CRANFIELD UNIVERSITY

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Bioproduction of Xylitol by Oleaginous Yeast *Yarrowia lipolytica*

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ABSTRACT

Xylitol is a commercially important chemical with multiple applications in the food and pharmaceutical industries. According to the US Department of Energy, xylitol is one of the top twelve platform chemicals that can be produced from biomass. The chemical method for xylitol synthesis is, however, expensive and energy-intensive. In contrast, the biological route using microbial cell factories offers a potentially cost-effective alternative process. The bioprocess occurs under ambient conditions and makes use of biocatalysts and biomass which can be sourced from renewable carbon originating from a variety of cheap waste feedstocks. In this study, the biotransformation of xylose to xylitol was investigated using *Yarrowia lipolytica*, an oleaginous yeast which, in this study was firstly grown on a glycerol/glucose medium for the screening of a co-substrate, followed by a media optimisation in shake flasks, scale-up studies in a bioreactor and then downstream studies where done on the processing of xylitol. A two-step medium optimization was employed using a central composite design and an artificial neural network coupled with a genetic algorithm. The yeast amassed a concentration of 53 g/L of xylitol whilst using pure glycerol (PG) and xylose media, with a bioconversion yield of 0.97 g/g. Similar results were obtained when PG was substituted with crude glycerol (CG) from the biodiesel industry (titre: 51 g/L; yield: 0.92 g/g). Even when xylose from sugarcane bagasse hydrolysate was used as opposed to pure xylose, a xylitol yield of 0.54 g/g was achieved. The xylitol was successfully crystallized from the PG/xylose and CG/xylose fermentation broths with a recovery yield of 40 and 35 %, respectively. To the best of the author's knowledge, this study demonstrates for the first time, the potential of using *Y. lipolytica* as a microbial cell factory for xylitol synthesis from inexpensive feedstocks. The results obtained are competitive with other xylitol producing organisms.

Keywords:

Bioconversion, Xylitol, *Yarrowia lipolytica*, Xylose reductase, Crude glycerol

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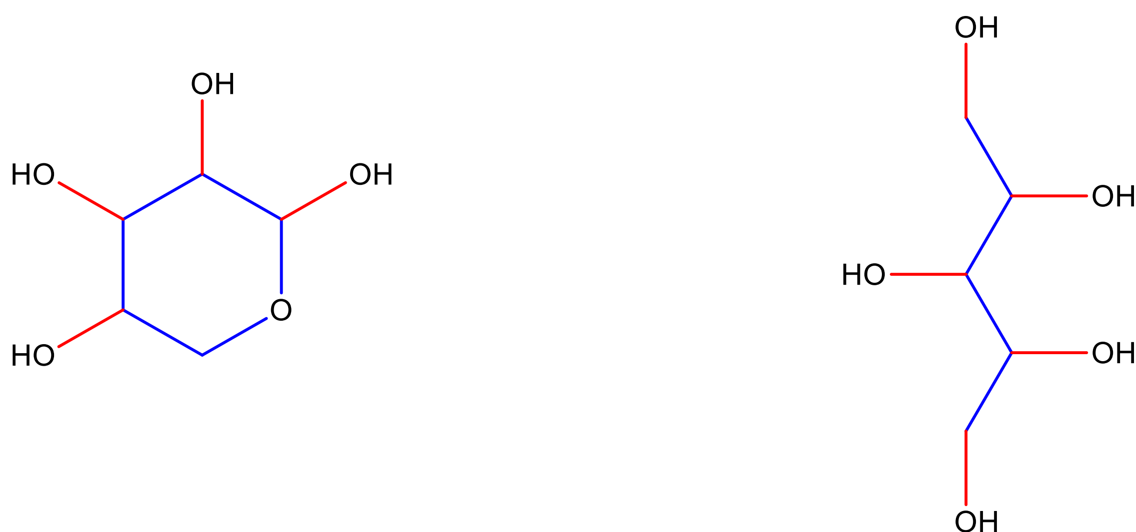
LIST OF ABBREVIATIONS

6-PGD	6-Phosphogluconate Dehydrogenase
Adj MS	Adjusted Mean Sum of Square
Adj SS	Adjusted Sum of Square
ANN	Artificial Neural Networks
ANN-GA	Artificial Neural Networks linked with Genetic Algorithm
ANOVA	Analysis of Variance
CCD	Central Composite Design
Coef	Coefficient
DF	Degrees of Freedom
F	F-Value
FDA	Food and Drug Administration (US Regulatory Body)
G3P	Glyceraldehyde 3 Phosphate
G3PDH	Glycerol-3-Phosphate Dehydrogenase
G6PD	Glucose-6-Phosphate Dehydrogenase
GA	Genetic Algorithm
GFX1	Glucose-Xylose Facilitator One
GK	Glycerol Kinase
GRAS	Generally Regarded as Safe (FDA Term)
GXS1	Glucose-Xylose Symporter One
His3	A Recombination Disruption Cassette
LM	Levenberg-Marquardt
MLP	Multilayer Perceptron
MS	Mean Sum of Square
MSE	Mean Square Error
NAD^+	The oxidized form of Nicotinamide Adenine Dinucleotide
$NADH^+$	The reduced form of Nicotinamide Adenine Dinucleotide
$NADP^+$	The oxidized form of Nicotinamide Adenine Dinucleotide Phosphate
NADPH	The reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NOP	Non-Oxidative Pathway
OD	Optical Density

OP	Oxidative Pathway
P	P-Value
PDVF	Polyvinylidene Fluoride
RID	Refractive Index Detector
RPM	Revolutions Per Minute
SE Coef	Square Error Coefficient
Seq SS	Sequential Sum of Square
SS	Sum of Square
SUT1	A Specialised Xylose Transport Protein
T	T-value
Trp	A Recombination Disruption Cassette
UK	United Kingdom
US/USA	United States/United States of America
UV	Ultraviolet/Ultraviolet Light
XDH	Xylitol Dehydrogenase
XK	Xylulose Kinase
XR	Xylose Reductase
YNB	Yeast Nitrogen Base
YPD	Yeast Peptone Dextrose

1 Introduction

The synthetic methods of producing many of the important chemicals in the fuel, food and pharmaceutical industries involve the use of petrochemicals, which are unsustainable resources and when utilised can contribute to global warming and environmental damage (Lee, 2013). This is why creating alternative methods of producing these chemicals is the focus of many intense research efforts. Often new and biological alternatives to these synthetic processes address these issues. This is because they often use renewable feedstocks and don't have the same problems with carbon emissions and environmental damage that the traditional methods do. This study focuses on replacing the inefficient, energy-intensive and expensive traditional synthetic method of xylitol production (Zhang *et al.*, 2018; L. Xu *et al.*, 2019), with the more efficient and environmentally friendly biological method of xylitol production.



<u>Figure 1.1</u>: A D-xylose molecule on the left and xylitol molecule on the right (NCBI, 2019a, 2019b).

As can be seen in Figure 1.1, xylitol is a 5 carbon sugar alcohol that contains a hydroxyl group bonded to each carbon (Albuquerque *et al.*, 2014). It is a valuable chemical because its unique chemical properties give it a large amount of utility

as a sweetener, drug ingredient and as a replacement for sucrose, in the pharmaceutical, nutraceutical, dental, food and beverage industries (Liu, Ji and Huang, 2015; Dasgupta *et al.*, 2017). In the food industry alone it is used as a sugar replacement for diabetics because it is metabolised through different metabolic pathways than other sugars such as sucrose, it is used as a healthier substitute for sucrose because it possesses a similar sweetness but with fewer calories, and it also has antimicrobial properties (Huang *et al.*, 2011; Ping *et al.*, 2013; Dasgupta *et al.*, 2017).

There are two ways of producing xylitol, the biological route and the synthetic route. The synthetic process involves the use of heat and pressure combined with synthetic catalysts to catalyse the reaction. The process has several issues that make it unnecessarily expensive and harmful to the environment. For instance the use of a toxic metal catalyst (usually rankey nickel) for the reduction of xylose into xylitol which is expensive and unsustainable (Li *et al.*, 2015; Jo *et al.*, 2017). The requirement for two expensive purification stages adds additional costs to the synthetic process. The reason for the additional purification step is that the synthetic process requires pure xylose and cannot utilise lignocellulosic material, as can be seen in Figure 1.2 (these stages can include processes like ion exchange, ultrafiltration, activated carbon and/or crystallisation) (Li *et al.*, 2015; Mohamad, Mustapa Kamal and Mokhtar, 2015). A large amount of water is also required for the synthetic process (Zhang *et al.*, 2018) making the process even more environmentally unfriendly. The pressure-temperature requirements (usually between 80-100 ^{o}C and at a pressure of 50 atm) also create further costs as they are expensive and energy-intensive to maintain (Li *et al.*, 2015; Jo *et al.*, 2017; Zhang *et al.*, 2018). In contrast the biological production method of xylitol, which uses biological catalysts such as enzymes, does not require high heat and pressure because the biological catalysts usually operate at ambient temperatures. The biological process doesn’t need a toxic, unsustainable and expensive metal catalyst. The process also only requires one purification step as the enzymes can utilise lignocellulosic material (Zhang *et al.*, 2018). These

combined make the biological process less expensive to maintain and less environmentally harmful.

There are a wide variety of bacteria and fungi that possess the pentose phosphate pathway necessary to ferment xylose, including *Enterobacter*, *Corynebacterium*, *Penicillium* and *Aspergillus* species (Yoshitake *et al.*, 1973; Rangaswamy and Agblevor, 2002; Sampaio *et al.*, 2003; Kang *et al.*, 2016). However, yeasts such as *Candida*, *Pichia*, *Kluyveromyces*, *Debaromyces* and *Hansunela* species are the organisms that most commonly express these pathways and hence are the most promising xylitol producers (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019). However, most of these suffer from one or more issues such as pathogenicity issues, chances of contamination issues and low yield issues. However there is a non-pathogenic organism that has shown high yields of xylitol and that possesses several advantages as a biological factory, the organism, is *Yarrowia lipolytica*.

Y. lipolytica is a non-pathogenic organism that has already demonstrated the ability to produce a xylitol yield of up to 92 % (Ledesma-Amaro *et al.*, 2016). *Y. lipolytica* has been extensively studied and is a model organism for several fields of study such as lipid metabolisms, dimorphism, secretion and saline tolerance (Zhu and Jackson, 2015; Qiao *et al.*, 2017; Madzak, 2018). It can also produce a wide variety of valuable by-products such as organic acids, proteins, lipids and fatty acids (Ledesma-amaro and Nicaud, 2016; Ledesma-Amaro *et al.*, 2016) and most importantly it can use cheap and prevalent waste materials such as wastewater, molasses, industrial fat and vegetable oil as carbon sources with lignocellulosic hydrolysates and crude glycerol being of particular interest to this study (Madzak, Gaillardin and Beckerich, 2004; Liu, Ji and Huang, 2015; Egermeier *et al.*, 2017). This ability to produce a similar yield of xylitol to other well-known xylitol producers like *Candida* strains from cheap and waste materials

like crude glycerol and lignocellulosic material whilst also being a non-pathogenic organism made *Y. lipolytica* worthy of investigation.

A sequential medium optimization strategy was adapted using central composite design (CCD) followed by an artificial neural network linked with a genetic algorithm (ANN-GA) to maximize xylitol production. Once the concentrations of an optimised media were obtained *Y. lipolytica* was also cultured using pure and crude glycerol from a biodiesel company called Greenergy in the UK as well as pure xylose and lignocellulosic material which was sugarcane bagasse from Nova Pangea Technologies in the UK. This was to examine the effect of these waste feedstocks on xylitol production. Then experiments were performed to examine the effect of resting cells on xylitol production. Further scale-up studies were also carried out in a batch bioreactor, moving the working volume of the experiments from 100ml to 1L. I did this using the optimized medium concentrations with pure and crude glycerol as primary carbon sources. Downstream processing methods such as activated charcoal treatment, alcohol precipitation and crystallization were where performed on the spent fermentation broth from the reactor studies.

1.1 Xylitol's utility

Xylitol is a sugar alcohol with a large global market demand. This demand is estimated to be between 125,000-190,900 tons per annum globally and is said to increase by 6% per year, some projections say that the demand will reach levels as high as 266,500 tonnes per annum globally by 2022 (Dasgupta *et al.*, 2017; Özüdoğru *et al.*, 2019). The reason for this demand is that, as can be seen in Table 1.1, xylitol possesses unique advantages as a substitute for sugar, as an anti-cariogenic and as a pharmaceutical (Rao *et al.*, 2016; Sapcı *et al.*, 2016). The US food and drug administration (FDA) states that xylitol is safe for consumption so long as the amount used is less than is required to produce its

intended effect (that being a laxative effect which requires the consumption of more than 60 g per day), it is also safe for consumption in foods for special dietary uses (Albuquerque *et al.*, 2014; FDA, 2019a). Its application in the food and beverage industries comes from the fact that its level of sweetness is comparable to that of sucrose whilst having only 40 % of its calories (Ping *et al.*, 2013; Firoozi and Kang, 2019). Due to this, it has been used as a low-calorie sweetener in cupcakes, chocolate, biscuits, chewing gums and other confectionery products (Rehman, Murtaza and Mushtaq, 2016; Sapcı *et al.*, 2016). Xylitol does not participate in Maillard reactions which cause browning in certain foods (Sapcı *et al.*, 2016). Xylitol is also used as a dietary supplement for people with metabolic conditions such as people with diabetes and people with enterocytic glucose-6-phosphate dehydrogenase (G6PD) deficiency, this is because it is metabolised through insulin-independent pathways in humans. (Huang *et al.*, 2011; Zhang *et al.*, 2012; Ping *et al.*, 2013; Dasgupta *et al.*, 2017).

Xylitol is also useful in the pharmaceutical industry (Zhang *et al.*, 2018). Xylitol has antimicrobial properties that allow it to simultaneously sweeten orally taken medication and negatively affect potential pathogens (Dasgupta *et al.*, 2017; Zhang *et al.*, 2018). Xylitol also has been shown to promote gastrointestinal health, prevent ear infection and can be a laxative in high concentrations (Albuquerque *et al.*, 2014; Godswill, 2017; Kumar *et al.*, 2018). As well as this, xylitol can be used to treat the following conditions: acute otitis media, colon diseases, haemolytic anaemia, inflammatory processes, osteoporosis, parenteral injuries, renal injuries and respiratory diseases (López-Linares *et al.*, 2018). Also, xylitol is less reactive and many organisms do not possess the pentose phosphate pathways necessary to metabolise it, thus rendering it harder to utilise as a carbon source for microorganisms (Dasgupta *et al.*, 2017). Xylitol also has utility in the field of dentistry (Zhang *et al.*, 2012; Ur-rehman *et al.*, 2015). Studies have demonstrated that xylitol is highly effective at treating oral infections that involve *Haemophilus influenzae*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*

(Albuquerque *et al.*, 2014). Studies also demonstrate xylitol's ability to prevent organisms from forming oral biofilms and when xylitol is used for its anti-cariogenic effects it demonstrates reductions in tooth decay of up to 100 %. It prevents the release of plaque acids. It increases calcification in teeth and it is used as a medication to help with dental caries (Albuquerque *et al.*, 2014; Zhang *et al.*, 2018). For these reasons, xylitol is used in many dental products, not only as a sweetener but also for its positive impact on oral health (Ur-rehman *et al.*, 2015; Chukwuma and Islam, 2018; Zhang *et al.*, 2018).

Table 1-1: The uses and functions of xylitol across various industries.

Industry	Function	Source
Food	Sweetener/ Sweet as sugar	(Ping *et al.*, 2013; Firoozi and Kang, 2019)
Food	Sweetener/ 40 % of the calories of sugar	(Ping *et al.*, 2013; Firoozi and Kang, 2019)
Food	Sweetener/ Metabolised through an insulin-independent pathway and can be consumed by diabetics	(Huang *et al.*, 2011; Zhang *et al.*, 2012; Ping *et al.*, 2013; Dasgupta *et al.*, 2017).
Food	Confectionary/ Does not participate in Maillard reactions	(Sapcı *et al.*, 2016)
Pharmaceuticals	Dental and drugs/ Hard for microorganisms to obtain energy from it	(Dasgupta *et al.*, 2017)
Pharmaceuticals	Sweetener/ Sweetens flavour of medication without encouraging pathogen growth	(Dasgupta *et al.*, 2017)
Pharmaceuticals	Nutritional/ Laxative	(Albuquerque *et al.*, 2014; Godswill, 2017; Kumar *et al.*, 2018)
Pharmaceuticals	Antimicrobial/ Used to prevent ear infection	(Albuquerque *et al.*, 2014; Godswill, 2017; Kumar *et al.*, 2018)
Pharmaceuticals	Nutritional/ Positive effect on gastrointestinal health	(Albuquerque *et al.*, 2014; Godswill, 2017; Kumar *et al.*, 2018)
Pharmaceutical	Medical/ Used to treat the following: acute otitis media, colon diseases, haemolytic anaemia, inflammatory processes, osteoporosis,	(López-Linares *et al.*, 2018).

	parenteral injuries, renal injuries, respiratory diseases	
Pharmaceutical	Antimicrobial and dental/ Used to treat oral infections of *Haemophilus influenzae, Streptococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus pneumoniae*	(Albuquerque *et al*., 2014; Zhang *et al*., 2018)
Pharmaceutical	Dental/ Prevents the formation of oral biofilms	(Albuquerque *et al*., 2014; Zhang *et al*., 2018)
Pharmaceutical	Dental/ 100 % reductions when used against tooth decay	(Albuquerque *et al*., 2014; Zhang *et al*., 2018)
Pharmaceutical	Dental/ Prevents the release of plaque acids	(Albuquerque *et al*., 2014; Zhang *et al*., 2018)
Pharmaceutical	Dental/ It increases calcification in teeth	(Albuquerque *et al*., 2014; Zhang *et al*., 2018)
Pharmaceutical	Dental/ Used as medication to help with dental caries	(Albuquerque *et al*., 2014; Zhang *et al*., 2018)

1.2 Xylitol production

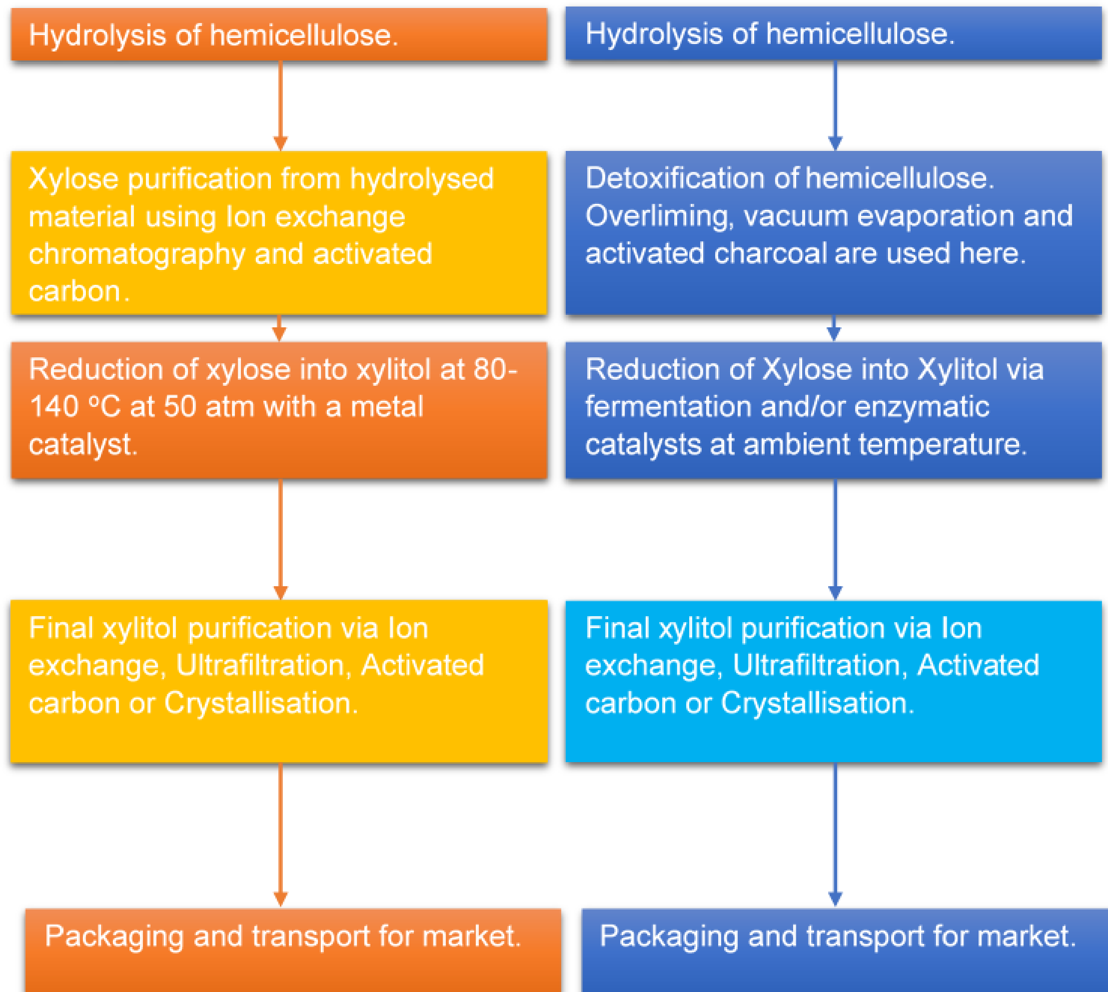


Figure 1.2: A comparison between biological xylitol production (on the right and in blue) and synthetic xylitol production (on the left and in orange). With the Purification steps in lighter shades of their respective colours (Mohamad, Mustapa Kamal and Mokhtar, 2015; Y. Xu *et al.*, 2019).

Currently, there are two methods of xylitol production, biological and synthetic (Delgado Arcaño *et al.*, 2018; Zhang *et al.*, 2018). The biotechnological method revolves around the fermentation of lignocellulosic material and the synthetic revolves around the catalytic hydrogenation of pure xylose. The former of which is cheaper as it requires detoxification as opposed to purification (Delgado Arcaño *et al.*, 2018; Zhang *et al.*, 2018). Despite the efforts of companies such as

Thomson Biotech (Xiamen) Co., Ltd, ZuChem Inc., Danisco and Xyrofin the biological method of xylitol production remains industrially unestablished and the synthetic process remains the only industrially established method of xylitol production (Delgado Arcaño *et al.*, 2018). This is because the process is advantageous in terms of yield and conversion efficiency (Dasgupta *et al.*, 2017; Ge *et al.*, 2018).

However, there is a growing interest in biological alternatives that use microorganisms to create the catalysts capable of reducing xylose into xylitol (Zhang *et al.*, 2018). This is due to many of the previously mentioned problems associated with the synthetic production of xylitol such as the high costs incurred when attaining and maintaining the heat and pressure required for the synthetic process (Wu *et al.*, 2018), the additional purification step involving ion-exchange chromatography and activated carbon before the reduction of the xylose into xylitol as can be seen in Figure 1.2 (Su *et al.*, 2015; Wu *et al.*, 2018), the toxic metal catalyst and the water requirement. Due to this, there have been many years of research into the biological production of xylitol (Li *et al.*, 2015; Mohamad, Mustapa Kamal and Mokhtar, 2015; Park *et al.*, 2016; Jo *et al.*, 2017; Zhang *et al.*, 2018).

A biological approach yields several advantages over a synthetic process. A biological process can be performed at ambient temperatures and pressures, thereby reducing costs (Chang *et al.*, 2018). A biological process does not require pure xylose as the microorganisms involved can utilise lignocellulosic hydrolysates directly, and therefore the biological process only requires one expensive and energy-intensive purification procedure before selling it. The organisms only require comparatively cheaper detoxification of the hydrolysed lignocellulosic hydrolysates as can be seen in Figure 1.2 (Mohamad, Mustapa Kamal and Mokhtar, 2015; Y. Xu *et al.*, 2019). There is also the potential with the biological process, to increase sustainability and economic viability through the

usage of cheap, renewable, and sustainable feedstocks with a high carbon content (Albuquerque *et al.*, 2014; Unrean and Ketsub, 2018).

As can be seen in Figure 1.3 when xylitol is produced in nature it utilises an enzyme pathway that relates to xylitol and consists of 3 steps. The first step involves xylose reductase (XR), which reduces xylose into xylitol and requires NADPH and/or NADH as a cofactor. The second step involves xylitol dehydrogenase (XDH), which converts xylitol into xylulose and is dependent on NAD^+ as a cofactor. Then there is a cofactor independent third step which involves xylulose kinase (XK), which phosphorylates xylulose into xylulose-5-phosphate. This xylulose-5-phosphate is then used in the pentose phosphate pathway to assist in the generation of biomass (Ledesma-Amaro *et al.*, 2016).

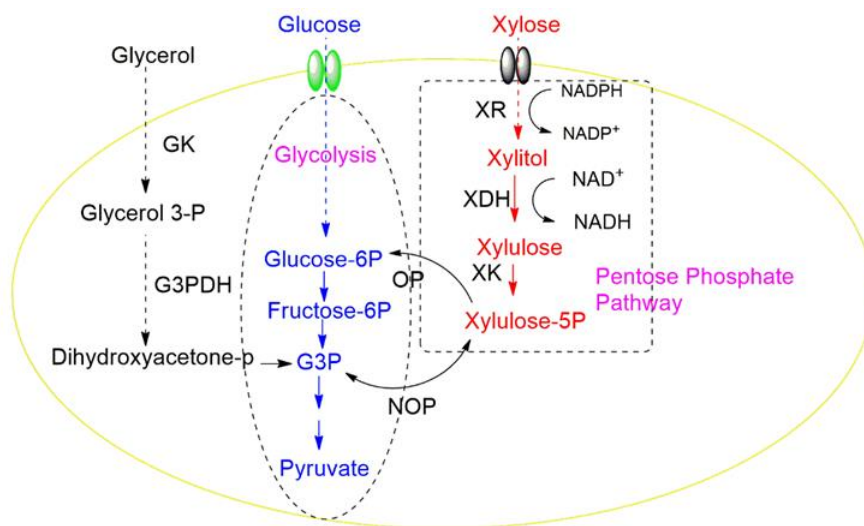


Figure 1.3: The xylose assimilation pathway and adjacent enzyme pathways. XR: xylose reductase, XDH xylitol dehydrogenase, XK: xylulose kinase, NOP: non-oxidative pathway, OP: oxidative pathway, G3P: glyceraldehyde-3-phosphate, GK: glycerol kinase, G3PDH: glycerol-3-phosphate dehydrogenase (Ledesma-Amaro *et al.*, 2016; Klein *et al.*, 2017).

The biotechnological production of xylitol relies on this enzyme pathway and there are two ways of utilising it for xylitol production 1). enzymatic production and 2). whole-cell fermentation (Chang *et al.*, 2018). Enzymatic production is where XR

enzymes and their cofactors are extracted from the cell, purified and utilised directly (Chang *et al.*, 2018). This has demonstrated high yields in laboratory conditions and can theoretically produce yields of up to 100 % (Dasgupta *et al.*, 2017; Chang *et al.*, 2018). However, the extraction and purification process, as well as the large cofactor requirements, render it uneconomical on a large industrial scale, for now (Dasgupta *et al.*, 2017). Whole-cell fermentation is where the organism that produces the enzymes is not purified and the enzymes are not extracted. This usually gives a lower yield because it is more difficult to avoid in this case xylitol being syphoned off using the XDH enzyme to assist in cell growth. However, this process does not require the expensive purification and extraction of enzymes and cofactors that enzymatic production does (Chang *et al.*, 2018).

There are also two feeding strategies utilised in the biological production of xylitol. 1). Where xylitol is used as the sole carbon source. 2). Where xylitol is used as a co carbon source with an additional carbon source (Akinterinwa, Khankal and Cirino, 2008; Sasaki *et al.*, 2010). Generally, the latter is preferred as the former has several issues that cause low yield and productivity, such as some organisms having a low preference for xylitol as a carbon source and the fact that if xylose is the only carbon source then the organism has to partition some for cell growth and some for xylitol production. Due to this, it is desirable to grow the organism and allow it to accumulate biomass initially on a carbon source preferred by that organism and then for it to start to produce xylitol from the remaining xylose. This approach allows for more growth and biomass accumulation in a shorter amount of time which in turn leads to higher productivities and yield in less time (Akinterinwa, Khankal and Cirino, 2008; Sasaki *et al.*, 2010).

<u>Table 1.2:</u> Xylitol yields and titres from different microorganisms. With bacteria in blue, fungus in orange and yeast in purple. A – symbol is used where the source neglects to provide the value for either the yield or the titre.

Organism (strain)	Carbon source	Yield (g/g)	Titre (g/l)	Reference
Enterobacter liquefaciens (553)	D-xylose	-	33.3	Yoshitake *et al.*, 1973
Corynebacterium sp. (B-4247)	D-xylose	0.48	40	Rangaswamy and Agblevor, 2002
Penicillium crustosom (CCT)	D-xylose	-	0.52	Sampaio *et al.*, 2003
Aspergillus niger (PY11)	D-xylose	0.101	1.139	Kang *et al.*, 2016
Candida sp. (559-9)	D-xylose	0.99	173	Ikeuchi *et al.*, 1999
Candida guilliermondii (FTI 20037)	Oat hull	0.87	54	Soleimani and Tabil, 2014
Pichia sp. (AM159106)	D-xylose	0.58	25	Rao and Shivaji, 2007
Kluyveromyces marxianus (CCA510)	Cashew apple bagasse hydrolysate	0.5	12.27	Albuquerque, Danielle, *et al.*, 2014
Debaromyces hansenii	Charcoal treated wood hydrolysate	0.47	-	Domínguez *et al.*, 1999
Debaryomyces nepalensis (NCYC 3413)	D-xylose	0.44	36	Kumdam, Murthy and Gummadi, 2012
Hansunela anomala (NCAIM Y.01499)	D-xylose	0.47	21.7	Mareczky *et al.*, 2016

There are several organisms with the ability to naturally ferment xylose into xylitol, some are filamentous fungi and bacteria, but most are yeasts (Y. Xu *et al.*, 2019). In biotechnological xylitol production, yeasts are the preferred organisms due to three main reasons, including, a high pentose assimilation rate, their high productivity, and their stable expression of XR. Several wild type and engineered yeasts have been reported as being capable of producing xylitol (Dasgupta *et al.*, 2017).

The most well documented of these yeasts are *Candida* strains (Aghcheh, Bonakdarpour and Ashtiani, 2016). This is because the average productivity of *Candida* yeasts is higher than other yeast strains (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019). Some *Candida* strains such as xylitol dehydrogenase deficient mutants of *Candida tropicalis* have demonstrated xylitol yields of up to 97 % (Jeon *et al.*, 2012) and some strains such as *Candida sp.* (559-9) have even demonstrated yields of up to 99 % (Ikeuchi *et al.*, 1999). One of the reasons for these yields is that *Candida* strains possess well defined specialised xylose transporters, and can utilise xylose as a sole carbon source (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019). Another is that the XR enzymes from certain *Candida* strains can use $NADH^+$ which is a more prevalent and stable source of cofactors than $NADP^+$ (Petschacher *et al.*, 2005). However, *Candida* are also some of the most prolific fungal pathogens and are the cause of most fungal infections worldwide (Turner and Butler, 2014).

Then there are *Pichia* strains, which on average are not as productive as *Candida* strains, having only demonstrated yields of up to 58 % (Rao and Shivaji, 2007). The advantage of *Pichia* species is that they are not as pathogenic yet they have pentose assimilation pathways (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019). Products that have been produced by *Pichia* species have been given a generally regarded as safe (GRAS) rating by the FDA (FDA, 2019d). However, there are also some more disadvantages. *Pichia* is not well documented as a xylitol producer as it is better at producing by-products from xylose, because of this the literature is mostly focused on these by-products such as ethanol production from xylose (Li, 2012). Cultures of *Pichia* species also have a higher risk of contamination by other organisms (Dasgupta *et al.*, 2017).

Kluyveromyces strains are seen as one of the better options for xylitol production (Dasgupta *et al.*, 2017). *Kluyveromyces* can produce xylitol at high temperatures and because of this, they are considered good candidates for industrial

fermentation (Hua *et al.*, 2019). Products produced by *Kluyveromyces* species have been given GRAS ratings from the FDA and *Kluyveromyces* species also have a low potential to be contaminated by other organisms, this is because they can ferment at higher temperatures (FDA, 2019c; Hua *et al.*, 2019). *Kluyveromyces* strains possess similar xylitol accumulation traits to *Candida* strains as well (Ikeuchi *et al.*, 1999; Albuquerque *et al.*, 2015). However, *Kluyveromyces* do not possess specialised xylose specific transporters, this affects their yields and their productivities (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019).

Hansenula strains are also promising candidates. Products produced by *Hansenula* strains have been given GRAS ratings from the FDA (FDA, 2019b). *Hansenula* strains are also methylotrophs meaning they can utilise alcohols as a primary carbon source (Dmytruk *et al.*, 2017).

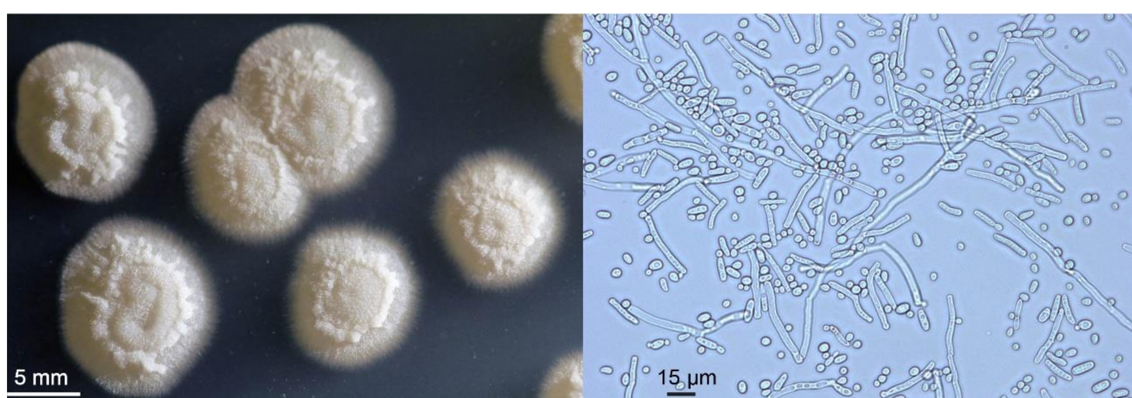
Then there are *Debaromyces* strains. Cultures of *Debaromyces* yeasts have a low chance of being contaminated by other organisms (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019). *Debaromyces* strains are also extremophiles meaning that they can ferment xylose in a broader variety of conditions (Buzzini, Turchetti and Yurkov, 2018). However both *Hansunela* and *Debaromyces* strains are poorly understood in terms of molecular biology and more research would need to be done before implementing them in an industrial process (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019).

Whilst most xylitol producers are yeasts, some organisms that produce xylitol are not yeasts or even fungi (Mohamad, Mustapa Kamal and Mokhtar, 2015). Bacteria such as *Corynebacterium* strains have been reported to produce yields of up to 40 %. *Enterobacteria* strains have also been known to produce xylitol, with *Enterobacter liquefaciens* obtaining a titre of 33.3 g/l (Yoshitake *et al.*, 1973;

Rangaswamy and Agblevor, 2002; Mohamad, Mustapa Kamal and Mokhtar, 2015). There are also filamentous fungi such as *Aspergillus niger*, which has produced a yield of 0.101 g/g with a titre of 1.139 g/l, and *Penicillium clostridium* which has produced titres of up to 0.52 g/l (Mohamad, Mustapa Kamal and Mokhtar, 2015). However, while there are non-yeasts that can produce xylitol, their yields and titres are not suitable and competitive enough for industrial-scale xylitol production.

1.3 Yarrowia lipolytica

Y. lipolytica is a dimorphic ascomycetous heterothallic saccharomycetous yeast. It has accrued decades of academic interest. In particular, it's application as a cell factory for the production of valuable metabolites (Zhu and Jackson, 2015; Qiao *et al.*, 2017; Madzak, 2018). It usually grows and can be isolated from environments with a high protein and lipid concentration, like meat, poultry, olive oil, cheese, yoghurt and other dairy products (Liu, Ji and Huang, 2015). It can also grow in other environments such as oil-polluted areas, mycorrhizae soils and aqueous environments (Madzak, 2018). Oxygen appears to have a large influence on its growth rate and it tends to grow in temperatures below 32-34°C (Liu, Ji and Huang, 2015).



<u>Figure 1.4:</u> (Left) A Photograph of *Y. lipolytica* colonies, (Right) A microscopy picture of *Y. lipolytica* cells at 1000x magnification (Wolfe, 2014).

Y. lipolytica, most importantly to this study, also has a high reported yield of xylitol when using a dual carbon source feeding strategy, producing 13.8 g/l of xylitol from 15 g/l of xylose, that being a yield of 0.92 g/g after only 4 days (Ledesma-Amaro *et al.*, 2016). This is most likely because *Y. lipolytica* poorly expresses the key xylose pathway enzymes XDH and XK. This is also indicated by the fact that *Y.lipolytica* is grown using xylose as a sole carbon source its ability to grow and produce xylitol is greatly diminished (Ledesma-Amaro *et al.*, 2016; Rodriguez *et al.*, 2016; Niehus *et al.*, 2018). This lack of expression of XDH and XK means that XR is still converting xylose into xylitol but once the xylitol is accumulated it cannot be converted into xylulose and xylulose-5-P and therefore it cannot contribute to cell growth and biomass accumulation via the pentose phosphate pathway (Pal *et al.*, 2013; Mohamad, Mustapa Kamal and Mokhtar, 2015; Ledesma-Amaro *et al.*, 2016; Rodriguez *et al.*, 2016; Dasgupta *et al.*, 2017). This accumulation effect is positive for a potential xylitol biofactory as it means that very little xylitol is being syphoned for cell growth.

However, *Y.lipolytica* also has several other features that make it a highly promising cell factory for the production of xylitol. It is known to produce a wide variety of valuable by-products (Ledesma-amaro and Nicaud, 2016) such as citric acid which can be used as an acidulant, and antioxidant and a flavouring agent (Sarris *et al.*, 2019), microbial oils which have utility in the biofuels industry (Ledesma-Amaro *et al.*, 2016), aromatic compounds which have utility as flavouring compounds in the food industry (Liu, Ji and Huang, 2015) and certain pigments that are antioxidants and have health benefits in humans (Zhu and Jackson, 2015). *Y. lipolytica* is also known for its single-cell protein production and its enzyme production (Juretzek *et al.*, 2001; Liu, Ji and Huang, 2015). This is not only beneficial in and of itself but it is also useful with regards to *Y.lipolytica* as a xylitol producer. This is because it makes an industrial scale bioprocess more attractive economically, as the extra revenue generated from the sale of these byproducts can offset the operating costs of running an industrial scale bioprocess.

Y. lipolytica is non-pathogenic and has a GRAS rating from the FDA (Juretzek *et al.*, 2001; Madzak, Gaillardin and Beckerich, 2004; FDA, 2015; Liu, Ji and Huang, 2015). With regards to xylitol production, this is useful because it makes any bioprocess that uses *Y. lipolytica* safer than a pathogenic yeast, such as a *Candida* yeast. *Y. lipolytica* has a level of tolerance to many environmental factors, including being able to operate within a broad pH range of 4-8, being resistant to salt solutions and metal ions (Liu, Ji and Huang, 2015; Egermeier *et al.*, 2017; Carsanba *et al.*, 2019). With regards to xylitol production, this is useful because it makes a bioprocess that uses *Y. lipolytica* more reliable, as there is less risk involved with regards to acidic, salt or metal contamination (Liu, Ji and Huang, 2015; Egermeier *et al.*, 2017; Carsanba *et al.*, 2019).

Y. lipolytica can also grow on a wide variety of substances, these include both hydrophilic and hydrophobic substances, as well as N-paraffins, fatty acids, oils, alkanes, sugars, sugar alcohols, fatty alcohol and alcohols (Madzak, Gaillardin and Beckerich, 2004; Liu, Ji and Huang, 2015; Egermeier *et al.*, 2017). This means that *Y. lipolytica* can grow on waste material, by-products, and other cheap materials such as molasses, triacylglycerols, wastewater, mannitol, industrial fats, arabitol, and erythritol. However most importantly for this study, it can also grow on crude glycerol and lignocellulosic material (Madzak, Gaillardin and Beckerich, 2004; Liu, Ji and Huang, 2015; Egermeier *et al.*, 2017).

1.4 The abundance of xylose and usage in biorefineries.

As previously stated, xylitol is obtained by reducing xylose. Xylose is a component of hemicellulose, which is obtained from, in particular, lignocellulosic biomass (Farhat *et al.*, 2017; Naidu, Hlangothi and John, 2018). Lignocellulosic biomass is plant biomass recovered from plants, it is one of the cheapest, most abundant, and most sustainable feedstocks available. Lignocellulosic biomass has three main components cellulose (54-25 % of total lignocellulosic biomass),

lignin (28-6 % of total lignocellulosic biomass) and hemicellulose (36-11 % of total lignocellulosic biomass) (Naidu, Hlangothi and John, 2018). Hemicellulose is a heteropolymer containing a wide variety of sugars including arabinose, mannose, galactose and glucose (Naidu, Hlangothi and John, 2018). However, xylose is the most abundant of these sugars (~90 %) and due to this fact, xylitol is the second most abundant sugar available after glucose (Cunha *et al.*, 2019). This means that fractions from various biomass sources usually contain, as can be seen in Figure 1.2, high percentages of xylose relative to their total biomass (Gírio *et al.*, 2010). This combined with the fact that 5 million tons of plant biomass residue is obtained from the agriculture sector every year makes xylitol a potentially very abundant and renewable resource (Naidu, Hlangothi and John, 2018). Although hemicellulose is one of the main components of lignocellulose, it is by far the most underutilised (Mugwagwa and Chimphango, 2019). This is due to many factors. The fact that it is a heteropolymer made up of five different sugars is one contributing factor. The other two lignocellulosic components are much more homogenous making them much easier to extract. For example, lignin consists mostly of phenylpropane units, and cellulose mostly consists of D-glucose units (Naidu, Hlangothi and John, 2018). This is why hemicellulose is underutilised when compared to cellulose and lignin (Farhat *et al.*, 2017; Naidu, Hlangothi and John, 2018). Another issue preventing the more widespread use of hemicellulose is the fact that there are not many microorganisms with a native pathway for xylose (Liu *et al.*, 2018). Many organisms also exhibit catabolite repression in favour of glucose as opposed to xylose (Kim *et al.*, 2019). This has resulted in hemicellulose and xylose being overlooked as a carbon source for use in industrial biotechnology. However, new biorefineries (which are facilities for the processing of biomass for the production of multiple products in a manner analogous to a petroleum refinery) are becoming more cost-effective and better

in their extraction of hemicellulose (Abate *et al.*, 2015; Lange, 2017; Sporck *et al.*, 2017).

<u>Table 1.3:</u> Composition of lignocellulosic biomass from different sources (Gírio *et al.*, 2010; Naidu, Hlangothi and John, 2018).

Biomass source	Cellulose (%)	Lignin (%)	Hemicellulose (%)	Xylose (%)
Harwood				
Poplar	50–53	16	26-29	18–21
Oak	40	24	36	22
Eucalyptus	54	21	18	18–19
Softwood				
Pine	42–50	20	24-27	5–11
Douglas fir	44	27	11	6
Spruce	46	28	23	5–10
Agricultural waste				
Wheat straw	35-39	12-16	23-30	19–21
Barely straw	36-43	6-10	24-33	15
Rice straw	29-35	17-19	23-26	15–23
Rice husks	29-35	15-20	15-20	18
Corn cob	33-41	6-16	32–36	28–35
Corn stalks	35-40	7-18	17-35	26
Sugarcane bagasse	25-45	15-25	28-32	21–26

1.5 Crude glycerol

Crude glycerol is often the result of (but not limited to) transesterification during biodiesel production (Ganigué *et al.*, 2019). In the biodiesel industry, 1 kg of crude glycerol is produced for every 10 kg of biodiesel (Ganigué *et al.*, 2019; Kumar *et al.*, 2019). As a by-product of biodiesel, the price of crude glycerol is highly linked to biodiesel production and an increase in biodiesel production over the past few decades has led to a significant reduction in the price of crude glycerol (Kumar *et al.*, 2019). For instance, in 2007 alone the price of xylitol decreased from $0.25 per pound to $0.05 per pound in the US alone (Fangxia, Milford and Runcang,

2012). This economic advantage makes crude glycerol a desirable feedstock for various bioprocesses.

Table 1.4: The composition of crude glycerol from different sources.

Component	Source	
	(Fangxia, Milford and Runcang, 2012)	(Kumar *et al.*, 2019)
Glycerol	38-96 %	45-55 %
Methanol	14-50 %	15-20 %
Ash	14 %	-
Soap	13 %	1-5 %
Salt	2-3 %	0.5-2 %
Water	-	25-35 %
Other impurities	2-3 %	-

However, although it usually contains a high glycerol concentration, it also contains other impurities such as methanol, ash, soap, salts and other impurities (Fangxia, Milford and Runcang, 2012; Kumar *et al.*, 2019). The percentages of these components, can vary wildly depending on several different factors, including the catalyst used, the recovery efficiency particularly of the catalysts, the efficiency of transesterification, and impurities in the feedstock (Fangxia, Milford and Runcang, 2012). While these disadvantages might present issues to other organisms, *Y. lipolytica* is a model organism for tolerance to salt and actively consumes lipids (Zhu and Jackson, 2015; Qiao *et al.*, 2017; Madzak, 2018). This means that *Y. lipolytica* is either resistant to, or can utilise many of the impurities in crude glycerol and can utilise crude glycerol just as effectively as pure glycerol (Liu, Ji and Huang, 2015).

1.6 Optimisation

Media optimisation is a methodology used in industry and is vital for increasing the yield of fermentative products (Prabhu, Mandal and Dasu, 2017). The traditional form of media optimisation or "one variable at a time analysis", which is where all variables remain the same and a single variable is varied within the experimental unit, to provide insight into how that variable affects the outcome/product (Prabhu and Jayadeep, 2017; Prabhu, Mandal and Dasu, 2017). The disadvantages of this are that it is both lab space and time-consuming as each variable requires an experiment and there is also no way to account for variable interaction (Prabhu and Jayadeep, 2017; Prabhu, Mandal and Dasu, 2017).

Instead, this paper opts to utilise multivariate analysis, which is where multiple variables are varied withing the same experimental unit, and the statistical relationships between the variables are the thing the provides insight into it (Olkin and Sampson, 2001). The type of multivariate analysis that this paper uses is called central composite design (CCD) which is a type of response surface methodology (RSM) (Asadi and Zilouei, 2017). RSM is where several variables are used to optimise a response variable (Asadi and Zilouei, 2017). CCD is an experimental design for RSM in which an experiment is carried out where input variables are varied according to levels (in this case of concentration) (Ahmadi *et al.*, 2005). The data from this experiment is then used to create a model whose fitness is then analysed using an analysis of variance (ANOVA) (Ahmadi *et al.*, 2005). This analyses the variance between the actual result and the predicted result to determine the fitness of the model (Ahmadi *et al.*, 2005).

However, the issue with RSM and CCD is that they both rely on linear regression (Prabhu and Jayadeep, 2017; Prabhu, Mandal and Dasu, 2017). Artificial neural networks (ANN) is a methodology based on a biological neuron that receives

signals and transmits outputs through nonlinear functions (Prabhu, Mandal and Dasu, 2017). The ANN neurones then form networks that align the neurones to a set of predetermined layers, input, hidden and output (Prabhu, Mandal and Dasu, 2017). These networks then utilise a feedforward backpropagation algorithm to transmit data from the input layer to the output layer and back again (Prabhu, Mandal and Dasu, 2017). These networks are usually used as training networks (Prabhu, Mandal and Dasu, 2017). ANN is usually also coupled with genetic algorithm (GA) (Prabhu and Jayadeep, 2017). GA is a methodology based on a form of Darwinian selection which creates a population of experiments and mutates them (Prabhu, Mandal and Dasu, 2017). This population then creates a new population with the best fitting and most optimised members surviving to the next generation, and so on a so forth until the best fitting most optimal member of the population is left (Prabhu, Mandal and Dasu, 2017).

There is a wide-scale industrial precedent for the use of these statistical methodologies (Olkin and Sampson, 2001; Asadi and Zilouei, 2017; Prabhu and Jayadeep, 2017). Multivariate analysis is used in finance for analysis financial instruments and in pharmaceuticals for measuring and relating multiple patient responses to toxicity (Olkin and Sampson, 2001). Statistical modelling methodologies such as RSM, CCD, ANN and GA are also routinely used in industries to check and optimise the operational efficiency of industrial processes as well as to explain phenomena (Asadi and Zilouei, 2017; Prabhu and Jayadeep, 2017).

1.7 Rationale

There are numerous rationales behind the various aspects of this study. The rationale behind the investigation of xylitol production is that there are many industrial uses for xylitol and the substance is in high demand. The biotechnological procedure has the potential to drastically reduce the costs and

the environmental impact of xylitol production. The rationale behind the use of *Y. lipolytica* in this study is its ability to produce xylitol yields similar to some of the most productive xylitol producing organisms whilst possessing several innate advantages over all of them such as non-pathogenicity, tolerances and the ability to consume a wide variety of cheap and abundant carbon sources. The rationale for scale-up tests is that there is a need to examine how applying industrial xylitol production techniques would affect xylitol production in *Y. lipolytica* if there is a potential for it to be used in the industry. These tests have not been done with regards to *Y. lipolytica* as a xylitol producer before making this data valuable for industry. The rationale behind the use of crude glycerol and lignocellulosic biomass lies in its cheapness and availability, this combined with the fact that *Y. lipolytica* can consume them provides a good rationale for its use in this study. crude glycerol and lignocellulosic biomass as feedstocks have the potential to drastically reduce the operating costs of a xylitol production process involving *Y. lipolytica* and providing novel data on this would be valuable for such an endeavour. The rationale behind the use of the optimisation methodologies in this study is because they can provide the optimum concentrations for multiple media components at once without the need for multiple experiments for each component. This not only saves time and lab space, but it also provides insight into all of the components and their interactions between each other.

2 Aims and Objectives

2.1 Aims

This studies aim is to investigate and optimise the ability of *Y. lipolytica* to produce xylitol.

2.2 Objectives

I. To compare the different co-substrates effect on xylitol production and cell biomass accumulation.
II. To optimise a production medium by utilising CCD along with ANN coupled with GA.
III. To perform scale-up studies in bench bioreactors.
IV. To perform downstream processing studies on the separation of xylitol from the fermentation broth.
V. To investigate the utilisation of resting cells for xylitol production.

3 Material and Methods

3.1 Microorganism and media preparation/maintenance

The current study made use of *Y. lipolytica* Po1d (*ΔUra ΔLeu*) derived from wild-type strain W29 (ATCC20460). The *Y. lipolytica* strain was preserved in 20 % glycerol (v/v) at - 80 °C and maintained on a petri dish containing a yeast peptone dextrose (YPD) agar medium (1 % yeast extract, 2 % peptone, 2 % dextrose and 2 % agar) at pH 7.0 and 30 °C. The seed culture was grown in a 250 mL Erlenmeyer flask containing 50 mL YPD broth. The final pH of the medium before sterilization was adjusted to 7.0. Cultivation was carried out for 24 h at 30 °C on a rotary shaker at an agitation speed of 250 rpm.

3.2 Shake flask cultivations

Before the optimisation the composition for the fermentation medium was based on a medium found in Ledesma-Amaro *et al.*, (2016). The media composition initially used was as follows: (g/L) pure or crude glycerol/glucose, 20; xylose, 20; yeast nitrogen base (YNB), 1.7; NH4Cl, 1.5. The medium was prepared in 50 mM phosphate buffer. This medium was used because in the aforementioned paper it achieved a yield of 0.92 g/g. Post optimisation the values of media components were as follows (g/L): pure/crude glycerol, 20; xylose, 55; YNB, 5.0; NH_4Cl, 3.94; phosphate buffer, 132.5 mM. The initial pH was adjusted to 6.8 before inoculation by using 5N NaOH in both cases. The submerged cultivations were carried out in 500 mL shake flasks containing a 100 mL working volume. The flask was inoculated with fresh inoculum at 600NM with an OD of 0.1 and kept at 28 °C under constant shaking at 220 rpm on a rotary shaker (Excella 24, New Brunswick). Crude glycerol used in this work was kindly provided by biodiesel company Greenergy, UK. The crude glycerol contained glycerol (72.8 %), non-glycerine material (soaps, fatty acids, esters, salts, other organic by-products (5.7 %), methanol (2.0 %), water (12.2 %) and ash (9.6 %) (these values are approximates and subject to variation of up to +/-5 %). The xylose (26.4 g/L) rich lignocellulosic hydrolysate from sugarcane bagasse was obtained from Nova

Pangea Technologies, UK. When the lignocellulosic hydrolysate was used the initial xylose, concentration was 10 g/L.

3.3 Optimisation studies

3.3.1 Central composite design (CCD) for media optimisation

The central composite design (CCD) optimisation was carried out, with the view of optimizing the variables and to give insight over the combined effect of the four variables (xylose, YNB, NH4Cl and phosphate buffer) at a constant glycerol concentration to maximise xylitol titre and yield. The Design-Expert software (version 7.0) was used to develop CCD for four independent variables and five levels (Table 3.1). The total number of experiments (*N*) was based on the equation (3.1).

$$N = 2^k + 2k + 6 \qquad (3.1)$$

k is the number of independent variables. The experiment comprises 2 axial points and 6 replicates for the centre points so that pure error could be evaluated. The second-order polynomial for predicting the optimal levels was expressed according to the equation (3-2).

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i<j} \sum \beta_{ij} X_i X_j + \varepsilon \qquad (3.2)$$

Where, Y_i: is the predicted response; β_0 β_i, β_{ij}, β_{ii} are constant and regression coefficients of the model, ε represents error, X and its variations represent the independent variables in coded form, X_i represents a linear term, $X_i{}^2$ represents the square term, $X_{i<j}$ represents the interaction term.

<u>Table 3.1:</u> Experimental codes, ranges and levels of the independent variables used for CCD experiments.

Independent Variables	Units	Symbol Code	Coded Value				
			-α	-1	0	1	+α
Xylose	g/l	X_1	5	20	35	50	65
YNB	%	X_2	0.05	0.2	0.35	0.5	0.65
NH_4Cl	%	X_3	0.05	0.2	0.35	0.5	0.65
Phosphate Buffer	mM	X_4	2.5	35	67.5	100	132.5

The 0 level values were based on the pre optimisation medium which itself is based on the media in Ledesma-Amaro *et al.*, 2016. There were a few modifications based on the experimental observations of how the yeast performed with regards to metabolite consumption and biomass accumulation. The xylose 0 value was increased to 35 g/l this was because in every fermentation that was run *Y. lipolytica* was capable of consuming all of the xylose in the media by halfway through the fermentation. During the initial experiments, a significant drop in pH was noticed so a higher concentration of buffer was used as the 0 value. The logic behind 0 value concentrations for YNB and NH_4Cl was that an increased concentration might result in an increased yield at the end. The maximum was based on the saturation point of xylose in *Y. lipolytica*. Which has been observed to be at 60g/l so the maximum was set at 65 to cover the whole range of potential xylose concentrations (Prabhu *et al.*, 2020).

The fitness of the created CCD model is analysed using an analysis of variance or ANOVA. This is expressed as a chart with several columns. The first, Degrees of freedom (DF) is the amount of information within a set of data and is determined by the number of observations in a term (eg. the term for xylose has a DF of 1 but the DF for the linear term is 4 as there are 4 components and therefor 4 observations). Sum of square (Seq SS) which is the variation in different parts of the model. This statistic comes in two forms the first is Seq SS or sequential SS which is affected by the order in which the terms are entered

into the model. The second is Adj SS or adjusted SS which is unaffected by this order. The Adjusted mean square (Adj MS) is a measure of how much variation is explained by the model or term, it is determined by Sum of square/Degrees of freedom. Fishers F Value is a measure of whether the term is associated with the response. The P Value is a measure of whether the differences in the data are statistically significant enough to provide evidence for the null hypothesis (Greater than 0.05 = significant, Less than 0.05 = not significant).

3.3.2 The artificial neural network linked genetic algorithm (ANN-GA)

To further optimise the media components, the artificial neural network methodology was adapted. This optimisation was done in addition to CCD because CCD is a linear methodology (Prabhu and Jayadeep, 2017; Prabhu, Mandal and Dasu, 2017) and biological processes are nonlinear in their nature. ANN is a biologically inspired model, which mimics neural systems and is a useful tool to optimize non-linear systems. The model uses a multi-layer perceptron method, this means that the CCD data was fed through a series of neurons or nodes arranged into multiple layers. In this case: the input layers which represent the media components (Prabhu, Mandal and Dasu, 2017), the output layers which represents the concentration of xylitol and the hidden layers which are several nodes that are determined via training that connect the input and output layers via a system of weights (which are the numbers associated between two neurons i.e. the data associated with the input neuron and the nonlinear function equation associated with the hidden layer) and biases (which are the parameters of the neural network) (Prabhu, Mandal and Dasu, 2017). The network was also based on feed-forward backpropagation. This means that the data is fed from the input layer to the output layer and back again (Prabhu, Mandal and Dasu, 2017). In the feed-forward training system, the data is channelised from input to output via the hidden layer, using the weights (w) and biases (b) parameters mentioned before. In this, work transfer functions such as tan sigmoid (f1: tansig) and Pure

linear (f2: Purelin) are situated between the hidden and output layers, respectively. Tansig (equation 3.4) sums up weighted input including the biases, and purelin (equation 3.3) carries out the linearization function for the output. The network architecture consisted of four input layers (xylose concentration, YNB, NH_4Cl, phosphate buffer), eight hidden layers and one output layer representing xylitol concentration. The rationale behind using the ANN topology is that it provides a nonlinear relationship between the input variable and output response making it useful for optimising nonlinear biological processes such as fermentation (Prabhu and Jayadeep, 2017).

$$Purelin = sum \qquad (3.3)$$

$$tansig = \frac{1+\exp(-sum)}{1-\exp(-sum)} \qquad (3.4)$$

The predicted output function can be represented by the equation (3.5)

$$Y_p = f2[w^0 \times f1 \times (w^H \times input\ vector + b^H) + b^0] \qquad (3.5)$$

Where Y_p is the predicted response, w^o, b^o and w^H, b^H are weights and biases of the output and hidden layer, respectively. The total set of experiments were distributed into three sets with a ratio of 70 %, 15 % and 15 % and each of these was subjected to training, validation, and testing, respectively. The network training was done using a Levenberg–Marquardt algorithm, which calculates the error function based on the difference between actual output and predicted output and is propagated backwards through the network layer to minimize the error by adjusting the weights. The algorithm is trained repeatedly until the subsequent minimisation in the error between the input and output layer is met. The

commonly used error function, mean squared error (MSE) is used in the present study and is given in equation (3.6):

$$MSE = \frac{1}{N}\sum_{i=1}^{N}(Y_a - Y_p)^2 \qquad (3.6)$$

Where Y_a is the actual output (experimental xylitol concentration), Y_p is the predicted output (ANN predicted xylitol concentration), N is the number of data points in this work. A Neural Network Toolbox of MATLAB (2010a) mathematical software was used to predict the xylitol production.

3.3.3 Genetic algorithm (GA)

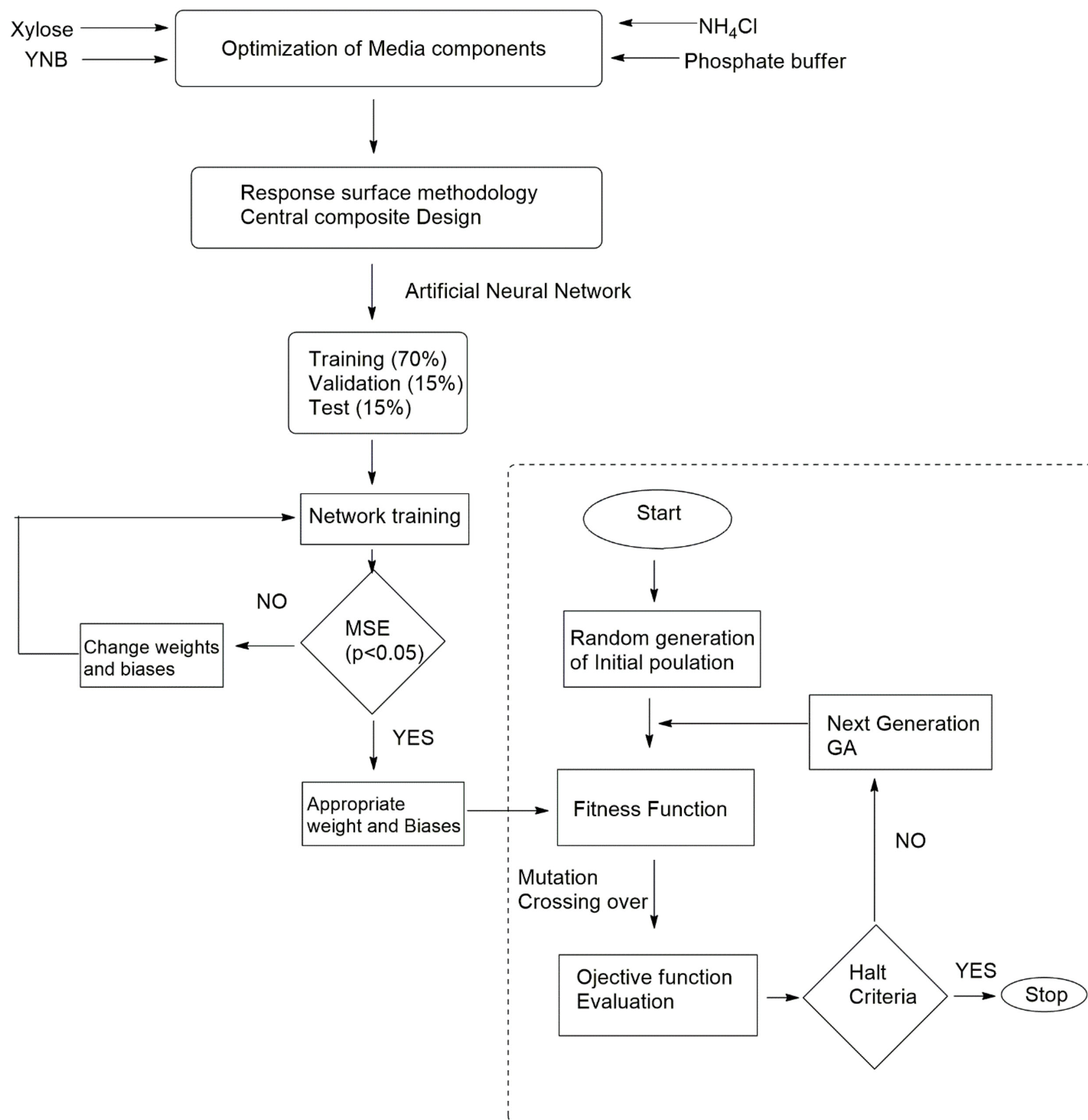
Genetic algorithm (GA) is a Darwinian methodology used to determine the global optimal solution for a non-linear problem and are independent of initial values; GA is often coupled with ANN to achieve precise optimization values. This made it useful for optimising a nonlinear biological process such as fermentation. GA follows four steps to find a global solution. In the first step, initialization of the solution for the population takes place followed by a fitness computation. The selected individual based on the fitness computation then undergoes crossing over and mutation, creating a new set of individuals (Yasin *et al.*, 2014; Prabhu, Mandal and Dasu, 2017). This process is repeated until a global optimum value is achieved. Figure 3.1 gives the generalized working of the optimization process, which includes RSM, ANN and GA. After the RSM the ANN will carry out further simulations the refined values of ANN with then subjected to GA where it uses different parameters and calculates the global optimum values (Pappu and Gummadi, 2017). The objective function of GA can be given by:

$$\text{Maximize } Y = f(x, w), x_i^L \leq x_i \leq x_i^u, i = 1,2,3 \cdots P \quad (3.7)$$

Where f is the objective function (ANN model): x denotes input vector: w denotes corresponding weight vector; Y refers to the xylitol experimental yield. X denotes operating conditions. P denotes no. of input variables and x_i^L&x_i^U lower and upper bounds of x_i fitness of each candidate solution were evaluated based on the following fitness function.

$$\text{error}_j = 1 - \frac{1}{Y^j_{pred}}; j = 1,2,3, \ldots, n \qquad (3.8)$$

Where error j denotes the fitness value of the candidate solution and Y_{pred} denotes the multilayer perceptron (MLP) model predicted xylitol yield of a given candidate solution.



<u>Figure 3.1:</u> Schematic representation of ANN-GA for achieving the global optimum value for the maximization of xylitol concentration in *Y. lipolytica.*

3.4 Resting cell experiments

In the first stage of this experiment *Y. lipolytica* was grown on an optimized medium with pure glycerol in 250 ml flasks containing a 20 % working volume.

The temperature, pH and agitation speed were the same as mentioned in section 3.2. In the second stage, the cells were harvested in the late exponential period (after 48 h) when the OD_{600} was somewhere between 20 and 25. Immediately after, the culture was centrifuged at 2800 g for 10 min, and the resulting pellet was washed with ice-cold 100 mM phosphate buffer (pH 7.0). The cells were then resuspended in a bioconversion medium containing xylose (30, 70 and 100 g/L) in phosphate buffer (100 mM). The bioconversion experiments were carried out at 30 °C with freshly prepared biomass.

3.5 Batch bioreactor fermentation for xylitol production

The batch experiments were performed in a 2.5 L bioreactor (Electrolab Bioreactors, UK) with a 1.0 L working volume. The inoculum was prepared using optimised media and the optimum values of media components were as follows (g/L): pure/crude glycerol, 20; xylose, 55; YNB, 5.0; NH_4Cl, 3.94; phosphate buffer, 132.5 mM. The starting pH was 6.8 and not controlled during the fermentation. The temperature and agitation speed were controlled at 30 ºC, 250 rpm respectively, while the aeration rate was maintained at 2 L/min for the initial 48 h and then changed to 1 L/min for the rest of the fermentation period. This experiment was done in duplicate.

3.6 Purification of xylitol from the fermentation broth

The purification protocol for xylitol was performed according to Rivas et al. (2006). For this, 100 ml of spent fermentation broth was subjected to centrifugation at 20,000 g to separate the cells and the clarified broth was treated with 5 % activated charcoal. The charcoal treated broth was precipitated by adding four-volume of 100 % ethanol and it was incubated at 4°C for 1h. After 1h, the precipitates were removed by centrifuging the mixture at 4000 g for 10 min. The supernatant was vacuum concentrated at 40 °C. The concentrated sample and ethanol were mixed at a ratio of 1:4 and incubated at -20 °C with slight agitation

(50 rpm) until crystals were observed. To improve the crystallization about 1 g/L of xylitol was mixed with the concentrated sample. To determine the concentration of xylitol, after crystallization the crystals were re-dissolved in 100ml of water and the samples were quantified using HPLC.

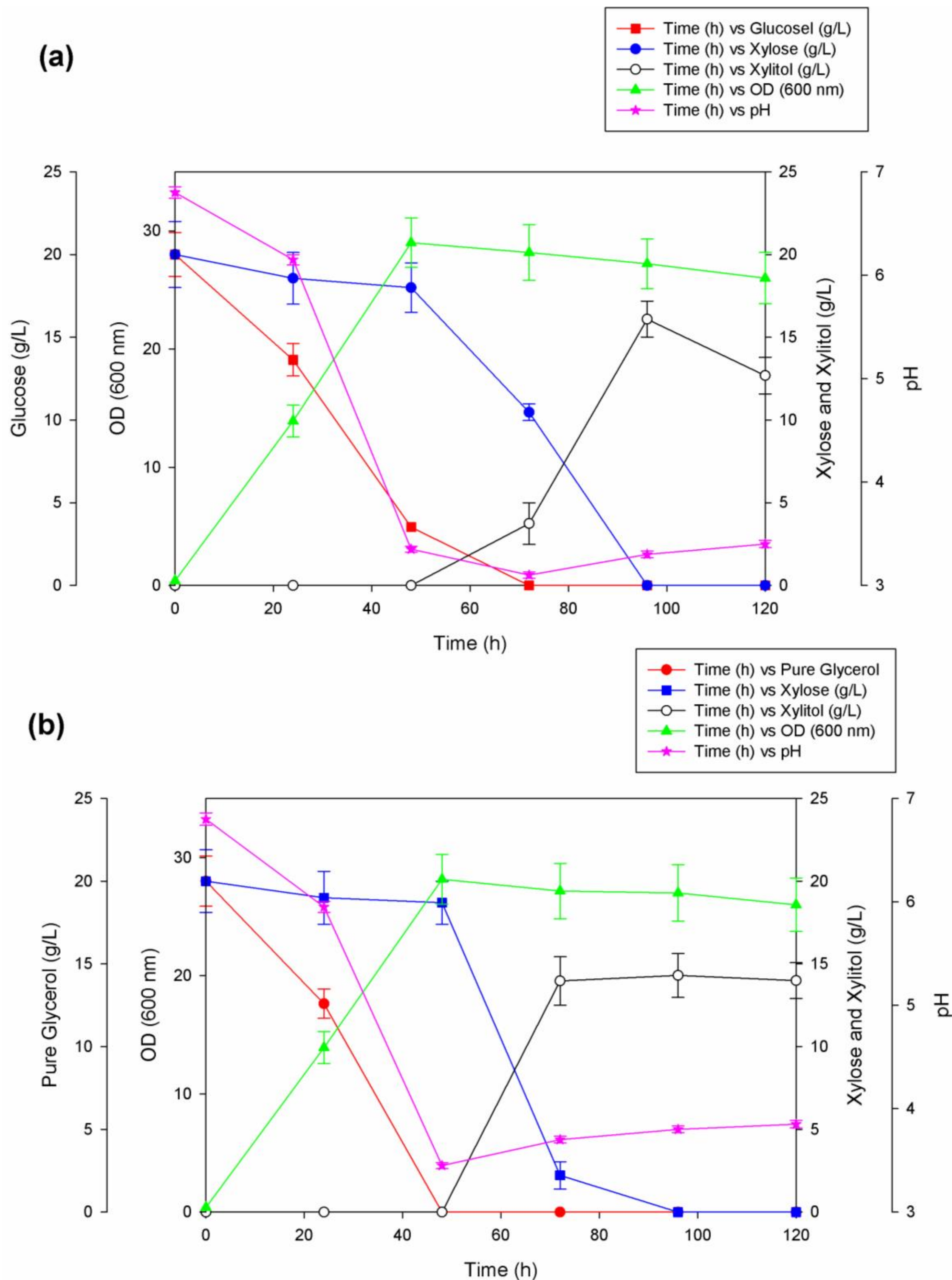
3.7 Analytical method

For all fermentation experiments, samples were taken at set intervals of either 12h or 24h (depending on the experiment) and analysed for optical density, pH, residual glycerol/glucose, xylose, and xylitol. Cell growth was quantified by measuring the optical density and was carried out using a double beam spectrophotometer (Jenway 6310, UK), at a wavelength of 600 nm, in a 1 mm-path-length cuvette. The concentrations of glycerol, glucose, xylose and xylitol were measured using high-performance liquid chromatography (Agilent Technologies 1200 series, USA). The supernatants were obtained using centrifugation of the culture samples at 10,000 g for 10 min, these were then filtered through a 0.22 µm PVDF membrane (Sartorius, Germany) and eluted using a Rezex ROA-Organic Acid H+ (Phenomenex, USA) column at 60 °C attached with refractive index detector (RID). The mobile phase and flow rates were 0.5 mM H_2SO_4 and 0.4 mL/min, respectively. All measurements were conducted in triplicates and the values were averaged. The standard deviation was not more than 10 %.

4 Results and Discussion

4.1 The co-substrate preference for xylitol bioconversion

In this study, glucose and glycerol were selected as the co-substrate for biomass accumulation before the commencement of the xylitol bioconversion. The reason for glucose is because the paper by Ledesma-Amaro et al., (2016) also utilised glucose and it achieved a yield of 0.92 g/g. The reason for glycerol is because glycerol and crude glycerol are more affordable materials that *Y. lipolytica* can utilise to accumulate biomass (Fangxia, Milford and Runcang, 2012; Liu, Ji and Huang, 2015; Ganigué *et al.*, 2019). This means that if *Y. lipolytica* can obtain a similar yield using these the costs of implementing a bioprocess for xylitol production are reduced significantly. The cell formation rate with glucose and glycerol were almost similar (Figure 4.1(a) and (b)). The drop in the pH was observed during the late log phase of the growth, with both the glucose and glycerol carbon sources indicating the secretion of organic acids such as citric acid, succinic acid and acetic acid etc (Abdel-Mawgoud *et al.*, 2018). The xylitol titre was 16.0 g/L with a yield of 0.8 g/g when glucose was the primary carbon source, while a titre of 14.5 g/L with a yield of 0.73 g/g was witnessed when glycerol was used as a primary carbon source. Most of the bioconversion of xylose to xylitol was observed between 96-120 h. In contrast, both glucose and glycerol would have ideally produced the same amount of biomass and converted the same amount of xylose to xylitol. In general, the biomass formation rate is accompanied by the consumption or formation of the NADH cofactor, which is necessary to maintain the redox and hydrogen balance. With glycerol as a carbon source, the main advantage is that it is a more reduced carbon source than glucose which results in a 1-mole net generation of the NADH molecule, while on the other hand the glucose results in the consumption of 1 mole of NADH, henceforth in further studies glycerol is used as the primary carbon source for biomass accumulation (Liu *et al.*, 2011).



<u>Figure 4.1:</u> Pre-optimisation shake flask fermentation profiles. (a) Shake flask fermentation profile with glucose and xylose as a carbon source (b) Shake flask fermentation profile with glycerol and xylose as a carbon source. All experiments were performed in triplicates and represented as Means±S.D.

4.2 The use of CCD for maximizing xylitol production

Multiple regression analysis is a powerful tool, which is frequently used to understand the effect of process parameters on the production of metabolites of interest. It also aids in reducing the amount of time and resources involved in experiments. Furthermore, the analysis performed can be easily examined, and experimental errors can be minimized (Prabhu *et al.*, 2019). Statistical methods measure the effects of changes in operating variables and their mutual interactions on the process through experimental design techniques. In the present study, experiments were performed according to the design matrix, to optimize the levels of process parameter components (xylose concentration, YNB, NH_4Cl and phosphate buffer) using the CCD. The design matrix and the corresponding results of observed and predicted responses (xylitol titre) are shown in Table 4.1.

Table 4.1: Experimental Design from central composite design as well as the predicted values from CCD and ANN.

Experimental, Central Composite Design, and ANN Results							
Experiment	**Xylose (g/l)**	**NH_4CL (%)**	**YNB (%)**	**Phosphate buffer (mM)**	**Xylitol titre (g/L)**	**CCD predicted xylitol titre (g/L)**	**ANN predicted xylitol titre (g/L)**
1	20	0.2	0.2	35	17.47	18.04	18.79
2	50	0.2	0.2	35	22.20	22.80	22.48
3	20	0.5	0.2	35	13.11	11.81	14.99
4	50	0.5	0.2	35	20.05	19.51	20.45
5	20	0.2	0.5	35	10.20	10.52	4.83
6	50	0.2	0.5	35	21.77	22.48	19.70
7	20	0.5	0.5	35	18.70	20.81	18.92
8	50	0.5	0.5	35	37.22	35.73	33.50
9	20	0.2	0.2	100	18.99	26.02	23.06
10	50	0.2	0.2	100	29.53	27.94	30.42
11	20	0.5	0.2	100	17.85	17.65	19.66
12	50	0.5	0.2	100	23.30	22.52	23.14
13	20	0.2	0.5	100	4.74	5.79	5.90
14	50	0.2	0.5	100	14.07	14.91	13.92
15	20	0.5	0.5	100	15.00	13.95	14.27
16	50	0.5	0.5	100	26.08	26.03	31.47
17	35	0.35	0.35	67.5	20.71	24.25	24.96
18	35	0.35	0.35	67.5	27.18	24.25	24.96
19	35	0.35	0.35	67.5	24.53	24.25	24.96

20	35	0.35	0.35	67.5	24.84	24.25	24.96
21	5	0.35	0.35	67.5	20.57	19.33	21.37
22	65	0.35	0.35	67.5	34.98	36.16	37.22
23	35	0.05	0.35	67.5	12.41	10.67	11.56
24	35	0.65	0.35	67.5	13.88	15.55	15.66
25	35	0.35	0.05	67.5	29.69	30.82	29.96
26	35	0.35	0.65	67.5	28.00	26.80	23.25
27	35	0.35	0.35	2.5	23.62	23.15	23.75
28	35	0.35	0.35	132.5	21.03	21.43	21.08
29	35	0.35	0.35	67.5	24.87	27.46	24.96
30	35	0.35	0.35	67.5	29.78	27.46	24.96

The observed values of xylitol titre were the mean values of duplicates

R-Sq = 94 %

R-Sq(pred) = 72 %

R-Sq(adj) = 88 %

The results were analysed using an ANOVA, shown in Table 4.2 for xylitol concentration. Table 4.2 also shows the error term, which indicates that the amount of variation in the response data is very low. According to the ANOVA, the regression model for the xylitol production showed high significance with the F value of 22.41. The high Fisher's F-value of the model indicates that most of the variation in the response can be explained by the model equation (Prabhu *et al.*, 2018). The lack of fit F-value of 0.92 implies the lack of fit for xylitol is not significant relative to the pure error.

Table 4.2: Analysis of Variance (ANOVA) for Xylitol Production.

Source	**Degrees of freedom (DF)**	**Sequential sum of square (Seq SS)**	**Adjusted sum of square (Adj SS)**	**Adjusted mean square (Adj MS)**	**F Value**	**P Value**
Blocks	1	68.61	68.61	68.607	15.35	0.002
Regression	14	1402.25	1402.25	100.161	22.41	0
Linear	4	489.28	489.28	122.321	27.37	0
Xylose	1	424.91	424.91	424.91	95.08	0
NH4Cl	1	35.71	35.71	35.712	7.99	0.013
YNB	1	24.22	24.22	24.221	5.42	0.035
Phosphate	1	4.44	4.44	4.44	0.99	0.336
Square	4	404.82	404.82	101.205	22.65	0

Xylose*Xylose	1	9.54	0.14	0.139	0.03	0.862
NH4Cl*NH4Cl	1	341.93	352.75	352.754	78.93	0
YNB*YNB	1	7.65	3.14	3.138	0.7	0.416
Phosphate*Phosphate	1	45.7	45.7	45.698	10.23	0.006
Interaction	6	508.15	508.15	84.692	18.95	0
Xylose*NH4Cl	1	8.73	8.73	8.725	1.95	0.184
Xylose*YNB	1	51.99	51.99	51.988	11.63	0.004
Xylose*Phosphate	1	8.09	8.09	8.085	1.81	0.2
NH4Cl*YNB	1	273.24	273.24	273.236	61.14	0
NH4Cl*Phosphate	1	4.56	4.56	4.562	1.02	0.329
YNB*Phosphate	1	161.56	161.56	161.555	36.15	0
Residual Error	14	62.57	62.57	4.469		
Lack-of-Fit	10	29.03	29.03	2.903	0.35	0.921
Pure Error	4	33.54	33.54	8.384		
Total	29	1533.43				

The goodness of fit model was further checked by the correlation coefficient (R^2) between experimental and model-predicted values of response variables. The regression coefficient (R^2) of the model was found to be 94 %, which indicates that the models are statistically significant and only 6 % of the total variation was not explained by the model (Table 4.1). The T distribution and the corresponding P values, along with the parameter estimate are shown in Table 4.3. Most of the interaction terms showed significance($P<0.05$), while the square terms including xylose and YNB showed insignificance. The second-order polynomial equation for xylitol production by CCD is given in Equation 4.1.

$$
\begin{aligned}
\mathrm{Y}_{\mathrm{xylitol}} = 25.90 + 4.45\mathrm{X}_1 + 1.46\mathrm{X}_2 - 0.75\mathrm{X}_3 - 0.68\mathrm{X}_4 + 0.008\mathrm{X}_1^2 - 3.64\mathrm{X}_2^2 \\
+ 0.27\mathrm{X}_3^2 - 1.35\mathrm{X}_4^2 + 0.36\mathrm{X}_1\mathrm{X}_2 + 1.42\mathrm{X}_1\mathrm{X}_3 - 0.33\mathrm{X}_1\mathrm{X}_4 + 3.7\mathrm{X}_2\mathrm{X}_3 \\
- 0.15\mathrm{X}_2\mathrm{X}_4 - 2.8\mathrm{X}_3\mathrm{X}_4
\end{aligned}
$$

(4.1)

X_1: Xylose concentration, X_2: NH_4Cl, X_3: YNB X_4: Phosphate buffer. The 25.9 figure is the Y-intercept point and the constant term in the linear model.

The response surface plot for the interactions (that being the statistical effect of two or more variables on the output between the media components) (Xylose, NH_4CL, YNB and Phosphate buffer) is depicted in Figure 4.2. The 3D surface plot gives an overview of the interaction between the two components on xylitol production by keeping the other parameters at central values. The interaction between xylose and NH4Cl (Figure 4.2a) showed a positive effect on xylitol production and a progressive increment in the xylitol titre was observed with the increasing concentration of both components. Further, higher xylose concentrations caused a steep reduction in xylitol titre. Similarly, the interactions between xylose & YNB (Figure 4.2b) and NH4Cl & YNB (Figure 4.2d) showed positive effects ($P < 0.05$) on xylitol production. This indicates that higher concentrations of YNB and NH4Cl will lead to the enhanced production of xylitol. On the other hand, the interaction between xylose & phosphate buffer (Figure 4.2c), phosphate buffer & NH4Cl (Figure 4.2e) and YNB & phosphate buffer (Figure 4.2f) showed statistically insignificant values, which indicate one of the components has to be kept at a minimum to enhance the xylitol production.

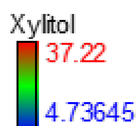
Table 4.3: Estimated Regression Coefficients for xylitol Production.

Term	Coef	SE Coef	T	P
Constant	25.9027	1.0443	24.803	0
Block	-1.754	0.4893	-3.585	0.003
Xylose	4.4577	0.5158	8.643	0
NH_4Cl	1.4698	0.5158	2.85	0.013
YNB	-0.7546	0.5158	-1.463	0.166
Phosphate	-0.6801	0.5158	-1.319	0.208
Xylose*Xylose	0.0088	0.4824	0.018	0.986
NH_4Cl*NH_4Cl	-3.6487	0.4824	-7.563	0
YNB*YNB	0.2757	0.4824	0.572	0.577
Phosphate*Phosphate	-1.3533	0.4824	-2.805	0.014
Xylose*NH_4Cl	0.3635	0.6317	0.575	0.574
Xylose*YNB	1.4276	0.6317	2.26	0.04
Xylose*Phosphate	-0.3359	0.6317	-0.532	0.603
NH_4Cl*YNB	3.7575	0.6317	5.948	0
NH_4Cl*Phosphate	-0.159	0.6317	-0.252	0.805
YNB*Phosphate	-2.8026	0.6317	-4.437	0.001

NH$_4$CL and Xylose

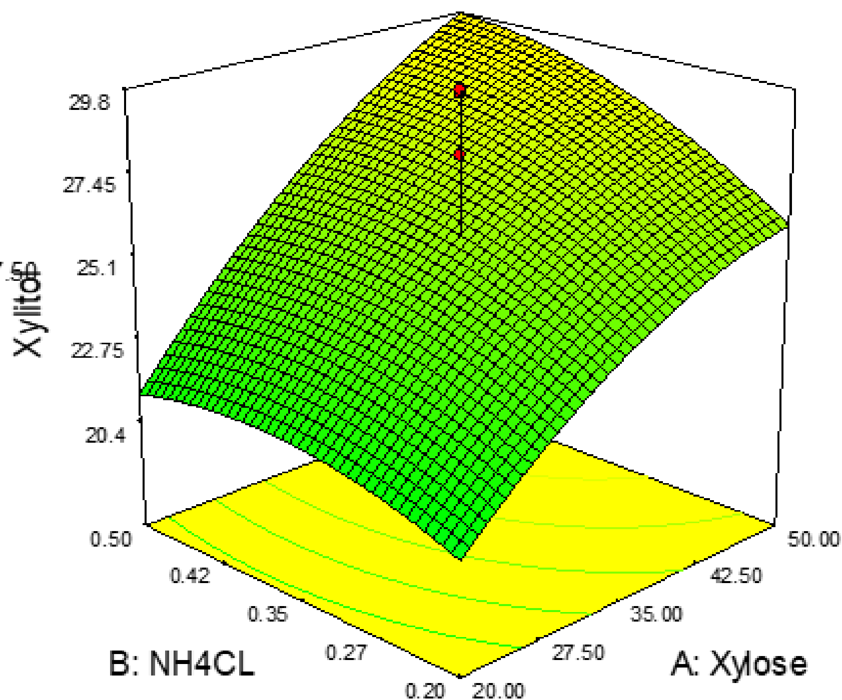
Design-Expert® Software

(a)



X1 = A: Xylose
X2 = B: NH4CL

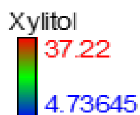
Actual Factors
C: YNB = 0.35
D: Phosphate bufffer = 67.50



YNB and Xylose

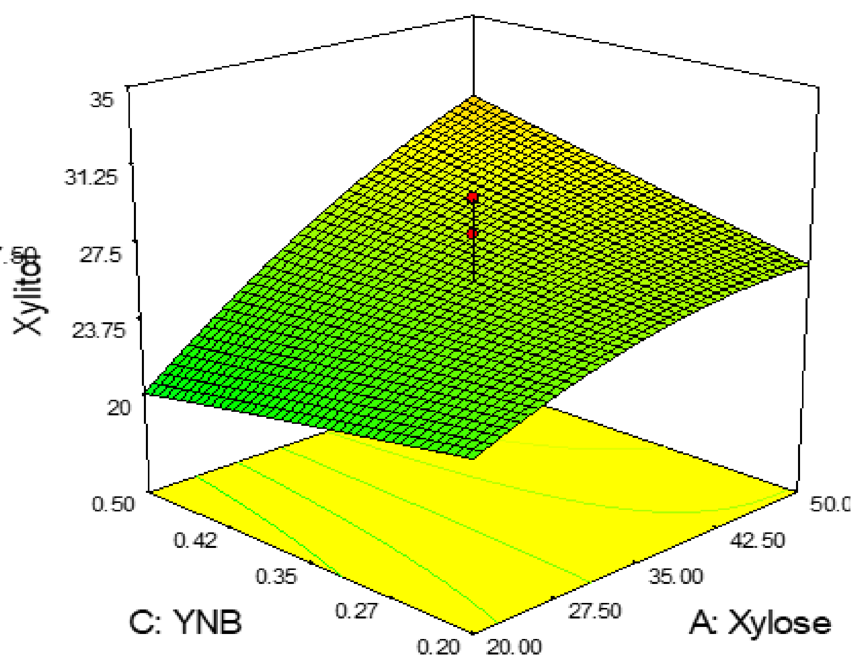
Design-Expert® Software

(b)

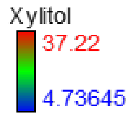


X1 = A: Xylose
X2 = C: YNB

Actual Factors
B: NH4CL = 0.35
D: Phosphate bufffer = 67.50



Phosphate buffer and Xylose

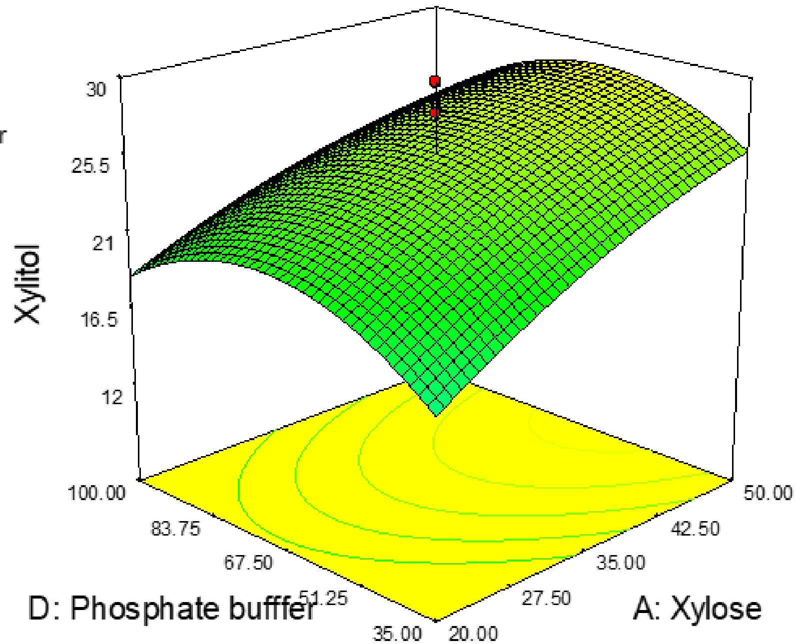


Design-Expert® Software

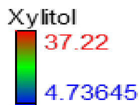
Xylitol
37.22
4.73645

X1 = A: Xylose
X2 = D: Phosphate buffer

Actual Factors
B: NH4CL = 0.35
C: YNB = 0.35



YNB and NH_4CL

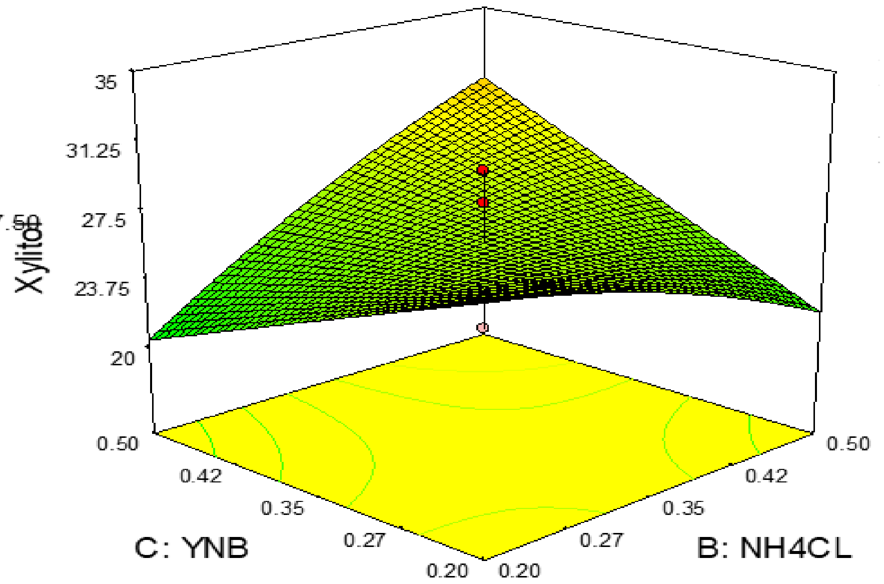


Design-Expert® Software

Xylitol
37.22
4.73645

X1 = B: NH4CL
X2 = C: YNB

Actual Factors
A: Xylose = 35.00
D: Phosphate buffer = 67.50



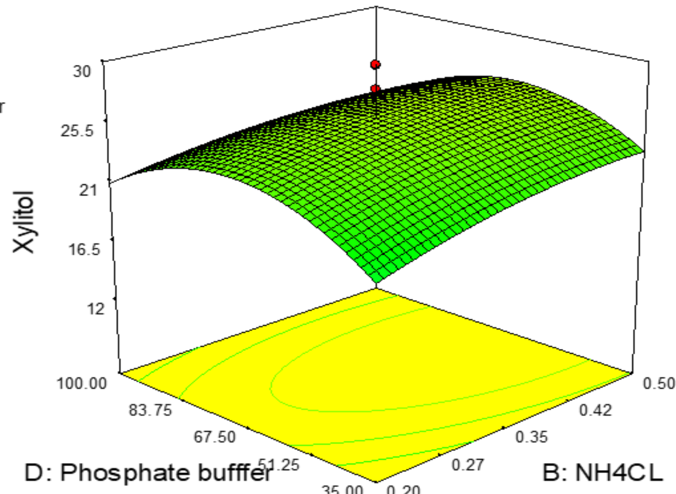
Phosphate buffer and NH_4CL

(e) Design-Expert® Software

Xylitol
37.22
4.73645

X1 = B: NH4CL
X2 = D: Phosphate bufffer

Actual Factors
A: Xylose = 35.00
C: YNB = 0.35



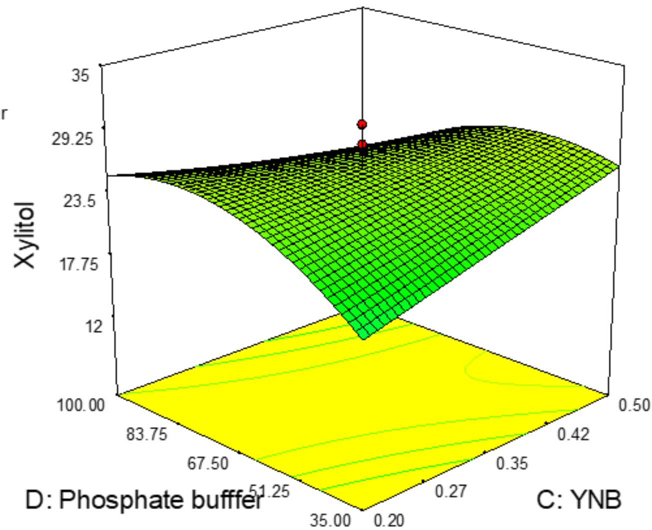
Phosphate buffer and YNB

(f) Design-Expert® Software

Xylitol
37.22
4.73645

X1 = C: YNB
X2 = D: Phosphate bufffer

Actual Factors
A: Xylose = 35.00
B: NH4CL = 0.35



<u>Figure 4.2:</u> Three-dimensional response surface plots for xylitol production showing the interactive effects of (a) Xylose and NH_4Cl (b) Xylose and YNB (c) Xylose and Phosphate buffer, (d) YNB and NH_4Cl, (e) Phosphate buffer and NH_4Cl, (f) YNB and Phosphate buffer with the remaining factors kept constant at their respective 0 levels from Table 3.1 Central composite experimental design. The yellow is a contour plot with the areas of highest xylitol concentration being in the middle and the green surface plot with those areas being the most raised sections.

4.3 Optimization of process parameters using ANN-GA

The experimental design generated by CCD was used as an input feed for the ANN algorithm. The data set comprising of 30 data points was randomly divided into three subsets: training (20 data points), validation (5 data points) and test (5 data points). Some may argue that these datasets are too small to make the model robust enough, however, there are numerous examples of this model being utilised to great effect to optimise systems with even fewer variables and hence even fewer data points in the training, validation and test datasets (Prabhu and Jayadeep, 2017; Prabhu, Mandal and Dasu, 2017; Sushma, Anand and Veeranki, 2017). It should also be argued that this is one of the advantages of this experimental design the fact that optimisation can be done with fewer experiments means that important resources like time and lab space can be saved and utilised for other experiments.

A Levenberg-Marquardt (LM) backpropagation algorithm was used for network training, which is a mere approximation of newtons method. The LM algorithm uses a second-order derivative of means squared error to calculate a better convergence between actual output data and predicted output data (Sivapathasekaran *et al.*, 2010). The training was carried out for 1000 epochs. The mean square error (MSE) as well as the R^2 values for the training, validation, and test points with regards to xylitol production are shown in Table 4.4. The data points apart from the training were used to examine the validation. Usually when the data overfits while training the data substantial error will be accumulated on the validation. When the error on the validation reaches the threshold point the weights and biases are adjusted to minimize the error. Network topology has important influences on the predicted results, in the present study, the number of input and output data resembles the input-output neuron of ANN, respectively. The number of neurons in the hidden layer was determined by a trial and error method to minimize MSE. The MSE, which is the difference between the actual and the predicted values, given as a statistical value as determined by equation

3.6, for xylitol production was found to be 7.425. The optimum value was achieved with 4 inputs, 8 hidden layers and 1 output layer. The predicted value of ANN for xylitol is shown in Table 4.1. Also, the regression correlation coefficient between the actual experimental production values and the ANN-simulated outputs was, found to be 0.938 (Figure 4.3).

Table 4.4: Statistical measures and performance of the ANN model for the training, testing, validation and all, data sets.

	Sets	MSE	R^2
Training	70 %	1.309	0.9457
Validation	15 %	12.605	0.9842
Test	15 %	26.709	0.9768
Overall		7.425	0.93887

The ANN trained values were subjected to training by GA to further optimize the input space. As previously sated GA utilises a methodology akin to natural selection and uses a series of parameters to represent a series of datapoints competing with each other to be the most optimal and the fittest. These are: population size which is the size of the population, cross over possibility represents the probability of members of that population mating and having offspring, mutation probability represents the probability of those offspring mutating and the number of generations represents the number of generations that this will go on for.

The values of GA specific parameters used in the optimization technique were as follows: population size = 20, cross over probability = 0.8, mutation probability=0.01, No. of generation = 100. The GA was repeated several times with a different initial parameter condition until there is a convergence towards an endpoint or a global optimum that can be seen in Figure 4.4. The maximum of 47.7 g/L of xylitol production was observed with 160 iterations. The best fitness plot for the GA of xylitol production (Figure 4.4) maps the gradual convergence

of the best fitness values of successive generations towards the final optimum value. The optimum values were found to be, xylose: 55 g/l, NH_4Cl: 0.394 %, YNB: 0.5 % and phosphate buffer: 132.5 mM.

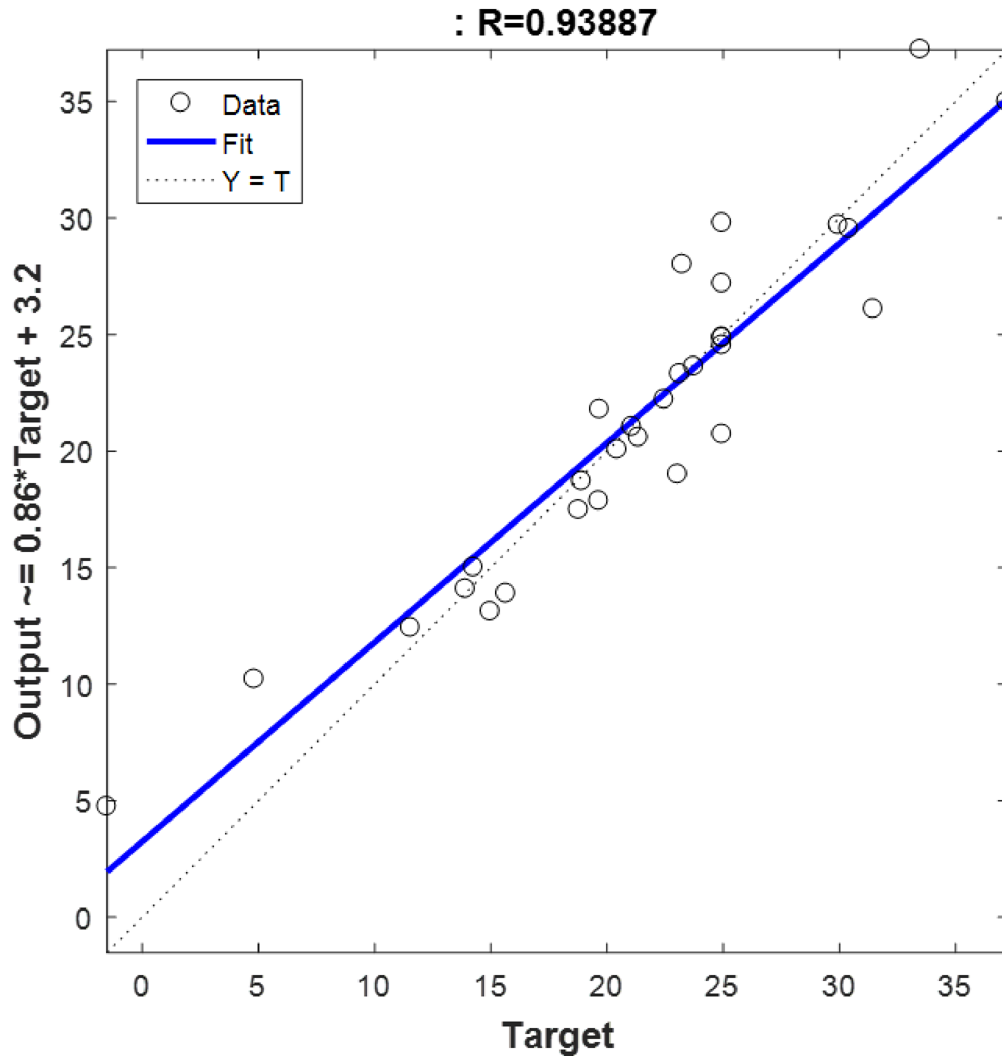


Figure 4.3: ANN-GA optimisation regression plot. The dashed line in each plot represents the perfect result – outputs = targets. The solid line represents the best fit linear regression line between outputs and targets. The R-value is an indication of the relationship between the outputs and targets. If R = 1, this indicates that there is an exact linear relationship between outputs and targets. If R is close to zero, then there is no linear relationship between outputs and targets. Here the Y-axis is the output result generated by the simulation of ANN, while the X-Axis represents the same but for the CCD data and both of these are related to xylitol production.

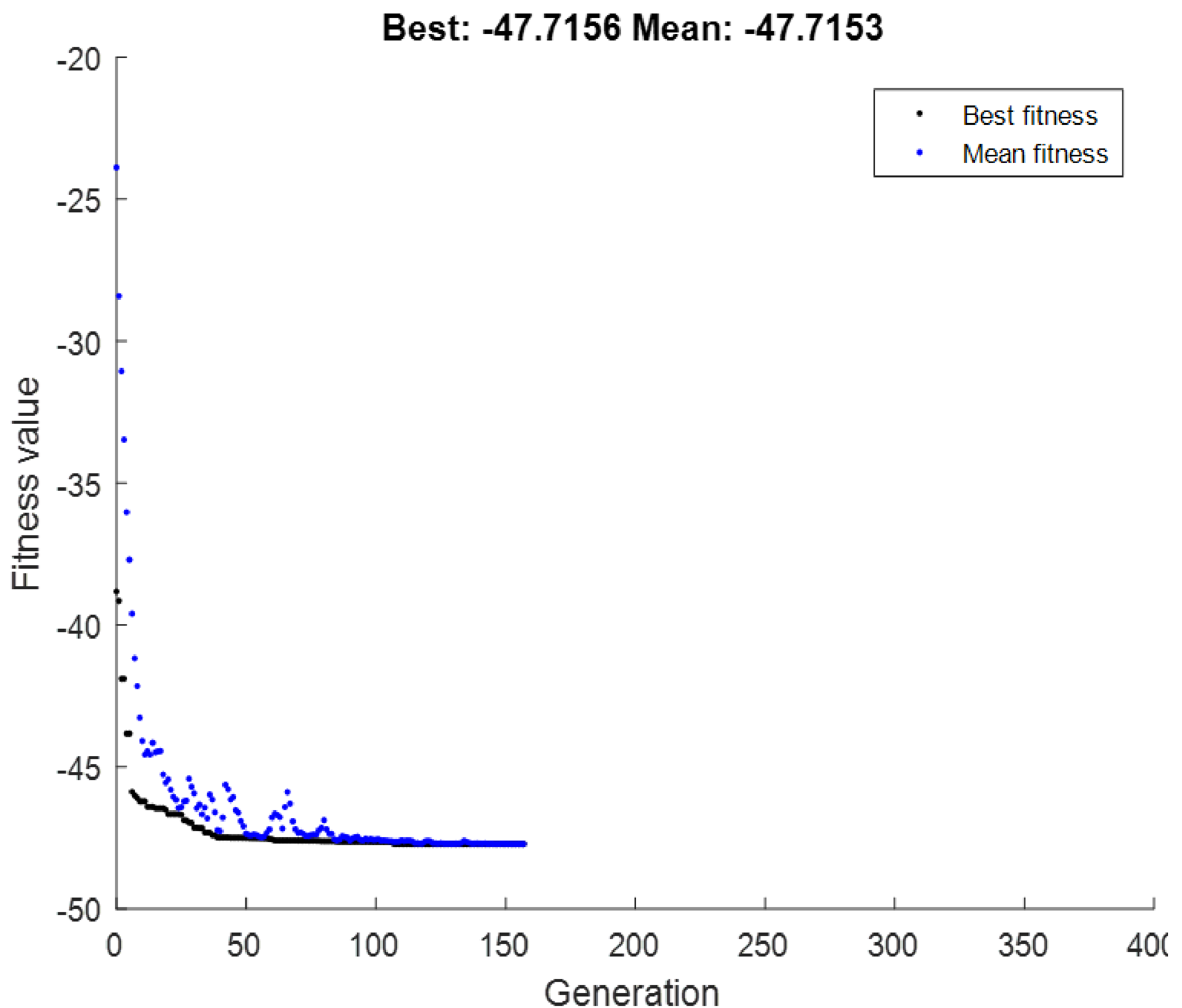


Figure 4.4: **ANN-GA generational fitness graph. Best and average fitness values with successive generations show a gradual convergence to the optimum value for xylitol production. The X-axis shows the number of generations and the Y-Axis is the fitness value which is related to the amount of Xylitol produced. It is given in negative values because When using "genetic algorithms" (GA) it is defined a scalar fitness function, say f(x), which is suitable for the optimization problem to which the GA is being applied. The GA evolves by iteration a population of chromosomes (the "x" population) trying to find the one which gets the "better" value for f(x). If the fitness function f(x) returns negative values when the "optimization is better" then you are getting negative values as your solution improves to the optimal. In general, the GA uses. Rank Selection also works with negative fitness values and is mostly used when the individuals in the population have very close fitness values The best and mean fitness are because this simulation was run multiple times and they are the mean of the results and the best results within the repeats.**

The optimum media values all lie in the upper ranges of their respective ranges from Table 3.1 but only 1 is at the upper limit of its range. That being the phosphate buffer, the reason for this is most likely because as can be seen in Figure 4.1, the pH experiences large drops towards the 20h mark and this pH drop can negatively impact biocatalytic activity. This can result in a reduction in xylitol production, this is most likely the reason that the buffer concentration is high so that the pH remains stable throughout the fermentation. Xylose was also high being between the +1 and alpha levels of concentration this was most likely because xylose is the resource required to create xylitol so it makes sense that this would need to be high but not beyond the saturation point of 60g/L (Prabhu *et al.*, 2020). The concentrations for both YNB and NH_4CL are also above the 0 level which also makes sense because a nitrogen source like NH_4CL and a source of amino acids like YNB are both beneficial for biocatalytic activity.

4.4 Validation of the ANN-GA optimization value

The validation experiments were performed based on the global values obtained after ANN-GA training. All these experiments were carried out in duplicates. Three different sets of experiments were conducted to illustrate the ability of the *Y. lipolytica* strain to utilize crude renewable feedstocks and convert them into a value-added product such as xylitol. Figure 4.6(a) depicts the fermentation time profile of *Y. lipolytica*, with the optimized concentration of the medium components whilst using pure glycerol and xylose as a carbon source. The major proportion of xylose is converted into xylitol, with a yield of 0.98 g/g and a titre of 54 g/L. The bioconversion took place during the 144 to 168 h time interval. Similarly, the validation experiment was performed with crude glycerol and pure xylose as carbon sources while maintaining all other components at an optimal level. However, the xylitol titre was 45 g/L with the yield of 0.82 g/g which is close to the optimized value achieved from the ANN-GA experiments. The difference between the two can be explained using the contents of crude glycerol. The contents of crude glycerol vary but they usually contain glycerol, soaps (lipids)

methanol, catalysts, salts, nonglycerol organic matter, and water impurities (Fangxia, Milford and Runcang, 2012). Salts, methanol and soaps are known to negatively impact bioproduction (Ganigué *et al.*, 2019). However, the difference between these two experiments is only minor relative to the difference between when lignocellulose was used and when it was not. This can be explained by the fact that *Y. lipolytica* is known to have a tolerance to saline conditions and is a potent lipid consumer (Madzak, 2018, Y. Xu *et al.*, 2019). These give *Y. lipolytica* a unique resistance to substances in crude glycerol that would normally inhibit its capabilities as a xylitol producer. As mentioned before, one set of validation experiments was performed with crude glycerol and xylose rich lignocellulosic hydrolysate from sugar cane bagasse. The yield in the lignocellulosic experiment was 58 % with the titre of 5.8 g/L. The reason for the comparatively low yield of the lignocellulose experiment can also be explained by the contents of lignocellulosic material. Lignocellulosic material often contains compounds such as phenols furan derivatives and aliphatic acid in considerable amounts, which tends to inhibit microbial growth and in turn, this reduces the productivity (Moreno *et al.*, 2019).

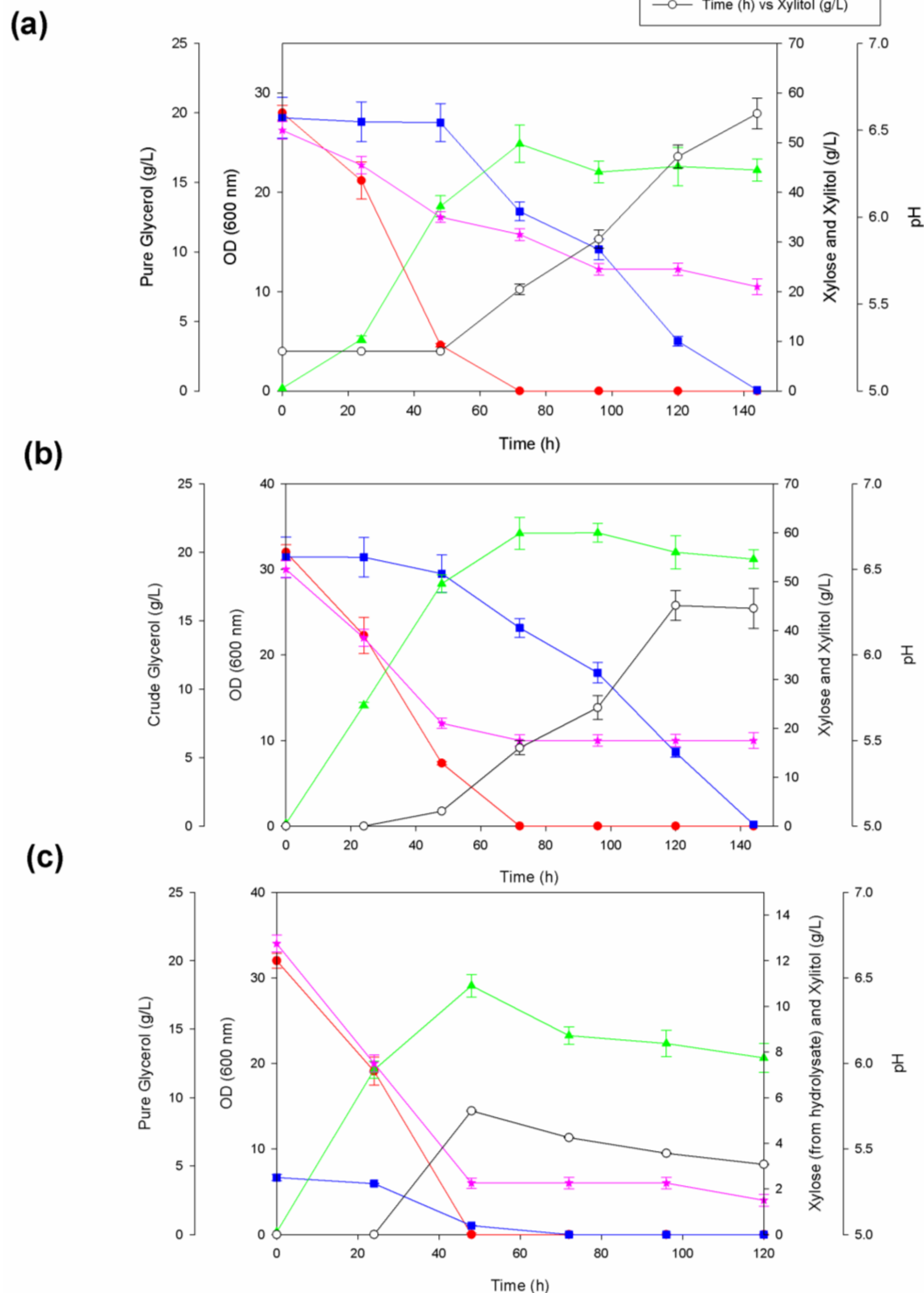
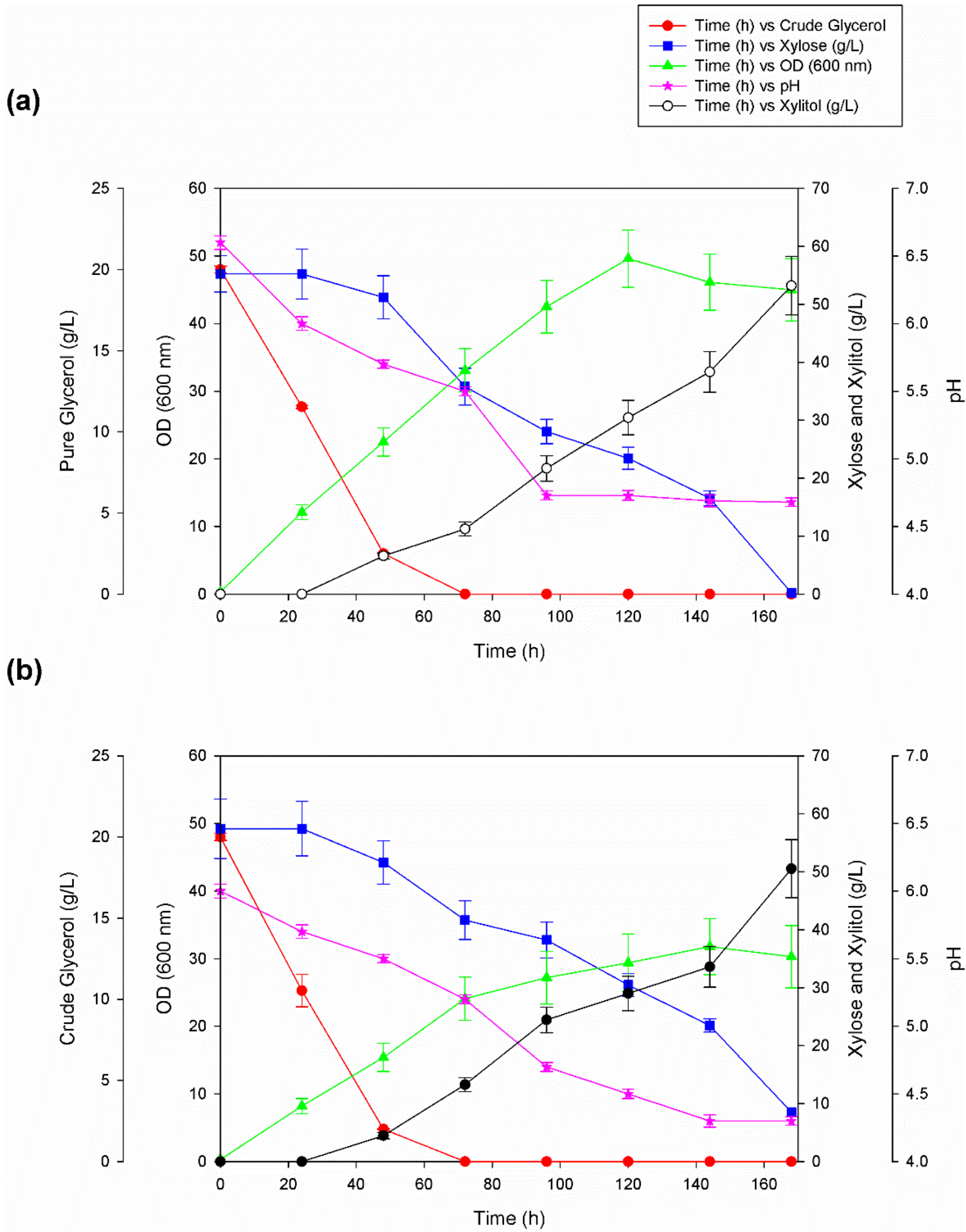


Figure 4.5: **Fermentation profiles of *Y. lipolytica* with an ANN-GA optimized media composition in shake flask cultivation conditions (a) with pure glycerol and xylose as a carbon source, (b) with crude glycerol and pure xylose as a carbon source, (c) with crude glycerol and xylose rich lignocellulosic hydrolysate as a carbon source.**

4.5 Batch fermentation in a bioreactor for the production of xylitol

To scale up the fermentation and validate the optimized medium composition, a batch fermentation was also carried out in a 2.5 L scale bench bioreactor with a 1L working volume. The process conditions where mimicked exactly like the shake flask studies. Two separated batch fermentations where run to understand the ability of *Y. lipolytica* to metabolize pure and crude glycerol and its ability to bio transform xylose to xylitol. It was quite evident from Figure 4.7(a) when pure glycerol and xylose is used as a carbon source. *Y. lipolytica* tends to prefer glycerol as a primary carbon source and it exhibits carbon catabolite repression over the consumption of xylose. When the glycerol was completely consumed after approximately 48h of fermentation, a gradual uptake of xylose was witnessed. A maximum cell OD of 49.6 was observed at 120 h, which was higher than the shake flask cultivation. The complete consumption of xylose was observed at 166h and a maximum of 53 g/L of xylitol was produced with a yield of 0.97 g/g. The fermentation profile of *Y. lipolytica* with crude glycerol and pure xylose is shown in Figure 4.7. The maximum cell OD was less, being 31.8, when compared with the shake flask studies that used pure glycerol, that being 48.6, this could be attributed to the presence of some of the inhibitory components, such as the methanol present in the crude glycerol. The yield of the xylitol was 0.92 g/g with the titre of 51 g/l, which is less than the pure glycerol fermentation. Further, the pH during fermentation and when using a pure carbon source fluctuated between 6.5-5.55, whereas during the fermentation with crude renewable glycerol the pH dropped to 4.5. The pH plays a crucial role in the transportation of xylose across the membrane (Chandel *et al.*, 2012). Low aeration was maintained in this fermentation because excess aeration causes re-oxidation of NADH, a co-factor necessary for xylitol production from xylose. Furthermore, enzymes down the pathway can metabolize the produced xylitol for cell growth (Pappu and Gummadi, 2018). The *Y. lipolytica* strain used in the current study cannot grow on xylose, therefore, we believe that the possibility of further metabolism of produced xylitol is low. Table 4.5 displays that the yield of

xylitol achieved in this study is highest with 0.97 g/g compared to the yield in the existing literature.



<u>Figure 4.6:</u> Fermentation profiles of xylitol production by *Y. lipolytica* under-optimized (bioreactor) fermentation conditions based on ANN-GA using (a) pure glycerol and xylose as a carbon source (b) Crude glycerol and pure xylose as a carbon source. The experiments were performed in duplicate, and these values the averages.

Table 4.5: Comparison of batch fermentation xylitol titre and yield from various xylose metabolizing yeast strains.

Strain	Initial Xylose (g L^{-1})	Xylitol (g L^{-1})	Yield (g g^{-1})	Reference
Candida guilliermondii FTI-20037	104	77.2	0.742	(Barbosa *et al.*, 1988)
Candida tropicalis	150	131	0.873	(Oh and Kim, 1998)
Candida boidinii NRRL Y-17213	150	53.1	0.354	(Vandeska *et al.*, 1995)
Candida sp.559-9	200	173	0.993	(Ikeuchi *et al.*, 1999)
Pichia sp.	40	25	0.58	(Rao *et al.*, 2007)
Debaryomyces hansenii UFV-170	100	76.6	0.73	(Sampaio *et al.*, 2008)
Hansenula polymorpha	125	58	0.62	(Suryadi *et al.*, 2000)
Y. lipolytica	15	13.8	0.92	(Ledesma-Amaro *et al.*, 2016)
Y. lipolytica	55	53	0.97	This study

4.6 Purification of xylitol from the fermentation broth

The crystallization method is the more commonly adopted method in the purification of polyols as it allows for the recovery of the xylitol in a purified form in a single step. In terms of energy consumption, crystallization is less energy-intensive when compared to the distillation process. Crystallization can be performed using various methods such as solvent evaporation, cooling, salting out etc (Martínez *et al.*, 2015). In the initial step, the coloured substance was clarified using a charcoal treatment, with 5 % activated charcoal, the fermentation broth almost became translucent and impurities such as residual xylose were removed, further, there was a slight decrease in the xylitol concentration and the recovery was about 76 and 77 % with crude glycerol and pure xylose respectively, both of which are shown in Table 4.6. The subsequent alcohol precipitation step reduced recovery of xylitol and final crystallization at -20 °C for 72h, this resulted

in a 35 and a 39 % recovery rate for crude glycerol and pure xylose respectively, these are also shown in Table 4.6. (Misra *et al.*, 2011) reported that a 44 % yield of xylitol was achieved using 15 g/L activated charcoal and a crystallizing of the solution at -20 °C. (Wei *et al.*, 2010) added an additional step for purification using two ionic exchange columns, using the same procedure they were able to enhance the recovery to 60 %.

Table 4.6: Downstream process data and recovery rate data.

	Crude Glycerol + Xylose		Pure Glycerol + Xylose	
Purification steps	Xylitol (g L^{-1})	Recovery (%)	Xylitol (g L^{-1})	Recovery (%)
Crude fermentation broth	48.7 ± 2.9	100	51.5±4.3	100
Activated charcoal treatment	37.1±1.7	76.2	39.7±2.3	77
Alcohol precipitation	24.2±1.9	49.7	27.1±1.5	53
Crystallization	17.2±0.9	35.3	20.3±1.0	40

4.7 Resting cell experiment for xylitol production

Metabolically active resting (non-growing) yeast cells display an excellent potential in co-factor dependent redox biotransformation. Resting cell experiments are when cells that have already accumulated biomass, are suspended in a media without the addition of carbon sources or energy sources and are used as a biocatalyst to produce value-added compounds. In contrast, active cells require a carbon source and a nitrogen source for biomass formation. Resting cells show advantages overactive cells such as being simple to operate, they require less nutrient medium for the bioconversion and they are more convenient for downstream processing (Carballeira *et al.*, 2009; Carvalho, 2011). In *Y. lipolytica* the conversion of xylose to xylitol is a one-step process catalysed by the enzyme xylose reductase (XR) which oxidizes NADPH into $NADP^+$, *Y. lipolytica* also displays weak XDH activity. These result in the considerable accumulation of xylitol in the fermentation broth. Hence to exploit this property

resting cell experiments were carried out. Initially, the cells were grown in pure and crude glycerol to obtain enough biomass and later the biomass was suspended in a media containing only xylose. From Figure 4.8 it is evident that the conversion of xylose to xylitol was not satisfactory. The probable cause for the ceasing of biocatalytic activity could be because *Yarrowia lipolytica* requires a primary carbon source even though it has already built biomass, maybe the cells cannot produce enough cofactors to perform the conversion without a primary carbon source and therefore cannot grow on xylose alone despite previous biomass accumulation (Ledesma-Amaro *et al.*, 2016; Li and Alper, 2019). The presence of xylose alone in the media appears to not be sufficient enough to produce the cofactors required for this bioconversion.

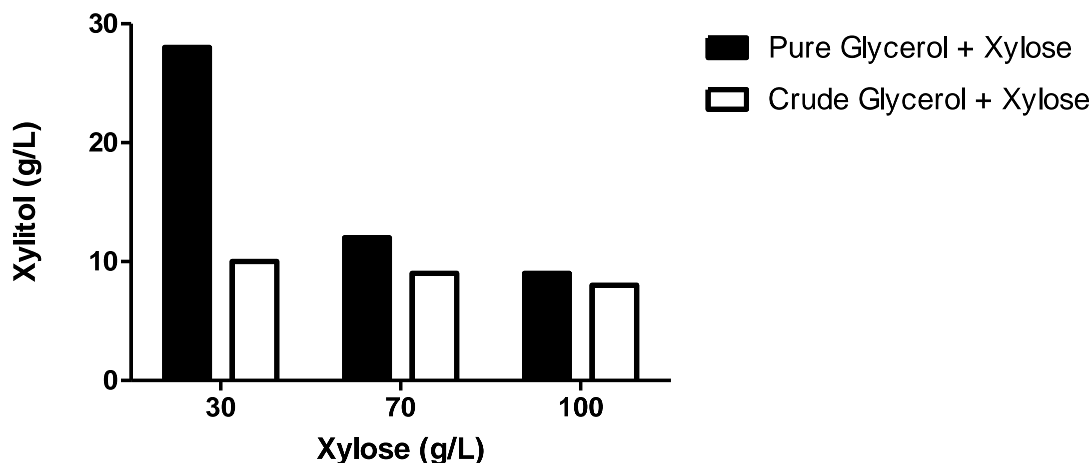


Figure 4.7: Titres of xylitol from fermentations using resting cells grown in crude glycerol and pure glycerol as a primary carbon source.

5 Conclusion

There is a multitude of ways of improving xylitol production. Transport systems and proteins are an important factor of all metabolisms involving sugars. Organisms that are pentose carriers use transport proteins that are designed and specialised for 6 carbon sugars, such as Gal2 as well as Hxt 4,5 and 7 (Saloheimo *et al.*, 2007). These can transport a five-carbon sugar like xylose but have a lower affinity for it (Albuquerque *et al.*, 2014; Dasgupta *et al.*, 2017). Pentose accumulators, on the other hand, have more specialised xylose transporters such as GFX1, GXS1 and SUT1 (Runquist, Hahn-Hägerdal and Rådström, 2010). These transporters can be found in *Candida* and *Pichia* yeasts and have been successfully expressed in other pentose assimilating microorganisms (Runquist, Hahn-Hägerdal and Rådström, 2010). The expression of these more specialised transporters in *Kluyveromyces marxianus* increased the uptake of xylose by 1.9 folds (Zhang *et al.*, 2015). The overexpression of these transporter proteins has also been shown to be effective in non-xylitol producing yeasts like *Saccharomyces cerevisiae* at dramatically increasing xylitol yield (Runquist, Hahn-Hägerdal and Rådström, 2010). Genes from *Candida* and *Pichia* yeasts have also been used to change the affinities of hexose transport proteins like Hxt 7 in *Saccharomyces* so that they are more specialised for pentose (Apel *et al.*, 2016). These strategies could be applied to *Y. lipolytica* as a way to improve its xylose uptake and potentially its xylitol production capabilities.

Another way to potentially improve xylitol production in *Y. lipolytica* would be through optimising the metabolic pentose enzyme pathway. One way of doing this would be to target the XR enzyme by increasing its expression levels in *Y. lipolytica*, or by expressing potentially more optimised versions of the XR enzyme (such as those from *Candida* and *Pichia* species) in *Y. lipolytica* (Dasgupta *et al.*, 2017). There are *Saccharomyces* strains that have been made to express XR enzymes from *Pichia* species, these have been shown to demonstrate yields between 90-100 % (Oh *et al.*, 2013; L. Xu *et al.*, 2019). This technique has also

been used in *E. coli* to allow for the production of xylitol in yields of up to 99 %. This was done by cloning XR and glucose dehydrogenase into a plasmid and then expressing them inside the *E. coli* strain BL21(DE3) (Chang *et al.*, 2018; Y. Xu *et al.*, 2019). Expressing the XR enzymes of *Candida* and *Pichia* species could improve the xylitol production capabilities of *Y. lipolytica* and if given more time and resources this could be a promising strategy to improve xylitol production in *Y. lipolytica*. Another target could be xylitol dehydrogenase (XDH), as it is the enzyme that converts xylitol into xylulose, therefore undermining it could lead to the accumulation of more xylitol (Ledesma-Amaro *et al.*, 2016). Mutagenesis would be a popular option for the pursual of this strategy. Chemical mutagenesis via UV stress has been tried on various *Candida* species (Kumar *et al.*, 2010; Moreno *et al.*, 2019). This induced an XDH point mutation that results in a 1.2-fold increase in yield (Kumar *et al.*, 2010). Disruption cassettes that encode for the knockout of XDH genes using homogenous recombination with URA, Trpl and His3 are also popular and have been demonstrated on xylitol producing species like *Kluyveromyces marxianus, Debaromyces hansenii, Candida tropicalis* and also species less known for their xylitol production like *Trichoderma reesei* (Dasgupta *et al.*, 2017; Y. Xu *et al.*, 2019). If these methods are effective in these organisms, they could be effective in *Y. lipolytica.*

Had this project had more time there are also several more experiments that would have provided valuable insight into xylitol production by *Y. lipolytica*. A one-parameter-a-time experiment on xylose specifically. This factor appeared to have the largest impact on xylitol production and having an experiment on how xylose as a single variable affects xylitol production would have been valuable. However, this would require time and lab space that was not available, and the multivariate analysis experiment had already provided a large degree of insight into the optimum concentrations of the media components. Another factor that would have provided valuable insight would be to include glycerol concentration in the list of variables to optimise. This would be informative on how the concentration of glycerol affects *Yarrowia lipolytica*. However, the more variables that are added

to the central composite design the more experiments that there are, and these experiments had to be done in multiples to ensure reliability. The lab was already struggling to accommodate the large number of experiments required for the optimisation and time was also an issue, because of this the decision was made to only optimise the other variables.

Xylitol is a platform chemical with vast commercial potential. This is the first detailed report of bioproduction of xylitol by *Y. lipolytica*. The current work demonstrates the enormous potential of *Y. lipolytica* to convert xylose to xylitol with a yield near to the theoretical (> 90 %). It produces similar concentrations of xylitol to some of the best xylitol producing organisms, such as *Candida* strains. Moreover, it is a safe organism to use with a GRAS status from the FDA and it exhibited high tolerance to CG and xylose. Employment of unconventional feedstocks as carbon sources is highly desirable for the economic viability of biorefineries and becomes a good destination for renewable carbon-rich wastes. The study demonstrated the feasibility of the simultaneous valorisation of two major wastes, CG, and xylose, which can be utilized as cheaper feedstocks. The strategy can be conducive towards the development of a bioprocess as an alternative to the commercial chemical route and could support the sustainability of biodiesel industries/lignocellulosic biorefineries. However, more work is required to optimise and scale-up, to improve the economics of this bioprocess.

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