

ISOLATION, PROPERTIES AND KINETICS OF GROWTH OF A THERMOPHILIC *BACILLUS*

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ABSTRACT

A strictly aerobic, thermophilic, spore-forming bacteria was isolated from a soil sample collected in Campos dos Goytacazes City, Rio de Janeiro, Brazil. The cells of this organism were Gram-positive, catalase positive, actively motile, 1.2 μm wide and 5.3 μm long. Growth occurred at pH values ranging from 6.5 to 9.0, and optimum growth occurred at about pH 7.0. The optimum temperature for growth was around 55°C, and the upper temperature limit for growth was around 70°C. Yeast extract stimulated growth on glucose and was obligately required at supraoptimal temperatures (65°C). The results of 16S rRNA sequence comparisons indicated that the isolate was closely related to *Bacillus caldoxylyticus* and *Bacillus* sp strain AK1, and these three organisms exhibited levels of ribosomal DNA sequence homology of 94%.

Key words: Thermophilic microorganism, *Bacillus*, isolation

INTRODUCTION

Several intensive studies were performed to isolate thermophilic carbohydrate-fermenting bacteria from several habitats, aiming the use of these microbes and their enzymes for biotechnological applications (1,5,6,8,12,20,22). The majority of the investigated thermophilic bacteria belong to the genus *Bacillus* and they have been isolated from thermophilic and mesophilic environments (3,4,8,11,13). According to Bergey's Manual of Systematic Bacteriology (5), this genus is phenotypically heterogeneous with members exhibiting an extremely wide range of nutritional requirements, growth conditions, metabolic diversity and DNA base composition (2).

The nutritional requirements of many thermophilic strains have been investigated and frequently found to be complex (3,4,5). Successful cultivation at higher temperatures depended upon the composition of the medium, the degrees of complexity usually being parallel to the increase of the growth temperature.

This study describes the isolation and characterisation of a thermophilic *Bacillus* strain based on morphological,

physiological, biochemical and phylogenetic studies. Furthermore, it also describes the effect of the temperature of incubation upon the nutritional requirements of the organism.

MATERIALS AND METHODS

Isolation of the organism

Soil samples (about 1 g) were suspended in 100 mL of sterile water, slightly agitated for 2h at 100 rpm, poured and spread onto TSY (Bacto-tryptone 10 g; Bacto-yeast extract 5 g; NaCl 10 g; agar 10 g; H₂O 1L) agar plates. These plates were sealed in plastic bags to reduce evaporation and incubated at 65°C for 24h. Some of the colonies that grew on the plates were purified by successive streaking on TSY plates. Finally, a few colonies of a homogeneous morphology were selected. All colonies isolated by this procedure proved to be thermophilic and did not grow in TSY medium at or below 44°C for as long as 7 days. The results reported here are related to one of these isolates.

Thermophilic bacteria were characterized according to Gordon *et al.* (7). All physiological and biochemical tests were carried out at 50°C.

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16S rRNA sequence analysis

The genomic DNA was extracted from the isolate and the amplification of the 16S rDNA was performed through PCR technique. The purified PCR products were sequenced on an automated DNA sequencer (ALFexpress, Pharmacia). The 16S rDNA sequence of isolate was aligned with the 16S rRNA gene sequences of various members of the genus *Bacillus* obtained from the Ribosomal RNA Database Project and from Genbank (10). Matrices of evolutionary distances were computed from the sequences alignments by calculating a pair wise Jukes-Cantor (9). From these distances, phylogenetic trees were inferred by a neighbour-joining method (16).

Growth conditions

The organism was grown on TSY agar plates for 12h at 55°C. Approximately 5 mL of the liquid medium containing (g/L of distilled water): NaH₂PO₄.2H₂O, 1.56; NH₄Cl, 5.35; KCl, 0.745; Na₂SO₄.10H₂O, 0.644; Citric acid, 0.42; MgCl₂.6H₂O, 0.25; CaCl₂, 2.2x10⁻³; ZnO, 2.5x10⁻³; FeCl₃.6H₂O, 2.7x10⁻²; MnCl₂.4H₂O, 1.0x10⁻²; CuCl₂.2H₂O, 8.5x10⁻⁴; CoCl₂.6H₂O, 2.4x10⁻³; NiCl₃.6H₂O, 2.5x10⁻⁴; H₂BO₃, 3.0x10⁻⁴; Na₂MoO₄, 1.0x10⁻³, pH 4.0 was pipetted into these agar plates and the cells scraped off using a sterile Pasteur pipette. The same liquid medium (50 mL contained in a 250 mL tightly closed Erlenmeyer flask) was inoculated with this suspension to give an initial absorbance at 470 nm of at least 0.1 and the cultures were incubated at temperatures starting at 50°C and going up to 65°C, with vigorous aeration in a shaker at 250 rpm. The pH was adjusted to 7.0 and the medium sterilised by autoclaving at 121°C for 30min. Glucose was sterilised separately and aseptically added to the flasks containing the liquid medium, after cooling. At time intervals, the turbidity of the cultures was determined by measuring the increase in optical density at 470 nm with a spectrophotometer Hitachi Model U-2000.

RESULTS

Strain characteristics: cell morphology

The isolate was a Gram-positive bacilli. It was actively motile, 1.2 µm wide and 5.3 µm long. It occurred singly or in chains and in monomorphic forms. Spores were subterminal to terminal, ellipsoidal in shape. Predominantly unswollen sporangia were seen. The isolate grew well on Nutrient Agar or in Nutrient Broth at 50°C for 24h, but it did not grow anaerobically in the same conditions. On Nutrient Agar, the colonies usually were circular with smooth edges, brightness, yellowish and were convex. Their size varied from small to 4 mm in diameter.

Growth characteristics and physiology

The isolate was a thermophilic, strictly aerobic bacterium. The optimal growth temperature of this strain was found to be around 55°C and no growth was obtained after 7 days below

44°C or above 70°C. The strain had a broad pH range for growth 6.5–9.0 with an optimum of 7.0 (Fig. 1).

The following sugars were fermented with acid production: ribose, D-glucose, D-fructose, D-mannose, cellobiose, amygdalin, esculin, salicin, N-acetyl-D-glucosaminemaltose, trehalose, starch, glycogen, gentiobiose, D-Galactose, Lactose, sucrose, melibiose, glycerol, L-xylose, β-m-xyloside, β-gentiobiose, α-methyl-D-glucoside, D-turanose, D-mannitol and xylitol. It was not observed acid production in Erythritol, D- and L-arabinose, adonitol, dulcitol, rhamnose, inositol, sorbitol, α-methyl-D-mannoside, arbutin, inulin, D-raffinose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate.

The strain grew in Nutrient Broth containing 5.0% NaCl (m/v), but was inhibited by 7.0% NaCl. Urea was not attacked; catalase was positive. Indole was not formed and acetoin formation was positive. Nitrate was reduced to nitrite.

When the organism was grown at 55°C in the absence of glucose but in the presence of yeast extract in the liquid medium, good growth was obtained, indicating that yeast extract can be used as one sole carbon and energy source. Also, good growth was observed when gelatine or casein replaced yeast extract.

Effect of temperature on growth

Growth obtained in the liquid medium supplemented with yeast extract (0.5%) at 50°C, 55°C, 60°C and 65°C is shown in Fig. 2.

It can be seen that the temperature determined the rate of growth. The shortest lag was observed at 55°C and the longest at 50°C. The highest turbidity was reached in cultures growing at the temperature of 55°C.

At 65°C, addition of peptone (1%) to the medium caused a further increase in growth, inducing higher growth rate. However, a small amount of growth was observed in the absence of yeast extract or peptone when glucose was used as an energy source (Fig. 3).

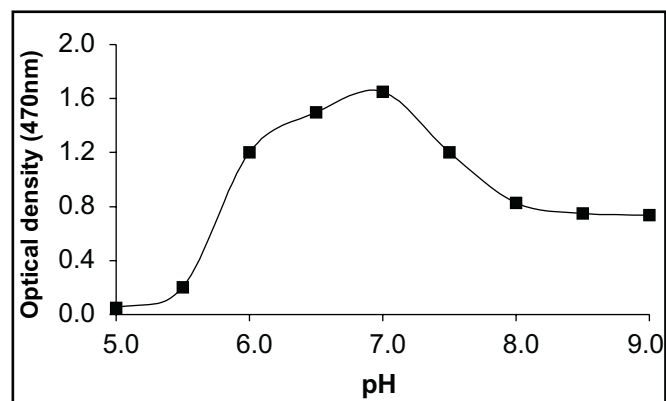


Figure 1. Effect of pH on growth of the endospore forming thermophile.

Phylogeny

16S rDNA sequence analysis indicated that the isolate is a member of the thermophilic *Bacillus* group. Based on the morphological, biochemical and physiological characteristics, the isolate was assigned to *Bacillus* rRNA group 5. The partial 16S rDNA sequence showed a similarity of 94% to *Bacillus caldxylolyticus* and *Bacillus* sp strain AK1 and 90% to 91% of similarity to *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus denitrificans*, *Bacillus flavothermus* and *Bacillus thermoglucosidasius*. Fig. 4 is the dendrogram generated by the neighbour-joining method from the evolutionary distance matrix and shows this relationship.

DISCUSSION

The strain isolated could be categorized as thermophilic since it required a temperature of about 55°C for optimum growth and was unable to grow outside the temperature range of 44°C–70°C.

The morphological and physiological characteristics and nutritional requirements of the isolate were consistent with the description of two species of the genus *Bacillus*: *B. circulans* and *B. macerans* (7). However, there are important differences between these organisms and the isolate. *B. circulans* and *B. macerans* grow at pH 5.7, the upper temperature limit for their growth is 50°C, acetoin is not formed, the sporangia is swollen and there is acid production from L-xylose, xylitol, arbutin, D-raffinose, L-arabinose. In addition, *B. macerans* does not grow in the presence of 5% and 7% of NaCl, does not hydrolyze casein and grows anaerobically.

An analysis of the 16S rRNA gene of the isolate revealed that this organism is phylogenetically closely related to members of the genus *Bacillus* rRNA group 5 (2,15). This group includes *Bacillus stearothermophilus* and other thermophilic *Bacillus* spp. The levels of similarity ranged from 90% to 91% from *B. stearothermophilus*, *B. thermocatenulatus*, *B. thermoleovorans*, *B. denitrificans*, *B. flavothermus* and *B. thermoglucosidasius* to 94% for *B. caldxylolyticus* and *Bacillus* sp strain AK1.

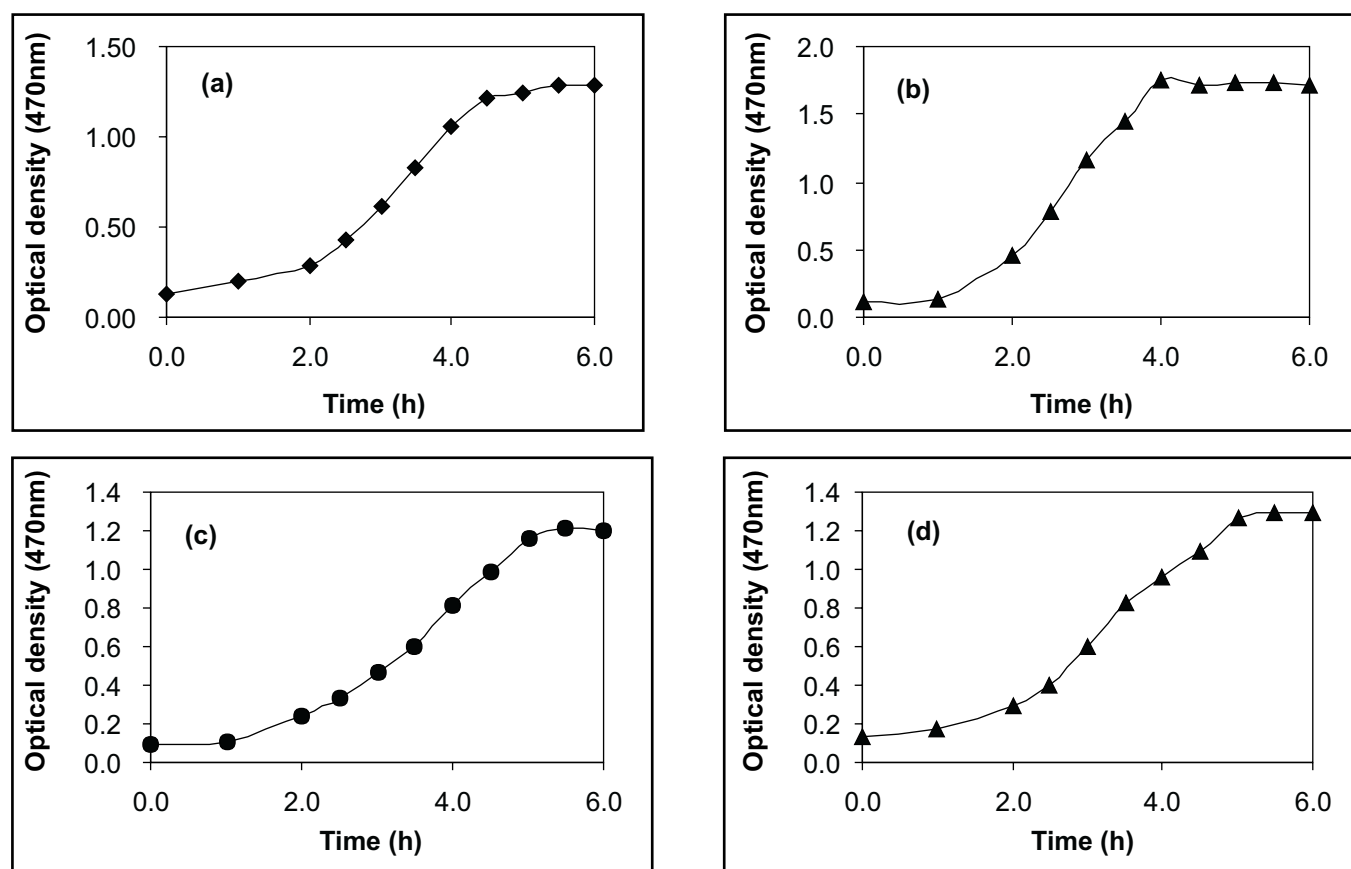


Figure 2. Growth curve of the endospore forming thermophile cultivated in minimal medium supplemented with yeast extract (0.5%) at 50°C (a), 55°C (b), 60°C (c) and 65°C.

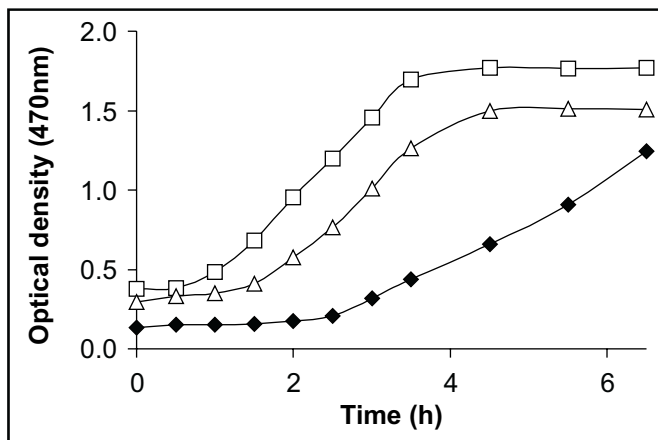


Figure 3. Growth curve of the endospore forming thermophile at 65°C. Symbols: ◆, minimal medium; △, minimal medium supplemented with yeast extract; □, minimal medium supplemented with yeast extract (0.5 %) and peptone (1.0 %).

B. thermocatenulatus is a facultatively anaerobic bacterium that does not hydrolyse starch and gelatine. It does not produce acid from xylose and lactose; acetoin is not formed. The temperature range for growth of this organism is 35°C – 78°C. *B. flavothermus* is also a facultatively anaerobic bacterium that does not hydrolyse gelatine and grows at 30°C. *B. thermoleovorans* does not form acetoin, degrades urea and hydrolyses casein weakly. Therefore, the isolate could not be identified as one of these species.

Another phylogenetically closely related species to the isolate is *B. thermodenitrificans*, which belongs to the “*B. stearothermophilus*” complex. However, the isolate was separated by a numerical study from typical strains of *B. stearothermophilus* (21).

The strain *Bacillus* sp. AK1 was the bacteria most closely related to the isolate for which 16S rRNA sequence data is available. On the other hand, the phylogenetically most closely related to *Bacillus* sp AK1 species is *B. thermoglucosidasius* (23). However, the isolate showed that levels of similarity to this organism ranged from 90% to 91%.

On the basis of morphological, biochemical, physiological and phylogenetic studies we concluded that the isolate is closely related to the members of the *Bacillus* rRNA group 5. However, even taking all data into account, it is not possible to identify the isolate as being one of the above mentioned species. Indeed, this approach led to the classification of the isolate as a thermophilic member of the genus *Bacillus* where all aerobic, endospore-forming organisms have been classified.

Reports in the literature dealing with the effect of temperature on the growth requirements of several

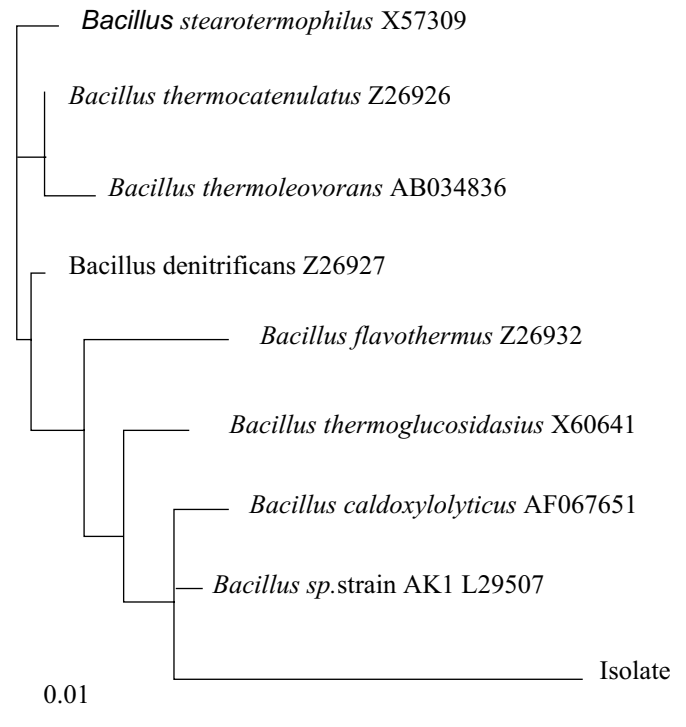


Figure 4. Phylogenetic dendrogram based on 16SrDNA sequence data indicating the position of the thermophile isolate among representatives of the genus *Bacillus*. All the sequences used in the analysis were obtained from the Ribosomal Database Project and Genbank. Scale bar indicates evolutionary distance.

thermophilic microorganisms have shown that some of them had additional requirements as the incubation temperature was increased and others showed no differences in growth requirements, regardless of the incubation temperature (6,24).

The thermophile isolate required yeast extract to grow at supraoptimal temperatures (65°C), suggesting that at higher temperatures the enzymes responsible for the synthesis of a particular metabolite present in this compound underwent thermal inactivation and thus the organism required it as an exogenous source. An alternative explanation is that the complete inactivation of a gene responsible for the synthesis of an essential metabolite imposed upon the organism a requirement of an external source of the substance which it could no longer produce.

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RESUMO

Isolamento, propriedades e cinética de crescimento de um *Bacillus* termofílico

Uma bactéria formadora de esporos, termofílica e aeróbica estrita foi isolada de amostras de solo coletadas na cidade de Campos dos Goytacazes, Rio de Janeiro, Brasil. As células deste organismo foram Gram-positivas, móveis e mediram 1,2 µm de largura e 5,3 µm de comprimento. O crescimento ocorreu a valores de pH variando de 6,5 a 9,0 e o crescimento ótimo ocorreu em torno de pH 7,0. A temperatura ótima de crescimento foi em torno de 55°C e a máxima de 70°C. O extrato de levedura estimulou o crescimento do organismo em glicose e foi obrigatoriamente requerido a supra-ótimas temperaturas (65°C). Os resultados da comparação de seqüências de 16SrRNA indicaram que o isolado foi proximamente relacionado com o *Bacillus caldoxylyticus* and *Bacillus* sp AK1 e estes três organismos exibiram níveis de homologia de 94% nas seqüências de DNA ribossomal

Palavras-chave: Microrganismo termófilo, *Bacillus*, Isolamento

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