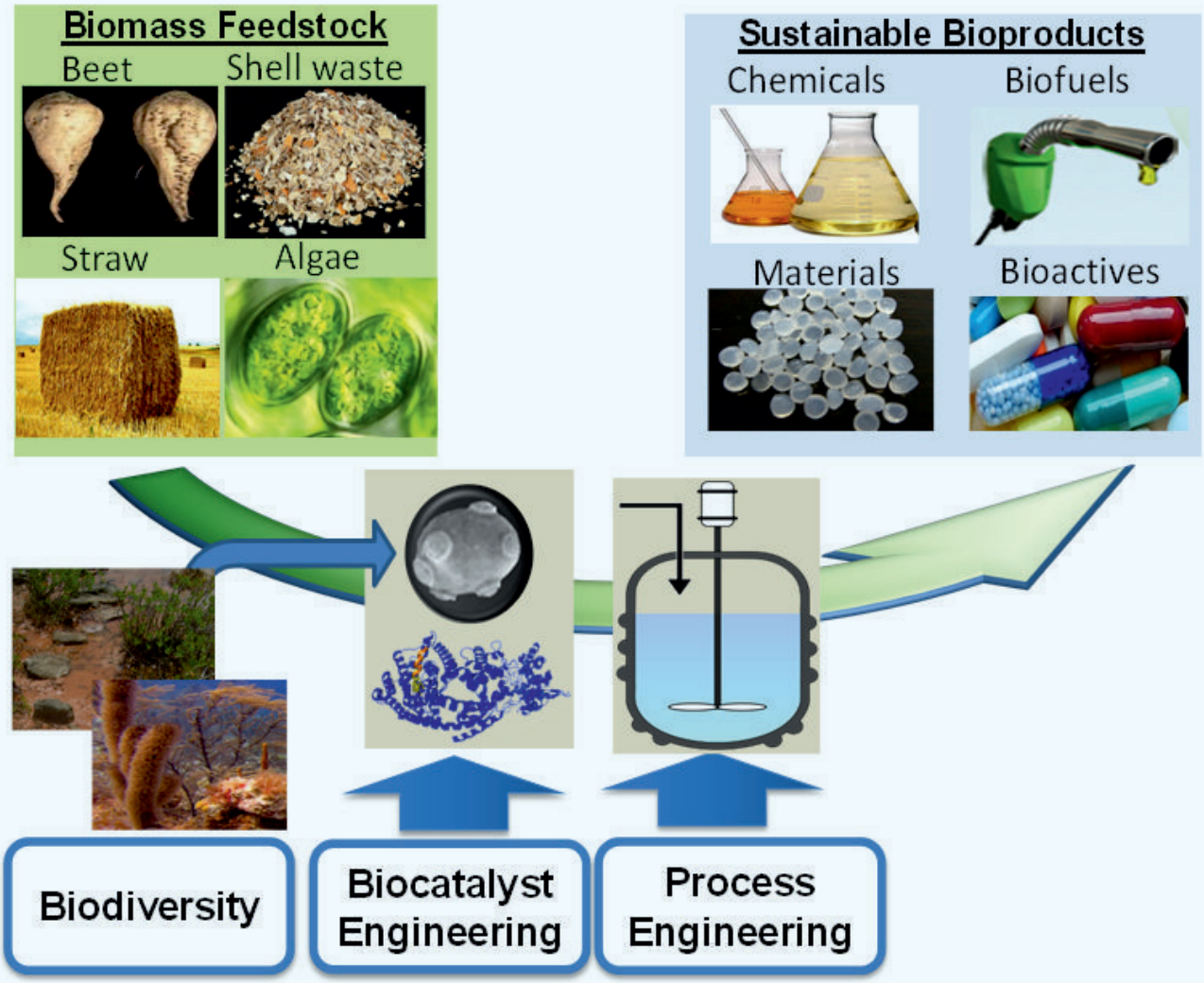
SciMedCentral

JSM

Biotechnology & Biomedical Engineering



Special Issue on

Industrial Biotechnology-Made in Germany: The path from policies to sustainable energy, commodity and specialty products

Edited by:

Dr. Thomas Brück

Professor of Industrial Biocatalysis, Dept. of Chemistry, Technische Universität München (TUM), Germany

Research Article

The Psychrophile *Shewanella arctica* sp. Nov: A New Source of Industrially Important Enzyme Systems

Qoura, F.[1], Brück, T.[2]* and Antranikian, G.[3]*

[1]*Department of Chemistry, Industrial Biocatalysis, Technical University of Munich, Germany*
[2]*Department of Chemistry, Industrial Biocatalysis, Technical University of Munich, Germany*
[3]*Institute of Technical Microbiology, Hamburg University of technology, Germany*

Keywords
Spitsbergen; Psychrophile; *Shewanella arctica*; Industrial enzymes

Abstract

A new psychrophilic, strictly aerobic bacterium, strain 40-3, was isolated from seawater samples collected at Spitsbergen in the Arctic. The cells are gram negative, straight or curved rod shaped and non-spore forming (2-3 µm long and 0.4-0.6 µm wide). Colonies on agar medium are slightly orange, circular, smooth and convex. 40-3 strain grows optimally over the temperature range of 10-15 °C and a pH range of 7-8 in media containing 8 to 9 % NaCl (w/v). Growth occurs with α cyclo-dextrin, dextrin, tween 80, N-acetyl-D-glucosamine, α-D-glucose, maltose, sucrose, methyl pyruvate, D,L-lactitate, succiniate , bromo succinic acid, inosine, esculin ferric citrate, L-arabinose, potassium gluconate, malic acid and trisodium citrate. In the presence of glucose H_2S was produced and nitrates are reduced to nitrites. The fatty acid methyl ester (FAME) are composed of 17.89 % straight chain saturated FAMEs, 14,85 % terminally branched saturated FAMEs and 17.73 % monounsaturated FAMEs. The DNA base ratio is 48 mol % G + C. Phylogenetic analysis reveals as close relationship to *Shewanella putrefaciens* with 99 % 16S rDNA composition identity and 50 % DNA-DNA similarity. The phylogenetic evidence, together with phenotypic characteristics, show that this psychrophilic strain constitute a novel species of the genus *Shewanella*. The name *Shewanella arctica* is proposed. Interestingly, when grown on glucose as a carbon source *Shewanella arctica* produced numerous industrially important enzyme systems including amylase, pullulanase, protease, ornithine decarboxylase, alkaline phospatase, esterase (C4), lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β– glucosaminidase. Due to the functional new physochrophilic *Shewanella* strainresource for the isolation of new enzyme system, which may operate at low reaction thereby increasing the energy efficiency of industrial processes.

***Corresponding authors**
Prof. Dr. Garabed Antranikian, Institute of Technical Microbiology, Hamburg University of technology, Kasernenstr, 12, 21073 Hamburg, Germany, Tel: 4904042878311; Fax: 49040428782582; Email: antranikian@tuhh.de
Prof. Dr. Thomas Brück, Department of Chemistry, Industrial Biocatalysis, Technical University of Munich, Lichtenbergstr, 4, 85748 Garching, Germany, Tel: 4908928913253; Fax: 4908928913255; Email: brueck@tum.de

Submitted: 19 March 2014
Accepted: 12 May 2014
Published: 14 May 2014
ISSN: 2333-7117

Keywords
- Spitsbergen
- Psychrophile
- *Shewanella arctica*
- Industrial enzymes

ABBREVIATION

FAME: Fatty Acid Methyl Ester

INTRODUCTION

Recently, increasing attention has been directed to cold-adapted microorganisms able to grow at/or close to the freezing point of water, namely psychrophiles [1]. Cold adapted microorganisms are found in both permanently and temporarily cold habitats, which comprise more than 80 % of the Earth's biosphere. The genus *Shewanella* MacDonell and Colwell 1985 comprises a ubiquitous group of gram negative, aerobic and facultatively anaerobic δ-*Proteobacteria*. This genus comprises more than 25 species inhabiting a wide range of environments including spoiled food, oil field wastes, redox interfaces in marine and freshwater, cold water and sediments of the deep sea [2-6]. During the last decade, the bacteria of this genus have received a significant amount of attention due to their important roles in co-metabolic bioremediation of halogenated organic pollutants [7], destructive souring of crude petroleum [8] and the dissimilatory reduction of magnesium and iron oxides [9].

Recently, a comprehensive study of the phylogenetic relationships and taxonomy of the genus provided an improved approach for identification of newly isolated wild strains [6]. In this study a novel psychrophilic bacterium was isolated from seawater samples obtained from the area of Spitsbergen, in the Arctic. The novel isolate was found to be similar to, but distinct from in a number of characteristics, a previously described genus, *Shewanella*. Taxonomic and physiological analysis of the newly isolated strain demonstrated that the represent is a novel specie of the genus *Shewanella*, and we propose the name *Shewanella arctica* sp. nov. For the species represented by the strain 40-3. Further, the isolated *Shewanella* strain secreted numerous industrially relevant enzyme systems including hydrolyases such, as amylase, protease and, esterases and lipases. Enzymes derived from physchiphilic organism have the potential to operate at low temperatures thereby improving the energy efficiency of biotechnological process [10-12]. Further, many physochophilic enzymes have shown the propensity to operate over a wider temperature profile than those derived from mesophilic organism [13,14]. More recently, various *Shewanella*

sp. have been flagged as excellent sources for novel industrially relevant enzyme systems [15-17]. Therefore, the enzymes systems that can be produced using the current bacterial strain have potential use in the biofuels and food (amylase, lipase) as well as the chemical industry (pullulanase, alkaline phospatase, leucine arylamidase, valine arylamidase).

MATERIALS AND METHODS

Sample collection, media and culturing conditions

Seawater samples were collected in 1998 during an expedition at Spitsbergen, Norway. Samples were transported to the laboratory at temperature between 2 to 10 °C. A 1 ml volume of liquid sample was incubated in a complex marine liquid medium. The complex marine medium consisted of a basal medium supplemented with a solution of different carbon sources. The basal medium contained (volume g l^{-1}): NaCl 28.13 g; KCl 0.77 g; $CaCl_2$ x2H_2O 0.02 g; $MgSO_4$ x7H_2O 0.5 g; NH_4Cl 1.0 g; iron-ammonium-citrate 0.02 g; yeast extract 0.5 g; 10-fold concentration trace element solution (DSM 141) 1 ml; 10-fold concentration vitamin solution (DSM 141) 1 ml; KH_2PO_4 2.3 g; Na_2HPO_4 x2H_2O 2.9 g. The carbon source mixture solution (volume g l^{-1}): NA-acetate 0.5 g; Na_2-succinate 0.5 g; Na-pyruvate 0.5 g; DL-malate 0.5 g; D-mannitol 0.5 g and glucose 2 g. The final pH of the complex medium was 7. Incubation was carried out at 4 °C for about 4-7 days, before growth and colonies on agar plates were observed. Colonies were selected on the basis of morphological differences. For the isolation of a pure culture, serial dilution and plating techniques were applied. The pure isolates were routinely cultivated on complex marine medium agar plates at 15 °C for 4 days.

Cellular characterization

Gram staining test was performed by staining cells using the Hucker method [18]. For the sporulation test cells were grown for up to 6 days in medium containing no carbon source other then 0.1 % (w/v) yeast extract. The presence of spores and cell morphology were determined by phase-contrast microscope (Zeiss/Axioskop).

Optimal temperature, pH and salt requirement for growth

The optimum growth temperature was tested between 4-37 °C and pH 7. The pH optimum for growth was tested between pH 2-10 at 15 °C. The salt requirement was determined on different NaCl concentration between 0 and 10 % (w/v), with no change of the other salts concentrations at pH 7 and 15 °C. Growth was measured by determining the optical density at 600 nm (1 – cm path length) using Schimadzu UV spectrophotometer.

Substrate spectrum

Substrates utilization from the new strain was tested on api 20 NE strips (20050) (Bio Merieux. Inc), api 20 E strips (20 100/20 160) (Bio Merieux. Inc) and Biolog GN2 micro-plates. api 20 NE strips (20050) (Bio Merieux. Inc) and api 20 E strips (20 100/20 160) were also used for testing the ability of the new strain to produce indol (tryptoPhane), acetoin and H_2S (from sodium thiosalfate) and the ability of the strain to reduce nitrates to nitrites and nitrites to nitrogen. The strips and micro-plates were inoculated with the new strain cells suspension (cells were resuspended in NaCl 0.85 % (w/v) medium to a final OD of 0.5 nm) and incubated at 15 °C overnight.

A large number of carbon source were tested: α cyclo-dextrin, dextrin, Tween 80, Tween 40, N-acetyl-D-glucosamine, α-D-glucose, maltose, sucrose, methylpyruvate, D,L-lactic acid, succinic acid, bromo succinic acid, inosine, esculin ferric citrate, L-arabinose, potassium gluconate, malic acid, trisodium citrate, glycogen, N-acetyl-D-galacto-samine, adonitol, capric acid, D-arabitol, cellobiose, L-erythol, D-fructose, L-fructose, D-galactose, gentiobiose, m-inositol, α-D-lactose, lactulose, D-mannitol, D-mannose, D-mellobiose, β-methyl-D-glucose, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, turanose, Xylitol, mono-methyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxy-butyric acid, β-hydroxy-butyric acid, γ-hydroxy-butyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, seabacic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenyl-alanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ-amino butyric acid, urocanic acid, uridine, thymidine, phenylthylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L α-glycerol phosphate, glucose 1-phosphate, glucose 6-phosphate, phenylacetic acid.

Screening for enzyme production

The ability of the new strain to produce: alkaline phospatase, arginine dihydrolase, esterase (C4), esterase lipase (C8), lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α chymotrpsin, acid phosphatase, omithine decarboxylase, lysine decarboxylase, urease, tryptophane deaminase, naphthol-AS-BI-phosphohydrolase, α and β – galactosidase, β – glucoronidase, α and β – glucosidase, N-acetyl-β – glucosaminidase, α – mannosidase and α – fucosidase was tested on api zym (25 200) strips (bio Merieux, Inc) by inoculation the strips wells with cell suspension of the new strain (cells were resuspended in water to a final OD of 5 nm). The strips were incubated at 15 °C for 6 hours.

Amylase, arabinase, arabinoxylanase, protease, HE-cellulase, glucanase, dextranase, galactanase, galactomannanase, β-glucanase, pullulanase, curdlanase xylanase and xyloglucanase production from the new strain was tested on diffusion agar plates containing the base medium and 0.1 % (w/v) of one of the following substrates: red pullulan (pullulanase), azo-casien (protease) , AZCL – pullulan (pullulanase), AZCL – HE – cellulose (HE – celulase), AZCL – arabinan (arabinase), AZCL – arabinoxylan (arabinoxylanase), AZCL – curdlan (curdlanase), AZCL – amylose (amylase), AZCL – dextran (dextranase), AZCL – galactan (galactanase), AZCL – galactomannan (galactomannanase), AZCL – β - glucan (β – glucanase) AZCL – xylan (xylanase) and AZCL – xyloglucan (xyloglucanase). Substrate degradation was detected by clearing zone/color diffusion halo around the colonies after

the new stain was grown on these substrates agar plates at 10 °C for 2-4 days. AZCL – polymers were purchased from Megazume, Bray, Ireland.

Fatty acids analysis

Cells of the new strain were harvested from a 2 l culture sample by centrifugation and were used for fatty acids analysis. Fatty acids methyl ester (FAME) was performed according to the modified method of Lepage and Roy (1984) [19]. Total lipids were extracted according to Bligh and Dyer (1959) [20]. The FAMEs were analyzed by capillary gas chrompack [21]. A fused silica capillary column D23, 40 m (Fisons) was used for the separation of fatty acid species.The chromatographic conditions were as follows: injector (PTV): 65 °C – 270 °C split ratio 15:1; carrier gas: helium at a 40 cm s^{-1} flow. Column oven: initial temperature : 60 °C for 0.1 min; from 60 °C to 180 °C at 40 °C min^{-1}; 180 °C for 2 min; from 180 °C to 210 °C for 3min; from 210 °C to 240 °C at 3 °C min^{-1}; 240 °C for 10 min. The spectra were recorded by a flame ionization detector at 280 °C.

16S rDNA amplification and sequencing

Cells of the new strain were harvested from 500 µl a cell culture sample by centrifugation and resuspended in 100 µl of water. A sub-sample of 1 µl was used as template for the amplification of the 16S rDNA. PCR-mediated amplification of the 16S rDNA was carried out according to Stakebrandt & Gloebel (1994) [22]. PCR products were purified using the QIA quick PCR purification kit (Qiagen). Purified PCR products were directly sequenced using the Tag Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems). Sequence reactions were electrophoresed using Applied Bio systems model 373S DNA sequence. Both strands of amplification product were sequenced using primers 8F, 518F and 1504R [23]. The complete 16S rDNA sequence of the new strain was discovered by the assembly of all sequence products using Pregap4 version 1.4bl and Gap4 version 4.8bl software.

Phylogenetic analysis

BLAST analysis was performed by NBCI online database on the new strain 16S rDNA sequence to determine the phylogenetic grouping to which the new strain was most closely related. Reference sequences utilized in phylogenetic analysis were retrieved from NCBI database and aligned with the newly determined sequence of the new strain by using CLUSTAL W (1.83) software. The phylogenetic and molecular evolutionary analyses was performed with neighbour joining method by using software from PHYLIP, version 3.57c [24]; the DNADIST program with Kimura-2 factor was used to compute the pairwise evolutionary distances for the above aligned sequences [25], the topology of the phylogenetic tree was evaluated by performing a bootstrap (algorithm version 3.6 b) with 1000 bootstrapped trials. The phylogenetic tree was drawn using Tree View 32 software. As out group the 16S rDNA of *Bacillus subtilis* was used.

The 16S rDNA sequence data was compared with all currently available sequences of organisms belonging to the genus *Shewanella*: *Shewanella putrefaciens* (U91552.1), *Shewanella affinis* (AF500080.1), *Shewanella alga* (U91544.1) *Shewanella denitrificans* (AJ457093.1), *Shewanella amazonensis* (AF005248.1), *Shewanella aquimarina* (AY485225.1), *Shewanella baltica* (AJ000214.1), *Shewanella benthica* (X82131.1) *Shewanella colwelliana* (AY653177.1), *Shewanella decolorationis* (AJ609571.1), *Shewanella fidelia* (AF420313.1), *Shewanella frigidimarina* (AJ300833.1), *Shewanella gaetbuli* (AY190533.1) *Shewanella gelidimarina* (U85907.1), *Shewanella hanedai* (X82132.1), *Shewanella japonica* (AF500079.1), *Shewanella kaireiae* (AB094598.1), *Shewanella livingstonis* (AJ300834.1), *Shewanella marinintestina* (AB081759.1), *Shewanella marisflavi* (AY485224.1), *Shewanella massilia* (AJ006084.1) *Shewanella pacifica* (AY366086.1), *Shewanella pealeana* (AF011335.1), *Shewanella saccharophilus* (AF033028.1), *Shewanella sairae* (AB081762.1), *Shewanella schlegeliana* (AB081761.1), *Shewanella surugaensis* (AB094597.1), *Shewanella violacea* (D21225.1), *Shewanella waksmanii* (AY170366.1), *Shewanella woodyi* (AF003548.1) and *Shewanella olleyana* (AF295592.1).

G + C content of genomic DNA

Cells of the new strain were harvested from a 2 l culture sample by centrifugation and were used for the determination of G + C content of the genomic DNA. The cells were disrupted with a cell French presser and purified by chromatography hydroxyapatit [26]. The mol % G + C of genomic DNA was determined by high performance liquid chromatography (HPLC) (Shimadzu corp, Japan) [27]. The analytical column was a VYDAC 201 SP 54, C18 5 µm (250 x 4.6 mm) equipped with a guard column 201 GD 54H (Vydac, Hesperia, USA).

DNA – DNA hybridization

DNA – DNA hybridization of the new strain with the strain *Shewanella putrefaciens* (DSM 6067 = ATCC 8071) was carried out from 3 g cell mass of each strain. DNA was isolated using a French pressure cell (Thermo spectroic) and was purified by chromatography on hydroxyapatite as described by Cashion. DNA – DNA hybridization was carried out as described by De Ley [28], with the modification described by Huss [29], using a model 2600 spectrophotometer equipped with a model 2527-R thermopropgrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER. BAS program of Jahnke [30].

RESULTS AND DISCUSSION

Physiological and morphological characteristics

Enrichment cultures (pH 7) containing glucose inoculated with seawater sample from Spitsbergen showed bacterial growth after one week of incubation at 4 °C. Microscopy revealed the presence of straight rod cells. After a number of transfers on marine medium agar plates at 15 °C, one culture was shown to exhibit the same characteristics and was selected as the culture for the strain 40-3.

Cells of the strain 40-3 were found to be gram negative. They were straight or curved rod - shaped (0.4-0.6 µm wide and 2-3 µm long). They occurred singly. Spores could never be detected. The strain 40–3 showed high similarity in morphological characterization to the strains belonging to the genus *Shewanella* identified as: straight or curved rod–shaped, 2-3 µm length, 0.4-0.7 wide, gram negative and non–spore forming [5,6,31-45]. The morphological characteristics of the strain 40-3 and the related strains are listed in Table 1.

No growth was observed under anaerobic condition with

Table 1: Comparative characteristics of the isolated strain 40-3 with related *Shewanella* sp. strains. 1. Strain 40-3, **2.** *Shewanella putrefaciens* [date reproduced from Venkateswaran]; **3.** *Shewanella baltica* [date reproduced from Ziemke]; **4.** *Shewanella frigidimarina* [date reproduced from Bowman]; **5.** *Shewanella pacifica* [date reproduced from Ivanova]; **6.** *Shewanella gaetbuli* [date reproduced from Yoon *et al* (2004)]; **7.** *Shewanella waksmanii* [date reproduced from Ivanova]. +, positive; -, negative; ND, not determined.

Character	***1***	***2***	***3***	***4***	***5***	***6***	***7***
Cell shape	Straight rod	Straight rod	Straight rod	Straight rod	Straight rod	Straight rod	Straight rod
Gram stain	-	-	-	-	-	-	-
Spore formation	-	-	-	-	-	-	-
Optimum growth temperature (°C)	10 -15	25 - 33	Growth at 4	20 - 22	20 - 25	30	20 - 22
Optimum pH for growth	7 - 8	7 - 8	ND	ND	ND	7 - 8	7,5
Optimum NaCl conscetration for growth (%, w/v)	8 - 9	0 - 6	ND	0 - 6	0.5 - 6	3 - 6	1 - 6
DNA G + C content (mol %)	48	47	46	40 - 43	40	42	43
Reduction of:							
NO3- to NO2-	+	+	+	+	+	-	ND
NO2- to N	-	-	ND	ND	ND	-	ND
Production of:							
Amylase	+	-	ND	-	+	+	-
Protease	+	ND	ND	ND	+	ND	ND
Pullulanase	+	ND	ND	ND	ND	ND	ND
Lipase	-	-	+	+	+	+	+
H2S from sodium thiosulfate	+	+	+	+	ND	-	+
Indole (tryptophane)	-	-	-	ND	-	ND	ND
Acetion	-	-	-	ND	-	ND	ND
Utilization of:							
α cyclo-dextrin	+	ND	ND	ND	ND	ND	ND
Dextrin	+	ND	ND	ND	+	ND	ND
Tween 80	+	ND	ND	ND	ND	ND	ND
N-acetyl-D-glucosamine	+	ND	ND	ND	+	ND	-
α-D-glucose	+	ND	ND	ND	ND	ND	+
Maltose	+	+	+	-	+	-	ND
Sucrose	+	+	+	-	ND	-	-
Methyl pyruvate	+	ND	ND	ND	ND	ND	ND
D,L-lactic acid	+	ND	ND	ND	ND	ND	ND
Succiniate	+	+	ND	+	+	-	-
Bromo succiniate	+	ND	ND	ND	ND	ND	ND
Inosine	+	ND	ND	ND	ND	ND	ND
Esculin ferric citrate	+	ND	ND	ND	ND	ND	ND
L-arabinose	+	ND	ND	ND	+	ND	ND
Potassium gluconate	+	ND	ND	ND	ND	ND	ND
Malic acid	+	ND	+	ND	ND	ND	ND
Trisodium citrate	+	ND	ND	ND	ND	ND	ND
D-galactose	-	+	ND	-	+	-	-
D-fructose	-	-	ND	-	-	-	-
Fumarate	ND	+	ND	+	-	-	-
Lactose	-	+	ND	ND	-	-	-
Citrate	-	-	+	ND	-	-	ND

glucose. Growth occurred only under aerobic conditions between 4 and 25°C. Optimal growth was optioned at 10-15°C where the growth rate reached its maximum. No growth was observed above 25°C (Data not shown). The strain 40-3 required seawater and grew well at salt (NaCl) concentration of 0–10 % (w/v) with optimum at 8-9 % NaCl (w/v) (Data not shown). The pH range for growth was 6–9, with an optimum at pH 7–8 (Data not shown). Under the optimum conditions using 2 g^{-1}glucose, the growth rate was 0.48 h^{-1}. The optimal growth temperature and pH of the strain 40–3 (10–15 °C and pH 7–8) were similar to the most related *Shewanella* species: *Shewanella putrefaciens* (25–35 °C and pH 7-8), *Shewanella frigidimarina* (20–22 °C), *Shewanella pacifica* (20–25 °C), *Shewanella gaetbuli* (30 °C and pH 7-8) and *Shewanella waksmanii* (20–22 °C and pH 7.5) [6,31,34,36,45]. However, *Shewanella baltica* grew at 4 °C [46]. The strain 40–3 grew in the presence of a wide range of NaCl concentration from 0 to 10 % (w/v) (optimum 8 to 9 % (w/v)) unlike the related *Shewanella* species: *S. putrefaciens, S. frigidimarina*, *S. pacifica*, *S. gaetbuli* and *S. waksmanii,* where no growth was observed above 6 % NaCl (w/v) [6,31,34,36,45]. The growth conditions of the strain 40-3 and the related strains are listed in Table 1

Substrate spectrum

The strain 40-3 grew on a variety of substrates. Good growth was observed on: α-cyclodextrin, dextrin, tween 80, N-acetyl-D-glucosamine, α-D-glucose, maltose, sucrose, methylpyruvate, D,L-lactic acid, succinic acid, bromo succinic acid, inosine, esculin ferric citrate, L-arabinose, potassium gluconate, malic acid or trisodium citrate. No growth was observed on glycogen, tween 40, N-acetyl-D-galacto-samine, adonitol, capric acid, D-arabitol, cellobiose, L-erythol, D-fructose, L-fructose, D-galactose, gentiobiose, m-inositol, α-D-lactose, lactulose, D-mannitol, D-mannose, D-mellobiose, β-methyl D-glucose, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, turanose, xylitol, mono-methyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxy-butyric acid, β-hydroxy-butyric acid, g-hydroxy-butyric acid, β-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, seabacic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenyl-alanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, g-amino butyric acid, urocanic acid, uridine, thymidine, phenylthylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L α-glycerol phosphate, glucose 1-phosphate, glucose 6-phosphate or phenylacetic acid. The strain 40–3 was able to produce H_2S. Indole (Tryptophane) and aceton were not produced. The strain was also able to reduce nitrates to nitrites but not nitrites to nitrogen. *Shewanella putrefaciens* and the strain 40–3 shared the ability to utilize: maltose, sucrose, succiniate, D–galactose, fumarate and lactose [6]. *Shewanella baltica* and the strain 40–3 on the other hand, shared the ability to utilize

Table 2: Comparative fatty acids composition (%) of the new isolated strain 40-3 and related *Shewanella* species. 1. Strain 40-3, **2.** *Shewanella putrefaciens* [date reproduced from Venkateswaran]; **3.** *Shewanella baltica* [date reproduced from Ziemke]; **4.** *Shewanella frigidimarina* [date reproduced from Bowman]; **5.** *Shewanella gaetbuli* [date reproduced from Yoon]; **6.** *Shewanella waksmanii* [date reproduced from Ivanova]. Both the strain 40 – 3 and *Shewanella frigidimarina* produce eicosapentaenoic acid, 20:5ω3. ND, not determined.

Fatty acids	1	2	3	4	5	6
Straight-chain fatty acids						
12:00	3,64	ND	ND	ND	3,1	2
13:00	0,07	ND	ND	ND	ND	ND
14:00	1,8	2,3	2,2	3,7	ND	1,7
15:00	0,31	3,2	7,8	2,5	3,8	5,3
16:00	11,24	19,1	4,3	11.8	8,4	6,2
17:00	0,31	1,5	0,6	1.2	ND	ND
18:00	0.52	2.1	ND	0.1	ND	0.3
Terminally branched saturated fatty acids						
13:0-iso	4.13	2.5	12.4	6.3	9.4	10
14:0-iso	0.18	0.3	1.6	0.6	2	ND
15:0-iso	9.4	21.1	14.3	9	ND	32.5
17:0-iso	1.03	1.7	0.5	0.3	ND	ND
18:0-iso	0.11	ND	ND	ND	ND	ND
Monounsaturated fatty acids						
15:1 ω6c	0.1	0.2	2.2	1.2	ND	ND
16:1 ω5c	0.28	ND	ND	ND	ND	ND
16:1 ω7c		29.6	24.1	51.1	21.1	9.8
16:1 ω9c	2.09	3.5	1.6	2.2	ND	ND
17:1 ω8c	0.89	6.7	11	3	6.4	ND
17:1 ω6c	0.24	0.9	1.4	ND	ND	ND
18:1 ω9c	4.5	3.8	0.3	1.7	ND	ND
18:1 ω7c	9.21	6.6	0.8	5.3	2.5	2
18:1 ω5c	0.35	ND	ND	ND	ND	ND

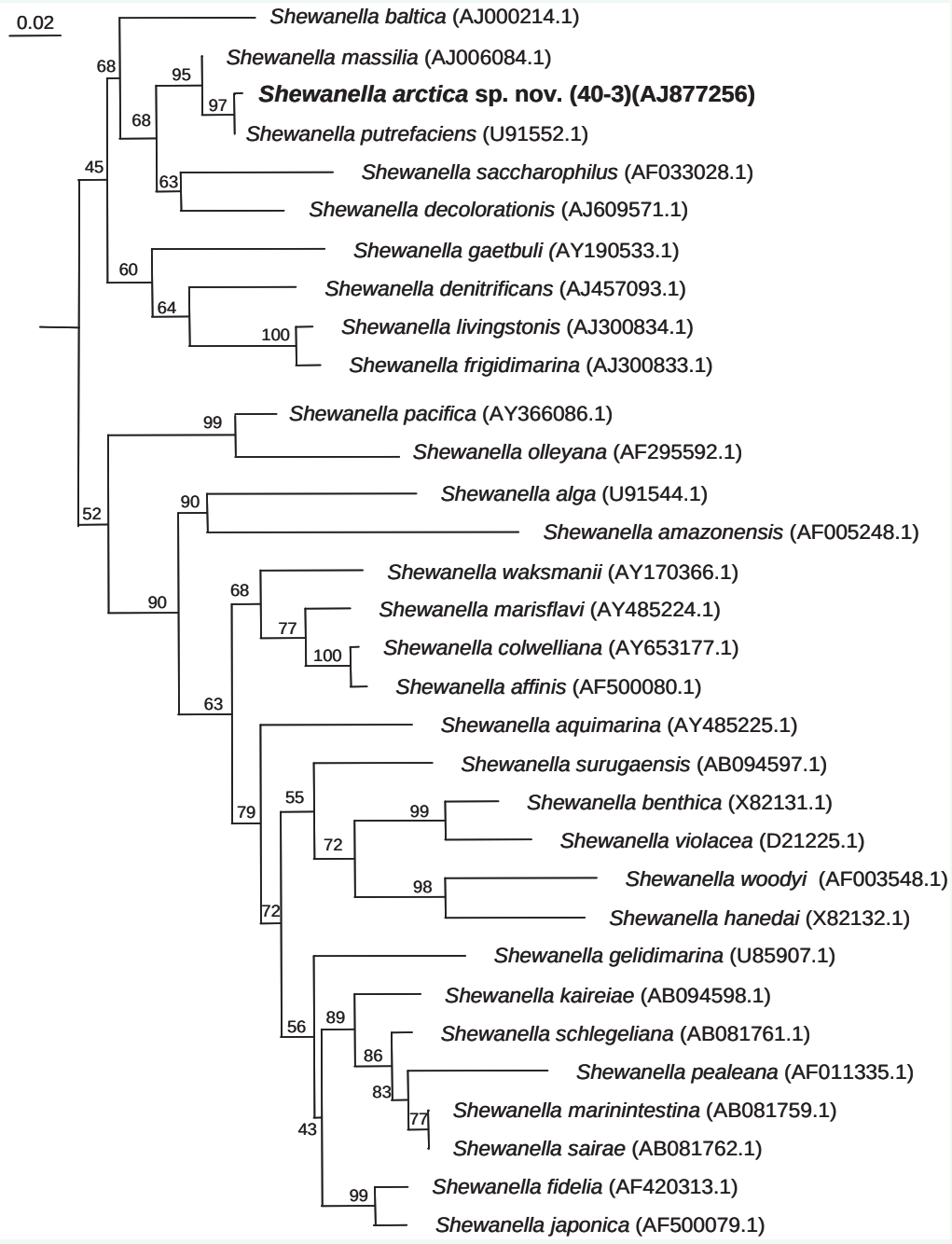


Figure 1 Phylogenetic dendrogram based on 16S rDNA gene sequence comparison indicating the position of the new strain *Shewanella arctica* sp. nov. 40-3 within the genes *Shewanella*. performed by the neighbor-joining method using software from PHYLIP, version 3.57c; the DNADIST program with Kimura-2 factor was used to compute the pair wise evolutionary distances for the above aligned sequences , the topology of the phylogenetic tree was evaluated by performing a bootstrap (algorithm version 3.6 b) with 1000 bootstrapped trials. The tree was draw using Tree View 32 software. Bar correspond to 2 nucleotide substitutions per 100 nucleotides.

maltose, sucrose, malic acid and citrate [46]. The strain 40–3 and the related strains *Shewanella putrefaciens, Shewanella baltica, Shewanella frigidimarina* and *Shewanella waksmanii* were able to produce H_2S (from sodium thiosulfate) and reduce nitrates to nitrites [6,31,36,46]. And finally the strain 40-3 and all previous *Shewanella* species were unable to produce indole or acetoin [6,31,36,45]. The ability of the strain 40–3 and related strains to grow on various substrates is listed in Table1.

Fatty acid analysis

The FAMEs profiles display only those fatty acids comprising ≥ 0.05 % of the total. Straight chain saturated FAMEs were 17.89 % total, terminally branched saturated FAMEs were 14.85 % total and monounsaturated FAMEs were 17.73 % total. 16:0 straight chain saturated FAME was the most abundant FAME found in the strain 40-3 (11.24 %) as well as, in the related

strains: *Shewanella putrefaciens* (19.1 %), *Shewanella baltica* (4.3 %), *Shewanella frigidimarina* (11.8 %), *Shewanella gaetbuli* (8.4 %) and *Shewanella waksmanii* (6.2 %) [6, 31, 36, 45, 46]. 15:0-iso terminally branched saturated FAME was the most abundant FAME found in the strain 40-3 (9.4 %) and in the related strains: *Shewanella putrefaciens* (21.1%), *Shewanella baltica* (14.3 %), *Shewanella frigidimarina* (9 %), and *Shewanella waksmanii* (32.5 %) [6, 31, 36, 45, 46]. However, 16:1 ω7c was the most abundant FAME in all *Shewanella* species mentioned above, but not in the strain 40–3 where, 18:1 ω7c was the most abundant FAME (Table 2).

DNA base composition and DNA - DNA hybridization

The G + C content of the DNA from the strain 40-3 was 48 mol %. High similarity was shown between the G + C content of strain 40-3 (48 mol %) and related strains: *Shewanella putrefaciens* (47 mol %), *Shewanella baltica* (46 mol %), *Shewanella frigidimarina* (40-43 mol %), *Shewanella pacifica* (40 mol %), *Shewanella gaetbuli* (42 mol %) and *Shewanella waksmanii* (42 mol %) [6,31,34-36,45,46]. The 16S rDNA sequence of the strain 40-3 (1.447 kb) was analyzed and compared to all currently available 16S rDNA sequences of organisms belonging to the genus *Shewanella* (Figure 1). The closest relationship was to *Shewanella putrefaciens* (U91552.1) with an identity on 16S rDNA level of 99 %. However, *Shewanella massilia* (AJ006084.1) and *Shewanella baltica* (AJ000214.1), showed 98% identity, *Shewanella decolorationis* (AJ609571.1) and *Shewanella frigidimarina* (AJ300833.1) showed 97 % identity, *Shewanella pacifica* (AY366086.1) and *Shewanella gaetbuli* (AY190533.1) showed 96% identity and *Shewanella marisflavi* (AY485224.1) and *Shewanella waksmanii* (AY170366.1) showed 95 % identity, on the 16S rDNA level.

Phylogenetic treeing placed the strain 40-3 (according to its 16S rDNA composition) among the species of the genus *Shewanella*. The strain 40-3 showed a stable relative branching order with *Shewanella putrefaciens* and was placed in between *Shewanella putrefaciens* and *Shewanella massilia* (Figure 1). DNA-DNA hybridization of the strain 40-3 and the most identical strain *Shewanella putrefaciens* (DSM 6067) showed only 50 % DNA–DNA similarity despite the fact that the 16S rDNA identity between both, the new strain and *Shewanella putrefaciens* was very high (99 %). This strongly indicates that the newly isolated strain (40-3) is a new species within the genus *Shewanella*.

All data mentioned previously demonstrate clearly that the new isolate 40-3 is a new species within the genus *Shewanella*. This fact was also supported by the DNA-DNA hybridization, phylogenetic, G+C content, morphological and fatty acid analysis data. Based on the phylogenetic analysis by 16S rDNA and DNA-DNA hybridization homology, the new isolated strain 40-3 was found to be closely related to *Shewanella putrefaciens*, but represents a new species within the genus *Shewanella*. we propose to assign the newly isolate to the genus *Shewanella* as *Shewanella arctica* sp. nov.

Screening for enzymes production

The strain 40–3 was able to produce several enzymes including amylase, pullulanase, protease, esterase (C4), esterase/lipase (C8), omithine decarboxylase, alkaline phospatase, , leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β–glucosaminidase. No activities for arabinase, arabinoxylanase, HE-cellulase, glucanase, dextranase, galactanase, galactomannanase, β-glucanase, curdlanase xylanase, xyloglucanase, α- and β–galactosidase, β–glucoronidase, α and β–glucosidase, α–mannosidase, α–fucosidase, acid phosphatase, cystine arylamidase, arginine dihydrolase, lysine decarboxylase, urease and tryptophane deaminase were detected. Amylase activities have also been reported for *Shewanella pacifica* and *Shewanella gaetbuli*, while protease activities are reported for *Shewanella pacifica* [34,45] (Table 1). This is the first time that a phsychrophilic *Shewanella* strain is reported to secrete hydrolyases with specific pullulanase (E.C. 3.2.1.XX), esterase (C4, E.C. 3.1.1.1), lipase (C8, E.C.3.1.1.3), phosphohydrolase (E.C. 3.1.3.XX) and arylamidase (E.C. 3.4.14.XX) activities. These specific enzyme activates are of industrial interest for the conversion of biomass to biofuels, food additives and platform chemicals [47-49]. Therefore, the new *Shewanella* strain represents an excellent resource to isolate new enzyme activities with potential industrial relevance. Since enzymes derived from phychrophilic organisms often operate at low temperatures, they can contribute sigficantly to enhynce the energy efficiency of an industrial process. The most prominent example is the use of phychrophilic protease in laundry powder formulation, which enable textile cleaning at temprastures as low as 15°C therby significantly contributing to household energy saving measures [50]. The same features will contribute to energy efficiency in starch based bioethanol production or the lipase catalysed transesterfication of plant oil derived fatty acids [13]. We have recently isolated and characterised the genetic elements encoding *Shewanella arctica* pullulanase and protease activities. Additionally we have purified and characterized the recombinant protein products, expressed in an *E. coli* system. The detailed description of these enzyme systems will be published elsewhere.

Description of *Shewanella arctica* sp. nov. and conclusion

Shewanella arctica (arc' ti.ca. L. fem. adj. *arctica* from the Arctic, referring to the site were the type strain was isolated). Cells are straight or curved rod- shaped, gram negative, 2-3 μm long and 0.4–0.6 μm wide, occur singly, non spore forming and strictly aerobic. Colonies on agar medium are slightly orange, circular, smooth and convex. Temperature range for growth is 4–25 °C, with an optimum between 10 and 15 °C. No growth above 25 °C was detected. Range of pH for growth 6–9, with an optimum at pH 7–8. Growth from 0-10 % NaCl (w/v) concentrations with an optimum between 8 and 9 % (w/v). Growth is observed with α-cyclo-dextrin, dextrin, tween 80, N-acetyl-D-glucosamine, α-D-glucose, maltose, sucrose, methylpyruvate, D,L-lactitate, succiniate , bromo succinic acid, inosine, esculin ferric citrate, L-arabinose, potassium gluconate, malic acid and trisodium citrate. In the presence of glucose, H_2S is produced and nitrates are reduced to nitrites. The fatty acid methyl esters (FAME) are composed of 17.89 % straight chain saturated FAMEs, 14,85 % terminally branched saturated FAMEs and 17.73 % monounsaturated FAMEs. Phylogenetic analysis reveals as close relationship to *Shewanella putrefaciens* with 99 % 16S rDNA composition identity and 50 % DNA-DNA similarty.

The DNA base ratio is 48mol % G + C. Habitat: artic seawater. The strain 40-3 (DSM 16509) is isolated from seawater samples taken from Spitsbergen. The *Shewanella arctica* strain was capable of secreting numerous industrially relevant enzyme systems when grown on glucose as the sole carbon source. The applied enzyme screening procedure could detect amylase, pullulanase, protease, esterase (C4), lipase (C8), ornithine decarboxylase, alkaline phospatase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β–glucosaminidase activities. This is the first time that a *Shewanella* strain is been reported to secrete hydrolases with specific pullulanase, alkaline phospatase, esterase (C4), lipase (C8), arylamidase, phosphohydrolase and glucosaminidase activities. The extreme functional diversity of the secreted enzyme systems provides a rich resource for the isolation industrially relevant biocatalysts, which can potentially optimize the energy efficiency of biotechnological processes producing biofuel, food additives and/or platform chemicals.

REFERENCES

1. Groudieva T, Grote R, Antranikian G. Psychromonas arctica sp. nov., a novel psychrotolerant, biofilm-forming bacterium isolated from Spitzbergen. Int J Syst Evol Microbiol. 2003; 53: 539-545.
2. MacDonell MT, Colwell RR. Pylogeny of the Vibrionaceae, and recommendation for two new genera, Listonella and Shewanella. Syst Appl Microbiol. 1985; 6: 171-182.
3. Garrity GM, Holt JG. The road map to the Manual. In Berger's Manual of Systematic Bacteriology. 2nd edn. Boone DR, Castenholz RW, editors. New York: Springer. 2001; 1: 119-166.
4. Gauthier G, Gauthier M, Christen R. Phylogenetic analysis of the genera Alteromonas, Shewanella, and Moritella using genes coding for small-subunit rRNA sequences and division of the genus Alteromonas into two genera, Alteromonas (emended) and Pseudoalteromonas gen. nov., and proposal of twelve new species combinations. Int J Syst Bacteriol. 1995; 45: 755-761.
5. Satomi M, Oikawa H, Yano Y. Shewanella marinintestina sp. nov., Shewanella schlegeliana sp. nov. and Shewanella sairae sp. nov., novel eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines. Int J Syst Evol Microbiol. 2003; 53: 491-499.
6. Venkateswaran K, Moser DP, Dollhopf ME, Lies DP, Saffarini DA, MacGregor BJ, et al. Polyphasic taxonomy of the genus Shewanella and description of Shewanella oneidensis sp. nov. Int J Syst Bacteriol. 1999; 49 Pt 2: 705-724.
7. Petrovskis EA, Vogel TM, Adriaens P. Effects of electron acceptors and donors on transformation of tetrachloromethane by Shewanella putrefaciens MR-1. FEMS Microbiol Lett. 1994; 121: 357-363.
8. Semple KM, Westlake DWS. Characterization of iron reducing Alteromonas putrefaciens from oil field fluids. Canadian journal of microbiology. 1987; 35: 925-931.
9. Myers CR, Nealson KH. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science. 1988; 240: 1319-1321.
10. Feller G, Gerday C. Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol. 2003; 1: 200-208.
11. Feller G. Psychrophilic enzymes: from folding to function and biotechnology. Scientifica (Cairo). 2013; 2013: 512840.
12. Struvay C, Feller G. Optimization to low temperature activity in psychrophilic enzymes. Int J Mol Sci. 2012; 13: 11643-11665.
13. Cavicchioli R, Charlton T, Ertan H, Mohd Omar S, Siddiqui KS, Williams TJ. Biotechnological uses of enzymes from psychrophiles. Microb Biotechnol. 2011; 4: 449-460.
14. Georlette D, Blaise V, Collins T, D'Amico S, Gratia E, Hoyoux A, et al. Some like it cold: biocatalysis at low temperatures. FEMS Microbiol Rev. 2004; 28: 25-42.
15. Kulakova L, Galkin A, Kurihara T, Yoshimura T, Esaki N. Cold-active serine alkaline protease from the psychrotrophic bacterium Shewanella strain ac10: gene cloning and enzyme purification and characterization. Applied and environmental microbiology. 1999; 65: 611-617.
16. Nichols D, Bowman J, Sanderson K, Nichols CM, Lewis T, McMeekin T, et al. Developments with antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes. Current opinion in biotechnology. 1999; 10: 240-246.
17. Fredrickson JK, Romine MF, Beliaev AS, Auchtung JM, Driscoll ME, Gardner TS, et al. Towards environmental systems biology of Shewanella. Nat Rev Microbiol. 2008; 6: 592-603.
18. Gerhardt P, Murray RGE, Wood WA, Krieg NR. Method of General and Molecular Microbiology. Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. Washington DC: American Society for Microbiology. 1994.
19. Lepage G, Roy CC. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res. 1984; 25: 1391-1396.
20. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37: 911-917.
21. Kohn G, van der Ploeg P, Möbius M, Sawatzki G. Influence of the derivatization procedure on the results of the gaschromatographic fatty acid analysis of human milk and infant formulae. Z Ernahrungswiss. 1996; 35: 226-234.
22. Stackebrandt E, Goebel BM. Taxonomic note: a place of DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol. 1994; 44: 846-849.
23. Buchholz-Cleven BEE, Rattunde B, Staub KL. Screening forgenetic diversity of anaerobic Fe(II)- oxidizing bacteria using DGGE and whole-cell hybridization. Syst Appl Microbiol. 1997; 20: 301-309.
24. Felsenstien J. PHYLIP (Phylogeny Inference Package), version 3.57c: Department of Genetics, University of Washington, Seattle, WA, USA; 1995.
25. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16: 111-120.
26. Cashion P, Holder-Franklin MA, McCully J, Franklin M. A rapid method for the base ratio determination of bacterial DNA. Anal Biochem. 1977; 81: 461-466.
27. Mesbah M, Whitman WB. Measurement of deoxyguanosine/thymidine ratios in complex mixtures by high-performance liquid chromatography for determination of the mole percentage guanine + cytosine of DNA. J Chromatogr. 1989; 479: 297-306.
28. De Ley J, Cattoir H, Reynaerts A. The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem. 1970; 12: 133-142.
29. Huss VA, Festl H, Schleifer KH. Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst Appl Microbiol. 1983; 4: 184-192.
30. Jahnke KD. Basic computer for evaluation of spectoscopic DNA renaturation data from GILFORD System 2600 spectrometer on a PC/XT/AT type personal computer. J Microbiol Methods. 1992; 15: 61-73.

31. Bowman JP, McCammon SA, Nichols DS, Skerratt JH, Rea SM, Nichols PD, et al. Shewanella gelidimarina sp. nov. and Shewanella frigidimarina sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5 omega 3) and grow anaerobically by dissimilatory Fe(III) reduction. Int J Syst Bacteriol. 1997; 47: 1040-1047.

32. Bozal N, Montes MJ, Tudela E, Jiménez F, Guinea J. Shewanella frigidimarina and Shewanella livingstonensis sp. nov. isolated from Antarctic coastal areas. Int J Syst Evol Microbiol. 2002; 52: 195-205.

33. Brettar I, Christen R, Höfle MG. Shewanella denitrificans sp. nov., a vigorously denitrifying bacterium isolated from the oxic-anoxic interface of the Gotland Deep in the central Baltic Sea. Int J Syst Evol Microbiol. 2002; 52: 2211-2217.

34. Ivanova EP, Gorshkova NM, Bowman JP, Lysenko AM, Zhukova NV, Sergeev AF, et al. Shewanella pacifica sp. nov., a polyunsaturated fatty acid-producing bacterium isolated from sea water. Int J Syst Evol Microbiol. 2004; 54: 1083-1087.

35. Ivanova EP, Nedashkovskaya OI, Sawabe T, Zhukova NV, Frolova GM, Nicolau DV, et al. Shewanella affinis sp. nov., isolated from marine invertebrates. Int J Syst Evol Microbiol. 2004; 54: 1089-1093.

36. Ivanova EP, Nedashkovskaya OI, Zhukova NV, Nicolau DV, Christen R, Mikhailov VV. Shewanella waksmanii sp. nov., isolated from a sipuncula (Phascolosoma japonicum). Int J Syst Evol Microbiol. 2003; 53: 1471-1477.

37. Ivanova EP, Sawabe T, Gorshkova NM, Svetashev VI, Mikhailov VV, Nicolau DV, et al. Shewanella japonica sp. nov. Int J Syst Evol Microbiol. 2001; 51: 1027-1033.

38. Ivanova EP, Sawabe T, Hayashi K, Gorshkova NM, Zhukova NV, Nedashkovskaya OI, et al. Shewanella fidelis sp. nov., isolated from sediments and sea water. Int J Syst Evol Microbiol. 2003; 53: 577-582.

39. Leonardo MR, Moser DP, Barbieri E, Brantner CA, MacGregor BJ, Paster BJ, et al. Shewanella pealeana sp. nov., a member of the microbial community associated with the accessory nidamental gland of the squid Loligo pealei. Int J Syst Bacteriol. 1999; 49 Pt 4: 1341-1351.

40. Makemson JC, Fulayfil NR, Landry W, Van Ert LM, Wimpee CF, Widder EA, et al. Shewanella woodyi sp. nov., an exclusively respiratory luminous bacterium isolated from the Alboran Sea. Int J Syst Bacteriol. 1997; 47: 1034-1039.

41. Nogi Y, Kato C, Horikoshi K. Taxonomic studies of deep-sea barophilic Shewanella strains and description of Shewanella violacea sp. nov. Arch Microbiol. 1998; 170: 331-338.

42. Reid GA, Gordon EH. Phylogeny of marine and freshwater Shewanella: reclassification of Shewanella putrefaciens NCIMB 400 as Shewanella frigidimarina. Int J Syst Bacteriol. 1999; 49 Pt 1: 189-191.

43. Skerratt JH, Bowman JP, Nichols PD. Shewanella olleyana sp. nov., a marine species isolated from a temperate estuary which produces high levels of polyunsaturated fatty acids. Int J Syst Evol Microbiol. 2002; 52: 2101-2106.

44. Venkateswaran K, Dollhopf ME, Aller R, Stackebrandt E, Nealson KH. Shewanella amazonensis sp. nov., a novel metal-reducing facultative anaerobe from Amazonian shelf muds. Int J Syst Bacteriol. 1998; 48 Pt 3: 965-972.

45. Yoon JH, Kang KH, Oh TK, Park YH. Shewanella gaetbuli sp. nov., a slight halophile isolated from a tidal flat in Korea. Int J Syst Evol Microbiol. 2004; 54: 487-491.

46. Ziemke F, Höfle MG, Lalucat J, Rosselló-Mora R. Reclassification of Shewanella putrefaciens Owen's genomic group II as Shewanella baltica sp. nov. Int J Syst Bacteriol. 1998; 48 Pt 1: 179-186.

47. Hii SL, Tan JS, Ling TC, Ariff AB. Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res. 2012; 2012: 921362.

48. Kirk O, Borchert TV, Fuglsang CC. Industrial enzyme applications. Curr Opin Biotechnol. 2002; 13: 345-351.

49. Lorenz P, Eck J. Metagenomics and industrial applications. Nat Rev Microbiol. 2005; 3: 510-516.

50. Kuddus M, Ramteke PW. Cold-active extracellular alkaline protease from an alkaliphilic Stenotrophomonas maltophilia: production of enzyme and its industrial applications. Canadian journal of microbiology. 2009; 55: 1294-1301.

Cite this article

Qoura F, Brueck T, Antranikian G (2014) The Psychrophile Shewanella arctica sp. Nov: A New Source of Industrially Important Enzyme Systems. JSM Biotechnol Bioeng 2(1): 1026.