

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

OPTIMIZATION OF 2,3-BUTANEDIOL PRODUCTION IN
STREPTOMYCES COELICOLOR



ADEM DEMİROĞLU

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ADVISOR: PROF. DR. SEDEF TUNCA GEDİK

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

STREPTOMYCES COELICOLOR'DA 2,3-
BÜTANDİOL ÜRETİMİNİN OPTİMİZASYONU

ADEM DEMİROĞLU

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM
DALI

DANIŞMAN: PROF. DR. SEDEF TUNCA GEDİK

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ABSTRACT

Streptomyces are gram-positive soil bacteria and are known for producing secondary metabolites mainly used in medical biotechnology. Most research on *Streptomyces* has focused on this subject due to their ability to produce secondary metabolites. To the best of our knowledge there are few studies that have focused on the primary metabolism of *Streptomyces* in the literature. 2,3-Butanediol (2,3-BDO) is a glycol alcohol that can be produced naturally and is dependent on primary carbon metabolism. It is used in the production of various industrial products, such as synthetic plasticizers, rubbers, fumigants, and as a fuel additive, octane booster and antifreeze agent. Its value lies in its use as a raw material in the plastic, flavor, cosmetic, and drug industries.

Previous studies conducted in our laboratory have shown that *Streptomyces coelicolor* is able to produce 2,3-BDO, but the production amount was found to be quite low. This study aims to optimize the production of 2,3-BDO in both the wild-type *S. coelicolor* strain and a recombinant strain containing an additional copy of the *glcP1* gene responsible for glucose transporter protein production in *S. coelicolor*. To enhance 2,3-BDO production, different kinds of media (YEME, TSB, and TSB+10% glucose) and growth conditions, including pH (pH 5.0, pH 6.0, pH 7.0, pH 8.0 and pH 9.0) and temperatures (25°C, 30°C and 35 °C) were tested. The HPLC results indicated that the optimal medium and conditions for producing 2,3-BDO by both wild-type and recombinant *Streptomyces* strains are TSB supplemented with 10% glucose, at a pH of 7.0 and a temperature of 30°C. The maximum amount of 2,3-BDO that could be produced in these conditions was found to be 0.033 mg/mL for *S. coelicolor* and 0.035 mg/mL for recombinant *S. coelicolor*. Although the values for industrial processes are currently low, higher production levels can be achieved through future optimization and genetic studies. This thesis is significant as it provides preliminary data supporting the use of *Streptomyces* for primary metabolite production.

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

ÖZET

Streptomyces'ler gram pozitif toprak bakterileridir ve esas olarak tıbbi biyoteknolojide kullanılan ikincil metabolitleri ürettiği bilinmektedir. Sekonder metabolit üretme yeteneklerinden dolayı *Streptomyces* üzerine yapılan araştırmaların çoğu bu konuya odaklanmıştır. Bildiğimiz kadarıyla literatürde *Streptomyces*'in primer metabolizmasına odaklanan çok az çalışma bulunmaktadır. Doğal olarak üretilen ve birincil karbon metabolizmasına bağlı olan 2,3-Butandiol (2,3-BDO), sentetik plastikleştiriciler, kauçuklar, fumigantlar gibi farklı türdeki endüstriyel ürünlerin üretiminde kullanılan bir glikol alkoldür. Ayrıca yakıt katkısı, oktan arttırıcı ve antifriz maddesi olarak da kullanıldığı bilinmektedir. Plastik, aroma, kozmetik ve ilaç endüstrilerinde hammadde olarak kullanılması 2,3-BDO'yu çok değerli kılmaktadır.

Laboratuvarımızda yapılan önceki çalışmalarda *Streptomyces coelicolor*'un 2,3-BDO üretebildiği kanıtlanmış ancak üretim miktarı oldukça düşük bulunmuştur. Bu çalışmanın amacı, hem yabanil tip *S. coelicolor* suşunun hem de glikoz taşıyıcı proteinin üretiminden sorumlu olan *glcP1* geninin ekstra bir kopyasını içeren recombinant *S. coelicolor* suşunun 2,3-BDO üretimini optimize etmektir. 2,3-BDO üretimini arttırmak için farklı besiyerleri (YEME, TSB ve TSB+10% glukoz), pH (pH 5.0, pH 6.0, pH 7.0, pH 8.0 ve pH 9.0) ve sıcaklıklar (25°C, 30°C ve 35°C) test edildi. HPLC sonuçlarına göre, hem yabanil tip hem de rekombinant *Streptomyces* suşları tarafından 2,3-BDO'nun üretilmesi için en uygun ortamın ve koşulların, pH 7.0 ve 30°C sıcaklıkta 10% glukoz içeren TSB olduğu saptanmıştır. Bu koşullarda üretilen maksimum 2,3-BDO miktarı yabanil tip *S. coelicolor* için 0,033 mg/mL, rekombinant *S. coelicolor* için ise 0,035 mg/mL olarak bulunmuştur. Endüstriyel proseslere göre değerler şu anda düşük olsa da gelecekte yapılacak optimizasyon ve genetik çalışmalarla daha yüksek üretim seviyelerine ulaşılabilir. Bu tez, *Streptomyces*'in birincil metabolit üretimi için ön veriler sağlaması açısından önemlidir.

Anahtar Kelimeler: 2,3-Bütandiol, *Streptomyces*, Fermentasyon.

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LIST OF ABBREVIATIONS

α	:	Alfa
Δ	:	Delta
μL	:	Microliter
μM	:	Micromolar
Apr	:	Apramycin
KOH	:	Potassium hydroxide
HCl	:	Hydrochloric acid
NaOH	:	Sodium hydroxide
2,3-BDO	:	2,3-Butanediol
mg	:	Milligram
mL	:	Milliliter
TSB	:	Tryptic Soy Broth
YEME	:	Yeast Extract Malt Extract broth
g	:	Gram
dH ₂ O	:	Distilled water
rpm	:	Round per minute
L	:	Litre
H ₂ SO ₄	:	Sulfuric acid
M	:	Molar
BGC	:	Biosynthetic gene cluster
TCA	:	Tricarboxylic acid
mM	:	Millimolar

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1. INTRODUCTION

Streptomyces are gram-positive soil bacteria with a high G:C content. They undergo a complex life cycle of mycelial growth and spore formation. With their secondary metabolism, *Streptomyces* are capable of producing a variety of bioactive compounds, such as antibiotics, antifungal and antiviral drug intermediates, during the differentiation process. They are commonly used in biotechnology. In addition to the compounds produced through their secondary metabolism, *Streptomyces* also have the ability to produce important compounds through their primary metabolism, such as primary alcohols. The resulting substances from primary metabolism, such as 2,3-butanediol (2,3-BDO), are mainly valuable biotechnological and industrial products. These substances can be used as platform substances in chemical reactions to obtain more complex chemical compounds.

There are several methods to enhance the production of primary and secondary metabolites. These include genetic manipulation and optimization of growth conditions. Optimization of the medium is necessary to maximize metabolite yield. This can be achieved through various techniques, ranging from classical methods, such as pH, carbon source and temperature optimizations, to modern statistical and mathematical techniques. It is important to note that every parameter has its own advantages and disadvantages. To achieve the highest metabolite yield, it is necessary to change each optimization parameter, and find the optimum fermentation conditions.

1.1 The Aim and Original Value of The Thesis

2,3-BDO is a valuable chemical compound used in the production of various industrial products such as synthetic plasticizers, rubbers, fumigants, and used as a fuel additive and antifreeze agent. Previous research has demonstrated that *S. coelicolor* is capable of producing 2,3-BDO [25]. The objective of this thesis was to optimize fermentation conditions to enhance 2,3-BDO production in both wild-type *S. coelicolor* and a recombinant strain of *S. coelicolor*, which contains an additional glucose transporter gene (*glcP1*) in its genome. To achieve this aim, various types of media and parameters, such as pH and temperature, were tested, and 2,3-BDO production was

measured using HPLC. The results of this study may contribute to further research on the development of 2,3-BDO production in *Streptomyces* as well as other primary metabolites produced by this organism.



2. STREPTOMYCES

Streptomyces are Gram-positive soil-dwelling, filamentous, aerobic bacteria, with high G:C content, and are famous for their secondary metabolism. Although they are mostly found in soil, it is also possible to isolate *Streptomyces* from aquatic regions [1]. The genetic infrastructure of *Streptomyces* is very suitable for producing natural pharmaceutical compounds, so much so that 70% of known antibiotics are produced by *Streptomyces*. The bioactive compounds synthesized by their secondary metabolism is one of the significant factors in studying *Streptomyces* as a model organism [2].

The most abundant Secondary metabolites produced by *Streptomyces* are antibiotics and *Streptomyces* uses these metabolites in order to block the growth of microorganisms living in the same environment. However, *Streptomyces* can also use secondary metabolites for cellular communication and signaling [3]. Secondary metabolites used by *Streptomyces* to survive in its environment are of great importance to humanity. The most important sources of antibiotics are known to be so far as *Streptomyces*. These antibiotics are mainly used in medicine, veterinary, and agriculture [4]. In addition to antibiotics, they also produce bioactive secondary metabolites such as antifungal, antiviral, antitumor, anti-hypertensive and immunosuppressant. Being a valuable source of bioactive compounds makes *Streptomyces* biotechnologically indispensable microorganisms [5].

The large part of the secondary metabolites are antibiotics in *Streptomyces* and these metabolites are polyketide metabolism. The production of secondary metabolites, as well as primary metabolites, are regulated by global regulators. Moreover, these global regulators are also responsible for regulating gene clusters related with differentiation and growth. Targeting these global regulators by genetic manipulation methods are mainly used strategies for secondary metabolite enhancement [6].

Biosynthetic gene clusters (BGCs), operonic sets of microbial genes, are responsible for the encoding of bioactive compounds in *Streptomyces*, like other microorganisms. Yet, many bioactive compounds that *Streptomyces* are capable of producing cannot be observed under laboratory conditions. Biosynthetic gene clusters in this situation are called cryptic clusters, and the existence of these cryptic clusters can be easily

discovered by today's technology, like sequencing technologies and bioinformatics. It is possible to obtain many new natural products with the discovery of these cryptic gene clusters, but in order to obtain these natural products that have the potential to be produced, these cryptic gene clusters must first be activated [7]. As with increasing secondary metabolite production, cryptic gene clusters can also be activated through genetic manipulation studies.

Another strategy used to increase secondary metabolite production and activate cryptic gene clusters is medium optimization studies. In order to trigger the cryptic gene cluster, environmental changes, such as changing nutrient source, pH and temperature, for *Streptomyces* can be achieved [8]. Determining the optimum growth conditions is one of the most important parameters for the production of secondary metabolites. The conditions in which each microorganism can grow optimally may vary. In addition, the substances that microorganisms can produce in which medium and under what conditions also vary [9]. For example, some components can be produced more at high pH, while some components can be produced more at low pH. In this case, microorganisms must first be grown in different media in order to observe the highest production of the target component. After the medium is determined, it is necessary to make changes in the parameters affecting the growth conditions. Changeable parameters are generally pH, temperature, carbon/nitrogen source and minerals. As a result, the appropriate medium and conditions are determined for high production of the target component [10].

2.1. Primary Carbon Metabolism

In order to understand the mechanism and functions of the secondary metabolism of *Streptomyces*, it is very crucial to have an idea about the primary metabolism of *Streptomyces* because the primary metabolism of *Streptomyces* provides the precursors required for secondary metabolism. This makes *Streptomyces* primary metabolism essential. The primary carbon metabolism in *Streptomyces* is responsible for anabolic and catabolic reactions. By harnessing energy, these reactions are mainly responsible for the synthesis of building blocks of lipids, proteins, polysaccharide storage and structural materials and nucleic acids, which results as an increase in the biomass [11].

There are three main metabolic pathways responsible for the production of energy and carbon resources required for *Streptomyces* to maintain its vital activities. These are; glycolysis, tricarboxylic acid cycle (TCA) and pentose phosphate pathway (PPP). Carbon and energy resources resulting from these metabolic pathways are used for the growth, differentiation, and secondary metabolite production of *Streptomyces* with the help of the primary metabolism [12]. *Streptomyces* have a unique and complex life cycle. The first step of this cycle is the development of spore germination, in which the vegetative hyphae (MI) grow and extends out from the spore formation in convenient conditions. After vegetative hyphae extend from the spore, the first programmed cell death (PCD) stage is initiated, and some parts of vegetative hyphae dies. The alive parts of vegetative hyphae differentiate to aerial hyphae (MII) form. This form of hyphae has the ability to produce secondary metabolites. Finally, the second PCD stage is initiated, and the remaining alive parts of the hyphae produces spore chains. All the required precursor substances for PCD during this growth and differentiation process are provided by primary metabolism [13]. Primary metabolism, which provides the necessary components for growth, differentiation and secondary metabolites, is also responsible for the production of volatile compounds that provide inter or intraspecies communication [14].

The carbon source to be used by primary metabolism can be provided by different carbohydrates. However, glucose, a monosaccharide that can be utilized by all microorganisms, is a significant carbon source for living organisms [11]. In many gram-positive and gram-negative bacteria, glucose is up taken to the cell by the phosphotransferase system (PTS). PTS is a signal transduction system, in which a phosphoryl group of phosphoenolpyruvate is transferred to the carbohydrate that will be up taken to the cell. This transfer process occurs by a set of biomolecular protein-protein complexes [15]. Nevertheless, the glucose uptake mechanism in *Streptomyces* is different. *Streptomyces* utilize a protein that belongs to the Major Facilitator Superfamily of sugar permeases, which is called GlcP protein, for glucose uptake. GlcP protein is encoded by two homologous genes, called *glcP1* and *glcP2*, both with similar sequence and encode same length of amino acid. Yet, in *Streptomyces*, the active protein for glucose uptake is only the one that is encoded by *glcP1* gene [16] [17].

2.2. 2,3-Butanediol and 2,3-Butanediol Metabolism in Bacteria

Using microorganisms for the production of industrial chemicals, is one of the most important topic in the field of biotechnology. These chemicals are produced using petroleum through conventional production methods. It is an undeniable fact that the use of petroleum for the production of these chemicals will be abandoned in the coming years, as petroleum is an expensive, exhaustible resource, and causes a lot of damage to the environment when used with conventional method. Considering these disadvantages of petroleum, the use of microorganisms for industrial chemical production has become very popular [18].

In this regard, 2,3-butanediol (2,3-BDO) has an important place among chemicals produced by using microorganisms. 2,3-BDO is an organic compound which is classified as vic-diol. Vic-diols are known as glycol and are found as 3 stereoisomers. These are, chiral pairs, D-(-)-2,3-BDO and L-(+)-2,3-BDO, and a meso-2,3-BDO isomer, which are found as colorless liquids. 2,3-BDO's are mainly used as precursors for various plastics and pesticides. Additionally, 2,3-BDO could be used for the production of cosmetic products. 2,3-BDO might be changed over to methyl ethyl ketone (MEK), a fluid fuel added substance and helpful modern dissolvable for lacquers and resins [19]. Usage as raw material for plastic, flavor, cosmetic and drug industries make 2,3-BDO very valuable. 2,3-BDO production by biotechnology with wastes and excessive biomass is a promising technique and is environment friendly than traditional chemical synthesis [20].

Some bacterial strains can naturally produce 2,3-BDO naturally, like *Enterobacter* and *Klebsiella* and *Bacillus*. Among natural producing bacteria, *Bacillus* is the most studied strain and is mainly used to achieve high production of 2,3-BDO through genetic studies [21]. The natural 2,3-BDO biosynthesis pathway starts with the condensation of two molecules of pyruvate to get one molecule of acetolactate, which is catalyzed by α -acetolactate synthase enzyme. Under aerobic conditions decarboxylation of acetolactate takes place to produce diacetyl. In the presence of NADH, diacetyl is reduced to form acetoin. The decarboxylation and conversion of acetolactate to acetoin is catalyzed by α -acetolactate decarboxylase. At this stage, if meso-2,3-BDO dehydrogenase (D-(-)-acetoin forming) is present in the cell, D-(-)-acetoin is formed or meso-2,3-BDO dehydrogenase (forming L-(+)-acetoin) is

present, L-(+)-acetoin is formed within the cell. In the final step, acetoin is reduced by NADH-dependent 2,3-BDO dehydrogenase to yield 2,3-BDO. This reaction is reversible, in some cases 2,3-BDO can be converted to acetoin [22]. 2,3-BDO is formed in different stereoisomers within the cell, depending on the type of the stereoisomer acetoin found in the cell [23].

There are mainly 2 parameters that effect the conversion of acetoin to 2,3-BDO within the cell. These are cellular NADH concentration and pH. NADH concentration does not affect the production rate of 2,3-BDO, instead the concentration of cellular NADH determines which stereoisomer of 2,3-BDO will be produced. As the enzymes that determines the isomers of acetoin are NADH dependent, according to cellular NADH concentration the type of stereoisomer of 2,3-BDO produced within the cell are determined. Conversely, cellular pH determines the production rate of 2,3-BDO within the cell. If the cellular pH is low, 2,3-BDO production is increased to get a neutralized cellular pH. On the other hand, if the cellular pH is high, acetoin is used to neutralize the cellular pH. In such cases, cells can convert 2,3-BDO to acetoin to get higher acetoin level [23]. Although there are not many examples, temperature change is also one of the factors affecting 2,3-BDO production. Research done with *Klebsiella pneumoniae* have shown that, a temperature decreases from 35 °C to 30 °C have stimulated a reduction in ethanol production and have blocked some byproducts formation. On the other hand, 2,3-BDO production have slightly increased [24].

According to the previous studies that have been conducted in our laboratory, it has been proven for the first time that *Streptomyces* can produce 2,3-BDO [33]. In order to see whether *Streptomyces* has the genetic infrastructure to produce 2,3-BDO, first the Butanoate metabolism of *Streptomyces* in the KEGG metabolism database was examined and it was found that *Streptomyces* has all the enzymes necessary to produce 2,3-BDO (Figure 2.2.1) [33].

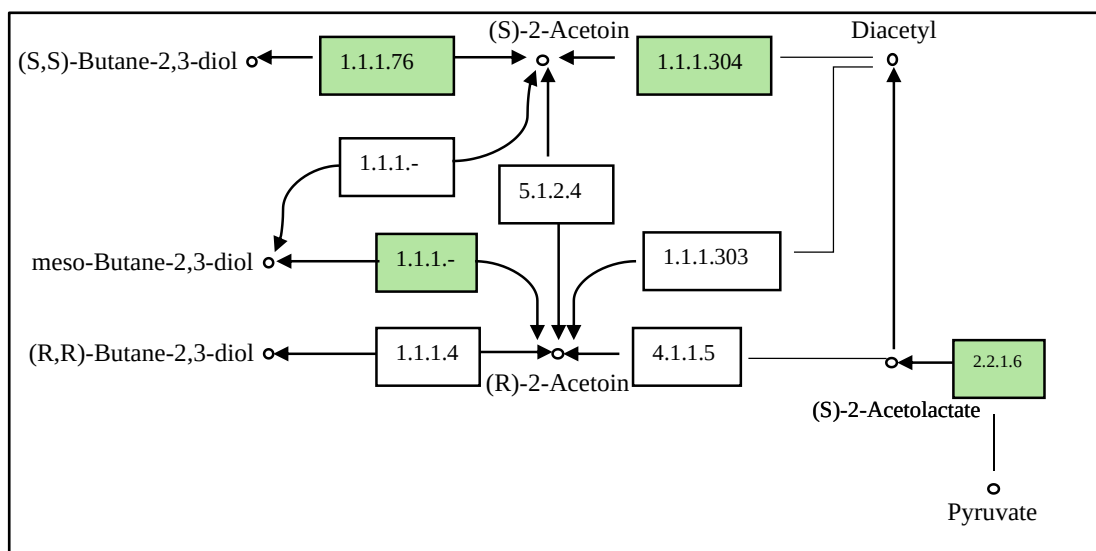


Figure 2.2.1: 2,3-BDO metabolism of *Streptomyces* from KEGG database, green boxes show the enzymes that are available in *Streptomyces* and white boxes show the enzymes not available in *Streptomyces*. The enzymes given in green boxes are: [EC:2.2.1.6]: acetolactate synthase, [EC:1.1.1.304]: diacetyl reductase ((S)-acetoin forming), [EC:1.1.1.76]: (S,S)-butanediol dehydrogenase, and [EC:1.1.1.-]: meso-butanediol dehydrogenase. The enzyme given in white boxes are: [EC:4.1.1.5]: (S)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase, [EC:1.1.1.303]: diacetyl reductase ((R)-acetoin forming), [EC:5.1.2.4]: acetoin racemase, [EC:1.1.1.-]: meso-butanediol dehydrogenase, and [EC:1.1.1.4]: (R,R)-butanediol dehydrogenase [33].

The KEGG analysis showed that *Streptomyces* is theoretically capable of producing 2,3-BDO, and this information was used to prove that *Streptomyces* can actually produce 2,3-BDO by performing GC-MS analysis (Figure 2.2.2). Once the GC-MS results showed that *S. coelicolor* was able to produce 2,3-BDO, HPLC analysis was carried out. In the first step, the retention time information for both 2,3-BDO and acetoin standards was determined by HPLC analysis. After determining the retention times of the standards (Figure 2.2.3A), the supernatants of wild-type *S. coelicolor* were analysed by HPLC and the peaks were observed at the retention time where acetoin (17.2), which is the precursor of 2,3-BDO, and 2,3-BDO (18.8) standards had formed peaks. However, the amount produced amount was found to be quite low [25] (Figure 2.2.3B).

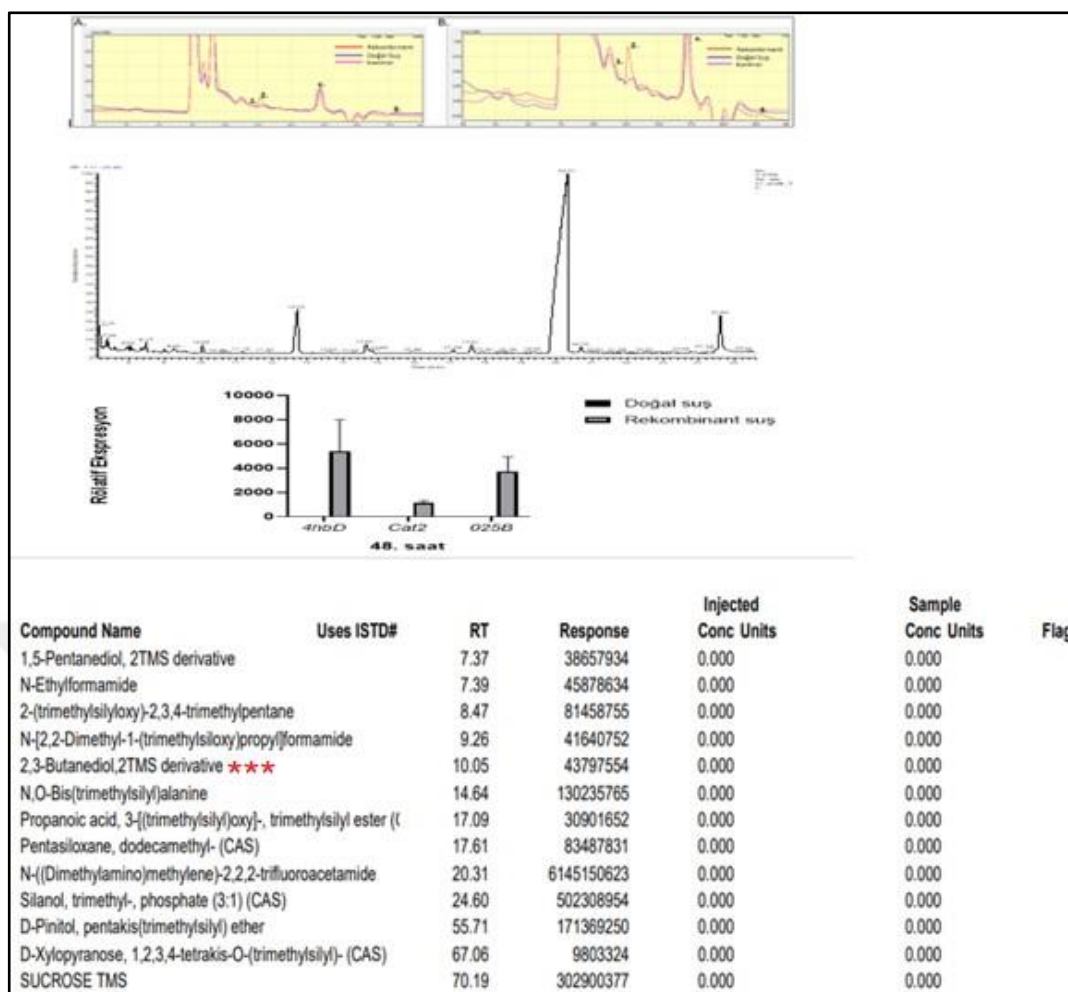


Figure 2.2.2: GC-MS result of *S. coelicolor* M145 strain. Red asterisks shows the 2,3-BDO production.

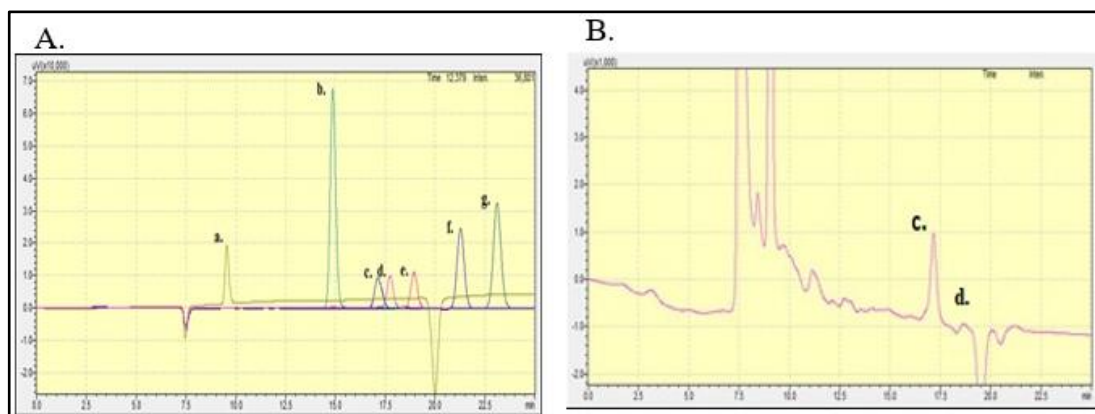


Figure 2.2.3: A) Chromatogram of different standards. Retention times of, a) Glucose b) Acetate, c) Acetoin, d) 2,3-BDO, e) Methanol, f) Ethanol, g) Propanol [25]. B) Chromatogram of *S. coelicolor* grown in TSB media, "c" determines acetoin and "d" determines 2,3-BDO.

In the same study, a recombinant *S. coelicolor* strain was constructed to express extra glucose transport protein (GlcP1), allowing it to take up more glucose and expected to increase the production of acetoin and 2,3-BDO. HPLC analysis showed that the recombinant cell was able to take up more glucose, but did not increase the production of 2,3-BDO and acetoin to the expected levels [25] (Figure 2.2.4).

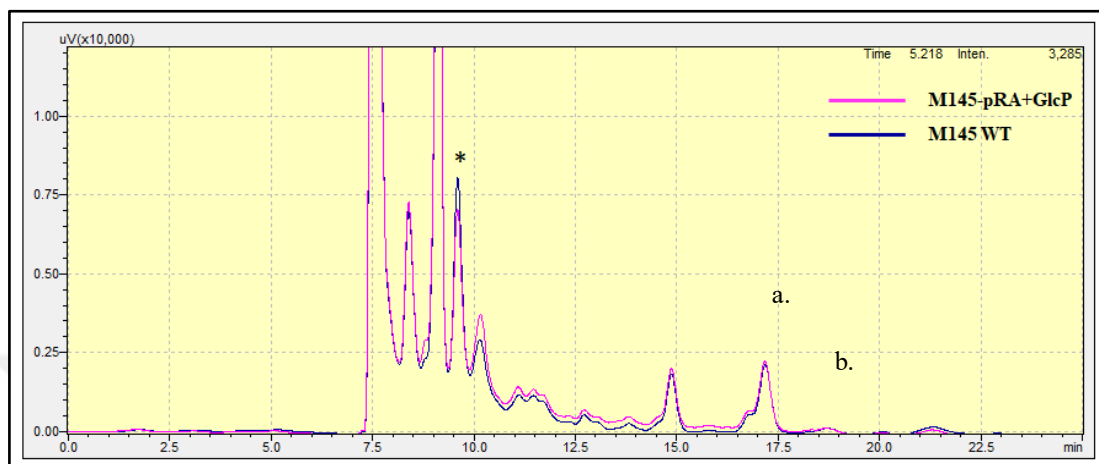


Figure 2.2.4: Chromatogram of wild-type and recombinant *S. coelicolor* strains grown in TSB+Glucose. "*" indicates glucose peak, which is decreased in recombinant strain, "a" indicates acetoin and "b" indicates 2,3-BDO.

Studies in the literature demonstrate that optimising fermentation conditions can increase 2,3-BDO production by different microorganisms [26], [27]. Our laboratory recently demonstrated the ability of *S. coelicolor* to produce 2,3-BDO [25]. The objective of this thesis is to optimize the fermentation conditions to increase the 2,3-BDO yield of both wild-type and recombinant strains.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Chemicals

The chemicals used in this study are; Yeast extract (Bacto™), Peptone (Merck), Malt extract (Bacto™), Sucrose (Merck), MgCl₂ (Merck), Glycine (Riedel de Haen), Tryptic Soy Broth (Merck), D-(+)-Glucose (Merck), KOH (Merck), HCl (Merck), %98 Sulfuric acid (Sigma Aldrich), Acetonitrile (Merck), Agar (Merck), %99 Glycerol (Sigma Aldrich).

3.1.2. Devices

Devices used are; pH-meter (Az Instruments, 86505), sterile cabinet (Tez-San), shaking incubator (Edmund Bühler, KS-15), oven (Binder), incubator (Wisecube), High Performance Liquid Chromatography (SHIMADZU) with BP-800 H⁺ organic acid column (7.8x300mm, 9μ, Benson Polymeric), centrifuges (Heraeus, Labofuge 400R and Beckman Coulter, Microfuge 22R), freezer -20 °C (Ariston), refrigerator +4 °C (Ariston), freezer -80 °C (Thermo, Forma -86 °C ULT Freezer), autoclave (Hirayama, Hiclave HVE-50).

3.1.3. Media

The culture media, with their ingredients, used in this research are shown in Table 3.1. All media were autoclaved at 121°C for 20 minutes before use.

Table 3.1: Media and their ingredients used.

Name of Media	Ingredients
Tryptic Soy Broth (TSB)- 1 L	30 g Tryptic Soy Broth (For Tryptic Soy Agar, 15 g of Agar is added.)
Yeast Extract Malt Extract Media (YEME)- 1 L	3 g Yeast extract, 5 g Peptone, 3g Malt extract, 10 g Glucose, 340 g Sucrose, 5mM MgCl ₂ .6H ₂ O, %0.5 Glycine
TBO Media- 1 L	20 g Tomato paste, 20 g Oat Bran, 25 g Agar
Tryptic Soy Broth+10% Glucose- 1 L	30 g Tryptic Soy Broth, 250 ml of %40 Glucose

3.1.4. Solutions

Solutions with their ingredients and antibiotics are given in Table 3.2 and 3.3, respectively.

Table 3.2: Solutions and their ingredients

Solutions	Ingredients
%40 Glucose- 1 L	400 g Glucose, 1 L dH ₂ O
5 M KOH- 200 mL	56.11 g KOH, 200 mL dH ₂ O
0.5 M HCl- 200 mL	9.85 mL %37 HCl, 190.15 mL dH ₂ O
%40 Glycerol- 50 mL	23 mL %99 Glycerol, 27 mL dH ₂ O

Table 3.3: Antibiotics

Antibiotic	Stock Concentration (mg/mL)	Final Concentration for <i>Streptomyces</i> (mg/mL)
Apramycin (in dH ₂ O)	50	50

3.1.5. Microorganisms

Streptomyces strains used in this study are given in Table 3.4.

Table 3.4: *Streptomyces* strains.

Bacterial Strain	Genotypic Properties	References
<i>Streptomyces coelicolor</i> M145	Wild Type	[28]
<i>Streptomyces</i> M145 + pRA/ <i>glcP1</i>	Recombinant strain containing glucose transporter gene (<i>glcP1</i>) cloned into pRA plasmid	[25]

3.2. Methods

3.2.1. Spore Preparation of *Streptomyces* Strains

50 mL of TSB media (antibiotics were added when necessary, Apramycin for recombinant strain) were inoculated separately with both *Streptomyces* strains. Inoculated media were incubated at 30 °C at 200 rpm for 48 hours. After incubation, bacterial cultures were transferred to sterile falcons and were centrifuged at 4000 rpm for 15 minutes. 45 ml of the supernatant was discarded, and the pellet is resuspended with the remaining 5 ml to obtain a dense cell suspension. 100 µL of cell suspension was inoculated on TBO agar (antibiotics were added when necessary, Apramycin for recombinant strain). Cells were incubated for 6-10 days at 30 °C. On the resulting spores, 5 mL of %40 glycerol was added and were collected via scratching by using sterile cotton swaps. The spore containing glycerol solution was transferred to sterile falcons and were centrifuged at +4 °C at 4000 rpm for 20 minutes. Supernatant was discarded until 1 mL remained, and the pellet was resuspended with remaining supernatant. Resulting spore solution was splitted as 200 µL into sterile Eppendorf tubes, and these tubes were stored at -80 °C. Serial dilution from 10⁻¹ to 10⁻⁹ were prepared to determine the number of spores. 100 µL of each dilution solution was inoculated on TSA and each plate were incubated for 3 days at 30 °C. Finally, resulting colonies were counted and spore numbers for each dilution were calculated with the given equation (3.1).

$$\text{Number of Spores (cfu/mL)} = (\text{colony number} \times \text{dilution factor}) \times (V_{\text{total}} / V_{\text{inoculation}}) \quad (3.1)$$

3.2.2. Optimization of Fermentation Conditions-Determination of Optimum Media

To understand at which media *S. coelicolor* can produce 2,3-BDO at highest, fermentation study has been started. Cells were inoculated to 100 mL of three different media, these were YEME, TSB and TSB+10% Glucose. Cell cultures were grown as pre-culture for 48 hours at 30 °C at 200 rpm. After incubation, bacterial cultures were transferred to sterile falcons and were centrifuged at 4000 rpm for 15 minutes. Supernatants were discarded, and pellet weights were measured to initiate

fermentation at same wet weight. Wild-type *S. coelicolor* pellets were resuspended with known amounts of sterile media, each with its initial media. Same wet weight including cell suspension amounts were inoculated to sterile 100 mL of TSB+%10 Glucose media. Three biological replications for each media with the same wet weight was prepared and incubated for 120 hours at 30 °C at 200 rpm. 5 mL of sample was collected to sterile falcons for every 24 hours. Collected samples were centrifuged at 4000 rpm for 15 minutes. 2 mL of supernatants of each sample were divided into sterile Eppendorf tubes and these tubes were stored at -20 °C until used for HPLC analyses.

3.2.3. Optimization of Fermentation Conditions-Determination of Optimum Temperature and pH Values

After optimum media for 2,3-BDO production in *S. coelicolor* was determined, temperature and pH optimization studies has been carried out in order to understand at which temperature and pH *S. coelicolor* can produce 2,3-BDO at highest. To understand the optimum temperature for 2,3-BDO production, wild-type and recombinant *S. coelicolor* strains were inoculated to 100 mL of TSB+10% Glucose media separately. Cell cultures were grown as pre-culture for 48 hours at 30 °C at 200 rpm. After incubation, bacterial cultures were transferred to sterile falcons and were centrifuged at 4000 rpm for 15 minutes. Supernatants were discarded, and pellet weights were measured to initiate fermentation at same wet weight. Wild-type and recombinant *S. coelicolor* pellets were resuspended with known amounts of sterile TSB+%10 Glucose media. Same wet weight including cell suspension amounts were inoculated to sterile 100 mL of TSB+%10 Glucose media. By using the prepared cell cultures, a fermentation process with three different incubation temperatures, which were 25°C, 30°C and 35°C, has been initiated. Three biological replications for each incubation temperature and strain with the same wet weight was prepared and incubated for 120 hours at 25°C, 30°C and 35°C at 200 rpm. 5 mL of sample was collected to sterile falcons for every 24 hours. Collected samples were centrifuged at 4000 rpm for 15 minutes. 2 mL of supernatants of each sample were divided into sterile Eppendorf tubes and these tubes were stored at -20 °C until used for HPLC analyses. To understand the optimum pH for 2,3-BDO production, wild-type and recombinant *S. coelicolor* strains were inoculated to 100 mL of TSB+10% Glucose media with five different pH, these were pH:5.0, pH:6.0, pH:7.0, pH:8.0 and pH:9.0, separately. Cell cultures were grown as pre-culture for 48 hours at 30°C at 200 rpm. After incubation,

bacterial cultures were transferred to sterile falcons and were centrifuged at 4000 rpm for 15 minutes. Supernatants were discarded, and pellet weights were measured to initiate fermentation at same wet weight. Wild-type and recombinant *S. coelicolor* pellets were resuspended with known amounts of sterile TSB+10% Glucose media, each with its initial media with different pH. Same wet weight including cell suspension amounts were inoculated to sterile 100 mL of pH:5.0, pH:6.0, pH:7.0, pH:8.0, pH:9.0 TSB+10% Glucose media. For pH:5.0 and pH:6.0, HCl was used until to reach the targeted pH and for pH:8.0 and pH:9.0, KOH was used until to reach the targeted pH. Three biological replications for each pH and strain with the same wet weight was prepared and incubated for 120 hours at 30°C at 200 rpm. 5 mL of sample was collected to sterile falcons for every 24 hours. Collected samples were centrifuged at 4000 rpm for 15 minutes. 2 mL of supernatants of each sample were divided into sterile Eppendorf tubes and these tubes were stored at -20 °C until used for HPLC analyses.

3.2.4. HPLC Analysis

According to HPLC methods adjusted by Champluvier et. al and Siemerink et. al [29], 2,3-BDO and acetoin peaks of samples were detected. Samples were filtered with 0.45 µm syringe filters to be ready for HPLC analysis. 1 mL of each sample was used for HPLC analysis. To detect 2,3-BDO and acetoin, Refractive index detector (Shimadzu) and organic acid column (Benson Polymeric BP-800 H⁺) were used. Method for analysis was as flow rate 0.6 mL/min, column temperature 65 °C, injection volume 15 µL, runtime 20 minute and mobile phase was 5 mM H₂SO₄.

3.2.5. Statistical Analysis

To understand whether there was a significant difference in the production of 2,3-BDO and acetoin between *S. coelicolor* M145 samples grown in TSB+10% glucose, TSB and YEME media, statistical analyzes were performed by using the two-way ANOVA test in GraphPad Prism, a tool used for statistical analysis and graphing. The mean of peak areas of each 24 hour collected samples of each media were calculated. The mean of same hour collected samples of each media were then compared to each other and a graph that shows the significant differences between compared samples was formed.

In order to understand whether there is a significant change in the production of 2,3-BDO and acetoin between *S. coelicolor* M145 and recombinant *S. coelicolor* grown at different temperature and pH, statistical analyzes were performed by using the two-way ANOVA test in GraphPad Prism. The mean of peak areas of each 24 hour collected samples of each temperature and pH were calculated for both *S. coelicolor* M145 and recombinant *S. coelicolor*. The mean of same hour collected samples of *S. coelicolor* M145 and recombinant *S. coelicolor* were then compared to each other, temperature samples were compared to each other and pH samples were compared to each other, and a graph that shows the significant differences between compared samples was formed.



4. RESULTS

4.1. Determination of Optimum Media for 2,3-BDO Production in *S. coelicolor* M145 Strain

S. coelicolor M145 strain was inoculated into YEME, TSB and TSB+10% glucose media and grown for 120 hours in three biological replicates. Samples were collected in every 24 hours and the supernatants of cultures were analyzed using HPLC, to determine the optimal media for 2,3-BDO production. The production amount of acetoin was also analyzed, as acetoin is the precursor of 2,3-BDO. Chromatographs of *S. coelicolor* grown in TSB+10% glucose (Figure 4.1), TSB (Figure 4.2), and YEME (Figure 4.3) were obtained. According to the chromatogram results, the highest 2,3-BDO production was in TSB medium supplemented with 10% glucose in the samples collected at 48th hour of fermentation (Figure 4.4 and Table 4.1). On the other hand, the highest acetoin production was seen in TSB medium in sample collected at 120th hour of fermentation (Figure 4.5 and Table 4.1). 2,3-BDO and acetoin production of *S. coelicolor* M145 in YEME media was at very low levels in contrast to TSB and TSB+10% glucose (Figure 4.6 and Table 4.1). Based on these results, TSB+10% glucose media was selected as the optimal media for 2,3-BDO production. Subsequently, pH and temperature optimization studies were conducted using this media.

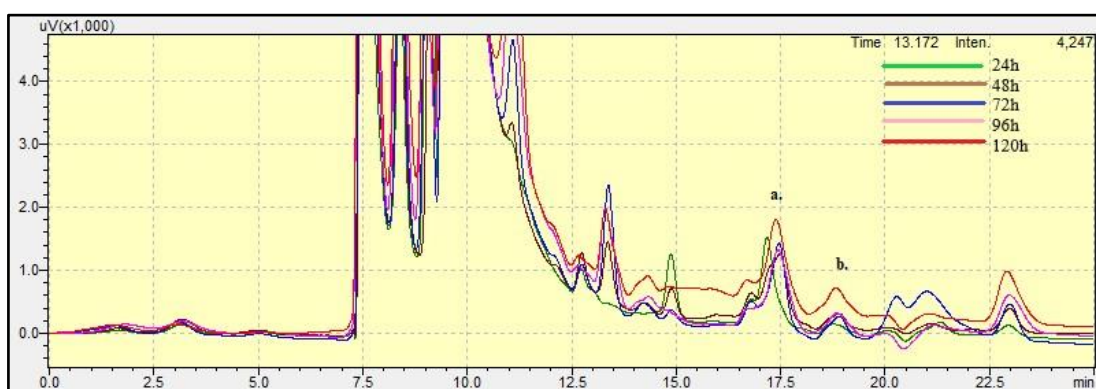


Figure 4.1: Chromatographs of *S. coelicolor* grown in TSB+10% glucose. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

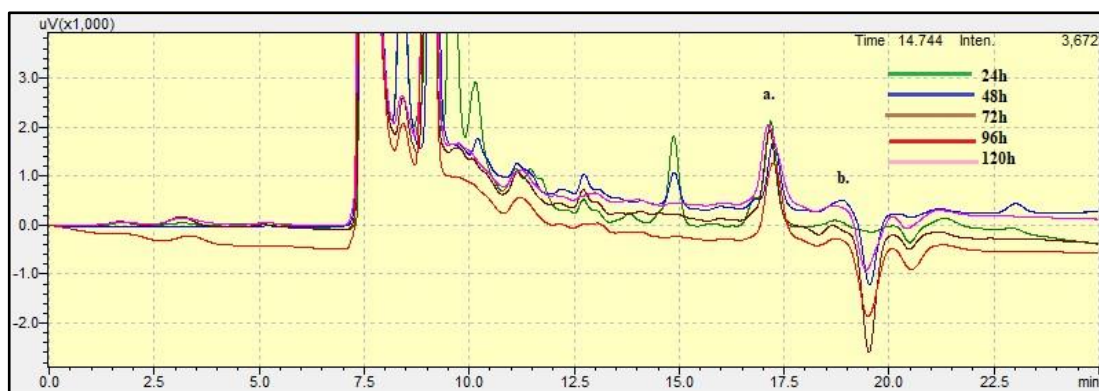


Figure 4.2: Chromatographs of *S. coelicolor* grown in TSB. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

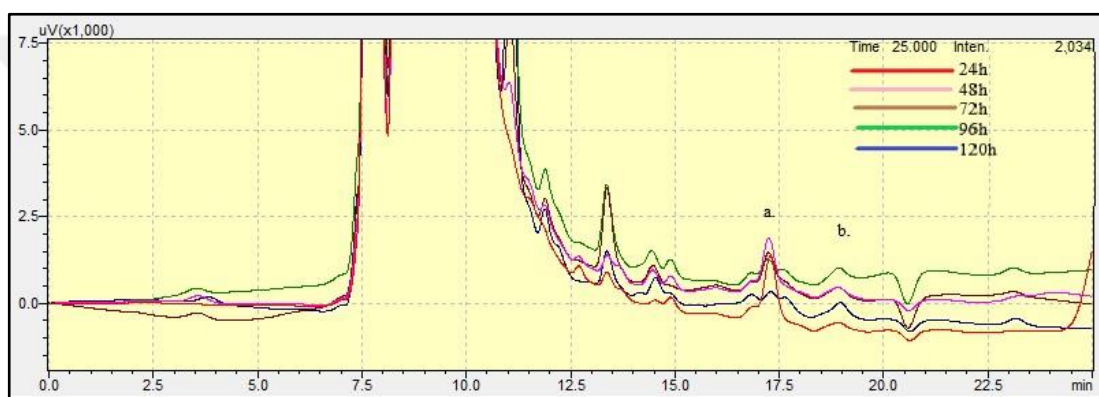


Figure 4.3: Chromatographs of *S. coelicolor* grown in YEME. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.



Figure 4.4: The 48th hour chromatogram of *S. coelicolor* M145 grown in TSB+10% glucose, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.5: The 120th hour chromatogram of *S. coelicolor* M145 grown in TSB, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.6: The 48th hour chromatogram of *S. coelicolor* M145 grown in YEME, “a” indicates acetoin and “b” indicates 2,3-BDO.

The peak area of each sample was measured and compared to the peak area of 251363 for 4.5 mg/mL acetoin and 98871 for 0.2 mg/mL 2,3-BDO, using acetoin and 2,3-BDO standards [25]. To understand whether there was a significant difference in the production of 2,3-BDO and acetoin between *S. coelicolor* M145 samples grown in TSB+%10 glucose, TSB and YEME media, statistical analyzes were performed using the two-way ANOVA test. According to this test, the mean of three biological replicates of each sample was measured and compared (Figure 4.7). The *Streptomyces* culture grown in TSB+10% glucose for 48 hours produced the highest amount of 2,3-BDO at 0.033 mg/mL (Table 4.1). In contrast, the *Streptomyces* culture grown in TSB for 120 hours produced the highest amount of acetoin at 2.09 mg/mL (Table 4.1).

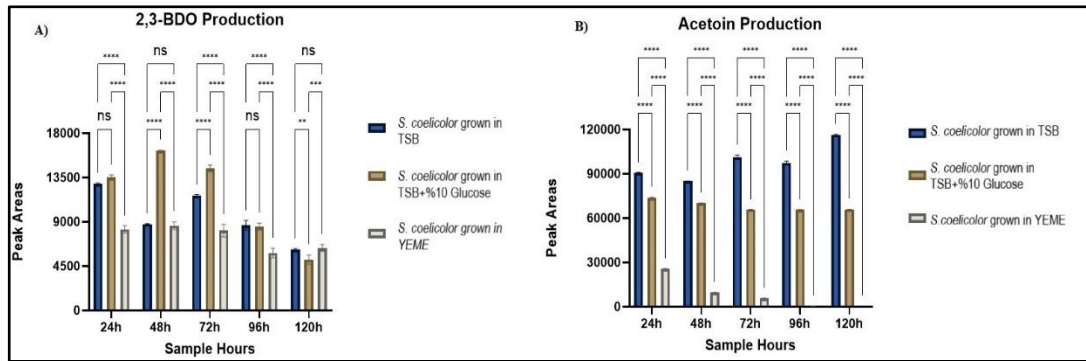


Figure 4.7: Two-way ANOVA test of *S. coelicolor* M145 samples grown in TSB, TSB+%10 glucose and YEME media. Each bar represents means of three biological replicates. A) 2,3-BDO production differences between *S. coelicolor* M145 samples grown in TSB, TSB+%10 glucose and YEME media (**** $p < 0.0001$, ** $p = 0.0017$, ns: non-significant). B) Acetoin production differences between *S. coelicolor* M145 samples grown in TSB, TSB+%10 glucose and YEME media (**** $p < 0.0001$).

Table 4.1: Production levels and peak areas of 2,3-BDO and acetoin for the *S. coelicolor* M145 strain grown in TSB, TSB+10% glucose and YEME. Each peak area represents the average of three biological replicates.

Time Media	2,3-BDO Peak Area 0.2 mg/mL=98871			Acetoin Peak Area 4.5 mg/mL=251363		
	<i>Streptomyces</i> grown in TSB	<i>Streptomyces</i> grown in TSB+%10 glucose	<i>Streptomyces</i> grown in YEME	<i>Streptomyces</i> grown in TSB	<i>Streptomyces</i> grown in TSB+%10 glucose	<i>Streptomyces</i> grown in YEME
24 th hour	12920 = 0.026 mg/mL	13767 = 0.028 mg/mL	8575 = 0.017 mg/mL	90969 = 1.63 mg/mL	73983 = 1.32 mg/mL	25735 = 0.46 mg/mL
48 th hour	8811 = 0.018 mg/mL	16276 = 0.033 mg/mL	8893 = 0.017 mg/mL	85168 = 1.52 mg/mL	70256 = 1.26 mg/mL	9715 = 0.17 mg/mL
72 nd hour	11756 = 0.024 mg/mL	14635 = 0.030 mg/mL	8502 = 0.017 mg/mL	102024 = 1.83 mg/mL	66012 = 1.18 mg/mL	5798 = 0.10 mg/mL
96 th hour	8980 = 0.018 mg/mL	8727 = 0.018 mg/mL	6123 = 0.012 mg/mL	98214 = 1.76 mg/mL	66040 = 1.18 mg/mL	650 = 0.011 mg/mL
120 th hour	6243 = 0.013 mg/mL	5652 = 0.011 mg/mL	6223 = 0.012 mg/mL	116661 = 2.09 mg/mL	66039 = 1.18 mg/mL	452 = 0.01 mg/mL

4.2. Production of 2,3-BDO by *Streptomyces* in different Temperatures

Once it was determined that TSB+10% glucose was the optimal medium for 2,3-BDO production, *S. coelicolor* and the recombinant strain [25], which overexpresses the glucose transporter gene (*glcP1*), were cultivated in three biological replicates at varying temperatures to determine the optimum temperature for 2,3-BDO production. Three temperatures were tested: 25°C, 30°C, and 35°C. Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at 25°C (Figure 4.8), 30°C (Figure 4.9) and 35°C (Figure 4.10), and wild-type *S. coelicolor* grown in TSB+10%

glucose at 25°C (Figure 4.11), 30°C (Figure 4.12) and 35°C (Figure 4.13) were obtained. In order to understand whether there is a significant change in the production of 2,3-BDO and acetoin between *S. coelicolor* M145 and recombinant *S. coelicolor*, statistical analyzes were performed using the two-way ANOVA test. According to this test, the mean of three biological replicates was measured and compared (Figure 4.14 and Figure 4.15). The chromatogram results of wild type strain have shown that the highest 2,3-BDO production was seen at 30°C in 48th hour samples (Figure 4.16 and Table 4.2). On the other hand, the highest acetoin production was seen at 25°C in 96th hour samples (Figure 4.17 and Table 4.2). In the case of recombinant strain, the chromatogram results have shown that the highest 2,3-BDO production was seen at 30°C in 72nd hour samples (Figure 4.18 and Table 4.3). On the other hand, the highest acetoin production was seen at 35°C in 24th hour samples (Figure 4.19 and Table 4.3).

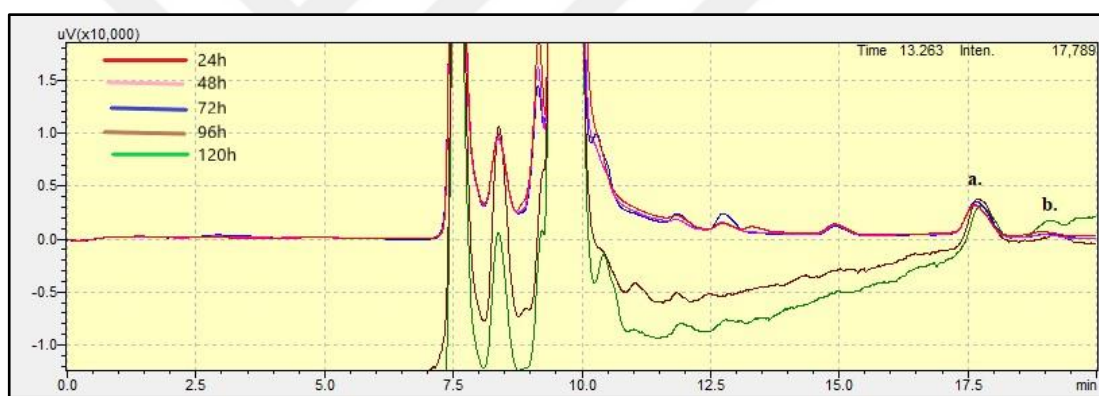


Figure 4.8: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at 25°C. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

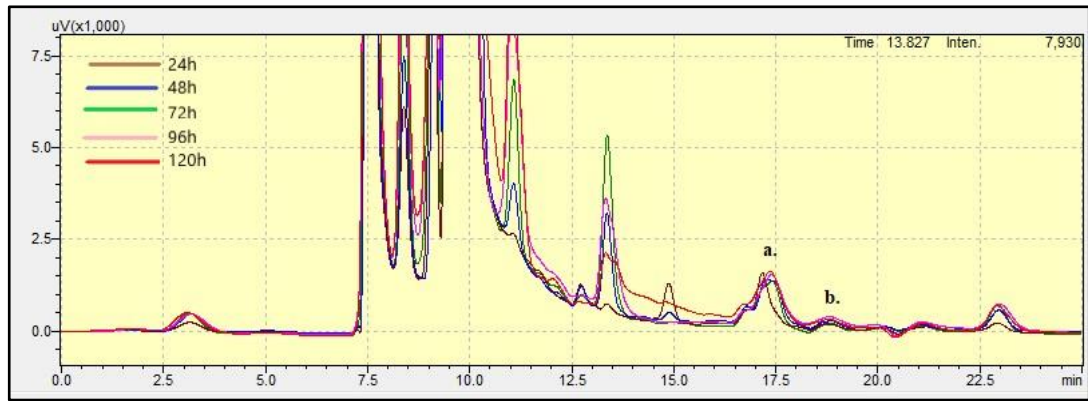


Figure 4.9: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at 30°C. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

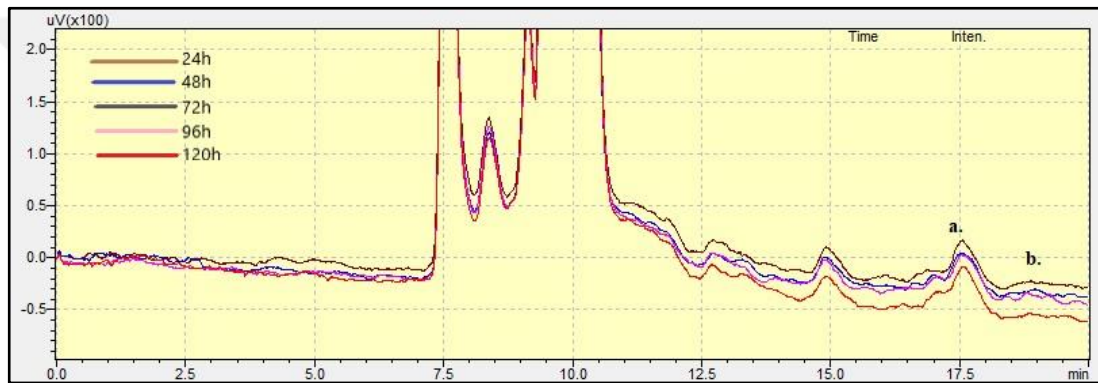


Figure 4.10: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at 35°C. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

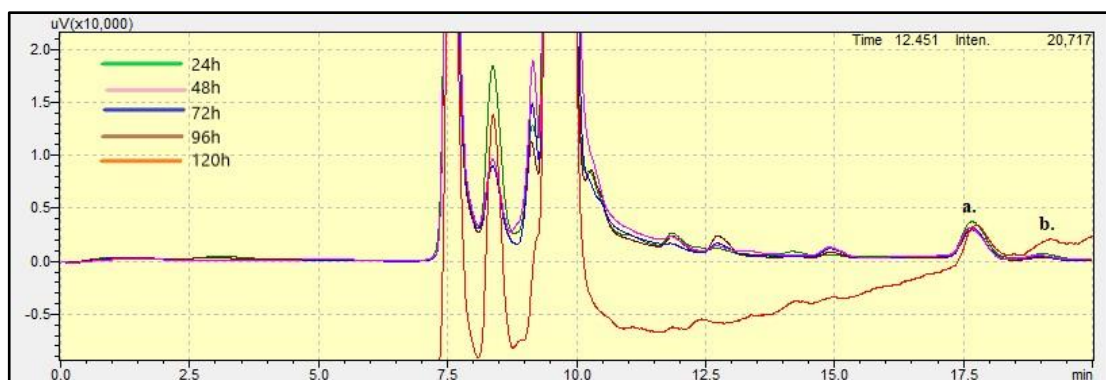


Figure 4.11: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at 25°C. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

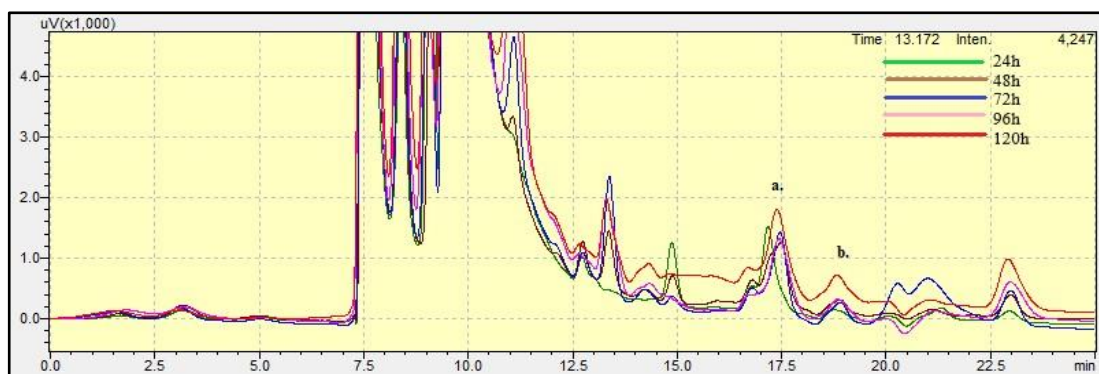


Figure 4.12: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at 30°C. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

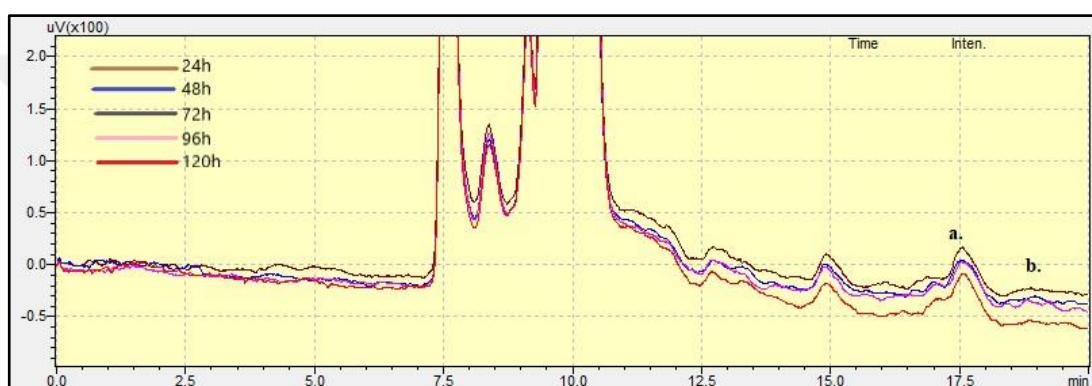


Figure 4.13: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at 35°C. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

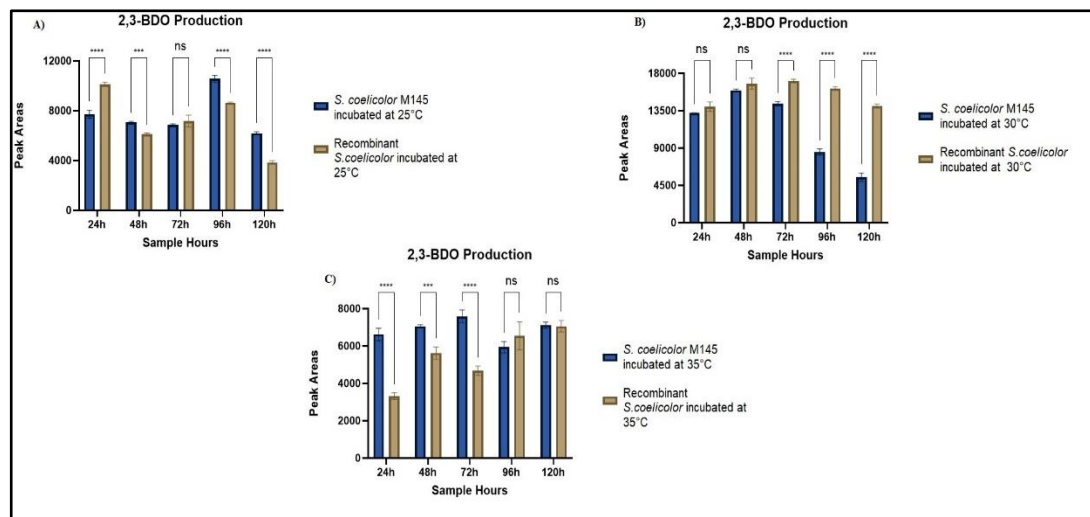


Figure 4.14: Two-way ANOVA test of *S. coelicolor* M145 and recombinant *S. coelicolor* samples grown in TSB+%10 glucose media and incubated at 25°C, 30°C and 35°C. Each bar represents means of three biological replicates. A) 2,3-BDO production differences between *S. coelicolor* M145 and recombinant *S. coelicolor* samples incubated at 25°C (**** $p < 0.0001$, *** $p = 0.002$, ns: non-significant). B) 2,3-BDO production differences between *S. coelicolor* M145 and recombinant *S. coelicolor* samples incubated at 30°C (**** $p < 0.0001$, ns: non-significant). C) 2,3-BDO production differences between *S. coelicolor* M145 and recombinant *S. coelicolor* samples incubated at 35°C (**** $p < 0.0001$, *** $p = 0.003$, ns: non-significant).

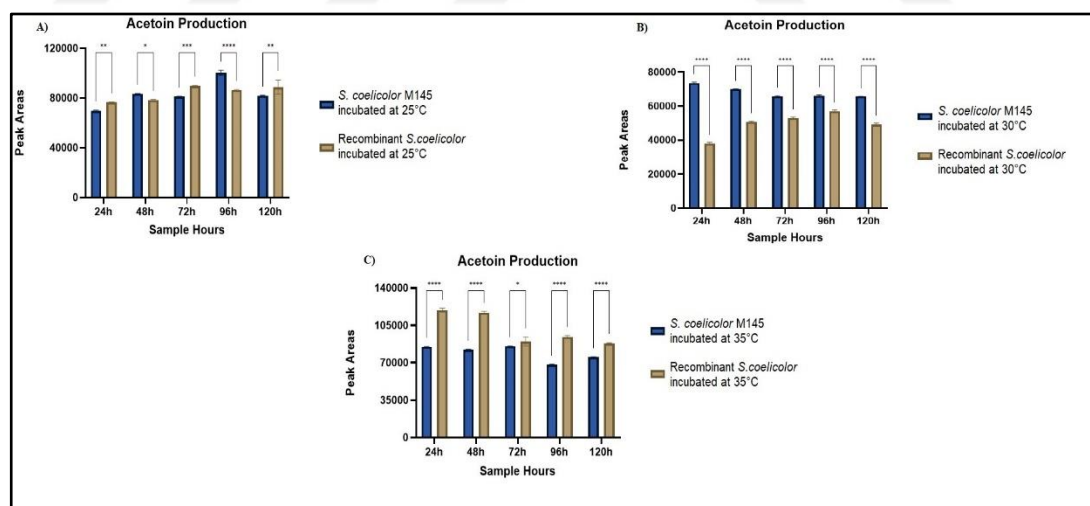


Figure 4.15: Two-way ANOVA test of *S. coelicolor* M145 and recombinant *S. coelicolor* samples grown in TSB+%10 glucose media and incubated at 25°C, 30°C and 35°C. Each bar represents means of three biological replicates. A) Acetoin production differences between *S. coelicolor* M145 and recombinant *S. coelicolor* samples incubated at 25°C (* $p = 0.0270$, ** $p = 0.0018$, *** $p = 0.0002$, **** $p < 0.0001$). B) Acetoin production differences between *S. coelicolor* M145 and recombinant *S. coelicolor* samples incubated at 30°C (**** $p < 0.0001$). C) Acetoin production differences between *S. coelicolor* M145 and recombinant *S. coelicolor* samples incubated at 35°C (* $p = 0.0136$, **** $p < 0.0001$).



Figure 4.16: The 48th hour chromatogram of *S. coelicolor* M145 grown in TSB+10% glucose at 30°C, “a” indicates acetoin and “b” indicates 2,3-BDO.

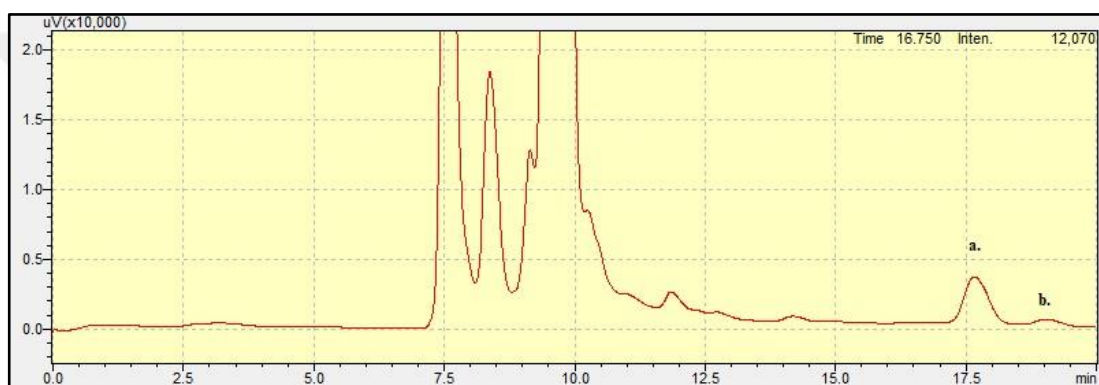


Figure 4.17: The 96th hour chromatogram of *S. coelicolor* M145 grown in TSB+10% glucose at 25°C, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.18: The 72nd hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at 30°C, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.19: The 24th hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at 35°C, “a” indicates acetoin and “b” indicates 2,3-BDO.

Based on the peak area measurements of chromatograms, it is evident that both the wild-type and recombinant *Streptomyces* strains produced the highest levels of 2,3-BDO at 30°C after 48 hours for the wild type (0.033 mg/mL) (Table 4.2) and after 72 hours for the recombinant strain (0.035 mg/mL) (Table 4.3). The highest acetoin production for the wild-type was observed at 25°C after 96 hours, with a production of 1.82 mg/mL (Table 4.2). In contrast, the recombinant strain showed the highest acetoin production at 35°C in the 24th hour, with a production of 2.17 mg/mL (Table 4.3).

Table 4.2: Production levels and peak areas of 2,3-BDO and acetoin for the *S. coelicolor* M145 strain grown in TSB+10% glucose at 25°C, 30°C, 35°C. Each peak area represents the average of three biological replicates.

Time Temperature	2,3-BDO Peak Area 0.2 mg/mL=98871			Acetoin Peak Area 4.5 mg/mL=251363		
	<i>Streptomyces</i> grown in TSB+%10 glucose at 25°C	<i>Streptomyces</i> grown in TSB+%10 glucose at 30°C	<i>Streptomyces</i> grown in TSB+%10 glucose at 35°C	<i>Streptomyces</i> grown in TSB+%10 glucose at 25°C	<i>Streptomyces</i> grown in TSB+%10 glucose at 30°C	<i>Streptomyces</i> grown in TSB+%10 glucose at 35°C
24 th hour	7979 = 0.016 mg/mL	13247 = 0.028 mg/mL	6835 = 0.014 mg/mL	70084 = 1.25 mg/mL	74009 = 1.32 mg/mL	85085 = 1.52 mg/mL
48 th hour	7130 = 0.014 mg/mL	16073 = 0.033 mg/mL	7149 = 0.014 mg/mL	83655 = 1.50 mg/mL	70129 = 1.26 mg/mL	82537 = 1.48 mg/mL
72 nd hour	6949 = 0.014 mg/mL	14531 = 0.030 mg/mL	7826 = 0.016 mg/mL	81437 = 1.46 mg/mL	65942 = 1.18 mg/mL	85813 = 1.52 mg/mL
96 th hour	10749 = 0.022 mg/mL	8793 = 0.018 mg/mL	6124 = 0.012 mg/mL	101910 = 1.82 mg/mL	66749 = 1.18 mg/mL	68522 = 1.23 mg/mL
120 th hour	6271 = 0.013 mg/mL	5932 = 0.011 mg/mL	7237 = 0.015 mg/mL	82153 = 1.47 mg/mL	65809 = 1.18 mg/mL	75700 = 1.36 mg/mL

Table 4.3: Production levels and peak areas of 2,3-BDO and acetoin for the recombinant *S. coelicolor* strain grown in TSB+10% glucose at 25°C, 30°C, 35°C. Each peak area represents the average of three biological replicates.

Time Temperature	2,3-BDO Peak Area 0.2 mg/mL=98871			Acetoin Peak Area 4.5 mg/mL=251363		
	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at 25°C	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at 30°C	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at 35°C	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at 25°C	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at 30°C	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at 35°C
24 th hour	10309 = 0.021 mg/mL	14624 = 0.029 mg/mL	3444 = 0.007 mg/mL	76933 = 1.37 mg/mL	38670 = 0.69 mg/mL	121360 = 2.17 mg/mL
48 th hour	6200 = 0.012 mg/mL	15750 = 0.031 mg/mL	5822 = 0.011 mg/mL	78972 = 1.38 mg/mL	51039 = 0.91 mg/mL	118408 = 2.12 mg/mL
72 nd hour	7702 = 0.016 mg/mL	17274 = 0.035 mg/mL	4838 = 0.009 mg/mL	89908 = 1.61 mg/mL	53429 = 0.95 mg/mL	92496 = 1.66 mg/mL
96 th hour	8700 = 0.018 mg/mL	16360 = 0.033 mg/mL	7414 = 0.015 mg/mL	86768 = 1.55 mg/mL	57358 = 1.02 mg/mL	95448 = 1.71 mg/mL
120 th hour	3944 = 0.008 mg/mL	14324 = 0.029 mg/mL	7242 = 0.015 mg/mL	92176 = 1.65 mg/mL	49922 = 1.18 mg/mL	88729 = 1.59 mg/mL

4.3. Production of 2,3-BDO by *Streptomyces* in different pHs

After determining that the optimum temperature for 2,3-BDO production was 30°C for both the wild type and recombinant strains, they were grown in TSB+10% glucose at different pHs to find out the optimum pH for 2,3-BDO production. The wild-type cells were grown in TSB+10% glucose with five different initial pH values and HPLC chromatograms of pH 5.0 (Figure 4.20), pH 6.0 (Figure 4.21), pH 7.0 (Figure 4.22), pH 8.0 (Figure 4.23) and pH 9.0 (Figure 4.24) were obtained and the recombinant cells

were grown in TSB+10% glucose with five different initial pH values and HPLC chromatograms of pH 5.0 (Figure 4.25), pH 6.0 (Figure 4.26), pH 7.0 (Figure 4.27), pH 8.0 (Figure 4.28) and pH 9.0 (Figure 4.29) were obtained.

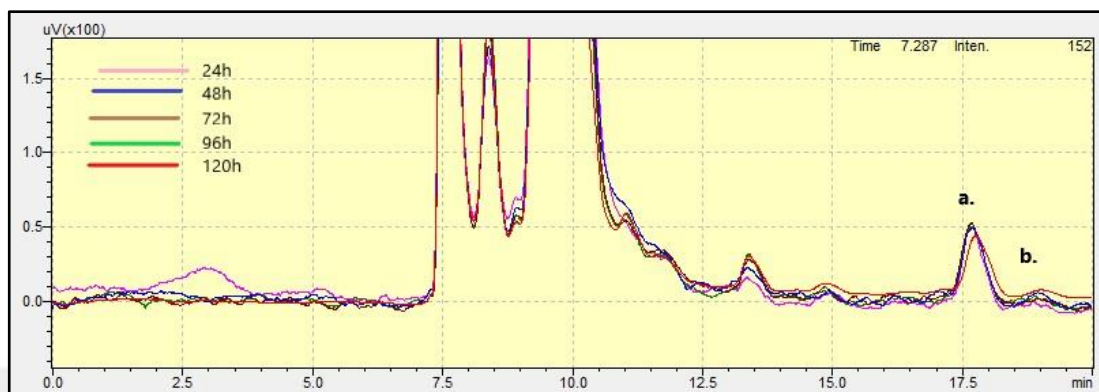


Figure 4.20: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at pH 5.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

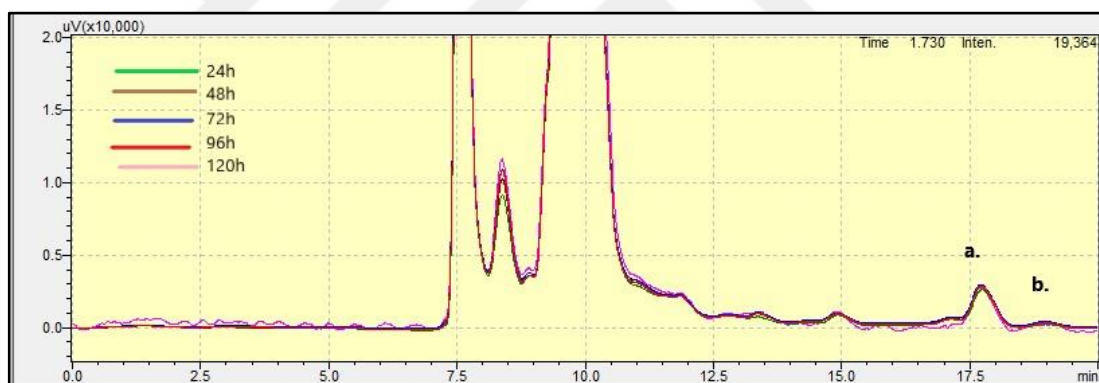


Figure 4.21: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at pH 6.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

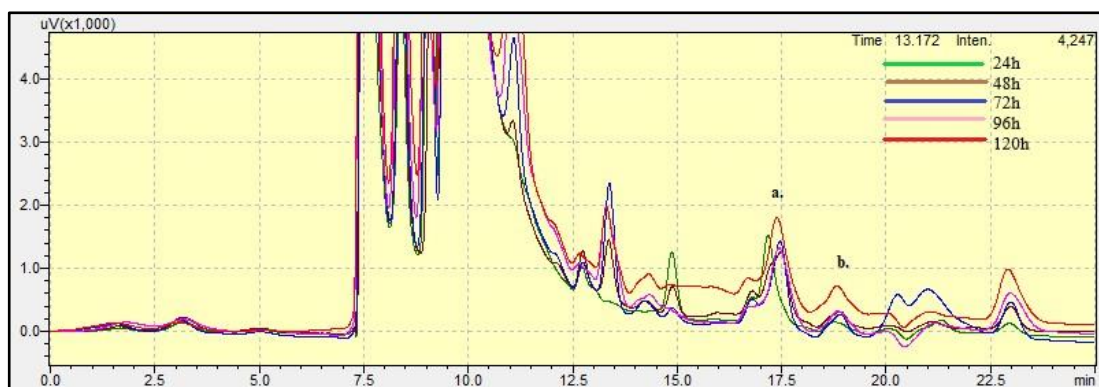


Figure 4.22: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at pH 7.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

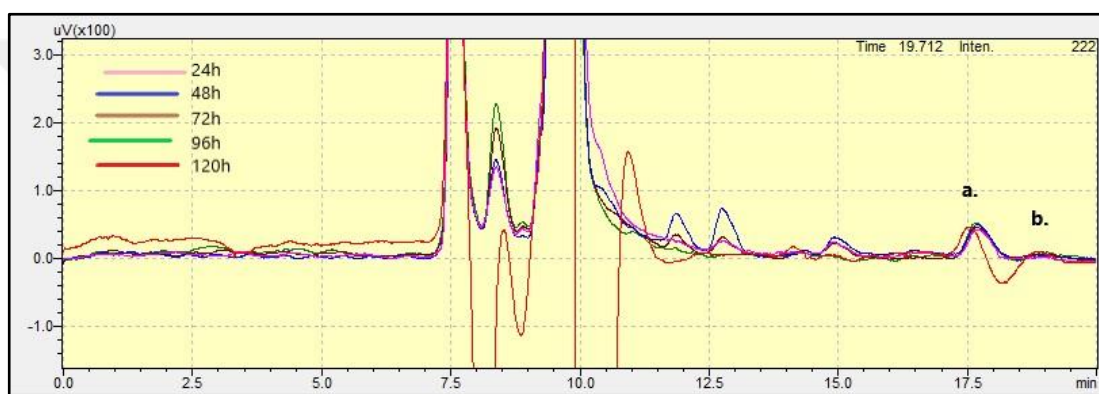


Figure 4.23: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at pH 8.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

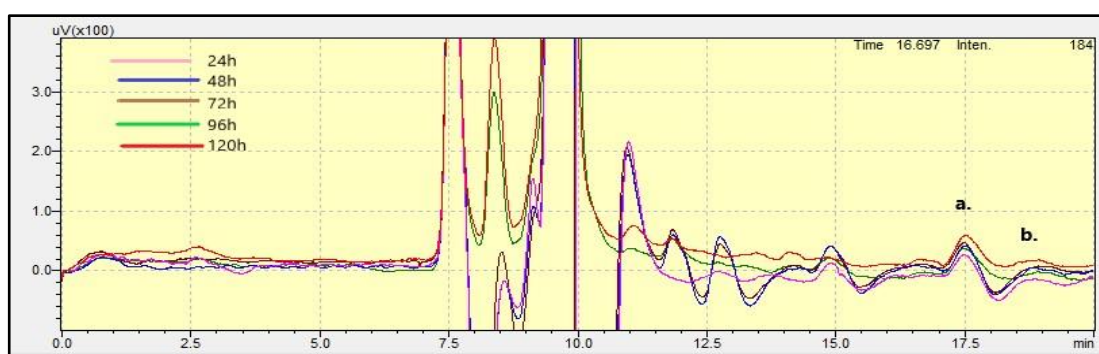


Figure 4.24: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at pH 9.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

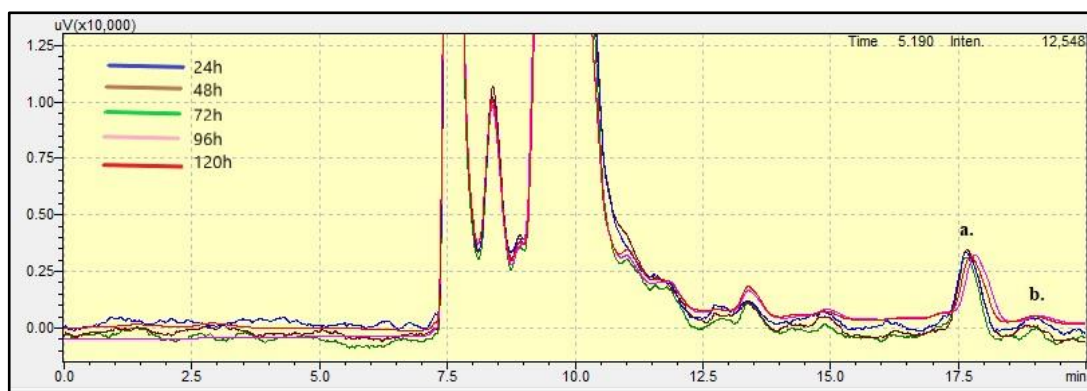


Figure 4.25: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at pH 5.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

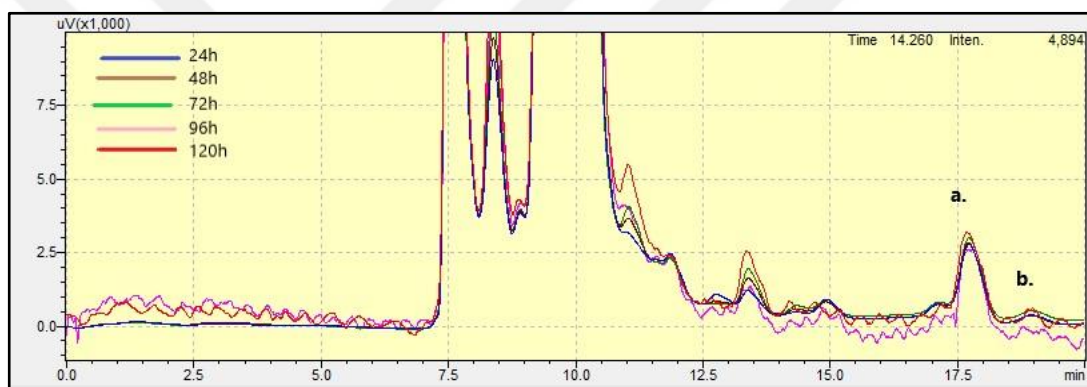


Figure 4.26: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at pH 6.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

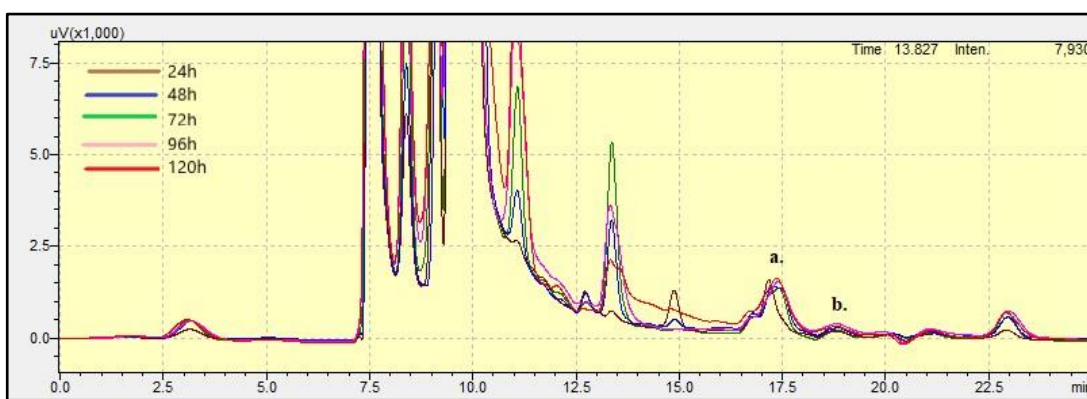


Figure 4.27: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at pH 7.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

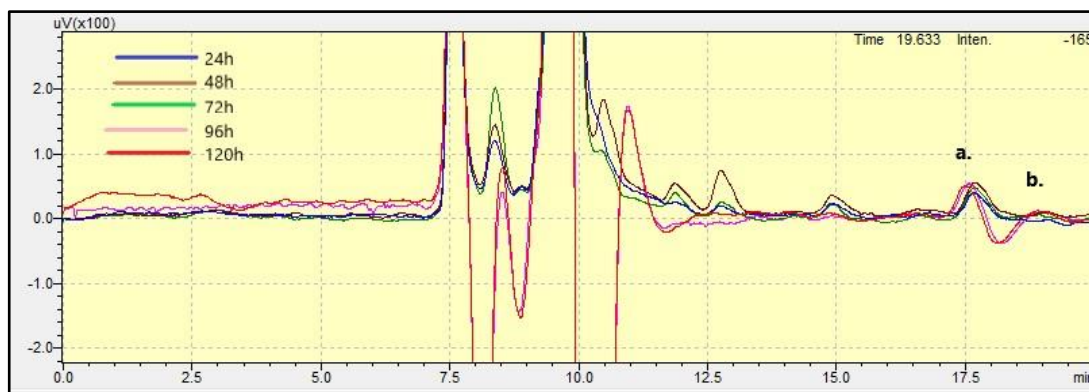


Figure 4.28: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at pH 8.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

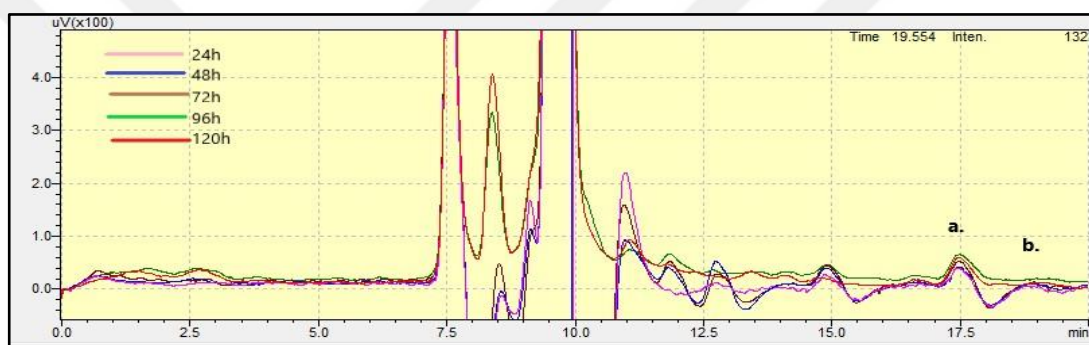


Figure 4.29: Chromatographs of recombinant *S. coelicolor* grown in TSB+10% glucose at pH 9.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

In order to understand whether there is a significant change in the production of 2,3-BDO and acetoin between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:5.0, pH:6.0, pH:7.0, pH:8.0 and pH:9.0 statistical analyzes were performed using the Two-way ANOVA test. According to this test, the mean of three biological replicates of each sample was measured. Samples collected at the same time were then compared with each other (Figure 4.30 and Figure 4.31). The results of the chromatogram analysis for the wild-type *Streptomyces* strain indicate that the highest production of 2,3-BDO was observed at pH 7.0, in the sample collected at 48th hours with a concentration of 0.033 mg/mL (Figure 4.32 and Table 4.4). Conversely, the highest production of acetoin was observed at pH 8.0, in the sample collected at 96th hours with a concentration of 1.89 mg/mL (Figure 4.33 and Table 4.4). The lowest 2,3-BDO production was at pH 8.0, which was the sample collected at 96th hour with 0.01

mg/mL (Figure 4.33 and Table 4.4) and the lowest acetoin production was seen at pH 6.0, which was the sample collected at 24th hour with 1.06 mg/mL (Figure 4.34 and Table 4.4). In the case of recombinant *Streptomyces* strain, the chromatogram results have shown us, that the highest 2,3-BDO production was seen at pH 7.0, which was the sample collected at 72nd hour with 0.035 mg/mL (Figure 4.35 and Table 4.5). On the other hand, the highest acetoin production was seen at pH 9.0, which was the sample collected at 120th hour with 1.66 mg/mL (Figure 4.36 and Table 4.5). The lowest 2,3-BDO production was at pH 8.0, which was the sample collected at 24th hour with 0.013 mg/mL (Figure 4.37 and Table 4.5) and the lowest acetoin production was seen at pH 7.0, which was the sample collected at 24th hour with 0.70 mg/mL (Figure 4.38 and Table 4.5).

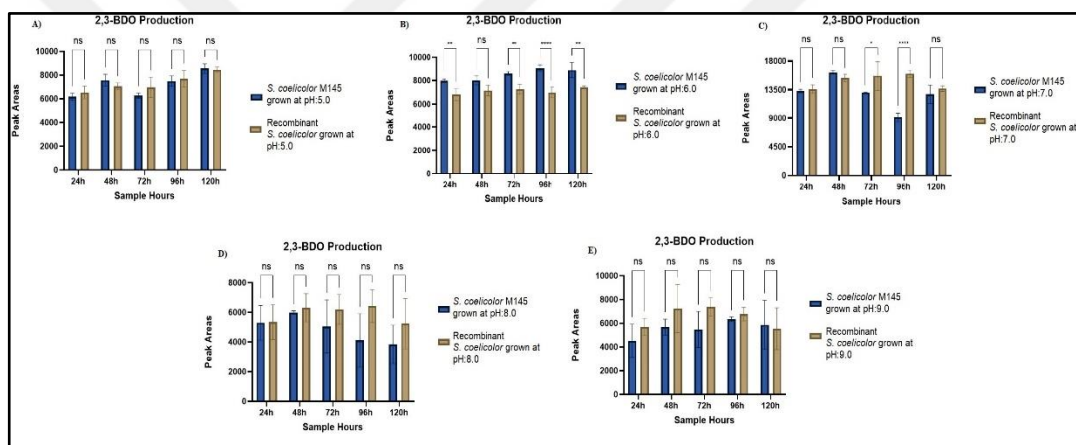


Figure 4.30: Two-way ANOVA test (drawn with GraphPad) of *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown in TSB+%10 glucose media at pH:5.0, pH:6.0, pH:7.0, pH:8.0 and pH:9.0. Each bar represents sample means of three biological replicates. A) 2,3-BDO production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:5.0 (ns: non-significant). B) 2,3-BDO production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:6.0 (*p=0.0166, **p=0.0027, ***p<0.0001, ns: non-significant). C) 2,3-BDO production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:7.0 (*p=0.0166, ***p<0.0001, ns: non-significant). D) 2,3-BDO production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:8.0 (ns: non-significant). E) 2,3-BDO production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:9.0 (ns: non-significant).

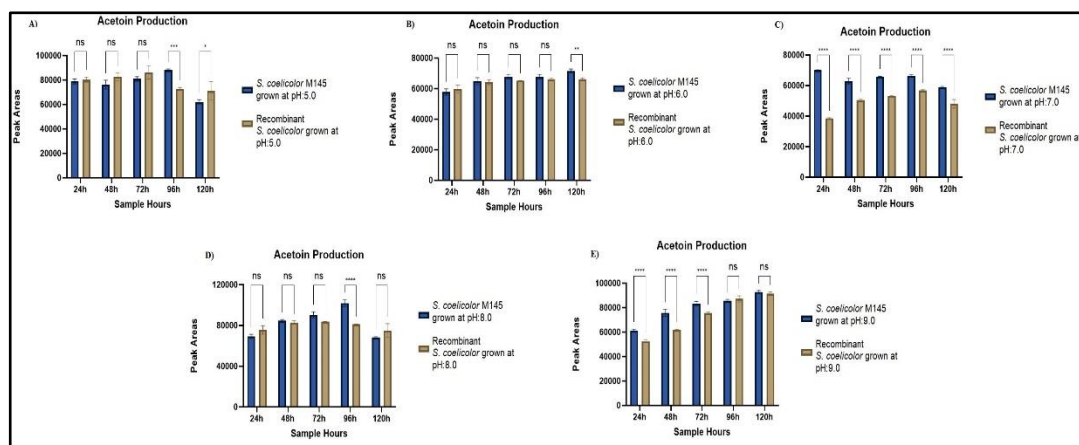


Figure 4.31: Two-way ANOVA test (drawn with GraphPad) of *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown in TSB+10% glucose media at pH:5.0, pH:6.0, pH:7.0, pH:8.0 and pH:9.0. Each bar represents sample means of three biological replicates. A) Acetoin production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:5.0 (* $p=0.0264$, *** $p=0.0002$, ns: non-significant). B) Acetoin production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:6.0 (** $p=0.0025$, ns: non-significant). C) Acetoin production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:7.0 (**** $p<0.0001$). D) Acetoin production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:8.0 (**** $p<0.0001$, ns: non-significant). E) Acetoin production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown at pH:9.0 (**** $p<0.0001$, ns: non-significant).



Figure 4.32: The 48th hour chromatogram of *S. coelicolor* M145 grown in TSB+10% glucose at pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.33: The 96th hour chromatogram of recombinant *S. coelicolor* M145 grown in TSB+10% glucose at pH 8.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

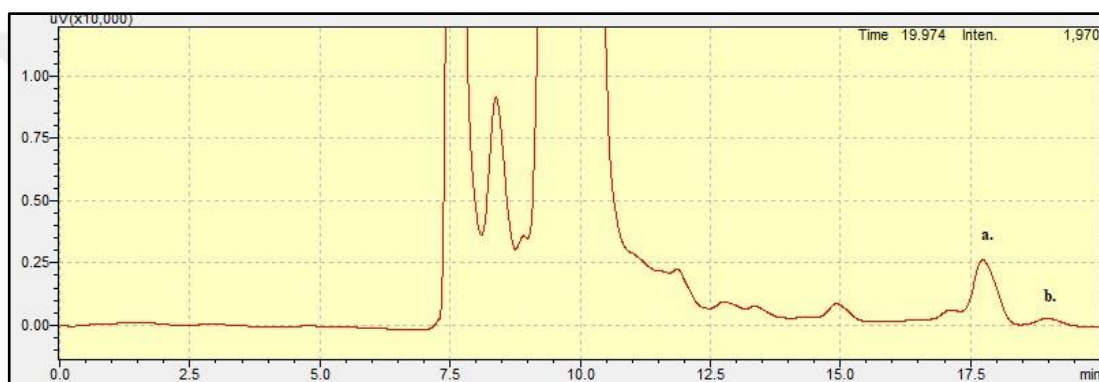


Figure 4.34: The 24th hour chromatogram of recombinant *S. coelicolor* M145 grown in TSB+10% glucose at pH 6.0, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.35: The 72nd hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

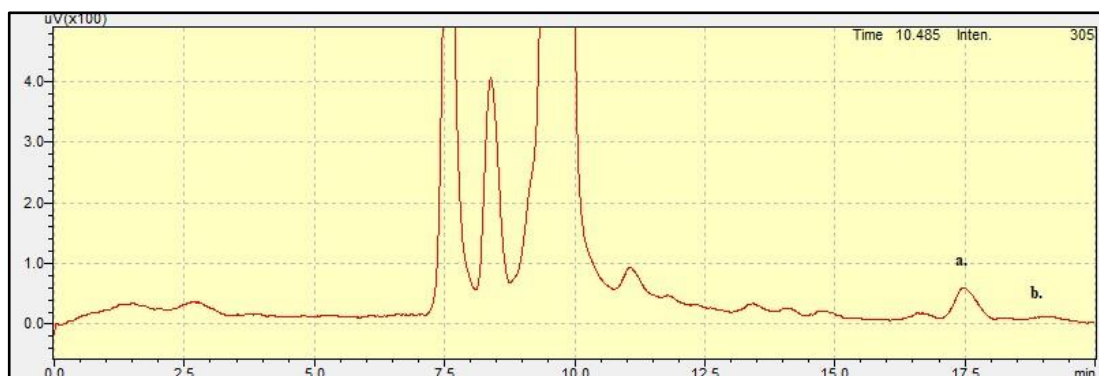


Figure 4.36: The 120th hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at pH 9.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

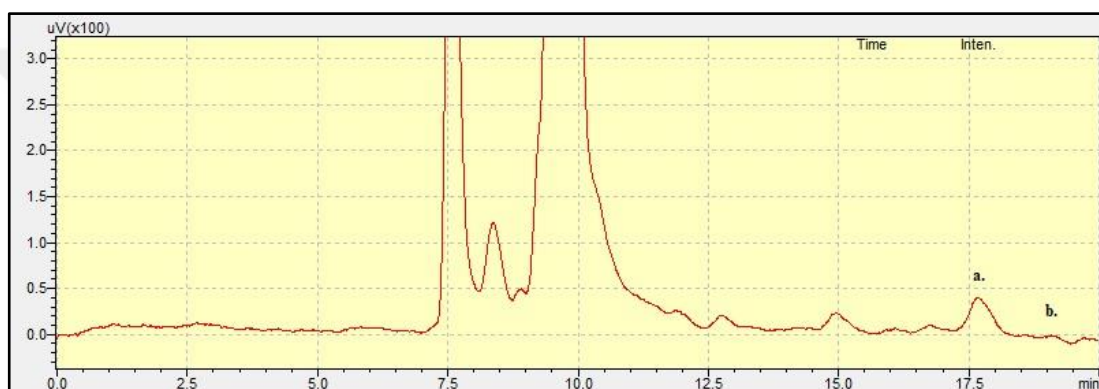


Figure 4.37: The 24th hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at pH 8.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

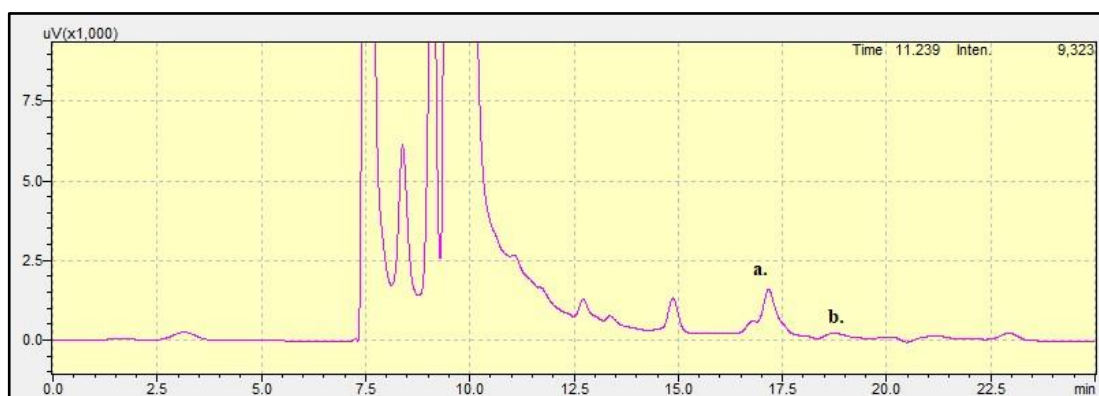


Figure 4.38: The 24th hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

Table 4.4: The production amounts and peak areas of 2,3-BDO and acetoin for the wild-type *S. coelicolor* M145 grown in TSB+10% glucose at pH 5.0, pH 6.0, pH 7.0, pH 8.0 and pH 9.0. Each peak area represents sample means of three biological replications.

Time pH	2,3-BDO Peak Area 0.2 mg/mL=98871					Acetoin Peak Area 4.5 mg/mL=251363				
	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:5.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:6.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:7.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:8.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:9.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:5.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:6.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:7.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:8.0	<i>Streptomyces</i> grown in TSB+%10 glucose at pH:9.0
24 th hour	6520= 0.013 mg/mL	8104= 0.016 mg/mL	13512= 0.028 mg/mL	6102= 0.012 mg/mL	5612= 0.011 mg/mL	80032= 1.43 mg/mL	59041= 1.06 mg/mL	70384= 1.26 mg/mL	70850= 1.27 mg/mL	62120= 1.11 mg/mL
48 th hour	7912= 0.016 mg/mL	8371= 0.016 mg/mL	16468= 0.033 mg/mL	6095= 0.012 mg/mL	6118= 0.012 mg/mL	78540= 1.41 mg/mL	66428= 1.19 mg/mL	64186= 1.16 mg/mL	85475= 1.53 mg/mL	79040= 1.42 mg/mL
72 nd hour	6392= 0.013 mg/mL	8728= 0.017 mg/mL	13120= 0.028 mg/mL	6760= 0.014 mg/mL	6620= 0.013 mg/mL	82984= 1.46 mg/mL	68962= 1.23 mg/mL	66040= 1.18 mg/mL	93665= 1.68 mg/mL	84659= 1.52 mg/mL
96 th hour	7800= 0.016 mg/mL	9391= 0.019 mg/mL	9800= 0.012 mg/mL	5230= 0.010 mg/mL	6512= 0.013 mg/mL	88672= 1.59 mg/mL	69178= 1.23 mg/mL	66803= 1.18 mg/mL	105820= 1.89 mg/mL	86515= 1.55 mg/mL
120 th hour	9008= 0.018 mg/mL	9510= 0.019 mg/mL	13779= 0.028 mg/mL	5323= 0.010 mg/mL	7112= 0.015 mg/mL	63752= 1.41 mg/mL	72653= 1.30 mg/mL	59353= 1.08 mg/mL	68510= 1.22 mg/mL	93535= 1.67 mg/mL

Table 4.5: The production amounts and peak areas of 2,3-BDO and acetoin for the recombinant *S. coelicolor* (pRA/*glcP1*) strain grown in TSB+10% glucose at pH 5.0, pH 6.0, pH 7.0, pH 8.0 and pH 9.0. Each peak area represents sample means of three biological replications.

Time pH	2,3-BDO Peak Area 0.2 mg/mL=98871					Acetoin Peak Area 4.5 mg/mL=251363				
	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:5.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:6.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:7.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:8.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:9.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:5.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:6.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:7.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:8.0	Recombinant <i>Streptomyces</i> grown in TSB+%10 glucose at pH:9.0
24 th hour	7091= 0.014 mg/mL	7225= 0.015 mg/mL	14324= 0.029 mg/mL	6200= 0.013 mg/mL	6450= 0.013 mg/mL	82348= 1.47 mg/mL	62608= 1.12 mg/mL	39012= 0.70 mg/mL	78080= 1.40 mg/mL	53200= 0.95 mg/mL
48 th hour	7362= 0.015 mg/mL	7436= 0.015 mg/mL	15932= 0.031 mg/mL	7380= 0.015 mg/mL	8422= 0.017 mg/mL	84705= 1.52 mg/mL	65264= 1.17 mg/mL	51400= 0.91 mg/mL	84360= 1.51 mg/mL	62080= 1.11 mg/mL
72 nd hour	7592= 0.017 mg/mL	7659= 0.015 mg/mL	17041= 0.025 mg/mL	6800= 0.014 mg/mL	8160= 0.016 mg/mL	89309= 1.60 mg/mL	65340= 1.17 mg/mL	53440= 0.95 mg/mL	84000= 1.51 mg/mL	76115= 1.36 mg/mL
96 th hour	8311= 0.017 mg/mL	7250= 0.015 mg/mL	16300= 0.033 mg/mL	7420= 0.015 mg/mL	7410= 0.015 mg/mL	73327= 1.31 mg/mL	66906= 1.20 mg/mL	57112= 1.02 mg/mL	81600= 1.46 mg/mL	88920= 1.59 mg/mL
120 th hour	8704= 0.018 mg/mL	7530= 0.015 mg/mL	14034= 0.029 mg/mL	6227= 0.013 mg/mL	6565= 0.014 mg/mL	75976= 1.36 mg/mL	66913= 1.20 mg/mL	50013= 0.90 mg/mL	79200= 1.41 mg/mL	92950= 1.66 mg/mL

4.4. Production of 2,3-BDO in Wild-Type and Recombinant *S. coelicolor* under Optimum Conditions

Following the determination of the optimum medium, temperature and pH for 2,3-BDO production, a fermentation experiment was conducted under these conditions. Both wild-type and recombinant *Streptomyces* were grown in TSB+10% glucose medium at 30°C and pH 7.0. Chromatograms of both wild-type (Figure 4.39) and recombinant *S. coelicolor* (Figure 4.40) grown in all optimum conditions were obtained. In order to understand whether there is a significant change in the production of 2,3-BDO and acetoin between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown in pH:7.0, TSB+%10 glucose media at 30°C, statistical analyzes were performed using the Two-way ANOVA test. According to this test, the mean of three biological replicates of each sample was measured. Samples collected at the same time were then compared with each other (Figure 4.41). The chromatogram results indicate under optimum conditions, the *S. coelicolor* M145 strain produced the highest amount of 2,3-BDO at the 48th hour of the fermentation, with a concentration of 0.033 mg/mL (Figure 4.42 and Table 4.5). Additionally, the highest concentration of acetoin was observed at the 24th hour of the fermentation, with a concentration of 1.32 mg/mL (Figure 4.43 and Table 4.6). In contrast, the recombinant *S. coelicolor* produced the highest levels of 2,3-BDO at the 72nd hour of the fermentation, with a concentration of 0.035 mg/mL (Figure 4.44 and Table 4.5) and the highest amount of acetoin at the 96th hour of the fermentation, with a concentration of 1.02 mg/mL (Figure 4.45 and Table 4.6).

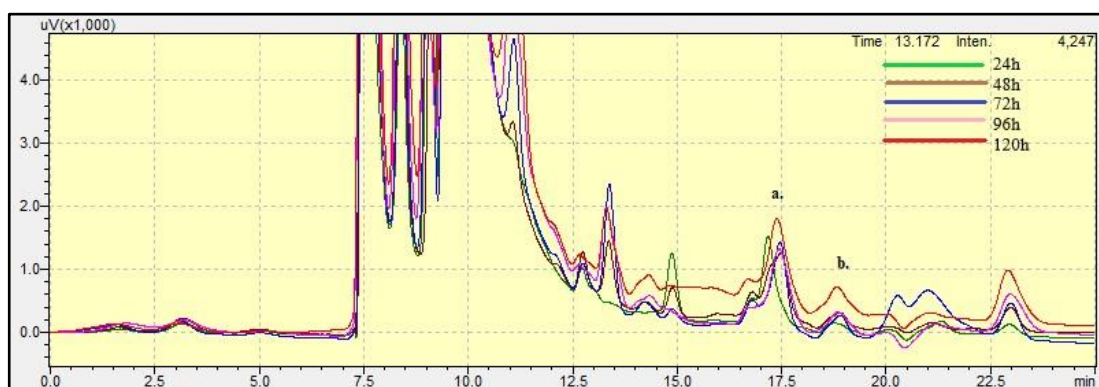


Figure 4.39: Chromatograms of wild-type *S. coelicolor* grown in TSB+10% glucose at 30°C and pH 7.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

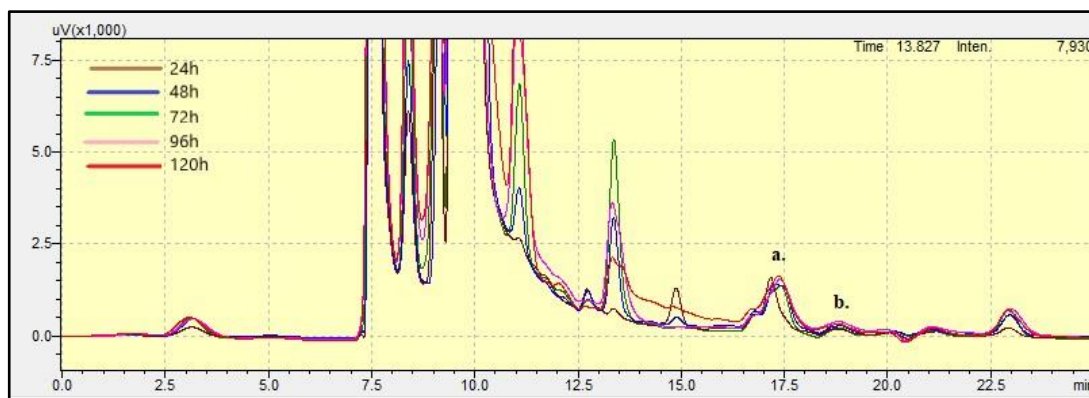


Figure 4.40: Chromatographs of wild-type *S. coelicolor* grown in TSB+10% glucose at 30°C and pH 7.0. Samples were taken in every 24 hour. “a” indicates acetoin peak and “b” indicates 2,3-BDO peak.

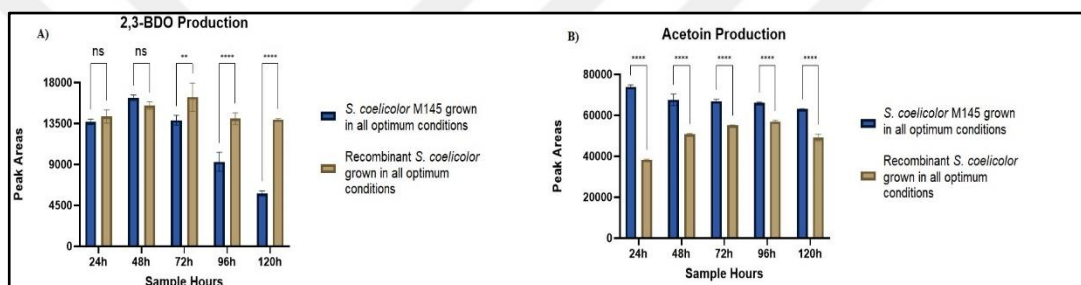


Figure 4.41: Two-way ANOVA test (drawn with GraphPad) of *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown in pH:7.0, TSB+%10 glucose at 30°C. Each bar represents sample means of three biological replicates. A) 2,3-BDO production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown in all optimum conditions (** $p=0.0017$, **** $p<0.0001$, ns: non-significant). B) Acetoin production differences between *S. coelicolor* M145 samples and recombinant *S. coelicolor* samples grown in all optimum conditions (**** $p<0.0001$).



Figure 4.42: The 48th hour chromatogram of *S. coelicolor* M145 grown in TSB+10% glucose at 30°C and pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.43: The 24th hour chromatogram of *S. coelicolor* M145 grown in TSB+10% glucose at 30°C and pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.



Figure 4.44: The 72nd hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at 30°C and pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

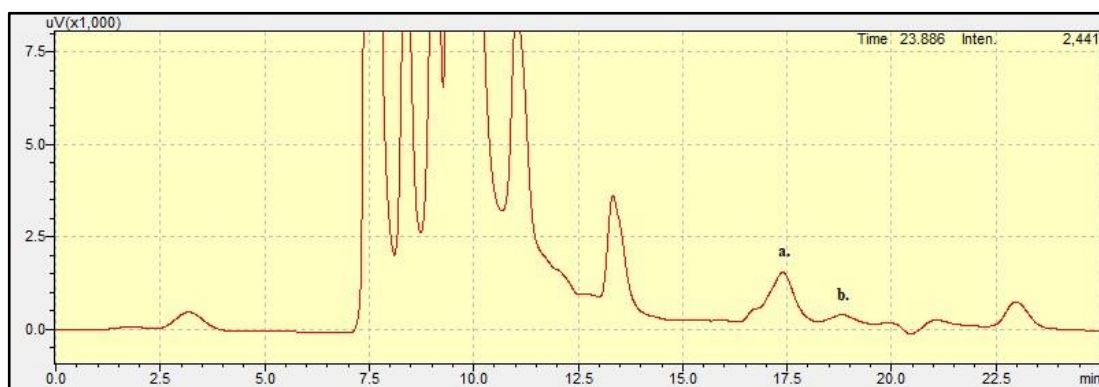


Figure 4.45: The 96th hour chromatogram of recombinant *S. coelicolor* (pRA/glcP1) grown in TSB+10% glucose at 30°C and pH 7.0, “a” indicates acetoin and “b” indicates 2,3-BDO.

Table 4.6: The production amounts and peak areas of 2,3-BDO and acetoin for the wild-type and recombinant *S. coelicolor* strains grown in TSB+10% glucose medium at 30°C and pH 7.0. Each peak area represents sample means of three biological replications.

		2,3-BDO Peak Area 0.2 mg/mL=98871		Acetoin Peak Area 4.5 mg/mL=251363	
Time	Optimum growth conditions	<i>S.coelicolor</i> M145 strain grown in pH 7.0 TSB+%10 glucose at 30°C	Recombinant <i>S.coelicolor</i> strain grown in pH 7.0 TSB+%10 glucose at 30°C	<i>S.coelicolor</i> M145 strain grown in pH 7.0 TSB+%10 glucose at 30°C	Recombinant <i>S.coelicolor</i> strain grown in pH 7.0 TSB+%10 glucose at 30°C
24 th hour		14009 = 0.029 mg/mL	14986 = 0.030 mg/mL	74829 = 1.32 mg/mL	38476 = 0.69 mg/mL
48 th hour		16623 = 0.033 mg/mL	15739 = 0.031 mg/mL	69329 = 1.24 mg/mL	51040 = 0.91 mg/mL
72 nd hour		14312 = 0.030 mg/mL	17528 = 0.035 mg/mL	67952 = 1.19 mg/mL	55229 = 0.97 mg/mL
96 th hour		9963 = 0.020 mg/mL	14568 = 0.030 mg/mL	66359 = 1.18 mg/mL	57400 = 1.02 mg/mL
120 th hour		6012 = 0.011 mg/mL	14002 = 0.029 mg/mL	63205 = 1.16 mg/mL	50086 = 1.18 mg/mL

5. DISCUSSION

A great deal of work has been done on the secondary metabolism of *Streptomyces*, as they can produce metabolites that are used in many important areas, such as medical and industrial biotechnology. There have been a few studies on the primary metabolism of *Streptomyces* so far and an example of these studies is the production of “butanal” using *S. coelicolor* [30], a patented work. Compounds produced by primary metabolism are generally starting materials for the production of more complex chemicals. Although they have simpler structures than secondary metabolites, they are very valuable in terms of their applications.

Raw materials of great industrial importance can be produced by bacteria. The use of bacteria as producers of valuable raw materials is becoming more and more popular instead of conventional methods, which are harmful to the environment and can be very expensive due to the use of petroleum. 2,3-BDO is another important raw material that can be produced naturally by bacteria. In our laboratory we have previously carried out a study showing that *S. coelicolor* is capable of producing 2,3-BDO. However, the amount produced was rather low [25]. Based on the previous study, fermentation optimization studies were carried out in this study to increase the production of 2,3-BDO.

The aim of the initial phase of the study was to identify the most appropriate medium for *S. coelicolor* to produce 2,3-BDO. According to the HPLC results, it was observed that *S. coelicolor* M145 produced the highest amount of 2,3-BDO at 0.033 mg/mL when grown in TSB+10% glucose medium, compared to YEME and TSB. These findings suggest that TSB+10% glucose medium is the most suitable for 2,3-BDO production by *S. coelicolor*. The data indicates that *S. coelicolor* M145 strain utilizes the extra glucose for primary metabolite production, resulting in an increase in 2,3-BDO production. In contrast, *S. coelicolor* grown in TSB produced the highest amount of acetoin at 2.09 mg/mL. Samples with additional glucose showed a decrease in acetoin and an increase in 2,3-BDO. This is because cellular enzymes can convert acetoin to 2,3-BDO. The results indicate that excess carbon sources in the environment can lead to the conversion of some of the acetoin in the cell into 2,3-BDO. Previous studies have investigated the use of glucose to enhance 2,3-BDO production [31]. Our results also indicate a slight increase in 2,3-BDO production with the uptake of

additional glucose. However, we found that the YEME medium was not suitable for the production of 2,3-BDO and acetoin. Despite containing both glucose and sucrose as carbon sources, this medium only yields a small amount of either chemical. This suggests that sucrose may not be an effective carbon source for 2,3-BDO production.

Once the optimal medium for 2,3-BDO production had been identified, we grew both the wild-type *S. coelicolor* M145 strain and the recombinant *S. coelicolor* strain (which contains an additional *glcP1* gene in its genome) at varying temperatures using the optimal medium to determine its impact on 2,3-BDO production. The results indicate that the highest 2,3-BDO production was seen in recombinant *Streptomyces* grown at 30°C with 0.035 mg/mL. It was observed that the amount of acetoin was lower and the amount of 2,3-BDO was higher in recombinant *Streptomyces* compared to wild-type *Streptomyces* grown at 30°C. Previous experiments conducted in our laboratory have demonstrated that the overexpression of the *glcP1* gene, which encodes glucose transport protein, enables recombinant *Streptomyces* to take in more glucose [25]. Recombinant *Streptomyces* was able to convert more acetoin to 2,3-BDO than wild-type *Streptomyces* due to its ability to take in more carbon sources into the cell. However when grown at 35°C, the production of 2,3-BDO remained quite low despite the high acetoin production of 2.17 mg/mL. This suggests that the 2,3-BDO dehydrogenase enzyme found in recombinant *Streptomyces* faces difficulties in converting acetoin into 2,3-BDO at this temperature. The amount of acetoin in wild-type *Streptomyces* grown at 35°C is higher than that in *Streptomyces* grown at 30°C, while the amount of 2,3-BDO is lower. However, wild-type *Streptomyces* grown at 35°C produced more 2,3-BDO from acetoin than recombinant *Streptomyces* grown at the same temperature. Both recombinant and wild-type *Streptomyces* grown at 25°C showed similar levels of acetoin and 2,3-BDO production. These results suggest that the optimal temperature for 2,3-BDO production is 30°C.

Based on pH experiments, the highest 2,3-BDO production was observed at pH 7.0 for recombinant *Streptomyces* (0.035 mg/mL) and wild-type (0.033 mg/mL). The highest amount of acetoin, 1.89 mg/mL, was observed in wild-type *Streptomyces* at pH 8.0. In addition, the highest 2,3-BDO production (0.019 mg/mL) was observed in wild-type *Streptomyces* at pH 6.0, except for pH 7.0. In previous fermentation studies with *Klebsiella* and *Bacillus*, it was observed that the production of 2,3-BDO increased as the pH was gradually lowered. It was observed that the production of acetic acid in

both organisms was switched to the production of 2,3-BDO. In this regard, it has been shown that *Klebsiella* and *Bacillus* can increase 2,3-BDO production to neutralise the acidic environment within the cell [23]. In the light of these studies and other studies conducted on *Enterobacter*, it was found that the most suitable pH range for 2,3-BDO production was 5.0-6.5 [23] [32]. However, our data did not support these findings. Although studies with *Klebsiella*, *Bacillus* and *Enterobacter* have shown that an acidic environment is more suitable for 2,3-BDO production, this situation was observed differently for *Streptomyces*. Among the pH values used in our study, the highest production was found at pH 7.0 for both wild-type and recombinant *Streptomyces*. This may be due to the fact that, *Streptomyces* does not increase 2,3-BDO production to neutralise the cellular pH, or it may increase 2,3-BDO production to neutralise cellular pH at much lower pHs. Regarding acetoin, if the cellular pH is high, acetoin is used to neutralize the cellular pH [23]. The pH optimization experiments with TSB+10% glucose at 30°C showed that the highest acetoin production was achieved at pH 8.0, with 1.89 mg/mL for wild-type *S. coelicolor*. However, the highest amount of acetoin, 2.17 mg/mL, was produced in pH 7.0 TSB+10% glucose media at 35°C for recombinant *S. coelicolor*. It was observed that, recombinant *Streptomyces* was unable to effectively convert acetoin into 2,3-BDO at 35°C, resulting in low levels of 2,3-BDO. The high amount of acetoin observed in recombinant *Streptomyces* grown at 35°C may be due to clustering within the cell. Based on our data, a fermentation process could be employed to achieve high acetoin production. This would involve growing the *Streptomyces* strains in TSB with 10% glucose at 35°C and a pH of 8.0.

The experiments showed that the optimal medium and conditions for producing 2,3-BDO by wild-type and recombinant *Streptomyces* were found to be TSB+10% glucose, pH 7.0, and 30°C. The maximum amount of 2,3-BDO that could be produced was found to be 0.033 mg/mL for *S. coelicolor* M145 and 0.035 mg/mL for recombinant *S. coelicolor*.

After optimizing the fermentation process, it was observed that the production of 2,3-BDO did not reach high levels, especially when compared to *Klebsiella* and *Bacillus* species which produced 2.1 g/L and 2.81 g/L, respectively. The maximum 2,3-BDO production achieved was quite low, at 0.035 g/L for recombinant *Streptomyces* and 0.033 g/L for wild-type *Streptomyces*. The addition of 10% glucose to the TSB increased the amount of 2,3-BDO from 0.024 mg/mL to 0.033 mg/mL. Based on these

results, addition of 10% extra glucose to the medium increased 2,3-BDO production by 37.5%. To further investigate the effect of high amounts of additional glucose on the production of 2,3-BDO further, future studies can conduct different percentage glucose assays.

Cost reduction is a crucial requirement for transitioning to bacterial production of 2,3-BDO. *Streptomyces*, commonly found in soil environments, can degrade plant and animal materials such as chitin and cellulose, which serve as alternative carbon sources [11]. Chitin and cellulose are two of the most abundant wastes in nature. Using these materials can meet the carbon source requirements of *Streptomyces* without additional cost and significantly reduce the production cost of important industrial products such as 2,3-BDO. Future studies can test other carbon sources, such as molasses, to determine their effects on 2,3-BDO production in *Streptomyces*. Previous studies have shown that *Enterobacter* can produce high amounts of 2,3-BDO, up to 10.24 g/L, using lignocellulosic waste [34]. Therefore, future studies could investigate the potential of *Streptomyces* to produce 2,3-BDO using waste materials as a carbon source. To improve the production of 2,3-BDO, future genetic experiments can be conducted to convert the accumulated acetoin in the cell into 2,3-BDO. This can be achieved by overexpressing 2,3-BDO dehydrogenase enzyme gene or by directing the the majority of the carbon source towards butanoate metabolism.

The study identified the optimal medium, temperature, and pH values for the production of 2,3-BDO by *Streptomyces*. *Streptomyces* has the potential to become a significant primary metabolite producer strain in the future. The data obtained in this study will provide valuable information for future research, particularly on 2,3-BDO production. This includes the parameters that can affect primary metabolite production and how they impact the production of primary metabolites by *Streptomyces*.

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