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Microbiological and physiochemical attributes of hot water sulphur spring of Unkeshwar

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ABSTRACT: A water sample was collected from hot sulphur spring of Unkeshwar for microbial and physiochemical study. Water sample is subjected to physiochemical analysis like pH, TDS, conductivity, chlorine, salinity, dissolved oxygen (DO), biological oxygen demand (BOD) and chemical oxygen demand (COD). The BOD and COD were found in lower amount. Four bacterial strains were isolated from the water sample and all they are spore former, gram positive, motile in nature. Among them one bacterial species was identified and confirmed by 16S r-RNA sequencing showed 97 % similarity to *Bacillus licheniformis*. The identified strain was observed under SEM showed an average length was 1.43 µm. The optimum pH and temperature for the growth of BWU-1 isolate was 9 and 50°C. The performed MPN method showed the absence of coliform bacteria in the water sample. The *B. licheniformis* BWU-1 having potential to produce the hydrolytic enzymes such as protease and amylase were analyzed on SMA and Starch agar plates respectively by observing zone of clearance.

Key words: Microbial and physiochemical analysis, SEM, Protease

Introduction

Springs are the places where ground water is discharged at specific locations on the earth and they vary dramatically as to the type of water they discharge. Many of the springs are the result of long cracks or joints in sedimentary rock (Brock, 1978). Hot springs are having the temperature of the water lies significantly above the mean of annual air temperature of that region (Sen et al., 2010). Microorganisms thriving in elevated temperature terrestrial and deep-sea hydrothermal systems have observed and inspect by several authors (Cadirci 2007). Temperature is one of the most important factors that govern species abundance and distribution. High temperatures in soil and/or water exert pressure on microbial species leading to the selection of specific flora capable of tolerating and surviving heat stress. Some species can survive at the elevated temperatures of hot springs, or in various other adverse environments. The defense mechanism cells utilize when confronted with high temperatures in their local environment is known as the heat shock response. This response has been described extensively in both eukaryotes and prokaryotes (Carlos, 1988). When thermal stress is applied, the most prominent physiological reactions are the production of a set of novel proteins or an increase in the quantity of certain types of existing proteins. In the present investigation, there are different parameters was analyzed such as microbiological and chemical analysis of water sample as well as the production of biotechnological important enzymes such as protease and amylase from the selected bacterial strain BWU-1 .

Materials and Methods

Sampling and Isolation of Bacteria: Water sample for viable bacterial counts were taken in sterile thermal containers from hot springs that are located at Unkeshwar, Dist. Nanded (M.S.) India. Temperature was measured in situ with a mercury bulb thermometer. The bottles were filled completely and then closed tightly to prevent the loss of dissolved gases. The water samples were brought back to the laboratory and analyzed within 24 h. Bacteria were isolated and enumerated using the standard plate method. 0.1 ml of the proper dilution was used to surface inoculate nutrient and thermus agar media (Thermus agar containing (%): 0.5 NaCl, 0.5 peptone, 0.4 beef extract, 0.2 yeast extract and 2 agar-agar, having pH 9.0 prepared in glycine-NaOH buffer) (Alane et al., 1996). From each dilution, five plates were inoculated at 45°C. Colonies were counted every day until maximal plate counts were obtained. Numbers were expressed as colony forming units (c.f.u.) ml^{-1}. Colonies obtained on thermus agar, were isolated and further purified on thermus medium. The isolates were stored at 2-8°C.

Chemical analysis: The water chemistry was determined in triplicate by using standard method and confirmed by water analyzer 371(Systronic make) (Kodarkar, 2006 and Aneja, 2003).

Microbial analysis: The obtained four isolates were tentatively identified as *Bacillus* sp. by analyzing its morphological, cultural and biochemical characters as per Bergey's Manual of Determinative Bacteriology. Among them one of the sample was identified by 16s r-RNA sequencing method. The presence of coliform bacteria was checked by Most Probable Number (MPN).

Effect of Incubation period on growth of the isolate: The BWU-1 isolate was incubated in thermus broth at 50°C for 18 hrs in rotary shaker incubator having 120 rpm.

Screening for extra cellular enzymes by plate assay method: Skim-milk agar (SMA) and starch agar plates were prepared for qualitative analysis of protease and amylase respectively. Both the medium was sterilized at 15 lb for 20 min. inoculate the activated culture of BWU-1 isolate over the medium and incubate it at 45°C for 24 hrs (Chandi et al., 2004 and Alane et al., 1996).

Results and Discussion

The water sample was collected from hot suphur spring of Unkeshwar in thermal containers. The total bacterial population of the hot spring of Unkeshwar was approximately 20 x 10^2 CFU ml^{-1} water. According to Reda the higher level of temperature affects the qualitative as well as quantitative structure of microbial communities and this was found in several studies that temperature influences microorganisms by adversely affecting their growth, morphology and biochemical activities, resulting in decrease biomass and diversity (Reda, 2007, Brock, 1978 and Ljungdahl, 1979). A total four morphologically distinct isolates were randomly selected from the thermus agar plate. All isolates were screened for their ability to grow at different temperature ranging from 20 to 60°C. At 20 and 60°C the growth of the isolate was drastically decreases but at 50°C the most advantageous growth of all the isolate was analyzed. The isolated four samples were biochemically analyzed and showed variable characters shown in table 1.

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Table 1 Biochemical characteristics of selected *Bacillus sp.*

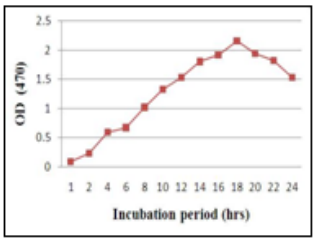
Test / Characteristics	BWU-1	BWU-2	BWU-3	BWU-4
Catalase	+	+	+	+
Indole	-	+	+	+
Nitrate reductase	-	-	+	+
Urease	-	-	-	+
Methyl red	+	+	-	-
Casein hydrolase	+	+	+	+
Vogus-proskaur test	+	-	+	-
Starch hydrolysis	+	+	+	+
Citrate Utilization	-	-	+	-
Glucose fermentation	+	+	+	+
Sucrose fermentation	+	+	+	+
Phenyl alanine deaminase production	+	+	+	-
Lysine decarboxylase activity	+	-	+	-
Lactose fermentation	-	+	+	+
Maltose fermentation	-	+	+	+
Mannitol fermentation	+	+	+	+
Melibiose	+	-	+	-
Rhamnose	+	+	+	+
Cellobiose	+	-	-	+

The analyzed BWU-1 isolate was confirmed by 16S r-RNA cataloging showed 97 % resembles to the *Bacillus licheniformis*. (Reda, 2007). The growth of the isolate was different from other organism (Fig 1).

Fig 1: Growth pattern of pure culture of *Bacillus licheniformis* BWU-1

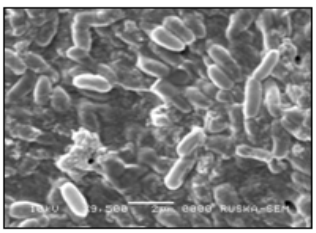
The *Bacillus licheniformis* BWU-1 having rapid growth property was tabulated in thermus broth, it starts from 2 hrs and reach utmost at 18 hrs of incubation at 50°C (Fig 2). After 18 hrs incubation the slight decrease in growth was observed.

Fig 2: Effect of incubation period on growth pattern of *B. licheniformis* BWU-1



The above results irritate examination of bacterial strain by scanning electron microscope (SEM) shown in Fig 3 and the average size of the isolate was measured about 1.43 µm.

Fig 3: SEM of *Bacillus licheniformis* BWU-1



Physiochemical analysis: The table 2 present physiochemical analysis of water sample was collected from hot spring of Unkeshwar.

Table 2 Physiochemical analysis of the water sample of hot spring

Tests	BOD	COD	DO	Chlorine	TDS	Salinity	pH	Conductivity
Concentrations	0.30 mg l^{-1}	3.95 mg l^{-1}	0.53 mg l^{-1}	0.6674 gm l^{-1}	3.32 ppt	3.73 ppt	8.4	6.79 ms

The WHO has suggested a limiting value of 500mg/L of TDS for potable water. In the present investigation this limit is not crossed on either side by the sample under study. Such value is acceptable for domestic use. The conductivity values of the water sample was 6.97 ms. According to Mishra an overwhelming value of TDS may be increases the conductivity values of the water sample (Mishra et al., 2008).

Dissolved oxygen present in drinking water adds taste and it is highly fluctuating factor in water. In this study dissolved oxygen content was 0.53 mg l^{-1}. The maximum allowed value of chemical oxygen demand (COD) is 10 mg/L in drinking water. The present samples have registered 3.95 mg/L. This value is much lower than the limit. According to WHO such water may be useful for the drinking purpose.

Most of the water samples contain significant amount of organic matter that provides nutrition for the growth and multiplication of microorganisms. The most probable number (MPN) is a suitable and widely used method to determine the microbial quality of water (Kodarkar, 2006 and Aneja, 2003). The present investigations have rendered all tests was negative indicates the absence of coliform bacteria.

The production of biotechnologically important enzymes was analyzed on SMA and Starch agar plates for protease and amylase respectively. The overnight incubated bacterial culture was streaked on SMA plate and inoculates a spot at the centre on Starch agar plate. After incubation the zone of hydrolysis was directly analyzed on SMA plate (Fig 4), where as grams iodine was spread on the surface of starch agar plate and observed the zone of hydrolysis against the dark blue surrounding (Fig 5). Proteolytic enzymes predominantly proteases, have become an important and fundamental part of the industrial processes including pharmaceuticals, food products and laundry detergents (Rao et al., 1998). The amylase is extensively used in many industries including starch liquification, brewing, food, paper, textile and pharmaceuticals (Ohdan et al., 2000).

Fig 4: Zone of hydrolysis on SMA plate

Fig 5: Effect of amylase on starch agar plate

In conclusion, the isolated thermostable *Bacillus licheniformis* BWU-1 have potential to produce a significant protease as well as amylase. The isolated enzymes from the *B. licheniformis* may produce an extremozyme like protease and amylase which could tolerate higher temperature as well as hydrolyse proteins and starch. Unkeshwar hot springs have large amount of potential microbial storages and can be used as a source for different thermostable biological products like enzymes.

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