ISOLATION AND CHARACTERIZATION OF THERMOPHILIC BACTERIA FROM GEOTHERMAL AREA IN VAJRESHWARI , MAHARASHTRA, INDIA AND PRELIMINARY RESEARCH ON INDUSTRIALLY IMPORTANT AMYLASE, OXIDASE AND CATALASE ENZYME PRODUCTION

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Article History: **Received: 15/12/2022** **Revised: 05/02/2023** **Accepted: 23/03/2023**

Abstract:
The aim of the present study was to isolate, identify, characterize, and screen the enzymatic activities of thermophilic bacteria from Vajreshwari hot spring, Maharashtra, India. The three isolates were characterized by morphological, biochemical, and microscopic characteristics. The optimum temperature for the growth was 50 °C, and the pH was 8. The sequencing of the 16S rRNA of the isolates followed by a BLAST search revealed that the three strains were *Pseudoxanthomonas* taiwanensis strain (NFC7-A), *Anoxybacillus salavatliensis* strain *(Y3MT21), and Bacillus licheniformis* strain *(HAZ6-17*). *Pseudoxanthomonas taiwanensis* and *Anoxybacillus salavatliensis were reported for the* first time from the study area, and both strains showed positive activity for amylase while all three bacteria showed positive activity for oxidase and catalase. The results showed that the isolated bacteria from hot springs could be a great source of biotechnologically important thermostable enzymes.

Keywords: Thermophiles, bacteria, Vajreshwari, hot spring, amylase, oxidase, catalase

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DOI: 10.48047/ecb/2023.12.si10.00316

Introduction

Microorganisms occupy all possible environments, including those that provide a suitable habitat for growth under extreme conditions. The microorganisms growing in such an environment can be classified into thermophiles (high temperature), acidophiles (low pH), alkaliphiles (high pH), halophiles (high salinity), and psychrophiles (low temperature) [1]. Geothermal areas are considered the source of the principal habitats of thermophile microorganisms [2]. It is not a common ecological characteristic, but they occur in some widely separated areas of the world where conditions are good for their occurrence. Due to the specific nature of geothermal sources, hot springs are available in only a few areas [3]. Hot springs, where geothermally heated water emerges from the earth's crust, are a reservoir of thermophilic organisms [4]. The hot springs from around the world were mostly researched in the United States, Iceland, Japan, Italy, Indonesia, New Zealand, Central America, and Central Africa (5-7). The discovery of different life forms at elevated temperature and the isolation of *Thermus aquaticus* bacteria producing *Taq DNA polymerase*, which has commercial uses in polymerase chain reaction (PCR) technology, led scientists to isolate and identify microorganisms from worldwide geothermal sources [8].

Over the past few years, extremophiles, and thermophiles in particular, have gained recognition due to their extensive use in biotechnology and also due to their ability to withstand and function under extreme conditions [9, 10]. Thermophilic bacteria surviving at 50^0C or above, have attracted great attention because they are sources of thermostable enzymes such as amylase, oxidase, catalase, cellulose, pectinases, lipases, xylanases, proteases, DNA polymerase, etc. that show unique characteristics that can be suitable for performing biotechnological processes at elevated temperatures [11]. In addition, thermophilic microorganisms are stable in detergents, many solvents, and acidic and alkaline pH [12]. The advantage of using thermophilic enzymes is well documented as industrial catalyst with potential revenue reflected on the rapid growth of the enzyme market [13]. Among the industrially important enzymes, amylases share 25–30% of the world enzyme market [14]. The most widespread applications of thermostable α-amylase are in the starch industry, which involves the processes of gelatinization, liquefaction, and scarification that are performed at high temperatures [15]. Glucose oxidase belongs to a large group of enzyme families called oxidoreductases. Glucose oxidase has wide use in various industries, like the pharmaceutical and food industries, and in biofuel cells. Catalase is a tetrameric protein found in aerobic organisms. It helps hydrogen peroxide decompose. Catalase is used to remove excess hydrogen peroxide from fabrics. This enzyme is mostly used along with other enzymes in the food processing industry [16].

The terrestrial hot springs exits on earth represents hot spots for unusual forms of life, genes and metabolites. Ever since a number of researchers have investigated similar environment all over the world [6] but more ever the researchers are still far away from being able to complete their identification and isolation that need extensive and holistic research to be carried out in order to fully investigate such promising microorganism In India, many hot springs are available in different regions across the country. In recent years, a number of publications have appeared on various aspects of the bacterial diversity of Indian hot springs: Manikaran, Tattapani-Himachal Pradesh [8,9]; Rajgir (Munger) [19]; Tarabalo and Atri - Odisha [5,10]; Bakreshwar-West Bengal [12]; Suryakund-Jharkhand [13]; Tulsi Shyam-Gujrat [14]; Solhar and Suryakund-Uttarakhand [20]; Unkeshwar-Maharashtra [16,17]; Vajreshwari-Maharashtra [17,18], etc. Most of these reports focused on the isolation and characterization of thermophilic bacteria as well as amylase activity.

Vajreshwari hot spring (190 29' 45" N, 730 2' 22" E) is located on the bank of the river Tansa in Thane district (M.S.) popular as a holy place near the metropolitan city of Mumbai. Earlier, Rathod et al. (2018) [21] investigated the isolation and characterization of antibiotic producers. George, et al. (2008) [22] isolated and characterised the sulphate-reducing bacteria *Desulfovibrio vulgaris*. Acharya, et al (2012) [23] investigated the effect of nutritional and environmental factors on cellulase production, specifically endoglucanase (CMCase) and exoglucanase (FPase) from *Bacillus licheniformis* MVS1 and *Bacillus sp.* MVS3. Scientists from the National Chemical Laboratory isolated a molecule that inhibits the AIDS-causing HIV-1 protease from a microbe that thrives in the hot, alkaline waters [24]. Pednekar et.al (2011) [25] investigated the anti-infective potential of bacteria, Vjay Kumar (2014) [26] Investigated the diversity of thermophilic actinomycetes and isolated the amylase enzyme for biotechnological applications from the Vajreshwari hot spring.

Although studies on thermophiles from the hot springs of Vajreshwari have been demonstrated in a few previous works, no sustainable research has focused on the utilisation of these thermophiles. The aim of the present study is to establish a continuous research line for the isolation and characterization of new thermophilic microorganisms that can possess high biotechnological and industrial potential.

Material and Method

Water Sampling

Stretching about 7 km along the riverbed of the Tansa River, the hot springs are mainly found in Akoli, Vajreshwari, Ganeshpuri and Satvalli in western Maharashtra, India. Samples were collected from three kundas at the hot spring origin (referred to as the surface).

Isolation of microorganisms

10 ml water samples were added in 100 ml nutrient broth and inoculated at 55^0C. After 3 day the growth of bacteria were observed and screened for further study.

Genomic DNA Isolation

The PCR reaction involved the use of a modified CTAB protocol to isolate DNA from a culture grown in nutrient broth. To ensure proper distribution of the culture, the pallet was suspended in 1.5ml Tris EDTA and centrifuged at 10,000 RPM for 5 min. A mixture of supernatant, 20µl Lysozyme, and proteinase K was added and mixed well, incubated for 1h at 37^0C. The mixture was then supplemented with 100µl of 5M NaCl and preheated at 65^0C before being mixed again after 10min. After incubation, the mixture was supplemented with chloroform: isoamyl alcohol (24:1) and mixed well. The developed upper aqueous layer was transferred to a fresh tube and accelerated at 0.6 vol. of isopropanol before undergoing centrifugation at 15 min. Supernatant was decanted and pellet washed with 70% ethanol and was centrifuged at maximum speed for 5 minutes. Supernatant was removed and pellet air dried by keeping it at room temperature for 5-10 min. On a dry pellet 20 µl TE was used for resuspension and stored at $–20^0$C until further use. The successful isolation of genomes was analyzed on 0.8 % agarose gels.[51,52].

PCR based 16S rRNA gene amplification and Sequencing

About 200 ng of bacterial DNA was used to amplify 16S rRNA gene applying following 16S universal primers:- 16S Forward primer: 5’ AGA GTT TGA TCC TGG CTC AG 3’ 16S Reverse primer: 5’ AAG GAG GTG ATC CAG CCG CA 3’ The PCR amplifications of bacterial isolates' 16S rRNA genes were performed for the entire 50 ml reaction mixture. 32 ml nuclease-free water, 5ml PCR buffer 10x, 2.0 l dNTP (10 mM), 4.0 l forward primer (10 mM), 4.0 l reverse primer (10 mM), 1.0 l Taq DNA polymerase enzyme (1U/l), and 200ng DNA template comprised the amplification mixture. The temperature range for each 50 l PCR reaction was programmed as follows: 58 º C, 57 ºC, 56 ºC, 54 ºC, 52 ºC, 50 ºC, and 48 ºC. Scale up cycle sequencing was performed at 54 º C using a thermal cycler (PTC 100, M J Research, Watertown, MA) under the following conditions: initial denaturation of 3 minutes at 94 degrees Celsius, denaturation of 1 minute at 94 degrees Celsius, primer annealing for 1 minute. Extension of 2 minutes at 72 º C, final extension of 5 minutes at 72 º C; total of 30 cycles and stored at 4 ºC. The amplified PCR products, along with 500 bp of DNA ladder, were separated on 1.2% agarose gel (NEB, Beverly, MA). DNA sequencing was performed with 50 ng of PCR product, 8:l of ready reaction mix (BDT v 3.0, Applied Biosystems, Foster City, CA), and 5 p Mol of forward primer. The following were the cycling conditions: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. After that, the samples were washed with 70% ethanol and suspended in Hi-Di formamide (Applied Biosystems). ABI prism 3100 Genetic Analyzer was used for the sequencing (Applied Biosystems). Using BLASTN search algorithms, the sequences were compared to microbial nucleotide databases.

BLAST Analysis

The BLASTN programme used sequences of 16S rRNA genes from the sequencer machine to convert them into FASTA format and pairwise alignment. The online BLAST programme was used to find homology for the given sequence by selecting the database of bacteria and archae 16S rRNA. The E - value, match/mismatch score, gap cost, and block cost were set to 10, 20, and 2 respectively. These sequences were then used in the ClustalW and Mega5 software to construct a phylogram.

Multiple Sequence Alignment

CLUSTALW was used to perform multiple sequence alignment on 16S rRNA gene sequences by accepting FASTA files with closely linked sequences. The top ten aligned sequences were downloaded in FASTA format and used in the analysis process. Alignment parameters in

ClustalW were set as DNA weight matrix, gap penalty, gap extension, gap distance and clustering method. The file was retrieved as an output result and a phylogram was constructed in MEGA5 software using DND.

Phylogenetic Analysis

For the phylogenetics study, the phylogram was constructed using the MEGA5 software and a DND file obtained from the CLUSTAL alignment. A phylogram with close homology of the bacteria isolated (showcased with the accession number and with the best-matched bacterial sequence) was documented in an output and highlighted by marking in a phylogram.

Enzyme Activity

Oxidase Test

Small piece of filter paper soaked in 1% Kovácsoxidase reagent and dried. Loop of bacterial culture rub onto treated filter paper and observed color changes. Microorganisms with oxidase positive shows color changes to dark purple within 5 to 10 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

Catalase Test

4 to 5 drops of 3% H_2O_2 were taken in a test tube. A small amount of 18-24 hrs isolated colony were placed into test tube for formation of bubbles. The positive reactions are evident by immediate effervescence (bubble formation). No bubble formation represents a catalase-negative reaction.

Amylase Test

The amylolytic properties were discovered using the starch hydrolysis method. Microbial isolates were used to streak the starch agar plates and they were then incubated at 37^0C for 24 hours. Following incubation, the starch agar plate was flooded with freshly prepared 1% iodine solution. Blue colour around the growth indicated unfavourable outcomes.

Results and Discussion:

1. Characterization of the samples

Water samples from three hot springs of Vajreshwari which were commonly called kund were analyzed to screen bacteria capable of producing thermophilic enzymes amylase, catalase and oxidase. In India there are several hot springs renowned for their medicinal and rejuvenating properties. The temperature of these hot springs ranges from 32^0C [27] to 90^0C [28]. In present investigation the temperature and pH of three kundas where ranging between 45^0C to 65^0C and 8.3 respectively that was alkaline in nature [Table 1]. In these conditions living organisms have to cope with extreme temperature, low humidity and low availability of nutritional compounds. The bacterial strains which are able to grow in such temperature could be classified as thermophilic bacteria according to Brock [29], Perry and Staley [30] Souza and Martins [31]. The temperature of the selected was previously reported 32 ^{0}C and pH 7.4 [27] while Rathod et.al [21] reported 65^0C which is similar to our findings.

2. Morphological and Biochemical Examination of the Isolates

A total four isolates of thermophilic bacilli designated S001V, S002V, S003V and S004V were isolated from water sample of study area. These isolates showed optimum growth at 55^0C and pH 8.3. Morphological, microscopic, observations and biochemical tests like catalase; oxidase and amylase are performed and presented in table 1. Morphologically the isolates showed some variation in the colour, margin, shape and texture of the colonies. They were yellow, white, opaque, rough or smooth with regular or irregular edges. Colonies might appear wrinkled and adherent to agar surface. The isolates S001V was gram negative, non-motile, rod shaped bacteria lacking flagella. The optimum growth temperature of the isolates was 45^0C while optimum pH was 8. The isolates S002V and S004V had same characteristics that may be same bacterial isolates. It was also rod shaped, round and irregular motile gram positive bacteria with optimum growth temperature 45 ^{0}C and pH 9. The isolates S003V were rod shaped, gram positive, spore forming, motile with optimum growth temperature 60 ^{0}C and pH 8. They were yellow, white, opaque, rough or smooth with regular or irregular edges. The colonies might appear wrinkled and adherent to agar surface.

3. Molecular Identification of the Isolates

The final identification and phylogenetic analysis of the isolates was assessed by the 16S r RNA sequencing. The 16S r RNA sequence from 4 haplotypes was aligned with their closely related reference bacterial sequences obtained from the GenBank by Basic Local Alignment Search Tool (BLAST) program. The sequence analysis showed high similarity with those of the reference strains available in the GenBank databases. Based upon 16 S rRNA sequence alignment, phylogenetic tree was constructed for all the isolated strains (Figure1).

The 16SrRNA sequence alignment revealed that the isolates S001V could be *Pseudoxanthomonas taiwanensis* (NFC7-A) with strong sequence similarity (99.30 %) isolates S002V and S004V could be *Bacillus licheniformis (strain HAZ6-17)* (99.60%) which is cosmopolitan and very common in study area [30-31] and isolates S003V could be *Anoxybacillus salavatliensis (*strain Y3MT21) (99.84%) (Table2).

The characterization of thermophilic bacteria which are able to grow at 70^0C was first time done by Miquel (1888) [32]. Since then many thermophilic and more than 20 different genera of hypothermic archaea have been isolated from geothermal and hydrothermal environment [33]. On the basis of morphological, microscopic and 16 S r RNA gene sequencing, 3 strain of bacteria viz. *Pseudoxanthomonas taiwanensis* NFC7-A, *Anoxybacillus salavatliensis*Y3MT21, *Bacillus licheniformisHAZ6-17 are* identified from study area. The sequence of the isolates has been deposited in GenBank with accession number EU250936.1, ON243957.1, AY162135.1 respectively.

The genus *Bacillus* is very common in the hot springs. The strain of *Bacillus* has been dominant in hot springs across the world [6, 34, 35]. Most of the isolated bacteria reported from five hot springs in Jordan were gram-positive rods (94.7%), which belong to the genus Bacillus [6]. Kiran et.al (2018) [7] isolated *Bacillus licheniformis* from Bihar hot springs. The dominance of species Bacillus in extreme conditions is attributed to their ability to resist environmental stress due to their spore-forming nature [36].

Anoxybacillus is a quite recent bacterial species isolated by [37] from animal manure based on 16S rRNA-DNA hybridization analysis. The phenotypic features of *Anoxybacillus K1's genus T* were proposed as new genera of the Bacillaceae. The name was derived from its anaerobic property, which can sustain depleted dissolved oxygen. Since then, a total of 19 species and 2 subspecies of *Anoxybacillus* have been reported [38]. A novel, moderately thermophilic, alkali-tolerant bacterium, *Anoxybacillus suryakundensis*, was isolated from hot springs in Jharkhand, India, by [13]. The *thermophilic bacterium Anoxybacillus salavatliensis*, a novel species that produces α-glycosidase was isolated from Salavatli, Turkey [39]. To the best of our knowledge, this bacterium strain was first reported not only from Vajreshwari hot spring but also from India.

Pseudoxanthomonas taiwanensis, a novel species, was isolated from Chi-ban hot springs in eastern Taiwan [40]. It is a thermophilic, N_2O-producing bacterium. The genus *Pseudoxanthomonas* was isolated from the caves and tunnels of the Naika underground system, Mexico [41], arsenic-contaminated ground water in India [42] and the midgut of *Anopheles stephensi* from Zahhar, Haryana, India [43]. But no *Pseudoxanthomonas taiwanensis* was isolated from Indian hot springs. To the best of our knowledge, this is the first report of *Pseudoxanthomonas* from the study area.

One of the important potentials of thermophilic microorganisms is their enzymatic activity due to their ability to function under severe conditions such as temperature, pH, and pressure. This will make their significance useful in industrial and biotechnological areas [6]

4. Production of Extracellular Enzymes

Bacterial isolates collected from hot springs were screened for amylase, oxidase, and catalase activity. In this study, *Pseudoxanthomonas taiwanensis* and *Anoxybacillus salavatliensis* showed positive results for amylase, oxidase, and catalase activities. But *Bacillus licheniformis* showed negative amylase activity, which is contrary to the results obtained by Mohammad et.al (2017) [6]; Ardhi et.al (2020)[34]; and Msarah et.al (2020)[35]. The thermophile *Bacillus thermoamylase* efficiently hydrolyzed the starch with amylase activity, which was studied in the same study area by [21]. This encourages further studies to carry on further enzyme analysis with this strain in the future. As it was observed, the variation of amylase activity of the S004 strain confirms the presence of a subspecies of *Bacillus licheniformis.*

Enzyme catalase is a central component of the detoxification pathways that prevent the formation of highly reactive hydroxyl radicals by catalysing the decomposition of H_2O_2 into water and oxygen by two electron transfer [44]. While oxidase is an enzyme that catalyses oxidation-reduction reactions, especially ones involving dioxygen (O_2) as the electron acceptor, In these reactions, various types of oxidase enzymes have been studied so far by many researchers, viz. D-amino acid oxidase obtained from thermophilic bacteria *Rubrobacter xylanophilus* (RxDAO) [45], cytochrome c oxidase [46] and quinol oxidase [47], and succinate oxidase from thermophilic bacteria PS3 and *Thermus* T351 [48]. In our study, all three bacteria showed positive results for catalase and oxidase. To the best

of our knowledge, no such catalase and oxidase activities have been studied for *Pseudoxanthomonas taiwanensis* NFC7-A, *Anoxybacillus salavatliensis Y3MT21,* and *Bacillus licheniformis HAZ6-17*. This is the first report of enzyme activity for *Anoxybacillus salavatliensis and Pseudoxanthomonas taiwanensis* from the study area and India as well.

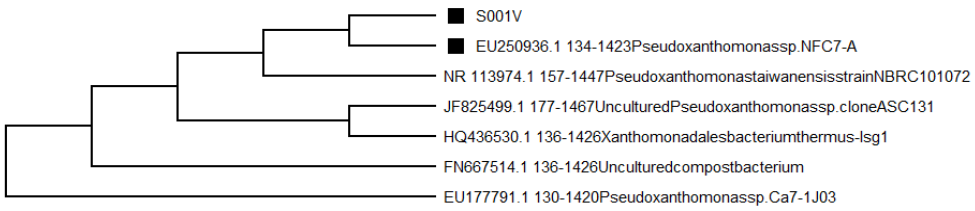
Conclusion

The thermophilic *Bacillus licheniformis HAZ6-17, Pseudoxanthomonas taiwanensis NFC7-A, and Anoxybacillus salavatliensis Y3MT21 were* isolated and characterised from Vajreshwari hot spring water samples from Thane, Maharashtra, India. This is the first report on the isolation of *Pseudoxanthomonas taiwanensis* NFC7-A and *Anoxybacillus salavatliensis Y3MT21* strains from Indian hot springs. Catalase and oxidase activities were present in all three bacterial strains, while *Bacillus licheniformis HAZ6-17* showed negative amylase activity. The contrast in enzymatic amylase activity for this strain indicates the presence of subspecies. This promising result can be exploited further for the production of biotechnologically important industrial enzymes. But some more biochemical and molecular analysis is still required.

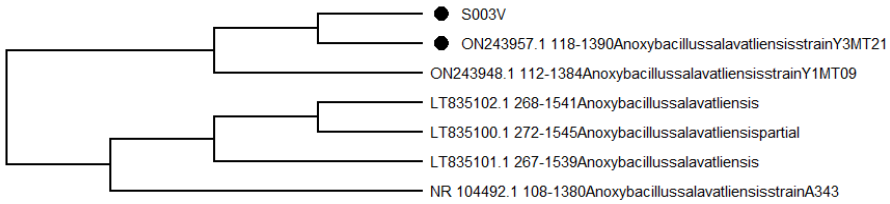
Table No. 1: Microscopic, Morphological and biochemical characteristics of thermophilic isolates from Vajreshwari hot springs, Thane, Maharashtra, India

Characteristics	**S001V**	**S002V**	**S003V**	**S004V**
Morphological	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Gram Nature	-ve	+ ve	+ ve	+ ve
Cell morphology	Absence of flagella	Round and irregular	Spore forming	Round and irregular
Motility	Non-motile	motile	motile	motile
pH range	6-11	8-10	6-11	8-10
Optimum pH	8	9	8	9
Temperature	30-60 ^{0}C	30-70 ^{0}C	37–69 ºC	30-70 ^{0}C
Optimum Temperature	45^0 C	45^0 C	60 °C	45^0 C
Amylase	+ve	-ve	+ve	-ve
Catalase	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	+ve

a)



b)



c)

Figure1: Phylogenetic trees of Isolate a) S001V (*Pseudoxanthomonas taiwanensis* NFC7-A), b) S003V (*Anoxybacillus salavatliensis*Y3MT21) and c) S002V and S004V *(Bacillus licheniformis HAZ6-17)* isolated from hot springs

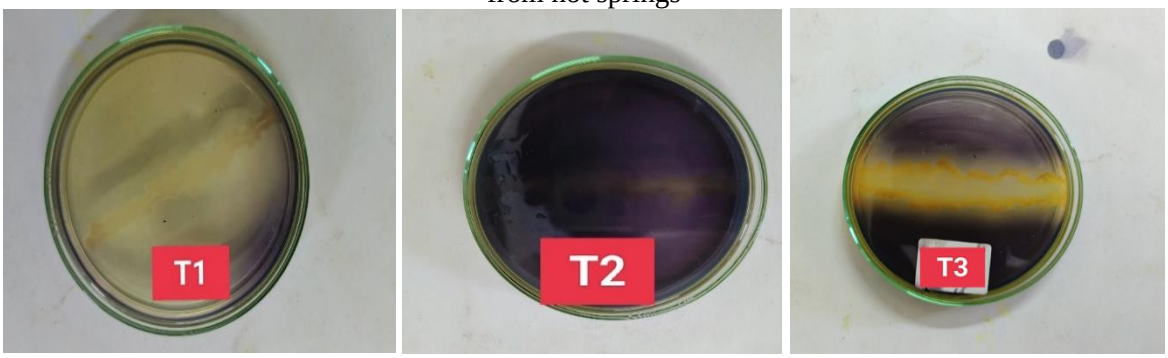


Figure 2: Amylase Activity Shown by Bacteria T1- *Pseudoxanthomonas taiwanensis* showing Amylase Activity , T2 – *Bacillus licheniformis* Amylase activity absent, T3- *Anoxybacillus salavatliensis* showing Amylase Activity

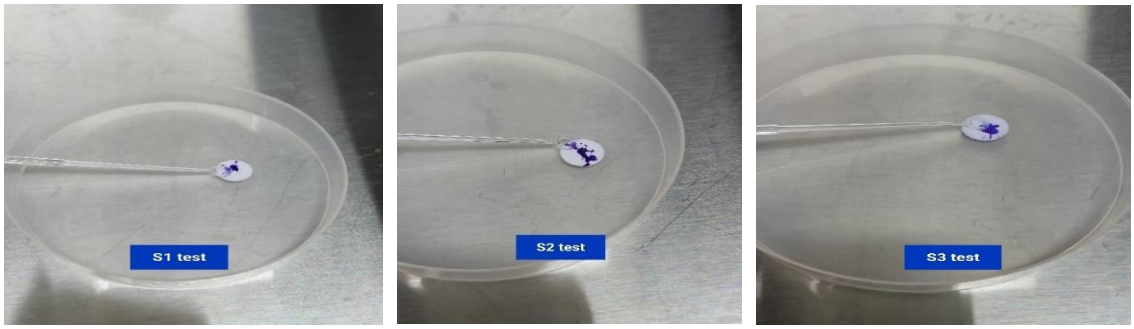


Figure 3: Oxidase Activity Shown by Bacteria T1- *Pseudoxanthomonas taiwanensis* showing Oxidase Activity , T2 – *Bacillus licheniformis* Oxidase activity absent,T3- *Anoxybacillus salavatliensis* showing Oxidase Activity.

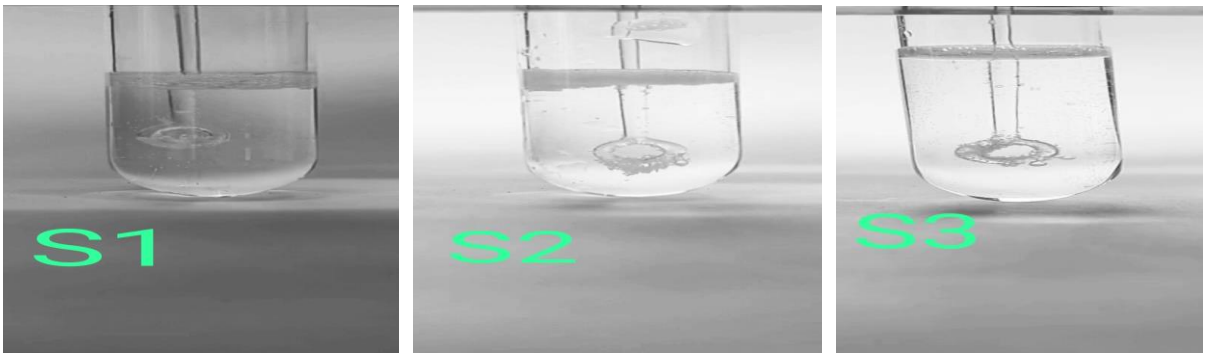


Fig.4: Catalase Activity Shown by Bacteria S1- *Pseudoxanthomonas taiwanensis*, S2 – *Bacillus licheniformis*, S3- *Anoxybacillus salavatliensis* showing Presence of Catalase enzyme

Acknowledgement:
We acknowledge Sai Bio systems Private Limited, Nagpur and SCITESLA laboratory, Mahape, Navi Mumbai for helping us in identification of thermophilic bacteria and further study. We would also like to thanks Principal and Head of Department of Zoology for providing necessary Laboratory facility.

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