Kaunas University of Technology

Faculty of Chemical Technology

Characterization and Application of Inducible Gene Expression System for Glycolic and Lactic Acids Quantification

Master's Final Degree Project

Samanta Šimaitytė

Project author

Prof. Naglis Malys

Supervisor

Kaunas, 2021

Kaunas University of Technology

Faculty of Chemical Technology

Characterization and Application of Inducible Gene Expression System for Glycolic and Lactic Acids Quantification

Master's Final Degree Project

Samanta Šimaitytė

Project author

Prof. Naglis Malys

Supervisor

Ernesta Augustinienė

Reviewer

Kaunas, 2021

Kaunas University of Technology

Faculty of Chemical Technology

Samanta Šimaitytė

Characterization and Application of Inducible Gene Expression System for Glycolic and Lactic Acids Quantification

Declaration of Academic Integrity

I confirm that the final project of mine, Samanta Šimaitytė, on the topic „Characterisation and Application of Inducible Gene Expression System for Glycolic and Lactic Acids Quantification“ is written completely by myself; all the provided data and research results are correct and have been obtained honestly. None of the parts of this thesis have been plagiarised from any printed, Internet-based or otherwise recorded sources. All direct and indirect quotations from external resources are indicated in the list of references. No monetary funds (unless required by Law) have been paid to anyone for any contribution to this project.

I fully and completely understand that any discovery of any manifestations/case/facts of dishonesty inevitably results in me incurring a penalty according to the procedure(s) effective at Kaunas University of Technology.

Samanta Šimaitytė

(name and surname)

Šimaitytė, Samanta. Characterization and Application of Inducible Gene Expression System for Glycolic and Lactic Acids Quantification. Master's Final Degree Project / supervisor Prof. Naglis Malys; Faculty of Chemical Technology, Kaunas University of Technology.

Study field and area (study field group): Biotechnology, Technological sciences.

Keywords: glycolic acid, lactic acid, genetic engineering, biosensor, *Escherichia coli*, *Pseudomonas putida*.

Kaunas, 2021. 64.

Summary

Nowadays the lactic acid is one of the most commercially utilized chemical compounds as it finds application in food, pharmaceutical, cosmeceutical and chemistry industries. The lactic acid is building block for one of the widely manufactured polimers, a polylactic acid (PLA) that can be used for developing various packaging materials, which are currently made from petrochemicals and are harmfull to the environment. In order to improve today's materials and to encourage industries to become greener, biobased strategies for production of lactic acid and structurally similar compounds are being developed. One of the lactic acid related compounds is glycolic acid that could be potential platform chemical to replace the PLA with a polyglycolic acid (PGA) or both, lactic and glycolic acid, can be combained into one polimer to develop environment friendly materials for various packaging solutions. Since microorganisms do not naturally produce glycolic acid under typical growth conditions, its biotechnological production requires metabolic engineering. To facilitate improvement of genetically engineered microorganisms or to examine their cultivation in industrial conditions, high-throughput analytical methods and techniques are required. Transcription factor-based biosensors are one of such possible tools, which could help to investigate microorganisms in laboratory in efficient way. They can be used in various applications including, for example, adaptive evolution or dynamic pathway control, to customize microorganism strains to industrial needs and to perform a real-time monitoring of chemical production in the high-throughput mode.

There has been no transcription factor-based biosensor responding to the glycolic acid developed so far. Therefore, the aim of this reseach project was to characterise and use glycolic acid-inducable gene expresion system for developing a whole-cell biosensor. To this end, several plasmids with inducable system variants were constructed and investigated. To better understand and identify genetic elements and genes involved in the glycolic acid catabolism, bioinformatics analyses were applied. Using information obtained by bioinformatics seraches and through the literature review, Glc operon was identified in *E. coli* MG1655 and *P. putida* KT2440 strains. Corresponding intergenic regions containing promoters and transcription regulator binding sequences, as well as genes encoding transcription regulator were determined and used for engineering the whole-cell biosensors. Subsequently, three different transcription factor-based biosensors where developed and evaluated. Whole-cell biosensor-based on pSS003 construct containg only intergenic regiono of *glcC/glcD* revealed the highest fold induction amongst all three biosensors. Whole-cell biosensor-based on pSS004 construct showed the strongest relative normalized fluorescence response to the glycolic acid. Whereas, the addition of synthehic P_{13} promoter to increase the transcription regulator expression did not improve characteristics of whole cell biosensor containing pSS004A construct. All three whole-cell biosensors were specific to glycolic acid and showed a minor response to D- lactic and 3-hydroxy propionic acids. However, they should be further improved to meat research and industrial application needs.

Šimaitytė Samanta. Indukuojamos genų ekspresijos sistemos apibūdinimas ir taikymas glikolio ir pieno rūgščių kiekybiniam įvertinimui. Magistro baigiamasis projektas / vadovas Prof. Naglis Malys; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

Studijų kryptis ir sritis (studijų krypčių grupė): Biotechnologija, Technologijų mokslai.

Reikšminiai žodžiai: glikolinė rūgštis, pieno rūgštis, genų inžinerija, biosensorius, *Escherichia coli*, *Pseudomonas putida*.

Kaunas, 2021. 64.

Santrauka

Pieno rūgštis yra viena iš labiausiai naudojamų rūgšių. Jos panaudojimo galimybės apima tokias pramonės sritis kaip farmacijos, kosmetikos, maisto ir chemijos. Vienas iš plačiai naudojamų junginių, gaunamų iš pieno rūgšties, yra poli-pieno rūšgtis, iš kurios yra gaminamos įvairios pakavimo medžiagos ir plėvelės. Šiomis dienomis kyla daug problemų su šios poli-pieno rūgšties naudojimu, nes ji nėra ekologiška ir „draugiška" aplinkai, todėl siekiant skatinti pramonę puoselėti žaliasias įdėjas, yra tiriamos strukūriškai panašios rūgštys, siekiant atrasti ekologiškešnes alternatyvas. Viena iš tokių struktūriškai panašių rūgšių yra glikolinė rūgštis, iš kurios gali būti gaminama poli-glikolinė rūgštis, kuri pasižymi panašiomis sąvybėmis į poli-pieno rūgštį, tačiau yra greičiau yranti medžiaga. Taip pat, poli-pieno ir poli-glikolinė rūgštys gali būti maišomos tarpusavyje, taip vystant ekologiškesnes technologijas pritaikomas pramoniniu mąstu. Mikroorganizmai natūraliai negamina glikolinės rūgšties esant normalioms aplinkos sąlygoms, todėl norint gauti glikolię rūgštį pramoniniu mąstu, tokie mikroorganizmai turi būti genetiškai modifikuoti. Siekiant pagerinti modifikuotus mikroorganizmus ir ištirti jų pritaikymo galimybes pramoninėmis sąlygomis, taikomi įvairūs tam skirti metodai. Vienas iš tokių įrankių galėtų būti biosensorius paremtas indukuojamos genų ekspresijos sistemos veikimo modeliu. Tai gana naujas ir dabar plačiai vystomas įrankis. Toks biosensorius galėtų būti pritaikomas nuo evoliucijos ir dinaminių kelių tyrimų laboratorijoje iki realaus laiko monitoringo ir glikolinės rūgšties koncentracijų matavimo pramoniniu lygmeniu, o tai būtų galima daryti dar greičiau nei naudojant įprastus metodus, tokius kaip HPLC.

Šiuo metu dar nėra aprašyto genų ekspresijos sistemos veikimo modeliu paremto biosensoriaus, kuris reaguotų į glikolinę rūgštį, tad šio tiriamojo projekto metu, genų inžinerijos pagalba, buvo sukonstruoti trys tokiu principu veikiantys biosensoriai. Norint geriau suprasti patį glc operoną ir jo veikimo galimą principinę schemą, buvo atlikta bioinformatinė analizė. Glc operonas buvo analizuotas *E. coli* MG1655 ir *P. putida* KT2440 mikroorganizmuose, pasitelkiant bioinformatinės analizės įrankius, o atrastų tarpgeninės sekos ir transkripcijos faktoriaus DNR buvo panaudoti konstruojant vektorius, indukuojamus glikoline rūgštimi. Pasitelkiant informaciją apie glc operoną, išanalizuotą literatūrinės analizės metu ir sintetinės molekulinės biologijos žinias, buvo surinkti trys skirtingi biosensoriai, o jų veikimas ištirtas. Biosensorius turintis pSS003 vektorių, kuriame yra tik tarpgeninė seka, tarp *glcC/glcD* genų, pasižymėjo didžiausiu dinaminiu diapazonu tarp visų trijų sukonstruotų biosensorių. Biosensorius turintis pSS004 vektorių, kuriame yra ne tik tarpgeninė seka, bet ir transkripcijos faktorius, išsiskyrė dižiausiu sąlyginiu normalizuotu fluorescencijos atsaku į glikolinę rūgštį. Biosensorius turintis patobulintą pSS004A vektorių, kuriame yra integruota ne tik tarpgeninė seka, transkripcijos faktorius, bet ir stipresnis papildomas P_{13} promotorius, teikė daugiausiai vilčių šiuose tyrimuose, nes buvo tikėtasi, pagerinti šio biosensoriaus veiklą ir atsaką į

glikolinę rūgštį, tačiau šis biosensorius pasižymėjo mažiausiu dinaminiu diapazonu, o sąlyginis normalizuotas fluorescencijos atsakas į glikolinę rūgštį buvo vidutinis, lyginant tarp visų tirtų biosensorių. Toks pSS004A biosensoriaus atsakas leido suformuluoti prielaidą, jog didelis transkripcijos faktoriaus kiekis ląstelėje daro neigiamą įtaką jos veiklai. Kaip parodė atlikti tyrimai, visi sukonstruoti biosensoriai buvo specifiški glikolinei rūšgčiai, o tai atskleidė potencialą toliau tirti ir tobulinti šiuos biosensorius ir , tikėtina, pritaikyti ne tik laboratoriniam naudojimui, bet ir pramoniniu mąstu.

Table of contents

List of abbreviations and terms

Abbreviations:

ATP - adenosine triphosphate

BLAST – basic local alignment search tool

DNA – deoxyribonucleic acid

gDNA – genomic DNA

FACS - fluorescence-activated cell sorting

GS – glyoxylate shunt

HPLC – high-performance liquid chromatography

LAB – lactic acid bacteria

NADH - nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (reduced form of nicotinamide adenine dinucleotide)

NCBI – national center for biotechnology information

PCR – polymerase chain reaction

PLA - poly lactic acid

PGA – poly glycolic acid

RFP – red fluorescent protein

RNA - ribonucleic acid

TCA cycle - tricarboxylic acid cycle

TF – transcription factor

Terms:

Plasmid - a small, often circular DNA molecule found in bacteria which is separate from the bacterial chromosome and replicate independently of it. Plasmid carry only a small number of genes, some of them are associated with antibiotic resistance.

Vector – a DNA molecule, equivalent to the plasmid, used for molecular cloning, which has been genetically enginnered using foreign DNA.

Whole-cell biosensor – competent cells containing transformed vector.

Introduction

Lactic acid is one of the most commercially useful acids and its applications range from mass production products, particularly polylactic acid (PLA); cheap materials, such as starchy and cellulosic materials, and renewable materials, which can be used as raw materials for lactic acid production, from the food industry to the pharmaceutical industry. Other structurally similar compounds are being used in many related areas as well as lactic acid and even in composition with lactic acid. One such acid is glycolic acid. Microorganisms do not naturally produce glycolic acid under typical growth conditions so their biotechnological production requires metabolic engineering, not like lactic acid-producing microorganisms. Polymerization of glycolic acid alone and with other acids, for example with lactic acid, can be suitable material for thermoplastic, packaging material, or even sutures, because of their capability of being hydrolyzed in aqueous environments. Polyglycolic acid (PGA) has a structure similar to PLA, so it has promising characteristics such as good biodegradability and barrier properties, which is potentially a beneficial supplement to PLA. By developing the production and compounding technology, PGA can be combined with PLA to play an essential role in the sustainable and environmentally friendly plastic industry, especially for single-used products requiring fast degradation at room temperature or in the natural environment.

As demand grows for various chemical compounds and genetic engineering becoming a more and more desirable tool in various industries, the production of lactic acid and other structurally related compounds needs to be optimized as much as possible to get a greater profit. For this purpose, the inducible gene expression systems could be used. Such inducible gene expression systems are called transcription factor-based biosensors and nowadays scientists are widely investigating such biosensors and trying to apply them to industrial needs. A transcription factor-based biosensors can detect amino or other acids, some secondary metabolites, measure their concentration, monitor and even regulate productivity of cellular metabolism. It is a very sensitive and specific tool, which produces results faster than HPLC, titration, or electrophoresis and according to database BiosensorDB.ucsd.edu, there are more than 750 different fluorescent biosensors listed.

The aim of this project was the identification and characterization of lactic acid or structurally similar compound such as glycolic acid-inducible gene expression system. To achieve this aim, the research work was based on the following six objectives:

1. Perform bioinformatics analysis to identify lactic or/and glycolic acid-inducible systems and analyze their gene sequences.

2. Using genetic elements of the inducible system and *E. coli*, develop the whole-cell biosensor responsive to a lactic or/and glycolic acid.

3. Evaluate the dose-response and dynamic range of the whole-cell biosensor.

4. Evaluate the performance of the biosensor in the presence of different carbon sources such as L- and D- lactic, glyoxylic, and glycolic acids.

5. Perform specificity analyses of the biosensor using compounds structurally similar to lactic acid.

6. Improve performance of the whole-cell biosensor by changing the strength of promoter that controls transcription factor glcC gene expression.

1. Literature review

1.1. History and properties of lactic acid

Lactic acid is an organic acid (CH3CH(OH)COOH) that was discovered and isolated from sour milk in 1780 by Carl Wilhelm Scheele. In Latin word lac means milk. Later, in 1808 Jöns Jacob Berzelius discovered lactic acid (L-lactate) in muscles during exertion, but only in 1873, its structure was established by Johannes Wislicenus. Louis Pasteur discovered the role of Lactobacillus in the synthesis of lactic acid in 1856 and this pathway was used commercially in 1895.

Lactic acid (2-Hydroxypropanoic acid by IUPAC) is widespread in nature. It is white in a solid state but it forms a colorless solution in a liquid state. Lactic acid is the simplest hydroxycarboxylic acid which has a chiral carbon atom and consists of two enantiomers. Enantiomers are known as L-(+)-lactic acid or (S)-lactic acid and its mirror image D-(−)-lactic acid or (R)-lactic acid (Fig. 1). Lactic acid with equal amounts of enantiomers is called racemic lactic acid (DL – lactic acid) [1].

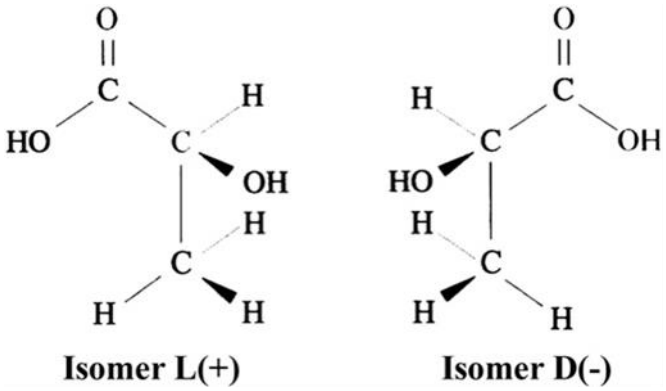


Fig. 1.1 Structure of enantiomers of lactic acid

Lactic acid is characterized by physicochemical properties such as bifunctional reactivity associated with the presence of a hydroxyl and carboxyl group, because of these groups lactic acid has great reaction versatility. Also, Lactic acid has an asymmetric optical activity of C2 and has acidic properties in an aqueous medium [1].

Lactic acid has many different properties useful in the industry. It is biodegradable, so it can be used as a material for packaging and labeling [2]. Its biocompatibility is useful in sutures, internal drug dosing, and prosthetic devices [1]. Lactic acid has antimicrobial, moisturizing, and rejuvenating effects, so it is used in the cosmetic industry [3]. The biggest amount of produced lactic acid is used in the food industry, in the production of cheese, yogurt, and other sour products.

1.2. Application and importance of lactic acid

Lactic acid is one of the most commercially useful acids. These hydroxycarboxylic acid applications range from mass production products, like PLA (polylactic acid), cheap materials, such as starchy and cellulosic materials, and renewable materials, which can be used as raw materials for lactic acid production, from the food industry to the pharmaceutical industry [4] Main applications in various fields of industry summarizes Table 1.1.

Table 1.1. Applications of lactic acid in different fields of industry.

Field	Applications
Food industry	Preservatives, acidulants, pH regulators, mineral fortification, bacterial inhibition
Cosmeceutical industry	Moisturizers, anti-acne agents, humectants, anti-tarter agents, pH regulators, skin-lightening agents
Pharmaceutical industry	Dialysis solution, mineral preparations, prostheses, surgical sutures, controlled drug delivery systems, parenteral/intravenous solution
Chemical industry	Neutralizers, chiral intermediates, green solvents, cleaning agents, descaling agents, pH regulators
PLA	Food containers, protective clothing, trach bags, rigid containers

1.2.1. Food Industry

Approximately 70% of the demand for lactic acid is used in food and food-related industries. Ant the rest of lactic acid is used in non-food industrial applications. In contrast with other acids used in food, lactic acid has a mild acidic taste, it is nonvolatile and odorless. It also has good properties as a preservative and pickling agent for olives, vegetables, and sauerkraut. Lactic acid is used as an acidulant, flavoring, pH buffering agent, or inhibitor of bacterial spoilage in various processed foods such as bread and other bakery products, drinks, soups, dairy products, candies, sherbets, jams, beer, processed eggs, mayonnaise, and many other processed food products. It can be used in conjunction with other acidulants. Lactic acid and its salts are also used in disinfection and when the packaging of fish and poultry. The addition of an aqueous solution of lactic acid and its salts during the processing of this food reduces the growth of anaerobic spoilage bacteria, such as Clostridium botulinum. More than 50% of food-related lactic acid is used to produce emulsifying agents, especially for a bakery. These emulsifying agents must be manufactured from heat-stable lactic acid [5], [6]. Lactic acid is also very important and takes a big part in the food industry because of yogurt and cheese production, it is the main product of Streptococcus thermophilus and Lactobacillus bulgaricus co-fermentation [7].

1.2.2. Cosmeceutical Industry

Lactic acid can be a natural ingredient in cosmetics. It has been used as a pH regulator, but also, it has other useful features, such as skin lightening, antimicrobial activity, and skin hydration. The moisturizing efficiency appears because of lactate moisture-detained capability. Lactic acid plays role in skin-lightening because it was produced by the repression of the formation of tyrosine. Because lactic acid is a natural constituent in the human body, so it also produces a good effect for skin renewal and illumination [8]. Lactic acid is useful in various types of moisturizers. It is mild enough for all skin types, so it can be used from sensitive to oily skin moisturizers, even in moisturizers from acne and rosacea. In some of the moisturizers, lactic acid promotes collagen production, which helps firm the skin, so it is an important ingredient in anti-aging moisturizers. A strong lactic acid lotion has a mild peeling effect, so such peeling removes damaged tissue. The lactic acid in cosmetics has fewer risks and side effects, but it takes longer to get wanted results on human skin [9], [10].

1.2.3. Pharmaceutical Industry

The lactic acid in pharmaceutics is also an important ingredient. Manufacturers can use just L(+) isomer of lactic acid because the D(-) isomer is not metabolized in the human body. Lactic acid also has many different applications reported in the literature. Iron, sodium, and calcium salts of lactic acid are used in several drug formulas because of their anti-tumor activity. Lactic acid can supply energy and volume for blood and regulate pH. 22-24 Applications of lactic acid in the pharmaceutical industry can be categorized in Table 1.2. [11]

Table 1.2. Applications of lactic acid in the pharmaceutical industry

Category	**Applications**	**Products**
Parenteral/I.V (intravenous solutions)	Used as an electrolyte	Dialysis, Lactated Ringer's, solutions
Dialysis solution	Dialysate fluid	CAPD (Continuous Ambulatory Peritoneal Dialysis
Controlled drug delivery system	Implantable drug delivery	Lactide-glycolide copolymers, polylactic acid (PLA)
Mineral lactate formulations for diseases	Anemia, hypertension, osteoporosis treatment	mineral lactates: ferrous lactate calcium lactate, manganese lactate, magnesium lactate zinc lactate
Chiral synthesis	Products with any desired stereochemistry	R and S isomers

1.2.4. Chemical Industry

Lactic acid is ubiquitous in nature so it has been producing as a fermentation by-product in many industries. Some of them use carbohydrate feedstock and fermentation technology to get lactic acid. Other industries use chemical technology. Such lactic acid is used in plastics as a monomer or as an intermediate in the synthesis of high-volume oxygenated chemicals. In the last decade, the production of lactic acid was growing and the production technology is mostly based on carbohydrate fermentation. Lactic acid is also used to make high molecular weight polymers of lactic acid (PLA), which are biodegradable thermoplastics. Solvents from lactic acid derivatives, such as ethyl, propyl, or butyl lactate are called environmentally friendly are used in electronics. Also, US Environmental Protection Agency classified lactate esters as an inert ingredient for use in the formulation of pesticides or other bioactive compounds. Large-volume oxygenated chemicals (propylene glycol, propylene oxide, acrylic acid, acrylate esters, lactate ester plasticizers can potentially be made from lactic acid as well [12].

1.3. Production of lactic acid

1.3.1. Fermentation by bacteria

Lactic acid bacteria are named so, because their major, and sometimes the only, product of sugar fermentation is lactic acid. Lactic acid bacteria also have enzymes for aerobic respiration but do not synthesize heme. So during fermentation, heme is added to the culture medium, because without it respiration chain is non-functional. Most lactic acid bacteria are immobile, catalase-negative, do not

form spores and their optimum growth temperature is between 20 and 45 °C. They also have a high tolerance to acidic conditions, when pH<5, so they are better to adapt than other bacteria and have an advantage over it [13]. Most of the production of lactic acid is from microbial fermentation because it has many advantages compared with chemical synthesis, it enables the production of pure isomers and the use of renewable resources as substrates. There are various microorganisms and materials used in the production of lactic acid, some of them are in Table 1.3.

Table 1.3. Microorganisms and materials used in the production of lactic acid.

Microorganism	**Material**	**Carbon source**
L.casei	Molasses	Saccharose
L. delbrueckii	Cow milk	Lactose
L. acidophilus	Whey	Lactose
L. amylophilus	Corn, potato, wheat	Starch
L. delbrueckii	Rice, soy fiber	Glucose
L. pentosus	Wheat straw	Xylose
L. plantarum	Tapioca, bamboo	Glucose

1.3.2. Biochemistry and metabolism of lactic acid bacteria

The largest and very diverse genus of lactic acid bacteria is Lactobacillus, which includes species with different physiological, biochemical properties and have special resistance against the acidic environment. These species have a high growth rate and productivity, so it is also very important in industrial production, where two main routes of fermentation exist [14], [15]. There can be homolactic and heterolactic fermentations. Homolactic fermentation takes place in two steps. The first step is called glycolysis or Embdene-Meyerhofe-Parnas pathway. At this step, glucose is transformed into pyruvic acid and later this is reduced to lactic acid by the reducing power previously produced in the form of NADH. Because of this, lactic acid is obtained as a sole product (Fig 1.2.) from glucose. Equation of this reaction:

$$\text{Glucose} \rightarrow 2\ \text{Lactic Acid} + 2\ \text{ATP}$$

During homolactic fermentation, theoretically, 2 mol of lactic acid per mole of consumed glucose should yield a theoretical yield of 1 g of product per g of substrate, but experimental yields are lower because some carbon is used for biomass production. Microorganisms that use this pathway are called Obligatory Homofermentative [16], [17], [18], [19].

Heterolactic fermentation process is characterized by the formation of co-products such as CO_2, ethanol, or acetic acid in addition to lactic acid (Fig. 1.2.). The first step of this process is called the pentose phosphate pathway and it leads to glyceraldehyde 3-phosphate, acetyl-phosphate, and CO_2. Glyceraldehyde 3-phosphate is transformed into lactic acid through glycolysis and also, acetyl-phosphate is converted into acetic acid or ethanol. Equations of these reactions:

$$\text{Glucose} \rightarrow \text{Lactic acid} + CO2 + \text{Ethanol} + \text{ATP}$$
$$\text{Glucose} \rightarrow \text{Lactic acid} + CO2 + \text{Acetic acid} + 2\ \text{ATP} + 2\ \text{NADH}$$

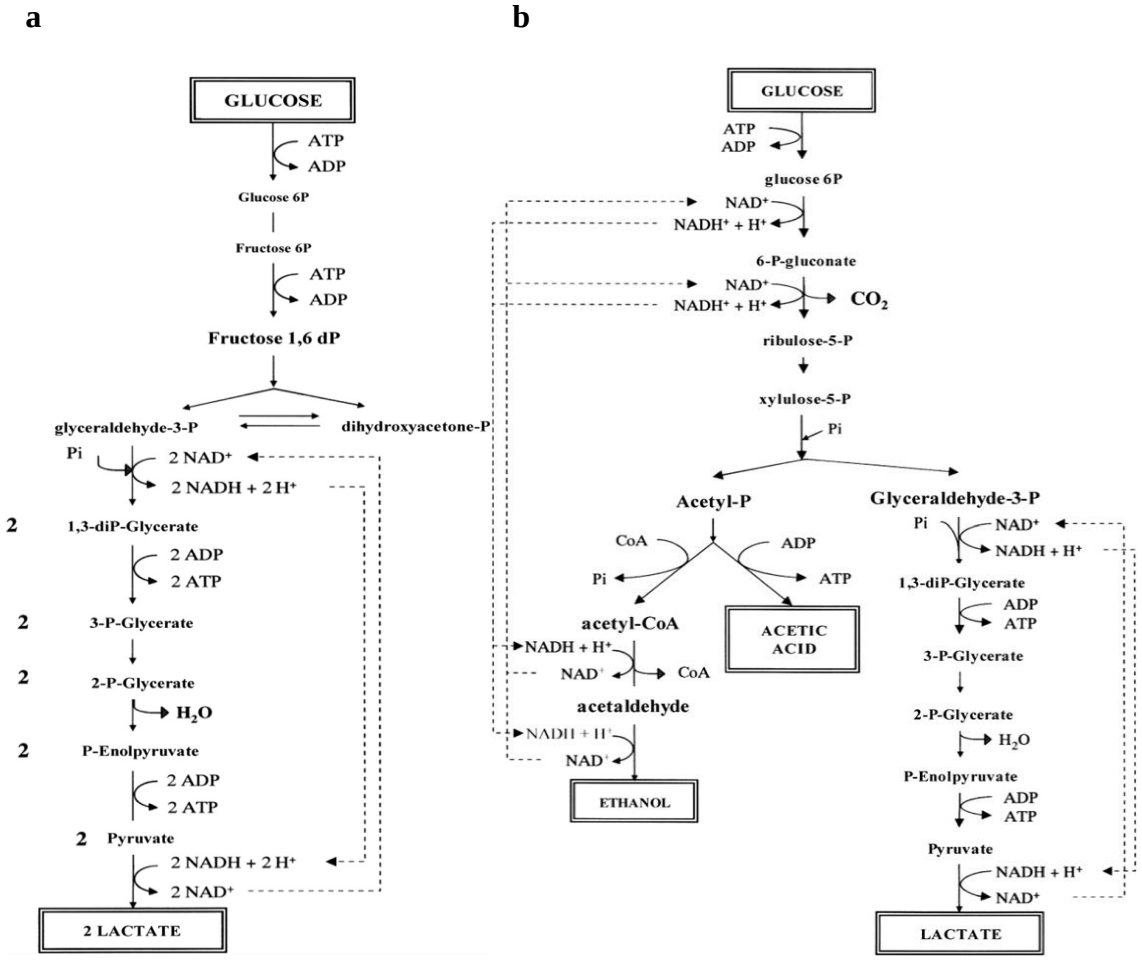


Fig. 1.2. Schemes of homofermentative and heterofermentative pathways (respectively) of glucose fermentation in lactic acid bacteria. (a) During homolactic glycolysis pathway, glucose is transformed into pyruvic acid and later this is reduced to lactic acid by the reducing power previously produced in the form of NADH. Because of this, lactic acid is obtained as a sole product from glucose. (b) During heterolactic fermentation process formation of co-products such as CO_2, ethanol, or acetic acid in addition to lactic acid occurs. Pentose phosphate pathway leads to glyceraldehyde 3-phosphate, acetyl-phosphate, and CO_2. Glyceraldehyde 3-phosphate is transformed into lactic acid through glycolysis and also, acetyl-phosphate is converted into acetic acid or ethanol. Adapted from **[1]**.

The amounts of ethanol and acetic acid depend on the ability of microorganisms to reoxidize the NADH generated in the early stages of this process along with its energy requirements. Some microorganisms use only this metabolic pathway and they are called Obligatory Heterofermentativ [18], [19], [20].

1.4. Compounds structuraly similar to lactic acid

1.4.1. Glycolic acid

1.4.1.1. Structure, biosynthesis and application

Glycolic acid (2-Hydroxyethanoic acid by IUPAC) is the smallest α-hydroxy acid (AHA) composed of alcohol and acid groups, its chemical formula C2H4O3 or HOCH2CO2H (Fig. 1.3.). Glycolic acid is colorless and odorless acid with hygroscopic properties and it is highly soluble in water.

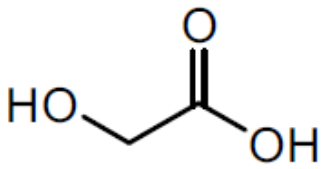


Fig. 1.3. A structure of glycolic acid

Glycolic acid is produced by some chemolithotrophic iron- and sulfur-oxidizing bacteria [21] and by some Alcaligenes sp. from glycolonitrile by hydrolyzation by nitrilase enzyme [22]. Glycolic acid can also be produced from ethylene glycol by oxidation by many different acetic acid bacteria and yeast [23], [24].

Microorganisms do not naturally produce glycolic acid under typical growth conditions so their biotechnological production requires metabolic engineering. Although some natural pathways of glycolic acid were studied, there are no known natural microbial pathways to directly produce glycolic acid from renewable and relatively cheap materials.

Glycolic acid is being used in many different areas, such as pharmaceutic, cosmetic, textile, and food industry (according to www.grandviewresearch.com). Polymerization of glycolic acid alone and with other acids, for example with lactic acid, can be suitable material for thermoplastic, packaging material, or even sutures, because of their capability of being hydrolyzed in aqueous environments [25], [26]. Poly glycolic acid (PGA) has a structure similar to PLA, so it has promising characteristics such as good biodegradability and barrier properties, which is potentially a beneficial supplement to PLA. Such combinational material has been widely studied in bio-medical applications, but not been well developed at large scales due to its relatively high production cost. PGA can be derived from industrial waste gases using an innovative production technology, which reduces carbon emissions and its production cost. By developing the production and compounding technology, PGA can be combined with PLA to play an essential role for a sustainable and environmental friendly plastic industry, especially for single-used products requiring fast degradation at room temperature or in the nature environment [27].

1.4.1.2. Metabolism and associated genes

As mentioned before, microorganisms do not produce glycolic acid under typical growth conditions so their biotechnological production requires metabolic engineering, so the naturally occurring glyoxylate shunt (GS) is the most studied metabolic pathway for glycolic acid production. This pathway consists of the TCA cycle from malate to isocitrate and the two uniquereactions of GS when formation of glyoxylate andsuccinate from isocitrate by isocitrate lyase and the formation of malate from glyoxylate and acetyl-CoA by malate synthase is in progress. Glycolic acid is formed from glyoxylate when it is catalysed by glyoxylate reductase. GS route is versatile as it involves metabolites of the central carbon metabolism such as pyruvate, oxaloacetate and citrate. Several compounds, such as ethanol, D-glucose, acetate and D-xylose have been used for glycolic acid production via described pathway by using *K. lactis, E. coli, S. cerevisiaeor, C. glutamicum* as hosts. The most common genetic modifications involved in the use of GS aim at accumulation of the TCA cycle metabolite isocitrate and include deletion of genes encoding isocitratedehydrogenase and malate synthase. Overexpression is necessary of isocitrate lyase and glyoxylate reductase encoding

genes to enhance the flux from isocitrate to glycolic acid. These modifications enabled glycolic acid production [28]. Sheme of a pathway shown in Fig. 1.4.

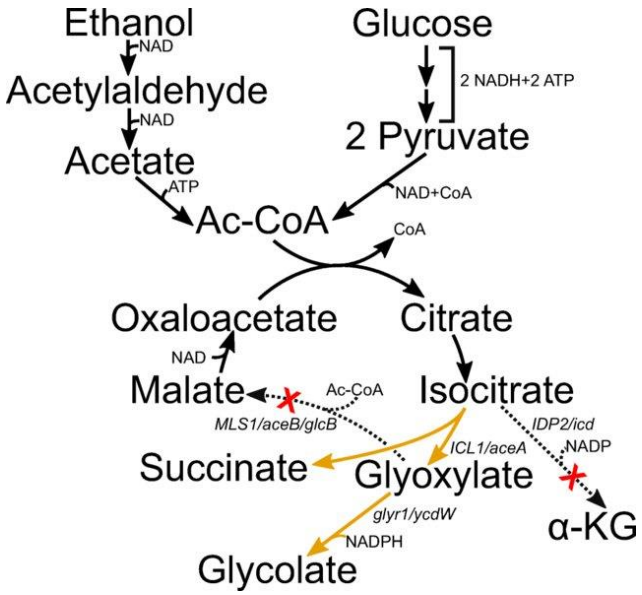


Fig. 1.4. Glyoxylate shunt pathway for glycolic acid production from D-glucose or ethanol. Adapted from [28].

The locus glc found in *Escherichia coli* is associated with the glycolate utilization trait. It is also known that locus contains *glcB*, encoding malate synthase G, and the gene(s) needed for glycolate oxidase activity, and the gene glcC, which encodes the glc regulator protein. Studies that included subcloning, sequencing, insertion mutagenesis, and expression research showed that glc locus has five genes: glcC in one direction and *glcD*, *glcE*, *glcF*, and *glcG* followed by *glcB* in the other direction [29].

1.4.2. 3-hydroxypropionic acid

3-Hydroxypropionic acid (3-Hydroxypropanoic acid by IUPAC) is a carboxylic acid, specifically a beta hydroxyl acid, its chemical formula C3H6O3 (Fig. 1.5.). It is a 3 – carbon, non – chiral organic molecule. 3-Hydroxypropionic acid is very soluble in water, ethanol, and diethyl ether. It naturally exists in some bacteria and thermophilic archaea and takes part in autotrophic carbon fixation cycles [30].

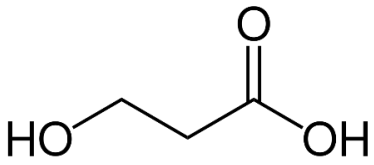


Fig. 1.5. A structure of 3-Hydroxypropionic acid

3-Hydroxypropionic acid is an important chemical in the industry with many useful applications. It is used in the industrial production of various acrylates, such as acrylic acid, 1,3 propanediol, methyl acrylate, acrylamide, ethyl 3- hydroxypropionic acid, acrylonitrile. Distilled 3-hydroxypropionic acid

from acrylic acid [31], [32]. 3-Hydroxypropionic can be used alone and be polymerized or in combination with other monomers and made poly-3-hydroxypropionate. It is a biodegradable polymer with good mechanical properties, such as ductility, rigidity, and sufficient tensile strength useful making biodegradable plastics and other packaging materials [33], [34].

Several microorganisms in nature incorporated 3-hydroxypropionic acid routes in their biochemical pathways, some of them can also convert resources such as glycerol or Acrylic acid to 3-hydroxypropionic acid under specific conditions (Table 1.4.).

Table 1.4. Natural 3-hydroxypropionic acid producers by conversion and metabolic processes.

Microorganism	**Resources**	**Products**
Lactobacillus sp.	Glycerol	3-hydroxypropionic acid and 1,3-propanediol
Byssochlamys sp., *Geotrichum* sp., *Trichoderma* sp., *Alcaligenes faecalis, Rhodococcus erythropolis*	Acrylic acid	3-hydroxypropionic acid
Metallosphaera sedula, Acidianus brierleyi, Acidianus ambivalens, Sulfolobus metallicus, Metallosphaera sedula, Crenarchaeota, Roseiflexus sp., *Chloroflexus aurantiacus*	CO_2	3-hydroxypropionic acid
Saccharomyces kluyveri	Uracil	3-hydroxypropionic acid

1.4.3. Glyoxylic acid

Glyoxylic acid (oxoacetic acid by IUPAC) is an organic compound that is one of the C_2 carboxylic acids. Its chemical formula C2H2O3 (Fig. 1.6.). Glyoxylic acid is a colorless crystalline solid that occurs naturally. It is mostly described as aldo-acid, because of the aldehyde functional group, but the aldehyde component is most common just in some situations. Glyoxylic acid can also exist as a cyclic dimer or as a hydrate.

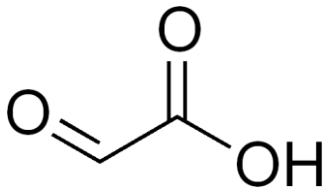


Fig. 1.6. A structure of glyoxylic acid

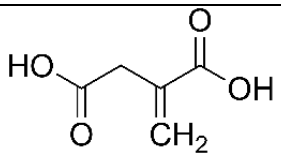
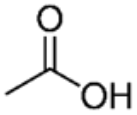
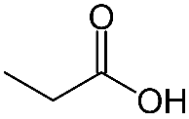
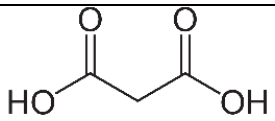
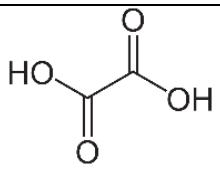
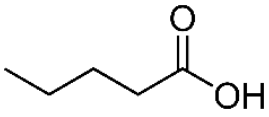
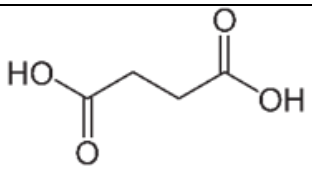
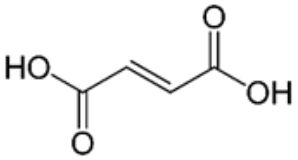
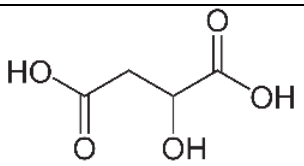
Glyoxylic acid is formed upon the oxidation of glycolic acid, metabolism of tricarboxylic acids in the Krebs cycle, or deamination of glycine in plants, bacteria, or fungi. In the human or other animal body, glyoxylic acid is a component of various metabolic processes. The conjugate base of glyoxylic acid is known as glyoxylate. Glyoxylate is an intermediate of the glyoxylate cycle, which is important in organisms such as plants, fungi, and bacteria by enabling them to convert fatty acids into carbohydrates. In plants, glyoxylate is also an intermediate in the photorespiration pathway.

Glyoxylic acid is important in various industries and is widely used in the pharmaceutical, food, perfume industry. Among most of the compounds obtained from glyoxylic acid, the most important are vanillin and allantoin. Vanillin is used in the pharmaceutical, food, and perfume industries. Glyoxylic acid is also useful in agrochemistry because of glyphosate production. [35]

1.4.4. Other compounds exhiniting structural features similar to the lactic acid

Some more lactic acid-related compounds are important in various industries, some of them are summarised in Table 1.5.

Table 1.5. Lactic acid-related acids, their formula, and appliance.

Compound	**Formula**	**Application***
Itaconic acid		Resins, paints, plastics, and synthetic fibers (acrylic plastic, superabsorbents, anti-scaling agents
Acetic acid		Food, medicine, solvents, reagent for the production of other chemical compounds (vinyl acetate monomer)
Propionic acid		Food
Malonic acid		Food, pharmacy, coating, electronics, fragrances, solvents
Oxalic acid		Mordant, food, cleaning and bleaching supplies, metallurgy
Valeric acid		Perfumes, cosmetics, food
Succinic acid		Food, dietary supplements, a precursor to polymers, resins, solvents
Fumaric acid		Food, medicine, resins, mordant
Malic acid		Food

* Research about application was conducted using https://pubchem.ncbi.nlm.nih.gov/

1.5. A Transcription factor-based biosensors

Living organisms have a variety of different sensor principles to monitor the intracellular or extracellular accumulation of ions, small molecules, or even changes in some physical parameters. Transcription factor-based biosensors play a major role in the physiological adaptation of prokaryotes, by controlling gene expression at the transcription level, most of the time, by interfering with the binding of the RNA polymerase to DNA. Transcriptional biosensors are built by linking environment-responsive promoters to engineered gene circuits for programmed transcriptional changes (Fig. 1.7.). Most biosensors are designed to focus on the promoters and their associated transcription factors on well-known and characterized prokaryotic environment-responsive promoters. Transcription factor N-terminal domain interacts wich promoter and C-terminal domain interacts with the ligand. The N-terminal domain binds to specific DNA sequences in the promoter region and is responsible for controlling the formation of RNA polymerase and promoter complex. Regulators can be activators or repressors (Fig. 1.7.), which means that activators bind to the operator and promotes the formation of stable RNA polymerase-promoter complex, while repressor bind to the operator and prevents the formation of RNA polymerase-promoter complex [36], [37]. Plasmids as genetically encoded biosensors are based on inducible gene expression systems, so plasmid is constructed with two main components which are sensing unit and reporting unit. Reporting unit mostly consists of the fluorescent protein and it can respond to the sensing unit to change its fluorescence activity [38], [37].

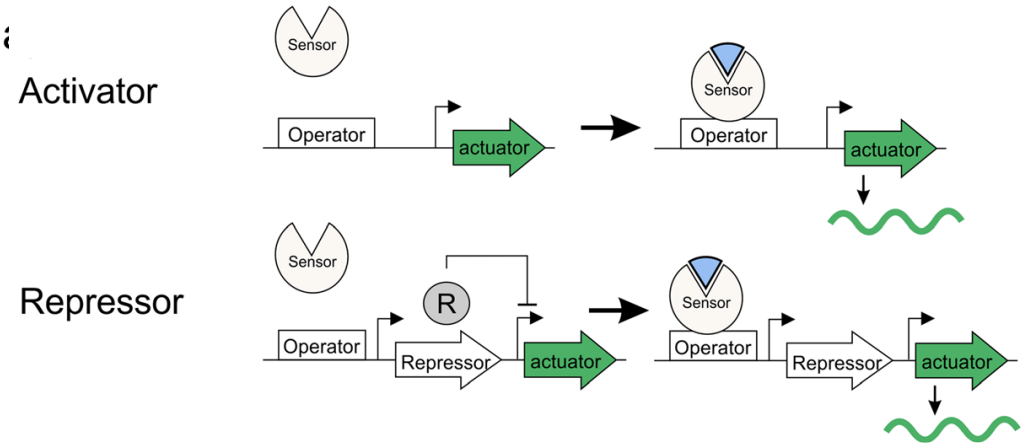


Fig. 1.7. Principles of transcription factor-based biosensors. Transcriptional activator is used to activate expression of an actuator gene in response to effector molecules. In contrast, repressors block the expression of actuators. By setting the expression of a second repressor under the control of the TF biosensor repressor, the signalling is inverted, resulting in a positive output of the actuator module. Adapted from [39].

1.5.1. Development and application of transcription factor-based biosensors

Genetically encoded biosensors are valuable for biotechnological applications and can detect amino or other acids, some secondary metabolites, measure their concentration, monitor and even regulate productivity of cellular metabolism. It is a very sensitive and specific tool, which produces results faster than HPLC, titration, or electrophoresis [40]. There are more than 750 different fluorescent biosensors listed in BiosensorDB.ucsd.edu [38]. So far there is no transcriptional factor-based lactic acid biosensor and they are just a few biosensors of related acids. It could be useful to have more lactic acid-related transcriptional factor-based biosensors in the biotechnology industry (Table 1.6.).

Transcription factor-based biosensors are very sensitive so parameters must be optimized before working with them to achieve the best results. Sensitivity, sensing range, and specificity need to be well studied and optimized for specific analysis. Other important parameters must include minimized leakiness to allow accurate measurements at low signal levels, minimized reporter expression level for signal detection in the presence of background noise, and a high dynamic range for more accurate identification [41], [42].

Table 1.6. Lactic acid-related compounds potentially possible or already having constructed transcriptional factor-based biosensors.

Compound	A Transcription Factor-Based Biosensor	Reference
Glycolic acid (glycolate)	-	-
3 – hydroxypropionic acid	+	[43]
Glyoxylic acid (glyoxylate)	-	-
Itaconic acid	+	[44]
Acetic acid (acetate)	-	-
Propionic acid (propionate)	-	-
Malonic acid (malonate)	-	-
Malic acid (malate)	-	-
Succinic acid (Succinate)	-	-
Fumaric acid (fumarate)	-	-

Although there are quite many described and characterized transcriptional biosensors but not many are adapted to a successful application. Such applications are for real-time monitoring, dynamic pathway control, phenotype screening, and adaptive evolution control [45].

Phenotype screening can be done by biosensors when the detection of a target metabolite or other compound is coupled to fluorescent protein expression. Screening is needed to identify improved bacteria from mutant libraries and selecting for suitable synthetic pathways. Biosensors constructed with FACS are used for ultra-high-throughput screening on a single cell level so it allows the isolation of a producing single cell from large libraries. Lactic acid-related 3-hydroxypropionic acid biosensor showed promising results when generated a fluorescent signal that was proportional to 3-hydroxypropionic acid production in presence of interfering components. Such biosensors could be used for enzyme and metabolic applications for 3-hydroxypropionic acid production [46].

1.6. Justification of the project aim and objectives

Literature review revealed that the lactic acid is one of the most commercially useful acids which is widely used in food, cosmeceutical, pharmaceutical, and chemistry industries. Because PLA is made of lactic acid, it is also very important to search for alternative compounds that would have similar properties, but would be more environmentally friendly. For such reason, structurally related compounds to lactic acid were investigated and one of these acids, which have promising properties and appliance possibilities, was a glycolic acid. To optimize the biosynthesis of various compounds, including lactic or glycolic acids, to gain the most profit, microorganisms should be examined and their application must be optimized. For a better selection of useful natural engineered microorganisms and examination of their performance, transcription factor-based biosensors could be used as a faster tool comparing with HPLC, because such biosensors allow examining a lot more

examples at the same time. For this reason, it was chosen to perform bioinformatics analyses for a better understanding of glc operon and metabolism of glycolic acid and to construct the whole-cell biosensors using information from the results of bioinformatics analyses.

2. Materials and methods

2.1. Chemical material

Most of the chemicals were bought from Thermo Ficher Scientific, Inc., USA, and Sigma-Aldrich, USA. A more detailed list of chemicals is provided in Table 2.1.

Table 2.1. Chemicals used in the research project.

Chemical	Application	Manufacturer	Catalog Number
L- lactic acid	Ligand sued to test inducible system	Thermo Fisher Scientific, Lithuania	984308
D- lactic acid	Ligand sued to test inducible system	Sigma-Aldrich, USA	L0625
3-hydroxypropionic acid	Ligand sued to test inducible system	Sigma-Aldrich, USA	166898
Glyoxylic acid monohydrate	Ligand sued to test inducible system	Sigma-Aldrich, USA	G10601
Sodium glycolate	Ligand sued to test inducible system	Sigma-Aldrich, USA	799254
10X FastDigest Green Buffer	Digestion buffer for restriction enzymes	Thermo Fisher Scientific, Lithuania	B72
Phusion™ High-Fidelity DNA Polymerase	Polymerase for PCR	Thermo Fisher Scientific, Lithuania	F-530XL
DreamTaq Green PCR Master Mix	Ready-to-use PCR master mix solution containing DreamTaq DNA Polymerase	Thermo Fisher Scientific, Lithuania	K1081
T4 DNA Ligase	4 DNA Ligase catalyzes the formation of phosphodiester bonds in the presence of ATP between double-stranded DNAs with 3´ hydroxyl and 5´ phosphate termini.	Thermo Fisher Scientific, Lithuania	EL0011
Midori Green Advance Safe DNA/RNA stain	Stain for agarose gel for DNA/RNA electrophoresis	NIPPO Genetics Europe, Germany	MG04
TriTrack DNA Loading Dye (6x)	Loading dye for PCR products for electrophoresis	Thermo Fisher Scientific, Lithuania	R1161
GeneRuler 1 kb DNA Ladder	DNA ladder for electrophoresis	Thermo Fisher Scientific, Lithuania	SM0313
NdeI (10 U/µL)	Restriction enzyme, recognizes CA^TATG sites.	Thermo Fisher Scientific, Lithuania	ER0581
AatII (10 U/µL)	Restriction enzyme recognizes GACGT^C sites.	Thermo Fisher Scientific, Lithuania	ER0991

BamHI (10 U/µL)	Restriction enzyme recognizes G^GATCC sites.	Thermo Fisher Scientific, Lithuania	ER0051
GeneJET Gel Extraction Kit	Purification kit of DNA from PCR, DNA extraction from standard or low-melting point agarose gels in Tris-acetate (TAE).	Thermo Fisher Scientific, Lithuania	K0691
GeneJET Plasmid Miniprep Kit	The kit recovers high copy plasmid DNA per isolation procedure.	Thermo Fisher Scientific, Lithuania	K0503
Zymoclean Gel DNA Recovery Kit	Purification kit of DNA from PCR, DNA extraction from standard or low-melting point agarose gels in Tris-acetate (TAE).	Zymo Research Corporation, USA	D4002
NEBuilder HiFi DNA Assembly Master Mix	One-step cloning of multiple fragments master mix	New England Biolabs, UK	E5520S
Lysogeny broth (LB)	Growth media	Sigma-Aldrich, USA	L3522
LB agar	Growth media	Sigma-Aldrich, USA	L3147
Agarose	Compound for electrophoresis gels.	Sigma-Aldrich, USA	A9539
TAE buffer	Buffer was used for electrophoresis of nucleic acids in agarose.	Thermo Fisher Scientific, Lithuania	B49
M9 media	Minimal salts for growth media	Sigma-Aldrich, USA	M6030

2.2. Equipment

The equipment used for this research is listed in Table 2.2.

Table 2.2. Equipment used in this project.

Equipement	**Manufacturer**
Autoclave	CertoCLAV, Austria
Block heater	VWR, USA
Centrifuge	Eppendorf, Hamburg, Germany
DNA electrophoresis apparatus, POWER PRO-300	Cleaver Scientific, UK
DNA electrophoresis bath	Cleaver Scientific, UK
Laminar flow cabinet	ESCO, USA
Shaking incubator, MaxQ 6000	Thermo Fisher Scientific, USA
Incubator	Memmert, Schwabach, Germany
Refrigerated centrifuge	Eppendorf, Hamburg, Germany
Heated bath	Grant Instruments, Cambridge
UVITEC Essential Imaging System	UVITEC, Cambridge, UK

UV lamp Safe Light-Box 20-blue Q9 PLUS	UVITEC, Cambridge, UK
Nanophotometer N60	Implen, Germany
Spectrophotometer	Thermo Fisher Scientific, USA
Tecan Infinite 200 Pro plate reader	Grödig, Austria
Thermal cycler, Nexus X2	Eppendorf, Hamburg, Germany
Vortex mixer, V-1 plus	Biosan, Latvia

2.3. Bacteria strains and plasmids

Two bacteria strains were used in this research project. *Escherichia coli* MG1655 was used as genomic DNA for targeted DNA sequences. *E. coli* TOP10 (Invitrogen, Thermo Fisher Scientific, Inc., USA) was used for heat-shock chemotransformation and cloning. All the plasmids (vectors) are summarized in Table 2.2.

Table 2.2. Plasmids were used and develop during this project.

Plasmid	**Characteristics**	**References**
pBRC1	Chloramphenicol resistance marker, contains the arabinose-inducible system, high copy number plasmid, fluorescent protein.	Augustiniene and Malys, unpublished data
pSS003	Chloramphenicol resistance marker, intergenic region of *glcC* from gDNA of *E. coli* MG1655 and cloned in pBRC1 by NdeI and AatII restriction endonucleases sites	This work
pSS004	Chloramphenicol resistance marker, an intergenic region with transcription factor of *glcC* from gDNA of *E. coli* MG1655 and cloned in pBRC1 by NdeI and AatII restriction endonucleases sites	This work
pSS004A	Chloramphenicol resistance marker, an intergenic region with transcription factor of glcC from gDNA of *E. coli* MG1655, synthetic p13 promoter before transcription factor, cloned in pBRC1 by BamHI and AatII restriction endonucleases sites	This work
pEA003	Chloramphenicol resistance marker, intergenic region of glcC from gDNA of E. coli MG1655 and cloned in pBRC1 by NdeI and AatII restriction endonucleases sites. Used as a control vector.	E.Augustiniene unpublished work
pEA004	Chloramphenicol resistance marker, an intergenic region with transcription factor of glcC from gDNA of *E. coli* MG1655, synthetic p13 promoter before transcription factor, cloned in pBRC1 by BamHI and AatII restriction endonucleases sites. Used as a control vector.	E.Augustiniene unpublished work

2.4. Primers

Oligonucleotide primers used in this research were synthesised by Thermo Fisher Scientific (Lithuania). Primers were diluted with water and prepared according to manufacturer

recommendations to achieve 100 µM concentration. The working stock was prepared by 1:10 dilution of water to achieve 10 µM concentration. Primers and their sequences are listed in Table 2.3.

Table 2.3. Oligonucleotide primers used in this research

Primer name	**Sequence (5' → 3')**
N221	ATATATcatatgTAGGCTTCGCTTTGTTGTGTTGTGTG
N222	ATATgacgtcTCCCGGACCTCGTGCACA
N223	ATATgacgtcCTAACTCAGGTTCATCTCCAGC
EG013	ccttactcgagtttggatcc
EG068	TTCCCTTTTAATCATCCGGCTCGTATAATGTGTGGAGACTTGAATTCACTAGTTT AACTTTAAGAAGGAGATATATCTATGAAAGATGAACGTCGCC
EG069	GCCGGATGATTAAAAGGGAATCCCGGACCTCGTGCACA
EG070	gggcctttcgttttatgacgtc

2.5. Growth media and cultivation

Bacterial cells were cultivated in different mediums which types, preparation, and cultivation conditions are described in Table 2.4.

Table 2.4. Mediums, preparation, and cultivation conditions.

Medium	**Preparation**	**Cultivation conditions**
LB (Lysogeny broth)	40 g of the LB powder is dissolved in 1L distilled water. Medium is autoclaved and cooled down to 60 ºC in a water bath. A required antibiotic is added. Medium is stored at room temperature (Sigma-Aldrich, USA commercial protocol).	*E. coli* TOP10 was cultivated at 37 ºC and 30 ºC overnight for ~ 14hours while shaking at 200 rpm with aeration.
LB agar	40 g of the LB agar powder is dissolved in 1L distilled water. Medium is autoclaved and cooled down to 60 ºC in a water bath. A required antibiotic is added. Before pouring the medium into Petri dishes, it has to be heated to 60 ºC again. Medium is poured into Petri dishes in a laminar flow cabinet and letting it harden. Then Petri dished are inverted and stored at +4 ºC (Sigma-Aldrich, USA commercial protocol).	E. coli TOP10 was cultivated at 37 ºC and 30 ºC overnight for ~ 14hours.
M9 minimal medium (with different carbon sources)	Listed components are dissolved in 7.748 ml distilled water: 2 ml 1x M9 minimal salts, 1µg/ml thymine, 0.4 mM leucine, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 25 µl/ml chloramphenicol, and required carbon source. There were made 5 different mediums using 5 different carbon sources: 22mM glucose, 44mM L-lactic acid, 44 mM D-lactic acid, 66 mM glyoxylic acid monohydrate, 66mM sodium glycolate.	E. coli TOP10 was cultivated at 37 ºC and 30 ºC overnight for ~ 14hours while shaking at 200 rpm with aeration.

All bacterial cultures were cultivated on Petri dishes with LB agar medium at 37 ºC overnight, for ~14h. 25 μg/mL of chloramphenicol was used as an antibiotic.

Overnight bacterial cultures (using one colony from Petri dish) were grown in 50 mL falcon centrifuge tubes, using 5 mL of LB medium with 25 μg/mL of chloramphenicol. Cultures were cultivated at 37 ºC while shaking at 200 rpm with aeration.

Cryobanks were prepared to store made plasmid constructs. For the preparation of the cryobank, a microbank cryovial system was used (Pro Lab Diagnostics Inc., Canada). This system consists of a vial with some treated beads and filled up with a cryopreservative solution. When preparing a cryobank, the solution that is in the cryovial should be removed. Then 0.5 mL of night cultures of bacteria is added in a vial, gently tur over a few times, and incubated at room temperature for a couple of minutes. After that, excess liquid is removed, and cryobanks are stored at –80 ºC.

2.6. Genomic DNA purification

Genomic DNA (gDNA) purification was performed by using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to the added commercial protocol. 1.5 mL of an overnight *E. coli* MG 1655 culture was centrifuged at 12000 g for 2 min. After that, the supernatant was removed and cell pellets were resuspended in 180 mL of Lysis Solution. 20 μL of RNase A solution was added for RNR removal in addition, and the mixture was incubated for 2 min at room temperature. After incubation, 20 μL of the Proteinase K solution was added followed by 200 μL of Lysis Solution C, vortexed thoroughly for homogeneous mixture about 15 seconds, and incubate at 55 °C for 10 minutes. 200 mL of Column Preparation Solution was adding to each pre-assembled GenElute Miniprep Binding Column and centrifuged at 12000 g for 1 min, then the flow-through was discarded. 200 mL of ethanol (95–100%) was added to the lysate and vortexed to achieve a homogeneous mixture. The entire content of the tube was transferred to the binding column and centrifuged at 6500 g for 1 min. The tube containing eluate was discard and the binding column was placed into a new 2 mL tube. 500 μL of the Wash Solution I was added to the column and centrifugated for 1 min at 6500 g, collection tube containing eluate was discard and the binding column was placed into a new 2 mL tube again. 500 μL of Wash Solution Concentrate was added and centrifugated for 3 min at 12000 g. The column was centrifuged for an additional 1 min to dispose of ethanol completely. The column was placed into a new 2 mL collection tube, and 200 mL of the Elution Solution was added directly onto the center of the column and incubated for 10 min at room temperature. Tubes were centrifuged for 1 min at 6500 g and stored in -20 ºC.

2.7. Plasmid DNA isolation

Plasmid DNA isolation was performed by using the DNA prep kit (GeneJET Plasmid Miniprep Kit, Thermo Fisher Scientific, Lithuania). Cultures of *E. Coli* TOP10 and isolation were grown overnight as recommended by the manufacturer. *E. Coli* TOP10 cultures with pBRC1 vector from cryobank were revived on LB agar medium with 25 μg/mL of chloramphenicol overnight in 37 ºC. Then, one colony from a Petri dish was placed in 5 mL of LB with the same amount of chloramphenicol and incubated overnight at 37 ºC with aeration while shaking at 200 rpm in 50 mL falcon centrifuge tubes. After ~14h falcon was centrifuged at 8000 g for 2 min at room temperature and the medium was removed. Cell pellets were resuspended in 250 μL of the Resuspension Solution, with RNase A, and transferred to a microcentrifuge tube. 250 μL of the Lysis Solution was added and tubes were mixed thoroughly by inverting ~6 times. Immediately 350 μL of the Neutralisation Solution was added, and

again, tubes were mixed thoroughly by inverting ~6 times. Tubes were centrifuged for 10 min at 12000 g. The supernatant was transferred to the GeneJET spin column by pipetting and avoiding to disturbing or transferring white precipitate. Then the content was centrifuged for 1 min at 12000 g and the flow-through was discarded. 500 µL of the Wash solution (diluted with ethanol) was added to the spin column and was centrifuged for 1min., and the flow-through was discarded. The Wash procedure was repeated one more time and then, an empty tube was centrifuged for an additional 2 min to remove residual Wash Solution. The spin column was transferred into a new 1.5 µL microcentrifuge tube. 30 µL of the elution buffer (heated up to 60 ºC for 10 min. for buffer yield) was added to the center of the GeneJET spin column membrane and incubated for 10 min at room temperature. Then, the tube was centrifuged for 2 min. and purified plasmid stored at -20 ºC.

2.8. Preparation of *Escherichia coli* competent cells

An overnight culture of *E. coli* Top10 was set by inoculating cells in 5 mL LB medium a day before. Cultivation was done without an antibiotic. 0.5 mL of the overnight culture was added to 50 mL of LB medium incubated with 200 rpm shaking with aeration at 37 ºC to OD600 of 0.4–0.8. To follow changes in the optical density, 1 mL of sample was measured after 1.5 hours using 1-cm-path-length cuvette and spectrophotometer (BioMate 160 UV-Visible Spectrophotometer, Thermo Fisher Scientific, Lithuania). Later, measurements of samples were taken every 30 min. not to miss the required OD. When the sample reached about 0.7 of optical density, all the culture was cooled on ice for 10 min, then transferred to 50 mL centrifuge tubes, and pelleted cells using a precooled centrifuge (4 ºC) for 6 min at 4000 g (Centrifuge 5804 R, Eppendorf, Germany). The supernatant was discarded, and cells were resuspended in 15 mL of 0.1 M MgCl2 by gentle shaking on ice, then centrifuged again as described before. After supernatant discarded again were resuspended in 15 mL of 0.1 M CaCl2 by gentle shaking on ice. Tubes have left on the ice for 20 min and later centrifuged as described before. The supernatant was discarded and cells were resuspended in 3 mL of 0.1 M CaCl2 – 15% glycerol solution by gentle shaking on ice. Cells were frozen as 200 µL aliquots at –80 ºC and kept at least 16h before the first transformation.

2.9. Polymerase chain reaction (PCR)

PCR was used to amplify wanted regions of genomic *E. coli* MG 1655 DNA. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Lithuania) was used for this amplification following the manufacturer's protocol listed in table 2.5. PCR conditions are listed in table 2.6.

PCR was also used for colony analysis. Colony PCR was done to detect positive colonies after heat-shock transformation from Petri dishes. DreamTaq PCR Master Mix (Thermo Fisher Scientific, Lithuania) was used for this PCR method. Separate colonies from a Petri dish were picked up with a sterile toothpick and suspended in the PCR mixture prepared according to the manufacturer's protocol described in table 2.7. PCR conditions are listed in table 2.6. as well as described in manufacturers protocol.

One more PCR was done to prepare fragments for HiFi DNA assembly. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Lithuania) was used for this amplification following the manufacturer's protocol already listed in table 2.5., and PCR conditions are listed in table 2.6. Vector pSS004 was used here as a DNA template.

Table 2.5. Components used for PCR for gDNA amplification and fragments to HiFi DNA assembly.

Component	Final concentration	20 µl reaction	
		gDNA amplification	Fragments amplification for HiFi DNA assembly
Nuclease-free water	to 20 µl	12.3 µl	12.3 µl
5X Phusion HF or GC Buffer	1 X	4 µl	4 µl
10 mM dNTPs	200 µM	0.4 µl	0.4 µl
10 µM Forward Primer	0.5 µM	1 µl	1 µl
10 µM Reverse Primer	0.5 µM	1 µl	1 µl
Template DNA	-	0.5 µl	0.5 µl
DMSO	3%	0.6 µl	0.6 µl
Phusion DNA Polumerase	0.02 U/ µl	0.2 µl	0.2 µl

Table 2.6. Components used for colony PCR with DreamTaq PCR Master Mix

Component	Final concentration	20 µL reaction
DreamTaq PCR Master Mix (2X)	1 X	10 µL
Forward Primer	0.1-1.0 µM	1 µL
Reverse Primer	0.1-1.0 µM	1 µL
DMSO	3 %	0.6 µL
Template DNA	10 pg – 1 µg	cells from the separate colony on the Petri dish was added directly to the mix by a sterile toothpick
Nuclease-free water	to 20 µL	7.4 µL

Table 2.7. Reaction conditions for PCR depending on the used method.

Step	Phusion for gDNA		Phusion for fragments for Hifi DNA assembly		DreamTaq		Cycles
	Temperature	Duration	Temperature	Duration	Temperature	Duration	
Initial denaturation	98 ºC	2 min	98 ºC	30 s	95 ºC	5 min	1
Denaturation	98 ºC	10 s	98 ºC	10 s	95 ºC	30 s	35
Annealing	63 ºC	30 s	63 ºC	30 s	54 ºC	30 s	
Extension	72 ºC	2 min	72 ºC	1 min	72 ºC	1.5 min	
Final Extension	72 ºC	15 min	72 ºC	10 min	72 ºC	15 min	1
Storage	15 ºC	Infinite	15 ºC	Infinite	15 ºC	Infinite	-

2.10. DNA digestion

DNA digestion was used for several purposes. One purpose was to prepare plasmid DNA and fragments by extraction of these components from agarose gel as described in section 2.12. It was used for ligation. The other one, to confirm successfully assembled vectors by agarose gel analyses. All the DNA was digested using two of FastDigest Restriction Enzymes (Thermo Fisher Scientific, Lithuania). Reactions were done using combined protocol by manufacturer's recommendation and

experimental data noticed during this work. The protocol is listed in table 2.8. All reactions after the recommended amount of time were thermally deactivated at 80 ºC in a block heater for 5 min.

Table. 2.8. DNA digestion protocol.

Component	Plasmid DNA	Fragments	Vector analyses
10 X FastDigest Green Buffer	3 µL	3 µL	1 µL
1 restriction enzyme	1 µL	1 µL	0.5 µL
2 restriction enzyme	1 µL	1 µL	0.5 µL
DNA	300 ng	300 ng	100 ng
nuclease-free water	to 30 µL	to 30 µL	to 10 µL
Total volume	30 µL	30 µL	10 µL
Reaction time	Using NdeI and AatII restriction enzyme combination, fist NdeI was added and left overnight in 37 ºC. In the morning, an additional 0.5 µL of NdeI and 1 µL of AatII were added and left for 3 h.	Using NdeI and AatII restriction enzyme combination, fist NdeI was added and left overnight in 37 ºC. In the morning, an additional 0.5 µL of NdeI and 1 µL of AatII were added and left for 3 h.	Reaction with NdeI and AatII was used as described in Plasmid DNA and Fragments section. The reaction using AatII and BamHI restriction enzymes was stored at 37 ºC for 1.5 hours.

2.11. DNA electrophoresis

DNA electrophoresis was done in 1% agarose gel. 1 g of agarose was diluted with 50 x TAE buffer and microwaved for about 5 min until agarose powder was melted in the buffer. The solution must be clear and transparent. Then, the solution was put in a water bath where the temperature was 60 ºC to cool it down. When the gel was at the required temperature it was poured into the gel casting tray (~50-70 ml) and immediately added 0.75 µL of Midori Green Advance Safe DNA/RNA stain (NIPPON Genetics Europe, Germany) and mixed with agarose gel. Wells was formed by added combs while the gel was left to solidify. TriTrack DNA Loading Dye 6x (Thermo Fisher Scientific, Lithuania) was used to dye PCR samples before loading them on the gel. Digested DNA already had dye in the used buffer for the reaction, so no additional dye was needed. For DNA size analyses GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Lithuania) was used. Electrophoresis was run at 120 V for ~ 20-30 min. Gel imaging was performed with Uvitec Essential Gel Imaging system (Uvitec Cambridge, UK) under the ultraviolet (UV) lamp.

2.12. DNA extraction from agarose gel

DNA plasmid and fragments were extracted from agarose gel with the aid of Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, USA) according to the manufacturer's protocol. When electrophoresis was finished, plasmid and fragments were cut out from the gel under UV light (SafeLight Box, Uvitec, UK) using the scalpel. DNA was cut from the gel as fast as possible to prevent damaging DNA under UV light. For better DNA extraction yield all agarose without DNA should be cut off. The excised gel was transferred to an empty (weighed before use) 1.5 mL microcentrifuge tube and weighed again with a gel slice in it. 3 µL of Binding Buffer was added to every 1 µg of gel weight. The mixture was incubated at 55 ºC for ~ 10 min until the gel was completely

dissolved. Then, the mixture was transferred to the Zymo-Spin column and centrifuged for 60 s at 10000 g. The flow-through fraction was discarded and 200 µL of DNA Wash Buffer was added. After 60 s of centrifugation at 10000 g the flow-through fraction was discarded and the wash step was repeated one more time. The column was centrifuged once more without Wash Buffer at the same conditions. The column was placed in a new 1.5 mL microcentrifuge tube and 12 µL of DNA Elution Buffer was added. For a better yield tube was kept at room temperature for ~5 min and then centrifugated for 60 s to elute DNA.

2.13. DNA ligation

DNA ligation reaction was done by using T4 DNA Ligase (Thermo Fisher Scientific, Lithuania). The reaction was performed following the manufacturer's protocol. The used amount of insert DNA was calculated by equation 2.1. The molar ratio of insert to vector was chosen to be 1 to 5. 20-100 ng of the linear vector was used for reaction mixture according to manufactures recommendations. The total volume of the mixture was 20 µL which included 2 µL of 10x T4 DNA Ligase Buffer, 1 µL of T4 DNA Ligase, and nuclease-free water filling up the rest of the volume. The tube was incubated at room temperature for 2 h. and left in the fridge at 4 ºC overnight. After incubation, 4 µL of the ligate was used for heat-shock transformation described in section 2.15.

Equation 2.1. The formula used to calculate the required amount of the insert DNA for ligation m_{vector}. – mass of the used vector, ng; l_{vector}– length of the used vector, bp; R – molar ratio of insert to vector; l_{insert} – length of the used insert, bp.

$$\mathrm{m_{insert}} = \frac{\mathrm{m_{vector} \cdot l_{vector} \cdot R}}{\mathrm{l_{insert}}}$$

2.14. HiFi DNA assembly

HiFi DNA assembly was done using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, UK) according to the manufacturer's protocol. Required amounts of inserts were calculated according to equation 2.1. The molar ratio of insert to vector was chosen to be 1 to 1. During this method, two fragments of inserts were used at the same time for constructing the vector. The total volume of the mixture was 5 µL which included 2.5 µL of NEBuilder HiFi DNA Assembly Master Mix, the calculated amount of plasmid and fragments, and nuclease-free water if needed.

2.15. Heat-shock transformation

The heat-shock transformation was done using *E. coli* Top10 competent cells and ligated or assembled by HiFi reaction vectors. The heat-shock transformation was done by standard method based on Inoue h., et al., [47] research and modified in our laboratory. *E. coli* Top10 competent cells were taken out from -80 ºC freezer and put on ice (4 ºC). 4 µL of the ligated mixture and 5 µL of HiFi assembled DNA were added to 50 µL of competent cells separately. 1.5 mL tube with competent cells and added mixtures containing built vector was incubated on ice for 5 min, then heat-shock was performed for 1.5 min at 42 ºC in block heater. After the heat-shock, the tube was placed on ice for more 5min. After incubation on ice, 1 mL of LB medium was added to the tube and incubated for 90 min at 37 ºC with 200 rpm shaking. Then the tube was centrifuged for 5 min at 800 g, the supernatant

was discarded leaving ~200 μL of a medium, and cells were resuspended in it. The suspension was plated on the LB agar plate with 25 μg/mL of chloramphenicol and spread on the surface using a spatula.

2.16. Absorbance and fluorescence assay

For growth and evaluation of fluorescence at a single time point first step was to prepare cells. Freshly transformed or revived cells from cryobank were grown in 2 mL of LB medium with 25 μg/mL of chloramphenicol in 50 ml tubes overnight, for ~16-18 hours, at 30 ºC with 200 rpm shaking and aeration. Three biological replicas were used from a single sample. After incubation, OD_{600} was measured and it should be ~ 1.6 per sample. Samples were diluted 1:50 into 2 mL fresh LB medium with 25 μg/mL of chloramphenicol and incubated for ~ 2 hours at 30 ºC with 200 rpm shaking and aeration. After incubation, OD_{600} reached ~0.1-0.2 log-phase. 142.5 μL of cells were transferred to a well of a 96-well plate and an additional 7.5 μL of required inducer at the desired concentration was added. Growth and fluorescence were measured using Tecan Infinite 200 Pro plate reader, with 585 nm as excitation wavelength and an emission wavelength of 620 nm (Grödig, Austria) every 10 min for 120 kinetic cycles. To determine the background autofluorescence, 142.5 μL of LB medium was transferred to a separate well containing 7.5 μL distilled water. Fluorescence and absorbance values were corrected by medium fluorescence and absorbance. Normalized fluorescence values for each measurement were obtained by dividing fluorescence by absorbance and subtracting the normalized background autofluorescence. Calculations additionally showed in equation 2.2.

Equation 2.2. The formula used to calculate absolute normalized fluorescence (A.U.)

$$\text{A.U.} = \frac{(\text{fluorescence+inductor})-(\text{fluorescence without inductor})}{(\text{absorbance}-\text{normalized background autofluorescence})}$$

2.17. Bioinformatics analysis of glcC and glcD homologous sequences

Bioinformatics analysis at https://www.ncbi.nlm.nih.gov/ was made to find more homologous sequences of selected DNA fragments. Per. Ident % was selected >70%. Forty homologous sequences were copied and saved as sequences of bases to compare the similarity of motifs. For comparison was used WebLogo tool which showed highly conservative nucleotides.

3. Results and discussion

3.1. Identification of glycolic acid-inducible systems in *E. coli* MG1655 and *P. putida* KT2440

Six enzymes have been identified to be involved in glycolic acid metabolism in *E. coli* MG1655 [29]. They are arranged in one operon (Fig. 3.1.) This operon consists of glc regulator protein (*glcC*), glycolate dehydrogenase, putative FAD-linked subunit (*glcD, glcE*), glycolate dehydrogenase, putative iron-sulfur subunit *(glcF*), putative heme-binding protein (*glcG*), malate synthase (*glcB*).

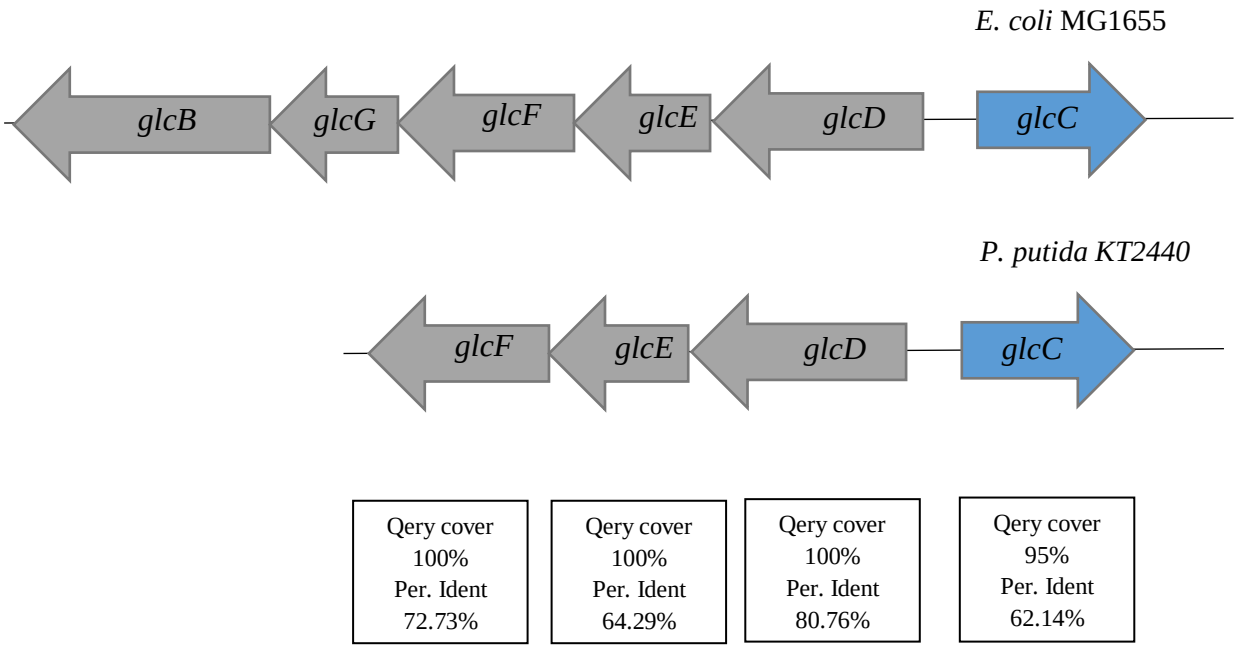


Fig. 3.1. Identification of genes involved in glycolic acid metabolism. Operons of enzymes involved in glycolic acid metabolism in *E. coli* MG1655 and *P. putida* KT2440. Alignment of genes involved in glycolic acid metabolism between operons of *E. coli* MG1655 and *P. putida* KT2440, using BLAST tool.

For the purpose of this research, it was important to identify the intergenic region (Fig. 3.2.) and the intergenic region with the transcription factor. The intergenic region and its transcription factor were identified using the NCBI database. Translation sites are noted in Fig. 3.2. Bioinformatic analyses were insufficient to determine TR binding sites or to identify the -35 and -10 sites of the promoter, because different *Escherichia* genus sequences were very conservative and did not show any significant differences.

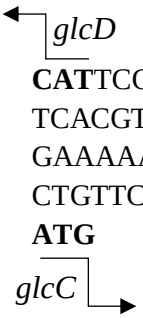
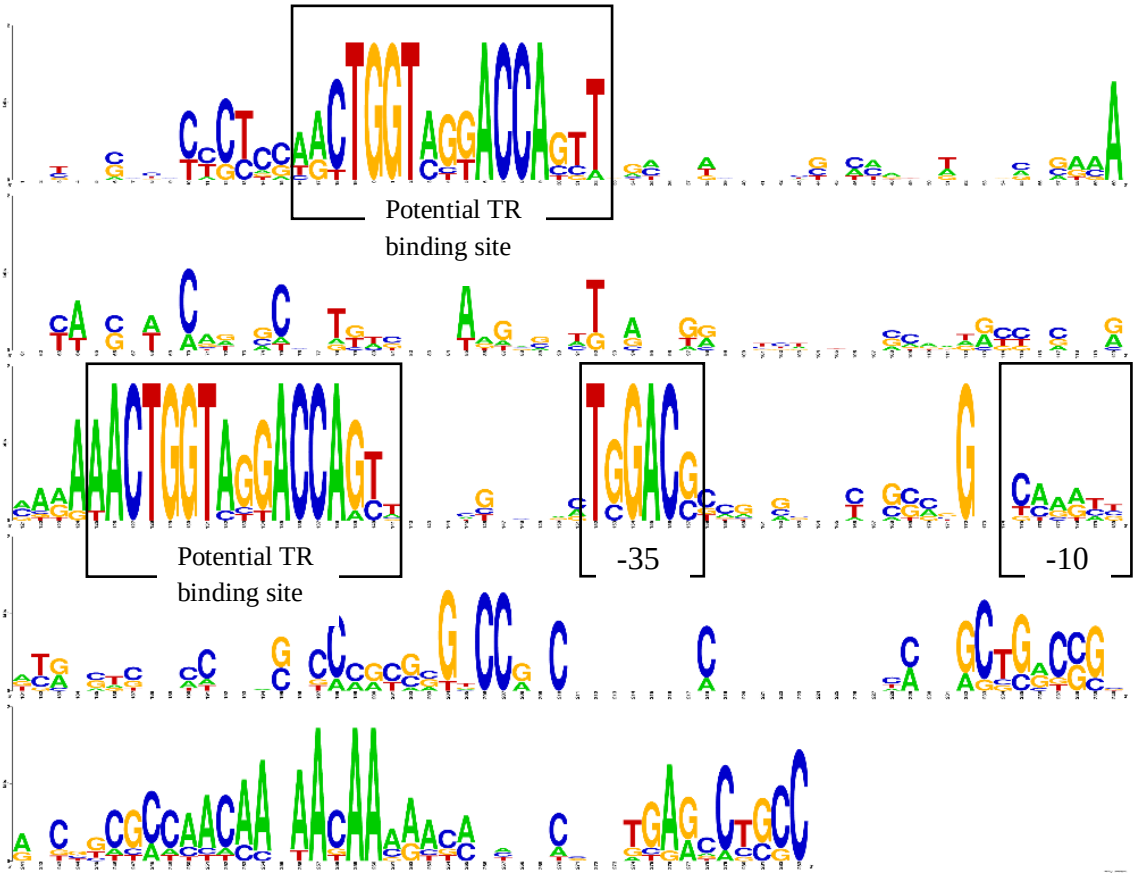


Fig. 3.2. *E. coli* MG1655 *glcD/glcC* intergenic region. Translation start sites are bold and italicized.

To increase understanding of the Glc operon in *E. coli*, it was analysed using the BLAST tool to find more such operons between different species. *P. putida* KT2440 was selected for subsequent analyses, due to having a similar Glc operon. Another BLAST analysis of corresponding genes from the Glc operon was done between *E. coli* MG1655 and *P. putida* KT2440 strands that revealed that *P. putida* KT2440 has quite a similar glc operon (Fig. 3.1.). This operon consists of a transcriptional regulator (*glcC*) and glycolate oxidase subunits (*glcD, glcE, glcF*). The intergenic region between *glcC* and *glcD* genes in *P. putida* KT2440 was also identified to perform more detailed analyses. Highly conserved nucleotides in the *glcD/glcC* intergenic region were discovered by using the WebLogo tool (https://weblogo.berkeley.edu/) and analyzing different species of the Pseudomonas genus. BLAST was performed using species that were >70% per ident (Table 3.1.).The obtained results suggested the potential transcription factor binding sites and the predicted -35 and -10 regions (Fig. 3.3.).

a



b

glcD

TTGACTTATATCGCCAATCTAGCCAGACAGAACCGTGAATAGACAGTACTGCCCGAAG

-35 -10

AAACTGGTAGGACCAGTCAACATCTCAC**TCGACC**CCAAACACTAGCACGC**GCACGC**T

GCATCGGTGACCGCTTCGCCAACAACAACAAAAACCGTTGCGAGTGAGCCG***ATG***

glcC

Fig. 3.3. *P. putida* KT2440 glcD/glcC intergenic region. (a) A sequence similarity motif. It represents highly conserved nucleotides in the *glcD/glcC* intergenic region of ten analysed Pseudomonas species. (b) Translation start sites are bold and italicized. The predicted glcC -35 and -10 regions are bold and highlighted in grey.

3.1. Table. Results of BLAST using *Pseudomonas putida* KT2440 intergenic region between *glcC* and *glcD*. Intergenic regions of listed species where used for WebLogo analyses.

	Species	glcC locus tag	Size aa	Query cover %	Per. Ident %	glcD	
						Query cover %	Per. Ident %
1	*Pseudomonas putida* KT2440	PP_3744	263	100	100	100	100
2	*Pseudomonas aeruginosa* M18	PAM18_5476	251	94	81.93	99	83.17
3	*Pseudomonas citronellolis*	A9C11_30995	257	95	81.75	99	86.97
4	*Pseudomonas denitrificans (nomen rejiciendum)*	F1C79_14910	256	94	80.40	99	90.38
5	*Pseudomonas monteilii* SB3078	X969_15460	254	96	95.28	99	94.59
6	*Pseudomonas resinovorans* NBRC 106553	PCA10_27990	257	95	73.41	99	87.78
7	*Pseudomonas stutzeri* DSM 10701	PSJM300_17500	256	93	78.63	99	86.77
8	*Pseudomonas frederiksbergensis*	BLL42_28340	256	93	70.45	99	85.77
9	*Pseudomonas mendocina* NK-01	MDS_0218	257	93	78.54	99	87.98
10	*Azotobacter vinelandii* DJ	Avin_43340	259	95	79.05	99	87.17

Further results of the sequence homology analyses using different sets of bacterial species are provided in appendixes.

3.2. Conctruction of plasmids containin inducible systems

Plasmids containing inducible systems were constructed using pBRC1 vector (Fig. 3.4.) (Augustiniene, and Malys, unpublished data) (Augustiniene, and Malys, unpublished data) and the regions of the glc locus identified by a bioinformatics search. pBRC1 contains RFP (Red Fluorescent Protein) reporter gene and chloramphenicol resistance gene. The first plasmid was constructed using the pBRC1 vector and the intergenic region between glcC and glcD sequences, which consists of 269 base pairs. This region was amplified by PCR using Phusion™ High-Fidelity DNA Polymerase, *E. coli* MG1655 DNA as a template and N221, N222 as primers. The pBRC1 plasmid was digested with NdeI and AatII restriction enzymes to remove the arabinose-inducible system. The prepared plasmid fragments and the intergenic region were assembled using T4 DNA ligase. Schematic representation of the construct is shown in Fig. 3.5. The new inducible plasmid was called pSS003.

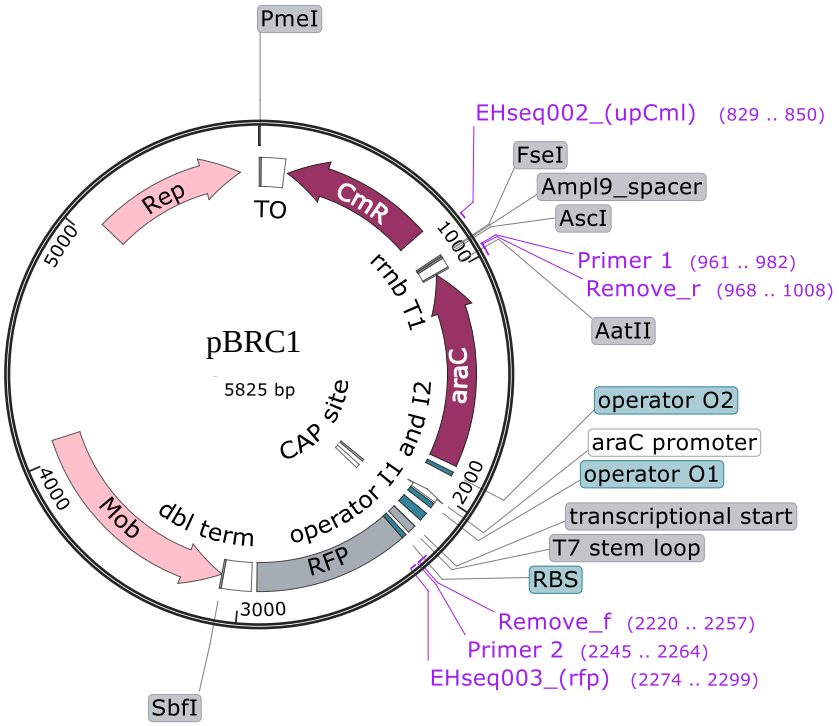


Fig. 3.4. Map of vector pBRC1. CmR represents chloramphenicol resistance gene, RFP – red fluorexcent protein, araC - the arabinose-inducible system. Adapted from Augustiniene and Malys unpublished.

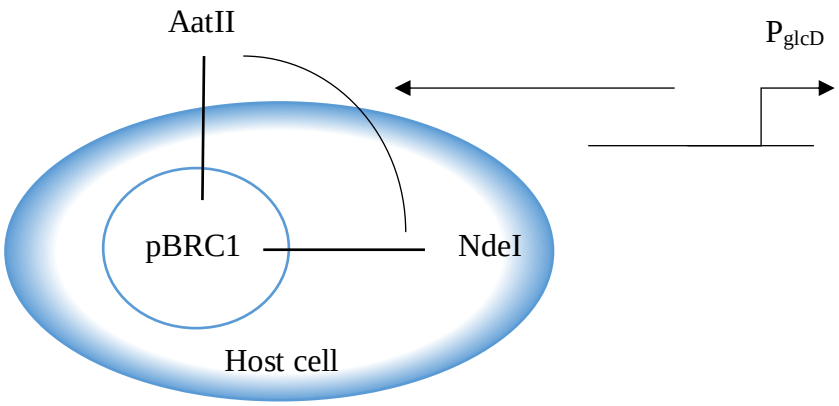


Fig. 3.5. Shematic representation of the construction of the plasmid pSS003.

The second plasmid was constructed using the pBRC1 vector and the intergenic region between *glcC* and *glcD* sequences with the transcription factor. This region consists of 1034 base pairs. The intergenic region with the transcription factor was amplified by PCR using Phusion™ High-Fidelity DNA Polymerase, *E. coli* MG1655 DNA as a template and N221, N223 as primers. The pBRC1 plasmid was digested with the same NdeI and AatII restriction enzymes, and assembled with DNA sequence using T4 DNA ligase. Schematic representation of the construct is shown in Fig. 3.6. The new inducible plasmid was called pSS004.

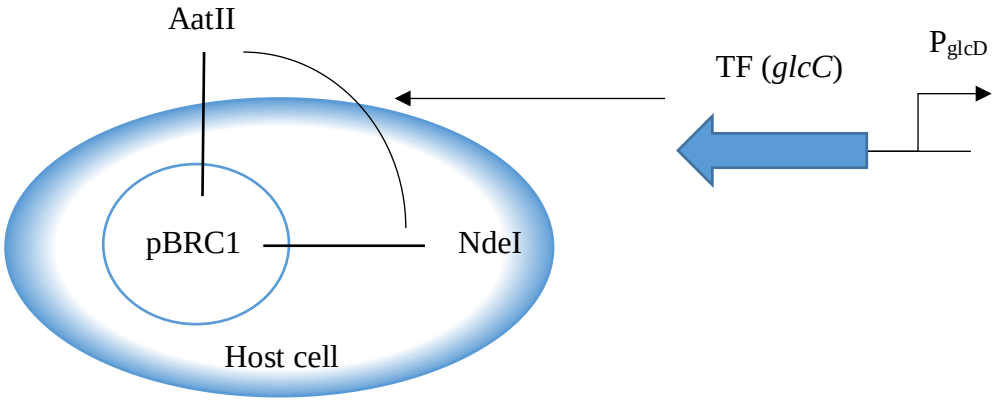


Fig. 3.6. Shematic representation of the construction of the plasmid pSS004.

The third plasmid was assembled using a different method. The plasmid, called pSS004A, was assembled by a HiFi assembly reaction using two different fragments and the pBRC1 plasmid. The purpose of this construct was to get a plasmid with a stronger (p13) promoter. Thefragments were amplified by PCR. The first fragment was amplified using EG070 and EG068 primers to multiply the transcription factor (glcC) and add a synthetic p13 promotor (865 bp) (46). The second fragment was amplified using EG069 and EG013 primers to multiply the intergenic region with RFP (Red Fluorescent Protein). The pBRC1 plasmid was digested with BamHI and AatII restriction enzymes. Schematic representation of construct is shown in Fig. 3.7.

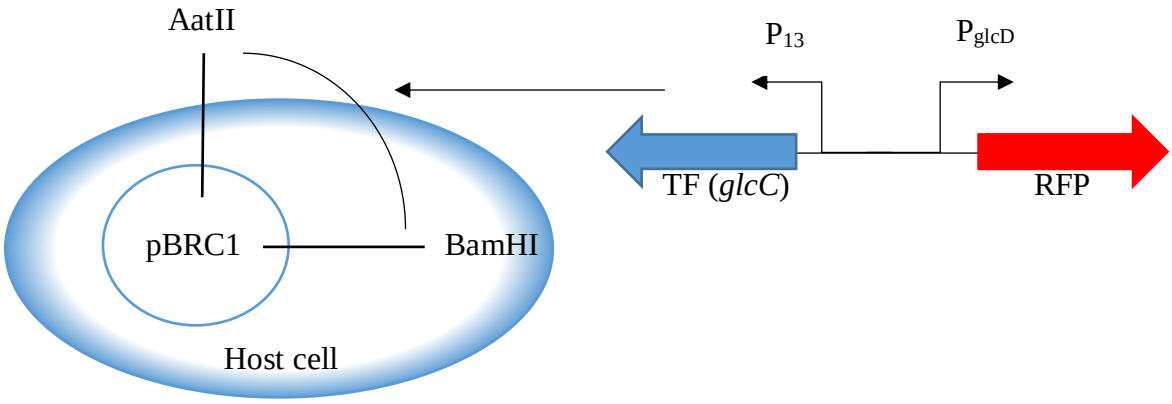


Fig. 3.7. Shematic representation of the construction of the plasmid pSS004A.

3.3. Preparation of the fragments and the plasmids for construction of the new vectors

The first step was to amplify fragments using PCR. This method is described in section 2.9. Fragments used for the construction of plasmid pSS003 were amplified using primers N221 and N222. Fragments used for the construction of plasmid pSS004 were amplified using primers N221 and N223. Fragments for plasmids pSS003 and pSS004 were amplified from genomic *E. Coli* MG1655 DNA. After amplification, all these fragments were loaded on an agarose gel to test if the PCR was successful. Results are shown in Fig. 3.5. Fragments used for the construction of vector pSS004A were amplified from pSS004 vector DNA. The first fragment was amplified using EG070 and EG068 primers to multiply the transcription factor (glcC) and add a synthetic p13 promotor (865 bp). The second fragment was amplified using EG069 and EG013 primers to multiply the intergenic region with RFP (Red Fluorescent Protein).

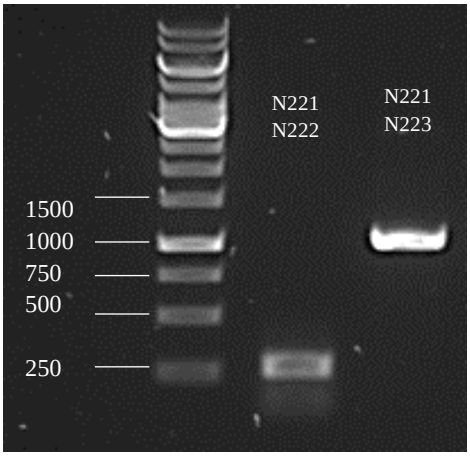


Fig. 3.5. Agarose gels of fragments for the construction of vectors after PCR. Fragment amplified using N221 and N222 primers has 269 bp. Fragment amplified using N221 and N223 primers has 1034 bp.

Prior to digestion with restriction enzymes, all fragments were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Lithuania). The plasmid was purified using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Lithuania).

3.4. Digestion, DNA purification and ligation

Purified plasmid and DNA fragments were digested using NdeI and AatII restriction enzymes (Thermo Fisher Scientific, Lithuania) to construct vectors pSS003 and pSS004. Vector pSS004A was constructed using a plasmid digested with AatII and BamHI restriction enzymes. Fragments assembled to this vector already had restriction sites attached by primers during the PCR. Digestion was performed as described in section 2.10. After digestion, the DNA fragments were loaded on an agarose gel to separate the fragments by electrophoresis. The plasmid and the digested fragments were extracted from the agarose gel with the Zymoclean Gel DNA Recovery Kit (Zymo Research Corporation, USA) according to the manufacturer's protocol as described in section 2.12. The plasmid and fragments were cut out from the gel with a scalpel under UV light (SafeLight Box, Uvitec, UK). DNA was cut from the gel as fast as possible to prevent damaging DNA under UV light so no photos were taken using Uvitec Essential Gel Imaging system (Uvitec Cambridge, UK) under the ultraviolet (UV) lamp. Purified DNA were measured by Nanophotometer N60 (Implen, Germany) to determine concentrations (ng/µl). After that, ligation was done as described in section 2.13. to construct vectors pSS003 and pSS004. Construction of vector pSS004A was performed by HiFi DNA assembly using NEBuilder HiFi DNA Assembly Master Mix as described in section 2.14. When vectors pSS003, pSS004 and pSS004A were assembled, heat-shock transformations were performed as described in section 2.15. using *E. coli* TOP10 cells. Transformed cell were cultivated overnight on LB agar plates with chloramphenicol. About 20 to 50 colonies were visible in every Petri dish after overnight cultivation, while Petri dishes with negative control were empty.

3.5. Bacteria screening after transformation

The first step to confirm if the constructed and ligated vectors were assembled correctly was to perform a colony PCR test. Three randomly chosen colonies were picked from the Petri dish and a colony PCR test was performed as described in section 2.9 using DreamTaq PCR Master Mix (Thermo Fisher Scientific, Lithuania). Fig. 3.6. presents colony PCR results after electrophoresis. Vector pSS003 was tested using primers N221 and N222 to see if the vector’s intergenic region is of the right size, which should be 269 bp long. Vector pSS004 was tested using primers N221 and N223 to see if this vector has an intergenic region with the transcription factor, which should be 1034 bp

long. As demonstrated by the results, all three colonies of each vector had the right size of DNA inserted.

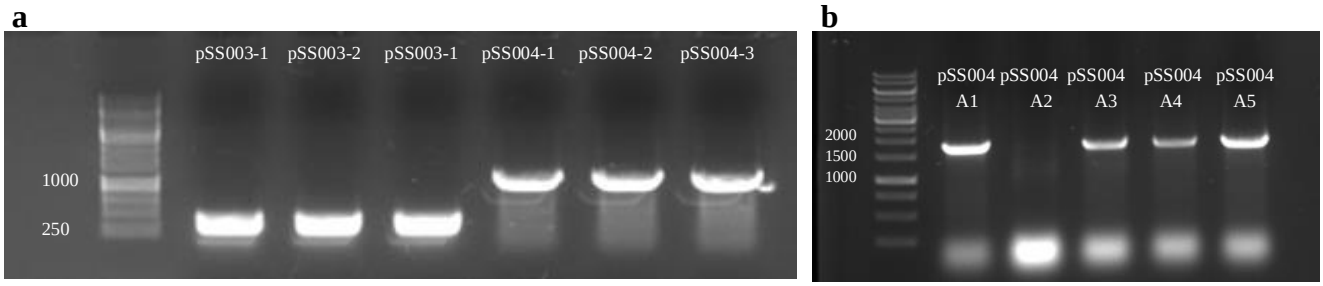


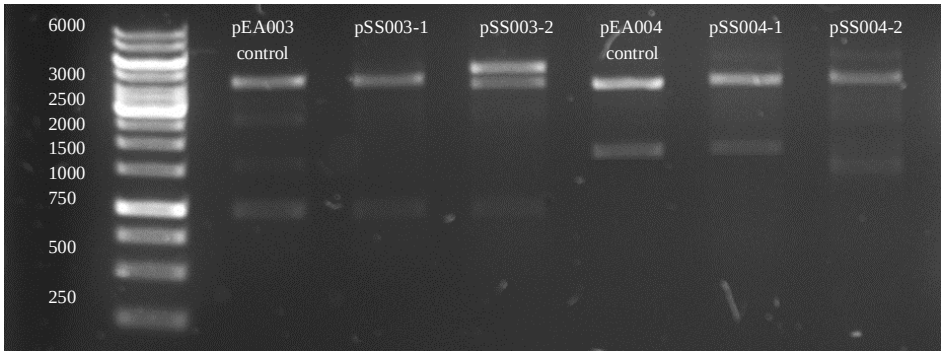
Fig. **3.6.** Electrophoresis results for colonies PCR. (a) Colonies PCR for vectors pSS003 (269 bp length) and pSS004 (1034 bp length). (b) Colonies PCR for vector pSS004A (1840 bp length).

Vector pSS004A was tested using primers EG070 and EG013 to see if both fragments were assembled to this vector. The expected length of the merged fragments was 1840 bp.As demonstrated in Fig. 3.6. (b), four out of five colonies had a correctly assembled vector.

3.6. Restriction analysis of constructed vectors

Restriction analysis was the next step needed to confirm whether the vectors were assembled correctly and to ensure that there were no false-positive colony PCR results. Digestion reaction mixtures were prepared as described in section 2.10. AatII and BamHI restriction enzymes were used for all vectors. As BamHI restriction enzyme works more efficiently than NdeI, it was used to test pSS003 and pSS004 vectors as well. After digestion, electrophoresis was performed and the results are presented in Fig. 3.7. After digestion with AatII and BamHI, the pSS003 vector was expected to a have 930 bp long fragment, vector pSS004 – a 1695 bp long fragment, and vector pSS004A – a 1840 bp long fragment. Vectors pEA003 and pEA004 (E. Augustiniene, unpublished) were used as control vectors because these vectors are analogous to pSS003 and pSS004, respectively. Results presented in Fig. 3.7. (a) demonstrate that vectors pSS003-1 and pSS004-1 were digested as expected,compared to controls. Vector pSS003-2 was not fully digested even though the same conditions were used. Vector pSS004-2 may have some errors compared to the control vector, as its digested fragment is a bit shorted than the control.

a



b

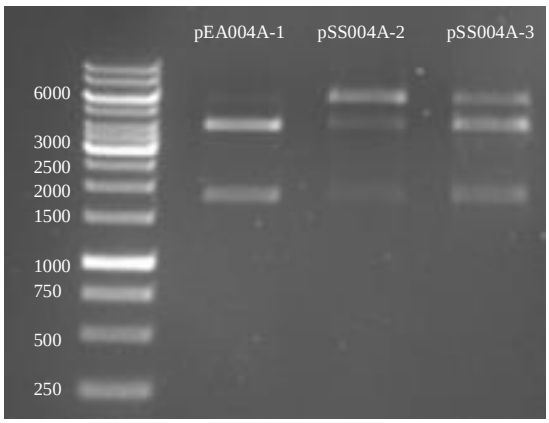


Fig. 3.7. Restriction analysis of vectors from colonies. (a) Restriction analysis of colonies of transformed cells with vectors pSS003 and pSS004 respectively. (b) Restriction analysis of colonies of transformed cells with vector pSS004A.

Restriction analysis of colonies containing vector pSS004A showed that all three randomly selected colonies had this vector with the putative sequence of DNA, which should be 1840 bp long. Vector pSS004A was almost fully digested, while other two vectors were not, even though the same conditions were used

3.7. RFP fluorescence assay using different concentrations of glycolic acid

An inducible system can be evaluated for its ability to control gene expression. The fluorescence output can be visualized and quantified using specific equipment; in this research Tecan Infinite 200 Pro plate reader (Grödig, Austria) was used. Other suitable equipment could include flow cytometry, fluorescence spectrophotometers or fluorescence microscopes. Plate readers allow to test a large number of samples and to determine the characteristics of the system, for example, specificity towards inductors or dose-response [49]. The induction level is usually the first indicator of the inducible system's performance. The method of this assay is described in section 2.16.

At first, whole-cell biosensors pSS003 and pSS004 were tested with only one inductor - 5mM of sodium glycolate - in order to establish whether the constructed inducible system works. Two isolates of each vector were tested after heat-shock transformation. Results presented in Fig. 3.8.a demonstrate that each isolate of vector pSS003 had a stable growth rate. The absolute normalized fluorescence value plots revealed that only one isolate responded to the inducer, despite a similar growth rate and a positive restriction analysis result (Fig. 3.8.b). Since one of the biosensors was responding to the inducer, analysis using different concentrations of glycolic acid was performed (Fig. 3.8.c) The results suggested that the optimal glycolic acid concentration for the pSS003 biosensor could be 20 mM. The fluorescence was weaker at 40 mM of glycolic acid, compared to 20 mM. The dose-response curve provides more information since it covers the full range of ligand concentration that results in a measurable change in reporter output—from the minimum compound concentration required to elicit an induction of gene expression, to the maximum ligand concentration that saturates the system. It correlates relative normalized fluorescence values and ligand concentrations at a specific time point **[49]**. The relative normalized fluorescence was calculated, by substracting the mean fluorescence response from the induced fluorescence response. The dose-response curve of the glycolic acid inducible system pSS003, 12h after the addition of the ligand, is presented in Fig.3.8.d. The results of this curve suggest that the whole-cell biosensor pSS003 works in a wide range of concentrations

and it is possible to evaluate the relative fluorescence response even when the sodium glycolate concentration is as low as 0.16 mM.

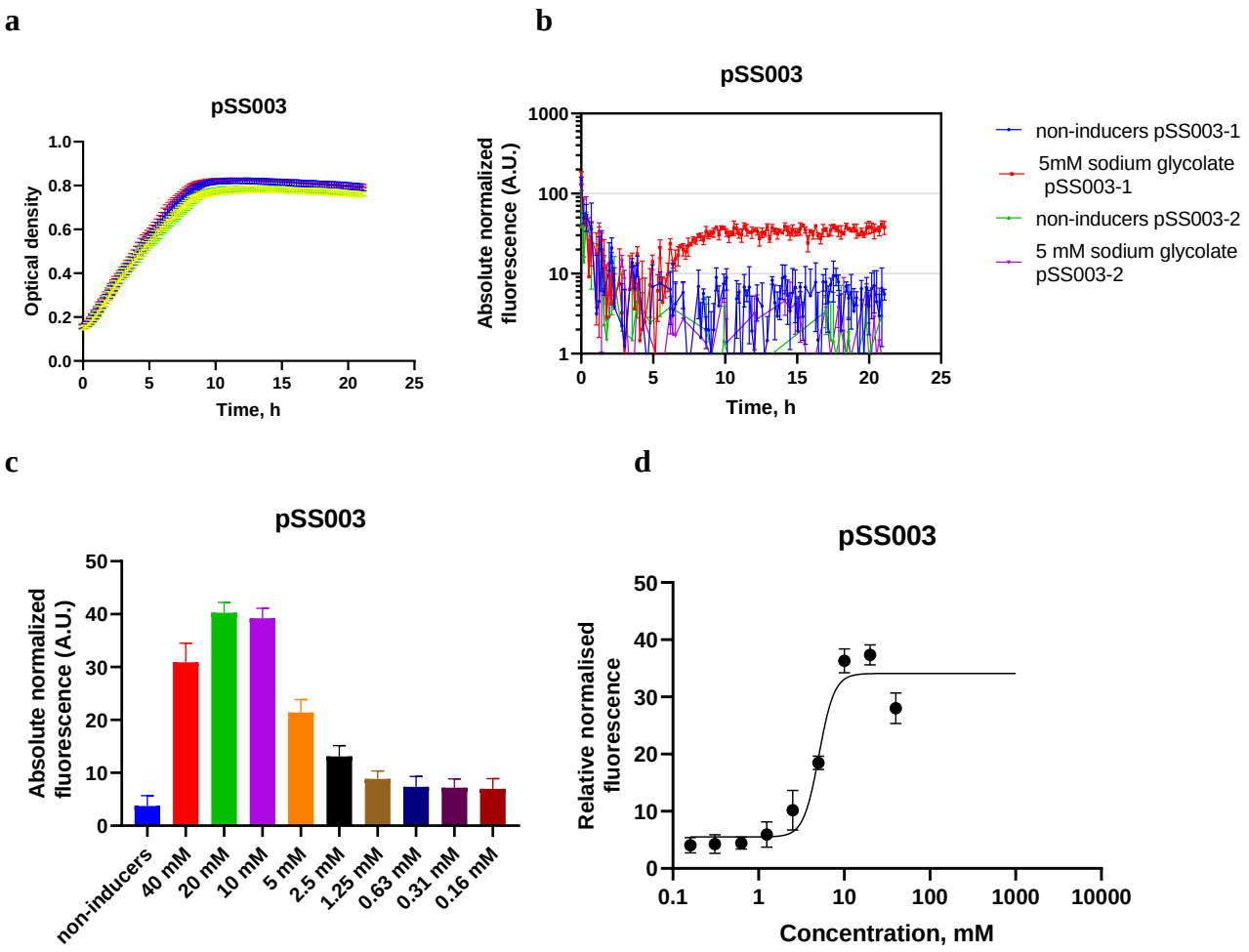


Fig. 3.8. A growth rate and fluorescence of the whole-cell biosensor pSS003 indusible system using sodium glycolate as an inducer. (a) Biosensor pSS003 uninduced and induced with 5 mM sodium glycolate. (b) Biosensor pSS003 uninduced and induced with 5 mM sodium glycolate. (c) Absolute normalized fluorescence using different concentrations of sodium glycolate 12 hours after addition of inductor. (d) The dose-response curve of sodium glycolate inducible system 12 hours after addition of the inductor.

When the two isolates of the whole-cell biosensor pSS004 were tested, similar growth rates to pSS003 were observed (Fig. 3.9.a), but only one isolate of pSS004 was responding to an inducer (Fig. 3.9.b.) The absolute normalized fluorescence test also revealed that the whole-cell biosensor pSS004 has a stronger fluorescence than pSS003, so its response to an inducer seemed stronger. An analysis of the working pSS004 biosensor using different concentrations of glycolic acid was performed as well (Fig. 3.9.c). The results suggested that the most optimal concentration of glycolic acid for pSS004 could be 20 mM. The fluorescence was weaker at 40 mM concentration of glycolic acid compared to 20 mM. The dose-response curve of the glycolic acid inducible system pSS004, 12h after the addition of the ligand, is presented in Fig.3.9.d. The results of this curve suggest that the biosensor pSS004 works in a wide range of concentrations, the same as the biosensor pSS003.

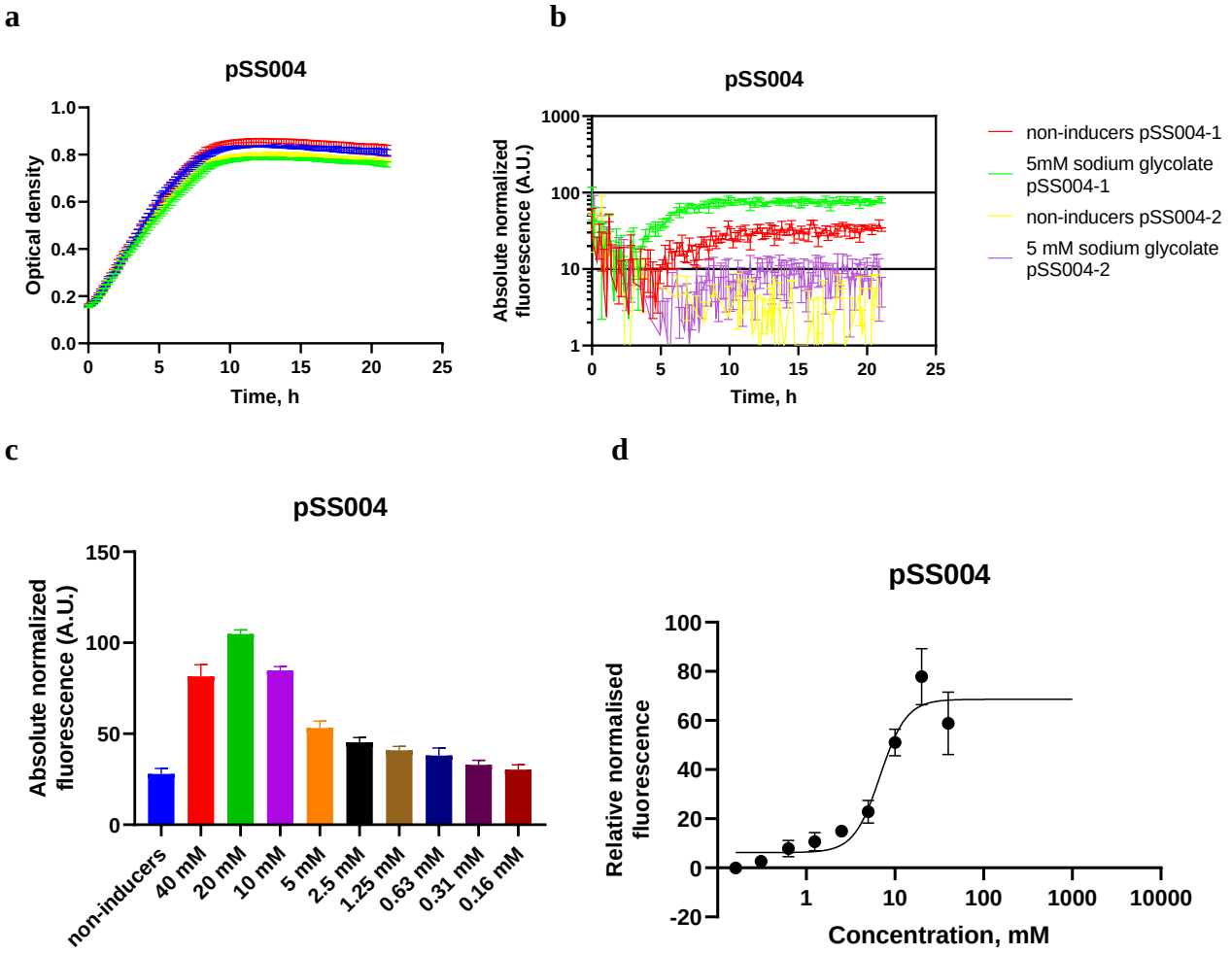


Fig. 3.9. A growth rate and fluorescence of the whole-cell biosensor pSS004 indusible system using sodium glycolate as an inducer. (a) The whole-cell biosensor pSS004 uninduced and induced with 5 mM sodium glycolate. (b) Isolates of vector pSS004 uninduced and induced with 5 mM sodium glycolate. (c) Absolute normalized fluorescence using different concentrations of sodium glycolate 12 hours after addition of inductor. (d) The dose-response curve of sodium glycolate inducible system 12 hours after addition of the inductor.

3.8. RFP fluorescence assay using different inductors

It was also important to evaluate the specificity of the whole-cell biosensor by using different acids such as D- and L- lactic, glyoxylic, 3-hydroxypropionic and glycolic acids. The results indicated that both whole-cell biosensors, with pSS003 and pSS004 vectors accordingly, could grow in LB media using all the listed acids as inductors (Fig. 3.10 a and Fig. 3.11 a), but the fluorescence response was strongest when using glycolic acid (Fig. 3.10 b and Fig. 3.11 b). To evaluate specificity, the relative fluorescence response was also calculated, and the results demonstrated that both whole-cell biosensors are able to respond to glycolic acid among other structurally related acids (Fig. 3.10 c and Fig. 3.11 c). The whole-cell biosensor with the pSS003 vector demonstrated a minor fluorescence response to all listed acids (Fig 3.10 c), but the biosensor with the pSS004 vector was only able to respond to D- lactic, 3-hydroxypropionic and glyoxylic acids, in descending order (Fig. 3.11 c).

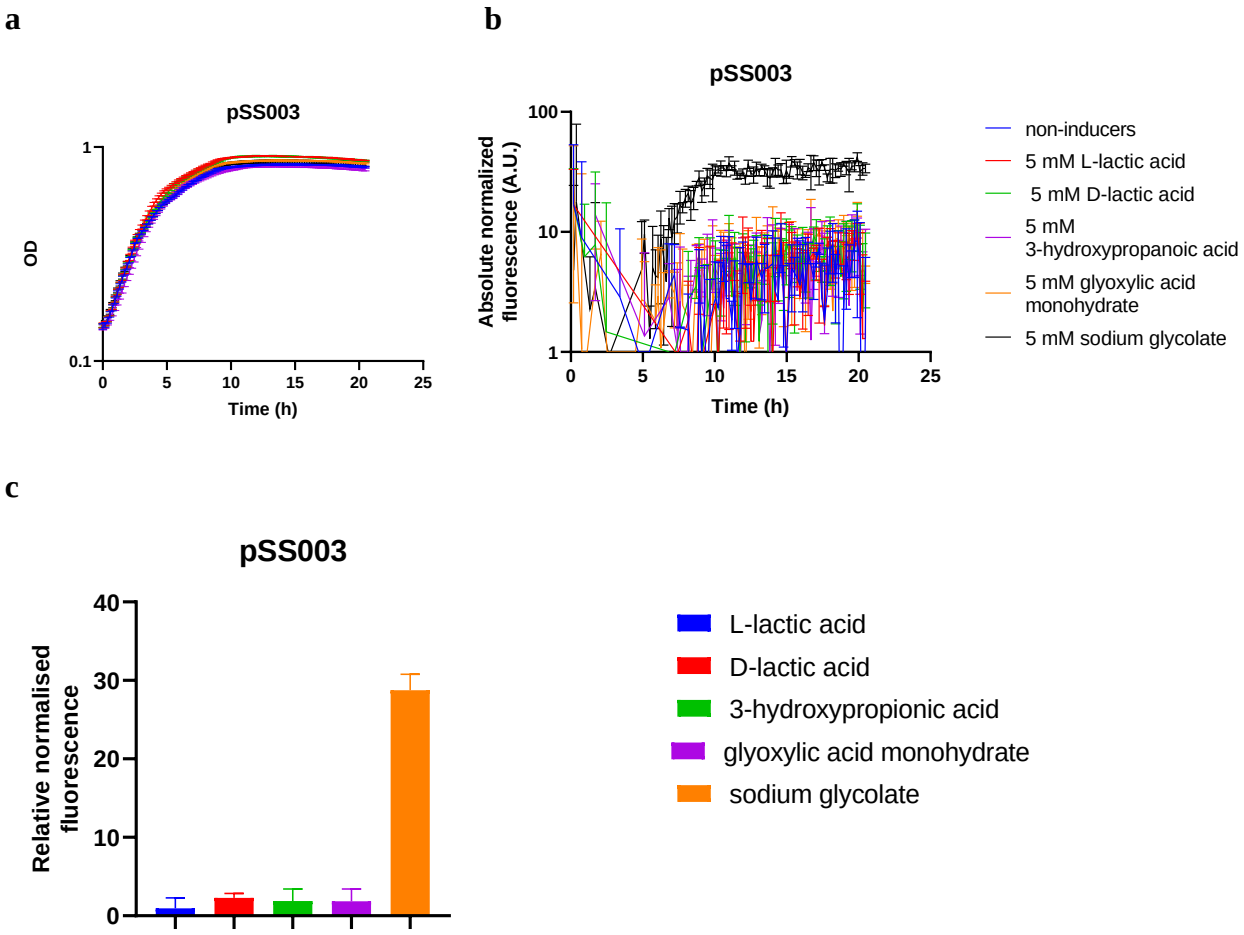
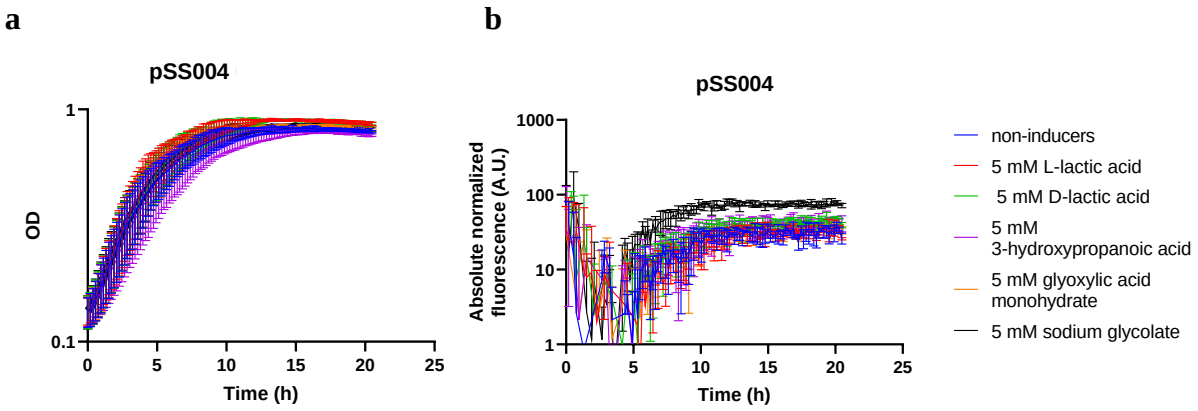


Fig. 3.10 A growth rate and fluorescence analyses with a whole-cell biosensor with vector pSS003 specificity analyses using L- and D- lactic, 3-hydroxypropionic, glyoxylic and glycolic acids. (a) A growth rate of non-iduced and induced biosensor. (b) Absolute normalized fluorescence of non-iduced and induced biosensor. (c) Relative normalized fluorescence of biosensor.



c

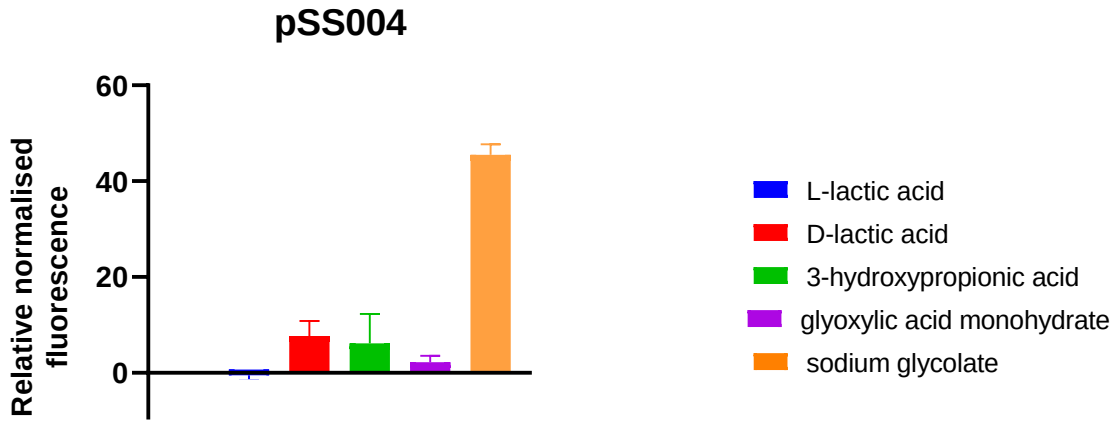


Fig. 3.11 A growth rate and fluorescence analyses with a whole-cell biosensor with vector pSS004 specificity analyses using L- and D- lactic, 3-hydroxypropionic, glyoxylic and glycolic acids. (a) A growth rate of non-iduced and induced biosensor. (b) Absolute normalized fluorescence of non-iduced and induced biosensor. (c) Relative normalized fluorescence of the biosensor.

3.9. Performance of the biosensor in the presence of different carbon sources

The performance of the biosensor in M9 minimal medium was important to test catabolite repression, as it is an important part of the control system of various microorganisms. Catabolite repression allows microorganisms to adapt quickly to a preferred carbon source. This is usually achieved by inhibiting synthesis of enzymes involved in the catabolism of carbon sources other than the preferred one. The catabolite repression can usually be initiated by glucose, but other carbon sources are known to induce catabolite repression as well**. [45]** The whole-cell biosensors pSS003 and pSS004 were tested in M9 minimal medium using glucose, D- and L- lactic, glyoxylic or glyxolic acids as a sole carbon source. Both whole-cell biosensors were incubated over night as described in section 2.5 using M9 minimal medium. OD_{600} was measured as described in section 2.16. Cells did not grow using glyoxylic and glycolic acids as a carbon source, but showed minor growth using D- and L- lactic acids. As expected, glucose was the most suitable carbon source (Fig. 3.12).

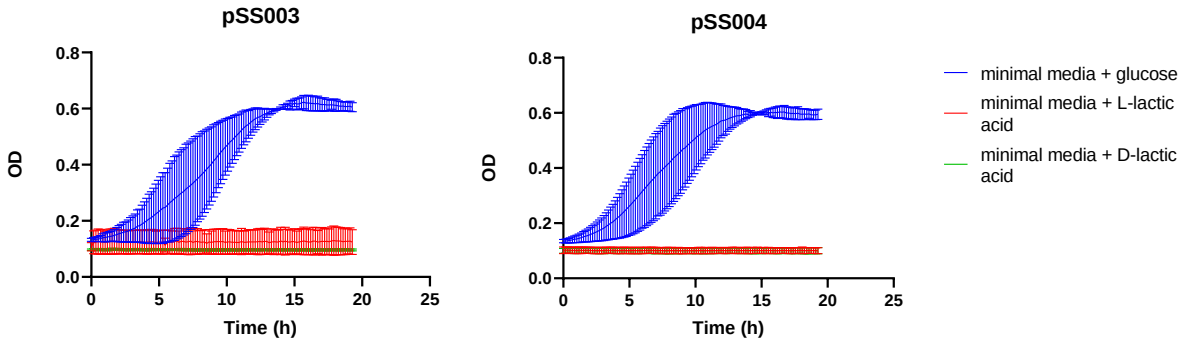


Fig. 3.12 OD_{600} measured as described in section 2.16.of the whole-cell biosensors pSS003 and pSS004.

It was suggested that a plate reader was not an appropriate tool to grow cells in M9 minimal medium with different carbon sources, besides glucose, as it was possible that cells did not get enough aeration. For this reason, whole-cell biosensors pSS003, pSS004, pSS004A and *E. coli* Top10 cells with plasmid pBRC1 as a control, were incubated for three nights and OD was measured after each night using a spectrophotometer. This test was performed to check whether better aeration, longer incubation time, and a larger medium volume could have a positive impact on growth rate. Results did not show any improvement (Fig. 3.13), so it should be further explored in future studies.

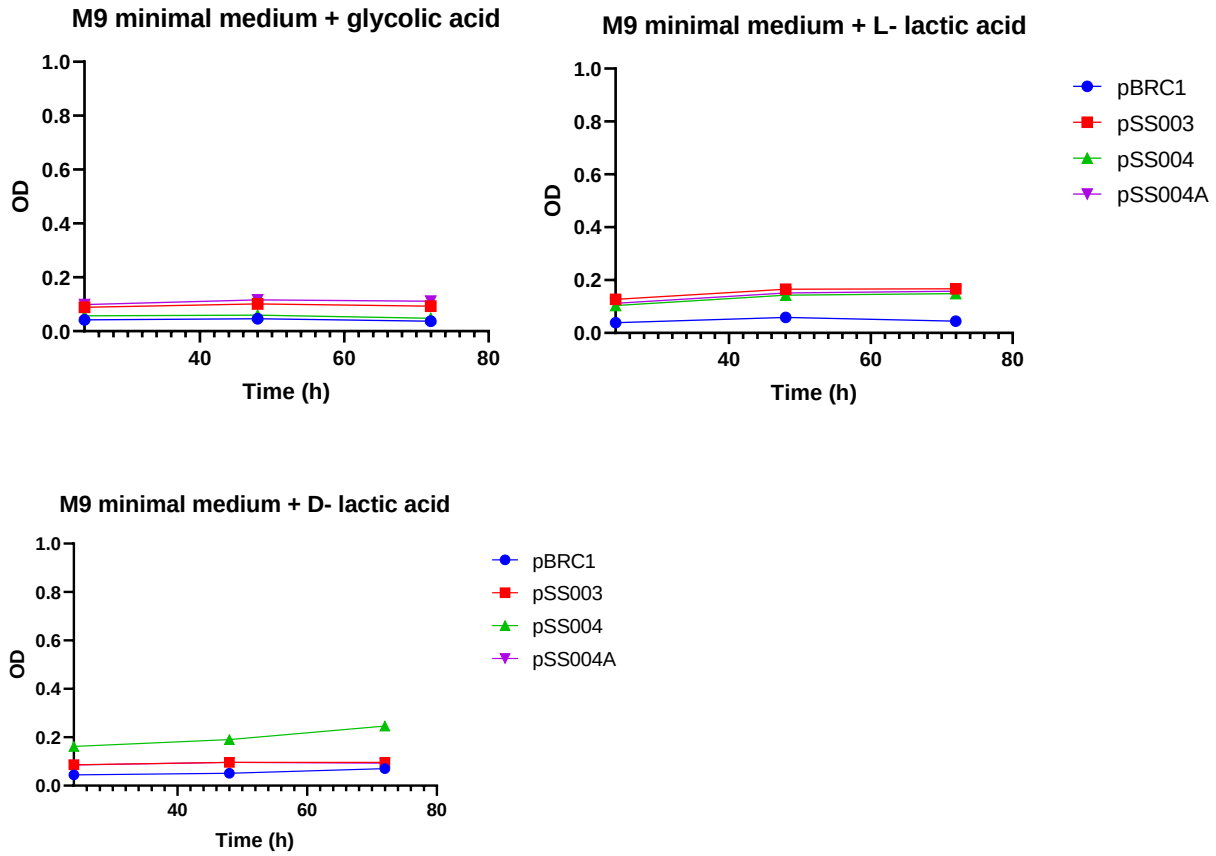


Fig. 3.13 OD_{600} of thw whole-cell biosensors pSS003, pSS004, pSS004A and control plasmid pBRC1 measured with spectrophotometer, after 24, 48 and 72 hours, using different source of carbom in M9 minimal medium.

3.10. RFP fluorescence assay of improved whole-cell biosensor using different concentrations of glycolic acid and using different inductors

Construction of an improved whole-cell biosensor pSS004A was described in section 3.2. The purpose of this improvement was to achieve better performance. It was expected that an additional, stronger promoter – P13, and an increased amount of transcription factors should give a stronger fluorescence effect. Construction of such a whole-cell biosensor was successful, and the results of its restriction analyses are described in section 3.6. The whole-cell biosensor pSS004A was tested the same way as earlier biosensors – pSS003 and pSS004. Results demonstrated that the improved biosensor was able to achieve a similar growth rate to previously constructed whole-cell biosensors (pSS003 and pSS004), indicated by optical density (Fig. 3.14 a), but its fluorescence was unexpectedly weaker. Results presented in Fig. 3.14 b confirmed that the whole-cell biosensor

pSS004A is specific to glycolic acid, but results listed in Fig. 3.14 c verified that its relative normalised fluorescence was not high enough. This biosensor has also a minor fluorescence to D-lactic and 3-hydroxypropionic acids used as inductors, same as the other constructed whole-cell biosensors. Different concentrations of glycolic acid were also used as an inductor to test performance of this new whole-cell biosensor. Results presented in Fig. 3.14 d demonstrate that this biosensor was able to respond to concentrations from 0,08 mM to 40 mM of glycolic acid, but, as demonstrated in Fig. 3.14 e, its relative normalized fluorescence was weaker than the fluorescence of the whole-cell biosensor pSS004 (Fig. 3.9 d).

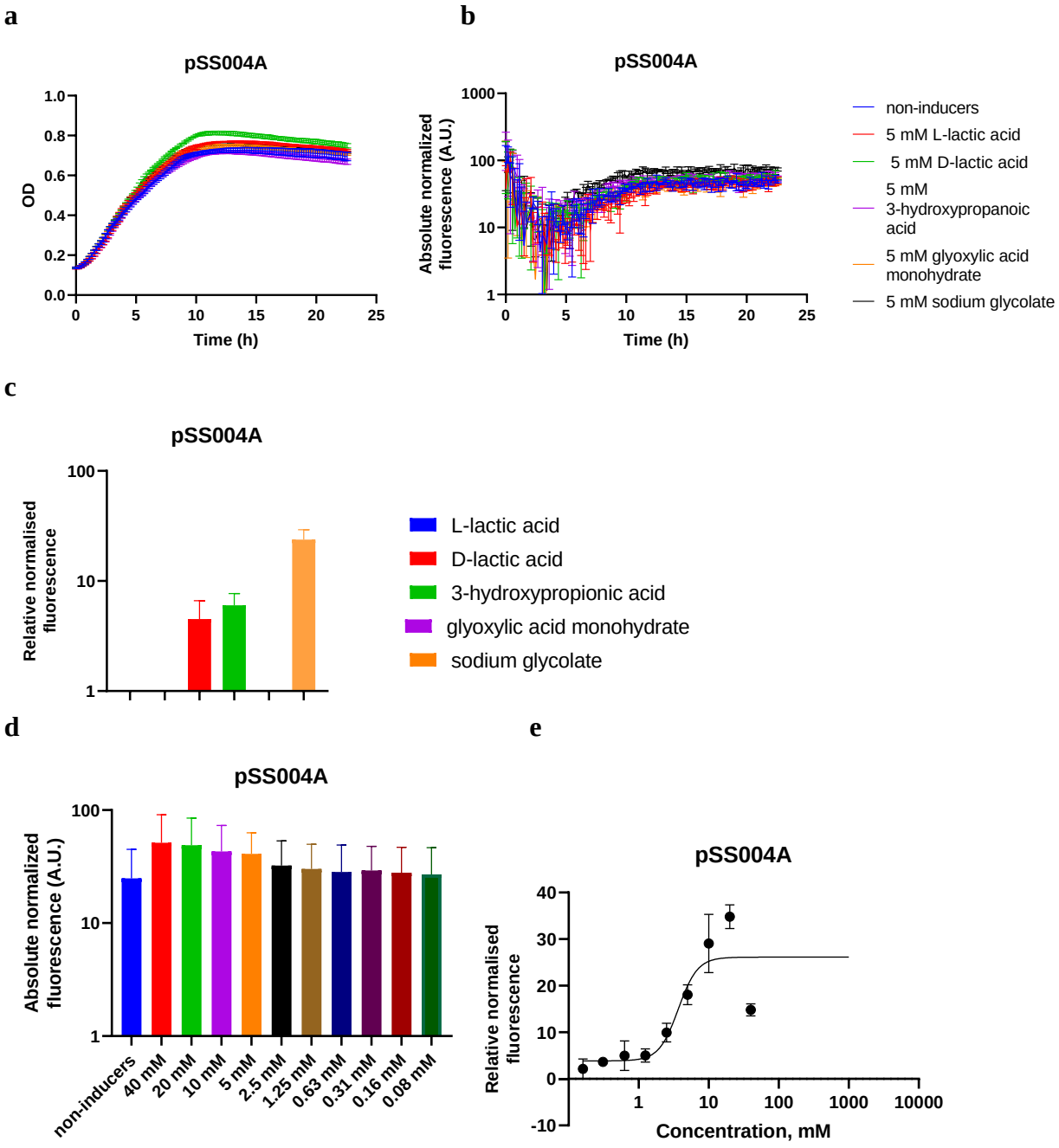


Fig. 3.14 Analyses of the improved whole-cell biosensor pSS004A. (a) A growth rate of non-iduced and induced the whole-cell biosensor pSS004A (b) Specificity analyses of the whole-cell biosensor pSS004A using L- and D- lactic, 3-hydroxypropionic, glyoxylic and glycolic acids. (c) Relative normalized fluorescence of specificity analyses. (d) Absolute normalized fluorescence using different concentrations of glycolic acid 12

hours after addition of inductor. (e) The dose-response curve of sodium glycolate inducible system 12 hours after addition of the inductor.

By comparing fold induction of all three whole-cell biosensors – pSS003, pSS004 and pSS004A (Fig. 3.15), it was demonstrated that the improvement of the biosensor pSS004A was not successful. These results suggest that the response to glycolic acid was most effective when the biosensor had the lowest concentration of TF, while higher concentration of TR lead to a weaker response to glycolic acid.

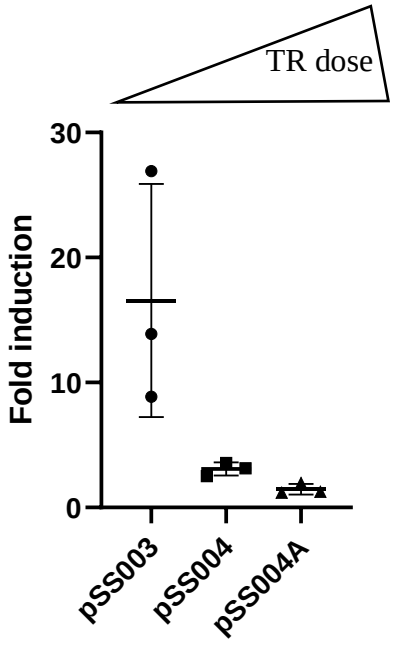


Fig. 3.15 Graphic of fold induction between three whole-cell biosensors – pSS003, pSS004 and pSS04A. Fold induction calculated using fluorescence response 8, 10 and 12 hours after induction.

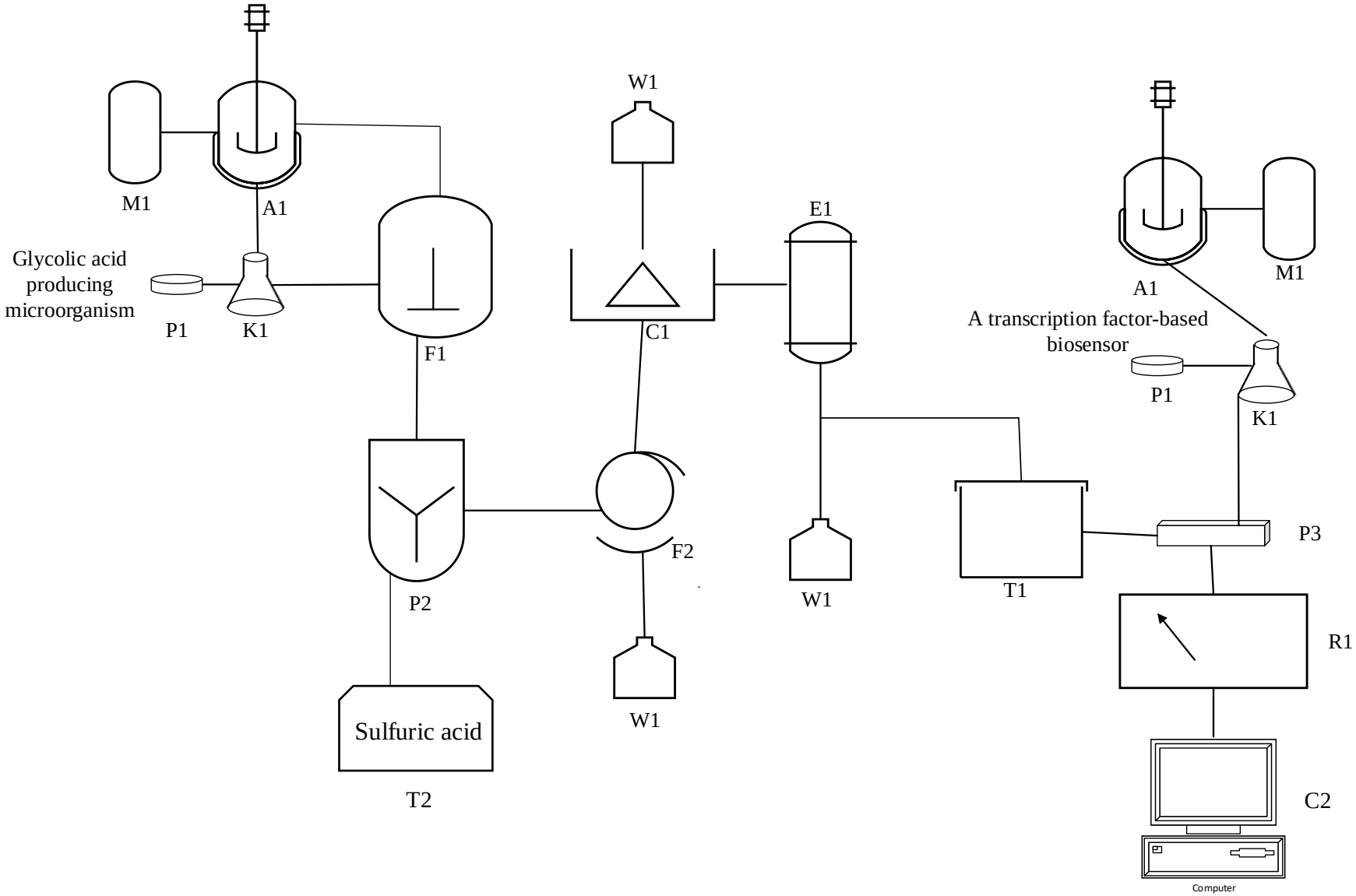
4. Recommendations part

Research of transcription factor-based biosensors is a novel and applicable field, which is widely developing. This research had a start for developing a transcription factor-based biosensor, which specifically responded to glycolic acid, but further investigation is needed to explore its full potential for various applications.

Investigation of constructed whole-cell biosensors in the presence of different carbon sources is needed for a better understanding of their catabolism. For this purpose, constructed vectors pSS003, pSS004, and pSS004A could be transformed into more suitable, than E. coli Top10, competent cells.

Constructed vectors (pSS003, pSS004, and pSS004A) could be transformed into other related species for more detailed metabolic analyses. Such studies could better describe the functionality of inserted intergenic region and transcription factor.

Improved and more investigated transcription factor-based biosensors could be gradually tested in industrial conditions. The fundamental technological scheme of such an example is presented in Fig. 4.1. This scheme consists of two parts: the first part describes the purification of glycolic acid, the second part describes the appliance of a transcription factor-based biosensor, using purified acid. Glycolic acid could be purified using a common method of lactic acid purification which includes neutralization and precipitation of salt with acid. The first step is to revive glycolic acid-producing culture on a Petri dish (P1) and when the colonies on a Petri dish are the right size, transfer it into the liquid medium (K1). Medium from the medium capacity (M1) must be autoclaved (A1) before any culture is transferred in it. When the colonies reach the required OD in K1, some of the inoculate must be transferred to the fermenter (F1). Excess calcium carbonate or calcium hydroxide needs to be added to the fermenter to neutralize the acid produced. Later, the fermentation broth is poured into the precipitator (P1) where this broth is treated with sulfuric acid (S1) to precipitate the calcium sulfate, which is later filtered using a rotary filter (F2) and gypsum is separated to the waste container (W1). The remaining mass, after filtration through a rotary filter (F2), is displaced to centrifuge (C1) to separate other biomass impurities before the evaporation process. Separated mass is transferred to an evaporator (E1) and purified glycolic acid is transferred to the product tanker (T1). The second step of the listed scheme consists of the appliance of the transcription factor-based biosensor. First, biosensors must revive the same way as glycolic acid-producing microorganisms. Then, the transcription factor-based biosensor needs to be transferred to a plate (P3) adapted to industrial needs. This whole-cell biosensor is induced in the same plate (P3) using purified glycolic acid and after that, the plate needs to be inserted into a plate reader (R1) immediately. Reader (R1) must measure OD and fluorescence under strict parameters. The specific computer programs process information from the reader (R1) and calculations of concentration of glycolic acid or other parameters is done (C2).


M1
A1
Glycolic acid producing microorganism
P1
K1
F1
W1
C1
E1
A transcription factor-based biosensor
P2
F2
W1
W1
T1
P3
R1
Sulfuric acid
T2
C2
Computer

Conclusions

Based on the results of the conducted research, it was possible to implement most of the objectives, and the following conclusions were made:

1. Glc operon in E. coli MG1655 and P. putida KT2440 was identified using bioinformatics tools. A sequence of the intergenic region between glcC and glcD genes and the transcription factor was identified in E. coli MG1655. Whereas in the case of P. putida KT2440 besides sequences of the intergenic region between glcC and glcD genes, and the transcription factor, the conservative sequences of potential transcription factor binding sites, as well as -35 and -10 motifs of the promoter, were identified.
2. Using genetic elements of the inducible system and E. coli as a host strain, three different whole-cell biosensors based on plasmids pSS003, pSS004, and pSS004A were constructed.
3. The dose-response and dynamic range of the whole-cell biosensors were measured.
4. Due to the lack of growth using L- and D- lactic, glyoxylic, and glycolic acids as sole carbon sources, it was not possible to evaluate the performance of biosensors in the presence of different carbon sources other than glucose.
5. The specificity analyses of biosensors using compounds structurally similar to lactic acid showed that all three constructed whole-cell biosensors were specific to glycolic acid and showed a minor response to D- lactic and 3-hydroxy propionic acids. All three constructed whole-cell biosensors were capable to work in a wide range of glycolic acid concentrations, from 0.08 mM to 40 mM.
6. To improve the performance of the whole-cell biosensor by changing the strength of promoter that controls transcription factor glcC gene expression, the construct pSS004A containing a strong promoter P13 in front of glcC gene was developed. However, a dynamic range investigation using whole-cell biosensors based on pSS003, pSS004, and pSS004A showed the following induction results: 9 to 27 – fold, 2.5 to 3.6 – fold, and 1.2 to 2 – fold, respectively, measured after 8, 10, and 12 hours after inducer addition. This revealed that having the least amount of transcription factor had the most effective response to glycolic acid.

List of references

1. F. A. CASTILLO, E. M. BALCIUNAS, J. M. SALGADOJOSÉ, M. SALGADO. Lactic acid properties, applications and production: A review. *Trends in Food Science & Technology* [online]. 2013, 30(1):70-83 [accessed 2021-02-20]. DOI: 10.1016/j.tifs.2012.11.007

2. M. SAN-MARTÍN, C. PAZOS, J. COCA. Reactive extraction of lactic acid with Alamine 336 in the presence of salts and lactose. *Journal of chemical technology and biotechnology* [online]. 1992, 54(1):1-6 [accessed 2021-02-25]. doi.org/10.1002/jctb.280540102

3. M. SINGHVI, D. JOSHI, M. ADSUL, A. VARMAB, D. GOKHALE. d-(−)-Lactic acid production from cellobiose and cellulose by Lactobacillus lactis mutant RM2-24. *Green chemistry* [online]. 2010 6:120-126 [accessed 2021-03-21]. https://doi.org/10.1039/B925975A

4. B. S. K. SAIBABA, K. V. SAIBABA, K.V. SAI NIKHILESH, G. S. N. GANTALA. Industrial production of lactic acid and its applications. *International journal og biotech research* [online]. 2018 1(1) [accessed 2021-02-04]. ISSN 2328-3505

5. SALMINEN, S. OUWEHAND, A. C. WRIGHT, A. VON DALY, C. Future aspects of research and product development of lactic acid bacteria. *Lactic acid bacteria: microbiology and functional aspects* [online]. Marcel Dekker, New York, No.Ed.3 pp.611-615 ref.2, 2004. [accessed 2021-02-02]. ISBN : 0824753321

6. S. NORTON, C. LACROIX, J. VUILLEMARD. Kinetic study of continuous whey permeate fermentation by immobilized Lactobacillus helveticus for lactic acid production. *Enzyme and Microbial Technology* [online]. 1994 16(6): 457-466. [accessed 2021-03-15] DOI:10.1016/0141-0229(94)90015-9

7. L. D. LEE, K. D.S. LEE. A kinetic model for lactic acid production in Kimchi, a Korean fermented vegetable dish. J*ournal of Fermentation and Bioengineering* [online].1993 75(5): 392-394. [accessed 2021-02-20]. doi.org/10.1016/0922-338X(93)90142-U

8. SUDHEER K. SINGH, SYED U. AHMED, A. PANDEY. Metabolic engineering approaches for lactic acid production. *Process Biochemistry* [online]. 2006 41(5): 991-1000. [accessed 2021-02-02]. doi.org/10.1016/j.procbio.2005.12.004

9. J. A. BOUWSTRA, M. PONEC. The skin barrier in healthy and diseased state. *Biochimica et Biophysica Acta (BBA) - Biomembranes* [online]. 2006 1758(12): 2080-2095. [accessed 2021-03-02]. doi.org/10.1016/j.bbamem.2006.06.021

10. K. E. BURKE, J. CLIVE, G. F. COMBS, J. COMMISSO, C. L. KEEN, R. M. NAKAMURA. The effects of topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet irradiation in Skh:2 hairless mice. *Nutrition and Cancer* [online]. 2000 38(1):87-97. [accessed 2020-03-03]. doi.org/10.1207/S15327914NC381_13

11. A. K. S. BASKAR. Lactic Acid Production and Its Application in Pharmaceuticals. *Bioactive Natural products in Drug Discovery* [online]. 2020 Springer, pp 467-484. [accessed 2021-02-12]. ISBN: 978-981-15-1394-7

12. R. DATTA, M. HENRY. Lactic acid: recent advances in products, processes and technologies – a review. *Journal of chemical technology and biotechnology* [online]. 2006 81(7): 1119-1129. [accessed 2021-02-26]. doi.org/10.1002/jctb.1486

13. MARTIN B. PEDERSEN, P. GAUDU, D. LECHARDEUR, M-A. PETIT, A. GRUSS. Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. *Annual Review of Food Science and Technology* [online]. 2012 3:37-58. [accessed 2021-02-02]. doi.org/10.1146/annurev-food-022811-101255

14. K. H. KYLA-NIKKILA. Metabolic engineering of Lactobacillus helveticus CNRZ32 for production of pure L-(þ)-lactic acid. *Lactic Acid in the Food Industry* [online]. 2017 Springer 33-34. [accessed 2021-03-25]. ISBN: 978-3-319-58146-0

15. CHAO GAO, CUIQING MA, PING XU. Biotechnological routes based on lactic acid production from biomass. *Biotechnology Advances* [online]. 2011 29(60): 930-939. [accessed 2021-03-02]. doi.org/10.1016/j.biotechadv.2011.07.022

16. CONCEPCIÓN N. BURGOS-RUBIO, MARTIN R. OKOS, PHILLIP C. WANKAT. Kinetic study of the conversion of different substrates to lactic acid using Lactobacillus bulgaricus. *Biotechnology progress* [online]. 2000 16(3): 305-314. [accessed 2021-03-25]. doi.org/10.1021/bp000022p

17. J. M. BRUNO-BÁRCENA, A. L. RAGOUT, P. R. CÓRDOBA & F. SIÑERIZ. Continuous production of L(þ)-lactic acid by Lactobacillus casei in two-stage systems. *Applied Microbiology and Biotechnology* [online]. 1999 51:316-324. [accessed 2021-04-01]. doi.org/10.1007/s002530051397

18. F. MOZZIRAÚL R. RAYAGRACIELA M. VIGNOLO. Updates in the metabolism of lactic acid bacteria. *Biotechnology of Lactic Acid Bacteria: Novel Applications, Second Edition* [online]. 2010 John Wiley & Sons, 1-24. [accessed 2021-03-02]. ISBN:9781118868409

19. M. E. SANDERS. Invited review: the scientific basis of Lactobacillus acidophilus NCFM functionality as a probiotic. *Journal of Dairy Science* [online]. 2001 84(2): 319-331. [accessed 2021-03-02]. doi.org/10.3168/jds.S0022-0302(01)74481-5

20. A. NIGATU. Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate Lactobacillus plantarum, Lact. isolated from kocho and tef. *Applied Microbiology* [online]. 2000 89(6): 969-978. [accessed 2021-04-05]. doi.org/10.1046/j.1365-2672.2000.01202.x

21. I. NANCUCHEO, D. BARRIE JOHNSON. Production of Glycolic Acid by Chemolithotrophic Iron- and Sulfur-Oxidizing Bacteria and Its Role in Delineating and Sustaining Acidophilic Sulfide Mineral-Oxidizing Consortia. *Microorganisms* [online]. 2010 8(7), 990. [accessed 2021-03-02]. doi.org/10.3390/microorganisms8070990

22. YU-CAI HE, JIAN-HE XU, LI ZHOU. Bioproduction of Glycolic Acid from Glycolonitrile with a New Bacterial Isolate of Alcaligenes sp. ECU0401. *Applied Biochemistry and Biotechnology* [online]. 2010 160:1428-1440. [accessed 2021-03-01]. https://doi.org/10.1007/s12010-009-8607-y

23. GUODONG WEI, XUEPENG YANG, TULA GAN, WENYU ZHOU, JINPING LIN, DONGZHI WEI. High cell density fermentation of Gluconobacter oxydans DSM 2003 for glycolic acid production. *Journal of Industrial Microbiology and Biotechnology* [online]. 2009 36(8):1029-1034. [accessed 2021-02-24]. doi.org/10.1007/s10295-009-0584-1

24. M. KATAOKA, M. SASAKI, A. GD HIDALGO, M. NAKANO, S. SHIMIZU. Glycolic Acid Production Using Ethylene Glycol-Oxidizing Microorganisms. *Bioscience, Biotechnology, and Biochemistry* [online]. 2001 65(10):2265-2270. [accessed 2021-03-01]. https://doi.org/10.1271/bbb.65.2265

25. T. M. GÄDDA, M. M. PIRTTIMAA. The industrial potential of bio-based glycolic acid and polyglycolic acid: Short survey. [Online]. Available: https://cris.vtt.fi/en/publications/the-industrial-potential-of-bio-based-glycolic-acid-and-polyglyco. [accessed 2021-03-20].

26. M. W. SUSANNE FREDENBERG, M. W. MATS, R. A. AXELSSONA.The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems—A review. *International Journal of Pharmaceutics* [online]. 2011 415(1-2): 34-52. [accessec 2021-03-02]. doi.org/10.1016/j.ijpharm.2011.05.049.

27. K. JIM JEM, B. TAN. The development and challenges of poly (lactic acid) and poly (glycolic acid). *Advanced Industrial and Engineering Polymer Research* [online]. 2020 3(2): 60-70. [accessed 2021-02-25]. doi.org/10.1016/j.aiepr.2020.01.002

28. L. SALUSJÄRVI, S. HAVUKAINEN, O. KOIVISTOINEN, M. TOIVARI. Biotechnological production of glycolic acid and ethylene glycol: current state and perspectives. *Applied Microbiology and Biotechnology* [online]. 2019 103:2525-2535. [accessed 2021-03-02]. doi.org/10.1007/s00253-019-09640-2

29. B. MARIA-TERESA PELLICER. glc Locus of Escherichia coli: Characterization of Genes Encoding the Subunits of Glycolate Oxidase and the glc Regulator Protein. *Journal of bacteriology* [online]. 1996 178(7):2051-2059. [accessed 2021-04-01]. doi: 10.1128/jb.178.7.2051-2059.1996

30. Y. D. CHANGSHUI LIU. Malonyl-CoA pathway: a promising route for 3-hydroxypropionate biosynthesis. *Critical Reviews in Biotechnology* [online]. 2017 37(7):933-941. [accessed 2021-02-25]. doi.org/10.1080/07388551.2016.1272093

31. YUN CHEN, JENS NIELSEN. Advances in metabolic pathway and strain engineering paving the way for sustainable production of chemical building blocks. *Current Opinion in Biotechnology* [online]. 2013 24(6): 965-972. [accessed 2021-03-01]. doi.org/10.1016/j.copbio.2013.03.008

32. V. KUMAR, S. ASHOK, S. PARK. Recent advances in biological production of 3-hydroxypropionic acid. *Biotechnology Advances* [online]. 2013 31(6): 945-961. [accessed 2021-02-25]. doi.org/10.1016/j.biotechadv.2013.02.008

33. B. ANDREEßEN, N. TAYLOR, A. STEINBÜCHE. Poly (3-hydroxypropionate): a promising alternative to fossil fuel-based materials. *Applied and environmental microbiology* [online]. 2014 80(21):6574-6582. [accessed 2021-02-25]. doi: 10.1128/AEM.02361-14

34. D. MENGA, Y. WANGA, LIN-PING WUB, R. SHENA, JIN-CHUN CHENA, QIONG WUA, GUO-QIANG CHEN. Production of poly (3-hydroxypropionate) and poly (3-hydroxybutyrate-co-3-hydroxypropionate) from glucose by engineering Escherichia coli. *Metabolic Engineering* [online]. 2015 29: 189-195. [accessed 2021-03-26]. doi.org/10.1016/j.ymben.2015.03.015

35. ANDREW C. CLARK. PAUL D. PRENZLER, GEOFFREY R. SCOLLARY. Impact of the condition of storage of tartaric acid solutions on the production and stability of glyoxylic acid. *Food Chemistry* [online]. 2007 102(3): 905-916. [accessed 2021-03-25]. doi.org/10.1016/j.foodchem.2006.06.029

36. H. ARIS, S. BORHANI, D. CAHN, C. O'DONNELL, E. TAN, P. XU. Modeling transcriptional factor cross-talk to understand parabolic kinetics, bimodal gene expression and retroactivity in biosensor design. *Biochemical Engineering Journal* [online]. 2019 144: 209-216. [accessed 2021-03-25]. doi.org/10.1016/j.bej.2019.02.005

37. AHMAD A. MANNAN, DI LIU, FUZHONG ZHANG, DIEGO A. OYARZÚN. Fundamental design principles for transcription-factor-based metabolite biosensors. *ACS Synthetic Biology* [online]. 2017 6(10): 1851-1859. [accessed 2021-03-29]. doi.org/10.1021/acssynbio.7b00172

38. E. C. GREENWALD, S. MEHTA, J. ZHANG. Genetically encoded fluorescent biosensors illuminate the spatiotemporal regulation of signaling networks. *Chemical Reviews* [online]. 2018 118(204):11707-11794. [accessed 2021-03-29]. doi.org/10.1021/acs.chemrev.8b00333

39. R. MAHR, J. FRUNZKE. Transcription factor-based biosensors in biotechnology: current state and future prospects. *Appl Microbiol Biotechnol* [online]. 2016 100:79–90. [accessed 2021-03-25]. DOI 10.1007/s00253-015-7090-3

40. C. S. PUNDIR, V. NARWAL, B. BATRA. Determination of lactic acid with special emphasis on biosensing methods: A review. *Biosensors and Bioelectronics* [online]. 2016 86: 777-790. [accessed 2021-04-02]. doi.org/10.1016/j.bios.2016.07.076

41. A. BEREPIKI, R. KENT, L. F. M. MACHADO, N. DIXON. Development of high-performance whole cell biosensors aided by statistical modeling. *ACS Synthetic Biology* [online]. 2020 9(3):576-589. [accessed 2021-04-12]. doi.org/10.1021/acssynbio.9b00448

42. N. DING, Z. YUAN, X. ZHANG, J. CHEN, S. ZHOU. Programmable cross-ribosome-binding sites to fine-tune the dynamic range of transcription factor-based biosensor. *Nucleic Acids Research* [online]. 2020 48(18): 10602–10613. [accessed 2021-04-05]. doi.org/10.1093/nar/gkaa786

43. E. K. R. HANKO, N. P. MINTON, N. MALYS. Characterisation of a 3-hydroxypropionic acid-inducible system from Pseudomonas putida for orthogonal gene expression control in Escherichia coli and Cupriavidus necator. *Scientific Reports* [online]. 2017 7:1724. [accessed 2021-02-22]. doi.org/10.1038/s41598-017-01850-w

44. E. K. R. HANKO, N. P. MINTON, N. MALYS. A Transcription Factor-Based Biosensor for Detection of Itaconic Acid. *ACS Synthetic Biology* [online]. 2018 7(5):1436-1446. [accessed 2021-02-22]. doi.org/10.1021/acssynbio.8b00057

45. A. GONZALEZ, N. DIXON. Genetically encoded biosensors for lignocellulose valorization. *Biotechnology for Biofuels* [online]. 2019 12:246. [accessed 2021-04-02]. doi.org/10.1186/s13068-019-1585-6

46. N. H. NGUYEN, J. KIM, S. PARK. Application of transcription factor-based 3-hydroxypropionic acid. *Biotechnology and Bioprocess Engineering* [online]. 2018 23:564-572. [accessed 2021-03-16]. doi.org/10.1007/s12257-018-0390-6

47. H. INOUEA, H. H. OKAYAMA. High efficinecy transformation of Escherichia coli with plasmids. *Gene* [online]. 1990 96(1): 23-28. [accessed 2021-03-25]. doi.org/10.1016/0378-1119(90)90336-P

48. S. ALAGESAN, E. K. R. HANKO, N. MALYS, M. EHSAAN, K. WINZER, N. P. MINTON. Functional genetic elements for controlling gene expression in Cupriavidus necator. *Applied Environmental Microbiology* [online]. 2018 84(19): e00878-18. [accessed 2021-02-22]. doi: 10.1128/AEM.00878-18

49. J. DEUTSCHER. The mechanisms of carbon catabolite repression in bacteria. *Current Opinion in Microbiology* [online]. 2008 11(2): 87-93. [accessed 2021-04-02]. https://doi.org/10.1016/j.mib.2008.02.007

50. E. K. R. HANKO, N. P. MINTON, N. MALYS. Design, cloning and characterization of transcription factor-based inducible gene expression systems. *Methods in Enzymology* [online]. 2019 612: 153-169. [accessed 2021-02-22]. doi.org/10.1016/bs.mie.2019.02.018

Appendices

Appendix 1. Table 1. *P. putida* KT2440 glcC and glcD homologues. List of glcC and glcD homologues from various species sharing at least 70 % protein sequence identity with *P. putida* KT2440 glcC and glcD, respectively.

	Species	glcC locus tag	Size aa	Query cover %	Per. Ident %	glcD	
						Query cover %	Per. Ident %
1	*Pseudomonas putida* KT2440	PP_3744	263	100	100	100	100
2	*Pseudomonas putida* ND6	YSA_09393	257	97	98.83	99	99.0
3	*Pseudomonas putida* F1	Pput_2019	254	96	99.21	99	98.40
4	*Pseudomonas putida* BIRD-1	PPUBIRD1_2018	254	96	98.82	99	97.78
5	*Pseudomonas putida* GB-1	PputGB1_2160	254	96	96.85	99	95.39
6	*Pseudomonas putida* H8234	L483_19590	254	95	97.22	99	95.79
7	*Pseudomonas putida* NBRC 14164	PP4_20450	254	95	96.83	99	95.79
8	*Pseudomonas monteilii* SB3078	X969_15460	254	96	95.28	99	94.59
9	*Pseudomonas putida* W619	PputW619_2203	255	95	95.24	99	92.79
10	*Pseudomonas putida* W619	PputW619_2307	259	93	86.29	99	87.58
11	*Pseudomonas citronellolis*	A9C11_30995	257	95	81.75	99	86.97
12	*Pseudomonas aeruginosa* M18	PAM18_5476	251	94	81.93	99	83.17
13	*Pseudomonas aeruginosa* c7447m	M802_5534	251	94	81.93	99	83.37
14	*Pseudomonas aeruginosa* B136-33	G655_28180	251	94	81.53	99	83.37
15	*Pseudomonas aeruginosa* RP73	M062_28205	251	94	81.53	99	82.97
16	*Pseudomonas aeruginosa* UCBPP-PA14	PA14_70710	251	94	81.53	99	83.57

17	*Pseudomonas denitrificans (nomen rejiciendum)*	F1C79_14910	256	94	80.40	99	90.38
18	*Pseudomonas aeruginosa* LES431	T223_29395	251	94	81.12	99	83.17
19	*Pseudomonas aeruginosa* PA7	PSPA7_6133	251	93	81.45	99	83.37
20	*Pseudomonas aeruginosa* DK2	PADK2_28515	251	94	81.12	99	83.37
21	*Pseudomonas stutzeri* A1501	PST_0430	257	93	82.11	99	88.38
22	*Pseudomonas stutzeri* DSM 4166	PSTAA_0483	257	93	82.11	99	88.18
23	*Pseudomonas stutzeri* CCUG 29243	A458_19230	257	93	81.71	99	88.58
24	*Azotobacter vinelandii* DJ	Avin_43340	259	95	79.05	99	87.17
25	*Pseudomonas stutzeri* RCH2	Psest_3842	257	93	81.30	99	88.38
26	*Pseudomonas mendocina* NK-01	MDS_0218	257	93	78.54	99	87.98
27	*Pseudomonas stutzeri* DSM 10701	PSJM300_17500	256	93	78.63	99	86.77
28	*Pseudomonas mendocina ymp*	Pmen_0204	257	93	77.73	99	87.37
29	*Pseudomonas resinovorans* NBRC 106553	PCA10_27990	257	95	73.41	99	87.78
31	*Pseudomonas frederiksbergensis*	BLL42_28340	256	93	70.45	99	85.77
32	*Pseudomonas sp.* JY-Q	AA098_17235	254	100	98,43	100	98,67
33	*Pseudomonas sp.* SWI36	C4Q27_00030	252	99	97,50	100	97,60
34	*Pseudomonas putida* DOT-T1E	T1E_0139	254	100	98,17	100	98,73
35	*Pseudomonas putida* strain B4	CHN49_10785	254	100	98,56	100	98,47

36	*Pseudomonas putida* B6-2	KKK_06340	254	100	98,30	100	98,80
37	*Pseudomonas putida* SJTE-1	A210_10485	252	100	98,68	100	98,60
38	*Pseudomonas putida* strain N1R	SAMN05216501_3188	252	100	96,83	100	97,13
39	*Pseudomonas putida* JB	Q5O_10710	254	100	97,12	100	97,27
40	*Pseudomonas putida* S12	RPPX_25400	254	100	96,99	100	97,27

Appendix 2. Similarity of intergenic sequences between different *Pseudomonas* species.

Fig. 1. A sequence similarity motif was generated using WebLogo tool. It represents highly conserved nucleotides in the glcC/glcD intergenic regions of five analysed *Pseudomonas stutzeri* species.

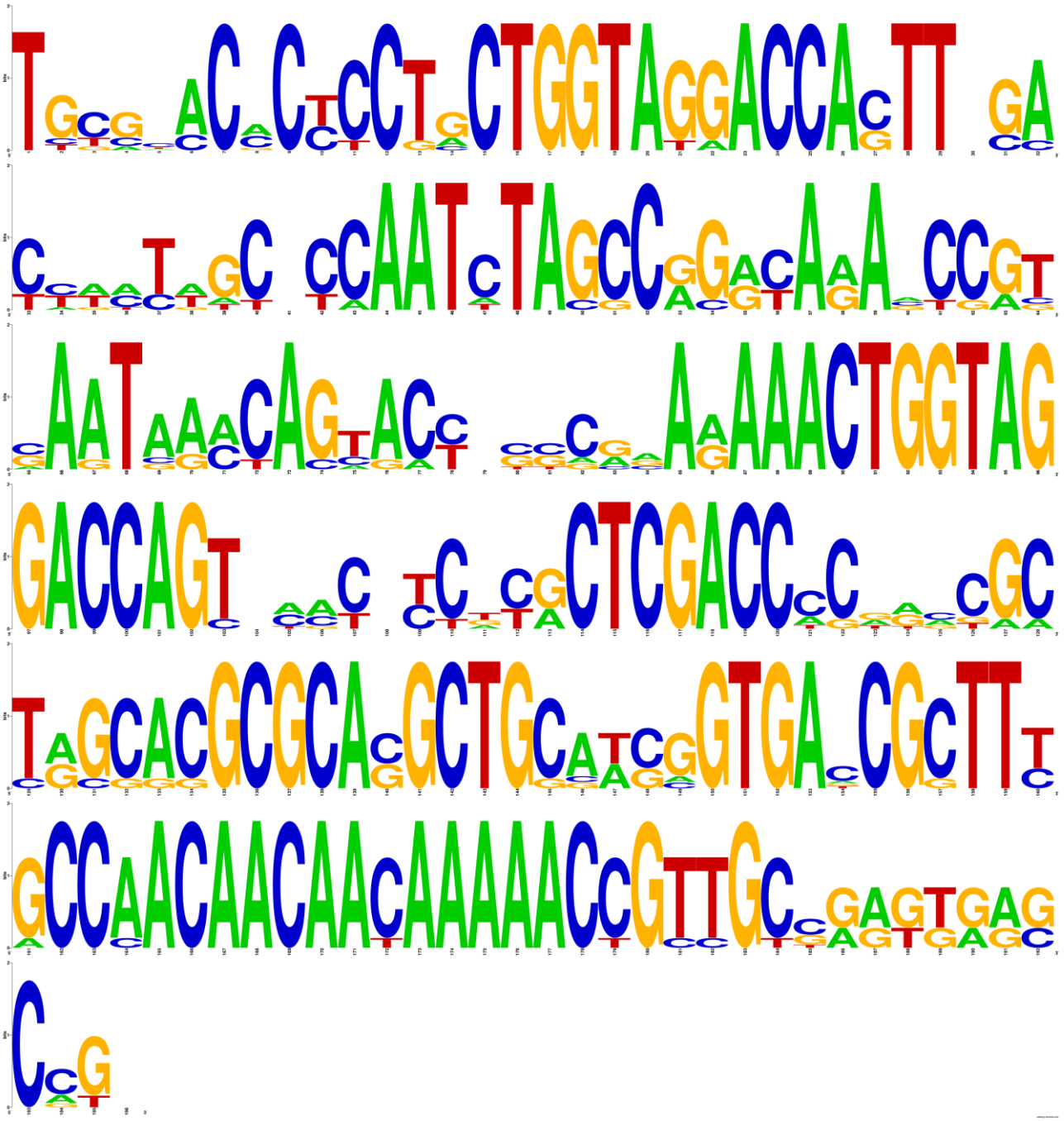


Fig. 2. A sequence similarity motif was generated using WebLogo tool. It represents highly conserved nucleotides in the glcC/glcD intergenic regions of fifteen analysed *Pseudomonas putida* species.

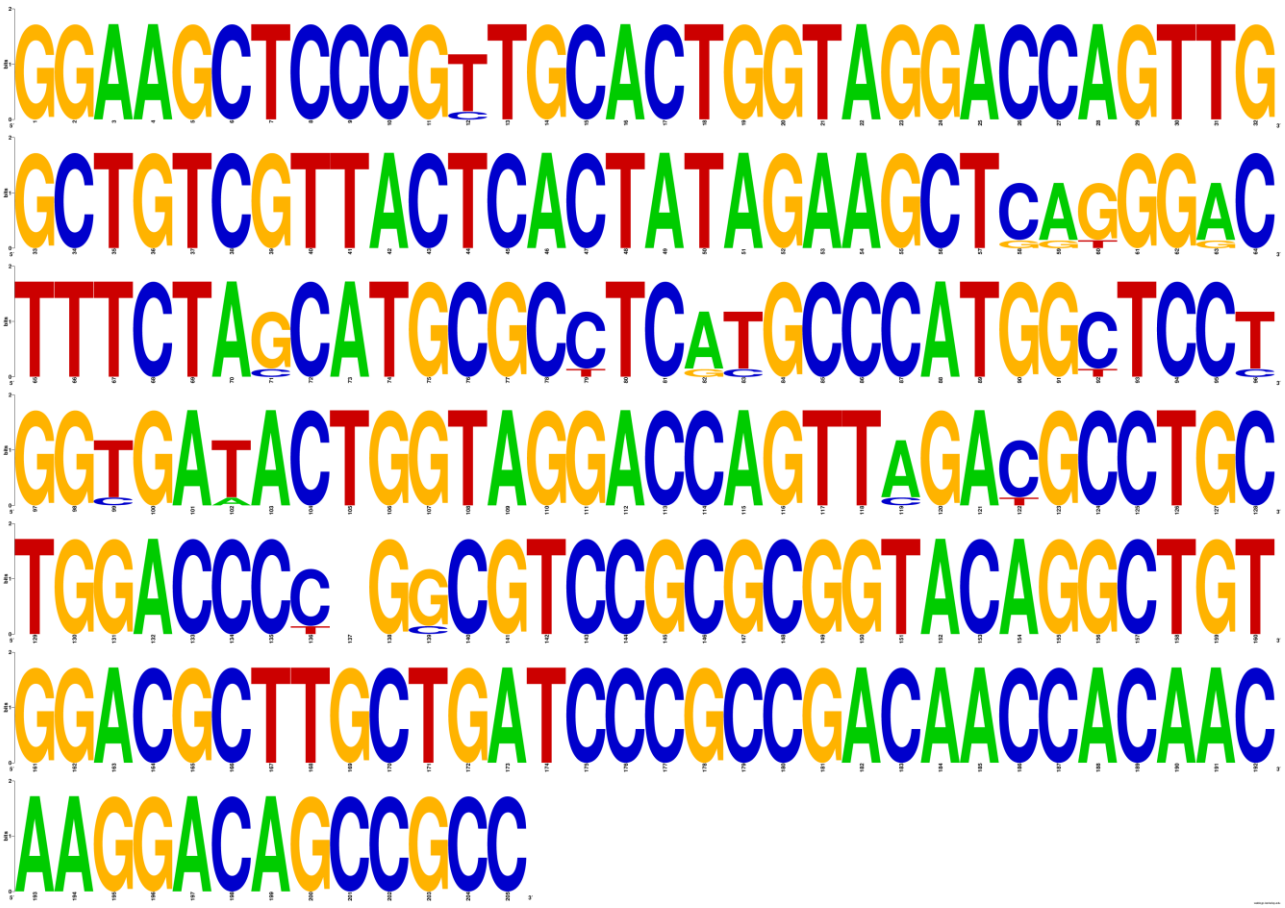


Fig. 3. A sequence similarity motif was generated using WebLogo tool. It represents highly conserved nucleotides in the glcC/glcD intergenic regions of seven analysed *Pseudomonas aeruginosa* species.

Appendix 3. List of used *glcC/glcD* intergenic region sequences for WebLogo analyses.

>glcC_glcD_Pseudomonas_stutzeri_A1501
gccaatctccagctggtaggaccagttgacgggcagtttacctcgaatgctgaaaagcacctagcggcgcggcctggaagggcttcaggtagtgctttgaac
gggatgactacaaaaaactggtaggaccagttggtgcgggcatggacggctcgcggccatcggcgctaaagatgcgtcattccaccgcagctgcccgcgc
tggcggtgactcacgaaagcggaacctgcc
>glcC_glcD_Pseudomonas_stutzeri_DSM4166
Gccacttctccagctggtaggaccagttggcgggcagtttacctcgaatgctgaaaagcacctaacggcgcggtctggaggggcttcaggtagtgctttgaa
cgggatgactacaaaaaactggtaggaccagttggtgcgggcatggacggctcgcggccatcggcgctaaagatgcgtcattccaccgcagctgcccgcg
ctggcggtgactcacgaaagcggaacctgcc
>glcC_glcD_Pseudomonas_stutzeri_CCUG29243
Gtccttcgttctcccactggtaggaccagtttggcgggagtttaccctgaatgcctgaataccagtaacagcaccgtctggaggactattcggtagtgcctttcat
cgggcgactacaaaaaactggtaggaccagttgtctaggtcatggactggggaccatcttcgacgttaaagatgcgtcattccaccgcagtcgttcgcgctgg
ctgcaacccacgagaaccggaacctgcc
>glcC_glcD_Pseudomonas_stutzeri_RCH2
Gtccttcgttctccactggtaggaccagtttgaggcgagtttaccctgaatggctggatagcagaaacagcgccgcttggaaggccgtttggtagtgcctttcat
cggctgactacaaaaaactggtaggaccagttgtctggggcatggacgggcgcccaccttcgacgttaaagatgcgtcattccaccgcagccgtgcgcgct
ggctgtggccccacgagaactggaacctgcc
>glcC_glcD_Pseudomonas_stutzeri_DSM10701
Gggcttttccaggtggtaggaccagtggcgagccagtatagctactttcgcgctttagtcacgccagccccgtgctcgctgcattccgggcggtcaaggactg
gctccagctttcacaaactggtacgaccagtggtttcgagggtggactggccgatccgttcaggctaatgatgcgtcactccgccgcagctggtcgcgctgcc
ggtgaacaacgagagcatccgactgcc

>glcC_glcD_Pseudomonas_putida_kt2440
tgcgcacactcctgctggtaggaccactttgacttatatcgccaatctagccagacagaaccgtgaatagacagtactgcccgaagaaactggtaggaccagt
caacatctcactcgaccccaaacactagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagccg
>glcC_glcD_Pseudomonas_putida_ND6
Tgcgtacacccctgctggtaggaccactttgacttatagcgccaatatagccagacagaaccgtgaataaacagtactgcccgaagaaactggtaggaccag
tcaacatctcgctcgaccccgaacgctagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagcca
>glcC_glcD_Pseudomonas_putida_F1
Tgcgtacacccctgctggtaggaccactttgacttatagcgccaatctagccagacagaaccgtgaataaacagtactgcccgaagaaactggtaggaccag
tcaacatctcgctcgaccccgaacgctagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagcca
>glcC_glcD_Pseudomonas_putida_BIRD_1
Tgcacacactcctgctggtaggaccactttgacttatagcaccaatctagccggacagagtcgtgagtaaacagcactgttcgaagaaactggtaggaccagt
caacatctcactcgaccccggccgctagcacgcgcacgctgcatcagtgaccgctttgccaacaacaataaaaaccgttgccgagtgagccg
>glcC_glcD_Pseudomonas_putida_GB_1
Ctcaacgcccctactggtagaaccagttgcatcactggcttcaatctagccggacaaagccggcaatagctacagcctggcagaaaaactggtaggaccag
tacctgccgcgctcgaccacagccgccggcacgcgcacgctggcagggtgatcgctttgccaacaacaacaaaaaccgttgccgagtgagccg
>glcC_glcD_Pseudomonas_putida_H8234
Tgtgccccctcctcctggtaggaccagttagcccatctatccaaatctagccgggtaaacccaccaataaccagtaccacggcgaaaaactggtaggaccag
tgcccgccatgctcgaccccaggcgctggcacgcgcaggctgcaaccgtgaccgctttgccaacaacaacaaaaaccgttgccgagtgagccg
>glcC_glcD_Pseudomonas_putida_NBRC_14164
Tgcgcaccctcctgctggtaggaccagttagaccacctgccccaatctagccgggtaaacccatcaatcaccagtacccggaagaaaaactggtaggacca
gtgcctgccatgctcgaccactgctgctggcacgcgcaggctgcaacggtgaccgctttgccaacaacaacaaaaaccgttgccgagtgagccg
>glcC_glcD_Pseudomonas_putida_W619_PputW619_2203
Gcgcacccctcgactggtaggaccacttccatagcttgcgccaatctacgcacgtaaaccggtaaatgaacagcaactggcacaaaaaactggtaggaccag
tgtgcttcgcgctcgacctgttgcgatgggggggcgcaggctgcgtgggtgaacggtttacccacaacaacaaaaactgccgttgagtgagcctg
>glcC_glcD_Pseudomonas_putida_DOT_T1E_ T1E_0139
Tgcgcacacccctgctggtaggaccactttgacttatagcgccaatctagccagacagaaccgtgaataaacagtactgcccgaagaaactggtaggacca
gtcaacatctcactcgaccccaaacgctagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagcca

>glcC_glcD_Pseudomonas_putida_B4_ CHN49_10785
Tgcgtacacccctgctggtaggaccactttgacttatagcgccaatctagccagacagaaccgtgaataaacagtactgcccgaagaaactggtaggaccag
tcaacatctcactcgaccccaaacgctagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagcca
>glcC_glcD_Pseudomonas_putida_B6_2_ KKK_06340
Tgcgcacacccctgctggtaggaccactttgactcatagcgccaatctagccagacataaccgtgaataaacagtactgcccgaagaaactggtaggacca
gtcaacatttcactcgaccccaaacgctagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagcca
>glcC_glcD_Pseudomonas_putida_SJTE_1_ A210_10485
Tgcgtacacccctgctggtaggaccactttgacttatagcgccaatatagccagacagaaccgtgaataaacagtactgcccgaagaaactggtaggaccag
tcaacatctcgctcgaccccgaacgctagcacgcgcacgctgcatcggtgaccgcttcgccaacaacaacaaaaaccgttgcgagtgagcca
>glcC_glcD_Pseudomonas_putida_N1R_ SAMN05216501_3188
Tgcacacactcctgctggtaggaccactttgacttatagcgccaatctagccggacagagccgtgagtaaacaccactgttcgaagaaactggtaggaccag
tcaacatctcactcgaccccggccgctagcacgcgcacgctgcatcagtgaccgctttgccaacaacaataaaaaccgttgccgagtgagccg
>glcC_glcD_Pseudomonas_putida_JB_ Q5O_10710
Tgcacacactcctgctggtaggaccactttgacttatagcaccaatctagccggacagagtcgtgagtaaacagcactgttcgaagaaactggtaggaccagt
caacatctcactcgaccccggccgctagcacgcgcacgctgcatcagtgaccgctttgccaacaacaataaaaaccgttgccgagtgagccg
>glcC_glcD_Pseudomonas_putida_S12_ RPPX_25400
Tgcacacactcctgctggtaggaccactttgacttatagcaccaatctagccggacagagccgtgagtaagcagcactgttcgaagaaactggtaggaccag
tcaacatctcactcgaccccggccgctagcacgcgcacgctgcatcagtgaccgctttgccaacaacaataaaaaccgttgccgagtgagccg

>glcC_glcD_Pseudomonas_aeruginosa_M18
Ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatact
ggtaggaccagttagacgcctgctggaccccggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_c7447m
Ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatact
ggtaggaccagttagacgcctgctggaccccggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_B136_33
Ggaagctcccgctgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatac
tggtaggaccagttagacgcctgctggaccccggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_RP73
Ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatact
ggtaggaccagttagacgcctgctggaccccggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_UCBPP_PA14
Ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatact
ggtaggaccagttagacgcctgctggaccctggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_LES431
Ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatact
ggtaggaccagttagacgcctgctggaccccggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_PA7
ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctggtgggctttctaccatgcgcttcgcgcccatggttcccggcgaaact
ggtaggaccagttcgatgcctgctggaccccggccgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc
>glcC_glcD_Pseudomonas_aeruginosa_DK2
Ggaagctcccgttgcactggtaggaccagttggctgtcgttactcactatagaagctcagggactttctagcatgcgcctcatgcccatggctcctggtgatact
ggtaggaccagttagacgcctgctggaccccggcgtccgcgcggtacaggctgtggacgcttgctgatcccgccgacaaccacaacaaggacagccgcc

Acknowledgment

I would like to express my special thanks of gratitude to my supervisor Prof. Naglis Malys who gave me the opportunity to do this interesting project, which also helped me in doing a lot of research, shared his knowledge, and lead to an understanding of the novel scientific approach.
Secondly, I would also like to thank my colleagues at the Bioprocess Research Centre who helped me a lot with their support and advice.