

**T.R.**  
**GEBZE TECHNICAL UNIVERSITY**  
**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

**THE EFFECT OF OVEREXPRESSION OF GLUCOSE  
TRANSPORTER ON 2,3-BUTANEDIOL PRODUCTION IN  
STREPTOMYCES COELICOLOR**

**OYTUN SARIGÖZ**  
**A THESIS SUBMITTED FOR THE DEGREE OF  
MASTER OF SCIENCE**  
**DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

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**THESIS SUPERVISOR  
PROF. DR. SEDEF TUNCA GEDİK**

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**FEN BİLİMLERİ ENSTİTÜSÜ**

**GLİKOZ TRANSPORTER 'ın YÜKSEK**  
**İFADESİNİN STREPTOMYCES**  
**COELICOLOR'UN 2,3-BÜTANDİOL**  
**ÜRETİMİNE ETKİSİ**

**OYTUN SARIGÖZ**  
**YÜKSEK LİSANS TEZİ**  
**MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI**

**DANIŞMANI**  
**PROF. DR. SEDEF TUNCA GEDİK**

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## YÜKSEK LİSANS JÜRİ ONAY FORMU

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 30/06/2022 tarih ve 2022/33 sayılı kararıyla oluşturulan jüri tarafından 22/07/2022 tarihinde tez savunma sınavı yapılan OYTUN SARIGÖZ'ün tez çalışması MOLEKÜLER BİYOLOJİ VE GENETİK Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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## SUMMARY

*Streptomyces* produce special kinds of secondary metabolites which are used as pharmaceutical or industrial intermediates. Therefore, primary metabolisms of *Streptomyces* have not been focused on as much as their secondary metabolisms. 2,3-butanediol, which is a primary alcohol, is intensively used as a platform chemical in polymer, cosmetics, food, and pharmaceutical industries. Homologues of enzymes, which are responsible for production of 2,3-butanediol, are theoretically shown in *Streptomyces coelicolor* according to their protein homology. Also, some *Streptomyces* strains, which produces 2,3-butanediol and its derivatives, are discovered. 2,3-butanediol production depends on primary carbon metabolism and the main precursor of 2,3-butanediol, acetoin, is synthesized from pyruvate by acetolactate synthase and butanediol dehydrogenase. The main carbon source that controls primary metabolism is glucose. Glucose uptake is mainly controlled by special 'glucose transporter protein' (GlcP) in *S. coelicolor*. GlcP is encoded by *glcP* gene which, has two homologues: *glcP1* and *glcP2* in this organism. Effect of GlcP1 on glucose uptake was shown by different studies. Depending on these studies, overexpression of *glcP1* gene was thought to have potential to enhance primary metabolite production in *Streptomyces*. In this study, *glcP1* gene was cloned into a multicopy pSPG plasmid under the control of glycerol inducible promoter and also into integrative pRA vector with its own promoter and transcriptional terminator. The effects of overexpression of this gene on primary metabolites were determined. According to our results primary carbon metabolism and related organic acid production have been enhanced by overexpression of *glcP1* gene. However, 2,3-butanediol production was not affected by the extra copies of *glcP1* gene. In future studies, it will be understood whether the recombinant strain has industrial importance by determining what organic acids are highly produced in this strain.

**Keywords: glcP, 2,3-Butanediol, *Streptomyces*, Fermentation.**

## ÖZET

*Streptomyces* 'ler endüstriyel ve farmasötik ara ürün olarak kullanılabilen birçok özel ikincil metabolit türünü üretebilmektedir. Bu sebeple, *Streptomyces*'lerin birincil metabolizmasına ikincil metabolizmalarına olduğu kadar odaklanılmamıştır. Birincil alkollerden olan 2,3-bütandiol platform kimyasalı olarak polimer, kozmetik, gıda ve farmasötik endüstrilerinde yoğun şekilde kullanılmaktadır. 2,3-bütandiol üretiminde görevli homolog enzimler *Streptomyces coelicolor* bakterisinde protein homoloji çalışmaları ile gösterilmiştir. Ayrıca, farklı 2,3-bütandiol türevlerini üretebilen *Streptomyces* türleri keşfedilmiştir. 2,3-bütandiol birincil karbon metabolizmasına bağlıdır ve ana öncül molekülü olan asetoin, asetolaktat sentaz ve bütandiol dehidrogenaz enzimleri ile pirüvattan üretilmektedir. Birincil metabolizmayı kontrol eden en önemli karbon kaynağı glikozdur. *S. coelicolor*'da glikoz alımı genel olarak özel glikoz transfer proteini, GlcP, ile kontrol edilmektedir. *Streptomyces* bakterilerinde, GlcP proteinini *glcP* geni kodlanmaktadır ve *glcP* geninin *glcP1* ve *glcP2* olmak üzere 2 homologu bulunmaktadır. *glcP1* geninin glikoz alımındaki etkisi farklı çalışmalarla gösterilmiştir. Bu çalışmalar ışığında, *glcP1* geninin aşırı ekspresyonunun, *Streptomyces*'te birincil metabolit üretimini artırma potansiyeline sahip olduğu düşünülmüştür. Bu çalışmada, *glcP1* geni çok kopyalı bir vektör olan pSPG vektörünün gliserol ile uyarılabilen promotörü altına ve kendi promotor ve transkripsiyonel terminatör sekansını içerecek şekilde de genomu entegre olabilen pRA vektörüne klonlanmıştır. *glcP1* geninin yüksek ekspresyonunun birincil metabolizma üzerindeki etkileri çalışılmıştır. Sonuçlarımıza göre, birincil metabolizma ve ona bağlı organik asitlerin üretimi *glcP1* geninin yüksek ekspresyonu ile artmıştır. Fakat, 2,3-bütandiol üretiminin ekstra *glcP1* geninden etkilenmediği bulunmuştur. Bu suşta hangi organik asitlerin yüksek oranda üretildiğinin belirlenmesi ile rekombinant suşun endüstriyel öneme sahip olup olmadığı ileride yapılacak çalışmalarla anlaşılacaktır.

**Anahtar Kelimeler: glcP, 2,3-Bütandiol, Streptomyces, Fermantasyon.**

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## LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
$\alpha$	: Alfa
$\Delta$	: Delta
$\mu\text{L}$	: Microlitre
$\mu\text{M}$	: Micromolar
Apr	: Apramycin
Amp	: Ampicillin
BamHI	: <i>Bacillus amyloliquefaciens</i> H endonuclease
bp	: Base pair
$\text{CaCl}_2$	: Calcium chloride
CCR	: Carbon Catabolite Repression
EcoRV	: <i>Escherichia coli</i> restriction endonuclease V
EDTA	: Ethylenediaminetetraacetic acid
h	: Hour
kb	: kilobase
KCl	: Potassium chloride
KOAc	: Potassium acetate
L	: Litre
LB	: Luria-Bertani broth
M	: Molar
Mg	: Milligram
$\text{Mg}^{+2}$	: Magnesium ion
mL	: Millilitre
mM	: Millimolar
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
NdeI	: <i>Neisseria denitrificans</i> endonuclease I

PCR	:	Polymerase chain reaction
PPP	:	Pentose phosphate pathway
SDS	:	Sodium dodecyl sulfate
SpeI	:	<i>Sphaerotilus</i> sp. Endonuclease I
TCA	:	Tricarboxylic acid
Tris	:	Tris (Hydroxymethyl) aminomethane



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# 1. INTRODUCTION

*Streptomyces* are gram-positive, and filamentous soil bacteria with high G:C ratio in their genome. They are capable of producing various bioactive compounds, which are antibiotics, antifungal or antiviral drugs or drug intermediates, by their secondary metabolism. *Streptomyces* has a great potential to be used in bioproduction of other bioactive chemicals because of their unknown cryptic biosynthetic gene clusters. Moreover, *Streptomyces* have biotechnological importance with also their primary metabolism, since they synthesize different types of organic acids, and primary alcohols. Products of primary metabolism, such as 2,3-butanediol (2,3-BDO), have an industrial importance, because these metabolites are utilized as platform chemical for further high-value chemical production in various industries.

Primary metabolism is mainly composed of Tricarboxylic acid (TCA) Cycle, pentose phosphate pathway (PPP), and glycolysis. Products of primary metabolism are primary alcohols (ethanol, 2,3-BDO etc.), and organic acids (acetate etc.). 2,3-BDO is directly branched from pyruvate, and acetoin is its intermediate precursor. Primary metabolism is strictly regulated by concentration and type of carbon source. Glucose is the main carbon source that regulate primary metabolism. Carbon sources are transported into the cell by conserved phosphoenolpyruvate sugar phosphotransferase system (PTS) pathways in bacteria. Other than PTS, a special “glucose transporter protein”, GlcP, was found to be responsible from uptake and utilization of glucose.

## 1.1. The Aim and Original Value of The Thesis

In this study, primary metabolism of *S. coelicolor* was targeted to enhance 2,3-BDO. As a primary metabolite, 2,3-BDO is a high-value chemical that can be used as a precursor or ingredient for industrial products, such as print ink, polymer, and fuel additives. This metabolite is mainly synthesized from pyruvate, therefore, enhancement of glucose utilization should have a direct effect on production of 2,3-BDO. Enhanced production of 2,3-BDO could give an enormous potential to *Streptomyces* for bioproduction of chemicals important for industrial applications. Since extracellular glucose is transferred into the cell by also GlcP transporter protein,

in order to increase glucose utilization, *glcP1* gene was overexpressed in *Streptomyces coelicolor* in the concept of this thesis. To the best of our knowledge, the effect of *glcP1* on primary metabolite production in *Streptomyces* was shown for the first time in this study. Recombinant strain with high glucose uptake and increased primary metabolite synthesis could have potential to be implemented for the production of other platform chemicals.



## 2. STREPTOMYCES

*Streptomyces* are gram-positive, aerobic, filamentous soil-dwelling bacteria, which produces valuable secondary metabolites. *Streptomyces* species can be isolated from different terrestrial or aquatic environments, and they have been emphasized according to their various secondary metabolites. *Streptomyces* are known to produce 70% of known antibiotics. Dilemma of using antibiotic is creating resistant pathogenic bacteria species, and that dilemma push forward scientists to discover more and unique antibiotics. From this aspect, unknown biosynthetic gene clusters of *Streptomyces* have more importance for discovering novel secondary metabolites [1].

*Streptomyces* can synthesize various bioactive molecules via their specific secondary metabolism, which are encoded by biosynthetic gene clusters (BGCs). However, some of BGCs are not expressed under normal cultivation conditions in *Streptomyces*, and these are called cryptic or silent clusters. The cryptic gene clusters are not active in general laboratory conditions, therefore new cryptic gene clusters are usually discovered by bioinformatic studies, which are based on promoter and gene homology [2]. Newly discovered BGCs have great potential to discover new bioactive compounds, which can be used as therapeutics. However, to produce new bioactive compounds, cryptic gene clusters must be activated or expressed. For expression of the cryptic gene clusters, some specific conditions must be generated for *Streptomyces* [3]. In general prospects, environmental changes can be applied to activate or trigger these clusters. In molecular genetic prospects, mutagenesis can be applied to overwhelm transcriptional regulation, which keeps silent the gene clusters at normal conditions. Mutations can be induced by chemical or physical agents, such as ethyl methanesulfonate or UV. Deletion of a negative regulator gene can also be used to achieve continuous expression of BGCs. Moreover, specific clusters can be overexpressed to enhance production of target metabolite [4].

Enhancement of secondary metabolite production is also achieved by genetic alterations, which affect metabolic pathways. Secondary metabolites, especially antibiotics, which are generally synthesized by polyketide metabolisms, are regulated with global regulators. These global regulators also regulate essential molecular pathways, which are related with growth and differentiation. Global regulators control transcription of gene clusters at different stages of life cycle [5]. Discovering the interconnection between growth or differentiation pathways and secondary

metabolism can be exploited to enhance production of high-value bioactive compounds. Differentiation is directly connected with both growth and secondary metabolism, in *Streptomyces*. Previously, global regulators and regulator molecules, such as ppGppp, have been discovered as parts of differentiation and secondary metabolite production [6].

Other than secondary metabolism, global regulators also regulate primary metabolism with same regulatory molecules or unique regulatory molecules. Utilized carbon source is generally participated as a regulatory molecule in primary metabolism and global regulatory cascade mechanisms [7]. Global regulators give more information to enlighten unknown parts of differentiation, carbon utilization, primary and secondary metabolisms as cellular mechanisms. Identified global regulators can down or up regulate some pathways, and the regulation system can be implemented to obtain desired outcome, for instance, increased antibiotic production [8].

Secondary metabolisms synthesize bioactive molecules, which are not essential for growth. Although they are not essential, secondary metabolites have positive effects on bacteria to gain additional benefits. In natural environments, bacteria gain advantages against other microorganism by their secondary metabolites to eliminate other competitors for limited energy and carbon supply [12].

To produce enhanced bioactive metabolites, all knowledge about circuits of global metabolic pathways have been used to obtain suitable host strain. *Streptomyces* have been studied for using as host strains because of they are commonly studied organism, and genomes of some strains were sequenced [9]. Although *Streptomyces* strains naturally synthesize several bioactive metabolites by their native BGCs, recombinant *Streptomyces* strains have been developed to produce various antibiotics or other bioactive metabolites [10].

## **2.1. Primary Carbon Metabolism**

*Streptomyces* have conserved primary carbon metabolisms as other bacteria to provide energy and carbon backbone for synthesis of essential amino acids and precursor molecules, which are necessary for biomolecules. Glycolysis, TCA cycle and PPP are main metabolic pathways for energy and carbon source utilization in *Streptomyces*. In addition, *Streptomyces* can utilize nitrite and nitrate by reduction in nitrogen metabolism. Moreover, *Streptomyces* are highly oxygen dependent

microorganism, which cannot grow on glucose without sufficient oxygen supply [7]. Growth of *Streptomyces* depends on a complex life cycle, and primary carbon metabolism is differently regulated at each part of the life cycle because of the energy demand is altered in different part. Life cycle of *Streptomyces* starts with germination of spore at suitable environmental conditions. During germination, first vegetative hyphae grow, and then colony formation is constituted. Next, aerial mycelium grown into air by their hydrophobic features. Finally, spore chains are produced by differentiation of cells in aerial mycelium. Differentiation of the cells is determined by programmed cell death (PCD), which is highly regulated with global mechanisms. Also, PCD interconnected with secondary metabolite production, especially with antibiotic synthesis [11]. Primary carbon metabolisms provide all necessary precursor molecules for PCD and other metabolic pathways as well as for secondary metabolisms.

For primary carbon metabolism, different types of carbohydrate can be utilized as a carbon source, such as sucrose. *Streptomyces* are mainly found in soil environments, so they have ability to degrade animal and plant materials, which includes complex polysaccharides, as cellulose and chitin [7]. Cellulose is the most abundant waste material, and chitin is the second abundant waste material in the world. Therefore, utilization of these type of materials, as carbon sources, is crucial for low cost and sustainable production processes [13], Polysaccharides are degraded into their monosaccharides and disaccharides by exogenous enzymes, which are secreted by *Streptomyces*. Various type of exogenous enzymes is secreted by *Streptomyces*. *Streptomyces* can digest starch by  $\alpha$ -amylase, cellulose by cellulase, and chitin by chitinases. In addition, *Streptomyces* digest agar by their agarase activity [7]. Monosaccharides or disaccharides are transferred into the cell for further utilization in catabolic pathways by various target specific transfer mechanism. Approximately, 50 permeases have been identified in *Streptomyces* for different carbohydrates according to homology research [14]. Some carbon sources, such as cellobiose, *N*-acetylglucosamine, and xylobiose, are identified to be utilized by various transport mechanisms, which are ATP-dependent ABC transport system or phosphotransferase system (PTS) in different *Streptomyces* species [15],[16],[17]. Glucose is important monosaccharide, which is universally utilized by all organisms. It affects various global metabolic pathways, such as carbon catabolic repression (CCR). CCR is a global mechanism, which regulates different parts of primary and secondary

metabolism. According to CCR, bacteria determine which carbon source is firstly utilized. BGCs are also down or up regulated according to carbon catabolite repression. If bacteria access appropriate carbon source, CCR is triggered, and secondary metabolism is delayed. CCR is related and connected with PTS system and other carbon utilization mechanism [18].

Glucose is transferred into the cell by PTS in several bacterial species [19]. In gram-negative and gram-positive bacteria, enzyme I, enzyme II complexes, and HPr are generally essential enzymes of PTS. Enzyme I and enzyme II are responsible for transferring of carbon source with phosphorylation, and HPr is a phosphoryl donor. Specificity of PTS for different carbon source is determined by enzyme II complex. EI and EII complexes also contain carbohydrate specific kinases. However, CCR is regulated differently by PTS in gram-negative and gram-positive bacteria. In gram-negative bacteria, such as *E. coli*, CCR is regulated with enzyme IIA<sup>Glc</sup> and adenylate cyclase. In gram-positive bacteria, such as *B. subtilis*, CCR is constituted by phosphorylation of HPr at a serine amino acid [20]. However, in *Streptomyces* glucose is not transferred into the cell by PTS [21]. Instead, GlcP protein is responsible for glucose uptake in this organism. GlcP belongs to Major Facilitator Superfamily of sugar permeases, and there are two homologous of *glcP* gene, which are *glcP1* and *glcP2*. These two homologues have similar sequences and encode same length of amino acid. It was shown that only *glcP1* gene product is active for glucose uptake in *Streptomyces*. [22].

## 2.2. 2,3-Butanediol and 2,3-Butanediol Metabolism

As a result of decreasing reservoir for petroleum and crude oil, new renewable production strategies are developing to overwhelm increased demand of worldwide industries on platform chemicals. Microbial chemical production is also one of the renewable production strategies [23]. Microbial production strategies can be implemented to produce different chemicals, and 2,3-Butanediol (2,3-BDO) is one of them. 2,3-BDO has a potential to be used as a platform chemical, because it can be used as a direct ingredient or a raw material for further applications in pharmaceutical, polymer and other industries. 2,3-BDO is a natural organic chemical, and there are 3 natural isomers of 2,3-BDO, which are meso-2,3-BDO, D(-)-2,3-BDO, and L(+)-

2,3-BDO. 2,3-BDO is produced naturally by different microorganisms which can be used in industrial production of this chemical [24].

Different microbial organisms can produce 2,3-BDO, such as *Bacillus*, *Klebsiella* and *Enterobacter*. *Klebsiella pneumoniae*, and *Klebsiella oxytoca* are most desirable producer strains because of their high yield in 2,3-butanediol production. However, *Bacillus* strains were developed for higher yield of 2,3-BDO [25],[26],[27]. 2,3-butanediol is synthesized from pyruvate by commonly three enzymes, which are  $\alpha$ -acetolactate dehydrogenase,  $\alpha$ -acetolactate decarboxylase and 2,3-butanediol dehydrogenase. Pyruvate is firstly converted into acetolactate by  $\alpha$ -acetolactate synthase, and acetolactate is further converted into acetoin by  $\alpha$ -acetolactate decarboxylase, then at the final step, 2,3-BDO is synthesized from acetoin by 2,3-butanediol dehydrogenase. Production of each isomer of 2,3-BDO is determined by production of acetoin isomer, which can be D- (-) or L- (+)- acetoin. Acetolactate is spontaneously converted into diacetyl, and D-(-) or L- (+) acetoin are synthesized from diacetyl by two different meso-2,3-BDO dehydrogenases, which form D-(-) acetoin, L-(+)-acetoin. Stereoisomers of 2,3-BDO are synthesized from same isomer of acetoin by isomer specific 2,3-BDO dehydrogenases. Conversion of acetoin to 2,3-BDO is reversible and the conversion is mainly based on pH and cellular NADH concentration. Cellular pH mainly determines yield of 2,3-BDO conversion from acetoin. In high pH, cellular mechanism tries to neutralize pH to 7 with acetoin instead of 2,3-BDO production. Reversibly, in low pH, 2,3-BDO production is increased to decrease cellular pH. In addition to pH, NADH concentration and NAD/NADH availability determine which isomer of 2,3-BDO is produced. In low NADH concentration, D-(-) form of 2,3-BDO is produced, and in high NADH concentration, racemic mixture of 2,3-BDO is produced. NADH determines the isomer formation because isomers of acetoin are produced by NADH-dependent enzymes [28],[29].

In *Streptomyces*, 2,3-BDO pathway is available and active according to butanoate metabolism of *Streptomyces* at KEGG Database [30]. Although production of 2,3-BDO precursor acetoin and acetoin derivatives have been demonstrated by various studies in *Streptomyces*, 2,3-BDO production have not been directly demonstrated. *Streptomyces* are known to produce various volatile compounds. Some of these compounds are produced by bacteria for inter or intraspecies communication in soil. Also, stimulation effect of bacterial volatile compounds on fungal or bacterial spore germination have been discovered [31],[32]. Volatile compounds may have

important tools to understand quorum sensing mechanism of soil bacteria, especially of *Streptomyces*. By quorum sensing mechanism, microorganism can respond to the presence of their own kind or other microorganisms [33]. It is a crucial mechanism that determines which global regulator will be activated according to population changes in the environment. Quorum sensing mechanism affects metabolic pathways and have been used to trigger silent metabolism for discovering novel secondary metabolites. Most popular quorum sensing molecules in *Streptomyces* are gamma-butyrolactones (GBLs) or GBL-like molecules [34]

*Streptomyces* can utilize different types of carbon sources. Some of these sources are renewable and waste of other industries, such as cellulose. Microbial production processes gain more focus to replace conventional production processes with a renewable and beneficial substitute. Renewable and waste materials can be utilized by *Streptomyces* to produce high-value platform chemical with low cost. All of these gives *Streptomyces* the potential to become a host strain for the production of 2,3-BDO or other valuable bioactive metabolites.

## 3. MATERIALS AND METHODS

### 3.1. Materials

#### 3.1.1. Media

Culture medias, which were used in this research, and their ingredients are listed in Table 3.1. All medium was sterilized by autoclave at 121 °C for 20 minutes before cultivation.

Table 3.1: Media and Ingredients.

MEDIA
<b>Luria Bertani (LB) Broth-800 ml</b> <ul style="list-style-type: none"><li>• 8 g NaCl</li><li>• 8 g Peptone</li><li>• 4 g Yeast Extract</li></ul>
<b>Mannitol Salt (MS) Agar-600 ml</b> <ul style="list-style-type: none"><li>• 12 g Mannitol</li><li>• 12 g Soy-bean Flour</li><li>• 12 g Agar</li></ul> <p>(MS agar was autoclaved twice at 121 °C for 20 minutes.) (10 mM MgCl<sub>2</sub> was added separately).</p>
<b>Tryptic Soy Broth (TSB)-800 ml</b> <ul style="list-style-type: none"><li>• 24 g Tryptic Soy Broth</li></ul> <p>12 g agar was added into the medium for Tryptic Soy Agar (TSA).</p>
<b>TBO Agar-800 ml</b> <ul style="list-style-type: none"><li>• 16 g Oat Bran</li><li>• 16 g Tomato Paste</li><li>• 20 g Agar</li></ul>
<b>2xYT Broth-800 ml</b> <ul style="list-style-type: none"><li>• 12.8 g Peptone</li><li>• 8 g Yeast Extract</li><li>• 4 g NaCl</li></ul>

### 3.1.2. Enzymes

Lysozyme (Sigma), RNase, Phusion DNA Polymerase (Thermoscientific), *NdeI* Restriction Endonuclease (NEB), *SpeI* Restriction Endonuclease (NEB), *BamHI* Restriction Endonuclease (Thermo Scientific), *EcoRV* Restriction Endonuclease (NEB), T4 DNA Ligase (Thermo Scientific).

### 3.1.3. DNA Isolation Kits and Markers

Plasmids and PCR products were purified from agarose gel by using PCR Clean-Up and Gel Extraction Kit (Machery-Nagel (MN)). GeneRuler 1 kb DNA Ladder (Thermo Scientific) was used as a reference for agarose gel electrophoresis (Figure 3.1)

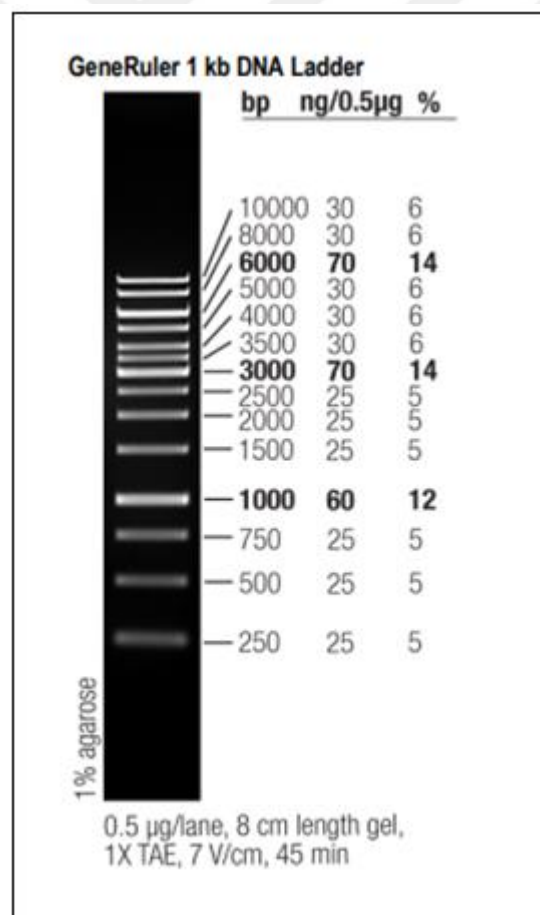


Figure 3.1: GeneRuler 1 kb DNA Ladder as a Marker (Thermo Scientific, SM0311).

### 3.1.4. Chemicals

The following chemicals were used in the experiments of the thesis; Peptone (Bacto™, Merck), NaCl (Sigma-Aldrich), Yeast extract (Bacto™), Agar (Merck), NaOH (Merck), SDS (Merck), Tris (Merck), EDTA (Merck), D-(+)-Glucose (Merck), KOAc (Sigma Aldrich), Isopropanol (Riedel de Haen), Agarose (Sigma Aldrich), Sucrose (Merck), CaCl<sub>2</sub> (Merck), KCl (Riedel de Haen), Acetic Acid (Riedel de Haen), 99.5% Ethanol (Riedel de Haen), Tryptic soy broth (Merck), K<sub>2</sub>HPO<sub>4</sub> (Merck), K<sub>2</sub>SO<sub>4</sub> (Merck), TES Buffer (Applichem), Mannitol (Merck), Bacto Casaminoacid (BD), EDTA (Merck), 98% H<sub>2</sub>SO<sub>4</sub> (Sigma Aldrich), 84-88% Glycerol (Sigma Aldrich), Phenol (Merck), Formic Acid (Merck), Ethyl Acetate (Merck), Acetonitrile (Merck), Phosphoric Acid (Merck).

### 3.1.5. Solutions

Solutions and antibiotics, which are used in this research, were prepared according to Table 3.2 and Table 3.3.

Table 3.2: Ingredients of Solutions.

<b>SOLUTIONS</b>	
<p><b>Solution I (+4° C)</b></p> <ul style="list-style-type: none"> <li>• 25 mM Tris (pH: 8.0)</li> <li>• 10 mM EDTA (pH: 8.0)</li> <li>• 50 mM Glucose</li> </ul> <p><b>Solution II</b></p> <ul style="list-style-type: none"> <li>• 0.2 N NaOH</li> <li>• 1% SDS</li> </ul> <p>(Must be prepared freshly).</p> <p><b>Solution III (+4° C)</b></p> <ul style="list-style-type: none"> <li>• 0.2 N</li> <li>• 60% 5 M KOAc</li> <li>• 11.5% Glacial Acetic Acid</li> </ul> <p><b>Tris-EDTA (TE) Buffer</b></p> <ul style="list-style-type: none"> <li>• 10 mM Tris</li> <li>• 1 mM EDTA</li> </ul>	<p><b>Blocking Solution:</b></p> <ul style="list-style-type: none"> <li>• 0.5 g Blocking Reagent</li> <li>• In 50 mL of Maleic Acid Buffer (10% w/v)</li> </ul> <p><b>Depurination Solution:</b></p> <ul style="list-style-type: none"> <li>• 0.25M NaCl</li> </ul> <p><b>Deneutralization Solution:</b></p> <ul style="list-style-type: none"> <li>• 1.5M NaCl</li> <li>• 0.5M NaOH</li> </ul> <p><b>Neutralization Solution:</b></p> <ul style="list-style-type: none"> <li>• 1.5M NaCl</li> <li>• 0.05M Tris-HCl (pH 7.2)</li> <li>• 1mM EDTA</li> </ul>

Table 3.2: Ingredients of Solutions (Continue).

<b>SOLUTIONS</b>	
<p><b>50x TAE</b></p> <ul style="list-style-type: none"> <li>• 50 mM EDTA</li> <li>• 2 M Tris</li> <li>• 1 M Glacial Acetic Acid</li> </ul> <p><b>TSS:</b></p> <ul style="list-style-type: none"> <li>• 10% Polyethylene glycol</li> <li>• 5% Dimethyl Sulfoxide</li> <li>• 20mM MgCl<sub>2</sub></li> <li>• Bring to 4.5 ml with sterile LB</li> </ul> <p><b>20X SSC:</b></p> <ul style="list-style-type: none"> <li>• 3M NaCl</li> <li>• 0.3M Sodium Citrate</li> </ul> <p><b>Maleic Acid Buffer:</b></p> <ul style="list-style-type: none"> <li>• 0.1M Maleic Acid</li> <li>• 5 M NaCl</li> <li>• pH 7.5</li> </ul> <p><b>Hybridization Solution:</b></p> <ul style="list-style-type: none"> <li>• DIG Labelled Probe is added in 50 mL of Prehybridization Solution</li> </ul> <p><b>CIA</b></p> <ul style="list-style-type: none"> <li>• 24:1 Chloroform: Isoamyl alcohol</li> </ul> <p><b>CTAB/NaCl:</b></p> <ul style="list-style-type: none"> <li>• 10% CTAB</li> <li>• 0.7 M NaCl</li> </ul>	<p><b>Washing Buffer:</b></p> <ul style="list-style-type: none"> <li>• 0.1M Maleic Acid</li> <li>• 0.15M NaCl pH 7.5</li> <li>• 0.3% Tween 20</li> </ul> <p><b>Washing Buffer I:</b></p> <ul style="list-style-type: none"> <li>• 2X SSC</li> <li>• 0.1% SDS</li> </ul> <p><b>Washing Buffer II:</b></p> <ul style="list-style-type: none"> <li>• 0.1% SSC</li> </ul> <p>0.1% SDS</p> <p><b>Detection Buffer:</b></p> <ul style="list-style-type: none"> <li>• 0.1M Tris-HCl</li> <li>• 0.1M NaCl pH 9</li> </ul> <p><b>Antibody Solution:</b></p> <ul style="list-style-type: none"> <li>• 1:5000 diluted (150 mU/mL) Anti-Digoxigenin AP in Blocking Solution</li> </ul> <p><b>Color-Substrate Solution:</b></p> <ul style="list-style-type: none"> <li>• 200 µL of NBT/BCIP is added into 10 mL of Detection Buffer</li> </ul> <p><b>Prehybridization Solution:</b></p> <ul style="list-style-type: none"> <li>• 5X SSC</li> <li>• 1% Blocking Solution</li> <li>• 0.1% Sarkosyl</li> <li>• 0.02% SDS</li> </ul>

Table 3.3: Concentration of Antibiotics.

Antibiotics	Stock Concentration (mg/mL)	Final Concentration for <i>Streptomyces</i> ( $\mu\text{g/mL}$ )	Final Concentration for <i>E. coli</i> ( $\mu\text{g/mL}$ )
<b>Apramycin</b> (in dH <sub>2</sub> O)	50	50	50
<b>Ampicillin</b> (in dH <sub>2</sub> O)	50	-	50
<b>Kanamycin</b> (in dH <sub>2</sub> O)	50	50	50
<b>Chloramphenicol</b> (in dH <sub>2</sub> O)	25	-	25
<b>Nalidixic Acid</b> (in 0.15 M NaOH)	25	-	25

### 3.1.6. Microorganisms

*E. coli* and *S. coelicolor* strains, which are used in this research, are shown in Table 3.4.

Table 3.4: Bacterial Strains and Their Characteristics.

Bacterial Stain	Genotypic Properties	Reference
<i>E. coli</i> ET12567/pUZ8002	<i>dam, dcm, hsdS, cat, tet/ tra, neo, RP4</i>	[3]
<i>S. coelicolor</i> A3(2) and M145	Wild Type	[24]
<i>S. coelicolor</i> M145 + pRA/ <i>glcP1</i>	Recombinant cells containing pRA/ <i>glcP1</i> plasmid	This Research
<i>S. coelicolor</i> M145+ pSPG/ <i>glcP1</i>	Recombinant cells containings pSPG/ <i>glcP1</i> plasmid	This Research
<i>E. coli</i> DH5 $\alpha$ + pRA/ <i>glcP1</i>	Recombinant cells containing pRA/ <i>glcP1</i> plasmid	This Research
<i>E. coli</i> DH5 $\alpha$ + pSPG/ <i>glcP1</i>	Recombinant cells containing pSPG/ <i>glcP1</i> plasmid	This Research
<i>E. coli</i> ET12567/pUZ8002 + pRA/ <i>glcP1</i>	Recombinant cells containing pRA/ <i>glcP1</i> plasmid	This Research
<i>E. coli</i> ET12567/pUZ8002 +pSPG/ <i>glcP1</i>	Recombinant cells containing pSPG/ <i>glcP1</i> plasmid	This Research

### 3.1.7. Plasmids

Plasmids, which were used in this research, are listed in Table 3.5.

Table 3.5: Characteristics of the plasmids.

NAME	Resistance	Size	Reference
pUZ8002	Kanamycin	70 kb	[25]
pRA	Apramycin	5769 bp	[26]
pSPG	Apramycin Ampicilin	8149 bp	[25]

pRA plasmid, which can integrate into the genome, is an *E. coli*-*Streptomyces* shuttle vector (Figure 3.2). Integration of pRA is constituted by  $\phi$ C31 integrase, which is a site-specific serine recombinase.  $\phi$ C31 integrase inserts the plasmid via site specific recombination between *attB* sites or pseudo-*attB* sites in the genome and *attP* site of the plasmid [35]. pSPG plasmid, which contains glycerol-inducible promoter ( $P_{glpF}$ ), is a high-copy number *E. coli*-*Streptomyces* shuttle vector (Figure 3.3).

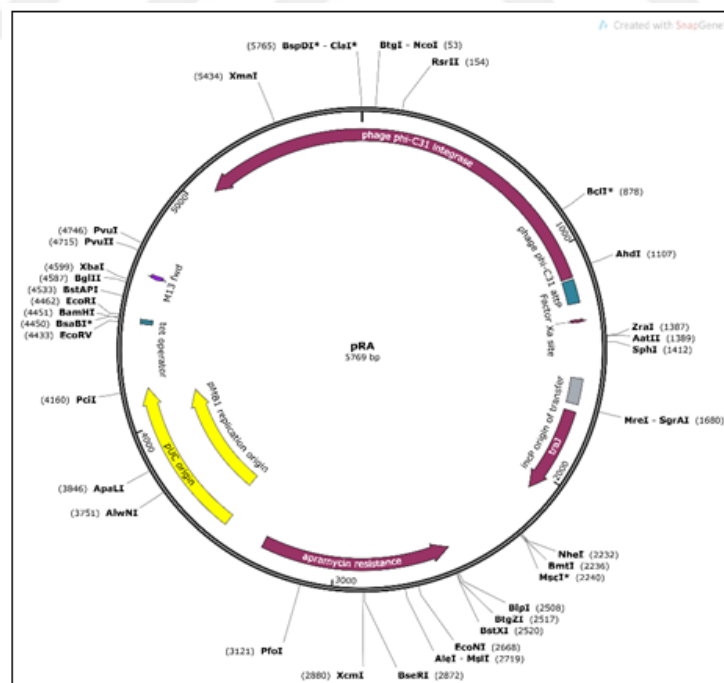


Figure 3.2: Map of pRA Plasmid.



## 3.2. Methods

### 3.2.1. Plasmid Isolation from *E. coli*

Isolation of pRA (integrative vector) and pSPG (multicopy vector) plasmids from *E. coli* cells were performed according to Sambrook et. al. [36]. 100 mg/mL of Apramycin and Ampicillin were used to grow pRA containing and pSPG containing *E. coli*, respectively. *E. coli* cells were grown in 100 mL of LB for 12-16 hours. 100 mL culture was divided into 50 mL falcon tubes, and the cell pellets were collected by centrifugation at 4 °C and 4000 rpm for 15 minutes. The pellets were dissolved in 800 µL of Solution I with vortex, then the cell suspensions were incubated at RT for 5 minutes. 200 µL of cell suspension were aliquoted into the Eppendorf tube. 400 µL of freshly prepared Solution II was added into each aliquot and incubated on ice for 5 minutes. Then, 300 µL of cold Solution III was added in each aliquot, the aliquots were mixed gently and incubated on ice for 10 minutes. After the incubation on ice, they were centrifuged at 4 °C and 13000 rpm for 20 minutes. Supernatants was collected into new Eppendorf tubes; propanol was added as 0.6 volume of supernatant and the mixture was incubated at RT for 1 hour. The mixtures were centrifuged at RT and 13000 rpm for 30 minutes. Supernatants were removed, and each pellet was dissolved in 25 µL of dH<sub>2</sub>O. 4 Eppendorf tubes were collected into 1 Eppendorf tube to obtain 100 µL volume. 10 µL of RNase (10 mg/mL) was added into each 100 µL mixture and the mixtures were incubated at 37 °C for 30 minutes to degrade RNA content. Finally, plasmid DNAs were purified by MN PCR and Gel Purification Clean-Up Kit and stored at -20 °C.

### 3.2.2 Amplification of *glcP1* by PCR

Primers were designed (Table 3.6) for glucose transporter gene (*glcP1*) (NO: SCO5578) of *S. coelicolor* A3(2) genome according to criteria as the length, G:C ratio, melting temperature <sup>TM</sup>, ΔG value for dimer formation, etc. For cloning into pSPG plasmid, sequence of *NdeI* restriction enzyme site was added into forward primer, and *SpeI* restriction enzyme site was added into reverse primer. For cloning into pRA plasmid, forward and reverse primers were designed for upstream and downstream of *glcP1* gene to cover *glcP1* coding sequence together with its promoter (2379 bp). The

DNA fragments were amplified by PCR (Table 3.7) and were purified from the agarose gel by using MN Gel and PCR Clean-Up kit for further cloning experiments.

Table 3.6: Primer sequences for *glcP1* gene. *NdeI* and *SpeI* restriction sites are indicated as underlined and bold.

Primer	Sequence	Length
F-pRA	5'- CTGGTGTCTCGATCTTCAAGGC – 3'	20
R-pRA	5'- AGCTTCATGGGGTCTTCAT – 3'	20
F-pSPG	5'- <u><b>GGAATTC</b></u> <b>ATATG</b> GTGGCCAGCACATCGCAG – 3' <i>NdeI</i> Restriction Site	31
R-pSPG	5'- <u><b>GGACTAGT</b></u> CTCAGCCCATTTCCCTCCAG -3' <i>SpeI</i> Restriction Site	27

Table 3.7: Components and Cycle Steps for PCR via Phusion Polymerase.

Reaction Content		Method				
Component	Quantity	Cycle Steps	2-Step Method		3-Step Method	
			Temp.	Time	Temp.	Time
5X Phusion Buffer	4 µL	<b>Initial Denaturation</b>	98 °C	30 s	98 °C	30 s
10 mM dNTPs	0.4 µL	<b>Denaturation</b>	98 °C	10 s	98 °C	10 s
F-Primer	1 µL	<b>Annealing</b>	-	-	T <sub>m</sub> of Primers	30 s
R-Primer	1 µL	<b>Extention</b>	72 °C	30 s/kb	72 °C	30 s/kb
Template	0.5 µL	<b>Final Extention</b>	72 °C	10 min	72 °C	10 min
DMSO	0.6 µL	<b>Hold</b>	4 °C	1 hour	4 °C	1 hour
Phusion polymerase	0.2 µL					
dH <sub>2</sub> O	up to 20 µL					

### 3.2.3. Agarose Gel Electrophoresis

PCR products and plasmid DNAs were separated on agarose gels which were prepared with 1 % (w/v) agarose in TAE (1X) Buffer. To obtain agarose gel, 0.5 g of agarose was dissolved in 50 mL of TAE (1X) Buffer by heating in microwave. Then, the gel liquid was cooled to ~50 °C, and 2,5 µL of RedSafe was added into the gel liquid. The gel was solidified in the gel tray with an appropriate well comb. Samples were loaded into the wells with Loading Dye (1X Final Concentration), and electrophoresis was performed at 100V for 30-40 minutes. UV was used to visualize and examine the fragments.

### 3.2.4. Gel and PCR Clean-Up Kit

Fragments, PCR products, and digested plasmid DNAs were purified from agarose gel by using MN Gel and PCR Clean-Up Kit. Purification was performed by following the official protocol of the kit, established by the manufacturer.

### 3.2.5. Restriction Enzyme Digestion

*NdeI* and *SpeI* restriction enzymes were used to digest PCR products and pSPG plasmid to perform sticky-end ligation. *EcoRV* restriction enzyme was used to digest pRA plasmid to perform blunt-end ligation. For digestion of DNA fragments and plasmids, manufacturer's protocols were used for each enzyme.

### 3.2.6. Cloning into Plasmid DNA

Cloning of the PCR products was performed according to the method developed by Sambrook et. al. [36]. For the cloning, concentration of DNA fragments and plasmid DNA was calculated by the equation (3.1).

$$insert (ng) = \frac{vector (ng) \times insert (kb)}{vector (kb)} \quad (3.1)$$

### **3.2.7. Competent Cell Preparation of *E. coli***

For using as a seed culture, *E. coli* cells were inoculated into 1 mL of LB broth, and the culture was incubated at 37 °C and 180 rpm for overnight. For competent cell preparation, 1% of the seed culture was inoculated into 10 mL of LB Broth, and the new culture was incubated at 37 °C and 180 rpm until the OD<sub>600</sub> value reached to 0.4. Then, the cells were centrifuged at 4000 rpm for 10 minutes, and the cell pellets were dissolved in 1 mL of TSS with slow mixing on ice. Then, competent cells were aliquoted in Eppendorf tubes as 100 µL, and they were stored at -80 °C.

### **3.2.8. Transformation of *E. coli* Cells**

*E. coli* DH5α and ET12567/pUZ8002 cells were transformed according to Sambrook et. al. [36]. 50 µL of competent cells were mixed with 3 µL of ligation reaction solution. Then, the cells were incubated on ice for 10 minutes, and later the cells were placed on heater at 42 °C for 45 seconds. After the heat-shock, the cells were placed again into the ice for 3 minutes. Then, 600 µL of cold LB was added on the cells and they were incubated for 45 minutes at 37°C and 180 rpm. Finally, 100 µL of the regenerated cells were inoculated on selective LB agar plates and incubated at 37 °C for overnight.

### **3.2.9. Conjugation of *E. coli* and *Streptomyces***

The plasmid DNA, containing the target gene, was transferred from *E. coli* cells to *Streptomyces* according to conjugation method developed by Kieser et. al. [37]. *E. coli* ET12567/pUZ8002 strain, containing pRA/*glcP1* recombinant vector, was inoculated in 10 mL of LB with apramycin (50 µg/mL), kanamycin (50 µg/mL), and chloramphenicol (25 µg/mL), and was incubated for overnight at 37°C at 180 rpm. Next day, 100 µL of pre-culture was inoculated into 10 mL of fresh LB with same antibiotics and incubated at 37 °C at 180 rpm until OD<sub>600</sub> value reached to 0.4. When OD value reached to 0.4, the cell pellet was collected by centrifugation at 4000 rpm for 10 minutes, and the pellet was washed twice with 10 mL of LB. Then, *E. coli* pellet was dissolved in 1 mL of LB. During the washing step of *E. coli* pellet, 10 µL (10<sup>8</sup>) of *S. coelicolor* spores were inoculated into 500 µL of 2x-YT Broth, and the spores were

heat-shocked at 50 °C for 10 minutes. After the heat-shock, 500 µL of *E. coli* suspension was added into the spore suspensions, and dilution series were made from  $10^{-1}$  to  $10^{-4}$  with dH<sub>2</sub>O. 100 µL of each dilution was plated into MS agar containing 10 mM MgCl<sub>2</sub>, and the cells were incubated for 20 hours at 30 °C. After the incubation, MS plates were overlaid with 1 mL of antibiotic solution, contained 0.5 mg of nalidixic acid, and 1.25 mg of apramycin, and the plates were incubated at 30 °C until the colonies were formed. The possible positive conjugants were selected from MS agar plates and were inoculated onto TSA or R2YE agar plates, which contains nalidixic acid and apramycin. This selection procedure was repeated for two or three times from TSA or R2YE agar plates to eliminate all *E. coli* cells. Then, the selected colonies were examined under the microscope to confirm their purity. One colony from freshly grown cells on TSA or R2YE (with nalidixic acid and apramycin) was inoculated in TSB or R2YE broth and incubated at 30 °C at 180 rpm for 2-3 days. The culture was examined with light microscope to confirm purity of the culture, and glycerol stocks of the pure cultures were prepared.

### **3.2.10. Preparation of Spore Stocks of *Streptomyces***

*Streptomyces* strains were inoculated into 50 mL of TSB (antibiotics were added when necessary). The bacterial culture was incubated at 30 °C for 48 hours and then it was centrifuged at 4000 rpm for 15 minutes. The pellet was resuspended in 5 mL of remaining supernatant to obtain dense cell suspension. 100 µL of the cell suspension was inoculated on TBO agar (antibiotics were added when necessary) and the cells were incubated at 30 °C for 7-10 days. The spores were collected via scratching by using sterile cotton swaps after addition of 5 mL of 40% glycerol. Then, spore containing glycerol solution was transferred in a sterile falcon tube by pipetting. The spore solution was centrifuged at 4000 rpm at +4 °C for 20 minutes, and the spore pellet was dissolved in remaining 1 mL of supernatant. 1 mL of spore solution was divided as 200 µL volume into the Eppendorf tubes, and they were stored at -80 °C. For the determination of the number of spores, serial dilutions were prepared from  $10^1$  to  $10^{-9}$ , and 100 µL of each dilution was inoculated into TSA. TSA plates were incubated at 30 °C for 3 days. Colonies were counted and spore numbers were calculated by using the equation (3.2)

$$\begin{aligned}
 & \text{Number of spores (cfu/mL)} \\
 & = (\text{colony number} \times \text{dilution factor}) \times (V_{\text{total}}/V_{\text{inoculation}})
 \end{aligned}
 \tag{5.2}$$

### 3.2.11. Genomic DNA Isolation from *Streptomyces*

*Streptomyces* strains were inoculated in 50 mL of YEME (antibiotics were added when necessary). This pre-culture was incubated at 30 °C for 48 hours. Then, the fresh 100 mL of YEME was inoculated with pre-culture and incubated at 30 °C for 48 hours. The culture was divided into 2 sterile falcon tubes as 50 mL volume, and the cells were collected by centrifugation at 4000 rpm for 15 minutes. The cell pellet was dissolved in 8.5 mL of TE Buffer containing lysozyme (2 mg/mL). 500 µL of 20% SDS and RNase (10 mg/mL) were added into the cell suspension, and the suspension was incubated at 37 °C for 1 hour. After the incubation, 1.5 mL of 5M NaCl and 1.2 mL of CTAB/NaCl were added into the suspension, and the suspension was incubated at 65 °C for 25 minutes. Then, the suspension was extracted with phenol/CIA. The suspension was centrifuged at +4 °C at 4000 rpm for 15 minutes to separate phases. Upper phases were collected into new falcon tubes, and the phenol/CIA extraction was repeated. 1 volume of CIA was added into the last upper layer to remove remaining phenol. The upper layers were collected again into new falcon tubes, and 0.6 volume of isopropanol was added. The suspensions were incubated at room temperature for 20 minutes and centrifuged at 4500 rpm for 30 minutes. The pellets were dissolved in 2 mL of TE buffer, and each 2 mL of suspension was aliquoted into two Eppendorf tubes as 1 mL volume. The suspensions were again extracted with Phenol/CIA for twice, and the upper layers were extracted with 1 volume of CIA to remove remaining phenol. Final upper layers were collected into new Eppendorf tubes, and 3 volume of 100% ethanol was added. The suspensions were incubated at -20 °C for overnight. Then, the suspensions were centrifuged at 13000 rpm for 30 minutes, and the pellets were dried. Finally, DNA pellets were dissolved in 50 µL of dH<sub>2</sub>O, and stored at -20 °C.

### 3.2.12 Southern Blot and Hybridization

Southern blot was used to confirm additional copy of *glcP1* in putative recombinant strain according to Sambrook et. al. [36]. For the preparation of probe, *glcP1* gene was amplified by PCR by using specific primers (FpRA and RpRA) (Table 3.5). Product of PCR was purified from agarose gel by using MN PCR Cleanup and DNA Extraction Kit. Then, probe of *glcP1* was synthesized according to the manual of DIG DNA Labelling and Detection Kit (Roche). Prepared probe was tested according to the kit manual. Serial dilutions (1 ng, 100 pg, 10 pg, 1 pg, and 0.1 pg) of the probe, standard and control were prepared, and 1  $\mu$ L of each serial dilution was imbrued in positively charged nylon membrane. Then, probes were fixed with UV, and the membrane was incubated with 20 mL of maleic acid buffer at 25 °C for 2 minutes. After that, the membrane was incubated with 10 mL of detection buffer at 25 °C for 5 minutes. For colouring, the membrane was covered with fresh 2 mL of Color-substrate Solution until the colouring reaction was sufficient, and the membrane was washed with sterile dH<sub>2</sub>O to stop the reaction.

After the probe preparation, chromosomal DNAs of putative recombinant strain, wild type strain, and pRA control strain were isolated, and DNAs were digested with *KpnI* and *XbaI* restriction enzymes. Then, digested DNA samples were run in 1% Agarose gel to separate the fragments. After the electrophoresis, DNA fragments were transferred onto the positively charged nylon membrane via vacuum blotting system (40 mBar) (Amersham). The gel was incubated with depurination solution, denaturalization solution and neutralization solution respectively for 30 minutes in each solution. Finally, the gel was covered with 20X SSC for 90 minutes. After the transfer, DNA fragments were fixed onto the membrane by exposure to the UV light for 4 minutes.

Later, the membrane was incubated in prehybridization solution at 42°C for 5 hours. Then, the membrane was hybridized with the labelled probe at 42 °C for overnight. After the hybridization, the membrane was washed two times with washing buffer I at 24°C for 15 minutes. Then, it was washed two times with washing buffer II at 42 °C for 25 minutes. The membrane was rinsed with maleic acid buffer for 5 minutes, and the membrane was incubated in 100 mL of blocking solution for 30 minutes. After that, the membrane was incubated in 20 mL of antibody solution for 2 hours. To remove remaining antibodies, the membrane was washed two times with

100 mL of maleic acid buffer for 15 minutes. Finally, the membrane was incubated in 20 mL of detection buffer for 5 minutes, and the membrane was covered with 10 mL of colour-substrate solution. The membrane was washed with sterile dH<sub>2</sub>O to stop the reaction.

### **3.2.13. MiniPrep Plasmid Isolation from *E. coli***

Screening of *E. coli* transformants were performed by MiniPrep plasmid isolation and digestion with restriction enzymes. Putative transformant colonies were inoculated into 1 mL of LB with required antibiotics, and cultures were incubated at 37 °C at 180 rpm for overnight. Incubated cultures were centrifuged at 13000 rpm for 10 minutes, and each pellet was dissolved in 250 µL of STET solution, containing lysozyme (1 mg/mL). The cell suspension was incubated at 37 °C for 30 minutes, and then it was incubated in boiling water for 15 minutes. Later, the suspension was centrifuged at 4 °C and 13000 rpm for 20 minutes, to remove any cell debris. The supernatants were transferred into new Eppendorf tubes. Plasmid DNA was precipitated by addition of 250 µL of isopropanol, and incubation at room temperature for 30 minutes. Plasmid DNAs were collected as pellets by centrifugation at room temperature at 13000 rpm for 20 minutes. Each pellet was dissolved in 25 µL of ddH<sub>2</sub>O.

### **3.2.14. Detection of 2,3-Butanediol, Acetoin, Glucose and Organic Acids by HPLC**

2,3-butanediol, acetoin, glucose and organic acids were detected by using High-Performance Liquid Chromatography (HPLC) according to Siemerink et. al and Champluvier et. al [38][39]. The bacterial cells were grown in TSB at 30 °C for 3 days. By using these pre-cultures, a fermentation study was started by inoculating equal wet weight from each cell type. Cells were inoculated into 100 mL of TSB and after 72 hours of growth at 30 °C, 1 mL of fermentation broth from each cell type was sampled and stored at -20 °C until the measurement. The samples were filtered with 0.45 µm syringe filter prior to HPLC analysis.

Refractive index detector (Shimadzu), UV-Vis Detector (Shimadzu) and an organic acid column (Benson Polymeric BP-800 H<sup>+</sup>) were used to analyse 2,3-

Butanediol, acetoin, glucose, and organic acids. Method was developed as flow rate 0.6 mL/min, injection volume 15  $\mu$ L, column temperature 65  $^{\circ}$ C, runtime 25 min and mobile phase was 5 mM H<sub>2</sub>SO<sub>4</sub>. Retention time of 2,3-Butanediol, acetoin, acetic acid, ethanol, methanol, 2-propanol, and glucose was determined by analysing reference standard solutions.



## 4. RESULTS

### 4.1. Analysis of 2,3-Butanediol Production of *Streptomyces coelicolor* M154 by HPLC

In order to determine if *S. coelicolor* M145 is able to produce 2,3-butanediol, it was grown both in TSB and TSB supplemented with glucose (15%). Samples were taken from 72 hours grown cultures and the supernatants were analysed by HPLC. By comparing the peaks of standard chemicals (Figure 4.1), it was shown that *S. coelicolor* is able to produce 2,3-butanediol and its precursor, acetoin (Figure 4.2). Retention times were determined as 17.2 min, and 18.8 minutes for acetoin, and 2,3-butanediol respectively. Chromatogram of culture supernatant of TSB supplemented with glucose has both acetoin and 2,3-butanediol peaks, however, chromatogram of culture supernatant of TSB has only acetoin peak (Figure 4.2). According to these results, glucose supplementation enhanced 2,3-butanediol production in this microorganism. Therefore, we hypothesized that increasing glucose concentration inside the cell can enhance 2,3-butanediol production in *Streptomyces*. For this purpose, glucose transporter protein gene (*glcP1*) was planned to be overexpressed in *Streptomyces* to increase glucose uptake.

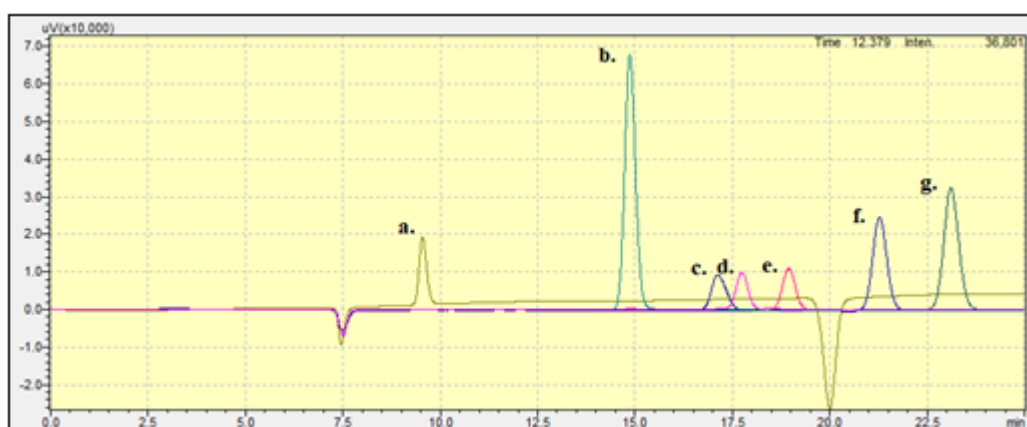


Figure 4.1: HPLC (RID) chromatogram of standard chemicals. a) Glycose b) Acetate, c) Acetoin, d) 2,3-BDO, e) Methanol, f) Ethanol, g) Propanol.

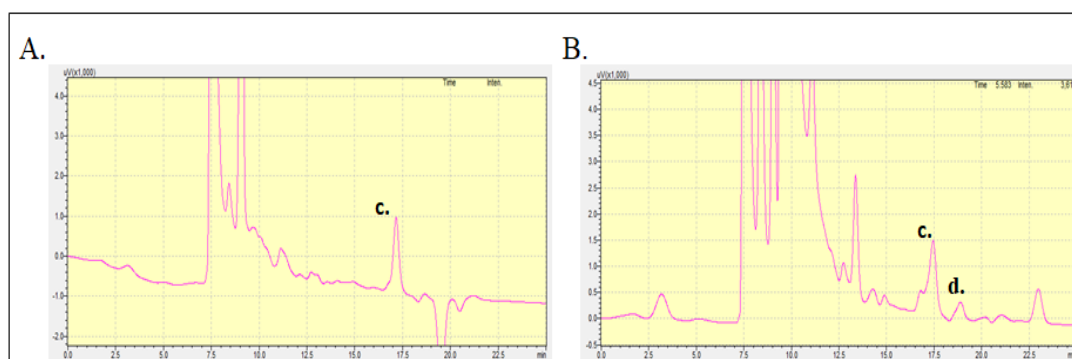


Figure 4.2: Chromatogram of supernatant of *S. coelicolor* M145 grown in TSB (A) and TSB supplemented with glucose (B). In each chromatogram acetoin and 2,3-butanediol were indicated by (c) and (d), respectively.

## 4.2. Effect of Overexpression of Glucose Transporter on 2,3-Butanediol Production in *Streptomyces coelicolor*

*glcP1* gene (SCO5578) have been overexpressed in *S. coelicolor* to enhance biosynthesis of 2,3-butanediol.

### 4.2.1. Amplification of *glcP1* Gene by PCR

*S. coelicolor* M145 genome has 2 homologue genes for glucose transporter protein, which are *glcP1* and *glcP2*. Glucose transporter protein is a permease and activity of GlcP1 was demonstrated by van Wezel et. al. [22]. According to BLAST analysis, *glcP1* and *glcP2* genes have similar coding sequences with only two nucleotide changes (Figure 4.3). However, *glcP2* does not have any activity of glucose utilization. To amplify *glcP1* gene (1419 bp), a larger DNA fragment (2379 bp) was chosen to design primers. This region, containing *glcP1* gene with its promoter and terminator, was amplified from genomic DNA of *S. coelicolor* M145 via PCR (Figure 4.4A). 2379 bp amplified region was purified from agarose gel and was used to be cloned into integrative pRA vector. The 2379 bp DNA fragment was also used as a template to amplify *glcP1* coding sequence (SCO5578) via PCR. Newly amplified fragment (*glcP1* coding sequence, 1419 bp) was used to be cloned into pSPG vector under the control of glycerol inducible promoter (Figure 4.4B).

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**Streptomyces coelicolor A3(2) chromosome, complete genome**  
 Sequence ID: [NC\\_003888.3](#) Length: 8667507 Number of Matches: 1

Range 1: 6078582 to 6080000 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
2610 bits(1413)	0.0	1417/1419(99%)	0/1419(0%)	Plus/Plus
Query 7946421	GTGGCCAGC	ATCGCAGGCGCCAGTCCAGGAGCCGGACGGCTCATCCCGATCATCTC		7946420
Sbjct 6078582	.....	.....		6078641
Query 7946481	GGGCACGTCATCTTCATCGCGCGCGCCCGCGATGGGCGTTTCTGTTCCGGTTACGAC			7946540
Sbjct 6078642	.....	.....		6078701
Query 7946541	ASTTCCGTGATCAACGGTSCGGTCCGAGCGATCCGGACCCGTACGACSTCGGTTCCGG			7946600
Sbjct 6078702	.....	.....		6078761
Query 7946601	GTGCTGGCGCAGGTATCGCCGTGGCTCTGATCGGCTGTGCCATCGGTGCCGCGACCCGG			7946660
Sbjct 6078762	.....	.....		6078821
Query 7946661	GGCCGATCGCGGACCGTATCGCCGTATCCGCTGCATGCAGATGCCGCGGTCCTGTTT			7946720
Sbjct 6078822	.....	.....		6078881
Query 7946721	ACGGTGAGCGCCGTCGGCTCCGCGCTGCCCTTCGCGCTGTGGACCTGGCCATGTGGCGG			7946780
Sbjct 6078982	.....	.....		6079041
Query 7946781	ATCATCGCGGTTTCGCGATCGGCATGGCTCGGTGATCGGCCCGCCACATCGCCGAG			7946840
Sbjct 6078942	.....	.....		6079001
Query 7946841	GTGTCCCGCCCGCTACCGCGCCCGCTCGGTTCTTCCAGCAGGCGCGATCGTCATC			7946900
Sbjct 6079002	.....	.....		6079061
Query 7946901	GGCATCGCCGTCCTCCAGCTGGTCAACTGGGTCGTCTGAACGCCGCCGGCGGTGACCA			7946960
Sbjct 6079062	.....	.....		6079121
Query 7947001	CGCGCGAGCTGATGGGCTGGAGGCCCTGGCAGGTCATGCTCGGCGTATGGTATCCCG			7947020
Sbjct 6079122	.....	.....		6079181
Query 7947021	GCCGCTCTGTACGGCCTGCTGTCTTCGCCATCCCGAGTCCCGCCGCTTCTTGATCTCG			7947080
Sbjct 6079182	.....	.....		6079241
Query 7947081	GTGGCAAGCSCGAGCGCCCAAGAAATCCTCGAGGAGTCCGAGGGCAAGGACGTGGAC			7947140
Sbjct 6079242	.....	.....		6079301
Query 7947141	TTCGACGCCCGCTCACCGATCGAGCACGCCATGCACCCGCGAGGAGGAGTCTCTCTTC			7947200
Sbjct 6079302	.....	.....		6079361
Query 7947201	AAGGACCTCTCGGCGGAGCTTCTTCTCAAGCGATCGTGTGGATCGGTATCGGCTG			7947260
Sbjct 6079362	.....	.....		6079421
Query 7947261	TCGGTCTTCCAGCAGTTCGTGGCATCAACGTGGCTTCTACTACTCTCGACGCTGTGG			7947320
Sbjct 6079422	.....	.....		6079481
Query 7947321	CAGTCGGTCCGCGTCGACCCCGCCGACTCGTTCTTCTACTCGTTCACGACGTCGATCATC			7947380
Sbjct 6079482	.....	.....		6079541
Query 7947381	AACATCGTGGCACCGTATCGCGATGATCTTCGTGGACCGCGTCCGCGCAAGCCGCTC			7947440
Sbjct 6079542	.....	.....		6079601
Query 7947441	GCCCTGATCGGCTCCGTCGGCATGGTATCGGCTGGCGCTGGAGCCCTGGGCTTCTCC			7947500
Sbjct 6079602	.....	.....		6079661
Query 7947501	TTCGACCTGGTCCAGCGGAACTCCCGCCACCCAGGCTGGGTCGCCCTGATCGCCGCC			7947560
Sbjct 6079662	.....	.....		6079721
Query 7947561	CAGCTTTCGCTCTTCTTCGCGCTGTGCGGGGTGTGGTCGTGTGGTCTTCTCTGGC			7947620
Sbjct 6079722	.....	.....		6079781
Query 7947621	GAGATGTTCCCAACCGGATCCGCGCCGCGCTGGGCTGGCGCCCTCCGCGAGTGG			7947680
Sbjct 6079782	.....	.....		6079841
Query 7947681	ATCGCCAACTGGGCCATCACCGGAGCTTCCCGTCACTGGCCGACTGGAACCTCTCCGGC			7947740
Sbjct 6079842	.....	.....		6079901
Query 7947741	ACCTACGTGATCTACACGATCTTCGCCGCCCTCTCCATCCCTTCGTCCTCAAGTTCGTG			7947800
Sbjct 6079902	.....	.....		6079961
Query 7947801	AAGGAGACCAAGGSCAAGGCCCTGGAGGAAATGGGCTGA		7947839	
Sbjct 6079962	.....	.....	6080000	

Figure 4.3: BLAST Analysis of *glcP1* and *glcP2* genes.

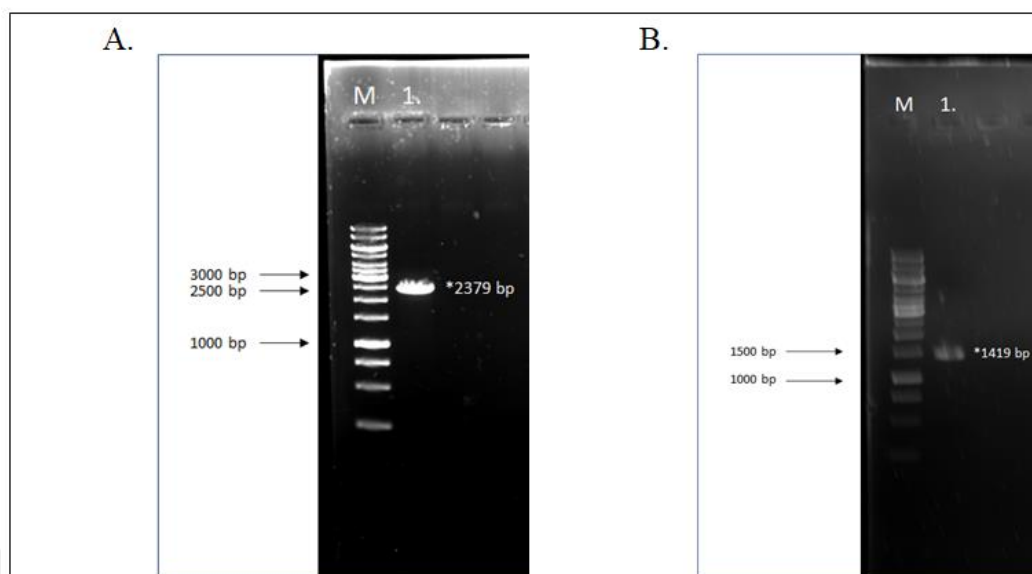


Figure 4.4: Amplification of *glcP1* Gene by PCR. A) 2379 bp PCR product which contains *glcP1* gene with its promoter and transcriptional terminator. B) *glcP1* coding sequence (1419 bp). M: GenRuler 1 kb DNA Ladder.

#### 4.2.2 Cloning of *glcP1* gene into pRA and pSPG Plasmids

To overexpress *glcP1* gene in *S. coelicolor* M145 the integrative pRA (5769 bp), and multicopy pSPG (8149 bp) vectors were isolated. pSPG was digested with *NdeI* and *SpeI* restriction enzymes and pRA was digested with *EcoRV* (Figure 4.5). For cloning into pSPG, *glcP1* fragment (1419 bp) was digested with *NdeI* and *SpeI* restriction enzymes. After the ligation reaction, *E. coli* DH5 $\alpha$  cells were used for transformation to screen for recombinant plasmids. Plasmids were isolated from putative transformants by MiniPrep plasmid isolation, and the isolated plasmids were used as template for PCR (Figure 4.6). 1419 bp DNA fragment (*glcP1*) was amplified by PCR and the recombinant plasmid was named as pSPG/*glcP1*.

2379 bp DNA fragment which contain *glcP1* gene with its promoter and transcriptional terminator was used to be cloned into *EcoRV* site of pRA. *E. coli* DH5 $\alpha$  cells were transformed with the ligation products and transformants were screened with plasmid isolation. One of the possible recombinant plasmids was confirmed by digestion with *BamHI* restriction enzyme (Figure 4.7). pRA has only one restriction site for *BamHI*, therefore 6479 bp and 1669 bp DNA fragments were expected after

digestion with *Bam*HI if the recombinant plasmid was correct. The correct recombinant plasmid was named as pRA/*glc*P1.

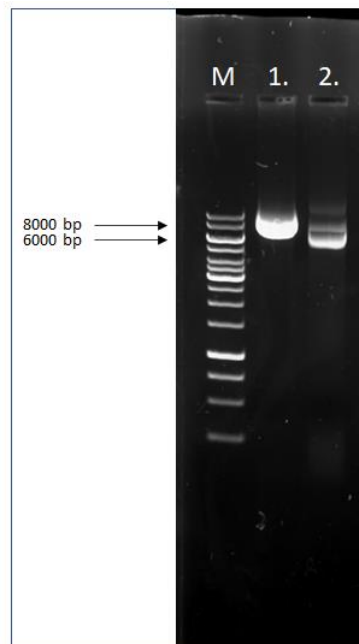


Figure 4.5: Cloning vectors: pSPG (1) and pRA (2). pSPG is digested with *Nde*I and *Spe*I, and pRA is digested with *Eco*RV M: GeneRuler 1 kb DNA Ladder.

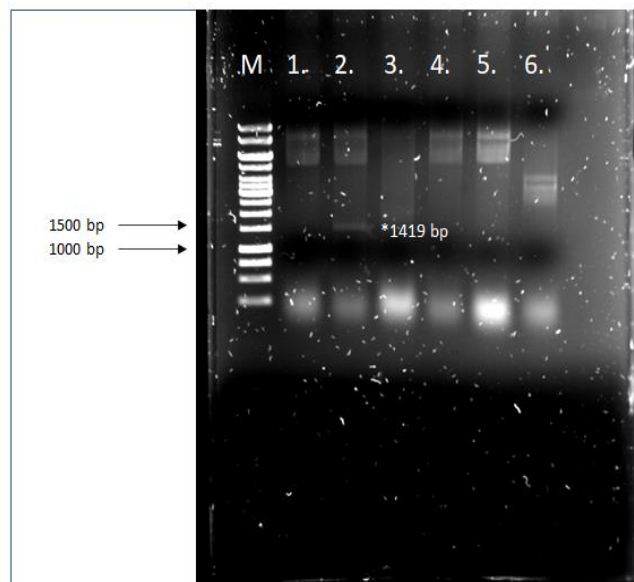


Figure 4.6: PCR result to screen for recombinant pSPG/*glc*P1 plasmid. The plasmid in the 2<sup>nd</sup> well has *glc*P1 coding sequence (1419 bp) M: GenRuler 1 kb DNA Ladder.

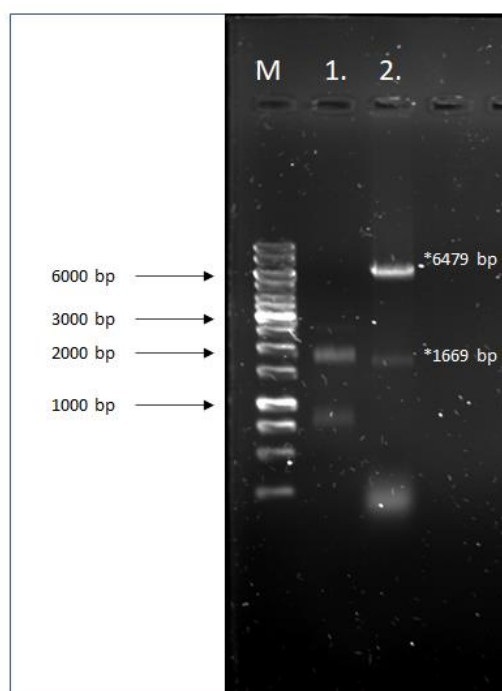


Figure 4.7: *Bam*HI Restriction Enzyme Digestion of 2379 bp DNA fragment (1), and putative pRA/*glc*P1 plasmid isolated from *E. coli* DH5 $\alpha$ . Digestion with *Bam*HI resulted in “1649 bp and 732 bp fragments” from 2379 bp DNA fragment and “6479 bp and 1669 bp fragments” from putative pRA/*glc*P1 plasmid as expected. M: GenRuler 1 kb DNA Ladder.

### 4.2.3. Transferring Recombinant Plasmids into *Streptomyces* via Conjugation

pRA/*glc*P1 and pSPG/*glc*P1 were transferred into *S. coelicolor* M145 via conjugation. First, recombinant plasmids isolated from *E. coli* DH5 $\alpha$  cells were transferred into *E. coli* ET12567/pUZ8002 cells via transformation. *E. coli* ET12567/pUZ8002 is a methylation deficient strain and has *tra* gene on pUZ8002, which is responsible for transferring the recombinant plasmid to *Streptomyces*.

In order to confirm pRA/*glc*P1 integration into the genome of *Streptomyces*, Southern Blot was carried out by using one of the apramycin resistant conjugant. For Southern Blot, probe was synthesized for *glc*P1 gene (1419 bp), and effectiveness of the probe was tested according to the manual of DIG DNA Labelling and Detection Kit (Roche) (Figure 4.8). As a control, conjugant that contain empty pRA plasmid was also used in Southern experiment. Genomic DNAs were isolated from possible conjugant colonies. Similar concentration of genomic DNAs for each strain were digested with *Xba*I and *Kpn*I restriction enzymes. Digested DNAs were run in agarose

gel (data not shown), and then the DNAs transferred onto nylon membrane. After hybridization experiment with labelled probe, 2395 bp *Xba*I and *Kpn*I fragment was only seen in the recombinant cell as expected (Figure 4.9).

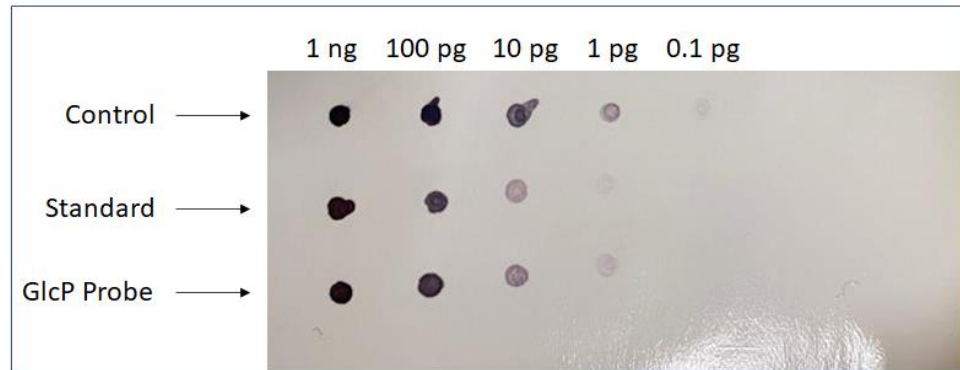


Figure 4.8: Labelling efficiency of glcP1 probe.

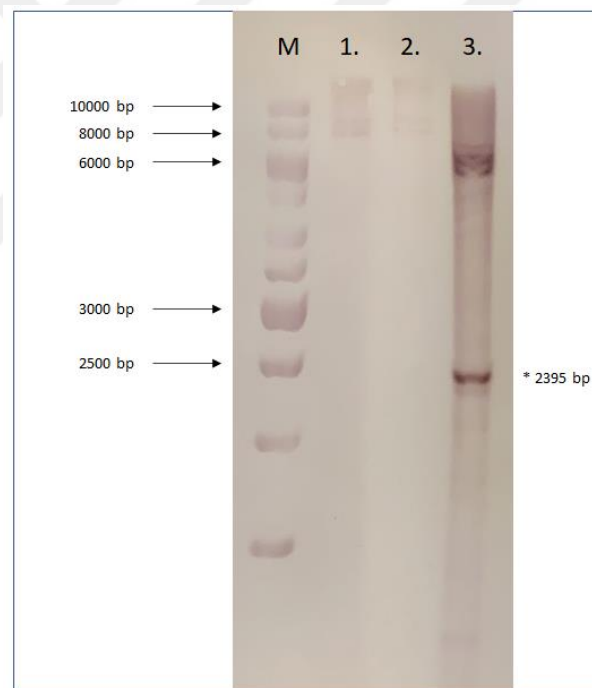


Figure 4.9: Southern Blot result. *S. coelicolor* M145 WT (1), *S. coelicolor* M145 with empty pRA (2), and possible conjugant with pRA/*glcP1* (3). M: GenRuler 1 kb DNA Ladder.

In order to find pSPG/*glcP1* containing *S. coelicolor* cells, conjugants were screened by colony PCR by using apramycin primers. Apramycin gene fragment was seen in the conjugants found in wells 6-11 (Figure 4.10).

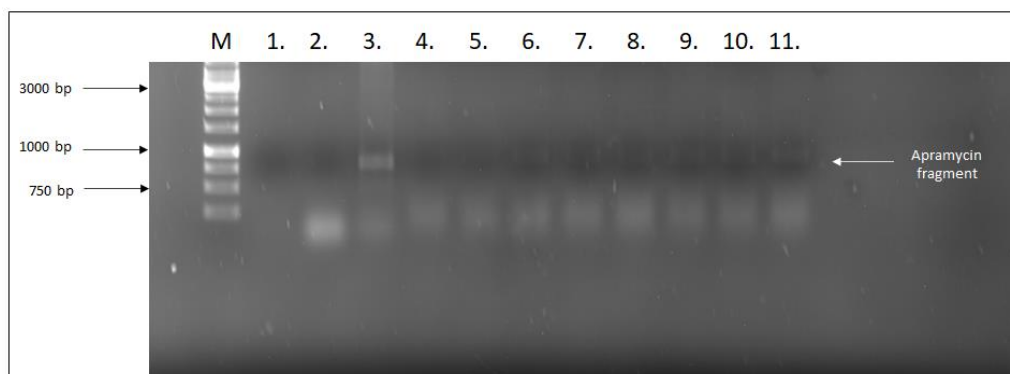


Figure 4.10: Colony PCR results of putative pSPG/*glcP1* containing *S. coelicolor* conjugants (4-11). 1, 2 and 3 shows the results of PCRs for *S. coelicolor* WT, *S. coelicolor* with empty pRA and pSPG/*glcP1* isolated from *E. coli*, respectively. M: GenRuler 1 kb DNA Ladder.

Although we didn't confirm the expression of the *glcP1* gene at the transcriptional or translational level, growth and antibiotic production of recombinant strains were different than the wild type. Surprisingly, recombinant strains containing pSPG/*glcP1* and pRA/*glcP1* did not produce any coloured antibiotics unlike the wild type strain (data not shown). Moreover, the growth of pSPG/*glcP1* containing recombinant strain was impaired compared to the wild type and pRA/*glcP1* containing strain. These results have indirectly shown that the cloned gene were expressed and overexpression of *glcP1* gene on a multicopy plasmid affected the growth negatively (data not shown).

#### 4.2.4. HPLC Analysis of Acetoin, 2,3-Butanediol and Organic Acids

Effect of overexpression of *glcP1* gene on 2,3-butanediol production was determined via HPLC analysis. *S. coelicolor* M145 wild type, pRA containing control and pRA/*glcP1* containing recombinant strains were grown in TSB and TSB supplemented with glucose (15%) for 72 hours. The supernatants of cultures grown in TSB (Figure 4.11) and TSB supplemented with glucose (Figure 4.12) were analysed by using HPLC. According to the results, glucose peak was more rapidly decreased in recombinant strain, showing an increased glucose uptake rate (Figure 4.13). It was also found that *glcP1* overexpression does not enhance 2,3-butanediol and acetoin (precursor of 2,3-butanediol) production in *Streptomyces* cells grown in both TSB and TSB supplemented with glucose. However, production of some of the metabolites noticeably increased in the recombinant strain grown in TSB supplemented with

glucose compared to the control strains. These results were accepted as direct evidence for *glcP1* expression in the recombinant strain. HPLC results of different chemicals (Figure 4.1) were compared with our results to identify the metabolites which were produced in large amount in the recombinant strain. None of the peaks were determined to be belong to acetate, ethanol, methanol, and 2-propanol according to their retention times.

General organic acids can be analysed at 210 nm wavelength by UV-Vis detector. In order to distinguish the metabolites produced in high amount in the recombinant strain, HPLC analysis was repeated with UV-Vis detector (Figure 4.14). Preliminary findings have been obtained from this results that metabolites produced in high amount may be organic acids. In another word, presence of one extra copy of *glcP1* gene in *S. coelicolor* stimulated the production of some organic acids, that needs to be identified in future studies.

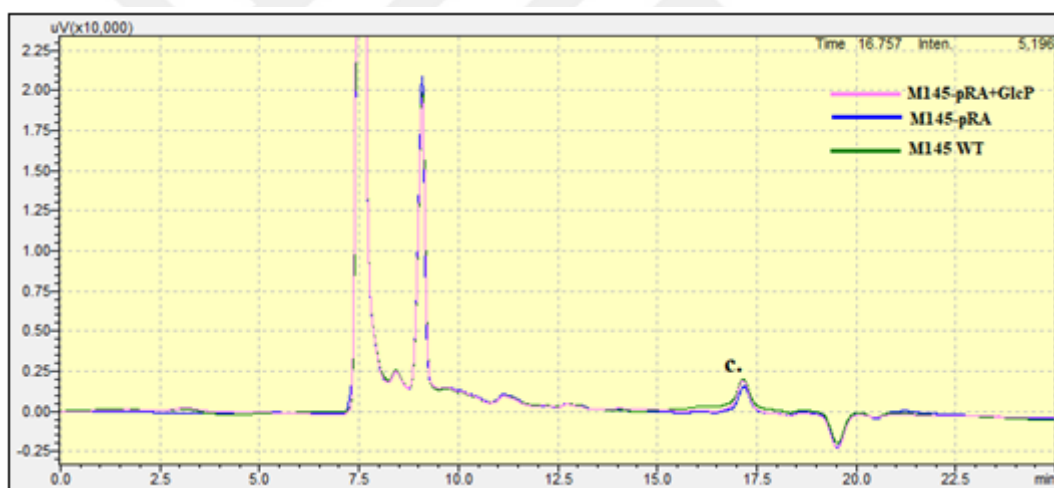


Figure 4.11: RID Chromatogram of TSB grown *Streptomyces* strains. “c” is acetoin.

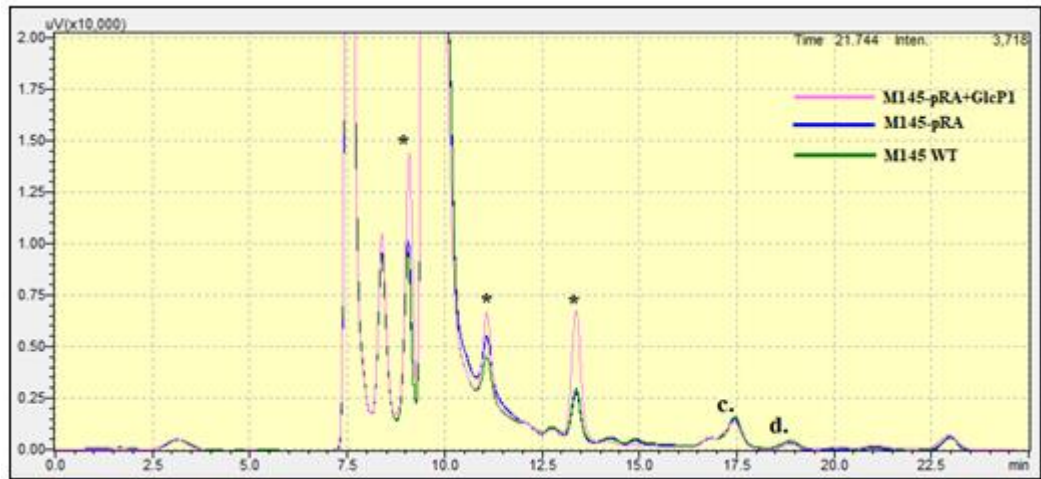


Figure 4.12: RID Chromatogram of TSB (+glucose) grown *Streptomyces* strains. “c” is acetoin, “d” is 2,3-butanediol. “\*” indicates metabolites produced at high amount compared to the control.

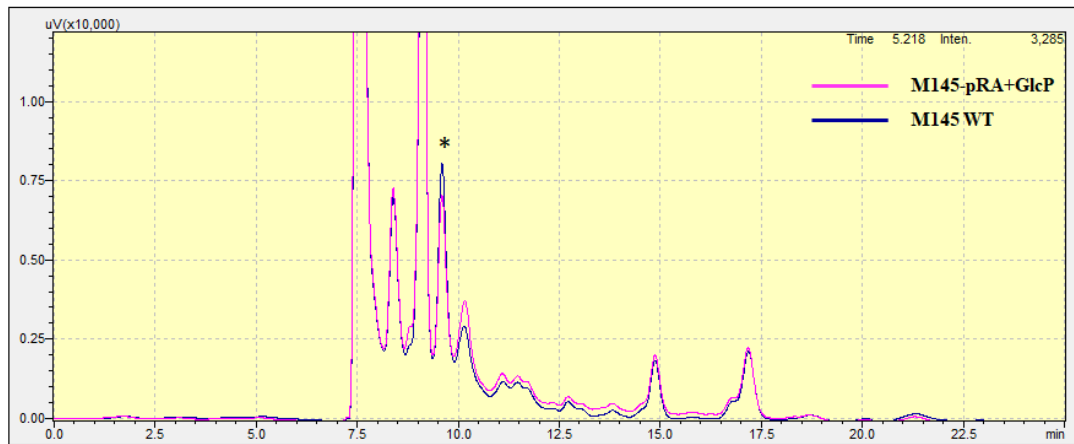


Figure 4.13: RID Chromatogram of TSB grown *Streptomyces* strains. “\*” indicates glucose decreased at higher rate compared to the control in 24-hour samples.

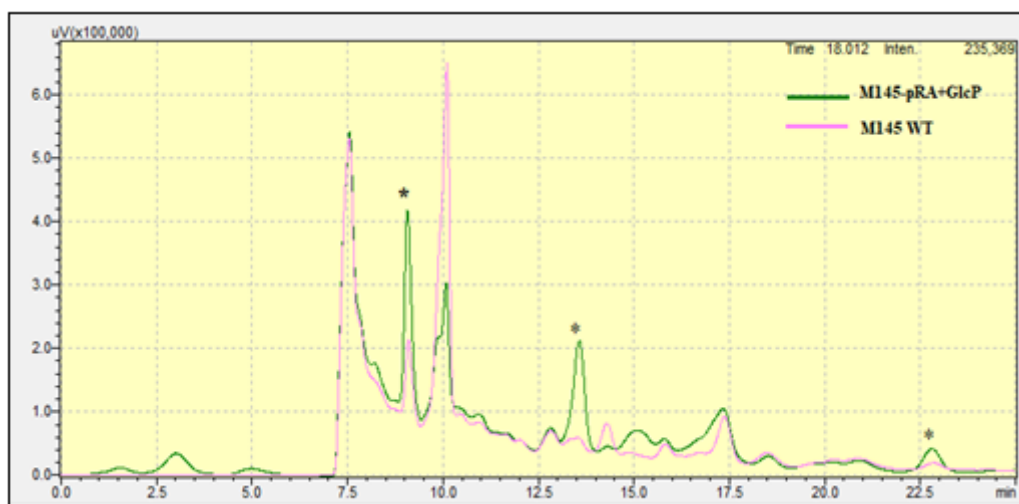


Figure 4.14: Chromatogram of TSB (+glucose) grown *Streptomyces* strains obtained with UV detector (210 nm). “\*” indicates metabolites produced at high amount compared to the control.

## 5. DISCUSSION

*Streptomyces* species are capable of producing various secondary metabolites, and some of these secondary metabolites can be used as therapeutics. Secondary metabolites produced by *Streptomyces* are so valuable in terms of medical biotechnology that little attention has been paid to studies on primary metabolites produced by this organism. There are only few studies related with primary metabolite production by *Streptomyces*. One of them is related with the production of “butanal” and “butanol” by *S. coelicolor* which they patented their results [40]. In this study, we planned to focus on primary metabolism of *Streptomyces*.

Primary metabolites are basic but versatile molecules, and they can be used as raw material for more complex chemicals in different industries. 2,3-BDO is one of the basic primary metabolites, and it can be used as platform metabolite to produce different valuable chemicals. 2,3-BDO is commonly produced as a natural product by different microorganisms. Natural stereoisomers of 2,3-BDO which are analysed by chiral chromatography (based on GC) have different applications [41].

2,3-BDO have not been studied or analysed directly in *S. coelicolor*, although its precursor acetoin have been shown to be produced by this microorganism. However, acetoin production is important evidence for 2,3-BDO production by *Streptomyces*. *Streptomyces* species have a great metabolite productivity, and they can metabolise different complex polysaccharides (cellulose and chitin etc.), generally waste materials [7]. These characteristics make *Streptomyces* potential producer of 2,3-BDO. In this study, to enhance the production of 2,3-BDO in *S. coelicolor* by stimulating the primary metabolism, native glucose transporter protein (GlcP) was overexpressed.

At the first part of this study, we analysed the production of 2,3-butanediol and its precursor acetoin by HPLC in the wild type *S. coelicolor* M145. For this, *S. coelicolor* M145 was grown in TSB and TSB supplemented with glucose. Standards of all target chemicals, which are glucose, 2,3-butanediol, acetoin, acetate, ethanol, methanol, and isopropanol were analysed with same method to obtain standard peaks for each chemical. Methanol, ethanol, isopropanol and acetate peaks were not noticeably detected in the samples. We observed acetoin peaks in TSB samples of both wild type and recombinant strain, however, distinguishable peaks were not observed for 2,3-BDO in the same samples. Moreover, glucose peak was diminished more rapidly in the sample of recombinant strain in respect to the wild type strain. In the

samples of TSB supplemented with glucose, a considerable peak was observed for 2,3-BDO. Although, we obtained one peak, which had similar retention time with standard 2,3-BDO, we could only distinguish between meso- form of 2,3-BDO and other stereoisomers of 2,3-BDO. D-(-) and L-(+) isomers of 2,3-BDO has same retention time in HPLC analysis method [39]. Observation of 2,3-BDO peak with addition of glucose supports that, increase in glucose concentration inside the cell enhances primary metabolite production. Glucose supplementation is also generally used to stimulate 2,3-BDO production in other bacteria [42][43]. After the confirmation of acetoin and 2,3-BDO production in *S. coelicolor*, we aimed to enhance 2,3-BDO production by overexpression of GlcP, glucose transporter protein. Increase in glucose uptake should provide more carbon and energy source for overall primary carbon metabolism, and that should also enhance 2,3-BDO production. Supporting this, it was shown that increased glucose uptake enhanced the productivity of *E. coli* in nitrogen starvation conditions [44].

Glucose is the main carbon source universally metabolized by all known organisms. Glucose uptake is an important parameter that determines rate of growth and yield of produced metabolites. Global mechanisms control switching off or on the other metabolisms with the accessibility of glucose. In *Streptomyces* glucose is transported inside the cell by GlcP and catabolized via primary carbon metabolism. GlcP encoded by two homologue genes, which are *glcP1* and *glcP2*. *Streptomyces glcP1* have been used to retrieve glucose utilization ability in other bacteria by heterologous expression [22].

For overexpression of *glcP1* gene, a multicopy pSPG and integrative pRA vectors were used. Recombinant pSPG/*glcP1* strain had impaired growth, however, recombinant pRA/*glcP1* strain has stable morphology and growth. Multi-copy of *glcP1* might have caused molecular stress on the recombinant cell. GlcP is known as a permease, and permeases are membrane bound proteins. Membrane bound proteins has a critical ratio in the cell, which is also called “protein to lipid” ratio. If the “protein to lipid” ratio is enormously high, the cellular integrity can be disrupted because there would be not enough space in membrane for proteins [45]. Recombinant pSPG/*glcP1* strain could not produce any antibiotics although wild type strain can produce coloured antibiotics at the same glucose concentration. This result is predictable because of carbon catabolic repression (CCR). CCR is induced by increased concentration of cellular glucose, and it regulates secondary and primary metabolism as a global

regulatory mechanism. It is clear that CCR down regulated antibiotic production because of the presence of sufficient carbon source. Moreover, antibiotic production is generally stimulated with stress. With high amount of carbon source, the recombinant microorganism may not be under stress. Interrupted antibiotic production can also be interpreted as carbon source is channelled into different metabolic pathways instead of secondary metabolism.

Recombinant pSPG/*glcP1* strain was not included to HPLC analysis since its growth was impaired. According to our HPLC results, there were negligible differences in 2,3-BDO production by recombinant pRA/*glcP1* and wild type strains. This unexpected result could be related with the initial glucose concentration in TSB. It is possible that 15% glucose was not enough to observe any alteration in cellular pathways in recombinant strain. Therefore, the effect of different glucose concentrations can be analysed in future studies.

Although 2,3-BDO production was not changed in the recombinant strain compared to the wild type, a dramatic alteration in other metabolites was detected. These results could be interpreted as increase in glucose uptake stimulated other metabolic pathways in recombinant strain. There were at least 3 peaks, which has inevitable alteration, for recombinant strain in respect to wild type. However, we could not be able to determine these metabolites with refractive index detector. For characterization of the metabolites, we repeated same HPLC analysis with UV detector instead of RID to determine organic acids in the samples. According to chromatogram for UV analysis of the same samples, we could detect two of the three peaks. This result indicated that these two metabolites were probably organic acids. For further accurate characterization of these metabolites, GC-MS or Prep-HPLC analysis can be performed in future studies.

With this study, we showed that *S. coelicolor* can produce 2,3-BDO and overexpression of *glcP1* gene encoding glucose transporter protein enhanced glucose uptake and stimulated primary metabolism. Although 2,3-BDO and acetoin production were not affected, the production of at least two organic acids were increased in the recombinant strain (pRA/*glcP1*) by the presence of extra *glcP1* gene. Increased organic acid production is evidence for stimulation of primary metabolism in *S. coelicolor*. Organic acids are generally intermediate metabolites, which are further catalysed to synthesize different metabolites, such as diols (1,3-propanediol, 1,3-butanediol) and hydroxy acids (4-hydroxybutyric acid) [46]. In future studies, the

organic acids produced in high amount by the recombinant strain has to be defined and metabolic fluxes have to be analysed to determine how carbon source was channelled to metabolic pathways in this microorganism. Then, critical enzymes for each targeted metabolic pathway should be determined to redirect the precursor pool to metabolic pathways of desired metabolites.



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