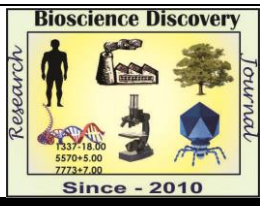
Print & Online, Open Access, Research Journal Available on http://jbsd.in
ISSN: 2229-3469 (Print); ISSN: 2231-024X (Online)
Research Article



Thermophiles: Isolation, Characterization and Screening for Enzymatic Activity

Jean Bosco Nshimiyimana[1,2], Sujan Khadka*[2,3], Esther Muhindo Mwizerwa[1], Nathan Akimana[1], Sanjib Adhikari[3], Antoine Nsabimana[1]

[1]Department of Applied Biology, College of Science and Technology, University of Rwanda, Kigali, Rwanda
[2]Department of Biochemistry and Molecular Biology, School of Life Sciences, Central China Normal University, Wuhan, Hubei, P.R. China
[3]Department of Microbiology, Birendra Multiple campus, Tribhuvan University, Chitwan, Nepal
*E-mail: sukha11@mails.ccnu.edu.cn

Article Info

Received: 19-04-2018,
Revised: 22-06-2018,
Accepted: 02-07-2018

Keywords:
Bacillus spp.,
Nyamyumba hot spring,
Thermophiles,
Thermostable enzymes,
Thermus aquaticus

Abstract

In biotechnology, the significance of several thermo-stable biomolecules has encouraged to perform many researches into organisms capable of growing at higher temperature. With the major objectives to isolate and characterize thermophiles from Nyamyumba hot spring, Rwanda, 8 hot water samples were collected in sterile vacuum flasks that allow samples to keep their temperature. Samples were processed in Nyarugenge campus laboratory within 2 hours after collection and their physiochemical properties were studied. A loopful of samples was streaked on both nutrient and thermus agar plates and incubated at 40ºC, 50ºC, 60ºC, 70ºC, 80ºC and 90ºC for 48 hours to evaluate their thermo-tolerant characteristics. Morphological and biochemical analysis (ortho-Nitrophenyl-β-D-Galactopyranosidase (ONPG), indole, catalase, cytochrome oxidase, urease and sugar fermentations tests-glucose, lactose, sucrose, mannitol, sorbitol, inositol and arabinose) were performed for two remarkably different isolates (Isolate-I and Isolate-II). In addition to this, Gram staining, capsule staining, spore staining, and wet mount tests were also performed. Enzymatic assays (protease and amylase tests) were done to investigate the ability of the isolates to break down proteins and carbohydrates. Both the isolates grew well at pH range 7–8.5, and temperature range of 60-70°C. With referencing Bergey's manual and by morphological, cultural, biochemical, thermotolerant and enzyme producing abilities isolate-I was found to be *Bacillus* spp. and isolate-II was found to be *Thermus aquaticus*.This finding indicates bacteria producing thermostable enzymes are present in Nyamyumba hot spring and study furthering strain selection and identification would pave the way for commercial enzyme productions.

INTRODUCTION

Climatic and geographical diversity in Rwanda has unquestionably gifted to multiple biological diversities including thermophilic microorganisms. Such microbial array is always a motivation for the scientific community to investigate for the remarkable microbial potency and of all; thermophilic microbes producing enzymes are highly emphasized which can be widely used for industrial and pharmacological purposes. Thermophiles are heat-loving bacteria; with an average optimum temperature of 70^0C (Zierenberg *et al.*, 2000), while hyperthermophiles require very high temperature between 80^0C to 105^0C for their growth (Bertoldo and Antranikian, 2002). Scientists took a great interest in thermophilic bacteria

since the discovery of *Thermus aquaticus* from Yellowstone national hot spring (Brock and Hudson, 1969).

Today, the researches including phenotypic and genotypic characterization of thermophilic bacteria have been exceled in different geothermal areas of this globe, including Turkey (Adiguzel *et al.*, 2009), Italy (Maugeri, 2001), Bulgaria (Derekova *et al.*, 2008), Greece (Sievert *et al.*, 2000), China (Lau *et al.*, 2009), India (Sharma *et al.*, 2008), Iceland (Takacs *et al.*, 2001) and Morocco (Aanniz *et al.*, 2014).

Thermophilic bacteria are less studied because they are difficult to isolate and handle in the laboratory. Hence their diversity, morphological, biochemical as well as molecular characteristics yet remain to be explored. During last three decades many species of thermophilic bacteria have been isolated and majority of them belong to domains of bacteria or archaea (Madgan and Martinko, 2010).

In recent days, several advanced techniques of molecular biology and particular sequencing development have boosted the further researches in the classification of thermophilic bacteria at species and subspecies level and also to study their evolutionary relationship with other organisms (Haki and Rakshit, 2003; Becker *et al.*, 1997). Many biochemical tests have been introduced with the objective of evaluating the enzyme activities of thermophilic bacteria and also their identification (Panda *et al.*, 2013). Several researchers are keen to explore them intensively for isolation of numerous thermostable enzymes which are not just limited to DNA polymerase, lipase, and protease etc. (Singh *et al.*, 2010). Further researches are going on to investigate their potency to produce several new biotechnologically significant thermozymes.

Very few studies have been done in Africa regarding isolation, characterization and screening of thermophiles for enzymatic activities before. This is probably the first report of isolation and characterization thermophilic bacteria from a hot spring at Nyamyumba. The aim of this study was to isolate and characterize thermophilic bacteria from hot spring of Nyamyumba, Rubavu district, Western province, Rwanda by cultural, morphological and physiological and biochemical methods.

MATERIALS AND METHOD

Study area

In Rwanda, there are numerous small to large hot water springs, among which two are the major type

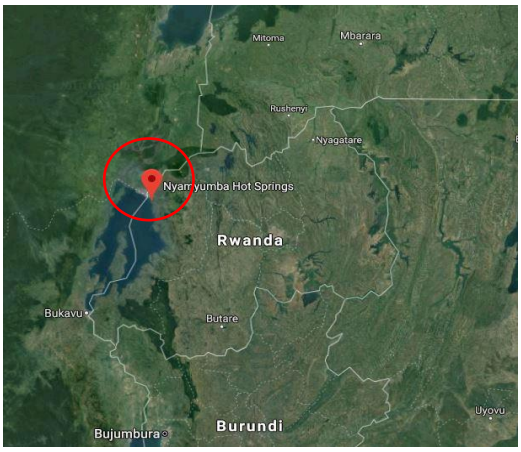


Figure 1: Showing the location of study area-Nyamyumba hot spring

of hot-water springs-Nyamyumba hot spring and Bugarama hot spring. Our study site was Nyamyumba hot spring located in the North-West part of Rwanda, Western province, Rubavu district. It is one of the largest hot springs of Rwanda. Rwanda is situated in central-East Africa and is surrounded by Uganda, Burundi, Tanzania, and Democratic Republic of Congo (DRC) in North, South, East and West respectively. Nyamyumba hot spring is situated at 20° 14' 49.38" E Longitude and 85° 19' 11.04"N Latitude. The temperature of the hot spring during the sampling period was about 55°C, and the pH was found to be 7-8. No scientific research has been carried out to find out why the water temperature is so high, however, it is believed to be caused by the neighboring active volcanoes from DRC.

Collection of samples

Eight hot water samples were collected from different sites of Nyamyumba hot spring in sterile vacuum flasks which are able to keep the temperature constant. The samples were brought in the laboratory within 2 hours of collection for processing. Physical and chemical properties of water samples were tested and presence of heavy metals and nutrients were also determined.

Isolation and determination for thermo-tolerance of isolates

Samples were initially inoculated under sterile conditions on both nutrient agar and thermus agar media. The nutrient agar (NA) is general media which allows the growth of a wide range of microorganism whereas thermus agar (TA) medium is specific for growth of thermophilic bacteria only. After culturing, the plates were incubated at different temperatures 40ºC, 50ºC, 60ºC 70ºC, 80ºC and 90ºC for 48-72 hrs.

Identification of isolates

Thermophilic bacteria were identified using different techniques such colony color, morphology observation and other biochemical tests such as Gram staining, spore staining, capsule staining and enzymatic activities like (ONPG, catalase, indole, urease, cytochrome oxidase tests) and their fermentative activities on sugars (glucose, sucrose, lactose, mannitol, sorbitol, inositol and arabinose).

Determination of OD density

Incubation was done at 40, 50, 60, 70, 80, 90 °C and temperature ranges for growth were determined. Isolates were incubated overnight (16 hours) at 60±1°C in Luria-Bertani (LB) medium maintained to different pH (4, 5, 6, 7, 8, 9 and 10) separately (Antón *et al.,* 2002) and finally pH ranges were studied. A double beam UV/VIS scanning spectrophotometer was used to determine optical density at 600nm.

Enzyme assay

The enzymatic activities of the isolates were determined by two tests: protease and amylase tests. To determine protease activity, gelatin agar (GA) medium was prepared and the nutrient broth culture of bacterium after 24 hrs of incubation was streaked on the gelatin agar plates. The GA plates were incubated at 50°C for 24-48 hrs after inoculation. After proper incubation, $HgCl_2$ solution (15.0% $HgCl_2$ in 20.0% HCl) was heavily flooded over the plates and was observed for clear zone around the colony. Similarly, amylase activity was determined in starch agar (SA) medium. SA plates were prepared and inoculated with the bacterial isolate. The plates were incubated at 50°C for 24-48 hours and were observed for clear zone near inoculated colony after treating with iodine solution.

RESULTS AND DISCUSSION

In our study, 8 water samples were collected from hot water spring of Nyamyumba and their physiochemical properties were analyzed. The temperature and pH of water at collection site were found to 7-8 and 40-51^{o}C respectively and were colorless with unpleasant smell. Various nutrients such phosphates, sulphates and heavy metals were also detected in the water samples as shown in the (Table 1). After 3 days of incubation, colonies were counted, observed and recorded. Colonies of different size and color were observed on the agar plates; including big white colonies and small brownish and yellowish colonies. All the colonies on culturing medium were found to be round, raised and entire. Selection of two isolates-I and II were based on the different colors and sizes of colonies; isolate I being whitish and bigger colony while isolate II being brownish and yellowish colored colony with smaller size than isolate I (Figure 2). These isolates were further subcultured on nutrient agar to obtain them in pure form.

On morphological analysis by wet-mount and Gram staining, both isolates-I and II were found to be rod shaped. Isolate-I was motile whereas isolate-II was found to be non-motile. Isolate I was Gram positive rods whereas isolate II is Gram negative rods. Capsules were absent in both isolates but only isolate-I possessed spores. Both isolates showed negative indole test. Similarly, both isolates gave positive ONPG (producing permease and β-galactosidase), catalase, oxidase and negative urease tests. In the same way, both isolates showed positive results for sugar fermentation tests like, glucose, lactose, sucrose, mannitol, inositol, arabinose fermentation. Both isolates grew well at temperature range of 60-70°C (Figure 3). Their optimum growth was noted at pH range 7-8.5 (Figure 4).

A study conducted by Sethy and Ray, 2011 from hot spring at Atri, Odisha, reported 20 isolates of which 11 (55.0%) were Gram negative rods, 6 (30.0%) were Gram positive bacilli and 3 (15.0%) were fungi. In the present study, isolate I was found to be Gram positive rod whereas isolate II was found to be Gram negative rod. Microorganisms play significant role in the bio-geochemical cycling of heavy metals and also in purifying metal contaminated environments (Nayak, 2013). In our study, the isolates were found to be well grown at different concentrations of metal ions such as Iron (0.06 mg/l), Copper (0.02 mg/l), Zinc (0.01 mg/l) and Manganese (0.0119 mg/l).

Thermophiles also produce several thermostable enzymes such as ά-amylase, cellulose, ß-glucosidase, ß-galactosidase, protease, pullulanase and xylanase (Nayak, 2013) which can be exploited for industrial purposes (Cowan, 1996). Similarly, a study conducted in the hot spring Tarabalo, Nayagarh District, Orissa, India by Mohanta and Rath showed that several extracellular enzymes were isolated from thermophilic bacteria (Mohanta and Rath 2010). In our study, both isolates were found to show proteolytic and amylolytic activity at 50°C as these isolates were capable of producing protease as well as amylase enzymes. The gelatin protein in gelatin agar was degraded by the gelatinase enzyme produced

by these thermophiles, therefore; on flooding $HgCl_2$ solution over the incubated plates, clear zones were observed around the growth of bacteria where gelatinase was diffused and remaining undiffused part showed clear precipitation zones **(**Figure 5). This is because $HgCl_2$ cannot precipitate degraded gelatin but can precipitate non-degraded gelatin. Forgarty and Kelly, 1979 and Iverson and Millis, 1974 found that the starch nutrient agar and iodine can be used for detecting amylase (hydrolytic enzyme) producing microorganisms that can hydrolyze starch on starch agar plates forming clear zone surrounding an area of colony. In the same way, these isolates also hydrolyzed starch by producing amylase so the clear zones were observed around the bacteria and remaining portion turned blue in color when treated with iodine solution as iodine can only physically combine with non-hydrolyzed starch to form starch-iodine complex and give blue color but it cannot combine with hydrolyzed starch (Figure 6). These thermo-stable amylase and protease are highly useful to particular industries that employ elevated temperatures. For example, pulp and paper, food, brewing, and feed processing industries (Urbieta *et al.*, 2015).

As described by Gordon *et al.* 1973, Souza and Martins 2001, and Mathew and Rathnayake 2014, morphological, biochemical and cultural characteristics for the isolate-I were similar to the characteristics of the genus *Bacillus* and isolate-II was similar to the isolate described by Brock and Hudson 1969 as thermophilic bacterium *Thermus aquaticus.* Based on these findings and with the help of Bergeys Manual, isolate-I was found to *Bacillus* spp. and isolate II *Thermus aquaticus.*

Advanced techniques like electron microscopy as well as comparison of base sequences of rRNA and other molecular biological tools can be used to determine the exact species of the isolates. Further, both of these isolates showed the potent capability to produce extracellular enzymes which could be exploited for pharmaceutical and industrial purposes.

Table 1. Physiochemical properties of Nyamyumba hot spring water

Attributes	**Parameters**	**Results**
Physical properties	Temperature	40-51^{o}c
	PH	7-8
	Color	Colorless
	Smell	Unpleasant
Chemical properties	Electrical conductivity (E.C)	1760 µs/cm
	Total Suspended Solids (TSS)	3mg/l
	Total Dissolved Solids (TDS)	880ppm
	Total Hardness	468mg/l
	Calcium Hardness	76mg/l
	Magnesium Hardness	19.34mg/l
	Salinity	0.89%
Nutrients	Phosphate	0.12 mg/l
	Nitrate	0.5 mg/l
	Nitrite	0.001 mg/l
	Sulphate	2.0 mg/l
	Fluoride	0.57 mg/l
Heavy metals	Iron	0.06 mg/l
	Copper	0.02 mg/l
	Zinc	0.01 mg/l
	Manganese	0.119 mg/l

Table 2. Observation of the primary culture

Samples	Plates	Number of colonies	Colonial shape	Colonial elevation	Colonial edge	Colonial color
A	1	89	Round	Raised	Entire	Brownish
	2	97	Round	Raised	Entire	Brownish/ yellowish
	3	53	Round	Raised	Entire	Brownish/yellowish
	4	32	Round	Raised	Entire	Whitish
	5	115	Round	Raised	Entire	Brownish
B	6	26	Round	Raised	Entire	Whitish
	7	75	Round	Raised	Entire	Brownish/yellowish
	8	43	Round	Raised	Entire	Brownish/ whitish
	9	97	Round	Raised	Entire	Whitish
	10	111	Round	Raised	Entire	Yellowish

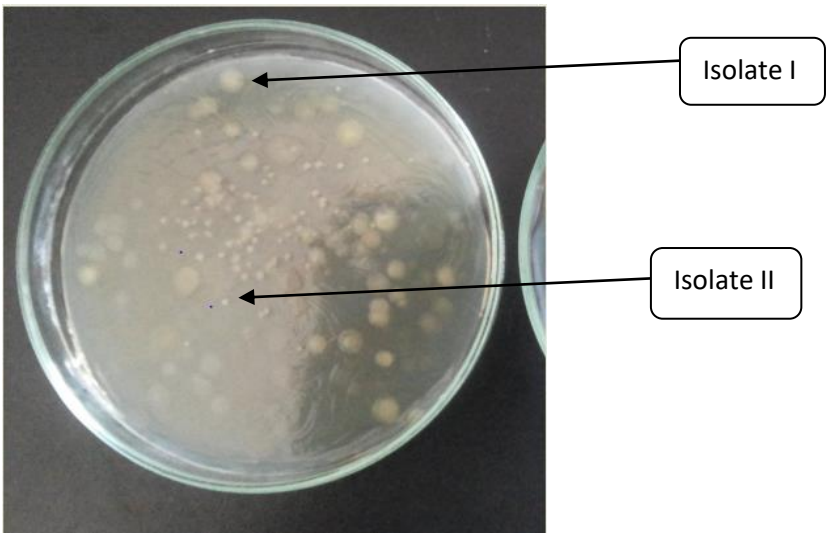


Figure 2: Plate of primary culture

Table 3: Summary of morphological and biochemical results

Test	Observation (Isolate I)	Observation (Isolate II)
Wet mount	Rod shaped (Motile)	Rod shaped (Non- motile)
Gram staining	+ rods	-rods
Capsule staining	Negative	Negative
Spore staining	Positive	Negative
Indole test	Negative	Negative
Catalase test	Positive	Positive
Sugar fermentation tests (glucose, lactose, sucrose, mannitol, sorbitol and inositol)	Positive	Positive
Ortho-Nitrophenyl-β-D-Galactopyranosidase (ONPG)	Positive	Positive
Cytochrome oxidase	Positive	Positive
Urease	Negative	Negative

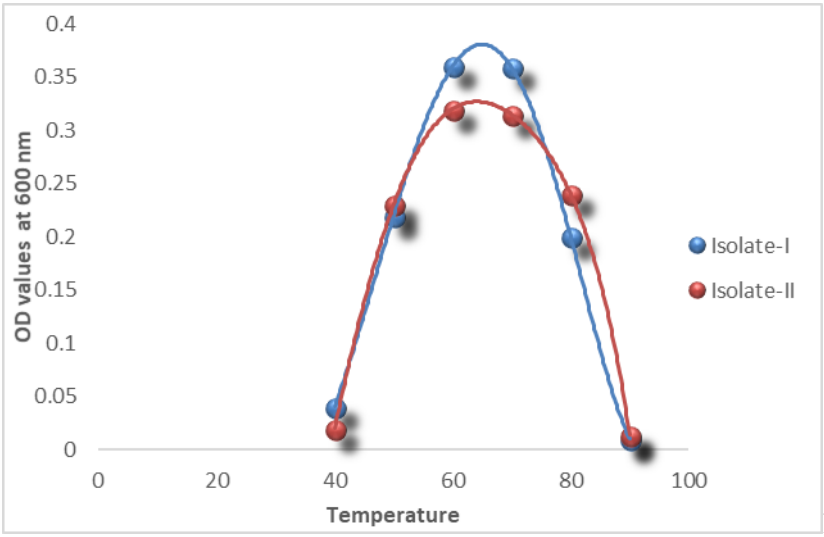


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Figure 3: Effect of Temperature on the growth of thermophilic bacterial isolates-I and I

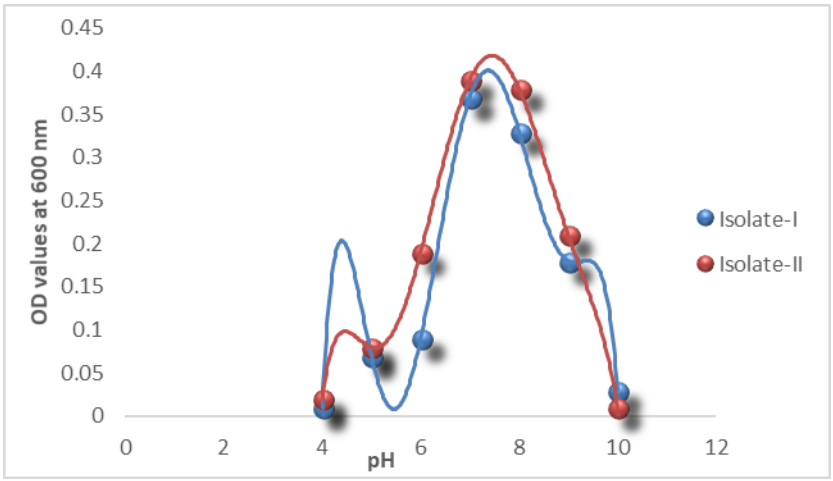

Figure 4: Effect of pH on the growth of thermophilic bacterial isolates-I and II

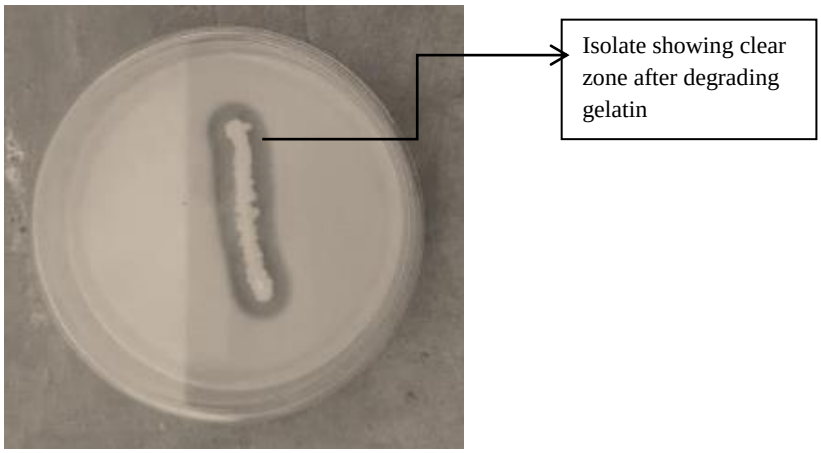

Figure 5: Clear zone formation around the growth area in Gelatin agar at 50°C.

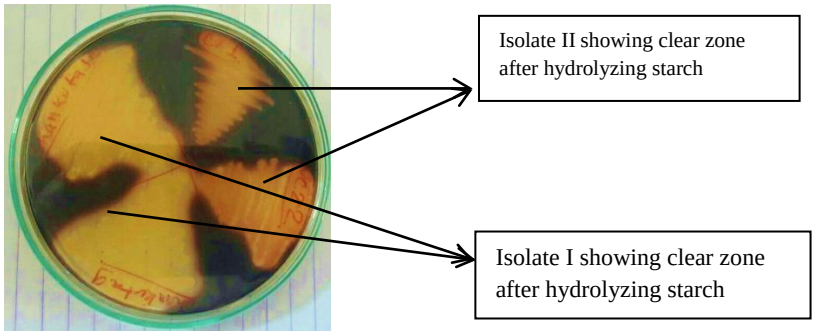

Figure 6: Clear zone formation around growth area of both isolates in Starch agar plates at 50°C.

REFERENCES

Aanniz T, Ouadghiri M,Melloul M, Swings J, Elfahime E, Ibijbijen J,Ismaili M, Amar M, 2015. Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. *Braz J Microbiol*, 46(2):443-53.

Adiguzel A , Ozkan H , Baris O , Inan K , Gulluce M , Sahin F, 2009. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *Journal of Microbiology Methods*, **79**(3):321-328.

Antón J, Oren A, Benlloch S, Rodriguez-Valera F and Rosello-Mora R, 2002. Salinibacter ruber gen. nov. sp. Nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. Int. J. Syst, Evol. Microbiol, **52**,485-491.

Becker P, Abu-Reesh I and Markossian S, 1997. Determination of the kinetic parameters durring continuos cultivation of lipase producing thermophile bacillus Sp IH-190. *Applied microbiology and biotechnology*, **48**(2):184-90.

Bertoldo C, and Antranikian G, 2002. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Current Opinion in Chemical Biology, **6**(2):151-60.

Brock DH and Hudson F, 1969. Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile. *J Bacteriol*.**98**(1): 289–297.

Cowan D, 1996. Industrial enzyme technology. *Trends in Biotechnology* **14**(6): 177-178.

Derekova A, Mandeva R and Kambourova M, 2008. Phylogenetic diversity of thermophilic carbohydrate degrading Bacilli from Bulgarian hotsprings. *World J. Microbiology and Biotechnology*. **24**(9):1697-1702

Forgarty W and Kelly C, 1979. Starch degrading enzymes of microbial origin. Journal of progress in industrial microbiology.15:87-150.

Gordon RE, Haynes WC, and Pang HN, 1973.*The Genus Bacillus. Agricultural Research Service, United State, Department of Agriculture*, Government Printing Office, Washington, DC, USA. 427.

Haki GD and Rakshit SK, 2003. Developments in industrially important thermostable enzymes. *BioresourTechnol*, **89**(1):17-34.

Iverson WG and Nancy FM, 1974. A method for the detection of starch hydrolysis by bacteria. *Journal of Applied bacteriology*, **37**(3):443-446.

Lau MC, Aitchison JC and Pointing SB, 2009. Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet. *Extremophiles*, **13**(1):139-49.

Madgan M and Martinko JM, 2005. *Biology of micoorganisms.* Brock: prentice hall.

Mathew CD and Rathnayake S, 2014. Isolation and characterization of alpha amylase isolated from a hot water spring in Sri Lanka. *International Research Journal of Microbiology*, **5**(4) 50-61.

Maugeri TL, Gugliandolo C, Caccamo D and Stackebrandt E, 2001. A Polyphasic Taxonomic Study of Thermophilic Bacilli from Shallow, Marine Vents. *Systematic and Applied Microbiology*.**24**: 572-587.

Mohanta H, and Rath C, 2010. Extracellular enzymatic activity of bacterial strains isolated from a local hotspringTarabalo, Nayagarh District, Orissa, India. *The Internet Journal of Microbiology*, **7**(2):1-5.

Nayak S.P. 2013. *Identification and Characterization of Thermophillic Bacteria from Hot Springs of Odisha and Their Potential Applications.* Orissa University of Agriculture and Technology Bhubaneswar-3, Odisha, India. 77.

Panda MK, Sahu MK, Tayung K, 2013. Isolation and characterization of a thermophilic Bacillus sp. with

protease activity isolated from hot spring of Tarabalo, Odisha, India. *Iran J Microbiol.* 5(2):159-65.

Sethy A, and Ray P, 2011. Isolation, Characterization and identification of thermophilic microorganisms of hot spring 'Atri' of Orissa. *J. Microb. World*. **13**(1):50-54.

Sharma A, Pandey A, Shouche YS, Kumar B and Kulkarni G, 2008. Characterization and identification of Geobacillus sp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. *Journal of Basic Microbiology*, **49**(2):187-94

Sievert SM, Ziebis W, Kuever J, and Sahm K, 2000. Hydrothermal Microbial systems. *Relative abundance of Archaea and Bacteria along a thermal gradient of a shallowwater hydrothermal vent quantified by rRNA slot-blot hybridization.* Microbiology. **146** (6):1287-93.

Singh SP, Purohit, MK, Aoyagi C, Kitaoka M, and Hayashi, K, 2010. Effect of growth temperature, induction and molecular chaperones on the solubilization of over109 expressed cellobiose phosphorylase from Cellvibrio gilvus under in-vivo conditions. *Biotechnology Bioprocess Engineering*, **15** (2): 273-276.

Souza AN and Martins ML, 2001. Isolation, properties and kinetics of growth of a thermophilic *Bacillus*. *Brazilian Journal of Microbiology*, **32**(4):1517, 2001.

Takacs CD, Ehringer M, Favre R, Cermola M, Eggertsson G, Palsdottir A, and Reysenbach A, 2001. Phylogenetic characterization of the blue filamentous bacterial community from an Icelandic geothermal spring. *FEMS Microbiology Ecology*, **35**(2):123-128.

Urbieta MS, Donati ER, Chan KG, Shahar S, Sin LL, Goh KM, 2015. Thermophiles in the genomic era: Biodiversity, science, and applications. *Biotechnol Adv.* **33**(6):633-47.

Zierenberg RA, Adams and Arp AJ, 2000. Life in extreme environments. *Proc Natl Acad Sci U S A*, **97**(24):12961-2.

How to cite this article

Jean Bosco Nshimiyimana, Sujan Khadka, Esther Muhindo Mwizerwa, Nathan Akimana, Sanjib Adhikari, Antoine Nsabimana, 2018. Thermophiles: Isolation, Characterization and Screening for Enzymatic Activity. *Bioscience Discovery*, **9**(3):430-437.