

***Halobiforma haloterrestris* Strain FS1 and *Halogeometricum borinquense* Strain FS2, Two New Extremely Halophilic Archaeal Members Isolated from Salted Fish Sauce in Aswan City, Egypt**

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Abstract: Two new extremely halophilic archaea, strain FS1 and strain FS2 were isolated from salted fish sauce in Aswan city, Egypt. These organisms were neutrophilic, motile, Gram negative and required at least 10 (% w/v) NaCl to grow. Their morphology, physiology and biochemical characteristics revealed that strain FS1 and strain FS2 were members within the family *Halobacteriaceae* and they belong to *Halobiforma haloterrestris* and *Halogeometricum borinquense* respectively.

Key words: *Halobiforma haloterrestris* • *Halogeometricum borinquense* • Halophilic • Archaea • Salted fish sauce

INTRODUCTION

Extremely halophilic archaea are microorganisms that can grow best in media containing 2.5-5.2 M (i.e. 15-32 % w/v) NaCl [1]. The extremely halophilic archaea are the dominant microbial populations of hypersaline environments [2]. Extremely halophilic archaea include neutrophilic and alkaliphilic halophilic members which are placed in the order *Halobacteriales*, family *Halobacteriaceae* [3]. At the time of writing the aerobic, extremely halophilic archaea are classified within 26 genera which are *Haloalkalicoccus*, *Haloarcula*, *Halobacterium*, *Halobaculum*, *Halobiforma*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halomicrobium*, *Halorhabdus*, *Halorubrum*, *Halosimplex*, *Halostagnicola*, *Haloterrigena*, *Halovivax*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronolimnobius*, *Natronomonas*, *Natronorubrum*, *Halopiger*, *Haladaptatus*, *Haloquadratum* and *Halosarcina* [4-26]. In the present study, an attempt was done to isolate and identify two new extremely halophilic archaea, strain FS1 and strain FS2 from salted fish sauce in Aswan city, Egypt.

MATERIALS AND METHODS

Source of organisms: salted fish sauce was collected from different salted fish shops in Aswan city, Egypt.

Nutrient Media Used in the Study: The following media were used for the isolation and the purification of the extremely halophilic archaeal bacteria: proteose peptone salt agar medium (g/l): NaCl, 250; KCl, 2; MgSO₄·7H₂O, 10; proteose peptone, 7.5; yeast extract, 5; agar, 20; distilled water up to 1000 ml. Modified Lockhead's skim milk agar medium (g/l): NaCl, 250; KCl, 2; MgSO₄·7H₂O, 10; Yeast extract, 1; Fresh milk, 100 ml.; agar, 20; distilled water up to 1000 ml. The basal salt medium was the salt minerals medium supplemented with 0.1 % (w/v) yeast extract and was used for biochemical characterization. The pH was adjusted to 7-7.5 with NaOH or HCl (1 N) by a digital pH meter with glass electrode (Hanna instruments, model HI 8418), then media were sterilized by autoclaving.

Morphology and Growth Characteristics: The colonies of the two strains were described on proteose peptone salt agar medium where forms, pigmentation, elevation, margins, opacity, consistency and diameters (in mm.) were determined. The cell motility and shapes were examined under a Phase-contrast light microscope (Olympus CH-2).

Gram staining was performed by using acetic acid fixed cells as described by [27]. The spore staining was performed following the method described by [28].

Physiological and Biochemical Characterization: The growth response to NaCl was examined in liquid and solid proteose peptone salt medium using different

concentrations of NaCl (% w/v): 0.0 (control), 1.0, 3.0, 5.0, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0, 27.5, 30.0 and 32.0. The growth response to pH was performed by testing growth at different pHs (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, 52 and 55°C).

Oxygen requirements, catalase activity, oxidase activity, hydrogen sulfide production, nitrate reduction, indole formation, acid production from sugars, Voges-Proskauer and methyl Red Reactions (VPMR) were performed according to [28].

Lipase activity was tested by streaking the bacterial isolates on the surfaces of the basal salt agar medium supplemented with 1 % (v/v) of tributyrin, tween 20, tween 40 or tween 80 following the method described by [29]. The amylolytic activity was determined following the method described by [30], using the basal salt agar medium supplemented with 1 % (w/v) soluble starch as a carbon and energy source. The proteolytic activity was determined following the method described by [31, 32], using the basal salt agar medium supplemented with 1% gelatin or casein.

Antibiotic Susceptibility: The susceptibility of the strains to different antibiotics was performed by placing discs impregnated with antibiotics (20 µg/disc) on agar plates of proteose peptone salt medium previously streaked uniformly with the bacterial isolates. The plates were incubated at 40°C for 72 hour and the diameters of inhibition zones were measured in mm. Antibiotics tested were Chloramphenicol, erythromycin, cotrimoxazole, novobiocin, tetracycline, sulphafurazole, streptomycin, ampicillin, amoxicillin, penicillin G, gentamicin and nitrofurantoin.

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, sodium acetate and sodium pyruvate as sole nitrogen and/or carbon and energy sources were performed in the basal salt medium supplemented with 1 % (w/v) from the substrate.

RESULTS

Morphology and Growth Characteristics: The colonies on agar plates of FS1 strain were opaque, entire, flat or slightly raised, circular and measured about 2-3 mm in diameter. Colonies were red-pigmented and pigmentation intensity increased when the agar plates cultivations were stored for along time in the refrigerator. It was observed that the growth on the agar plates was strongly sticky and it was difficult to streak on agar surface or even to be distributed in liquid media when subcultured. The colonies of Strain FS2 were pink-pigmented, flat to raised, entire, opaque and circular and measured about 4 mm. in diameter. Agar growth of Strain FS2 was butyrous in consistence and it was easy to streak on agar plates. The cells of both strains were motile, Gram negative and were non-spore forming. The cells of Strain FS1 growing in liquid medium under optimum growth conditions showed rod and pleomorphic shapes, the cells from solid cultivations were coccus-shaped with the presence of some rods (Fig. 1 a and b).

The cells of Strain FS2 were rods in the liquid cultivations, while on the solid medium were extremely pleomorphic showing rods, squares, sphericals and irregular shapes (Fig. 2 a and b).

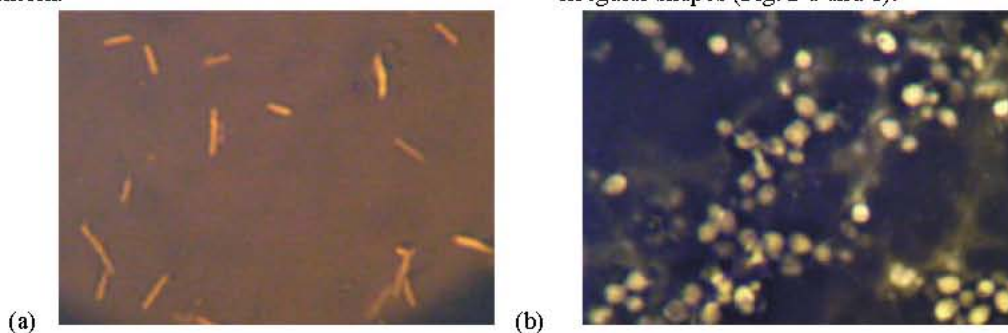


Fig. 1a: A microphotograph of nigrasin stain of Strain FS1 showing the cell shapes in the liquid cultivation (Phase contrast microscope-X3000)

Fig. 1b: A microphotograph of nigrasin stain of Strain FS1 showing the cell shapes on the solid medium (Phase contrast microscope-X3000)

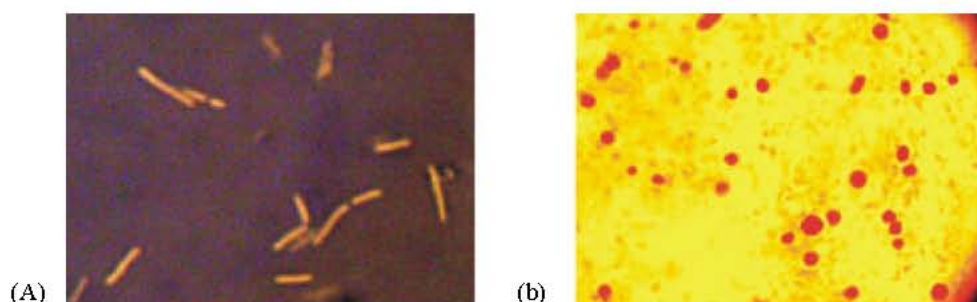


Fig. 2a: A microphotograph of nigrosin stain of Strain FS2 showing the cell shapes in the liquid cultivation (Phase contrast microscope-X3000)

Fig. 2b: A microphotograph of nigrosin stain of Strain FS2 showing the cell shapes on the solid medium (Phase contrast microscope-X3000)

Table 1: The morphological, physiological and growth characteristics for strain FS1 and strain FS2 isolated from salted fish sauce

characteristics	Strain FS1	Strain FS2
Temperature for growth (oC):		
Minimum	<10	<10
Optimum	40	40
Maximum	52	52
pH for growth:		
Minimum	5.5	5.5
Optimum	7-7.5	7-7.5
Maximum	9	9
NaCl concentration for growth (% w/v):		
Minimum conc.	10	10
Optimum conc.	20-25	20-25
Maximum conc.	32	32
Replacement of NaCl by KCl:		
12% KCl+ 8% NaCl	+	+
14% KCl+ 6% NaCl	+	+
17% KCl+3% NaCl	+	+
Pigmentation	Red	Pink
Oxygen relationship	Strictly aerobic	Strictly aerobic
Lysis in water	Rods only lyse	Rods only lyse
Cell forms:		
In liquid medium	Rods	Rods
On solid medium	Cocci and pleomorphic	Rods, squares, sphericals and irregular
Cell size (µm):		
Rods	1.4x2.8-7.1	1-3x1-2
Cocci	2.8-4.2	1.4-4.2
Cell motility	Rods are motile	Rods are motile
Gram stain	-ve	-ve
Spore formation	-ve	-ve

Table 2: Biochemical characteristics and enzymatic activities for FS1 and Strain FS2s isolated from salted fish sauce

characteristics	Strain FS1	Strain FS2
Catalase production	+	+
Oxidase test	+	+
Reduction of nitrate to nitrite	-	+
Reduction of nitrate till gas	-	+
Indole formation	+	+
H ₂ S production from cysteine	+	+
Acetylmethylcarbinol formation	-	-
Methyl red test	-	-

Table 2:

Degradation of:		
Starch	-	-
Gelatin	+/-	+
Casein	-	+
Tributyrin	-	-
Tween 20	-	-
Tween 40	-	-
Tween 80	-	-
Cholesterol	-	-
Acid and gas from:		
Glucose	-	Acid and gas
Fructose	-	Acid and gas
Sucrose	-	Acid and gas
Maltose	-	-
Lactose	-	-
Arabinose	-	Acid and gas
Galactose	-	Acid and gas
Mannose	-	Acid and gas
Mannitol	-	-
Starch	-	-

Physiological and Biochemical Characteristics:

The response of FS1 and FS2 strains to temperature, pH, NaCl concentration, NaCl replacement by KCl, oxygen relationship, lysis of cells in water and other characteristics were summarized in Table (1).

The biochemical activities of both FS1 and Strain FS2s i.e. catalase production, oxidase activity, indole formation as well as enzymatic activities were summarized in Table (2).

Antibiotic Susceptibility: Both of the two strains were resistant to the known eubacterial inhibitors i.e. chloramphenicol, erythromycin, cotrimoxazole, tetracycline, sulphafurazole, streptomycin, ampicillin, amoxicillin, penicillin G and gentamicin, while they were sensitive to the inhibitors which act against haloarchaea i.e. novobiocin and nitrofurantoin.

Utilization of Different Substrates as Nitrogen And/or Carbon and Energy Sources: Both of the two strains could use organic nitrogenous compounds e.g. casein, gelatin, tryptone, beef extract, casaminoacid, peptone, yeast extract and proteose peptone for growth. None of the two strains exhibited detectable growth when carbohydrates or inorganic compounds were used.

DISCUSSION

Agar colonies of Strain FS1 were red-pigmented, opaque, flat, entire and circular and measured about 2-3 mm. in diameter, while the colonies of Strain FS2 were

pink-pigmented, flat, entire, opaque and circular and measured about 4 mm. in diameter. The intensity of pigmentation of both of the two strains was increased when the agar cultivations were stored in the refrigerator; this was previously observed by [33]. Increasing of the pigmentation intensity was mostly related to the slowly growth of the organisms in the refrigerator. Agar growth of Strain FS1 was sticky and strongly attached to each other, so that harvesting of the cells and streaking on agar plates was difficult. It was previously reported that some halophilic archaea secrete extracellular polymers such as poly glutamic acid (PGA) and polysaccharides which cause the sticky consistence of these archaea [34-40]. In contrast, Strain FS2 was butyrus in its consistence and easy to be streaked on agar surface.

The cells of Strain FS1 in the liquid cultivations were mostly motile rods with the presence of few pleomorphic shapes and were non motile cocci on solid medium. Variation in cell shape for the same species have been described for some members of the family *Halobacteriaceae* [4, 14, 41,] and were extensively studied for the halophilic archaeon *Halobiforma haloterrestis* by [17]. The cells of Strain FS2 in liquid cultivations were mostly motile rods and showed pleomorphic shapes. On solid medium most of the cells were strongly pleomorphic showed square, spherical, triangular and irregular shapes, these shapes were usual for rod-shaped extremely halophilic archaea [11, 14, 23]. The cells of both of the two strains were non spore forming and stained Gram negative. All extremely halophilic archaea isolated until now were Gram negative and non spore forming [4-26, 41-43].

Both of the two strains have absolute NaCl requirements, which need at least 10 % (w/v) NaCl to grow and also can grow even in saturated NaCl (i.e. 32 %, w/v), showing optimum growth in the presence of 20-25 % (w/v) NaCl, NaCl could be partially replaced by KCl. The most interesting feature of halobacteria was their absolute requirement for high concentrations of NaCl. Although some strains may grow at salt concentrations as low as 1.5 M NaCl (9 %, w/v), most of the strains grow best at concentrations of 3.5-4.5 M (20-26 %, w/v) and can grow in saturated NaCl i.e. 5.2 M (32 %, w/v). To compensate for the high salt concentrations in the environment, the organisms accumulate mainly KCl and may be growth limited by the amount of KCl [11, 14, 17, 23-25, 33, 40-47]. For both of the two strains, the pH range for growth was 5.5-9, with an optimum at pH 7-7.5. The two strains could grow in a wide range of temperatures i.e. <10-52°C, with an optimum at 35-40°C.

The two strains were catalase and oxidase positive, indole was produced from tryptophane and hydrogen sulphide was produced from cysteine. Gelatin was hydrolyzed but starch, tributyrin, tween 20, 40, 80 and cholesterol not. Casein was hydrolyzed by only Strain FS2 using fresh milk as a substrate.

Strain FS1 could not produce neither acid nor gas from any of the tested sugars, but Strain FS2 could do this from glucose, fructose, sucrose, arabinose, galactose and mannose, while maltose, lactose, mannitol and starch were not fermented.

Both of the two strains were only sensitive to antibiotics which act against the extremely halophilic archaea i.e. novobiocin and nitrofurantoin and were resistant to the other known eubacterial inhibitors such as chloramphenicol, erythromycin, cotrimoxazole, tetracycline, sulphafurazole, streptomycin, ampicillin, amoxicillin, penicillin G and gentamicin as previously reported for extremely halophilic archaea [11, 14, 17, 23, 33, 40, 45, 46]. Both of the two strains exhibited good growth when organic nitrogenous compounds were used in growth media e.g. casein, gelatin, tryptone, beef extract, casamino acid, peptone, yeast extract and proteose peptone. No growth could be detected when using inorganic compounds or carbohydrates as substrates for growth. This may be due to the nature of nutrients available in salted fish and salted fish sauce.

It was thought that the extremely halophilic archaea do not utilize carbohydrates or utilize carbohydrates only slightly [42, 48]. This may be right for those that were isolated from salted meat, hides and salted fish. But it was found that many members of extremely halophilic archaea can grow at expense of carbohydrates [11, 45, 46, 49-52].

As a sort of adaptation to substrates, these organisms should have a well enzymatic system for hydrolyzing proteins and metabolizing amino acids to grow.

Based on the absolute requirement of NaCl for growth, pigmentation, antibiotic susceptibility, in addition to that, the two strains were Gram negative and non spore forming, it was suggested that the two strains were members within the family *Halobacteriaceae* which included the extremely halophilic archaea.

From all the above data, it was found that strain FS1 was strongly related to *Halobiforma haloterrestris* [17]. It was suggested to be given the name *Halobiforma haloterrestris* strain FS1.

It was also found that Strain FS2 was strongly related to *Halogeometricum borinquense* [11]. It was suggested to be given the name *Halogeometricum borinquense* Strain FS2.

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