Microbiology, Vol. 73, No. 4, 2004, pp. 372–377. Translated from Mikrobiologiya, Vol. 73, No. 4, 2004, pp. 443–448.

EXPERIMENTAL ARTICLES

The Effect of Temperature and pH on the Growth of Aerobic Alkalithermophilic Bacteria from Hot Springs in Buryatia

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Received October 13, 2003; in final form, December 29, 2003

Abstract—Growth parameters (temperature and pH) were determined for collection cultures of aerobic heterotrophic bacteria. Analysis of the experimental data with the use of the Rosso model made it possible to calculate the extreme values of temperature and pH permissive for culture growth. The cultures examined were subdivided into three groups with respect to their growth temperature and pH. The first group is represented by cultures with minimum, maximum, and optimal growth temperatures of <20, 60–64, and 38–40°C, respectively, and with the optimal growth pH 8.0–8.5. Bacteria of the second group are true alkalithermophilic organisms with a temperature optimum of 45–50°C and a pH optimum of 8.5–9.0. The third group includes a culture of a thermophilic alkalitolerant bacterium.

Key words: hydrothermal vents, alkalithermophiles, mathematical models.

In the last two decades, the microbial communities of extreme habitats have attracted the consistent attention of researchers [1]. The microbial communities of alkaline hydrothermal vents are of special interest. In alkaline hot springs of Yellowstone National Park and in springs of Japan, Iceland, and New Zealand, the species composition of microbial communities has been studied and a number of new taxa have been identified [2–4].

Until recently, the microbial communities of the alkaline (pH 8.5–9.95) nitrogen-rich hot springs (30–79°C) located in Buryatia in the Baikal rift zone were only occasionally studied. Phototrophic microbial communities have been studied in some alkaline hot springs on the Lake Baikal shore, and strains of thermophilic microorganisms have been isolated [5]. The cell number and species diversity of microorganisms involved in the production and degradation of organic matter were determined and the rates of these processes were measured [6, 7].

We isolated bacterial strains of various physiological groups and developed a collection of aerobic heterotrophic bacteria capable of growing within wide ranges of temperature and pH. However, it remained unclear whether these isolates were alkalithermophilic or only thermo- and alkalitolerant microorganisms.

In this work, the growth of collection cultures of aerobic heterotrophic bacteria from the microbial communities of alkaline hot springs of Buryatia was studied as dependent on temperature and pH. The Rosso mathematical model [8] was used to describe the dependence of bacterial growth on temperature and pH.

MATERIALS AND METHODS

Physicochemical parameters of thermal waters were measured using portable devices. The temperature was determined by an alcohol thermometer, pH was measured with a precision PRO pH meter (Singapore), and mineralization was evaluated with a TDS-4 conductometer (Singapore). Samples of water, silt, and microbial mats taken during expeditions of 1998 and 1999 from the hot alkaline springs Alla, Garga, Gusikha, Goryachinsk, and Seya were used to obtain microbial cultures.

The strains of aerobic thermophilic bacteria were isolated on four different media of the following compositions: (1) (g/l): sodium citrate, 1.29; $(NH_4)_2SO_4$, 1.92; KH_2PO_4, 9.3; $MgSO_4 \cdot 7H_2O$, 0.2; glucose, 19.0; (2) (g/l): KH_2PO_4, 0.33; NH_4Cl, 0.33; $CaCl_2 \cdot 6H_2O$, 0.33; $MgCl_2$, 0.33; yeast extract, 0.5; a solution of microelements prepared according to Lippert, 1 ml; glucose, 1% (1.5% agar was added to obtain agarized medium [10]); (3) nutrient broth; and (4) nutrient agar. The bicarbonate/carbonate buffer was used to attain a certain pH value of the medium taking into account the in situ natural pH. For this purpose, the following reagents were added to the medium (g/l): $NaHCO_3$, 10.0 (pH 8.0); $NaHCO_3$, 4.5, and Na_2CO_3, 2.0 (pH 9.0); $NaHCO_3$, 10.0, and Na_2CO_3, 2.0 (pH 9.5). Sample dilutions were plated onto solid agarized media (2) and (4); bacteria of the enrichment cultures obtained by inoculation of liquid media were also plated, which was fol-

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lowed by incubation at 55°C for two to five days. The strain morphology was studied under a PZO SK14 microscope (Poland) at a magnification of 1250×. The dependences of bacterial growth on NaCl concentration, casein and starch hydrolysis, catalase reaction, and acid and sugar utilization were studied by conventional methods [11]. The tested substrates—ethanol, glycerol, rhamnose, xylose, sucrose, cellobiose, arabinose, glucose, and mannitol—were added to the basic medium at a concentration of 2%. The inoculated media were incubated at 55°C for two to seven days. The temperature effect on the growth of aerobic heterotrophs was studied at a temperature gradient within the range of 30–60°C at the pH of the medium optimal for each strain. The pH effect was analyzed at 55°C and medium pH values of 6, 7, 8, 8.5, 9, 9.5, and 10. To obtain the required medium pH, bicarbonate/carbonate buffer or 1 M HCl solution was used. During cultivation, pH was controlled by an Ekspert-001 device (NPP Ekoniks-Ekspert, Russia). The intensity of bacterial growth was estimated from the optical density measured at 540 nm. The growth experiments were performed in two replicates, and average data were used as final results. The specific growth rates of bacterial cultures were calculated by a conventional formula [12]. To determine the optimal, maximum, and minimum temperature and pH values, the experimental data were approximated in accordance with the Rosso model [8], which describes changes in the specific growth rate as a function of temperature and pH by the following equation:

$$\mu_{max}(T, \mathrm{pH}) = \mu_{opt}\tau(T)\rho(\mathrm{pH}),$$

where $\tau(T)$ is a function of temperature and $\rho(\mathrm{pH})$ is a function of pH.

Transformation of this equation leads to the following form:

$$\mu_{max}(T) = \begin{cases} 0, & T < T_{min} \\ \mu_{opt}\tau(T), & T_{min} < T < T_{max}, \\ 0, & T > T_{max} \end{cases}$$

where

$$\tau(T) = \frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)]}$$

is known as *the model of basic temperatures* and relates the optimal specific growth rate μ_{opt} at a temperature of T_{opt}, as well as the minimum, optimal, and maximum temperatures (T_{min}, T_{opt}, and T_{max}), with the maximum specific growth rate μ_{max} at a temperature T. T_{opt} was defined as the temperature at which the highest average specific growth rate was determined.

The pH-dependent changes in μ_{max} are described by *the model of basic pH*, which operates with three pH values: pH_{min}, pH_{opt}, and pH_{max}. The equation is as follows:

$$\mu_{max}(\mathrm{pH}) = \begin{cases} \mathrm{pH} < \mathrm{pH}_{min}, & 0.0 \\ \mu_{opt}\rho(\mathrm{pH}), & \mathrm{pH}_{min} < \mathrm{pH} < \mathrm{pH}_{max}, \\ \mathrm{pH} > \mathrm{pH}_{max}, & 0.0 \end{cases}$$

where

$$\rho(\mathrm{pH}) = \frac{(\mathrm{pH} - \mathrm{pH}_{min})(\mathrm{pH} - \mathrm{pH}_{max})}{(\mathrm{pH} - \mathrm{pH}_{min})(\mathrm{pH} - \mathrm{pH}_{max}) - (\mathrm{pH} - \mathrm{pH}_{opt})^2}.$$

T_{min}, T_{opt}, and T_{max} and pH_{min}, pH_{opt}, and pH_{max} were calculated using the least squares method; the sum of squares was minimized. The approximation accuracy rating was determined by calculation of the standard square deviation σ expressed in percent.

RESULTS AND DISCUSSION

The following physicochemical parameters of the examined alkaline hot springs in Buryatia were determined. At sites of sampling, the temperature ranged from 30 to 79°C and the pH ranged from 8.1 to 9.9. The maximum temperature was in Alla and Garga spring water. The water pH ranged from low-alkaline (8.1–8.5) in Gusikha and Garga springs to high-alkaline (9.9) in Alla spring. The lowest Eh values were in high-temperature springs. The level of water mineralization was not high in all springs (0.1–0.8 g/l).

In total, 37 strains of aerobic heterotrophic bacteria were isolated from all samples. In this study, nine strains exhibiting active growth in a strongly alkaline medium at high temperature were under consideration. These strains were isolated from hot springs with different temperature and pH (from low-alkaline to strongly alkaline). Table 1 provides a characterization of the sites of sampling.

The cells of the isolates were gram-positive nonmotile rods 6–8 μm long and 0.2–0.8 μm wide, which formed terminal spores; in some cultures, filaments were observed (Se-1, Se-2, Se-3, and Se-4). On solid medium, the strains formed round yellow colonies of one and the same type. Strains Bg-24, Se-1, Se-2, Se-3, and Bg-50 were obligate aerobes, whereas strains Bg-10, Bg-26, Bg-44, and Se-4 were facultative anaerobes. The physiological and biochemical properties of the isolates are presented in Table 2. Based on our tests, the

Table 1. Brief characterization of the sites of sampling

Spring	Sample type	*T*, °C	pH	Eh	Mineralization	Culture
Alla	White-gray mat	45	9.20	–	0.3	Bg-10
Garga	Griffon, medium-grained sand	74	8.19	–	0.8	Bg-24
Garga	Yellow-green cyanobacterial mat	68	8.40	–	0.6	Bg-26
Gusikha	Yellow-green cyanobacterial mat	48	9.00	+128	0.1	Bg-44
Goryachinsk	Gray sand	45	8.50	+65	0.4	Bg-50
Seya	Near-bottom cyanobacterial mat	49	9.85	–74	0.3	Se-1
Seya	Surface cyanobacterial mat	49	9.85	–74	0.3	Se-2
Seya	Thin orange-green mat	45	9.80	+22	0.3	Se-3
Seya	Thick multilayered mat	43	9.75	+60	0.3	Se-4

Table 2. Physiological and biochemical characteristics of the isolated strains

Culture	Bg-10	Bg-24	Bg-26	Bg-44	Bg-50	Se-1	Se-2	Se-3	Se-4
T_{opt}	40	45	38	40	45	45	50	40	45
pH_{opt}	8.0	7.5	8.0	8.0	9.0	8.5	9.0	8.5	8.0
Gram staining	+	+	+	+	+	+	+	+	+
Utilization of alcohols and sugars:									
ethanol	+	+	+	+	–	+	–	–	+
glycerol	+	+	+	–	–	–	–	–	+
rhamnose	–	+	+	–	–	–	–	+	+
xylose	+	+	+	+	+	–	+	+	+
sucrose	+	+	+	+	+	+	+	+	+
cellobiose	+	+	+	+	+	+	+	+	+
arabinose	–	–	–	–	–	–	–	–	–
glucose	+	+	+	+	–	+	+	+	+
mannitol	+	+	+	–	+	–	+	–	+
Reaction to [NaCl]									
2.5%	+	+	+	+	+	+	+	+	+
6.5%	+/–	+/–	–	–	–	–	–	–	+
Reaction to O_2	fac. an.	obl. aer.	fac. an.	fac. an.	obl. aer.	obl. aer.	obl. aer.	fac. an.	obl. aer.
Hydrolysis of casein	–	–	+	+	+	–	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+	+	+	+

Note: obl. aer., obligate aerobe; fac. an., facultative anaerobe.

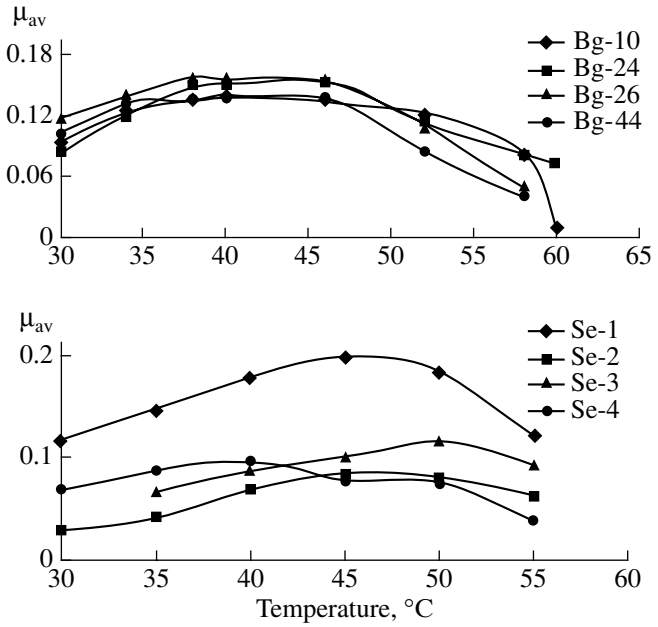

Fig. 1. Plots of average specific growth rates of bacteria versus temperature.

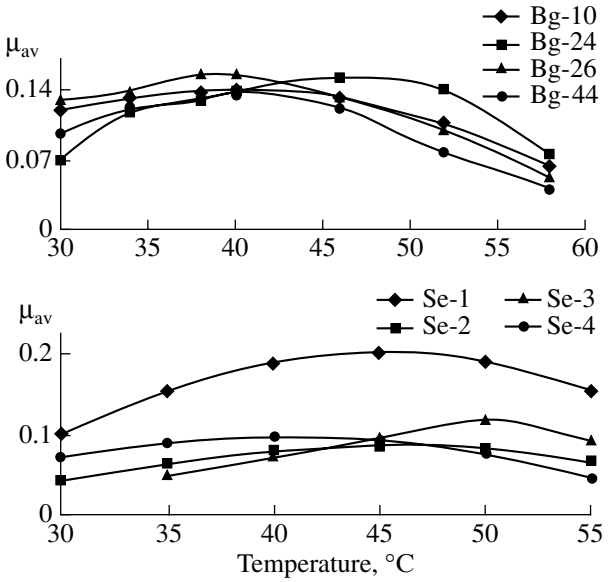

Fig. 2. Plots of maximum specific growth rates of bacteria versus temperature.

strains were assigned to the genus *Bacillus*. As determined experimentally, the strain growth occurred within a temperature range from 30 to 60°C, with an optimal temperature of 40–50°C. The pH of range was determined to be from 5.5 to 10.0, and the optimal pH was 7.5–9.0.

Thermophilic bacilli that form group 5 (according to the classification of Ash *et al.* [13]) grow at higher temperatures: the temperature minimum is 35–48°C; the maximum, 65–78°C; and optimum, 55–65°C. Their pH range is narrower, 6.0–8.5, with an optimal pH of 6.2–7.5 [14]. An exception to the rule is *Bacillus flavothermus*, isolated from hot springs of New Zealand, which grows in a wide range of pH (5.5–9.0) [15]. The strains isolated from hot springs of Buryatia differ from the previously described aerobic thermophilic bacilli by a lower optimal temperature and a higher optimal pH.

The dependence of bacterial growth on the temperature and pH was determined by comparison of average specific growth rates (μ_{av}), which were calculated during exponential culture growth at all studied temperatures and pH (Figs. 1 and 3). However, this approach does not secure against errors, and the μ_{av} values cannot be used to calculate the optimal specific growth rates (μ_{opt}). Therefore, to verify our experimental data, we used a mathematical model that describes the microorganism growth dependence on temperature and pH. Models of microbial growth usually describe changes in the maximum specific growth rate, which reflects the metabolic activity of a certain microorganism under given ambient conditions. There are several models that

Table 3. Growth temperatures and pH calculated for the studied bacterial cultures

Culture	T_{min}	T_{opt}	T_{max}	Standard deviation σ, %	pH_{min}	pH_{opt}	pH_{max}	Standard deviation σ, %
Bg-10	15	40	64	18.0	5.5	8.0	10.2	15.4
Bg-24	24	45	64	23.0	5.0	8.0	10.3	18.6
Bg-26	15	38	62	24.0	5.1	8.0	10.5	12.1
Bg-44	19	40	61	14.0	5.1	8.0	10.0	3
Bg-50	–	–	–	–	5.0	8.0	10.2	12.8
Se-1	23	45	65	12.3	7.2	9.0	10.6	10.3
Se-2	23	45	65	23.0	7.0	8.5	10.0	8.2
Se-3	30	50	68	21.1	7.3	8.8	10.1	9.2
Se-4	20	40	60	9.7	7.0	8.5	10.0	20.3

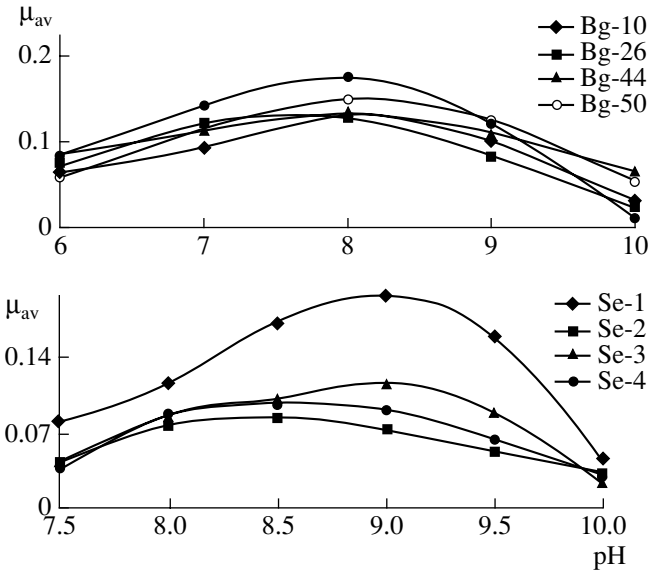

Fig. 3. Plots of average specific growth rates of bacteria versus pH of the medium.

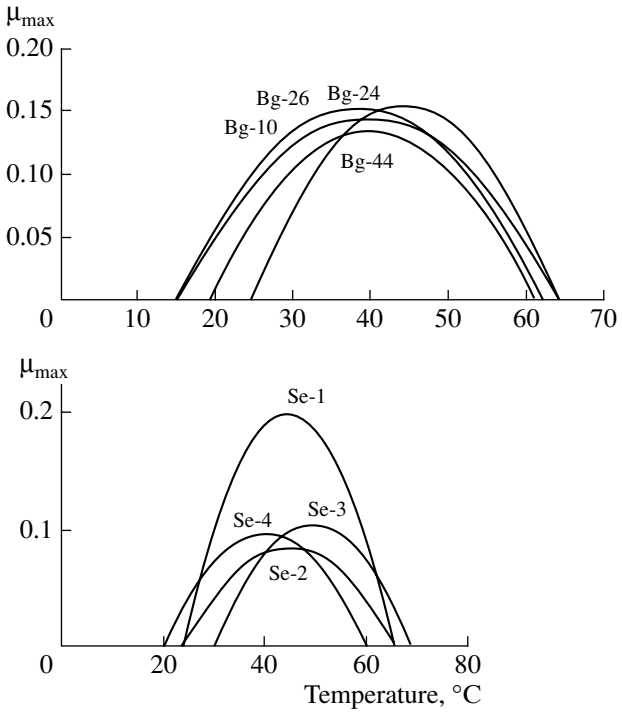

Fig. 4. Plots of maximum specific growth rates of bacteria versus temperature.

take into account the combined effect of temperature and pH on the maximum specific growth rate (μ_{max}) [8, 16–18].

We chose the Rosso model [8] for two reasons. First, models with multiple parameters were undesirable. Second, we needed a model without significant structural correlations of parameters, which impede their calculation. The Rosso model satisfies both conditions. In addition, with the Rosso model, pH_{min}, pH_{opt}, pH_{max}, T_{min}, T_{opt}, and T_{max} can be estimated from the real properties of microorganisms, whereas, in other models, the coefficients of regression are difficult to estimate.

The Rosso model describes the typical trend of a curve (T, pH) with the optimum shifted to the maximum values still ensuring microorganism growth. Similar behavior was characteristic of the cultures Se-1, Se-2, and Se-4 (Fig. 2). However, other cultures displayed different behavior.

Extrapolation showed that the isolated cultures can be divided into two groups with respect to temperature (temperature optima 38–40 and 45–50°C) and with respect to pH (pH optima 8.0 and 8.5–9.0) (Table 3; Figs. 1–4). In general, the approximation is in good agreement with the experimental data. By Wiegel's definition [19], microorganisms with an optimal growth temperature above 40–45°C belong to thermophiles.

The optimal pH for alkaliphilic and alkalitolerant organisms is above 8.5 and 8.0, respectively. Hence, the isolated cultures can be divided into several groups. The first group of thermoalkalitolerant bacteria includes the Bg-10, Bg-26, Bg-44, and Se-4, cultures, with a minimum growth temperature <20°C; optimal and maximum temperatures of 38–40°C and 60–64°C, respectively; and optimal pH values of 8.0–8.5. On the plots of their maximum specific growth rate versus temperature, a plateau is observed within the temperature range from 35 to 45°C, which is a typical feature of thermotolerant species. The second group includes true alkalithermophiles with a temperature optimum of 45–50°C and an optimal pH of 8.5–9.0. These are cultures Se-1, Se-2, and Se-3, isolated from a microbial community of the high-alkaline Seya spring. The third group is represented by the Bg-24 culture, the growth temperatures of which are higher (Table 3, Fig. 2) than those of the first group of microorganisms, though with respect to medium pH this strain belongs to alkalitolerant bacteria. This culture was isolated from a Garga spring sample taken at the outlet of the very hot (74°C) low-alkaline thermal water. Note that all springs are fresh and therefore, unlike soda lakes, were thought to contain only alkalitolerant strains. However, our studies showed that true alkaliphiles are also present in microbial communities of alkaline hydrothermal vents. The calculated temperature and pH growth parameters of the isolated strains testify to the wide ecological spectra of bacteria in microbial communities of alkaline hot springs of Buryatia and characterize these communities as resistant systems.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 03-04-48047); the Presidium of the Siberian Division, Russian Academy of Sci-

ences (project no. 170); and the Ministry of Education of the Russian Federation (project no. E 02-6.0-294).

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