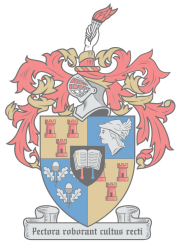
Engineered yeast and microalgae mutualisms: Synthetic ecology applied to species isolated from winery wastewater

by

Zoë Faith Simpson



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Declaration

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Summary

Large volumes of winery wastewater, classified as biodegradable industrial effluent, are generated annually. The development of a cost-effective treatment system is difficult due to the variable and batch nature of winery wastewater. Research has focused on the development of dynamic biological treatment systems using microorganisms including yeast and microalgae, as it has become apparent that these microbes have bioremediation capabilities in various wastewaters. However, no biological winery wastewater treatment system, employing multi-species ecosystems with known species of yeast and microalgae, currently exists.

In this study, yeast and microalgae were previously isolated from natural winery wastewater. The first aim of this study was to characterise the bioremediation potential of the yeast, *Saccharomyces cerevisiae*, and microalga, *Parachlorella beijerinckii*, in synthetic and raw winery wastewater. *P. beijerinckii* was physiologically characterised and was able to tolerate salinity and ethanol levels commonly associated with winery wastewater, making it a suitable candidate for further bioremediation studies. Both *S. cerevisiae* and *P. beijerinckii* were able to decrease the chemical oxygen demand of winery wastewater and *P. beijerinckii* monoculture was able to increase the pH of the acidic wastewater. *S. cerevisiae* out-competed *P. beijerinckii* in co-culture growth experiments. Interestingly, yeast growth was improved in the presence of the microalgae in this system, suggesting a potential for symbiotic association. The increased yeast growth however had no impact on the bioremediation potential of the co-culture system.

To overcome this drawback, a synthetic ecology approach was used to engineer stable symbiotic associations between these evolutionarily unrelated strains of yeast and microalgae. Engineered mutualisms between *S. cerevisiae* and *P. beijerinckii* were established under strongly selective conditions based on the nutrient exchange of carbon and nitrogen. These mutualistic associations were relatively easy to establish as the complementary metabolic abilities of each organism were key elements in the mutualism design. The impact of temperature and pH were assessed in these obligatory mutualistic conditions to determine whether the co-culture functions optimally in specific environmental conditions and whether such conditions are similar or different from the optimal conditions required for single species growth. Experiments were first conducted in small scale and continued in larger scale bioreactor studies. The bioreactor conditions were evaluated to generate a more constant continuous culture system. Such continuous culture system would provide an ideal tool to conduct studies on the evolutionary development of mutualistic associations, and may be the first step in developing a multi-species approach to winery wastewater treatment with enhanced bioremediation capabilities. We propose that in the long run such co-culture systems might

serve to overcome the limitations associated with single culture system and might improve biotechnological processes.

Opsomming

Groot volumes kelderafvalwater, geklassifiseer as bioafbreekbare nywerheidsafvloeisel, word jaarliks gegenereer. Die ontwikkeling van 'n koste-effektiewe behandelingstelsel is moeilik as gevolg van die konstante variasie en groep-aard van kelderafvalwater. In die verlede het navorsing gefokus op die ontwikkeling van dinamiese biologiese behandelingstelsels deur mikroörganismes te gebruik, insluitend gis en mikroalge. Dit het duidelik geword dat hierdie mikrobes bioremediëringsvermoëns in verskeie afvalwater het. Daar bestaan egter geen biologiese kelderafvalwaterstelsel wat bekende spesies gis en mikroalge gebruik as multi-spesiesekosisteme nie.

In hierdie studie is gis en mikroalge, voorheen geïsoleer uit natuurlike kelderafvalwater, gebruik. Die eerste doelwit van die studie was om die bioremediëringspotensiaal van die gis, *Saccharomyces cerevisiae*, en die mikroalge, *Parachlorella beijerinckii*, in sintetiese en rou kelderafvalwater te karakteriseer. *P. beijerinckii* is fisiologies gekarakteriseer en was in staat om die sout- en etanolvlakke in kelderafvalwater te weerstaan en dus is *P. beijerinckii* ‘n geskikte kandidaat vir verdere bioremediëringsstudies. Beide *S. cerevisiae* en *P. beijerinckii* was in staat om die chemiese suurstofaanvraag te verminder in kelderafvalwater en *P. beijerinckii* monokultuur was in staat om die suur pH van die water te verhoog. *S. cerevisiae* het in multikultuur groei-eksperimente beter as *P. beijerinckii* gegroei. Interessant genoeg is gisgroei verbeter in die teenwoordigheid van die mikroalge, wat 'n potensiaal vir simbiotiese assosiasie voorstel. Die verhoogde gisgroei het egter geen impak gehad op die bioremediëringspotensiaal van die ko-kultuurstelsel nie.

Om hierdie nadeel te oorkom, is 'n sintetiese ekologiebenadering gebruik om stabiele simbiotiese assosiasies tussen hierdie evolusionêr onverwante stamme van gis en mikroalge te bou. *S. cerevisiae* en *P. beijerinckii* is onder sterk selektiewe toestande toegelaat om ‘n mutualistiese verhouding te vorm, gebaseer op die uitruiling van koolstof en stikstof. Hierdie mutualistiese assosiasies was relatief maklik om te vestig, aangesien die komplementêre metaboliese vermoëns van elke organisme sleutelelemente in die mutualisme-ontwerp was. Die impak van temperatuur en pH op hierdie obligate mutualistiese kondisies is geëvalueer om vas te stel of die ko-kultuur optimaal funksioneer onder spesifieke omgewingskondisies en of sulke kondisies ooreenstem of verskil van die optimale toestande wat benodig word vir enkelspesiegroei. Die eksperimente is eers op klein skaal uitgevoer, gevolg deur bioreaktorstudies op groot skaal. Die bioreaktor kondisies is uitgevoer om 'n meer konstante aaneenlopende kultuurstelsel te genereer. So 'n deurlopende kultuurstelsel sal 'n ideale hulpmiddel wees om studies uit te voer oor die evolusionêre ontwikkeling van mutualistiese assosiasies. Hierdie kan die eerste stap wees in die ontwikkeling van 'n multi-spesie benadering

tot kelderafvalwaterbehandeling met verhoogde bioremediëringsvermoëns. Ons stel voor dat hierdie ko-kultuurstelsels op die langtermyn die beperkinge geässosieer met enkelkultuurstelsel kan oorkom en sodoende biotegnologiese prosesse verbeter.

This thesis is dedicated to my parents

Reginald Charles Simpson & **Bonita Heather Simpson**

My mentors, my teachers, my idols and my heroes.
Thank you for always motivating, supporting and loving me.
Ek is lief vir Mom en Dad.

Biographical sketch

Zoë was born in Cape Town on 23 December 1990 and grew up in the beautiful Tulbagh valley. She attended Tulbagh High and matriculated in 2009. She started her University studies at Stellenbosch in 2010 but discovered her passion for science in 2011. In 2012 she enrolled for a BSc-degree in Molecular Biology and Biotechnology at the University of Stellenbosch and obtained her degree in 2014. Thereafter re-enrolled for a HonsBSc-degree in Wine Biotechnology the Institute for Wine Biotechnology, Stellenbosch University and continued with her MSc in Wine Biotechnology in 2016 at the same institution.

Zoë contributed to the following local and international conference presentations during her studies:

- Simpson, Z.F., Naidoo, R.K., Bauer, F.F., 2016. Engineered yeast and microalgae mutualism for the potential bioremediation of winery waste water. Presented at the Stellenbosch University Water Institute (SUWI) Symposium, Stias, Stellenbosch.

- Simpson, Z.F., Naidoo, R.K., Bauer, F.F., 2017. Engineered yeast and microalgae mutualism for the potential bioremediation of winery waste water [Poster] Exhibited at the 12th International Congress on Microbial Interaction and Applications of Beneficial Microbes, Munich, Germany.

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Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal *South African Journal of Oenology and Viticulture*.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Yeast and Bacteria: Candidates for microalgal mutualisms and potentially improved biotechnological processes.

Chapter 3 **Research results**
The bioremediation potential of *Parachlorella beijerinckii* and *Saccharomyces cerevisiae* isolated from winery wastewater.

Chapter 4 **Research results**
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Chapter 1

Introduction and project aims

Chapter 1 - General introduction and project aims

1.1 Introduction

Jan van Riebeeck, the first governor of the Cape, planted the first vineyard in 1655 and produced the first wine in February 1659. Today, South Africa is one of the great wine countries of the world. According to the South African Wine Industry Information and Systems (SAWIS), 1089 million litres of wine were produced during harvest 2016. With this vast wine production, large amounts of wastewater, which is classified as biodegradable industrial effluent, are generated every year (Sheridan *et al.,* 2011). For each litre of wine produced between 0.2 and 14 L of wastewater is generated (Ioannou *et al*., 2015; Oliveira & Duarte, 2010; Welz *et al.,* 2016) resulting in a billion litres of winery wastewater which requires disposal every year. Presently, winery wastewater is either discharged into municipal wastewater or irrigated on agricultural land; and both methods are governed by restrictions stated in Section 39 of the South African National Water Act (Act No. 36 of 1998).

The chemical composition of winery wastewater fluctuates seasonally, and depend on factors such as cellar activities (crushing of grapes, fermentation, maturation/stabilisation, decanting and bottling of wine), grape varietal and cleaning products used in the cellar (Bolzonella & Rosso, 2013; Bories & Sire, 2010; Sheridan *et al.,* 2011; Vlyssides *et al.,* 2005; Welz *et al.,* 2016). The variable nature of this wastewater makes the development of cost-effective treatment systems difficult, especially for smaller wineries, and many studies have explored the development of dynamic biological treatment systems with re-use of agricultural-industrial wastewater (Daffonchio *et al.,* 1998; Markou & Georgakakis, 2011; Petruccioli *et al.,* 2002; Welz *et al.,* 2016). Previous studies have shown that yeast and microalgae have bioremediation capabilities in various wastewaters (municipal, industrial and agricultural) (De-Bashan *et al.,* 2003; Liu *et al.,* 2016; Malandra *et al.,* 2003; Oswald *et al.,* 1953). In addition, these species have significant biotechnological potential with regards to value-added by-product production, making them candidates for the bioremediation of winery wastewater.

In recent years there has been considerable amount of interest in co-cultivation systems incorporating microalgae, yeast and bacteria to increase productivity of biotechnological processes by providing advantages such as increased biomass production, enhanced lipid production and decreased nutrient/energy inputs leading to more cost effective and environmentally friendly processes (Dong & Zhao, 2004; Cheirsilp *et al.,* 2011; Grant *et al*., 2014; Papone *et al.,* 2012; Pisman & Somova, 2003; Puangbut & Leesing, 2012; Rivas *et al.,* 2010; Santos *et al.,* 2013; Xue *et al.,* 2010). Most biotechnological processes are monoculture systems which are by nature unstable, prone to contamination by unwanted organisms,

sensitive to environmental change and produce inconsistent biomass. Multispecies ecosystems, comprised of two or more organisms, can provide more functionalities and environmental resilience, but remain difficult to control and therefore of limited usefulness in biotechnological processes such as biological wastewater treatment (Cai *et al.,* 2007). To overcome this drawback, we propose a synthetic ecology approach, as multi-species systems with complementary metabolic capabilities have previously proven to enhance productivity (Kazamia *et al.,* 2014; Li *et al.,* 2017). These engineered multi-species systems have been designed to improve growth and survival of the partners involved, improve functional and metabolic capabilities and can perform more complex tasks (Brenner *et al.,* 2008; de-Bashan *et al.,* 2016; Dolinšek *et al.,* 2016). Recently, de-Bashan *et al.* (2016) defined synthetic ecology as the development of a "cooperative and steady-state microbial community that performs a desirable biotechnological function". Furthermore, synthetic ecology incorporates the use of engineering principles such as species specific selection, engineered symbiosis and tailored growth conditions; which allows the study of microbial interactions under carefully controlled conditions (Kazamia *et al.,* 2014).

Hom and Murray (2014) recently established an obligate mutualism between the yeast *Saccharomyces cerevisiae* and the alga *Chlamydomonas reinhardtii* using a synthetic ecology approach. In this system, the reciprocal exchange of carbon and nitrogen, was used to establish an obligate mutualism between *S. cerevisiae* and *C. reinhardtii. S. cerevisiae* ferments glucose with the release of carbon dioxide, which is assimilated photosynthetically by *C. reinhardtii*, which in turn metabolises the sole nitrogen source, nitrite, releasing ammonia as a nitrogen source for *S. cerevisiae.* This study demonstrated that yeast and microalgae mutualisms are relatively easy to establish when a strong selection pressure is applied (Hom & Murray, 2014). In this study, we propose the use of a synthetic ecology approach to engineer stable associations between yeast and microalgae which are indigenous to winery wastewater. These engineered mutualisms may be the first step in developing a community ecology approach to winery wastewater treatment. Yeast (Malandra *et al.,* 2003) and microalgae (Liu *et al.*, 2016) have been previously investigated for the bioremediation of winery wastewater, however no biological treatment systems using multi-species ecosystems with known species of yeast and microalgae currently exists. Identifying and characterising potential yeast and microalgae species isolated from winery wastewater, will be the first step in developing this multi-species system. Using synthetic ecology approaches to identify conditions which promote the formation of engineered mutualistic associations between selected yeast and microalgae, optimising growth conditions in terms of temperature and pH and developing a protocol for an up-scaled bioreactor system, can be the first steps in building an ecosystem, which is more efficient at bioremediation with improved biomass production.

1.2 Aims and objectives

In this study, yeast and microalgae were isolated from the natural winery wastewater environment. The yeast, *Sachharomyces cerevisiae*, and microalga, *Parachlorella beijerinckii,* were identified and selected for further study. *P. beijerinckii* was selected for further characterisation because limited data exists on physiological responses to environmental changes, especially within a wine wastewater environment. The two main aims of this project are to (1) characterise the bioremediation potential of *S. cerevisiae* and *P. beijerinckii* in synthetic and raw winery wastewater; (2) to engineer stable yeast and microalgae mutualisms under strongly selective conditions which promote the reciprocal exchange of carbon and nitrogen.

To achieve above mentioned aims, the following objectives were pursued:

1. Investigate optimal temperature and pH; and the effect of salinity and ethanol stress on uncharacterised *P. beijerinckii*, as these are key parameters which contribute to the variable nature of winery wastewater.

2. Investigate the proliferation, decontamination ability and biomass production of both *S. cerevisiae* and *P. beijerinckii* mono- and co-culture in synthetic and raw winery winery wastewater.

3. Engineer yeast and microalgae mutualisms between *S. cerevisiae* and *P. beijerinckii*, with the reciprocal exchange of carbon and nitrogen, by identifying carbon sources which can be easily metabolised by the yeast, but not the microalgae.

4. Investigate optimal obligate mutualisms between *P. beijerinckii* with *S. cerevisiae* in terms of temperature and pH; and under non-obligatory conditions.

5. Develop a protocol for up-scaled obligate mutualism in bioreactor set-up, to allow continuous sampling without the disruption of the mutualism, to control and monitor different parameters, to decrease the risk of contamination and to build the foundation for future co-evolutionary studies.

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Chapter 2

Literature review

Yeast and Bacteria: Candidates for microalgal mutualisms and improved biotechnological processes

Chapter 2 - Yeast and Bacteria: Candidates for microalgal mutualisms and potentially improved biotechnological processes

2.1 Introduction

All living systems interact with and depend on other species. In 1877, Albert B. Frank used the term symbiosis, which means living together, to define the mutualistic relationship of lichens. Similarly, in 1879, Heinrich A. de Bary described symbiosis as “the living together of unlike organisms and the interaction between different species". Symbiotic relationships can be obligate, where both symbionts depend entirely on each other for growth and survival, or facultative, meaning that organisms can, but do not have to live with each other (Douglas, 2010; Willey *et al*., 2011). These associations can be broadly categorised as relationships that include mutualism, cooperation, commensalism, predation, parasitism, amensalism and competition (Fig. 2.1). Symbiosis is a common phenomenon in nature and there are a number of factors which influence the formation of symbiotic associations within the natural environment. These can include physical (secure habitat) and biochemical factors (nutrient exchange); however, nutrient exchange has been identified as the driving force behind most symbiotic associations (Oksanen, 2006).

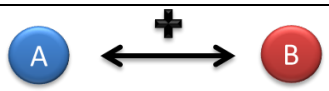
Symbiosis	Interaction
Mutualism (Obligatory) / Positive bidirectional	
Cooperation (Non-obligatory) / Positive bidirectional	
Commensalism / Positive unidirectional	
Predation	
Parasitism	
Amensalism / Negative unidirectional	
Competition	One outcompetes the other for site resources
	Both co-exist at lower levels, because they share limiting resource

Figure 2.1 Types of symbiotic interactions common in nature. These associations include mutualism, cooperation, commensalism, predation, parasitism, amensalism and competition (adapted from Dolinšek *et al.,* 2016 and Willey *et al.,* 2011).

Mutualism (Latin *mutuus* – “together”) can be defined as a “relationship in which reciprocal benefit accrues to both partners” (Willey *et al.,* 2011). Mutualism, as an area of research, has received less attention compared to other interactions such as parasitism and predation. This is due mainly to the challenging and highly complex nature of these ecosystems which impedes interaction studies between microorganisms in their natural environment (Grant *et al.,* 2014; Willey *et al.,* 2011).

Lichens are perhaps the oldest and most well studied example of mutualistic symbiosis and are widely used in biotechnology today. For 140 years lichens have been defined as composite organisms involving a close association between a photosynthetic alga/cyanobacterium and a fungus. In this mutually beneficial relationship the alga fixes CO_2 through photosynthesis and supplies the fungus with organic nutrients, while the fungus protects the alga and supplies minerals and water (Oksanen, 2006). Recently, it was discovered that lichens consist in fact of three species, with newly discovered Basidiomycota yeasts being part of the mutualistic association. All three members were seen over six continents in 52 lichen genera, however, the role of the yeast is still uncertain (Spribille *et al.,* 2016). These newly discovered yeasts are embedded in the cortex of the lichen which is generally made up of a unitary vegetative body that grows on tree trunk, rocks and other habitats (Fig. 2.2). Additionally, bacterial communities were identified to be integrated partners within the lichen symbiosis (Grube *et al.,* 2015). The metabolic potential of the bacteria in this stable and specific partnership was identified by using lichen *Lobaria pulmonaria* as a model organism. In this study, more than 800 bacterial species had the potential to contribute numerous functions including (1) nutrient supply (N, P and S), (2) resistance against abiotic factors, (3) resistance against biotic stress factors (pathogens), (4) provision of vitamin B_{12}, (5) supply of hormones, (6) detoxification of metabolites and (7) the degradation of old lichen thalli. This partnership is essential for longevity of lichens under severe and changing ecological conditions (Grube *et al.,* 2015).

This mutualistic species assembly allows lichens to persist in extreme environments and to occupy habitats that would not accommodate any of the individual species on their own. The combined species also produce a wide range of metabolites that can be extracted from these composite organisms (Calcott *et al.,* 2017). Historically, most compounds extracted from lichens were predominately from the fungal partner, but in recent years it has been shown that the alga/cyanobacterium partner within the lichen produce unique molecules within the symbiotic association, which differ from free living cyanobacteria (Calcott *et al.,* 2017). Metabolites that originate from lichens include α-glucan, which has bioactivity against macrophages (Schepetkin & Quinn, 2006) and antifreeze proteins which are used to homogenise milk in the dairy industry and are extracted from the lichen thalli (Berry *et al.,* 2001). Thus, lichens provide a good model system for studying mutualistic associations and demonstrate the biotechnological potential of obligate mutualistic systems, including the production of a wider range of metabolites (Oksanen,

2006) and the persistence in ecological niches that would be unfavourable for the individual species. However, factors which underlie associations between phylogenetically remote species such as microalgae and yeast/bacteria are still largely underexplored. Microalgae have high commercial potential, but are limited in their ability to persist in many biotechnologically relevant environments. An improved understanding of mutualistic associations and the development of new mutualistic systems to exploit complementary metabolic abilities of organisms in biotechnological processes, is therefore highly desirable.

Figure 2.2 Lichen on tree trunk makes up a unitary vegetative body (Photographed by Z.F. Simpson, Tulbagh, South Africa, 2016).

2.2 Microalgae: The most important "plants" in the world?

Microalgae are defined as unicellular photosynthetic protists that possess a cell wall. They can live individually, in chains or groups in freshwater and marine environments (Willey *et al.,* 2011). Microalgae differ in size, and species can range between a few to hundreds of micrometers (µm). These photosynthetic microorganisms are important for life on earth, as they provide approximately half of the atmospheric oxygen and simultaneously sequester carbon dioxide for photoautotrophic growth (Starckx *et al.,* 2012). Furthermore, their ability to sequestrate CO_2 makes them good candidate species in the fight against global warming.

The biodiversity of microalgae is enormous and only 50,000 species out of the estimated 200 000 – 800 000 species have been described (Cardozo *et al.,* 2006). More than 15,000 novel compounds have originated from microalgal biomass as most microalgae species produce unique products such as antioxidants, enzymes, fatty acids, nutraceuticals (PUFA, β-carotene, astaxanthins), peptides, polymers, sterols and toxins (Fig. 2.3) (Cardozo *et al.,* 2006; Rosenberg *et al.,* 2008). Traditionally omega-3 fatty acids have been obtained from fish which ingest invertebrates which ingest microalgae, the source of the omega-3 fatty acids. However, these fatty acids can also be obtained directly from microalgae in a nutritious and healthy

manner. Algal biomass is also used in the production of syngas, for nutritional purposes and in aquaculture (Rosenberg *et al.,* 2008).

Microalgae require light, water and nutrients (N, P, S and trace elements) for photosynthesis and growth, however they are able to grow heterotrophically by utilising organic compounds such as glucose and acetic acid (Juneja *et al*., 2013). There has been considerable interest in microalgae for the production of biofuels as they are efficient producers of biomass (Kazamia *et al.,* 2012). Many algal strains also produce tremendous amounts of storage lipids which can be easily converted into biofuels/liquid transportation including biodiesel, biogas and gasoline equivalents (Fig. 2.3) (Kazamia *et al.,* 2012). This combined with their ability to adapt and tolerate a wide variety of environmental conditions makes them useful as environmentally friendly biofuel feed-stock (Jones & Mayfield, 2012). However, the production of algae biofuel/ bio-products has not been successful and one possible factor is the high costs associated with water and nutrient input at industrial scale. A potential solution to this problem is the growth of microalgae in various wastewaters which provides a low-cost source of water and nutrients for algal biomass production. This can be seen as a promising avenue for the production of bio-energy and bio-products in an environmentally friendly way.

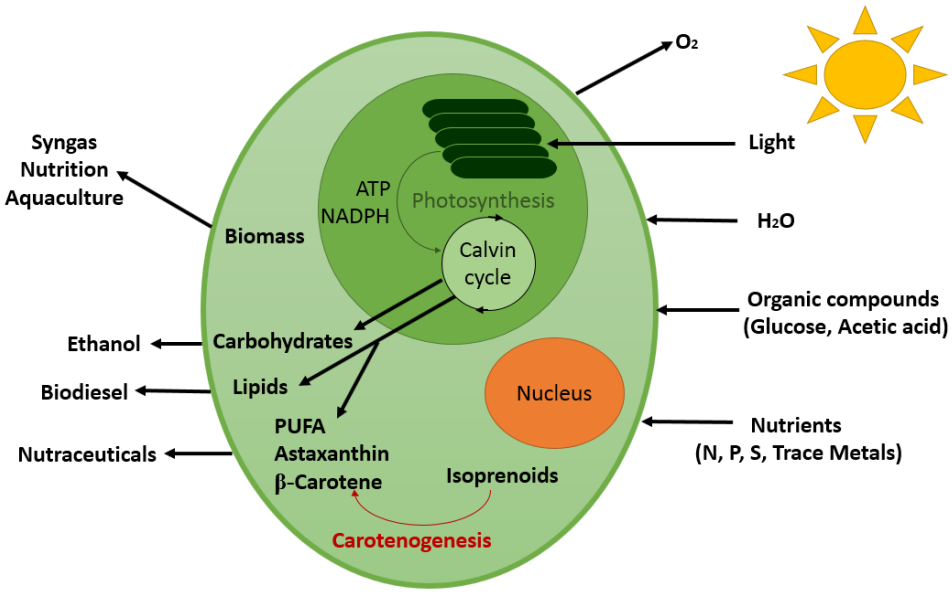


Figure 2.3 Commercially important metabolic pathways in microalgae. This schematic representation shows the simplified metabolic pathways of microalgae for commercial purposes (Adapted from Rosenberg *et al.,* 2008).

While microalgae studies have been predominantly centred on biofuel research, the focus has shifted in recent years to the development of co-culture systems for industrial use. These integrated processes could help to overcome some of the cost challenges currently associated with bio-product production. A good example of an integrated system is the carbon and

dissolved organic carbon exchange between microalgae *Botryococcus braunii* and *Rhizobium* sp. bacteria for the production of hydrocarbons for biofuel (Rivas *et al.,* 2010).

In 2007, more than $1 billion was already invested in the algae industry in the United States of America (Carlsson *et al.,* 2007). Commercial production has grown significantly in the last decade and is conducted mainly with the species belonging to the genera *Isochrysis, Chaetoceros, Chlorella, Arthrospira* and *Dunaliella.* A major challenge with industrial cultivation of microalgae is the increased risk for contamination which often occurs in monoculture cultivation systems. More stable mixed culture systems incorporating microalgae, yeast and/or bacteria could serve to alleviate this problem as multispecies systems are known to provide structure and stability in fluctuating environmental conditions; and minimise the risk for invasive species as more ecological niches are already occupied (Burmolle *et al.,* 2006; Cai *et al.,* 2007). Thus, the development of mixed culture systems including the identification of good candidate species for microalgal mutualism forms an integral part of the strategies to improve the biotechnological application of microalgae. There has been multiple studies which have explored microalgal mutualisms with a range of species, however there is still much to be discovered.

2.3 Mutualism: Beneficial interactions between microorganisms

Mutualism is defined as “any long-term association between two species that confers mutual fitness benefits to individual members of both species” and these interactions play a key role in ecology (Kazamia *et al.,* 2012). For instance in the terrestrial ecosystem, plants are dependent on mycorrhizal relationships with filamentous fungi, as this mutualistic interaction provides them with inorganic compounds and trace elements (Kazamia *et al.,* 2012). In addition, mutualisms are considered to have driven biological diversity through co-evolution of species (Brenner *et al.,* 2008; Kazamia *et al.,* 2012).

Previous studies have shown that beneficial mutualistic associations within an ecosystem can provide a number of advantages to the organisms involved. These include robustness to withstand environmental fluctuations, resistance to invasive species (Burmolle *et al.,* 2006) and the ability to withstand periods of nutrient limitation (La Para *et al.,* 2002). This can be overcome by the division of labour i.e. the exchange of metabolites and information between microorganisms in mixed cultures (Santos & Reis, 2014). Despite the many advantages conferred on organisms by mutualistic associations, there are a few disadvantages which should be mentioned as well. In the process of evolution, organisms can become dependent on one another, which may be a successful evolutionary strategy in a specific setting. The Black Queen Hypothesis (BQH) proposes that co-evolution could lead to adaptive gene loss which promotes the evolution of dependency. Certain biological functions are costly and therefore unwanted by an organism; therefore it is advantageous to lose this function if it can be provided

by helper organisms in the communities (Kazamia *et al.,* 2016; Morris *et al.,* 2012). However, the evolution of these fitter organisms would also make them more vulnerable if conditions change. Additionally, mutualistic associations can be affected by disease affecting one species in the relationship, but both will suffer. Alternatively, if a predator kills one species in the mutualistic relationship, the other species will be equally disadvantaged (Willey *et al.,* 2011).

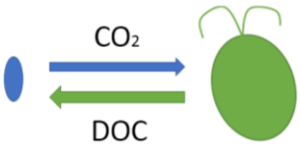
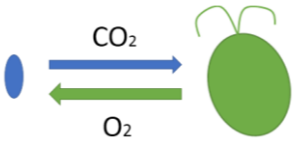
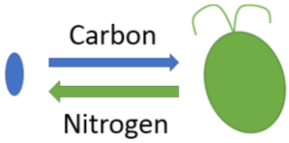
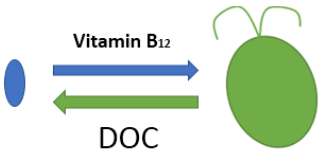
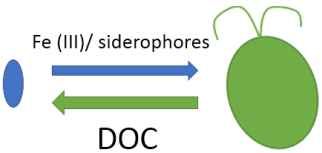
Measuring the exact fitness benefit to individual members in a mutualistic association is not simple, as individuals can receive benefits from multiple sources and several species within an ecosystem (Willey *et al.,* 2011). It is therefore important to consider the closeness of the association when categorising mutualisms. Terminology such as obligate and facultative are used as these refer to mutual dependency. Obligate mutualism refers to two organisms that are completely dependent on each other for survival and each species cannot live without its partner, whereas facultative mutualisms refer to a non-obligatory positive interaction between two organisms. Facultative mutualisms are more common in nature than obligate mutualisms and require longer periods of time to evolve as they are not completely dependent on each other (Willey *et al.,* 2011). Mutually beneficial symbiosis between microalgae and cyanobacteria/ yeast/bacteria will be discussed in the following sections as current research indicates that these associations have the potential to improve biotechnological processes by maximising productivity and minimising the risk for contamination.

2.4 Beneficial interactions between microalgae and bacteria

Algae are the primary producers in aquatic ecosystems, and heterotrophic bacteria utilise organic compounds produced by microalgae. Previous studies have shown a co-occurrence of specific species of algae and bacteria, suggesting the presence of specific mutualistic interactions. Interactions between bacteria and microalgae are difficult to study, as in the natural environment they occur together, but when isolated in a laboratory setting, the partner has often been discarded. However, it is common for bacteria and microalgae to grow together and in recent years there have been a number of interesting studies describing these interactions (De-Bashan *et al.,* 2003; De-Bashan *et al.,* 2016; Kazamia *et al.,* 2012; Rivas *et al.,* 2010; Villa *et al*., 2013) (Table 2.1).

This review will focus on nutrient exchange, as it has been identified as the most common factor in establishing mutualistic interactions between microalgae and bacteria (Cooper & Smith, 2015). In the following sections studies which have explored microalgae and bacterial interactions within different industrial contexts will be discussed; and important benefits of these co-culture systems will be highlighted. Furthermore, nutrient exchange between bacteria and microalgae can be broken down further into the following subsections: (1) carbon and dissolved organic carbon exchange, (2) oxygen and carbon dioxide exchange, (3) carbon and nitrogen exchange, (4) micronutrients and (5) competitive exclusion.

Table 2.1: Symbiotic association studies between microalgae and bacteria for industrial purposes.

Exchange (example)	Interaction	Application
Carbon and dissolved organic carbon (DOC) *(Botryococcus braunii / Rhizobium* sp.)		Hydrocarbons for biofuel (Rivas *et al.,* 2010)
Oxygen and carbon dioxide (methane-oxidizing bacteria and microalgae)		Sustainable carbon neutral methane oxidation (Van der Ha *et al.,* 2011)
Carbon and Nitrogen (*Chlorella vulgaris* or *C. sorokiniana* / *Azospirillum brasilense* strain Cd and & *Neochloris oleoabundans*/ *Azotobacter vinelandii*)		Remove nutrients (P & N) from municipal wastewater (de-Bashan *et al.*, 2003; de-Bashan *et al.,* 2016) CO_2 fixation without fertilizer added (Villa *et al.,* 2013)
Micronutrients: **Vitamin B12 and photosynthate** (*Lobomonas rostrate* / *Mesorhizobium loti*)		Model algae for interaction assays (Grant *et al.,* 2014)
Iron/ siderophore and DOC (γ–proteobacteria *Marinobacter* and α-proteobacteria *Roseobacter*)		Sustain microalgae-bacteria equilibrium in ocean communities (Amin *et al.,* 2009)

2.4.1 Carbon and dissolved organic carbon

Microalgae and bacteria form part of the planktonic community, involved in the global carbon cycle. Microalgae are known to convert carbon dioxide to organic material dissolved in water i.e. dissolved organic carbon (DOC) through photosynthesis, whereas heterotrophic bacteria supply CO_2 while assimilating and decomposing algal DOC (Kouzuma *et al.,* 2015). These heterotrophic bacteria also promote microalgae/plant growth by nutrient exchange and communication mechanisms (Philippot *et al.,* 2013). Kim *et al.* (2014) described the mutualistic relationship between plant growth promoting bacteria (PGPB) and microalgae *Chlamydomonas reinhardtii*, *Chlorella vulgaris, Scenedesmus* sp. and *B. braunii*. This study showed that the most dominant and prevalent phycosphere bacterium isolated from *C. vulgaris* was a *Rhizobium* sp. (between other bacterium including *Mesorhizobium*, *Shinella*, *Flavobacterium*, *Pseudomonas*).

The *Rhizobium* sp. promoted the growth of *C. vulgaris* by 72% when grown in co-culture. The study also revealed that the growth rates of microalgae and bacteria increased by 11% and 110%, respectively (Kim *et al.,* 2014). Cho *et al.* (2015) showed that when selected microalgae are grown with mutualistic bacteria in an artificial consortium, the algae supplies fixed organic carbon. These examples confirm the mutualistic relationship between microalgae and bacterium with carbon and dissolved organic carbon exchange. *Botryococcus braunii* and *Rhizobium* species are currently used in industry to produce hydrocarbons for biofuel (Rivas *et al.,* 2010).

2.4.2 Oxygen and carbon dioxide

Microalgae and bacteria have different respiratory requirements and can often work together in a complementary manner to increase productivity in biotechnological processes. Microalgae provide aerobic bacteria with the oxygen necessary to degrade organic contaminants while consuming CO_2 released from bacterial respiration (Munoz, 2006). Heterotrophic bacteria utilise the oxygen produced by microalgae as an electron acceptor. This is needed to break down organic substances, including aromatic pollutants and organic wastes (Munoz, 2006). In turn, algae assimilate CO_2 that is released by bacteria and can be used to mitigate CO_2 emission from certain industrial processes. Van der Ha *et al.* (2011) reported that a mutualistic interaction between methane-oxidizing bacteria and algae allows methane oxidation, with lower CO_2 emissions and external oxygen supply. Thus, a sustainable, carbon neutral methane oxidation is possible by the mutualistic partnership between methane oxidizing bacteria and microalgae (Van der Ha *et al.*, 2011). All these studies suggest that the synergistic interactions between microorganisms can be applied in biotechnology processes producing a more green production process.

A symbiosis based on reciprocal metabolisms between microbes was first studied in 1958 as a means to enhance wastewater treatment. Oxygen in oxidation ponds was increased by exploiting algal/bacterial symbiosis (Oswald *et al.,* 1953). Microorganisms oxidises organic matter and this can be used for effective secondary wastewater treatment processes. However, this process requires large amounts of oxygen. This process is also energy intensive and expensive and microalgae could provide an alternative option as photosynthetic oxygenation by microalgae would supply sufficient oxygen for bacterial growth, avoiding intensive consumption of energy (Oswald *et al.,* 1953). This symbiotic relationship between autotrophic microalgae and heterotrophic bacteria is a good example of mutualistic symbiosis as both organisms benefit from each other (CO_2 and O_2) with an accompanying increase in growth rate. Wastewater treatment using symbiosis in high-rate oxidation ponds is a well-known technology since the 1960s (Oswald *et al.,* 1953). Further information can be found in reviews by other authors (Abeliovich, 1986; Larsdotter, 2006; Su *et al.,* 2011).

2.4.3 Carbon and nitrogen

Nitrogen is an essential requirement for microalgal growth. The bacterium *Azotobacter vinelandii* has the ability to fix nitrogen under aerobic conditions when a reduced carbon source (including sucrose or glycerol) is available (Villa *et al.,* 2013). *A. vinelandii* also has the ability to produce siderophores to scavenge different metals from an environment. Villa *et al.* (2013) showed that when *A. vinelandii* is co-cultured with two strains of green algae, *Neochloris oleoabundans* and *Scenedesmus* sp. BA032, algal growth was improved. The microalgae were able to utilise the siderophore azotobactin produced by *A. vinelandii*, as a source of nitrogen to support microalgae growth. This interaction between bacteria and microalgae may be applied to industrial culture of microalgae, as it reduces the nitrogen input costs (Villa *et al.,* 2013).

De-Bashan *et al.* (2003) described a co-culture between growth-promoting bacterium, *Azospirillum brasilense* strain Cd and microalgae (*Chlorella vulgaris* or *C. sorokiniana*), developed to remove nutrients (nitrogen and phosphorus) from municipal wastewater. This co-culture was co-immobilised in small alginate beads and was used to treat municipal wastewater. *A. brasilense* Cd significantly increased growth of both *C. vulgaris* and *C. sorokiniana* when these co-immobilised microbes were grown in wastewater. It should be noted that *A. brasilense* is incapable of removing nutrients from wastewater, but both microalgae are able to. However, when a consortium of microalgae and bacteria were used, it resulted in increased growth and nutrient reduction for 100% ammonium, 36% phosphorus and 15% nitrate after 6 days, compared to 75% ammonium, 19% phosphorus and 6% nitrate by microalgae monoculture (De-Bashan *et al.,* 2003). In a more recent study using stable isotope enrichment and high-resolution secondary ion mass spectrometry (SIMS) imaging it was demonstrated that this association is based on the transfer of carbon and nitrogen. A stable physical association was observed for 10 days after cells were released. The bacterium was not able to grow in the medium without the support of the microalgae and in turn the bacteria increased the growth of the microalgae (De-Bashan *et al.,* 2016).

A similar trend has been reported by Lau *et al.* (1995), with bacteria that consume organic nitrogen, supplying microalgae with ammonium and thus preventing nitrogen limitation at the beginning of cultivation. The presence of microalgae could also enhance bacterial activity due to the release of extracellular compounds, which are used as substrates by the native bacteria (Wolfaardt *et al.,* 1994). Wolfaardt *et al.* (1994) observed that the removal of diclofop methyl increased up to 36% when using a bacterial-microalgal consortium or if microalgal metabolites were added to the bacterial culture. These studies clearly demonstrate that microalgae and bacterial co-culture systems are a much better option than single strain culture systems, which may be attributed to the division of labour which allows for better productivity (Brenner *et al.,* 2008).

2.4.4 Micronutrients

Bacteria can produce compounds that promote (or inhibit) microalgal growth. Microalgae are auxotrophic for vitamin B_{12} (cobalamin), which can be produced by prokaryotes. Vitamin B_{12} is one of the most complex primary metabolites in nature and is needed for an isoform of methionine synthase enzyme to function properly in microalgae. This complex production process requires more than 20 enzymatic catalysed reactions and some microalgae have lost their ability to produces this important metabolite, however bacteria is able to synthesis this vitamin (Grant *et al.,* 2014). Recent studies have described mutualistic relationship between heterotrophic bacteria and vitamin B_{12}-dependent microalgae (Grant *et al.,* 2014; Kazamia *et al.*, 2012; Xie *et al*., 2013), and that 171 out of 326 algal species require exogenous vitamin B_{12} (cobalamin), suggesting that half the algal kingdom is cobalamin auxotrophic (Croft *et al.,* 2005). The freshwater green alga *Lobomonas rostrata* and bacteria *Mesorhizobium loti* provide a good example of this mutualistic interaction. In this system, *M. loti* supplies *L. rostrata* with vitamin B_{12} and in return the bacterium receives fixed carbon. This fundamental study was further able to distinguish between the different mechanisms of nutrient exchange, and found that *M. loti* regulates the production levels of vitamin B_{12} resulting in a true mutualism with *L. rostrata*. The growth dynamics of interacting species in all populations is of great importance, both for the understanding of the natural aquatic ecosystems and for cultivation of these organisms for industrial purposes (Grant *et al.,* 2014). It has also been reported that ±25% of the microalgae are auxotrophic for vitamin B_1, and ± 8% for vitamin B_7 (Durham *et al.,* 2015).

In addition to vitamins as micronutrient for microalgae, the contribution of iron to mutualistic associations has been elucidated. Bacteria can regenerate and fix inorganic iron for microalgae to use (Amin *et al.,* 2009). *Scrippsiella trochoidea* needs siderophores (an iron-chelating compound) produced by the bacterial species (γ–proteobacteria *Marinobacter* and α-proteobacteria *Roseobacter*). The latter produces siderophore vibrioferrin that binds to Fe (III). This interaction makes the iron bioavailable for the microalgae to use. The microalgae use this iron during photosynthesis (of inorganic carbon fixation), and exchange forms DOC for bacterial growth (Amin *et al.,* 2009).

2.4.5 Competitive exclusion

Finally, bacterial contamination is a significant problem in algal cultivation, and can be addressed by the incorporation of mixed culture systems especially during the process of up-scaling. The presence of selected bacteria in the co-culture system will minimise the risk of other bacteria invading the environment, as the ecological niche is already occupied. A good example of competitive exclusion can be described with the mutualistic relationship between microalgae, *Emiliania huxleyi* and bacterium *Phaeobacter gallaeciensis*. The latter produces antibiotic molecules to prevent invasive bacteria from invading the niche (Seyedsayamdost *et*

al., 2011). This example is referred to as community ecology and relies on the competitive exclusion principle, which states that if species from the same niche are chosen, a stable synthetic community will grow without the presence of contaminants (Santos & Reis, 2014). This is an important concept, as in order for algae and bacteria co-cultivation practices to be fully exploited for biotechnological processes, a better understanding of mechanisms underlying algae/bacteria interactions are required.

2.5 Beneficial interactions between microalgae and yeast

Microalgae and yeast are commercially valuable organisms. Yeasts are widely used in scientific research, and are commercially relevant for the production of alcoholic beverages (beer and wine), baking, bioremediation, ethanol production (biofuel), nutritional supplements, and probiotics. They are also amenable to genetic engineering, and frequently developed for specific production processes referred to as biofactories (Chambers & Pretorius, 2010). Yeasts have been shown to have the potential for bioremediation in different wastewater types, including winery wastewater (Malandra *et al.,* 2003). It has been suggested that the co-occurrence of specific species of algae and yeasts can increase productivity of biotechnological processes, and in recent years a number of interesting studies describing these interactions have appeared (Dong & Zhao, 2004; Cheirsilp *et al.,* 2011; Papone *et al.,* 2012; Pisman & Somova, 2003; Puangbut & Leesing, 2012; Santos *et al.*, 2013; Xue *et al.,* 2010). Co-cultures of microalgae and yeast may provide some answers as to how these symbiotic associations are formed and provide insight into the factors which drive these mutualistic interactions (Hom & Murray, 2014). A number of studies have investigated important symbiotic relationships between microalgae and yeasts for industrial purposes and these are listed in Table 2.2. In the following sections previous studies which have investigated microalgae and yeast interactions with different industrial application, will be discussed and important benefits of these co-culture systems will be highlighted. This section can be broken down into two subsections: (1) oxygen and carbon dioxide exchange and (2) carbon and nitrogen exchange interactions.

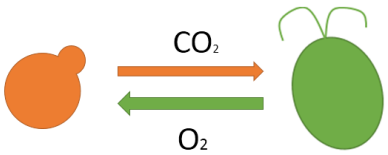
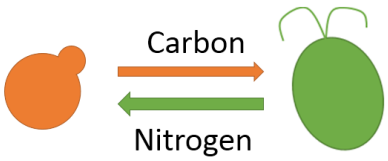
2.5.1 Oxygen and carbon dioxide

Papone *et al.* (2010) described a mutualism between the microalga *Chlorella* sp. KKUS2 and the oleaginous yeast *Torulaspora maleeae* or *Torulaspora globosa*. Here, the microalga supplied oxygen to the yeast, and in return the yeast provided CO_2 to the microalga (Papone *et al.,* 2012). In this study, the lipid yields were improved by 96% in co-culture compared to monocultures. This study demonstrated the advantage of using mixed culture systems for enhanced lipid production.

The oleaginous yeast, *Rhodotorula glutinis* has been reported to grow faster and produce higher lipid contents when co-cultured with *Chlorella vulgaris* (Cheirsilp *et al.,* 2011). *C. vulgaris* acts as an oxygen generator for the yeast, while the yeast provides CO_2 to the microalga resulting in

faster grow rates and enhanced lipid production. This study provides a good example of cooperation as both microbes are advantageous towards each other with improved biotechnological process for industrial application (Cheirsilp *et al.,* 2011). Moreover, higher concentrations of CO_2 and O_2 that have detrimental effects on the microalgae and yeast are avoided. Similar studies conducted by Chi *et al.* (2011) and Santos *et al.* (2013) have further demonstrated mutualistic symbioses between different species. Similarly, Xue *et al.* (2010) stated that a mixed culture of the microalga *Spirulina platensis* and yeast *R. glutinis* increased total lipid yield and the accumulation of total biomass. Collectively, these studies demonstrate how mutually beneficial symbiotic associations can be exploited to develop improved strategies for lipid production (Chi *et al.,* 2011; Santos *et al.,* 2013; Xue *et al.,* 2010). Alternatively, recent studies on yeast and microalgae co-cultures have included finding alternative aquaculture feed for fish cultivation (Cai *et al.,* 2007). Another example is fine chemical production by using mixed cultures of *Haematococcus pluvialis* and *Phaffia rhodozyma*, in the production of astaxanthin (Dong & Zhao, 2004).

Table 2.2 Symbiotic association studies between microalgae and yeast for industrial purposes.

Exchange (example)	Interaction	Application
Oxygen and carbon dioxide *Haematococcus pluvialis/ Phaffia rodozyma*; *Chlorella vulgaris/ Rhodotorula glutinis*; *Spirulina platensis/ R. glutinis*; *Chlorella* sp. KKU-S2/ *Torulaspora globosa* YU5; *Chlorella* sp./ *Torulaspora maleeae* Y30; *Chlorella* sp. KKU-S2/ *T. globosa* YU5/2; *Chlorella* sp./ *T. maleeae* Y30; *Chlorella protothecoides/ Rhodosporidium turoloides; C. vulgaris/ Candida utilis*		Astaxanthin (Dong & Zhao, 2004) Lipids for biodiesel (Cheirsilp *et al.,* 2011; Papone *et al.,* 2012; Puangbut & Leesing, 2012; Xue *et al.,* 2010) Lipid for biodiesel; carotenoids (Santos *et al.,* 2013) Model of micro-ecosystem (Pisman & Somova, 2003)
Carbon and Nitrogen (*Saccharomyces cerevisiae/ Chlamydomonas reinhardtii*)		Model of micro-ecosystem (Hom & Murray, 2014)

2.5.2 Carbon and Nitrogen

An interesting recent study demonstrated that obligate mutualisms between the alga *Chlamydomonas reinhardtii* and yeast *Saccharomyces cerevisiae* are relatively easy to establish when a strong selection pressure such as reciprocal carbon and nitrogen exchange is applied (Fig. 2.4, Hom & Murray, 2014). This study demonstrated how two model organisms with very different life histories are able to become obligate mutualists when a strong selection

pressure is applied. They also demonstrated that these mutualisms can be phylogenetically broad as they were established with 4 *Chlamydomonas* species and many different ascomycetous yeasts spanning 300 million years of evolutionary divergence in each clade. The fact that these fungal-algal mutualisms were created between two eukaryotes with distinctive life histories suggest that these interactions are relatively easy to establish (Hom & Murray, 2014). Physical interaction between *Chlamydomonas* algal cells with filamentous fungi was also observed (Hom & Murray, 2014). The study demonstrates that "under specific conditions, environmental change induces free-living species to become obligate mutualists and establishes a set of experimentally tractable, synthetic systems for studying the evolution of symbiosis" (Hom & Murray, 2014).

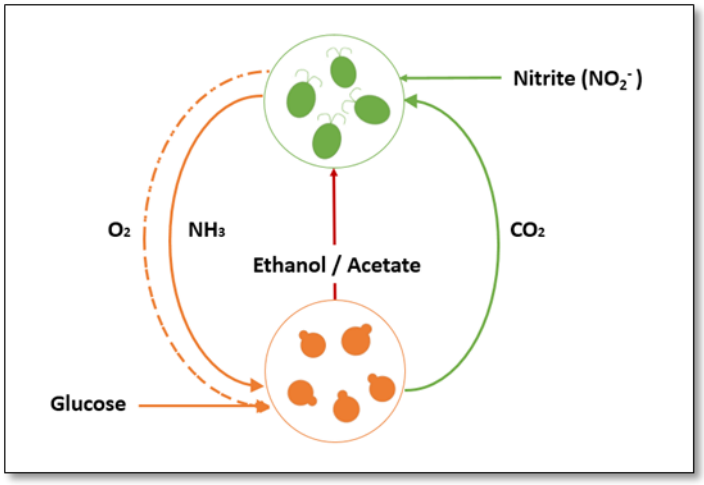


Figure 2.4 A synthetic mutualism between *C. reinhardtii* and *S. cerevisiae*. *S. cerevisiae* (orange) metabolises glucose and releases carbon dioxide (CO_2), which is utilsed photosynthetically by *C. reinhardtii* (green), to release oxygen (O_2); *C. reinhardtii* metabolises nitrite (NO_2^-) and releases ammonia (NH_3) as a nitrogen source for *S. cerevisiae* (Adapted from Hom & Murray, 2014).

From Table 2.2, the majority of the research studies have explored the interaction between microalgae and yeasts, which indicate that they could be ideal partners for forming mutualisms with microalgae. However, these studies have mostly focussed on improving lipid productivity and more research efforts have to be concentrated on investigating the interactions which occur between microalgae and yeast and how these interactions can be manipulated for specific purposes such as wastewater bioremediation.

2.6 Synthetic microbial ecology

The interdisciplinary field of synthetic biology involves the use of engineering principles to synthesise biologically complex systems, which possess functions that do not exist in nature. This engineering approach can be applied in all biological structures - from individual molecules to whole cells, tissues and organisms (Serrano *et al*., 2007).

The emerging field of synthetic ecology has highlighted the increasing need for more research on beneficial mutual symbioses within ecosystems. Synthetic ecology, a sub-discipline of synthetic biology, can be defined as the rational design and theory driven manipulation of artificial microbial ecosystems to enable, control or optimise a desired biotransformation (De Roy *et al.,* 2014; Dolinšek *et al.,* 2016; Stenuit & Agathos, 2015). Recently, Dolinšek *et al.* (2016) extended on this definition to include aspects of design, construction and analysis of the behaviour of artificial ecosystems. While, synthetic biology focuses largely on designing, building, quantifying, analysing, and predicting the dynamic behaviour of regulatory and metabolic circuits, synthetic microbial ecology attempts to understand the dynamic behaviour of artificial ecosystems. Thus, synthetic biology aims to understand how cellular-level properties emerge as a consequence of molecular interactions whereas synthetic microbial ecology wants to understand how community-level properties emerge as a consequence of microbial interactions (Dolinšek *et al.,* 2016). Recently, de-Bashan *et al.* (2016) proposed that 'the specific aim' of synthetic ecology is to "develop a cooperative and steady-state microbial community that performs a desirable biotechnological function". The goal is to design systems wherein both organisms benefit from this association combined with the maintenance of this association over multiple generations (de-Bashan *et al.,* 2016).

In the natural environment it is difficult to understand microbial interactions due to the level of complexity as well as the constantly changing environment. Synthetic ecology approaches such as species specific selection, engineered symbiosis and tailored growth condition provides an alternative to this situation (Kazamia *et al.,* 2014). Informed decisions can be used to build artificial ecosystems where the conditions can be controlled, monitored and manipulated as required. The generated data can be mathematically modelled to understand the interactions between organisms and to build prediction models for upscale to industrial level. The latter is important because in order to up-scale a system, one needs to understand the process and outcome before investing time and money into industrialisation. An understanding of the system may answer some fundamental questions regarding microbial interactions and we can then harness this information to develop improved industrial applications.

Recently, LaSarre *et al.* (2017) developed a synthetic environment to assess the reciprocal exchange of carbon (organic acid) and nitrogen (ammonium) between photoheterotrophic *Rhodopseudomonas palustris* and fermentative *Escherichia coli*. The obligate mutualism coexisted immediately, even after the disruption of increased ammonium production of *R. palustris* (through genetic engineering), which led to the amplification of organic acid production (LaSarre *et al.,* 2017). Additionally, Li *et al.* (2017) demonstrated a mutualistic interaction between phototrophic sucrose-secreting cyanobacteria (*Synechococcus elongatus*) and heterotrophic yeast strains (*Cryptococcus curvatus*, *R. glutinis*, or *S. cerevisiae*) within an artificial 'lichen-like' environment. In this synthetic mutualism cyanobacteria provided the yeast

with a carbon source and in return, the yeast removed oxidative stress from the environment and improved cyanobacterium growth. This synthetic mutualism resulted in improved growth and survival, improved reactive oxygen species (ROS) removal and lipid production (Li *et al.,* 2017). These studies show that by incorporating synthetic ecological principles, like engineered symbiosis with reciprocal metabolic capabilities, biotechnological processes can be improved and our understanding of complex communities can be broadened.

2.7 Winery wastewater

The South African wine industry produces 0.2 - 14 L winery wastewater per litre wine produced (Bolzonella & Rosso, 2013; Vlyssides *et al.,* 2005; Welz *et al.,* 2016), which is classified as biodegradable industrial effluent (Sheridan *et al.,* 2011). This can translate into a billion litres of winery wastewater that requires disposal every year. Winery wastewater originates from seasonal winemaking production and cleaning activities in cellars. This effluent typically has a low pH and high chemical oxygen demand (COD) concentration (Malandra *et al.,* 2003; Vlyssides *et al.,* 2005; Welz *et al.,* 2016). Moreover, the variable nature of this wastewater makes the development of cost-effective treatments difficult especially for smaller wineries, as it varies in volume and chemical composition (organic and inorganic). The chemical composition can depend on a number on factors which are influenced by cellar activities, grape varietal and cleaning products used in the cellar (Bolzonella & Rosso, 2013; Sheridan *et al.,* 2011; Vlyssides *et al.,* 2005; Welz *et al.,* 2016). The organic composition of the wastewater originates from the crushing of grapes, fermentation, maturation/stabilisation, decanting and bottling of wine and is seasonally dependent (Bories & Sire, 2010).

South African Water Act no. 36 (1998) indicates that winery wastewater should have a pH between 5.5 and 7.5, and a COD <75 mg/l when discharged. However, most winery effluent has a pH between 3 and 4 (Petruccioli *et al.,* 2002), with a COD average of 11 886 mg_{COD}/l (between 320 and 49 105 mg_{COD}/l) (Iaonnou *et al.,* 2015). South African discharge standards for wastewater are based on COD limits (and other parameters) per daily volume applied via irrigation. South African legislative states that “<5,000 mg COD/L for volumes <500 m3/day or < 400 mg COD/L for volumes >500 m3/day” (Welz *et al.,* 2016). Most wineries exceed these regulations and this can result in toxicity to the soil and soil microbiome (Mosse *et al.,* 2011). Thus, winery wastewater should undergo treatment before being discharged to the environment via irrigation to limit deleterious effects on the environment.

There are a number of factors that can influence the COD in winery wastewater and these include sugars, organic acids and alcohols. Malandra *et al*. (2003) took a ‘snapshot’ of different wineries and used this information to construct a synthetic winery wastewater recipe. This recipe is useful for experimental purposes, as it is consistent and not as variable as raw winery wastewater. More recently, Iaonnou *et al.* (2015) reviewed the treatment of winery wastewater

by biochemical, physicochemical and advanced processes. There are several treatment processes for winery wastewater, however it not efficient enough to meet discharge requirements. Iaonnou *et al.* (2015) divided winery wastewater treatments from bench- and industrial-scale processes into five categories: i.e., 'physicochemical, biological, membrane filtration and separation, advanced oxidation processes, and combined biological and advanced oxidation processes' (Fig. 2.6). The advantages and disadvantages are discussed in Iaonnou *et al*. (2015) review. The qualitative characteristics of winery wastewater was also summarised (Table 2.3).

Table 2.3 Qualitative characteristics of winery wastewater (Adapted from Iaonnou *et al.* (2015).

Parameter	**Unit**	**Min**	**Max**	**Mean**
Chemical Oxygen Demand	mgL^{-1}	320	49105	11886
Biochemical Oxygen Demand	mgL^{-1}	203	22418	6570
Total Organic Carbon	mgL^{-1}	41	7363	1876
pH		2.5	12.9	5.3
Electrical Conductivity	$mScm^{-1}$	1.1	5.6	3.46
Total Solids	mgL^{-1}	748	18332	8660
Total Volatile Solids	mgL^{-1}	661	12385	5625
Suspended Solids	mgL^{-1}	66	8600	1700
Total Phosphorous	mgL^{-1}	2.1	280	53
Total Nitrogen	mgL^{-1}	10	415	118
Total Phenolic Compounds	mgL^{-1}	0.51	1450	205

Presently, some wineries have no pre-treatment measures for winery wastewater prior to disposal, even though there are strict regulations regarding wastewater disposal. This is often attributed to the high costs and multiple treatment steps associated with conventional treatment systems which makes the costs prohibitive for smaller wineries. Biological treatment systems (i.e outdoor ponds) provide an alternative solution as these could potentially serve to alleviate this problem if systems are able to 'self-balance' nutrients (Welz *et al.,* 2016). Recently, Welz *et al.* (2015) reported that during January 2015 an algal bloom in a winery wastewater stabilisation pond decreased the COD levels substantially. Thus, it was suggested that this low COD could be attributed to degradation of organic contaminants by the microalgae.

Currently there is no biological treatment system for winery wastewater using multi-species ecosystems with known species of yeast and microalgae. Previous studies have shown that yeast and microalgae have bioremediation capabilities in various wastewater types and with their high biotechnological potential, it makes them the suitable candidates for the bioremediation of winery wastewater coupled with the production of valuable bio-products (De-Bashan *et al.,* 2003; Malendra *et al.,* 2003; Oswald *et al*., 1953). Designing a 'green' process which is self-sustaining by using wastewater as a nutrient source and developing an integrated treatment process can possibly decrease nutrient inputs and enhance productivity. Synthetic ecology will enable us to understand the interactions between yeast and microalgae and this information could have implications on building an ecosystem specific for wastewater treatment.

Figure 2.6 All components involved in winery wastewater.

2.8 Conclusion

The emerging field of synthetic ecology aims to unlock the potential of microbial consortia and to develop ecological systems that can be useful to generate products beyond that of single strain culture systems (Escalante *et al.,* 2015). This review has focussed on mutually beneficial symbiosis between microalgae and yeast/bacteria and how these associations can be used to potentially improve productivity and decrease economic inputs in biotechnological processes. While it is clear that co-culture systems offer significant advantages over single strain systems, there is a need for more intensive research into the complex and advantageous interactions between species within artificial microbial ecosystems. Mutualistic symbioses between yeast and algae could prove to be beneficial in biotechnological processes as systems relying solely on monocultures are by nature unstable and prone to perturbations. Exploration of the complementary interactions between microalgae and bacteria/ yeast could provide significant insights for the development of efficient biotechnological processes, as it has been established that microbial consortia provide a number of advantages to ecosystems and including robustness to environmental fluctuations, resistance to invasions and short periods of nutrient limitation. The use of directed selection approaches to engineer stable yeast-algae mutualisms provide the first building block for designing microbial consortia for use in biological winery wastewater bioremediation.

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Chapter 3

Research results

The bioremediation potential of *Parachlorella beijerinckii* and *Saccharomyces cerevisiae* isolated from winery wastewater

Chapter 3 - The bioremediation potential of *Parachlorella beijerinckii* and *Saccharomyces cerevisiae* isolated from winery wastewater

3.1 Introduction

The agricultural sector generates considerable amounts of wastewater every year, most of which originates from the food and beverage industry (Liu *et al.,* 2016); including the wine industry that generates a billion liters of winery wastewater every year (Ioannou *et al*., 2015; Oliveira & Duarte, 2010; Welz *et al.,* 2016). Winery wastewater consists of various organic (ethanol, organic acids and residual sugars) and inorganic components (ammonium, metal ions and phosphate). However, the nature of the winery effluent changes continuously and dramatically both in composition and volume throughout the year because of the batch nature of the wine production process (Liu *et al.,* 2016). Currently, there are strict regulations regarding winery wastewater disposal but most wineries have no treatment measures for winery effluent. There are various suitable treatment methods for winery wastewater, including physicochemical, biological and mechanical treatments, however treatment systems with multiple treatment steps are not always cost-effective for smaller wineries. Thus, research has focused on the development of efficient biological treatment systems with re-use of agricultural-industrial wastewater (Daffonchio *et al.,* 1998; Markou & Georgakakis, 2011; Petruccioli *et al.,* 2002).

Previous studies have shown that yeast and microalgae have bioremediation capabilities in various wastewaters and this combined with their high biotechnological potential, makes them useful for the bioremediation of winery wastewater coupled with the production of valuable bio-products (De-Bashan *et al.,* 2003; Liu *et al.,* 2016; Malandra *et al.,* 2003; Oswald *et al.,* 1953). Yeast (Malandra *et al.,* 2003) and microalgae (Liu *et al.,* 2016) have been previously investigated for winery wastewater bioremediation, however no biological treatment systems using multi-species ecosystems with known species of yeast and microalgae currently exists. Recently, it has become apparent that microalgae and yeast co-cultivation systems with complimentary metabolisms have the potential to increase productivity of biotechnological processes (Dong & Zhao, 2004; Cheirsilp *et al.,* 2011; Li *et al*., 2017; Papone *et al.,* 2012; Pisman & Somova, 2003; Puangbut & Leesing, 2012; Santos *et al.,* 2013; Xue *et al.,* 2010). However, there are challenges that need to be addressed before establishing a multi-species system for the bioremediation of winery wastewater. This includes the selection of appropriate species which are able to persist in the variable wine wastewater environment with the natural microflora and inconsistent biomass production of all species involved.

Yeast and microalgae were previously isolated from natural winery wastewater. The aim of this study was to characterise selected yeast and microalga isolates in synthetic and natural winery wastewater. Firstly, the physiological responses of selected *Sachharomyces cerevisiae* and *Parachlorella beijerinckii* in winery wastewater was characterised, with a particular focus on *P. beijerinckii*, as this microalgal species has not been evaluated in this context. The objectives of this study was to (1) identify previously isolated yeast and microalga from winery wastewater; (2) to investigate physiological optima and responses of *P. beijerinckii* to changes in key environmental variables associated with the winery wastewater environment; (3) to investigate the proliferation, decontamination ability and biomass production of both *S. cerevisiae* and *P. beijerinckii* in synthetic and raw winery wastewater. This included yeast and microalga mono-, co-culture as well as sequential inoculation experiments.

3.2 Materials and methods

3.2.1 Isolation and identification of microalgae and yeast from winery wastewater

3.2.1.1 Water sampling and isolation of microalgae and yeast

Winery wastewater was collected in November 2014 at a winery in the Stellenbosch area, South Africa. The wastewater had a pH of 4 with a chemical oxygen demand (COD) of 4140 mg/ml. Standard microbiological techniques were used to culture microalgae and yeast species from winery wastewater samples. Bold Basal medium with 3 fold nitrogen (3N BBM-V) (Bold, 1949), a selective nutrient-rich media, was used for the isolation of microalgae and Dichloran Rose Bengal agar (DCRB, Sigma) with chloramphenicol for selective isolation of yeasts. Microalgae single colonies were cultured on tris-acetate phosphate (TAP) agar plates (Gorman & Levine, 1965) under continuous light at 25°C. Microalgal isolate KW 4.29 was selected for further characterisation and maintained in TAP liquid medium and preserved on TAP agar slopes. Yeast isolate KW 15 was selected for further studies and maintained on Yeast Peptone Dextrose (YPD) agar plates (Biolab, SA) and preserved as glycerol stocks at -80°C.

3.2.1.2. Genomic DNA extraction, Polymerase chain reaction and sequencing

Genomic DNA was extracted from each isolate according to Hoffman and Winston (1987) and used as a template for 18S rRNA (Algae) and ITS-5.8S (Yeast) PCR reactions. PCR reactions (50 µl) contained 50 ng of yeast genomic DNA, 0.5 µM ITS1 primer (5'TCCGTAGGTGAACCTGCGG 3'), 0.5 µM ITS4 primer (5' TCCTCCGCTTATTGATATGC 3'), 1× buffer (10× colorless ExTaq Buffer®), 0.25 mM dNTPs, 2 mM $MgCl_2$ and 1.25 U Ex Taq (Promega®) as described by Ghosh *et al.* (2015). PCR reactions for microalgae isolates was as described for the yeast isolates, however, 0.5 µM 18S rRNA primers (5' ACCTGGTTGTCCTGCCAGT 3' and 5' TCAGCCTTGCGACCATAC 3') were used instead of the ITS primers (Perreira *et al.,* 2013). PCR cycling conditions were as follows: 3 min at 94°C,

30 seconds at 94°C, 54°C for 30 seconds, 72°C for 45 seconds for 30 cycles and a final elongation step for 10 min at 72°C. The PCR products were separated by gel electrophoresis on a 0.8% gel and were excised and purified with the Zymoclean™ Gel DNA Recovery Kit. Sequencing was performed, using the primers listed above, on an ABI Prism 377 automated DNA sequencer at the Central Analytical Facility at Stellenbosch University. ITS-5.8S rRNA and 18S rRNA gene sequences were assembled using DNAMAN analysis software version 4.15 (Lynnon BioSoft). The nucleotide sequences obtained from each of the isolates were compared using the BLAST (Basic local alignment search tool) algorithm with the available sequences in GeneBank at National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1997).

3.2.2 Microalgae growth conditions

Microalga KW 4.29 was cultured in TAP medium, containing ammonium chloride (NH_4Cl) as the nitrogen source supplemented with Hom's vitamins (4 mg/l myo-inositol, 0.4 mg/l 4-amino benzoic acid, 0.4 mg/l calcium D-pantothenate, 0.4/l mg niacin, 0.4 mg/l pyridoxine hydrochloride, 0.4 mg/l thiamine hydrochloride, 0.002 mg/l biotin, 0.002 mg/l cyanocobolamin, 0.002 mg/l folic acid, pH at 6.8). Experiments were conducted under continuous light (2500 lux) with agitation at 110 rpm. Microalga starter cultures used as an inoculum were prepared prior to growth experiments in 10 ml TAP medium containing NH_4Cl and 1× Hom's vitamins and incubated at 25°C for 24 hours (till mid-log) with agitation. All experiments were conducted in 250 ml Erlenmeyer flasks containing 50 ml TAP medium with a starting optical density of 0.1. Growth was determined spectrophotometrically at 750 nm every 3 h over a period of 72 h. Triplicate growth curves were generated and the specific growth rates were calculated. The latter was determined with the formula $\mu = (\ln N_1 - \ln N_0) / t_1 - t_0$, where μ indicated specific growth rate, N_0 the optical density from three OD measurements during exponential growth phase at a particular time (t_0) and N_1 a second point selected during exponential growth phase (t_1) (Levasseur *et al.,* 1993). Growth was evaluated at different temperatures, pH, salinity and ethanol concentrations. The pH of the TAP medium was adjusted with HCl and NaOH solutions.

3.2.3 Growth of yeast and microalgae in synthetic winery wastewater and decontamination efficiency

Microalgae and yeast cells were cultured in 50 ml synthetic winery wastewater (1.8 g/l glucose, 1.8 g/l fructose, 1 mg/l citric acid, 2 mg/l tartaric acid, 2 mg/l malic acid, 1.7 µl/l lactic acid, 1.6 µl/l propanol, 1.2 µl/l butanol, 4.7 µl/l iso-amyl alcohol, 238 µl/l acetic acid, 12.7 µl/l ethanol, 4.5 µl/l ethyl acetate, 7.5 µl/l propionic acid, 1.0 µl/l valeric acid, 0.8 µl/l hexanoic acid, 0.5 µl/l octanoic acid, 1.7 g/l YNB and 5 g/l NH_4SO_4, pH 5) supplemented with Hom's vitamins. Yeast and algal cells were inoculated to an OD of 0.1. Growth was determined spectrophotometrically at 600 nm (yeast) and 750 nm (algae) and with microscopic counts using a haemocytometer every 12 h for four days, and chemical oxygen demand (COD) was measured after 0 h, 24 h, 48 h and 96 h. A 2 ml sample was spun down and the supernatant was used to measure COD

decrease using the COD Cell Test (Merck KGaA, Germany), Thermoreaktor TR300 and Spectroquant Nova 60. Percentage of COD removal was calculated as following % COD = [(COD at t_0) – (COD at t_{96})]/ (COD at t_0) × 100. The decontamination ability of yeast and microalgae monoculture, co-culture and sequential inoculation were investigated. Sequential inoculation refers to a co-culture condition, where the microalgae were first cultured for 24 h before the yeast was inoculated. Experiments were conducted in triplicate.

3.2.4 Characterisation and decontamination of raw winery wastewater

3.2.4.1 Raw winery wastewater sampling and chemical analysis

Raw winery wastewater (RWW) was obtained during 2017 harvest season from the same winery as previously mentioned (Figure 3.1). Centrifugation (20 min at 4000 rpm) was used to remove all solid particles, and the winery wastewater was filter-sterilised using 0.22 µm filters (Whatman™, GE Healthcare Life Sciences). Glucose and fructose concentrations were determined using the Glucose/Fructose enzymatic kit (Megazyme, USA). The sterile RWW was chemically analysed using High Performance Liquid Chromatography (HPLC) to determine the type and relative concentrations of monosaccharides and organic acids (Eyéghé-Bickong *et al.,* 2012). COD was determined as described in section 3.2.3.2. The RWW sample was chemically analysed for elemental concentrations (carbon, hydrogen, nitrogen and sulphur), metals and several non-metals using inductively coupled plasma mass spectrometry (ICP-MS) (Central Analytical Facility, Stellenbosch University). The RWW was stored at -20°C.

A  B 

Figure 3.1 Sampling site of winery wastewater (A) and winery wastewater before filtration (B). (Photographed by Z.F. Simpson, Stellenbosch, South Africa, 2017).

3.2.4.2 Decontamination of raw winery wastewater

Microalga KW 4.29 and yeast KW 15 monocultures were cultivated in 50mL RWW supplemented with Hom's vitamins (personal communication, Erik F. Y. Hom, 2015). Experiments were conducted under continuous light conditions with agitation at 110 rpm at 30°C. Pre-cultures were prepared as described in section 3.2.2. Algae and yeast cells were inoculated to an OD of 0.2 and growth, and COD were measured as outlined in section 3.2.3.2.

The difference in pH was measured at the beginning and end of the experiment. Experiments were conducted in triplicate.

3.2.5 Statistical analysis

All data obtained were statistically analysed by performing a one-way analysis of variance (ANOVA) followed by the Tukey's honest significant difference (HSD) test for pairwise comparison using XLSTAT (version 2017, Addinsoft). The differences between treatments were statistically significant when the *p*-values were below 0.05. Data in tables and graphs are presented as means ± standard error of the mean.

3.3 Results

3.3.1 Isolation and identification of microalgae and yeast from winery wastewater

Microalgae KW 4.29 was observed as spherical green algal cells when viewed using light microscopy (Appendix A, Fig. 1b), and was identified as *Parachlorella beijerinckii* (100% identity) using 18S rRNA sequencing. Yeast KW 15 was identified as *Saccharomyces cerevisiae* isolate 10-1358 (100% identity). The well-known yeast, *S. cerevisiae* isolate MEA9 (CBS strain) has previously shown bioremediation potential in a winery wastewater environment (Malandra *et al.,* 2003), making *S. cerevisiae* isolate 10-1358 an ideal candidate for this study.

3.3.2 Impact of abiotic stressors on *P. beijerinckii* growth

3.3.2.1 The effect of temperature on the growth of *P. beijerinckii*

To determine optimal growth temperature for *P. beije*rinckii, different temperatures (25ºC, 30ºC and 37ºC) were investigated. *P. beijerinckii* reached stationary phase after ±30 h at both 25°C and 30°C (Fig 3.2A) and grew to the same OD after 36 h (~1.6 OD_{750nm}), with optimal growth observed at 30°C. Although there was no difference between the optical densities for microalgal growth at 25°C and 30°C after 36 h, there was a significant difference between 25/30°C and 37°C ($p < 0.05$). The growth of *P. beijerinckii* at 37°C was slower, with a longer lag phase, however the same OD_{750nm} was attained after 72 h at all temperatures (1.64 OD_{750nm}), with no statistically significant difference between 25°C, 30°C and 37°C. The specific growth rate of *P. beijerinckii* at 30°C was 0.0968 h^{-1} compared to 0.0887 h^{-1} and 0.0429 h^{-1} at 25°C and 37°C, respectively (Appendix A, Table 1).

3.3.2.2 The effect of pH on the growth of *P. beijerinckii*

The growth of *P. beijerinckii* at different pH levels (between pH 5 and 9) was evaluated. *P. beijerinckii* was able to grow between pH 6 and 9 (Figure 3.2B) with a longer lag phase observed at pH 6 and 9. However, the same final OD (±1.45 OD_{750nm}) is attained after 72 h for pH 6 compared to pH 6.5, 7, 7.5, 8 and 8.5, with no statistically significant difference between these groups ($p > 0.05$). The end-point growth of *P. beijerinckii* at pH 9 was significantly

different from all other pH levels, however, *P. beijerinckii* was still able to grow to an OD_{750nm} of 1.29. There was no growth observed when *P. beijerinckii* was cultured at pH 5 and pH 5.5. The specific growth rate of *P. beijerinckii* was 0.105 h^{-1} at pH 7 and 0.094 h^{-1} at pH 7.5, with highest specific growth rate observed at pH 7 (Appendix A, Table 1).

3.3.2.3 The effect of salt and ethanol stress on the growth of *P. beijerinckii*

The effect of salinity on *P. beijerinckii* was investigated, by the addition of sodium chloride (0, 5, 7.5, 10, 12.5 and 15 g/l) in TAP medium. The higher the salinity, the lower the specific growth rate (Appendix A, Table 1) and lower the biomass (optical density) after 72h (Fig. 3.2C). However, there is no significant difference between 5 g/l and 7.5 g/l optical density after 72h. A similar trend was observed for ethanol, with lower growth and specific growth rates at higher ethanol concentration (Appendix A, Table 1). *P. beijerinckii* was not able to proliferate in 5% ethanol (Fig. 3.2D).

3.3.3 Proliferation and decontamination of synthetic winery wastewater

Preliminary studies included testing the growth of *P. beijerinckii* in SWW with different pH values. Growth was most favourable at pH 5 and all further experiments were performed under these conditions (data not shown). Both *P. beijerinckii* and *S. cerevisiae* monocultures were able to proliferate in SWW, pH 5 (Figure 3.3A), growing to a cell count of 34.4×10^6 cells/ml yeasts and 14.1×10^6 cells/ml microalgae. *P. beijerinckii* was able to decrease COD by 59% compared to 91% for the yeast after 96 h (Fig. 3.3A). *S. cerevisiae* had a higher cell count compared to *P. beijerinckii* and was more efficient at reducing COD over time. The yeast-microalga co-culture was able to reduce COD by 51% within 24 h, and 88% after 96 h (Fig. 3.3B) compared to yeast monoculture with 44% decrease in COD within 24 h and 91% after 96 h (Table 3.1). Additionally, there was a significant difference ($p < 0.0001$) between the cell count of the yeast monoculture (34.4×10^6 cells/ml) and yeast in co-culture (40.6×10^6 cells/ml), with an observed increase in biomass for the yeast in co-culture (Table 3.1). *P. beijerinckii* was present in low cell numbers in co-culture with 1.67×10^6 cell/mL after 96 h (Fig. 3.3B). The percentage COD decrease for the sequential condition was similar to microalgae monoculture after 24 h (Fig. 3.3C). There were more algae cells present in the sequential condition after 96 h (6.9×10^6 cells/mL) compared to the co-culture (1.67×10^6 cell/mL), but COD decrease was more efficient in the co-cultures with a reduction of 88% in co-culture compared to 84% for sequential inoculation.

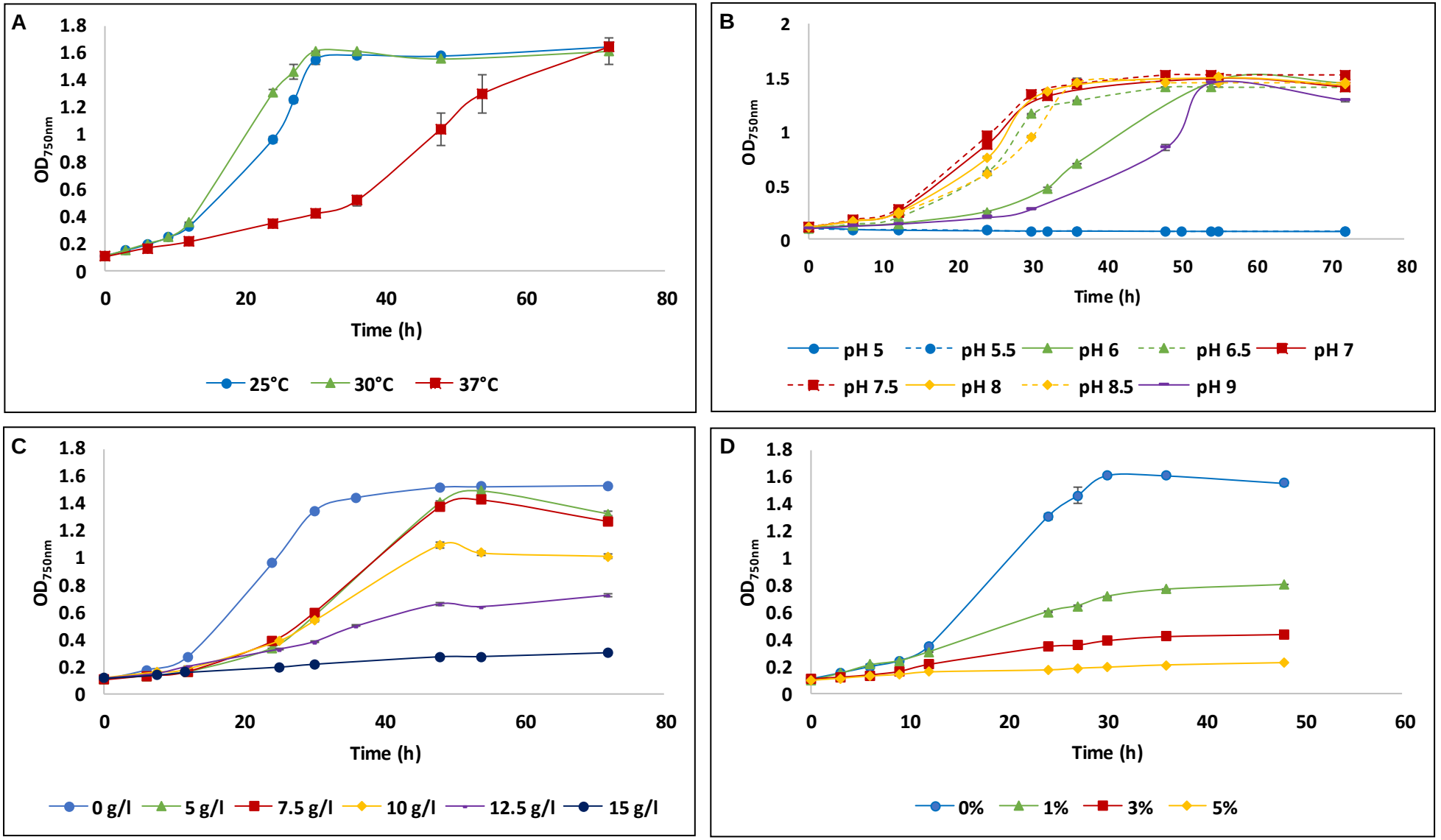

Figure 3.2 *Parachlorella beijerinckii* growth at different temperatures (A), pH (B), salinity (C) and ethanol concentrations (D) conducted under continuous light with agitation. Data represent the mean ± standard error (*n*=3).

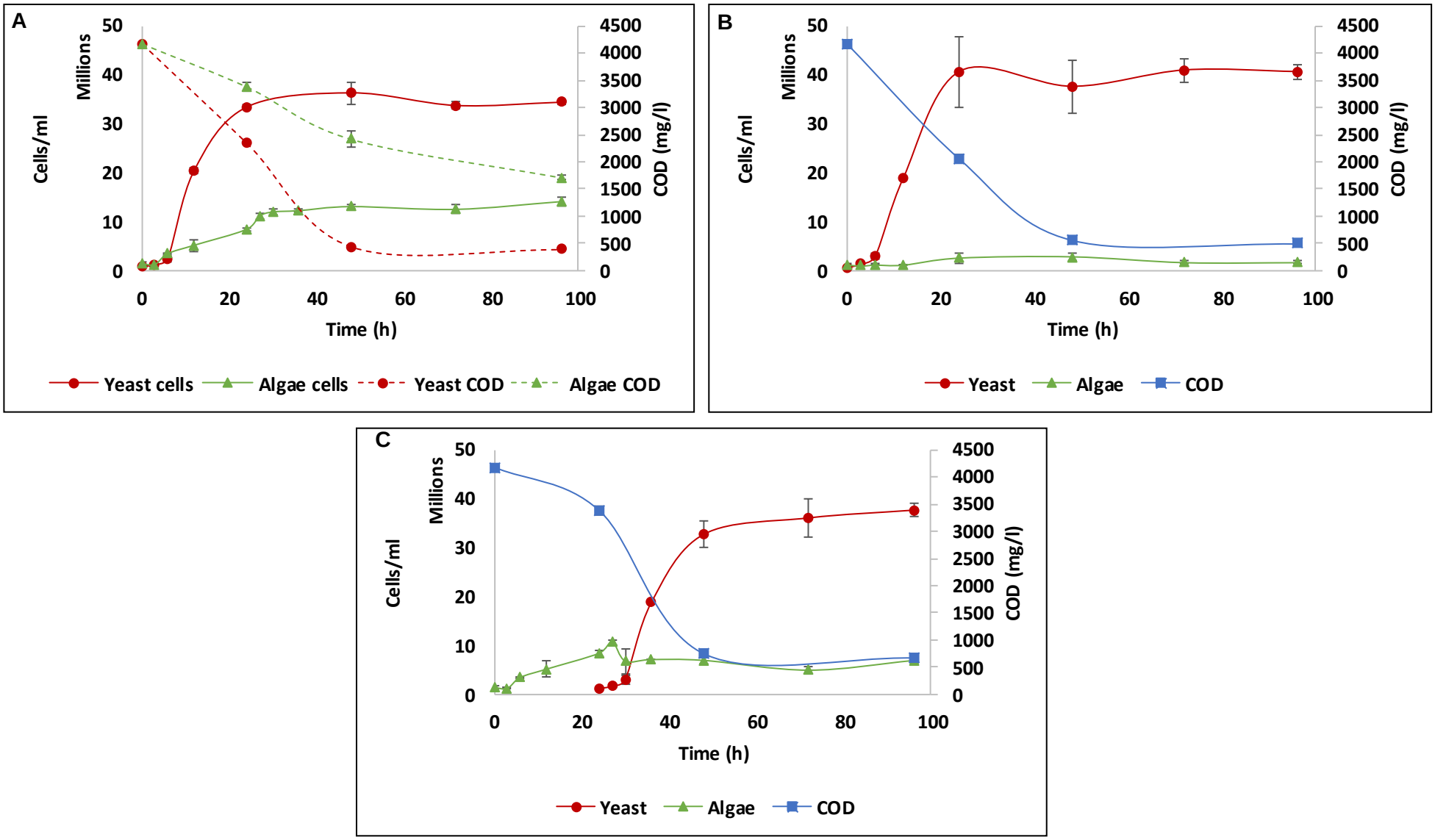

Figure 3.3 *Parachlorella beijerinckii* (green) and *Saccharomyces cerevisiae* (red) monoculture (A), co-culture (B) and sequential inoculation (C) growth and decontamination of synthetic winery wastewater (pH 5) at 30°C. Data represent the mean ± standard error (*n*=3).

Table 3.1 Summary of *S. cerevisiae* and *P. beijerinckii* growth and decontamination ability in synthetic winery wastewater, pH 5. Data represent a mean ± standard error (*n* = 3).

Condition	Parameter	Cell/ml (×10^6)		COD
Yeast mono-culture	Initial concentration	0.73 ± 0.04^a		4180 ± 0.00^a
	Final concentration	34.4 ± 0.44^d		392 ± 14.53^e
	Removal %			91%
Microalgae mono-culture	Initial concentration	1.40 ± 0.25^a		4180 ± 0.00^a
	Final concentration	14.1 ± 0.77^c		1715 ± 40.1^b
	Removal %			59%
		Yeast	Microalgae	
Co-culture	Initial concentration	0.62 ± 0.04^a	1.22 ± 0.10^a	4180 ± 0.00^a
	Final concentration	40.6 ± 1.04^e	1.67 ± 0.71^a	498 ± 14.24^d
	Removal %			88%
Sequential	Initial concentration	1.15 ± 0.06*^{a}	1.40 ± 0.35^a	4180 ± 0.00^a
	Final concentration	37.7 ± 1.37^{de}	6.92 ± 0.71^b	668 ± 11.67^c
	Removal %			84%

Values with different letters in the same column are statistically different when compared with Tukey's HSD post-hoc test at 95 % confidence level.
*Yeast was inoculated after 24.

3.3.4 Characterisation and decontamination of raw winery wastewater

3.3.4.1 Raw winery wastewater sampling and chemical analysis

The chemical composition of the raw winery wastewater is displayed in Table 3.2. The chemical oxygen demand of the raw winery wastewater was 8335 mg/l with a pH of 4.46. The RWW had 0.360 g/l glucose, 0.505 g/l fructose and 1.71 g/l ethanol present, with 37.5% of dry weight comprised of carbon and 0.8% nitrogen. There were no citric acid, malic acid, tartaric acid, succinic acid, acetic acid, glycerol and sulphur above the limit of quantification (LOQ) in the RWW sample. Different elements were evaluated using IPC-MS analysis and results are displayed in Table 3.2. According to the South African water quality guidelines for agricultural irrigation, Iron (Fe), Zinc (Zn), Chromium (Cr) and Manganese (Mn) were present in the RWW were above the target water quality range (Appendix A, Table 2). Other elements were below toxicity threshold.

Table 3.2: Organic and inorganic composition of raw winery wastewater

Components	Concentration^a	Element	Concentration^b	Element	Concentration^b
COD (mg/l)	8335	%Hydrogen	6.2	Mn	0.131
pH	4.46	%Nitrogen	0.8	B	0.113
Glucose	0.360	%Sulphur	< 0.05	Ni	0.079
Fructose	0.505	K	88.12	Mo	0.047
Citric acid	0	Ca	37.36	Pb	0.018
Malic acid	0	Na	10.34	Co	0.004
Tartaric acid	0	Fe	9.877	As	0.001
Succinic acid	0	Zn	6.24	Cu	< 0.01
Acetic acid	0	Mg	5.49	Li	< 0.01
Ethanol	1.71	P	4.92	V	< 0.01
Glycerol	0	Al	2.897	Cd	< 0.01
%Carbon	37.5	Cr	0.131	Be	< 0.01

[a] Concentrations are given as g/l unless indicated otherwise.
[b] Concentrations are given in mg/l.

3.3.4.2 Decontamination of raw winery wastewater

Winery wastewater isolates, *S. cerevisiae* and *P. beijerinckii,* were able to proliferate in pH 4.46 RWW when inoculated at an OD of 0.2 (Fig. 3.4). *S. cerevisiae* reduced COD from 8335 mg/l to 4063 mg/l (51%) after 24h compared to 4550 mg/l (45%) by *P. beijerinckii*. *P. beijerinckii* was able to lower COD with 53% compared to 63% for *S. cerevisiae* after 96 h (Table 3.3). Both yeast and microalgae were able to reduce COD more efficiently in SWW, with 91% decrease for *S. cerevisiae* in SWW and 63% in RWW; and 59% decrease for *P. beijerinkcii* in SWW compared to 53% in RWW. *S. cerevisiae* biomass was lower in RWW with a final optical density at 1.11 OD_{600nm} compared to 3.78 OD_{600nm} in SWW. The growth of *P. beijerinckii* was less affected with final optical density at 1.39 OD_{600nm} in RWW compared to 1.56 OD_{600nm} in SWW, with a significant difference between the two wastewater types. The initial pH for the microalgal mono-culture in RWW was 4.46. There was a pH increase with end-point pH at 7.2 after 96h.

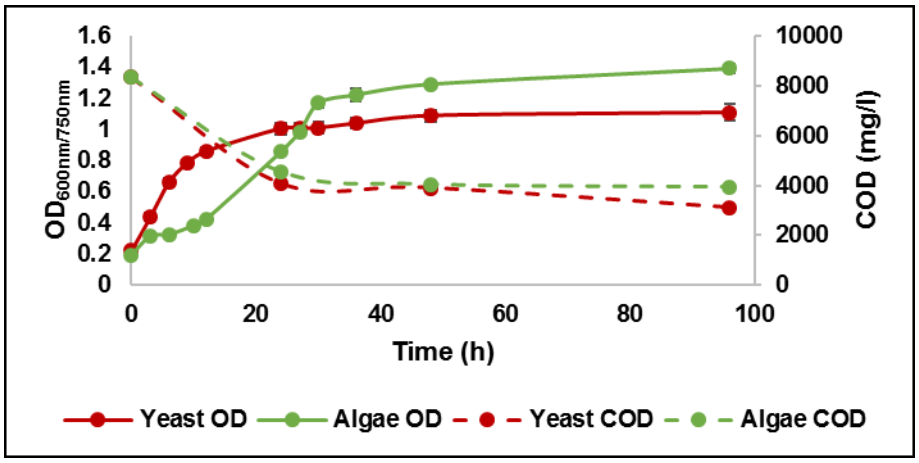

Figure 3.4 *P. beijerinckii* (green, 750nm) and *S. cerevisiae* (red, 600nm) growth and decontamination of raw winery wastewater at 30°C. Data represent the mean ± standard error (n=3).

Table 3.3 Summary of *S. cerevisiae* and *P. beijerinckii* growth in pH 5 synthetic and pH 4.46 raw winery wastewater. Data represent a mean ± standard error (*n* = 3).

Wastewater type	Isolate	Parameter	OD	COD
Synthetic	Yeast	Initial concentration	0.1 ± 0.00^{a}	4180 ± 0.00^{b}
		Final concentration	3.78 ± 0.04^{e}	392 ± 14.53^{f}
		Removal %		91%
	Microalgae	Initial concentration	0.13 ± 0.00^{a}	4180 ± 0.00^{b}
		Final concentration	1.56 ± 0.02^{d}	1715 ± 40.1^{e}
		Removal %		59%
Raw	Yeast	Initial concentration	0.22 ± 0.00^{a}	8335 ± 14.43^{a}
		Final concentration	1.11 ± 0.05^{b}	3100 ± 55.07^{d}
		Removal %		63%
	Microalgae	Initial concentration	0.19 ± 0.00^{a}	8335 ± 14.43^{a}
		Final concentration	1.39 ± 0.03^{c}	3940 ± 62.45^{c}
		Removal %		53%

Values with different letters in the same column are statistically different when compared with Tukey's HSD post-hoc test at 95 % confidence level.

3.4 Discussion

3.4.1 Impact of abiotic stressors on *P. beijerinckii* growth

This study was the first attempt to phenotypically characterise the response of *P. beijerinckii* to temperature, pH, salinity and ethanol stress. *P. beijerinckii*, like other microalgal species, can grow and tolerate a wide temperature (Kessler *et al.,* 1985; Lehana, 1990; Li *et al.,* 2013) and pH (Azov, 1982; Juneja *et al.,* 2013) range. *P. beijerinckii*, like other microalgal species, is sensitive to high levels of salt (Sudhir & Murthy, 2004; Zhang *et al.,* 2010) and ethanol (El Jay, 1996). However, it has been previously reported that winery effluent has low sodium (0.007 g/l - 0.470 g/l) and low ethanol (0.126% (v/v) - 0.630% (v/v)) concentrations (Mosse *et al.,* 2011), making *P. beijerinckii* a potentially suitable candidate for this environment.

3.4.2 Proliferation and decontamination of synthetic winery wastewater

In order to reduce the variability commonly associated with winery wastewater, a constant synthetic environment was maintained by employing the use of a synthetic winery wastewater protocol (Malandra *et al.,* 2003). *S. cerevisiae* is known to have optimal growth at pH ranging between 4 and 6 depending on the strain, temperature and the presence of oxygen (Narendranath & Power, 2005), confirming growth of *S. cerevisiae* isolate 10-1358 in the SWW.

P. beijerinckii, like other microalgal species, has the ability to grow heterotrophically with organic carbon (acetic acid and glucose) as an alternative carbon source (Juneja *et al*., 2013). There are a few advantages afforded to microalgal heterotrophic growth in comparison to photoautotrophic cultivation including (1) increased growth rate and biomass production; (2) increased stress tolerance (3) and light is not required for cultivation, eliminating the effects of low light intensity (Cheng *et al.,* 2009; Morales-Sánchez *et al.,* 2013; O'Grady & Morgan, 2010; Perez-Garcia *et al.,* 2011). Juneja *et al*. (2013) also reported that there is a reduction in photosynthesis when microalgae are exposed to a low pH environment. However, when the microalgae grow heterotrophically with additional carbon sources, photosynthesis is no longer needed; explaining the growth of *P. beijerinckii* in pH 5 SWW compared to no growth in pH 5 TAP medium. There are some disadvantages associated with heterotrophic growth, including the cost associated with the addition of carbon and the increased risk of contamination by heterotrophic invasive species.

However, the focus of this study was to determine whether *S. cerevisiae* and *P. beijerinckii* had bioremediation capabilities in synthetic and natural winery wastewater. There is currently no literature available on the decontamination of SWW (pH 5) using *P. beijerinckii* and *S. cerevisiae*, and this study provides the first account of this. However, Malandra *et al.* (2003) showed that *S.*

cerevisiae MEA9 (CBS strain) reduced COD with 88% in SWW (pH 4) after 120 h under aerated conditions. Previous studies have also shown that decontamination ability is species-specific (Liu *et al.,* 2016; Malandra *et al.,* 2003) and we suggest that this particular environment was more favourable towards *S. cerevisiae* (an acidophile) compared to *P. beijerinckii* (neutraphil) (Juneja *et al.,* 2013); explaining the increased growth and improved reduction of chemical oxygen demand by *S. cerevisiae*.

Since *P. beijerinckii* and *S. cerevisiae* monocultures were both able to decontaminate synthetic winery wastewater, the microalgae-yeast co-culture was evaluated. The increase in yeast cell numbers and growth rate in co-culture after 24 h, led to improved COD reduction at the specific time. Numerous studies have shown that the presence of microalgae improves the growth of yeast species (Dong & Zhao, 2004; Cheirsilp *et al.,* 2011; Papone *et al.,* 2012; Pisman & Somova, 2003; Puangbut & Leesing, 2012; Santos *et al.,* 2013; Xue *et al.,* 2010), echoing the increase of *S. cerevisiae* cell numbers, growth rate and decontamination ability in the presence of *P. beijerinckii* compared to yeast monoculture. However, after 96 h, lower COD removal was observed; possibly associated with competition for nutrients and resources between microorganisms. Sequential inoculation experiments were performed as *P. beijerinckii* was outcompeted by *S. cerevisiae* in the co-culture condition. This experiment showed that although the microalgae were present in higher cell numbers, the yeast monoculture was still more efficient at bioremediation. In this study, both *S. cerevisiae* and *P. beijerinckii* were able to proliferate and bioremediate synthetic winery wastewater but the yeast were more effective in this context. The co-culture condition favoured the growth of the yeast as it dominated the co-culture, but no improvement of bioremediation capability was observed. The yeast and microalgae co-culture described in this study does not work optimally together and therefore we suggest the use of ecosystem engineering to create functional communities, which are more efficient at wastewater bioremediation. Before this can be applied, the next step was to determine whether *S. cerevisiae* and *P. beijerinckii* had bioremediation capabilities in natural winery wastewater.

3.4.3 Characterisation and decontamination of raw winery wastewater

The COD in natural winery effluent varies between 320 and 49105 mg/l (mean 11886 mg/l) and pH between 2.5 and 12.9 (mean 5.3), with a peak in COD and low pH during harvest (Ioannou *et al.,* 2015). Additionally, there are primary metabolites in a grape berry, and this include sugars (e.g. glucose and fructose) and organic acids (e.g. citric acid, tartaric acid, malic acid, succinic acid and acetic acid); and ethanol and glycerol are by-products of fermentation during winemaking (Musingarabwi *et al.,* 2016). All these metabolites were present in the sampled winery wastewater and within the ranges described in literature (Malandra *et al.,* 2003; Mosse *et al.,* 2011). Therefore,

the growth and bioremediation potential of *P. beijerinckii* and *S. cerevisiae* were evaluated in the raw winery wastewater. The biomass production and similarity between the yeast and microalgae bioremediation capabilities was a result of the nutrient status of the environment i.e. less organic carbon (glucose and fructose) and lower pH compared to SWW. Secondly, the low levels of nitrogen and phosphorus in the winery effluent had a negative impact on the growth of *P. beijerinckii* and *S. cerevisiae* (Abdel-Raouf *et al.*, 2012). Despite the reduction in bioremediation capabilities, both yeast and microalgae were still able to reduce COD. *P. beijerinckii* also had the ability to increase the pH of the raw winery wastewater within the requirements stipulated in the South African Water Act (1998), as microalgae are known to have this ability due to their photosynthetic CO_2 metabolism (Chi *et al.,* 2011).

Certain limitations were apparent during the progress of this study. Firstly, this study only accounts for inoculated strains in synthetic and natural winery wastewater under experimental conditions and does not account for other microbes present in the wastewater. Secondly, the bioremediation potential of *P. beijerinckii* and *S. cerevisiae* were only investigated under one 'snapshot' of raw winery wastewater isolated from a winery. Future studies should include multiple sampling points during harvest and the rest of the year with the natural microflora present. However, this study provides insight to the possibility of using *S. cerevisiae* and *P. beijerinckii* for the bioremediation of winery wastewater, including the decrease of COD and increase of pH within requirements. Both yeast and microalgae showed bioremediation potential in both model SWW and RWW. However, in co-culture there is no cumulative effect, as both organisms have this ability but the yeast and microalgae compete for nutrients in this environment, instead of working together. Therefore, we suggest the engineering of stable associations between *S. cerevisiae* and *P. beijerinckii*, as multi-species systems with complementary metabolic capabilities has previously been shown to enhance productivity (Kazamia *et al.,* 2014; Li *et al.,* 2017). We suggest that such complimentary engineered associations may be the first step in the development of a multi-species approach to winery wastewater treatment.

3.5 References

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3.7 Appendix A

Figure 1 Microalgae isolated from winery wastewater, *Parachlorella beijerinckii*: (a) colour and shape of alga colonies on Tris-acetate phosphate media agar plates grown at 25°C, under continuous light (b) and microalgae cells (marked x) observed under light microscopy at 400× magnification (Photographed by Z.F.Simpson, Stellenbosch, 2017).

Table 1 Specific growth rate (μ) of *Parachlorella beijerinckii* at different temperatures and pH, salinity and ethanol concentrations. Data represent the mean ± standard error (n=3).

Temperature (°C)	μ (h^{-1})
25	0.0887
30	0.0968
37	0.0429
pH	μ (h^{-1})
5	-
5.5	-
6	0.055
6.5	0.087
7	0.105
7.5	0.094
8	0.080
8.5	0.071
9	0.034
Salinity (g/l)	μ (h^{-1})
0	0.094
5	0.0627
7.5	0.0560
10	0.0500
12.5	0.0329
15	0.0152
Ethanol (%)	μ (h^{-1})
0	0.0968
1	0.0241
3	0.0177
5	-

Table 2 A summary of the South African water quality guidelines for agricultural irrigation compared to sampled raw winery wastewater.

Element	Water Act guidelines (mg/l)	Raw winery wastewater (mg/l)
K	-	88.12
Ca	-	37.36
Na	< 70	10.34
Fe	< 5.0	9.877
Zn	< 1.0	6.24
Mg	-	5.49
P	-	4.92
Al	< 5.0	2.897
Cr	< 0.10	0.131
Mn	< 0.02	0.131
B	< 0.50	0.113
Ni	< 0.20	0.079
Mo	< 0.01	0.047
Pb	< 0.2	0.018
Co	< 0.05	0.004
As	< 0.10	0.001
Cu	< 0.20	< 0.01
Li	< 2.50	< 0.01
V	< 0.10	< 0.01
Cd	< 0.01	< 0.01
Be	< 0.10	< 0.01

Chapter 4

Research results

Engineered yeast and microalgae mutualisms: Synthetic ecology applied to species isolated from winery wastewater

Chapter 4 - Engineered yeast and microalgae mutualisms: Synthetic ecology applied to species isolated from winery wastewater

4.1 Introduction

All living systems occupy habitats that are shared with other species. Symbiosis refers to species that specifically interact with each other in these habitats. There are a number of factors which influence the formation of symbiotic associations within the natural environment, including physical (secure habitat) and biochemical (nutrient exchange) factors. Nutrient exchange has been identified as a major driving force behind most symbiotic associations (Oksanen, 2006). Mutualism, a form of symbiosis, can be defined as any long-term association between two species that confers mutual fitness benefit to individual members of both species (Kazamia *et al.,* 2012). Studies on mutualistic interactions between microalgae/cyanobacteria and bacteria/ yeasts have increased over the last decade, as it has become apparent that these associations could potentially benefit industrial processes (Dong & Zhao, 2004; Cheirsilp *et al.,* 2011; Li *et al.*, 2017; Papone *et al*., 2012; Pisman & Somova, 2003; Puangbut & Leesing, 2012; Santos *et al.,* 2013; Xue *et al.*, 2010). These multispecies systems can provide more functionalities to an ecosystem and are often better able to cope with environmental perturbations, however, remain difficult to control and understand in the natural environment due to the level of complexity and fluctuating environmental conditions (Brenner *et al.,* 2008; Santos & Reis, 2014). To overcome this drawback, we suggest a synthetic ecology approach, which can be defined as the rational design and theory driven manipulation of artificial microbial ecosystems to enable, control or optimise a desired biotransformation (De Roy *et al.,* 2014; Dolinšek *et al.,* 2016; Stenuit & Agathos, 2015). Multi-species systems with complementary metabolic capabilities has previously proven to enhance productivity (Kazamia *et al.,* 2014; Li *et al.,* 2017), as these engineered multi-species systems often improve growth and survival of the partners involved, improve functional and metabolic capabilities, can perform more complex tasks and are often more resilient to change (Brenner *et al.,* 2008; de-Bashan *et al.,* 2016; Dolinšek *et al.,* 2016). Thus, synthetic ecology allows us to study microbial interactions under controlled artificial conditions, using engineering principles such as species specific selection, engineered symbiosis and tailored growth condition to generate robust and functional ecosystems (Kazamia *et al.,* 2014).

Recently, Li *et al.* (2017) demonstrated a mutualistic interaction between heterotrophic yeast strains (*Cryptococcus curvatus*, *Rhodotorula glutinis*, or *Saccharomyces cerevisiae*) with phototrophic

sucrose-secreting cyanobacteria (*Synechococcus elongatus*) in an artificial environment. Here, the cyanobacteria provided the yeast with carbon and in return, the yeast improved photosynthetic cyanobacteria growth by using the provided oxygen and removing oxidative stress from the environment. By engineering symbiosis between organisms with complimentary metabolic capabilities which facilitates the establishment of the symbiosis, an improvement in growth and survival, improved reactive oxygen species (ROS) removal and lipid production was observed in co-culture condition (Li *et al.,* 2017). This strategy was also used by Hom and Murray (2014) when they demonstrated that an obligate mutualism between the yeast *S. cerevisiae* and the alga *Chlamydomonas reinhardtii* are relatively easy to establish when a strong selection pressure such as reciprocal carbon and nitrogen exchange is applied. In this system, *S. cerevisiae* fermented glucose and released carbon dioxide, which was assimilated by photosynthetic *C. reinhardtii*, with the release of oxygen. In return, *C. reinhardtii* metabolised nitrite and released ammonia as a nitrogen source for *S. cerevisiae* (Hom & Murray, 2014).

In this study, the aim was to establish and optimise culture conditions which enforce an obligatory mutualism, while optimising growth of both organisms. Once established, such conditions will be used for the co-evolution of the two species. Previously, *S. cerevisiae* and *P. beijerinckii*, isolated from winery wastewater, were identified as potential candidates for winery wastewater treatment and biomass production (Chapter 3). In the second part of this study, the complimentary metabolic capabilities of these organisms were exploited to facilitate the reciprocal exchange of carbon and nitrogen; leading to the establishment of a synthetic obligate mutualism between *S. cerevisiae* and *P. beijerinckii.* The impact of temperature and pH, which are key environmental parameters which contribute to the variable nature of winery wastewater, were determined on the mutualistic association. Different carbon and nitrogen nutrient sources were used to form non-obligatory associations and the effect of co-culture growth on biomass production were evaluated. Finally, a protocol was developed to up-scale the mutualism for the use in continuous culture systems which will offer a more constant environment, allow for the control and monitoring of different parameters and provide an environment for co-evolutionary studies.

4.2 Materials and methods

4.2.1 Mono- and co-culture growth in modified synthetic winery wastewater

Synthetic winery wastewater (SWW) was prepared according to the method described in chapter 3 (Malandra *et al.,* 2013) with a few modifications. Glucose and yeast nitrogen base were excluded from the medium and yeast nitrogen base without ammonium and amino acids (Sigma, USA) was

added to supplement the medium with vitamins and trace elements. Nitrite (KNO_2) was added as the nitrogen source. Yeast and microalgae starter cultures, were prepared prior to inoculation. *S. cerevisiae* was cultured in log phase for 12 hours and *P. beijerinckii* for 24 h in 10 ml Tris-acetate phosphate (TAP) medium (Gorman & Levine, 1965), 2% fructose and 1× Hom's vitamins (personal communication, Erik F. Y. Hom, 2015). Prior to co-culturing yeasts and microalgae cultures of individual organisms were centrifuged at 2000 x g (alga) or 4000 x g (yeast) for 3 min to pellet cells. Cells were re-suspended in a volume of TAP media without nutrients equivalent to the pre-culture volume and cells were washed twice. Cell densities were determined using microscope counts using a haemocytometer and a stock concentration of each cell type was prepared in fresh TAP medium lacking nutrients for co-culture inoculation.

Cells were inoculated to a density of 0.1×10^6 cells/ml. All single and co-culture experiments were conducted at 25°C at pH 7 without agitation under continuous light (2500 lux) unless otherwise indicated. All co-culture experiments were performed without agitation, as cell to cell proximity has been shown to be important in establishing mutualistic relationships for certain species of yeast and microalgae (Hom & Murray, 2014). Three replicate experiments were performed and at appropriate experimental time-points (Day 0, 5, 7, 11 and 14) single and co-cultures were thoroughly mixed by vortexing and the cell densities were measured using microscopic haemocytometer cell counts to evaluate mutualistic behaviour.

4.2.2 Obligate mutualistic growth condition in TAP medium

An obligate mutualism was established between *S. cerevisiae* and *P. beijerinckii* using a methodology similar to that described by Hom and Murray (2014) with some modifications. Cells were co-cultured in modified TAP medium, which consists of TAP medium lacking ammonium (NH_4Cl), supplemented with 3.6% carbon source and 16 mM KNO_2. Carbon sources included glucose, fructose, sucrose, maltose, galactose, mannose, ethanol, glycerol and acetic acid. Cell densities were measured after seven days using microscopic haemocytometer cell counts to identify a carbon source which *P. beijerinckii* is unable to utilise.

The effect of temperature (25°C, 30°C and 37°C) and pH (pH 5, 6, 7, 8 and 9 at 25°C) were assessed under obligate mutualistic conditions. At appropriate experimental time-points (Day 0, 5, 7, 11 and 14) co-cultures were evaluated for mutualistic behaviour by means of microscopic haemocytometer counts. For these experiments, three biological replicate experiments were performed. A co-culture was sacrificed for each experimental measurement that was made.

4.2.3 Non-obligatory growth conditions in TAP medium

TAP medium lacking NH_4Cl supplemented with 3.6% mannose, 16 mM KNO_2, 1× Hom's vitamins and 0.1% acetic acid was used for co-culture growth under semi-selective conditions. For co-culture growth under non-selective conditions, TAP medium supplemented with 3.6% mannose, 16 mM NH_4Cl, 1× Hom's vitamins and 0.1% acetic acid was used. The single and co-culturing conditions were the same as described above. Three replicate experiments were performed and at appropriate experimental time-points (Day 0, 5, 7, 11 and 14) single and co-cultures cell densities were measured using microscopic haemocytometer counts to evaluate mutualistic behaviour.

4.2.4 Obligate mutualistic growth in 1L bioreactor

Both microalgae *P. beijerinckii* and *Chlorella sorokiniana* were co-cultured with *S. cerevisiae* in a bioreactor set-up. Starter cultures were prepared with a methodology similar to that described in section 4.2.2. Cells were co-cultured in modified TAP medium (pH 8), which consisted of 500 mL TAP medium lacking ammonium (NH_4Cl), supplemented with 3.6% mannose, 16 mM KNO_2 and 1× Hom's vitamins. Cells were inoculated to a cell density of 1×10^6 cells/ml. Co-culture experiments were conducted at 25°C, with 50 rpm agitation under continuous light (~11 000 lux) in a BioFlo 110 Vessel bioreactor system (New Brunswick Scientific). The batch bioreactor system was set-up as described in the Guide to Operations manual no. M1273-0054 (Figure 4.1, New Brunswick Scientific). At appropriate experimental time-points (12-hour interval during the day), co-cultures were thoroughly mixed by increasing agitation, and 5 mL were sampled every 12 h for seven days. Yeast and microalgae growth was monitored by means of haemocytometer cell counts (cell/mL) and combined dry weight (g/l). Mannose (D-Mannose/D-Fructose/D-Glucose Assay kit, Megazyme) and nitrite (Nitrite/Nitrate Assay Kit, colourimetric, Merck) consumption were monitored throughout the experiment. The production of organic acids (citric acid, tartaric acid, malic acid, succinic acid and acetic acid), glycerol and ethanol were measured using High-Performance Liquid Chromatography (HPLC) analysis (Eyéghé-Bickong *et al.,* 2012). The HPLC method (SUG_PY5) was run using an isocratic gradient of 5 mM H_2SO_4 at a flow rate of 0.5 ml/min for 39 min, and the injection volume was 10 µl. After HPLC analysis, the integrated standard area and known concentrations were used to plot each standard accordingly, to determine unknown sugar and organic acid concentrations.

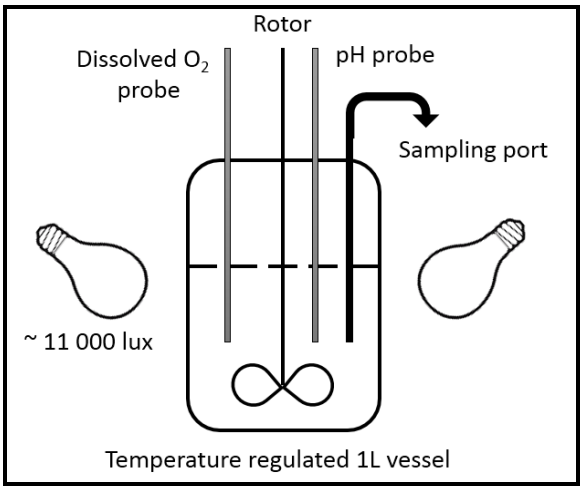

Figure 4.1 Graphic illustration of 1 L BioFlo 110 vessel bioreactor including temperature and agitation control; pH and dissolved oxygen monitoring and sampling port.

4.2.5 *Statistical analysis*

All data obtained was analysed by performing a one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test for pairwise comparison using XLSTAT (version 2017, Addinsoft). Differences between treatments were regarded as statistically significant when the p-values were below 0.05. Data in tables and graphs are presented as means ± standard error, unless otherwise indicated.

4.3 Results

4.3.1 Mono- and co-culture growth in modified synthetic winery wastewater

S. cerevisiae was unable to grow in modified synthetic winery wastewater (SWW) as a single culture, as this yeast species was unable to assimilate nitrite as a nitrogen source (Fig. 4.2). *P. beijerinckii* monoculture grew well in this closed system, as *P. beijerinckii* was able to utilise fructose as a carbon source, reaching a cell density of 2.7×10^6 cells/ml after 14 days (Fig. 4.2). In co-culture, the yeast fermented fructose with the release of carbon dioxide, providing an additional carbon source to the microalgae and *P. beijerinckii* metabolised nitrite (NO_2^-) into ammonia (NH_3^+) to provide *S. cerevisiae* with a nitrogen source. *S. cerevisiae* grew to a cell count of 1.0×10^6 cells/ml after 14 days in co-culture with a ~2 fold improvement of *P. beijerinckii* growth in co-culture compared to monoculture. In this system, *P. beijerinckii* was not dependent on *S. cerevisiae* for growth, therefore it was important to identify a carbon source which *S. cerevisiae* can ferment but *P. beijerinckii* cannot utilise, to initiate an obligate mutualism between *S. cerevisiae* and *P. beijerinckii*.

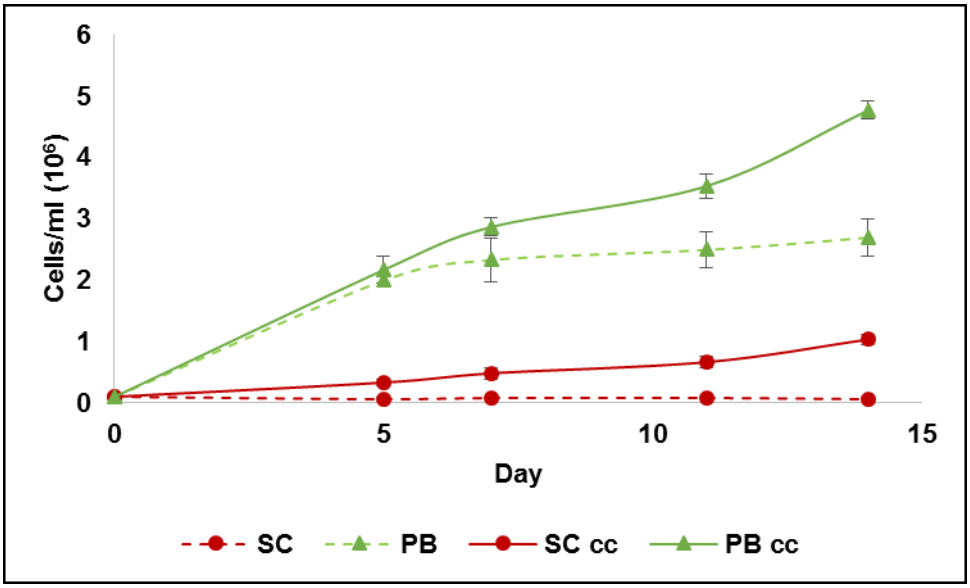

Figure 4.2 Non-obligate growth of *S. cerevisiae* (SC) and *P. beijerinckii* (PB) under single and co-culture (cc) growth conditions in synthetic winery wastewater (pH 7). The growth temperature was kept constant at 25°C with no agitation under continuous light. Data represent the mean ± standard error (*n*=3).

4.3.2 Carbon source selection to induce obligate mutualistic growth

S. cerevisiae and *P. beijerinckii* single and co-culture growth were evaluated with different carbon sources and nitrite as sole nitrogen source, to select a carbon source that the yeast can ferment but the microalgae cannot utilise (Fig. 4.3). *P. beijerinckii* was able to grow heterotrophically under monoculture growth conditions in the presence of glucose, fructose, sucrose, galactose and maltose, in this order of preference (Fig. 4.3). *P. beijerinckii* monoculture was unable to grow in mannose, ethanol, glycerol and acetic acid. As expected, *S. cerevisiae* was unable to grow under monoculture conditions with nitrite as nitrogen source. Of all the carbon sources that did not allow microalgal growth in single culture, only mannose induced an obligate mutualism between *S. cerevisiae* and *P. beijerinckii* in co-culture, with improved yeast (0.28×10^6 cells/ml) and microalgae (0.38×10^6 cells/ml) growth in co-culture (Fig. 4.3).

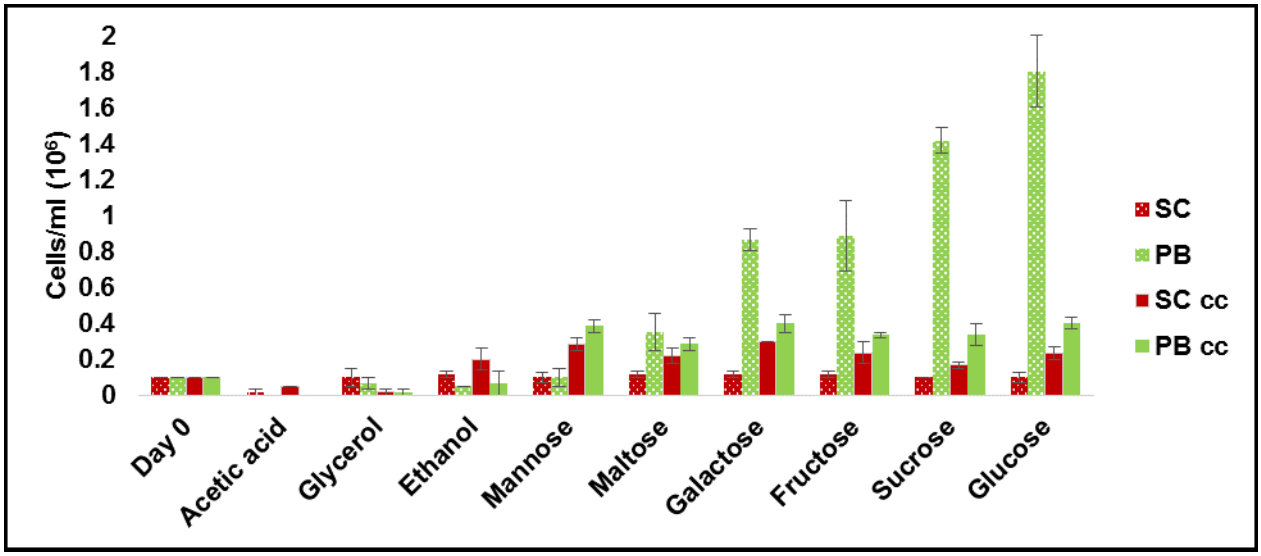

Figure 4.3 Mutualistic growth of *Parachlorella beijerinckii* (PB) and *Saccharomyces cerevisiae* (SC) under single and co-culture (cc) conditions after 7 days with different carbon sources at 25°C. The carbon source was added at a concentration of 3.6%, nitrite at 16 mM and the cells were inoculated to a cell density of 0.1 × 10^6 cells/ml. Data represent the mean ± standard error (n = 3).

4.3.3 Impact of temperature on mutualistic association

The optimal temperature for *P. beijerinckii* and *S. cerevisiae* obligate mutualism was evaluated under co-culture conditions. Yeast and microalgae monocultures were unable to survive under the engineered conditions with little to no growth observed at all temperatures (Fig. 4.4). The yeast and microalgae cell numbers reached 0.55 × 10^6 cells/ml and 0.7 × 10^6 cells/ml, respectively, at 25°C. The co-culture growth of *S. cerevisiae* and *P. beijericnkii* were higher at 30°C, with 1.35 × 10^6 cells/ml for both yeast and microalgae (Fig. 4.4A and Fig. 4.4B). There was a significant difference between the co-culture growth of *P. beijerinckii* and *S. cerevisiae* at the different temperatures (25°C, 30°C and 37°C) after 14 days (p < 0.05). The mutualism did not form at 37°C, with no significant growth observed (Fig. 4.4C). Co-culture growth for *P. beijerinckii* and *S. cerevisiae* was optimal at 30°C for the tested temperature range, with the cells still growing exponentially after 14 days.

4.3.4 Impact of pH on mutualistic association

The optimal pH for the engineered *P. beijerinckii* and *S. cerevisiae* mutualism was evaluated under co-culture conditions. *P. beijerinckii* and *S. cerevisiae* were able to form mutualistic associations at different pH levels, including pH 6, 7 and 8 (Fig. 4.5). Optimal co-culture growth associations at different pH levels, including pH 6, 7 and 8 (Fig. 4.5). Optimal co-culture growth was observed at

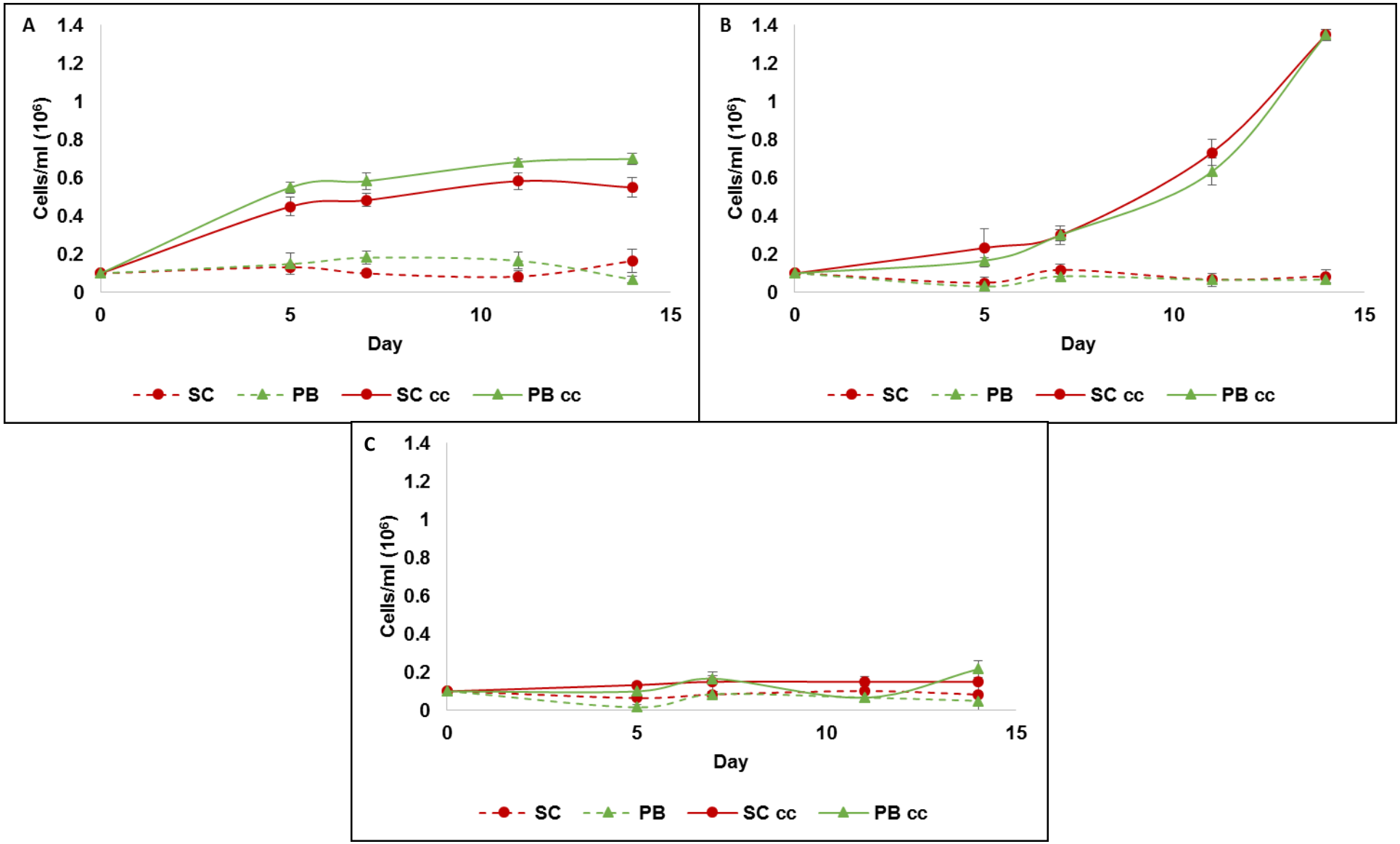

Figure 4.4 Mutualistic growth of *Parachlorella beijerinckii* (PB) and *Saccharomyces cerevisiae* (SC) under single and co-culture (cc) conditions over 14 days grown from an initial inoculum of ~0.1 × 106 cells/ml for each species at temperatures of (A) 25°C, (B) 30°C and (C) 37°C. Data represent the mean ± standard error (*n*=3).

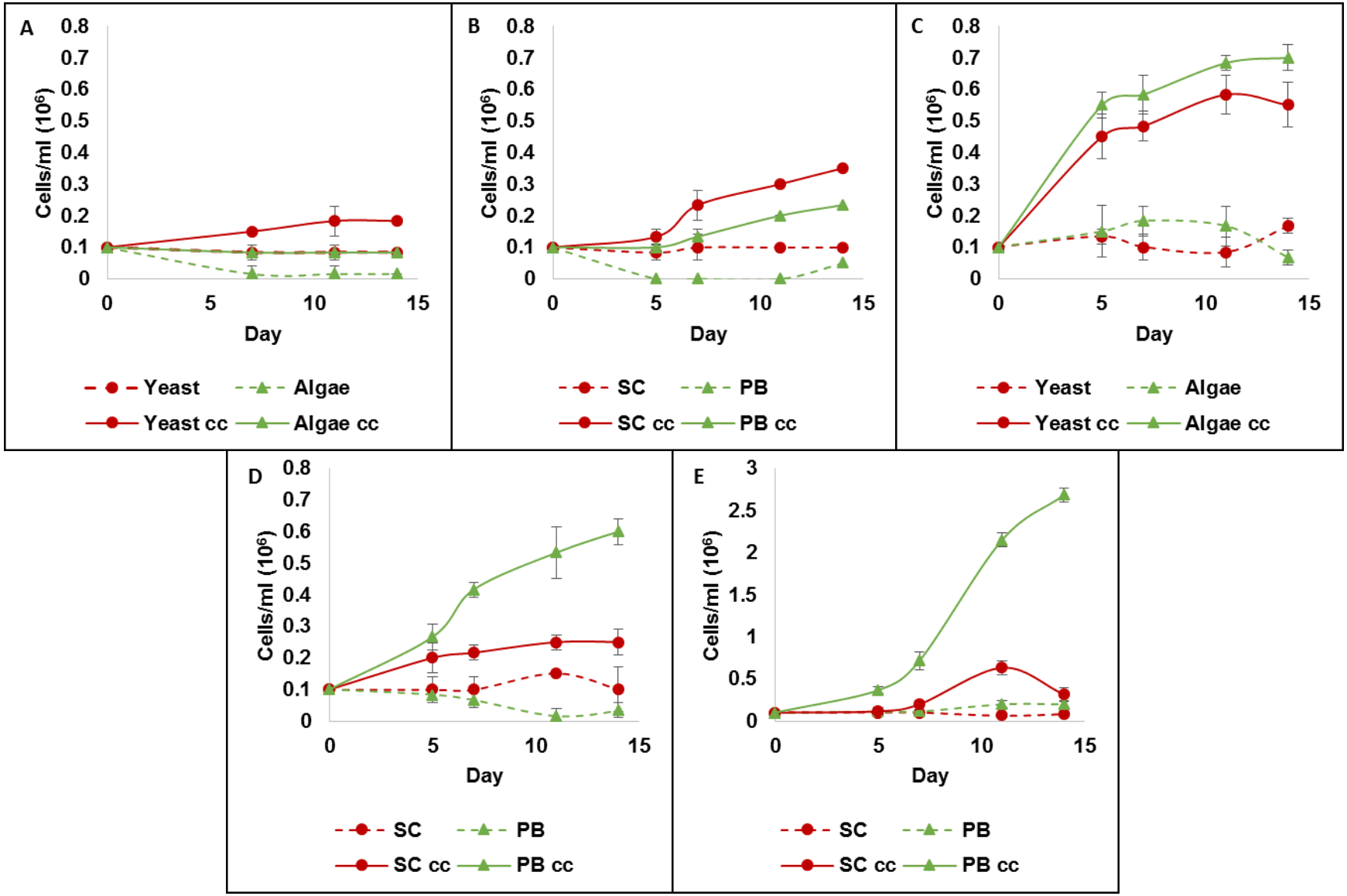

Figure 4.5 Mutualistic growth of *Parachlorella beijerinckii* (PB) and *Saccharomyces cerevisiae* (SC) at 5 different pH levels: (A) pH 5, (B) pH 6, (C) pH 7, (D) pH 8 and (E) pH 9. The growth temperature was kept constant at 25°C with no agitation under continuous light. Cells were inoculated to a cell density of 0.1 X 10^6 cells/ml. Data represent the mean ± standard error (*n*=3).

pH 7, with *S. cerevisiae* at a cell count of 0.55 × 10^6 cells/ml after 14 days, and *P. beijerinckii* at 0.70 × 10^6 cells/ml (Fig. 4.5C). At pH 6 and 8, *P. beijerinckii* and *S. cerevisiae* displayed mutualistic growth under co-culture conditions, even though cell numbers were lower compared to pH 7 (Figs. 4.5B and 4.5D). Higher algal cell numbers were observed at pH 9 (2.15 × 10^6 cells/ml), with lower yeast growth (0.63 × 10^6 cells/ml) after 11 days (Fig. 4.5E); and lower microalgae growth (0.23 × 10^6 cells/ml) with higher yeast (0.35× 10^6 cells/ml) cell numbers were observed at pH 6 after 14 days (Fig. 4.5B). *P. beijerinckii* increased more than ~3 fold at pH 9 compared to pH 7 (Fig. 4.5E). There was no mutualism observed at pH 5, however, there was a small increase in yeast growth (Fig. 4.5A).

4.3.5 Non-obligatory growth conditions in TAP medium

To evaluate if these engineered associations provide a growth advantage to one or both species when grown as co-cultures, semi-selective growth conditions were designed. The first condition provided the microalgae with acetic acid as an additional carbon source, but the yeast is still reliant on the conversion of nitrite to ammonia, by the microalgae. Under these semi-selective conditions, *P. beijerinckii* and *S. cerevisiae* showed improved growth when in co-culture, with a ~ 5 fold increase in growth for *P. beijerinckii* and ~ 40 fold increase for *S. cerevisiae* compared to monoculture growth (Fig. 4.6A). There was a statistically significant difference between co- and monoculture growth from 5 days onwards ($p < 0.05$). Furthermore, compared to the selective condition in section 4.3.4 (Fig. 4.5C), there was a ~ 10 fold increase in growth for *P. beijerinckii* and a ~ 9 fold increase for *S. cerevisiae* when grown in co-culture in semi-selective conditions after 14 days (Fig. 4.6A).

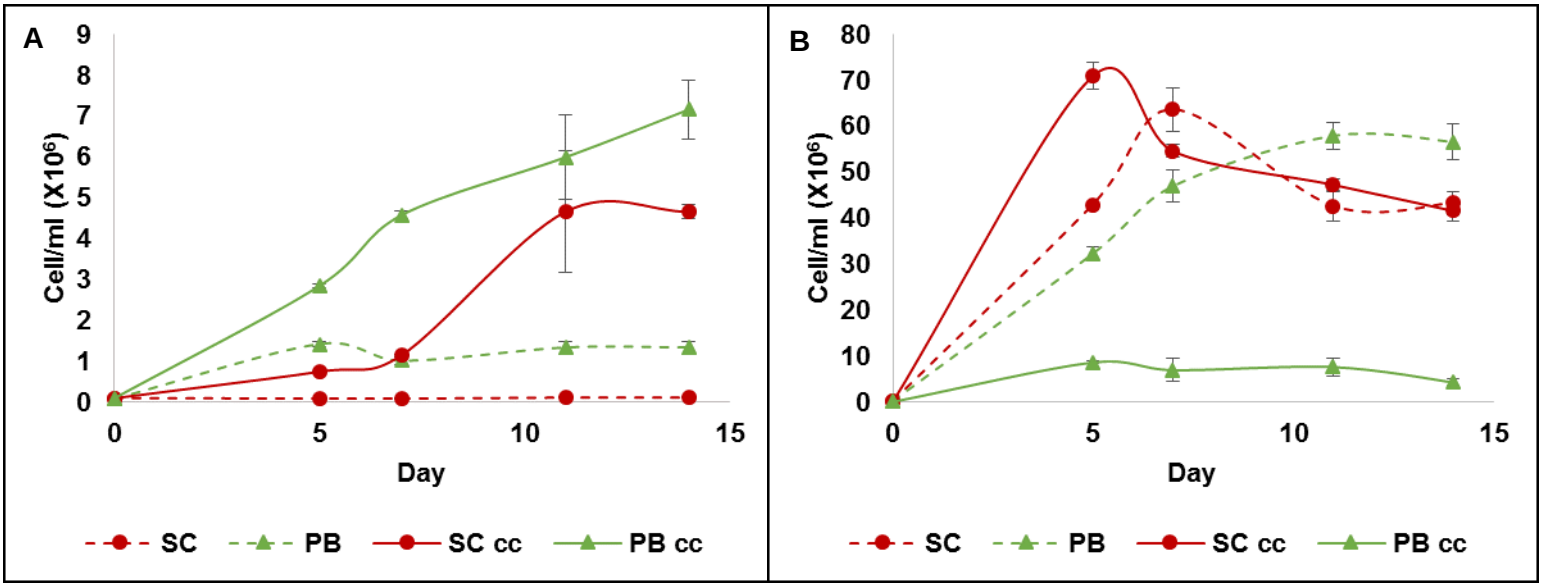

Figure 4.6 Non-obligate mutualistic growth patterns of *S. cerevisiae* (SC) and *P. beijerinckii* (PB) under single and co-culture (cc) growth conditions with acetic acid as the carbon source and either nitrite (A) (semi-selective) or ammonium (B) (non-selective) as the nitrogen source in TAP medium. Data represent the mean ± standard error (*n*=3).

Non-selective growth conditions in which the microalgae was provided with acetic acid as a source of carbon and ammonium (NH_4Cl) as a source of nitrogen for the yeast, was developed to allow the microorganisms to proliferate independently. *P. beijerinckii* was out-competed by *S. cerevisiae* in a non-selective environment with 41.7 × 10^6 cells/ml yeast and 4.3 × 10^6 cells/ml microalgae after 14 days (Fig. 4.6B). *P. beijerinckii* had improved growth under single culture conditions compared to co-cultures. Although there is no difference in mono- and co-culture growth of *S. cerevisiae* after 14 days, improved yeast biomass (71 ×10^6 cells/ml) was observed in co-culture after 5 days compared to yeast mono-culture (43 × 10^6 cells/ml). However, it is clear that there was no growth advantage for *P. beijerinckii* when grown in co-culture.

4.3.6 Obligate mutualistic growth in 1L bioreactor

The engineered obligate mutualisms between *S. cerevisiae* and *P. beijerinckii* was cultured in the batch bioreactor system, but little to no growth was observed for both yeast and microalgae (data not shown). The microalgal pre-culture was investigated and bacterial contamination was observed. Consequently, an additional obligate mutualism between *S. cerevisiae* and *C. sorokiniana,* which was developed using the same strategy as described above, was selected for further study due to time constraints. *C. sorokiniana* was isolated from the same winery wastewater as *P. beijerinckii* and small-scale growth conditions were previously optimised by R.K. Naidoo (Appendix B, Fig. 1). *S. cerevisiae* and *C. sorokiniana* co-cultures were performed in TAP medium (pH 8) as this was the optimal growth condition for this pairing, with a cell count of 5.63 × 10^6 cells/ml for *C. sorokiniana* and 6.97 × 10^6 cells/ml for *S. cerevisiae* after 11 days (R.K. Naidoo, unpublished data). When up-scaled to the bioreactor, there was a significant difference between the growth of *C. sorokiniana* and *S. cerevisiae* in co-culture, with a higher cell count observed for *C. sorokiniana* (17.6 × 10^6 cells/ml) compared to *S. cerevisiae* (7.2 × 10^6 cells/ml) after 168 h (Figure 4.7A). Variation in cell numbers and dry weight was observed in the three biological repeats for both yeast and microalgae (Figure 4.7B), but generally the same trends were observed. All biological repeats entered exponential phase after 60 h (Fig. 4.7). The combined dry weight was ~2.125 mg/ml for all biological repeats after 168 h (Fig. 4.7A).

Mannose and nitrite was consumed by *S. cerevisiae* and *P. beijerinckii,* respectively. Mannose concentrations decreased from ~ 34 g/l to ~ 11.6 g/l within 168 h, while nitrite decreased from an initail concentration of 0.18 g/l to 0.05 g/l. The production of organic acids, glycerol and ethanol were measured using HPLC analysis (Table 4.1). There was no citric acid, tartaric acid, malic acid and acetic acid produced above the limit of quantification (LOQ). Production of succinic acid, glycerol and ethanol were observed above the LOQ in all biological repeats. Succinic acid was present with ~ 0.121 g/l after 168 h and glycerol concentration was above LOQ in stationary phase after 132 h in all biological repeats. There was an increase in ethanol concentration observed over the LOQ after 72 h. Variation was observed between biological

repeats, but succinic acid and glycerol production were detected at the same time points. However, ethanol production was different between all biological repeats. The change in dissolved oxygen and pH were measured throughout the experiment and differences between biological repeats were observed (Appendix B, Fig. 2 and Fig. 3). However, dissolved oxygen decreased with the increase in yeast cell numbers after 60 h and the pH decreased from pH 8 to ~6 after 168 h in all biological repeats.

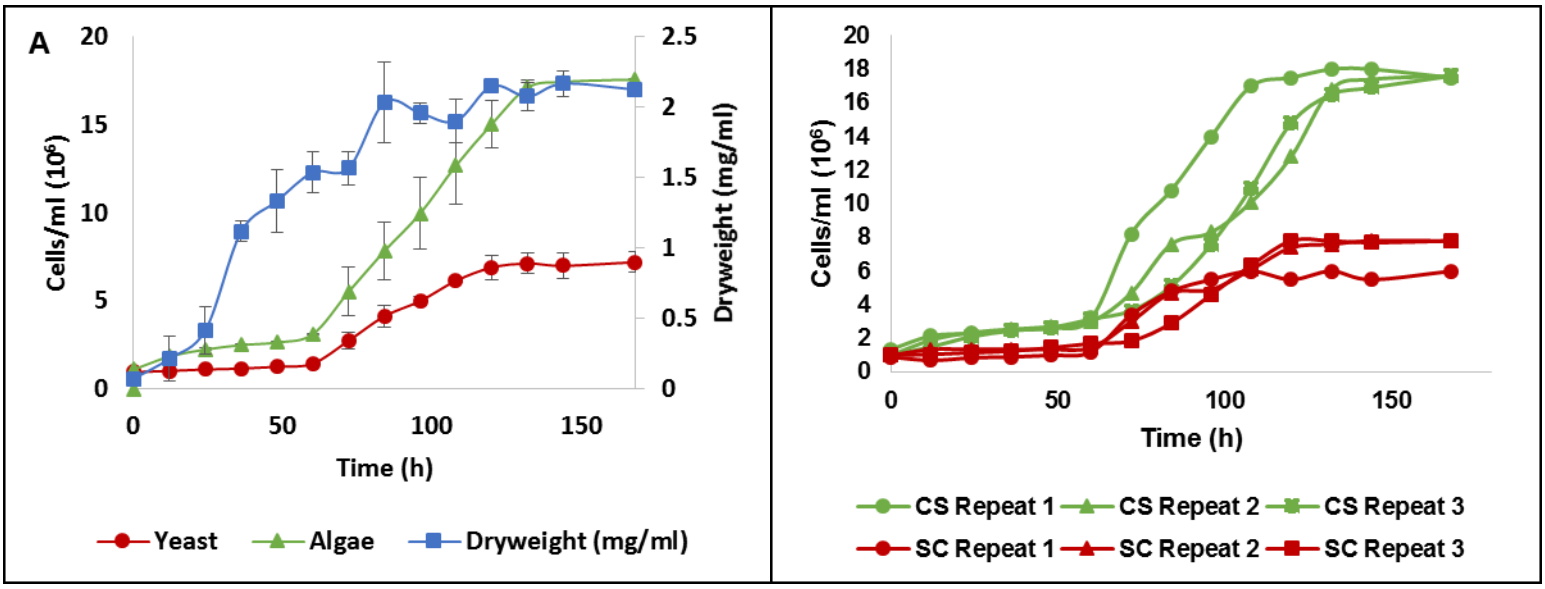

Figure 4.7 *S. cerevisiae* (SC) and *C. sorokiniana* (CS) co-culture cell growth (cells/ml) and combined dry weight (mg/ml) (A) in pH 8 TAP medium, 3.6% mannose and 16mM nitrite in 1 L bioreactor. Data represent the mean ± standard error (*n* = 3). (B) Cell counts of *S. cerevisiae* and *C. sorokiniana* in 1 L bioreactor but data represents 3 biological repeats.

Table 4.1 Mannose and nitrite consumption and succinic acid, glycerol and ethanol production detected above limit of quantification (LOQ) in *S. cerevisiae* and *C. sorokiniana* co-culture in 1 L bioreactor. Data represents mean ± standard error of mean.

Time	Mannose*	Nitrite*	Succinic acid*	Glycerol*	Ethanol*
0	33.77 ± 2.54	0.180 ± 0.02	0	0	0
72	22.87 ± 1.30	0.097 ± 0.024	0	0	0.736 ± 0.387
96	21.86 ± 0.490	0.078 ± 0.022	0	0	1.349 ± 0.439
132	18.23 ± 0.833	0.063 ± 0.021	0	0.545 ± 0.121	2.896 ± 0.222
168	11.62 ± 0.858	0.051 ± 0.021	0.121 ± 0.008	1.009 ± 0.206	5.234 ± 0.980

*Concentrations are given as g/l

4.4 Discussion

4.4.1 Carbon and Nitrogen sources for obligate mutualistic growth

To establish this synthetic obligate mutualism between *S. cerevisiae* and *P. beijerinckii*, it was important to identify a carbon source which could be easily fermented by the yeast, but not utilised by the heterotrophic microalgae; and to identify a nitrogen source which the yeast

cannot use but the microalgae can metabolise to a usable form. In this study, *S. cerevisiae* was unable to use nitrite as a sole nitrogen source (Siverio, 2002) and mannose was the only carbon source which led to the development of an obligate mutualism between *P. beijerinckii* and *S. cerevisiae*, based on the reciprocal exchange of carbon and nitrogen (Hom & Murray, 2014). Thus, mannose and nitrite was used as the carbon and nitrogen sources in further experiments to assess obligate mutualistic growth in differing environmental conditions.

4.4.2 Temperature and pH optimisation of the mutualistic association

Temperature and pH plays a significant role in mutualistic associations, as different organisms have different growth requirements. *P. beijerinckii* can proliferate between 25°C and 37°C, with optimal temperature at 30°C (chapter 3, Figure 3.2A). Similarly, *S. cerevisiae* can grow between 3°C and 45°C with optimal growth for some strains at 32°C (Salvadó *et al.,* 2011). The temperature preference for both *P. beijerinckii* and *S. cerevisiae* is ~ 30°C, supporting the optimal growth temperature of the synthetic mutualism. Furthermore, *S. cerevisiae* is an acidophile (prefers between pH 4 and 6) (Narendranath & Power, 2005) and *P. beijerinckii* a neutrophile (between pH 6 and 9, optimal at 7) (chapter 3, Fig. 3.2B). The engineered mutualism between *P. beijerinckii* and *S. cerevisiae* was most stable at pH 7, as both organisms were able to proliferate at a neutral pH. The pH preference of both *P. beijerinckii* and *S. cerevisiae* influenced the number of yeast and microalgae cells in the synthetic mutualistic association. We observed that the yeast dominates the mutualism at lower pH, whereas the microalgae dominate at higher pH. This was demonstrated by the mutualism at pH 6, where *S. cerevisiae* had a growth advantage with higher cell number compared to *P. beijerinckii* and vice versa at pH 8. At pH 5, *P. beijerinckii* was unable to grow and consequently couldn't metabolise NO_2^- to NH_3^+ to allow growth of the yeast. The variation in co-culture growth at the different temperatures and broad pH specifity illustrates how simple environmental changes can influence mutualistic associations.

4.4.3 Non-obligatory growth conditions in TAP medium

To evaluate if these engineered associations provide a growth advantage to one or both species when grown in co-culture, semi-selective growth conditions were investigated. In this study, *P. beijerinckii* proliferation was improved by the presence of mannose-fermenting, CO_2-generating budding yeast; while *S. cerevisiae* growth was improved by the presence of the photosynthetic, ammonia-generating microalgae. A similar result was observed by Hom and Murray (2014), when *S. cerevisiae* and the alga *C. reinhardtii* was cultured under semi-selective growth conditions, with an additional carbon source. It was observed that the faster growing species had to be obligately dependent on nutrients produced by the slower growing partner, for a stable semi-selective metabolic mutualism (Hom & Murray, 2014).

Furthermore, Hom and Murray (2014) used ammonium chloride to allow non-selective growth of *S. cerevisiae* and *C. reinhardtii*. This study showed that *S. cerevisiae,* with a ~4 h doubling time, out-proliferated *C. reinhardtii*, with ≥12 h doubling time and drove the microalga to near extinction (Hom & Murray, 2014). A similar observation was made when *P. beijerinckii* and *S. cerevisiae* was co-cultured under non-selective conditions. However, the improved yeast growth after 5 days indicated that the presence of the microalgae improved the growth of *S. cerevisiae* in a non-selective environment. A similar result was observed in SWW (Chapter 3, Fig. 3.5). The growth experiments conducted in this study demonstrate how a change in nutrients (carbon and nitrogen) can influence the formation of synthetic co-culture systems, driving the organisms towards mutually beneficial interactions or non-beneficial interactions. These results suggest that even though non-obligatory co-culture conditions can lead to the dominance of one species, it can also lead to an increase in growth performance of the dominant organism.

4.4.4 Obligate mutualistic growth in 1L bioreactor

The optimised obligate mutualism was up-scaled to a 1 L bioreactor system, to allow continuous sampling without the disruption of the mutualism, to control and monitor different parameters (temperature, pH, agitation, dissolved oxygen) and to decrease the risk of contamination. A true mutualism was observed between *C. sorokiniana* and *S. cerevisiae* in the bioreactor, with the reciprocal exchange of carbon and nitrogen. Zheng *et al.* (2013) reported that *C. sorokiniana* is able to grow well between pH 6 and 8 under monoculture conditions and *S. cerevisiae* is an acidophilic microorganism, as previously discussed (Narendranath & Power, 2005). The pH of the system was thus more favourable towards *C. sorokiniana,* explaining the high algal cell numbers compared to yeast cell numbers. *S. cerevisiae* was still present in sufficient cell numbers to support algal growth. The difference in light intensity between small-scale (~2500 lux) and up-scale (~11 000 lux) also played a pivotal role in the improvement of microalgal growth (Wahidin *et al.,* 2013; Xu *et al.,* 2016).

Sufficient mannose was consumed to support the growth of *S. cerevisiae;* and *C. sorokiniana* indirectly. Excess mannose after experimentation suggested that carbon was not the limiting factor for growth; and a similar trend was observed for nitrite consumption. However, the tris-acetate phosphate (TAP) medium used in this study contains different salts and caused interference in the testing of nitrite concentrations. The Nitrite/Nitrate Assay Kit (colorimetric), used for detection of nitric oxide metabolites, is very sensitive and could not determine nitrite concentrations accurately (Smith, 2016). We suggest that a nitrite probe should be used in future studies, for a more accurate representation of nitrite utilisation.

In yeast metabolism, *S. cerevisiae* is able to breakdown mannose into fructose-6-phosphate, which can be utilised during glycolysis (Lobo & Maitra, 1977). During the breakdown of mannose, carbon atoms become available for biosynthetic reactions (growth and reproduction)

and the rest are by-products of these reactions, such as carbon dioxide, ethanol, glycerol and succinic acid (Pretorius, 2000). Succinic acid is produced during the tricarboxylic acid cycle and fermenting yeasts can produce succinic acid during the exponential and stationary phase, supporting the findings in this study (Arikawa *et al.,* 1999; Heerde & Radler, 1978). Microalgae are able to produce oxygen through photosynthesis (Willey *et al.,* 2011) and yeast utilise oxygen for growth (Haukeli & Lie, 1976), explaining the decrease in dissolved oxygen during the exponential growth of *S. cerevisiae.* In return, oxidative stress is reduced, as the latter inhibits the growth and biomass production of microalgae (Li *et al.,* 2017; Raso *et al.,* 2012, Ugwu *et al.,* 2007). Additionally, acetic acid and other acids produced by the yeast may have contributed to the decrease of pH, even under the LOQ. These acids, especially acetic acid, can also serve as additional carbon source for the microalgae (Juneja *et al.,* 2013; Perez-Garcia *et al.,* 2011).

Three biological replicate experiments were performed with the bioreactor system, but differences in (1) growth, (2) consumption of mannose and nitrite, (3) production of organic acids, glycerol and ethanol, (4) dissolved oxygen and (5) pH were observed between replicates. The difference in yeast and microalgae growth patterns had a direct impact on all parameters. For example, the variation between biological repeats for succinic acid, glycerol and ethanol could be attributed to differences in yeast and microalgal growth patterns for each replicate experiment. Biological systems are inherently variable environments and in a co-culture system, this variation may be amplified as compared to single culture systems. Although there were differences in the absolute values for each biological repeat, similar trends were observed between repeats.

In this study, *S. cerevisiae* and *P. beijerinckii* were engineered to form obligate mutualistic associations, based on the reciprocal exchange of carbon and nitrogen. This study illustrates how tailored growth conditions can initiate mutualistic associations between unrelated organisms with complementary metabolic capabilities. We can confirm that these engineered mutualistic organisms interact with one another by cross-feeding carbon and nitrogen but we have no knowledge of other interactions that may occur. However, we have created a system which could keep these organisms in prolonged ecological associations and these systems allow us to monitor and understand these interactions better. The growth dynamics and nutrient (mannose and nitrite) utilisation of these mutualistic associations can be used to design co-evolutionary studies in continuous culture systems to generate superior strains that have improved capabilities. These synthetic systems could have implications for wastewater treatment and the construction of functional ecosystems which are more efficient can be coupled to the production of valuable by-products by the microalgae and yeast.

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4.7 Appendix B

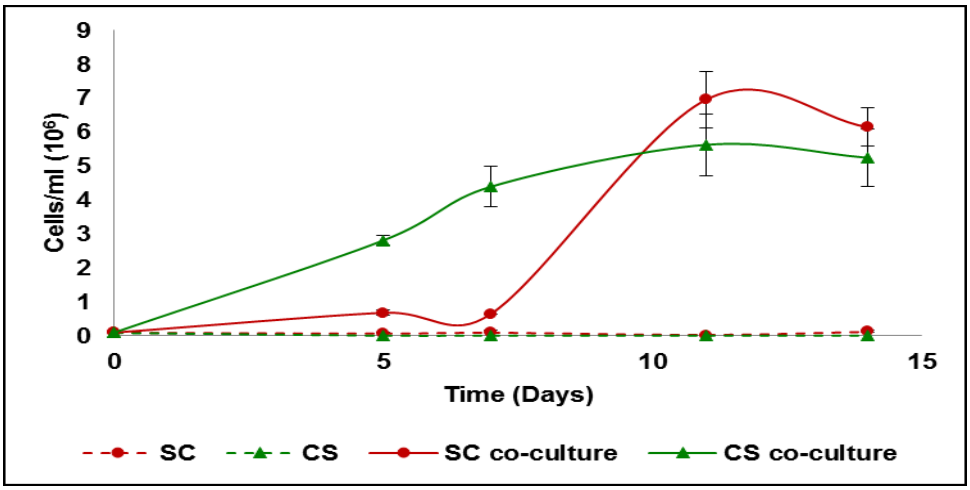

Figure 1 Mutualistic growth of *Chlorella sorokiniana* (CS) and *Saccharomyces cerevisiae* (SC) under single and co-culture conditions in pH 8 TAP medium. The growth temperature was kept constant at 25°C with no agitation under continuous light. Cells were inoculated to a cell density of 0.1 X 10^6 cells/ml. Data represent the mean ± standard error (*n*=3).

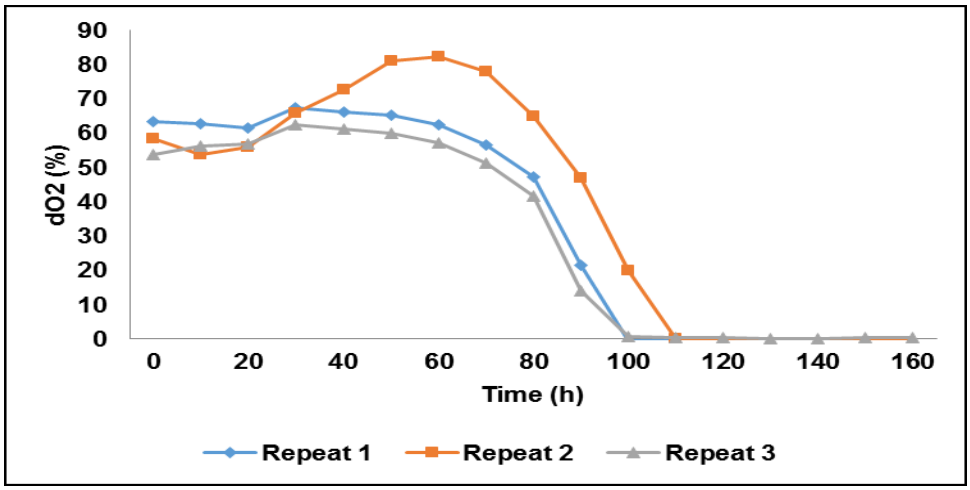

Figure 2 The change in dissolved oxygen during *P. beijerinckii* and *S. cerevisiae* co-culture in 1 L bioreactor. Data represents 3 biological repeats.

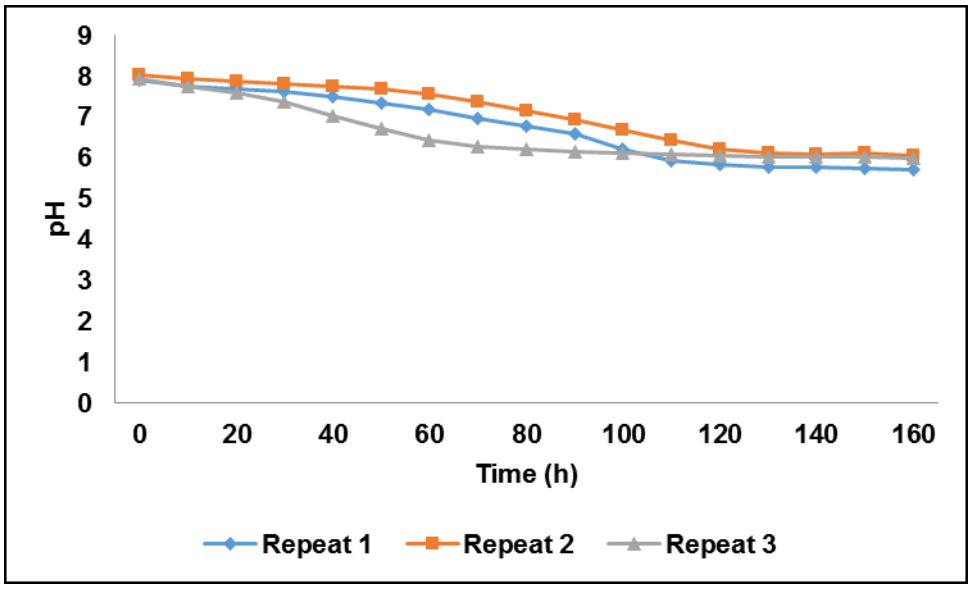

Figure 3 The change in pH during *P. beijerinckii* and *S. cerevisiae* co-culture in 1 L bioreactor. Data represents 3 biological repeats.

Chapter 5

General discussion and conclusions

Chapter 5 - General discussion and conclusions

5.1 Concluding remarks

In this study, it was shown that the microalgae *Parachlorella beijerinckii* and the yeast *Saccharomyces cerevisiae,* isolated from natural winery wastewater, had bioremediation potential in synthetic and raw winery wastewater. Both microorganisms were able to decrease the chemical oxygen demand, however not to levels within the requirements stipulated in Section 39 of the South African National Water Act (Act No. 36 of 1998). *P. beijerinckii* monocultures were however able to increase the pH within these requirements. In co-culture, *S. cerevisiae* out competed *P. beijerinckii* and yeast growth was improved by the presence of the microalgae. However, the increased yeast growth had no impact on the bioremediation potential of the co-culture system. The nutrient balance in the co-culture system favoured the growth of one organism over the other, leading to a non-optimal co-culture system. To overcome this, we suggested an environment where both organisms work optimally together, to possibly create a cumulative bioremediation effect. Thus, we developed an engineered mutualism between *S. cerevisiae* and *P. beijerinckii*, as multi-species systems with complementary metabolic capabilities has previously proven to enhance productivity (Kazamia *et al.,* 2014; Li *et al.,* 2017). In this study, an obligatory mutualism was established between yeast and microalgae, with the reciprocal exchange of carbon and nitrogen and compared to non-selective conditions, as both organisms derived benefit from the association resulting in a true mutualism.

This engineered system allows us to evolve microalgae and yeast co-culture systems to increase dependency among the partners when co-evolved for a significant amount of generations. This could result in fitter, more robust stains that are more resistant to the variable nature of winery wastewater. The bioreactor protocol, growth dynamics and nutrient utilisation laid the foundation for future co-evolutionary studies because this system provides a constant environment to keep strong selection pressure on the co-culture system. The information generated in this study, can be used to design a continuous culture system to evolve mutualistic association to generate strains with improved traits. Additionally, these systems can be used to understand the mechanisms involved in the process of co-evolution, and what impact it might have on yeast and microalgae populations over a relatively short time. Different evolutionary studies including yeast and microalgae have proven to generate improved strains for biotechnological use (Helliwell *et al.,* 2015; Tilloy *et al.,* 2014; Zhou *et al.*, 2017). The co-evolved strains will not only broaden our understanding of co-evolution, but also how these microorganisms can adapt to change in response to environmentally variable conditions.

Such artificial microbial ecosystems may be the first step in developing a multi-species approach to winery wastewater treatment. Yeast and microalgae co-cultures holds great promise for winery wastewater treatment because of their heterotrophic organic removal, photoautotrophic nutrient utilisation while producing biomass and increasing pH (Lee *et al.,* 2017). We suggest designing a 'green' process which is self-sustaining by using wastewater as a low cost nutrient source, and developing an integrated treatment process with bioremediation and enhance productivity. We propose that in the long run these co-culture systems might serve to overcome the limitations associated with single culture system and might improve biotechnological processes by creating cost-effective integrated winery wastewater treatment systems.

Future research should focus on identifying a condition where both yeast and microalgae monocultures are able to proliferate, to identify whether co-culture conditions provide increased stability in different environmental (temperature and pH) conditions. Currently, temperature and pH experiments were only performed in conditions of obligate mutualism. Furthermore, there is a need to understand the mechanisms that are involved in establishing these mutualistic associations on a molecular level. System-wide approaches using tools such as transcriptomics, proteomics and metabolomics, would allow investigation of the mechanisms involved. In this study, the reciprocal exchange of carbon and nitrogen is applied but it is unclear whether the establishment of the obligate mutualism is solely because of this nutrient exchange. Therefore, future studies should focus on identifying whether other metabolic or regulatory factors are involved. The bioremediation potential of *P. beijerinckii* and *S. cerevisiae* were investigated by means of chemical oxygen demand removal, but these organisms might be able to remove other pollutants present in winery wastewater. Therefore, future studies should focus on characterising pollutants within winery wastewater and their individual and combined effects on yeast and microalgae growth. Furthermore, the present study provides information of nutrient utilisation and biomass production, which could be used to mathematically model the data to predict the behaviour of organisms in microbial consortia in response to changing environmental conditions. Additionally, co-evolutionary studies with yeast and microalgae mutualisms could produce mutualistic associations independent of the selective conditions and potentially generate strains with improved traits such as enhanced biomass production and improved detoxification abilities. Yeast and microalgae mutualisms can be encapsulated in alginate beads to serve as a novel tool in winery wastewater treatments. This study provides the first attempt to engineer a synthetic mutualistic association between *P. beijerinckii* and *S. cerevisiae* under different environmental conditions; and also provides the first attempt in using a 1 L bioreactor system for an engineered yeast and microalga mutualism.

5.2 References

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