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Full Length Research Paper

Isolation and characterization of a protease-producing thermophilic bacterium from an African hot spring

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The aim of this study was to screen for bacterial isolates from a hot spring in Eastern Zimbabwe (conditions in the hot spring: temperature 53 to 54°C and pH 9.3) for organisms of biotechnological interest. Screening of water samples from the hot spring resulted in the isolation of a novel bacterium with interesting protease activity. The bacterium was rod-shaped, Gram negative, motile, non-sporulating with 0.5 to 0.6 µM width and 2.0 to 4.0 µM length. It was oxidase and catalase positive and did not produce acid from glucose. The G+C content of the DNA was 71.7 mol%. A detailed examination of the isolate using conventional biochemical, physiological tests, fatty acid methyl ester analyses and 16S rDNA analysis showed that the strain code named EP1001 is a new genus within the *γ-Proteobacteria* that was distantly related to genera *Stenotrophomonas*, *Xanthomonas, Xylella*, and *Lysobacter*. The strain EP1001 produces an exoprotease enzyme when cultured in standard thermophilic M162 medium. The growth and protease production by the strain was also studied in shake flask cultivations to investigate optimum carbon and nitrogen sources. The results from physiological and biochemical characteristics, as well as fatty acids analysis, the 16S rDNA data and the chemotaxonomic data provided independent support for the novel nature of the isolate EP1001. In general, the results show that EP1001 isolate is a new bacterium in a new genus related to genera *Stenotrophomonas*, *Xanthomonas, Xylella* and *Lysobacter*, and has possibilities for use as a protease-producing bacterium.

Key words: γ-Proteobacteria, moderate thermophile, hot spring.

INTRODUCTION

The potential of thermophilic microorganisms as bio-technological sources of industrially relevant enzymes has stimulated a continued interest in the exploration of microorganisms for extracellular enzymes such as proteases. The enzymes are both active and stable at high temperatures and are hence of great technological potential (Tayyb et al., 2011; Synowiecki, 2010). One of the natural habitats of the thermophilic bacteria is the hot spring where the temperature of the water is between 50 to 100°C. A hot spring is a spring that is produced by the emergence of geothermal-heated groundwater from the earth's crust (Kazue et al., 2006).

The phylum *Proteobacteria* was circumscribed on the basis of phylogenetic analysis of 16S rRNA gene sequences. The phylum contains Gram-negative bacteria in the classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria* (Krieg et al., 2005). The class *Gammaproteobacteria* contains the orders

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Acidithiobacillales, *Aeromonadales*, *Alteromonadales*, *Cardiobacteriales*, *Chromatiales*, *Enterobacteriales*, *Legionellales*, *Methylococcales*, *Oceanospirillales* and *Pasteurellales*. The majority of the bacteria species belonging to these lines of descent are mesophilic; however, some are slightly thermophilic, moderately thermophilic and even extremely thermophilic (Moreira et al., 2000). The genera *Stenotrophomonas* and *Xanthomonas* are phylogenetically placed in the class *Proteobacteria* where they form a deep branch located at the root of the gamma-subclass together with the genus *Xylella* (Finkmann et al., 2000).

Meanwhile, the microflora from African geothermal environments has not been studied. We recently isolated one moderately thermophilic strain from a hot spring in the eastern province of Zimbabwe. The strain is aerobic, motile and produces a protease with interesting properties. Phylogenetic analysis showed that the strain belonged to the γ-*Proteobacteria*, but was unrelated to any of the known genera. On the basis of these results and from the biochemical, physiological and chemotaxonomic characteristics, we proposed that the strain EP1001 represents a new genus and species within the class γ-*Proteobacteria*. In this study, the morphological, physiological and genetic characteristics of this new species in a new genus are described.

MATERIALS AND METHODS

Isolation and growth conditions

Samples of water were collected from a hot spring in Chimanimani District, located in the eastern province of Zimbabwe. The temperature of the hot spring ranged from 53 to 54°C and the pH was approximately 9. The water samples were diluted with sterile distilled water, spread on to 10% Luria-Bertani (LB) agar plates [1.0 g tryptone, 0.5 g yeast extract, 1.0 g NaCl, and 15 g agar (L^{-1}) and incubated at 50°C]. Skim milk medium [10 g of skim milk and 20 g agar (L^{-1})] was used to assess protease production, which was evidenced by the development of a clear halo zone surrounding the bacterial colonies. The novel bacterium strain with protease activity thus recovered was designated EP1001.

Morphological and physiological tests

Strain EP1001 was grown on 10% LB medium for 24 h at 50°C. The cell morphology was observed under a light microscope (Zeiss Axioskop, Jena, Germany) and the gliding or motility was examined on Cytophaga agar. Flagellum staining was performed using Spot Test Flagella Stain (Difco); Gram staining was performed using the Gram stain Set S (Difco. NJ, USA). In addition, catalase, oxidase, citrate utilization and acid formation from glucose were performed using standard microbiological procedures.

Lipoquinones, polar lipids and fatty acid methyl ester analyses

Respiratory lipoquinones and polar lipids were extracted from 100 mg of freeze-dried cells using a two-stage method described by Tindall (1990a, 1990b). Briefly, cell material was stirred in hexane: methanol (1:2, v/v), placed on ice and the respiratory lipoquinones recovered into hexane layer by addition of one volume of cold hexane. The hexane phase was removed, and respiratory lipoquinones extracted a second time from the methanol phase by addition of two volumes of hexane and two volumes of 0.3% aqueous NaCl. The hexane phases were pooled and used for lipoquinone analysis.

Polar lipids were extracted by adjusting the methanol: 0.3% aqueous NaCl phase to give chloroform: methanol: 0.3% aqueous NaCl mixture (1: 2: 08, v/v/v). The extraction solvent was stirred and the cell debris removed by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform: methanol: 0.3% aqueous NaCl mixture to 1:1:0.9 (v/v/v).

16S rDNA sequencing and phylogenetic analysis

Approximately 95% of the 16S rDNA gene sequence of EP1001 strain was determined by direct sequencing of polymerase chain reaction (PCR)-amplified 16S rDNA. Genomic DNA extraction, PCR mediated amplification of the 16S rDNA and purification of the PCR products was carried out as described by Sambrook and Russell (2001). Purified PCR products were sequenced using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Germany) as described in the manufacturer's protocol. Sequence reactions were subjected to electrophoresis using the Applied Biosystems 373A DNA Sequencer. The resulting sequence data from the strain EP1001 was put into the alignment editor Ae2, aligned manually and compared with representative 16S rRNA gene sequences of organisms belonging to the γ-*Proteobacteria* (Maidak et al., 1999). For comparison, 16S rRNA sequences were obtained from the EMBL data base or RD. The results are presented as a similarity matrix and phylogenetic tree. The 16S rRNA gene similarity values were calculated by pair-wise comparison of the sequences within the alignment.

For construction of the phylogenetic dendrogram, operations of the phylogeny inference package (PHYLIP) were used (Felsenstein, 1993). Pair-wise evolutionary distances were computed from percent similarities by the correction of Jukes and Cantor (1969) and based on the evolutionary distance values; the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). The root of the tree was determined by including the 16S rRNA gene sequence of *Acidithiobacillus caldus* into the analysis. Bootstrap estimation was used to confirm the authenticity of the results in replica.

Shake flask cultivations

Optimum growth conditions: Temperature and pH

The pH range of growth was determined by measuring optical densities (wavelength 600 nm) of the culture grown in 10% LB medium whose pH was adjusted to values between 3 and 11 with appropriate biological buffers. To determine growth temperature ranges, cells were incubated in 10% LB medium at temperatures between 25 and 80°C, while growth was determined by measuring the optical density of the culture with respect to time. Growth was considered to have occurred when the observed absorbance value exceeded twice the initial value after 120 h incubation. Uninoculated control media had not shown increased absorbance value after 120 h incubation.

Effects of varying carbon and nitrogen sources in M162 medium on growth and protease production by EP1001 isolate

Effects of different single-carbon-source (glucose, fructose, maltose, galactose, sucrose at 2.5 g/L) and varying yeast extract

Table 1. Distinctive phenotypic properties of the strain EP1001.

Characteristic	Result
Shape of cells	Rods
Width (μm)	0.5 to 0.6
Length (μm)	2.0 to 4.0
Gliding on Cytophaga agar	-
Growth on Cytophaga agar	+
Gram reaction	-
Lysis by 3% KOH	-
Aminopeptidases (Cerny)	-
Spores	-
Oxidase	+
Catalase	+
Colonies on CASO	Slimy
Hydrolysis of casein	+
Gelatin	+
Nitrate reduction	+
Acid formation from glucose	-
Citrate utilization	-
Hemolysis	-
Growth on blood agar	+
Growth at 25, 30, 35, 40, 45, 50, 55°C (Cytophaga agar)	+
Growth on CASO from 35 to 50°C	+
G+C mole %	71.7

concentrations (0.5 to 6.0g/L) in M162 medium on growth and protease production was investigated in shake flask cultivations. The cultivation was done in 500 ml Erlenmeyer flasks with working volume of 200 ml incubated at 45°C. The medium pH was adjusted to 8.0. The experiments were done in duplicates.

Experimental analyses

Biomass determination

Biomass was determined by measuring the absorbance at 600 nm using a UV-120-02 spectrophotometer (Shimadzu). All samples giving an absorbance higher than 0.7 were diluted with distilled water for accurate measurement.

Protease assay

The protease activity was assayed in duplicate with cell-free culture supernatants, using azocasein as the substrate (Kole et al., 1988). Enzymatic hydrolysis of azocasein produces stable dye-labeled peptides and amino acids into the reaction mixture which can be measured easily. Azocasein protease activity was measured by incubating 1 ml of culture supernatant and 1 ml of 0.5% (w/v) azocasein (Sigma) in 0.2 M tris-hydroxymethyl amino methane hydrochloride (Tris-HCl) buffer (pH 7.4) in an incubator (Innova, New Brunswick Scientific) at 75°C for 1 h. The reaction was stopped by adding 2 ml of 10% (w/v) trichloroacetic acid. The test tubes were allowed to stand for 30 min at room temperature. The mixture was thoroughly mixed using a vortex mixer (VF2, Janke and Kunkel IKA-Labortechnik) before being centrifuged at 3 000 rpm for 10 min to remove a yellow precipitate. The absorbance of the supernatant was measured at 440 nm using a Shimadzu UV-120-2 spectrophotometer. The activity of the protease was expressed in arbitrary units, where 1 unit of activity is equivalent to change in optical density of 0.01 nm per minute at 440 nm. The enzyme assays were done in duplicate for each sample.

Analysis of respiratory lipoquinones

Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by thin layer chromatography on silica gel thin layers (Macherey-Nagel Art No. 805 023), using hexane: *tert*-butylmethylether as solvent. Ultraviolet (UV) absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analyzed by high performance liquid chromatography (HPLC). HPLC analysis was carried out on a LDC Analytical (Thermoseparations) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm × 125 mm, 3 μM, RP18). Methanol served as the eluant and respiratory lipoquinones were detected at 269 nm.

Analysis of polar lipids

Polar lipids were separated by two dimensional silica gel thin layer chromatography (Macherey-Nagel Art. No. 818 135), developed in chloroform:methanol:water (65:25:4, v/v/v) for first direction, and chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v), second direction. Total lipid material and specific functional groups were detected using dodeca molybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), Dragendorff (quaternary nitrogen), anisaldehyde-sulphuric acid and α-naphthol-sulphuric acid (glycolipids).

Fatty acid analysis

Fatty acids were analysed as methyl ester derivatives prepared from 20 mg of dry cell material. The methods used allowed for the selective hydrolysis of ester and amide linked fatty acids (Tindall, unpublished). Fatty acid methyl esters were analysed by gas chromatography using a 0.2 μM × 25 μM non-polar capillary column and flame ionization detection. The run conditions were injection and detector port temperature 300°C, inlet pressure 80 KPa, split ratio 50:1, injection volume 1 μL, and temperature program of 130 to 310°C at a rate of 4°C/min.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics

The morphological and biochemical characteristics of the strain EP1001 are shown in Table 1. Cells were non-sporulating, Gram-negative, motile, rod-shaped, 0.5 to 0.6 μM in diameter and 2.0 to 4.0 μM in length, with one polar flagellum (Figure 1). The strain EP1001 was catalase and oxidase positive. The following biochemical characteristics were negative: acid formation from glucose, citrate utilization and hemolysis. The isolate EP1001 had an optimum growth temperature of 45°C and pH 8.0 and thus a moderate thermophile. Furthermore, the morphological and biochemical characteristics results indicated that strain EP1001 is a member of the *γ-Proteobacteria*. Meanwhile, the physiological tests did not

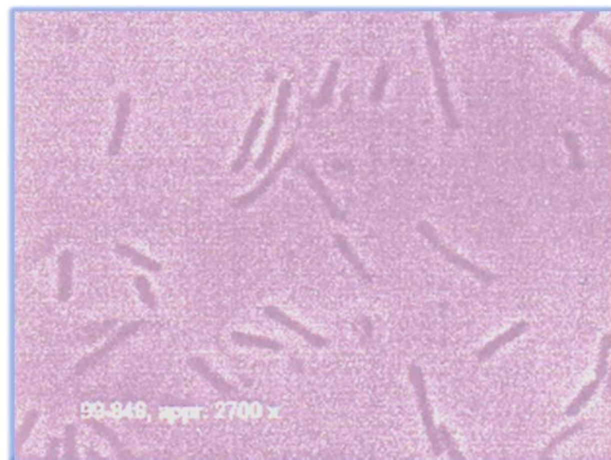

Figure 1. Photograph showing typical cell morphology of strain EP1001. Magnification: Approximately x 2700.

Table 2. Percentage (%) 16S rRNA gene sequence similarity values for strain EP 1001 (ID 00-464, ID 99-846) and related taxa.

S/N	Strain	1	2	3	4	5	6	7	8	9	10	11	12
1	Strain EP 1001 (ID 00-464, ID 99-846)	-											
2	*Lysobacter enzymogenes*	93.0	-										
3	*Lysobacter antibioticus*	93.3	97.0	-									
4	Strain N4-7 (acc.nr. U89956)	91.5	96.9	95.7	-								
5	Strain PVB 47 (acc.nr. U15113)	93.7	98.9	97.3	97.2	-							
6	Strain PVB OTU 1(acc.nr. U15111)	93.7	99.1	97.5	97.4	99.6	-						
7	Strain ES-1 (acc.nr. AF012541)	93.7	99.3	97.7	97.6	99.6	99.8	-					
8	*Stenotrophomonas maltophilia*	91.5	94.2	94.5	92.8	94.5	94.7	95.0	-				
9	*Xanthomonas campestris*	92.0	94.5	96.2	93.2	94.8	95.0	95.2	97.3	-			
10	*Xylella fastidiosa*	91.5	92.2	93.5	90.6	92.8	92.7	92.9	94.2	95.3	-		
11	*Azotobacter vinelandii*	86.4	85.8	86.5	84.4	86.3	86.2	86.3	84.9	85.6	84.4	-	
12	*Acidithiobacillus caldus*	83.4	82.3	83.0	80.7	82.7	82.5	82.7	82.7	83.5	82.7	83.3	-

point any member of a certain genus or species.

Lipoquinones, polar lipids and fatty acid methyl ester analyses

Examination of the respiratory lipoquinone composition of the strain showed that only ubiquinones were present. The major respiratory lipoquinone present was ubiquinone 8 (UQ-8), and this indicated that aerobic isolate EP1001 was probably a member of the ß- or γ-subclass of the Proteobacteria (Moreira et al., 2000). The sum of the chemical data indicated that isolate showed a number of features typical of members of the *γ-Proteobacteria*, in particular members of the genera *Xanthomonas* and *Stenotrophomonas*. However, this strain could not be clearly assigned to any of the species within these two genera, and certain features supported the possibility of placing the new isolate in a new genus.

On the other hand, the polar lipid patterns showed that phosphatidyl glycerol and phosphatidyl ethanolamine were the major phospholipids present. The fatty acid composition was rather complex, although relatively few components could be described as the major fatty acids. Both straight and branched chain fatty acid, together with hydroxyl fatty acids were present; a feature not uncommon in certain groups within the γ-subclass of the Proteobacteria. The characteristic presence of branched chain fatty acid pattern and a ubiquinone with eight isoprenoid units (UQ-8) is restricted to the genera *Stenotrophomonas* and *Xanthomonas* of the class Proteobacteria (Finkmann et al., 2000).

Phylogenetic analysis

The complete 16S rRNA gene sequence data for the isolate EP1001 together with its related strains are shown in Table 2. The complete 16S rRNA gene sequence data indicated that the new strain showed a high degree of

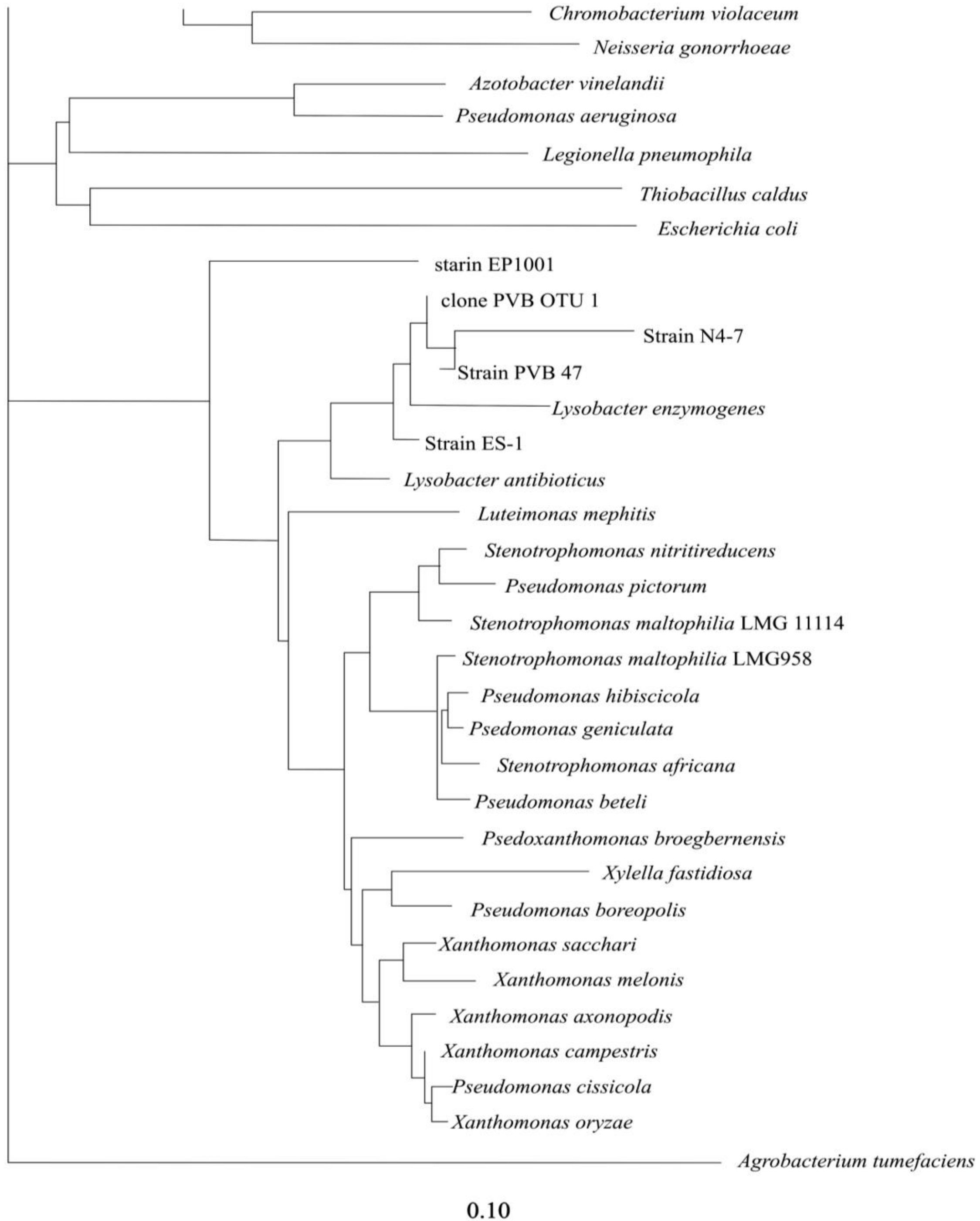

Figure 2. Phylogenetic relationships of strain EP1001 and other closely related species of the *Proteobacteria* based on 16S rDNA sequencing. The tree was generated using the neighbor-joining method and the root was determined by including the 16S rRNA gene sequence of *Acidithiobacillus caldus*.

similarity to three environmental strains, (strain PVB 47, strain PVB OUT 1 and strain ES -1, 93.7%) and to members of the genera *Xanthomonas* and *Stenotrophomonas.* Similarity values between the new isolate and members of these genera were in the range 90 to 92%. The highest similarity to validated species was to *Lysobacter antibioticus*, while the lowest level of similarity was 83.4% to *A. caldus* (93.3%). The phylogenetic tree constructed from the 16S rDNA-based sequence data by neighbor-joining method (Figure 2) showed the detailed

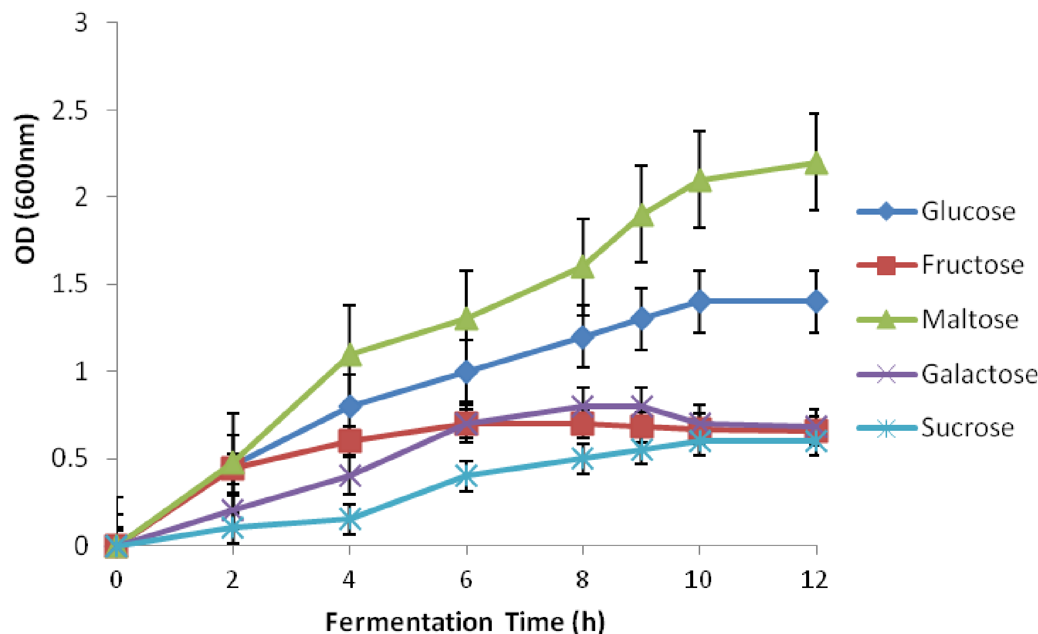

Figure 3. The effect of different carbon sources (2.5 g/L) on biomass formation by strain EP1001 in M162 medium during shake flask cultivations.

evolutionary relationships between strain EP1001 and other related microorganisms.

Although the highest similarity to a validated species was to *L. antibioticus* (93.3%), however, the physiological tests did not point to the genus *Lysobacter* (no gliding). Certain structural features of the 16S rDNA gene also supported the assignment of the strain to this group of microorganisms. The phylogenetic tree also indicated that strain EP1001 represented a distinct lineage within the *γ-Proteobacteria* that is distantly related to the *Xanthomonas* group.

Description of strain EP1001

The isolate forms rod-shaped cells, 0.5 to 0.6 μM in diameter and 2.0 to 4.0 μM in length that is Gram-negative. It does not form spores and is moderately thermophilic. The isolate is strictly aerobic, oxidase- and catalase-positive. It does not form acid from glucose but prefers glucose for protease production to other sugars; yeast extract was required for growth. The G+C content of the DNA was 71.7 mol%.

Growth and protease production during shake flask cultivations

The effect of different carbon sources on growth of strain EP1001 during shake flask cultivations is shown in Figure 3. The isolate was able to grow on most of the carbon sources, with maltose promoting the highest biomass formation. Furthermore, the effect of the different carbon sources on protease production by strain EP1001 is shown in Figure 4. The highest protease production was promoted by glucose followed by fructose. The protease production by the strain in all carbon sources was observed during the exponential phase.

The effects of varying yeast extract concentration on growth of the isolate in M162 medium during shake flask is shown in Figure 5. Tryptone and other components of the M162 medium were kept constant. The results show that the biomass increased with increasing yeast concentration, with the highest biomass recorded in M162 medium containing 6 g/L yeast extract. The effects of varying yeast extract concentration in M162 medium on protease by the strain is shown in Figure 6. Highest protease production (0.9 U/ml) was obtained at 2.0 g/L yeast extract concentration followed by 4 g/L yeast extract. The highest protease production by the isolate at yeast extract concentration of 6 g/L was 0.42 U/ml compared to 0.78 U/ml at 4.0 g/L yeast extract. There was probably repression of protein synthesis at yeast concentration above 2 g/L as evidenced by the decrease in protease production above 4.0 g/L yeast extract concentration.

The source and concentration of carbon and nitrogen in the medium are critical in exoprotease production by bacteria (Moon and Parulekar, 1993). Excess or deficiency of carbon and nitrogen may cause repression of extracellular protease production. Protease productions by isolate EP1001 was repressed at lower yeast extract concentration (0.5 g/L) and at the highest (6 g/L) yeast extract concentration investigated. Yeast extract

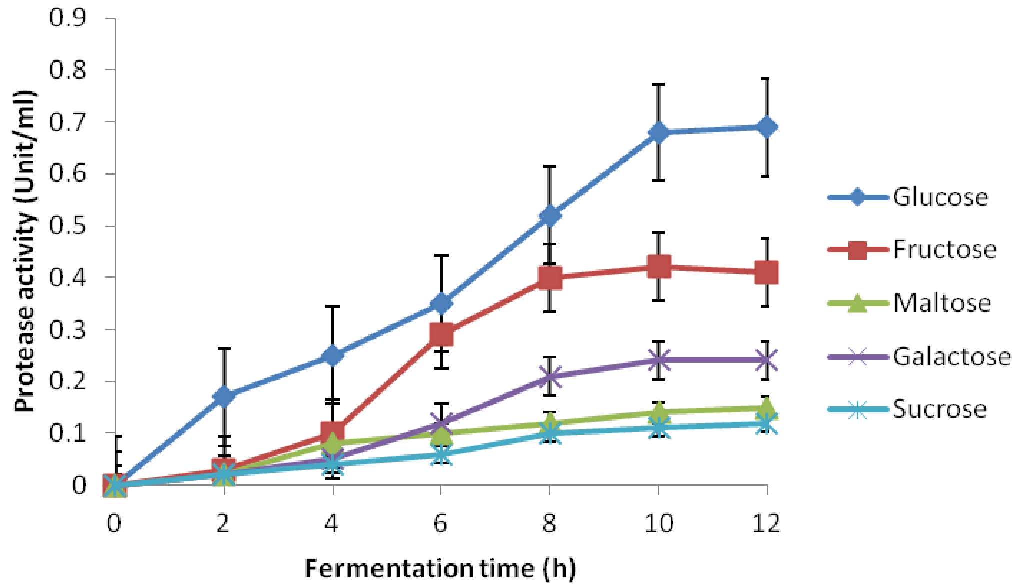

Figure 4. Effect of different carbon sources (2.5 g/L) in M162 medium on protease production by strain EP1001 during shake flask cultivation.

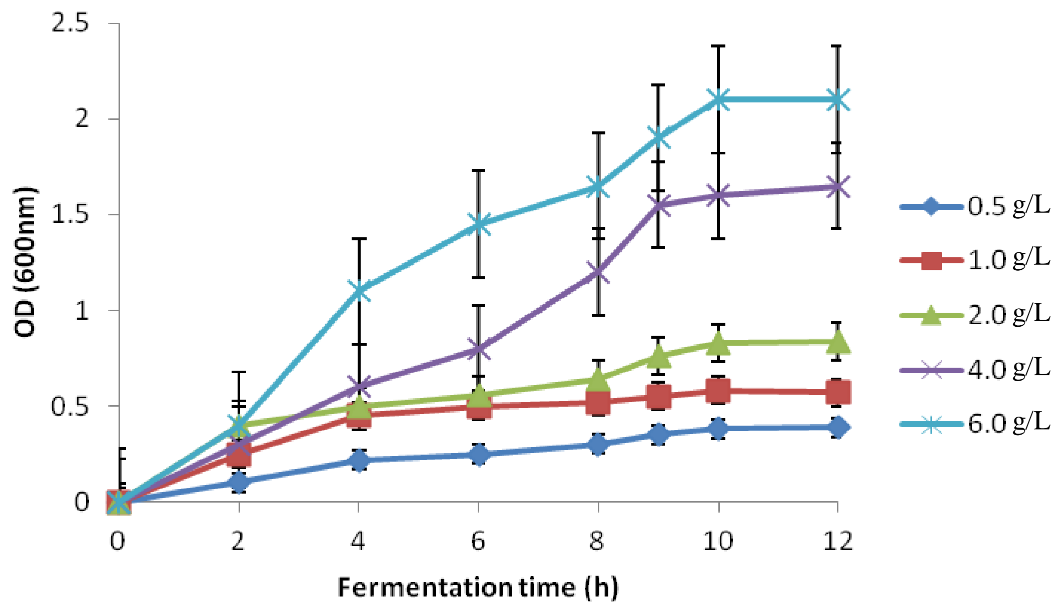

Figure 5. Effect of varying yeast extract only in M162 medium on biomass formation by EP1001 isolate during shake flask cultivation.

is therefore required for protease production by the strain EP1001. The protease production by the strain in all carbon sources was observed during the exponential phase.

Conclusion

Taken together, the physiological, biochemical characteristics, as well as fatty acids analysis, the 16S rDNA data and the chemotaxonomic data provide independent support for the novel nature of the isolate EP1001. Considering that 16S rDNA data alone is not sufficient to delineate taxa, the inclusion of the chemical data and the biochemical data showed that it is possible to recognize the isolate EP1001 as a new species. Furthermore, with reference to certain features of the 16S rDNA sequence, the chemical data and the biochemical

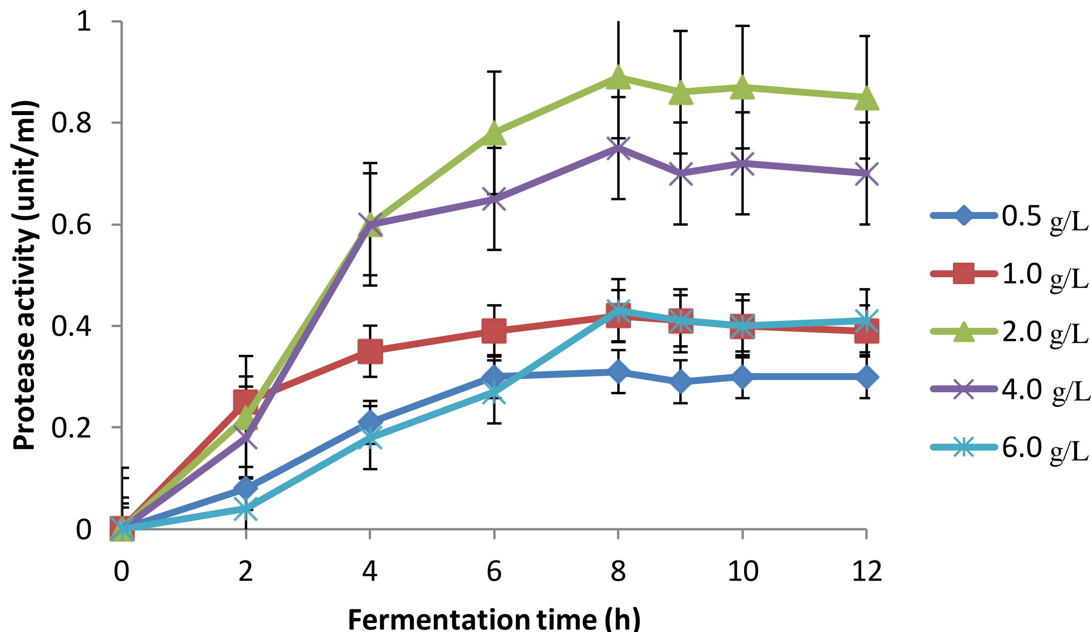

Figure 6. Effect of varying yeast extract in M162 medium on protease production by strain EP1001 during shake flask cultivation.

data, it is possible to delineate a new genus, similar to but distinct from currently recognized members of the genera *Xanthomonas* and *Stenotrophomonas*.

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