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Methods in Microbiology of Extremophiles (Volume II)

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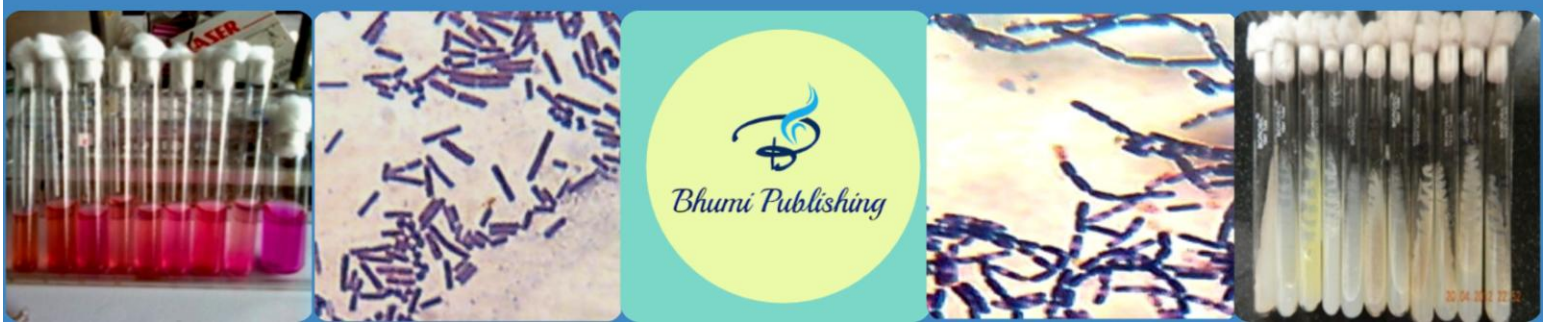

Methods in Microbiology of Extremophiles (Volume-II)

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- Working on halophilic bacterial diversity in Bordi region.
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- Pursuing Ph.D. in biotechnology at School of Life sciences, S.R.T.M. University, Nanded under the guidance of Prof. Dr. (Mrs.) A. P. Pathak.
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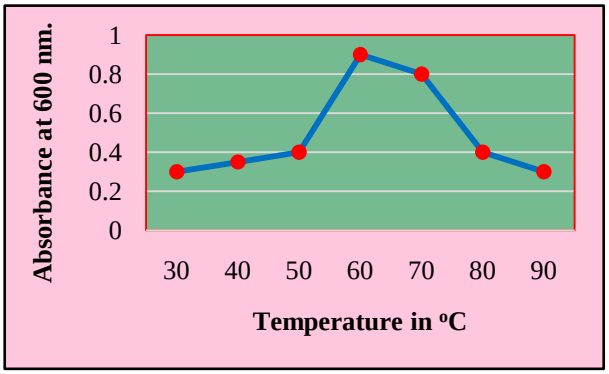

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Message from a Renowned Scientist

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Re-accredited by NAAC with 'A' Grade

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Message

I am happy to note that Prof. Dr. A. P. Pathak and Colleagues have authored a book on Methods in Microbiology of Extremophiles. Prof. Pathak has been working on the biology of extremophiles for the last more than fifteen years. This will be a very useful book for students and researchers on extremophile biology.

Best Wishes!

(Prof.S.Mohan Karuppayil)

Preface

We are pleased while publishing second volume of 'Methods in Microbiology of Extremophiles'. Microbiology of extremophiles is studied by a certain group of researchers and scientists worldwide on the various mesmerizing extreme habitats that are geographically distributed in the continents and sub-continents of the Earth. The ability of some microorganisms growing in extreme environmental conditions such as a very high temperature, pH, pressure and salinity, which is not normal for mesophiles, provides the basis of continuous research and the interest about them gives ideas of their exploitation for wellness of human being. Thousands of important bioactive compounds such as antibiotics, enzymes, proteins, pigments, metabolites, neutraceuticals etc. have been isolated from extremophiles having pharmaceutical, agricultural and medicinal applications in different industries.

This book introduces second volume of Microbiology of extremophiles. In this volume the authors report the deliberate isolation of extremophiles from some extraordinary habitats such as mangrove region and Vajreshwari hot spring, and their biotechnological importance have been discussed. This volume exactly covers the isolation and screening of industrially important extremophiles in precise manner. Moreover the ideas of *in situ* sample collection, temperature-optima determination and isolation of thermophilic fungi and actinomycetes have been put-forth scientifically by designing the simple experiments which can be easily performed in any microbiological laboratory not only by the researchers but also U.G. and P.G. students for their dissertations. In second volume also, sufficient space has been provided to students for recording their individual observations and results. In second volume field study, site visiting and sample collection have been emphasized along with some practical application of extremophiles.

- Prof. Dr. (Mrs.) Anupama P. Pathak

Acknowledgements

With the seamless efforts taken by us on the second volume of 'Methods in Microbiology of Extremophiles', we eager to acknowledge some dignitaries and many other people who directly and indirectly guided and supported us.

We would like to show our gratitude to Hon. Dr. Uddhav Bhosale, Vice Chancellor, S.R.T.M. University, Nanded, Hon. Dr. Dayanand Agsar, Vice Chancellor, Gulbarga University, Karnatak, Hon. Dr. Yogesh S. Shouche, Scientist G and Director, National Centre for Microbial Resource, Prof. Dr. Mohan S. Karuppayil Former Director of School of Life Sciences and now, Professor & Head, Department of Stem cell and Regenerative medicine at Dept. of Medical Biotechnology, D.Y. Patil Medical College and University, Kolhapur for giving us a good guideline for assignment throughout their numerous consultations.

We would also like to expand our deepest gratitude to, Hon. Dr. Rafiq Shaikh, President of Muslim Educational, Cultural, Health and Welfare Society's Yeshwant College of Information Technology, Parbahni (affiliated to SRTM University, Nanded), Dr. (Mrs.) Anjali Kulkarni, Principal of N.B. Mehta Science college, Bordi, Palghar (affiliated to Mumbai University, Mumbai) Dr J N Kulkarni ,Director, KRC, SRTMUN and Dr. Narayan Totawad, B.K. Birla college of Arts, Science and Commerce (Autonomous), Kalyan, Mumbai and Many people, especially our colleagues and team members themselves, have made valuable suggestions on this proposal which gave us an inspiration to improve our book. We thank to all the people and our family members for their help directly and indirectly to complete our book

Authors and serial editors

Prof. Dr. (Mrs.) Anupama P. Pathak

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Mr. Vikas Joshi

Ms. Sonali Girdhari

Dedicated

to

Mother of Universe

Ultimate Source of Energy

Goddess BHAVANI

Index

[A] Chapters

1. Physicochemical analysis of the water sample collected from Vajreshwari hot spring, Maharashtra, India

Abstract:

A hot water spring is produced by the emergence of geo-thermally heated groundwater that rises from the Earth's crust, some of them are found in the vicinity of volcanic region**.** Hot spring depending on the temperature can be classified, as euthermal (warm) (30-50°C), acrothermal (hot) (50-70 °C), and hyperthermal (steaming) (70 °C or higher) [1]. Hot springs differ in geothermal characters and are not similar to each other, suggesting they may be of independent occurrences. Hot springs that look same appearance may differ in characteristics like pH, temperature and physicochemical properties of water [2]. From ancient times people have found them useful for bathing and other recreational activities [3]. Hot water from these springs is believed to have medicinal value and curing of many ailments like skin diseases. These springs in India are situated in the vicinity of various temples and are considered for religious importance. Therefore, people visit them for holy baths and religious rituals. Studies of hot springs reveal quality of water in terms of physicochemical and biological characteristics and can indicate its suitability for irrigation, agriculture, drinking and industrial purposes [4]. It also adds up to the environmental and ecological database of the region as well useful for the public who uses these spring water for health purposes [5]. Biotechnological and microbiological importance of hot springs is shown by many reports confirming that they harbor microbes capable of producing biomolecules which find a vast number of applications in various industries like food and beverages, waste disposal and management, paper and pulp and so on [6]. Some of the examples of hot spring are Limpopo province, South Africa [7], Yellowstone national park, USA [8], Chinoike Jigoku , Japan [9], Unkeshwar , India [10], and Kamchatka hot springs, Russia [11]. Temperature and pH data reported for few of them are as 55.9°C and pH 8.6 for Atrihot spring, orissa [12], 42°C and pH range 7.37 - 7.65 for Unkeshwar hot spring, India [13], 45°C and pH range 8-9 for Tattapani hot spring, Jammu, 47°C and pH 12 for Jakrem, Meghalaya [14]. Abiotic or physicochemical analysis of hot spring water is important to understand the hot spring ecosystem. In our studies, we selected Vajreshwari hot spring which is still under investigation by various research experts in the field of extremophiles. Vajreshwari town (19°29′12″N 73°1′33″E) is situated on the bank of the river Tansa, in Bhiwandi city of Thane district, Maharashtra, India. There are approximately 21 hot springs within a 5 km radius of Vajreshwari shrine. Many people for religious purposes and tourists for hot spring baths visit this place [15].

Important features of this chapter are site visit, water sample collection and determination of selected physicochemical parameters.

Objective: To collect water sample from Vajreshwari hot spring and determination of selected physicochemical parameters.

Materials:

1. Portable thermometer
2. pH paper strips [Hi-media]
3. White PVC autoclaved or new sterile bottles as showed in the following figure.

Methods:

1. Plan to visit Vajreshwari hot spring kund preferably at low rush time.
2. Rinse the sampling bottles with spring water three-four times before water collection and then proceed for the water sample collection by keeping some air space in the bottle.

3. Record water temperature immediately on the site manually by a portable thermometer and pH with a broad range pH paper strip. pH strips should be used instead of a pH meter due to high temperature of water.
4. Transport capped bottles to the lab and store at 4°C in a refrigerator until further processing for microbiological studies.
5. Individual bottles should be used for physicochemical studies which need to be stored at room temperature for the analysis purpose, as prescribed in the standard methods. [16,17,18].

Expected observation: Temperature of hot spring is expected to be greater than the normal water which is generally used for the drinking, domestic and irrigation purposes.

Model observation: The observed temperature and pH was in average 50 ^{o}C and around near neutral respectively.

Expected result: It is expected to collect hot spring water samples properly that to be used further for analysis.

Model results: Water sample was collected and stored at proper temperature in the laboratory. Selected physicochemical parameters were recorded.

Space for students:

(i) Figure:

(ii) Result:

Figure: A photograph of Vajreshwari kund, Maharashtra, India.

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2. Isolation of thermophilic actinomycetes from Vajreshwari hot spring, Maharashtra, India

Abstract:

Actinomycetes are a group of gram-positive bacteria with filamentous and branching growth patterns. They are mostly found in soil and produce extracellular enzymes capable of breakdown of many types of substrate. Actinomycetes are attractive due to production of a wide array of enzymes and their high substrate specificity. Hence they can be exploited in many biotechnological industries [1]. Recently, thermophilic actinomycetes have been the subject of intensive investigations since they can produce thermostable enzymes with activity at high temperature (50-65°C). Actinomycetes found in unique and rare ecological habitats like hot spring water that can be a source of novel species; moreover some biomolecules from them can serve a wide purpose since many of them have not been tested as prospective enzymes and as other biomolecule producers [2,3,4]. Examples of thermophilic actinomycetes include the species of genera *Saccharomonospora*, *Thermoactinomyces*, *Streptomyces*, *Micromonospora* and *Actinomadura*. Enzyme production examples include xylanase from *Thermoactinomyces thalophilus* with temperature stability at 80°C, *Streptomyces* producing both amylase and cellulase with temperature optimum at 55°C, and amylase from *Thermoactinomyces Vulgaris* [5-10]. Actinomycetes are also well-known antibiotic producers and some of the isolates from hot springs are capable of producing them. Few names of growth media for actinomycetes include Kenknight and Munaier's agar, chitin agar, and starch casein agar [11]. Basic morphological characteristics include circular and opaque colonies with different pigmentations. Microscopically cells are Gram-stain positive and the mycelium has different types of spore arrangement [4]. This chapter deals with isolation of thermophilic actinomycetes from Vajreshwari hot spring water sample.

Materials:

1. Hot spring water sample
2. Starch casein agar: (composition in g/L)

Starch	10
Casein	0.3
Potassium Nitrate	2.0
Magnesium sulfate hydrated	0.05
Dipotassium hydrogen phosphate	2.0
Sodium chloride	2.0
Calcium carbonate	0.02
Ferrous sulfate	0.01
Agar	25

3. Digital weighing machine
4. Distilled water
5. Autoclave
6. Bacteriological incubator (Remi make, Mumbai)
7. Bunsen Burners

Methods:

1. Prepare starch casein agar plates.
2. Inoculate 0.1 mL Vajreshwari hot spring water sample on nutrient agar plates and spread the sample thoroughly with the help of a glass spreader aseptically.
3. Incubate all the plates in a bacteriological incubator at 50^{o}C or above for 2 to 3 days.
4. Observe the plates and record the colony characteristics.
5. Pick up single colony and subculture on starch casein agar slants.

6. Proceed for the Gram-stain reaction to distinguish and discriminate them as actinomycetes.

Expected observation:

Colonies having different shapes and sizes are expected to appear on starch casein agar plates. Students are expected to visit following web links to observe morphological features of actinomycetes.
https://doi.org/10.1111/j.1365-2672.1968.tb0039.x
https://doi.org/10.1016/B978-0-12823414-3.00021-6

Model observations:

Morphologically distinct colonies were observed on starch casein agar plates. The following table describes some characteristics of common actinomycetes example, *Streptomyces* sp.

Character	**Model observation**
Size	2-3mm
Shape	Circular
Margin	Entire
Pigmentation	White
Opacity	Opaque
Surface	Smooth
Consistency	Butyrous
Elevation	Raised
Mycelium	Present
Spores	Non-motile
Gram-stain reaction	Positive

Expected results:

Thermophilic actinomycetes are expected to be isolated from Vajreshwari hot spring water samples on starch casein agar plates.

Model results:

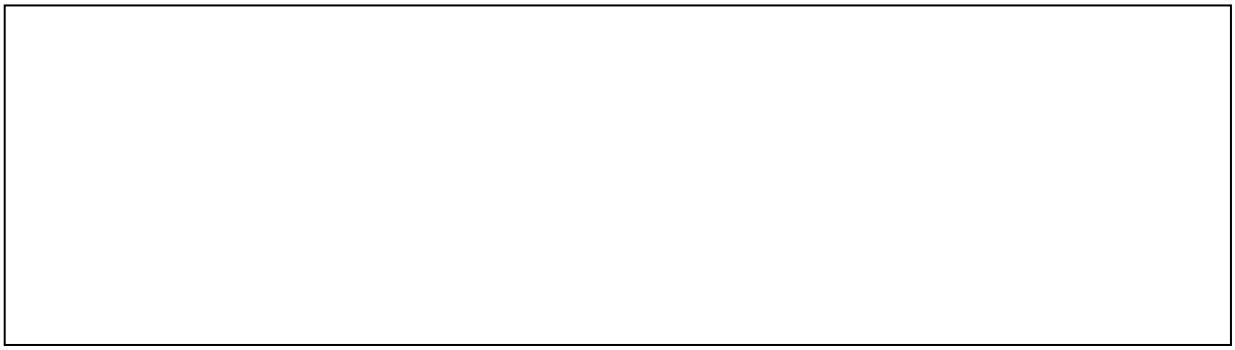
Thermophilic actinomycetes were isolated from Vajreshwari hot spring water samples and preserved on starch casein agar slants at low temperature.

Space for students:

Table for recording characteristics of an isolate:

Character	**Model observation**
Size	
Shape	
Margin	
Pigmentation	
Opacity	
Surface	
Consistency	
Elevation	
Mycelium	
Spores	
Gram-stain reaction	

(i) Figure:



(ii) Result:

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3. Isolation of thermophilic fungi from Vajreshwari hot spring, Maharashtra, India

Abstract:

Thermophilic microbes grow at temperatures above 45°C. Thermophiles and their products such as enzymes, proteins and bioactive compounds have several applications in industries [1]. Moderate thermophilic organisms with a growth range between 20 °C to 60 °C include the prokaryotic domains (bacteria and archaea) and also eukaryotic organisms, mainly filamentous fungi. Hyperthermophilic microorganisms grow in the temperature range between 65°C and 110 °C and contain several representatives of bacteria, archaea but not eukaryotes due membrane composition limitations [2,3,4].

Fungi morphologically have spores containing filamentous structure and can be identified tentatively from colony and microscopic examination. Examples of thermophilic fungi include*Thermomyces thermophiles, T. lanuginosus, Thermoascus aurantiacus, Rhizomucor miehei*, *Thermomucor indaticae* etc. They produce thermostable enzymes having application in a number of industrial processes for the production and processing of food, in the textile industry, in paper production and biofuels production. General purpose media used for fungal growth are Sabouraud dextrose, malt extract, potato dextrose, Czepek Dox agar which have acidic pH to support fungal growth. Some of the reported examples are polygalacturonase from *Thermoascus auranticus*, thermostable xylanases from *Thermomyces lanuginosus*, mannanases and pectinases from the thermophilic fungus *Thermomyces lanuginosus*, and β-glucosidases from *Thermoascus aurantiacus* [5-10]. Thermophilic fungi are found in diverse habitats including thermal springs and isolated by employing potato dextrose agar and Czapek Dox agar.

Objective: To isolate thermophilic fungi from Vajreshwari hot spring

Materials:

1. Vajreshwari hot spring water sample
2. Potato Dextrose agar (HiMedia, Mumbai)
3. Czapek Dox agar (HiMedia, Mumbai)
4. Digital weighing machine
5. Autoclave
6. Bacteriological incubator (Remi make, Mumbai)
7. Bunsen Burners

Methods:

1. Prepare Potato Dextrose agar and Czapek Dox agar plates in accordance with the instructions given by the manufacturer.
2. Inoculate 0.1 mL of Vajreshwari water sample on the surface of agar plates and spread each sample thoroughly with the help of a glass spreader aseptically.
3. Incubate all the plates in a bacteriological incubator at 45^{o}C or above for 3 to 4 days.
4. Maintain sufficient moisture in the incubation chamber for avoiding cracking of agar medium at high temperatures.
5. Observe the plates, record the growth characteristics and observe fungal hyphae and spores under microscope.
6. Pick up isolated fungal growth and subculture on respective agar media slants.
7. Preserve the slants at a low temperature in a refrigerator.

Expected observation:

Fungi having different shapes, sizes and structure are expected to appear on agar plates.

Model observation:

Morphologically distinct fungal colonies were observed on agar plates.

Expected results:

Thermophilic fungi are expected to be isolated from Vajreshwari hot spring water samples on selected agar media plates.

Model results:

Thermophilic fungi were isolated from Vajreshwari hot spring water samples and preserved at a low temperature.

Space for students:

(i) Figure:

(ii) Result:

References:

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4. Isolation of thermophilic bacteria from Vajreshwari hot spring, Maharashtra, India

Abstract:

Thermophiles are organisms capable of growing at a high temperature and have temperature optima for growth above 45°C [1]. They have several modifications in their structural components and biomolecules. In thermophiles, the biomolecules such as proteins, lipids, enzymes, ribosome, RNA and DNA have higher intrinsic stability [2]. Thermophiles and their products such as enzymes, proteins and bioactive compounds have several applications in industries [3]. Various kinds of thermostable enzymes play a vital role in industrial processes. The biocatalytic, detergents, food, feed, starch, textile, leather, pulp and paper, and pharmaceutical industries are the major users of thermostable enzymes. Microbial diversity study of hot springs was rapidly initiated after the discovery of *Thermus aquaticus* from the thermal vents of Yellowstone National Park, U.S.A. [4]. Researchers started to explore similar environments of hot springs in different parts of the world namely North America, China, Philippines, Japan, India, Russia and several other countries [5-9]. In India, there are approximately 400 geothermal springs found either solitary or in groups [10, 11]. Many species of the genera viz. *Pseudomonas*, *Clostridium*, *Staphylococcus*, *Serratia* and *Bacillus* including *B. licheniformis, B. pumilus, B. subtilis, B. cereus* secrete extracellular thermo-stable enzymes like amylase, protease, lipase, cellulase, pectinase and gelatinase [12-21]. Few of them have shown an application in antibiotic production also [22].

Objective: To isolate thermophilic bacteria from Vajreshwari hot spring, Maharashtra, India

Materials:

1. Vajreshwari hot spring water sample
2. Nutrient agar (g/L)

Peptone	5
Beef extract	3
NaCl	3
Agar powder	27

3. Digital weighing machine
4. Distilled water
5. Autoclave
6. Bacteriological incubator (Remi make, Mumbai)
7. Bunsen Burners

Methods:

1. Prepare nutrient agar plates.
2. Inoculate 0.1 mL of Vajreshwari water sample on nutrient agar plates and spread the sample thoroughly with the help of a glass spreader aseptically.
3. Incubate all the plates in a bacteriological incubator at 45 to 50^{o}C for 24 to 48 h.
4. Observe the plates and record the colony characteristics.
5. Pick up a single colony and subculture on nutrient agar slants.
6. Preserve the slants at a low temperature in a refrigerator.

Expected observation:

Colonies having different shapes and sizes are expected to appear on nutrient agar plates.

Model observation:

Morphologically distinct colonies were observed on nutrient agar plates.

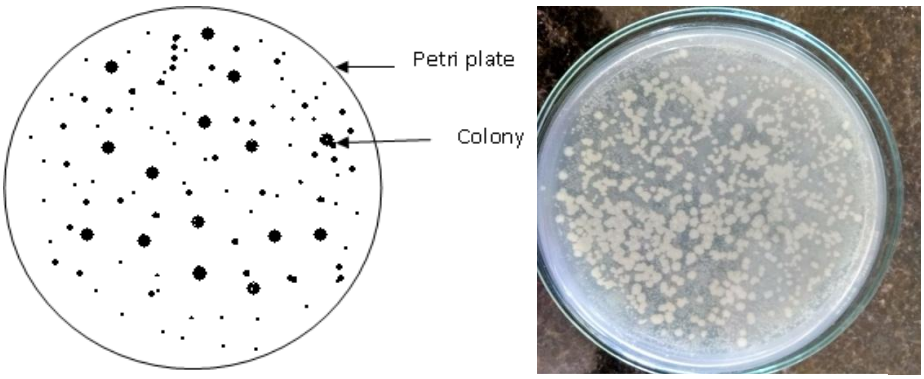

Figure: Colonies of thermophiles on a nutrient agar plate

Expected results:

Thermophilic bacteria are expected to be isolated from Vajreshwari hot spring water samples on nutrient agar plates.

Model result:

Thermophilic bacteria were isolated from Vajreshwari hot spring water samples and preserved on nutrient agar slants at a low temperature.

Space for students:

Figure:

Result:

References:

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5. Determination of temperature optima for the growth of thermophilic bacteria

Abstract:

Temperature influences metabolic activities of life. Every microorganism has an optimum temperature range at which it is stable and grows fast. Based on the temperature limits, the microorganisms are termed as psychrophiles (16-25°C), mesophiles (20-45°C), thermophiles (45- 70°C), and hyperthermophiles (more than 75°C). Being extremophiles with respect to temperature, psychrophiles, thermophiles and hyperthermophiles are important from an industrial point of view as they produce highly stable biomolecules like enzymes which can withstand high stress conditions [1,2,3]. Examples of thermophiles include *Sulfolobus acidocaldarius* (75-80°C) from the solfataric hot springs, *Thermus aquaticus* (70°C) from Yellowstone national park, U.S.A. and many species of *Bacillus* and *Geobacillus*. It is generally observed that stability of biomolecules produced by microorganisms lies in the range of their optimum values of growth parameters [4-11]. In order to study and make use of these thermophiles for various applications it is necessary to know their stability and growth at different temperature values. In this chapter, we have assessed the growth of selected thermophilic isolate at various temperature values.

Objective: To determine optimum temperature for the growth of selected thermophilic bacteria.

Materials:

1. Nutrient broth (Composition in g/L)

Peptone	5
Beef extract	3
NaCl	3
(pH adjusted to 7)	

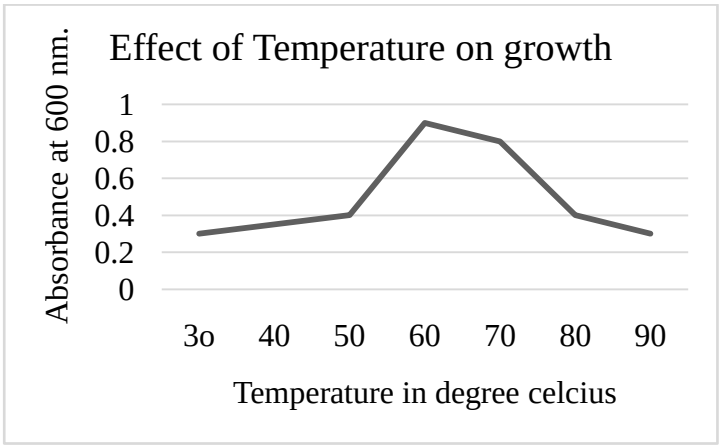
2. Digital weighing machine
3. Digital pH pen/meter
4. Autoclave
5. Bacteriological incubator (Remi make, Mumbai)
6. Bunsen burners
7. Colorimeter

Methods:

1. Prepare a fresh liquid culture (in nutrient broth) of selected thermophilic isolate.
2. Inoculate 0.1 mL of this freshly grown culture in 7 different conical flasks containing equal volume of nutrient broth media (sterile).
3. Incubate each flask at a specific temperature value in the range of 30-90^{o}C (with an increment of 10^{o}C) at a predetermined optimum incubation time period of the selected isolate.
4. After completion of incubation period, record the absorbance at 600 nm against sterile nutrient broth (blank).
5. Note down all the readings and plot the values on a graph by taking temperature on X-axis and absorbance (optical density) values on Y-axis.
6. Find the optimum temperature value for the growth of the selected thermophilic isolate.

Expected observation:

Absorbance value (optical density) of the selected culture will be the highest at an optimum temperature value as compared to other temperatures in the tested range.



(If peak absorbance noted at 60^{o}C)

Model observation:

Maximum growth (absorbance) of the selected thermophilic bacterial culture was observed at 60^{o}C temperature.

Expected results:

Optimum temperature for the growth of thermophilic bacteria is expected to have greater than 45°C.

Model result:

Optimum temperature for the growth of selected thermophilic isolate was 60°C.

Space for students:

Figure:

Result:

References:

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6. Determination of pH optima for the growth of thermophilic bacteria

Abstract:

Extremophiles are organisms that not only survive but grow in detrimental environments. Extreme conditions of temperature, pH, pressure and salinity challenge the physical and metabolic functions of a typical life. Acidophiles and alkaliphiles survive at acidic and alkaline pH conditions, respectively. Many bioprocesses perform normally under neutral pH conditions. However, extremophiles and their products are employed in various biotechnological operations under acidic and alkaline conditions [1, 2]. Alkaliphiles are capable of growing at a high alkaline pH value e.g. *Anditalea andensis* grows in the pH range of 7 to 11. Alkalistable enzymes isolated from alkaliphiles are important commercially. Thermophilic acidophiles belong to groups of iron and sulfur oxidizing bacteria (e.g., *Leptospirillum* sp., pH optima 2.0-2.3), sulfur-oxidizing archaea (e.g., *Metallosphaera* sp., pH optima 2.0-3.0), facultative anaerobes (e.g., *Acidithiobacillus ferrooxidans,* pH optima 1.2-2.0), and sulfur-reducing heterotrophs (e.g., *Acidianus* sp., pH optima 1.0-5.0). Acidophiles are mainly found in the volcanic areas and coal and metal mining industries lying in geothermal and coastal areas. Acidophiles can be used to treat acidic waste water produced from fermentation and nitrification processes. *Thermus aquaticus* has a pH optima of 7.5-7.8. Many species of thermophilic *Bacillus* and *Anoxybacillus* has alkaline 7.5- 9.0 pH range as optimum value for their growth [1, 3-8].

Microbes like fungi grow well in acidic conditions while many but not all bacteria require neutral to alkaline pH for growth. For bioprocess standardization it is necessary to know the pH optima for the growth of thermophilic isolates. In order to study thermophiles and use them for applications involving desired pH range, it is necessary to know their stability and growth at different pH values. In this chapter, we have assessed the growth of selected thermophilic isolate at different pH values.

Objective: To determine optimum pH for the growth of selected thermophilic bacteria.

Materials:

1. Nutrient broth (Composition in g/L)
   Peptone 5
   Beef extract 3
   NaCl 3
   (pH range from 3 to 11 with an increment of 1.0 pH unit need to be adjusted with 0.1N NaOH and HCL solution)
2. Digital weighing machine
3. Digital pH pen/meter or pH paper strips
4. Autoclave
5. Bacteriological incubator (Remi make, Mumbai)
6. Bunsen burners
7. colorimeter

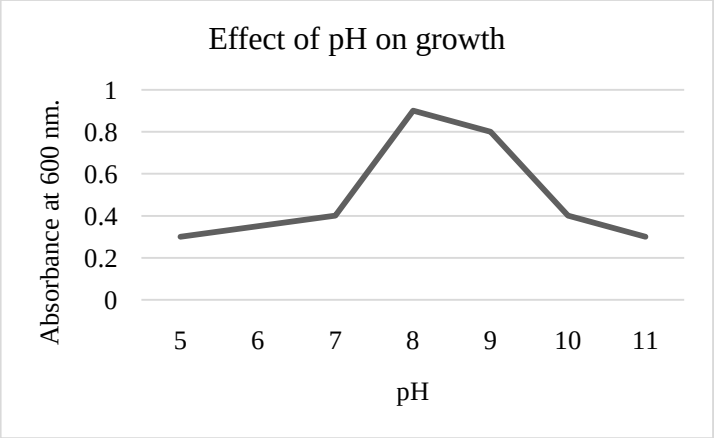
Methods:

1. Prepare 100 mL of nutrient broth in different conical flasks.
2. Adjust pH of the growth medium in each flask at a specific pH value in the range of 5-11 (with an increment of 1 pH unit) with the help of a few drops of diluted HCl or NaOH solution.
3. Check pH of growth media after sterilization and adjust again if necessary at the desired pH values.
4. Inoculate 0.1 mL of fresh liquid culture of a thermophilic bacterial isolate in each flask.
5. Incubate all the flasks at a predetermined optimum temperature and incubation period of the selected isolate.

6. After completion of incubation period, record the absorbance of the growth medium at 600 nm against sterile nutrient broth of the specific pH value (blank).
7. Note down all the readings and plot the values on a graph by taking pH range on X-axis and absorbance (optical density) values on Y-axis.
8. Find the optimum pH value for the growth of the selected thermophilic isolate.

Expected observation:

Absorbance value (optical density) of the selected culture will be the highest at an optimum pH value.



(If a peak absorbance noted at pH 8)

Model observation:

Maximum growth (absorbance) of the selected thermophilic bacterial culture was observed at pH 8.

Expected result:

Optimum pH for the growth of selected thermophilic bacteria is expected to be similar with the pH of its habitat sample.

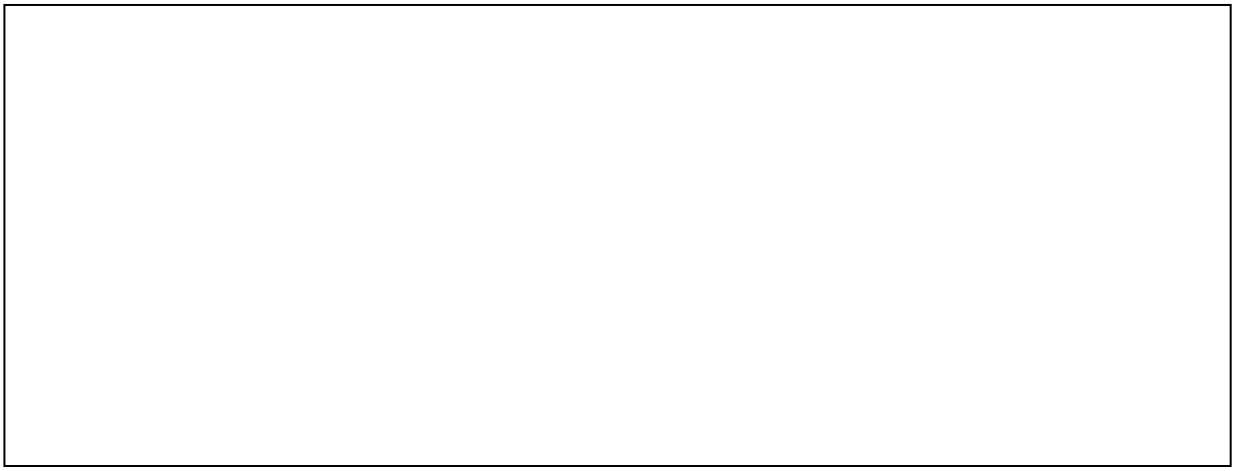
Model result:

Optimum pH for the growth of the selected thermophilic isolate was 8.

Space for students:

Figure:

Result:



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7. Screening for antibiotic production ability of thermophilic bacteria

Abstract:

Antibiotics or antimicrobials are produced by various microbes like bacteria, fungi and are a very important part of the health industry for humans as well as veterinary science. Since the discovery of penicillin many new groups of antibiotics have emerged and served the purpose in various disease control and management. Recent emergence of antibiotic resistance phenomena in pathogens is limiting our options for treatment of infections demanding discovery of new types of antibiotics. Much effort is being directed towards developing new antibiotics to overcome this problem. Discovery of novel chemical entities from microbial sources depends on the novelty of its habitat along with other factors. The diversity of geographical location decides the type of micro-flora which in turn gives rise to novel biomolecules. Several reports have suggested terrestrial and aqueous microorganisms providing several novel bioactive secondary metabolites of pharmaceutical importance. Comparatively hot-springs have not been as extensively exploited as other terrestrial resources [1,2,3]. Antibiotics producers reported mostly belong to fungi as well filamentous actinomycetes and bacilli [4,5]. According to literature hot springs are habitats of many actinomycetes, bacilli as well other microbes. *Streptomyces* sp., *Micromonospora* sp., *Microbispora* sp. and *Planosporangium* sp. are antibiotic producing actinomycetes reported from hot springs [6-9]. There are previous reports of thermophilic actinomycetes from Vajreshwari hot springs as well as antimicrobial compounds producing bacteria [10-12]. The methods used for screening of antibiotic producers include crowded plate technique and agar well/disc diffusion method [13,14]. In this chapter the efforts have been taken to screen thermophilic bacteria for antibiotic production by crowded plate technique.

Objective: To perform screening for antibiotic production ability of thermophilic bacteria

Materials:

1. Hot spring water sample
2. Nutrient agar (composition in g/L)

Peptone	5
Beef extract	3
NaCl	3
Agar powder	27

3. Digital weighing machine
4. Distilled water
5. Autoclave
6. Bacteriological incubator (Remi make, Mumbai)
7. Bunsen burners

Methods:

1. Prepare nutrient agar plates.
2. Inoculate 0.1 mL to 1.0 ml Vajreshwari water sample on nutrient agar plates and spread the sample thoroughly with the help of a glass spreader aseptically.
3. Incubate all the plates in a bacteriological incubator at 45 to 50^{o}C for 24 to 48 h.
4. Observe for colonies showing growth inhibition zone on plate and record the size of zone radius or diameter.
5. Pick up colony and subculture on nutrient agar slants.
6. Preserve the slants at a low temperature in a refrigerator.

Expected observation:

Colonies of microbes showing zone of inhibition for the test organism are expected to be seen on agar plates.

Model observation:

Morphologically distinct colonies were observed on plates showing zone of growth inhibition due to production of antibiotics or antimicrobial compounds.

Expected results:

Thermophilic antibiotic producing bacteria are expected to be isolated from Vajreshwari hot spring water samples.

Model results:

Thermophilic antibiotic producing bacteria were isolated from Vajreshwari hot spring water samples and preserved on nutrient agar slants at a low temperature.

Space for students

Figure:

Result:

References:

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8. Isolation of phosphate solubilizing halophilic bacteria from Bordi beach, Palghar, Maharashtra, India

Abstract:

Phosphorus (P) is one of the major essential macronutrients for plants as it has several roles in the plants. Phosphorus is involved in functioning of nucleic acids, proteins, photosynthesis and in the formation of oils, sugars and starches etc. [1]. Phosphate solubilizers are economical, ecofriendly and have greater agronomic utility to compensate the expensive inorganic sources of P fertilizers [2]. Phosphate solubilizing microorganisms (PSM) have attracted attention in semi arid regions and endowed to enhance the crop yields [3,4]. The growth of phosphate-solubilizing bacteria (PSB) often causes soil acidification, playing a key role in phosphorus solubilization [5]. Since the beginning of last century, many PSB have been isolated including, for example, those in *Bacillus*, *Pseudomonas*, *Erwinia*, *Agrobacterium*, *Serratia*, *Flavobacterium*, *Enterobacter*, *Micrococcus*, *Azotobacter*, *Bradyrhizobium*, *Salmonella*, *Alcaligenes*, *Chromobacterium*, *Arthrobacter*, *Streptomyces*, *Thiobacillus*, and *Escherichia* [6]. A moderately halophilic bacterium was isolated from the sediment of Daqiao saltern on the eastern coast of China, which also performs phosphate-solubilizing ability [7]. P-solubilizing activity is determined by the microbial biochemical ability to produce and release metabolites such as organic acids through their hydroxyl and carboxyl groups, to chelate the cations (mainly calcium) bound to phosphate and that will be converted into soluble forms [8]. Among the variety of microorganisms, phosphate solubilising bacteria plays a vital role in the regeneration of phosphates through solubilisation of unavailable inorganic phosphorous to available from [9]. In this chapter we have focused on isolation of phosphate solubilizing halophilic bacteria from Bordi beach sediment sample.

Objective: To isolate phosphate solubilizing halophilic bacteria from Bordi beach sediment sample

Materials:

Media

1. Modified marine agar medium [10]

Tryptone	3 gm
Yeast extract	1 gm
Agar agar	15 gm
Sea water aged	1000 ml
pH	7.5

2. Media supplemented either with lecithin or $Ca_3(PO_4)_2$.

a) Modified $Ca_3(PO_4)_2$ agar medium [11]

Glucose	10 gm
$(NH_4)_2SO_4$	0.5 gm
NaCl	30 gm
KCl	0.3 gm
$FeSO_4 \cdot 7H_2O$	0.03gm
$MnSO_4 \cdot 4H_2O$	0.03gm
$MgSO_4 \cdot 7H_2O$	0.3gm
$Ca_3(PO_4)_2$	10 gm
Agar powder	20 gm
Distilled water	1000ml
pH	7.0–7.5.

b) Lecithin agar medium [12]

Glucose	10 gm
$(NH_4)_2SO_4$	0.5 gm
NaCl	30 gm
KCl	0.3 gm
$FeSO_4 \cdot 7H_2O$	0.03gm
$MnSO_4 \cdot 4H_2O$	0.03gm
$MgSO_4 \cdot 7H_2O$	0.3 gm
lecithin	0.2 gm
$CaCO_3$	5.0 gm
yeast extract	0.4 gm
Agar powder	20 gm
Distilled water	1000 mL
pH	7.0–7.5.

3. Pikovskaya's agar medium [6]

4. Pikovskaya's broth [6]

Miscellaneous:

1. Digital weighing machine
2. Digital pH pen/meter or pH paper strips
3. Autoclave
4. Bacteriological incubator
5. Bunsen burners

Methods:

1. Prepare modified marine agar, modified $Ca_3(PO_4)_2$ agar , Lecithin agar and Pikovskaya's agar media (pH 7.5) plates.
2. Approximately 1gm sediment sample suspended in 100 mL of sterilized seawater and vortexed for 10min.
3. Prepare 10-fold serial dilution of the sediment suspension.
4. Inoculate 0.1 mL of the diluted sample on all the agar plates and spread the sample thoroughly with the help of a glass spreader aseptically.
5. Incubate all the plates in a bacteriological incubator at 28^oC for 7 days.
6. Observe the zones of phosphate-solubilizing around the colonies and record the colony characteristics.
7. Subculture desired colonies on agar slants of respective media and preserve at a low temperature.

Expected observations:

Colonies having different shapes and sizes are expected to appear on selected agar plates and some of the bacterial colonies may show clear zones around them on media containing insoluble mineral phosphate such as tri-calcium phosphate.

Model observation:

Morphologically distinct colonies were observed on marine agar plates and a few colonies have shown clear zones around them.

Expected results:

Phosphates solubilizing halophilic bacteria are expected to be isolated from sediment samples on selected agar (pH 7.5) plates.

Model result:

Phosphate solubilizing halophilic bacteria were isolated from Bordi beach sediment samples and preserved at a low temperature.

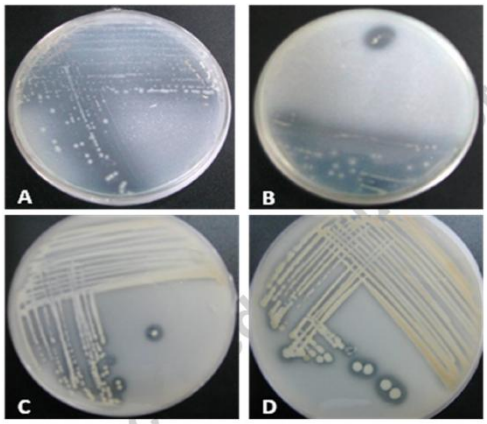


Figure: Phosphate solubilization by bacteria [13]

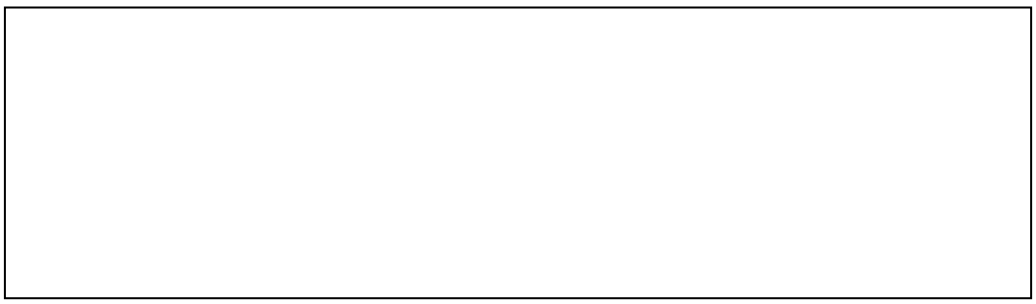
Space for students

Figure :

Morphological characterization of the isolates:

Biochemical characterization of the isolates:

Result:



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9. Production and extraction of antimicrobial compounds from halophilic bacteria and actinobacteria

Abstract:

Life exists over the whole range of salt concentrations encountered in natural habitats from freshwater environment to hypersaline lakes, such as the Dead Sea, saltern crystallizer ponds and other places saturated with respect to sodium chloride. The diversity in the properties of saline and hypersaline habitats on the arth is reflected in the great diversity within the microbial communities adapted to life under the prevailing conditions [1]. Infectious diseases and multidrug resistance of clinically relevant pathogens are threatening global human health care system. Multidrug resistant bacteria have become a severe threat to community well being. Conventional antibiotics are getting progressively more ineffective as a consequence of resistance, making it imperative to realize improved antimicrobial option [2]. Natural products especially microbial metabolites are fertile source of bioactive scaffolds and serve as the foundation for the development of several life saving antibiotics [3]. Marine Actinobacteria evolved with unique physiological, chemical and structural features that enable them to survive under the varying pressure, salinity and temperature occurring in marine ecosystem [4, 5]. In fact such marine organisms are further endowed with the ability to produce novel molecule with interesting therapeutic applications not observed in their terrestrial counterparts, which is evident from various finding reporting the production of diverse bioactive compounds [6-13].

Some halophilic bacterial examples and their antimicrobial compounds from various findings are given in the following table.

Isolation source	**Genus**	**Compound/ molecules**	**Reference**
Saline soil of Kovalam solar salterns India	*Nocardiopsis* sp. AJ1	Actinomycin C2	[16]
Sediments of mangrove Nizampatnam, Bay of Bengal, Andhra Pradesh, India	*Pseudonocardia endophytica* VUK-10	N-(4aminocyclooctyl) -3,5dinitrobenzamide,	[17]
Condenser water, solar salt works in Thamaraikulam, Kanyakumari district, Tamil Nadu, India	*Bacillus* sp. BS3	Lipopeptide biosurfactants, 13-Docosenamide, (Z), Mannosamine, 9-Octadecenamide, (Z)	[18]
Solar salt condenser, Thamaraikulam solar saltern, Kanyakumari district, Tamil Nadu, India	*Halomonas salifodinae* MPM-TC	Perfluorotributylamin	[19]
Seashore soil, Bigeum Island,SouthWest coast of South Korea	*Streptomyces hygroscopicus* BDUS 49	7-Demethoxy rapamycin	[20]
Marine sediment of Mission Bay, San Diego, South California	*Marinispora* sp. NPS12745	Lynamicin A, Lynamicin B, Lynamicin C, Lynamicin D	[21]
Platinum Coast on the Mediterranean Sea, north of Egypt	*Streptomyces* sp. Merv8102	Essramycin	[22]

Marine sediment, La Jolla,California	*Streptomyces* sp. CNQ-418	Marinopyrroles A, Marinopyrroles B	[23]
Sediment of Bay of Bengal,India	*Streptomyces chibaensis* sp. AUBN1/7	1-Hydroxy-1-norresistomycin	[24]
Sandy sediment, coastal site of Mauritius, Indian Ocean	*Streptomyces* sp. B6921	Fridamycin D, Himalomycin A, Himalomycin B	[25]
Saline soil, Qaidam Basin, north-west China	*Nocardiopsis terrae* YIM 90022	p-hydroxybenzoic acid, N-acetyl-anthranilic acid, Indole-3-carboxylic acid	[26]

In this chapter we have explained a simple methodology for the isolation of crude antimicrobial extract from halophiles and further given a simple protocol for qualitative screening of its antimicrobial activity against some test pathogens

Objective: Production and extraction of antimicrobial compounds from halophilic bacteria and actinobacteria

Materials:

A] Media:

a. Sea water medium (for halophilic bacteria) (SWM): [14]

Peptone:	5 gm
Yeast extract	2.5 gm
Glucose	1.0 gm
K_2HPO_4	0.2 gm
$MgSO_4$	0.05 gm
Sea water aged	500 ml
Distilled water	500 ml
pH	7.1

b. Starch Casein broth (for actinobaceria) (SCB): [15]

Soluble starch	10 gm
Casamino acid	0.3 gm
Calcium carbonate	0.02 gm
Fe_3SO_4. $7H_2O$	0.01 gm
KNO_3	2.0 gm
$MgSO_4$. $7H_2O$	0.05 gm
NaCl	5.0 gm
Aged sea water	1000 ml
pH	7.1

(Note: a and b both media can be used for inoculum preparation and fermentation)

c. Muller Hinton agar (HiMedia, Mumbai)

B] Chemicals:

a.	1N NaOH	10 ml
b.	Phosphate buffer (pH7.4)	100 ml
c.	Ethyl acetate	100 ml
d.	Ammonium sulphate	100 gm
e.	70% Alcohol	20 ml

C] Miscellaneous:

i. Shaking incubator
ii. Centrifuge (Remi RM-12C Micro Centrifuge)
iii. Separating funnel
iv. Magnetic stirrer
v. Digital weighing machine
vi. Digital pH pen/meter
vii. Autoclave
viii. Bacteriological incubator (Remi make, Mumbai)
ix. Bunsen burners
x. Swabs
xi. Whatman filter paper No.1 discs
xii. Forceps

Methods:

1. Inoculate freshly cultured halophilic bacterial and/or actinobacterial isolates in the medium SWM and SCB respectively at 30^{o}C for 24 h in shaking incubator at 120 rpm agitation speed.
2. Add 5% of inoculum in the fermentation medium.
3. Incubate all the flasks containing fermentation medium in a shaking incubator for 10 days at 30^{o}C with the agitation speed of 120 rpm.
4. After 10 days of incubation, adjust pH of the fermented medium to 7 using 1N NaOH and centrifuge at 3000Xg for 20 min.
5. Collect cell free extract from broth (supernatant) and keep at 4^{o}C for 24 h.
6. Take about 20 ml of cell free extract in to a separating funnel, add equal volume of ethyl acetate, and mix both phases thoroughly for 30 min.
7. Separate out the lower organic phase from the funnel and store in a refrigerator for 24 h.
8. Mix lower organic phase with ammonium sulphate (55% w/v) and stir the mixture on magnetic stirrer for 20 min.
9. Remove the mixture from magnetic stirrer and centrifuge at 10000Xg at 4^{o}C for 20 min.
10. The pellets obtained are suspended in phosphate buffer (pH 7.4).
11. Use this partially purified crude suspension for antimicrobial activity testing by well diffusion or disc diffusion method against some selected clinical pathogens.
12. Spread 0.1 mL liquid culture of the selected test pathogen on Muller Hinton agar plates in aseptic condition.
13. Soak/dip Whatman filter paper no.1 disc (3-4 mm in size) in crude extract and place on Muller Hinton agar.
14. Keep the plate in a refrigerator for better diffusion of liquid from the disc for about 20 min.
15. Incubate plates in an incubator at 37^{o}C for 24-48 h and observe the zone of growth inhibition after incubation period.

Expected observation:

The extract containing partially purified antimicrobial compound is expected to show a zone of growth inhibition against selected clinical pathogen.

Model observation:

Zone of growth inhibition has been shown by the extract containing antimicrobial compound(s) against the selected pathogenic organism(s).

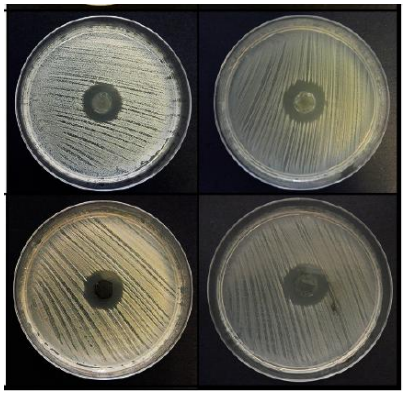

Fig: Antimicrobial activity [27]

Expected results:

The extract containing partially purified antimicrobial compound is expected to give zone of growth inhibition against selected clinical pathogen like *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* etc.

Model results:

Partially purified extract containing antimicrobial compounds obtained from halophilic bacteria and actinobacteria was stored in a refrigerator at 4^{o}C and its antimicrobial activity was tested against the selected clinical pathogens.

Space for students

Figure:

Result:

References:

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10. Screening for organic acid production ability of halophiles isolated from a mangrove region

Abstract:

Organic acids are a long chained organic carbon containing compounds with their attached functional carboxylic groups showing acidic properties. Organic acids are produced exogenously by a number of microorganisms [1]. Organic acids from microbial origin serve as the carbon sources for microorganisms during the activity of solubilization of phosphorus which gets consumed and reduced. This postulates that secretion of those organic acids must be of a continuous process that secreted downwards [2]. Several strains of extremely halophilic bacteria produced pyruvate when grown in the presence of glucose [3]. An alkaliphilic and halophilic bacterium was isolated from a commercial *Spirulina* culture. Pyruvate is widely used as a starting material in the industrial biosynthesis of pharmaceuticals, and is employed for production of crop protection agents, polymers, cosmetics, and food additives [4]. The alkaliphilic and halophilic bacterium *Halomonas* sp. KM-1 can utilize both hexose and pentose sugars for the intracellular storage of bioplastic poly-(*R*)-3-hydroxybutyric acid (PHB) under aerobic conditions [5]. Oxaloacetate is an important intermediate chemical in the TCA cycle, glycogenesis, and aspartic acid biosynthesis. It is used as a health food based on its role in energy synthesis; however, it can easily be decarboxylated to pyruvate in ambient water solutions [6]. Microbial production of organic acid is a promising approach in recent day's biotechnology. Production of organic acid through an economically commercial process opens new markets by providing new opportunities for the chemical industries [7]. Non growing cells of *Halobacterium saccharovorum* oxidized lactose to a product identified as lactobionic acid by thin-layer, paper, and column chromatography, and by identification of the galactose and gluconic acid produced from it after acid hydrolysis [8]. A rising chemical segment of industrial production including fumeric, propionic and itaconic acids are chiefly attributed to organic acid production through microbial fermentation of carbohydrates and similar substrates [9]. In this chapter we have focused on the isolation of organic acid producing halophilic organisms from sediment samples.

Objective: To perform screening for organic acid production ability of halophiles.

Materials:

1. Fresh suspension culture of selected halophilic bacteria isolated from a mangrove region
2. Sabouraud dextrose agar (SDA) (HiMedia, Mumbai)
3. Phenol red
4. Sodium chloride
5. Digital weighing machine
6. Digital pH pen/meter
7. Autoclave
8. Bacteriological incubator (Remi make, Mumbai)
9. Bunsen burners
10. Glass spreader

Methods:

1. Prepare Sabouraud dextrose broth containing 5 % NaCl, adjust pH to 7.2 and add a pinch amount of phenol red; ensure the formation of red colour of the broth.
2. Add agar powder (2.5 %) in the above medium, proceed for the sterilization by moist heat method and then prepare solid-agar plates
3. Inoculate diluted 0.1 ml of the test halophilic culture onto the surface of agar plates and spread aseptically with the help of a glass spreader.
4. Incubate all the plates in a bacteriological incubator at 30^{o}C temperature for 48 to 72 h.

5. Record the acid unitage value which gives effective production of organic acid by the colonies of selected isolates [11].
6. To calculate acid unitage values (AU) use the following formula.

$$\text{Acid Unitage} = \frac{\text{Diameter of a yellow-orange colored zone around the promising colony}}{\text{Diameter of the colony}}$$

Expected observations:

It is expected to form a yellow or orange colored zone around the promising bacterial colony as the color of phenol red containing medium turns into orange in presence of moderate acidic condition (pH) and yellow in presence of high acidic pH. Acid unitage value may be used to find the efficiency of a specific colony for organic acid production.

Table-1 Acid Unitage value of isolates [11]

Colony number/ name	Diameter of the zone in mm	Diameter of a colony in mm	Acid Unitage value (AU)
Colony-1			
Colony-2			
Colony-3			
Colony-4			
Colony-5			

Model observation:

Yellow or orange colored zones around the promising bacterial colonies were observed.

Expected results:

Screening for organic acid production ability of halophiles is expected by using modified Sabouraud dextrose agar medium.

Model result:

Screening for organic acid production ability of selected halophiles was performed.

Space for students:

Figure:

Results:

References:

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11. Qualitative screening for auxin (IAA) production from halophiles

Abstract:

Indole-3-acetic acid (IAA) is a phytohormone which is one of the important compounds for plant growth and development, affecting cell elongation, cell enlargement and cell division. IAA synthesizes both in the plants and microorganisms. For microorganisms, production of a phytohormone, IAA, is one of the essential criteria for plant growth promotion [1]. IAA is a naturally occurring auxin produced through L-tryptophan metabolism pathway. IAA increases root growth and length, expands the root surface area, facilitates the absorption of soil nutrients by plants [2,3,4]. Effect of inoculation of bacteria producing IAA on the growth of canola seedlings in the greenhouse experiment also showed that these strains have enhanced growth of canola seedlings [5]. IAA positively influences root growth and development, thereby enhancing nutrient uptake [6]. IAA triggers the enzyme H^+-ATPase which is elementary for synthesis of energy in the nodule of leguminous plant roots [7]. Since the beginning of last century, many IAA producing microorganisms have been isolated including, for example, those in *Stryptomyces* sp., *Bacillus subtilis*, *Enterobacter cloacae*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Agrobacterium tumefaciens*, *Alcaligenes faecalis* and *Azotobacter tumefaciens* [9-14]. In this chapter we have focused on the qualitative detection and quantitative estimation of IAA from halophiles.

Objective: To perform qualitative and quantitative detection of auxin (IAA) from selected halophiles

Materials:

1. 24 hr old culture of the selected halophilic isolates
2. 1% Tryptone broth (5% NaCl)
3. Nutrient broth (5% NaCl)
4. Xylene solution
5. Ehrlich's reagent
6. Digital weighing machine
7. Digital pH pen/meter
8. Autoclave
9. Bacteriological incubator (Remi make, Mumbai)
10. Bunsen Burners

Methods:

1. Inoculate about 0.1 mL of 24 h old culture of selected halophile into 5 mL of tryptone broth and incubate at 28^o C for 5 days.
2. After incubation, add 3-4 drops of xylene and mix vigorously and separate the two layers.
3. Slowly add 1 mL of Ehrlich's reagent to form a layer on the surface of xylene **[8]**.
4. Observe and record the results.

Expected observation:

Formation of pink colored ring at the lower surface of xylene layer is expected which confirms presence of IAA.

Model observation:

Formation of a pink colored ring at the lower surface of xylene layer was observed.

Model Result:

The isolates exhibit their positive reaction by developing pink colour.

Model result:

Formation of a pink colored ring at the lower surface of xylene layer indicated the production of IAA and thus qualitative screening for IAA production was performed.

[Note: In qualitative screening only presence of desired product is tested; however in quantitative screening concentration of desired product is recorded.]

Space for students

Figure:

Result:

References:

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12. Screening for HCN production ability of halophilic bacteria isolated from mangrove rhizosphere, Bordi, Maharashtra, India

Abstract:

Halophiles are microorganisms that grow in or can tolerate saline conditions [1]. They have been isolated from diverse saline environments such as natural brines and hypersaline lakes. Hypersaline habitats favor microbial species having complexity in their composition and nature [2]. Bordi ($72^{o}44'24.0468''$ E) is located in Palghar district of Maharashtra where a coastal region (Beach) of mangrove ecosystem is present. Over the past few decades, halophiles have been considered for biotechnological applications. Diverse response mechanisms of halophiles under high-salinity conditions cause the production of various valuable biomolecules. It has been recognized that halophiles are also major sources of stable enzymes that function in very high salinity, an extreme condition that results in denaturation and aggregation of most conventional proteins [3,4]. Furthermore, halophiles are also considered to be potential sources for discovery of bioactive compounds, compatible solutes, and unique enzymes including other potential biotechnological uses [5,6,7]. Salinity is one major limiting factor to plant growth and crop productivity [8]. A new biological approach 'plant-microbe interaction' to conquer salinity troubles has recently gained a great interest from many workers throughout the world. The use of rhizobacteria is one of the most acceptable approaches to reduce the effect of salt-stress on plants as they are endowed with mechanisms which either modulate or ameliorate the salt stress [9]. To overcome this problem, application of plant growth promoting halophilic bacteria (PGPB) in salt-affected soil, bioinoculant with salt-tolerant property is required for better survival and performance in the field. Plant growth promoting rhizobacteria produce chemical compounds with different benefits for the plant. Among them, hydrogen cyanide (HCN) is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens. Production of HCN by certain species of *Pseudomonas, Bacillus,* and *Rhizobium* has been involved in suppression of soil borne pathogens. HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens. Cyanide forms stable complexes with the essential elements (Cu^{2+}, Fe^{2+}, and Mn^{2+}) for the protein function and therefore is considered a toxic substance to most living organisms [10,11,12,13]. So this HCN production characteristic may help to discriminate species of *Pseudomonas, Bacillus,* and *Rhizobium* from other organisms by inhibiting other pathogens present in the rhizosphere. Hydrogen cyanide (HCN) is a volatile secondary metabolite that is synthesized by many rhizobacteria and has a powerful effect on many organisms. In this chapter we have focused on isolation of halophilic bacteria producing HCN from the Bordi region mangrove associated soil sample.

Objective: To perform screening for HCN production ability of halophilic bacteria isolated from mangrove rhizosphere, Bordi, Maharashtra, India

Materials:

1. Modified nutrient agar (Composition in g/L)

Peptone	10
Beef extract	10
Sodium chloride	5
Glycine	4.4
Agar	12
pH after sterilization	7.3±0.1

2. Whatman filter paper No.1
3. Reagent A (sodium carbonate 2% and picric acid 0.5%)
4. Parafilm
5. Digital weighing machine
6. Autoclave
7. Bunsen burners

Methods:

1. Prepare modified nutrient agar plates.
2. Inoculate a spot of loop full suspension of halophilic bacteria isolated from mangrove rhizosphere (Bordi) on modified nutrient agar plates.
3. Soak filter papers in reagent A and place in the upper lid of Petri dishes.
4. Seal the plates with parafilm to prevent volatilization and incubate at 30°C for 5-6 days.
5. Observe and record the results.

Expected observations:

Yellow filter paper changes to cream, light brown, dark brown and eventually turn into reddish-brown depending upon amount of HCN produced.

Model Observation:

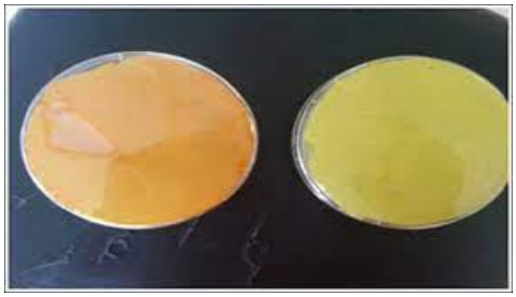

Figure : Left side plate shows HCN production while right side plate is a negative control.

Color of the filter paper was changed from yellow to dark brown which indicated HCN production (Figure 1).

Expected results:

HCN producing halophiles are expected to be isolated from Bordi region mangrove associated soil sample.

Model result:

Halophiles isolated from Bordi region mangrove associated soil sample were screened for the production of HCN.

Space for students

Figure:

Result:

Figure: Mangrove forest at Bordi Beach, Palghar, Maharashtra, India

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13. Isolation of halophiles from mangrove rhizosphere and screening for ammonia production

Abstract:

Halophilic microorganisms from a group of soil microorganisms are able to grow in habitats with high salt concentration and they are adapted to these conditions. [9]. A group of bacteria that can grow very well in the alkaline conditions and in the presence of salt is known as haloalkalophiles. This dual haloalkalophilic characteristic is making them interesting for basic studies of different aspects of biotechnology [2][5]. Haloalkalophiles can be isolated from diverse habitats such as alkaline lakes, saltern lakes, salt brines, carbonate springs and sea salt and some from saline and alkaline environments. [7]. Endophytic bacteria from genus *Bacillus* isolated from leaves of halophytes from the coastal region of Gujarat have shown their ability to produce ammonia. [1]. Ammonium produced during nitrogen fixation in soda lakes can be oxidized to nitrate via nitrite by haloalkalophilic nitrifers. In soda lakes and soda soils ammonium oxidation to nitrite is performed by an extremely alkali tolerant sub population of *Nitosomonas halophila,* while nitrite oxidation can be performed by moderately alkali tolerant *Nitrobacter alkalicus* [8]. *Halomonas* sp. MAN 5, *Halobacillus* sp. MAN 6 and *Halobacillus* sp. are known to produce HCN and ammonia in the presence of a wide range of NaCl [4]. This chapter is focused on qualitative detection of ammonium production by some indigenous extremophiles (halophiles, alkalophiles and haloalkalophiles).

Objective: Isolation of halophiles from mangrove rhizosphere soil sample and their screening for ammonia production.

Materials:

1. Sediment soil sample from mangrove rhizosphere
2. Nutrient agar containing 5% NaCl (pH 8.5)
3. Peptone water
4. Nessler's reagent
5. Centrifuge (Remi make, Mumbai)
6. Bacteriological incubator (Remi make, Mumbai)
7. Bunsen burners

Methods:

1. Collect the soil sample from mangrove rhizosphere.
2. Weigh 10 gm of soil and add into 100 ml of saline water (0.85% w/v NaCl).
3. Serially dilute saline suspension of soil sample and spread over the sterile nutrient agar plate containing 5% NaCl and incubate on room temperature for 8 days.
4. Select morphologically different colonies and cultivate them in sterile peptone water containing 5 % Nacl at a room temperature for 24 h.
5. Centrifuge the peptone broth at 150 rpm for 10 min and use supernatant for ammonia detection.
6. Add 0.5 ml of Nessler's reagent in the supernatant sample and keep for 30 min at a room temperature.
7. Record the observations and results.

Expected observation:

Change in supernatant color from pale yellow to reddish brown is expected.

Model observation:

The color of supernatant has changed from pale yellow to reddish brown.

Expected results:

Halophiles, Haloalkalophiles and/or Alkaliphiles are expected to be isolated from mangrove rhizosphere sediment soil samples on saline nutrient agar plates (pH-8.5) that shows considerable amount of ammonia production.

Model Result:

Halophiles were isolated from mangrove rhizosphere soil samples and ammonia production was recorded.

Space for students

Figure:

Result:

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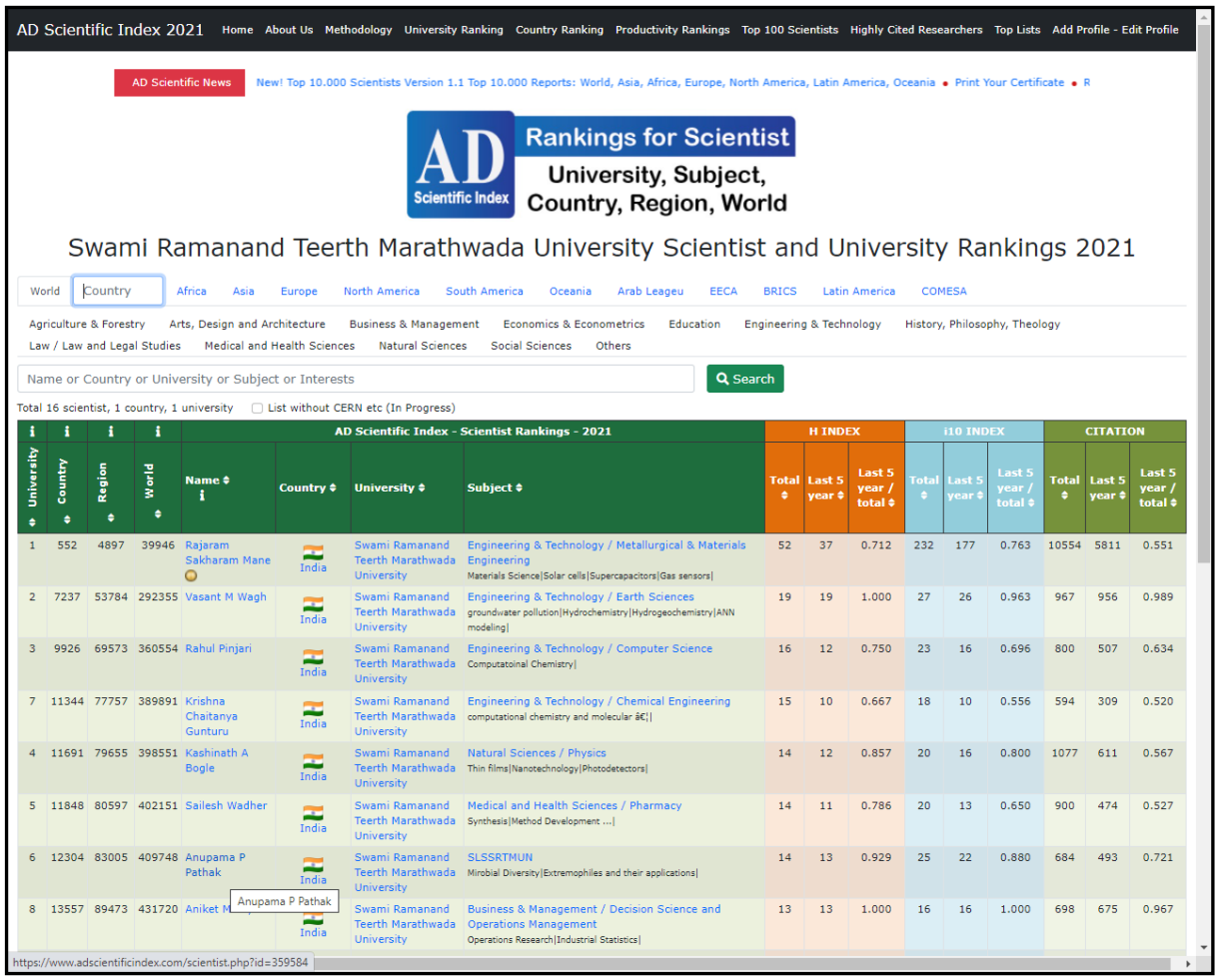

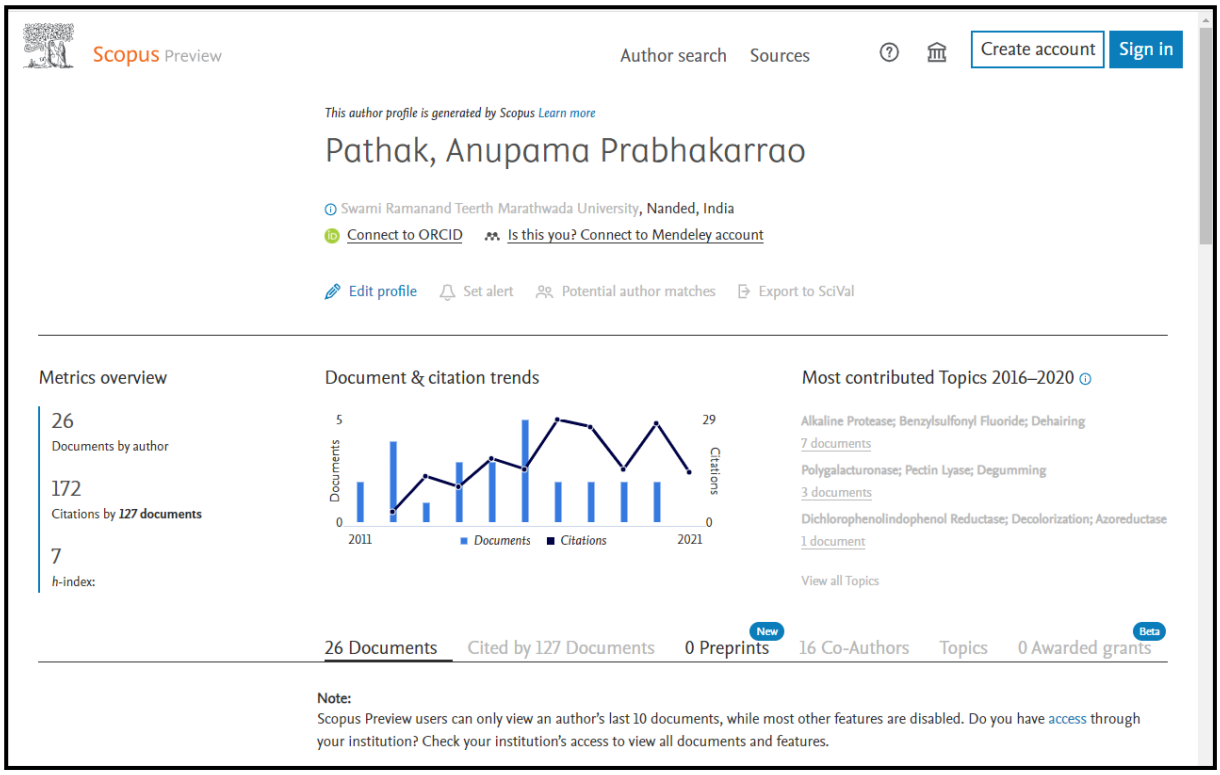
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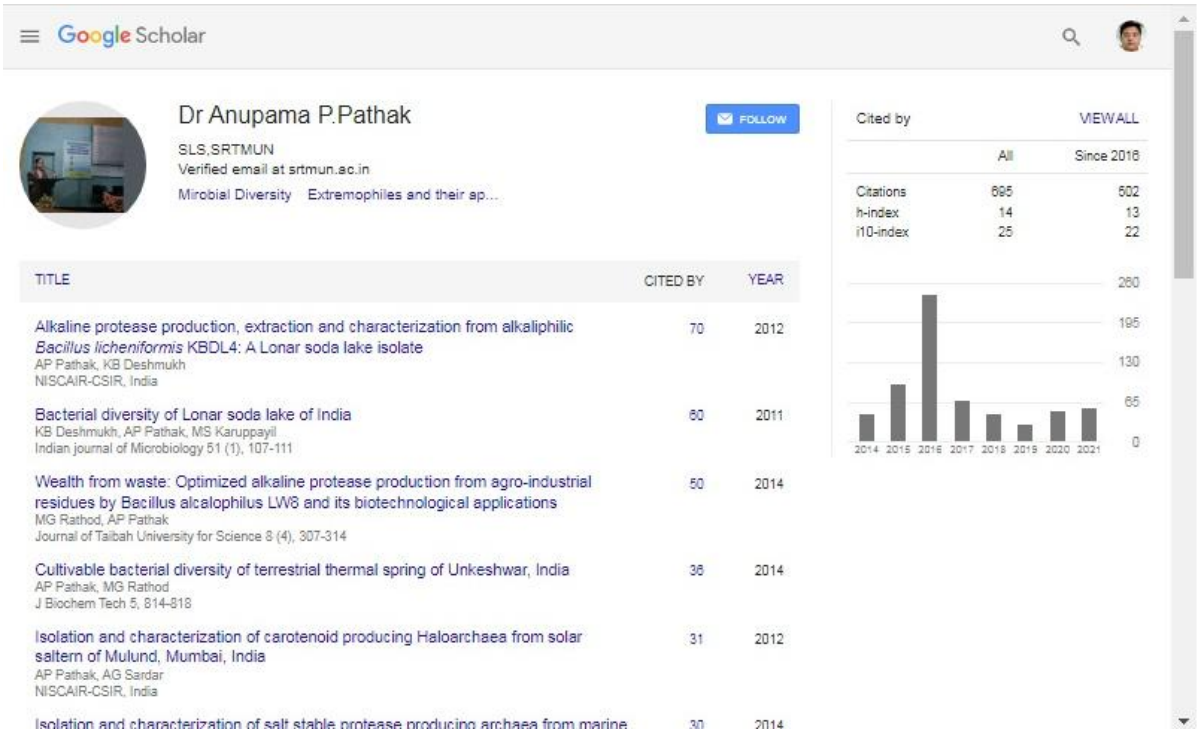

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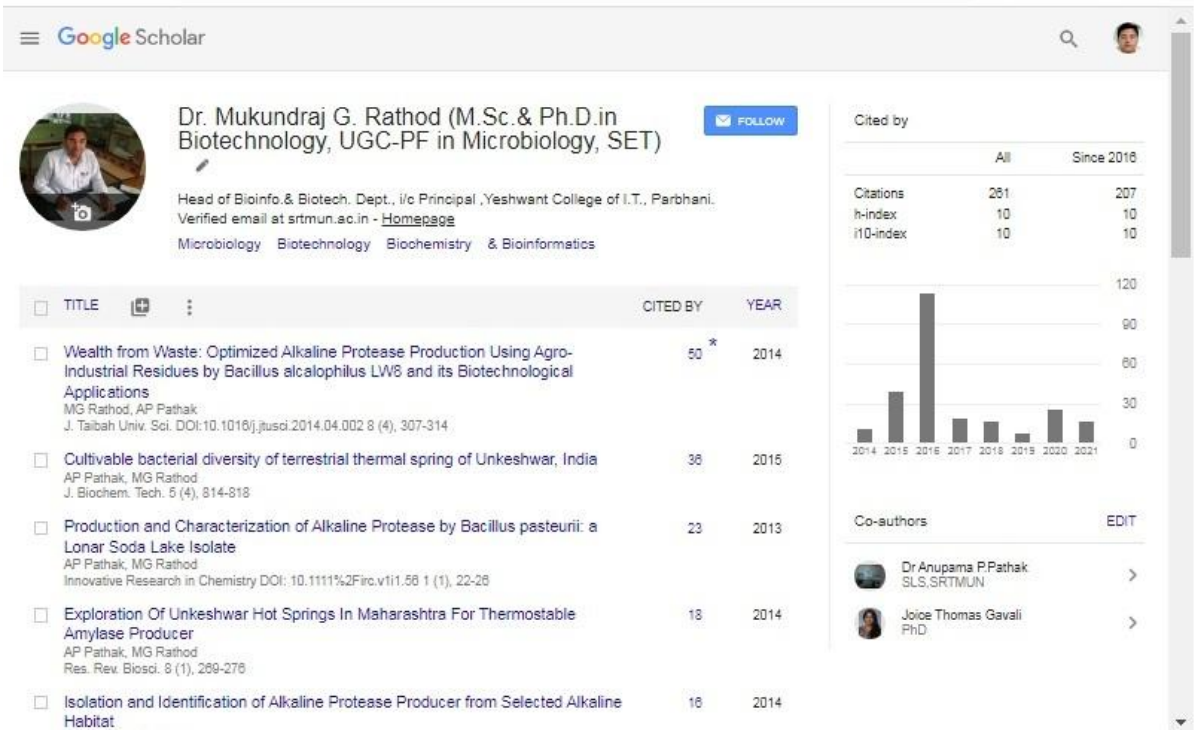

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TITLE	CITED BY	YEAR
Wealth from Waste: Optimized Alkaline Protease Production Using Agro-Industrial Residues by Bacillus alcalophilus LW8 and its Biotechnological Applications MG Rathod, AP Pathak J. Taibah Univ. Sci. DOI:10.1016/j.jtusci.2014.04.002 8 (4), 307-314	50 *	2014
Cultivable bacterial diversity of terrestrial thermal spring of Unkeshwar, India AP Pathak, MG Rathod J. Biochem. Tech. 5 (4), 814-818	36	2015
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CERTIFICATE OF GRANT

INNOVATION PATENT

Patent number: 2021104201

The Commissioner of Patents has granted the above patent on 13 October 2021, and certifies that the below particulars have been registered in the Register of Patents.

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Title of invention:

AN ORGANIC FOOD PRESERVATIVE COMPOSITION

Name of inventor(s):

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Eight years from 15 July 2021

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Patent number: 2021107011

The Commissioner of Patents has granted the above patent on 8 December 2021, and certifies that the below particulars have been registered in the Register of Patents.

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Title of invention:

A METHOD FOR ECOFRIENDLY RECYCLING LIGNITE FLY ASH FOR IMPROVEMENT OF GROWTH AND BIOCHEMICAL CONSTITUENTS OF SPIRULINA PLATENSIS

Name of inventor(s):

Saranraj, P.; Nivetha, M.; Nisha, R.; Totewad, Narayan Dattatraya; Balasaheb, Joshi Vikas and Feliciano, Gláucio Diré

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position in the top most leading scientists of S.R.T.M. university, Nanded based on the last five year's total H index analysis as calculated by AD scientific index Ltd. (2021). Recently, the Australian government has granted her a patent (patent no. 2021104201) for inventing the organic food preservation composition. She is now actively engaged in research by filing many patents. She has planned the organization of many national and international conferences in future for providing a wide platform for all the knowledge-seekers. Recently she has authored, edited and published the first volume of this book.

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Ms. Sonali Vishwanath Girdhari

(Ph.D. Student)

Ms. Girdhari has teaching and research experience in the subjects of Microbiology and Biotechnology, currently working on isolation, identification and exploration of thermophilic bacteria for commercial applications and pursuing PhD in biotechnology at School of life sciences, S.R.T.M. University, Nanded [supervisor- Dr. (Mrs.) Anupama P Pathak (Director of School of Life sciences)]

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Ms. Sonali Girdhari has teaching and research experience in the subjects of Microbiology and Biotechnology, currently working on isolation, identification and exploration of thermophilic bacteria for commercial applications and pursuing PhD in biotechnology at School of life sciences, S.R.T.M. University, Nanded [supervisor- Dr. (Mrs.) Anupama P Pathak (Director of School of Life sciences)]

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