

***Selenomonas lipolytica* sp. nov., an obligately anaerobic bacterium possessing lipolytic activity**

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A novel, obligately anaerobic bacterium capable of hydrolysing lipids was isolated from a tropical anaerobic lagoon receiving waste water from an edible oil mill. The isolate had many characteristics similar to those of members of the genus *Selenomonas*. The isolate showed lipolytic activity on tributyrin, triolein and groundnut oil in qualitative plate clearance assays, which has not been reported for the type strain of the genus *Selenomonas*. It did not require n-valerate supplementation for growth on glucose. Acetate and propionate were the only volatile fatty acids produced from glucose fermentation with propionate as the major end product. The isolate could grow optimally at pH 6.8 and at a temperature of 40 °C. It could tolerate NaCl concentrations of up to 40 g l⁻¹. The G + C content of the DNA was 40 mol% as determined by thermal denaturation analysis. Comparison of partial 16S rRNA gene sequences revealed that the isolate was most closely related to genus *Selenomonas* with 91% sequence similarity (250 bp compared) to *Selenomonas ruminantium* strain GA 192. On the basis of the results obtained in the present investigation, it is suggested that a new species of *Selenomonas* should be created for this novel isolate and the name *Selenomonas lipolytica* is proposed for this new species. The type strain is strain CF1B^T (= MCMB 505^T).

Keywords: anaerobic bacterium, lipase, lipid degradation, *Selenomonas lipolytica* sp. nov.

INTRODUCTION

Amongst the various microbial extracellular enzymes with commercial applications, lipase is an important one. Lipase is used on a large scale in cheese production, detergent manufacture and organo-conversions. Micro-organisms producing lipase play a significant role in degradation of complex organic matter containing lipid, both in the natural environment and in waste-treatment plants. These micro-organisms are also said to be responsible for spoilage of pickles and vegetable oils (Sarnaik *et al.*, 1989).

Different aerobic bacteria and fungi have been reported as lipase-producing microbes. Commercial

production of lipase relies solely on aerobic bacteria and fungi. Most of the knowledge about the biochemical nature of lipase and the process of lipolysis is also based on enzyme preparations obtained from aerobic organisms. Obligate anaerobes possessing lipolytic activity have received less attention. Much of the available information on lipid degradation under anaerobic conditions is in relation to activities in the rumen. Hobson & Mann (1961) isolated a glycerol-fermenting and lipolytic bacterium from the rumen of sheep. Later, Hungate (1966) designated this lipolytic strain, which could hydrolyse linseed oil triglycerides to glycerol and free fatty acids, *Anaerovibrio lipolytica*. Henderson (1971) studied the lipase produced by *A. lipolytica* and partially purified the enzyme. Schauder & Schink (1989) described a new species of the genus *Anaerovibrio* as *Anaerovibrio glycerini*, which could ferment glycerol and glycerol residues of diolein. Four species of the genus *Clostridium* (*Clostridium auranti-butyricum*, *Clostridium botulinum*, *Clostridium novyi* and *Clostridium sporogenes*) are reported to produce

Abbreviations: PY, peptone/yeast extract medium; PYG, peptone/yeast extract/glucose medium; RDP, Ribosomal Database Project; S_{abr} , similarity value; T_m , midpoint of DNA melting temperature. The GenBank accession number for the sequence reported in this paper is AF001901.

lipase, but very little is known about their actual lipolytic ability (Cato *et al.*, 1986). Generally, there are hardly any other obligately anaerobic bacteria that have been isolated and identified which possess true lipolytic activity (P. N. Hobson, personal communication).

Nowadays, anaerobic bacteria and their activities are gaining importance. This is mainly due to the fact that these bacteria do not require aeration during growth and hence energy can be saved. Attempts have been made to express 'lipase' genes of aerobes when the culture is grown under O_2 -limited conditions, to save the cost of aeration and agitation. Winteler *et al.* (1996) tried to manipulate a strain of *Pseudomonas aeruginosa* (PAO 1) so that it could grow under anaerobic conditions (nitrate respiration or anaerobic fermentation using L-arginine) and would overproduce lipase.

Secondly, anaerobic bacteria, along with methanogens, bring about degradation of complex matter and simultaneously produce gaseous fuel in the form of methane. With this background, a search for anaerobes having extracellular enzymes is essential. In this report, one strain of an obligate anaerobe showing strong lipolytic activity was isolated and its properties were investigated.

METHODS

Bacterial strain. Strain CF1B^T was isolated from an anaerobic lagoon receiving waste-water from an edible oil mill near Pune, India. The *in situ* temperature of the lagoon was 33 °C and the pH was 6.95. A sample of semi-solid sediment was collected from the lagoon. During the collection and transport period of 2 h, care was taken to maintain anoxic conditions.

Media and culture conditions. The solid and liquid media were prepared according to the anaerobic techniques described by Hungate (1969) and subsequently modified by Miller & Wolin (1974).

The emulsions of groundnut oil, triolein and tributyrin were prepared in distilled water as well as in 2% (w/v) polyvinyl alcohol solution in distilled water. The distilled water (or polyvinyl alcohol solution) was boiled and cooled to room temperature under a constant stream of O_2 -free N_2 to expel all the dissolved O_2 . Cysteine.HCl (0.5 g l⁻¹) was then added as the reducing agent in the distilled water. The pH of the distilled water was adjusted to 7.0 with 1 M NaOH solution. The substrate was mixed with anaerobic distilled water prepared as above to give final concentration of substrate of 200 g l⁻¹. It was then emulsified in an ultrasonicator until a milky white homogeneous emulsion was obtained. The emulsified solution was dispensed in 65 ml capacity serum vials under a continuous passage of O_2 -free N_2 . The vials were sealed under N_2 using butyl rubber stoppers and aluminium seals. The vials were sterilized by autoclaving at 121 °C for 30 min.

The strain was enriched, subsequently isolated and maintained on nutrient medium, henceforth referred to as OA-1 medium, having the following composition (l⁻¹): 0.3 g KH_2PO_4 ; 1.0 g NH_4Cl ; 0.6 g NaCl; 0.1 g $MgCl_2 \cdot 6H_2O$; 0.2 g $CaCl_2$ anhydrous; 5.0 g yeast extract; 5.0 g tryptone;

1 ml trace element solution (Touzel & Albagnac, 1983); 1 ml trace vitamin solution (Wolin *et al.*, 1963); 1 ml resazurin solution (0.1%, w/v, in distilled water); and 0.5 g cysteine.HCl. The medium was boiled and cooled to room temperature under a stream of O_2 -free N_2 . The pH of the medium was adjusted to 7.0 with 10 M NaOH. It was then distributed anaerobically as 20 ml aliquots in 65 ml capacity serum vials which were sealed with butyl rubber stoppers and crimped with aluminium seals. Sterilization was carried out by autoclaving at 121 °C for 20 min. The vials were then fortified with sterile groundnut oil emulsion, prepared as described above, to have final oil concentration of 1% (w/v).

PY medium (peptone/yeast extract medium) of the following composition (l⁻¹) was prepared (Holdeman *et al.*, 1977): 10 g peptone; 10 g yeast extract; 40 ml salt solution; 1 ml resazurin solution (0.1%, w/v, in distilled water); and 0.5 g cysteine.HCl. Composition of the salt solution (100 ml⁻¹) was as follows: 0.1 g K_2HPO_4 ; 0.1 g KH_2PO_4 ; 1.0 g $NaHCO_3$; 0.2 g NaCl; 0.02 g $CaCl_2$ anhydrous; and 0.02 g $MgSO_4 \cdot 7H_2O$. The medium was boiled and cooled to room temperature under a stream of O_2 -free N_2 . The pH of the medium was adjusted to 7.0 with 10 M NaOH solution. It was then distributed anaerobically and sealed using butyl rubber stoppers and aluminium seals. PYG medium (peptone/yeast extract/glucose medium) was prepared by adding 10 g glucose per litre of the PY medium.

All experiments using the solid media were performed in an anaerobic glove box (Forma Scientific). The solid media were prepared using agar/agar as the solidifying agent at a concentration of 30 g l⁻¹ and poured into glass Petri plates inside the anaerobic glove box.

Unless mentioned otherwise, the incubation temperature was 35 °C.

Isolation and purity. As direct isolation failed to yield bacterial growth on solid medium, enrichment was set up by inoculating the sample (10%, v/v) in the OA-1 medium containing 1% (w/v) groundnut oil. The enriched sample was streaked onto the OA-1 agar containing 1% (w/v) triolein. After the incubation period of 5 d, the colonies showing a clearance zone were subcultured and their purity and lipolytic activity were confirmed. The purity of the isolate was confirmed by the streak-plate method using PYG agar plates and the serially diluted culture. The characteristics of all the colonies obtained in this manner were identical. Furthermore, randomly selected colonies obtained from different dilutions showed rod-shaped, Gram-negative bacteria that had identical morphological characteristics by microscopic examination. The subculture of each colony showed a clearance zone on triolein agar plates, confirming the purity and lipolytic ability of the isolated culture.

Cellular characterization. Gram-staining characteristics were determined with a standard Gram-stain kit (Hi Media). Presence or absence of spore formation in the culture was examined by phase-contrast microscopy (Nikon) at the end of growth in different media including PY, PYG, OA-1 and Robinson's cooked meat medium (Hi Media). The broth culture in PYG medium was heated in a water bath at 85 °C for 2 h and then stored at 4 °C for 4 h to check whether heat shocks could stimulate spore formation. Motility was determined under the phase-contrast microscope by making observations of wet mounts of the broth culture using a glass cavity slide. After putting the cover-slip on the culture drop, the edges of the cover-slip were immediately sealed by molten wax. This helped to keep the culture active for

Table 1. Comparative characteristics of strain CF1B^T with other closely related anaerobes from the family Bacteroidaceae

Information obtained from Hespell *et al.* (1992); Holdeman *et al.* (1984); Lee *et al.* (1978); and Schauder & Schink (1989) except for strain CF1B^T. N, Data not available; V, variable.

Characteristic	<i>S. ruminantium</i> subsp. <i>lactilytica</i>	CF1B ^T	<i>P. cerevisiphilus</i>	<i>A. lipolytica</i>
Habitat	Rumen	Tropical anaerobic lagoon	Spoiled packaged beer	Rumen
Morphology	Curved rods	Curved rods	Slightly curved rods	Curved rods
Size (µm)	0.9–1.1 by 3–6	0.55–0.77 by 3.3–4.5	0.7–0.8 by 2–32	0.5 by 1.2–3.6
Gram staining	Gram-negative	Gram-negative	Gram-negative	Gram-negative
Sporulation	Non-spore-forming	Non-spore-forming	Non-spore-forming	Non-spore-forming
Flagellar arrangement	Linear arrangement as a tuft near the centre of the concave side	Linear arrangement as a tuft near the centre of the concave side	Attached only on the concave side but not limited to the central portion	Single polar flagellum
Growth temperature:				
Optimum temperature (°C)	37	40	32	
Temperature range	Does not grow below 20–30 °C	30–47 °C	15–40 °C	Growth occurs at 38 °C but not at 20, 30 or 50 °C
End-products of glucose fermentation	Acetate and propionate	Acetate and propionate	Acetate and propionate	Does not utilize glucose
Requirement of n-valerate for glucose utilization	Required	Not required	Not required	–
DNA G + C content (mol%)	48–60	40	39.8	31.4
Utilization of carbon sources:				
Lactate	+	–	+	+
Cellobiose	+	+	+	–
Mannitol	+	–	+	–
Sucrose	+	+	–	–
Galactose	+	+	+	–
Lactose	+	+	–	–
Glucose	+	+	+	–
Mannose	V	+	+	–
Arabinose	+	+	+	–
Maltose	+	+	+	–
Inositol	V	+	–	–
Dulcitol	+	–	+	–
Inulin	V	+	–	–
Salicin	+	+	–	–
Starch	V	+	–	–
Aesculin	+	+	–	–
Raffinose	+	+	–	–
Rhamnose	N	–	+	N
Melibiose	+	+	–	N
Melizitose	N	+	–	N

7–8 min due to the presence of reductant in the medium which helped in temporarily maintaining anaerobiosis.

Growth and metabolic properties. The ability of the isolate to grow under aerobic and obligately anaerobic conditions was tested using the PYG agar plates and the PYG broth. The catalase, indole and gelatin-liquefaction tests were performed according to the methods of Holdeman *et al.* (1977). The proteolysis was tested on milk agar plates (Hi Media); a clearance zone was taken as a positive result.

Determination of optimum pH and temperature. PYG medium was used to determine growth of the isolate at different pH and temperature values. A cell suspension was prepared by suspending cell growth from PYG agar plate in sterile anaerobic saline of pH 7.0 and used as the inoculum (10%, v/v). The optical density (OD₆₀₀) of the cell suspension, determined using a Bausch & Lomb spectrophotometer, was 0.3.

Requirement for NaCl. PYG broth (pH 7.0) was prepared with varying concentrations of NaCl (0–100 g l⁻¹). The vials were inoculated with the well-grown culture in PYG (10%, v/v) and incubated for 5 d. At the end of the incubation

period, the culture broth was analysed for volatile fatty acid content.

Utilization of carbon source. The test was performed in anaerobic vials containing 18 ml PY broth plus the respective test substrate (Table 1), added to give final concentration of 1% (w/v). A vial containing PY broth without any addition of substrate served as the control. The culture grown in PYG was used as the inoculum (10%, v/v). All the inoculated vials were incubated for a period of 4 d. At the end of the incubation period, the pH of each vial was measured and only those vials which showed a pH drop by a value greater than 0.5 units over the control vial were taken as positive, indicating that the test compound was utilized (Holdeman *et al.*, 1984). It is worthwhile to mention that growth of the isolate in the plain PY broth resulted in a drop in pH by only 0.2 units.

Utilization of nitrogen source. The ability of the isolate to use different nitrogenous compounds as nitrogen source was tested on agar plates. To test the utilization of nitrogen sources, peptone and yeast extract were excluded from the PYG medium. To achieve desired anaerobiosis in this medium, cysteine.HCl was added as the reducing agent at a

concentration of 0.5 g l⁻¹. The PYG agar modified in this manner served as the control medium. The modified PYG agar thus contained glucose, reductant, salt solution, resazurin and agar/agar. The test compounds were added separately at a concentration of 0.5% (w/v). The culture grown on PYG agar was streaked onto the test and control media. The agar plates were incubated for 5 d. Growth of the isolate during three successive transfers on the medium containing the specific nitrogen source was taken as a positive result. The control medium showed poor growth of the isolate; this could be due to the presence of cysteine at a very low concentration.

Requirement for n-valerate. The test was carried out in Hungate tubes containing PYG medium with (500 mg l⁻¹) and without n-valerate supplementation. Growth curves were plotted by taking OD₆₀₀ at regular time intervals. At the end of the incubation, the culture broth was analysed for volatile fatty acid content.

Antibiotic sensitivity pattern. Sensitivity of the culture towards different antibiotics was checked by disk assay using standard antibiotic disks (Hi Media). The culture grown in OA-1 medium containing 1% (w/v) emulsified tributyrin was spread on agar plates prepared using the same medium. The antibiotic disk was placed at the centre of each plate. The plates were incubated for 5 d and the zone of inhibition of growth around the antibiotic disk was measured. Referring to the manufacturer's protocol, the response of the culture towards different antibiotics was classified as resistant, intermediate and sensitive depending upon the diameter of the inhibition zone.

Determination of G + C content of the DNA. The DNA was isolated by the method of Marmur (1961) with a modification in the method for lysis of the cells. The cells were lysed using 10% (v/v) Triton X-100 in Tris/EDTA buffer of pH 8.0. The G + C content was determined by the thermal denaturation (*T_m*) method (Mandel & Marmur, 1968), using a spectrophotometer (DU-8B; Beckman) with a thermal programming facility.

Plasmid isolation was carried out by the alkaline lysis method (Birnboim & Doly, 1979).

16S rDNA sequence analysis. The DNA was extracted from 3 ml culture. Amplification of 16S rRNA gene was carried out using PCR (Perkin Elmer) as described by Moore *et al.* (1993). Conserved priming sites were used for the amplification. This gave a fragment of around 1.5 kb which was then purified by filtration on Microcon 100 columns to remove unincorporated primers and dNTPs. The concentration of purified PCR product was checked on an agarose gel.

The purified product was sequenced directly by Gibco-BRL ds Cycle Sequencing kit using the manufacturer's protocol. The gel was run on IBI-Kodak Base Runner 100 and an electrolyte gradient was used to enable reading of longer sequences (Ausubel, 1995). Using a single primer that binds to position 8–27 (*Escherichia coli* numbering), the sequence of 250 bases at the 5' end of the molecule was determined. The primer used was F27 and had the following sequence: AGAGTTTGATC(A/C)TGGCTCAG. This region was selected for the analysis because it includes the hypervariable region V2. An additional sequence at the 3' end was also determined. Here, the primer used was R1525-XP, which had the sequence TTCTGCAGTCTAGAAGGAGGTG-(T/A)TCCAGCC. The sequence was read manually and analysis was done at the RDP (Ribosomal Database Project)

(Maidak *et al.*, 1996). The analysis gave a partial 16S rRNA sequence (250 bases) of isolate CF1B^T (GenBank accession no. AF001901).

The alignment, similarity and distance estimations were done using CLUSTAL v (Higgins *et al.*, 1992). The *S_{ab}* values were obtained by similarity check program at RDP. To determine the correct extent of sequence similarity between strain CF1B^T and other closely related organisms, all available sequences of species of the genera *Selenomonas* and *Pectinatus* were retrieved from the database. The sequence of strain CF1B^T was then aligned with these sequences using CLUSTAL v. These aligned sequences were then used to determine the similarity values and distance. The phylogenetic tree was constructed using distance values.

Lipolytic activity. Agar plates of OA-1 medium were used for qualitative plate clearance assays. Tributyrin, triolein and refined groundnut oil were used individually to check the lipase activity. The substrate stocks were prepared as described above. The substrates were sterilized separately and added to the molten agar medium. The culture grown in OA-1 medium containing 1% (w/v) groundnut oil was spot-inoculated on the plates. The clearance zone from the centre of the colony was measured after incubation for 5 d. Results were confirmed by giving successive transfers on the same substrate.

Analytical techniques. Volatile fatty acids in the broth cultures were analysed by GC (8510; Chemito) equipped with a flame ionization detector (oven temperature 150 °C, injector temperature 170 °C, detector temperature 190 °C). The column used was Chromosorb W(HP) (1.83 m × 3.2 mm, SS) packed with 10% FFAP and 2% H₃PO₄. The carrier gas used was N₂ at the flow rate of 30 ml min⁻¹.

Lactic acid was estimated after esterification of the sample using the same GC with a flame ionization detector (oven temperature 110 °C, injector temperature 150 °C, detector temperature 190 °C). The esters were prepared by adding 1 ml of the sample to 2 ml 15% (v/v) H₂SO₄ in methanol. The reaction mixture was kept in a boiling water bath for 3 h. Esters were extracted by adding 1 ml chloroform. The chloroform layer was used for GC analysis. A fused silica capillary column, BP-1 (25 m × 0.32 mm), was used. The carrier gas used was H₂ at the split ratio of 30:1.

H₂S was detected using a GC (3800; Chemito) equipped with a thermal conductivity detector (oven temperature 75 °C, injector temperature 90 °C, detector temperature 90 °C). The column used was Porapak Q (1.83 m × 3.2 mm, SS). The carrier gas used was H₂ at the flow rate of 25 ml min⁻¹.

In all the GC analyses, data analysis was done using an integrator (SP 4270; Spectra Physics). All experiments were done in duplicate.

RESULTS

Isolation

One pure culture of an obligately anaerobic bacterium showing a degradation zone on tributyrin, triolein and groundnut oil was obtained from the enrichment bottle, which was set up using the sample of semi-solid sediment from an anaerobic lagoon and OA-1 medium containing 1% (w/v) groundnut oil. The culture was designated CF1B^T and characterized further. The

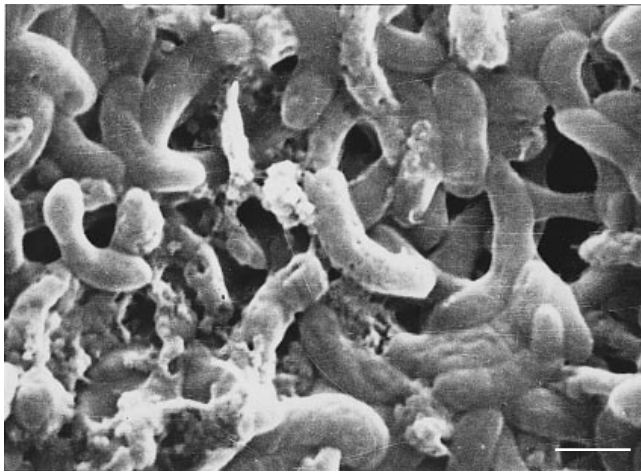


Fig. 1. Scanning electron micrograph of strain CF1B^T. Cells from an exponentially growing culture were fixed using 2% (w/v) glutaraldehyde solution for 12 h. The cells were then dehydrated by gradual washing with an increasing grade of ethanol by a standard method. The sample was allowed to air dry. The gold coating (20 nm thick) of the sample was done using an E 5200 Auto Sputter Coater (Bio-Rad). The observations were made on a Stereoscan S 120 scanning electron microscope (Cambridge Instruments). Bar, 1.16 μ m.

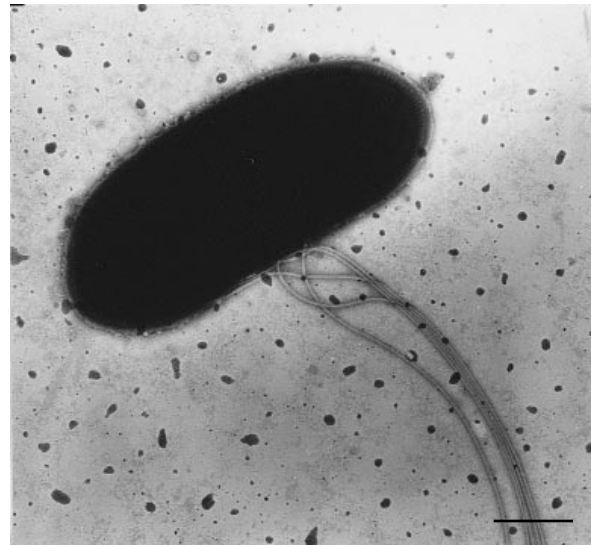


Fig. 2. Transmission electron micrograph of strain CF1B^T. Cells were harvested from an actively growing culture and fixed in 1% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2 for 10 min at 4 °C. The cells were then washed with one change of the cacodylate buffer and negatively stained with 1% (w/v) potassium phosphotungstic acid solution by direct on-grid staining. Electron microscopy was done using a Philips EM 301 transmission electron microscope. The samples were seen under 80 kV operating voltage in a standard fixed high-resolution stage and photographed on Kodak 4489 thick-base EM film (Eastman Kodak). Bar, 400 nm.

colonies of this organism on PYG agar were light brown, spherical, 2 mm in diameter, opaque, convex and had defined borders. The fully grown colonies possessed a more dense and elevated pointed centre. The bacterium showed confluent mat growth on tributyrin agar but not on PYG agar.

Cellular properties

Cells of the strain CF1B^T were typical curved rods measuring 0.55–0.77 by 3.3–4.5 μ m in size and occurred singly (Fig. 1). When the culture was stained with safranin, central large granules were prominently seen under oil emulsion (magnification \times 1000). The strain was Gram-negative, non-spore-forming and actively motile. The motility was very poor in PYG broth, but was markedly enhanced when the cells were grown in glycerol- and phosphate-containing medium. Transmission electron microscopy revealed the presence of 4–5 flagella, arranged linearly as a tuft near the centre of the concave side (Fig. 2).

Growth and metabolic properties

The strain could not grow on the PYG agar plates as well as in the PYG broth under aerobic conditions. Similarly, it failed to grow if the reducing agent, namely cysteine.HCl, was removed from the medium. These results confirmed that the strain was obligately anaerobic. It was catalase-negative and indole-negative. Strain CF1B^T could not hydrolyse gelatin, but showed proteolytic activity on milk agar. Acetate and propionate were the only volatile fatty acids produced from glucose fermentation with production of carbon

dioxide and propionate as the major end product. Strain CF1B^T did not require *n*-valerate when grown on glucose, which was evident from the growth curves plotted using the medium with and without *n*-valerate supplementation (data not shown). It produced almost equal amounts of volatile fatty acid in the presence and absence of *n*-valerate and also, the added *n*-valerate remained unutilized in the test. The optimum temperature for growth was 40 °C and growth occurred at temperatures of 30–47 °C (Fig. 3). The culture showed growth over a pH range of 5.0–7.4 with maximum growth at pH 6.8 (Fig. 3). The strain could tolerate a NaCl concentration of up to 40 g l⁻¹, but optimum growth of the strain occurred in the absence of NaCl in the PYG medium. This was evident from maximum volatile fatty acid production in the absence of NaCl in the PYG broth. The absence of both growth and volatile fatty acid production clearly indicated that the strain could not tolerate NaCl concentrations greater than 40 g l⁻¹.

Fermentable substrates included cellobiose, sucrose, galactose, lactose, glucose, mannose, arabinose, maltose, inositol, inulin, salicin, starch, aesculin, raffinose, melibiose, melizitose and glycerol (Table 1). The following compounds were not utilized as sole carbon and energy source: mannitol, xylose, sorbose, rhamnose, dulcitol, sorbitol or DL-lactate. The utilization of lactate was checked by estimating the concentration of DL-lactate from the culture broth using GC. It was

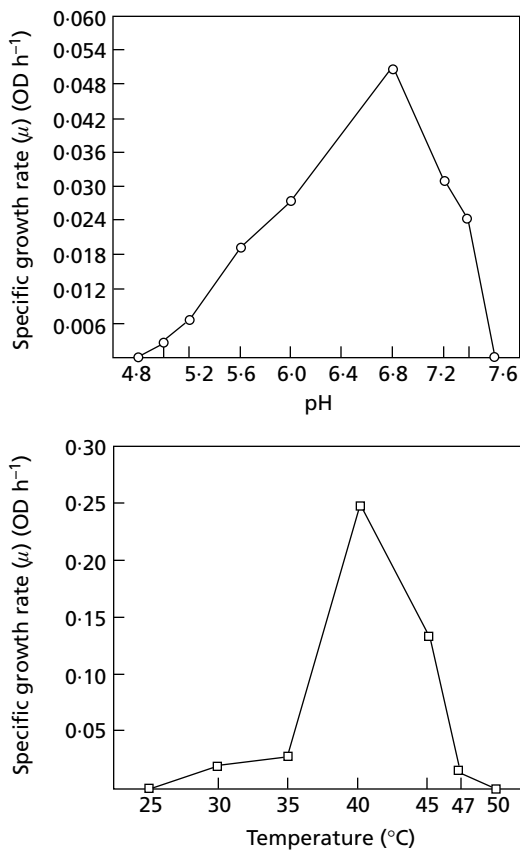


Fig. 3. Effect of initial pH and temperature on growth.

clearly seen that the lactate remained totally unutilized in the inoculated vials.

Strain CF1B^T could utilize NH₄Cl, KNO₃, L-cysteine, histidine, serine and aspartate as sole nitrogen source. It produced H₂S from L-cysteine.

Antibiotic sensitivity pattern

The strain was resistant to gentamicin, amikacin, neomycin, tobramycin, kanamycin, streptomycin, erythromycin, sisomycin, trimethoprim, co-trimoxazole, sulphaphenazole, cloxacillin, norfloxacin, novobiocin and rifampicin. The strain showed an intermediate response to cephotaxime, ofloxacin, ciprofloxacin and nalidixic acid. It was sensitive to chloramphenicol, tetracycline, chlortetracycline, metronidazole, nitrofurantoin, amoxycillin, ampicillin, carbenicillin, cephaloridine, penicillin, cephalosin and cephalixin. Thus, the strain showed a considerable resistance to antibacterial compounds that affecting protein synthesis, but it was sensitive to most of the antibiotics that affect cell-wall synthesis.

DNA base composition

The mean DNA G + C composition, as determined by T_m analysis, was 40 mol%. The strain did not contain any plasmid.

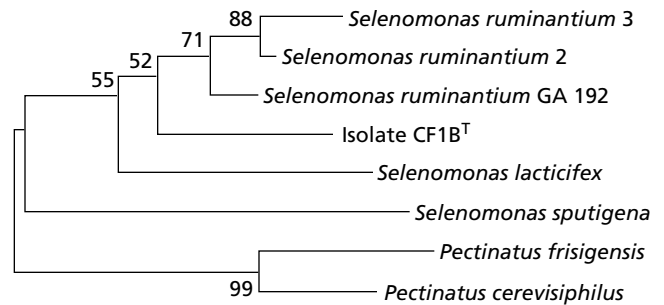


Fig. 4. Unrooted phylogenetic tree derived from the distance values (data not shown). The neighbour-joining method was used for generation of the tree. The numbers at the branches indicate the bootstrap values (percentages) after 1000 replications.

16S rDNA sequence analysis

The partial 16S rDNA sequence of strain CF1B^T (250 bp) was compared with those available in the RDP. The estimated S_{ab} values for the partial 16S rDNA sequence of the isolate CF1B^T with different species of the genera *Selenomonas* and *Pectinatus* were as follows: *Selenomonas ruminantium* strain GA 192, 0.674; *S. ruminantium* strain HD4, 0.661; *S. ruminantium* subsp. *lactilytica*, 0.552; *Selenomonas lactificifex*, 0.448; *Selenomonas sputigena*, 0.310; *Pectinatus cerevisiphilus*, 0.305; and *Pectinatus frisingensis*, 0.259. The results clearly indicated that isolate CF1B^T was most closely related to *S. ruminantium* strain GA 192 with a maximum S_{ab} value of 0.674. Furthermore, the isolate showed maximum similarity of 91% with *S. ruminantium* strain GA 192, followed by 89% with other strains of *S. ruminantium*. Different strains of *S. ruminantium* had the similarity of minimum 95%. The similarity values of the strain CF1B^T with other species of *Selenomonas* were 79–90% and those with the species of the genus *Pectinatus* were below 80%. Even when the analysis included all the organisms for which sequences are available at RDP, the isolate showed highest similarity with only *S. ruminantium*.

The phylogenetic tree constructed using the distance values grouped this isolate with the genus *Selenomonas* but it was distinct from *S. ruminantium* (Fig. 4).

Lipolytic activity

Strain CF1B^T possessed true lipolytic activity. It could hydrolyse tributyrin, triolein and groundnut oil to show prominent clearance zones in qualitative plate clearance assays (Fig. 5). It was observed that tributyrin and triolein were hydrolysed even when emulsified in plain distilled water. Groundnut oil was completely hydrolysed only when it was emulsified in 2% (w/v) polyvinyl alcohol solution in distilled water. The presence or absence of trace element solution did not have any effect on lipolytic activity of the culture when tributyrin and triolein were used as the substrates. The presence of the trace element solution had

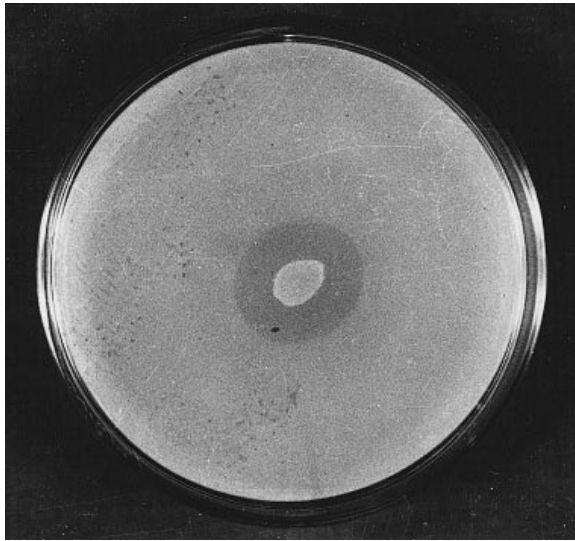


Fig. 5. Strain CF1B^T showing degradation zone on triolein agar plate.

a markedly positive effect when groundnut oil was used as the substrate.

DISCUSSION

On the basis of different cellular and physiological characteristics, strain CF1B^T was classified as a species belonging to the family *Bacteroidaceae*. It shared some characteristics, including the ability to ferment glycerol, with three different genera of the family *Bacteroidaceae*, namely, *Selenomonas*, *Anaerovibrio* and *Pectinatus*. Table 1 shows the comparative characteristics of these organisms with the isolated strain CF1B^T. Among these three genera, only *Anaerovibrio* has been reported to possess lipolytic activity in the species *A. lipolytica* (Hungate, 1966). Other major characteristics of strain CF1B^T, however, were significantly different to those of *Anaerovibrio*, particularly G+C content of the DNA, flagellar arrangement and utilization of different substrates for growth. The strain, therefore, could not be classified as a species of *Anaerovibrio*.

The strain resembled *Pectinatus* with respect to some characteristics like morphology, volatile fatty acid produced after glucose fermentation and G+C content of the DNA. The strain showed a higher optimum temperature for growth than *Pectinatus*. A striking difference was observed in flagellar arrangement. Strain CF1B^T showed a tuft of flagella near the centre of the concave side, which is a peculiar characteristic of the genus *Selenomonas* (Holdeman *et al.*, 1984; Kingsley & Hoeniger, 1973). In the case of *Pectinatus*, the flagella are typically located all over the surface of the concave side and never occur as a tuft (Holdeman *et al.*, 1984; Lee *et al.*, 1978). Moreover, the isolated strain showed true lipolytic activity. There is not a

single strain of genus *Pectinatus* that is reported to possess lipolytic activity (Holdeman *et al.*, 1984; Haikara, 1992). The 16S rDNA sequence analysis showed that strain CF1B^T shared only 77.4% sequence similarity with *P. cerevisiphilus* and 76% sequence similarity with *P. frisigensis*. These findings confirmed that the strain CF1B^T did not belong to the genus *Pectinatus*.

The strain showed striking morphological similarity, including the flagellar arrangement, with the genus *Selenomonas*. Excess glucose in the nutrient medium causes loss of flagella in *Selenomonas* (Kingsley & Hoeniger, 1973). Similar results were observed for the isolated strain. Addition of phosphate and glycerol to the growth medium enhanced the motility of strain CF1B^T. This again is a typical characteristic of the genus *Selenomonas* (Holdeman *et al.*, 1984). The strain also showed greater resemblance to the genus *Selenomonas* with respect to the spectrum of utilization of carbon sources and temperature requirement for growth. Like *Selenomonas*, the strain produced acetate and propionate as the end products of glucose fermentation, with propionate as the major end product. The strain also resembled the genus *Selenomonas* with respect to production of H₂S from L-cysteine.

The 16S rDNA sequence analysis showed that strain CF1B^T was closely related to genus *Selenomonas* with 91% sequence similarity over the 250 bp sequence and an estimated *S_{ab}* value of 0.674 for *S. ruminantium* strain GA 192. The isolated strain was resistant to most of the inhibitors of protein synthesis, including kanamycin (30 µg per disk). The isolate was highly sensitive to antibiotics like ampicillin (10 µg per disk) which affect cell-wall synthesis. *S. ruminantium* strain GA 192 has also been reported to be kanamycin-resistant and ampicillin-sensitive (Ricke *et al.*, 1996). No plasmids were detected in *S. ruminantium* strain GA 192 (Ricke *et al.*, 1996). Similarly, strain CF1B^T did not contain any plasmids.

Strain CF1B^T did not require n-valerate supplementation for glucose utilization. *S. ruminantium*, with which the strain showed highest similarity, requires n-valerate for glucose utilization. The strain could utilize glycerol like *S. ruminantium* subsp. *lactilytica*. Increased utilization of lactate in presence of *p*-aminobenzoate and DL-aspartate is an important character of *S. ruminantium* subsp. *lactilytica*. Strain CF1B^T could not utilize lactate even when the medium was supplemented with *p*-aminobenzoate and DL-aspartate. No species of the genus *Selenomonas* has been reported to possess lipolytic activity (Holdeman *et al.*, 1984; Hespell *et al.*, 1992). The strain CF1B^T showed a strong and true lipolytic activity. The G+C content of the DNA of the isolated strain was 40 mol%, whereas the G+C content for the genus *Selenomonas* is reported to be 48–60 mol%.

Thus, the results clearly indicate that strain CF1B^T is most closely related to the genus *Selenomonas*. At the same time, the strain shows some significant differences

from the typical characteristics of the reported species of *Selenomonas*. Therefore, it could not be accommodated in the existing species of genus *Selenomonas*. Based on these findings, it is therefore proposed that a new species of the genus *Selenomonas* should be created to accommodate strain CF1B^T. The name *Selenomonas lipolytica* sp. nov. is proposed for this isolate, with description given below.

Description of *Selenomonas lipolytica* sp. nov.

Selenomonas lipolytica (li.po.ly'ti.ca. Gr. n. *lipos* fat; Gr. adj. *lytikos* dissolving; M.L. adj. *lipolytica* fat-dissolving).

Cells are curved rods, 0.55–0.77 by 3.3–4.5 µm in size and actively motile. Gram-negative, non-spore-forming, obligately anaerobic, catalase-negative, indole-negative. Strain CF1B^T cannot hydrolyse gelatin. The habitat is tropical anaerobic lagoon. The type strain is CF1B^T, which was isolated from a tropical anaerobic lagoon of an edible oil mill near Pune, India. Flagella (4–5) are arranged linearly as a single tuft near the centre of the concave side. Growth occurs at temperatures of 30–47 °C, with optimum growth occurring at 40 °C. The optimum pH is 6.8. The strain could tolerate NaCl concentration up to 40 g l⁻¹ with optimum growth occurring in the absence of NaCl in PYG broth. The organism is sensitive to many antibiotics affecting cell-wall synthesis. It shows considerable resistance to antibacterial compounds affecting protein synthesis. It shows luxurious growth on glucose without any supplementation of n-valerate. Acetate and propionate are the only volatile fatty acids produced after fermentation of glucose, with propionate as the major end product. The strain shows strong and true lipolytic activity. It shows degradation zones on tributyrin, triolein and groundnut oil in qualitative plate clearance assays. The strain utilizes glycerol but not lactate. The G+C content of the DNA is 40 mol%. The strain has maximum similarity with the genus *Selenomonas* with respect to a 16S rDNA sequence with a similarity of 91% and *S*_{ab} value of 0.674 for *S. ruminantium* strain GA 192.

The type strain, CF1B^T, has been deposited as MCMB 505^T, in the MCM culture collection centre (Maharashtra Association for Cultivation of Science Culture Collection of Microorganisms) established at Agharkar Research Institute, Pune, India.

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