MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES

UNDERSTANDING ALKALIPHILIC ADAPTATION OF *Bacillus marmarensis* sp. nov. USING OMIC TOOLS

FATMA ECE ALTINIŞIK KAYA

MASTER THESIS
Department of Bioengineering

Thesis Supervisor
Prof. Dr. Berna SARIYAR AKBULUT

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Fatma Ece ALTINIŞIK KAYA, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled "Understanding Alkaliphilic Adaptation of *Bacillus marmarensis* sp. nov. Using OMIC Tools", on December 26, 2017 and has been found to be satisfactory by the jury members.

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APPROVAL

Marmara University Institute for Graduate Studies in Pure and Applied Sciences Executive Committee approves that Fatma Ece ALTINIŞIK KAYA be granted the degree of Master of Science in Department of Bioengineering, Bioengineering Program on 08.01.2018..... (Resolution no: 2018/01-03).

Director of the Institute Prof. Dr. Bülent EKİCİ

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Bacillus marmarensis sp. nov. Türkiye'nin Marmara Bölgesine yakın mantar kompostlarından izole edilen alkalifilik bir mikroorganizmadır. pH 12.5 a kadar olan ekstrem pH değerlerinde yaşayabilirler. Potansiyel bir hücre dışı hidrolaz üreticisi olmasına ek olarak yüksek pH koşullarında yaşayabilmesi (pH 8.0 ve pH 12.5 arasında) onu endüstriyel uygulamalar için önemli bir mikroorganizma yapmaktadır. Yapılan bu çalışmada, mikroorganizmanın alkali koşullara adaptasyon stratejisi transkriptomik ve proteomik yöntemler kullanılarak araştırılmıştır. Belirlenen iki farklı pH değerine sahip besi yerinde (pH 9.8 ve 10.6) mikroorganizma büyütülmüş ve büyüyen kültürden elde edilen hücrelere ait toplam mRNA'dan RNA dizilemesi (RNA-seq) yapılmış ve farklı pH değerlerine sahip ortamlarda büyürken mikroorganizmanın geliştirdiği adaptasyon mekanizması transkriptom seviyesinde araştırılmıştır. Ayrıca kültürden elde edilen tüm proteinler ile proteom haritaları çıkarılmış ve farklı pH koşullarındaki protein regülasyonu araştırılmıştır. Elde edilen tüm sonuçlar birleştirilerek *B. marmarensis* sp. nov.'un alkalifilik ortama adaptasyonu için Krebs döngüsünde tükenen metabolitleri tamamlayıcı olarak glioksilat döngüsünü devreye soktuğu gözlemlenmiştir. Glioksilat döngüsünde görev alan iki enzime ait genlerin ekspresyonunun artmıştır. Ayrıca transkriptom analizi sonucunda hücre zarının oluşumunda görevli çeşitli protein ve enzimlere ait genlerin yüksek pH ortamında farklı olarak indüklenmesi, membranın aşırı alkalafilik ortamda hücreyi korumak üzere yeniden düzenlendiğine yorumlanmıştır. Tüm bu bilgilerin ışığında, organizmanın belirli mekanizmalarının (Krebs döngüsü, membran yapısı, stres proteinleri ve transport proteinleri) alkalifilik ortama adaptasyonunu sağlamak amacıyla regüle olduğu tahmin edilmiştir.

Anahtar Kelimeler: *Bacillus marmarensis* sp. nov., alkalifilik, RNA-seq, proteom, transkriptom

ABSTRACT

UNDERSTANDING ALKALIPHILIC ADAPTATION OF *Bacillus marmarensis* sp. nov. USING OMIC TOOLS

Bacillus marmarensis sp. nov. is an extreme obligate alkaliphile isolated from mushroom compost near Marmara Region of Turkey. It can survive at extreme pH values up to 12.5. In addition to being a potential extracellular hydrolase producer, its ability to survive in the high pH range of 8.0 to 12.5 makes it an attractive microorganism for different industrial applications. In the current study, the adaptation strategy of *B. marmarensis* sp. nov. to alkaline conditions were investigated using transcriptomic and proteomic tools. The organism was grown in two different pH values, 9.8 and 10.6. Total cellular mRNAs were analysed using RNA sequencing (RNA-seq) technology. Protein maps were obtained for the same cells. In the light of all the results, glyoxylate cycle was found to be up-regulated as a replenishing pathway for the TCA cycle of the organism. The genes encoding the two enzymes specific for the glyoxylate shunt were both found to be up-regulated. Transcriptomic analysis has also shown that the genes encoding a number of proteins and enzymes involved in cell membrane biosynthesis were differentially expressed under high pH condition. These results suggest the re-organization of cell membrane as a defence mechanism against alkaliphilic conditions to protect the cells. In the light of these results, the organism was found to regulate different pathways (TCA cycle, cell membrane, transport proteins, and stress proteins) for adaptation to the alkaline condition.

Keywords: *Bacillus marmarensis* sp. nov., alkaliphilic, RNA-seq, proteome, transcriptome

SYMBOLS and ABBREVATIONS

OD **:** Optical density

NB **:** Nutrient Broth

IEF **:** Isoelectric Focusing

2D-PAGE **:** Two Dimensional Poly Acrylamide Gel Electrophoresis

IPG **:** Immobilized pH Gradient

TBP **:** Tributylphosphine

HPLC **:** High Pressure Liquid Chromatography

pI **:** Isoelectric Point

RT **:** Room temperature

SDS **:** Sodium Dodecyl Sulfate

CBB **:** Coommassie Brilliant Blue

BSA **:** Bovine Serum Albumin

ABC **:** ATP-Binding Cassette

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1. INTRODUCTION

High environmental pH is hostile for many cells. Interestingly, a group of extremophilic microorganisms, classified as alkaliphiles, require high pH to live and multiply. Microorganisms from different kingdoms, such as archaea, prokaryotes, and eukaryotes could display alkaliphilic characteristic. Alkaliphilic organisms do not have a long history.

The first alkaliphilic *Bacillus* strain was reported by Vedder in 1934 and by 1968 there were only 16 scientific papers on these organisms (Horikoshi, 2008). This group of microorganisms is an interesting research area for scientist because of their ability to grow under conditions which are hostile for most microorganisms. Most alkaliphilic organisms require growth environments with pH values above 8.0 and cannot grow or can grow only very slowly at neutral pH (Horikoshi, 1999). In contrast to most bacteria they need alkali environment for germination and sporulation. Introduction of alkaliphiles to industrial processes has brought many advantages that include elimination of contamination risks, decreased sterilization, and reduced chemical costs.

In order to understand the importance of alkaliphilic microorganisms, some areas where they find important applications are summarized below.

1.1. Application Areas of Alkaliphiles

1.1.1. Wastewater treatment

Many industrial processes generate waste materials with alkaline or acidic characteristics (Noack *et al.*, 2014). Plants are responsible for refining their wastes before they are discharged back into nature. This process constitutes a significant part of process costs. Many industrial applications commonly use NaOH for cleaning and sanitization of chromatographic systems of industrial applications. Its efficacy, ease of detection and removal, and economic feasibility are the major advantages of utilization of NaOH for cleaning purposes (N Adner, 1994). These cleaning solutions are then discharged into nature as a part of waste. The presence of NaOH in wastewater results in wastes with high pH values. Biological or chemical methods may be applied to treat wastewater to remove NaOH. Usually biological remediation strategies are preferred

over chemical ones because of their low cost and easy-to-use nature. Furthermore, they are natural. For many biological treatment processes to be effective, pre-treatment steps are required to bring wastewater pH to neutral values since most microorganisms used in bioremediation processes can survive at neutral pH. In that case, alkaliphilic microorganisms emerge as important alternatives in wastewater treatment.

1.1.2. Biorefining

Biorefining is another important area where alkaliphiles play a critical role for the environment. Sustainable energy or sources, such as fuels or chemicals can be produced by biorefining using cellulosic biomaterials. However risks such as contamination, pentose or disaccharide co-fermentation, and water footprint emerge during utilization of cellulosic biomaterials (Somerville *et al.*, 2010). These processes necessitate sterilization pre-treatment techniques that require high costs. Utilization of alkaliphiles in biorefining processes reduces process costs by eliminating sterilization steps.

Neutral pH (at or near 7.0) is the predominant growth condition in the bacterial world; thus most microorganisms used in biorefining are not tolerant to high pH. When mesophilic organisms are used in biorefining, contamination risk with unwanted organisms is very high. To obtain pure cultures, material or chemical specific sterilization techniques are needed. However, if these processes could be conducted under alkaline conditions, only alkaliphilic microorganisms would survive, mesophilic organisms would die even without sterilization. Therefore utilization of alkaliphilic microorganisms could be considered as an important advantage to reduce process costs and consequently process time in industrial applications (Alper and Stephanopoulos, 2009; Lin *et al.*, 2014).

1.1.3. Washing-agent industry

Washing agent industry is one of the largest application areas of alkaliphilic organisms and their enzymes. Alkaline enzymes, especially proteases, are essential ingredients of cleaning agents, mostly detergents (Takami and Horikoshi, 2000; Gupta, Beg and Lorenz, 2002; Saeki *et al.*, 2002). Alkaliphilic organisms are rich sources of industrially important enzymes with optimal working conditions lying in the alkaline range.

To date, many alkaliphilic *Bacillus* strains have been reported to already produce alkaline proteases. The genome sequences of many alkaliphilic *Bacillus* strains clue the presence of extracellular protease enzymes (Takami and Horikoshi, 2000; Denizci, Kazan and Erarslan, 2010).

1.2. *Bacillus marmarensis* sp. nov.

Bacillus marmarensis sp. nov. is an alkaliphilic organism which was isolated from mushroom compost in Marmara Region of Turkey. Growth characteristics of the microorganism were determined by Denizci *et al*. in 2010 and the obtained results are presented in Table 1.1. Genome analysis of *B. marmarensis* sp. nov. was performed by Wernick DG *et al*. (2016). In their report, based on its genome sequence, this group has annotated 7 proteases, 6 amylases, 2 cellulases, a lipase, an n-butanol and a biodegradable plastic poly β 3-hydroxybutyrate in its different metabolic pathways. *B. marmarensis* sp. nov. has been found to exhibit closely related similarities with another alkaliphilic *Bacillus* species, *B. pseudofirmus* DSM 8715^{T}, in its cellular membrane structure and genome sequence.

Table 1.1. *B. marmarensis* sp. nov. physical and biochemical characteristics

	Range
Temperature	15-45 ^{o}C
Salt Tolerance	12 % (w/v)
pH	8.0-12.5 (9.8 optimum)
O_2 Demand	Aerobic
Colony Morphology	Creamy-yellowish, circular
Gram-staining	Gram-positive

The peptidoglycan cell wall of *B. marmarensis* sp. nov. contains meso-diaminopimelic acid. The strain is heterotrophic utilizing D-arabinose, L-arabinose, ribose, glucose, fructose, mannose, α-methyl-D-mannoside, α-methyl-D-glucoside, cellobiose, N-acetylglucosamine, salicin, maltose, trehalose, saccharose, glycogen, xylitol, gentiobiose, D-turanose, D-arabitol, and 2- ketogluconate, but not utilizing glycerol, erythritol, D-xylose, L-xylose, adonitol, β-methyl-D- xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, amygdalin, arbutin, aesculin, lactose, melibiose, inulin, melezitose, raffinose, D-lyxose, D-tagatose, D- fucose, L-fucose, L-arabitol, gluconate and 5-ketogluconate (Denizci, Kazan and Erarslan, 2010; Wernick *et al.*, 2013).

B. marmarensis sp. nov. can be considered as an important candidate for industrial applications that require growth at extremely high pH values. Characteristics that lead to maintenance of biological activities at high pH may make this organism an attractive target.

In a recent study by Wernick *et al.* (2016) *B. marmarensis* sp. nov. was used in contamination-resistant wastewater for biorefining of a wide range of carbohydrates. They have simply used unsterilized and non-potable seawater for their process (Wernick *et al.*, 2016). In the same study the gene(s) responsible for ethanol production were inserted into *B. marmarensis* sp. nov. In the unsterilized seawater and wastewater that was contaminated by algae, *B. marmarensis* sp. nov. was able to grow and produce ethanol at a high yield (50 %) from glucose (Wernick *et al.*, 2016). Overall, this would help to reduce the cost of biorefining using this microorganism.

1.3. OMIC Approaches

It is believed that for survival under extreme alkaline conditions, alkaliphilic microorganisms have developed specialized systems or adaptation strategies. In order to evaluate alkaliphiles for different types of industrial processes, the organisms should be well-defined in their biological activities. Furthermore, this information is especially useful when it is necessary to extend their capabilities to other microorganisms.

In the last two decades, OMIC approaches combined with high-throughput analysis techniques have proven to be very powerful in the characterization of microorganisms.

Especially different OMIC techniques and their combinatorial studies allowed us to get deep insight into microbial adaptation strategies. An example for this kind of a study was on *B. subtilis* following its genome sequencing in 1997 (Kunst *et al.*, 1997). The study of the genome of this microorganism allowed the elucidation of its spore formation genes (Eichenberger, Bate and Bonneau, 2014). Then a transcriptomic study helped in understanding the regulation of the defined sporulation genes using a comparative analysis. This analysis identified factors that influenced the expression of these genes. Finally, proteomic tools enabled the identification of the function of the genes. For its further phenotypic characterization, metabolomics analysis has been performed (Eichenberger, Bate and Bonneau, 2014). This study clearly shows how OMIC approaches collectively can be used in microbial characterization studies.

A schematic representation summarising the relation between different OMIC approaches is given in Figure 1.1.

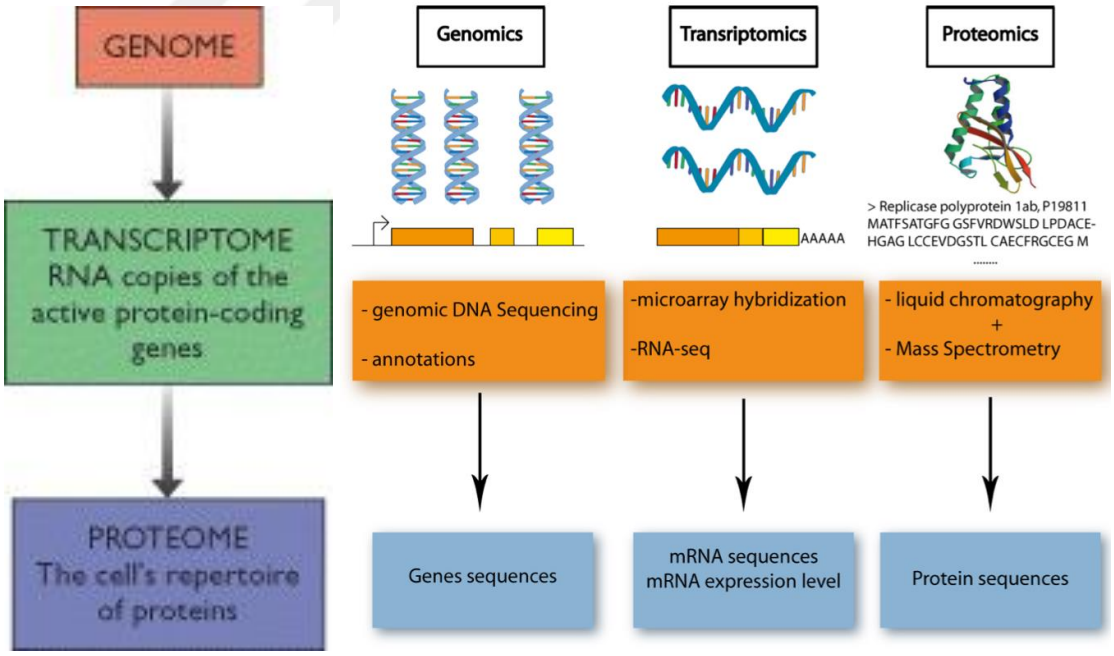

Figure 1.1. Relationship between proteomic and transcriptomic analysis (Brown, 2002)

When considered alone, it is clear that proteome should reflect the transcriptome. The transcriptome can be defined as the precursor of the proteome results. Genome sequence contains the codes for the expression of desired proteins. The up-regulated proteins should have over-expressed RNA short sequences to be expressed.

1.4. Transcriptomics and the Study of the Transcriptome

Transcriptome represents the set of all RNA molecules in one cell or tissue under a specific physiological condition or development stage. Different from the genome data, transcriptome data is not fixed for a specific organism, generally it is highly sensitive to changing environmental conditions. Expression profiles of mRNAs obtained from transcriptome analysis clearly define the cellular conditions in its environment. DNA microarrays and RNA-seq as next-generation sequencing technologies are commonly used transcriptomic techniques (Gupta and Gupta, 2013).

1.5. Proteomics and the Study of the Proteome

The cellular proteome is the collection of proteins found in a particular cell type under a particular set of environmental conditions. Its analysis is the investigation of all the proteins present in a cell, tissue or organism at any time. It is much more complex, than both the genome and the transcriptome. This simply is because the genome is the complete set of genes, which is placed on the organism but expression needs to make sense of a gene to translate a protein. Moreover, each protein can be chemically modified in different ways after being synthesized (Wilkins, 2009). Cells create responses according to their environment and changes in their environment by adapting their synthesized proteins. The differences in expression levels of different proteins may help to understand the metabolic changes leading to adaptation to extreme conditions such as high alkalinity. By using carefully controlled growth conditions, it is also possible to obtain detailed information about the molecular biology of the microorganism (Altelaar, Munoz and Heck, 2012).

There are many techniques to understand proteins and their features. Gel based 2D-PAGE analysis is the most widely used protein separation technique (Chandramouli and Qian, 2009). Immunoassays with antibodies and mass spectrometry could be used for the detection of proteins. Matrix assisted Laser desorption/Ionization (MALDI) and

Electrospray Ionization (ESI) are protein identification techniques that do not use antibodies. Mass spectrometry is a powerful high-throughput protein analysis tool. Protein chips and reverse-phased protein microarrays are also used for proteome analysis (Westont and Hood, 2004).

2. MATERIALS AND SOLUTIONS

2.1. Equipment List

Table 2.1. List of laboratory equipment that were used during the experimental study

Equipment	Purpose of Usage	Supplier
Autoclave	Sterilization	Nüve OT 032, TURKEY
Spectrophotometer	Optical density measurement	BioRad, California, USA
Scientific Balance	Weighing	Mettler Toledo, USA
Refrigerators	+4°C and -20°C	Arçelik and Uğur, TURKEY
Deepfreezer	-80°C	Thermo Fisher Scientific, USA
1st Dimensional Electrophoresis System and Power Supply	Protein separation	BioRad, California, USA
2nd Dimensional Electrophoresis System and Power Supply	Protein separation	BioRad, California, USA
Gel Imaging System	Gel Imaging and Quantification Analysis	BioRad, California, USA
Laboratory water distiller	Preparation of distilled water and HPLC grade distilled water	Elga PureLab, UK
MPBio FastPrep	Mechanical cell disruption	MPBIO, USA
Etuves	Incubator	Nüve, TURKEY
Centrifuge	Separation	Sigma, Germany
pHmeter	pH measurement	Mettler Toledo, USA
Microcentrifuge	Separation	Sigma, Germany
Horizantal Laminar Flow Cabinet	Achieving a sterile environment	ESCO Class II type A2, USA
Incubator Shaker	Incubator for growth	Nüve, TURKEY
Magnetic Stirrer	Media and solutions preparation	Sigma, Germany
Vortex	Mixing homogenizing of solutions	Mettler Toledo, USA
Automatic Pipettes	Experimental studies	Thermo Fisher Scientific, USA

2.2. List of Solutions

Table 2.2. Optimized protein rehydration buffer

Urea	8 M	24.04 g
Thiourea	2 M	7.612 g
Chaps	4 %	2 g
DTT	50 mM	385.62 mg
Bromophenol Blue	0.002 %	Trace Amount
Total Volume: 50 mL mL w/ddH_20		

2.2.1. 2D- PAGE solutions

Table 2.3. Chemicals of gel solution A

Tris-Base	110.408 g
Tris-HCl	45.436 g
Sodium Dodecyl Sulfate (SDS)	3.2 g
TEMED	960 µL
Total Volume: 1400 mL w/dH_20	

Table 2.4. Chemicals of gel solution B

Acrylamide	498 g
Bis-acrylamide	45.436 g
Serdolit	3.2 g
Total Volume: 1660 mL w/dH_20	

Solution A and B were prepared by mixing all the given components. Serdolit was filtered from solution B using filter paper. To prepare the polyacrylamide gel, 1400 mL of solution A and 1600 mL of solution B were mixed with the help of a magnetic stirrer. This 2D-PAGE gel solution was divided into 40-50 mL aliquots in falcon tubes and then stored at -20 ^{o}C.

Table 2.5. Chemicals of overlay agarose

Agarose	0.5 %
Tris	25 mM
Glycine	192 mM
SDS	0.1 %
Bromophenol-blue	0.002 %
Total Volume: 50 mL w/dH_20	

Table 2.6. Chemicals of equilibration buffer

Urea	6 M
SDS	2 %
Tris-HCl (pH 8.8)	0.375 M
Glycerol	20 %
Total Volume: 50 mL w/dH_20	

2% (0.04 g for 2 mL) DTT was added to Equilibration Buffer to prepare Equilibration Buffer 1 and 2.5% (0.05 g for 2 mL) iodeacetamide was added to Equilibration Buffer to prepare Equilibration Buffer 2. 2D-PAGE equilibration buffers were stored in 2 mL aliquots in eppendorf tubes and stored at -20 ^{o}C until further experiments.

2.3. 2D-PAGE Midi System Tank Buffers

Table 2.7. Chemicals of 2D-PAGE midi system outer tank buffer

Glycine	55.44 g
SDS	3.6 g
Tris Base	10.8 g
Total Volume: 3200 mL w/dH_20	

Table 2.8. Chemicals of 2D-PAGE midi tank buffer

Glycine	154 g
SDS	10 g
Tris Base	30 g
Total Volume: 1000 mL w/dH_20	

2.4. Coomassie Brilliant Blue (CBB)-G250 Gel Staining Solutions

Table 2.9. Chemicals of CBB-G250 fix solution

Ethanol	1 L
Phosphoric Acid (H_3PO_4)	28 mL
Total Volume: 2000 mL w/dH_20	

Table 2.10. Chemicals of CBB-G250 wash solution

Methanol	680 mL
Ammonium Sulfate	310 g
Phosphoric Acid (H_3PO_4)	28 mL
Total Volume: 2000 mL w/dH_20	

Table 2.11. Chemicals of CBB-G250 dye solution

Methanol	680 mL
Ammonium Sulfate	310 g
Phosphoric Acid (H_3PO_4)	28 mL
CBB-G250	1.32 g
Total Volume: 2000 mL w/dH_20	

3. METHODS

3.1. EXPERIMENTAL STUDY

3.1.1. Growth of microorganism

3.1.1.1. Sterilization techniques

All experiments were conducted with sterile equipment and media. Sterilization was achieved by autoclaving at 121 ^{o}C and 1.06 bar for 15 minutes. When required, buffers or solutions were filter sterilized.

3.1.1.2. Preparation of frozen stocks

A single colony of *B. marmarensis* sp. nov. was inoculated into sterile 5 mL Nutrient Broth (NB) (Merck, Germany) prepared in Na-sesquicarbonate buffer with pH 9.7 and grown overnight (approximately 16 hours). Grown cells were mixed in 1:1 ratio with 60% glycerol solution and stored at -86 ^{o}C in sterile eppendorf tubes. Stocks were renewed regularly during the study.

3.1.1.3. Growth of microorganism

B. marmarensis sp. nov. growth was achieved in NB media prepared in buffers with different pH values. For the final growth, Na-sesquicarbonate buffer with pH 9.7 and pH 10.6 were prepared with mixtures of 0.1 M Na_2CO_3 and 0.1 M Na_2HCO_3 using the amounts given in Table 3.1. To prepare 1 L NB, the media contents were dissolved in 900 mL high purity water and then autoclaved. 10X concentrated Na-sesquicarbonate buffer was separately prepared and filter sterilized. Then the autoclaved NB was mixed with 100 mL filter sterilized Na-sesquicarbonate buffer.

All flasks containing growth media were incubated at 37^{o} C and 180 rpm in shaking incubators.

Table 3.1. Reference table for preparation of Na-sesquicarbonate buffer (Wood, 1987)

pH		x ml 0.1M-Na_2CO_3	y ml 0.1M-$NaHCO_3$
20 °C	**37 °C**		
9.2	8.8	10	90
9.4	9.1	20	80
9.5	9.4	30	70
9.8	9.5	40	60
9.9	9.7	50	50
10.1	9.9	60	40
10.3	10.1	70	30
10.5	10.3	80	20
10.8	10.6	90	10

3.1.1.4. Cell growth for protein extraction

1 mL preculture of *B. marmarensis* sp. nov. was used to inoculate 100 mL NB media with selected pH (pH 9.7 or 10.6). Cells were incubated at 37 ºC and 180 rpm for 8 hours. Growth was monitored spectrophotometrically at 600 nm. pH was also measured during the incubation period. After 8 hours, cells were collected by centrifugation at 10,000 rpm for 10 minutes. Grown cells were washed three times with Tris-EDTA buffer and were removed from the wash buffers by centrifugation at 10,000 rpm for 10 minutes. Cells were stored at -20 ºC until later analysis.

3.1.1.5. Protein extraction by mechanical cell disruption

Mechanical disruption of the cells was achieved using silica beads with the MP-BIOMEDICALS FastPrep®-24 Classic Instrument. Cells were resuspended in 0.5 mL rehydration buffer and then protease inhibitor mix (GE Healthcare, Little Chalfont, UK)

was added at a concentration of 10 µL/mL. The resuspended cells were mixed in the lysing matrix tubes with lysing matrix beads for cell disruption. These cells in tubes were mechanically disrupted at a rate of 4 m/s for 40 seconds in FastPrep®-24 Classic Instrument. After the lysing step, cells were centrifuged at 10,000xg for 10 min and then 20000xg for 45 min to remove matrix beads. Supernatants were transferred to Lobind Eppendorf tubes. After measuring the protein concentration, 10 µL/mL nuclease mix (GE Healthcare, UK) was added to the sample solution according to the manufacturer's protocol.

3.1.1.6. Protein concentration measurement

Protein concentration was measured with the Bradford method after protein extraction (Bradford, 1976). The protein samples were mixed with 1 mL Bradford reagent (Thermo Fisher Scientific, USA) and incubated for 5 min in darkness. The change in absorbance was measured at 595 nm. Protein concentrations were calculated using the calibration curve prepared with 1000 µL Bradford reagent using Bovine Serum Albumin (BSA) as the standard. The standard curve used is given in Figure 3.1.

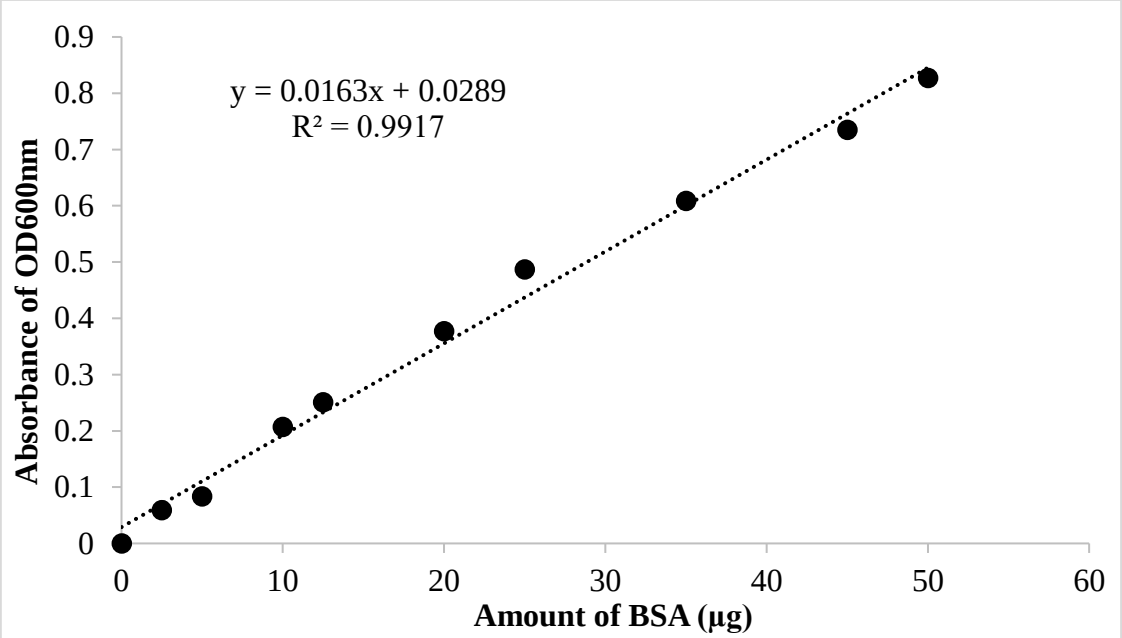

Figure 3.1. Bradford calibration curve graph

3.1.2. 2D gel electrophoresis

3.1.2.1. Cleaning up samples using Micro Bio-Spin® columns with Bio-Gel® P-30

Samples from protein extraction were cleaned with Micro Bio-Spin® Columns with Bio-Gel® P-30 (Bio-Rad, California, USA) to remove salts, nucleotides, dyes, and other small-undesired molecules from protein samples which could be risky for protein imaging with 2D gel electrophoresis. Column matrix was regenerated by washing the matrix several times with rehydration sample buffer. A predetermined amount of a protein sample was loaded to the column and the column was centrifuged at 1000xg for 4 min. Cleaned protein samples were collected inside a collection tube after centrifugation.

3.1.2.2. First dimensional isoelectric focusing (IEF)

Initial protein separation was achieved based on Isoelectric Focusing. 17 cm pH 3-6 gradient immobilized pH gradient (IPG) strips and rehydration sample buffer given in Table 2.2 were used. 600 µg of a protein sample in 300 µL rehydration buffer was mixed with 3 µL Tributylphosphine (TBP) (BioRad, California, USA) and 3 µL Bio-Lyte 3-6 ampholytes (BioRad, California, USA) for better visualization of separated proteins. Resuspended protein samples were pipetted onto the rehydration/equilibration tray and 17 cm IPG strips (Bio-Rad, pH 3-6) were carefully placed on each sample. The IPG strips were covered with 2-3 ml of mineral oil after the strips completely absorbed protein samples. Strips were incubated overnight (approximately 16 h). The 17 cm IPG strips that absorbed rehydrated proteins were placed on the PROTEAN IEF focusing tray to initiate the first dimensional separation. 250V for 20 minutes (linear), 10,000V for 3 hours (linear), and 10,000V for 45,000V-h (rapid) were applied to the strips at 20°C.

3.1.2.3. Second dimensional protein separation

2D-PAGE gels were prepared using the chemicals listed in Table 2.3 and Table 2.4. Gel solutions were degassed for 10 minutes in sonic water bath. Gel solutions were mixed with 1.28% APS to start polymerisation and then were directly poured into the space between the two glass plates. The gels were overlaid with isopropanol. After

solidification, isopropanol was removed from the gel by washing the gel with HPLC grade water. IPG strips were immersed in Equilibration buffers I and II, 10 minutes each. Then strips were placed on top of the gels carefully avoiding air bubbles and were fixed by overlaying with agarose solution. Once the agarose solution solidified, the gel cast was inserted into the electrophoresis tank and the tank was filled with running buffer. 30 mA for 15 minutes and 120 mA for 280 minutes were applied to separate the proteins on the SDS polyacrylamide gels.

3.1.2.4. Coomassie Brilliant Blue G250 gel staining

Gels removed from the 2D-PAGE cast system were stained with CBB-G250 dye to visualize protein spots (Neuhoff *et al.*, 1988). For this, proteins in the gels were first fixed overnight in CBB-G250 fixing solution. Then gels were washed for 2 hours in CBB-G250 wash solution. After the wash solution was removed, gels were stained with CBB-G250 dye solution for 3-5 days. The dye residues were removed by washing the gel with HPLC grade water.

3.1.2.5. Gel imaging and analysis

2D-PAGE stained gels' images were captured with Bio-Rad Chemi-Doc MP Imaging System (Bio-Rad, USA). Proteomic spot detection was performed with Bio-Rad Image-Lab 5.2.1 Software. Gels were analysed with PDQuest Advance software (BioRad, USA) to compute fold changes in the expression levels of proteins under two different conditions. Local regression model was used for the normalization of spot quantity and student's t-test was used for the statistical significance of image analysis. Data were assumed as significant when the p-value was lower than 0.1. Selected protein spots were excised by ExQuest Spot-cutter (BioRad, USA) for protein identification analysis. In-gel digestion kit from Pierce (USA) was used for the in-gel tryptic digestion of spots according to the manufacturer's protocol. Samples were concentrated and detracted from the salts by 10 μL ZipTipC18 according to the recommended protocol of Millipore (USA). Spot analysis and protein identification was performed at Kocaeli University DEKART proteomics laboratory by using ABSCIEX MALDI-TOF/TOF 5800 system. Peptide mass fingerprints were analysed in the MASCOT v.2.5 (Matrix Science) using a manually created library database from the network because the target organism was

novel. Data restriction was applied for the results with p-value values lower than 0.05 for significant matches.

3.1.3. RNA isolation

3.1.3.1. Total RNA extraction

RNeasy Mini Kit (QIAGEN, Germany) was used for total RNA isolation. Bacterial culture sample and RNAprotect Bacteria Reagent (QIAGEN, Germany) were mixed in 1:2 ratio according to the manifacturer's protocol. 10 mL of bacterial culture was added to the test tube containing 20 mL of RNAprotect Bacteria Reagent and the tube was mixed for 5 sec. Tube was incubated for 5 min at room temperature then the sample was centrifuged at 5,000xg for 10 min. Pellets were collected from the tubes (and stored at -80^{o}C when needed). Pellet was resuspended in 700 µL RLT buffer using a vortex. Cell disruption was achieved with acid washed beads and FastPrep device (MPBio, USA) by mechanical disruption at 6 m/s for 40 sec. Cell lysate was separated from beads by centrifugation at maximum speed (app 12,000xg) for 1 min. Supernatant was transferred to a new reaction tube. Equal volume ethanol (70%) was mixed with the sample and mixed thoroughly. Formed lysate from the kit was loaded to the column and the column was centrifuged at 10,000xg for 15 sec. After centrifugation, flow-through was discarded. 350 µL of RW1 Buffer was added to the column and column was centrifuged at 12,000xg for 15 sec. In another Eppendorf tube 10 µL DNase I and 70 µL RDD Buffer were mixed properly by inverting and then loaded to the column. The column was left for 30 min at RT. 350 µL RW1 Buffer was added to the column and column was incubated for 5 min. After 5 min, column was centrifuged at 8,000xg for 15 sec. Flow-through was discarded with the collection tube and a new collection tube was placed under the column. 500 µL RPE Buffer was loaded on the column and column was centrifuged at 8,000xg for 15 sec. These steps were repeated with 2 min centrifugation using 500 µL RPE Buffer. After discarding the flow-through, the column was centrifuged at max speed for 1 min. As a final step of RNA isolation, 40 µL of RNase free water was loaded to the column and sample was centrifuged at 8,000xg for 1 min. Aliquots of 5 µL RNA samples stored at -80^{o}C (QIAGEN, 2012).

3.1.3.2. RNA concentration measurement

RNA concentration was measured using NanoPhotometer P330 Device (Implen, Germany). The $A_{260/280}$ ratio represented the purity of the RNA sample and a value of 2.0 was accepted as pure RNA sample. Furthermore, the $A_{230/260}$ ratio was a measure of the purity of the sample. 2.0-2.2 for this value was acceptable for the purity of the RNA sample.

3.2. RNA-Seq analysis or transcriptome

3.2.1. Rockhopper

For studying the RNA-seq data of *B. marmarensis* sp. nov. grown at two different pH conditions, the reads from Illumina Hiseq were comparatively analysed using the de novo tool of Rockhopper version 2.03 (Tjaden, 2015). Local regression was used to get a smooth forecast of differential expressed transcripts. Statistical test for defining the null hypothesis was performed by Rockhopper. P-value of the expression was computed by the negative binomial distribution and q-values were computed using Benjamini-Hochberg procedure. Sequence, expression levels of the two different conditions, and q values (the adjusted p values) were found in the output file of the analysis. Data were accepted as significant and reliable with q-values less than 0.01.

3.2.2. Blastx

Following Rockhopper analysis, expression values were obtained for each read. Values obtained for the same read for the two different conditions (pH 9.7 and 10.6) were divided by each other and then log2 values were computed. 4-fold expressional changes were considered as significant. These nucleotide reads were not paired with any protein since there was no full annotation for this novel organism. Therefore analysis was performed without a reference organism (de novo analysis).

Rockhopper reads were aligned using Blastx 2.7.1 to identify potential protein products. Protein database was searched for the query nucleotide sequence with Blastx and findings were listed based on their scores, which were calculated from query coverage, E value, and identity of the sequence. Matches and sequence IDs from Blastx results

were added to the Rockhopper read list. Redundant and unmatched reads were removed from the list. Some reads were matched with a protein but they were not related with *B. marmarensis* sp. nov. These results were also removed from the final list. Proteins in these lists were categorized according to their functions in the cell and possible metabolic activities.

4. RESULTS

4.1. Selection of Growth Medium and Initial Growths

B. marmarensis sp. nov. is an extreme alkaliphilic organism which can grow at high pH values. Denizci *et al*. at 2010 has reported the optimum pH value for the growth of this organism as 9.8 (Denizci, Kazan and Erarslan, 2010). DSMZ 31 medium was recommended for its growth (Denizci, Kazan and Erarslan, 2010). This is an alkaline medium where NB is prepared in Na-sesquicarbonate buffer. The final pH of this preparation without any pH adjustment is 9.7.

This work aimed to study the protein expression of *B. marmarensis* sp. nov. during growth at elevated pH values. Therefore, the initial work involved the preparation of NB in different buffer solutions of basic pH. In order to select the suitable buffers for this purpose, those solutions with a pH around 11-12 were investigated. In literature, many buffer solutions for high pH values such as KCl-NaOH, and Na_2HPO_4-NaOH are reported. Unfortunately, when NB was prepared in these buffers, following autoclaving, pH always dropped to values below 11. Thus, their buffering capacities were not strong enough to keep medium pH constant.

Since autoclaving lowered the pH of the growth medium, filter sterilization was tested as an alternative to the autoclave. When NB was prepared in filter sterilized buffers, the pH of the medium remained constant around 12. Unfortunately, when growth was monitored in these buffered solutions, significant growth of *B. marmarensis* sp. nov. never initiated before pH was lowered around to 10. Thus, preliminary findings have shown that although *B. marmarensis* sp. nov. is tolerant to high pH, its exponential growth does not start before the pH is below 11.

Since there was no significant growth above 11, finally Na-sesquicarbonate buffer, which has been reported to have a buffering capacity between pH 8.8 and 10.6 was selected. Using a buffer solution prepared only from Na_2CO_3 and $NaHCO_3$ would also eliminate the metabolic changes resulting from the presence of different salts and ions in the growth medium since, changes gene expression could also be due to changes in the buffer composition. Filter sterilization was more efficient in keeping the pH stable,

therefore for all differential expression studies filter sterilized buffers were used to prepare growth media.

Na-sesquicarbonate buffer with different pH values was prepared by mixing different combinations of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ as described in Materials and Methods. Growth and pH were monitored during growth in NB medium prepared in the filtered buffer solutions. Initially 3 different pH values were selected for this work; the three extreme values that could be prepared with Na-sesquicarbonate buffer. These pH values were 8.8, 9.7 and 10.6.

Growth of *B. marmarensis* sp. nov. in the NB prepared in buffers of different pH values is presented in Figure 4.1, Figure 4.2, and Figure 4.3. These figures also give the changes in pH during growth.

Growth was first monitored in the media with the lowest pH. Unfortunately, it was not possible to prepare the buffer with pH 8.8 under the conditions used in this work. When the recipe given in Table 3.1 was used, pH never reached 8.8. It always remained in the 9.2-9.4 range, even after filter sterilization. Therefore, for growth in this medium the initial pH was always slightly above 9.0.

When growth and pH were monitored as a function of time in this buffer, it was seen that as cells grew, pH gradually dropped and reached a final value of about 8.7. The lag phase for adaptation was rather short and the cells reached stationary phase after 10 hours of growth. The final value of OD_{600} nm was around 6.0 (Figure 4.1).

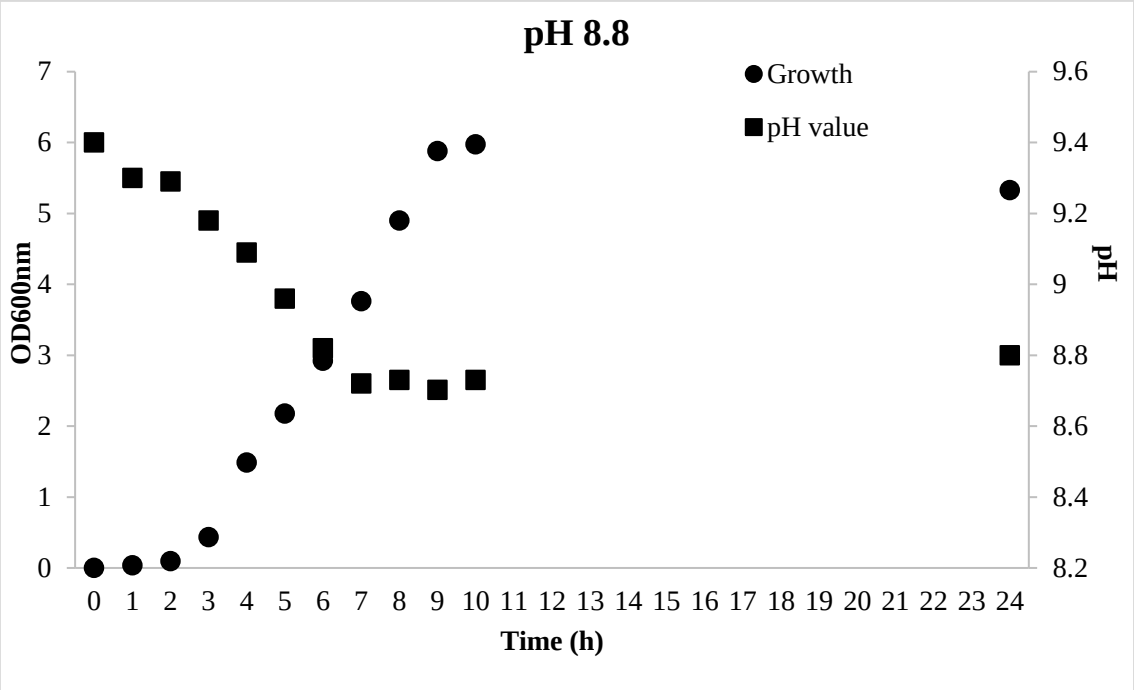

Figure 4.1. *B. marmarensis* sp. nov. growth in NB prepared in the buffer solution prepared with 90 ml 0.1M-Na_2CO_3 and 10 ml 0.1M-$NaHCO_3$ (pH 8.8, 37 °C)

The second growth was achieved in the buffered solution with pH 9.7. The optimum pH for the growth of *B. marmarensis* sp. nov. was reported as 9.8 by Denizci *et al*. (2010). Based on this information, growth was expected to be the fastest in the NB medium with an initial pH of 9.7.

Interestingly, growth pattern in this media (Figure 4.2) was similar to the growth achieved in the media with a lower initial pH (Figure 4.1). The final pH achieved was 8.7 after 24 hours and the maximum OD_{600} was around 5.5. The pH of this sample also decreased with time and reached the same value.

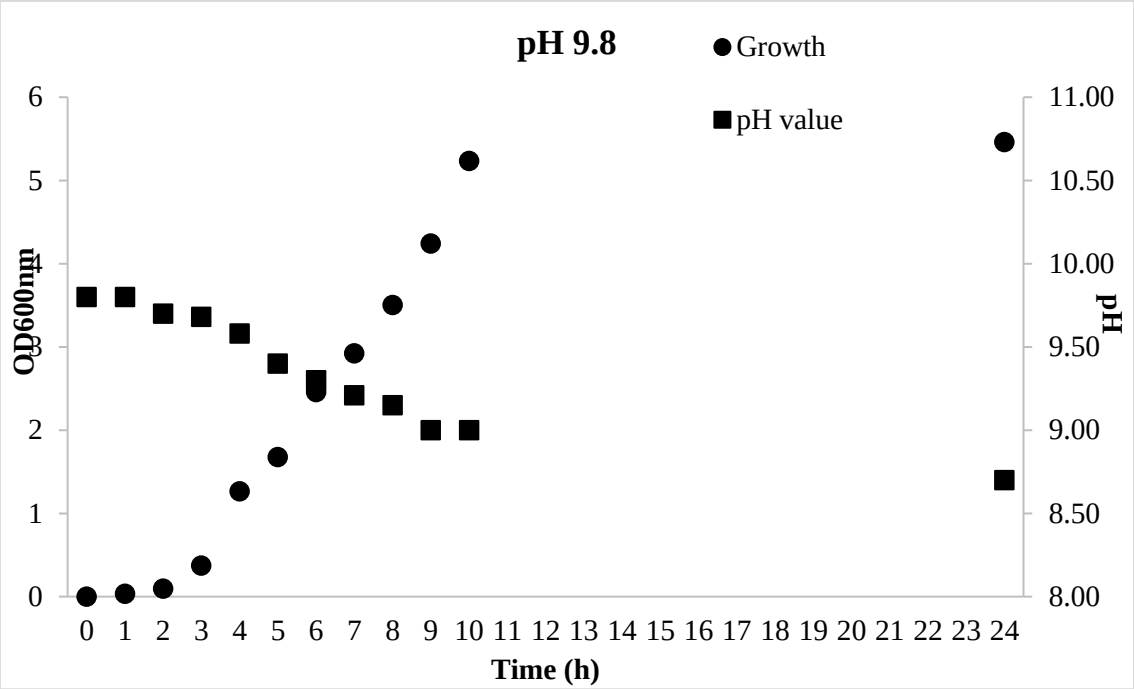

Figure 4.2. *B. marmarensis* sp. nov. growth in NB prepared in the buffer solution prepared with 50 ml 0.1M-Na_2CO_3 and 50 ml 0.1M-$NaHCO_3$ (pH 9.8, 37°C)

The final pH selected for the analysis of *B. marmarensis* sp. nov. growth was 10.6. Interestingly, although the initial pH of the NB prepared in this Na-sesquicarbonate buffer was rather high when compared to the other two conditions, the final pH at the end of 24 hours of growth was around 9.1 (Figure 4.3), similar to the final pH of the other two conditions (Figure 4.1 and Figure 4.2). The length of the lag phase was also similar to the lag phases found for growth in media with a pH around 9, but growth was much slower.

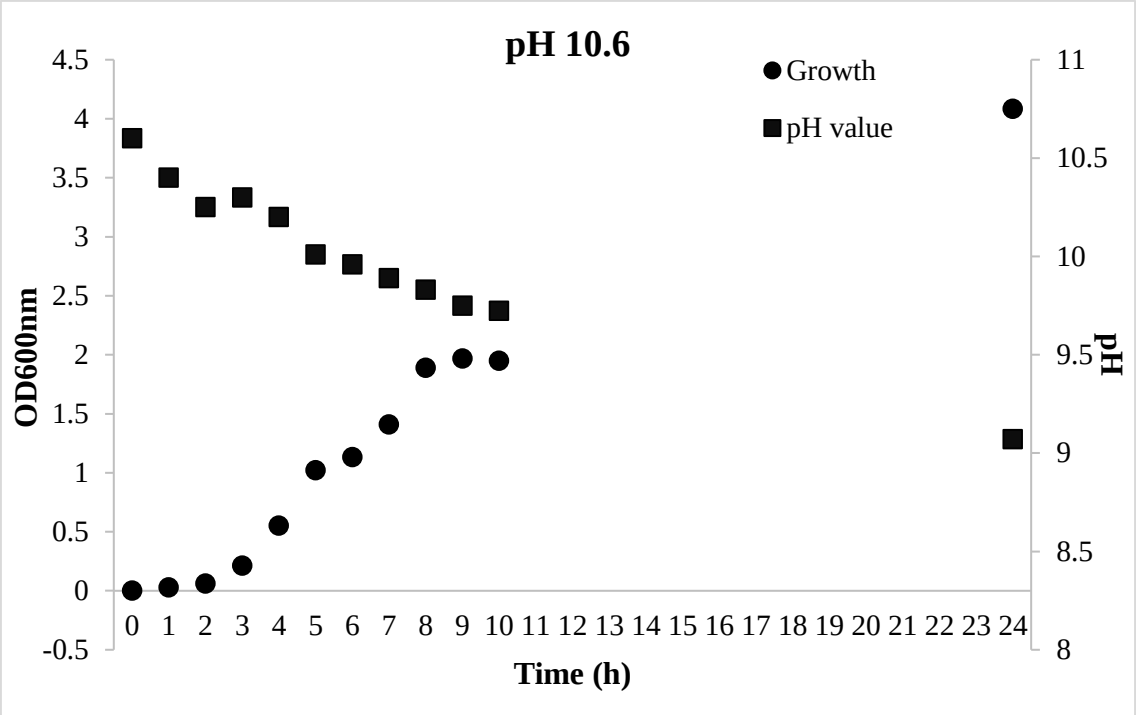

Figure 4.3. *B. marmarensis* sp. nov. growth in NB medium prepared in the buffer solution prepared with 10 ml 0.1M-Na_2CO_3 and 90 ml 0.1M-$NaHCO_3$ (pH 10.6, 37 °C)

4.2. Growth of *B. marmarensis* sp. nov. in Different pH Conditions

After achieving initial growth in Na-sesquicarbonate buffered NB with three different initial pH values, Na-sesquicarbonate buffered media was selected for further differential expression analysis. Growth experiments were conducted to determine the time of sampling for expressional analysis. First the growth characteristics of the cultures were evaluated under the three selected pH conditions. Repeated growth curves were constructed for *B. marmarensis* sp. nov. The growth curves are plotted in Figure 4.4.

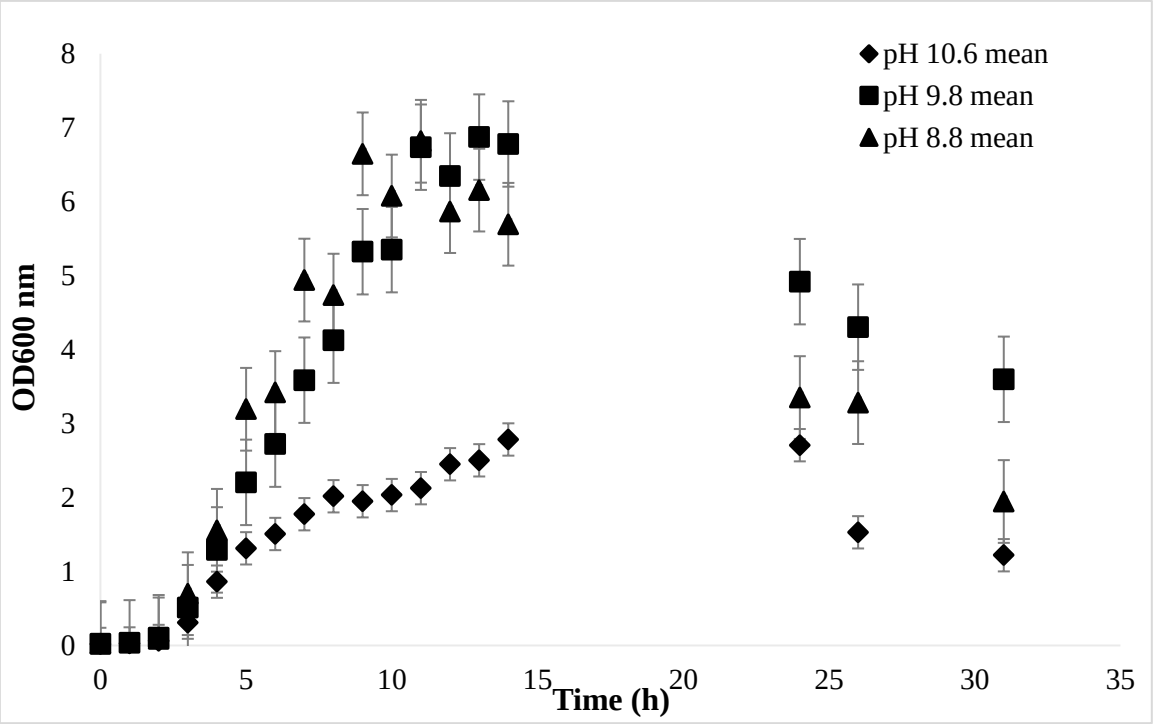

Figure 4.4. Growth curves of *B. marmarensis* sp. nov. in NB with different pH

Fine proteome and transcriptome analysis requires good quality samples in high amounts. When the samples are not good enough for analysis, misleading results can be obtained. It is also possible not to get any signals with poor quality samples. In this sense, in addition to sample preparation techniques, time of sampling is of crucial importance to get protein and RNA samples in quantities enough for analysis. Furthermore, selection of timing could also be important parameter in understanding adaptation strategies. Proteins required for adaptation would build up with time in the cell.

In all three conditions, after 8 hours of growth cells were found to be in late-log phase (Figure 4.4). The number of cells was sufficient at this point for both protein and total RNA extraction. Moreover, the 8-hour period was also long enough for adaptation. Therefore 8 hours of growth was fixed for collecting samples.

When growth in the two lower-pH media were compared, it was seen that the growth patterns and the change in pH were highly similar for the two conditions (Figure 4.4). This could be mainly because of the similar initial pH of the growth media. The difficulty in getting a pH buffer of 8.8 complicated the situation. Thus for further

comparative analysis only two conditions were selected: growth media prepared with Na-sesquicarbonate buffer of pH of 9.7 and 10.6.

4.3. Transcriptomic Analysis

Total RNA was isolated from cells grown for eight hours. The RNA concentration of samples are summarized in Table 4.1. The quality of the RNA was confirmed by BGI (Beijing, China) and only samples with good quality were used for RNA-sequencing (RNA-seq).

Table 4.1. RNA concentration of samples that were grown in 3 different pH conditions

Sample Name	RNA Concentration (ng/µL)
pH 9.8	840
pH 10.6	989

Initial analysis of the data with Rockhopper has returned a total of 2986 reads. Taking a value of $-1 \leq \log_2 \leq 1$ for the expressional change, 1498 reads were found to have a significant change in expression. Taking pH 9.8 as the base condition, expressional changes were evaluated. Among the reads, 715 were up-regulated and 783 were down-regulated when pH increased from 9.8 to 10.6. Since this was a huge list and de novo analysis was performed, the analysis was restricted to expressional changes with $-2 \leq \log_2 \leq 2$, which contained 304 up and 258 down-regulated reads.

Among the 304 up-regulated reads, 161 were found as repeats, which left only 143 reads that could be aligned to discrete genes. Following BlastX search, 102 of the reads were aligned to well-defined genes of *B. marmarensis* sp. nov. and 33 were aligned to hypothetical genes of *B. marmarensis* sp. nov. 8 of these reads were either aligned to genes of different organisms or were not aligned at all.

Among the 258 down-regulated reads, 72 were found as repeats, which left only 186 reads that could be aligned to discrete genes. Following BlastX search, 129 of the reads were aligned to well-defined genes of *B. marmarensis* sp. nov. and 45 were aligned to

hypothetical genes of *B. marmarensis* sp. nov. 12 of these reads were either aligned to genes of different organisms or were not aligned at all.

Differentially up-regulated genes are given in Table 4.2 and differentially down-regulated genes are given Table 4.3.

Table 4.2. List of up-regulated genes

			10.6	**9.8**
Log2	**protein name**	**sequence ID**	**Expression**	
6.2	peptidase S8	WP_022628745.1	1010	14
5.6	isocitrate lyase	WP_012959587.1	199	4
5.6	kynureninase	WP_022629778.1	49	1
5.3	hypothetical protein	WP_022629257.1	2802	70
5.3	sodium-dependent transporter	WP_022629775.1	40	1
	tryptophan 2,3-dioxygenase	WP_022629776.1		
5.1	peptidoglycan endopeptidase	WP_022626564.1	302	9
	dipeptide epimerase	WP_022626563.1		
4.8	spore coat protein	WP_022627091.1	141	5
4.8	hypothetical protein	WP_022629452.1	28	1
4.8	cytochrome ubiquinol oxidase subunit I	WP_022629436.1	307	11
	cytochrome D ubiquinol oxidase subunit II	WP_022629437.1		
4.7	carbonic anhydrase	WP_022627653.1	369	14
4.7	magnesium transporter	WP_022628656.1	26	1
4.5	hypothetical protein	WP_022626735.1	22	1
4.2	choline ABC transporter ATP-binding protein	WP_041203335.1	19	1
4.2	heme ABC transporter ATP-binding protein	WP_022629696.1	18	1
4.1	outer membrane lipoprotein carrier protein LolA	WP_022626605.1	17	1
4.1	hypothetical protein	WP_022629223.1	17	1
4.0	lactate dehydrogenase	WP_022629003.1	661	40
4.0	ABC transporter permease	WP_022627329.1	16	1
4.0	hypothetical protein	WP_022629212.1	16	1
3.9	mechanosensitive ion channel protein	WP_022628328.1	15	1
3.9	ABC transporter	WP_022627475.1	15	1
3.9	DUF523 domain-containing protein	WP_022627860.1	15	1
3.9	aminobenzoyl-glutamate transporter	WP_012958599.1	15	1

			10.6	**9.8**
Log2	**protein name**	**sequence ID**	**Expression**	
3.9	hypothetical protein	WP_022627988.1	15	1
3.9	hypothetical protein	WP_022629037.1	134	9
3.9	methionine ABC transporter substrate-binding protein	WP_022629042.1	59	4
3.9	transcriptional regulator	WP_022629681.1	707	49
3.9	alanine racemase	WP_022629679.1		
3.8	ABC transporter permease	WP_022629340.1	14	1
3.8	glycine/betaine ABC transporter permease	ERN53107.1	28	2
3.8	Methionine import system permease MetP (ABC transporter permease)	WP_012960351.1	14	1
3.8	hypothetical protein	WP_022629669.1	28	2
3.8	MBL fold metallo-hydrolase	WP_022628958.1	293	21
3.8	sodium-independent anion transporter (sulfate transporter)	WP_022627791.1	27	2
3.7	aminodeoxychorismate lyase	ERN55030.1	13	1
3.7	radical SAM/SPASM domain-containing protein	WP_022629246.1	13	1
3.7	hypothetical protein	ERN54311.1	13	1
3.7	YlbF family regulator	WP_022626453.1	102	8
3.7	hypothetical protein	WP_022629258.1	714	56
3.5	ABC transporter permease (branched-chain amino acid ABC transporter permease)	WP_012957398.1	46	4
3.5	hypothetical protein	ERN53698.1	891	78
		WP_031311500.1		
3.5	recombinase family protein	WP_022629575.1	90	8
3.5	NupC/NupG family nucleoside CNT transporter	WP_022626915.1	22	2
3.4	aldo/keto reductase (D-threo-aldose 1-dehydrogenase)	WP_022629969.1	43	4

			10.6	9.8
Log2	**protein name**	**sequence ID**	**Expression**	
3.3	hypothetical protein	ERN52011.1	111	11
3.2	hypothetical protein	WP_022628554.1	28	3
3.2	HU family DNA-binding protein	WP_012960273.1	109	12
3.2	ribonucleotide-diphosphate reductase	WP_022628406.1	160	18
3.1	universal stress protein	WP_022627790.1	17	2
3.1	ABC transporter ATP-binding protein (Macrolide ABC transporter ATP-binding protein)	WP_022627474.1	25	3
3.1	hypothetical protein	WP_022627293.1	25	3
3.0	peptide ABC transporter permease	WP_022627473.1	33	4
3.0	peptidase S8	ERN55058.1	33	4
3.0	hypothetical protein	WP_022630016.1	33	4
3.0	cold-shock protein	WP_012958600.1	32	4
3.0	spore germination protein	WP_022627050.1	16	2
3.0	tRNA pseudouridine(55) synthase TruB	WP_022628135.1	16	2
3.0	flagellar hook-associated protein 3	WP_022629139.1	16	2
3.0	hypothetical protein	WP_083477561.1	24	3
		ERN55102.1		
2.9	DUF1049 domain-containing protein	WP_012957768.1	77	10
2.9	citrate transporter	WP_022629449.1	23	3
2.9	hypothetical protein	ERN52306.1	23	3
2.9	Spo0E family sporulation regulatory protein-aspartic acid phosphatase	WP_012957183.1	15	2
2.9	polysaccharide deacetylase	WP_083477628.1	15	2
2.9	hypothetical protein	WP_022628073.1	15	2
2.9	phosphoenolpyruvate carboxylase	WP_022626697.1	22	3
2.8	ATP-dependent helicase (DEAD/DEAH box helicase)	WP_022629514.1	50	7
2.8	hypothetical protein	WP_022628039.1	100	14

			10.6	9.8
Log2	**protein name**	**sequence ID**	**Expression**	
2.8	hypothetical protein	ERN54331.1	92	13
		ERN54332.1		
2.8	gas vesicle protein GvpU	ERN51587.1	21	3
2.8	peptidoglycan-binding protein	WP_022628079.1	381	55
2.8	peptidase	WP_022629221.1	48	7
2.8	general stress protein	WP_012960839.1	143	21
2.7	peptidase U32	WP_022626539.1	20	3
2.7	NAD(P)-dependent oxidoreductase	WP_022629284.1	20	3
2.7	hypothetical protein	ERN51588.1	20	3
2.7	CTP synthase	WP_022629390.1	73	11
2.7	hypothetical protein	WP_022629438.1	79	12
2.7	hypothetical protein	WP_022629184.1	105	16
2.7	adenylosuccinate synthase	WP_022628424.1	111	17
2.7	zinc ribbon domain-containing protein	WP_022629339.1	97	15
2.7	thiol reductant ABC exporter subunit CydD	WP_022627862.1	161	25
2.7	hypothetical protein	WP_022627248.1	32	5
2.6	acetate kinase	WP_012960043.1	75	12
2.6	BMP family ABC transporter substrate-binding protein	WP_012957395.1	212	35
2.6	phosphoenolpyruvate carboxykinase	WP_022628921.1	2787	461
2.6	sodium:alanine symporter family protein	WP_022629682.1	54	9
2.6	cysteine desulfurase (aminotransferase V)	WP_022628858.1	12	2
2.6	S-layer homology domain-containing protein	WP_083477664.1	54	9
2.6	hypothetical protein	WP_022627301.1	90	15
		WP_022627302.1		
2.5	3-oxoacyl-ACP reductase	WP_022627783.1	17	3
2.5	STAS/SEC14 domain-containing protein	WP_083477588.1	51	9
2.5	hypothetical protein	WP_022628282.1	72	13

			10.6	9.8
Log2	**protein name**	**sequence ID**	**Expression**	
2.4	hypothetical protein	WP_022626928.1	812	149
2.4	PTS glucose transporter subunit IIABC	WP_022629808.1	32	6
2.4	aspartate--tRNA ligase	WP_022626520.1	88	17
2.4	alanine dehydrogenase	WP_022628082.1	459	89
2.3	rhodanese-like domain-containing protein	WP_031311345.1	20	4
2.3	hypothetical protein	WP_055725547.1	15	3
		ERN51629.1		
2.3	hypothetical protein	ERN51860.1	15	3
2.3	ectoine hydroxylase (multidrug DMT transporter permease)	WP_022626640.1	84	17
2.2	phosphate ABC transporter substrate-binding protein	ERN52786.1	19	4
2.2	catalase HPII (hydroperoxidase II)	WP_022629546.1	66	14
2.2	GMP reductase (guanosine 5'-monophosphate oxidoreductase)	WP_022626653.1	37	8
2.2	sodium:phosphate symporter	WP_022626877.1	23	5
2.2	23S rRNA (adenine(2503)-C(2))-methyltransferase RlmN	WP_022629559.1	78	17
2.2	type 1 glutamine amidotransferase domain-containing protein	WP_012960772.1	32	7
2.2	protease modulator HflC (tail fiber protein)	WP_022628830.1	99	22
2.2	bifunctional murein DD-endopeptidase/murein LD-carboxypeptidase	WP_022626557.1	36	8
2.2	hypothetical protein	WP_022626549.1	18	4
2.1	phosphate/phosphite/phosphonate ABC transporter substrate-binding protein	WP_022628071.1	22	5
	phosphonate ABC transporter substrate-binding protein	ERN53427.1		

			10.6	9.8
Log2	**protein name**	**sequence ID**	**Expression**	
2.1	DoxX family protein (Crp/Fnr family transcriptional regulator)	WP_022629092.1	664	152
2.1	stage II sporulation protein E	WP_041203446.1	13	3
2.1	type 1 glutamine amidotransferase (general stress protein)	WP_022628678.1	188	44
2.1	thiamine biosynthesis protein ThiI	ERN52316.1	34	8
2.1	GGDEF domain-containing protein	WP_083477671.1	17	4
2.1	undecaprenyl-diphosphatase UppP	WP_022627021.1	38	9
2.1	peptidoglycan-binding protein	WP_022628555.1	21	5
2.1	penicillin-binding protein	WP_022627199.1	147	35
2.1	Dipeptide ABC transporter ATP-binding protein	WP_022627938.1	100	24
2.1	malate synthase G	WP_022628344.1	108	26
2.1	sodium:phosphate symporter	WP_022628665.1	54	13
2.1	DNA topoisomerase I	WP_022628183.1	58	14
2.1	hypothetical protein	WP_022626471.1	29	7
2.0	methionine adenosyltransferase	WP_022628920.1	121	30
2.0	TatD family deoxyribonuclease	WP_022627953.1	12	3
2.0	DUF302 domain-containing protein	WP_022626726.1	20	5
2.0	hypothetical protein	WP_022628193.1	12	3
2.0	hypothetical protein	WP_022629266.1	36	9
2.0	30S ribosomal protein S20	ERN54693.1	468	119
2.0	phenylalanine--tRNA ligase subunit beta	WP_022628814.1	35	9
2.0	N-acetyltransferase (GCN5 family acetyltransferase)	WP_022629864.1	31	8
1.9	D-amino-acid transaminase	WP_022628407.1	23	6
1.9	cytochrome P450	WP_022629519.1	19	5
1.0	preprotein translocase subunit SecY	WP_022630055.1	463	229
	DNA-directed RNA polymerase subunit	ERN51067.1		

			10.6	**9.8**
Log2	**protein name**	**sequence ID**	**Expression**	
	alpha			
	50S ribosomal protein L2	WP_022630054.1		

Table 4.3. List of down-regulated genes

			10.6	**9.8**
log2	**protein name**	**sequence ID**	**Expression**	
6.1	capsid protein	ERN52791.1	1	68
	phage portal protein	WP_022628463.1		
	phage major capsid protein	WP_031311568.1		
6.0	phage tail tape measure protein	WP_051320839.1	1	64
	hypothetical protein	ERN52825.1		
5.8	NAD(P)H:quinone oxidoreductase (TrpR binding protein WrbA)	WP_022627892.1	2	111
5.5	N-acetylmuramoyl-L-alanine amidase	WP_022628439.1	1	44
5.1	capsid protein	ERN52791.1	2	68
5.0	phage tail family protein	WP_022628451.1	1	32
4.5	N-acetylglucosamine-6-phosphate deacetylase	WP_031311491.1	1	22
4.3	phage terminase small subunit P27 family	WP_022628467.1	1	20
4.3	terminase large subunit	WP_022628466.1	1	20
4.2	DNA-binding protein	WP_022628485.1	3	56
4.1	alpha-glycosidase (cyclomaltodextrinase)	WP_022628604.1	1	17
4.1	VanZ family protein	WP_083477598.1	1	17
		ERN53977.1		
4.0	Rrf2 family transcriptional regulator	WP_022627637.1	2	33
3.9	alpha-glucosidase (oligo-1,6-glucosidase)	WP_022628603.1	1	15
3.9	ATP-dependent helicase	WP_022627255.1	1	15
3.9	anion transporter	WP_022629122.1	1	15
3.9	permease	WP_022627745.1	1	15
3.9	DUF2507 domain-containing protein	WP_022628798.1	1	15
3.9	formate dehydrogenase	WP_022628345.1	16	238
3.8	D-2-hydroxyacid dehydrogenase (3-phosphoglycerate dehydrogenase)	WP_022629921.1	1	14
3.8	EamA/RhaT family transporter	WP_022627516.1	1	14

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
3.8	TIGR01212 family radical SAM protein	WP_022628910.1	1	14
3.7	iron export ABC transporter permease subunit FetB (ABC transporter permease)	WP_022626907.1	1	13
3.7	FmtA	WP_022627680.1	1	13
3.7	SMI1/KNR4 family protein	WP_022627522.1	1	13
3.7	STAS domain-containing protein	WP_083477645.1	2	26
3.6	dihydrodipicolinate synthase family protein	WP_022629276.1	1	12
3.6	tripartite tricarboxylate transporter TctB family protein	WP_022629272.1	1	12
3.6	anthranilate synthase component I	WP_022627159.1	14	167
	tryptophan synthase subunit beta	ERN54189.1		
	histidinol-phosphate transaminase	WP_022627165.1		
3.5	PTS acetylglucosamine transporter subunit IIB	WP_022627836.1	2	23
3.5	tryptophan synthase subunit beta	WP_031311397.1	15	169
3.5	primase	WP_022628483.1	1	11
3.4	MMPL family transporter	WP_022629745.1	27	289
3.3	DUF418 domain-containing protein	WP_022627600.1	2	20
3.3	XRE family transcriptional regulator	WP_022629588.1	4	39
3.2	MFS transporter	WP_083477683.1	2	18
		ERN51709.1		
3.2	LysR family transcriptional regulator	WP_022627501.1	2	18
3.1	N-acetyltransferase (GNAT family acetyltransferase)	WP_022627332.1	3	26
3.1	N-acetylglucosamine-6-phosphate deacetylase	WP_031311491.1	2	17
3.1	PspC domain-containing protein (ohage shock protein)	WP_022627805.1	2	17
	hypothetical protein	WP_022627806.1		

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
3.1	antibiotic biosynthesis monooxygenase	WP_022626488.1	3	25
3.0	DUF2200 domain-containing protein	WP_022627558.1	10	82
3.0	amidase	WP_022629800.1	2	16
3.0	MFS transporter	WP_022628221.1	19	149
2.9	alpha/beta hydrolase (phospholipase)	WP_022628915.1	2	15
2.9	YitT family protein	WP_012960778.1	23	169
2.8	integrase	WP_022627318.1	11	79
2.8	ATP-dependent helicase DinG (DNA polymerase III subunit epsilon)	WP_022627187.1	2	14
2.8	dephospho-CoA kinase (shikimate kinase)	WP_022628417.1	2	14
2.8	group II intron reverse transcriptase/maturase	WP_022629923.1	2	14
2.8	transcriptional regulator NadR	WP_022626499.1	3	21
2.8	HxlR family transcriptional regulator	ERN53755.1	13	90
2.8	DUF402 family protein	WP_012957594.1	34	234
2.8	peptidoglycan editing factor PgeF	WP_022628239.1	8	54
	laccase	ERN53221.1		
2.7	DUF1453 domain-containing protein (membrane protein)	WP_022628084.1	24	161
2.7	DUF1751 domain-containing protein (S54 family peptidase)	WP_022628757.1	8	52
2.7	MFS transporter	WP_083477683.1	2	13
2.7	sporulation histidine kinase inhibitor Sda	WP_022629918.1	4	26
2.7	TetR/AcrR family transcriptional regulator	WP_022629928.1	4	26
2.7	aromatic acid exporter family protein	WP_022626942.1	2	13
2.7	CoA pyrophosphatase (NUDIX hydrolase)	WP_022626713.1	10	64
2.6	ligand-binding protein SH3	WP_022626511.1	12	74
2.6	phytoene desaturase	WP_022626991.1	6	37
2.6	nitronate monooxygenase (2-nitropropane	WP_022628299.1	2	12

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
	dioxygenase)			
2.6	membrane protein	WP_022628530.1	2	12
2.6	molybdopterin molybdenumtransferase	ERN52658.1	3	18
2.5	endonuclease	WP_031311785.1	4	23
		ERN51664.1		
2.5	GGDEF domain-containing protein	WP_022628864.1	17	97
2.5	beta-N-acetylhexosaminidase	WP_022627743.1	6	34
2.5	PAS domain-containing sensor histidine kinase	WP_083477646.1	3	17
2.5	hypothetical protein	ERN52601.1		
2.5	GNAT family acetyltransferase	ERN54412.1	8	45
2.5	ABC transporter ATP-binding protein (multidrug ABC transporter ATP-binding protein)	WP_022629915.1	22	121
2.5	bacillithiol biosynthesis cysteine-adding enzyme BshC	WP_022628259.1	2	11
2.5	dihydrodipicolinate synthase family protein	WP_022629276.1	2	11
2.5	N-acetyltransferase	WP_022627683.1	2	11
2.4	DUF2071 domain-containing protein	WP_022626662.1	5	27
2.4	cysteine hydrolase (isochorismatase hydrolase)	WP_012960266.1	4	21
2.4	glutathione peroxidase	WP_022629794.1	5	26
2.4	2-dehydropantoate 2-reductase	WP_083477625.1	5	26
2.4	DUF309 domain-containing protein	WP_022627064.1	5	26
2.4	ChrA protein	ERN52199.1	6	31
2.3	HlyC/CorC family transporter	WP_022627470.1	60	305
2.3	3-hydroxyacyl-CoA dehydrogenase	WP_022629047.1	3	15
2.3	GNAT family N-acetyltransferase	WP_022628081.1	8	40
2.3	DUF2197 domain-containing protein	WP_022628294.1	3	15

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
2.3	DUF3196 domain-containing protein	WP_022628778.1	3	15
2.3	DUF997 domain-containing protein	WP_022627843.1	3	15
2.3	glycerophosphodiester phosphodiesterase	WP_022627913.1	2	10
2.3	N-acetylmuramoyl-L-alanine amidase	WP_022628439.1	2	10
2.3	NUDIX domain-containing protein	ERN54404.1	2	10
	N-acetyltransferase	WP_022627372.1		
2.3	glucosamine-6-phosphate deaminase	WP_022627834.1	15	73
2.3	MMPL family transporter	WP_022629745.1	35	170
	type II secretion system protein	WP_022629747.1		
2.2	veg	ERN53520.1	29	137
2.2	phosphatidylglycerophosphatase A	WP_022626913.1	25	118
2.2	type II toxin-antitoxin system PemK/MazF family toxin (PemK family transcriptional regulator)	WP_012957256.1	23	108
2.2	isovaleryl-CoA dehydrogenase	WP_022627247.1	4	18
2.2	LysR family transcriptional regulator	WP_022629709.1	4	18
2.2	zinc metallopeptidase	WP_012958521.1	136	605
2.1	carotenoid biosynthesis protein	WP_022626997.1	5	22
2.1	3-phosphoshikimate 1-carboxyvinyltransferase	WP_022627167.1	32	140
2.1	ATP-dependent DNA helicase RecQ	WP_022627116.1	8	35
	hypothetical protein	WP_022627115.1		
2.1	inositol monophosphatase	WP_022628296.1	44	191
2.1	excinuclease ABC subunit UvrC	WP_022628800.1	3	13
2.1	phytoene desaturase (capsular polysaccharide biosynthesis protein CpsH)	WP_022626992.1	15	65
	phytoene/squalene synthase family protein	WP_022626993.1		
2.1	pyrroline-5-carboxylate reductase	WP_022626974.1	3	13
2.1	thioredoxin	WP_022628801.1	152	658

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
2.1	LLM class flavin-dependent oxidoreductase	WP_022629540.1	11	47
2.1	TraB/GumN family protein	WP_083477591.1	11	47
	hypothetical protein	ERN54446.1		
2.1	esterase	WP_022628267.1	4	17
2.1	acyl-CoA thioesterase	WP_022628796.1	4	17
2.1	glycine dehydrogenase (aminomethyl-transferring)	WP_022628410.1	136	575
2.1	ABC transporter substrate-binding protein	WP_022628602.1	12	50
2.0	methylmalonyl-CoA epimerase	WP_022626945.1	122	495
2.0	prepilin peptidase	WP_022628689.1	3	12
2.0	potassium/proton antiporter	WP_012961218.1	14	56
2.0	lipoate-protein ligase A	WP_031311329.1	3	12
		ERN54613.1		
2.0	PaaI family thioesterase	WP_041203489.1	6	24
2.0	sigma-54-dependent Fis family transcriptional regulator (ATPase AAA)	WP_022628378.1	3	12
2.0	EamA/RhaT family transporter	WP_022627516.1	5	20
	hypothetical protein	WP_022627517.1		
2.0	LacI family transcriptional regulator	WP_022629275.1	4	16
2.0	N-acetyltransferase	WP_022627664.1	3	12
	HGG motif-containing thioesterase	ERN53073.1		
	phage tail tape measure protein	WP_051320839.1	0	53
	hypothetical protein	ERN52825.1		
	hypothetical protein	ERN52820.1	0	39
	hypothetical protein	WP_022628455.1	0	43
	hypothetical protein	ERN52822.1	0	35
	hypothetical protein	ERN52816.1	0	30
2.2	hypothetical protein	WP_012958188.1	5	23
2.9	hypothetical protein	WP_012958455.1	5	38

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
3.2	hypothetical protein	WP_022627719.1	4	37
3.2		WP_022627718.1		
3.2	hypothetical protein	WP_022627300.1	2	18
3.2		WP_022629842.1		
2.9	hypothetical protein	WP_022627719.1	6	45
2.9		WP_022627718.1		
2.9	hypothetical protein	WP_022627944.1	2	15
2.5	hypothetical protein	WP_022629753.1	4	23
2.7	hypothetical protein	WP_022629909.1	2	13
2.3	hypothetical protein	WP_022626550.1	2	10
2.1	hypothetical protein	WP_022626598.1	10	43
2.5	hypothetical protein	WP_022626680.1	3	17
2.9	hypothetical protein	WP_022626918.1	2	15
3.7	hypothetical protein	WP_022626954.1	1	13
3.6	hypothetical protein	WP_022627177.1	1	12
2.3	hypothetical protein	WP_022627216.1	7	34
3.2	hypothetical protein	WP_022627306.1	2	19
3.2		ERN54336.1		
2.5	hypothetical protein	WP_022627524.1	2	11
2.0	hypothetical protein	WP_031311458.1	11	44
2.0		ERN53981.1		
3.5	hypothetical protein	WP_022627565.1	1	11
2.3	hypothetical protein	WP_022627570.1	5	24
3.7	hypothetical protein	WP_022627601.1	1	13
2.3	hypothetical protein	WP_022627602.1	2	10
2.4	hypothetical protein	WP_022627644.1	9	48
2.7	hypothetical protein	WP_083477604.1	3	19
2.7		ERN53890.1		
3.0	hypothetical protein	WP_022627667.1	2	16

			10.6	9.8
log2	**protein name**	**sequence ID**	**Expression**	
3.2	hypothetical protein	WP_022627676.1	2	18
2.8	hypothetical protein	WP_022627701.1	3	21
2.5	hypothetical protein	WP_022627734.1	6	34
2.7	hypothetical protein	WP_022627739.1	34	222
2.2	hypothetical protein	WP_022628067.1	8	36
2.2	hypothetical protein	WP_022628444.1	7	32
3.2	hypothetical protein	WP_022628473.1	2	18
2.7	hypothetical protein	WP_022628627.1	2	13
2.2	hypothetical protein	WP_022628695.1	3	14
3.2	hypothetical protein	WP_022628773.1	2	18
2.4	hypothetical protein	WP_022628795.1	10	54
2.1	hypothetical protein	WP_022628948.1	3	13
2.3	hypothetical protein	WP_022628964.1	2	10
2.8	hypothetical protein	WP_031311847.1	2	14
2.5	hypothetical protein	WP_083477604.1	2	11
2.3	hypothetical protein	WP_031311845.1	5	25
2.1	hypothetical protein	WP_022627546.1	3	13
3.1	hypothetical protein	ERN53980.1	2	17
2.4	hypothetical protein	WP_012957711.1	37	202
2.5	hypothetical protein	WP_049779949.1	6	34
2.4	hypothetical protein	WP_031311357.1	16	82

4.4. Proteome

Total cellular proteins were extracted from cells grown for 8 hours. Then 2D-PAGE was used to evaluate expressional changes in the proteome. The changes were evaluated taking 9.8 pH as the base case. Following Coommassie staining, the representative proteome maps obtained for the two conditions are given in Figure 4.4 and Figure 4.5. The analysis of these two maps with PDQuest Advance Software (BioRad, USA) returned a total of 929 spots on the protein map of the cells grown at pH 9.8 and a total of 662 spots on the protein map of the cells grown at pH 10.6.

When the protein maps for the two conditions were compared, 45 protein spots were selected for further analysis and excised from gels. Among the 45 spots, only 27 of them match-up with a protein sequence. The results obtained following MALDI-TOF analysis showed that only 20 of these spots had differential expression, while in the remaining 7 spots there was no significant change in expression as pH increased. Only 5 of the up-regulated and 15 of the down-regulated spots that have been excised returned matches to proteins of *B. marmarensis* sp. nov. Except for the 2 down-regulated proteins, all were matched to specific *B. marmarensis* sp. nov. proteins. Unfortunately, the expressional change between the two conditions was not very high. Furthermore, the coverage and score of the results were not satisfying.

pH 3 pH 6

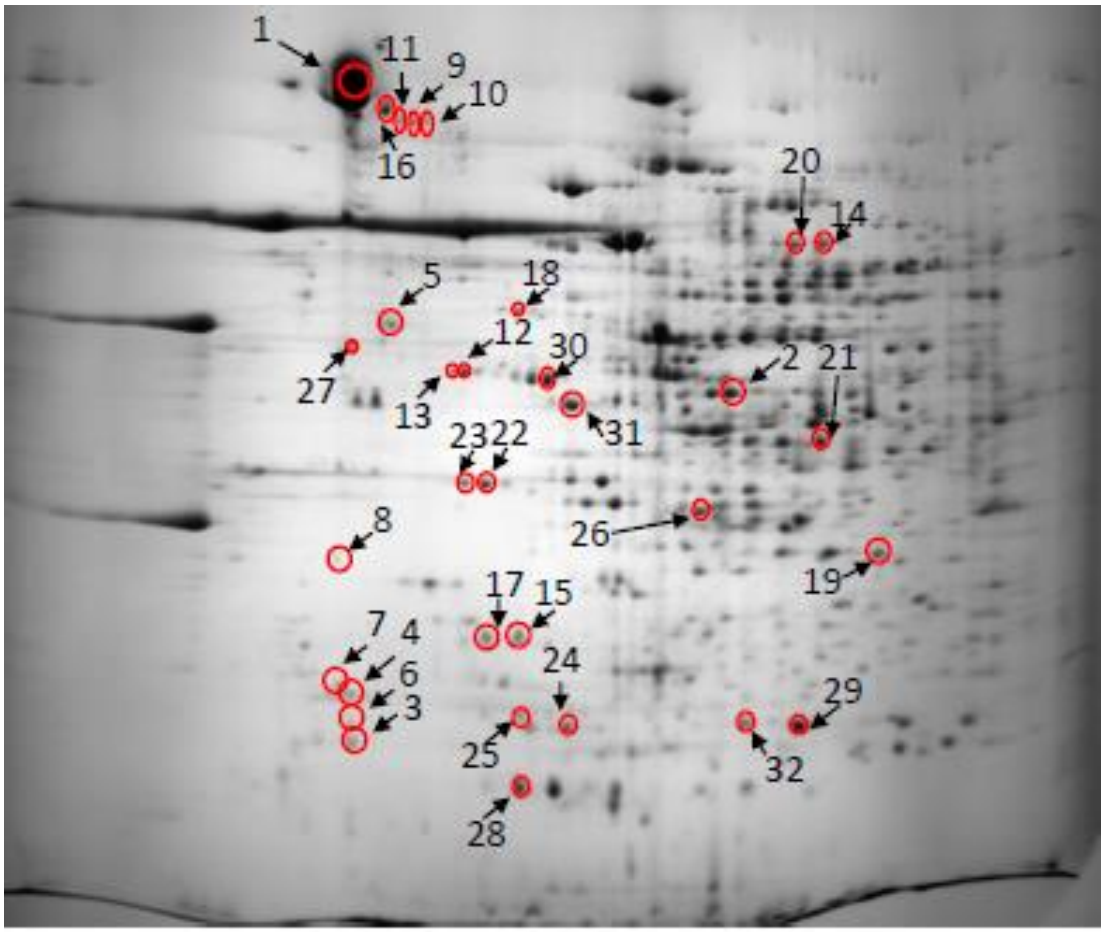

Figure 4.5. Representative proteome map for the cells grown in media with an initial pH of 9.8

pH 3 pH 6

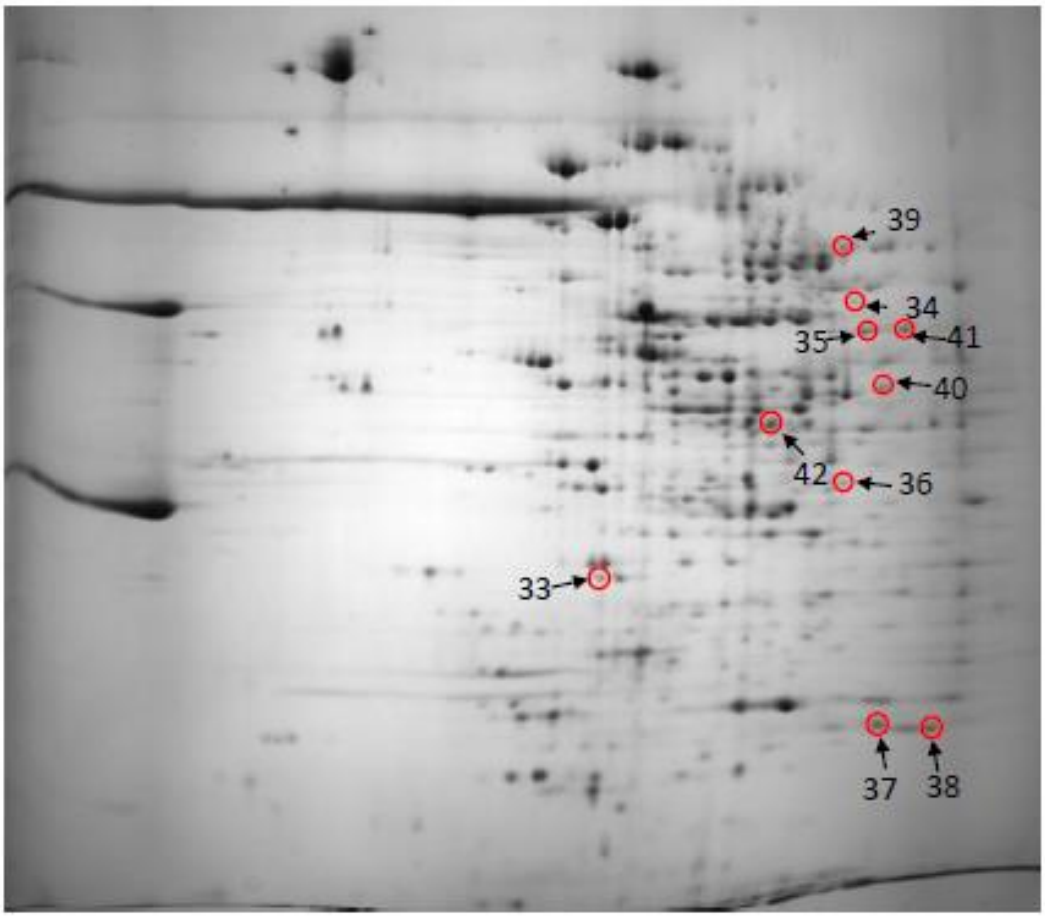

Figure 4.6. Representive proteome map for the cells grown in media with an initial pH of 10.6

Table 4.4. Proteome results of MALDI-TOF analysis

Spot ID	AC no.	Best Protein Accession	Best Protein Mass	Best Protein Score	Matches	Calc pI	Seq. Cov. (%)	FOLD	UP/ DOWN	Best Protein Description
J24	U6SN43	WP_022628393.1	49556	745	28	5.26	51	1,14	Down	acetyl-CoA carboxylase biotin carboxylase subunit
J8	U6SL31	ERN52313.1	22829	59	13	9.94	46	1,36	Up	30S ribosomal protein S4
J1	U6SSW7	ERN54804.1	22452	174	7	5.18	42	1,84	Up	superoxide dismutase
J4	U6SQS5	WP_022628374.1	35889	607	23	4.91	38	1.14	Down	alpha-ketoacid dehydrogenase subunit beta
	U6SQS5	ERN52996.1	35899	607	23	4.91	38			2-oxoisovalerate dehydrogenase subunit beta
J5	U6SJR3	ERN51954.1	41625	533	25	5.07	38	1.43	Down	acyl-CoA dehydrogenase
J16	U6SLL1	WP_022629257.1	95965	659	29	4.41	38	0		hypothetical protein, partial
J2	U6SNK2	WP_022628802.1	34196	213	17	4.73	35	1.33	Down	electron transfer flavoprotein subunit alpha
J23	U6SJR3	ERN51954.1	41625	521	21	5.07	34	1.58	Up	acyl-CoA dehydrogenase
J10	U6SKC7	WP_022629220.1	39413	491	18	4.57	34	1.35	Down	hypothetical protein
J15	U6SLL1	WP_022629257.1	95965	554	25	4.41	31	0		hypothetical protein, partial
	U6SLN6	ERN51541.1	38003	253	13	5.0	30			chromosome partitioning protein ParA
J7	U6SJ50	ERN51613.1	20631	132	6	4.6	27	1.05	Up	alkyl hydroperoxide reductase subunit C
J12	U6STB1	WP_022626723.1	55870	386	24	4.62	25	2.0	Down	hypothetical protein
J14	U6SLL1	WP_022629257.1	95965	523	20	4.41	23	10.0	Down	hypothetical protein, partial
J21	U6SLL1	WP_022629257.1	95965	663	23	4.41	23	0		hypothetical protein, partial
J6	Q9KG45	GLMS_BACHD	65836	485	20	5,22	21	1.56	Down	Glucosamine--fructose-6-phosphate aminotransferase [Bacillus halodurans]
K1	U6SHJ3	ERN51043.1	20044	19	7	5.15	20	3.7	Down	transcription antitermination protein NusG
J3	U6SMV9	WP_022629396.1	31266	184	7	5.18	19	1.69	Down	3-hydroxybutyryl-CoA dehydrogenase
J11	U6SM75	WP_022629184.1	129447	453	22	4.33	11	5.88	Down	hypothetical protein
J20	U6SLL1	WP_022629257.1	95965	198	11	4.41	9	0		hypothetical protein, partial
K2	B1HVR0	CLPP_LYSSC	21614	116	4	5,43	8	1.08	Down	ATP-dependent Clp protease proteolytic subunit [Lysinibacillus sphaericus]

J9	U6SH11	ERN51014.1	19977	31	2	9.4	4	2.22	Down	50S ribosomal protein L5
J13	U6SH11	ERN51014.1	19977	35	2	9.4	4	1.42	Up	50S ribosomal protein L5
J22	U6SH11	ERN51014.1	19977	34	2	9.4	4	1.49	Down	50S ribosomal protein L5
J17	U6SKD4	WP_022629230.1	97865	66	7	3.84	4	0		hypothetical protein
J18	U6SKD4	WP_022629230.1	97865	75	9	3.84	3	0		hypothetical protein
J19	U6SKD4	WP_022629230.1	97685	44	9	3.84	3	0		hypothetical protein
K3	U6SNW7	ERN53313.1	39843	601	28	**0**	**0**	2.13	Down	alanine dehydrogenase

5. DISCUSSION

The transcriptome and the proteome were analysed to understand and evaluate the alkaliphilic adaptation mechanism of *B. marmarensis* sp. nov.

Although significant changes have been expected between the two conditions, proteomic results have shown that the gels obtained for the two different pH conditions were not very different. Consistent with this observation, among the 27 protein spots selected for analysis, only 6 proteins had 2-fold or more expressional change. Furthermore, only one of these proteins had a sequence coverage over 50%. The low coverage obtained for the identification results could be explained by the novelty of *B. marmarensis* sp. nov. but this further questioned the reliability of the identification results.

In order to get further details on the differential expression a different approach, RNA-seq was performed. Examination of the RNA-seq results using Rockhopper has returned a total of 2985 differentially expressed genes with a fold change over 4. This expressional difference from the proteome analysis can simply be explained by the inefficiency of 2D-PAGE based proteomic tools for the detection of proteins with low expression levels. Thus, the limit of detection in proteomics is restricted to highly expressed proteins; therefore many proteins with low expression levels were missed on the 2D gels.

In either OMIC approach, the analysis is further complicated by the novelty of the organism. Many proteins and genes that have been identified were tagged as 'hypothetical protein'. The only common protein of the two approaches was the down-regulated hypothetical protein with ID of WP_022629257.1.

Based on the RNA-seq results, below discussed are the groups of proteins or pathways that had a significant role in the adaptation of *B. marmarensis* sp. nov. to high alkalinity. The categorized proteins are summarized in Table 5.1.

Table 5.1. Categorization of genes form RNA-seq that were important for pH adaptation

Protein Name	Fold change (log_2)	Up/Down
Stress Proteins		
General stress protein (WP_012960839.1)	2.8	Up
Type1 glutamine amidotransferase (general stress protein) (WP_022628678.1)	2.1	Up
Universal stress protein (WP_022627790.1)	3.1	Up
Cold-shock protein (WP_012958600.1)	3.0	Up
Ectoine hydroxylase (WP_022626640.1)	2.3	Up
Glycine Betaine ABC transporter permease (WP_022628306.1)	3.8	Up
Choline ABC transporter ATP binding protein (WP_041203335.1)	4.2	Up
Proteins associated with oxidative stress		
Glutathione peroxidase (WP_022629794.1)	2.4	Down
Hydroperoxidase II (ERN51585.1)	2.2	Up
Aldo-keto reductase (WP_022629969.1)	3.4	Up
Proteins associated with cell wall reorganisation		
N-acetylmuramoyl-L-alanine amidase (WP_022628439.1)	5.5	Down
Amidase (WP_022629800.1)	3.0	Down
Bifunctional murein DD-endopeptidase/murein LD-carboxypeptidase (WP_022626557.1)	2.2	Up
Glucosamine-6-phosphate deaminase (WP_022627834.1)	2.3	Down

N-acetylglucosamine-6-phosphate deacetylase (WP_031311491.1)	3.1	Down
N-acetylglucosamine-6-phosphate deacetylase (WP_031311491.1)	4,5	Down
PTS acetylglucosamine transporter subunit IIB (WP_022627836.1)	3.5	Down
Peptidoglycan editing factor PgeF	2.8	Down
Peptidoglycan-binding protein (WP_022628079.1)	2.8	Up
Peptidoglycan-binding protein (WP_022628555.1)	2.1	Up
Peptidoglycan endopeptidase (WP_022626564.1)	5.1	Up
S54 family peptidase (ERN52618.1)	2.7	Down
Penicillin-binding protein (WP_022627199.1)	2.1	Up
Membrane protein (WP_022628530.1)	2,6	Down
S-layer homology domain-containing protein (WP_083477664.1)	2.6	Up
Flagellar hook-associated protein 3 (WP_022629139.1)	3.0	Up
Polysaccharide deacetylase (WP_083477628.1)	2.9	Up
Phospholipase	2.9	Down
Outer membrane lipoprotein carrier protein LolA (WP_026626605.1)	4.1	Up
Lipoate-protein ligase A (WP_031311329.1)	2,0	Down
Cytochrome P450	1.9	Up
Cytochrome ubiquinol oxidase subunit I-II	4.8	Up
Transport Proteins		

ABC transporter permease (WP_022629340.1)	3.8	Up
ABC transporter permease (WP_022627329.1)	4.0	Up
ABC transporter ATP Binding Protein (WP_022629915.1)	2.5	Down
ABC transporter ATP Binding Protein (WP_022627474.1)	3.1	Up
ABC transporter permease (WP_022627329.1)	4.0	Up
ABC transporter (WP_022627475.1)	3.9	Up
MFS transporter (WP_083477683.1)	3.2	Down
MFS transporter (WP_026628221.1)	3.0	Down
MMPL family transporter (WP_026629745.1)	2.3	Down
Permease (WP_026627745.1)	3.9	Down
Iron export ABC transporter permease subunit FetB (WP_022626907.1)	3.7	Down
Potassium/proton antiporter (WP_012961218.1)	2.0	Down
Sodium-independent anion transporter (WP_022627791.1)	3.8	Up
Sodium:alanine symporter family protein (WP_022629682.1)	2.6	Up
Sodium:phosphate symporter (WP_022626877.1)	2.2	Up
Sodium:phosphate symporter (WP_022628665.1)	2.1	Up
Sodium-dependent transporter (WP_022629775.1)	5.3	Up
Magnesium transporter (WP_022628656.1)	4.7	Up
Heme ABC transporter ATP-binding protein	2.1	Up

(WP_022629696.1)		
Mechanosensitive ion channel protein (WP_022628328.1)	3,9	Up
Aminobenzoyl-glutamate transporter (WP_012958599.1)	3.9	Up
Proteins of primary carbon flux		
Phosphoenolpyruvate carboxykinase (ATP) (WP_022628921.1)	2.6	Up
Phosphoenolpyruvate carboxylase (WP_022626697.1)	2.9	Up
Isocitrate lyase (WP_012959587.1)	5.6	Up
Malate synthase G (WP_012959587.1)	2.1	Up
Formate dehydrogenase (WP_022628345.1)	3.9	Down
Lactate dehydrogenase (WP_022629003.1)	4.0	Up
Citrate Transporter (WP_022629449.1)	2.9	Up
PTS glucose transporter subunit IIABC (WP_022629808.1)	2.4	Up
Proteins associated with spore formation		
Spore coat protein (WP_022627091.1)	4.8	Up
Spore germination protein (WP_022627050.1)	3.0	Up
Stage II sporulation protein E (WP_041203446.1)	2.1	Up
Sporulation histidine kinase inhibitor Sda (WP_022629918.1)	2.7	Down

Stress Proteins

The ability of survival in different environmental conditions could be challenging for organisms thus specialized strategies might be necessary for preserving vital activities.

Specifically under extreme conditions such as high or low heat, salt, pH, O_2 etc. most *Bacillus* species induce the expression of stress proteins. These commonly are classified as general stress proteins (Gsp) and heat-specific stress proteins (Hsp) (Bernhardt *et al.*, 1997). In line with these expectations, a major group of genes with differential expression levels belonged to stress proteins. These were all up-regulated upon increasing the pH of the growth medium. These were the genes of two proteins classified as general stress proteins (WP_012960839.1 and WP_022628678.1) and the gene of a universal stress protein (WP_022627790.1) in *B. marmarensis* sp. nov.

Cold shock proteins (Csp) constitute another group of proteins, which are indicators of stress conditions. Despite initial studies that reported their induction during rapid temperature downshift, some of them were also found to be non-cold inducible. Thus they promote both normal growth and stress adaptation responses. Csps have been shown to contribute to osmotic, oxidative, starvation, pH and ethanol stress tolerance as well as to host cell invasion (Keto-Timonen *et al.*, 2016). The gene that encodes the cold shock protein (WP_012958600.1) in *B. marmarensis* sp. nov. was found to be significantly induced with increasing growth medium pH.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) and hydroxyectoine ((4S,5S)-5-Hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) are well known small organic molecules that are present in bacteria but more general for microorganisms that are commonly exposed to osmotic stress in their environments (e.g. halophiles). They act as compatible solutes adjusting cell volume and fluid balance under high osmotic pressure. The initial discovery of ectoine was in *Ectothiorhodospira halochloris*, but from that day on its presence has been confirmed in many different bacterial species (Pastor *et al.*, 2010; Janto *et al.*, 2011). It was shown to protect enzymes, membranes, and in general the cells against changing conditions such as salt concentration, temperature, and dehydration. A number of ectoine producers are capable of converting ectoine into 5-hydroxyectoine through hydroxylation. Unlike ectoine, this compatible solute was reported to possess stress-protective and function-preserving properties (Widderich *et al.*, 2014). The up-regulation of the enzyme, ectoine hydroxylase that hydroxylates ectoine clues the conversion of ectoine to hydroxyectoine during growth in a higher pH environment, most probably to cope with stress (Lippert and Galinski, 1992).

Another commonly known compatible solute is glycine betaine. Its presence has been confirmed in different organisms including different species of plants and bacteria that are exposed to stress factors like osmotic pressure, temperature or radiation etc. Its uptake has also been reported in *E. coli* while dealing with stress conditions. While it can be accumulated through its up-take from the environment, different pathways are also available in bacteria for its synthesis. One of these biosynthetic pathways oxidizes choline to get glycine betaine (Kempf and Bremer, 1998; Van Kessel *et al.*, 2015). In *B. marmarensis* sp. nov., when growth was achieved in a higher pH medium, along with the induction of the expression of glycine/betaine ABC transporter permease, the genes encoding choline ABC transporter also was induced.

Overall, the finding on these compatible solutes, clue their accumulation upon exposure to higher alkalinity. Many studies confirmed that these osmolytes behave as chaperons and assist cell for the refolding of unfolded or misfolded proteins and also protect proteins in their native forms from adverse effects of stress conditions (Diamant *et al.*, 2001). Thus their chaperon-like function suggests that these organic molecules are required for cell survival.

The analysis of the stress-related proteins have shown that, they were only up-regulated in response to increasing environmental pH, which is an indication of significant stress conditions imposed on the cells at higher alkalinity.

Sporulation proteins

Various bacterial species are capable of forming endospores, which are dormant and non-reproductive structures. Its formation is usually triggered by a lack of nutrients. These resistant structures are required for survival under unfavourable conditions. *Bacilli* are among the known endospore forming species. Its formation in *B. subtilis* is initiated by the sensor histidine kinase (KinA). Sda is the known as the inhibitor of this kinase (Jacques *et al.*, 2009).

A careful investigation of the differentially regulated genes have shown that there is a tendency in *B. marmarensis* sp. nov. to form endospores in response to increasing environmental pH. The genes of three major sporulation related proteins, spore germination protein, stage II sporulation protein E, and spore coat protein, were induced

with pH. Furthermore, the expression of the sporulation histidine kinase inhibitor was decreased, which is an indicator for an active KinA.

Re-organisation of the cell wall structure

Similar to other *Bacilli* species, *B. marmarensis* sp. nov. possesses Gram-positive cell wall features. A major structure of the cell wall is the peptidoglycan layer, which is the thick and rigid layer evolved for protection. Its major constituents are linear polymers of alternating monosaccharides, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), cross-linked by linked by short peptides, the exact structure of which depends on the bacterial species (Salton Milton RJ, 1996). It covers the cell and thus regulates the transportation of pathway intermediates, chemicals, and cellular wastes for cellular activities (Woese, 1987; Ghuysen and Hakenbeck, 1994). Being the outer-layer of the cell wall, it constitutes the major target of many attacks. Its collapse leads to cell death. Below this peptidoglycan is found the lipid bilayer, the major component of phospholipids such as phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Van Meer, Voelker and Feigenson, 2008). The fatty acids tails are variable, as long/short, branched/un-branched, and saturated/unsaturated. The distribution of different fatty acids' tails and phosphate groups in addition to the different surface peptides are known to be a part of adaptation strategy to different environmental conditions (Murínová and Dercová, 2013).

On the genome of a closely related alkaliphilic species, *B. pseudofirmus* OF4, 3 different cardiolipin synthase genes were located, which were believed to be important for its alkaliphilic adaptation. High levels of cardiolipin in the alkaliphilic organisms promote oxidative phosphorylation via facilitating rapid proton transfer along the membrane (Clejan *et al.*, 1986; Haines and Dencher, 2002; Haines, 2009).

In another alkaliphilic microorganism, *B. halodurans* C-125, the teichurono surface peptide was shown to contribute to its adaptation mechanism (Fujinami and Fujisawa, 2010). Its recently completed genome sequence showed the presence of an operon of poly-g-L-glutamate synthetase genes. These two results indicate that *B. halodurans* C-125 are enclosed by at least two acidic polymers as teichuronic acid and poly-g-L-glutamic acid with highly negative charges. Furthermore, whole genome analysis of

another alkaliphilic *Bacillus* strain, *Oceanobacillus iheyensis* also revealed the presence of a putative protein showing significant similarity to the gene product involved in teichuronopeptide biosynthesis in *B. halodurans*. Therefore, acidic polymers of poly-g-L-glutamic acid and teichuronic acid are important components of the cell wall that contributes to alkaliphilic adaptation.

In the case of *B. marmarensis* sp. nov., a significant number of proteins related to peptidoglycan synthesis were differentially expressed. The reorganisation of the preexisting cell wall requires the old cell wall material to first be metabolized by lytic transglycosylases, endopeptidases, and amidases. Thus changes in the expression of endopeptidases, and amidases were as anticipated when cells were exposed to a higher pH media. Among the differentially expressed genes of the proteins directly involved in peptidoglycan biosynthesis were peptidoglycan-binding proteins, peptidoglycan editing factor, peptidoglycan endopeptidase and penicillin-binding protein. Some of these were down- and others were up-regulated. Furthermore, the change in the expression of glucosamine-6-phosphate deaminase and N-acetylglucosamine-6-phosphate deacetylase also hint the structural changes in the peptidoglycan layer. The up-regulation of the gene for polysaccharide deacetylase may also contribute to the maintenance in cell shape (Arnaouteli *et al.*, 2015). The changes in the cell wall are not restricted to the peptidoglycan layer; the variation in the expression of genes encoding a phospholipase, lipoate-protein ligase A, and outer membrane lipoprotein carrier protein LolA are persuasive proofs for the structural changes taking place in the whole cell wall structure.

The evaluation of differentially expressed genes related to the complete cell wall underlines its importance for adaptation to alkalinity. However with the current changes, it is not possible to suggest a specific regulation pattern.

Carbon Flux Redirection

In all microorganisms central carbon flow provides the major energy supply. It also serves as the central pathway providing precursor metabolites. Impaired carbon flow may lead to endospore formation and eventually to death.

Among the investigated proteins and pathways, a striking and consistent activity was observed in the glyoxylate cycle. This is a two-step pathway where isocitrate is cleaved

by isocitrate lyase to form succinate and glyoxylate and malate synthase subsequently hydrolyzes acetyl-coenzyme A and glyoxylate to form malate and CoA. It can be seen as a shortcut for tricarboxylic acid cycle and schematic representation of this pathway shown in Figure 5.1. Two major functions have been associated with this cycle; (i) it is essential for utilization of acetate and fatty acids as carbon sources under conditions requiring gluconeogenesis, (ii) it acts as a replenishing pathway (anaplerotic pathway), refilling missing metabolic intermediates. Its up-regulation has specifically been reported for degradation of fatty acids, for which acetyl-CoA is the direct product. Recent reports suggest its involvement in response to oxidative stress and virulence (Ahn *et al.*, 2016).

The genes of the two major enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, were both induced with increasing environmental pH. Considering the activation of glyoxylate cycle under different physiological conditions, there could be various explanations for this.

The requirement for continuous re-organisation of the membrane structure may generate fatty acids from phospholipid breakdown. These in turn will be directed to glyoxylate cycle for degradation. These produced metabolites could be used for other biosynthetic reactions but they could as well be used for production of glucose through gluconeogenesis. The simultaneous induction of the gene encoding the first enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase could be seen as a proof for the latter case. This enzyme converts oxaloacetate into phosphoenolpyruvate and carbon dioxide to eventually produce glucose.

On the other hand, the increase in the expression of the citrate transporter suggests that this metabolite taken up from the environment could be funnelled to cycle. The activity of the glyoxylate cycle as an anaplerotic pathway, coupled with the increased expression of phosphoenolpyruvate carboxylase, also known as an anaplerotic enzyme suggests that they could function to make up for the missing intermediates.

A more recent perspective suggests the involvement of glyoxylate cycle in oxidative stress. By using glyoxylate cycle as an alternative to tricarboxylic acid cycle, the organism by-passes CO_2 production steps (Ahn *et al.*, 2016). This shortcut may disturb cellular redox potentials, trying to lower the formation of radicals as a defence

mechanism. The increased expression of catalase HPII (hydroperoxidase II) and aldo-keto reductase from RNA-seq and superoxide dismutase from the proteomic analysis are strong indicators of elevated levels of oxidative stress in *B. marmarensis* sp. nov. during growth in extreme alkaline conditions.

With all the summarised possibilities for the involvement of glyoxylate cycle in various metabolic pathways suggests that its exact function in the adaptation to alkalinity remains to be largely unclear at this stage.

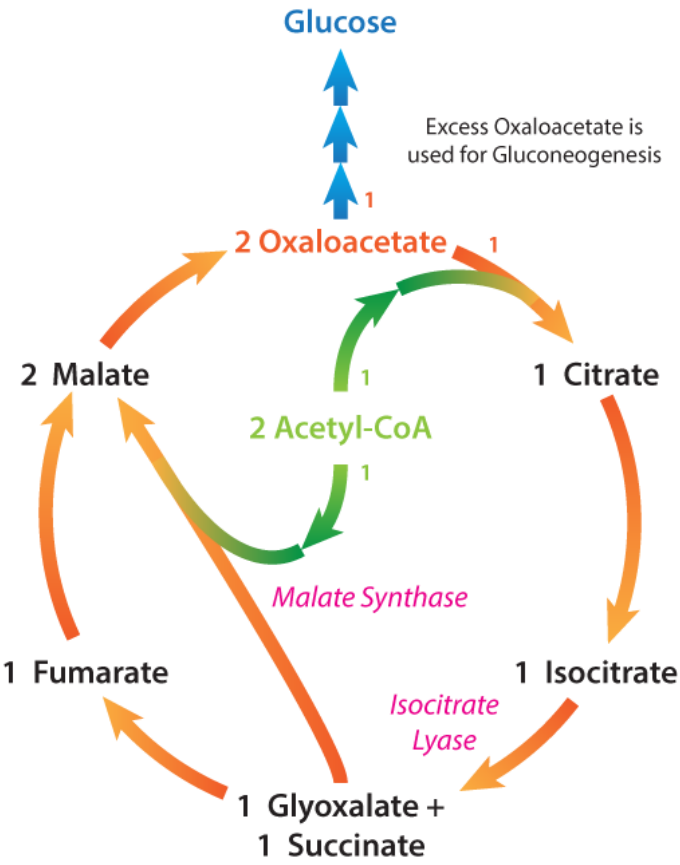

Figure 5.1. Schematic comparison of Glyoxylate shunt and TCA cycle

Transport Proteins

Transport proteins regulate trafficking of ions, small molecules, or macromolecules across biological membranes (Figure 5.2). Thus these proteins are vital to the growth and life of all living cells. The increasing pH in the growth medium triggered the

expression and repression of a huge number of transport proteins, varying from general transporters to specific ion transporters for adaptation.

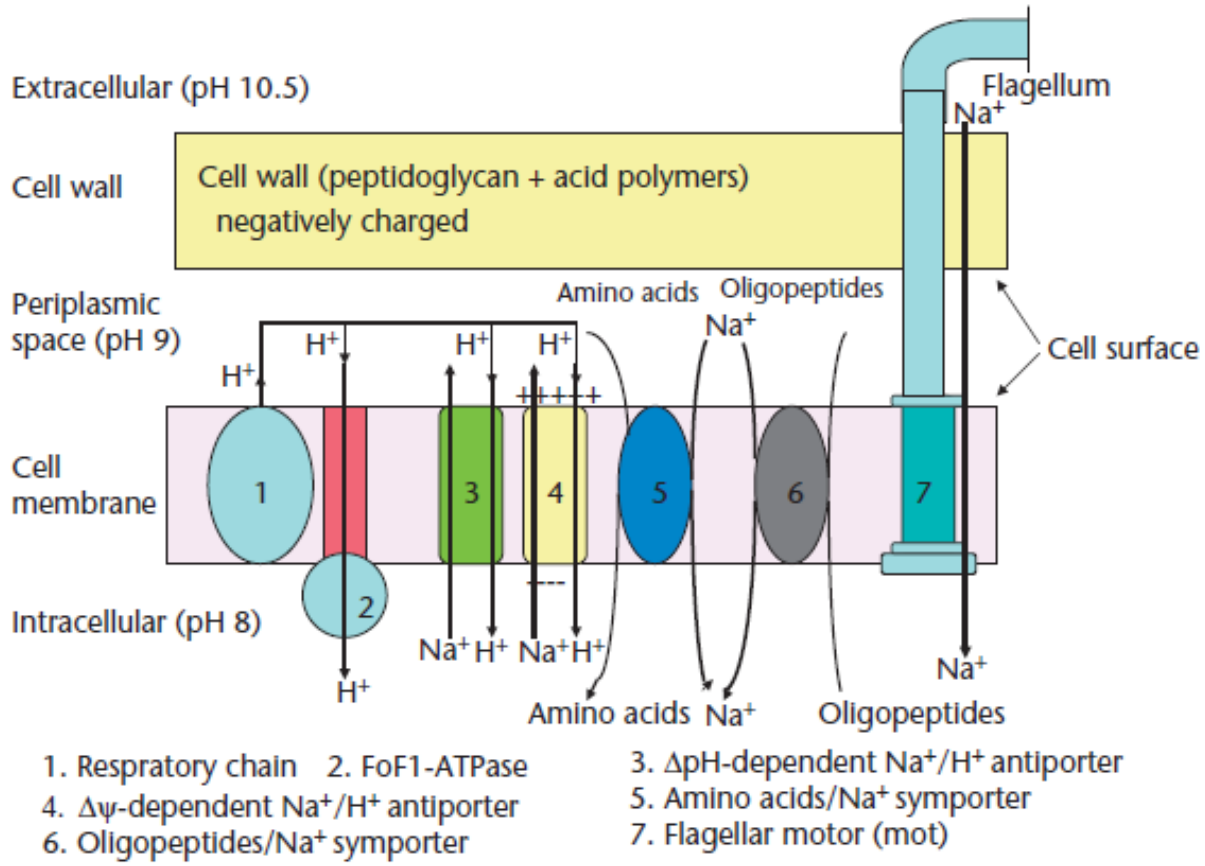

Figure 5.2. Cytoplasmic pH regulation and transport mechanisms (Horikoshi, 2008)

Bacterial ATP-binding cassette (ABC) transporters are among the oldest transporter families, which are energised by ATP for the translocation of various substrates across membranes, either for uptake or export of the substrate. They mediate the uptake of nutrients including ions, amino acids, sugars, etc. Exporters are commonly involved in the extrusion of toxic compounds. In contrast to ABC-type transporters, transporters belonging to the major facilitator superfamily (MSF) transfer small solutes without utilizing cellular energy. This passage depends on the difference in ion concentrations between the two faces of the membranes. Although the favourable ion transfer direction is from high to lower ion concentration but MFS may also work as uniporter, antiporter

and symporter to achieve the transfer of target molecules or substrates (Reddy *et al.*, 2012; Quistgaard *et al.*, 2016).

Among the general transporters, there were 6 ATP-binding cassette **(**ABC) transporters, most of which were with induced expression and there were 2 major facilitator superfamily (MFS) transporters, which were both down-regulated with increasing pH.

Apart from these general transporters there were a significant number of differentially expressed transporters specifically requiring the involvement of sodium. These were sodium dependent/independent transporters, sodium/phosphate symporters, and sodium/alanine symporters. These were all up-regulated with increasing pH, thus evidencing a sodium gradient in high pH growth media, which energises the transport process.

On the other hand, while a potassium/proton antiporter and a permease subunit of an iron export ABC transporter were repressed, a magnesium transporter was induced with increasing environmental pH.

In a previous study by Wei *et al.* (2007) the importance of the ion transporters for organism grown in alkaline lakes was reported (Wei *et al.*, 2007). *Bacillus* possesses two layers to cover the cytoplasm; these two different layers also protect the organism internal pH homeostasis. Considering the presence of high ion concentrations at high pH, it is highly probable that the regulation of the expression of transporter proteins is responsible not only for nutrient uptake but also for adjusting ion balances to keep its intracellular pH homoeostasis under these conditions in *B. marmarensis* sp. nov. (Ito *et al.*, 1997).

The increase in the expression of one transport protein reserves special attention: the mechanosensitive ion channel protein. Mechanosensitive channels act as biological force-sensing systems in response to environmental conditions. Under normal conditions, these channels are semipermeable. When they sense mechanical force in their external environment (for example, shear force, gravity, touch) and in their internal environment (including osmotic pressure and membrane deformation), they respond for proper growth and development. They function as emergency valves releasing excess osmolyte upon hypo-osmotic stress to which bacterial cells become exposed in their

living environments (Levina *et al.*, 1999; Booth and Blount, 2012). This should be an active ion channel in *B. marmarensis* sp. nov. to prevent the detrimental effects of the high amount of ions in the environment (Kung, Martinac and Sukharev, 2010).

6. CONCLUSION

Many bacilli species are used for different experimental purposes but among them the most popular is *Bacillus subtilis*. The popularity of *B. marmarensis* sp. nov. is based on its easy growth with short half–time and its being tolerant to alkali environments (Turnbull PCB, 1996). This extremely alkaliphilic organism can grow in high pH environments. The underlying mechanism behind this interesting and rare feature has not been fully understood yet.

In the current thesis, transcriptomic and proteomic approaches were used to understand the survival of *B. marmarensis* sp. nov. in media with high pH. The transcriptomic results have shown that among the up-regulated genes were mostly those that code for transporter proteins, stress proteins (like universal stress proteins, ectoine hydroxylase, choline ABC transporter ATP binding protein) and among the down-regulated genes were mostly those that code for membrane proteins, sporulation and transport proteins like MFS transporter, potassium transporter CPA, etc. In contrast to transcriptomic analysis, the analysis of the proteome has shown that, those proteins that could be detected on the 2D-gels have not significantly changed in abundance. Both approaches have returned a significant number of hypothetical proteins after analysis. This was not unexpected since *B. marmarensis* sp. nov. is a novel microorganism for which the genome sequence has recently been published.

The findings of this work suggest that *B. marmarensis* sp. nov. protects itself via redesigning the membrane structure, using alternative carbon flux pathways, and by over-expressing stress proteins. The up-regulation of the transport proteins, especially ion transporters, is possibly to maintain internal pH homeostasis.

This information obtained regarding the alkaliphilic adaptation of *B. marmarensis* sp. nov. can be used for the improvement of the properties of other strains, which could be used under alkaliphilic conditions.

Further analysis with real-time PCR will help to verify the findings of this work. Analysis following the gene annotation of *B. marmarensis* sp. nov. could provide clues on the involvement of other proteins for alkaliphilic adaptation which were defined as hypothetical.

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APPENDIX

Table A1. Absorbance and pH values of B. *marmarensis* sp. nov. in pH 8.8 medium condition

	Time (h)	OD Value			OD mean	Dilution Factor (DF)	OD Final	pH Value
8.8 pH sample	0							9.4
	1	0.037	0.039	0.039	0.038	1	0.038	9.30
	2	0.097	0.098	0.097	0.097	1	0.097	9.29
	3	0.435	0.436	0.438	0.436	1	0.436	9.18
	4	0.296	0.297	0.298	0.297	5	1.485	9.09
	5	0.432	0.436	0.437	0.435	5	2.175	8.96
	6	0.581	0.584	0.588	0.584	5	2.921	8.82
	7	0.742	0.751	0.765	0.765	5	3.763	8.72
	8	0.481	0.491	0.498	0.490	10	4.900	8.73
	9	0.581	0.59	0.593	0.588	10	5.880	8.70
	10	0.593	0.599	0.600	0.597	10	5.973	8.73
	24	0.530	0.533	0.536	0.533	10	5.330	8.8

Table A2. Absorbance and pH values of B. *marmarensis* sp. nov. in pH 9.8 medium condition

	Time (h)	OD Value			OD mean	Dilution Factor (DF)	OD Final	pH Value
9.8 pH sample	0							9.8
	1	0.340	0.350	0.370	0.0353	1	0.035	9.7
	2	0.092	0.094	0.095	0.094	1	0.094	9.68
	3	0.363	0.372	0.380	0.372	1	0.372	9.58
	4	0.628	0.636	0.635	0.633	2	1.266	9.53
	5	0.335	0.331	0.340	0.335	5	1.677	9.4
	6	0.488	0.492	0.493	0.491	5	2.455	9.3
	7	0.581	0.585	0.586	0.584	5	2.920	9.21
	8	0.352	0.352	0.347	0.350	10	3.503	9.15
	9	0.418	0.427	0.427	0.424	10	4.240	9.0
	10	0.525	0.521	0.524	0.523	10	5.233	9.0
	24	0.543	0.547	0.548	0.546	10	5.460	8.7

Table A3. Absorbance and pH values of B. *marmarensis* sp. nov. in pH 10.6 medium condition

	Time (h)	OD Value			OD mean	Dilution Factor (DF)	OD Final	pH Value
10.6 pH sample	0							10.6
	1	0.026	0.029	0.027	0.027	1	0.027	10.4
	2	0.058	0.061	0.062	0.060	1	0.0603	10.25
	3	0.212	0.210	0.213	0.212	1	0.212	10.3
	4	0.545	0.549	0.559	0.551	1	0.551	10.2
	5	0.200	0.206	0.206	0.204	5	1.020	10.01
	6	0.223	0.228	0.228	0.226	5	1.131	9.96
	7	0.281	0.288	0.277	0.282	5	1.410	9.89
	8	0.374	0.381	0.379	0.378	5	1.890	9.83
	9	0.201	0.195	0.194	0.197	10	1.967	9.75
	10	0.390	0.385	0.412	0.408	5	1.950	9.724
	24	0.402	0.411	0.412	0.408	10	4.083	9.07

Table A4. PDQuest Software Fold Ratio Results of the Detected Protein Spots

SSP number	pH10.6	Ratio	pH9.8	SSP number	pH10.6	Ratio	pH9.8
1903		0.00	167588032	**6707**	4593407.5	0.64	7146874
5408		0.00	26518496	**3204**	3105081.5	0.67	4663399.5
1002		0.00	25891236	**7614**	1904360.4	0.68	2820034.5
1104		0.00	16237120	**6416**	10710042	0.70	15262788
1201		0.00	8091361	**2302**	9975183	0.74	13470709
1101		0.00	7361474.5	**2301**	10029922	0.75	13372884
1506		0.00	7301662	**3307**	18796986	0.75	25059556
1105		0.00	6108088.5	**7510**	8314771	0.88	9491346
1909		0.00	4124481	**3304**	16211297	0.88	18324254
1910		0.00	3701243	**8001**	12719292	0.93	13682099
1908		0.00	3373627.8	**7309**	2950998.3	0.96	3089112.3
1603	2802469.8	0.10	28094664	**2013**	13840448	1.05	13159484
2501	2539842.3	0.17	15119125	**8004**	8341966	1.29	6465073
1510	1276841.3	0.19	6769529.5	**2110**	9950098	1.36	7342633
7611	5094319.5	0.27	19026144	**5202**	10121892	1.40	7207448

6712	5091770	0.29	17592284	**1504**	6440139	1.42	4551270
2106	5646564	0.32	17395676	**6406**	21008026	1.58	13279302
1907	2660256	0.37	7176673	**2111**	17080466	1.72	9955850
2109	6928576	0.45	15252236	**6108**	26921416	1.84	14649944
8404	5570080	0.47	11866267	**2509**	39836508	2.08	19140016
2607	2985885.3	0.50	5955514.5	**3401**	36070716	2.12	17014146
7209	7059006	0.59	12023236	**5108**	40990148	2.32	17688040
8508	6962326	0.62	11318830				

Table A5. Gel spot numbers and SSP numbers of analyzed spots with PDQuest and MALDI-TOF

SSP number	**Gel spot number**	**SSP number**	**Gel spot number**	**SSP number**	**Gel spot number**	**SSP number**	**Gel spot number**
1903	1	**6707**	20*	**1603**	5*	**2013**	24
5408	2	**3204**	33	**1510**	13	**8004**	38
1002	3	**7614**	34	**7611**	39	**2110**	25
1104	4	**6416**	21	**6712**	14	**5202**	26
1201	5	**2302**	22	**2106**	15	**1504**	27
1101	6	**2301**	23	**1907**	16	**6406**	42
1506	7	**3307**		**2109**	17	**2111**	28
1105	8	**7510**	35	**8404**	40	**6108**	29
1909	9	**3304**		**2607**	18	**2509**	30
1910	10	**8001**	37	**7209**	19	**3401**	31
1908	11	**7309**	36	**8508**	41	**5108**	32
2501	12						

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PUBLICATIONS

Ayyildiz D, Arga K, Avci FG, **Altinisik FE**, Gurer C, Gulsoy Toplan G, Kazan D, Wozny K, Brügger B, Mertoglu B, Sariyar Akbulut B (2017). Transcriptomic analysis displays the effect of (-)-roemerine on the motility and nutrient uptake in *Escherichia coli*. *Current Genetics*, 63(4), 709-722.

Avci FG, **Altinisik FE**, Vardar Ulu D, Ozkirimli Olmez E, Sariyar Akbulut B (2016). An evolutionarily conserved allosteric site modulates beta-lactamase activity. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 31(sup3), 33-40.

POSTER PRESENTATIONS

Altinisik FE, Kazan D, Sariyar Akbulut B. Adaptation of *B. marmarensis sp. nov.* to extreme alkaline conditions. *7th Congress of European Microbiologists (FEMS)*, 9-13 July 2017, Valencia, Spain.

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Alaybeyoglu B, **Altinisik FE**, Irvali D, Sariyar Akbulut B, Ozkirimli E. Biophysical Characterization and Celullar Transport of Inhibitor/Antimicrobial Peptides. *From Cell-Penetrating Peptides to Nanoparticles for Cellular Delivery-CPP*, 1-3 July, 2015, Paris-France.

Alaybeyoglu B, **Altinisik FE**, Irvali D, Sariyar Akbulut B, Ozkirimli E. A Novel Approach to Deliver Peptides that Target Intracellular Enzymes. *3rd International BAU Drug Design Congress*. 1-3 Oct 2015, Istanbul-Turkey.

Altinisik FE, Avci FG., Sariyar Akbulut B, Denizci AA, Kazan D. Investigation of proteome profile of alkaliphilic *B. marmarensis sp. nov.* under different pH conditions. *FEBS-IUBMB Workshop on Biointeractomics: from bimolecular interactions to networks.* 17-20 May 2016, Sevilla-Spain.

Altinisik FE, Avci FG., Sariyar Akbulut B, Denizci AA, Kazan D. Understanding alkaliphilic adaptaion of B.marmarensis sp. nov. (Poster), *41st Federation of European Biochemical Societies (FEBS) Congress*, 2016, Kuşadası-Turkey.

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