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Oceanobacillus aswanensis Strain FS10 sp. nov., an Extremely Halotolerant Bacterium Isolated from Salted Fish Sauce in Aswan City, Egypt

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Abstract: A halophilic-extremely halotolerant- bacterium, strain FS10, was isolated from a fish sauce sample that was collected from salted fish shop in Aswan City, Egypt. The isolate FS10 was a Gram positive, ferments several sugars and has motile, rod-shaped cells that were not spore formers. The isolate was facultative anaerobic, slightly acidophilic that grows at pH 5.5 to 8.5. The strain grows over a wide range levels of salinity [3 to 27.5 (%, w/v) NaCl]. The strain FS10 showed 98%, 93% and 95% 16S rRNA gene partial sequence similarity to 16S rRNA gene sequence of *Oceanobacillus iheyensis* strain S8-19, *Oceanobacillus oncorhynchi* strain LS22 and *Oceanobacillus oncorhynchi* subsp. *incaldanensis*, respectively. On the basis of 16S rRNA gene partial sequence similarities, the strain FS10 was a member of the genus *Oceanobacillus* and closely related to *Oceanobacillus iheyensis* strain S8-19. The many clear differences in physiological and biochemical characteristics between both strains indicated that strain FS10 should be designed as a new species of *Oceanobacillus* and the name *Oceanobacillus aswanensis* was proposed.

Key words: *Oceanobacillus* · Extremely halotolerant · Fish sauce

INTRODUCTION

Extremophiles are microorganisms that grow under hostile to most organisms [1]. Some of them, such as bacteria which thrive in hypersaline environments have been recognized for their use in biotechnological applications [2-5]. On the other hand, [6] studied an extracellular halophilic ribonuclease from halotolerant *Pseudomonas sp.* No. 3241 that was isolated from fish sauce in Thailand. Moreover, [7] studied the production and characterization of NaCl-activated proteinases from the halotolerant *Virgibacillus sp.* SK33 isolated from fish sauce in Thailand. Thus, extensive studies have been made in recent years into hypersaline environments, resulting in a large number of new halophilic species being isolated [8-11].

Halotolerant bacteria are those that can tolerate a broad range of NaCl concentrations (0-32 %, w/v) [12-15]. According to the definition of [16], there are different categories of halotolerant microbes: non-tolerant, those which tolerate only a small concentrations of salt about 1 (%, w/v); slightly tolerant, tolerating up to 6-8%; moderately tolerant, up to 18-22%; and extremely tolerant, those microbes that grow over the whole range of salt concentrations (0-32 %, w/v). Salt normally means NaCl and distinction between tolerance for salt and requirement for salt should be noted.

Examples for Gram positive halotolerant bacteria are comprised in numerous genera such as *Salinococcus, Halobacillus, Virgibacillus, Gracilibacillus, Oceanobacillus, Thalassobacillus* [17-22]. The family *Halomonadaceae* was designed to comprise the Gram negative genera such as *Halomonas* and *Deleya.* [23- 24] isolated two halotolerant bacteria from the gills of salted fish, the Gram-positive *Staphylococcus saprophyticus* and the Gram-negative *Neisseria elongata.* Also, halotolerant bacteria were placed within other different genera [25-26].

Production of fish sauce involves the addition of salt to uneviscerated fish at a ratio of approximately 1:3 and requires about 1 to 1.5 years to complete fermentation [7].

Our aim has been to look for halophilic bacteria that grow in salted fish and consequently to accelerate the fermentation process and to describe phenotypically and to some extent its 16S rRNA Partial sequence and analysis and to screen its enzymatic activity.

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MATERIALS AND METHODS

Source of the Strain: Salted fish sauce sample that was collected from a salted fish shop in Aswan city, Egypt.

Nutrient Media Used in the Study: The organism was isolated, grown and maintained on proteose peptone-salt medium, which contained (gl^{-1}): NaCl, 250; KCl, 2; $MgSO_4.7H_2O$, 5; proteose peptone, 7.5; and yeast extract, 5. [27]. To prepare agar plates, the medium was solidified with 20 g of agar per liter. Basal medium contained the following (gl^{-1}): NaCl, 225; KCl, 2; $MgSO_4.7H_2O$, 5; yeast extract, 1 and was used for testing growth on different substrates as well as testing some enzymatic and biochemical activities. The pH was adjusted to 7-7.5 with 1 N NaOH or HCl using digital pH meter with glass electrode (Hanna instruments, model HI 8418), then media were sterilized by autoclaving.

Morphological Tests: The colonies of the isolate FS10 were described on proteose peptone salt agar medium. Forms, pigmentation, elevation, margins, opacity, consistency and diameters (in mm.) were determined. Gram staining and motility were determind in 24 - hour cultures. The spore staining was performed following the method described by [28] in 48 - hour cultures.

Physiological Tests : The growth response to NaCl was examined in liquid and solid proteose peptone salt media using different salt concentrations: 0.0, 1, 3, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30 and 32 (%, w/v). The growth response to pH was determined by testing growth at different pH's (4,4.5, 5, 6, 7, 8, 8.5, 9, 9.5 and 10).The growth response to temperature was determined by testing the growth of organisms on proteose peptone salt agar medium at different temperatures (10, 15, 18, 20, 25, 30, 35, 37, 40, 45, 50, 52and 55 °C). Oxygen requirements were determined by stabbing the bacterial isolate in a deep proteose peptone salt agar medium and incubating them at 40 °C for 4 days.

Biochemical Tests: Oxidase activity was performed according to [29]. Catalase activity was shown by adding drops of 3 % hydrogen peroxide to agar colonies; effervescent of oxygen bubbles indicated a positive result. Hydrogen sulfide production, nitrate reduction, indole formation, acid production from the sugars (glucose, fructose, sucrose, maltose, lactose, arabinose, galactose, mannose and mannitol), Voges-Proskauer and Methyl Red reactions (VPMR) were performed according to [28] in the presence of 10 (%, w/v) NaCl.

Lipase activity was tested by streaking the bacterial isolate on the surfaces of the basal salt agar medium supplemented with 1 (%, v/v) of either tributyrin, tween 20, tween 40 or tween 80 following the method described by [30]. The amylolytic activity was determined following the method described by [31] using the basal salt agar medium supplemented with 1 (%, w/v) soluble starch. The proteolytic activity was determined following the method described by [32,33] using the basal salt agar medium supplemented with 1 (%, w/v) gelatin or casein.

Utilization of Different Substrates as Nitrogen and/or Carbon and Energy Sources: Utilization of glucose, sucrose, maltose, starch, casein, gelatin, tryptone, beef extract, casamino acid, sodium citrate, peptone, yeast extract, proteose peptone, DL-Asparagin,glycine, sodium acetate and sodium pyruvate as sole nitrogen and/or carbon and energy sources was performed in the basal salt medium supplemented with 1 % (w/v) from the substrate.

Antibiotic Susceptibility: The susceptibility of the isolate to the antibiotics, chloramphenicol, erythromycin, cotrimoxazole, novobiocin, tetracycline, sulphafurazole, streptomycin, ampicillin, amoxicillin, penicillin G, gentamicin and nitrofurantoin was determind by placing discs impreginated with the antibiotics (20 µg/disc) on the surfaces of agar plates of proteose peptone salt medium previously seeded uniformly with the bacterial isolate. The plates were incubated at 40°C for 72 hours and the diameters of inhibition zones were measured in mm.

16s rRNA Gene Sequencing: Partial 16S rRNA gene sequence of strain FS10 was determined in Mubarak city for scientific research and technological applications, genetic engineering center, Alexanderia, Egypt, following the method of molecular cloning described by [34].

Analysis of the Sequencing Data: The sequencing data were analysed according to the computer program DNA strider. The alignments, homology and comparison studies were done by using the blast programs [20], which are available at the home page of the National Center for Biotechnology Information (NCBI;// www.ncbi.nlm.nih.gov).

RESULTS

Morphology, Physiology and Growth Characteristics: Colonies of strain FS10 after three days incubation on agar plates of proteose peptone salt medium were circular, buff, umbonate, entire, opaque, butyrus and measured

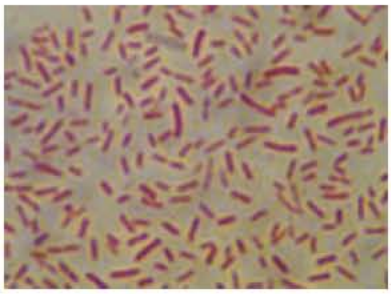

Fig. 1: A microphotograph of nigrosin stain of strain FS10 showing cell shapes (Phase contrast microscope- X3000).

Table 1: The morphological, physiological and growth characteristics for the isolate FS10

Characteristics	Isolate FS10
Temperature for growth (°C):	
Minimum	10
Optimum	35-40
Maximum	52
pH for growth:	
Minimum	5
Optimum	7-7.5
Maximum	8.5
NaCl concentration for growth (%, w/v):	
Minimum	3
Optimum	10-15
Maximum	27.5
Colony morphology Circular, buff, umbonate, entire opaque and butyrus	
Oxygen relationship	FA
Cell forms	Rods
Cell size (μ m):	
Width	1.4-2.1
Length	1.4-3.5
Cell motility	Motile
Gram stain	+ve
Spore formation	-ve

about 1-2 mm. in diameter. The cells of the strain FS10 were rods appeared as single cells and measured 1.4-2.1x1.4-3.6 μm. The cells were motile, Gram positive and non- spore forming (Fig.1).

Physiology and growth characteristics of the isolate FS10 were summarized in Table (1). Growth occured at 10°C until 52°C showing optimum growth at a temperature range of 35-40°C. pH for growth was 5-9 with an optimum at 7-7.5. The isolate FS10 was facultative anaerobic and tolerated NaCl concentration from 3 to 27.5 % (w/v).

Table 2: Biochemical characteristics and enzymatic activities for the isolate FS10

Characteristics	Isolate FS10
Catalase production	+
Oxidase test	+
Reduction of nitrate to nitrite	-
Reduction of nitrate till gas	-
Indole formation	-
H2S production from cysteine	+
Acetylmethylcarbinol	-
Methyl red test	-
Degradation of:	
Starch	-
Gelatin	+/-
Casein	-
Tributyrin	-
Tween 20	-
Tween 40	-
Tween 80	-
Cholesterol	-
Acid and gas form:	
Glucose	Weak acid
Fructose	Weak acid
Sucrose	-
Maltose	-
Lactose	-
Arabinose	Weak acid
Galactose	-
Mannose	Acid
Mannitol	-
Starch	-

Biochemical Characteristics and Enzymatic Activities: Biochemical and enzymatic activities of the strain FS10 are summarized in Table (2). The strain FS10 was catalase and oxidase positive, Hydrogen sulphide was formed from cystein, only gelatin was weakly hydrolyzed and acid was produced only from glucose, fructose, arabinose and mannose.

Utilization of Different Substrates as Nitrogen and/or Carbon and Energy Sources: The ability of strain FS10 to grow on either, glucose, sucrose, maltose, starch, casein, gelatin, tryptone, beef extract, casamino acid, sodium citrate, peptone, yeast extract, proteose peptone, DL-Asparagin, glycine, sodium acetate or sodium pyruvate as sole nitrogen and/or carbon and energy source was tested. The isolate FS10 could grow on each of casein, gelatin, tryptone, beef extract, casamino acid, peptone, yeast extract and proteose peptone.

Antibiotic Susceptibility: Sensitivity against chloramphenicol, erythromycin, cotrimoxazole, novobiocin, tetracycline, sulphafurazole, streptomycin, ampicillin, amoxicillin, penicillin G, gentamicin and nitrofurantoin was tested and the strain was sensitive to nitrofurantoin only.

16s rRNA Gene Sequencing Data: The partial 16S rRNA sequence [321 base-pair -bp-] was determind as follows:
CTGGGCTACACGTGCTACAATGGATGGAAAAAGG
GAAGCGAACCCGCGAGGTCAAGCAAATCCCACAA
AACCATTCTCAGTTCGGATTGTAGGCTGCAACTCG
CCTACATGAAGCCGGAATCGCTAGTAATCGCGGAT
CAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCACGAGAGTTGGTAACAC
CCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCG
CCGAAGGTGGGACTAATGATTGGGGTGAAGTCGTA
ACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCA
CCTCCTT

DISCUSSION

Strain FS10 was isolated from a salted fish sauce sample. Colonies on agar plates of strain FS10 were buff, opaque, entire, umbonate, butyrus, circular and measured about 1-2 mm. in diameter. The cells of the strain FS10 were rods and appeared as single cells. The cells were motile, Gram positive and non- spore forming. The strain could grow in NaCl concentration ranged from 3 up to 27.5% (w/v). Based on the NaCl tolerance, the strain FS10 was classified as an extremely halotolerant bacterium [16-23]. The strain FS10 could grow optimally at pH 7-7.5, temperature 35-40°C and NaCl 10-15% (w/v). The isolate FS10 could produce acid from glucose, fructose, arabinose and mannose but not from sucrose, maltose, lactose, galactose or mannitol. The strain was catalase and oxidase positive, hydrogen sulphide was formed from cystein, neither indole nor acetylmethylcarbinol was formed, gelatin was hydrolyzed but not starch, casein, tributyrin, tween 20, tween 40, tween 80 or cholesterol.

The isolate FS10 could grow on each of casein, gelatin, tryptone, beef extract, casamino acid, peptone, yeast extract and proteose peptone but not on non-nitrogenous organic compounds such as starch and glucose. This may be because of the protein nature of its habitat [fish sauce].

The partial sequence of 16S rRNA gene of the isolate FS10 was determined, (321 bp) and was compared using the ncbi data-base, blast search http://www.ncbi.nlm.nih.gov. The isolate showed the highest degree of similarity [98%] with the 16S rRNA gene sequence of the type strain of *Oceanobacillus iheyensis* strain S8-19 [7] and the bases from 1-292 were corresponding to the bases from 1220 to 1528. Also it showed similarities with the other members of the genus *Oceanobacillus*, 93% and 95% with *Oceanobacillus oncorhynchi* strain LS22 and *Oceanobacillus oncorhynchi* subsp. *incaldanensis* [35, 36] corresponding to the bases from 1231 to 1420 and from 1220 to 1509 respectively. This indicates that strain FS10 was a member in the genus *Oceanobacillus* and closely related to the species *Oceanobacillus iheyensis* strain S8-19. The isolate was also far from the other species of the genus *Oceanobacillus* [35-41].

The isolation locality of both strains was completely differ, consequently, this will affect the behaviour of both strains hence the surroundings form, to large extent, the characteristics of the individuals. *Oceanobacillus iheyensis* was isolated from deep-sea sediment collected at a depth of 1050 m [7] while the isolate FS10 was isolated from salted fish sauce sample. There are many clear differences between both strains. The most distinct feature was that *Oceanobacillus iheyensis* form endospores where FS10 strain was not. In contrast to FS10 strain, cells of *Oceanobacillus iheyensis* measure 0.6-0.8×2.5-3.5 μm. grow at 0-21% (w/v) NaCl, with optimum growth at 3% NaCl. Growth occurs at temperatures of 15-42°C (optimum 30°C). The pH range for growth was 6.5-10. Also *Oceanobacillus iheyensis could* hydrolyze casein, Tween 40 and Tween 60 while the strain FS10 couldn't. *Oceanobacillus iheyensis* was susceptible to gentamycin, tetracycline, chloramphenicol, novobiocin, penicillin G, while FS10 was not.

Based on the above mentioned significant differences between strain FS10 and *Oceanobacillus iheyensis* it was proposed that strain FS10 represents a new species within the genus *Oceanobacillus* and was given the name *Oceanobacillus aswanensis* strain FS10.

[27] identified *Natrialba aegyptiaca* as a new species although its 16S rRNA gene sequence showed 99.2% similarity with that of *Natrialba* asiatica B1T and [42] identified *Halopiger aswanensis* as a new species although the similarity of its 16S rRNA gene sequence to that of the type strain of *Halopiger xanaduensis* was 99%. This was determined based on distinct physiological and biochemical characteristics in addition to DNA-DNA hybridization relatedness.

Description of *Oceanobacillus aswanensis* strain FS10 sp. nov.: *Oceanobacillus aswanensis* sp. nov. (as.wan.en`sis. M.L. gen. n. *aswanensis was* isolated from

a salted fish sauce sample in Aswan (Egypt)). The cells were motile, Gram positive and non-spore forming rods measuring 1.4-2.1x1.4-3.5 μm under optimum conditions. Colonies were buff and small. Grow in NaCl concentration ranged from 3 up to 27.5 %.

The strain grow on each of casein, gelatin, tryptone, beef extract, casamino acid, peptone, yeast extract and proteose peptone but not on non-nitrogenous organic compounds such as starch and glucose. Catalase and oxidase positive, Hydrogen sulphide was formed from cystein, only gelatin was weakly hydrolyzed but not starch, casein or lipids and acid was produced from glucose, fructose, arabinose and mannose. It was resistant to chloramphenicol, erythromycin, cotrimoxazole, novobiocin, tetracycline, sulphafurazole, streptomycin, ampicillin, amoxicillin, penicillin G, gentamicin and sensitive against nitrofurantoin. Other characteristics were recorded [Tables 1, 2].

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