JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 743-747

Molecular Characterization and Identification of an Alkalophilic Bacterial Strain Isolated from a Local Hotspring Atri, Khurda District, Odisha, India

S.P. Nayak[1]*, P. Ray[1], P.K. Sahoo[2]

[1]Department of Microbiology, Orissa University of Agriculture & Technology, Bhubaneswar 751003, Odisha, India.
[2]Fish Health Management Division, CIFA, India.

(Received: 09 April 2013; accepted: 31 May 2013)

The present study was conducted to isolate, identify, characterize and to determine the enzymatic activities of the thermophiles from Atri hot spring of odisha. The optimal temperature for growth of these isolates was 55 °C and the optimal pH was 8. These bacterial cells were Gram positive rods and endospore forming. All the strains were amylase, catalase and oxidase positive but gelatinase and caseinase negative but isolate A1 showed the best amylase producing. The phenotypic characterization of those isolates was confirmed by genotypic method using 16S rDNA sequence analysis. Maximal homology of isolate A1 to genus *Geobacillus* was observed. Isolate A1 showed 96% homology with Geobacillus *sp. WCH 70* (Accession no. NC 012793.1). Therefore, 16S rDNA gene sequence analysis can be considered as a valuable genotypic tool for the identification and characterization of this thermophilic bacterium at genus level. Moreover, enzymatic products of these isolates could receive considerable attention due to their potential applications in biotechnology.

Key words: Thermophiles, Enzymes, Hot spring, 16S rDNA.

Microorganisms occupy all possible environments including habitats offering ideal conditions for growth and extreme environments. Extremophiles, thermophiles in particular, are getting recognition mainly due to their attractive attributes in biotechnology. The discovery of different life forms at elevated temperatures and the isolation of *Thermus aquaticus* bacterium producing *Taq* DNA polymerase, which has a commercial success because of its uses in polymerase chain reaction (PCR) technology, led scientists to isolate and identify microorganisms from the worldwide geothermal sources. Thermophilic microorganisms are adapted to grow at high temperatures and they are separated into three categories; moderate thermophiles, extreme thermophiles and hyperthermophiles[1, 2]. Thus, they can be isolated from high temperature terrestrial and marine habitats including volcanically and geothermally heated hydrothermal vent systems[2].

Interest in thermophilic bacteria from the genus *Bacillus* has clearly emerged during the past two decades because of their significant potential for biotechnological applications including their importance as sources of thermostable enzymes (such as proteases, amylases, lipases, xylanases, cellulases and DNA restriction endonucleases), and other products of industrial interest[3-6]. Thermophilic bacilli grow best at temperatures between 45 and 70°C, and were isolated from different environments including hot springs, petroleum reservoirs, deep-sea hydrothermal vents and deep ocean-basin cores [7, 8]. Recently, Nazina

* To whom all correspondence should be addressed.
E-mail: saritanayak01@gmail.com

et al. (2001) grouped Gram positive, rod-shaped, endospore-forming thermophilic bacilli into the genus *Geobacillus*. The *Geobacillus* species are widely distributed and readily isolated from different habitats [9, 10], with a continually increasing industrial interest for their thermostable gene products [11, 12]. Therefore, studying phylogenetic relations and diversity in this novel bacterial genus is not only a taxonomical concern, but also a necessity in order to exploit its biotechnological potential completely.

MATERIALS AND METHODS

Isolation of the strains

Water sample was collected from Atri hot spring and analyzed microbiologically by 10-fold serial dilution method and spread on pre-sterilized Nutrient Agar (NA-HiMEDIA Ltd., Mumbai, India) plates and then, the plates were packed in polythene bags to avoid drying and were incubated at 55 °C for 24–48 h. After incubation, different colonies developed in the media which were selected based on their morphological characteristics and purified by sub-culturing. Isolated and purified bacterial strains were made glycerol stock and stored at -20°C for further studies.

Conventional identification tests

All isolates were initially evaluated by conventional tests i.e. Gram stain, growth and morphometric characteristics on nutrient agar medium, growth at different temperature and pH, and various biochemical tests.

Screening of thermoalkaline amylase producing bacterial cultures

The water sample of atri hot spring is slightly alkaline, which supports rich and diverse microflora. All the 12 isolates were assessed for their extracellular amylase activity. The strains were screened on solid agar media at 55°C in triplicates. Freshly grown cultures of test bacteria were spot inoculated on starch agar plates by the help of a sterile loop. Amylase activity was observed by incubating the plates and exposing to iodine vapours[13].

Genomic DNA isolation and quantification

1.5 ml of bacterial culture was centrifuged at 11,700rpm for 2 min. Supernatant was decanted. The pellet was collected and 1ml of TENS (10Mm Tris-cl, 1Mm EDTA, 0.5%SDS, 0.1M NaoH) was added and vortex properly. Incubated at 37°C for 30 mins. 4 µl RNase A(10mg/ml) was added and incubated at 37°C for 30min. To it, 25µl proteinase k was added and incubated at 37°C for 30 min. 0.5ml Tris saturated phenol was added and mixed by inverting, centrifuged at 10,000rpm for 10 mins. Upper aqueous phase was collected to a fresh micro-centrifuge tube and 0.5ml of equal volume of phenol/chloroform/isoamyl alcohol was added and centrifuged at 10,000rpm for 10mins.upper aqueous phase was collected and to it 1/10 vol of sodium acetate and 2.5 vol of chilled ethanol was added. Inverted twice and stored at -30°C for 1 hr. Again centrifuged at 13,000rpm for 15mins. pellet was washed with 70% ethanol and dried. Finally the pellet was dissolved in TE buffer (50µl). Total DNA concentration and purity were determined by measuring the OD at 260 and 280 nm using Nano Drop ND1000 (Nano Drop Technologies Inc., USA).

PCR amplification, PCR product purification and cloning

The 16S rDNA sequence was amplified using 16S universal primers. Each PCR reaction consisted of 40.70µl dH_2O, 5µl 10× PCR buffer (Genei, India), 1µl 10mM dNTPs (Chromous Biotech), 1µl (10pmol) of each forward and reverse primer, followed by 0.3µl(1.5U) *Taq* DNA polymerase(Genei, India) and 1µl genomic DNA. The amplification profile was 95^0C for 3 min followed by 45 cycles of denaturatuon for 30s at 95^0C, at an annealing temperature of 46°C for 1 min and extension at 72°C for 1 min, followed by a final extension for 10 min at 72°C. The generated PCR product (8µl) was then analysed by electrophoresis on 1% agarose gel. The PCR product was purified using PCR product purification kit (Genei, India). The generated amplicons for newly synthesized genes were purified by PCR product purification kit (Genie, India) and subcloned into pGEMT vector (Promega) as per manufacturer's instructions. The plasmid DNA from the above was further purified by standard phenol-chloroform extraction method before sequencing. Sequencing was done using the cycle sequencing kit (Bigdye Terminator V.3.1, ABI, USA) with T7 universal primer (New England Biolab) in 310 Genetic Analyzer, ABI, U.S.A.

RESULTS

A total 12 strains of thermophilic bacilli were isolated from water samples of Atri hot spring in odisha. These isolates showed optimum growth at 55 °C and pH 8. The investigation of these strains in terms of morphology, biochemical and physiological properties were shown in Table.1.

Detection of thermoalkaline amylase secretion

Twelve bacterial isolates producing variable amylolytic zones on starch agar plates were isolated from the water samples. The zones of clearance by isolates reflect their extent of amylase activity. All the bacterial isolates exhibited good

Table 1. Morphological, biochemical and physiological characteristics of thermophilic bacterial isolates from Atri hot spring in odisha

Profiling	Bacterial isolates											
Morphological	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Gram nature	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Cell morphology	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Motility	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
Endospore forming	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
BiochemicalIndole	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
MRVP	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
VP	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
ONPG	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Anaerobic Growth	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Amylase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Urease	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Caseinase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Gelatinase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Growth profiling												
Growth at Temp- 45°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
50°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
55°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
60°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
65°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
70°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
80°C	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
90°C	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Growth at pH - 7	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
8	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
9	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
10	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Sugar utilisation												
Fructose	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Trehalose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Sucrose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Inulin	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Xylose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Galactose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Note: + positive; - negative

amylase activity on starch agar plates (pH 8) and at temperature 55 °C. Among all bacterial isolates, the isolate A1, showing maximum clearance zone diameter was selected for molecular studies.

Molecular studies

Genomic DNA extraction and quantification

Genomic DNA was extracted from isolate A1and the quality of the DNA from the sample(A1) isolated from Atri hot spring as examined by taking OD260 nm/OD280 nm was found to be 1.6 using Nano Drop ND1000 (NanoDrop Technologies Inc., USA).

16s rDNA data analysis

Amplified 16S ribosomal DNA fragment of about 1500 bp size was obtained from strain A1 using 16S F/R universal primers. The obtained sequence was then compared to sequences available in GenBank using the NCBI-BLAST program. Nucleotides BLAST search of the 16S rDNA sequences showed that the isolate had 96% identity with *Geobacillus sp. WCH 70*(Accession no. NC 012793.1).

DISCUSSION

Our earth harbors a huge number of harsh environments that are considered as "extreme" from an anthropocentric point of view, as far as temperature, pH, osmolarity or pressure are concerned. However, these peculiar biotopes have been successfully colonized by numerous organisms, mainly extremophilic bacteria and archaea. The potential biotechnological use of thermophilic bacteria and their thermostable enzymes has led to extensive isolation studies in a wide variety of thermophilic environments. Among these environments, hot springs were explored as a potential source of thermophilic bacteria by many researchers all over the world. Narayan *et al*., (2008) isolated aerobic thermophilic bacteria from the Savasavu hot spring in Fiji. Lu *et al*., (2009) isolated thermophilic anaerobic bacteria from hot springs in Tengchong Rehai. Al- Batayneh *et al*., (2011) isolated *Geobacillus pallidus* and *Anoxybacillus flavithermus* from different hot springs in Jordan. So in this study, an alkalophilic thermophilic bacterium from Atri hot spring, Odisha was isolated. The isolate (A1) was identified by morphological analysis, biochemical tests and 16S rDNA sequence analysis. Strain A1 was gram positive rod. Strain A1 gave positive tests for amylase activity. The results of biochemical tests were compared with the characteristics given in Bergey's manual and they indicated that the strain A1 belongs to *Geobacillus.* 16S rRNA sequence analysis revealed the strain A1 shared 96% sequence similarity with its closest relative i.e., *Geobacillus WCH70.* So sequence similarity of 96% suggests that the strain A1 characterizes a new species of *Geobacillus* but some more biochemical and molecular analysis is still required.

CONCLUSION

In conclusion, the thermal environment, Atri hotspring, Odisha, is a good source of thermophilic micro-organisms with promising biotechnological potential since the isolated thermophilic and alkalitolerant bacterial strain that produce hydrolytic enzymes under thermophilic conditions.

ACKNOWLEDGEMENTS

The authors would like to acknowledge laboratory staff of P.G Department of Microbiology (OUAT) and Fish Health Management Division, CIFA, Bhubaneswar, for their co-operation.

REFERENCES

1. Baker G, Gaffar S, Cowan DA, Suharto AR., Bacterial community analysis of Indonesian hotsprings. *FEMS Microbiol. Lett*. 2001; **200**: 103-109.
2. Bertoldo C, Antranikian G., Starch-hydrolyzing enzymes from thermophilic archea and bacteria. *Curr. Opin. Chem. Biol.* 2002; **6**: 151-160.
3. Bergquist PC, Morgan HW., The molecular genetics and biotechnological application of enzyme from extremely thermophilic eubacteria. In: Herbert RA, Sharp RJ (eds) Molecular biology and biotechnology of extremophiles. Chapman and Hall, New York, 1992; 44-45.
4. Maugeri TL, Gugliandolo C, Caccamo D, Stackebrandt E., A polyphasic taxonomic study of thermophilic bacilli from shallow, marine vents. *Syst. Appl. Microbiol*. 2001; **24**: 572-587.
5. Rainey FA, Fritze D, Stackebrandt E., The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. *FEMS Microbiol. Lett.* 1994; **115**: 205-212.

6. Sharp RJ, Riley PW, White D., Heterotrophic thermophilic bacilli. In: Kristjansson JK (ed) Thermophilic Bacteria. CRC Press, Boca Raton, 1992; 19-50.
7. Bae SS, Lee JH, Kim SJ., *Bacillus alveayuensis* sp. nov., a thermophilic bacterium isolated from deep-sea sediments of the Ayu Trough. *Int. J. Syst. Evol. Microbiol*. 2005; **55**: 1211-1215.
8. Rahman TJ, Marchant R, Banat IM., Distribution and molecular investigation of highly thermophilic bacteria associated with cool soil environments. *Biochem. Soc. Trans*. 2004; **32**: 209-213.
9. Meintanis C, Chalkou KI, Kormas KA, Karagouni AD, Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island. Biodegradation, 2006; **17**: 105-111.
10. Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV., Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov., and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans*. *Int. J. Syst. Evol. Microbiol.* 2001; **51**: 433-446.
11. Lama L, Calandrelli V, Gambacorta A, Nicolaus B., Purification and characterization of thermostable xylanase and beta-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus. Res. Microbiol*. 2004; **155**: 283-289.
12. Schallmey M, Singh A, Ward OP., Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol*. 2004; **50**: 1-17.
13. Rath, C.C.; Subramanyam, V.R., Enhanced protease and beta-Lactamase activity by immobilization of a thermophilic *bacillus sps.* Isolated from a local hotspring in Orissa, India. Proceeding of national conference in *recent trends in biotechnology,* 2000.
14. Narayan, V.V., M.A. Hatha, H.W. Morgan and D. Rao., Isolation and characterization of aerobic thermophilic bacteria from Savusavu hot spring in Fiji. *Microbes Environ.* 2008; **23**(4): 350-352.
15. Lu, Y., B. Chen, X. Liu, X. Ji, Y. Wei and L. Lin., Isolation and identification of seven thermophilic and anaerobic bacteria from hot springs in Tengchong Rehai. *Wei Sheng Wu Xue Bao*., 2009; **49**(9): 1234-1239.
16. Al-Batayneh, K. M., J.H. Jacob and E.I. Hussein., Isolation and molecular identification of new thermophilic bacterial strains of *Geobacillus pallidus* and *Anoxybacillus flavithermus*. *Int. J. Integ. Biol.*, 2011; **11**(1): 39-43.