NORTH-WEST UNIVERSITY
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NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

Production of ethanol from tropical sugar beet

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BSc. (Hons)

Dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in Engineering Sciences (Chemical and Minerals Engineering) at the Potchefstroom Campus of North-West University

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2010

Abstract

The concern over depleting fossil fuel resources and increasing greenhouse gas emissions has prompted the research into alternative and renewable energy resources. Bioethanol is seen as a potential alternative to petroleum fuels and is mainly produced from sugar and starch containing crops such as sugar cane and maize. In South Africa the use of maize for ethanol production has been prohibited due to food security concerns; therefore, alternative feedstocks need to be investigated. Tropical sugar beet, a new variety of sugar beet, is a potential alternative as it is able to grow in tropical and subtropical climates using much less water than sugar cane. The main objective of this study was to determine the potential of using tropical sugar beet for ethanol production. The study focused on the effects of dilution ratio, pH, yeast concentration and the addition of a nitrogen supplement on the ethanol yield. The maximum ethanol yield of 0.47 $g.g^{-1}$ which is a conversion efficiency of 92% and a glycerol yield of 0.08 $g.g^{-1}$ was obtained when no additional water was added to the juice. The best dilution ratio was found to be 1:4 which gave a maximum ethanol yield of 0.48 $g.g^{-1}$ which is a conversion efficiency of 94% and a glycerol yield of 0.07 $g.g^{-1}$. An ethanol yield of 0.48 $g.g^{-1}$ which is a conversion efficiency of 94% was achieved at a yeast concentration of 5 $g.L^{-1}$ after four hours of fermentation. Nitrogen supplements such as urea, peptone, yeast extract and ammonium sulphate were added during fermentation. The addition of a nitrogen supplement to fermentation had a positive effect on the ethanol yield. The maximum ethanol yield of 0.47 $g.g^{-1}$ which is a conversion efficiency of 92% was achieved when urea was added to the fermentation. The addition of a nitrogen supplement also decreased the amount of glycerol formed from 0.15 $g.g^{-1}$ to 0.08 $g.g^{-1}$. Ammonium sulphate was chosen as the preferred nitrogen source as it is a simple component that can enter the cell directly. A maximum ethanol yield of 0.45 $g.g^{-1}$ which is a conversion efficiency of 88%, was achieved when 750 mg $N.L^{-1}$ ammonium sulphate was added. Adjusting the pH prior to fermentation had no real effect on the ethanol yield. The maximum ethanol yield of 0.45 $g.g^{-1}$ was achieved at all the pH values investigated.

Therefore the natural pH of the juice, or pH values between 4 and 5.5, could be used. Adjusting the pH was done to merely reduce the risk of contamination. The optimal fermentation parameters were found to be pH 4, yeast concentration 5 $g.L^{-1}$ and a ammonium sulphate concentration of 750 mg $N.L^{-1}$. At these conditions, a maximum ethanol of 0.45 $g.g^{-1}$ was achieved. These results show that tropical sugar beet with a sugar content of approximately 21.8% ($w.w^{-1}$) is a good feedstock for ethanol production in South Africa.

Keywords: Alternative energy resource, tropical sugar beet, fermentation, ethanol, glycerol

Opsomming

Die afname in die beskikbaarheid van fossiel brandstof en die toename van kweekhuis gasse is ‘n bron van kommer wat die navorsing van hernubare en alternatiewe energie bronne aangehits het. Die produksie van bio-etanol vanaf suiker- en styselbevattende gewasse word gesien as ‘n moontlike alternatiewe voermateriaal wat petroleum brandstowwe kan vervang. Die gebruik van mielies as voermateriaal vir etanolproduksie word tans verbied in Suid-Afrika weens voedselsekuriteit-bekommernisse en dus moet alternatiewe bronne nagevors word. Een so ‘n alternatief is ‘n nuwe variëteit suikerbeet naamlik tropiese suikerbeet. Hierdie beetvariëteit is in staat om in tropiese en sub-tropiese klimate te groei en gebruik heelwat minder water as suikerriet onder dieselfde omstandighede. Die hoofdoel van hierdie studie was om die potensiaal van tropiese suikerbeet vir die produksie van etanol te bepaal. Die uitwerking van die verdunningsverhouding, pH, giskonsentrasie en die byvoeging van stikstofaanvullings op die etanolopbrengs was fokusareas van die studie. ‘n Maksimum etanolopbrengs van 0.47 $g \cdot g^{-1}$, wat gelykstaande is aan ‘n omskakelingseffektiwiteit van 92%, is behaal sonder die byvoeging van enige addisionele water tot die beetsap. Hierdie opbrengs het gepaard gegaan met ‘n gliserolopbrengs van 0.08 $g \cdot g^{-1}$. Dit is bevind dat die optimale verdunningsverhouding 1:4 was, wat ‘n maksimum etanolopbrengs van 0.48 $g \cdot g^{-1}$ en ‘n omskakelingseffektiwiteit van 94%, sowel as ‘n gliserol opbrengs van 0.07$g \cdot g^{-1}$ gelewer het. ‘n Etanolopbrengs van 0.48 $g \cdot g^{-1}$ en ‘n omskakelingseffektiwiteit van 94% is behaal na vier ure se fermentasie deur gebruik te maak van ‘n giskonsentrasie van 5 $g \cdot L^{-1}$. Stikstofaanvullings soos ureum, peptoon, gis-ekstrak en ammoniumsulfaat is gedurende fermentasie bygevoeg. Die etanolopbrengs was positief beïnvloed deur die toevoeging van stikstof aanvullings by die fermentasie media. Die toevoeging van ureum tot die fermentasiemedia het ‘n maksimum etanolopbrengs van 0.47 $g \cdot g^{-1}$ en ‘n omskakelingseffektiwiteit van 92% gelewer. Die hoeveelheid gliserol wat geproduseer is, het afgeneem van 0.15 $g \cdot g^{-1}$ tot 0.08 $g \cdot g^{-1}$ met die toevoeging van stikstofaanvullings.

Weens ammoniumsulfaat se eenvoudige struktuur wat die direkte opname deur selle bevoordeel, was dit die voorkeur stikstofbron. ‘n Maksimum etanolopbrengs van 0.45 $g \cdot g^{-1}$ en ‘n omskakelingseffektiwiteit van 88% is behaal met die toevoeging van ammoniumsulfaat gelykstaande aan 750 mg $N \cdot L^{-1}$. Geen noemenswaardige verandering in etanolopbrengste is waargeneem met die aanpassing van die pH voor fermentasie nie. ‘n Maksimum etanolopbrengs van 0.45 $g \cdot g^{-1}$ is behaal vir alle pH waardes wat ondersoek is. Dit is dus bevind dat die natuurlike pH van die sap of ‘n pH van tussen 4 en 5.5 gebruik kan word. Die pH-aanpassings is dus gedoen slegs om die moontlikheid van kontaminasie te verminder. Die optimale fermentasie kondisies was pH 4, giskonsentrasie van 5 $g.L^{-1}$ en ‘n ammoniumsulfaat konsentrasie van 750 mg $N.L^{-1}$. ‘n Maksimum etanolopbrengs van 0.45 $g.g^{-1}$ was bereik onder hierdie kondisies. Die gebruik van tropiese suikerbeet met ‘n suikerinhoud van ongeveer 21.8% ($w \cdot w^{-1}$) is dus goeie voermateriaal vir die produksie van etanol in Suid-Afrika, soos getoon deur die bevindings.

Sleutelwoorde: Alternatiewe energie bron, tropiese suikerbeet, fermentasie, etanol, gliserol

Declaration

I, Janine Ellen Brandling, hereby declare that I am the sole author of the dissertation entitled Production of ethanol from tropical sugar beet.

Janine Ellen Brandling

December 2010

Acknowledgements

‘*The most exciting phrase to hear in science, the one that heralds the most discoveries, is not “Eureka!” (I found it!) but “ That’s funny….” Issac Asimov.*

‘*Men love to wonder, and that is the seed of science’ Ralph Waldo Emerson.*

I would like to thank my supervisor, Prof. Sanette Marx, for allowing me this opportunity to further my studies, as well as all her guidance, support, advice and leadership. I would also like to thank Dr Percy van der Gryp for all his support, advice and recommendations.

I would like to acknowledge the financial assistance of SANERI with regard to this work.

To all my fellow master students and staff of the School of Chemical and Minerals Engineering thank you for all the support and recommendations.

To my parents, Eric and Ina Brandling, thank you for all your love and encouragement and for always believing in me.

To my sisters, Chanel and Tarryn, thank you for all your love and motivation

And finally to my friend, Lia, thank you for all your support and inspiration and for making me laugh when life gets tough

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Chapter 1

General Introduction

Overview

This chapter is divided into three sections. Section 1.1 gives a background and motivation for this study. The objectives of this study are given in section 1.2 and section 1.3 outlines the scope of this investigation.

1.1 Background and motivation

Fossil fuels provide 80% of the primary energy needed worldwide and the combustion of fossil fuels accounts for 73% of worldwide carbon dioxide emissions (Nigam and Singh, 2011; Balat *et al*., 2008). The progressive depletion of fossil fuel resources, increasing energy demand and concern over the greenhouse gas emissions have increased the research and development of alternative and renewable energy sources (Nigam and Singh, 2011).

Transportation plays a major role in the economic activity of South Africa and transport costs constitute approximately 20% of the gross domestic product (Singh, 2006). Globally, transportation accounts for 30% of the energy demand and is responsible for 21% of global greenhouse gas emissions (Markevičius *et al.,* 2010). Currently there are 700 million motor vehicles on the roads worldwide and this is set to increase to 1.3 billion by 2030 and 2 billion by 2050, with most of the increase coming from developing countries (Balat and Balat, 2009).

South Africa has an estimated oil reserve of 16 million barrels which is not enough to meet the country's needs, and thus South Africa is reliant on oil imports (Wabiri and Amusa, 2010) and a proven coal reserve of 30 408 million tons of coal (BP statistical energy survey, 2010). Biofuels such as a bioethanol are seen as potential alternatives to petroleum fuels. Biofuels account for 1.5% of the transport fuel demand and according to the International Energy Agency the use of biofuels will rise to 5% by 2030 (IEA, 2008).

The benefits associated with biofuel use are a reduced reliance on foreign oil imports, which can lead to long-term energy security, economic growth in rural areas such as job creation and providing an additional income stream for farmers and environmental benefits such as reduced greenhouse gas emissions (Chakauya *et al.,* 2009). The challenges of biofuels are the lack of storage and collection of feedstocks, food fuel competition, technology cost and limitations and lack of governance and clear policies into biofuel use (Nigam and Singh, 2010).

Bioethanol, which is a biofuel seen as an alternative to petroleum fuels, has a long history as a transportation fuel. It had been used as early as 1894 in internal combustion engines in Germany and France. Its use was widespread until after World War II when it became too expensive to produce and cheaper petroleum fuels became available. The oil crisis of the 1970s prompted a renewed interest in its use. Bioethanol is an oxygenated fuel which means its combustion is cleaner and more efficient, has a higher octane number, broader flammability limit, higher flame speeds and higher heats of vaporization compared to petrol-based fuels. The disadvantages of bioethanol use are that it has a lower energy density compared to gasoline, it is corrosive, has a low flame luminosity and low vapour pressure and it is toxic to the ecosystem (Balat *et al.,* 2008).

Bioethanol is produced from feedstocks that contain sugars or materials that can be converted into sugars such as starch or cellulose. Bioethanol feedstocks are classified into three types: 1) sucrose-containing crops such as sugar cane, sugar beet and sweet sorghum, 2) starchy materials such as maize, cassava and wheat, 3) lignocellulosic materials such as wood, straw and agricultural waste (Balat *et al.,* 2008). About 60% of the global ethanol production comes from sugar cane and 40% from other crops (Balat *et al.,* 2008). The United States and Brazil are the top producers of bioethanol, using maize and sugar cane respectively and account for 70% of the world's production, while South Africa only produces about 1% of the world's ethanol (Balat *et al.,* 2008).

The biofuel strategy of South Africa proposes a 2% market penetration of biofuels by 2013 and states that bioethanol will be produced from sugar cane and sugar beet and excludes the use of maize due to food security concerns (Department of Energy, 2007). Sugar cane is a water intensive crop and as South Africa is already a water-stressed country the cultivation of sugar cane will be limited to certain areas of the country. Sugar beet, however, has a higher tolerance to a wide range of climatic variations, requires 30-40% less water and fertilizer compared to sugar cane and has a similar sugar yield as sugar cane (Chakauya *et al., 2009*).

A variety, known as tropical sugar beet, that is able to grow in tropical and subtropical areas has been developed and is undergoing trials and has the potential to be an efficient feedstock for ethanol production. There has been some investigation into the production of ethanol from sugar beets and their processing products with great success (Pavlečić *et al.,* 2010, Dodić *et al.,* 2009, Ranković *et al.,* 2009, Hinková and Bubnik, 2001, Ogbonna *et al.,*2001, Roukas, 1996, El-Refai *et al.,*1992 and Zayed and Foley, 1987). Table 1.1 presents previous work done on sugar beets.

Table 1.1 Previous work done on the production of ethanol from sugar beets and their processing products

Substrate	Fermentation conditions	Observation	Reference
Intermediate processing products (thin and thick juice and molasses)	14- 16% ($g.g^{-1}$) initial sugar, 28˚C , pH 5, 10 $g.L^{-1}$ yeast concentration	An ethanol concentration of 59.89 $g.L^{-1}$ was achieved with a production efficiency of 78.85%	Pavlečić *et al.,* (2010)
Thick juice	200 $g.L^{-1}$ initial sugar concentration, 30 ˚C, pH 5 and 10 $g.L^{-1}$ yeast concentration	An ethanol concentration of 12 % ($v.v^{-1}$) was achieved	Dodic *et al.,(*2009)
Intermediate processing products (thin and thick juice and molasses)	13 $g.L^{-1}$ initial sugar concentration, 30 ˚C, 10 $g.L^{-1}$ yeast concentration	An ethanol yield of 0.485 – 0.494 $g.g^{-1}$ was achieved	Ranković *et al.,* (2009)
Raw juice	16- 20 % ($w.w^{-1}$) initial sugar concentration 30 ˚C, pH 5	A conversion efficiency of 94.4 % was achieved	Hinková and Bubnik , (2001)
Raw juice	16.5% ($w.w^{-1}$) initial sugar concentration, 30 ˚C, pH 4.5	An ethanol yield of 0.40 $g.g^{-1}$ was achieved	Ogbonna *et al.,*(2001)

Table 1.1 Previous work done on the production of ethanol from sugar beets and their processing products

Substrate	Fermentation conditions	Observation	Reference
Molasses	250 $g.L^{-1}$ initial sugar concentration, 30 ˚C, pH 4.5	An ethanol yield of 53 $g.L^{-1}$ was achieved	Roukas (1996)
Molasses	200 $g.L^{-1}$ initial sugar concentration , 30 ˚C, pH 5, Urea concentration 1.08 $g.L^{-1}$	An ethanol yield of 10%($v.v^{-1}$) was achieved	El-Refai *et al.* (1992)
Molasses	20% ($w.w^{-1}$) initial sugar concentration , 30 ˚C, pH 4.5, Urea concentration 1.2 $g.L^{-1}$	An ethanol yield of 10% ($v.v^{-1}$) was achieved	Zayed and Foley (1987)

1.2 Objectives

The main objective of this study was to investigate the potential of using tropical sugar beet for ethanol production. The study focused on the effects of 1) dilution ratio, 2) pH, 3) yeast concentration and 4) the addition of nitrogen supplement on the ethanol yield.

1.3 Scope of investigation

- Chapter 2 is an overview of the literature on the ethanol production process using sugar beet.
- Chapter 3 discusses the experimental procedures used in this study. A schematic representation of the experimental scope of this study is presented in Figure 1.1.

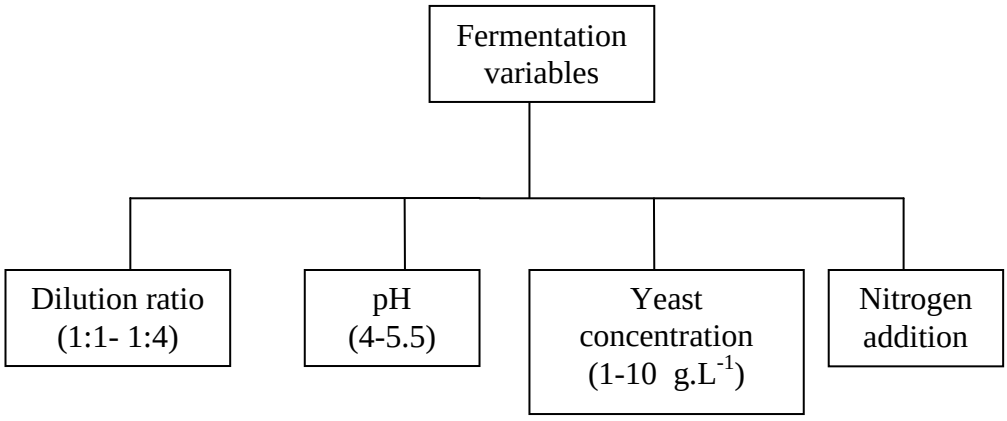

Figure 1.1 A schematic representation of the experimental scope in this study

- Chapter 4 presents and discusses the data obtained from this study.
- Chapter 5 discusses the conclusions and recommendations arising from this study.

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CHAPTER 2

Background and Literature survey

Overview

In this chapter, the terminology, principles and data required to understand this study are introduced. A general overview of sugar beet and tropical sugar beet is given in section 2.1 and 2.2, respectively. In these sections the cultivation, harvesting and composition of sugar beet and tropical sugar beet are discussed. In section 2.3 the terms and principles of ethanol production are discussed as research relevant to this study is evaluated (section 2.3.2). The microorganisms used in fermentation are discussed in section 2.4.

2.1 Sugar beet

2.1.1 General Information

Sugar beet (*Beta vulgaris ssp.vulgaris*) is a biennial sugar producing tuber crop mainly grown in moderately cold climates. It is a halophyte belonging to the *Chenopodiaceae* family, which originates from areas around the Mediterranean (Milford, 2006). During the first year of growth the beet stores sucrose in the root and during the second year the root shrinks in size and flowers and seeds are produced (Asadi, 2007). Figure 2.1 shows a typical sugar beet tuber.

Figure 2.1: *Beta vulgaris ssp.vulgaris* (Sugar beet)

It was a relatively unknown crop in Europe in the 17th century, but with the Napoleonic wars restricting the import of cane sugar to Europe, alternate sources of sugar needed to be investigated (Francis, 2006). Two scientists, by name of Margraff and Achard, developed the process whereby sucrose could be extracted from the sugar beet and since then sugar beet has been selectively bred for sugar production (Francis, 2006).

Sugar beet is grown in 50 countries and in 2007 the top producing country was France with a production of 33 million tons (FAOSTAT, 2010). Worldwide, sugar beet accounts for 30% of sugar production (Draycott, 2006).South Africa does not grow sugar beet on a commercial scale because the South African sugar industry is based on sugar cane as primary feedstock. There are approximately 38 200 cane growers farming predominately in KwaZulu-Natal, Mpumalanga and the Eastern Cape. The industry produces an estimated 2.3 million tons of sugar per season (SASA, 2010). Sugar beet has been successfully grown in trials in the Eastern Cape of South Africa (Tyrer, 2006).

2.1.2 Cultivation

Sugar beet is mainly planted in the spring and harvested in early winter. It grows well on sandy loam, clay loam and peaty loam as these soils hold water well. Sugar beet is sensitive to soil compaction so the soil needs to be carefully prepared for cultivation. Sugar beet plants are very vulnerable in the first six to eight weeks of cultivation, but once the root has been established the plant is quite hardy (Asadi, 2007).

Sugar beet has a long tap root that can reach up to two meters and is able to extract nutrients and water from a considerable depth enabling it to use 30-40% less water than sugar cane (Balat *et al.,* 2008). Fertilizers rich in nitrogen, phosphorous and potassium need to be added during cultivation for efficient sugar beet production. Sugar beet is also susceptible to diseases and therefore pesticides need to be used during cultivation (Asadi, 2007).

Sugar beet requires about 1 400 hours of sunshine during its growth period. This is important because sucrose is synthesized in the leaves by photosynthesis before it is moved to the root (Asadi, 2007).

2.1.3 Harvesting and processing

Sugar beet is harvested when the leaves start to yellow and the brix reading of the root is 15-18%. The beets are harvested by digging them out of the ground and, therefore, contain more dirt than sugarcane and need to be cleaned before processing takes place (Asadi, 2007). The process of sugar and/or ethanol production is presented in Figure 2.2 (Krajnc and Glavic, 2009).

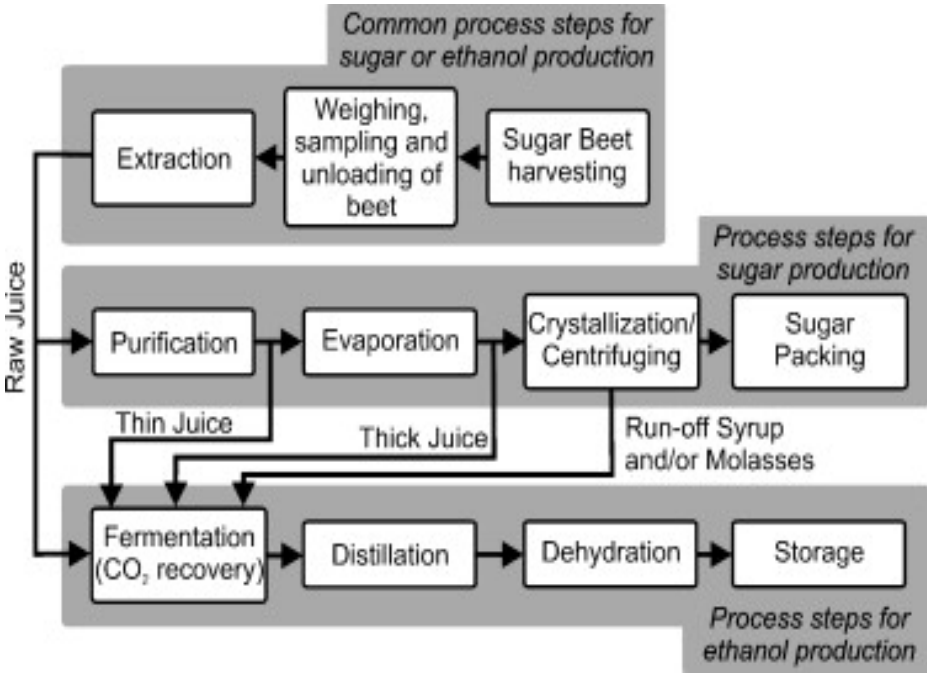

Figure 2.2: Basic process flowsheet for sugar and /or ethanol production (Krajnc and Glavic, 2009)

In order to extract the sugar, the beet is cut up into small slices, known as cossettes, and hot water is added to diffuse the sucrose out of the cell in a diffuser. The resultant pulp is pressed to recover as much of the juice as possible and then dried and used as animal feed (Krajnc and Glavic, 2009).

For sugar production the raw juice is purified by adding lime to precipitate the impurities and then carbon dioxide is added to convert the lime to calcium carbonate which is then filtered off. The resultant filtrate, known as thin juice, has a sugar concentration of 12-14 wt%; this can be increased to 65-70 wt% by evaporation. The end product, known as thick juice, is then crystallized to form sugar (Kranjc and Glavic, 2009). All the products of sugar beet processing such as the raw, thin and thick juice, as well as the molasses can be used for ethanol production.

2.1.4 Sugar beet yield and composition

Sugar beet produces a root of between 0.5 to 2kg where the majority of the sugar is stored. The sugar beet juice consists mainly of sucrose (15 to 20 wt%), raffinose (0.2% to 0.5 wt%), glucose and fructose (0.05% to 0.1 wt%) and planteose, stachyose and verbascose (Asadi, 2007). The sugar concentration depends on the variety and growth conditions and the average yield of sugar beet is 50-60 tons per hectare (Asadi, 2007). In Table 2.1 the chemical composition of sugar cane and sugar beet juice is given.

Table 2.1 presents a good comparison between the world's two major sugar producing crops. From Table 2.1 it can be seen that the main component of the juice of both crops is sucrose with sugar beet containing more than sugar cane, making it an ideal feedstock for ethanol production. Sugar beet already contains nitrogen components and thus additional nitrogen sources do not need to be added during the fermentation step.

Table 2.1: Composition of sugar cane and sugar beet juice (Drapcho *et al.,* 2008)

Components	**Sugar cane juice (g/100g)**	**Sugar beet juice (g/100g)**
Solids	13.7	17.3
Sucrose	12	16.5
Raffinose		0.07
Monosaccharides	0.63	0.15
Polysaccharides	0.028	0.019
Lactate	0.016	
Acetate	0.033	
Sulphate	0.039	0.02
Phosphate	0.033	0.047
Nitrate		0.015
Nitrite		0.005
Aconitate	0.09	
K	0.11	0.125
Na	0.005	0.015
Cl		0.003
Ca	0.04	
Mg	0.028	
Total –N		0.105
Betaine-N		0.046
Amino acid-N		0.026
Ammonia-N		0.006
Amide-N		0.011

2.1.5 Sugar beet pulp

Sugar beet pulp is a by-product of the sugar industry and consists of the plant fiber after sucrose has been extracted. On average about 63.5kg (dry weight) of pulp is produced from one ton of sugar beets. The pulp is generally used as animal feed but due to its low lignin content it is a potential feedstock for bioethanol production (Foster *et al.,* 2001).

Sugar beet pulp consists of 20-24 wt% cellulose, 25-36 wt% hemicellulose of which arabinose is the main sugar present, 20-25 wt% pectin, 1-2 wt% lignin and 7-8 wt% protein (Foster *et al.,* 2001). Table 2.2 shows the composition of sugar beet pulp compared to other lignocellulosic feedstocks.

Table 2.2: Selected lignocellulosic biomass compositions (% dry weight) (Doran-Peterson *et al.,* 2008).

	Wood		**Grass**		**Agricultural residues**		
Feedstock	**Hardwood (poplar)**	**Softwood (pine)**	**Switch-Grass**	**Bermuda grass**	**Sugar beet pulp**	**Wheat Straw**	**Corn stover**
Cellulose	44.7	44.6	32.0	32.4	24.0	45.0	37.4
Hemicellulose	18.6	21.9	25.2	25.1	29.2	25.7	27.6
Xylan 5C	14.6	6.3	21.1	19.4	2.0	20.0	21.1
Arabinan 5C	0.8	1.6	2.8	4.6	21.0	3.5	2.9
Mannan 6C	2.2	11.4	0.3	ND	1.1	0.0	1.6
Galactan 6C	1.0	2.6	1.0	1.1	5.1	2.2	2.0
Lignin	26.4	27.7	18.1	20.3	2.0	18.0	20.4
Pectin		ND	ND	ND	24.0	ND	1.1

Table 2.2 shows that sugar beet pulp has a much lower cellulose content compared to other lignocellulosic materials. Therefore, the amount of fermentable sugars that can be released will probably be much lower compared to the other lignocellulose materials. Due to its low lignin content the release of the sugars is much simpler and less expensive compared to the other crops. It has been suggested that sugar beet pulp could be used in the food industry due to its high concentration of pectin, which has been found to have prebiotic properties (Martinez *et al.,* 2009).

As a potential feedstock for ethanol production sugar beet pulp is not as attractive as the other lignocellulosic materials but it can be used in other applications. It has been found that sugar beet pulp has high levels of galaucturonic acid, arabinose and rhamnose which can be released by enzymatic hydrolysis. The galautronic acid can be converted to ascorbic acid by enzymes, while the arabinose has the potential to treat Parkinson's disease the rhamnose can be converted to aromas such as furanol used in the food industry (Bonnin *et al.,* 2000).

2.2 Tropical sugar beet

Traditionally sugar beet is grown in the colder temperate regions, but it was observed that the crop is quite adaptable. Through research and development Syngenta developed a variety of sugar beet that could be grown in tropical and subtropical areas.

Tropical sugar beet has been successfully introduced in India and currently there are trials being conducted in other tropical countries such as China, Australia, Kenya, South Africa, Brazil and United States (Syngenta, 2007).

2.2.1 Characteristics of tropical sugar beet

Tropical sugar beet is a promising alternative crop for bioethanol production. It can be grown in relatively dry areas, can be harvested after 5-6 months and is an excellent rotational crop. Due to its short growth period the farmer can plant a second crop, thereby increasing agricultural output and the farmer's income (Tamil Nadu Agricultural University, 2009).The crop requires an optimum temperature of 20 to 25˚C for germination, 30 to 35˚C for growth and 25 to 35˚C for sugar accumulation. It is able to grow on all types of soil which are well drained as it is sensitive to water stagnation. The optimum soil pH is from 6.5 to 8.0 but it can tolerate saline and alkaline conditions (Tamil Nadu Agricultural University, 2009).

In Table 2.3 the characteristics of tropical sugar beet are compared to the two other major sugar producing crops, i.e. sugar cane and sweet sorghum.

Table 2.3: Comparison of Sugarcane, Tropical Sugar Beet and Sweet Sorghum (Prasad *et al.,* 2007)

	Sugar cane	**Tropical sugar beet**	**Sweet sorghum**
Crop duration	12-13 months	5-6 months	3.5 months
Growing season	One season	Through the year	All season
Soil requirement	Loamy soil	Sandy loam; also tolerates alkalinity	All types of drained soil
Water management	Requires water throughout the year	40-60% less water compared to sugarcane	Can be grown as rain-fed crop
Crop management	Requires good management	Greater fertilizer requirement; moderate management	Less fertilizer needed; easy management
Yield per acre	25-30 tons	30-40 tons	20-25 tons
Sugar content	8-12%	15-16%	8-10%
Sugar yield	2.5 -4.8 tons/acre	4.5-7.2 tons/acre	2-3 tons/acre
Ethanol production directly from juice	1700-2700 L/acre	2800-4100 L/acre	1140-1640 L/acre
Harvesting	Difficult and laborious	Very simple	Very simple

Table 2.3 shows that tropical sugar beet can potentially be a very efficient feedstock for ethanol production. It produces more ethanol compared to the other two crops and can be grown throughout the year. It can also be exclusively grown for bioethanol production, thereby not interfering with food production. Tropical sugar beet is a very efficient water user and utilizes 40-60% less water compared to sugar cane (Prasad *et al.,* 2007). The average yield is similar to sugarcane and it has higher sugar content than sugar cane (Prasad *et al.,* 2007).

2.3 Ethanol production

2.3.1 Introduction

Ethanol is produced from biological feedstocks that contain sugar or materials that can be converted to sugar such as starch and cellulose. The way in which ethanol is produced, depends on the raw material used. The general process of ethanol production is discussed in section 2.3.2 and the production of ethanol from sugar beet is discussed in section 2.3.3

2.3.2 General process

The feedstocks for ethanol production are classified as: sucrose-containing materials (sugar cane, sugar beet and sweet sorghum), starchy materials (maize, wheat and cassava) and lignocellulosic materials such as agricultural residues, forestry residues and energy crops (Balat *et al.,* 2008). A general scheme for ethanol production is presented in figure 2.3

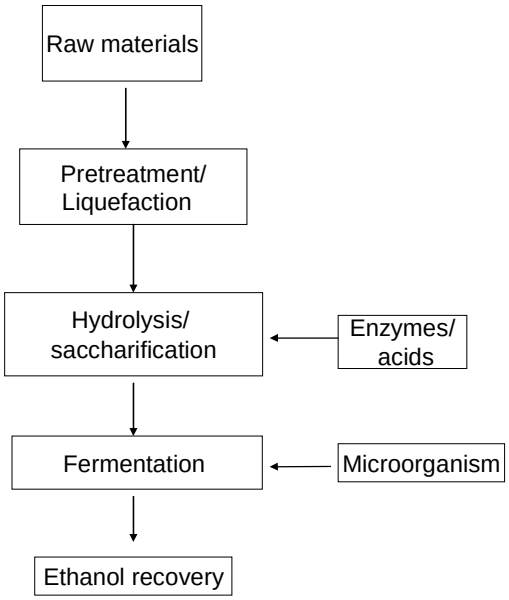

Figure 2.3: A general scheme for ethanol production (adapted from Taherzadeh and Karimi, 2008).

The production of ethanol from sucrose containing raw materials is easier compared to starch and lignocelluosic raw materials as pretreatment and hydrolysis are not required, because the sucrose is hydrolysed by the yeast itself (Demirbas, 2009). Starch, which is a polymer of glucose, cannot be fermented directly by the microorganisms and, therefore, needs to be converted to fermentable sugars. This is achieved in two stages, liquefaction and saccharification. During liquefaction the starch polymer is broken down into dextrins and in the saccharification step the dextrins are converted to glucose (Taherzadeh and Karimi, 2008).

Lignocellulosic materials such as sugar beet pulp consist of a mixture of cellulose and hemicellose which also need to be converted to fermentable sugars. Therefore, these materials undergo hydrolysis prior to fermentation (Taherzadeh and Karimi, 2008). After hydrolysis the sugars are converted to alcohol and other by-products by the process known as fermentation. Fermentation can be expressed by the following equation:

$$2C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 + \text{By-products}$$

In theory, 1 gram of glucose can produce 0.51g of ethanol and 0.48g of CO_2. In practice, however, this yield is not achieved as not all of the glucose is converted to ethanol. Some is used for cell maintenance, cell mass synthesis and the production of by-products such as acetic acid, glycerol, formic acid, lactic acid, sorbitol and levan (Drapcho *et al.,* 2008). The average ethanol yield obtained using sugar beet products is $0.48 g.g^{-1}$ (Rankovic *et al.,* 2009).

2.3.3 Ethanol Production from sugar beet

Sugar beet is not as widely used for ethanol production as sugarcane. Therefore the details of the commercial processes are not as readily available. Only 29% of the ethanol produced in Europe is from sugar beet even though the ethanol yield per hectare is more than that of wheat. The potential ethanol yield from sugar beet is 5 145 L/hectare (Balat and Balat, 2009).

Sugar beet and the intermediate products produced during the production of sugar can be used as materials for ethanol production. These materials do not require hydrolysis as the sugar content is mainly sucrose which is easily utilized and fermented *by Saccharomyces cerevisiae* and various other yeasts. Beet molasses is a commonly used feedstock and is usually diluted to the required sugar concentration and pH for ethanol production. (Dodić *et al.,* 2009).

Through a survey of the available literature (Amin and Khalar, 1992; Beckers *et al.,*1999; Dodic *et al.,* 2009; El-Refai *et al.,*1992; Gőksungur and Zorlu, 2001; Hinková and Bubnik, 2001; Ogbonna *et al.,*2001; Ranković *et al.,* 2009; Roukas, 1996 and Zayed and Foley, 1987) it is seen that the production of ethanol from tropical sugar beet has so far not been reported; however, there have been studies into the use of sugar beet for ethanol production. These studies investigated the influence of different fermentation parameters on the ethanol yield. The parameters investigated, include initial sugar concentration, temperature, pH and addition of nutrients and biomass form. There has also been investigation into the use of different strains and forms of the yeast such as commercial baker's yeast, beverage yeasts and wine yeasts as well as other microorganisms.

Dodic *et al.* (2009) investigated the use of thick juice for ethanol production and investigated the effect of initial sugar concentration on the ethanol yield. The disadvantages of using these intermediate products such as thin and thick juice and molasses are that it is difficult to store and it is prone to contamination.

The effect of sugar concentration on the ethanol yield was examined by dilution of the thick juice to give a total sugar concentration of 5, 10, 15, 20 and 25% ($w.w^{-1}$) and fermentation by commercial baker's yeast for 72 hours at 30˚C.

During fermentation the concentration of sugars present in the thick juice and molasses decreased over time, indicating that biomass and ethanol were being formed. The ethanol concentration increased as the available sugar concentration increased, but the fermentation time was also increased. In all substrates it was seen that if the sugar concentration was increased from 20 to 25% ($w.w^{-1}$) the ethanol concentration started to decrease and this showed that the initial sugar concentration did have an effect on the ethanol yield. This study (Dodic *et al*., 2009) showed that intermediate products such as thick juice could be used for ethanol production and was just as efficient as molasses.

Similar results were seen by El-Refai *et al.* (1992) and Zayed and Foley (1987), who both investigated the influence of fermentation parameters on the ethanol yield from sugar beet molasses. El-Refai *et al.* (1992) investigated the effect of sugar concentration, pH and the addition of nutrients such as urea and magnesium sulphate. It was observed that as the sugar concentration increased so too did the ethanol yield, but the ethanol yield decreased when the sugar concentration was increased further from 200 $g.L^{-1}$ to 250 $g.L^{-1}$ and 350 $g.L^{-1}$. At a high sugar concentration the yeast could experience osmotic pressure which led to plasmolysis and could influence the ethanol yield.

Zayed and Foley (1987) investigated the use of three different yeast strains in the production of ethanol using sugar beet molasses, as well as the effect of sugar concentration, fermentation temperature, pH and addition of nutrients. It was found that different yeast strains had a different optimum sugar concentration but as seen in Dodic *et al.* (2009) and El-Refai *et al.* (1992) the increase in sugar concentration led to an increase in ethanol concentration. The highest ethanol yield was obtained at a sugar concentration of 20.8% ($w.v^{-1}$).

Roukas (1996) and Gőksungur and Zorlu (2001) investigated the use of immobilized yeast cells in the production of ethanol from beet molasses and examined the effect of the initial sugar concentration on the yield. Roukas (1996) found that the maximum ethanol yield was obtained at a sugar concentration of 250 $g.L^{-1}$ and increasing the sugar concentration led to a decrease in ethanol yield which was also seen by Gőksungur and Zorlu (2001).

Zayed and Foley (1987) found that the optimum pH for ethanol production was 4.5 which was in contrast to El-Refai *et al.* (1992) who found it to be 5. This was perhaps because two different yeast strains were investigated, each with their own optimum. Zayed and Foley (1987) observed that when the pH was increased to 5.5 the ethanol yield decreased; this was due to the fact that the yeasts favoured the production of glycerol at a pH of 5.0 and above.

The addition of a nitrogen source, like urea, at a concentration of 1.2 $g.L^{-1}$ as well as the addition of phosphorus at 0.4 $mL.L^{-1}$, sulphur and magnesium at a concentration of 0.3 $g.L^{-1}$ substantially increased the ethanol yield. Therefore, it can be concluded that even though nitrogen is found in sugar beet molasses it is not freely available for the yeasts to utilize (Zayed and Foley, 1987). An increase in the concentrations of these nutrients resulted in a decrease in the ethanol yield. This was explained by the possible breakdown of the urea, which increased the pH to above the optimum for yeast growth.

The results of Zayed and Foley (1987) are in contrast to that of Ogbonna *et al.* (2001) who investigated the potential of producing ethanol from raw sugar beet juice. The ethanol production from a) unmodified juice, b) juice supplemented with nitrogen sources and pH adjusted to 6.5, c) juice supplemented with nitrogen sources and d) a synethic sucrose medium was compared and no significant increase to the ethanol yield was seen with the addition of nitrogen or the adjustment of the pH and it was concluded that sugar beet juice contained all the nutrients needed for cell growth and ethanol production and did not contain any inhibitory substances.

Ogbonna *et al.* (2001) showed that raw sugar beet juice could be used for ethanol production without any adjustment to the pH or supplementing with nitrogen sources and was perhaps a better substrate than molasses which needed pH adjustment and addition of vital nutrients for efficient ethanol production.

Zayed and Foley (1987) also studied the effect of the fermentation temperature on the ethanol yield and when the temperature increased from 20 to 30˚C the ethanol yield also increased, but when the temperature was increased further to 35˚C the ethanol yield started to decrease. This could be explained by the fact that at this temperature intracellular ethanol was more rapidly produced and the yeast could not transport it through the cell membrane fast enough leading to cell death.

A similar effect was seen in the investigation done by Amin and Khalar (1992) who investigated the use of *Zymomonas mobilis* in the production of ethanol from sugar beet. It was seen that when the temperature was increased from 30 to 35˚C the ethanol yield also increased. This is in contrast to study done by Zayed and Foley (1987) who found that the ethanol yield decreased at 35˚C, suggesting that *Z. mobilis* has a higher temperature tolerance compared to *S. cerevisiae*. At higher temperatures (above 35℃) the ethanol yield decreased as cell viability was decreased. The ethanol yield also decreased at temperatures below 30℃, because the production of by-products such as levan was favoured at lower temperatures.

There are different forms and strains of *Saccharomyces cerevisiae* that can be used in ethanol production. Commercial preparations of yeasts, the dried form as well as pressed blocks containing 70% moisture were used by Rankovic *et al*. (2009) to investigate the fermentation of raw, thin and thick sugar beet juice, as well as sugar beet molasses. Five different strains of the yeasts were investigated: a) a yeast for beverage production, b) two wine yeasts and c) two bakery yeasts. Rankovic *et al*. (2009) showed that all the strains investigated were able to effectively utilized 98-99% of all the sugars present in the raw

materials. Similar results were observed by Hinkova and Bubnik (2001). The ethanol yields ranged from 0.485 to 0.494 $g.g^{-1}$ with the wine yeasts giving higher ethanol yields. There was no significant difference in the ethanol yields between the different forms of the yeasts which was also seen by Dodic *et al.* (2009).

Therefore, this study (Ranković *et al.,* 2009) showed that ethanol production from raw, thin and thick juice and molasses is possible and that the best results are obtained by yeasts in the dried form. Hinková and Bubnik (2001) also investigated the use of different yeast strains for the fermentation of raw juice at different initial sugar concentrations. Hinková and Bubnik (2001) showed that sugar concentration did have an effect on the ethanol yield and the optimum concentration was determined to be 20% ($w.v^{-1}$). The dried yeasts performed better at sugar concentrations of 20% ($w.v^{-1}$) while the distillery yeast performed better at the higher sugar concentration of 25%. Therefore, each yeast strain had its own tolerance to osmotic pressure, with the distillery yeast having the highest tolerance.

Saccharomyces cerevisiae is the most commonly used microorganism for fermentation but there is growing interest in using other microorganisms such as *Zymomonas mobilis* for ethanol production. There have been some investigations into the use of *Z. mobilis* for ethanol production using sugar beet substrates. Bekers *et al*. (1999) investigated the use of *Z. mobilis* in the production of ethanol from sugar beet juice and syrup as well as the effect the addition of mineral salts and yeast extract would have on the ethanol yield. Bekers *et al*. (1999) showed that the addition of mineral salts and yeast extract was necessary as the ethanol yield of juice supplemented with these nutrients was 7.11% ($w.v^{-1}$) compared to the syrup without any supplements which only yielded 3.6% ($w.v^{-1}$).

During the processing of sugar beets the extracted juice undergoes evaporation, this thermal treatment can degrade some growth factors, which can explain the lower ethanol yield. Bekers *et al*. (1999) showed that for efficient ethanol production using sugar beet syrup, additional growth factors need to be added.

2.4 Fermentation organisms

2.4.1 Yeast

The organism most commonly used for ethanol production is *Saccharomyces cerevisiae.* This organism is able to metabolize the sugars glucose, fructose, mannose, galactose, sucrose, maltose and maltotriose (Drapcho *et al.,* 2008). Ethanol is produced via the metabolic pathway known as glycolysis, through which one molecule of glucose is metabolized to two molecules of pyruvate. Under anaerobic conditions the pyruvate is further metabolized to produce ethanol and carbon dioxide (Bai *et al.,* 2008).

Of all the sugars that *Saccharomyces cerevisiae* can metabolize, it prefers to utilize glucose and sucrose. The sucrose is hydrolyzed by the enzyme invertase which is found in between the cell membrane and cell wall and is then taken up by the cell through the sugar transporters which are controlled by a system of 20 genes (Picataggio and Zhang, 1996).

During the hydrolysis of starch the sugars maltose and maltotriose are formed, both of which can be taken up by yeasts and broken down into simpler sugars by the enzyme α-glucosidase, but *S. cerevisiae* cannot metabolize the higher polysaccharides such as dextrins (Drapcho *et al.,* 2008). One of the disadvantages of using *S. cerevisiae* is that it cannot ferment the pentose sugars such as xylose and arabinose. Therefore, it cannot be used to ferment agricultural residues such as sugar beet pulp which contains a high concentration of these sugars.

2.4.2 Bacteria

Certain bacteria are able to produce ethanol in addition to other products. The bacterium *Zymomonas mobilis* is considered an ideal organism for ethanol production. It is a Gram negative anaerobic bacterium that produces ethanol via the Entner-Doudoroff pathway (Drapcho *et al.,* 2008). It was originally discovered in fermenting sugar-rich plant saps such as palm wines and honey (Bai *et al.,* 2008).

The advantages of using *Z. mobilis* are that a yield of 97% of the theoretical value can be achieved, it produces less biomass, has higher sugar uptake and ethanol production rates and has a higher tolerance to ethanol. The disadvantage *of Z. mobilis* use is that it can only ferment glucose, sucrose and fructose, and its cell biomass is also not regarded as acceptable to be used as animal feed, which makes its disposal problematic (Bai *et al.,* 2008).

Another bacterium that is being investigated is for potential use in ethanol production is *Escherichia coli* as it is able to ferment a wide variety of sugars, has no requirements for complex growth factors and has been used in the pharmaceutical industry before. The major disadvantage of *E. coli* is that it has a narrow pH growth range and it is less hardy than yeast cultures. The ethanol yield is also lower as a number of by-products such as acetic and succinic acid are also produced during fermentation (Dien *et al.,* 2003).

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CHAPTER 3

EXPERIMENTAL

Overview

In this chapter all the experimental work done in this study will be discussed in detail. All the materials, chemicals and equipment, as well as the preparation of the feedstock and yeast, are discussed in section 3.1. The experimental setup and procedure followed, is shown in section 3.2. The composition of the residual pulp is presented in section 3.3. The analysis procedure followed, is described in section 3.4. The optimization of the fermentation parameters is discussed in section 3.5.

3.1 Materials, Chemicals and Equipment

A brief discussion on the preparation of the feedstock and yeast is presented in this section. The materials and chemicals used in this study are summarized in Table 3.1 and the information for the equipment used, is listed in Table 3.2.

Table 3.1: Information of Materials and Chemicals used in this study

Chemical	Supplier	Purity	Cas-no	Purpose
Glucose	Fluka	99 %	-	Preparation of glucose standard solution
Fructose	Associated chemical enterprises	-	-	Preparation of fructose standard solution
Sucrose	Associated chemical enterprises	-	57-50-1	Preparation of sucrose standard solution
Ethanol	Rochelle chemicals	99 %	-	Preparation of ethanol standard solution
Ammonium sulphate	Fluka	99 %	-	Nitrogen source for yeast
Peptone	Fluka	>8% total nitrogen	-	Nitrogen source for yeast
Urea	Sigma	98%	57-13-6	Nitrogen source for yeast
Yeast extract	Sigma	9-12% Nitrogen content	8013-01-2	Source of growth factors
Sodium hydroxide	Fluka	98%	-	pH adjustment
Sulphuric acid	Labchem	98%	-	pH adjustment
Microorganism				
S. cerevisiae	Anchor Yeasts, South Africa	-	-	Yeast for fermentation
Materials				
Tropical sugar beet	Agricultural Research Council	-	-	Feedstock for ethanol production

Table 3.2 Information of equipment used in this study

Equipment name	Supplier	Model	Purpose
Autoclave	D&E International	HL-341	Sterilization of equipment
Balance	Scientech	ZSP 250	Weighing of materials
Glassware	Boeco	-	Fermentation
Hammer mill	Trapp	TRF 70	Milling of dried sugar beet pulp
HPLC	Agilent systems	Model 1206 series	Analysis of fermentation samples
Kitchen juicer	Moulinex	Type 753	Extraction of juice from sugar beet
Oven dryer	Scientific	Series 2000	Drying of sugar beet pulp
pH meter	Hanna instruments	HI 0925	pH measurement
Shaking incubator	Labcon	FSIE-SPO 8-35	Fermentation

3.1.1 Feedstock

Tropical sugar beets were received from the Agricultural Research Council in Rustenburg. The tropical sugar beets were washed by hand to remove any soil residue and then chopped into smaller pieces. A kitchen juicer was used to extract the juice from the chopped sugar beets and the juice was then stored in a freezer and thawed when needed. The residual pulp was oven dried at 90˚C for 24 to 72 hours. The dried pulp was then milled and sieved using a Trapp hammer mill and a 1.5 mm screen and then stored in air tight containers.

3.1.2 Preparation of yeasts

Commercial Baker's yeast (*Saccharomyces cerevisiae*) in dried form was used in this study. The fermentation broth was used to reconstitute the yeast.

3.2 Experimental procedure

Figure 3.1 and 3.2 show the experimental procedure and apparatus used in the fermentation of tropical sugar beet. The tropical sugar beet was harvested, washed and chopped and the juice was extracted using a kitchen juicer. The remaining pulp was dried in the oven and then milled with a hammer mill. The extracted juice then underwent fermentation where fermentation variables such as dilution ratio, pH, yeast concentration and addition of a nitrogen supplement were manipulated. Samples were taken periodically and using HPLC analysis the response to this manipulation was measured.

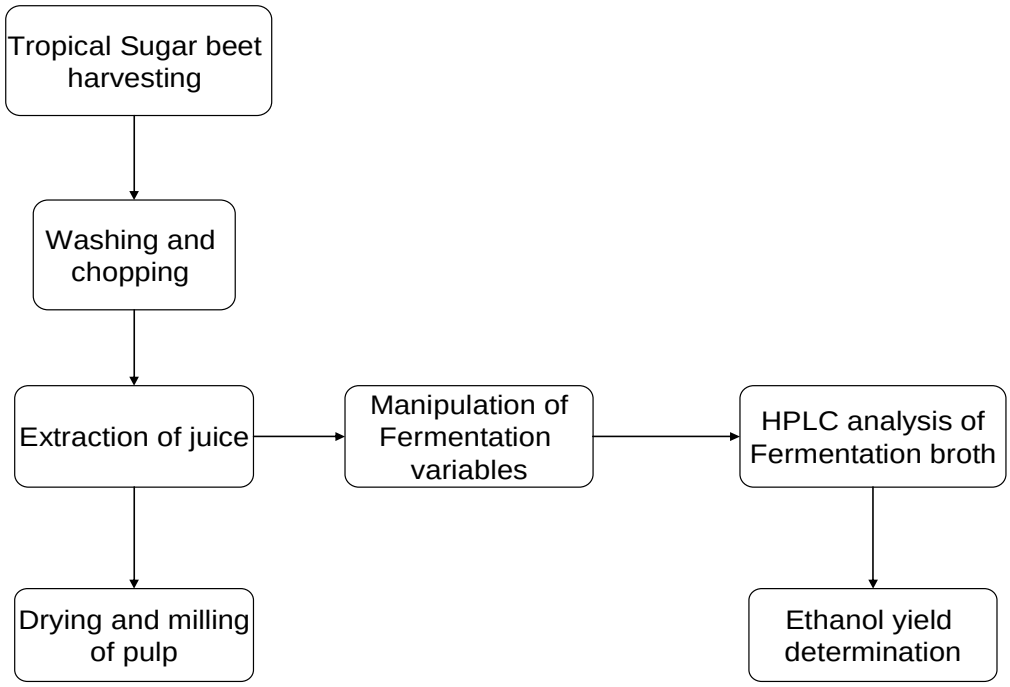

Figure 3.1: Flow diagram of experimental procedure followed in the fermentation of tropical sugar beet

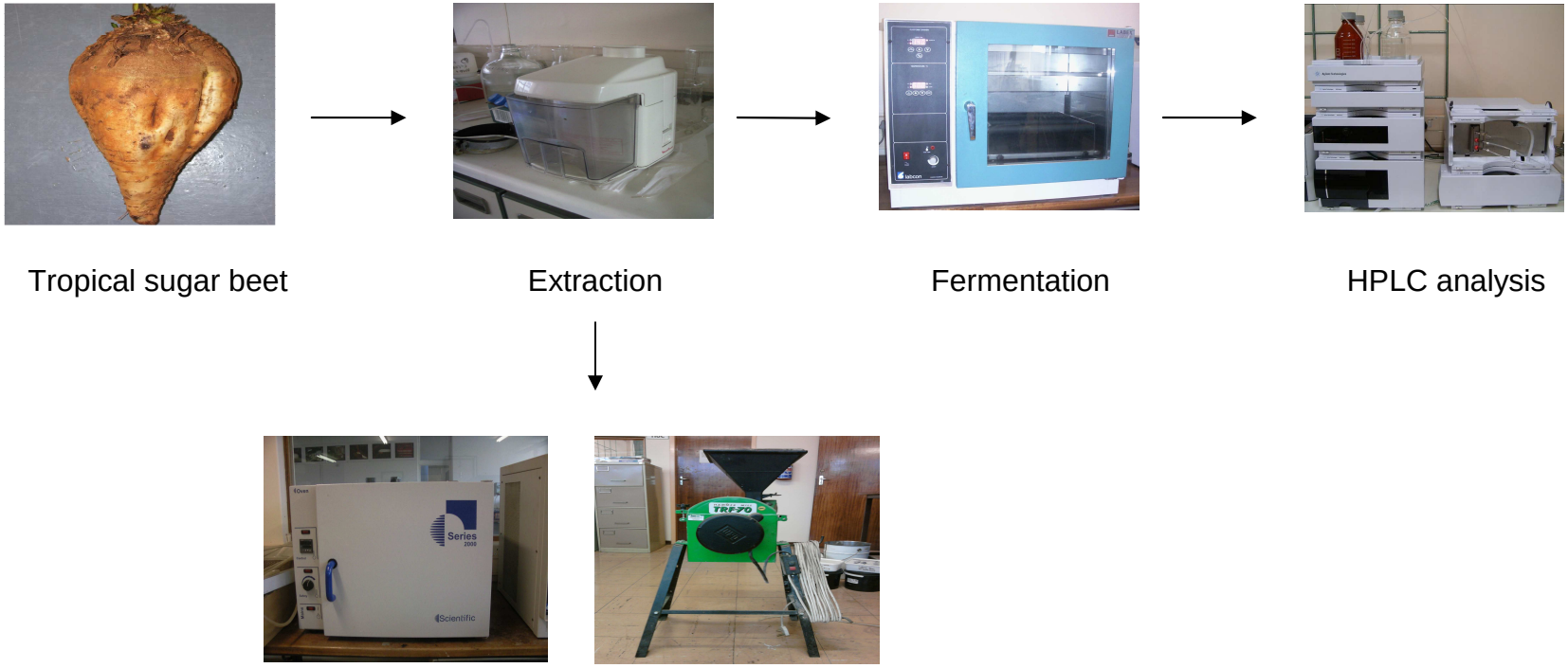

Figure 3.2 Flow diagram of experimental procedure followed showing the standard apparatus used in the fermentation of tropical sugar beet

3. 3. Analysis procedures

All samples were filtered through a Pall life sciences 0.2 µm GHP membrane micro pore syringe filter and analysed by High Performance Liquid Chromatography (HPLC). Calibration curves constructed with known concentrations of ethanol and sugar were used to quantify the amount of ethanol and different sugars present in each sample. An evaluation of the chromatographs obtained in this study showed the presence of only sucrose, fructose and glucose as sugars and only ethanol and glycerol as fermentation products. The method used to prepare and construct the calibration curves to quantify the sugars present and the products formed, is presented in Appendix A. The analysis parameters used for the HPLC analysis are presented in Table 3.3.

Table 3.3: Parameters of the HPLC columns

Parameter	Shodex SP0810	Zorbax Carbohydrate
Mobile phase	HPLC grade water	75% Acetonitrile, 25% HPLC grade water
Column temperature	75 ˚C	30 ˚C
Detector temperature	55 ˚ C	30 ˚C
Flow rate	0.75 $ml.min^{-1}$	1.4 $ml.min^{-1}$
Injection volume	5 µL	3 µL

3.4 Compositional analysis of pulp

A compositional analysis of the pulp was done by ARC-Irene analytical services. The results are presented in Table 3.4.

Table 3.4: Composition (wt %) of tropical sugar beet pulp

Component	Accreditation number	Sample number	
		1	2
Dry matter	ASM 013	96.47	98.89
Moisture	ASM 013	3.53	1.11
Ash	ASM 048	2.61	3.42
Fat (ether extraction)	ASM 044	0.17	0.27
Fiber (crude)	ASM 059	8.94	8.15
Protein	Not accredited	4.14	4.39
Cellulose	Not accredited	6.05	8.44
Hemicelluloses	Not accredited	10.12	7.25

The protein content is lower than that which was reported by Martinez *et al* (2009). The cellulose and hemicellulose content is also lower than that reported by Doran-Peterson *et al*. (2008) who found the cellulose content to be 24% (w.w.$^{-1}$) and the hemicellulose to be 29.2% (w.w^{-1}).

3.5 Fermentation experiment

The effect of manipulating fermentation variables was investigated according to the methods employed by Zayed and Foley (1987), Breisha (2010) and Harding *et al* (1984) with some modifications. Screening fermentations showed that all the sugars present in the initial raw juice were consumed after approximately 24 hours and thus all fermentation experiments described in this study was for a maximum fermentation period of 24 hours.

3.5.1 Fermentation conditions

The fermentation process was performed using a 100 mL Erlenmeyer flask with a working volume of 50 mL. All fermentations were carried out under anaerobic conditions for 24 hours at a temperature of 30 ˚C and an agitation rate of 120 rpm. Samples were taken at predetermined time intervals of 0, 2, 4, 8, 12, and 24 hours. Sugar, ethanol and glycerol yields were determined by HPLC analysis.

3.5.2 Effect of dilution ratio

The effect of the initial sugar concentration on the ethanol yield was investigated by diluting the juice with distilled water. The dilution ratios investigated were 1:1, 1:2, 1:3 and 1:4. The pH of the juice was adjusted to 4.5 prior to fermentation and used without sterilization and nutrient addition. A yeast concentration of 1 $g.L^{-1}$ was used in this experiment.

3.5.3 Effect of pH

The effect of pH on the ethanol yield was investigated, using the optimal dilution ratio as determined in section 3.5.2. The pH of the juice was adjusted to 4, 4.5, 5 and 5.5 with sulphuric acid (H_2SO_4) or sodium hydroxide (NaOH).

3.5.4 Effect of yeast concentration

The effect of yeast concentration on the ethanol yield was investigated, using the optimized dilution ratio and pH determined in section 3.5.2 and 3.5.3. Different yeast concentrations (1, 3, 5 and 10 $g.L^{-1}$) were investigated.

3.5.5 Effect of Nitrogen supplementation

The effect of nitrogen supplementation on the ethanol yield was investigated, using the optimal dilution ratio, pH and yeast concentration as determined in section 3.5.2, 3.5.3 and 3.5.4. Different nitrogen sources (urea, peptone, yeast extract and ammonium suphate ($(NH_4)_2SO_4$) were evaluated to determine the effect of the addition of a nitrogen supplement on the ethanol yield. The effect of varying the concentration of ammonium sulphate ($(NH_4)_2SO_4$) on the ethanol yield was investigated at three different concentrations, i.e. 250 mg $N.L^{-1}$; 500 mg $N.L^{-1}$ and 750 mg $N.L^{-1}$.

3.6 References

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CHAPTER 4

Results and Discussion

Overview

In this chapter the results of the investigation into the production of ethanol using tropical sugar beet, is presented. An introduction into the study is presented in section 4.1. Section 4.2 presents the results obtained from this study and concluding remarks are presented in section 4.3.

4.1. Introduction

This study investigated the potential of using tropical sugar beet as a feedstock for ethanol production. The study particularly focused on the effect of fermentation variables on the ethanol yield obtained from the fermentation of tropical sugar beet juice. The fermentation variables and their ranges that were investigated, are presented in Figure 4.1.

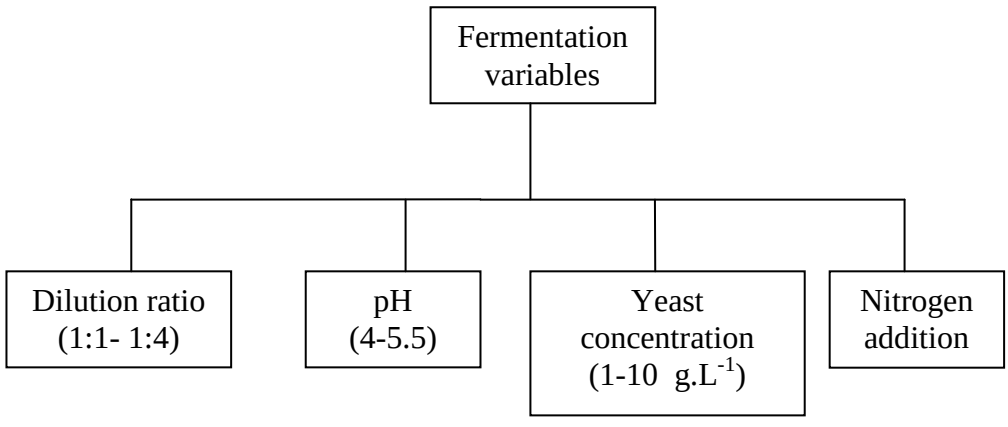

Figure 4.1: Schematic representation of the study

4.2 Fermentation results

4.2.1 Effect of dilution ratio

The effect of diluting the sugar concentration in the tropical sugar beet juice, by adding water on the ethanol yield, was investigated according to the experimental procedure outlined in section 3.5.2. The experimental error associated with dilution was determined to be 6.37% (confidence level of 95%). Detailed calculations can be found in Appendix B. The influence of dilution on the sucrose, glucose and fructose utilization is presented in Figure 4.2, 4.3 and 4.4.

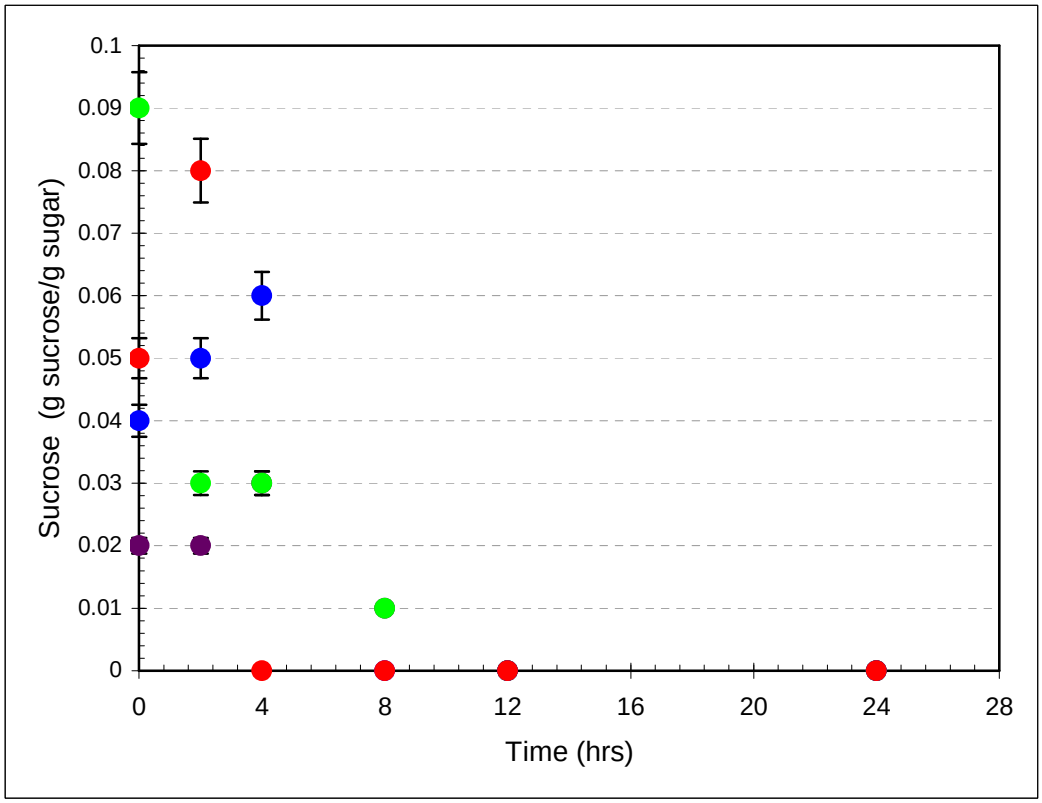

Figure 4.2 Effect of dilution on the sucrose utilization

(● No dilution, ● Dilution ratio 1:1, ● Dilution ratio 1:2, ● Dilution ratio 1:3)

(pH 4.5, yeast concentration 1.g.L^{-1})

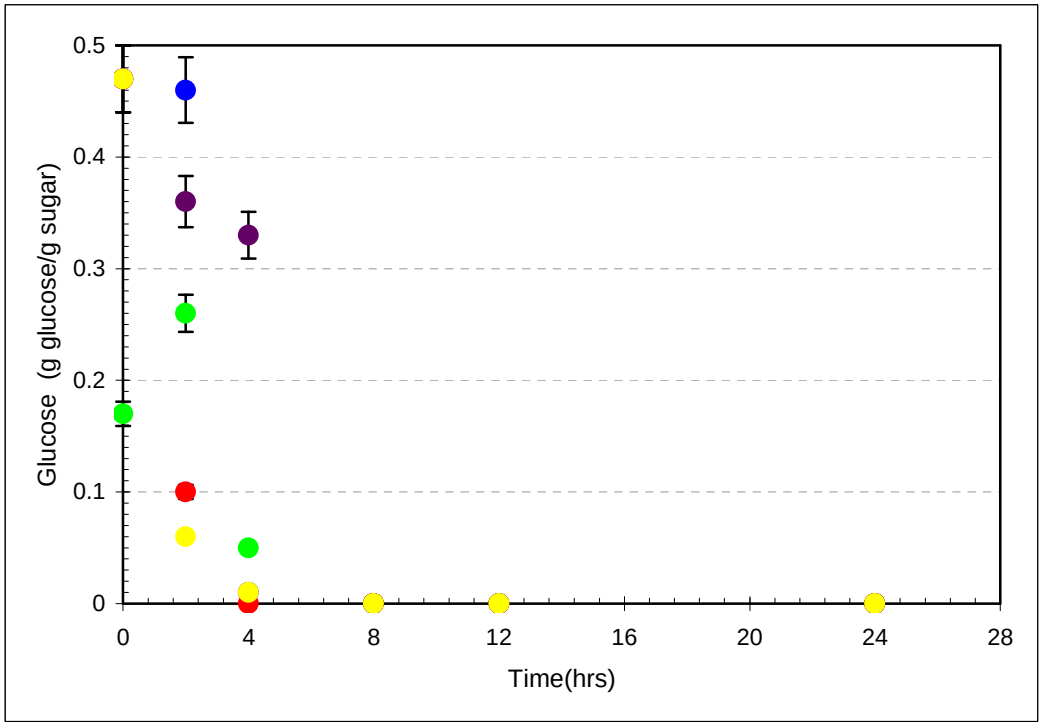

Figure 4.3 Effect of dilution on glucose utilization

(● No dilution, ● Dilution ratio 1:1, ● Dilution ratio 1:2, ● Dilution ratio 1:3

● Dilution ratio 1:4)

(pH 4.5, yeast concentration 1.g.L^{-1})

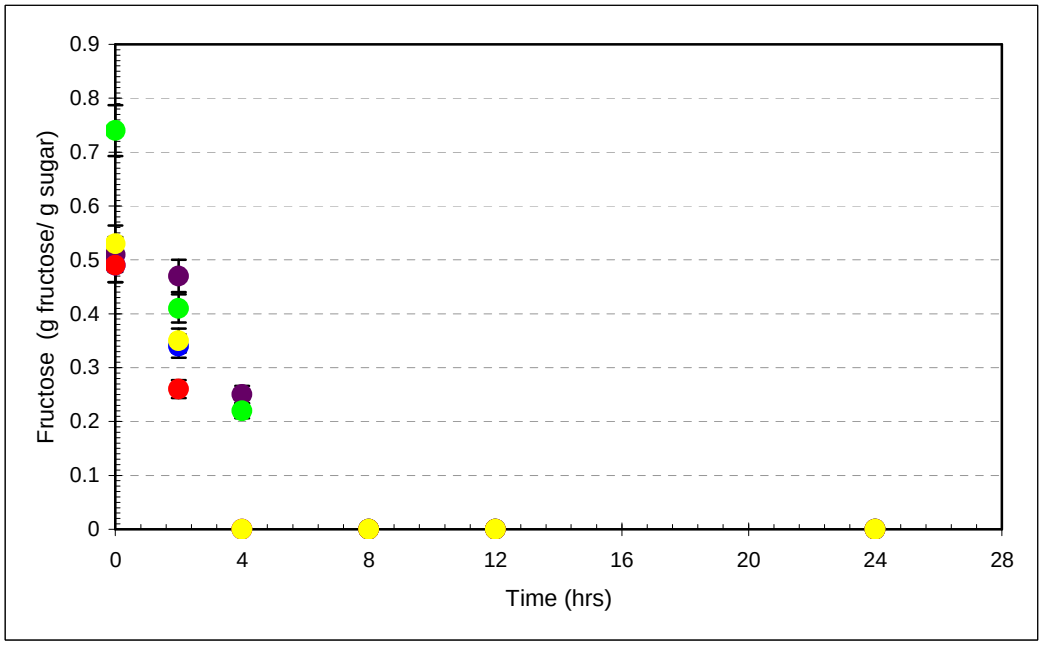

Figure 4.4 Effect of dilution on fructose utilization

(● No dilution, ● Dilution ratio 1:1, ● Dilution ratio 1:2, ● Dilution ratio 1:3

● Dilution ratio 1:4)

(pH 4.5, yeast concentration 1.g.L^{-1})

Figure 4.2, 4.3 and 4.4 show that dilution did not have any effect on the rate of sugar utilization. Figure 4.2 shows that there was very little sucrose present indicating that most of the sucrose has been hydrolyzed to glucose and fructose as seen in Figure 4.3 and 4.4. At all the dilution ratios investigated all the sugars had been consumed after 8 hours of fermentation.

The influence of dilution ratio on the ethanol yield per gram of sugar is presented in Figure 4.5 and the effect on the glycerol yield is presented in Figure 4.6.

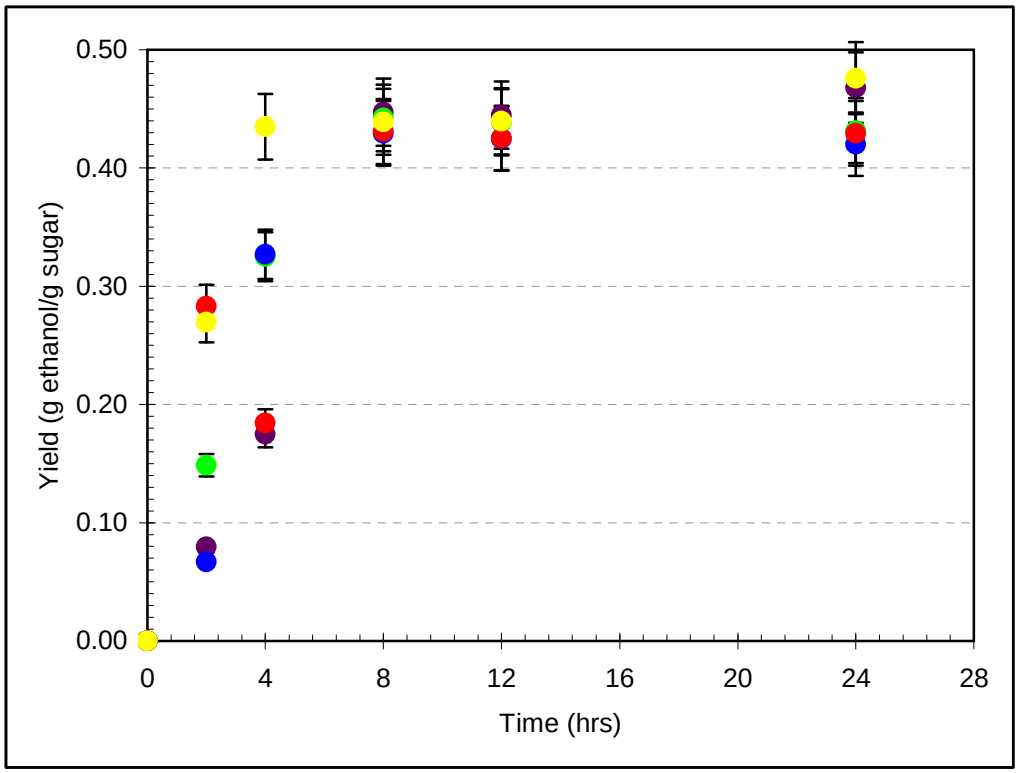

Figure 4.5: Effect of dilution on the ethanol yield

(● No dilution, ● Dilution ratio 1:1, ● Dilution ratio 1:2, ● Dilution ratio 1:3 ● Dilution ratio 1:4)

(pH 4.5, Yeast concentration 1.g.L^{-1})

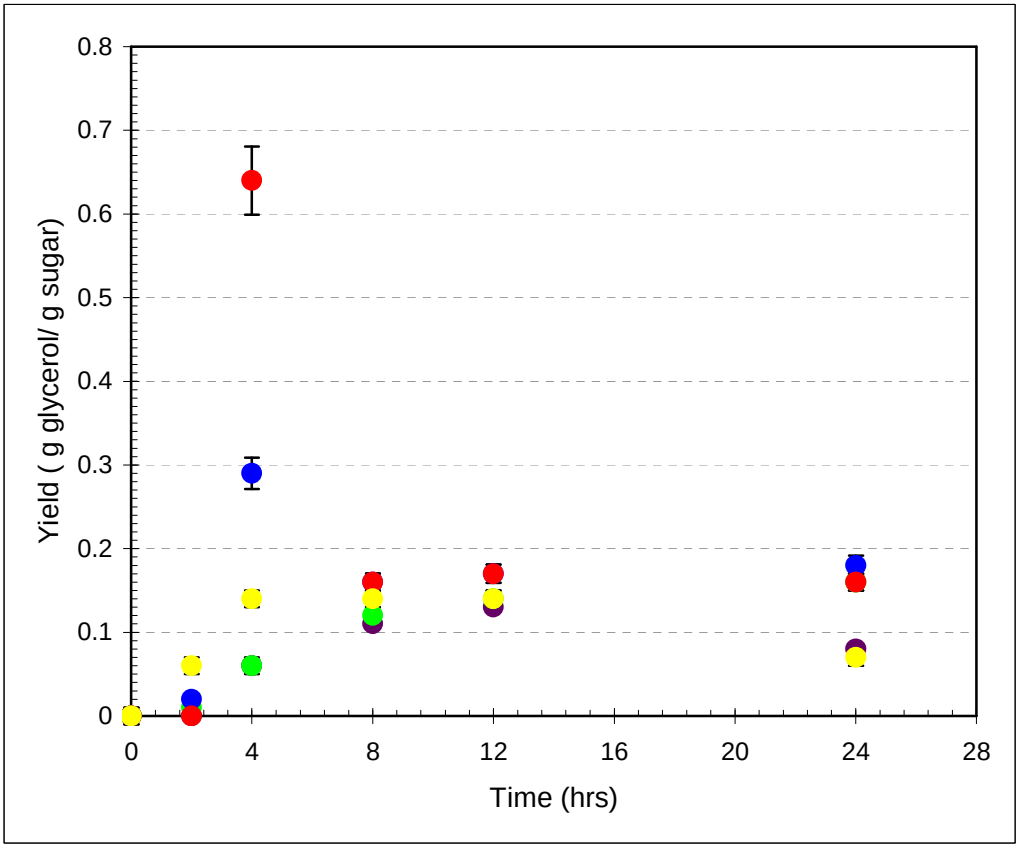

Figure 4.6 Effect of dilution on the glycerol yield.

(● No dilution, ● Dilution ratio 1:1, ● Dilution ratio 1:2, ● Dilution ratio 1:3

● Dilution ratio 1:4)

(pH 4.5, yeast concentration 1.g.L^{-1})

Figure 4.5 and 4.6 show that diluting the juice did have a significant effect on the ethanol yield as well as the glycerol yield. The significant effect is clearly illustrated in Figure 4.7

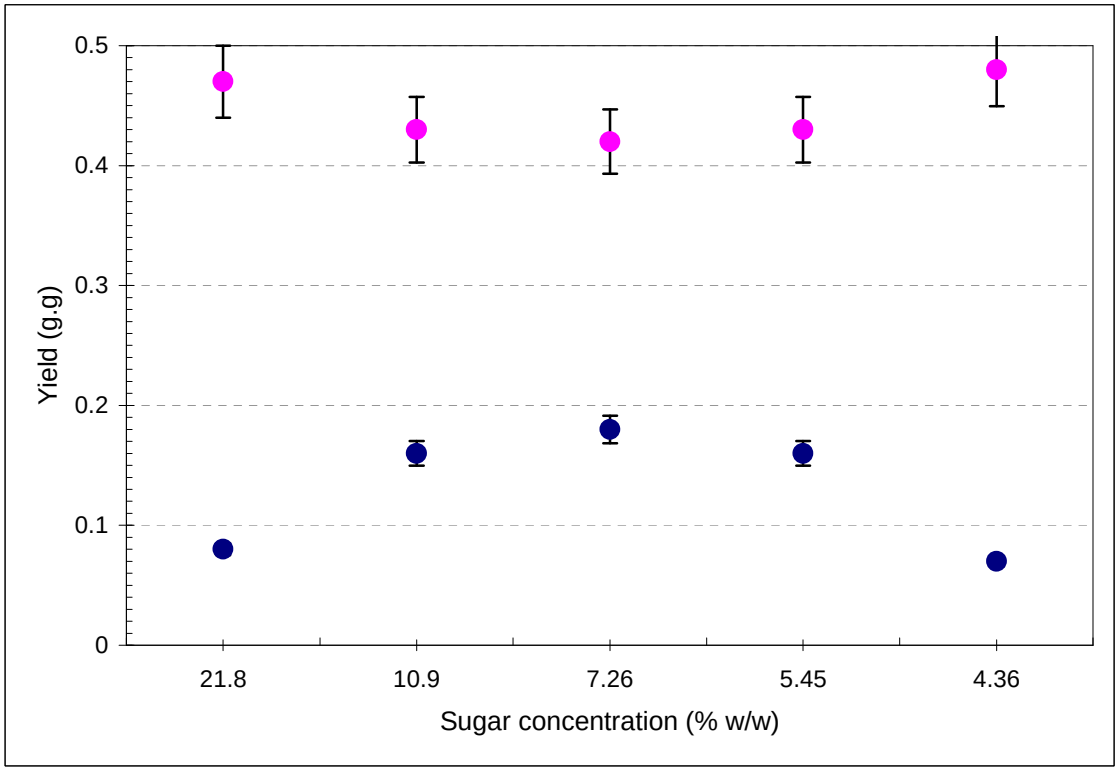

Figure 4.7 Effect of dilution on fermentation after 24 hours.
(● Ethanol, ● Glycerol)
(pH 4.5, yeast concentration 1.g.L^{-1})

Figure 4.7 shows that an increase in dilution ratio initially caused a decrease in the ethanol yield up to a minimum of 0.42 g.g^{-1} while also causing an increase in the glycerol yield up to a maximum of 0.18 g.g^{-1}. A maximum ethanol yield of 0.47 g.g^{-1}, which corresponds to a conversion efficiency of 92%, and a glycerol yield of 0.08 g.g^{-1} was achieved when no additional water was added to the sugar juice to dilute the sugar concentration. At a dilution ratio of 1:4 a maximum ethanol yield of 0.48 g.g^{-1}, which corresponds to a conversion efficiency of 94%, and a glycerol yield of 0.07 g.g^{-1} was achieved. Pavlečić *et al.* (2010) found that the ethanol production of raw sugar beet juice with a sugar content of 14-16% sucrose was only 78.8% efficient. The effect of dilution on the ethanol concentration is presented in Figure 4.8.

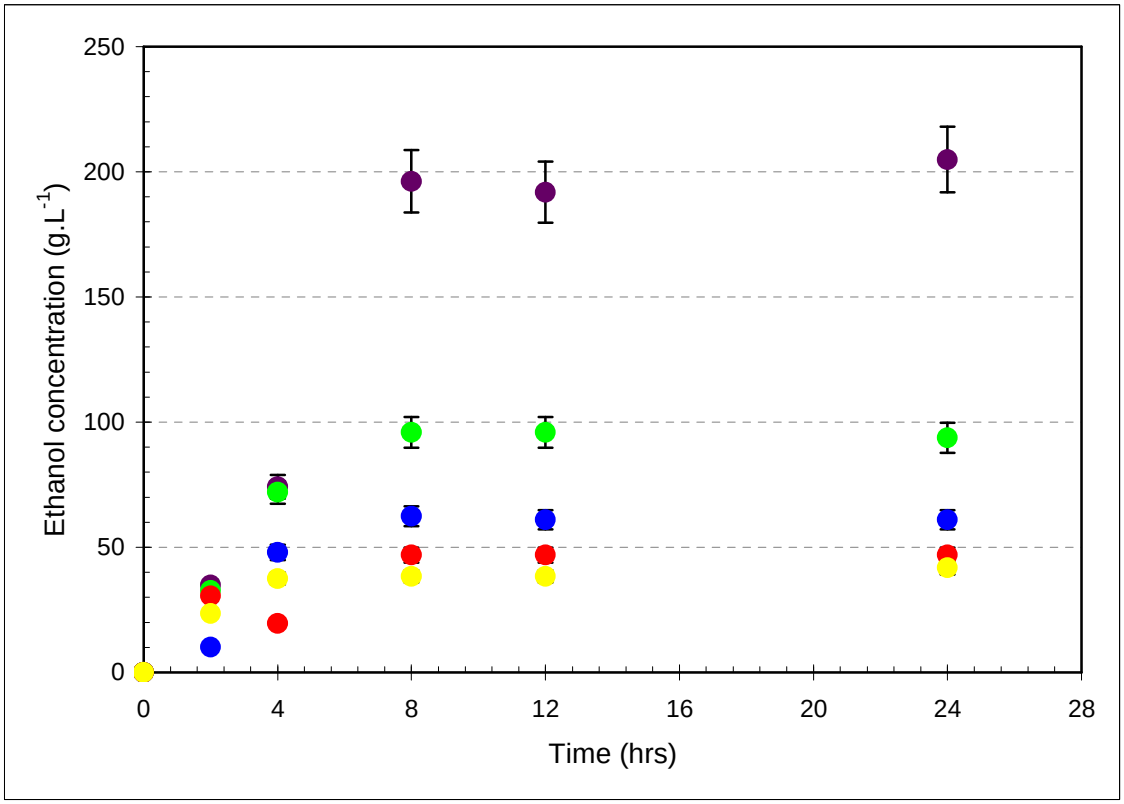

Figure 4.8 Effect of dilution on the ethanol concentration
(● No dilution, ● Dilution ratio 1:1, ● Dilution ratio 1:2, ● Dilution ratio 1:3
● Dilution ratio 1:4)
(pH 4.5, yeast concentration 1.g.L^{-1})

Figure 4.8 shows that dilution of the juice causes a decrease in the ethanol concentration. This is expected as the sugar concentration is reduced by dilution therefore there is less sugar available for conversion to ethanol.

Saccharomyces cerevisiae experiences osmotic pressure that hampers cell growth when exposed to a very high sugar concentration. Yeast cells will compensate for the effect of osmotic pressure by producing glycerol as main product instead of ethanol (Munene *et al.,* 2002). Dodić *et al* (2009) and Hinková and Bubnik (2001) found that *Saccharomyces cerevisiae* can tolerate sugar concentrations as high as 20% ($w.w^{-1}$) without suffering the effects of osmotic pressure. The initial Brix index of the tropical sugar beet juice used for investigating the influence of dilution ratio on ethanol yield was determined to be 21.8 %($w.w^{-1}$).

It is expected that as the sugar concentration is increased the glycerol yield will also increase but this was not seen in Figure 4.7 where the glycerol yield increased as the sugar concentration decreased. This phenomenon can be explained by using the metabolic pathways illustrated in Figure 4.9.

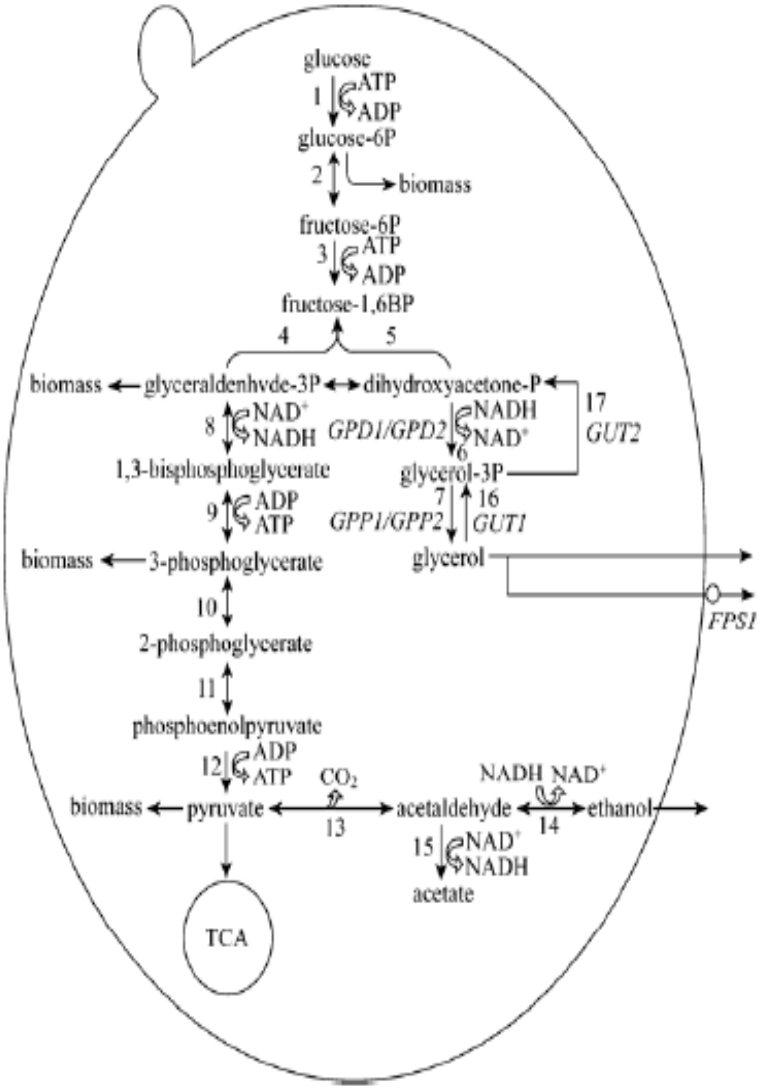

Figure 4.9 Important pathways of glycerol and ethanol metabolism in *S.cerevisiae.* Important enzymes: 1:hexokinase (glucokinase);
2: phosphoglucose isomerase; 3: phosphofructokinase; 4: aldolase;
5: triose phosphate isomerase; 6: NAD-dependent glycerol-3-phosphate dehydrogenase; 7: glycerol-3-phospatase; 8: glyceraldehyde-3-phosphate dehydrogenase; 9: phosphoglycerate kinase; 10: phosphoglycerate mutase;
11-enolase; 12: pyruvate kinase; 13:pyruvate decarboxylase; 14: alcohol dehydrogenase; 15: aldehyde dehydrogenase, 16: glycerol kinase;
17: FAD-dependent glycerol-3-phosphate dehydrogenase. (Aili and Xun, 2008).

Figure 4.9 illustrates the pathways involved in glycerol and ethanol metabolism in *S. cerevisiae.* The first step in fermentation involves the transport of the sugars into the cell. Sucrose can not be transported into the cell but is instead hydrolyzed to glucose and fructose outside the cell by the enzyme invertase (Kőnig *et al.,* 2009). The glucose and fructose is then transported across the plasma membrane by a process known as facilitated diffusion. *S. cerevisiae* metabolizes sugar by the alcoholic fermentation pathway and the first step is glycolysis which is a common pathway found in most organisms (Kőnig *et al.,* 2009). The main purpose of the glycolysis pathway is to produce energy for the cell. The individual steps involved in glycolysis ,which leads to pyruvate and eventually ethanol, will be discussed using Figure 4.9.

The first step in glycolysis is the irreversible phosphorylation of glucose and fructose, which is catalyzed by the enzyme kinase, to form glucose-6-phosphate and fructose-6-phosphate. The glucose-6-phosphate is then converted to fructose-6-phosphate catalyzed by the enzyme isomerase. Fructose-6-phosphate is then irreversible phosphorylated to fructose-1,6-*bis*phosphate, this reaction is catalyzed by the enzyme phosphofructokinase which is a important enzyme involved in glycolysis. Fructose-1,6-*bis*phosphate is then cleaved into two fragments, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, this reaction is catalyzed by the enzyme aldolase. Both these fragments are interchangeable with one another, i.e. isomerization of dihydroxyacetone phosphate to glyceradehdyde-3-phosphate can take place. It is at this step in glycolysis where glycerol can be produced.

The conversion of glyceraldehyde-3-phosphate through glycolysis depends on the availability of the electron acceptor NAD^+ which is usually regenerated from NADH at the last step of fermentation. If this step is inhibited the NAD^+ can be regenerated by respiration or glycerol production. Glyceraldehyde-3-phosphate is then converted to 1,3-*Bis*phosphoglycerate by the transfer of a phosphate group and the oxidation of NAD^+ to NADH.

1,3-*Bis*phosphoglycerate is then converted to 3-phosphoglycerate by the transfer of a phosphate group to ADP. This is the first reaction whereby energy is generated in the form of ATP for the cell. The enzyme phosphoglycerate mutase catalyzes the isomerization of 3-phosphoglycerate to 2-phosphoglycerate. The enzyme enolase catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. The final step in glycolysis is the conversion of phosphoenolpyruvate to pyruvate by the transfer of a phosphate group to ADP forming ATP. This is the second energy forming reaction and this is catalyzed by the enzyme pyruvate kinase.

The final two steps leading to ethanol production are catalyzed by two enzymes pyruvate decarboxylase and alcohol dehydrogenase. Pyruvate decarboxylase catalyzes the decarboxylation of pyruvate to acetaldehyde which releases CO_2 in the process. Alcohol dehydrogenase catalyzes the reduction of acetaldehyde to ethanol while at the same time regenerating NAD^+ which is required for the conversion of glyceraldehyde-3-phosphate. A lack of alcohol dehydrogenase can cause a redox imbalance which could then lead to glycerol production (Kőnig *et al.,* 2009)

Glycerol production has two main functions during fermentation, firstly it protects the cell from lysis under osmotic stress conditions and under anaerobic conditions when respiration does not take place it regenerates NAD^+ and therefore helps maintain the NAD^+/NADH balance (Aili and Xun, 2008). Glycerol is produced when the intermediate glycolysis product dihydroxyacetone phosphate is converted to glycerol-3-phosphate and then to glycerol. These two reactions are catalyzed by NAD-dependent glycerol-3-phosphate dehydrogenase and glycerol-3-phospatase. During the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate NAD^+ is regenerated which can then be used in the later steps of fermentation.

As stated previously it is expected that at a higher sugar concentration, the ethanol yield will decrease and the glycerol yield will increase but this was not seen. Therefore this indicates that the yeast was able to tolerate the sugar concentration of the tropical sugar beet juice. Therefore the increase in glycerol yield and the decrease in the ethanol yield as the juice was diluted could be due to an imbalance in the NAD^+/NADH ratio.

Figure 4.7 shows that at a dilution ratio of 1:4 the ethanol yield starts to increase and the glycerol yield decreases. During anaerobic growth alcoholic fermentation is the only form of energy production as seen in Figure 4.9 and when there is a very low sugar concentration the yeast will favor the production of ethanol as it produces energy for the cell while glycerol production, which is not associated with energy production, will be reduced.

4.2.2 Effect of yeast concentration

The effect of varying the yeast concentration during fermentation of tropical sugar beet juice was investigated according to the experimental procedure in section 3.5.4. The experimental error associated with yeast concentration was determined to be 2.57% (confidence level of 95%); detailed calculations can be found in Appendix B. The effect of yeast concentration on the utilization of sugars is shown in Figure 4.10 and 4.11

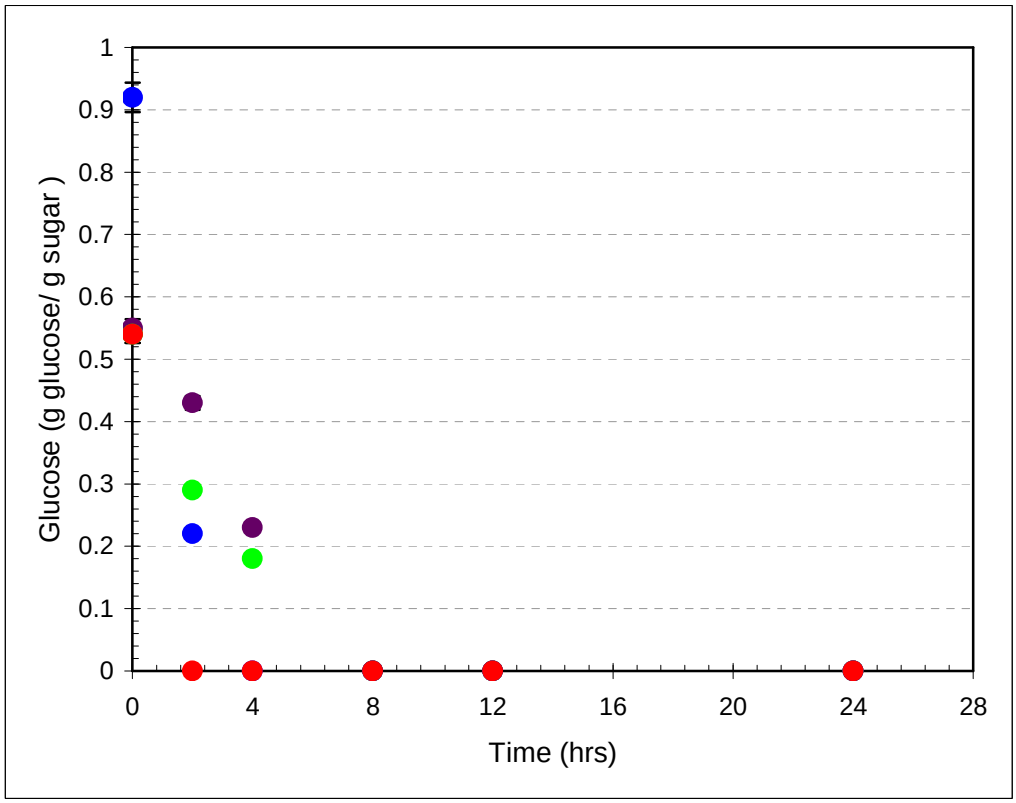

Figure 4.10 Effect of yeast concentration on glucose utilization

(● 1 $g.L^{-1}$; ● 3 $g.L^{-1}$; ● 5 $g.L^{-1}$; ● 10 $g.L^{-1}$)

(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5)

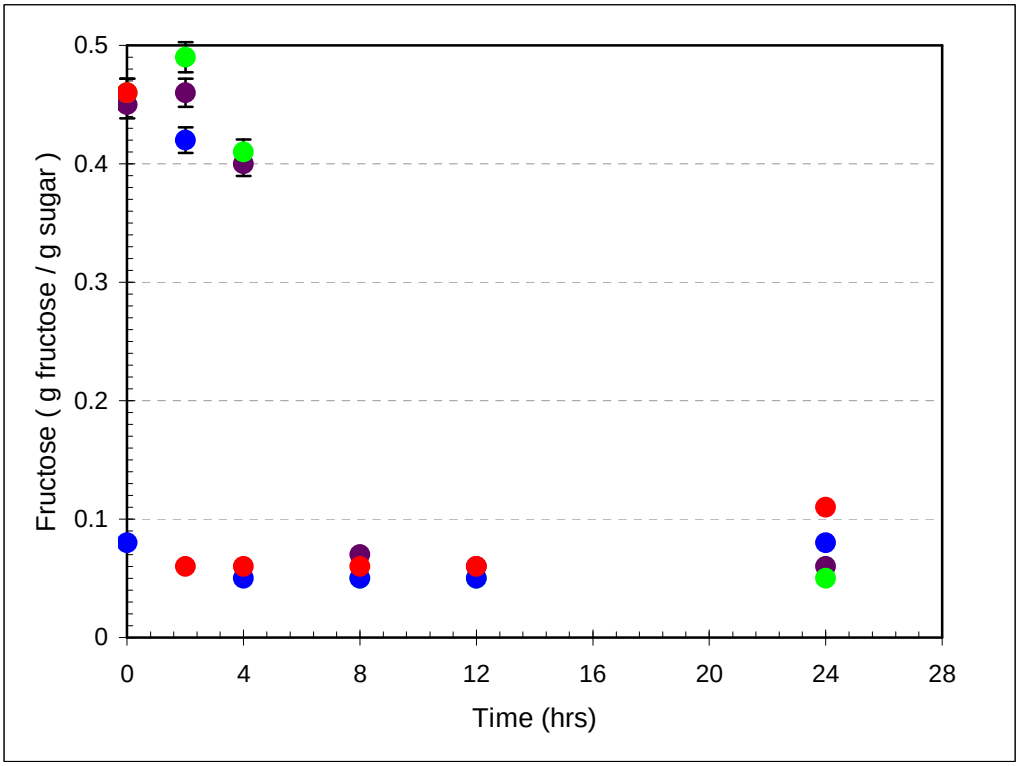

Figure 4.11 Effect of yeast concentration on fructose utilization
(● 1 g.L^{-1}; ● 3 g.L^{-1}; ● 5 g.L^{-1}; ● 10 g.L^{-1})
(Initial sugar concentration 10.9% (w.w^{-1}), pH 4.5)

Figure 4.10 shows that increasing the yeast concentration did have an effect on the utilization of glucose. At a yeast concentration of 10 g.L^{-1} almost all of the glucose was utilized within 2 hours and at yeast concentration of 5 g.L^{-1} the glucose was utilized within 4 hours. After 8 hours of fermentation no glucose could be detected at all of the yeast concentrations investigated. A similar effect is seen in Figure 4.11 which shows that at a yeast concentration of 10 g.L^{-1} almost all of the fructose has been utilized within 2 hours. Figure 4.11 also shows that after 8 hours of fermentation no more fructose is being utilized and a residual fructose yield of 0.06 g.g^{-1} is being detected.

The effect of yeast concentration on the ethanol yield and concentration is presented in Figure 4.12 and 4.13.

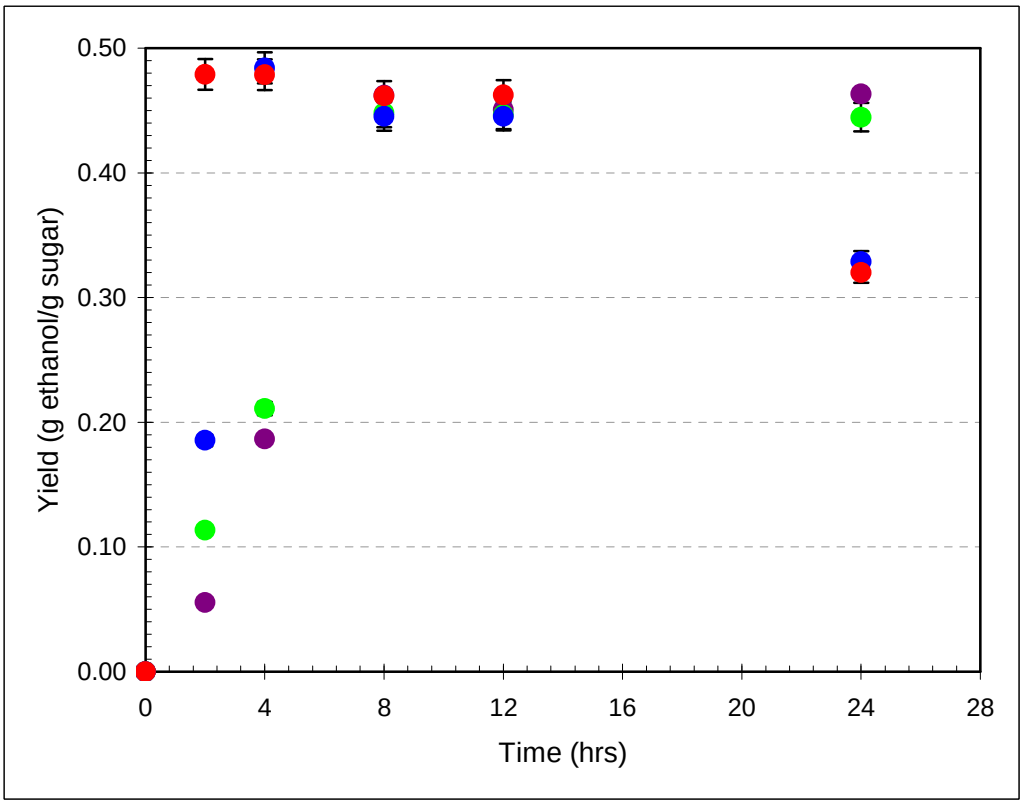

Figure 4.12 Effect of yeast concentration on the ethanol yield.

(● 1 g.L^{-1}; ● 3 g.L^{-1}; ● 5 g.L^{-1}; ● 10 g.L^{-1})

(Initial sugar concentration 10.9% (w.w^{-1}), pH 4.5)

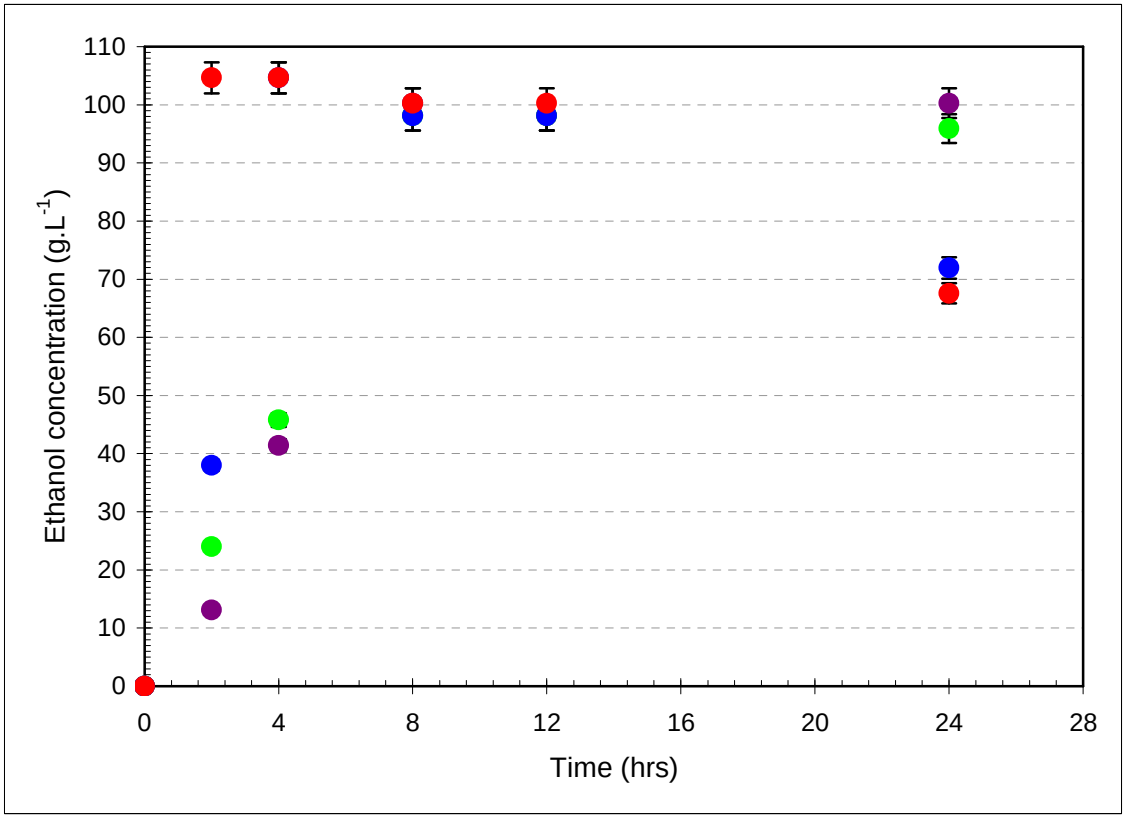

Figure 4.13 Effect of yeast concentration on ethanol concentration
(● 1 $g.L^{-1}$; ● 3 $g.L^{-1}$; ● 5 $g.L^{-1}$; ● 10 $g.L^{-1}$)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5)

Figure 4.12 and 4.13 show that increasing the yeast concentration did have a significant effect on the ethanol yield and concentration. A maximum ethanol yield of 0.48 $g.g^{-1}$, which corresponds to a conversion efficiency of 94% and a ethanol concentration of 104.64 $g.L^{-1}$ was achieved after 4 hours of fermentation, using a 5 $g.L^{-1}$ yeast concentration. Both figures also show that after 2 hours an ethanol yield of 0.48 $g.g^{-1}$ was achieved using a yeast concentration of 10 $g.L^{-1}$. Increasing the yeast concentration means that there are more yeast cells available to convert the glucose into ethanol at a faster rate and this can be seen in Figures 4.12 and 4.13. Breisha (2010) also found that when the inoculum size was increased from 3% to 6% the ethanol concentration increased to 9.3% and the fermentation time was reduced from 72 hours to 48 hours. It was concluded that this could be attributed to the short or neglible lag phase during growth.

Arshad *et al.* (2010) found that increasing the inoculum size from 10% to 30% increased the ethanol concentration to 7.8% but also decreased the formation of by-products such as methanol, fusel alcohols and acetic acid. It is also seen in Figures 4.12 and 4.13 that as the fermentation progresses the ethanol yield starts to decrease at the higher yeast concentrations of 5 and 10 $g.L^{-1}$. The effect of yeast concentration on the glycerol yield is presented in Figure 4.14

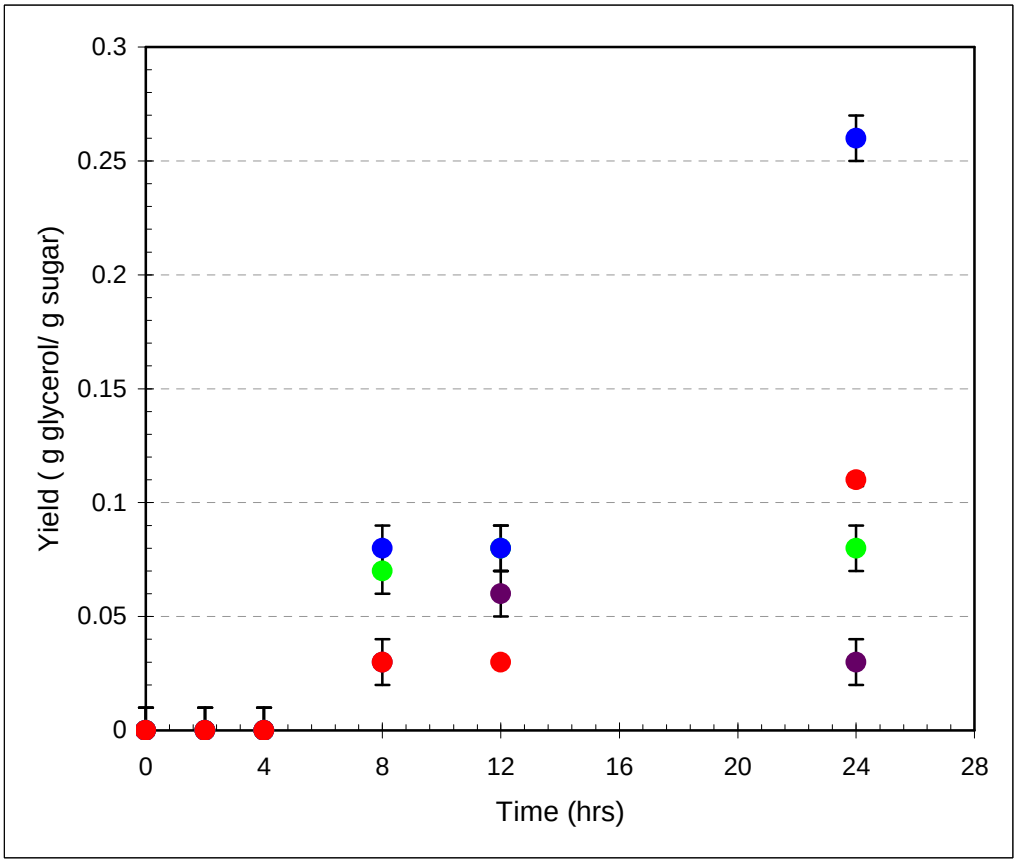

Figure 4.14 Effect of yeast concentration on glycerol yield
(● 1 $g.L^{-1}$; ● 3 $g.L^{-1}$; ● 5 $g.L^{-1}$; ● 10 $g.L^{-1}$)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5)

Figure 4.14 shows that increasing the yeast concentration did have a significant effect on the glycerol yield. At the higher yeast concentrations of 5 and 10 $g.L^{-1}$ more glycerol was produced compared to the lower yeast concentrations of 1 and 3 $g.L^{-1}$. A maximum glycerol yield of 0.26 $g.g^{-1}$ was achieved at a yeast concentration of 5 $g.L^{-1}$ after 24 hours of fermentation.

Figures 4.12 and 4.13 show that after 24 hours the ethanol yield starts to decrease at the higher yeast concentrations of 5 and 10 $g.L^{-1}$. At the same time the glycerol yield also starts to increase as seen in Figure 4.14. The significant effect is more clearly illustrated in Figure 4.15

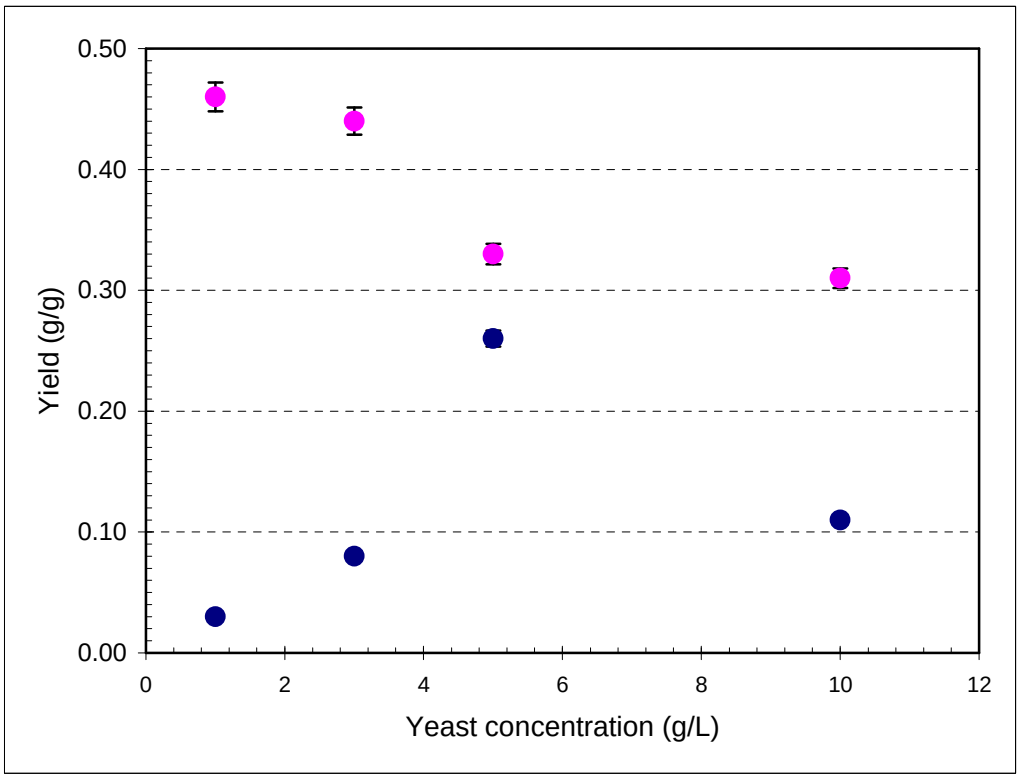

Figure 4.15 Effect of yeast concentration on fermentation after 24 hours.
(● Ethanol, ● Glycerol)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5)

Figure 4.15 shows that after 24 hours of fermentation the ethanol yield starts to decrease at the higher yeast concentrations while the glycerol yield starts to increase. This indicates that some of the sugar is being diverted to glycerol production. When there are more yeast cells the sugar is depleted at a faster rate and after some time all the sugar initially present has been consumed, but the cell still needs a energy source and will start to use the ethanol. Zayed and Foley (1987) found that yeast undergoes an internal metabolic adjustment to use ethanol as an energy source when sugar becomes depleted and Munene *et al*. (2002) found that at high yeast concentrations more glycerol is formed.

The time for each yeast concentration to produce the maximum amount of ethanol is shown in Table 4.1

Table 4.1 Optimal time for maximum ethanol production

Concentration ($g.L^{-1}$)	**Conversion ($g.g^{-1}$)**	**Ethanol concentration ($g.L^{-1}$)**	**Glycerol ($g.g^{-1}$)**	**Time (hrs)**
1	0.91	100.28	0.03	24
3	0.88	98.1	0.07	8
5	0.95	104.64	0	4
10	0.94	104.64	0	2

From Table 4.1 it can be seen that the highest yeast concentration resulted in the highest ethanol yield and concentration in the shortest time. At the time of maximum ethanol production for a yeast concentration of 5 and 10 $g.L^{-1}$, no glycerol has started to form, indicating that if the fermentation was to be stopped at this point, a high ethanol yield with no side-products will be obtained. If the ethanol yields for the different yeast concentrations were compared at the same time, as in Figure 4.15, the higher yeast concentrations will show more glycerol formed because the fermentation was allowed to continue beyond the time for maximum ethanol production.

In conclusion this study showed that increasing the yeast concentration did increase the ethanol yield and at the same time reduced the fermentation time. Therefore, to achieve the maximum ethanol yield in the shortest possible time, with no by-product formation, as well as keeping production costs at a minimum, a yeast concentration of 5 $g.L^{-1}$ is suggested as the optimum yeast concentration to use for the production of ethanol from tropical sugar beet juice.

4.2.3 Effect of a nitrogen supplementation

The effect of supplementing the fermentation medium with a nitrogen source on the ethanol yield was investigated according to the experimental procedure outlined in section 3.5.5. Nitrogen sources such as peptone, urea, yeast extract and ammonium sulphate were investigated as possible nitrogen supplements for the fermentation broth. The experimental error associated with this variable is 1.42% (confidence level of 95%); detailed calculations can be found in Appendix B. These nitrogen sources were investigated, using a concentration of 750 mg $N.L^{-1}$ and the results of this investigation on the glucose and fructose utilization are shown in Figure 4.16 and Figure 4.17

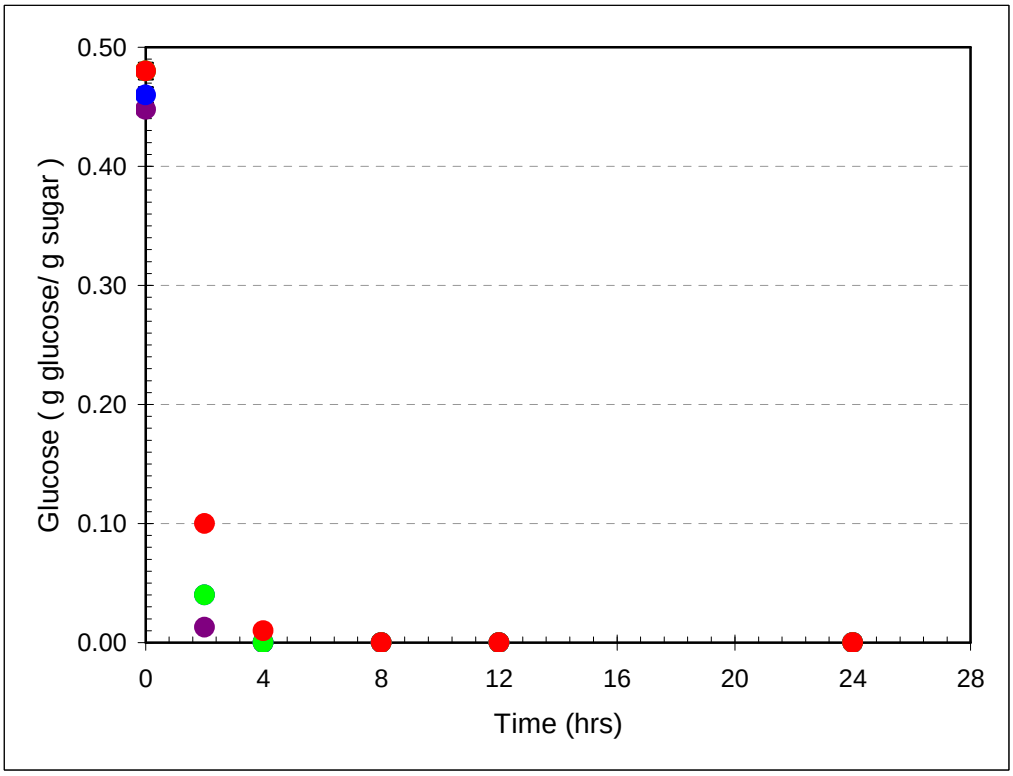

Figure 4.16 Effect of adding a nitrogen source on the glucose utilization (● No addition, ● Urea, ● Peptone, ● Yeast extract, ● Ammonium sulphate) (Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$, nitrogen concentration 750 mg $N.L^{-1}$)

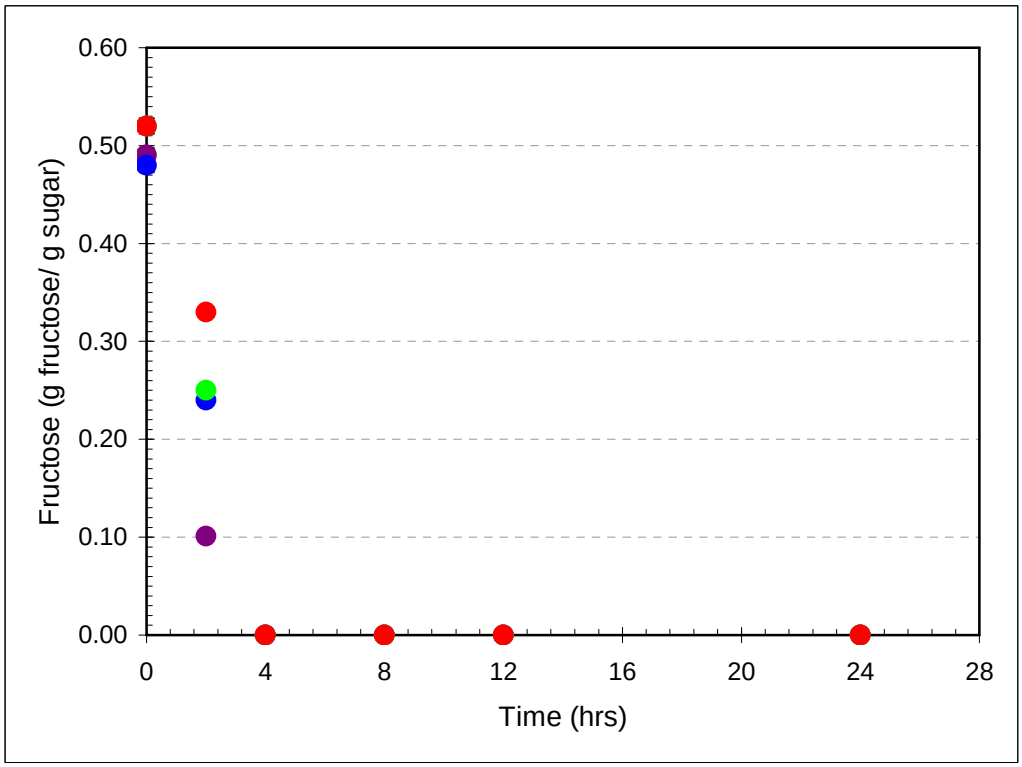

Figure 4.17 Effect of adding a nitrogen source on the fructose utilization (● No addition, ● Urea, ● Peptone, ● Yeast extract, ● Ammonium sulphate) (Initial sugar concentration 10.9% (w.w^{-1}), pH 4.5, yeast concentration 5 g.L^{-1}, nitrogen concentration 750 mg N.L^{-1})

Figure 4.16 and 4.17 show that adding a nitrogen source did effect the rate of sugar uptake. Both figures show that when no nitrogen was added more glucose and fructose were consumed within 2 hours compared to when a nitrogen source was added however it is seen that after 4 hours of fermentation no glucose or fructose is detected at all nitrogen sources investigated.

The effect of adding a nitrogen source on the ethanol yield and concentration is presented in Figure 4.18 and 4.19.

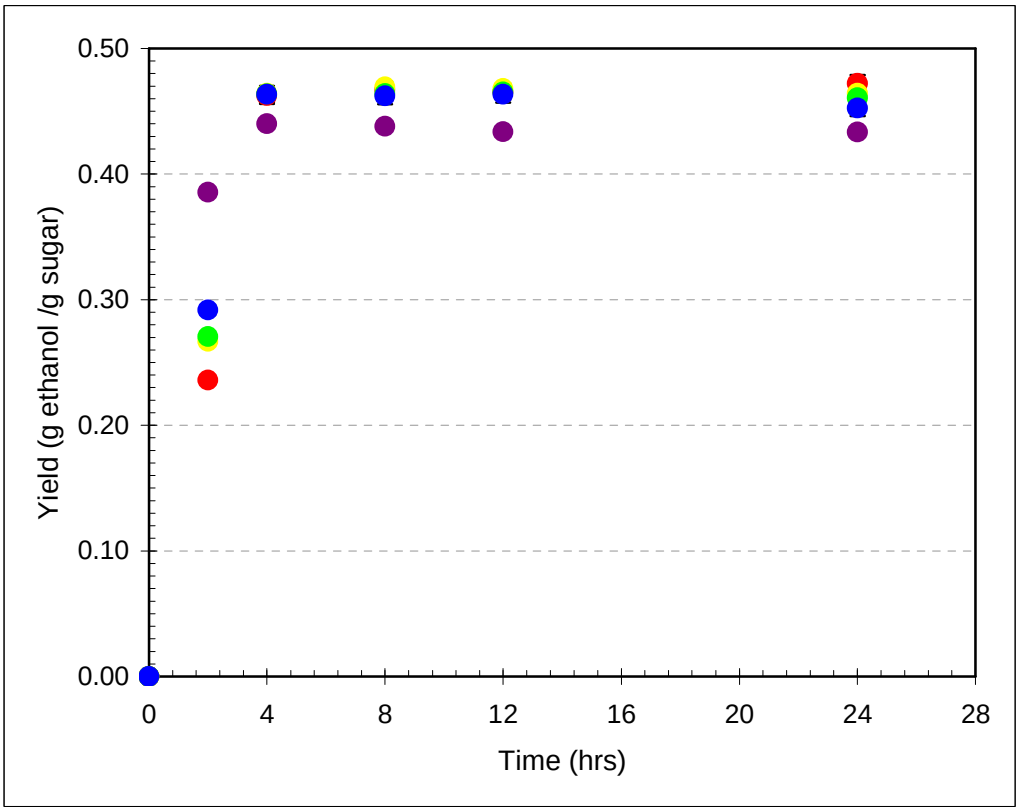

Figure 4.18 Effect of adding a nitrogen source on the ethanol yield (● No addition, ● Urea, ● Peptone, ● Yeast extract, ● Ammonium sulphate) (Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$, nitrogen concentration 750 mg $N.L^{-1}$)

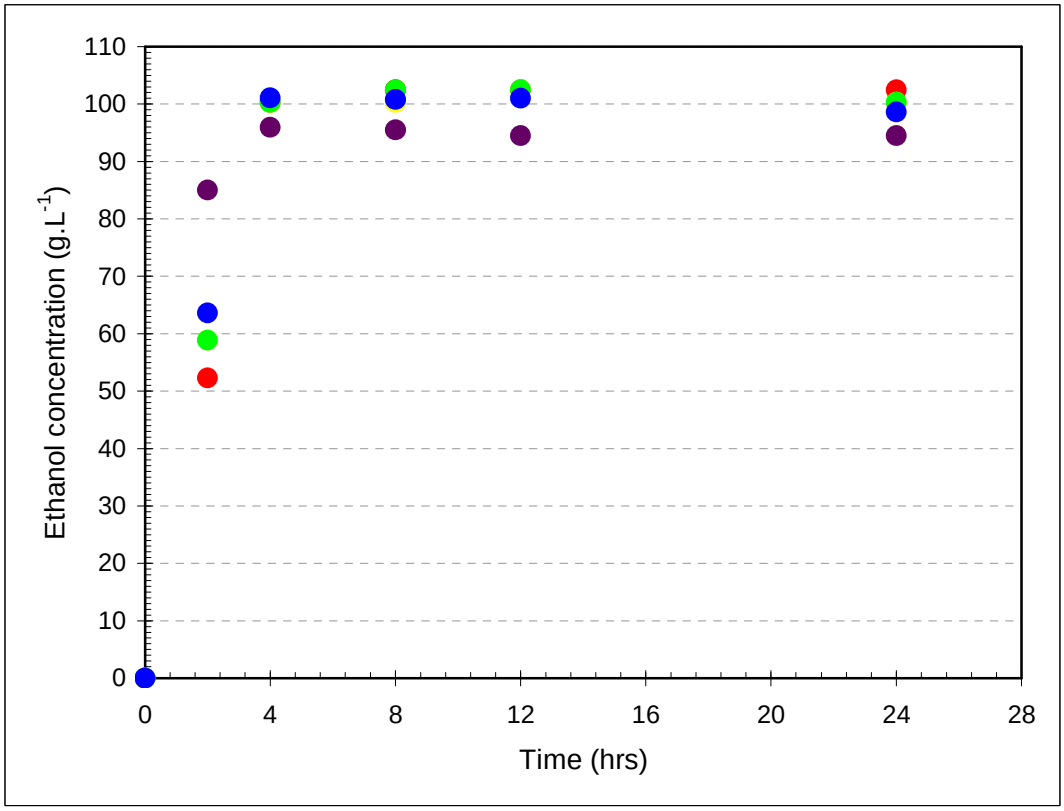

Figure 4.19 Effect of adding a nitrogen source on the ethanol concentration (● No addition, ● Urea, ● Peptone, ● Yeast extract, ● Ammonium sulphate) (Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$, nitrogen concentration 750 mg $N.L^{-1}$)

Figure 4.18 and 4.19 show that all nitrogen sources investigated had a significant positive effect on the ethanol yield. The yield increased from 0.44 $g.g^{-1}$ to 0.47 $g.g^{-1}$ and the concentration increased from 95.96 $g.L^{-1}$ to 102.46 $g.L^{-1}$ when nitrogen was added. The effect on the glycerol yield is presented in Figure 4.20.

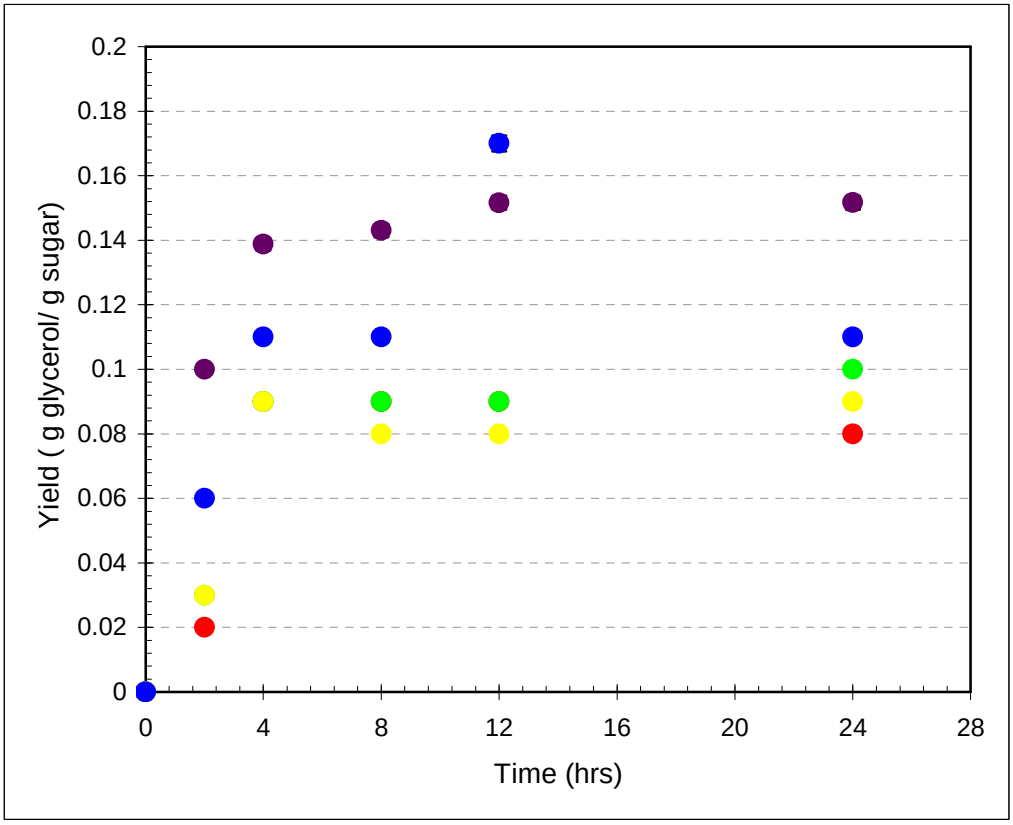

Figure 4.20 Effect of adding a nitrogen source on the glycerol yield (● No addition, ● Urea, ● Peptone, ● Yeast extract, ● Ammonium sulphate) (Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$, nitrogen concentration 750 mg $N.L^{-1}$)

Figure 4.20 shows that adding a nitrogen source had a significant positive effect on the glycerol yield. The glycerol yield decreased from 0.15 $g.g^{-1}$ when no nitrogen was added to 0.08 $g.g^{-1}$ when nitrogen was added. The significant effect on the ethanol and glycerol yield is clearly illustrated in Figure 4.21.

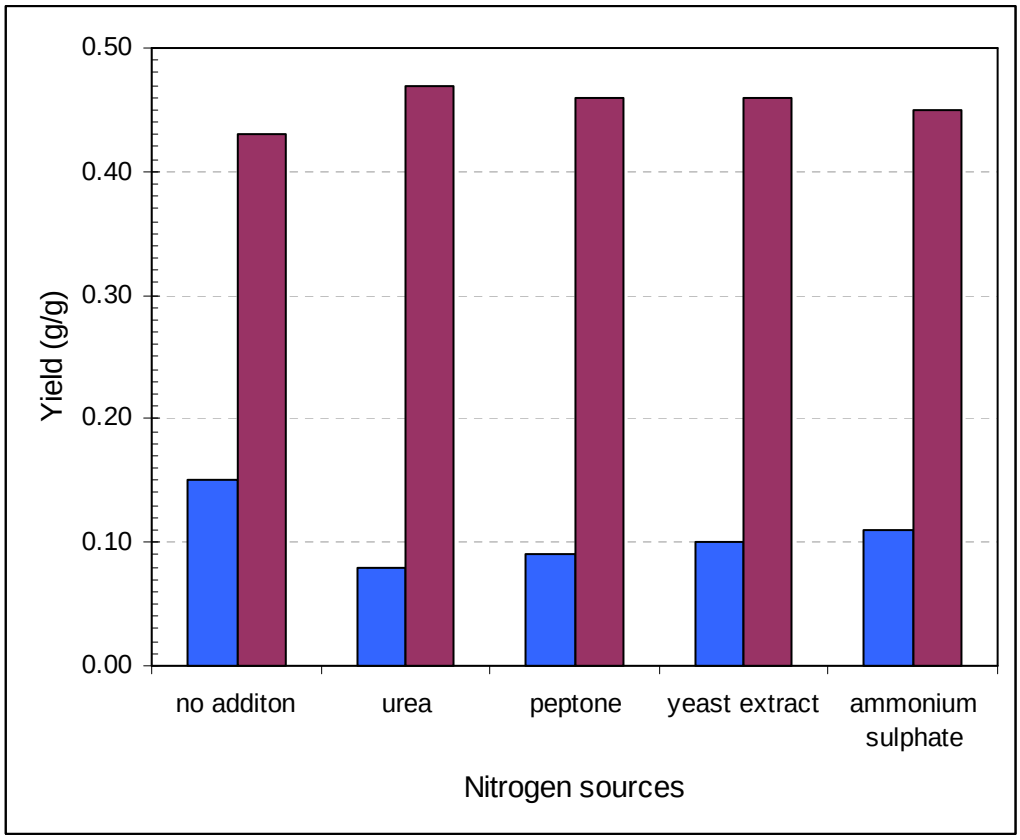

Figure 4.21 Effect of adding a nitrogen source after 24 hours of fermentation (■ Ethanol, ■ Glycerol)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$, nitrogen concentration 750 mg $N.L^{-1}$)

Figure 4.21 shows that the maximum ethanol yield of 0.47 $g.g^{-1}$ was obtained when urea was used as a nitrogen source. Figure 4.21 also shows that not only did nitrogen have a significant positive effect on the ethanol yield but it also reduced the amount of glycerol formed as seen in Figure 4.20.

Zayed and Foley (1987) and El-Refai *et al.* (1992) both found that the addition of urea at a concentration of between 1.08 to 1.2 $g.L^{-1}$ significantly improved the ethanol yield. Ortiz-Muniz *et al.* (2010) found that adding yeast extract at varying concentrations of 1 to 2.5 $g.L^{-1}$ increased the ethanol yield, as well as stimulating glucose consumption. Junior *et al*. (2008) found that peptone improved the fermentation performance of the yeast.

In contrast Ogbonna *et al.* (2001) found that the addition of peptone and yeast extract at a concentration of 4 $g.L^{-1}$ did not have any effect on ethanol production and it was concluded that the sugar beet juice had no inhibitory substances.

In conclusion, this study showed that the addition of a nitrogen source to the fermentation broth significantly increases the efficiency of ethanol production and that any of the nitrogen sources investigated, could be utilized.

Ammonium sulphate was chosen as the nitrogen source for future experiments as it is a simple nitrogen source that can enter the cell directly (Mendes-Ferreira *et al.,* 2004) and from Figure 4.18 it can be seen that a better ethanol yield is obtained with ammonium sulphate after 2 hours of fermentation, compared to the other nitrogen sources.

Yeasts can be affected in two ways by the addition of nitrogen: it can increase biomass production and can also increase the sugar utilization rate (Beltran *et al.,* 2005). Yeasts require a constant supply of assimilable nitrogen as it plays a role in the structure and function of the cell (Junior *et al.,* 2008). It has also been found that yeast might require this extra nitrogen to cope with osmotic pressure (Thomas *et al.,* 1996) and it has been reported that the minimal amount of freely assimible nitrogen (FAN) required for an adequate fermentation process is 140 $mg.L^{-1}$ increasing as the sugar concentration increased (Breisha, 2010).

The results in Figures 4.18 and 4.21 suggest that the tropical sugar beet juice used in this study was slightly deficient in freely assimilable nitrogen (FAN) which can lead to stuck or sluggish fermentation.

The effect of varying the ammonium sulphate concentration on the ethanol yield was investigated according to the experimental procedure outlined in section 3.5.5. The results of this investigation on the glucose and fructose utilization is presented in Figure 4.22 and 4.23 respectively.

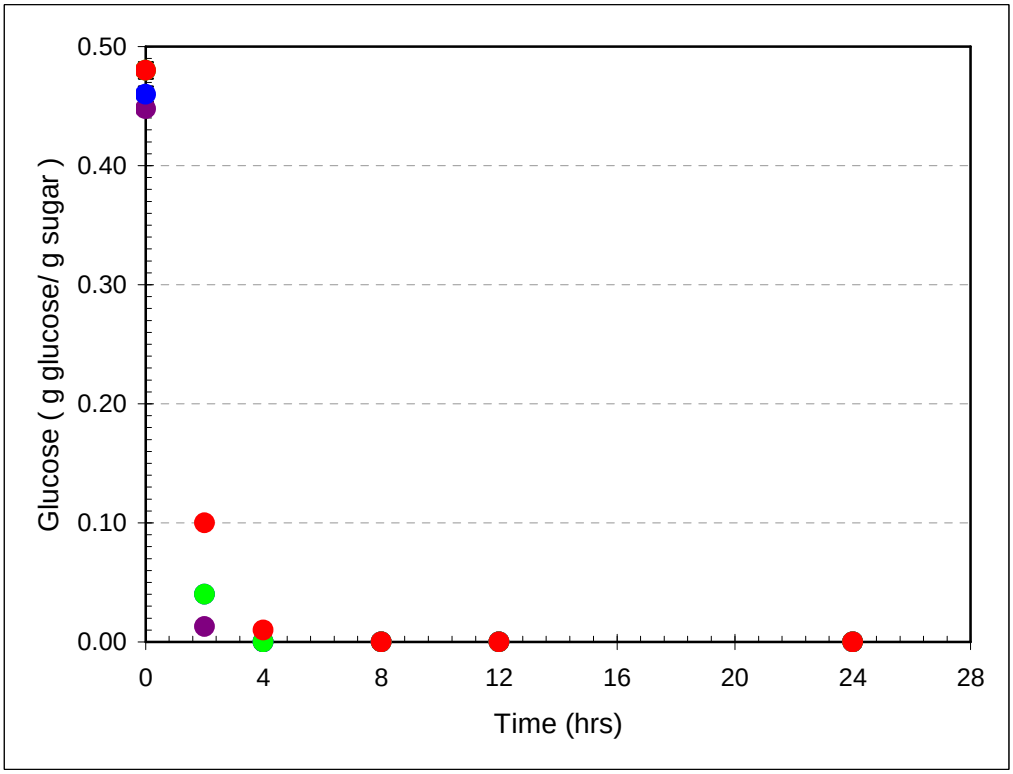

Figure 4.22 Effect of adding ammonium sulphate on the glucose utilization
(● No addition ; ● 250 mg $N.L^{-1}$; ● 500 mg $N.L^{-1}$; ● 750 mg $N.L^{-1}$)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5,
yeast concentration 5 $g.L^{-1}$)

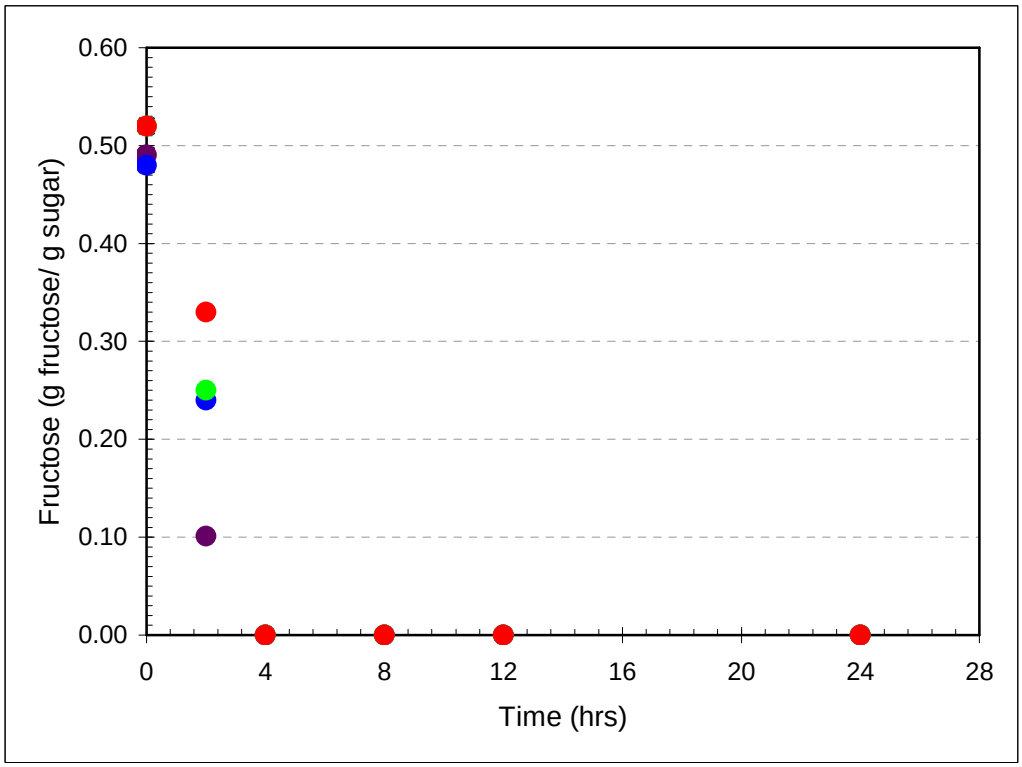

Figure 4.23 Effect of adding ammonium sulphate on fructose utilization (● No addition ; ● 250 mg $N.L^{-1}$; ● 500 mg $N.L^{-1}$; ● 750 mg $N.L^{-1}$) (Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$)

Figure 4.22 and 4.23 shows that the addition of ammonium sulphate did effect the rate of sugar uptake. Similar to what was seen in Figure 4.16 and 4.17 more sugar was consumed within 2 hours when no nitrogen was added. However after 4 hours of fermentation all the sugars had been consumed.

The effect of adding ammonium sulphate at varying concentrations on the ethanol yield and concentration is presented in Figure 4.24 and 4.25.

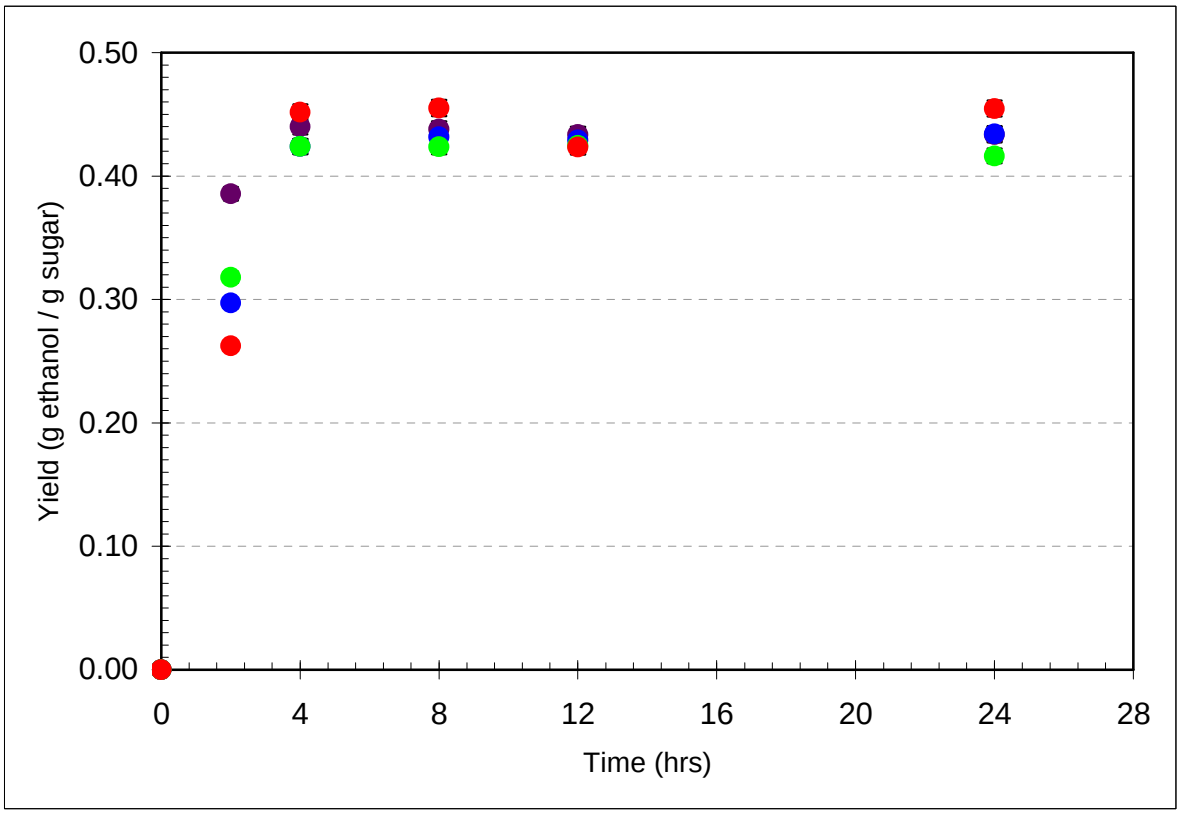

Figure 4.24 Effect of adding ammonium sulphate on the ethanol yield
(● No addition ; ● 250 mg $N.L^{-1}$; ● 500 mg $N.L^{-1}$; ● 750 mg $N.L^{-1}$)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5,
yeast concentration 5 $g.L^{-1}$)

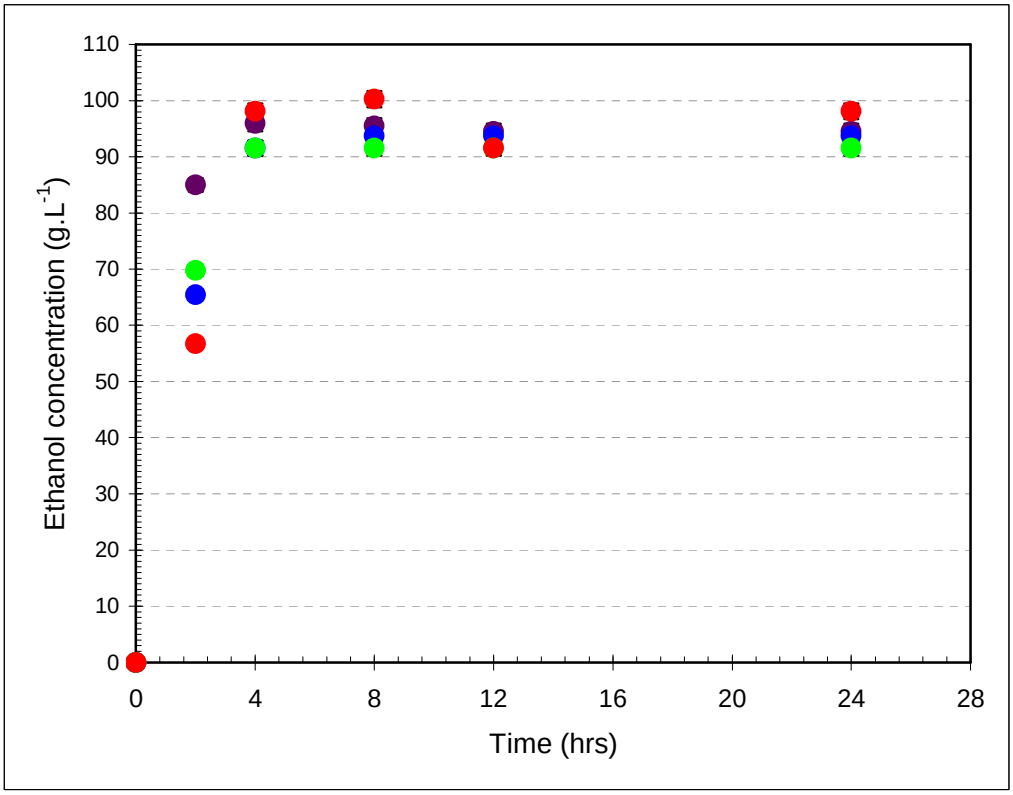

Figure 4.25 Effect of adding ammonium sulphate on the ethanol concentration (● No addition ; ● 250 mg $N.L^{-1}$; ● 500 mg $N.L^{-1}$; ● 750 mg $N.L^{-1}$) (Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5, yeast concentration 5 $g.L^{-1}$)

From Figure 4.24 and 4.25 it can be seen that the addition of a nitrogen source such as ammonium sulphate to the fermentation medium had a significant effect on the ethanol yield and concentration. A maximum ethanol yield of 0.45 $g.g^{-1}$ and a concentration of 98.1 $g.L^{-1}$ was obtained when ammonium sulphate was added to the fermentation broth at a concentration of 750 mg $N.L^{-1}$. The addition of the ammonium nitrogen source thus added the necessary assimilable nitrogen to increase the ethanol yield. The effect of the addition of ammonium sulphate on the glycerol yield is presented in Figure 4.26.

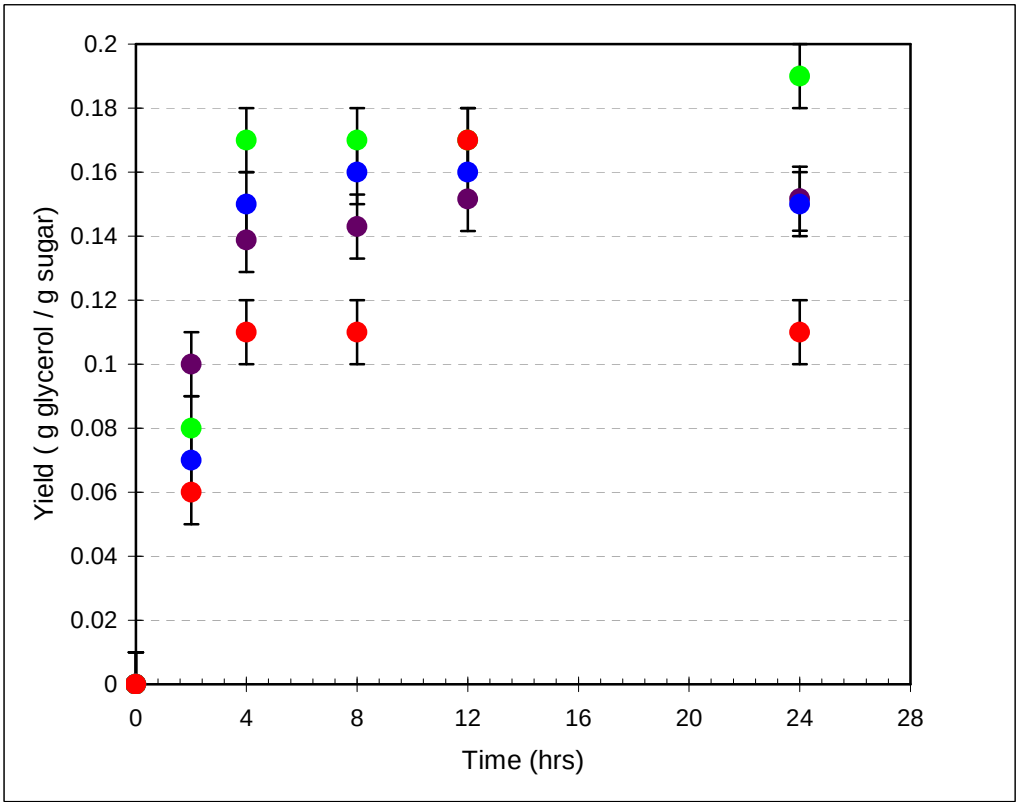

Figure 4.26 Effect of adding ammonium sulphate on the glycerol yield
(● No addition ; ● 250 mg $N.L^{-1}$; ● 500 mg $N.L^{-1}$; ● 750 mg $N.L^{-1}$)
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5,
yeast concentration 5 $g.L^{-1}$)

Figure 4.26 shows that the addition of a nitrogen source such as ammonium sulphate had an effect on the glycerol yield. The addition of ammonium sulphate decreased the glycerol yield and increased the ethanol yield as seen in Figure 4.24. The significant effect on the ethanol and glycerol yield is more clearly presented in Figure 4.27.

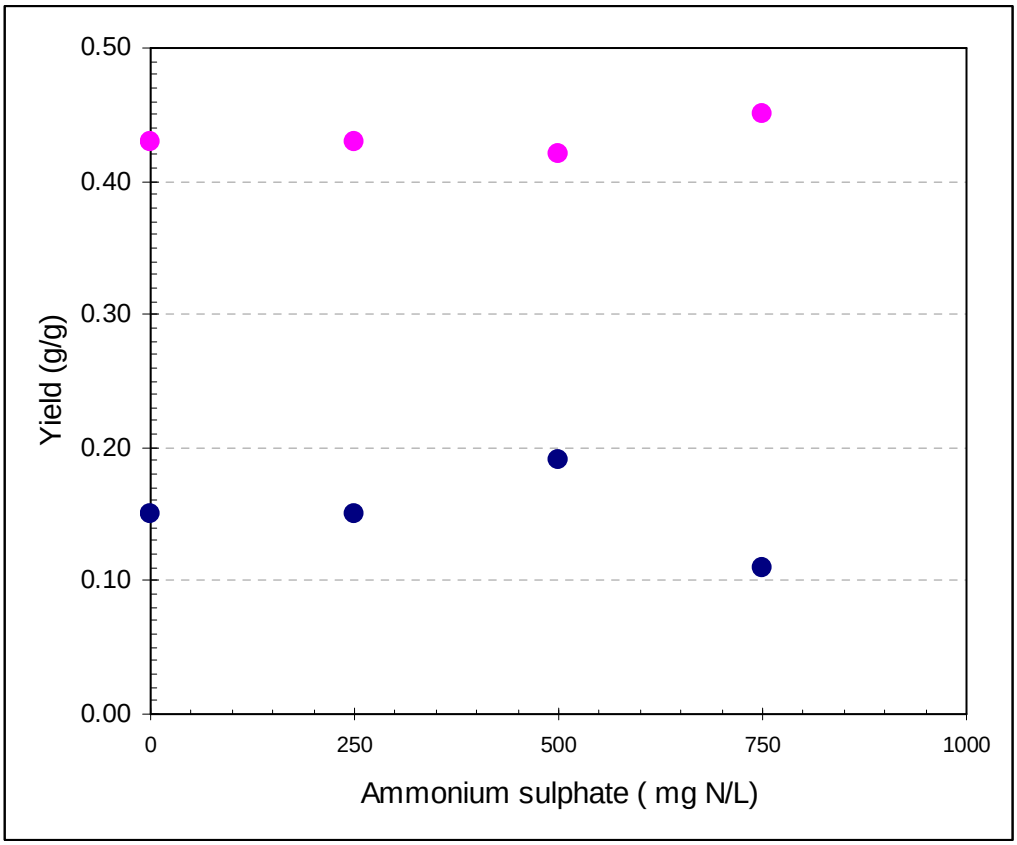

Figure 4.27 Effect of varying the ammonium sulphate concentration on fermentation after 24 hours
(Initial sugar concentration 10.9% ($w.w^{-1}$), pH 4.5,
yeast concentration 5 $g.L^{-1}$)

Figure 4.27 shows that adding ammonium sulphate increased the ethanol yield from 0.43 $g.g^{-1}$ to 0.45 $g.g^{-1}$ and decreased the glycerol yield from 0.15 $g.g^{-1}$ to 0.11 $g.g^{-1}$ with the addition of 750 mg $N.L^{-1}$ ammonium sulphate. These results in are in agreement with the work done by Devine and Slaughter (1980) and Harding *et al.* (1984) who both found that the ethanol yield was the highest at an ammonium sulphate concentration of 750 mg $N.L^{-1}$.

4.2.4 Effect of pH

The effect of the pH on the ethanol yield was investigated according to the experimental procedure outlined in section 3.5.3. The experimental error associated with this variable is 4.17% (confidence level of 95%); detailed calculations can be found in Appendix B. The effect of pH on the utilization of sucrose, glucose and fructose is shown in Figure 4.28, 4.29 and 4.30.

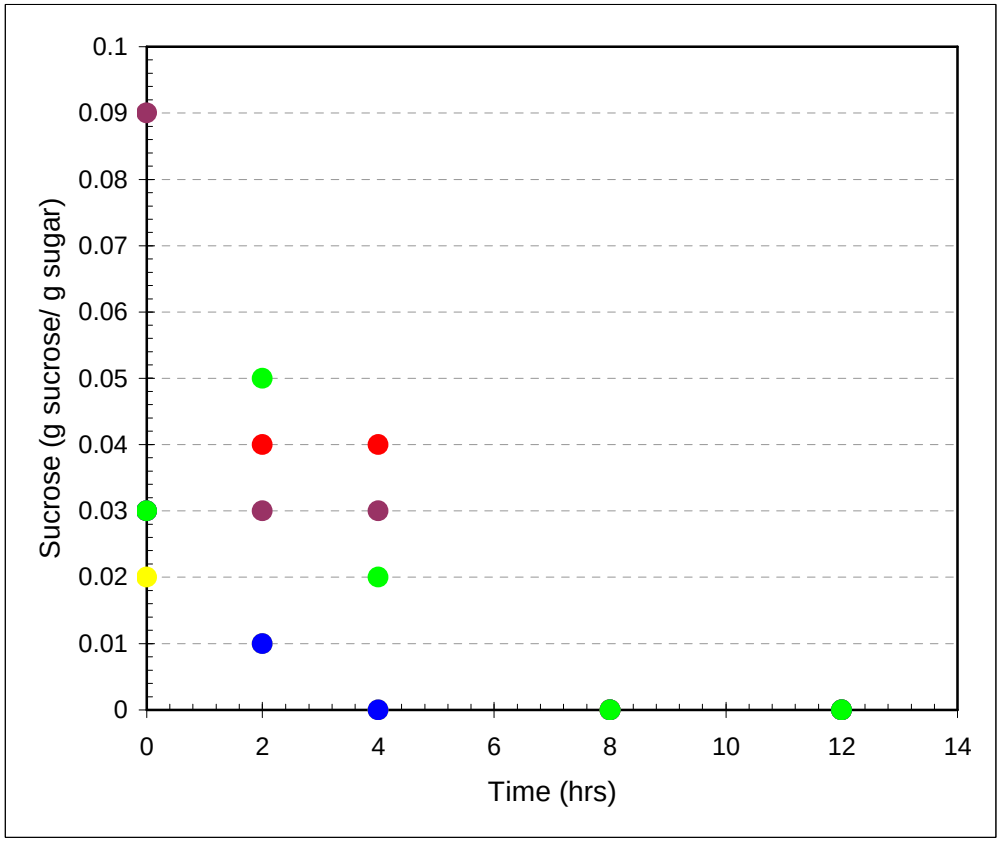

Figure 4.28 Effect of pH on the sucrose utilization

(● no adjustment, ● ph 4, ● pH 4.5, ● pH 5, ● pH 5.5)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

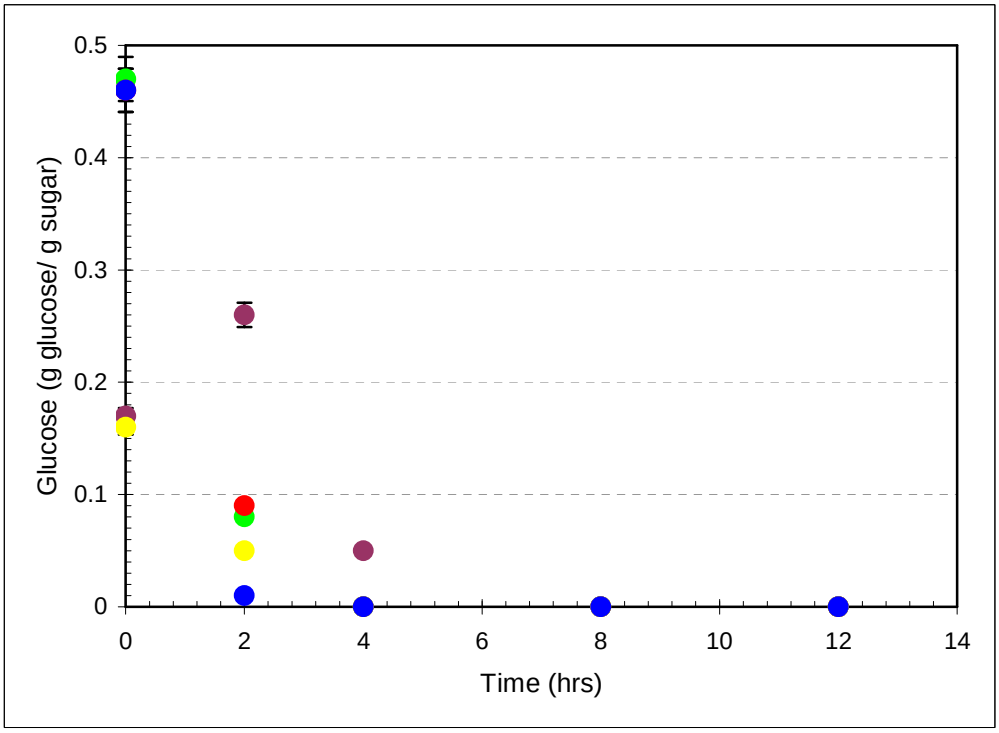

Figure 4.29 Effect of pH on the glucose utilization

(● no adjustment, ● ph 4, ● pH 4.5, ● pH 5, ● pH 5.5)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

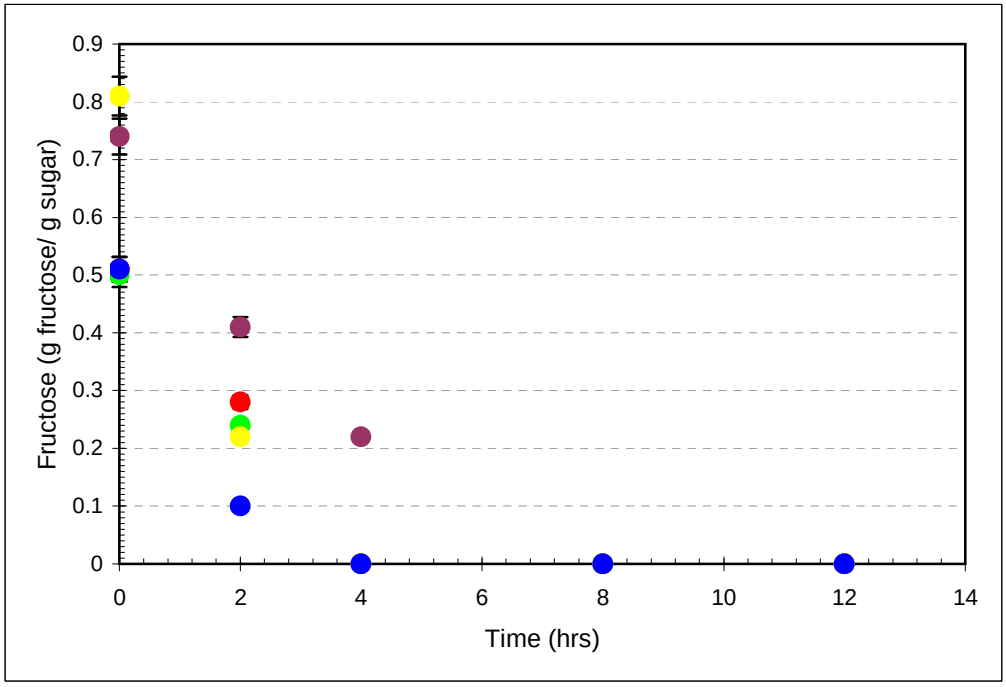

Figure 4.30 Effect of pH on the fructose utilization

(● no adjustment, ● ph 4, ● pH 4.5, ● pH 5, ● pH 5.5)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

From the figures it can be seen that varying the pH had no effect on the assimilation of the sugars. From Figure 4.28 it is seen that there is very little sucrose present which indicates that most of the sucrose has been converted to glucose and fructose which can be seen in Figure 4.29 and 4.30. All of the figures show that most of the sugars were consumed within 4 hours and that the yeast is able to utilize both glucose and fructose. The effect of pH on the ethanol yield and concentration is shown in Figure 4.31 and 4.32.

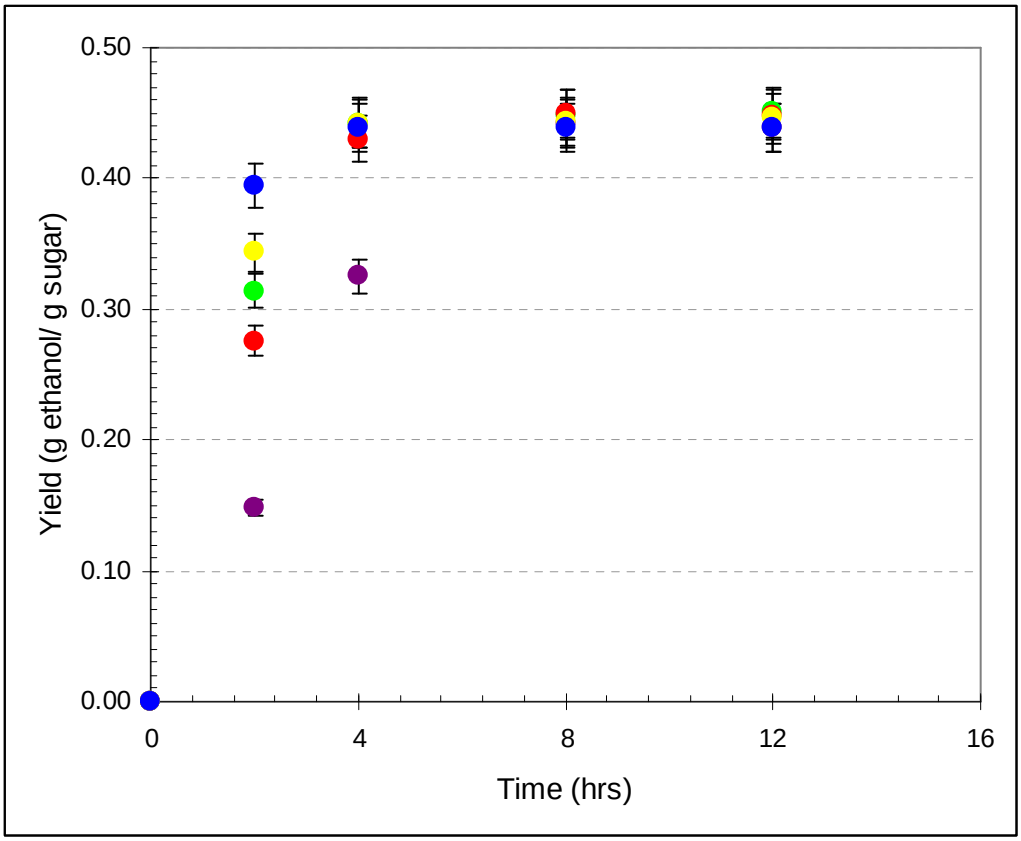

Figure 4.31 Effect of pH on the ethanol yield

(● no adjustment, ● ph 4, ● pH 4.5, ● pH 5, ● pH 5.5)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

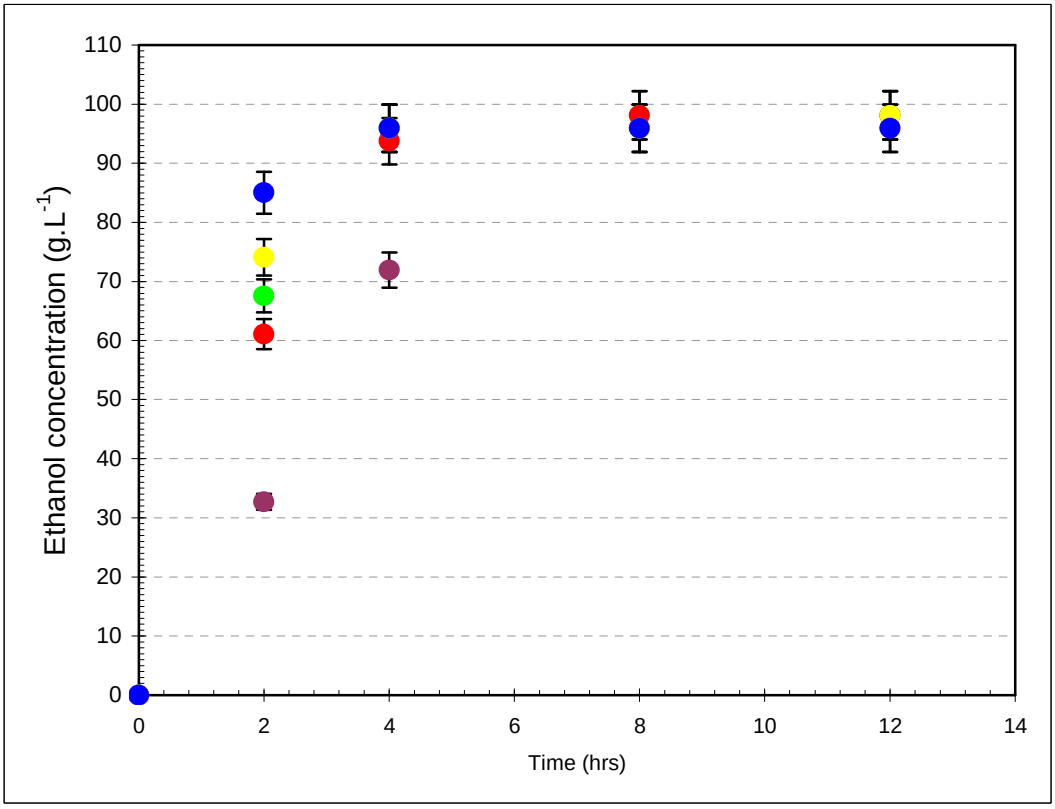

Figure 4.32 Effect of pH on ethanol concentration

(● no adjustment, ● ph 4, ● pH 4.5, ● pH 5, ● pH 5.5)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

From Figures 4.31 and 4.32 it can be seen that varying the pH did not have any significant effect on the ethanol yield and concentration. The maximum ethanol yield of 0.45 $g.g^{-1}$ and a concentration of 98.1 $g.L^{-1}$ were still achieved at all the pH values investigated. The effect of pH on the glycerol yield is shown in Figure 4.33

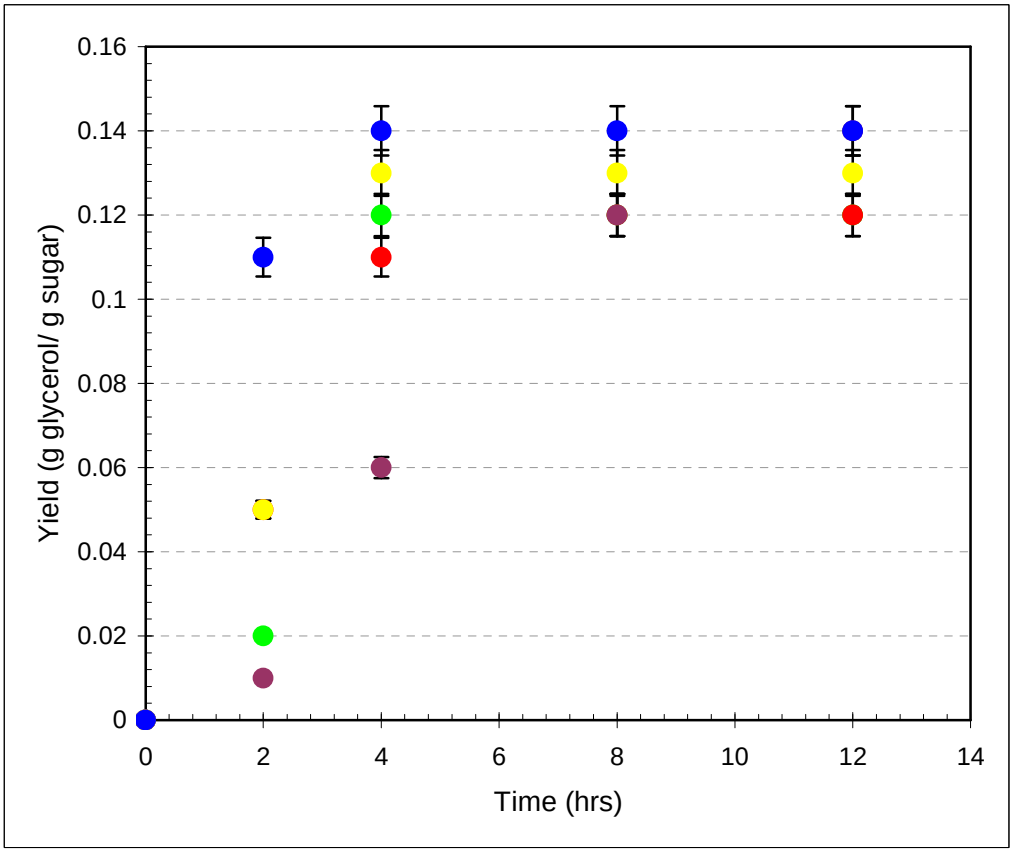

Figure 4.33 Effect of pH on the glycerol yield

(● no adjustment, ● ph 4, ● pH 4.5, ● pH 5, ● pH 5.5)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

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Figure 4.33 shows that varying the pH had no significant effect on the glycerol yield. All the pH values investigated gave similar glycerol yields with the maximum of 0.14 $g.g^{-1}$ been achieved at pH 5.5. The significant effect on the ethanol and glycerol yield is more clearly illustrated in Figure 4.34.

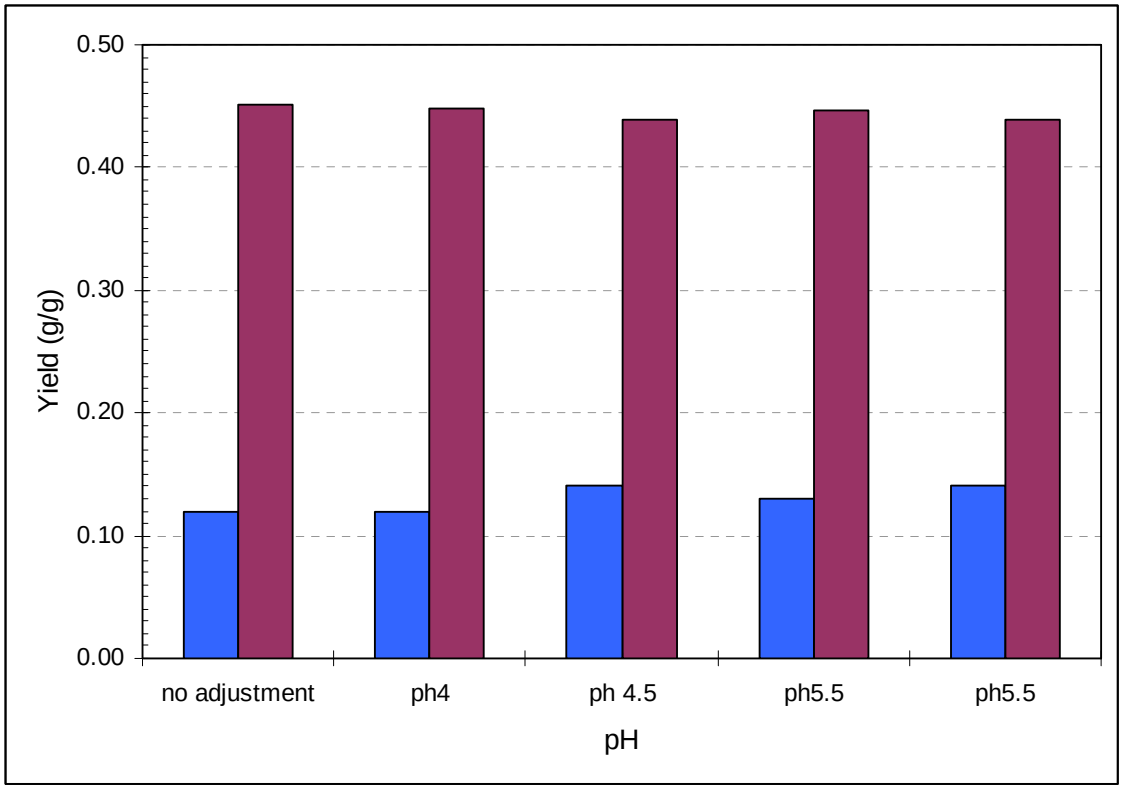

Figure 4.34 Effect of pH after 12 hours of fermentation

(■ Ethanol, ■ Glycerol)

(Initial sugar concentration 10.9% ($w.w^{-1}$), yeast concentration 1 $g.L^{-1}$)

Figure 4.34 shows that adjusting the pH did not have a significant effect on the ethanol yield. It is also seen that there was no significant effect on the glycerol production as seen in Figure 4.31. Ogbonna *et al* (2001) also found that adjusting the pH of the juice had no effect on the ethanol yield.

Therefore, adjusting the pH of the juice prior to fermentation is not necessary but it might be beneficial to control the pH as it could reduce the risk of contamination (Ortiz-Muniz *et al.,* 2010). Therefore, the juice could be adjusted to any pH between 4 to 5.5.

4.3 Concluding Remarks

This study found that the manipulation of some fermentation variables had an effect on the ethanol yield. It was found that the yeast is able to tolerate the sugar concentration of the juice and that diluting the juice caused the ethanol yield to decrease. Therefore, it was concluded that dilution is not necessary. This study showed adjusting the pH had no significant effect on the ethanol yield, but to reduce the risk of contamination the pH can be adjusted to 4. The best conditions for fermentation of the raw juice were: pH 4, yeast concentration 5 $g.L^{-1}$ and the addition of ammonium sulphate at a concentration of 750 mg $N.L^{-1}$. This study showed that tropical sugar beet has the potential to be an excellent feedstock for ethanol production. It was determined that from 1 ton of sugar beet 126 L of ethanol can be obtained.

4.4 References

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Chapter 5

Conclusions and Recommendations

Overview

In this study the potential of tropical sugar beet as a feedstock for ethanol production was investigated. This study investigated the manipulation of fermentation variables such as dilution ratio, pH, yeast concentration and the addition of a nitrogen source. From this study some conclusions and recommendations were reached which will be presented in section 5.1 and section 5.2 respectively.

5.1 Conclusions

- This study showed that tropical sugar beet has a high sugar content which could be converted to ethanol.
- The sugar content of the juice was determined to be 21.8% ($g.g^{-1}$)
- It was found that dilution did have an effect on the ethanol yield and diluting the juice caused the ethanol yield to decrease.
- It was concluded that the yeast is able to tolerate the sugar concentration of 21.8 % ($g.g^{-1}$) and, therefore, no dilution is necessary.
- It was found that increasing the yeast concentration did affect the ethanol yield and reduced the fermentation time.
- The optimal yeast concentration was determined to be 5 $g.L^{-1}$.
- Adding nitrogen sources such as urea, peptone, yeast extract and ammonium sulphate had a positive effect on the ethanol yield and also reduced the amount of glycerol formed.
- Therefore, adding a nitrogen source will be beneficial to ethanol production.

- The optimal ammonium sulphate concentration was found to be 750 mg $N.L^{-1}$.
- Adjusting the pH of the juice prior to fermentation is not necessary as no significant effect to the ethanol yield was seen.
- Controlling the pH is beneficial to ethanol production as the risk of contamination could be reduced.
- Therefore, from 1 ton of sugar beet 126 L of ethanol can be obtained

5.2 Recommendations

There is still a need for further investigation into the use of tropical sugar beet and this should focus on the following

- The hydrolysis and fermentation of the tropical sugar beet pulp.
- The fermentation of other intermediate products associated with sugar beet processing such as thin and thick juice and molasses.
- The use of other microorganisms in ethanol production that are able to ferment a wider variety of sugars.

Appendix A

HPLC Analysis

Overview

This appendix describes the preparation of calibration curves for the determination of the sugar and ethanol content of an unknown sample. It also describes the calculations used to determine the ethanol yield. Section A1 describes the construction of the sugar standards, section A2 the construction of the ethanol standards and section A3 the construction of glycerol standards. Section A4 describes the calculations used to determine the ethanol yield.

In order to use high performance liquid chromatography for analysis, calibration curves needed to be constructed. Standard mixtures were prepared by combining two substances together into one standard for example sucrose and glucose. The standards were analyzed by HPLC and the response peak areas of the two components present in each standard mixture were reported and compared. A peak area ratio was then calculated and this ratio was plotted against the weight percentage ratio of the two standards. A straight line was fitted to the data and a constant (k) was obtained which was used in the calculation of the sugar and ethanol yields (Section A4).

A1 Sugar calibration curves

The sugars that are available for fermentation in tropical sugar beet juice are sucrose, glucose and fructose. The way in which the sugar standards were prepared, are presented in Table A1

Table A1: Preparation of sugar standards

Sugar A (g)	Sugar B (g)	Water (g)	Total (g)
0.025	0.15	0.325	0.5
0.05	0.125	0.325	0.5
0.075	0.075	0.35	0.5
0.1	0.1	0.3	0.5
0.125	0.05	0.325	0.5
0.15	0.025	0.325	0.5

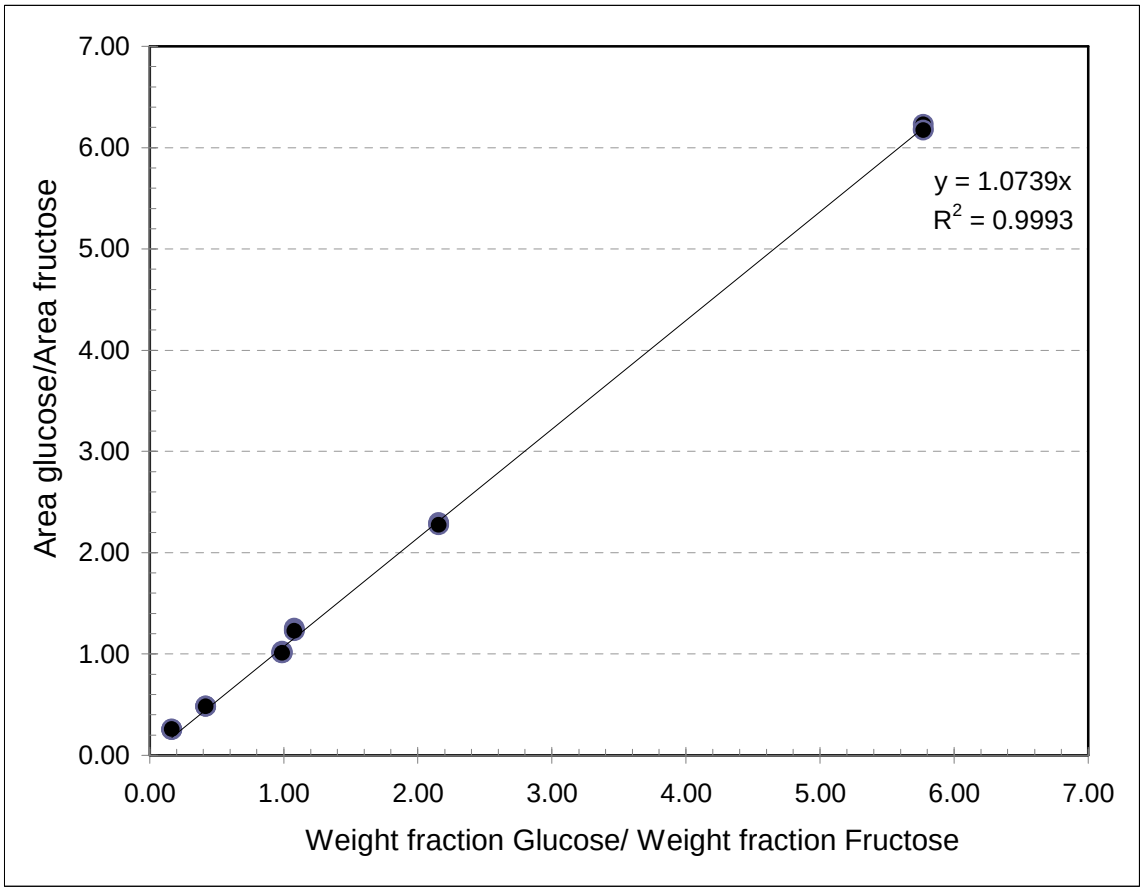

Figure A.1 Glucose/Fructose calibration curve

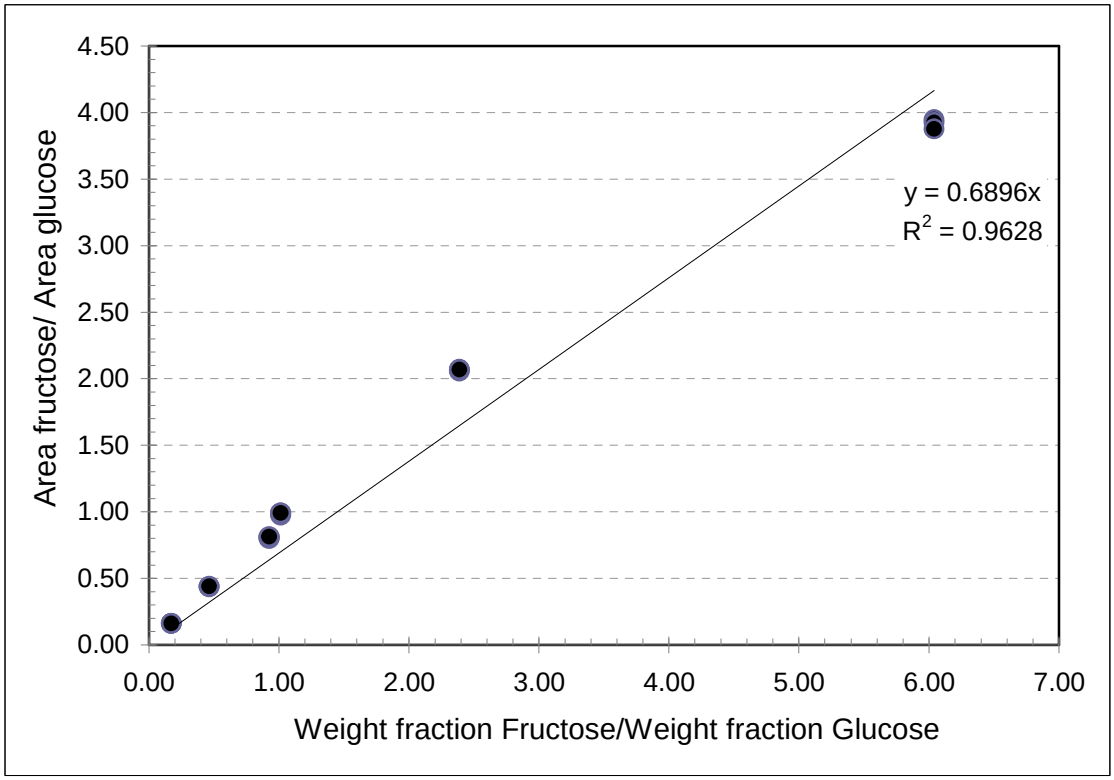

Figure A.2 Fructose/Glucose calibration curve

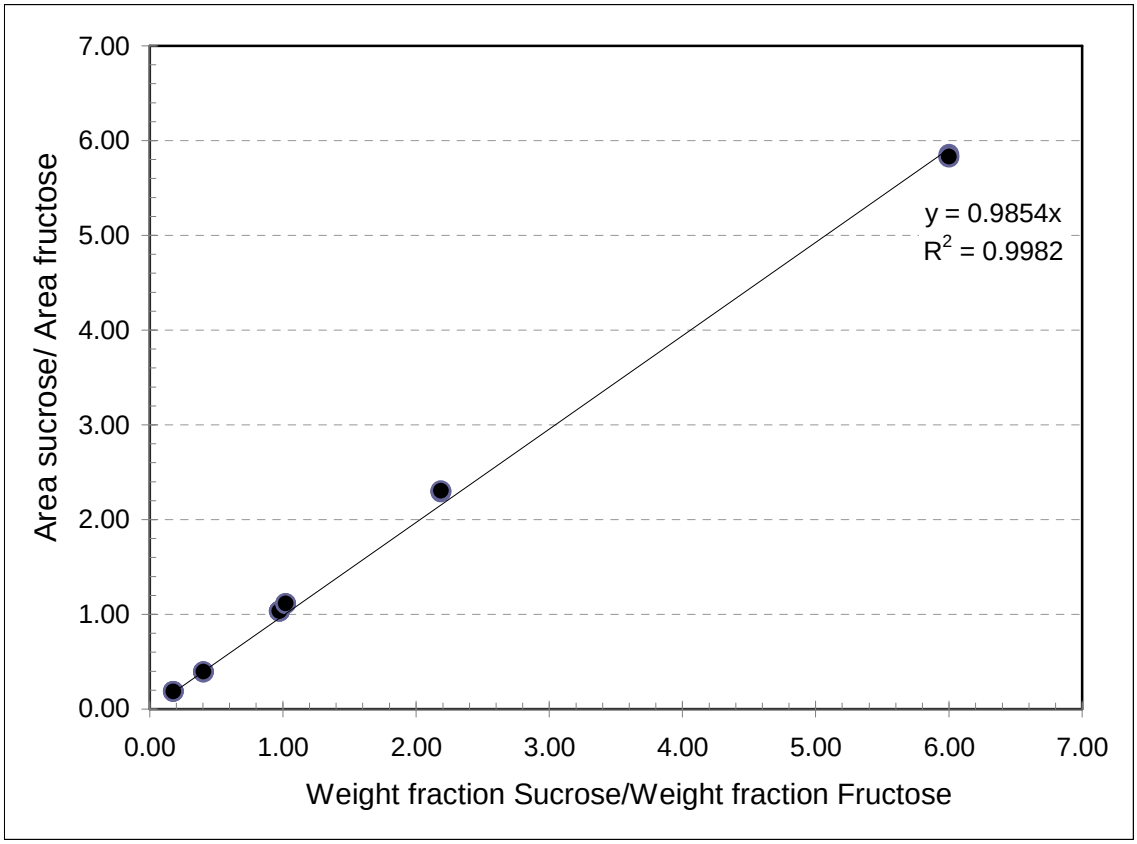

Figure A.3 Sucrose/Fructose calibration curve

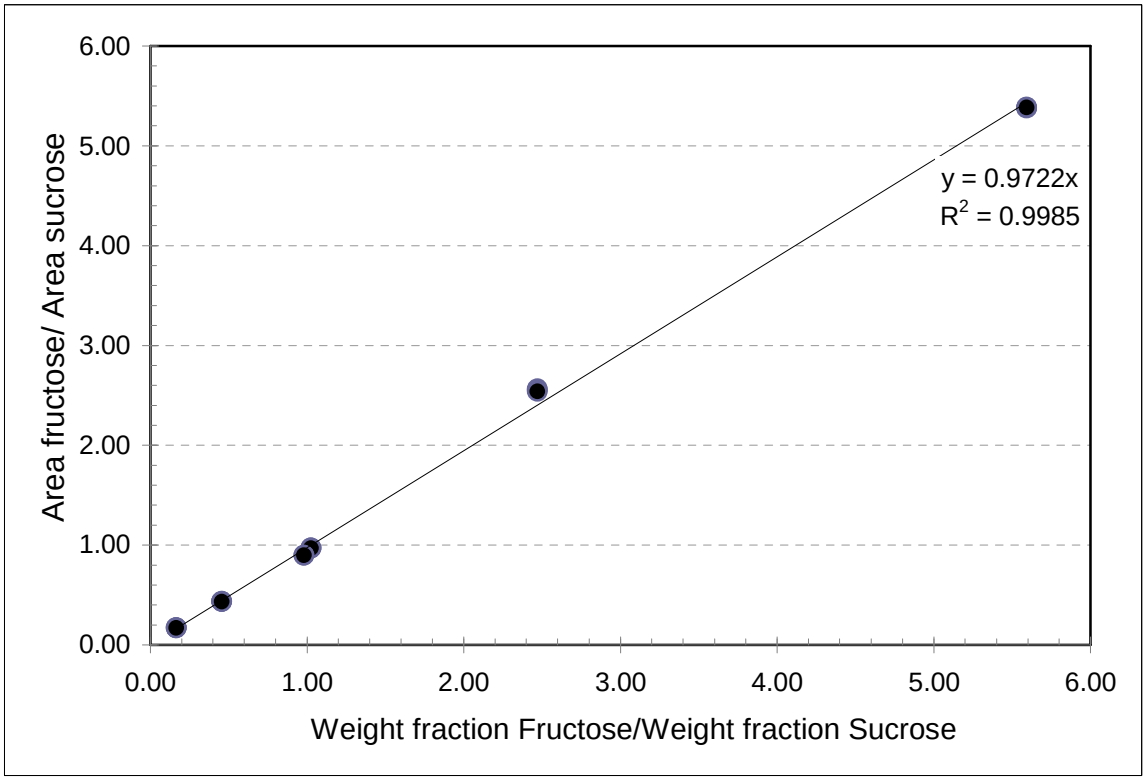

Figure A.4 Fructose/Sucrose calibration curve

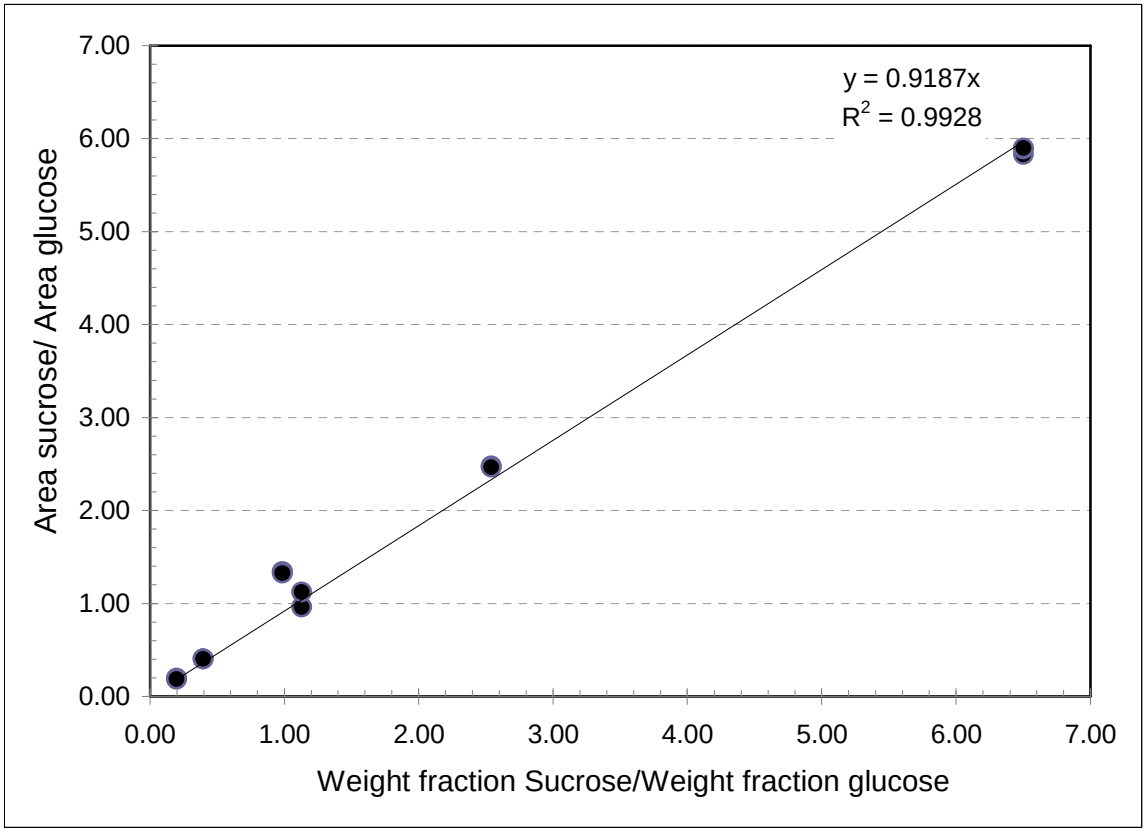

Figure A.5 Sucrose/Glucose calibration curve

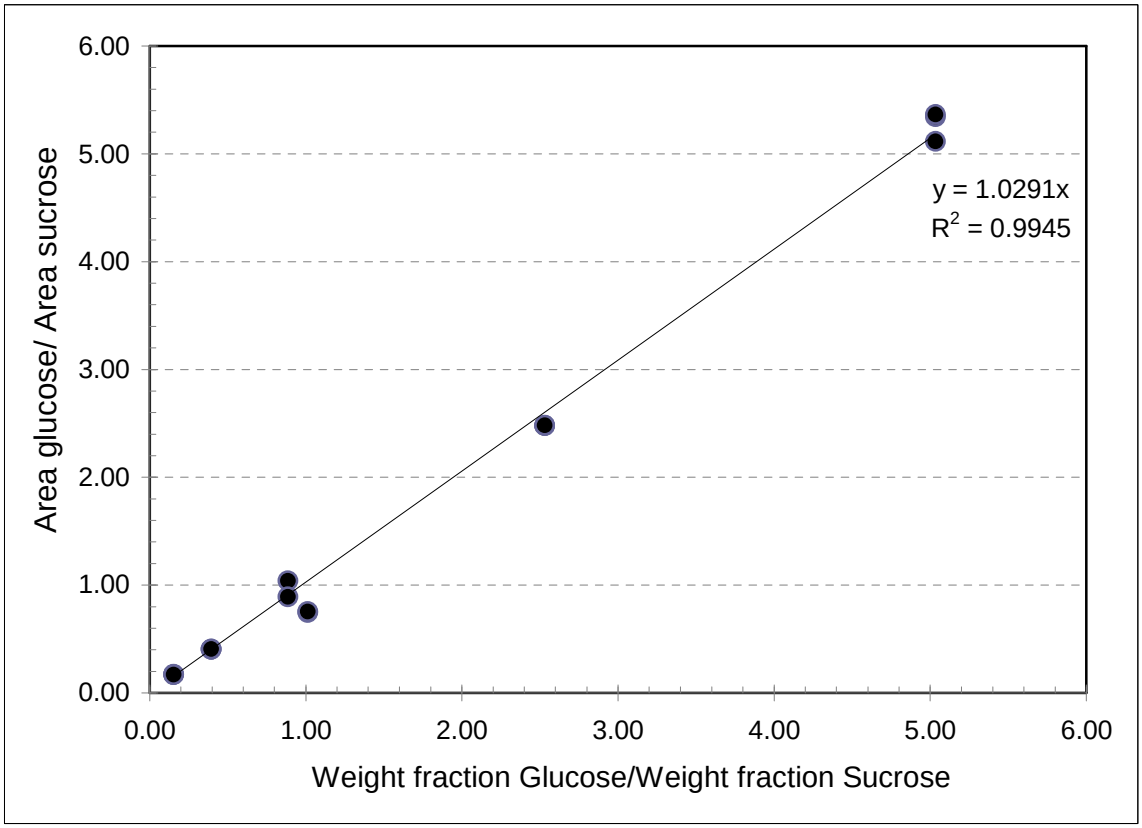

Figure A.6 Glucose/Sucrose calibration curve

A2 Ethanol calibration curves

The ethanol standard was prepared with each sugar available for fermentation. The method of preparation is presented in Table A 2.

Table A2 Preparation of ethanol standard

Sugar(g)	Ethanol (g)	Water (g)	Total (g)
0	0.5	0.5	1.00
0.025	0.475	0.475	1.00
0.05	0.45	0.45	1.00
0.075	0.425	0.425	1.00
0.1	0.4	0.4	1.00
0.125	0.375	0.375	1.00
0.15	0.35	0.35	1.00
0.175	0.325	0.325	1.00

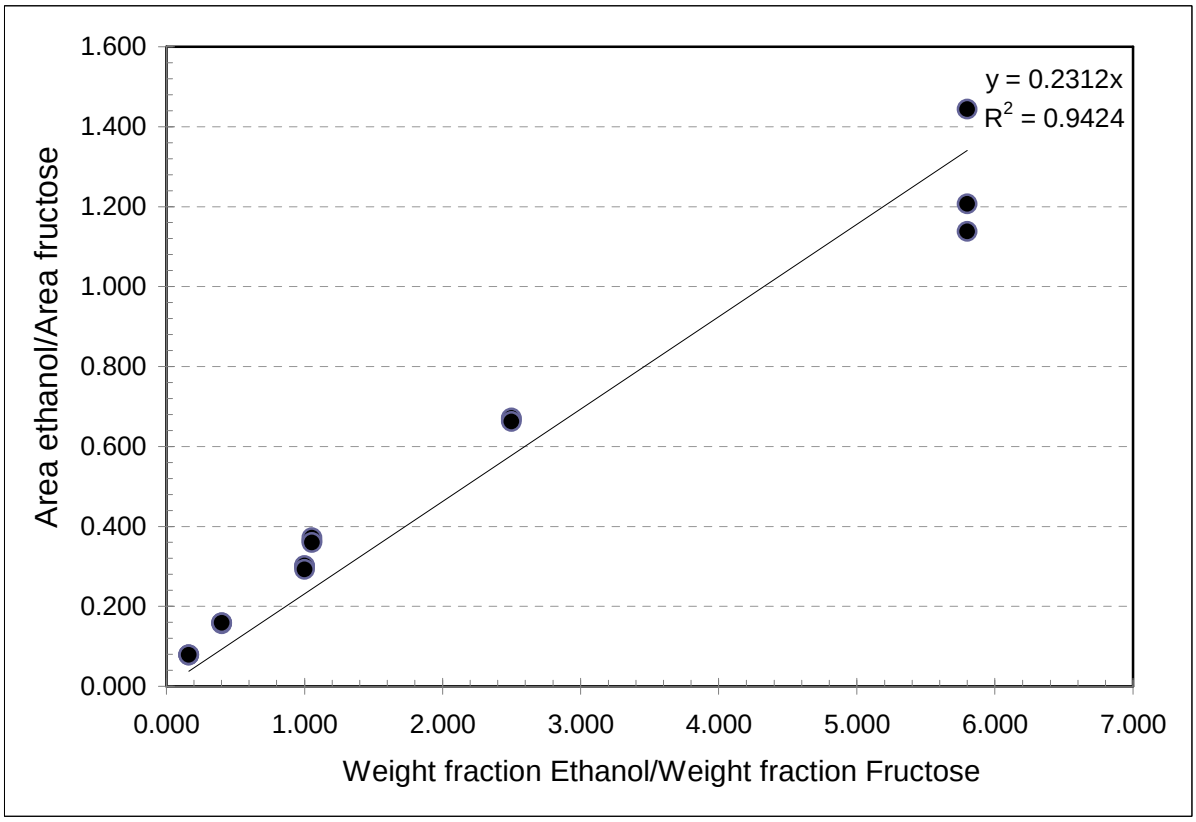

Figure A.7 Ethanol/Fructose calibration curve

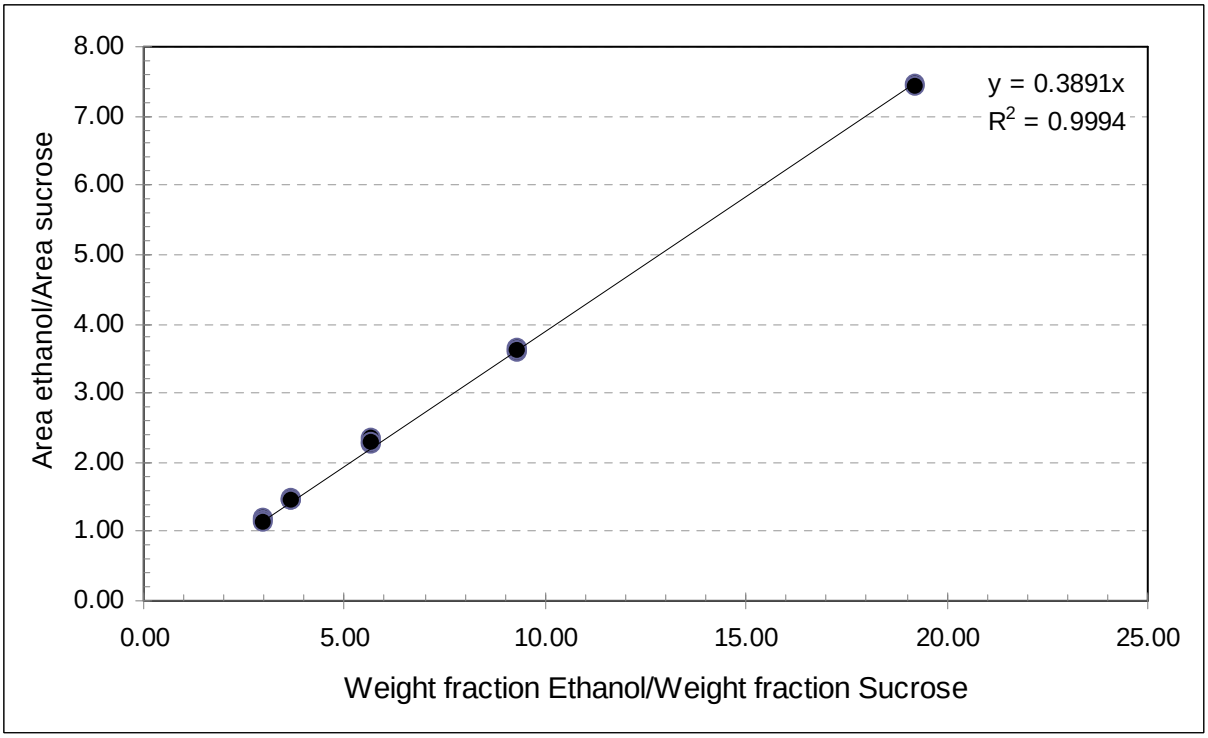

Figure A.8 Ethanol/Sucrose calibration curve

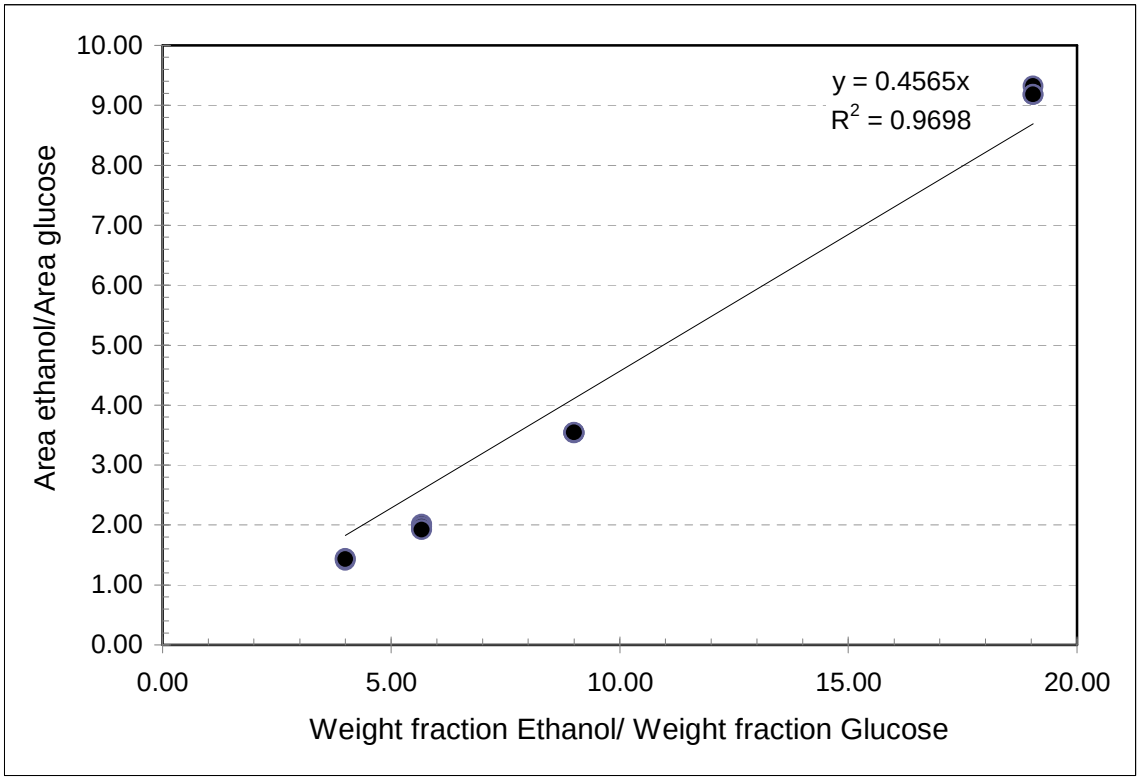

Figure A9 Ethanol/Glucose calibration curve

A3 Glycerol Standards

Glycerol standards were prepared with each sugar and ethanol. The method of preparation is presented in Table A3

Table A3: Preparation of glycerol standards

Sugar/Ethanol (g)	Glycerol (g)	Water (g)	Total (g)
0.025	0.15	0.325	0.5
0.05	0.125	0.325	0.5
0.075	0.075	0.35	0.5
0.10	0.1	0.3	0.5
0.125	0.05	0.325	0.5
0.15	0.15	0.325	0.5

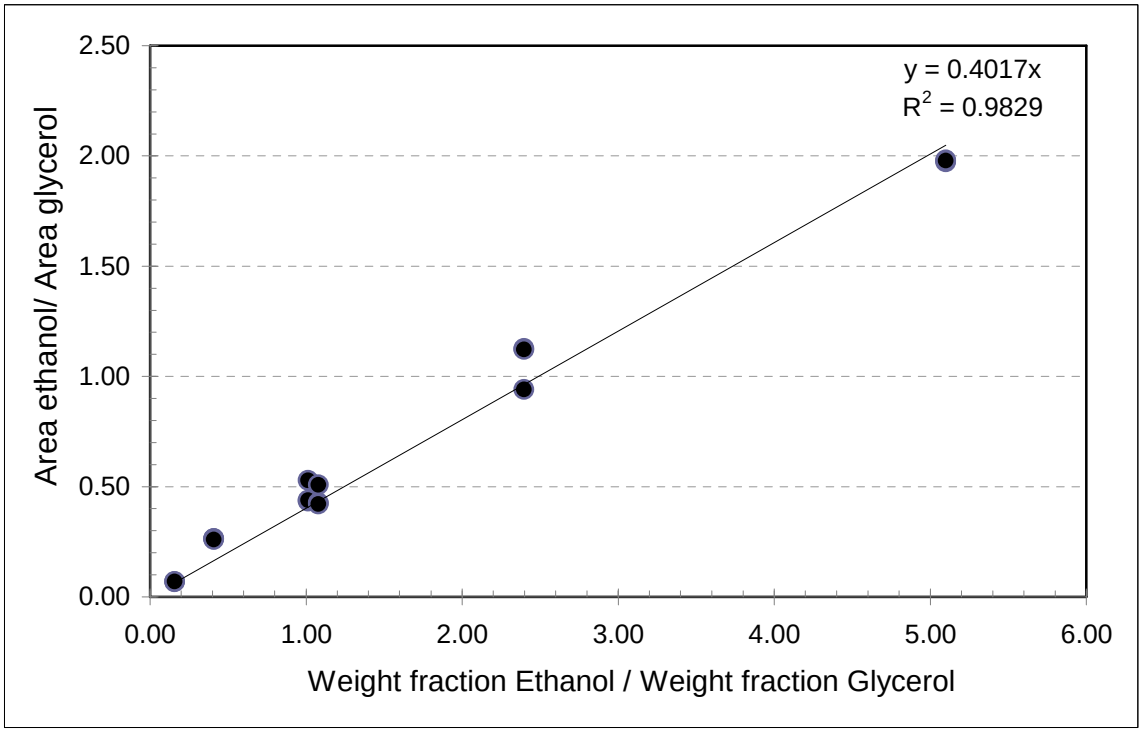

Figure A10 Ethanol/Glycerol calibration curve

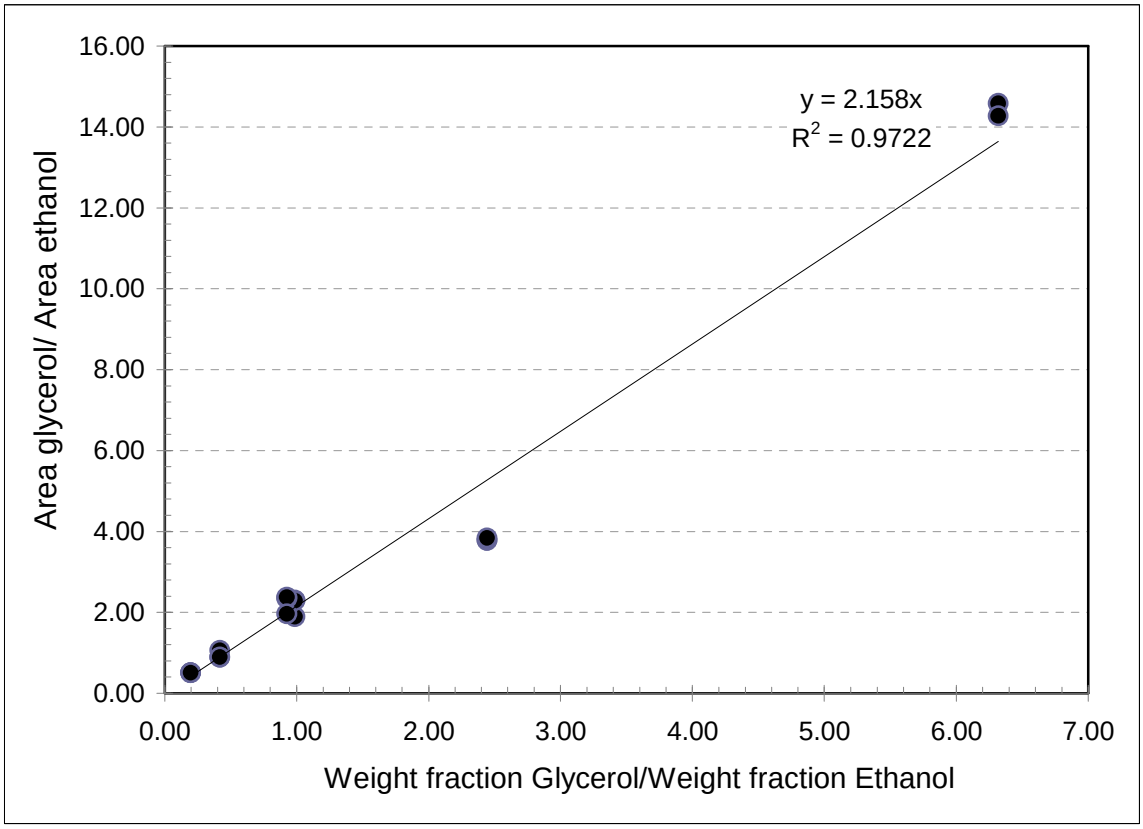

Figure A11 Glycerol/Ethanol calibration curve

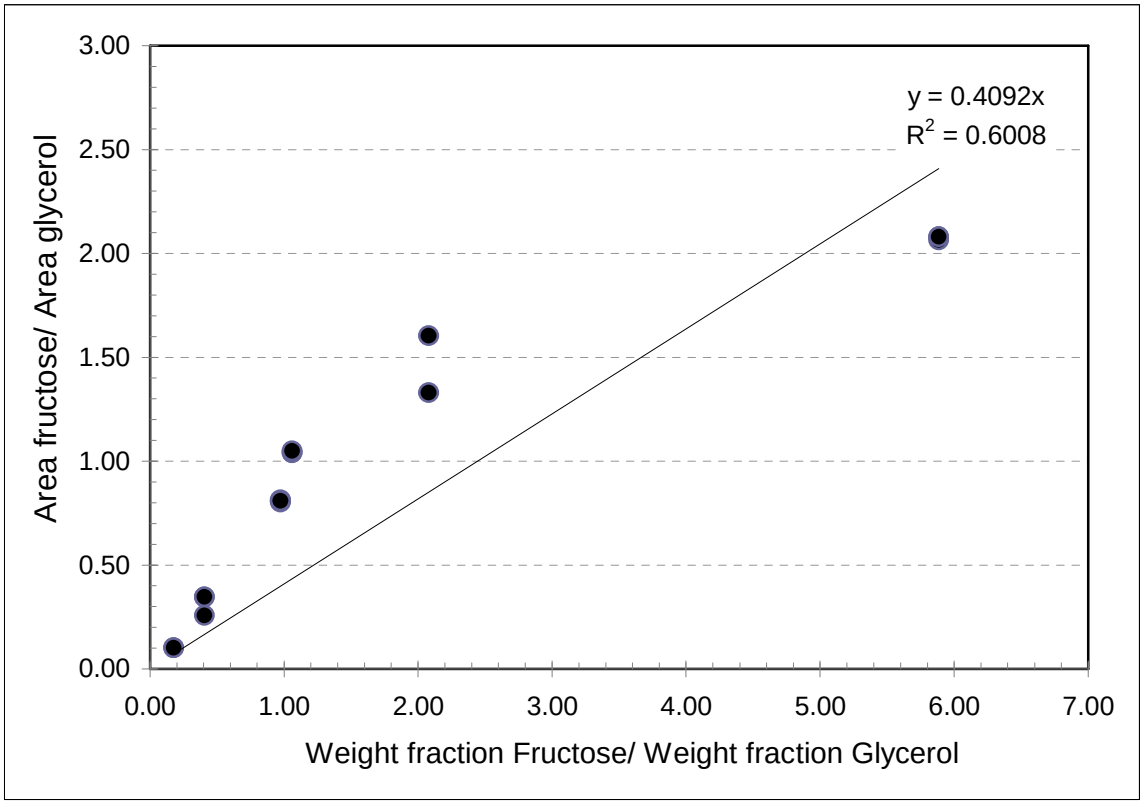

Figure A12 Fructose/Glycerol calibration curve

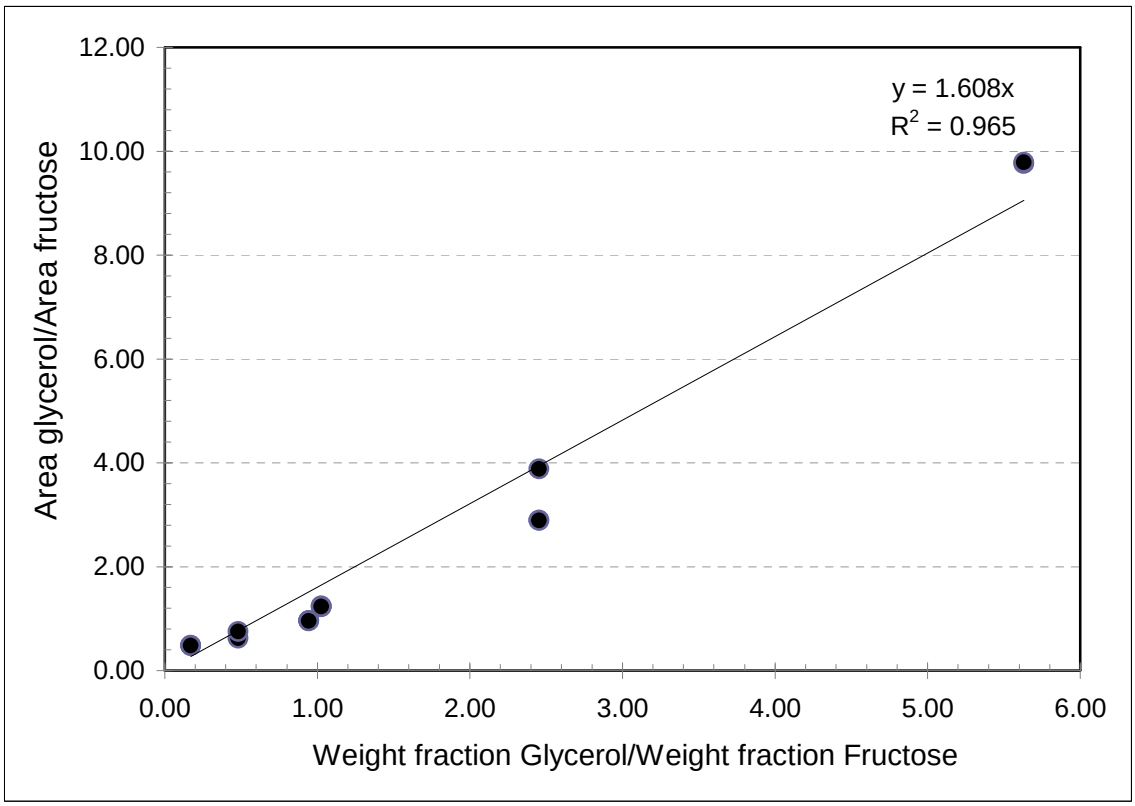

Figure A13 Glycerol/Fructose calibration curve

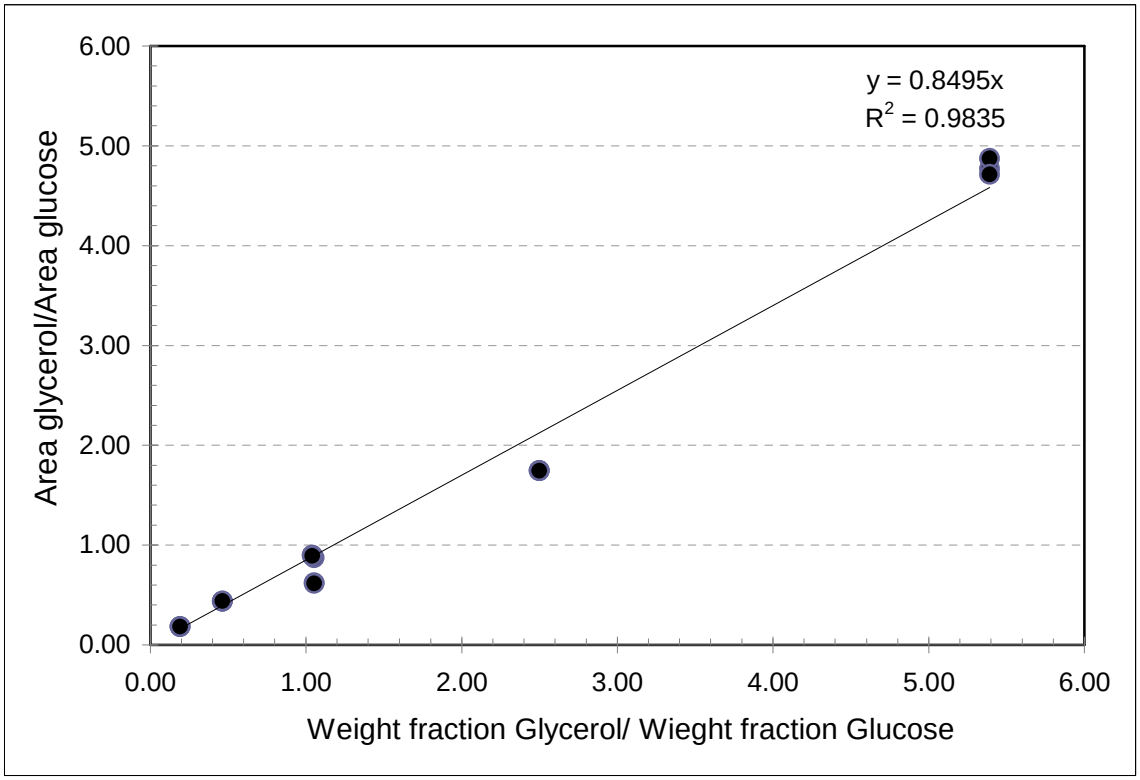

Figure A14 Glycerol/Glucose calibration curve

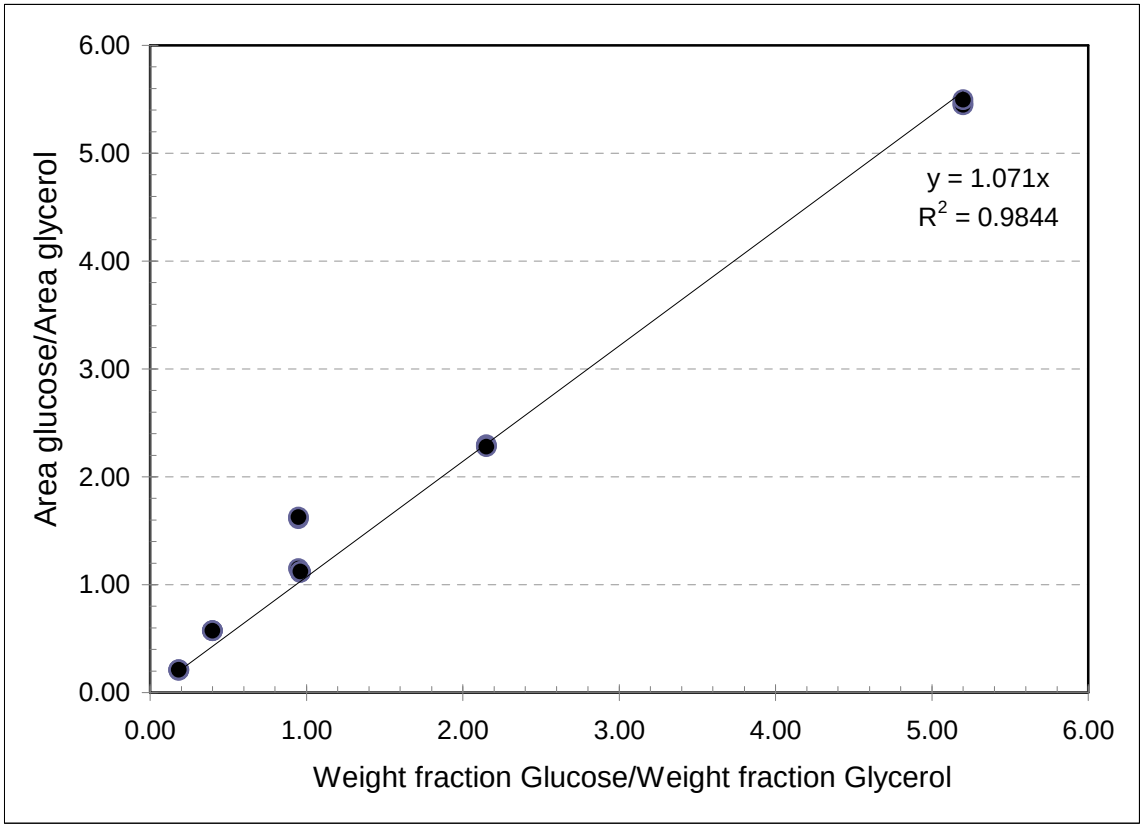

Figure A15 Glucose/Glycerol calibration curve

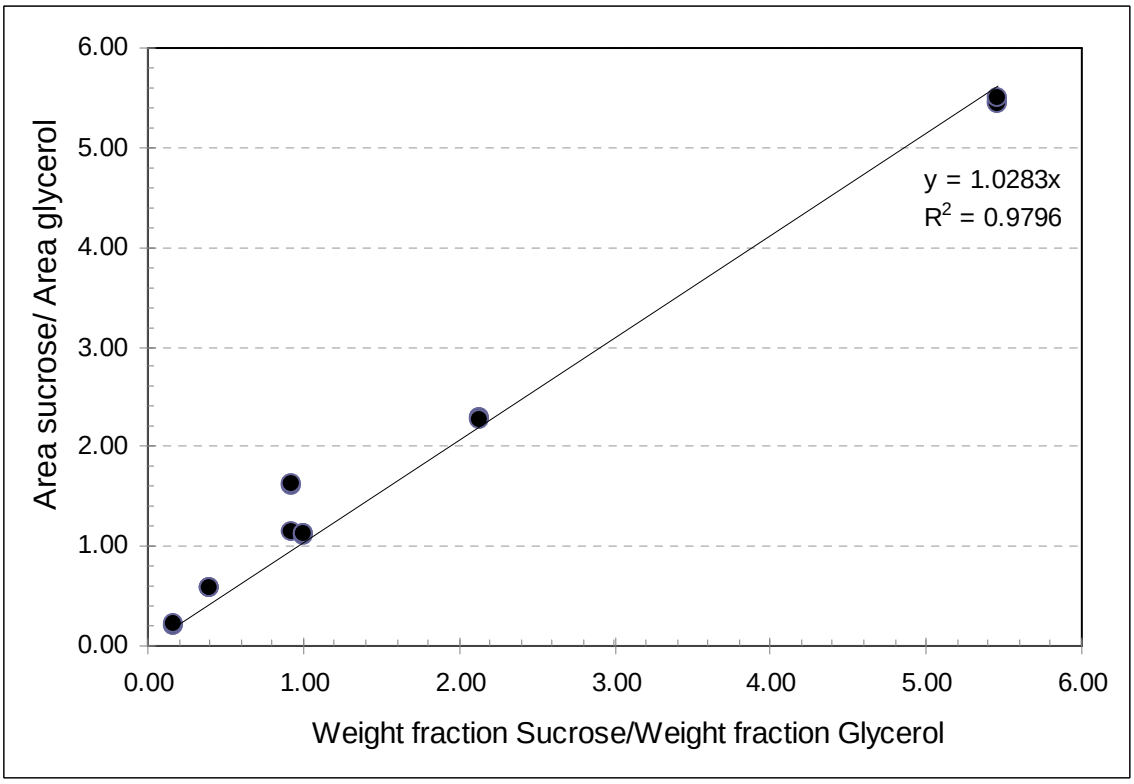

Figure A16 Sucrose/Glycerol calibration curve

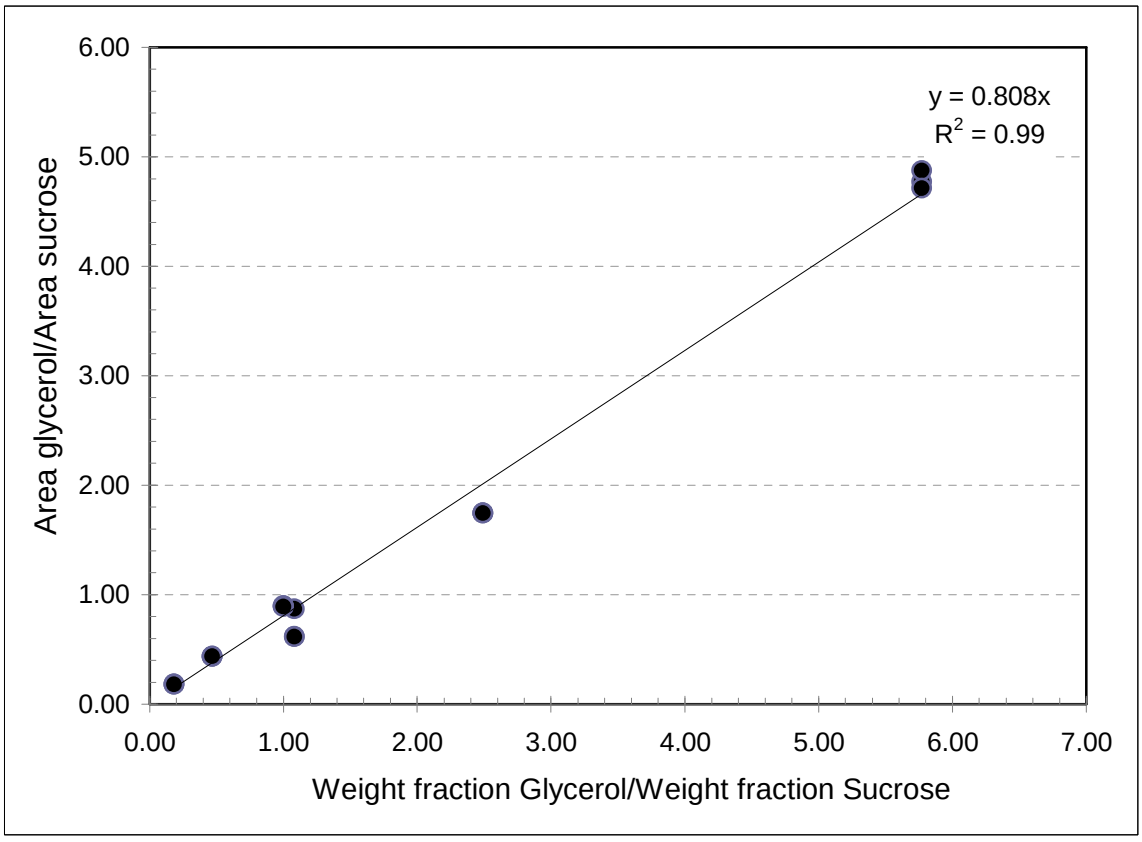

Figure A17 Glycerol/Sucrose calibration

A4 Calculations

From the calibration curves the constant (k) was obtained for each standard and is presented in Table A4

Table A4: Constant (k) obtained from calibration curves

Standard	Constant (k)
Ethanol/Fructose	0.231
Fructose/ Sucrose	0.972
Fructose/Glucose	0.689
Ethanol/ Sucrose	0.389
Glucose/Sucrose	1.029
Ethanol/Glucose	0.456
Glucose/Fructose	1.073
Sucrose/ Glucose	0.918
Ethanol/ Glycerol	0.401
Glycerol/Fructose	1.608
Glycerol/Sucrose	0.808
Glycerol/Glucose	0.849
Fructose/Glycerol	0.409
Sucrose/Glycerol	1.028
Glucose/Glycerol	1.071
Sucrose/ Fructose	0.985

The following equation was used to determine the ethanol and sugar content of each sample

$$x_1 + x_2 + x_3 + x_4 + x_8 = 1$$

where

x_1 is the ethanol + CO_2 mass fraction

x_2 is the fructose mass fraction

x_3 is the sucrose mass fraction

x_4 is the glucose mass fraction

x_8 is the glycerol mass fraction

Therefore each component could be determined by rearranging the equation
For example the mass fraction of fructose can be determined as follows:

$$\frac{X_1}{X_2} + \frac{X_2}{X_2} + \frac{X_3}{X_2} + \frac{X_4}{X_2} + \frac{X_8}{X_2} = \frac{1}{X_2}$$

Therefore

$$x_2 = \frac{1}{1 + \frac{X_1}{X_2} + \frac{X_3}{X_2} + \frac{X_4}{X_2} + \frac{X_8}{X_2}}$$

But

$$\frac{X_1}{X_2} = \frac{1}{k_1} \times \frac{\text{Peak area of ethanol}}{\text{Peak area of fructose}}$$

Therefore

$$X_2 = \frac{1}{1 + (\frac{1}{k_1}) \times \frac{\text{Peak area of ethanol}}{\text{Peak area of fructose}}}$$

The same principle applies to the other ratios.

The ethanol and CO_2 mass fraction indicates the conversion efficiency of the process but to determine the ethanol yield the following equation was used.

Ethanol yield ($Y_{p/s}$) = $\frac{M_r \text{ Ethanol}}{M_r \text{ Ethanol} + CO_2}$ x Mass fraction

CO2 yield ($Y_{CO2/s}$) = $\frac{M_r \ CO_2}{M_r \text{Ethanol} + CO_2}$ x Mass fraction

Where

M_r Ethanol (molecular mass) = 46 $g.mol^{-1}$

M_r CO_2 (molecular mass) = 44 $g.mol^{-1}$

M_r Ethanol + CO_2 (molecular mass) = 90 $g.mol^{-1}$

Ethanol (g) = Ethanol yield (g ethanol/ g sugar) x initial sugar (g)

Ethanol concentration ($g.L^{-1}$) = $\frac{\text{g ethanol}}{\text{volume (L)}}$

Appendix B

Experimental error

Overview

In this appendix the experimental error associated with each fermentation variable is given. The appendix is divided into two sections: In section B1 the equations used to calculate the experimental error is shown and in section B2 the experimental error for each fermentation variable will be given.

B1 Experimental error equations

The experimental error associated with each variable was calculated using the following principles (Varderman,1994)

Average ($\bar{x}$): Also known as the arithmetic mean, it is calculated by adding all the values together and dividing by the number of samples.

$$\bar{x} = \frac{1}{n} \cdot \sum_{i=1}^{n} x_i \qquad (1)$$

Standard deviation (σ): It is a measurement that shows how much the data varies or is dispersed from the mean.

$$\sigma = \sqrt{\frac{\Sigma(\bar{x} - x)^2}{n - 1}} \qquad (2)$$

Confidence limit (±): It is the upper and lower values of the confidence interval. The confidence interval is an estimated range of values which is likely to include the sample mean.

$$95\% \text{ confidence level} = 1.96 \, (\sigma/\sqrt{n}) \qquad (3)$$

Experimental Error: It is the discrepancy between an exact value and some approximation to it

$$\% \text{ Error} = \frac{\text{Confidence level}}{\bar{x}} \times 100 \qquad (4)$$

B2 Experimental error

The experimental error for each fermentation variable was determined by repeating each experiment three times. Using the equations as discussed in section B1 the experimental error could be calculated.

The experimental error associated with dilution was determined by repeating the dilution ratio of 1:1 three times at a pH 4.5, yeast concentration of 1 $g.L^{-1}$ and a temperature of 30 ˚C. The ethanol yields ($g.g^{-1}$), obtained at different time intervals for the three experiments are presented in Table B1. The statistical parameters calculated from this data are presented in Table B2. It should be noted that the experimental error was calculated using the ethanol yield obtained after 8 hours of fermentation.

Table B1 Ethanol yield ($g.g^{-1}$) for repeated dilution ratio experiments

Time (hours)	Experiment 1	Experiment 2	Experiment 3
0	0	0	0
2	0.15	0.08	0.08
4	0.33	0.20	0.18
8	0.44	0.49	0.48
12	0.44	0.49	0.48
24	0.43	0.48	0.51

Table B2 Statistical parameters used to calculate experimental error of dilution ratio

	Ethanol yield ($g.g^{-1}$)
Experiment 1	0.44
Experiment 2	0.49
Experiment 3	0.48
Mean	0.47
Standard deviation	0.03
Confidence limit (95%)	0.03
% error	6.37

The experimental error of pH was determined by repeating the experiment three times at the pH of 4, a dilution ratio of 1:1, yeast concentration of 1 $g.L^{-1}$ and a temperature of 30 ˚C. The ethanol yield ($g.g^{-1}$) of each experiment at different time intervals is given in Table B3. The statistical parameters calculated from this data are presented in Table B4

Table B3 Ethanol yield ($g.g^{-1}$) for repeated pH experiments

Time (hours)	**Experiment 1**	**Experiment 2**	**Experiment 3**
0	0	0	0
2	0.28	0.08	0.08
4	0.43	0.14	0.14
8	0.45	0.48	0.48
12	0.45	0.46	0.45

Table B4 Statistical parameters used to calculate experimental error of pH

	Ethanol yield ($g.g^{-1}$)
Experiment 1	0.45
Experiment 2	0.48
Experiment 3	0.48
Mean	0.47
Standard deviation	0.02
Confidence limit (95%)	0.02
% error	4.17

The experimental error associated with yeast concentration was determined by repeating the experiment three times at the optimal yeast concentration of 5 $g.L^{-1}$, dilution ratio 1:1, pH 4 and a temperature of 30˚C. The ethanol yield ($g.g^{-1}$) obtained from each experiment at different time intervals are given in Table B5. The statistical parameters calculated from the data are presented in Table B5

Table B5 Ethanol yield ($g.g^{-1}$) for repeated yeast concentration experiments

Time (hours)	**Sample 1**	**Sample 2**	**Sample 3**
0	0	0	0
2	0.19	0.23	0.24
4	0.48	0.40	0.43
8	0.45	0.44	0.43
12	0.45	0.45	0.44
24	0.33	0.44	0.41

Table B6 Statistical parameters used to calculate experimental error of yeast concentration

	Ethanol yield ($g.g^{-1}$)
Experiment 1	0.45
Experiment 2	0.44
Experiment 3	0.43
Mean	0.44
Standard deviation	0.01
Confidence limit (95%)	0.01
% error	2.57

The experimental error associated with the addition of a nitrogen source was determined by repeating the experiment three times at the optimal concentration of 750 mg $N.L^{-1}$ ammonium sulphate, dilution ratio 1:1, pH 4, yeast concentration of 5 $g.L^{-1}$ and a temperature of 30 ˚C. The ethanol yield ($g.g^{-1}$) at different time intervals is presented in Table B7. The statistical parameters calculated from the data are presented in Table B8

Table B7 Ethanol yield ($g.g^{-1}$) for repeated addition of ammonium sulphate experiments

Time (hours)	**Experiment 1**	**Experiment 2**	**Experiment 3**
0	0	0	0
2	0.26	0.29	0.23
4	0.45	0.46	0.45
8	0.46	0.46	0.45
12	0.42	0.46	0.45
24	0.45	0.45	0.45

Table B8 Statistical parameters used to calculate the experimental error of addition of ammonium sulphate

	Ethanol yield ($g.g^{-1}$)
Experiment 1	0.46
Experiment 2	0.46
Experiment 3	0.45
Mean	0.46
Standard deviation	0.01
Confidence limit (95%)	0.01
% error	1.42

B3 Reference

Vardeman, S. B. 1994. Statistics for engineering problem solving. Boston: PSWPublishing Company, 712 p.

Appendix C

Experimental data

Overview

In this appendix all the experimental data obtained during this investigation is presented. The ethanol, CO_2, sucrose, glucose, fructose and glycerol mass fractions obtained for each fermentation variable is presented. The experimental data obtained from the investigation into the effect of dilution ratio is given in section C1 while section C2 presents all the data obtained from investigating the effect of pH. Section C3 gives the data obtained from investigating the effect of yeast concentration and Section C4 gives the data obtained from investigating the effect of adding a nitrogen source.

C1 Effect of dilution ratio

Table C1 Yields ($g.g^{-1}$) obtained from fermentation with no dilution

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.02	0.47	0.51	0
2	0.08	0.08	0.02	0.36	0.47	0
4	0.17	0.17	0.03	0.33	0.25	0.06
8	0.45	0.43	0.01	0	0	0.11
12	0.44	0.43	0	0	0	0.13
24	0.47	0.45	0	0	0	0.08

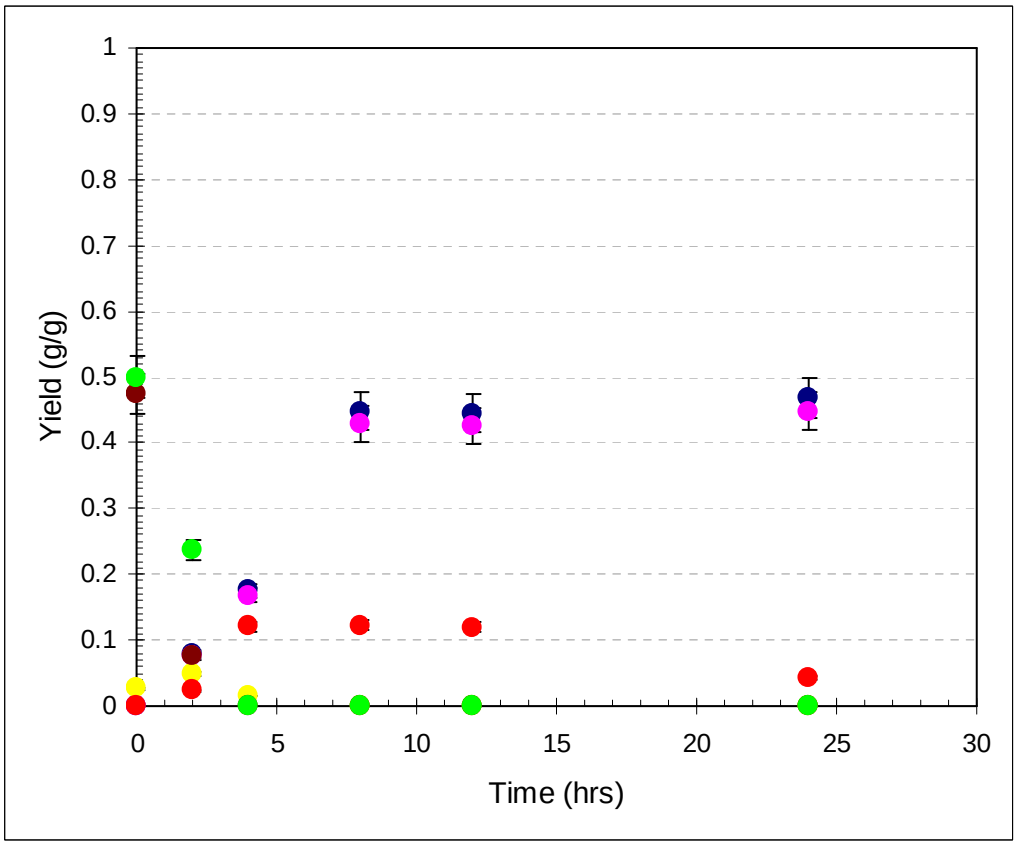

Figure C1 Fermentation with no dilution (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C2 Yields ($g.g^{-1}$) obtained from fermentation at a dilution ratio 1:1

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.09	0.17	0.74	0
2	0.15	0.14	0.03	0.26	0.41	0.01
4	0.33	0.31	0.03	0.05	0.22	0.06
8	0.44	0.42	0.01	0	0	0.12
12	0.44	0.42	0	0	0	0.14
24	0.43	0.41	0	0	0	0.16

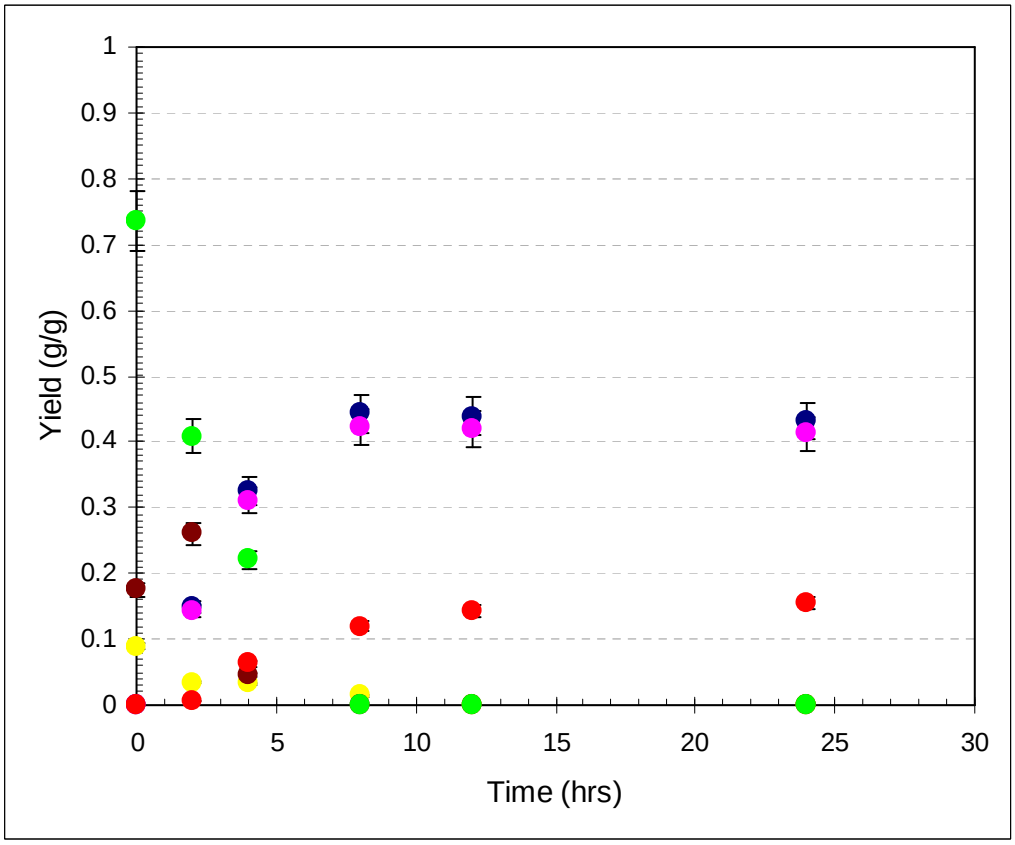

Figure C2 Fermentation at a dilution ratio 1:1 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C3 Yields ($g.g^{-1}$) obtained from fermentation at a dilution ratio of 1:2

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.04	0.47	0.49	0
2	0.07	0.06	0.05	0.46	0.34	0.02
4	0.33	0.31	0.06	0.01	0	0.29
8	0.43	0.41	0	0	0	0.16
12	0.42	0.41	0	0	0	0.17
24	0.42	0.40	0	0	0	0.18

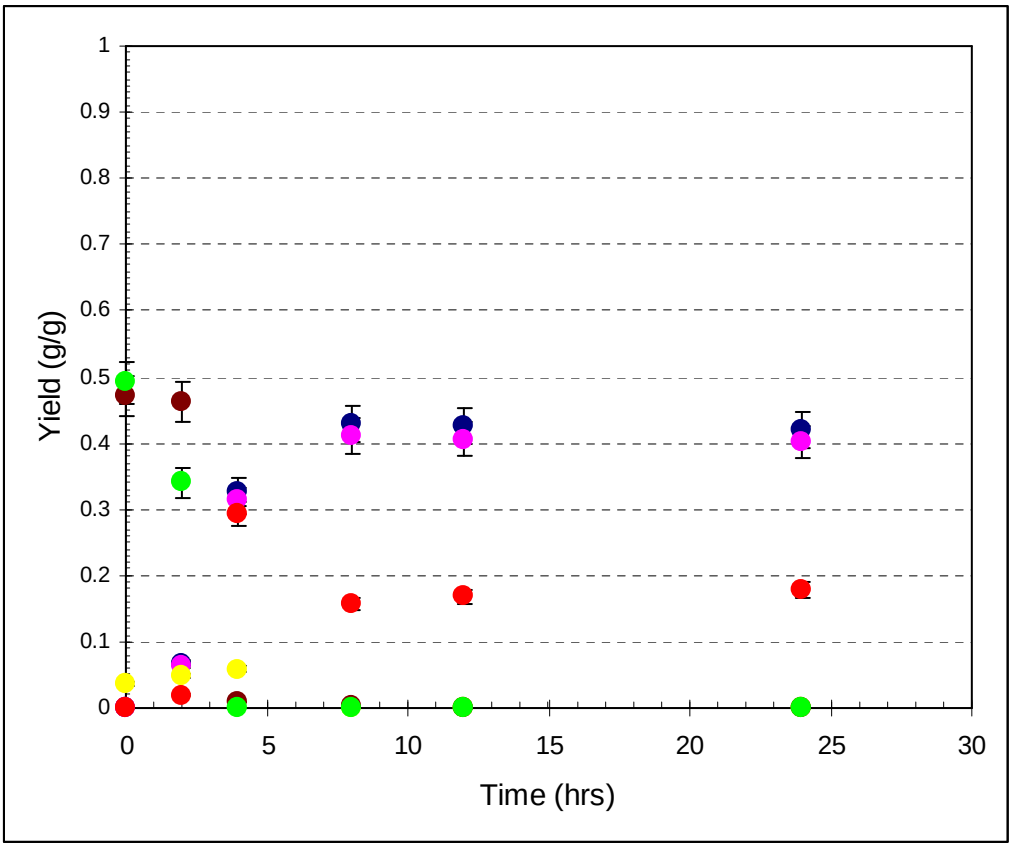

Figure C3 Fermentation at a dilution ratio of 1:2 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C4 Yields ($g.g^{-1}$) obtained from fermentation at a dilution ratio of 1:3

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.05	0.47	0.49	0
2	0.28	0.28	0.08	0.10	0.26	0
4	0.18	0.18	0	0	0	0.64
8	0.43	0.43	0	0	0	0.16
12	0.43	0.43	0	0	0	0.17
24	0.43	0.43	0	0	0	0.16

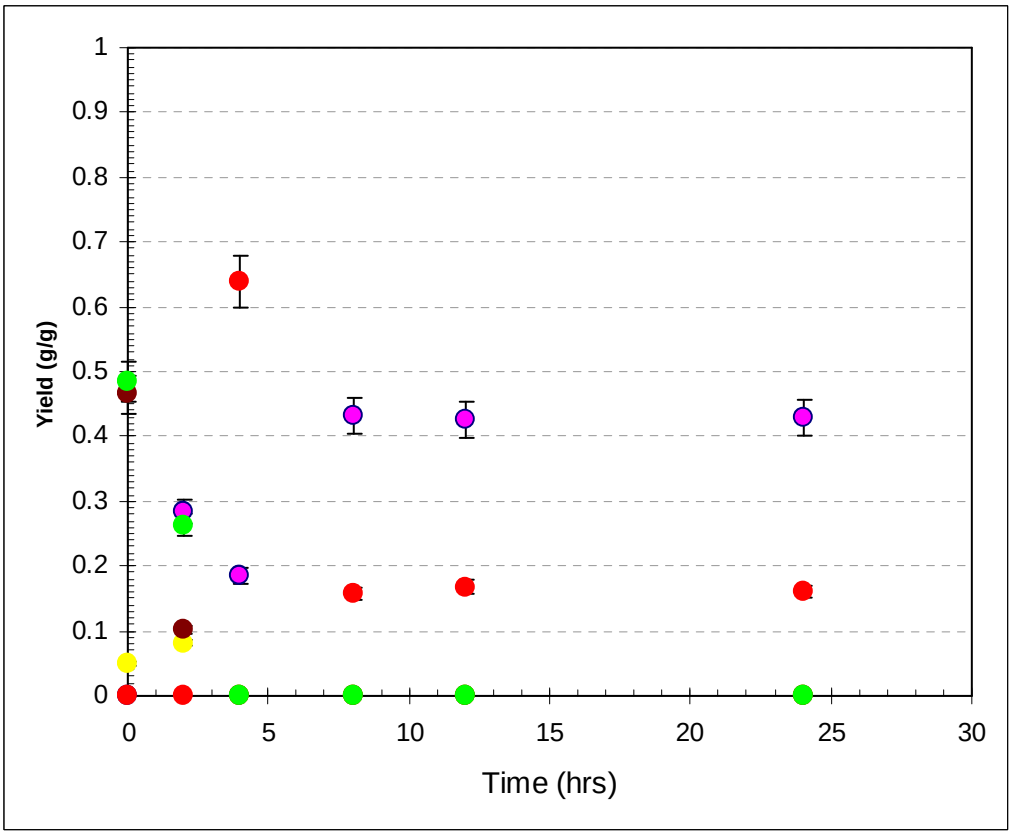

Figure C4 Fermentation at a dilution ratio of 1:3 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C5 Yields ($g.g^{-1}$) obtained from fermentation at a dilution ratio of 1:4

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.47	0.53	0
2	0.27	0.26	0	0.06	0.35	0.06
4	0.43	0.42	0	0.01	0	0.14
8	0.44	0.42	0	0	0	0.14
12	0.44	0.42	0	0	0	0.14
24	0.48	0.46	0	0	0	0.07

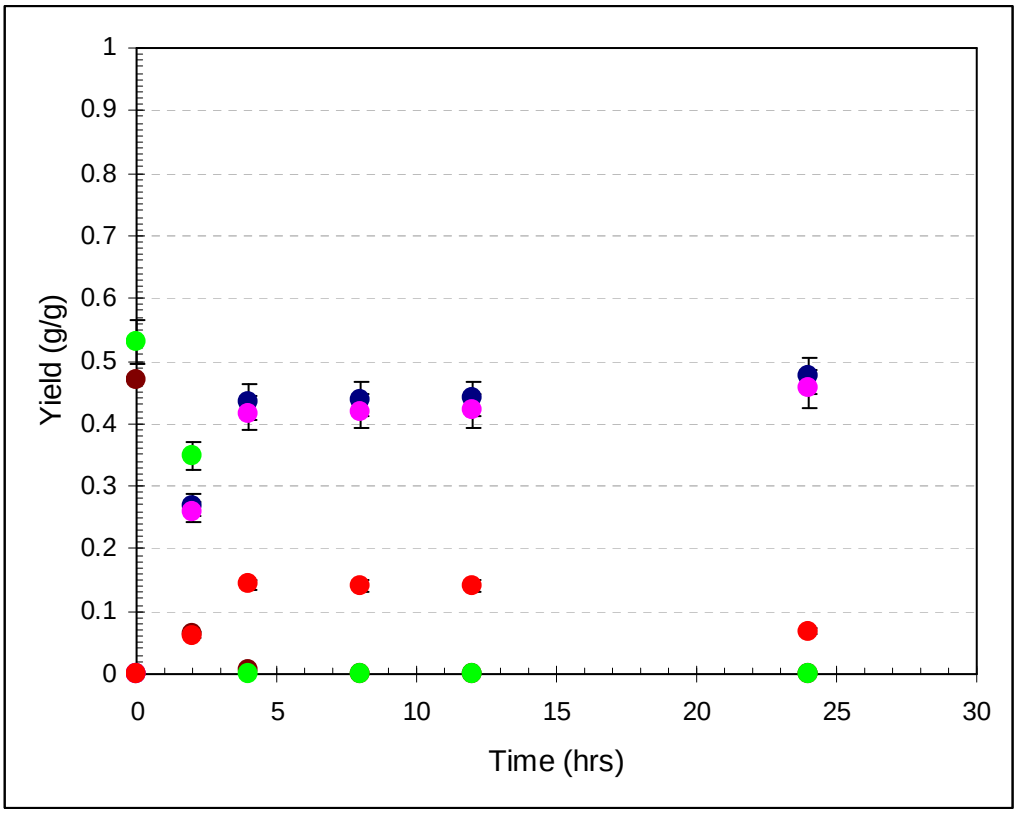

Figure C5 Fermentation at a dilution ratio 1:4 (● Ethanol, ● CO_2, ● Glucose, ● Fructose, ● Glycerol)

Table C6 Ethanol concentration ($g.L^{-1}$) obtained from the investigation into the effect of dilution

Time (hrs)	no dilution	1:1	1:2	1:3	1:4
0	0	0	0	0	0
2	34.88	32.7	10.16	30.52	23.54
4	74.12	71.94	47.92	19.62	37.50
8	196.2	95.92	62.44	46.87	38.37
12	191.84	95.92	60.98	46.87	38.37
24	204.92	93.74	60.98	46.87	41.86

Table C7 pH values measured during the investigation into the effect of dilution

Time (hrs)	No dilution	1:1	1:2	1:3	1:4
0	4.13	4.14	4.15	4.17	3.72
2	4.44	4.38	4.33	4.43	4.17
4	4.39	4.25	4.25	4.42	4.30
8	4.31	4.25	4.36	4.47	4.38
12	4.32	4.42	5.12	4.75	4.40
24	4.78	5.77	5.92	6.01	4.63

C2 Effect of pH

Table C8 Yields ($g.g^{-1}$) obtained using the natural pH of the juice

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.03	0.47	0.50	0
2	0.31	0.30	0.05	0.08	0.24	0.02
4	0.44	0.42	0.02	0	0	0.12
8	0.45	0.43	0	0	0	0.12
12	0.45	0.43	0	0	0	0.12

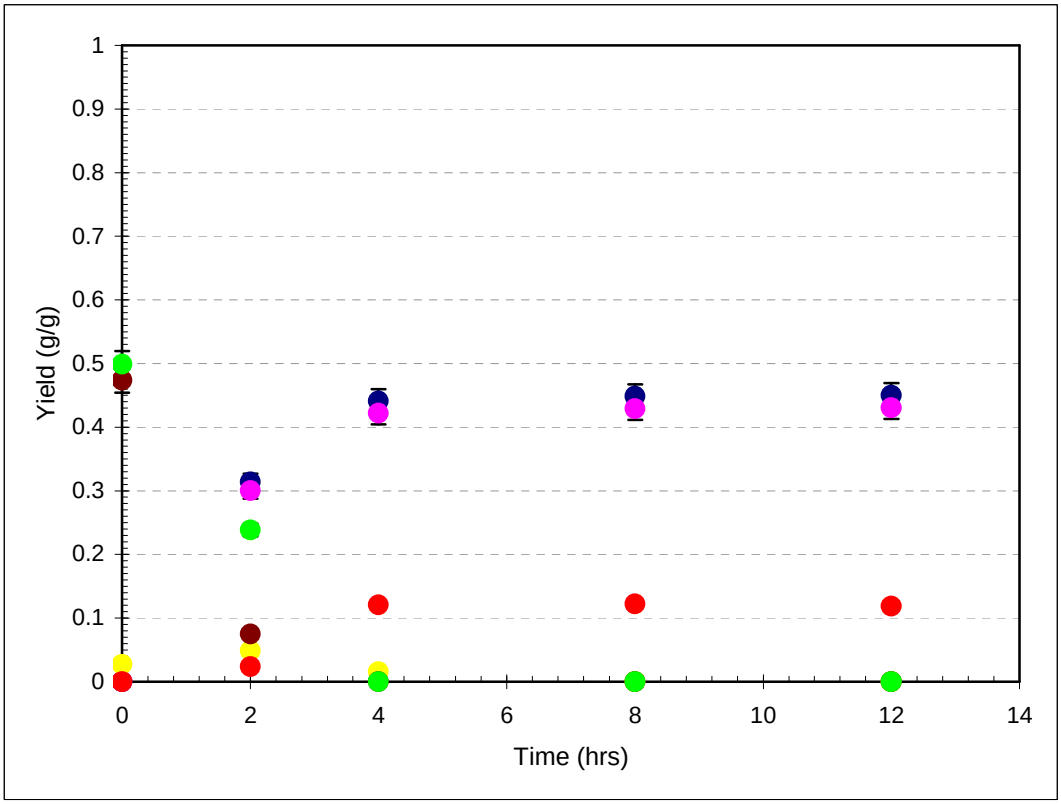

Figure C6 Fermentation at the natural pH of the juice (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C9 Yields ($g.g^{-1}$) obtained at a pH of 4

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.03	0.46	0.51	0
2	0.28	0.26	0.04	0.09	0.28	0.05
4	0.43	0.41	0.04	0	0	0.11
8	0.45	0.43	0	0	0	0.12
12	0.45	0.43	0	0	0	0.12

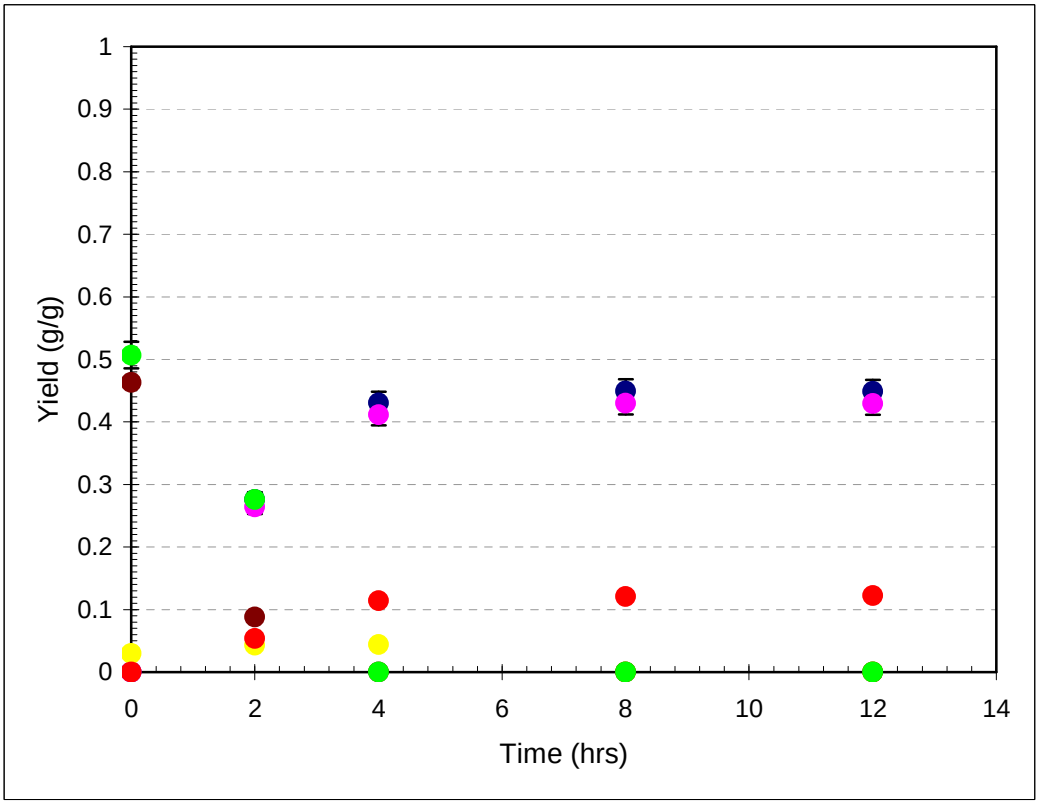

Figure C7 Fermentation at a pH of 4 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C10 Yields ($g.g^{-1}$) obtained from fermentation at pH of 4.5

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.09	0.17	0.74	0
2	0.15	0.14	0.03	0.26	0.41	0.01
4	0.33	0.31	0.03	0.05	0.22	0.06
8	0.44	0.42	0	0	0	0.12
12	0.44	0.42	0	0	0	0.14

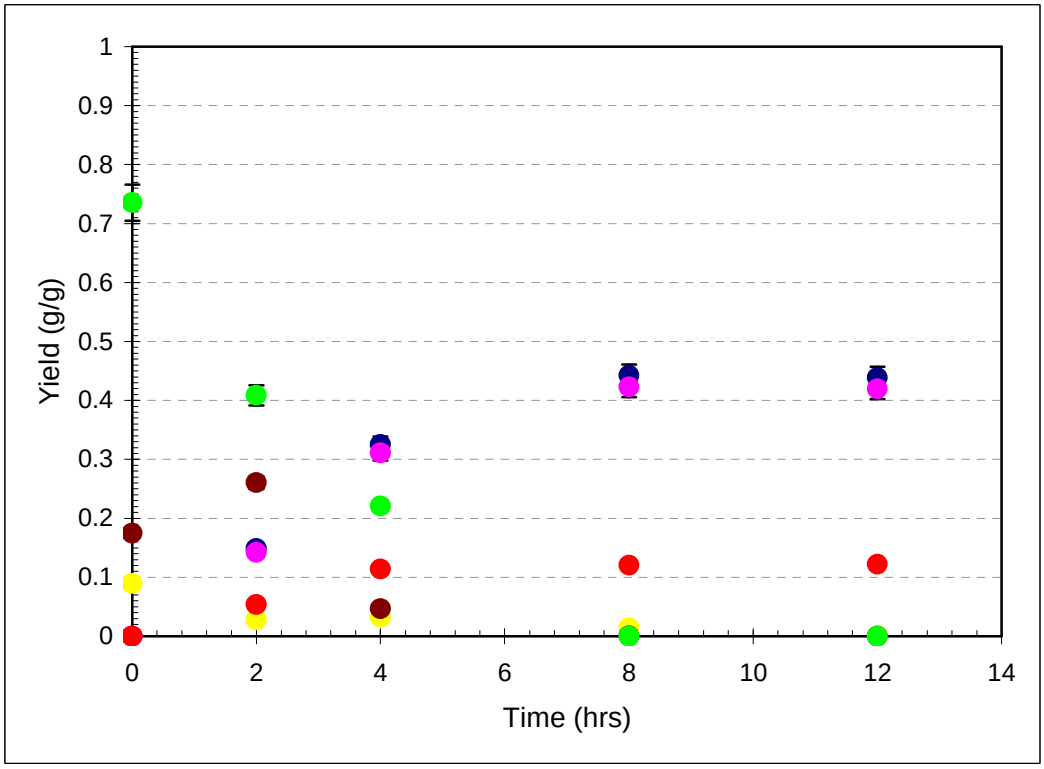

Figure C8 Fermentation at a pH of 4.5 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C11 Yields ($g.g^{-1}$) obtained from fermentation at pH of 5

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.02	0.16	0.81	0
2	0.34	0.33	0.01	0.05	0.22	0.05
4	0.44	0.42	0	0	0	0.13
8	0.44	0.42	0	0	0	0.13
12	0.45	0.43	0	0	0	0.13

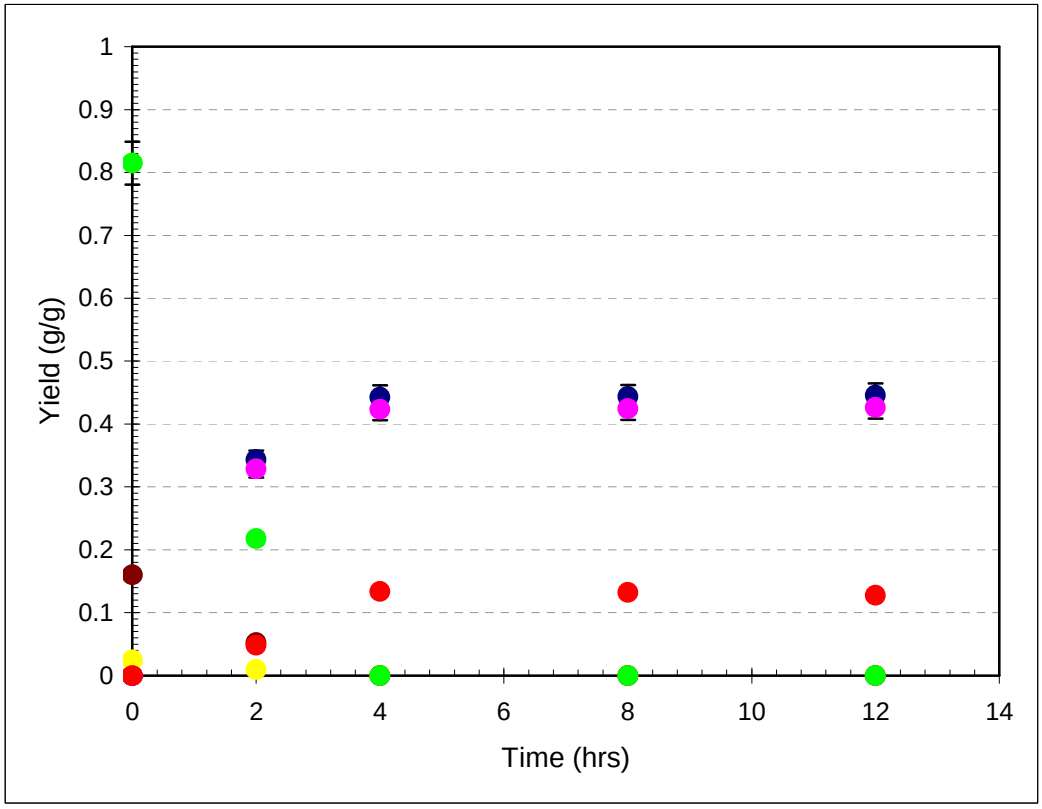

Figure C9 Fermentation at a pH of 5 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C12 Yields ($g.g^{-1}$) obtained from fermentation at pH of 5.5

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.03	0.46	0.51	0
2	0.39	0.38	0.01	0.01	0.10	0.11
4	0.44	0.42	0	0	0	0.14
8	0.44	0.42	0	0	0	0.14
12	0.44	0.42	0	0	0	0.14

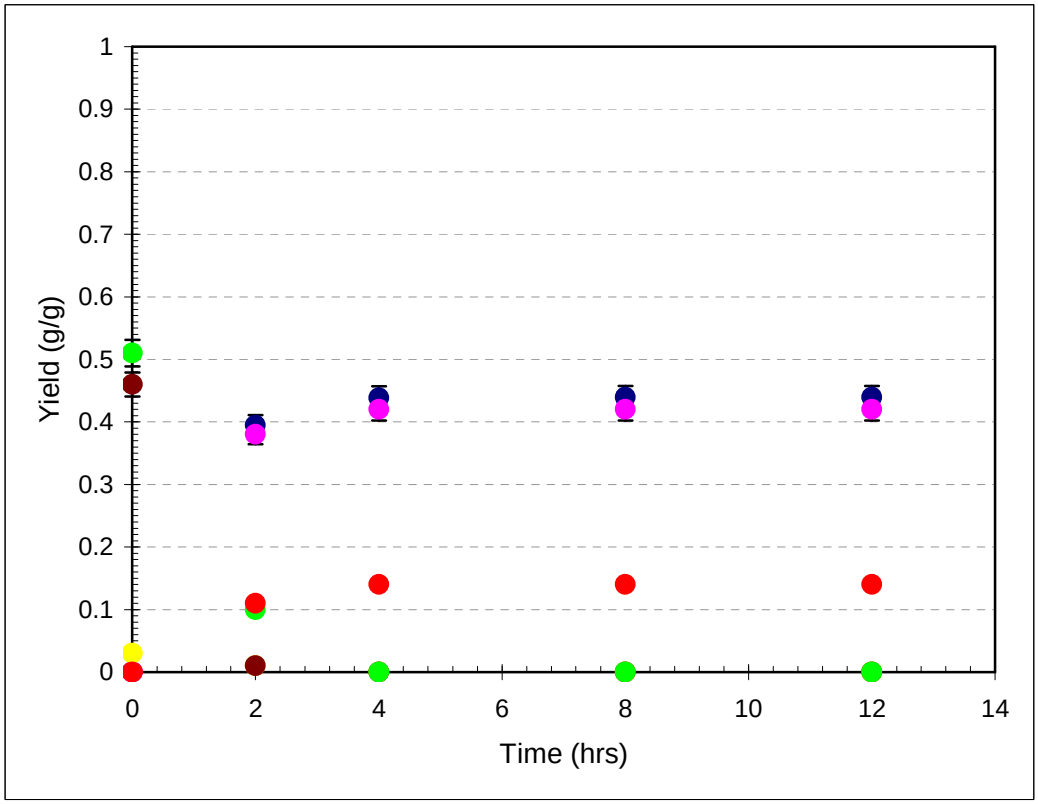

Figure C10 Fermentation at a pH of 5.5 (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C13 Ethanol concentration ($g.L^{-1}$) obtained during the investigation into the effect of pH

Time (hrs)	no adjustment	pH 4	pH 4.5	pH 5	pH 5.5
0	0	0	0	0	0
2	67.58	61.04	32.7	74.12	85.02
4	95.92	93.74	71.94	95.92	95.92
8	98.1	98.1	95.92	95.92	95.92
12	98.1	98.1	95.92	98.1	95.92

Table C14 pH values measured during the investigation into the effect of pH

Time (hrs)	No adjustment	pH 4.0	pH 4.5	pH 5	pH 5.5
0	4.17	3.85	4.09	5.0	5.5
2	3.91	4.20	4.33	4.72	4.82
4	3.97	3.83	4.43	4.63	4.90
8	4.23	4.41	4.67	4.64	5.20
12	4.96	5.09	5.30	5.65	6.38

C3 Effect of yeast concentration

Table C15 Yields ($g.g^{-1}$) obtained using a 1 $g.L^{-1}$ yeast concentration

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.55	0.45	0
2	0.06	0.05	0	0.43	0.46	0
4	0.19	0.18	0	0.23	0.40	0
8	0.46	0.44	0	0	0.07	0.03
12	0.45	0.43	0	0	0.06	0.06
24	0.46	0.44	0	0	0.06	0.03

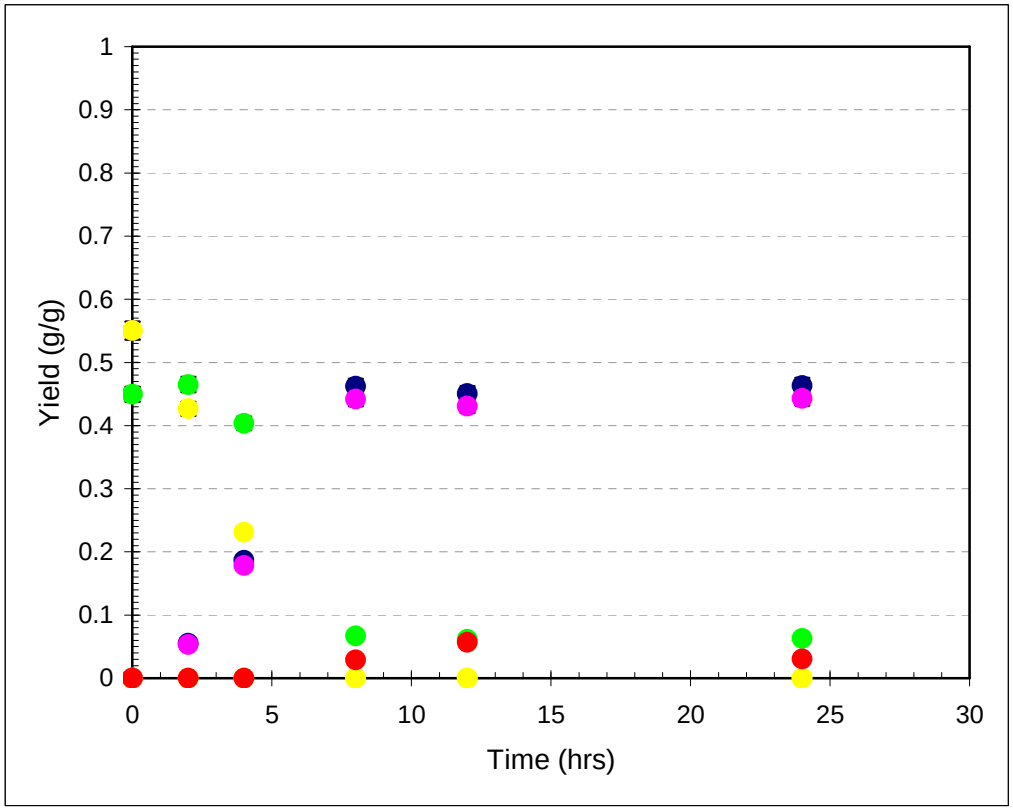

Figure C11 Fermentation using a 1g.L^{-1} yeast concentration

(● Ethanol, ● CO_2, ● Glucose, ● Fructose, ● Glycerol)

Table C16 Yields (g.g^{-1}) obtained using a 3 g.L^{-1} yeast concentration

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.54	0.46	0
2	0.11	0.11	0	0.29	0.49	0
4	0.21	0.20	0	0.18	0.41	0
8	0.45	0.43	0	0	0.05	0.07
12	0.45	0.43	0	0	0.05	0.08
24	0.44	0.43	0	0	0.05	0.08

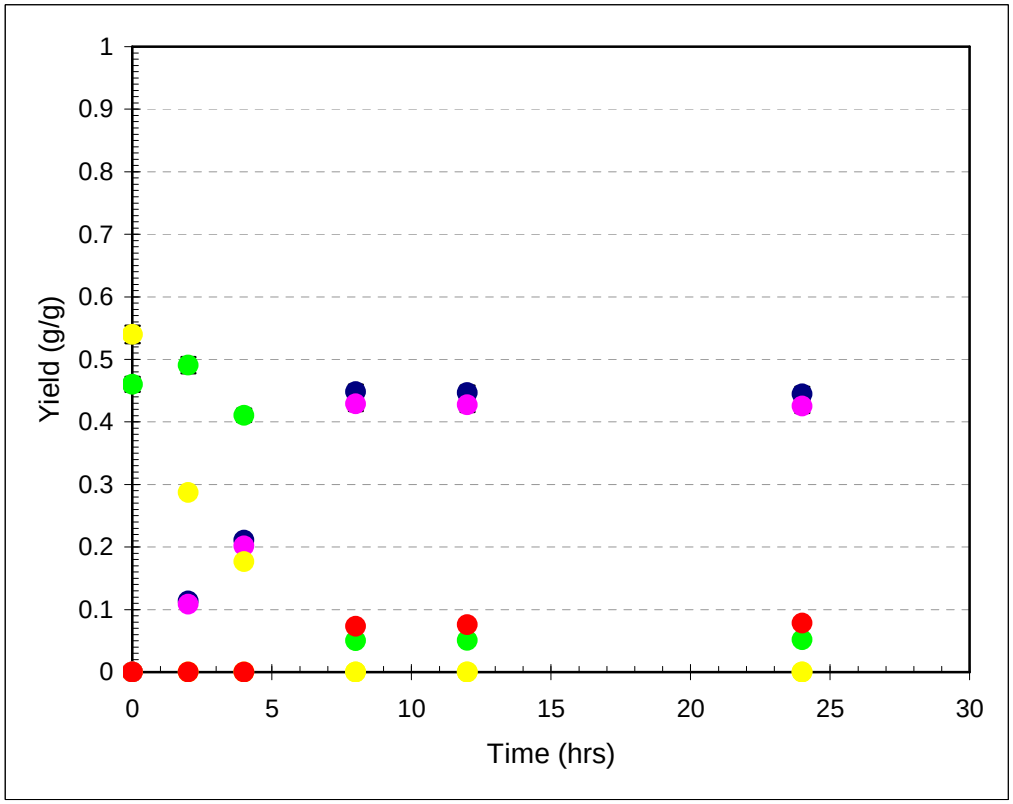

Figure C12 Fermentation using a 3g.L^{-1} yeast concentration

(● Ethanol, ● CO_2, ● Glucose, ● Fructose, ● Glycerol)

Table C17 Yields (g.g^{-1}) obtained using a 5 g.L^{-1} yeast concentration

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.92	0.08	0
2	0.19	0.18	0	0.22	0.42	0
4	0.48	0.46	0	0	0.05	0
8	0.45	0.43	0	0	0.05	0.08
12	0.45	0.43	0	0	0.05	0.08
24	0.33	0.31	0	0	0.08	0.26

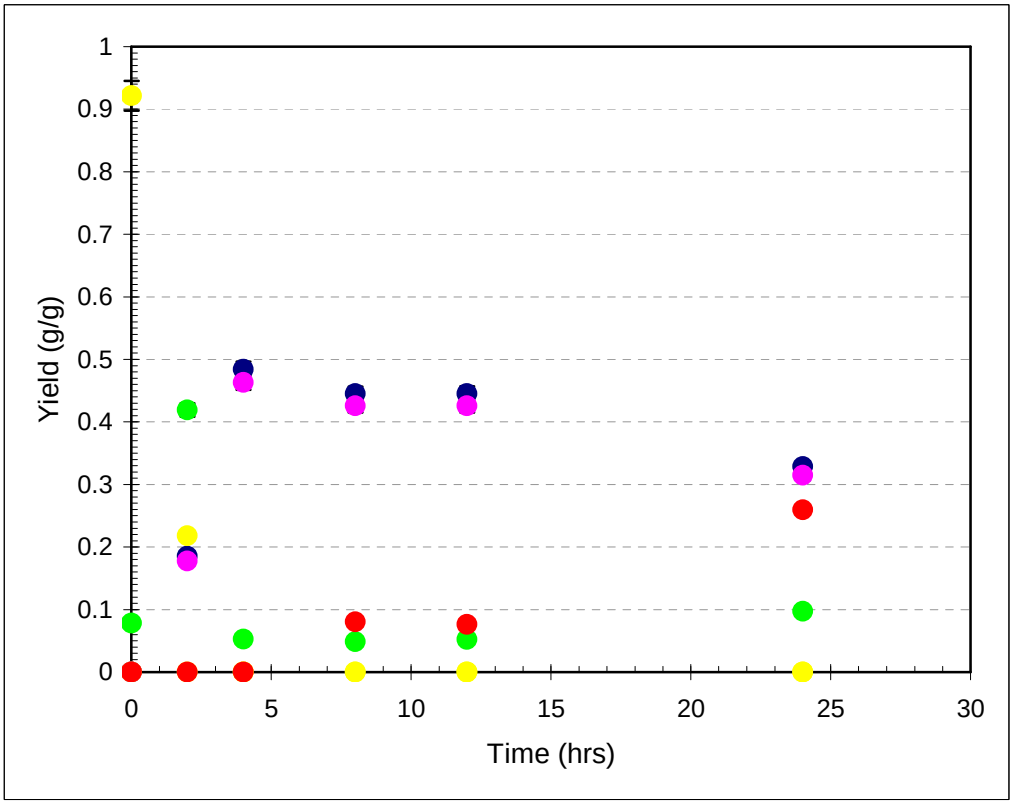

Figure C13 Fermentation using a 5g.L^{-1} yeast concentration

(● Ethanol, ● CO_2, ● Glucose, ● Fructose, ● Glycerol)

Table C18 Yields (g.g^{-1}) obtained using a 10 g.L^{-1} yeast concentration

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.54	0.46	0
2	0.48	0.46	0	0	0.06	0.
4	0.48	0.46	0	0	0.06	0
8	0.46	0.44	0	0	0.06	0.03
12	0.46	0.44	0	0	0.06	0.03
24	0.32	0.30	0	0	0.11	0.11

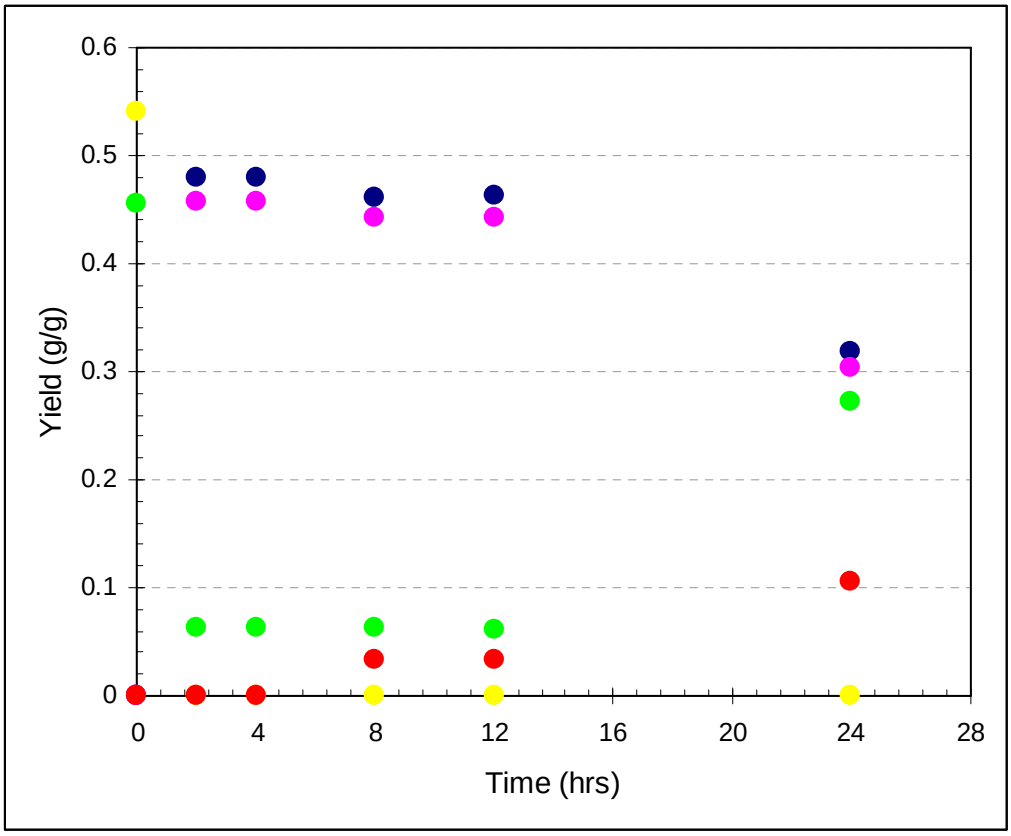

Figure C14 Fermentation using a 10 $g.L^{-1}$ yeast concentration

(● Ethanol, ● CO_2, ● Glucose, ● Fructose, ● Glycerol)

Table C19 Ethanol concentration ($g.L^{-1}$) obtained during the investigation into the effect of yeast concentration

Time (hrs)	**1 $g.L^{-1}$**	**3 $g.L^{-1}$**	**5 $g.L^{-1}$**	**10 $g.L^{-1}$**
0	0	0	0	0
2	13.08	23.98	38	104.64
4	41.42	45.78	104.64	104.64
8	100.28	98.1	98.1	100.28
12	98.1	98.1	98.1	100.28
24	100.28	95.92	71.94	67.58

Table C 20 pH values measured during the investigation of the effect of yeast concentration

Time (hrs)	1 g.L^{-1}	3 g.L-1	5 g.L^{-1}	10 g.L^{-1}
0	4.17	4.16	4.19	4.5
2	4.44	4.43	4.56	4.74
4	4.24	4.44	4.71	4.83
8	4.31	4.53	4.77	5.03
12	4.41	4.63	4.85	5.03
24	4.53	4.71	4.84	4.89

C4 Effect of nitrogen supplementation

Table C21 Yields (g.g^{-1}) obtained with addition of urea

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.49	0.51	0
2	0.24	0.23	0	0.15	0.37	0.02
4	0.46	0.44	0	0	0	0.09
8	0.47	0.45	0	0	0	0.09
12	0.47	0.45	0	0	0	0.09
24	0.47	0.45	0	0	0	0.08

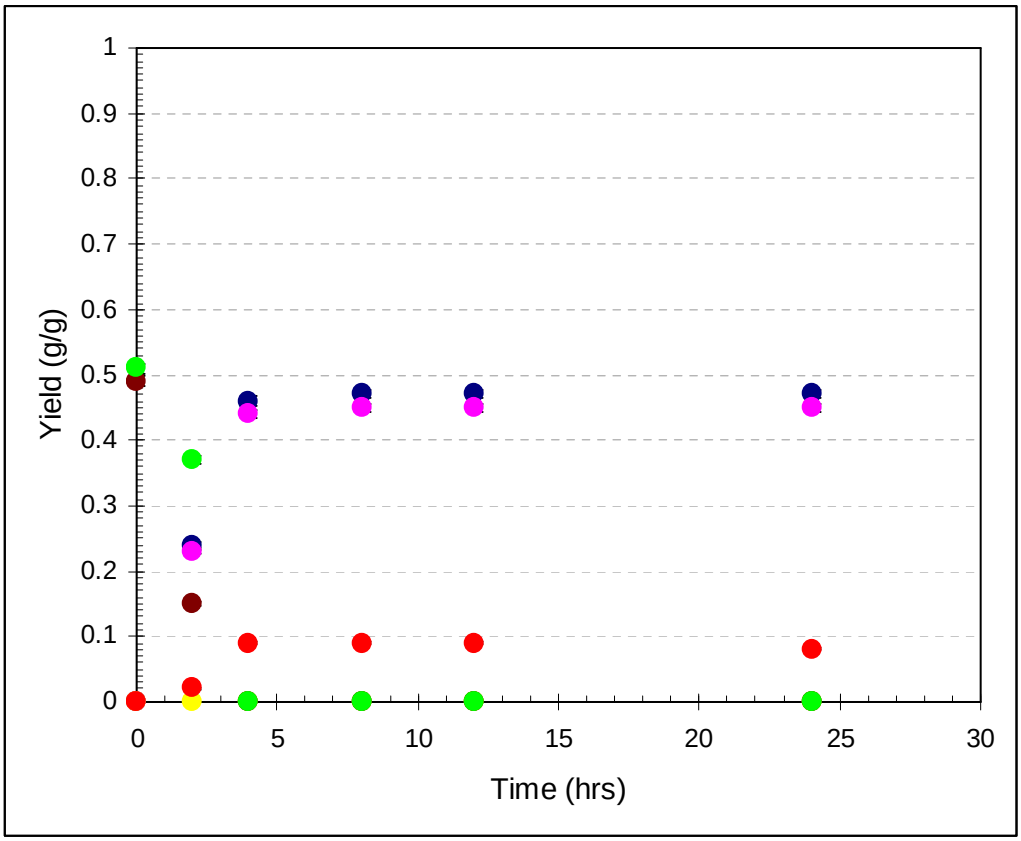

Figure C15 Fermentation with the addition of urea

(● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C22 Yields ($g.g^{-1}$) obtained with the addition of peptone

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.48	0.52	0
2	0.27	0.26	0	0.11	0.34	0.03
4	0.46	0.44	0	0	0	0.09
8	0.47	0.45	0	0	0	0.08
12	0.47	0.45	0	0	0	0.08
24	0.46	0.44	0	0	0	0.09

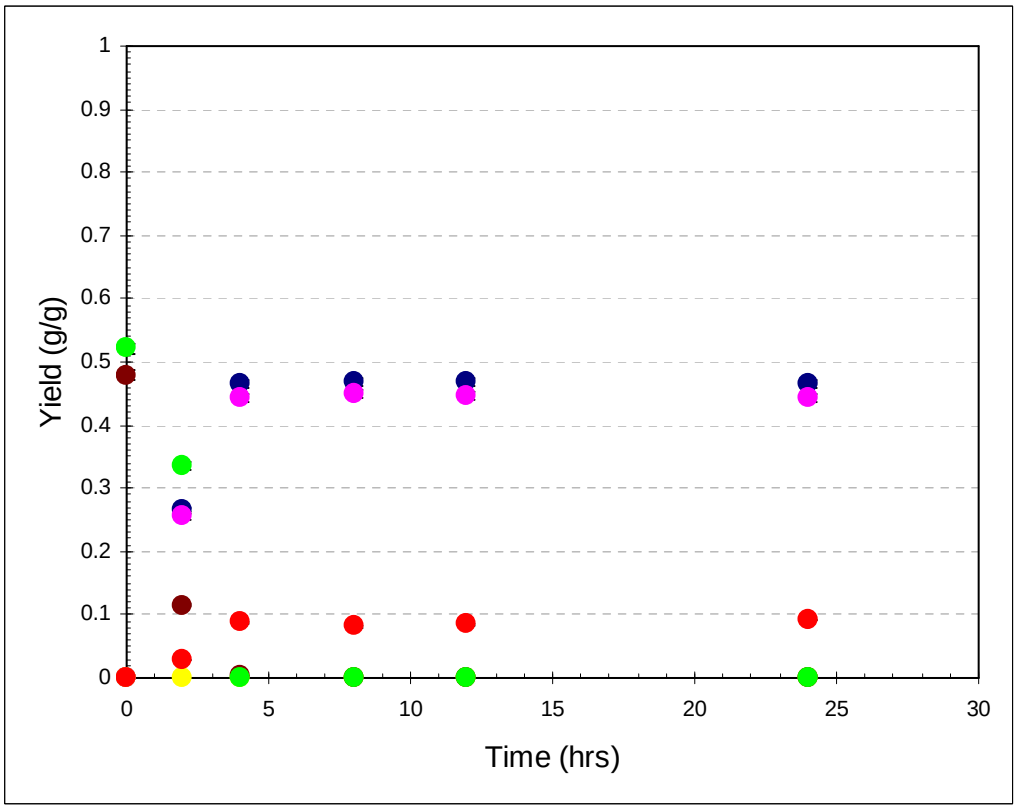

Figure C16 Fermentation with the addition of peptone

(● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C23 Yields ($g.g^{-1}$) obtained with the addition of yeast extract

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.48	0.52	0
2	0.27	026	0	0.11	0.33	0.03
4	0.46	0.44	0	0	0	0.09
8	0.46	0.44	0	0	0	0.09
12	0.47	0.45	0	0	0	0.09
24	0.46	0.44	0	0	0	0.10

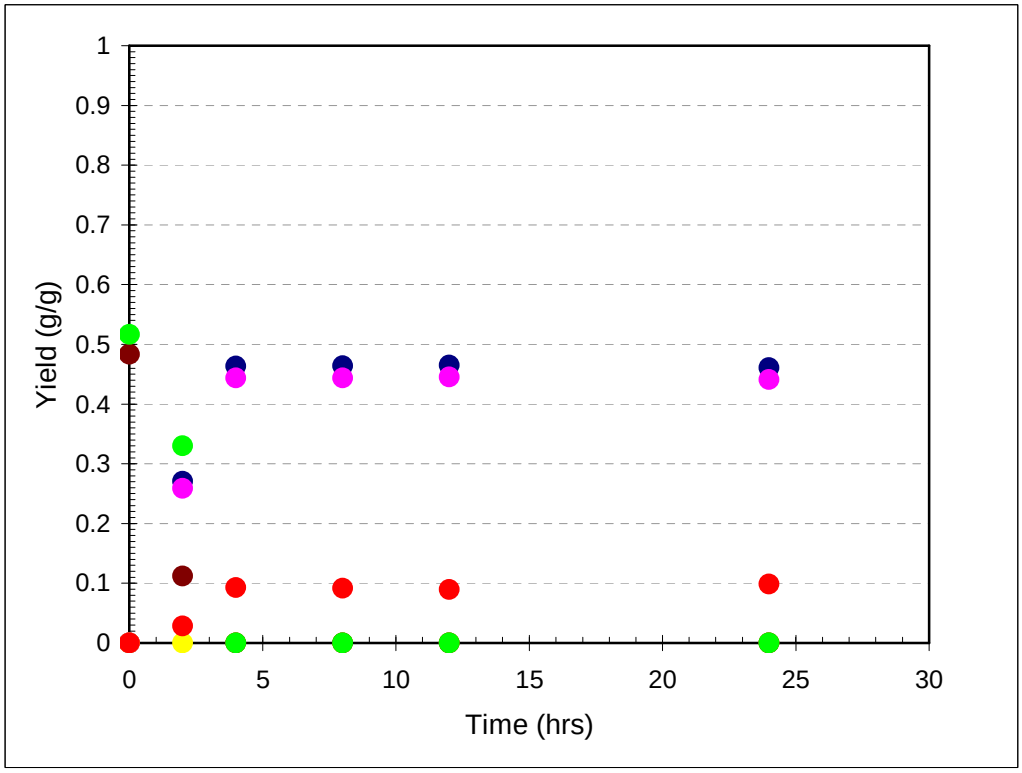

Figure C17 Fermentation with the addition of yeast extract

(● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C24 Ethanol concentration ($g.L^{-1}$) obtained during the investigation into the effect of adding a nitrogen source.

Time (hrs)	no addition	Urea	yeast extract	Peptone	ammonium sulphate
0	0	0	0	0	0
2	85.02	52.32	58.86	58.86	63.61
4	95.96	100.28	100.28	100.28	101.08
8	95.48	102.46	100.28	102.46	100.78
12	94.53	102.46	102.46	102.46	101.03
24	94.52	102.46	100.28	100.28	98.65

Table C25 pH values measured during the investigation of the effect of adding a nitrogen source

Time (hrs)	Urea	Peptone	Yeast extract	Ammonium sulphate
0	3.57	3.63	3.65	3.64
2	4.00	4.00	4.02	4.02
4	4.37	4.02	4.07	4.00
8	3.84	3.77	3.83	3.80
12	4.04	4.09	4.12	4.11
24	4.14	4.21	4.32	4.31

Table C26 Yields ($g.g^{-1}$) obtained with no addition of ammonium sulphate

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.45	0.52	0
2	0.39	0.28	0		0.30	0.03
4	0.46	0.44	0	0.44	0	0.09
8	0.46	0.44	0	0.44	0	0.10
12	0.46	0.44	0	0.44	0	0.09
24	0.45	0.43	0	0.43	0	0.11

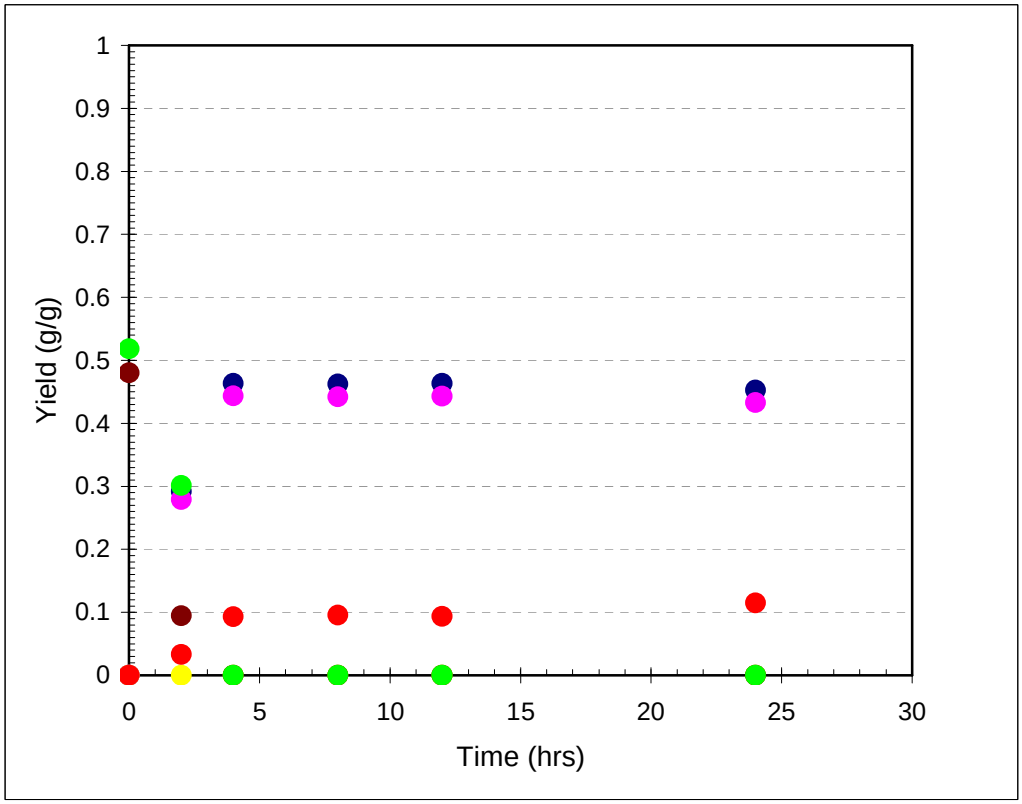

Figure C18 Fermentation with no addition of ammonium sulphate (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C27 Yields ($g.g^{-1}$) obtained with the addition of 250 mg $N.L^{-1}$ ammonium sulphate

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0.06	0.46	0.48	0
2	0.30	0.28	0.07	0.04	0.24	0.07
4	0.42	0.41	0.02	0	0	0.15
8	0.43	0.42	0	0	0	0.16
12	0.43	0.42	0	0	0	0.16
24	0.43	0.42	0	0	0	0.15

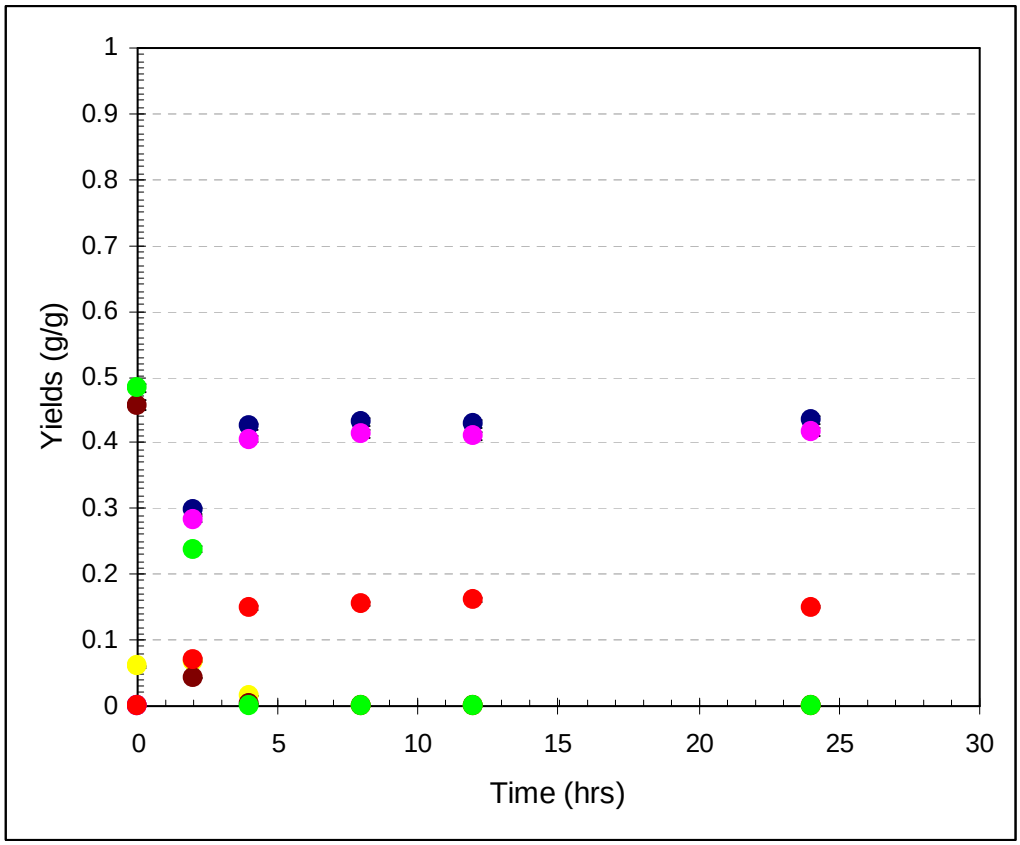

Figure C19 Fermentation with the addition of 250 mg $N.L^{-1}$ Ammonium sulphate (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C28 Yields ($g.g^{-1}$) obtained with the addition of 500 mg $N.L^{-1}$ ammonium sulphate

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.48	0.52	0
2	0.32	0.30	0	0.04	0.25	0.08
4	0.42	0.41	0	0	0	0.17
8	0.42	0.41	0	0	0	0.17
12	0.42	0.41	0	0	0	0.17
24	0.42	0.40	0	0	0	0.19

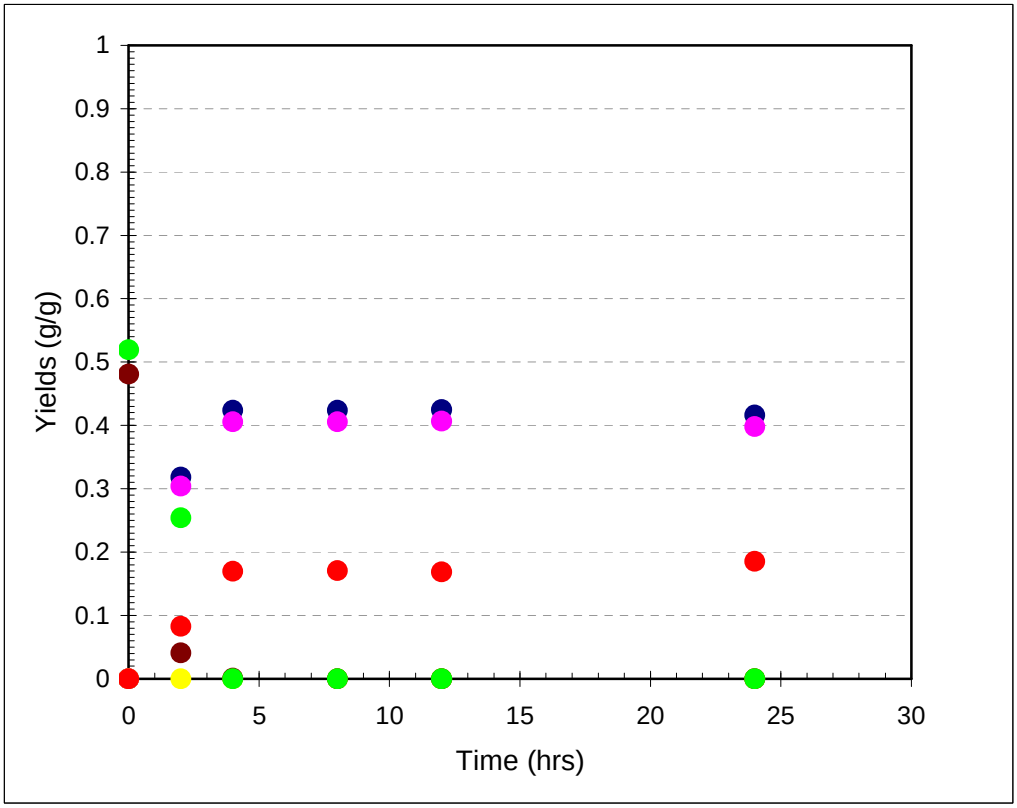

Figure C20 Fermentation with the addition of 500 mg $N.L^{-1}$ ammonium sulphate (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C29 Yields ($g.g^{-1}$) obtained with the addition of 750 mg $N.L^{-1}$ ammonium sulphate

Time (hrs)	Ethanol	CO_2	Sucrose	Glucose	Fructose	Glycerol
0	0	0	0	0.48	0.52	0
2	0.26	0.25	0	0.10	0.33	0.06
4	0.45	0.43	0	0.01	0	0.11
8	0.46	0.44	0	0	0	0.11
12	0.42	0.41	0	0	0	0.17
24	0.45	0.43	0	0	0	0.11

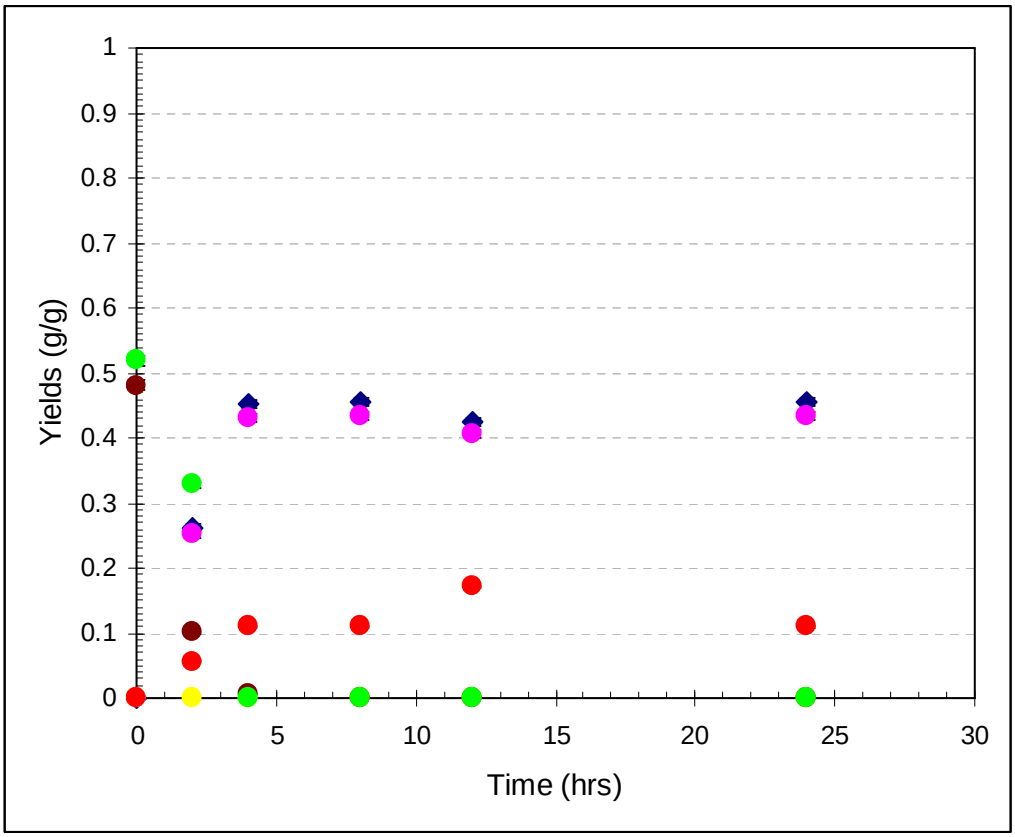

Figure C21 Fermentation with the addition of 750 mg $N.L^{-1}$ ammonium sulphate (● Ethanol, ● CO_2, ● Sucrose, ● Glucose, ● Fructose, ● Glycerol)

Table C30 Ethanol concentration ($g.L^{-1}$) obtained from the investigation into the effect of adding ammonium sulphate

Time (hrs)	no addition	250 mg $N.L^{-1}$	500 mg $N.L^{-1}$	750 mg $N.L^{-1}$
0	0	0	0	0
2	85.02	65.4	69.76	56.68
4	95.96	91.56	91.56	98.1
8	95.48	93.74	91.56	100.28
12	94.53	93.74	91.56	91.56
24	94.52	93.74	91.56	98.1

Table C31 pH values measured during the investigation into the effect of adding ammonium sulphate

Time (hrs)	No addition	250 mg $N.L^{-1}$	500 mg $N.L^{-1}$	750 mg $N.L^{-1}$
0	3.71	3.70	3.72	3.68
2	3.87	3.85	3.78	3.88
4	4.04	4.06	4.01	3.90
8	4.12	4.08	4.02	4.01
12	4.02	4.03	4.02	4.07
24	4.13	4.24	4.14	4.20