al. 2007) was used for all analyses. The 16S rDNA gene sequence determined in this study was deposited in GenBank of NCBI data library under accession number EF675625.

Enzyme assays

The culture supernatants from xylan-grown culture were screened for the extracellular enzymes xylanase and cellulase after centrifuging at 7,000×*g* at 4°C for 20 min. Xylanase was assayed according to Biely et al. (1985) using culture supernatant (0.1 ml), 0.9 ml of 1% (w/v) oat

spelt xylan in 0.05 M phosphate buffer (pH 7.0) in a total volume of 1 ml and incubated at 60°C for 15 min. The reducing sugar released was measured according to Miller (1959). One unit of xylanase was defined, as the amount of enzyme required releasing 1 μmol of xylose/min. The reaction conditions for assaying cellulase activity were same as those of the xylanase assay except that the substrates used contained 1.0% carboxymethylcellulose (CMC). The reducing sugars that were released were assayed using the dinitrosalicylic acid method using glucose as the standard. One unit of cellulase activity was defined as the enzyme amount necessary to release 1 μmol of glucose equivalent per minute at 60°C. All the experiments were performed in triplicates.

Results and discussion

Physico-chemical properties of red mud

Red mud samples were collected over 3 years in different seasons from the National Aluminum Company (NALCO), Damanjodi, Orissa. The pH of the bauxite residue was 11.1; electrical conductivity (EC) 4.9 μs/cm; organic carbon 0.0036 mg/g; available P: 0.0014 mg/g and total nitrogen was below detectable level. Predominantly, iron oxide (47%) and aluminium oxide (~7%) were two major constituents of red mud (Krishna et al. 2005).

The high EC, pH and exchangeable Na values are indicative of the extreme salinity, alkalinity and sodicity of the red mud (Hunt and Gilkes 1992). A high concentration of heavy metal oxides makes this environment extremely unfavorable and different from natural alkaline soda lakes. Low levels of organic carbon and essential micronutrients are limiting conditions to grow any heterotrophic population of microbes. Moreover, microbiological analysis of red mud is very scarce. The maximum number of cultivable bacteria was very low and contained a small number of metabolically inactive (injured) bacterial cells that grew in enriched medium but not in minimal media (Hamdy and Williams 2001). In order to isolate metabolically active bacteria, rich medium was used. The isolates unveiled the existence of a few xylan-degrading bacteria in this extreme ecosystem and were phenotypically, biochemically and phylogenetically distinct.

Isolation, characterization and identification of bacteria

A total of 57 bacteria were isolated using different media and growth condition. Out of these, three isolates were prominent in extracellular xylanase production; one strain has been chosen based on its better growth pattern and xylanase production. The isolate was designated as RM1. Pigmentation was rather discrete and appeared pink in color. No diffusible pigments were produced on any media. The RM1 was aerobic, gram variable, catalase and nitrate reduction positive and negative for the oxidase, H_2S production, citrate utilization, urease, dnase production, esculin, lysine, ornithine, TDA hydrolysis and ONPG test. Acid production was noted with maltose, dextrose and ribose sugars. This isolate was able to grow in nutrient media amended with different buffers and at pH 10.5 (Table 1). When subjected to salinity and temperature tests, RM1 survived 0–5% NaCl concentration and a temperature range of 25 to 37°C (Table 1). The RM1 was able to utilize carbon substrates, dextrin, tween 40, tween 80, D-fructose, D-gluconic acid, D-glucose, maltotriose, D-mannitol, D-mannose, palatinose, D-psicose, D-sorbitol, sucrose, D-trehalose, pyruvic acid, glycerol, adenosine, inosine, thymidine, α-cyclodextrin, acetic acid, α-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketovaleric acid, L-lactic acid, D-malic acid, L-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alanine, L-asparagine, L-glutamic acid, L-serine, putrescine, 2′-hydroxyadenosine. Detailed physiological properties of the red mud isolate and the other similar species are given in Table 1. These physiological features show marked differences among the red mud isolate and other similar species.

Carbon substrate utilization pattern of this strain was carried out on Biolog GN/GP plates. Homology searching of the RM1 isolate had low similarities (1%) and no probability match with the existing database of Biolog. Biolog, which is based on carbon substrate utilization pattern to identify microbes faster than the routine cultivation based approach, was used for the identification of the microbes and to analyse the physiological and biochemical diversity of the isolates. The effectiveness of Biolog in identifying Actinomycetes strains was tested and the accuracy of substrate utilization patterns for identification was correct 70% of the time with about 10% error. This is consistent with the previous reports indicating that the identification of non-clinical isolates is often wrong, although the Biolog system is very useful for their phenotypic characterization. In many cases, >70%, but less than 100%, of isolates could be identified (Miller et al. 1993). No similarity match of the RM1 with the existing database revealed the differences in metabolism of different carbon substrates of that of existing *Kocuria* sp. in the Biolog databse. The discrete physiology of this strain may be due to the adaptiveness to the alkaline environment, which is an indication of attainment of novel physiological characteristics in the isolate.

The nucleotide BLAST and RDP-II similarity showed that RM1 belongs to phylum Actinobacteria and family

Table 1 Characteristic physiological features of the aerobic bacterial isolate RM1 of the red mud and comparison with other *Kocuria* species

Characteristics	Red mud isolate RM1	*K. rosea*[a]	*K. aegyptia*[a]
Colony color (Nutrient agar)	Pink	Pink or red	Pink
Catalase	+	+	+
Oxidase	−	−	−
Nitrate reduction	+	+	−
Citrate utilization	−	ND	ND
H_2S	−	−	−
Salinity survival (% NaCl)			
0–5	+	+	+
6–7.5	−	+	−
10	−	−	−
Temperature tolerance (°C)			
25	+	ND	ND
37	+	+	+
45	−	ND	ND
Alkalinity (pH tolerance)			
7–10.5	+	ND	ND
>11.0	−		
Acid phosphatase	+	−	ND
Alkaline phosphatase	+	−	ND
DNase	−	ND	ND
Cellulase	+	ND	ND
Urease	−	−	−
Acid production			
Maltose	+	−	−
Dextrose	+	−	−
Ribose	+	−	−

[a] Data taken from Li et al. (2006)

+, Positive; −, negative; ND, no data available

Micrococcaceae. Phylogenetic analysis revealed that RM1 showed 98% similarity with *Kocuria aegyptia* (Fig. 1). Many actinomycete genera show morphologic variation at different stages of growth and when cultured on different media (Goodfellow 1989). Reliable suprageneric classification of actinomycetes may not be possible using traditional approaches based on a few morphologic and physiologic features. Homology analysis of 16S rRNA provides suitable genetic data that can be used to determine both close and very distant relationships (Bull et al. 1992). It is noteworthy that RM1 16S rRNA did not show high identity with sequences in the database. The clones that had sequence identities of over 98% to a known organism may represent the same species. The sequences that share an identity 88–98% are usually considered to be part of the same genus (Sadowsky et al. 1996). On this basis, the red mud isolate described here probably represents a new member of a known genus, which are capable of xylan hydrolysis. Several physiological features as depicted in Table 1 show its distinction at the phenotypic level while 16S rDNA sequence data reveals its distinction at the genetic level with other *Kocuria* sp.

Enzyme characteristics

The extracellular enzymes produced by this isolate showed xylanase, cellulase, acid and alkaline phosphatase activity. It had xylanase and cellulase activity when grown in xylan as a substrate. This isolate was able to produce 311 U/ml of xylanase and 54.15 U/ml of CMCase when grown in nutrient media supplemented with 0.2% oat spelt xylan.

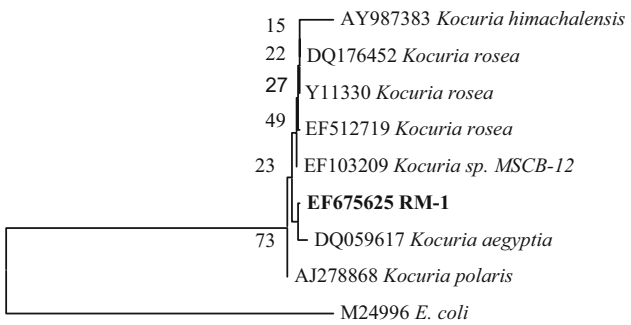

Fig. 1 Neighbor-joining tree based on 16S rDNA sequences of RM1 of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1,500 replicates

Xylanase was reported very low in nutrient media or minimal media supplemented with glucose and oat spelt xylan as substrate (Fig. 2). Both xylanases and cellulases are induced by monosaccharides and disaccharides. Cellobiose is thought to be the inducer of these enzymes in natural conditions (Royer and Nakas 1989; Nogawa et al. 2001). Our results also showed induction of this enzyme by cellobiose. The production of xylanase/CMCase activity was low in nutrient medium which indicates secretion at basal level. When nutrient media was supplemented with CMC, the production of this enzyme was very low. This is probably due to it not being preferred over nutrient medium and the hydrolysis of the CMC was not achieved, which can serve as an inducer of the enzyme, as the rate of enzyme synthesis depends on the hydrolysis of cultivation substrates (Ooshima et al. 1990). Production of xylanase and CMCase were 163 and 119 U/ml when cellobiose was used as substrate. The production of CMCase on cellobiose was higher compared to other substrates. The crude xylanase preparation from RM1 showed activity over a wide range of pH (4.5–9.0) and temperature (30–85°C) optima while retaining 90% of its activity (Fig. 3a, b). The initial pH of the medium has played an important role for the production of xylanase. At neutral pH it produced more xylanase as compared to pH 10.0. This isolate showed a broad range of pH and temperature optima of extracellular xylanase. Some actinomycetes have shown xylanase pH stability between 6.5 and 10.0 (40°C) and 5.0–8.0 (30°C) from *Micrococcus* sp. AR-135 (Gessesse and Mamo 1998) and *Streptomyces chattanoogensis* CECT 3336 (Fernández López et al. 1998), respectively. The optimum activity and stability of crude xylanase in *Bacillus subtilis* C 01 was observed at 50°C (Ayyachamy and Vatsala 2007). Similar temperature optimum for xylanases has also been reported in *Bacillus* spp. and *Staphylococcus* sp. (Kulkarni and Rao 1996; Gupta et al. 2000; Damiano et al. 2003). The crude enzyme also had broad pH optima and stability between 6.0 and 8.0 and retained 77 and 56% of enzyme activity at pH 9 and 10, respectively. The optimum pH for xylanase activity from 7.0 to 9.0 has been found in many bacterial strains (Duarte et al. 2003). Interestingly, xylanase from RM1 showed wider range of pH (4.5–9.0) optima. Xylanase from this isolate had thermal stability above 70°C.

Several physiological features as depicted in Table 1 shows its distinction at the phenotypic level, while the 16S rDNA sequence data reveals its distinction at the genetic level with other *Kocuria* sp. No *Kocuria* sp. has been reported to degrade xylan. Isolation of novel alkaliphilic bacteria from different environments may result in the finding of extracellular enzymes with novel properties that could be useful for diverse industrial applications. These results confirm the identity of the new strain of *Kocuria* sp. from red mud, which is the first report of isolation and production of xylanase from *Kocuria* sp. Further, studies are required to clone and characterize the xylanase gene and its product for application in various industries.

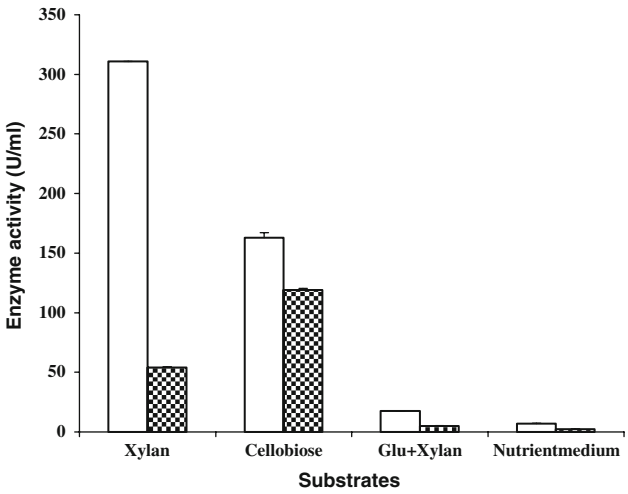

Fig. 2 Xylanase (empty bar) and CMCase (filled bar) production by red mud isolate RM 1 with different substrates. Error bars are ±SD

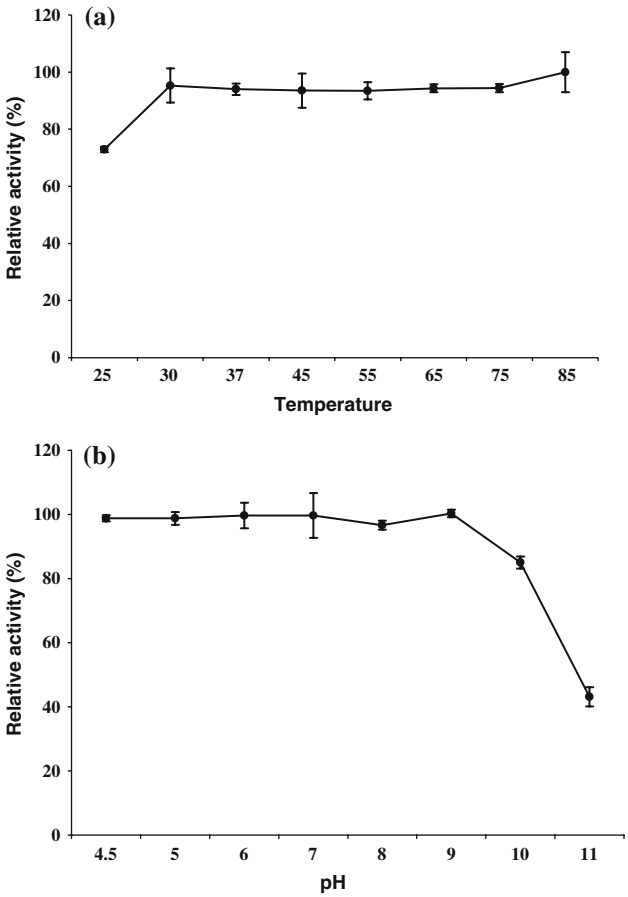

Fig. 3 (**a**) Thermal stability and (**b**) Alkaline stability profile of xylanase from the red mud isolate RM1. Error bars are ±SD

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