Investigation regarding the Biosolvent Yield of Industrially Relevant Biocatalysts, and an Economic Evaluation for the Valorisation of Agricultural Waste

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Declaration

I do declare that no part of this thesis has been submitted in support of an application for any degree or qualification at the University of Kent, any other university or institute of higher education.

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Abstract

There are 3.3 million tonnes of agricultural waste a year produced at the farm stage of the food production chain in the UK (1). Waste agricultural product is sent for composting, to landfill, to be spread on fields or sent for anaerobic digestion producing biogas for electricity. There are 650 anaerobic digestors in the UK (2), which produce ~300kWh per tonne of food waste (2). This project is investigating the economic viability of producing bio-solvents from waste agricultural produce by fermentation using *S. cerevisiae*, *S. stipitis*, or *C. saccharoperbutylacetonicum*, or whether it is more economically viable to send the waste for anaerobic digestion.

The amount of bio-solvent produced by these microorganisms when supplemented with different carbon sources has been analysed by GC-MS. With the bioethanol yield produced by *S. cerevisiae* being greater than that of *S. stipitis*, whilst *S. stipitis* has a higher biomass yield and can utilise a wider variety of carbon sources than *S. cerevisiae.* The biosolvent yield achieved by *C. saccharoperbutylacetonicum* was low in comparison to *S. cerevisiae* or *S. stipitis*, as well as being an unreliable and temperamental strain, but does have the advantage over *S. cerevisiae* and *S. stipitis* of producing acetone and butanol as well as ethanol.

An economic evaluation has been undertaken to determine the economic viability of bioethanol production and how it compares financially to anaerobic digestion. Bioethanol fermentation requires a high upfront investment, due to the large amount of infrastructure, and once the feedstock costs are considered bioethanol produced by fermentation only becomes financially viable once the by-product of ‘Dried Distillers Grains’ is sold as a protein rich animal feed. Whereas anaerobic digestion is simpler in terms of operation and feedstock requirements, and is much more scalable than bioethanol fermentation.

The conclusion of this investigation has been that the disposal of agricultural waste by means of anaerobic digestion is preferable to the fermentation of said waste to produce biosolvents. This is due to the high upfront investment because of the large amount of infrastructure required for bioethanol production. As well as the simplicity of anaerobic digestion in terms of operation and feedstock requirements, together with its scalability.

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Presentations

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Talk given, which was entitled “Investigating the economic viability of the large-scale production of bio-solvents by the valorisation of agricultural waste.”.

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Introduction

Organic waste is a growing problem globally, with developed nations producing more waste per capita than developing nations and LDCs (Least Developed Countries). In the United Kingdom there is an estimated 12.8 million tonnes of food waste produced annually. With 9.5 million tonnes of this waste being produced by the hospitality, wholesale, household, and retail sectors (3). The majority of which ends up in landfill sites (4).

The remaining 3.3 million tonnes of food waste is produced before the farm gate (1), meaning that 25.8% of the food waste produced annually in the United Kingdom is present on farms. Meaning that there is presently a large supply of organic waste material being produced on farms in the United Kingdom, which could be utilised to produce commodities such as; electricity, heat, biomethane, and biosolvents. This organic waste could be utilised locally using farm scale anaerobic digestors, or at a community level by a collective of farmers owning and operating an anaerobic digestion plant. As of 2023 there are 650 anaerobic digestors operating across the United Kingdom (2).

This organic waste could also be used for large scale bulk batch fermentation to produce biosolvents such as ethanol. This bioethanol can then be used to supplement fossil fuels in petrol, which would reduce the amount of fossil fuels used.

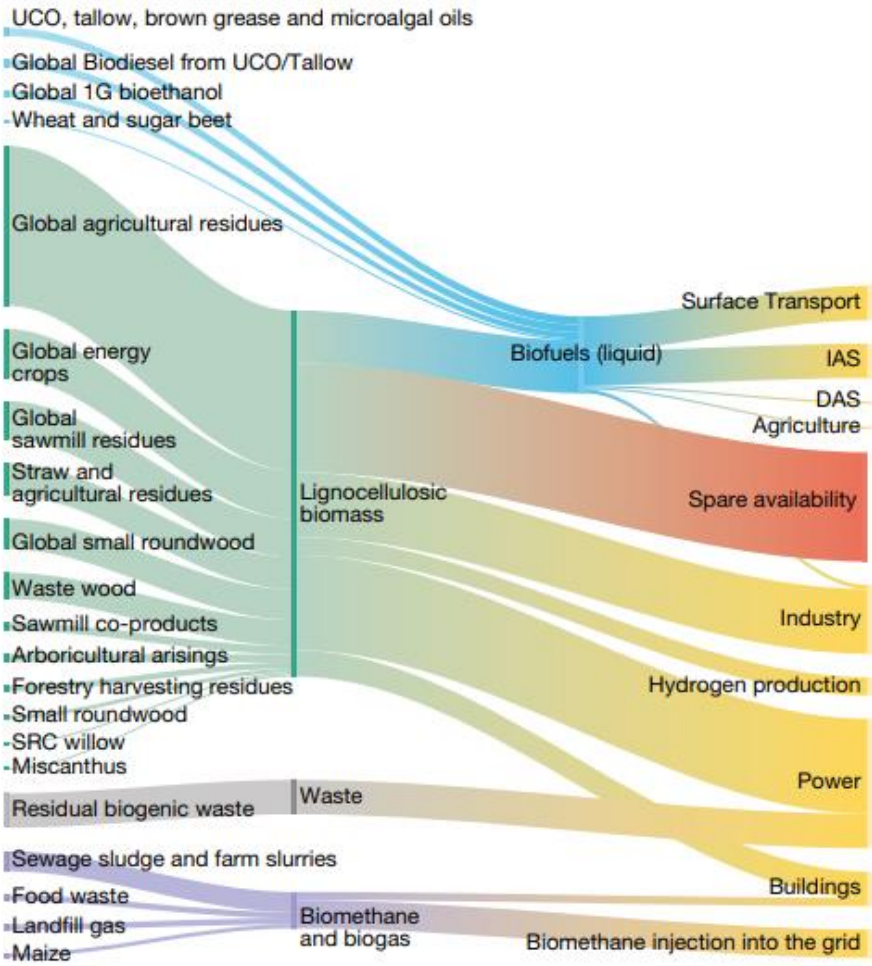


*Figure 1 - **Sankey diagram of the origin of biomass and the end-use sectors.** In an ambitious scenario for biomass supply to the United Kingdom in 2035.* (5).

Figure 1 shows the potential biomass supply to the United Kingdom in an ambitious scenario in 2035. A large amount of the biomass is being imported from abroad, but there is a large amount of 'Spare availability' which could be funnelled into biofuel production, or green energy production by Anaerobic Digestion.

There's a wide range of potential feedstocks available for these processes. Such as, agricultural waste, be it sugar beet leaves which have been removed prior to harvest, hedgerow trimmings, vine prunings or slurry and manure from livestock. Commercial food waste is another potential feedstock, with 18% of annual food waste being produced by the hospitality sector (6). In Kent 36,000 tonnes of municipal food waste was collected and sent to a local Anaerobic digestion plant to be turned into green electricity (7). Silage, and purpose grown energy crops such as corn grain or sugar beet can also be used to produce green energy and biosolvents.

In recent years there has been a push on the national level for energy independence due to price shocks in the energy sector due to the Covid Pandemic and the war in Ukraine. As well as encouragement for the economy to transition into a more circular economy which is more environmentally friendly, where products last longer, and are repaired. As well as, extracting all we can from raw materials, and finding a use for waste materials. This is known as 'Valorisation' which is the concept of increasing the economic value of a waste, by transforming the waste into a useful product. The purpose of this work is to investigate the potential of valorising organic waste, into energy or biosolvents.

Anaerobic Digestion:

Anaerobic digestion to produce biomethane for electricity is carried out in Anaerobic Digesters (Fig. 2). This can be carried out at different scales, be it a small scale farm anaerobic digester (2000m^3, producing 100kW (8), up to a multi-digester anaerobic digestion facility, such as the 20MW capacity AD plant in Penkun Germany (9) anaerobic digester which is used to generate electricity for homes and industries. The average price of an anaerobic digestion plant per kW of capacity is £4,500 (10).

*Figure 2 - **Anaerobic Digestion Plant**, located in Ireland* (11)***.***

In 2020 there were 579 anaerobic digestion plants in the UK. 418 of these plants were farm fed, with a cumulative installed capacity of 222MWe. The remaining 161 plants were fed using waste, such as municipal waste or waste sourced from catering businesses, these 161 plants had a combined installed capacity of 244MWe. In 2020 there were 331 anaerobic digestion plants in development, showing that this is a rapidly growing industry (12).

Anaerobic digestion uses bacteria to break down organic matter in the absence of oxygen. This process produces biogas. This biogas can subsequently be purified to produce biomethane (biogas is 50-70% biomethane) (13).

The biomethane produced can be sold by direct injection into the grid, used as a component in industrial processes, or to fuel some vehicles (specially designed buses or lorries). The biomethane can also be burnt on site to generate electricity.

In 2020 1.021 million tonnes of Oil Equivalent Energy was produced in the United Kingdom by anaerobic digestion (14).

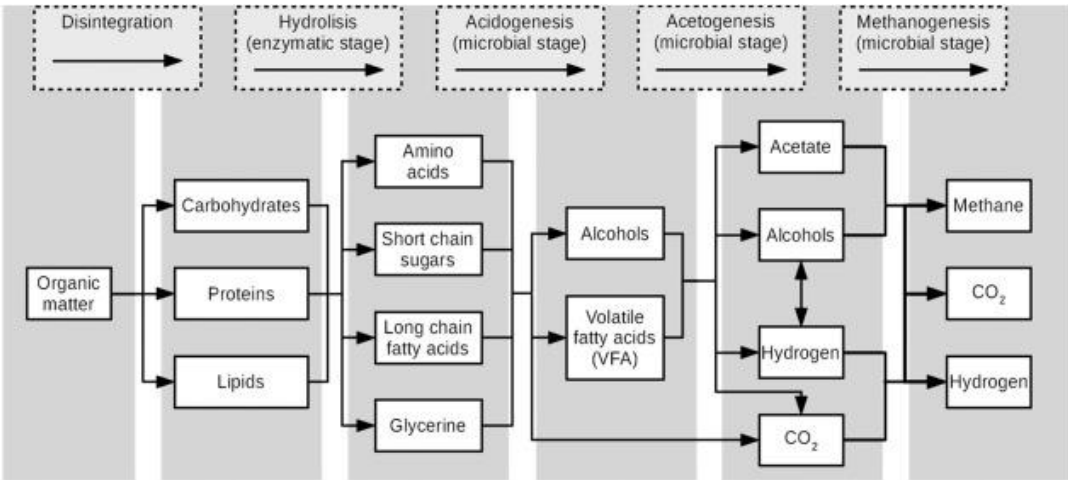


Figure 3 - ***Flow chart demonstrating the multistep biochemical process of the breakdown of feedstock to produce methane which occurs within an Anaerobic Digester*** (15)***.***

Figure 3 shows the biochemical process which takes place in an Anaerobic Digester. Figure 3 demonstrates that the digestion of feedstock to methane is a complex multistep process. Organic matter is broken down to macromolecules through abrasive action caused by the stirring of the substrate within the digester. These macromolecules are then broken down into their constituent parts, such as short chain sugars and amino acids. This process is undertaken by extracellular enzymes hydrolysing the macromolecules, the hydrolyses step is the rate limiting step in the process of digestion of feedstock to biomass (16).

These compounds are then transformed into fatty acids by acidogenic bacteria, such as bacteria of the genus; *Clostridium*, *Butyirbacterium*, *Eubacterium* and *Ruminococcus* (17). The facultative acidogens also consume the remaining oxygen in the process, which protects the methanogens which are oxygen-sensitive (17).

Acetogens catabolise the metabolites of acidogenic bacteria into acetate. Acetogenesis is carried out by bacteria such as; *Acetitomaculum*, *Eggerthella* and *Tepidanaerobacter*, amongst others (18). Acetogenesis is the conversion of volatile fatty acids and alcohols to hydrogen gas, carbon dioxide, acetate and shorter chain alcohols which are then transformed into methane gas by methanogenic bacteria such as species of *Methanospirillium*, and *M. harundinacea* (19).

The efficiency of anaerobic digestion plants is modulated by numerous factors, such as the operating temperature of the plant. Whether the operating temperature of the plant is optimum for the bacteria present will affect the performance of the anaerobic digestion plant since the enzymes produced by the bacteria present are responsible for the majority of the catabolise of the feedstock, through to the production of the biomethane.

If the anaerobic digester is supplemented with nutrients will impact the performance, because the nutrients may aid in the growth and functioning of the bacteria. The nutrients could be cofactors for enzymes which are vital for the catabolise of the feedstock.

The microbial composition of the anaerobic digester will affect the performance of the plant. There is a diverse range of anaerobic digester microbial composition used globally. The composition will be optimised to the primary feedstock of the anaerobic digester. To increase the efficiency and effectiveness of the anaerobic digester (20).

The pretreatment of feedstocks can lead to an improvement in the digestion efficiency. However, the increase in efficiency of the breakdown and transformation of the feedstock into biomethane may not outweigh the increase in cost of the pretreatment, or the cost of downstream processing which may be required due to the pretreatment method (21).

Anaerobic digestion plants can agree to fixed 20 year tariffs for the electricity or biomethane they generate (UK, (22)). This means that the operator will know what their revenue will be for the next 20 years, and will be able to plan loans, purchasing of additional feedstock, and whether the operation will be financially viable and what the payback time will be. There is also a premium on green electricity over energy generated by fossil fuels (2).

Feed in tariffs were available between 2010 and 2019. Feed in tariffs were available to facilities which had a capacity <5MW, and the value of the electricity produced decreased in a bracketed system, meaning an above market rate was paid per kWh to small scale renewable energy producers. This was to incentivise investment in small scale renewable energy sources, such as farm based anaerobic digesters (2).

The Green Gas Support Scheme (GGSS) is a government scheme running from 2021 to 2028. The GGSS pays producers of biomethane quarterly, based on the amount of biomethane which the producer directly injects into the grid (22).

Operators of anaerobic digesters can gain an additional revenue stream by charging a gate fee per tonne of organic waste to municipalities and members of industries, such as the catering industry. By charging a gate fee operators gain an additional source of revenue, as well as receiving feedstock. The benefit for municipalities and industry of sending their organic waste to an anaerobic digestion plant instead of landfill is that they are able to be more environmentally friendly, which is popular with constituents and consumers.

The gate fee charged at an anaerobic digestion plant (the average price in the UK in 2023/2024 was £26/tonne (23), but this value can be variable, due to location, and AD feedstock supply), is often lower for industry than disposing of their waste at a landfill (~£130.70/tonne, this is the average landfill fee (£27/tonne) (23), as well as the standard rate landfill tax in the UK (£103.70/tonne) (24)), this is due to the price of using a landfill increasing due to taxation to discourage landfill usage.

<u>Bioethanol:</u>

Bioethanol is ethanol which has been produced from organic matter, such as corn grain, sugarcane, or sugar beet. Bioethanol is ordinarily produced by the fermentation of a sugar rich feedstock, by the eukaryotic microorganism *S. cerevisiae*, by batch production, in very large fermenters.

Globally 104.5 billion litres of bioethanol was produced in 2021 (25). In the United States corn grain is the primary feedstock for the production of bioethanol, with 34% (135.7 million tonnes (26)) of all the corn grain produced being used to produce bioethanol.

Large scale batch fermentation is a complex multistage process (Fig. 4). This process requires multiple stages, and due to economies of scale is done on a very large scale to make the process economically viable, therefore there is a high start-up infrastructure cost to this process.

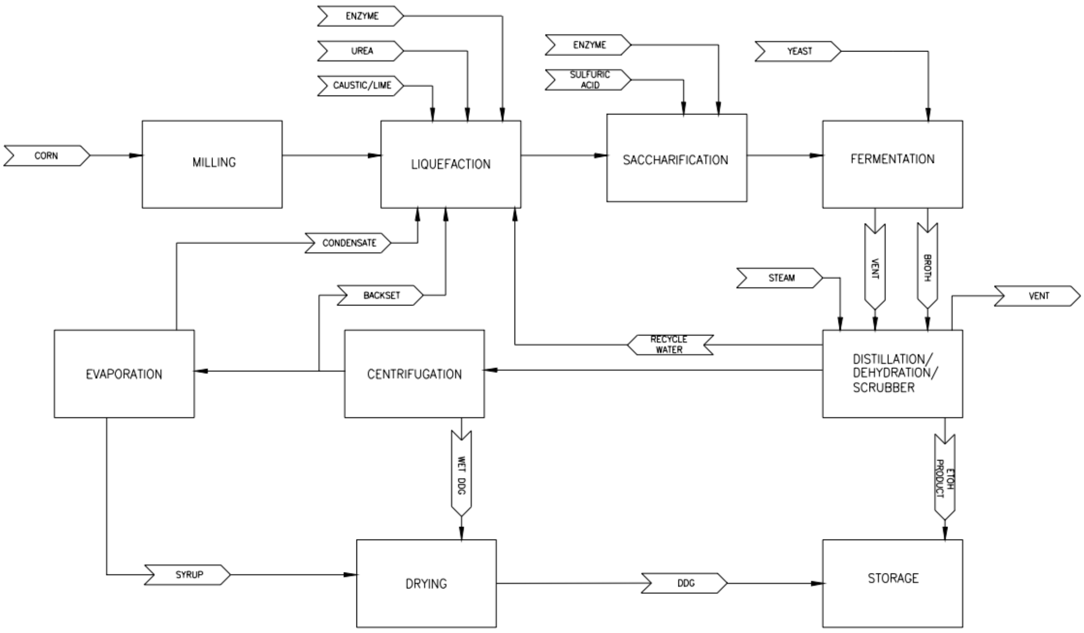


Figure 4 - ***Process of the bulk batch fermentation of corn grain*** (27).

Figure 4 shows the process by which bioethanol is produced from corn grain. A by-product of this process is Dried Distiller's Grain (DDG) which can be utilised as animal feed. The first step in the process is milling, this is where the corn grain is powdered.

Then the powdered corn is mixed with water to produce a slurry, as well as urea (which provides nitrogen), lime (helps to maintain the pH of the mix, sulphuric acid, α-amylase and glucoamylases. The α-amylase converts the starch present in the ground corn to dextrins, which are a mix of glucose polymers, which are then broken down into glucose by glucoamylases (28). This process is done in the liquefaction and saccharification steps (Fig. 4).

The slurry which contains a high concentration of free glucose monomers then has yeast added, and is left for 46 hours, and the ethanol concentration at the end of the fermentation time is 12% by volume (27). Gas which is produced during fermentation is vented.

After fermentation the slurry is distilled to extract the ethanol from the mix, increasing the concentration of ethanol. The distillation step requires steam, and the wastewater produced is directed to the liquefaction step to be reused.

The remaining 'cake' of the fermentation slurry is centrifuged using a decanter centrifuge, the wet DDG is sent for drying. The backset which is an acidic fluid is directed back to the liquefaction and helps to catalyse the fermentation and reduce the risk of bacterial contamination. The 'supernatant' remaining after centrifugation is heated to remove water, which is also directed back to the liquefaction step, and the syrup which remains is sent for drying alongside the wet DDG.

Once the DDG is dried then it can be sold as a protein rich animal feed, and the bioethanol produced can be sold to supplement petroleum based fuel.

Biofuel Usage and Projections:

Bioethanol can be used to supplement petroleum based fuels at different blend levels. The blend being the ratio of bioethanol to petroleum based fuel.

E10 petrol is unleaded petrol which contains 10% bioethanol, and 90% petroleum based fuel. In September 2021 the Government of the United Kingdom mandated that E10 petrol would be the standard petrol grade.

E10 petrol is commonly used across Europe, the United States of America and Australia.

Brazil has historically been using fuel with a high blend of bioethanol to petroleum based fuel. The average blend rate for Brazil in 2018 was 52% (Global Demand for Fuel Ethanol Through 2030), although this blend proportion is skewed upwards due to the use of bioethanol directly as fuel.

There is a continuing growing demand for petroleum based fuel. In 1975 37.85EJ of oil was used for transport, rising to a maximum of 111.18EJ in 2019, then dropping 4.3% to 106.41EJ in 2022 (IEA). In the International Energy Agencies net zero scenario, by 2030 the oil used for transport will need to drop a further 23.8% from the 2022 total to 81.13EJ.

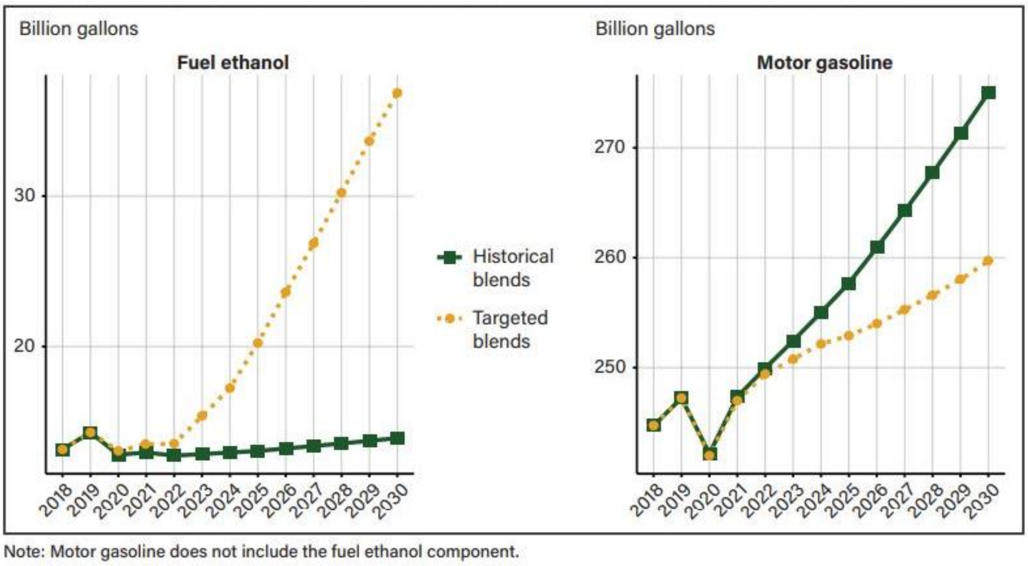


*Figure 5 - **Projected Fuel Ethanol and Petroleum based fuel consumption, 2018-2030.*** (29)*.*

As the targeted blends thresholds are reached, the demand for bioethanol will increase drastically, as the global blend increases from a few percent, up to 17 percent. The overall demand for fuel is projected to increase as more countries become developed, but if the targeted blends are reached then less petroleum based fuel will be used than if historical blend rates are maintained.

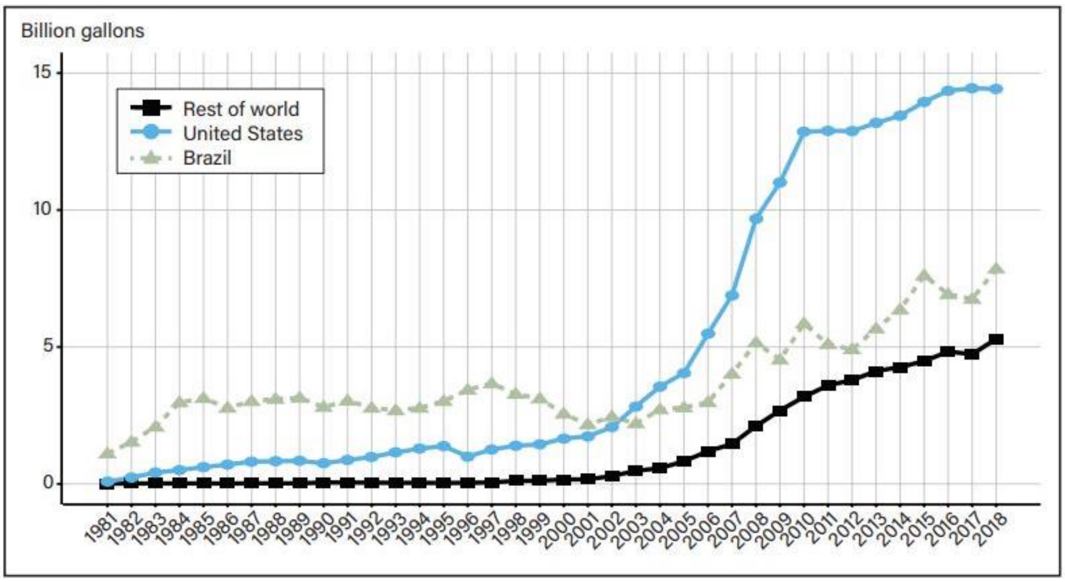


*Figure 6 - **The Consumption of Fuel Ethanol Globally, between 1981 and 2018.*** (29)*.*

Figure 6 shows the combined effect of increasing fuel usage, alongside increasing blend ratios. Which is the case in America, and rest of the world, whereas Brazil is just using more fuel, and the blend ratio isn't due to increase further. Brazil has consistently been targeting a blend rate of 50% (2008 – 2021), however the actual blend rate has fluctuated.

The demand for bioethanol may increase further than expected if more countries mandate blend targets, as well as countries with pre-existing blend targets increasing said targets.

However, it should also be considered when projecting the demand for bioethanol is the uptake of electric vehicles, in developed nations and globally. This is because electric vehicles do not require petroleum based fuels, or bioethanol. Being able to be ran with electricity generated from sources which are generated without requiring plant-based or fossil-based fuels, such as by nuclear energy, wind, or solar power.

Feedstocks:

There are various potential feedstocks available for Anaerobic digestion or the production of bioethanol, such as corn silage, corn grain, sugar beet and food waste. Different potential feedstocks have pros and cons regarding their seasonal availability, cost, and the carbohydrate to mass ratio of the feedstock.

Different feedstocks have different carbohydrate compositions, which may mean that some feedstocks would require pre-processing to enable the biocatalysts to fully utilise the feedstocks. Such as if the feedstock is rich in complex carbohydrates such as starch. Different carbohydrate monomers may impact the biocatalysts differently in respects to biomass yield, and product yield.

The bio solvent yield and biomass yield determined from testing individual carbon sources, could then be used to estimate the potential bio solvent and biomass yield of different real feedstocks, based upon their sugar composition.

Composition of Corn Silage:

Corn silage is produced by harvesting corn whilst the stem and leaves are still green, and harvesting/shredding it using a forage harvester. By harvesting it when it is still green the water content will still be high, meaning that when it is compressed and covered it will ferment anaerobically well. When producing silage from corn the whole plant is utilised as biomass, instead of just the corn grain. Corn silage is more beneficial as a feed for cattle than hay, since silage is already partially digested, and is more nutritious. Corn silage is also a good feedstock for anaerobic digestion, as a lot of biomass is produced when corn is grown.

Corn silage is rich in starch, with the starch content being 328g/kg of dry corn silage (30). Corn silage has 128g of pectin and other sugars per kg of dry corn silage, resulting in 457 g/kg of nonfiber carbohydrates (NFC) (30)(31). Corn silage also has a high content of NDF (neutral detergent fibre), at 406g/kg (30)(32). Corn silage contains 247 g/kg of cellulose and lignin which is quantified under the label of ADF (Acid Detergent Fibre) (30)(33).

Composition of Corn grain:

Depending on when corn is harvested the kernel can be considered to be a fruit if the corn is harvested at maturity when the ear of corn has reached optimum ripeness and has a water content of ~70% (34). Whereas if corn is harvested later when the kernels have dried, then the harvest is considered to be a grain. Corn grain should be harvested when the average moisture is between 26% and 34% (35). Starch is the most abundant constituent of corn grain, comprising 72-73% of the kernel by weight (36), followed by 8-10% of the mass being protein, followed by 4-5% being oil (37). Glucose, fructose, and sucrose is also present in corn grain at amounts of 1-3% (36).

Composition of sugar beet:

Sugar beet is a root vegetable which is commonly grown for sugar production. Sugar beet is very rich in sucrose, with the free sucrose present in fresh sugar beet being 18.77% w/w. Galactose, ketose, and glucose are present in much lower quantities than sucrose in fresh sugar beet, at 0.54%, 0.30%, and 0.26% w/w respectively (38). The conditions in which the sugar beet is stored changes the carbohydrate content of the sugar beet. With sugar beet which has been stored in an uncovered mound containing 35% more sucrose than sugar beet which has been stored undercover (38).

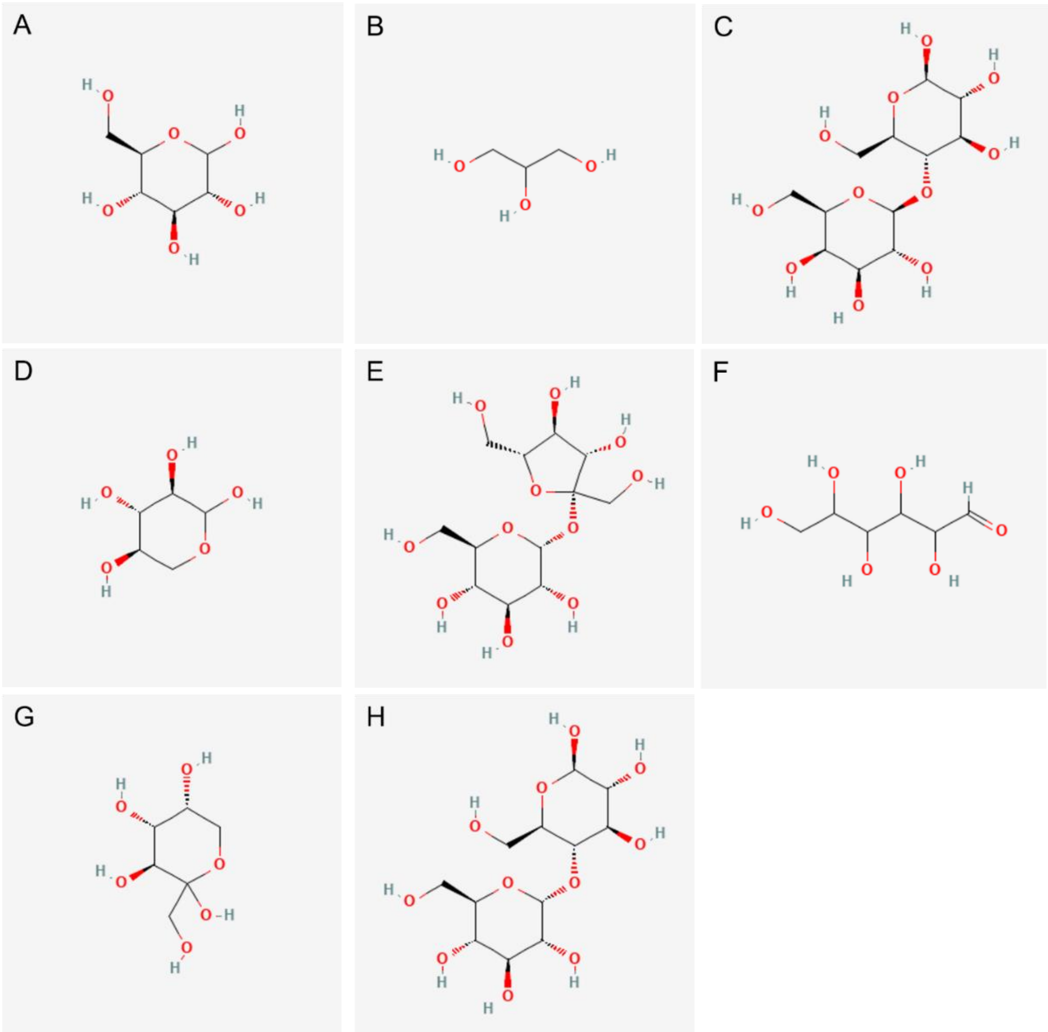


*Figure 7 - **Individual Carbon Sources Used as Experimental Feedstocks.** Glucose (A), Glycerol (B), Lactose (C), Xylose (D), Sucrose (E), Mannose (F), Fructose (G), and Maltose (H). Sourced from PubChem.*

Glucose is a hexose monomer, which is the most abundant monosaccharide on earth. Glucose is the monomer, which is formed into the polymer cellulose in plants, which is the most abundant carbohydrate, and is the main constituent in plant cell walls.

Glycerol (Fig. 7B) is a three carbon triol, which is a waste product of industrial processes such as the production of biodiesel (39). Meaning that as the production of biodiesel increases (the global production of biodiesel is expected to reach 50 billion litres by 2030 (40), there will be an increase in the supply of crude glycerol available.

Lactose is a disaccharide comprised of a galactose monomer, and a glucose monomer. Bovine milk is 4.8% lactose (41), whereas goats' milk is 5.1% lactose (42). It is estimated that 3.2% of all milk produced in France is lost at the farm stage (43). Milk wastage on farms is evidently a large problem, given that there is guidance from the UK government for farmers on how to dispose of their unsold/waste milk (44). 490 million pints of milk is wasted in the UK each year across the supply chain (45).

Xylose is a pentose sugar, which contains an aldehyde functional group, meaning xylose is classified as an aldopentose. Xylose is the second most abundant sugar in the nature, and a major constituent of lignocellulosic biomass (46). Xylose is found in angiosperm plants (flowering plants).

Sucrose is a disaccharide comprised of glucose and fructose monomers connected by a glycosidic bond between the first carbon on the glucosyl subunit and the second carbon on the fructosyl subunit. Sucrose is found naturally occurring in sugar beets, sugar cane, and in honey at values of <5% of the total composition of the honey.

Mannose is an aldohexose molecule, meaning it is a six-carbon molecule which has an aldehyde functional group. Free mannose can be found in oranges, peaches and apples (47). Mannose can form polymers called mannans which are a component of yeast cell walls (48) and are found in the seeds of legumes and in the cell wall of gymnosperms (49).

Fructose is a ketohexose monomer, which when in the linear form has a ketone functional group on the second carbon atom. Fructose is a monosaccharide which is found in fruits and honey. Fructose is used by plants to form sucrose, as well as being used to give the fruit and flowers a sweet taste which encourages animals to ingest them, promoting seed dispersal.

Maltose (Fig. 7H) is a disaccharide comprising of two glucose monomers. Maltose is a product from the breakdown of starch, which can be produced industrially by saccharification (50). Maltose can be found in germinating grains (51).

Strains (Biocatalysts):

Saccharomyces cerevisiae is a species of budding yeast which has historically been used for the baking of bread, and the fermentation of grapes for wine, and grain for beer. There is evidence that S. cerevisiae has been used since 7,000 BC to produce wine-like beverages (52). *S. cerevisiae* is used globally to produce fermented beverages such as beer and wine, as well as to cause the dough to proof in leavened doughs, which is then baked into bread, which is a staple food eaten globally. *S. cerevisiae* is also used industrially to produce bioethanol, chosen because it has the natural yeast with the highest ethanol tolerance (53).

YDL022W is a mutant strain of *S. cerevisiae*. YDL022W has the gene 'GPD1' knocked out. GPD1 encodes for NAD-dependent glycerol-3-phosphate dehydrogenase (54). This enzyme produces glycerol from the same precursor metabolites as ethanol is produced from. So, it was hypothesized that the knocking out of GPD1 would result in an increased ethanol yield, due to a redirection of metabolic flux.

Scheffersomyces stipitis (formerly known as *Pichia stipitis*) is an ascomycetous yeast, meaning it forms spores which are developed in asci. *S. stipitis* can produce ethanol by aerobic fermentation, or by fermentation in microaerophilic environments. *S. stipitis* is known for its ability to ferment the pentose sugar xylose, which makes this biocatalyst an interesting organism to investigate its potential application in bioethanol production. However, has a low ethanol tolerance (53), meaning that a fermentation using just *S. stipitis* wouldn't result in a high bioethanol yield, meaning that *S. stipitis* should be a constituent in a mixed culture, where maybe *S. cerevisiae* is inoculated into the reaction vessel once the ethanol content has reached a threshold where *S. stipitis* is becoming less effective. The use of *S. stipitis* in this way would allow for an increased variety of feedstocks to be utilised, whilst still producing a high enough bioethanol yield for the process to be economically viable.

Clostridium spp. are rod-shaped gram variable bacteria, which begin as gram positive, however present more as gram negative as the *clostridium* culture ages. *Clostridium spp.* are endospore forming bacterium, and are responsible for tetanus and botulism. Botulism being due to the botulin toxin produced by *C. botulinum* (55), and tetanus being caused by *C. tetani* (56). *C. perfringens* is a bacterium which can be the cause of food poisoning (57). *Clostridium spp.* can be found in various locations ranging from the human intestine, to the soil, to decaying vegetation (58)(59).

Clostridium saccharoperbutylacetonicum is an industrial anaerobic microbial strain, which produces Acetone, Butanol and Ethanol (ABE) by solventogenesis. ABE fermentation was first industrially used in the mid-1910s, but there was a decline in this method of production of ABE in favour of using fossil fuels in the 1950s, and then a resurgence in the 1970s due to the increase in the price of oil.

Metabolic Pathways:

The conversion of monosaccharides such as glucose into biosolvents by microorganisms acting as biocatalysts is often a complex multi-step process which produces numerous intermediates as well as secondary products which can reduce the efficiency of the process in regards to quantity of desired product produced.

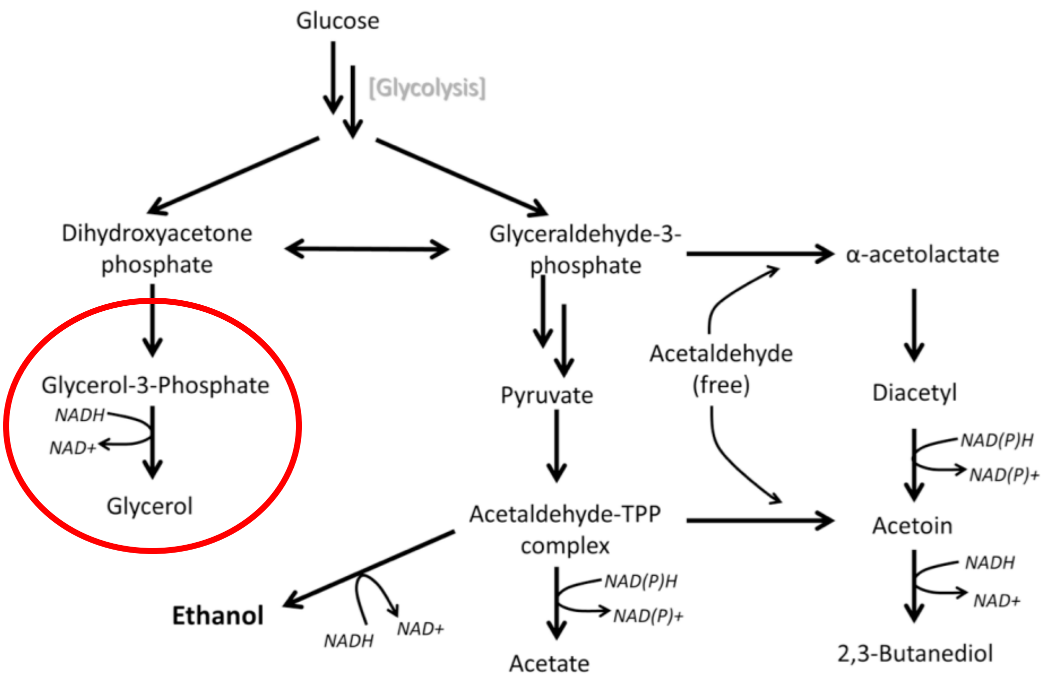


*Figure 8 - **Metabolic Pathway of the Production of Ethanol by S. cerevisiae.*** (60).

Figure 8 shows the metabolic pathways involved in the production of ethanol by *S. cerevisiae* starting from the input of the monosaccharide glucose. *S. cerevisiae* produces ethanol as a by-product of anaerobic respiration. Glycolysis converts glucose into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, the latter of which can be converted into glyceraldehyde-3-phosphate.

Glyceraldehyde-3-phosphate is then transformed into pyruvate, which is the second half of the glycolytic pathway (also known as the Embdem – Meyerhof pathway (61)).

Pyruvate is then converted into acetealdehyde by pyruvate decarboxylase (62), which is then converted into ethanol, which is catalysed by the enzyme alcohol dehydrogenase I (63).

Alongside the desired product of ethanol produced, the side product acetate is produced by aldehyde dehydrogenase converting acetaldehyde into acetate from acetaldehyde (64). Glycerol is also produced as a by-product by the catalysis of dihydroxyacetone phosphate into glycerol-3-phosphate by the cytosolic enzyme Glycerol-3-phosphate dehydrogenase (65). Glycerol-3-phosphate is dephosphorylated into glycerol. The production of glycerol by yeast forms part of the response when the yeast cell undergoes osmotic stress (66).

2,3-Butanediol is another product of anaerobic respiration by yeast, where Glyceraldehyde-3-phosphate is converted to 2,3-Butanediol, via the intermediates α-acetolactate, diacetyl, and acetoin. 2,3-Butanediol is produced in very small quantities in *S. cerevisiae,* 2,3-Butanediol has uses for the production of drugs, as well as synthetic rubber as an alternative to fossil fuel based petroleum (67).

The YDL022W mutant *S. cerevisiae* knocks out the enzyme glycerol-3-phosphate dehydrogenase, which converts glycerol-3-phosphate to glycerol (Fig. 8, circled in red). The knocking out of genes related to the redirection of metabolic flux away from the end product of ethanol has been shown to increase ethanol yield (68), so it was hypothesised in this work that by knocking out glycerol-3-phopshate dehydrogenase the ethanol yield would be increased.

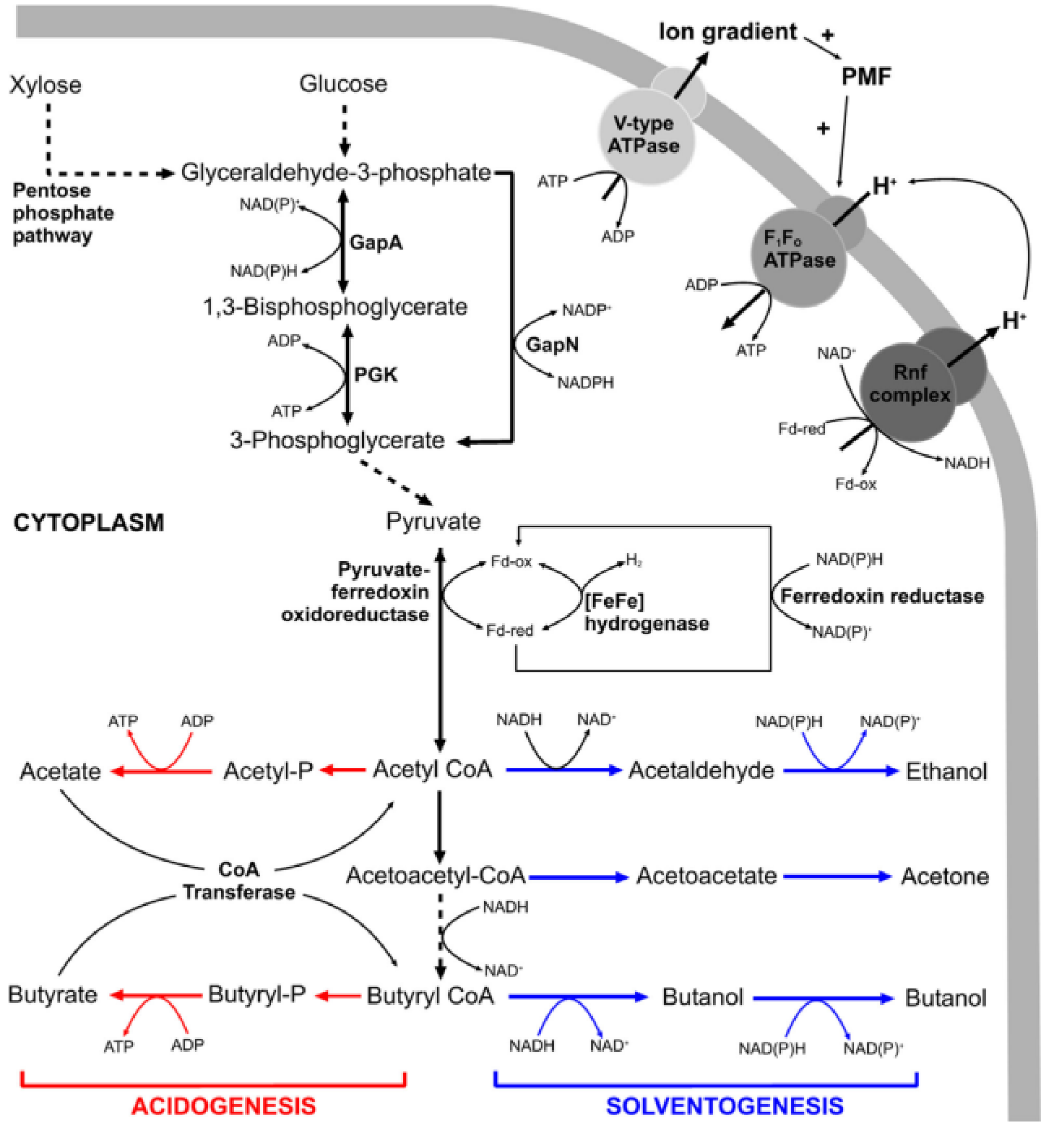


Figure 9 - ***Metabolic Pathways of the Production of Acetone, Butanol and Ethanol by C. saccharoperbutylacetonicum.*** (69).

Figure 9 shows how glucose and xylose can be metabolised by *C. saccharoperbutylacetonicum* into acetone, butanol, and ethanol. This is by the pentose phosphate pathway catalysing the conversion of xylose into glyceraldehyde-3-phosphate, this is where the pentose phosphate pathway links into glycolysis where glucose is being transformed into glyceraldehyde-3-phosphate, leading to pyruvate.

Pyruvate-ferredoxin oxidoreductase then catalyses the conversion of pyruvate to acetyl CoA, by using reduced ferredoxin as an electron donor (70), which becomes oxidised ferredoxin, which is then re-reduced by ferredoxin hydrogenase.

Acetyl CoA can then be converted into acetate via acetyl phosphate, or converted into acetoacetyl CoA by cytosolic thiolase (71), or transformed into acetaldehyde which is then converted into Ethanol. Acetoacetyl CoA is converted into acetone via acetoacetate, or into Butyryl CoA by butyryl CoA dehydrogenase (71) and NADH. The butyryl CoA can undergo solventogenesis to produce butanol, or acidogenesis to produce butyrate.

The production of acetate and butyrate is acidogenesis, whereas the production of Ethanol, acetone and butanol is solventogenesis.

<u>Research Aims:</u>

The aims of this research project are to:

- Quantify the biomass yield of different yeast strains (*S. cerevisiae, and S. stipitis)*, when grown in minimal and complex medias, supplemented with a variety of carbon sources.
- Quantify the ethanol yield of different yeast strains (*S. cerevisiae, and S. stipitis)*, when grown in minimal and complex medias, supplemented with a variety of carbon sources.
- Evaluate if the YDL022W mutant strain of *S. cerevisiae* has a higher ethanol yield than the wildtype *S. cerevisiae* strain.
- Quantify the biomass, and ABE (Acetone, Butanol and Ethanol) yield of *C. saccharoperbutylacetonicum*, when supplemented with a variety of carbon sources.
- Evaluate the economic viability of the industrial processes of Anaerobic Digestion and Bulk Batch Fermentation.

Materials

Yeast Media components:

- Adenine, Thermo Fisher Scientific, CAS: 73-24-5.
- Uridine, Thermoscientfic, CAS: 58-96-8.
- Bacto Peptone, Gibco, 211677.
- Difco Agar Bacteriological, 214530.
- Yeast Extract Powder, Melford, CAS: 8013-01-2.
- Synthetic Complete Mixture (Kaiser) Drop-Out: Complete, Formedium, DSCK1000.
- Difco Yeast Nitrogen Base w/o Amino Acids, 291940.

Clostridium media components:

- Clostridial nutrient medium, Sigma Aldrich, Millipore, 27546-500G-F.

Supplementary Carbon sources:

- Glucose, Fisher Scientific, CAS: 50-99-7.
- Glycerol, Fisher Scientific, CAS: 56-81-5.
- Lactose, Fisher Chemicals, CAS: 63-42-3.
- Xylose, Sigma Aldrich, CAS: 58-86-6.
- Sucrose, Fisher Scientific, CAS: 57-50-1.
- Maltose, Thermo Scientific, CAS: 6363-53-7.
- Fructose, Thermo Scientific, CAS: 57-48-7.
- Mannose, Sigma Life Sciences, CAS: 3458-28-4.

Strains:

- Wildtype *S. cerevisiae,* S288C, sourced from the Kent Fungal Group.
- Wildtype *S. stipitis,* ATCC 58785, sourced from the Kent Fungal Group.

- Wildtype *C. saccharoperbutylacetonicum*, ATCC 27021, sourced from the Shepherd Lab at the University of Kent.
- *S. cerevisiae* deltaGPD1 knockout. Strain YDL022W from the MATa library (72).

GC-MS (machines, column, software):

- Agilent 5973 Network Mass Selective Detector.
- Agilent 6890N Network GC System.
- Agilent 7683 Series Injector.
- Agilent 10µl Autosampler Syringe, Part Number: 5181-1267.
- Agilent MassHunter Qualitative Analysis, Version 10.0.10305.0.
- Agilent MassHunter GCMS Data Acquisition 10.2.489.0.
- Phenomenex, Zebron ZB-WAXplus, Serial Number: 313997.
- BOC Regulator, Model: 1702B.

GC-MS (consumables):

- HPLC Grade Methanol, Fisher Scientific, CAS: 67-56-1.
- HPLC Grade Hexane, Fisher Scientific, CAS: 110-54-3.
- BOC UN1046 Compressed Helium.

Solvents for the GC-MS Standards:

- Acetone, Fisher Scientific, CAS: 67-64-1.
- Butanol, Fisher Scientific, CAS:71-36-3.
- Ethanol, Fisher Scientific, CAS: 64-17-5.

Other Equipment / Softwares used:

- Agilent Technologies Cary 60 UV-Vis.
- Cary WinUV, Simple Reads Application Version 5.0.0.999.
- BMG LABTECH SPECTROstar Nano.
- BMG LABTECH SPECTROstar Nano, Software Version 5.70.

- BMG LABTECH MARS, Version 3.42 R6.
- Microsoft Office 365 (Excel, Word).
- BOC Series 8000 Nitrogen Regulator.
- BOC UN1066 Compressed Nitrogen.
- Butyl Rubber Stoppers, Merck, Z166065.
- Crimp Caps Aluminum, Merck, 508500.
- Wheaton Glass Serum Bottles, Merck, Z114014.

Methods

Media recipes:

YPD:

YPD (Broth):

- 20g/L Bacto Peptone (Gibco).
- 10g/L yeast extract (Melford).
- Autoclave.
- 0.04g/L Adenine (Thermo Fisher Scientific).
- 0.08g/L Uridine (Thermoscientific).

YPD (Plates):

- 20g/L Bacto Peptone (Gibco).
- 10g/L yeast extract (Melford).
- 20g/L agar (Difco).
- Autoclave.
- 0.04g/L Adenine (Thermo Fisher Scientific).
- 0.08g/L Uridine (Thermoscientific).

Minimal Media:

Minimal Media (Broth):

- 2.002 g/L Kaiser Complete SC Mixture (Formedium).
- 6.7g/L Yeast Nitrogen Base without amino acids (Difco).
- Autoclave.
- 0.08g/L Uridine (Thermoscientific).

Minimal Media (Plates):

- .002 g/L Kaiser Complete SC Mixture (Formedium).
- 6.7g/L Yeast Nitrogen Base without amino acids (Difco).
- 20g/L agar (Difco).
- Autoclave.
- 0.08g/L Uridine (Thermoscientific).

Clostridial Nutrient Medium:

Clostridial Nutrient Medium (Broth):

- 33g/L of Clostridial Nutrient Medium mix (Sigma Aldrich).
- Autoclave.

GC-MS protocols:

GC-MS Equipment Set Up:

An ‘Agilent 5973 Network Mass Selective Detector’ was used in conjunction with an ‘Agilent 6890N Network GC System’ and an ‘Agilent 7683 Series injector’. An ‘Agilent 10 µl Autosampler Syringe (Part number: 5181-1267)’ was used in the series injector. The column used was a ‘Phenomenex, Zebron ZB-WAXplus (Serial Number: 313997)’. The carrier gas used was Helium UN1046.

The GC-MS system was operated using Agilent MassHunter GCMS Data Acquisition 10.2.489.0.

For all samples a 200:1 Split ratio was used, which had a split flow rate of 200ml/min.

For all samples the MSD transfer line temperature was 250ºC.

Detection of Ethanol in Samples:

For the detection of only Ethanol in samples 0.2 μl of sample was injected at an inlet temperature of 150ºC. The oven temperature remained constant at 40ºC for the 10 minute duration of the run.

Detection of; Acetone, Butanol and Ethanol in Samples:

For the detection of Acetone, Butanol and Ethanol; 0.2 μl of sample was injected at an inlet temperature of 150ºC. The initial oven temperature was 40ºC which was held for 5 minutes, and then ramped up at a rate of 8ºC/min to 150ºC. Then the oven temperature increased at a rate of 25ºC/min to 200ºC, and held for one minute.

Baking the Column:

A bake sequence was used to clear the column at the beginning and end of a batch of samples being ran. 0.2 μl of HPLC grade hexane was injected at an inlet temperature of 200ºC.

The initial temperature of the oven was 40ºC, which held for one minute, and then increased to 220ºC at a rate of 25ºC/min. Then the oven held at 220ºC for 35 minutes, and returned to a temperature of 50ºC post run.

Analysis of the Raw GC-MS Data:

The raw GC-MS data was analysed using ‘Agilent MassHunter Qualitative Analysis, Version 10.0.10305.0.’.

Standard samples of known concentrations of Ethanol, and (Acetone, Butanol and Ethanol), were run on the GC-MS. The standards ranged between 0% and 10% of the respective solvents, (0%, 0.05%, 0.1%, 0.25%, 0.5%, 1.0%, 2.5%, 5.0%, 7.5% and 10.0%).

The solvents were identified in the GC-MS trace by their retention time (Acetone has a retention time of 2.3 minutes, Ethanol of 3.7 minutes, and Butanol of 9.0 minutes), and mass ion.

The standards traces were integrated, and the area under the peak was plotted against the concentration of the Ethanol / (Acetone, Butanol and Ethanol), to produce a standard curve.

The peaks in the samples were identified by the retention time, and mass ion. The peaks were then integrated, and the equation from the standard curve was used to calculate the amount of Ethanol / (Acetone, Butanol and Ethanol) present in the sample.

Sample production:

Samples produced using *S. cerevisiae* and *S. stipitis* were grown in a rotary incubator at 180 rpm, at a temperature of 30ºC, in aerobic conditions.

Samples produced using *C. saccharoperbutylacetonicum* were grown in a rotary incubator at 180 rpm, at a temperature of 32ºC. *C. saccharoperbutylacetonicum* cultures were grown anaerobically in 100ml serum bottles, in 25ml of the respective medium. The serum bottles were purged with compressed nitrogen, then autoclaved, and then inoculated. Which produced an anaerobic environment within the serum bottles.

The sample cultures were supplemented with the relevant carbon source, at the relevant concentration. Then grown for 24 hours. Samples were taken from the cultures and centrifuged at 13,200 rpm for 7.5 minutes. The supernatant was extracted and stored at -20ºC.

Each supplementation / biocatalyst condition was grown in biological triplicate, and the GC-MS samples were produced in technical duplicate from each biological replicate.

Growth curves:

Growth curves were produced using a BMG LABTECH SPECTROstar Nano, running BMG LABTECH SPECTROstar Nano software version 5.70.

Overnight cultures were diluted at a dilution of 1 in 1000. Each well was filled with 100µl of diluted culture, and unused wells were filled with 100µl of sterile media.

The plate was incubated at 30ºC, with OD_{600} readings being taken every 7 minutes. The plate was set to shake at 200rpm (double orbital) for 120 seconds before each reading.

CFU protocol (for S. cerevisiae and S. stipitis):

Overnight cultures were diluted to a range of OD_{600} values between 0.1 and 1.0. These underwent 1 in 10 serial dilutions. Until dilutions ranging from $1x10^{-5}$ to $1x10^{-7}$ was reached. 100 µl of these dilutions were spread onto YPD agar plates, and incubated in a stationary incubator at 30^{o}C c for 24 hours.

The colonies present on the plates were counted manually, averaged, and a cells/ml at the various OD_{600} values was derived from the colony counts. These values were then plotted on a standard curve, to obtain a formula for the number of cells/ml for OD_{600} values in the range of 0.1 to 1.0.

Results

Investigation into the growth, biomass and ethanol yield of *S. cerevisiae* and *S. stipites* using standard media (YPD and Minimal) and conditions:

A growth curve of *S. cerevisiae* and *S. stipitis* in minimal and YPD medium was produced. This was to confirm that the two strains in both media types reached stationary phase, and thus their maximum density, within 24 hours. This was used as evidence in the decision to grow the samples of the different strains/medias/carbon sources, for 24 hours.

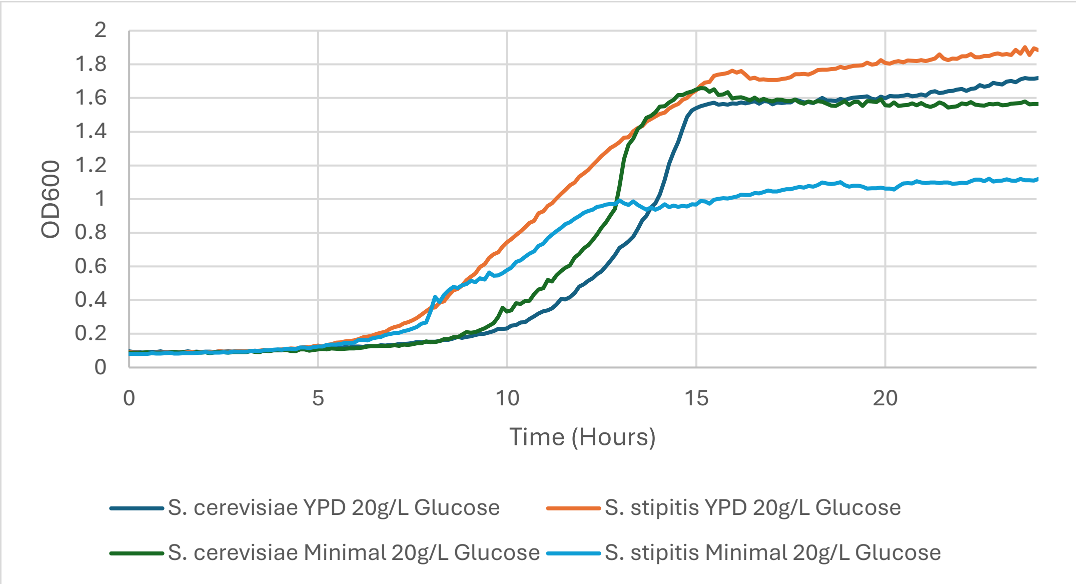


*Figure 10 – **Growth Curve** of S. cerevisiae, and S. stipitis in minimal and YPD media, grown at 30ºC, supplemented with 20g/L of Glucose. Data points are an average value of 3 technical replicates.*

Table 1 – ***Statistics derived from the Growth Curve of S. cerevisiae and S. stipitis in minimal and YPD media, when grown at 30°C, (Figure 10).***

Organism	Media (Supplemented with 20g/L Glucose)	Growth Rate (AU /Hour)	OD_{600} of the Stationary Phase	Time of the Start of the Exponential Phase (Hours)
S. cerevisiae	Minimal	0.2442	1.6	11
S. stipitis	Minimal	0.1386	1.1	7.5
S. cerevisiae	YPD	0.2927	1.6	11.5
S. stipitis	YPD	0.1953	1.8	8

Figure 10 shows that *S. stipitis* grows at a slower rate than *S. cerevisiae*, with the exponential growth phase lasting for longer. It can also be seen that *S. stipitis* has a shorter lag phase than *S. cerevisiae.* With *S. stipitis* in minimal media reaching exponential phase after 7.5 hours, and *S. stipitis* in YPD media reaching exponential phase after 8 hours (Table 1). *S. cerevisiae* grown in minimal media reached exponential phase after 11 hours, and when grown in YPD media *S. cerevisiae* reached exponential phase after 11.5 hours.

At 15 hours *S. stipitis* and *S. cerevisiae*, in minimal media and YPD media, have reached stationary phase. The OD_{600} at stationary phase of *S. stipitis* when grown in YPD media is comparable to that of *S. cerevisiae* when grown in minimal media or YPD media. However, the OD_{600} at stationary phase of *S. stipitis* is significantly lower (Table 1).

S. stipitis grew at about half the rate of *S. cerevisiae* when both were grown in minimal media, 0.1386 (AU/hour) and 0.2442 (AU/hour) respectively (Table 1). When grown in YPD media *S. stipitis* grew at about two-thirds the rate of *S. cerevisiae* at 0.1953 (AU/hour) and 0.2927 (AU/hour) respectively (Table 1).

When grown in minimal media supplemented with 20g/L of glucose, *S. stipitis* to a lower OD_{600} value at stationary phase, compared to when grown in YPD media supplemented with 20g/L of glucose, at OD_{600} 1.1 and OD_{600} 1.6 respectively.

S. cerevisiae grew to an OD_{600} value in stationary phase of 1.6, when grown in minimal media supplemented with 20g/L of glucose, and when grown in YPD media supplemented with 20g/L of glucose. This is lower than when *S.* stipitis was grown in YPD supplemented with 20g/L of glucose (OD_{600} 1.8), but higher than when *S.* stipitis was grown in minimal media supplemented with 20g/L of glucose (OD_{600} 1.1).

Colony Forming Units (CFU) Standard Curves

CFU standard curves were produced, so the number of cells present in the samples can be detected from their optical density values at 600nm. This is so the ethanol produced per CFU can be calculated, so it can be identified whether some feedstocks lead to more ethanol produced per CFU, rather than as a culture as a whole, therefore eliminating the growth variable.

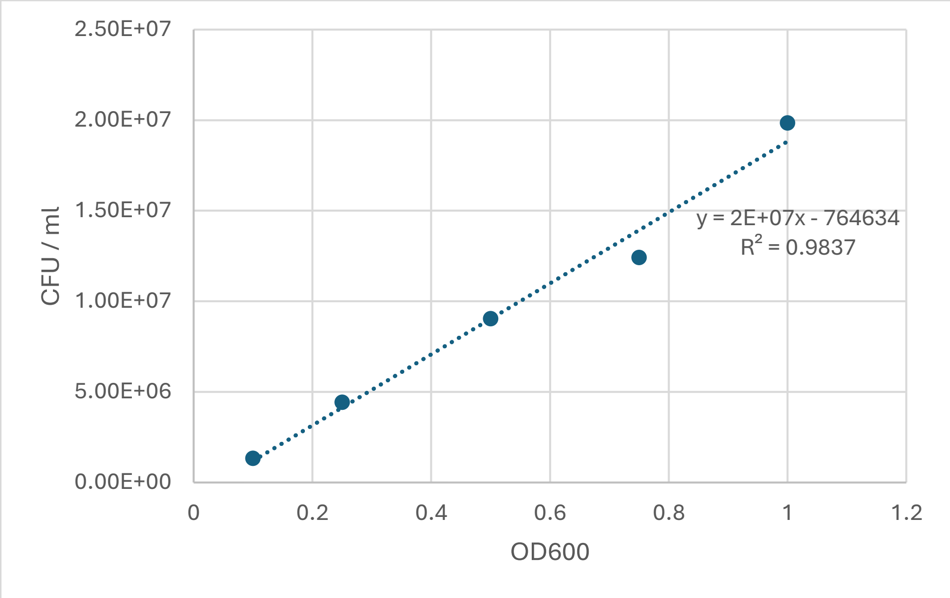


*Figure 11 – **Standard curve of S. cerevisiae CFU/ml against OD600 readings.** Data points are an average value of 3 biological replicates.*

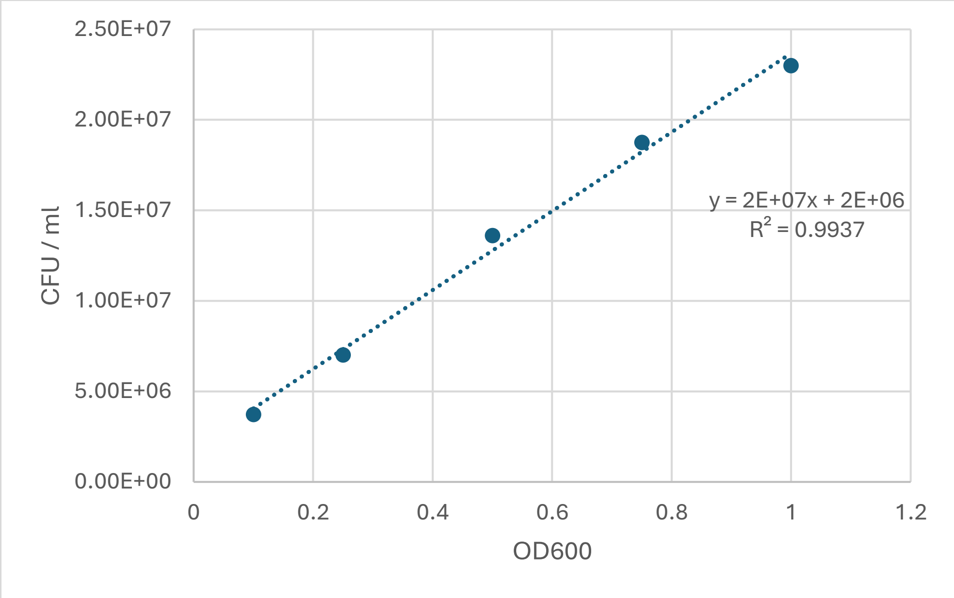


Figure 12 - ***Standard curve of S. stipitis CFU/ml against OD600 readings.*** *Data points are an average value of 3 biological replicates.*

The equation for the CFU/ml of *S. cerevisiae* from an absorbance value measured at 600nm, is CFU/ml = $2\times10^7(OD_{600}) - 764634$ (Fig. 11). Meaning at an OD_{600} value of 1.0, there are 1.924×10^7 CFU per millilitre of *S. cerevisiae* culture.

The equation for the CFU/ml of *S. stipitis* from an absorbance value measured at 600nm, is CFU/ml = $2\times10^7(OD_{600}) + 2\times10^6$ (Fig. 12). Meaning at an OD_{600} value of 1.0, there are 2.2×10^7 CFU per millilitre of *S. stipitis* culture.

Ethanol Curves

An ethanol production curve was produced at 20g/L and at 100g/L of glucose supplementation with *S. cerevisiae* and *S. stipitis*. This was to identify whether all the supplemented carbon source could be fermented within a 24-hour period, as part of the process for guiding the decision to grow the samples for 24 hours. 20g/L was chosen because this is a standard concentration of glucose to supplement media with for the growth of various bacterial and fungal strains. 100g/L was chosen as to saturate the media with glucose, and to aid in identifying whether a 24-hour growth period for the samples was appropriate.

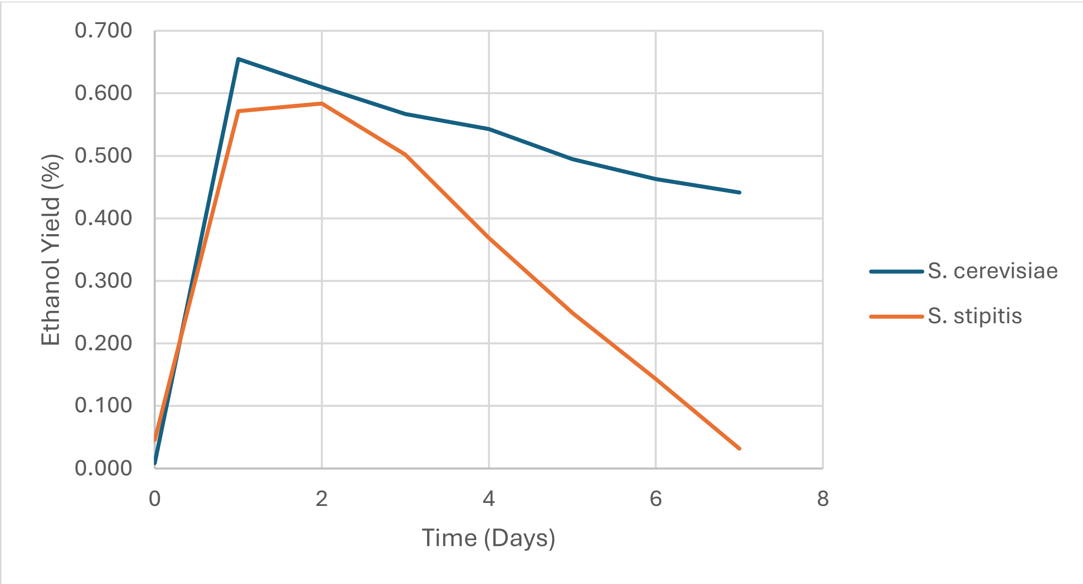


Figure 13 - ***Ethanol Production Curve.*** *Ethanol production by S. cerevisiae and S. stipitis in YPD medium, grown at 30°C, supplemented with 20g/L of Glucose, over a 7-day period. Readings taken every 24-hours. Data points are an average value of 3 biological replicates.*

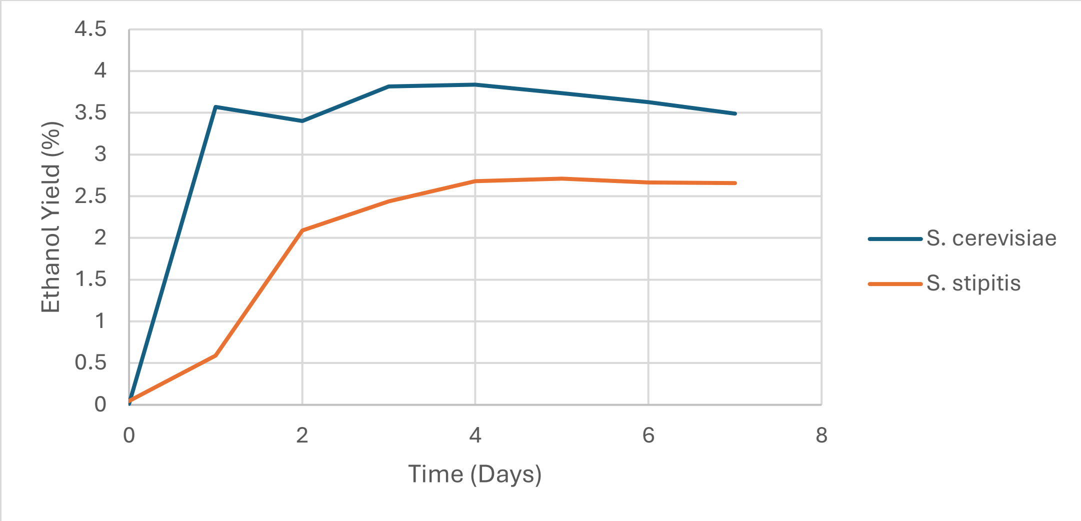


*Figure 14 – **Ethanol Production Curve.** Ethanol production by S. cerevisiae and S. stipitis in YPD medium, grown at 30ºC, supplemented with 100g/L of Glucose, over a 7-day period. Readings taken every 24-hours. Data points are an average value of 3 biological replicates.*

S. cerevisiae reaches its highest ethanol yield within 24 hours, when grown in YPD medium supplemented with 20g/L of glucose (Fig. 13). As well as nearly reaching it's highest ethanol yield within 24 hours when grown in YPD medium when supplemented with 100g/L of glucose (Fig. 14).

S. stipitis appears to ferment most of the sugar present when grown in YPD medium supplemented with 20g/L of glucose within the first 24-hour period (Fig. 13). However, when *S. stipitis* is grown in YPD medium supplemented with 100g/L of glucose the highest ethanol yield is reached within the first 24 hours, and there is a 4-fold increase in between 24 hours and 48 hours (Fig. 14). This may be because of a slower rate of fermentation by *S. stipitis* compared to *S. cerevisiae*, or due to the slow rate of growth exhibited by *S. stipitis* as seen in figure 10.

Analytical Quantification of Target Analytes using GC-MS:

Solvent standard curves were produced so when the samples were ran the solvent peak identified by the retention time and the mass ions could be integrated to calculate the peak area. The peak area is then converted into the percentage of the solvent present in the sample using the equation of the line of the standard curve.

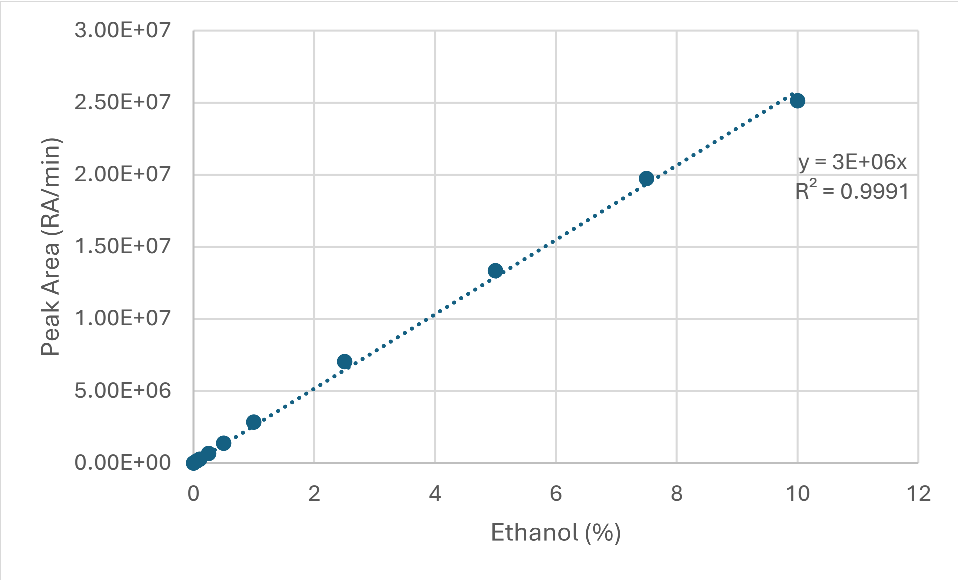


*Figure 15 – **Standard curve of Peak area against Ethanol (%).** Ethanol concentrations ranging between 0% and 10%. (RA) is Relative Abundance.*

The equation for converting the peak area of the ethanol peak identified on the GC-MS trace to the percentage ethanol present within the sample is Ethanol (%) = Peak Area (RA/min) / $3x10^6$ (Fig. 15).

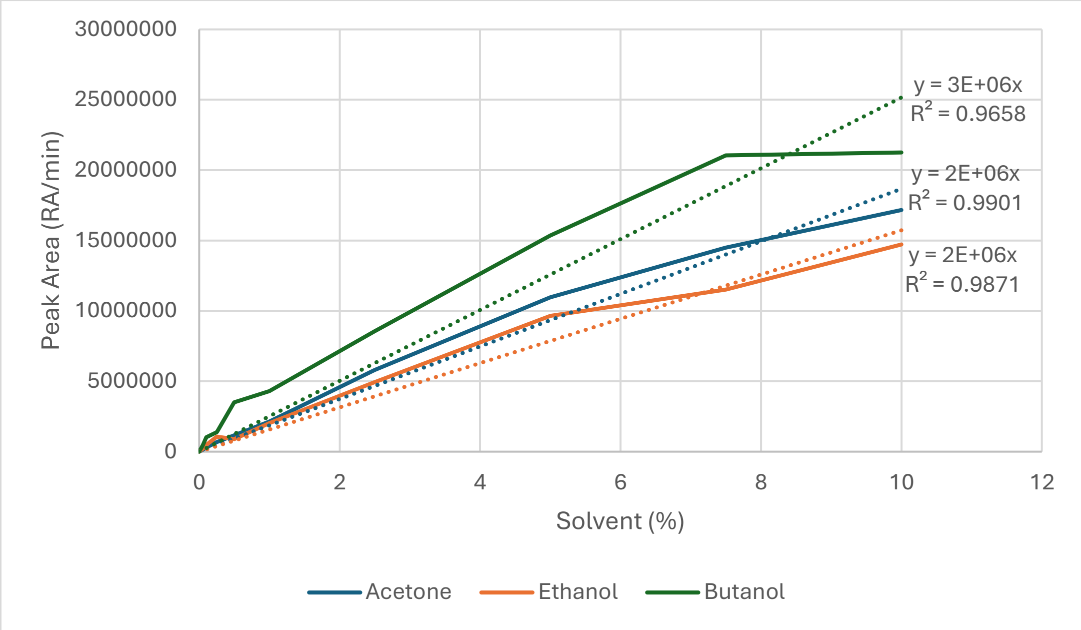


*Figure 16 - **Standard curve of Peak area against Solvent (%).** Standard curve showing peak integration against percentage of Acetone, Ethanol, and Butanol. Concentrations ranging between 0% and 10%. (RA) is Relative Absorbance.*

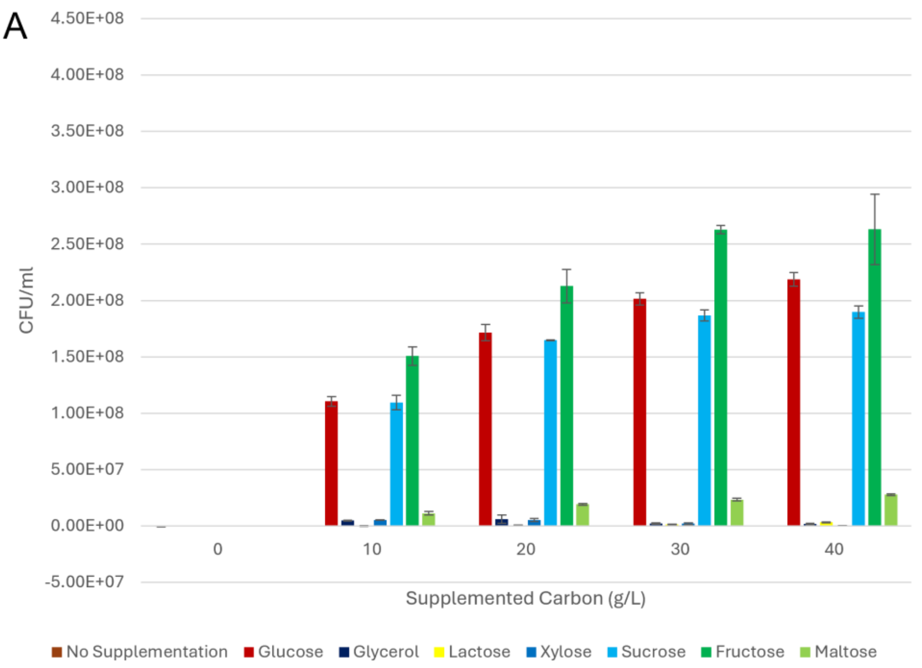
For samples produced by *C. saccharoperbutylacetonicum* a standard curve was produced using standards containing acetone, ethanol, and butanol. The equation for calculating the percentage of acetone in the sample is ‘Acetone (%) = Peak Area (RA/min) / 2x10^6’. The equation for calculating the percentage of ethanol in the sample is ‘Ethanol (%) = Peak Area (RA/min) / 2x10^6’. The equation for calculating the percentage of butanol in the sample is ‘Butanol (%) = Peak Area (RA/min) / 3x10^6’. These three equations were derived from figure 16.

Biomass yield for *S. cerevisiae* and *S. stipitis* on a range of prospective feedstocks:

The yield of biomass per condition was measured by taking an OD600 reading at the end of the 24-hour sample growth period. This OD600 value was converted to CFUs, using the equations (Fig. 10). This was done so variations in biomass yield between carbon source supplementations and concentration of said supplementations could be identified.

Biomass yield is an important metric for the effectiveness of the feedstock. This is because the higher the CFUs the more cells there are to produce the desired product. In this case ethanol.

A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3.50E+08
3.00E+08
2.50E+08
2.00E+08
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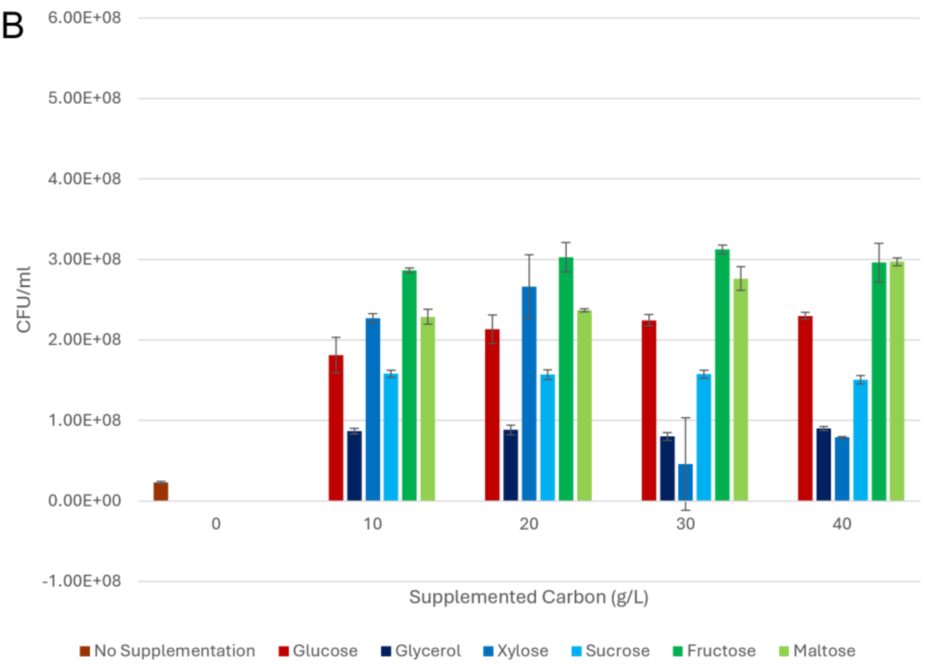
1.00E+08
5.00E+07
0.00E+00
-5.00E+07
CFU/ml
0
10
20
30
40
Supplemented Carbon (g/L)
No Supplementation
Glucose
Glycerol
Lactose
Xylose
Sucrose
Fructose
Maltose

B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5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
-1.00E+08
CFU/ml
0
10
20
30
40
Supplemented Carbon (g/L)
No Supplementation
Glucose
Glycerol
Xylose
Sucrose
Fructose
Maltose

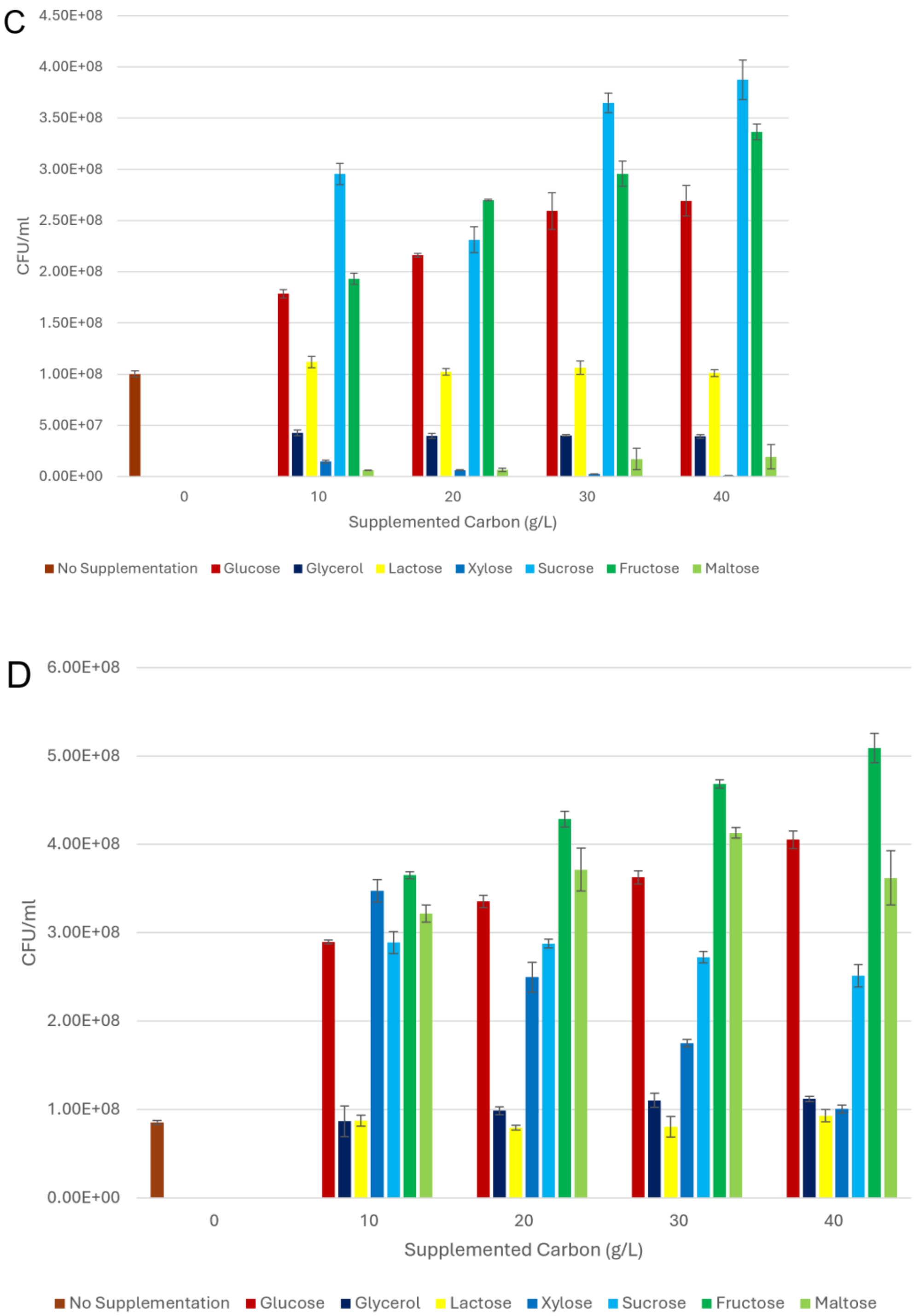


Figure 17 - ***Biomass yield (CFU/ml) when S. cerevisiae (A & C) and S. stipitis (B & D) were grown in minimal (A & B) or YPD (C & D) media (24 hours at 30°C) containing a range of different substrates (Glucose, Glycerol, Lactose, Xylose, Sucrose, Fructose, and Maltose). Substrate concentration ranged from 10-40 g/L.***

Figure 17a shows that *S. cerevisiae* grows well in minimal media, when supplemented with glucose, sucrose, and fructose. As well as growing to a marginal amount when supplemented with maltose. However, S. *cerevisiae* doesn't grow in minimal media which has been supplemented with, Glycerol, lactose, and xylose.

Figure 17b shows that *S. stipitis* can utilise all the provided carbon sources, to grow to a higher CFU/ml value than the control cultures grown in minimal media which was not supplemented. *S. stipitis* grows to the highest CFU/ml value in minimal media when supplemented with fructose, at each respective concentration of the supplemented carbon source. Other than at 40g/L of supplementation where *S. stipitis* grew to the highest CFU/ml value when supplemented with Maltose, however this value is only marginally greater than the CFU/ml value when *S. stipitis* was supplemented with fructose at 40g/L (297306667 CFUs/ml, compared to 296183333.3 CFUs/ml) (Fig. 17b).

S. stipitis in minimal media supplemented with 10g/L of xylose grows to 79.3% the density of the highest CFU/ml condition at 10g/L (which was fructose supplementation). And when supplemented at 20g/L of xylose, *S. stipitis* grows to 87.9% the highest CFU/ml value at 20g/L, which was fructose supplementation. However, at 30g/L, and 40g/L xylose supplementation the CFU/ml drops drastically, to 14.7% and 26.6% of the highest CFU/ml value at their respective supplementation concentrations.

Figure 17c shows that when *S. cerevisiae* is grown in YPD medium supplemented with Glucose, Fructose, and Sucrose a high CFUs/ml value is reached. However, when *S. cerevisiae* is grown in YPD medium supplemented with lactose the biomass yield (CFUs/ml) is equivalent to the no supplementation control sample, across the range of concentrations lactose was supplemented at (10g/L, 20g/L, 30g/L, and 40g/L).

Figure 17c also shows that when *S. cerevisiae* is grown in YPD medium supplemented with glycerol, maltose or xylose, the biomass yield is significantly worse than the no supplementation control. Although, the biomass yield across the glycerol supplemented samples is lower than the biomass yield of the no supplementation sample, the biomass yield is consistent across the supplemented glycerol concentrations compared to the no supplementation sample (42.6%, 39.6%, 40.2% and 39.3% respectively).

The biomass yield of *S. cerevisiae* grown in YPD medium supplemented with glycerol, is higher than the biomass yield observed when *S. cerevisiae* is grown in YPD medium supplemented with xylose or maltose.

It can be seen in figure 17c that *S. cerevisiae* grown in YPD medium supplemented with sucrose or fructose, has a higher biomass yield than when supplemented with glucose. With the biomass yield of *S. cerevisiae* having an observable upwards trend as the concentration of the supplementation of sucrose, fructose, or glucose increases. However, the increases of the biomass yield are not proportional to the increased concentration of the supplemented carbon source (Fig. 20c), meaning that the supplemented carbon source isn't the limiting factor of the biomass yield.

Figure 17d shows that when *S. stipitis* is grown in YPD medium supplemented with glucose or fructose, the biomass yield increases consistently alongside the increasing concentration of glucose or fructose.

The level of growth exhibited by *S. stipitis* in YPD medium, when not supplemented with an additional carbon source, is consistent with the biomass yields of *S. stipitis* when supplemented with the four concentrations of lactose or glycerol.

The biomass yield of *S. stipitis* is seen to decrease as the concentration of supplemented maltose increases, however the biomass yield does not reduce past the biomass yield of the no supplementation control sample, when maltose is at its highest tested concentration of 40g/L.

S. stipitis is shown to have a biomass yield higher than that of the no supplementation control, when grown on low concentrations of xylose (10g/L, and 20g/L). Whereas *S. cerevisiae* when grown on any tested xylose concentration in YPD medium, grows to a lower biomass yield than that of the no supplementation control of *S. cerevisiae* in YPD medium. However, in minimal media, *S. cerevisiae* grows marginally better than the no supplementation *S. cerevisiae* in minimal media control sample, when supplemented with xylose at all tested concentrations, although the *S. cerevisiae* no supplementation minimal media control sample doesn't exhibit growth.

In YPD medium *S. stipitis* grows comparatively well when supplemented with 10g/L of sucrose, to that of *S. stipitis* supplemented with 10g/L of fructose (79.1%). However, as the concentration of the supplemented sucrose increases then biomass yield compared to that of the equivalent concentration of supplemented fructose decreases, until at 40g/L where the biomass yield of *S. stipitis* supplemented with sucrose is 49.4% that of the equivalent fructose sample (Fig. 17d).

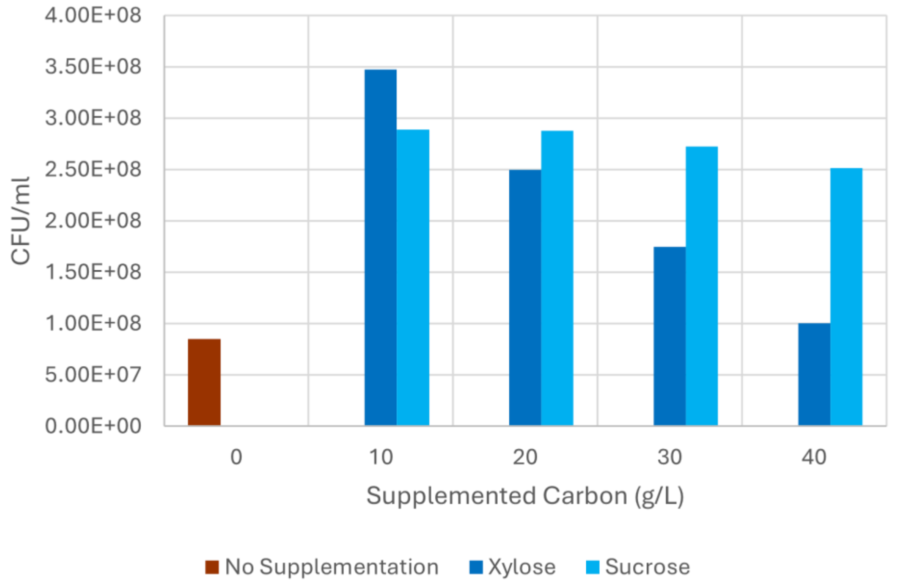


Figure 18 – ***Biomass yield (CFU/ml) when S. stipitis grown in YPD media (24 hours at 30ºC), supplemented with Xylose or Sucrose, ranging from 10-40g/L.***

As is observed when *S. stipitis* is grown YPD medium supplemented with 40g/L maltose, when *S. stipitis* is grown in YPD medium supplemented with a high concentration of xylose (40g/L) the biomass yield does not drop below that of the no supplementation control sample. The biomass yield of *S. stipitis* grown in YPD medium supplemented with xylose decreases compared to the no supplementation control sample as the concentration of xylose increases, with 407.2%, 292.3%, 204.8%, 117.7% respectively (Fig. 17d and Fig. 18).

When *S. stipitis* is grown in YPD medium supplemented with sucrose, the biomass yield can be seen to decrease as the concentration of the supplemented sucrose increases, this is comparable to when *S. stipitis* is supplemented with xylose, however the drop off in biomass yield exhibited is less drastic when *S. stipitis* is supplemented with sucrose compared to xylose (Fig. 18).

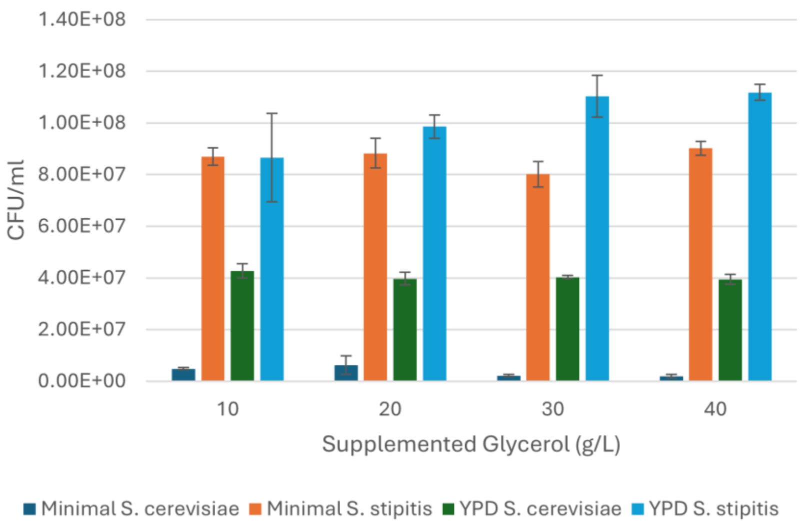
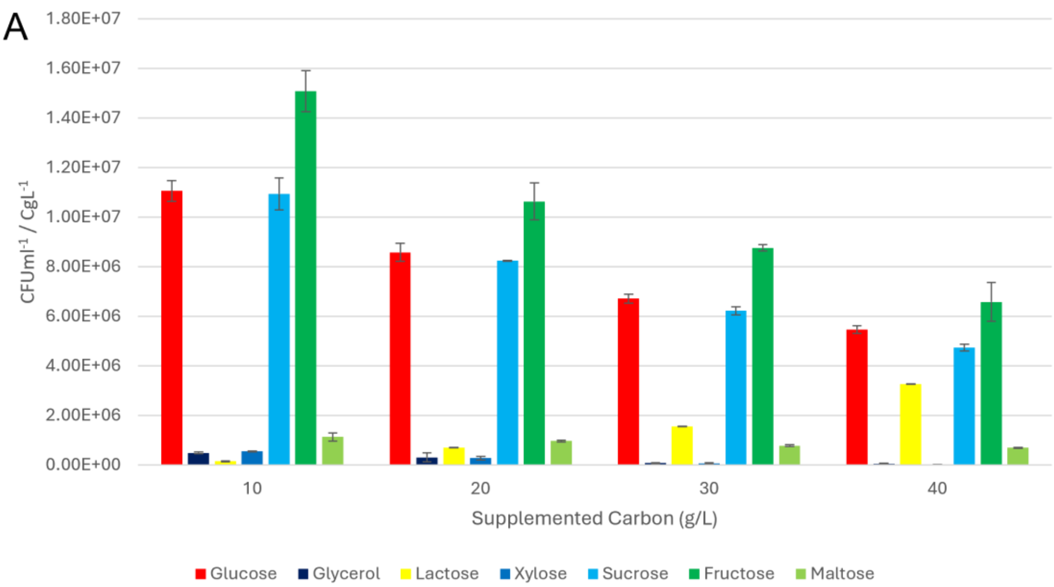


Figure 19 – ***Biomass yield (CFU/ml) when S. cerevisiae and S. stipitis were grown in minimal and YPD media (24 hours at 30°C) containing glycerol at concentrations ranging from 10-40g/L.***

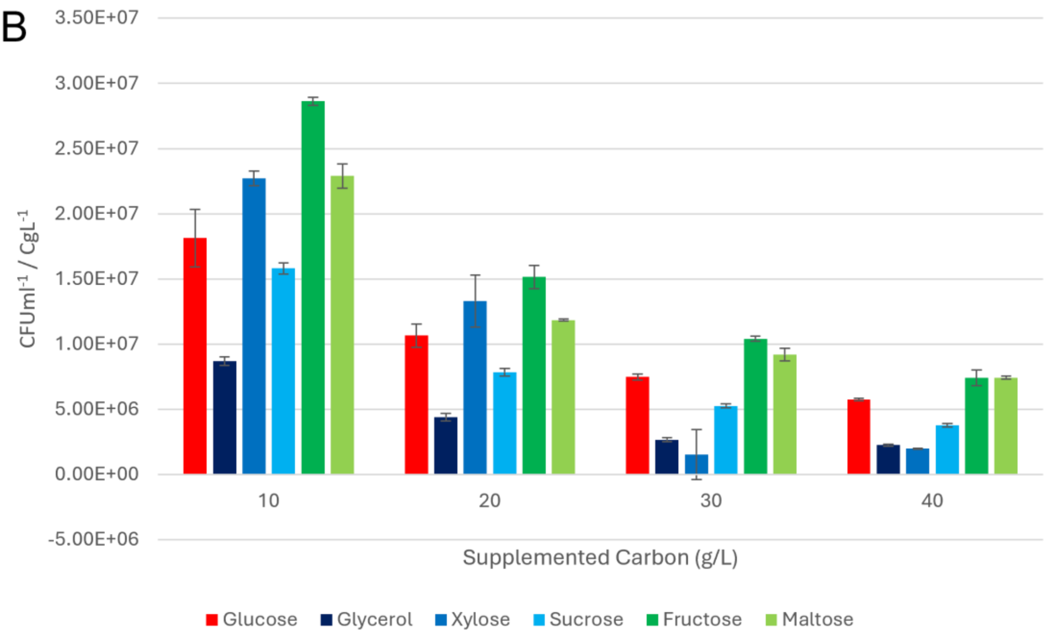
Figure 19 shows that *S. cerevisiae* has a lower biomass yield than *S. stipitis* when grown in media supplemented with glycerol. *S. cerevisiae* has a higher biomass yield when supplemented with glycerol in the complex YPD medium, than in minimal media. *S. stipitis* also has a greater yield when grown in YPD medium supplemented with glycerol, than minimal media supplemented with glycerol, however the difference in biomass yield is less pronounced than it is for *S. cerevisiae*.

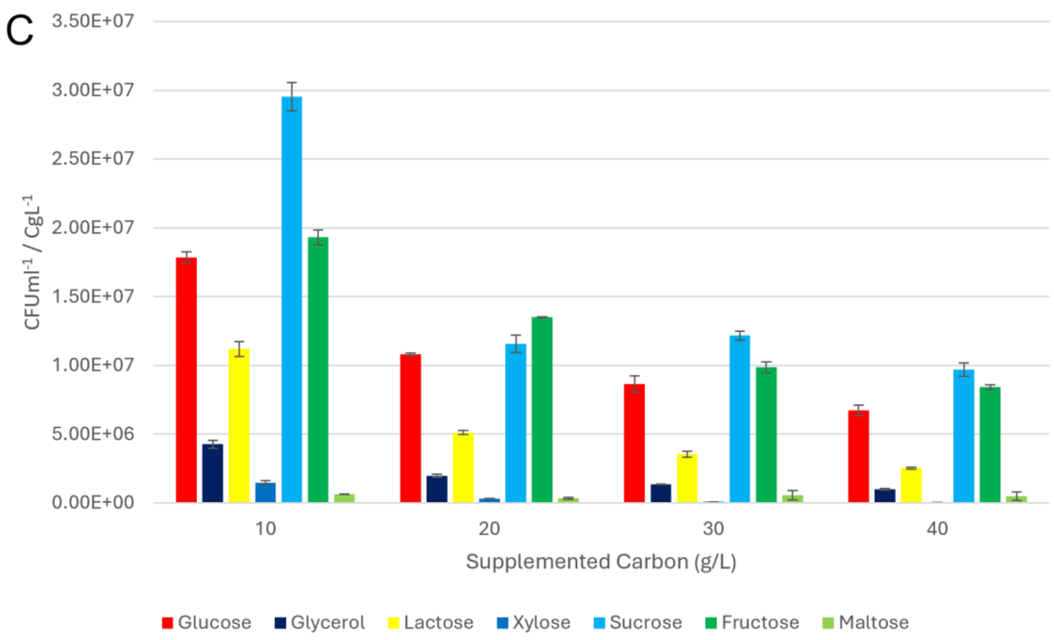
However, both *S. cerevisiae* and *S. stipitis* have substantially better biomass yields when grown in a carbon source such as glucose, in minimal or YPD medium, compared to when supplemented with glycerol (Fig. 17).

A


1.80E+07
1.60E+07
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
CFUml-1 / CgL-1
10
20
30
40
Supplemented Carbon (g/L)
Glucose
Glycerol
Lactose
Xylose
Sucrose
Fructose
Maltose

B


3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
-5.00E+06
CFUml-1 / CgL-1
10
20
30
40
Supplemented Carbon (g/L)
Glucose
Glycerol
Xylose
Sucrose
Fructose
Maltose



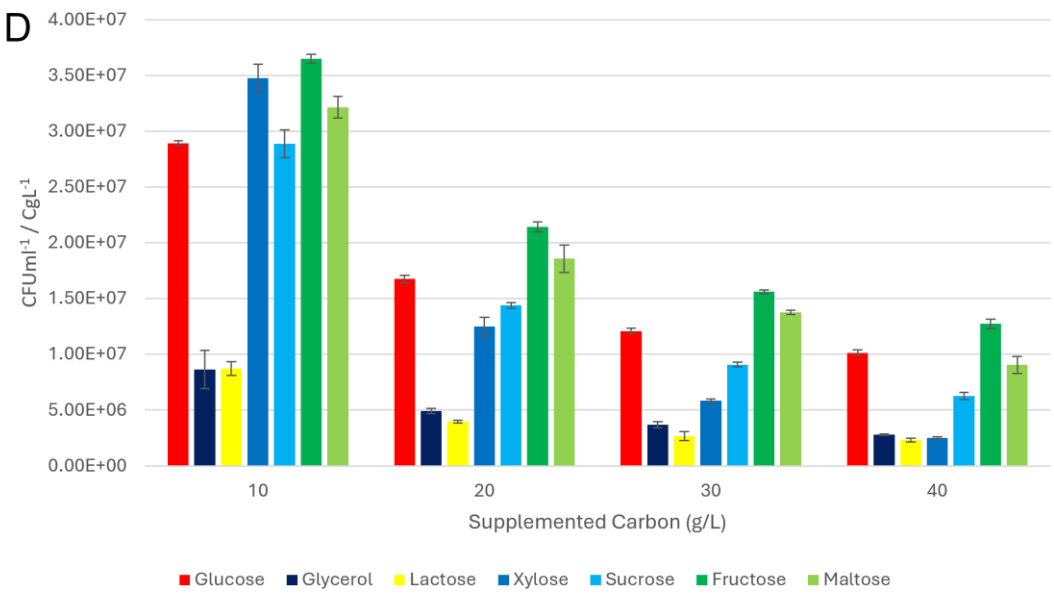


Figure 20 - ***Biomass yield (CFUml^{-1} / CgL^{-1}) when S. cerevisiae (A & C) and S. stipitis (B & D) were grown in minimal (A & B) or YPD (C &D) media (24 hours at 30^{o}C) containing a range of different substrates (Glucose, Glycerol, Lactose, Xylose, Sucrose, Fructose, and Maltose). Substrate concentration ranged from 10-40 g/L.***

It can be seen across Figure 20 that the general trend which is present across supplemented carbon sources, medias, and organisms, is that as you increase the concentration of the supplemented carbon source there is diminishing returns on the biomass yield.

This observation is pronounced and easily observed in Figure 20a, as the concentration of supplemented glucose, sucrose, and fructose is increased, the ((CFUs/ml / C (g/L)) decreases. The rate at which the '(CFUs/ml / C (g/L)) of *S. cerevisiae* grown in minimal media supplemented with glucose' decreases at is $1.864x10^5$ ((CFUs/ml / C (g/L)) / C (g/L)). The rate of decrease of '(CFUs/ml / C (g/L))' for when *S. cerevisiae* is grown in minimal media supplemented with fructose or sucrose is $2.740x10^5$ ((CFUs/ml / C (g/L)) / C (g/L)) and $2.061x10^5$ ((CFUs/ml / C (g/L)) / C (g/L)) respectively.

Meaning that per gram of supplemented carbon source, at higher concentrations of the supplemented carbon source, there is a lower return on the supplemented carbon source in terms of biomass yield of *S. cerevisiae* grown in minimal media.

This trend can also be readily observed in Figure 20d, whereas the concentration of Glucose, fructose, and maltose increases, the ((CFUs/ml) / C (g/L)) of *S. stipitis* decreases.

Table 2 - ***Summary of whether there is a substantial increase in biomass yield from the supplementation of each carbon source compared to the no supplementation control. Upon S. cerevisiae and S. stipitis, when grown in Minimal media and YPD media, for 24 hpurs at 30°C.*** *A green arrow signifies that the biomass yield is increased when the respective biocatalyst is supplemented with the carbon source, with said arrow being large when an increased concentration of the carbon source supplemented results in an increased biomass yield, whereas a small arrow is when the supplementation increases the biomass yield, but doesn't lead to an increased yield as the concentration of the supplemented carbon source increases. Whereas a red arrow signifies that the biomass yield is decreased when the respective biocatalyst is supplemented with the carbon source. With a horizontal black line signifying that the samples of that condition exhibited no change compared to the no supplementation control. A large red arrow is displayed when the increasing concentration of the carbon source supplemented leads to an increased biomass yield. A horizontal black line signifies that the when the respective biocatalyst is supplemented with the carbon source no change was exhibited in respect to the biomass yield compared to the no supplementation control.*

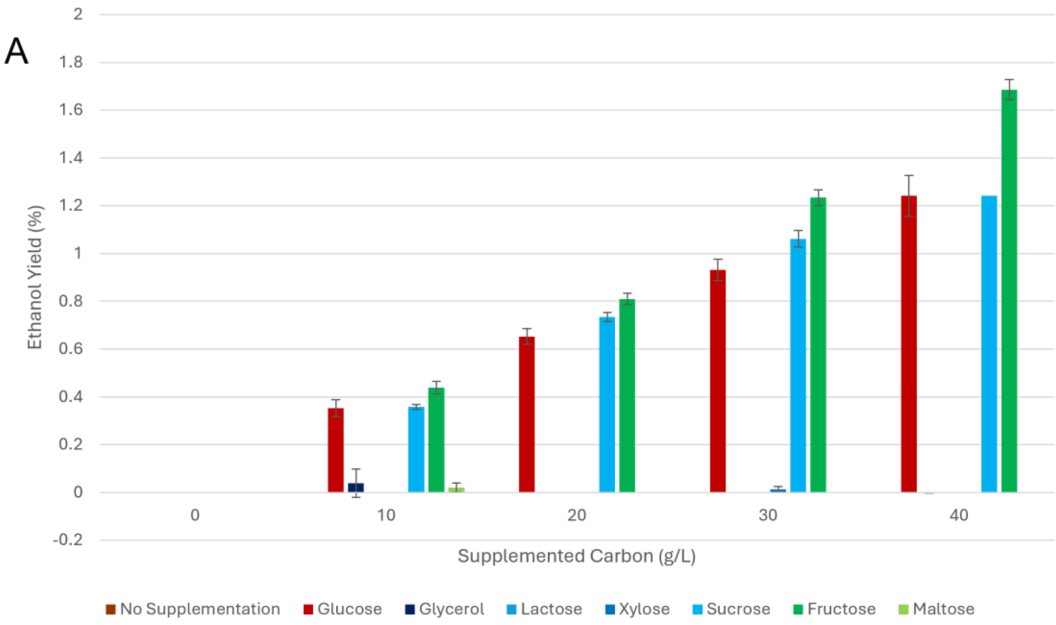
	S. cerevisiae		*S. stipitis*	
Supplemented Carbon	Minimal Media	YPD Media	Minimal Media	YPD Media
Glucose	↑	↑	↑	↑
Glycerol	—	↓	↑	—
Lactose	—	—	N/A	—
Xylose	—	↓	↑	↑
Sucrose	↑	↑	↑	↑
Fructose	↑	↑	↑	↑
Maltose	—	↓	↑	↑

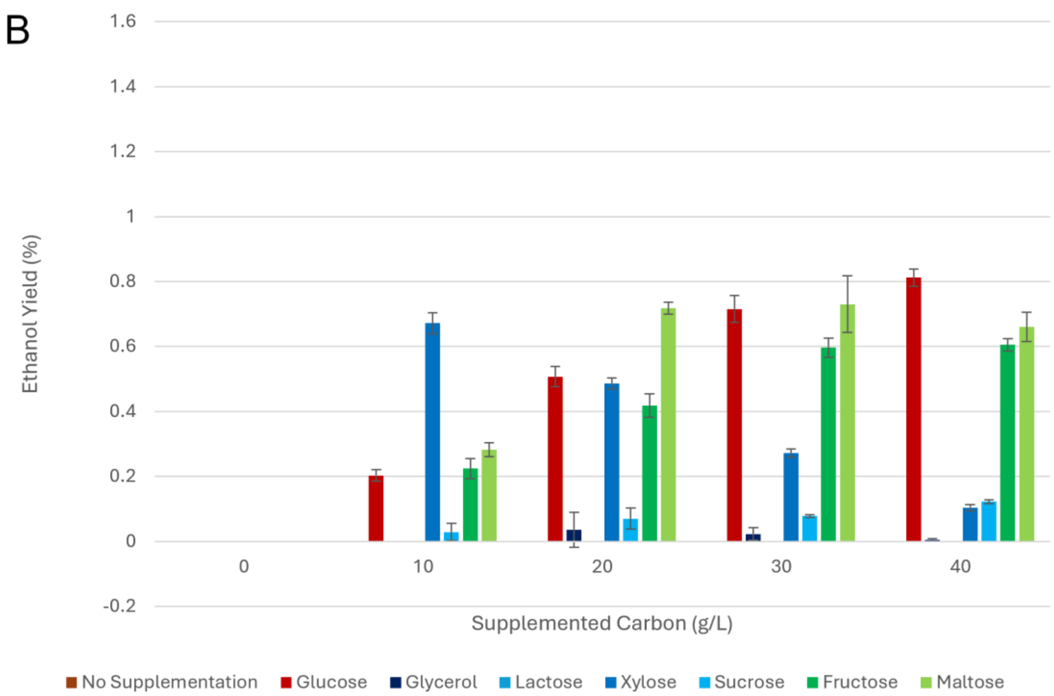
Ethanol Yield of *S. cerevisiae* and *S. stipitis,* when supplemented with a range of prospective feedstocks:

The ethanol yield is important to determine because ethanol is the desired product from the process. Meaning the quantification of the ethanol yield relative to the amount of feedstock supplementation (Percentage Ethanol produced per gram of supplemented carbon), is vital for performing an economic analysis on which feedstocks are economically viable for large scale production of bio-solvents.

Ethanol yield has been reported as a %, because then the ethanol produced by the culture as a whole, regardless of culture density and volume can be compared. This is important because as shown in the previous section different feedstocks lead to vastly different biomass yields, which would, in turn lead to varying ethanol yields.

Ethanol yield per CFU has been reported as Ethanol (g)/CFU. This is because then the grams of ethanol, produced by a single CFU can be compared. This eliminates the impact of the different biomass yields produced by the different feedstocks, allowing a comparison of solely then yield of ethanol by a particular strain, grown in a particular feedstock, supplemented with a particular carbon source.


A
Ethanol Yield (%)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
10
20
30
40
Supplemented Carbon (g/L)
No Supplementation
Glucose
Glycerol
Lactose
Xylose
Sucrose
Fructose
Maltose


B
Ethanol Yield (%)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
10
20
30
40
Supplemented Carbon (g/L)
No Supplementation
Glucose
Glycerol
Lactose
Xylose
Sucrose
Fructose
Maltose

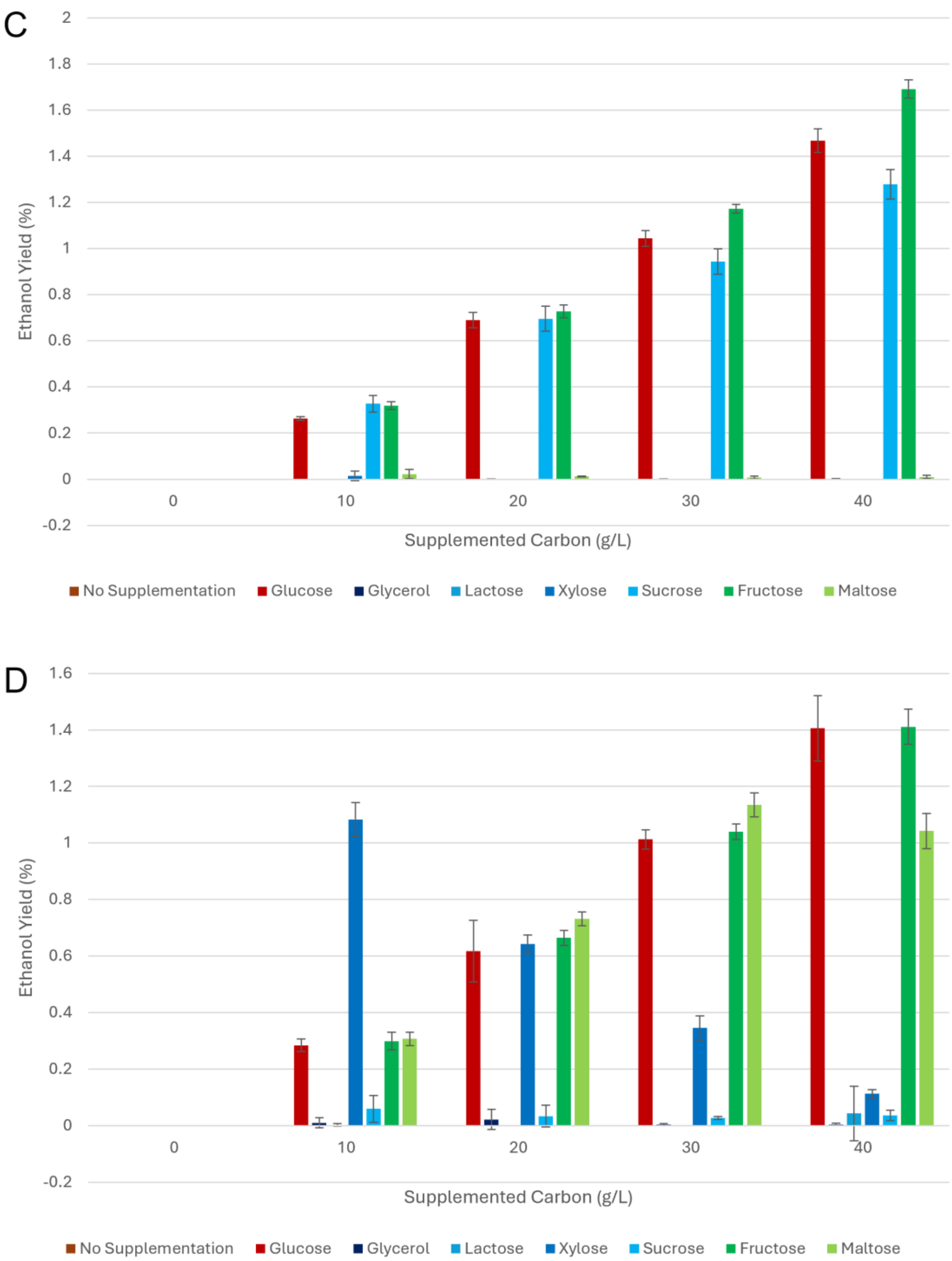


Figure 21 - ***Ethanol yield (%) when S. cerevisiae (A & C) and S. stipitis (B & D) were grown in minimal (A & B) or YPD (C & D) media (24 hours at 30ºC) containing a range of different substrates (Glucose, Glycerol, Lactose, Xylose, Sucrose, Fructose, and Maltose). Substrate concentration ranged from 10-40 g/L.***

Figure 21a shows that when *S. cerevisiae* is grown in minimal media, ethanol is produced when the media is supplemented with glucose, sucrose, and fructose. Glucose and fructose are both six carbon compounds, and sucrose is comprised of a glucose monomer bound to a fructose monomer by a α-1, β-2 glycosidic linkage. Ethanol is not produced when the media is supplemented with Glycerol, lactose, xylose, and maltose.

At the four concentrations of supplemented carbon source tested (10g/L, 20g/L, 30g/L, and 40g/L), more ethanol was produced when the minimal media was supplemented with fructose, rather than glucose or sucrose. With the samples supplemented with fructose producing 124.4%, 123.9%, 132.4% and 135.9% more than the samples supplemented with glucose at each concentration respectively (Fig. 21a).

S. stipitis produces ethanol when grown in minimal media supplemented with glucose, xylose, sucrose, fructose, and maltose. The amount of ethanol produced increases as the concentration of glucose, sucrose, or fructose increases. *S. stipitis* produces more ethanol when supplemented with maltose at concentrations of 20g/L or 30g/L than is produced when at 10g/L or 40g/L. As the concentration of xylose increases, the amount of ethanol produced decreases (Fig. 21b).

Both *S. cerevisiae* and *S. stipitis* produce ethanol in minimal media which has been supplemented with glucose or fructose. However, *S. cerevisiae* produces on average 42.0% more ethanol when supplemented with glucose than *S. stipitis*, and 126.2% more ethanol when supplemented with fructose than *S. stipitis* in minimal media (Fig. 21a, 21b).

As is the case in minimal media, when *S. cerevisiae* is grown in YPD medium, more ethanol is produced when the medium is supplemented with fructose, than when the medium is supplemented with glucose or sucrose (Fig. 21c).

The ethanol produced by *S. stipitis* grown in YPD medium follows a similar pattern as to when *S. stipitis* was grown in minimal media. With more ethanol being produced when the media is supplemented with maltose instead of glucose or fructose at concentrations of 20g/L and 30g/L, and then when *S. cerevisiae* is grown in media supplemented with glucose or fructose at 40g/L more ethanol is produced than when the media is supplemented with maltose at 40g/L. The trend that *S. stipitis* produces less ethanol at higher concentrations of supplemented xylose, is present when *S. stipitis* is grown in YPD media as well as minimal media.

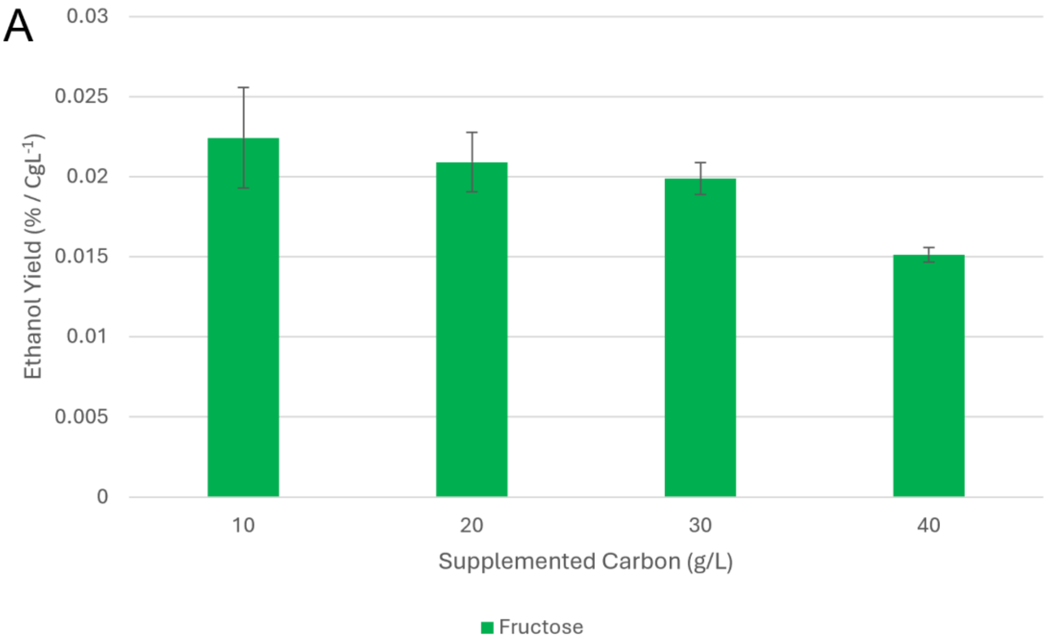
When *S. stipitis* is grown in minimal or YPD medium supplemented with xylose, the highest ethanol yield is reached when the supplementation is at the lowest tested concentration of 10g/L, which is a higher ethanol yield than the no supplementation controls. The ethanol yield decreases drastically as the concentration of supplemented xylose increases (Fig. 21b, 21d).

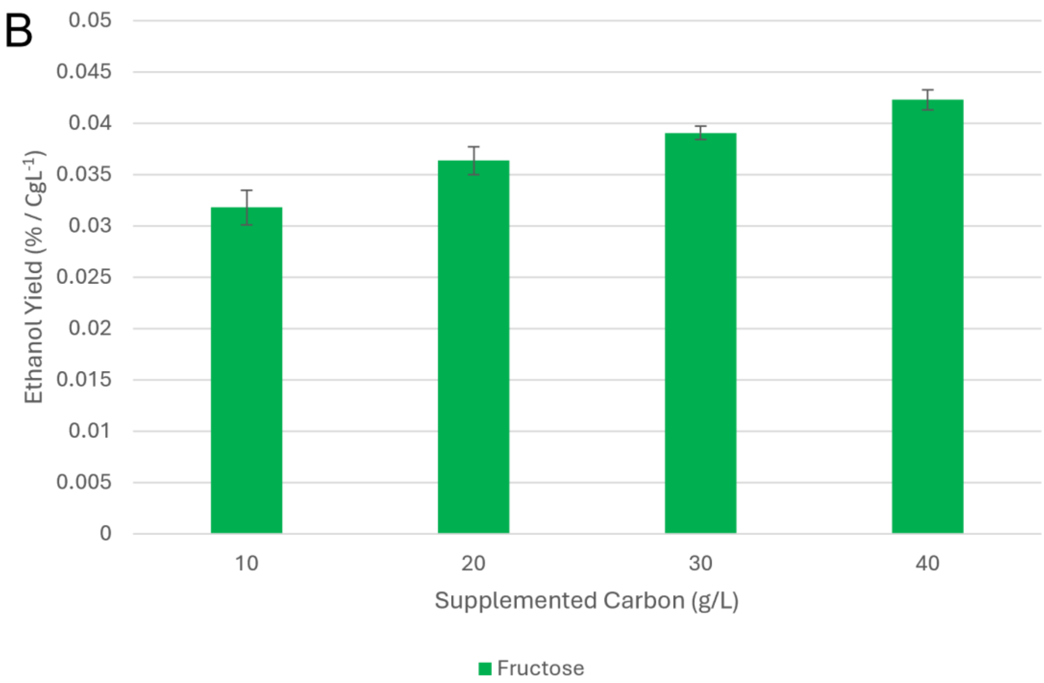
When *S. stipitis* is grown in YPD medium, the ethanol produced with supplemented glucose or fructose is very similar at the respective concentrations. Compared to in minimal media where more ethanol was produced by *S. stipitis* when supplemented with glucose than fructose in the three higher concentrations (20g/L, 30g/L, and 40g/L).

As was the case in minimal medium, in YPD medium *S. cerevisiae* produces more ethanol than *S. stipitis* when both are supplemented with the same carbon source at the same concentrations, although to a much lesser extent than was observed in minimal medium. With *S. cerevisiae* producing 4.4% more ethanol than *S. stipitis* on average across the concentrations, when supplemented with glucose. Then when supplemented with fructose, *S. cerevisiae* produced 14.5% more ethanol than *S. stipitis*.

In minimal media *S. cerevisiae* produces 8.53g of ethanol, across the various carbon supplementations, similarly when *S. cerevisiae* is grown in YPD media supplemented with the various carbon sources at the four concentrations 8.43g of ethanol is produced. Whereas when *S. stipitis* is grown in YPD medium supplemented with the range of carbon sources and concentrations, 47.9% more ethanol is produced. With *S. stipitis* in YPD medium producing 9.76g of ethanol, compared to 6.60g of ethanol when *S. stipitis* is grown in minimal media.

A





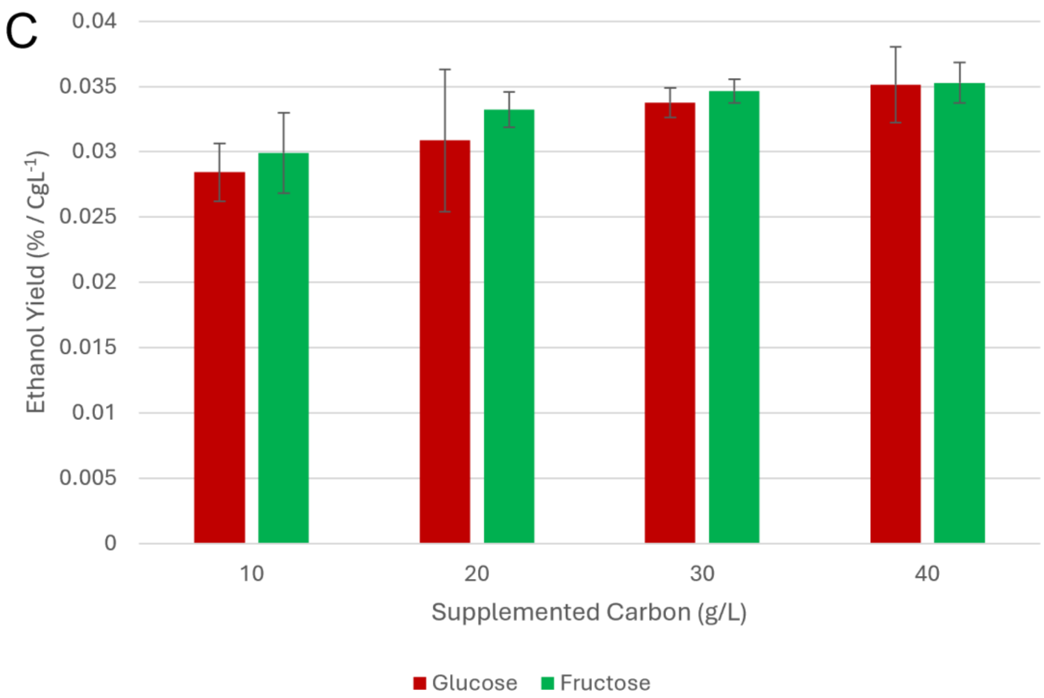
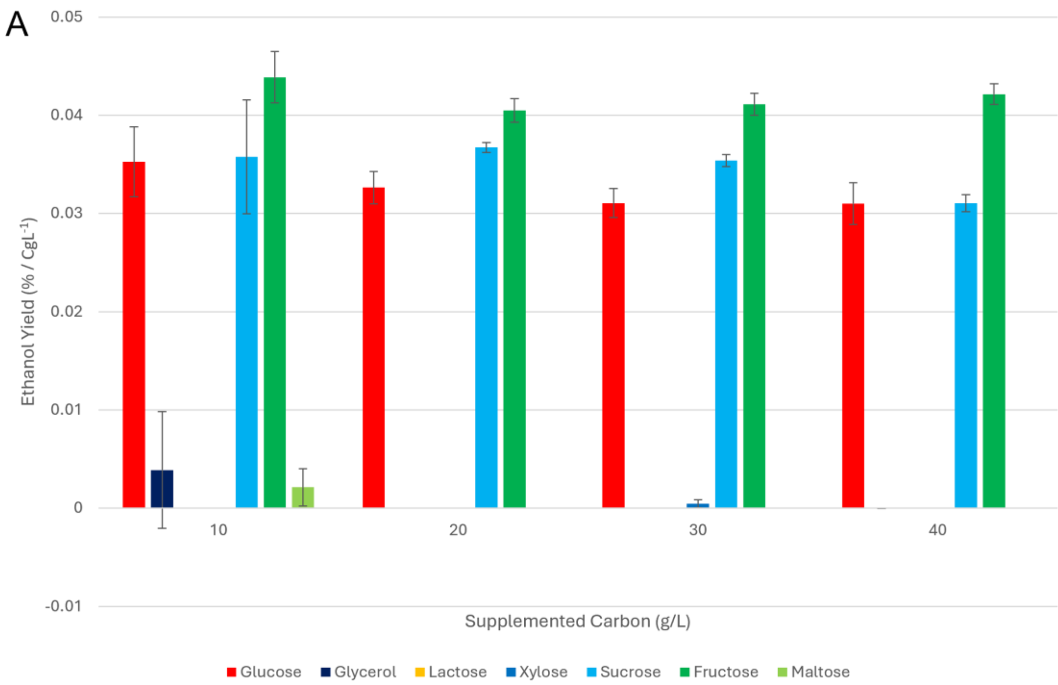
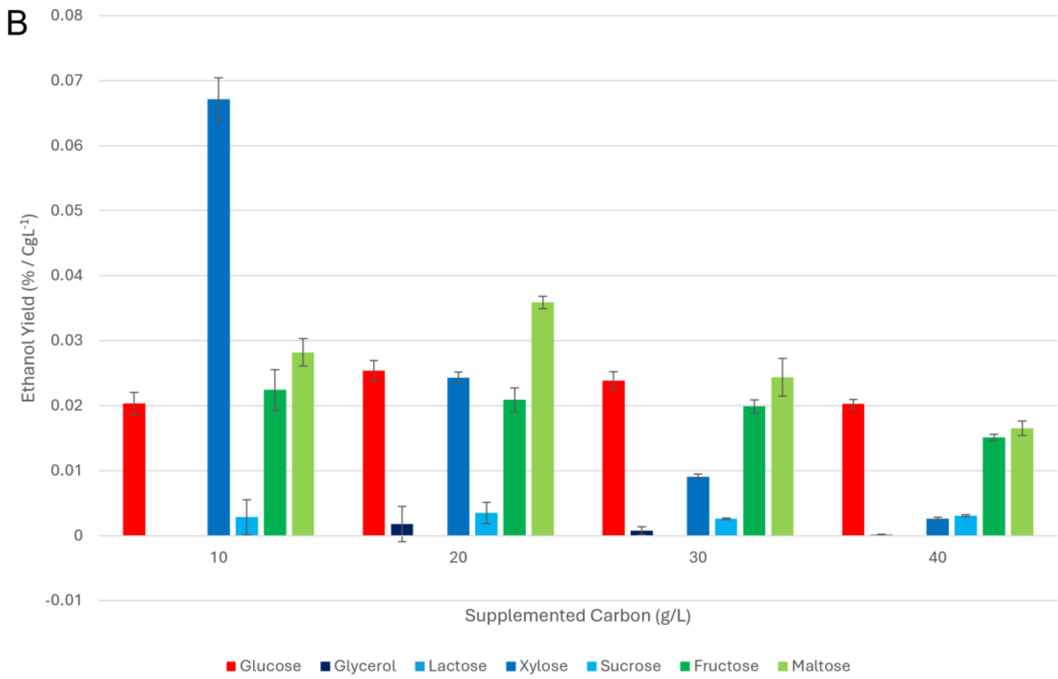


Figure 22 – ***Ethanol yield (% / gCL^{-1}) when S. stipitis (A &C) or S. cerevisiae (B) is grown in minimal (A) or YPD (B & C) media (24 hours at 30^{o}C) containing fructose (A, B &C) or glucose (C) at concentrations ranging from 10-40g/L.***

Figure 22a shows diminishing returns in regards to the ethanol yield of *S. stipitis* in minimal media, upon supplementation with fructose. Whereas figure 22c shows that when *S. stipitis* is grown in YPD media the ethanol yield per gram of supplemented carbon increases as the concentration of fructose or glucose increases. Meaning that when *S. stipitis* is grown in minimal media supplemented with fructose, diminishing returns is exhibited, whereas increasing returns to scale is exhibited when *S. stipitis* is grown in YPD media supplemented with fructose.

Figure 22b shows that increasing returns to scale is also exhibited when *S. cerevisiae* is grown in YPD media which has been supplemented with fructose.


A
0.05
0.04
0.03
0.02
0.01
0
-0.01
Ethanol Yield (% / CgL-1)
10
20
30
40
Supplemented Carbon (g/L)
Glucose
Glycerol
Lactose
Xylose
Sucrose
Fructose
Maltose


B
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
Ethanol Yield (% / CgL-1)
10
20
30
40
Supplemented Carbon (g/L)
Glucose
Glycerol
Lactose
Xylose
Sucrose
Fructose
Maltose

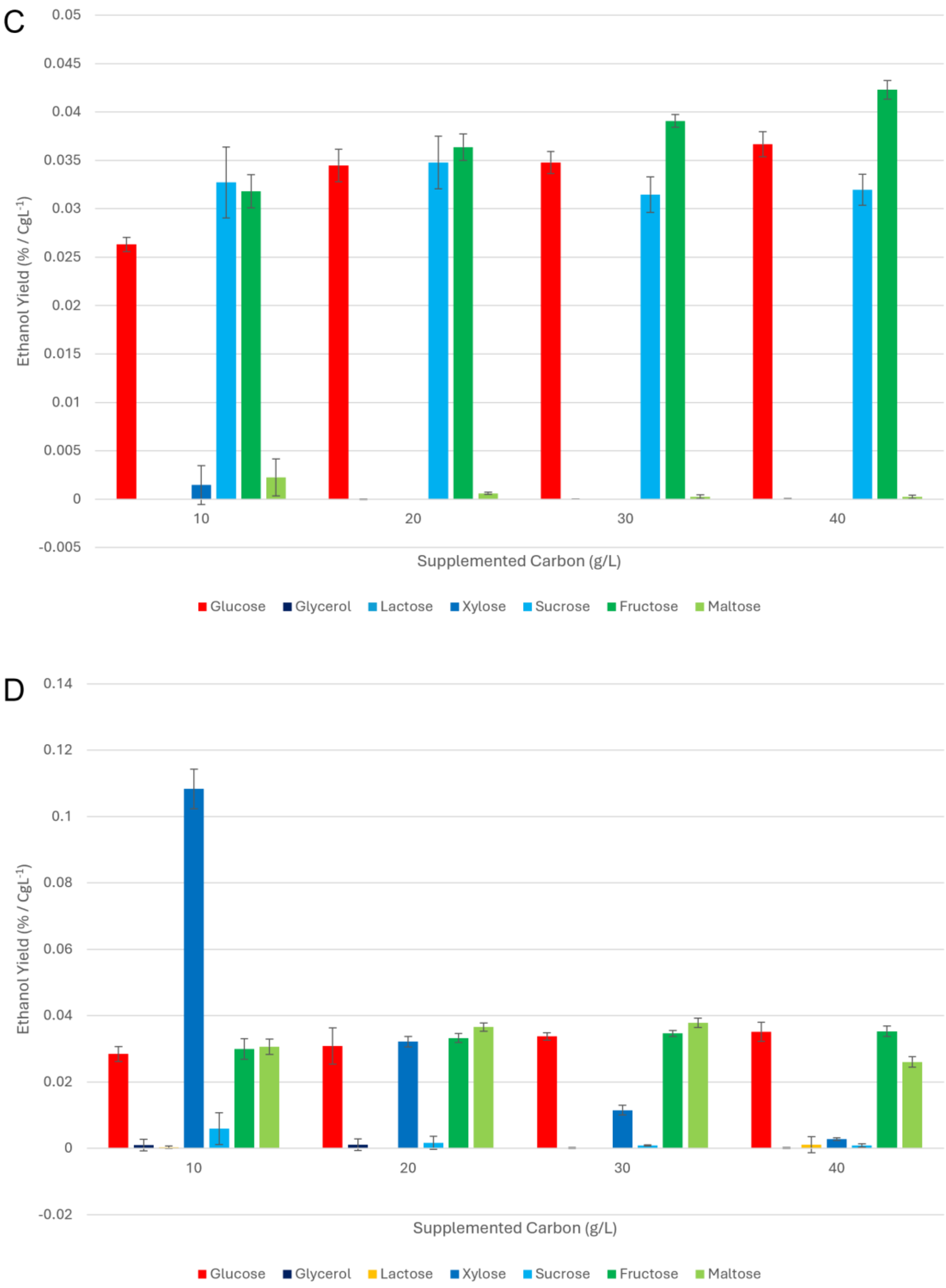


Figure 23 – ***Ethanol yield (% / CgL^{-1}) when S. cerevisiae (A & C) and S. stipitis (B & D) were grown in minimal (A & B) or YPD (C & D) media (24 hours at 30°C) containing a range of different substrates (Glucose, Glycerol, Lactose, Xylose, Sucrose, Fructose, and Maltose). Substrate concentration ranged from 10-40 g/L.***

The ethanol yield per gram of supplemented glucose, sucrose, and fructose by *S. cerevisiae* in minimal media can be seen in figure 23a to be reasonably consistent across the four concentrations of the supplementation. As can be seen in figure 23b, where the ethanol yield per gram of supplemented glucose, fructose and maltose is fairly consistent across the concentrations of supplementations tested, when *S. stipitis* is grown in minimal media.

However, it can be observed in figure 23a and figure 23c, that when *S. stipitis* is grown in minimal media and YPD media respectively. The ethanol yield per gram of supplemented xylose, decreases rapidly as the concentration of supplemented xylose increases. Meaning that as you supplement more xylose, you experience diminishing returns on the ethanol yield.

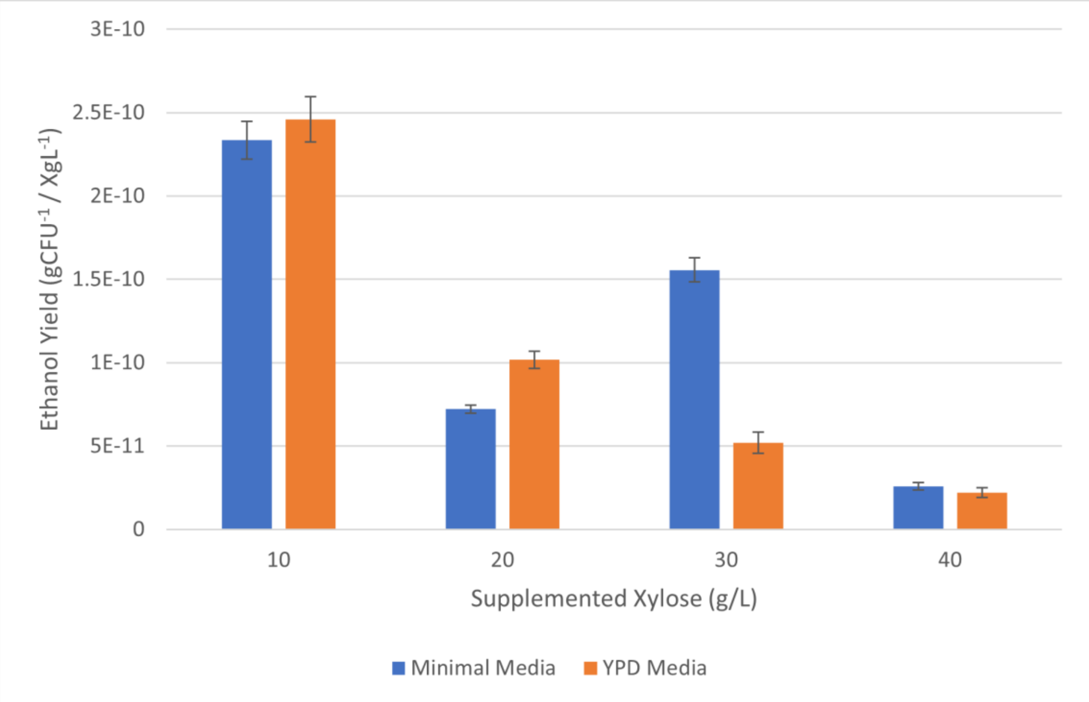


Figure 24 – ***Ethanol yield (gCFU^{-1} / XgL^{-1}) when S. stipitis is grown in minimal or YPD media (24 hours at 30°C) containing xylose at a range of 10-40g/L.***

It can be seen in figure 24 that the ethanol yield per CFU decreases as the concentration of xylose increases. Figure 24 is taking into account the lower CFU count of S. stipitis when grown in media supplemented with higher concentrations of xylose, which was observed in section 4, figure X. Figure 4 shows that the ethanol yield is decreasing not just because of the lower CFU count, but also due to a lower ethanol yield per CFU.

Across the range of concentrations tested *S. cerevisiae* is shown to produce similar levels of ethanol, whether grown in minimal media or complex YPD medium (Fig. 23a, 23c).

However, at lower concentrations of supplemented glucose (10g/L, and 20g/L), *S. cerevisiae* has a higher ethanol yield when grown in minimal media, compared to YPD media. Although at the higher concentrations of supplemented glucose (30g/L, and 40g/L), *S. cerevisiae* produces more ethanol when grown in YPD media, rather than minimal media. The is also observed when *S. cerevisiae* is grown in minimal, or YPD media which has been supplemented with fructose (Fig. 23a, 23c).

Table 3 - ***Summary of whether there is a substantial increase in ethanol yield from the supplementation of each carbon source compared to the no supplementation control. Ethanol produced by S. cerevisiae and S. stipitis, grown in Minimal media and YPD media.***

A green arrow signifies that the bioethanol yield is increased when the respective biocatalyst is supplemented with the carbon source, with said arrow being large when an increased concentration of the carbon source supplemented results in an increased bioethanol yield, whereas a small arrow is when the supplementation increases the bioethanol yield, but doesn't lead to an increased yield as the concentration of the supplemented carbon source increases. A horizontal black line signifies that the when the respective biocatalyst is supplemented with the carbon source no change was exhibited in respect to the bioethanol yield compared to the no supplementation control.

	S. cerevisiae		*S. stipitis*	
Supplemented Carbon	Minimal Media	YPD Media	Minimal Media	YPD Media
Glucose	↑	↑	↑	↑
Glycerol	—	—	—	—
Lactose	—	—	—	—
Xylose	—	—	↑	↑
Sucrose	↑	↑	—	—
Fructose	↑	↑	↑	↑
Maltose	—	—	↑	↑

An Investigation into Redirecting Metabolic Flux to Increase Ethanol Production by *S. cerevisiae*:

The effect of the redirection of metabolic flux upon ethanol production was investigated by using the *S. cerevisiae* strain YDL022W. YDL022W, wildtype *S. cerevisiae* and wildtype *S. stipitis*, were supplemented with glucose at 10g/L, 20g/L, 30g/L, and 40g/L. With ethanol production being determined by measuring the ethanol present in samples by GCMS. And the biomass yield was measured converting OD600 readings to CFUs.

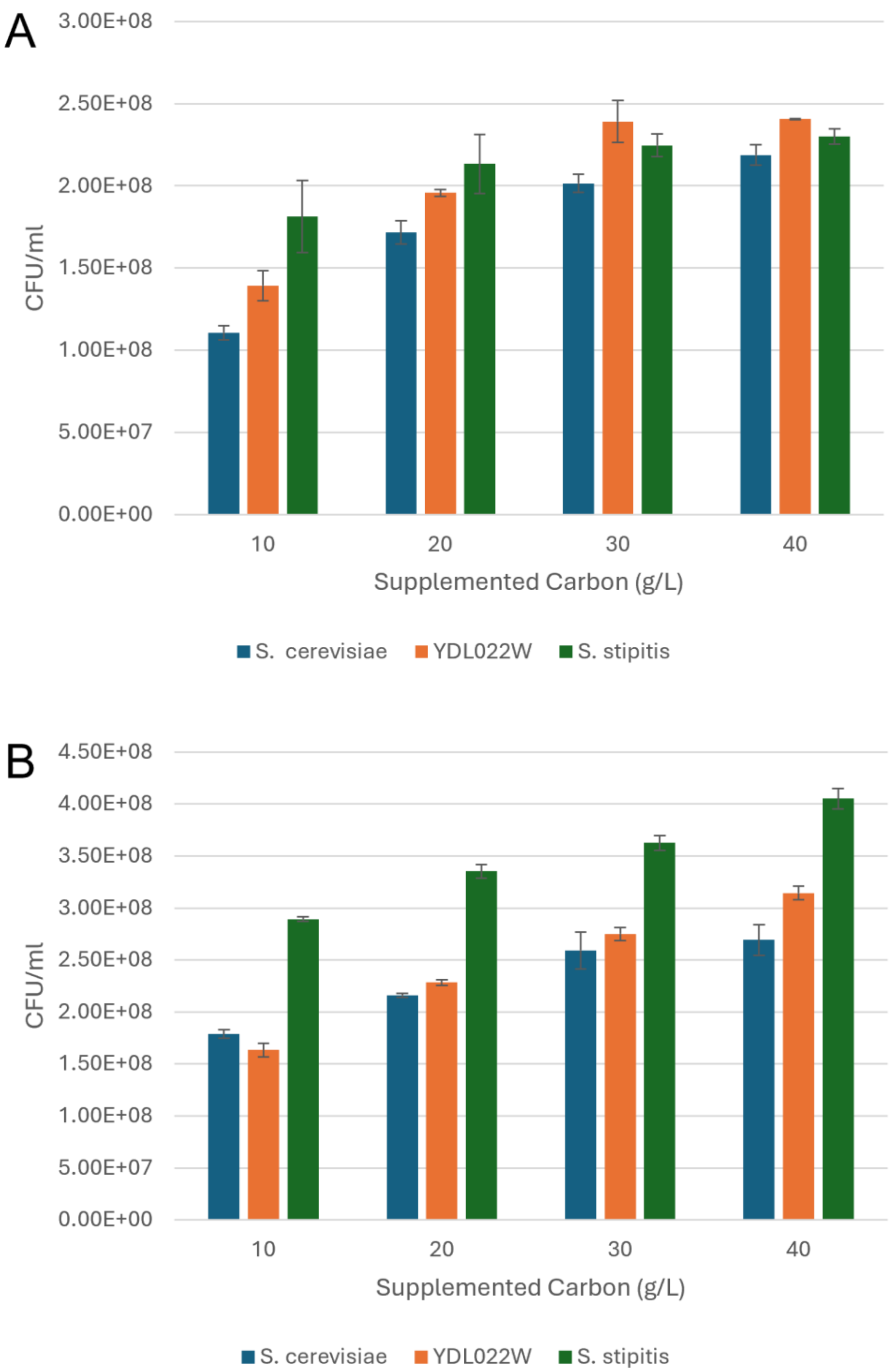


Figure 25 – ***Biomass yield (CFU/ml) when S. cerevisiae Wildtype, S. cerevisiae YDL022W, or S. stipitis Wildtype were grown in minimal (A) or YPD (B) media (24 hours at 30°C) containing glucose, at concentrations ranging from 10-40 g/L.***

Figure 25A shows that in minimal media YDL022W grew to a higher biomass yield than wildtype *S. cerevisiae*. With the amount of the increase in CFUs being consistent regardless of the amount of supplemented glucose.

When grown in minimal media YDL022W grew to a greater biomass than wildtype *S. stipitis* when supplemented with glucose at 30g/L and 40g/L.

In minimal media, the increase in CFUs decreases at the higher incremental increases of glucose supplementation. At 20g/L there is a 55.2%, 40.6%, and 17.7% increase (*S. cerevisiae,* YDL022W, and *S. stipitis* respectively), whereas from 30g/L to 40g/L there is only an 8.6%, 0.6%, and 2.4% increase (*S. cerevisiae,* YDL022W, and *S. stipitis* respectively) (Fig. 25A).

When grown in YPD media, YDL022W grew to a greater biomass yield than wildtype *S. cerevisiae* in every glucose supplementation other than 10g/L. Whilst wildtype *S. stipitis* grew to a greater biomass yield than YDL022W and wildtype *S. cerevisiae* in every quantity of supplemented glucose (Fig. 25B).

Wildtype *S. cerevisiae,* wildtype *S. stipitis*, and YDL022W all grew to a greater biomass in YPD media than in minimal media, at each of the tested levels of glucose supplementation. With the general trend observed for all three strains, in both minimal and YPD media, being that CFUs increase as the amount of supplemented glucose increases (Fig. 25A, 25B).

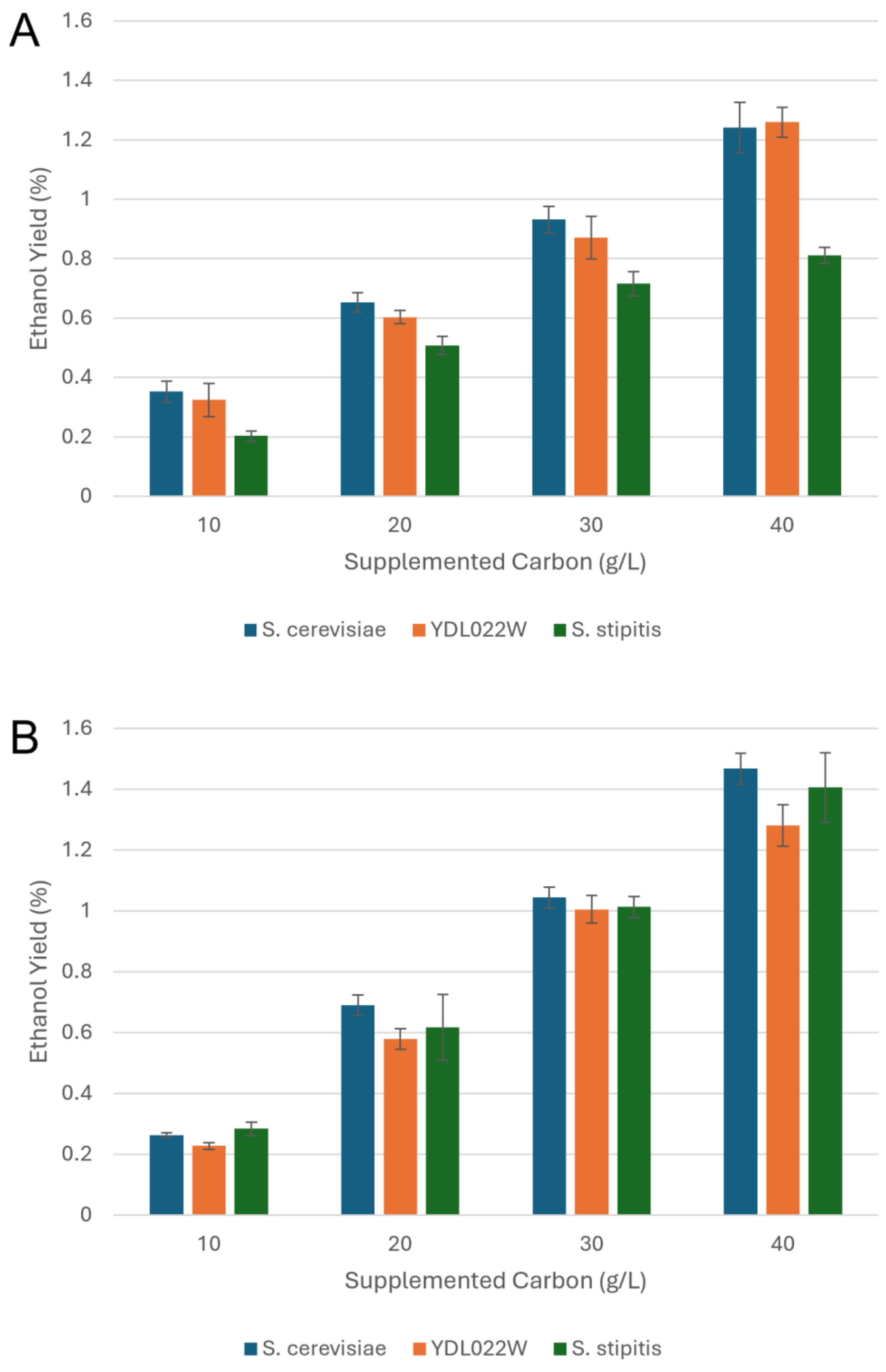


Figure 26 – ***Ethanol yield (%) when S. cerevisiae Wildtype, S. cerevisiae YDL022W, and S. stipitis Wildtype were grown in minimal (A) or YPD (B) media (24 hours at 30°C) containing glucose at concentrations ranging from 10-40 g/L.***

Across the three organisms tested, in both medias the ethanol yield increases as the amount of supplemented glucose increases (Fig. 26A, 26B).

Figure 26A shows that when grown in minimal media wildtype *S. cerevisiae* and YDL022W produce similar amounts of ethanol. Whilst *S. stipitis* produces significantly less ethanol than either.

When grown in YPD media *S. cerevisiae* produces consistently more ethanol than YDL022W in each level of supplemented glucose. With *S. cerevisiae* producing 15.7%, 19.0%, 3.8%, and 14.5% more ethanol (in 10g/L, 20g/L, 30g/L, and 40g/L of supplemented glucose) respectively than YDL022W (Fig. 26B).

Despite YDL022W having a higher biomass yield than wildtype *S. cerevisiae* (ranging from $1.39x10^8$ to $2.41x10^8$, compared to $1.11x10^8$ to $2.19x10^8$ respectively, in minimal medium, and ranging from $1.63x10^8$ to $3.14x10^8$, compared to $1.79x10^8$ to $2.69x10^8$ respectively, in YPD medium), (Fig. 25A, 25B), the ethanol yield of YDL022W is equal to or less than that of wildtype *S. cerevisiae* (ranging from 0.32% to 1.26%, compared to 0.35% to 1.24% respectively, in minimal medium, and ranging from 0.23% to 1.28%, compared to 0.26% to 1.47% respectively, in YPD medium), (Fig. 26A, 26B).

Meaning that the ethanol yield per CFU is less by YDL022W, than that of *S. cerevisiae*. Therefore, the knocking out of the GPD1 gene, redirecting the metabolic flux, has in fact been detrimental to the ethanol yield, rather than increasing it.

Biomass and Biosolvent yield of *C. saccharperbutylacetonicum*, when supplemented with a range of prospective feedstocks:

Clostridium saccharperbutylacetonicum is an industrial microorganism which is used to produce Acetone, Butanol and Ethanol.

Here the biomass yield has been measured and reported, because the biomass yield of the organism is vital when comparing the solvent yield. This is because the more of the microorganism present in the culture, the more solvent that can be produced within a given time frame, by the culture as a collective. The optimum use of bioreactor time is essential for the economic viability of the process, this is because bioreactors are a very expensive infrastructure component of the process, so their time must be utilised in the most efficient way.

The solvent produced has been reported by comparing the amount of acetone, butanol, and ethanol, produced when *C. saccharoperbutylacetonicum* is supplemented with different carbon sources. As well as comparing the acetone, butanol, and ethanol yields independently to each other, in respect to the feedstock supplemented.

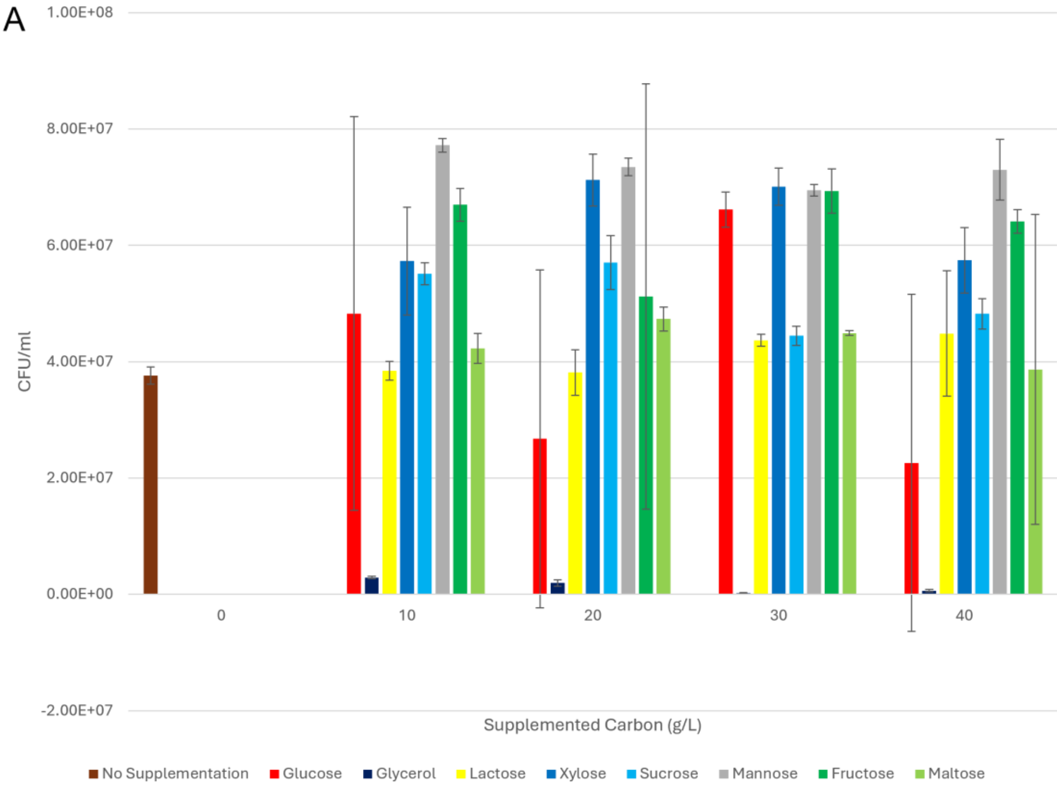
C. saccharoperbutylacetonicum was grown in a complex medium. Meaning that there will have been other carbon sources present in the medium, as well as the supplemented carbon source of interest.

C. saccharoperbutylacetonicum experienced inconsistent growth between biological replicates, when grown on a small laboratory scale in serum bottles. Due to this a wide range of CFU values were obtained for each condition tested, leading to large standard deviations.

The solvent yield of each solvent produced by *C. saccharoperbutylacetonicum* is low, meaning that when the raw GC-MS data was being analysed, the peak integration function in ‘Agilent MassHunter Qualitative Analysis’ software did not necessarily always detect the solvent peak, due to the size of the peak being below the detection threshold.

<u>Biomass yield of *C. saccharperbutylacetonicum*, when supplemented with a range of prospective feedstocks.</u>

A



B

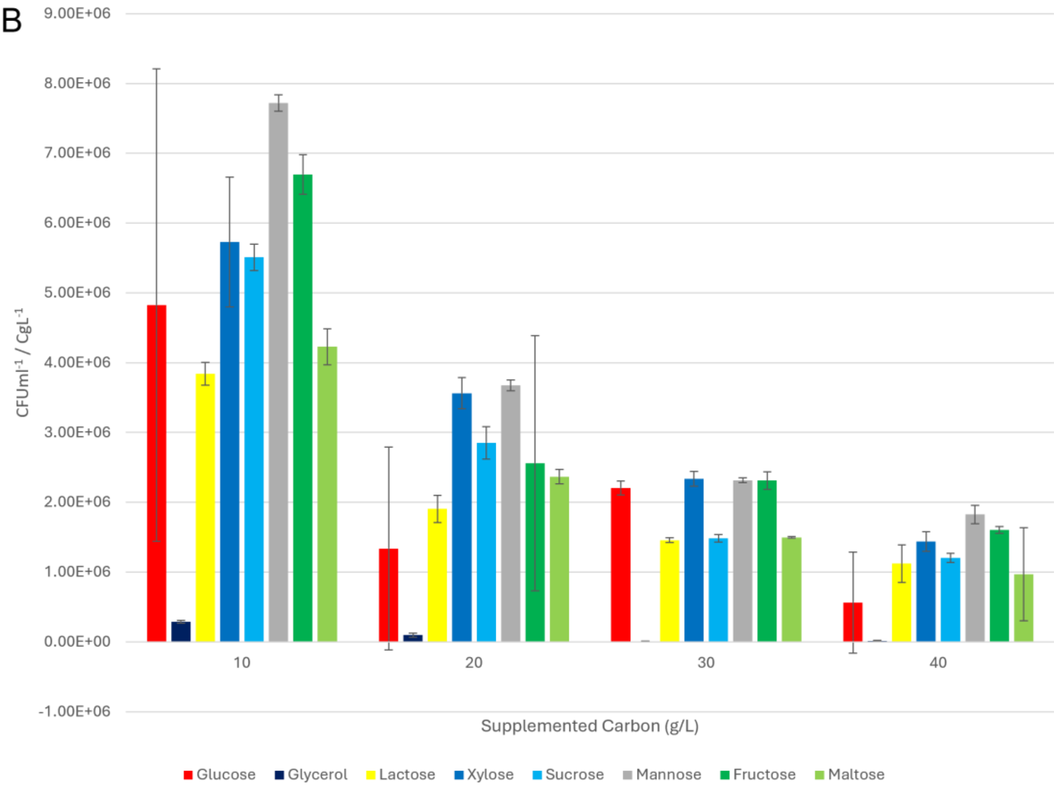


Figure 27 – ***Biomass yield (CFU/ml) (A), and Biomass yield ($CFUml^{-1}$ / gCL^{-1}), when C. saccharoperbutylacetonicum was grown in Clostridial nutrient medium (24 hours at 32^{o}C) containing a range of different substrates (Glucose, Glycerol, Lactose, Xylose, Sucrose, Mannose, Fructose, and Maltose). Substrate concentration ranged from 10-40g/L.***

There is no increase in CFUs as the amount of supplementation increases, this is the case for all the carbon sources tested (Fig. 27A). Figure 27B shows diminishing returns is experienced in respects to biomass yield as the concentration of the supplemented carbon source increases.

C. saccharoperbutylacetonicum can be seen not to grow when grown in the presence of glycerol. Whilst when supplemented with lactose, maltose, and sucrose (at 30g/L and 40g/L) there is a biomass yield comparable to the no supplementation control sample. Meaning that *C. saccharoperbutylacetonicum* is likely not utilising lactose and maltose for growth.

When supplemented with xylose, mannose, and fructose, *C. saccharoperbutylacetonicum* grows to higher CFUs than the no supplementation control sample, meaning the supplementation of xylose, mannose and fructose leads to a greater biomass yield. Although the biomass yield does not increase at higher levels of supplementation of xylose, mannose, and fructose (Fig. 27A, 27B).

Biosolvent yield of *C. saccharperbutylacetonicum*, when supplemented with a range of prospective feedstocks.

Figure 28 – ***Acetone, Ethanol and Butanol yield (%) when C. saccharoperbutylacetonicum was grown in clostridial nutrient medium (24 hours at 32°C) containing a range of different substrates (Glucose (A), Glycerol (B), Lactose (C), Xylose (D), Sucrose (E), Mannose (F), Fructose (G), Maltose (H), and no additional substrate (I)). Substrate concentration ranged from 10-40g/L.***

Figure 28A shows that when supplemented with glucose, the solvent which *C. saccharoperbutylacetonicum* produces the most of is butanol. With the concentration within the sample reaching 0.083%, when *C. saccharoperbutylacetonicum* is supplemented with glucose at 10g/L. However higher supplementation of glucose is shown to be detrimental to butanol production.

As was observed in figure 27A *C. saccharoperbutylacetonicum* had a very low biomass yield when supplemented with glycerol. This leads to minimal solvent production (Fig. 28B). The value of 0.064% for ethanol produced at a supplementation level of glycerol at 20g/L is likely to be an anomalous result, given the exceedingly large error bar.

When supplemented with lactose *C. saccharoperbutylacetonicum* produces a minimal amount of solvent across the board. With all the values being less than 0.05% (Fig. 28C).

The highest yields of biosolvent when *C. saccharoperbutylacetonicum* is supplemented with xylose is seen at the lowest concentration of supplementation (10g/L), with the lowest yield being at the highest concentration of xylose supplementation (40g/L), meaning that xylose is not a valid feedstock for biosolvent production by *C. saccharoperbutylacetonicum* (Fig. 28D).

As was seen with lactose, when *C. saccharoperbutylacetonicum* is supplemented with sucrose there is minimal biosolvent produced across the board, with all values being less than 0.07% (Fig. 28E).

Mannose results in the greatest biosolvent yield at 40g/L of supplementation to *C. saccharoperbutylacetonicum*. With a butanol yield of 0.3% at 40g/L of supplementation. At 10g/L, 20g/L and 30g/L the levels of acetone and butanol produced are comparable across concentrations, then within each concentration the butanol yield is slightly higher than the acetone yield (40%, 10%, and 14%, respectively), whilst at 40g/L supplementation the yield of butanol is 70% higher than that of acetone (Fig. 28F).

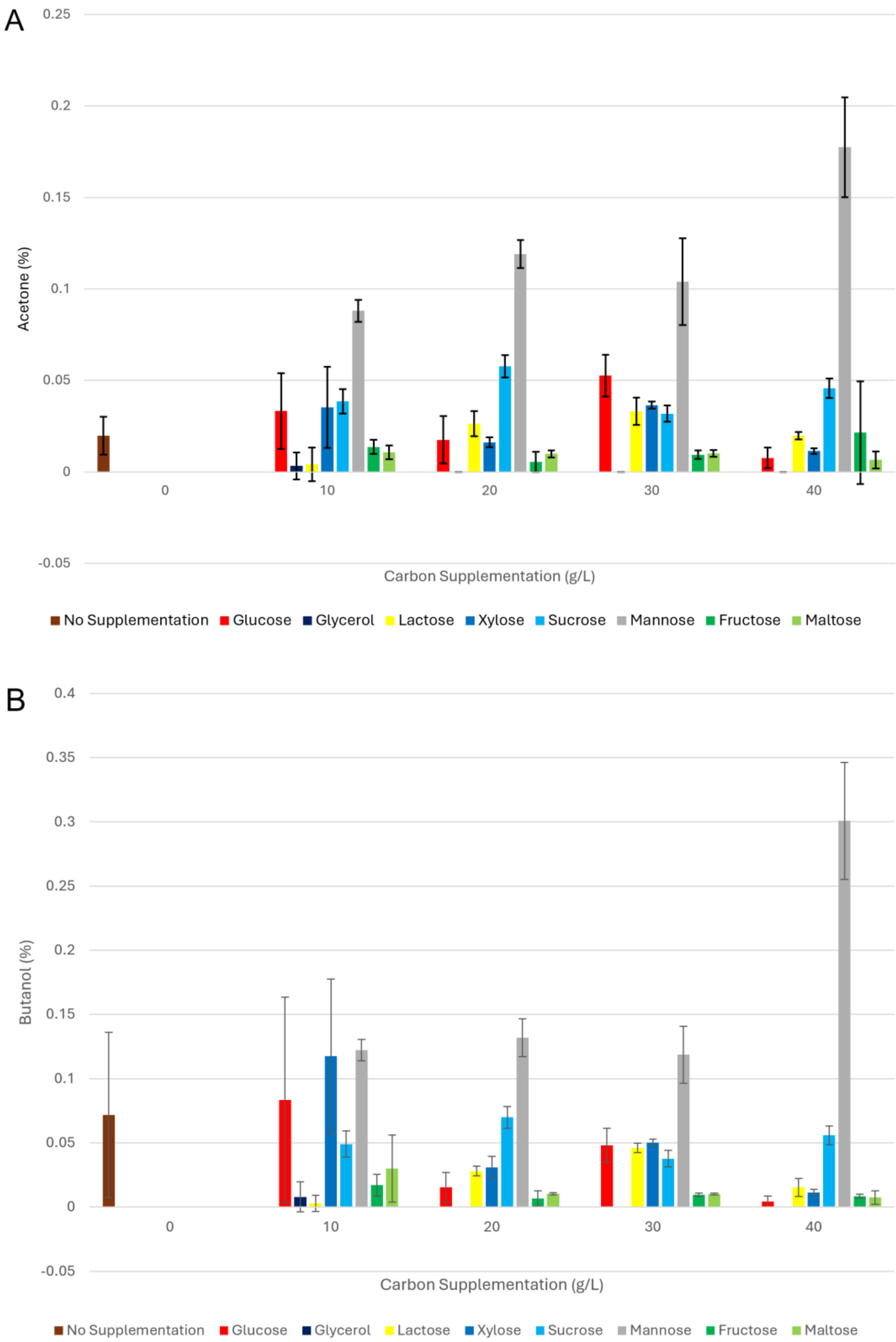
When *C. saccharoperbutylacetonicum* is supplemented with fructose, across the four concentrations of supplementation a minimal amount of biosolvent is produced (Fig. 28G).

Figure 28H shows that the supplementation of *C. saccharoperbutylacetonicum* with maltose leads to a minimal biosolvent yield across the tested concentrations of supplemented maltose.

Figure 28I shows that when *C. saccharoperbutylacetonicum* is grown in Clostridial Nutrient Medium, without additional supplementation butanol is the highest yielding biosolvent, 29% higher than the ethanol yield, which itself has a 180% higher yield than acetone, which is the lowest yielding at 0.02% of the sample after 24 hours of growth.

The general trend across figure 28 is that ethanol is produced in the lowest quantity by *C. saccharoperbutylacetonicum* and butanol in the highest. With it also being seen that the biosolvent yield doesn’t increase in line with supplementation.

The levels of Acetone, Butanol and Ethanol produced compared to each other does vary depending on the supplemented carbon source.


A
0.25
0.2
0.15
0.1
0.05
0
-0.05
Acetone (%)
0
10
20
30
40
Carbon Supplementation (g/L)
No Supplementation
Glucose
Glycerol
Lactose
Xylose
Sucrose
Mannose
Fructose
Maltose
B
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
Butanol (%)
0
10
20
30
40
Carbon Supplementation (g/L)
No Supplementation
Glucose
Glycerol
Lactose
Xylose
Sucrose
Mannose
Fructose
Maltose

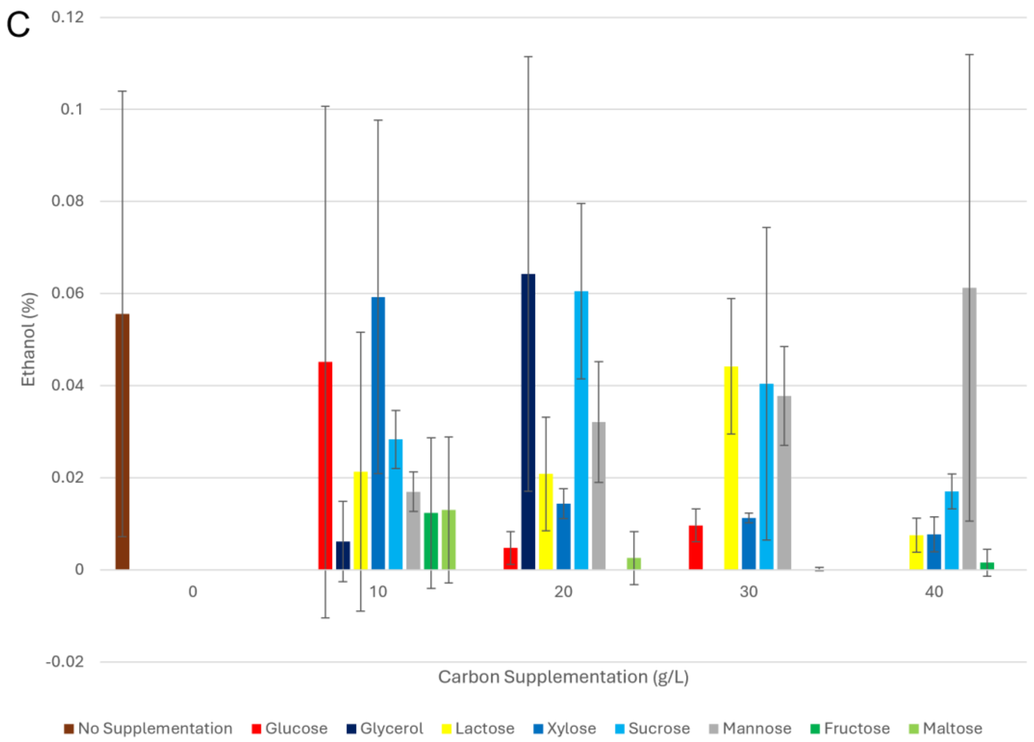


Figure 29 – ***Acetone (A), Butanol (B), and Ethanol (C) yields (%) when C. saccharoperbutylacetonicum is grown in clostridial nutrient medium (24 hours at 32°C) containing a range of different substrates (Glucose, Glycerol, Lactose, Xylose, Sucrose, Mannose, Fructose, and Maltose). Substrate concentration ranged from 10-40g/L.***

The acetone yield doesn't increase in a consistent manner as the supplemented carbon increases. Mannose is the only supplemented carbon source which sees a significant increase in the yield of acetone, with 40g/L yielding 101.5% greater amount of acetone than when *C. saccharoperbutylacetonicum* is supplemented with 10g/L of mannose (Fig. 29A).

Less acetone is produced when *C. saccharoperbutylacetonicum* is supplemented with glycerol, fructose, and maltose at all concentrations of supplementation, when compared to the no supplementation control sample (Fig. 29A).

Figure 29A shows that the greatest acetone yield reached is 0.18%, which is when *C. saccharoperbutylacetonicum* is supplemented with mannose at 40g/L.

All yields of acetone remain below 0.05%, apart from when *C. saccharoperbutylacetonicum* is supplemented with mannose (apart from sucrose at 20g/L, and glucose at 30g/L, which yield 0.058% and 0.053% acetone respectively) (Fig. 29A).

Mannose is the best supplemented carbon source in terms of butanol yielded by *C. saccharoperbutylacetonicum* over a 24-hour period (Fig. 29B).

In general, as the concentration of supplemented carbon source increases there is either a decrease in butanol produced, or no significant difference to the butanol yielded by *C. saccharoperbutylacetonicum* over a 24-hour period. The exception to this being when *C. saccharoperbutylacetonicum* is supplemented with mannose (Fig. 29B).

In terms of butanol yield, all supplemented carbon sources tested (apart from mannose) at a concentration of 20g/L or above, yield less butanol than the no supplementation control sample. With less butanol being produced than the no supplementation control sample at all tested concentrations when *C. saccharoperbutylacetonicum* was supplemented with glycerol, lactose, sucrose, fructose and maltose (Fig. 29B).

It can be seen in figure 29B, that the highest butanol yield is obtained when *C. saccharoperbutylacetonicum* is supplemented with mannose at 40g/L (0.30%). The second highest butanol yield is only 0.13% (56.7% less than the highest), this is reached when *C. saccharoperbutylacetonicum* is supplemented with mannose at 20g/L.

Ethanol is the lowest yielding solvent out of the three produced by *C. saccharoperbutylacetonicum*. With the highest ethanol yield (0.064%) being when *C. saccharoperbutylacetonicum* is supplemented with glycerol at 20g/L (Fig. 29C). However, as previously noted this may be an anomalous result.

Figure 29C exhibits very sizeable error bars, as detailed previously this is due to the inconsistent growth of *C. saccharoperbutylacetonicum* when grown at a laboratory scale. Combined with the very low ethanol proportions within the samples, meaning that the ethanol peak in the GC-MS trace was not substantial enough for the peak integration function to detect it.

The no supplementation control sample yielded a greater amount of ethanol than when *C. saccharoperbutylacetonicum* was supplemented with; glucose, lactose, fructose, and maltose at all tested concentrations (Fig. 29C).

Table 4 - ***Summary of whether there is a substantial increase in biomass yield and ABE yield from the supplementation of C. saccharoperbutylacetonicum with different carbon sources compared to the no supplementation control, when grown in clostridial nutrient medium (24 hours at 32°C.*** *A green arrow signifies an improved yield, whereas a red arrow signifies that the yield has decreased. With a large arrow meaning the yield is greatly changed, whereas a small arrow means that the yield is on the whole changed by the supplementation. A horizontal black line signifies that there has been no change compared to the no supplementation control.*

Supplemented Carbon	Biomass Yield	Acetone Yield	Butanol Yield	Ethanol Yield
Glucose	↑	↑	↓	⬇
Glycerol	⬇	⬇	⬇	⬇
Lactose	—	—	⬇	↓
Xylose	⬆	—	⬇	↓
Sucrose	↑	⬆	—	—

Mannose	↑	↑	↑	↑
Fructose	↑	—	↓	↓
Maltose	—	—	↓	↓

An Investigation into the Economic Viability of Large-Scale Batch Fermentation and Anaerobic Digestion:

In this section the economic viability of Large-Scale Batch Fermentation and Anaerobic Digestion will be analysed. By comparing the volume of product produced per tonne of feedstock, and the cost of the feedstock per tonne, resulting in a comparison of the difference between the value of the electricity generated against the cost of the feedstock, for the various feedstocks.

The unit costs for various feedstocks for anaerobic digestion or large-scale batch fermentation have been identified. Alongside the volume of methane produced per unit of feedstock. The revenue per tonne of feedstock has been calculated, using the base rate tariff, of 5.51p per kWh, set by the UK government (22).

Then there will be a more detailed comparison between Large-Scale Batch Fermentation and Anaerobic Digestion, using Maize as the feedstock.

Considering; start-up costs, ongoing expenses, feedstock costs, value of the product produced and government policies/incentives.

Overview of various potential feedstocks for anaerobic digestion, cost of said feedstocks, and volume and value of the methane produced.

Table 5 – ***Cost of potential feedstocks per tonne, alongside the volume of product (methane gas) produced, as well as the revenue and profit from the sale of the methane gas produced from the digestion of the feedstock in the anaerobic digester.***

Feedstock	Cost of feedstock (wet mass) midpoint (GBP/tonne)	Volume of biogas produced (m^3/tonne of feedstock) (2)	Volume of methane produced (m^3/tonne of feedstock)	Amount of electricity generated from the methane (kWh), (10kWh per m^3 of methane). (73)	Difference between the electricity sale price and cost of feedstock (GBP/tonne of feedstock).
Cattle Slurry	-	20	12	120	£6.61
Grass Silage	£52.22 (74)	180	108	1080	£7.29
Maize Silage	£31.68 (75)	210	126	1260	£37.75
Crude Glycine	£217.60 (76)	790	474	4740	£43.57
Sugar Beet	£40.00 (77)	293.50	176.1	1761	£57.03
Potatoes	£167.50 (78)	338	202.8	2028	£55.76
Straw	£55.00 (79)	283	169.8	1698	£38.56

The cost of potential feedstocks for anaerobic digestion varies greatly. With cattle slurry being freely and readily available to dairy farmers, and maize silage being £31.68 per tonne. Whereas potatoes and crude glycine are the most expensive per tonne, at £167.50 and £217.60 respectively. Likely due to potatoes being primarily grown as a food crop, rather than an energy crop, and crude glycine being a by-product of industrial processes, making it different from the other potential feedstocks which are agricultural products.

The biogas produced by anaerobic digestion is ~60% methane (13). This methane is stripped from the biogas and can be sold as biomethane, or burnt on-site to generate electricity which can be sold directly to the National Grid, as well as being used by the anaerobic digester parasitically.

The volume of methane produced also varies widely between the feedstocks, with cattle slurry only producing 12m^3 per tonne. Whereas maize silage and potatoes produce 126m^3 and 202.8m^3 of methane respectively.

Due to the variety of feedstock costs, and biomethane yields, there is also a wide range in the 'value of the electricity sold minus the cost of the feedstock'. With cattle slurry only yielding £6.61 per tonne, whereas maize silage yields £37.75 per tonne, and sugar beet has the highest yield at £57.03 per tonne.

Overview of various potential feedstocks for large-scale batch fermentation digestion, cost of said feedstocks, and volume and value of the bioethanol produced.

Table 6 - ***Cost of various feedstocks per tonne, alongside the volume of product (ethanol) produced, and the revenue and profit from the sale of the ethanol produced from the fermentation of the feedstock in the bioreactor.***

Feedstock	Cost of feedstock (GBP/tonne)	Ethanol produced (L) / tonne of feedstock.	Value of ethanol (GBP) / L (80)	Value of Ethanol (GBP) / tonne of feedstock	(Ethanol value (GBP) / tonne of feedstock) – (cost of feedstock (GBP)/tonne)
Potatoes	£167.50 (78)	135 (81)	£0.46	£62.10	£-105.40
Sugarcane	£28.41 (82)	90 (83)	£0.46	£41.40	£12.99
Sugar Beet	£40.00 (77)	110 (84)	£0.46	£50.60	£10.60
Corn (Grain)	£187.90 (85)	445 (86)	£0.46	£204.70	£16.80
Corn Stover	£43.36 (87)	205 (86)	£0.46	£94.30	£50.94

The product of fermentation is bioethanol, which has a value of £0.46 per litre. Corn grain has the highest yield of ethanol per tonne, at 445 litres, and costs £187.90 per tonne. Leading to the value of the ethanol produced after the cost of the feedstock being £16.80 per tonne of feedstock.

Whereas corn stover which is a waste product from the production of corn grain, is 23.1% the cost of corn grain per tonne, at £43.36 per tonne, whilst producing 46.1% the amount of ethanol. Meaning that the value of the ethanol, produced after the cost of the feedstock is £50.94 per tonne of feedstock.

However not all feedstocks are economically viable. Potatoes cost £167.50 per tonne, but only yield 135 litres of ethanol per tonne. Leading to a value of the ethanol produced after the cost of the feedstock of £-105.40.

In depth evaluation of the economics involved in Anaerobic Digestion.

- The average OLR (organic loading rate) for anaerobic digestion is 2.5kg/m^3/day (88).
- If the plant is 6000m^3, then the daily processing capacity is 15 tonnes.
- This would produce 18.9MWh/day of electricity. Which being sold at the rate of 5.51p per kWh (which is the rate on tariff 1 (22)), would generate £1,041.39/day of revenue.

- The average start-up cost is £4,500 per kWe (10), in this example the kWe is 788. Therefore, the cost is £3,546,000.
- The maintenance cost is ~1.5%, therefore the maintenance cost is £53,190/year. Over 10 years this will be £531,900. Resulting in an overall cost of £4,077,900.
- The repayment time will be 10.73 years, without costs being considered.

- However, there is the feedstock cost which would be £475.20/day.
- With an average transport cost of £7/tonne (89), this would cost £105.00/day.
- However, a £26/tonne gate fee (23) can be charged, for the processing of organic waste, be this from industrial or municipal sources.

In depth evaluation of the economics involved in Large-Scale Batch Fermentation.

- A 4,000m^3 Plant to process corn grain into ethanol would cost ~£32 million (90).
- 1m^3 of corn is 0.76 tonnes.
- Corn comprises 31.1% of the slurry which is fermented (91).
- 80% of the volume of the fermenter is usable (92), therefore in one batch 756.4 tonnes of corn grain can be fermented.

- One batch would take 56 hours, including 6 hours to remove the ethanol, and clean the fermenter. The plant can run 24/7, for 330 days a year (to allow for downtime / maintenance) (90).
- One tonne of corn grain produces 445 litres of ethanol (86).
- 144,256 litres of ethanol would be produced a day.
- Daily revenue would be £66,358. Therefore, annually revenue would be £21,898,061.
- The annual cost of the feedstock would be £20,100,898.

- DDGS (Dried Distillers Grains with Solubles) is a waste product from this process, and can be used as an animal feed, which has a high protein content.
- The conversion rate from corn grain to DDG (Dried Distillers Grains) is 32.2% (92).

- The average value of DDG is £145.28/tonne (93).
- Annually 34,446.34 tonnes of DDG would be produced, which would have a value of £5,004,365.14 per annum.

- However, there are high labour costs involved, which have been estimated to be in the region of £3.6 million, alongside repayments for the cost of the plant and other costs involved (90).

- These calculations assume that the yield of 445L/tonne of corn grain will be reached.
- These calculations are also heavily dependent on a consistent large supply of feedstock, allowing the plant to run consistently for the projected 330 days a year.

To conclude anaerobic digestion could be economically viable for a farmer, who has a ready supply of feedstock. Such as cattle slurry if they were a livestock farmer, which could be subsidised with grass or maize silage which could be produced on the farm. As well as municipal/industrial vegetative waste, which alongside providing more feedstock, would be able to provide an additional revenue stream for the farmer due to the charging of a gate fee, for the processing of the municipal/industrial vegetative waste. The locality of the feedstock is vital for the enterprise to be economically viable, due to the aforementioned high cost of transport at £7 / tonne (89).

Government schemes/incentives could be implemented/utilised which could increase the economic viability of anaerobic digestion for the farmer. Be it tax relief, low/no interest rate loans, or subsidies/grants. Currently grants to assist with the capital investment required to purchase an anaerobic digester are available through the GGSS (Green Gas Support Scheme) (94).

On a larger scale than anaerobic digestion, bulk batch fermentation of corn grain, in conjunction with corn stover could be economically viable. However, a very large supply of consistent feedstock will be required (106,976 tonnes of corn grain annually, being 324 tonnes a day as per the previous example), as well as a high initial investment (~£32 million (90)). Bulk batch fermentation of corn grain becomes economically viable when the Dried Distillers Grains is sold for animal feed, meaning a consistent large-scale buyer/buyers will need to be available for the Dried Distillers Grains, such as cattle feedlots.

However, for both processes, there are other factors to consider. On the downside there are other costs to consider such as staffing costs (a full time employee paid living wage would cost the employer £24,612 (95)), and insurance costs. But on the upside, with an increasing demand for energy, in particular green energy, the prices which the value of the products may increase, increasing the overall revenue of both processes, making them more financially viable.

Discussion

(a) The effect of different carbon source supplementation on the single-cellular eukaryotic organisms *S. cerevisiae* and *S. stipitis*, with regard to biomass yield and bioethanol production:

Table 7 - ***Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with glucose at 10g/L, 20g/L, 30g/L, and 40g/L.***

Glucose supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisiae	Minimal Medium	Biomass yield	0.000	1.106×10^8	1.717×10^8	2.014×10^8	2.188×10^8
		Bioethanol yield	0	0.353	0.653	0.932	1.241
	YPD Medium	Biomass yield	8.538×10^7	1.787×10^8	2.161×10^8	2.593×10^8	2.693×10^8
		Bioethanol yield	0	0.263	0.69	1.044	1.467
S. stipitis	Minimal Medium	Biomass yield	2.294×10^7	1.993×10^7	2.313×10^7	2.426×10^7	2.480×10^7
		Bioethanol yield	0	0.203	0.508	0.716	0.812

	YPD Medium	Biomass yield	1.002x 10^8	3.073x 10^7	3.533x 10^7	3.806x 10^7	4.233x 10^7
		Bioethanol yield	0	0.284	0.617	1.013	1.405

Table 8 - ***Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with fructose at 10g/L, 20g/L, 30g/L, and 40g/L.***

Fructose supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisi-ae	Minimal Medium	Biomass yield	0.000	1.509x 10^8	2.128x 10^8	2.629x 10^8	2.632x 10^8
		Bioethanol yield	0	0.439	0.809	1.234	1.686
	YPD Medium	Biomass yield	8.538x 10^7	1.932x 10^8	2.700x 10^8	2.956x 10^8	3.365x 10^8
		Bioethanol yield	0	0.318	0.727	1.172	1.692
S. stipitis	Minimal Medium	Biomass yield	2.294x 10^7	3.043x 10^7	3.209x 10^7	3.305x 10^7	3.142x 10^7
		Bioethanol yield	0	0.224	0.418	0.596	0.605
	YPD Medium	Biomass yield	1.002x 10^8	3.832x 10^7	4.465x 10^7	4.865x 10^7	5.270x 10^7
		Bioethanol yield	0	0.299	0.665	1.040	1.411

S. cerevisiae grown in media supplemented with glucose or fructose has a much greater biomass yield and bioethanol yield than when *S. cerevisiae* is grown in minimal media or YPD media without additional carbon supplementation (Table 7, 8), (Fig. 17, 21).

Table 9 - ***Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with sucrose at 10g/L, 20g/L, 30g/L, and 40g/L.***

Sucrose supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisiae	Minimal Medium	Biomass yield	0.000	$1.094x10^8$	$1.649x10^8$	$1.869x10^8$	$1.898x10^8$
		Bioethanol yield	0.000	0.358	0.734	1.062	1.242
	YPD Medium	Biomass yield	$8.538x10^7$	$2.954x10^8$	$2.312x10^8$	$3.650x10^8$	$3.874x10^8$
		Bioethanol yield	0.000	0.327	0.696	0.944	1.278
S. stipitis	Minimal Medium	Biomass yield	$2.294x10^7$	$1.761x10^7$	$1.750x10^7$	$1.757x10^7$	$1.689x10^7$
		Bioethanol yield	0.000	0.029	0.07	0.078	0.122
	YPD Medium	Biomass yield	$1.002x10^7$	$3.068x10^7$	$3.056x10^7$	$2.903x10^7$	$2.694x10^7$
		Bioethanol yield	0.000	0.059	0.033	0.028	0.036

When *S. cerevisiae* is supplemented with sucrose there is an increase in both the biomass yield and bioethanol yield (Fig. 17, 21), (Table 9). *S. cerevisiae* can metabolise the disaccharide sucrose into a fructose monomer and a glucose monomer, because of *S. cerevisiae's* ability to produce the invertase enzyme '*β* – fructofuranosidase' (96). Meaning that *S. cerevisiae* can utilise sucrose for growth and ethanol production.

However, when *S. stipitis* is supplemented with sucrose the biomass yield increases, but ethanol is not produced (Fig. 17, 21), (Table 9). This is perplexing because the production of ethanol is a product of respiration by yeast, so *S. stipitis* is growing to a greater extent, but with only a marginal increase in the amount of ethanol produced, compared to when *S. stipitis* is not supplemented with an additional carbon source.

Mastella *et al. (2023)* showed bioinformatically that *S. stipitis* does not contain an enzyme homologous to SUC2, which is the invertase present in the *S. cerevisiae* reference strain (97). However, three potential invertases have been identified in the *S. stipitis* genome (97). These invertases may be intracellular, because Kobayashi *et al. (2021)* showed that when *S. stipitis* is supplemented with sucrose in minimal media, there is no significant build up of glucose or fructose monomers in the supernatant (98).

*Table 10 - **Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with xylose at 10g/L, 20g/L, 30g/L, and 40g/L.***

Xylose supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisiae	Minimal Medium	Biomass yield	0.000	5.545x 10^7	5.519x 10^7	2.162x 10^7	4.754x 10^7
		Bioethanol yield	0.000	0.000	0.000	0.014	0.000
	YPD Medium	Biomass yield	8.538x 10^7	1.467x 10^7	6.435x 10^7	2.399x 10^7	9.520x 10^7
		Bioethanol yield	0.000	0.015	0.000	0.000	0.000
S. stipitis	Minimal Medium	Biomass yield	2.294x 10^7	2.450x 10^7	2.842x 10^7	6.399x 10^7	9.697x 10^7
		Bioethanol yield	0.000	0.672	0.486	0.272	0.104
	YPD Medium	Biomass yield	1.002x 10^7	3.656x 10^7	2.676x 10^7	1.929x 10^7	1.185x 10^7
		Bioethanol yield	0.000	1.083	0.643	0.346	0.112

It is well known that *S. cerevisiae* is unable to utilise the pentose monosaccharide xylose (99), as evidenced in (Fig. 17a, 17c), (Table 10), showing that in minimal media there is no significant growth when supplemented with xylose. With a lower biomass yield when *S. cerevisiae* is supplemented with xylose in YPD medium, than when not supplemented with an additional carbon source (Fig. 17c), (Table 10). Suggesting that xylose may have a detrimental effect on the ability of *S. cerevisiae* to function ordinarily, resulting in a lower biomass yield.

S. stipitis however is known for its ability to utilise the pentose monosaccharide xylose (100), due to the presence of the enzymes xylose reductase, and xylitol dehydrogenase (101). Figure 17b and figure 17d show that the biomass yield is significantly greater when *S. stipitis* has been supplemented with xylose in minimal and YPD medium. Supplementation of *S. stipitis* with xylose also significantly increases the bioethanol yield, as shown in figure 21b and 21d. Demonstrating that *S. stipitis* can utilise the pentose monosaccharide xylose.

Table 11 - ***Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with glycerol at 10g/L, 20g/L, 30g/L, and 40g/L.***

Glycerol supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisiae	Minimal Medium	Biomass yield	0.000	4.865x 10^7	6.115x 10^7	2.192x 10^7	1.925x 10^7
		Bioethanol yield	0.000	0.039	0.000	0.000	0.000
	YPD Medium	Biomass yield	8.538x 10^7	4.266x 10^7	3.968x 10^7	4.030x 10^7	3.938x 10^7
		Bioethanol yield	0.000	0.000	0.000	0.000	0.001
S. stipitis	Minimal Medium	Biomass yield	2.294x 10^7	1.049x 10^7	1.063x 10^7	9.812x 10^7	1.082x 10^7
		Bioethanol yield	0.000	0.000	0.036	0.023	0.004
	YPD Medium	Biomass yield	1.002x 10^7	1.046x 10^7	1.166x 10^7	1.283x 10^7	1.298x 10^7
		Bioethanol yield	0.000	0.010	0.022	0.003	0.004

Glycerol is considered “non-fermentable” by *S. cerevisiae* (102). Figures 17 and 21 support this notion. Figures 17a and 21a, shows that when *S. cerevisiae* is supplemented with glycerol the biomass yield is inconsequential when grown in minimal media, and in the complex YPD medium the biomass yield is less than when *S. cerevisiae* is not supplemented with additional carbon source (Table 11).

Whereas, *S. stipitis* in minimal media grows to a slightly higher biomass yield than when not supplemented, and equivalent to the not supplemented control when grown in YPD medium. Meaning that *S. stipitis* isn’t as negatively impacted by the presence of glycerol as *S. cerevisiae* (Table 11), but the presence of glycerol cannot be considered beneficial to *S. stipitis* when you compare it to the effect which glucose or fructose has upon an *S. stipitis* culture in terms of biomass yield.

Figure 21, shows that when *S. cerevisiae* or *S. stipitis* are supplemented with glycerol ethanol is not produced, meaning fermentation isn’t taking place, supporting that glycerol is unfermentable by wildtype yeasts.

However, Aßkamp *et al*. *(2019)* (103) has shown that it is possible to modify *S. cerevisiae* to include a glycerol catabolic pathway, which leads to a small ethanol yield, then there is a 6-fold increase in the ethanol yield when a heterologous aquaglyceroporin is expressed, likely due to the increased ability of the modified *S. cerevisiae* to uptake glycerol.

There are however wildtype anaerobic bacterial strains such as *Anaerobium acetethylicum* (104)(which was isolated from a biogas plant, which was fed corn silage (105)), which are known to metabolise glycerol into primarily ethanol, with small quantities of formate, acetate and propylene glycol also being produced (when a culture of *Anaerobium acetethylicum* is supplemented with 48.7mM of glycerol, 3.6mM, 0.8mM, and 1mM of the aforementioned compounds are produced), (104).

Table 12 - ***Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with maltose at 10g/L, 20g/L, 30g/L, and 40g/L.***

Maltose supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisiae	Minimal Medium	Biomass yield	0.000	1.132x 10^7	1.929x 10^7	2.338x 10^7	2.780x 10^7
		Bioethanol yield	0.000	0.021	0.000	0.000	0.000
	YPD Medium	Biomass yield	8.538x 10^7	6.342x 10^7	6.622x 10^7	1.718x 10^7	1.942x 10^7
		Bioethanol yield	0.000	0.023	0.012	0.008	0.010
S. stipitis	Minimal Medium	Biomass yield	2.294x 10^7	2.469x 10^7	2.549x 10^7	2.944x 10^7	3.153x 10^7
		Bioethanol yield	0.000	0.282	0.718	0.730	0.661
	YPD Medium	Biomass yield	1.002x 10^7	3.395x 10^7	3.894x 10^7	4.310x 10^7	3.800x 10^7
		Bioethanol yield	0.000	0.307	0.732	1.135	1.042

S. cerevisiae which is grown in media which has been supplemented with maltose grows very minimally, whereas *S. stipitis* in media supplemented with maltose does grow, and grows to a much higher biomass than when not supplemented with an additional carbon source (Fig. 17), (Table 12). This is also the case when you investigate the bioethanol yield of *S. cerevisiae* and *S. stipitis* which has been supplemented with maltose. The *S. cerevisiae* does not produce ethanol, whereas the *S. stipitis* does (Fig. 21), (Table 12).

The carbon source supplementation is based off weight, rather than on a molar basis, meaning that with the biomass and bioethanol yields of *S. stipitis* being similar when supplemented with maltose or glucose, at the same concentration (g/L), shows that the maltose is being fully hydrolysed into two glucose molecules per maltose molecule, and then being utilised to produce ATP, resulting in the biomass and bioethanol produced (Table 12).

However, *S. cerevisiae* does encode for genes which enable maltose utilisation. Being a maltase enzyme which hydrolysis the glycosidic bond between the two glucose monomers which comprise the maltose disaccharide, and maltose permeases which enable maltose to cross the cell membrane (106). Meaning you would expect *S. cerevisiae* to be able to metabolise maltose and utilise it for growth and respiration. However, when *S. cerevisiae* is exposed to high extracellular concentrations of maltose cell death can occur (107). Zhang *et al*. *(2024)* has suggested that this maltose induced cell death could be due to hypotonic stress acting upon the cells (108).

Possibly *S. stipitis* is less susceptible to osmotic stressors than *S. cerevisiae*, meaning that the *S. stipitis* doesn’t undergo maltose induced cell death, therefore is able to survive to utilise maltose for growth and respiration.

Table 13 - ***Biomass yield (CFU/ml) and Bioethanol yield (%) of S. cerevisiae and S. stipitis in minimal medium and YPD medium, when supplemented with lactose at 10g/L, 20g/L, 30g/L, and 40g/L.***

Lactose supplementation			0g/L	10g/L	20g/L	30g/L	40g/L
S. cerevisiae	Minimal Medium	Biomass yield	0.000	N/A	N/A	N/A	N/A
		Bioethanol yield	0.000	0.000	0.000	0.000	0.000
	YPD Medium	Biomass yield	8.538×10^7	1.119×10^7	1.024×10^7	1.065×10^7	1.012×10^7
		Bioethanol yield	0.000	0.000	0.000	0.000	0.000
S. stipitis	Minimal Medium	Biomass yield	2.294×10^7	N/A	N/A	N/A	N/A
		Bioethanol yield	0.000	0.000	0.000	0.000	0.000
	YPD Medium	Biomass yield	1.002×10^7	1.051×10^7	9.728×10^7	9.822×10^7	1.109×10^7
		Bioethanol yield	0.000	0.000	0.000	0.000	0.000

When either *S. cerevisiae* or *S. stipitis* is supplemented with lactose the biomass yield is no different than when *S. cerevisiae* or *S. stipitis* is grown in media which isn't supplemented with an additional carbon source. This means that when grown in minimal media there is no growth exhibited, and in the complex YPD medium the growth is the same as the no supplementation control (Fig. 17), (Table 13).

The inability of *S. cerevisiae* and *S. stipitis* to utilise lactose as seen when investigating the biomass yield, is also reflected in the bioethanol yield of the two catalysts when supplemented with lactose (Fig. 21), with neither producing a bioethanol yield greater than the no supplementation control (Table 13).

The findings presented within this study regarding the ability of the investigated biocatalysts are supported by literature, where it is often noted that wildtype *S. cerevisiae* is unable to utilise lactose due to a lack of lactose permease and lactase (109). However, *S. cerevisiae* can metabolise galactosidase (by means of the Leloir pathway (109)), which is one of the monomers comprising the disaccharide lactose (110).

There have been *S. cerevisiae* strains which have been able to utilise lactose for bioethanol production (111)(109), by bioengineering *S. cerevisiae* to contain the genes *LAC4* and *LAC12* from *K. marxianus*, which encode for β-galactosidase (112) and lactose permease (113) respectively (109). The purpose of these mutants is to be able to ferment the lactose present in dairy waste into bioethanol.

(b) The impact of knocking out gene '*GPD1*' in *S. cerevisiae S288C*, regarding biomass yield and bioethanol production:

Table 14 – ***Percentage which the biomass yield of YDL022W is greater than that of wildtype S. cerevisiae, and the percentage of which the bioethanol yield produced by YDL022W is less than that of wildtype S. cerevisiae.***

	Minimal Medium				YPD Medium			
Glucose supplementation	10g/L	20g/L	30g/L	40g/L	10g/L	20g/L	30g/L	40g/L
Biomass yield	25.93	14.06	18.74	9.99	-8.60	5.65	6.06	16.74
Bioethanol yield	7.88	7.57	6.63	-1.14	13.56	15.98	3.70	12.67

Despite *S. cerevisiae* YDL022W growing to a greater number of CFUs/ml than wildtype *S. cerevisiae,* as shown in Table 14. YDL022W typically produced a lower bioethanol yield than the wildtype in both minimal media, and YPD medium at the four tested concentrations of glucose (10g/L, 20g/L, 30g/L and 40g/L) (Table 14). This indicates that, under the conditions tested herein, knocking out *GPD1* is not redirecting the metabolic flux towards a greater amount of ethanol being produced in return for the absence of glycerol being produced.

However, Yang *et al*. *(2022)* found that when *GPD2*, *FPS1*, *ADH2*, and *DLD3* were knocked out, there was a 18.58% increase in the ethanol produced by *S. cerevisiae* (114), with a decrease in the amount of acetic acid, lactic acid and glycerol produced by 8.87%, 16.82% and 22.32% respectively (114). The use of a mutant strain such as this to produce bioethanol would lead to a more efficient process, as a greater amount of the feedstock will be converted into the desired product rather than by-products. Although the financial benefit of an increased product yield from the same amount of feedstock may be outweighed by the increased regulatory requirements for the disposal of waste which the operator will be held to, due to the use of a genetically engineered microbial strain.

(c) The effect of different carbon source supplementation on the industrial anaerobe *C. saccharoperbutylacetonicum*, regarding biomass yield and biosolvent production:

Table 15 - ***Biomass yield (CFU/ml) and Biosolvent yield (%) of C. saccharoperbutylacetonicum, when supplemented with various carbon sources at 10g/L, 20g/L, 30g/L, and 40g/L.***

Carbon Source	Amount of supplementation (g/L)	Biomass yield	Acetone (%)	Butanol (%)	Ethanol (%)
Glucose	10	$4.826x10^7$	0.033	0.083	0.045
	20	$2.675x10^7$	0.018	0.015	0.005
	30	$6.616x10^7$	0.053	0.048	0.010
	40	$2.259x10^7$	0.008	0.004	0.000
Glycerol	10	$2.884x10^6$	0.003	0.008	0.006
	20	$1.946x10^6$	0.000	0.000	0.064
	30	$2.077x10^5$	0.000	0.000	0.000
	40	$6.289x10^5$	0.000	0.000	0.000
Lactose	10	$3.841x10^7$	0.004	0.003	0.021
	20	$3.813x10^7$	0.026	0.028	0.021
	30	$4.368x10^7$	0.033	0.046	0.044
	40	$4.484x10^7$	0.020	0.015	0.008
Xylose	10	$5.729x10^7$	0.035	0.118	0.059
	20	$7.124x10^7$	0.016	0.031	0.014
	30	$7.007x10^7$	0.036	0.050	0.011
	40	$5.742x10^7$	0.011	0.011	0.008

Sucrose	10	$5.510x10^7$	0.038	0.049	0.028
	20	$5.706x10^7$	0.058	0.070	0.061
	30	$4.446x10^7$	0.032	0.038	0.040
	40	$4.823x10^7$	0.046	0.056	0.017
Mannose	10	$7.722x10^7$	0.088	0.122	0.017
	20	$7.348x10^7$	0.119	0.132	0.032
	30	$6.947x10^7$	0.104	0.119	0.038
	40	$7.299x10^7$	0.177	0.301	0.061
Fructose	10	$6.698x10^7$	0.014	0.017	0.012
	20	$5.120x10^7$	0.005	0.007	0.000
	30	$6.935x10^7$	0.009	0.010	0.000
	40	$6.412x10^7$	0.021	0.008	0.002
Maltose	10	$4.228x10^7$	0.011	0.030	0.013
	20	$4.735x10^7$	0.010	0.010	0.003
	30	$4.492x10^7$	0.010	0.010	0.000
	40	$3.866x10^7$	0.007	0.007	0.000
No supplementation	0	$3.761x10^7$	0.020	0.072	0.056

C. saccharoperbutylacetonicum is able to utilise supplemented mannose, xylose, or fructose to yield a greater amount of biomass than when grown in unsupplemented media (Fig. 27a, Table 15). When *C. saccharoperbutylacetonicum* is supplemented with glucose, lactose, sucrose or maltose, the biomass yield is similar to the yield when *C. saccharoperbutylacetonicum* is grown in unsupplemented media (Table 15), but these values are variable due to inconsistencies in culture growth, showing that *C. saccharoperbutylacetonicum* is a bacterial strain which is challenging to grow in a repeatable manner, which could limit its usefulness in an industrial setting (Fig. 27a). Figure 27a shows that *C. saccharoperbutylacetonicum* is unable to grow when in media which has been supplemented with glycerol.

Figure 28 shows that the yields of ABE vary depending on the supplemented carbon source being utilised by *C. saccharoperbutylacetonicum* and the concentration of said carbon source. With all carbon sources having a detrimental effect on the yields of butanol and ethanol, other than sucrose which makes minimal difference, and mannose which increases the amounts of butanol and ethanol produced by *C. saccharoperbutylacetonicum* significantly (Fig. 28, Table 4). Whereas the acetone yield is increased when *C. saccharoperbutylacetonicum* is supplemented with sucrose and mannose, and slightly increased when cultured in the presence of glucose (Table 4, Table 15).

The media used for growing *C. saccharoperbutylacetonicum* in these carbon source utilisation screening tests was 'Clostridium Nutrient Medium', which is a complex medium, meaning that there are other sources of carbon already within the medium, such as the 10g/L of meat extract, 5g/L of glucose, 3g/L of yeast extract, and 1g/L of starch (115). Therefore, the ABE produced by *C. saccharoperbutylacetonicum* in these samples can't be solely contributed to the supplemented carbon, however the use of the 'no supplementation' control should help account for this.

As can be seen the biosolvent yields produced by *C. saccharoperbutylacetonicum* are very low, often in the region of 0.05% - 0.1% (Table 15). This is much lower than the potential bioethanol yield of *S. cerevisiae* which can be up to 21% (116), as so it is important that the most appropriate biocatalyst is utilised for the desired product, where the benefit of *C. saccharoperbutylacetonicum* over *S. cerevisiae* is its ability to produce acetone and butanol.

With biosolvent production there are also high infrastructure costs to consider, which will subsequently have high annual maintenance costs. With the production of ABE by *C. saccharoperbutylacetonicum* compared to producing bioethanol by *S. cerevisiae*, the three biosolvent products are mixed together so there will need to be an additional step of separating the solvents by distillation. Given the already very low biosolvent yields, the cost of feedstocks, and infrastructure. Additional steps are likely to make the process of producing biosolvents using biocatalysts such as *C. saccharoperbutylacetonicum* less attractive compared to petroleum based alternatives, despite the push for moving away from petroleum based products.

(d) The benefits, drawbacks, and economic viability of Anaerobic Digestion and Bioethanol Production:

Anaerobic digesters can be fed multiple feedstocks at the same time, which provides the operator a degree of flexibility regarding the feedstock they utilise. Meaning the operator can input livestock waste, or purpose grown energy crops, or municipal waste depending on what will be most appropriate for them at the time. The ability for anaerobic digester operators to handle municipal waste is a benefit to them because they can charge a gate fee to local councils, (on average £26/tonne (23)), providing the operator with an additional revenue stream alongside the value of electricity/biogas produced. Whereas the fermentation of a biomass for bioethanol requires more specific homogenous feedstocks such as corn grain, because a side product of bioethanol production is distiller's dried grains with solubles, which does provide the operator with another revenue stream, but does limit the operator to the feedstocks which can be used for the process. The distiller's dried grains with solubles produced cannot necessarily be seen as a bonus from the process either, because the value of the distiller's dried grains with solubles for livestock feed has already been priced into the process and is what enables the operation of bulk fermentation for bioethanol to be an economically viable proposition.

Anaerobic digesters also produce digestate which can be used as a nutrient rich organic fertiliser. Digesters also produce heat which could be used to heat homes in local communities, or buildings on the farm.

Bioethanol fermenters also require the feedstock to undergo pretreatments such as liquefaction, and saccharification where additional digestion agents are added such as enzymes and sulphuric acid (27) to enable the feedstock to be fermented by the yeast added. Whereas anaerobic digestion feedstocks do not need to undergo pretreatments, which simplifies the process, making the operation of an anaerobic digester simpler, ergo cheaper to run than a bioethanol fermenter.

A drawback of both bioethanol production, and anaerobic digestion plants is that land which could be used for growing food for human consumption, or animal feed is being used to produce energy crops. However, on this point an anaerobic digester is preferable to a bioethanol fermenter due to the increased variety of feedstocks which can be used, as discussed before.

Bioethanol fermenters ordinarily operate on a batch fermentation system, meaning that the feedstock is fed into the fermenter, allowed to ferment for 46 hours (27), and then emptied and processed. The fermenter is then cleaned, and the process is run again. Whereas anaerobic digesters operate on a continuous culture method, where the biogas, and digestate is removed from the digester during operation, and feedstock is continuously fed in at regular intervals by an auger connected to a hopper which on a farm scale anaerobic digestion plant only needs to be refilled daily, which isn't a time consuming task. This supports the notion that biofermenters are far more complex, and costly in terms of operating costs than anaerobic digestion plants.

Bioethanol demand is projected to increase, as detailed in the introduction. With demand increasing its reasonable to conclude that price could increase as well (depending on the increase in production capacity of bioethanol). If the value of bioethanol increases, then that will increase the economic viability of the process to produce bioethanol by fermentation. Whereas, anaerobic digesters in the United Kingdom are often on 20-year electricity price tariffs, although this can be seen as a benefit, because it insulated the operator from fluctuations in the electricity market, meaning they can plan in the long term. Bioethanol demand is also dependent on the uptake of electric vehicles, this is because if there is above expected uptake of electric vehicles then the main market for bioethanol, which is petroleum fuel blends, will not be nearly as large as expected, meaning that bioethanol will not be as valuable as projected. Making the prospect of investing into the building of an expensive bioethanol fermentation facility is less appealing to investors. However, this could be mitigated by government intervention, where a government would guarantee a minimum price for bioethanol over a set period of time, which would make an investment into a bioethanol plant less risky, therefore more appealing.

Given the higher costs involved in the set up of a bioethanol fermenter, compared to an anaerobic digester, less fermenters will be built but in more centralised locations. Whereas, anaerobic digesters could be built on farms, where they are close to the production of energy crops, and livestock waste. Both operations require feedstock to be transported to them which comes at a cost, but anaerobic digesters produce biogas which can be burnt on site to produce electricity, which can be directly injected into the grid, or used on site by the farm. But fermenters produce bioethanol, which will need to be removed most likely by road (unless the fermenter is very large scale, and built nearby to a railway line, in which the product and the feedstock could be transported by train, which on a large enough scale may be more cost effective than using road transport). Meaning, that as fewer fermenters would be developed due to their high cost, there will be a greater distance between them, meaning there will be a greater distance for the feedstock and product to be transported, increasing the cost, making the process less economically attractive.

The previously discussed benefit of anaerobic digesters over bioethanol fermenters of being able to take multiple feedstocks, is also beneficial because to ensure financial viability both operations need to be producing product year-round. So, the ability to utilise multiple types of feedstocks is important because then feedstock doesn't need to be stored to feed the facilities when it isn't the time of the year in which the feedstock (corn grain, in the case of bioethanol fermenters) is harvested.

Future Work:

- Investigate the rate at which a supplemented carbon source is consumed by the biocatalyst, and comparing this to the change in biomass of the biocatalyst and the quantity of biosolvent produced over time.
- Investigate the biomass yield and biosolvent yield, when the biocatalyst is supplemented with a combination of carbon sources. As well as monitoring the rate of consumption of the carbon sources, alongside which is preferred by the biocatalyst.
- Investigate the effect of coculture of biocatalysts on biosolvent production. With inoculation of a secondary biocatalyst being trialled at different points of initial biocatalyst culture maturity.
- Investigating whether the biocatalyst *S. stipitis* prioritises the consumption of xylose over the consumption of glucose, and how the rate of biosolvent production differs depending on which carbon source is actively being utilised.
- Investigating the impact on yields of different pretreatment methods for polysaccharide breakdown, and comparing the cost effectiveness of the different pretreatment methods when conducted on an industrial scale.
- Investigating the biomass yield and biosolvent yield of the investigated biocatalysts when supplemented with ‘real world’ feedstocks, such as sugar beet pulp, potato mash, corn grain or corn silage.
- Scale up select biocatalyst experiments, into a 5 litre lab scale metal fermentation vessel, which will subject the reaction to different airflow conditions compared to the small scale experiments which have already taken place. With a view of investigating if the biosolvent yield is consistent with the small scale experiments.
- Investigate the effect on biomass yield and biosolvent yield, if the glucose metabolism pathway is knocked out of *S. stipitis*, requiring *S. stipitis* to only utilise xylose, and if the knockout of the genes involved has unexpected impacts of the viability of the biocatalyst.

- Investigate the effect on ethanol yield if *FPS1*, *ADH2*, and *DLD3* were knocked out of *S. cerevisiae* individually.
- Quantify how much glycerol is produced by wildtype *S. cerevisiae* and YDL022W.
- Investigate whether YDL022W produces a greater amount of acetate, or 2,3 – butanediol, to identify where the redirected metabolic flux is going.
- Further investigation into the economic aspect of this project could be:
    - Evaluating public perception on the use of bioethanol to produce fuel blends.
    - Investigating the public perception of various energy sources, such as Anaerobic digestion versus fossil fuels versus wind/solar versus nuclear energy.

Concluding Remarks:

Anaerobic digestion plants are a large capital expenditure, but over the long term if a consistent supply of feedstock can be maintained then the owning and operating of an anaerobic digestion plant can be a useful diversified revenue stream for farmers. Anaerobic digestion plants can act as an environmentally friendly and economically beneficial method for the disposal of surplus/waste organic matter.

With revenue being generated from the sale of the biogas / biomethane / electricity, as well as charging a gate fee for external entities to dispose of their waste. Farmers can also use the digestate produced by local anaerobic digesters as a nutrient rich organic fertiliser, and farmers who own anaerobic digesters could sell the digestate to local farmers, or fertilise the crops of neighbouring farms on a contract basis, where they would supply their own equipment and fertiliser (digestate). Government incentives can further increase the economic feasibility of farmers and local communities owning and operating anaerobic digestion plants, to generate green energy on a localised scale.

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