MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES

Investigation of the Fructose Uptake System in *Halomonas smyrnensis* AAD6 to Design Strategies for Enhanced Levan Biosynthesis

BÜŞRA AYDIN

M.Sc. THESIS
Department of Bioengineering

ADVISOR

Assoc. Prof. Dr. KAZIM YALÇIN ARĞA

ISTANBUL, 2014

MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES

Investigation of the Fructose Uptake System in *Halomonas smyrnensis* AAD6 to Design Strategies for Enhanced Levan Biosynthesis

BÜŞRA AYDIN

524212003

M.Sc. THESIS
Department of Bioengineering

ADVISOR

Assoc. Prof. Dr. KAZIM YALÇIN ARĞA

ISTANBUL, 2014

MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES

Büşra AYDIN, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled "**Investigation of the fructose uptake system in *Halomonas smyrnensis* AAD6 to design strategies for enhanced levan biosynthesis**", on December 18, 2014 and has been found to be satisfactory by the jury members.

Jury Members

Assoc. Prof. Kazım Yalçın ARĞA (Advisor)
Marmara University ....(SIGN) ....

Prof. Dr. Ebru TOKSOY ÖNER (Jury Member)
Marmara Üniversitesi ....(SIGN) ....

Assist. Prof. Elif DERELİ EKE (Jury Member)
Nişantaşı Üniversitesi..(SIGN) ....

APPROVAL

Marmara University Institute for Graduate Studies in Pure and Applied Sciences Executive Committee approves that Büşra AYDIN be granted the legree of Master of Science in Department of Bioengineering, Bioengineering Program on 22.12.2014 . (Resolution no: 2014/27-03.

Director of the Institute
Prof. Dr. Uğur YAHŞİ

ACKNOWLEDGMENTS

At first, I would like to express my deepest sense of gratitude to my supervisor also my mentor Assoc. Prof. Kazım Yalçın ARĞA for his continuous advice and encouragement throughout this thesis. I thank him for the instilling me the qualities of being not only a good scientist but also a good person. I thank also for his systematic guidance and his infectious enthusiasm that has been a really good driving force to accomplish this study. It was a real pleasure to work with him.

I would like to express my sincere appreciation to Prof. Ebru TOKSOY ÖNER for her encouragement, continuous guidance, support, and valuable advices during all stages of this thesis.

I express my gratitude to other members of my thesis committee, for spending their precious time to read and for their valuable comment on my thesis.

This thesis was conducted under the financial support by The Scientific and Technological Research Council of Turkey(TUBITAK) through grant MAG/110M613, and by Marmara University Research Fund through grant FEN-C-YLP-101013-0404. I am thankful to TUBITAK for support of the scholarship program with 2210-C Bursary Program for Primary Areas of Turkey. I wish to thank all of my friends Esra GÖV, Songül YAŞAR YILDIZ, Deniz KÖŞEBENT, Beste ÇALIMLIOĞLU and Merve ERGİNER that have worked with me in the Industrial Biotechnology & System Biology Laboratory for their everlasting support. I owe my much gratitude to Melek AKENGİN and Esra GÖV who are special-bestfriends, sisters, colleagues and source of moral support of mine. I love you girls!

I also owe deepest gratitude to my loving parents Asiye and Bahattin ERCAN, my sister Kübra ERCAN, my brother Hasan ERCAN, my extended family Hatice, Mehmet Zeki and Rukiye AYDIN who have always been there to support and love me throughout my life. I am thankful for their unwavering support and encouragement. They have been a source of unconditional love, endless patience, inspiration and motivation for me.

This thesis is gratefully dedicated to my incredibly wonderful husband, *Muhammed*, thank you for being there whenever I need, for making me laugh, and for providing great joy, beauty, and truth to my life. This thesis and the pursuit of my goals would not have been

possible without you. I cannot possibly thank you enough for all of your love, support, and encouragement over the many years we have been together. I am eternally grateful. Final and deepest dedication goes to my baby who grows with me. I am extremely fortunate to going to be your mother. You and your father are everything to me. I love you, *always* and *forever*.

İSTANBUL, December, 2014 **Büşra AYDIN**

TABLE OF CONTENTS

ÖZET

ARTIRILMIŞ LEVAN BİYOSENTEZİ STRATEJİLERİ TASARLAMAK İÇİN *Halomonas smyrnensis* AAD6^T deki FRUKTOZ ALIMININ ARAŞTIRILMASI

Mikrobiyal biyopolimerler gösterdikleri farklı fizikokimyasal ve reolojik özellikleri ile yeni dönem biyomateryaller olarak kabul edilmektedirler. Bu polimerler biyobozunurluk ve biyouyumluluk özellikleri sayesinde çevre ve insan dostu bileşiklerdir, bu nedenle mikrobiyal biyopolimerler tekstil, deterjan, yapıştırıcı madde, atık su arıtımı, mayalama, kozmetoloji, ilaç bilimi ve gıda katkı maddeleri gibi geniş endüstriyel uygulama alanlarına sahiptirler. *Halomonas smyrnensis* AAD6^T gram-negatif, aerobik, ekzopolisakkarit üreten ve orta derecede halofilik bir bakteridir. Bu bakteri yüksek miktarda levan biyopolimeri üretmektedir. Levan, suda ve yağda iyi çözünebilirliği, güçlü adezivite, biyouyumluluk ve filmleşme özellikleri nedeniyle gıda, besicilik, kozmetik, ilaç ve kimya sanayisinde birçok potansiyel kullanım alanına sahip bir fruktoz homopolimeridir. Son yıllarda *Halomonas smyrnensis* AAD6^T 'nin metabolik ağ yapı modeli tüm genom sekansı baz alınarak oluşturulmuş ve polimer sentezini artıracak stratejiler araştırıldığında levan üretiminin artırılmasının organizmadaki fruktoz alımı sistemine bağlı olduğu gösterilmiştir. Bu sonuçlara dayanarak fruktoz spesifik fosfotranferaz sisteminin bütün bileşenleri *in silico* analizlerle belirlenmiştir. Bileşenlerin belirlenmesi bir metabolizma mühendisliği stratejisi kurulmasını ve transposonların kullanılacağı bir mutasyonun gerçekleştirilmesini sağlamıştır. Fruktoz alım sistemin önemli parçalarından biri olan HPr bileşeni fruktozun hücre içine alımını engellemek için organizma genomundan omega interpozunu ile mutasyonu sağlanmıştır. Mutasyonun başarılı olması *Halomonas smyrnensis* AAD6^T 'de fruktozun hücre içine alınamayıp yalnızca levan polimeri sentezinde kullanılmasını sağlamış ve levan üretimi %40'lık bir verimle artırılmıştır. Buna ek olarak bu mutasyon *Halomonas smyrnensis* BMA14 olarak isimlendirilen yeni bir suşu oluşturmuştur. Son olarak, bu çalışmanın çıktıları halofilik bakteriler tarafından sentezlenen diğer polimerlerin üretimini artırmak için sistem biyolojisi yaklaşımı ve metabolizma mühendisliği stratejilerinin tasarlanmasını içeren çalışmaları hızlandıracaktır.

ABSTRACT

INVESTIGATION OF the FRUCTOSE UPTAKE SYSTEM in *Halomonas smyrnensis* AAD6^T to DESIGN STRATEGIES FOR ENHANCED LEVAN BIOSYNTHESIS

Microbial biopolymers are accepted as new biomaterials due to their different physicochemical and rheological characteristics with novel functionality. Biopolymers are more biocompatible, biodegradable, and both environmental and human friendly compounds. There are wide range of applications of microbial exopolysaccharides in many industrial sectors like textiles, detergents, adhesives, wastewater treatment, brewing, cosmetology, pharmacology, and food additives. *Halomonas smyrnensis* AAD6^T is a gram-negative, aerobic, exopolysaccharide-producing, and moderately halophilic bacterium that produces levan, a fructose homopolymer with many potential uses in various industries like foods, feeds, cosmetics, pharmaceutical and chemical industries. Levan has outstanding properties like high solubility in oil and water, strong adhesivity, good biocompatibility and film-forming ability. Based on the whole genome sequence of *Halomonas smyrnensis* AAD6^T, a metabolic network of the microorganism was reconstructed and strategies were designed to enhance levan biosynthesis recently. Enhancement strategies for the biosynthesis of levan biopolymer by *Halomonas smyrnensis* AAD6^T basically depends on the fructose uptake system of the organism. Therefore, in the present study, the fructose specific phosphotransferase system (PTS^{fru}) of *H. smyrnensis* AAD6^T was investigated via *in silico* analysis. Investigation of all components of this system provided to construct a metabolic enginnering strategy which hinges basically on mutagenesis with using transposons. Fructose specific phoshocarrier protein- HPr is one of the most important component of PTS^{fru} system was mutated in the bacteial genome in order to prevent uptake of fructose into the cell. Achievement of the mutagenesis on genome of *Halomonas smyrnensis* AAD6^T contributed to utilize fructose monomers only in biopolymer production which resulted in elevated production of the polymer with 40% efficiency. Also, a new strain of *Halomonas smyrnensis* was constructed which has been named as *Halomonas smyrnensis* BMA14. Outcomes of this study will accelerate the research on enhanced production of any other biopolymers from halophilic bacteria towards systems biology approaches and design of metabolic engineering strategies.

SYMBOLS

g **:** gram

s : second

h **:** hour

L **:** liter

M **:** Molar

min **:** minute

v/v **:** Volume per volume

w/v **:** Weight per volume

µL **:** Micro liters

mL **:** Mili liters

µg **:** Micro grams

mg **:** Mili grams

mM **:** milli molar

rpm **:** Round per minute

nm **:** Nanometers

µm **:** Micro meters

Mw **:** Molecular weight

ABBREVIATIONS

ADP : Adenosine diphosphate

ATP : Adenosine triphosphate

Amp : Ampicillin

bp : Base pair

DNA : Deoxyribonucleic acid

dNTP : Deoxyribonucleotide triphosphate

EC number : Enzyme Commission number

EPS : Exopolysaccharide

HPr : Heat resistant protein or Histidine carrying protein

Kb : Kilo base pairs

Km : Kanamycin

LB : Luria Broth

PTS : Phoshotransferase System

RNA : Ribonucleic acid

Str : Streptomycin

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Aim

The main objective of this thesis was investigation of the system that lied behind the production of levan biopolymer in order to gain information about metabolic and genetic targets for modification of levan producing *Halomonas smyrnensis* AAD6 with metabolic engineering techniques to produce levan at enhanced levels.

The ultimate goal in this research was to construct a metabolically engineered, *Halomonas sp.* strain works like a microbial cell factory by design and perform genetic manipulations and mutagenesis for overproduction of industrially important levan biopolymer via systems biology and genetic engineering techniques. Based on the available genomic (Sogutcu et al.,2012) and system-based (Ozer, 2013) informations of *H. smyrnensis* AAD6, metabolic engineering experiments were successfully conducted and completed with new improved levan-producer strain *Halomonas smyrnensis* BMA14.

In literature, metabolic engineering of EPS-producing strains has been attempted, for example, for the production of xanthan (Rehm, 2009), gellan (Bajaj et al., 2007), bacterial cellulose (Bae et al., 2004). For some of these polymers (e.g. bacterial cellulose), this approach has been successful and has led to higher EPS production (Rehm, 2009; Bae et al., 2004). Also, in this thesis metabolic engineering approach has concluded with improved EPS production.

This thesis is the first attempt to make a metabolically engineered halophilic strain in Turkey via genetic manipulations and modification of the biological mechanisms related to EPS production which aims to overproduce of industrially important levan biopolymer.

1.2 General Background

1.2.1 Halophiles

Most extremophiles inhabit in microbial world and can survive in different extreme conditions. Extremophiles may be divided into some subclasses like; thermophiles, acidophiles, alkophiles, halophiles, and psychrophiles. Many extremophilic microorganisms possess properties suitable for biotechnological and commercial uses (Tango and Islam, 2002).

Halophiles are microorganisms that require high salt concentration for their survival. They are observed among all kingdoms of life: bacteria, eukarya, and archaea (Kamekura, 1998; Oren, 2013). Halophiles are classified as slightly halophilic, moderately halophilic and extremely halophilic depending on their salt requirements for optimal growth. The halotolerant microorganisms can survive and even grow in relatively high concentrations of salt but rather live in the absence of it.The moderate halophiles achieve their optimal growth at salt concentrations from 0.5 M (25 g/l) to 2.5 M (150 g/l) and the extreme halophiles at salt concentrations over 2.5 M to saturation (340 g/l) (Joo and Kim, 2005; Ventosa et al., 1998).

The name “Halophile” comes from the Greek roots “hals”, meaning salt, and “phil”, meaning friendly with, so halophile means that salt is required for survive (Madern et al., 2000).

Halophiles flourish in saline environments which are found all over the world, in arid, coastal, and deep-sea locations, underground salt mines, and artificial salterns. Halophilic microorganisms include a variety of heterotrophic, phototrophic, and methanogenic archaea, photosynthetic, lithotrophic, alkaliphilic and heterotrophic bacteria, and photosynthetic and heterotrophic eukaryotes. Halophilic organisms either accumulate internal organic compatible solutes to balance the osmotic stress of the environment or produce acidic proteins to increase solvation and improve function in high salinity (DasSarma et al., 2006).

It was reported that both the moderately halophilic bacteria and the extremely halophilic archaea have a broader catabolic versatility and capability. Halophiles can be also used for bioremediation studies. Different type of contaminating compounds is susceptible to be degraded by halotolerant and halophile bacteria (Le Borgne et al., 2008).

Moreover, *Halomonas campisalis* denitrifying bacterium that is both moderately halophilic and alkaliphilic hold promise for the treatment of saline, alkaline waste (Mormile et al.,1999).

1.2.2 Halomonadaceae

The family Halomonadaceae, within the gama subclass of proteobacteria, consists mostly of marine and moderately halophilic microorganisms that are phenotypically rather diverse (Arahal et al.,2006).

They are Gram-negative, motile by flagellation or non-motile and shapes are like straight rods. Members of this family require at least 75 mM sodium for growth with some strains capable of growth in media containing 30% salts. All species can utilize succinate, lactate and alanine for growth and most utilize glucose. Members of the family have been isolated from temperate and Antarctic saline lakes, solar salt facilities, saline soils and marine environments (Franzmann et al.,1988).

Family of Halomonadaceae includes *Aidingimonas, Carnimonas, Chromohalobacter, Cobetia, Halomonas, Halotalea, Kushneria, Modicisalibacter, Salinicol* and *Zymobacter* genera and 76 species, mostly halophilic bacteria. (Argandoña et al.,2012).

Bacteria which are in this family act like most halophilic bacteria at hypersaline environments. In order to adjust to increased external NaCl, cells accumulate a variety of small molecules in the cytoplasm to counteract the external osmotic pressure (Roberts et al.,2005). Polyols and derivatives, sugars and derivatives,amino acids and derivatives, betaines, and ectoines and occasionally peptides are organic molecules for osmotic balance and to remove charges (Galinski,1995). There were some studies for production of ectoines from bacteria of halomonadaceae family (Pastor et al.,2010 and Cánovas et al.,1998).

Some species such as *Halomonas elongata* and *Chromohalobacter salexigens* have been extensively used in recent years for the study of molecular osmoadaptation mechanisms in halophilic bacteria (Ventosa et al.,2008)

Halomonas eurihalina is a member of this family and F2-7 strain of this bacterium produces large amounts of an anionic exopolysaccharide called polymer V2-7, with a number of promising applications in industry such as emulsifying agent (Martínez-Checa et al.,2007).

One of the members of this family is *Halomonas maura* which produces an exopolysaccharide mauran (Arco et al.,2005). Mauran is a biodegradable and biocompetible polymer which is used recently in biomedical applications like drug delivery and tissue engineering. Mauran based biocompatible nanofibers were produced by *Halomonas maura*. This nanofiber enhance cell growth, migration, proliferation and differentiation of mammalian cells (Raveendran et al.,2013).

Halomonas smyrnensis AAD6 is also member of this group and it produces an extracellular biopolymer levan (Poli et al., 2009; Poli et al., 2013) with distinguished applications like cyto-toxicity agent, emulsifier and stabilizer.

1.2.2.1 Halomonas

The genus Halomonas belonging to the family Halomonadaceae within the class of Gammaproteobacteria, comprised 89 species at time of writing (http://www.bacterio.net/halomonas.html). Halomonas bacteria strains are halophiles, inhabit salt lakes, marine environments, saltern areas, as well as saline sand and soils.They require high sodium chloride concentrations for growth.

Members of this genus vary from moderate halophiles such as Halomonas smyrnensis to extreme halophiles such as Halobacterium salinarum. This genus has been considered as a model system among the moderate halophiles and has been used extensively for studies concerning both osmoregulatory mechanisms and physiological adaptations (Ventosa et al., 1998; Nieto et al., 2000). On the other hand, the genus Halomonas is a taxon comprising strains that are metabolically versatile: some Halomonas species have been described as denitrifying halophilic bacteria useful in the reduction of nitrate during the treatment of saline waste (Berendes et al., 1996; Mormile et al., 1999; Romano et al., 1996; Dobson & Franzmann, 1996). They are highly versatile in terms of their ability to successfully grow in a variety of temperature and pH conditions. This versatility may eventually lead to Halomonas species being used as a substitute for the utilization of starch-derived raw materials (Quillaguamán et al.,2005).

In addition, there are also a few reports on Halomonas strains able to degrade aromatic pollutants (Maltseva et al., 1996; Muñoz et al., 2001).

Phenol degradation has been also observed during the growth of several Halomonas strains on phenol witha using the ortho pathway (Alva and Peyton, 2003; Hinteregger and Schreiber, 1997; Maskow and Kleinsteuber, 2004)

1.2.2.2 *Halomonas smyrnensis* AAD6

H. smyrnensis AAD6 is a novel halophilic isolate from soil samples taken from Camaltı Saltern Area, in Sasalı, Izmir, Turkey . Halomonas sp. AAD6 (JCM 15723) strain, was found to produce high levels of exopolysaccharides (EPS), in the presence of sucrose in defined media.

H. smyrnensis AAD6 was placed in genus *Halomonas* based on the sequence (GenBank ID DQ131909) of 16S rRNA gene (Poli et al., 2012). This microorganism was described as a Gram-negative, non-motile, rod-shaped, oxidase negative and catalase positive bacteria forming cream-yellowish-coloured, circular and slightly irregular colonies. The optimum growth conditions were reported as 10% (w/v) NaCl concentration, pH 7.0, and temperature of 37°C. *H. smyrnensis* AAD6^T can utilize sucrose, glucose, fructose, maltose, galactose and mannose as sole carbon sources. Despite, these carbon sources can be used for biomass production, sucrose is a must for levan polymer production (Poli et al., 2009).

The bacterium is known to produce high levels of levan, which is a fructose homopolymer with many potential uses in foods, feeds, cosmetics, pharmaceutical and chemical industries due to its outstanding properties like high solubility in oil and water, strong adhesivity, good biocompatibility and film-forming ability.

The draft genome was analyzed phylogenetically and revealed a rather close relationship to other species of the genus *Halomonas*, where *Halomonas salina* (99.5%) and *Halomonas halophila* (99.5%) were found to be the closest species on the basis of 16S rRNA sequence (GenBank accession no. DQ131909.2) comparison. However, whole-genome comparison with genome sequences indicated that the closest similarity *to Chromohalobacter salexigens* DSM 3043 (score, 524), followed by *Halomonas elongata* DSM 2581 (score, 346) which was available at the RAST server (Sogutcu et al., 2012).

There are different studies carried out to investigate characteristic behaviors and associated potential biotechnological applications of levan synthesized by *H. smyrnensis* AAD6^T. First, optimization studies were done for improving production yields and decreasing the production costs.

Table 1.1 : Characteristics of *H. smyrnensis* AAD6 strain (Poli et al., 2013)

Characteristics	*Halomonas smyrnensis* AAD6
Motility	-
EPS Production	+
Colony pigmentation	Dark yellow
NaCl range (%, w/v)	3-25
NaCl optimum (%, w/v)	10
Temperature range (^oC)	5.0-40
pH range	5.5-8.5

Substrate causes about half of price of the fermentation broth. Substrates like sugar beet molasses and starch molasses as substitutes for sucrose was used as carbon source. Almost ten-fold increase of levan yields from *H. smyrnensis* AAD6 cultures and low cost production was obtained at the same time (Kucukasik et al., 2011).

In addition to experimental studies, there were computational based studies carried on *H. smyrnensis* AAD6^T. Sogutcu and co-workers (2012) reported the draft genome sequence of *H. smyrnensis* AAD6^T, which accelerated research on the rational design and optimization of microbial levan production. Bioinformatic based metabolic network reconstruction of construction of *H. smyrnensis* AAD6^T were also carried out by Ozer (2013) and this model accounts for 429 reactions, and 383 metabolites. Also, it offers strategies for enhanced levan biosynthesis.Another metabolic network studies were carried out according to the genome-scale metabolic network of *Chromohalobacter salexigens* DSM3043 (Ates et al., 2011), as a model organism for halophilic bacteria and then, it was refined via integration of the available biochemical, physiological, and phenotypic features of *H. smyrnensis* AAD6^{T} (Ates et al., 2013). As a result of this study, it was found that mannitol has a stimulatory effect on levan biosynthesis, and it was verified experimentally via supplementation of mannitol to the fermentation medium. The optimal concentration was determined as 30 g/L mannitol supplemented to the 50 g/L sucrose-based medium with result of a two-fold increase in levan production (Ates et al., 2013).

1.2.3 Exopolysaccharides (EPSs)

Polysaccharides that are classified according with their location relative to the cell. Those that are excreted outside the cell wall are called exocellular polysaccharides or EPSs. These can form an adherent cohesive layer and are called capsular polysaccharides (Ruas-Madiedo,2002). The EPSs also can either be loosely attached or be completely excreted into the environment as slime (Cerning, 1994). The bacterial EPSs are not used as energy sources by the producer microorganism. They probably have a protective function in the natural environment, e.g. against desiccation, phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds and osmotic stress. EPSs also have a role in cell recognition, in adhesion to surfaces and in formation of biofilms facilitating the colonisation of various ecosystems (Looijesteijn, Trapet, de Vries, Abee, & Hugenholtz, 2001; Whitfield & Valvano, 1993).

Bacterial polysaccharides synthesized and secreted into the external environment or are synthesized extracellularly by cell all-anchored enzymes referred to as EPS (Jaiswall et al.,2014). Polysaccharides can be divided into two groups: homopolysaccharides composed of one monosaccharide moiety such as dextran (Barker & Ajongwen, 1991; Funane et al., 1995; Monchois, Remaud-Simeon, Monsan, & Willemot, 1998) or levan (van Geel-Schutten et al., 1999), and heteropolysaccharides composed of different sugar moieties, e.g. glucose, galactose, rhamnose, mannose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid (Cerning, Bouillanne, Landon, & Desmazeaud, 1990; Faber, Zoon, Kamerling, & Vliegenthart, 1998; Grobben et al., 1997; Marshall, Cowie, & Moreton, 1995; Stingele, Neeser, & Mollet, 1996).

The composition and structure of the polysaccharides determines their primary conformation. Further, ordered secondary configuration frequently takes the form of aggregated helices. In some of these polymers, the backbone composition of sequences of 1,4-β- or 1,3-β-linkages may confer considerable rigidity, as is seen in the cellulosic backbone of xanthan from Xanthomonas campestris. Other linkages in polysaccharides may yield more flexible structures. These can be exemplified by the 1,2-α- or 1,6-α-linkages found in many dextrans. The transition in solution from random coil to ordered helical aggregates is often greatly influenced by the presence or absence of acyl substituents such as O-acetyl or O-succinyl esters or pyruvate ketals (Sutherland, 1997).

EPSs, produced by both prokaryotes (eubacteria and archaebacteria) and eukaryotes (phytoplankton, fungi, and algae), have been of topical research interest. Newer approaches are carried out today to replace the traditionally used plant gums by their bacterial counterparts. The bacterial exopolysaccharides represent a wide range of chemical structures, but have not yet acquired appreciable significance. Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30 kDa) and have heteropolymeric composition. They have new-fangled applications due to the unique properties they possess. Owing to this, exopolysaccharides have found multifunctional applications in the food, pharmaceutical and other industries (Suresh Kumar et al.,2007).

EPS's which synthesized by lactic acid bacteria (LAB) may act both as texturizers and stabilizers, firstly increasing the viscosity of a final product, and secondly by binding hydration water and interacting with other milk constituents, such as proteins and micelles, to strengthen the rigidity of the casein network. As a consequence EPS can decrease syneresis and improve product stability. Furthermore it has been reported that EPS can positively affect gut health (Duboc et al.,2001).

In addition, health benefits have been attributed to some of these EPSs, particularly antitumor and immunomodulating activities (Ruas-Madiedo et al.,2002). EPSs have many other novel properties to offer, and the discovery of immune modulation and tumouristasis by β-d-glucans, and the use of bacterial cellulose in audio membranes and of hyaluronic acid in cosmetics provide some novel applications (Sutherland, 1998)

Bacterial EPSs have been extensively used in high-value applications, such as food, pharmaceutical, medical and cosmetic products or processes, wherein they are mostly used as thickening, stabilizing, binding and structure creation agents as a result of their non-Newtonian behavior and high viscosity in aqueous media.When applied in food products, they must be able to maintain their properties when incorporated into formulations, in which they might experience significant variability of pH and ionic strength, along with the influence of other food components (Freitas et al.,2011).

EPSs are also used in controlled release of drugs studies. Recently, research has turned to the creation of novel structures (micro/nanospheres, polymer beads and capsules) with edible safe materials, wherein bioactive compounds (e.g. antioxidants, vitamins, probiotics or prebiotics) are encapsulated (Aguleria, 2007). Encapsulation techniques are designed to protect the bioactive substances and to promote their controlled release.

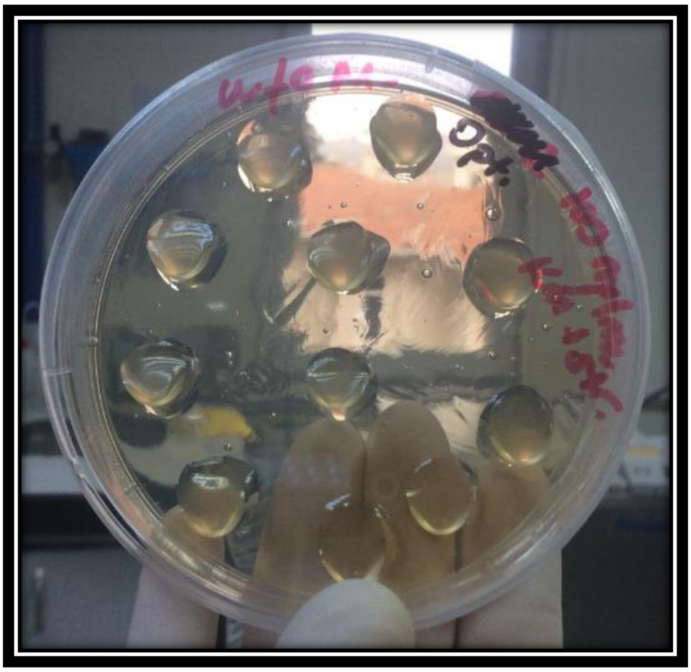


Figure 1.1: Mucoid colony formation of *H. smyrnensis* AAD6 since it produces EPSs

Some polysaccharides (e.g. bacterial alginate, gellan FucoPol or GalactoPol) possess the capacity to establish physical and/or chemical intermolecular interactions, which result in a cohesive polymeric matrix that is able to form a film. Recent research has focused on developing polymeric matrices with tuned properties (e.g. transparency, barrier and mechanical properties, biocompatibility or bioactivity) for several applications, namely edible coatings for food products (Oms-Oliu et al., 2008; Matsumoto et al., 2010) and packaging purposes (Alves et al., 2011 and Nguyen et al.,2008).

Environmental applications of the EPS compounds have been focused so far on the degradation process of organic substances, the denitrification of wastes, phosphate ion elimination from manufacturing and municipal wastes. The studies of Skłodkowska and Matlakowska (1998) showed that the extracellular substances also bind heavy metals from different environments.

The usage of EPS compounds in the food industry have been intensively investigated. The novel properties of microbial exopolysaccharides such as xanthan, curdlan, pulluan and alginate, may improve food viscosity, hydration of products and low calories food production. It is also considered to apply the microbial extracellular polysaccharides for food edible coating production that effectively would protect products from spoilage (Becker et al., 1998; Banik et al., 2000)

A better understanding of the structure-function relationship of EPS remains a challenge to further improve applications of EPS to better satisfy the consumer demand for almost all industrial products.

1.2.3.1 Levan

The homopolysaccharide levan is synthesized by levan-sucrase (EC 2.4.1.10). Levan whose synthesis and polymerization occur outside the cells by the action of secreted enzymes that convert the substrate into the polymer in the extracellular environment (Rehm, 2009).

Levan is composed of β-(2-6) linked D-fructofuranosyl units as 6-kestose of the basic trisaccharide, with extensive branching through β-(2-1) linkages (Kang et al., 2009). Levan polymer is a distributed component in plants, yeasts, fungi and bacteria.

Recently, the interest in the production of microbial levan for commercial use has been intensified, because it offers a variety of industrial applications in the fields of cosmetics, foods and pharmaceuticals; it can be used as industrial gums, blood plasma extender, sweeteners, hypocholesterolemic agent and antitumor agent (Leibovici et al., 1984; Yamamoto et al., 2000). Potential applications of levan have also been proposed as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent, and carrier for flavour and fragrances (Han, 1990; Jang et al., 2001); in addition, it is biodegradable and can be an ideal raw material for the production of green plastics (Wu et al., 2013).

H. smyrnensis AAD6, was found to excrete high levels of levan EPS which is a long linear homopolymer of ß(2-6)-linked fructose residues (Poli et al., 2009) and has many potential uses in foods, feeds, cosmetics, pharmaceutical and chemical industries. Recently, research on the potential uses of levan produced by Halomonas sp. as a bioflocculating agent (Sam et al., 2011), its nanostructured thin films (Sima et al., 2011), its suitability for peptide and protein based drug nanocarrier systems (Sezer et al., 2011) and an economical production scheme via cheap carbon resources (Küçükaşık et al., 2011) were reported. Moreover, via a systems-based approach, the first comprehensive genome-scale metabolic model of C. salexigens DSM 3043 was reported which in turn formed the basis for reconstruction of a geneic in silico metabolic network of levan-producing *Halomonas* sp. (Ates et al., 2011).

Due to its exceptionally high costs of production and purification processes, levan are not used in the current polymer market as much as foreseen. To reduce levan production cost, it is important to optimize the fermentation conditions, or engineering of new strains (e.g. by mutagenesis or genetic manipulation) via system biology results related to metabolic pathways, and to develop novel downstream processes resulting with levan production rate and yields.

1.2.3.2 Microbial EPS Biosynthesis

Most bacterial EPSs are synthesized intracellularly and exported to the extracellular environment as macromolecules (Rehm, 2009; Ullrich, 2009). There are a few known exceptions (e.g. levans and dextrans) whose synthesis and polymerization occur outside the cells by the action of secreted enzymes that convert the substrate into the polymer in the extracellular environment (Rehm, 2009).

The EPS synthesized by microbial cells vary greatly in their composition and hence in their chemical and physical properties. Some are neutral macromolecules, but the majority are polyanionic due to the presence of either uronic acids (D-glucuronic acid being the commonest, although D-galacturonic and D-mannuronic acids are also found) or ketal-linked pyruvate (Sutherland, 2009).

The synthesis of extracellular biopolymers by microbial cells depends on the carbon and nitrogen availability in the culture medium. Most exopolymer-producing microorganisms utilize carbohydrates as their carbon and energy source and either ammonium salts and amino acids as their source of nitrogen (Gandhi et al., 1997; CzaCzyK et al., 2003).

The biosynthesis of EPSs occurs in different growth phases, depends on environmental conditions and the organism used for the production (Cerning, 1995). However, very little is known on biosynthesis of EPSs and extensive study is required to understand the mechanism involved. The EPSs are synthesized by extracellular enzymes which are secreted from the bacteria or located on the cell surface whereas the synthesis of EPSs is complex (Welman and Maddox, 2003). They are produced by utilizing precursors formed intracellularly at the cytoplasmic membrane.

The synthesis of EPSs involves a larger number of genes coding for enzymes and regulatory proteins which are not unique to EPS production and secretion. Nucleotide diphosphates serve as precursors for EPS biosynthesis which are intermediates of the central carbon metabolism that starts from the transport of the sugar from surrounding medium (Boels et al., 2001).

In Gram negative bacteria, undecaprenyl phosphate is a lipid carrier for EPS assembly. The assembly of basic repeating unit occurs at the cytoplasmic membrane and involves sequential transfer of sugar nucleotide diphospho precursors to an isoprenoid lipid carrier, undecaprenyl phosphate (Sutherland, 2001). Once the basic repeating unit is assembled, the lipid-linked intermediates are usually translocated across the membrane and polymerized outside of the cell. Then, the EPS may be covalently linked to the cell surface to form a capsule, or released into the medium as slime. The undecaprenyl phosphate plays a significant role in the EPS biosynthesis in Gram positive bacteria also. The biosynthetic pathway is divided into four separate reaction sequences. These are the sugar transport into the cytoplasm, the synthesis of sugar-1- phosphates, activation of and coupling of sugars, and the processes involved in the export of the EPS (De Vos et al., 1999).

The energy used for the elongation of the levan chains is provided by the hydrolysis of sucrose. By contrast, synthesis of heteropolysaccharides involves several biosynthetic steps and it is linked to the central carbon metabolism of the producing cell (de Vos, 1996; De Vuyst & Degeest, 1999; Hutkins & Morris, 1987; Ramos, Boels, de Vos, & Santos, 2001).

1.2.4 Fructose Uptake System in Halomonas smyrnensis AAD6

The monosaccharides and disaccharides are the principal carbon sources of microorganisms. The movement of these carbon sources into the cytoplasm from surrounding medium is tightly regulated by a number of different control proteins. The transport of sugar molecules from the surrounding medium into the cytoplasm is basically carried out by three different transport systems. In the primary transport systems, ATP hydrolysis is directly coupled to sugar translocation via sugar transport ATPase. In secondary transport systems, the import is coupled to transport of ions and other solutes. Phosphoenolpyruvate (PEP)-sugar phosphotransferase system (PTS) is the third and most important sugar transport machinery in microorganisms. The PEP–PTS system contains a group of proteins that are responsible for binding, transmembrane transport, and phosphorylation of a variety of sugar substrates. In this system, a phosphate group is released from the conversion of PEP into pyruvate catalyzed by pyruvate kinase (Madhuri et al., 2014).

Components of PTS^{Fru}, which includes the membrane-bound permease FruA, consisting of a fusion of $EIIB^{Fru}$ and $EIIC^{Fru}$ domains. It is generally believed that P-EIIB phosphorylates the carbohydrate bound to EIIC. FruA thus transports extracellular fructose through a phosphorylation-dependent process to yield fructose 1-P (F1P), which is then channeled towards the central metabolism following the action of FruK to yield fructose 1,6-bisphosphate (FBP). The second component of the PTS^{Fru} is FruB, a fusion of $EIIA^{Fru}$, HPr, and EI modules into a single polypeptide, the last domain of which is responsible for conveying high-energy phosphates from phosphoenolpyruvate (PEP) into the system.

1.2.5 Industrial Systems Biology (Systems Biology of Industrial Microorganisms)

Systems biology relies on a multidisciplinary approach to integrate data from various disciplines of biology (Friboulet and Thomas, 2005) bringing together molecular disciplines (e.g., genetics, biochemistry, molecular biology) with those involving more complex systems (e.g., cell biology, microbiology, plant or human physiology). The aim of systems biology is to link the quantitative data in a mathematically defined sense across the different scales of biological organization (from DNA, RNA, protein to cell, tissue, organs). Mathematical modelling is used to drive integration with an aim of reaching a unified understanding of biological systems (You, 2004).

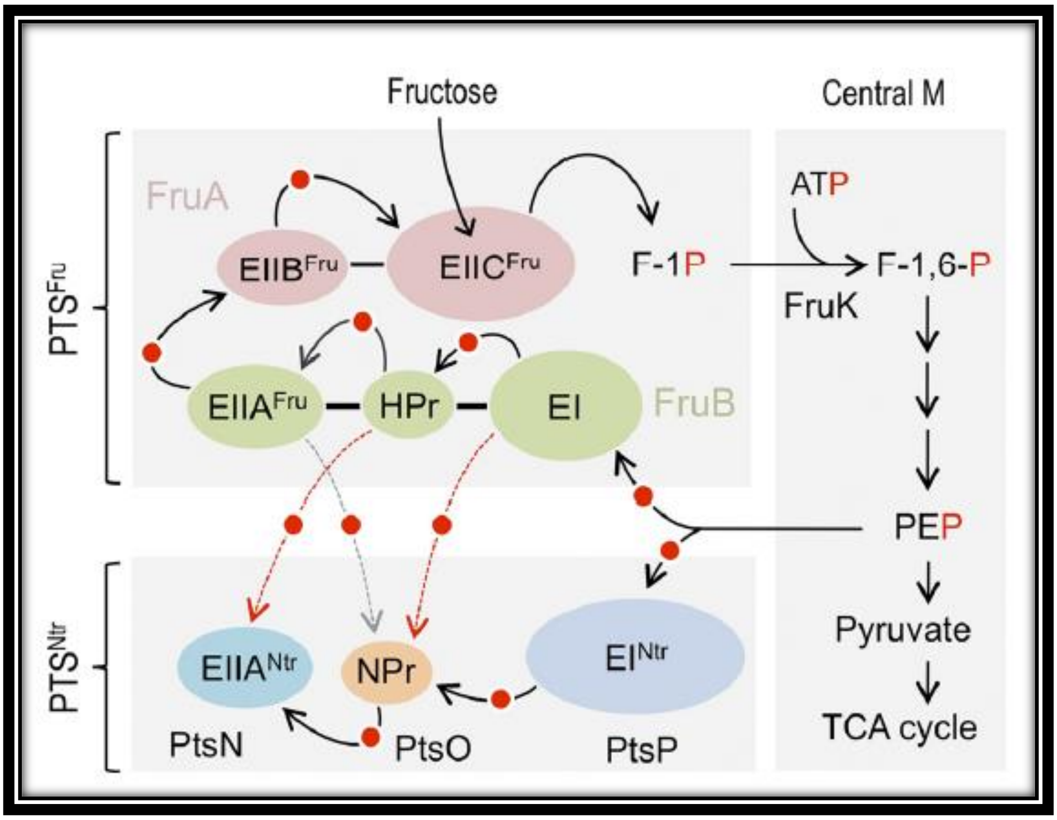
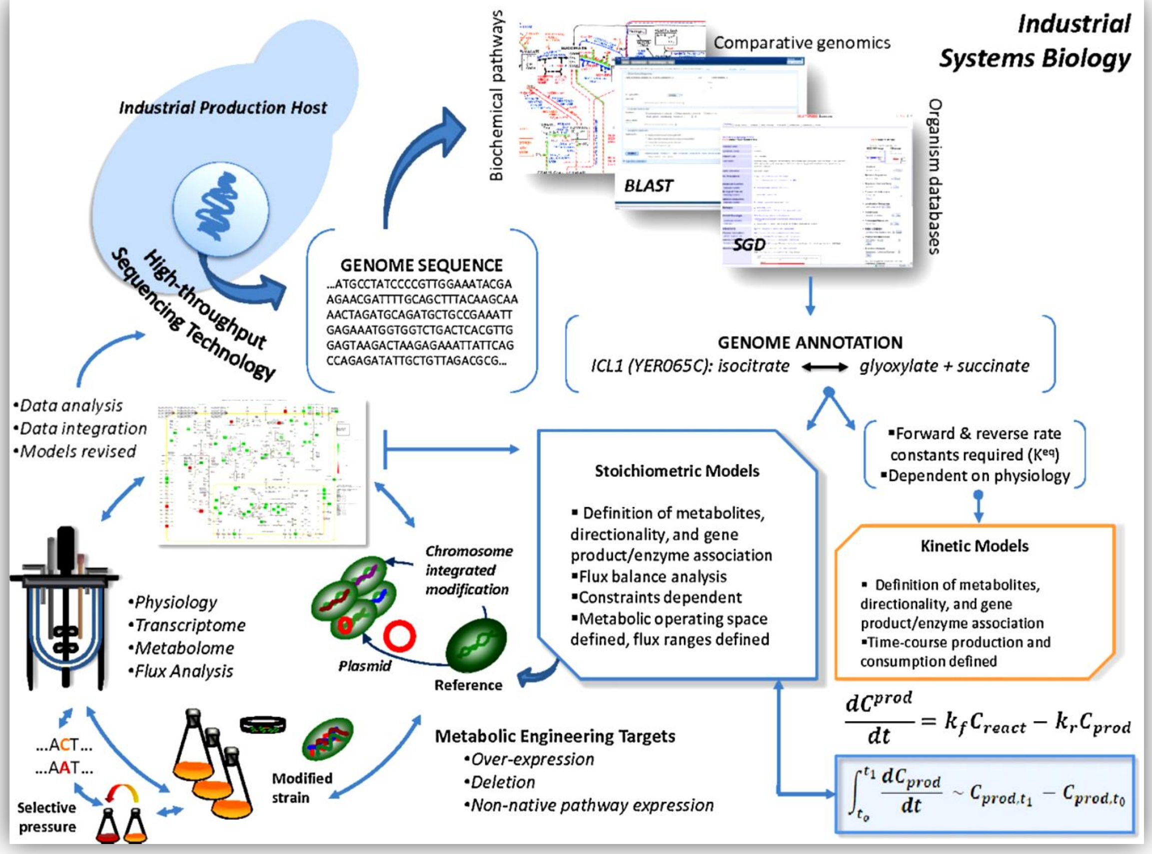


Figure 1.2: Organization of fructose-specific phosphotransferase system ($\mathrm{PTS}^{\mathrm{fru}}$)(Chavarria et al., 2012)

The field of industrial biotechnology is expanding rapidly as the chemical industry is looking towards more sustainable production of chemicals that can be used as fuels or building blocks for production of solvents and materials. In connection with the development of sustainable bioprocesses, it is a major challenge to design and develop efficient cell factories that can ensure cost efficient conversion of the raw material into the chemical of interest (Papini et al., 2010). This is achieved through metabolic engineering, where the metabolism of the cell factory is engineered such that there is an efficient conversion of sugars, the typical raw materials in the fermentation industry, into the desired product. However, engineering of cellular metabolism is often challenging due to the complex regulation that has evolved in connection with adaptation of the different microorganisms to their ecological niches. In order to map these regulatory structures and further de-regulate them, as well as identify ingenious metabolic engineering strategies that full-fill mass balance constraints, tools from systems biology can be applied. This involves both high-throughput analysis tools like transcriptome, proteome and metabolome analysis, as well as the use of mathematical modeling to simulate the phenotypes resulting from the different metabolic engineering strategies.



$$\frac{dC^{prod}}{dt} = k_f C_{react} - k_r C_{prod}$$

$$\boxed{\int_{t_o}^{t_1} \frac{dC_{prod}}{dt} \sim C_{prod,t_1} - C_{prod,t_0}}$$

Figure 1.3 : Industrial systems biology (Otero and Nielsen, 2009)

It is in fact expected that systems biology may substantially improve the process of cell factory development, and we therefore propose the term Industrial Systems Biology for how systems biology will enhance the development of industrial biotechnology for sustainable chemical production.

The goal of systems biology therefore is to define the structure, dynamics and control of biological systems. The expected impact from systems biology focuses on an improved integrative understanding of biological systems (Kitano, 2001), to subsequently allow for better control and prediction of the behaviour of existing biological systems (e.g., improved human disease treatments; (Westerhoff and Palsson, 2004) and then to mediate the design of new biological systems with desired properties (Dixon, 2005; Drubin et al., 2007 and Sweetlove et al., 2003)

Systems biology accelerates the classic metabolic engineering cycle and thereby aids industrial biotechnology. If there is a good collaboration between systems biology and industrial biotechnology, it will be resulted in unique studies on substrate utilization and the production of organic acids, enzymes and natural products by desired organism.

1.2.6 Metabolic Engineering

Metabolic engineering, which is the introduction of rational changes in the genetic makeup of an organism to alter the metabolic profile or improve biosynthetic capabilities (Nielsen et al.,2001), has gained increasing interest as a way to develop high titer bioprocesses and produce new “non-natural” natural products. Advances in molecular biology techniques and knowledge of metabolic pathways have enabled unprecedented amount of control over these complex biosynthetic processes. The dramatic decreases in the cost of sequencing technology has resulted in an exponential amount of genomic data available and led to the identification of many genes involved in natural product pathways. Genome-scale metabolic reconstructions have been developed for many important organisms and can be used to guide metabolic engineering by identifying distal targets for genetic modification and avoiding unintended consequences which may not be clear by local pathway analysis alone (Oberhardt et al., 2009).

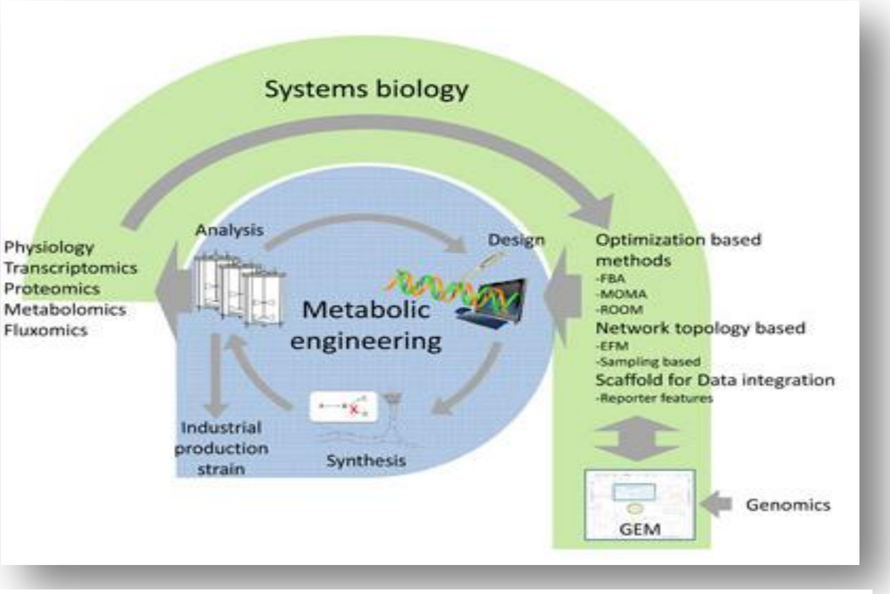


Figure 1.4 : Collaboration of metabolic engineering and systems biology (Knuf and Nielsen, 2012)

Metabolic engineering has developed as a very powerful approach to optimising industrial fermentation processes through the introduction of directed genetic changes using recombinant DNA technology. Successful metabolic engineering starts with a careful analysis of cellular function; based on the results of this analysis, an improved strain is designed and subsequently constructed by genetic engineering. In recent years some very powerful tools have been developed, both for analysing cellular function and for introducing directed genetic changes(Nielsen et al., 2001).

Metabolic engineering involves the use of recombinant DNA technology to selectively alter cell metabolism and improve a targeted cellular function (Bailey et al., 1990). Traditionally, metabolic engineering has been performed on a small scale through manipulation of a few genes to affect yield of a target metabolite. Enzymatic targets are chosen through analysis of literature-derived central metabolic pathway maps, or intuitive engineering based on local metabolic knowledge.

The main goal in this area to be able to link gene functions and chemical EPS structure to physico-chemical behavior. Approaches for the reduction of production costs might involve using cheaper substrates, improving product yield by optimizing fermentation conditions, or developing higher yielding strains (e.g. by mutagenesis or genetic manipulation), and optimizing downstream processing (Rehm, 2009 and Yang, 2011). It is crucial to lower their production costs.

Traditional strategies to improve the fermentative production of bacterial EPS include improved strain selection and optimization of cultivation conditions. However, these strategies allow only marginal bioprocess improvement due to the physiological limits for each given bacterium(Freitas et al., 2001). The most interesting prospects for increasing bacterial EPS production are through metabolic engineering either by manipulation of the genes that encode the enzymes that catalyze the reactions in the pathways, or by altering the regulatory pathways that affect gene expression and enzyme activity (Yang,2007)

1.2.7 Genetic Manipulations via Special vectors

Traditionally, improvement of industrially important cell factories has been done using classical methods, that is, random mutagenesis and screening; however, advances in methods for performing directed genetic modifications has enabled the use of metabolic engineering strategies. Previously, the whole genome sequence analysis of H. smyrnensis AAD6 was performed using two different next-generation sequencing technologies (Sogutcu et al., 2012). Genome sequencing of *H. smyrnensis* AAD6 was originally trailing behind developments in the field of metabolically improvement of bacteria, it has become possible to implement systems biology tools to advance metabolic engineering. It was also supported by a research thesis in our group. Ozer (2013) constructed a genome-scale metabolic models of *H. smyrnensis* AAD6. As a cocnclusion of this study, optimal strategy was the knock-out of the gene associated with the PTS system of fructose uptake seems to be the most possible strategy for levan overproduction. According to this results, it was seem that if some modifications were performed for this PTS system, levan production will have affected in desired way. Therefore, a strategy was constituted for modification of this system with using genetic engineering techniques.

This strategy mainly aimed to modification of one of the PTS system component (HPr region) via transposon mutagenesis. So, strategy was composed of three main stages based on the main objective . First one was the construction of recombinant plasmids that were carrying target region, second one included an introduction of mutagenesis agent to target region into another constructed recombinant plasmid. Genetic transfer of this final construction via conjugation into recipient *H. smyrnensis* AAD6 was the third stage. These constructions was carried out via special vectors such as clonning vector, transposon mutagenesis vector, suicide plasmid vector and helper plasmid vector that will be explained below.

1.2.7.1 pJET 1.2/ blunt vector

pJET1.2/blunt is a novel positive selection cloning vector.This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate, eliminating the need for expensive blue/white screening. It was a component of CloneJET PCR Cloning Kit (Thermo Scientific, USA). Vector map of pJET1.2/blunt is showed in Figure 1.5.

The pJET1.2/blunt cloning vector has been linearized with Eco32I (EcoRV) and this linearized cloning vector accepts inserts from 6 bp to 10 kb. The 5'-ends of the vector contain phosphoryl groups, therefore, phosphorylation of the PCR primers is not required. Blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated with the vector in just 5 min. PCR products with 3’-dA overhangs generated using Taq DNA polymerase or other nonproofreading thermostable DNA polymerase are blunted in with a proprietary thermostable DNA blunting enzyme (included in the CloneJET kit)

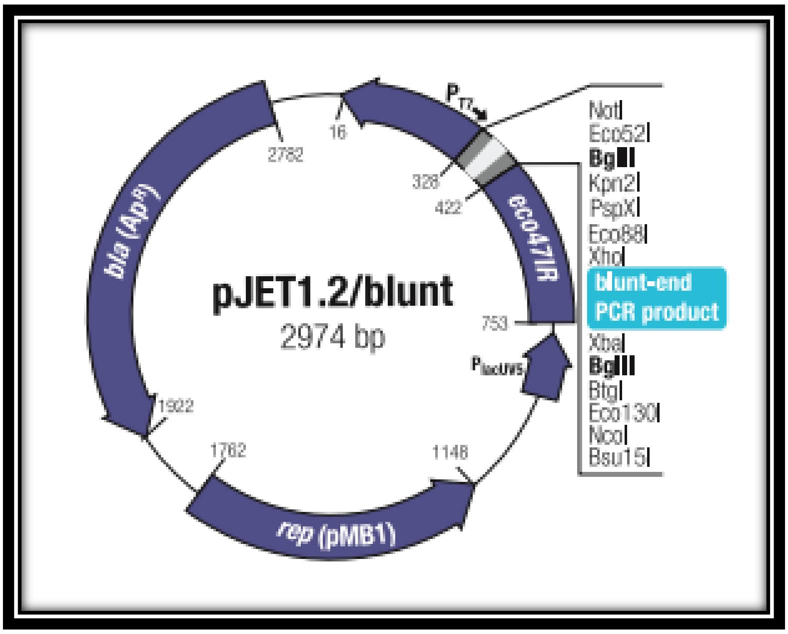


Figure 1.5: pJET1.2/ blunt vector map (Thermo Scientific)

prior to ligation. All common laboratory E.coli strains can be directly transformed with the pJET1.2/blunt vector. Amplified DNA fragment of target gene region HPr approximately 567 bp that was cloned into pJET1.2/blunt vector.

1.2.7.2 pHP45-omega/ Kanamycin (Km)

Ideally, a DNA fragment to be used for insertional mutagenesis should have three properties: (i) be selectable; (ii) abolish the expression of the genetic unit into which it has been inserted; and (iii) be easy to localize. It was reported that (Prentki and Krish, 1984) the construction and the use of a plasmid, called pHP45-omega which carries a DNA fragment ("Ω") incorporating these three properties.

The most important aspect of Ω mutagenesis is the selectable introduction of translational and transcriptional stop signals. This allows the definition of both translational and transcriptional units within cloned DNA. Ω can also be used when inactivation of RNA or protein synthesis downstream from a given location, such as a restriction site, is desired.

A series of derivatives of the Ω interposon have been constructed. These can be used for in vitro insertional mutagenesis. Each of these DNA fragments carries a different antibiotic or Hg2+ resistance gene (ApR, CmR, TcR, KmR or HgR) which is flanked, in inverted orientation, by transcription and translation termination signals and by synthetic polylinkers. The DNA of these interposons can be easily purified and then inserted, by in vitro ligation, into a plasmid linearized either at random by DNase I or at specific sites by restriction enzymes. Plasmid molecules which contain an interposon insertion can be identified by expression of its drug resistance (Fellay et al., 1987). In the scope of this thesis, Ω interposon with Km resistance was selected as mutagenesis agent, it will be cloned into resultant HPr carrying recombinant plasmid. Vector map of pHP45-omega/ Km was given in Figure 1.6.

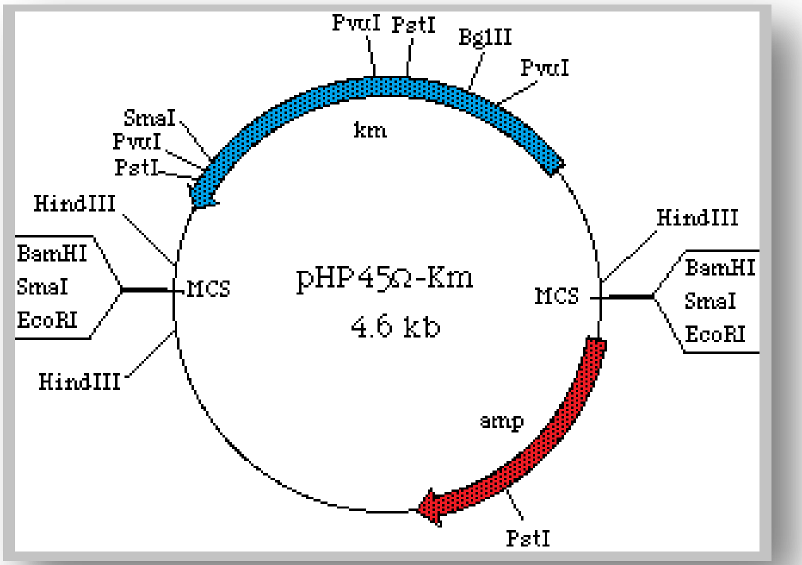


Figure 1.6: Vector map of pHP45-omega/ Km plasmid

1.2.7.3 pUK4134 suicide vector

One of the most common strategies to generate knockout mutants in Gram-negative bacteria are suicide vectors for allelic exchange, such as those carrying the replication origin of R6K that replicate only in strains producing the π protein from λ phage (Miller and Mekalanos, 1988). Maintenance of R6K derivatives under selective pressure requires plasmid integration by homologous recombination. These vectors can be used to carry out allelic exchange of wild-type genes by plasmid-encoded disrupted alleles. This is a two-step procedure with plasmid integration within the target gene by a recombination event, followed by its excision via a second crossover, which renders allelic exchange. Integration of the plasmids into the chromosome can be selected by means of the antibiotic marker used to disrupt the target gene (first recombination event). Excision of the integrated plasmid that results in allelic exchange (second recombination event), may be either selected using counter-selectable markers (Donnenberg and Kaper, 1991; Selbitschka et al., 1993), or screened for on the basis of antibiotic resistance (Miller and Mekalanos, 1988). This method requires cloning and disruption of the target gene into the suicide vector prior to allelic exchange.

pUK4134 was constructed to enlarge the repertoire of vectors available for allelic exchange of mutated sequences in gram-negative bacteria. pUK4134 is a suicide vector, carrying the origin of replication of the plasmid R6K and thus requiring the product of the pir gene for its stable maintenance. The *rpsL* gene encoding Escherichia coli ribosomal protein S12 confers streptomycin sensitivity on streptomycin-resistant strains and provides a positive selection for bacteria that have lost the plasmid following allelic exchange. The bla gene provides for selection by ampicillin (Amp) resistance (Amp^R) . Other features are a unique Eco RV cloning site, *oriT* of plasmid RK2, and the bacteriophage λ cos sequence. This vector was successfully used several times to carry out allelic exchange in Gam negative bacteria. pUK4134 vector can be useful for a wide spectrum of Gram negative bacteria, the limiting features being rpsL and bla need to function (and of course that the host cannot be Amp resistant).

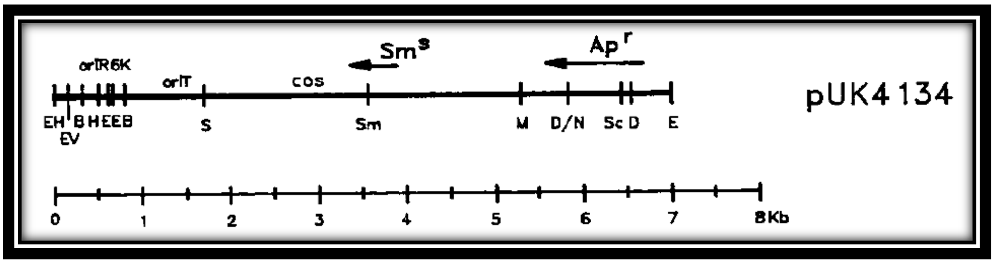


Figure 1.7 : pUK4134 vector map in linearized form (Skrzypek et al, 1993). Capital letters refer restriction enzymes. B, BamHI; D, DraI; E, EcoRI; Ev, EcoRV; H, HindIII; M, MscI; S, SalI; Sc, ScaI; Sm, SmaI.

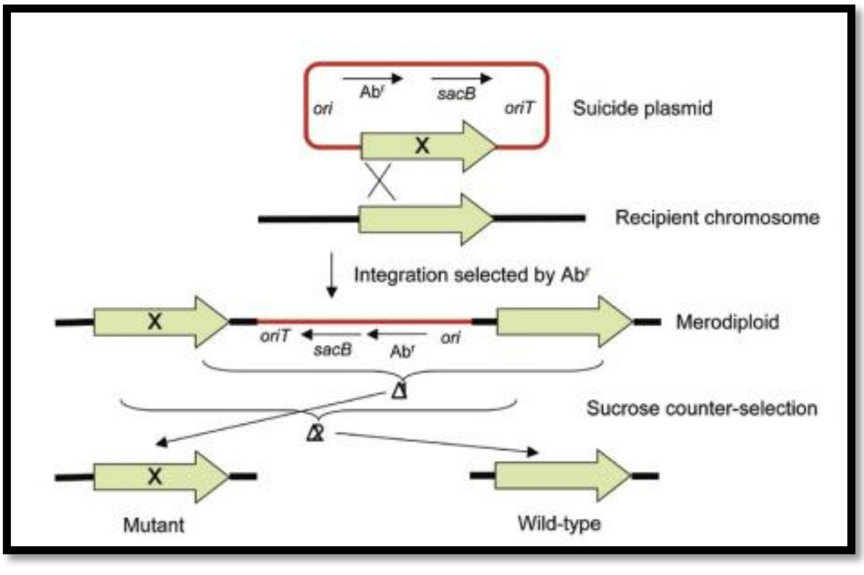


Figure 1.8: Strategy for allelic replacement using a suicide vector (Schwizer, 2008) For allele replacement, the indicated suicide plasmid containing an origin of conjugal transfer *(oriT*), an antibiotic resistance selection (*Ab*^{r}), a narrow-host-range of replication, which is suicidal in most, a counter-selection marker, and the mutated gene (the mutation is indicated with an X) is

1.2.7.4 pRK2013 helper plasmid

pRK2013 is a helper plasmid for mobilization of non-self-transmissible plasmids. Such plasmids are capable of conjugal self-transfer to a wide variety of Gram-negative bacteria (Phadnis and Dus, 1987; Knauf and Nester, 1984). This unique property has been used as the basis for development of a plasmid cloning system in E. coli with widespread applicability.

Detection of a transposition event in a bacterial cell is possible after the elimination of the vehicle that is used to carry the transposon into the cell. To accomplish this, one of the possible approach is to use a plasmid vehicle which can be introduced into the cells by conjugation or transformation, but which is not replicated in the particular host. The lack of replication can be ascertained by the absence of the antibiotic resistance, the gene(s) for which is resident in the vehicle (Ditta et al., 1980).

This plasmid was derived from RK2 plasmid. Although native RK2 DNA can be used directly as a recombinant DNA cloning vector, its large size (56 kilobase (kb)) is a serious drawback to routine use. In order to reduce the size and still retain overall broad host range transfer capability, a cloning system has been devised that separates RK2 transfer and replication functions onto separate plasmids. The tetracycline-resistant plasmid component of this system, pRK290, contains a functional RK2 replicon and can be mobilized at high frequency by using a helper plasmid, but is non-self-transmissible. pRK290 contains single EcoRI and BglII sites where DNA can be inserted without loss of essential functions. The kanamycin-resistant helper plasmid, pRK2013, is a self-transmissible derivative of the naturally occurring plasmid RK2, but the replication origin has been replaced by that of *Col*El l replicon (Figurski and Helinski,1979). Its sole function in this system is to trans-complement the vector for mobilization. It contains *tra* gene which refers to regions containing genes required for conjugal transfer (Barth et al., 1978; 1979). This plasmid a way to introduce transposon into *H. smyrnensis* AAD6 cells and provides plasmid stability.

It will be used as a vehicle for transposition of omega region into the chromosome of *H. smyrnensis.*

It was thought that the plasmid pRK2013 will not be detected in *H. smyrnensis*, inspite of the successful mobilization. It was assumed that pRK 013 enters into *H. smyrnensis* by conjugation, but is unstable because of its narrow host range replication origin.

1.2.8 Genetic Transfer Mechanisms

Genetic manipulation of bacteria requires transfer of recombinant DNA molecules, either circular plasmids or linear DNA fragments, into the intended host(s). The four basic methods used for transfer of DNA molecules into bacteria are: (i) natural transformation; (ii) transformation using chemical competent cells; (iii) electroporation into electrocompetent cells; and (iv) conjugation (Schweizer, 2008).

Conjugation, transformation, and transduction have been used as available genetic transfer mechanisms for some *Halomonadaceae*. Within the scope of this thesis, conjugation and transformation methods will be explained.

1.2.8.1 Transformation

Transformation is the process by which foreign DNA is introduced into a cell. Transformation of bacteria with plasmids is important not only for studies in bacteria but also because bacteria are used as the means for both storing and replicating plasmids. Because of this, nearly all plasmids, even those designed for use in mammalian cells, carry both a bacterial origin of replication and an antibiotic resistance gene for use as a selectable marker in bacteria.

Transformation is the process by which an organism acquires exogenous DNA. Transformation can occur in two ways: natural transformation and artificial transformation. Natural transformation describes the uptake and incorporation of naked DNA from the cell’s natural environment. Artificial transformation encompasses a wide array of methods for inducing uptake of exogenous DNA. In cloning protocols, artificial transformation is used to introduce recombinant DNA into host bacteria (*E. coli*). The most common method of artificial transformation of bacteria involves use of divalent cations (e.g., calcium chloride) to increase the permeability of the bacterium’s membrane, making them chemically competent, thereby increasing the likelihood of DNA acquisition. Another artificial method of transformation is electroporation, in which cells are shocked with an electric current, to create holes in the bacterial membrane. With a newly-compromised cell membrane, the transforming DNA is free to pass into the cytosol of the bacterium. The technique is fast, simple, reproducible, frequently gives very high transformation frequency and appears to be applicable to a wide range of bacterial types previously thought untransformable (Chassy et al., 1988).

Regardless of which method of transformation is used, outgrowth of bacteria following transformation allows repair of the bacterial surface and selection of recombinant cells if the newly acquired DNA conveys antibiotic resistance to the transformed cells.

Transformation occurs when naked DNA is released on lysis of an organism and is taken up by another organism. The antibiotic-resistance gene can be integrated into the chromosome or plasmid of the recipient cell (Furuya and Lowy, 2006).

1.2.8.2 Conjugation

The transfer of DNA mediated by conjugal plasmids or conjugal transposons; requires cell to cell contact but can occur between distantly related bacteria or even bacteria and eukaryotic cells; can transfer long fragments of DNA.

Conjugation occurs by direct contact between two or more bacteria:plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of antibiotic-resistance genes by the recipient cell. Transposons are sequences of DNA that carry their own recombination enzymes that allow for transposition from one location to another; transposons can also carry antibiotic-resistance genes (Furuya and Lowy, 2006).

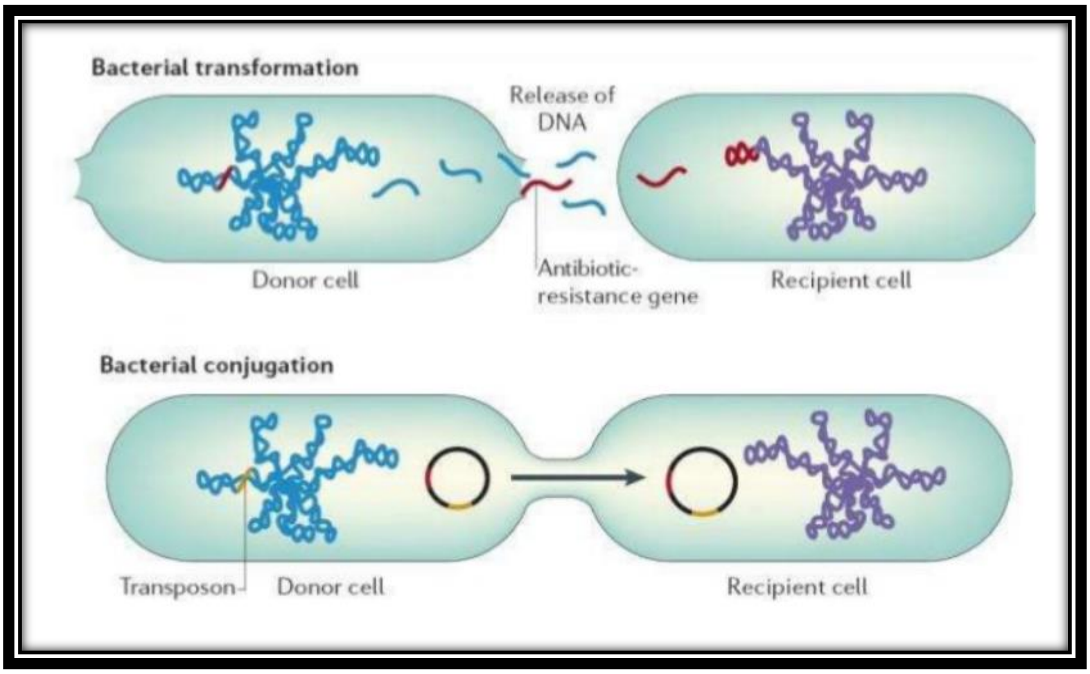


Figure 1.9: Bacterial Conjugation and Transformation (Furuya and Lowy,2006)

Conjugation has been successfully used to transfer both vectors derived from native plasmids (Vargas et al., 1995; Osman et al., 2010) , and broad-host range vectors (Jörg Kunte and Galinski, 1995; Vargas et al., 1997) from E. coli to *Chromohalobacter* and *Halomonas*. Several factors affecting the efficiency of conjugation (cell growth phase, mating time, donor–recipient ratio, and composition and salinity of the mating medium) were evaluated to optimize the conditions for conjugation between E. coli and moderate halophiles.

Triparental Mating :

Depending on how the transfer functions are supplied, biparental, triparental, or fourparental matings can be used for conjugal plasmid transfer. In biparental mating, specialized E. coli strains (De Lorenzo and Timmis,1994; Simon et al., 1989) supply the transfer functions in trans via a chromosomally integrated copy of RP4. In triparental mating, the transfer functions used for transfer of the mobilizable plasmid from a separate donor strain to a recipient strain are provided by a helper plasmid (Figurski and Helinski, 1979).

Triparental mating is a form of bacterial conjugation where a conjugative plasmid present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third bacterial strain. Plasmids are introduced into bacteria for such purposes as cloning or transposon mutagenesis. Triparental matings can help to cope with some of the barriers to efficient plasmid mobilization. For instance, if the conjugative plasmid and the mobilizable plasmid are members of the same incompatibility group they do not need to stably coexist in the second bacterial strain for the mobilizable plasmid to be transferred. A helper strain, carrying a conjugative plasmid (such as the F-plasmid) that codes for genes required for conjugation and DNA transfer. A donor strain, carrying a mobilizable plasmid that can utilize the transfer functions of the conjugative plasmid. A recipient strain, you wish to introduce the mobilizable plasmid into.

1.2.9 Selection Markers

Many bacteria are disobedient to genetic manipulation because of the lack of suitable selection markers, necessitating either identification of novel selection markers or use of markers that can be repeatedly used (“recycled”).

Selective Markers:

Once established in the respective host(s), many plasmids are stably maintained. However, selection markers are required for selection of plasmid-containing colonies after transformation or conjugation experiments and to ensure that each cell in a bacterial population contains a plasmid. Useful selection markers include, in order of decreasing importance, those conferring resistance to antibiotics, other antimicrobials, heavy metals, and metabolic markers.Numerous plasmids have been developed that serve as sources for various antibiotic resistance cassettes (Schweizer et al., 1993; Alexeyev, 1995; EcoRI and AccI, 1996; Dennis and Zylstra,1998; Poteete et al., 2006). The choice of antibiotic selection marker(s) depends largely on the susceptibility of the host, and intrinsic resistance to many antibiotics quite often hampers use of plasmids in bacteria. Intrinsic resistance or regulatory restrictions quite often leave only a few useful and approved antibiotic selection markers. In these instances, one can consider implementation of broad-host-range site-specific recombinase systems that allow recycling of the precious few markers remaining for some bacteria. Removal of antibiotic resistance markers is often also required during construction of genetically modified organisms in the food industry (Schweizer , 2008).

Non-selective Markers:

In contrast to selection markers, counter-selection markers serve to eliminate unwanted elements. These elements can be helper strains used during conjugation experiments or DNA segments introduced during genetic manipulation experiments, for example, as with transposon delivery.

Counter-selection against bacteria can be achieved by exploiting intrinsic properties present in one bacterium but not another. These include intrinsic resistance to antimicrobials or unique metabolic properties.

Several counter-selection methods can be applied to eliminate unwanted DNA sequences. Plasmids containing temperature-sensitive (Ts) replicons are maintained at permissive temperatures (usually 30°C) and eliminated at temperatures around 37°C (Le Borgne et al., 2008; Phillips et al., 1998; Weaver et al., 1998). In many instances, such Ts replicons are easily obtained by error-prone PCR (Maier et al., 2004; Choi et al., 2008). Other methods involve incorporation of sequences that encode enzymes whose expression is toxic in the presence of certain substrates. Some examples include the following: (i) sequences containing the sacB gene from Bacillus subtilis confer sucrose-sensitivity when expressed in E. coli and other Gram-negative bacteria. Selection of sucrose-resistant derivatives eliminates sacB-containing sequences (Hynes et al., 1989; Golovliov et al., 2003; Quénée et al., 2005); (ii) the wild-type *rpsL* gene confers streptomycin susceptibility in a *rpsL* mutant background. This marker can therefore also be used to counter-select against unwanted DNA sequences in any background in which streptomycin resistance can be selected (Stibitz, 1994; Skrzypek et al., 1993).

Choice of the counter-selection marker(s) depends largely on the physiology of the host bacterium. For example, the *sacB* and *rpsL*-based counter-selection strategies are not applicable in wild-type *Burkholderia spp*. because they contain endogenous sacB (Nierman et al., 2004) genes and are intrinsically streptomycin-resistant (Moore et al., 1999).

1.2.10 Mutagenesis

Mutagenesis and complementation analyses are powerful techniques in the characterization of operons, genes, and proteins. The creation of mutants is an essential step in determining gene function. In general, two main approaches are available to generate bacterial mutants: (1) in vivo random mutagenesis using chemical agents or transposons or (2) gene replacement of previously cloned DNA that has been manipulated in vitro by introducing insertions, deletions or base replacements. It was described below this kind of approach carried out in *Halomonadaceae*.

1.2.10.1 Transposon Mutagenesis

DNA transposons are discrete pieces of DNA with the ability to change their positions within the genome via a ‘‘cut and paste’’ mechanism called transposition. In nature, these elements exist as single units containing the transposase gene flanked by terminal inverted repeats (TIRs) that carry transposase binding sites (Ivics et al., 2011).

It allows generation of a large number of mutant plasmids with minimal effort. This is particularly useful for sequencing large DNAs that require more than one sequencing reaction to determine the entire sequence, and provides a faster alternative to primer walking, random subcloning, and nested deletion methods. It can also be used for mapping projects (Brune, 2002).

Transposition has also become a powerful tool in genetic analysis, with applications in creating insertional knockout mutations, generating gene–operon fusions to reporter functions, providing physical or genetic landmarks for the cloning of adjacent DNAs, and locating primer binding sites for DNA sequence analysis (Goryshin et al., 2000).

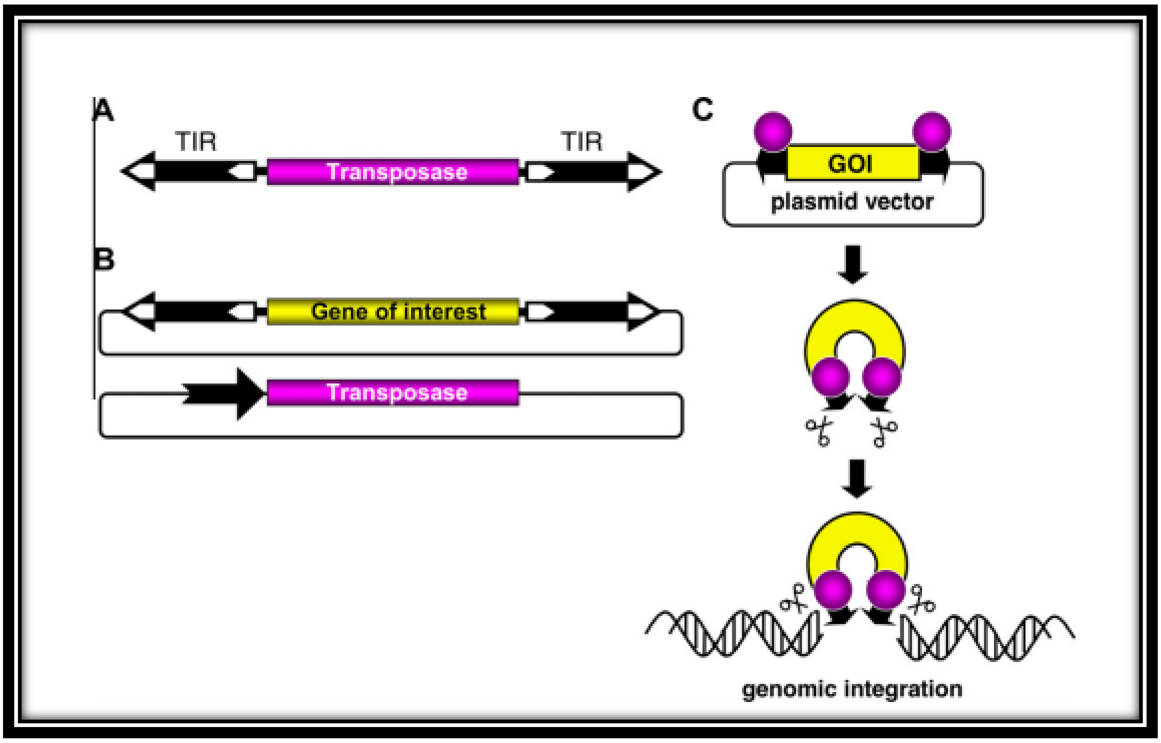


Figure 1.10: General organization and use of transposable elements as gene vectors. (a) Autonomous transposable elements consist of terminal inverted repeats (TIR),) that flank the transposase gene. (b) Bi-component transposon vector system for delivering transgenes that are maintained in plasmids. One component contains a DNA of interest between the transposon TIRs carried by a plasmid vector, while the other component is a transposase expression plasmid, in which the black arrow represents the promoter driving expression of the transposase. (c) The transposon carrying a DNA of interest is excised from the donor plasmid and is integrated at a chromosomal site by the transposase (Ivics et al, 2011).

Tn 5 is a kind of tranposon , which has been widely used for mutagenesis in Gram negative bacteria, was tested by Kunte and Galinski (1995) for insertional mutagenesis in *H. elongata*. Transposon was successfully transferred from *E. coli* SM10 containing the suicide vector pSUP101::Tn 5 to *H. elongata* by biparental mating.

Mini-Tn5 has been used for insertional mutagenesis in *H. eurihalina* , obtaining both exopolysaccharide-defective mutants (Llamas et al., 2000) and mutants that were dependent on arginine and uracil for growth (Llamas, 2003) , and in *H. maura*, obtaining mutants incapable of anaerobic respiration with nitrates (Argandona et al., 2006).

2. MATERIALS

2.1. Bacterial Strains and Plasmids

Halomonas smyrnensis AAD6 strain (GenBank accession number DQ131909) used and genetically modified in the present study was isolated from Çamaltı Saltern Area, Izmir, Turkey (Poli et al., 2012). All genetic manipulations on *H. smyrnensis* AAD6 were planned and carried out based on its available whole genome sequence data (Sogutcu et al., 2012), which has been deposited at DDBJ/EMBL/GenBank under the accession numbers AJKS02000001 to AJKS02000034.

Escherichia coli TOP10 strain (F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ) was used for competent cell preparation.

Both bacterial strains, *H. smyrnensis* AAD6 and *E. coli* TOP10, are provided from the culture depository of Department of Bioengineering laboratories, Marmara University.

pHP45omega-Km (ATCC 37653) plasmid with a vector size of 4.5 kb, which shows resistance and markers for ampicillin (amp^R) and kanamycin (kan^R), was employed in interposon mutagenesis studies.

A kanamycin-resistant helper plasmid, pRK2013 (ATCC 37159), was used for mobilization of non-selftransmissible plasmids (consists of the RK2 transfer genes cloned onto a *Col*El replicon). It contains sequences for ribosomal protein S12 and origin of transfer replication.

pUK4134 (ATCC 77480) shuttle vector was employed as conjugative agent for which permits positive selection of integration. This suicide and gene replacement vector for *Yersinia* and other gram-negative hosts permits positive selection for loss of the plasmid after allelic exchange. The vector also contains cos sites and oriT, and can be used for cosmid cloning or conjugative transfer. RP6 is a suicide replicon in hosts not producing the *pir* protein. *RpsL* (streptomycin sensitive) is dominant to streptomycin resistance, so loss of the plasmid can identified by streptomycin resistance and ampicillin sensitivity. The order of the major features in the plasmid is: EcoRV - R6K ori - *oriT* - SalI - *cos* - *rpsL* - ampR.

pJET1.2/blunt is linearized positive selection cloning vector with a lethal insert that allows for efficient recovery of blunt-ended PCR products. As a result, only cells with recombinant plasmids are able to propagate, eliminating the need of blue/white screening. The vector contains an expanded multiple cloning site, as well as a T7 promoter for in vitro transcription and ampicillin resistant gene.

The plasmid vectors, pHP45omega-Km, pRK2013, and pUK4134, were purchased from ATCC (UK). pJET1.2/blunt vector was purchased from supplier as a component of CloneJET PCR Cloning Kit (THERMO SCIENTIFIC, K1231).

2.2. Chemicals

All chemicals used in this study were supplied from MERCK (Germany), and SIGMA (USA). All experiments were carried out in the laboratories of Bioengineering Department at Marmara University.

2.3. Kits

Kits employed in the present study are presented in Table 2.1.

2.4. Oligonucleotide Primers

Oligonucleotide primers designed, synthesized and employed in the present study are presented in Table 2.2.

Table 2.1: Kits employed in the present study

Kit	Supplier, Catalog Number	Purpose
Wizard® Genomic DNA Purification Kit	PROMEGA (USA), A1120	Isolation of the genomic DNA of *H. smyrnensis* AAD6
GoTaq® Flexi DNA Polymerase Kit	PROMEGA (USA), M8291	Amplifications of target gene regions
Wizard® SV Gel and PCR Clean-Up System	PROMEGA (USA), A9281	Purification of PCR and gel products
High Pure Plasmid Isolation Kit	ROCHE (Switzerland), 11754777001	Isolation of plasmid DNA
CloneJET PCR Cloning Kit	THERMO SCIENTIFIC (USA), K1231	Cloning of targeted gene (HPr) region
Glucose assay kit	SIGMA (USA), GAGO20	Quantification of extracellular glucose concentrations
Fructose assay kit	SIGMA (USA), FA20	Quantification of extracellular fructose concentrations

Table 2.2 : Oligonucleotide primers designed, synthesized and employed in the present study

Amplification region	**Forward primer sequence (5' to 3')**	**Reverse primer sequence (3' to 5')**
HPr region of *H. smyrnensis* AAD6	TTACTTCACGGCGGCGAC	GTTGACCCTACGCCAGGAT
Omega region of pHP45omega-Km plasmid	GAATCACCGGCGATCCGGTG	GAATCGCCGGTGATCCGGTG
HPr + Omega Region From HOM2 plasmid	AGCTAGATCTACTTCACGGCGG	AAAGATCTTGACCCTACGCCAGGA

2.5. Buffers and Solutions

2.5.1. Bacterial Growth Media

2.5.1.1. Trace Element Solution

For preparation of 100 ml solution,
$MnCl_2.4H_2O$: 36 mg
$ZnSO_4.7H_2O$: 44 mg
$FeSO_4.\ 7H_2O$: 230 mg
$CuSO_4.5H_2O$: 5 mg
distilled water 100 ml

2.5.1.2. *H. smyrnensis* Optimum Medium

NaCl : 137.2 g/L
Sucrose : 50 g/L
K_2HPO_4 : 7 g/L
KH_2PO_4 : 2 g/L
$MgSO_4.7H_2O$: 0.1 g/L
$(NH_4)_2SO_4$: 1 g/L
Peptone : 0.5 g/L
Trace elements : 1 mL/L
Thiamine : 800 ul/L (1 mg/mL stock solution)
Per liter of distilled water

2.5.1.3. *H. smyrnensis* Growth Medium with Boric Acid

NaCl : 137.2 g/L
Sucrose : 50 g/L
K_2HPO_4 : 7 g/L
KH_2PO_4 : 2 g/L
$MgSO_4.7H_2O$: 0.1 g/L
$(NH_4)_2SO_4$: 1 g/L
Peptone : 0.5 g/L
Trace elements : 1 mL/L

Thiamine : 800 ul/L (1 mg/mL stock solution)
Boric Acid : 3.0916 g/L
Per liter of distilled water

2.5.1.4. LB Broth

Tryptone : 10 g/L
Yeast extract : 5 g/L
NaCl : 5 g/L
Per liter of distilled water

2.5.1.5. LB Agar Medium

Trypton : 10 g/L
Yeast extract : 5 g/L
NaCl : 5 g/L
Agar : 18 g/L
Per liter of distilled water

2.5.1.6. S.O.C Medium

Tryptone : 20 g/L
Yeast extract : 5 g/L
NaCl : 10 mM
KCl : 2.5 mM
$MgCl_2$: 10 mM
$MgSO_4$: 10 mM
Glucose : 20 mM
Per liter of distilled water

2.5.1.7. Sea-Water Stock Solution

NaCl : 240 g/L

$MgSO_4.7H_2O$: 35 g /L
$MgCl_2.6H_2O$: 30 g/L
KCl : 7 g/L
$CaCl_2.2\ H_2O$: 5mM
Per liter of distilled water
pH adjusted to 7.5

The solution was sterilized at 121^{o}C 1.06 bar for 15 minutes and stored at +4^{o}C.

2.5.1.8. SW-10 Medium for Conjugation

SW-10 is a saline medium containing 10% (w/v) total salts and 0.5 % (w/v) yeast extract.

NaCl : 81 g/L
$MgCl_2$: 7 g/L
$MgSO_4$: 9.6 g/L
$CaCl_2$: 2 g/L
KCl : 0.06 g/L
$NaHCO_3$: 0.0026 g/L
NaBr : 0.0026 g/L
Yeast extract : 5 g/L
Per liter of distilled water
pH of all media was adjusted to 7.2

2.5.1.9. SW-2 Medium for Conjugation

The composition of SW-2 medium is the same with that of SW-10 medium with a difference in total salt concentration. In preparation of SW-2 medium, the SW-10 saline medium was diluted five times with distilled water. Thus, SW-2 medium contains 2% (w/v) salt.

2.5.2. Buffers and Solutions

2.5.2.1. Agarose Gel Electrophoresis Tris-Borate-EDTA (TBE) Buffer

For 5X solution

Tris base : 54 g/L
Boric acid : 27.5 g/L
EDTA : 10 mM (pH= 8.0)

2.5.2.2. Buffers Used for pH Adjustment

NaOH Buffer (1M)

NaOH : 40 g
dH2O : 1000 ml

NaOH was dissolved in 1000 ml distilled water. The buffer was sterilized at 121°C, 1.06 bar for 3 minutes and stored at +4°C.

HCl Buffer (1M)

HCl (37%) : 83 ml
dH2O : 917 ml

83 ml of HCl (37%) was added to 917 ml of distilled water. The buffer was sterilized at 121°C, 1.06 bar for 3 minutes and stored at +4°C.

2.5.2.3. Buffers and Solutions used in Plasmid DNA Isolation

Mini-Prep Plasmid Isolation Solutions

Alkaline Lysis Solution I

Glucose : 50 mM
Tris-Cl : 25 mM (pH = 8.0)
EDTA : 10 mM (pH= 8.0)
Sterilized by autoclaving and stored at 4°C.

Alkaline Lysis Solution II

NaOH : 0.2 N (freshly diluted from 10 N stock)
SDS : 1 % (w/v)
Used at room temperature

Alkaline Lysis Solution III
CH_3COOK : 3 M
Glacial Acetic Acid : 11.5 ml
dH_2O : 28.5 ml

TE Buffer
Tris-Cl : 100 mM
EDTA : 10 mM
RNase A : 20 ug/ml

- **Phenol:Chloroform Extraction Solutions**

TE Buffer
Tris-Cl : 10 mM
EDTA : 1 mM

Phenol	50 mM TE saturated phenol
Phenol / Chloroform	1:1 (v/v)
Ammoium acetate	3 M
Ethanol	100 %

- **Solutions for Isolation of DNA from Agarose Gels**

Tris- HCl	20 mM
EDTA	1 mM
Phenol	50 mM TE saturated phenol
Chloroform	50 ml
Ammonium Acetate	0.3 M

Ethanol 100%

TE Buffer
Tris-Cl : 10 mM
EDTA : 1 mM

- **Solutions for Competent Cell Preparation**

  CaCl$_2$ Solution
  CaCl$_2$ 1 M
  DMSO and PEG Solutions
  DMSO or PEG (40% v/v)

2.5.3. Restriction and Modification Enzymes

2.5.3.1. SgrA1

Recognition Site 5'… CR'CCGGYG…..3'
3'… GYGGCC'RC…..5'

This restriction enzyme supplied from New England Biolabs (USA).

2.5.3.2. Alkaline Phosphotase

This modification enzyme used for preventing re-circularization of plasmid vector. Calf intestine alkaline phosphatase enzyme was purchased from Promega (USA).

2.5.3.3. T4 DNA Ligase

1. Ligase enzyme, which catalyzes ligation of two DNA strands via repairing single-stranded discontinuities in double stranded DNA molecules, was purchased from (Promega, USA) and used in ligation process of vector and insert.

3. METHODS

3.1 Sterilization

In the course of this study, sterilizations were carried out by autoclave and filtration processes. Antibiotics, thiamine and trace element solution were sterilized by filters with pore size of 0.45 µm. All glass equipments, pippet tips, eppendorf tubes, graduated cylinders, centrifuge tubes and liquid solutions were sterilized by autoclave at 1.02 atm and 121^{o}C for 15 minutes. Liquid solutions including the carbon sources were autoclaved at 1.02 atm and 121^{o}C for 3 minutes in order to prevent their degradation and precipitation.

3.2 Preparation of Preculture

To prepare *H. smyrnensis* preculture, 50 mL of sterile optimum medium (described in 2.5.1.2) was inoculated with 1% (v/v) frozen culture in 250 mL Erlenmeyer flask incubated at 37^{0}C at 180 rpm in the orbital shaker for overnight. In order to obtain optimum aeration, one fifth of the flask volume was accepted as the maximum culture volume. In preparation of *E. coli* preculture, the same procedure was followed, however this time LB medium was employed instead of optimum medium. Besides, media were supplemented with antibiotics such as ampicillin, kanamycin (Km) and streptomycin (Str), when required.

3.3 Growth Conditions

Microoorganisms were cultured in both liquid and solid media. Cells were grown in Erlenmeyer flasks with 20% working volume. 1% (v/v) of preculture was used as an inoculum for growth. Cultures were incubated at 37^{0}C at 180 rpm in the orbital shaker. Furthermore, solid media of *H. smyrnensis* and *E. coli* were spreaded with cells and incubated at 37^{0}C in the incubator.

3.4 Genomic DNA Isolation of *H. smyrnensis*

50 ml optimum medium inoculated with 1% frozen stock was grown at 37^{o}C at 180 rpm in the orbital shaker for overnight. Then, 2 ml culture was centrifuged at 10000 rpm for 20 min at 4^{o}C. Genomic DNA was isolated via the procedure given in the manual of Wizard Genomic DNA Purification Kit (Promega) (Appendix C).

3.5 Cloning of Hpr Gene Region into pJET1.2/Blunt Vector

The construction of HPr carrying recombinant plasmid was carried out by amplification of HPr region from *H. smyrnensis* bacterial genome with specifically designed primers (Table 2.2) and ligation of the amplified HPr insert into pJET1.2/Blunt Vector.

3.5.1 Amplification of Target Gene Region (HPr) and PCR Optimization

After isolation of the genomic DNA from *H. smyrnensis,* target gene region HPr, which is a crucial component of the PTS^{fru} system, was amplified for further genetic manipulations. The amplification of target gene region from the template DNA (i.e., *H. smyrnensis* bacterial genome) was carried out by polymerase chain reaction (PCR) using Taq DNA Polymerase. All reactions were carried out using thermal cycler and visualized by UV transilluminator. To obtain high quality of amplicons, PCR conditions and reaction mixtures were optimized as a result of systematic trials. Optimum PCR reaction mixture and conditions were as in the Table 3.1. All PCR reactions were carried out in 25 µl reaction volume. Then, agarose gel electrophoresis was performed and visualized under UV transilluminator.

Table 3.1: PCR Reaction of HPr Region

PCR mixture	
Components	**1X reaction**
dH_2O	13 µl
Buffer 5X	5 µl
$MgCl_2$ (25 mM)	1.5µl
dNTP (10 mM)	1 µl
Forward primer (25 pmol/ul)	1 µl
Reverse primer (25 pmol/ul)	1 µl
Taq DNA polymerase	0.5µl
Template DNA	1 µl
DMSO	1 µl
Total	**25 µl**

Table 3.2: PCR Conditions for HPr Region

PCR Conditions	
Temperature	**Duration**
95° C	3 min
94° C	1 min
60.5° C	30 sn
72° C	30 sn
72° C	5 dk
4° C	∞

3.5.2 Separation of DNA Fragments via Agarose Gel Electrophoresis

In order to separate DNA fragments by their size and molecular weights, agarose gel electrophoresis technique was employed. In this technique, an electric current is employed to move the DNA molecules across an agarose gel, which is a polysaccharide matrix that functions as a kind of sieve to help separation of the molecules according to their size when they are transported by the electric current. Gels were prepared by boiling proper amount of agarose in order to make 0.7 % gel in 0.5 TBE buffer. Ethidium bromide was added when gel reaches approximately 50°C - 60°C. After gel formation, lanes were loaded with sample and dye mixture (5:1 v/v). Gel electrophoresis was performed at 100 V for 30 min. Then, DNA fragments were visualized by UV transilluminator.

3.5.3 Cleaning of PCR Products

In order to purify PCR product via eliminating the buffer and DMSO based impurities, the procedure presented in the manual of the Wizard® SV Gel and PCR Clean-Up System Kit was employed (Appendix D).

3.5.4 Cloning of HPr Region with CloneJET PCR Cloning Kit

After cleaning PCR products of HPr region, cloning of these products were performed by assisting of CloneJET PCR Cloning Kit (Appendix E), which is an advanced positive selection system for high-efficiency cloning of PCR products generated with any thermostable DNA polymerase. The kit was composed of pJET 1.2/blunt Cloning Vector, reaction buffers, T4 DNA Ligase, DNA blunting enzyme, forward and reverse sequencing primers, control PCR product and nuclease free water.

Any other blunt or sticky-end DNA fragment can be cloned via the CloneJET PCR Cloning Kit as well. The kit can be used for phosphorylated or non-phosphorylated DNA fragments, and provides positive selection of recombinants by vector pJET1.2/blunt. This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate. DNA blunting enzyme, which is a proprietary thermostable DNA polymerase with proofreading activity, was employed for removing 3'- overhangs and fill-in 5'-overhangs on sticky ends. Blunt-end PCR products generated by proofreading DNA polymerases were directly ligated in 5 min with the pJET1.2/blunt cloning vector. Re-circularized pJET1.2/blunt vector expresses a lethal restriction enzyme which kills the host *E. coli* cell after transformation. As a result, only recombinant clones containing the insert appear on culture plates.

There are two strategies for clonning. One for blunt- ended and the other one for sticky-end DNA products. Since Taq DNA polymerase was employed for amplification, DNA fragments of HPr were sticky-ended. So, sticky-end protocol was followed for cloning. The protocol which is recommended by supplier was followed.

3.5.5 Preparation of Competent Cells by Chemical Method

500 µL overnight *E. coli* culture was used as inoculation for 50 ml LB medium. Growth was performed vigorous shaking at 37^{o}C and 180 rpm, and followed until cell density reaches mid-log growth phase (i.e., OD$_{600}$ between 0.4-0.6). Then, growth was terminated and cells were chilled on ice for 15 min. For further steps, all equipment was sterilized.

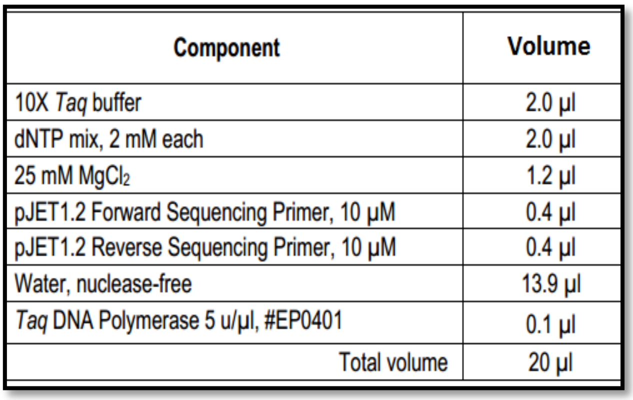
Cells were harvested at 4^oC and 4000 rpm for 10 min. The cell pellet was dissolved in 20 ml of 0.1 M $CaCl_2$ and stored on ice for 30 min. Cells were centrifuged, dissolved in 2 ml of 0.1 M $CaCl_2$ and stored on ice. At the end of 2 hours, chemically competent cells were ready to use. They were stored at -80 ^oC.

3.5.6 Transformation

After preparation of blunting and ligation reactions, pJet1.2/blunt vector carrying HPr region was transformed into competent cells. The ligation mixture was directly used for transformation. So, 50 µL of competent cells and 5 µL of ligation mixture were combined and incubated on ice for 30 min. Cells were heated at 42°C for 30 seconds without shaking, then transferred immediately to ice again for 5 min. Then, 800 µL of pre-warmed LB broth was added and tube was closed tightly. Cells were incubated at 37°C and 125 rpm for 1.5 – 2 hours. Meanwhile, plates were heated to 37°C before spreading of the cells to prevent cells from heat shock. After 2 hours, 200 µL of cell mixture was spreaded on warmed selective and control LB plates and incubated overnight at 37°C. Next day, colonies were transferred immediately to a new plate and incubated overnight again. For screening of recombinant colonies, colony PCR and nucleotide sequencing were employed.

3.5.7 Colony PCR

50 µL of PCR grade water was added into the sterile 1.5 mL eppendorf tubes. By using either micropipette tip or sterile toothpick, cells were transferred into tubes and were gently mixed until water became blurry. Cells were heated at 100^0C for 2 minutes in order to disrupt the cell wall and then centrifuged at 14.000 rpm for 3 min. Supernatant was collected and used as a DNA template for PCR. The protocol followed in colony screening by PCR was given in Figure 3.1. Each reagent was mixed gently and 5 µL of template DNA was added finally. PCR were performed at 95°C for 3 min; 94°C for 30 s, 60°C for 30 s, 72°C for 1 min/kb; 25 cycles. Presence of the PCR product was analyzed on agarose gel.



Component	Volume
10X *Taq* buffer	2.0 µl
dNTP mix, 2 mM each	2.0 µl
25 mM $MgCl_2$	1.2 µl
pJET1.2 Forward Sequencing Primer, 10 µM	0.4 µl
pJET1.2 Reverse Sequencing Primer, 10 µM	0.4 µl
Water, nuclease-free	13.9 µl
Taq DNA Polymerase 5 u/µl, #EP0401	0.1 µl
Total volume	20 µl

Figure 3.1: Colony PCR Reaction

3.5.8 Sequencing

Recombinant colonies were also screened via nucleotide sequencing. pJET1.2 Forward and Reverse Sequencing Primers, which were supplied with the kit, were used to analyze sequence of the cloned insert, which was HPr region in this study. Sequences of forward and reverse primers of the kit are presented in Appendix F. These sequencing analyses were purchased from BGI (Beijing Genome Institution, China). At the end of sequencing, the plasmid with correct insert, which was named as H4, was selected for further studies.

3.6 Plasmid DNA Isolation of Transposon Agent (pHP45omega-Km Plasmid)

In the scope of this study, transposon mutagenesis was employed to delete target region of the fructose uptake system. In order to accomplish this, isolation of desired omega transposon was performed via two alternative methods; alkaline lysis method for small quantities and kit aided method (High Pure isolation Kit, Roche).

3.6.1 pHP45omega-Km Plasmid DNA isolation with Kit

Kit aided isolation was preferred in order to avoid use of hazardous organic compounds such as phenol, chloroform, as well as to make quick purifications with improved reliability and reproducibility in downstream applications because of removing RNA and other impurities that cause plasmid DNA to behave unpredictably. The kit contents were suspension buffer, Rnase A (in powder form), lysis buffer, binding buffer, wash buffers, elution buffer, spin filter tubes and collection tubes.

The kit relies on releasing plasmid DNA from bacteria. RNase removes all RNA in the lysate. After cellular debris and (entrapped) genomic DNA were removed by centrifugation, the remaining supernatant was mixed with a chaotropic salt and applied to the glass fiber fleece in a High Pure Spin Filter Tube. Under the buffer conditions used in the procedure, the plasmid binds to the glass fiber fleece, while contaminating substances (salts, proteins, and other cellular contaminants) cannot. Spin steps readily remove these contaminants. Once purified, the plasmid was easily eluted in a small volume of low-salt buffer or water.

5 mL of LB-medium were inoculated with powder form of pHP45omega-Km. Bacteria were grown overnight at 37°C and 180 rpm. Remaining steps and the protocol which was employed, was recommended by supplier and shown in Figure 3.2.

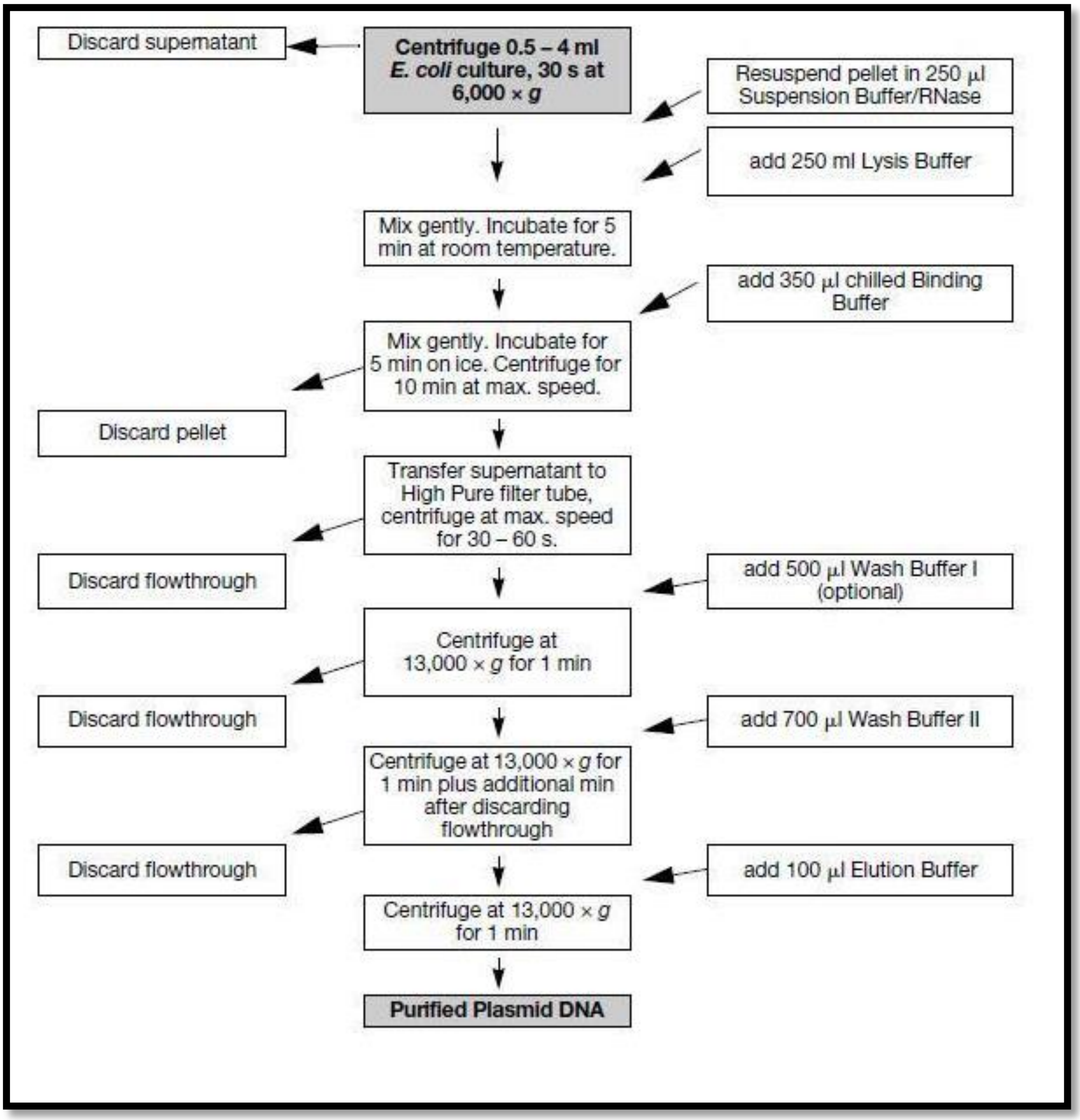


Figure 3.2: High Pure Plasmid Isolation Kit Procedure

3.6.2 pHP45omega-Km Plasmid DNA isolation with Alkaline Lysis Method

This method was employed in order to compare the isolation yield of the kit and manually performed alkaline lysis method. The kit provided highly pure products but yields were very low. For further analysis DNA yield was very essential, therefore alkaline lysis method were used, too.

100 mL LB medium containing the appropriate antibiotic inoculated with powder form of pHP45omega-Km and incubated overnight at 37°C with vigorous shaking. 2 ml of the culture was poured into a microfuge tube, centrifuged at maximum speed for 30 seconds at 4°C in a microfuge. The unused portion of the original culture was stored at 4°C. The supernantant medium was removed; the bacterial pellet was leaved as dry as possible.The pellet was resuspended in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing. 200 µl of freshly prepared Alkaline lysis solution II was added to each bacterial suspension. The tubes were closed tightly, and the contents were mixed well by inverting the tube. The tubes were stored in ice. Then, 150 µl of ice-cold Alkaline lysis solution III was added. Alkaline lysis solution III was dispersed through the viscous bacterial lysate by inverting the tube several times. Tubes were stored in ice for 3-5 minutes. The bacterial lysate was centrifuged for 5 minutes at maximum speed at 4°C in a microfuge. The supernatant was collected to a fresh tube. Equal volume of phenol: chloroform were added. Organic and aqueous phases were mixed by vortexing and then the emulsion was centrifuged at maximum speed for 2 minutes at 4°C in a microfuge. The aqueous upper layer was transferred to a fresh tube. Nucleic acids were precipitated from the supernatant by adding two volumes of ethanol at room temperature. The solutions were mixed by vortexing and then the mixture was standed for 2 minutes at room temperature. The precipitated nucleic acids were collected by centrifugation at maximum speed for 5 minutes at 4°C and supernatant was discarded by aspiration. Tubes were standed in an inverted position on a paper towel to allow all of the fluid to drain away. 1 ml of 70% ethanol was added to the pellet and tubes were inverted several times. DNA was recovered by centrifugation at maximum speed for 2 minutes at 4°C. All of the supernatant were removed by aspiration. Tubes were stored at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5-10 minutes). Nucleic acids were dissolved in 50 µl of TE (pH 8.0)

containing 20 µg/ml DNase free RNase A. The final solution was vortexed gently for a few seconds. The DNA was ready and it was stored at -20°C for further analysis.

3.7 Cloning of Omega Transposon into Recombinant H4 vector

The construction of transposon carrying recombinant HPr plasmid was carried out by amplification of omega transposon from pHP45-omega-Km plasmid with specifically designed OMEGA primers (Table 2.2) and by ligating the amplified omega insert into the recombinant HPr carrying H4 vector.

3.7.1 Amplification of Omega Transposon from pHP45omega-Km vector

The amplification of omega transposon was carried out by OMEGA primers with using isolated pHP45omega-Km plasmid DNA as the template. All PCR reactions were implemented in 25 µl reaction volume. Then, agarose gel electrophoresis and visualization of the gel under UV transilluminator were performed. Reaction mixture and conditions are as in the Table 3.3 and 3.4.

Table 3.3: PCR mixture for Amplification of Omega Region

PCR Mixture	
Components	**1X reaction**
dH_2O	13 µl
Buffer 5X	5 µl
$MgCl_2$ (25 mM)	1.5µl
dNTP (10 mM)	1µl
Forward primer (25 pmol/µl)	1µl
Reverse primer (25 pmol/µl)	1µl
Taq DNA polymerase	0.5µl
Template DNA	1µl
DMSO	1µl
Total	**25 µl**

Table 3.4: PCR conditions for Omega Region

PCR Conditions	
Temperature	**Duration**
95°C	3 min
94°C	30 sec
50°C	30 sec
72°C	90 sec
72°C	5 dk
4°C	∞

3.7.2 Isolation of PCR Products From Gel

Amplification of Omega products were run in agarose gel. and visualized. Then, bands with expected lenghts were isolated from gel using Wizard® SV Gel and PCR Clean-Up System (Promega). DNA fragments were extracted from standard or low-melt agarose gels in either Tris acetate (TAE) or Tris borate (TBE) buffers, and PCR products were purified. DNA purity was checked to be used for DNA sequencing, cloning or restriction enzyme digestion without further manipulation. The instructions that were suggested by manufacturer, were performed. Gel was loaded and run with TBE buffer. 1.5 ml microcentrifuge tubes were weighed for each DNA fragment and the weights were recorded. Gel was irradiated for a duration as minimum as possible. The interested fragments were excised in a minimal volume of agarose using a razor blade. Gel slices were transferred weighed microcentrifuge tubes and weighed again. Membrane binding solution was added to each tube at a ratio of 10 μl of solution per 10 mg of agarose gel slice. Mixtures were vortexed and incubated at 60°C for 10 minutes. Tubes were centrifuged and final mixtures were transferred to mini-column and incubated for 1 minute at room temperature. Mini-columns were centrifuged at 14000 rpm for 1 minute. Liquid part discarded and columns were washed with once 700 μl membrane wash solution, then once with 500 μl same solution for 1 minute at 14000 rpm. Then columns were transferred to new sterile centrifuge tubes and 50 μl nuclease free water was added to columns. The columns were incubated at room temperature for 1 minute and centrifuged at same conditions. Purified DNA fragments (Omega region) were ready to use for the next step restriction digestion.

3.7.3 Digestion of Vector and Insert with SgrA1

Before ligation, both vector (H4) and insert (Omega) were digested with the same restriction enzyme, i.e., SgrA1 (NEB, England). Since both of them include a restriction site for SgrA1, only one enzymatic restriction digestion was required. Enzymatic reaction components were showed in Table 3.5 and 3.6.

Table 3.5: Digestion Reaction for Vector

Digestion Reaction of Vector	
Plasmid	**60 µL**
10X Buffer	**6 µL**
Enzyme	**2,5 µL**
dH$_2$0	**11,5 µL**
Total	**80 µL**

Table 3.6: Digestion Reaction for Insert

Digestion Reaction of Insert	
PCR Product	**80 µL**
10X Buffer	**8 µL**
Enzyme	**3 µL**
dH$_2$0	**9 µL**
Total	**100 µL**

Every component was added and mixtures were incubated at 37 ^{o}C for 3 hours. At the end of 3 hours, heat inactivation of enzyme was performed at 65 ^{o}C for 20 minutes. There was no need to inactivate enzyme with phenol: chloroform method. After inactivation, products were loaded and run on agarose gel to confirm cutting of enzyme within expected bands. Products were purified from gel with using Wizard® SV Gel and PCR Clean-Up System Kit.

3.7.4 Ligation Reaction

Ligation is a method for the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. In this study, T4 DNA Ligase (Promega, USA) was employed in the ligation reaction as joining agent. Reaction buffers, conditions and components were summarized in Table 3.7.

Table 3.7: Ligation Reaction Components

Ligation Reaction	
Vector (H4 Plasmid DNA)	**4 µL**
Insert (Omega Region)	**12 µL**
10X Buffer	**2 µL**
Enzyme	**1 µL**
dH$_2$0	**1 µL**
Total	**20 µL**

The manufacturer (Thermo Scientific) recommends using a 1:1, 1:3 or 3:1 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. In the present study a vector:insert DNA ratio of 1:3 was chosen. All components were added according to Table 3.7 and reaction was incubated at room temperature for 3 hours. Heat inactivation was applied at 70^{o}C for 10 minutes. The reaction was ready at the end of 3 hours for transformation.

3.7.5 Transformation

After preparation of ligation reaction, HPr carrying H4 vector and omega mixture was transformed into competent cells. Transformation protocol was same as previous one with some exceptions. For this transformation 200 µL competent cell and 5 µL ligation mixture were mixed.

Following steps were the same prior transformation until spreading the final product on LB agar plates. This time, kanamycin was added as a selective agent in addition to ampicillin in LB agar plates. Also, it is important for screening of recombinant colonies.

3.7.6 Screening of Recombinant Colonies

Screening of recombinant colonies were carried out with selective antibiotics, colony PCR and sequencing techniques. For this screening step, kanamycin had an essential role for selection of colonies, because omega transposon had an antibiotic resistance gene (kanamycin resistance - KmR), which was employed for selection of recombinants with correct insert. Survived colonies on agar plates were transferred to new plates and colony PCR was performed with both HPr and omega primers. Resultant colonies, which gave positive results for both regions, were selected for further analysis. They were grown on liquid antibiotic aided LB medium and plasmid DNA was isolated for sequencing with HPr and Omega primers.

3.8 Isolation of pUK4134 (suicide vector) Plasmid DNA

Suicide vectors deliver a transposon into the host cells in which the vector itself cannot replicate but the transposon can be maintained and used for transposon mutagenesis. In order to complete transposon mutagenesis, isolation of suicide vector was required, and it was carried out by High Pure Isolation Kit (Roche). The protocol was mentioned in section 2.6.1.

3.9 Clonning of HPr + Omega Region into Suicide Vector

After cloning of Omega region into H4 vector, one of the recombinant colonies was selected and named as HOM2. This recombinant had both HPr and Omega at the same time in its plasmid DNA. The next step was cloning of this region into suicide vector, therefore amplification of HPr + Omega region (HOM region) was required. Then, this amplicon was introduced into suicide vector using BamH1 and Bgl11 restriction endonucleases.

3.9.1 Amplification of Hpr + Omega (HOM) Region from Recombinant HOM2 vector

Target region was amplified with specifically designed primers (HOM forward and reverse primers) thereafter isolation of HOM2 plasmid DNA. Amplification of HOM region was carried out with same reaction conditions that were mentioned in section 2.5.1. Only difference was the primers which was specific for binding to the HOM region. Also, PCR conditions were adjusted to melting temperature (Tm) of the primers. After refinement of annealing temperature, the following PCR conditions were applied: initial denaturation step at 95^{o}C for 3 minutes followed by subsequently 30 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 57.5 ^{o}C for 1 minute, extention at 72 ^{o}C for 2 minutes, ending with 5 minutes incubation at 72 ^{o}C and cooling was performed at 4 ^{o}C for 15 minutes. PCR products were loaded and run on agarose gel and products with expected band lenghts were purified for further analysis using Wizard® SV Gel and PCR Clean-Up System Kit (Promega) .

3.9.2 Restriction Digestion of Vector (pUK4134) and Insert (HOM Region)

To be able to clone a DNA insert into a cloning or expression vector, both have to be treated with two restriction enzymes that create compatible ends. At least one of the enzymes used should be a sticky-end cutter to ensure that the insert is incorporated in the right orientation. Therefore, two restriction enzymes, BamH1 (Thermo Scientific) and Bgl11 (Thermo Scientific), were employed. They were fast digest enzymes and made us quick digestion. Insert (HOM region) has recognition site for Bgl11 and vector (PUK4134) has a site for BamH1. Also, these two enzymes make compatible ends with each other.

Vector Preparation

Vector DNA was digested using BamH1 restriction enzyme with protocol that shown in Table 3.8. All components were mixed and incubated at 37^{o}C for 5 minutes. Reaction was inactivated at 80^{o}C for 5 minutes.

Table 3.8: Restriction Digestion of Vector

Digestion of Vector	
Plasmid	**45 µL**
10X Buffer	**6 µL**
Enzyme	**2,5 µL**
dH$_2$0	**6,5 µL**
Total	**60 µL**

In order to prevent recircularization of vector DNA, the ends were dephosphorylated using calf intestine alkaline phosphatase (CIAP, Promega). This enzyme reduces the background of non-recombinants due to self-ligation of the vector (when a single site was used for cloning). Purification of the digested vector by agarose electrophoresis was performed to remove residual nicked and supercoiled vector DNA and the small piece of DNA that was cut out by the digestions. This usually reduces strongly the background of non-recombinants due to the very efficient transformation of undigested vector. Digested vector product was extracted from gel with Wizard® SV Gel and PCR Clean-Up System Kit.

Insert Preparation:

Insert DNA was the amplification product of HOM region. It was digested via Bgl11 restriction enzyme according to components that are shown in Table 3.9.

Table 3.9: Restriction Digestion of Insert

Digestion of Insert	
Plasmid	**50 µL**
10X Buffer	**6 µL**
Enzyme	**2,5 µL**
dH$_2$0	**1,5 µL**
Total	**60 µL**

Purification of the digested insert was carried out by phenol: chloroform extraction. Then, agarose gel electrophoresis was performed and products were extracted from gel using Wizard® SV Gel and PCR Clean-Up System Kit.

3.9.2.1 Phenol / Chloroform Extraction

Sample that will be purified was mixed with 2 volumes of tris-saturated phenol and chloroform mixture(1:1 v/v), and the resulting mixture was centrifuged at 10,000 rpm for 5 min at room temperature. After centrifugation, the upper aqueous phase was transferred to a fresh tube and an

equal volume of chloroform was added, mixed and centrifuged at 10,000 rpm for 5 min at room temperature. This step was repeated twice. The upper aqueous phase transferred again to a fresh tube and 1/10 the volume of 3 M Sodium Acetate solution was added into tube. Then, 2.5 volumes of ethanol was added to precipitate DNA. The mixture was incubated for 30 min at -20°C. Then, the mixture was centrifuged for 10 min at 10,000 rpm. The supernatant was discarded and the pellet was rinsed with 70% cold ethanol, air-dried and dissolved in TE buffer.

3.9.3 Ligation of pUK4134 and HOM Region

The next step was the ligation of the insert into the linearized vector. This involves the formation of phosphodiester bonds between adjacent 5'-phosphate and 3'-hydroxyl residues, which is catalyzed by T4 DNA ligase. Since the efficiency of ligation reaction depends on the ratio between insert and vector DNA, 3:1 (vector:insert) ratio was chosen. The vector DNA had a slight band on gel, so it was less concentrated than the insert DNA. 12 µL vector DNA, 4 µL insert DNA, 2 µL buffer and 2 µL ligation enzyme were added. Then, reaction was incubated at room temperature for 3 hours. At the end of three hours, ligation mixture was ready for transformation. If mixture is not used at that moment, it was stored at -20 ^{o}C for not more than 2 weeks.

3.9.4 Transformation of *E.coli* competent cells

The same procedure was applied in transformation process. Ligation reaction was used directly for transformation. HPr + Omega (HOM) region and pUK4134 vector were transformed into competent *E. coli* cells. For this transformation, 200 μL competent cell and 5 μL ligation mixture were mixed.

Following steps were the same prior transformation except spreading the final product on LB agar plates. Because pUK4134 is a suicide and gene replacement vector that permits positive selection for loss of the plasmid after allelic exchange. So, after allelic exchange happens loss of the plasmid can be identified by streptomycin resistance and ampicillin sensitivity. This time, streptomycin was added as a selective agent in addition to kanamycin in LB agar plates. It is important for screening of recombinant colonies, as well.

3.9.5 Screening and Selection of Recombinant Colonies

In this step, selection of recombinant colonies was carried out with specific antibiotics, colony PCR and sequencing techniques. For this screening, streptomycin was used as selection agent. Because pUK4134 suicide vector carrying the rpsL gene encoding E. coli ribosomal protein S12 confers streptomycin sensitivity on streptomycin-resistant strains and provides a positive selection for bacteria that have lost the plasmid following allelic exchange. Therefore, the colonies which survived on agar plates with streptomycin and kanamycin, had correct inserts. Colony PCR was performed with these survived colonies with both HPr and omega primers. Resultant colonies, which gave positive results for both regions, were selected for triparental mating studies. They were grown on liquid antibiotic aided LB medium and plasmid DNA was isolated for sequencing with HPr and Omega primers.

3.10 Conjugal Transfer of Plasmids via Triparental Mating

Triparental conjugation is a method which is used for transposon mutagenesis or gene replacement in the recipient chromosome. Triparental mating is a form of bacterial conjugation which permits to assist of a conjugative plasmid present in one bacterial strain for transferring of a mobilizable plasmid present in a second bacterial strain into a recipient bacterial strain. As the name implies there are three requirements for triparental conjugation, which were helper plasmid (pRK2013 in this study) carrying a conjugative plasmid that codes for genes required for conjugation and DNA transfer, donor strain carrying a mobilizable plasmid that can utilize the transfer functions of the conjugative plasmid, and recipient strain which aimed to introduce the mobilizable plasmid into.

3.10.1 Donor, Recipient and Helper Plasmid

In this study, plasmid transfers were performed by helper plasmid selected as pRK2013 (ATCC) whose function in this system is to trans-complement the vector for mobilization. Donor strain was a recombinant which carries omega+HPr region and pUK4134 vector that named as pUKHom4. Recipient strain was H. smyrnensis AAD6. The protocol that followed for conjugation was inoculation of each strain into required medium (for pUKhom4 LB Medium with Km and Str, for pRK2013 LB medium with Km, for H. smyrnensis AAD6 optimum medium) and incubation of them at 37oC for overnight. The next day these cultures used as inoculum for subcultures with 1:10 (v/v) ratio. Growth of each strain was followed until optical densities reach to 0.4 – 0.6 at different wavelengths 660 nm, 600 nm and 600 nm for H. smyrnensis, pRK2013 and pUKHom4, respectively. Then, 100 µl of logarithmic cultures of each donor and helper strain, 400 µl of logarithmic culture of donor were mixed, centrifuged and washed twice with SW-2 liquid mating medium, and re-suspended in 100 µl of the same medium. The mating mixture was then placed on the surface of a sterile 0.45 µm pore-membrane filter (Sigma) onto a plate of the corresponding solid mating medium (SW-2 agar plates without antibiotics) and incubated at 37oC for overnight. This step was repeated once without filter membrane.

3.10.2 Screening and Selection of Transconjugants

The collected cells were then re-suspended in SW-2 medium and, after appropriate dilutions, they were plated onto SW-2 and optimum medium agar plates with corresponding antibiotics (Km and Str) to counter-select the donor strain, and to select for transconjugants carrying the plasmid. Optimum medium agar plates with antibiotics provided the selection of only H. smyrnensis cells with correct insert.

3.11 Determination of Growth Profiles and Levan Production of Wild Type and Mutant Organism

3.11.1 Growth Profiles

All fermentation media components (explained in 1.5.1.2), NaCl and the rest of the medium components were dissolved in distilled water, and then sterilized separately by autoclaving at 1.06 bar and 121ºC for 15 minutes. Carbon sources were autoclaved at 1.06 bar and 121ºC for 3 minutes. The pH was adjusted to 7.0-7.5 using sterile 1 M NaOH or with 1 M HCl. Sterile salt and carbon source solutions were combined to 1 L final volume. 200 ml of optimum medium was inoculated with *H. smyrnensis* AAD6 (wild type) and 200 ml of optimum medium with corresponding antibiotics (Km and Str) was inoculated with mutant transconjugant, which was named as H. smyrnensis BMA14. The growth profiles of AAD6 and BMA14 were monitored by measuring the optical densities (OD) at 660 nm. Cell-free medium was used as the blank. Growth was terminated at the end of 200 hours.

3.11.2 Levan Production

Fermentation was terminated after 200 hours. Cells were harvested at 10.000 rpm for 20 min. The supernatant phases, consisting of EPS, were treated with two volumes of ethanol, held at −20 °C overnight, and then centrifuged at 12.000 rpm and 4°C for 30 min. The pellets were dissolved in warm distilled water. Dissolved polymer was dialyzed against several runs of distilled water for 3-5 days. After dialysis, 10 ml of each polymer that was produced by BMA14 and AAD6 were incubated to dry at 60oC for overnight.

3.12 Sucrose Assay

In order to obtain differences between sucrose consumption rates in AAD6 and in BMA14, extracellular sucrose was measured by High Pressure Liquid Chromatography (HPLC) with ZORBAX Carbohydrate Analysis (4.6 x 250 mm) column, which was purchased from Agilent (USA). 1 ml samples were collected form the liquid cultures of fermentation, cells were harvested at 10000 rpm for 20 minutes, and excess components were removed by filter sterilization (45µm pore size filter, TPP). 100 µl sample was mixed with 100 µl acetonitrile:water (1:1 v/v) solution. Also, mobile phase was prepared as 75:25 acetonitrile:water. Samples were run at 1.4 ml/min flow rate with 3 µl injection volume. Retention time was set to 10 minutes. There were 10 different samples from taken at different times of fermentation. Standarts were also prepared in order to determine the calibaration chart.

3.13 Glucose Assay

Measurement of extracellular glucose was carried out with glucose assay kit, which was purchased from Sigma. Kit was composed of Glucose Oxidase/Peroxidase Reagent, o-Dianisidine Reagent and Glucose Standard Solution.

Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a colored product. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The reaction is showed at Figure 3.3.

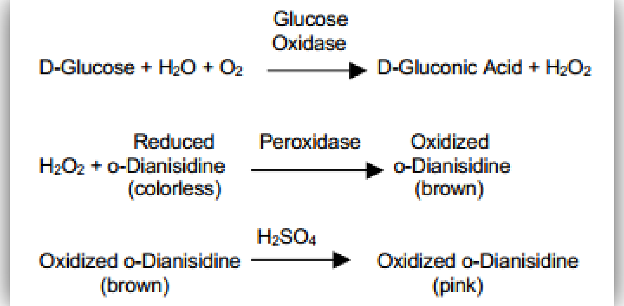


Figure 3.3: Principles of glucose measurement reaction

The intensity of the pink color measured at 540 nm was proportional to the original glucose concentration. Distilled water was used as blank. If necessary, samples were diluted to keep the readings within reliable limits of device, which is between 0.2 and 0.8. The protocol was given in Appendix G.

3.14 Fructose Assay

Fructose measurements were carried out with fructose assay kit that was supplied from Sigma. Kit contents were Phosphoglucose Isomerase, Glucose Assay Reagent and Fructose Standard Solution.

Fructose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Fructose 6-phosphate is converted to glucose 6-phosphate by phosphoglucose isomerase (PGI). Glucose-6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) in the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm was directly proportional to fructose concentration. The steps were showed Figure 3.4.

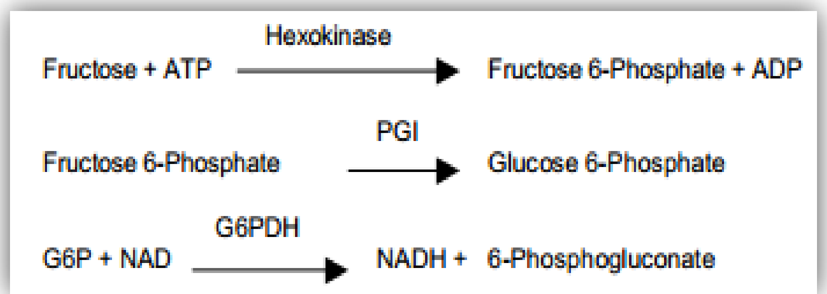


Figure 3.4: Reaction steps of fructose concentration measurement

4. RESULTS and DISCUSSION

4.1 *In silico* Analysis of Fructose Uptake System in *Halomonas smyrnensis* AAD6

The investigation of the fructose uptake system was one of the main objectives of the present study, which aims to develop strategies to enhance the polymer production. Since levan is a fructan polymer, levan biosynthesis depends on utilization of the fructose molecules at the extracellular environment. So, understanding the mechanism behind fructose metabolism, its utilization, consumption or transportation, is crucial. Therefore, in the first step of this study we investigated the fructose metabolism in *H. smyrnensis* AAD6.

Whole genome sequencing of *H. smyrnensis* AAD6 was performed (Sogutcu et al., 2012) successfully, so, only need was determination of the systems that were responsible for the fructose metabolism, indicated in annotation results of bacterium. There were almost 5 mechanisms which were associated with fructose metabolism, utilization and transport. Also, the thesis study, which was carried on analysis of levan biosynthesis via metabolic network model of *H. smyrnensis* AAD6 reported that fructose spesific phosphotransferase system had an essential role for levan biosynthesis (Ozer, 2013). It was also reported that the knock-out of the gene associated with the PTS system of fructose uptake is the most possible strategy for levan overproduction. Hereby, the system that will be investigated and manipulated with molecular and genetic methods for elevated levan biosynthesis, selected as fructose uptake system.

After selection of the target region, nucleotide sequence and amino acid sequence of fructose uptake system were extracted from annotation results. Then, subunit/domain analyses of this system were performed via web-based softwares such as Interproscan (EMBL) and Conserved Domain Architecture Tool (NCBI). Complete name of the system in annotation result was phosphoenolpyruvate-protein phosphotransferase of PTS system, fructose-specific phosphocarrier protein HPr and fructose-specific IIA component (EC 2.7.1.69). At the end of the domain analysis, it was obtained that this system composed of IIA component and HPr protein as the name of the system implies. This analysis also gave us order of each component. According to domain analysis, IIA component comes first when it slides through 5’ to 3’. Since, it was important to know functions of each component; function analysis of each subunit was also inquired.

PTS system fructose specific IIA subunit is a component of a cytoplasmic enzyme, necessary for the uptake of carbohydrates across the cytoplasmic membrane and their phosphorylation. Histidine-containing phosphocarrier protein (HPr) is a central component of the bacterial phosphoenolpyruvate sugar phosphotransferase system (PTS). The PTS catalyses the phosphorylation of sugar substrates during their translocation across the cell membrane. The phosphoryl group from phosphoenolpyruvate is transferred to HPr by enzyme I (EI). Phospho-HPr then transfers the phosphoryl group to one of several sugar-specific phosphoprotein intermediates. The conserved histidine in the N-terminus of HPr serves as an acceptor for the phosphoryl group of EI. In addition to the phosphotransferase proteins HPr and E1, this family also includes the closely related Carbon Catabolite Repressor (CCR) proteins which use the same phosphorylation mechanism and interact with transcriptional regulators to control expression of genes coding for utilization of less favored carbon sources.

Components of phosphoenolpyruvate-protein phosphotransferase system, fructose-specific phosphocarrier protein HPr and fructose-specific IIA component, were shown in Figure 4.1.

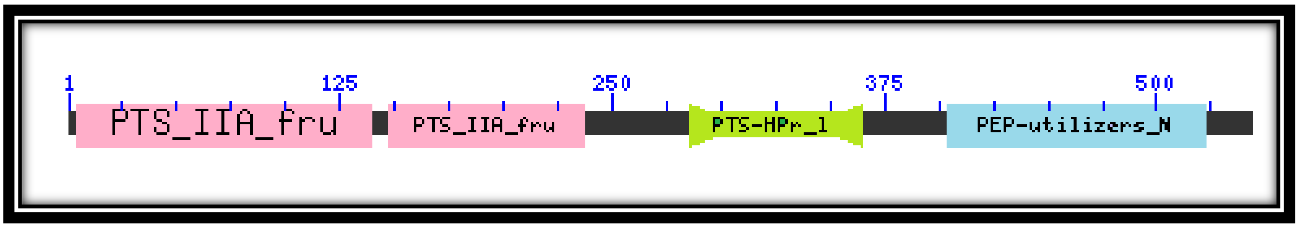


Figure 4.1: PTSfru system that composed of EIIA, HPr ve EI (PEP utilisers) components. (NCBI- Conserved domain architecture retrival tool, http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi)

After the determination of target gene region and establishment of all components belong to this region, the central component of this PTS^{fru} system, HPr was chosen as insert area for further genetic manipulations.The strategy that will be followed was constituted by three subsequent constructions: Construction of a vector that carrying the HPr region which was then used for second construction by insertion of omega region. Third construction was carried out with transfer of HPr+Omega region into a suicide vector. After all constructions performed, final product was used as donor strain for conjugal mating with target organism *H. smyrnensis* AAD6 via a helper plasmid.

4.2 Amplification of the HPr region and Construction of First Vector

4.2.1 PCR Optimization

At first, genomic DNA isolation was performed from *H. smyrnensis* AAD6 in order to be used as template DNA for PCR. The amplification of HPr region was performed specifically designed HPr-F and HPr-R primers as explained in section 3.5.1. To decide optimum PCR reaction mixture and conditions, a lot of trials were designed and performed. High on the list of optimization variables are Mg++ concentrations, buffer pH, and cycling conditions (Roux, 2009).

The most critical parameter for successful PCR is optimal oligonucleotide primer design. A poorly designed primer can result in little or no product, due to nonspecific amplification and/or primer-dimer formation leading to reaction failure, even when all the other parameters are properly optimized (Apte et al., 2009). The forward and reverse primers must have an exact base match with the beginning and end of the target region since a single-base mismatch lowers the Tm by ~5°C (Roux, 2009). Thus, the design of the primer is the most essential point. Concentration of primers is important, as well. Using an excessive concentration of primers can increase the chance of primers binding nonspecifically to undesired sites on the template or to each other. If the primer concentration is too low, annealing may be inefficient. Well-designed primers should be used at 0.2–1 μM concentration in the final reaction. Contaminants in primers may inhibit PCR, too. Desalted primers or more highly purified primers should be employed.

Magnesium Chloride ($MgCl_2$) concentration is also important for PCR reaction. Magnesium ion ($Mg^{++)}$ concentration is the easiest to manipulate because all concentration variations can be run simultaneously in separate tubes. Insufficient or omitted magnesium will result in no or reduced PCR product. Also, using high concentrations of magnesium increases the likelihood of nonspecific primer binding and unwanted product formation. Suppliers of Taq polymerase now provide MgCl2 solution separate from the rest of the standard reaction buffer to simplify its adjustment. A typical two-step optimization series might first include Mg^{++} at 0.5 mM increments from 0.5 to 5.0 mM and, after the range is narrowed, a second round covered by several 0.2 or 0.3 mM increments. Generally, Mg^{++} usage at 1.5 mM gives good amplicons in the final reaction.

dNTP is another important component that effects amplification. If the dNTP concentration is too high, Mg^{++} depletion occurs. An increase in the concentration of dNTPs decreases the concentration of free Mg^{++} available to influence polymerase function (Roux, 2009). Usage of impure dNTP results in contaminants which can lead to incomplete or incorrect amplification or PCR inhibition. With regard to the last, to prevent dNTP-based contamination, usage of high-quality dNTPs is suggested.

There are also PCR condition based failures such as annealing temperature and cycling times. Excessive cycling increases the opportunity for nonspecific amplification and errors (Bell et al., 1991). Cycles can be adjusted at interval between 20 and 35. Fewer cycles should be used when template concentration is high, and more cycles used when template concentration is low. If the annealing temperature is too low, primers may bind nonspecifically to the template. The rule of thumb is to use an annealing temperature that is 5°C lower than the Tm of the primer. Excessive annealing time may increase spurious priming also. Generally, annealing time for 30 sec is employed for amplifications. Besides, excessive extension time can allow nonspecific amplification. Generally, extension time is adjusted with ratio of 1 min/kb.

Some enhancers like dimethylsulfoxide (DMSO) , polyethyleneglycol (PEG) or glycerol were also employed to increase the amplification yield. Such additives can be incorporated into the reaction to increase specificity and yield (Pomp and Medrano 1991).

It was very difficult to set optimum PCR components and conditions for amplification of HPr region. It took almost 2 months to get desired bands with expected lenght without smear and nonspecific bindings. Low yield or no yield amplifications were overcome with adjusting annealing temperature via employing thermal gradient PCR. Moreover, Mg^{++} and template DNA concentrations were adjusted with titration trials. Finally, best results were observed at annealing temperature of 60.5°C with 1.5 mM and 1 mM, Mg^{++} and template DNA, respectively. All reaction components and conditions were optimized after all these trials and finally carried out as in section 3.5.1.

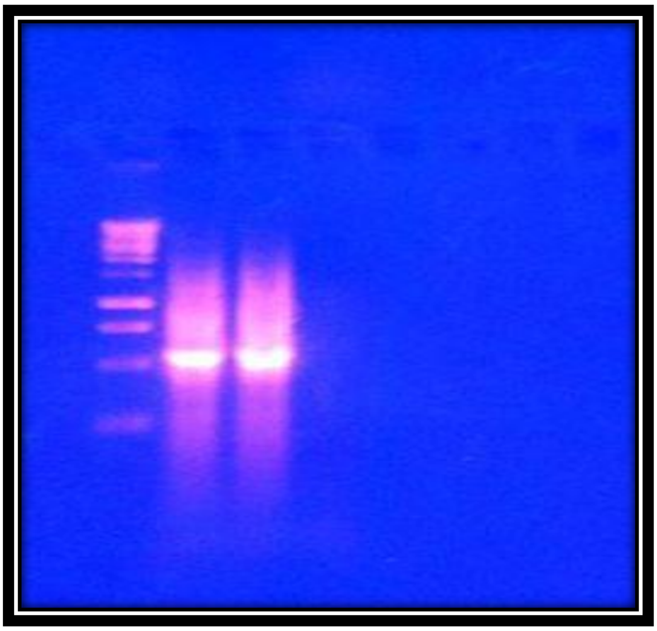


Figure 4.2: Amplification products of HPr Region (bands with smear), before optimization of electrophoresis buffer

After all of PCR optimizations, HPr region was amplified with one undesired exception which one was bands with smear. It was thought that if there were no nonspecific bands, this smear image could source by low ionic capacity of buffer or high voltage. Thus, electrophoresis optimization was required.

4.2.2 Gel Electrophoresis Optimization

At the end of optimized amplification, PCR products were run with agarose gel electrophoresis. It was faced some troubles like smeared, curved or mountain shaped DNA and low intensity of DNA. All of these problems could be based on improper electrophoresis conditions. For instance, an excessively low voltage during the entire run may result in diffusion of bands during electrophoresis. Also, excessively high voltage may result in gel heating and DNA denaturation. To calculate the optimal electrophoresis conditions (voltage) and to use the recommended V/cm value one has to measure the distance between electrodes (cathode and anode) – X, cm, and multiply that X, cm value by the recommended voltage (Y, V/cm), the result (X, cm x recommended Y, V/cm) is Z – recommended voltage to be applied. According to this calculation the voltage that can be applied to device in our lab was between 100V and 120V. Furthermore, excessive electrophoresis run times or voltage may result in migration of small DNA fragments off of the gel. Very short or slow electrophoresis may result in incompletely resolved bands. So, gels should be run at 5-8 V/cm until the dye passes

2/3 of the gel. Plus, in order not to get curved or mountain shaped DNA, electrophoresis buffer should completely cover the entire gel during sample loading and run.

During the running of DNA fragments through the gel buffer has also an essential role. There are two cwell known buffers which are Tris-Borate-EDTA (TBE) and Tris-Acetate-EDTA (TEA). When comparing these two by the reduced current, temperature generation, and gel resolution, TBE was a better conductive medium than TAE (Brody et al, 2004). It was reported that, in continuous voltage electrophoresis, acetate separates large DNA fragments better than borate, although borate resolves smaller DNA fragments (2 kb and lower) well. This has led some investigators to favor TAE in the resolution of larger DNA, and to favor TBE for smaller fragments. This difference among the conductive media may be due to borate–DNA complexes and is partially mitigated by large (greater than 45 mM) concentrations of borate (Stellwagen et al, 2000). Therefore, TBE was employed as electrophoresis buffer during whole study.

At last, all of optimizations were performed and amplification of HPr region without any undesired condition was obtained succesfully. Amplicons of HPr region showed in Figure 4.3.

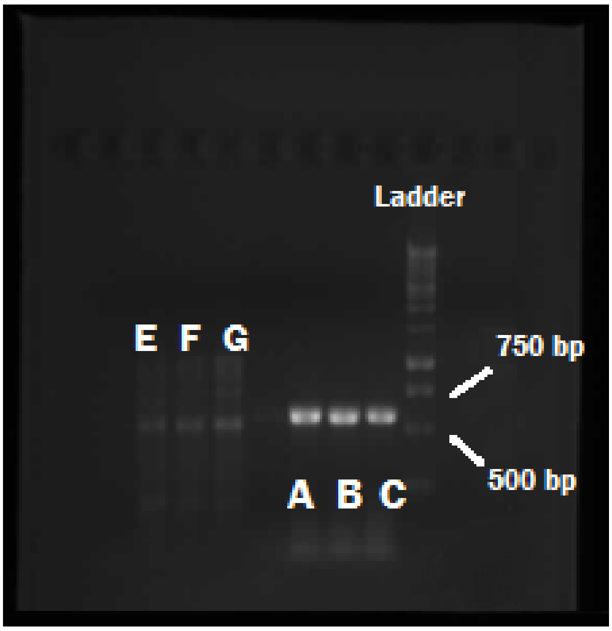


Figure 4.3 : Amplicons of HPr Region after all optimizations. As Lane A,B,C, bands with expected length of 567 bp. In Lane E,F,G bands amplified with *Pfu* DNA Polymerase and some nonspesific bands are seen. GeneRuler 1 kb DNA ladder was used as marker.

4.2.3 pJET1.2/Blunt Cloning Vector

After amplification of target region, it is need to clean the excessive remainings such as enhancers PEG, DMSO or excessive buffer. For this purpose, PCR clean up kit was used before cloning and ligation mixture for transformation. Wizard SV Gel and PCR Clean-Up Kit were used according to manufacturer´s instructions. Cloning of HPr region was carried out by pJet1.2/blunt cloning vector.

The pJet1.2/blunt vector contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. Any DNA fragment, either blunt or sticky-end, can be successfully cloned using this vector. The pJET1.2/blunt vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Recircularized pJET1.2 sequence/blunt vector molecules lacking an insert express a lethal restriction enzyme, which kills the host E. coli cell after transformation. This positive selection drastically accelerates the process of colony screening and eliminates additional costs required for blue/white selection. Plus, the vector contains a T7 promoter for in vitro and in vivo transcription as well as sequencing of the insert. Sequencing primers are included for convenient sequencing of the cloned insert.

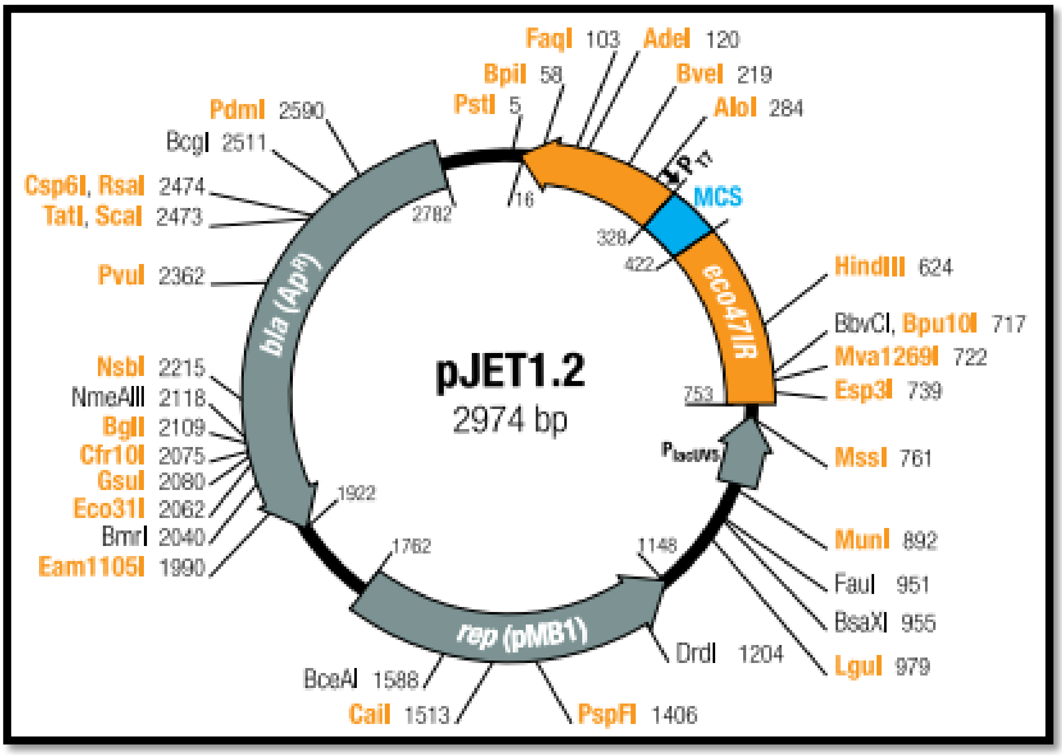


Figure 4.4: Vector map of pJET 1.2

Amplification of HPr region was performed by Taq DNA polymerase, amplicons were sticky-ended. So, sticky-end cloning protocol was followed. The DNA blunting enzyme was a thermostable DNA polymerase with proofreading activity like *Pfu* DNA polymerase. Also, it removed 3’- overhangs and fil-in 5’- overhangs with using nucleotides which are included in reaction buffer of the kit for blunting reaction. Transformation was performed by using of ligation reaction directly. According to manufacturer’s instructions 50 µl competent cell was mixed with maximum 5 µl ligation mixture. Otherwise formation of background colonies which did not carry the insert, could be observed after transformation. At the end of transformation, it was expected to be cloned and insert HPr region into multiple cloning site (MCS) of pJET vector. After overnight incubation, survived colonies since their eco47IR gene provides growing only recombinant colonies that are carrying the HPr region, selected and re-grown on new plates.

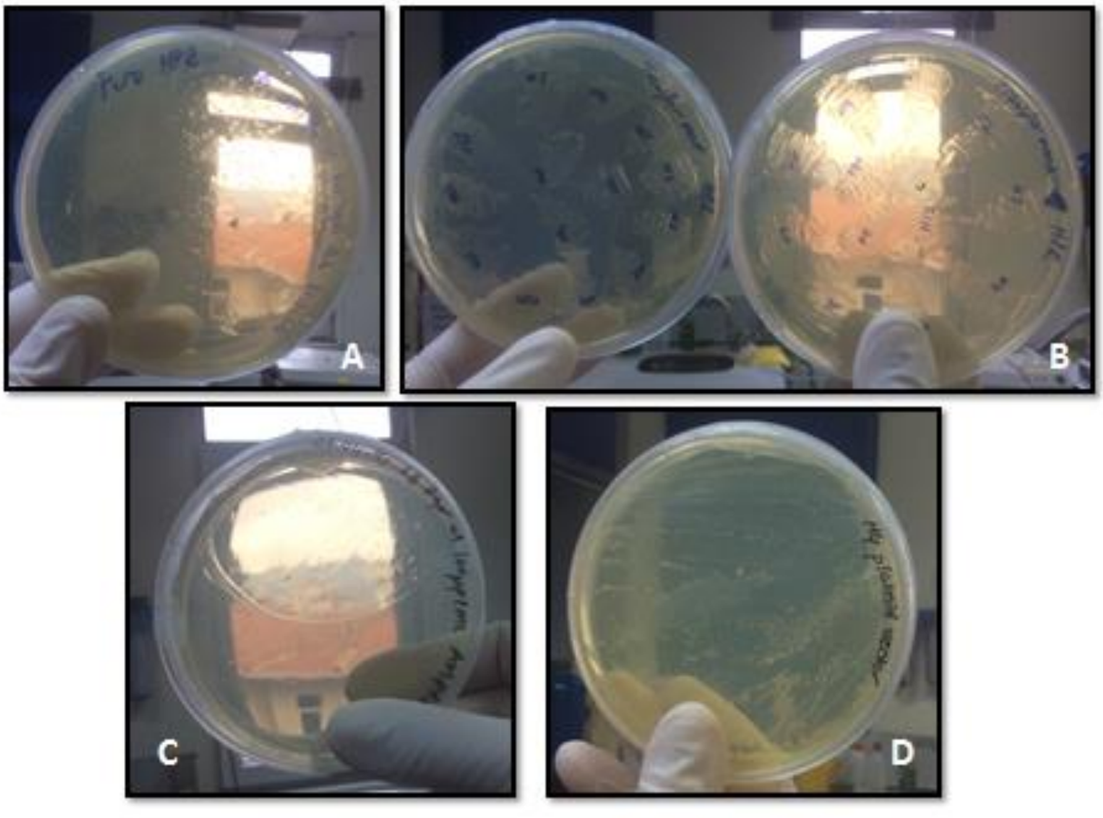


Figure 4.5: Plates of HPr region transformation.

A: Recombinant HPr carrying plasmids,

B: plasmids that were transferred new plates,

C : Negative Control,

D : H4 plasmid which was selected after analyses for other constructions

Then colony PCR were performed for 26 colonies and recombinants that gave the positive HPr bands (567 bp) both with HPr specific and with pJET specific primers, were selected. Three of 26 colonies were chose for sequencing analysis.

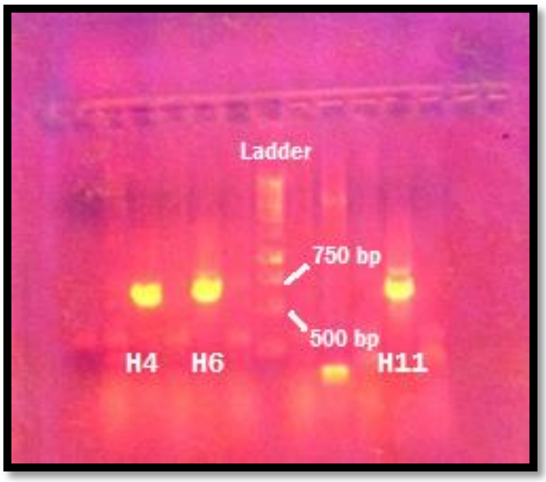


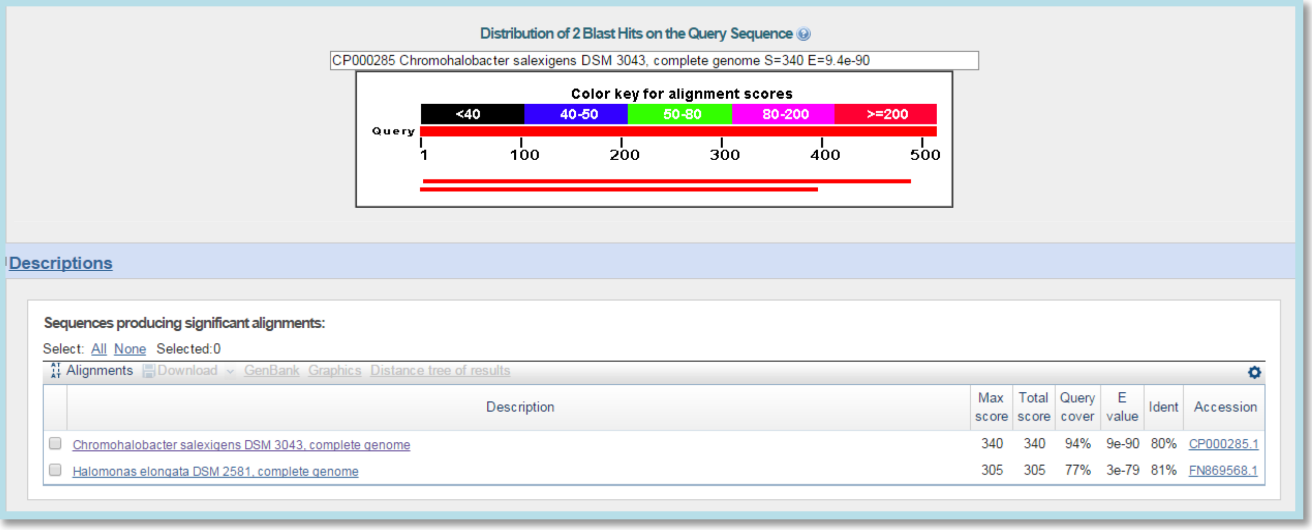
Figure 114.6: Colony PCR of recombinants with pJET-F/R Primers. Marker was GeneRuler 1 kb DNA ladder.

4.2.4 Nucleotide Sequencing Results

Three colonies which were shown in Figure 4.5, H4, H6 and H11 were sent to BGI (Beijing Genome Institution, China) for sequencing. Sequencing is a very precise screening method for recombinants to control whether it contains wrong or right inserts. BGI is using Sanger sequencing method for DNA sequencing.

The ABI3730 sequencer were employed and it uses AB sequencing reagents and interprets bases sequencing using its own judgment value (Quality Value). Depending on the size of the QV value, it is possible to determine the accuracy of each base. ABI’s commitment to the sequencing length of the standard sample is 929bp. Results of analysis consist of a sequencing peak figure, basic analysis, and the final report. BGI sends the results as an .abi chromatogram file which can be analyzed and visualized as peaks by Finch Tv software (Geospiza, Perkin Elmer).

Nucleotide blast were done for these three colonies through their FASTA sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). According to BLAST analyses only H4 plasmid carries the complete HPr region with correct orientation. Also, alignments of original HPr region and HPr region in H4 plasmid were performed with using Clustal omega (ClustalΩ, EMBL) which was a web-based analysis program for multiple sequence alignment that uses seeded guide trees and hidden Markov model (HMM profile-profile) techniques to generate alignments between three or more sequences. Clustal omega results indicated very high resemblance between original HPr sequence and HPr that was carried by H4. Also, megablast analysis gave good results for H4 plasmid. Since, only draft genome information of *H. smynrensis* AAD6 were reported (Sogutcu et al.,2012) , blast analysis resulted in HPr region in *Chromohalobacter salexigens* DSM 3043 and *Halomonas elongata* DSM 2581 which are closest species with regards to their whole genomes. The results of Megablast analyses were shown in Figure 4.7 and Figure 4.8.


Distribution of 2 Blast Hits on the Query Sequence

CP000285 Chromohalobacter salexigens DSM 3043, complete genome S=340 E=9.4e-90

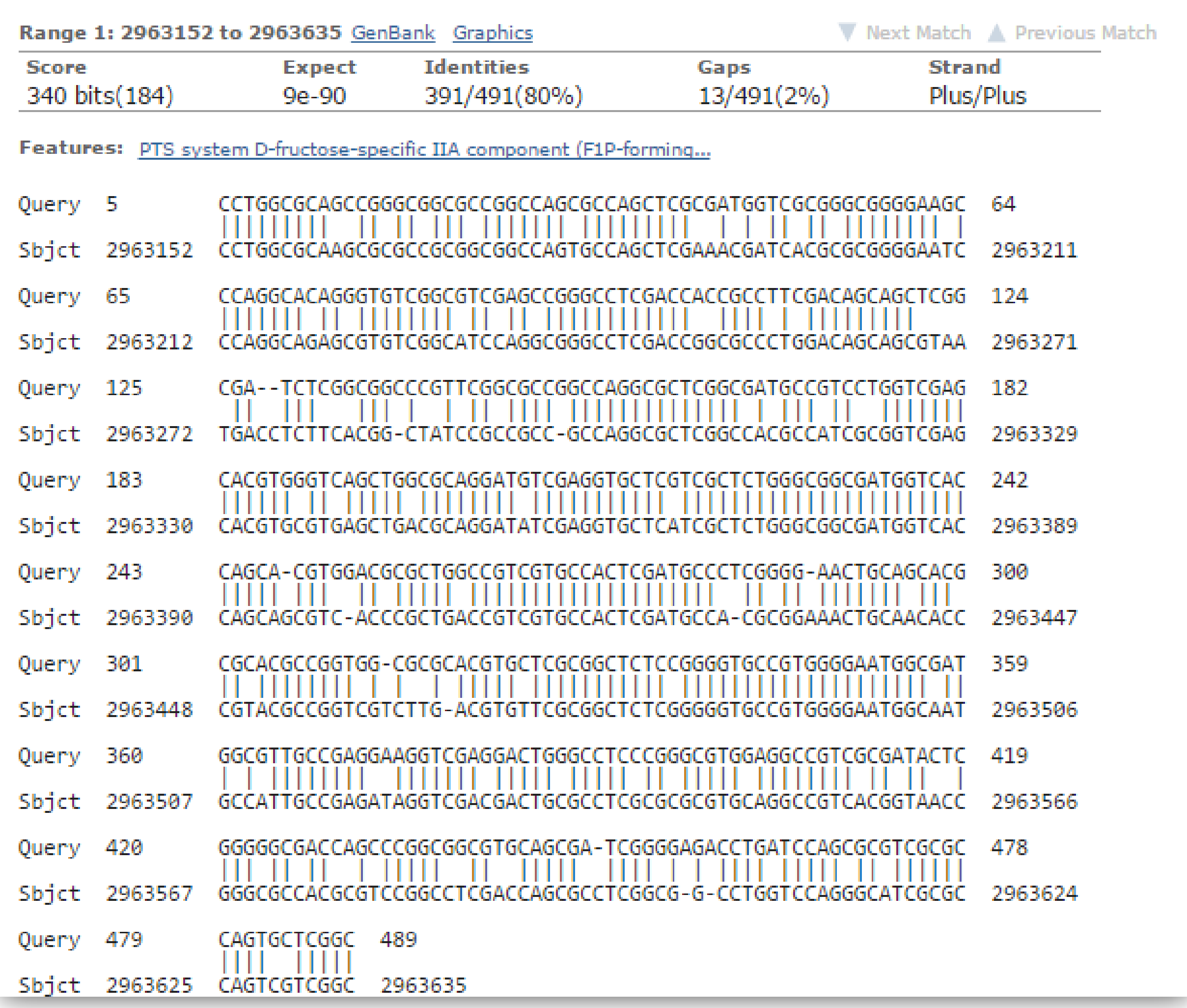
Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Chromohalobacter salexigens DSM 3043, complete genome	340	340	94%	9e-90	80%	CP000285.1
☐	Halomonas elongata DSM 2581, complete genome	305	305	77%	3e-79	81%	FN869568.1

Figure 4.7: Blast analysis results of H4 Plasmid



Chromohalobacter salexigens DSM 3043, complete genome

Sequence ID: gb|CP000285.1| **Length:** 3696649 **Number of Matches:** 1

Range 1: 2963152 to 2963635 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
340 bits(184)	9e-90	391/491(80%)	13/491(2%)	Plus/Plus

Features: PTS system D-fructose-specific IIA component (F1P-forming...

```
Query  5        CCTGGCGCAGCCGGGCGGCGCCGGCCAGCGCCAGCTCGCGATGGTCGCGGGCGGGGAAGC  64
                |||||||||  || || ||| ||||||| |||||||||  | | || || |||||||| |
Sbjct  2963152  CCTGGCGCAAGCGCGCCGCGGCGGCCAGTGCCAGCTCGAAACGATCACGCGCGGGGAATC  2963211

Query  65       CCAGGCACAGGGTGTCGGCGTCGAGCCGGGCCTCGACCACCGCCTTCGACAGCAGCTCGG  124
                ||||||| || |||||||| || || ||||||||||||  |||| | |||||||||    
Sbjct  2963212  CCAGGCAGAGCGTGTCGGCATCCAGGCGGGCCTCGACCGGCGCCCTGGACAGCAGCGTAA  2963271

Query  125      CGA--TCTCGGCGGCCCGTTCGGCGCCGGCCAGGCGCTCGGCGATGCCGTCCTGGTCGAG  182
                 ||  |||   ||| |  | || |||| |||||||||||||| | ||| ||  |||||||
Sbjct  2963272  TGACCTCTTCACGG-CTATCCGCCGCC-GCCAGGCGCTCGGCCACGCCATCGCGGTCGAG  2963329

Query  183      CACGTGGGTCAGCTGGCGCAGGATGTCGAGGTGCTCGTCGCTCTGGGCGGCGATGGTCAC  242
                |||||| || ||||| |||||||| ||||||||||| |||||||||||||||||||||||
Sbjct  2963330  CACGTGCGTGAGCTGACGCAGGATATCGAGGTGCTCATCGCTCTGGGCGGCGATGGTCAC  2963389

Query  243      CAGCA-CGTGGACGCGCTGGCCGTCGTGCCACTCGATGCCCTCGGGG-AACTGCAGCACG  300
                ||||| |||  || ||||| ||||||||||||||||||||  || || ||||||| ||| 
Sbjct  2963390  CAGCAGCGTC-ACCCGCTGACCGTCGTGCCACTCGATGCCA-CGCGGAAACTGCAACACC  2963447

Query  301      CGCACGCCGGTGG-CGCGCACGTGCTCGCGGCTCTCCGGGGTGCCGTGGGGAATGGCGAT  359
                || |||||||| | |  | ||||| ||||||||||| |||||||||||||||||||| ||
Sbjct  2963448  CGTACGCCGGTCGTCTTG-ACGTGTTCGCGGCTCTCGGGGGTGCCGTGGGGAATGGCAAT  2963506

Query  360      GGCGTTGCCGAGGAAGGTCGAGGACTGGGCCTCCCGGGCGTGGAGGCCGTCGCGATACTC  419
                | | ||||||||  ||||||| ||||| ||||| || ||||| |||||||| || ||  |
Sbjct  2963507  GCCATTGCCGAGATAGGTCGACGACTGCGCCTCGCGCGCGTGCAGGCCGTCACGGTAACC  2963566

Query  420      GGGGGCGACCAGCCCGGCGGCGTGCAGCGA-TCGGGGAGACCTGATCCAGCGCGTCGCGC  478
                ||| || ||  | |||||  ||  |||||  |||| | | |||| ||||| || ||||||
Sbjct  2963567  GGGCGCCACGCGTCCGGCCTCGACCAGCGCCTCGGCG-G-CCTGGTCCAGGGCATCGCGC  2963624

Query  479      CAGTGCTCGGC  489
                ||||  |||||
Sbjct  2963625  CAGTCGTCGGC  2963635
```

Figure 4.8: Nucleotide Megablast sequence analysis of H4 plasmid

According to nucleotide blast results HPr region was correctly inserted in H4 plasmid, since blast analysis gave 80% identity with PTS system IIA component and with %94 query cover. Also, insertion was succeeded with correct orientation (insert in plus/plus strand). At the end of these analysis, construction of first vector that was carrying the HPr region was completed, therefore, H4 plasmid was selected for the next steps. Vector map of H4 plasmid was constructed as shown in Figure 4.9.

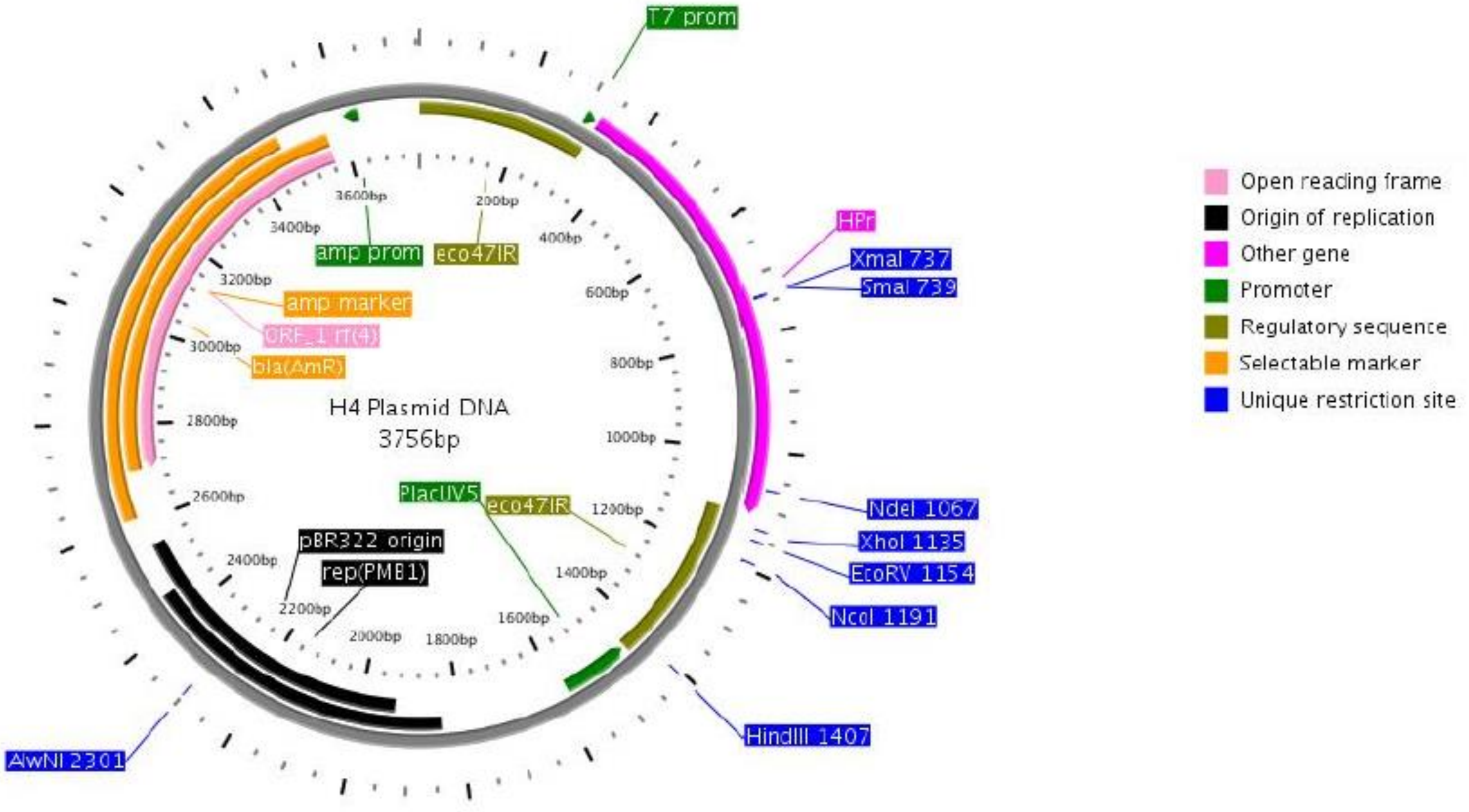


Figure 4.9: Vector construction of H4 plasmid. It was created with Plasmapper web-based software which can be found in this link **http://wishart.biology.ualberta.ca/PlasMapper/**

4.3 Genetic Manipulations on H4 vector for Construction of Second Recombinant Plasmid

After the construction of HPr carrying H4 vector, the next step was amplification of the deletion agent which was chosen as omega transposon. Amplification of Omega transposon from pHP45-omega plasmid was conducted with the same PCR procedure as in the previous one except some points. For instance, primers that were designed specifically for Omega amplification had different annealing temperatures from HPr primers. Also, length of amplicons would be almost 2000 bp, thus extension time should be increased when comparing to HPr amplicons (567 bp). PCR mixture components and conditions were explained in details in section 3.7.1. Moreover, there were some trials for isolation of pHP45-omega plasmid DNA. Firstly, DNA was isolated with High Pure Plasmid Isolation Kit but yield was too low for further manipulations also there were some nicked or linearized DNA fragments above the supercoiled DNA on the gel after isolation. Therefore, template DNA isolation was carried out with alkaline lysis method in small scale. The yield was higher than kit isolation also any other bands were not observed. The comparison of kit isolation and alkaline lysis isolation was shown in Figure 4.10.

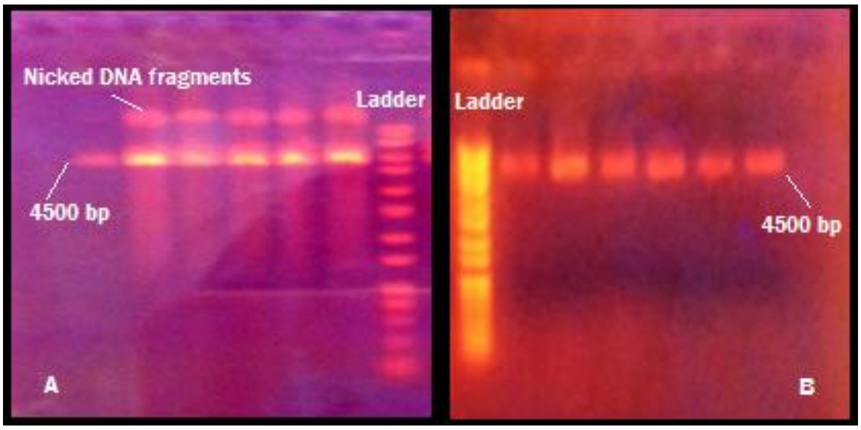


Figure 4.10: Comparison of plasmid DNA isolation, A: plasmid DNA isolation with Kit, nicked or linearized DNA fragments observed, B: Alkaline lysis plasmid isolation there were no other bands except supercoiled DNA bands. (Wide range DNA marker, Sigma was used as ladder)

As in the Figure 4.10 nicked circles are the slowest migrating form in an agarose gel. The differences between two isolations might be sourced due to nuclease contamination or harsh treatment during purification. Since supercoiled plasmid have a slightly better transformation efficiency than its extended open circular form which are transformed at around 75% efficiency of supercoiled ones (Hanahan, 1983) , alkaline lysis method was preferred for isolation of plasmid DNA in order to get rid of relaxed and nicked DNA fragments.

After isolation optimizations, pHP45-omega plasmid DNA was ready to be used as template DNA for omega transposon amplification. Annealing temperature and extension time were adjusted as 57.5^{o}C and 2 min, respectively. Gel image of amplicons were in Figure 4.11. At the end of omega amplification, it was needed to plan a way that provide introduction of omega transposon and H4 vector to each other. It was logical to use restriction enzymes. Thus, an enzyme which had restriction sites for both omega and HPr region was selected. This enzyme has been named as SgrA1 will provide to cut HPr region once from its center and cut omega region from its beginning and end nucleotides. So, only one enzyme could digest HPr from center and omega from both ends. This enzyme made it possible to insert omega region into center of HPr region.

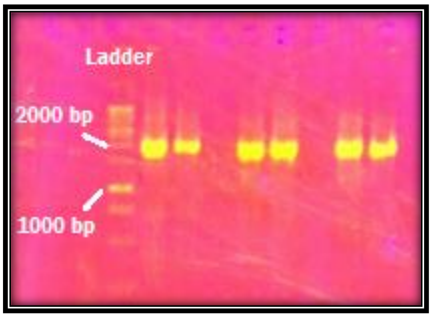


Figure 4.11 : Omega bands on the gel after amplification. Bands have expected length of 2000 bp. GeneRuler 1 kb DNA ladder was used as marker.

After digestion and ligation of H4 vector and omega insert, transformation was performed. At the end of this transformation, it will be possible to obtain a plasmid which carries HPr region and omega at the same time.

4.3.1 Factors That Affect Transformation Efficiency

In previous transformation (transformation of HPr region), manufacturer’s instructions were followed, since they gave the optimized conditions, experiments were performed no need to any replication. But this time for omega transformation into HPr carrying H4 vector, some trials were performed with different variables; such as competent cell amount, enhancer agents, re-treatment or cell recovery medium.

Competent cell amount: 50 μl, 100 μl and 200 μl of competent cell were mixed with 5 μl ligation mixture. Efficiency of transformation depends largely on the competent cells. It was reported that increasing the plasmid to cell ratios 200-fold, from 1:1 to 200:1, only increases the number of transformed cells by six to eight fold (Hanahan, 1983). Transformation which performed with 200 μl gave more transformant colonies so it was decided to carry on experiments with this ratio (competent cell : ligation mixture, 200 μl: 5 μl).

Enhancer agents:

Transformation experiments showed that polyethylene glycol (PEG) also increase the transformation efficiency. In literature, it was reported that depending on the strain of E. coli used, transformation efficiency can be improved by addition of other chemicals such as rubidium chloride, magnesium, manganese, hexamine cobalt, dimethyl sulfoxide, and dithiothreitol to the calcium chloride buffer (Ausubel et al., 2002, Hanahan et al., 1996, Maniatis et al.,1982).

Cell recovery medium:

Transformation trials also indicated that the composition of the medium significantly affects the efficiency of transformation. Another type of broth is now the preferred broth for transformation. SOC broth was employed instead of LB medium for transformation. Because it is superior to LB for transformation purposes because of the refined salt balance and in particular the inclusion of di-valent and mono-valent cations. This broth stands for super optimal broth with catabolite repression and it is a common medium used to transform bacteria.

4.3.2 Screening of HPr+ Omega Carrying Recombinants

After all these trials, transformation was optimized with using SOC medium for growing, with adding PEG to mix of competent cell and ligation mixture and with using 200 μl of competent cell for 5 μl ligation mixture. These optimizations ended up with increased colony formation.

At the end of overnight incubation, almost 60 colonies formed. These colonies were transferred new plates. As mentioned before, the reaction in which 200 μl competent cell used with 5 μl ligation mixture gave more colonies than the reaction that contained 100 μl competent cell. This difference showed in Figure 4.12- A and B.

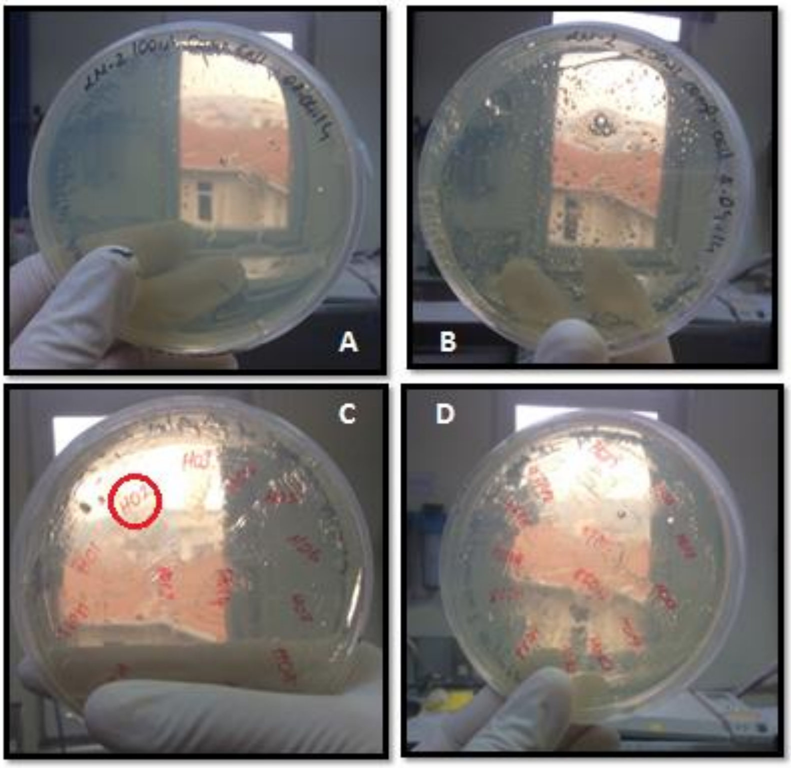


Figure 4.12: Recombinants after second transformation for construction of second vector.

A: Transformation reaction that contains 100 ul competent cell,

B: Reaction that contain 200 ul competent cell,

C: Resultant colonies after retransferring from first plate (HOM 1-HOM14)

D : Resultant colonies after retransferring from first plate (HOM15 – HOM28)

Colony PCR was performed with these colonies using both omega and HPr specific primers. It was needed to select transformants that were carrying HPr and omega at the same time. Colonies were named with HOM initials that refer HPr and omega. Colony PCR results showed that only two colonies which were HOM1 and HOM2 carried omega region (2041 bp). If these colonies carrying omega region, they should also carry HPr region since omega was inserted in the center of HPr region. In order to understand whether omega inserted in the middle of HPr region in these colonies, one another PCR was performed with HPr primers. After amplification with HPr primers, length of amplicons should be approximately 2700-2800 bp since HPr has 567 bp and Omega has 2041 bp. Amplification products were loaded on electrophoresis gel and run, amplicons on agarose gel resulted in bands with expected lengths as in Figure 4.13. Thus, this analysis provided to find a recombinant which carried both HPr and Omega regions (HOM region). The next step for this recombinant should be the determination of insertion direction of HOM region into plasmid DNA. That could only be possible with sequencing of this region with HPr primers. Amplification products were sent to BGI and analysis results showed that insertion was performed with positive strand

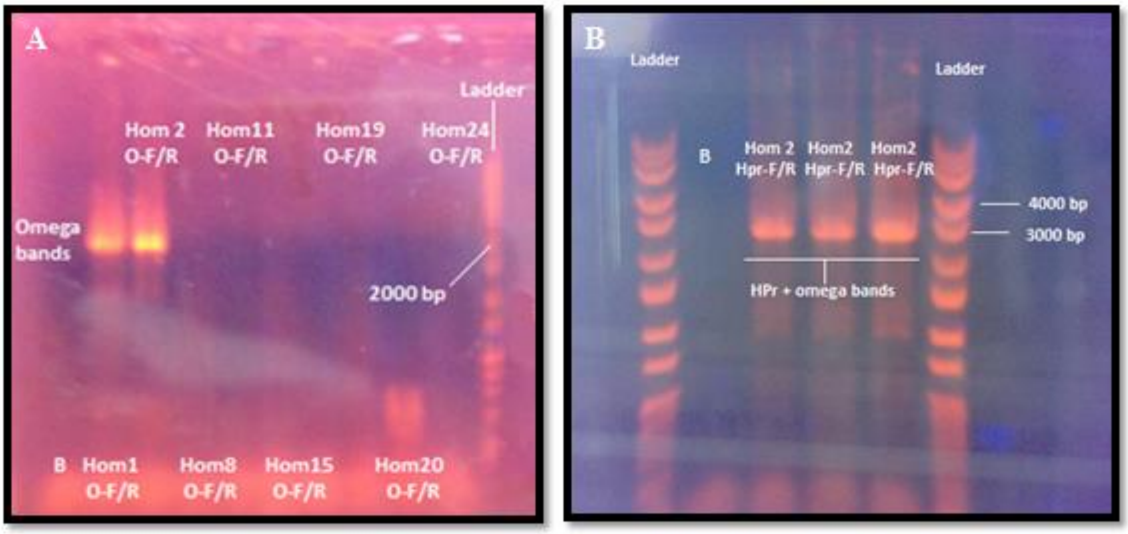


Figure 4.13 : Gel image of recombinants that amplified by colony PCR and HPr + Omega region after recombinant HOM2 amplification with HPr specific primers. Direct-load wide range DNA marker (Sigma) was used as ladder. O-F/R: Omega forward and reverse primers, B: blank. Hpr-F/R : HPr forward and reverse primers

Sequencing analyses were also performed in order to be sure the order of the components in HOM region. According to nucleotide sequence of HOM2, HPr region came first until beginning of SgrA1 cutting site, then omega region was present. At the end of omega region, the remaining HPr region was observed. Since the size of amplicon that was sequenced was too big for sequencing at one run and also it was necessary to know the direction of the regions, forward primer and reverse primer were both used for sequencing. The employment of two primers allowed us to know sequences that could not be read with one primer. Neither forward nor reverse primer could not read entire amplicon until the end of strands. Thus, sequencing by two primers provided to read the regions that was missed by another. Presence of both HPr and omega regions were verified, too.

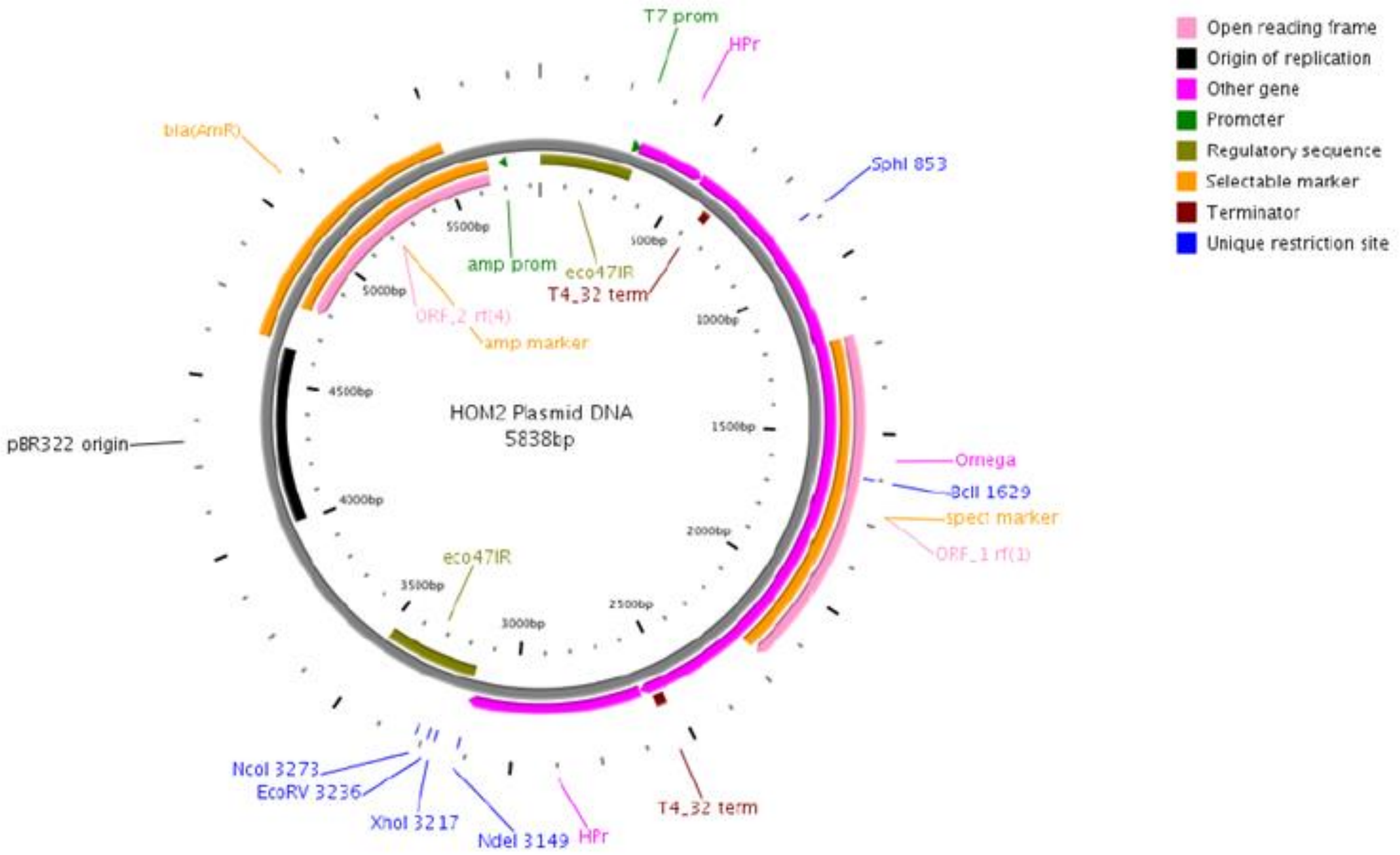


Figure 124.14: HOM2 plasmid vector map (drawn with using Plasmapper)

After sequence analysis, insertion of two regions into HOM 2 plasmid was confirmed, thus construction of second vector was achieved and vector map was drawn as in Figure 4.14.

4.4 Construction of Third Recombinant Plasmid with Using Transformant HOM2

The ability to easily introduce specific point mutations, deletions, or gene fusions into an organism's chromosome to assess the function of a gene in its normal chromosomal context is a powerful method to facilitate the study of complex biological processes. In order to perform insertional mutagenesis for Gram negative bacteria, usage of suicide vector required to obtain positive selection after allelic exchange. Bacterial allelic exchange mediated by recombinant suicide vectors has been used extensively to introduce recombinant or mutated alleles into the chromosomes of both gram-positive and gram-negative bacteria (Maloy et al, 1996, Snyder et al., 1997).

In addition, to facilitate selection for mutated alleles, many suicide vectors contain genes for counter-selection, such as *tetAR* by fusaric acid selection (Maloy et al., 1986), *sacB* by sucrose sensitivity (Ried et al., 1987, Gay et al., 1985) and *rpsL* by streptomycin sensitivity (Stibitz et al., 1986). Recombinant suicide plasmids can be delivered to recipient strains by transduction, electroporation, or conjugation.

In this study, suicide vector was pUK4134 which can be used for allelic exchange in a wide variety of bacterial species because their R6K origin of replication function only in bacteria engineered to produce the replication protein. In addition, it was reported that this vector express the *rpsL* gene form *Escherichia coli* that is responsible for encoding ribosomal protein S12, which provides a positive selection for bacteria that have exchanged cloned plasmid sequences with the corresponding chromosomal sequences. Also, this vector contains *bla* gene provides to select by ampicillin resistance, EcoRV cloning site, *oriT* of plasmid RK2 and *cos* sequence (Skorupski et al.,1996). The construction of gene knockout mutant is carried out by allelic exchange of the chromosomal gene with a mutant allele disrupted by the insertion of a kanamycin resistance cassette (Ortiz et al., 2006).

Constructed HPr + omega region will have been used as an insertion for pUK4134 suicide vector. However it was needed to extract HPr + Omega region from HOM2 recombinant plasmid. The extraction of this region was carried out by firstly designing a couple of primers based on HOM2’s sequence analysis that amplifies HPr+ Omega region only. They were named as HOM forward and reverse primers. Secondly, a strategy was designed for introduction of insert and vector in ligation process. According to this strategy, since pUK4134 vector has a cutting site for BamHI restriction enzyme, another enzyme for insert should have a relationship with BamHI somehow. Thus, BglII enzyme was selected for this mission because the overhang sequences resulting from BamHI and BglII digest are complementary and base-pairs of them anneal perfectly with one-another. In brief, these restriction endonucleases produce compatible cohesive ends during ligation. In order to binding of BglII to HPr + Omega region primers were designed including cutting sites for BglII. Therefore, amplification of HPr + omega region provides creation of a site that is specific for BglII recognition.

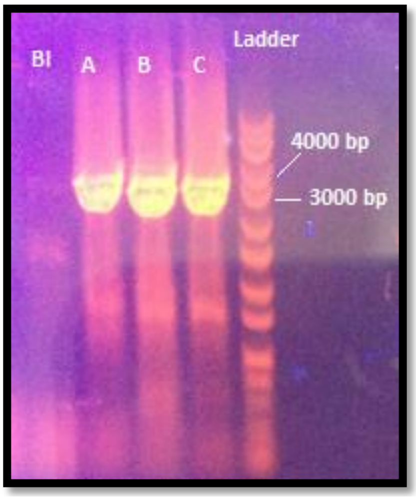


Figure 4.15: Amplification of HPr+ omega region with newly designed HOM primers and using HOM2 plasmid as template DNA. Direct-load Wide Range DNA Marker (Sigma) was employed as ladder.

Lane A, B, C: HPr+ Omega amplicons that include BglII restriction site.

Lane Bl: Blank of amplification

For ligation, pUK4134 was digested with BamHI and amplified HPr + Omega region was cut by BglII. These two were ligated by T4 DNA Ligase then transformation performed with previously optimized conditions. Survived transformants were shown in Figure 4.16. Then, colony PCR was performed with these survivor colonies with using HOM primers. Sequencing analysis was performed with colonies which gave positive results for HPr+Omega region amplification with HOM primers.

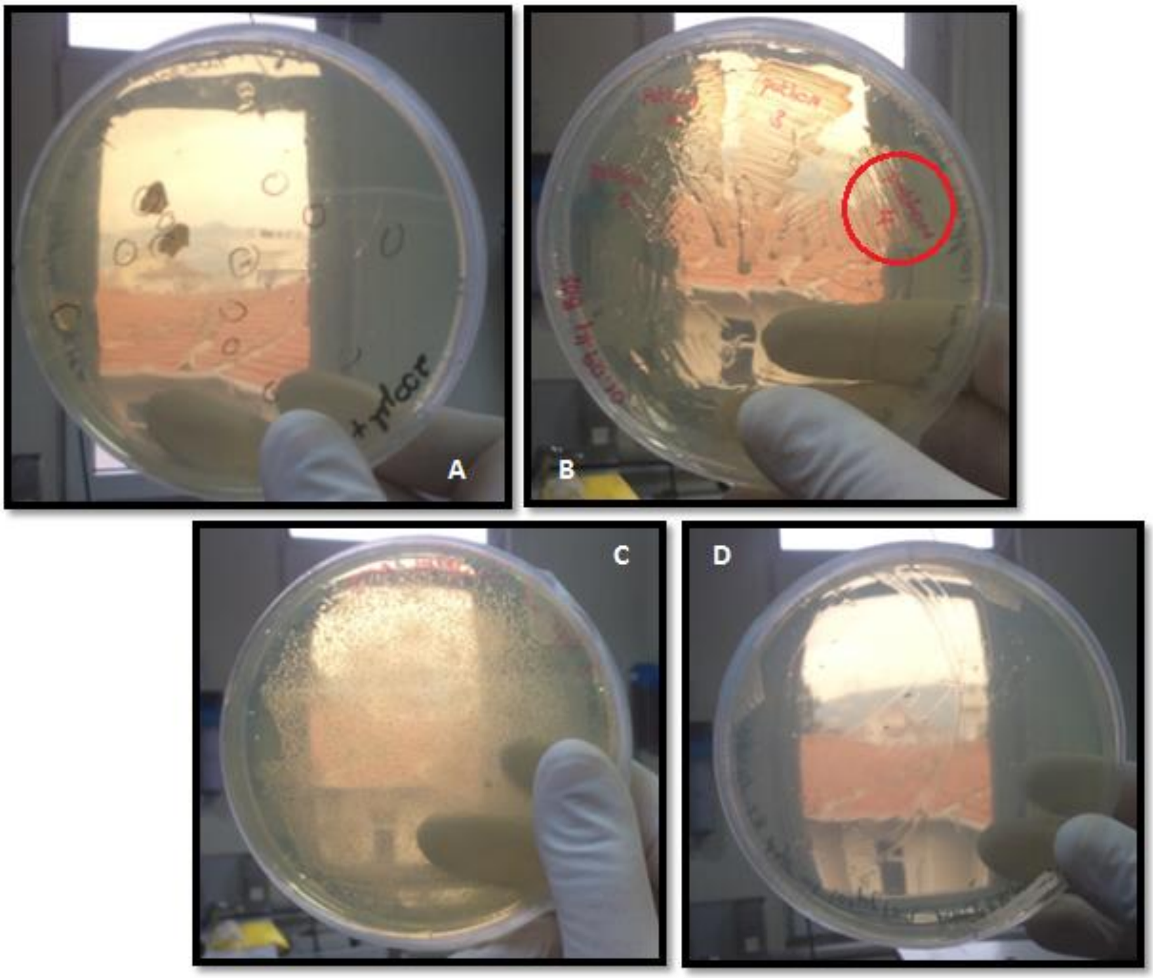


Figure 4.16 : Transformation plates of third construction**.**

A: First transformation plate,

B: Colonies after retransferring to new plate only few of them survived,

C: Plate contain only Amp,

D: Negative control

As in the Figure 4.16-A, there were less colonies than plate contain only amp (Figure 4.16-C). This transformation was based on selection with kanamycin so main plate was aided with Km+ Amp antibiotics for good selection of recombinants. That’s why the colony number increased in plates with only amp, since surviving with one antibiotic is easier than two. In Figure 4.16-D, there was no colony formation as expected. If there is colony formation in negative control, this means antibiotics probably did not work or loss its activity because of adding in medium at high temperatures. Re-transferring of colonies into new plates was required to obtain colonies with real resistance to Km. Resistance to an antibiotic could be determined at least after a passage. Increasing passages allows obtaining plasmid with good resistance. For this reason, colony number may decrease with re-transferring as in the Figure 4.16-A and 4.16-B. There were 13 survived colonies after transformation and 10 of them were re-transferred to new plates again with Km+ Amp and only 4 of them could survive. Moreover, colonies were labeled as PukHOM. Colony PCR was performed with these 4 colonies using HOM primers and gel electrophoresis of these products were run on agarose gel showed in Figure 4.17.

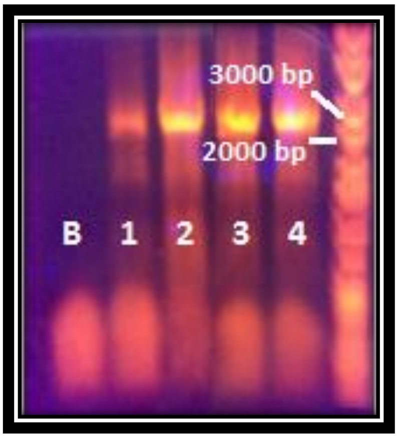


Figure 4.17: Colony PCR products of recombinant PukHOM colonies for third construction.

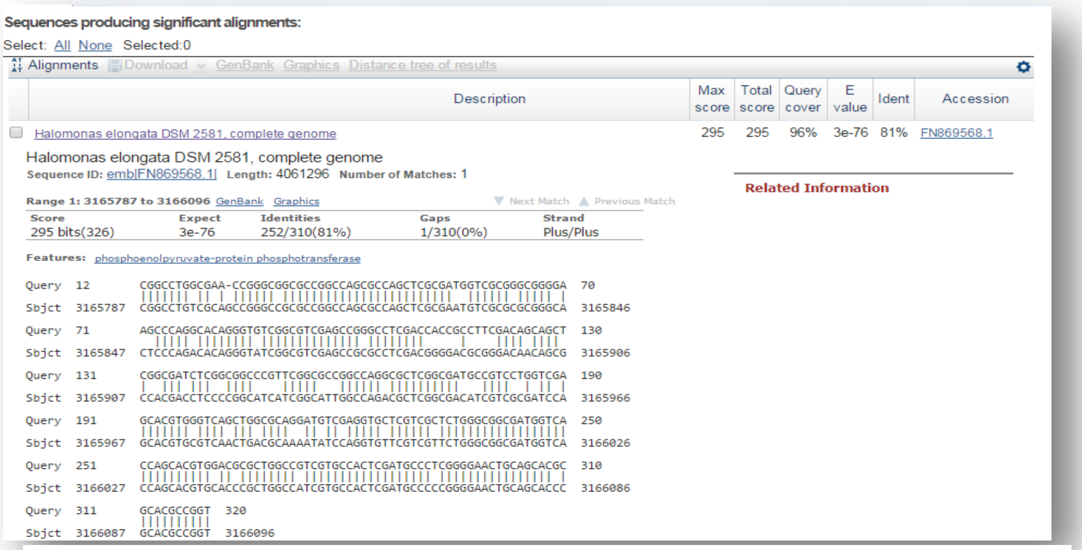
Lane 1: Colony PCR with using PukHOM1 plasmid DNA as template with HOM primers

Lane 2: PukHOM2 plasmid DNA was used as template

Lane 3: PukHOM3 plasmid DNA was used as template

Lane 4: PukHOM4 plasmid DNA was used as template

Colony PCR turned out with correct amplicons with expected length for four colonies. All of them were carrying HOM region. In order to decide which one of them used for further manipulations, sequencing was also performed for PukHOM2 and PukHOM4 colonies. It was expected that sequencing should result in two regions when blast analysis performed.



Sequences producing significant alignments:

Select: All None Selected:0

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Halomonas elongata DSM 2581, complete genome	295	295	96%	3e-76	81%	FN869568.1

Halomonas elongata DSM 2581, complete genome

Sequence ID: emb|FN869568.1| **Length:** 4061296 **Number of Matches:** 1

Range 1: 3165787 to 3166096 GenBank Graphics

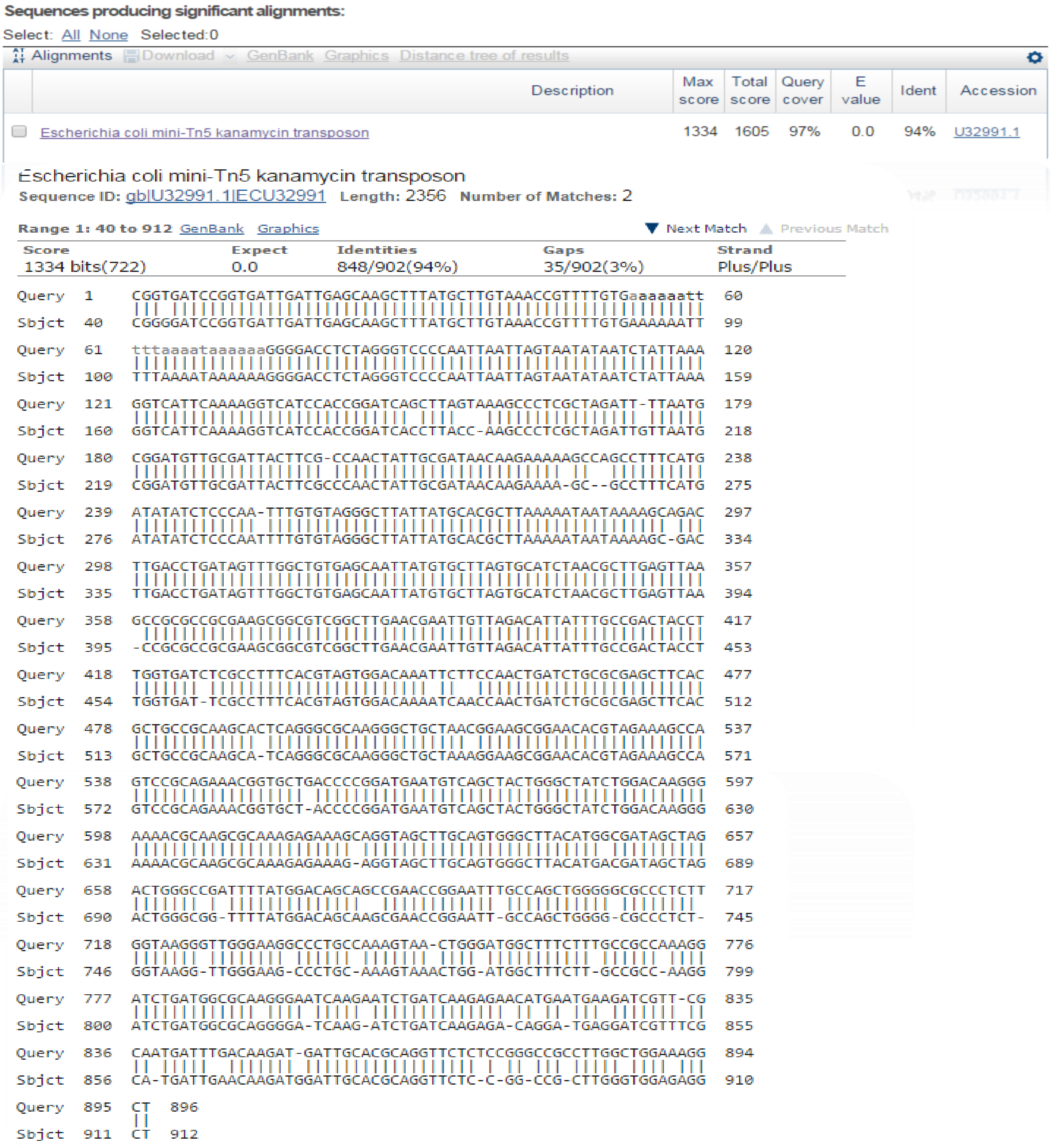
Score	Expect	Identities	Gaps	Strand
295 bits(326)	3e-76	252/310(81%)	1/310(0%)	Plus/Plus

Features: phosphoenolpyruvate-protein phosphotransferase

```
Query  12       CGGCCTGGCGAA-CCGGGCGGCGCCGGCCAGCGCCAGCTCGCGATGGTCGCGGGCGGGA  70
                ||||||| || | |||||| ||||||||||||||||||||||||  |||||| ||||| |
Sbjct  3165787  CGGCCTGTCGCAGCCGGGCCGCGCCGGCCAGCGCCAGCTCGCGAATGTCGCGCGCGGGCA  3165846

Query  71       AGCCCAGGCACAGGGTGTCGGCGTCGAGCCGGGCCTCGACCACCGCCTTCGACAGCAGCT  130
                  ||||| |||||||| |||||||||||||| ||||||||     |    |||| |||| 
Sbjct  3165847  CTCCCAGACACAGGGTATCGGCGTCGAGCCGCGCCTCGACGGGGACGCGGGACAACAGCG  3165906

Query  131      CGGCGATCTCGGCGGCCCGTTCGGCGCCGGCCAGGCGCTCGGCGATGCCGTCCTGGTCGA  190
                |  ||| |||  ||||    |||||   |||||| ||||||||||   ||||  | || |
Sbjct  3165907  CCACGACCTCCCCGGCATCATCGGCATTGGCCAGACGCTCGGCGACATCGTCGCGATCCA  3165966

Query  191      GCACGTGGGTCAGCTGGCGCAGGATGTCGAGGTGCTCGTCGCTCTGGGCGGCGATGGTCA  250
                ||||||| |||| ||| ||||  |  || ||||| |||||| ||||||||||||||||||
Sbjct  3165967  GCACGTGCGTCAACTGACGCAAAATATCCAGGTGTTCGTCGTTCTGGGCGGCGATGGTCA  3166026

Query  251      CCAGCACGTGGACGCGCTGGCCGTCGTGCCACTCGATGCCCTCGGGGAACTGCAGCACGC  310
                |||||||||| || |||||||| |||||||||||||||||| |||||||||||||||| |
Sbjct  3166027  CCAGCACGTGCACCCGCTGGCCATCGTGCCACTCGATGCCCCCGGGGAACTGCAGCACCC  3166086

Query  311      GCACGCCGGT  320
                ||||||||||
Sbjct  3166087  GCACGCCGGT  3166096
```

Figure 4.18: Blast analysis of sequenced PukHOM4 colony showed high resemblance with HPr region

According to sequencing results, PukHOM4 eventuated with inevitable these two regions with correct orientation. Blast results were given in Figure 4.18 and Figure 4.19. Query coverage and identity value of the analyzed sequences were also good for both omega and HPr region.



Sequences producing significant alignments:

Select: All None Selected:0

Description	Max score	Total score	Query cover	E value	Ident	Accession
☐ Escherichia coli mini-Tn5 kanamycin transposon	1334	1605	97%	0.0	94%	U32991.1

Escherichia coli mini-Tn5 kanamycin transposon

Sequence ID: gb|U32991.1|ECU32991 **Length:** 2356 **Number of Matches:** 2

Range 1: 40 to 912 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1334 bits(722)	0.0	848/902(94%)	35/902(3%)	Plus/Plus

```
Query  1    CGGTGATCCGGTGATTGATTGAGCAAGCTTTATGCTTGTAAACCGTTTTGTGaaaaaatt  60
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  40   CGGGGATCCGGTGATTGATTGAGCAAGCTTTATGCTTGTAAACCGTTTTGTGAAAAAATT  99

Query  61   tttaaaataaaaaaGGGGACCTCTAGGGTCCCCAATTAATTAGTAATATAATCTATTAAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100  TTTAAAATAAAAAAGGGGACCTCTAGGGTCCCCAATTAATTAGTAATATAATCTATTAAA  159

Query  121  GGTCATTCAAAAGGTCATCCACCGGATCAGCTTAGTAAAGCCCTCGCTAGATT-TTAATG  179
            ||||||||||||||||||||||||||||| ||||   |||||||||||||||| ||||||
Sbjct  160  GGTCATTCAAAAGGTCATCCACCGGATCACCTTACC-AAGCCCTCGCTAGATTGTTAATG  218

Query  180  CGGATGTTGCGATTACTTCG-CCAACTATTGCGATAACAAGAAAAAGCCAGCCTTTCATG  238
            |||||||||||||||||||| |||||||||||||||||||||||| ||  ||||||||||
Sbjct  219  CGGATGTTGCGATTACTTCGCCCAACTATTGCGATAACAAGAAAA-GC--GCCTTTCATG  275

Query  239  ATATATCTCCCAA-TTTGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGAC  297
            ||||||||||||| |||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  276  ATATATCTCCCAATTTTGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGC-GAC  334

Query  298  TTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTAACGCTTGAGTTAA  357
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335  TTGACCTGATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTAACGCTTGAGTTAA  394

Query  358  GCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCT  417
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  395  -CCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTGCCGACTACCT  453

Query  418  TGGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAACTGATCTGCGCGAGCTTCAC  477
            ||||||| ||||||||||||||||||||||| ||  ||||||||||||||||||||||||
Sbjct  454  TGGTGAT-TCGCCTTTCACGTAGTGGACAAAATCAACCAACTGATCTGCGCGAGCTTCAC  512

Query  478  GCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAACGGAAGCGGAACACGTAGAAAGCCA  537
            ||||||||||||| ||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  513  GCTGCCGCAAGCA-TCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCA  571

Query  538  GTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGG  597
            |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  572  GTCCGCAGAAACGGTGCT-ACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGG  630

Query  598  AAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAG  657
            ||||||||||||||||||||||| ||||||||||||||||||||||||| ||||||||||
Sbjct  631  AAAACGCAAGCGCAAAGAGAAAG-AGGTAGCTTGCAGTGGGCTTACATGACGATAGCTAG  689

Query  658  ACTGGGCCGATTTTATGGACAGCAGCCGAACCGGAATTTGCCAGCTGGGGGCGCCCTCTT  717
            ||||||| | ||||||||||||||  |||||||||||| ||||||||||| |||||||| 
Sbjct  690  ACTGGGCGG-TTTTATGGACAGCAAGCGAACCGGAATT-GCCAGCTGGGG-CGCCCTCT-  745

Query  718  GGTAAGGGTTGGGAAGGCCCTGCCAAAGTAA-CTGGGATGGCTTTCTTTGCCGCCAAAGG  776
            ||||||| |||||||| |||||| ||||||| |||| ||||||||||| |||||| ||||
Sbjct  746  GGTAAGG-TTGGGAAG-CCCTGC-AAAGTAAACTGG-ATGGCTTTCTT-GCCGCC-AAGG  799

Query  777  ATCTGATGGCGCAAGGGAATCAAGAATCTGATCAAGAGAACATGAATGAAGATCGTT-CG  835
            ||||||||||||| |||| ||||| |||||||||||||| || || ||| ||||||| ||
Sbjct  800  ATCTGATGGCGCAGGGGA-TCAAG-ATCTGATCAAGAGA-CAGGA-TGAGGATCGTTTCG  855

Query  836  CAATGATTTGACAAGAT-GATTGCACGCAGGTTCTCTCCGGGCCGCCTTGGCTGGAAAGG  894
            || |||||  ||||||| |||||||||||||||||| | || ||| ||||| |||| |||
Sbjct  856  CA-TGATTGAACAAGATGGATTGCACGCAGGTTCTC-C-GG-CCG-CTTGGGTGGAGAGG  910

Query  895  CT  896
            ||
Sbjct  911  CT  912
```

Figure 4.19: Blast analysis of sequenced PukHOM4 colony gave also omega region

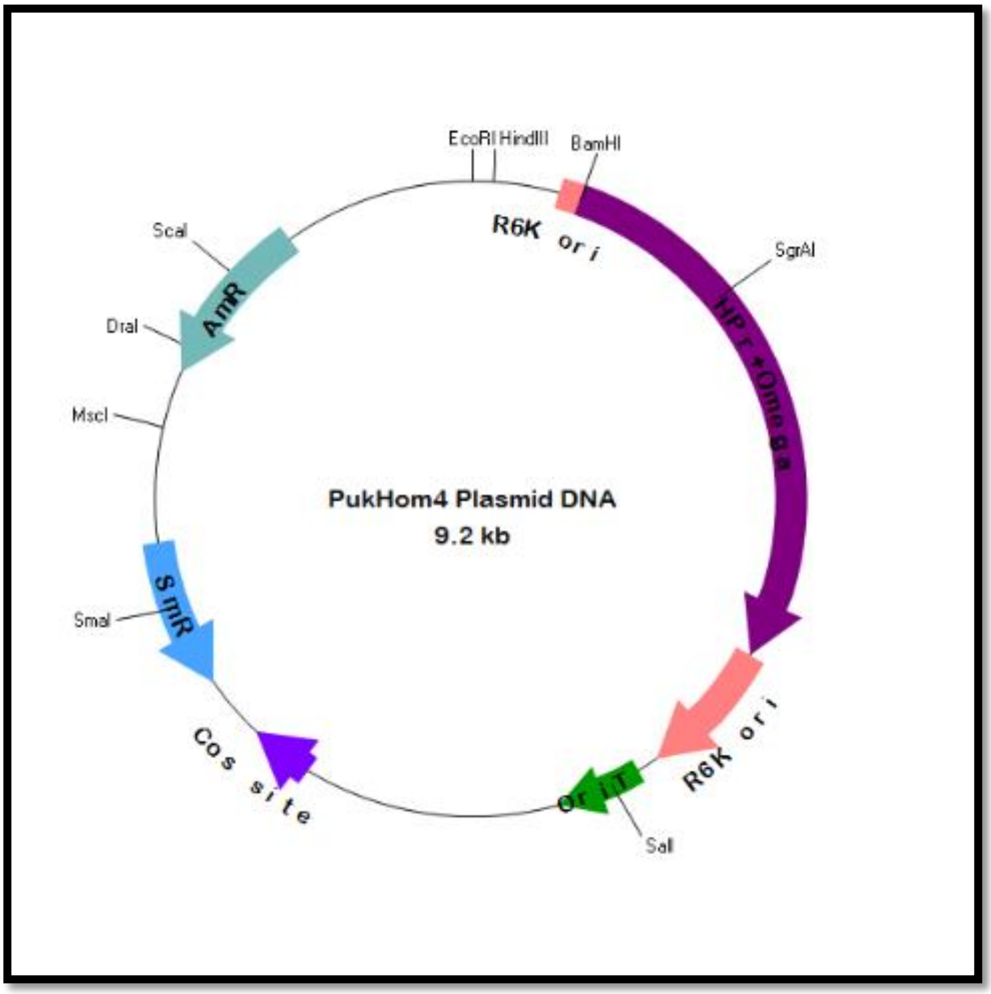


Figure 4.20: Vector map of constructed plasmid PukHOM4 drawn with pDRAW32 DNA Analysis Software (This program can be downloaded from http://www.acaclone.com/download/download.html)

Correspondingly, construction of third recombinant plasmid was completed with certain results. Vector map of third construct which was pUKHOM4 showed in Figure 4.20. At the beginning of this thesis, it was aimed to make a construct for inactivation or deletion of HPr region in *H. smyrnensis* AAD6. This objective came into real when these three constructions were completed accurately. That was the hardest part of whole work and took really long time. As a conclusion for this part, the final construct which was PukHOM4 will be culminated with deletion in the following step. PukHOM4 will be employed for conjugal transformation by triparental mating as donor plasmid.

4.5 Bacterial Conjugation of PukHOM4 and *H. smyrnensis* via Helper Plasmid

After completion of desired construction that had both HPr and omega regions on a suicide plasmid, the next step was transformation of this region into recipient *H. smyrnensis* AAD6. For transmission of PukHOM4 plasmid DNA into target genome, bacterial conjugation was employed. Gram-negative conjugation can be considered as a specialized replicative event that increases the population size of the plasmid during its horizontal transfer between organisms (Matson et al., 1993). The process can be divided into two stages. The first involves the formation of a specialized bridge between the plasmid-containing donor and the recipient cell, which are brought into connection by the extracellular conjugative pilus. The second stage concerns the transfer and processing of DNA. This stage is initiated by nicking of the plasmid at the specific origin of transfer (*oriT*) site and is followed by unwinding of the duplex form by one or more DNA helicases and the transfer of the open DNA strand to the recipient cell. The transferred DNA is then circularized by a mechanism involving (Matson et al., 1993).

There was a requirement for the pilus in the mating process. Conjugative systems form a filamentous appendage called pilus. The pilus was considered to have a role in attachment to the recipient. The pilus itself worked as a channel inside which the DNA would travel from one cell to the other. The recent finding shows that although Ti plasmid Vir mutants that do not produce pili for conjugation, they can manage the transfer (Sagulenko et al., 2001), there are many argues against a role for the pilus as a DNA channel.

Bacterial conjugation may differ with corresponding to different species. Regardless of the type of system, the secreted substances or other possible functions in attachment or transport, it is known that pili are associated with systems intended to reach the cytoplasm of either plants or Gram-negative bacterial cells, because they show differences among their surface structures. Moreover, they are recipients that present additional barriers in their envelopes when compared with the single lipid bilayer present in animal cells. As reported in a study (Llosa and Zambryski, 1998), pili could be required for opening the way through additional barriers and envelopes that are present in the recipient, therefore allowing the entrance of DNA and proteins through a bacterial secretion system.

In this thesis, conjugation was carried out by triparental mating method (Vargas et al., 1997). It was composed of donor strain which is PukHOM4 contains mobilizable plasmid of interest, helper strain that is pRK2013 has conjugative plasmid that can mobilize desired plasmid into recipient strain and a recipient strain *H. smyrnensis* AAD6.

Conjugation machinery works actively with four components which were origin of transfer (*oriT*) site, gene for relaxase protein, type IV coupling protein and type IV secretion system (DiLaruenzio,1990). In this study, these components were provided by pRK2013 since it is a self-transmissible and conjugative plasmid in this process, it was reported that self-transmissible vectors code for the four components of a conjugative apparatus: an origin of transfer (oriT), a relaxase (R), a type IV coupling protein (T4CP), and a type IV secretion system (T4SS) (Smillie et al., 2010).

Plasmid mobility types were also important for designing conjugation process. It was essential to know selected plasmid were whether conjugative (self-transmissible), mobilizable or non-mobilizable. It was reported that pRK2013 was conjugative (Figurski et al., 1979, Ditta et al., 1980).

Conjugation process generally start with relaxase which catalyzes nicking of *oriT* in donor plasmid and finalizes ligation of transported DNA in recipient and DNA replication occurs by rolling circle replication (Brown et al.,2002). In proposed study, this *oriT* provided by pRK2013. Also, pRK2013 was used for mobilization of non-self-transmissible plasmids. Helper plasmids are widely applicable and have distinct advantages for plasmid or chromosome mobilization (Klein et al.,1992, Nassif et al., 1989, Reimman et al., 1988, Simon et al., 1984 and 1989, Tomcsanyi 1990). The mobilization is commonly mediated by cointegration; the replicon to be mobilized is fused to a conjugative plasmid. In this study, PukHOM4 was mobilizable donor strain and pRK2013 was the mobilization vector.

Triparental conjugation involves two steps, first one helper-donor conjugation in which conjugation initiated by self-mobilizable helper plasmid pRK2013 and helper plasmid is trans-conjugated into donor strain that contains plasmid with desired functions, in second step donor plasmid is mobilized by helper and transfers desired donor plasmid into recipient *H. smyrnensis* AAD6 cell. However, the helper plasmid was not found in trans-conjugants at the end of conjugation, presumably because of its inability to replicate itself (Shoemaker et al., 1986) pHP45-omega also is a transposon vector which can be used as shuttle vector for transfer genes between distantly related bacteria in other studies by promoting chromosome mobilization in E. coli and Rhizobium sp. (Johnson et al.,1988).

Conjugation and plasmid mobilizations were carried out in this study for member of *Halomonas sp.*, these methods also have been useful as methods for the introduction of DNA into the Bacteroides group (Macrina, 1993).

Two types of nutrient solid medium plates were used during conjugation. One of them was100 μg/ml Km and Str antibiotic aided optimum medium plates for selection of only halophile trans-conjugants since this medium contains high amount of NaCl and it was suitable for only salt-like bacteria. Second of them was again 100 μg/ml Km and Str supplemented SW-2 medium which was used for suspension of cells and recovery of these cells after suspension. SW-2 medium contained only %2 NaCl, therefore it allowed tosurvive PukHOM and pRK2013 cells before conjugation.

The optimum growth medium of *H. smyrnensis* AAD6 was predicted (Poli et al., 2009), which was explained in section 2.5.2. In addition to this, *H. smyrnensis* AAD6 utilizes sucrose and produces levan polymer at the presence of optimum medium. Thus, it was expected that cells that formed after conjugation should produce levan when they grow in optimum medium plates. On contrary, it was expected that wild type *H. smyrnensis* AAD6 cells will have not grown on antibiotic aided SW-2 plates since wild type does not have resistance for Str.

Antibiotic susceptibility test were also run for determination of antibiotic resistance of wild type bacterium somehow has it. Amp, Km and Str antibiotics were essential for the experiments that were used for constructions. Thus, only these antibiotic resistances were examined with 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml concentrations, respectively. Wild type *H. smyrnensis* AAD6 has a resistance for Km till 50 μg/ml concentration but showed no resistance to Amp and Str. Eventually, selective media were individually designed according to the strain and the minimal inhibitory concentration (MIC) of the antibiotic in the corresponding medium.

It was predicted that if conjugation were performed successfully, trans-conjugants should grow in antibiotic aided both optimum medium and SW-2 Medium. Plus, it was expected that obtaining no growth on these antibiotic plates in where wild type *H. smyrnensis* AAD6 inoculated.

Bacterial conjugation was performed via filter conjugation, too. Filter mating is also frequently used method for conjugation (Chopin et al., 1984, McKay et al., 1984, Pappas et al., 1996). It was performed with method that reported (Arvanitis et al., 1995). Constructed plasmid PukHOM4 was mobilized by pRK2013 to moderately halophilic strains by triparental mating. 100 μl volumes of logarithmic-phase cultures of each of the donor, helper, and recipient strains were mixed on a nitrocellulose filter (0.22-mm pore diameter) on a plate of a modified SW-2 medium, in which the final percentage of the total salt solution was decreased to 2% to permit the growth of E. coli. Filters were incubated overnight at 37^{o}C, and the trans-conjugants of moderate halophiles were selected on SW-2 containing Km and Str (to counter-select the donor strain). Both filter mating and conventional agar mating were performed and there were no differences that could be observed on agar plates by naked eye (Figure 4.21) after conjugation. In molecular trials, both of them grew well and integrated the insertion correctly.

In literature, transposons used for with the aim of making a new construction in Gram negative bacterium *Zymomonas mobilis* (Pappas et al., 1996) and employed for control of ectoine mechanism in halophilic bacterium *Chromohalobacter salexigens* (Rodríguez-Moya et al.,2010). Transposons were also used for increasing exopolysaccharide producing capacity of a moderately halophilic microorganism *Halomonas eurihalina* with biotechnological interest (Llamas et al., 2000). Moreover, pRK2013 preferred in cloning and expression studies of a gene involved in the production of EPS alginate in *Pseudomonas aeruginosa* (Goldberg et al., 1984). The identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa* was screened with pRK2013 (Whiteley et al., 1999). Use of the plasmid pRK2013 as a vehicle for transposition in *Azotobacter vinelandii* reported, too. (Phadnis et al., 1987).

After overnight incubation of conjugation, colonies were transferred into antibiotic aided plates and incubated for 2 days. At the end of two days incubation, resultant colonies were selected and then re-transferred again to be sure their resistance with respect to passaging. Colonies were shown in Figure 4.21.

PCR amplification was performed with these survivor colonies with omega primers in order to be sure the insertion of transposon agent omega. It was the crucial part of this study since whole work designed based on this transposon mutagenesis. Hopefully, expected omega band was observed with the PCR product which was used isolated mutant *H. smyrnensis* AAD6 DNA as template DNA. Gel electrophoresis results of trans-conjugants were shown in Figure 4.22.

As a result of the whole work, insertion of transposon into recipient bacteria was achieved, and a new mutant strain with its Omega region constructed finally. At the end of verifications, this mutant strain of *H. smyrnensis* AAD6 has been re-named as *H. smyrnensis* BMA14.

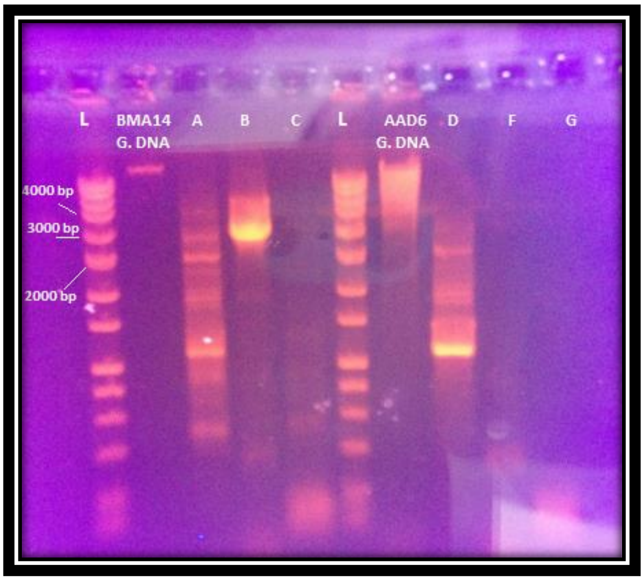


Figure 134.21: PCR products of mutant trans-conjugants.

BMA14 G. DNA, represents the genomic DNA of *H. smyrnensis* BMA14.

AAD6 G. DNA, represents the genomic DNA of *H. smyrnensis* AAD6.

Lane A: amplification of HPr region with HPr primers using BMA14 DNA as template at omega specific annealing temperature (un-optimized PCR conditions).

Lane B: amplification of Omega region with omega primers using BMA14 DNA as template at optimized annealing temperature.

Lane C: amplification of HOM region with HOM primers using BMA14 DNA as template at omega specific annealing temperature (un-optimized PCR conditions).

Lane D : amplification of of HPr region with HPr primers using AAD6 DNA as template at omega specific annealing temperature (un-optimized PCR conditions). Band of HPr region could be clearly seen with un-spesific bands.

Lane F : amplification of omega region with omega primers using AAD6 DNA as template at omega specific annealing temperature (optimized PCR conditions). Since there is no omega region inside bacterial genome, this region must not be amplified as in the lane.

Lane G : amplification of HOM region with HOM primers using AAD6 DNA as template at omega specific annealing temperature (un-optimized PCR conditions). Since bacterial genomic DNA does not contain HOM region, no amplification occurs.

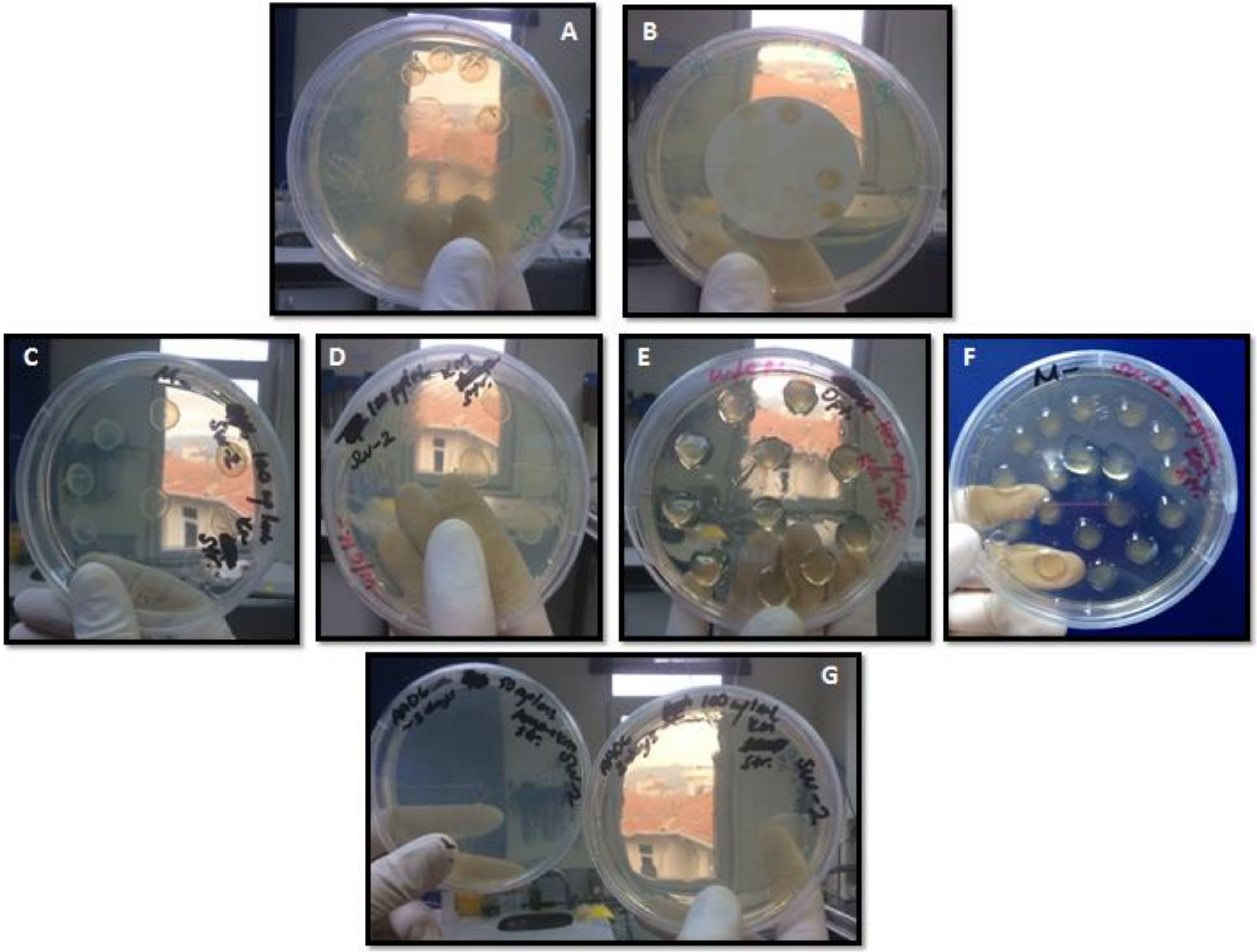


Figure 4.22 : Colonies after bacterial conjugation

4.6 Comparison of *H. smyrnensis* BMA14 and *H. smyrnensis* AAD6 by Their Growth Profiles and Levan Production

Mutant organisms may behave differently in some mechanisms when comparing to wild type. This is because there is a perturbation on its genetic material and mutant strains were designed generally with this purpose acting not like itself. Some experiments were designed hereafter mutant strain *H. smyrnensis* BMA14 was completed and verified its originality. The differences between mutant strain BMA14 and wild type strain AAD6 were tried to be indicated by their growth, levan production, sucrose consumption and glucose utilization profiles.

4.6.1 Bacterial Growth Profiles

The growth profiles of AAD6 and BMA14 were monitored by measuring the optical densities (OD) at 660 nm. Cell-free medium was used as the blank. Three data were collected for each strain in a day. Growth was terminated at the end of 200 hours. At the end of analysis, there were no significant differences between BMA14 and AAD6 strains according to their growth profiles. It was expected, since bacteria utilize both fructose and glucose for cell maintenance and survival, but primarily prefer glucose. Prevention of fructose uptake into intracellular environment did not affect main energy source of bacteria. They kept growing same as wild type strain, i.e. both the adaptation durations and maximum biomass concentrations were not

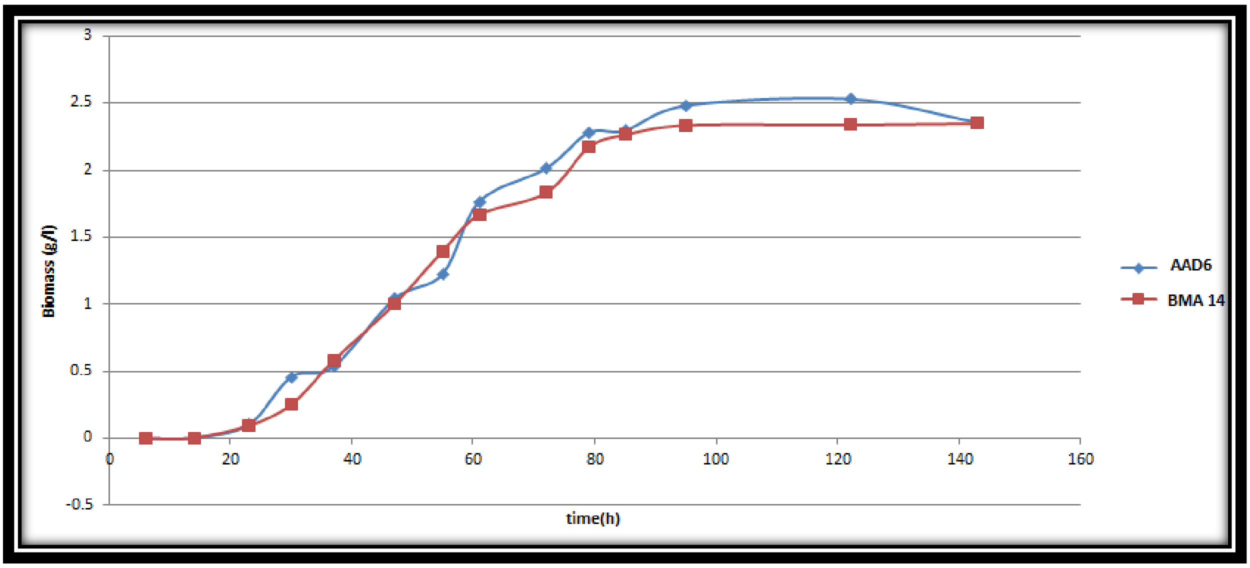


Figure 4.23: Biomass values of *H. smyrnensis* AAD6 and BMA14

different. Generally mutant strains grow more difficult than wild type strain. Thus, this result was a one of the positive outcomes of mutant strain since it can grow easily as wild type strain.

4.6.2 Levan Production

Main objective of proposed thesis was to increase levan production by mutagenesis. Therefore, a difference between levan productions of the mutant strain and wild type strain was expected. Since PTS^{fru} system of BMA14 strain was re-structured by transposon mutagenesis, levan production should be affected in this strain. Levan production of BMA14 was 14 g/L at the end of fermentation whereas it was 10 g/L in AAD6. AAD6 consumes fructose for intracellular requirements although it is also possible with glucose. In BMA14 bacterial genome, the system that was responsible for the uptake of fructose into cell was deleted out. Thus, BMA14 can use fructose monomers only at extracellular environment. Increasing of levan polymer production mostly depends on this situation. Yield for mass of product per unit mass of biomass formed ($Y_{x/p}$) was determined for each strain. Yield of mutant strain calculated as 5.82 whereas yield of wild type strain 4.33.

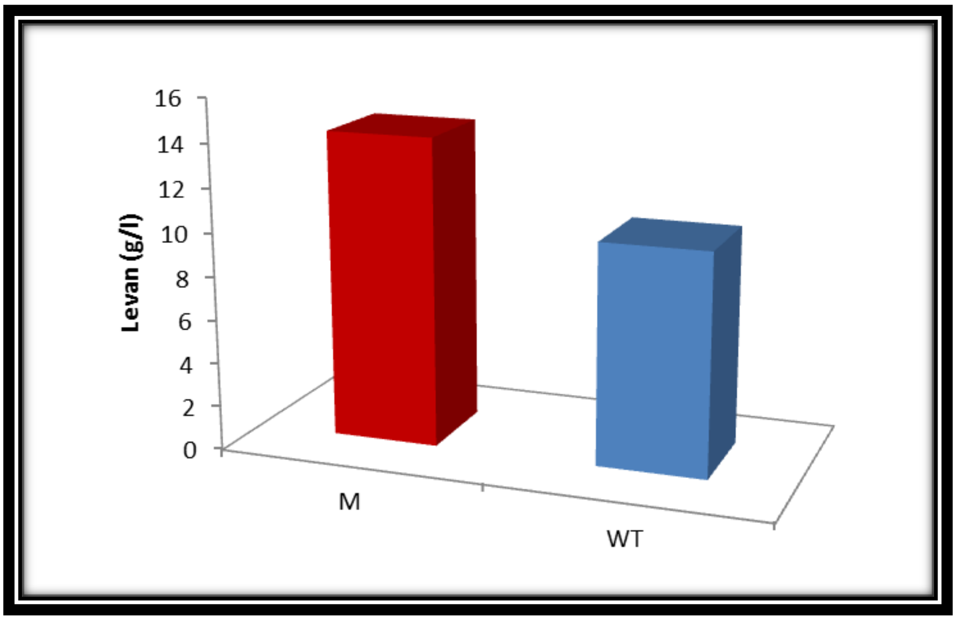


Figure 4.24: Levan productions of BMA14 (M) and AAD6 (WT) strains

4.6.3 Sucrose Consumption

Difference between sucrose consumption rates in AAD6 and in BMA14 was measured as extracellular sucrose by High Pressure Liquid Chromatography (HPLC) tecnique with ZORBAX Carbohydrate Analysis (4.6 x 250 mm) column. At the beginning of analysis, calibration curve was set with sucrose standard solutions with known concentrations. This calibration curve was given in Appendix A. Analyses resulted that sucrose is metabolized more in AAD6 strain whereas levan production was lower compared to BMA14. At the end of fermentation, sucrose consumption was 36.58 g for BMA14 and 41.16 g for AAD6. Yield for mass of substrate per unit mass of biomass formed ($Y_{X/S}$) was determined for BMA14 as 15.60 whereas AAD6 16.89. According to these results, wild type strain uses much sucrose than mutant strain.

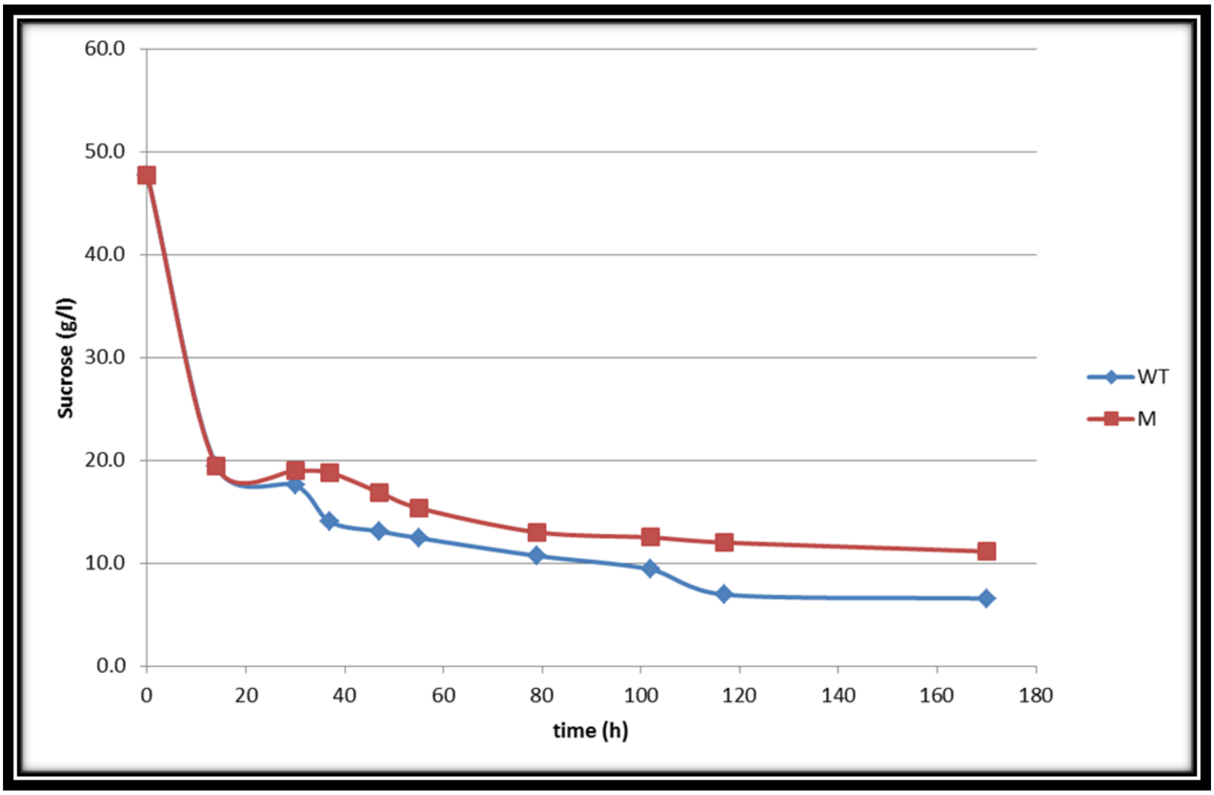


Figure 4.25: Measurement of extracellular sucrose concentration with HPLC

4.6.4 Glucose Consumption

Glucose consumption was not measured by HPLC since the column was not suitable for measuring of glucose and fructose concentrations at the same sample. *H. smyrnensis* AAD6 and mutant BMA14 utilizes glucose both at intracellular and extracellular environments. In order to obtain whether there is a difference between AAD6 and BMA14 in case of utilizing glucose, glucose assay were performed to measure extracellular glucose concentrations. Measurement of glucose concentration was carried out with glucose assay kit and the intensity of the pink color measured at 540 nm which then calculated by proportional to the standard glucose concentrations (Glucose calibration chart is given in Appendix B). Standard glucose was measured by prepared glucose standards with known concentration then calibration curve was plotted and glucose concentrations of samples were calculated by using equation that was derived from calibration curve. According to these results, the extracellular glcuse concentrations were higher in AAD6, meaning that BMA14 utilizes more glucose during fermentation when compared to AAD6 (Figure 4.26). Glucose monomers that derived from extracellular sucrose might be utilized more efficiently at intracellular environment in BMA14. Since BMA14 could not utilize fructose internally, it needs more intracellular glucose for survival.

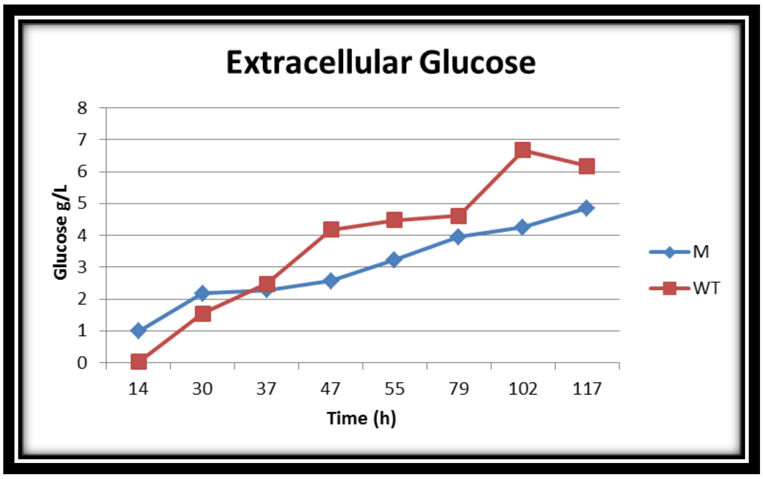


Figure 4.26 : Extracellular glucose concentrations of strains that was measured by Glucose Kit.

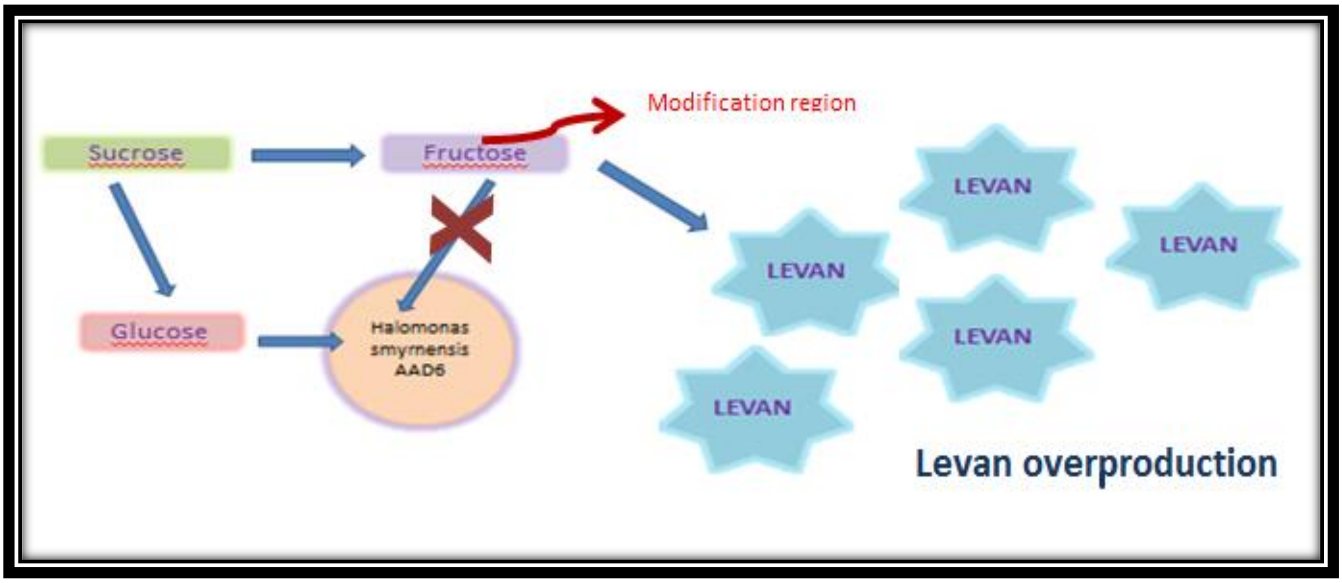


Figure 4.27: Levan production scheme in BMA14

If we gather all outcomes of these analyses, it was obtained that BMA14 is more efficient strain for levan production. BMA14 consumed 36.581 g sucrose according to analysis, if sucrose degraded into fructose and glucose equally with respect to conservation of mass, it was assumed that almost 18 g fructose was at extracellular environment. BMA14 utilized fructose and synthesized 14 g levan. Whereas AAD6 consumed 41.157 g sucrose and produced 10 g levan, AAD6 has 20 g fructose at extracellular environment but produced levan only half mass of fructose. BMA14 consumes less sucrose and produced more levan than AAD6. Based on these results, it can be said that, BMA14 utilizes sucrose more efficiently than AAD6 for production of levan. Also, BMA14 is a one of the producers of levan with % 40 efficiency with respect to AAD6.

5. CONCLUSION and RECOMMENDATIONS

This thesis aims to produce levan biopolymer at enhanced levels by modification of fructose uptake mechanism in *Halomonas smyrnensis* AAD6^T. Levan that is produced by *H. smyrnensis* AAD6^T is highly valuable. Because it is produced by a halophilic organism that means it is suitable for production at high scale in industrial conditions since the organism can resist harsh conditions. Furthermore, source of the organism is Turkey, so it is a national product which has an international importance, too.

Within the scope of the study, using the results of the whole genome sequence of *Halomonas smyrnensis* AAD6, all components of the fructose uptake system were identified. After determination of the essential mechanisms in phosphotransferase system, it was thought that making some alterations to the fructose uptake system may end up with increment in biopolymer production. As a result of the study, a metabolic engineering strategy was designed on blocking the fructose uptake system of the organism to enhance levan production.

Gene deletion was made on HPr component for 300-350 bp length of sequence. Transposons were employed for deletion process. After mutation studies, new valuable strain was constructed which has been named as *Halomonas smyrnensis* BMA14. Achievement of the deletion ended up also with production of levan at enhanced level by new strain. Since deletion led to utilization of fructose monomers only at extracellular environment, fructose levels increase here and it is resulted in increment of levan biosynthesis and overproduction of the levan polymer.

Considering the results obtained in this thesis, further studies will be conducted to investigate polymer production in details. Since *H. smyrnensis* BMA14 produces more levan than AAD6 strain, the mechanisms that lie behind this outcome should be studied elaborately. In this context, bioreactor experiments should be designed and consumption profiles of sugars (glucose, fructose, sucrose) should be determined precisely. Moreover, levansucrase enzyme which is only multifunctional enzyme for fructan synthesis in bacteria, can be studied for its isolation, cloning, characterization and activity. Also, new strain BMA14 may have genomic potentials for producing biological industrial supplies such as osmolytes, Pel polysaccharide and PHA as well as levan. For instance, the production cost and time may be reduced with the developing new co-production methods for these commercially important products.

Outcomes of this study will accelerate the research on enhanced productions of any other biopolymers from halophilic bacteria towards systems biology approaches and design of metabolic engineering strategies. These genetic and metabolic engineering strategies will facilitate future studies on the diversity of halophilic bacteria.

As a conclusion, it was achieved to construct a novel levan producer strain *Halomonas smyrnensis* BMA14 and it has provided the starting points for various present and future studies.

REFERENCES

- Aguilera, J. M., & Lillford, P. J. (2007). Food materials science: principles and practice, 1st Edition, Springer, New York.
- Alexeyev, M. F., Shokolenko, I. N., & Croughan, T. P. (1995). Improved antibiotic-resistance gene cassettes and omega elements *for Escherichia coli* vector construction and in vitro deletion/insertion mutagenesis. Gene,160(1), 63-67.
- Alva, V. A., & Peyton, B. M. (2003). Phenol and catechol biodegradation by the haloalkaliphile *Halomonas campisalis*: influence of pH and salinity. Environmental science & technology, 37(19), 4397-4402.
- Alves, V. D., Ferreira, A. R., Costa, N., Freitas, F., Reis, M. A., & Coelhoso, I. M. (2011). Characterization of biodegradable films from the extracellular polysaccharide produced by *Pseudomonas oleovorans* grown on glycerol byproduct. Carbohydrate Polymers, 83(4), 1582-1590.
- Arahal, D. R., & Ventosa, A. (2006). The family *Halomonadaceae,* 3rd Edition, Springer, New York.
- Arco, Y., Llamas, I., Martínez-Checa, F., Argandoña, M., Quesada, E., & del Moral, A. (2005). epsABCJ genes are involved in the biosynthesis of the exopolysaccharide mauran produced by *Halomonas maura*. Microbiology, 151(9), 2841-2851.
- Argandona, M., Martínez-Checa, F., Llamas, I., Arco, Y., Quesada, E., & del Moral, A. (2006). A membrane-bound nitrate reductase encoded by the *narGHJI* operon is responsible for anaerobic respiration in Halomonas maura. Extremophiles, 10(5), 411-419.
- Argandona, M., Vargas, C., Reina-Bueno, M., Rodríguez-Moya, J., Salvador, M., & Nieto, J. J. (2012). An extended suite of genetic tools for use in bacteria of the *Halomonadaceae*: an overview, 3rd Edition ,Humana Press, Newyork.
- Arvanitis, N., Vargas, C., Tegos, G., Perysinakis, A., Nieto, J. J., Ventosa, A., & Drainas, C. (1995). Development of a gene reporter system in moderately halophilic bacteria by employing the ice nucleation gene of *Pseudomonas syringae*. Applied and environmental microbiology, 61(11), 3821-3825.
- Apte, A., & Daniel, S. (2009). PCR primer design, 2nd Edtion, Cold Spring Harbor Protocols, New York.

- Ates, O., Arga, K. Y., & Oner, E. T. (2013). The stimulatory effect of mannitol on levan biosynthesis: Lessons from metabolic systems analysis of *Halomonas smyrnensis* AAD6T. *Biotechnology progress*, *29*(6), 1386-1397.
- Ausubel, F. M. (2002). Short protocols in molecular biology: a compendium of current protocols in molecular biology. 5th ed. John Wiley & Sons, New York.
- Bae, S. O., Sugano, Y., Ohi, K., & Shoda, M. (2004). Features of bacterial cellulose synthesis in a mutant generated by disruption of the diguanylate cyclase 1 gene of *Acetobacter xylinum* BPR 2001. Applied microbiology and biotechnology, 65(3), 315-322.
- Bajaj, I. B., Survase, S. A., Saudagar, P. S., & Singhal, R. S. (2007). Gellan gum: fermentative production, downstream processing and applications. Food Technology and Biotechnology, 45(4), 341-354.
- Bailey, J. E., Birnbaum, S., Galazzo, J. L., Khosla, C., & Shanks, J. V. (1990). Strategies and Challenges in Metabolic Engineering. Annals of the New York Academy of Sciences, 589(1), 1-15
- Banik, R. M., Kanari, B., & Upadhyay, S. N. (2000). Exopolysaccharide of the gellan family: prospects and potential. World Journal of Microbiology and Biotechnology, 16(5), 407-414.
- Barker, P. E., & Ajongwen, N. J. (1991). The production of the enzyme dextransucrase using nonaerated fermentation techniques. Biotechnology and bioengineering, 37(8), 703-707.
- Barth, P. T., Grinter, N. J., & Bradley, D. E. (1978). Conjugal transfer system of plasmid RP4: analysis by transposon 7 insertion. Journal of bacteriology,133(1), 43-52.
- Barth, P. T., Timmis, K. N., & Puhler, A. (1979). Plasmids of medical, environmental and commercial importance.Volume 1, Elsevier/North-Holland Biomedical Press, Amsterdam.
- Becker, A., Katzen, F., Pühler, A., & Ielpi, L. (1998). Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Applied microbiology and biotechnology, 50(2), 145-152.
- Bell, D. A., & DeMarini, D. M. (1991). Excessive cycling converts PCR products to random-length higher molecular weight fragments. Nucleic acids research, 19(18), 5079.

- Berendes, F., Gottschalk, G., Heine-Dobbernack, E., Moore, E. R. B., & Tindall, B. J. (1996). *Halomonas desiderata* sp. nov, a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works.Systematic and applied microbiology, 19(2), 158-167.
- Boels, I. C., Kranenburg, R. V., Hugenholtz, J., Kleerebezem, M., & de Vos, W. M. (2001). Sugar catabolism and its impact on the biosynthesis and engineering of exopolysaccharide production in lactic acid bacteria. International Dairy Journal, 11(9), 723-732.
- Brody, J. R., & Kern, S. E. (2004). History and principles of conductive media for standard DNA electrophoresis. Analytical biochemistry, 333(1), 1-13.
- Brown, T. A. (2002). Genome Replication. 2nd Edition, Wiley- Liss, Oxford.
- Brune, W. (2002). Random transposon mutagenesis of large DNA molecules in Escherichia coli. 2nd Edition, Humana Press, New York.
- Cánovas, D., Vargas, C., Calderón, M. I., Ventosa, A., & Nieto, J. J. (1998). Characterization of the Genes for the Biosynthesis of the Compatible Solute Ectoine in the Moderately Halophilic Bacterium *Halomonas elongata* DSM 3043. Systematic and applied microbiology, 21(4), 487-497.
- Chavarría, M., Kleijn, R. J., Sauer, U., Pflüger-Grau, K., & de Lorenzo, V. (2012). Regulatory tasks of the phosphoenolpyruvate-phosphotransferase system of *Pseudomonas putida* in central carbon metabolism. *MBio*, *3*(2), e00028-12.
- Cerning, J. (1994). Polysaccharides exocellulaires produits par les bactéries lactiques. Bacteries lactiques, 1, 309-329.
- Cerning, J., Bouillanne, C., Landon, M., & Desmazeaud, M. J. (1990). Comparison of exocellular polysaccharide production by thermophilic lactic acid bacteria. Sciences des aliments, 10(2), 443-451.
- Chassy, B. M., Mercenier, A., & Flickinger, J. (1988). Transformation of bacteria by electroporation. Trends in Biotechnology, 6(12), 303-309.
- Choi, K. H., Mima, T., Casart, Y., Rholl, D., Kumar, A., Beacham, I. R., & Schweizer, H. P. (2008). Genetic tools for select-agent-compliant manipulation of Burkholderia pseudomallei. Applied and environmental microbiology, 74(4), 1064-1075.

- Chopin, A., Chopin, M. C., Moillo-Batt, A., & Langella, P. (1984). Two plasmid-determined restriction and modification systems in *Streptococcus lactis* Plasmid, 11(3), 260-263.
- CzaCzyK, K., & Wojciechowska, K. (2003). Formation of bacterial biofilms the essence of the matter and mechanisms of interactions. Biotechnologia, (3), 180-192.
- DasSarma, S., & Robb, F. T., (1995). Archaea: A laboratory manual. Halophiles. Cold Spring Harbor Laboratory Press, New York.
- De Lorenzo, V., & Timmis, K. N. (1994). Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5 and Tn10 derived minitransposons. Methods in enzymology, 235, 386-405.
- de Vos, W. M. (1996). Metabolic engineering of sugar catabolism in lactic acid bacteria. Antonie van Leeuwenhoek, 70(2-4), 223-242.
- Dennis, J. J., & Zylstra, G. J. (1998). Improved antibiotic-resistance cassettes through restriction site elimination using *Pfu* DNA polymerase PCR. BioTechniques, 25(5), 772-776.
- Ditta, G., Stanfield, S., Corbin, D., & Helinski, D. R. (1980). Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proceedings of the National Academy of Sciences, 77(12), 7347-7351.
- Dobson, S. J., & Franzmann, P. (1996). Unification of the genera Deleya (Baumann et al. 1983), Halomonas (Vreeland et al. 1980), and Halovibrio (Fendrich 1988) and the species Paracoccus halodenitrificans (Robinson and Gibbons 1952) into a single genus, Halomonas, and placement of the genus Zymobacter in the family Halomonadaceae. International journal of systematic bacteriology, 46(2), 550-558.
- Donnenberg, M. S., & Kaper, J. B. (1991). Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infection and immunity, 59(12), 4310-4317.
- Duboc, P., & Mollet, B. (2001). Applications of exopolysaccharides in the dairy industry. International Dairy Journal, 11(9), 759-768.
- EcoRI, K., & AccI, S. (1996). Broad-host-range cloning and cassette vectors based on the R388 trimethoprim resistance gene. BioTechniques, 20(5), 762-764.
- Faber, E. J., Zoon, P., Kamerling, J. P., & Vliegenthart, J. F. (1998). The exopolysaccharides produced by *Streptococcus thermophilus* Rs and Sts have the same

repeating unit but differ in viscosity of their milk cultures. Carbohydrate research, 310(4), 269-276.

- Fellay, R., Frey, J., & Krisch, H. (1987). Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. Gene, 52(2), 147-154.
- Franzmann, P. D., Wehmeyer, U., & Stackebrandt, E. (1988). *Halomonadaceae* fam. nov., a New Family of the Class *Proteobacteria* to Accommodate the Genera *Halomonas* and *Deleya*. Systematic and Applied Microbiology, 11(1), 16-19.
- Freitas, F., Alves, V. D., & Reis, M. A. (2011). Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends in biotechnology, 29(8), 388-398.
- Funane, K., Yamada, M., Shiraiwa, M., Takahara, H., Yamamoto, N., Ichishima, E., & Kobayashi, M. (1995). Aggregated form of dextransucrases from Leuconostoc mesenteroides NRRL B-512F and its constitutive mutant. Bioscience, biotechnology, and biochemistry, 59(5), 776-780.
- Furuya, E. Y., & Lowy, F. D. (2006). Antimicrobial-resistant bacteria in the community setting. Nature Reviews Microbiology, 4(1), 36-45.
- Galinski, E. A. (1995). Osmoadaptation in bacteria. Advances in microbial physiology, 37, 273-328.
- Gandhi, H. P., Ray, R. M., & Patel, R. M. (1997). Exopolymer production by *Bacillus* species. Carbohydrate polymers, 34(4), 323-327.
- Goldberg, J. B., & Ohman, D. E. (1984). Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. Journal of bacteriology, 158(3), 1115-1121.
- Golovliov, I., Sjöstedt, A., Mokrievich, A., & Pavlov, V. (2003). A method for allelic replacement in Francisella tularensis. FEMS microbiology letters, 222(2), 273-280.
- Goryshin, I. Y., Jendrisak, J., Hoffman, L. M., Meis, R., & Reznikoff, W. S. (2000). Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. Nature biotechnology, 18(1), 97-100.
- Grobben, G. J., Van Casteren, W. H. M., Schols, H. A., Oosterveld, A., Sala, G., Smith, M. R., ... & De Bont, J. A. M. (1997). Analysis of the exopolysaccharides produced by

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Applied Microbiology and Biotechnology, 48(4), 516-521.

- Grossiord, B., Vaughan, E. E., Luesink, E., & De Vos, W. M. (1998). Genetics of galactose utilisation via the Leloir pathway in lactic acid bacteria. Le Lait,78(1), 77-84.
- Han, Y. W. (1990). Microbial levan. Advances in applied microbiology, 35, 171-194.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. Journal of molecular biology, 166(4), 557-580.
- Hanahan, D. O. U. G. L. A. S., & Bloom, F. R. (1996). Mechanisms of DNA transformation. *Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology. ASM Press, Washington.
- Hinteregger, C., & Streichsbier, F. (1997). *Halomonas* sp., a moderately halophilic strain, for biotreatment of saline phenolic waste-water. Biotechnology Letters, 19(11), 1099-1102.
- Hutkins, R. W., & Morris, H. A. (1987). Carbohydrate metabolism by Streptococcus thermophilus: a review. Journal of food protection, USA
- Hynes, M. F., Quandt, J., O'Connell, M. P., & Pühler, A. (1989). Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the Bacillus subtilis *sacB* gene. Gene, 78(1), 111-120.
- Ivics, Z., Izsvák, Z., Chapman, K. M., & Hamra, F. K. (2011). Sleeping Beauty transposon mutagenesis of the rat genome in spermatogonial stem cells. Methods, 53(4), 356-365.
- Jaiswal, P., Sharma, R., Sanodiya, B. S., & Bisen, P. S. (2014). Microbial Exopolysaccharides: Natural Modulators of Dairy Products. Journal of Applied Pharmaceutical Science Vol, 4(10), 105-109.
- Jang, K. H., Song, K. B., Kim, C. H., Chung, B. H., Kang, S. A., Chun, U. H., ... & Rhee, S. K. (2001). Comparison of characteristics of levan produced by different preparations of levansucrase from Zymomonas mobilis. Biotechnology letters, 23(5), 339-344.
- Johnson, D. A. (1988). Construction of transposons carrying the transfer functions of RP4. Plasmid, 20(3), 249-258.Figurski, D. H., & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proceedings of the National Academy of Sciences, 76(4), 1648-1652.

- Joo, W. A., & Kim, C. W. (2005). Proteomics of halophilic archaea. Journal of Chromatography, 815(1), 237-250.
- Jörg Kunte, H., & Galinski, E. A. (1995). Transposon mutagenesis in halophilic eubacteria: conjugal transfer and insertion of transposon Tn5 and Tn1732 in Halomonas elongata. FEMS microbiology letters, 128(3), 293-299.
- Klein, S. H. O. S. H. A. N. A., Lohman, K. A. R. I. N., Clover, R. A. L. P. H., Walker, G. C., & Signer, E. R. (1992). A directional, high-frequency chromosomal mobilization system for genetic mapping of Rhizobium meliloti. Journal of bacteriology, 174(1), 324-326.
- Knauf, V. C., & Nester, E. W. (1982). Wide host range cloning vectors: a cosmid clone bank of an Agrobacterium Ti plasmid. Plasmid, 8(1), 45-54.
- Knuf, C., & Nielsen, J. (2012). Aspergilli: systems biology and industrial applications. Biotechnology journal, 7(9), 1147-1155.
- Küçükaşik, F., Kazak, H., Güney, D., Finore, I., Poli, A., Yenigün, O., ... & Öner, E. T. (2011). Molasses as fermentation substrate for levan production by *Halomonas* sp. *Applied microbiology and biotechnology*, *89*(6), 1729-1740.
- Kushner, D.J., Kamekura, M., (1988). Halophilic Bacteria, 1st Edition, CRC Press Inc, Florida.
- Laurenzio, L., Frost, L. S., & Paranchych, W. (1992). The TraM protein of the conjugative plasmid F binds to the origin of transfer of the F and *Col*E1 plasmids. Molecular microbiology, 6(20), 2951-2959.
- Leibovici, J., & Stark, Y. (1984). Increase in cell permeability to a cytotoxic agent by the polysaccharide levan. Cellular and molecular biology, 31(5), 337-341.
- Le Borgne, S., Paniagua, D., & Vazquez-Duhalt, R. (2008). Biodegradation of organic pollutants by halophilic bacteria and archaea. Journal of molecular microbiology and biotechnology, 15(2-3), 74-92.
- Le Borgne, S., Palmeros, B., Valle, F., Bolivar, F., & Gosset, G. (1998). pBRINT-T : a plasmid family with a temperature-sensitive replicon, designed for chromosomal integration into the *lacZ* gene of *Escherichia coli*. Gene, 223(1), 213-219.
- Llamas, I., Argandoña, M., Quesada, E., & del Moral, A. (2000). Transposon mutagenesis in *Halomonas eurihalina*. Research in microbiology,151(1), 13-18.

- Llamas, I., Suarez, A., Quesada, E., Béjar, V., & del Moral, A. (2003). Identification and characterization of the carAB genes responsible for encoding carbamoylphosphate synthetase in *Halomonas eurihalina*. Extremophiles, 7(3), 205-211.
- Llosa, M., & Zambryski, P. (1998). On the origin and function of pili. Trends in microbiology, 6(3), 98-99.
- Looijesteijn, P. J., Trapet, L., de Vries, E., Abee, T., & Hugenholtz, J. (2001). Physiological function of exopolysaccharides produced by *lactococcus lactis*. International journal of food microbiology, 64(1), 71-80.
- Macrina, F. L., & Archer, G. L. (1993). Conjugation and broad host range plasmids in streptococci and staphylococci. 1st Edition, Springer US, New York.
- Madern, D., Ebel, C., & Zaccai, G. (2000). Halophilic adaptation of enzymes. Extremophiles, 4(2), 91-98.
- Madhuri, K. V., & Prabhakar, K. V. (2014). Microbial Exopolysaccharides: Biosynthesis and Potential Applications. Oriental Journal of Chemistry, 30(3), 1401-1410.
- Maier, T. M., Havig, A., Casey, M., Nano, F. E., Frank, D. W., & Zahrt, T. C. (2004). Construction and characterization of a highly efficient *Francisella* shuttle plasmid. Applied and environmental microbiology, 70(12), 7511-7519.
- Maltseva, O., McGowan, C., Fulthorpe, R., & Oriel, P. (1996). Degradation of 2, 4-dichlorophenoxyacetic acid by haloalkaliphilic bacteria. Microbiology,142(5), 1115-1122.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring, New York.
- Marshall, V. M., Cowie, E. N., & Moreton, R. S. (1995). Analysis and production of two exopolysaccharides from *Lactococcus lactis* subsp. cremoris LC330. Journal of Dairy Research, 62(04), 621-628.
- Maskow, T., & Kleinsteuber, S. (2004). Carbon and energy fluxes during haloadaptation of *Halomonas* sp. EF11 growing on phenol. Extremophiles, 8(2), 133-141.
- Martínez-Checa, F., Toledo, F. L., El Mabrouki, K., Quesada, E., & Calvo, C. (2007). Characteristics of bioemulsifier V2-7 synthesized in culture media added of

hydrocarbons: chemical composition, emulsifying activity and rheological properties. Bioresource technology, 98(16), 3130-3135.

- Matson, S. W., Nelson, W. C., & Morton, B. S. (1993). Characterization of the reaction product of the oriT nicking reaction catalyzed by *Escherichia coli* DNA helicase I. Journal of bacteriology, 175(9), 2599-2606.
- Matsumoto, Y., & Kuroyanagi, Y. (2010). Development of a wound dressing composed of hyaluronic acid sponge containing arginine and epidermal growth factor. Journal of Biomaterials Science, Polymer Edition, 21(6-7), 715-726.
- McKAY, L. L., & Baldwin, K. A. (1984). Conjugative 40-megadalton plasmid in *Streptococcus lactis* subsp. diacetylactis DRC3 is associated with resistance to nisin and bacteriophage. Applied and environmental microbiology, 47(1), 68-74.
- Miller, V. L., & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. Journal of bacteriology, 170(6), 2575-2583.
- Monchois, V., Remaud-Simeon, M., Monsan, P., & Willemot, R. M. (1998). Cloning and sequencing of a gene coding for an extracellular dextransucrase (DSRB) from *Leuconostoc mesenteroides* NRRL B-1299 synthesizing only a α (1–6) glucan. FEMS microbiology letters, 159(2), 307-315.
- Moore, R. A., DeShazer, D., Reckseidler, S., Weissman, A., & Woods, D. E. (1999). Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrobial agents and chemotherapy, 43(3), 465-470.
- Mormile, M. R., Romine, M. F., Garcia, M. T., Ventosa, A., Bailey, T. J., & Peyton, B. M. (1999). *Halomonas campisalis* sp. nov., a Denitrifying, Moderately Haloalkaliphilic Bacterium. Systematic and applied microbiology,22(4), 551-558.
- Muńoz, J. A., Pérez-Esteban, B., Esteban, M., De La Escalera, S., Gomez, M. A., Martínez-Toledo, M. V., & Gonzalez-Lopez, J. (2001). Growth of moderately halophilic bacteria isolated from sea water using phenol as the sole carbon source. Folia microbiologica, 46(4), 297-302.
- Nassif, X. A. V. I. E. R., Fournier, J. M., Arondel, J., & Sansonetti, P. J. (1989). Mucoid phenotype of Klebsiella pneumoniae is a plasmid-encoded virulence factor. Infection and immunity, 57(2), 546-552.

- Nguyen, V. T., Gidley, M. J., & Dykes, G. A. (2008). Potential of a nisin-containing bacterial cellulose film to inhibit *Listeria monocytogenes* on processed meats. Food Microbiology, 25(3), 471-478.
- Nielsen, J. (2001). Metabolic engineering. Applied Microbiology and Biotechnology, 55(3), 263-283.
- Nierman, W. C., DeShazer, D., Kim, H. S., Tettelin, H., Nelson, K. E., Feldblyum, T., ... & Fraser, C. M. (2004). Structural flexibility in the Burkholderia mallei genome. Proceedings of the National Academy of Sciences of the United States of America, 101(39), 14246-14251.
- Nieto, J. J., Vargas, C., & Ventosa, A. (2000). Osmoprotection mechanisms in the moderately halophilic bacterium Halomonas elongata. Recent research developments in microbiology, 43-54.
- Oberhardt, M. A., Palsson, B. O., & Papin, J. A. (2009). Applications of genome-scale metabolic reconstructions. Mol Syst Biol. (5), 320-334.
- Oms-Oliu, G., Soliva-Fortuny, R., & Martín-Belloso, O. (2008). Edible coatings with antibrowning agents to maintain sensory quality and antioxidant properties of fresh-cut pears. Postharvest Biology and Technology, 50(1), 87-94.
- Ozer, T., Bioinformatics Based Metabolic Network Reconstruction of Levan Producing *Halomonas smyrnensis* AAD6. Master Thesis, Marmara University, Institute of Natural Science, Istanbul, Turkey, 55-57.
- Oren, A. (2013). Life at High Salt Concentrations. 4th Edition,Springer, Berlin Heidelberg.
- Osman, O., Tanguichi, H., Ikeda, K., Park, P., Tanabe-Hosoi, S., & Nagata, S. (2010). Copper-resistant halophilic bacterium isolated from the polluted Maruit Lake, Egypt. Journal of applied microbiology, 108(4), 1459-1470.
- Papini, M., Salazar, M., & Nielsen, J. (2010). Systems biology of industrial microorganisms. Springer, Berlin Heidelberg.
- Pappas, K. M., Galani, I., & Typas, M. A. (1997). Transposon mutagenesis and strain construction in *Zymomonas mobilis*. Journal of applied microbiology,82(3), 379-388.
- Pastor, J. M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L. N., ... & Cánovas, M. (2010). Ectoines in cell stress protection: uses and biotechnological production. Biotechnology advances, 28(6), 782-801.

- Phadnis, S. H., & Das, H. K. (1987). Use of the plasmid pRK 2013 as a vehicle for transposition in *Azotobacter vinelandii*. Journal of Biosciences, 12(2), 131-135.
- Phillips, G. J. (1999). New cloning vectors with temperature-sensitive replication. Plasmid, 41(1), 78-81.
- Poli, A., Kazak, H., Gürleyendağ, B., Tommonaro, G., Pieretti, G., Öner, E. T., & Nicolaus, B. (2009). High level synthesis of levan by a novel *Halomonas* species growing on defined media. Carbohydrate Polymers, 78(4), 651-657.
- Poli, A., Nicolaus, B., Denizci, A. A., Yavuzturk, B., & Kazan, D. (2013). *Halomonas smyrnensis* sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium. International journal of systematic and evolutionary microbiology, 63(Pt 1), 10-18.
- Pomp, D., & Medrano, J. F. (1991). Organic solvents as facilitators of polymerase chain reaction. BioTechniques, 10(1), 58-59.
- Poteete, A. R., Rosadini, C., & StPierre, C. (2006). Gentamicin and other cassettes for chromosomal gene replacement in *Escherichia coli*. Biotechniques, 41(3), 261-264.
- Prentki, P., & Krisch, H. M. (1984). In vitro insertional mutagenesis with a selectable DNA fragment. Gene, 29(3), 303-313.
- Quénée, L., Lamotte, D., & Polack, B. (2005). Combined *sacB*-based negative selection and cre-lox antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. Biotechniques, 38(1), 63-67.
- Quillaguaman, J., Hashim, S., Bento, F., Mattiasson, B., & Hatti-Kaul, R. (2005). Poly (β-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. Journal of applied microbiology, 99(1), 151-157.
- Ramos, A., Boels, I. C., de Vos, W. M., & Santos, H. (2001). Relationship between glycolysis and exopolysaccharide biosynthesis in *Lactococcus lactis*. Applied and environmental microbiology, 67(1), 33-41.
- Raveendran, S., Dhandayuthapani, B., Nagaoka, Y., Yoshida, Y., Maekawa, T., & Sakthi Kumar, D. (2013). Biocompatible nanofibers based on extremophilic bacterial polysaccharide, Mauran from *Halomonas maura*. Carbohydrate polymers, 92(2), 1225-1233.
- Rehm, B. H., (2009). Microbial production of biopolymers and polymer precursors: applications and perspectives. 1st Edition, Horizon Scientific Press, Norfolk.

- Reimmann, C., Rella, M., & Haas, D. (1988). Integration of replication-defective R68. 45-like plasmids into the *Pseudomonas aeruginosa* chromosome. Journal of general microbiology, 134(6), 1515-1523.
- Roberts, M. F. (2005). Organic compatible solutes of halotolerant and halophilic microorganisms. Saline systems, 1(5), 1-30.
- Rodríguez-Moya, J., Argandoña, M., Reina-Bueno, M., Nieto, J. J., Iglesias-Guerra, F., Jebbar, M., & Vargas, C. (2010). Involvement of *EupR*, a response regulator of the NarL/FixJ family, in the control of the uptake of the compatible solutes ectoines by the halophilic bacterium *Chromohalobacter salexigens*. BMC microbiology, 10(1), 256.
- Roux, K. H. (2009). Optimization and troubleshooting in PCR. 1st Edition, Cold Spring Harbor Protocols, New York.
- Ruas-Madiedo, P., Hugenholtz, J., & Zoon, P. (2002). An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. International Dairy Journal, 12(2), 163-171.
- Ruffing, A., & Chen, R. R. (2006). Metabolic engineering of microbes for oligosaccharide and polysaccharide synthesis. Microbial cell factories, 5(1), 25.
- Sagulenko, E., Sagulenko, V., Chen, J., & Christie, P. J. (2001). Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. Journal of bacteriology, 183(20), 5813-5825.
- Schweizer, H. P. (1993). Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. BioTechniques,15(5), 831-832.
- Schweizer, H. P. (2008). Bacterial genetics: past achievements, present state of the field, and future challenges. BioTechniques, 44(5), 633.
- Selbitschka, W., Niemann, S., & Pühler, A. (1993). Construction of gene replacement vectors for Gram − bacteria using a genetically modified *sacRB* gene as a positve selection marker. Applied microbiology and biotechnology,38(5), 615-618.
- Shoemaker, N. B., Getty, C. O. L. I. N., Gardner, J. F., & Salyers, A. A. (1986). Tn4351 transposes in *Bacteroides spp*. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. Journal of bacteriology,165(3), 929-936.
- Simon, R. (1984). High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn5-Mob transposon. Molecular and General Genetics MGG, 196(3), 413-420.

- Simon, R., Quandt, J., & Klipp, W. (1989). New derivatives of transposon tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene, 80(1), 161-169.
- Sklodowska, A., & Matlakowska, R. (1998). Relative surface charge, hydrohobicity of bacterial cells and their affinity to substrate during copper bioleaching from post-flotation wastes. Biotechnology letters, 20(3), 229-233.
- Skrzypek, E., Haddix, P. L., Plano, G. V., & Straley, S. C. (1993). New suicide vector for gene replacement in *yersiniae* and other gram-negative bacteria. Plasmid, 29(2), 160-163.
- Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P., & de la Cruz, F. (2010). Mobility of plasmids. Microbiology and Molecular Biology Reviews,74(3), 434-452.
- Stellwagen, N. C., Bossi, A., Gelfi, C., & Righetti, P. G. (2000). DNA and buffers: are there any noninteracting, neutral pH buffers?. Analytical biochemistry, 287(1), 167-175.
- Stibitz, S. (1994). Use of conditionally counterselectable suicide vectors for allelic exchange. Methods in enzymology, 235, 458-465.
- Stingele, F., Neeser, J. R., & Mollet, B. (1996). Identification and characterization of the eps (Exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. Journal of bacteriology, 178(6), 1680-1690.
- Suresh Kumar, A., Mody, K., & Jha, B. (2007). Bacterial exopolysaccharides– a perception. Journal of Basic Microbiology, 47(2), 103-117.
- Sutherland, I. W., (1990). Biotechnology of microbial exopolysaccharides. 1st Edition, Cambridge University Press, Cambridge.
- Sutherland, I. W. (1997). Microbial exopolysaccharides-structural subtleties and their consequences. Pure and applied chemistry, 69(9), 1911-1918.
- Sutherland, I. W. (1998). Novel and established applications of microbial polysaccharides. Trends in biotechnology, 16(1), 41-46.
- Sutherland, I.W. (2001) Microbial polysaccharides from Gram -negative bacteria, International Dairy Journal. 11, 663–674.
- Tango, M.S.A., Islam, M.R., (2002). Potential for extremophiles for biotechnological and petroleum applications. Energy Sources, 24, 543-559.

- Tomcsanyi, T., Berg, C. M., Phadnis, S. H., & Berg, D. E. (1990). Intramolecular transposition by a synthetic IS50 (Tn5) derivative. Journal of bacteriology, 172(11), 6348-6354.
- Ullrich, M., (2009). Bacterial polysaccharides: current innovations and future trends. 1st Edition, Horizon Scientific Press, Poole.
- Van Geel-Schutten, G. H., Faber, E. J., Smit, E., Bonting, K., Smith, M. R., Ten Brink, B., ... & Dijkhuizen, L. (1999). Biochemical and Structural Characterization of the Glucan and Fructan Exopolysaccharides Synthesized by the *Lactobacillus reuteri* Wild-Type Strain and by Mutant Strains. Applied and environmental microbiology, 65(7), 3008-3014.
- Vargas, C., Argandoña, M., Reina-Bueno, M., Rodríguez-Moya, J., Fernández-Aunión, C., & Nieto, J. J. (2008). Unravelling the adaptation responses to osmotic and temperature stress in *Chromohalobacter salexigens*, a bacterium with broad salinity tolerance. Saline Systems, 4(1), 14.
- Vargas, C., Coronado, M. J., Ventosa, A., & Nieto, J. J. (1997). Host range, stability and compatibility of broad host-range-plasmids and a shuttle vector in moderately halophilic bacteria. Evidence of intrageneric and intergeneric conjugation in moderate halophiles. Systematic and applied microbiology,20(2), 173-181.
- Vargas, C., Fernández-Castillo, R., Cánovas, D., Ventosa, A., & Nieto, J. J. (1995). Isolation of cryptic plasmids from moderately halophilic eubacteria of the genus Halomonas. Characterization of a small plasmid from *H. elongata* and its use for shuttle vector construction. Molecular and General Genetics MGG, 246(4), 411-418.
- Ventosa, A., Nieto, J. J., & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. Microbiology and Molecular Biology Reviews, 62(2), 504-544
- Vuyst, L., & Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. FEMS microbiology reviews, 23(2), 153-177.
- Weaver, K. E., Walz, K. D., & Heine, M. S. (1998). Isolation of a Derivative of *Escherichia coli Enterococcus faecalis* Shuttle Vector pAM401 Temperature Sensitive for Maintenance in *E. faecalis* and Its Use in Evaluating the Mechanism of pAD1*par* Dependent Plasmid Stabilization. Plasmid, 40(3), 225-232.
- Welman, A. D., & Maddox, I. S. (2003). Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends in biotechnology, 21(6), 269-274.

- Whiteley, M., Lee, K. M., & Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences, 96(24), 13904-1390.
- Whitfield, M.A. Valvano.(1993). Biosynthesis and expression of cell-surfaces polysaccharides in gram-negative bacteria. Advances in Microbiology and Physiology, 35, 135–146
- Wu, F. C., Chou, S. Z., & Shih, I. L. (2013). Factors affecting the production and molecular weight of levan of *Bacillus subtilis* natto in batch and fed-batch culture in fermenter. Journal of the Taiwan Institute of Chemical Engineers, 44(6), 846-853.
- Yamamoto, Y., Takahashi, Y., Kawano, M., Iizuka, M., Matsumoto, T., Saeki, S., & Yamaguchi, H. (2000). Hypercholesterolemic effects of levan in rats. Hydrocolloids (Part II). Elsevier Science, The Netherlands, 399-400.
- Yang, S. T., (2011). Bioprocessing for value-added products from renewable resources: new technologies and applications. 1st Edition, Elsevier, Amsterdam.

APPENDIX A : Sucrose Calibration Curve

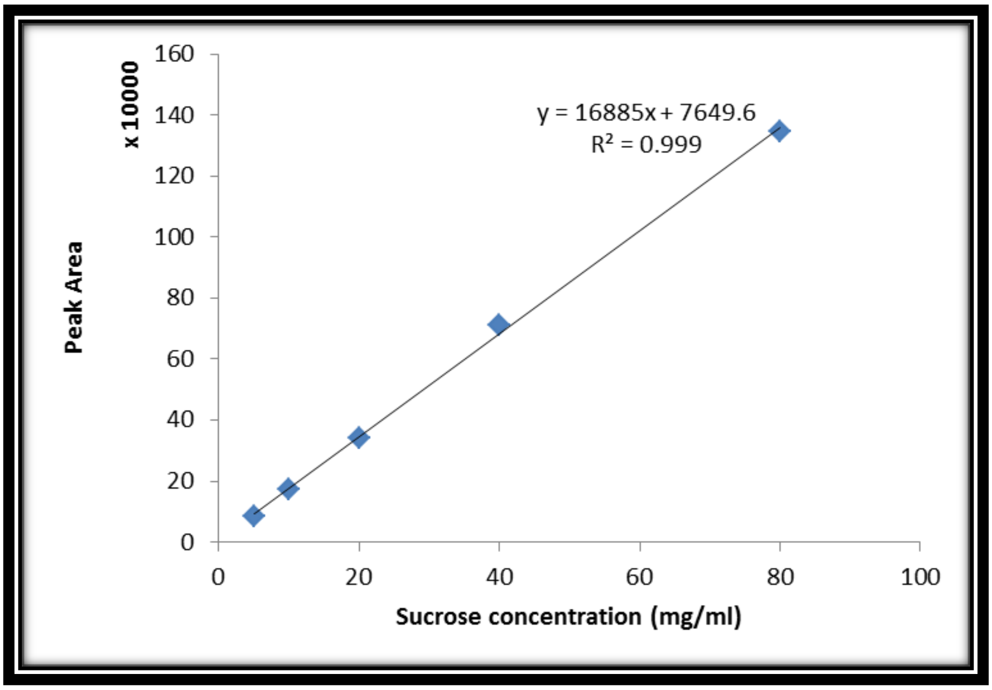


Figure A : Sucrose calibration curve for concentration convertion

APPENDIX B : Glucose Calibration Curve

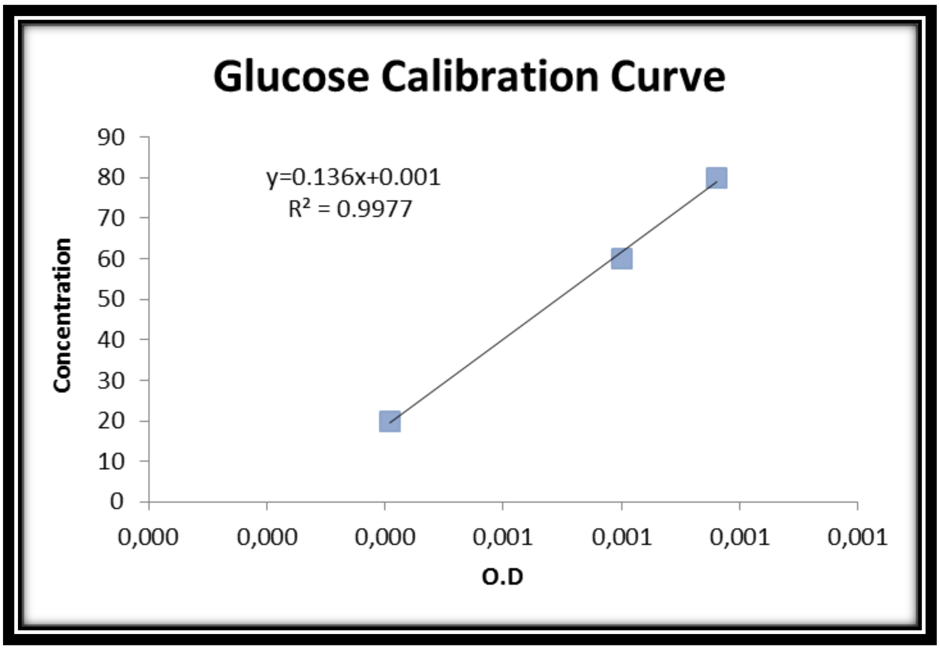
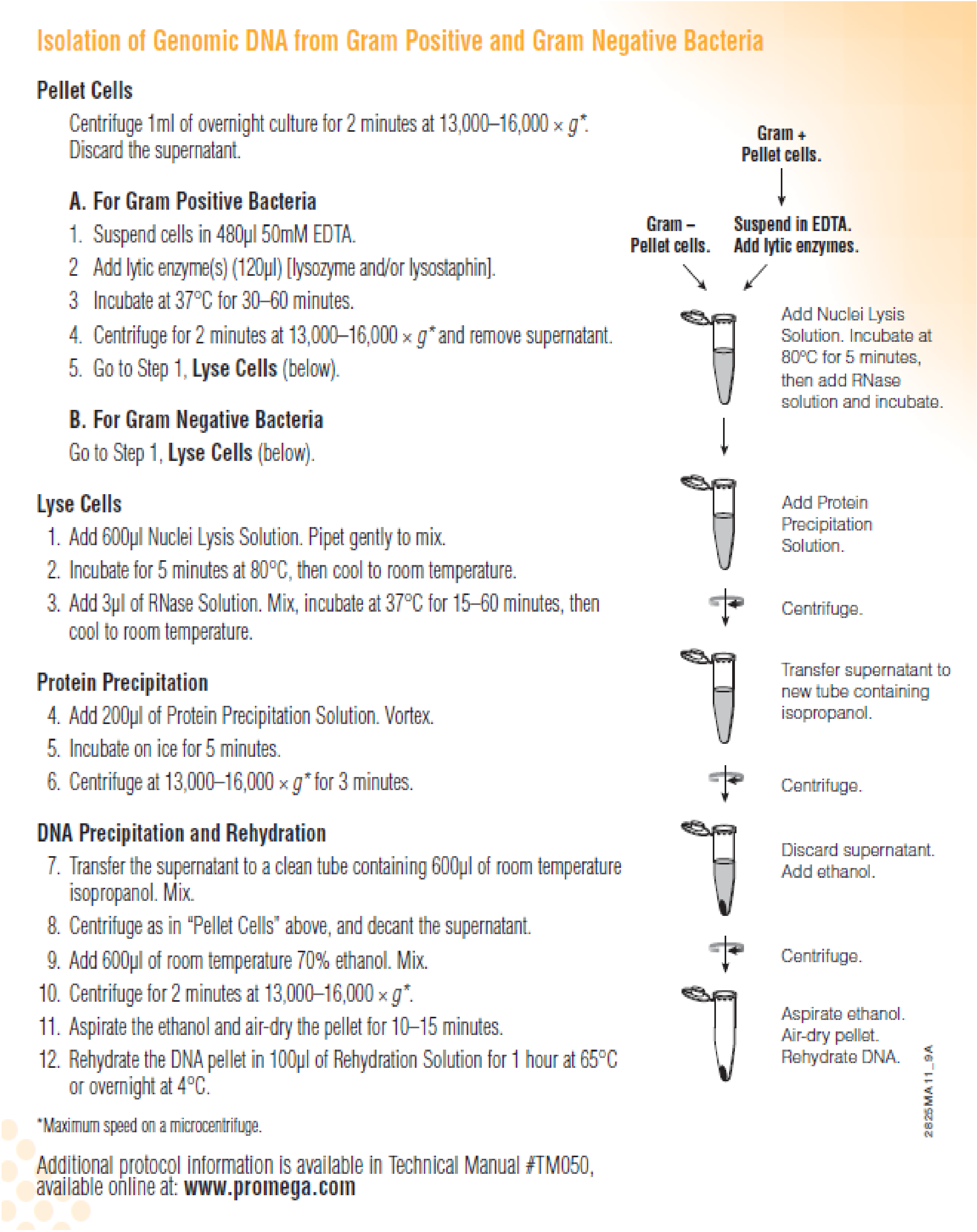


Figure B: Glucose calibration curve for calculation of concentration

APPENDIX C: Genomic DNA Isolation Kit Protocol



Isolation of Genomic DNA from Gram Positive and Gram Negative Bacteria

Pellet Cells

Centrifuge 1ml of overnight culture for 2 minutes at 13,000–16,000 × *g**. Discard the supernatant.

A. For Gram Positive Bacteria

1. Suspend cells in 480µl 50mM EDTA.
2. Add lytic enzyme(s) (120µl) [lysozyme and/or lysostaphin].
3. Incubate at 37°C for 30–60 minutes.
4. Centrifuge for 2 minutes at 13,000–16,000 × *g** and remove supernatant.
5. Go to Step 1, **Lyse Cells** (below).

B. For Gram Negative Bacteria

Go to Step 1, **Lyse Cells** (below).

Lyse Cells

1. Add 600µl Nuclei Lysis Solution. Pipet gently to mix.
2. Incubate for 5 minutes at 80°C, then cool to room temperature.
3. Add 3µl of RNase Solution. Mix, incubate at 37°C for 15–60 minutes, then cool to room temperature.

Protein Precipitation

4. Add 200µl of Protein Precipitation Solution. Vortex.
5. Incubate on ice for 5 minutes.
6. Centrifuge at 13,000–16,000 × *g** for 3 minutes.

DNA Precipitation and Rehydration

7. Transfer the supernatant to a clean tube containing 600µl of room temperature isopropanol. Mix.
8. Centrifuge as in "Pellet Cells" above, and decant the supernatant.
9. Add 600µl of room temperature 70% ethanol. Mix.
10. Centrifuge for 2 minutes at 13,000–16,000 × *g**.
11. Aspirate the ethanol and air-dry the pellet for 10–15 minutes.
12. Rehydrate the DNA pellet in 100µl of Rehydration Solution for 1 hour at 65°C or overnight at 4°C.

*Maximum speed on a microcentrifuge.

Additional protocol information is available in Technical Manual #TM050, available online at: **www.promega.com**

Gram +
Pellet cells.

Suspend in EDTA.
Add lytic enzymes.

Gram –
Pellet cells.

Add Nuclei Lysis Solution. Incubate at 80°C for 5 minutes, then add RNase solution and incubate.

Add Protein Precipitation Solution.

Centrifuge.

Transfer supernatant to new tube containing isopropanol.

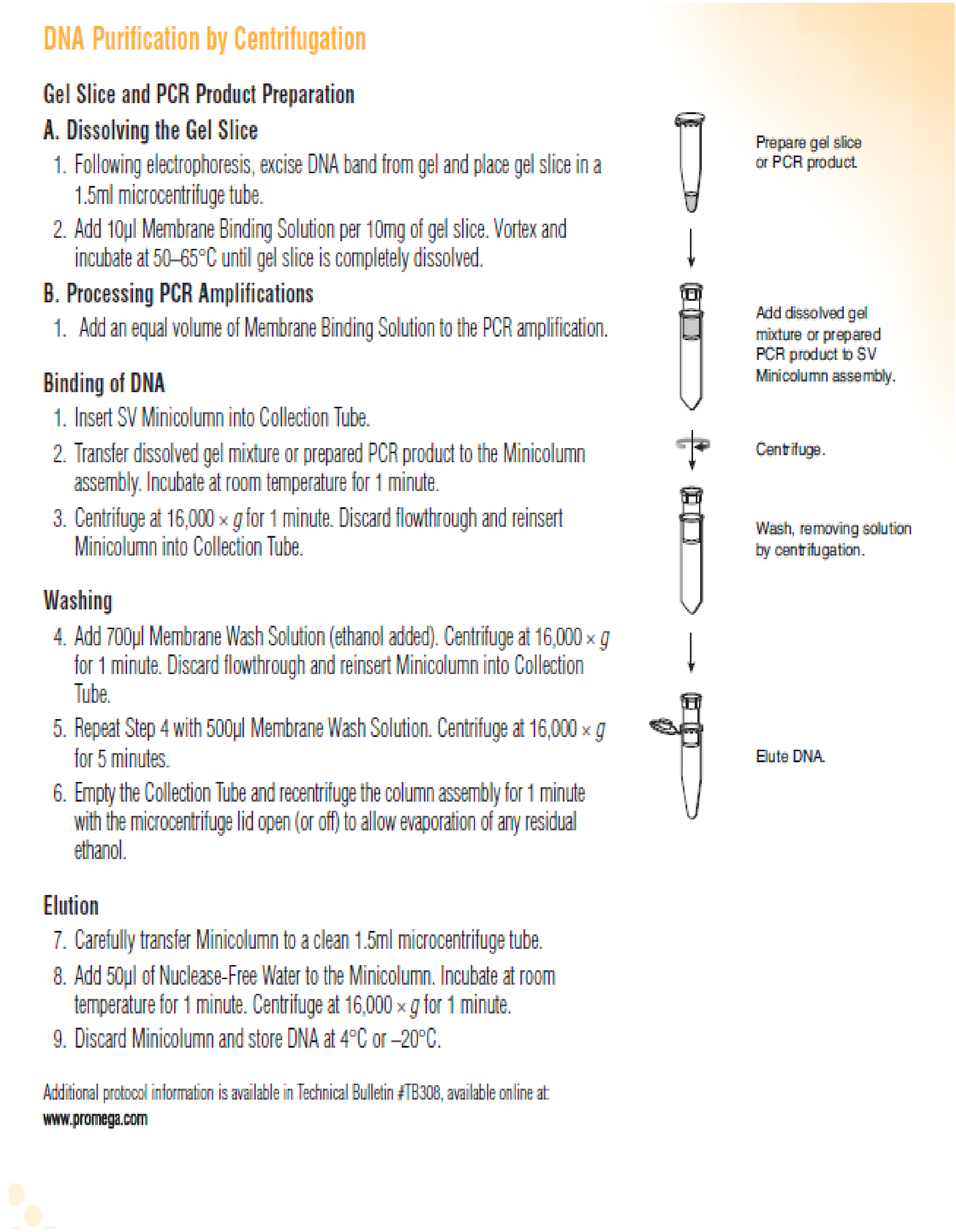
Centrifuge.

Discard supernatant. Add ethanol.

Centrifuge.

Aspirate ethanol. Air-dry pellet. Rehydrate DNA.

APPENDIX D : Wizard Gel and PCR Clean Up Kit Protocol



DNA Purification by Centrifugation

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

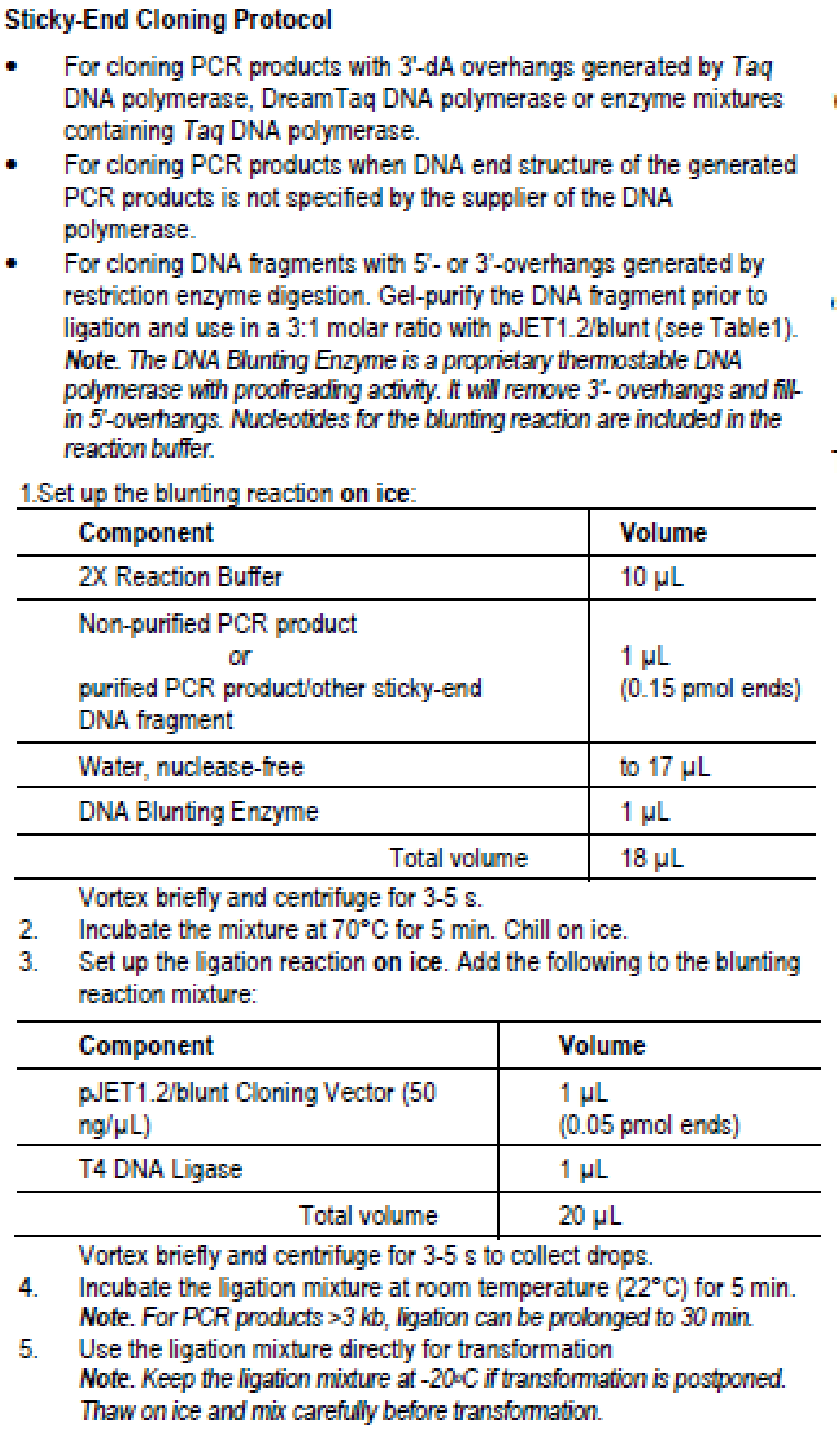
4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.

Additional protocol information is available in Technical Bulletin #TB308, available online at:
www.promega.com

APPENDIX E: CloneJET Sticky-End Cloning Protocol



Sticky-End Cloning Protocol

- For cloning PCR products with 3'-dA overhangs generated by *Taq* DNA polymerase, DreamTaq DNA polymerase or enzyme mixtures containing *Taq* DNA polymerase.
- For cloning PCR products when DNA end structure of the generated PCR products is not specified by the supplier of the DNA polymerase.
- For cloning DNA fragments with 5'- or 3'-overhangs generated by restriction enzyme digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt (*see* Table1).
  Note*. The DNA Blunting Enzyme is a proprietary thermostable DNA polymerase with proofreading activity. It will remove 3'- overhangs and fill-in 5'-overhangs. Nucleotides for the blunting reaction are included in the reaction buffer.*

1.Set up the blunting reaction **on ice**:

Component	**Volume**
2X Reaction Buffer	10 µL
Non-purified PCR product *or* purified PCR product/other sticky-end DNA fragment	1 µL (0.15 pmol ends)
Water, nuclease-free	to 17 µL
DNA Blunting Enzyme	1 µL
Total volume	18 µL

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the mixture at 70°C for 5 min. Chill on ice.
3. Set up the ligation reaction **on ice**. Add the following to the blunting reaction mixture:

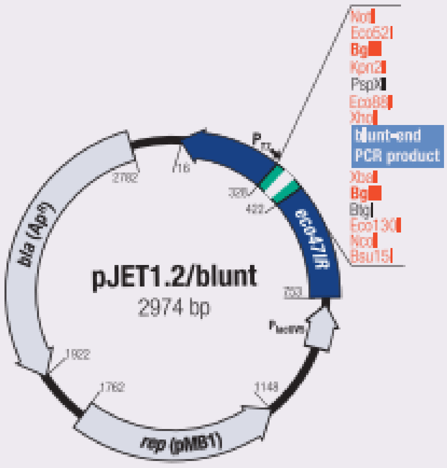
Component	**Volume**
pJET1.2/blunt Cloning Vector (50 ng/µL)	1 µL (0.05 pmol ends)
T4 DNA Ligase	1 µL
Total volume	20 µL

Vortex briefly and centrifuge for 3-5 s to collect drops.

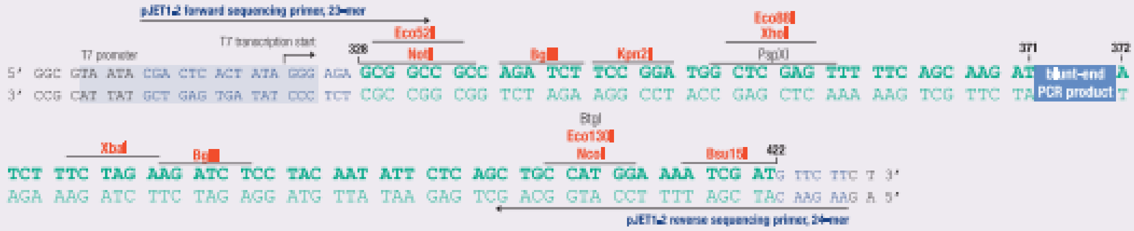
4. Incubate the ligation mixture at room temperature (22°C) for 5 min.
   Note*. For PCR products >3 kb, ligation can be prolonged to 30 min.*
5. Use the ligation mixture directly for transformation
   Note*. Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.*

APPENDIX F: pJET1.2/Blunt Vector Information

pJET1.2/blunt vector map



DNA sequence of MCS region



Genetic elements of the pJET1.2/blunt cloning vector

Element	Function	Position, bp
rep (pMB1)	Replicon (rep) from the pMBI plasmid is responsible for the replication of pJET1.2	1762-1148
Replication start	Initiation of the replication	1162 ±1
bla (Ap^R)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells	2782-1922
eco47IR	Lethal gene *eco47IR* enables positive selection of the recombinants	753-16
P$_{lacUV5}$	Modified P$_{lac}$ promoter for expression of the *eco47IR* gene at a level sufficient to provide positive selection	892-769
T7 promoter	T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert	305-324
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	422-328
Insertion site	Blunt DNA ends for ligation with insert	371-372
Primer binding sites:		
pJET1.2 forward sequencing	Sequencing of insert, colony PCR	310-332
pJET1.2 reverse sequencing	Sequencing of insert, colony PCR	428-405

Enzymes which cut pJET1.2/blunt DNA once

AasI	1204	**GsuI**	2080
AdeI	120	**HindIII**	624
AloI	284	**Kpn2I**	343
BfiI	2040	**LguI**	979
BglI	2109	**MssI**	761
BplI	58	**MunI**	892
Bpu10I	717	**Mva1269I**	722
Bsu15I	417	**NcoI**	408
BveI	219	**NotI**	328
CaiI	1513	**NsbI**	2215
Cfr10I	2075	**PdmI**	2590
Csp6I	2474	**PstI**	5
Eam1105I	1990	**PvuI**	2362
Eco130I	408	**RsaI**	2474
Eco31I	2062	**ScaI**	2473
Eco52I	329	**SmuI**	951
Eco88I	352	**TatI**	2473
Esp3I	739	**XbaI**	377
FaqI	103	**XhoI**	352

Primer sequences

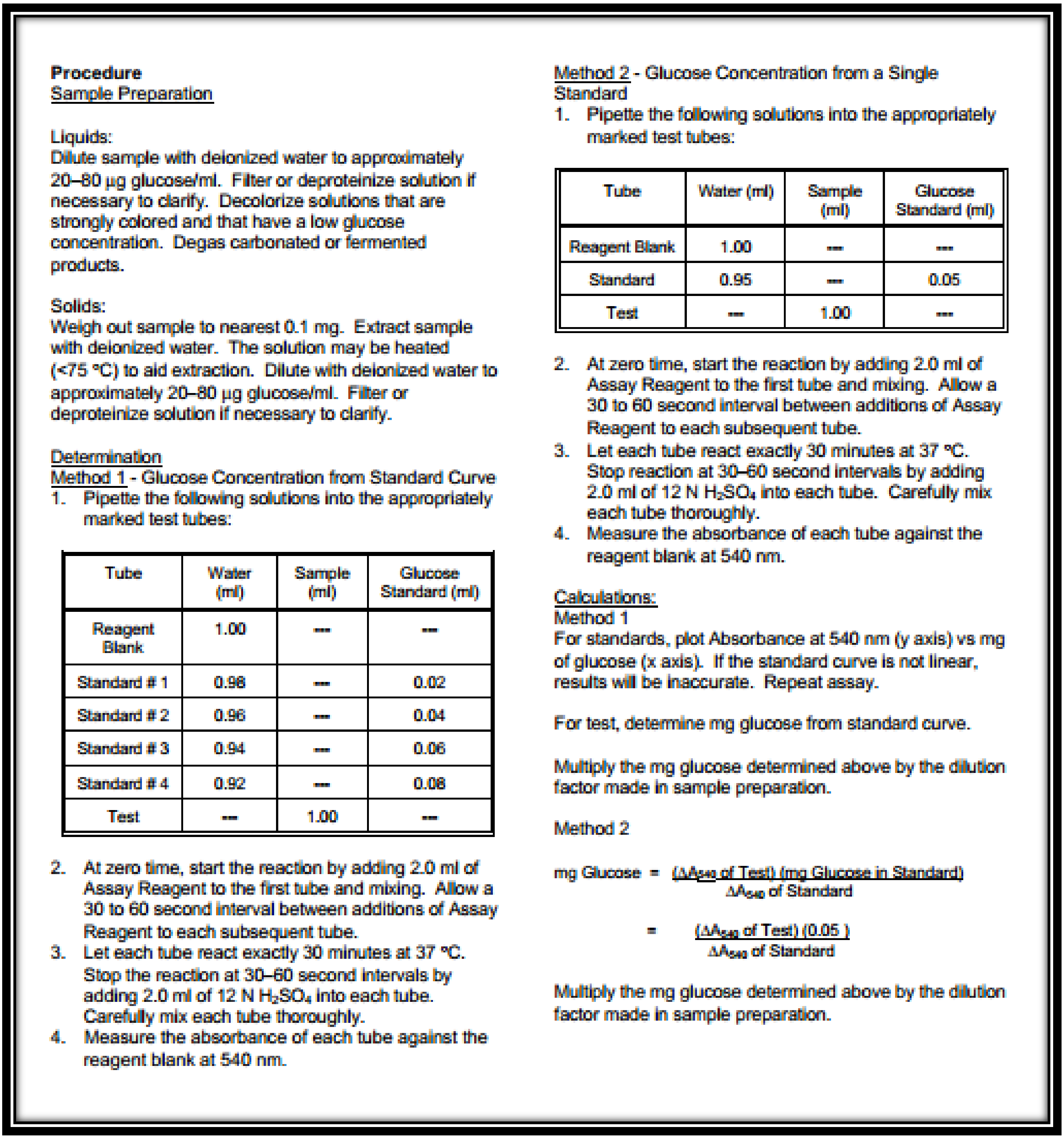
Primer	Sequence
pJET1.2 Forward Sequencing Primer, 23-mer	5'-CGACTCACTATAGGGAGAGCGGC-3'
pJET1.2 Reverse Sequencing Primer, 24-mer	5'-AAGAACATCGATTTTCCATGGCAG-3'

Enzymes that do not cut pJET1.2/blunt DNA

AarI, Acc65I, AjiI, AjuI, AlfI, ApaI, BamHI, BclI, BcuI, BoxI, BpiI, Bpu1102I, BseJI, BshTI, Bsp68I, Bsp119I, Bsp120I, Bsp1407I, BspTI, Bst1107I, BstXI, Cfr9I, Cfr42I, CpoI, Ecl136II, Eco24I, Eco32I, Eco47III, Eco72I, Eco81I, Eco91I, Eco105I, Eco147I, Eco0109I, EcoRI, EheI, FspAI, HincII, KpnI, KspAI, MlsI, MluI, Mph1103I, NdeI, NheI, OliI, PaeI, PasI, PauI, PdiI, Pfl23II, PfoI, Ppu21I, Psp5II, PsyI, SacI, SalI, SdaI, SfiI, SgfI, **SgsI, SmaI, TstI, Van91I, XagI**, XcmI, **XmaJI**.

Enzymes produced by Fermentas are shown in orange.

APPENDIX G: Glucose Assay Kit Protocol



Procedure

Sample Preparation

Liquids:
Dilute sample with deionized water to approximately 20–80 µg glucose/ml. Filter or deproteinize solution if necessary to clarify. Decolorize solutions that are strongly colored and that have a low glucose concentration. Degas carbonated or fermented products.

Solids:
Weigh out sample to nearest 0.1 mg. Extract sample with deionized water. The solution may be heated (<75 °C) to aid extraction. Dilute with deionized water to approximately 20–80 µg glucose/ml. Filter or deproteinize solution if necessary to clarify.

Determination

Method 1 - Glucose Concentration from Standard Curve

1. Pipette the following solutions into the appropriately marked test tubes:

Tube	Water (ml)	Sample (ml)	Glucose Standard (ml)
Reagent Blank	1.00	---	---
Standard # 1	0.98	---	0.02
Standard # 2	0.96	---	0.04
Standard # 3	0.94	---	0.06
Standard # 4	0.92	---	0.08
Test	---	1.00	---

2. At zero time, start the reaction by adding 2.0 ml of Assay Reagent to the first tube and mixing. Allow a 30 to 60 second interval between additions of Assay Reagent to each subsequent tube.
3. Let each tube react exactly 30 minutes at 37 °C. Stop the reaction at 30–60 second intervals by adding 2.0 ml of 12 N H_2SO_4 into each tube. Carefully mix each tube thoroughly.
4. Measure the absorbance of each tube against the reagent blank at 540 nm.

Method 2 - Glucose Concentration from a Single Standard

1. Pipette the following solutions into the appropriately marked test tubes:

Tube	Water (ml)	Sample (ml)	Glucose Standard (ml)
Reagent Blank	1.00	---	---
Standard	0.95	---	0.05
Test	---	1.00	---

2. At zero time, start the reaction by adding 2.0 ml of Assay Reagent to the first tube and mixing. Allow a 30 to 60 second interval between additions of Assay Reagent to each subsequent tube.
3. Let each tube react exactly 30 minutes at 37 °C. Stop reaction at 30–60 second intervals by adding 2.0 ml of 12 N H_2SO_4 into each tube. Carefully mix each tube thoroughly.
4. Measure the absorbance of each tube against the reagent blank at 540 nm.

Calculations:

Method 1

For standards, plot Absorbance at 540 nm (y axis) vs mg of glucose (x axis). If the standard curve is not linear, results will be inaccurate. Repeat assay.

For test, determine mg glucose from standard curve.

Multiply the mg glucose determined above by the dilution factor made in sample preparation.

Method 2

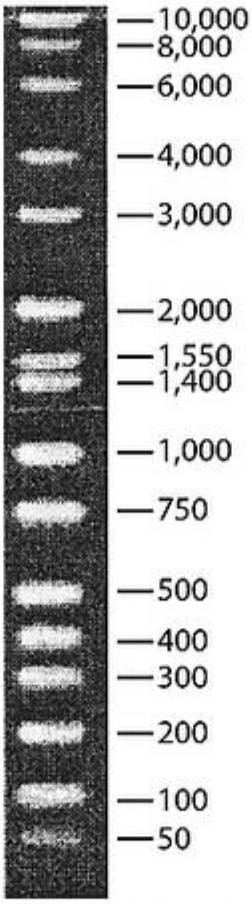
$$\text{mg Glucose} = \frac{(\Delta A_{540}\text{ of Test})\ (\text{mg Glucose in Standard})}{\Delta A_{540}\text{ of Standard}}$$

$$= \frac{(\Delta A_{540}\text{ of Test})\ (0.05\ )}{\Delta A_{540}\text{ of Standard}}$$

Multiply the mg glucose determined above by the dilution factor made in sample preparation.

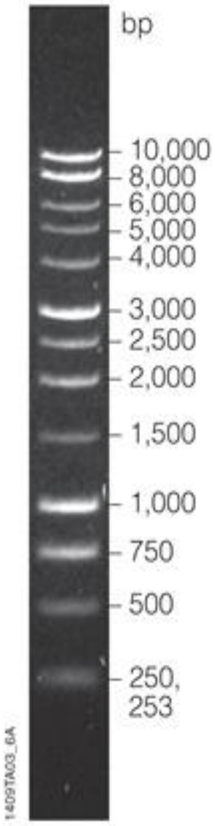
APPENDIX H: Markers

Sigma Wide Range DNA Marker



D7058 on a 0.75% agarose gel

Promega- 1kb DNA Ladder



0.7% agarose

AUTOBIOGRAPHY

Name Surname **:**Büşra AYDIN

Place and Date of Birth **:**Ankara /15.10.1989

Foreign Language **:**English

E-mail **:** busra.ercan@marun.edu.tr

Education

Degree	**Department/Program**	**University/High School**	**GraduationYear**
High School	Science	Samanyolu Cemal Şaşmaz Lisesi	2007
Bachelor of Science	Genetics and Bioengineering	Fatih University	2012

Work Experience

Year	**Company/Institution**	**Position**
July-September, 2009	Fatih University, Faculty of Medicine, Medical Microbiology Department	Internship
August-September, 2010	Fatih University, Faculty of Engineering, Biofluids Dynamics Research Laboratory	Internship
August-September, 2010	Gazi University, Faculty of Medicine, Medical Genetics Department	Internship

Publications :

Presentations:

1. Aydin B., Calimlioglu B., Arga K.Y. “Comparative Genomic Approach to Halophilic Bacteria”, The 15th International Conference on Systems Biology (ICSB2014), 14-18 September 2014, Melbourne, Australia (accepted as a poster presentation)
2. Aydin B., Toksoy Oner E., Arga K.Y. “Investigation of the fructose uptake system in *Halomonas smyrnensis* AAD6^T to design strategies for enhanced levan biosynthesis”, 38th FEBS Congress, 6-11 July 2013, Saint Petersburg, Russia (poster presentation)
3. Aydin B., Toksoy Oner E., Arga K.Y. “Metabolizma mühendisliği ile mikrobiyal levan biyosentezinin optimizasyonu”,2th Bio&Nano Technology Congress, 1 -3 October 2013, Istanbul, Turkey (poster presentation).

Projects:

1. 2013-2014 Marmara University Research Fund Project no: FEN-C-YLP-101013-0404 and title: “Investigation of the fructose uptake system in *Halomonas smyrnensis* AAD6^T to design strategies for enhanced levan biosynthesis”.
2. 2013-2014 TUBITAK research Project no: MAG 110M613 and title: “System Based Optimization of Microbial Biopolymer Production”.

Awards and Fellowships:

1. Bursary winner, TUBITAK 2210-C Öncelikli Alanlara Yönelik Yüksek Lisans Burs Programı, 2014.

2. Bursary winner, 38th FEBS Congress, 6-11 July 2013, Saint Petersburg, Russia.

3. Poster award as 3rd winner, Bio&Nano Technology Congress, 1 -3 October 2013, Istanbul, Turkey