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Isolation and characterization of lipid-degrading *Bacillus thermoleovorans* IHI-91 from an icelandic hot spring

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Abstract An efficient lipid-degrading thermophilic aerobic bacterium was isolated from an icelandic hot spring and classified as *Bacillus thermoleovorans* IHI-91. The aerobic bacterium grows optimally at 65°C and pH 6.0 and secretes a high level of lipase (300 U l^{-1}). The newly isolated strain utilizes several lipids such as palmitic acid, stearic acid, lanolin, olive oil, sunflower seed oil, soya oil, and fish oil as sole carbon and energy source without an additional supply of growth factors. The degradation of about 93% of triolein, which is present in olive oil, was observed after only 7 h of fermentation at a maximal growth rate of 1.0 h^{-1}. During growth at optimal conditions on yeast extract, the doubling time was only 15 min. Based on 16S rDNA studies, DNA–DNA hybridization and morphological and physiological properties, the isolate IHI-91 was identified as *Bacillus thermoleovorans* IHI-91 sp. nov. Because of its production of high concentrations of thermoactive lipases and esterases and the capability of degrading a wide range of lipids at high temperatures, the isolated strain is an ideal candidate for application in various biotechnological processes such as wastewater treatment.

Key words *Bacillus thermoleovorans* · Esterase · Fat · Fatty acid · Lipase · Lipid · Oil

Introduction

Lipid-rich wastes are produced during several industrial processes such as oil refining, soap and leather production. These waste streams are characterized by a high concentration of oxidizable substances, such as oils, fats, fatty acids, and sulfates, in addition to low pH values and high temperatures. The quantities and the composition of these wastes depend on the amount, applied technology, and raw materials employed at each manufacturing plant (Pöppinghaus et al. 1994; Meinck et al. 1968; Heinrich et al. 1992). The increased demands and the strict jurisdiction in the realm of environmental protection require the development of modern biotechnological processes for the treatment of such industrial wastes.

The application of thermophilic microorganisms for purification of lipid-containing wastes has obvious advantages. At temperatures above 50°C, the lipid aggregates melt, and stable emulsions of substrates with a large surface area are formed during agitation. Under such conditions, the bioavailability of such hydrophobic substrates for enzymes and microorganisms is significantly increased. From a technological point of view, high temperatures are also desirable, because the viscosity of the streams decreases, and thus diffusion and mass transfer are accelerated. Furthermore, the hygienization of the waste sludge is achieved at temperatures above 60°C (Becker et al. 1997, 1999).

A number of thermophilic microorganisms produce thermoactive lipases and esterases (Handelsman and Shoham 1994; Schmidt-Danert et al. 1994; Ikeda and Clark 1998). Potential industrial applications of such thermoactive enzymes include production of mono- and diacylglycerides, fatty acids, and glycerol via hydrolysis of oils and fats, modification of the composition and physical properties of triacylglycerides, synthesis of chemicals in organic solvents, paper manufacturing, and application for biochemical catalysis in supercritical fluids. Some of these applications are highly advantageous because the enzymes can be produced at low cost and exhibit improved stability in general (Fischer et al. 1993; Nakamura 1990).

In this study, we describe the isolation and characterization of a new thermophilic *Bacillus* strain, which is able to degrade a broad spectrum of lipids and produce thermoactive lipases and esterases.

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Materials and methods

Media and growth conditions

Enrichment medium for the isolation of lipid-degrading bacteria contained basal salt solution (Atlas 1993), supplemented with trace elements (Balch et al. 1979) and vitamin solutions (Wolin et al. 1964), yeast extract 0.1% (w/v), tryptone 0.05% (w/v), and fatty acids 0.4% (w/v). The basal salt solution contained (in $g l^{-1}$) K_2HPO_4, 0.8; KH_2PO_4, 0.6; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \times 7H_2O$, 0.2; $CaCl_2 \times 2H_2O$, 0.05; NaCl, 3.0; and $FeCl_3$, 0.001. The pH value of the medium was adjusted at room temperature at pH 6.8. In further experiments, fatty acids (0.4%) were replaced with the same amount of olive oil (Sigma, St. Louis, MO, USA). The lipids were distributed directly into the vials due to their water immiscibility. The medium was sterilized for 20 min at 121°C.

Isolation and storage

Numerous enrichment cultures that were derived from samples of hot springs and soils from different regions were screened for their ability to grow on lipids. The strain described here was isolated from a sample from a hot spring in the Hverageroi area in South Iceland.

Samples were inoculated (5%–10% v/v) into 10-ml vials containing enrichment media and incubated in a water bath at 70°C for several days. The enriched culture was subcultured several times under the same conditions in the same medium. Agar plates containing olive oil (4% v/v) and the fluorescent dye rhodamine B were prepared according to Kouker and Jaeger (1987). Olive oil hydrolysis causes the formation of orange fluorescent halos around the colonies that can be made visible with UV radiation. Lipid-degrading microorganisms were isolated from these plates after growth at 65°C under aerobic conditions. The most active colony was then purified by repeated streaking onto the agar plates and further cultivated in liquid culture at 65°C and pH 6.8.

The ability of the isolated bacterium to grow under microaerophilic conditions with nitrate as a terminal electron acceptor was used for its storage to avoid strain contamination. Storage was accomplished on enrichment medium supplemented with $10 g l^{-1}$ KNO_3 and without the addition of lipids. 25 ml of the medium was distributed into 50-ml serum bottles (Pierce, Beijerland, Holland), the remaining volume of the vials was gassed for 1–3 min with nitrogen, and the vials were closed with rubber stoppers and sealed with aluminum cups. The strain was stored at 4°C and subcultured once every 3–4 months. The type strain *Bacillus thermoleovorans* (DSM 5366) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig).

Physiological studies

The determination of optimal pH and temperature for growth was carried out under aerobic conditions in a 1-l glass fermentor (BCC, Göttingen, Germany) on a medium composed of mineral elements and $4.5 g l^{-1}$ of yeast extract. For each pH value in the range 5.0–7.5 (with 0.5 pH steps at 65°C) and each temperature value in the range 55°–75°C (with 5°C steps at pH 6.0), fermentation experiments were performed. The estimation of the optimal NaCl concentration for growth was carried out under aerobic conditions in 100-ml Erlenmeyer flasks in the same medium supplemented with $0–30 g l^{-1}$ of NaCl. Every 30 min, samples were taken, cells were counted, and the doubling time during the exponential growth phase was calculated.

The substrate spectrum of the newly isolated strain IHI-91 was investigated under aerobic conditions in test tubes or shake flasks in a medium containing mineral elements and $4 g l^{-1}$ of each substrate without the addition of yeast extract. The consumption of the lipids by strain IHI-91 and by type strain *B. thermoleovorans* (DSM 5366) was studied under identical conditions in 100-ml Erlenmeyer flasks. After subculturing twice, the growth on different carbon sources was evaluated. In addition, substrate consumption was also tested by using the commercial test tubes API 50 CHB (Bio-Merieux, Marcy l'Etoile, France). Nitrate and nitrite reduction was studied in the medium used for enrichment with $10 g l^{-1}$ KNO_3 without any lipids. Formation of molecular N_2 was observed with Durham vials, and nitrate and nitrite concentration was determined by HPLC using an LCA A03 column (100 mm × 4 mm, Sykam, Gilching, Germany). The sulfate reduction was studied as described by Süßmuth et al. (1987).

Morphological studies

Gram staining, spore staining, a KOH test, and an aminopeptidase test were performed according to the methods of Bartolomew (1962), Schaeffer and Fulton (1933), Gregersen (1978), and Cerny (1978), respectively.

Strain identification

The partial sequencing of 16S rDNA, DNA–DNA hybridization, and estimation of membrane fatty acid composition were carried out at the DSMZ. The DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al. (1977). DNA–DNA hybridization was carried out as described by DeLey et al. (1970) with the modifications of Huss et al. (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories, Oberlin, OH, USA). Renaturation rates were computed with the TRANSFER.BAS programme described by Janke (1992). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of the PCR products were carried out as described by Rainey et al. (1992). Purified PCR products were sequenced using the Taq Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacturer's protocol. Sequence reactions were applied to electrophoresis using the Applied

Biosystems 373 A DNA Sequencer. The partial 16S rDNA sequences were aligned against representative sequences of members of the genus *Bacillus.*

Membrane fatty acids

Strain IHI-91 was grown on trypticase soy broth agar (Becton Dickinson, Cockeysville, MD, USA) at 65°C. Fatty acids were obtained from biomass by saponification, methylation, and extraction using the minor modification by Kuykendall et al. (1988) of the method of Miller (1982). The fatty acid methylester mixtures were separated using a model 5898A microbial identification system (Microbial ID, Newark, DE, USA) as described by Vainshtein et al. (1992).

Lipid utilization

To study the ability of the new isolate to grow on lipids, experiments on agar plates with rhodamin B and in liquid cultures with and without lipids as carbon source were performed. To determine the lipid degradation quantitatively, a batch fermentation was carried out under aerobic conditions in a 2-l foil fermentor (Bio Engineering, Wald, Switzerland) in a medium containing basal salt and vitamin solutions, trace elements, and 10 ml l^{-1} of olive oil as the sole carbon source. The fermentation was performed at 65°C and pH 6.0 with an aeration rate of 100 l h^{-1}. Before taking samples, the stirring speed was increased to 2000 rpm to spread the lipids equally in the medium. The samples were extracted with the solvent (*n*-hexane/ether 1:4) and evaporated with N_2. The residues were then dissolved in *n*-hexane/acetone (7:3), dried with Na_2SO_4, and finally silylated with 0.5 ml of N-Methyl-N-trimethylsilyl-heptafluorobutyramide (MSHFBA; Macherey-Nagel, Düren, Germany) at 60°C for 10 min. The samples were analyzed on GC with a classical configuration using a packed glass column (1 m × 2 mm). Lipase activity was determined by hydrolyzing para-nitrophenyllaurate (Sigma). One unit of lipase activity is defined as the amount of enzyme that releases 1 µmol of para-nitrophenol in 1 min under optimal assay conditions (Sigurgisladottir et al. 1993).

Results and discussion

Morphology

The cells of the isolated strain IHI-91 are nonmotile, gram-negative, endospore-forming rods 0.7–0.8 × 2.5–5.0 µm. Chains of two or more cells could be observed in growing cultures. In the stationary phase, cells tend to aggregate depending on the growth conditions (Fig. 1). In some cases they showed variable Gram staining independent of their growth phase. Such behavior has been also described for other *Bacillus* sp. by Handelsman and Shoham (1994), Hollocher and Kristjansson (1992), and Zarilla and Perry (1987). The spores are ellipsoidal and located terminally. The sporangium is slightly swollen.

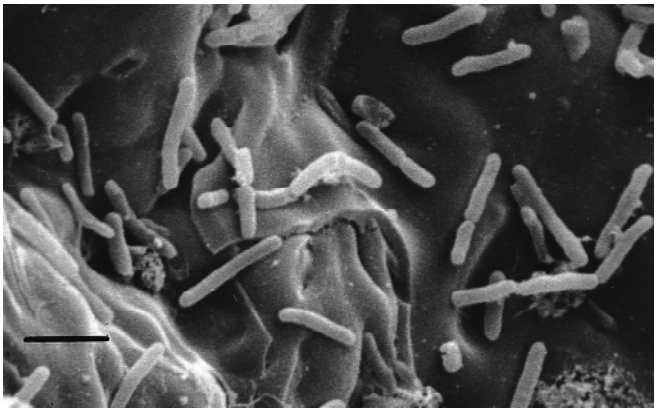

Fig. 1. Scanning electron photomicrograph showing the morphology of the strain *Bacillus thermoleovorans* IHI-91. *Bar* 2.4 µm

Growth requirements and physiology

The estimation of optimal pH and temperature values for growth was first carried out under aerobic conditions in Erlenmeyer flasks. The significant shift of the adjusted pH of the medium after less than 2 h of cultivation was observed in all flasks. The same was also true when the experiments were carried out in a medium buffered with 30 mM phosphate. Reliable results could be obtained after cultivation of the new isolate in temperature and pH-controlled bioreactors. To estimate the doubling time, fermentations in 1-l glass reactor were performed for each pH and temperature value. The strain IHI-91 is able to grow at a pH range of 5.0–7.5 and a temperature range of 45°–70°C. At values outside the mentioned ranges, no growth was observed. The optimal values for the growth are 65°C (Fig. 2A) and pH 6.0 (Fig. 2B). The strain IHI-91 grows optimally in the presence of 5 g l^{-1} NaCl and tolerates up to 20 g l^{-1} of salt (Fig. 2C). Under optimal conditions (65°C, pH 6.0, and 5 g l^{-1} NaCl), strain IHI-91 has a doubling time of only 15 min.

Strain IHI-91 is able to grow on various substrates as a sole carbon and energy source without the requirement of growth factors. Great attention has been paid to the consumption of fatty acids, natural oils, and synthetic substances such as Tween. The following substrates were found to support growth: ribose, glucose, fructose, mannose, arabinose, sucrose, maltose, cellobiose, trehalose, melezitose, turanose, gluconate, 2-ketogluconate, mannitol, α-methyl-D-glucoside, starch, xylan, pullulan, peptone, tryptone, yeast extract, pyruvate, gum arabic, glycerol, acetic acid, butyric acid, and oleic acid. The following substrates were found to be ideal for growth: palmitic acid, stearic acid, olive oil, sunflower seed oil, soya oil, fish oil, lanolin, antifoam M30, Tween 20, 40, 60, and 80, triacetin, and tributyrin. The strain IHI-91 was not able to grow on the following substrates: lactose, galactose, sorbose, rhamnose, melibiose, raffinose, gentiobiose, lyxose, tagatose, fucose, 3-ketogluconate, arabitol, dulcitol, inositol, adonitol, sorbitol, xylitol, erythritol, α-methyl-D-mannoside, β-methyl-xyloside, amygdalin, arbutin, esculin, salicin, amidon, *N*-

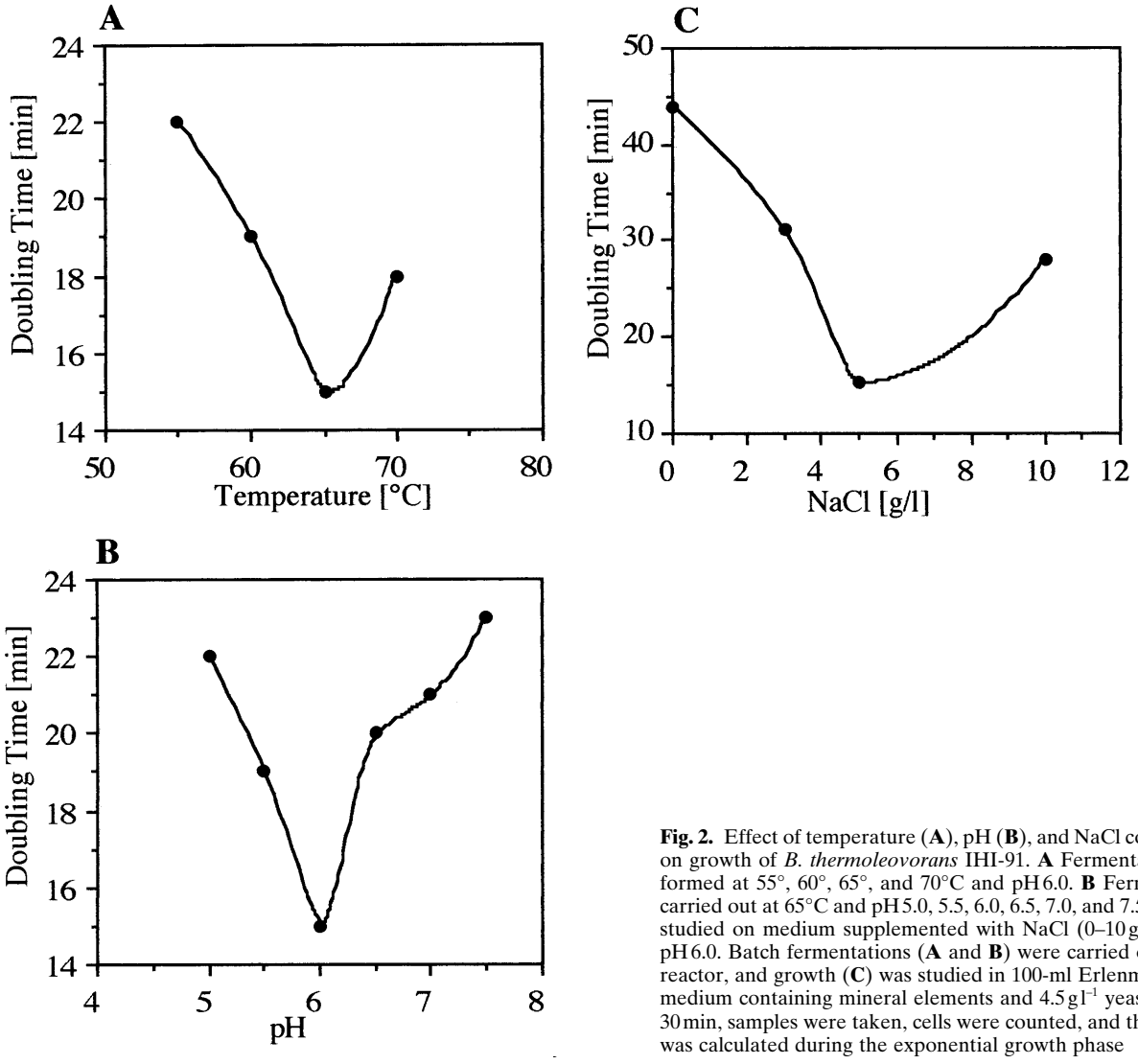

Fig. 2. Effect of temperature (**A**), pH (**B**), and NaCl concentration (**C**) on growth of *B. thermoleovorans* IHI-91. **A** Fermentations were performed at 55°, 60°, 65°, and 70°C and pH 6.0. **B** Fermentations were carried out at 65°C and pH 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. **C** Growth was studied on medium supplemented with NaCl (0–10 $g l^{-1}$) at 65°C and pH 6.0. Batch fermentations (**A** and **B**) were carried out in a 1-l glass reactor, and growth (**C**) was studied in 100-ml Erlenmeyer flasks on a medium containing mineral elements and 4.5 $g l^{-1}$ yeast extract. Every 30 min, samples were taken, cells were counted, and the doubling time was calculated during the exponential growth phase

acetylglucosamine, inulin, glycogen, pectin, cellulose, gelatin, casein, bactopeptone, propionic acid, lauric acid, myristic acid, Triton-100, and silicon oil DC 200. Table 1 summarizes some of the physiological properties of strain IHI-91.

Classification

The partial sequencing of 16S rDNA resulted in grouping of strain IHI-91 to group 5 of the genus *Bacillus* (Ash et al. 1991). A homology of 99.8% was found between the isolated strain IHI-91 and the species *B. kaustophilus, B. thermolevorans*, *B. caldotenax*, *and B. caldolyticus*. Based on its physiological and biochemical properties, strain IHI-91 has the most resemblance to *B. thermoleovorans.* This ordering was also confirmed by DNA–DNA hybridization with a value of 82.5%. Much lower values, 34.0% and 28.2%, respectively, were obtained with the other reference strains *B. thermoruber* (DSM 7064) and *B. thermoglucosidasius* (DSM 2542). Comparison of biochemical, morphological, and physiological properties of the strain IHI-91 and other representatives of group 5 of the genus *Bacillus* (Table 2) shows the genotypic homogeneity and phenotypic heterogeneity of this group.

Accordingly, and as suggested by Sunna et al. (1997), all these strains (*B. caldotenax*, *B. caldolyticus*, *B. caldovelox*, *B. thermocatenulatus*, and *B. kaustophilus*) should be combined into the emended species *Bacillus thermoleovorans.*

Fatty acid composition

The main fatty acids of the strain IHI-91 are the *iso*-branched pentadecanoic acid (*iso*-C15, 45.88%), heptadecanoic acid (*iso*-C17, 31.79%), and hexadecanoic acid (*iso*-C16, 8.81%); these account for 86.48% of the total fatty acids. In particular, *iso*-C15 is the most abundant, representing 45.9% of the total fatty acids. Similar to strain IHI-91, in *B. caldolyticus*, *B. caldovelox*, *B. caldotenax*, *B. thermoclocae*, and *Bacillus* strain HSR, the branched *iso*-C15, *iso*-C16, and *iso*-C17 acids have been reported to

Table 1. Physiological properties of strain IHI-91

Properties	
Anaerobic growth	–
Catalase	+
Voges–Proskauer reaction	–
Reduction of NO_3 to NO_2	+
Reduction of SO_4	–
Growth in the presence of:	
2% NaCl	+
5% NaCl	–
7% NaCl	–
10% NaCl	–
Lysozyme-medium	–
Acid production from:	
Glucose	+
Arabinose	+
Xylose	–
Mannitol	+
Fructose	+
Gas formation from glucose	–
Hydrolysis of:	
Starch	+
Gelatin	–
Casein	–
Esculin	–
Tween 80	+
Utilization of:	
Citrate	+
Propionate	+
Indole	–
Phenylalanine desaminase	–
Arginine hydrolase	–
Urease	+
Lecithinase	–

+, positive; –, negative

represent about 80% of the total membrane fatty acids (Heinen and Heinen 1972; Weerkamp and Heinen 1972; Demharter and Hensel 1989; Sunna et al. 1997). Adaptation to high growth temperatures resulted in an increase in the relative amount of the high melting point *iso*-fatty acids and a decrease of the low melting point *anteiso*-fatty acids. This biochemical adaptation seems to be necessary to maintain membrane fluidity at elevated growth temperatures (Kaneda 1977, 1991). Recently, seven thermophilic strains were isolated by Nicolaus et al. (1995) from the Antarctic continent. Growth of the new isolates at temperatures between 40°C and 71°C showed that at higher temperatures all strains preferred the synthesis of the higher melting point fatty acid (*iso*-C17).

Lipid degradation

Although the isolated strain IHI-91 has 82.5% DNA–DNA homology to the type strain *B. thermoleovorans* (DSM 5366), an obvious difference can be seen in substrate consumption, especially in the case of oils and *n*-alkanes (Table 3). The type strain *B. thermoleovorans* is more "specialized" for the degradation of alkanes and shows poor growth on olive oil and Tween 80. The newly isolated strain IHI-91 does not grow on alkanes, but shows very good growth on several lipids and fatty acids including oleic acid, palmitic acid, stearic acid, olive oil, sunflower seed oil, soya oil, fish oil, and lanolin. To study oil degradation in detail, a fermen-

Table 2. Comparison of biochemical, morphological, and physiological properties of strain IHI-91 and related thermophilic bacilli

Characteristics	IHI-91	*B. thermoleovorans*[b]	*B. kaustophilus*[c]	*B. caldotenax*[d]	*B. caldolyticus*[d]
G + C mol % of DNA[a]	n.d.	53.7	51.0	56.2	54.5
Growth at:					
30°C	–	–	–	n.d.	n.d.
70°C	+	+	+	+	+
Optimal growth Temperature, °C	65	55–65	60–65	80	72
Spore:					
Shape	O	O	O/C	O	C
Position	T	T	T	T	T
Catalase	+	+	n.d.	–	–
Oxidase	+	+	–	+	+
Anaerobic growth	–	–	–	w	w
Acid from glucose	+	+	+	+	+
Formation of:					
Acetoin	n.d.	–	–	–	–
Indole	–	–	–	–	–
H_2S	n.d.	n.d.	n.d.	–	–
Citrate utilization	+	–	+	+	+
Hydrolysis of:					
Starch	+	+	+	+	+
Casein	–	w	–	+	+
Gelatin	–	n.d.	+	+	+
Urea	+	+	–	n.d.	n.d.
Nitrate reduction	+	n.d.	+	+	+
Denitrification	–	n.d.	+	–	–

+, positive; –, negative; w, weak reaction; n.d., not determined; O, oval; C, cylindrical; T, terminal

[a] G + C mol % of DNA was redetermined for *Bacillus thermoleovorans, B. caldotenax*, and *B. caldolyticus* by Sunna et al. (1997)

[b] Zarilla and Perry (1987)

[c] Priest et al. (1988)

[d] Heinen and Heinen (1972), Sharp et al. (1980), and Wolf and Sharp (1981)

Table 3. Lipid consumption of strain IHI-91 compared to the type strain *Bacillus thermoleovorans* (DSM 5366)

Carbon sources	IHI-91	*B. thermoleovorans* (DSM 5366)
Palmitic acid	+	–
Stearic acid	+	–
Olive oil	+	+
Sunflower seed oil	+	–
Soya oil	+	–
Fish oil	+	–
Lanolin	+	–
Tween 80	+	+
Dodecane	–	+[a]
Hexadecane	–	+[a]
Heptadecane	–	+[a]
Eicosane	–	+[a]

+, positive; –, negative
[a] Data were obtained from Zarilla and Perry (1987)

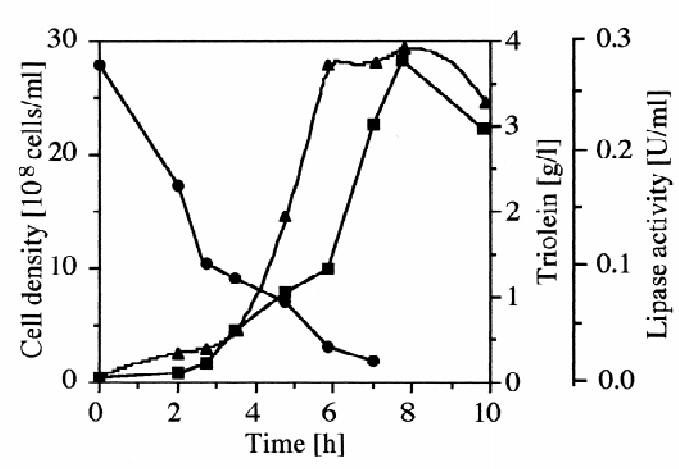

Fig. 3. Time course during batch fermentation of *B. thermoleovorans* IHI-91. Fermentation was carried out in a 2-l foil fermentor on a medium containing mineral elements and 1% (v/v) olive oil as the sole carbon source at 65°C and pH 6.0. Profiles of cell density (■), lipase activity in the supernatant (▲), and triolein concentration (●)

tation experiment was carried out with olive oil as the carbon source ($10\,ml\,l^{-1}$). During 7-h fermentation, about 93% of olive oil was utilized, and lipase activity of $0.3\,U\,ml^{-1}$ was detected in the supernatant. A maximum cell density after 7 h of fermentation and a maximal growth rate during the exponential growth phase was determined to be 2.8×10^9 cells ml^{-1} and $1.0\,h^{-1}$, respectively (Fig. 3). Growth was completed after 8 h of cultivation, demonstrating the high efficiency of this process.

In spite of high similarity to the other strains, *B. thermoleovorans* IHI-91 has unique features that are not found in the already described *Bacillus* strains (Schmidt-Danert et al. 1994); these include broad substrate specificity, especially for lipids, high lipase and esterase activity, and rapid growth rate.

The icelandic hot springs are a rich source of thermophilic microorganisms, and a number of thermophilic denitrifying bacteria have been already isolated and characterized by Hollocher and Kristjansson (1992). Sigurgisladottir et al. (1993) have reported on the presence of lipolytic activity in these strains. The isolation of a new strain of *B. thermoleovorans* from the spring of the Hverageröi area of South Iceland, which has a similarity to already described icelandic strains, supports the expressed view of Hollocher and Kristjansson (1992) that *Bacillus* represents a widely dispersed genus of thermophilic denitrifiers which seem to have a similar role in thermal as in nonthermal ecosystems.

The newly isolated thermophilic strain is an ideal candidate for application in various biotechnological processes. Because of its unique features, the strain *B. thermoleovorans* IHI-91 has been deposited at DSMZ as DSM 10561. The cloning and expression of its lipase and esterase is under current investigation.

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