Yeast producing Biofuel

The modification of yeast through genetic engineering to enhance biofuel production

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Figure 1 A yeast cell

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1 Preface:

1.1 Why did we choose this topic?

We both like to bake, in many recipes we use yeast – this is how we got the idea of looking into yeast as our topic. We were curious about how exactly yeast functions in baked goods. Furthermore, we enjoyed the idea of combining part of our leisure interests with a scientific school task.
Especially intriguing is how easily yeast can be modified to suit our needs. Another aspect that sparked our interest is the fact that the production of ethanol by yeast is a sustainable source for biofuel and might be a viable choice for many to come in the future. We wondered whether the production of biofuel with yeast is applicable to our everyday life and if yes, how is it done. We will mainly be discussing *Spathaspatra Passalidarum* and *Saccharomyces cerevisiae*. *S.cerevisiae* also commonly known as baker's yeast, is the most efficient yeast and versatile producer of ethanol, accordingly it has been researched a lot. *S. passalidarum* is the yeast that can convert xylose into ethanol. For a general overview of the production process, see part 8 in the appendix.

1.2 Questions for the chosen topic

We started out by asking ourselves a handful of question. With time the number of questions increased, as we learned more about our topic. All of our final questions can be found in the interview, which is attached to the appendix.

These are the questions we started out with:
How can yeast be modified to produce more bioethanol?
Can biofuel be produced on a large scale?
Are there other microbes that produce bioethanol?
Could every form of yeast be used to produce biofuel?
Why is Saccharomyces Cerevisiae the most commonly used yeast in bioethanol production?

2 Introduction

2.1 Context of the chosen topic

As climate change threatens to change our future, scientists have tried to find ways to replace petroleum, which upon combustion emits high amounts of CO_2, and to develop biofuels instead, which do not emit as high amounts of CO_2 during the overall process. At least 35% less greenhouse gases are produced with biofuels.[1][2] Biofuels also burn more cleanly, emitting smaller amounts of carbon dioxide than fossil fuels, because combustion is more complete. Bioethanol is one of those biofuels, currently it is the most important one. Biorefineries use biomass as a feedstock to produce biofuels instead of fossil sources. Biorefineries can use a wide variety of biomasses, from microalgae to wood, as long as they contain sugar or starch. As yeast ferments sugar to ethanol, scientists hope to modify yeast genetically, so that it can be used in a large-scale production of biofuel. When yeast ferments sugar into ethanol, it is exposed to various stressors during this process. These cause the yeast to reduce ethanol production or stop it entirely, if the yeast tolerance limits are exceeded. You can see some of the major stressors which yeast experiences during the process of fermenting sugar to alcohol in Figure 2). When starch is fermented to ethanol it undergoes hydrolysis and saccharification. Hydrolysis is the addition of water to a compound; H_2O is used to break up the chemical bonds of the compound. Saccharification turns complex carbs into sugars. [3] [4]

[1] (Umweltbundesamt GmbH Wien, 2012)
[2] (Shazmi, 2011)
[3] (Müller-Langer F. , 2014)
[4] (University of Hawaii, 2007)

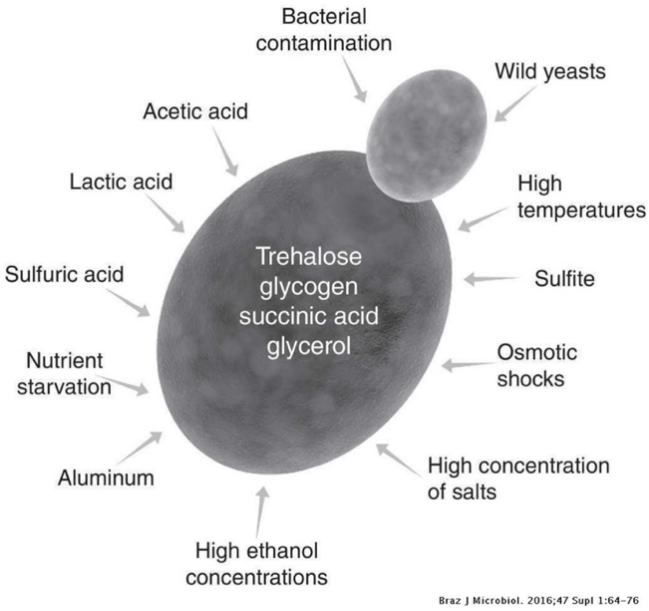


Figure 2 An overview of the major stressors during fermentation (trehalose, glycogen, succinic acid and glycerol. Are part of the cell defense mechanisms and reactions to the stressors)

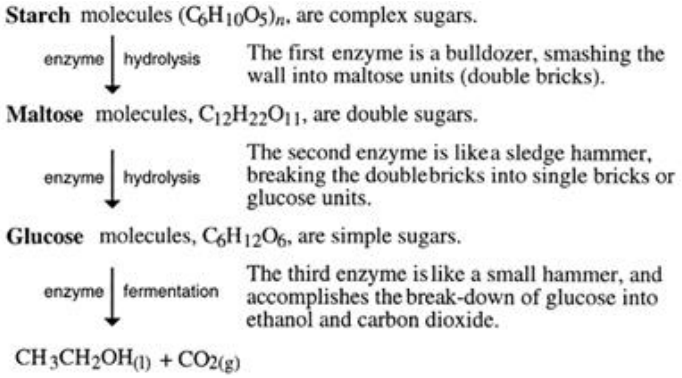


Figure 3 A step by step process showing how starch is converted into ethanol and carbon dioxide

At this point in time a type of yeast that meets all the requirements to be used as a bioethanol producer does not yet exist. For instance, many yeasts do not operate at a temperature above 30°C, which increases cooling costs. Also, the ethanol tolerance poses a problem, by decreasing or even stopping the fermentation process, if the yeast's ethanol tolerance limits are exceeded. Additionally, the baker's yeast *Saccharomyces Cerevisiae* can only convert hexose sugars, which limits the usable sugars for fuel supply.[5]

So, in order for yeast to be utilized in biofuel production, it must be modified using various techniques. Genetic engineering is applied for making the yeast more heat-resistant, to develop a tolerance for a high ethanol concentration and to increase the ability to process also pentose sugar. As the idea of using yeast as biofuel producer is still being researched, there are several different approaches to genetically improve yeast to be applicable.

If the thermal tolerance is enhanced, the cooling costs can be lowered, the rate of fermentation increased, and the risk of contamination is minimized. Increasing the fermentation temperature from 30°C to 40°C already increases the efficiency and reduces the costs significantly. The yeast *S.cerevisiae* does not function properly as converter of sugar to ethanol at such a high temperature, so genetic engineering techniques can be applied to enhance its thermotolerance. Although many interesting engineering techniques are currently being used to modify yeast, we focused our attention on one.[6]

2.2 Recent scientific history:

A handful of chemicals serve as a catalyst to speed up the breakdown of plants for the production of biofuels, for example ethanol are toxic for yeasts.

This creates a significant problem during the biofuel production, because the yeast decreases its ethanol production once it has reached its ethanol tolerance. On one hand the yeast stops operating at 35°C, however the saccharification and hydrolysis creates heat, so the tanks that contain the yeast must be cooled down to. On the other hand, enzymes that are being added to speed up the process, function their best at 40-45°C.[7]

[5] (Ching-Sung Tsai, 2015)
[6] (Li, 2019)
[7] (Li, 2019)

Scientists from MIT (Massachusetts Institute of Technology, USA) have stepped over a major milestone by solving both problems at once. They exposed yeast three months long to a temperature of 40°C, during this exposure it developed ways to cope with the heat. The yeast mutated. At the end of this trial, they had developed seven yeast strains that had grown twice as fast at 40 °C than normal yeast.
After isolating these seven yeast strains, they checked the whole genome to determine which genes were responsible for the heat tolerance. They discovered that all the strains had mutations in the same gene, indicating that a single gene can drastically change the heat tolerance.
The mutation takes place in a gene which is involved in production of sterols. Normally yeast produces the sterol ergosterol, but the mutation produces fecosterol instead. These sterols are being incorporated into the fat layer of the cell membrane. They influence how liquid the cell membrane is at different temperatures. Fecosterol creates a firmer membrane at higher temperature than ergosterol. The ergosterol makes the cell membrane too liquid at higher temperatures, which affects the well-being of the yeast. This discovery has only been tested in a laboratory and has not faced any field trials yet.[8]

Another study tackled the ethanol tolerance problem from a different angle. Scientists tried to manipulate the gene that controls the ethanol tolerance, so it can be increased. Recently Researchers from the University of Wisconsin-Madison and Department of Energy laboratories have identified two changes to a single gene that can make the yeast tolerate the pre-treatment chemicals. Pre-treatment chemicals are used to increase the rate of reactions. Manufactures catalyse the process by pre-treating the raw plant biomass. For example, applying ammonia gas, acids, heat and pressure and salts called ionic liquids.
Following pre-treatment, the cellulose that creates the plant cell wall and fibres are broken down with enzymes to release sugar.
The ionic liquids serve as starter to get the whole process going. The only problem they face is that even after retrieving as much of the ionic liquid from the biomass, the amount that is left is toxic for many microbes.
Based on their findings they looked into different kinds of *Saccharomyces cerevisiae* strains. They compared 136 yeast strains, of these strains they discovered one strain that had an impressive tolerance to ionic liquids. Surveying DNA sequences, they were able to identify a pair of genes essential to surviving the pre-treatment chemicals. One of those genes, named SGE 1, produces a protein that lays in the yeast cell membrane and serves as a pump to get rid of toxins. They realised that having more of those pumps at the cell surface, helps removing more of the ionic liquid molecules from the cell.
Interestingly an alteration of only two individual nucleotides suffices to increase the creation of those cellular pumps. Researchers made use of the gene-editing tool CRISPR to change a strain of a yeast that is susceptible to ionic liquids, introducing the two nucleotide changes and thereby producing a yeast that survives and ferments alongside amounts of ionic liquid that are usually toxic.
This is an uncomplicated engineering procedure, which does not require much time, is not costly and can be done in a matter of a week or two.[9]

[8] (Felix H. Lam, 2014)
[9] (Hadhazy, 2019)

3 Description of genome shuffling

3.1 General Information

We chose to focus on a study (Development and genomic elucidation of hybrid yeast with improved glucose-xylose co-fermentation at high temperature from Yuping Lin et al. *FEMS Yeast Research 2019*)[10] about the fermentation rate of two sugars (glucose and xylose) at high temperatures of a hybrid yeast strain. In this publication, they applied the techniques or genome shuffling and adaptive evolution. We will discuss the two applied techniques used during that study. Scientists enhanced the capability of co- fermenting glucose and xylose at high temperature by combining genome shuffling and adaptive evolution. [11] [12]

First, the intergeneric hybrids of naturally glucose-xylose co-utilizing yeast *S.passalidarum* and a thermotolerant *S. cerevisiae* industrial strain were generated by using genome shuffling. (Intergeneric means, that the parental DNA-strands are derived from individuals of different genera)
After the genome shuffling, one hybrid was chosen to undergo adaptive evolution. In Figure 3) an overview of the breeding of thermotolerant xylose-utilizing yeast with the applied techniques is shown. [13]

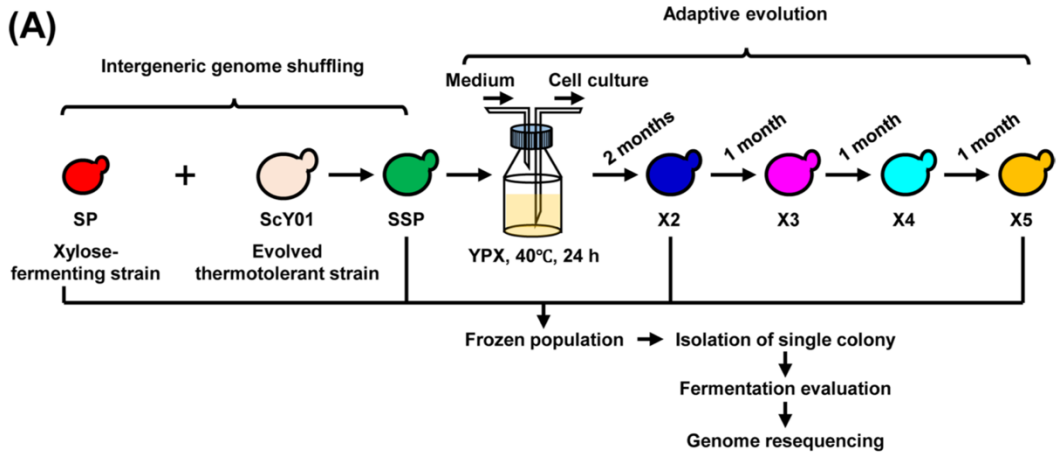


Figure 4 The breeding of thermotolerant xylose-utilizing yeast strains using genome shuffling and adaptive evolution

3.2 Genome shuffling:

Genome shuffling is used for hybridization of two or more genes. W. Stemmer developed this technique in 1994. The result is a mixture of genes that have recombined different segments from different original genes. In our case the genes from *Saccharomyces passalidarum* SP and *Saccharomyces cerevisiae* ScY01.

Genome shuffling consists of four steps:

1) preparation of genes to be shuffled,
2) fragmentation with DNase I,
3) reassembly by thermocycling in the presence of a DNA polymerase,
4) amplification of reassembled products by a conventional PCR.[14] [15]

[10] (Yuping Lin Y. C., 2019)
[11] (Borgne, 2012)
[12] (Li Wua, 2020)

[14] (Basu, 2016)
[15] (Li Wu, 2020)

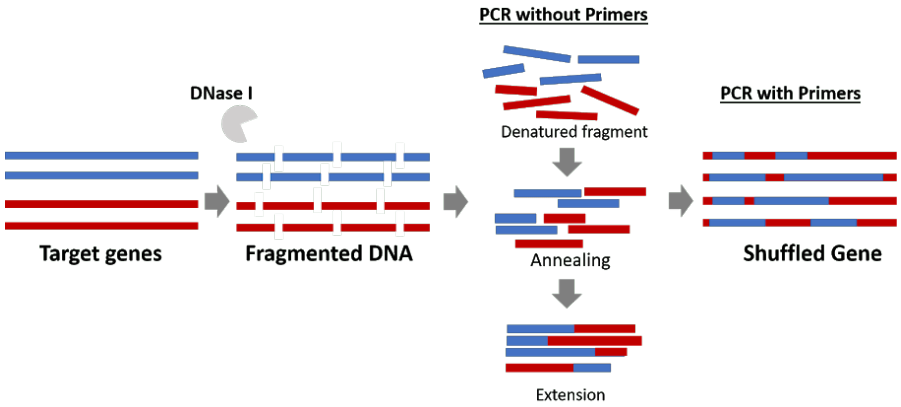


Figure 5 Genome shuffling explained

1) First, the parent strain library must be constructed. Therefore, the initial strain is engineered to generate more genotypes or genetic material from strains of different genera is used. The desired strains are then collected to form the parental library for protoplast fusion. [16](A genomic library is a collection of the total genomic DNA from a single organism.

In our example, intergeneric genome shuffling was performed with the two yeast strains *Spathaspora passalidarum NRRL Y-27907* (abbreviated as SP) and *Saccharomyces cerevisiae ScY01* (abbreviated as ScY01) as parental strands. The non-conventional yeast *S. passalidarum* is a newly identified species capable of producing ethanol more proficiently from xylose, but it mistranslates the CUG codon as serine instead of leucine, which can disrupt protein folding. The other parental strain *Saccharomyces cerevisiae* ScY01 is an evolved thermotolerant strain derived from an industrial strain ScY01 previous modified in their laboratory (Shui *et al*. 2015), which can grow and ferment well at 40°C. Both strains are diploid.
Yeast cells from both generations were grown separately, then mated and harvested by centrifugation. The cells were collected and then resuspended in a buffer containing an enzyme for enzymatic digestion of the cell wall.[17] Protoplast formation involves the removal of the cell wall, leaving a fatty membranous sac containing the genetic material of the cell. The protoplasts from the two yeast strains are fused by a specific technique so that the genetic material from both strains is being mixed.[18] [19]

2) Then DNase is used to fragment (cut) the set of parent genes into pieces of 50-100 bp in length.

3) The third step is a polymerase chain reaction (PCR) without primers
DNA fragments with sufficient overlapping homologous sequence will anneal to each other and are then extended by DANN Polymerase.
In this reassembly step, the fragments are heated to a high temperature to promote denaturation of the DNase I-digested fragments. They will act as self-primers. Then the temperature is lowered to allow the fragments to randomly anneal to other fragments. The annealing leads to the recombination of different templates. While randomly recombining the DNA sequences, the technique also introduces new point mutations at a relatively high rate (0.7%).

[16] (JixianGongHuijieZhengZhijunWuTaoChenXuemingZhao, 2016)
[17] (Wang, 2019)
[18] (Yuping Lin Y. C., FEMS Yeast Research, 2019)
[19] (K. Leja, 2014)

4) Then, primers specific for the gene to be engineered are used to PCR amplify the full-length sequences.
The yeast cells regenerate their walls and are screened for the desired phenotypes. Strains from the regenerated protoplasts are pooled, resulting in the strain library for a second-round fusion. The strains in the fusion library are grown as a population and used to prepare protoplasts which are similarly fused and regenerated. This process can be repeated several times, so that various hybrids occur.
In our example, the fused protoplasts were plated on YP agar plates containing 20 g /L xylose (YPX agar plates), and the plates were incubated at 40°C until visible colonies appeared. The colonies were later transferred by replica plating to the YP agar plates containing 20 g/L glucose (YPD agar plates) and incubated at 30°C for 3 days. The grown colonies were then transferred back to YPX agar plates and incubated at 40°C for 4 days. The transfers between YPX agar plates at 40°C and YPD agar plates at 30°C were repeated three times to select hybrid strains with the capability of utilizing xylose and at the same time stable tolerance to elevated temperature. The finally selected colonies were maintained on YPX agar plates for further characterization.[20]

As a second method, the scientists relied on adaptive evolution:
After the intergeneric genome shuffling, a total of 21 hybrid colonies were tested in terms of xylose fermentation and cell growth at 40°C. The colony 18 (SSP) showed the best fermentation capacity, so it was selected as the starting strain for adaptive evolution experiments for 5 months. It was further evolved at high temperature solely using xylose as a carbon source. Single colonies were isolated from the evolved cell population every month, and successfully named X2 through 2-month evolution, X3 through 3-month evolution, X4 through 4-month evolution and X5 through 5-month evolution. They were stored in a freezer and used for fermentation evaluation and genetic stability at 40°C.[21]

4 Interview

4.1 Introduction of our expert

We chose Dr. Poonam Singh (Ulster University UK), because we were searching for a Microbiology expert. We contacted her before we decided on genome shuffling as our main topic. That is why she does not mention genome shuffling. We are very thankful for her expertise, regarding biofuel. All her degrees are attached to the appendix.

4.2 Interview with the expert

Summarized, Dr. Singh explained to us the process of how yeast goes through the process of producing bioethanol, how yeast can be modified to use pentose sugars as fuel and the sustainability of yeast as well as the application of yeast, which we partly used to create a list of advantages and disadvantages.

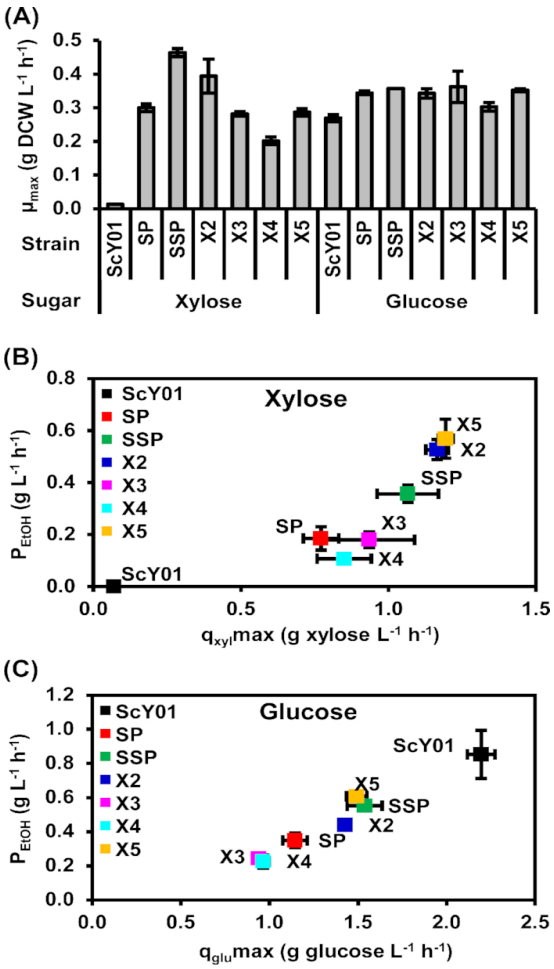
The entire interview can be found in the appendix.

5 Discussion:

5.1 Advances in genome shuffling and adaptive evolution

This is visualized in Figure 6 and discussed below. With this innovative method, yeast strains have been generated with greatly increased fermentation capacities and greater biofuel output.[22]

[20] (Yuping Lin Y. C., Development and genomic elucidation of hybrid yeast with improved glucose-xylose co-fermentation at high temperature, 2021)
[21] (Yuping Lin Y. C., Development and genomic elucidation of hybrid yeast with improved glucose-xylose co-fermentation at high temperature, 2021)
[22] (Yuping Lin Y. C., Development and genomic elucidation of hybrid yeast with improved glucose-xylose co-fermentation at high temperature, 2021)



(A) The maximum growth rates (μ_{max}) with the parental, genome shuffled and evolved strains. The strain SSP showed a 1.5-fold increase of cell growth than the parental strain SP when grown on xylose. The maximum growth rates of the genome-shuffled and evolved strains on glucose at 40°C were more similar to that of SP.

(B) Xylose consumption rates ($q_{xyl}max$) and ethanol productivities (P_{EtOH}) with the strains in **(A)** The strain SSP showed in contrast to SP a 1.39-fold increase of maximum xylose consumption rate and a 2-fold increase of ethanol productivity, respectively.

(C) Glucose consumption rates ($q_{glu}max$) and ethanol productivities (P_{EtOH}) with the strains in **(A)** The parental strain ScY01 had still the best glucose fermentation, but the genome-shuffled strain SSP had compared to SP a 1.35-fold increase of glucose consumption rate and a 1.57-fold increase of ethanol productivity.

All experiments in (A–C) were performed at 40°C using 30 g/L glucose or xylose as a sole carbon source.

Figure 6. Evaluation of high-temperature xylose or glucose fermentation by genome-shuffled and evolved strains.

In summary, the genome-shuffled strain SSP showed better high-temperature xylose and glucose fermentation capacities than the parental strain SP. The glucose fermentation capability of SSP was lower than the one of the parental strain ScY01, but the xylose fermentation capability was much higher. The lower glucose fermentation rate might result from the intergeneric hybridization. The cell growth and fermentation capacities on xylose as well as the fermentation capacity on glucose of the evolved strains adapted to high temperature first decreased and then rose again. This might be due to the underlying genetic changes which occurred during adaptive evolution. Also, because large parts of the dominant parental strain SP were inherited, it could cause a mistranslation of the CUG codon[23] as serine instead of leucine, which interferes with protein folding. But all in all, the hybrid strain shows an improvement considering the bioethanol production.

5.2 Pro and contra of gene shuffling

Benefits of this technique in our study:

- Co-fermentation of both Xylose(pentose) and Glucose(hexose) are made possible
- A higher fermentation rate is achieved
- possible fermentation at high temperatures
- DNA segments can be deleted or magnified for the hybrid strain, making the yeast more healthy

[23] (Yuping Lin Y. C., Development and genomic elucidation of hybrid yeast with improved glucose-xylose co-fermentation at high temperature, 2021)

- 1.5-fold increase in cell growth can be achieved

General advantages of genome shuffling:

- Genome shuffling has the advantage of exploiting the full genetic diversity in a population and makes it possible to combine useful mutations from many different individuals[24]
- Recursive genomic recombination within a population of bacteria can efficiently generate combinatorial libraries of new strains
- Can be done with multiple genes resulting in almost limitless number of hybrids/mutations
- Relatively easy to perform
- Simple and low in costs
- Significant improvement of the quality of industrially important microbiological phenotypes

Downsides of this technique in our study:

- CUG codon could be translated as serine instead of leucine, which could disrupt protein folding
- Environmental and nutritional stress can change the modification of the tRNA pool thereby possibly causing mistranslation

General disadvantages of genome shuffling:

- Ethical dilemma, limitations are natural and should not be changed, "god's work" – this applies to all kinds of introduced genetic modifications
- Shuffling can also also break up favorable combinations of genes

5.3 The future in this area of research:

Future research will be done in several areas of this subject. Nonetheless, we tried to highlight one of the most important ways of increasing Biofuel production, by looking at the fuel itself.
Ethanol is not the only product of yeast fermentation that can be used as fuel. Yeast does not only produce ethanol while fermenting, but it also creates isobutanol as well. Usually, yeast produces a small amount of isobutanol, but scientists have discovered a genetic switch that increases its production. Benefits of creating more isobutanol are that it has a 25% greater energy density than ethanol and it does not require as much water during production, however it is 10 times more toxic to the yeast. In the yeast cell isobutanol functions as a signal for starvation thereby telling the yeast to cease growth. Researchers from the University of Princeton have determined a gene involved in this process. They found out that deleting this gene remarkably improved the tolerance towards isobutanol. Productivity intensified 5-fold. The gene responsible for this change is GLN3. By simply deleting this gene, the yeast increases its performance. [25]

5.4 Advantages and Disadvantages of Biofuel:[26] [27]

Advantages of Biofuel:

The costs to produce biofuel are lower in relation to fossil fuels. The production method is environmentally more friendly, biofuels do not emit as many toxins as fossil fuels do and the amount of CO_2 released in the production process is the same amount as crops previously absorbed during photosynthesis. Biofuels burn more cleanly, as there is more complete combustion and It can be produced locally, because of its wide range of materials that can be used for the production, they only have to contain sugar and starch. This gives countries which lack their own fossil fuel industry a chance to become less dependent on energy import. [28]

[24] (Jan Steensels, 2014)
[25] (Wess, 2019)
[26] (Rinkesh, 2021)
[27] (Müller-Langer F. M., 2014)
[28] (Shazmi, 2011)

Disadvantages of Biofuel:

Biofuel production requires land conversion, to meet the fuel supply (biomass), which competes with human food crops and could lead to food shortage and higher prices for food in comparison to fossil fuels. Also, the biofuel production requires enormous amounts of water. It drains water supplies from humans. On top of that biofuels have a lower out-put of energy than traditional fossil fuels, which increases the fuel supply needed to produce the same amount of energy. Maybe the biggest problem is that large scale production is challenging, making it hard for biofuel to substitute fossil fuels.

6 Summary

We introduced the recent achievements towards enhancement of biofuel production.After that we focused on one technique, we explained the genetic engineering technique called gene shuffling using a published example (Development and genomic elucidation of hybrid yeast with improved glucose-xylose co-fermentation at high temperature from Yuping Lin et al. *FEMS Yeast Research 2019*) In that publification, the application of said techniques enhanced the capability of fermenting glucose and xylose at high temperatures has been enhanced. After learning about the topic of yeast fermenting sugar to ethanol, we searched for an expert, who answered our questions to help us get a better in-depth knowledge about yeast. We also pointed out the benefits and downsides of bioethanol as future engine fuel. At the moment it is not possible for yeast to be the sole fuel producer on a large scale and therefore it cannot fully replace our current needs for fossil fuel, but the biofuel industry is being heavily researched and shows promise for a more sustainable future.

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https://science.sciencemag.org/content/346/6205/75.full

Recent Scientific History: (visited between December 2020-January 2021)
How to make better biofuels? Convince yeast it's not starving (princeton.edu)
Biofuel breakthrough: scientists use GMO yeast to produce fuel (sciencenordic.com) Scientists discover how to protect yeast from damage in biofuel production – BIOENGINEER.ORG

Gene Shuffling: (visited January 2021)

Example for Genome shuffling Publication: https://academic.oup.com/femsyr/article/19/3/foz015/5333307
Other helpful sources:
https://www.researchgate.net/publication/51870752
https://www.researchgate.net/publication/230793138_Genome_shuffling_A_method_to_improve_biotechnological_processes
https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-019-1631-4#Sec9
https://www.researchgate.net/figure/Genome-shuffling-by-recursive-protoplast-fusion-1-Protoplasts-are-prepared-cell-walls_fig2_280329318
https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-019-1631-4#Sec9
https://bitesizebio.com/29721/dna-shuffling-like-pro/#attachment_29730

Bioethanol:
https://energsustainsoc.biomedcentral.com/articles/10.1186/s13705-014-0020-x
http://www2.hawaii.edu/~khanal/fungal/biofuels.html
https://www.carsdirect.com/green-cars/the-advantages-of-biofuels-over-fossil-fuels
https://www.conserve-energy-future.com/advantages-and-disadvantages-of-biofuels.php

Figures: (visited January 2021)
Figure1) 5561507098_2964a82752.jpg (333×500) (thinkingmomsrevolution.com)

Figure 2) https://www.elsevier.es/en-revista-brazilian-journal-microbiology-490-articulo-ethanol-production-in-brazil-bridge-S1517838216310346
Figure 3) https://academic.oup.com/femsyr/article/19/3/foz015/5333307
Figure 4)https://lifescience.canvaxbiotech.com/product/pickmutant-dna-shuffling-kit/
Figure 5) © FEMS 2019.This article is published and distributed under the terms of the Oxford University Press, Standard Journals Publication Model
https://academic.oup.com/journals/pages/open_access/funder_policies/chorus/standard_publication_model
Figure 6) https://academic.oup.com/femsyr/article/19/3/foz015/5333307

Appendix

Interview with Dr. Poonam Singh
She is a senior lecturer in Biotechnology at the School of Biomedical Sciences, Ulster University, in the UK. [29]

All of Dr. Poonam Singh's degrees:
DSc PhD CBiol SFHEA FRSB FCHERP FBRS FIFIBiop FAMSc
Director- MSc Program Biotechnology Research
Ulster University, Northern Ireland
Senior Fellow-Higher Education Academy, UK
Fellow-Royal Society of Biology, London
Fellow-Centre for Higher Education Research & Practice,UK
Fellow-Biotechnology Research Society of India
Fellow-International Forum on Industrial Bioprocesses
Fellow-Academy of Microbiological Sciences, India
Associate Advisor Biotechnology (Ex), British Council, UK

4.2 Interview

Could we completely replace our current needs of crude oil in the transport industry with sustainable bioethanol in the future, even though it requires an extensive use of land and cause a deficiency of crops? Ethanol is an immediately available solution to climate and air quality issues. Ethanol has experienced unseen levels of attention due to its value as fuel alternative to gasoline, the increase of oil prices, and the climatic changes, besides being a renewable and sustainable energy source, efficient and safe to the environment. Currently, worldwide ethanol production is in high levels. The future of bioethanol appears to be bright as the need for renewable energy sources to replace dependence on foreign oil is in high demand.

And if it is possible to use this biofuel from yeast, is it difficult to produce it on a large scale? Yes

Is the S. cerevisiae the commonly used microbe in bioethanol production and why is it easier to genetically modify it to suit our needs and how is it done? S. cerevisiae is the most commonly used in bioethanol production. However, it can only ferment hexoses but not

[29] (Ulster University, 2021)

pentoses. Only some yeasts from genera Pichia, Candida, Schizosaccharomyces and Pachysolen are capable of fermenting pentoses to ethanol.

Could every form of yeast be utilized for producing biofuel? Yes, natural and genetically modified both forms for different carbon source

or is there something particular in S. cerevisiae which makes it possible for us to modify it? Most industrial ethanol fermentations use the yeast Saccharomyces cerevisiae, as it exhibits fast sugar consumption, high yields, and ethanol tolerance.

Why can S. cerevisae only convert hexose sugars and not pentose sugars? The engineering of a *Saccharomyces cerevisiae* strain has been studied to make it able to utilize the pentose sugar for growth and to ferment it to ethanol. Expanding the substrate fermentation range of *S. cerevisiae* to include pentoses is important for the utilization of this yeast in economically feasible biomass-to-ethanol fermentation processes

Are there different possibilities for the ethanol fuel in the future?
Bioethanol is a fuel produced from plant sources, such as sugar. As a plant based fuel, bioethanol can provide an alternative to fossil fuels such as petrol, and can have lower emissions, depending on how it is produced.The EU Renewable Energy Directive includes a statutory target that 10% of transport fuel by 2020 must come from renewable sources such as electricity, hydrogen, and biofuels like bioethanol. Due to concerns about land use change to produce biofuels, the proportion of biofuels that can count towards the target is limited to 7%.

Escheria Coli or Zymomonas mobilis are also used during this ethanol production, in which way to they differentiate from S.cerevisae? S. cerevisiae is the most commonly employed yeast in industrial ethanol production as it tolerates a wide range of pH, thus making the process less susceptible to infection. *Baker's yeast* was traditionally used as a starter culture in ethanol production due to its low cost and easy availability.

If we compare Algae and land plants, 5-10 times more biomass can be produced by Algae, why is that and isn't it a more sustainable option than yeast ? Algae can be used as carbon source, still yeast are needed as microorganism for fermentation to produce ethanol

Why is it more useful to ferment pentose sugars than hexose sugars? Because it is a more simple sugar? Genetic engineering has been used to establish a D-xylose-utilizing pathway in *S.cerevisiae* by inserting heterologous genes encoding D-xylose reductase and xylitol dehydrogenase and by increasing the expression of its endogenous xylulokinase. This process has resulted in yeast strains able to utilize the pentose D-xylose and to ferment it to ethanol. However, efforts to establish an L-arabinose-fermenting *S.cerevisiae* strain have failed so far

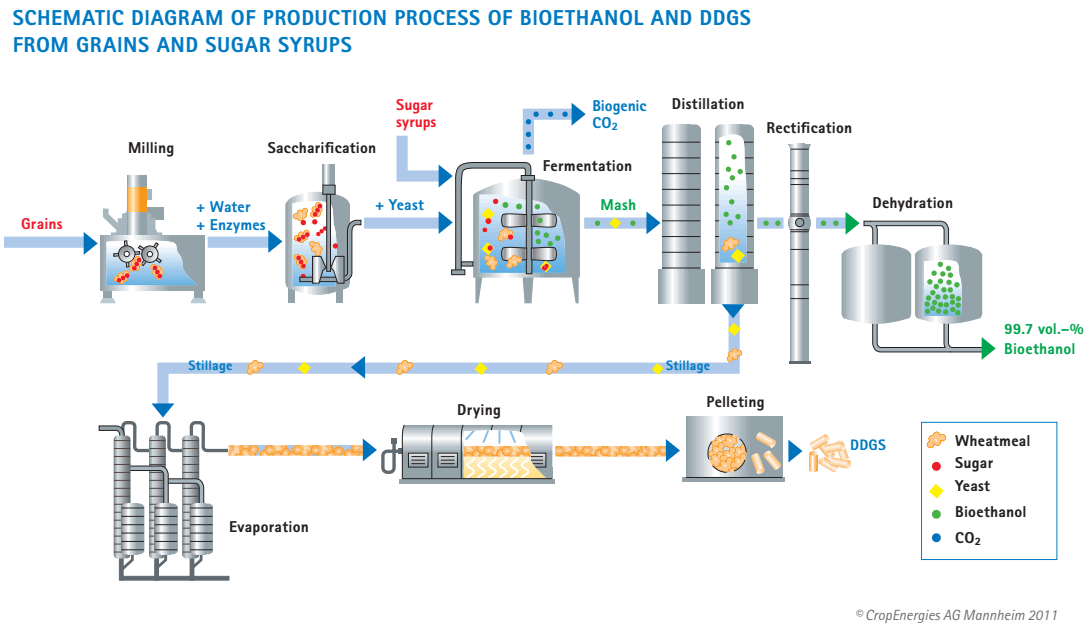
Which methods are being used to modify yeast? Gene modification of laboratory yeast strains is currently a very straightforward task thanks to the availability of the entire yeast genome sequence and the high frequency with which yeast can incorporate exogenous DNA into its genome. Unfortunately, laboratory strains do not perform well in industrial settings, indicating the need for strategies to modify strains to enable strain development for industrial applications.

Which process takes part in which part of the cell to convert glucose into ethanol? In the absence of oxygen, alcoholic fermentation occurs in the cytosol of yeast. Alcoholic fermentation begins with the breakdown of sugars by yeasts to form pyruvate molecules, which is also known

as glycolysis. Glycolysis of a glucose molecule produces two molecules of pyruvic acid. The two molecules of pyruvic acid are then reduced to two molecules of ethanol and $2CO_2$.

8 From biomass to bioethanol

This part is copied from the website of Crop Energies. It contains a general overview of the bioethanol production and should be helping for the general understanding of the process. Here is the link to the website: https://www.cropenergies.com/en/Ethanol/Produktionsverfahren/



At this plant the production of bioethanol from starch-containing cereals takes place in five steps:

1. Milling, i.e., the mechanical crushing of the cereal grains to release the starch components
2. Heating and addition of water and enzymes for conversion into fermentable sugar
3. Fermentation of the mash using yeast, whereby the sugar is converted into bioethanol and CO_2
4. Distillation and rectification, i.e. concentration and cleaning the ethanol produced by distillation
5. Drying (dehydration) of the bioethanol

Bioethanol can also be produced directly from sugar syrups. This dispenses with steps 1 and 2, which serve to prepare the grain for fermentation.