UTILISATION OF NOVEL MARINE YEAST AND SEAWATER-BASED MEDIA FOR THE PRODUCTION OF BIOETHANOL

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Abstract

Bioethanol can be considered as one of the best replacements for petrol because of its positive impact on environment and many other advantages. Currently, bioethanol accounts for around 2% of the global road fuels and this is projected to increase to around 10% within the next few decades. Bioethanol is a very high water consuming product, with an average global water footprint of 2855 L H_2O/L ethanol. A growing worldwide demand for bioethanol has raised concerns over the use of freshwater resources. This PhD project aimed to establish a marine fermentation strategy for bioethanol production where seawater replaced freshwater for the preparation of fermentation media in conjunction with use of marine yeast as a biological catalyst, and potentially utilising a marine biomass (i.e. seaweed) as a carbon source substrate.

Yeasts that are present in marine environments have evolved to survive hostile environments. Therefore, yeast isolated from marine environments could have potentially interesting characteristics for industrial applications. Current methods for marine yeast isolation suffer several limitations as they usually encourage the growth of filamentous fungi and produce low number of yeast isolates. A new method was developed in this study, which included: a 3-cycle enrichment step followed by an isolation step and a confirmation step. By applying this method on 14 marine samples (collected in the UK, Egypt and the USA), a large number of marine yeast isolates was obtained without any bacterial or filamentous fungal contamination. Amongst these isolates, 116 marine yeast isolates were evaluated for their capacity for utilising monomeric fermentable sugars (glucose, xylose, mannitol and galactose) using a seawater-based media, this assessment of sugar utilisation was performed in a phenotypic microarray assay. Following determination of sugar utilisation, 21 isolates that representing the best sugar utilisers were further characterised using YT-plates (BioLog) and identified by DNA sequencing using ITS and D1/D2 primers. The identified isolates belonged to 8 species: *Saccharomyces cerevisiae* (5 strains), *Candida tropicalis* (4 strains), *Candida viswanathii* (4 strains), *Wickerhamomyces anomalus* (3 strains), *Candida glabrata* (2 strain), *Pichia kudriavzevii* (1 strain), *Issatchenkia orientalis* (1 strain) and *Candida albicans* (1 strain).

Out of the 21 identified yeasts, 9 strains representing different species were screened for ethanol production using YPD media containing 6% (w/v) glucose and prepared by freshwater (ROW) and seawater (SW). Results revealed that 3 marine *S. cerevisiae* strains (S65, S71, and S118) had the best fermentation rates when using SW media. These yeasts were therefore taken forwarded for investigation into their growth performance under high concentrations of glucose

and seawater salts (the components of synthetic seawater). Results determined that these marine strains were significantly more tolerant when compared with a reference terrestrial *S. cerevisiae* strain. Fermentation experiments using YPD media containing 6% glucose were prepared using synthetic seawater (SSW), 2x SSW and different sodium chloride (NaCl) concentrations (3, 6 and 9%) and results confirmed that the marine strain S65 was a highly halotolerant and osmotolerant yeast with high fermentative capacity.

In a batch fermentation using 15 L bioreactors, strain S65 produced 73 g/L ethanol from 165 g/L of glucose within 20 h of fermentation, with ethanol productivity of approximately 4 g/L/h. In a batch fermentation, using sugarcane molasses (about 14% sugar) prepared in SW, strain S65 produced 52.23 g/L of ethanol after 48 h.

According to literature, determination of sugars in samples which contain chloride salts was inaccurate when applying an existing HPLC method because chloride ions and sugars (especially glucose and sucrose) elute at a similar retention time. In this study seawater - which contains high concentration of NaCl (about 2.8%) - was used for preparing the fermentation media and therefore, developing a new method for sugar determination was necessary. Subsequently, an accurate and reliable HPLC method for the simultaneous quantification of chloride salts, sugars, organic acids and alcohols was developed. The method was validated for the accurate quantification of NaCl and successfully applied on fermentation samples as well as variety of food samples from retail market.

The results obtained in this study highlighted the potential for using marine yeasts and the suitability of seawater-based media for the production of bioethanol. They also provide a new strategy for increasing the efficiency of bioethanol production at the industrial level with positive impact on food and freshwater scarcity issues.

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List of Abbreviations

%	Percentage
°C	Degrees centigrade
AFDC	Alternative Fuels Data Center of The USA Energy
CFU	Colony Forming Unit
CV	Coefficient of Variation (Precision)
DEV%	Percentage Deviation (accuracy)
DNA	Deoxyribo-nucleic acid
DOE	The U.S. Department of Energy
EDTA	Ethylene-diamine-tetra-acetic acid
EIA	The U.S. Energy Information Administration
EISA	Independence and Security Act of The USA Energy
EtOH	Ethanol
FAO	The Food and Agriculture Organization of the United Nations
FP	Flame Photometer
FW	Freshwater
g/L/h	Volumetric ethanol productivity
GHG	Greenhouse Gas
GM	Gene Modified
GJ	Giga Joule
h	Hour
HPLC	High Performance Liquid Chromatography
IC	Ion Chromatography
ID	Identification
IEA	International Energy Agency
IL	Ionic Liquid
IPCC	The Inter-Governmental Panel on Climate Change
IRENA	International Renewable Energy Agency
ITS	Internal Transcribed Spacers
IWMI	International Water Management Institute
L	Litre
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
MEA medium	Malt Extract Agar medium
MFVs	Mini Fermentation Vessels
mg	Milligram
Min.	Minute
mL	Millilitre

MWS	Molasses Working Solution
N/A	Not Applicable
NCYC	The National Collection of Yeast Cultures
Non-OECD	All countries which are not members of the OECD
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PM	Phenotypic Microarray
REN21	The Renewable Energy Policy Network for the 21st Century
RFA	Renewable Fuels Association
ROW	Reverse Osmosis Water
rpm	Revolutions Per Minute
RSI	Redox signal intensity
R_t	Retention Time
S/N	Signal to-noise ratio
SD	Standard Deviation
SDA medium	Sabouraud's Dextrose Agar medium
sp.	Species
SSW	Synthetic Seawater
SW	Seawater
UNEP	United Nations Environment Programme
USDA	United States Department of Agriculture
v/v	volume per volume
VW	Virtual Water
w/v	weight per volume
WEF	World Economic Forum
WF	Water Footprint
WS	Water Scarcity
YM medium	Yeast Malt medium
YPD medium	Yeast-extract Peptone Dextrose medium
YVC	Yeast Viable Count
µg	Microgram
µL	Microliter

CHAPTER 1:

1. General Introduction

1.1 Global demand for energy and the impact on the environment

An ever-growing population and shifting demographics have led to a continuous increase in global demand for energy. Total world energy consumption is predicted to rise from 549 quadrillion British thermal units (Btu) in 2012 to 815 quadrillion Btu in 2040 with an increase of 48% (EIA, 2016). The rise in energy consumption is mostly caused by non-OECD nations (Figure 1.1). As a consequence, world CO_2 emissions related to energy will rise from 32.2 billion metric tons in 2012 to around 43.2 billion metric tons in 2040 with an increase of 34% over the projection period (Figure 1.2) (EIA, 2016). The Inter-Governmental Panel on Climate Change (IPCC) reported that, among greenhouse gases, CO_2 accounts for nearly 55% of the global warming, and therefore, reduction of CO_2 emissions from fossil fuels is an urgent issue in order to reduce the global warming trend (Yeh and Bai, 1999).

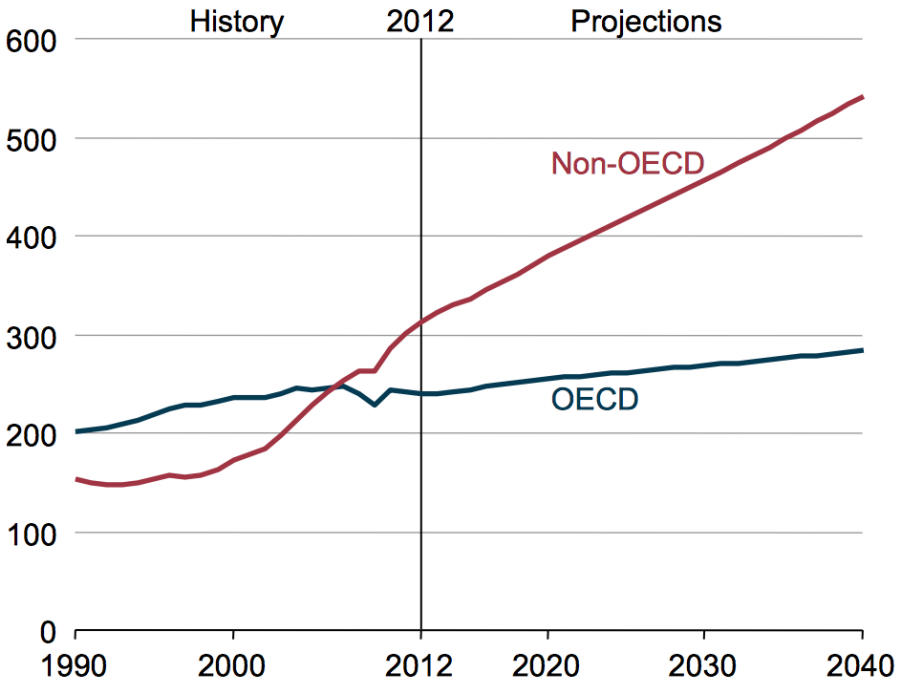


Figure 1.1: World energy consumption by region, 1990–2040 (quadrillion Btu)

OECD: Organization for Economic Cooperation and Development. The organization started on 14 December 1960 with 20 countries (18 EU countries plus the United States and Canada). Members od OECD now is 35 countries.
Non-OECD: all countries which are not members of the OECD.
Source: U.S. Energy Information Administration: International Energy Outlook 2016, Report Number: DOE/EIA-0484(2016)

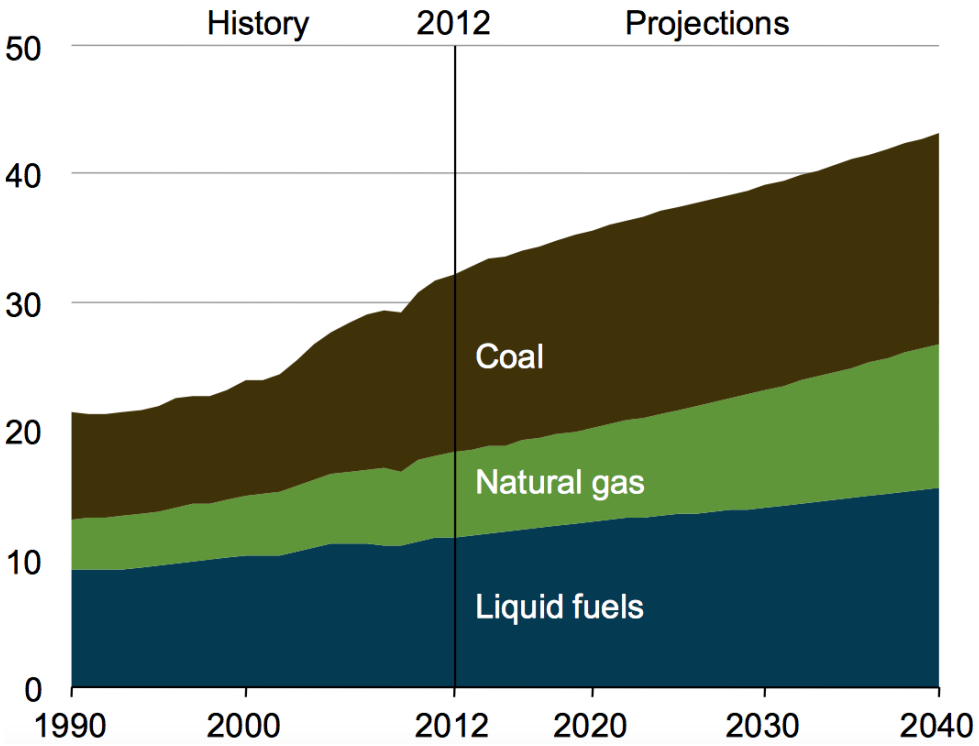


Figure 1.2: World energy-related carbon dioxide emissions by fuel type, 1990-2040 (billion metric tons)

Source: U.S. Energy Information Administration: International Energy Outlook 2016, Report Number: DOE/EIA-0484(2016)

Price rises of petrol along with the environmental issues has led to the search for alternative sustainable sources of energy. Bioethanol has been considered to be one of the best fuel alternatives because it is a liquid fuel and has similar characteristics to petrol. Hence, governments in many countries have implemented policies to increase the percentage of bioethanol in their fuel mixes. These policies have promoted a three-fold increase in bioethanol production over the past decade (2000 - 2010) (REN21, 2014).

1.2 Bioethanol, a promising renewable biofuel

Ethanol, also called ethyl alcohol, (C_2H_5OH) is a clear colourless liquid, biodegradable and low in toxicity. Bioethanol, produced from biological materials such as sugarcane or maize, has been used for few decades as an alternative fuel to replace petrol. Ethanol burns producing CO_2 and water. Ethanol is a high octane fuel and therefore, it has replaced lead as a safe octane enhancer in petrol. Bioethanol blended petrol can also be oxygenated, so it burns more completely and reduces waste gas emissions. Bioethanol blended petrol is widely sold in the United States with the most common blend being E10, which contains 10% ethanol and 90%

petrol. Vehicle engines do not require modifications to use E10 and vehicle warranties are also unaffected. Flexible fuel vehicles only can use up to 85% ethanol and 15% petrol blends (E85) (Božiková and Hlaváč, 2013, Kim and Lee, 2015).

The main advantage of using bioethanol is that it can substantially reduce greenhouse gas (GHG) emissions in the transport sector by 70-90% when compared with gasoline, with only minor changes to vehicle and the existing infrastructure of fuel distribution (RFA, 2015). The other advantages of using bioethanol as a renewable fuel include: the increase in energy supply security, the reduction of depletion risks and the improvement of resource diversification (de Vries et al., 2007).

Biofuels - especially bioethanol - are expected to be used as an alternative fuel in the coming decades, along with the increasing awareness on global warming, climate change and depletion of the fossil resources (Zaky et al., 2014b, Domínguez de María, 2013). Worldwide bioethanol production in 2015 exceeded 97 billion litres/year (RFA, 2015, AFDC, 2015), contributing approximately 2.4% to the world's fuel consumption for transportation. Due to the increasing demand on energy with an ever growing world population and the limited supply of fossil fuels, the contribution of bioethanol is expected to increase further over the next decades. The USA Energy Independence and Security Act (EISA) of 2007 mandates annual production of biofuel in the USA to be 136 billion litres by 2022, most of which is likely to be bioethanol (IEA, 2012). The International Energy Agency (IEA) estimated that bioethanol could contribute for up to 9.3% of the world transportation fuels by 2030 and up to 27% by 2050 (IRENA, 2013).

1.3 Challenges facing bioethanol production

The increasing demand for bioethanol has led to a large area of arable land being dedicated to the growth of biofuel targeted crops instead of food and feed. This has resulted in rising food prices and has thus limited the further development of bioethanol industry. The Food and Agriculture Organization (FAO) of the United Nations reported that biofuel production has increased food insecurity (FAO, 2008). The World Bank considered biofuel production as a major factor in food price increases. Although it is arguable, one report estimated that about 75% of the increase in food prices, in the period between 2002 and 2008, was related to the production of biofuels (Mitchell, 2008).

Besides land usage issue, significant amounts of water are being used for the production of bioethanol, which is much more than other renewable energy systems, such as solar energy and

wind energy (King and Webber, 2008, Fingerman et al., 2010). With the increasing concern on water shortage, the consumption of freshwater could be a potential barrier for the expansion of bioethanol production. Gerbens-Leenes and Hoekstra (2012) concluded that freshwater resources in the globe are limited and allocation of water for bio-ethanol production on a large scale will be at the cost of water allocation for food and other usages. Therefore, the water usage issue could soon be included in the food and land usage debate.

1.4 Objectives of the current study

This PhD project was conducted to investigate a new strategy for bioethanol production aiming to reduce freshwater consumption during the production of bioethanol. The project focused on the use of seawater as a replacement for freshwater and the use of novel marine yeast as a replacement for the conventional industrial yeast strains for the production of bioethanol. This strategy was validated by studying the conversion of molasses, as a carbohydrate substrate for bioethanol production. Molasses is one of the preferred substrate for fermentation because it is a relatively cheap industrial by-product and it contains high concentration of fermentable sugars (about 50% (w/v) of sucrose, glucose and fructose). Molasses is produced in a large quantity, as by-product from the sugar industry, and it is currently being used for bioethanol production in many countries such as Egypt.

The objectives of this project are listed below:

a) To develop an efficient and non-laborious method for marine yeast isolation (chapter 4)
b) To investigate a new evaluation strategy for screening the newly isolated marine yeasts for potential application in bioethanol production using seawater-based media (chapter 5)
c) To establish and validate a convenient HPLC method for sugar quantification in samples containing high amounts of chloride salts (chapter 6)
d) To explore the potential of using seawater-based media in fermentation industry using marine yeasts (for bioethanol production as an example) (chapter 7)
e) To evaluate the new fermentation approach (using SW-based media & marine yeast) using molasses as substrate in SW-based media (chapter 7)

1.5 Thesis outline

The thesis is presented as the following:

Chapter 1. Introduction: this chapter introduces the global demand for energy and its impact on the environment. It also points out the objectives of this project, and provides an overview of this thesis.

Chapter 2. Literature review: this chapter provides a detailed review on marine yeasts and their applications in industrial fermentation. Also, it covers a survey on the water consumption related to bioethanol production.

Chapter 3. Material and methods: this chapter describes all Materials and Methods applied in this project including the procedures that have been developed in this study.

Chapter 4. Development of an efficient method for the isolation of marine yeasts strains: in this chapter, a new method for marine yeast isolation was developed. The new method was applied on 14 marine samples (collected in the UK, Egypt and the USA). A large number of marine yeast isolates were obtained without any bacterial or fungal contamination. 116 marine isolates, representing different sample sources, different isolation media, and different cell and colony morphology, were selected for further study.

Chapter 5. Evaluation and identification of novel marine yeasts: The new marine isolates obtained in the previous chapter were evaluated for their ability of utilising monomeric fermentable sugars (glucose, xylose, mannitol and galactose) in a seawater-based media using a phenotypic microarray assay. 21 isolates, representing the best utilisers for each sugar, were further characterised using YT-plates and identified by DNA sequencing using ITS and D1D2 primers.

Chapter 6. The simultaneous determination of Cl^- salts, sugars and fermentation metabolites using HPLC: In this chapter, an accurate and reliable HPLC method for the simultaneous quantification of chloride, sugars, organic acids and alcohols was developed and validated. The method was also tested for its suitability of analysing various food samples as a demonstration for its potential to be used in a wider field.

Chapter 7. Bioethanol production using marine yeast and seawater-based media: in this chapter, nine representative marine yeasts were screened for ethanol production using YPD media prepared using freshwater (ROW) and seawater (SW). The best three strains were taken

forwarded to investigate their growth performance under high concentrations of glucose and seawater salts. Two strains were then assessed for their fermentation capabilities under high concentrations of glucose and salts. The best strain was then used for the production of bioethanol from seawater-based media using 15 L bioreactors.

Chapter 8. Conclusion and future work: This chapter summarises the results obtained in this study and proposes plans for future work.

CHAPTER 2:

2. Literature Review

Over the last century, terrestrial yeasts have been widely used in various industries, such as baking, brewing, wine, bioethanol and pharmaceutical protein production. However, little attention has been given to yeasts isolated from the marine environment. Recent research has showed that marine yeasts have several unique and promising features over the terrestrial yeasts, e.g. higher osmotic tolerance, higher special chemical productivity and production of industrial enzymes (Kutty and Philip, 2008, Zhenming et al., 2006, Zaky et al., 2014). These features indicate that marine yeasts have great potential to be applied in various fermentation industries including the production of bioethanol.

Traditionally bioethanol production has been carried out using media made with distilled or tap water. Seawater, which accounts for 97% of the world's water, could be a promising alternative in coastal cities, especially in the Middle East where freshwater is increasingly precious. Seawater contains a spectrum of minerals which when used in a fermentation may avoid the essential addition of commercial nutrients which are currently required for fermentations. In addition, the use of seawater in fermentations could potentially improve the overall economics of the process by both reducing freshwater intake and producing freshwater through distillations in the bio-refinery. Therefore, the development of seawater based bioethanol strategy can have an impact on overcoming freshwater and energy crises.

The special features of marine yeasts, particularly being highly osmotolerant and halotolerant yeast, make them an ideal choice for bioethanol production especially when seawater is used instead of freshwater for preparing the fermentation medium. However, use of seawater rather than freshwater for fermentation system is still a relatively unexplored area of research, therefore, the aim of this study was to establish a new strategy for bioethanol production using seawater instead of freshwater and novel marine yeast strains instead of the conventional terrestrial yeast for the production of bioethanol. Part of the literature review in this chapter formed a review paper published in FEMS Yeast Research, (Zaky et al., 2014).

2.1 Marine yeast

Coastal environments have been identified as being amongst the most diverse and rich microbial environments (Danovaro et al., 2009). Fungi have been reported to have an active

role in the utilisation of available nutrients in marine environments (Gao and Liu, 2010), however, their suitability for fermentations under osmotic stress inducing conditions has not been extensively investigated previously. Yeasts isolated from a marine environment have been shown to produce commercially relevant extracellular enzymes (Chi et al., 2009) indicating that the diversity in environments alongside the shifting panorama in terms of available nutrients makes the commercial use of marine derived yeast an interesting biosystem to explore.

Yeasts are eukaryotic unicellular microorganisms belonging to the fungal kingdom, currently there are around 1,500 described species, which represent about 1% of the total fungal species existing in nature (Kurtzman and Fell, 2006, Kurtzman and Piškur, 2006). Yeasts, such as *Saccharomyces cerevisiae*, have been successfully exploited in various industries such as ethanol production, bakery, wine making and brewing. In order to satisfy the growing demand for a fermentation-based economy, researchers have been trying to isolate novel yeast strains with promising properties, such as, high fermentation capability, high stress tolerance and producing novel products (Zaky et al., 2014, Cadete et al., 2014). The vast majority of existing yeasts have been isolated from terrestrial resources such as sugar refineries, breweries, wineries, bakeries, beet and cane molasses, as well as from various fruits and vegetables.

2.1.1 Marine Yeast Isolation Methods

Over the years, microbiologists have developed several methods for marine yeast isolation. These methods differ in their sampling, sample preparation, medium composition and strain maintenance. This variation is required to cope with the diverse marine habitats, the target properties required in the isolates (e.g. the ability of utilizing xylose) and the likely cell density of the sample (Zaky et al., 2014).

Surface seawater samples can be collected using simple plastic or glass bottles (1-5 litres). Bottles should have screw caps for easy handling as well as for preventing contamination and leaks. For aseptic reason, bottles should be opened under water and washed thoroughly using the seawater 3-5 times before filling with sample. Sterilized plastic bags, jars and vials can also be employed in collecting surface samples. Surface seawater samples are suitable for isolating aerobic and facultative anaerobic yeasts (Fell, 2001, Zaky et al., 2014). A "near shore" location is more suitable for sampling yeasts that are capable of carbohydrate fermentation. Samples of 250 mL are generally enough when they are taken near shore, whereas samples from the open ocean should be at least 1 litre as a lower microorganism density is expected. 50 mL of sediment

samples are generally considered adequate. Experiment design and replication should be taken into account for the required sample volume (Fell, 2001).

More advanced devices have been designed and used to collect deep sea samples (water and sediments). The first water sampler that was able to maintain *in situ* hydrostatic pressure was reported by Jannasch et al. (1973). Generally, Niskin, Van Dorn and Kemmerer samplers are the most common apparatuses that have been used for deep sea sampling, as shown in Figure 2.1. Niskin samplers can be used singly or in series or in a rosette of up to 12 samplers per rack. Van Dorn is a horizontal sampler while Kemmerer is a vertical sampler so that it could fit narrow areas. These devices can collect samples from as deep as 6000 meters. However, those devices do not maintain *in situ* hydrostatic pressure. These samplers usually consist of cylindrical tube(s) with a stopper at each end (between 1 and 121 tubes per frame). These stoppers could be controlled remotely from the surface (Dorschel, 2011, Singh, 2011). Research submarines can also be used to collect deep-sea samples. These devices are larger in size, very complicated and massively expensive. On the other hand, research submarines allow the collection of large amounts of samples, good observation of the sample environment and instant work on the samples as it can also carry all the laboratory equipment needed (Singh, 2011).

Sample preparation for marine yeast isolation is dependent on two main factors (a) the desired characteristics of the isolates and (b) the expected number of yeast cells per mL. Samples collected from the open sea usually contain around 10 or fewer cells per mL (Kutty and Philip, 2008, Fell, 2001). Therefore, filtration of 5 litres seawater is required followed by the re-suspension of the cells remaining on the filter in 15 mL of the same seawater filtrate. In contrast samples collected from the high organic matter containing surface near shore can contain thousands of yeast cells per mL (Kutty and Philip, 2008). So, filtration of 100 mL is usually enough. Alternatively, samples could be subject to an enrichment step for a couple of days before isolation to select desirable strains with specific characteristics. For extraction from solid samples, such as seaweed, sea sand, dead marine plant and animal material, a known weight of solid particles can be transferred into a broth medium for enrichment, or be placed directly on an agar plate. Serial dilution is required prior to isolation if more than 300 isolates are expected per mL (Fell, 2001).

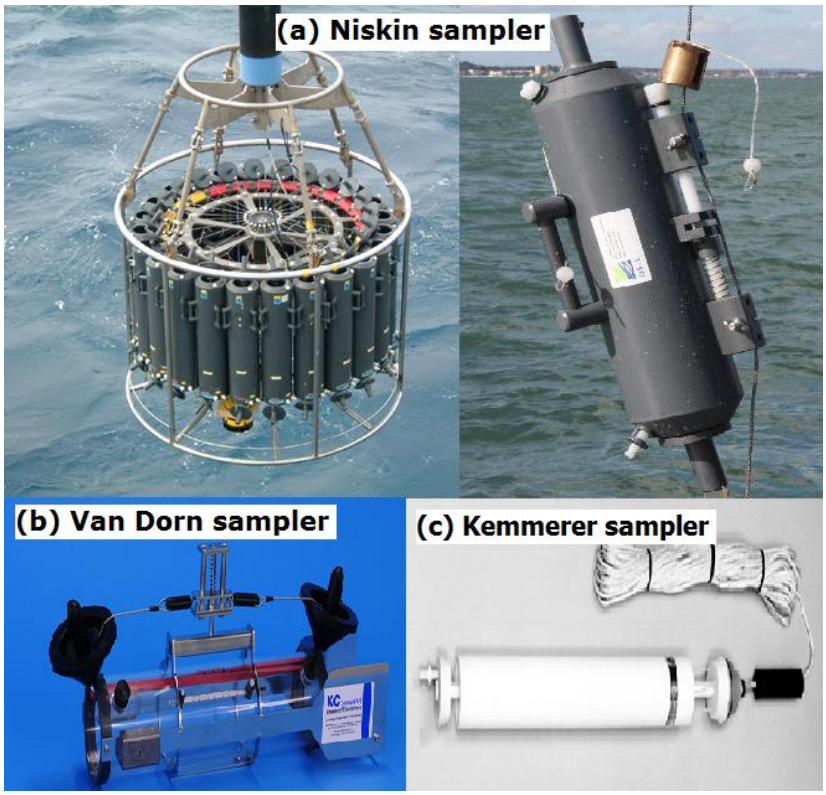


Figure 2.1: The commonly used samplers for deep seawater sampling

(a) Niskin sampler (http://www.godac.jamstec.go.jp/darwin/instrument/mirai/e)
(b) Van Dorn sampler (http://www.kc-denmark.dk/products/water-sampler/van-dorn-water-sampler.aspx)
(c) Kemmerer sampler (http://www.rickly.com/as/kemmerer.htm)

Several different medium recipes have been used for the isolation of marine yeasts. Although both natural and artificial seawater have been used for preparing medium, natural seawater is preferable as it is closer to the natural environment that the yeasts inhabit. A mixture of broad-spectrum antibiotics has been used in isolation media, which have been shown to be more effective than single antibiotic in inhibiting the growth of bacteria and were less harmful to yeast cells (Beuchat, 1979, Thomson, 1984). Different inhibitors including; rose bengal (Jarvis, 1973, King et al., 1979), dichloran (Jarvis, 1973) and propionate (Bowen and Beech, 1967) have been added to the media in order to inhibit the growth of moulds (Kutty, 2009). Usually the same medium as used for isolation is also used for maintenance but without added antibiotics. Plates should be incubated at a temperature similar to the environment where the samples were collected. The optimum temperature for marine yeasts varies (Watson, 1987). For taxonomic tests, yeasts are usually incubated at 25ºC (Buhagiar and Barnett, 1971). The following list gives some media and incubation conditions suggested by researchers for the isolation of marine yeasts:

1. Wickerham's yeast malt (YM) medium (Wickerham, 1951): This medium is widely used for marine yeast isolation. It contains (w/v) 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2% agar. All the chemicals are prepared in seawater at a salinity equivalent to the sample site. 200 mg/L of chloramphenicol was added into the medium prior to autoclaving and the final pH was adjusted to 7.0. Alternatively, an antibiotic mixture of penicillin G and streptomycin sulphate (each at 150-500 mg/L) can be added to the autoclaved, cooled (below 45°C) medium.
2. Chi et al., (2007) modified a liquid YPD medium (2.0% glucose, 2.0% poly-peptone and 1.0% yeast extract, w/v) by preparing the medium with natural seawater instead of fresh water. 0.05% (w/v) chloramphenicol was also added. This medium should be prepared immediately after sampling and cultivated at the natural temperature for five days.
3. Wang et al., (2007) prepared a seawater nutrient agar medium consisting of (w/v) 2.0% glucose, 2.0% peptone, 1.0% yeast extract, 2.0% agar. Components were dissolved in half-strength artificial seawater, and the pH of the medium was then adjusted to 4.5. The agar plates were incubated for 5 days at 20°C. The composition of the artificial seawater was (per litre); NaCl, 20 g; KCl, 0.35 g; $MgCl_2 \bullet 6H_2O$, 5.4 g; $MgSO_4 \bullet 7H_2O$, 2.7 g; $CaCl_2 \bullet 2H_2O$, 0.5 g.
4. Masuda et al., (2008) used an YPD agar, containing (w/v) 2.0% glucose, 2.0% peptone, 1.0% yeast extract and 2.0% agar, supplemented with 3.0% NaCl and 100 ng/µl of chloramphenicol at pH 6.0. The plates were incubated at 25°C for 5-7 days.
5. Hernandez-Saavedra et al., (1995) used an isolation medium consists of (w/v) 2.0% glucose, 1.0% peptone, 5.0% yeast extract and 2.0% agar prepared in filtered seawater. The pH was adjusted to 4.5 with 0.1 N HCI.
6. Loureiro et al., (2005) used a modified Sabouraud's Dextrose Agar (SDA) medium (0.5% peptic digest of animal tissue, 0.5% pancreatic digest of casein, 4.0% dextrose and 1.5% agar, w/v). They added yeast extract and chloramphenicol to the medium and incubated the plates at 28 ±1ºC. The concentrations of yeast extract and chloramphenicol were not reported.
7. Dinesh et al., (2011) used SDA medium prepared in 50% seawater. The plates were incubated at 35ºC for 48 hours.
8. Nagahama et al., (1999) prepared an YM agar medium from Difco, which was dissolved in artificial seawater (3% NaCl, 0.07% KCl, 1.08% $MgCl_2$, 0.54% $MgSO_4$, 0.1% $CaCl_2$, w/v). This was used for isolating yeast from a cold marine habitat. Medium was supplemented

with 0.01% (w/v) chloramphenicol and 0.002% (w/v) streptomycin. The plates were incubated at a low temperature (5-10ºC) for 2 weeks and then at 20ºC for 1 month.

9. Sarlin and Philip (2011) suggested a Malt Extract Agar (MEA) medium containing (w/v) 2.0% malt extract, 0.5% mycological peptone and 2.0% agar. It was suspended in around 50% diluted seawater at pH 6.0.
10. Kodama (1999) described a medium consisting of (w/v): 20% sucrose, 3% polypeptone, 0.3% yeast extract, chloramphenicol 100 ppm, 1.5% agar. The medium was prepared using filtered seawater, and the pH was adjusted to 5.6.
11. Khambhaty et al., (2013) suggested a method combining filtration followed by enrichment before isolation. The enrichment medium was GYP broth consisting of (w/v): 1% glucose, 0.5% peptone, 0.5% yeast extract and 2.5% sodium chloride. Enrichment was carried out for 24 h at 30°C in a shaking incubator. A loopful of the suspension was spread on GYP plates consisting of (w/v): 1% glucose, 0.5% peptone, 0.5% yeast extract, 2.5% sodium chloride and 2.5% agar. The plates were incubated at 30°C for 2-3 days.

The above mentioned incubation conditions including temperature were suggested by the researchers and could be changed according to the experiment requirement. Growth on plates should be observed daily. Parts of any suspected yeast colonies should be picked up and transferred onto a microscope slide for inspection. Streak plate technique should be applied on confirmed yeast colonies using YPD seawater agar medium without antibiotics. Streak plate should be repeated to ensure the purity of the isolate. Colonies of interest can be transferred into a slant culture tube for further study.

2.2 Use of Marine Yeast for bioethanol production

Over the last few decades, halo-tolerant yeasts have been investigated as promising alternative candidates for bioethanol production. Urano et al. (2001) isolated several marine yeasts from various aquatic environments. Most of these isolates belonged to two genera *Candida* and *Debaryomyces*. These isolates were preliminary tested for their fermentation capabilities by observing gas production in a media containing sodium chloride. But the production of ethanol was not reported. Limtong et al., (1998) hybridized *Saccharomyces cerevisiae* M30, a high ethanol producing strain, with *Zygosaccharomyces rouxii* TISTR1750, a halo-tolerant strain, using polyethylene glycol induced protoplast fusion. Compared with the parental strains, one of the derived strains (Fusant RM11) exhibited higher ethanol producing capacity in terms of both ethanol concentration and yield, in glucose broth media containing 1.5, 3, 5 or 7% sodium

chloride. By using the medium containing (w/v) 18% glucose and 3% sodium chloride, the Fusant RM11 showed maximal ethanol production of 68.5 g/L while the parental strains, *S. cerevisiae* M30 and *Z. rouxii* TISTR1750, produced 65.0 g/L and 63.6 g/L bioethanol, respectively. The fermentations were carried out at 30ºC for 60 hours.

Kathiresan et al. (2011) isolated 10 marine yeast strains from mangrove sediments on the southeast coast of India. These isolated strains were *Candida albicans, Candida tropicalis, Debaryomyces hansenii, Geotrichum sp., Pichia capsulata, Pichia fermentans, Pichia salicaria, Rhodotorula minuta, Cryptococcus dimennae* and *Yarrowia lipolytica*. They reported that *Pichia salcaria* was the best strain for ethanol production with 12.3 ± 0.8 g/L bioethanol from sawdust filtrates at 2% concentration after 120 hours of incubation. When 2% sawdust hydrolysis (hydrolyzed by dilute phosphoric acid) was used as the carbohydrate source, 26.2 ± 8.9 g/L bioethanol was produced by *Pichia salcaria.* Follow on studies, Senthilraja et al., (2011) reported that in fermentations using free cells, *Pichia salicaria* produced the highest ethanol concentration of 28.5 ± 4.32 g/L among these 10 isolates. When these yeast cells were immobilized in sodium alginate, improved ethanol production was observed in fermentations using all strains. *Candida albicans* exhibited the highest ethanol production of 47.3 ± 3.1 g/L.

Obara et al. (2012) studied bioethanol production from the hydrolysate of paper shredder scrap using a marine yeast isolated from Tokyo Bay. It was found that the marine yeast - *S. cerevisiae* (strain C-19) showed high osmotic tolerance and high ethanol production. It produced 122.5 g/L of ethanol from a medium containing 297 g/L of glucose. The maximum bioethanol concentrations for the control strains, *S. cerevisiae* NBRC 10217 and *S. cerevisiae* K-7, were 37.5 g/ L and 98.5 g/L, respectively. Moreover, the fermentation using the marine yeast C-19 reached peak ethanol production at day 3, while both control strains required 7 days to achieve their maximum bioethanol production. The high osmotic tolerance of the marine yeast strain was considered to contribute to its promising performance. As this strain belonged to *S. cerevisiae* species, it could be amenable to the existing genetic modification tools that developed based *Saccharomyces* sp. for further improvement.

Saravanakumar et al. (2013) compared bioethanol production using a marine *S. cerevisiae* strain and a terrestrial *S. cerevisiae* strain. In fermentations using the hydrolysate of sawdust as the substrate, the marine strains showed maximum ethanol production of 25.1 g/L, while the terrestrial strain produced only 13.8 g/L ethanol.

Khambhaty et al. (2013) isolated a marine yeast strain (*Candida* sp.) from Veraval, on the West coast of India. This strain was able to convert galactose, sugar cane bagasse hydrolysate as well as the hydrolysate of a red seaweed *Kappaphycus alvarezii* into bioethanol under a wide range of pH (2.0 - 11.0) conditions and in the presence of high salt concentration (2.5%-15% w/v). Sugarcane bagasse hydrolysates were prepared using H_2SO_4 and HCl, resulting in 7.17% and 7.57% reducing sugar, respectively. Around 22.8 and 18.9 g/L ethanol were obtained, equating to conversion efficiencies of 66% and 55%, respectively. In a seaweed hydrolysate containing 5.5% reducing sugar with 11.25% salt concentration, around 12.3 g/L ethanol was produced after 72 h of incubation, representing 50% conversion efficiency. When the seaweed hydrolysate was diluted by fresh water with a ratio of 3:1 or 1:1, 100% carbohydrate conversions were observed within 48 h. Moreover, approximately 21 to 24 g/L bioethanol was produced in fermentation using a GYE broth media containing 5% (w/v) galactose in the presence of 0-10% of KCl, $CaCl_2$, and NaCl.

Khambhaty et al. (2013) concluded that the presence of 2 - 13% salt benefited the growth of their isolate. Although fermentation efficiency was relatively low in a medium containing 11.25% salt, 100% fermentation efficiency could be achieved in fermentations using media containing 6.25 to 9% salt. Their isolate could also tolerate a wide range of pH from 4.0 up to 10.0 with very little growth difference. They claim that the pH and salt tolerance of the marine yeast made it a promising candidate for fermentations under different environmental conditions. Khambhaty's findings were in line with a study conducted by Gupta (1996) who reported that various species of yeasts such as *Debaryomyces*, *Rhodotorula*, *Candida*, and *Saccharomyces* could tolerate up to 16% (w/v) NaCl. In addition, yeasts that could tolerate NaCl up to 3.5 M (20.5%) have also been reported (Kutty & Philip, 2008).

Various biological materials have been investigated for the generation of bioethanol, such as wheat straw (Pensupa et al. 2013), sugarcane bagasse (Chandel et al., 2013) and corn stover (Bondesson et al., 2013). Recently, various marine biomass sources, e.g. seaweed (Khambhaty et al., 2013) and sea lettuce (Yanagisawa et al., 2011), have attracted increasing attention as a promising non-food material for bioethanol production, as they do not compete with edible crops in terms of land and fresh water resources. The hydrolysis of marine biomass could result in a hydrolysate containing salt, which would require desalting (e.g. electrodialysis) before fermentation when terrestrial yeasts are used (Mody et al., 2015). However, halophilic yeasts, especially yeasts isolated from a marine environment, would be able to directly ferment the

salty hydrolysate to bioethanol (Khambhaty et al., 2013). Therefore, the energy intensive step, desalting, could be avoided, making the whole fermentation process more economically competitive (Khambhaty et al., 2013). Table 2.1 compares the bioethanol produced using various yeast strains isolated from the marine environment and respective fermentation conditions.

The recent research has shown great potential of marine yeasts in bioethanol production, however, more investigation should be conducted to further demonstrate the benefits of using marine yeasts in bioethanol industry, especially in bioethanol fermentations using marine biomass based substrate. Subsequently more marine yeasts should be isolated to explore their potential. The isolates should be selected based on their capability for utilising and fermenting a wide range of sugars that are presented in marine biomass hydrolysate, including galactose, xylose, mannitol and fucose. Also, the isolates should have high tolerance capacity to salts and inhibitors that may be generated during the hydrolysis of marine biomass.

Table 2.1: Ethanol Production by marine yeasts

Ref.	Yeast Name	Isolation Source	Substrate	Hydrolysis method	Fermentation condition (Sugar con., Temp., Incubation time)	Ethanol Con. g/L
Khambhaty et al., 2013	*Candida sp.*	Veraval, the West coast of India	Seaweed	2.5% H_2SO_4, cooked at 100°C for 1 h.	3.77% sugar, 30°C, 48 h.	17.6
			Sugarcane Bagasse		2.28 % sugar, 30°C, 48 h	7.7*
			Galactose	N/A	5 % galactose, 30°C, 0 - 10 % of KCl, 24 h.	21–24
Saravanakumar et al.,2013	*S. cerevisiae*	Mangrove soil, southeast coast of India	Sawdust	0.8% H_3PO_4	6.84 mg/L sawdust, 30°C, 89 h	0.0024*
Obara et al., 2012	*S. cerevisiae*	Tokyo Bay, Japan	paper shredder scrap	3 % H_2SO_4 at 121 °C for 1 h then enzymatic saccharification	29.7% of glucose from paper shredder scrap, 30°C, 72 h	122.5
				Enzymatic saccharification only (cellulase for 2 days at 50°C and 150 rpm)		
					Immobilized	13
			Sawdust	NaOH 4% at 121°C for 30 min.	2% of sawdust, 28°C, 120 rpm for 72 h.	7.6
[1]Kathiresan et al., 2011 [2]Senthilraja et al., 2011	*C. albicans* *C. tropicalis* *D. hansenii* *Geotrichum sp.* *P. capsulata* *P. fermentans* *P. salicaria* *R. minuta* *C. dimennae* *Y. lipolytica*	sediments, southeast coast of India	[1]Glucose	N/A	28°C, 120 rpm for 96 h. Non immobilized	9.8 - 28.5
					28°C, 120 rpm for 96 h. Immobilized	13 - 47.3
			[2]Sawdust	NaOH 4% at 121°C for 30 min.	2% of sawdust, 28°C, 120 rpm for 72 h.	1.7 - 12.3

* No ethanol concentration was reported in the original papers. This value was estimated based on the conversation efficiencies reported by the references.

2.3 Biology of salt tolerance in yeast

Salt tolerance has been highlighted as an important yeast phenotype in industrial applications. Research has been carried exploring yeast response and tolerance to salt (Lenassi et al., 2007, Kejžar et al., 2015, Talemi et al., 2016). Yeasts vary in their tolerance to salt from those deemed sensitive to those deemed extremely halotolerant. *S. cerevisiae* has been classified as a salt sensitive or moderately halotolerant yeast (Plemenitaš et al., 2008, Silva-Graça et al., 2003). Prista et al., (2007) reported that exposure to 0.5 M NaCl inhibited *S. cerevisiae*, however, halotolerant mutants of *S. cerevisiae* have been shown to tolerate up to 2.0 M NaCl (Gaxiola et al., 1996, Prista et al., 1997). On the other hand, black yeast *Hortaea werneckii* has been reported to be an extremely halotolerant yeast which can tolerate an almost saturated NaCl solution (5.2 M). *H. werneckii* grows well in the absence of NaCl but the optimum growth condition requires the presence of NaCl at concentrations ranging from 1.0 to 3.0 M depending on the strain (Plemenitaš et al., 2008, Gunde-Cimerman et al., 2000).

Zygosaccharomyces rouxii is haploid yeast which has also been identified as a halotolerant yeast because it grows in a medium containing up to 3.0 M NaCl (Martorell et al., 2007, Radecka et al., 2015). *Pichia sorbitophila* was shown to tolerate a mineral medium containing high concentrations of NaCl (4 M) when glycerol or glucose was used as the sole carbon source (Lages and Lucas, 1995). *Debaryomyces hansenii* is another halotolerant yeast which can tolerate a medium containing up to 5.0 M NaCl. For optimal growth, *D. hansenii* requires NaCl at a concentration of 0.5 M in the propagation media (Plemenitaš et al., 2008). It has also been reported that *D. hansenii* can survive on a saturated NaCl solution (Zalar et al., 2005). Strains of *D. hansenii* have been isolated worldwide from natural hypersaline environments (Gunde-Cimerman et al., 2009).

Candida krusei, *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata* are all halotolerant yeasts which have been isolated from the hypersaline seawater of the Dead Sea. Furthermore, *H. werneckii* as well as *Phaeotheca triangularis* and *Trimmatostroma salinum* have not been isolated from any terrestrial sources. Therefore, hypersaline water has been suggested to be their natural ecological niche (Gunde-Cimerman et al., 2000).

Exposing yeast cells to a medium containing high salt concentration leads to a massive release of cellular water into the medium. This process is regulated by the difference in water activity of the cell and the environment. Osmoregulation has been defined as the cellular response that directed at restoring and maintaining important physiological cellular parameters such as turgor

pressure, cell volume and normal biological activities of the cell (Nevoigt and Stahl, 1997). These parameters are essential for the proper cellular functions, such as cell cycle progression and protein synthesis. Moreover, these cellular processes are interlinked to each other and therefore, the maintenance of these parameters within a narrow range is one of the key aspects of cellular adaptation to salt stress (Ke et al., 2013, Ariño et al., 2010). The yeast tolerance to salt stress could be explained by three mechanisms, including: i) ion homeostasis, ii) compatible solutes strategy, and iii) signalling pathways.

2.3.1 Ion homeostasis:

Monovalent ions, such as H^+, K^+ and Na^+ play multiple essential roles within yeast cells. Nevertheless, the regulation and maintenance of these ions within a restricted concentration range is vital in order to avoid toxicity (Rothstein, 1964, Mulet et al., 1999, Ke et al., 2013). Yeasts maintain suitable intracellular ion concentrations in hypersaline medium via complex homeostatic pathways. K^+ is the major cellular cation and it is actively retained intracellularly at high concentrations. K^+ concentration is the principal factor controlling the important cellular physiological parameters including; turgor, cell volume, and cytoplasmic ionic (Mulet et al., 1999). In addition, the threshold for the toxicity of other monovalent cations such as Na^+ is lower than that of K^+. Hence, the accumulation of Na^+ in the cytosol must be the prevented in order to protect sensitive enzymes i.e. phosphatases and nucleotidase of the gene *HAL2* family (Mulet et al., 1999, Murguía et al., 1996).

D. hansenii is the most studied halotolerant yeast with regard to ion homeostasis (Gunde-Cimerman et al., 2009). Previous publications on marine yeasts reported that *D. hansenii* was slightly affected when cultivated on media containing high concentrations of NaCl (Norkrans and Kylin, 1969). Later studies have revealed that *D. hansenii* grew better and accumulated more Na^+ when exposed to NaCl compared to *S. cerevisiae* and therefore, Na^+ was not toxic to *D. hansenii* (Prista et al., 1997, Neves et al., 1997, González-Hernández et al., 2005). Moreover, intracellular H^+ homeostasis was also reported to have a role in *D. hansenii* tolerance to salt. Mortensen et al. (2006) found that, the closer *D. hansenii* strain can maintain its intracellular pK (value of the potential of potassium) to its intracellular pH homeostasis level, the better the strain can manage NaCl stress (Mortensen et al., 2006).

2.3.2 Compatible solutes strategy

Most yeasts use the strategy of accumulating "compatible solutes" to control their intracellular Na^+ at a concentration below the toxic level. *S. cerevisiae* - which is a salt sensitive yeast tends to accumulate glycerol as an osmolyte, while other yeasts are known to produce and/or accumulate polyols such as arabinitol, erythritol, galactitol, mannitol, sorbitol, ribitol and xylitol, in addition to glycerol (Gunde-Cimerman et al., 2009, Hohmann, 2002).

The three model yeasts, *D. hansenii*, *S. cerevisiae* and *H. werneckii*, synthesise and accumulate glycerol as their main compatible solutes (Hohmann, 2002). Glycerol is accumulated intracellularly in *S. cerevisiae* when cells are exposed to decreased extracellular water activity. Nevoigt and Stahl (1997) suggested that increasing intracellular glycerol could be a result of the enhanced anabolism, restricted catabolism, increased retention by the plasma membrane and/or increased uptake of glycerol from the medium by the yeast cells (Nevoigt and Stahl, 1997).

Similar to *S. cerevisiae*, glycerol synthesis in *D. hansenii* is mainly stimulated by the presence of high Na^+ (Nilsson and Adler, 1990, André et al., 1991) and is partly due to the increased expression of the *GPP2* and *GPD1* genes coding for a glycerol-3-phosphatase and a glycerol-3-phosphate dehydrogenase, respectively (Thomé, 2005, Gori et al., 2005). It has been reported that, unlike *S. cerevisiae*, *D. hansenii* probably has an active process of glycerol transport mediated by a sodium–glycerol symporter (Lages et al., 1999)

Although *D. hansenii* accumulates glycerol as its main compatible solute, small amounts of arabitol, trehalose, glutamic acid and alanine have also been detected (Jovall et al., 1990). However, recent research reported that *D. hansenii* accumulated more trehalose than glycerol under moderate NaCl stress, however, this strain accumulated more glycerol than trehalose in the presence of high concentrations of salt (2.0–3.0 M salt) (González-Hernández et al., 2005). In addition, glutamate worked as an additional salt-tolerance determinant in *D. hansenii* as it was observed that the activity of NADP-glutamate dehydrogenase was increased when cells were grown in high salt media (Alba-Lois et al., 2004).

H. werneckii was also reported to accumulate a mixture of polyols such as erythritol, arabitol and mannitol as well as glycerol when grown in hypersaline media (Gunde-Cimerman et al., 2009). The amounts of these osmolytes are dependent on the salinity of the growth medium and the growth phase of the yeast culture. Although, the total amount of polyols in yeast cells is

mostly dependent on the amount of glycerol, glycerol accumulates mainly during the exponential phase and decreases steeply during the stationary phase. In contrast, the concentration of erythritol increases gradually in the yeast cells during the exponential phase and reaches its maximum level during the stationary phase. The amount of other polyols decreases during the stationary phase and thus remains at a low concentration comparing to glycerol (Gunde-Cimerman et al., 2009).

2.3.3 Signalling pathways

The ability of yeast cells to sense the changes of Na^+ concentrations in the environment is very important for cell survival. The main pathway involved in sensing and responding to these changes in *S. cerevisiae* is called the high osmolarity glycerol (HOG) signalling pathway (Gunde-Cimerman et al., 2009, Talemi et al., 2016). HOG pathway maintains cell volume and restores their turgidity and adaptation to high osmolarity conditions by involving several activities including; cell cycle arrest, enzymes activities change, glycerol channel closure and gene expression, as well as complex metabolic adaptation processes. These activities lead to both producing the osmolyte glycerol and maintaining its intracellular concentration within the yeast cell (Talemi et al., 2016).

Exposing yeast cells to high salt concentration results in reduced cell volume as water outflow to equilibrate internal and external water osmosis potential differences. *S. cerevisiae* survives in high salt concentration media by activating the HOG signalling cascade, which controls glycerol accumulation. The Hog1 kinase stimulates transcription of the genes (*GPD1, GPP2*) encoding enzymes required for glycerol production and the gene (*STL1*) encoding enzyme required glycerol import as well as activating a regulatory enzyme in glycolysis (Pfk26/27). In addition, glycerol outflow is prevented by closure of the Fps1 glycerol facilitator (Lee et al., 2013, Petelenz-Kurdziel et al., 2013). Increase of intracellular glycerol concentration increases the internal osmolarity of the yeast cells which forces water back into the cells restoring their original volume (Talemi et al., 2016).

The identification of the homologue of MAP kinase in *H. werneckii* (HwHog1) confirmed the existence of a signalling pathway similar to the HOG pathway in *S. cerevisiae* (Lenassi et al., 2007, Kejžar et al., 2015). Although HwHog1 protein shows high homology to *S. cerevisiae* Hog1, HwHog1 is fully active only at extremely high salt concentrations while Hog1 in *S. cerevisiae* is activated even at very low salt concentrations (Turk and Plemenitaš, 2002,

Kejžar et al., 2015). Kejžar et al. (2015) found that inhibiting the activity of HwHog1 kinase along with the ATP analogue BPTIP restricted the growth of *H. werneckii* at 3.0 M KCl, NaCl and sorbitol. However, the survival of *H. werneckii* at moderate KCl and NaCl concentrations was not dependent on the activity of HwHog1. Therefore, HOG pathway in *H. werneckii* is vital for its extreme osmotolerance ability but has a different regulation comparing to the other homologous pathways described in other halotolerant and mesophilic fungi.

2.4 Water consumption in bioethanol production

Bioethanol production consumes large amount of water. This water is consumed along the biofuel supply chain including substrate cultivation, bioethanol fermentation and bioethanol distribution. Figure 2.2 shows the major uses of water in agricultural production and industrial processing phases of bioethanol production.

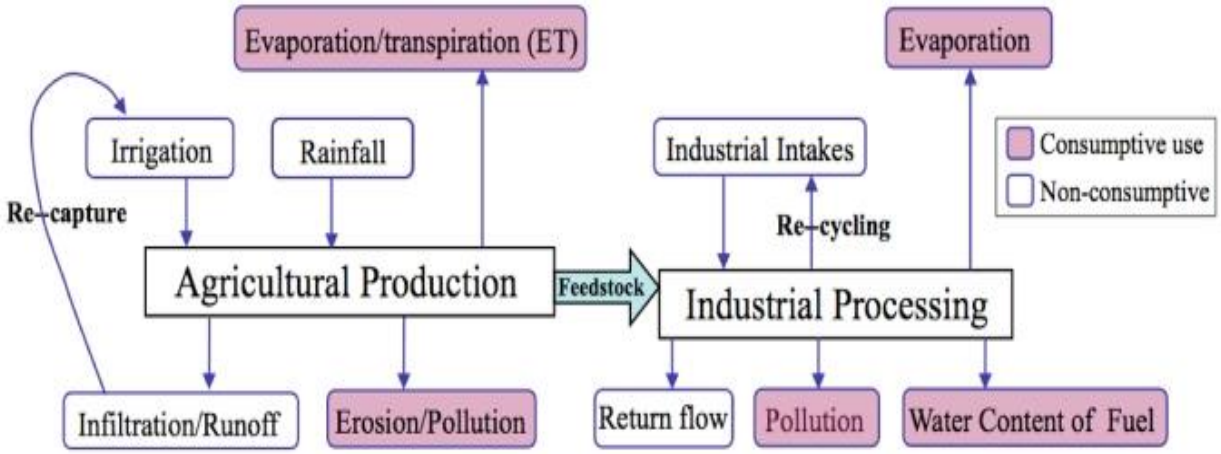


Figure 2.2: Schematic diagram of water consumption in biofuel life cycle

The figure shows the flows of water in and out of the bioenergy production system.
Source: (Fingerman et al., 2010)

A study conducted by Fingerman (2012) on water consumption for corn ethanol in California showed that over 1,000 litres of water was required to produce 1 litre of ethanol; 99% of the water was consumed during the feedstock cultivation phase (Fingerman, 2012).

The total water required for bioethanol production can be measured using the indicator virtual water (VW). The concept of VW was proposed by Allan in 1994. VW has been defined as the total volume of water required to produce a unit commodity or service (Allan, 1994). VW can be used to recognize products that have more influence on the water resource system and provide a way of water saving (Hoekstra, 2003). A relatively recent concept, Water footprint

(WF) which was initially developed by Hoekstra and Hung (2002) has become more popular in evaluating the total water consumed during bioethanol production. WF is the total volume of water needed during production processes, accounted over the whole supply chain (Yang et al., 2013, Yang et al., 2016). The concept of WF can be used to measure the direct and indirect water use of an individual, business, region or a nation through consumption of commodities and services (Hoekstra et al., 2009). Both indicators - VW and WF - are closely linked and the main difference between them is that VW is defined from the perspective of production while WF is defined from the perspective of consumption (Zhao and Chen, 2014). In other words, the term VW is mostly used in the context of interregional or international trade, whereas the term WF is generally used in the context where producers or consumers of products are concerned (Yang et al., 2013).

2.4.1 Water footprint (WF) for bioethanol

WF of bioethanol depends on many parameters including but not limited to the type of crops being used for production and the region of production. The total global weighted average WF of bioethanol ranges from 1,388 to 9,812 litre of water for each litre of ethanol produced. This is dependent on the crop being used for ethanol production. Table 2.3 shows the total weighted-global average WF for 10 crops providing ethanol, expressed as litres of water required to produce one litre of bioethanol and cubic meters of water required to produce one Giga Joule energy of ethanol (Gerbens-Leenes et al., 2009).

The region where bioethanol production takes place is also a major factor affecting the total WF of bioethanol. Table (2.4) shows the variation between different countries for the value of total WF calculated as cubic meters of water required to produce one Giga Joule energy equivalent of ethanol. France recorded the lowest WF for bioethanol with 41.8 m^3/GJ while China recorded the highest WF for bioethanol with 124.8 m^3/GJ (Rulli et al., 2016).

Both the region where the crops are cultivated and the ethanol being produced, play an important role for the total amount of water required for bioethanol production. Figure 2.3 shows the highest value, the lowest value and the weighted-average global WF values for 10 crops in terms of bioethanol production. The figure clearly shows the enormous variation in the total WF among these crops especially for sorghum which mainly affected by the unfavourable production conditions in Niger and the highly efficient production conditions in Egypt

(Gerbens-Leenes et al., 2009). It can be concluded that, using current approach of production, bioethanol is a heavy water consuming product.

Table 2.3: Total weighted global average water footprint (WF) for 10 crops providing bioethanol

Crop	Total water WF (L/L)*	Total water WF (m^3/GJ)**
Sugar beet	1,388	59
Potato	2,399	103
Sugar cane	2,516	108
Maize	2,570	110
Cassava	2,926	125
Barley	3,727	159
Rye	3,990	171
Paddy rice	4,476	191
Wheat	4,946	211
Sorghum	9,812	419

The data is the average figures for 5 countries (Brazil, Guatemala, Nicaragua, India and Indonesia)

* (Litres of water/litre of bioethanol); ** (cubic meters of water/Giga Joule of ethanol)

Source: (Gerbens-Leenes et al., 2009)

Table 2.4: Total water footprint of bioethanol in the major consuming countries

Country	Total water WF (m^3/GJ)
France	41.8
Colombia	58.8
Brazil	59.7
Germany	61.2
Poland	64.1
Italy	73.5
USA	76.1
Sweden	79.3
UK	89.3
Spain	94.3
Canada	98.9
Netherlands	118.1
India	122
China	124.8

Source: (Rulli et al., 2016)

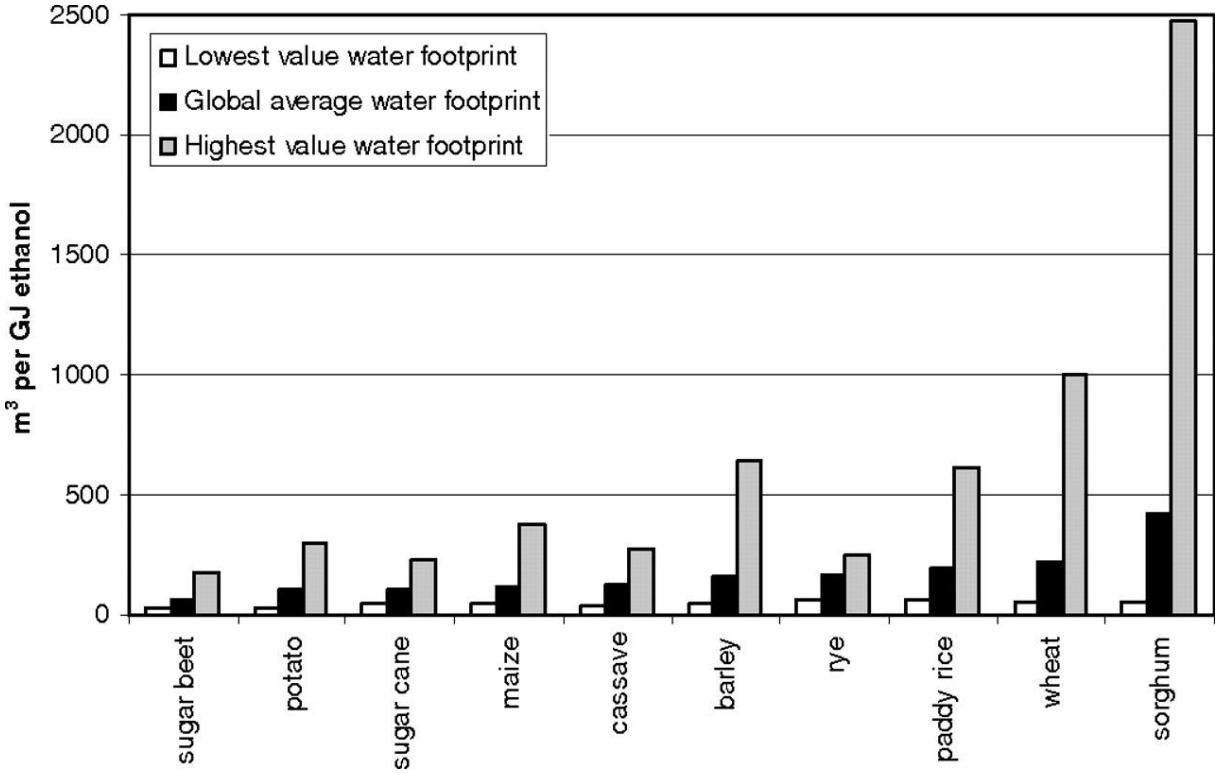


Figure 2.3: Lowest and highest value, and the weighted-average global value of the WF for bioethanol from 10 crops

Source: (Gerbens-Leenes et al., 2009)

The amount of water required for cultivating feedstock crops for bioethanol production vary depending on the crop, the region where it is being cultivated and the water efficiency of the irrigation system that being used. For example, one litre of bioethanol from corn consumes around 160 L of water for corn cultivation and 3 to 11 L of water for biorefinery (Wu et al., 2009a).

Growing crops that require minimal water or planting them in regions that receive considerable amounts of rainfall can greatly reduce water requirements for the purpose of irrigation. Rain-fed crops grown in Brazil and Southeast Asia, for example, generally make lower demands on water resources than irrigated crops grown in parts of the United States or Middle Eastern countries.

2.4.2 Direct water requirements for bioethanol production

Bioethanol production requires water manly for irrigating the feedstock crops and for industrial processing (which includes converting the feedstock into sugars then converting sugars into ethanol via fermentation process). Although the majority of water consumption goes to the

irrigation of feedstock crops, considerable amounts of water is still required for the refining of bioethanol.

2.4.2.1 Direct water requirements for ethanol production from crops

Historically, biofuels have been produced from grain-based crops with water supplied by precipitation and/or irrigation. According to De Fraiture et al., 2007, the total global irrigation withdrawal was 2,630 billion cubic metre (bm^3) in 2005 (De Fraiture et al., 2007; De Fraiture et al., 2008). 44 bm^3 of the 2,630 bm^3 is consumed for the growth of biofuel crops. On average, around 830 L of irrigation water withdrawn are required to produce one litre of biofuel. However, regional variation is large. In Brazil, the main crop for bioethanol production is sugarcane, which is mostly grown under rain-fed conditions. Hence, the required irrigation water is less than 100 L for producing one litre of ethanol in Brazil. In the USA, where maize is mainly rain-fed cultivated, 3% of the total irrigation withdrawals are devoted to the production biofuel crops, corresponding to about 400 L of irrigation water withdrawals per litre of bioethanol. China withdraws 2,400 L of irrigation water to produce the same amount of maize needed for one litre of bioethanol. With high sugarcane yields and efficient conversion, Brazil yields more than 6,200 L bioethanol per hectare. In India, where conversion efficiency is lower, one hectare yields only 4,000 L of bioethanol (De Fraiture et al., 2008). Table 2.5 shows in details the water requirements for irrigation and bioethanol production in different countries investigated in the above-mentioned report.

In a bioethanol production plant, ethanol from corn requires water for drying, grinding, liquefaction, fermentation, separation and cooling. Water requirements for biorefining in the new dry mill plants has been estimated to be 3 L of freshwater for each litre of ethanol produced (Wu and Chiu, 2011). However, older dry mill ethanol plants use up to 11 L of freshwater to produce 1 L of bioethanol (Shapouri, 2005). Table 2.6 shows examples of water requirements for different crops during biorefinery.

Table 2.5: Irrigation withdrawals for biofuel crops in 2005

Bioethanol	Bioethanol (million litres) [a]	Main feedstock crop	Feedstock used (million tonnes) [b]	Irrigation withdrawals for biofuel crops (Million m^3) [b]	Littre of irrigation water per Littre of ethanol
Brazil	15098	Sugarcane	167.8	1310	86.77
USA	12907	Maize	33.1	5440	421.48
Canada	231	Wheat	0.6	80	346.32
China	3649	Maize	9.4	9430	2584.27
India	1749	Sugarcane	19.4	6480	3704.97
Thailand	280	Sugarcane	3.1	1550	5535.71
Indonesia	167	Sugarcane	1.9	910	5449.10
S. Africa	416	Sugarcane	4.6	1080	2596.15
World ethanol	36800	-	-	30600	831.52

[a] (Dufey, 2006); [b] (De Fraiture et al., 2008)

Table 2.6: Water consumption for ethanol produced in California

Feedstock	Refinery water (L H_2O/L EtOH)
Corn grain	3.6[a]
Sugar beets	3.6[a]
Low-yield biomass	6[b]
High-yield biomass	6[b]

[a] (Wu et al., 2009b); [b] (Aden et al., 2002)

2.4.2.2 Direct water requirements for cellulosic ethanol production

Nowadays, lignocellulosic raw materials, such as herbaceous biomass, forest wood and many other agricultural residues are being considered as the substrate for cellulosic bioethanol. Cellulosic bioethanol is believed to be the biofuel solution for the long term (Wu and Chiu, 2011). According to a study led by USDA and DOE, around 285 million tonnes of biomass could be available by 2017 for conversion to ethanol, and 329 million tonnes could be available by 2030 (Perlack et al., 2011). Although, a small amount of cellulosic ethanol are currently

being produced, it has been projected that more than 60 billion litre of cellulosic bioethanol will be produced annually by 2022 (Perlack et al., 2011).

It has been suggested that advanced biofuels which are derived from waste products does not require water for cultivation as a fuel feedstock because the water consumed by these crops would have been allocated to the activity of primary value (food or feed production) (IEA, 2012). This would also apply to lignocellulosic materials used for bioethanol production. Normally, water for cellulosic ethanol is required for pretreatment, hydrolysis and fermentation processes. It has been reported that producing one litre of cellulosic ethanol via fermentation process consumes 9.8 L of water (Wu et al., 2009a). Aden and others (2002) estimated that water consumption for cellulosic ethanol will be reduced to 5.9 L of water per L of ethanol providing the expected advancement in ethanol yield (Aden et al., 2002). Table 2.7 shows a comparison of the water requirements for production between corn ethanol, cellulosic and petroleum gasoline. Production of cellulosic ethanol consumes less amount of water when compared with petroleum gasoline, 1.9–4.6 L water/L ethanol and 2.6–6.6 L water/L gasoline. However, corn ethanol consumes very large amounts of water when compared with either cellulosic ethanol or petroleum gasoline.

Due to the large water requirements for bioethanol production from conventional substrate, a number of research studies have been carried out using marine biomass, such as seaweed, for bioethanol production (Falter et al., 2015, Kostas et al., 2016). This will reduce the impact of water usage in feedstock cultivation and the arable land will be saved for the production of crops for food and feed purposes.

Table 2.7: Water consumption for ethanol and petroleum gasoline production, a comparison

Fuel (feedstock)	Net Water Consumed	Major Factors Affecting Water Use
Switchgrass ethanol	1.9 - 4.6 L/L ethanol	Production technology
Gasoline [a]	3.4 - 6.6 L/L gasoline	Age of oil well, production technology, and degree of produced water recycle
Gasoline [b]	2.8 - 5.8 L/L gasoline	Same as above
Gasoline [c]	2.6 - 6.2 L/L gasoline	Geologic formation, production technology

[a] U.S. conventional crude; [b] Saudi conventional crude; [c] Canadian oil sands.

Source: (Wu et al., 2009a)

2.5 Seawater as a replacement of freshwater for bioethanol production

A shift from fossil fuel based energy profile towards biofuel puts an additional pressure on the limited freshwater resources in many regions of our overpopulated planet. Hence, the use of alternative water resources is essential to meet future bioethanol targets (De Fraiture et al., 2008, Zaky, 2017). Oceans and seas are abundant sources of water which can be easily accessed in most arid and semi-arid zones. They can also provide marine biomass as a substrate for bioethanol production, which does not compete with food and feed for water and land usage. However, seawater contains a spectrum of minerals that inhibit the growth and fermentation ability of the conventional yeast (Zaky et al., 2014). But, the implementation of marine yeasts in bioethanol industry will facilitate the use of seawater as a replacement for freshwater in bioethanol production (Zaky et al., 2016). In addition, seawater composition -which is not favourable for terrestrial microorganisms- may play a role as a selective agent against microbial contamination in bio-refineries. Hence, the development of seawater based media along with the usage of marine yeast in bioethanol production can make a valuable impact on overcoming both the freshwater crisis and energy crisis (Zaky, 2017).

2.5.1 Water scarcity

Water covers about 71% of the surface of Earth, however, the vast majority of the world's water is saline water (seawater) that is located in the oceans and seas. Freshwater is required for all sort of human uses, only accounts for a very small proportion of the world's water (Hinrichsen and Tacio, 2002, Jensen et al., 1998, OECD, 2005). Hence, water is considered as limited resources on Earth. Figure (2.4) shows the distribution of water on Earth and human freshwater use.

Availability of freshwater is uneven due to wide differences in climatic patterns, geography and human use. In Brazil, water is abundant in most parts as they receive relatively high rainfall. By contrast, many countries in North Africa and the Middle East suffer chronic water scarcity, receiving low water flow from outside their borders as well as receiving minimal rainfall (IEA, 2012). Such countries therefore must turn to other sources of water supply, such as desalination and non-renewable aquifers. However, it is expected that the problem of water scarcity will be further aggravated due to the growing world population combined with rising per capita water use. Also, a reduction of water availability is expected due to climate change (Rockström et al., 2009, Lotze-Campen et al., 2008, de Fraiture and Wichelns, 2010).

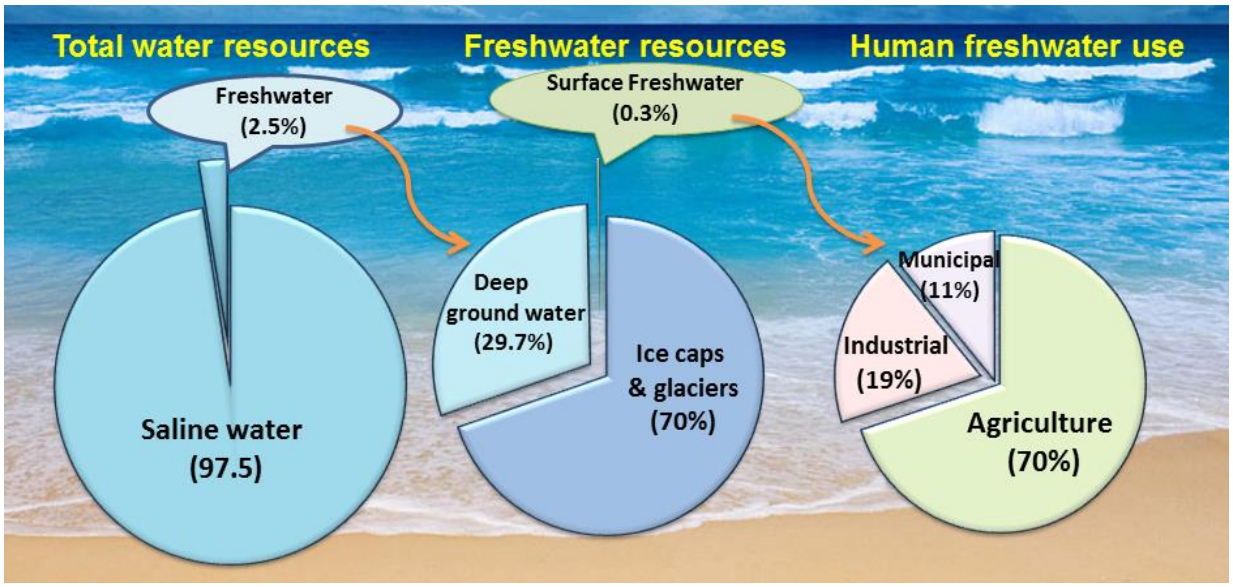


Figure 2.4: Distribution of water on Earth and human freshwater use

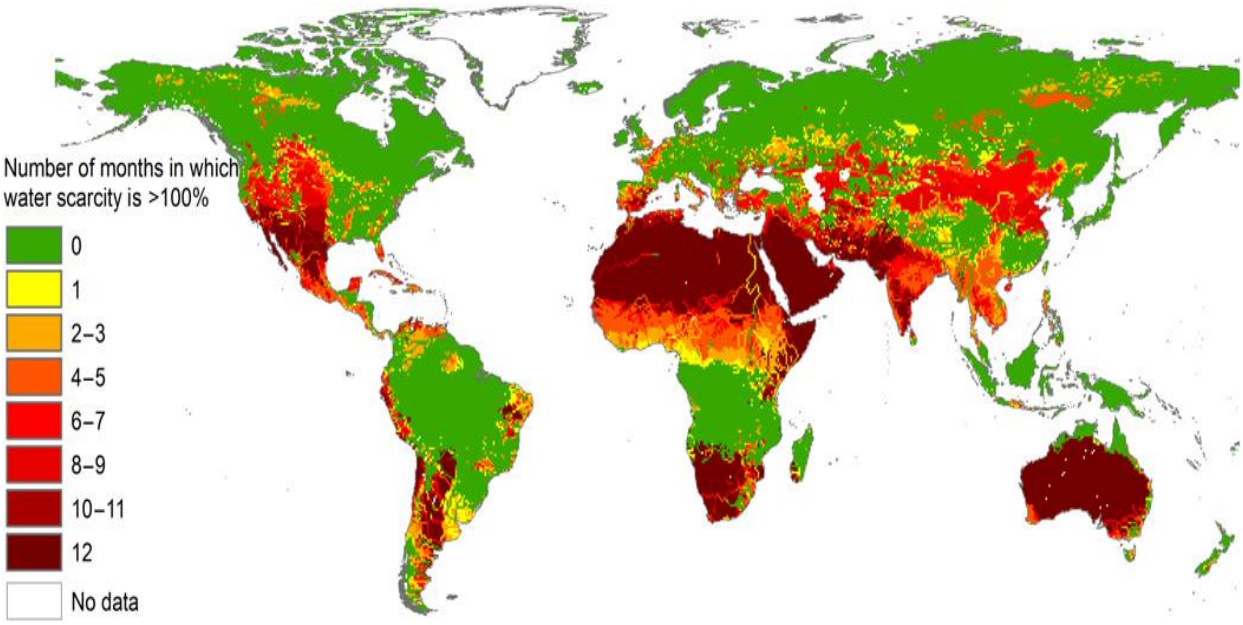
The percentage of human freshwater use vary depending on different countries and regions. For uptodate figure on water consumption visite this website (http://www.worldometers.info/water/)

The World Economic Forum (WEF) listed water crises as the largest global risk for its potential impact (Mekonnen and Hoekstra, 2016). Freshwater, therefore, should be considered as an economic commodity and therefore should be treated economically because water is scarce in many regions of the world (Gleick et al., 2002).

The International Water Management Institute (IWMI) estimated that 1.4 billion people will experience severe water scarcity within the first quarter of the 21st century. This water crisis will affect quarter of the world's population or a third of the population in the developing countries (Seckler et al., 1999, Seckler et al., 1998). Similarly, United Nations Environment Programme (UNEP) estimated that 1.8 billion people around the world are predicted to be living in absolute water scarcity by 2025, and 2/3 of the world population will experience some water stress (UNEP, 2007).

A recent study by Mekonnen and Hoekstra (2016) reported that currently four billion people around the world are suffering from facing severe water scarcity at least once month per year (Figure 2.5). Of these four billion, 1.0 billion live in India and 0.9 billion live in China. Considerable populations face severe water scarcity during at least part of the year live in the United States (130 million), Bangladesh (130 million), Pakistan (120 million), Nigeria (110 million), and Mexico (90 million). When compiled the number of people facing severe water scarcity for four to six months per year has been estimated at 1.8 to 2.9 billion. In addition, half a billion people are facing severe water scarcity all year round; 180 million live in India, 73

million in Pakistan, 27 million in Egypt, 20 million in Mexico, 20 million in Saudi Arabia, and 18 million in Yemen. Saudi Arabia and Yemen were considered in an extremely vulnerable position because all people in those two countries are suffering severe water scarcity year-round. Also, there are anther 6 countries where more than 50% of the population experiences severe water scarcity all year round. Those countries are Libya and Somalia (80 to 90% of the population); Morocco, Niger Jordan and Pakistan (50 to 55% of the population) (Mekonnen



and Hoekstra, 2016).

Figure 2.5: The number of months per year in which blue water (fresh surface water and groundwater) scarcity (WS) exceeds 1.0

WS < 1.0 (low); 1.0 < WS < 1.5: (moderate); 1.5 < WS < 2.0 (significant); WS > 2.0 (severe).
Source: Mesfin M. Mekonnen, and Arjen Y. Hoekstra Sci. Adv. 2016;2:e1500323

2.5.2 Bioethanol production using Seawater

Seawater accounts for about 97% of world's water and covers approximately 71% of the world's surface in form of connected network of aquatic ecosystem. It is a renewable source of water and accessible easily in most countries around the world. But seawater contains approximately 35 g/L of dissolved salts, mainly NaCl (27.13 g/L), $MgCl_2$ (2.50 g/L), KCl (0.74 g/L), $CaCl_2$ (1.17 g/L), $MgSO_4$ (3.38 g/L), and $NaHCO_3$ (0.21 g/L) (Fang et al., 2015). Because of its high salt content, the direct application of seawater for agricultural, industrial and domestic activates is very limited. Usually seawater requires desalination before being used, however, desalination is usually a costly and intensive energy consuming process. Therefore,

investigating approaches where seawater replaces freshwater (totally or partly) in heavy water consuming industries, such as bioethanol production, is of prime importance.

It was reported that, the action of inorganic salt added during the strong inorganic acid catalysis of cellulose breakdown is believed to be analogous to that of Ionic Liquids (IL). More interestingly, adding saline water (e.g., NaCl, 30%) or concentrated (about 5x) seawater, the organic-acid-catalysed cellulose depolymerisation was able to proceed efficiently under mild temperature (100 -125^{o}C) (Fang et al., 2015, vom Stein et al., 2010). Investigations on variety of chloride and sulphate salts showed significant improvement in cellulose and hemicellulose hydrolysis, and xylose and xylotriose degradation (Liu and Wyman, 2006, Yu et al., 2011).

A recent study by Fang et al (2015) on the use of seawater of the pretreatment of date palm leaflets for bioethanol production reported that leaflets pre-treated with seawater showed lower cellulose crystallinity comparing with those pre-treated with freshwater. Pretreatment obtained using seawater produced similar amounts of digestible and fermentable sugars in comparison with those obtained with freshwater. They also found that there was no significant difference of inhibition to *S. cerevisiae* between hydrolysates from pretreatment with seawater and freshwater. They concluded that seawater could be a promising alternative to freshwater for cellulosic ethanol especially in coastal and/or arid/semiarid zones (Fang et al., 2015).

Very recently, Ren et al (2016) studied the use of seawater, as an alternative to freshwater, for Ionic Liquid (IL) pretreatment of lignocellulosic biomass (Ren et al., 2016). They reported that when grass was pre-treated by a mixture of cholinium IL and seawater (1:1) at 90^{o}C for 6 h then washed by seawater, the treated grass became highly accessible to enzymatic hydrolysis and 54 - 72% reducing sugar yields was obtained using subsequent enzymatic hydrolysis of the residues. They also studied the microbial lipid production from wheat straw hydrolysate using seawater. They reported that *Trichosporon fermentans* yielded 4.5 g/L of lipid, with a lipid coefficient of 0.21 g/g of sugar, after 3 days of cultivation on wheat straw hydrolysates with the initial sugar concentration of approximately 30 g/L. They concluded that the use of seawater had no negative effect on neither IL pretreatment nor enzymatic hydrolysis.

Concerning the use of seawater in microbial fermentation, Lin et al (2011) conducted a proof-of-concept study using seawater in fermentation for succinic acid production (Lin et al., 2011). Using synthetic seawater-based media, they reported that no significant inhibition of cell growth of *Actinobacillus succinogenes* was observed and the production of succinic acid was not affected. In a fermentation using natural seawater, a concentration of 49 g/L of succinic acid

was produced from a wheat-derived medium, with a yield of 0.94 g/L and a productivity of 1.12/L/h. Their results revealed that seawater can also be used to form a nutrient-complete medium for succinic acid production avoiding the cost of adding mineral supplement to the wheat-derived media.

2.6 Concluding remarks

Marine yeasts live in harsh environments, which provide the potential for several unique desirable properties to be used in various industries. The latest development in the methodology of marine yeast isolation and cultivation offers the opportunity of discovering novel marine yeasts. Various media have been proposed by different research groups in order to suit the different requirement of marine yeasts. However, a standard method for the efficient isolation of marine yeast is still to be developed. Although using marine yeasts in bioethanol production shows distinctive advantage on the osmotic tolerance, the possibility of utilising seawater instead of freshwater as well as the potential of using marine biomass (i.e. seaweed) as a substrate. Yet, the commercial application of marine yeasts is still limited. The current research, however, indicates the promising features of the marine yeasts for the potential industrial application and their superiority over the terrestrial ones in certain fields.

Although a few researchers have suggested the positive impact of using seawater for fermentation, up to now, no research has investigated the production of bioethanol using natural seawater-based media. As discussed in section 2.5, bioethanol requires large amount of freshwater for production. Using seawater instead of freshwater for bioethanol production will reduce the WF of bioethanol and potentially convert this process from a high water consuming process to a water producing process. On industrial scale, bioreactors usually contain around 12% ethanol, 12% of solids and 76% water by the end of ethanol fermentation. Theoretically, if seawater was used in the fermentation, roughly 7.5 litre of freshwater can be obtained with each litre of produced ethanol. The produced water will be of very high quality and therefore can be used in industries that require high quality water or it can be used to enhance lower quality water to produce acceptable drinking water. Further advantages of using seawater in the fermentation for bioethanol production include; a) the minerals in seawater will potentially reduce the need for adding minerals to the fermentation media, b) the production of sea salt as a by-product, c) producing salted animal feed that can be used to eliminate the cost of adding minerals to the animal diets. Thus, using seawater in fermentations could potentially improve

the overall economics of the process and make a strong impact on overcoming both the freshwater and energy crises (Zaky, 2017).

CHAPTER 3:

3. Materials and Methods

3.1 Marine samples

Fourteen marine samples were obtained from different locations in the UK, Egypt and USA as listed in Table 3.1. All samples were collected near the shore and were taken from a depth of a maximum of 1 meter using 1 L sterilised plastic bottles with screw caps to prevent contamination. Samples were then transported immediately to the laboratory or sent by a courier to the laboratory. When samples were received at the laboratory, they were stored immediately in a fridge at 4°C until isolation was performed. The isolation was performed within 2 weeks of sampling.

Table 3.1: Distribution of new marine Yeast Isolates

Samples	Sample type	Sources	No of Isolates
A	Seawater	Mediterranean Sea, Alexandria, Egypt	10
B	Seawater	Suez, Gulf of Suez, Egypt	7
C	Seawater	Ras Seder, Gulf of Suez, Egypt	12
D	Seawater	Lake Timsah, Ismailia, Egypt	10
E	Seawater	Irish Sea, Northern Wales, UK	3
F	Seawater	Irish Sea, Northern Wales, UK	10
G	Seawater	English Channel, Plymouth, UK	12
H	Seawater	English Channel, Plymouth, UK	9
I	Sea sand	Mediterranean Sea, Alexandria, Egypt	6
J	Seaweed	English Channel, Plymouth, UK	13
K	Rotten Seaweed	English Channel, Plymouth, UK	5
L	Seawater	North Sea, Whitby, UK	3
M	Seawater	Atlantic Ocean, New York, USA	8
N	Seawater	Pacific Ocean, San Diego, USA	8

3.2 Seawater (SW)

Seawater was used in this study for media preparation and dilutions. The seawater was obtained from a seaside town in the UK called Skegness, which is located on the North Sea coast of Lincolnshire. The seawater was filtered using glass microfiber filters (pore size, 1.2 μm; Whatman®) and autoclaved at 121°C for 15 min, then stored at 4°C till required.

3.3 Determination of total dissolved solids (TDS) in SW

Natural SW was filtered using glass microfiber filters (pore size, 1.2 μm; Whatman®) then it was filtered again using disposable filters (pore size, 0.45 μm; Millipore, UK). 50 mL of the filtered SW were transferred into clean dry beakers (size 100 mL). Beakers were placed in a drying oven at 100°C until water fully evaporated. Beakers were weighed using a 4-digit balance after 24 h of drying and then every 2 h until constant weight.

3.4 Molasses

The molasses used in this study was a commercial product of sugarcane molasses (horse feed supplement grade) called 'NAF Molasses' that was purchased online from Amazon.co.uk. The total sugar content in this product was about 45% (w/w) as provided on the label.

3.4.1 Preparation of the molasses working solution (MWS)

Crude molasses contains certain amount of undissolved inorganic particles and some microorganisms from the sugar industry. These unwanted materials need to be removed before using molasses as a fermentation medium. Thus, a clarification step was applied on the crude molasses to prepare the Molasses Working Solution (MWS). Clarification is a heating process used to remove the unwanted particles and pasteurize the molasses. In this study, seawater was used for molasses clarification to prepare a MWS contained 50% (w/v) of molasses which contains about 22.5% (w/v) sugars. The MWS was prepared as following:

500 g of molasses was transferred into a measuring cylinder (1000 mL) then filled up to 1 L with seawater to achieve final concentration of 50% molasses (w/v). The diluted molasses was then transferred into a 2 L Duran bottle and supplemented by 5-10 mL of concentrated H_2SO_4 (98%) to lower the pH to 3.5, then 2 mL of 50% sterilised antifoam A (Sigma Aldrich, UK) was added. The bottle was then placed in autoclave and heated at 100^{o}C for 45 min. Heated bottle was left to cool down to 55^{o}C then transferred into a cold room (4^{o}C) and left to stand

overnight. Under control condition, the clear solution on the top of the molasses (about 70% of the total volume) was transferred into a new sterilized Duran bottle and the sediment was discarded.

3.5 Microorganisms

The terrestrial yeast *Saccharomyces cerevisiae* NCYC2592 strain (www.ncyc.co.uk) was used in this study as a reference strain. It was maintained on YPD agar slopes containing (w/v) 1% yeast extract, 2% peptone, 2% glucose and 2% agar at pH 6.0 ± 0.2. The slopes were kept at 4°C and used as a working stock culture. Propagation was carried out aerobically using YPD broth medium (as above, no agar) in an orbital shaker (150 rpm) at 30°C.

Marine-derived microorganisms used in this study were isolated from 14 samples collected from different locations as mentioned below in section 3.1. These isolates were propagated using modified YM medium, containing (w/v) 2% dextrose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone dissolved in natural seawater (designated as SW-YM broth). The pH of medium was adjusted to 5.0 before autoclaving at 121°C for 15 min. All marine yeast isolates were maintained on SW-YM agar slopes, containing the above-mentioned SW-YM broth with the addition of 2% (w/v) agar.

Glycerol stocks of each marine isolate was prepared using 1:1 glycerol and yeast broth that was cultured in SW-YM broth for 48 hours. The glycerol stocks were stored at −80°C.

3.6 Isolation methods for marine yeast

3.6.1 List of microbiological media used for isolation and maintenance:

a) SW-YM broth (w/v): 2% dextrose, 0.3% malt extract, 0.3 % yeast extract, 0.5% peptone.
b) SW-YM agar (w/v): 2% dextrose, 0.3% malt extract, 0.3 % yeast extract, 0.5% peptone, 2% agar.
c) YM medium (w/v): 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2% agar.
d) Sabouraud's Dextrose Agar (SDA) (Oxoid, UK): 65 g of commercial medium was dissolved in 1 litre of water to reach the following concentrations: 4% glucose 1% mycological peptone and 1.5% agar (w/v) with a final pH of 5.6.

e) Zaky's enrichment medium (w/v): 3% glucose, 3% xylose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 0.1% $(NH_4)_2SO_4$ and 0.025% KH_2PO_4.
f) Zaky's isolation broth medium (w/v): 6% glucose or alternative carbohydrate source (i.e. xylose, galactose, starch or cellulose), 0.3% yeast extract, 0.5% peptone, 0.1% $(NH_4)_2SO_4$ and 0.025% KH_2PO_4.
g) Zaky's isolation agar medium (w/v): 6% glucose or alternative carbohydrate source (i.e. xylose, galactose, starch or cellulose), 0.3% yeast extract, 0.5% peptone, 0.1% $(NH_4)_2SO_4$ and 0.025% KH_2PO_4 and 2% agar. Cellulose used in this study was a microcrystalline, powder (20 μm) from Cotton linters.

All components were dissolved in seawater or deionized water and adjusted to the required pH using either NaOH (1N) or HCl (1N), then autoclaved at 121°C for 15 minutes.

3.6.2 Isolation method 1: Kutty method (2009)

Isolation of marine yeasts was carried out by filtration and pour plate using the method described by Kutty et al. (2009) with some modifications. 30 mL of seawater was filtered through nitrocellulose filters (pore size 0.45 μm) using an autoclaved filter apparatus (Fisher). For solid marine samples (sea sand or seaweed), 10 g of each sample was suspended and vortexed in 30 mL of sterilized seawater. Then, the seawater containing the solid marine sample was filtered through nitrocellulose filters (pore size 0.45 μm) using an autoclaved filter apparatus (Fisher). The filters then were placed face up on an empty petri dish followed by the addition of YM medium (using seawater and supplemented with chloramphenicol (200 mg/L) after autoclaving) and incubated at room temperature 23 ±1°C for 14 days.

3.6.3 Isolation method 2: Dinesh method (2011)

Isolation of marine yeast was carried out by serial dilution and the pour plate technique using the method described by Dinesh et al. (2011) with some modifications. 1 mL of seawater was serial diluted by sterilized seawater to reach a dilution factor range of 10^{-1} to 10^{-6}. Then 1 mL of the original sample or the diluted sample was transferred to an empty Petri Dish followed by pouring of melted and cooled Sabouraud's Dextrose Agar (SDA), prepared using 50% seawater. For solid samples, 1 g of the sample was suspended and vortexed in 10 mL of sterilized seawater. Then it was serial diluted by sterilised seawater to reach a dilution factor range from 10^{-1} to 10^{-6}. After that, 1 mL from each dilution was transferred into an empty Petri Dish

followed by pouring of melted and cooled SDA prepared using 50% seawater followed by incubation at 35ºC for 48 hours.

3.6.4 Isolation method 3: The new method developed in this study

The new method developed in this study involves three steps (Figure 3.1). The first step was three cycles of enrichment, including a Primary Enrichment cycle, a Scale Up Enrichment cycle and Differential Enrichment cycle. In the second step, the isolation of yeasts from the final enriched culture was carried out using the pour-plate technique. The final step was conducted using the streak-plate technique and microscopic examination of selected colonies to confirm the purity of the new isolates.

In the Primary Enrichment cycle, 100 mL of seawater samples was transferred into a 500-mL conical flask containing 100 mL of 2x Zaky's enrichment medium. For solid samples, 20 g of the sample was transferred into a 500-mL conical flask containing 100 mL of Zaky's enrichment medium. Then the flasks were incubated in a shaking incubator (180 rpm) at 30ºC for 48 hours.

In the Scale Up Enrichment cycle, 20 mL of culture from the Primary Enrichment cycle was transferred into a 500-mL conical flask containing 180 mL of Zaky's enrichment medium. Then the flasks were incubated in a shaking incubator (150 rpm) at 30ºC for 48 hours.

In the Differential Enrichment cycle, 10 mL of culture from the Scale up Enrichment cycle was transferred into a 250-mL conical flask contained 90 mL of Zaky's isolation broth medium. Then the flasks were incubated in a shaking incubator (150 rpm) at 30ºC for 48 hours.

All media used in this step were prepared using seawater and adjusted to pH 5.0 using HCl (1N). A mixture of antibiotics (Penicillin-G 500 mg/L and streptomycin sulphate 500 mg/L) was added to all enrichment media after autoclave. Microscopic inspection was carried out after each enrichment cycle to monitor the type of growth after the period of cultivation.

In the second step (isolation step), a 10-fold serial dilution was carried out from the Differential Enrichment cycle and 1 mL of diluted broth (with a dilution factor of 10^{-4} to 10^{-7}) was transferred into a petri dish then poured with 10 mL of Zaky's isolation agar medium. The plates were then incubated for 48 hours at 30ºC.

In the third step (confirmation step), selected single colonies from the isolation step were streaked on a fresh SW-YM agar plates and incubated for 48 hours at 30ºC followed by microscopic examination of smear slide stained with methylene blue.

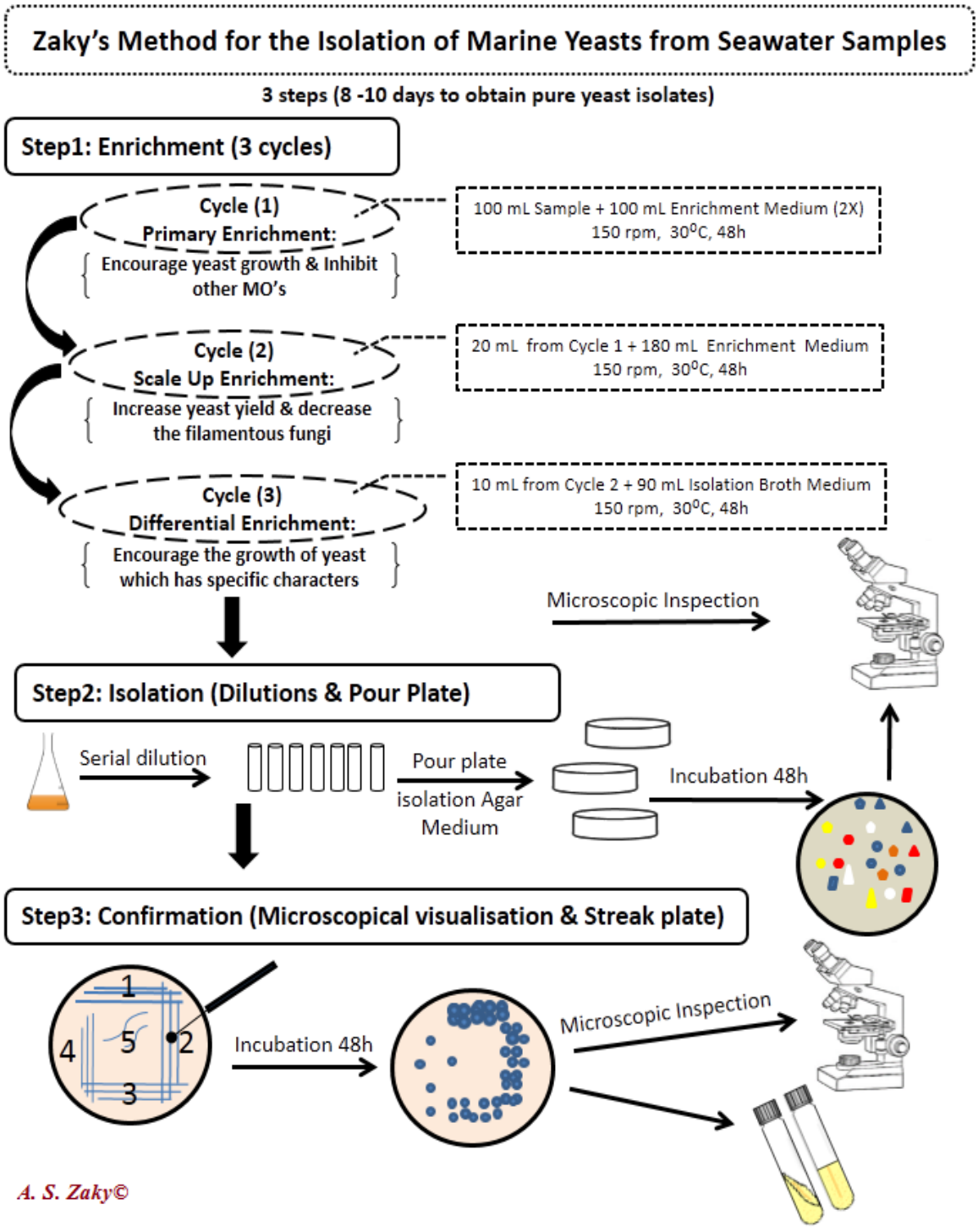


Figure 3.1: Schematic diagram of the new marine yeast isolation method proposed in this study

3.6.5 Microscopic examination

Purity and cell morphology of all new isolates were inspected under microscope using fixed smears on glass slides that stained with methylene blue (0.1%, w/v). Methylene blue solution was prepared by dissolving 0.1 g of methylene blue in 50 mL of distilled water. Then 2.0 g of sodium citrate dehydrate was added. The solution was thoroughly mixed until all compounds were completely dissolved. Distilled water was added to adjust the final volume to 100 mL.

Simple staining was done according the following procedure; i) a loopful of sterilised water was transferred on a clean glass slide, ii) a small amount of yeast growth was dispersed in the drop of water and smeared on the slide then let to dry, iii) dry smear was fixed by passing the underside of the slide through a Bunsen flame for a few times, iv) after cooling, slide was flooded with methylene blue 1% solution for 5 min., v) the excess strain was rinsed gently with running water and slide was dried. Slides were inspected under the oil immersion lens (100X) using light microscope (Optika) attached with camera (VWR).

3.7 Screening and metabolism evaluation using phenotypic microarray (PM) assay.

PM assays were carried out using sterilised empty BioLog 96-well plates based on the method developed by Greetham et al. (2014) with minor modifications. Each well in the plate was supplied with a mixture of growth medium (30 µL) and buffered cell suspension (90 µL). Stock solutions of (40% w/v) of each sugar (glucose, mannitol, xylose, galactose) and (28.7% w/v) of yeast nitrogen base (YNB) were prepared individually in seawater then filter sterilised. Sugars were prepared from the stock solutions to give a 6 % (w/v) final concentration per well and 0.67 % (w/v) of YNB in the well's final volume (120 µL). Hence, 18 µL stock solution's sugar, 2.8 µL of YNB stock solution, 9 µL of seawater and 0.2 µL of dye D (BioLog, Hayward, CA, USA) were aliquoted to individual wells in the BioLog 96-well plate.

Marine isolates and the reference strain were prepared for inoculation into the PM assay plates as following; a fresh culture (48 h) on SW-YM agar slope was prepared from stock cultures stored at 4°C. A small amount of yeast growth was transferred into test tubes (20 × 100 mm), containing 10 mL sterile seawater and transmittance adjusted to 62% (~5×10^6 cells/mL) (turbidimeter, BioLog). Then, 1 mL of the cell suspension was added to 5 mL of IFY buffer™ (BioLog) to obtain the cell suspension for the inoculum. Next, 90 µL of the above cell

suspension was inoculated into each well of the BioLog plate. Semi-anaerobic conditions were created by placing the plates inside PM gas bags (BioLog) and vacuum sealed using

DMC 260PD Vacuum Packaging Machine. Inoculated plates were then incubated at 30°C in the OmniLog plate reader (BioLog, CA, US) and reading was recorded every 15 minutes for 24 hours. By the end of the run, the signal data was compiled and exported from the BioLog software using Microsoft® Excel. Only the isolate that had a final BioLog reading (Redox signal intensity) of 20 or over was counted. All experiments were performed in triplicates and the mean signal values were presented.

3.8 PCR-based method (D1/D2 and ITS primers) for yeast identification

Identification of selected marine isolates was carried out based on the sequence of D1/D2 region of the large 26S rDNA subunit using primers NL-1 (5̀-GCATATCAATAAGCGGAGGAAAAG -3̀) and NL-4 (5̀-GGTCCGTGTTTCAAGACGG-3̀). Identification was also performed by sequencing the Internal Transcribed Spacer (ITS) region using primers ITS1 (5̀-TCCGTAGGTGAACCTGCGG-3̀) and ITS4 (5̀-TCCTCCGCTTATTGATATG-3̀) as described by White et al. (1990) and Mitchell et al. (1992).

The PCR master mix for PCR amplification, each reaction contained 8 µL of 10X PCR buffer, 1 unit of *Taq* DNA polymerase, 25 pmol of each forward and reverse primer, 100 µM of each deoxynucleoside triphosphate and enough distilled water up to a total reaction volume of 50 µL. A small amount of yeast growth from a culture (24-48 h) was picked using a micropipette tip and suspended in 50 µL of deionized water then incubated for 10 min at 95ºC. 4 µL of the preheated yeast suspension was transferred into the PCR tubes as a DNA template. Tubes were then placed in a thermo cycler (TECHNE TC-5, UK), using the following settings: initial denaturation at 98°C for 30 s followed by 35 cycles of 98°C for 15 s, 52°C (for D1/D2 primers) or 54°C (for ITS primers) for 45 s, and 72°C for 90 s, with a final extension step of 72°C for 5 min. PCR products were detected by electrophoresis separation using 1% (w/v) agarose gel containing 4 µL of ethidium bromide in TBE buffer (0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA, pH 8.3). A PCR cleaning kit (Sigma-Aldrich, UK) was used for purification before sequencing. The sequences obtained were aligned using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) for comparison with currently available sequences.

3.9 YT-MicroPlate (BioLog system) for yeast identification

BioLog YT MicroPlates (BioLog, Hayward, CA) were used to identify 21 of the marine isolates and the reference strain. YT Micro-Plates contained pre-prepared substrates for 94 different tests in a ready to use 96 BioLog micro-plate with 2 reference wells (Figure 3.1). Fresh cultures (48 h) were prepared on malt extract agar slopes using the working cultures stock that were stored at 4°C. For each strain, a small amount of yeast broth was aseptically transferred into a test tube (20 × 100-mm), contain sterile deionized water. Then the test tube was placed on BioLog turbidimeter to reach a transmittance of 48%. Using a multichannel micro pipette, one YT MicroPlate was used for each strain by loading 100 µL of the cell suspension into each well. Plates were incubated at 26°C and the reads were taken after 24, 48 and 72 hours at a wavelength of 450 nm for oxidation tests (the upper 3 rows in the plate) and a wavelength of 590 nm for assimilation tests (the other 5 rows in the plate) using a TECAN (Infinite® 200 PRO) plate reader. Reads were generated and manually converted to values (positive, negative and weak). All reads below 0.1 were considered negative, reads between 0.1 and 0.3 were considered weak reaction, while any reads above 0.3 were considered positive. The results were sent to Technopath (Surrey, UK) for identification using MicroLog® manual microbial ID system.

A1 Water	**A2** Acetic Acid	**A3** Formic Acid	**A4** Propionic Acid	**A5** Succinic Acid	**A6** Methyl Succinic	**A7** L-Aspartic Acid	**A8** L-Glutamic Acid	**A9** L- Proline	**A10** D-Gluconic Acid	**A11** Dextrin	**A12** Inulin
B1 Cellobiose	**B2** Mentiobiose	**B3** Maltose	**B4** Maltotriose	**B5** D-Melezitose	**B6** D-Melibiose	**B7** Palatinose	**B8** D-Raffinose	**B9** Stachyose	**B10** Sucrose	**B11** Trehalose	**B12** Turanose
C1 N-Acetyl-DGlucosamie	**C2** α-D-Glucose	**C3** D-Galactose	**C4** D-Psicose	**C5** L-Sorbose	**C6** Salicin	**C7** D-Mannitol	**C8** D-Sorbitol	**C9** D-Arabitol	**C10** Xylitol	**C11** Glycerol	**C12** Tween 80
D1 Water	**D2** Fumaric Acid	**D3** L-Malic Acid	**D4** D4 Methyl Succinate	**D5** Bromosuccinc Acid	**D6** L-Gluamic Acid	**D7** γ-Aminobutyric Acid	**D8** α-Keto Glutaric Acid	**D9** 2- Keto-DGluconic Acid	**D10** D-Gluconic Acid	**D11** Dextrin	**D12** Inuilin
E1 Cellobiose	**E2** Gentiobiose	**E3** Maltose	**E4** Maltotriose	**E5** D-Melexitose	**E6** D-Melibiose	**E7** Palatinose	**E8** D-Raffinose	**E9** Stachyose	**E10** Sucrose	**E11** Trehalose	**E12** Turanose
F1 N-Acetyl DGlucosamie	**F2** DGlucosamine	**F3** α-D-Glucose	**F4** D-Galactose	**F5** D-Psicose	**F6** L-Rhamnose	**F7** L-Sorbose	**F8** α-Methyl-DGlucoside	**F9** β- Methyl-DGlucoside	**F10** Amygdalin	**F11** Arbutin	**F12** Salicin
G1 Maltitol	**G2** D-Mannitol	**G3** D-Sorbitol	**G4** Adonitol	**G5** D-Arabito	**G6** G6 Xylitol	**G7** i-Erythritol	**G8** Glycerol	**G9** Tween 80	**G10** L-Arabinose	**G11** D-Arabinose	**G12** D-Ribose
H1 D-Xylose	**H2** Methyl Succinate +D-Xylose	**H3** N-Acetyl-LGlutamic Acid +D-Xylose	**H4** Guinic Acid +D-Xylose	**H5** D-Glucuronic Acid+D-Xylose	**H6** Dextrin +D-Xylose	**H7** α-D-Lactose +D-Xylose	**H8** D-Melibiose +D-Xylose	**H9** D-Galactose +D-Xylose	**H10** m-Inositol +D-Xylose	**H11** 1,2-Propanediol +D-Xylose	**H12** Acetoin +DXylose

Figure 3.1: Chemical substrates in YT-MicroPlate

A1 to C12: For oxidation tests; D1 to H12: For assimilation tests.

3.10 Fermentations using Mini Fermentation Vessels (MFVs)

Fermentations were conducted at miniature scale (100 mL) to allow applying maximum number of experiments for yeast screening and for the optimising the condition of ethanol production.

3.10.1 Microorganisms

S. cerevisiae 2592 which was obtained from NCYC Norwich, UK was used in all experiment as a reference and control strain. 9 marine yeast strains (Table 3.2) were selected from the isolated marine yeasts for the fermentation tests in this study.

Table 3.2: Newly isolated marine yeasts with potiencial application in fermentation industry

Source	ID Name	Strain
Meditation Sea, Egypt	*Candida viswanathii*	S8
Irish Sea, UK	*Saccharomyces cerevisiae*	S65
English Channel, UK	*Saccharomyces cerevisiae*	S71
	Wickerhamomyces anomalus	S80
	Pichia kudriavzevii	S83
	Issatchenkia orientalis	S88
	Saccharomyces cerevisiae	S118
North Sea, UK	*Candida glabrata*	S127
Pacific Ocean, USA	*Candida albicans*	S142

3.10.2 Preparation of yeast inoculum

Yeast inoculum of each strain in this study was prepared as following; i) cryopreserved yeast was streaked on YPD slant agar and incubated at 30°C for 48 hours, ii) 20 mL of YPD broth in a 50 mL conical flask was inoculated by a loopful of the 48 hours yeast slant culture then incubated in an orbital shaker at 30ºC and 150 rpm for 48 h, iii) The culture was then transferred into a 500 mL conical flask containing 200 mL of YPD and incubated for another 48 h under the same conditions, iv) Yeast cells were harvested using a benchtop centrifuge at 3000 rpm for 3 minutes (Eppendorf, UK). The yeast pellets were then washed three times by dissolving and

harvesting them with sterile deionised water. Clean yeast pellets were re-suspended in around 5 mL of sterile water to form a concentrated liquid yeast inoculum with OD_{600} of around 500 and used as an inoculum. Depending on the experiment, SW or Reverse Osmosis Water (ROW) was used for media and inoculum preparation.

3.10.3 Preparation of Mini fermentation vessels (MFVs) for anaerobic fermentation

150 mL glass serum bottle (Wheaton, USA) containing magnetic fleas were autoclaved at 121°C for 15 min. Under controlled conditions, 100 mL of the required fermentation medium was transferred into the sterile MFVs. MFVs were then inoculated with around 100 µL of the yeast inoculum to reach a starting concentration of 0.5 OD_{600}. Anaerobic conditions were established using an autoclaved sealed butyl plug (Fisher, Loughborough, UK) and aluminium caps (Fisher Scientific). An autoclaved handmade hypodermic needle attached to a Bunsen valve was purged through a rubber septum of each MFV to facilitate the release of CO_2 during the fermentation (Figure 3.2). The MFVs were then placed on a 15-position magnetic stirring plate set at 80 rpm and incubated in a static incubator at 30°C for the required fermentation time.

The rates of the fermentation were monitored by measuring the weight loss at regular time points during the fermentation until it reached constant weight. At the end of the fermentation, samples from each MFV were prepared for HPLC analysis (see 3.13.2) to quantify the concentrations of ethanol, glycerol and glucose residues.

Based on the purpose of the experiment, the composition of the fermentation media was designed as following:

3.10.4 Screening for high ethanol producing marine yeast

Fermentation media containing 6% glucose, 2% peptone and 1 % yeast extract were prepared in SW and ROW. The pH of the media was adjusted to 5.5 using 1N HCl and/or NaOH then autoclaved at 121ºC for 15 min. 9 marine yeast strains and the reference strain (*S. cerevisiae* NCYC2592) were used in this experiment.

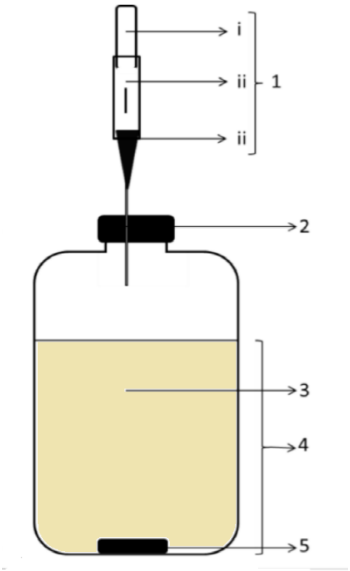


Figure 3.2: Schematic diagram of mini fermentation vessel (MFV) for anaerobic fermentation

1, Bunsen valve (i, Durham tube; ii, Silicon tubing; iii, Hypodermic needle), 2, rubber septum with metal crimp; 3, glass bottle; 4, working volume (100 mL); 5, magnetic flea.

3.10.5 Ethanol fermentation under osmotic stress by marine *S. cerevisiae* using seawater-based media with high glucose concentrations

The fermentation was conducted using SW-YPD media containing 2% peptone, 1 % yeast extract and different concentrations of glucose (10, 15, 20, and 25%). Also, YPD medium containing 2% peptone, 1 % yeast extract and 10% glucose in ROW was used for comparison. The starting pH of all media was adjusted to 6.0 using 1N HCl and/or NaOH then autoclaved at 121^{o}C for 15 min. Marine *S. cerevisiae* strains (S65 and S118) and the reference strain (*S. cerevisiae* NCYC2592) were used in this experiment.

3.10.6 Ethanol fermentation by marine *S. cerevisiae* under salt stress using YPD media with high NaCl concentrations

Salt solutions at 3, 6 and 9% NaCl was prepared and used for preparing the fermentation media. The media components were dissolved in the salt solutions to reach 6 % glucose, 2 % peptone, 1 % yeast extract. Volumes of media were adjusted to account for the addition of NaCl to ensure that all fermentations began with the same carbon load. The fermentation media was prepared also using ROW and SW at the same YPD concentration and used for comparison. The starting pH of all media was adjusted to 6.0 using 1N HCl and/or NaOH then autoclaved at 121^{o}C for 15 min. Marine *S. cerevisiae* strains (S65 and S118) and the reference strain (*S. cerevisiae* 2592) were used in this experiment.

3.10.7 Ethanol fermentation by marine *S. cerevisiae* under salt stress using synthetic seawater- (SSW) based media

Synthetic seawater of 35.22 and 70.44 g/L total salts was prepared as described in Table 3.3. The fermentation media were prepared by dissolving YPD components in the SSW to reach 6% glucose, 2% peptone and 1 % yeast extract. Also, SW-YPD and ROW-YPD at similar concentrations were used for comparison. The starting pH of all media was adjusted to 6.0 using 1N HCl and/or NaOH then autoclaved at 121^{o}C for 15 min. Marine *S. cerevisiae* strains (S65 and S118) and the reference strain (*S. cerevisiae* 2592) were used in this experiment.

Table 3.3: Composition of SSW according to the Oceano-graphical Table, Stewart and Munjal (1970), and a doubled concentration as used in this experiment

	Concentration g/L	
Component	**1-strength**	**2-strength**
NaCl	27.133	54.266
$MgCl_2$	2.504	5.008
$MgSo_4$	3.382	6.764
$CaCl_2$	1.167	2.334
KCl	0.742	1.484
$NaHCO_3$	0.207	0.414
NaBr	0.085	0.17
Total salts	35.22	70.44

3.11 Assessing the tolerance of marine *S. cerevisiae* to osmotic and salt stress

YPD media of 5, 10, 15 and 20% glucose were prepared in ROW and SSW of different compositions (1x 2x and 3x) (Table 3.4). A 96-well microliter plates were loaded with the media at 200 µL per well. 3 marine *S. cerevisiae* strains and the reference strain *S. cerevisiae* NCYC2592 were grown aerobically to exponential phase in YPD broth at 30°C using the working stock cultures. Cells were harvested by centrifugation at 3000 rpm for 3 min and washed 3 times using ROW. Suspension of yeast at concentration of OD_{600} of 2 was prepared from each strain using ROW. 5 µL of the yeast suspension was pipetted into each well to reach a starting OD_{600} of ≈0.05.

Plates were incubated in a TECAN Infinite M200 Pro plate reader (Mannedorf, Switzerland) at 30°C. The plate reader records OD_{600} every 30 minutes using Magellan (7.1, SP1) software. The plates were orbitally shaken for 1 minute before converting pixel density to a signal value reflecting cell growth. After completion of the run, the signal data was compiled and automatically converted into Microsoft® Excel compatible data by Magellan software. The assay was performed in triplicate and the average reading was plotted.

Table 3.4: Components (g/L) of YPD media with different glucose concentrations in ROW and SSW of different salt concentrations

	Glucose	**Peptone**	**Yeast Extract**	**NaCl**	**$MgCl_2$**	**$MgSO_4$**	**$CaCl_2$**	**KCl**	**$NaHCO_3$**	**NaBr**
YPD in ROW	50.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	100.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	150.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	200.00	20.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
YPD in 1x SSW	50.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
	100.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
	150.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
	200.00	20.00	10.00	27.13	2.50	3.38	1.17	0.74	0.21	0.09
YPD in 2x SSW	50.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
	100.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
	150.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
	200.00	20.00	10.00	54.27	5.01	6.76	2.33	1.48	0.41	0.17
YPD in 3x SSW	50.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26
	100.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26
	150.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26
	200.00	20.00	10.00	81.40	7.51	10.15	3.50	2.23	0.62	0.26

3.12 Fermentation using 15 L bioreactors

3.12.1 Bioreactor, description and operation

Large scale fermentations were conducted using 15 L, in-situ sterilisable, stainless steel bioreactors (Techfors-S, Infors-HT, Bottmingen, Switzerland) with 10 L working volumes (Figure 3.3). The cylindrical body of the bioreactor (200 mm diameter and 505 mm depth) with central rotor from the top plate was equipped with three 6-bladed impellers of 66 mm diameter around the shaft used for stirring. The vertical distance between impellers was 78 mm, and the lowest impeller was positioned 78 mm above the reactor bottom. Temperature probes were inserted through a metal jacketed port from the top of the plate and the sensor was positioned 78 mm from the bottom of each vessel.

The compressed gases (air or nitrogen) were injected into the vessel through the Novasip™ steam-in-place capsule (Pall, U.K.) inlet filter then into the fermentation media through a ring sparger with a pore size of 0.5 mm, located 30 mm above the reactor bottom. Flow rate of the gas passing into the bioreactor was regulated using a rotameter (V100, Vogtlin Instruments, Germany). Gases exited the bioreactor first through a condenser, to ensure there was no loss of media, and then through an exit filter (Novasip™). An extension tube was attached to the exit tube of the bioreactors to facilitate the safe removal of the gas coming out the vessels into the environment through the nearest ventilated exhaust.

Before operating the bioreactors, pH probes (Mettler-Toledo, U.K.) were calibrated using buffers (pH 7.0 and pH 4.0) and inserted into an ingold port of the bioreactor. Sterilisation was achieved by direct injection of steam into the double jacket surrounding the bioreactor, maintaining a temperature of 121°C for 15 minutes. Sterilisation step requires at least 5 L of water or media inside the bioreactor before operation. All experiments were conducted in triplicate and aseptic procedure was followed.

The bioreactor was either controlled from a control panel attached to the body of the vessel or remotely using a laptop connected to the bioreactor processing units. Fermentation parameters, such as temperature, pH and stirring rate were controlled remotely and all measurements were recorded using Iris NT (version 5.02.709.0997, Infors, Bottmingen, Switzerland).

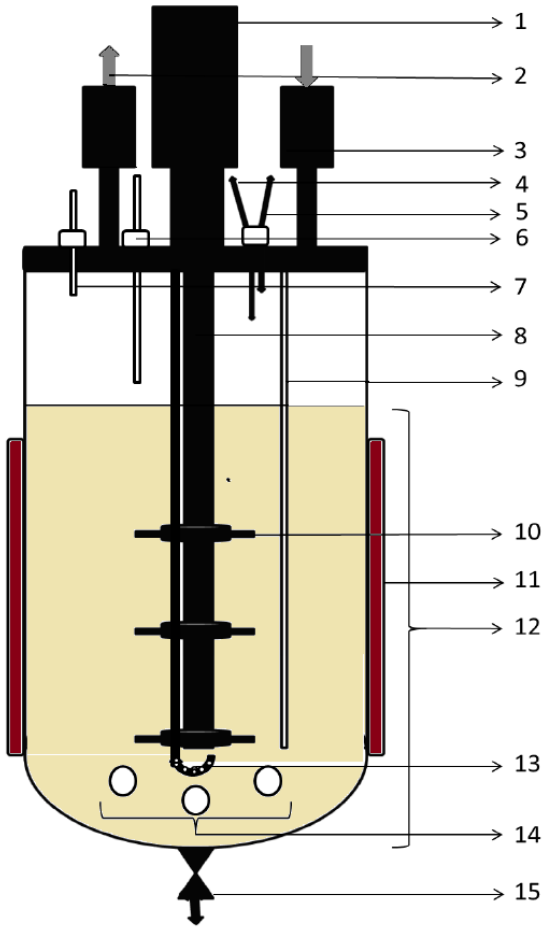


Figure 3.3: Schematic diagram of a 15 L Bioreactor used for 10 L fermentation

1, motor; 2, Air outlet (through a condenser and HEPA filter); 3, Air inlet (through HEPA filter), 4, probe for inlet of acid; 5, probe for inlet of base; 6, probe for antifoam; 7, probe for inlet of gas into head space through metal plate; 8, Agitator shaft; 9, probe for temperature; 10, impeller; 11, double metal jacket; 12, working volume; 13, Gas sparger; 14, three Ingold ports, one utilized for a pH probe and another for a dissolved oxygen probe/sensor, 15, sampling port (8 mm manual valve with tri-clamp connector).

3.12.2 Preparation of yeast inoculum for 15 L bioreactors

Inoculum of the marine yeast *S. cerevisiae* S65 were prepared for 10 L fermentation media using the following protocol; i) cryopreserved yeast was streaked on DMYP slope and incubated at 30°C for 48 hours, ii) 20 mL of YPD broth in a 50 mL conical flask was inoculated by a loopful of the 48 hours yeast slant culture then incubated in an orbital shaker at 30ºC and 150 rpm for 48 h, iii) The culture was then transferred into a 500 mL conical flask containing 200 mL of YPD and incubated for another 48 h under the same conditions, iv) 100 mL of the yeast culture was then used to inoculate 1 L of YPD broth in a 2 L conical flask and incubated

at 30ºC and 150 rpm for 48 h, v) yeast cells were harvested by centrifugation (Beckman, Model-J2-21) at 5000 rpm and 10°C for 5 minutes, vi) harvested yeast was washed three times by suspending and re-harvesting them with sterile SW, vii) Clean yeast pellets were re-suspended in a similar volume of sterilised SW to form a concentrated slurry yeast inoculum with OD_{600} of around 500 that was used as inoculum. All steps were conducted under aseptic conditions.

3.12.2.1 Batch fermentation for ethanol production using marine yeast and SW-YPD medium containing 22% glucose

Bioreactors were filled with 10 L of SW-YPD fermentation media containing 1% yeast extract, 2% peptone, and 22% glucose dissolved in seawater at a starting pH of 6.0. Sterilisation for the media and the bioreactor was conducted at 121°C for 15 min (as in section 3.12.1). Fermentation medium was aerated for an hour using compressed air at a rate of 10 L/min. Aerated media was aseptically inoculated with yeast to achieve final cell density of about 0.5 OD_{600} (about $3x10^6$ cells/mL). Fermentation was carried out at 30ºC and a stirring rate of 200 rpm. Samples were collected at regular time points over 54 hours to analyse the kinetics of the fermentation by measuring glucose residue, glycerol and ethanol production. In order to achieve anaerobic condition during ethanol fermentation, no additional air or oxygen were injected into the vessels after the initial oxygen was consumed by yeast.

3.12.2.2 3-Stage batch fermentation for ethanol production using marine yeast and SW-YPD medium

The first stage was starting by transferred 8 L of the propagation medium, composed of 1% yeast extract, 2% peptone, and 4% glucose dissolved in seawater, into the bioreactors. The pH of the medium was adjusted to 6.0 using NaOH (50% w/v) and the sterilisation, for the media and bioreactor, was carried out as described in section 3.12.1. Vessels were cooled down till 30ºC and aseptically inoculated using the yeast inoculum to achieve a final cell concentration of about 2.0 OD_{600}. The propagating stage (1^{st} stage) was carried out aerobically using compressed air (at a rate of 10 L/min) at 30ºC and stirring rate of 200 rpm for 10 h. Samples were collected at regular time points to determine the growth rate in terms of OD using spectrophotometer. By the end of the propagation stage, air supply was stopped and the bioreactors were flushed with nitrogen (at a rate of 10 L/min) for 30 min to establish anaerobic condition for the second stage. The temperature of the reactors was also increased to 35ºC.

The second stage was started by adding 2 L of sterilised glucose solution (100% w/v) prepared in seawater to the bioreactors in order to obtain a glucose concentration around 20% (w/v) in the fermentation medium. A sample was taken at the 0-time point to analyse the concentration

of glucose, glycerol ethanol and yeast cell density at the beginning of the ethanol fermentation stage. Then, samples were withdrawn at regular time intervals for 20 h to assess the fermentation kinetics by monitoring the changes in the concentration of glucose, glycerol ethanol and yeast cell density. The 3rd stage started by adding 1 L of the glucose solution to the fermenters. The fermentation conditions and sampling were conducted as in the 2nd stage. The pH was adjusted to 6.0 at the beginning of each stage by adding concentrated NaOH (50% w/v).

3.12.2.3 Batch fermentation for ethanol production from sugarcane molasses medium (30%) prepared in seawater.

Bioreactors were filled with 4 L of seawater then sterilised at 121°C for 15 min. (as in 3.12.1). 6 L of the MWS (section 3.4.1) was transferred aseptically into the bioreactors to obtain a molasses medium concentration of 30% (w/v). The medium was supplemented with 3 mL of antifoam (50% v/v, in seawater) and 10 mL of urea solution (20% w/v, in seawater) and the pH was adjusted to 5.5 using NaOH (50% w/v). The medium was aerated for an hour using compressed air at a rate of 10 L/h before inoculating with yeast to achieve final cell density of about 1.0 OD_{600} (about $3x10^6$ cells/mL). Fermentation was carried out at 30°C and stirring rate of 200 rpm. To assess the kinetics of the fermentation, samples were withdrawn at a regular time points for 48 hours to monitor sugars (sucrose, glucose and fructose), glycerol and ethanol. Anaerobic condition was achieved in the bioreactor as no air or oxygen has been injected into the vessels during the fermentation.

3.12.2.4 2-stage batch fermentation for ethanol production from sugarcane molasses medium prepared in seawater.

Bioreactors were filled with 4 L of seawater then sterilised at 121°C for 15 min (as in section 3.12.1). 1 L of MWS (section 3.4.1) was transferred aseptically into the bioreactors to obtain a propagation medium of 10% (w/v) molasses concentration. The medium was supplemented with 3 mL of antifoam (50% w/v in seawater) and 10 mL of urea solution (20% w/v, in seawater). The medium was aerated for an hour using compressed air at a rate of 10 L/h after adjusting the pH to 5.5 using NaOH (50% w/v). The propagation stage (1st stage) was started by inoculating the fermentation medium with yeast to achieve a cell density of an OD_{600} of about 2.0. The fermentation was carried out for 14 h at 30°C with a stirring rate of 200 rpm. Samples were collected at a regular time points to determine the growth rate in terms of cell density at OD_{600} using spectrophotometer. By the end of the propagation stage, air supply was stopped and the bioreactors were flushed with nitrogen (at a rate of 10 L/min) for 30 min to establish anaerobic condition for the second stage, also the temperature of the reactors were increased to

35ºC. The second staging (ethanol production) was started by adding 1 L of sterilized seawater and 4 L of the MWS (section 3.4.1) to the bioreactors in order to obtain a fermentation media with molasses concentration of 20% (w/v). A sample was taken at the 0-time point to analyse the concentration of glucose, glycerol ethanol and yeast cell density at the beginning of the ethanol fermentation stage. Samples were withdrawn at regular time intervals for 30 h to assess the fermentation kinetics by mentoring the changes in the concentration of sugars (glucose, fructose, and sucrose), glycerol and ethanol as well as the pH yeast cell density.

3.12.3 Sampling from FVs

Samples were collected manually through a sampling port manual valve (8 mm) with a tri-clamp connector. The valve was sterilised with 70% (v/v) ethanol before sampling. The pH and OD_{600} were recorded directly after sampling (when required) and samples were centrifuged at 3000 rpm for 5 min. The supernatant was collected and store at -20°C until required for analysis.

3.13 Development new HPLC method for measuring sugars and fermentation metabolites in samples containing high concentrations of chloride salts

3.13.1 Chemicals

Chemicals and solvents used in this study were HPLC or analytical grade purchased from Fisher or Sigma-Aldrich. Distilled water was used as a solvent for preparing the mobile phase and samples.

3.13.2 Chromatography

The HPLC system consisted of a JASCO AS-2055 Intelligent auto sampler (JASCO, Tokyo, Japan) and a JASCO PU-1580 Intelligent HPLC pump (JASCO). Chromatographic separation of sodium chloride (NaCl) as well as all other components under investigation in this study (organic and inorganic salts, sugars, organic acids and alcohols) was achieved at 35ºC using a Hi-Plex H column (7.7 x 300 mm, 8 µm) (Agilent Technologies, Inc., UK) and a Jasco RI-2031 Intelligent refractive index detector (Jasco). The mobile phase was 0.005 N H_2SO_4 at a flow rate of 0.4 mL/min. The mobile phase solution was also used for flushing the syringe of the auto sampler. The injected volume was 10 µL and the analysis was completed in 12 min. for determination of Cl^- salts only, 16 min for determining Cl^- salts and sugars and 32 min to include the determination of organic acids and ethanol. A blank sample of distilled water was used to

verify the purity of the water being used as solvent. The goodness-of-fit of various calibration models were evaluated by visual inspection and the correlation coefficient as well as intra and inter-run accuracy and precision values (section 3.13.6).

3.13.3 Preparing a stock solution of NaCl for peak detection

Stock solutions at the concentration of 40.00 g/L from 3 different NaCl grades (analytical grade from Fisher 99.85%, rock salt Lab grade from Fisher and salt food grade from SAXA) were prepares at 4 levels (40.00, 20.00, 10.00, 5.00 g/L) to identify the peak under investigation.

3.13.4 Preparation of different stock solutions from various components for peak detection

Stock solutions of 29 different salts, sugars, organic acids and alcohols (Table 3.5) were prepared at a concentration of 20 g/L. Each component was injected separately into the system at a concentration of 10 g/L in order to test the ability of the method for detecting and determining the retention time (Rt) of each component. The stock solutions were used to prepare 5 mixed solutions (A, B, C, D, and E) from components that had different Rt to test the separation efficiency of the method. Mixture A was prepared from 9 components (sodium chloride, maltose, sodium citrate, glucose, mannitol, sodium succinate, glycerol, sodium acetate and ethanol) at a concentration of 2.22 g/L for each component. Mixture B was prepared from 8 components (potassium chloride, citric acid, galactose, arabinose, succinic acid, formic acid, acetic acid and ethanol) at a concentration of 2.5 g/L for each component. Mixture C was prepared from 4 components (magnesium chloride, lactose, xylose and lactic Acid) at a concentration of 2.5 g/L for each component. Mixture D was prepared from 3 components (sodium sulphate, sucrose and sorbitol) at a concentration of 3.33 g/L for each component. Mixture E was prepared from 3 components (sodium bromide, sodium phosphate and fructose) at a concentration of 3.33 g/L for each component.

Table 3.5: Different chemical compounds tested using HI-Plex H column

No.	Substance
1	Sodium bicarbonate ($NaHCO_3$)
2	Sodium carbonate (Na_2CO_3)
3	Sodium fluoride (NaF)
4	Sodium sulphate (Na_2SO_4)
5	Sodium bromide (NaBr)
6	Potassium chloride (KCl)
7	Magnesium chloride ($MgCl_2$)
8	Sodium chloride (NaCl)
9	Maltose ($C_{12}H_{22}O_{11}$)
10	Sucrose ($C_{12}H_{22}O_{11}$)
11	Tri-sodium phosphate (Na_3PO_4)
12	Lactose ($C_{12}H_{22}O_{11}$)
13	Tri-sodium citrate ($Na_3C_6H_5O_7$)
14	Citric acid ($C_6H_8O_7$)
15	Glucose ($C_6H_{12}O_6$)
16	Galactose ($C_6H_{12}O_6$)
17	Xylose ($C_5H_{10}O_5$)
18	Fructose ($C_6H_{12}O_6$)
19	Mannitol ($C_6H_{14}O_6$)
20	Sorbitol ($C_6H_{14}O_6$)
21	Arabinose ($C_5H_{10}O_5$)
22	Succinic acid ($C_4H_6O_4$)
23	Sodium succinate ($C_4H_4Na_2O_4$)
24	Lactic acid ($C_3H_6O_3$)
25	Glycerol ($C_3H_8O_3$)
26	Formic acid (CH_2O_2)
27	Sodium acetate ($C_2H_3NaO_2$)
28	Acetic acid (CH_3COOH)
29	Ethanol (C_2H_6O)

3.13.5 Preparation of stock solutions of NaCl for calibration standards, and quality control samples

Calibration solutions of NaCl (Fisher, UK) at concentrations of 40.00, 20.00, 10.00, 5.00, 2.50, 1.00, 0.50 and 0.25 g/L were prepared in distilled water. These solutions were freshly prepared in triplicate on the day of analysis and the experiment was repeated on four different days. The results were used to generate a standard curve as well as to investigate intra- and inter-run variation.

Quality control samples of NaCl in distilled water at concentrations of 30.00, 15.00, 7.50, 3.00, 2.00 and 1.00 g/L were prepared in quadruplicate. The quality control samples were used to validate the accuracy and reproducibility of the standard curves.

3.13.6 Validation of the procedures

The selectivity of the methodology (the validation of the peak and retention time) was evaluated by using different NaCl solutions of different grades (analytical grade, rock lab grade and food grade). Different NaCl grades were used to make sure the peak at 10.90 min. correlate to the Cl- in the seawater and not any other organic substances from the sea.

The validation was carried out following formerly reported procedures (Marin et al., 2007; Shah et al., 2000). Validation of the chromatographic method was carried out for two concentration ranges; high concentration range (40.00 to 5.00 g/L) and low concentration range (2.50 to 0.25 g/L) and was determined by applying 4 sets of calibrations in triplicate at 4 levels for each concentration range. Quality control samples at 6 concentrations and from different stock solutions were also applied.

Calibration graphics in the range of 5.00 - 40.00 g/L and the range of 2.50 - 0.25 g/L NaCl were plotted based on the peak areas of NaCl on axis y against the respective nominal concentrations on axis x. All calibration curves were required to have a correlation value of at least 0.998. The intra-run and inter-run accuracy and precision of the assay were assessed by the average relative percentage deviation (DEV%) from the nominal concentrations and the coefficient of variance (CV) values, respectively, based on reported guidelines (Marin et al., 2007; Shah et al., 2000). Precision (CV) and accuracy (DEV%) were calculated by the following Equations 3.1 and 3.2:

CV (%) = (SD/ Average calculated concentration) x 100 ---------- (3.1)

DEV (%) = (1- Average calculated concentration / Nominal concentration) x 100 -------- (3.2)

Intra-run (n = 3) and Inter-run (n = 12) precision and accuracy of the analytical method were determined from the results of 2 groups of calibration curves run on 4 different days. Quality control samples containing the 6 concentrations of NaCl were evaluated from the obtained calibration curves. The intra-run precision and accuracy measurements were also performed on the quality control samples (n = 4).

3.13.7 Limit of quantification (LOQ) and Limit of detection (LOD)

LOQ was determined by considering the lowest concentration with a precision expressed by CV of lower than 20% and accuracy expressed by DEV% also lower than 20%, and a Signal to-noise ratio (S/N) greater than 10.0. LOD was determined at the lowest detectable concentration with S/N greater than 3.0 (Marin et al., 2007; Shah et al., 2000).

3.13.8 Application of the method

Salt content in 15 food samples, purchased from a retail market, was determined as NaCl concentration using our new method. The results were compared with three other methods as listed below in sections 2.8.3 and 2.8.4.

3.13.8.1 Sample preparation

Liquid samples (1 to 10) were filtered using a 0.45 µm syringe filter (Millipore, UK). Four grams of each solid sample (11 - 15) were placed into a 50-mL falcon tube. Then, 40 mL of a hot deionised water (85^{o}C) were added to each falcon tube. The samples were dissolved by vortexing the falcon tube for 5 min. The tubes were then incubated in a water bath at 85^{o}C for 10 min, then vortexed again for 1 min. The suspensions were filtered using a glass microfiber filter (pore size, 1.2 µm; Whatman®) then they were filtered again using a 0.45 µm syringe filters (Millipore, UK). Cheese samples (11 &12) were mashed in a porcelain mortar before salt extraction.

3.13.8.2 Using the new HPLC method for simultaneously measuring NaCl, sugars, organic acids and alcohols in food samples

Prepared food samples were injected (directly without dilution) into the HPLC system using an auto sampler with an injection volume of 10 µL as described in section 2.3. The total running time was 32 min. All measurements were carried out in triplicate. Means with standard deviations of triplicate determinations were reported.

3.13.8.3 Using ATAGO salt meter to measure salt content in food samples

ATAGO pocket salt meter (PAL-ES2, Japan) is a typical equipment used for testing the level of salinity in a solution, which is based on the conductivity of the sample. Prepared food samples were used directly (without dilution) as the equipment states that the measurement range from 0.1 to 50 g/L. All measurements were carried out in triplicate and the measuring cell was rinsed with distilled water after each test. Means with standard deviations of triplicate determinations were reported.

3.13.8.4 Using Ion Chromatography (IC) to measure NaCl content in food samples

Prepared food samples were diluted to an expected Cl^- range between 10 and 200 ppm. The Dionex ICS-1100 ion chromatography System (Thermo Scientific) was used to measure the Cl^- in the samples. Separation of Cl ions was achieved by using IonPac AG14A Carbonate Eluent Anion-Exchange Column while IonPac AS14A Carbonate Eluent Anion-Exchange Column was used as a guard column. The flow rate of the mobile phase was 1.4mL/min containing; a) 3.5 mM Na_2CO_3 and 0.1 mM $NaHCO_3$. The separation was achieved at 30ºC and the total running time was 12 min.

The concentration of sodium chloride in the liquid samples was calculated using Equation 3.3. The concentration of sodium chloride in the solid samples was calculated using Equation 3.4.

$$\boldsymbol{C_{NaCl} = C_{Cl^-} \times \frac{molecular\ weight\ of\ NaCl}{molecular\ weight\ of\ Cl^-} \times D}$$ ---------- Equation (3.3)

$$\boldsymbol{C_{NaCl} = C_{Cl^-} \times \frac{molecular\ weight\ of\ NaCl}{molecular\ weight\ of\ Cl^-} \times D \times \frac{0.040\ L}{4.0\ g} \times 1000}$$ ---------- Equation (3.4)

C_{NaCl} is the concentration of NaCl in the sample (g/L for liquid sample, g/kg for solid sample)

C_{Cl^-} is the concentration Chloride ion obtained from the IC method (g/L)

D is the dilution factor for IC analysis

3.13.8.5 Using Flame Photometer (FP) to measure NaCl content in food samples

Prepared food samples were diluted to several concentrations to achieve the suitable concentration range of sodium (1 – 20 ppm). Diluted samples were then injected manually in a flame photometer (Sherwood 410, Halstead, UK) to determine the sodium content. The method for using this flame photometer was previously described by Helrich (1990). Butane and air were supplied as the source of flame in this experiment. The flow rate of fuel was adjusted to obtain the maximum sensitivity. Standard curve of sodium in the concentration range from 5 to

20 ppm was prepared and the signal of the 20 ppm standard was checked several times during the analysis.

The concentration of sodium chloride in the liquid samples was calculated using Equation 3.5. The concentration of sodium chloride in the solid samples was calculated using Equation 3.6. Means with standard deviations of triplicate determinations were reported.

$$\boldsymbol{C_{NaCl} = C_{Na} \times \frac{molecular\ weight\ of\ NaCl}{molecular\ weight\ of\ Na} \times D}$$ ---------- Equation (3.5)

$$\boldsymbol{C_{NaCl} = C_{Na} \times \frac{molecular\ weight\ of\ NaCl}{molecular\ weight\ of\ Na} \times D \times \frac{0.040\ L}{4.0\ g} \times 1000}$$ ---------- Equation (3.6)

C_{NaCl} is the concentration of NaCl in the sample (g/L for liquid sample, g/kg for solid sample)

C_{Na} is the concentration sodium ion obtained from the FP method (g/L)

D is the dilution factor for FP analysis.

3.14 Statistical analysis

3.14.1 Mean and standard deviation

Most experiments throughout this thesis have been carried out in triplicate; therefore, the data reported are the mean values with standard deviation. The statistical analysis was carried out using Excel (Microsoft, USA).

3.14.2 R statistical computing environment

Data from the 24-hour time points from PM assays were analysed using Linkage analysis with jQTL (http://churchill.jax.org/software/jqtl.shtml), a java graphical interface for R/qtl package x86_64-w64-mingw32/x64 (R Development Core Team, 2008). The data was converted into comma delimited files and operated on a R workspace application. RGui 64 bit is a free to use software for statistical analysis package (http://ww1.rproject.org/). This package was used to compare the sugar utilisation in ROW, SW and SSW based media using 116 newly isolated marine yeasts.

Chapter 4:

4. Development of an efficient method for the isolation of marine yeasts strains

4.1 Introduction

The marine environment, which accounts for 71% of the planet's surface area, has not been widely explored for yeast isolation. Recently, marine yeasts have been identified as potential sources for producing valuable compounds such as biofuels, amino acids, proteins, vitamins, polysaccharides, fatty acids, phospholipids and enzymes (Chi et al., 2009, Sarkar et al., 2010). These products have been shown to have potential for commercial exploitation. Research on marine yeasts has highlighted several promising features over terrestrial yeast strains, e.g. higher tolerance to extreme environments and higher productivity (Zaky et al., 2014, Obara et al., 2015). Urano et al. (2001) has suggested that yeasts which inhabit a marine environment would have developed a mechanism to tolerate high osmotic stress (Urano, 2001). However, utilisation of marine yeasts for commercial purposes has not been widely established, this is principally due to a lack of research and limited availability of marine yeast isolates (Zaky et al., 2014, Chi et al., 2009, Zaky and Du, 2014).

Torula sp. and *Mycoderma* sp. were the first yeasts isolated from the marine environment in 1894 by Bernhard Fischer (Kutty and Philip, 2008). Since then various marine yeasts have been isolated from different marine sources, including seawater, sea sand (Wang et al., 2008, Khambhaty et al., 2013), seaweeds (Seshadri and Sieburth, 1975), fish and different marine animals (Burgaud et al., 2010). According to a recent report, the number of marine yeast species which have been classified and described has reached 213 species (Jones et al., 2015).

In this study, isolation of marine yeast strains using the existing methods was initially planned. However, the application of two existing methods to isolate marine yeasts using our samples did not give satisfactory results (as described in chapter 4.2.1 and 4.2.2 below). This led to the adjustment of the project orientation to develop an effective marine yeast isolation method in the first place. The development of an efficient and reliable isolation method for marine yeast will advance the employment of marine yeasts by introducing new species and strains with desired properties for research projects and industry. In general, the development of a marine yeast isolation method involves three aspects: (i) specify a sample collection technique; (ii) design a yeast isolation medium and (iii) propose a strain isolation protocol. The main challenges in marine yeast isolation are the relatively low yeast population present in marine

samples (Kutty and Philip, 2008, Fell, 2001) and the presence of filamentous fungi (Fell et al., 2011). To maximize the chance of obtaining a higher number of yeast strains from the marine samples, isolation was conducted within 2 days of sampling for most of the reported marine yeast isolation methods (Kutty and Philip, 2008, Ahearn et al., 1968). The growth of bacteria contained in the marine samples can be inhibited by the addition of antibiotics (Fell, 2001). However, growth of filamentous fungi could not be prevented, as there is no medium which is yeast specific and does not support the growth of filamentous fungi. Therefore, several strategies have been proposed in the isolation protocol development stage to minimize filamentous fungi growth, including reducing the incubation period (Dinesh et al., 2011, Khambhaty et al., 2013); reducing the amount of the sample to be used in the strain isolation (Dinesh et al., 2011); or reducing the incubation temperature (Fell et al., 2011, Nagahama et al., 1999). As a consequence of reducing the presence of filamentous fungi, yeast growth was also negatively affected.

The current methods for marine yeast isolation have several limitations, including (i) requirement of recent collected samples; (ii) utilising growth media that encourage mould growth; and (iii) producing low numbers of yeast isolates. Therefore, the objective of this chapter was to develop an efficient method for marine yeast isolation which included the design of a new enrichment and isolation media and the construction of a new isolation protocol. Particularly, the new method enables the usage of an aging marine sample for the potential isolation of marine yeasts. The results in this chapter constitute part of a paper published in journal of Microbiology and Biotechnology (Zaky et al., 2016)

4.2 Results

In this chapter, the development of a new and efficient method for marine yeast isolation was described. Initially, two methods from literature; Kutty method (Kutty, 2009) and Dinesh method (Dinesh et al., 2011) were applied - with a small modification as described in chapter 3 - on 14 marine samples which were collected for the current study. Results revealed no yeast colonies obtained from any sample using either method (as described in detailed in sections 4.2.1 and 4.2.2 below); as a result, a new method for the isolation of yeasts from marine environments was developed. The sample type, location and sampling season for the marine samples are listed in Table 4.1.

Table 4.1: Marine samples for yeast isolation

	Sample Type	Locations	Habitat	Sampling Time
A	Seawater	Alexandria, Egypt	Mediterranean Sea	March
B	Seawater	Suez, Egypt	Gulf of Suez	March
C	Seawater	Ras Sedr, Egypt	Gulf of Suez	March
D	Seawater	Ismailia, Egypt	Timsah Lake	March
E	Seawater	Wales 1, UK	Irish Sea	April
F	Seawater	Wales 2, UK	Irish Sea	April
G	Seawater	Plymouth1, UK	English Channel	March
H	Seawater	Plymouth2, UK	English Channel	March
I	Sea sand	Alexandria, Egypt	Mediterranean Sea	March
J	Seaweed	Plymouth3, UK	English Channel	March
K	Rotten Seaweed	Plymouth4, UK	English Channel	March
L	Seawater	Whitby, UK	North Sea	April
M	Seawater	New York, USA	North Atlantic Ocean	August
N	Seawater	San Diego, USA	North Pacific Ocean	August

4.2.1 The isolation of marine yeasts using the method described by Kutty et al (2009)

In this method, seawater samples and suspension of the solid samples were filtered using 0.45 μm nitrocellulose filters then the filters were placed face up on petri dishes that supplied with SW-YM medium. The plates were then incubated at room temperature (23 ±1°C). The plates were inspected daily to observe the growth of yeast colonies. No growth was observed from 3 samples (B, C or F) during 14 days of incubation while medium to heavy growth of mould colonies was obtained from the other 11 samples (Figure 4.1a & Table 4.2a).

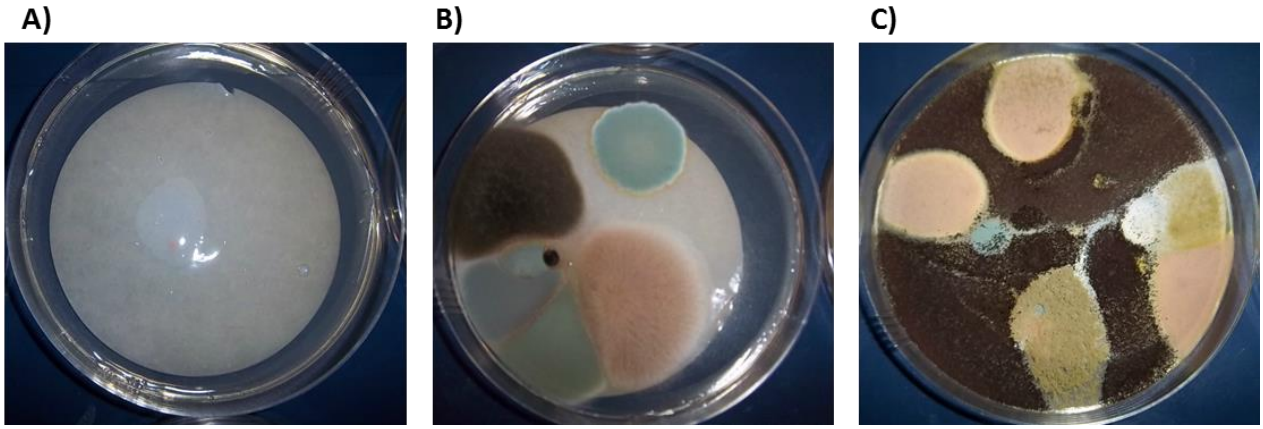


Figure 4.1a: Moulds load after applying Kutty method for yeast isolation from marine samples

A) No mould colonies (sample B); B) Low number of mould colonies (sample D); C) High number of mould colonies (sample G).

Table 4.2a: The Mould load on YM agar plates from marine samples using Kutty method for yeast isolation

Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Moulds Load	++	-	-	+	+	-	++	++	++	+	+	++	+	++

'-' No mould colonies; '+' Low number of mould colonies; '++' High number of mould colonies.

4.2.2 The isolation of marine yeasts using the method described by Dinesh et al (2011)

This method uses a pour plate technique using Sabouraud's Dextrose Agar (SDA) medium which was prepared using 50% seawater and incubation at 35°C. No growth of any kind of microorganisms was observed after 2 days incubation. The inhibition of bacterial growth was achieved by adding antibiotic to the medium. The incubation time of this method (2 days) was not enough for the formulation of mould colonies. The absence of yeast colonies maybe due to the very low number of yeast cells in the sample - especially the samples were 2-3 weeks old - that made it impossible to isolate yeasts from 1 mL of the sample. The incubation of the plates was continued for another 5 days. Inspection of the plates after 7 days of incubation revealed that mould colonies was present on 10 out of 14 samples (Figure 4.1b and Table 4.2b).

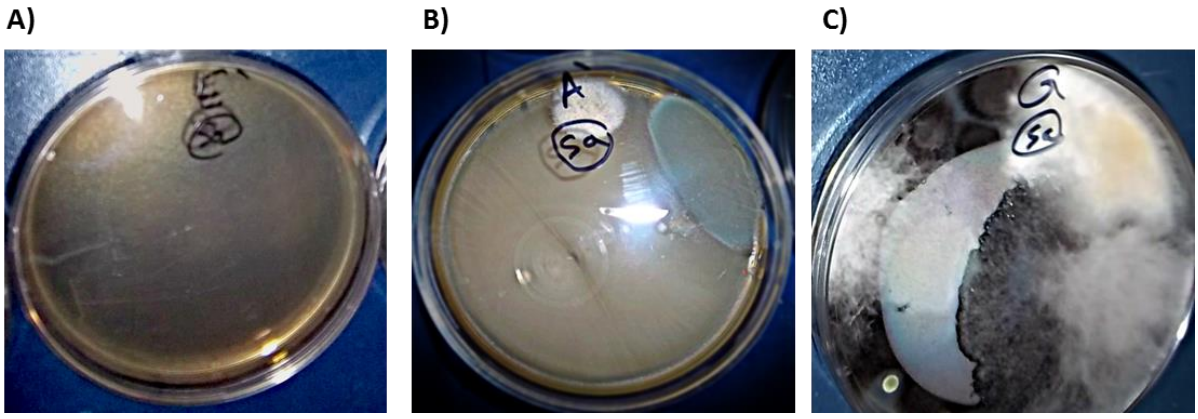


Figure 4.1b: Filamentous fungal load on SDA medium from marine samples after 7 days of incubation

A) No mould colonies (sample E); B) Low number of mould colonies (sample A) ; C) High number of mould colonies (sample G).

Table 4.2b: Mould load on SDA plates from marine samples after 7 days of incubation

Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Moulds Load	+	-	-	+	-	-	++	++	++	+	+	+	+	+

'-' No mould colonies; '+' Low number of mould colonies; '++' High number of mould colonies.

4.2.3 Development of a new methodology for the isolation of yeasts from a marine environment

The results obtained from the above-mentioned isolation methods revealed that the samples contained very low number of yeast cells and relatively high number of moulds. Hence, these two methods for marine yeast isolation were not applicable to our samples and there was a need for a new isolation method.

Based on the results obtained above (Figure 4.1a and 4.1b), the main challenges of marine yeast isolation seem to be the lack of yeast growth in the isolation medium and the presence of moulds in the samples. Therefore, in the first step, an enrichment medium (Zaky's enrichment medium) was prepared using high sugar concentration supplied with nutrients that support the faster growth of yeast when compared with filamentous fungi. Enrichment has been suggested in this study to increase the number of yeast cells and reduce moulds in the samples at the same time, as yeast cells are expected to grow faster than moulds in a liquid medium containing high sugar concentration (Zaky et al., 2016). Therefore, a 100 mL seawater sample was mixed with equal amount of Zaky's enrichment medium (double strength) or 20 g of each solid sample (sea sand or seaweed) was inoculated in 180 mL of Zaky's enrichment medium; and then all inoculated

media were incubated aerobically at 30°C, at 150 rpm for 48 hours. Microscopic inspection for the enriched culture after 2 days of incubation revealed that high numbers of yeast cells were obtained but filamentous fungi were also present in the yeast culture (Figure 4.2A). The media and culturing conditions have certainly succeeded to encourage the growth of yeast cells and produced large number of yeasts. The presence of filamentous fungi in the enriched cultures was due to their presence in the original samples. In order to reduce the number of filamentous fungi in the yeast cultures, there followed a second enrichment cycle. The second cycle of enrichment used the same enrichment medium and incubation condition as with the first enrichment cycle using a 10% (v/v) inoculation from the first enrichment cycle to the second.

Inspection for the culture obtained from the second enrichment cycle revealed that the number of yeast cells had increased and the presence of moulds had decreased (Figure 4.2B).

A third enrichment cycle was then conducted to further decrease the occurrence of filamentous fungi in the yeast cultures and to screen for yeast strains with potentially desirable sugar utilisation. Therefore, in the third cycle of enrichment, different types of sugar were used in preparing the enrichment media (Zaky's isolation broth media). The microscopic inspection on the cultures obtained from the third enrichment cycle showed high numbers of yeast cells without any presence of filamentous fungi. By the end of the enrichment step, yeast cell numbers reached 10^5 - 10^8 cells/mL, while the growth of bacteria and filamentous fungi was eliminated.

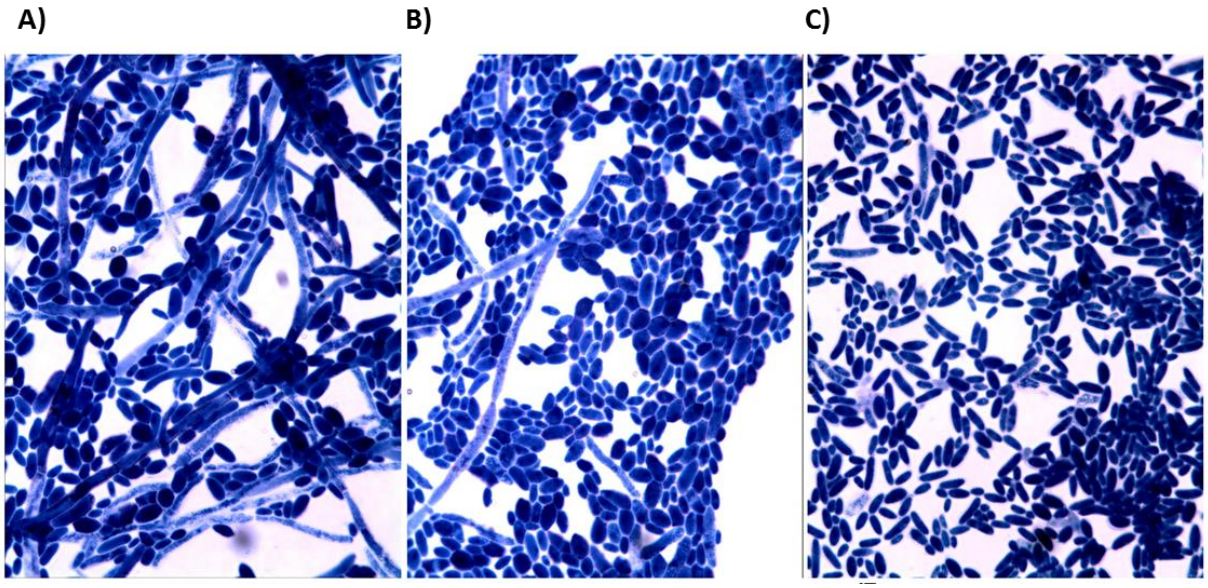


Figure 4.2: Microscopic image (1000x) of the cultures after each enrichment cycle

A) primary enrichment cycle; B) scale up enrichment cycle; C) differential enrichment cycle. Each cycle of enrichment was conducted for 48 h.

Following three cycles of enrichment, the isolation of yeasts was conducted using Zaky's isolation using serial dilution and pure plate technique. Isolation plates were incubated at 30°C. Large numbers of yeast colonies were obtained with no obvious bacterial or mould colonies were observed on the agar plates after 48 hours of incubation (Figure 4.3).

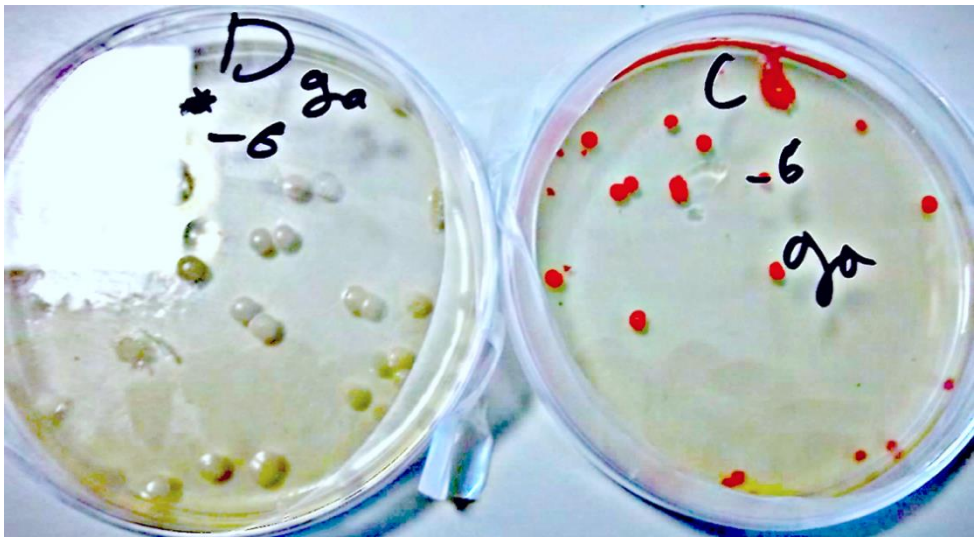


Figure 4.3: Example of yeast colonies on the isolation plates

Table 4.3 shows the yeast viable count (YVC) of the isolates from the samples under investigation. When glucose was used as the carbon source in the third cycle of enrichment and isolation media, the YVC ranged from 24.7±5.03 to 220±20.82 million CFU/mL. Similar YVC range was obtained when galactose was used as the carbon source for the final cycle of enrichment and isolation media. In comparison with glucose and galactose, using xylose as the carbon source resulted in a significant reduction of YVC in almost all samples, indicating glucose and galactose were the preferred substrates for marine yeasts. Starch was also used as a sole carbon source in the last step of enrichment. Isolates were obtained in 5 samples, and YVC ranged from 0.25±0.13 to 1.80±0.15 million CFU/mL. When cellulose was used, isolates were detected in 3 samples (F, H, & J), and YVCs were 2.0±0.46, 0.55±0.13 and 0.5±0.46 million CFU/mL, respectively (Table 4.3).

Table 4.3: Yeast viable count (YVC) of the isolates for 14 marine samples using the new isolation method that was developed in this study

Isolation media	Glucose	Xylose	Galactose	Starch	Cellulose
Samples	**CFU/mL (million[a])**				
A	206.67±20.8	32.67±4.0	230.00±20.0	N/A	N/A
B	220.00±20.0	5.53±0.5	45.33±4.5	N/A	N/A
C	49.00±3.6	N/A	25.67±6.0	0.75±0.1	N/A
D	24.67±5.0	14.33±4.0	86.33±4.0	1.60±0.3	N/A
E	42.67±5.0	1.77±0.6	17.33±5.0	N/A	N/A
F	129.00±5.6	7.97±2.3	83.67±7.8	N/A	2.00±0.5
G	24.33±4.5	12.33±2.5	68.33±3.5	1.80±0.2	N/A
H	91.67±3.5	34.67±5.0	20.67±3.1	N/A	0.50 ±0.1
I	73.33±6.7	25.33±4.5	38.67±9.2	N/A	N/A
J	38.33±5.7	39.33±5.5	61.67±7.8	N/A	0.55±0.1
K	27.00±4.6	14.67±3.5	N/A	N/A	N/A
L	122.67±7.4	30.33±6.1	84.67±6.1	N/A	N/A
M	106.33±12.1	4.37±0.8	33.67±6.5	0.75±0.1	N/A
N	169.33±14.4	31.00±15.1	33.33±7.6	0.25±0.1	N/A

N/A: Data Not Applicable because the test was not conducted (as in starch and cellulose) or the colonies were not present on the plates of the 5^{th} dilution (as with xylose and galactose).

Different colony morphologies such as colour (white, cream, yellowish, red), shape (circular, oval, irregular, spindle, star, triangle), size (small, medium, large), surface (smooth, fluffy, rough, dry) and elevation (flat, raised, convex) were observed on the isolation plates. Colonies that were present on the same plate and had the same appearance were considered to be the same strain. A total of 116 yeast colonies were selected from all samples. The selected colonies represent different sample sources, different isolation media and different colony morphology (Table 4.4).

Table 4.4: Distribution of new marine yeast isolates

	Numbers of isolates using different substrates					
Sample	**Glucose**	**Xylose**	**Galactose**	**Starch**	**Cellulose**	**Total Isolates**
A	4	3	3	N/A	N/A	10
B	3	2	2	N/A	N/A	7
C	7	0	2	3	N/A	12
D	4	2	2	2	N/A	10
E	1	0	2	N/A	N/A	3
F	3	2	2	N/A	3	10
G	4	3	2	3	N/A	12
H	4	2	2	N/A	1	9
I	3	0	3	N/A	N/A	6
J	3	1	3	N/A	6	13
K	2	1	2	N/A	N/A	5
L	3	0	0	N/A	N/A	3
M	2	3	2	1	N/A	8
N	3	2	2	1	N/A	8
Total	46	21	29	10	10	116

Following the third enrichment step, purity of the 116 selected colonies was confirmed by streaking them on an agar plate containing SW-YM agar medium followed by microscopic examination using methylene blue slides and preparing agar slants and glycerol stocks of the pure isolates. Microscopic images showed that some of the isolates were polymorphological yeast like cells (Figure 4.4).

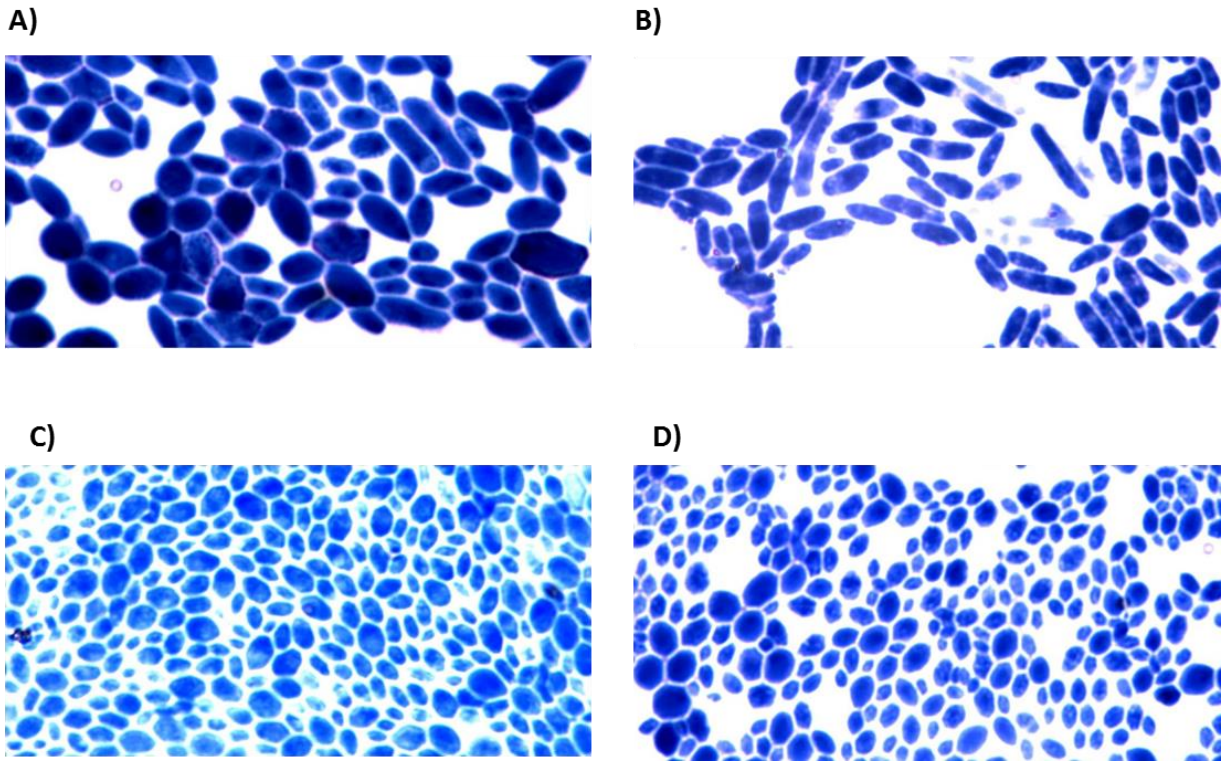


Figure 4.4: Example of polymorphological yeast like cells of some strains isolated from different marine samples

A) Strain S11 from sample A; B) Strain S126 from sample L; C) Strain S130 from sample M; D) Strain S131 from sample M.

4.2.4 Comparison between three methods for the isolation of yeasts from some marine environments

Three methods of isolation were used in this study. They are different in sample preparation, isolation media, incubation conditions and the time required for isolation. Table 5, compares the three isolation methods which were used in this study.

Table 4.5: Comparison of the three marine yeast isolation methods used in this study

	Zaky's Method (developed in this study)	Kutty's method[a]	Dinesh's method[b]
Sample Preparation	Enrichment 3 cycles (48 hours each)	Filtration filters (0.45 µm)	-
Media[c]	Zaky's Media (enrichment, isolation broth & isolation agar)	YM agar (Wickerham's medium)	Sabouraud's Dextrose Agar (SDA)
Antibiotic	penicillin G + streptomycin sulphate (500 mg/L each)	Chloramphenicol (200 mg/L)	-
pH	5.0	7.0	5.6
Incubation Temp.	30°C	18 ±2°C	35°C
Total Time	Maximum of 10 Days	At least 16 Days	At least 4 Days
Sample size	10 g or 100 mL	30 - 100 mL	1 g
Sample	Up to 3 weeks old samples	New samples only	New samples only
Number of isolates	Very high	Low	Very low
Specificity	Specific to yeasts	Specific to fungi	Not specific
Applicability	Suitable for both liquid and solid marine sample	Fresh liquid samples	Fresh solid samples

[a] This method was carried out based on the procedure suggested by Kutty et al, 2009.

[b] This method was carried out based on the procedure suggested by Dinesh et al, 2011.

[c] Detailed medium composition reported in Materials and Methods.

4.3 Discussion

The samples used for marine yeast isolation in the current study were collected from coastal water within a one-meter depth. Near shore was chosen as a location for sampling in this study because it was expected to contain a larger number of yeasts compared with off shore sites (Kutty and Philip, 2008, Ahearn et al., 1968) and to have a higher possibility of obtaining marine yeasts with potential characteristics for industrial application. Most reported marine yeast isolation methods were carried out using fresh samples, e.g. usually within 2 days of sampling, to avoid the reduction of yeast cell number in the sample (Fell et al., 2011, Wang et

al., 2008). This limited the potential of finding novel yeast strains from marine samples out of the local area as well as studies requiring samples from diverse marine habitats. The new isolation protocol reported in this study successfully overcame this limiting factor and maintained a high number and variety of yeast strains. It has been reported that filamentous fungi are present in high numbers in samples taken near the shore (Fell et al., 2011). This was confirmed by the results obtained in this study, since filamentous fungal colonies were observed from 10 out of 14 marine samples when Kutty's method was used. Kutty's method utilizes Wickerham's yeast medium (YM medium), which is a rich medium suitable for mould growth as well as for yeasts (Zaky et al., 2016). There was no yeast, bacterial or fungal growth when Dinesh's method was used for isolation. This was probably due to the small volume of the samples (1 mL) recommended by the Dinesh's method as well as the samples being old. It was reported that near shore seawater samples only contain 10-10000 yeast cells per litre (Kutty and Philip, 2008, Fell, 2001). Therefore, 1 mL of the sample may not contain any yeast cells for isolation.

The fact that our new isolation method generated a large number of pure yeast colonies from relatively old samples was mainly because of the 3 enrichment cycles. Generally, filamentous fungi propagate slower than yeasts. The incubation period in fermentations using filamentous fungi is generally 3-7 days; whilst for yeasts it is normally 1-2 days (Cavka and Jo¨nsson, 2014, Nasr et al., 2010). In the new marine yeast isolation method, the sub-culturing time was selected to be 2 days so that moulds did not have enough time to increase their number. So, when the enriched culture was used to inoculate the next enrichment cycle, the number of moulds was reduced. Throughout the 3 cycles of enrichment step, the reproduction of filamentous fungi was inhibited, while a rapid yeast growth was maintained.

In our new isolation method, 30^{o}C was used for the cell growth, which is higher than the normal temperature of seawater. This was due to the following considerations: (a) 30^{o}C is the preferred temperature for the industrial application of the potential isolated yeasts, higher temperatures correlates with a faster fermentation process and therefore higher productivity; (b) It has been demonstrated that although marine yeast strains' habit temperature is relatively low, their optimum growth temperature could be higher. Kutty (2009) studied the effect of temperature on the growth of marine yeasts obtained from slope sediments of Arabian Sea at different depths (up to 100 m), where the temperatures range between 6 - 16°C. It was concluded that the maximum growth was observed at 30°C for almost all isolates (Kutty, 2009).

4.4 Conclusion

Two methods for marine yeast isolation were tested on the marine samples collected for this study but no yeast isolates were obtained. Hence, an efficient and selective method for marine yeast isolation was developed. The new method includes; 3-cycle enrichment step followed by isolation step and confirmation step. By applying this method on marine samples, a large number of marine yeast isolates were obtained without any bacterial or filamentous fungal growth. 116 marine yeast isolates were selected for further investigations. These isolates were selected to represent different sample sources, different isolation media, and different cell and colony morphology. The new method for marine yeast isolation was easily adapted to either liquid or solid marine samples. This method took 8-10 days to obtain a large number of pure yeast isolates. It was successfully applied to samples up to 3 weeks old so fresh samples are not necessary. This methodology allows for the isolation of yeasts that are present at very low numbers in the original sample.

CHAPTER 5:

5. Evaluation and identification of novel marine yeasts

5.1 Introduction

New yeast isolates should be evaluated for their potential importance for industrial applications. Sugar utilisation and tolerance to inhibitory compounds are important criteria for industrial fermentations, especially for biofuel fermentation. Phenotypic microarray analysis of fermentable monomeric sugars has revealed to be a suitable technique for screening yeast isolate for bioethanol fermentations using hydrolysates derived from lignocellulosic materials (Greetham et al., 2014)

Yeast isolates were identified according to a number of different criteria such as cell morphology (e.g., spore shape and mode of cell division), immunology, physiology (e.g., sugar fermentation). Currently, there are many commercial yeast identification kits (e.g., API bioMe´rieux and BioLog YT MicroPlate) which are based on yeast growth and cell metabolism. However, use of molecular techniques e.g., amplified fragment length polymorphism (AFLP) of Domains 1 and 2 (D1/D2) and the internal transcribed spacer (ITS) is being increasingly used to categorise new species (Pincus et al., 2007).

The improvement of D1/D2 and ITS database in GenBank (GenBank) allows laboratories around the world to easily and accurately identify more yeast species. Furthermore, phylogenetic analysis of the gene sequences is leading to a major modification of yeast systematics that will result in redefinition of almost all genera (Kurtzman et al., 2015).

In the previous chapter, 116 marine yeast isolates were obtained using the new method for marine yeast isolation, which was developed in this study. In this chapter, the new isolates were assessed for their ability for utilising fermentable sugars in seawater-based media. Yeasts which displayed improved sugar utilisation were identified by sequencing the ITS and D1/D2 domains and by using YT Micro-plate technique (BioLog). The results in this chapter constitute part of a paper published in journal of Microbiology and Biotechnology (Zaky et al., 2016)

5.2 Results

5.2.1 Determination of yeast metabolic output on monomeric fermentable sugars using a phenotypic microarray assay

The ability of the 116 marine yeast isolates to utilize glucose, mannitol, xylose and galactose in Reverse Osmosis Water (ROW), seawater (SW) or double synthetic seawater (2x SSW) based media was assessed using a Phenotypic Microarray (PM) assay. The terrestrial yeast *S. cerevisiae* NCYC2592 was included in this study as a reference strain for its high sugar utilization rate and high tolerance to inhibitors. *S. cerevisiae* NCYC2592 had been well defined previously for sugar utilisation and tolerance to the presence of inhibitory compounds (Zaki et al., 2014, Oshoma et al., 2015). BioLog 96-well plates were loaded with different biological media containing Yeast Nitrogen Base (YNB), dye D (tetrazolium violet) and one of the sugars under investigation (glucose, mannitol, xylose or galactose) at the concentration of 6% (w/v). Plates were then inoculated with the yeast cells and incubated at 30°C in the OmniLog plate reader (BioLog, CA, US) for 24 hours (detailed procedure in section 3.8). The results of sugar utilization were considered positive only if the redox signal intensity was higher than 20.

5.2.1.1 Glucose utilisation

The ability of glucose utilisation by the 116 newly isolated marine yeasts and the reference strain were tested in SW, ROW and 2x SSW based medium. Results revealed that 96 out of the 116 marine isolates were able to utilise glucose in a SW-based medium and 10 of the marine isolates utilised higher amount of glucose than the reference strain (Figure 5.1A). Similarly, 96 marine isolates were able to utilise glucose using ROW-based medium but 10 marine isolates utilised higher amount of glucose than the reference strain (Figure 5.1B). Using 2x SSW-based medium, 100 marine isolates were able to utilize glucose and 13 of them were able to utilised higher amount of glucose than the reference strain (Figure 5.1C).

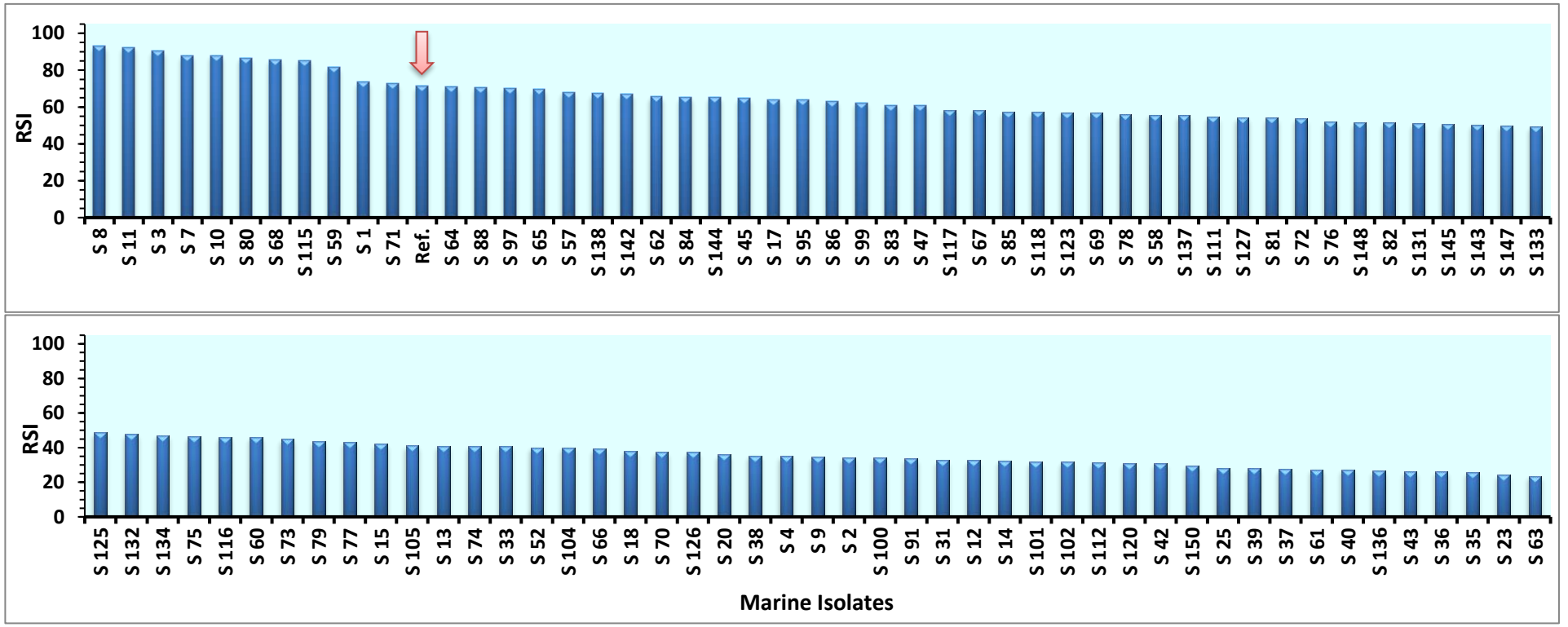


Figure 5.1A: Screening and evaluating 116 new marine yeast isolates for their ability to utilise glucose in SW-based mediun using PM assay

RSI: Redox signal intensity.

The small arrows point out the position of the reference strain *S. cerevisiae* NCYC2592.

Strains with RSI below 20 were considered not glucose utilisers and they were not presented in the graph.

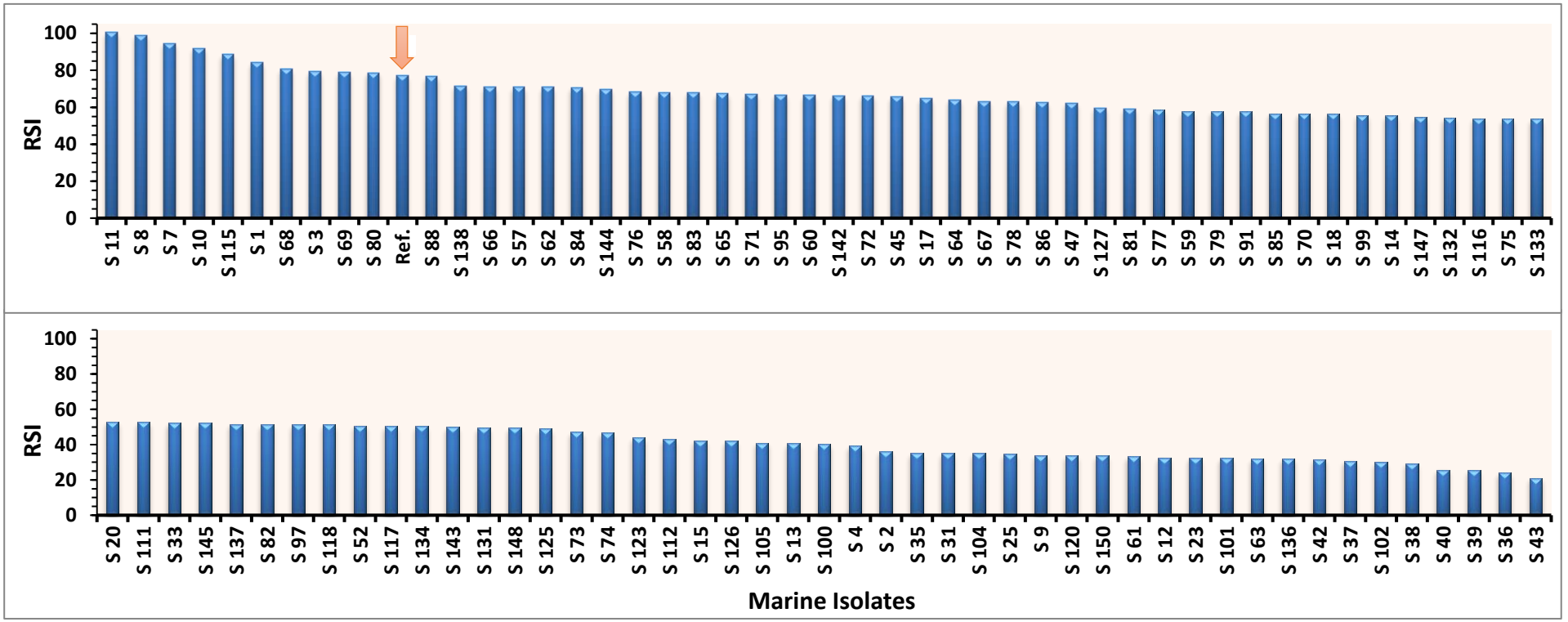


Figure 5.1B: Screening and evaluating 116 new marine yeast isolates for their ability to utilise glucose in ROW-based mediun using PM assay

RSI: Redox signal intensity.
The small arrows point out the position of the reference strain *S. cerevisiae* NCYC2592.
Strains with RSI below 20 were considered not glucose utilisers and they were not presented in the graph.

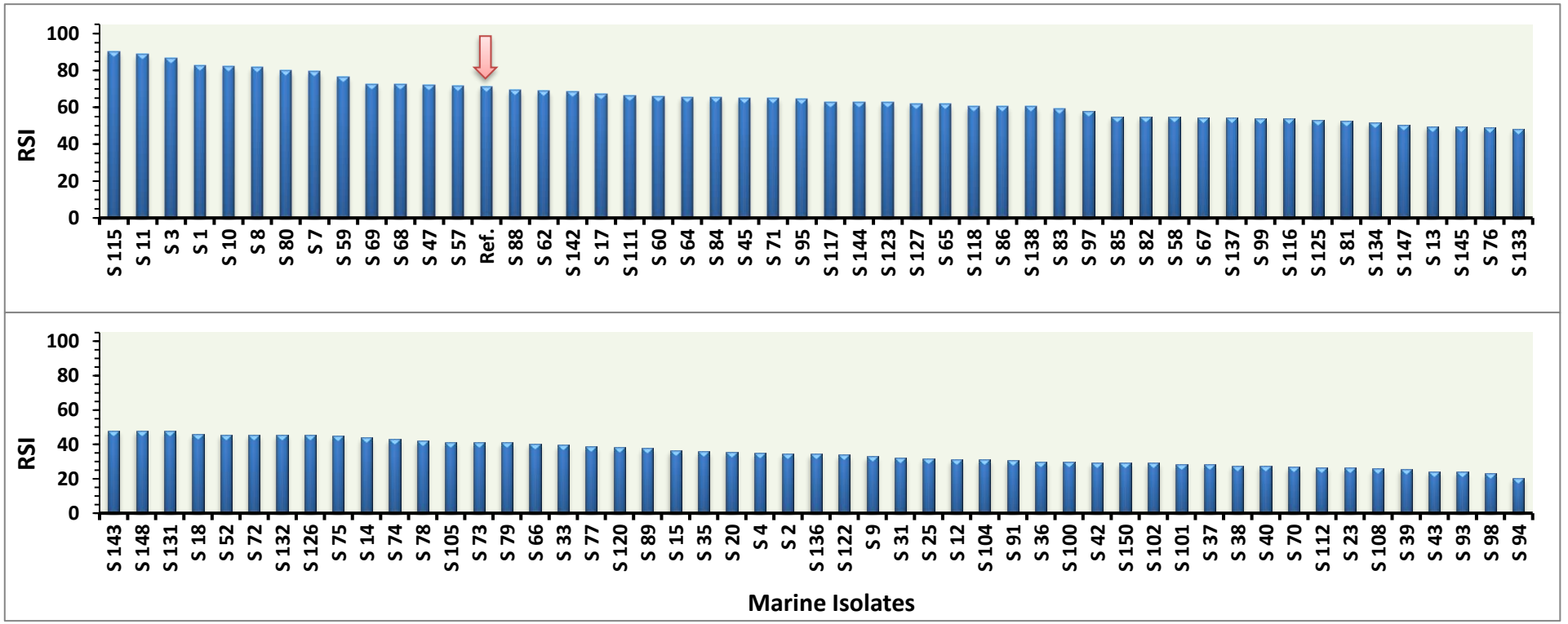


Figure 5.1C: Screening and evaluating 116 new marine yeast isolates for their ability to utilise glucose in 2xSSW-based mediun using PM assay

RSI: Redox signal intensity.

The small arrows point out the position of the reference strain *S. cerevisiae* NCYC2592.

Strains with RSI below 20 were considered not glucose utilisers and they were not presented in the graph.

5.2.1.2 Mannitol utilisation

The 116 newly isolated marine yeasts were screened for their ability to utilise mannitol as a sole carbon source in a SW-based medium with the reference strain, *S. cerevisiae* NCYC2592, also included for comparison.

38 out of the 116 marine isolates were able to utilise mannitol of these, 26 displayed improved utilisation when compared with the reference strain (NCYC2592) (Figure 5.2).

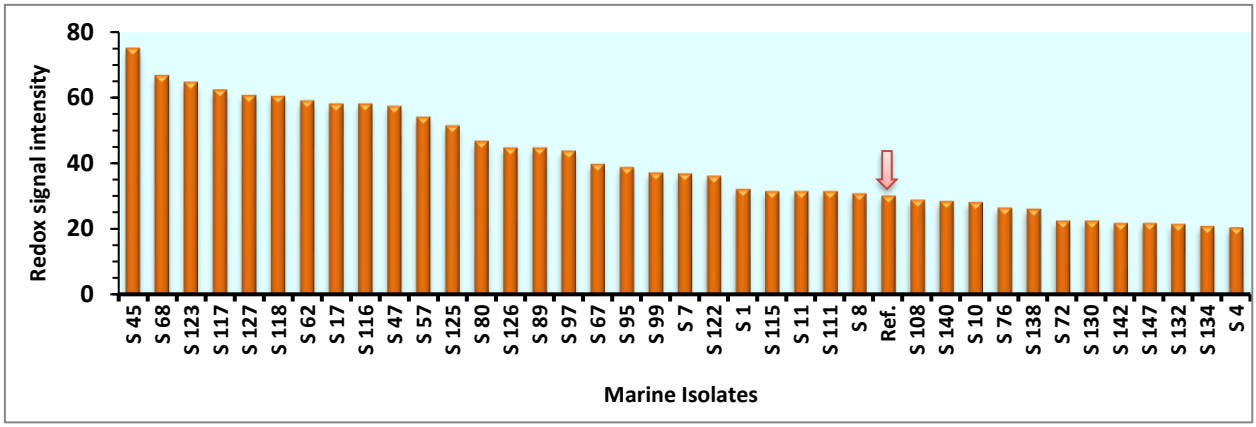


Figure 5.2: Screening and evaluating 116 newly isolated marine yeast isolates for their ability to utilise mannitol (6%) in seawater-based medium using a phenotypic microarray assay

RSI: Redox signal intensity.
The small arrows point out the position of the reference strain *S. cerevisiae* NCYC2592.
Strains with RSI below 20 were considered not mannitol utilisers and they were not presented in the graph.

5.2.1.3 Xylose utilisation

The 116 newly isolated marine yeasts were screened for their ability to utilise xylose as a sole carbon source in a SW and ROW based medium. 20 marine strains were able to utilise xylose whereas the reference strain (NCYC2592) showed no xylose utilisation capabilities (Figure 5.3).

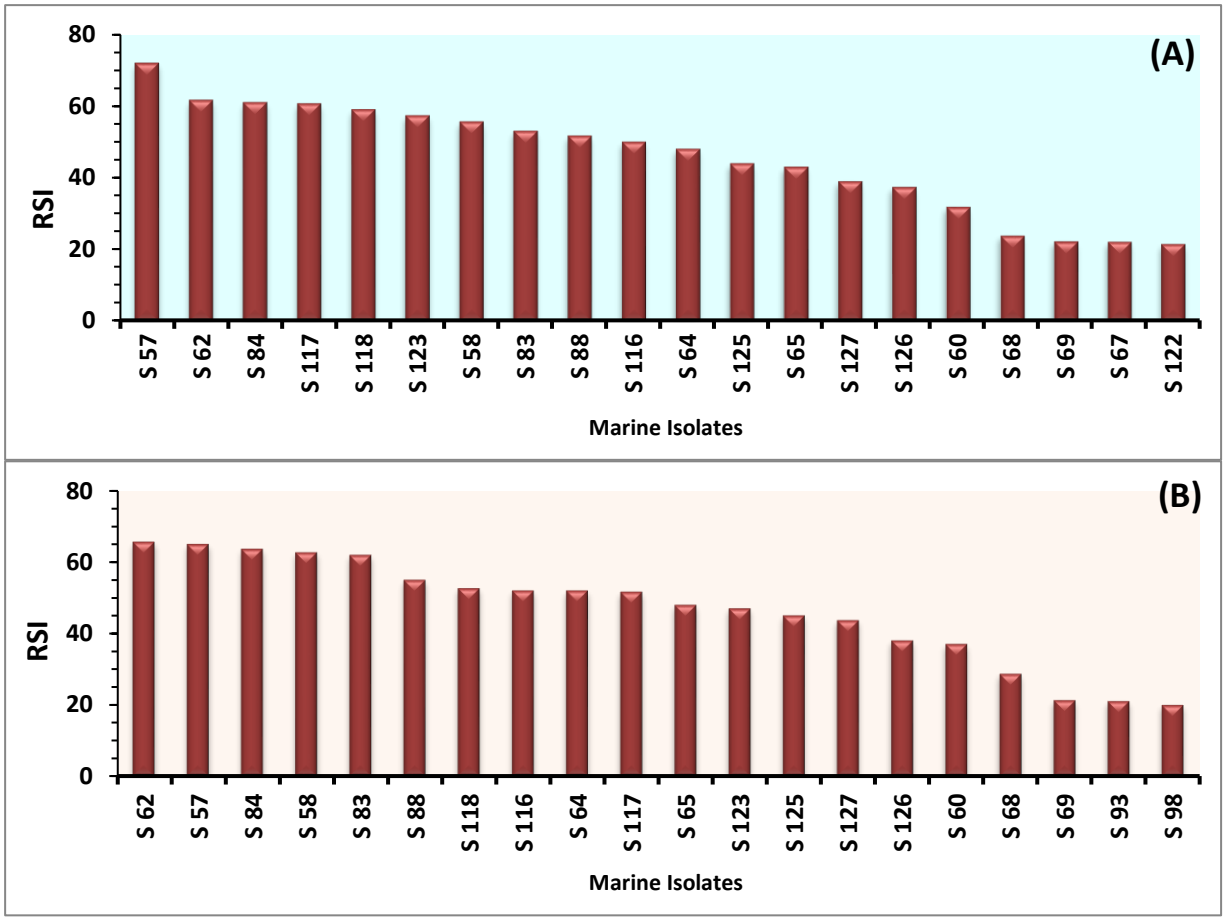


Figure 5.3: Screening and evaluating 116 newly isolated marine yeast isolates for their ability to utilise xylose using a phenotypic microarray assay

(A) Xylose 6% in SW-based medium; (B) Xylose 6% in ROW-based medium.
RSI: Redox signal intensity.
Strains with RSI below 20 were considered not xylose utilisers and they were not presented in the graph.

5.2.1.4 Galactose utilisation

The 116 newly isolated marine yeasts were screened for their ability to utilise galactose as a sole carbon source in a SW and ROW based medium. 39 out of the 116 marine isolates were able to utilise galactose and of these, 3 displayed improved utilisation of galactose when compared with the reference strain (NCYC2592) (Figure 5.4).

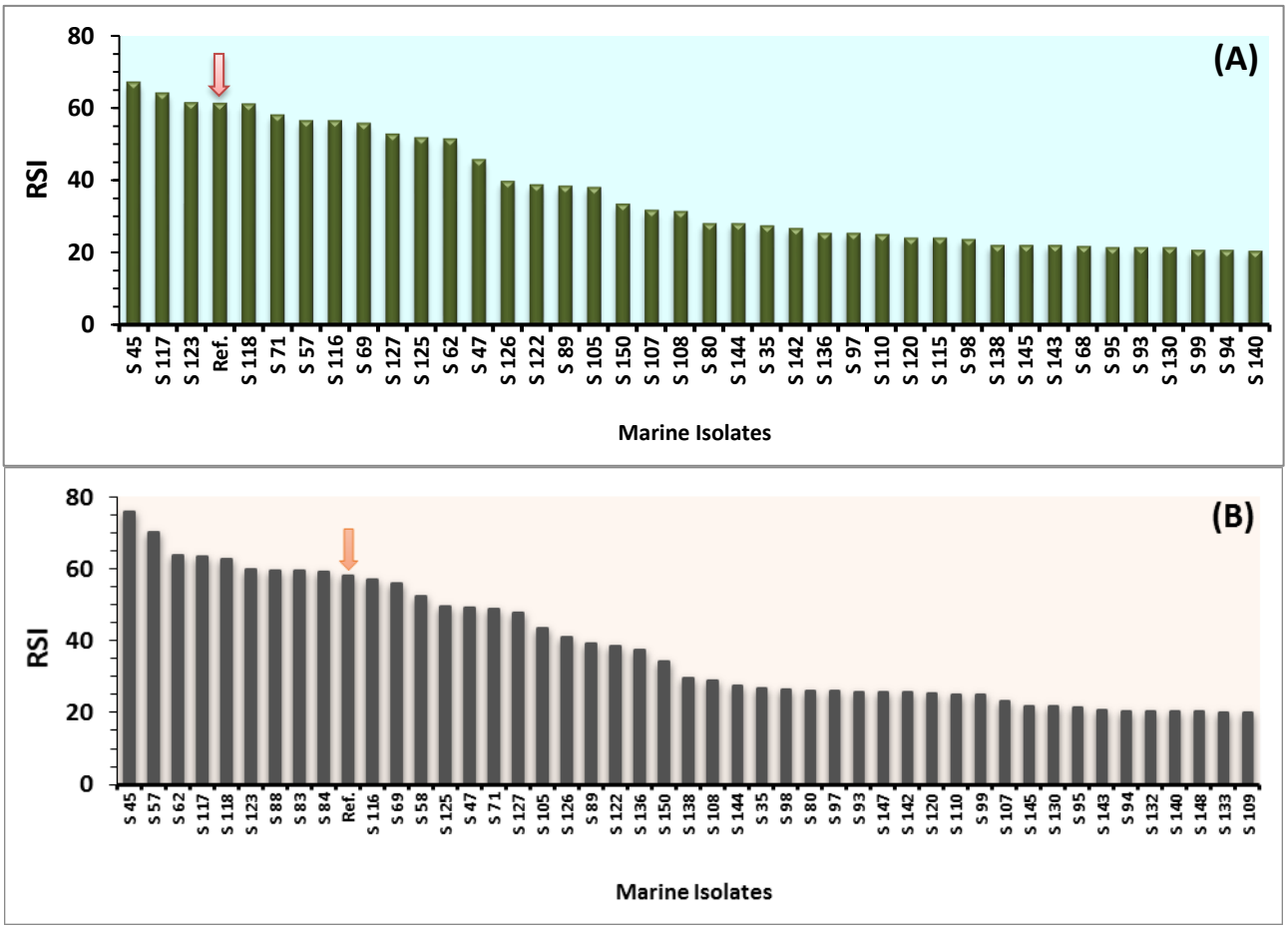


Figure 5.4: Screening and evaluating 116 newly isolated marine yeast isolates for their ability to utilisegalactose using a phenotypic microarray assay

(A) Galactose 6% in SW–based media; (b) Galactose 6% in ROW–based media
RSI: Redox signal intensity.
The small arrows point out the position of the reference strain *S. cerevisiae* NCYC2592.
Strains with RSI below 20 were considered not galactose utilisers and they were not presented in the graph.

5.2.2 Comparing the utilisation of sugars in ROW, SW and 2xSSW based medium using marine yeasts

Figure 5.5 shows the statistical comparison (using R) for the yeast population's (116 isolates) ability for utilising different sugars (glucose, mannitol, xylose, or galactose) in media prepared using ROW, SW and 2xSSW. Results were presented in the figure as squares with a number ($\leq$1.0) indicate similarity between populations of yeast using the sugars in question, R = 1 indicates identical response to those two sugars. Ranking of the marine yeast isolates according to their ability of utilising different sugars at a relatively high concentration (6%) using freshwater (ROW) or salt water (SW or 2xSSW) allowed us to estimate their ability to tolerate osmotic stress induced by the presence of sugars and salts in the culture media. Using this approach it was observed that yeast populations utilised glucose in ROW, SW and 2xSSW at very similar rates indicated by the high R score (0.90, 0.94 and 0.95). Xylose utilisation in ROW and xylose utilisation in SW was very similar with an R score of 0.97. Galactose utilisation differed between ROW and SW with an R score of 0.83. These results suggested that marine yeasts can utilise sugars in freshwater and salt water in a similar manner and therefore, they have high tolerance to the osmotic stress induced by the presence of sugars and salts (Figure 5.5).

Using this approach, we were able to estimate the similarity of utilisation between different types of sugars. Little similarity was observed between glucose utilisation in ROW and the utilisation of other sugars with similarity values 0.28, 0.26, 0.27, 0.17 and 0.066 for mannitol in SW, xylose in ROW, xylose in SW, galactose in ROW, and galactose in SW respectively. The similarity was increased when using glucose in SW to 0.35, 027, 0.21 and 0.13 respectively. The similarity was further increased when using glucose in 2xSSW to 0.43, 0.34, 037, 0.33 and 0.25 respectively (Figure 5.6). Mannitol utilization was conducted in SW only and it recorded medium similarity with the utilisation of xylose-ROW, xylose-SW, galactose-RO and galactose-SW at values of 0.44, 0.48, 0.59 and 0.69 respectively. Xylose and galactose utilisation showed medium similarity ranging from 0.39 to 0.69 based on the type of water and sugar being compared (Figure 5.5).

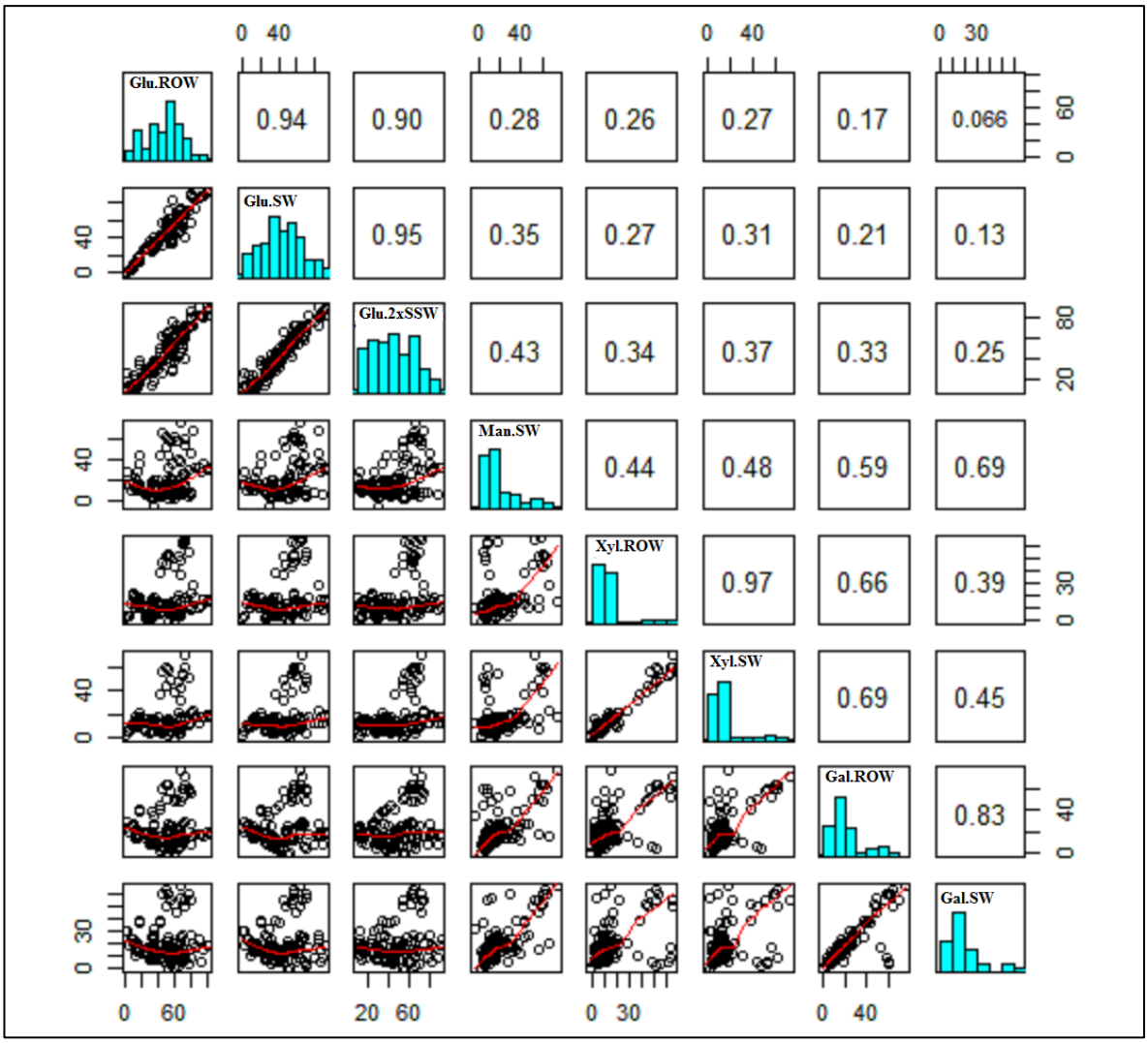


Figure 5.5: Statistical comparison (using R) for the utilisation of fermentable sugars by 116 newly isolated marine yeasts

This analysis was done based on PM assays using 8 conditions as following; Glucose in ROW (Glu.ROW), Glucose in SW (Glu.SW), Glucose in 2xSSW (Glu.2xSSW), Mannitol in SW (Man.ROW), Xylose in ROW (Xyl.ROW), Xylose in SW (Xyl.SW), Galactose in ROW (Gal.ROW), Galactose in SW (Gal.SW)
Squares, with a bar chart, across the figure represent the different media conditions, indicating response to that sugar in a yeast population. Squares with a number indicate similarity between populations of yeast using the sugars in question, R = 1 indicates identical response to those two sugars. The squares on the left-hand side, with line chart, show the difference in response in yeast populations to the pair of sugars tested. Squares on mirror sides of the bar charts are the R score on the right and the cluster analysis on the left. For example, comparing yeast populations using either galactose in SW media (Gal.SW) or glucose in ROW media (Glu.ROW) gave an R score of 0.066 (square in the top right-hand corner) with the cluster analysis in the bottom left-hand corner square showing that the populations of yeast had very little similarities when using galactose in sea-water and glucose in RO-water.

5.2.3 Identification of new marine yeast isolates

Based on the results of the PM assay, 21 isolates with interesting sugar utilisation abilities were selected for identification (Figure 5.6). Those isolates were the top 10 isolates for utilising glucose, mannitol, xylose and galactose in seawater based media (Figure 5.6). Biochemical identification approach was carried out using YT-MicroPlate for yeast identification but the results were not satisfactory (detailed results in section 5.2.3.1). Therefore, a genetic identification approach using DNA sequencing using ITS and D1/D2 primers was carried out which gave satisfactory results (detailed results in section 5.2.3.2).

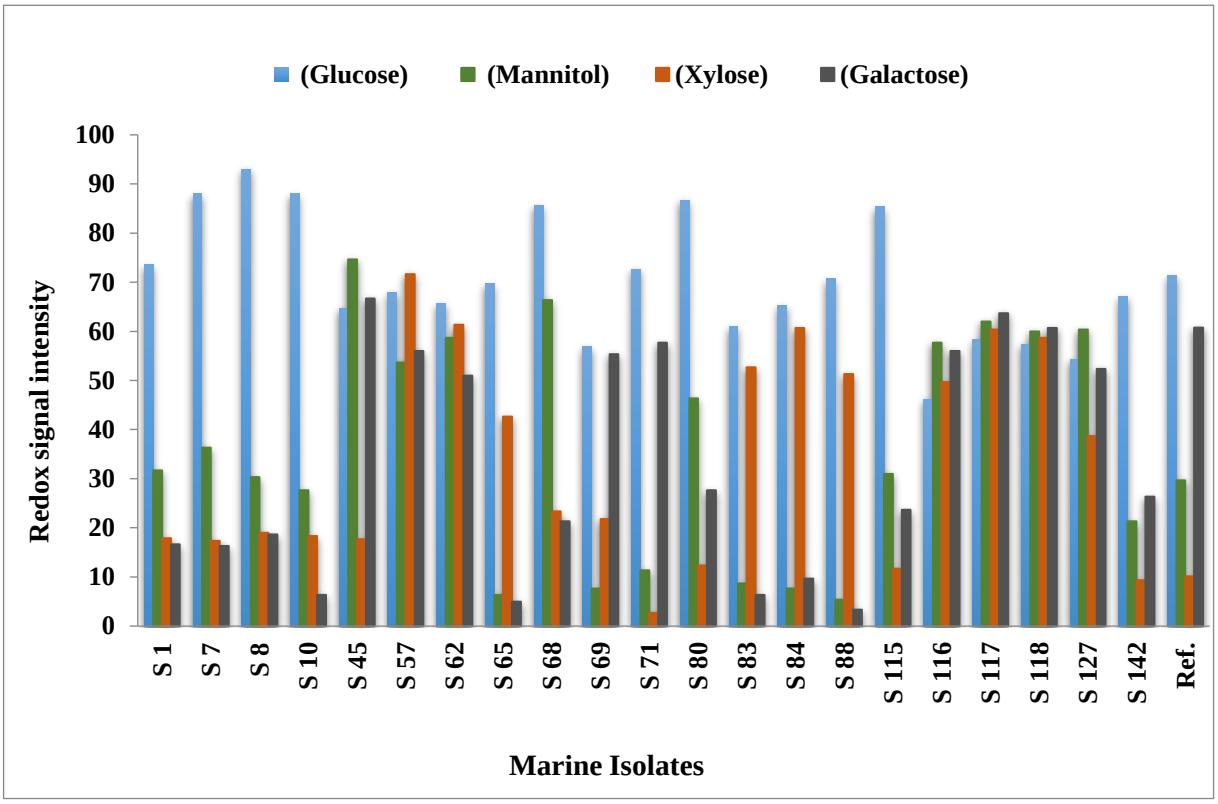


Figure 5.6: Metabolic output of selected marine yeast isolates on monomeric fermentable sugars using a phenotypic microarray assay

These results were obtained using PM assys. Results of the reference strain *S. cerevisiae* NCYC2592 (Ref.) is presented at the right end of the graph for comparison.

5.2.3.1 Identification of marine yeast using YT-MicroPlate

Identification of 21 selected yeast isolates was carried out using YT-MicroPlates. YT-MicroPlate is a BioLog plate that has 96 wells containing a solid media of specific substrate/s for operating 35 oxidation tests and 65 assimilation tests to evaluate the ability of the inoculated isolate for identification purpose (Figure 5.7). Each YT-MicroPlate was inoculated with a yeast suspension of one of the selected yeast isolates. Plates then were incubated at 25^{o}C in a static incubator. TECAN (Infinite® 200 PRO) plate reader was used for reading the plates after 24, 48 and 72 hours of incubation (detailed procedure in section 3.10). Reads were generated and manually converted to values (positive '+', negative '-' and weak 'w') (Tables 5.1 & 5.2). The results of the 48 hours were then sent to Technopath (Surrey, UK) for identification using MicroLog® manual microbial ID system.

A1 Water	**A2** Acetic Acid	**A3** Formic Acid	**A4** Propionic Acid	**A5** Succinic Acid	**A6** Methyl Succinic	**A7** L-Aspartic Acid	**A8** L-Glutamic Acid	**A9** L- Proline	**A10** D-Gluconic Acid	**A11** Dextrin	**A12** Inulin
B1 Cellobiose	**B2** Mentiobiose	**B3** Maltose	**B4** Maltotriose	**B5** D-Melezitose	**B6** D-Melibiose	**B7** Palatinose	**B8** D-Raffinose	**B9** Stachyose	**B10** Sucrose	**B11** Trehalose	**B12** Turanose
C1 N-Acetyl-DGlucosamie	**C2** α-D-Glucose	**C3** D-Galactose	**C4** D-Psicose	**C5** L-Sorbose	**C6** Salicin	**C7** D-Mannitol	**C8** D-Sorbitol	**C9** D-Arabitol	**C10** Xylitol	**C11** Glycerol	**C12** Tween 80
D1 Water	**D2** Fumaric Acid	**D3** L-Malic Acid	**D4** D4 Methyl Succinate	**D5** Bromosuccinc Acid	**D6** L-Gluamic Acid	**D7** γ-Aminobutyric Acid	**D8** α-Keto Glutaric Acid	**D9** 2- Keto-DGluconic Acid	**D10** D-Gluconic Acid	**D11** Dextrin	**D12** Inuilin
E1 Cellobiose	**E2** Gentiobiose	**E3** Maltose	**E4** Maltotriose	**E5** D-Melexitose	**E6** D-Melibiose	**E7** Palatinose	**E8** D-Raffinose	**E9** Stachyose	**E10** Sucrose	**E11** Trehalose	**E12** Turanose
F1 N-Acetyl DGlucosamie	**F2** DGlucosamine	**F3** α-D-Glucose	**F4** D-Galactose	**F5** D-Psicose	**F6** L-Rhamnose	**F7** L-Sorbose	**F8** α-Methyl-DGlucoside	**F9** β- Methyl-DGlucoside	**F10** Amygdalin	**F11** Arbutin	**F12** Salicin
G1 Maltitol	**G2** D-Mannitol	**G3** D-Sorbitol	**G4** Adonitol	**G5** D-Arabito	**G6** G6 Xylitol	**G7** i-Erythritol	**G8** Glycerol	**G9** Tween 80	**G10** L-Arabinose	**G11** D-Arabinose	**G12** D-Ribose
H1 D-Xylose	**H2** Methyl Succinate +D-Xylose	**H3** N-Acetyl-LGlutamic Acid +D-Xylose	**H4** Guinic Acid +D-Xylose	**H5** D-Glucuronic Acid+D-Xylose	**H6** Dextrin +D-Xylose	**H7** α-D-Lactose +D-Xylose	**H8** D-Melibiose +D-Xylose	**H9** D-Galactose +D-Xylose	**H10** m-Inositol +D-Xylose	**H11** 1,2-Propanediol +D-Xylose	**H12** Acetoin +DXylose

Figure 5.7: Chemical substrates and Carbon sources in YT-MicroPlate

A1 to C12: For oxidation tests; D1 to H12: For assimilation tests.

Table 5.1: Oxidation tests (using YT plates for yeast identification) of 21 marine yeast isolates and the terrestrial *S. cerevisiae* NCYC2592

Test	Ref.[a]	Marine yeast isolates																				
		S1	S7	S8	S10	S45	S57	S62	S65	S68	S69	S71	S80	S83	S84	S88	S115	S116	S117	S118	S127	S142
B1	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	w	w	-	-	-	-
C1	-	-	w	+	w	w	+	w	-	w	-	-	-	-	-	-	-	-	-	-	-	w
A2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B2	-	-	-	-	-	-	-	-	w	-	-	-	-	-	w	-	w	-	-	-	-	-
C2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-
B3	+	+	+	+	+	+	+	+	-	+	-	-	+	-	w	w	w	+	+	+	-	+
C3	+	+	w	+	+	+	w	w	+	w	+	+	-	-	w	w	w	w	+	+	-	+
A4	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-
B4	-	+	+	+	+	w	+	w	w	w	-	-	+	-	w	-	+	+	-	-	-	+
C4	-	-	-	w	w	-	w	-	w	-	-	w	-	-	w	w	w	-	-	w	w	-
A5	-	-	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B5	-	-	w	w	w	-	w	w	-	-	-	-	w	-	-	-	+	w	-	-	-	-
C5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-
A6	-	-	-	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-
C6	-	-	-	-	w	-	-	-	-	-	-	-	w	-	w	-	+	+	-	-	-	-
A7	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B7	-	-	w	w	w	+	+	+	+	+	+	+	w	-	w	-	+	w	-	-	-	w
C7	-	-	w	+	w	+	+	w	-	+	-	-	-	-	w	-	+	-	-	-	-	-
A8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-
B8	w	-	-	-	-	-	-	-	+	-	+	+	+	-	w	-	+	+	+	+	-	-
C8	-	w	w	+	w	+	w	w	-	w	-	-	-	-	w	-	w	-	-	-	-	-
A9	-	w	w	+	w	-	-	w	-	-	-	-	-	-	-	-	w	-	-	-	-	-
B9	w	-	-	-	-	-	-	-	w	-	-	w	-	-	w	-	w	-	w	w	-	-
C9	-	-	-	-	w	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-
A10	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B10	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	w
C10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-
A11	-	w	w	w	w	w	w	w	w	-	-	w	w	-	w	-	w	-	-	w	-	-
B11	-	-	-	w	-	+	+	+	-	+	-	-	-	-	+	+	w	-	w	-	+	w
C11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-
A12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B12	+	w	w	+	+	+	+	+	-	+	-	-	+	-	-	-	+	+	+	+	-	w
C12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] The reference strain, *S. cerevisiae* NCYC2592.

B1 to C12: refer to figure 5.7 for the name of substrate/s used in each test.

"+" positive response, "-" negative response, and "w" weak positive response.

Table 5.2: Assimilation tests (using YT plates for yeast identification) of 21 marine yeast isolates and the terrestrial *S. cerevisiae* NCYC2592

	Ref.[a]	Marine yeast isolates																				
		S1	S7	S8	S10	S45	S57	S62	S65	S68	S69	S71	S80	S83	S84	S88	S115	S116	S117	S118	S127	S142
E1	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	w	w	-	-	-	-
F1	-	w	w	+	w	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	w	w
G1	-	-	-	w	-	w	w	w	-	w	-	-	w	-	-	-	w	w	-	-	-	-
H1	-	-	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D2	-	w	w	w	w	w	w	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-
E2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-
F2	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G2	-	-	w	w	w	w	w	+	-	+	-	-	-	-	-	-	w	w	-	-	-	-
H2	-	w	w	w	w	-	-	-	-	-	-	-	-	w	-	w	w	w	-	-	-	-
D3	-	w	w	w	w	w	w	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-
E3	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	-	+	+	+	+	-	+
F3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G3	-	-	-	w	-	w	-	w	-	w	-	-	-	-	-	-	w	-	-	-	-	-
H3	-	-	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D4	-	-	-	+	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E4	-	+	+	+	+	+	+	+	-	+	-	-	+	-	-	-	+	+	-	-	-	+
F4	+	w	w	w	w	+	w	w	+	w	+	+	-	-	-	-	w	w	+	+	-	+
G4	-	-	w	w	w	w	w	w	-	w	-	-	-	-	-	-	w	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
D5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E5	-	w	w	w	-	w	w	w	-	w	-	-	w	-	-	-	w	w	-	-	-	-
F5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D6	-	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-
D7	-	-	-	-	-	w	w	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-
E7	-	w	-	+	+	+	+	+	+	+	+	+	w	-	-	-	w	w	-	-	-	w
F7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-
H7	-	-	w	-	w	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-

(See the rest of this table in the next page)

Table 5.2 (continued): Assimilation tests (using YT plates for yeast identification) of 21 marine yeast isolates and the terrestrial *S. cerevisiae* NCYC2592

	Ref.[a]	S1	S7	S8	S10	S45	S57	S62	S65	S68	S69	S71	S80	S83	S84	S88	S115	S116	S117	S118	S127	S142
D8	-	-	-	-	-	w	w	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-
E8	w	-	-	-	-	-	-	-	w	-	w	w	w	-	w	-	+	+	w	w	-	-
F8	-	w	-	w	w	w	w	w	-	w	-	-	w	-	-	-	w	w	-	-	-	-
G8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D9	-	w	+	+	+	w	w	+	-	w	-	-	-	-	-	-	-	-	-	-	-	w
E9	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-
F9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-
G9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	+	w	w	w	w	+	w	w	+	w	+	+	-	-	+	-	w	w	+	+	-	w
D10	-	-	-	-	-	w	-	w	-	w	-	-	-	-	-	-	-	-	-	-	-	-
E10	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+
F10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D11	-	-	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E11	-	-	w	w	-	+	+	+	-	+	-	-	-	-	+	+	w	-	-	-	+	w
F11	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	+	+	-	-	-	-
G11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H11	-	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E12	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	-	+	+	+	+	-	w
F12	-	-	-	-	-	-	w	-	-	-	-	-	w	-	-	-	w	w	-	-	-	-
G12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	-	w	-	-	w	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-

[a] The reference strain, *S. cerevisiae* NCYC2592.

E1 to H12: refer to figure 5.7 for the name of substrate/s used in each test.

"+" positive response, "-" negative response, and "w" weak positive response.

Processing the results from Tables 5.1 and 5.2 using MicroLog® manual microbial ID system revealed that no identification was obtained for 8 isolates; S10, S62, S69, S83 S84, S88, S127 and S124. Identification was obtained for 13 isolates as following; *Hyphopichia burtonii C* (S1), *Rhodotorula acheniorum* (S7), *Candida parapsilosis* B (S8), *Candida albicans* (S45, S57& S68), *Kluyveromyces marxianus* (S71), *Endomyces fibuligera* (S80), *Pichia onychis* (S115), *Pichia subpelliculosa* (S116) and *Saccharomyces boulardii* (S117 & S118) (Table 5.3). Despite the fact that this method was not able to identify all isolates, the similarities that were given to the identified isolates were very low. Also, the probability of the majority of the identified isolates was less than 0.90 which is not satisfactory for identification (Table 5.3).

5.2.3.2 Genetic identification of marine yeast isolates

In order to obtain a satisfactory identification for our newly isolated marine yeasts, genetic identification was carried out on the 21 selected isolates using ITS and D1/D2 primers. Primers sequences and the detailed procedure for identification was explained in chapter 3 (section 3.9).

Using genetic identification, one isolate (S127) did not produce sufficient sequences length from PCR using D1/D2 primer while three isolates (S65, S88 & S142) did not produce sufficient sequences length from PCR using ITS primers. The length of PCR sequences of the identified isolates ranged between 553 and 586 nucleotides with D1/D2 primers, and 342 and 839 nucleotides with ITS primers, which was sufficient for identification (Table 5.4). Full sequences output for the 21 isolates are shown in Appendix 1. All sequences obtained were blasted against sequences in GenBank (http://blast.ncbi.nlm.nih.gov/). Out of the 21 isolates, 11 isolates belonged to the genus *Candida* as following; *Candida tropicalis* (4 isolates), *Candida viswanathii* (4 isolates), *Candida glabrata* (2 isolates) and *Candida albicans* (1 isolate). The rest of the isolates were *Saccharomyces cerevisiae* (5 isolates), *Wickerhamomyces anomalus* (3 isolates), *Pichia kudriavzevii* (1 isolate) and *Issatchenkia orientalis* (1 isolate) (Table 5.4). Results obtained using genetic identification were satisfactory with a 99% or better level of identification using to different primers. Matching the results obtained from the biochemical identification (YT-MicroPlates) with the confirmed results from the genetic identification, there were 7 isolates that matched the genetic identification at the genus level but none matched at the species level.

Table 5.3: Identification outcome of 21 marine yeast isolates using YT MicroPlate

Isolate source	Sample	Strain	Genus	Species	Probability	SIM
Mediterranean Sea Alexandria Egypt	**A**	S 1	*Hyphopichia*	*burtonii C*	0.69	0.55
		S 7	*Rhodotorula*	*acheniorum*	0.6	0.50
		S 8	*Candida*	*parapsilosis B*	0.64	0.55
		S 10	N/A	N/A	N/A	N/A
Red Sea Ismailia Egypt	**D**	S 45	*Candida*	*albicans*	0.91	0.77
Irish Sea Wales U.K.	**E**	S 57	*Candida*	*albicans*	0.79	0.67
	F	S 62	N/A	N/A		
		S 65	*Kluyveromyces*	*marxianus*	0.87	0.67
		S 68	*Candida*	*albicans*	0.91	0.77
English Channel Plymouth U.K.	**G**	S 69	N/A	N/A	N/A	N/A
		S 71	*Kluyveromyces*	*marxianus*	0.88	0.67
		S 80	*Endomyces*	*fibuligera*	0.64	0.53
	H	S 83	N/A	N/A	N/A	N/A
		S 84	N/A	N/A	N/A	N/A
		S 88	N/A	N/A	N/A	N/A
	J	S 115	*Pichia*	*onychis*	0.70	0.59
		S 116	*Pichia*	*subpelliculosa*	0.95	0.81
	K	S 117	*Saccharomyces*	*boulardii*	0.99	0.85
		S 118	*Saccharomyces*	*boulardii*	0.98	0.84
North Sea Whitby, UK	**L**	S 127	N/A	N/A	N/A	N/A
Pacific Ocean San Diego, USA	**N**	S 142	N/A	N/A	N/A	N/A

Probability: the possibility of providing a correct identification for the tested isolate.

SIM: similarity index value, which was calculated based on the records obtained after 48 h of incubation.

N/A: No identification obtained because the recorded Probability and/or SIM was less than 0.5.

Table 5.4: Identification outcome of 21 marine yeast isolates using ITS and D1/D2 primers

Isolate source	Sample	Strain	Identified name		ITS		D1/D2	
			Genus	Species	Length	% ID	Length	% ID
Mediterranean Sea. Alexandria, Egypt	**A**	S 1	*Candida*	*viswanathii*	507	99	564	99
		S 7	*Candida*	*viswanathii*	509	99	571	100
		S 8	*Candida*	*viswanathii*	418	100	567	99
		S 10	*Candida*	*viswanathii*	498	99	568	99
Red Sea. Ismailia, Egypt	**D**	S 45	*Candida*	*tropicalis*	489	99	561	99
Irish Sea. Wales, UK	**E**	S 57	*Candida*	*tropicalis*	480	99	567	100
	F	S 62	*Candida*	*tropicalis*	568	99	553	100
		S 65	*Saccharomyces*	*cerevisiae*	N/A	-	570	100
		S 68	*Candida*	*tropicalis*	520	99	567	100
English Channel Plymouth, UK	**G**	S 69	*Saccharomyces*	*cerevisiae*	781	99	571	100
		S 71	*Saccharomyces*	*cerevisiae*	784	99	571	100
		S 80	*Wickerhamomyces*	*anomalus*	579	99	571	99
	H	S 83	*Pichia*	*kudriavzevii*	468	100	562	100
		S 84	*Candida*	*glabrata*	839	99	586	99
		S 88	*Issatchenkia*	*orientalis*	N/A	-	559	100
	J	S 115	*Wickerhamomyces*	*anomalus*	577	99	564	100
		S 116	*Wickerhamomyces*	*anomalus*	579	99	566	100
	K	S 117	*Saccharomyces*	*cerevisiae*	679	99	570	100
		S 118	*Saccharomyces*	*cerevisiae*	677	99	570	100
North Sea Whitby, UK	**L**	S 127	*Candida*	*glabrata*	342	100	N/A	-
Pacific Ocean San Diego, USA	**N**	S 142	*Candida*	*albicans*	N/A	-	566	99

N/A: No identification obtained due to lack of PCR product.

5.2.4 Phenotypic characterization of marine yeasts using YT-MicroPlate

The genetic identification using ITS or D1D2 primers for marine yeasts gave reliable identification up to the species level but could not distinguish between strains. Biochemical phenotyping using YT- MicroPlates could be useful because the media in those plates contain variety of different substrates to test the oxidation ability of the inoculated microorganism on 35 different substrates; and to test the assimilation ability of the inoculated microorganism on 65 different substrates. Hence, YT-Plates results obtained in section 5.2.3.1 were used as a biochemical phenotyping analysis for the strains that belonged to the same species, these results also facilitated differences to be observed in the phenotypic output of strains belonging to different species.

Table 5.5 shows differences in substrate utilisation were recorded for isolates of the same species. For example, *Candida viswanathii* isolate S8 could oxidise mannitol but isolate S1 was not, while isolates S7 and S10 recorded weak oxidation. For *S. cerevisiae* isolates, there was a difference in oxidation results between the reference strain (NCYC2592) and marine derived yeasts. However, there were also differences in the oxidation results among the marine yeasts, indicating that different *S. cerevisiae* strains were isolated. In general, results suggested that all isolates could utilise glucose and inulin. Sucrose cannot be utilised by the isolates belong to *Candida glabrata*, *Pichia kudriavzevii* and *Issatchenkia orientalis.* None of the strains could utilise acetic acid or Tween 80 as a sole carbon source.

Table 5.6 shows the differences in the assimilation capability of these yeasts on 65 different substrates. Similar to the results obtained from the oxidation tests, the assimilation ability of the yeast isolates varied within the same species. The results showed that none of the tested isolates could assimilate xylose except of S7 and S10 which had a weak ability to assimilate xylose. However, many yeast isolates - including S65 and S118 - showed ability to utilise xylose using PM assay, those isolates could not assimilate xylose using YT-MicroPlate. This could be due to the ability of these isolates to utilise xylose producing intermediary metabolites that cause reduction changing the colour of the tetrazolium violet redox dye. The assimilation tests in YT-MicroPlate does not contain the tetrazolium violet redox dye.

Identification results obtained from both methods (PCR and BioLog) suggested that genetically similar marine and terrestrial yeasts differ phenotypically. This would explain the marine environmental impact on the yeast as most of marine yeasts have terrestrial origin and reached a marine environment through wind, rivers or human activity. Even though the BioLog

approach using YT-MicroPlate was not an ideal method for marine yeast identification, it remains useful for biochemical phenotyping and identifying strains within a certain species.

Table 5.5: Comparison of oxidation tests (using YT-MicroPlate for yeast identification) of 21 marine yeast isolates and the terrestrial *Saccharomyces cerevisiae* NCYC2592

Oxidation Tests	*S. cerevisiae*						*C. tropicalis*				*C. viswanathii*				*W. anomalus*			*C. glabrata*		*P. kudriavzevii*	*I. orientalis*	*C. albicans*
	Ref[a]	S65	S69	S71	S117	S118	S45	S57	S62	S68	S1	S7	S8	S10	S80	S115	S116	S84	S127	S83	S88	S142
B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-
C1	-	-	-	-	-	-	w	+	w	w	-	w	+	w	-	-	-	-	-	-	-	w
A2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B2	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-
C2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-
B3	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	w	+	w	-	-	w	+
C3	+	+	+	+	+	+	+	w	w	w	+	w	+	+	-	w	w	w	-	-	w	+
A4	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B4	-	w	-	-	-	-	w	+	w	w	+	+	+	+	+	+	+	w	-	-	-	+
C4	-	w	-	w	-	w	-	w	-	-	-	-	w	w	-	w	-	w	w	-	w	-
A5	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-
B5	-	-	-	-	-	-	-	w	w	-	-	w	w	w	w	+	w	-	-	-	-	-
C5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-
A6	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-	-	-	-	-
B6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-
C6	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	+	+	w	-	-	-	-
A7	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-
B7	-	+	+	+	-	-	+	+	+	+	-	w	w	w	w	+	w	w	-	-	-	w
C7	-	-	-	-	-	-	+	+	w	+	-	w	+	w	-	+	-	w	-	-	-	-
A8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-
B8	w	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	w	-	-	-	-
C8	-	-	-	-	-	-	+	w	w	w	w	w	+	w	-	w	-	w	-	-	-	-
A9	-	-	-	-	-	-	-	-	w	-	w	w	+	w	-	w	-	-	-	-	-	-
B9	w	w	-	w	w	w	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-
C9	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	w	-	-	-	-
A10	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-
B10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	w
C10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-
A11	-	w	-	w	-	w	w	w	w	-	w	w	w	w	w	w	-	w	-	-	-	-
B11	-	-	-	-	w	-	+	+	+	+	-	-	w	-	-	w	-	+	+	-	+	w
C11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-
A12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B12	+	-	-	-	+	+	+	+	+	+	w	w	+	+	+	+	+	-	-	-	-	w
C12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] The reference strain, S. cerevisiae NCYC2592.

B1 to C12: Refer to figure 5.7 for the name of substrate/s used in each test.

"+" positive response, "-" negative response, and "w" weak positive response.

Table 5.6: Comparison of assimilation tests (using YT plates for yeast identification) of 21 marine yeast isolates and the terrestrial *Saccharomyces cerevisiae* NCYC2592

Assimilation Tests	*S. cerevisiae*						*C. tropicalis*				*C. viswanathii*				*W. anomalus*			*C. glabrata*		*P. kudriavzevii*	*I. orientalis*	*C. albicans*
	Ref[a]	S65	S69	S71	S117	S118	S45	S57	S62	S68	S1	S7	S8	S10	S80	S115	S116	S84	S127	S83	S88	S142
E1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-
F1	-	-	-	-	-	-	+	+	+	+	w	w	+	w	-	-	-	-	w	-	-	w
G1	-	-	-	-	-	-	w	w	w	w	-	-	w	-	w	w	w	-	-	-	-	-
H1	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-	-	-	-
D2	-	-	-	-	-	-	w	w	w	w	w	w	w	w	-	-	-	-	-	-	-	-
E2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-	-
F2	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-
G2	-	-	-	-	-	-	w	w	+	+	-	w	w	w	-	w	w	-	-	-	-	-
H2	-	-	-	-	-	-	-	-	-	-	w	w	w	w	-	w	w	-	-	w	w	-
D3	-	-	-	-	-	-	w	w	w	w	w	w	w	w	-	-	-	-	-	-	-	-
E3	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+
F3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G3	-	-	-	-	-	-	w	-	w	w	-	-	w	-	-	w	-	-	-	-	-	-
H3	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-
D4	-	-	-	-	-	-	w	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
E4	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+
F4	+	+	+	+	+	+	+	w	w	w	w	w	w	w	-	w	w	-	-	-	-	+
G4	-	-	-	-	-	-	w	w	w	w	-	w	w	w	-	w	-	-	-	-	-	-
H4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-
D5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E5	-	-	-	-	-	-	w	w	w	w	w	w	w	-	w	w	w	-	-	-	-	-
F5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D6	-	-	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-
E6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	w	-	-	-	-	w	w	w	-	-	w	-	-	-	-	-
D7	-	-	-	-	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-
E7	-	+	+	+	-	-	+	+	+	+	w	-	+	+	w	w	w	-	-	-	-	w
F7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
H7	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	w	-	-	-	-	-	-

(See the rest of this table in the next page)

Table 5.6 (continued): Comparison of assimilation tests (using YT plates for yeast identification) of 21 marine yeast isolates and the terrestrial *Saccharomyces cerevisiae* NCYC2592

Tests	Ref[a]	S65	S69	S71	S117	S118	S45	S57	S62	S68	S1	S7	S8	S10	S80	S115	S116	S84	S127	S83	S88	S142
D8	-	-	-	-	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-
E8	w	w	w	w	w	w	-	-	-	-	-	-	-	-	w	+	+	w	-	-	-	-
F8	-	-	-	-	-	-	w	w	w	w	w	-	w	w	w	w	w	-	-	-	-	-
G8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D9	-	-	-	-	-	-	w	w	+	w	w	+	+	+	-	-	-	-	-	-	-	w
E9	w	-	-	-	w	w	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
F9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-	-
G9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	+	+	+	+	+	+	+	w	w	w	w	w	w	w	-	w	w	+	-	-	-	w
D10	-	-	-	-	-	-	w	-	w	w	-	-	-	-	-	-	-	-	-	-	-	-
E10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+
F10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-
D11	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-
E11	-	-	-	-	-	-	+	+	+	+	-	w	w	-	-	w	-	+	+	-	+	w
F11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	+	+	-	-	-	-	-
G11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H11	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-
D12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E12	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	w
F12	-	-	-	-	-	-	-	w	-	-	-	-	-	-	w	w	w	-	-	-	-	-
G12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	-	-	-	-	-	-	-	-	-	-	w	-	-	w	-	w	-	-	-	-	-	-

[a] The reference strain, S. cerevisiae NCYC2592.

E1 to H12: Refer to figure 5.7 for the name of substrate/s used in each test.

"+" positive response, "-" negative response, and "w" weak positive response.

5.3 Discussion

5.3.1 Screening and metabolism evaluation using Phenotypic Microarray (PM) assay

Conversion of monomeric sugars into commercially valuable products is a desirable trait for yeasts for potential industrial application. In this study, PM assays have been used to screen for novel marine-derived yeasts with high capability for the utilisation of the monomeric sugars, including glucose, xylose, galactose and mannitol, in a seawater based medium. *S. cerevisiae* NCYC2592, a strain that has high metabolism capacity and high tolerance to a wide range of inhibitors presented in the lignocellulosic hydrolysate (Oshoma et al., 2015, Wimalasena et al.,

2014), was also used in the PM assays as a control to evaluate the isolated marine yeast for their metabolism capacity. The results showed that *S. cerevisiae* NCYC2592 grew well in the seawater based medium. However, 11 marine isolates have been identified which performed better than *S. cerevisiae* NCYC2592 in terms of glucose utilisation in seawater based media. This may be due to the fact that the marine yeasts acquired high tolerance to salts and other inhibitors, which exist in seawater; therefore, their growth was not disturbed by the presence of high level of salts in the medium. As expected, the xylose utilisation experiments revealed that the reference strain did not grow (Wimalasena et al., 2014, Wenger et al., 2010). However, many of our marine isolates show good xylose utilisation and they could be potential candidates for bioethanol fermentations using xylose as the substrate.

Isolating yeast strains with the capacity for utilising mannitol is of particular interest. Mannitol was considered the most abundant sugar alcohol in nature (Bieleski, 1982) and has been highlighted as a potential bulk marine sugar (Reed et al., 1985) as it is found in different types of marine algae (brown, golden and red algae) (Karsten et al., 1997). PM assays results revealed that reference strain *S. cerevisiae* NCYC2592 cannot utilise mannitol. However, many marine yeasts isolated in the current study had high mannitol utilisation ability, especially isolate S45, which showed the highest mannitol consumption, this strain may have potential to be used as an industrial strain for converting mannitol to value added products. It was reported that mannitol is rapidly metabolised by bacteria in marine habitats (Koop et al., 1982). White et al. (2010) suggested that several herbivorous fish cannot assimilate mannitol directly but it can be utilised as an indirect nutrient via fermentation in the hindgut (White et al., 2010). It has been reported that mannitol plays an osmo-regulatory role in algae that experience significant changes in salinity (Bieleski, 1982, Karsten et al., 1997). Therefore, we suggest that marine yeasts that are able to ferment mannitol are of particular importance as candidates for marine fermentation (fermentations that use marine yeast, marine biomass and seawater) not only because mannitol is widely available in marine biomass but also because mannitol is expected to weaken the inhibitory effects of seawater's salinity.

Galactose is another monomeric sugar, which is present in marine biomass substrates such as red seaweed (Kumar et al., 2013). Isolate S45 showed the highest utilisation level amongst the marine isolates and was higher than the reference strain. This makes it a potential candidate for bioethanol production for marine substrates such as seaweed. Galactose utilisation is of particular interest as in most yeast galactose utilisation is suppressed by the presence of glucose (Ostergaard et al., 2000), research may lead to identification of marine yeast which are capable of efficient utilisation of both glucose and galactose simultaneously. Biomass hydrolysates are

characterised by a complex mixture of sugars. Efficient conversion of these sugars into end-metabolites has been highlighted as a desirable phenotype. PM assays revealed that marine derived yeasts have a good overall sugar utilisation capabilities and several marine yeast isolates have the capacity for utilising all four of these sugars.

Freshwater is traditionally used in fermentations. However, many parts of the world struggle to produce enough potable water for human consumption. There are only limited studies using seawater for fermentation (Lin et al., 2011). PM Assays revealed that the sugar utilisation by marine yeasts was almost identical after 24 hours of incubation in experiments using seawater and using freshwater. These results indicated that the presence of salt was not deleterious to sugar utilisation by marine yeasts.

5.3.2 Genetic identification of marine yeast isolates

New isolates of marine yeasts were identified using the same procedures as used for terrestrial yeasts as no specific procedure for marine yeast identification has yet been reported. Genetic identification methods by DNA sequencing using ITS and D1/D2 successfully identified our marine-derived yeasts and that proves they have terrestrial origin (as they share the same genotype). This finding supported previous reports that the majority of marine yeasts including those isolated from deep-sea regions, are not indigenous (Burgaud et al., 2010, Kohlmeyer and Kohlmeyer, 1979). We found that genus *Candida* was more common in the marine environment when compared with other yeast genera, which agrees with several previous studies (Rhishipal and Philip, 1998, Fell et al., 2011). *S. cerevisiae*, *C. tropicalis*, *W. anomalus*, and *C. glabrata* were previously isolated from different marine habitats (Guo et al., 2013, Obara et al., 2015). *Candida viswanathii* was recently isolated from deep-sea hydrothermal animals (Burgaud et al., 2010). *Pichia kudriavzevii* has not previously been isolated from a marine environment.

5.3.3 Phenotypic characterization

In this study, the identification of the 21 isolates using YT-MicroPlates did not match the genetic identification. This could be due to the fact that the BioLog database was based on substrate utilisation by terrestrial yeast strains only. YT-Plates is commercially available and is a mature method for yeast identification (Pincus et al., 2007, Praphailong et al., 1997). It was reported that 49 out of 72 yeasts that have been isolated from food and beverage were correctly identified using the BioLog system (Praphailong et al., 1997). However, in order to obtain a valid identification, a larger database is needed. Marine yeasts have inherited new characteristics for living in a marine habitat and so have altered phenotypes. This means that

available biochemical based identification methods such as YT-Plates from BioLog maybe not suitable for the identification of marine yeasts using the terrestrial yeasts based database. Foschino et al. (2004) reported that MicroPlate YT BioLog failed to identify yeast isolates from sourdoughs samples probably because their database was based on clinical yeast isolates (Foschino et al., 2004). If an intensive database could be built based on substrate utilisation in marine yeasts, the YT-Biology plate could be used for marine yeast identification as well.

In general, identification using sequence data has been shown to be more robust than using phenotypic data. However, it has been useful in highlighting the metabolic requirements of marine microorganisms with interesting industrial capabilities (Silvi et al., 2013). The development of D1/D2 and ITS database in GenBank (http://www.ncbi.nlm.nih.gov/genbank) allows laboratories around the world to easily and accurately identify more yeast species. Furthermore, phylogenetic analysis of the gene sequences is leading to a major modification of yeast systematics that will result in redefinition of almost all genera (Kurtzman et al., 2015). It is worth mentioning that PM screening assay revealed that several marine yeast isolates had the capacity for utilising xylose (Figure 5.6). But, use of YT-Plates revealed no utilisation of xylose (Tables 5.3 and 5.4). This inconsistency could be due to the differences of the culturing conditions, including the concentrations of xylose in the medium, the incubation temperature, and the inoculation level. In addition, the PM assay used liquid media while the YT MicroPlate used solid media.

5.4 Conclusion

In this chapter, a framework for screening large numbers of yeast isolates using microarray technique was designed and it was applied to 116 selected isolates. Out of these, 21 strains representing the best utilizer for each sugar were subjected to identification using PCR-based method. These 21strains belonged to 8 different species including *Pichia kudriavzevii* which has not previously been reported as a marine yeast. Phenotypic microarray assay using YT-BioLog micro plate was found to be a useful technique for strain discrimination but not for the identification of marine yeast.

Chapter 6:

6. The simultaneous determination of chloride ions (Cl^-), sugars and fermentation metabolites using HPLC

6.1 Introduction

HPLC analysis using Rezex ROA organic acid H^+ column is a conventional method for measuring sugar, ethanol and organic acids in fermentation samples (Oshoma et al., 2015, Greetham et al., 2014). Applying this method on fermentation samples of seawater-based media resulted in unclear peaks and inadequate results (Figure 6.1).

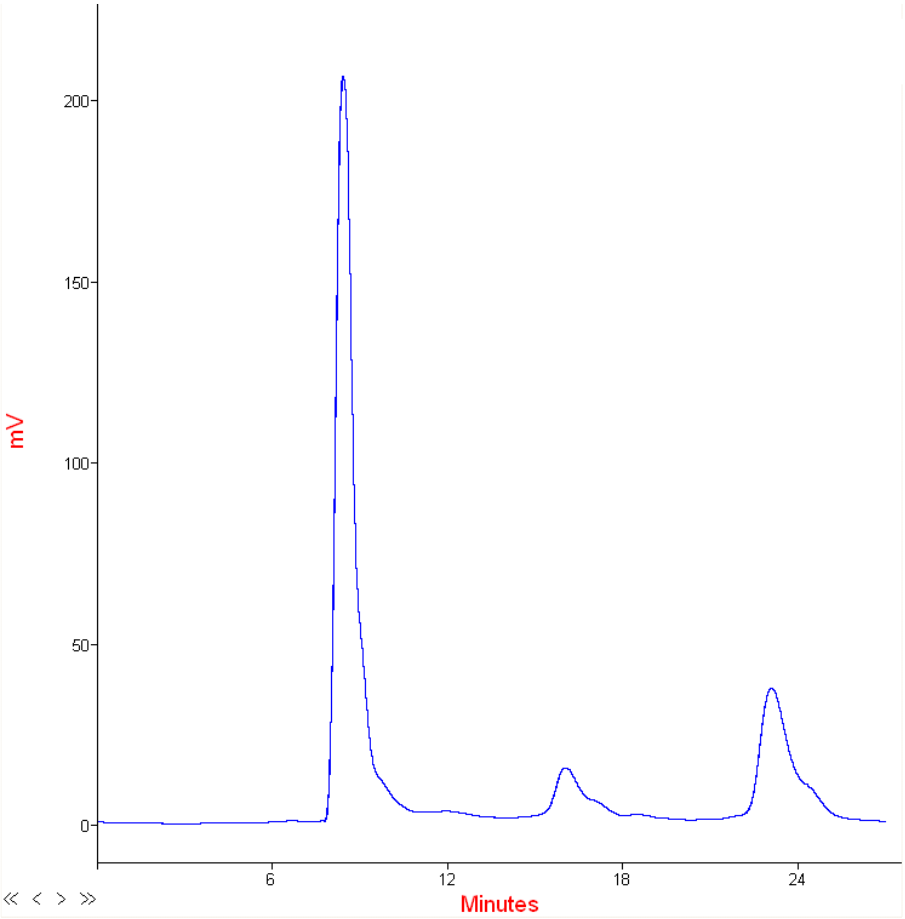


Figure 6.1: Peaks present on a HPLC chromatogram from fermentations media using seawater based media using Rezex ROA column.

Tbe peaks are not sharp and the first peak overestimated the concentration of glucose.

The main objective of this PhD project was to establish the use of seawater to replace freshwater in fermentation industry, particularly for bioethanol production. Hence, there is a necessity for investigating a suitable analysis method for sugar and alcohol contents derived from media containing high concentration of salts. An ideal method should be able to accurately determine

substrates and fermentation metabolites (i.e., sugars, organic acids and alcohols) in a fermentation broth containing high concentrations of chloride salts. HPLC is the preferred method for sugar quantification according to the guidelines of the Association of Official Analytical Chemists (Sims, 1995, AOAC, 1993). HPLC is a convenient and accurate analytical method suitable for the quantification of organic and inorganic compounds for a variety of samples. Chromatographic methods are the best analytical techniques for the quantification and identification of mono and oligosaccharides in food products (Duarte-Delgado et al., 2015). However, obtaining an accurate quantification of sugars using HPLC in samples containing NaCl has proven difficult due to similar retention times for chloride ions (Cl^-) and sugars especially glucose and sucrose (Sims, 1995).

Research was therefore conducted to investigate the suitability of using a Hi-Plex H column for analysing sugars and fermentation metabolites in samples derived from a fermentation using seawater-based media. In this chapter, an accurate method for the simultaneous determination of Cl^-, sugars, organic acids and alcohols in samples that contain high concentrations of Cl^- will be described. The accuracy of the method for quantifying Cl^- was also investigated. The application of the method on variety samples of commercial food products was carried out. The salt content obtained from the developed method had been compared with 3 other existing commonly used methods for salt determination. The majority of the content in this chapter formed the paper recently accepted by the Journal of Food Composition and Analysis (Zaky et al., 2017).

6.2 Results and discussion

6.2.1 Chromatography and peak identification

HPLC analysis was conducted as described in section (3.15.2). The method was applied on a sample from fermentation using medium prepared from natural seawater. Four peaks were obtained from the chromatogram analogous to standards for glucose, glycerol, acetic acid and ethanol. In addition, unidentified peak with a R_t of 10.90 min. appeared before the expected fermentation products (Figure 6.2).

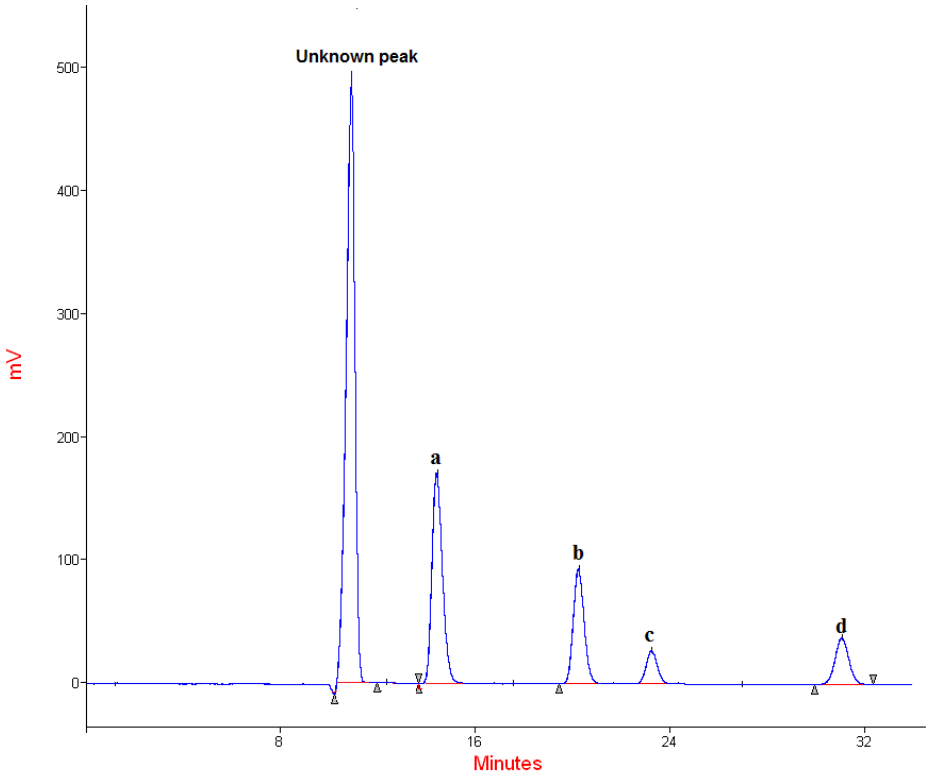


Figure 6.2: Peaks present on a HPLC chromatogram from fermentations media using seawater based media using HI-Plex H column

Peaks analogous to standards for glucose (a), glycerol (b), acetic acid (c) and ethanol (d) are labelled along with an unknown peak which eluted after 10.9 minutes.

Normally, seawater from open seas and oceans contains around 34 g/L of different salts (Yen et al., 2016, Zaky et al., 2016). NaCl represents about 85% (about 28 g/L) of the seawater's total salts and therefore Cl^- was a potential candidate for the unknown peak.

Using analytical grade NaCl at 4 concentrations revealed a peak at R_t 10.90 min. whose peak area correlated with the peak areas observed for the NaCl standards. This was confirmed when peak areas of different grades of NaCl (rock salt (lab grade) and table salt (food grade)) were compared with the retention time and peak areas of the unknown peak (Figure 6.3).

These findings along with the sharpness and symmetrical resolution of the peak indicated the potential of this method for the quantitative measurement of Cl^- simultaneously with sugars and fermentation metabolites when they are present together in a sample.

Substantially, the elution order of the analytes (in figure 6.2), which was Cl^-, glucose, glycerol, acetic acid and ethanol can be explained. These analytes have a mixed polarizability and they elute as a function of their size, and their electrical charge; apart from Cl^-, the first to elute are the large neutral molecules (glucose then glycerol) and subsequently the charged molecules (acetic acid) and smaller neutral molecules such as ethanol.

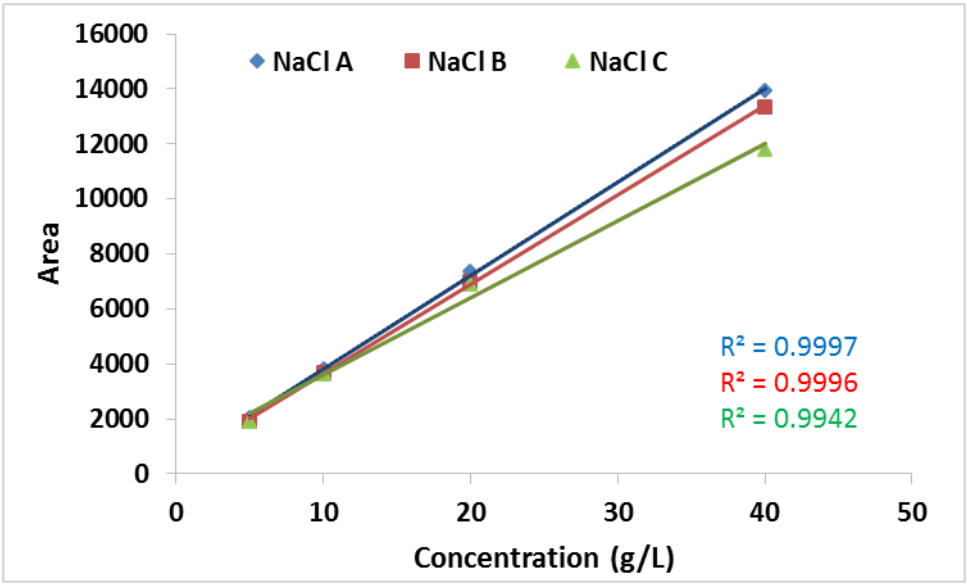


Figure 6.3: Quantification of standard solutions of different NaCl grades

This figure shows the linearity of a standard solutions of 3 NaCl grades at 4 concentrations: NaCl A, analytical grade NaCl from Fisher 99.85%; NaCl B, rock salt Lab grade from Fisher; NaCl C, table salt food grade from SAXA.

6.2.2 The method's ability to detect different salts, sugars, organic acids and alcohols

In order to test the ability of the method in detecting different chemical compounds, 29 different salts, sugars, organic acids and alcohols were tested using the new HPLC method investigated in this study, as described in section (3.15.4). The results (Table 6.1) revealed that sodium carbonate, sodium bicarbonate and sodium fluoride were not detectable by this method. On the other hand, sodium chloride, potassium chloride and magnesium chloride were all detected and gave a peak with a similar R_t of around 10.55 min. These results verified that the peak obtained corresponded to chloride anions (Cl^-) and not sodium cations (Na^+) and therefore that this methodology could be applied to the efficient quantification of chloride salts in the presence of sugars, alcohols and acetic acid. The results also revealed that other inorganic salts like sodium sulphate and sodium bromide can be detected with a similar R_t to chloride salts. Di-saccharides (maltose, sucrose and lactose) eluted with a R_t of around 12.5 min, followed by mono-saccharides and sugar-alcohols (glucose, galactose, xylose, fructose, mannitol, sorbitol and arabinose) which had a R_t of between 14.45 and 16.7 min. Low carbon organic acids and alcohols eluted after 20 min (Table 6.1 and Figure 6.4).

This method can distinguish between different organic acids (citric acid, succinic acid, lactic acid acetic acid) as they eluted at different R_t. But, this method cannot distinguish between organic acids and their sodium salts (sodium citrate, sodium succinate and sodium lactate) - if the acid and its salt are present together in the same sample - as these salts elute with similar R_t

to their organic acids. However, this finding gives other evidence that this method does not depend on the presence of sodium cations in the compound for detection.

Although this methodology cannot distinguish between chloride salts present in one sample, it would prove useful for the determination of total chloride salts. The current colorimetric methods for measuring NaCl, such as Volhard, Mohr and Chloride analyser, are also not NaCl specific but they measure all chloride ions present in the sample under investigation (Stankey et al., 2015).

The results obtained as regards the unambiguous determination of chloride were expected for the kind of analytical column used in this study. The dimensions of the column were; 300 mm length and 7.7 mm I.D., as well as the size particle (as great as 8 μm). These dimensions favour the development of low back pressures and consequently the elution of non-polarizable analytes like those included in this paper, as a function of properties other than polarizability. The results confirm conclusions published for chromatographic and solid phase fractionations by Andrade-Eiroa et al. (2014). On the other hand, small cations cannot be retained most likely due to two reasons: a) most of them are too small and b) the column is positively charged and the cations are strongly repelled (Andrade-Eiroa et al., 2011).

Although the manufacturer claims that the retention mechanism of the column is a mixture between size exclusion and anion-exchange, the results do not support this statement. As a matter of fact, and due to the pH of the mobile phase (about 2.3), the target analytes are most likely positively charged (a higher positive charge of a molecule correlates with presence of OH groups) and consequently are repelled by the positively charged column. As charge on the molecule increases, the more significant the repulsion force of the column and the smaller the retention time. This might explain the short retention times and the elution order: glucose (6 –OH groups), glycerol (3 –OH groups), acetic acid and finally ethanol (1 –OH group).

Table 6.1: Detection and retention time (R_t) of different chemical compounds using HI-Plex H column

No.	Substance	Rt (min)
1	Sodium bicarbonate ($NaHCO_3$)	NA
2	Sodium carbonate (Na_2CO_3)	NA
3	Sodium fluoride (NaF)	NA
4	Sodium sulphate (Na_2SO_4)	10.48
5	Sodium bromide (NaBr)	10.5
6	Potassium chloride (KCl)	10.55
7	Magnesium chloride ($MgCl_2$)	10.55
8	Sodium chloride (NaCl)	10.55
9	Maltose ($C_{12}H_{22}O_{11}$)	12.5
10	Sucrose ($C_{12}H_{22}O_{11}$)	12.52
11	Tri-sodium phosphate (Na_3PO_4)	12.65
12	Lactose ($C_{12}H_{22}O_{11}$)	12.7
13	Tri-sodium citrate ($Na_3C_6H_5O_7$)	13.38
14	Citric acid ($C_6H_8O_7$)	13.42
15	Glucose ($C_6H_{12}O_6$)	14.45
16	Galactose ($C_6H_{12}O_6$)	15.35
17	Xylose ($C_5H_{10}O_5$)	15.35
18	Fructose ($C_6H_{12}O_6$)	15.73
19	Mannitol ($C_6H_{14}O_6$)	16.08
20	Sorbitol ($C_6H_{14}O_6$)	16.65
21	Arabinose ($C_5H_{10}O_5$)	16.7
22	Succinic acid ($C_4H_6O_4$)	19.25
23	Sodium succinate ($C_4H_4Na_2O_4$)	19.27
24	Lactic acid ($C_3H_6O_3$)	20.08
25	Glycerol ($C_3H_8O_3$)	20.33
26	Formic acid (CH_2O_2)	21.28
27	Sodium acetate ($C_2H_3NaO_2$)	23.28
28	Acetic acid (CH_3COOH)	23.28
29	Ethanol (C_2H_6O)	30.63

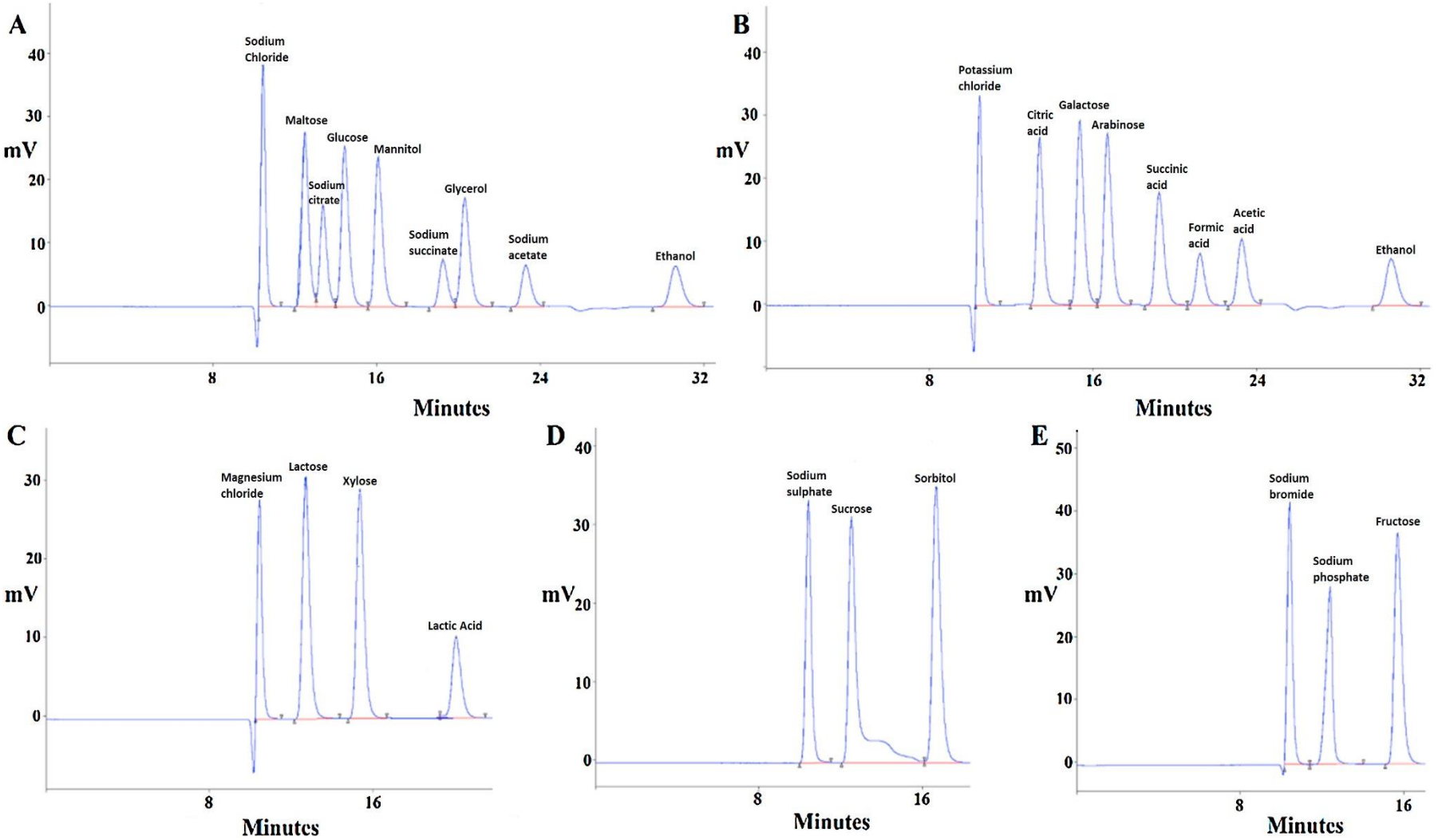


Figure 6.4: HPLC chromatograms from 5 mixed solutions of different chemical components

6.2.3 Validation of the method for the accurate quantification of NaCl

These experiments were conducted to validate the ability of the method for measuring NaCl. The detailed procedures were descried in sections (3.13.5), (3.13.6) and (3.13.7).

6.2.3.1 Assay linearity, accuracy, precision, and sensitivity

A set of NaCl standards in the range of 5.00 - 40.00 g/L was used to establish the calibration curves at high concentration range. The data from 4 different sequences of standard NaCl samples run on separate occasions are shown in Table 6.2. The relationship between the NaCl concentration and the peak areas was described by the linear regression equation: $y = 289.49x + 485.74$ ($n = 12$, $R = 0.999$), in which x is the NaCl concentration in (g/L) and y is the chromatogram peak area of NaCl. The precision and the accuracy of the results were within an acceptable level with CV and DEV values of ≤ 5.34% for all standards (Table 6.2).

Similarly, NaCl standards at the low concentrations range of 2.50 – 0.25 g/L were used to build calibration curves that suit samples containing 5 g/L NaCl or below. The linear regression equation: $y = 409.67x + 24.853$, $R^2 = 0.9996$ ($n = 12$, $R = 0.999$) was used to calculate the NaCl concentrations for 4 different sequences of NaCl standard samples run on separate occasions as shown in Table 6.2. The precision and the accuracy of the results were within an acceptable level with CV and DEV values ≤ 7.80% for all samples (Table 6.2).

Table 6.3 shows the inter-run average results of all standard curve samples. The accuracy of the assay was demonstrated by DEV values ≤ 7.80% and by precision CV values less than 9.43% for all samples representing both high and low standard ranges.

6.2.3.2 LOD and LOQ

The lower LOD was determined as the sample whose S/N was just greater than 3 and corresponded to 0.2 g/L NaCl. On the other hand, the lowest LOQ was estimated at 0.25 g/L NaCl, which displayed an S/N ratio equal to 10. The accuracy (DEV%) and precision (CV) values were within 10% of the nominal concentration values (see Tables 6.2 and 6.3).

Table 6.2: Intra variation of four separate assays[a] - accuracy, precision, and linearity of the standard curve samples

Nominal concentration (g/L)		Intra-run of each assay							
		Calculated concentration (g/L) (*n*=3)				Ave.	SD (g/L)	CV (%)[b]	DEV (%)[c]
		1	2	3	4				
High concentrations	**40**	40.49	39.98	39.77	39.80	40.01	0.33	0.83	-0.03
	20	20.49	20.69	20.53	19.72	20.36	0.44	2.14	-1.78
	10	9.50	10.42	10.24	10.15	10.08	0.40	3.95	-0.77
	5	5.42	4.94	5.52	5.18	5.27	0.26	4.87	-5.34
	R	**0.9992**	**0.9995**	**0.9998**	**0.9999**	**0.9999**	**0.00031**	**0.0305**	**0.01**
Low concentrations	**2.5**	2.55	2.54	2.48	2.56	2.53	0.03	1.30	-1.21
	1	1.08	1.11	1.09	1.1	1.10	0.01	1.18	-9.50
	0.5	0.48	0.51	0.52	0.49	0.50	0.02	3.79	-0.40
	0.25[d]	0.25	0.24	0.21	0.25	0.24	0.02	7.46	4.64
	R^2	**0.999**	**1**	**0.9994**	**0.999**	**0.9999**	**0.000473**	**0.047263**	**0.01**

[a] A linear curve was fitted to the data for response of NaCl versus theoretical concentration as described in Section 3. The calculated concentration was derived from reading the response for the standard sample against calibration curve. Each entry (assays 1–4) corresponds to the average value of triplicate analysis.

[b] CV (coefficient of variation, precision) = calculation according to Eq. (3.1) in section 3.13.6.

c Accuracy (DEV%) = the deviation of the calculated concentration from the nominal value. Calculated according to Eq. (3.2) in section 3.13.6.

[d] (LOQ) limit of quantification.

Table 6.3: Inter-run variation of four separate assays[a] - accuracy, precision, and linearity of the standard curve samples

Nominal concentration (g/L)		Inter-run			
		Mean (*n*=12)	SD (g/L)	CV (%)[b]	DEV (%)[c]
High concentrations	**40**	40.03	0.88	2.19	-0.06
	20	20.62	0.11	0.53	-3.10
	10	10.07	0.59	5.82	-0.73
	5	5.28	0.30	5.63	-5.62
	R	**1.00**	**0.00**	**0.00**	**0.03**
Low concentrations	**2.5**	2.53	0.17	6.74	-1.21
	1	1.1	0.01	-0.32	-9.06
	0.5	0.50	0.04	7.71	-0.40
	0.25[d]	0.24	0.02	7.63	3.12
	R^2	**0.999**	**0.00**	**0.00**	**0.1**

For [a], [b], [c], and [d], see the legend in Table 6.2.

The reproducibility of the method was evaluated by analysing the quality control samples of NaCl made up at concentrations of 1, 2, 3, and 7.5, 15 and 30 g/L (n=4). The accuracy and precision of the assay are demonstrated by DEV values ≤ 8.2% and by CV values ≤ 6.51% for all samples (Table 6.4)

Table 6.4: Quality Control Samples[a]

Nominal concentration (g/L)	Average (n=4)	SD	CV (%)	DEV (%)
30	29.50	0.83	2.82	1.68
15	15.86	0.18	1.14	-5.75
7.5	8.07	0.53	6.51	-7.64
3	2.93	0.16	5.34	2.21
2	2.14	0.09	4.21	-7.08
1	1.08	0.06	5.55	-8.20

[a)] The data are shown as averages, SD, accuracy (percent deviation, DEV%), and CV (precision). Accuracy and precision calculations were carried out by Eqs. (3.1) and (3.2), respectively.

6.2.4 Application of the HPLC assay to the quantification of chloride salts in food and beverages

In this section, the HPLC method, investigated in this study, was applied to 15 food and drink samples obtained from a retail market. Furthermore, three existing methods for salt analysis were used for comparison. Sample preparation and analysis procedures were described in section (3.13.8).

6.2.4.1 Analysis of NaCl content using the HPLC method and comparison with three existing NaCl determination methods

Table 6.5 shows the results of NaCl content in 15 food and drink samples which were measured using the HPLC method developed in this study, as compared with three existing methods for sodium chloride quantification. The expected salt content in 3 types of energy drinks was around 2 g/L. The results shown in Table 6.5 revealed that the majority of the salts in these drinks were sodium-based salts. Hence, only Flame Photometer (FP) could provide the labelled salt content in those samples. Results obtained by our HPLC method were close to those results obtained by the IC in the case of 8 samples including; milk, whey (a) and (b), feta cheese, cheddar cheese, pickle solution (a) and (b) and Peri-Peri sauce. Also, the results from HPLC were close to the results obtained by ATAGO meter in the case of 6 samples (5, 6, 7, 10, 13 and 14) and close to the results obtained by FP in the case of 5 samples (6, 7, 10, 11 and 15).

As expected, the results from IC were closer to that obtained from the HPLC method reported in this study, as both methods are Cl^- based methods. However, looking at the expected salts content and the results from the other methods, the HPLC method recorded better results comparing with IC in the case of 5 products (samples; 4, 5, 6, 10 and 15). ATAGO meter is a simple method and can provide a rapid measurement of the salt content in a sample but the results maybe not be very accurate as the conductivity is usually affected by other components in the solution. Sodium chloride (NaCl) is a common flavour and preservative component present in many food products such as cheeses, sauces and pickles. Also, media used in fermentations could contain high salt concentrations as seawater has been suggested as an alternative to the use of freshwater in some fermentations such as bioethanol production (Lin et al., 2011; Zaky et al., 2014). Hence, there is a need for an accurate and rapid method for NaCl determination during the manufacturing processes.

Classical titration methods, Mohr (Doughty, 1924), and Volhard (modified) (Schales and Schales, 1941), which are based on the use of silver nitrate ($AgNO_3$), are still widely used for the determination of NaCl (Leong et al., 2014, Rajković, 2010). However, those methods are associated with several limitations such as: a) time consuming, b) results are sensitive to the pH and the presence of heavy metals in the sample, c) they can have false end points, d) difficult to automate, and e) the safe disposal of silver compounds after testing (Wolfbeis and Hochmuth, 1924). Silver nitrate is considered a very toxic and corrosive compound even at very low concentrations (Zhao and Wang, 2011). Hence, the chloride analyser has been suggested as a method, this is a rapid test but still requires $AgNO_3$ to operate (Johnson and Olson, 1985). The HPLC method investigated in this study provided an accurate quantification for NaCl with the possibility for quantifying variety of chemical components in one analysis. However, the main limiting factor of this method is that NaCl cannot be accurately quantified in samples containing other chloride salts such as KCl and $MgCl_2$ because these salts elute at the same R_t as NaCl. Also, some inorganic anions such as SO_4^- and Br^- elute at a similar R_t as Cl^- and therefore, NaCl cannot be quantified accurately in the presence of these anions.

Table 6.5: Determination of salt content in food samples using HPLC method with comparison with 3 other methods

No	Sample	Salt content on the label	HPLC	Ion Chromatography (IC)		Flame Photometer (FP)		ATAGO Meter
			NaCl	Cl-	as NaCl	Na+	as NaCl	NaCl
		g/L (samples 1 to 10) or g/kg (samples 11 to 15)						
1	**Energy Drink (a)**	2	0.56 ±0.01	0.0077	0.013	0.85 ±0.04	2.15	0.80 ±0.01
2	**Energy Drink (b)**	2	0.31 ±0.01	0.0075	0.012	0.66 ±0.05	1.67	0.80 ±0.00
3	**Energy Drink (c)**	2.3	0.31 ±0.00	0.0078	0.013	0.85 ±0.04	2.15	1.23 ±0.02
4	**Lime soda**	0.3	0.29 ±0.01	0.0043	0.007	0.32 ±0.06	0.81	0.47 ±0.01
5	**Tomato Juice**	5.5	5.73 ±0.2	2.3775	3.920	1.19 ±0.04	3.03	6.33 ±0.04
6	**pickle solution (a)**	NA	40.98 ±0.22	21.6337	35.665	14.57 ±1.06	37.03	39.00 ±0.17
7	**pickle solution (b)**	NA	38.58 ±0.65	22.2017	36.602	15.37 ±0.39	39.06	38.33 ±0.06
8	**Milk**	1.5	1.77 ±0.12	0.9704	1.600	0.41 ±0.01	1.05	2.63 ±0.01
9	**Whey (a)***	NA	2.32 ±0.01	1.1046	1.821	0.42 ±0.03	1.07	3.53 ±0.01
10	**Whey (b)***	NA	36.73 ±0.62	26.8964	44.341	13.97 ±0.53	35.50	36.00 ±0.00
11	**Feta Cheese**	25	17.55 ±0.23	9.5582	15.758	6.40 ±0.51	16.27	26.00 ±0.00
12	**Cheddar Cheese**	18	22.77 ±0.02	13.5634	22.361	7.30 ±0.25	18.56	30.00 ±0.00
13	**Humus**	8.1	11.35 ±0.01	4.5170	7.447	3.33 ±0.39	8.47	13.00 ±0.00
14	**Peri-Peri Sauce**	15	16.90 ±0.01	9.9333	16.376	5.27 ±0.15	13.39	17.67 ±0.01
15	**Tomato Sauce**	7.2	8.71 ±0.01	3.8774	6.392	2.23 ±0.15	5.68	12.00 ±0.00

Except IC, data were presented as a mean value of 3 replicates ±SD.

*Whey (a) was obtained from out of date milk sample (8), while whey (b) was obtained by adding 35 g/L NaCl, 11 g/L lactic acid and 11 g/L acetic acid to the milk sample (sample 8) and incubated a at 35ºC for 3 days.

6.2.4.2 Analysis of food sample using the HPLC method

Table 6.6 shows sugars, organic acids and ethanol content in salty food samples using the HPLC method developed in this study. The method was able to quantify different types of sugars (sucrose, glucose, lactose and galactose) that exist in the samples under investigation. The results obtained for the sugars were similar to the total sugar content showed on the labels of these samples. There was clear separation between the sugar peaks and between the sugars and the Cl^- salt peak. This was one of the major objectives of this study because obtaining accurate quantification for sugars, especially glucose and sucrose, in samples contain Cl^- salts was reported difficult due to similar retention times for Cl^- and sugars (Sims, 1995).

Citric acid was found in most of the products with around 10 g/L or g/kg in 5 samples including; energy drinks (a, b and c), humus and tomato sauce. Acetic acid was detected in 6 samples and recorded at 10.61 g/L in pickle solution (a) and at 33.19 g/kg in Peri-Peri sauce. The presence of high amounts of acetic acid in Peri-Peri sauce was expected as vinegar is one of the main components in its recipe. Lactic acid was detected in 6 samples (6, 7, 9, 10, 11 and 12) and recorded at about 18 g/kg in both cheese samples. The presence of lactic acid in cheese is normal due to the fermentation of lactose during cheese maturation (Olson, 1990). Lactic acid in cheese was reported at 1.5 to 2.0% by many researchers (Blake et al., 2005, Macedo and Malcata, 1997).

Ethanol was detected in small amounts (0.24 ± 0.01 - 0.74 ± 0.02 g/L) in 4 samples including pickle solutions (a) and (b), energy drink (b), and lime soda. Few studies have reported the presence of ethanol in non-alcoholic foods and beverages (Goldberger et al., 1996, Logan and Distefano, 1998, Lutmer et al., 2009).

Table 6.6: HPLC analysis of food products from retail market

No	Samples*	Organic compounds									
		Sugars						Organic acids			Ethanol
		Sucrose	Lactose	Fructose	Glucose	Galactose	Total	Citric acid	Lactic Acid	Acetic acid	
		g/L (samples 1 to 10) or g/kg (samples 11 to 15)									
1	Energy Drink (a)	57.90 ±3.68	0.00	15.79 ±0.66	25.43 ±4.16	0.00	99.12	9.69 ±1.31	0.00	0.00	0.00
2	Energy Drink (b)	58.60 ±1.66	0.00	10.16 ±0.51	32.33 ±3.46	0.00	101.09	10.93 ±1.53	0.00	0.00	0.24 ±0.01
3	Energy Drink (c)	0.00	0.00	0.00	0.00	0.00	0.00	9.26 ±1.30	0.00	0.00	0.00
4	Lime soda	0.00	0.00	0.00	0.00	0.00	0.00	3.42 ±0.19	0.00	0.00	0.34 ±0.01
5	Tomato Juice	1.18 ±0.04	0.00	12.93 ±0.47	12.88 ±0.49	0.00	26.99	4.38 ±0.56	0.00	0.00	0.00
6	pickle solution (a)	0.00	0.00	5.10 ±0.34	3.40 ±0.31	0.00	8.50	3.50 ±0.04	6.68 ±0.06	10.61 ±0.09	0.36 ±0.00
7	pickle solution (b)	0.00	0.00	0.00	4.50 ±0.11	0.00	4.50	0.29 ±0.00	2.23 ±0.07	1.59 ±0.05	0.74 ±0.02
8	Milk	0.00	60.91 ±3.24	0.00	0.00	0.00	60.91	0.00	0.00	0.35 ±0.04	0.00
9	Whey (a)	0.00	58.14 ±0.86	0.00	0.00	0.29 ±0.01	58.43	0.00	0.42 ±0.00	0.55 ±0.01	0.00
10	Whey (b)	0.00	42.72 ±0.75	0.00	0.00	0.00	42.72	0.17 ±0.00	9.99 ±0.23	12.00 ±0.18	0.00
11	Feta Cheese	0.00	6.27 ±0.02	0.00	0.00	0.00	6.27	2.31 ±0.06	18.19 ±0.27	0.00	0.00
12	Cheddar Cheese	0.00	0.00	0.00	0.00	0.00	0.00	2.60 ±0.01	18.80 ±0.03	0.00	0.00
13	Humus	7.48 ±0.68	0.00	2.27 ±0.06	1.93 ±0.35	0.00	11.68	11.91 ±1.04	0.00	0.00	0.00
14	Peri-Peri Sauce	0.00	0.00	6.33 ±0.11	4.78 ±0.05	0.00	11.11	5.56 ±0.15	0.00	33.19 ±0.69	0.00
15	Tomato Sauce	15.51 ±0.42	0.00	32.17 ±0.86	29.28 ±0.79	0.00	76.96	11.78 ±0.18	0.00	0.00	0.00

Data were presented as a mean value of 3 replicates ±SD.

6.3 Conclusion

A simple, rapid and reproducible chromatographic methodology has been developed and successfully applied for the determination of chloride in the presence of sugars from food and beverage samples. The results obtained by this method were compared with those obtained using a salt meter (ATAGO), flame photometer and ion chromatography. The results suggested that the new method can be applied to a wide variety of food products such as milk, cheese, whey, pickles, sauces and juices as well as samples from fermentations using seawater. For the first time, the simultaneous determination of Cl^-, sugars, organic acids and ethanol in food and beverages has been achieved in a rapid HPLC assay. The efficient separation of the aforementioned compounds was achieved by using an HPLC system equipped with a Hi-Plex H column and RI detector. The column was capable of fractionating the compounds in approximately 32 minutes.

CHAPTER 7:

7. Bioethanol production using marine yeast and seawater-based media

7.1 Introduction

With the growing demand for bioethanol, issues like freshwater shortage and competition with food and arable land are expected to rise. Water consumption for bioethanol production matters more than feedstock consumption because the production of crops is dependent on water availability. Gerbens-Leenes and Hoekstra (2012) concluded that global freshwater resources are limited and allocation of water for bio-ethanol production on a large scale will be at the cost of water allocation for food and other usages (Gerbens-Leenes and Hoekstra, 2012).

Bioethanol production is a heavily water-consuming process, and the water footprint of bioethanol depends on the type of feedstock being used in the production. It has been estimated that the global weighted average Water Footprint (WF) of ethanol (for each litre of ethanol produced) is 1400 L from sugar beet, 2500 L from sugar cane, and 2600 L from maize (Gerbens-Leenes et al., 2009).

Water requirements for biorefining in the new dry mill plants has been estimated at 3 L of freshwater for each litre of ethanol produced (Wu and Chiu, 2011). However, older dry mill ethanol plants use up to 11 L of freshwater to produce 1 L of bioethanol (Shapouri, 2005). Based on this, ethanol production currently consumes between 0.28 - 1.02 billion cubic meter of freshwater a year for biorefining only and this could increase at least 10 times by 2050 in line with the expected increase of world population and world demand on bioethanol. Hence, amongst other challenges associated with biofuel production, water consumption is an important consideration for large scale operations to achieve sustainability.

Seawater accounts for about 97% of world's water and covers approximately 71% of the world's surface, in form of connected network of aquatic ecosystem. It is a renewable water source and readily accessible in most countries around the world. Hence, the use of seawater for preparing media for fermentation an important approach for bioethanol production (Zaky et al., 2014). Additionally, seawater contains a spectrum of minerals and as such may avoid the addition of essential nutrients currently required for commercial fermentation medium (Lin et al., 2011). Thus, using seawater in fermentations could potentially improve the overall

economics of the process and make a strong impact on overcoming both the freshwater and energy crises (Zaky et al., 2014, Serra et al., 2016).

Using industrial yeast strains for bioethanol production from seawater media could be challenging as seawater has a high salt content (≈35 g/L) and other inhibitors resulting from the biological activities of marine organisms. Marine ecosystem contains 80% of the world's biological resources in a harsh and extreme habitat (DeLong et al., 2006), these factors make marine environment appealing for discovery of microorganisms with desired phenotypic traits. Hence, in the last two decades, there have been continuous efforts to explore useful marine microorganisms and the identification of unique biologically active molecules (Ramesh et al., 2009, Zaky et al., 2014). Marine yeasts are considered a promising biocatalyst system in fermentation with improved ecological footprints and smart properties (Domínguez de María, 2013). Research on yeast isolated from marine environments has revealed that these organisms have several promising features over terrestrial yeast strains (Sarkar et al., 2010, Zaky et al., 2014). For example; enzymes generated from marine yeast found to have salt tolerance, baro-philicity, cold adaptability, hyper-thermo-stability, chemo-selectivity, regio-selectivity, and stereo-selectivity (Lima and Porto, 2016). Hence, marine yeasts have been recently used for the production of enzymes (Raj et al., 2016), metallic nanoparticles (Manivasagan et al., 2016) and microbial pigments (Muthezhilan et al., 2014)

Recently, research has been conducted on the use of marine biomass for bioethanol production (Falter et al., 2015, Kostas et al., 2016). This will certainly reduce the impact of water used in feedstock cultivation and arable land will be saved for the production of food and feed crops, however, considerable amounts of water will be required for the refining of bio-ethanol. Hence, the aim of this chapter is to establish the first step towards marine fermentation strategy for bioethanol production where seawater substitutes freshwater in the fermentation media with the use of marine yeast as a biological catalyst.

7.2 Results:

In the previous chapters, 116 new marine yeasts were isolated using a method that was developed in this study (chapters 3 and 4). The new isolates were evaluated for the utilisation of mono-saccharides (glucose, xylose, galactose and mannitol) in reverse osmosis water (ROW) and seawater (SW) media. 21 isolates that represented the best utilisers for each sugar were further characterized using YT-plates and identified by DNA sequencing using ITS and D1D2 primers. The identified isolates belonged to 8 species: *Saccharomyces cerevisiae* (5), *Candida tropicalis* (4), *Candida viswanathii* (4), *Wickerhamomyces anomalus* (3), *Candida glabrata* (2),

Pichia kudriavzevii (1), *Candida albicans* (1) and *Issatchenkia orientalis* (1). In this chapter, out of the 19 identified yeasts, we selected 9 representative strains to be screened for ethanol production using media prepared with ROW and SW. Three marine *S. cerevisiae* strains were taken forward to investigate their tolerance to the presence of high concentrations of glucose and salt. The industrial distiller strain *S. cerevisiae* NCYC2592 was used as a reference terrestrial strain for comparison. The marine strain (*S. cerevisiae* S65) which showed the best performance was applied for ethanol production from YPD-SW and molasses-SW media using 15 L bioreactors.

7.2.1 Screening for high ethanol producing marine yeast

Using small scale (100 mL) fermentation technique, 9 marine yeasts were screened for ethanol production from YPD media containing 6% (w/v) glucose which had been prepared using ROW and SW. The industrial strain *S. cerevisiae* NCYC2592 was used as reference for comparison. The fermentation was carried out anaerobically at 30^{o}C for 30 h. The fermentation rate was monitored as a weight loss over the fermentation period. Glucose utilisation and fermentation output (ethanol, glycerol and acetic acid) were determined using HPLC.

7.2.1.1 Fermentation rate

In fermentations using ROW, marine *S. cerevisiae* (S65, S71, and S118) had a faster fermentation rate when compared with the terrestrial strain NCYC2592. In general, non *S. cerevisiae* yeasts had a slower fermentation rates (Figure 7.1A), however, two of them (*I. orientalis* S88 and *C. glabrata* S127) had a faster fermentation rates in the first 8 hours of the fermentation when compared with NCYC2592 (Figure 7.1A). Fermentations using SW revealed that marine *S. cerevisiae* strains (S65, S71, S118) had significantly faster rates of fermentation when compared with NCYC2592 (Figure 7.1B). Two non *S. cerevisiae* marine yeasts (S88 and S127) had a fast rate of fermentation initially; but after 12 hours, their fermentation rates slowed in comparison with the reference strain (Figure 7.1B).

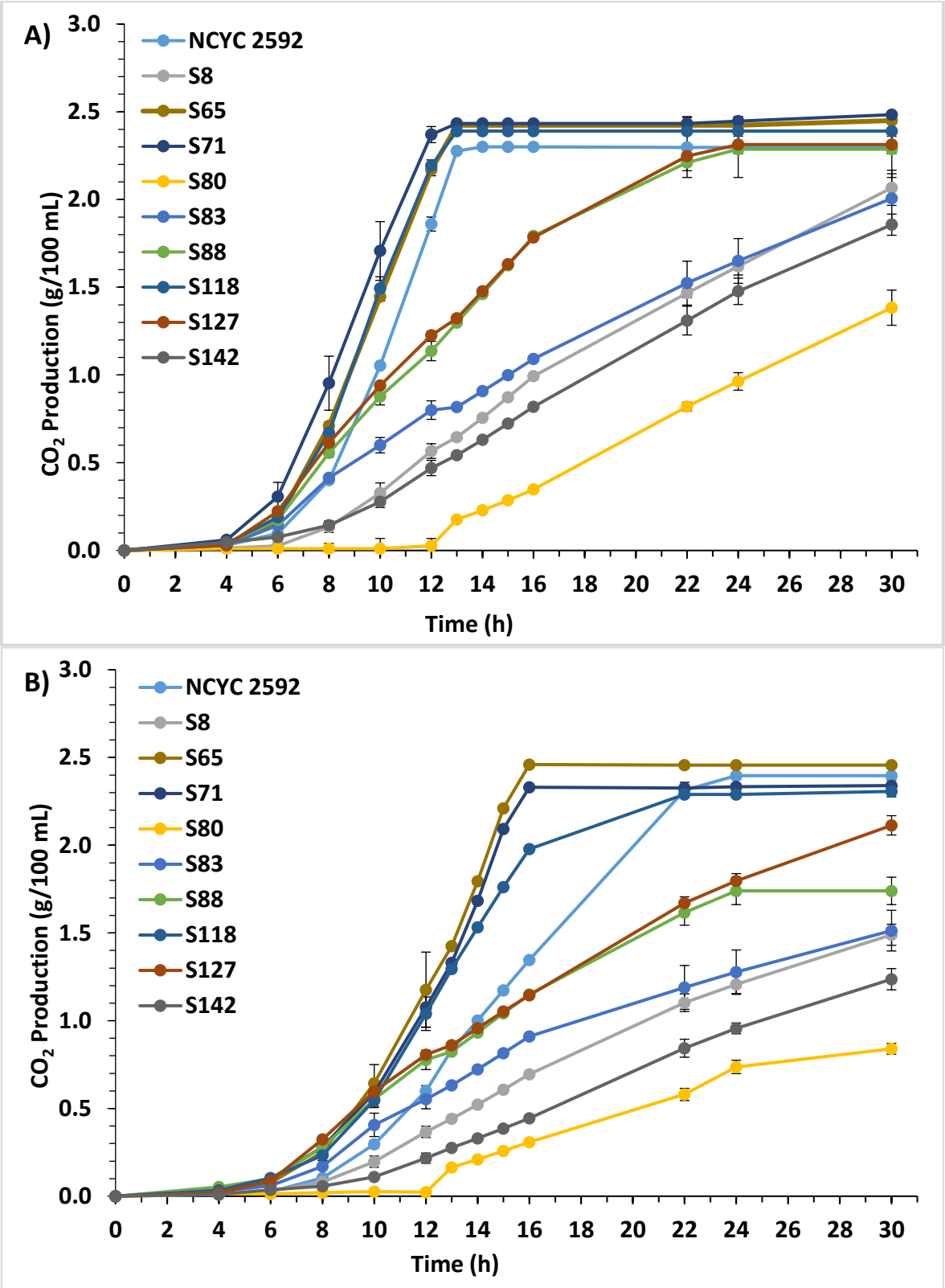


Figure 7.1: Fermentation rates of marine yeast strains in seawater and ROW based media
A) Fermentation profiles using ROW based medium.
B) Fermentation profiles using SW based medium.

7.2.1.2 Analysis of Fermentation output using HPLC

Tables 7.1a, b and Figure 7.2 showed that all *S. cerevisiae* strains - including the reference strain - utilised all of the glucose in the fermentation media within 30 hours regardless of the type of water being used (ROW or SW). Two of the non *S. cerevisiae* marine strains (S88 and S142) were only able to completely utilise the glucose present when ROW was used. The other non *S. cerevisiae* strains were not able to completely utilise all the glucose available in the media with either ROW or SW. Generally, glucose utilisation was faster when ROW was used in the fermentation media for all strains. In fermentations using ROW, ethanol productivity (g/L/h) of the marine *S. cerevisiae* strains was slightly higher in comparison with NCYC2592. However, when SW was used, the ethanol productivity of the marine strains S65 and S71 was much higher than that obtained using NCYC2592. Interestingly, the ethanol yields were always higher when SW was used with all strains used in this study. The highest ethanol yield using ROW was 82.36%, which was achieved by the reference strain while the highest ethanol yield using SW was 92.48%, which was achieved by the marine *S. cerevisiae* S65. The production of ethanol by 6 strains, including the reference strain, was higher using SW-based medium; while, 4 strains (S8, S83, S88 and S142) produced more ethanol using ROW- based medium. The production of glycerol was slightly higher when SW was used comparing with using ROW and ranged from 2.49 to 8.00 and from 2.29 to 7.46 g/L respectively; however, two marine strains (S8 and S88), produced slightly higher amounts of glycerol using SW in comparison with ROW.

Table 7.1a: HPLC analysis for fermentations samples of marine yeasts using ROW under screening for ethanol production

ID Name	Strain No.	Time (h)	Glucose (g/L)	Glucose utilised (%)	Glycerol (g/L)	EtOH (g/L)	EtOH Yield (%)[a]	EtOH Yield (%)[b]	EtOH prod. (g/L/h)
S. cerevisiae	NCYC 2592	14	0.00 ±0.00	100.00	1.92 ±0.05	23.10 ±0.25	82.36	82.36	1.65
C. viswanathii	S8	30	6.75 ±1.72	87.73	1.54 ±0.09	19.99 ±1.71	71.26	81.22	0.67
S. cerevisiae	S65	13	0.00 ±0.00	100.00	2.20 ±0.19	22.98 ±0.47	81.93	81.93	1.77
S. cerevisiae	S71	13	0.00 ±0.00	100.00	2.13 ±0.16	22.86 ±0.78	81.50	81.50	1.76
W. anomalus	S80	30	23.16 ±1.20	57.90	1.40 ±0.01	12.99 ±0.37	46.31	79.98	0.43
P. kudriavzevii	S83	30	10.29 ±1.27	81.28	1.92 ±0.13	18.71 ±0.80	66.69	82.05	0.62
I. orientalis	S88	30	0.00 ±0.00	100.00	3.67 ±0.14	21.44 ±0.37	76.44	76.44	0.71
S. cerevisiae	S118	13	0.00 ±0.00	100.00	1.89 ±0.14	22.39 ±0.14	79.82	79.82	1.72
C. glabrata	S127	30	0.00 ±0.00	100.00	3.73 ±0.10	20.54 ±0.33	73.24	73.24	0.68
C. albicans	S142	30	12.75 ±1.86	76.82	1.14 ±0.03	17.18 ±0.21	61.24	79.71	0.57

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total glucose (60 g).

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised glucose.

Table 7.1b: HPLC analysis for fermentations samples of marine yeasts using SW under screening for ethanol production

ID Name	Strain No.	Time (h)	Glucose (g/L)	Glucose utilised (%)	Glycerol (g/L)	EtOH (g/L)	EtOH Yield (%)[a]	EtOH Yield (%)[b]	EtOH prod. (g/L/h)
S. cerevisiae	**NCYC 2592**	**24**	0.00 ±0.00	100.00	2.38 ±0.10	25.75 ±0.58	91.80	91.80	1.07
C. viswanathii	**S8**	**30**	22.05 ±2.00	59.91	1.46 ±0.05	16.14 ±0.21	57.54	96.05	0.54
S. cerevisiae	**S65**	**16**	0.00 ±0.00	100.00	2.54 ±0.04	25.94 ±0.52	92.48	92.48	1.62
S. cerevisiae	**S71**	**16**	0.00 ±0.00	100.00	3.03 ±0.08	23.59 ±0.29	84.09	84.09	1.47
W. anomalus	**S80**	**30**	22.57 ±1.54	58.96	2.05 ±0.18	13.83 ±0.34	49.31	83.65	0.46
P. kudriavzevii	**S83**	**30**	13.82 ±1.51	74.87	2.29 ±0.20	17.78 ±1.90	63.39	84.66	0.59
I. orientalis	**S88**	**30**	13.06 ±2.07	76.25	3.33 ±0.20	19.17 ±0.81	68.34	89.62	0.64
S. cerevisiae	**S118**	**22**	0.00 ±0.00	100.00	2.93 ±0.22	23.72 ±1.16	84.58	84.58	1.08
C. glabrata	**S127**	**30**	10.83 ±1.55	80.31	4.00 ±0.17	21.42 ±0.77	76.38	95.11	0.71
C. albicans	**S142**	**30**	26.63 ±1.13	51.58	1.25 ±0.11	13.99 ±0.49	49.87	96.67	0.47

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total glucose (60 g).

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised glucose.

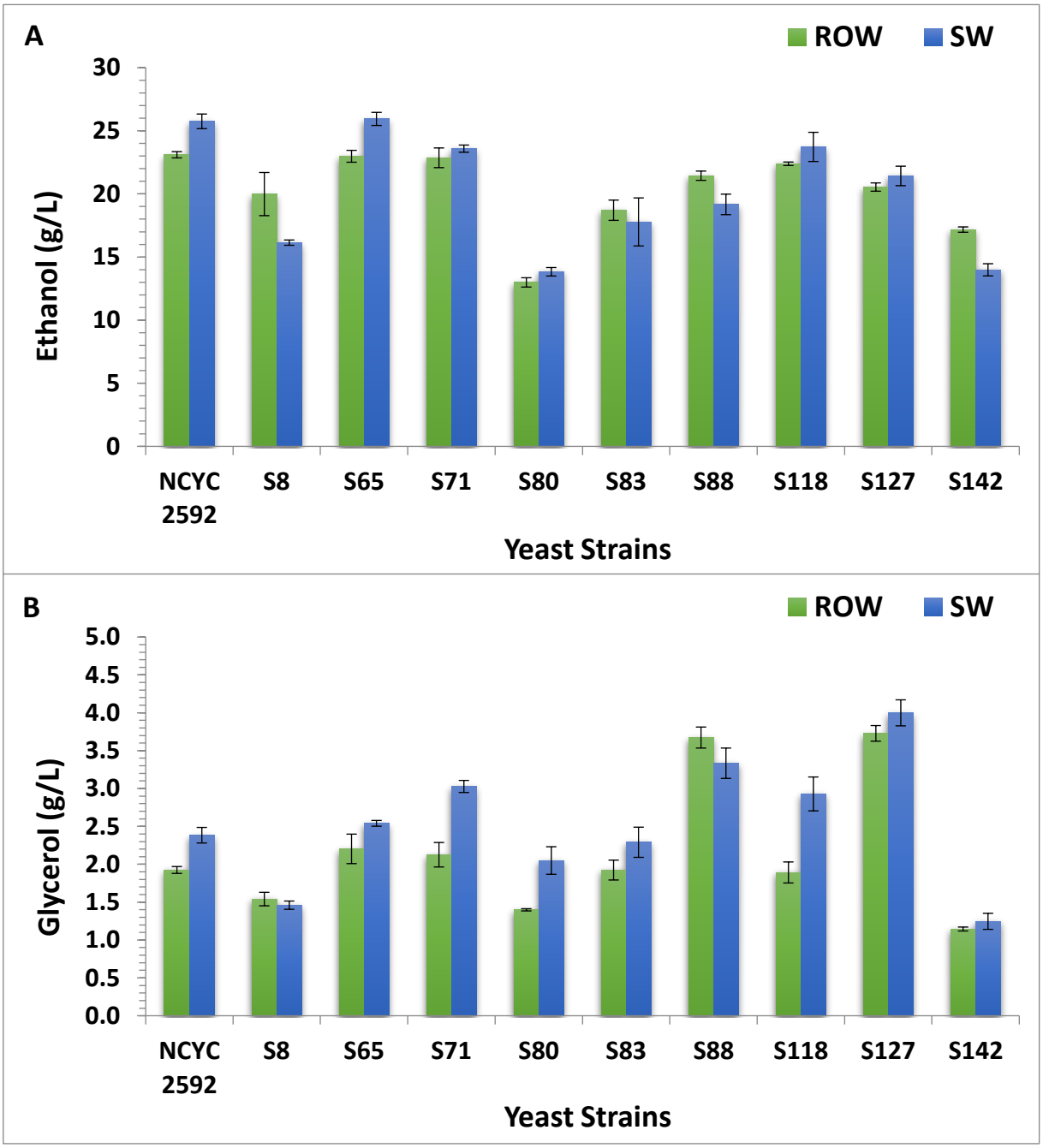


Figure 7.2: Ethanol and glycerol production by 9 marine yeasts in YPD media containing 6% glucose and prepared in seawater (SW) and freshwater (ROW)

7.2.2 Growth of marine yeast when exposed to increased salt and osmotic stress

Marine *S. cerevisiae* strains (S65, S71, and S118), which performed well in ethanol fermentation using media prepared with natural seawater, were selected to investigate their ability to tolerate high concentrations of glucose and seawater salts (the component of synthetic seawater). The reference strain *S. cerevisiae* NCYC2592 was also used for comparison. Growth media were prepared using four glucose concentrations (5, 10, 15, and 20%) dissolved in either ROW water or synthetic seawater (SSW) at different strengths (1x, 2x and 3x) to form 16 different conditions (Table 3.4 in chapter 3).

Results of fermentations using increasing osmotic stress (up to 20% glucose) in ROW water revealed that there were no differences in growth between the reference and the marine strains. All strains maintained a good growth rate and reached stationary phase in less than 20 h of propagation (Figure 7.3 A, B, C & D). When SSW was used for preparing the media, marine strains grew faster than the reference strain especially with medium contain 20% glucose (Figure 7.3 E, F, G & H). Fermentations using 2X SSW with 20% glucose showed a significant reduction in the growth of NCYC2592 when compared with the marine strains (Figure 7.4 D). Results of fermentations using 2x SSW with 20% glucose and 3x SSW demonstrated that the marine strain S118 was less tolerant comparing with the other marine strains (S65 and S71) (Figure 7.4D - H). Using 3x SSW and 20% glucose almost completely inhibited the growth of the reference strain (Figure 7.4H).

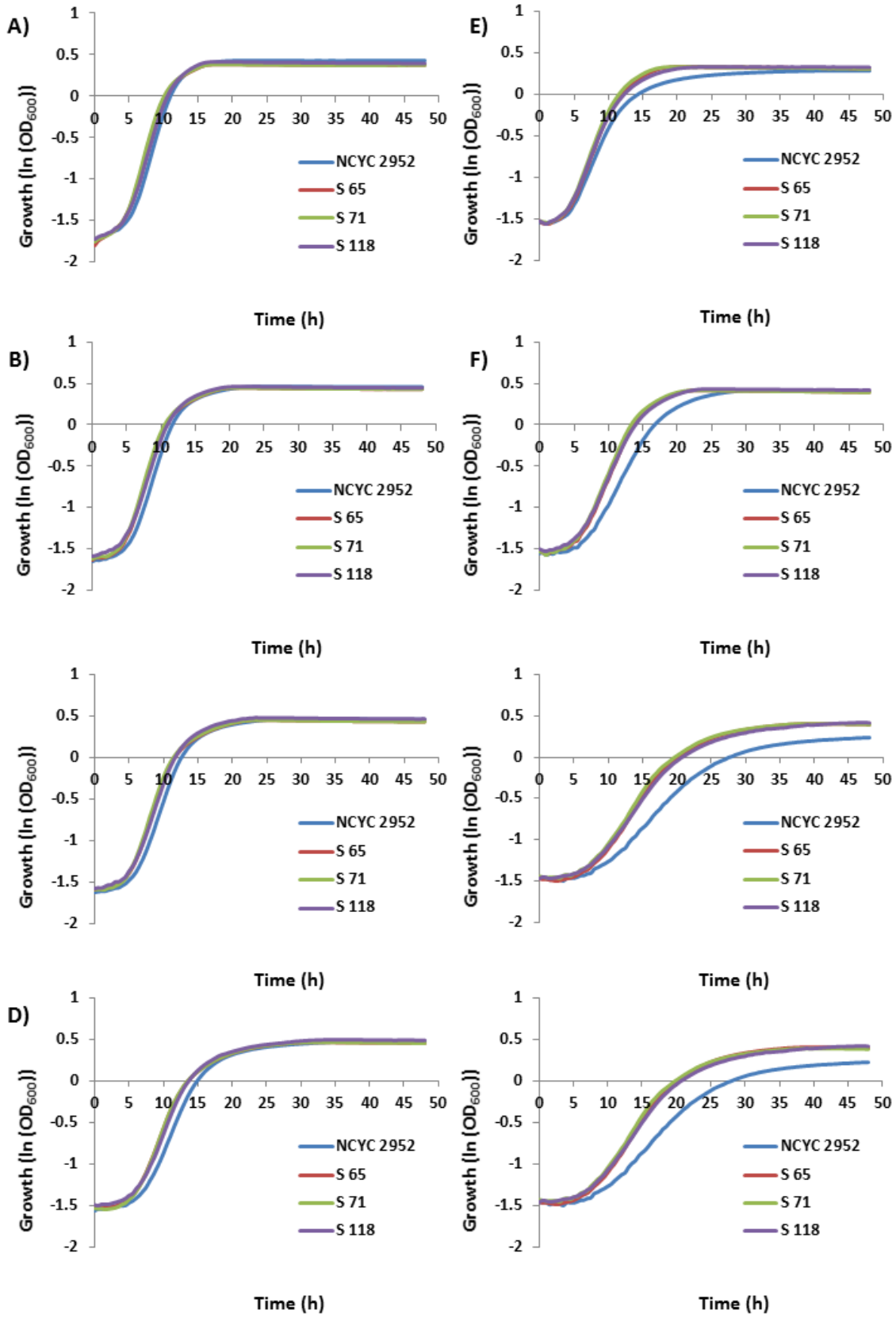


Figure 7.3: Growth of marine yeast strains when exposed to increased osmotic stress (up to 20% glucose) using ROW-based media, and SSW-based media

A) ROW contains 5% glucose, B) ROW contains 10% glucose, C) ROW contains 15% glucose, D) ROW contains 20% glucose, E) SSW contains 5% glucose, F) SSW contains 10% glucose, G) SSW contains 15% glucose, H) SSW contains 20% glucose. ln (OD) values were plotted on a linear scale.

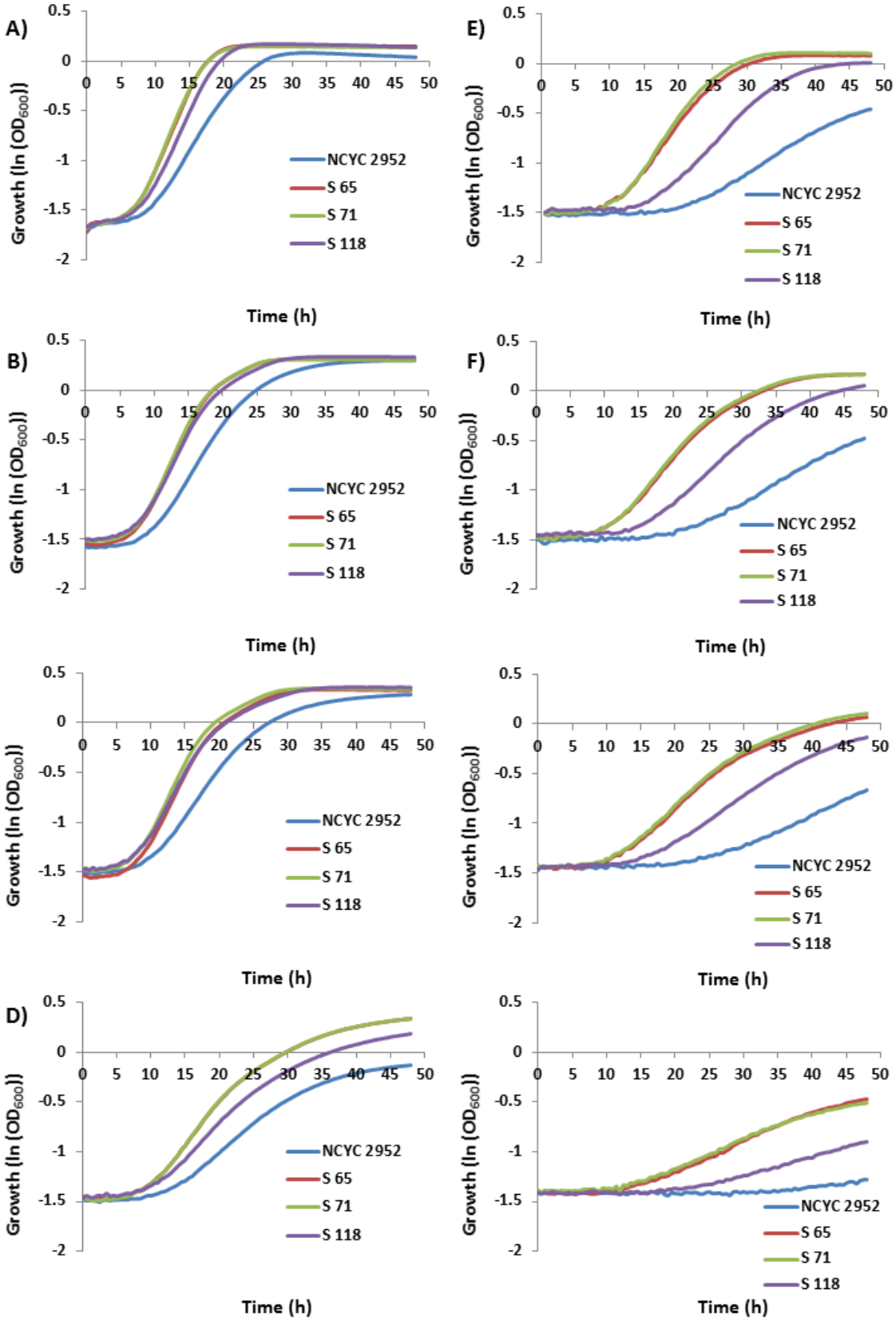


Figure 7.4: Growth of marine yeast strains when exposed to increased osmotic stress (up to 20% glucose) using 2XSSW-based media, and 3XSSW-based media

A) 2XSSW contains 5% glucose, B) 2XSSW contains 10% glucose, C) 2XSSW contains 15% glucose, D) 2XSSW contains 20% glucose, E) 3XSSW contains 5% glucose, F) 3XSSW contains 10% glucose, G) 3XSSW contains 15% glucose, H) 3XSSW contains 20% glucose. ln (OD) values were plotted on a linear scale.

7.2.3 The fermentation capacity of marine *S. cerevisiae* under high salt concentrations

This experiment investigated the fermentation ability of marine *S. cerevisiae* in the presence of high concentrations of salt. The fermentation media (YPD of 6% glucose) were prepared using SSW, 2x SSW and NaCl solutions of different concentrations (3, 6 and 9%). The reference strain NCYC2592 was used for comparison. Fermentations using media prepared in SW and ROW were used in this experiment for the purpose of comparison.

7.2.3.1 Ethanol fermentation using different concentrations of NaCl

Using ROW fermentation media revealed no differences in the fermentation rate of all strains under investigation (Figure 7.5A). Fermentations under different NaCl concentrations revealed that NCYC2592 had a slower rate of fermentation in media containing 3 and 6% of NaCl when compared with the marine strains (Figures 7.5B and 7.5C). In addition, the results showed that fermentation media containing 9% NaCl inhibited the fermentation of the reference strain (NCYC2592) while the marine strains had a long lag phase of 15 hours but then the fermentations did commence (Figure 7.5D).

7.2.3.2 Ethanol fermentation using synthetic seawater media of different strengths

Fermentations using SSW media were completed within 14 hours using marine strains while they took 24 hours with the reference strain (Figure 7.5F). When 2x SSW fermentation medium was used, marine strains were able to complete the fermentation within 24 hours, however, the reference strain required 36 hours to reach completion (Figure 7.5G). Generally, the rate of fermentations using SW media was slower than SSW but faster than 2x SSW.

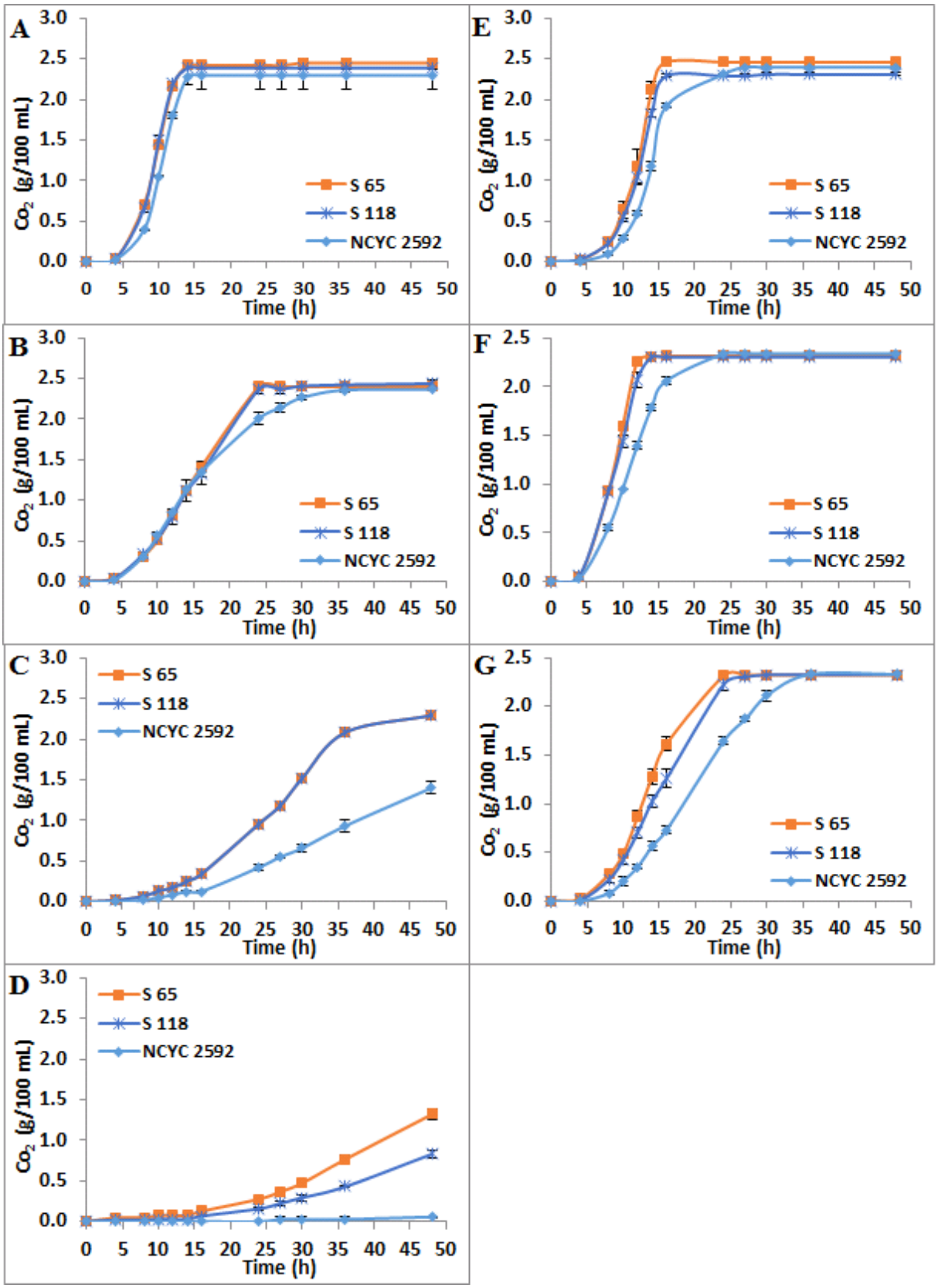


Figure 7.5: Fermentation rate of marine yeast strains when exposed to increased salt stress using YPD media containing 6%% glucose dissolved in different saline solutions

A) ROW, B) NaCl 3%, C) NaCl 6%, D) NaCl 9%, E) SW, F) SSW, G) 2x SSW.

7.2.4 The fermentation capacity of marine *S. cerevisiae* under high glucose concentration

This experiment was conducted to investigate the fermentation ability of two marine *S. cerevisiae* (S65 and S118) using SW-YPD fermentation media containing high glucose concentrations (10, 15, 20, and 25%). The reference strain *S. cerevisiae* NCYC2592 was used for comparison.

7.2.4.1 Fermentation rate

The rate of fermentation was accessed by assessing weight loss which is analogous to the production of CO_2 during the fermentation period. The fermentation was considered completed when there was no further weight loss. Marine strain S65 gave the best fermentation rate with all glucose concentrations, while the reference strain recorded the lowest fermentation rate (Figure 7.5). Using media containing 10% glucose, all strains were able to finish the fermentation within 68 hours, however, NCYC2592 required 36 hours, S118 required 24 hours and S65 required 20 hours to complete their fermentations (Figure 7.6A). In case of media containing 15% glucose, all strains were also able to complete the fermentation, however, the reference strain (NCYC2592) required the maximum scheduled fermentation time of the experiment (Figure 7.6B). When 20% glucose was added to the fermentation media, NCYC2592 was not able to complete the fermentation and S118 required the maximum time of the experiment while S65 was able to complete the fermentation within 36 hours (Figure 7.6C). Fermentations using SW-YPD media supplemented with 25% glucose, no strain was capable of completing the fermentation. However, S65 produced the highest amount of CO_2 (9.95 g/100 mL) while S118 and NCYC produced 7.56 and 4.89 g/100 mL CO_2, respectively (Figure 7.6D). The results clearly indicated that the marine strain S65 was more halo-osmo tolerant comparing with the reference and the other marine strain S118.

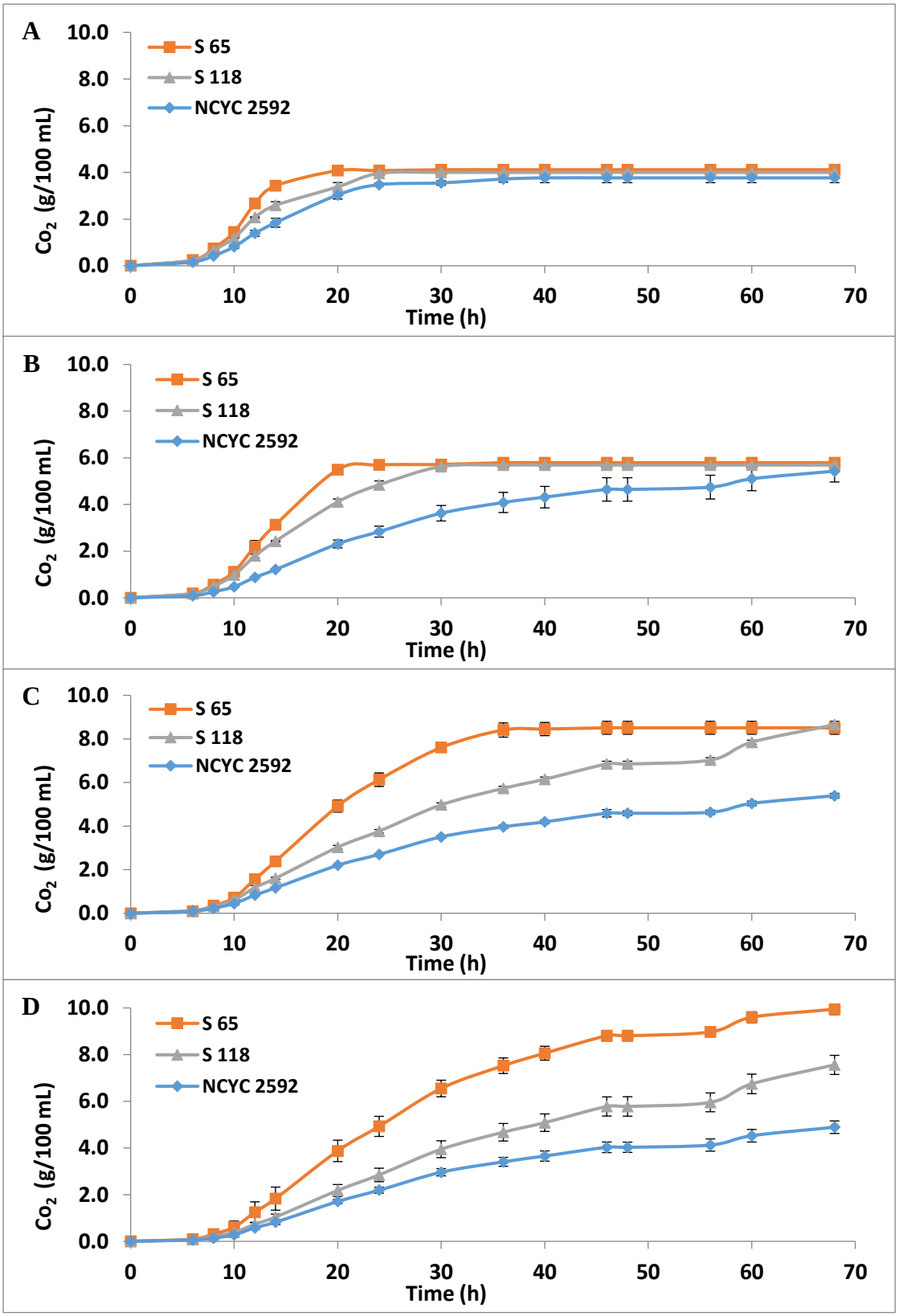


Figure 7.6: Fermentation rates of 3 yeast strains when exposed to osmotic stress using YPD media containing increased glucose concentrations (10, 15, 20, 25%) dissolved in seawater

A) glucose 10%, B) glucose 15%, C) glucose 20%, D) glucose 25%.

7.2.4.2 Analysis of Fermentation output using HPLC

Samples were prepared for analysis using HPLC after MFVs stopped losing weight for 3 time points continuously or after 68 h of fermentation. Ethanol production was the main parameter for strain evaluation. The marine strain S65 produced significantly higher concentrations of ethanol using SW medium which contained 25% of glucose when compared with the other marine strain and the reference strain. The results revealed that marine strains produced significantly higher amount of ethanol when compared with the reference strain when using SW media containing 20 or 25% glucose but slightly higher amount of ethanol when SW media containing 10 or 15% glucose was used. No significant difference in ethanol production was observed when ROW medium containing 10% glucose was used.

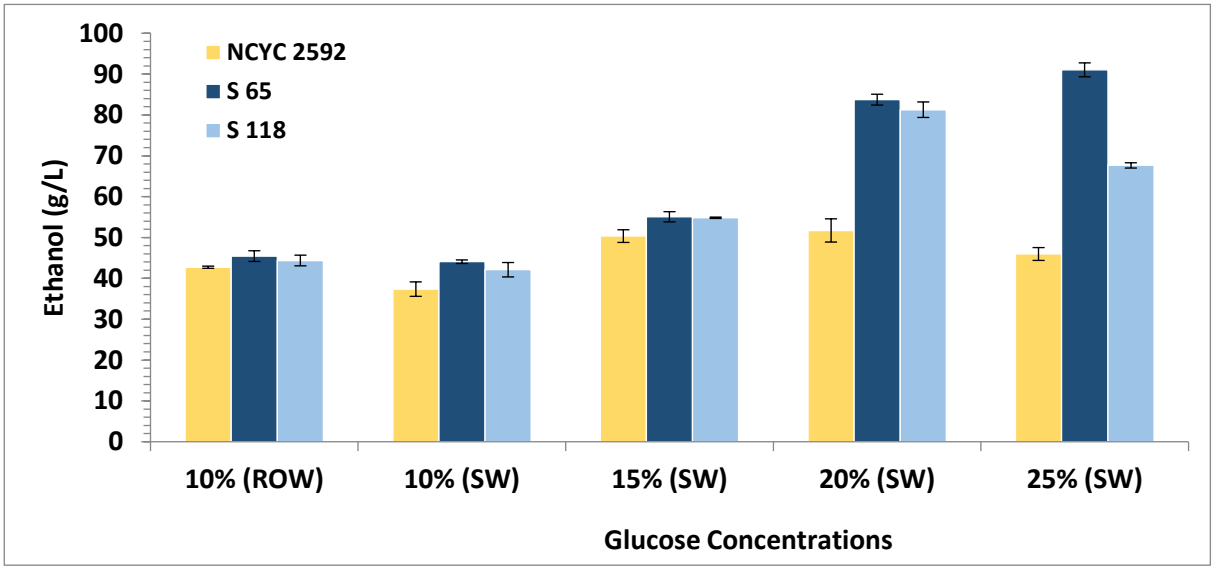


Figure 7.7: Comparing ethanol production by 2 marine strains and the reference strain using SW-YPD media containing increased glucose concentrations (10, 15, 20, 25%) dissolved in seawater

The reference strain was able to utilise 100% of the glucose in the fermentation media during the experiment time at the concentration of 10% only. The highest ethanol yield was 83.77% and highest ethanol productivity was 1.19 g/L/h which were obtained when ROW media containing 10% glucose was used. Ethanol yield and productivity generally decreased as the concentration of glucose in the SW fermentation media increased, the lowest yield and productivity were 36.05% and 1.19 g/L/h respectively. The lowest glycerol and acetic acid were produced from ROW medium and recorded 4.92±0.20 and 0.37±0.01 g/L respectively. The production of glycerol and acetic acid were increased as the glucose was increased in the SW fermentation media and reached 10.50±0.25 and 1.10±0.04 g/L respectively.

The marine strain S118 recorded better results comparing with the reference strain. The strain could utilise 100% of the glucose in the fermentation media that contained 15% glucose. The highest ethanol yield and ethanol productivity were obtained from ROW medium and recorded 87% and 1.85 g/L/h respectively. Ethanol yield and ethanol productivity in SW media ranged from 82.56 - 53.06% and 1.85 – 0.99 g/L/h respectively. The lowest glycerol and acetic acid were produced from ROW medium and recorded 3.93 ± 0.34 and 0.16 ± 0.02 g/L respectively. The production of glycerol and acetic acid were increased as the glucose was increased in the SW fermentation media and reached 13.34 ± 0.89 and 1.36 ± 0.09 g/L respectively (Table 7.2).

The best performance was obtained by the marine strain S65. This strain could utilise 87% of the glucose present in a fermentation medium which contained 25% glucose after 86 h of fermentation. In addition, this strain utilised 100% of the glucose present in all other fermentation media (10, 15, 20% glucose) in less than 50 h of fermentation. The highest ethanol yield and ethanol productivity were obtained from ROW medium and recorded 89.10% and 1.89 g/L/h respectively. The lowest ethanol yield was 71.98% from SW with 15% glucose while the ethanol yield from the other SW media was above 80%. The best ethanol productivity from SW-based media was 1.82 g/L/h and obtained using SW media of 20% glucose, while the lowest ethanol productivity was 1.34 g/L/h. The lowest glycerol and acetic acid were produced from ROW medium and recorded 7.41 ± 1.25 and 0.37 ± 0.01 g/L respectively. The production of glycerol and acetic were increased as the glucose was increased in the fermentation media and reached 15.45 ± 0.84 and 1.08 ± 0.02 g/L respectively. The production of glycerol by this strain was significantly higher when compared with the reference strain and the marine strain S118 (Table 7.2).

Table 7.2: HPLC analysis for fermentations using SW-based media containing increased glucose concentrations (10-25%)

Strain	Medium Glucose (g/L)	Time (h)	Glucose (g/L)	Glucose utilised (%)	Glycerol (g/L)	Acetic (g/L)	EtOH (g/L)	EtOH Yield[a] (%)	EtOH Yield[b] (%)	EtOH Produc. (g/L/h)
NCYC2592	10% (ROW)	36	0.00 ±0.00	100.00	4.92 ±0.20	0.37 ±0.01	42.72 ±0.27	83.77	83.77	1.19
	10%	40	0.00 ±0.00	100.00	7.60 ±0.31	0.66 ±0.01	37.36 ±1.77	73.25	73.25	0.93
	15%	68	38.35 ±1.49	74.44	9.36 ±0.49	0.74 ±0.03	50.34 ±1.55	65.80	88.40	0.74
	20%	68	85.55 ±4.43	57.22	10.00 ±0.66	0.94 ±0.06	51.73 ±2.83	50.71	88.62	0.76
	25%	68	142.42 ±4.25	43.03	10.50 ±0.25	1.10 ±0.04	45.96 ±1.57	36.05	83.77	0.68
S 65	10% (ROW)	24	0.00 ±0.00	100.00	7.41 ±1.25	0.37 ±0.04	45.44 ±1.31	89.10	89.10	1.89
	10%	30	0.00 ±0.00	100.00	7.91 ±0.91	0.59 ±0.02	44.07 ±0.43	86.41	86.41	1.47
	15%	36	0.00 ±0.00	100.00	9.57 ±0.35	0.64 ±0.02	55.07 ±1.25	71.98	71.98	1.53
	20%	46	0.00 ±0.00	100.00	12.57 ±0.28	0.83 ±0.03	83.75 ±1.33	82.11	82.11	1.82
	25%	68	30.89 ±4.32	87.64	15.45 ±0.84	1.08 ±0.02	91.04 ±1.70	71.40	81.47	1.34
S 118	10% (ROW)	24	0.00 ±0.00	100.00	3.93 ±0.34	0.16 ±0.02	44.37 ±1.31	87.00	87.00	1.85
	10%	30	0.00 ±0.00	100.00	6.78 ±0.17	0.50 ±0.04	42.10 ±1.77	82.56	82.56	1.40
	15%	36	0.00 ±0.00	100.00	8.85 ±0.42	0.70 ±0.04	54.83 ±0.17	71.67	71.67	1.52
	20%	46	16.02 ±1.87	91.99	12.58 ±0.24	1.12 ±0.04	81.28 ±1.89	79.69	86.63	1.77
	25%	68	77.48 ±2.03	69.01	13.34 ±0.89	1.36 ±0.09	67.65 ±0.64	53.06	76.89	0.99

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total glucose used in the fermentation medium.

[b] Calculated as a percentage of the theoretical yield (0.51) based on the amount of utilised glucose by the end of the experiment.

7.2.5 Assessing ethanol production of marine *S. cerevisiae* S65 using seawater-based media in a 15 L bioreactor

7.2.5.1 Batch fermentation

Fermentations at a small scale (0.1 L) revealed that the marine strain *S. cerevisiae* S65 was capable of efficiently converting high concentrations of glucose (20 – 25%) into ethanol using seawater-based media. After this initial assessment, the performance of this strain in 15 L bioreactors was carried out. YPD (20% glucose) fermentation media was prepared using natural seawater and inoculated with the marine strain *S. cerevisiae* S65 at a rate of 0.86 ± 0.09 OD. Fermentation was conducted anaerobically at 30^{o}C and 200 rpm for 48 hours. Yeast growth, ethanol concentration, glycerol concentration and the remaining glucose concentration were monitored at regular time intervals, and the results are shown in Figure 7.8 and Table 7.3. Yeast growth reached the maximum value of OD 17.25 ± 0.66 after 42 hours. All available glucose was utilised by 48 hours and there was a concurrent conversion of the glucose into ethanol. The maximum ethanol production (93.50 g/L) was recorded at 48 hours with 83.33% of the theoretical yield. Ethanol productivity increased during the first 24 hours and reached 2.49 g/L/h, and then decreased during the second 24 hours and reached 1.95 g/L/h by the end of fermentation time. Glycerol production showed a consistent trend of increase and reached the maximum of 13.66±0.43 g/L after 48 hours of fermentation. By The end of the fermentation time, the concentration acetic acid reached 0.69 ±0.02 g/L. This experiment was repeated two times and the results from individual experiments were shown in Figure 7.9.

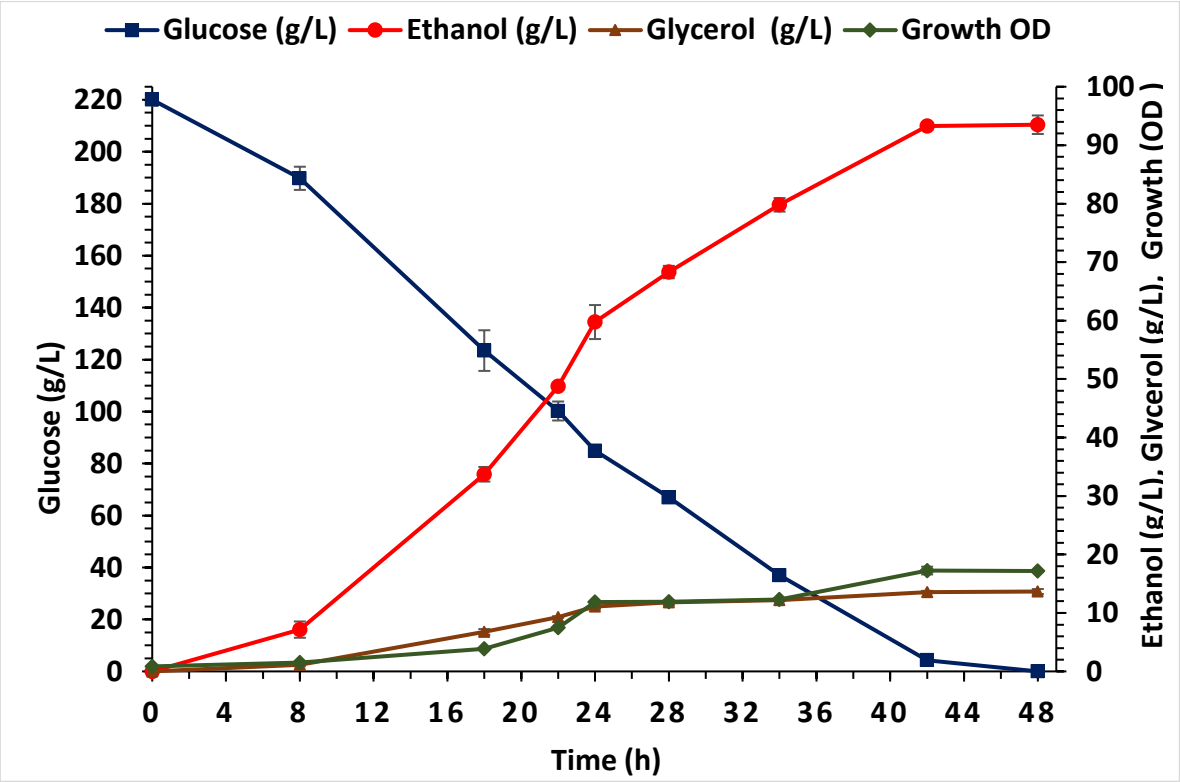


Figure 7.8: Changes in the concentration of glucose, ethanol, glycerol and biomass in a batch fermentation using SW media using marine *S. cerevisiae* S65

Table 7.3: HPLC analysis for batch fermentation using SW-based media containing 22% glucose

Time (h)	Growth OD	Glucose (g/L)	Utilised Glucose (%)	Glycerol (g/L)	Acetic (g/L)	EtOH (g/L)	EtOH Yield[a] (%)	EtOH Yield[b] (%)	EtOH Prod. (g/L/h)
0	0.86	220.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	1.52	189.76	13.75	1.15	0.07	7.15	6.38	46.16	0.89
18	3.88	123.47	43.88	6.76	0.32	33.72	30.06	69.01	1.87
22	7.48	100.22	54.45	9.30	0.44	48.74	43.44	79.79	2.22
24	11.84	84.92	61.40	11.12	0.55	59.75	53.24	86.71	2.49
28	11.92	66.92	69.58	11.78	0.61	68.31	60.89	87.49	2.44
34	12.31	37.02	83.18	12.19	0.65	79.81	71.13	85.51	2.35
42	17.25	4.27	98.06	13.57	0.69	93.30	83.16	84.80	2.22
48	17.18	0.00	100.00	13.66	0.69	93.50	83.33	83.33	1.95

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total glucose (220 g).

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised glucose at the time of analysis.

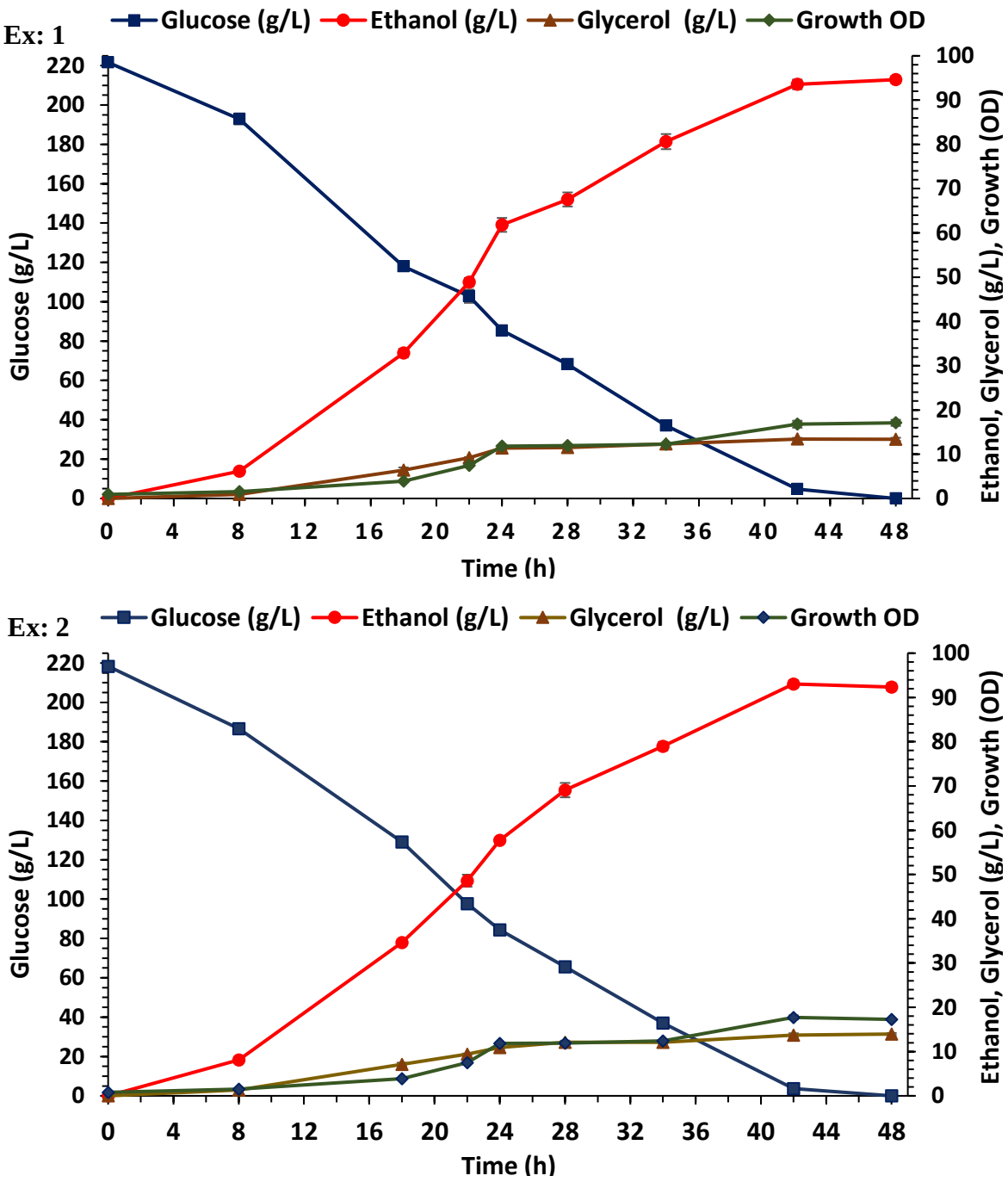


Figure 7.9: Two repeated experments show the changes in the concentration of glucose, ethanol, glycerol and biomass by the marine strain *S. cerevisiae* S65 in bach fermentation using SW-based media

7.2.5.2 3-stage batch fermentation

The fermentation experiment was conducted in 3 stages. The first two stages (yeast propagation and ethanol production) were conducted to simulate the industrial process. The 3rd stage was conducted to test the tolerance of the yeast strain to the presence of high sugar and ethanol in seawater-based fermentation media. The first fermentation stage was operated aerobically at 30°C and 200 rpm for 10 hours using SW-YPD media containing 4% (w/v) of glucose to allow maximum yeast propagation. In the second stage, glucose concentration in the fermentation medium was adjusted at about 20% (w/v) using glucose solution (100%, prepared using seawater) and fermentation was operated anaerobically at 35°C and 200 rpm for 20 hours. The third stage was conducted by increasing the glucose concentration in the fermentation medium to test the tolerance capabilities of the yeast strain. The third stage was operated anaerobically at 35°C and 200 rpm for 24 hours. Figure 7.10 shows the relation between the glucose utilisation, yeast growth, ethanol production and glycerol production though out the 3 stages of the fermentation.

In the first stage, yeast density increased rapidly from an OD of 2.22 ±0.11 to an OD of 17.75 ±0.92 after 10 hours when the glucose had been fully utilised. The highest ethanol production, ethanol yield and productivity were achieved after 8 hours of the fermentation and were 16.08 ±0.63 g/L, 74.10% and 2.01 g/L/h respectively. By the end of this stage, glycerol and acetic acid concentration reached 4.08 ±0.05 and 0.21 ±0.01 g/L respectively (Table 7.4).

In the second stage, the addition of the glucose solution diluted the concentrations of yeast cells, glycerol, acetic acid and ethanol that were obtained from the first stage. Yeast cells continued to increase and reached an OD of 24.65 ±0.78 by the end of this stage. During 20 hours of fermentation in this stage, around 73.32 g/L of ethanol was produced making the total ethanol in the reactor 86.72 ±1.33 g/L. glucose was not fully utilised by the end of this stage so, the maximum ethanol yield was only 73.26% but the yield based on the utilised glucose ranged between 89.64 - 85.84% throughout the second stage. Ethanol productivity ranged between 3.67 - 4.15 g/L/h. By the end of this stage, glycerol and acetic acid concentrations recorded 15.16 ±20 and 0.86 ±0.06 g/L respectively (Table 7.4).

The third stage of this fermentation experiment started with 129.62 ±0.62 g/L of glucose, 13.24 ±0.91 g/L of glycerol, 0.76 ±0.02 g/L of acetic acid and 75.50 ±1.79 g/L of ethanol. The reduction of the concentrations of yeast cells, glycerol, acetic acid and ethanol was a result of adding the glucose solutions. Yeast continued to produce ethanol, but at a slower rate comparing with the second stage, and reached 113.52 ±0.01 g/L after 24 hours of fermentation. Only 65.25% of the glucose had been utilised during the time of the 3rd stage hence, the maximum

ethanol yield in this stage was 57.51%, however, the yield based on the utilised glucose ranged between 74.31 and 90.73% throughout the stage. The highest ethanol productivity was 1.86 g/L/h which recorded after 4 hours of fermentation, then slightly decreased by time and reached 1.58 g/L/h by the end of the stage. Yeast growth stayed constant at around an OD of 21.5. By the end of this stage, glycerol and acetic acid concentrations were 18.31 ±0.77 and 0.98 ±0.13 g/L respectively (Table 7.4). This experiment was done in duplicate and the results from individual experiments were shown in figure 7.11.

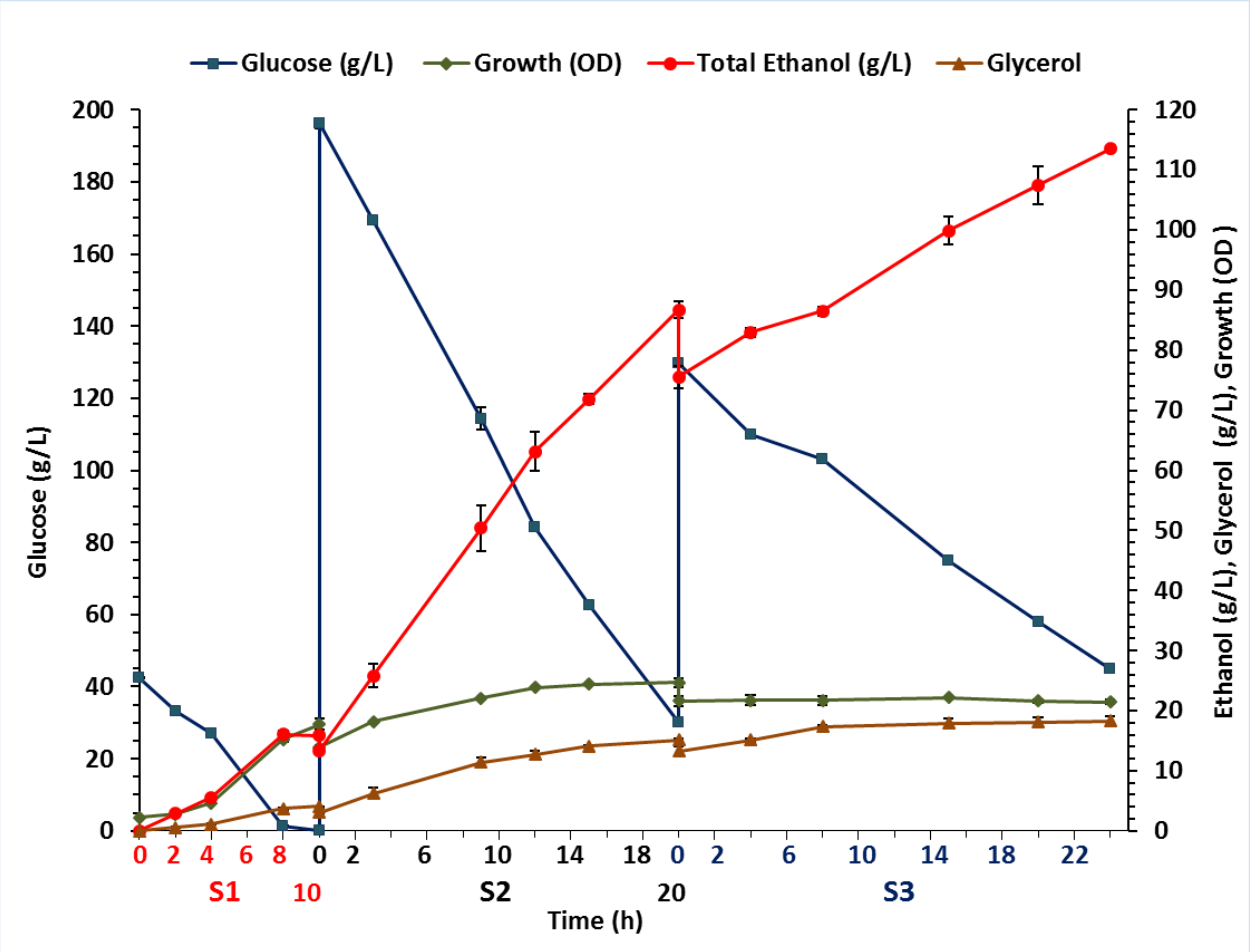


Figure 7.10: Changes in the concentration of glucose, ethanol, glycerol and biomass by the marine strain *S. cerevisiae* S65 using SW-based media in 3-stage fermentation

Table 7.4: HPLC analysis for 3-stage batch fermentation using SW-based media and high concentrations of glucose

Stage	Time (h)	Growth (OD)	Glucose (g/L)	Utilised Glucose (%)	Glycerol (g/L)	Acetic (g/L)	EtOH (g/L)	EtOH/ Cycle (g/L)	EtOH Yield[a] (%)	EtOH Yield[b] (%)	EtOH Produc. (g/L/h)
S1	**0**	2.22	42.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2	2.82	9.45	0.58	0.58	0.06	2.89	2.89	13.34	59.96	1.45
	4	4.65	27.12	36.27	1.13	0.09	5.60	5.60	25.81	71.14	1.40
	8	15.25	1.21	97.15	3.70	0.18	16.08	16.08	74.10	76.28	2.01
	10	17.75	0.00	100.00	4.08	0.21	15.92	15.92	73.36	73.36	1.59
S2	**0**	13.95	196.23	0.00	3.03	0.18	13.41	0.00	0.00	0.00	0.00
	3	18.20	169.22	13.76	6.22	0.34	25.78	12.37	12.36	89.64	4.12
	9	22.05	114.32	41.73	11.41	0.66	50.36	36.95	36.91	88.36	4.11
	12	23.85	84.26	57.06	12.73	0.77	63.15	49.75	49.70	87.10	4.15
	15	24.45	62.71	68.04	14.15	0.80	71.86	58.45	58.41	85.84	3.90
	20	24.65	29.99	84.72	15.16	0.86	86.72	73.32	73.26	86.48	3.67
S3	**0**	21.60	129.62	0.00	13.24	0.76	75.50	0.00	0.00	0.00	0.00
	4	21.75	110.00	15.14	15.13	0.86	82.95	7.45	11.27	74.31	1.86
	8	21.70	101.60	21.61	17.39	1.00	88.47	12.97	19.62	90.73	1.62
	15	22.15	75.05	42.10	17.87	1.05	99.83	24.33	36.80	87.43	1.62
	20	21.60	58.00	55.25	18.07	1.08	107.46	31.95	48.34	87.51	1.60
	24	21.45	45.05	65.25	18.31	0.98	113.52	38.02	57.51	88.13	1.58

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total glucose of each stage.

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised glucose.

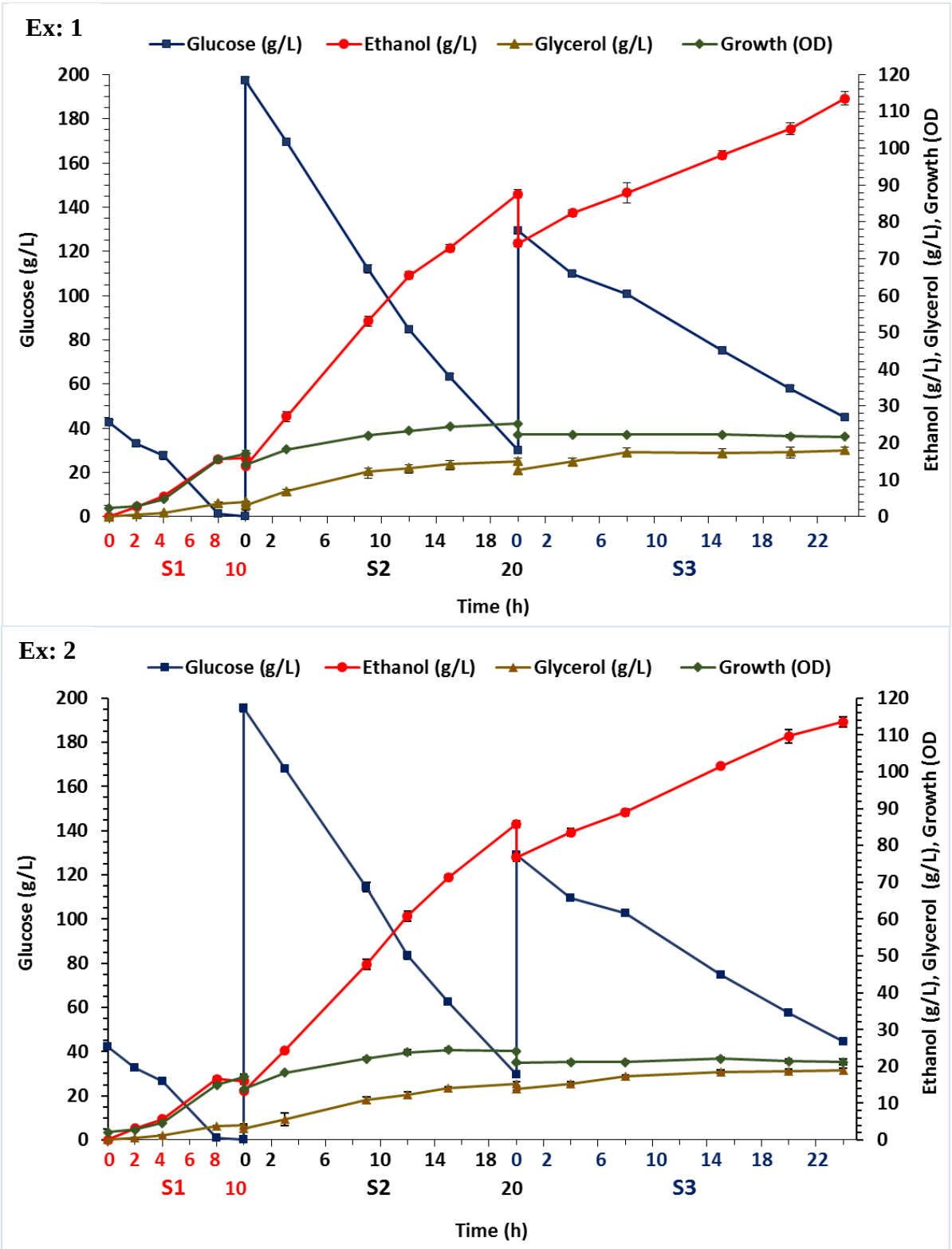


Figure 7.11: Two repeated experiments show the changes in the concentration of glucose, ethanol, glycerol and biomass by the marine strain *S. cerevisiae* S65 using SW-based media in 3-stage fermentation

7.2.6 The production of bioethanol from sugarcane molasses prepared in seawater using marine *S. cerevisiae* S65

7.2.6.1 Batch fermentation

The production of bioethanol using sugarcane molasses instead of commercial sugar was carried out. The fermentation was conducted anaerobically at 30^{o}C and 200 rpm for 48 hours. The fermentation medium was prepared using sugarcane molasses at a concentration of 30% (w/v). The clarification and dilution for the molasses was done using natural seawater and the total sugars were measured at 138.8 ±2.37 g/L. Yeast growth, ethanol concentration, glycerol concentration and the remaining sugars concentration were monitored at regular time intervals, and the results are shown in Figure 7.12 and Table 7.5. Yeast growth reached a maximum value of OD 12.44 ± 0.29 after 48 hours of the fermentation. Almost all available sugars (99.33%) were utilised by the end of the fermentation time and there was a concurrent conversion of sugars into ethanol. It was noticed that the rate of sucrose utilisation was slower than that of glucose but faster than fructose. The final ethanol production was 52.23 ±2.19 g/L with a yield of 73.80% of the theoretical yield. Ethanol productivity increased by time and reached a maximum of 1.43 g/L/h after 20 hours then decreased to 1.09 g/L/h by the end of fermentation. Glycerol production showed a consistently increased throughout the fermentation period and reached a maximum of 13.17 ±1.15 g/L. This experiment was done in triplicate and the results from individual experiments were shown in Figure 7.13.

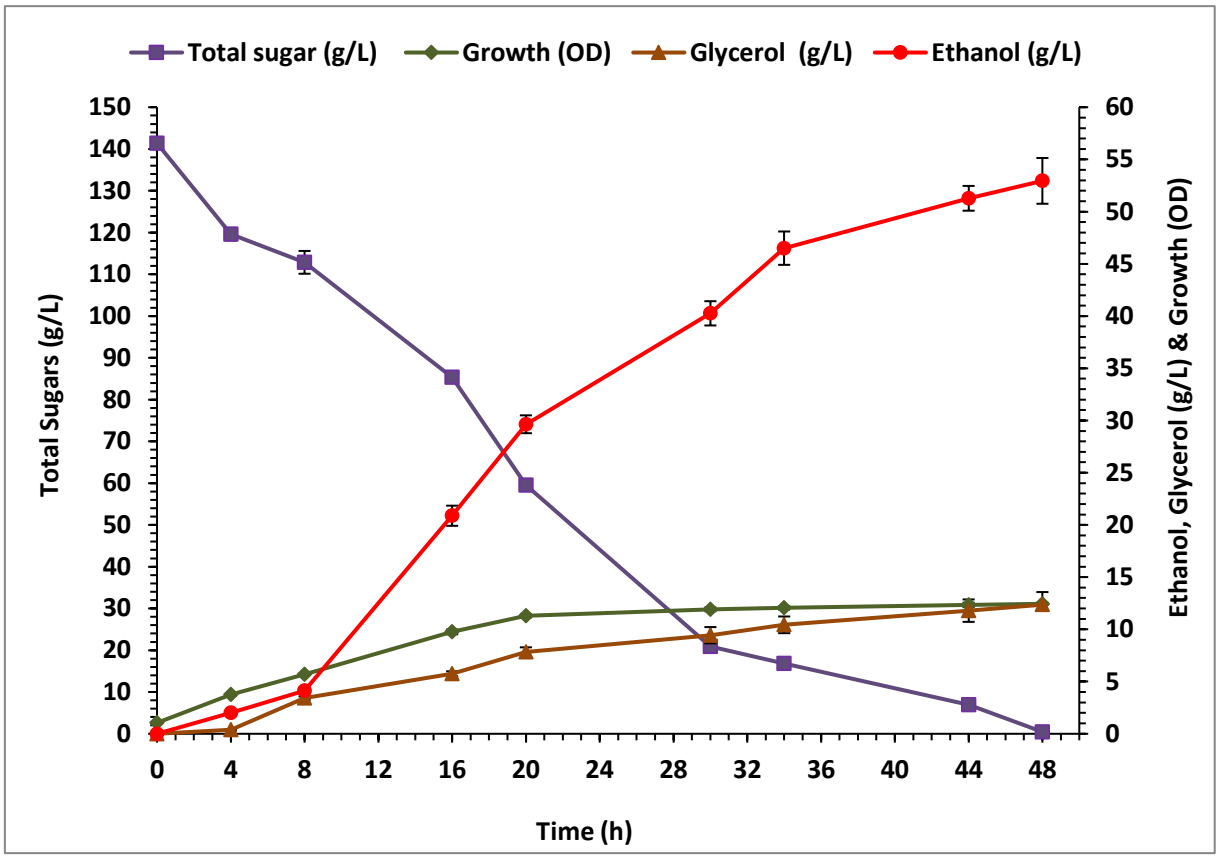


Figure 7.12: Changes in the concentration of total sugars, ethanol, glycerol and biomass in a batch fermentation by the marine strain *S. cerevisiae* S65 using sugarcane molasses prepared in seawater

Table 7.5: HPLC analysis for batch fermentation by marine *S. cerevisiae* S65 using sugarcane molasses prepared in seawater

Time (h)	Growth OD	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)	Total Sugars (g/L)	Utilised Sugars (%)	Glycerol (g/L)	EtOH (g/L)	EtOH Yield (%)[a]	EtOH Yield (%)[b]	EtOH Prod. (g/L/h)
0	1.05 ±0.05	18.03 ±2.29	60.86 ±0.40	59.92 ±049	138.80 ±2.37	0.00	0.00	0.00	0.00	0.00	0.00
4	3.74 ±0.12	14.22 ±2.41	53.30 ±1.39	53.33 ±2.42	120.84 ±1.97	12.91	0.66 ±0.27	1.88 ±0.11	2.65	21.14	0.47
8	5.70 ±0.21	13.27 ±2.91	47.04 ±2.75	49.65 ±2.68	109.97 ±6.54	20.79	2.74 ±0.58	4.10 ±0.51	5.79	28.94	0.51
16	9.76 ±0.18	12.45 ±3.06	29.80 ±2.38	44.34 ±2.09	86.60 ±1.63	37.59	5.22 ±0.65	18.36 ±2.30	25.91	68.94	1.15
20	11.30 ±0.17	10.13 ±0.36	14.32 ±1.97	35.53 ±3.39	59.98 ±5.38	56.79	7.88 ±0.95	28.51 ±2.90	40.25	71.36	1.43
30	11.91 ±0.07	6.23 ±0.25	3.33 ±0.48	10.89 ±2.57	20.45 ±3.27	85.27	9.48 ±0.40	41.87 ±2.73	59.16	69.47	1.40
34	12.06 ±0.10	5.53 ±0.79	1.77 ±1.54	6.90 ±2.36	14.21 ±4.68	89.79	10.67 ±0.35	48.44 ±2.86	68.48	76.21	1.42
44	12.34 ±0.26	1.69 ±0.28	0.75 ±1.30	1.93 ±1.65	4.37 ±2.47	96.87	12.54 ±1.19	51.76 ±4.82	73.15	75.48	1.18
48	12.44 ±0.29	0.87 ±0.71	0.05 ±0.09	0.00	0.92 ±0.65	99.33	13.17 ±1.15	52.23 ±2.19	73.80	74.31	1.09

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total sugar (138.8 g).

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised sugars.

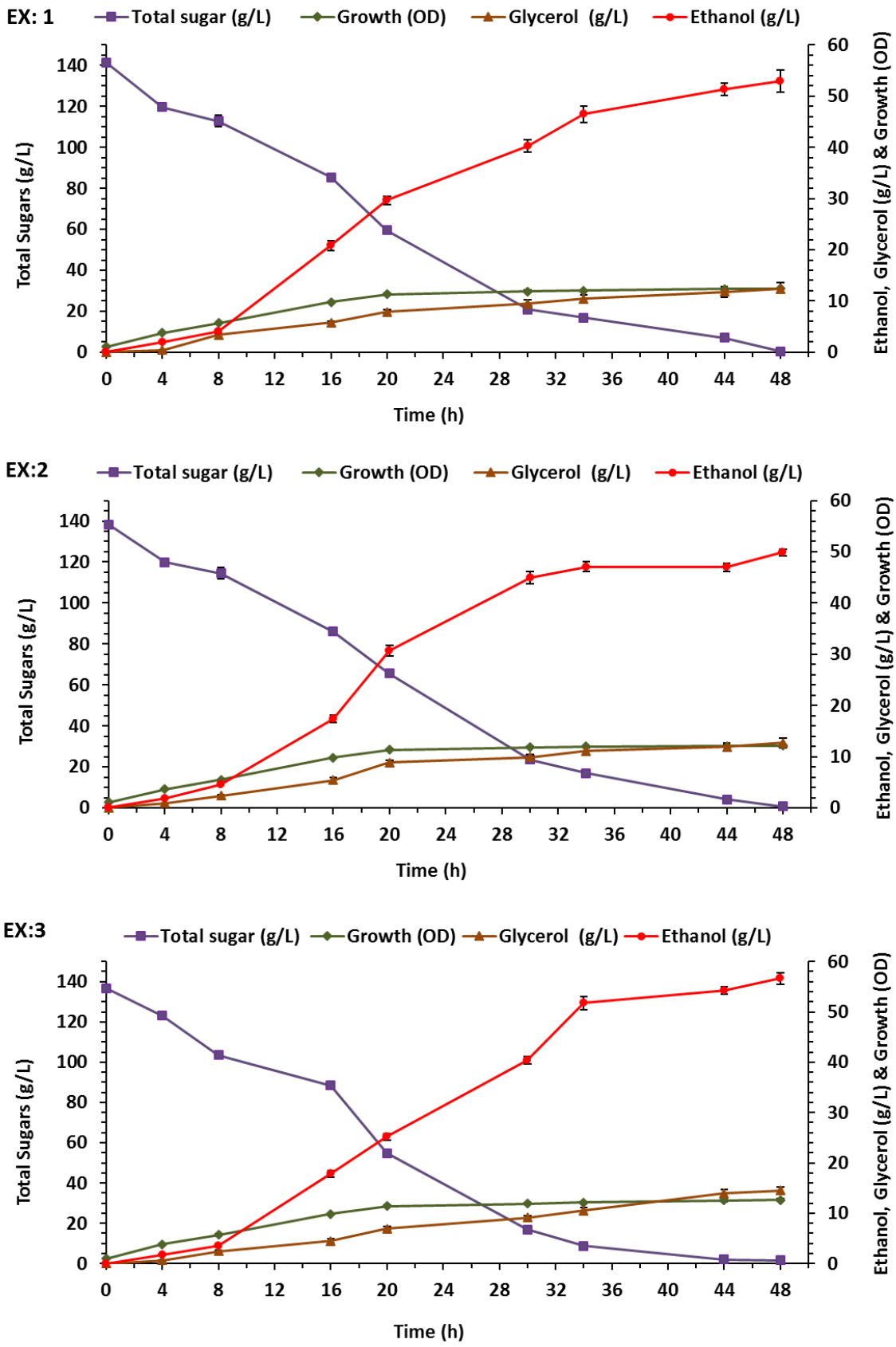


Figure 7.13: Three experiments show the changes in the concentrationof total sugars, ethanol, glycerol and biomass in a batch fermentation by the marine strain *S. cerevisiae* S65 using sugarcane molasses prepared in seawater

7.2.6.2 2-stage batch fermentation

In this experiment, a 2-stage batch fermentation process was investigated to improve the bioethanol productivity. The first stage of this experiment was conducted to propagate the yeast to obtain high yeast cell numbers for the main stage for ethanol production. The first stage was operated aerobically at 30^{o}C and 200 rpm for 14 hours using sugarcane molasses prepared at a concentration of 10% using natural seawater. The second stage was operated anaerobically at 35^{o}C and 200 rpm for 30 hours using sugarcane molasses prepared using natural seawater at a concentration of 20% (w/v). Yeast growth was measured as an OD value using spectrophotometer at regular time intervals. The concentrations of the sugars (sucrose, glucose & fructose), glycerol and ethanol were monitored using HPLC at regular time intervals, and the results were shown in Figure 7.14 and Table 7.6.

In the first stage, yeast density increased from an OD of 1.65 ±0.04 to an OD of 9.79 ±0.02 in 14 hours where 98.68% of sugars had been utilised. By the end of this stage, 11.32 ±1.67 g/L of ethanol was produced which accounted for 49.13% of the theoretical yield and the productivity was 0.81 g/L/h, and the concentration of glycerol reached 3.40 ±0.10 g/L.

In the second stage, 4 L of 50% (w/v) molasses replaced 4 L of the culture obtained of the 1st stage and as sequences; the total sugars at the start of this stage was 91.27 ±5.90 g/L while the concentrations of yeast cells, glycerol and ethanol were diluted to 5.89 ±0.83, 2.73 ±0.19 and 7.97 ±0.60 g/L respectively. During 30 hours of fermentation, yeast cells continued to increase and reached an OD of 10.39 ±1.44 by the end of this stage. Around 42.35 g/L of ethanol was produced during this stage making the total ethanol in the fermenter to be 50.32 ±1.95 g/L when 98.12% of the sugars were utilised. Ethanol yield reached 95.35% after 30 hours of fermentation and ethanol productivity recorded 2.46 g/L/h after 3 hours of fermentation then decreased by time to 1.41 g/L/h at the end of the fermentation time of this experiment. The production of glycerol recorded was 10.55 ±0.14 g/L (Table 7.6). This experiment was done in duplicate and the results from individual experiments were shown in Figure 7.15.

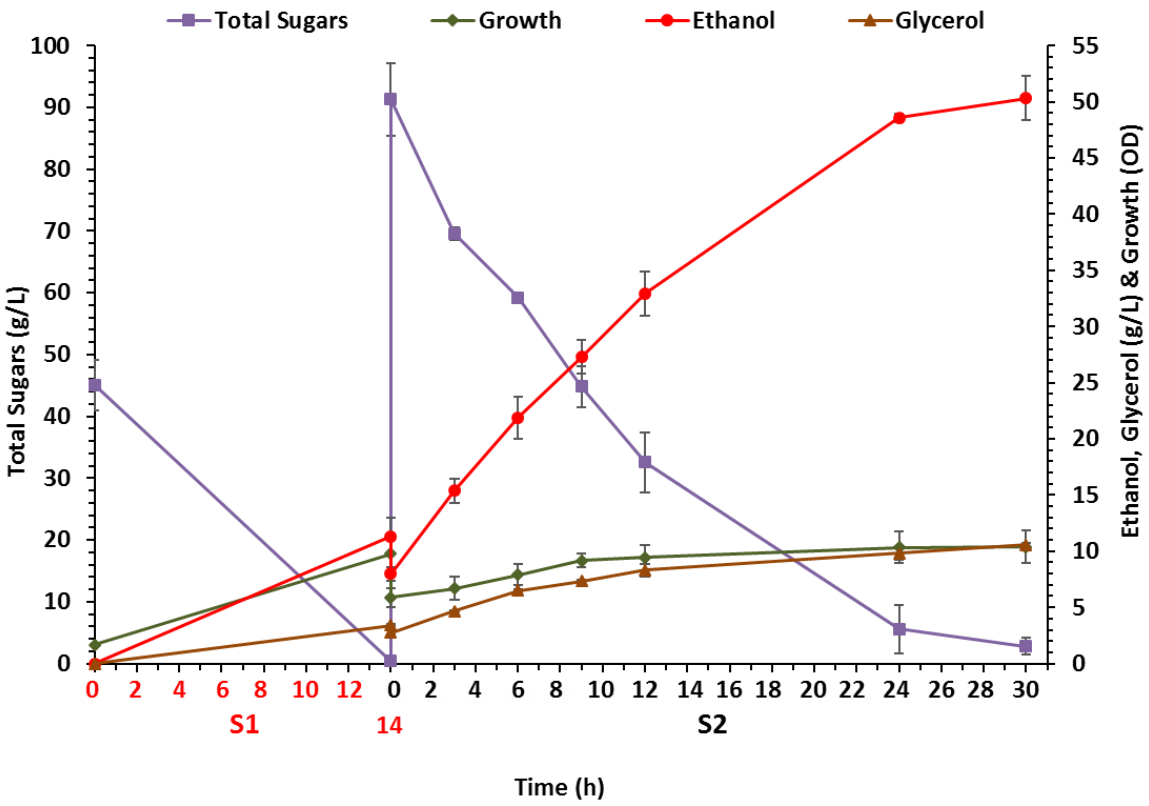


Figure 7.14: Changes in the concentrationof total sugars, ethanol, glycerol and biomass in a 2-stage batch fermentation by the marine strain *S. cerevisiae* S65 using sugarcane molasses prepared in seawater

Table 7.6: HPLC analysis for 2-stage batch fermentation by marine *S. cerevisiae* S65 using sugarcane molasses prepared in seawater

Stage	Time (h)	(OD)	Sugars (g/L)				Utilised Sugars (%)	Glycerol (g/L)	EtOH (g/L)	EtOH/ Cycle (g/L)	EtOH Yield[a] (%)	EtOH Yield[b] (%)	EtOH Prod. (g/L/h)
			Sucrose	Glucose	Fructose	Total							
S1	**0**	1.65 ±0.04	12.90 ±0.97	14.96 ±1.51	17.22 ±1.57	45.07 ±4.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	14	9.79 ±0.02	0.17 ±0.06	0.00	0.21 ±0.20	0.38 ±0.26	98.68	3.40 ±0.10	11.32 ±1.67	11.32	49.13	49.56	0.81
S2	**0**	5.89 ±0.83	24.91 ±4.93	33.45 ±2.28	32.91 ±1.32	91.27 ±5.90	0.00	2.73 ±0.19	7.97 ±0.60	0.00	0.00	0.00	0.00
	3	6.68 ±1.05	22.65 ±2.94	15.24 ±0.23	31.68 ±3.76	69.57 ±1.05	27.88	4.69 ±0.00	15.36 ±1.05	7.39	16.64	71.09	2.46
	6	7.88 ±0.93	18.02 ±3.70	11.10 ±1.14	30.06 ±2.83	59.19 ±0.26	38.43	6.50 ±0.10	21.90 ±1.88	13.93	31.35	85.24	2.32
	9	9.17 ±0.62	9.25 ±0.65	7.76 ±1.55	27.78 ±2.42	44.79 ±3.32	55.52	7.33 ±0.12	27.30 ±1.50	19.33	43.51	83.57	2.15
	12	9.44 ±1.13	5.49 ±0.72	4.00 ±2.36	23.06 ±1.76	32.55 ±4.84	69.50	8.30 ±0.60	32.93 ±1.94	24.96	56.18	83.97	2.08
	24	10.35 ±1.43	3.16 ±0.96	0.00	2.39 ±2.90	5.55 ±3.86	97.05	9.83 ±0.62	48.56 ±0.73	40.59	91.38	93.43	1.69
	30	10.39 ±1.44	2.17 ±0.53	0.00	0.60 ±0.85	2.77 ±1.38	98.12	10.55 ±0.14	50.32 ±1.95	42.35	95.35	94.28	1.41

[a] Calculated as a percentage of the theoretical yield (0.51) based on the total sugar in each stage.

[b] Calculated as a percentage of the theoretical yield (0.51) based on the utilised glucose.

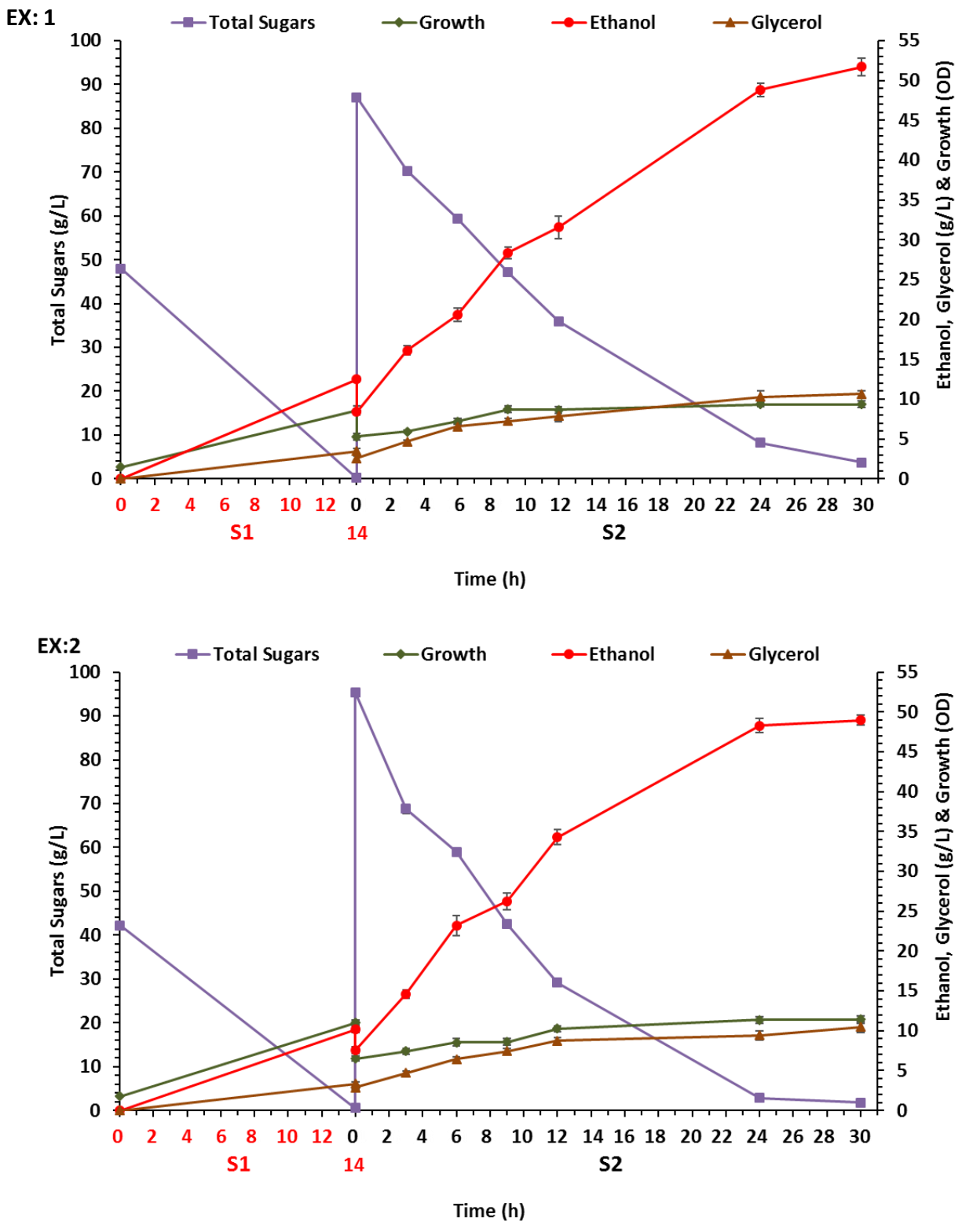


Figure 7.15: Two experments show the changes in the concentration of total sugars, ethanol, glycerol and biomass in a 2-stage batch fermentation by the marine strain *S. cerevisiae* S65 using sugarcane molasses prepared in seawater

7.3 Discussion

Coastal environments have been identified as being amongst the most diverse and rich microbial environments (Danovaro et al., 2009). Fungi have been reported to have an active role in the utilisation of available nutrients in marine environments (Gao and Liu, 2010), however, their suitability for fermentations under osmotic stress inducing conditions has not been extensively investigated previously. Yeasts isolated from a marine environment have been shown to produce commercially relevant extracellular enzymes (Chi et al., 2009), indicating that the diversity in environments, the shifting panorama in terms of available nutrients makes the commercial use of marine derived yeast an interesting bio-system to explore.

In this chapter, the suitability of marine *S. cerevisiae* for the production of bioethanol was confirmed. Comparing the fermentation profile of 3 newly isolated marine *S. cerevisiae* (S65, S71 & S118) with the terrestrial reference strain *S. cerevisiae* NCYC2592 indicated the potential of marine *S. cerevisiae* in the bioethanol industry. *S. cerevisiae* NCYC2592 is an industrial distiller strain which is well known for its high fermentation capacity and high tolerance to various inhibitors. Therefore, *S. cerevisiae* NCYC2592 was expected to perform better than wildtype marine *S. cerevisiae* isolated in this study. However, the results in this chapter revealed that marine *S. cerevisiae* strains performed significantly better than *S. cerevisiae* NCYC2592 when seawater was used to prepare the fermentation medium. In addition, marine *S. cerevisiae* strains performed slightly better that the reference strain when freshwater was used to prepare the fermentation medium. On the other hand, it was observed that fermentation rates of all non *S. cerevisiae* strains were significantly lower than that of the reference strain, regardless the type of fermentation media being used. This finding explains why *S. cerevisiae* is the preferred microorganism for bioethanol production.

Osmotic stress induced by the presence of salts is an important factor that affects yeast's performance during fermentation (Casey et al., 2010). Presence of salts of any kind has been shown to reduce glucose utilisation, cellular growth and production of ethanol (Casey et al., 2013). Improving salt tolerance has been highlighted as an important parameter for improving yeast performance in fermentation (Wei et al., 1982, Ramos et al., 2013). In this chapter, results obtained from cell growth and bioethanol fermentation experiments revealed that marine *S. cerevisiae* strains had a higher tolerance to the presence of salts of the SSW when compared with the terrestrial yeast strain NCYC2592. *S. cerevisiae* is only moderately tolerant and other yeast such as *Zygosaccharomyces rouxii* have been shown to be more tolerant to the presence of salt than *S. cerevisiae* (Dakal et al., 2014). However, the sequencing of the *S. cerevisiae*

S288C genome (Goffeau et al., 1996) has led to insights into how *S. cerevisiae* responds to high external salt concentrations leading to conclusions on how the yeast modifies its genetic make-up to accommodate the changes in the extracellular environment (de Nadal et al., 2011). It was observed, in this study, that fermentation rates using SSW-based medium was higher than fermentation rates using SW-based medium even though, both media contained similar amount of salts. This indicates that natural seawater may contain inhibitors in the form of trace minerals or other chemical substances. Study of these inhibitors may lead to avoiding their effect (by water treatment or yeast modification) on yeast growth and fermentation activity. We also observed that fermentation rates using a medium containing 3% NaCl was slower than fermentation rates using SSW-based medium (3.5% total salts) and similar to the fermentation rate using 2X SSW-based medium. This indicates that salts other than NaCl may have a positive role in moderating the inhibitory effect of NaCl on yeast.

In fermentations using high sugar concentration in natural seawater, it was observed that the marine *S. cerevisiae* strains performed significantly better than the reference terrestrial strain. In addition, it was observed that the reference strain produced less CO_2 within 68 hours of fermentation when the initial concentration of glucose exceeded 15%. Urano et al (2001) studied the fermentation ability and salt tolerance of yeasts isolated from various aquatic environments (upper stream of Arakawa river, middle and lower streams of Tamagawa rivers and sea coasts of Kemigawa in Chiba prefecture and of Chemigahama in Choshi city) and concluded that yeasts with high salt tolerance and high fermentation ability were marine yeasts (Urano et al., 2001). Khambhaty et al (2013) validated the ability and efficiency of a marine isolate - *Candida* sp. - to grow and ferment galactose to ethanol in the presence of different types of salts (NaCl, $CaCl_2$, and KCl) and at different concentrations (0 - 15%). This yeast strain yielded 1.23 to 1.76% ethanol from seaweed hydrolysates, containing different concentrations of sugar (2.7 to 5.5%) and salt (6.25 to 11.25%).

Low ethanol yield was obtained by the reference strain from high gravity (≥15 %) fermentation using medium prepared in seawater; but, improved ethanol yield was achieved using the marine strains especially S65. Liu et al (2016) found that fermentations conducted in stressful environments involving very high gravity medium results in incomplete utilisation of glucose at the end of fermentation. In addition, the stressful condition will lead to slow yeast growth and low cell viability which will lead to lower ethanol production (Liu et al., 2016).

The 3-stage experiments were conducted to maintain high cell number during the aerobic stage (the 1st stage) in order to achieve high ethanol yield and productivity during the anaerobic stages

(the 2nd and the 3rd stages). High ethanol yield and productivity were achieved during the 2nd stage but they dropped down during the 3rd stage. This could be due to the increasing stress occurred in the 3rd stage from adding more glucose to the medium which already contained high concentrations of salts and ethanol and the absence of oxygen which was perhaps completely utilised during the 2nd stage. Liu et al., (2016) found that supplying low amounts of oxygen during a very high gravity ethanol fermentation enhanced the yield and productivity of ethanol (Liu et al., 2016). Oxygen advances cell recovery through TCA cycle and respiration pathway by retaining vital cellular components during synthesis and carbon utilisation. Oxygen helps yeast synthesise sterols and unsaturated fats required for maintaining a healthy cell membrane. (Fornairon-Bonnefond et al., 2002).

Although, sucrose is the major sugar in raw molasses and account for about 50% of the total sugars, the chemical analysis of our clarified molasses showed lower amounts of sucrose when compared with glucose and fructose. This was due to the addition of concentrated sulphuric acid (H_2SO_4) and heating for an hour during molasses preparation (Bower et al., 2008). In line with results obtained by D'Amore et al., (1989), we noticed that yeast favours utilising glucose then sucrose and lastly fructose when they are present together in the fermentation medium.

7.4 Conclusion

In this study, we examined the suitability of marine yeast in bioethanol fermentation using media prepared by freshwater and seawater. The results were compared with the fermentations using terrestrial strain, *S. cerevisiae* NCYC2592. The results revealed that 3 marine *S. cerevisiae* strains showed fermentation ability similar to the industrial terrestrial strain in freshwater-based media and higher fermentation ability in seawater-based media. These three strains where taken forward to investigate their performance under high concentrations of glucose and salts (especially NaCl). In general, marine strains performed significantly better than the reference terrestrial strain. Amongst the marine yeast, *S. cerevisiae* S65 recorded the best growth rate as well as the best ethanol production, yield and productivity in the presence of high salt (9% NaCl and 3X SSW) and high glucose concentrations (20 and 25%). Hence assessment of fermentation capacity for S65 was conducted in 15 L bioreactors using seawater based media. The results revealed that 93.5 g/L ethanol was produced within 48 h using YPD media contained 220 g/L glucose and prepared in natural seawater. Applying 3-stage experiment, where the second stage was applied for ethanol production, the strain S65 produced 73 g/L of ethanol from 165 g/L of glucose within 20 h of fermentation with an ethanol

productivity of around 4 g/L/h. Using sugarcane molasses (30%) prepared in SW, this strain produced 54.23 g/L of ethanol after 48 h in a batch fermentation.

CHAPTER 8:

8. Conclusion and Future work

8.1 Conclusion

The production of bioethanol requires large amounts of water during the cultivation of crops (substrates) as well as during industrial processing. With the increasing demand for bioethanol, there is increasing concern over the use of freshwater resources. In order to address this concern, a seawater-based biorefinery concept was investigated in this study as a new approach for bioethanol production. One of the main challenges of utilising seawater is that seawater contains high amount of salts. High concentration of salt is unfavourable for conventional terrestrial yeasts which have been used for the industrial production of bioethanol. In addition, the current analytical methods for measuring sugars and fermentation metabolites are not suitable for samples containing high concentration of salts such as seawater derived fermentation samples. Therefore, developing an isolation and screening method for halotolerant ethanol producing yeast as well as investigating an accurate analytical method for seawater-fermentation samples are the main prerequisites for the development of this approach.

Firstly, an efficient method for marine yeast isolation was developed. The new method includes: a 3-cycle enrichment step followed by an isolation step and a confirmation step. By applying this method on 14 marine samples (collected in the UK, Egypt and the USA), a large number of marine yeast isolates were obtained without any bacterial or filamentous fungal contamination. 118 marine yeast isolates were selected for further evaluation and screening. These isolates were selected to represent different sample sources, different isolation media, and different cell and colony morphology.

Next, a new technique for screening large number of yeasts for potential application in ethanol production was investigated. The new marine isolates were screened for their utilisation ability of monomeric fermentable sugars (glucose, xylose, mannitol and galactose) in a seawater-based media using a phenotypic microarray assay. 21 isolates that representing the best sugar utilisers were further characterised using YT-plates and identified by DNA sequencing using ITS and D1D2 primers. The identified isolates belonged to 8 species, including *S. cerevisiae* (5), *C. tropicalis* (4), *C. viswanathii* (4), *W. anomalus* (3), *C. glabrata* (2), *P. kudriavzevii* (1), *C. albicans* (1) and *I. orientalis* (1). The ability of these strains for improved sugar utilisation using seawater-based media was confirmed and therefore, they could potentially be utilised in fermentations using seawater-based media for the production of bioethanol.

This study also developed a new method suitable for the analysis of fermentation samples that contain high concentration of salt. Because the main objective of this study is to establish a fermentation process using seawater instead of freshwater, investigating an accurate analytical method for simultaneous analysis of sugars and salt was a necessity. HPLC is the preferred method for sugar quantification according to the guidelines of the Association of Official Analytical Chemists. However, obtaining an accurate quantification of sugars using HPLC in samples containing NaCl was proven difficult due to similar retention times for Cl^- and some sugars especially glucose and sucrose. Hence, an accurate and reliable HPLC method for the simultaneous quantification of chloride salts and sugars (i.e. glucose, xylose, mannitol, or sucrose) in samples containing chloride salts was developed using a HI-Plex H column. The separation was achieved at 35ºC using H_2SO_4 (0.005 N) as the mobile phase at a flow rate of 0.4 mL/min and the column effluent was monitored by a Refractive Index (RI) detector. The peak correlated with the concentration of chloride salts eluted early at R_t of 10.65 minutes followed by the sugar peaks then glycerol and ethanol peaks. The (HI-Plex H) column was designed for determining the concentrations of sugars, organic acids and alcohols when in solution. In addition, the accurate quantification of NaCl using this method was validated. A linear response was achieved over NaCl concentrations of 0.1 – 2.5 g/L and 5 – 40 g/L. The analytical method inter- and intra-run accuracy and precision were better than ±10%.

Following this, an investigation was carried out to establish the use of seawater to replace freshwater for ethanol production using halotolerant marine yeast strains. Out of the 21 identified yeasts, 9 representative strains were selected to be screened for ethanol production using YPD media containing 6% glucose and prepared by freshwater (ROW) and seawater (SW). It was found that the marine *S. cerevisiae* strains (S65, S71, and S118) recorded the best fermentation rates using SW media. Hence, they were taken forwarded to investigate their growth performances under high concentrations of glucose and seawater salts (the components of synthetic seawater). The marine strains were clearly more tolerant to these stresses when compared with the reference strain. Fermentation experiments using YPD media containing 6% glucose were prepared using synthetic seawater (SSW), 2x SSW and NaCl solutions at different concentrations (3, 6 and 9%) confirming that the marine-derived strain S65 was a halo-osmotolerant when compared with the other yeasts in the study.

Initial experiments using small scale fermentations (0.1 L) revealed that marine yeast strain *S. cerevisiae* S65 was capable of converting glucose effectively into ethanol in the presence of seawater. To scale up this process, the production of bioethanol from seawater-based media by marine yeast strain S65 was performed in 15 L bioreactors. Using YPD media containing 220

g/L glucose and prepared in natural seawater, strain S65 produced 93.5 g/L bioethanol within 48 h in a batch fermentation at a low yeast pitching rate. Using a high yeast pitching rate, strain S65 produced 73 g/L of ethanol from 165 g/L of glucose within 20 h of fermentation with ethanol productivity of around 4 g/L/h. Fermentation using sugarcane molasses (30% molasses, about 15% sugars) prepared in natural SW, strain S65 produced 52.23 g/L of ethanol after 48 h. The results obtained in this study indicated the potential of marine yeasts and seawater-based media in bioethanol production. They also provide a new strategy for increasing the efficiency of bioethanol production at the industrial level with positive impact on food and freshwater scarcity issue. However, the economics of replacing freshwater by seawater in bioethanol production has still to be fully explored and therefore, an intensive investigation is required.

8.2 Future work

This study has investigated the use of marine yeast and seawater-based media for bioethanol production with the aim to reduce the water footprint of bioethanol.

The current study validated the possibility of using seawater efficiently in place of freshwater for bioethanol production. It also validated the superiority of marine yeast over the current industrial terrestrial yeast in bioethanol production under certain stress conditions. This study also validated the application of marine yeast and SW-based media for the production of bioethanol using molasses which is currently being used as substrate industrially for the production of bioethanol. However, in order to shift from freshwater to seawater based fermentation technology, seawater should be involved in all steps of bioethanol production cycle from substrate production through industrial processing. This can be achieve if marine biomass is used as a substrate for bioethanol production.

Corn and sugarcane are currently the preferred industrial feedstocks for the production of bioethanol. However, “food versus fuel” concerns may limit their use as non-food crops in the foreseeable future (Wargacki et al., 2012). Lignocellulosic biomass and agricultural residues have been intensively investigated as a replacement for edible crops in terms of ethanol production. However, the current hydrolysis technologies have yet to overcome the high cost associated with the complex processes required to liberate fermentable sugars from the complex polysaccharides present in the plant cell walls for the subsequent fermentation into bioethanol or other value-added products (Stephanopoulos, 2007). In addition, cultivation of terrestrial biomass requires resources (land and freshwater) that could otherwise be allocated for growing edible crops (Lee et al., 2014). Therefore, focused strategies are required for the efficient

conversion of sustainable non-lignocellulosic containing feedstocks, such as seaweed as biofuel feedstocks (Wargacki et al., 2012).

Seaweeds grow in the marine environment and therefore they do not require arable land, fertilizer or freshwater resources. Cultivation of these marine biomass eliminates the economic concerns associated with water and land management and does not impact on existing food supply chains. In addition, seaweeds do not contain lignin and have little cellulose content, which lowers the costs associated with pretreatment. Therefore, seaweed is currently being considered as a potential sustainable source of biomass for the production of bioethanol (Lee et al., 2014). Seaweeds encompass few thousand species (El-Said and El-Sikaily, 2013, Lee et al., 2014). Seaweeds are classified according to their nutrient value and chemical composition to; red seaweed (*Rhodophyta*), brown seaweed (*Phaeophyta*), and green seaweeds (*Chlorophyta*) (Dawczynski et al., 2007).

Carbohydrates comprise 50% of seaweed dry weight (El-Said and El-Sikaily, 2013, Lee et al., 2014), however, composition varies between types and species (Dawczynski et al., 2007). Green seaweed contains the highest average carbohydrate concentration consisting mainly of cellulose, starch, mannans, xylans, uranic acids and sulphated polysaccharides. Monomeric, potentially fermentable, sugars such as rhamnose, xylose, galactose and arabinose were also found in green seaweed (Lahaye, 1991, Lee et al., 2014). Red seaweeds varieties consist of different carbohydrates types including; floridean starch (α-1,4-binding glucan), xylan, mannan and cellulose. In addition, their water-soluble fibre fraction consists of sulphur-containing galactans, (carrageenan and agar) (Jiménez-Escrig and Sánchez-Muniz, 2000, Dawczynski et al., 2007). On the other hand, the main reserve polysaccharides of brown seaweeds are laminaran (β-1,3-glucan) and mannitol (El-Said and El-Sikaily, 2013, Kolb et al., 1999). Fucoidan, alginates and cellulose also exist in brown seaweeds (Dawczynski et al., 2007).

Because seaweed does not contain lignin, sugars can be extracted by simple procedures such as milling and crushing. This feature gives seaweed a distinct advantage over lignocellulosic biomass by avoiding the complex and energy-intensive processes (pretreatment and hydrolysis) that are required before fermentation (Wargacki et al., 2012). However, application of seaweeds as feedstocks for bioconversion into bioethanol is limited primarily by the availability of microorganisms that can utilise and ferment seaweeds carbohydrates (Wargacki et al., 2012, Enquist-Newman et al., 2014).

Wargacki et al., (2012) discovered DNA fragment (36–kilo–base pair) encoding enzymes for alginate transport and metabolism in *Vibrio splendidus*. The integration of this DNA fragment in *E. coli*, along with an engineered depolymerisation system for extracellular alginate, allowed the GM strain to simultaneously degrade, uptake, and metabolize alginate. Further engineering of the GM *E. coli* strain for ethanol synthesis showed that bioethanol produced directly from seaweed via a consolidated process achieving 4.7% (v/v) with yield equivalent to ~80% of the maximum theoretical yield from the sugar composition in seaweed. Although this was a promising result, the full potential of seaweed as feedstocks for the production of bioethanol on a commercial-scale requires extensive re-engineering of the alginate and mannitol catabolic pathways in an industrial *S. cerevisiae* strain (Enquist-Newman et al., 2014).

Enquist-Newman et al. (2014) discovered an alginate monomer (4-deoxy-L-erythro-5-hexoseulose, DEHU) transporter from the *Asteromyces cruciatus*. By integrating this transporter in the genome of an industrial *S. cerevisiae* strain, along with the necessary bacterial alginate and mannitol catabolism genes, the new GM *S. cerevisiae* strain was able to efficiently metabolize DEHU and mannitol. This platform was further adapted to grow on DEHU and mannitol under anaerobic conditions and then, it was capable of producing 36.2 g/L ethanol from mannitol and DEHU with yields up to 83% of the maximum theoretical yield from seaweed sugars (Enquist-Newman et al., 2014).

The annual world harvested wild stock of seaweed reached about 1.1 million wet metric tons in 2006 (Roesijadi et al., 2010). The annual world production from seaweed farming accounted increased from 10 million tons in 2000 to 28 million tons in 2013 with estimated value of 5.8 billion US Dollars (FAO, 2016) (Capuzzo and McKie, 2016). Intensive cultivation of seaweed on a large-scale is practiced in about 50 countries around the world. However, about 95% of seaweeds production comes from Asian countries especially China, Philippines and Indonesia (Roesijadi et al., 2010). It was reported that Chana and Indonesia produced more than 80% of the world's seaweed from seaweed farms. Farming of seaweed has increased rapidly in certain countries around the world. The most obvious example is Indonesia where seaweed production increased 42-fold between 2000 and 2013 (FAO, 2014). Cultivation of seaweed in the UK is limited to several pilot facilities which were established mainly for research and development. The largest pilot farm in the UK is a one hectare seaweed farm developed in 2016 by a Scottish company called SAMS. This pilot establishment could produce up to 25 tonnes/year (http://www.sams.ac.uk/globalseaweed) (Capuzzo and McKie, 2016).

It has been estimated that bioethanol productivity of 19,000 L/ha/year can be obtained from seaweed which is 5 times higher than bioethanol productivity from corn and 2 times higher than bioethanol productivity from sugarcane (Wargacki et al., 2012). This figure shows the prospective of seaweed in bioethanol production and a role it may play in the 'food vs fuels' debate.

Therefore, seaweed seems an ideal substrate for bioethanol production as a complementary to this study. In this regard, the following objectives are suggested for further investigation in the future:

- Screening marine isolates from this study to select the best strain for ethanol production from hydrolysates derived from seaweed using seawater as a water source.
- Genetically modify the marine *S. cerevisiae* S65 strain, isolated and investigated in this study, for the production of bioethanol directly from seaweed by integrating DEHU transporter and the respective genes for alginate and mannitol catabolism.
- Investigating the optimal conditions for cultivating seaweed to accumulate the highest amount of fermentable sugars in order to be used as a substrate for bioethanol production.
- Developing an efficient hydrolysis procedure for sugars extraction from seaweed by using seawater.
- Assessing the financial and environmental impact of replacing freshwater by seawater in bioethanol industry.
- Collaboration with material engineers to manage the corrosion risk of seawater.

We hope to develop a demonstration biorefinery plant near a coastline, which will benefit from many advantages including: a) easy access to the abundant water in the sea for preparing the fermentation media and other uses, b) easy access to a suitable place for waste disposal, c) reduce the shipping cost especially when marine substrates being used, and d) reduce the time and cost of shipping the products and by-products after fermentation.

Appendices

Appendix 1: Complete DNA sequences of the PCR product from 21 newly isolated marine yeasts using ITS and D1/D2 primers

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
1	S 1	TGCCCACATGTGTTTTTTACTGGACAGCTGCTTTG GCGGTGGGGACTCGTTTCCGCCGCC AGAGGTCACAACTAAACCAAACTTTTTATTACCAG TCAACCATACGTTTTAATAGTCAAA ACTTTCAACAACGGATCTCTTGGTTCTCGCATCGA TGAAGAACGCAGCGAAATGCGATAC GTAGTATGAATTGCAGATATTCGTGAATCATCGA ATCTTTGAACGCACATTGCGCCCTTT GGTATTCCAAAGGGCATGCCTGTTTGAGCGTCAT TTCTCCCTCAAGCCCGCGGGTTTGGT GTTGAGCAATACGCCAGGTTTGTTTGAAAGACGT ACGTGGAGACTATATTAGCGACTTAG GTTCTACCAAAACGCTTGTGCAGTCGGCCCACCA CAGCTTTTCTAACTTTTGACCTCAAA TCAGGTAGGACTACCCGCTGAACTTAAGCATATC AATAAGCGGGAGGAAAAAGGATCATT ACTGATTTGCTTAATTGCACACATGTG	CCTTAGTAGCGGCGAGTGAGCGGCAAAAGCTCAA ATTTGAAATCTGGCTCTTTCAGAGTC CGAGTTGTAATTTGAAGAAGGTATCTTTGGGCCT GGCTCTTGTCTATGTTTCTTGGAACA GAACGTCACAGAGGGTGAGAATCCCGTGCGATG AGATGACCCAGGTCCGTGTAAAGTTCC TTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCT AAGTGGGTGGTAAATTCCATCTAAAG CTAAATATTGGCGAGAGACCGATAGCGAACAAGT ACAGTGATGGAAAGATGAAAAGAACT TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTT GAAAGGGAAGGGCTTGAGATCAGACT TGGCATTTTGCATGTTGCTTCTTCGGGGGCGGCCT CTGCGGTTTGTCGGGCCAGCATCAG TTTGGGCGGCAGGACAATCGCGTGGGAATGTGG CACGGCCTCGGCTGTGTGTTATAGCCC GCGTGGATACTGCCAGCCTAGACTGAGGACTGCG GTTTATACCTAGGATGTTGGCATAAT GATCTTAAGTCGCCCGTCTTGGCAA
2	S 7	CATACCTGATTTGAGGTCAAAGTTAGAAAAGCTG TGGTGGGCCGACTGCACAAGCGTTTT GGTAGAACCTAAGTCGCTAATATAGTCTCCACGT ACGTCTTTCAAACAAACCTGGCGTAT TGCTCAACACCAAACCCGCGGGCTTGAGGGAGA AATGACGCTCAAACAGGCATGCCCTTT GGAATACCAAAGGGCGCAATGTGCGTTCAAAGA TTCGATGATTCACGAATATCTGCAATT CATACTACGTATCGCATTTCGCTGCGTTCTTCATC GATGCGAGAACCAAGAGATCCGTTG TTGAAAGTTTTGACTATTAAAACGTATGGTTGACT GGTAATAAAAAGTTTGGTTTAGTTG TGACCTCTGGCGGCGGAAACGAGTCCCCACCGCC AAAGCAGCTGTCCAGTAAAAAACACA TGTGGTGCAATTAAGCAAATCAGTAATGATCCTT CCGCAGGTTCACCCTACGGAAGAAAG ATCATTACTGATTTGCTGAATTGCACACAC	TGCACATCCTAGGTATAAACCGCAGTCCTCAGTCT AGGCTGGCAGTATCCACGCGGGCTA TAACACACAGCCGAGGCCGTGCCACATTCCCACG CGATTGTCCTGCCGCCCAAACTGATG CTGGCCCGACAAACCGCAGAGGCCGCCCCCGAAG AAGCAACATGCAAAATGCCAAGTCTG ATCTCAAGCCCTTCCCTTTCAACAATTTCACGTACT TTTTCACTCTCTTTTCAAAGTTCT TTTCATCTTTCCATCACTGTACTTGTTCGCTATCGG TCTCTCGCCAATATTTAGCTTTAG ATGGAATTTACCACCCACTTAGAGCTGCATTCCCA AACAACTCGACTCGTCGAAGGAACT TTACACGGACCTGGGTCATCTCATCGCACGGGATT CTCACCCTCTGTGACGTTCTGTTCC AAGAAACATAGACAAGAGCCAGGCCCAAAGATA CCTTCTTCAAATTACAACTCGGACTCT GAAAGAGCCAGATTTCAAATTTGAGCTTTTGCCGC TTCACTCGCCGCTACTAAGGCAATC CCTGTTGGTTTCTTTTCCTCCGCTATTGGATA

Table continue in the next pages:

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
3	S 8	CGCCAGAGGTCACAACTAAACCAAACTTTTTA TTACCAGTCAACCATACGTTTTAATAGT CAAAACTTTCAACAACGGATCTCTTGGTTCTC GCATCGATGAAGAACGCAGCGAAATGCG ATACGTAGTATGAATTGCAGATATTCGTGAAT CATCGAATCTTTGAACGCACATTGCGCC CTTTGGTATTCCAAAGGGCATGCCTGTTTGAG CGTCATTTCTCCCTCAAGCCCGCGGGTT TGGTGTTGAGCAATACGCCAGGTTTGTTTGAA AGACGTACGTGGAGACTATATTAGCGAC TTAGGTTCTACCAAAACGCTTGTGCAGTCGGC CCACCACAGCTTTTCTAACTTTTGACCT CAAATCAGGTAGGACTACCCGCTGAACTTAA GCATATCAATAAAGCGGAGGAAGAAAGA	CCTTAGTAGCGGCGAGTGAGCGGCAAAAGCTCAAAT TTGAAATCTGGCTCTTTCAGAGTC CGAGTTGTAATTTGAAGAAGGTATCTTTGGGCCTGGC TCTTGTCTATGTTTCTTGGAACA GAACGTCACAGAGGGTGAGAATCCCGTGCGATGAGA TGACCCAGGTCCGTGTAAAGTTCC TTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAG TGGGTGGTAAATTCCATCTAAAG CTAAATATTGGCGAGAGACCGATAGCGAACAAGTAC AGTGATGGAAAGATGAAAAGAACT TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGA AAGGGAAGGGCTTGAGATCAGACT TGGCATTTTGCATGTTGCTTCTTCGGGGGCGGCCTCT GCGGTTTGTCGGGCCAGCATCAG TTTGGGCGGCAGGACAATCGCGTGGGAATGTGGCAC GGCCTCGGCTGTGTGTTATAGCCC GCGTGGATACTGCCAGCCTAGACTGAGGACTGCGGT TTATACCTAGGATGTTGGCATAAT GATCTTAAGTCGCCCGTCTTGAAAACAG
4	S 10	TTGCACCACATGTGTTTTTTACTGGACAGCTG CTTTGGCGGTGGGGACTCGTTTCCGCCG CCAGAGGTCACAACTAAACCAAACTTTTTATT ACCAGTCAACCATACGTTTTAATAGTCA AAACTTTCAACAACGGATCTCTTGGTTCTCGC ATCGATGAAAAACGCAGCGAAATGCGAT ACGTAGTATGAATTGCAGATATTCGTGAATCA TCGAATCTTTGAACGCACATTGCGCCCT TTGGTATTCCAAAGGGCATGCCTGTTTGAGCG TCATTTCTCCCTCAAGCCCGCGGGTTTG GTGTTGAGCAATACGCCAGGTTTGTTTGAAA GACGTACGTGGAGACTATATTAGCGACTT AGGTTCTACCAAAACGCTTGTGCAGTCGGCCC ACCACAGCTTTTCTAACTTTTGACCTCA AATCAGGTAGGACTACCCGCTGAACTTAAGC ATATCAATAAGCGGAGGAAAAAGATCATT ACTGATTTGCTTAATTGCA	CCTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAA TTTGAAATCTGGCTCTTTCAGAGT CCGAGTTGTAATTTGAAGAAGGTATCTTTGGGCCTGG CTCTTGTCTATGTTTCTTGGAAC AGAACGTCACAGAGGGTGAGAATCCCGTGCGATGAG ATGACCCAGGTCCGTGTAAAGTTC CTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAA GTGGGTGGTAAATTCCATCTAAA GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTA CAGTGATGGAAAGATGAAAAGAAC TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTG AAAGGGAAGGGCTTGAGATCAGAC TTGGCATTTTGCATGTTGCTTCTTCGGGGGCGGCCTCT GCGGTTTGTCGGGCCAGCATCA GTTTGGGCGGCAGGACAATCGCGTGGGAATGTGGCA CGGCCTCGGCTGTGTGTTATAGCC CGCGTGGATACTGCCAGCCTAGACTGAGGACTGCGG TTTATACCTAGGATGTTGGCATAA TGATCTTAAGTCGCCCGTCTTGACCACAG
5	S 45	ATTGCCCACATGTGTTTTTTATTGAACAAATTT CTTTGGTGGCGGGAGCAATCCCACCGC CAGAGGTTATAACTAAACCAAACTTTTTATTT ACAGTCAAACTTGATTTATTATTACAAT AGTCAAAACTTTCAACAACGGATCTCTTGGTT CTCGCATCGATGAAGAACGCAGCGAAAT GCGATACGTAATATGAATTGCAGATATTCGTG AATCATCGAATCTTTGAACGCACATTGC GCCCTTTGGTATTCCAAAGGGCATGCCTGTTT GAGCGTCATTTCTCCCTCAAACCCCCGG GTTTGGTGTTGAGCAATACGCTAGGTTTGTTT GAAAGAATTTAACGTGGAAACTTATTTT AAGCGACTTAGGTTTATCCAAAAACGCTTATT TTGCTAGTGGCCACCACAATTTATTTCA TAACTTTGACCTCAAATCAGGTAGGACTACCC GCTGAACTTAAGCATATCAATAAGCGGG AGGAAAAAAG	TGCCTTAGTAGCGGCGAGTGAGCGGCAAAAGCTCAA ATTTGAAATCTGGCTCTTTCAGAG TCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGTCTG GCTCTTGTCTATGTTTCTTGGAA CAGAACGTCACAGAGGGTGAGAATCCCGTGCGATGA GATGATCCAGGCCTATGTAAAGTT CCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTA AGTGGGTGGTAAATTCCATCTAA AGCTAAATATTGGCGAGAGACCGATAGCGAACAAGT ACAGTGATGGAAAGATGAAAAGAA CTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTT GAAAGGGAAGGGCTTGAGATCAGA CTTGGTATTTTGTATGTTACTTCTTCGGGGGTGGCCTC TACAGTTTATCGGGCCAGCATC AGTTTGGGCGGTAGGAGAATTGCGTTGGAATGTGGC ACGGCCTCGGTTGTGTGTTATAGC CTTCGTCGATACTGCCAGCCTAGACTGAGGACTGCGG TTTATACCTAGGATGTTGGCATA ATGATCTTAAGTCGCCCATCTT

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
6	S 57	TACCTGATTTGAGGTCAAGTTATGAAATAAAT TGTGGTGGCCACTATGCAAAATAAGCGT TTTTGGATAAACCTAAGTCGCTTAAAATAAGT TTCCACGTTAAATTCTTTCAAACAAACC TAGCGTATTGCTCAACACCAAACCCGGGGGTT TGAGGGAGAAATGACGCTCAAACAGGCA TGCCCTTTGGAATACCAAAGGGCGCAATGTG CGTTCAAAGATTCGATGATTCACGAATAT CTGCAATTCATATTACGTATCGCATTTCGCTGC GTTCTTCATCGATGCGAGAACCAAGAG ATCCGTTGTTGAAAGTTTTGACTATTGTAATA ATAAATCAAGTTTGACTGTAAATAAAAA GTTTGGTTTAGTTATAACCTCTGGCGGTAGGA TTGCTCCCGCCACCAAAGAAATTTGTTC AATAAAAAACACATGTGGTGCAATTAAGCAA ATCAGTAATGATCCTTCCGCAGGTTACCC T	CACTCCTAGGTATAAACCGCAGTCCTCAGTCTAGGCT GGCAGTATCGACGAAGGCTATAA CACACAACCGAAGCCGTGCCACATTCCAACGCAATTC TCCTACCGCCCAAACTGATGCTG GCCCGATAAACTGTAGAGGCCACCCCCGAAGAAGTA ACATACAAAATACCAAGTCTGATC TCAAGCCCTTCCCTTTCAACAATTTCACGTACTTTTTCA CTCTCTTTTCAAAGTTCTTTT CATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCTC GCCAATATTTAGCTTTAGATG GAATTTACCACCCACTTAGAGCTGCATTCCCAAACAAC TCGACTCTTCGAAGGAACTTTA CATAGGCCTGGATCATCTCATCGCACGGGATTCTCAC CCTCTGTGACGTTCTGTTCCAAG AAACATAGACAAGAGCCAGACCCAAAGATACCTTCTT CAAATTACAACTCGGACTCTGAA AGAGCCAGATTTCAAATTTGAGCTTTTGCCGCTTCACT CGCCGCTACTAAGGCAATCCCT GTTGGTTTCTTTTCCTCCGCTTTTTGAT
7	S 62	TGCACCACATGTGTTTTTTATTGAACAAATTTC TTTGGTGGCGGGAGCAATCCTACCGCC AGAGGTTATAACTAAACCAAACTTTTTATTTA CAGTCAAACTTGATTTATTATTACAATA GTCAAAACTTTCAACAACGGATCTCTTGGTTC TCGCATCGATGAAGAACGCAGCGAAATG CGATACGTAATATGAATTGCAGATATTCGTGA ATCATCGAATCTTTGAACGCACATTGCG CCCTTTGGTATTCCAAAGGGCATGCCTGTTTG AGCGTCATTTCTCCCTCAAACCCCCGGG TTTGGTGTTGAGCAATACGCTAGGTTTGTTTG AAAGAATTTAACGTGGAAACTTATTTTA AGCGACTTAGGTTTATCCAAAAACGCTTATTT TGCTAGTGGCCACCACAATTTATTTCAT AACTTTGACCTCAAATCAGGTAGGACTACCCG CTGAACTTAAGCATATCAATAAGCGGAG GAAAAAGGATCATTACTGATTTGCTTAATTGC ACCCATGTGTTTTTTATTGAACAAATTT CTTTGGTGGCGGGAGCAATCCTACGCCAG	TATAACCGCAGTCCTCAGTCTAGGCTGGCAGTATCGA CGAAGGCTATAACACACAACCGA AGCCGTGCCACATTCCAACGCAATTCTCCTACCGCCCA AACTGATGCTGGCCCGATAAAC TGTAGAGGCCACCCCCGAAGAAGTAACATACAAAAT ACCAAGTCTGATCTCAAGCCCTTC CCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCA AAGTTCTTTTCATCTTTCCAT CACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTT AGCTTTAGATGGAATTTACCAC CCACTTAGAGCTGCATTCCCAAACAACTCGACTCTTCG AAGGAACTTTACATAGGCCTGG ATCATCTCATCGCACGGGATTCTCACCCTCTGTGACGT TCTGTTCCAAGAAACATAGACA AGAGCCAGACCCAAAGATACCTTCTTCAAATTACAAC TCGGACTCTGAAAGAGCCAGATT TCAAATTTGAGCTTTTGCCGCTTCACTCGCCGCTACTA AGGCAATCCCTGTTGGTTTCTT TTCCTCCGCTTTTT
8	S 65		TGCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCA GCTGGCAGTATTCCCACAGGCT ATAATACTTACCGAGGCAAGCTACATTCCTATGGATTT ATCCTGCCACCAAAACTGATGC TGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGG AGCAGAGGGCACAAAACACCATG TCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTA CTTTTTCACTCTCTTTTCAAAG TTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCG GTCTCTCGCCAATATTTAGCT TTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCC AAACAACTCGACTCTTCGAAGG CACTTTACAAAGAACCGCACTCCTCGCCACACGGGAT TCTCACCCTCTATGACGTCCTGT TCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTG CCCTCTCCAAATTACAACTCGGGC ACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCG CTTCACTCGCCGTTACTAAGGCA ATCCCGGTTGGTTTCTTTTCCTCCGCTTTTG

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
9	S 68	CACCACATGTGTTTTTTATTGAACAAATTTCTT TGGTGGCGGGAGCAATCCTACCGCCAG AGGTTATAACTAAACCAAACTTTTTATTTACA GTCAAACTTGATTTATTATTACAATAGT CAAAACTTTCAACAACGGATCTCTTGGTTCTC GCATCGATGAAGAACGCAGCGAAATGCG ATACGTAATATGAATTGCAGATATTCGTGAAT CATCGAATCTTTGAACGCACATTGCGCC CTTTGGTATTCCAAAGGGCATGCCTGTTTGAG CGTCATTTCTCCCTCAAACCCCCGGGTT TGGTGTTGAGCAATACGCTAGGTTTGTTTGAA AGAATTTAACGTGGAAACTTATTTTAAG CGACTTAGGTTTATCCAAAAACGCTTATTTTG CTAGTGGCCACCACAATTTATTTCATAA CTTTGACCTCAAATCAGGTAGGACTACCCGCT GAACTTAAGCATATCAATAAGCGGAGGA AAAAAGATCATTACTGATTTGCTTAATTGCAC CCATGTGTT	TGCACATCCTAGGTATAAACCGCAGTCCTCAGTCTAG GCTGGCAGTATCGACGAAGGCTA TAACACACAACCGAAGCCGTGCCACATTCCAACGCAA TTCTCCTACCGCCCAAACTGATG CTGGCCCGATAAACTGTAGAGGCCACCCCCGAAGAA GTAACATACAAAATACCAAGTCTG ATCTCAAGCCCTTCCCTTTCAACAATTTCACGTACTTTT TCACTCTCTTTTCAAAGTTCT TTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCT CTCGCCAATATTTAGCTTTAG ATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAAC AACTCGACTCTTCGAAGGAACT TTACATAGGCCTGGATCATCTCATCGCACGGGATTCT CACCCTCTGTGACGTTCTGTTCC AAGAAACATAGACAAGAGCCAGACCCAAAGATACCT TCTTCAAATTACAACTCGGACTCT GAAAGAGCCAGATTTCAAATTTGAGCTTTTGCCGCTT CACTCGCCGCTACTAAGGCAATC CCTGTTGGTTTCTTTTCCTCCGCTTTTG
10	S 69	ACTGATTTGAGGTCAACTTTAAGAACATTGTT CGCCTAGACGCTCTCTTCTTATCGATAA CGTTCCAATACGCTCAGTATAAAAAAGATTAG CCGCAGTTGGTAAAACCTAAAACGACCG TACTTGCATTATACCTCAAGCACGCAGAGAAA CCTCTCTTTGGAAAAAAAACATCCAATG AAAAGGCCAGCAATTTCAAGTTAACTCCAAAG AGTATCACTCACTACCAAACAGAATGTT TGAGAAGGAAATGACGCTCAAACAGGCATGC CCCCTGGAATACCAAGGGGCGCAATGTGC GTTCAAAGATTCGATGATTCACGGAATTCTGC AATTCACATTACGTATCGCATTTCGCTG CGTTCTTCATCGATGCGAGAACCAAGAGATCC GTTGTTGAAAGTTTTTAATATTTTAAAA TTTCCAGTTACGAAAATTCTTGTTTTTGACAAA AATTTAATGAATAAATAAAATTGTTTG TGTTTGTTACCTCTGGGCCCCGATTGCTCGAA TGCCCAAAGAAAAAGTTGCAAAGATATG AAAACTCCACAGTGTGTTGTATTGAAACGGTT TTAATTGTCCTATAACAAAAGCACAGAA ATCTCTCACCGTTTGGAATAGCAAGAAAGAA ACTTACAAGCCTAGCACGACCGCGCACTT AAGCGCAGGCCCGGCTGGACTCTCCATCTCTT GTCTTCTTGCCCAGTAAAAGCTCTCATG CTCTTGCCAAAACAAAAAAATCCATTTTCAAA ATTATTAAATTTCTTTAATGATCCTTCG CA	TGCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCA GCTGGCAGTATTCCCACAGGCT ATAATACTTACCGAGGCAAGCTACATTCCTATGGATTT ATCCTGCCACCAAAACTGATGC TGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGG AGCAGAGGGCACAAAACACCATG TCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTA CTTTTTCACTCTCTTTTCAAAG TTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCG GTCTCTCGCCAATATTTAGCT TTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCC AAACAACTCGACTCTTCGAAGG CACTTTACAAAGAACCGCACTCCTCGCCACACGGGAT TCTCACCCTCTATGACGTCCTGT TCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTG CCCTCTCCAAATTACAACTCGGGC ACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCG CTTCACTCGCCGTTACTAAGGCA ATCCCGGTTGGTTTCTTTTCCTCCGCTTTTGA

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
11	S 71	TACTGATTTGAGGTCAACTTTAAGAACATTGT TCGCCTAGACGCTCTCTTCTTATCGATA ACGTTCCAATACGCTCAGTATAAAAAAGATTA GCCGCAGTTGGTAAAACCTAAAACGACC GTACTTGCATTATACCTCAAGCACGCAGAGAA ACCTCTCTTTGGAAAAAAAACATCCAAT GAAAAGGCCAGCAATTTCAAGTTAACTCCAAA GAGTATCACTCACTACCAAACAGAATGT TTGAGAAGGAAATGACGCTCAAACAGGCATG CCCCCTGGAATACCAAGGGGCGCAATGTG CGTTCAAAGATTCGATGATTCACGGAATTCTG CAATTCACATTACGTATCGCATTTCGCT GCGTTCTTCATCGATGCGAGAACCAAGAGAT CCGTTGTTGAAAGTTTTTAATATTTTAAA ATTTCCAGTTACGAAAATTCTTGTTTTTGACAA AAATTTAATGAATAAATAAAATTGTTT GTGTTTGTTACCTCTGGGCCCCGATTGCTCGA ATGCCCAAAGAAAAAGTTGCAAAGATAT GAAAACTCCACAGTGTGTTGTATTGAAACGGT TTTAATTGTCCTATAACAAAAGCACAGA AATCTCTCACCGTTTGGAATAGCAAGAAAGA AACTTACAAGCCTAGCACGACCGCGCACT TAAGCGCAGGCCCGGCTGGACTCTCCATCTCT TGTCTTCTTGCCCAGTAAAAGCTCTCAT GCTCTTGCCAAAACAAAAAAATCCATTTTCAA AATTATTAAATTTCTTTAATGATCTTTC GCAGA	TGCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCA GCTGGCAGTATTCCCACAGGCT ATAATACTTACCGAGGCAAGCTACATTCCTATGGATTT ATCCTGCCACCAAAACTGATGC TGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGG AGCAGAGGGCACAAAACACCATG TCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTA CTTTTTCACTCTCTTTTCAAAG TTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCG GTCTCTCGCCAATATTTAGCT TTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCC AAACAACTCGACTCTTCGAAGG CACTTTACAAAGAACCGCACTCCTCGCCACACGGGAT TCTCACCCTCTATGACGTCCTGT TCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTG CCCTCTCCAAATTACAACTCGGGC ACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCG CTTCACTCGCCGTTACTAAGGCA ATCCCGGTTGGTTTCTTTTCCTCCGCTTTTGG
12	S 80	TATTGCAGCGCTTATTGCGCGGCGATAAACCT TACACACATTGTCTAGTTTTTTTGAACT TTGCTTTGGGTGGTGAGCCTGGCTTACTGCCC AAAGGTCTAAACACATTTTTTTTAATGT TAAAACCTTTAACCAATAGTCATGAAAATTTTT AACAAAAATTAAAATCTTCAAAACTTT CAACAACGGATCTCTTGGTTCTCGCAACGATG AAGAACGCAGCGAAATGCGATACGTATT GTGAATTGCAGATTTTCGTGAATCATCGAATC TTTGAACGCACATTGCACCCTCTGGTAT TCCAGAGGGTATGCCTGTTTGAGCGTCATTTC TCTCTCAAACCTTCGGGTTTGGTATTGA GTGATACTCTGTCAAGGGTTAACTTGAAATAT TGACTTAGCAAGAGTGTACTAATAAGCA GTCTTTCTGAAATAATGTATTAGGTTCTTCCAA CTCGTTATATCAGCTAGGCAGGTTTAG AAGTATTTTAGGCTCGGCTTAACAACAATAAA CTAAAAGTTTGACCTCAAATCAGGTAGG ACTACCCGCTGAACTTAAGCATATCAATAAGC GGAGGAAA	GCCTCAGTACGGCGAGTGAAGCGGCAAAAGCTCAAA TTTGAAATCTAGCACCTTCGGTGT TCGAGTTGTAATTTGAAGATGGTAACCTTGGGTTTGG CTCTTGTCTATGTTCCTTGGAAC AGGACGTCATAGAGGGTGAGAATCCCGTCTGATGAG ATGCCCATTCCTATGTAAGGTGCT ATCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAA GTGGGTGGTAAATTCCATCTAAAG CTAAATATTGGCGAGAGACCGATAGCGAACAAGTAC AGTGATGGAAAGATGAAAAGAACT TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGA AAGGGAAGGGCATTAGATCAGACT TGGTGTTTTACGATTATCTTCTCTTCTTGAGTTGTGCA CTCGTATTTCACTGGGCCAGCA TCGATTCGGATGGCAAGATAATGGCAGTTGAATGTG GCTTCACTTCGGTGGAGTGTTATA GCTTCTGCTGATATTGCCTGTCTGGATCGAGGGCTGC GTCTTTTGACTAGGATGCTGGCG TAATGATCTAATGCCGCCCGTCTTGACCCCCC

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
13	S 83	AGTACTACACTGCGTGAGCGGAACGAAAACA ACAACACCTAAAATGTGGAATATAGCATA TAGTCGACAAGAGAAATCTACGAAAAACAAA CAAAACTTTCAACAACGGATCTCTTGGTT CTCGCATCGATGAAGAGCGCAGCGAAATGCG ATACCTAGTGTGAATTGCAGCCATCGTGA ATCATCGAGTTCTTGAACGCACATTGCGCCCC TCGGCATTCCGGGGGGCATGCCTGTTTG AGCGTCGTTTCCATCTTGCGCGTGCGCAGAGT TGGGGGAGCGGAGCGGACGACGTGTAAA GAGCGTCGGAGCTGCGACTCGCCTGAAAGGG AGCGAAGCTGGCCGAGCGAACTAGACTTT TTTTCAGGGACGCTTGGCGGCCGAGAGCGAG TGTTGCGAGACAACAAAAAGCTCGACCTC AAATCAGGTAGGAATACCCGCTGAACTTAAG CATATCAATAAGCGGAGG	CCTCAGTAGCGGCGAGTGAGCGGCAAGAGCTCAGAT TTGAAATCGTGCTTTGCGGCACGA GTTGTAGATTGCAGGTTGGAGTCTGTGTGGAAGGCG GTGTCCAAGTCCCTTGGAACAGGG CGCCCAGGAGGGTGAGAGCCCCGTGGGATGCCGGC GGAAGCAGTGAGGCCCTTCTGACGA GTCGAGTTGTTTGGGAATGCAGCTCCAAGCGGGTGG TAAATTCCATCTAAGGCTAAATAC TGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGG AAAGATGAAAAGCACTTTGAAAAG AGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAG GGTATTGCGCCCGACATGGGGATT GCGCACCGCTGCCTCTCGTGGGCGGCGCTCTGGGCTT TCCCTGGGCCAGCATCGGTTCTT GCTGCAGGAGAAGGGGTTCTGGAACGTGGCTCTTCG GAGTGTTATAGCCAGGGCCAGATG CTGCGTGCGGGGACCGAGGACTGCGGCCGTGTAGGT CACGGATGCTGGCAGAACGGCGCA ACACCGCCCGTCTTGACACACAC
14	S 84	ATTATTGATTTGTCTGAGCTCGGAGAGAGACA TCTCTGGGGAGGACCAGTGTAGACACTC AGGAGGCTCCTAAAATATTTTCTCTGCTGTGA ATGCTATTTCTCCTGCCTGCGCTTAAGT GCGCGGTTGGTGGGTGTTCTGCAGTGGGGG GAGGGAGCCGACAAAGACCTGGGAGTGTGC GTGGATCTCTCTATTCCAAAGGAGGTGTTTTA TCACACGACTCGACACTTTCTAATTACT ACACACAGTGGAGTTTACTTTACTACTATTCTT TTGTTCGTTGGGGGAACGCTCTCTTTC GGGAGGGAGTTCTCCCAGTGGATGCAAACAC AAACAAATATTTTTTTAAACTAATTCAGT CAACACAAGATTTCTTTTAGTAGAAAACAACT TCAAAACTTTCAACAATGGATCTCTTGG TTCTCGCATCGATGAAGAACGCAGCGAAATG CGATACGTAATGTGAATTGCAGAATTCCG TGAATCATCGAATCTTTGAACGCACATTGCGC CCTCTGGTATTCCGGGGGGCATGCCTGT TTGAGCGTCATTTCCTTCTCAAACACGTTGTGT TTGGTAGTGAGTGATACTCTCGTTTTT GAGTTAACTTGAAATTGTAGGCCATATCAGTA TGTGGGACACGAGCGCAAGCTTCTCTAT TAATCTGCTGCTCGTTTGCGCGAGCGGCGGG GGTTAATACTGTATTAGGTTTTACCAACT CGGTGTTGATCTAGGGAGGGATAAGTGAGTG TTTTGTGCGTGCTGGGGCAGACAGACGTC TTTAAGTTTGACCTCAAATCAGTAGGGTTACC CGCTGAACTTAAGCATATCAATAGCCGG	GCCTTAGTACGGCGAGTGAGCGGCAAAAGCTCAAAT TTGAAATCTGGTACCTTTGGTGCC CGAGTTGTAATTTGGAGAGTACCACTTTGGGACTGTA CTTTGCCTATGTTCCTTGGAACA GGACGTCATGGAGGGTGAGAATCCCGTGTGGCGAG GGTGTCAGTTCTTTGTAAAGGGTGC TCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGT GGGTGGTAAATTCCATCTAAAGC TAAATACAGGCGAGAGACCGATAGCGAACAAGTACA GTGATGGAAAGATGAAAAGAACTT TGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAA AGGGAAGGGCATTTGATCAGACAT GGTGTTTTGCGCCCCTTGCCTCTCGTGGGCTTGGGAC TCTCGCAGCTCACTGGGCCAGCA TCGGTTTTGGCGGCCGGAAAAAACCTAGGGAATGTG GCTCTGCGCCTCGGTGTAGAGTGT TATAGCCCTGGGGAATACGGCCAGCCGGGACCGAGG ACTGCGATACTTGTTATCTAGGAT GCTGGCATAATGGTTATATGCCGCCCGTCTTGAACCA ACGGACCAAA

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
15	**S 88**		CAGCATCCGTGACTACACGGCCGCAGTCCTCGGTCCC CGCACGCAGCATCTGGCCCTGGC TATAACACTCCGAAGAGCCACGTTCCAGAACCCCTTCT CCTGCAGCAAGAACCGATGCTG GCCCAGGGAAAGCCCAGAGCGCCGCCCACGAGAGGC AGCGGTGCGCAATCCCCATGTCGG GCGCAATACCCTTCCCTTTCAACAATTTCACGTGCTGT TTCACTCTCTTTTCAAAGTGCT TTTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCT CTCGCCAGTATTTAGCCTTAG ATGGAATTTACCACCCGCTTGGAGCTGCATTCCCAAA CAACTCGACTCGTCAGAAGGGCC TCACTGCTTCCGCCGGCATCCCACGGGGCTCTCACCCT CCTGGGCGCCCTGTTCCAAGGG ACTTGGACACCGCCTTCCACACAGACTCCAACCTGCA ATCTACAACTCGTGCCGCAAAGC ACGATTTCAAATCTGAGCTCTTGCCGCTTCACTCGCCG CTACTGAGGCAATCCCTGTTGG TTTCTTTTCCTCCGCTTTTT
16	**S 115**	TATTGCAGCGCTTATTGCGCGGCGATAAACCT TACACACATTGTCTAGTTTTTTTGAACT TTGCTTTGGGTGGTGAGCCTGGCTTACTGCCC AAAGGTCTAAACACATTTTTTTTAATGT TAAAACCTTTAACCAATAGTCATGAAAATTTTT AACAAAAATTAAAATCTTCAAAACTTT CAACAACGGATCTCTTGGTTCTCGCAACGATG AAGAACGCAGCGAAATGCGATACGTATT GTGAATTGCAGATTTTCGTGAATCATCGAATC TTTGAACGCACATTGCACCCTCTGGTAT TCCAGAGGGTATGCCTGTTTGAGCGTCATTTC TCTCTCAAACCTTCGGGTTTGGTATTGA GTGATACTCTGTCAAGGGTTAACTTGAAATAT TGACTTAGCAAGAGTGTACTAATAAGCA GTCTTTCTGAAATAATGTATTAGGTTCTTCCAA CTCGTTATATCAGCTAGGCAGGTTTAG AAGTATTTTAGGCTCGGCTTAACAACAATAAA CTAAAAGTTTGACCTCAAATCAGGTAGG ACTACCCGCTGAACTTAAGCATATCAATAAGC CGGAAG	GCCTCAGTACGGCGAGTGAAGCGGCAAAAGCTCAAA TTTGAAATCTAGCACCTTCGGTGT TCGAGTTGTAATTTGAAGATGGTAACCTTGGGTTTGG CTCTTGTCTATGTTCCTTGGAAC AGGACGTCATAGAGGGTGAGAATCCCGTCTGATGAG ATGCCCATTCCTATGTAAGGTGCT ATCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAA GTGGGTGGTAAATTCCATCTAAAG CTAAATATTGGCGAGAGACCGATAGCGAACAAGTAC AGTGATGGAAAGATGAAAAGAACT TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGA AAGGGAAGGGCATTAGATCAGACT TGGTGTTTTACGATTATCTTCTCTTCTTGAGTTGTGCA CTCGTATTTCACTGGGCCAGCA TCGATTCGGATGGCAAGATAATGGCAGTTGAATGTG GCTTCACTTCGGTGGAGTGTTATA GCTTCTGCTGATATTGCCTGTCTGGATCGAGGGCTGC GTCTTTTGACTAGGATGCTGGCG TAATGATCTAATGCCGCCCGTCTTG

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
17	S 116	TATTGCCAGCGCTTATTGCGCGGCGATAAACC TTACACACATTGTCTAGTTTTTTTGAAC TTTGCTTTGGGTGGTGAGCCTGGCTTACTGCC CAAAGGTCTAAACACATTTTTTTTAATG TTAAAACCTTTAACCAATAGTCATGAAAATTTT TAACAAAAATTAAAATCTTCAAAACTT TCAACAACGGATCTCTTGGTTCTCGCAACGAT GAAGAACGCAGCGAAATGCGATACGTAT TGTGAATTGCAGATTTTCGTGAATCATCGAAT CTTTGAACGCACATTGCACCCTCTGGTA TTCCAGAGGGTATGCCTGTTTGAGCGTCATTT CTCTCTCAAACCTTCGGGTTTGGTATTG AGTGATACTCTGTCAAGGGTTAACTTGAAATA TTGACTTAGCAAGAGTGTACTAATAAGC AGTCTTTCTGAAATAATGTATTAGGTTCTTCCA ACTCGTTATATCAGCTAGGCAGGTTTA GAAGTATTTTAGGCTCGGCTTAACAACAATAA ACTAAAAGTTTGACCTCAAATCAGGTAG GACTACCCGCTGAACTTAAGCATATCAATAAG CGGAAGGA	
18	S 117	CTTTTGAATGGATTTTTTTGTTTTGGCAAGAG CATGAGAGCTTTTACTGGGCAAGAAGAC AAGAGATGGAGAGTCCAGCCGGGCCTGCGCT TAAGTGCGCGGTCTTGCTAGGCTTGTAAG TTTCTTTCTTGCTATTCCAAACGGTGAGAGATT TCTGTGCTTTTGTTATAGGACAATTAA AACCGTTTCAATACAACACACTGTGGAGTTTT CATATCTTTGCAACTTTTTCTTTGGGCA TTCGAGCAATCGGGGCCCAGAGGTAACAAAC ACAAACAATTTTATCTATTCATTAAATTT TTGTCAAAAACAAGAATTTTCGTAACTGGAAA TTTTAAAATATTAAAAACTTTCAACAAC GGATCTCTTGGTTCTCGCATCGATGAAGAACG CAGCGAAATGCGATACGTAATGTGAATT GCAGAATTCCGTGAATCATCGAATCTTTGAAC GCACATTGCGCCCCTTGGTATTCCAGGG GGCATGCCTGTTTGAGCGTCATTTCCTTCTCA AACATTCTGTTTGGTAGTGAGTGATACT CTTTGGAGTTAACTTGAAATTGCTGGCCTTTTC ATTGGATGTTTTTTTTCCAAAGAGAGG TTTCTCTGCGTGCTTGAGGTATAATGCAAGTA CGGTCGTTTTAGGTTTTACCAACTGCGG CTAATCTTTTTTAATACTGA	TGCTTAGTACGGCGAGTGAGCGGCAAAAGCTCAAAT TTGAAATCTGGTACCTTCGGTGCC CGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGT TCCTTGTCTATGTTCCTTGGAACA GGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGG AGTGCGGTTCTTTGTAAAGTGCCT TCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGT GGGTGGTAAATTCCATCTAAAGC TAAATATTGGCGAGAGACCGATAGCGAACAAGTACA GTGATGGAAAGATGAAAAGAACTT TGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAA AGGGAAGGGCATTTGATCAGACAT GGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGAA TCTCGCATTTCACTGGGCCAGCA TCAGTTTTGGTGGCAGGATAAATCCATAGGAATGTAG CTTGCCTCGGTAAGTATTATAGC CTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCG ACGTAAGTCAAGGATGCTGGCATA ATGGTTATATGCCGCCCGTCTTGAAAAAGGA

No	Strain	Sequence (ITS primers)	Sequence (D1/D2 primers)
19	**S 118**	TGAATGGATTTTTTTGTTTTGGCCAAGAGCAT GAGAGCTTTTACTGGGCAAGAAGACAAG AGATGGAGAGTCCAGCCGGGCCTGCGCTTAA GTGCGCGGTCTTGCTAGGCTTGTAAGTTT CTTTCTTGCTATTCCAAACGGTGAGAGATTTCT GTGCTTTTGTTATAGGACAATTAAAAC CGTTTCAATACAACACACTGTGGAGTTTTCAT ATCTTTGCAACTTTTTCTTTGGGCATTC GAGCAATCGGGGCCCAGAGGTAACAAACACA AACAATTTTATCTATTCATTAAATTTTTG TCAAAAACAAGAATTTTCGTAACTGGAAATTT TAAAATATTAAAAACTTTCAACAACGGA TCTCTTGGTTCTCGCATCGATGAAGAACGCAG CGAAATGCGATACGTAATGTGAATTGCA GAATTCCGTGAATCATCGAATCTTTGAACGCA CATTGCGCCCCTTGGTATTCCAGGGGGC ATGCCTGTTTGAGCGTCATTTCCTTCTCAAACA TTCTGTTTGGTAGTGAGTGATACTCTT TGGAGTTAACTTGAAATTGCTGGCCTTTTCAT TGGATGTTTTTTTTCCAAAGAGAGGTTT CTCTGCGTGCTTGAGGTATAATGCAAGTACG GTCGTTTTAGGTTTTACCAACTGCGGCTA ATCTTTTTTTATACTAAC	GCCTTAGTACGGCGAGTGAAGCGGCAAAAGCTCAAA TTTGAAATCTGGTACCTTCGGTGC CCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCG TTCCTTGTCTATGTTCCTTGGAAC AGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAG GAGTGCGGTTCTTTGTAAAGTGCC TTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAG TGGGTGGTAAATTCCATCTAAAG CTAAATATTGGCGAGAGACCGATAGCGAACAAGTAC AGTGATGGAAAGATGAAAAGAACT TTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGA AAGGGAAGGGCATTTGATCAGACA TGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGA ATCTCGCATTTCACTGGGCCAGC ATCAGTTTTGGTGGCAGGATAAATCCATAGGAATGTA GCTTGCCTCGGTAAGTATTATAG CCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGC GACGTAAGTCAAGGATGCTGGCAT AATGGTTATATGCCGCCCGTCTTGACCCCCC
20	**S 127**	TCATAAAATCTTTGAACGCACATTGCGCCCTC TGGTATTCCGGGGGGCATGCCTGTTTGA GCGTCATTTCCTTCTCAAACACGTTGTGTTTGG TAGTGAGTGATACTCTCGTTTTTGAGT TAACTTGAAATTGTAGGCCATATCAGTATGTG GGACACGAGCGCAAGCTTCTCTATTAAT CTGCTGCTCGTTTGCGCGAGCGGCGGGGGTT AATACTGTATTAGGTTTTACCAACTCGGT GTTGATCTAGGGAGGGATAAGTGAGTGTTTT GTGCGTGCTGGGCAGACAGACGTCTTTAA GTTTGACCTCAAATCAGGTAGGGTTACCCGCT GAACTTAAGCA	
21	**S 142**		CCACATCCTAGGTAAACCGCAGTCCTCGGTCTAGGCT GGCAGTATCGTCAGAGGCTATAA CACACAGCAGAAGCCGTGCCACATTCCTCCGCCATTA TCCTGCCGCTCCAAACCGATGCT GGCCCGGTAAACCGCAGCGGCCGCCCCCGAGAGAGC AACATGCAAAATACCAAGTCTGAT CTCAAGCCCTTCCCTTTCAACAATTTCACGTACTTTTTC ACTCTCTTTTCAAAGTTCTTT TCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCT CGCCAATATTTAGCTTTAGAT GGAATTTACCACCCACTTAGAGCTGCATTCCCAAACA ACTCGACTCGTCGAAGGAACTTT ACACAGACCCGGGTCATCTCATCGCACGGGATTCTCA CCCTCTGTGACGTCCTGTTCCAA GGAACATAGACAAGAGCCGGGCCCAAAGATACCTTC TTCAAATTACAACTCGGACGCCAA AGACGCCAGATTTCAAATTTGAGCTTTTGCCGCTTCAC TCGCCGCTACTGAGGCAATCCC TGTTGGTTTCTTTTCCTCCGCTTTTGG

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