Conversion of the biodiesel by-product glycerol by the non-conventional yeast Pachysolen tannophilus

Liu, Xiaoying

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Conversion of the biodiesel by-product glycerol by the non-conventional yeast

*Pachysolen tannophilus*

Ph.D. thesis

Xiaoying Liu

Supervisors:
Assoc. prof. Mhairi Workman
Prof. Peter Ruhdal Jensen

Center for Systems Microbiology
Department of Systems Biology
Technical University of Denmark
Lyngby, Denmark

2012, March
Konvertering af biodiesel affald glycerol ved ikke-konventionelle gær *Pachysolen tannophilus*

Ph.D. afhandling

Xiaoying Liu

vejledere:
Assoc. prof. Mhairi Workman
Prof. Peter Ruhdal Jensen

Center for Systems Microbiology
Institut for Systembiologi
Danmarks Tekniske Universitet
Lyngby, Danmark

2012, mart
Preface and Acknowledgements

This dissertation includes the results of my PhD study carried out at the Center for Systems Microbiology (CSM), Department of Systems Biology in Technical University of Denmark (DTU) during the period from March 2009 to March 2012. The PhD study was funded by the European Community’s 7th Framework Research Programme under Grant Agreement Number 213506 (Project GLYFINERY).

First of all, I would like to thank for my supervisor Peter Ruhald Jensen who gave the great opportunity to pursue my PhD study at DTU and guide me along the entire PhD project. I would like to express my special gratitude to my supervisor Mhairi Workman. Whenever I had problems with my project or in my daily life, she was always so kind to lead me to the right direction and solve the problems. During my PhD study, her guidance, good advice, support, encouragement and discussions regarding problems accompany me along with my PhD. I also very appreciate that Mhairi helped me a lot with my publications and the PhD thesis. In addition, thanks for the scientific discussions and help from Uffe Hasbro Mortensen and Morten Kielland-Brandt at the Center for Microbial Biotechnology.

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Finally, I would like to thank the support and comfort from my family and my boyfriend Tian Ran Sun when I was struggling with my project and listen to my voice and give me so much encouragement and confidence.

Without all those lovely and helpful people around, my PhD could not be completed!

Copenhagen, March 2012
Xiaoying Liu
Summary

The focus on developing new renewable energy in the transportation sector by the EU has boosted the production of biodiesel from rapeseed and other vegetable oils in Europe. This has led to an immense increase in the production of glycerol, which is an inevitable by-product from the biodiesel production process. Since the volume of the glycerol by-product has exceeded the current market need, biodiesel producers are looking for new methods for sustainable glycerol management and improving the competitiveness of the biodiesel industries. The EU Commission funded GLYFINERY project is one initiative targeted to development of a novel technology based on biological conversion of the glycerol feedstocks into known and new advanced liquid biofuels, bioenergy and valuable green chemicals in an integrated biorefinery concept.

As part of the GLYFINERY project, the objective of this PhD project was to develop a process for bioconversion of waste glycerol into biofuel ethanol, characterize and optimize the process. The present thesis comprises of eight chapters. The project background, scope and aims are introduced in Chapter 1. Besides, the related background knowledge for better understanding the studies in the following chapters is also introduced in this chapter. Chapter 2-7 are comprised of the experimental results obtained during the whole PhD study.

The well characterized yeast *Saccharomyces cerevisiae* has been used for fermentation of alcoholic beverages throughout thousands of years of human history, and is applied in many areas of modern biotechnology. In this project the interest was in investigating non-conventional yeasts which had the capability of conversion of glycerol primarily to liquid biofuels. Chapter 2 is about the initial results for screening of the potential candidates for glycerol fermentation. Two candidates *Pachia pastoris* and *Pachysolen tannophilus* were shown to be capable of producing ethanol with glycerol as the sole carbon source. After growth comparison on glycerol and tests for extracellular metabolites in agitated flasks, *P. tannophilus* was selected as the object of further studies for conversion of glycerol to ethanol.

In chapter 3, physiology studies in lab scale fermentation of the ethanol production process with *P. tannophilus* were investigated on glycerol. The effect of aeration, pH and nitrogen source was studied for improving the ethanol production and yield and designing a
competitive ethanol production process. The ethanol tolerance of *P. tannophilus* on glycerol was studied for further characterizing the ethanol production process. A growth comparison on crude glycerol and pure glycerol was performed to test if the impurities in the crude glycerol inhibit the growth of *P. tannophilus* and affect product formation. Based on optimized parameters, 28.1 g/L ethanol was produced by a staged batch process, which was the maximum achieved so far for conversion of glycerol to ethanol by a microbial bioprocess.

The physiology study of ethanol tolerance of *P. tannophilus* showed that the ethanol tolerance of this strain was relatively low. The low ethanol tolerance of *P. tannophilus* might be the factor which inhibits further improvement of ethanol production process. Chapter 4 describes adaptive evolution studies performed to enhance the ethanol tolerance of *P. tannophilus* on glycerol. The adapted strains isolated during the evolution process were characterised according to the ethanol tolerance, growth rate on glycerol, ethanol production and growth profile on glycerol.

For better understanding the genetic background, the genomic DNA of *P. tannophilus* CBS4044 was isolated and sequenced. The draft genome sequencing results of *P. tannophilus* are summarized in chapter 5. Raw data of short reads from genome sequencing results were assembled together. The protein-coding genes were identified and the putative amino acid sequences were analysed for the gene function annotation. Pulsed field gel electrophoresis was performed to predict the chromosome numbers and approximate chromosome sizes in *P. tannophilus*.

For the purpose of further improving the yields and production levels of ethanol produced, it would be beneficial if *P. tannophilus* could be genetically engineered and the ethanol synthesis pathway in *P. tannophilus* could be investigated. The whole-genome sequencing of *P. tannophilus* also makes it possible to perform genetic engineering of this strain. Chapter 6 describes the attempts to set up the transformation system in *P. tannophilus* in order to know more about the genetic background and further improve the ethanol production process. The commonly applied methods using antibiotic resistance and auxotrophic markers *URA3* were used for transformation selection.

Since the genome of *P. tannophilus* CBS4044 was sequenced and the mechanism behind glycerol metabolism is poorly understood in this strain. In chapter 7 focusses on studying the
genes involved in glycerol metabolism in *P. tannophilus*, which were predicted by blasting with the sequences of genes known to have these functions in *S. cerevisiae*. Quantitative real-time PCR was performed to unveil the expression pattern of the genes during growth on glycerol. The glycerol metabolism and pathways in *P. tannophilus* are discussed. The genes involved in glycerol transport in *P. tannophilus* have been cloned and expressed in *S. cerevisae* (CEN.PK 113-5D) strains to validate the function of the predicted glycerol transporter genes.

Finally, the most relevant results from all the studies during the PhD are summarised and future perspectives for continuing these studies are presented in Chapter 8.
Dansk Sammenfatning

Den øgede fokus på udvikling af nye vedvarende energikilder til transportsektoren fra europeisk side har medført en øget produktion af biodiesel fra raps og andre vegetabilske olier. Dette resulteret i en betydelig stigning i produktionen af glycerol, som er et uundgåeligt biprodukt fra produktionen af biodiesel. Mængden af glycerol har efterhånden overskredet det aktuelle behov på markedet, derfor er producenter af biodiesel på udkig efter nye metoder til glycerol anvendelse og dermed forbedre konkurrenceevnen for biodiesel producenterne.

EU-kommissionen har finansieret GLYFINERY Projektet, et initiativ rettet mod udvikling af nye teknologier baseret på biologisk omdannelse af glycerol til kendte og nye biobrændstoffer, bioenergi og værdifulde grønne kemikalier i et integreret bioraffinaderi koncept.

Som en del af GLYFINERY projektet, var formålet med dette ph.d.-projekt at udvikle en proces til biologiskomdannelse af uraffineret glycerol til ethanol, samt karakterisere og optimere processen. Denne afhandling består af otte kapitler; Kapitel 1 introducerer projektets baggrund, omfang og mål, desuden er den tilhørende baggrundsviden for bedre at forstå de undersøgelser i de følgende kapitler også indført i dette kapitel. Kapitel 2-7 er sammensat af de eksperimentelle resultater opnået i løbet af hele ph.d.-studiet.

Den velkarakteriserede gær *Saccharomyces cerevisiae* er blevet brugt til fermentering af alkoholholdige drikkevarer gennem tusinde år af menneskets historie, og anvendes inden for mange områder af moderne bioteknologi. I dette projekt var interessen for at undersøge ikke-konventionelle gær, som havde evnen til omdannelse af glycerol primært til flydende biobrændstoffer. Kapitel 2 om handler de første resultater for screening af de potentielle kandidater til glycerol gæring. To kandidater *Pachia pastoris* og *Pachysolen tannophilus* viste sig at være i stand til at producere ethanol på baggrund af glycerol som eneste kulstofkilde. Efter vækst sammenligning af glycerol og tests for eksterne metabolitter i rystekolber blev *P. tannophilus* valgt som undersøgelsen genstand for yderligere undersøgelse til omdannelse af glycerol til ethanol proces.

Kapitel 3 om handler fysiologiske undersøgelser af *P. tannophilus* vokset på glycerol i laboratorieskala kultiveringer, med henblik på ethanol produktion. Virkningen af beluftning,
pH og nitrogenkilde blev undersøgt for at forbedre produktionen, og udbyttet af ethanol samt
designe en konkurrencedygtig proces til ethanol produktion. *P. tannophilus’ ethanol
tolerancemen den vokser på glycerol blev undersøgt for yderligere karakterisering af ethanol
produktionsprocessen. En vækst sammenligning af uraffineret glycerol og ren glycerol blev
udført for at teste om de urenheder i den uraffinerede glycerol har en inhiberende effekt på
væksten, og ethanol produktionen af *P. tannophilus*. Baseret på optimerede parametre blev
28.1 g/L ethanol fremstillet ved en trinvis batchproces, hvilket var den maksimale hidtidige
til omdannelse af glycerol til ethanol ved en mikrobio bioprocess.

De fysiologiske undersøgelser af *P. tannophilus’ ethanol tolerancviste at denne er relativt
lav, den lave ethanol tolerance *P. tannophilus* udviser, kan være den faktor der besværliggøre
en yderligere forbedring af ethanol produktionsprocessen. Kapitel 4 beskriver adaptive
evolution eksperimenter udført for at forbedre ethanol tolerancen hos *P. tannophilus*. De
tilpassede stammer der er blevet isoleret under eksperimentet karakteriseres ifølge ethanol
tolerance, væksthastighed på glycerol, ethanol og vækst profil glycerol.

For bedre forståelsen af den genetiske baggrund blev det genomiske DNA af *P. tannophilus*
CBS4044 isoleret og sekventeret. Udkastet genomsekvensering. Resultaterne af
sekvenseringen af *P. tannophilus* er sammenfattet i kapitel 5. Rådata med korte læsninger fra
genomsekvenseringen blev samlet sammen, de protein-kodende gener identificeret, og de
formodede aminosyresekvenser analyseret for genfunktion, og efterfølgende annoteret.
Pulsfeltgelelektroforese blev udført for at estimere kromosomtal og tilnærmelsesvise
kromosom størrelser i *P. tannophilus*.

Med henblik på yderligere at forbedre udbyttet og produktions niveauer af ethanol, vil det
være fordelagtigt, hvis *P. tannophilus* kan optimeres genetisk, og ethanol syntesevejen i *P.
tannophilus* undersøges. En komplet genom sekventering af *P. tannophilus* vil muliggøre
genetisk manipulation stammen. Kapitel 6 beskriver forsøg på at etablere et transformations
system til *P. tannophilus* for at bidrage yderligere til den tilgængelige viden om den genetiske
baggrund samt yderligere forbedre ethanol produktionen. De almindeligt anvendte metoder
såsom anvendelse af antibiotika resistens og den auxotrofe markør *URA3* blev anvendt til
selektion for transformanter.
Kapitel 7 fokuserer på at forstå og undersøge mekanismen bag glycerol metabolismen i stammen, vedpå at studere de gener der er involveret i glycerol metabolisme i *P. tannophilus*. Disse gener blev forudsagt ved blast mod sekvenser af gener, der vides at have disse funktioner i *S. cerevisiae*. Kvantitativ real-time-PCR blev udført for at afsløre ekspRESSIONSMØNSTERET af generne under vækst på glycerol. Desuden er generne der forventeligt er involveret i glycerol transport i *P. tannophilus* blevet klonet og udtrykt i *S. cerevisiae* (CEN.PK 113-5D) for at validere deres funktion. Afslutningsvis diskuteres glycerol metabolismen, og pathways i *P. tannophilus*.

I det afsluttende kapitel 8 a dresseres de relevante resultater fra hele ph.d. forløbet, og perspektiver for resultaterne, samt fremtidige studier diskuteres.
List of publications

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Chapter 1  Introduction

1.1 Project overview and aim

My PhD project forms part of the Europe Commission funded project GLYFINERY. The background of the whole GLYFINERY project will be introduced in this chapter. The aim and scope of my PhD project will be pointed out.

Among the different sources for the substitution of fossil fuels, bioethanol and biodiesel are the most promising sources for biofuels. Biodiesel is a renewable fuel produced from vegetable oils such as rape seed oil, sunflower seed oil, soybean oil and also used frying oils or animal fats. Biodiesel has been demonstrated to have great environmental benefits compared to fossil fuels. It can reduce CO₂ emissions, particulate emissions and other harmful emissions and decrease global warming impacts. In addition, biodiesel has a high lubricity and fast biodegradability. It can be effectively used either by blending with fossil diesel fuel or in a pure form as a transport fuel. In Europe, motor manufacturers have tested biodiesel blended with diesel oil up to 5-10%, or 25-30% or 100% pure. But normally for use at 100% pure biodiesel, minor modifications to seals and piping in existing engines are required.

The European Union is the leading biodiesel production region worldwide, representing about 55-60% of worldwide output and biodiesel accounted for over 75% of the total biofuels production in Europe in 2009 (Biofuels platform http://www.biofuels-platform.ch/en/infos/eu-biodiesel.php). Due to increasing interest in biofuels, the European biodiesel production has accelerated from approximately 500,000 tons in 1998 to 22 million tonnes in July 2011 with 254 existing biodiesel facilities. Figure 1 presents the biodiesel production trends in the EU from 1998 to 2011 according to the European Biodiesel Board (EBB) 2011 press release.
Figure 1. The biodiesel production trends in the EU from 1998 to 2011 (EBB press release).

Glycerol is the inevitable by-product in biodiesel production. It has seen a proportionally dramatic increase in production with raising biodiesel production in recent years. In a given biodiesel production process, approximately 10% of the reaction volume ends up as glycerol. With the increased global biodiesel production, the problem with glycerol-waste will continue to grow. The glycerol generated during biodiesel production contains impurities such as salts, heavy metals, alcohols and water. Glycerol could be utilized in many industries including cosmetic, soaps, pharmaceuticals, food, drinks and other uses. However, the crude glycerol from biodiesel production must be purified for the traditional processing of glycerol, which is not economically feasible for many biodiesel manufacturers. The market for glycerol now has become saturated and it is considered as a waste by many biodiesel producers. There is a lack of reliable methods for the efficient management of glycerol waste. Bioconversion of glycerol into liquid biofuels, green chemicals and bioenergy on the basis of fermentation processes might provide an efficient solution for sustainable management of glycerol, which can improve the economics of biodiesel industries.

The GLYFINERY project aims at finding a solution for sustainable management of the glycerol from both environmental and economic point of view. The target bioproducts developed by the GLYFINERY concept are liquid biofuels, bioethanol and the long-chain alcohol biobutanol, the green chemical (1,3-propanediol) and bioenergy in form of biomethane. The concept of GLYFINERY is analogous to the concept of biorefinery, where it integrates low cost biomass conversion processes and equipment to produce fuels, power, heat, and value-added chemicals by virtue of microbes as the cell factory. As part of the
GLYFINERY project, the aim of my PhD project was developing a process for bioconversion of waste glycerol into biofuel ethanol, characterization and optimization of the process.

1.2 Biodiesel production and the glycerol glut

Biodiesel production process

The simplified biodiesel production process is shown in the following flowchart (Figure 2). The main reaction for converting oil to biodiesel is called transesterification. In the transesterification process, the triglyceride oils contained in vegetable oils, animal fats, or recycled greases react with an alcohol (typically methanol or ethanol) to form fatty acid alkyl monoesters (biodiesel) and glycerol. The reaction requires heat and acid, alkali or enzymatic catalysts.

![Figure 2. An overview of a standard biodiesel production process. Biodiesel and glycerol are the two main products produced in this process.](image)

The basic biodiesel producing process is a chemical transesterification reaction converting triglycerides into fatty acid alkyl monoesters in the presence of a catalyst. An overview of the stoichiometry of the transesterification reaction can be seen in Figure 3. Since the reaction is a reversible reaction, the alcohol must be added in excess to drive the reaction towards the right and ensure complete conversion.
Figure 3. Chemical process for methyl ester biodiesel production. The R groups represent fatty acid radicals.

The most commonly used oils for biodiesel production are from rape seed, sunflower, palm, canola, cotton seed and jatropha and soy bean. Other low cost feedstocks including waste cooking oils or animal fats are also used for biodiesel production (Singh & Singh, 2010). The feedstocks used for biodiesel production should fulfil two requirements: low production costs and large production scale (Singh & Singh, 2010). In principle, any oleaginous material can be used as a feedstock. The source of biodiesel usually depends on the crops amenable to the regional climate. In Europe, rapeseed oil is the most common source for biodiesel while in tropical countries palm oil is the most common source. In the United States, biodiesel is mostly made from soybean oil or recycled cooking oils. Animal fats, other vegetable oils, and other recycled oils can also be used to produce biodiesel, which depends on their costs and availability. In reality biodiesel producers use a mixture of different vegetable oils. In the future, blends of all kinds of fats and oils may be used to produce biodiesel.

Before the transesterification process some feedstocks must go through pretreatment, which depends on the free fatty acid (FFA) concentration. Feedstocks having higher levels of FFA and water might lead to problems with saponification during biodiesel production with an alkali catalyst. If the feedstocks contain less than 4% free fatty acids, pretreatment is not required, this is the case for vegetable oils and some food-grade animal fats. If the feedstocks contain more than 4% free fatty acids, they must be pretreated in an acid esterification process, such as when inedible animal fats and recycled greases are used. In this step, the feedstock is reacted with an alcohol converting the free fatty acids into biodiesel in the presence of a strong acid catalyst (sulfuric acid). The remaining triglycerides are converted to biodiesel in the transesterification reaction.
Generally there is no difference in the biodiesel yield with various types of alcohols. The choice of alcohol to use is mainly an economic concern. Since methanol is often the cheapest alcohol available and can be obtained in a very anhydrous formulation, it is often the alcohol of choice. There are three major types of catalysts used for biodiesel production: acid catalysts (e.g. HCl, H₂SO₄), alkali catalysts (e.g. NaOH, KOH) and enzymatic catalysts (lipase). On an industrial scale only the acid and alkali catalysts are used. Alkali catalysts are the predominant catalyst as they are cheap and give high yields and fast reaction times. However, if the feedstock contains high levels of FFA’s and water, it might risk of saponification during the reaction. Acid catalysts have higher tolerance for FFA’s and water but require higher alcohol to oil ratios and have slower reaction times. Compared to chemical catalysts, enzymatic catalysts have moderate reaction conditions and do not require the same amount of excess alcohol ratios. The use of enzymatic catalysts also makes it easier for downstream processing and product recovery. But, the cost of enzymatic catalysts is still very high for application on a commercial scale. Alkali catalyzed transesterification with methanol is the most prevalent process on an industrial scale.

**The waste glycerol from biodiesel production**

The main source of glycerol on the market is associated with the increasing production of biodiesel. In the biodiesel production, the two main by-products are glycerol and esters (biodiesel). The glycerol phase and biodiesel phase can be separated by settling for several hours or in some cases by centrifugation since the glycerol is much denser than the biodiesel. Biodiesel is separated from the glycerol fraction after neutralization, washing and drying, and then it is ready for use as a fuel. The crude glycerol stream leaving the separator typically is about 50% glycerol or less and also contains unused catalyst, alcohol, soap, water and salts (Singh & Singh, 2010). In some cases, the glycerol stream is treated by an inorganic acid whereby soaps are split into salts. The alcohol in the glycerol phase could be removed by vacuum flashing. The water will also be removed to form the concentrated glycerol (approximately 80 - 88% purity) which can be sold to glycerol refiners. The concentrated glycerol is normally regarded as crude glycerol, which comprises impurities like catalyst and soaps and it was normally brown colour. Pure glycerol could be used in a variety of applications in the food and pharmaceutical, cosmetic, textiles, paint, tobacco, waxes, pulp and paper, leather industries (Wang et al., 2001). However, the purification of this crude glycerol is not industrially feasible due to the high cost. Currently, only combustion is
considered as the utilized way of disposing crude glycerol. However, the salts present in the crude glycerol create significant amounts of ash in the boiler and formation of acrolein by thermal decomposition of glycerol is a concern. In view of the economic and environmental concerns, therefore, utilization of crude glycerol in biorefineries can represent an alternative and environmental friendly way to improve the economics of the biodiesel industry as well as providing a means to handle the increasing increment of glycerol waste streams (Yazdani & Gonzalez, 2007). Bioconversion of glycerol into liquid biofuels, green chemicals and bioenergy on the basis of biological fermentation processes can provide an efficient solution for sustainable management of glycerol (Lynd et al., 2005).

**Composition of biodiesel and crude glycerol**

The composition of biodiesel is closely related to the composition of the feedstocks used since biodiesel production is a simple chemical transesterification reaction. Therefore, it means that the biodiesel produced from animal fats which contain higher saturated fatty acid or other saturated sources often have difficulty in achieving desired cold flow properties. This problem can be partly remedied by the right choice of alcohol.

The composition of the crude glycerol depends on the parent feedstocks employed and the biodiesel production process used. These characteristics together with the extent of post-production purification of the crude glycerol, determines the final characteristics of the commercially available glycerol feedstock. In general, the composition of the glycerol depends on the process used and is varied from different biodiesel producers. Chemical and physical properties of the crude glycerol from seven different vegetable oils: mustard, rapeseed, canola, crambe, soybean, and waste cooking oils (WCO) were characterized by Thompson and He (2006). In the final analysis there was very little variation with the exception of WCO. The viscosity of the crude glycerol prior to any treatment ranged from 8.46 to 8.80 cs (centistokes) and 26.5 cs for the WCO. The carbon content averaged about 25% and the metals Ca, K, Mg, Na, P, and S were present in small quantities from 4 to 163 ppm with the exception of sodium, which averaged just over 1%. Protein levels ranged from 0.06% to 0.44%. Fat content ranged from 1% to 13% and carbohydrates ranged from 75% to 83%. Most crude glycerol feedstock falls within the range of 60-90% glycerol w/w with varying amounts of the other components.
In the GLYFINERY project, three different batches of crude glycerol Batch 1 (B1), Batch 2 (B2), Batch 3 (B3) were received from the Slovakian biodiesel producer MEROCO A.S. The details of the batches used for biodiesel production are listed below:

- B1: Based on 100% rape seed oil feedstock
- B2: Based on a mix of 90% rape seed oil and 10% waste cooking oil
- B3: Based on 100% rape seed oil feedstock

Each batch was derived from different production runs. The crude glycerol feedstock contains up to 7% ash, 0.5% methanol, 10% water and 82 ± 5% glycerol. A picture of the three batches can be seen in Figure 4. Although the B1 and B3 are both based on 100% rape seed oil, B1 seems much lighter, and thus possibly cleaner, compared to B3 and B2. This is an indication that the composition of the crude glycerol varied even between different processes of the same biodiesel producer. The three batches were subjected to ion chromatography, gas chromatography and high pressure liquid chromatography analytical procedures (HPLC). The analysis with ion chromatography quantifies the amount of compound present in the crude glycerol. The results can be seen in Table 1.

Table 1: Ion chromatography analysis results of the three batches of crude glycerol: B1, B2 and B3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>B1 [g/kg]</th>
<th>B2 [g/kg]</th>
<th>B3 [g/kg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>0.34</td>
<td>0.47</td>
<td>0.41</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.073</td>
<td>0.10</td>
<td>0.083</td>
</tr>
<tr>
<td>Chloride</td>
<td>36.95</td>
<td>31.12</td>
<td>32.35</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>Sulphate</td>
<td>0.01</td>
<td>0.57</td>
<td>0.36</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.014</td>
<td>0.063</td>
<td>0.15</td>
</tr>
<tr>
<td>Citrate</td>
<td>6.81</td>
<td>6.57</td>
<td>5.73</td>
</tr>
</tbody>
</table>

It can be seen in Table 1 that chloride and citrate are present in fairly large amounts compared to other components in all the three batches. From the analysis results of gas chromatography, one peak is identified in sample B2 which was not present in the other
samples B1 and B3 (results not shown here). Further analysis would be needed to determine the identity of this peak. Based on HPLC analysis, more peaks are evident in samples B2 and B3 that are not present in B1 (figure not shown), which indicates that more complexity exists in the samples B2 and B3 compared to B1. This corresponds to the visual inspection that the first batch B1 looks much cleaner while the batches B2 and B3 appear more turbid (Figure 4). More analysis is needed to determine the identity of the unknown compounds. There seems to be a fair amount of variance within the batches of glycerol received from MEROCO A.S.

1.3 Biotechnology

Industrial biotechnology and the microbial cell factory

With the development of human civilization, the world in the future will be faced with an energy crisis arising from rapid consumption of fossil fuel, global environmental pollution and food crisis. Biotechnology could be one of the solutions to alleviate the problems for sustainable development. Industrial biotechnology is often known as “white biotechnology”, which is the application of microbial technology for processing of renewable resources to produce biofuels, biomaterials and biochemical. Biotechnology could diminish our dependency on fossil fuel, save energy in production processes and significantly reduce CO₂ emissions (Sabra et al., 2010).

Long before the discovery of microorganisms, the yeast *Saccharomyces cerevisiae* had been used for baking and fermentation of alcoholic beverages throughout thousands years of human history. Different microorganisms were also exploited to produce cheeses, pickled food, vinegar and soy sauce to meet human needs. Modern industrial biotechnology is utilized to produce a bulk of fine chemicals, biofuels, biodegradable plastics, enzymes, pharmaceuticals and food ingredients by microbial fermentation. The multi-step chemical synthesis by the traditional chemistry industry could be replaced with a single step with microbial as the cell factory. In recent years, several widely used microorganisms like the filamentous fungi *Aspergillus oryzae* and *Aspergillus niger*, the bacterium *E. coli* and the yeast *S. cerevisiae* have been used as cell factories for production of desired products. With realizing the importance of bio-based production, the biorefinery concept analogous to the petroleum refinery has emerged, where it integrates low cost biomass conversion processes and equipment to produce fuels, power, heat, and value-added chemicals by virtue
of microbes as cell factories. The GLYFINERY project aims to achieve conversion of the low cost glycerol feedstock into new bioproducts, which fits to the concept of biorefinery. In addition, lignocellulosic biomass particularly agricultural and forestry residues is very important low cost biomass used in the biorefinery. These are abundant, readily available and renewable resources. To develop a robust and efficient production process in the biorefinery, the characteristics concerned with the potential cell factory are:

- Growth rate on the low cost substrate;
- Tolerance towards elevated concentrations of substrate;
- Production of the desirable value-added products;
- Tolerance towards elevated concentrations of products;
- Substrate conversion efficiency;
- Products yields;
- Limited formation of by-products.

**Process optimization and Strain improvement**

The microbial fermentation platform is employed in biorefinery schemes to achieve the conversion process. By controlling the process operating conditions and the medium composition, it is possible to regulate the pattern of fermentation products in the fermentation process. The bioreactor performance could be monitored under different conditions. In order to optimize the production process, fermentation can be performed under different conditions and at a variety of scales. In addition, different modes of fermentation such as batch, fed-batch, continuous operation in chemostats could be operated for improving the production of the compound of interest.

In order to further optimize the process and improve the strain performance for the desired cellular properties, it is suitable to manipulate the microbial strains by genetic modification. Traditional mutagenesis strategies by random mutagenesis and screening have been applied for decades to increase product yield, production level, productivity, and elimination of the side products. Mutagens such as chemical agents and short wavelength radiation (UV), X-ray ionizing radiation have been extensively used. However, the screening and selection process for isolating of stable mutants with desired characteristics demand in terms of time and labor. Even though the mutants with desired properties may be obtained, the underlying
mechanisms may still be unknown, which is not beneficial for further research and development.

The development of metabolic engineering has facilitated the expansion of industrial biotechnology, which utilizes genetic tools to manipulate microbial metabolism to improve the production of desired compounds. Metabolic engineering is through the modification of biochemical reactions to improve the product formation and cellular properties by the use of recombinant DNA technology. The applications of metabolic engineering greatly depend on available molecular or functional knowledge. Compared to the traditional random mutagenesis and screening, metabolic engineering is based on rational and directed genetic modification, which improves the microbial production process in a more efficient way. In addition, adaptive evolution, as a complementary strategy for strain development and process optimization, simulates nature’s engineering principle where populations of cells adapt to their environment over many generations by natural selection. During the evolutionary process, the microbial cells undergo random mutation, recombination and continuous evolution.

1.4 Bioconversion of glycerol

**Biological processes for conversion of glycerol by microorganisms**

A variety of (typically anaerobic) bacteria such as *Klebsiella, Citrobacter, Enterobacter, Clostridium, Lactobacillus, Bacillus, Propionibacterium, and Anaerobiospirillum* have been shown to be capable of fermenting glycerol. Glycerol can be fermented to 1,3-propanediol, acetate, butyrate, acetone, butanol, ethanol, succinate and lactate by various species of clostridia (Chatzifragkou et al., 2011; Dabrock et al., 1992). Anaerobic fermentative production of 1,3-propanediol was considered as the most promising option for the biological conversion of glycerol. It can be used for the synthesis of polyester with the necessary properties for application in the textile industries. *Pseudomonas aeruginosa* was shown to produce rhamnolipid which is a kind of biosurfactant (Silva et al., 2010). *E. coli* has been genetically engineered for efficient conversion of crude glycerol into ethanol with the coproducts hydrogen and formate (Shams Yazdani & Gonzalez, 2008). However, application in industry of some of those organisms has been limited due to issues that include pathogenicity, the need for strict anaerobic conditions and supplementation with rich
nutrients. The lack of genetic tools and physiological knowledge necessary for their effective manipulation has also hampered progress.

A number of yeast strains also exhibit the ability to grow on glycerol and produce value added products. Glycerol can be assimilated into organic acids and single cell oil by Yarrowia lipolytica (Makri et al., 2010; Papanikolaou et al., 2002). The basidiomycete yeast Pseudozyma antarctica was reported to efficiently produce mannosylerythritol lipids as glycolipid biosurfactants from glycerol (Morita et al., 2007). Glycerol has also been used as the carbon source for producing biomass and recombinant proteins in Pichia pastoris (Celik et al., 2008; Fieldhouse et al., 2009). Resting cells of Candida magnolia produce mannitol from glycerol under aerobic conditions (Khan et al., 2009). Arabitol can be produced from glycerol by Debaryomyces hansenii SBP-1 (Koganti et al., 2011). The industrial work horse S. cerevisiae has been genetically engineered to produce and improve ethanol production from glycerol (Yu et al., 2010). S. cerevisiae was also engineered to be as a platform for production of fatty acid ethyl esters (FAEEs) from glycerol by esterifying exogenous fatty acids with endogenously produced ethanol (Yu et al., 2012).

Roles of glycerol in yeast metabolism

Glycerol is involved in different metabolic pathways and plays an important role in yeast metabolism. Glycerol can be utilized as the sole carbon and energy source for cell growth under aerobic conditions and also can be formed as a product by many different types of yeast. In S. cerevisiae, glycerol is formed with ethanol production as a by-product to maintain the cytosolic redox balance and consume cellular NADH especially under anaerobic conditions (Albertyn et al., 1994; Nevoigt & Stahl, 1997; van Dijken & Scheffers, 1986). In addition to the function for maintaining the redox balance, the production of glycerol also has the function of protecting against osmotic stress (Blomberg & Adler, 1989; Van Eck et al., 1993). When cells are exposed to osmotic stress, the response appears to be the formation and intracellular accumulation of osmoprotective solutes (osmolytes) such as glycerol, arabitol, mannitol, and erythritol to survive in stressful environments (Kayingo et al., 2001; Van Eck et al., 1993; Yancey et al., 1982). For example, Zygosaccharomyces rouxii releases glycerol and also arabitol as the osmolytes upon hypo-osmotic shock (Kayingo et al., 2001). Yeast cells have developed adaptive mechanisms to control glycerol flux within limits suitable for growth. These mechanisms include modulation of glycerol formation, degradation, and
increased retention by regulation glycerol transport under osmotic stress (Kayingo et al., 2001). For instance, the production of glycerol is stimulated and accumulated intracellularly under hyper-osmotic stress, and accumulated glycerol is secreted extracellularly when the external osmolarity decreases (Hohmann, 2002). Rapid export of glycerol is an important event in adaptation to low osmolarity.

In addition, glycerol is also involved in lipid metabolism in some yeasts (Meesters et al., 1996; Nevoigt & Stahl, 1997) as well as in the regulation of inorganic phosphate recycling (Alonso-Monge et al., 2003; Ansell et al., 1997). Moreover, glycerol appears to play a protective role against heat shock (Siderius et al., 2000). It is crucial to understand the ways glycerol crosses the cell membrane and how it is involved in different metabolic pathways and cell responses.

**Glycerol metabolism**

The cell membrane is the first and also the last barrier for glycerol movement through the cells. The existence of specific systems responsible for glycerol transport across the cell membrane has been shown by several studies (Ferreira et al., 2005; Kayingo et al., 2009; Sutherland et al., 1997; Tamás et al., 1999).

In *E. coli*, it was investigated that glycerol was transported into the cells by facilitated diffusion with the glycerol facilitator. It was thought that glycerol facilitator and glycerol kinase are involved in the transporting of external glycerol into cellular metabolism (Voegele et al., 1993). The glycerol facilitator GlpF is thought to function as a carrier to form a selective pore in the cytoplasmic membrane, whereas the kinase traps the glycerol inside the cell as glycerol-3-phosphate (Voegele et al., 1993). GlpF is also responsible transporting poly alcohols and urea. The influx of glycerol through GlpF is 100- to 1000-fold greater than expected for a transporter. Glycerol degradation may occur through oxidative and reductive pathways in *Klebsiella, Citrobacter, Clostridium* and *Enterobacter*. In the oxidative pathways, glycerol could be assimilated by a glycerol dehydrogenase and a dihydroxyacetone kinase to dihydroxy-acetone phosphate (DHAP), which finally reaches the glycolytic pathway (Daniel et al., 1995; Macis et al., 1998). In the reductive branch, glycerol is converted into 3-hydroxypropionaldehyde (3-HPA) by coenzyme B₁₂-dependent glycerol dehydratase and related diol dehydratases (Talarico et al., 1990). The 3-HPA can be subsequently reduced to
1,3-propanediol (1,3-PDO) by a NADH-linked 1,3-propanediol dehydrogenase (Talarico et al., 1990) or can be oxidized to 3-hydroxypropionic acid (3-HP) (Lonvaud-Funel, 2002).

For glycerol metabolism in yeast, *S. cerevisiae* was utilized as a model to investigate the glycerol metabolism and regulation mechanisms (Brisson et al., 2001). It has been shown that glycerol accumulation and export during osmoregulation in *S. cerevisiae* was controlled by a MIP family membrane channel protein Fps1p (Tamás et al., 1999). The physiological role of the facilitator Fps1p in *S. cerevisiae* was described to be glycerol export rather than uptake during hypo-osmotic shock. It appears that Fps1p channel closes during hyper-osmotic conditions and opens during hypo-osmotic conditions thereby controlling the accumulation and release of glycerol during osmoregulation. Similar transport systems and Fps1p homologues have been found in many other yeasts (Kayingo et al., 2001; Neves et al., 2004). Besides, Lages and Lucas (1997) discovered an active transport system with a high affinity uptake for glycerol in *S. cerevisiae*. The active transport was described to be the proton symport type and was shown to be under glucose repression and inactivation. The glycerol proton symporter in *S. cerevisiae* is found to be encoded by *STL1* which is a member of the sugar transporter family (Ferreira et al., 2005). It was demonstrated in *S. cerevisiae* that glycerol enters the cell by two different mechanisms: a low affinity transport system with facilitated diffusion encoded by *FPS1* and a high affinity proton symport system encoded by *STL1* (Figure 5). Active glycerol uptake systems have also been shown to be present in other yeasts species like halotolerant yeasts *Debaryomyces hansenii* (Lucas et al., 1990), *Pichia sorbitophila* (Lages & Lucas, 1995), *Candida versatilis* (Silva-Graça & Lucas, 2003) and in *Zygosaccharomyces rouxii* (Zyl et al., 1990) and *Candida albicans* (Kayingo et al., 2009). In these yeasts, glycerol was found to be actively transported along with protons or sodium ions.

For glycerol dissimilation it is known that two pathways exist in yeasts: the respiratory pathway and the fermentative pathway. The first pathway is started by a phosphorylation of a glycerol kinase and followed by a FAD-dependent glycerol 3-phosphate dehydrogenase on the outer surface of the mitochondrial inner membrane (Klingenberg, 1970). The fermentative pathway includes glycerol dehydrogenase and dihydroxyacetone kinase (May et al., 1982). Dihydroxyacetone phosphate is formed as the glycolytic intermediate after the two-branch pathways. In some yeast strains, all four enzymes in both pathways are present, but only one pathway functions for glycerol dissimilation. In some strains, the enzyme activity related with glycerol catabolism could be measured, but the strains are unable to
ferment glycerol or consume glycerol at very low rate. The wine yeast *S. cerevisiae* is able to utilize glycerol as sole source of carbon. The catabolic pathway of glycerol involves a glycerol kinase encoded by *GUT1* (Pavlik et al., 1993) and a glycerol 3-phosphate dehydrogenase encoded by *GUT2* (Rønnow & Kielland-Brandt, 1993) (Figure 5). So far, it has been shown that glycerol is degraded by the respiration pathway and the mutants lacking one of the two genes are incapable of utilizing glycerol. The *GUT1* and *GUT2* genes were repressed on glucose at the transcriptional level (Pavlik et al., 1993; Rønnow & Kielland-Brandt, 1993). Although the fermentative pathway was also discovered to be present in *S. cerevisiae*, the function of them is unknown.

Figure 5. Glycerol Metabolism pathways in *S.cerevisiae*
Glycerol is synthesized with two steps by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by a NAD dependent cytosolic G3P dehydrogenase, followed by dephosphorylation by a specific phosphatase (Gancedo et al., 1968). In S. cerevisiae, each enzyme has two isoenzymes and encoded by GPD1/2 and GPP1/2 (Figure 5). The expression of GPD1 at the mRNA level was induced under osmotic stress, however GPD2 gene was constitutively expressed at a particularly low level (Ansell et al., 1997; Ohmiya et al., 1995). The two genes GPP1 and GPP2 show 95% amino acid identity. Gpp2p was induced under osmotic stress, while the concentration of Gpp1p in cells was not affected by changes of external osmolarity (Norbeck et al., 1996). The synthesis of glycerol via glycerol-3-phosphate is very common in yeasts since GPD1 or GPD2 genes have been cloned and sequenced from many types of yeasts (Wang et al., 2001). Glycerol-3-phosphate and dihydroxyacetone phosphate also function as the important metabolic intermediates for synthesis of other substances besides glycerol. For instance, glycerol-3-phosphate and dihydroxyacetone phosphate could be used as precursors for glycerolipids and phospholipids synthesis (Athenstaedt et al., 1999; Daum et al., 1998; Racenis et al., 1992).
1.5 *P. tannophilus* as a non-conventional cell factory

**Nature of *P. tannophilus***

*P. tannophilus* was selected as the host for ethanol production from glycerol after screening of several yeast strains, discussed in detail in Chapter 2. Here a general overview of this species is summarized as basic knowledge for further study.

*P. tannophilus* was initially isolated from wood extracts used in leather tanning and described by Boidin and Adzet in 1957 (Kurtzman, 1983). *P. tannophilus* was identified as a yeast and the vegetative growth of *P. tannophilus* is mainly by budding and usually has one or two buds as do many other types of yeast. Under certain conditions, it may form pseudohyphal. However, *P. tannophilus* is one of the most unusual yeasts because of the unique way of ascospore formation (Jeffries & Kurtzman, 1994). Asco formation starts when a vegetative cell produces a stout tube which may be quite short or up to 60 µm in length and either straight or curved (Figure 6a and 6b). The tip of the tube enlarges to form the ascus and the tube could be regarded as an ascophore (Slininger et al., 1987). *P. tannophilus* is the only yeast known so far to produce this unique structure. Four hemispheroidal ascospores are released from the asci by the deliqueses of the ascus wall. Once this happens, the ascus has formed within a V-shaped notch at the end of the ascophore. Ascophore walls normally are greatly thickened and refractile, which make ascopores easily identified under microscope (Figure 6b). Ascii may be conjugated or unconjugated, and this is strain dependent to some extent. Cells forming conjugated asci are haploid whereas unconjugated asci result from diploid cells. The species of *P. tannophilus* is homothallic because single isolated ascospores produce sporogenous colonies (Slininger et al., 1987).
Substrate diversity of *P. tannophilus* in biotechnology

Plant biomass is considered as one of the most abundant renewable resources and refers largely to lignocellulosic material as this makes up the majority of the cheap and abundant nonfood materials available from plants. Plant biomass is seen as a promising source of material for fuels and raw materials. Furthermore, glycerol is also regarded as a cheap substrate because of the increasing production of biodiesel as explained in section 1 and 2 in this chapter.

*P. tannophilus* was the first yeast shown to be capable of fermenting xylose sugars to ethanol (Slininger et al., 1982) and the xylose utilisation pathway has been extensively studied in this organism (Sathesh-Prabu & Murugesan, 2011; Slininger et al., 1987; Zhao et al., 2010). *P. tannophilus* is one of the few yeasts that can ferment xylose to ethanol. Additionally, it was reported that *P. tannophilus* also could ferment all the common sugars glucose, mannose and galactose except L-arabinose occurring in the hemicelluloses hydrolysate mixture (Slininger et al., 1987). Besides, it was reported that *P. tannophilus* could accumulate 4g/L ethanol on glycerol under aerobic growth in a previous study (Maleszka et al., 1982). However, the conditions for ethanol production were not precisely defined or controlled and the physiology during growth on glycerol has not been extensively studied in this organism. *P. tannophilus* has certain unique qualities that make it as the potential cell factory and worth of study as it can naturally utilize xylose and glycerol, while the wild type of the brewing and baking yeast
S. cerevisiae cannot utilize xylose (Olsson & Hahn-Hägerdal, 1993) and uses glycerol very slowly (Ochoa-Estopier et al., 2011).

**P. tannophilus as an ethanol producer**

As *P. tannophilus* was the first yeast identified to have a significant capacity to convert xylose to ethanol, most of previous studies of this species were about the ethanol production process from xylose.

Ethanolic fermentation is traditionally regarded as occurring anaerobically like in *S. cerevisiae*. It was noted that the cell growth of the xylose fermentation by *P. tannophilus* depended on aerobic conditions (Slininger et al., 1987). A functioning mitochondrion is essential to the growth of *P. tannophilus*. None of the xylose fermenting strains has been shown capable of significant anaerobic growth on any substrate including glucose. By addition of oxygen the fermentation rate of many yeasts could be enhanced and this phenomenon is referred to as either a negative Pasteur effect or Custer’s effect (Slininger et al., 1987). It was hypothesized that Custer’s effect might be due to a shortage of NAD brought about by an imbalanced intracellular redox system. *P. tannophilus* displays a Custer’s effect and an NAD shortage also diminished production of ethanol from xylose by *P. tannophilus* (Slininger et al., 1987). Like in many yeasts and filamentous fungi, xylose is converted to xylitol by a NADPH-linked reductase. By an NAD-linked reductase xylitol dehydrogenase xylitol is further converted to xylulose in *P. tannophilus*. Xylulose is then phosphorylated to xylulose-5-phosphated by xylulose kinase. It was shown that aeration was necessary for successful induction of xylose reductase, xylitol dehydrogenase and xylulose kinase (Neirinck et al., 1982). It was demonstrated that with the decreasing of aeration rate the ratio of NADH- to NADPH-linked xylose reductase activity increased (Verduyn et al., 1985).

For the ethanol production process, the fermentation rate and the maximum ethanol concentration accumulated ultimately depend on cellular resistance to ethanol toxicity. For the xylose fermentation process by *P. tannophilus*, the specific ethanol production and xylose consumption rates were affected when ethanol concentration was greater than 20 g/L. The maximum growth rate of *P. tannophilus* on xylose without ethanol was 0.31 h⁻¹. However, it dropped to 0.08 with 34 g/L ethanol present in the medium. The maximum of ethanol
produced on xylose was 38 g/L at 25°C and *P. tannophilus* was able to produce > 50g/L ethanol when glucose was the substrate.

Studies referring to the genetic engineering for further enhancing the ethanol production of ethanol are few. Only one article has been published about transformation of the yeast *P. tannophilus* by Wdelock and Thornton (1989). It was shown that lithium treated hexose negative mutants of *P. tannophilus* have been successfully transformed with YRp7 plasmid. However, the transformants lost the ability to grown on glucose after sub-culturing on non-selective medium (Wedlock & Thornton, 1989). More information about the genetic background and the effective genetic engineering tools of *P. tannophilus* are needed for learning more about the strains and further improving the process. So far, a useful transformation system is still absent in *P. tannophilus*. Work in this project on developing a transformation system in *P. tannophilus* is described in chapter 6.

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Chapter 2 Initial screening results for ethanol producers on glycerol

Abstract

This chapter describes the initial results for screening of the potential candidates for glycerol conversion to products of interest. Two candidates *Pachia pastoris* and *Pachysolen tannophilus* were shown to be capable of producing ethanol with glycerol as the sole carbon source. After growth comparison on glycerol and tests for ethanol production in shake flasks, *P. tannophilus* was selected as the organism for further study for conversion of glycerol to ethanol in the experimental work conducted in this PhD study.

Keywords: Screening; glycerol conversion; non-conventional yeasts.
1. Objective

In order to find new hosts capable of producing value-added products from glycerol, studies focused on the group of organisms known as non-conventional yeasts. In this project, the interest was in investigating non-conventional yeasts which had the capability of converting glycerol primarily to liquid biofuels. It is known from the literature that several non-conventional yeasts can grow on glycerol and a number of these strains exhibit the ability to produce value-added products, as was already introduced in Chapter 1. Screening for the potential candidates for glycerol conversion was done by a thorough literature search, with a shortlist of relevant organisms being subsequently tested for growth on agar plates with glycerol as the sole carbon source. Then the potential candidates which showed growth on glycerol plates were further tested in liquid medium. Potentially interesting strains were assessed based on their profiles of secreted metabolites. Metabolites with primary interest were alcohols.

2. Materials and Methods

2.1 Medium and strains

All the strains used in were obtained from CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) named as CBS plus number or from the culture collection of the Center for Microbial Biotechnology, Technical University of Denmark, named as IBT plus number. The growth experiments were performed in defined medium, containing (per liter) 0.67g yeast nitrogen base w/o amino acids (Difco, USA) and glycerol at % (v/v) as stated. Agar plates were prepared by adding 2% w/v agar. The strains were maintained in 15% glycerol in frozen vials (-80°C).

2.2 Submerged cultivation

The strains were cultivated in 250ml shake flasks at 28°C, 150rpm with defined medium, plus 2.5% (v/v) or 5% (v/v) glycerol as the sole carbon source.

2.3 Analytical methods

Growth was monitored by measuring OD450nm with a Shimadzu UV mini-1240 spectrophotometer (Shimadzu, Japan). Samples were taken periodically from the flasks and
filtered through a 0.22 µm syringe filter, and supernatants were preserved at -20°C for later HPLC analysis. Concentrations of the substrate glycerol in supernatants were measured by HPLC refractive index detector RID-10A using an Aminex87H column (Bio-Rad, USA). Separations were performed at 60°C, flow rate of 0.6ml/min and 5mM H2SO4 as mobile phase.

3. Results and Discussion

3.1 Initial screening results

For the purpose of selecting non-conventional yeast strains which could grow on glycerol and produce biofuels, a thorough literature search was done in order to find the potential candidates. The potential strains of interest were then obtained from different culture collections and are listed in Table 1. The initial growth results on agar plates with different concentrations of glycerol as the sole carbon source are also shown in Table 1. Results from agar plates showed that all the yeast strains could grow with 1%, 2% or 5% glycerol as the sole carbon source, except *Zygosaccharomyces rouxii* and *Schizosaccharomyces pombe*. The strains which showed growth on agar plates were cultivated in liquid medium for further physiological characterization and analysis of secreted metabolites.

From HPLC analysis, ethanol was only constantly produced from two strains *Pichia pastoris* CBS704 and *Pachysolen tannophilis* CBS4044. *Candida tropicalis, Hansenula polymorpha, Pichia stipitis, Debaromyces hansenii* and *Candida magnoliae* exhibited growth on 2% glycerol but without obvious products accumulated in defined medium except the biomass. *C. tropicalis* and *P. stipitis* consumed only small amounts of glycerol, so the maximum growth rate (µmax) on glycerol was not calculated with those two strains. *P. pastoris* and *H. polymorpha* could produce relatively high levels of biomass compared to other screened yeasts. The OD450nm of *H. polymorpha* reached 47 and *P. pastoris* reached 53 at 72 hours’ cultivation, while the OD450nm range of other yeasts was from 10 to 30 (Figure 1). *Candida utilis* produced acetate on glycerol. *Yarrowia lipolytica* grew relatively fast compared to other strains with µmax 0.33 h⁻¹ and could generate mannitol, citric acid, erythritol in defined medium, which indicated that *Y. lipolytica* has the potential to be developed for producing biochemicals from glycerol. The products produced by the strains tested are listed in Table 1.
Figure 1. 250ml shake flask cultivations on 2.5% (v/v) glycerol as the sole carbon source with 100ml medium showing OD\textsubscript{450nm} of the screened yeasts over 72 hours of cultivation. Results represent the mean of at least duplicate experiments.
Table 1. Results of initial screening of potential non-conventional yeast cell factories for a glycerol biorefinery (NC*: Not calculated).

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Strain no.</th>
<th>Growth 1/2/5% glycerol plates</th>
<th>Products from glycerol (literature)</th>
<th>Products in this study</th>
<th>$\mu_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida magnoliae</td>
<td>IBT235</td>
<td>Yes</td>
<td>Mannitol (Khan et al., 2009)</td>
<td>Biomass</td>
<td>0.1</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>IBT249</td>
<td>Yes</td>
<td>Ethanol produced from xylose/Glucose (Hahn-Hägerdal et al., 1985)</td>
<td>Biomass</td>
<td>NC*</td>
</tr>
<tr>
<td>Candida utilis (Lindnera jadinii)</td>
<td>CBS 841</td>
<td>Yes</td>
<td>Biomass (Fieldhouse et al, 2009)</td>
<td>Biomass, Acetate</td>
<td>0.2</td>
</tr>
<tr>
<td>Debaromyces hansenii</td>
<td>IBT24</td>
<td>Yes</td>
<td>Arabitol (Koganti et al., 2011)</td>
<td>Biomass</td>
<td>0.16</td>
</tr>
<tr>
<td>Hansenula polymorpha (Pichia angusta)</td>
<td>CBS 1976</td>
<td>Yes</td>
<td>Biomass, phytase, alcohol oxidase (Eggeling &amp; Sahm, 1980; Mayer et al., 1999)</td>
<td>Biomass</td>
<td>0.15</td>
</tr>
<tr>
<td>Pachysolen tannophilus</td>
<td>CBS4044</td>
<td>Yes</td>
<td>Ethanol (Maleszka et al, 1982b)</td>
<td>Ethanol, Acetate, 2,3-Butanediol, Biomass</td>
<td>0.33</td>
</tr>
<tr>
<td>Pichia pastoris</td>
<td>CBS704</td>
<td>Yes</td>
<td>Biomass, recombinant protein (Celik et al., 2008)</td>
<td>Ethanol, Acetate, Biomass</td>
<td>0.28</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>IBT446</td>
<td>Yes</td>
<td>Biomass, organic acids, lipids, $\alpha$-amylase (Papanikolaou and Aggelis, 2002; Rywina &amp; Rymowicz, 2010)</td>
<td>Mannitol, Erythritol, Citric acid, Biomass</td>
<td>0.33</td>
</tr>
<tr>
<td>Pichia stipitis</td>
<td>CBS5773</td>
<td>Yes</td>
<td>Ethanol produced from xylose/Glucose (Silva et al., 2010)</td>
<td>Biomass</td>
<td>NC*</td>
</tr>
<tr>
<td>Zygosaccharomyces rouxii</td>
<td>IBT565</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>IBT8</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The products produced by the organisms cultivated in previous studies, are dependent on the mode of cultivation and the medium used. For instance, it was shown that mannitol can be produced from glycerol in aqueous solution under aerobic condition without any other nutrient by the resting cells of *C. magnolia* (Khan et al., 2009). However, another study showed that *C. magnoliae* could only produce mannitol from fructose and sucrose but did not produce mannitol from glycerol (Baek et al., 2003). The production of arabitol from glycerol (Koganti et al., 2011) was not noticed in this study, probably because of differences in the medium composition. *S. pombe* did not grow on the glycerol agar plate in this study, whereas it has been shown that it can utilize glycerol in the presence of small amounts of ethanol and 1-propanol, which were recognized as inducers of glycerol metabolism. These results varied between studies. In this study, a defined medium was used for all submerged cultivations, in order to allow a relevant quantitative physiological comparison of results obtained under different cultivation conditions.

**3.2 Growth comparison of two ethanol producers on glycerol in shake flasks**

For the purpose of selecting one strain which could be optimized for ethanol production from glycerol, *P. pastoris* and *P. tannophius* were cultivated in defined medium on 5% (v/v) glycerol as the sole carbon source in shake flasks.

*P. pastoris* was cultured with 50ml, 100ml, 150ml and 200ml of medium in 250 ml shake flasks. This method allows a rough estimate of the influence of oxygen transfer in submerged cultivation. It was shown (Figure 2) that the lower the volume of medium in the flasks, the more biomass was accumulated and the faster glycerol was consumed. With regard to the ethanol production level, the cells in 150ml medium produced the highest level of ethanol (3.4 g/L and a yield of 0.13 g ethanol g⁻¹ glycerol), which indicated that the ethanol production level was likely to be related to oxygen availability.
Figure 2. Results from 250ml shake flask cultivations of *P. pastoris* on 5% (v/v) glycerol as the sole carbon source with 50ml, 100ml, 150ml and 200ml of medium showing (A) OD$_{450nm}$ (B) Glycerol consumption (g/L) (C) ethanol production (g/L) over 9 days of cultivation.
*P. tannophilus* was cultured with 100ml medium in 250ml shake flasks. It produced 22.7g/L ethanol with a yield of 0.36 g ethanol g\(^{-1}\) glycerol after 6 days cultivation (Figure 3). Under the same conditions, *P. pastoris* only accumulated 2.5 g/L ethanol with a yield of 0.1 g ethanol g\(^{-1}\) glycerol over 8 days of cultivation. *P. tannophilus* grew on glycerol with \(\mu_{\text{max}}\) 0.33 h\(^{-1}\) while *P. pastoris* grew on glycerol with \(\mu_{\text{max}}\) 0.28 h\(^{-1}\) (Table 1). By comparing the ethanol yield, production level and growth rate on glycerol, *P. tannophilus* showed more potential to be selected as the ethanol production host on glycerol. The physiology studies and characterization of the potential ethanol producer *P. tannophilus* was further investigated in fermentor, where all the cultivation conditions, such as pH, dissolved oxygen concentration, off-gas composition, temperature, stirrer speed and so on could be monitored and controlled during the whole cultivation process. The results obtained from these studies are presented in Chapter 3.

![Figure 3](image.png)

*Figure 3.* Results from 250ml shake flask cultivations of *P. tannophilus* and *P. pastoris* on 5% (v/v) glycerol as the sole carbon source with 100ml medium showing OD\(_{450\text{nm}}\), glycerol consumption (g/L) and ethanol production (g/L).
In addition, prior to further investigation in the controlled fermentor, cultivation of *P. tannophilus* on 2.5 % (v/v) glycerol were performed for confirmation of the previous shake flask results. It was shown that *P. tannophilus* could produce 6.5 g/L ethanol with a yield of 0.282 g ethanol g⁻¹ glycerol over 72 hours cultivation (Figure 4). The results from studies to optimize this process are presented in Chapter 3.

**Figure 4.** Results from 250ml shake flask cultivations of *P. tannophilus* on 2.5% (v/v) glycerol as the sole carbon source with 100ml medium showing OD₄₅₀nm, glycerol consumption (g/L) and ethanol production (g/L). Results represent the mean of triplicate experiments.
Reference


Chapter 3 Bioconversion of crude glycerol feedstocks into ethanol

by *Pachysolen tannophilus*

Xiaoying Liu, Peter Ruhdal Jensen, Mhairi Workman*

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Bioconversion of crude glycerol feedstocks into ethanol by Pachysolen tannophilus

Xiaoying Liu, Peter Ruhdal Jensen, Mhairi Workman*

Center for Systems Microbiology, Department of Systems Biology, Building 301, Matematiktorvet, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

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ABSTRACT

Glycerol, the by-product of biodiesel production, is considered as a waste by biodiesel producers. This study demonstrated the potential of utilising the glycerol surplus through conversion to ethanol by the yeast Pachysolen tannophilus (CBS4044). This study demonstrates a robust bioprocess which was not sensitive to the batch variability in crude glycerol dependent on raw materials used for biodiesel production. The oxygen transfer rate (OTR) was a key factor for ethanol production, with lower OTR having a positive effect on ethanol production. The highest ethanol production was 17.5 g/L on 5% (v/v) crude glycerol, corresponding to 56% of the theoretical yield. A staged batch process achieved 28.1 g/L ethanol, the maximum achieved so far for conversion of glycerol to ethanol in a microbial bioprocess. The fermentation physiology has been investigated as a means to designing a competitive bioethanol production process, potentially improving economics and reducing waste from industrial biodiesel production.

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1. Introduction

In recent years, increasing focus on renewable and sustainable energy has boosted the production of biodiesel from rapeseed oil, cooking oil and animal fats, especially in Europe. Glycerol, a by-product of biodiesel production, has seen a proportionally dramatic increase in production. As approximately 10% of the reaction volume in a given biodiesel production process ends up as glycerol (Johnson and Taconi, 2007), the market for glycerol has become saturated and it is now being considered as a waste by many biodiesel producers. Bioconversion of glycerol into liquid biofuels, green chemicals and bioenergy on the basis of fermentation processes can provide an efficient solution for sustainable management of glycerol, which can improve the economics of biodiesel industries (Lynd et al., 2005). Among the different types of biofuels for replacement of fossil fuels, bioethanol is currently most used, and bioethanol is the only alcohol produced in large scale fermentation processes. Presently, ethanol is primarily produced from sugarcane in Brazil, from corn starch in the USA and from sugar beets in Europe (da Silva et al., 2009). Glycerol is considered as an inexpensive feedstock for biotechnology industries, and thus the possibility for the production of sustainable biofuels from this substrate is attractive.

The composition of crude glycerol generated from biodiesel processes varies dependent on the oil feedstock used and the catalysts employed in the transesterification process. The common feedstocks are soybean (USA), rapeseed (Europe), sunflower, safflower, canola, and palm oils. In order to reduce the cost, waste cooking oil, animal fats, soapstocks and greases are also utilized (Canakci and Sanli, 2008). Oil from microalgae is also a competitive feedstock for biodiesel production (Pokoo-Aikins et al., 2010). Increasing demand for sustainable biofuels such as biodiesel ensures that glycerol will continue to be a potential substrate for further conversion into value-added products in the foreseeable future. This offers considerable scope for designing biorefineries based on glycerol and developing microbial cell factories for biological conversion of this substrate on large scale. The organisms employed to convert the crude glycerol should be robust enough to withstand the variability in glycerol stock, thus ensuring the applicability of the conversion process to the widest range of biodiesel producers.

It has been shown that a number of (typically anaerobic) bacteria are capable of growing on glycerol as the sole carbon and energy source. Glycerol can be converted to a wide range of biochemicals and biofuels such as ethanol, butanol, 1,3-propanediol, succinate, dihydroxyacetone, propionic acid and pigments (da Silva et al., 2009). The newly isolated bacterium, Kluvyera cryocrescens can produce up to 27 g/L ethanol from crude glycerol under microaerobic batch fermentation (Choi et al., 2011). Escherichia coli has been investigated to be an ethanol production platform on glycerol, with up to 10 g/L achievable by engineered E. coli growing on 22 g/L crude glycerol and with hydrogen and formate as byproducts under anaerobic condition (Shams Yazdani and Gonzalez, 2008). An engineered Klebsiella pneumoniae strain has been shown to achieve 25 g/L ethanol on crude glycerol (Oh et al., 2011). However, these processes require a controlled anaerobic environment, maintained through sparging with nitrogen.

* Corresponding author. Present address: Center for Microbial Biotechnology, Department of Systems Biology, Building 223, Saltofts Plads, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark. Tel.: +45 4525 2700; fax: +45 4588 4148. E-mail address: mwo@bio.dtu.dk (M. Workman).

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In order to find new hosts capable of producing value-added products from renewable substrates, it may be interesting to look to non-conventional hosts for the production of biofuels and biochemicals. Here, a wider substrate range can be naturally exploited, and more diverse product profiles are observed, without the need for genetic engineering or expensive operating requirements. It is known that several non-conventional yeasts can grow on glycerol and a number of these strains exhibit the ability to produce value-added products. For example, glycerol can be assimilated into organic acids and single cell oil by _Yarrowia lipolytica_ (Makri et al., 2010; Papanikolaou et al., 2002) and has been exploited as carbon source for producing biomass and recombinant proteins in _Pichia pastoris_ (Celik et al., 2008; Fieldhouse et al., 2009). For ethanol production from glycerol, only two genetically engineered yeasts have been reported which can convert glycerol into ethanol. The industrial workhorse _Saccharomyces cerevisiae_ has been genetically engineered to produce ethanol from glycerol, but only up to 4.4 g/L (Yu et al., 2010). The methylotrophic yeast _Hansenula polymorpha_ has been genetically engineered to produce ethanol from glycerol, and has been exploited as carbon source for producing biomass and recombinant proteins in _Yarrowia lipolytica_ (Makri et al., 2010; Papanikolaou et al., 2002) and has been engineered to improve ethanol production by expression of varied genes from bacteria, however after several rounds of genetic engineering, the production level achieved was only 3.1 g/L (Hong et al., 2010).

_Pachysolen tannophilus_ was the first yeast shown to be capable of fermenting xylose sugars to ethanol (Kurtzman, 1983) and the xylolose utilisation pathway has been extensively studied in this organism (Sathesh-Prabu and Murugesan, 2011; Slininger et al., 1987; Zhao et al., 2010). In a previous study, it was reported that _P. tannophilus_ could accumulate 4 g/L ethanol on glycerol under aerobic growth (Maleszka et al., 1982), however, the conditions for ethanol production were not precisely defined or controlled and the physiology during growth on glycerol has not been extensively studied in this organism. The possibility for studying the physiology of glycerol conversion to ethanol in this organism provides an interesting prospect for the future production of biofuels. In this study, therefore, the glycerol metabolism has been further investigated in this strain as a means of defining the parameters which affect ethanol production in controlled submerged cultivations. The results may be used to design an optimised production protocol for ethanol production at lab scale, which could be implemented and tested at larger scale. In addition, we aim to provide the basis for an investigation of glycerol metabolism in _P. tannophilus_, with a view to highlighting targets for further improvements of this strain as a cell factory through genetic engineering.

### 2. Methods

#### 2.1. Microorganism and culture medium

_P. tannophilus_ CBS4044 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was used throughout this study. The growth experiments were performed in defined medium, containing (per liter) 0.67 g yeast nitrogen base w/o amino acids (Difco, USA) and glycerol at % (v/v) as stated. The strain was maintained in 15% glycerol in frozen vials (–80°C).

#### 2.2. Substrate source

Pure glycerol (Sigma–Aldrich, USA) or crude glycerol from biodiesel production (MEROCO a.s., Leopoldov, Slovakia) was used as the sole carbon source in media used in this study. Three different batches of crude glycerol were obtained from MEROCO, based on different raw materials for biodiesel production. Batches 1 and 2 were based on rapeseed oil (from production in December 2008 and October 2009, respectively) and Batch 3 was based on 90% rapeseed oil and 10% cooking oil (from production in December 2009).

The crude glycerol feedstock contains up to 7% ash (including formic acid, citric acid, acetic acid, nitrate and chloride), 0.5% methanol, 10% water and 82 ± 5% glycerol.

#### 2.3. Submerged batch cultivations

Unless otherwise stated, experiments were carried out in fully instrumented and automatically controlled BIOSTAT® Q plus fermentors (Sartorius Stedim Biotech S.A., Germany). Temperature, stirrer speed, pH and dissolved oxygen tension (DOT) were constantly monitored and automatically controlled on-line. Carbon dioxide and oxygen concentrations in the off-gas were monitored by a Bruel and Kjær acoustic gas analyzer (Bruel and Kjaer, Nærum, Denmark). The working volume was 600 mL.

All cultivations were performed at 28 °C. All batch processes contained 100 µL/L silicone oil to control the foaming during fermentation. Duplicates of all batches were performed; results shown are mean values (and standard error) for two identical processes.

To study the effect of aeration rate on growth and ethanol production, cultivations were performed on 2% (v/v) glycerol, at pH 5 with a stirrer speed of 450 rpm with the following airflow (aeration) rates: 0.5 L/min (0.83 vvm), 0.2 L/min (0.33 vvm), 0.1 L/min (0.17 vvm), 0.05 L/min (0.083 vvm), and at 350 rpm with 0.05 L/min (0.083 vvm), and one process where the dissolved oxygen tension (DOT) controlled at 20%. To convert from flow rate (L/min) to aeration rate (vvm) the following conversion factor was used: 1 L/min = 1.7 vvm (the working volume for the fermentor was 0.6 L).

The oxygen transfer rate (OTR) was assumed to be equal to the oxygen uptake rate (OUR) under dissolved oxygen limited conditions. From a mass balance, the OUR in batch fermentations could be calculated according to:

\[
\text{OUR} = \frac{F_{\text{in}}^\text{O}_2 - F_{\text{out}}^\text{O}_2}{V}
\]

where _F_\text{in} and _F_\text{out} are the molar flow rates based on the O\text{2} concentration measured at the fermentor inlet and outlet, and _V_ is working volume in the fermentor (Garcia-Ochoa and Gomez, 2009).

The effect of pH on ethanol production was investigated in submerged batch cultivations on 2% (v/v) glycerol. Cultivations were performed at pH 3, 4, 5, 6 and 7 with airflow (aeration) at 0.05 L/min (0.083 vvm) and 450 rpm stirring. In all cases, pH was automatically controlled by addition of 2 M HCl or 2 M NaOH.

To determine how ethanol production was affected by different nitrogen sources, cultivations were performed in defined medium with (NH_4)_2SO_4 or NaNO_3 as the nitrogen source on 5% (v/v) glycerol in 1 L fermentors (Biostat, B. Braun Biotech International, Germany) at 150 rpm, 0.2 vvm, without pH control and 10 g succinic acid and 6 g NaOH (per liter) were supplemented as buffer.

The effect of three different batches of glycerol from biodiesel production at Meroco was investigated in batch cultivations at pH 6, 450 rpm and 0.1 L/min (0.17 vvm) on 2% (v/v) crude glycerol. The effect of different concentrations of crude glycerol on cell growth and ethanol production was tested at 2% (v/v), 5% (v/v), and 10% (v/v) using Batch 3 crude glycerol based on 90% rapeseed oil and 10% cooking oil, pH 5, 450 rpm and 0.05 L/min (0.083 vvm).

A staged batch fermentation was performed on 5% (v/v) of Batch 3 crude glycerol. At the end of batch phase where glycerol was exhausted, concentrated medium containing crude glycerol and YNB were added the fermentor to a final concentration of 5% (v/v).

#### 2.4. Ethanol tolerance studies

The cells were grown in defined medium on 2% (v/v) glycerol (Sigma–Aldrich, USA) in 250 mL Erlenmeyer flasks with 100 mL culture media with 0.5% (v/v), 1.7% (v/v), 2.6% (v/v), 3.4% (v/v),
4.2% (v/v), 5.2% (v/v) ethanol added at the beginning of fermentation to determine the ethanol tolerance by *P. tannophilus*.

### 2.5. Analytical methods

Growth was monitored by measuring OD$_{450}$nm with a Shimadzu UV mini-1240 spectrophotometer (Shimadzu, Japan). Samples were taken periodically from the fermentor, filtered through a 0.22 μm syringe filter and supernatants were preserved at -20°C for later HPLC analysis.

Cell dry weight (CDW) was measured by centrifuging 2 mL of culture at 8000 g for 20 min in a preweighed Eppendorf tube. The cells were washed twice with distilled water and then were dried in a 70°C oven until a constant weight. Cell dry weight was correlated with OD$_{450}$nm using a standard curve (1 OD$_{450}$ = 0.163 g/L cell dry weight, CDW).

Concentrations of the substrate glycerol and all the main products: ethanol, acetic acid, acetoin and 2,3-butanediol in supernatants were measured by HPLC using an Aminex87H column (Bio-Rad, USA). Separations were performed at 60°C, flow rate of 0.6 mL/min and 5 mM H$_2$SO$_4$ as mobile phase.

### 3. Results and discussion

#### 3.1. *P. tannophilus* conversion of glycerol

*P. tannophilus* has been previously shown to be capable of conversion of glycerol to small amounts of ethanol (Maleszka et al., 1982), and with increasing focus on glycerol as an abundant substrate; this process was selected for characterisation and optimisation. This organism is known to convert xylose to ethanol, the genetics and physiology of which have been studied in detail (Sathesh-Prabu and Murugesan, 2011; Slininger et al., 1987). The goal was to further investigate the possibilities for glycerol conversion with a view to developing this organism as a versatile cell factory for biorefineries, where substrate feed stocks may be based on plant biomass (containing xylose) or other waste streams (for example glycerol).

#### 3.2. Submerged cultivation

##### 3.2.1. Effect of aeration rate on ethanol production

Initial results in shaking flasks showed that *P. tannophilus* could grow on 2.5% (v/v) pure glycerol and produce 6.5 g/L ethanol: a yield of 0.282 g ethanol g$^{-1}$ glycerol (details not shown). Several studies have reported that aeration and stirrer speed have great influence on ethanol production on xylose with this organism (Fu and Peiris, 2008; Silva et al., 2011). In order to investigate the aeration and agitation effect on ethanol production on glycerol by *P. tannophilus*, six batch cultivations were performed with different combinations of aeration rates and stirrer speeds, where all other conditions were constant. The oxygen transfer rate (OTR), maximum specific growth rate, yield of biomass and ethanol and ethanol productivity were determined for each of the processes. The results are shown in Table 1. The time course for one of the processes, at 450 rpm and 0.083 vvm aeration showing cell dry weight (g/L) ( ), ethanol concentration (g/L) ( ), dissolved oxygen tension (%) ( ) and off-gas carbon dioxide concentration (v/v %) ( )

---

### Table 1

<table>
<thead>
<tr>
<th>Stirrer speed (rpm)</th>
<th>Airflow (L/min)</th>
<th>Oxygen transfer rate (g/L/h)</th>
<th>Max. growth rate (h$^{-1}$) 1st phase</th>
<th>Biomass yield (g/g) 2nd phase</th>
<th>Ethanol yield (g/g)</th>
<th>Ethanol produced (g/L)</th>
<th>Vol. Ethanol productivity (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>0.5 0.27</td>
<td>0.28 ± 0.00</td>
<td>0.38 ± 0.01</td>
<td>N</td>
<td>0.87 ± 0.13</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>0.2 0.23</td>
<td>0.27 ± 0.00</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>4.30 ± 0.62</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>0.1 0.08</td>
<td>0.30 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>5.25 ± 0.86</td>
<td>0.09 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>0.05b 0.04</td>
<td>0.29 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.27 ± 0.00</td>
<td>6.33 ± 0.15</td>
<td>0.08 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>0.05a 0.02</td>
<td>0.28 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>4.49 ± 0.45</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

N – Not calculated.
the point where DOT decreased to zero until all glycerol was consumed. This phase was considered to be microaerobic phase. Similar trends were observed for all processes, with the aerobic phase continuing until approximately 10 h after inoculation, typically without ethanol production in this phase. The off-gas CO2 trace in the microaerobic phase clearly demonstrates oxygen limitation in the culture, and was accompanied by a lower growth rate and ethanol accumulation.

The results showed that the maximum specific growth rate was not greatly influenced by the change in OTR in the range studied. For the set of processes operated at 450 rpm, the biomass yield on the second phase on glycerol decreased as OTR decreased, with a maximum of 0.18 g cells g\(^{-1}\) glycerol at 0.5 L/min airflow, while ethanol yield increased to a maximum of 0.27 g ethanol g\(^{-1}\) glycerol at 450 rpm and 0.05 L/min airflow.

One additional process was performed where the DOT was controlled at a stable value of 20% throughout the cultivation; the cells grew at maximum growth rate of 0.28 h\(^{-1}\) with a biomass yield of 0.51 g cells g\(^{-1}\) glycerol. Only 0.87 ± 0.13 g/L of ethanol was produced at the last 8 h fermentation.

The tendency for ethanol production from glycerol was that with increasing OTR the ethanol yield declines (Fig. 2A). It has been reported that the aeration rate is one of the most important parameters concerned with alcoholic fermentation in yeasts (Jeffries, 1983b; Visser et al., 1990). In the ethanol production phase in this study, the DOT was around 0% for all processes. Some studies have shown that the optimal DOT values for ethanol production under microaerobic condition are below 1% of saturation (Agbogbo and Coward-Kelly, 2008). The cells begin to accumulate ethanol under microaerobic conditions when their growth is oxygen limited. Otherwise the cells will grow at maximum growth rate. High OTR has negative effect on ethanol yield and production and determines the carbon flow distribution between cell growth and ethanol production.

From HPLC analysis results, at higher airflow rates (0.5 L/min, 0.2 L/min) the main products on glycerol were ethanol, acetic acid, and small amounts of acetoin and 2,3-butanediol. At lower aeration rates (0.1 L/min, 0.05 L/min), the products were mainly ethanol and acetic acid.

![Fig. 2.](image1) Ethanol yield from batch cultivations with *P. tannophilus* at different aeration and stirring rates on 2% (v/v) pure glycerol at pH 5. (B) Ethanol yields from batch cultivations with *P. tannophilus* at different pH on 2% (v/v) pure glycerol and at pH 6 on different batches of crude glycerol from biodiesel production. Note: 0.05a – 0.05 L/min, 350 rpm; 0.05b – 0.05 L/min, 450 rpm.

![Fig. 3.](image2) Time course for submerged batch cultivations of *P. tannophilus* on (A) 2% (v/v) pure glycerol and (B) 2% (v/v) Batch 3 crude glycerol at the optimal process conditions: pH 5, 450 rpm, and 0.083vvm aeration, showing glycerol concentration (g/L) (●), ethanol concentration (g/L) (■), and cell dry weight (g/L) (▲).
3.2.2. Effect of pH on ethanol production

Previous studies investigating the effect of pH on xylitol production in *P. tannophilus* have shown that the optimum to be between pH 6 and pH 7.5 (Converti et al., 1999). With the purpose of optimization ethanol production on glycerol, the effect of pH on ethanol production was investigated. Results are shown in Fig. 2B. pH 5 was found to be the optimal pH for ethanol yield and production on glycerol by *P. tannophilus*. The deviation from optimal pH affected the ethanol yield to varying degrees. Only very slow growth of the culture was observed at pH 7 on glycerol. These results were different from the value (pH 6) reported for the optimal growth and ethanol production on sorghum stove for this yeast (Sathesh-Prabu and Murugesan, 2011), however, the optimal value is likely to change with varied substrates, products, medium and strains utilized for different processes. The pH effects probably resulted from the different enzyme activity in the metabolic pathways for the specific process (Sathesh-Prabu and Murugesan, 2011).

3.3. Conversion of crude glycerol

3.3.1. Growth comparison on crude glycerol and pure glycerol

Due to a wide variety of feedstocks being utilized in biodiesel production, the composition of crude glycerol can vary due to residues and impurities being carried over to the by-product. Chemicals used in the transesterification process can also be present in the glycerol. These residues can inhibit the growth of micro-organisms and thus affect product formation (GLYFINERY project report, results not shown). The process for converting glycerol should be robust to the impurities and variability of crude glycerol offering a cost-effective solution for converting this waste. In order to determine the sensitivity of the strain *P. tannophilus* and the ethanol production process to impurities and variations in crude glycerol supply, three different batches of glycerol from a biodiesel producer were tested. The growth and ethanol yield varied only slightly compared to the values on pure glycerol at pH 6 and no great difference was observed on the three different crude glycerol batches (Fig. 2B).

Previous results (Table 1) showed that the optimal conditions for batch ethanol production on pure glycerol were pH 5, 0.05 L/min airflow (0.083vvm), and 28 °C. Ethanol production using these conditions and the third batch of crude glycerol from rapeseed oil and cooking oil, which is likely to contain more impurities and residues, was almost the same as on the pure glycerol (Fig. 3A and B). The ethanol yield on crude glycerol was 0.26 g ethanol g⁻¹ glycerol and on pure glycerol was 0.27 g ethanol g⁻¹ glycerol. The ethanol production was around 6.3 g/L for both processes. Therefore, from the results we can conclude that the impurities (ash, methanol, salts, etc.) and variability of crude glycerol have no negative effect on the growth and ethanol production of *P. tannophilus*. The *P. tannophilus* process is thus robust and can be adapted for ethanol production from different sources of crude glycerol feedstocks from biodiesel production. This means that for large scale production of ethanol, it would not be necessary to purify the crude glycerol which will greatly reduce the production cost.

<table>
<thead>
<tr>
<th>Glycerol source</th>
<th>pH</th>
<th>Airflow (L/min)</th>
<th>Glycerol conc. (v/v%)</th>
<th>Glycerol utilized (g/L)</th>
<th>Ethanol produced (g/L)</th>
<th>Ethanol yield (g/g)</th>
<th>Dry cell weight (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure</td>
<td>5</td>
<td>0.05</td>
<td>2</td>
<td>24.2 ± 1.41</td>
<td>6.33 ± 0.15</td>
<td>0.27 ± 0.00</td>
<td>4.67 ± 0.26</td>
</tr>
<tr>
<td>Batch 3</td>
<td>5</td>
<td>0.05</td>
<td>2</td>
<td>26.1 ± 0.71</td>
<td>6.28 ± 0.15</td>
<td>0.26 ± 0.02</td>
<td>4.40 ± 0.02</td>
</tr>
<tr>
<td>Batch 3</td>
<td>5</td>
<td>0.05</td>
<td>5</td>
<td>62.9 ± 0.95</td>
<td>17.5 ± 0.88</td>
<td>0.28 ± 0.03</td>
<td>5.90 ± 0.11</td>
</tr>
<tr>
<td>Batch 3</td>
<td>5</td>
<td>0.05</td>
<td>10</td>
<td>113.8 ± 1.02</td>
<td>17.5 ± 1.15</td>
<td>0.16 ± 0.04</td>
<td>5.40 ± 0.03</td>
</tr>
</tbody>
</table>

Note: Glycerol conc. 1 (v/v%) = 1.261 g/cm³ = 12.61 g/L.

Fig. 4. Time course of the batch fermentation with *P. tannophilus* on 5% (v/v) pure glycerol with different nitrogen sources (A) NO₃⁻ (B) NH₄⁺, showing glycerol concentration (g/L) (■), ethanol concentration (g/L) (▼), and cell dry weight (g/L) (●).
3.3.2. The effect of different concentrations of crude glycerol on ethanol yield and production

With the aim to investigate the elevated substrate concentration effect on ethanol production and to enhance the ethanol production, the cells were cultivated on 2% (v/v), 5% (v/v) and 10% (v/v) crude glycerol. Ethanol production levels of 17.5 g/L were achieved both on 5% (v/v) and 10% (v/v) crude glycerol, however, growth was much slower as the concentration of glycerol was increased above 5% (v/v). The ethanol yield on 5% (v/v) crude glycerol was 0.28 g/g, slightly higher than the yield on 2% (v/v) crude glycerol (0.26 g/g). However, the ethanol production on 10% (v/v) crude glycerol represents a yield of only 0.12 g/g. The results are summarised in Table 2.

3.4. Effect of different nitrogen sources on ethanol production

*P. tannophilus* was cultivated on NaNO₃ and (NH₄)₂SO₄ to investigate the effect of nitrogen source on ethanol production. The results are shown in Fig. 4. Cells grown on NO₃⁻/C₀³ had higher biomass than NO₃⁺ grown cells. Cells grown on NO₃⁻ assimilated glycerol faster than NO₃⁺ grown cells. The final biomass yield with NO₃⁻ grown cells was 62% higher than NO₃⁺ grown cells. The specific rate of ethanol production by NO₃⁻ was almost the same as the rate obtained with NO₃⁺. The yield of ethanol in the presence of NO₃⁻ was 44% higher than NO₃⁺ as nitrogen source. Results suggest that NO₃⁻ stimulates higher carbon flow to ethanol but a lower flow to biomass. However, NO₃⁺ accelerates the rate of ethanol production. It is in accordance with previous research that NO₃⁻ stimulates the rate ethanol production from xylose under aerobic condition by *P. tannophilus* (Jeffries, 1983a).

3.5. Ethanol tolerance on glycerol

There have been some previous reports regarding ethanol tolerance of *P. tannophilus* on xylose (Zhao et al., 2010). However, as with pH, the ethanol tolerance is likely to change dependent on the carbon source and was thus investigated during growth on glycerol. Small scale batch cultivations were performed in shake flasks with

![Graph](image1.png)

Fig. 5. Shake flask cultivations of *P. tannophilus* on 2% (v/v) glycerol with different added ethanol concentrations, showing (A) cell dry weight (g/L) over 72 h of cultivation and (B) maximum specific growth rates.

![Graph](image2.png)

Fig. 6. Time course of staged-batch fermentation with *P. tannophilus* on 5% (v/v) Batch 3 crude glycerol, concentrated medium was added to the fermentor after the glycerol was depleted in 2 staged additions to give a final concentration of 5% (v/v). The figure shows glycerol concentration (g/L) (●), ethanol concentration (g/L) (◆), and cell dry weight (g/L) (○). I, II and III indicates the three staged-batch phases used for calculation of quantitative fermentation data.
varying amounts (0–5.1%) of ethanol being added to the cultures at the point of inoculation. As is shown in Fig. 5A, biomass increased more slowly as the ethanol concentration increased. When the organism was cultivated without ethanol addition, 3.11 g/L biomass was achieved after 72 h cultivation, where only 0.097 g/L organism was cultivated without ethanol addition, 3.11 g/L more slowly as the ethanol concentration increased. Results show no growth occurred on glycerol with 5.2% (41 g L−1) ethanol in the medium, which is consistent with the study where growth of *P. tannophilus* ceased when 40 g L−1 ethanol was included in xylose medium (Zhao et al., 2010). The ethanol tolerance for *P. tannophilus* could be improved in further studies for example through UV mutagenesis and selection for more ethanol tolerant strains (Watanabe et al., 2011).

### 3.6. Staged-batch fermentation on crude glycerol

In order to further improve glycerol conversion to ethanol and increase yields in the fermentation processes, a staged-batch fermentation was performed (Fig. 6). In this process, medium was added to the fermenter after the glycerol was depleted in the initial batch phase, and a second batch addition was made again on glycerol depletion. After 2 stages of batch addition of medium, an ethanol production of 28.1 g/L was achieved on crude glycerol (Fig. 6, Table 3). This value is the highest reported so far in the literature for microbial conversion of glycerol to ethanol. However, as Table 3 shows the productivity was still very low compared to bacterial processes for ethanol production from glycerol. In order to further optimise conditions with a view to industrial scale production, further improvements in ethanol production could be achieved by designing a fed-batch process where feeding follows growth rate and substrate utilisation. Improving ethanol tolerance in the strain (e.g. by adaptive evolution) would be a further requirement prior to larger scale development of this process, in order to achieve the higher ethanol concentrations required for the currently used recovery methods. However, this study represents considerable steps towards developing a process for conversion of crude glycerol to ethanol, which could improve the economic and environmental burden for future biofuel production.

### 4. Conclusions

*P. tannophilus* is known for its ability to convert xylose, a component of plant biomass, to ethanol. This investigation shows that crude glycerol, the biodiesel by-product, can also be utilized as a potential low cost substrate by *P. tannophilus*. The organism was not sensitive to variations in crude glycerol batches from biodiesel production based on different oil feedstocks. An ethanol production of 28.1 g/L was achieved in a staged-batch process, the highest value for glycerol conversion to ethanol reported to date. *P. tannophilus* can, therefore, be considered as a potential versatile host for biorefineries based on a variety of feedstocks.

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Chapter 4 Adaptive evolution studies for improving ethanol tolerance of *Pachysolen tannophilus* CBS4044

Abstract

*P. tannophilus* CBS4044 can be considered as a potentially versatile host for biorefineries as it can utilize both glycerol and pentoses (mainly xylose) in bioconversions. The studies in this thesis have focused on the production of ethanol. Ethanol toxicity has been found to be a limiting factor in ethanol production and it is therefore hypothesized that fermentation performance could be improved in more ethanol tolerant strains. In the present study, ethanol-tolerant mutants were obtained by adaptive evolution by increasing concentrations of ethanol in the growth medium with glycerol as the sole carbon source. Two adapted cultures, Evo4 and Evo2 of *P. tannophilus* CBS4044 were obtained by sequentially transferring 20 and 43 times respectively. Adapted mutant E243 was isolated by streaking out Evo2 culture on YPD plates, and selecting a single colony. The ethanol tolerance and maximum specific growth rate ($\mu_{\text{max}}$) of Evo4, Evo2 and E243, were improved at higher concentrations of ethanol (> 4 % v/v) compared to the wild type. However, the $\mu_{\text{max}}$ and the initial glycerol consumption rate of both adapted cultures and mutant when ethanol was not present in the medium were decreased compared to the wild type strain. Additionally, the ethanol production from glycerol when ethanol was absent was decreased with the mutant and adapted cultures. E243 only produced 4.5 g/L ethanol while the wild type strain could produce 5.8 g/L ethanol.

**Keywords:** *Pachysolen tannophilus*; ethanol tolerance; adaptive evolution; mutant.
1. Introduction

The yeast *Pachysolen tannophilus* has been investigated for ethanol production in previous studies due to its ability to convert xylose and glycerol into ethanol (Liu et al., 2012; Slininger et al., 1987). Any potential cell factory to be employed in a biorefinery should be robust and tolerant to high concentrations of substrate and product. *P. tannophilus* was shown not to be sensitive to variations in the crude glycerol batches supplied from one biodiesel producer and can grow on relatively high concentrations of glycerol (up to 20% v/v). However, the strain was sensitive to higher ethanol concentrations with the maximum specific growth rate dropping to 0.058 h⁻¹ when 4.2% v/v ethanol was present in the medium, compared to 0.28 h⁻¹ without ethanol present. The accumulation of ethanol during cultivations causes stress to yeast cells, leading to a decrease in cell growth and reduced the production of desired products (Dinh et al., 2008). The low ethanol tolerance of *P. tannophilus* might be the factor which has inhibited further improvement in ethanol production in the work presented in this thesis. It is believed that improving ethanol tolerance will lead to improved ethanol productivities and yields (van Voorst et al., 2006).

Using genetic engineering to improve the ethanol tolerance of yeast is limited by the lack of knowledge and the complexity of the ethanol stress-related mechanisms that inhibit cell performance (Hu et al., 2007). The approaches for generating ethanol-tolerant yeast mutants could be mutagenic or non-mutagenic adaptive evolution with ethanol stress as a selection pressure (Stanley et al., 2010). Isolation of stable mutants with desired characteristics from random mutagenesis could be very difficult, since the isolation procedure is time consuming and mutants always contain undesired characteristics in addition to the desired traits. An effective means of obtaining ethanol-tolerant yeast strains is by exposing yeast cells to a stepwise increase in the level of ethanol stress (Dinh et al., 2008). Adaptive evolution works on the principle that populations of cells adapt to their environment over time by natural selection. Previous investigations about adaptive evolution for improved ethanol tolerance in yeast (Dinh et al., 2008) showed that the ethanol-adapted cultures of *S. cerevisiae* had slightly higher specific growth rates at higher concentrations of ethanol. By observing the morphology and analyzing the fatty acid compositions of the cell membranes it was revealed that the cells from the ethanol adapted cultures were larger and had a lower proportion of C₁₆₀ fatty acids in their cytoplasmic membrane (Dinh et al., 2008). Stanley et al. (2010)
generated ethanol tolerant mutants by chemical mutagenesis and adaptive evolution with ethanol stress as a selective pressure. It was concluded that the mutants from both approaches had increased growth rates when the cells were cultivated in sub-lethal ethanol concentrations. It was suggested that the increased ethanol tolerance of the mutants might be due to their elevated glycerol production rates (Stanley et al., 2010).

In this study, adaptive evolution with ethanol stress as the selection pressure was used as a strategy to obtain the ethanol tolerant-mutants of *P. tannophilus*. The cells were cultivated in medium without ethanol and then transferred to fresh medium containing a low ethanol concentration followed by repetitive cultivation. After the cells adapted to the low ethanol concentration, they were inoculated into medium containing a higher ethanol concentration followed by repetitive cultivation. Adaptive evolution was used as an approach for increasing ethanol tolerance of *P. tannophilus* CBS4044, potentially leading to improvement of fermentation performance, high ethanol yield and productivity.

2. Materials and Methods

2.1 Microorganism and culture medium

*Pachysolen tannophilus* CBS4044 was used in this study. Both the adapted evolution and growth experiments with the mutants were performed in defined medium, containing (per liter) 0.67g yeast nitrogen base w/o amino acids (Difco, USA), 10g succinic acid and 6g NaOH as a buffer system, and glycerol at 2% (v/v) as the sole carbon source.

2.2 Adaptive evolution under ethanol stress

All the cultivations were performed in test tubes with a working volume of 8ml defined medium at 28°C under aerobic conditions. Adaptive evolution of the yeast was performed by sequentially transferring and growing cells in defined media containing incrementally increased concentrations of ethanol step by step (0%, 2%, 4%, 4.5%, 5%, 5.5%, 6% v/v). The wild type strain of *P. tannophilus* was inoculated without ethanol in the medium and cultivated overnight. Then the cells were transferred into a new medium with 2 % (v/v) ethanol present with 2.5 % (v/v) inoculating rate. When the cells grew up after three days (OD$_{450nm}$ around 10), the cells were transferred into medium with 4 % (v/v) ethanol present with 2.5 % (v/v) inoculating rate. To obtain a strain with improved ethanol tolerance on
glycerol, the strain was repeatedly transferred into fresh minimal medium containing the same ethanol concentration. After that, the cells were transferred to medium containing a higher ethanol concentration followed by repetitive cultivations. As a back-up cells continued to be cultivated at the lower ethanol concentration, while sequential transfer to higher ethanol concentrations was performed. As a standard, achieving an OD$_{450}$nm of 10 was required after 3 days of growth at the higher ethanol concentration. In some cases, growth took longer. If the cells were not grown after one week in the medium with the higher concentration of ethanol, further transfers were performed at the lower concentration before trying again to increase the ethanol concentration. Four independent test tube parallels were performed for adaptive evolution from 0% to 6% v/v of ethanol concentration, the respective culture series obtained were named as Evo1, Evo2, Evo3, and Evo4.

2.3 Ethanol tolerance studies

The cells were grown at 28°C in defined medium on 2% (v/v) glycerol in 250ml Erlenmeyer flasks with 100ml culture media with 0% (v/v), 2% (v/v), 4% (v/v), 5% (v/v), 6% (v/v) ethanol added at the beginning of cultivation to characterize the growth profile and determine the ethanol tolerance of adaptive cultures and mutant. All the growth experiments last for 72 hours with 100ml medium in the flasks.

2.4 Analytical methods

Growth was monitored by measuring OD$_{450}$nm with a Shimadzu UV mini-1240 spectrophotometer (Shimadzu, Japan). Samples were taken periodically from the fermentor, filtered through a 0.22 µm syringe filter and supernatants were preserved at -20°C for later HPLC analysis. Concentrations of the substrate glycerol and all the main products: ethanol, acetic acid, acetoin and 2, 3-butanediol in supernatants were measured by HPLC using an Aminex87H column (Bio-Rad, USA). Separations were performed at 60°C, flow rate of 0.6ml/min and 5mM H$_2$SO$_4$ as mobile phase.

3. Results and discussion

3.1. Adaptive evolution to generate ethanol-tolerant mutants

In order to enhance the ethanol tolerance of *P. tannophilus* CBS 4044, an adaptive evolution approach was used to generate ethanol-tolerant yeast mutants. The strain was repeatedly
transferred (up to 43 times) into fresh minimal medium with incremental increases in ethanol concentration as shown in Figure 1. Variants with an advantageous phenotype are likely to emerge when a microorganism is growing under a strong pressure of selection (for example high temperature, uncommon substrate) (Dykhuizen, 1990). Along the adaptive evolution, the cultures obtained after 20 transfers (Evo4) and 43 transfers (Evo2) were analyzed with respect to their ethanol tolerance, maximum growth rate, glycerol consumption and ethanol production in submerged batch cultivation in shake flasks. The “adapted strain” used for the ethanol tolerance analysis was isolated from the Evo2 culture by single colony selection from colonies grown on YPD agar plate inoculated from the last cultivation (43 transfer no.). The strain was named as E243.

Figure 1. Adapted evolution was performed for enhancing ethanol tolerance with Pachysolen tannophilus CBS4044. 4 independent lineages of P. tannophilus were transferred in defined medium with increasing concentration of ethanol for 43 transfers. No ethanol was added to the defined medium in the first transfer.

3.2. Growth characterization of adapted cultures Evo4, Evo2 and mutant E243

Figure 2 shows the biomass concentration after 72 hours cultivation of the 4 strains in the presence of ethanol up to 6% (v/v). Higher biomass concentrations were obtained for the adapted cultures and mutant in the presence of higher concentration of ethanol. For example,
at 5.2 % (v/v) ethanol the wild type strain showed almost no growth, while Evo4 produced 0.17g/L biomass at 5 % (v/v) ethanol and Evo2 produced 0.95g/L biomass at 4.9 % (v/v) ethanol present. The mutant strain E243 produced 0.82 g/L biomass at 5.2 % (v/v) ethanol. Figure 3 shows the maximum specific growth rates for each of the strains at each of the ethanol concentrations added to the growth medium. At low ethanol concentrations the adapted cultures and mutant performed generally poorer than the wild type strain. At higher concentrations, however, the adapted cultures and mutant strain performed better compared to the wild type.

Figure 2. Comparison of biomass concentrations obtained in shake flask cultivations with P.tannophilus CBS4044 wild type strain, adapted cultures Evo4, Evo2 and mutant E243 after 72 hours cultivation in defined medium containing 2% (v/v) glycerol and a range of ethanol concentrations at 28°C. Results represent the means of duplicate experiments.
Figure 3. $\mu_{\text{max}}$ comparison of *P.tannophilus* CBS4044 wild type strain, adapted cultures Evo4, Evo2 and mutant E$_2$43 in defined medium containing 2% (v/v) glycerol and a range of ethanol concentrations at 28°C in agitated flasks. Results represent the means of duplicate experiments.

With the continuation of adaptive evolution, the ethanol tolerance of *P.tannophilus* was indeed improved when higher concentrations of ethanol were present in the medium. However, the ethanol yield on glycerol and ethanol production levels were decreased (Figure 4B). When the organism was cultivated without ethanol addition, the yield of ethanol from glycerol was around 0.25 g/g, while the yield for Evo4 was 0.23 g/g, Evo2 was 0.19 g/g and E$_2$43 was also 0.19 g/g. The ethanol production level for wild type strains was 5.8 g/L, while for Evo4 it was 5.5 g/L, Evo2 was 4.3 g/L and E$_2$43 was 4.5 g/L. It was noticed that for the wild type strain and Evo4 at 24 hours after inoculation, there was some amount of ethanol produced in the medium. However, Evo2 culture and E$_2$43 mutant started to produce ethanol after 24 hours. For glycerol consumption (Figure 4A), it seems that the mutants consume glycerol at lower rate at the initial 24 hours after inoculation compared to the wild type strain. Evo2, E$_2$43 and wild type strain consumed all the glycerol at 72h cultivations. For the biomass, the adaptive cultures Evo2, Evo4 and mutant E$_2$43 accumulated biomass at slower rate compared to the wild type strain at the beginning of fermentation, however at 72 hours cultivation they will end up with almost the same level of biomass (Figure 4C). The mutant E$_2$43 accumulates a slightly higher biomass compared to wild type and adaptive cultures Evo4 and Evo2.
Figure 4. A, Glycerol consumption; B, Ethanol production comparison; C, Cell dry weight (CDW) of *P. tannophilus* CBS4044 wild type strain, adapted cultures Evo4 Evo2 and mutant E243 in defined medium containing 2% (v/v) glycerol as the sole carbon source at 28°C in agitated flasks. Results represent the means of duplicate experiments.
4. Conclusion

In this study, the adaptive evolution of *P. tannophilus* towards improved ethanol tolerance was investigated by exposing the cells to a stepwise increase of ethanol concentration in the medium with repetitive cultivations. Using this adaptive evolution strategy two adapted cultures (Evo4 and Evo2) and one mutant (E243) were investigated. Increases in biomass concentration after 72 hours of growth were seen when the strains were cultivated in shake flasks in the presence of increased concentrations of ethanol. The lethal ethanol concentration for the wild type strain was 5.2% (v/v), however, the mutant and adapted cultures were able to grow at this concentration.

The present study mainly focuses on characterization of the adapted cultures and mutant of *P. tannophilus*. It has been shown in other studies that the lipids in the cell membrane and ergosterol, which is one of the components of cell surface, are responsible for ethanol stress tolerance in *S. cerevisiae* and other yeasts (Aguilera et al., 2006; Alexandre et al., 1994; Thomas et al., 1978). Some studies also showed that the addition of ethanol causes a cell-cycle delay and results in an increase in cell size (Dinh et al., 2008; Kubota et al., 2004). Due to the time limitation of the PhD study period, the mechanism of ethanol tolerance has not been investigated further in with *P. tannophilus*. It would be interesting to look into the lipid composition, cell size and perform genome and transcriptional analysis in order to unveil the mechanism behind the performance of ethanol-tolerant mutants of *P. tannophilus*.

References


Chapter 5 Draft genome sequence of the yeast *Pachysolen tannophilus* CBS 4044

Xiaoying Liu, Rolf Sommer Kaas, Peter Ruhdal Jensen, Mhairi Workman*

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Abstract

A draft genome sequence of the non-conventional yeast *P. tannophilus* CBS 4044 is presented. The organism has potential to be developed as a cell factory for biorefineries, due to its ability to utilize agricultural and industrial wastes. The sequenced genome size was 12,238,196 bp, consisting of 34 scaffolds. 4,463 genes were annotated with function from 5,346 predicted open reading frames. The genome sequence data provides a better understanding of the genetic background of this strain and will facilitate improved industrial applications of *P. tannophilus*.

**Keywords:** *Pachysolen tannophilus*; genome; annotation; chromosome.
The yeast, *Pachysolen tannophilus* was first isolated from wood extracts used in leather tanning by Boidin and Adzet in 1957 (Kurtzman, 1983). This yeast has gained considerable attention due to its ability to ferment D-xylose, the main component in hemicellulose plant residues (Fu & Peiris, 2008; Schneider et al., 1981; Slininger et al., 1987). A number of studies have investigated fuel ethanol production from D-xylose by *P. tannophilus* (Sathesh-Prabu & Murugesan, 2011; Slininger et al., 1987; Zhao et al., 2010). Additionally, it has been reported that *P. tannophilus* could ferment the common sugars (glucose, mannose and galactose) occurring in hemicellulose hydrolysate mixtures, with the exception of L-arabinose (Slininger et al., 1987). It has been shown recently that *P. tannophilus* could produce ethanol from glycerol, indicating the potential application of this organism in converting other waste streams (Liu et al., 2012). The whole-genome sequencing of *P. tannophilus* was performed in this study to provide genetic information on this yeast. This is a necessary step towards genetic engineering of the strain and its potential further development as a cell factory for industrial applications.

The genome of *Pachysolen tannophilus* CBS4044 was sequenced by a whole-genome shotgun sequencing strategy with an Illumina Genome Analyser at Beijing Genomics Institute (BGI, Shenzhen, China). Raw data of short reads was assembled into 279 contigs and the contigs were ordered into 34 scaffolds (>2kb) with N50 size of 1.1Mb by using the SOAPdenovo package (Li et al., 2010) which is self-developed by BGI. Augustus v2.5 software (Stanke et al., 2004), which was trained for predicting genes in *Debaryomyces hansenii*, was utilized to identify protein-coding genes in the genome and the putative amino acid sequences were used for subsequent gene functional annotation analysis. The functional annotation was accomplished by BlastP analysis (E-value < 1x10⁻⁵) of protein sequences in the databases (COG, KEGG) and the best hit was selected. Pulsed field gel electrophoresis (PFGE) (Maringele & Lydall, 2006) was performed to predict the chromosome numbers and approximate sizes in *P. tannophilus*. The program for PFGE was 48h at 3V/cm with a 500s switch time at an included angle of 106°, 0.5xTBE on 0.75% LMP (Low melting point) Agarose and at 14°C.

The total length of the sequenced genome was 12,238,196 bp (without N) with a GC content of 29.82%. Total 1970.8 Mb raw data was sequenced representing around 145 fold coverage of the *P. tannophilus* genome. 5,346 protein-coding genes (CDSs) set were predicted, with 4,463 (83.5%) genes annotated with function.
Four coding sequences of *P. tannophilus* were retrieved from Genbank to compare with the annotated CDS sequences in the sequenced genome. There were 2 bp differences among 4803bp length. Furthermore, 13 full-length coding sequences were PCR amplified and resequenced to estimate the accuracy of the genome. The sequencing results showed that the genome sequencing results were very accurate. The total resequenced lengths were 21,111bp.

Based on PFGE results, six chromosomal bands were separated with two of the bands probably migrating as doublets. The sizes of chromosomal bands were estimated to be around $2.9\pm0.05\text{Mb}$, $2.1\pm0.04\text{Mb}$, $1.9\pm0.05\text{Mb}$, $1.6\pm0.08\text{Mb}$ (doublet), $1.3\pm0.07\text{Mb}$ (doublet), $0.98\pm0.02\text{Mb}$ based on comparison with the yeast marker *Hansenula wingei*. The estimated genome size of *P. tannophilus* (CBS4044) was approximately $13.6\pm0.4\text{Mb}$ with an estimated 8 chromosomes in *P. tannophilus*.

*P. tannophilus* was characterized during ethanol production from the uncommon carbon sources xylose and glycerol. It produced ethanol, acetic acid, acetoin and 2, 3-butanediol on glycerol as the sole carbon source (Liu et al., 2012). The genome analysis revealed that all the genes coding for enzymes involved in glycolysis, xylose degradation, glycerol assimilation and formation, products synthesis were present in the genome sequences.

**Nucleotide sequence accession number.** The draft genome sequences of *P. tannophilus* CBS 4044 were deposited in GenBank.

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Chapter 6 Development of a transformation and selection system for \textit{Pachysolen tannophilus}

Abstract

\textit{Pachysolen tannophilus} has been studied in this PhD project as a host for production of ethanol from glycerol. Much time has been devoted to performing submerged cultivations with this strain at a variety of scales and under different cultivation conditions with a view to optimizing the ethanol production process. Certain progress has been made by this process optimization strategy. However, in order to further improve the organism as a cell factory, genetic engineering could be a useful tool. Specifically, for the purposes of this study, it would be advantageous to investigate the glycerol consumption and ethanol synthesis pathways. A first step for genetic engineering is the development of a suitable transformation and selection system in \textit{P. tannophilus}, as such genetic tools do not exist for this strain at present. In this chapter, the creation and selection of antibiotic (Geneticin) resistance mutants and uracil auxotrophic mutants is described. Unfortunately, the strategies and methods employed here did not yield successful results. Much more effort and knowledge about the genetic background of \textit{P. tannophilus} is needed in order to develop a successful transformation procedure.

\textbf{Keywords:} \textit{Pachysolen tannophilus}; G418; \textit{URA3}; transformation.
1. Introduction

*P. tannophilus* was considered in this study as a potential versatile host for biorefineries based on its ability to convert the carbon sources xylose and glycerol to ethanol. While a lot of research has been performed on physiological characterization of the strain, very little is known about the genetic background of *P. tannophilus*. With the aim of further improving ethanol production, it is necessary to better understand the ethanol synthesis pathway in *P. tannophilus* and to design tools for subsequent genetic engineering of this yeast species. The whole-genome sequencing of *P. tannophilus* aided a better understanding of the genetic background, which opens the possibility of genetically engineering the strain.

In order to work towards metabolic engineering of the yeast strain, a first step is development of an efficient transformation and selection system in *P. tannophilus*. Such genetic tools do not exist for this strain at present. However, it was reported once that that the lithium treated hexose negative mutants of the yeast *P. tannophilus* survived in the presence of D-glucose or D-fructose by the introduction of a *S. cerevisiae* HXK2 gene (Wedlock & Thornton, 1989). It indicates the possibility of transformation of this strain.

With regard to development of a transformation selection system, antibiotic resistance is a simple and powerful tool. The only requirement is that the wide type cells should be killed or prevented from growing by the antibiotic. The fact that the transformed cells harbouring the antibiotic resistance gene grow in the presence of antibiotic could be utilized as the selection principle. It has been shown that the Kan$^f$ gene from the bacterial transposable element Tn903 confers resistance to the antibiotic geneticin (G418) in yeast (Jimenez & Davies, 1980). Many prokaryotes and eukaryotes including plants and yeasts are sensitive to G418. Jimenez and Davies (1980) demonstrated that *Saccharomyces cerevisiae* expressing the Kan$^f$ gene of Tn903 (Tn601) become resistant to G418. The aminoglycoside phosphotransferase encoded by Kan$^r$ inactivates the antibiotic G418 and renders transformed cells resistant to the G418 (Sreekrishna et al., 1984). The similar antibiotic resistance selection has been successfully employed in many different types of yeasts and fungi (Sreekrishna et al., 1984; Suárez & Eslava, 1988). In developing the G418 selection procedure, we have constructed the recombinant shuttle vectors containing the KanMX module, which are hybrids of the coding sequence of the kan$^f$ gene of transposon Tn903 and transcriptional and translational control sequences from the TEF gene of the filamentous fungus *Ashbya gossypii* (Steiner &
Philipppsen, 1994). The KanMX module with long flanking sequences homologous to *P. tannophilus GUT1* was also constructed in order to obtain G418-resistant *P. tannophilus*.

In addition to the antibiotic selection system, most transformation systems are based on auxotrophic markers. The most commonly used method is based on the orotidine-5-phosphate decarboxylase (URA3) gene which is widely used in yeast and fungi (Boeke et al., 1984; François et al., 2004; Goosen et al., 1987). URA3 is a gene that encodes orotidine 5-phosphate decarboxylase (ODCase), which is an enzyme that catalyzes the last step in the synthesis of pyrimidine ribonucleotides in yeast RNA (Rose et al., 1984). The *URA3* gene works as a counter selectable marker and allows positive and negative selections. The yeast cells which cannot grow lack ODCase activity (ura3) unless uracil or uridine is added into the medium. After the *URA3* gene was reintroduced into cells, the auxotrophic cells can start to grow (positive selection). In contrast, if 5-Fluoroorotic acid (5-FOA) is added into the media, the cells could convert 5-FOA into the toxic compound 5-fluorouracil leading to death (negative selection). Since *URA3* allows two different selection systems, it is one of the most important genetic markers in yeast genetic modification. Here the methods and results for making *URA3* selection marker in *P. tannophilus* are also described.

2. Materials and Methods

2.1 Strains and plasmids

*Pachysolen tannophilus* CBS4044 was used throughout this study. *S. cerevisiae* CEN.PK 113-5D (*MATa MAL2-8c SUC2 ura3-52*) was used for the control experiments of the transformation procedure. Plasmid PUG6 (Güldener et al., 1996) harbouring the KanMX module (1473bp) was used as a template for amplification of the G418 resistance cassette. A centromeric plasmid pCM188 (Garí et al., 1997) was used for constructing the G418 resistance vector used for transformation.

2.2 Medium and culture conditions

*P. tannophilus* was cultivated on yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) and synthetic dextrose medium (SD) (0.67% yeast nitrogen base without amino acid and 2% glucose) media supplemented with 0.03% Uracil and 0.15% 5-FOA were used for selection of mutants. Different concentrations of G418 were
supplemented in YPD agar medium for selection of G418 resistance transformants. For the preparation of solid plates, 2% agar was added.

2.3 Sensitivity of *P. tannophilus* to G418 and 5-FOA

The sensitivity of *P. tannophilus* with antibiotic G418 was determined by plating cell suspensions (approximately 1x 10^6 cells/plate, 100µl OD_450=1) on YPD containing various concentrations of G418 (0, 25, 50, 75, 100 µg/ml) and plates were incubated at 30°C for 2-3 days. The sensitivity of *P. tannophilus* with 5-FOA was performed by plating cell suspensions (approximately 1x 10^7 cells/plate, 1 ml OD_450=1) on SD media containing various concentrations of 5-FOA (0, 0.5, 1, 1.5, 2 mg/ml) and plates were incubated at 30°C for 5-10 days.

2.4 Construction of vectors for selection of G418 resistance transformants

In this study, two vectors carrying the KanMX cassette were constructed. The first vector was constructed by combination of the pCM188 plasmid and KanMX cassette. The pCM188 plasmid was digested with BamHI/SalI enzymes (Fermentas). The Phusion® High-Fidelity DNA Polymerase (Fermentas) was used for amplification of the KanMX cassette (1473bp) with the PUG6 plasmid as the template. The amplified KanMX cassette was then digested with BamHI/SalI enzymes for cloning into the digested pCM188 plasmid. The digested KanMX and pCM188 plasmid were ligated and the reaction mix was used directly to transform chemically competent *E. coli* DH5α cells. The construct was named as pCM188- KanMX and was verified by sequencing. Lithium chloride (Gietz et al., 1995) treated cells were transformed with the pCM188- KanMX vector. After transformation, the cells were cultivated in YPD liquid medium without G418 for 4 hours and then plated on YPD + G418 agar plates for transformants with G418 resistance selection. Meanwhile, *S. cerevisiae* CEN.PK 113-5D was used for control experiments.

The second vector was constructed as an integrative vector with the KanMX cassette flanking with PtGUT1 partial sequences. Gene targeting substrates were constructed based on PCR methods using the cloning-free PCR-based allele replacement strategy (Erdeniz et al., 1997). In total, 4 individual pieces of DNA need to be fused to create the substrates as is shown in Figure 1.
The primers Pt_GUT1_Up_F and D_Pt_GUT1_Up_R, C_Pt_GUT1_Dw_F and Pt_GUT1_Dw_R were used to amplify the PtGUT1 upstream sequences and downstream sequences. The primers dKanMX_5" and KanMX_3"_int, KanMX_5"_int and cKanMX_3" were utilized to amplify the KanMX N-term and KanMX C-term sequences. To construct the first bipartite substrate, the fragment upstream of PtGUT1 was fused to the KanMX N-term fragment using primers Pt-GUT1-Up_F and KanMX_3"_int. The second bipartite substrate was obtained by fusion of the KanMX C-term fragment with the downstream PtGUT1 fragment using primers Pt-GUT1-Dw_F and cKanMX_3". The transformation was performed by the lithium acetate method (Gietz et al., 1995) for chromosomally homologous integration. After transformation, the cells were cultivated in YPD liquid medium without G418 for 4 hours and then plated on YPD + G418 agar plates for transformants with G418 resistance selection.
Table 1. Primers used for constructing vectors

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5′ to 3′</th>
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<tr>
<td>KanMX_F_BamHI</td>
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<td>KanMX_R_SalI</td>
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<tr>
<td>Pt_GUT1_Up_F</td>
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<td>D_Pt_GUT1_Up_</td>
<td>GCAGGGATGCGGCAGCCGCTGACTCAACAAGGAAGGTTCACACG</td>
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<tr>
<td>Pt_GUT1_Dw_R</td>
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<tr>
<td>KanMX_3&quot;_int</td>
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<tr>
<td>KanMX_5&quot;_int</td>
<td>AAAAGACTCACGTTCGAG</td>
</tr>
<tr>
<td>cKanMX_3&quot;</td>
<td>CACGCGCGCTAGCAGCGGGAGTCAGTGCAGCGAGGAA</td>
</tr>
</tbody>
</table>

2.5 Ura- Mutants selection

*P. tannophilus* was grown overnight in YPD liquid medium, centrifuged, washed twice with sterile water, and resuspended in 1 ml of sterile water. Aliquots (200 µl) of cells were then spread on 5-FOA plates and exposed to 0, 30J and 40J UV (around 20 – 40% killing efficiency). Colonies were picked after 5 days at 28°C and tested for a uracil requirement by assessing growth on minimal medium with and without uracil.

3. Results

3.1 G418 antibiotics as the transformation marker

*P. tannophilus* showed no growth when the concentration of antibiotic G418 in medium was greater than 50 µg/ml. Therefore, G418 was chosen for being used as a transformation marker. It has been shown that the ARS (autonomously replication sequences) of *S. cerevisiae* in YRp7 plasmid functioned in the lithium treated hexose negative mutants of the yeast *P. tannophilus* (Wedlock & Thornton, 1989). The ARS of the pCM188 plasmid is also from *S. cerevisiae*, which indicates that the ARS should also work in *P. tannophilus*. Lithium chloride treated cells were transformed with the pCM188-KanMX vector. Meanwhile, *S. cerevisiae* was used for control experiments. KanMX gave resistance to G418 if this cassette
could be successfully expressed. It was shown from the results that *S. cerevisiae* with the KanMX grew on the YPD+G418 (100 µg/ml) plates. However, no transformants of *P. tannophilus* were obtained on YPD+G418 (50 µg/ml) selection plates. Lithium chloride treated cells were transformed with KanMX flanking PtGUT1 upstream and downstream fragments of *P. tannophilus* for homologous recombination. However, no transformants were obtained on YPD+G418 (50 µg/ml) selection plates.

### 3.2 URA3 as the transformation marker

The possibility of making a uracil auxotrophic marker was also investigated. The first step in the development of the transformation system was the isolation of a stable ura3 auxotrophic mutant. The mutants could be selected on media containing 5-FOA. The growth of wide-type *P. tannophilus* was inhibited when 1.5 mg/ml 5-FOA was supplemented in the medium. The cells of *P. tannophilus* were UV mutated and then selected onto SD supplemented with uracil and 5-FOA plates for 5 days cultivation (Figure 2). The mutants from the plates were re-streaked onto the same plates for isolating single colonies and confirming the mutants’ resistance to 5-FOA. Then the single colonies were streaked onto SD plates to check if they were ura3 auxotrophic mutants. The ura3 auxotrophic mutants are expected to have no growth on SD plates and grow only on the SD + Uracil plates. However, from the results obtained here, several mutants exhibited growth when the cells were streaked out on SD plates. Several mutants which lacked of growth on SD plates also exhibited no growth when the cells were streaked out onto SD + Uracil plates.

![Figure 2. *P. tannophilus* cells were mutated with different doses of UV radiation on SD + Uracil + 5FOA plates.](image)

### 4. Discussion
The aim of this study was the development of a transformation and selection system for the ethanol producing yeast *P. tannophilus* based on the G418 antibiotic resistance marker and uracil auxotrophic marker. However, no right mutants were obtained during the time frame of the work.

Many factors might influence the transformation procedure including the vector composition, replicons, genetic markers, transformation methods and efficiency, vector stability and copy numbers, the promoters and the host strain (Wang et al., 2001). The success of a transformation procedure depends on all the factors working properly. It has been shown that the ARS from *S. cerevisiae* functioned in the lithium treated hexose negative mutants of the yeast *P. tannophilus* (Wedlock & Thornton, 1989). Since the ARS in the pCM188 plasmid is also from *S. cerevisiae*, the vector of pCM188-KanMX should replicate in the cells of *P. tannophilus* once the vector gets inside of the cells. For the chromosomally homologous integration, it also can eliminate the ARS factor. However, the vector stability, TEF promoter activity, transformation methods and efficiency and the incubation time for expression G418 resistance might still influence the transformation results. In order to successfully transform *P. tannophilus*, the strategy employed could be further improved. This could involve constructing a new plasmid with the original automatic replication sites, and original promoter from *P. tannophilus* and trying to transform *P. tannophilus* with this plasmid. This may eliminate the uncertain factors influencing the transformation procedure. In addition, other transformation methods such as electroporation of *P. tannophilus* could also be tested.

For obtaining the ura3 auxotrophic mutants, selection of more 5-FOA mutants might be necessary for obtaining mutants with right phenotype. The difficulty of obtaining auxotrophic mutants might be related to the complex life cycle of *P. tannophilus*. For instance, low concentrations of carbon source in the medium leads to vegetative cells transition to the mating process (Bolotnikova et al., 2005). An excess of microelements, vitamins, potassium and phosphorous activates the sexual stage of the yeast (Bolotnikova et al., 2005). In comparison, yeasts of the genera *Saccharomyces* and *Schizosaccharomyces* which have a simple life cycle are subject to genetic and biotechnological studies.

Since the approaches of trying to set up a transformation and selection system did not yield successful results, an alternative strategy could be transferring the glycerol assimilation pathways in *P. tannophilus* into the well characterized yeast *S. cerevisise*, where the genetic
background are well understood and the genetic engineering tools are available. Heterologous expression of the enzyme or the metabolic pathways involved in glycerol metabolism in *S. cerevisiae* was investigated in the following chapter.

**References**


Chapter 7 Functional studies of genes involved in transport and metabolism of glycerol in *Pachysolen tannophilus*

Xiaoying Liu, Peter Ruhdal Jensen, Mhairi Workman*

Submitted to *Applied and Environmental Microbiology*

**Abstract**

*Pachysolen tannophilus* is considered as a potential microbial cell factory since it can metabolize a variety of the carbon sources found in low cost feedstocks including glycerol and xylose. The xylose utilisation pathways have been extensively studied in this organism. However, the mechanism behind glycerol metabolism is poorly understood. The genome sequence of *P. tannophilus* CBS4044 has become available recently. The genes related to glycerol transport and metabolism in *P. tannophilus* have been identified here by blasting the genome sequence with the sequences of genes known to have these functions in *S. cerevisiae*. Quantitative real-time PCR was performed to unveil the expression pattern of the genes during growth on glycerol and glucose as sole carbon source. The genes involved in glycerol transport in *P. tannophilus* have been cloned and successfully expressed in *S. cerevisiae* (CEN-PK 1135D) strains. The transformed *S. cerevisiae* strains with heterologous genes showed improved growth and glycerol consumption rates with glycerol as the sole carbon source.

**Key works:** *P. tannophilus*; genes involved in glycerol metabolism; glycerol transporter; *FPS1; STL1*. 
1. Introduction

*Pachysolen tannophilus* is known for its ability to ferment D-xylose, one of the major components in hemicellulose plant residues, to ethanol (Kurtzman, 1983). In recent years, due to the dramatic increase in production of biodiesel, glycerol (a by-product of biodiesel production) has also been considered as a potential alternative carbon source for industrial bioprocesses. *P. tannophilus* has been shown to be capable of converting this crude glycerol to ethanol under microaerobic conditions (Liu et al.). Further optimization of the previously described processes would be beneficial and could be achieved through genetic modification of the organism directed towards improved glycerol uptake and processing.

The whole genome of *P. tannophilus* CBS4044 has been sequenced (Chapter 5), and with this, the possibility for genetic comparison of *P. tannophilus* with other better characterized yeasts is possible. A great number of studies have investigated glycerol transport, consumption and production by many different types of yeast, but the glycerol transport and metabolic pathways in *P. tannophilus* have not been studied so far. Since the genome sequence is now known, it is possible to look at the mechanism behind glycerol metabolism in *P. tannophilus*.

Glycerol transport is the first barrier for glycerol utilization in a microbial cell. In *Saccharomyces cerevisiae*, glycerol enters the cell by two different mechanisms: a low affinity transport system and a high affinity proton symport system (Lages and Lucas, 1997). When glucose is present, glycerol diffuses into the cells through a glycerol channel by facilitated transport, a process encoded by the *FPS1* gene (Sutherland et al., 1997; Tamás et al., 1999). However, during growth on non-fermentable carbon sources (glycerol, acetate, ethanol) an active uptake system driven by a proton motive force ensures the uptake of glycerol (Holst et al., 2000; Lages & Lucas, 1997). Two multi-membrane-spanning proteins encoded by *GUP1* and *GUP2* (Glycerol Uptake Protein), were first identified as being involved in active glycerol uptake in *S. cerevisiae* (Holst et al., 2000). However, in later studies Gup1/2p were proposed to have different roles than glycerol transport (Neves et al., 2004). A gene involved in active glycerol uptake in *S. cerevisiae* was identified by screening for genes encoding membrane proteins involved in glycerol assimilation. It was concluded that the glycerol proton symporter in *S. cerevisiae* is encoded by *STL1*. The protein which is localized in the plasma membrane, is a member of the sugar transporter family and is glucose
repressed (Ferreira et al., 2005). Stl1p is present in the plasma membrane of *S. cerevisiae* when the glycerol symport is functional. Stl1p has also been shown to have a function in glycerol uptake in several other yeasts. It has been shown that in *Candida albicans* glycerol was actively transported into the cells by a proton symporter encoded by the *C. albicans STL1* (Kayingo et al., 2009). It was also reported that *S. cerevisiae* strains harboring the *STL1* gene from *D. hansenii* improved their growth and doubling times slightly (González-Hernández, 2010).

After glycerol is transported into the cells, two different routes have been identified in yeasts for further assimilation: phosphorylation route and oxidation route. Within the first pathway, glycerol is dissimilated by glycerol kinase encoded by *GUT1* and then by mitochondrion-located glycerol-3-phosphate dehydrogenase encoded by *GUT2* (Nevoigt & Stahl, 1997). Another pathway is catalyzed via glycerol dehydrogenase encoded by *GCY1* and followed by dihydroxyacetone kinase encoded by *DAK1* and *DAK2* to dihydroxyacetone phosphate and then enter the glycolysis. The pathways involved in glycerol utilization differ in different yeasts. For glycerol formation, glycerol is commonly produced in the cytosol of yeasts from the glycolytic intermediate dihydroxyacetone phosphate in two steps that are catalyzed by glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp), respectively. Each enzyme activity has two isoenzymes, Gpd1p and Gpd2p, Gpp1p and Gpp2p (Wang et al., 2001). The production of glycerol has two functions in *S. cerevisiae*: redox balance and protection against osmotic stress as a compatible solute (osmolyte).

Although there have been several studies on glycerol metabolism in *S. cerevisiae*, many industrially used baker’s yeast strains grow poorly on glycerol. For example, the specific growth rate of *S. cerevisiae* CBS 8066 was found to be very low at 0.010 ± 0.002 h⁻¹ during shake flask cultivations in mineral medium containing glycerol as sole carbon source under aerobic conditions (Ochoa-Estopier et al., 2011). It was reported that the specific growth rate of *S. cerevisiae* BY 4741 was 0.05 h⁻¹ grown on glycerol in a minimal medium (Lu et al., 2005). Adapted evolution, overexpression or knock out of the genes involved in glycerol metabolism have been performed to increase the host strain’s growth on glycerol and production of different value-added products (Ochoa-Estopier et al., 2011; Yu et al., 2010). However, one possible rate-limiting step during glycerol metabolism, glycerol transport, was not addressed in those approaches (Burd et al., 2009).
In this study, a comparison of the genome sequences of *S. cerevisiae* and *P. tannophilus* has been performed to compare the genes involved in glycerol transport and glycerol metabolism in these two organisms. Quantitative real-time PCR was performed to compare transcript levels during growth on glycerol. The genes involved in glycerol transport in *P. tannophilus* have been heterologously expressed in a *S. cerevisiae* (CEN.PK 113-5D) to assess their function and also the possible contribution of transporter genes to the growth of *S. cerevisiae* on glycerol.

2. Materials and methods

2.1 Strains and plasmids

The *Pachysolen tannophilus* strain used in this study was CBS4044. *S. cerevisiae* CEN.PK 113-5D was used as a host for heterologous expression of glycerol transporters from *P. tannophilus*. The integrative USER vector pXI-5 (Mikkelsen et al., 2012) was used in this study for constructing the expression vectors in *S. cerevisiae*. Plasmid pSP-G1 (Partow et al., 2010) was used as a template for amplifying the bidirectional promoter *TEF1*/*PGK1*. All plasmids were propagated in *Escherichia coli* strain DH5α. *S. cerevisiae* strains and plasmids used in this study were listed in Table 1.

Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pSP-G1</td>
<td>2µ-based URA3 plasmid with <em>TEF1</em>/<em>PGK1</em> promoter</td>
<td>Partow et al., 2010</td>
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<tr>
<td>pXI-5</td>
<td>Integrative USER vector, with reusable <em>URA3</em> Marker</td>
<td>Mikkelsen et al., 2012</td>
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<tr>
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<td>This study</td>
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<td><em>S. cerevisiae</em> Strains</td>
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<tr>
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<td>Peter Kötter</td>
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2.2 Medium and culture conditions

For quantitative real-time PCR (qPCR) experiments, *P. tannophilus* was grown at 30°C in defined medium, containing (per liter) 0.67g yeast nitrogen base w/o amino acids, 10g succinic acid and 6g NaOH as buffer system, 2% (v/v) glycerol or 2% (w/v) glucose as the carbon source. Cells were harvested at mid-log exponential growth phase at the same optical density by centrifugation at 5000g, 4°C for 5mins, resuspended in 1ml ice cold ddH2O, and the cell pellet was stored at -80°C. *S. cerevisiae* CEN.PK 113-5D transformants harbouring glycerol transporter genes from *P. tannophilus* were cultivated at 30°C for 96 hours in agitated flasks with defined medium, containing (per liter) 7.25g synthetic complete (SC) powder, 10g succinic acid and 6g NaOH as buffer system, 2% (v/v) glycerol as the sole carbon source.

2.3 Sequence retrieval and analysis

The genome sequence from *P. tannophilus* CBS4044 was obtained from previous work and is available in the GenBank database. The sequences of genes involved in glycerol transport and metabolism in *S.cerevisiae* *FPS1, STL1, GUT1, GUT2, GCY1, Dak1/2, GPD1/2, GPP1/2* were blasted against the genome sequence of *P. tannophilus*. The genes with high similarities and high identities were listed as potential orthologous genes. Gene sequences were registered in GeneBank at NCBI. The multiple sequence alignments with the amino acid sequences of *FPS1* and *STL1* transporters from different yeast strains and *E. coli* were performed by using ClustalW2 free program at PDBe. Phylogenetic analyses were performed by using PAUP* 4.0b10 (*Swofford, 2003*). Unweighted parsimony analysis was performed. Trees were inferred using the heuristic search option with tree bisection-reconnection branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Clade stability was assessed in a bootstrap analysis with 1000 replicates. Trees were visualized in Treeview (*Page, 1996*).

2.4 Quantitative real-time PCR

Total RNA isolation and cDNA synthesis

Total RNA was isolated from frozen cells using an RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer’s protocol. The quantity and quality of the isolated RNA were measured by NanoDrop ND-1000. The total RNA was treated with DNasel (Fermentas) prior
to cDNA synthesis. Five µg total RNA were used to synthesize cDNA employing the RevertAid™ First Strand cDNA Synthesis kit (Fermentas) following the manufacturer’s recommendations using oligo(dT)$_{18}$ primer. The cDNA was used as the template for quantitative real-time PCR for determining the transcript levels of the target genes under different growth conditions.

qPCR was performed on a Stratagene Mx3005P using the SYBR Green technology. The qPCR reaction mixture was prepared with 5µl of 5 times dilution of cDNA as template, 10µl SYBR Green master mix, 2µl of 1µM forward primer and 2µl of 1µM reverse primer and ddH$_2$O to 20µl. The PCR program for qPCR was as follows: 10 min of incubation at 95°C, followed by 40 cycles of 95°C for 30s, 58°C for 30 s and 72°C for 30s, and finally the temperature was increased from 55°C to 94°C to check for unspecific products. The number of fluorescence threshold cycles (Ct) was calculated with the set threshold value by using Mx3005P software. Results presented are mean values of three independent experiments. Suitable primer pairs for all the genes investigated were designed using the Primer Express software v3.0 (Applied Biosystems) software with the following parameters: product size 140-180bp and melting temperature (Tm) 57-59°C. The primers used in this work are listed in Table 2. In addition, control reactions which include all components for qPCR except for the reverse transcriptase were performed to detect the genomic DNA contaminating. The absence of products under these conditions meant the absence of enough genomic DNA for successful amplification.

The relative expression levels (RE) were calculated approximately based on $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_T$ gene of interest - $C_T$ internal control) sample A - (C$_T$ gene of interest - C$_T$ internal control) sample B. C$_T$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data were normalized to the relative expression value of the housekeeping gene TAF12 in each sample, thus giving normalized relative expression for a target gene.
Table 2. Primers used for Semi-quantitative RT-PCR and USER Cloning.

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<td>PtSTL1_qF</td>
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<tr>
<td>PtFPS2_F_U</td>
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76
PtFPS2_R_U  CACGCGAUTTAGTATTGATTTTCTACGTCTTTGT
PtSTL1_F_U  ACTAACGCUATGTCCAAAAATCGATAAAAATTG
PtSTL1_R_U  CACGCGAUTCCTCTTCTTTTCGGGTT
PtSTL2_F_U  ACTAACGCUATGGATTCGAATATAGATGATACAG
PtSTL2_R_U  CACGCGAUCTAATGACGACCCTATTT

2.5 Constructs harboring glycerol transporters from *P. tannophilus*

In this study, four expression plasmids carrying the glycerol transporter genes from *P. tannophilus* were constructed. The USER vector pXI-5 was digested with AsSI and then the nicking endonuclease Nb.BsmI for making the AsSI/Nb.BsmI USER cassettes (Hansen et al., 2011). The proofreading PfuX7 (Norholm, 2010) was used for amplification of fragments *PtFPS1*, *PtFPS2*, *PtSTL1*, *PtSTL2* and bidirectional promoter *TEF1/PGK1* with appropriate USER tails for insertion into the designated USER cassette AsSI/Nb.BsmI. PCR was performed with PfuX7 DNA polymerase according to manufacturer’s instructions. DNA mixtures were prepared from purified digested vector, glycerol transporter gene, and bidirectional promoter *TEF1/PGK1*, 5× Phusion HF buffer (Fermentas), and 1 U of USER enzyme (New England BioLabs), adjusted to 10μl by adding ddH2O. The mixture was incubated at 37°C for 20 min, followed by 25°C for 20 min. The 10μl reaction mix was used directly to transform chemically competent *E. coli* DH5α cells. All the glycerol transporter genes were cloned under control of the *PGK1* promoter. Constructs were named as: pXI-5-PtFPS1, pXI-5-PtFPS2, pXI-5-PtSTL1, pXI-5-PtSTL2 for plasmids (Table 1). The plasmids were verified by sequencing and digested with NotI (Fermentas) for 1h at 37°C and then subjected to gel purification. Each linearized construct was homologously integrated into the *S. cerevisiae* CEN.PK 113-5D genome by transformation (Gietz et al., 1995); five yeast strain lines were constructed as summarized in Table 1. Transformants were plated onto SC-Ura for selection. Sequences and descriptions of primers are presented in Table 2.

2.6 Analysis of *S. cerevisiae* transformants by colony PCR and RT-PCR

The *S. cerevisiae* transformants were restreaked on SC-Ura plates for single colonies, and the desired homologous integration was checked by colony PCR. Cells of confirmed integrants grown in SC minimal medium at 30°C for 96 hours were harvested and used for total RNA extraction and cDNA synthesis. RT-PCR was used to analyze expression of the transporter
genes from *P. tannophilus*. The program for RT-PCR: 94°C for 2min, followed by 34 cycles: 50°C for 30s, 72°C for 2min, finally 72°C for 5min.

2.7 Analytical methods

Growth was monitored by measuring ODnad450nm with a Shimadzu UV mini-1240 spectrophotometer (Shimadzu, Japan). Samples were taken periodically from the flasks and filtered through a 0.22 µm syringe filter, and supernatants were preserved at -20°C for later HPLC analysis. Concentrations of the substrate glycerol in supernatants were measured by HPLC refractive index detector RID-10A using an Aminex87H column (Bio-Rad, USA). Separations were performed at 60°C, flow rate of 0.6ml/min and 5mM H2SO4 as mobile phase.

3. Results

3.1 Identification of genes in glycerol metabolism and analysis of glycerol transporters in *P. tannophilus*

In order to find genes related to glycerol transport and metabolism in *P. tannophilus*, the genome sequence was blasted with the sequences of genes known to have these functions in *S. cerevisiae*. In *P. tannophilus*, two glycerol facilitator and two glycerol symporter genes were found related with glycerol transport and named respectively *PtFPS1*, *PtFPS2*, *PtSTL1* and *PtSTL2*. In the glycerol consumption pathways, the genes with high similarity to *S. cerevisiae* were found and named *PtGUT1*, *PtGUT2*, *PtGCY1*, *PtGCY2*, *PtDAK* (Table 3). In the glycerol production pathways, the genes found were named *PtGPD* and *PtGPP*. It was noticed that the genes *PtFPS2* and *PtGUT1* were located close to each other in the *P. tannophilus* genome. With the aim of predicting the functions of the putative genes, bioinformatics tools Blastx and Blastp were used, and functional domain predictions were applied. The results are presented in Table 3.
Table 3. Discovery of genes potentially involved in glycerol metabolism in *P. tannophilus* by a BLAST search with known *S. cerevisiae* genes.

<table>
<thead>
<tr>
<th>Annotated function</th>
<th>Gene Name</th>
<th>Gene Name</th>
<th>Size (bp)</th>
<th>Identity / Similarity</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transport</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol Faciliator</td>
<td>ScFPS1</td>
<td>PtFPS1</td>
<td>1170</td>
<td>35/49</td>
<td>JQ481631</td>
</tr>
<tr>
<td></td>
<td>PtFSP2</td>
<td></td>
<td>972</td>
<td>32/51</td>
<td>JQ481632</td>
</tr>
<tr>
<td>Glycerol Symporter</td>
<td>ScSTL1</td>
<td>PtSTL1</td>
<td>1728</td>
<td>34/51</td>
<td>JQ481633</td>
</tr>
<tr>
<td></td>
<td>PtSTL2</td>
<td></td>
<td>1905</td>
<td>31/50</td>
<td>JQ481634</td>
</tr>
<tr>
<td><strong>Consumption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>ScGUT1</td>
<td>PtGUT1</td>
<td>1848</td>
<td>53/67</td>
<td>JQ481635</td>
</tr>
<tr>
<td>G3P dehydrogenase</td>
<td>ScGUT2</td>
<td>PtGUT2</td>
<td>1998</td>
<td>52/66</td>
<td>JQ481636</td>
</tr>
<tr>
<td>Glycerol dehydrogenase</td>
<td>ScGCY1</td>
<td>PtGCY1</td>
<td>936</td>
<td>54/73</td>
<td>JQ481637</td>
</tr>
<tr>
<td></td>
<td>PtGCY2</td>
<td></td>
<td>933</td>
<td>51/70</td>
<td>JQ481638</td>
</tr>
<tr>
<td>Dihydroxyacetone kinase</td>
<td>ScDAK1</td>
<td>PtDAK</td>
<td>1767</td>
<td>42/58</td>
<td>JQ481639</td>
</tr>
<tr>
<td></td>
<td>ScDAK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Production</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3P dehydrogenase</td>
<td>ScGPD1</td>
<td>PtGPD</td>
<td>1314</td>
<td>67/80</td>
<td>JQ481640</td>
</tr>
<tr>
<td></td>
<td>ScGPD2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3-phosphatase</td>
<td>ScGPP1</td>
<td>PtGPP</td>
<td>705</td>
<td>33/53</td>
<td>JQ481641</td>
</tr>
</tbody>
</table>

In order to investigate the phylogenetic relationship among putative glycerol transporters in *P. tannophilus* and their homologues from other yeast strains, an alignment was performed with Ptfps1p, Ptfps2p, Pptst1p, Pptstl2p as well as other predicted or published transporter proteins available from GenBank, Génolevures, SGD (Saccharomyces Genome Database), CGD (Candida Genome Database) databases of other yeasts. Unrooted phylogenetic trees are presented in Figure 1.

For *FPS1*, the lengths of PtFps1p, Ptfps2p, PpFps1p, YlFps1p and GlpF were relatively short compared to other Fps1p proteins. PtFps1p was shown to be 54% identical to PpFps1p, 45%
to YlFps1p, 35% to ScFps1p and only 31% to GlpF. PtFps2p was shown to be 60% identical
to PpFps1p, 49% to YlFps1p, 34% to GlpF and only 32% to ScFps1p. PtFps1p and PtFps2p
were grouped together with YlFps1p and PpFps1p, while they were in separate branch with
ScFps1p. For STL1, the identity between PtStl1p and ScPtStl1p was only 34%. However, the
identity between PtStl1p and YlPtStl1p was 56%.

Fps1

![Fps1 diagram]

Stl1

![Stl1 diagram]
Figure 1. Unrooted phylogenetic tree based on alignment of predicted protein sequences. The tree was bootstrapped 1000 times. Accession numbers are presented after protein names. Ca, Candida albicans; Dh, Debaryomyces hansenii; Kl, Kluyveromyces lactis; Km, Kluyveromyces marxianus; Pp, Pichia pastoris; Pt, Pachysolen tannophilus; Sc, Sacchromyces cerevisiae; Yl, Yarrowia lipolytica; Zr, Zygosaccharomyces rouxii.

3.2 Quantitative real time RT-PCR

qPCR was used to compare the levels of transcripts during growth on glycerol to that on glucose as the sole carbon source. It was found that the genes involved in glycerol transport and assimilation PtFPS1, PtFPS2, PtGUT1, PtGUT2, PtGCY1, PtDAK were up-regulated on glycerol to different degrees compared to on glucose, while the genes involved with glycerol production PtGPD and PtGPP were down-regulated on glycerol (Figure 2). Among all the genes PtFPS2 and PtGUT1 were most highly over-expressed and were up-regulated by a factor of 19.6 ± 2.3 and 17.6 ± 1.3, respectively, on glycerol compared to glucose. The transcript levels of PtFPS1, another facilitator, was up-regulated by a factor of 5.4 ± 0.9 on glycerol compared to glucose, while the glycerol symporter PtSTL1, PtSTL2 genes were expressed almost at the same level on glycerol and glucose. PtGUT2, which is a putative mitochondrial glycerol phosphate ubiquinone oxidoreductase, was also up-regulated at 4.2 ± 0.04 fold on glycerol.
Figure 2. Relative expression levels (RE) of glycerol metabolism related genes in *P. tannophilus* on glycerol compared to that on glucose. RE estimation was based on $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_T$ gene of interest - $C_T$ internal control) sample A - $(C_T$ gene of interest - $C_T$ internal control) sample B. $C_T$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. All the experiments were performed in triplicate.

3.3 Performance of *S. cerevisiae* harboring glycerol transporter genes from *P. tannophilus* on glycerol

All the predicted transporter genes from *P. tannophilus* were heterologously expressed under a strong and constitutive promoter *PGK1* in *S. cerevisiae*. To analyze the performance of *S. cerevisiae* recombinant strains, growth in defined medium with 2% (v/v) glycerol as the sole carbon source of CEN.PK 113-5D strain harboring plasmids pXI-5PtFPS1, pXI-5PtFPS2, pXI-5PtSTL1 and pXI-5PtSTL2 were tested under aerobic conditions in shake flasks. CEN.PK 113-5D containing the empty plasmid pXI-5 was grown aerobically in the same medium as a control.

The recombinant strains expressing the transporter genes *PtFPS1*, *PtFPS2* and *PtSTL1* from *P. tannophilus* showed improved growth on glycerol to different degrees compared to the
control strain after 96 hours of cultivation, while the growth of the recombinant strain harbouring *PtSTL2* was not increased (Figure 3). CEN.PK 113-5D harboring the empty plasmid pXI-5 grew very slowly on glycerol with the doubling time of 42.8 ± 5.7 h. At 96 hours, the strain reached a final OD 2.9±0.62 (Table 4). The strains expressing *PtFPS1*, *PtFPS2* genes grew on glycerol with a doubling time of 26.7±0.8 h and 22.7±1.4 h, and achieved of a final OD of 6.4±0.57 and 9.6±0.35 at 96 hours cultivation. The strain expressing *PtSTL1*, grew on glycerol with the doubling time 24.7±2.3, and achieved of a final OD of 8.3±1.4. The final OD with recombination strains PtFPS1_XY1, PtFPS2_XY2 and PtSTL1_XY3 were 2.2fold, 3.3fold and 2.9fold higher respectively compared to the control strain XI-5_XYC1.

**Figure 3.** Growth of recombinant *S. cerevisiae* strains expressing different glycerol transporter genes from *P. tannophilus* in defined medium containing 2% (v/v) glycerol as the sole carbon source at 30°C in agitated flasks. Results represent the mean of at least duplicate experiments.

For glycerol consumption, the recombinant strains harboring the genes PtFPS1, PtFPS2 and *PtSTL1* assimilated glycerol much faster than the control strain (Figure 4). The recombinant strains expressing the gene *PtSTL2* consumed glycerol almost the same as the control strain. The consumption rate of the control strain was 0.028g/L/h, while the strains harboring *PtFPS1*, *PtFPS2*, *PtSTL1*, *PtSTL2* utilized the glycerol at 0.069 g/L/h, 0.093 g/L/h and 0.1
The consumption rate of glycerol of the recombinant strains was 1.5fold, 2.3fold, 2.6fold higher than the control strain. The final glycerol consumed for the control strain was only 2.1±0.59 g/L at 96h cultivation, while the strains expressing the different transporter genes PtFPS1, PtFPS2 and PtSTL1 utilized 4.9±0.55 g/L, 7.6±0.33 g/L and 7.5±1.4 g/L glycerol respectively (Table 4).

![Figure 4](image-url)  
**Figure 4.** Glycerol consumption of recombinant *S. cerevisiae* strains expressing different glycerol transporter genes from *P. tannophilus* in defined medium containing 2% (v/v) glycerol as the sole carbon source at 30°C in agitated shake flasks. Results represent the means of at least duplicate experiments.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Doubling time on glycerol (h)</th>
<th>OD450 (96h)</th>
<th>Glycerol consumed (96h)</th>
<th>Glycerol consumption rate (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XI-5_XYC1</td>
<td>42.8±5.7</td>
<td>2.9±0.62</td>
<td>2.1±0.59</td>
<td>0.028±0.01</td>
</tr>
<tr>
<td>PtFPS1_XY1</td>
<td>26.7±0.8</td>
<td>6.4±0.57</td>
<td>4.9±0.55</td>
<td>0.069±0.00</td>
</tr>
<tr>
<td>PtFPS2_XY2</td>
<td>22.7±1.4</td>
<td>9.6±0.35</td>
<td>7.6±0.33</td>
<td>0.093±0.01</td>
</tr>
<tr>
<td>PtSTL1_XY3</td>
<td>24.7±2.3</td>
<td>8.3±1.4</td>
<td>7.5±1.4</td>
<td>0.1±0.02</td>
</tr>
</tbody>
</table>

*Table 4.* Summary of results from shake flask cultivations with *S. cerevisiae* CEN.PK 113-5D recombination strains grown on defined medium with 2% (v/v) glycerol as the sole carbon source.
3.4 Analysis of *S. cerevisiae* transformants by PCR and RT-PCR

To validate the function of glycerol transporters from *P. tannophilus*, four expression vectors harboring genes *PtFPS1*, *PtFPS2*, *PtSTL1* and *PtSTL2* under control of the *PGK1* promoter were constructed and integrated into *S. cerevisiae* CEN.PK 113-5D. The engineered strains carrying the plasmid pXI-5-PtFPS1, pXI-5-PtFPS2, pXI-5-PtSTL1 and pXI-5-PtSTL2 were confirmed by colony PCR to have undergone correct insertion of the target genes. Expression of the target genes in the recombinant strains was confirmed by RT-PCR. The colony PCR and RT-PCR showed that all the transporter genes from *P. tannophilus* were successfully inserted and transcribed in the recombination strains of *S. cerevisiae* CEN.PK 113-5D (Figure 5 a and b).

![Figure 5 a. Results for colony PCR performed on the S.cerevisiae recombinant strains. The expected PCR products are 1170bp for PtFPS1 (lane1), 972bp for PtFPS2 (lane2/3) and 1728bp for PtSTL1 (lane4/5), 1905bp for PtSTL2 (lane6/7). M represents marker.](image)

**Figure 5 a.** Results for colony PCR performed on the *S.cerevisiae* recombinant strains. The expected PCR products are 1170bp for PtFPS1 (lane1), 972bp for PtFPS2 (lane2/3) and 1728bp for PtSTL1 (lane4/5), 1905bp for PtSTL2 (lane6/7). M represents marker.

![Figure 5 b. Results for RT-PCR performed on the control and recombinant strains. mRNA was extracted from the cells grown in defined medium at 30°C for 96 h. PtFPS1(lane3/4); PtFPS2(lane5/6); PtSTL1(lane1/2); PtSTL2(lane7/8). Lane2/4/6/8 was the results from the control strain XI-5_XYC1.](image)

**Figure 5 b.** Results for RT-PCR performed on the control and recombinant strains. mRNA was extracted from the cells grown in defined medium at 30°C for 96 h. PtFPS1(lane3/4); PtFPS2(lane5/6); PtSTL1(lane1/2); PtSTL2(lane7/8). Lane2/4/6/8 was the results from the control strain XI-5_XYC1.

4. Discussion

With regard to glycerol transport, two glycerol facilitator homologues *PtFPS1*, *PtFPS2* and two glycerol symporter homologues *PtSTL1*, *PtSTL2* were found in the genome of *P. tannophilus*. Based on qPCR results, all the genes involved in glycerol transport and assimilation were induced on glycerol compared to glucose except the predicted glycerol symporters *PtSTL1* and *PtSTL2* which were constitutively expressed on both glycerol and glucose as carbon source. It was found that the two most strongly up-regulated genes *PtFPS2* and *PtGUT1* (19.6 fold and 17.6 fold) were located nearby each other in the scaffold of
genome sequences, which could fit with the possibility that those two genes might be functionally related and thus possibly most relevant for glycerol assimilation in *P. tannophilus*. Since attempts to investigate the function of those genes by knock-out in *P. tannophilus* have failed so far (data not shown), the pathway actually responsible for glycerol assimilation in *P. tannophilus* remains uncertain. Therefore, further focus has been placed on glycerol transport. In this paper the function of the two types of glycerol transporters from *P. tannophilus* was addressed by transferring them individually to the well characterized yeast *S. cerevisiae*.

The low affinity transporter Fps1p is a glycerol facilitator protein and belongs to the major intrinsic protein (MIP) family of channel proteins with six putative transmembrane domains (TMDs). Fps1p is responsible for transporting water, small molecules like glycerol, urea, NH₃, CO₂ or ions without consuming energy. The physiological role of the facilitator Fps1p in *S. cerevisiae* was described to be glycerol export rather than uptake during hypo-osmotic shock and the Fps1p channel closed and retained the glycerol inside the cells in response to hyperosmotic shock (Tamás et al., 1999). An N-terminal domain 225LYQNPQTPTVLP236 and a C-terminal domain 535HESPVNWSLPVY546 were found to have an important role in controlling Fps1p function. The N-terminus was required for closing of the channels and restricted transport through Fps1p. It was found that the rate of glycerol efflux was higher than that for uptake (Tamás et al., 1999). In the present study, the similarity of PtFps1p and PtFps2p to ScFps1p were 35% and 32% respectively, but the homology was only restricted to the core of the protein with the six putative TMDs. ScFps1p (669 amino acid residues) was much longer than PtFps1p (389 residues) and PtFps2p (323 residues), the size of which was more similar to the *E. coli* GlpF (281 amino acid). The size difference was mainly due to long N- and C-terminal domains of ScFps1p. By searching the protein sequences and also the alignment results, both the N- and the C-terminal hydrophilic extensions in ScFps1p were missing in the sequences of PtFps1p, PtFps2p and GlpF (Figure 6). By expressing PtFPS2 and PtFPS1 in *S. cerevisiae* the facilitators from *P. tannophilus* increased the glycerol influx and glycerol consumption, presumably due to improved glycerol transport ability. Previously it was also reported that the glycerol transport was approximately 2.5-fold increased in *S. cerevisiae* by introduction of the bacterial gene GlpF (Tamás et al., 1999).
The high-affinity transporter Stl1p was identified as the glycerol proton symporter in *S. cerevisiae* (Ferreira et al., 2005), which is a member of the sugar permease family of the major facilitator superfamily (MFS) (Zhao et al., 1994). It was demonstrated that the transcription of *STL1* gene was significantly induced with glycerol as the sole carbon source, and the *STL1* gene was subject to glucose repression based on microarray-based transcriptome analysis (Roberts and Hudson, 2006; Roberts and Hudson, 2009). However, the glycerol uptake by *STL1* from *C. albicans* was not affected by the carbon source and salt stress (Kayingo et al., 2009). In *D. hansenii*, it was shown that the active glycerol transport system was constitutively expressed and not subject to glucose repression (Lucas et al., 1990). In our study, the glycerol symporters PtSTL1 and PtSTL2 in *P. tannophilus* also showed constitutive expression on glycerol and glucose based on qPCR expression analysis. However, the presence of the PtSTL2 gene had no obvious effect on the physiology of *S. cerevisiae*, while the glycerol consumption and growth with *PtSTL1* were improved. The symporters PtStl1p showed a low degree of sequence identities to ScStl1p with 34%. However, the functional PtStl1p from *P. tannophilus* exhibited 52% identity to the Stl1p from *D. hansenii* (DEHA2A12364g) and 56% to the Stl1p from *Yarrowia lipolytica* (YALI0C16522g).

It has been reported that *Y. lipolytica* can grow on glycerol with $\mu_{max}$ around 0.3h$^{-1}$ (Papanikolaou & Aggelis, 2002; Papanikolaou et al., 2002). *P. tannophilus* can grow on glycerol with $\mu_{max}$ around 0.29h$^{-1}$ (Liu et al.), while *S. cerevisiae* grows relatively slowly on glycerol with $\mu_{max}$ of 0.017h$^{-1}$ (CEN.PK 113-5D). Both the facilitator and symporter similarity between *P. tannophilus* and *Y. lipolytica* were higher than that compared to *S. cerevisiae*. The protein sequence similarity to some degree was relevant to the biological
functions. The facilitator and symporter from *P. tannophilus* might have higher affinity and efficiency for transporting glycerol through cell membrane into the cells. However, more protein sequence data with verified functions are needed to provide a definitive conclusion.

Heterologous expression of the glycerol transporters from yeasts growing well on glycerol was used as the approach for efficient glycerol assimilation in *S. cerevisiae* in this paper. Besides, many consider that the glycerol transport might be the rate-limiting step for glycerol utilization. Improved glycerol transport ability was demonstrated here to increase the glycerol consumption rate and growth rate under aerobic conditions with *S. cerevisiae*. PtFps2 and PtStl1 had similar effects on growth and glycerol consumption in the recombinant strains. It is known that the secondary active transport (glycerol active symporter) consumes more energy than facilitation. Although, with the exception of PtSTL2, all the putative transporters from *P. tannophilus* improved the growth and glycerol consumption to different degrees, the quantitative contribution of each transporter gene to the growth of *P. tannophilus* on glycerol has not yet been ascertained and it may be relevant to investigate this under different growth conditions and metabolic regulation of the cells. The current study demonstrates the influence of the glycerol transporter from *P. tannophilus* on growth and glycerol utilization with *S. cerevisiae*. It would be of interest to further study whether there is a synergy of different transporters. Furthermore, all the experiments were performed under aerobic conditions, and it could be relevant to investigate the physiological performance under anaerobic conditions. Our studies open new possibilities for further improving glycerol fermentation in industrial yeast strains. Heterologous expression of glycerol transporters from the glycerol utilising *P. tannophilus* could aid development of glycerol-based bioprocesses in *S. cerevisiae*.

Acknowledgement

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Chapter 8 Conclusions and future perspectives

In this PhD project, possible hosts for conversion of the biodiesel by-product glycerol were selected through screening of the literature and were studied with regard to growth and external metabolite production on glycerol. *Pachysolen tannophilus* was selected as the investigation subject for further study of ethanol production on glycerol.

In chapter 3, the fermentation physiology was investigated as a means to designing a competitive bioethanol production process by *P. tannophilus*. It was shown that the oxygen transfer rate (OTR) was a key factor for ethanol production, with lower OTR having a positive effect on ethanol production. The effect of pH on ethanol production showed that pH 5 was the optimal pH for ethanol yield and production on glycerol by *P. tannophilus*. The deviation from optimal pH affected the ethanol yield to varying degrees. Compared to NO₃⁻ as the nitrogen source, NH₄⁺ stimulated higher carbon flow to ethanol but a lower flow to biomass. However, NO₃⁻ accelerated the rate of ethanol production. The highest ethanol production with batch fermentation was 17.5 g/L on 5% (v/v) crude glycerol, corresponding to 56% of the theoretical yield. This study demonstrated that the ethanol production process from crude glycerol was a robust bioprocess and was not sensitive to the batch variability in crude glycerol dependent on raw materials used for biodiesel production. A staged batch process achieved 28.1 g/L ethanol, the maximum achieved so far for conversion of glycerol to ethanol in a microbial bioprocess.

In chapter 4, adaptive evolution was performed to enhance the ethanol tolerance of *P. tannophilus* on glycerol by exposing the cells to a stepwise increase of ethanol concentration in the medium with repetitive cultivations. The growth performance and the ethanol tolerance of the mutants were investigated. The ethanol tolerance and maximum specific growth rate ($\mu_{\text{max}}$) of isolated mutant E₂₄₃, were improved at higher concentrations of ethanol (> 4 % v/v) compared to the wild type. However, the $\mu_{\text{max}}$ and the initial glycerol consumption rate of the mutant when ethanol was not present in the medium were decreased compared to the wild type strain. The ethanol yield on glycerol and ethanol production levels of E₂₄₃ were also decreased.
A draft genome sequence of the non-conventional yeast \textit{P. tannophilus} CBS 4044 was presented in Chapter 5. The total length of the sequenced genome was 12,238,196 bp (without N) with a GC content of 29.82%. Total 1970.8 Mb raw data was sequenced representing around 145 fold coverage of the \textit{P. tannophilus} genome. 5,346 protein-coding genes (CDSs) set were predicted, with 4,463 (83.5%) genes annotated with function. Based on PFGE results, the estimated genome size of \textit{P. tannophilus} (CBS4044) was approximately 13.6 ± 0.4Mb with an estimated of 8 chromosomes.

Chapter 6 was mainly about the attempts to set up the transformation and selection system in \textit{P. tannophilus} in order to know more about the genetic background and further improve the ethanol production process. Antibiotic (Geneticin) resistance marker and uracil auxotrophic marker were tested for creating the transformation and selection system. Unfortunately, the strategies and methods employed did not yield successful results.

In chapter 7, based on genome sequencing results the genome of \textit{P. tannophilus} has been blasted to find genes similar to \textit{FPS1}, \textit{STL1}, \textit{GUT1}, \textit{GUT2}, \textit{GPD1/2}, \textit{GPP1/2}, \textit{GCY1}, \textit{DAK1/2} in \textit{S. cerevisiae}. Eleven genes were found with the predicted function in glycerol metabolism of \textit{P. tannophilus}. Quantitative real-time PCR showed that the genes \textit{PtFPS1}, \textit{PtFPS2}, \textit{PtGUT1}, \textit{PtGUT2}, \textit{PtGCY1}, \textit{PtDAK} involved in glycerol transport and assimilation were up-regulated on glycerol to different degrees compared to on glucose, while the genes involved with glycerol production \textit{PtGPD} and \textit{PtGPP} were down regulated on glycerol. It was shown that the two most strongly up-regulated genes \textit{PtFPS2} and \textit{PtGUT1} (19.6 fold and 17.6 fold) were located close each other in the \textit{P. tannophilus} genome and might be functionally related and possibly most relevant for glycerol assimilation in \textit{P. tannophilus}. A phylogenetic analysis with the transporter genes revealed that the similarity of PtFps1p and PtFps2p to ScFps1p were 35% and 32% respectively. The symporters PtStl1p also showed a low degree of sequence identities to ScStl1p with 34%. The genes involved in glycerol transport in \textit{P. tannophilus} have been cloned and successfully expressed in \textit{S. cerevisiae} (CEN-PK 1135D) strains. The transformed \textit{S. cerevisiae} strains with heterologous genes showed improved growth and glycerol consumption rates with glycerol as the sole carbon source.

Based on the results of my PhD study, the following suggestions could be recommended for further developing the glycerol utilization platform and ethanol production process:
1. Rational fed-batch fermentation should be designed as growth and ethanol production is correlated to oxygen transfer rate. This could further increase ethanol yields and production level.

2. More effort should be focused on mapping the 34 scaffolds from genome sequencing results into the chromosomes of *P. tannophilus*.

3. More experiments such as testing different transformation methods would be needed for setting up a suitable transformation and selection system in *P. tannophilus*.

4. The physiological performance of the *S. cerevisiae* transformants under anaerobic conditions should be investigated.

5. The *S. cerevisiae* recombination transformant harboring the combination transporters of *PtFPS1* and *PtSTL1* from *P. tannophilus* should be constructed to test if there is any synergistic effect for growth and glycerol assimilation with *S. cerevisiae*.

Hopefully this thesis will help clarify the glycerol metabolism in yeast and provide readers with more understanding in the area of conversion of glycerol by different microorganisms and particularly *P. tannophilus*. 