4 METHODS

ISOLATION OF MICROORGANISMS FROM SALTERN AREAS

COLLECTION OF SOIL SAMPLES

Soil samples were collected from Çamaltı saltern area, which is located in Aegean area (Anatolia) in the city border of Izmir (Ancient Smyrna).

HUMIDITY PERCENTAGE DETERMINATION OF THE SOIL SAMPLES

10 grams of soil sample was dried at 100ºC (Pasteur oven) until it reached a constant mass. Humidity percentage of the soil sample was determined according to the equation below:

$$\% \text{ Humidity} = \frac{\text{Mass}_{\text{first}} - \text{Mass}_{\text{last}}}{\text{Mass}_{\text{first}}} \times 100$$

ISOLATION OF MICROORGANISMS AND COLONY COUNTING FROM SOIL SAMPLES

The isolation and colony counting of microorganisms were done by “soil dilution plaque method”. In this method samples were diluted using known percentages of Ringer solution and with amounts of organism in the sample decreasing.

25 grams of soil sample was mixed with 225 ml of Ringer solution in a beaker. 10 ml of this solution was mixed with 90 ml of Ringer solution in the second beaker (10^{-2} dilution factor). From the second beaker 10 ml was taken and diluted in the third beaker with 90 ml of Ringer solution (10^{-3} dilution factor). This dilution steps were made until a dilution factor of 10^{-7}.

Petri dishes were prepared using agar and Brown (1963) medium containing 25% NaCl under sterile conditions (sterilization at 121ºC for 15 minutes). Then 1 ml of sample from each of these dilutions was inoculated in the petri dishes. 5 copies of each sample were made and the dishes were incubated at 39ºC for 13 days [109].

After 13 days of incubation, petri dishes containing 30 – 300 colonies were counted. Microorganisms' amount was determined as given:

Direct counting x Dilution factor = Number/gram

CULTURING OF MICROORGANISMS

At the end of the incubation period (13 days at 39ºC), colonies grown in agar medium in petri dishes were separately cultured under sterile conditions in Agar-Brown (1963) medium containing 5 g/L yeast extract, 3 g/L sodium citrate, 20 g/L magnesium sulphate x $7H_2O$, 2 g/L potassium chloride and 250 g/L sodium chloride. This period was lasted until pure cultures of microorganisms were supplied. 11 new colonies were chosen depending on their morphology and numbered [109].

From all these 11 microorganisms No 6 was chosen for this work.

After preparing microorganisms under sterile conditions on agar-brown Petri dishes, they were transferred into the 30 % glycerol stock solutions in eppendorf tubes by the help of spreading glass rods and tubes were tightly closed. These prepared stocks were stored at - 80ºC. All pre-cultures were prepared from these sterile stocks.

PREPARATION OF PRE-CULTURES AND INOCULATION

Pre-cultures of a new isolate No 6 and type strain *Halomonas salina (*DSMZ No. 5928) were prepared in glass tubes containing 5 ml of Brown medium under sterile conditions. Each microorganism was grouped in 4 groups:

Group 1: Cultured in Brown medium containing 5 % NaCl at 37ºC

Group 2: Cultured in Brown medium containing 20 % NaCl at 37ºC

Group 3: Cultured in Brown medium containing 5 % NaCl at 45ºC

Group 4: Cultured in Brown medium containing 20 % NaCl at 45ºC

4 different groups for each microorganism (new isolate No 6 and *Halomonas salina*) were prepared. 5 ml of medium was placed in glass tubes and sterilized at 121ºC for 15 minutes. For each glass tube, 50µl of stock microorganism is added under hood in sterile conditions. The pre-cultures were incubated with a shaking of 180 rpm at indicated temperatures (37ºC and 45ºC) and their densities controlled at 600 nm spectrometrically. Cultures grown to about 0.7 at 600 nm, were inoculated to 100 ml of appropriate culture medium. Inoculation ratio was 1/100 for each microorganism. 8 samples (2 microorganisms for 4 groups) were incubated with a shaking of 180 rpm at indicated temperatures. The cells were harvested at their late exponential phase by centrifugation at 5000 rpm at 4ºC for 10 minutes.

SAMPLE PREPARATION

Harvested cells were washed two times with 50 mM Tris buffer. They were re-suspended in lysis buffer supplied from Sigma (Total protein extraction kit) with externally addition of 0.04 % protease inhibitor cocktail containing 0.1 µM pepstatin, 1mM PMSF, 0.08 % benzamidine, 2.5 mM leupeptine and 1.5mM EDTA. The cells were lysed by 5 cycles of freezing in liquid nitrogen and thawing at room temperature. Total freezing and thawing steps lasted half an hour. Then the suspensions were centrifuged at 15,000 x g for 30 minutes at 15 ºC. The supernatants were decanted into clean tubes, which contained the extracted proteins after they were reduced by addition of 5 mM tributylphosphine. Reduction was completed at room temperature with an incubation of 1 hour and time to time shaking. After reducing, the solutions were alkylated by addition of 15 mM iodoacetamide with an incubation of 1.5 hours at room temperature. Finally reduced and alkylated sample solutions were centrifuged at 20,000 x g for 10 minutes at room temperature. Total protein in the final protein solutions was determined using Bradford protein assay using BSA as a standard protein.

PROTEIN CONCENTRATION DETERMINATION USING BRADFORD

To determine the protein concentration of the bacterial samples, rapid and reliable dye-based Bradford assay was used [110]. A standard curve with BSA (1mg/ml stock solution) was prepared in parallel with unknown protein solutions. Both standard and sample readings were done in duplicate.

The standard curve was determined using concentrations of 3µg, 5µg, 8µg, 10µg ,13µg , 15µg, 18µg, 20µg, 22µg, 24µg, 28µg, 30µg BSA, respectively in each of the duplicate test tubes. Then known samples were all filled up to 100µl with

experimental buffer (So they were all equal in volume but contain increasing amounts of BSA). Then, 1 ml of Bradford working solution was added in each test tube and vortexed very carefully. After 2 minutes all samples were read in a spectrophotometer at an absorbance of 595 nm. Duplicate samples were calculated and a standard curve was prepared plotting concentrations of BSA in the x-axis and the absorbance at 595 nm values in the y- axis. Taking the standard curve into consideration, unknown samples were read in the same manner and the concentrations were determined placing the read OD_{595nm} values on the curve (the values were in the linear range of the curve) and correlating these to the x-axis.

The readings of the samples were done after the standard curve was developed and all the absorbance readings were done within 10 minutes after mixing with Bradford working buffer.

HIGH RESOLUTION TWO DIMENSIONAL ELECTROPHORESIS

Bacterial proteins were separated by large gel 2 – DE technique where the gels size is 30 x 23 x 0.15 cm (HxWxT) as described by Klose and Kobalz [59]. Using this very popular technique, proteins are separated according to their isoelectric points in the first dimension; in the second dimension the proteins of the first dimension, were further separated according to their molecular masses by sodium dodecylsulfate polyacrylamide gel electrophoresis. At the end, with this technique, proteins are separated according to these two physico - chemical properties.

NEPHGE TECHNIQUE (FIRST DIMENSION)

The nonequilibrium pH gradient electrophoresis was developed by two famous scientists, namely J. Klose and P. O'Farrell, independently [61]. The proteins in the NEPHGE gels were separated in the nonequlibrium state thereby prevented to reach their isoelectric points and thus, kept from their precipitation point.

In the first dimension, bacterial samples were separated on capillary rod acrylamide gels containing 9 M urea, 3.5 % acrylamide, 0.3% piperazine diacrylamide and 4% ampholyte mixture providing pH range 3-7.5. Besides separation gels, also rough gels were used as cap gels to inhibit some artificial side effects. Cap gels were composed of 9 M urea, 12 % acrylamide, 0.13 % piperazine diacrylamide and 4 % ampholyte mixture providing pH range 3-7.5. For analytical gels capillaries with inner diameter of 0.9 mm, and for preparative gels 1.7 mm inner diameter was used with lengths of 23 cm.

The separation gels were first degassed for 10 minutes and then 0.8 % ammonium persulphate (APS) was added. After addition of APS, the gel solution was filled into the capillaries with the help of vacuum supplied by syringes with careful handling to avoid air bubbles in the polymerisation gel solutions. The gel solution was filled up to 22 cm into the capillaries leaving free space at the cathodic side of the gel tubes. After waiting nearly 30 minutes for polymerisation of the separation gel, cap gel solution under addition of 0.8 % APS was filled at the cathodic side by a syringe. Following full polymerisation, a drop of water was loaded into both ends of the capillary rods leaving an air bubble between the gel surface and the water drop and then sealed with a parafilm piece to protect the gels against dryness. The capillaries containing gels were stored in the dark at room temperature for 3 nights providing mechanical strength to the gels.

The capillary rods were prepared for the run by removing the water drops and then attaching them to the electrophoresis chamber. The samples were loaded onto the anodic side of the gels pointing the cap gel sides towards the cathodic side. At the anodic side, 10 µl of sephadex solution previously mixed with 108 mg urea and 10 µl Servalyte 2-4 were added. For the analytical gels 80 µg of protein, and for preparative gels 150 µg, respectively, were loaded. After addition of the samples an overlay solution of 10 µl was loaded on top of each gel and then all capillaries were placed into the anodic buffer. The electrophoresis run was started with the anodic side pointing upwards and the cap gel cathodic side pointing down, by applying 100 V for 60 minutes, 20 V for 60 minutes, 400 V for 1050 minutes, 600 V for 60 minutes and 1000 V for 45 minutes, respectively. At the end of the run, samples were transferred to incubation solution and gently shaken for 10 minutes at room temperature. Following the incubation, the solution was dispersed and samples were stored at –80ºC until the start of the second dimensional run.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SECOND DIMENSION)

The second dimensional separation of bacterial proteins was performed by using sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). The Laemmli system was used lacking the original stacking gel part. The gel sizes were 30 x 23 x 0.9 cm for analytical gels, and 30 x 23 x 1.5 cm for preparative gels.

SDS- PAGE was run in a DESAVOR VA large gel system. SDS-PAGE gel solution containing 15% acrylamide and 0.2% bisacrylamide was degassed for 10 minutes and then, 1.28% APS was carefully added avoiding air bubbles. The gel solution was

then poured into the electrophoresis chamber up to 10 mm below the top of the glass plates. After ensuring that all the air bubbles were removed within the gel, the surface of the gel was covered with isopropanol to supply a flattened surface for loading the first dimensional gel. The gel was left at room temperature for polymerisation for about 45 minutes. After this period, isopropanol was removed from the upper part and overlaying solution composed of diluted SDS was loaded. The gels were then stored overnight in a refrigerator.

On the day of second dimension run, the electrophoresis chamber was filled with electrophoresis buffer equipped with a circulating cooling system (cooled to 18 ºC). The gel casting was taken out from the refrigerator and the first dimension gels were taken from –80ºC where they were stored. The upper overlaying solution was removed from the top of the SDS-PAGE gels and the thawed gels of the first dimension were loaded carefully on top of the second dimensional gels. Extra care was taken to remove all air bubbles appearing between the first and the second dimension gels. After placing the first dimensional gel, the free upper part of the chamber was filled with liquidified agarose solution in order to fix the first dimensional gel on the second dimension gel surface by direct contact. When the agarose solution was solidified the gel cast was inserted to the electrophoresis chamber. After filling up the upper buffer reservoir the system was run for 15 minutes at 65 mA and 400 minutes at 100 mA for analytical gels, and 15 minutes at 80 mA and 450 minutes at 120 mA for preparative gels.

At the end of the run, the agarose gel and the first dimensional gel were removed from the top part and the gels were carefully placed into the trays and stained with an appropriate method.

STAINING

SILVER STAINING

At the end of the electrophoresis run, the gels were transferred into the staining trays and were incubated overnight in the fixation solution by gentle shaking. The fixation solution was discharged and the gels gently shaken in incubation solution for two hours. The incubation step was followed by 3 times 20 minutes washing with distilled water. After the washing steps, gels were incubated in silver staining solution for 45 minutes. Thereafter, the gels were washed with distilled water for 20 seconds and then transferred to the developer solution. They were kept in developer solution until stained fully (3 to 15 minutes). After satisfactory colouring, the

developer solution was carefully removed; the gels were immediately covered with stop solution and incubated by gentle shaking for 15 minutes [111, 112]. For longer preservation in solution, gels were taken to another stop solution containing only 50 mM EDTA and kept in 4ºC in the dark until they were dried.

COLLOIDAL COOMASSIE G-250 STAINING

The gels were transferred to staining trays containing fixation solution. They were incubated overnight by gentle shaking. Following the fixation step, the gels were taken into the incubation solution and incubated for 2 hours with a gentle shaking. After this step, the gels were taken into the staining solution and incubated for 5 days in the dark by gentle stirring at the first day, then without. At the end of the staining step, the gels were transferred to a clean tray and washed gently with a solution containing 34 % methanol, 17 % ammonium sulphate, and 2 % phosphoric acid. When the gels were clean enough, they were covered with a plastic envelope and kept at 4 ºC for longer storage.

GEL DRYING

The stained gels were placed between two cellophane sheets and dried under vacuum. The drying was optimised at 80ºC for 2 hours 25 minutes for 23 x 30 cm big analytical gels, and 3.5 hours for 23 x 30 cm big preparative gels.

SEMI DRY BLOTTING

Following the two-dimensional electrophoresis, samples were blotted on PVDF membranes for identification.

Five minutes before the end of electrophoresis PVDF membranes were wetted in methanol and then, soaked into Towbin buffer. After electrophoresis, on the wetted anodic side, 3 layers of 3M filter papers, which were soaked in Towbin buffer, were placed. On top of these layers, the wetted membrane was placed very carefully avoiding air bubbles. Onto the membrane the gel was placed and the sandwich was closed by another 3 layers of 3M filter paper. Extra care was taken to avoid air bubbles between each layer.

The gels were divided in two pieces to sizes of each 15 x 20 cm and the blotting conditions were 300 mA for two hours in the cold room.

After the run, the membranes were air dried in a clean area and stored until usage.

STAINING OF BLOTTED PVDF MEMBRANES

Dried membranes were first wetted in methanol and then gently shaken in the staining solution (CBB-R250) for 5 -10 minutes. Then the membranes were shaken in destaining solution for nearly 15 minutes and then dried at room temperature avoiding any dust contamination.

IN-GEL DIGESTION; TRYPTIC DIGEST OF PROTEINS

The coomassie stained spots were excised from the gels with a scalpel. They were transferred to clean Eppendorf tubes. Most of the spots were small enough not to need cutting but bigger spots were divided into smaller pieces before the digestion.

Spot samples were incubated with nearly 100 µl of shrinking buffer at 30ºC for 15 minutes in a thermomixer. After 30 minutes of incubation the solution was removed and 100 µl of swelling buffer was added; the gel pieces were incubated another 15 minutes at 30ºC in a thermomixer. Those two steps were repeated 2 times to remove all the coomassie dye from the gel pieces.

After removing all the stain from the gel pieces, trypsin solution at a concentration of 0.4 µg/µl was used to digest the protein samples. The gel pieces were covered with tryptic solution (approximately 20µl) and digestion took place at 37ºC overnight. The next day the thermomixer was set to 60ºC and 3 µl of 5 % TFA per 10 µl enzyme mix was added to each sample and incubated for 1 hour at 60ºC. Trypsin-digested proteins were eluted by extraction buffer. Extraction was done using Millipore Zip-Tips with recommended application. Roughly, Zip-Tips were first washed with 10µl of 60 % MeCN / 0.1 % TFA three times, then three times with 10 µl of 0.1 % TFA solution. An automatic pipette was adjusted to 10µl and by several withdrawals and pour down steps the peptide mixture was bound to the zip tip. After binding, the automatic pipette was adjusted to 5 µl and moved to an Eppendorf tube that contained elution buffer (85 % MeCN/0.1 % TFA). Approximately after 10 successive up and down steps the peptides that attached to the zip tips were released into the elution solution. Elution solutions containing peptides were kept at -20ºC until MS measurements.

MALDI-TOF MS

One of the mass spectrometer used in this work was matrix assisted laser desorption/ionization time of flight (MALDI-TOF) - MS that was a positive ion mode reflectron system.

The samples, digested with trypsin were mixed 1:1 ratio with freshly prepared matrix solution. First, one drop of MeCN was spotted carefully onto the sample cell of the clean MALDI-target plate and then just before all MeCN was evaporated 1 µl of sample-matrix mixture was spotted onto the cell. The target plate was left at room temperature until it became dry. After dryness, sample cells were washed once with an ice-cold drop of ethanol. The plate was then introduced into the mass spectrometer.

The mass correction of the instrument was done with Glu-Fibrinogen with an average molecular mass (amu) of 1570.64. Before sample application the intensities were controlled and the mass spectrometer was calibrated using mono-alcohol dehydrogenase (mono ADH) using automatic calibration. From the reference file for ADH with a peak list of 968.48, 1013.61, 1136.57, 1251.67, 1312.67, 1386.74, 1447.80, 1618.84, 2019.07, 2312.14, 2465.19, 2700.39, minimum 9 peaks of the given 12 peaks were matched within the tolerance limits.

After calibrating, the samples were run with the same voltage parameters and the results were evaluated by the Protein Lynx software.

NANO LC-ESI-QTOF MS/MS

The selected spots were digested with trypsin as described in section 4.9 yet, having some modifications in overall procedure. Interested CBB-G 250 stained spots were cut out of the gels and placed in centrifuge compatible 96 well microplate and trypsin digestion procedure (section 4.9) was fallowed. The elution steps through the procedure were done by centrifugation. The last elutions that contain tryptically-digested proteins were collected at the bottom part of the plate. Prepared samples were then transferred to the micro eppendorf tubes and then frozen on dry ice. The solvents of frozen samples were evaporated by the speedvac till dryness. The peptides were resolved with 0.1 % formic acid solution and transferred to bottles of LC system. A voltage of approximately 700-1000 V was applied to the capillary. Argon was used as the collision gas and the kinetic energy was set around -20 -40 V for fragmentation. Daughter ions acquired were then separated by the time-of-flight mass analyser. Mass spectra of the samples were recorded in the Nano LC ESI-QTOF MS/MS mass spectrometer, Micromass, Manchester, England.

N-TERMINAL SEQUENCING

CBB-R 250 stained protein spots on PVDF membranes were cut very carefully with a scalpel and placed into the sequencers cartridge. After applying the membrane to the cartridge, Applied Biosystems Model 492A Procise sequencer (WITA GmbH, Teltow, Germany) was run automatically according to the manufacturer's directions.