Genetic manipulations of Serratia marcescens with a goal of industrial application

Kozarić, Marta

Master's thesis / Diplomski rad

2021

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Food Technology and Biotechnology / Sveučilište u Zagrebu, Prehrambeno-biotehnološki fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:159:274300

UNIVERSITY OF ZAGREB

FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

GRADUATE THESIS

Zagreb, September 2021. Marta Kozarić

1335/MB

GENETIC MANIPULATIONS OF *Serratia marcescens* WITH A GOAL OF INDUSTRIAL APPLICATION

Experimental work for this Graduate thesis was done at the Department of Biotechnology, Institute of Microbiology and Microbial Biotechnology, BOKU – University of Natural Resources and Life Sciences, Vienna, Austria. The thesis was made under the guidance of associate professor Michael Sauer, Assoc. Prof. Priv.-Doz. Dipl. Natw. ETH Dr., and with the help of Hannes Russmayer, Bakk.techn. Dipl.-Ing. Dr.

ACKNOWLEDGMENT

I would like to thank the associate professor Michael Sauer for giving me the opportunity to be a part of his lab team and for all the help and advice he provided.

In particular, I want to thank Hannes Russmayer for guidance, patience, technical support, great knowledge and experience he shared with me while working in the lab.

It was a great pleasure and honor to work with all the members of the lab. Thank you for making my stay in Vienna perfect.

Zahvaljujem se i profesoru Božidaru Šanteku što mi je pristao biti mentor na matičnom fakultetu u Zagrebu. Hvala na svim savjetima, pomoći i podršci.

Htjela bih se zahvaliti i svim prijateljima koji su bili uz mene kako u lijepim, tako i u teškim trenucima tijekom studiranja.

Naposlijetku, najveće hvala mojim roditeljima i sestri koji su uvijek bili tu za mene i poticali me, te mi bili oslonac tijekom svih godina studiranja i boravka u Beču.

BASIC DOCUMENTATION CARD

Graduate Thesis

University of Zagreb
Faculty of Food Technology and Biotechnology
Department of Biochemical Engineering
Laboratory for Biochemical Engineering, Industrial Microbiology and Melting and Brewing Technology

Scientific area: Biotechnical Sciences
Scientific field: Biotechnology

GENETIC MANIPULATIONS OF *Serratia marcescens* WITH A GOAL OF INDUSTRIAL APPLICATION

Marta Kozarić, 1335/MB

Abstract: 2,3-butanediol (2,3-BD) is one of the promising bulk chemicals that has a great potential for a variety of industries. *Serratia marcescens* is a Gram negative bacterium capable of producing specific meso-2,3-BD isoform. The main aim of the thesis was genetic engineering of genes connected to glucose uptake in *S. marcescens*. As target genes, *ptsG* gene and *man* operon were chosen and the method of choice for gene knock-out was CRISPR-Cas9. An efficient DNA transformation method for *S. marcescens* was established in the laboratory where electroporation showed bigger efficiency comparing to chemical competent process. Potential *man* operon knocked-out clones, grown on xylose as a carbon source, have been revealed but the further examination is required. *PtsG* gene knocked- out clones were not obtained.

Keywords: genetic transformation, *Serratia marcescens*, CRISPR-Cas9, gene knock-out
Thesis contains: 56 pages, 23 figures, 20 tables, 42 references, 4 supplements

Original in: English

Graduate Thesis in printed and electronic (pdf format**) version is deposited in:** Library of the Faculty of Food Technology and Biotechnology**,** Kačićeva 23, Zagreb.

Mentor at Faculty of Food Technology and Biotechnology: full professor, Božidar Šantek

Principal mentor: Assoc. Prof. Priv.-Doz. Dipl.Natw. ETH FH-Prof. Dr., Michael Sauer

Technical support and assistance: Bakk.techn. Dipl.-Ing. Dr. Hannes Russmayer

Reviewers:

1. PhD. Anamarija Štafa, Assistant professor
2. PhD. Božidar Šantek, Full professor
3. PhD. Michael Sauer, Associate professor, University of Natural Resources and Life Science, Vienna
4. PhD. Mario Novak, Assistant professor (substitute)

Thesis defended: September 1st 2021

TEMELJNA DOKUMENTACIJSKA KARTICA

Diplomski rad

Sveučilište u Zagrebu
Prehrambeno-biotehnološki fakultet
Zavod za Biokemijsko inženjerstvo
Laboratorij za biokemijsko inženjerstvo, industrijsku mikrobiologiju i tehnologiju slada i piva

Znanstveno područje: Biotehničke znanosti
Znanstveno polje: Biotehnologija

GENETIČKE MODIFIKACIJE *Serratia marcescens* S CILJEM INDUSTRIJSKE PRIMJENE

Marta Kozarić, 1335/MB

Sažetak: 2,3-butandiol (2,3-BD) kemikalija je s potencijalnom primjenom u raznim granama industrije. *Serratia marcescens* je Gram negativna bakterija koja može proizvesti specifičnu mezo-2,3-butandiol izoformu. Glavni cilj rada bio je genetički modificirati gene povezane s transportom glukoze u *S. marcescens*. Kao ciljane regije odabrani su gen *ptsG* i *man* operon , a za inaktivaciju gena izabrana je CRISPR- Cas9 metoda. U laboratoriju je uspostavljena učinkovita metoda genetičke transformacije bakterije *S. marcescens*, te je elektroporacija pokazala veću efikasnost u usporedbi s kemijski kompetentnim postupkom. U konačnici, dobiveni su klonovi u kojima je došlo do moguće inaktivacije *man* operona, izrasli na ksilozi kao izvoru ugljika, međutim potrebna su daljnja istraživanja. Klonovi u kojima je došlo do inaktivacije gena *ptsG* nisu dobiveni.

Ključne riječi: genetička transformacija, *Serratia marcescens*, CRISPR-Cas 9, inaktivacija gena

Rad sadrži: 56 stranica, 23 slike, 20 tablica, 42 literaturnih navoda, 4 priloga

Jezik izvornika: engleski

Rad je u tiskanom i elektroničkom (pdf format) obliku pohranjen u: Knjižnica Prehrambeno-biotehnološkog fakulteta, Kačićeva 23, Zagreb

Mentor na Prehrambeno-biotehnološkom fakultetu: prof. dr. sc. Božidar Šantek

Neposredni mentor: Assoc. Prof. Priv.-Doz. Dipl.Natw. ETH FH-Prof. Dr., Michael Sauer

Pomoć pri izradi: Bakk.techn. Dipl.-Ing. Dr. Hannes Russmayer

Stručno povjerenstvo za ocjenu i obranu:
1. Doc. dr. sc. Anamarija Štafa
2. Prof.dr.sc. Božidar Šantek
3. Izv. prof.dr.sc. Michael Sauer, Sveučilište za prirodne resurse i znanost o životu, Beč
4. Doc.dr.sc. Mario Novak (zamjena)

Datum obrane: 01. rujan 2021.

TABLE OF CONTENTS

1. INTRODUCTION

The economic growth and industrialisation during the 20 th century were mainly based on fossil fuels. Given the growing climate change and concerns about the energy security, renewable alternatives are being encouraged as desirable alternatives. Of the total global energy consumption, renewables and bioenergy account for approximately 10 %. Bioenergy is produced from biological raw materials (biomass) and includes different categories such as biofuels for transport, products for electricity production or biogas (Ho et al., 2014).

Among commonly used biofuels today, biobutanol is becoming a promising alternative. There are many advantages of its use and four different isomers are known. 2-butanol is for its caracteristics, comparing to other butanol isomers, of the big interest for biofuel production. 2-butanol can be produced from meso-2,3-butanediol during anaerobic sugar fermentation (Russmayer et al., 2019).

Recently, many studies showed a potential role of 2,3-butanediol for industrial application. It is also known that 2,3-BD isomers show different physicochemical characteristics especially in the pharmaceutical industry, agriculture and other industries. Traditional chemical production of 2,3-BD has many dissadvantages. In the chemical process 2,3 -BD is produced using butene which needs to be pre-separated from cracked gas. Also, upon production, 2,3-BD exists as three isomers and a chemical catalyst cannot recognize these isomers. To obtain stereospecific 2,3-BD, expensive separation and purification processes are required. Biological process is environmentally-friendly, simple and economic in relation to chemical processes, so the interest in the biological production of 2,3-BD is growing (Song et al., 2019). There is a number of microorganisms that have the ability to produce 2,3-butanediol. Only a few, such as species belonging to the genera *Klebsiella*, *Enterobacter*, *Bacillus* and *Serratia*, are currently described to do it in significant quantities. *Klebsiella pneumoniae* or *K. oxytoca* and *Paenibacillus polymyxa* have been demonstrated to be potentially applicable in the industrial production of 2,3-BD (Ji et al., 2011). *Serratia marcescens* compared to other strains, has advantages including better resistance to bacteria pollution, relatively simple metabolic pathways, broad substrate spectrum, etc. (Zhang et al., 2010). For these reasons, it was an interesting host organism for this project.

S. marcescens is a Gram negative bacterium, part of the family *Enterobacteriaceae* which is capable of producing meso-2,3-butanediol (meso-2,3-BD) with a yield on glucose of over

98 % (Zhang et al., 2010). Meso-2,3-BD is a specific isoform from which 2-butanol is further produced, so *S. marcescens* seems as an ideal producing organism. *S. marcescens* is capable of production meso-2,3-BD from glucose. It is known that some *Lactobacillus* spp. are capable of naturally producing 2-butanol from meso-2,3-butanediol during anaerobic sugar fermentations. A two step cultivation process was developed where *S. marcescens*, after producing stereo specifically meso-2,3-BD from glucose, was heat inactivated. Meso-2,3-BD was subsequently converted during anaerobic fermentation with glucose into 2-butanol by *Lactobacillus diolivorans* (Russmayer et al., 2019).

Previously mentioned two step cultivation process works, but one way to further improve it is to engineer *S. marcescens*. The goal was to knock-out the genes connected to glucose uptake which should stop the growth on glucose. The idea was to separate growth of both organisms. If every organism could use a carbon source, that the other organism can not use, it is possible to decide which organism to feed, how and when, without influencing the other.This would be more efficient with potential higher titers of 2,3-BD and 2- butanol produced. In that way, later co-cultivation of *S. marcescens* and *L. diolivorans* could be done without heat inactivation of *S.marcescens*. Instead of using glucose, *S.marcescens* will use some other carbon source for producing 2,3-BD and thus, no heat inactivation would be needed for later 2-butanol production.

The method of choice for gene knock-out was CRISPR Cas9 technology. CRISPR Cas9 is a dominant genetic engineering tool that enables with high precision the introduction of different genomic changes such as gene knockout, knock in, tagging etc. It is a fast, highly efficient method and, in contrast to other programmable DNA nucleases such as ZFNs and TALENs, is based on specific binding of the Cas9 nuclease which is mediated by a small guide RNA (sgRNA). In that way specific changes in the genome are more achievable (Neldeborg et al., 2019). To apply genetic engineering techniques to *S. marcescens*, an efficient DNA transformation method is required. *S. marcescens* was successfully transformed using a $CaCl_2$-mediated genetic transformation (Reid et al., 1981) and an electroporation method (Sakural and Komatsubara, 1996). The reproducible genetic transformation is a prerequisite for all further steps and should be established in the lab.

2. THEORETICAL PART

2.1. Renewable sources of energy

Fossil fuels in their basic form are hydrocarbons based on coal, petroleum or natural gas. The main concern regarding fossil oils is the negative side effects coming from their combustion (Shahzad, 2015). Although, fossil fuels are very effective in terms of energy production quality, the negative impact of fossil fuels on the environment culminates in a growing need for finding renewable alternatives for obtaining fuels. With technological advances, people have become more aware of the disadvantages associated with burning fossil fuels. Global warming is a potential global crisis in part caused by the burning of oil, coal and natural gas. Fossil fuels are finite, and therefore it is necessary to turn to renewable energy sources. Renewable energy has become an important sector in recent years. It can be defined as "*energy derived from natural processes that are replenished at a faster rate than they are consumed"* (Harjanne and Korhonen, 2019). Renewable technologies are considered as clean and sustainable sources of energy regarding current and future economic and societal needs (Panwar et al., 2011). The main renewable energy sources are solar energy, wind, biomass, geothermal energy and tidal energy. Non-renewable energy, such as coal, natural gas, oil, require expensive exploration and potentially dangerous mining and drilling, and will become increasingly expensive as supply decreases while energy demand increases. Renewable energy produces only small levels of carbon emissions, and is therefore very effective in combating climate change caused by the burning of fossil fuels (Shahzad, 2015).

2.1.1. Biofuels

In recent years, the production of biofuels from renewable sources has been increasing due to fewer fossil resources, fluctuations in oil prices, but also due to growing concerns about the environmental problems (Zhang et al., 2018). Biofuels such as biodiesel, biobutanol, bioethanol, biohydrogen, biogas derived from biomass are a desirable substitutes for fossil fuels since they are currently the only path to sustainable development. Biofuels are today considered a major source of bioenergy and provide an adequate alternative to conventional forms of energy for populations around the world. In order to produce economically viable and environmentally friendly fuels, it is necessary to use an efficient and suitable microorganism. Some examples of biomass resources are agricultural products, biological waste, industrial waste, etc. An efficient and convenient microorganism can now be created by applying genetic

engineering methods. Furthermore, by using an optimal process and developing efficient microbial cell factories, it is possible to improve biofuel production. Biofuels from microalgae are considered to be third generation fuels. They have potentially significant economic, environmental and social benefits for the whole population from individual to industry level (Srivastava, 2019).

2.2. Biobased production

Biodiesel is most often produced from various vegetable oils or waste cooking oil. The production of biodiesel from waste cooking oil is environmentally friendly and has great economic meaning (Degfie et al., 2019). Using genetic engineering techniques microbial strains can be transformed into the desired microbial cell reactors with enhanced capacity of producing desiderable product (Srivastava, 2019). Conversion of lignocellulosic biomass into simple sugars is the first step of production of biofuels. Engineered microbes then convert these simple sugars into different kinds of biofuels (figure 1). Bioethanol is a fuel derived from microbial fermentation using starch as a substrate. It is a first-generation biofuel obtained from corn carnels as feedstock and in the USA it is present in over 90% of gasoline pumps (Kung et al., 2012). It is characterized with high hygroscopicity, high vapor pressure and low energy density which makes it unsuitable as a long -term solution for existing infrasctructure. Furthermore, usage of corn as feedstock is in competiton with food industry supply and growing corn requires significant quantities of water, pesticides and other chemicals which presents a threat for the environment.

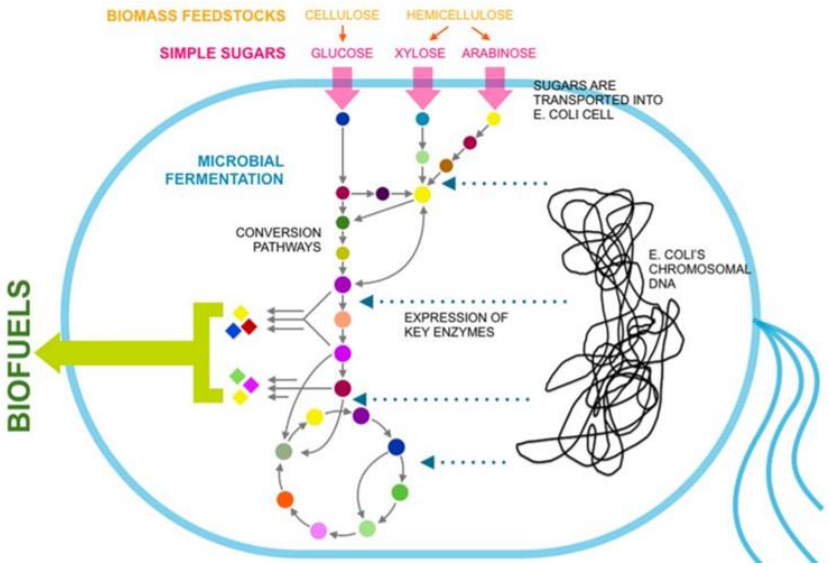

Figure 1. Production of biofuels using microbial cell factory (Kung et al., 2012).

Isopropanol and 1-butanol are short-chain alcohols that have higher energy density and lower hygroscopicity than ethanol and have been produced in engineered microbial hosts. Synthesis of isopropanol was achieved using *Escherichia coli* as a host organism in a fermentative pathway that starts with condensation of two molecules of acetyl-CoA. In the last step decarboxylation of acetoacetate results in acetone which is then reduced in the final product isopropanol. 1-butanol is also a short-chain alcohol with high energy density and low higroscopicity. The first step of the metabolic pathway for natural 1-butanol biosynthesis is the same as for isopropanol. In the final step, dehydrogenation of butyryl-CoA to 1-butanol takes place (figure 2) (Kung et al., 2012). There are three more isoforms of butanol that have substantial potential as biofuels, 2-butanol (sec-butanol), iso-butanol and tert-butanol. 2-butanol, in comparison to other isomers, has the highest octane number, lowest boiling point and lower toxicity compared to 1-butanol.

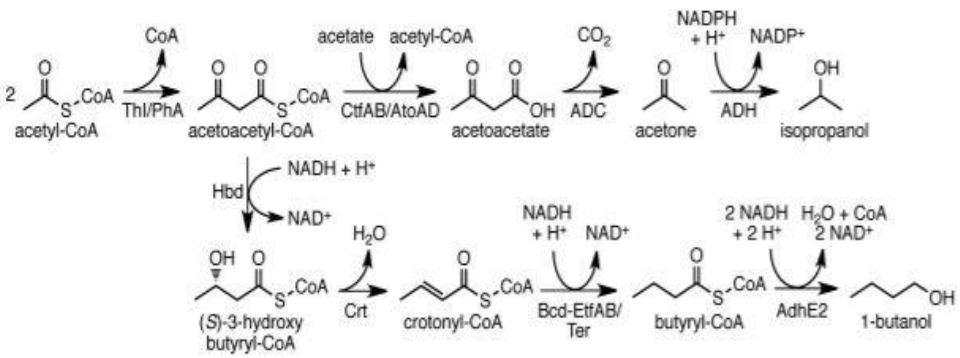

Figure 2. Metabolic pathways for isopropanol and 1-butanol product (Kung et al., 2012)

2.2.1. Microbial based production of 2-butanol

2-butanol is one of the butanol isomers that is widely used as a solvent, cleaning agent or potential biofuel. Generally, to develop strains that will produce nonnatural molecules, four approaches are potentially of importance: first approach is *in-silico* design, using computer methods to develop potential metabolic pathways. Second, is to engineer enzymes required to catalize the desired reaction. Third, is to assemble designed metabolic pathways and final level is to further engineer the strains with aim to increase product yield and productivity. It is important to emphasize that not always all four approaches are needed. Potential metabolic pathways can be predicted using different algorithms, since the field of synthetic biology is rising fast. Some of the factors which need to be taken into consideration are the pathway length, the toxicity of intermediates, and the theorectical yield. Until now, three metabolic pathways for 2-butanol production have been proposed. All of the pathways start with the same molecule, acetoin, which can be produced from acetolactate by decarboxylation. In the first pathway acetoin is converted into 2,3-butanediol in the reduction reaction which is then dehydrated to 2-butanone. Butanone can be further reduced to 2-butanol using alcohol dehydrogenase. In the second pathway, 3-amino-2-butanol is obtained from acetoin by transaminase or a reductive aminase. This product is phosphorylated to 3-amino-2-butanol phosphate which can be then dephosphorylated to butanone which is finally reduced to 2-butanol. In the third pathway, phosphoacetoin is first obtained from acetoin which is then converted into 3-amino-2-butanol phosphate. 3-amino-2-butanol phosphate can further be dephosphorylated to butanone which is finally reduced to 2-butanol (figure 3). 2-butanol has very similar chemical and physical properties as 1-butanol and isobutanol (figure 4) (Zhang et al., 2019). It has the lowest value of logP among the three butanol isomers. Log P is a logarithm of octanol - water partition coefficient and it is a mesaure of lipophilicity. Therefore, since the toxicity of butanol is mainly caused by its hydrophobic nature logP can be used as an important indicator of solvent toxicity (Zhang et al., 2019).

Naturally occuring 2-butanol can be produced by some *Lactobacillus spp.* in reaction of 2,3-butanediol (2,3-BD) reduction during anaerobic fermentation. 2- butanol is obtained from meso-2,3-BD which is dehydrated to 2-butanone by vitamin B_{12} dependent glycerol dehydratase and subsequently reduced to 2-butanol by a secondary alcohol degydrogenase (Russmayer et al., 2019). Currently, it is not shown that reactions of pyruvate reduction to meso-2,3-BD and the conversion of meso-2,3-BD to 2-butanol occur within the same natural organism. The metabolic pathway, which is required for conversion of meso-2,3-BD to 2-

butanol, can be found in organisms capable of glycerol fermentation where it is needed for regeneration of the redox balance (Mar, 2020.).

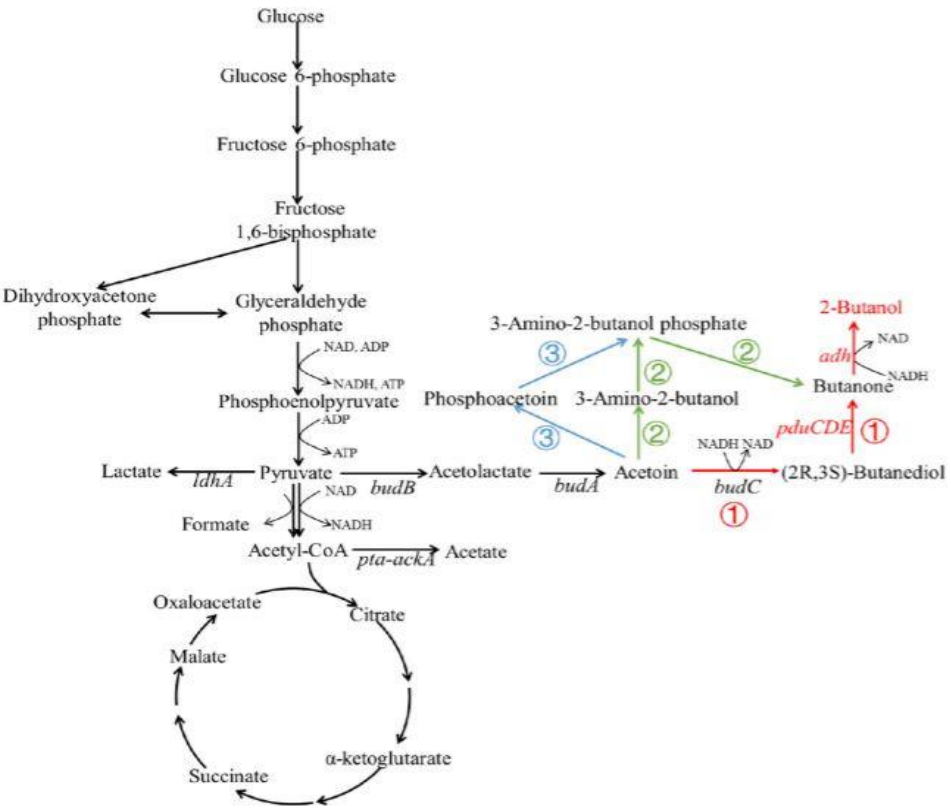

Figure 3. Different metabolic pathways for 2-butanol production (Zhang et al., 2019)

Property	Gasoline	Ethanol	n-Butanol	Isobutanol	2-Butanol
Chemical formula	C_4–C_{12}	C_2H_5OH	C_4H_9OH	C_4H_9OH	C_4H_9OH
Octane number	0–10	108	96	113	110
Density(g/mL) at 20°C	0.72–0.78	0.789	0.808	0.802	0.806
Flash point (°C)	−45 to −38	13	35	28	26.7
Autoignition temperature (°C)	300	363	343	415	406
Boiling temperature (°C)	25–215	78.4	117.4	108.1	99.5
Melting temperature (°C)	−56.6	−114.1	−89.8	−108	−114.7
Viscosity at 25°C	0.6	1.074	2.573	4	4.21
Heat of vaporization (MJ/kg)	0.36	0.92	0.34	0.43	0.56
Lower heating value (MJ/kg)	43.4	26.9	34.3	34.3	-
Solubility in water (%) at 25°C	-	100	6.32	8.5	13
Oxygen content	0	34.8	21.6	21.6	21.6
LogP	-	−0.31	0.88	0.79	0.61
Energy density (MJ/kg)	44.4	26.8	36.6	36.6	36.6

Figure 4. Different properties of butanol isomers (Zhang et al., 2019)

2.2.2. Microbial producers of 2-butanol

Lactic acid bacteria are a group of microorganisms that show sufficient promise as production organisms for different compounds, such as 1,3 propanediol, lactic acid, or 2-butanol. Many lactic acid bacteria are able to utilise various hexoses and pentoses and this could lead also to the exploitation of lignocellulosic sugars which are abundant renewable resources. Since lactic acid bacteria have certain characteristics such as tolerating low pH, high alcohol concentration, growing on low-cost, renewable feedstocks they are considered as promising industrial cell factories. To have a balanced and optimised process the most important element is to achieve butanol tolerance in production host. To produce 2-butanol as a final product, researchers developed a two-step co-cultivation process using *S. marcescens* and *L. diolivorans*. *S. marcescens* was used to produce meso-2,3-BD from glucose after which bacterium was heat-inactivated. In the second step during anaerobic fermentation with glucose the accumulated meso-2,3-BD was converted to the final 2-butanol by *L. diolivorans* (Russmayer et al., 2019).

2.3. Microbial based production of 2,3- butanediol

2,3-butanediol (2,3-BD) is one of the promising bulk chemicals that has great potential for a variety of industries, including chemical, cosmetic, agricultural and pharmaceutical. 2,3-BD has two stereoactive centers, so there are three different isomers in nature. These are the optically active R- and S-form and the meso-form. Chemically, 2,3-BD is produced in very small quantities using butene, which must first be separated from the cracked gas. In the biological process, optically pure isomers of 2,3-BD are obtained from monosaccharides using microorganisms (figure 5). All three isomers can be produced by naturally occurring microbial strains. However, the ratio between the different isomers they produce varies (Song et al., 2019). The ratio of produced stereoisomers can vary considerably depending on the type of microorganism and the fermentation conditions. The synthesis of 2,3-BD usually follows a pyruvate-diacetyl / acetoin metabolic pathway that can be found in lactic acid bacteria and in microorganisms that ferment citrate and glucose and the metabolic pathway itself consists of 3 enzymatic steps. The metabolic pathway begins by condensing two pyruvate molecules into alpha-acetolactate with the enzyme alpha-acetolactate synthase. Then in the next step the alpha-acetolactate is decarboxylated to acetoin which is then used as a precursor to produce 2,3-BD. This reaction is catalyzed by various acetoin reductases or 2,3-BD dehydrogenases.

Bacterial species such as *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Bacillus polymyxa* and *S. marcescens* are capable of 2,3-BD production. For *K. pneumoniae*, *K. terrigena*, *E. aerogenes*, *Vibrio cholerae*, *B. subtilis*, and *S. marcescens* regulation mechanism of 2,3-BD is well known (Yang et al., 2017). *K. pneumonia* mainly produces meso-2,3-BD and has some advantages such as more complete fermentation, capability of using variety substrates and cultural adaptability. *B. polymyxa* produces only the (R,R)-2,3- BD and is capable of anaerobic growth (Zhang et al., 2010). The titers of 2,3-BD produced by lactic acid bacteria are low and a racemic mixture of 2,3-BD is formed. In order to produce a larger amount of 2-butanol, a higher titer of meso-2,3-BD is required, and so far the highest production of 150 gL^{-1} and 152 gL^{-1} 2,3-BD has been achieved with *S. marcescens* H30 and *K. pneumoniae* SDM (Bai et al., 2015).

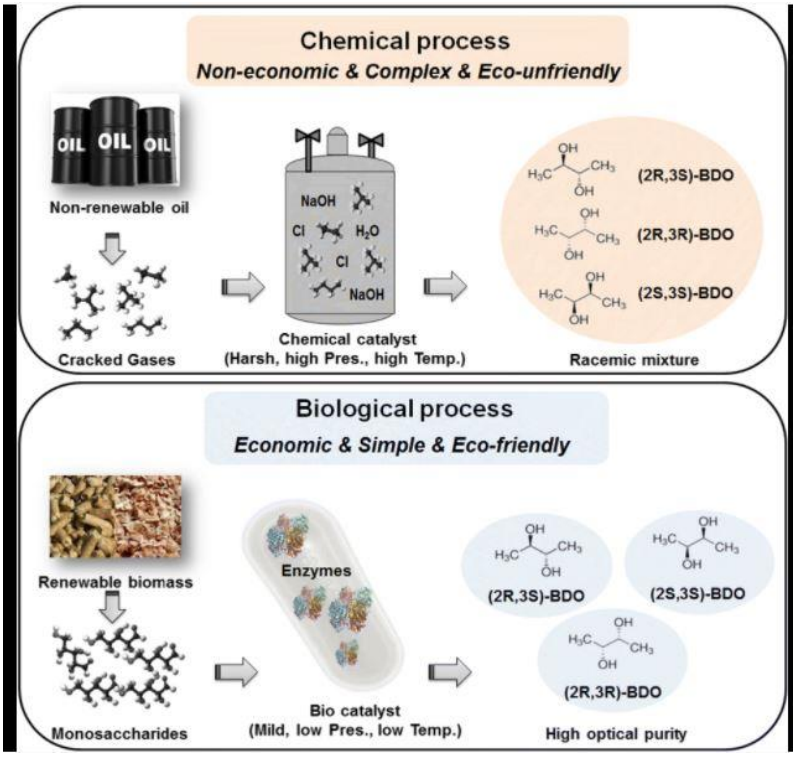

Figure 5. Comparison of chemical and biological processes for 2,3-BD production. In the chemical process, a racemic mixture of isomers is obtained from the cracked gases by a chemical catalyst, while in the biological process, microorganisms produce optically pure isomers of 2,3-BD from monosaccharides (Song et al., 2019).

2.3.1. *Serratia marcescens* as an efficient producer of 2,3-BD

S. marcescens showed a great potential for 2,3- BD production. It grows rapidly with a wide range of substrates (Shi et al., 2013) and is capable of producing only meso-2,3-BD which is an additional advantage for further 2-butanol production (Russmayer et al., 2019). *S. marcescens* is a Gram-negative bacterium, member of the genus *Serratia* (Hejazi and Falkiner, 1997). The bacterium is part of the family *Enterobacteriaceae* and it is capable of producing a large quantity of 2,3-BD in the presence of sugar (Bai et al., 2015). *Serratia spp*. in general are motile, non-endospore forming bacteria, widely distributed and can be isolated from plants, soil, water and air. Species of *Serratia* genus share very close genotypic and phenotypic characteristics with each other. *S. marcescens* is an opportunistic pathogen and it is noticed that currently eight of 14 species of *Serratia*, which are recognized within the genus, are associated with human infection. Of all *Serratia* strains, *S. marcescens* is the most common clinical isolate and the most important human pathogen (Mahlen, 2011). *S. marcescens* produces a red pigment, prodigiosin (Figure 6) at room temperature. They secrete several enzymes like lipase, gelatinase, DNase, hemolysin, chitinase, proteases and multiple isozymes of alkaline phosphatase. Within the *Enterobacteriaceae* family, *Serratia* is phenotypically one of the easiest genera to differentiate. Moreover they are resistant to the antibiotics cephalothin and colistin and *Serratia* is ubiquitous genus in nature. It can utilise a lot of nutrients which helps its ability to grow and survive under extreme conditions (Buckle, 2015).

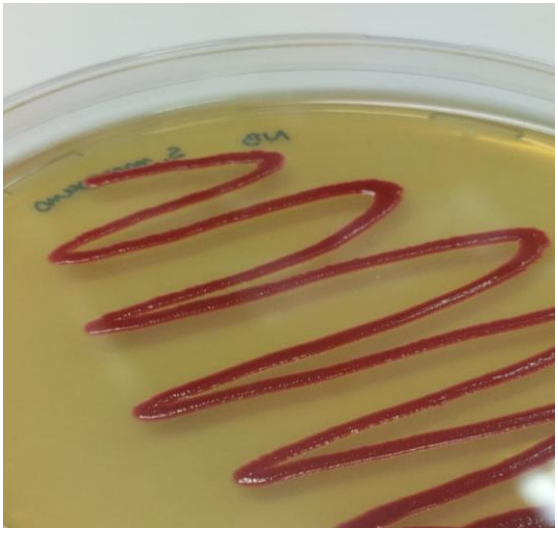

Figure 6. *S. marcescens* growing on the NB medium, overnight culture. The red colour indicates a production of a pigment, prodigiosin (laboratory photo).

2.3.2. Engineered *S. marcescens* for 2,3-butanediol production

Successful microbial production of 2,3-BD is possible to achieve using native microbial hosts. Engineering strategies, that include genetic modifications and optimisation of the process itself, can lead to strain improvement and reaching industrially-relevant levels. The metabolic pathway and genes for 2,3-BD synthesis have recently been identified in *S. marcescens* MG1. *SlaA* (encoding alpha acetolactate decarboxylase), *slaB* (encoding acetolactate synthase) and *slaC* (encoding meso-2,3-butanediol dehydrogenase) with two regulators encoded by *slaR* and *swrR* were detected as key genes for production. *SlaR* has been shown to be an activator of *slaA* and *slaB* while deleting *swrR* has led to earlier production of 2,3-BD in regard to wild type (Yang and Zhang, 2019). *S. marcescens* can produce high yield of meso -2,3-BD using glucose as a substrate (figure 7). The metabolism of glucose by *S. marcescens H30* is a mixed acid and solvent fermentation. In the experiments, various carbon and nitrogen sources as well as inorganic salts were examined to see the possibility of high 2,3-BD production from *S. marcescens H30*. The results showed that sucrose has a large effect on 2,3-BD production (Zhang et al., 2010).

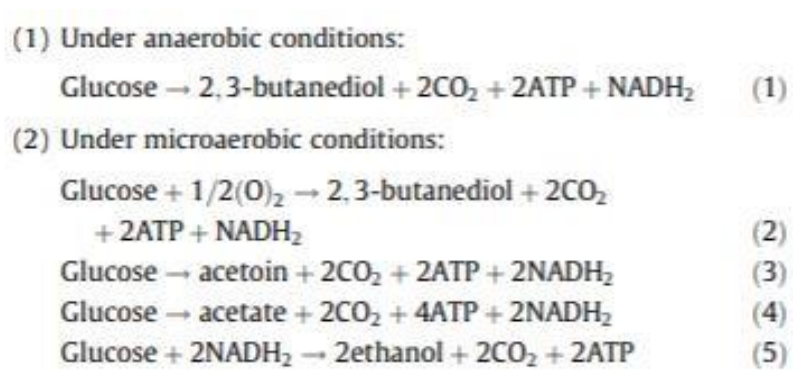

Figure 7. The metabolism of glucose under anaerobic and microaerobic conditions (Zhang et al., 2010)

2.4. Glucose transport systems in eubacteria

Glucose is often used as a carbon source by many microorganisms and over the years many different transport systems have been studied. Most widespread is PTS (phosphotransferaze) system. Except PTS system nutrient can be taken up by proton or cation gradient -driven transporters, through ATP-dependent ATP binding cassette (ABC) permeases, SGLT or MFS system (figure 8). The activity of the glucose transport systems often determines the rate of the metabolic flux (Jahreis et al., 2008).

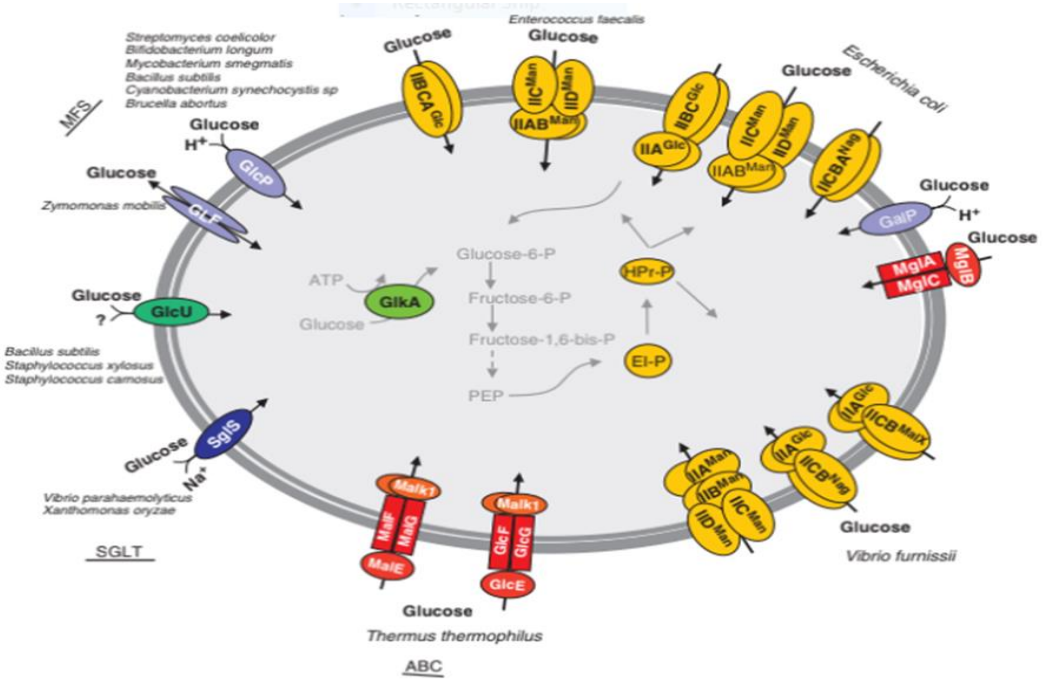

Figure 8. Different glucose transport systems in bacteria (Jahreis et al., 2008)

2.4.1. Glucose transport systems in Gram-negative bacteria

There are different sugar transport systems known in Gram-negative bacteria and *Enterobacteriaceae*. The transport of carbon sources, especially glucose, and molecular knowledge of glucose uptake is an important field which was widely examined in *E. coli*. In *E. coli* exist several transport systems which are capable of glucose transport into the cytoplasm (figure 9). The main glucose uptake systems are glucose PTS and mannose PTS while galactose and maltose transporters are also capable of glucose translocation. All the systems consist of specific proteins encoded by genes which are regulated at multiple levels with several global transcriptional regulators involved in controlling carbohydrate uptake and metabolism (Steinsiek and Bettenbrock, 2012). In the *E. coli* genome, the genes for HPr (*ptsH*), enzyme I (*ptsI*), and enzyme IIAGlc (*crr*) are the parts of the pts operon. *S.marcescens* is also a member of ***Enterobacteriaceae*** and it appears it has a *pts* operon that is very similar to that of *E. coli* (Uchiyama et al., 2003).

Transport systems:

1. Glucose -PTS- glucose transport by $EIICB^{Glc}$ (encoded by *ptsG*)

2.Mannose -PTS (encoded by *manXYZ*)- aerobic conditions with glucose as sole carbon source

3.Galactose transport systems: galactose ABC transporter (encoded by *mglBAC*)

galactose permease (encoded by *galP*)

4.Mgl-system has been reported to be active under conditions

Figure 9. Different transport systems connected with glucose uptake (Steinsiek and Bettenbrock, 2012)

2.5. Genetic engineering on *S. marcescens*

2.5.1. Genetic transformation

Genetic transformation in general is a process which involves the introduction and expression of foreign genes in a host organism (Lorenz and Wackernagel, 1994). Using genetic engineering techniques, strain construction and improvement can be achieved nowadays. A working transformation procedure is a prerequisite for applying genetic engineering techniques to any organism (*S. marcescens)*. There were not many genetic manipulations done on *S. marcescens*. Research was conducted on *S. marcescens Sr41* with aim of determining the adequate conditions for the electrotransformation (Sakural and Komatsubara, 1995). Electrotransformation is a process used for the introduction of plasmid DNAs into various cells which are made permeable using a brief pulse of high voltage electricity. This method is often used because it showed higher efficiency compared to other transformation methods.

2.5.2. CRISPR Cas-9 method

The CRISPR Cas-9 is a genome editing technology used as a powerful molecular tool for targeting or modifying DNA. CRISPR is an acronym for "clustered regularly interspaced short palindromic repeats". It has been discovered as part of the bacterial immune system. The main principle of the method is that a specific protein Cas9 is able to localize and cleave a target DNA via a guide RNA molecule which is complementary to the target sequence and consisting of a protospacer-adjacent motif (PAM) at the 3′ end (Tycko et al., 2016). To disrupt or cut a certain gene from the genome of some organism it is essential to have a match between the CRISPR RNA and the DNA sequence as well as the presence of the Cas 9 protein and the tracrRNA (Doudna and Sternberg, 2018).

3. MATERIALS AND METHODS

3.1. Working strains and plasmids

S. marcescens DSMZ 14187 was used as a main host organism (table 1). It was streaked, from a permanently frozen at -80 ^{o}C glycerol stock, on a plate. The plate was incubated at 30 ^{o}C. *Escherichia coli* JM110 as well as *Escherichia coli* DH10B were used for plasmid construction and plasmid propagation (table 1). Plasmids used for genetic transformation protocols were made in the lab using Golden Gate assemblies (table 2). The genetic elements required for plasmid construction such as origin of replication, promoter and terminator were from *E.coli* strains. All the sequences coding for promotors and terminators were from the iGEM Parts Registry (http://parts.igem.org/Part:BBa_K1468000). Promoters used in the experiment were constitutive promoters and plasmids contained gene coding for kanamycin resistance.

Table 1. Bacterial strains used during the experiment

Organism	**Strain**	**Source**
Serratia marcescens	DSMZ 14187	DSMZ*
Escherichia coli	DH10B	DSMZ
Escherichia coli	JM110	DSMZ

* German Collection of Microorganisms and Cell Cultures

Table 2. Plasmids made for transformation process

Plasmid	**Source**
BB3_AB_p105_mCherry_t1001	Made in this work
BB3_AB_p105_GFP_t1001	Made in this work
BB3_AD_p109_Cas9_p105_ptsG5'_ptsG3'_t1001	Made in this work
BB3_AD_p109_Cas9_p105_man5'_man3'_t1001	Made in this work
BB3_AB_p105_eGFP_t1001	In-house

3.2. Primers

Specific primers with overlapped sequences were ordered to assemble DNA fragments as the templates of sgRNAs for CRISPR-Cas9 gene targeting (table 3). To knock out target genes, for each *ptsG* gene and *man* operon it have been designed and ordered four primers (see table 3). The rest of the primers required for the assembly of DNA fragments as the templates of tracRNA (constant part of sgRNA) were made *in-house*. Ordered primers were delivered as pellets (25 nMoles), diluted after with water to gain 100 μM stock solution, and then to final 10 μM and 2μM working solutions and stored at -20 ^{o}C. Four ordered primers were connected in turn by overlapping parts. The DNA templates for sgRNAs were assembled by an overlapping PCR reaction with the ordered PCR primers (see apendix). Gained oligonucleotides after PCR reaction were cloned into individual vectors. For colony PCR reaction it have been designed primers complemented to 5' and 3' end of *ptsG* gene and *man* operon and additional "in" primers as well (table 4).

Table 3. Primers used for the assembly of DNA fragments as the templates of sgRNAs

Gene	**Target sequence +PAM 5'- 3'**	**cr RNA primers**	**tracRNA primers**
*ptsG*_5	ATCACGTGGGAGACTACCGCAGG	1_ptsG_5_fw	1gRNA_all_rev
		2_ptsG_5_fw	
*ptsG*_3	CTGGTCGACTTTGGACACGTCGG	1_ptsG_3_fw	2gRNA_all_rev
		2_ptsG_3_fw	
*man*_operon_5	GGTCGACAAAGAAAACTATGAGG	1_man_operon_5_fw	3gRNA_all_rev
		2_man_operon_5_fw	
*man*_operon_3	ATCGAGGATGGTCTGCACCGTGG	1_man_operon_3_fw	4gRNA_all_Fw
		2_man_operon_3_fw	

Table 4. Primers used for colony PCR reaction

Primer	**Sequence**
ptsG_check_fw	5' GAATCATCGCGACAAGAAG 3'
ptsG_check_out	5' CACAGAGCAAACTAGATGG 3'

ptsG_check_in	5’ GTTTGGCCGCAATCTCTTC 3’
MAN_check_fw	5’ CGGTCAGTGACATCTCTTG 3’
MAN_check_out	5’ GTGAGAAATGTTAGGCGTG 3’
MAN_check_in	5’ CGATATCGTCAACGACATG 3’

3.3. Chemicals, culture medium and cultivating conditions

Chemicals were purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and were of the highest purity and analytical grade available.

3.3.1. *Serratia marcescens*

For *S. marcescens* growth medium was Nutrient broth (NB) medium. This medium contained soy peptone, meat extract,yeast extract, NaCl and glucose as a carbon source (table 5). Nutrient broth medium provides best conditions for growth according to the literature data (Sakural and Komatsubara, 1996). Nutrient broth was used for the growth of pre-cultures and main cultures of bacteria. Flasks were incubated at 30 ^{o}C, shaken at 180 rpm for 24 hours.

3.3.2. *Escherichia coli*

Growth media used for *E. coli* strains was Luria-Bertani Medium (LB). It contained bacto tryptone, yeast extract and NaCl (table 6). Liquid cultures contained LB medium and antibiotic kanamycin. Volume in incubated test tubes was 2 mL, they were shaken at 180 rpm at 37 °C for 24 hours.

Table 5. Preparation for 1000 g NB-medium

Component	**Concentration(gL^{-1})**	**Initial weight (g)**	**±2.5 % (g)**
Yeast extract	10.0	10.0	0.25
Meat extract	3.0	3.0	0.075
Hy soy peptone	10.0	10.0	0.25
NaCl	5.0	5.0	0.125
Glucose	10.0	10.0	0.25
RO-water	Up to 1000 g		
For solid medium: Agar-Agar (10.0 g +/- 0.25 g) was added to a 500 mL bottle before autoclaving. It was heated in microwave for liquefaction before pouring.			
Autoclave and aliquot			

Table 6. Preparation of 1000 g LB medium

Component	**Initial weight (g)**	**±2.5 % (g)**
Bacto tryptone	10.0	0.3
NaCl	5.0	0.13
Yeast extract	5.0	0.13
Set pH to 7.4 – 7.6 with 4 M NaOH/8 M NaOH		
RO-water	Up to 1000	
For solid medium: Agar-Agar (10.0 g +/- 0.25 g) was added to a 500 mL bottle before autoclaving. It was heated in microwave for liquefaction before pouring.		
Autoclave and aliquot		

3.4. Determination of antibiotic concentration for selection process

To establish reproducible genetic transformation protocol for *S. marcescens* the first step was determining the antibiotic concentration needed for selection of positive transformants. Antibiotic concentration was determened by several experiments where range of concentrations of kanamycin antibiotic were used according to the literature data (Sakural and Komatsubara, 1996) (table 7). Resistance gene was placed on the backbone of every recombinant plasmid which was transformed into the cell prior to the cultivation. Only cells that have received the correct plasmid are able to survive, others are killed by the antibiotic. The antibiotic resistance of a strain is the highest level of antibiotic where a wildtype strain without the plasmid can grow. Positive and negative control have also been done and it was examined both for chemical competent process and for electroporation.

Table 7. Determination of required antibiotic concentration

Kanamycin concentration (mgL^{-1})	**Volume (µL)**
25	10 100 REST*
50	10 100 REST*
75	10 100 REST*
100	10 100 REST*
150	10 100 REST*
200	10 100 REST*
250	10 100 REST*
300	10 100 REST*

3.5. Genetic transformation protocol

There are a lot of organisms using different types of genetic transformation methods. In the case with *S. marcescens* two types of genetic transformation protocols have been examined, chemical competent protocol and electroporation. Both methods required the use of competent cells, either chemically competent or electrocompetent cells.

3.5.1. Chemically competent protocol

Rubidium Chloride (RbCl) protocol, which is the standard protocol used in the lab, was used for preparation of competent cells in *S. marcescens* and in *E. coli*.

Steps required for preparation of chemically competent cells:

1. Preparation of pre-culture of *S. marcescens* - 50 mL of NB medium has been inoculated with a single colony and incubated the flask overnight in a shaker-incubator (30 ^{o}C, 180 rpm)

2. On the following day, a main culture was inoculated from the overnight pre-culture for transformation

3. Culture was incubated untill OD_{600} reached 0.6

4. The cells were then cooled down on the ice for 10 minutes and after that were pelleted in a centrifuge (5 min, 4 ^{o}C, 4000 g)

5. Cell pellets were washed in ice-cold buffers (TFB1 and TFB2) (see table 8)

6. 100 μL aliquots were taken in tubes and the cells were placed on -80 ^{o}C untill further use

After competent cells are prepared they are mixed together with plasmid DNA to perform the transformation process. After the mixture is prepared it was put on ice for 30 minutes. The next step was to heat shock cells at exactly 42 ^{o}C for 90 seconds. After recovering cells on ice for 5 minutes, 1 mL of room temperature LB medium was added into the mixture and placed at 30 ^{o}C for 2h for kanamycin expression**.** The final step was to plate the cells on selective NB agar. Cells were spread on three plates containing 10 μL, 100 μL and 890 μL cell culture. To achieve better evaporation of the excess liquid, the 890 μL were centrifuged for 15 sec, the supernatant was discarded and the cell pellet resuspended in 100 μL (rest volume*). The process was tested using three different amounts of DNA (100, 200 and 400 ng), 5 different antibiotic concentrations (25, 50, 75, 100 and 150 mgL^{-1}) and in all cases plates were plated with 10 μL, 100 μL and the rest volume.

Table 8. Composition of TFB1 and TFB2 solutions

Solution TFB1	30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM MnCl2, 100 mM RbCl and 15 % glycerol; adjust to pH 5.8 (with 1M acetic acid)
Solution TFB2	100 mM MOPS (or PIPES), 75 mM $CaCl_2$, 10 mM RbCl and 15 % glycerol; adjust to pH 6.5 (with 1 M KOH)

3.5.2. Electroporation protocol

Electroporation protocol was used for *S. marcescens* and the first step was also preparing competent cells. In preparation of electrocompetent cells first two steps were the same as for chemical competent protocol. Competent cells have been incubated untill OD_{600} reached value 1. After centrifugation of the culture, the resulting pellets were washed twice with ice-cold 100 gL^{-1} glycerol. Then 80 µl aliquots of the suspension were dispensed into cold 1.5-ml Eppendorf tubes and immediately frozen at - 80°C. After competent cells have been prepared they were mixed together with DNA to perform the transformation process.

Electroporation protocol was performed using Gene Pulsed device in a cooled 0.2-cm cuvette at 2500 $kVcm^{-1}$, the capaticance was 25 µF and the resistance of the Pulse Controller was set at 600 Ω. After the electrical discharge, NB broth (1.0 ml) was immediately added to the cuvette. The resulting suspension was kept at 30 ^oC for 2h to allow expression of the kanamycin (Sakural and Komatsubara, 1996). Protocol was tested using three different amounts of DNA (100, 200 and 400 ng), 5 different antibiotic concentrations (25, 50, 75, 100 and 150 mgL^{-1}) and all plates were plated with 10 µL, 100 µL and the rest volume. After transformation, selected colonies grown on plates were transferred with toothpicks on prepared master-plates and then incubated in the liquid cultures. Liquid cultures contained NB medium and antibiotic kanamycin. Volume in incubated test tubes was 2 mL, they were shaken at 180 rpm at 30 °C for 24 hours.

3.5.3. Effect of different carbon sources on genetic transformation

Different types of carbon sources have been examined to see the effect on the transformant cells growth during transformation of *S. marcescens* while doing CRISPR Cas9 gene targeting. Different carbon sources have been added in the standard NB medium (table 9).

Table 9. Different carbon sources used for examination

Carbon source (part of NB medium)	**Concentration (gL $^{-1}$)**
Glucose	20
Sucrose	20
Fructose	20
Xylose	20
Glycerol	20
Natrium Acetate	10

3.6. Golden Gate assembly

Golden Gate cloning was applied for the plasmid preparation in a way that individual expression cassettes were assembled into a single vector. Inserts were assembled into a destination vector containing an antibiotic resistance marker. The Golden Gate system consisted of three hierarchical backbone (BB) levels. In the lowest cloning level, individual elements such as promoters, coding sequences and transcription terminators were incorporated into backbone 1 (BB1) plasmids, which were subsequently assembled to one expression cassette in backbone 2 (BB2) plasmid. This was followed by the assembly of multiple expression cassettes into final backbone 3 (BB3) vector. Table 10 shows all Golden Gate assemblies used for the preparation of plasmids made in this thesis. Genetic elements from *E. coli* (promoters, terminators, origin of replication) were used for construction and distribution of the plasmid in *S. marcescens* and heterologous protein expression. The method was applied also for the assembly of plasmids containing DNA templates of sgRNA molecules required for CRISPR Cas-9 technology. Coding sequences in each back bone 1 vector (BB1) varied depending on the final plasmid. T4 DNA ligase was diluted with Cut Smart buffer and together with proper enzyme added to the mixture to initiate the reaction. The mixture was incubated on a thermocycler in a single PCR tube.

Table 10. Golden Gate assemblies for plasmid preparation, tables (a, b, c) present hierarchical backbone (BB) levels needed for plasmids construction; a) BB1 assembly; b) BB2 assembly, c) BB3 assembly

a) BB1 assembly

BB1_assembly	**Volume (µL)**
BB1_L_23	1
CDS (coding sequence, variable part*)	2
ATP	2
Cut Smart	2
Ligase (1:10)	2.5
BsaI	1
H_20	Up to 20 µL

*CDS – *gfp*, *mCherry*, *ptsG* 5', *ptsG* 3', *man 5*', *man 3*'

b) BB2 assembly

BB2_assembly	**Volume (µL)**
BB2_AB (empty backbone)	1
BB1 (expression cassette)	1
BB1_p	1
BB1_t	1
ATP	2
Cut Smart	2
Ligase (1:10)	2.5
BbsI	1.5
H_20	Up to 20 µL

c) BB3 assembly

BB3_ assembly	**Volume (µL)**
BB3_*(empty backbone)	1
BB2_ * (quantity depended on the number of expression cassettes)	1
ATP	2
Cut Smart	2
Ligase (1:10)	2.5
BsaI	1
H_2O	Up to 20 µL

3.7. Plasmid preparation

The main aim of plasmid preparation was to isolate and purify plasmid DNA (BB3 vectors) from *E. coli* cells after liquid cultivation. It have been done mini-preps with elution volume of 50 µL using elution buffer and Midi-preps from 100 mL liquid cultivations. It was used SLG® Hi Yield® Plasmid Mini DNA Kit Süd-Laborbedarf GmbH for Mini-preps. HiSpeed®Plasmid Midi Kit by QIAGEN was used to isolate plasmid DNA from 100 mL overnight cultures. The preparations were performed according to the manufacturer's protocols. In the first step bacterial cells were harvested by centrifugation and re-suspended in a PD1 buffer (EDTA/RNasaA) to stabilize DNA and to degrade bacterial RNA. After steps of extraction, the plasmid DNA was bound on a solid phase, washed with specific buffer and eluted. All fragments used for assemblies preparation were in concentration of 40 nM. The accurate plasmid concentrations have been determined with NanoDrop™ 1000 Spectrophotometer.

3.7.1. Restriction analysis

After each plasmid preparation, restriction analysis was done. It means DNA was cut with specific restriction enzymes that have been determened using *in silico* analysis. The

prepared mixture was incubated at 37 ºC for one hour (table 11). Gained DNA fragments were then separated with gel-electrophoresis to identify the sizes of unknown fragments relative to a standard ladder. Fragments of interest were then isolated and ligated into a plasmid vector for cloning.

Table 11. Standard reaction mixture for restriction analysis

Component	**Volume (µL)**
DNA	2
Cut Smart buffer	2
Enzyme	0.5
H_2O	Up to 20

3.8. Fluorescent microscopy

Fluorescent microscopy (Zeiss Observer Z1, AX10) was used to check whether transformants growing on the plate were positive and contain genes expressing the fluorescent proteins (GFP and mCherry). Transformants grown on plates were streaked on new plates to get single clones. One colony per sample was then picked after one day incubation and put in 2 mL NB medium, centrifuged and washed with Phosphate Buffered Saline (PBS). Appropriate sample preparation is necessary to ensure high quality images are captured. Cells were fixated using PBF buffer again and watched under fluorescent microscope.

3.9. CRISPR Cas-9

For CRISPR Cas-9 gene targeting it was required to have a custom single guide RNA (sgRNA). It was contained of a targeting sequence (crRNA) which is a 20 nucleotides long variable part of the sgRNA, homologous to a region in the gene of interest and will direct Cas9 nuclease activity, and Cas9 nuclease-recruiting sequence (tracRNA) which is constant for every sgRNA. To perform a complete gene knock out two DNA templates for sgRNAs were used and cutting was done at the 5' and at the 3' end using specific primers (see table 3). Cas9-mediated DNA cleavage should cause a double-strand break within the target DNA and subsequently inactivate genes.

3.9.1. Assembly of plasmids

In the first step, oligonucleotides for sgRNAs obtained in PCR reaction were cloned into individual plasmids (back bone vector 1). These back bone vectors, containing ligation product,

were used for genetic transformation of competent *E.coli* cells. After picking the colonies from the plates, plasmid DNA was isolated using commercial Mini prep kit. To verify recombinant colonies it was used Sanger sequencing. Inserts were identified using sequencing primers appropriate for the selected vector. The same steps, except the Sanger sequencing, have taken place to get the back bone 2 vector (BB2) and the back bone 3 vector (BB3). The final assembly (BB3) contained three expression cassettes for each of two final samples ptsG 5'-3' and man operon 5'-3'(table 10; c).

3.10. Polymerase chain reaction

A polymerase chain reaction is an *in vitro* method that allows some specific region of DNA to be amplified multiple times. In the experiment, two subtypes of PCR reaction had been used, overlapping PCR and colony PCR.

The overlapping PCR reaction was used for fusing gene elements together. It was used to generate a template DNA expressing the sgRNAs. For each of *ptsG* gene and *man* operon it have been designed four overlapping primers (see table 3). The rest of the primers required for the DNA template for tracRNA (constant part of sgRNA) were made *in-house*. Primers were designed using CHOP-CHOP tool and were delivered as pellets (25 nMoles), diluted after with water to gain 100 μM stock solution, and then to final 10 μM and 2μM working solutions and stored at -20 ^{o}C. Four ordered primers per gene were connected in turn by overlapping parts (see appendix). In the PCR reaction, it was used a specific type of DNA polymerase enzyme, the high fidelity, high-processivity Q5 DNA polymerase. In the table 12 it is shown which components were put in the PCR reaction tube. The main principles of the method are the same as the one in regular PCR reaction (see table 13 for conditions). After steps of denaturation, annealing and extension, a final DNA product was synthesized.

The colony PCR reaction was used to check gene knockout of the *ptsG* gene and the *man* operon after transformation of *S. marcescens.* For transformation were used BB3 _AD_p109_Cas9_p105_ptsG5'_ptsG_3'_t1001 and BB3_AD_p109_Cas9_p105_ MAN5'_ MAN3'_ t1001 plasmid constructs (see table 2). Primers were designed to specifically target fragments of interest (*ptsG* gene and *man* operon) (see table 4). First step was to pick a single colony using a sterile pipette tip and pipette it up and down in 20 μL of nuclease- free water. After that 5 μL of prepared mixture was transfered into the empty PCR tube. In the table 14 are listed all the components put in the colony PCR reaction tube and in table 15 conditions used in the reaction.

Table 12. Standard components mixture for an overlapping PCR reaction containing 50 µL total reaction volume in PCR tubes.

Component	**Volume (µL)**	**Final concentration**
10x Q5 Buffer	10	1x
10 mM dNTPs	1	200 µM each
Q5-polymerase	1	
1_ordered forward primer	2.5	10 µL
2_ordered reverse primer	0.5	2 µL
1_gRNA primer	0.5	2 µL
2_gRNA primer	0.5	2 µL
3_gRNA primer	0.5	2 µL
4_gRNA reverse primer	2.5	10 µL
H_2O	Up to 50 µL	

Table 13. Conditions used for an overlapping PCR reaction

Step	**Temperature (ºC)**	**Time**	**Cycles**
Initial denaturation	98	30 s	
Denaturation	98	10 s	35
Annealing	60	20 s	
Elongation	72	10 s	
Final elongation	72	2 min	

Table 14. Standard components mixture for a colony PCR containing 20 µL total reaction volume in PCR tubes.

Component	**Volume (µL)**
Template DNA	variable
Q5 polymerase	0.2
Q5 buffer	4
dNTPs	0.4
Forward primer	1
Out primer	1
H_2O	Up to 20

Table 15. Conditions used for a colony PCR reaction

Step	**Temperature (ºC)**	**Time**	**Cycles**
Initial denaturation	98	30 sec	
Denaturation	98	10 sec	35
Annealing	66	10 s	
Elongation	72	15-30 sec per kb	
Final elongation	72	10 min	

3.11. DNA sequencing

After sgRNA DNA templates were obtained in PCR reaction, synthesized regions were subcloned into individual back bone 1 vectors. After transformation of *E.coli* competent cells, few obtained colonies were chosen for Sanger sequencing. Plasmids were purified from cells and assembled regions in back bone vector 1 were sequenced by Microsynth AG using the Sanger sequencing method. For sequencing by Microsynth, the sample template needed to be prepared in certain way. It was premixed in a 1.5 mL vial tube containing a total volume of 12 µL (table 16). After sequencing results were delivered, they have been compared and approved with *in silico* cloning sequences using CLC Main Workbench computer software.

Table 16. Standard mixture for sequencing with total volume of 12 μL

Component	**Volume (μL)**	**Concentration**
DNA template	5	200 ng μL^{-1}
RO-water	7	

3.12. DNA purification from the gel

For most of the DNA methods it is very important to provide purified samples of DNA. It was used Wizard® SV Gel and PCR Clean-Up System from Promega. It is a commercially available kit for DNA purification. The whole procedure was done according to the manufacturer's protocol. PCR reaction products on an agarose gel have been cut out prior to purification. Primary step when purifying DNA from an agarose gel is to melt the agarose. Therefore, the purified DNA is available for binding to the silica membrane. This entire process was done after the polymerase chain reaction of regions for sgRNAs expression. Purified primers have then been used for cloning and sequencing.

3.13. Agarose gel-electrophoresis

The method was applied after PCR reaction and it was performed with a sample amount up to 50 μL sample per well. Also, it was used for the verification during the cloning process to detect the presence of DNA fragments and in this case it was performed with 20 μL sample per well. Electrophoresis was performed in a buffer solution (1xTris-Acetate-EDTA) (TAE). Depending on the size of DNA fragments different percentage of agarose can be used. It was used 1% (w/v) agarose in done experiments. After that the mixture was heated in the microwave untill the agarose was dissolved. The following step was adding 15 μL of stain to the mixture and pouring it into the prepared form to get solid. It was used SYBR® Safe by Life Technologies Corporation. Therefore, it was illuminated by an ultraviolet light in a BIORAD universal hood II with the help of computer software (QuantityOne 4.6.1- GelDoc; BIORAD)**.** Another stain was also used which binds to the DNA fragments with aim to visualise the results after an electrophoresis. It was 6x MassRuler DNA Loading dye by ThermoScientific which contained glycerol to prevent the DNA from floating out of the wells. 4 μL of loading dye were used per 20 μL sample. Also, 7 μL of Mass Ruler ^{TM} DNA ladder mix by Thermo Scientific was used (see figure 10). MassRuler^{TM} DNA ladder mix is a mixture of DNA fragments of known sizes.

It was ranged between fragment sizes of 80 bp to 10 kb for fragments sizes between 300 bp and 4 kb.

(a) **(b)**

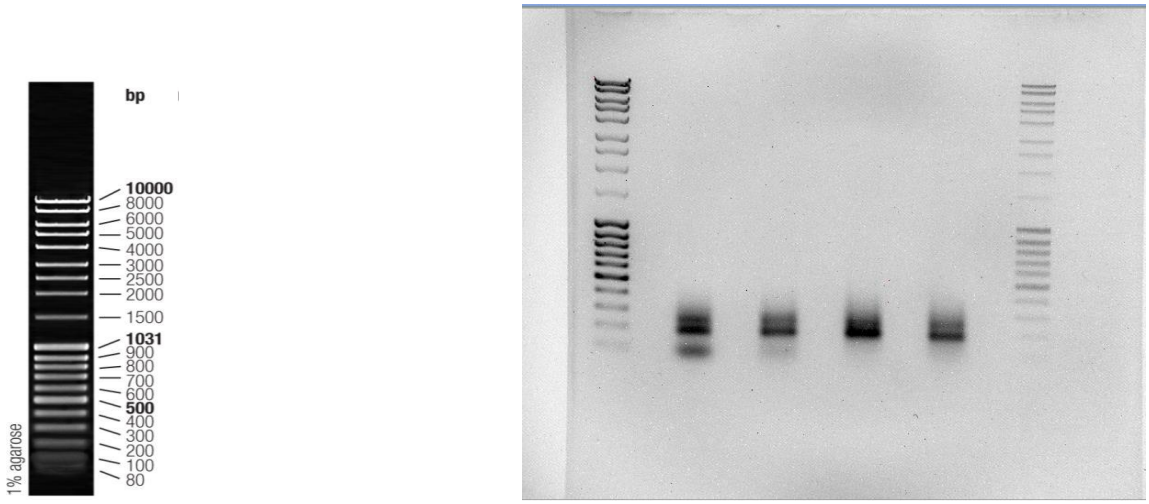

Figure 10 a) MassRuler^{TM} DNA ladder mix (ISO 9002, 2016); b) example of gel -band 1-ladder, band 2,3,4 and 5 PCR fragments size app. 240 bp, band 6-ladder (laboratory image).

3.14. In silico analysis

Certain bioinformatic research was done using a computer simulation program (CLC Main Workbench). Using this software all cloning steps such as detection of correct enzymes capable of restriction digestion have been previously performed. Using CLC software were also found genes coding for transport systems proteins in *S.marcescens* that could be potentially knock-out. It was also used to confirm the accuracy of the sequences obtained by Sanger sequencing and to display template strands for sgRNAs. Another tool, CHOP CHOP, was used to design templates for sgRNAs and primers.

4. RESULTS AND DISCUSSION

The main focus of the thesis was to abolish the glucose uptake systems in *S. marcescens*. The goal was to knock-out the genes connected to glucose uptake which should stop the growth on glucose. The genes of interest were *ptsG* and the *man* operon. The method of choice for gene knock-out was CRISPR Cas9 strategy. The prerequisite for this process is an efficient DNA transformation method for *S. marcescens* which was established using *E. coli* genetic elements for plasmid construction.

4.1. Determination of antibiotic concentration

The antibiotic of choice was kanamycin. For determination of antibiotic resistance, 5.52 x10^8 cells mL^{-1} were plated on NB agar containing various levels of the antibiotic. First it have been examined concentrations of 150, 200, 250 and 300 mg L^{-1} of kanamycin to see the effect on bacterial cells growth. Since no growth has been observed, concentrations for examination were lowered to 25, 50, 75, 100 and 150 mg L^{-1} and still there was no growth seen. These were the preliminary experiments to see the effect of antibiotic on *S. marcescens* cells before doing genetic transformation. After the examination of the antibiotic concentration, both in chemical competent protocol and electroporation, it was shown that 100 mg L^{-1} of kanamycin was the lowest concentration at which cells did not grow on negative control and results were quite similar with 150 mg L^{-1} (table 17). According to the literature data, the proper antibiotic for use was kanamycin in concentration of 200 mg L^{-1} (Sakural and Komatsubara,1996).

Table 17. Number of cells using different antibiotic concentrations and different amounts of DNA after chemical competent process and electroporation.

Antibiotic concentration (mgL^{-1})	Volume (µL)	Mass DNA (ng)	Number of cells	
			Chemical competent process	Electroporation
25	10	0	0	0
	100		5	8
	REST		49	17
25	10	100	4	4
	100		8	8
	REST		n.c.	n.c.
25	10	200	2	2
	100		8	8
	REST		n.c.	n.c.
25	10	400	7	7
	100		n.c.	n.c.
	REST		n.c.	n.c.
50	10	0	0	0
	100		7	7
	REST		38	38
50	10	100	0	0
	100		n.c.	n.c.
	REST		n.c.	n.c.
50	10	200	3	3
	100		n.c.	n.c.
	REST		n.c.	n.c.
50	10	400	1	1
	100		n.c.	n.c.
	REST		n.c.	n.c.
75	10	0	0	0
	100		0	0
	REST		33	9
75	10	100	9	n.c.
	100		91	n.c.
	REST		335	n.c.
75	10	200	13	n.c.
	100		110	n.c.
	REST		374	n.c.
75	10	400	16	n.c.
	100		130	n.c.
	REST		380	n.c.
100	10	0	0	0

	100		0	0
	REST		9	9
	10		2	n.c.
	100		89	n.c.
100	REST	100	503	n.c.
	10		7	n.c.
	100		55	n.c.
100	REST	200	537	n.c.
	10		3	n.c.
	100		63	n.c.
100	REST	400	386	n.c.
	10		0	0
	100		0	0
150	REST	0	0	0
	10		0	n.c.
	100		9	n.c.
150	REST	100	165	n.c.
	10		2	n.c.
	100		16	n.c.
150	REST	200	178	n.c.
	10		18	n.c.
	100		270	n.c.
150	REST	400	n.c.	n.c.

n.c. not counted (overgrown plates)

4.2. Genetic transformation of *S. marcescens*

Two genetic transformation protocols have been examined, chemically competent process and electroporation. Chemically competent process resulted in lower transformation efficiency then electroporation when using the same amount of DNA and antibiotic concentration (table 18). It was not possible to calculate the exact transformation efficiency for the electroporation process in the same way as for the chemical competent protocol since the cells have overgrown on the plates. A bigger efficiency in the electroporation process corresponds to literature data and results gained by other groups (Sakural and Komatsubara, 1996). Transformation efficiency is expressed as the number of transformants per μg of plasmid DNA. For further experiments only the electroporation process was used with some improvements. Lower amount, of 50 ng plasmid DNA, was examined along with the 100 ng to see the effect on the transformation efficiency. As a result, 50 ng of DNA was chosen as final amount of DNA since the number of colonies was similar as with 100 ng in the same conditions.

To calculate the exact efficiency 1 μL of cells was plated. Tables 19 and 20 show the comparison of the transformation efficiencies using fresh competent cells and one week old batches of competent cells . The efficiency is similar in both cases which corresponds to literature data (Sakural and Komatsubara, 1996). Furthermore, in the publication, no loss in transformation efficiency was reported with cells frozen at - 80°C for up to 3 months. Moreover, different electroporation buffers (glycerol, sucrose and mannitol) and their concentrations were tested (100 and 300 g L^{-1}) to see the effect on transformation efficiency. The highest efficieny was observed when using glycerol in concentration of 100 g L^{-1} and that combination was used in this experiment too (Sakural and Komatsubara, 1996).

Table 18. Transformation efficiency for chemically competent process

Antibiotic concentration (mgL $^{-1}$)	**Mass DNA (ng)**	**Transformation efficiency (number of transformants/ μg of plasmid DNA**
100	100	5,45 x 10^{3}
	200	3,125 x 10^{3}
	400	1,163 x 10^{3}

Table 19. Transformation efficiency using fresh electrocompetent cells

Plasmid (DNA)	**Mass DNA (ng)**	**Antibiotic concentration (mgL^{-1})**	**Volume (µL)**	**Number of cells**	**Efficiency (trans./ µg DNA)***
-	0	100	1	0	-
			10	0	
			100	0	
			REST	0	
BB3_AB_p105_GFP_ t1001	50	100	1	338	$6{,}76 \times 10^{6}$
			10	n.c.	
			100	n.c.	
			REST	n.c.	
BB3_AB_p105_mCherry _t1001	50	100	1	2	$4{,}15 \times 10^{4}$
			10	17	
			100	252	
			REST	n.c.	
BB3_AB_p105_eGFP _t1001	50	100	1	12	$1{,}73 \times 10^{5}$
			10	48	
			100	908	
			REST	n.c.	

n.c. not counted (overgrown)

*calculated mean value

Table 20. Transformation efficiency using one week old batch of electrocompetent cells

Plasmid (DNA)	**Mass DNA (ng)**	**Antibiotic concentration (mgL $^{-1}$)**	**Volume (µL)**	**Number of cells**	**Efficiency (trans./ µg DNA) ***
-	0	100	1	0	-
			10	0	
			100	0	
			REST	0	
BB3_AB_p105_GFP _t1001	50	100	1	1	1,373 x10^5
			10	34	
			100	n.c.	
			REST	n.c.	
BB3_AB_p105_ mCherry_t1001	50	100	1	5	9,6911 x10^4
			10	12	
			100	297	
			REST	n.c.	
BB3_AB_p105_ eGFP_t1001	50	100	1	245	7,84667 x10^6
			10	n.c.	
			100	n.c.	
			REST	n.c.	

n.c. not counted (overgrown plates); * calculated mean value;

4.2.1. Transformation efficiency

Chung et al. (1989) in their work examined the different effects on transformation efficiency in the case of *E.coli*. It was shown that transformation efficiency was not significantly changed as the amount of DNA increased from 10 pg to 1 ng. However, transformation efficiency decreased as the concentration of DNA increased from 1 ng to 1 µg. They showed that optimal results were obtained with 100 pg to 1 ng of plasmid DNA for transformation. Also, quality and number of competent cells needs to be taken in consideration. The conclusion was that cells from the early exponential phase (OD_{600} 0.3-0.4) yielded more transformants per µg of plasmid DNA than those later in the growth stage (Chung et al., 1989).

Genetic transformation of *S. marcescens* was described in a few publications where chemical competent as well as electroporation processes have been tested. Reid et al. (1981)

described a transformation procedure using *S. marcescens* strain. To determine the antibiotic resistence, cells were plated on LB agar containing different amounts of antibiotic. Competent cells were grown untill cell density reached 2 to 5 $x10^8$ cells mL^{-1} and after that the culture was centrifuged for 10 min at 12000 x g at 5 ^oC. Heat treated *S. marcescens* cells were transformed with plasmid pBR322 using the method described by Cohen et al. (1972). It was shown that transformation using the $CaCl_2$- mediated procedure and selection of transformants while using 400 μg mL^{-1} of ampicilin were unsuccessful. Potential interference of endonuclease was suggested in that matter. For that reason, various heat treatments were incorporated as pretreatments with the aim to inactivate the endonuclease. The procedure examined and described in the publication (Reid et al., 1981) could be applicable to most *S. marcescens* strains.

Palomar et al. (1990) examined the effect of Dnase (nuclease) on the transformation efficiencies of *S. marcescens*. The nuclease enzyme of genus *Serratia* was known from previous studies and the ability of *S. marcescens* to degrade DNA as well as RNA has been described. Plasmids used for the experiment contained genes encoding the kanamycin resistence. Three different methods have been tested (by Cohen, Merrick and Hanahan) and no significant difference in transformation efficiency was seen. Transformation efficiencies have been compared using *S. marcescens* 2170, *S. marcescens* 2170c and *E.coli* 5K. The results also showed no big difference in transformation efficiency in the nuclease defective strain which was the opposite of the expected result (Palomar et al., 1990).

The experiment conducted by Sakural and Komatsubara (1996) provided useful guidelines for the transformation procedure described in this thesis. Electroporation was done using a Gene Pulser and *S. marcescens* competent cells were grown in NB broth untill O.D. reached value of 1 at 660 nm. Electroporation was carried out in a cooled 0.2-cm cuvette at 12.5 kV cm^{-1}. They used the capacitance of 25 pF and the resistance was set at 600 Ω. Instead of using genetic elements from *E. coli* for plasmid construction and propagation, plasmid DNA in their work was from *S. marcescens*. It was isolated using an alkali-SDS lysis method. As a final result, the highest transformation efficiency for *S. marcescens* was 1,4 x 10^6 colonies per μg DNA and was obtained with cells harvested during the mid-exponential growth phase (Sakural and Komatsubara, 1996).

4.3. Plasmids construction

For plasmid construction genetic elements from *E. coli* were used. Plasmids were obtained using the Golden Gate cloning method and used for transformation of *S. marcescens*. Since there are not many known genetic elements from *S. marcescens*, *E. coli* cells were chosen for plasmid construction and propagation. Plasmids made in the first part of the work contained coding sequences (CDS) for genes encoding the green fluorescent protein (GFP) and red fluerescent protein (mCherry) together with other genetic elements (promoter, terminator, plasmid origin of replication, kanamycin resistance marker). In the second part of the work, plasmids contain DNA templates of sgRNAs together with gene encoding Cas9 protein and other genetic elements mentioned above. Altogether they contained three expression cassettes for both *ptsG* 5'-3' and *man* operon 5'-3'. Plasmid purification and restriction-digestion analysis were accompanied with gel-electrophoresis for all plasmids. Correct band sizes for restriction-digestion analysis were calculated using the CLC Main Workbench (figure 11; a,b). Plasmids were confirmed by restriction- digestion analysis and visualized on the gel.

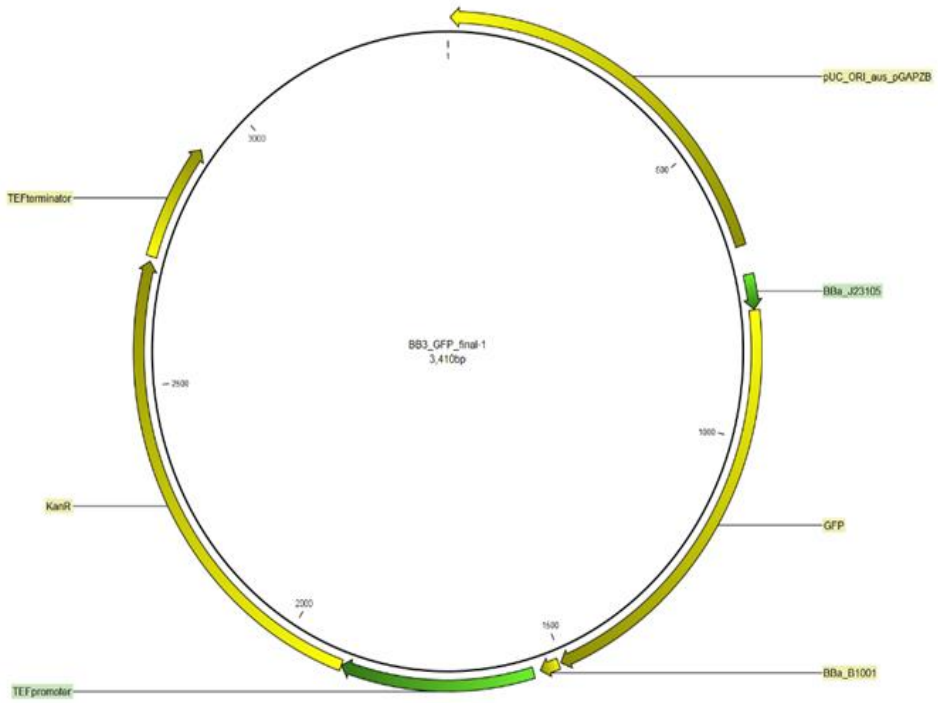

Figure 11. a) BB3_AB_p105_GFP_t1001 plasmid constructed in the lab using CLC Main Workbench. Figure shows all genetic elements required for the plasmid construction. BB3_AB_p105_mCherry_t1001 plasmid was constructed in the same way.

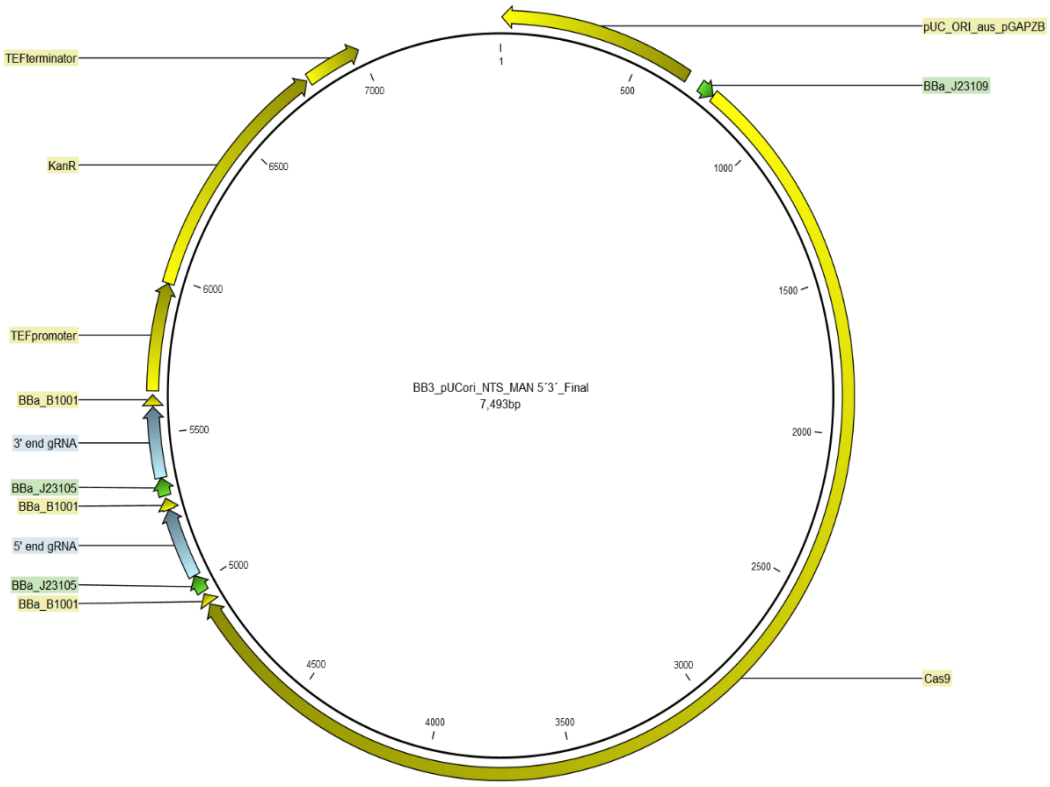

Figure 11. b) BB3_AD_p109_Cas9_p105_MAN5'_MAN3'_t1001 plasmid constructed in the lab using CLC Main Workbench. BB3_AD_p109_Cas9_p105_ptsG5'_ptsG3'_t1001 plasmid was constructed in the same way.

In the work of Orruño et al. (2014), researchers managed to transform *S. marcescens* strain, Spanish Type Culture Collection (CECT) 159, with the plasmid DNA pGEN222 using a standard electroporation technique. Transformants were screened using green fluorescence emission. This was a guidance for a plasmid construcion in this thesis as well.

4.4. Fluorescence microscopy

After transforming *S. marcescens* cells, a chosen number of clones was picked for verification. Clones were put in liquid culture containing Nutrient Broth (LB) medium and

antibiotic kanamycin. With fluorescent microscope (Zeiss Observer Z1, AX10) it was confirmed that checked clones are positive and express fluorescent proteins. Fluorescence measured for plasmid-bearing strains is directly related to synthesis of fluorescent proteins. Verification was done in comparison with negative control samples.

4.4.1. Green fluorescent protein (GFP)

In the figure 12 it can be seen that clones, checked under fluorescent microscope, are positive and express green fluorescent protein (GFP). These clones contained BB3_AB_p105_GFP_t1001 plasmid. Figure 13 also shows positive clones but in the test tubes without UV light.

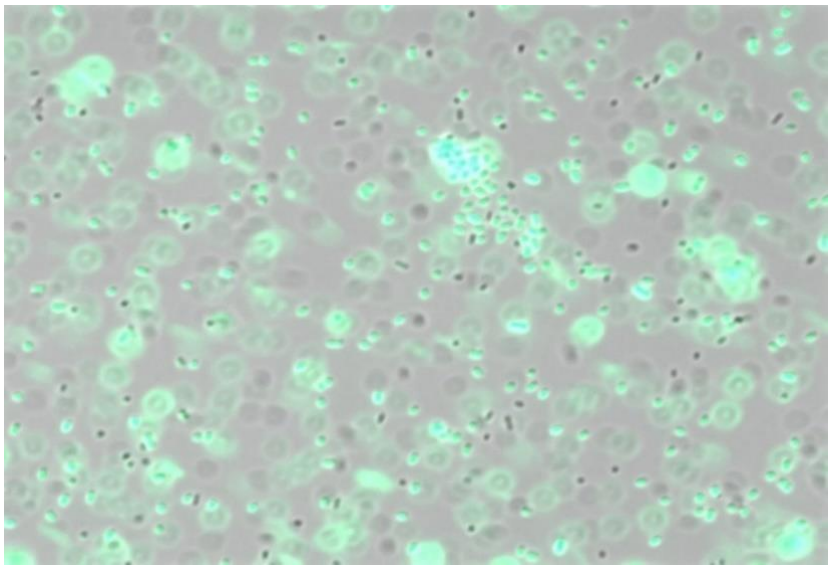

Figure 12. Clones containing plasmid BB3_AB_p105_GFP_t1001 with coding sequence (CDS) for the green fluorescent protein (GFP) seen under the fluorescent microscope.

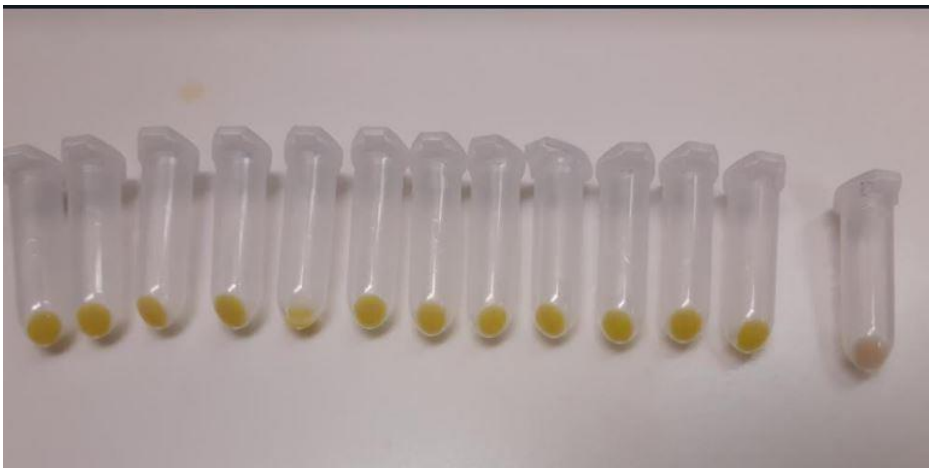

Figure 13. Image of test tubes without UV light with clones containing plasmid BB3_AB_p105_GFP_t1001 with CDS encoding green fluorescent protein (GFP); left GFP clones, the last tube negative control sample;

4.4.2. Red fluorescent protein (mCherry)

Figure 14, taken under the fluorescent microscope, also confirms clones are positive. Clones contained plasmid BB3_AB_p105_mCherry_t1001 with coding sequence (CDS) encoding red fluorescent protein (mCherry). In the figure 15 are shown positive clones in the test tubes without UV light.

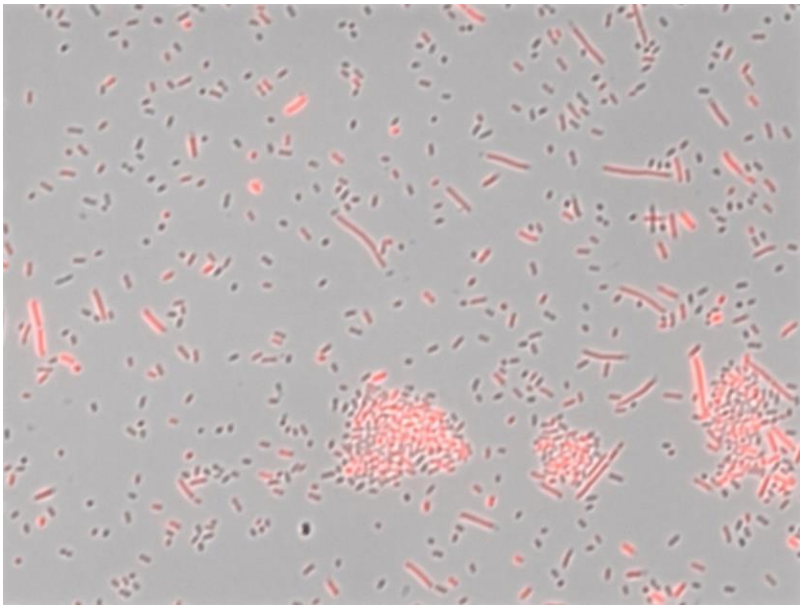

Figure 14. Clones containing plasmid BB3_AB_p105_mCherry_t1001 with coding sequence (CDS) encoding the red fluorescent protein (mChrry) seen under the fluorescent microscope.

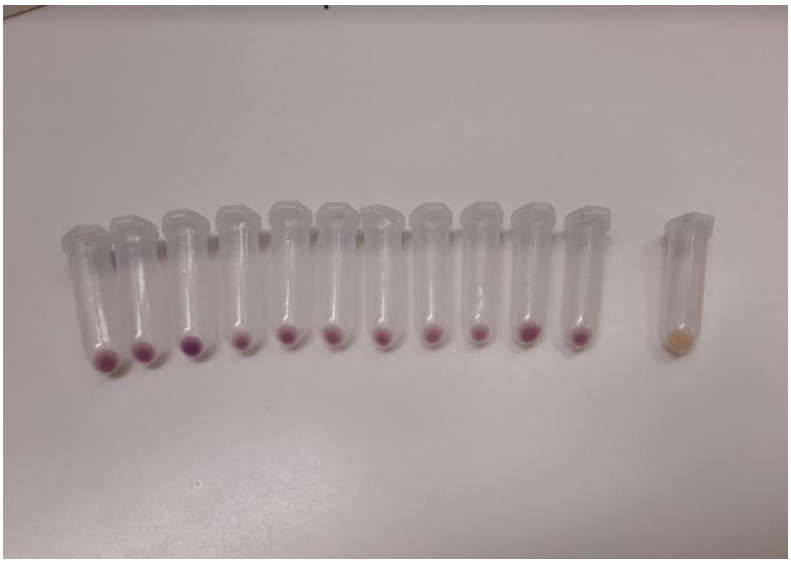

Figure 15. Image of test tubes without UV light with clones containing plasmid BB3_AB_p105_mCherry_t1001 with CDS encoding red fluorescent protein (mCherry); left mCherry clones, the last tube negative control sample;

4.5. Engineering of the glucose uptake

After establishing a reproducible genetic transformation method, the second part was focused on disruption of glucose transport related genes in *S. marcescens*. For gene knock-out, *ptsG* and *man* operon were chosen as target genes which are both part of the phosphotransferase transport system (PTS). Figure 16 shows other proteins which are part of different transport systems found in *S. marcescens.* Glucose transport systems of *S. marcescens* are not so well examined as those in *E. coli* and since these two bacteria showed certain similarities *E.coli* was chosen for comparison.

In their work, Steinsiek and Bettenbrock (2012) analyzed glucose transport in *E. coli* mutant strains with defects in sugar transport systems. The strains they used were derivatives of *E. coli* K12 MG1655 and transcription of gene coding for several glucose transporters was analysed under aerobic and anaerobic conditions. They showed that deletion of the galactose transporters, the maltose transporters or the mannose-PTS did not cause major changes in the growth rate if glucose is used as a carbon source. PtsG mutant strains showed significantly reduced growth rates in aerobic conditions. In a further analysis of ptsG mutants, the galactose ABC transporter was disrupted which did not reduce the growth rate either. Finally, deletion of the mannose-PTS (*manx*, *many*, *manz* genes organized in operon) in the ptsG mutant led to a

significant decrease in the growth rate (μ <0.01 h^{-1}) which means that disruption of both the glucose and the mannose-PTS almost abolished aerobic growth on the glucose.

***Serratia marcescens* subsp. *marcescens* atcc 13880**
PTS glucose transporter subunit IIBC (***ptsG***)
PTS mannose transporter subunit IIAB (***manX***)
PTS mannose/fructose/sorbose transporter subunit IIC (***manY***)
"PTS mannose transporter subunit IID (***manZ***)
galactose/glucose ABC transporter (*mglB*)
galactose/methyl galactoside ABC transporter ATP-binding protein (*mglA*)
galactose/methyl galactoside ABC transporter permease (*mglC*)
maltose/maltodextrin ABC transporter substrate-binding protein (*malE*)
maltose ABC transporter permease (*malF*)
maltose ABC transporter permease *(malG*)

Figure 16. Genes and proteins connected with different transport systems found in *S. marcescens* using NCBI database. Bolded are shown target genes for CRISPR Cas-9.

4.5.1. CRISPR-Cas9

CRISPR Cas9 technology was used to inactivate the genes of interest in *S. marcescens* genome by inducing the double strand breaks in the target genes. With specific overlapping primers PCR products were obtained. DNA templates of sgRNAs for each gene at 5' and 3' end were assembled in a PCR reaction and then cloned into back bone 1 vector (BB1). The product size was approximately 240 bp which is shown in figure 17. There are some additional bands present on the gel image. Reasons for that could be nonspecific primer annealing, primer dimerization, or contamination of either DNA sample, dNTPs, water or some other component. It can potentially be connected with the number of PCR cycles (more than 30) which could cause multiple bands due to the increased chance of error with each cycle. After four DNA templates of sgRNAs were assembled into each individual vector (BB1), additional conformation was accomplished with Sanger sequencing. For the assembly of the final plasmid with three expression cassettes the Golden Gate system was used. The final backbone (BB3) assemblies contained three expression cassettes for Cas9 endonuclease, each of *ptsG* 5'-3' gene and *man* operon 5'-3'. Desired assemblies containing mentioned expression cassettes were

created via transformation of *E.coli* DH10B cells. They were subjected to restriction-digestion analysis and further confirmed and visualized using gel-electrophoresis. Restriction digestion was conducted with specific restriction enzyme. Enzyme (SpeI) cut at three specific places on plasmids, thus four fragments were obtained differentiating in size which is shown on the gel (figure 18).

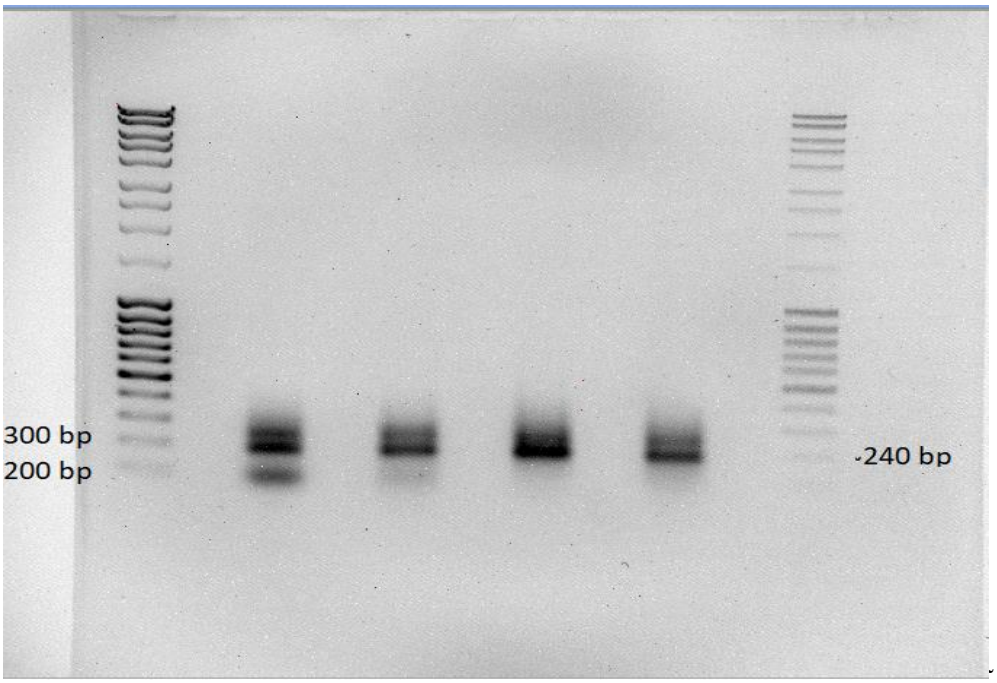

Figure 17. Four DNA templates of sgRNAs obtained in the PCR reaction, fragments size approximately 240 bp.

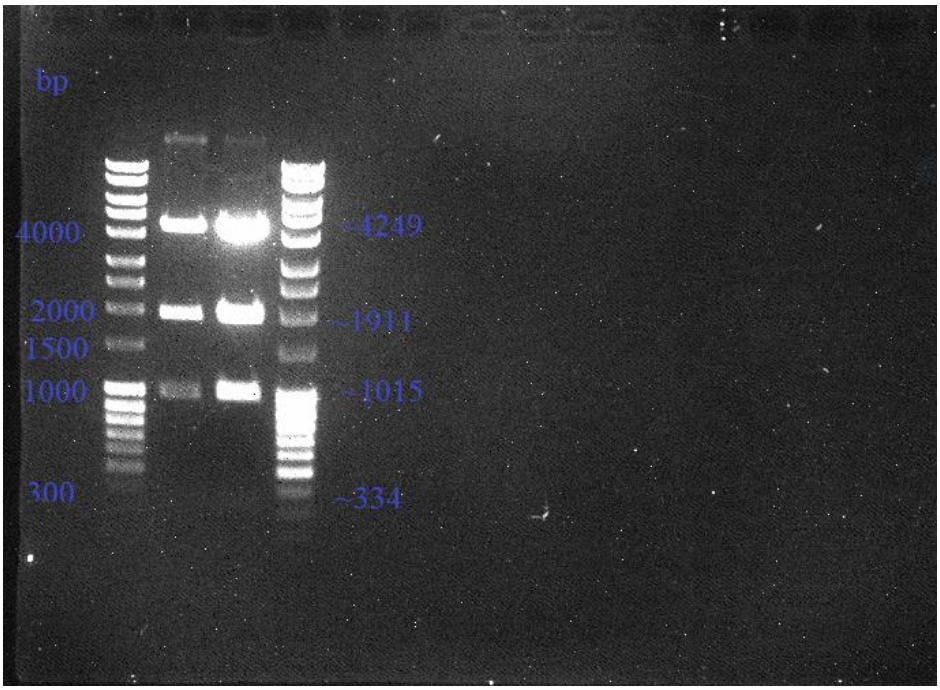

Figure 18. Results of restriction-digestion analysis . First line - ladder samples; second line- four fragments obtained after digestion of BB3_AD_ p109_Cas9_p105_ ptsG5'_ptsG3'_t1001 plasmid with SpeI enzyme; Third line- four fragments obtained after digestion of BB3_AD_p109_ Cas9_p105_man5'_ man3'_ t1001 plasmid with SpeI enzyme; Fourth line- ladder samples. Fragments sizes (4249, 1911, 1015 and 334 bp) were checked in CLC Main Workbench.

4.5.2. Gene knock-out

To knock out targeted genes electrocompetent cells of *S. marcescens* were transformed with prepared plasmids. The first transformation with established transformation conditions showed very low efficiency. Since the goal was to knock out glucose transport genes and carbon source in nutrient broth medium is glucose possible solution was that genes are knocked out and cells can not grow on glucose anymore. To check wheter this is a case carbon source in the medium was changed and transformation was repeated. Additional carbon sources examined were sucrose, fructose and xylose (see table 9). Transformation again resulted in very low efficiency. No growth was noticed on plates containing fructose as a carbon source.

Transformation was repeated with same sugars using higher amount of DNA. It was prepared 100 ng of DNA and transformation resulted in slightly higher efficiency. Gained

clones were checked using colony PCR and results did not show targeted genes are knocked-out. "Forward", "out" and "in" primers, required for verification, were designed as shown in the figure 19.

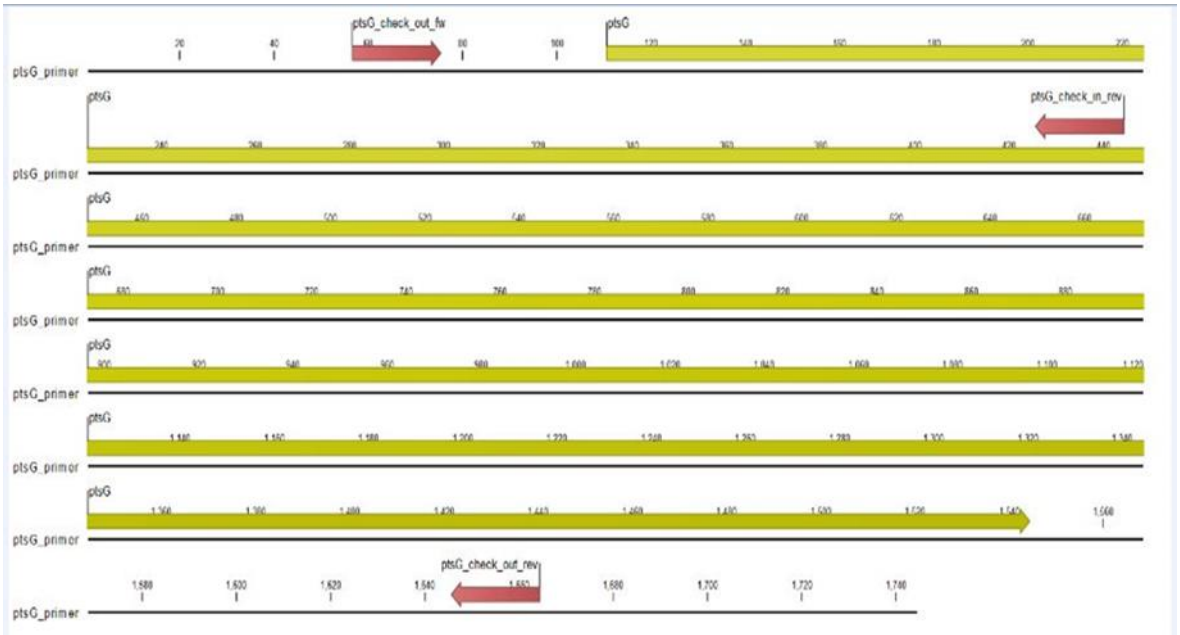

Figure 19. "Forward", "in" and "out" primers designed for *ptsG* gene knock-out verification.

4.5.2.1. *ptsG* gene transformants

Picked transformants shown on the figure 20 were grown on the NB medium containing either glucose or xylose as a carbon source. Expected fragment size using "forward" and "out" primers was around 1700 bp. For the gained ptsG transformants it was not clear whether *ptsG* gene was knocked-out bacause neither the wild type or other transformants showed expected size. For that reason, further examinations were done with an additional "in" primer to get a shorter fragment with an expected fragment size of around 450 bp.

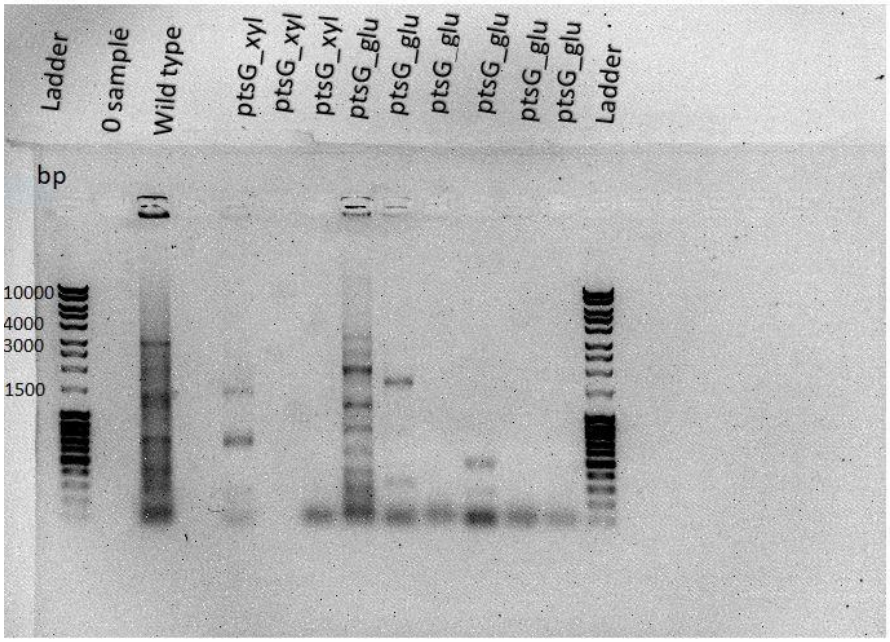

Figure 20. *ptsG* gene transformants grown on plates picked for the colony PCR. First line-ladder samples; second line- 0 sample (no DNA); third line- wild type sample; fifth to thirteenth lines-picked clones; fourteenth line-ladder samples;

4.5.2.2. *man* operon transformants

Figure 21 shows obtained *man* operon transformants and it is clear that none of clones has knocked-out genes because all the clones, including wild type, have expected size for *man* operon (3000 bp). Picked transformants have been grown on NB medium containing either sucrose, xylose or glucose as a carbon source.

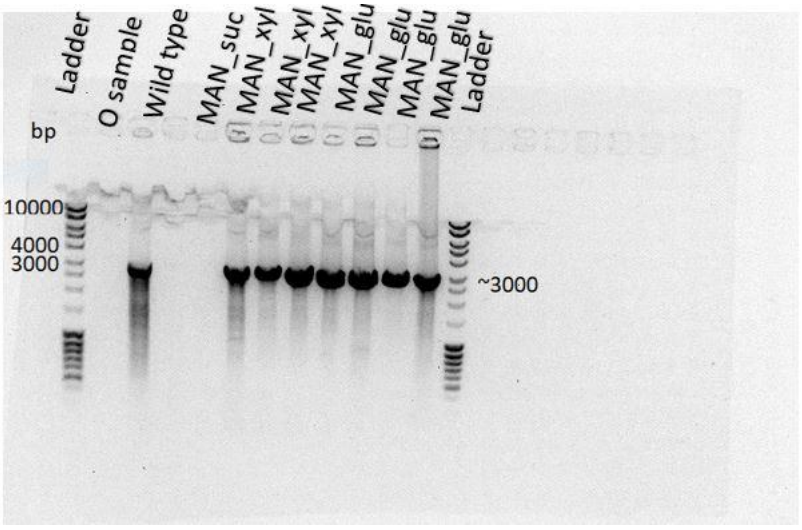

Figure 21. *man* operon transformants grown on plates picked for the colony PCR. First line- ladder samples; second line- 0 sample (no DNA); third sample-wild type; fifth to twelfth lines- picked clones; thirteenth line-ladder samples.

4.5.3. Results obtained with different carbon sources

For further experiments glucose, sucrose and xylose were chosen as carbon sources. Targeted genes were not knocked-out in that way either (figure 22). For man operon transformants, fragments size were around 3000 bp. For *ptsG* gene transformants, besides usual two primers ("forward" and "out"), additional "in" primer was used and fragment size of around 450 bp was obtained which corresponds to short fragment prior visualized with CLC software. The final attempt to increase transformation efficiency, and thus chance of obtaining wanted clones, was to increase amount of DNA at the most possible value. It was used an absolute amount of DNA (15 μg) and even though increasing the amount of DNA can have a different effect on the efficiency, it resulted in higher efficiency. Also, additional carbon sources were examined, glycerol and acetate. While cells on acetate as a carbon source did not show any growth, on glycerol on the other hand, bigger growth of *man* operon transformants was noticed. As a result of final electroporation of *S. marcescens* cells, with applied absolute amount of DNA (15 μg) using glucose, xylose and glycerol as carbon sources, the highest transformation efficiency till then was achieved.

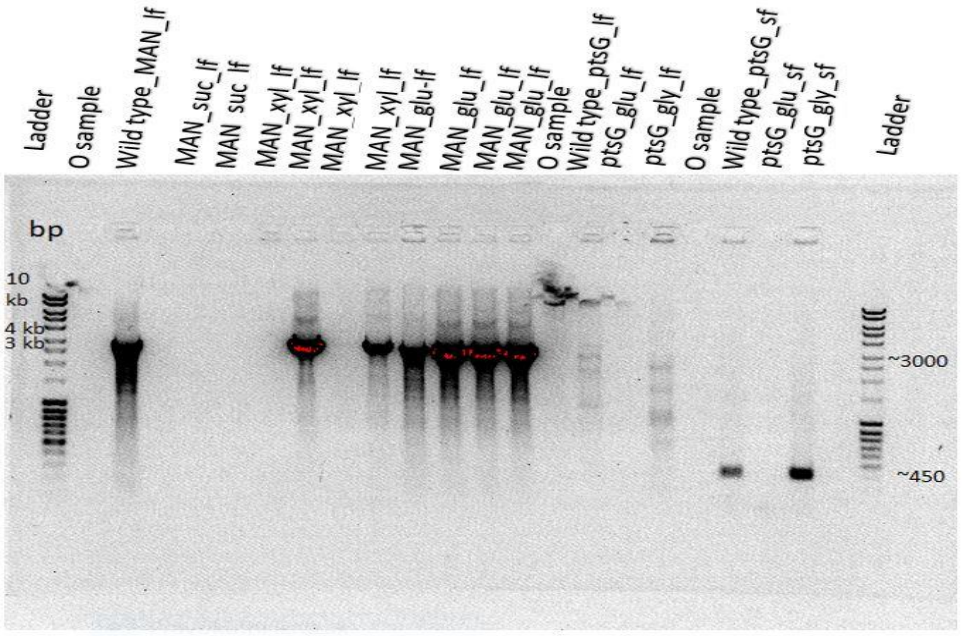

Figure 22. *man* operon and *ptsG* transformants after colony PCR grown on differen carbon

sources. Line 1-ladder samples; line 2 – 0 sample (no DNA); third line-wild type (*man*, long fragment); fifth to fourteenth samples – *man* operon transformants (long fragment); fifteenth line- 0 sample (no DNA); sixteenth line- wild type (*ptsG*, long fragment); seventeenth and eighteentg samples - *ptsG* gene transformants (long fragment); nineteenth line- 0 sample (no DNA); twentieth line- wild type (*ptsG*, short fragment); twenty-first and twenty second lines- *ptsG* gene transformants (short fragment); twenty-fourth line- ladder samples;

*lf- long fragment (3000 bp), sf- short fragment (450 bp);

4.5.3.1. *ptsG gene* transformants

For *ptsG* gene transformants, the desired results were not accomplished. Bands have been seen in the expected fragment size which means *ptsG* gene was not knocked-out. A possible additional step in that matter could be extracting bacterial genomic DNA for colony PCR to see if different results can be obtained. In the context of these results, transformation efficiency can additionally be discussed. This experiment showed that higher DNA concentration yielded higher efficiency. The literature data examined the effect of the amount of DNA on the transformation efficiency of competent cells. Liu et al. (2014) examined the optimal concentration of plasmid in *E.coli* bacteria. The results showed that the plasmid amount of 5 ng caused the highest transformation efficiency. When they increased the concentration further, efficiency decreased. Higher concentrations had negative and toxic effects on the cells (Lie et al., 2014).

4.5.3.2. *man* operon transformants

Picked clones were examined with colony PCR and figure 23 shows *man* operon transformants grown on xylose with possible knocked out genes. On the mentioned figure it can be seen few clones which do not show bands in the expected fragment size, of around 450 bp, which could be potential knocked-out clones for *man* operon.

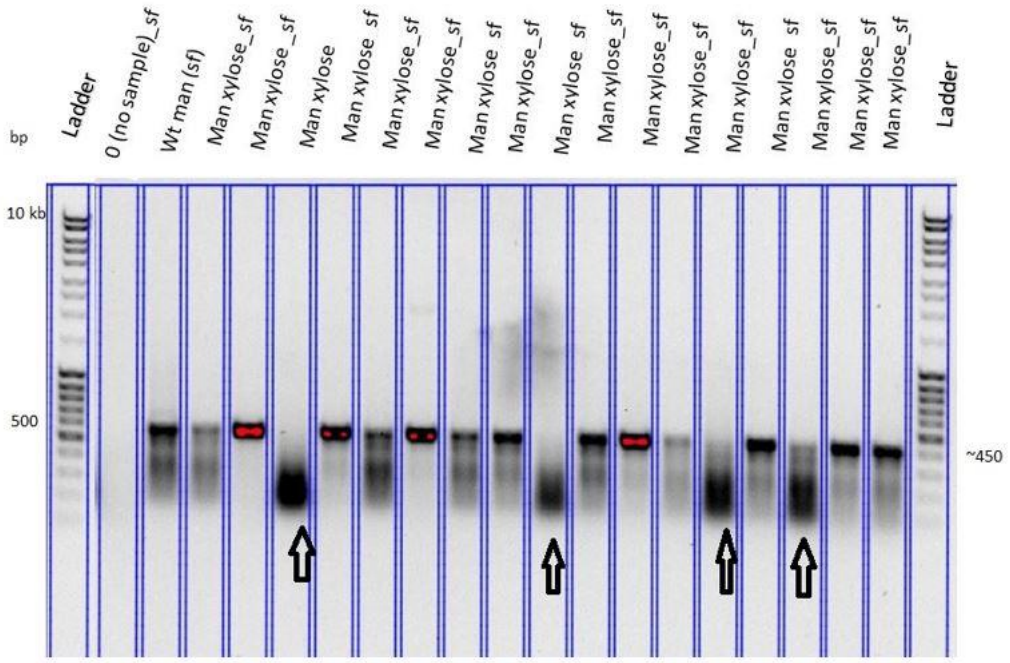

Figure 23. *man* operon transformants grown on xylose as a carbon source (short fragments made using 'forward' and 'in' primers). Arrows on the figure are showing potential knocked-out clones. Transformants examined on other carbon sources are not shown for technical reasons (wild type samples were not shown on the same gel image).

4.5.4. Cas9 optimization

Cas9 endonuclease used in the experiment was from *Streptococcus pyogenes*, codon optimized for yeast. The DNA sequence encoding Cas9 protein can be either native, human codon-optimized or yeast codon-optimized (Stovicek et al., 2017). It could be discussed whether using a bacterial Cas9 would cause the knockout experiment to work. Moreover, codon optimisation of used Cas9 endonuclease and sgRNAs for *S. marcescens* could be the possible solution. In the publication (Ren, F. et al., 2019) was used Cas9 that contained 35.1% GC-content which was much lower than that of the genome of organism in the experiment. The genome of *Myxococcus xanthus* DK1622 contained 68.9% GC content. To develop a CRISPR Cas9 editing system in *M. xanthus*, they employed a codon-optimized *cas9* gene, which had been used in similarly high-GC-possessing *Streptomyces* species. The potential factor affecting the genome editing could also have been the quantity of Cas9 protein. It was shown that findings and proposals described in their paper were workable in different organisms, at least the Gram negative bacteria with high GC content. One of the factors that also needs to be taken into consideration is the GC content of sgRNAs used to target genes of interest. It was shown that

CRISPR Cas9 editing efficiency dropped significantly when using sgRNAs with low GC content (25 and 30%) comparing those with high GC content (50 and 65%). Results suggested that the GC content of sgRNAs might be the limiting factor for the genome editing.

The most commonly used Cas nuclease, in genetic engineering methods, is *Streptococcus pyogenes* Cas9 (SpCas9). It has some limitations like the big protein size of SpCas9, certain off target mutations are possible, but mainly in therapeutic applications. Considering possible limitations, certain advances in improving Cas9 nuclease have been done. One of the methods typically used to alter the protein function is aminoacid substitution of critical domain. Some highly specific Cas9 variants have been developed which contain several mutations to reduce the non-specific interactions between Cas9 protein and target DNA. PAM-altered SpCas9 variants have also been created by amino acid substitutions. Several Cas9 homologs from other bacterial species have been identified and used. Each homolog is specific with different PAM and tracrRNA structures. Cas9 homologs often have particular features different from SpCas9. *Staphylococcus aureus* Cas9 (SaCas9) has the target restriction with the PAM sequence quite strict, but the variant with more relaxed PAM sequence was also developed. Among different Cas9 reported, BlatCas9s PAM showed broad targeting ranges which makes it interesting for future applications in different organisms (Yao et al., 2018).

There are different Cas9 orthologs designed, for instance for *E. coli*, *Francisella novicida etc*. The potential for CRISPR – based genome manipulations is in increasing use of engineered Cas9 proteins and libraries of orthologs. Functions like gene knock-out, gene knock-in or fine-tuning of gene expression could be enriched with those proteins. It was shown that the genomic context has an important role in CRISPR-mediated technology and the goal is to carry out precise editing at intended sites with no off-target effects (Mitsunobu et al., 2017).

Genetic elements, like promoter and terminator, used to assembly CRISPR in this experiment were from *E.coli*. Cas9 gene was under the control of constitutive promoter. The Cas9 gene is most often expressed under the control of constitutive promoters. They can be different strengths, from self-replicating low-copy centromeric vectors to high-copy 2μ vectors or they can be integrated into the genome (Stovicek et al., 2017). It was shown that engineered promoters together with dCas9-effector-mediated gene regulation result in selectively redirected carbon flux in *E. coli (*Mitsonubu, 2017*).* In the publication (Yang, Y. et al., 2017), the *cas9* gene was promoted by the constitutive promoter (PpilA) or the copper-inducible promoter (PcuoA).The plasmid, in which the *cas9* gene was driven by the constitutive promoter (PpilA), produced no deletion mutant. Possible explanation for that could be the lethality of

high expressions of Cas9. In contrast, transformation of plasmid containing the *cas9* gene controlled by the PcuoA promoter into the *M. xanthus* DK1622 produced colonies after the induction of copper at the concentration of 25 μM.

As for further improvement, CRISPR- Cas9 technology itself can also be discussed. The gRNA directs Cas9 protein to a specific target locus in the genome. Molecules of sgRNA bind both to Cas9 and PAM-proximal target sequence, which results in a double-stranded break (DSBs) in the genome of target organism. The DSBs created by the CRISPR technology can be solved by either homology-directed repair (HDR) or non-homologous end joining (NHEJ). The NHEJ process is error-prone with low fidelity and could create some random insertions or deletions within the gene at a break site, thus forming frameshift mutations. This type of mutation enables gene knock-out. But a possible unwanted situation is the establishment of stop codon which will stop further protein synthesis and thus, bringing the cell to death. One more challenge needs to be taken into consideration and that is some off-target effects Cas9 nuclease can have. Some of the approaches to reduce the off-target effects are using self-regulatory systems attenuating Cas9 expression, chemical modification of the sgRNA backbone with incorporation of 2′-O-methyl-3′-phosphonoacetate at special sites of sgRNA and also, modification of Cas9 protein. In that way, some Cas9 variants have been developed, like high-fidelity SpCas9 for instance (Ebrahimi and Hashemi, 2020).

5. CONCLUSIONS

Following conclusions can be drawn regarding the results gained and processed in this graduate thesis:

1. Antibiotic of choice for the transformation process of *S. marcescens* cells was kanamycin. After examination of series of different concentrations it has been concluded that 100 mg mL^{-1} is the proper concentration.

2. Comparing chemical competent process and electroporation, electroporation showed bigger efficiency in the same conditions. Electrocompetent cells were prepared in the lab and no big difference in the transformation efficiency was noticed when manipulating with fresh or one week old cells.

3. Gained transformant colonies were checked using colony PCR and first results did not show targeted *man* operon was knocked-out. Using only "forward" and "out" primers in the case of *ptsG* transformants, it was not clear whether genes were knocked-out. Even with an additional "in" primer desirable results were not achieved. Reasons for that could be unexpressed sgRNAs, non adequate Cas9 endonuclease or targeting the wrong gene similar in the sequence.

4. No growth was noticed on plates containing fructose or acetate as a carbon source. The possible explanation regarding usage of acetate could be that it was too toxic for the cells.

5. Final electroporation of *S.marcescens* cells with absolute amount of DNA (15 μg), using glucose, xylose and glycerol as carbon sources, showed the biggest transformation efficiency.

6. The last colony PCR revealed possible *man* operon knocked-out clones grown on xylose. Additional experiments should be conducted for further verification, such as positive control of potential knocked-out clones. For *ptsG* gene transformants, wanted results were not accomplished.

6. LITERATURE

Bai, F., Dai, L., Fan, J., Truong, N., Rao, B., Zhang, L., Shen, Y. (2015) Engineered Serratia marcescens for efficient (3R)-acetoin and (2R,3R)-2,3-butanediol production. *J. Ind. Microbiol. Biotechnol.* **42**, 779-786.

Buckle, J. (2015) Clinical Aromatherapy, 3th edition, Churchill Livingstone, Philadelphia, pages 130-167, chapter 7 - Infection

Chung, C.T., Niemela, S.L., Miller, R.H. (1989) One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U. S. A.* **86 (7)**, 2172-2175.

Degfie, T., Mamo, T., Mekonnen, Y. (2019) Optimized Biodiesel Production from Waste Cooking Oil (WCO) using Calcium Oxide (CaO) Nano-catalyst. *Sci. Rep.* **9**, 18982, doi: 10.1038/s41598-019-55403-4

Doudna J and Sternberg, S. (2018) A crack in creation, 1 st edition, Penguin Random House, London

Ebrahimi, V., Hashemi, A. (2020) Challenges of in vitro genome editing with CRISPR/Cas9 and possible solutions: A review. *Gene Gene*. **753**, doi:10.1016/j.gene.2020.144813

Engler, C., Kandzia, R., Marillonnet, S. (2008) A One Pot, One Step, Precision Cloning Method with High Throughput Capability. *Plos One*. **3(11),** 3647. doi: 10.1371/journal.pone.0003647

Galen, J.E., Nair, J., Wang, J.Y., Wasserman, S.S., Tanner M.K., Sztein M.B. (1999) Optimization of plasmid maintenance in the attenuated live vector vaccine strain Salmonella typhi CVD 908-htrA. *Infect. Immun.***67**, 6424–6433.

Harjanne, A., Korhonen, J. (2019) Abandoning the concept of renewable energy. *Energy Policy.* **127,** 330-340.

Hejazi, A., Falkiner, F.R. (1997) The Pathological Society of Great Britain and Ireland. *J. Med. Microbiol.* **46,** 903-912.

Ho, D.P., Ngo, H.H., Guo, W. (2014) A mini review on renewable sources for biofuel.

Bioresour. Technol. **169**, 742-749.

ISO 9002, Thermo Scientific MassRuler DNA Ladder Mix, ready-to-use.

Jahreis, K., Pimentel-Schmitt, E.F., Brückner, R., Titgemeyer, F. (2008) Ins and outs of glucose transport systems in eubacteria. *FEMS Microbiol. Rev.* **32**, 891–907.

Ji, X. J., Huang, H., Ouyang, P. K. (2011) Microbial 2,3-butanediol production: A state-of-the-art review. *Biotechnol. Adv.* **29 (3)**, 351-264.

Kung, Y., Runguphan, W., Keasling, J.D. (2012) From fields to fuels: Recent advances in the microbial production of biofuels. *ACS Synth. Biol.* **11**, 498–513.

Liu, X., Liu, L., Wang, Y., Wang, X., Ma, Y., Li, Y. (2014) The Study on the factors affecting transformation efficiency of E. coli competent cells. *Pak. J. Pharm. Sci.* **27**, 679-684.

Lorenz, M.G., Wackernagel, W. (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol. Rev.* **58 (3**), 563–602.

Mahlen, S. D. (2011) Serratia Infections: from Military Experiments to Current Practice. *Clin. Microbiol. Rev.* **24(4)**, 755–791.

Mar, M. J. (2020) Biological production of 2-butanol (PhD thesis), Technical University of Denmark

Mitsunobu, H., Teramoto, J., Nishida, K., Kondo, A. (2017) Beyond Native Cas9: Manipulating Genomic Information and Function. *Trends in Biotechnology.* **35 (10)**, 983-996.

Neldeborg, S., Lin, L., Stougaard, M., Luo, Y. (2019) Rapid and Efficient Gene Deletion by CRISPR/Cas9. CRISPR Gene Editing. Methods in Molecular Biology, Humana Press, New York, NY, pages 233-247.

Orruño, M., Garaizabal, I., Bravo, Z., Parada, C., Barcina, I., Arana, I. (2014) Mechanisms involved in Escherichia coli and Serratia marcescens removal during activated sludge wastewater treatment. *Microbiologyopen*. **3(5)**, 657-667.

Palomar, J., Guasch, J. F., Regue, M., Vinas, M. (1990) The effect of nuclease on transformation efficiency in *Serratia marcescens*. *FEMS Microbiol. Lett.* **69 (3)**, 255- 258.

Panwar, N., Kaushik, S., Kothari, S. (2011) Role of renewable energy sources in environmental protection: A review. *Renew. Sustain. Energy Rev.* **15 (3),** 1513-1524.

Reid, J. D., Stoufer, S.D., Ogrydziak, D.M. (1982) Efficient transformation of Serratia marcescens with pBR322 plasmid DNA. *Gene Gene.* **17**, 107-112.

Ren, F., Ren, C., Zhang, Z., Duan, W., Lecourieux, D., Li, S., Liang, Z. (2019) Efficiency Optimization of CRISPR/Cas9-Mediated Targeted Mutagenesis in Grape. *Front. Plant Sci.* **10**, 612.

Russmayer, H., Marx, H., Sauer, M. (2019) Microbial 2-butanol production with Lactobacillus diolivorans. *Biotechnol. Biofuels.* 12(1). doi: 10.1186/s 13068-019-1594-5

Sakural, N and Komatsubara, S. (1996) Simple and versatile electrotransformation of *Serratia marcescens* Sr41. *Lett. Appl. Microbiol.* **23**, 23-26.

Shahzad, U. (2015) The Need for Renewable Energy Sources. *Int. J. Inf. Technol. Electr. Eng.* **4**, 16-19.

Shi, L., Gao, S., Yu, Y., Yang, H. (2014) Microbial production of 2,3-butanediol by a newly-isolated strain of *Serratia marcescens*. *Biotechnol. Lett.* **36**, 969–973.

Song, C.W., Park, J.M., Chung, S.C., Lee, S.Y., Song, H. (2019) Microbial production of 2,3-butanediol for industrial applications. *J. Ind. Microbiol. Biotechnol.* **46**. 1583- 1601.

Srivastava, R.K. (2019) Bio-energy production by contribution of effective and suitable microbial system. *Mater. Sci. Energy Technol.* **2**, 308-318.

Steinsiek, S. and Bettenbrock, K. (2012) Glucose transport in Escherichia coli mutant strains with defects in sugar transport systems. *J. Bacteriol.* **194(21)**, 5897–5908.

Stovicek, V., Holkenbrink, C., Borodina, I. (2017) CRISPR/Cas system for yeast genome engineering: advances and applications. *FEMS Yeast Res.* 17 (5), 28505256. doi: 10.1093/femsyr/fox030

Tycko, J., Myer, V.E., Hsu, P.D. (2016) Methods for Optimizing CRISPR-Cas9 Genome Editing Specificity. *Mol. Cell.* **63 (3)**, 355-370.

Uchiyama, T., Kaneko, R., Yamaguchi, J., Inoue, A., Yanagida, T., Nikaidou, N., Regue, M., Watanabe, T. (2003) Uptake of N,N'-diacetylchitobiose [(GlcNAc)2] via the phosphotransferase system is essential for chitinase production by Serratia marcescens 2170. *J. Bacteriol.* **185(6)**, 1776–1782.

Yang, T., Rao, Z., Zhang, X.H., Xu, M. (2017) Metabolic engineering strategies for acetoin and

2,3-butanediol production: advances and prospects. *Crit. Rev. Biotechnol.* **37(8**), 1-16.

Yang, Y., Wang, Y., Li, Z., Gong, Y., Zhang, P., Hu, W., Sheng, D., Li, Y. (2017) Increasing on-target cleavage efficiency for CRISPR/Cas9-induced large fragment deletion in Myxococcus xanthus. *Microb. Cell Factories.* **16**, 142.

Yang, Z., Zhang, Z. (2019) Recent advances on production of 2, 3-butanediol using engineered microbes. *Biotechnol. Adv*. **37(4)**, 569-578.

Yao, R., Liu, D., Jia, X., Zheng, Y., Liu, W., Xiao, Y. (2018) CRISPR-Cas9/Cas12a biotechnology and application in bacteria. *Synth. Syst. Biotechnol.* **3**, 135-149.

Zhang, L., Yang, Y., Sun, J., Shen, Y., Wei, D., Zhu, J., Chu, J. (2010) Microbial production of 2,3-butanediol by a mutagenized strain of Serratia marcescens H30. *Bioresour. Technol.* **101 (6)**, 1961–1967.

Zhang, Y., Liu, D., Chen, Z. (2018) Synthetic biology approaches for the production of 2 butanol. In: Current Developments in Biotechnology and Bioengineering: Synthetic Biology, Cell Engineering and Bioprocessing, (Singh, S. P., Pandey, A., Du, G., Kumar, S., editors), Elsevier, Amsterdam/Oxford/ Cambridge, pages 295-309.

7. APPENDIX

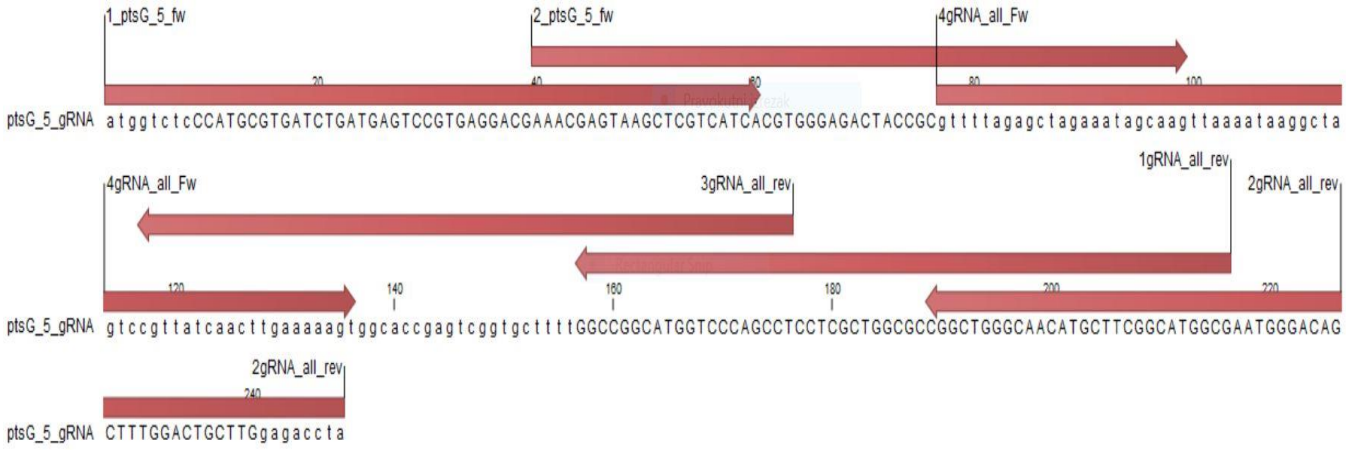

Figure 1. PtsG_5_gRNA

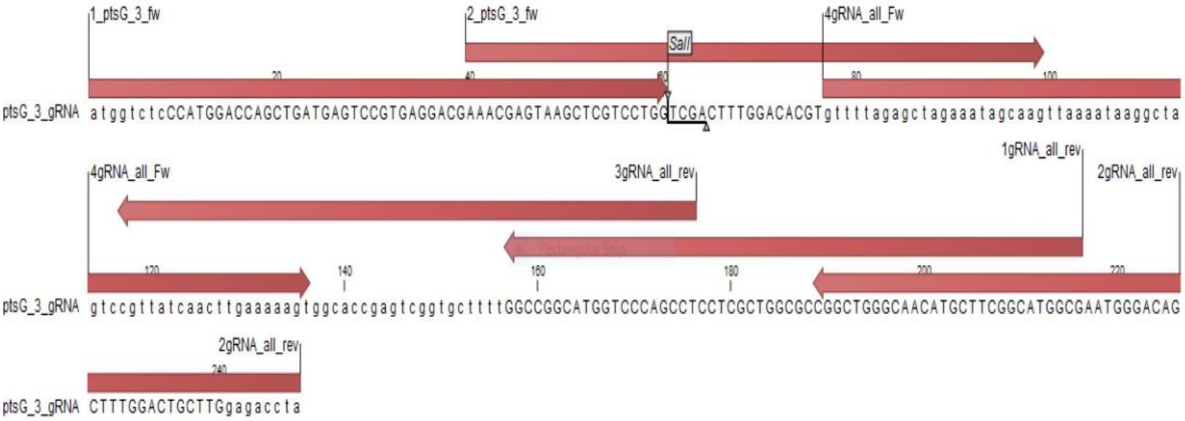

Figure 2. PtsG_3_gRNA

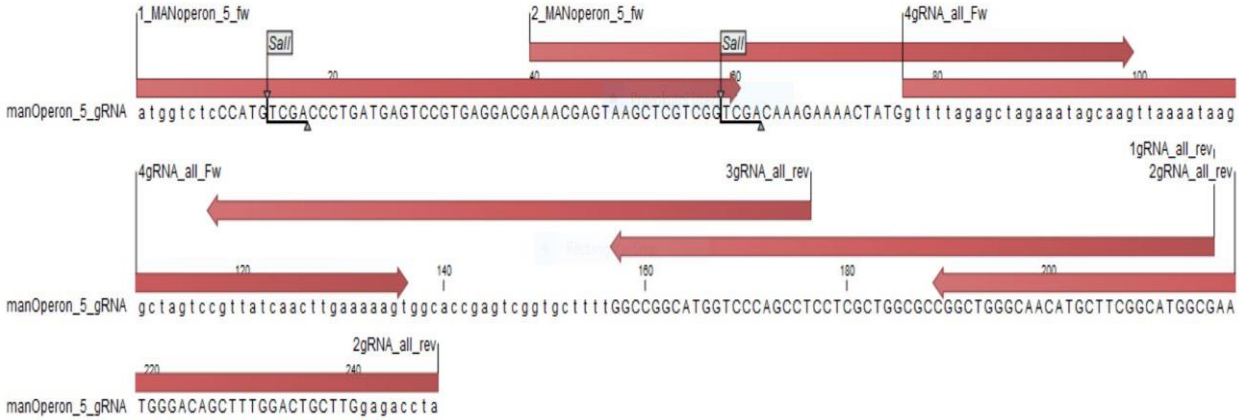

Figure 3. MAN operon_5_gRNA

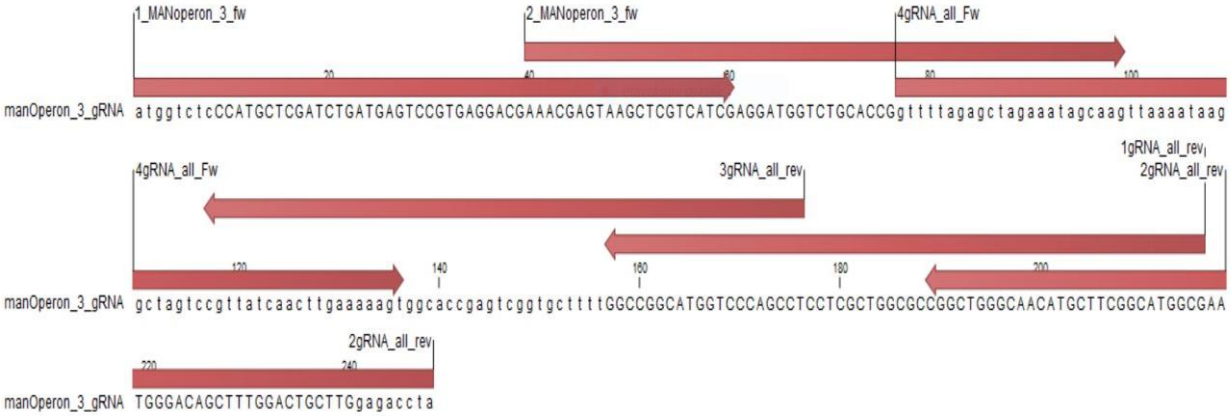

Figure 4. MAN operon_3_gRNA

STATEMENT OF ORIGINALITY

This is to certify, that the intellectual content of this thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

Marta Kozarić

Marta Kozarić