

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**RECOMBINANT PRODUCTION AND CHARACTERIZATION OF
AQUAPORIN PROTEIN ISOLATED FROM *GEOBACILLUS*
THERMOLEOVORANS ARTR1 AND *VIRGIBACILLUS* SP. AGTR STRAINS**

M.Sc. THESIS

Şevval UYSALCAN

Department of Molecular Biology - Genetics and Biotechnology

Molecular Biology - Genetics and Biotechnology Programme

FEBRUARY 2022

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**RECOMBINANT PRODUCTION AND CHARACTERIZATION OF
AQUAPORIN PROTEIN ISOLATED FROM *GEOBACILLUS*
THERMOLEOVORANS ARTR1 AND *VIRGIBACILLUS* SP. AGTR STRAINS**

M.Sc. THESIS

**Şevval UYSALCAN
(521191138)**

Department of Molecular Biology - Genetics and Biotechnology

Molecular Biology - Genetics and Biotechnology Programme

**Thesis Advisor: Prof. Dr. Nevin Gül KARAGÜLER
Thesis Co-Advisor: Dr. N. Esra ATEŞ GENÇELİ**

FEBRUARY 2022

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

***VIRGIBACILLUS SP. AGTR, GEOBACILLUS THERMOLEOVORANS* ARTRİ
SUŞLARINDAN İZOLE EDİLEN AKUAPORİN PROTEİNİNİN
REKOMBİNANT ÜRETİMİ VE KARAKTERİZASYONU**

YÜKSEK LİSANS TEZİ

**Şevval Uysalcan
(521191138)**

Moleküler Biyoloji - Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji - Genetik ve Biyoteknoloji Programı

**Tez Danışmanı: Prof. Dr. Nevin Gül KARAGÜLER
Eş Danışman: Dr. N. Esra ATEŞ GENCELİ**

ŞUBAT 2022

Şevval UYSALCAN, a M.Sc. student of ITU Graduate School student ID 521191138, successfully defended the thesis/dissertation entitled “RECOMBINANT PRODUCTION AND CHARACTERIZATION OF AQUAPORIN PROTEIN ISOLATED FROM *GEOBACILLUS THERMOLEOVORANS* ARTR1 AND *VIRGIBACILLUS SP.* AGTR STRAINS”, which he/she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Nevin GÜL KARAGÜLER**
Istanbul Technical University

Co-advisor : **Assis. Prof. Dr. Esra ATEŞ GENÇELİ**
Istanbul Technical University

Jury Members : **Prof. Dr. İsmail KOYUNCU**
Istanbul Technical University

Assos. Prof. Dr. Nagehan ERSOY TUNALI
Istanbul Medeniyet University

Asst. Prof. Dr. Emel ORDU BIÇAKÇI
Yıldız Technical University

Date of Submission : 13 January 2022
Date of Defense : 09 February 2022





To my family, fiance and best friends,



FOREWORD

Firstly, I want to thank my both thesis advisors Prof. Dr. Nevin GÜL KARAGÜLER and Dr. Esra ATEŞ GENÇELİ for their scientific advice and moral support and I would like to thank you for wonderful cooperation as well. I would also like to thank Prof. Dr. İsmail KOYUNCU, who allowed me to use MEMTEK laboratories to complete our experimental studies.

I also have to thank all present members of Protein Engineering Laboratory to special thanks to Aycan KAYRAV, Hande MUMCU, Şeyma YILMAZ for their endless help and patience. And also, thanks to Enise PEKGENÇ, Şebnem SEHERLER, Prof. Dr. Fatma Neşe KÖK, Prof. Dr. Melek ÖZKAN and Dr. Hilal YILMAZ for their endless support in the experimental processes.

Many thanks to my fiance Mehmet Can JANAT who kept me always motivated and always be there for me, and my dear family my mother Hülya ETÇİ, my father Okan ETÇİ, my uncle Ersin TAMŞEN, my aunt Hatice TAMŞEN and my little sisters Elif TAMŞEN and Eflin TAMŞEN and also my grandfather Kenan TAMŞEN, my grandmother Fatma TAMŞEN for their faith in me and supports to me throughout my life and my closest friends Evrim KAPUCU, Cemresu SARIHAN, Serim ERDEM for not leaving me alone in this process and listening to all my scientific talks. And another special thanks to my grandfather Nurettin UYSALCAN who passed away, for his support from my undergraduate to master degree.

In addition, I would like to thank to my second family Aslı JANAT and Mustafa JANAT for being with me with their moral support.

I would like to express my endless thanks to my mentor Asst. Prof. Özgür GÜL, who we lost last year tragically but touched my undergraduate university life and still many times. I hope, I made him proud.

This work was supported by Istanbul Technical University Scientific Research Foundation (Project ID: 42954).

February 2022

Şevval UYSALCAN
(Bioengineer)



TABLE OF CONTENTS

	<u>Page</u>
FORWARD.....	ix
TABLE OF CONTENTS.....	xi
FOREWORD.....	ix
TABLE OF CONTENTS.....	xi
ABBREVIATIONS.....	xiii
LIST OF TABLES.....	xv
LIST OF FIGURES.....	xvii
SUMMARY.....	xxiii
ÖZET.....	xxvii
1. INTRODUCTION.....	1
1.1 History of Cell Membrane.....	1
1.1.1 Overview of biological membrane proteins.....	1
1.1.2 Classification of biological membrane proteins.....	2
1.2 Aquaporin as a Biological Membrane Proteins.....	5
1.2.1 Usage field of aquaporin.....	7
1.2.2 Extremophilic aquaporins.....	8
1.3 Liposome as a Support Lipid Membrane.....	10
1.3.1 Liposome classification.....	11
1.3.2 Liposome composition.....	12
1.4 Aim of the Study.....	13
2. MATERIAL AND METHODS.....	15
2.1 Material.....	15
2.1.1 Laboratory equipments.....	15
2.1.2 Culture medium.....	15
2.1.3 Buffer, gel and solution.....	15
2.1.4 Chemicals, enzymes and kits.....	15
2.1.5 Bacterial strains and vectors.....	15
2.2. Methods.....	16
2.2.1 Bacterial strains and template DNA.....	16
2.2.2 Designing primers.....	16
2.2.3 Polymerase chain reaction (PCR) for Virgibacillus and Geobacillus Aqp genes.....	18
2.2.4 Agarose gel electrophoresis and PCR product clean up.....	19
2.2.5 Digestion for the vector and PCR product.....	20
2.2.6 Ligation.....	20
2.2.7 Competent cell preparation.....	21
2.2.8 Transformation.....	22
2.2.9 Colony PCR.....	22
2.2.10 Plasmid isolation and control digestion.....	23
2.2.11 Sanger sequencing.....	24

2.2.12 Optimizing protein expression.....	25
2.2.13 Purification of his-tagged proteins.....	26
2.2.14 SDS-PAGE.....	27
2.2.15 Western Blot analysis.....	28
2.2.16 Ultrafiltration.....	28
2.2.17 Liposome production.....	29
2.2.18 Proteoliposome production.....	29
2.2.19 Characterization of liposome and proteoliposome.....	30
3. RESULTS AND DISCUSSION.....	33
3.1 Genomic DNA Isolation and Primer Designing.....	33
3.2 Polymerase Chain Reaction (PCR) for Virgibacillus and Geobacillus Aqp Genes.....	34
3.3 PCR Product.....	35
3.4 Ligation.....	36
3.5 Transformation.....	36
3.6 Colony PCR.....	37
3.7 Plasmid Isolation and Control Cleavage.....	40
3.8 Sanger Sequencing Analysis.....	41
3.9 Optimizing Protein Expression in E. coli C43 (DE3)	42
3.10 His-tag Purification and Ultrafiltration.....	48
3.12 Liposome and Proteoliposome Production.....	53
4. CONCLUSION.....	57
REFERENCES.....	61
APPENDICES.....	65
CURRICULUM VITAE.....	73

ABBREVIATIONS

APS	: Ammonium Persulfate
BLAST	: Basic Local Alignment Tool
bp	: Base Pair
dH₂O	: Distilled Water
DNA	: Deoxyribonucleic Acid
dNTP	: Deoxynucleotide Triphosphate
<i>E. coli</i>	: <i>Escherichia coli</i>
EDTA	: Ethylene
g	: Gravitational force
Geo	: <i>Geobacillus thermoleovorans</i> ARTR1 Aquaporin coding sequence
Geo24	: <i>Geobacillus thermoleovorans</i> ARTR1 colony 24th
His-tag	: Histidine tag
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
kb	: Kilo base
kDa	: Kilo Dalton
LB	: Luria Broth
PCR	: Polymerase Chain Reaction
PMSF	: Phenylmethanesulfonyl fluoride
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
STAT	: Signal Transducers and Activators of Transcription
TBS	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline-Tween 20
TEMED	: Tetramethylethylenediamine
UV	: Ultraviolet
V13	: <i>Virgibacillus sp.</i> AGRT strain colony 13th
V19	: <i>Virgibacillus sp.</i> AGRT strain colony 19th
μg	: Microgram



LIST OF TABLES

	<u>Page</u>
Table 2.1 : Features and sequences of gene specific primers.....	17
Table 2.2 : PCR mixtures.	17
Table 2.3 : Thermocycling conditions for <i>Virgibacillus</i> 1 Aqp.	17
Table 2.4 : Thermocycling conditions for <i>Virgibacillus</i> 2 Aqp.	18
Table 2.5 : Thermocycling conditions for <i>Geobacillus</i> Aqp.....	18
Table 2.6 : Double Digestion Protocol for pET28a (+) and PCR Products.	19
Table 2.7 : Components and volumes for Ligation as a ratio of 1:5.	20
Table 2.8 : The Colony PCR reaction components and volume.	22
Table 2.9 : The Colony PCR conditions.	22
Table 2.10 : The Fast Digestion of isolated plasmid DNA.....	23
Table 3.1 : The concentrations of insert DNA and vector.....	34
Table 3.2 : Comparison of the water transfer rates (k) of liposomes and proteoliposomes.	53
Table A.1 : Used equipments in this study.....	64
Table B.1 : The Luria Bertani (LB) medium is composed of the following components for 1 liter.....	65
Table B.2 : The Luria Bertani (LB) agar medium is composed of the following components.	65
Table B.3 : The Nutrient Broth (NB) Medium is composed of the following components.	65
Table B.4 : The Nutrient Broth (NB) Agar Medium is composed of the following components.	65
Table B.5 : The MagicMedia™ (150ml) is composed of the following components.....	65
Table C.1 : The Agarose gel components.....	66
Table C.2 : His-tag Purification Lysis, Solubilization, Wash, Buffers.	66
Table C.3 : The 50X TAE buffer is composed of the following components.	66
Table C.4 : The 1X TBS buffer is composed of the following components.....	66
Table C.5 : The 10X running buffer is composed of the following components.	66
Table C.6 : The 10X transfer buffer is composed of the following components.....	66
Table C.7 : The 1X transfer buffer is composed of the following components.....	67
Table C.8 : The 10X TBS buffer is composed of the following components.....	67
Table C.9 : The 1X TBS-T buffer is composed of the following components.....	67
Table C.10 : The Coomassie Brilliant Blue stain solution is composed of the following components.	67
Table C.11 : The 10% Ammonium Persulfate (APS) solution is composed of the following components.....	67
Table C.12 : The Destaining solution is composed of the following components. ..	67
Table C.13 : The stock 500 mM isopropyl-β-D-thiogalactopyranoside (IPTG) is...	67
Table C.14 : The 30 mg/mL Kanamycin stock solution is composed of the following components.	68

Table C.15 : The components of 10% and 15% SDS polyacrylamide separating gels.	68
Table C.16 : The components of SDS polyacrylamide stacking gel.....	68
Table D.1 : Used chemicals in this study.....	69
Table D.2 : Used enzymes in this study.....	69
Table D.3 : Used lipid in this study.....	69
Table D.4 : The kits used in this study.....	70



LIST OF FIGURES

	<u>Page</u>
Figure 1.1 : General picture of biological membrane and membrane proteins respectively (Membrane Proteins, 2021).....	2
Figure 1.2 : E. Bitopic membrane proteins; F. Polytopic membrane proteins; G. Multimeric assemblies of membrane proteins (channels or transporters) (Ariöz, C., 2014).....	3
Figure 1.3 : Monotopic membrane proteins with an amphipathic helix; B. Monotopic membrane proteins; C. GPI-anchored membrane proteins; D. Lipid-anchored membrane proteins (Ariöz, C., 2014)	4
Figure 1.4 : The representative picture for liposome structure formed by phospholipids in an aqueous solution (Leung, A. W., Amador, C., 2019)	11
Figure 1.5 : Liposomes are categorized according to their size and number of lamellae (Wood, M. H., Milan, D. 2021)	12
Figure 1.6 : Chemical structure of the structural phospholipids. Abbreviations: PC phosphatidylcholine, PE phosphatidylethanolamine, PG phosphatidylglycerol, PS phosphatidylserine (Furt, F., Simon-Plas, F., 2011)	13
Figure 2.1 : Genomic map of pET-28a (+) expression vector.....	16
Figure 2.2 : Liposome production by thin film method (Jaradat, E., Weaver, E., 2021)	28
Figure 2.3 : SX20 Stopped Flow Light Scattering Spectrometer.....	29
Figure 3.1 : Agarose gel electrophoresis image of genomic DNA isolation from original organisms. <i>Virgibacillus</i> and <i>Geobacillus</i> represented in respectively 1st, 2nd Lane.M:Marker, GeneRuler, DNA Ladder.....	31
Figure 3.2 : Agarose gel electrophoresis image of PCR product Virgi1 and Virgi 2. The gradient PCR was performed using three different temperatures 60 °C, 63 °C and 70 °C represented in respectively 1st, 2nd and 3rd Lane. M: Marker, GeneRuler 1 kb Plus DNA Ladder.	32
Figure 3.3 : Agarose gel electrophoresis image of PCR product Virgi 1. The gradient PCR was performed using two different temperatures 55 °C, and 73 °C represented in respectively 1st, 2nd M: Marker, GeneRuler 1 kb Plus DNA Ladder.	32
Figure 3.4 : Agarose gel electrophoresis image of PCR product Geo. The gradient PCR was performed using three different temperatures 65 °C, and 70 °C and 73 °C represented in respectively 1st, 2nd, 3rd M: Marker, GeneRuler 1 kb plus DNA Ladder.3.3 PCR Product	33
Figure 3.5 : Agarose gel electrophoresis image of double digested PCR products and vector. <i>Virgibacillus</i> 1, <i>Virgibacillus</i> 2 and <i>Geobacillus</i> , pET28a (+) were represented in respectively 1st, 2nd, 3rd, 4rt M: Marker, GeneRuler 1 kb plus DNA Ladder	33

Figure 3.6 : Kanamycin screening plates with C43 including colonies after transformation for Virgi 1 containing a plasmid with a 1:5 ratio (vector: insert) of ligation.....	34
Figure 3.7 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to <i>Virgibacillus</i> 2. Six colonies were selected from this petri plate.....	35
Figure 3.8 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to <i>Virgibacillus</i> 2. Four colonies were selected from this petri plate.....	35
Figure 3.9 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to <i>Virgibacillus</i> 1. Five colonies were selected from this petri plate.....	36
Figure 3.10 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to <i>Virgibacillus</i> 1. Five colonies were selected from this petri plate.....	36
Figure 3.11 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to <i>Geobacillus</i> . Seven colonies were selected from this petri plate.....	36
Figure 3.12 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to <i>Geobacillus</i> . Five colonies were selected from this petri plate.....	37
Figure 3.13 : The colony PCR result for <i>Virgibacillus</i> 1. Ten colonies were selected and one positive colony was obtained. 3rd well showed positive colony respectively. Marker: GeneRuler 1 kb plus DNA Ladder	37
Figure 3.14 : The colony PCR result for <i>Virgibacillus</i> 2. Ten colonies were selected and two positive colonies were obtained. 13th and 19th wells showed positive colony respectively. Marker: GeneRuler 1 kb plus DNA Ladder.	37
Figure 3.15 : The colony PCR result for <i>Geobacillus</i> . Twelve colonies were selected and five positive colonies were obtained. 23th, 24th, 25th, 28th and 31th wells showed positive colonies respectively. Marker: GeneRuler 1 kb plus DNA Ladder.....	38
Figure 3.16 : Agarose gel electrophoresis control cleavage of plasmid DNA including insert. <i>Virgibacillus</i> 1-13th colony, <i>Virgibacillus</i> 1-19th colony and <i>Virgibacillus</i> 2-3th colony were showed in the wells respectively. Marker: GeneRuler 1 kb plus DNA Ladder.	38
Figure 3.17 : Agarose gel electrophoresis control cleavage of plasmid DNA including insert. <i>Geobacillus</i> 23th, 24th, 25th , 28th and 31th colony were showed under UV light. Marker: GeneRuler 1 kb plus DNA Ladder.	39
Figure 3.18 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V13 supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 2: V13 pellet treated with 0.5 mM IPTG and 6M urea. Lane 3: V13 after 4 hours withouth IPTG. Lane 4: V13 pellet after 4 hours withouth IPTG and 6M urea. Lane 5: V19 supernatant treated with 0.5 mM IPTG for 4 hours. Lane 6: V19 pellet treated with 0.5 mM IPTG and 6M urea. Lane 7: V19 after 4 hours withouth IPTG. Lane 8: V19 pellet after 4 hours withouth ...	40
Figure 3.19 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V3 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 2: V13 after 4 hours withouth IPTG.	

Lane 3: V3 pellet after 4 hours without IPTG and 6 M urea. Lane 4: C43 pet supernatant treated with 0.5 mM IPTG for 4 hours. Lane 5: C43 pet pellet treated with 0.5 mM IPTG and 6 M urea. Lane 6: C43 host after 4 hours without IPTG. Lane 7: C43 host pellet after 4 hours without IPTG and 6 M urea. Lane 8: V13 supernatant treated with 0.5 mM IPTG for 6 hours. Lane 9: V13 pellet treated with 0.5 mM IPTG and 6 M urea 6 hours. 41

Figure 3.20 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V13 after 6 hours without IPTG. Lane 2: V13 pellet after 6 hours without IPTG and treated with 6 M urea. Lane 3: V19 supernatant treated with 0.5 mM IPTG for 6 hours induction. Lane 4: V19 pellet treated with 0.5 mM IPTG and treated with 6 M urea. Lane 5: V19 after 6 hours without IPTG. Lane 6: V19 pellet after 6 hours without IPTG and treated with 6 M urea. Lane 7: V3 supernatant treated with 0.5 mM IPTG for 6 hours induction. Lane 8: V3 pellet treated with 0.5 mM IPTG and treated with 6 M urea 6 hours induction. Lane 9: V3 after 6 hours induction without IPTG. 42

Figure 3.21 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V3 pellet after 4 hours without IPTG and 6 M urea. Lane 2: C43pet supernatant treated with 0.5 mM IPTG for 6 hours. Lane 3: C43 pet pellet treated with 0.5 mM IPTG and 6 M urea. Lane 4: C43 host after 6 hours without IPTG. Lane 5: C43 host pellet after 6 hours without IPTG and 6 M urea. . 42

Figure 3.22 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: Geo 24 supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 2: Geo24 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 3: Geo24 after 4 hours without IPTG. Lane 4: Geo24 pellet after 4 hours without IPTG and 6 M urea. Lane 5: Geo28 supernatant treated with 0.5 mM IPTG for 4 hours. Lane 6: Geo28 pellet treated with 0.5 mM IPTG and 6M urea. Lane 7: Geo28 after 4 hours without IPTG. Lane 8: Geo28 pellet after 4 hours without IPTG and 6 M urea. Lane 9: V3 supernatant treated with 0.5 mM IPTG for 4 hours induction. 43

Figure 3.23 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: C43 pet supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 2: C43 pet pellet treated with 0.5 mM IPTG and 6M urea. Lane 3: C43 after 4 hours without IPTG. Lane 4: C43 pellet after 4 hours without IPTG and 6M urea. Lane 5: Geo24 supernatant treated with 0.5 mM IPTG for 6 hours. Lane 6: Geo24 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 7: Geo24 after 4 hours without IPTG. Lane 8: Geo24 pellet after 6 hours without IPTG and 6 M urea. Lane 9: V3 supernatant treated with 0.5 mM IPTG for 6 hours induction..... 43

Figure 3.24 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: Geo28 supernatant treated with 0.5 mM IPTG for 6 hours. Lane 2: Geo28 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 3: Geo28 after 6 hours incubation without IPTG. Lane 4: Geo28 pellet after 6 hours incubation without IPTG and 6 M urea. Lane 5: C43 pet supernatant treated with 0.5 mM

IPTG for 4 hours induction. Lane 6: Geo28 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 7: C43 host after 6 hours incubation without IPTG. Lane 7: C43 host pellet after 6 hours incubation without IPTG and 6 M urea. 44

Figure 3.25 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V13 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 2: V13 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 3: V3 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 4: V3 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 5: Geo24 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 6: Geo24 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 7: C43 pellet and treated with 150 mM OG detergent. Lane 8: C43 supernatant and treated with 150 mM OG detergent. Lane 9: V13 supernatant with 1 mM IPTG and treated with 150 mM OG detergent. 45

Figure 3.26 : Western Blot Analysis of supernatant and pellet for IPTG optimization. Marker: PageRuler 26619. Lane M: Molecular weight marker. Lane 1: V13 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent and dialysed. Lane 2: V13 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent and dialysed. Lane 3: V3 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent dialysed. Lane 4: V3 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent dialysed. Lane 5: Geo24 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent dialysed. 46

Figure 3.27 : SDS-PAGE analysis of V13 purification. Lane M: Molecular weight marker. Lane 1: V13 protein flow through. Lane 2: V13 protein Wash 1 with 10 mM Imizadole. Lane 3: V13 protein Wash 2 with 20 mM Imizadole. Lane 4: V13 protein Wash 3 with 30 mM Imizadole. Lane 5: V13 protein Elution 1 with 100 mM Imizadole. Lane 6: V13 protein Elution 2 with 150 mM Imizadole. Lane 7: V13 protein Elution 3 with 250 mM Imizadole. 47

Figure 3.28 : SDS-PAGE analysis of V3 purification. Lane M: Molecular weight marker. Lane 1: V3 protein flow through. Lane 2: V3 protein Wash 1 with 10 mM Imizadole. Lane 3: V3 protein Wash 2 with 20 mM Imizadole. Lane 4: V3 protein Wash 3 with 30 mM Imizadole. Lane 5: V3 protein Elution 1 with 100 mM Imizadole. Lane 6: V3 protein Elution 2 with 150 mM Imizadole. Lane 7: V3 protein Elution 3 with 250 mM Imizadole. 47

Figure 3.29 : SDS-PAGE analysis of Geo24 purification. Lane M: Molecular weight marker. Lane 1: Geo24 protein flow through. Lane 2: Geo24 protein Wash 1 with 10 mM Imizadole. Lane 3: Geo24 protein Wash 2 with 20 mM Imizadole. Lane 4: Geo24 protein Wash 3 with 30 mM Imizadole. Lane 5: Geo24 protein Elution 1 with 100 mM Imizadole. Lane 6: Geo24 protein Elution 2 with 150 mM Imizadole. Lane 7: Geo24 protein Elution 3 with 250 mM Imizadole. 48

Figure 3.30 : SDS-PAGE analysis of Geo24 ultrafiltration. Lane M: Molecular weight marker. Lane 1: Collection of Wash 2 and Wash 3 samples after

	purification exposed to ultrafiltration (30k-10k). Lane 2: Collection of Elution 1, Elution 2 and Elution 3 samples after purification exposed to ultrafiltration (30k-10k).....	49
Figure 3.31 :	Protein sequencing comparing for three aquaporin aminoacids in Clustal Omega.....	51
Figure 3.32 :	The colour description for Clustal Omega.	50
Figure 3.33 :	The HPLC tubes with volumes of 1.5 mL and 1 mL including stock solution and chloroform. 5 mL of diluted lipid soluiton was prepared.	51
Figure 3.34 :	The system for liposome and proteoliposome production. The number 1 is the mini extruder, number 2 is PTFE and number 3 is nut, number 4 is syringe respectively.	52
Figure 3.35 :	Curves of water permeation rates of aquaporins obtained by light scattering spectrometry. 0.85M NaCl was used. The control liposome does not contain aquaporin and is shown in gray. The proteoliposome contains aquaporin from <i>Geobacillus thermoleovorans</i>	53
Figure 3.36 :	Curves of water permeation rates of aquaporins obtained by light scattering spectrometry. 0.85M NaCl was used, and samples were incubated 5 minutes at 40°C. The control liposome does not contain aquaporin and is shown in red. The proteoliposome contains aquaporin from <i>Geobacillus thermoleovorans</i> ARTR1 and is shown in black.....	54



**RECOMBINANT PRODUCTION AND CHARACTERIZATION OF
AQUAPORIN PROTEIN ISOLATED FROM *GEOBACILLUS*
THERMOLEOVORANS ARTR1 AND *VIRGIBACILLUS* SP. AGTR STRAINS**

SUMMARY

Cells have a fluid mosaic model that provides the passage of organic and inorganic molecules, ions and water, which are survival for the cell, that form the structure of cell membranes and contain protein, carbohydrates and cholesterol. Proteins located in the membrane of the cell are called biological membrane proteins and they constitute approximately 25-30 % of all proteins. However, the partially hydrophobic surfaces of membrane proteins are still a largely unconquered area due to their lack of flexibility and stability. Biological membrane proteins have three subgroups depending on their location are analyzed in category. Integral membrane proteins, one of the biological membrane proteins which are permanently attached to the cell membrane. Detergents, non-polar solvents, or sometimes denaturing agents are used to separate this protein from the biological membrane. Integral membrane proteins are a permanent part of the membrane and can penetrate the membrane to form transmembrane proteins. Peripheral membrane proteins, another biological membrane protein, temporarily bind to the membrane or integral membrane proteins by hydrophobic, electrostatic, and other non-covalent interactions. These proteins can dissociate from the membrane after treatment with a solution containing a high pH or high salt concentration. Lipid-anchored proteins, on the other hand, are proteins on the surface of the cell membrane that covalently bind to lipids embedded in the cell membrane.

Today, the structural and functional studies of the above-mentioned biological membrane proteins have been largely hampered by the difficulties in their production and overexpression, which are necessary for the structural studies of membrane proteins. Compared to other protein classes, the determination of the structure, expression, and purification of membrane proteins is greatly hampered by difficulties. Aquaporin, a biological membrane protein of great medical and industrial importance, are integral proteins that form pores in cell membranes and facilitate the transport of water between cells. This protein is the membrane protein that allows the passage of water molecules and rejects all other solutes. In the literature, there is a number of researches on the discovery of aquaporin from different cell membranes. While aquaporins, which are at the forefront of biomimetic membrane production in prokaryotic cells in the industrial area, are known for the passage of molecules that provide the vital conditions of the cell in plant cells and their necessity during some stress conditions (drought), in mammals aquaporins are associated with many diseases, especially leukemia. The biggest difficulty encountered during the studies carried out in this direction is the obstacles in ensuring the overexpression of the integral biological membrane protein aquaporins and the optimization of their purification afterwards.

Aquaporins have a similar basic structure: aquaporin monomers consist of six transmembrane helical segments and two short helical segments surrounding cytoplasmic and extracellular vestibules connected by narrow aqueous pore. Between the helices are five regions (A-E) with an asparagine-proline-alanine ("NPA motif") pattern, two of which are hydrophobic (B, E) that fold in or out of the cell membrane. Another part of the channel is the "ar/R selectivity filter", a set of amino acids that allows aquaporin to selectively allow or block the passage of different molecules.

Aquaporin monomers can assemble as tetramers in membranes, with each monomer functioning independently. The primary function of most aquaporins is to transport water across cell membranes in response to osmotic gradients created by active solute transport. Aquaporins provide a 10-100-fold increase in water transfer across the cell membrane. Because of their unique properties, they need to be produced in large quantities for different purposes, including structure and activity analysis or new biomimicry materials. Different species of aquaporins may have different permeability and rejection properties for the same solute. While aquaporin may vary depending on solute concentrations, the selectivity of aquaporin also varies depending on load and size. It creates a positively charged energy barrier due to the amino acid Arginine in its structure. In this way, aquaporin prevents the passage of loaded or neutral solvents. Over the past 5 years, there has been great interest in the biology of aquaporins (AQPs) in over 500 studies published on aquaporin cloning, genetics, tissue localization, developmental and regulated expression, transgenic mouse models, and structure/function analyzes. When aquaporins, which are found in all cells from bacteria to mammals, are investigated in the literature, it is seen that mostly AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 species are water specific. AQP3, AQP7 and AQP9 are called aquaglyceroporins and also carry glycerol and/or other small uncharged molecules.

Most studies on recombinant aquaporins have so far been functional, regulatory or structural studies of aquaporins. In addition to these studies, the application of aquaporins with high water permeability and high solute rejection with biomimetic membranes for water desalination and reuse has attracted great interest in recent years.

Prokaryotic aquaporins were first identified in *Escherichia coli*, and microorganisms such as *Rhodobacter sphaeroides*, *Lactococcus lactis*, *Saccharomyces cerevisiae*, *Mathanothermobacter marburgensis*, *Photobactetrium profundum* SS9 and *Halomonas elongate* have been used in studies to purify aquaporin protein. Also the recombinant production of aquaporin is being studied by using cell-free expression systems. Studies with prokaryotic AQPs show that bacteria help cope with osmotic, oxidative stress and nutritional fluctuations. However, there are limited studies in the literature for aquaporins produced from prokaryotic organisms and especially prokaryotes under extreme conditions.

Virgibacillus sp. AGTR strain isolated from Acıgöl (Burdur, Turkey), which is a lake with high salinity has taken its place in the literature as a new halophilic bacterium strain. The genome information of this organism was obtained, and it was determined that it contains two aquaporin-encoding genes, one of which is 831 bp and the other 795 bp. Also, a new thermophilic bacterium *Geobacillus thermoleovorans* ARTR1 was isolated from another extreme environment, Armutlu (Yalova). The gene sequence encoding aquaporin with a length of 819 bp was determined in this organism. To analyze the stability of extremophilic aquaporins in industrial processes requiring harsh conditions (high temperature, high pH), in total, three extremophilic aquaporin

genes were isolated from both halophilic *Virgibacillus sp.* AGTR and thermophilic *Geobacillus thermoleovorans* ARTR1 strains. These isolated genes were transferred to the pET28a (+) vector and produced recombinantly in the *Escherichia coli* C43 host cell. In this process, genes related to the PCR method with appropriate primers were amplified and ligated with a pET28a (+) vector. IPTG induction was subjected to time, temperature, and molarity trials, respectively. Then the optimal condition of 1 mM IPTG, 37 °C and 4 hours induction was selected. Under this optimal condition, gene expression was visibly achieved. Since it was observed that the protein remained in the inclusion body formed in the centrifuge after sonication, it was continued with the pellet after centrifugation and examined by SDS-PAGE in the control supernatant. After this process, the protein was obtained purely by various His-tag purification methods. Proteins expected to appear at an average size of 29 kDa were confirmed by SDS-PAGE and Western Blot Analysis.

Liposomes are small artificial vesicles of spherical shape that can be formed from cholesterol and non-toxic natural phospholipids. Because of their size and hydrophobic and hydrophilic character (as well as biocompatibility), liposomes are promising systems for drug delivery. Liposomes can mimic the cell membrane. Different lipid compositions can be used to obtain the desired liposome type. DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), an example of Phosphatidylcholines (PC), was used in this study. The proteoliposome consists of liposomes containing aquaporin. For 2.5 mg/ml DOPC lipid, aquaporin with a concentration of 0.5 mg/ml was dissolved in 10 ml of PBS and 0.05M OG detergent was added to obtain a proteoliposome, and aquaporin-free liposome was obtained. Control liposome and proteoliposome were treated with 0.85M NaCl and the water transfer rate of aquaporins was measured with a steady flow light scattering spectrometer device. Here, it was determined that the water passage of liposomes with aquaporin was faster. Aquaporin-bearing proteoliposomes lose water at a much higher rate than control liposomes. This was explained as an indication that aquaporin obtained from the *Geobacillus Thermoleovorans* ARTR1 strain was working.

The identification and characterization of novel aquaporin proteins from both halophilic and thermophilic bacteria within the scope of this project are of great importance in terms of their contribution to the literature.



***VIRGIBACILLUS SP. AGTR, GEOBACILLUS THERMOLEOVORANS* ARTR1
SUŞLARINDAN İZOLE EDİLEN AKUAPORİN PROTEİNİNİN
REKOMBİNANT ÜRETİMİ VE KARAKTERİZASYONU**

ÖZET

Hücreler, hücre zarlarının yapısını oluşturan ve içerisinde proteini, karbonhidratı, kolesterolü bulunduran hücre için hayati öneme sahip organik ve inorganik moleküllerin, iyonların ve suyun geçişini sağlayan akışkan mozaik modele sahiptir. Hücrenin zarında bulunan bu proteinler biyolojik zar proteinleri olarak adlandırılırlar ve tüm proteinlerin yaklaşık % 25-30'unu oluştururlar. Fakat, zar proteinlerinin kısmen hidrofobik yüzeyleri, esneklikleri ve stabilite eksiklikleri nedeniyle hala büyük ölçüde aydınlatılmamış bir alandır. Biyolojik zar proteinleri bulundukları yere bağlı olarak iki alt kategoride incelenirler. Biyolojik zar proteinlerinden biri olan integral zar proteinleri, hücre zarına kalıcı olarak bağlanır. Bu proteini biyolojik zardan ayırmak için deterjanlar, polar olmayan çözücüler veya bazen denatüre edici maddeler kullanılır. Periferik zar proteinleri ise zara veya integral zar proteinlerine hidrofobik, elektrostatik ve diğer kovalent olmayan etkileşimler ile geçici olarak bağlanırlar. Bu proteinler yüksek pH veya yüksek tuz konsantrasyonu içeren bir çözelti ile muamelenin ardından zardan ayrışabilirler.

Günümüzde yukarıda bahsi geçen biyolojik zar proteinlerinin yapısal ve fonksiyonel çalışmaları, zar proteinlerinin yapısal çalışmaları için gerekli olan aşırı ekspresyonu ve üretimindeki zorluklar nedeniyle büyük ölçüde engellenmiştir. Diğer protein sınıflarıyla karşılaştırıldığında, zar proteinlerinin yapılarının belirlenmesi, ekspresyonundaki ve saflaştırılmasındaki zorluk nedeni ile büyük ölçüde engel ile karşılaşmaktadır.

Tıbbi ve endüstriyel açıdan büyük öneme sahip bir biyolojik zar proteini olan aquaporinler hücre zarlarında gözenekler oluşturan, hücreler arasında suyun taşınmasını kolaylaştıran transmembran integral proteinlerdir. Su moleküllerinin geçişine izin verirken diğer tüm çözünen maddeleri reddeden zar proteini. Farklı hücre zarlarından aquaporin keşfi üzerine çok sayıda araştırma yapılmıştır. Birçok canlı organizma, zarlarında yüksek su hareket kapasitesi sağlayan aquaporin kanal proteinlerine sahiptir. Prokaryotik hücrelerde endüstriyel alanda biyomimetik membran yapımı ile ön planda olan aquaporinler, bitki hücrelerinde hücrenin hayati koşullarını sağlayan moleküllerin geçişini ve bazı stres koşulları (kuraklık) sırasında gerekliliği ile bilinirken, memeli aquaporinleri ise birçok hastalıkla özellikle lösemi ile ilişkilendirilmektedir. Bu doğrultuda yapılan çalışmalar sırasında karşılaşılan en büyük zorluk integral biyolojik zar proteini olan aquaporinlerin aşırı ekspresyonunun sağlanması ve sonrasında saflaştırılmasının optimizasyonunun sağlanmasındaki engellerdir.

Aquaporinler, altı transmembran sarmal bölümden oluşan aquaporin monomerleri ve dar sulu gözenekle bağlanan sitoplazmik ve hücre dışı vestibülleri çevreleyen iki kısa sarmal bölümden oluşan temel yapıya sahiptirler. Sarmallar arasında, asparagin-prolin-alanin ("NPA motifi") desenine sahip, ikisi hidrofobik (B, E) olmak üzere hücre

zarının içine veya dışına kıvrılan beş bölge (A-E) vardır. Kanaldaki bir diğer kısım, akuaporinin seçici olarak farklı moleküllerin geçişine izin vermesini veya engellemesini sağlayan bir amino asitler kümesi olan "ar / R seçicilik filtresi" dir.

Akuaporinler, hücre zarından su transferinde 10-100 kata kadar artış sağlarlar. Ayrıca bu proteinler, hücre dışı sinyallerin iletimi veya maddelerin zar yoluyla taşınması gibi çeşitli kritik roller de oynarlar. Benzersiz özellikleri nedeniyle, yapı ve aktivite analizi veya yeni biyo-taklit materyalleri dahil olmak üzere farklı amaçlar için yüksek miktarda üretilmelerine ihtiyaç duyulmaktadır. Yapısında bulunan Arginin aminoasidinden dolayı pozitif yüklü enerji bariyeri oluşturmaktadır. Bu şekilde, akuaporin yüklü veya nötral özellikte olan çözücülerin geçişini engellemektedir.

Son 5 yılda akuaporinlerin rekombinant üretimi, genetik, doku lokalizasyonu, gelişimsel ve düzenlenmiş ekspresyonu, transgenik fare modelleri ve yapı / fonksiyon analizleri üzerine yayınlanan 500'den fazla çalışmada akuaporinlerin biyolojisine (AQP'ler) karşı büyük ilgi oluşmuştur. Bakterilerden memelilere kadar bütün hücrelerde bulunan akuaporinler literatürde araştırıldığında, çoğunlukla AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 ve AQP8 türlerinin suya özel olduğu görülmektedir. AQP3, AQP7 ve AQP9 ise akuagliseroproteinler olarak adlandırılır ve ayrıca gliserol ve / veya diğer küçük yüksüz molekülleri taşımaktadır.

Şu ana kadar rekombinant akuaporinle ilgili yapılan çoğu çalışmada, akuaporinlerin fonksiyonel, düzenleyici veya yapısal çalışmaları gerçekleştirilmiştir. Bahsedilen bu çalışmaların yanı sıra yüksek su geçirgenliği ve yüksek çözünen madde reddine sahip akuaporinlerin, suyun tuzdan arındırılması ve yeniden kullanılması için biyomimetik membranlarla kullanılması son yıllarda büyük ilgi görmektedir.

Prokaryotik akuaporinler ilk olarak *Escherichia coli* 'de tanımlanmıştır ve bugüne kadar akuaporin proteini saflaştırmak için yapılan çalışmalarda *Rhodobacter sphaeroides*, *Lactococcus lactis*, *Saccharomyces cerevisiae*, *Mathanothermobacter marburgensis*, *Photobacterium profundum* SS9 ve *Halomonas elongate* gibi mikroorganizmalar kullanılmıştır. Aynı zamanda, hücre içermeyen protein ekspresyon sistemleri (cell-free expression systems) gibi yöntemler kullanılarak akuaporinlerin rekombinant olarak üretilmesinde avantaj sağlayacak çalışmalarda bulunmaktadır.

Prokaryotik AQP'ler ile yapılan çalışmalar bakterilerin, ozmotik, oksidatif stres ve beslenme dalgalanmalarıyla başa çıkmasına yardımcı olduğunu göstermektedir. Fakat literatürde prokaryotik organizmalardan ve özellikle ekstrem koşullardaki prokaryotlardan üretilen akuaporinler için sınırlı sayıda çalışma bulunmaktadır.

Yüksek tuz oranına sahip bir göl olan Acıgöl'den (Burdur, Türkiye) izole edilen *Virgibacillus sp.* AGTR suşu, halofilik yeni bir suş olarak literatürde yerini almıştır. Bu organizmanın genom bilgileri elde edilmiş ve biri 831 diğeri 795 baz uzunluğu olmak üzere iki adet aquaporin kodlayan gen bulundurduğu tespit edilmiştir. Bir başka ekstrem ortama sahip Armutlu'dan (Yalova) sediment örnekleri alınmış ve buradan da termofilik bir bakteri olan *Geobacillus thermoleovorans* ARTR1 izole edilmiştir. Bu organizmanın da 819 baz uzunluğunda akuaporin kodlayan gen dizisi belirlenmiştir. Ekstremofilik aquaporinlerin zorlu şartlar gerektiren endüstriyel kararlılıklarını analiz etmek amacıyla literatürde herhangi bir çalışması bulunmayan bu ekstrem organizmalardan toplamda 3 adet ekstremofilik akuaporin geni izole edildi. İzole edilen bu genler pET28a (+) vektörüne aktarılarak, *Escherichia coli* C43 konakçı hücresinde rekombinant olarak üretildi. Bu süreçte, uygun primerler ile yapılan PZR yöntemi ile ilgili genler çoğaltıldı ve pET28a (+) vektörü ile ligasyonu sağlandı. Transformasyon işlemi ile konakçı hücreye transfer edilen ilgili geni içeren plazmitler

daha sonrasında *SacI* ve *NotI* restriksiyon enzimleri ile kontrol kesimi ve koloni PZR kurularak doğruluğu sağlandı. Uygun koşullarda büyütülen hücreler, IPTG ile indüklenerek proteinin aşırı ekspresyonu sağlanmıştır. Optimal koşul olan 1 mM IPTG, 37 °C ve 4 saat indüksiyon seçildi. Bu optimal koşulda görünür bir şekilde gen ifadesi sağlandı. Protein sonikasyon işleminden sonra uygulanan santrifüjde oluşan inklüzyon cisminde kaldığı gözlemlendiği için, santrifüj sonrası pellet ile devam edilmiş ve kontrol amaçlı ayrıca süpernatant SDS-PAGE ile incelenmiştir. Bu süreçten sonra ise protein çeşitli His-tag saflaştırma yöntemi kullanılarak saf olarak elde edildi. Ortalama 29 kDa boyutunda görülmesi beklenen proteinler SDS-PAGE ve Western Blot Analizleri ile teyit edilmiştir.

Lipozomlar, kolesterol ve toksik olmayan doğal fosfolipidlerden oluşturulabilen, küresel şekilli küçük yapay keseciklerdir. Boyutları ve hidrofobik ve hidrofilik karakterleri (biyoyumluluğun yanı sıra) nedeniyle, lipozomlar ilaç dağıtımı için umut verici sistemlerdir. Lipozom özellikleri, lipid bileşimi, yüzey yükü, boyutu ve hazırlama yöntemi ile önemli ölçüde farklılık gösterir. Ayrıca, çift katmanlı bileşenlerin seçimi, çift katmanın 'sertliğini' veya 'akışkanlığını' ve yükünü belirler. Lipozomlar hücre zarını taklit edebilir. Lipozomların hazırlanması için doğal veya ilgili lipidler kullanılır. Akuaporinin uygun koşullarda lipozoma entegrasyonu ile oluşan proteolipozom sayesinde proteinler karakterizasyona hazır hale gelir. Lipozomlar, insan hücrelerinin lipid çift katmanlı zarlarında bulunan ve fizyolojik olarak kabul edilebilir doğal veya sentezlenmiş fosfolipidlerdir. Bu çalışmada fosfatidilkolinlerin (PC) bir örneği olan DOPC (1,2-dioleoyl-sn-glisero-3-fosfokolin) kullanılmıştır. Proteolipozom ise aquaporin içeren lipozomlardan oluşmaktadır. 2.5 mg/ml DOPC lipidi için 0.5 mg/ml konsantrasyonu olan aquaporin 10 ml PBS ile çözülüp 0.05M OG deterjanı eklenerek proteolipozom elde edilmiştir. Kontrol lipozomu ve proteolipozomu 0.85 M NaCl ile muamele edilip durağan akışlı ışık saçılım spektrometre cihazı ile aquaporinlerin su transfer hızı ölçülmüştür. Burada, aquaporinli lipozomların su geçişinin daha hızlı olduğu belirlenmiştir. Aquaporin taşıyan proteolipozomlar kontrol lipozomlarına kıyasla çok daha yüksek bir hızda su kaybetmektedirler. Bu da *Geobacillus Thermoleovorans* ARTR1 suşundan elde edilen aquaporinin çalıştığına göstergesi olarak açıklanmıştır.

Bu proje kapsamında akuaporin proteinlerinin halofilik ve termofilik bakteriler gibi ekstrem koşullarda yaşayan bakterilerden tanımlanması ve karakterizasyonunun gerçekleştirilecek olması literatüre sağlayacağı katkı açısından büyük önem taşımaktadır.



1. INTRODUCTION

1.1 History of Cell Membrane

In the early 19th century after the discovery that cells being separated from plant cells, it turned out that Robert Hooke's Cell Membrane Theory in 1665 was actually a misdirection (Edidin, M., 2003). Until then, the cell wall was known as the cell membrane, as plants have cell walls and are the only organisms that can be studied. In 1890, however, many scientists stated that the cell membrane exists, but that it has only a secondary structure. Until 1972, Davson and Danielli continued their studies on the lipid bilayer hypothesis and also Gorter and Grendel continued their study about membrane structure (Gorter E, Grendel F, 1925). Finally, in 1972, Singer and Nicolson proposed the Fluid Mosaic Model, which is still accepted today (S. J. Singer and G. L. Nicolson, 1972). According to this model, the cell membrane consists of a lipid bilayer, including cholesterol, carbohydrates, proteins, which sits between phospholipids to maintain this fluid at various temperatures. This membrane is dynamic and a molecule in a certain position on the membrane may move to the right or left after a while and move to a different position. Thanks to the structures it contains, the selective permeability feature of the membrane ensure the passage of water, ions, or organic molecules between cells (Alberts B, Johnson A, 2002). Proteins located in this structure are called membrane proteins and are divided into classes within themselves (T. Herrmann, S.Sharma, 2019).

1.1.1 Overview of biological membrane proteins

Membrane proteins are proteins that are part of biological membranes. They are also known as common proteins that interact with biological membranes. Membrane proteins are located on or embedded in the cell membrane in Figure 1.1. Membrane proteins, which make up approximately 50% of the entire membrane, have the highest content on the cell membrane (J. Gray, S. Groeschler; T. Le, 2002). These proteins are needed for the performance of vital functions for the survival of organisms. They are responsible for ensuring intercellular substance transport, regulating the shape of

organelles and cells, and transporting ions, metabolites, and proteins across plasma membranes and RNA across the nuclear membrane (Lin, S. H., & Guidotti, G., 2009). For example, membrane receptor proteins in the membrane are responsible for intracellular and extracellular signals. Another example is the capacity of transport proteins to carry ions across the entire membrane (Almén MS, Nordström KJ, 2009).

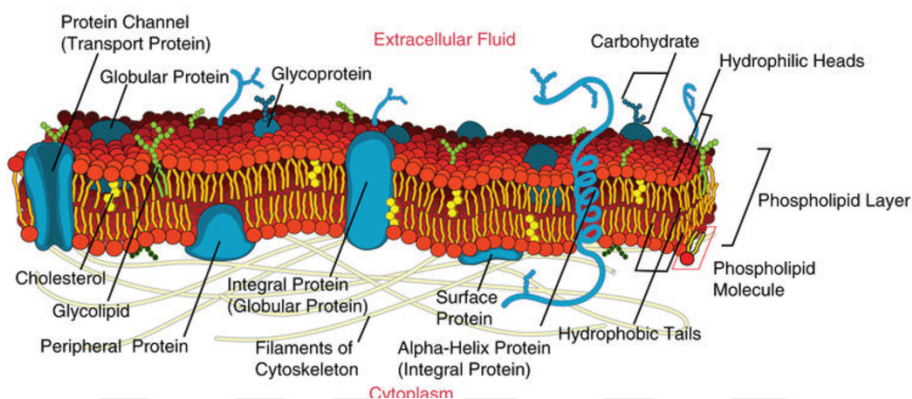


Figure 1.1 : General picture of biological membrane and membrane proteins respectively (Membrane Proteins, 2021).

In addition to being responsible for many biological activities, these proteins have specific tasks when they are grouped. To understand how a membrane protein works, the preparation of the protein for characterization and the purification of the protein for this is very important. But today, the amount of membrane proteins is still very low among the proteins obtained biochemically pure (Almén MS, Nordström KJ, 2009). The reason for this is that the expression and purification processes of membrane proteins are quite difficult compared to other proteins. However, the low level of membrane protein, its embeddedness in the lipid layer, and the requirement for denaturing conditions make these proteins difficult to study. At this point, the correct setting of the denatured conditions and the selection of the right detergent is critically important.

1.1.2 Classification of biological membrane proteins

As mentioned above, membrane proteins are examined under three main headings. These proteins are named according to the position they are in or on the membrane, and the molecules they carry into or out of the cell. Some of them are used as ion channels and proton pumps, while the other part is responsible for the passage of some enzymes and hormones.

1.1.2.1 Integral membrane proteins

Integral membrane proteins (IMPs) are biological membrane proteins that are permanently attached to the membrane. Since these proteins are surrounded by a lipid layer, denaturing agents, non-polar solvents or detergents are used to separate them from the membrane (Lin, S. H., & Guidotti, G., 2009). IMPs make up a quarter of the entire proteome (Whitelegge, J. P., 2013) shown in Figure 1.2 and they control the flux of molecules, energy and information between cell membranes. All transmembrane proteins are examples of integral proteins but not all integral proteins are known as transmembrane proteins. In addition to that, IMPs constitute a significant portion of the proteins encoded in an organism's genome.

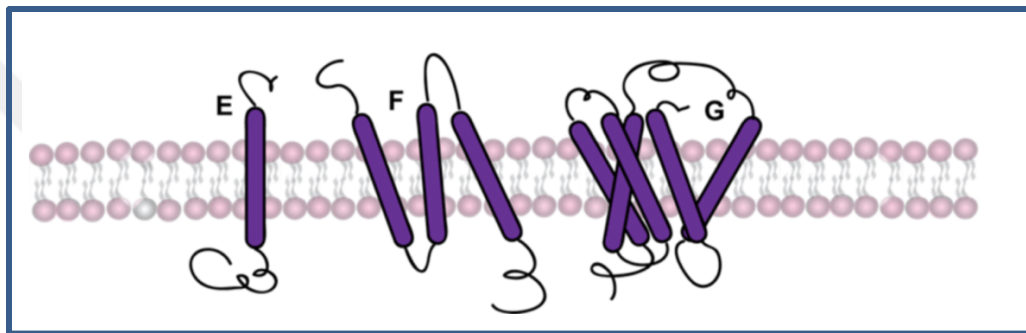


Figure 1.2 : E. Bitopic membrane proteins; F. Polytopic membrane proteins; G. Multimeric assemblies of membrane proteins (channels or transporters) (Ariöz, C., 2014).

Integral proteins are divided into two different groups;

- Integral polytopic proteins (Transmembrane proteins)
- Integral monotopic proteins

Integral polytopic proteins span the membrane only once. The carboxyl terminals of these single-pass proteins towards the cytosol are called Type I, and if the amino terminals are towards the cytosol, they are called Type II. Type III proteins, on the other hand, have more than one transmembrane domain in only one polypeptide. Type IV consists of different polypeptides, while Type V is located in the lipid bilayer with covalently bonded lipids. Finally, Type VI has both a transmembrane domain and lipid anchors (Nelson, D. L., & Cox, M. M., 2008). Integral monotopic proteins, on the other hand, are partially associated with the membrane but are not completely found in the lipid bilayer.

To sum up, integral proteins are proteins of critical importance such as proteins responsible for cell adhesion, channels, receptors and transporters, and intercellular communication is ensured thanks to the transport of molecules that are vital for the cell with these proteins.

1.1.2.2 Peripheral membrane proteins

Peripheral membrane proteins are temporarily bound to either the lipid bilayer or integral proteins by a combination of hydrophobic, electrostatic, and other non-covalent interactions shown in Figure 1.3. Generally, they have an affinity for binding sites on the membrane, and these sites are usually on the integral membrane protein. Peripheral proteins can be dissociated from the membrane by mild treatments such as a polar reagent, a solution with high pH or high salt concentration (The Membranes of Cells, 2016). For example, subunits of integral proteins such as many ion channels and transmembrane receptors can be defined as peripheral membrane proteins.

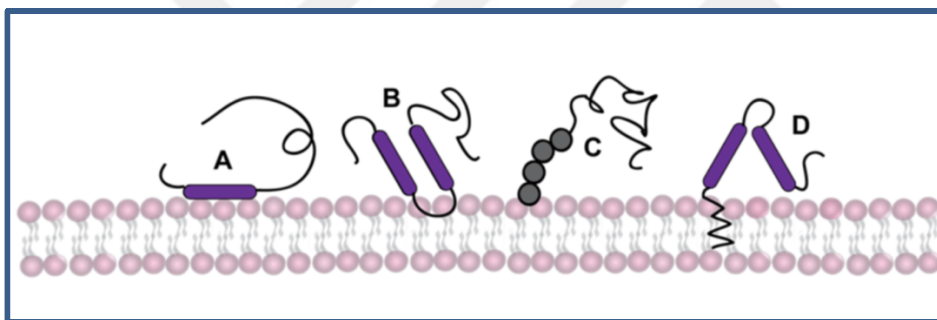


Figure 1.3 : Monotopic membrane proteins with an amphipathic helix; B. Monotopic membrane proteins; C. GPI-anchored membrane proteins; D. Lipid-anchored membrane proteins (Ariöz, C. ,2014).

1.1.2.3 Lipid anchored proteins

These are proteins that covalently bind to lipids embedded in the cell membrane. Lipid-anchored proteins are located on the lipid layer and play a role in the lipid bilayer structure of the membrane (Gerald Karp, 2009). However, the portion that is covalently bound to one or more lipid molecules is not the protein itself. The lipid groups present here to play a critical role in protein interactions, such as increasing hydrophobicity. These lipid anchors consist of long-chain acyl or prenyl groups, glycosyl-phosphatidylinositol (GPI) and cholesterol (Nalivaeva N.N., Turner A.J., 2009). However, it should not be forgotten that the region where the lipid binds varies both according to the lipid group and according to the protein.

Membrane proteins play a critical role in biological processes. Understanding how these proteins work will also help to purify these proteins for characterization. For example, Cytochrome c-hemeprotein is being studied as “the most up-to-date peripheral membrane protein” (Stillwell, W., 2016). This peripheral protein has a significant role in cell apoptosis. That is why, expression, purification, and characterization of Cytochrome c should be clarified to understand its function and biochemical characterization. Cytochrome c can be separated from the mitochondrial membrane by using simple solvent KCl (Potassium Chloride) because peripheral proteins dissociate more easily from the membrane compared to integral membrane proteins. Another example of a membrane protein is MBP which is bound to the membrane with electrostatic interactions. This protein also plays a role in the myelination of the nerves in the central system and the deficiency of this protein causes multiple sclerosis disease (Stillwell, W., 2016).

1.2 Aquaporin as a Biological Membrane Proteins

Aquaporins are integral membrane proteins that facilitate the diffusion of water and uncharged solutes across the cellular membrane and are widely distributed in all organisms, from humans to bacteria. These water channels (aquaporins) were first described by Agre et al during studies on the Rh (D) factor in erythrocytes (Akyüz, S., Büyüköztürk, H., Öztürk, A.). The protein of interest weighs 28 kDa and is named Channel-forming Integral Protein (CHIP28). CHIP28 is a member of the Major Intrinsic Protein (MIP) family found in the eye cell. As a result of experiments on *Xenopus laevis* oocytes, it was observed that each channel has very high-water permeability. These channels were named aquaporin by the Human Genome Organization in 1997.

There is a lot of research has been done on the discovery of aquaporin from different cell membranes. Many living organisms have aquaporin channel proteins in their membranes that provide high water movement capacity (Çalicioğlu, N. G., Özdemir, 2018). Aquaporins have a similar basic structure, containing aquaporin monomers; six transmembrane helical segments and two short helical segments surrounding cytoplasmic and extracellular vestibules associated with narrow aqueous pore (Verkman, A. S., 2013). Among the alpha helix, there are asparagine-proline-alanine proteins called NPA pattern which also has two hydrophobic (B, E) conserved areas.

Aquaporin has five special regions that span the cell membrane both inner and outer sides. The primary function of most aquaporins is to transport water across cell membranes in response to osmotic gradients created by active solute transport. Aquaporins provide a 10-100-fold increase in water transfer across the cell membrane (Yılmaz, H., Özdemir, F. İ., 2020). In addition, these proteins play a variety of critical roles, such as the transmission of extracellular signals or the transport of substances across the membrane. Because of their unique features, they are required to be produced in large quantities for various purposes such as structure, function, activity analysis or new biomimic materials. Different types of aquaporins might have different selectivity and rejection features for the same solvent. As aquaporin may vary depending on solute concentrations (Yılmaz, H., Özdemir, F. İ., 2020) the selectivity of aquaporin also changes depending on charge and size. It creates a positively charged energy barrier due to the arginine amino acid in its structure. In this way, aquaporin prevents the passage of charged or neutral solvents. Prokaryotic aquaporins were first identified in *Escherichia coli*, and in studies conducted to purify aquaporin protein to date, *Rhodobacter sphaeroides* (Erbakan, M., 2015), *Lactococcus lactis* (Froger, A., Rolland, 2001), *Saccharomyces cerevisiae* (André, B., 1995), *Mathanothermobacter marburgensis* (Kozono, D., 2003), *Photobactetrium profundum SS9* (Wei, P., Wang, Q., 2017) and *Halomonas elongate* (Sengur-Tasdemir, R., Aydin 2016) were used. There are also studies to produce aquaporin recombinantly using methods such as cell-free expression systems (Tong, H., 2019).

Over the past 5 years, there has been great interest in the biology of aquaporins (AQPs) in more than 500 studies published on recombinant production of aquaporin, genetics, tissue localization, developmental and regulated expression, transgenic mouse models, and structure/function analyzes (Verkman, AS, & Mitra, AK, 2000). When aquaporins, which are found in all cells from bacteria to mammals, are investigated in the literature, it is seen that mostly AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 species are water specific. The others such as AQP3, AQP7 and AQP9 are called aquaglyceroporins and carry glycerol and/or other small uncharged molecules (Johansson, I., Karlsson, 2000). Therefore, aquaporins are not only water passage proteins but also they are specific to glycerols, urea, ion and different type of molecules that are required for the survival of the cell.

1.2.1 Usage field of aquaporin

Aquaporins, called water channels, are generally found in mammals, prokaryotes, and plants. Aquaporins, which are divided into different groups among themselves, are not only specific for water passage, but also responsible for the passage of urea, glycerol, some organic molecules, and ion passage, which are vital for the cell. Aquaporin, which is categorized into 13 types in mammals, has more than 200 members in the living world (Akyüz, S., Büyüköztürk, H.) Aquaporins with the aid of special amino acid sequences, cross the membrane 6 times and allow 5-9 water molecules to pass through the channel. The distribution and expression patterns of aquaporin family members differ according to tissues (Akyüz, S., Büyüköztürk, H., Öztürk, A.). 0, 1, 2, 4, 5, 6 and 8 of the Aquaporin family only function as water channels. Although it is similar to the others in amino acid sequence, AQP6 functions as anion and AQP8 as urea channel. AQP 3, 7, 9 is aquaglyceroporin and recently identified AQP11 and AQP12 are superaquaporins. Mutations of these channels, whose transporter properties are controlled by different mediators, especially hormones, cause diseases ranging from cataracts to heart failure. While the relationship between aquaporin family members and solid tumours has been determined before, it has been shown that there is a relationship between aquaporins and haematological neoplasms recently. These proteins, which have more functions than simple water channels, will be targeted in the treatment development strategies of other cancer types, especially leukaemias in the future, and will offer new possibilities.

In plants, aquaporins exist in multiple isoforms, reflecting their wide variety of cellular localization and transport selectivity (Maurel C, Boursiac Y, 2015). Plant aquaporins are localized in the plasma membrane, endoplasmic reticulum, vacuoles, plastids, and in some species, membrane compartments that interact with symbiotic organisms. They are responsible for the passage of many physiological substrates in addition to water. They also provide the transport of vital gases of plants, such as carbon dioxide and ammonia or metalloids (Maurel C, Boursiac Y, 2015). For most of the AQPs identified in the tonoplast and plasma membranes, regulation of AQP activity and gene expression is also considered as part of the adaptation mechanisms to stress conditions (Kapilan, R., Vaziri, 2018). The main role of plant aquaporins is to ensure the passage of water and other small neutral molecules towards the cellular biological membrane. Plant AQPs are grouped into five major subfamilies consisting of the plasma

membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26 like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and X intrinsic proteins (XIPs) (Kapilan, R., Vaziri, 2018). To give an example, Terrain plants have evolved to cope with sudden changes in water availability by regulating aquaporins in their plasma membranes. The SoPIP2;I structure in spinach allows the passage of water molecules in a continuous row, connected by seven hydrogen bonds, similar to those in mammals (Akyüz, S., Büyüköztürk, H.). Environmental stresses for example salinity, drought and low root temperature may rapidly reduce water transport rates. That is why plants resistant to these environmental stresses can be produced with genetically modified aquaporins.

When looking at prokaryotic aquaporins, they remain largely unknown (Tong, H., 2019). Studies with prokaryotic AQPs show that they help bacteria cope with osmotic, oxidative stress and nutritional fluctuations. Prokaryotic aquaporins were first identified in *Escherichia coli* (Tong, H., 2019) and named AqpZ or glpF. Here, AqpZ with six transmembrane domains is known as the water channel, and glpF is known as the glycerol facilitator. These two proteins also form tetramers containing four monomers and have an ar/R region and two NPA motifs (Savage, D. F., 2003).

AqpZ studies are generally important in the field of industry. Functional, regulatory or structural studies of aquaporins have been performed in most studies on recombinant aquaporins so far (R. Kaldenhoff, 2007). In addition to these studies, the application of aquaporins with high water permeability and high solute rejection with biomimetic membranes for water desalination and reuse has attracted great interest in recent years (Tang, C. Y., Zhao, 2013). Thanks to the membrane-mimicking liposome integration of these proteins, they can be used in wastewater treatment. To emphasize the importance of aquaporin, a recent study revealed that an aquaporin from catalase-negative *Streptococcus* facilitates the efflux of endogenous H₂O₂ and therefore plays important role in H₂O₂ detoxification as well as in interspecies competition (Johansson, I., Karlsson., 2000).

1.2.2 Extremophilic aquaporins

Extremophiles are organisms that can survive under extreme conditions (high/low pH, high salt ratio, salinity, high/low temperature) where other organisms cannot survive (Zhu, D., Adebisi, 2020). Extremophiles are classified as plants, animals, insects,

fungi, and bacteria. Examining the extremophilic bacteria in detail here, they are defined in many categories such as acidophilic, alkaliphilic, halophilic, thermophilic, hyperthermophilic, psychrophilic, piezophilic/barophilic, oligotrophic (Zhu, D., Adebisi, 2020). These types of bacteria can defer the environment that lives in it. For example, alkaliphilic bacteria can survive at high pH, while halophilic bacteria can survive in a high salt environment. At the same time, thermophilic bacteria love high temperatures, hyperthermophilic bacteria survive at extremely high temperatures, and piezophilic/barophilic bacteria can survive under high pressure. Researchers are generally interested in describing the metabolic cycles of enzymes or proteins for potential industrial use due to their extreme properties, as they support high stability, good solubility of substrates, high mass transfer rate, and reduce the risk of contamination during industrial processes (Chen and Jiang, 2018). Aquaporins that are isolated from extremophilic bacteria can be used for various membrane types such as thin film composite membranes, nanofiltration membranes, biomimetic membranes in the industry.

For example, *Halonomas elongata*, a gram-negative bacterium first isolated from a sun salt plant in Bonaire, Netherlands Antilles, is a halophilic bacterium (Vreeland RH, Litchfield CD, 1980). *H. elongata* aquaporin is a suitable alternative for biomimetic membrane production. With this study, a remarkable increase in the water permeability of the aquaporin membrane obtained from Halomonas was observed. In another study, it was observed that the glucose rejection capacity of *E.coli* aquaporin added to the membrane structure increased by 27% and the water permeability potential increased at the same rate (Öztürk, A., Yıldız, A., 2020). As seen in these examples, as a result of the use of aquaporin obtained from organisms on the membrane, the transit capacity of substances such as water and glucose increases depending on the type of aquaporin. Another example is; *Geobacillus Thermoleovorans* is a thermophilic gram-positive bacteria which has a character that survive under high temperatures (Oztug, M., Cebeci, A., Mumcu, 2020). Purified aquaporin from this thermophilic organism can be active under high temperatures and be used for harsh conditions within the appropriate membrane in the industry. On the other side, *Virgibacillus sp.* is a halophilic bacterium that thrives at high concentration of salt. Purified aquaporin from this halophilic organism can be active under high salinity conditions and this is also useful for industrial applications such as halophilic

aquaporin within the membrane can remove the waste from wastewater under harsh conditions.

Since aquaporins are difficult to work with membrane proteins, it is very challenging to produce sufficient quantities of aquaporin in applications such as creating biomembranes for water filtration. Recently, with the developments in materials science, various artificial membranes can be produced for the integration of aquaporins (Li X, Wang R., 2012). In this way, aquaporins with different properties will be integrated into this liposome layer, their characterization will be examined, and they will be used in the industrial field.

1.3 Liposome as a Support Lipid Membrane

Liposomes are vehicles formed by the combination of layered phospholipids (Yurdakul, A., & Rıza, 2007). Bangham discovered and described them as inflated phospholipid structures called Banghasomes in 1965 (Nkanga, C. I., Bapolisi, 2019). Liposomes were first described as small devices made up of one or more closed phospholipid bilayers a few years later. Liposomal vesicles are classified as either nanoparticles or microparticles, depending on their particle size, which ranges from 20 nanometers to several micrometres, and their ability to encapsulate molecules of diverse origin and polarity. Liposomes can mimic the cell membrane. Natural or related lipids are used for the preparation of liposomes. Different lipid compositions can be used to obtain the desired type of liposome. Liposomes are classified as monolayer, bilayer and multilayer. As mentioned above liposomes are mainly composed of phospholipids; It has been used as a model membrane which shown in Figure 1.4 by scientists for many years because it is similar to the cell membrane in terms of structure and content, is not toxic, and their chemical content can be determined by the researcher. Recently, there has been an increase in interest in the use of this natural cellular membrane made by integrating aquaporins into liposomes for water filtration applications (Li X, Wang R., 2012). This is why aquaporin integrated into liposomes can provide water reuse and desalination. For the preparation of AQP biomimetic membranes, AQPs should be embedded in an amphiphilic bilayer and block copolymers (M.J. Borgnia, 1999). In this step it is important to use detergents with appropriate amount of critical micelle concentration for the detergent

to place or encapsulate the aquaporins into empty liposome. Then detergents might be remove using different applications to proteoliposome.

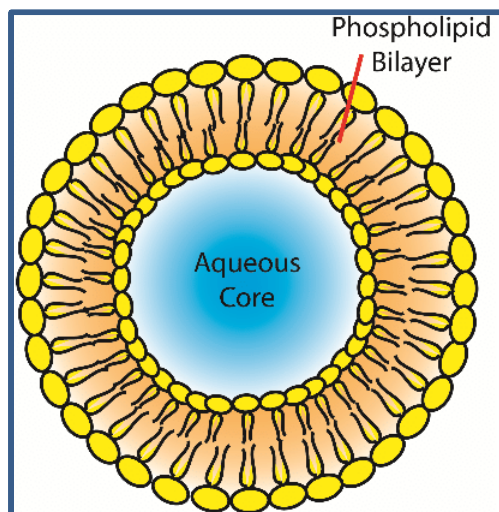


Figure 1.4 : The representative picture for liposome structure formed by phospholipids in an aqueous solution (Leung, A. W., Amador, C., 2019).

There are many successful strategies for the reconstitution of aquaporins to the biomimetic membrane (Li, X., Wang, R., 2012). Choosing the correct lipid is primarily important in liposome production. Proteins are ready for characterization thanks to the proteoliposome formed by the integration of aquaporin into the liposome under suitable conditions.

As an advantage, the stability of liposomes can increase by encapsulation and reduce toxicity, while liposomes can increase the efficiency of some therapeutic drugs. But besides these advantages, it also has some disadvantages such as low solubility, short half-life or production cost can be high (Immordino, M. L., 2006).

1.3.1 Liposome classification

Liposomes are classified depending on their particle size and bilayer numbers for forming vesicles as given below Figure 1.5; Liposomes are classified depending on their particle size and bilayer numbers for forming vesicles as given below;

- **Small Unilamellar Vesicles (SUV) (20-100nm)**

Sonication with a cuphorn, bath, or probe tip sonicator is commonly used to prepare.

- **Large Unilamellar Vesicles (LUV) (>100 nm)**

Extrusion, detergent dialysis, SUV fusion, reverse evaporation, and ethanol injection are some of the methods used to make them.

- **Multilamellar Large Vesicles (MLV) (>500 nm)**

Freeze-drying lipid mixtures used for liposome production.

- **Giant Unilamellar Vesicles (GULV) (>1000 nm)**

In research, GUVs are commonly utilized as models for biological membranes. Using water-oil interfaces as a scaffold to create lipid layers, a range of approaches for encapsulating biological reactants within GUVs exist (Wesołowska, O., Michalak, K., 2009)

- **Oligolamellar Vesicles (OLV) (100-1000 nm)**

Oligolamellar morphology refers to vesicles with between two and five concentric lamellae, whereas multilamellar vesicles are liposomes with more than five lamellae (MLVs).

- **Multivesicular Vesicles (1000 nm to several thousand nanometers)**

Smaller liposomes are enclosed in larger vesicles in multivesicular liposomes.

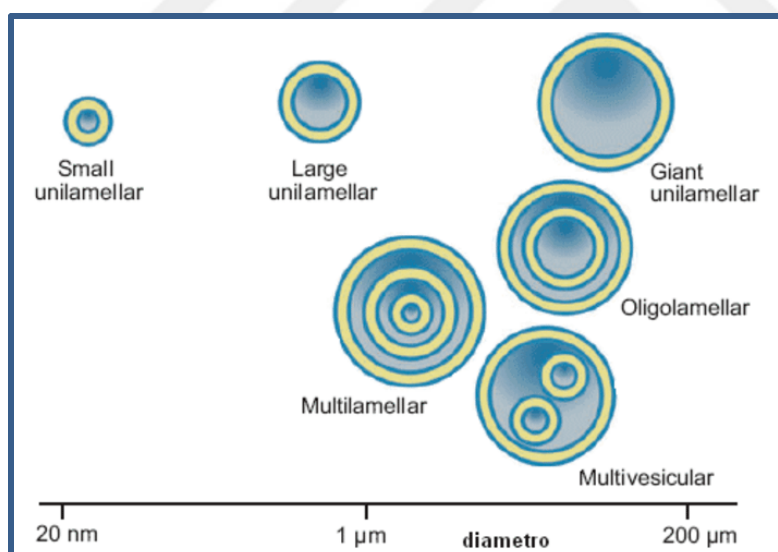


Figure 1.5 : Liposomes are categorized according to their size and number of lamellae (Wood, M. H., Milan, D. 2021).

1.3.2 Liposome composition

Liposomes are phospholipids found in the lipid bilayer membranes of human cells and are physiologically acceptable natural or synthesized phospholipids which are shown in Figure 1.6. Phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and phosphatidylglycerols (PG) are the most commonly

preferred phospholipids for liposome production. DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), an example of Phosphatidylcholines (PC), was used in this study.

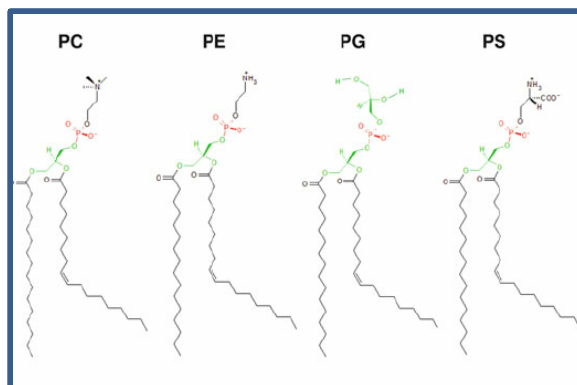


Figure 1.6 : Chemical structure of the structural phospholipids. Abbreviations: PC phosphatidylcholine, PE phosphatidylethanolamine, PG phosphatidylglycerol, PS phosphatidylserine (Furt, F., Simon-Plas, F., 2011).

1.4 Aim of the Study

Within the scope of this project, *Virgibacillus sp.* AGTR strain isolated from Acıgöl (Burdur, Turkey), which is a lake with high salinity has taken its place in the literature as a new halophilic bacterium strain. The genome information of this organism was obtained and it was determined that it contains two aquaporin-encoding genes, one of which is 831 bp and the other 795 bp. Also, a new thermophilic bacterium *Geobacillus thermoleovorans* ARTR1 was isolated from another extreme environment, Armutlu (Yalova). The gene sequence encoding aquaporin with a length of 819 bp was determined in this organism. In our previous studies, the whole genome sequence and basic phenotypic characteristics of these new strains were identified.

Here we aimed the recombinant production and characterization of these three aquaporin proteins. For this purpose, the following steps have been carried out;

- i) the design of primer sets suitable for the gene of interest sequences,
- ii) the amplification of the gene of interest by using Polymerase Chain Reaction (PCR),
- iii) cloning into the expression vector,
- iv) production, purification and characterization of the proteins.



2. MATERIAL AND METHODS

2.1 Material

2.1.1 Laboratory equipments

The equipments used in this study are given in Appendix A.

2.1.2 Culture medium

The compositions of culture medium are given in Appendix B.

2.1.3 Buffer, gel and solution

The compositions of buffers, gels and solutions are given in Appendix C.

2.1.4 Chemicals, enzymes and kits

The chemicals, enzymes and kits used in the experiments are given in Appendix D.

2.1.5 Bacterial strains and vectors

pET-28a (+) bacterial expression vector (Figure 2.1) was used to produce recombinant aquaporin protein in *Escherichia coli* C43 host. Length of pET-28a (+) vector is 5369 base pairs. The vector contains T7 lac promoter, thrombin cleavage site, internal T7 epitope tag and also restriction enzyme cleavage sites for *XhoI*, *NotI*, *HindIII*, *Sall*, *SacI*, *EcoRI*, *BamHI*, *NheI*, *NdeI*, *BmtI*, *NcoI* and *EcoRV*. Also, pET-28a (+) vector has lac operon, N- and C-terminal Histidine tags sequences for protein purification, kanamycin resistance gene (KanR).

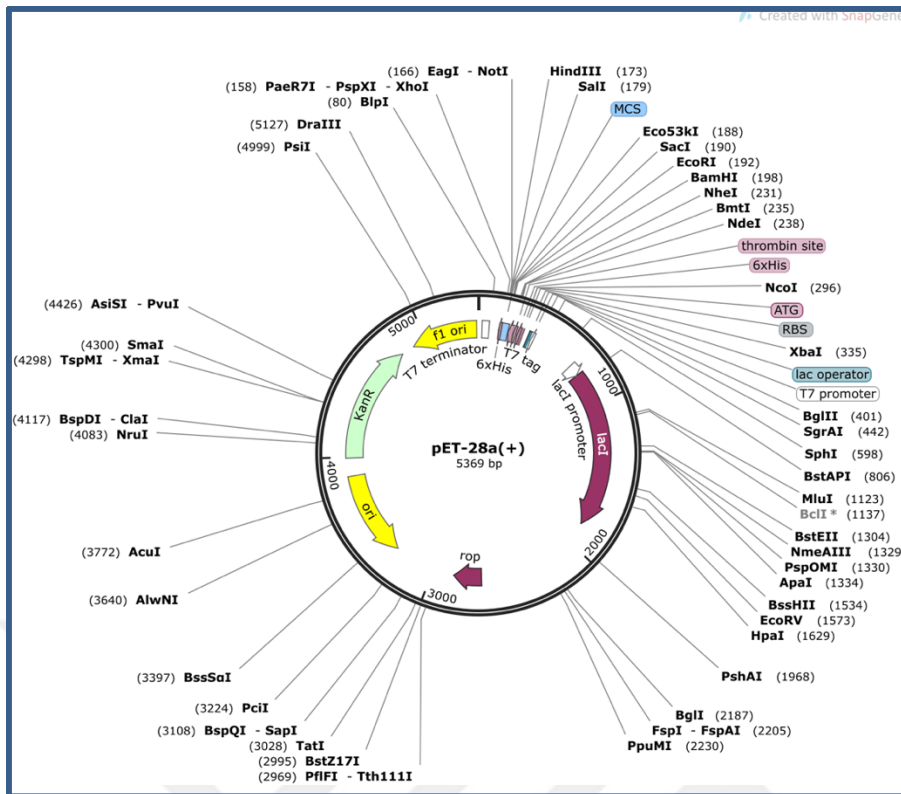


Figure 2.1 : Genomic map of pET-28a (+) expression vector.

2.2. Methods

2.2.1 Bacterial strains and template DNA

Aquaporin coding sequences which are 831 bp and 795 bp isolated from *Virgiballus sp.* AGTR strain and 819 bp isolated from *Geobacillus thermoleovorans* ARTR1 strain used as template DNA. They were isolated using GeneJET Genomic DNA Purification Kit (#K0721).

2.2.2 Designing primers

The Oligo Analyzer Tool was used to determine the GC content percent and T_m (°C) of gene-specific primers. When developing gene-specific primers, heterodimer and homodimer formation, hairpin structures, and secondary structures were avoided. The halophilic aquaporin gene from *Virgibacillus sp.* AGTR strain and thermophilic *Geobacillus thermoleovorans* AGTR1 were complementary to these primers. These primers for *Virgibacillus* 1 named as V.Aqp1_Forward_Primer_SacI and V.Aqp1Reverse_Primer_NotI. And also *Virgibacillus* 2; V.Aqp2_Forward_Primer_SacI and V.Aqp2_Reverse_Primer_NotI. For the

Geobacillus it is named as a GeoAqp_Forward_Primer_*SacI* and GeoAqp_Reverse_Primer_*NotI* which are shown in the Table 2.1.

Table 2.1 : Features and sequences of gene specific primers.

Name	Sequence (5' to 3')
R(V1) <i>NotI</i>	GCGAGCTCATGTCTGAATTTTTCAGCAGAAC
F (V2) <i>SacI</i>	GCGAGCTCATGTCTGCATTTTTCGAGAGTAGCG
R (V2) <i>NotI</i>	CCGCAGCCTTTTTCAGTTCATTACCTAAGGGAGC
F(Geo) <i>SacI</i>	TCATGTCGCCATTTTTCAGGGGAAAAGCG
R(Geo) <i>NotI</i>	GCCGCGAGAGAACGGCGGCTTGCG

2.2.3 Polymerase chain reaction (PCR) for *Virgibacillus* and *Geobacillus* Aqp genes

Polymerase Chain Reaction is the process of amplifying a specific region of DNA in vitro. In general, it includes three separate steps that are repeated which are shown in the Table 2.2. These steps are high temperature denaturation of the DNA to be amplified (94 °C), annealing-association reaction at a temperature that will allow specific hybridization of primers with target sites on DNA, extension of primers and complementary chain at 72 °C, the temperature at which Taq DNA polymerase enzyme shows high activity. PCR mixture was prepared in the below Table 2.2. The designed gene specific primers are used for each of them.

Table 2.2 : PCR mixtures.

Components	Volume
Phusion HF Buffer	5 µL
DMSO	0.6 µL
dNTP	0.5 µL
Template DNA	0.5 µL
Forward Primer	1.25 µL
Reverse Primer	1.25 µL
dH ₂ O	15.65 µL
Total	25 µL

Table 2.3 : Thermocycling conditions for *Virgibacillus* 1 Aqp.

Step	Temperature	Time	Cycle Number
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	1 min	
Annealing	55°C, 65°C, 72°C	40 sec	30-40
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1
Final Hold	4°C	∞	

Table 2.4 : Thermocycling conditions for *Virgibacillus* 2 Aqp.

Step	Temperature	Time	Cycle Number
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	1 min	
Annealing	60°C, 63°C, 70°C	40 sec	30-40
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1
Final Hold	4°C	∞	

The PCR mixture was prepared 25 µL for each tube and different temperatures are arranged for the annealing step. PCR conditions were prepared according to each specific primers for each gene; *Virgibacillus* 1 forward and reverse primers, *Virgibacillus* 2 forward and reverse primers shown in Table 2.3 and Table 2.4., and *Geobacillus* forward and reverse primers shown in Table 2.5. Nine mixtures were prepared according to annealing temperatures for each of them and also 30 to 40 cycle were arranged and as a initial denaturation step the temperature was 98°C, final extension was 72°C. Finally, the final hold was 4°C for three aquaporin PCR products.

Table 2.5 : Thermocycling conditions for *Geobacillus* Aqp.

Step	Temperature	Time	Cycle Number
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	1 min	
Annealing	65°C, 70°C, 73°C	40 sec	30-40
Extension	72°C	90 sec	
Final Extension	72°C	10 min	1
Final Hold	4°C	∞	

2.2.4 Agarose gel electrophoresis and PCR product clean up

All PCR products were load on the agarose gel to see the size of DNA molecules of the PCR product and agarose gel electrophoresis was performed by using 1 % agarose gel. For the preparation of agarose gel, 0.4 agarose was weighed and also 40 mL of 1X TAE buffer poured onto agarose. Both of them together boiled microwave oven until all mixture was seemed pure. After that, allowing the mixture became little cold, the mixture was poured into electrophoresis tank. Finally, agarose gel was incubated at room temperature and allowed to solidify then, 1X TAE buffer poured onto this gel. 5 µL PCR product was added 1µL of 6X DNA loading dye (EZ vision, #N472-1ML). As a marker GeneRuler 1 kb Plus (Thermo Scientific, #SM1331) was used. After the gel was run 120 V, 35 minutes, it was observed under UV light.

After seeing correct size of DNA, PCR product CleanUp was applied using ROCHE–High Pure PCR Product Purification Kit (Sigma-Aldrich). The steps were including; 125 μ L Binding Buffer and centrifuged at maximum speed for 30-60 seconds and discarding the flow through. After that 125 μ L Wash Buffer was added and centrifuged at 13.000 x g for 1 minute and discard the flow through. 50 μ L Wash Buffer added into the tube and again centrifuge at 13.000 x g for 1 minute and discard the flow through. Lastly, 40 μ L Elution Buffer placed and waited 2-3 minutes centrifuged at 13.000 x g for 1 minute and kept at -20 °C until used. Finally, PCR products were ready for the digestion and ligation steps.

2.2.5 Digestion for the vector and PCR product

Three aquaporin genes and vector pET28a (+) were consisting of *NotI* and *SacI* restriction sides. 10 U/ μ L *SacI* enzymes (Thermo Scientific, #ER1135) and 10 U/ μ L *SacI* enzymes (Thermo Scientific, #ER0591) were used to cleave and make sticky ends to vector pET28a (+) and three PCR products. According to Double Digest Calculator (Thermo Scientific) website, optimal digestion protocol was prepared and performed. Firstly, mixture was prepared for both pET28a (+) and PCR Products according to Table 2.6. After incubation for 1 hour 37 °C, Tango buffer and *NotI* are added to both reactions at 1/8 of the volume and again mixtures were incubated for 1 hour 37 °C. Again, using ROCHE–High Pure PCR Product Purification Kit, samples were cleaned previously like described. After that using NanoDrop digested vector and samples which includes inserts were calculated for quantification. Also, to check NanoDrop values, DNA concentration was calculated by using agarose gel according to marker and amount of samples that I load on the agarose gel.

Table 2.6 : Double Digestion Protocol for pET28a (+) and PCR Products.

pET28a (+) Vector Digestion		PCR Product Digestion	
Components	Volume	Components	Volume
10X Tango Buffer	2 μ L	10X Tango Buffer	2 μ L
<i>SacI</i>	1 μ L	<i>SacI</i>	1 μ L
<i>NotI</i>	1.5 μ L	<i>NotI</i>	1.5 μ L
pET28a (+) Vector	4 μ L	PCR Product	10 μ L
dH ₂ O	13 μ L	dH ₂ O	7 μ L

2.2.6 Ligation

In this step the purpose was combining vector and gene of interests. For ligation, firstly the vector:insert ratio was calculated according to quantities using NEB tool. 1:5 ratio was found correct ratio and mixture was prepared according to Table 2.7 volumes. It is known that *Virgibacillus* 1 is 831 bp and *Virgibacillus* 2 is 795 bp and *Geobacillus* is 819 bp. The ligation was checked according to agarose gel and marker. The mixtures were prepared based on nanogram on the agarose gel.

- *Virgibacillus* 1 → 6ng,
- *Virgibacillus* 2 → 3ng,
- *Geobacillus* → 3ng

Based on the calculations mixtures were prepared shown as Table 2.7. T4 DNA For the 17 μ L total volume Ligase enzyme (Roche, #10 716 359 001), 10X Ligation Buffer (Roche, #11 243 292 001) was used to combine insert and vector. After that, incubation was done 15 °C for 16 hours. Now, samples were ready for the ligation processes which is important for ligate the genes of interest to the expression vector in this case pET28a (+) vector.

Table 2.7 : Components and volumes for Ligation as a ratio of 1:5.

Components	Volumes
Buffer	2 μ L
DNA (insert)	8 μ L
Vector	8 μ L
Ligase	1 μ L
dH ₂ O	1 μ L
Total	20 μ L

2.2.7 Competent cell preparation

In this project, *E.coli* R2, BL21 and C43 (DE3) host organisms were tested and C43 was used because it was more efficient. Stocks from -80 °C were first spread over the LB plate. A single colony was selected from the colonies growing on the agar plate and left in 5 mL LB containing the relevant antibiotic (kanamycin) overnight at 37 °C. 5 mL of the preculture was taken and placed in 100 mL LB medium. It is kept at 37 °C until OD₆₀₀ reaches 0.4. Then, when the OD₆₀₀ reaches 0.4, the culture is placed in the falcon kept at -20 °C and kept on ice for 10 minutes. After incubation, it is centrifuged at 5000 rpm for 10 minutes at +4 °C. The supernatant is discarded. A total

of 30 mL of 80 mM MgCl_2 and 20 mM CaCl_2 is added onto the pellet. The samples are kept on ice for 5 minutes and centrifugation is applied under the above-mentioned condition. Finally, 100 mM (825 μL) CaCl_2 and 85 % glycerol (175 μL) are added onto the pellets. It is divided into 100 μL and stored at -80°C .

2.2.8 Transformation

The C43 (DE3) cell line from -80°C was kept on ice for 20-30 minutes. 25 mL of LB and 25 μL of 40mg/ml kanamycin were placed on the plate. DNA samples (5-10 μL) were added onto the host cells. Then, the samples were kept on ice for 30 minutes and kept at 42°C for 1 minute. Samples from 42°C were kept on ice for 5 minutes. 1000 μL LB was added on it and left at 37°C in a shaker at 200 rpm for 1 hour. It was then precipitated at $10,000 \times g$ for 5 minutes. The pellet was thawed with 200 μL LB and spread 100 μL -100 μL on two agar plates. After incubating overnight at 37°C , colonies are selected from the plates and incubated overnight at 200 rpm at 37°C . 32 colonies selected among all colonies and to clarify the plasmid is in the host I made Colony PCR.

2.2.9 Colony PCR

Colony PCR is a high-throughput method that quickly screening the colonies of bacteria that have grown up on selective media following a transformation stage to check for the presence of the desired genetic construct or to amplify a part of the construct. Individual transformants can be lysed in water after a brief heating step, or they can be introduced directly to the PCR reaction and lysed during the first heating step. In this step chosen single colony is lysed in water approximately 20 μL water and 1 μL from this solution was added directly to the PCR reaction. In the initial heating step plasmid was released from the cell so that in the amplification DNA could be served as a template. As mentioned before, using specific primers usual PCR reaction was occurred. After that, to understand the presence or absence of insert including plasmid samples load onto agarose gel electrophoresis with using appropriate marker. The chosen 32 single colonies were subjected to Table 2.8 and applied conditions shown in Table 2.9. 10 colonies were from Virgi 1, 10 colonies were from Virgi 2 and finally 12 colonies were from Geo and all put into PCR reaction based on their annealing temperatures.

Table 2.8 : The Colony PCR reaction components and volume.

Components	Volumes
Buffer	2.5 μ L
DNA (insert)	1 μ L
dNTP	0.5 μ L
Taq	0.25 μ L
Forward Primer	1.25 μ L
Reverse Primer	1.25 μ L
dH ₂ O	18.25 μ L
Total	25 μ L

Here the aim was amplifying the gene of interests of whole colonies including plasmids and observing the correct molecular size of gene of interests. In this case they were 831 bp, 795 bp and 819 bp for three aquaporin genes and two extreme organisms.

Table 2.9 : The Colony PCR conditions.

Conditions	Temperature	
Initial Denaturation	4 min.	94 °C
Cycle/denaturation	30 min.	94 °C
Annealing	45 min.	55 °C -63 °C -70°C
Elongation	60 sec.	68 °C
Final Elongation	7 min	68 °C

2.2.10 Plasmid isolation and control digestion

As an extra control step, plasmid isolation and fast digestion was done to double check the insert DNA was present or not. To do that, High Pure Plasmid Isolation Kit (Roche, Catalog #11 754777001) was used plasmid isolation for three genomic DNA including colonies. First of all, Binding buffer is placed on ice and 2mL bacterial culture was centrifuged at 6000 g for 30 sec and supernatant was removed. 250 μ L Suspension Buffer with RNase added the bacterial pellet and dissolved well. After that, 250 μ L Lysis Buffer was added into the tube and mixed gently by inverting the tube 3-5 times. Then tube was incubated for 5 minutes at room temperature. After that, 350 μ L of Binding Buffer was placed and mixed gently and waited into ice for 5 minutes. Using standard tabletop microcentrifuge, it was centrifuged for 10 min at 13000 x g. Filter tube was placed into one collection tube. The supernatant was placed into the filter tube and centrifuged for 1 minute at full speed. The filter tube was re-inserted in the same collection tube after the flow through was removed. To remove high nuclease

content, 500 μL of Wash Buffer I was added and centrifuged at full speed for 1 minute. Flow-through was removed from the equation. After that, 700 μL of Washing Buffer II was added to the filter, and the tube was centrifuged at full speed for 1 minute before the flow through was removed. Additional centrifugation for 1 minute at maximum speed was used to verify that any remaining Wash Buffer was removed. A new 2 mL micro-centrifuge tube was used to transfer the filter tube. 50 μL of elution buffer was added to the filter membrane. For one minute, the tube was centrifuged at full speed. For further examination, the eluted plasmid was kept at $-20\text{ }^{\circ}\text{C}$. Finally, plasmid was isolated from *E. coli* C43 (DE3) strains. An agarose gel electrophoresis was used to confirm plasmid isolation. After that, each plasmid was digested using 10X FastDigest Buffer (Thermo Scientific, #00101053) and Fast Digest *SacI* (Thermo Scientific, #FD1133). Table 2.10 lists the components and quantities used in one digestive reaction. The reaction tube was incubated at $37\text{ }^{\circ}\text{C}$ for 30 minutes after the reaction mixture was prepared. Agarose gel electrophoresis was used to check the DNA size. The vector pET28a (+) is 5369 bp, and the aquaporin gene of interests are, 831 bp, 795 bp, and 819 respectively. That is why the expected DNA molecule sizes were, 6200 bp, 6164 bp, 6188 bp. After that plasmids were chosen to be sequenced based on the correct molecular size of the three aquaporin genes which are expected to be seen 831 bp, 795 bp, and 819 bp gene of interests ligated to expression vector pET28a (+), size of 5369 bp. The selected plasmids sent to Sanger Sequencing for correcting the sequence of three aquaporin genes.

Table 2.10 : The Fast Digestion of isolated plasmid DNA.

Components	Volumes
10X FDBuffer	2 μL
DNA	2 μL
<i>SacI</i>	1 μL
dH ₂ O	15 μL
Total	20 μL

2.2.11 Sanger sequencing

Using universal T7 Promoter (5'- TAATACGACTCACTATAGGG-3') and T7 Terminator (5'- GCTAGTTATTGCTCAGCGG-3') primers, isolated DNA was sequenced in the Genometri Biotechnology Limited Company (Izmir, Turkey).

Also, using the wild type, sequenced plasmids were controlled whether mutations occurred or not. If there were not any mutations or frame shift the selected colonies moved for further applications.

2.2.12 Optimizing protein expression

E. coli C43 (DE3) transformants including inserts were taken from -80 °C glycerol stock solution and were spread through 40 µg/mL Kanamycin containing LB agar plate. When 16 hours 37 °C incubation occurred, single colony chosen from agar plate and let them in to incubation for 16 hours 37 °C with 10 mL LB including 40 µg/mL Kanamycin. Cultures were left in the 16 hours at 37 °C with 200 rpm orbital shaking. The next day, first of all MagicMedia™ which is *E. coli* Expression Medium auto induction growth media was prepared. For preparing 150mL MagicMedia™ 5.85 mg component A and 142.5 mL water was mixed. After that all media was autoclaved in 121 °C 20 minutes. After it was at room temperature, 7.5 mL component B was added and 250 µL preculture was added into media. When OD₆₀₀ reached 6.0 at 30 °C put them into 25 °C for 24 hours. After that, precipitated cultures put into -20 °C. This media was used for facilitating protein expression without IPTG addition.

Also, besides MagicMedia™ the IPTG (Isopropyl-β-D-thiogalactopyranoside) induction was tried with different temperatures and different molarities. First of all, the stock cells were taken from -80 °C and grown 10 mL LB including 40 µg/mL Kanamycin at 37 °C. Then, precultures were transferred to larger volume (50 mL preculture for 1L LB) and left in the at 37 °C with 200 rpm orbital shaking until OD₆₀₀ reached to 0.4-0.6. After that, when OD₆₀₀ reached to 0.4-0.6, IPTG is added with different values (0.5mM-1mM- 1.5mM) with different temperatures (30 °C and 37 °C). Cells were precipitated by centrifugation at 9000 rpm for 15 minutes according to different time intervals (1hour- 4 hours- 6 hours) and best IPTG molarity, time and temperature were chosen after optimization. After, supernatant was removed, pellets were resuspended with lysis buffer 1 which includes 20 mM TRIS (pH 7.4), 100 mM NaCl, 0.5 mM PMSF, 5 mM β-mercaptoethanol and with 100 mM detergent OG (Octyl-beta-Glucoside Detergent) and lysed by sonication. The cultures were sonicated with MS72 probe for 10 seconds pulse on / 20 seconds pulse off in total 10 cycles. After sonication, samples were centrifuged at 17000 x g for 30 minutes at 4 °C. After supernatant was removed, the pellet was solubilized in buffer 2 20 mM TRIS

(pH 7.4), 100 mM NaCl, 5 mM β -mercaptoethanol and with 150 mM detergent OG, 20 mM imidazole by overnight shaking for 300 rpm at 4 °C. After solubilization for using His-tag purification method, resin first checked for detergent molarity. Because of that, samples were incubated with dialysis by overnight. For the dialysis, membrane cut into pieces of convenient length (10-20 cm) and boil the membrane for 10 minutes in a large volume of (500 mL) 10 mM sodium bicarbonate and 1mM EDTA (pH 8.0). Rinse the membrane throughly in dH₂O, then boil the membrane for 10 minutes in 500 mL 1 mM EDTA (pH 8.0) and allow the membrane cool. Samples were treated with dialysis at 4 °C by overnight and then analyzed on 10 % SDS-PAGE for observing overexpressed proteins each of them.

2.2.13 Purification of his-tagged proteins

After cells prepared from 1 L of culture and lysed with 1.5 mL buffer 1 per 100 mL were sonicated, centrifuged, and pellet resuspended and finally left by overnight shaking, they were ready for his-tag purification. First of all, buffers were prepared for wash and elution steps and also for the aquaporin purification three different his-tagged purification were applied. For the buffer content, wash buffer contained 20 mM TRIS (pH 7.4), 500 mM NaCl, 5 mM β -mercaptoethanol, 30 mM OG detergent, 10% glycerol with different imidazole molarities (10mM, 20mM, 30mM). For the elution buffers, 20 mM TRIS (pH 7.4), 500 mM NaCl, 5 mM β -mercaptoethanol, 30 mM OG detergent, 10% glycerol and also different imidazole molarities were tried (100 mM, 150 mM, 200 mM). Dialysed samples were treated with 750 μ L Ni-NTA Agarose (Qiagen, #30210) at least 12 hours. 25 μ L sample was put into another tube as a clear result. Then purification steps were started.

- **Gravity his-tagged purification**

Samples treated with Ni-NTA Agarose, put into column and let them dropped as a flow through. It was named as a FT and kept at 4 °C for load to SDS-PAGE. After collecting FT, wash steps were applied (5 mL, 4 mL, 3 mL) with different imidazole containing wash buffers. They were also named W1, W2, W3 respectively 10 mM imidazole, 20 mM imidazole, 30 mM imidazole. After collecting washes, the specific protein was eluted with 2 mL elution buffer in elution buffer for three times. Each sample loaded to SDS-PAGE gel to check purification.

- **His-trap purification**

Dialysed samples were placed into his-trap column and using syringe wash and elution buffers were applied. First of all samples were collected as a FT and then they washed with same amounts mentioned above. After that they eluted with elution buffer with the same amounts before. This method was tried to optimize the binding and not losing proteins in the FT step. Again, each sample loaded onto SDS-PAGE gel to check purification.

- **Centrifuge purification**

This technique aims to purification by using centrifugal force. Samples treated with Ni-NTA Agarose, put into 15 mL falcon and centrifuged at 3000 x g, 2 minutes at 4 °C. In the first spin, FT was collected and again wash buffer was added. The steps were continued until protein was washed. After that, protein was eluted using elution buffer with same conditions as centrifuged at 3000 x g 2 minutes at 4 °C. Again, each sample loaded onto SDS-PAGE gel to check purification.

2.2.14 SDS-PAGE

After protein expression, purification and ultrafiltration stacking gel concentration was prepared as 5 % and resolving gel concentration was prepared as 12 %. SDS-PAGE was loaded every step to check protein sizes. First of all, glass plates were washed and cleaned with distilled water and then they were placed into casting frames. Prepared resolving gel was poured into glass plates. To make gel smooth isopropanol was added to top of the gel. After separation gel polymerized, top of the gel was cleaned from isopropanol and washed with distilled water. Using filter paper, glasses were dried and stacking gel was poured over resolving gel and combs were placed. When whole gel became polymerized, combs were removed and casting frames were placed into electrophoresis tank. 1X Running Buffer is poured through the tank using proper amount according to gel number. 20 µL sample and 5 µL of sample application buffer and incubated at 95 °C using heater for 15 minutes. Samples were loaded into wells and tank was loaded now the voltage on the power were arranged to 95 V until marker (Thermo Scientific PageRuler Plus, #26616) became separated. When it separated, the voltage were increased to 120V. When this process was done, frames were removed from the electrophoresis tank and gel was carefully removed from glass. First, gel was washed with distilled water and then transferred into container and incubated with

Coomassie Brilliant Blue R-250 stain on shaker for 1 or 2 hours. For removing excessive stain, the stain solution was taken from the container and destaining buffer was added into container. Gel was incubated with destaining buffer for 2 hours. Then the gel was illustrated using Chemidoc Imaging System.

2.2.15 Western Blot analysis

After analyzing proteins in the SDS-PAGE, Western blot analysis was done to understand protein in the SDS-PAGE was desired protein or not. The PVDF membrane was cut an appropriate gel size and incubated 100 % methanol for 10 minutes on a shaker. 1X Transfer Buffer was prepared and cool for 1-2 hours in the 4 °C. Then buffer was poured through tray, gel and membrane was placed into this tray. Also, the sponge and system (Mini Trans-Blot Electrophoretic Transfer Cell, 170- 3930, 170-3935, Bio-Rad) are wetted with 1X Transfer Buffer. Then gel and membrane were sandwiched by using sponges and filter paper that wetted. It was rolled over with a cylindrical material for avoiding air bubbles formation. After that, sandwiched system placed into transfer apparatus and they were placed into tank properly. Cold 1X Transfer Buffer was added into tank and ice battery placed into it. The transfer was performed 95 V 1,5 hours at 4 °C. After transfer step, 5 % skim milk was solubilized into 10 mL 1X TBST for the blocking step. Membrane was placed into container contains 5 % skim milk and incubated 1 hour at room temperature. After this, 1X TBST was removed, and 5 mL 2.5 % skim milk was prepared. 6x-His-tag monoclonal 34 antibody (3D5), AP (Thermo Scientific, #R932-25) was added into 1X TBST buffer with 2.5 % skim milk. After that, membrane was placed into falcon that contains both antibody and 2.5 % skim milk and left by overnight at 4 °C. The membrane was treated with TBST for 5 minutes on a shaker four times after the buffer was withdrawn. The manufacturer's method was followed to detect the antibody and colored bands using 1-Step NBT/BCIP SubstrateTM (Thermo Scientific, #34042).

2.2.16 Ultrafiltration

After his-tagged purification was done, ultrafiltration was applied to concentrate and purify the eluted proteins. The tubes were centrifuged at 3000 x g for 30 minutes with distilled water first and all eluted proteins were transferred into ultrafiltration tube includes membrane with 30 kDa molecular weight cut-off. The samples were centrifuged at 3000 x g for 30 minutes and the passed proteins from the membrane

were collected and placed another ultrafiltration tube containing 10 kDa molecular weight cut-off. Again, the samples were centrifuged at 3000 rpm for 30 minutes and samples under 10 kDa membrane was collected to determine aquaporin in the 28 kDa. The ultrafiltered protein was transferred to a 2 mL fresh sterile tube and the results were analyzed with SDS-PAGE and if it necessary analyzed by Western Blot. After that, proteins were stored at -20 °C.

2.2.17. Liposome production

In this process, Dipalmitoylphosphatidylcholine (DOPC) was used as a lipid and 10 mg/ml stock solution was prepared. To do this, 130 mg DOPC was solubilized 13 mL chloroform. In order to obtain a liposome concentration of 0.5 mg/ml, 0.5 mL of DOPC was taken from the stock solution and DOPC was added using a round bottomed flask shown in Figure 2.2 (250 ml or 500 ml) (bottle for short) in the cold room at +4 °C, and then the bottle is added under the fume hood at 45°. It is rotated by holding at an angle of until the chloroform in it evaporates.

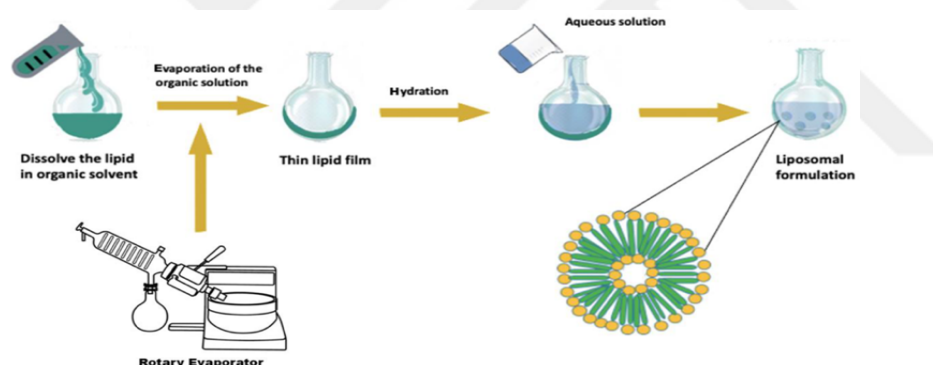


Figure 2.2 : Liposome production by thin film method (Jaradat, E., Weaver, E., 2021).

It is important at this stage that the lipids were evenly distributed on the bottle surface. Then the bottle was passed through nitrogen and kept in the refrigerator at +4 °C until the next day. 10 mL of the prepared buffer is added into the lipid spread bottle taken from the refrigerator. Then, the lipid solution added to the buffer is vortexed for 10-15 minutes or longer depending on its density. Again, sonication can be applied to remove the lipid layer from the surface according to the density. After the lipid layer was removed from the surface and large-sized liposomes were formed, the desired size liposomes were produced by passing them through 200 nm PCTE membranes 21 times.

2.2.18. Proteoliposome production

The same procedure was obtained in liposome production. The dispersed lipids were kept at +4 °C overnight, and the next day, PBS buffer was added and vortexed for about 5 minutes. It is then subjected to sonication (approximately 15 min). Then AqpZ was added into it. It was then vortexed again for about 10 minutes to form liposomes. Afterwards, this solution is taken from the bottle and placed in the falcon and left to mix at +4 °C until the next day. The next day, AqpZ added liposome solution was taken and while performing the redhydration process to obtain proteoliposomes, aquaporin protein and 1 % Octyl β D-glucopyranoside (OG) detergent (Sigma-Aldrich, USA) was added at a protein:lipid ratio of 1:100 shown in equation 2.1 and equation 2.2. The proteins loaded with detergent were allowed to enter the liposomes. The detergent was removed using BioBeads (Bio-Rad Laboratories, Inc., USA). 200 mg Beads were removed by using 0.45 μ m filter. The proteoliposome mixture was extruded using the above method to obtain vesicles of 200 nm size with 21 times extrusion. The amount of AqpZ to be used is determined according to the lipid concentration. 2.5 mg/ml DOPC x 10 mg/ml stock solution. In order to have a 1:100 AqpZ:lipid ratio;

$$1:100 = \frac{2.5 \frac{\text{mg DOPC}}{\text{ml Tris}}}{100} = 0.025 \text{ mg} \frac{\text{AqpZ}}{\text{ml}} \text{ PBS} \quad (2.1)$$

$$1.00 \text{ mg} \frac{\text{AqpZ}}{\text{ml}} \times ? = 0.025 \frac{\text{mg AqpZ}}{\text{ml tris}} \times 10 \text{ ml PBS} \quad (2.2)$$

$$? = 0.250 \text{ ml} = 250 \mu\text{l}$$

2.2.19.Characterization of liposome and proteoliposome



Figure 2.3 : SX20 Stopped Flow Light Scattering Spectrometer.

The water permeability of the liposomes was measured with the SX20 Stopped Flow Light Scattering Spectrometer (Applied Photophysics, UK) shown in Figure 2.3 using an osmotic solution containing 0.85 M NaCl. Due to the difference in osmolarity, when the liposomes are mixed with the hypertonic NaCl solution, they release the water they contain and shrink, this shape change increases the light scattering. Water release rates of proteoliposomes containing aquaporins, which are expected to facilitate water passage, are expected to be higher than those of empty protein-free liposomes. The water output velocity is directly proportional to the k value obtained from the measurement. Increasing k value in the proteoliposome indicates faster water passage.



3. RESULTS AND DISCUSSION

3.1 Genomic DNA Isolation and Primer Designing

Three Aquaporin gene regions were determined from both the *Virgibacillus sp.* AGTR strain and *Geobacillus Thermoleovorans* ARTR1 strain using whole genome sequencing. 831 bp and 795 bp are from *Virgibacillus sp.* AGTR strain and 819 bp is from *Geobacillus thermoleovorans* AGTR. According to BLASTn, there were only one similarity which was 100 % similar to *Virgibacillus sp.* Bac332. However, there were no any literature information about aquaporin in *Virgibacillus sp.* AGTR strain and *Geobacillus thermoleovorans* ARTR1 strain. Firstly, primers were designed based on the genes. The organisms were grown with their special medium for salty medium for halophilic bacterium and hight temperature for thermophilic bacterium and genomic DNA isolation was done shown in below Figure 3.1. Both DNA size was correct molecular size. Therefore, genes were amplified with PCR using specific primers as a next step. In the PCR rections, the conditions were arranged according to different annealing temperatures for three aquaporin genes which are mentioned in method parts.

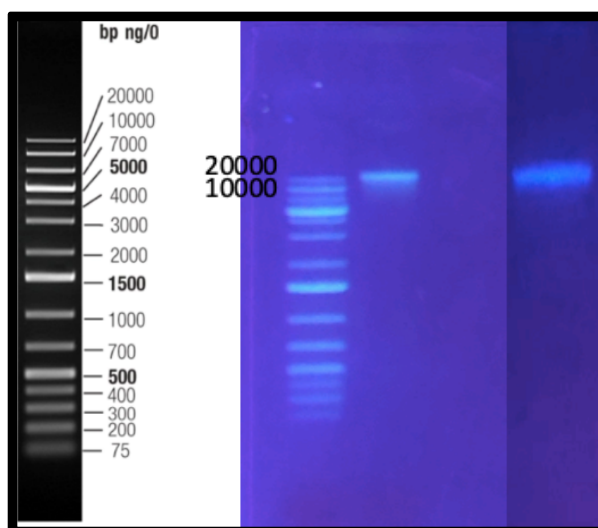


Figure 3.1 : Agarose gel electrophoresis image of genomic DNA isolation from original organisms. *Virgibacillus* and *Geobacillus* represented in respectively 1st, 2nd Lane. M: Marker, GeneRuler 1 kb Plus DNA Ladder.

3.2. Polymerase Chain Reaction (PCR) for *Virgibacillus* and *Geobacillus* Aqp Genes

Gene-specific primers were used for each aquaporin gene and PCR conditions were optimised. Gene-specific primers were used each aquaporin gene and PCR conditions were optimised. *Virgibacillus* sp AGTR 831 bp gene was named as Virgi 1, *Virgibacillus* sp AGTR 731 bp gene was named as Virgi 2 and *Geobacillus Thermoleovorans* ARTR1 was named as Geo. For the Virgi 1, it was showed clear result at 55 °C shown in Figure 3.2. For the Virgi 2 was showed clear result for 60 °C, 63 °C and 70 which are shown in Figure 3.2. Geo was showed clear at 70 °C and 73 °C shown in Figure 3.4.

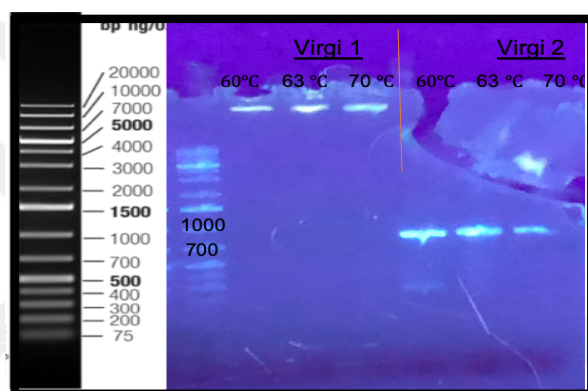


Figure 3.2 : Agarose gel electrophoresis image of PCR product Virgi1 and Virgi 2. The gradient PCR was performed using three different temperatures 60 °C, 63 °C and 70 °C represented in respectively 1st, 2nd and 3rd Lane. M: Marker, GeneRuler 1 kb Plus DNA Ladder.

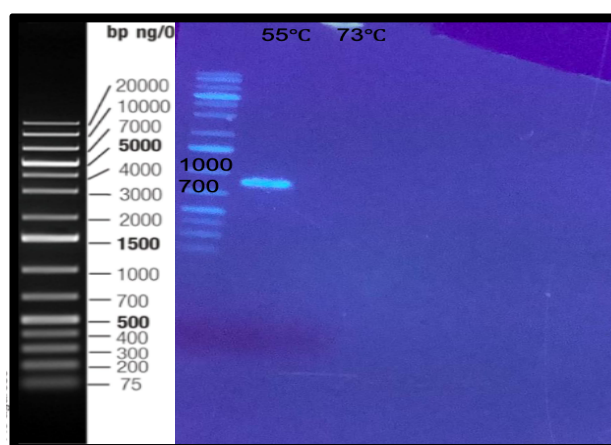


Figure 3.3 : Agarose gel electrophoresis image of PCR product Virgi 1. The gradient PCR was performed using two different temperatures 55 °C, and 73 °C represented in respectively 1st, 2nd M: Marker, GeneRuler 1 kb Plus DNA Ladder.

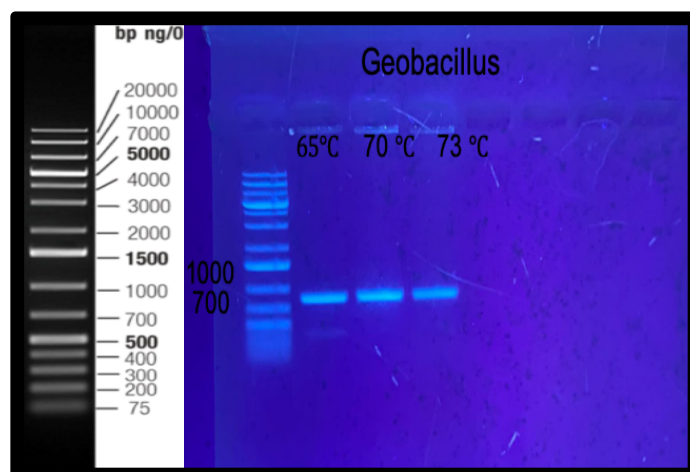


Figure 3.4 : Agarose gel electrophoresis image of PCR product Geo. The gradient PCR was performed using three different temperatures 65 °C, and 70 °C and 73 °C represented in respectively 1st, 2nd, 3rd M: Marker, GeneRuler 1 kb plus DNA Ladder.3.3 PCR Product

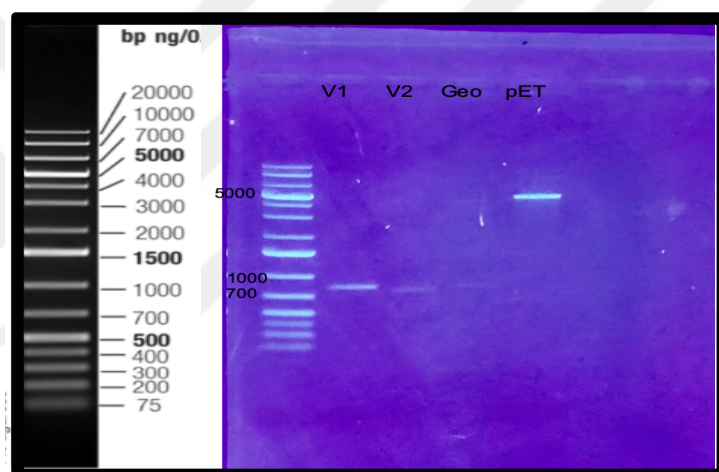


Figure 3.5 : Agarose gel electrophoresis image of double digested PCR products and vector. *Virgibacillus* 1, *Virgibacillus* 2 and *Geobacillus*, pET28a (+) were represented in respectively 1st, 2nd, 3rd, 4rt M: Marker, GeneRuler 1 kb plus DNA Ladder.

PCR products of *Virgibacillus* and *Geobacillus* and the pET-28a (+) vector were digested with *NotI* and *SacI* enzymes to form sticky ends shown in Figure 3.5. Using Double Digest Calculator (Thermo Scientific) online website tool, optimal double digestion conditions were arranged. After double digestion, using High Pure PCR Product Purification Kit, digested vector and PCR products purified. 5 μ L of *Virgi* 1, *Virgi* 2, *Geo* and vector samples were mixed with 1 μ L 6X DNA loading dye (EZ vision, #N472-1ML) and loaded to agarose gel. As a result, V1, V2 and Geo were observed Marker between 1000 bp and 700 bp.

3.4 Ligation

In the digestion step, as a result, sticky-ends were created. The sticky ends generated in the pET-28a (+) vector and insert DNA are compatible when using the same restriction enzymes. The two DNA fragments are joined together by the T4 ligase enzyme and so fused by the ligation reaction because the overhanging base pairs on the vector and the insert are complementary. Therefore, *NotI* and *SacI* restriction sites were used in the ligation. The Neb Ligation Calculator online tool were used for deciding concentrations of insert DNA and vector. Both NanoDrop and agarose gel DNA concentrations were examined and conditions made based on both of them. The ratio was selected 1:5 for insert DNA and vector which are shown in Table 3.1.

Table 3.1 : The concentrations of insert DNA and vector.

Virgi 1	Virgi 2	Geo	Vector
7.739 ng/ μ L	7.4 ng/ μ L	7.6 ng/ μ L	19.1 ng/ μ L

3.5 Transformation

Ligated insert DNAs and vector were transformed into *C43* host cell. Cells were spread on LB agar including kanamycin antibiotics and incubated by overnight at 37 °C. Because the kanamycin resistance gene is present in the pET-28a (+) vector used in this experiment, transformants formed following successful ligation form colonies on LB Agar plates containing kanamycin.

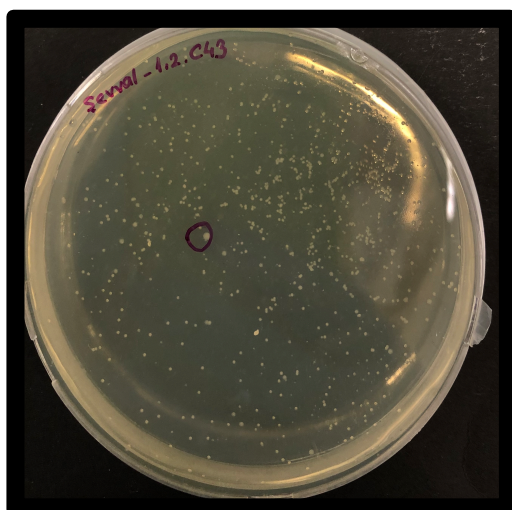


Figure 3.6 : Kanamycin screening plates with C43 including colonies after transformation for Virgi 1 containing a plasmid with a 1:5 ratio (vector: insert) of ligation.

Figure 3.6 shows Kanamycin screening plates. 1:5 ratio was used as a ligation rate and chosen transformants had the same condition. After that, chosen colonies were performed to colony PCR to check they had insert DNA or not.

3.6 Colony PCR

After transformation, colony PCR was applied to selected colonies. This procedure is important for understanding insert DNA is in the selected colony or not.

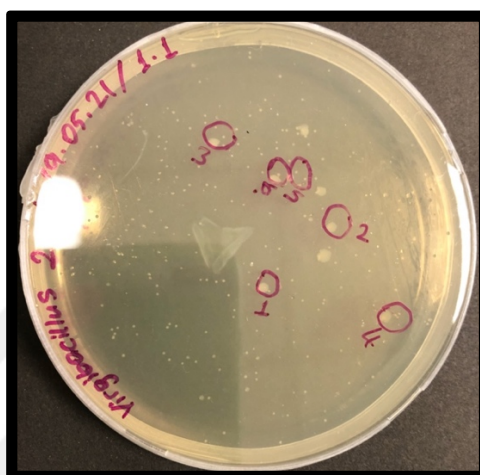


Figure 3.7 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to *Virgibacillus* 2. Six colonies were selected from this petri plate.

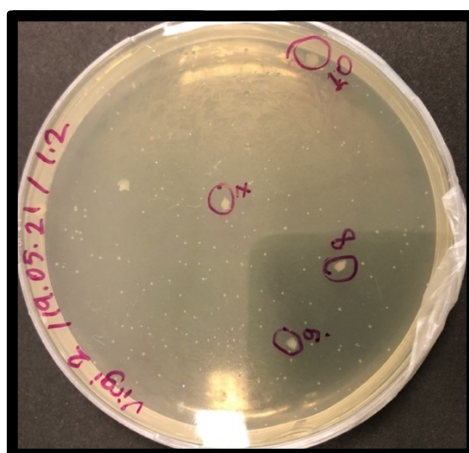


Figure 3.8 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to *Virgibacillus* 2. Four colonies were selected from this petri plate.

Conditions were arranged like mentioned in the Methods part. In total, 32 colonies were selected which are shown in below Figure 3.7, Figure 3.8, Figure 3.9, Figure 3.10, Figure 3.11, Figure 3.12 and performed colony PCR with arranged conditions.

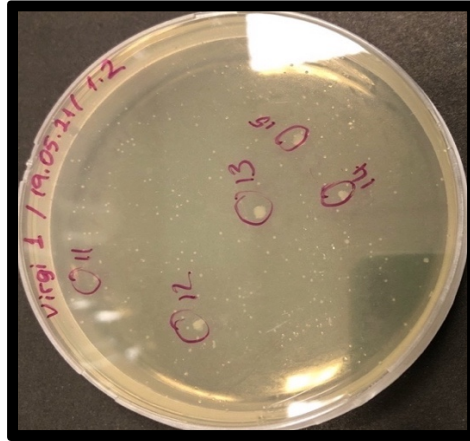


Figure 3.9 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to *Virgibacillus* 1. Five colonies were selected from this petri plate.

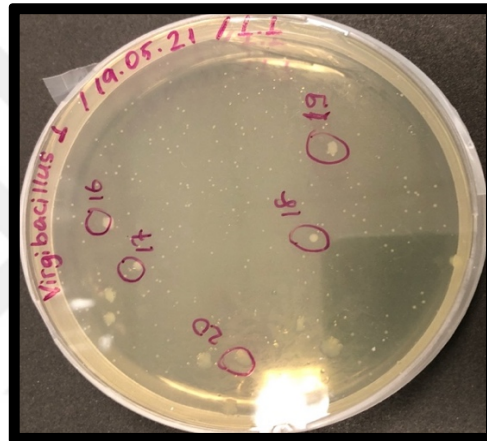


Figure 3.10 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to *Virgibacillus* 1. Five colonies were selected from this petri plate.

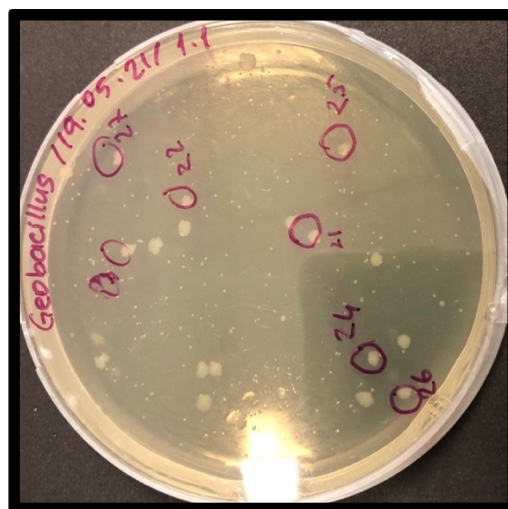


Figure 3.11 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to *Geobacillus*. Seven colonies were selected from this petri plate.

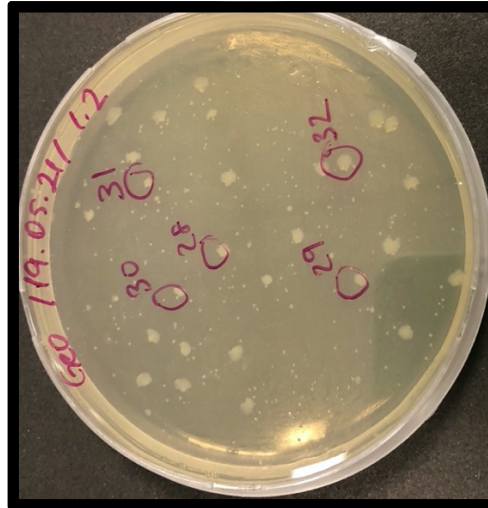


Figure 3.12 : The colonies were selected from transformation petri plates. The colony PCR was performed for these colonies belongs to *Geobacillus*. Five colonies were selected from this petri plate.

After choosing single colonies (32) they did under go colony PCR with designed same primers and to show the results, agarose gel electrophoresis were applied.

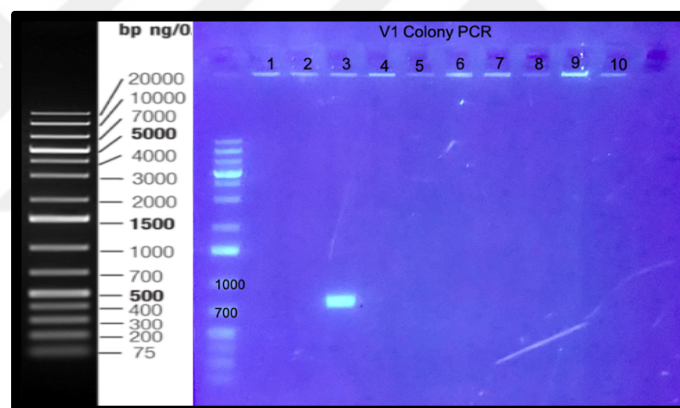


Figure 3.13 : The colony PCR result for *Virgibacillus* 1. Ten colonies were selected and one positive colony was obtained. 3rd well showed positive colony respectively. Marker: GeneRuler 1 kb plus DNA Ladder

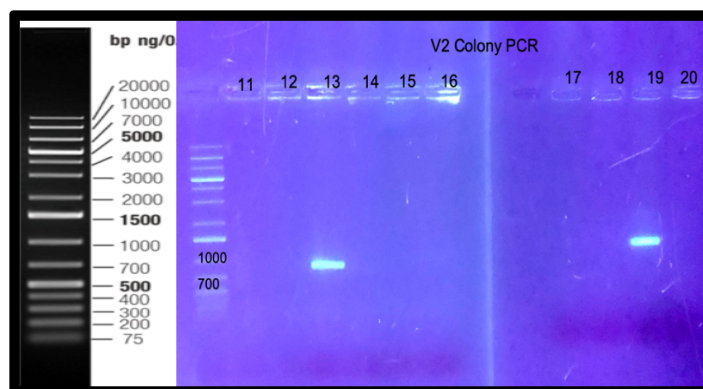


Figure 3.14 : The colony PCR result for *Virgibacillus* 2. Ten colonies were selected and two positive colonies were obtained. 13th and 19th wells showed positive colony respectively. Marker: GeneRuler 1 kb plus DNA Ladder.

The samples were loaded to agarose gel and the expected result was showing the desired insert DNA molecular size which are shown in Figure 3.13, Figure 3.14, Figure 3.15.

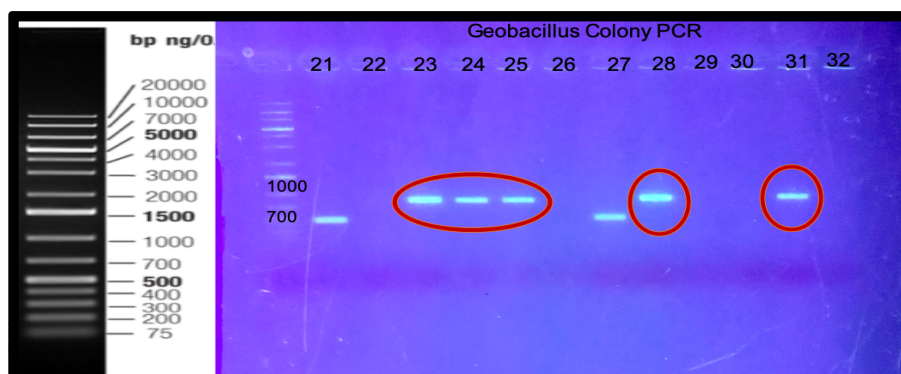


Figure 3.15 : The colony PCR result for *Geobacillus*. Twelve colonies were selected and five positive colonies were obtained. 23th, 24th, 25th, 28th and 31th wells showed positive colonies respectively. Marker: GeneRuler 1 kb plus DNA Ladder.

Among all 32 colonies, one colony in the *Virgibacillus* 1 was showed positive colony which consisting of insert DNA with correct molecular size. Two colonies in the *Virgibacillus* 2 were showed positive colonies which consisting of insert DNA with correct molecular size and also five colonies in the *Geobacillus* were showed positive colonies which consisting of insert DNA with correct molecular size. After that, control cleavage was performed to make sure colony PCR results were correct.

3.7. Plasmid Isolation and Control Cleavage

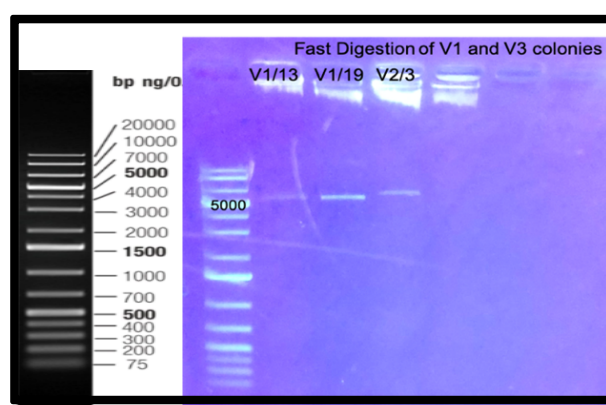


Figure 3.16 : Agarose gel electrophoresis control cleavage of plasmid DNA including insert. *Virgibacillus*1-13th colony, *Virgibacillus*1-19th colony and *Virgibacillus* 2-3th colony were showed in the wells respectively. Marker: GeneRuler 1 kb plus DNA Ladder.

After confirming the insert DNA with colony PCR, and plasmid isolation was done, the control digestion was performed. Samples were digested with using fast digestion enzyme *EcoRI* and linearized as a plasmid.

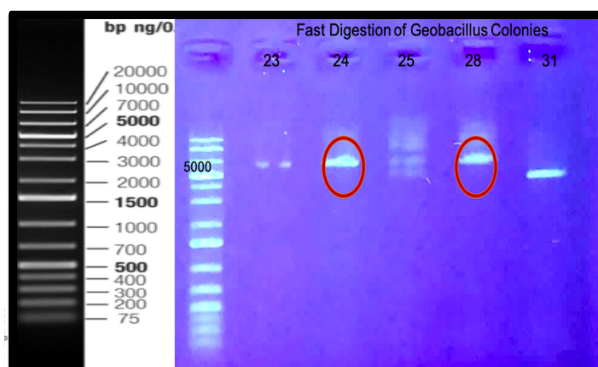


Figure 3.17 : Agarose gel electrophoresis control cleavage of plasmid DNA including insert. *Geobacillus* 23th, 24th, 25th , 28th and 31th colony were showed under UV light. Marker: GeneRuler 1 kb plus DNA Ladder.

The expected bands were higher than pET28a (+) because samples were including aquaporin genes and also pET28a (+). 10 % agarose gel was used to visualize insert DNA and showed under UV light as seen in Figure 3.16 and Figure 3.17. The samples, whose size was confirmed by colony PCR, were examined once again with the fast digestion and it was seen that only 5 of the 8 selected colonies in total were in the right place. These; *Virgibacillus1-13th* colony, *Virgibacillus1-19th* colony and *Virgibacillus2-3th* colony, *Geobacillus 24th* colony, 28th colony and they named as, *V13*, *V19*, *V3*, *Geo24*, *Geo28* respectively. The vector pET-28a (+) has a 5369 bp. length and *Virgi 1* has 831 bp, *Virgi 2* is 795 bp and *Geo* is 819 bp. Therefore, the expected plasmid molecular sizes were, 6200 bp, 6164 bp, 6188 bp and corrected in the agarose gel. After that, they sent to sequencing to conform there were no any mutations while making PCR and ligation steps.

3.8 Sanger Sequencing Analysis

Sanger sequencing is a DNA sequencing technology that uses electrophoresis and is based on DNA polymerase's random incorporation of chain-terminating dideoxynucleotides during in vitro DNA replication. According to sequencing results, DNA sequences were put into ExPASy tool and translated into amino acid sequences. After that, protein size were obtained for three of them 30 kDa, 28 kDa and 28 kDa respectively for the *V1*, *V2* and *Geo*.

3.9 Optimizing Protein Expression in *E. coli* C43 (DE3)

Expressing in the MagicMedia™ without using IPTG was performed. First of all, cells from -80°C glycerol stock were grown in LB medium with kanamycin. This preculture was in the 5-10 mL range. Then, this preculture, which was left overnight, was poured into 100 mL magic media prepared as mentioned in the method section and kept at 25 °C for about 24 hours. The cells were then centrifuged at 9000 x g for 30 minutes at 4 °C. Centrifuged cells were first thawed with lysis buffer and sonicated (10 on/ 20 off). Then, the supernatant and pellet of the samples, which were centrifuged for 20 minutes at 4 °C at 9000 x g, were loaded into SDS-PAGE. Pellets were incubated in 6 M urea for 1 hour and loaded into SDS-PAGE. Results for expression was very small. This could be due to magic media not working on this host. Therefore, IPTG optimization has been started. In IPTG optimization, firstly, samples were kept at 30 °C for 1 hour, 2 hours, 4 hours and 6 hours using 0.5 mM IPTG. Expired samples were sequentially centrifuged at 9000 x g for 30 minutes and precipitated. Precipitated cells were thawed with lysis buffer and sonicated under the same conditions. After sonication, the samples were centrifuged again at 9000 x g for 30 minutes at 4 °C and the supernatants were separated from the pellets. The pellets were again kept in 6 M urea for 1 hour.

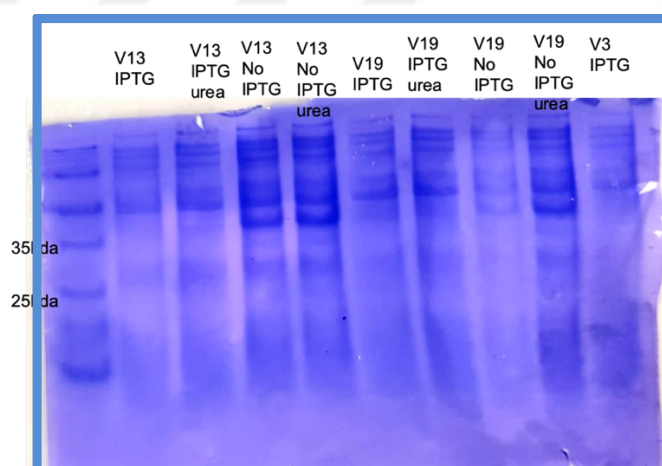


Figure 3.18 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V13 supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 2: V13 pellet treated with 0.5 mM IPTG and 6M urea. Lane 3: V13 after 4 hours without IPTG. Lane 4: V13 pellet after 4 hours without IPTG and 6M urea. Lane 5: V19 supernatant treated with 0.5 mM IPTG for 4 hours. Lane 6: V19 pellet treated with 0.5 mM IPTG and 6M urea. Lane 7: V19 after 4 hours without IPTG. Lane 8: V19 pellet after 4 hours without

Results viewed on SDS-PAGE which are shown in Figure 3.18, Figure 3.19, Figure 3.20, Figure 3.21 belongs to *Virgibacillus 1* and *Virgibacillus 2* colonies. These colonies were labeled as V3 which is from *Virgibacillus 1*, V13, V19 which are from *Virgibacillus 2*. Then, colonies that belongs to *Geobacillus thermoleverans* were labeled as a Geo24, Geo28 which are shown in Figure 3.22, Figure 3.23 and Figure 3.24. The protein, which should be seen in the range of approximately 28-30 kDa, could not be expressed in each colonies.

Firstly, when getting the 13th colony and 19th colony which are belongs to *Virgibacillus 1*, the results were not clear. Aquaporins couldnot be expressed in this colonies which are including a plasmid with gene of interest. The expected overexpressed bands were 28 kDa, but according to SDS-PAGE results the inducted gene could not be able to expressed. Therefore, I continued with *Virgibacillus 2* colony which is a 3rd colony and applied same conditions with IPTG and urea to 3rd colony. However, still there was no any overexpressed bands.

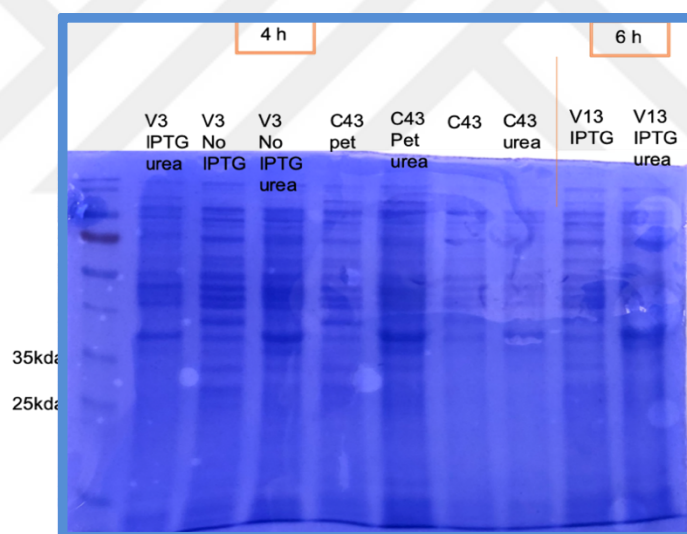


Figure 3.19 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V3 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 2: V13 after 4 hours without IPTG. Lane 3: V3 pellet after 4 hours without IPTG and 6 M urea. Lane 4: C43 pet supernatant treated with 0.5 mM IPTG for 4 hours. Lane 5: C43 pet pellet treated with 0.5 mM IPTG and 6 M urea. Lane 6: C43 host after 4 hours without IPTG. Lane 7: C43 host pellet after 4 hours without IPTG and 6 M urea. Lane 8: V13 supernatant treated with 0.5 mM IPTG for 6 hours. Lane 9: V13 pellet treated with 0.5 mM IPTG and 6 M urea 6 hours.

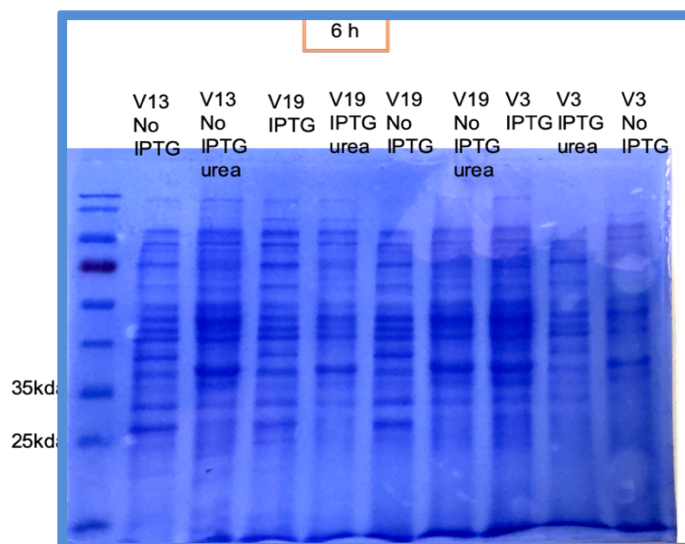


Figure 3.20 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V13 after 6 hours without IPTG. Lane 2: V13 pellet after 6 hours without IPTG and treated with 6 M urea. Lane 3: V19 supernatant treated with 0.5 mM IPTG for 6 hours induction. Lane 4: V19 pellet treated with 0.5 mM IPTG and treated with 6 M urea. Lane 5: V19 after 6 hours without IPTG. Lane 6: V19 pellet after 6 hours without IPTG and treated with 6 M urea. Lane 7: V3 supernatant treated with 0.5 mM IPTG for 6 hours induction. Lane 8: V3 pellet treated with 0.5 mM IPTG and treated with 6 M urea 6 hours induction. Lane 9: V3 after 6 hours induction without IPTG.

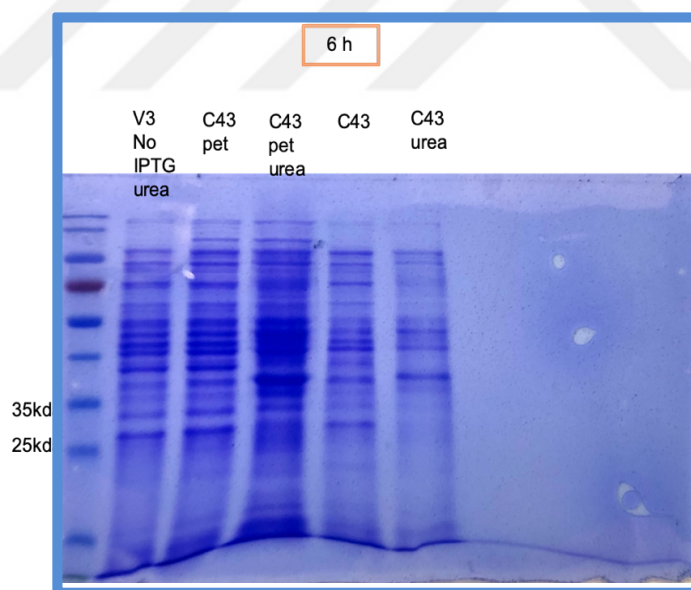


Figure 3.21 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: V3 pellet after 4 hours without IPTG and 6 M urea. Lane 2: C43pet supernatant treated with 0.5 mM IPTG for 6 hours. Lane 3: C43 pet pellet treated with 0.5 mM IPTG and 6 M urea. Lane 4: C43 host after 6 hours without IPTG. Lane 5: C43 host pellet after 6 hours without IPTG and 6 M urea.

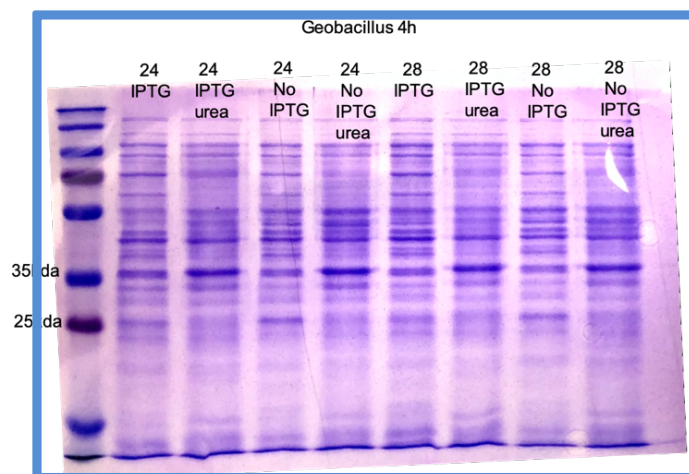


Figure 3.22 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: Geo 24 supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 2: Geo24 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 3: Geo24 after 4 hours without IPTG. Lane 4: Geo24 pellet after 4 hours without IPTG and 6 M urea. Lane 5: Geo28 supernatant treated with 0.5 mM IPTG for 4 hours. Lane 6: Geo28 pellet treated with 0.5 mM IPTG and 6M urea. Lane 7: Geo28 after 4 hours without IPTG. Lane 8: Geo28 pellet after 4 hours without IPTG and 6 M urea. Lane 9: V3 supernatant treated with 0.5 mM IPTG for 4 hours induction.

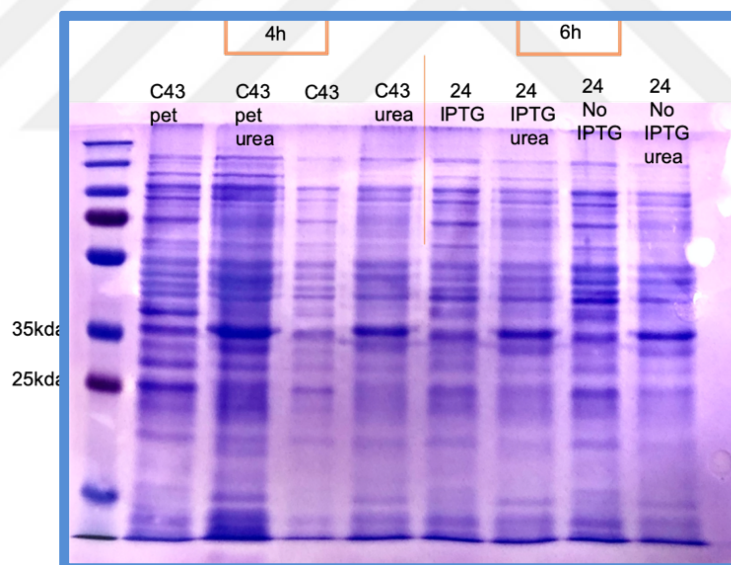


Figure 3.23 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: C43 pet supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 2: C43 pet pellet treated with 0.5 mM IPTG and 6M urea. Lane 3: C43 after 4 hours without IPTG. Lane 4: C43 pellet after 4 hours without IPTG and 6M urea. Lane 5: Geo24 supernatant treated with 0.5 mM IPTG for 6 hours. Lane 6: Geo24 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 7: Geo24 after 4 hours without IPTG. Lane 8: Geo24 pellet after 6 hours without IPTG and 6 M urea. Lane 9: V3 supernatant treated with 0.5 mM IPTG for 6 hours induction.

C43, the cell host, was changed because no expression was found. Transformation into BL21 and R2 cells took place. The same IPTG optimization procedure was applied. However, no expression was observed as a result in the 28 kDa - 30 kDa size. After that, as an alternative solution, buffer contents were changed and IPTG molarity was increased, and also optimal temperature was arranged to 37 °C.

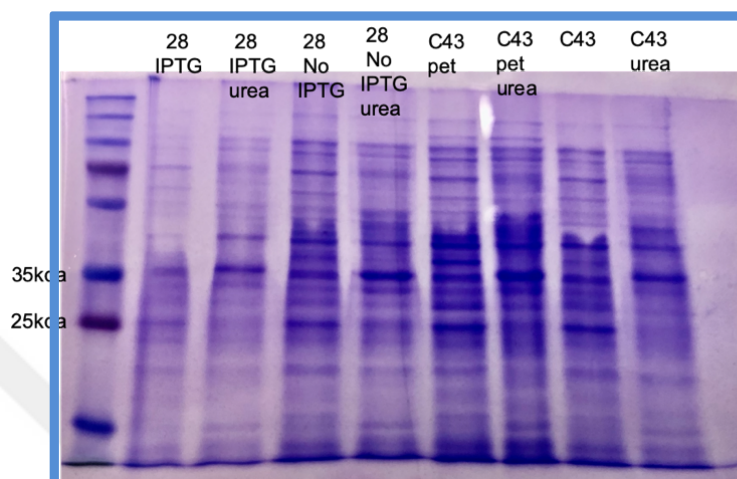


Figure 3.24 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1: Geo28 supernatant treated with 0.5 mM IPTG for 6 hours. Lane 2: Geo28 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 3: Geo28 after 6 hours incubation without IPTG. Lane 4: Geo28 pellet after 6 hours incubation without IPTG and 6 M urea. Lane 5: C43 pet supernatant treated with 0.5 mM IPTG for 4 hours induction. Lane 6: Geo28 pellet treated with 0.5 mM IPTG and 6 M urea. Lane 7: C43 host after 6 hours incubation without IPTG. Lane 8: C43 host pellet after 6 hours incubation without IPTG and 6 M urea.

First of all, cells from -80 °C glycerol stock were grown in LB medium with kanamycin. Preculture was poured into larger volume and OD₆₀₀ was followed until it reached to 0.4-0.6. After that 1 mM IPTG was added, and 4 hours incubation was started. At the end of the incubation, cells were centrifuged at 9000 x g for 30 minutes at 4 °C. The buffer contains 20 mM TRIS (pH 7.4), 100 mM NaCl, 0.5 mM PMSF, 5 mM β-mercaptoethanol and with 100 mM detergent OG (Octyl-beta-Glucoside Detergent) used as a lysis buffer and named as buffer 1. After sonication, supernatant was removed, the pellet was solubilized in buffer which is called buffer 2 contains 20 mM TRIS (pH 7.4), 100 mM NaCl, 5mM β-mercaptoethanol and with 150 mM detergent OG, 20mM imidazole by overnight shaking for 300 rpm at 4 °C. The important point was trying the different molarity of detergents to find optimal detergent amounts for protein solubilization and it decided that 100 mM OG detergent in a lysis solution of 150 mM OG detergent in a solubilization buffer.

Results were clear and the expression was observed and they were shown in Figure 3.25 and Figure 3.26.

It should not be forgotten that, when we use the detergents in the buffers sometimes, SDS-PAGE results become blurry. Therefore, some of the detergents should be removed from the samples before loaded to SDS-PAGE gel. Here, there should be an extra step to remove the some of the detergents; dialysis. Samples were left in the membrane with a 7k pores at 4 °C with occasionally shaker by overnight. After removing detergents now we can load to SDS-PAGE gel to see the overexpressed bands.

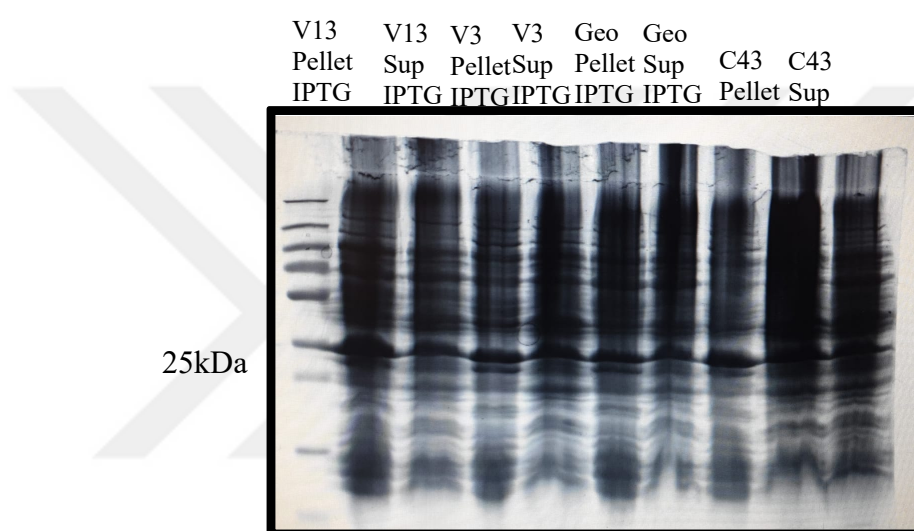


Figure 3.25 : SDS-PAGE analysis of lysate and pellet samples for IPTG optimization. Lane M: Molecular weight marker. Lane 1:V13 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 2: V13 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 3: V3 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 4: V3 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 5: Geo24 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent. Lane 6: Geo24 supernatant with 1mM IPTG induction and treated with 150 mM OG detergent. Lane 7: C43 pellet and treated with 150 mM OG detergent. Lane 8: C43 supernatant and treated with 150 mM OG detergent. Lane 9: V13 supernatant with 1 mM IPTG and treated with 150 mM OG detergent.

In the western blot analysis, when samples were in the detergent including buffers, dye interacting with antibody cannot make the protein visible. Therefore, before western blot analysis, detergent again should be removed using dialysis mebranes. Samples should be incubated at 4 °C by overnight.

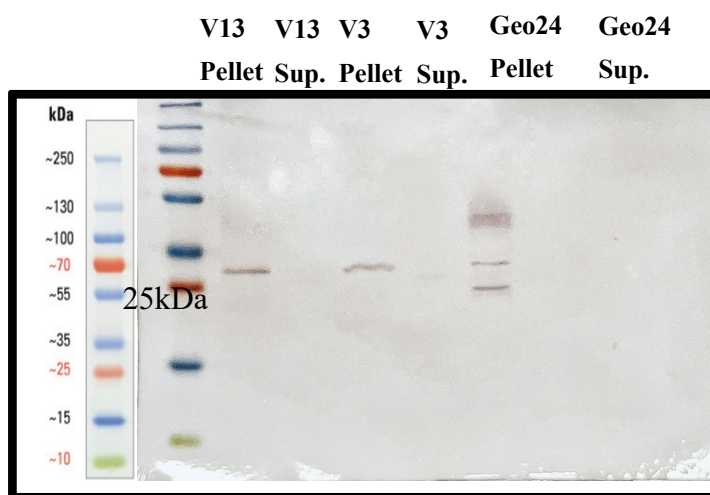


Figure 3.26 : Western Blot Analysis of supernatant and pellet for IPTG optimization. Marker: PageRuler 26619. Lane M: Molecular weight marker. Lane 1: V13 pellet with 1mM IPTG induction and treated with 150 mM OG detergent and dialysed. Lane 2: V13 supernatant with 1 mM IPTG induction and treated with 150 mM OG detergent and dialysed. Lane 3: V3 pellet with 1mM IPTG induction and treated with 150 mM OG detergent dialysed. Lane 4: V3 supernatant with 1mM IPTG induction and treated with 150 mM OG detergent dialysed. Lane 5: Geo24 pellet with 1 mM IPTG induction and treated with 150 mM OG detergent dialysed.

According to expression results, 1 mM IPTG at 37 °C and 4 hours incubation time were selected as a optimal conditions. Protein expression was visualized SDS-PAGE and to control the proteins correct or not Western Analysis were applied. Samples were cultured at 500 mL and solubilized 8 mL buffer in total. They were ready for the purification step.

3.10 His-tag Purification and Ultrafiltration

In the beginning of experiments, the vector used had his-tag sequences which was 6X His protein; CATCATCACCATCACCAC. These sequences are found both at the beginning and at the end of the vector and our restriction sites were planned based on this information. After the ligation process is complete, the target gene contains these his-tag sequences in the beginning and end. And thus, an ideal result is obtained when the protein is desired to be visualized by Western Blot Analysis or when his-tag protein purification was required. In this project, firstly gravity purification was tried and all purification steps different imidazole amount were tried such as 10 mM, 20 mM, 30 mM.

- **Gravity his-tagged purification results**

First of all, V13 colony was selected and tried to be optimized. Wash buffers and Elution buffers were arranged based on imidazole amounts and OG amounts.

Wash and Elution buffers include 30 mM OG detergents. Results were shown in Figure 3.27.

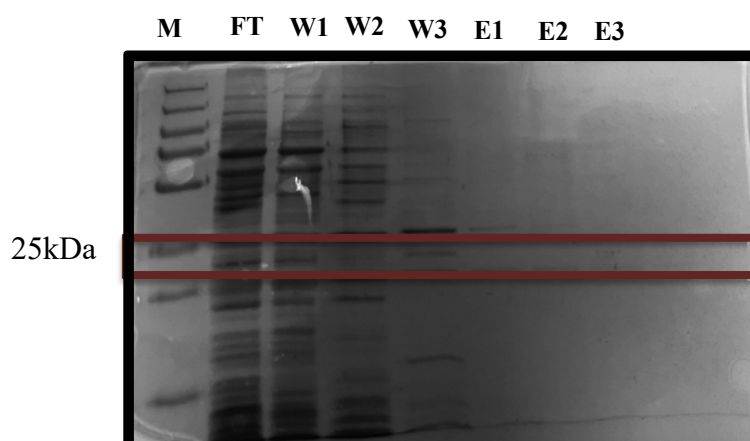


Figure 3.27 : SDS-PAGE analysis of V13 purification. Lane M: Molecular weight marker. Lane 1: V13 protein flow through. Lane 2: V13 protein Wash 1 with 10 mM Imizadole. Lane 3: V13 protein Wash 2 with 20 mM Imizadole. Lane 4: V13 protein Wash 3 with 30 mM Imizadole. Lane 5: V13 protein Elution 1 with 100 mM Imizadole. Lane 6: V13 protein Elution 2 with 150 mM Imizadole. Lane 7: V13 protein Elution 3 with 250 mM Imizadole.

Grown V3 colonies were expressed and dialysed for purification step. Firstly, Wash buffers and Elution buffers were prepared based on imidazole amounts and OG amounts. Wash and Elution buffers include 30 mM OG detergents. Results were shown in Figure 3.28. However, after purification, SDS-PAGE gel results which are belongs to *Virgibacillus* 2 showed lower binding affinity to resin shown in Figure 3.28.

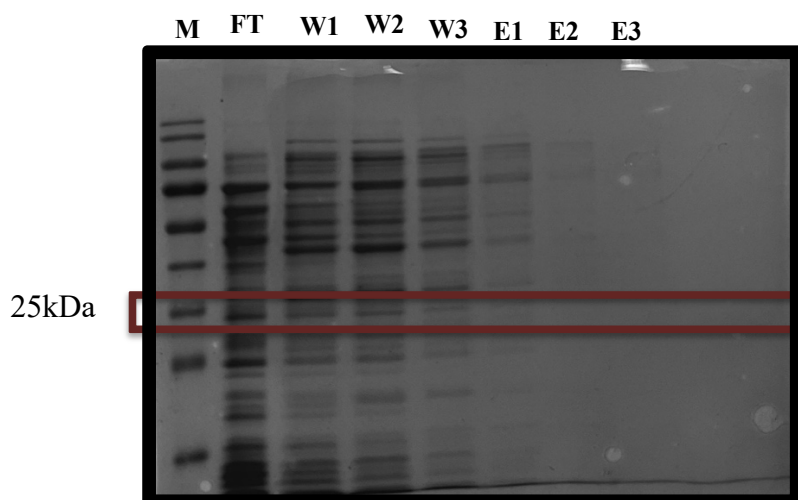


Figure 3.28 : SDS-PAGE analysis of V3 purification. Lane M: Molecular weight marker. Lane 1: V3 protein flow through. Lane 2: V3 protein Wash 1 with 10 mM Imizadole. Lane 3: V3 protein Wash 2 with 20 mM Imizadole. Lane 4: V3 protein Wash 3 with 30 mM Imizadole. Lane 5: V3 protein Elution 1 with 100 mM Imizadole. Lane 6: V3 protein Elution 2 with 150 mM Imizadole. Lane 7: V3 protein Elution 3 with 250 mM Imizadole.

The his-tagged protein could not be able to bind the resin and lost in the wash steps. Optimization of V13 and V3 clones were tried with different detergent molarities and different imidazole molarities. However, *Virgibacillus sp.* could not bind resin properly in any case. Because of the difficulty of *Virgibacillus* purification due to its optimization, *Geobacillus* samples were selected and continued with them. The His-trapped and centrifuge purification techniques were applied to *Geobacillus* samples and results were more clear according to *Virgibacillus* samples. That is why, aquaporins which are expressed from *Virgibacillus sp* AGTR were not be able to be purified.

- **His-trapped purification results**

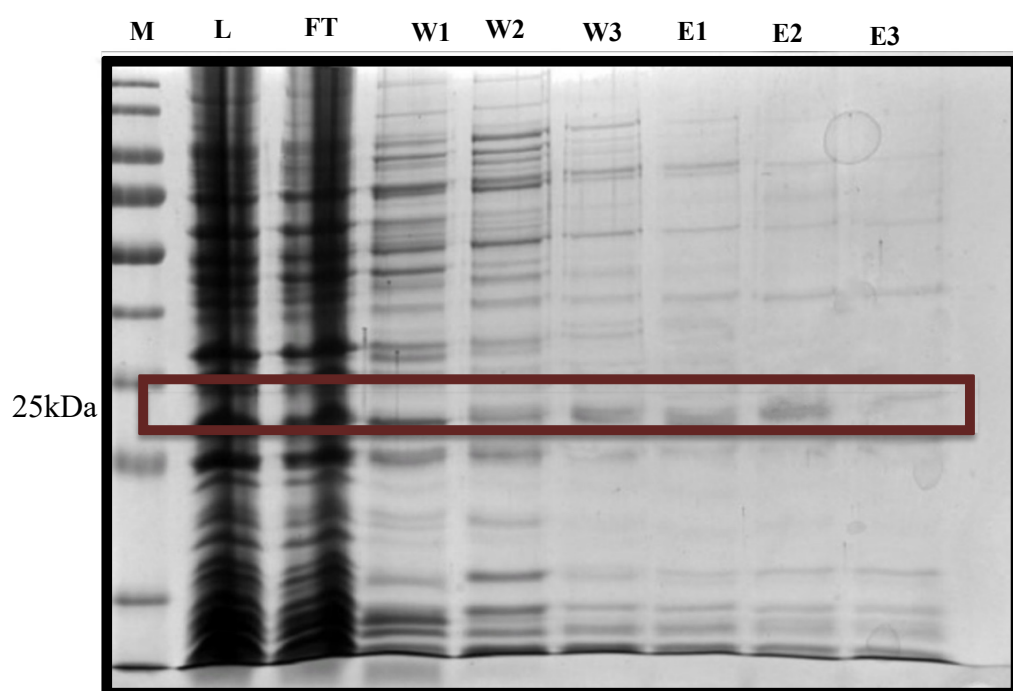


Figure 3.29 : SDS-PAGE analysis of Geo24 purification. Lane M: Molecular weight marker. Lane 1: Geo24 protein flow through. Lane 2: Geo24 protein Wash 1 with 10 mM Imizadole. Lane 3: Geo24 protein Wash 2 with 20 mM Imizadole. Lane 4: Geo24 protein Wash 3 with 30 mM Imizadole. Lane 5: Geo24 protein Elution 1 with 100 mM Imizadole. Lane 6: Geo24 protein Elution 2 with 150 mM Imizadole. Lane 7: Geo24 protein Elution 3 with 250 mM Imizadole.

According to Figure 3.29, the his-tagged protein tried to purify due to different imidazole amounts. This figure shows that the unwanted or not specific proteins also bound to the resin. Therefore, ultrafiltration which is another step to concentrate and getting rid of the unwanted proteins was applied. In the ultrafiltration step, the membranes were chosen based on the impurity. First of all, 30k membrane used for getting rid of the higher than 30 kDa proteins. After that, solution under the 30k

membrane was collected and put into 10k membrane to have the protein. Here, the aim was making the purified protein even more clear and removing unwanted proteins and impurities which are found in the upper kDa or lower kDa. This step was required for having pure aquaporin protein shown in Figure 3.30.

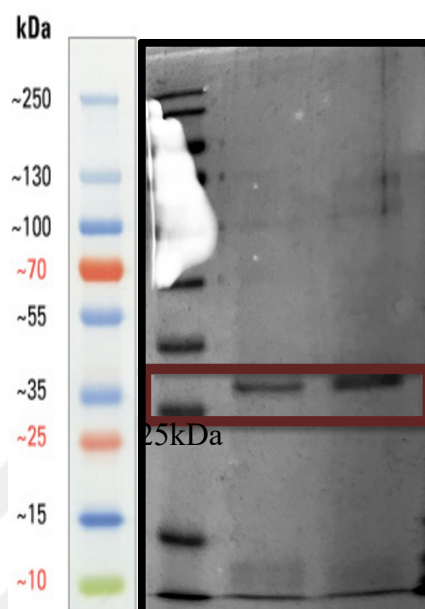


Figure 3.30 : SDS-PAGE analysis of Geo24 ultrafiltration. Lane M: Molecular weight marker. Lane 1: Collection of Wash 2 and Wash 3 samples after purification exposed to ultrafiltration (30k-10k). Lane 2: Collection of Elution 1, Elution 2 and Elution 3 samples after purification exposed to ultrafiltration (30k-10k).

- **Centrifuge purification**

Samples which were in buffer 2 was dialysed and left in 14 hours with resin. After that, samples centrifuged at 3000 x rpm for 2 min at 4 °C and collected as a flow through. Secondly, wash 1 was added and again centrifuged at 3000 x rpm for 2 min at 4 °C. This procedure was followed until elution 4 was added. After that, all samples were load to SDS-PAGE and visualized the result.

3.11 Comparing Protein Sequences

In cell membranes, AQPs form tetrameric holes. Six membrane-spanning helices and five loops (A to E) are found on each subunit; the amino and carboxyl terminal domains are on the cytosolic side. Seven Loops B and E fold inward to span the membrane as short helices and also, each having a conserved asparagine-proline-alanine (NPA) signature motif on the intracellular and extracellular sides, respectively. These conserved sequences are shown the Figure 3.31 with yellow colour in each three

aquaporin protein sequences. The symbols “*” (asterisk), “.” (period) and “:” (colon) are shown in Figure 3.32. According to the asterisk symbol, it determines the positions that have a single, fully conserved residue and column symbol indicates strongly similar properties and period symbol indicates weakly similar properties. It can be said that three of them have strongly similar sequences including conserved sequences. Also, hydrophobic regions (red) are found in high amounts as they are integral proteins.

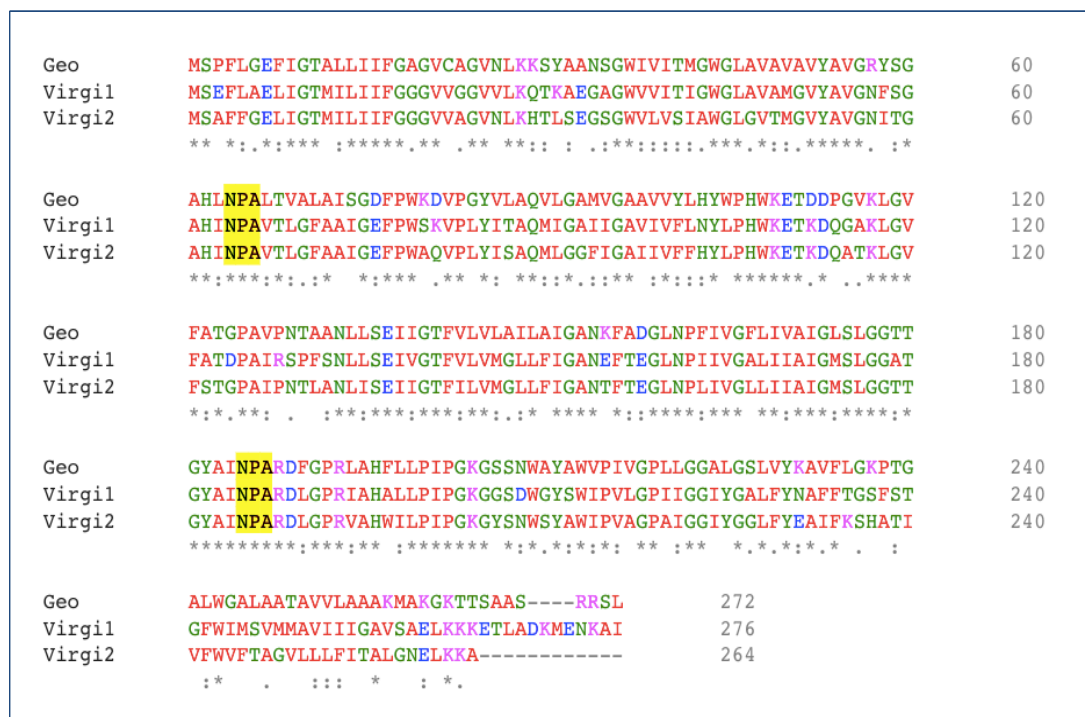


Figure 3.31 : Protein sequencing comparing for three aquaporin aminoacids in Clustal Omega.

Residue	Colour	Property
AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
Others	Grey	Unusual amino/imino acids etc

Figure 3.32 : The colour description for Clustal Omega.

Based on the above information, we can say that three aquaporin protein have hydrophobic sides and some acidic sides which are shown with red color and also blue color in the Figure 3.32 belongs to Clustal Omega Tool.

3.12 Liposome and Proteoliposome Production

As a result, DOPC lipid stock solution was diluted with using chloroform. HPLC tubes shown in Figure 3.33 were prepared based on 1.5 mL and 1 mL and the chloroform and also lipid amounts from DOPC 25 mg/mL were calculated based on equation 3.1 and equation 3.2. In total 5 mL diluted solution was observed.

For 1.5 mL;

$$\begin{aligned} \frac{10\text{mg}}{\text{mL}} \times 1.5 \text{ mL} &= \frac{25\text{mg}}{\text{mL}} \times ? & (3.1) \\ &= 0.6 \text{ mL stock} \\ &= 0.9 \text{ mL chloroform} \end{aligned}$$

For 1 mL;

$$\begin{aligned} \frac{10\text{mg}}{\text{mL}} \times 1 \text{ mL} &= \frac{25\text{mg}}{\text{mL}} \times ? & (3.2) \\ &= 0.4 \text{ mL stock} \\ &= 0.6 \text{ mL chloroform} \end{aligned}$$

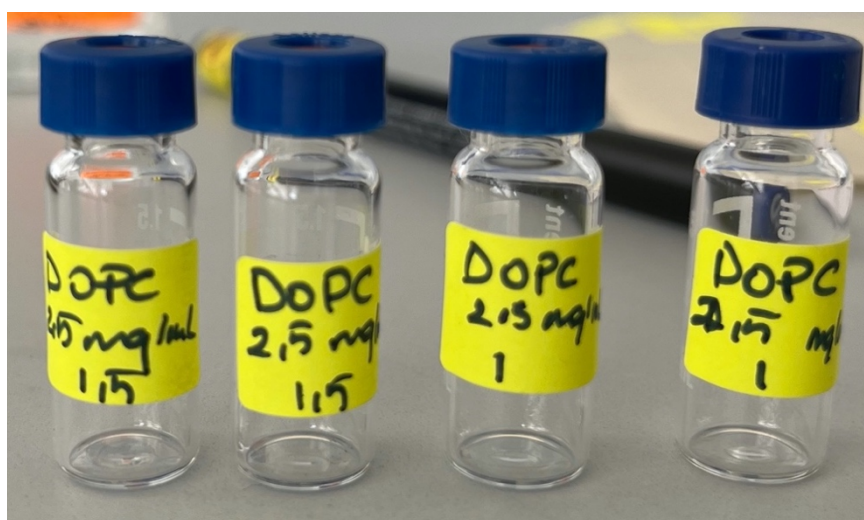


Figure 3.33 : The HPLC tubes with volumes of 1.5 mL and 1 mL including stock solution and chloroform. 5 mL of diluted lipid solution was prepared.

After preparing these lipid solutions in +4 °C, they were ready for using liposome and proteoliposome production. In the control liposome production, after placing lipid 45° angle to liposome it incubated at +4 °C by overnight, it was exposed to vortex

approximately 15 minutes. Collected liposomes were passed through 200 nm membrane and they were prepared.

In the proteoliposome which includes aquaporin solution in the lipid solutions were placed into balloon joje and added 10 mL PBS with including OG detergent (cmc: 0.025) were exposed to 5 minutes vortex and 15 minutes sonication and 10 minutes vortex again. Biobeads were used to remove the detergent and collected proteoliposomes were passed through 200 nm membrane and they were prepared.



Figure 3.34 : The system for liposome and proteoliposome production. The number 1 is the mini extruder, number 2 is PTFE and number 3 is nut, number 4 is syringe respectively.

1 mL of 10 mL liposome and proteoliposome solutions were passed through syringe with 21 times for the liposome extrusion step which is shown in Figure 3.34 and collected into 15 mL falcon. 0.85 M NaCl solution was mixed with liposomes and proteoliposomes. Using stopped flow light scattering spectrometer, the water transfer rates of the aquaporins were measured using 0.85 M NaCl solution and 0.5 M NaCl solution. For each measurement, approximately 100 μ l of sample and NaCl were delivered to the spectrometer from different syringes. In this way, the activities of the control liposome and the proteoliposome at room temperature were checked first, and then the measurements of the control liposome at 40 $^{\circ}$ C and proteoliposome at 40 $^{\circ}$ C were taken. The aim here is to show that aquaporin, which is resistant to high temperature, works even at 40 $^{\circ}$ C, but the liposome starts to denature, and the water velocity decreases. Shrinkage of aquaporins occurs with water passage and this shrinkage is directly related to the water passage rate called k-value. In other words, if the control liposome with a certain k-value is lower than the k-value of the

proteoliposome, it is believed that the aquaporins shrinkage and thus the water velocity increases.

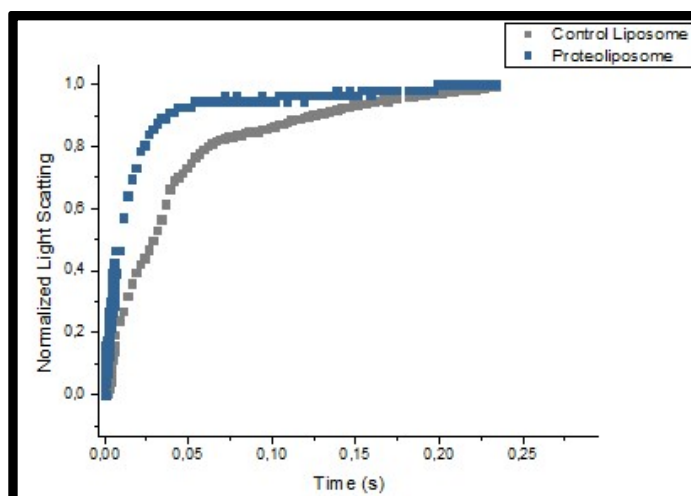


Figure 3.35 : Curves of water permeation rates of aquaporins obtained by light scattering spectrometry. 0.85M NaCl was used. The control liposome does not contain aquaporin and is shown in gray. The proteoliposome contains aquaporin from *Geobacillus thermoleovorans*

As a result, liposome and proteoliposome outcomes of which water velocities were calculated using 0.85 M NaCl were obtained as in Figure 3.35. The k-values taken from the measurement made here were entered on the y-axis and normalized against time to obtain this graph. The k-values for liposome and proteoliposome are given in the Table 3.2 below.

Table 3.2 : Comparison of the water transfer rates (k) of liposomes and proteoliposomes.

Samples	Temperature	k (s ⁻¹)
Control	Room	22
Proteoliposome	Temperature	78
Control		11
Proteoliposome	40 °C	44

In the control liposome and proteoliposome at room teperature, the difference was obtained in k-values. Control liposome has 22 s⁻¹ k value and proteoliposome has 78 s⁻¹. This can be explained based on the velocity of water. Aquaporin channels should allow more water to pass through. That is why, what is expected from the proteoliposome is that the water transfer rate should be higher because it contains aquaporin. In the stopped flow which measures the k value, results were confirmed this argument. It would be correct to say that the proteoliposome provided faster water passage. In addition, control liposome and proteoliposome were exposed to 40 °C.

Here the purpose was to find out whether the *Geobacillus thermoleovorans* that are thermophilic bacteria can be active or not at this temperature.

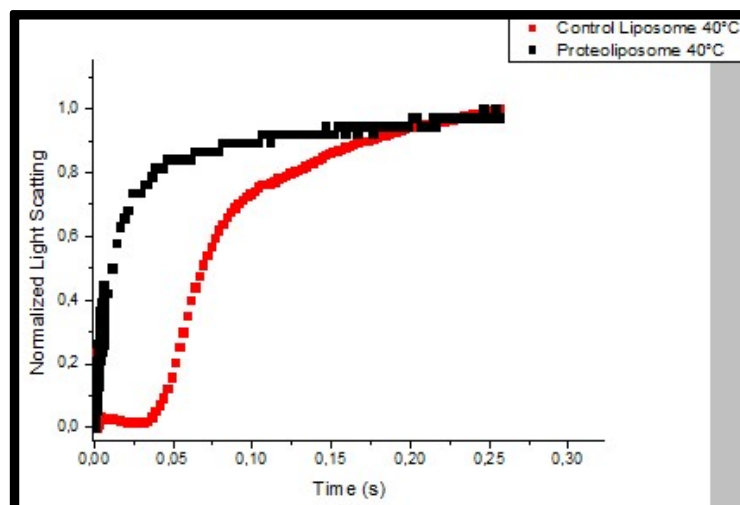


Figure 3.36 : Curves of water permeation rates of aquaporins obtained by light scattering spectrometry. 0.85M NaCl was used, and samples were incubated 5 minutes at 40°C. The control liposome does not contain aquaporin and is shown in red. The proteoliposome contains aquaporin from *Geobacillus thermoleovorans* ARTR1 and is shown in black.

Again, as a result, liposome and proteoliposome outcomes of which water velocities were calculated using 0.85 M NaCl and at 40 °C were obtained as in Figure 3.36. The k-values taken from the measurement made here were entered on the y-axis and normalized against time to obtain this graph. The k-values for liposome and proteoliposome are given in the Table 3.1. According to the result in Figure 3.36, it was seen that the proteoliposome was active at 40 °C with higher k value and allow the water transfer but the k value decreased in the empty liposome.

In this study, water passage was ensured as a result of the liposome integration of aquaporin produced recombinantly from *Geobacillus thermoleovorans* ARTR1 and the data were recorded according to the water passage rate. In line with these data in Table 3.1, it has been observed that aquaporin produced from *Geobacillus thermoleovorans* ARTR1 provides a higher k value (78 s^{-1} and 44 s^{-1}) than the control liposome (22 s^{-1} and 11 s^{-1}), both at room temperature and at 40 °C.

4. CONCLUSION

In recent years, the high-quality recombinant production of aquaporins using *E.coli* host cells has been in high demand. Since these proteins are biological membrane proteins, disadvantages in their expression and purification is a challenge to work with these proteins. However, if they are successfully produced, they can be used in different industrial applications such as health, environment, chemistry. These aquaporins, which differ according to their usage areas, have become very popular in the last 20 years in the industrial field. Specifically, through the biomimetic membranes, aquaporins are used in today's important processes such as wastewater treatment.

In recent years, another important area that has been studied is extreme microorganisms. Proteins, enzymes, metabolites obtained from these organisms contribute to the literature, and also these molecules can be used in many different fields. Since these organisms can grow in harsh conditions and the molecules isolated from them are expected to have the same characteristics as the living conditions of the organism. For this reason, the obtained proteins and enzymes will have properties such as high temperature, high or low pH, drought, halophilic and will have an advantage in their use such as bacteria living in halophilic and thermophilic environments can survive in harsh conditions such as high pH, high salt concentrations and high temperature. The metabolites produced by these can remain active under the same conditions. So, the extreme organisms and their products would be significant for industrial processes. The aquaporins which are produced recombinantly from halophilic and thermophilic bacteria can be integrated into biomimetic membranes and work under harsh stress conditions.

In our previous studies, *Virgibacillus sp. AGTR* strain isolated from Acıgöl that is a lake with high salinity has taken its place in the literature as a new strain of *Virgibacillus* species. Optimum growth conditions of the isolated organism were determined as 37 °C, 5 % salt and pH 8. The whole-genome analysis of the obtained strain was performed by next-generation sequencing. It was determined that this

species contains two aquaporin encoding genes: one 831 bp and the other 795 bp long. On the other hand, Armutlu is a region where tectonic movements are active in terms of their location. Thermal water resources in the region are widely used for different purposes (treatment, industry). It was found out that *Geobacillus species* were dominant amongst the sediment samples taken from Armutlu. As a result of the genome studies, this species was named *Geobacillus thermoleovorans* ARTR1. The aquaporin gene sequence of this microorganism, which has optimum growth conditions at 60 °C and pH 7, is 819 bp long. In the literature, there is no information describing the detailed biochemical characterization of the aquaporin protein isolated from this organism yet.

These strains were grown in their medium; LB medium for *Virgibacillus sp.* AGTR and NB medium for *Geobacillus thermoleovorans* ARTR1. After they have reached the required amount, genomic DNA was isolated. After isolation, gene-specific primers were designed for three aquaporin genes. The genes of interest were amplified by using the PCR at optimum temperatures. The amplified genes were purified using the PCR clean up kit. Then, the pET28a (+) vector was ligated using the T4 ligase enzyme. After ligation, the *E.coli* host cell, C43 (DE3), was grown for the transformation step and allowed to grow overnight at 37 °C. The ligated plasmid was transferred to the growing cells and transformation was performed. Then it was confirmed by establishing control cut and colony PCR with *SacI* and *NotI* restriction enzymes. After the transformed cells were spread on the agar plate and left at 37 °C, a single colony was selected and the selected colonies were grown again in LB medium containing kanamycin at 37 °C. Growing cells were reserved for stock, while the remainder was induced with IPTG to overexpress the protein. IPTG induction was subjected to time, temperature, and molarity trials, respectively. These cells were expressed for 1 hour, 2 hours, 4 hours, 6 hours, and at 37 °C and 30 °C with 0.5 mM IPTG and 1 mM IPTG. Then the optimal condition of 1 mM IPTG, 37 °C and 4 hours of induction was selected. Under this optimal condition, gene expression was visibly achieved. Since it was observed that the protein remained in the inclusion body formed in the centrifuge after sonication, it was continued with the pellet after centrifugation and examined by SDS-PAGE in the control supernatant. After this process, the protein was obtained pure by various His-tag purification methods. Proteins expected to appear at an average size of 29 kDa were confirmed by SDS-PAGE and Western Blot

Analysis. Looking at the general sequence, there are histidine amino acids at the C-terminal and N-terminus of the protein coding sequence. The antibodies used in the Western blot analysis are specific for the histidine amino acids in the C-terminus. Thus, these antibodies recognized the histidine at the C-terminus of the sequence encoding the protein and showed bands in 29 kDa size by irradiating with the dye used in the membrane. Proteins were kept at -20 °C as a stock solution.

In the liposome production, DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) lipids were prepared as a stock solution 10 ml/mg with using chloroform. The final liposome concentration was arranged to 2.5 mg/ml. 10 ml PBS buffer was used for liposome production. After placing lipid at a 45° angle to the liposome, it was incubated at +4 °C overnight and was exposed to vortex for approximately 15 minutes. After placing lipid at a 45° angle to the liposome, it was incubated at +4 °C overnight and was exposed to vortex for approximately 15 minutes. Collected liposomes were passed through a 200 nm membrane and they were prepared. In the proteoliposome which includes aquaporin solution in the lipid solutions were placed into balloon joje and added 10 mL PBS including OG detergent (cmc: 0.025) were exposed to 5 minutes vortex and 15 minutes sonication and 10 minutes vortex again. The detergent removed by Biobeads and collected proteoliposomes were passed through a 200 nm membrane and they were prepared. The water transfer rate was measured with the prepared control liposome and proteoliposome 20SX stopped-flow spectrometer. The aim here was to see the shrinkage of the liposomes as water passed and to calculate the k value that is directly proportional to this shrinkage. The expected result was the water transfer rate was greater than in the empty liposome, due to the shrinkage in the proteoliposome. Two types of liposomes were studied in 0.85 M NaCl medium. According to osmosis, water transfer is expected from a less concentrated medium to a high concentrated medium. Since NaCl is highly concentrated, only water will pass through, so only water will be transferred from the aquaporin water channels. The test result was confirmed in line with these expected data. A faster water transfer was achieved from the aquaporin-containing proteoliposome and a higher k value was obtained compared to the empty liposome. Since it is a thermophilic organism, the maximum temperature that the liposome can withstand was tried and it was observed that the proteoliposome gave a higher k value.

Within the scope of this thesis, the identification and preliminary characterisation of aquaporin genes from both halophilic and thermophilic bacteria, are of tremendous value in terms of its contribution to the literature. In near future, it is planned to improve the production of these three extreme aquaporins in large quantities by molecular biology techniques. The high amount of production would allow us to characterize these three proteins in detail for industrial purposes.



REFERENCES

- Akyüz, S., Büyüköztürk, H., Öztürk, A., & Turanoğlu, M. A. Aquaporinler: Su Kanallarından Fazlası
- Alberts B, Johnson A, Lewis J, et al. (2002). *Molecular Biology of the Cell* (4th ed.). New York: Garland Science. Archived from the original on 2017-12-20
- Almén MS, Nordström KJ, Fredriksson R, Schiöth HB. (August 2009). Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin". *BMC Biology*. 7: 50. doi:10.1186/1741-7007-7-
- Almén MS, Nordström KJ, Fredriksson R, Schiöth HB. (August 2009). "Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin". *BMC Biology*. 7: 50. doi:10.1186/1741-7007-7-
- André, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast*, 11(16), 1575-1611.
- Ariöz, C. (2014). *Exploring the Interplay of Lipids and Membrane Proteins* (Doctoral dissertation, Department of Biochemistry and Biophysics, Stockholm University).
- Çalicioğlu, N. G., Özdemir, G. Ö., Öztürk, A., Yıldız, A., Yılmaz, H., Ergenekon, P., ... & Özkan, M. (2018). Use of halophilic aquaporin for preparation of biomimetic thin film composite membrane. *Journal of Membrane Science*, 568, 105-112
- Chen, G. Q., and Jiang, X. R. (2018). Next generation industrial biotechnology based on extremophilic bacteria. *Curr. Opin. Biotechnol.* 50, 94–100. doi: 10.1016/j.copbio.2017.11.016
- Edidin, M. (2003). Lipids on the frontier: a century of cell-membrane bilayers. *Nature Reviews Molecular Cell Biology*, 4(5), 414-418.
- Erbakan, M., Curtis, B. S., Nixon, B. T., Kumar, M., & Curtis, W. R. (2015). Advancing *Rhodobacter sphaeroides* as a platform for expression of functional membrane proteins. *Protein expression and purification* 15, 109-117.
- Froger, A., Rolland, J. P., Bron, P., Lagrée, V., Le Cahérec, F., Deschamps, S., & Delamarche, C. (2001). Functional characterization of a microbial aquaglyceroporin. *Microbiology*, 147(5), 1129-1135.
- Furt, F., Simon-Plas, F., & Mongrand, S. (2011). Lipids of the plant plasma membrane. In *The plant plasma membrane* (pp. 3-30). Springer, Berlin, Heidelberg.

- Gorter E, Grendel F.** (March 1925). "On Bimolecular Layers of Lipoids on the Chromocytes of the Blood". *The Journal of Experimental Medicine*. 41 (4): 439–43.
- Gray J; Groeschler S.; Le T; Gonzalez Z.** (2002). "Membrane Structure" (SWF) Davidson College. Archived from the original on 2007-01-08. Retrieved 2007-01-11.
- Herrmann, T., Leavitt, L., & Sharma, S.** (2021). Physiology, Membrane. StatPearls
- Immordino, M. L., Dosio, F., & Cattell, L.** (2006). Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *International journal of nanomedicine*, 1(3), 297.
- Jaradat, E., Weaver, E., Meziane, A., & Lamprou, D. A.** (2021). Microfluidics Technology for the Design and Formulation of Nanomedicines. *Nanomaterials*, 11(12), 3440.
- Johansson, I., Karlsson, M., Johanson, U., Larsson, C., & Kjellbom, P.** (2000). The role of aquaporins in cellular and whole plant water balance. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1465(2), 324-342.
- Kaldenhoff, R., Bertl, A., Otto, B., Moshelion, M., & Uehlein, N.** (2007). Characterization of plant aquaporins. *Methods in enzymology*, 428, 505-531. S J Singer and G L Nicolson. "The fluid mosaic model of the structure of cell membranes." *Science*. (1972) 175. 720-731.
- Kapilan, R., Vaziri, M. & Zwiazek, J.J.** (2018). Regulation of aquaporins in plants under stress. *Biol Res* 51, 4.
- Karp G.** (2009). *Cell and Molecular Biology: Concepts and Experiments*
- Kozono, D., Ding, X., Iwasaki, I., Meng, X., Kamagata, Y., Agre, P., & Kitagawa, Y.** (2003). Functional expression and characterization of an archaeal aquaporin: AqpM from *Methanothermobacter marburgensis*. *Journal of Biological Chemistry*, 278(12), 10649-10656.
- Li, X., Wang, R., Tang, C., Vararattanavech, A., Zhao, Y., Torres, J., & Fane, T.** (2012). Preparation of supported lipid membranes for aquaporin Z incorporation. *Colloids and Surfaces B: Biointerfaces*, 94, 333-340.
- Lin, S. H., & Guidotti, G.** (2009). Purification of membrane proteins. *Methods in enzymology*, 463, 619-629.
- M.J. Borgnia, D. Kozono, G. Calamita, P.C. Maloney, P. Agre, J.** *Mol. Biol.* 291 (1999) 1169.
- Maurel, C., Boursiac, Y., Luu, D. T., Santoni, V., Shahzad, Z., & Verdoucq, L.** (2015). Aquaporins in plants. *Physiological reviews*, 95(4), 1321-1358.
- Nalivaeva N.N., Turner A.J.** (2009) Lipid Anchors to Proteins. In: Lajtha A., Tettamanti G., Goracci G. (eds) *Handbook of Neurochemistry and Molecular Neurobiology*. Springer, Boston, MA. 978-0-387-30378-9_14
- Nelson, D. L., & Cox, M. M.** (2008). *Principles of Biochemistry* (5th ed., p. 377). New York, NY: W.H. Freeman and Company.

- Nkanga, C. I., Bapolisi, A. M., Okafor, N. I., & Krause, R. W. M.** (2019). Liposomes-Advances and Perspectives.
- Oztug, M., Cebeci, A., Mumcu, H., Akgoz, M., & Karaguler, N. G.** (2020). Whole genome sequence of *Geobacillus thermoleovorans* ARTRW1, isolated from Armutlu Geothermal Spring, Turkey. *Microbiology Resource Announcements*, 9(24), e00269-20.
- Öztürk, A., Yıldız, A., Yılmaz, H., Ergenekon, P., & Özkan, M.** (2020). Organic solute rejection capacities of *Escherichia coli* and *Halomonas elongata* aquaporin-incorporated nanofiltration membranes. *Separation Science and Technology*, 55(13), 2362-2368.)
- Savage, D. F., Egea, P. F., Robles-Colmenares, Y., III, J. D. O. C., Stroud, R. M., & Simon, S.** (2003). Architecture and selectivity in aquaporins: 2.5 ÅX- ray structure of aquaporin Z. *PLoS biology*, 1(3), e72.
- Sengur-Tasdemir, R., Aydin, S., Turken, T., Genceli, E. A., & Koyuncu, I.** (2016). Biomimetic approaches for membrane technologies. *Separation & Purification Reviews*, 45(2), 122-140
- Stillwell, W.** (2016). Chapter 6-membrane proteins. *An Introduction to Biological Membranes*, 2nd ed.; Stillwell, W., Ed. ISO 690
- Tang, C. Y., Zhao, Y., Wang, R., Hélix-Nielsen, C., & Fane, A. G.** (2013). Desalination by biomimetic aquaporin membranes: Review of status and prospects. *Desalination*, 308, 34-40.
- Tong, H., Hu, Q., Zhu, L., & Dong, X.** (2019). Prokaryotic aquaporins. *Cells*, 8(11), 1316.
- Verkman, A. S.** (2013). Aquaporins. *Current Biology*, 23(2), R52-R55.
- Verkman, A. S., & Mitra, A. K.** (2000). Structure and function of aquaporin water channels. *American Journal of Physiology-Renal Physiology*, 278(1), F13-F28.
- Vreeland RH, Litchfield CD, Martin EL, Elliot E** (April 1980). "*Halomonas elongata*, a New Genus and Species of Extremely Salt-Tolerant Bacteria". *International Journal of Systematic Bacteriology*. 30 (2): 485–495
- Wei, P., Wang, Q., Hang, B., Shi, F., Cai, J., Huang, L., & Xu, Z.** (2017). High level cell-free expression and functional characterization of a novel aquaporin from *Photobacterium profundum* SS9. *Process Biochemistry*, 59, 172-179.
- Wesolowska, O., Michalak, K., Maniewska, J., & Hendrich, A. B.** (2009). Giant unilamellar vesicles-a perfect tool to visualize phase separation and lipid rafts in model systems. *Acta Biochimica Polonica*, 56(1).
- Whitelegge, J. P.** (2013). Integral membrane proteins and bilayer proteomics. *Analytical chemistry*, 85(5), 2558-2568.
- Wood, M. H., Milan, D. C., Nichols, R. J., Casford, M. T., & Horswell, S. L.** (2021). A quantitative determination of lipid bilayer deposition efficiency using AFM. *RSC Advances*, 11(32), 19768-19778.

- Yılmaz, H., Özdemir, F. İ., Ergenekon, P., & Özkan, M.** (2020). Affinity tag effect on the salt rejection potential of Halomonas elongata aquaporin incorporated in thin film nanocomposite membrane. *Protein expression and purification*, 173, 105664
- Yurdakul, A., & Rıza, A. T. A. V.** (2007). Structures and classification of liposomes *Textile and Apparel*, 17(4), 243-247.
- Zhu, D., Adebisi, W. A., Ahmad, F., Sethupathy, S., Danso, B., & Sun, J.** (2020). Recent development of extremophilic bacteria and their application in biorefinery. *Frontiers in Bioengineering and Biotechnology*, 8, 483.



APENDICES

APPENDIX A : Laboratory equipments

APPENDIX B: Culture media composition

APPENDIX C: Buffers and solution

APPENDIX D: Chemicals, enzymes and kits



APPENDIX A : Laboratory equipments

Table A.1 indicates all the laboratory equipments in this study

Table A.1 : Used equipments in this study.

Equipment	Supplier Company
20SX Stopped Flow Spectrometer	Applied Photophysics
Analytical Balances	Precisa BT 610C
Autoclave	TOMY SX-700E
Centrifuges	Beckman Coulter.
Deep freezers and refrigerators	1061 M refrigerator, Arcelik
Electrophoresis equipment	Horizon 11.14, Whatman,
Gel Documentation System:	GAS7000; Biometra
Heat Block	BioSan Bio TDB-100
Ice machine	AF 10, Scotsman.
Laminar flow cabinet	Faster Laminar Flow
Magnetic stirrer	Heidolph Standard
Microplate reader	Bio-Rad; CLARIOstar
Nanodrop	Scientific
Orbital shaker	Thermo Electron Corporation
pH meter	GmbH & Co KG
Pipettes	Eppendorf
Sonicator	Sonoplus, Bandelin
Thermal Cycler	MiniAmp Plus
Thermomixer	Biometra.
UV-Visible light spectrophotometer	UV Star Biometra
Vortex	Scientific Industries

APPENDIX B: Culture media composition

Culture media compositions are given in below :

Table B.1 : The Luria Bertani (LB) medium is composed of the following components for 1 liter.

Compound	Amount
Bacterial Peptone	10 g
Yeast Extract	5g
NaCl	10 g

Table B.2 : The Luria Bertani (LB) agar medium is composed of the following components.

Compound	Amount
Bacterial Peptone	10 g
Yeast Extract	5g
NaCl	10 g
Agar	15 g
dH ₂ O	up to 1 L

Table B.3 : The Nutrient Broth (NB) Medium is composed of the following components.

Compound	Amount
Beef Extract	3 g
Peptone	5 g
NaCl	5 g
dH ₂ O	up to 1 L

Table B.4 : The Nutrient Broth (NB) Agar Medium is composed of the following components.

Compound	Amount
Beef Extract	3 g
Peptone	5 g
NaCl	5 g
Agar	15 g
dH ₂ O	up to 1 L

Table B.5 : The MagicMedia™ (150ml) is composed of the following components.

Compound	Amount
Component A	5.85 mg
Component B	7.5 mL
dH ₂ O	142.5 mL

APPENDIX C: Buffers and solution

Buffers and solutions are given in Table C.1 to Table C.16

Table C.1 : The Agarose gel components.

Compound	Amount
Agarose	0.40 g
TAE (1X)	40 mL

Table C.2 : His-tag Purification Lysis, Solubilization, Wash, Buffers.

Lysis Buffer	Wash Buffer	Solubilization Buffer
20 mM Tris-HCl (pH: 7.4)	20 mM Tris-HCl (pH: 7.4)	20 mM Tris-HCl (pH: 7.4)
0.5 mM PMSF	0.5 mM PMSF	0.5 mM PMSF
5 mM BME	5 mM beta-mercapto	5 mM BME
100 mM OG	100 mM OG	100 mM OG
	10 % Glycerol	10 % Glycerol
	20 mM Imidazole	20 mM Imidazole

Table C.3 : The 50X TAE buffer is composed of the following components.

Compound	Amount
2 M Tris Base	242 g
1 M Acetic Acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
dH ₂ O	up to 1 L

Table C.4 : The 1X TBS buffer is composed of the following components.

Compound	Amount
10X TBS Buffer	100 mL
dH ₂ O	up to 1 L

Table C.5 : The 10X running buffer is composed of the following components.

Compound	Amount
Tris Base	30.3 g
Glycine	144 g
SDS	10 g
dH ₂ O	up to 1 L

Table C.6 : The 10X transfer buffer is composed of the following components.

Compound	Amount
Tris Base	30.3 g
Glycine	144 g
dH ₂ O	up to 1 L

Table C.7 : The 1X transfer buffer is composed of the following components.

Compound	Amount
10X Transfer Buffer	100 mL
Methanol	200 mL
dH ₂ O	up to 1 L

Table C.8 : The 10X TBS buffer is composed of the following components.

Compound	Amount
Tris Base	24.22 g
NaCl	87.66 g
dH ₂ O	up to 1 L
pH	adjust to 7.6 with HCl

Table C.9 : The 1X TBS-T buffer is composed of the following components.

Compound	Amount
Tween-20	1 mL
1X TBS	up to 1 L

Table C.10 : The Coomassie Brilliant Blue stain solution is composed of the following components.

Compound	Amount
Coomassie Brilliant Blue	1 g
Methanol	400 mL
Acetic Acid	100 mL
dH ₂ O	up to 1 L

Table C.11 : The 10% Ammonium Persulfate (APS) solution is composed of the following components.

Compound	Amount
APS	1 g
dH ₂ O	up to 10 mL

Table C.12 : The Destaining solution is composed of the following components.

Compound	Amount
Methanol	400 mL
Acetic Acid	100 mL
dH ₂ O	up to 1 L

Table C.13 : The stock 500 mM isopropyl- β -D-thiogalactopyranoside (IPTG) is composed of the following components.

Compound	Amount
IPTG	1.19 g
dH ₂ O	up to 10 mL

Table C.14 : The 30 mg/mL Kanamycin stock solution is composed of the following components.

Compound	Amount
Kanamycin	0.3 g
dH ₂ O	up to 10 mL

Table C.15 : The components of 10% and 15% SDS polyacrylamide separating gels.

Content	Amount	
	10 % Gel	12 % Gel
dH ₂ O	4.79 mL	2 mL
40% Acrylamide/Bis-acrylamide	2.5 mL	1.5 mL
1.5 M Tris-HCl (pH 8.8)	2.5 mL	1.3 mL
10% SDS	100 µL	50 µL
10% APS	100 µL	50 µL
100% TEMED	10 µL	5 µL

Table C.16 : The components of SDS polyacrylamide stacking gel.

Content	Amount
dH ₂ O	1.4 mL
40% Acrylamide/Bis-acrylamide	250 µL
1.5 M Tris-HCl (pH 6.8)	250 µL
10% SDS	20 µL
10% APS	20 µL
100% TEMED	5 µL

APPENDIX D: Chemicals, Enzymes And Kits

Chemicals, enzymes and kits are in below Table D.1 to Table D.4.

Table D.1 : Used chemicals in this study.

Chemical	Supplier Company
Acetic Acid	Bio-Rd
Acrylamide/Bis-Acrylamide (40%)	Sigma-Aldrich
Agar Bacteriological Grade	MultiCell
Agarose Molecular Biology Grade	MultiCell
Ammonium Persulfate (APS)	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
Bradford Reagent (1X)	Bio-Rad
Calcium Chloride	ZAG Kimya
Dimethyl Sulfoxide (DMSO)	BioMatik
Dithiothreitol (DTT)	Thermo Fisher
Ethanol Absolute	Merck
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich
Glycine	Sigma-Aldrich
Glycerol	Merck
Hydrochloric Acid (HCl)	Sigma-Aldrich
Imidazole	Sigma-Aldrich
Isopropanol	Merck
Kanamycin	Merck
Methanol	Sigma-Aldrich
Ni-NTA Agarose	Qiagen
Non-Fat Dry Milk	Santa Cruz
PhosSTOP	Roche
Phosphate-Buffered Saline (PBS) Tablets	Thermo Fisher
Sodium Chloride (NaCl)	Merck
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich
Sodium Hydroxide (NaOH)	Merck
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris Base	Sigma-Aldrich
OG	Thermofisher

Table D.2 : Used enzymes in this study.

Content	Company
Fast Digest <i>EcoRI</i>	Thermo Fisher Scientific
Fast Digest <i>SacI</i>	Thermo Fisher Scientific
Fast Digest <i>NotI</i>	Thermo Fisher Scientific
<i>NotI</i>	Thermo Fisher Scientific
Phusion DNA Polymerase:	Thermo Fisher Scientific
T4 DNA Ligase Enzyme	Roche

Table D.3 : Used lipid in this study.

Compound	Amount
DOPC	2.5 mg

Table D.4 : The kits used in this study.

Content	Amount
Bradford Assay Kits	Bio-Rad
High Pure Plasmid Isolation Kit	Roche
High Pure PCR Product Purification Kit	Roche

CURRICULUM VITAE

Name Surname : Şevval Uysalcan

--

EDUCATION :

B.Sc. : 2019, İstanbul Bilgi University, Faculty of Engineering and Natural Sciences, Department of Genetics and Bioengineering

PROFESSIONAL EXPERIENCE AND REWARDS :

- 2019 Istanbul Bilgi University , Best Senior Design Project Award : Engineering Lipase BTL-2 for *Geobacillus Thermocatenulatus* with Reduced Dimerization Behavior
- 2020-2022 Istanbul Technical University, Bilimsel Araştırma Projeleri : Ekstrem Organizmalardan İzole Edilmiş Aquaporinlerin Rekombinant Üretimi ve Karakterizasyonu, ID: 42954

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS :

- **Uysalcan Ş.,** Karagüler Gül N., (2022). Ekstrem Organizmalardan İzole Edilmiş Aquaporinlerin Rekombinant Üretimi ve Karakterizasyonu,1. Ulusal Biyolojik Bilimler Kongresi