School of Public Health

Development of a Biological Technique to Produce a Bioactive-Rich Food Ingredient from Carob Kibble

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date: 30th May 2017

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ABSTRACT

Carob kibble is a rich source of D-pinitol and phenolic compounds, but considered as by-product of carob seed production. Current methods for obtaining these compounds result in low yields and poor quality caused by co-extraction of interfering compounds. The main objective of the present research project was to develop a biological approach to improving the content of D-pinitol and phenolic compounds, and associated bioactivity of the carob. Specific objectives were: 1) to develop a fermentation process, and establish the initial pH and time parameters for removing unwanted sugars, and increasing D-pinitol content in carob kibble; 2) to develop a fermentation process, and establish the initial pH and time parameters for increasing the total phenolic content and associated bioactivity in carob kibble; 3) to optimise fermentation conditions using response surface methodology (RSM) to enhance the content of D-pinitol and antioxidant activity in carob kibble.

For the first objective, *S. cerevisiae* (Sc) and *L. plantarum* (Lp) singly and in a mixture (LS) at (1:1 v/v) were used. The substrate was prepared in a 500-mL flask containing 60 g kibble and 240 mL water. The fermentation was carried out at original carob pH (5.0), 35^0C and under shaking condition for 70 h. During the fermentation, samples were withdrawn every 5 h for analysing microbial population, Brix and pH values. The culture which removed the most sugar content was selected for next steps. This aspect established that while sugar content was unchanged by Lp (Brix of 14 % for the whole 70 h), this content was significantly decreased to 8.0 % at 15 h by Sc and at 20 h by LS. Based on these results, Sc was selected for the next part of the study. Sc was used to establish the effect of the initial pH and fermentation time on the sugar removal and D-pinitol content. A similar submerged

fermentation to that above was performed, but at a range of pH 5.0 - 7.0, at 30^0C for 50 h only. The content of total carbohydrates and D-pinitol were determined by phenol-sulphuric acid and high-performance liquid chromatography (HPLC) methods, respectively. The results showed that changes in total carbohydrate and D-pinitol content were not affected by the tested pHs. After 15 h of fermentation, up to 70 % of the total carbohydrates were removed by Sc, without any negative effect on D-pinitol content. The content of D-pinitol was concentrated from 42.9 mg/gdw to 71.8 mg/gdw at 15 h and remained constant until 50 h. Three main sugars present in the kibble, namely sucrose, fructose and glucose, were quantified. The data showed that sucrose was largely removed at 10 h, while fructose and glucose were completely removed at 15 h.

For the second objective, a submerged fermentation for improving total phenolic content and related bioactivity in carob kibble was developed. As for the previous part of the study Sc and Lp, and their combination (LS) were used. The fermentation was performed at original carob pH (5.0), at 35^0C under a shaking condition for 70 h. Samples were taken for chemical analyses at every 5 h. The total phenolic content (TPC) determined by the Folin-Ciocaltue colorimetric method were increased to their highest level by 75.7 and 64.1 % in acetone and water extracts, respectively, by Sc. In contrast, Lp resulted in no change to TPC in the acetone extract, while there was a noticeable decrease in these compounds in the water extracts throughout the fermentation process ($p > 0.05$). Two methods, namely the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) for examination of antioxidant activity were used in the present study. The results indicated Lp alone increased the antioxidant activity to its highest level of

63.9 and 50.7 % for ABTS and DPPH, respectively, while this trend was not observed for carob treated with Lp. Inhibition of α-glucosidase activity was investigated by using the pNPG substrate and indicated no significant difference among the carob samples fermented with Sc, Lp and LS. Sc was therefore selected as the most appropriate organism for further study. A range of initial pHs from 5.5 - 7.5 and time period during 50 h were established for characterising the fermentation process. The results showed that the tested pHs did have a significant effect on the content of total phenolics and antioxidant activity of the carob extracts. At a carob substrate of pH 5.5 the TPC increased by 53.5 %, while the sample adjusted to pH 7.5 had a relatively higher increase by 66.3 % at 15 h. Data from the Vanillin assay indicated no statistically significant effect of initial pH on total condensed tannin content (TCT) throughout the fermentation process. The results of total flavonoid content (TFC) analysed by the colorimetric method showed a similar result to TFC, reaching 48.9 mg CE/gdw at pH 7.0. Antioxidant activity of the carob kibble was also determined as described above. In general, the antioxidant activity, for both ABTS and DPPH assays, increased to their highest activity at 15 h, then decreased at the end of the process ($p < 0.05$). In the ABTS assay, the activity of the pH 7.5 sample improved to 78.5 % at 15 h before decreasing to 49.7 % ($p < 0.05$). In the DPPH assay, on the other hand, the same pH sample inhibited the highest scavenging activity at 66.2 % at 15 h ($p < 0.05$). The initial pH 7.5 was the most effective for improving the ABTS scavenging activity ($p < 0.05$), but not for the DPPH free radical ($p < 0.05$) as compared to pH 5.5. The influence of initial pH on inhibitory activity to diabetic enzymes was investigated in *in vitro* models. The samples fermented at pH 5.5 and 7.5 were tested. The inhibitory activity against α-glucosidase was not affected by the Sc fermentation at pH 5.0 as presented above.

Again, this activity did not change significantly for both pHs of 5.5 and 7.5 ($p < 0.05$). A similar result was apparent in the α-amylase inhibition assay, where a fluctuation of around 44.8 and 58.9 % was seen at pH 5.5 and a small change at pH 7.5. It was concluded that fermentation with Sc at pH 5.0 -7.5 had no effect on the activity of carob kibble against α-glucosidase and α-amylase in *in vitro* models.

For the third objective, optimization of fermentation conditions to maximise the content of D-pinitol and antioxidant activity in carob kibble was achieved using RSM. Design expert (dx10) was used to design and analyse the experiments. Fermentation conditions, namely, the amount of inoculum (8 – 20 %), temperature (25 - 37^{0}C), the amount of substrate (15 - 39 g) and agitation rate (50 - 200 rpm) were selected for investigation. Of the chosen parameters, only the amount of substrate had a positive effect on the D-pinitol content, additionally, there were no interactions among these parameters including the amount of inoculum, temperature and agitation rate. However, a combined effect of the amount of inoculum and substrate on antioxidant activity was observed, with an increase in the inoculum of yeast a corresponding amount of kibble decreased and its antioxidant capacity increased. The results showed the fermented carob contains 96.2 mg/gdw of D-pinitol and 71.5 % inhibition of antioxidant activity. Based on these results we suggest a fermentation regime using a temperature of 28^{0}C, agitation rate of 162.5 rpm, inoculum amount of 17 % and carob amount of 21 g.

The study suggests a potential biologically approach to produce a rich bioactive carob kibble as a healthy ingredient for potential use in the pharmaceutical and food industries.

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List of Abbreviations

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid	ABTS
2,2-diphenyl-1-picrylhydrazyl	DPPH
Analysis of variance	ANOVA
Anthocyanidins	ACN
Butylated hydroxyanisole	BHA
Butylated hydroxytoluene	BHT
Cell counts of yeast	CFU
Central composite design	CCD
Deoxyribonucleic acid	DNA
Dry weight	dw
Gram	g
Half maximal inhibitory concentration	IC_{50}
Herpes simplex virus 1 and 2	HSV1-2
High performance liquid chromatography	HPLC
High-density lipoproteins	HDL
Hours	h

Human immunodeficiency virus	HIV
Lactobacillus plantarum	Lp
Liquid fermentation	LF
locust bean gum	LBG
Low-density lipoprotein	LDL
Microgram/millilitre	µg/mL
Mililitre	mL
Milimolar concentration	mM
Minute	min
p-nitropheynyl-alpha-D-glucopyranoside	pNPG
Response surface methodology	RSM
Revolutions per minute	rpm
Saccharomyces cerevisiae and *Lactobacillus plantarum*	LS
Saccharomyces cerevisiae	Sc
Second	s
Solid state fermentation	SSF
Southern Australia	SA

Standard derivation	SD
Streptozotocin	STZ
Submerged fermentation	SmF
Total condensed tannins	TCT
Total flavonoids content	TFC
Total phenolic content	TPC
Ultra violet	UV
Ultra violet-visible	UV-VIS
United State of America	USA
Volt	V
Volume/volume	v/v
Weight/volume	w/v

Chapter 1 Introduction

1.1 Introduction

The carob tree is grown predominantly in Mediterranean countries for its seeds. In 2014, the estimated production of carob worldwide was about 156,800 tonnes, and mainly from Spain, Italy, Portugal, Greece and Morocco (Food and Agriculture Organization of the United Nations, 2017). Carob seed has been used as substitute for chocolate powder, and for production of locust bean gum. Carob kibble is the left-over material after removal of the seeds from carob pod, and contains a high level of sugars (45 - 52 %), mainly sucrose, fructose, and glucose (Turhan, 2014; Nasar-Abbas et al., 2016). Although making up to 90 % the pod, this left-over part has been considered as a by-product of carob seed production. To date, the main use of carob kibble is for animal feed and bioethanol production (Obeidat et al., 2012; Nasar-Abbas et al., 2016). Recently, carob kibble has been reported to be a rich source of D-pinitol and polyphenols which have various potential health benefits (Owen et al., 2003; Ruiz-Roso et al., 2010; Turhan, 2011, 2014; Nasar-Abbas et al., 2016).

D-pinitol, a plant bioactive compound, has a number of health benefits, such as anti-inflammatory, anti-hyperlipidemic and anti-hyperglycaemia properties (Bates et al., 2000; Singh et al., 2001; Geethan & Prince, 2008). D-pinitol can be found in a variety of plant species, particularly in the leguminous family such as soybean (Kim et al., 2005 and Turhan 2014). In comparison to other plants, carob kibble has been reported to be a less expensive, but rich source of D-pinitol (Turhan, 2011; Chafer & Berna, 2014; Tetik & Yüksel, 2014; Turhan, 2014; Nasar-Abbas et al., 2016). However, aqueous extraction of D-pinitol from carob kibble has several drawbacks related to other sugars present in the carob that have similar molecular weight with

that of D-pinitol. These sugars are highly water soluble, and are therefore co-extracted with D-pinitol. They not only cause difficulty for purification of carob D-pinitol, but also interfere with the exhibition of its bioactivity (Ruiz-Aceituno et al., 2013). Current methods for removal of these interfering sugars, such as using ion-exchange resins (Camero & Merino, 2004) or carbon activated chromatography, are costly and time-consuming (Kim et al., 2005b). Subsequently, the production of D-pinitol from carob kibble remains limited.

Phenolics, another bioactive group of antioxidant compounds, constitute up to 1.9 - 2.48 mg/gdw of carob kibble, and are highly dependent on cultivar and environment (Kumazawa et al., 2002; Makris & Kefalas, 2004; El Hajaji et al., 2010; Ouzounidou et al., 2012; Vekiari et al., 2012). The main phenolic compounds identified in carob pod include gallic acid, flavonol-glycosides, hydrolysable and condensed tannins (Papagiannopoulos et al., 2004). Tannins account for a major proportion of total phenolics in carob pods (up to 9.5 % of the dry sample weight) (Custódio et al., 2015). The condensed tannin compounds in the kibble account for 40 - 55 % of the total polyphenols. Tannins are known to be anti-nutrient factors, so the reduction of these compounds in the final carob product is considered a positive outcome. Flavonoids are less abundant but still contribute to 10 – 20 % of polyphenolic components of the kibble (Kumazawa et al., 2002; Makris & Kefalas, 2004; Custódio et al., 2011; Roseiro et al., 2013a). The content and bioactivity of carob polyphenols may be improved if simple and active compounds are released. There is no existing practicable method to improve content and activity of phenolic compounds in carob kibble.

Fermentation has been widely used in food technology. For instance, fermentation can be used to remove carbohydrates from an agriculture product, or to convert carbohydrates into more valuable compounds. Fermenting micro-organisms use the carbohydrates for their metabolism and growth (Rodríguez-Sánchez et al., 2013). Depending on the micro-organisms used and experimental conditions, fermentation can remove 40 - 95 % of sugars from a substrate (Roukas, 1994, 1999; Lima-Costa et al., 2012). Furthermore, during fermentation process, some polyphenols can be converted from one form to another. Subsequently, this process can alter polyphenol content and bioactivity of a plant product (Dueñas et al., 2005; Đorđević et al., 2010; Juan & Chou, 2010; Xiao et al., 2014).

Saccharomyces cerevisiae (S. cerevisiae) is a safe fermenting micro-organism and has been widely used in food processing. Rodríguez-Sánchez et al. (2013) demonstrated that monosaccharides, disaccharides, and oligosaccharides in carob pod extracts could be significantly reduced by liquid fermentation with *S. cerevisiae* without any negative effect on inositols, derivatives of D-pinitol. Furthermore, *S. cerevisiae* is able to improve the content and properties of polyphenols in several other plants (Đorđević et al., 2010; Rashad et al., 2013). However, there is no published studies on the use of this organism for submerged fermentation of carob kibble.

Lactobacillus plantarum (*L. plantarum*) is a lactic acid bacterium which produces lactic acid as an end product of the fermentation process. Similarly to *S. cerevisiae*, this bacterial specie is also capable of enhancing both the antioxidant capacity and inhibitory enzyme activity in several plants, such as black olives, soybean and wheat (Dueñas et al., 2005; Pistarino et al., 2013). Again, no publications were found

regarding the use of *L. plantarum* with the aim of improving the content of phenolics and their associated bioactivity in carob kibble.

1.2. Objectives of the research project

In order to maximise the extraction of D-pinitol from carob kibble as well as to improve the potential health benefits of this by-product, it is necessary to address thoroughly the current drawbacks related to interfering sugars and high content of tannins in carob kibbles. Submerged fermentation may be a promising approach to address this. Therefore, the present research project was conducted to address the three main objectives as below:

1. Development of a fermentation process, and establishment of the initial pH and time parameters for removing unwanted sugars and increasing D-pinitol content in carob kibble (Chapter 3).
2. Development of a fermentation process, and establishment of the initial pH and time parameters for increasing the total phenolic content and associated bioactivity in carob kibble (Chapter 4 and 5).
3. Optimisation of fermentation conditions using response surface methodology (RSM) to enhance the content of D-pinitol and antioxidant activity in carob kibble (Chapter 6)

Chapter 2 Literature review

Some of the materials presented in this chapter have been published in the following journal article:

Syed, MNA, Zill-e-Huma, **Thi-Huong**, V, Muhammad, KK, Henry E, & Vijay, J. (2016). Carob kibble: a bioactive-rich food ingredient. *Comprehensive Reviews in Food Science and Food Safety*, 15, 63-72. http://dx.doi.org/10.1111/1541-4337.12177.

2.1 Overview

This chapter presents an up-to-date review of the literature on major bioactive compounds and their bioactivity associated with different parts of the carob tree, with a particular focus on carob kibble. The chapter consists of six main sections. Section 2.2 presents an overview of the carob pod such as chemical compositions, health benefits, and its current use. Sections 2.3 and 2.4 provide more insight into D-pinitol and polyphenols from carob, respectively. Section 2.5 discusses the application of fermentation for improving contents of the major bioactive compounds and their bioactivity from plants as well as from carob pods. Section 2.6 reviews the application of response surface methodology (RSM) to determine optimal fermentation parameters for maximising targeted products. Finally, the present chapter ends with a summary of key findings and gaps in the literature related to extraction of carob bioactive compounds, and their bioactivity.

2.2 Carob tree

Carob (*Ceratonia siliqua* L.) is an evergreen specie which belongs to the *Leguminosae* family. It grows in most Mediterranean countries (Hajib et al., 2015). The carob tree can reach fifteen meters in height and can live for over 200 years (Battle & Tous, 1997).

Global production of carob pod is estimated to be on average 150,000 ton per year (2010-1014) (Food and Agriculture Organization of the United Nations, 2017). The yield of carob pods is strongly dependent on the gender of carob tree, environment and cultivars (Makris & Kefalas, 2004; Custódio et al., 2011; Turhan, 2014).

In Australia, carob trees were first introduced in the early 1980's. Currently, carob is grown in five states of the country, but predominantly in South and Western Australia (Tous & Esbenshade, 2014). In 2007, the total area of carob cultivation in Australia was approximately 170 hectares (Carobs Austrlia). Since then the area of carob growing has increased drastically due to improvement in cultivation technology and an increased interest in the health benefits of the plants. The contribution of Australian carob to the global market is about 1,000 tons per year, and continue to increase (Carobs Austrlia).

2.2.1 Carob pod

A carob tree can produce about 10 kg of pod (per year) at the age of 5 - 7 years, but a mature tree can produce about 250 - 500 kg pods (Battle & Tous, 1997). The highest yield of carob pod is achieved when the tree reaches 15 years (Battle & Tous, 1997). The carob pod consists of seed (10 %) and kibble (90 %) which is leftovers after removal of seed (Bouzouita et al., 2007).

(a) (b)

(Carobs Austrlia)

Figure 2.1 Carob pod (a) and kibble (b)

Carob seed is composed of three major components, namely husk, endosperm, and germ, making up 30 – 33 %, 23 – 25 % and 42 – 46 % of the total, respectively (Bouzouita et al., 2007). Carob seeds are a good source of gum, called locust bean

gum (LBG) which contains mainly galactomannans (80 %) and protein (5 %) (Durazzo et al., 2014). The main protein constituents in LBG include albumin (68 %) and globulin (32 %). LBG has a high level of fiber (80 %) which consists of 20 % insoluble and 60 % soluble fiber (Durazzo et al., 2014). Carob seeds have also been found to contain high level of phenolic compounds which are important source of antioxidants. Carob germ flour contains 19.25 mg/gdw total phenolic content, has antioxidant activity equivalent to 439.25 µmol FRAP/gdw (Durazzo et al., 2014). Recently, increasing attention has been paid to LBG for its industrial uses. In the food industry, carob seed powder, which is a white to creamy color, has been commonly used as natural food additives (e.g. as a thickener, stabiliser or gelling agent) (Ünal et al., 2003; Bouzouita et al., 2007). This powder is also utilised in other industries, such as paper, cosmetics, pharmaceutics and textile (Barak & Mudgil, 2014). Due to its ability to regulate the release of excipients in tablets, LBG is commonly used in pharmaceutical industry (Beneke et al., 2009). LBG is also used in the production of solid monolithic matrix systems, films and in viscous liquid and gel formulations. For biopharmaceutical applications, the gelling capacity and synergies with other polysaccharides of LBG have been discussed (Beneke et al., 2009). Currently, LBG is of increasing interest in terms of health. The viscous characteristics of its soluble fiber lead to effects on both the viscosity and structure of food and in turn alter carbohydrate degradation during digestion (Barak & Mudgil, 2014). This may be beneficial to the management of cholesterol level and reduction of postprandial blood glucose and insulin levels. These are key approaches for the prevention and treatment of diabetes, obesity and cardiovascular diseases (Ferguson & Harris, 2005).

2.2.2 Carob kibble

Carob kibble makes up 90 % the weight of carob pods, and is the left-over material after removal of seeds. It has been reported to contain high concentration of D-pinitol and polyphenols which are attributed to various potential health benefits (Turhan, 2014; Nasar-Abbas et al., 2016). Given the yield of about 160,000 tons a year (Food and Agriculture Organization of the United Nations, 2017), carob kibble may be a valuable natural source of raw materials for various industries, such as food technology or ethanol production.

2.2.2.1. Chemical composition

The most abundant chemical components in carob kibble are carbohydrates, crude fibre and phenolics. As shown in Table 2.1, carbohydrates contribute to 52 - 85 % of the total weight of carob kibble. Among them, sucrose is the most predominant compound which accounts for 38 – 63 %, followed by fructose (5 – 12 %) and glucose (2 – 6 %). D-pinitol also contributes to 3 – 4 % of the kibble. The second major chemical constituent of carob kibble is fibre, with a crude content of 70 - 400 mg/gdw. Table 2.2 shows that dietary fibre in carob mainly consists of insoluble components (68 %) and dietary constituents account for 75 % of the total carob fibre. Details of minerals, protein and phenolics in carob kibble are presented in the Table 2.3, 2.4 and 2.5. Table 2.1 also shows a large variation in the content of carob kibble's chemical compositions. This is because of the effects of harvest time, carob gender and cultivar, storage conditions and climatic factors (Ayaz et al., 2007; Biner et al., 2007; Khlifa et al., 2013).

Table 2.1. Chemical composition of carob kibble

Constituents	Content (g/100gdw)
Total carbohydrates	52 – 85
Sucrose	38 – 63
Fructose	5 – 12
Glucose	2 – 6
Pinitol	3 – 4
Crude fibre	7 – 40
Protein	2 – 7
Phenolics	2 – 12
Ash	2 – 3
Fat	< 1

Source: (Kumazawa et al., 2002; Ayaz et al., 2007; Biner et al., 2007; Khlifa et al., 2013; Turhan, 2014; Nasar-Abbas et al., 2016)

Table 2.2 Typical composition of carob fibre

Components	Content (g per 100gdw)
Moisture	5.0
Total dietary fibre	75
Insoluble dietary fibre	68.4
Soluble dietary fibre	6.0
Carbohydrates	6.0
Protein	5.0
Fat	0.2
Ash	3.4

Source: (Haber, 2002; Owen et al., 2003; Zunft et al., 2003)

Interestingly, carob kibble contains important minerals such as K, Ca and Mg (Ayaz et al., 2007; Khlifa et al., 2013; Youssef et al., 2013). Table 2.3 shows that the content of K in carob pods ranges from 986.7 - 1010.9 mg/100gdw which is much higher than those in cereals (e.g. wheat flour and white rice) and fruits (e.g. apple and bilberry) (Heinemann et al., 2005; Ekholm et al., 2007; Khlifa et al., 2013; Youssef et al., 2013). The contents of Ca, P and Mg are also at higher levels in carob kibbles as compared to cereals and fruits (Table 2.3).

Table 2.3 Mineral content in carob pod and several cereals and fruits

Mineral	Content (mg/100gdw)				
	Carob	Wheat flour	Rice	Apple	Bilberry
K	986.7 - 1010.9	133.3 - 200.0	127 – 160	900 - 980	508.3 - 680
Ca	212.3 - 300.0	18.9 - 29.1	60 – 79	40 - 48.3	119.9 - 140
P	71.2 - 125.5	130.5 - 140.0	98 – 150	80 - 91.0	109.9 - 130
Mg	60.0 - 94.7	39.5 - 118.0	35 – 55	43.5 - 50	55.6 - 60
Fe	1.8 - 3.8	1.5 - 5.1	0.7 - 0.8	1.1 - 3.3	1.7 - 3.1
Mn	0.29 - 1.29	0.96 - 3.1	0.7 - 1.2	0.2 - 0.33	2.8 - 10.4
Zn	0.46 - 2.4	1.07 - 3.5	0.8 - 1.92	0.1 - 0.7	0.8 - 1.1
Cu	0.29 - 0.85	0.21 - 0.48	0.13 - 0.2	0.3 - 0.63	0.4 - 0.5

Source: (Lovis, 2003; Heinemann et al., 2005; Ekholm et al., 2007; Elisabetta et al., 2013)

Protein content accounts for 4 – 7 % (w/w) of carob kibble, and varies depending on harvest season, geography, cultivar, climate and storage conditions (Khlifa et al., 2013; Youssef et al., 2013; Nasar-Abbas et al., 2016). Carob pods contain up 18 amino acids, predominantly aspartic acid (18.25 g/100gdw protein) and alanine (10.55 - 18.25 g/100 gdw protein) (Ayaz et al., 2007). Interestingly, up to 9 essential amino acids were detected (Table 2.4) (Ayaz et al., 2007). Valine and leucine were recorded at a relatively higher level at 9.05 and 9.30, compared to other components.

Table 2.4 Comparison of amino acid composition of carob pod versus apple and mushroom

Amino acid	Content		
	Carob (g/100g protein)	Apple (mg/100g fresh weight)	Mushrooms (mg/100g fresh weight)
Aspartic acid	18.25	31.1	293
Glutamic acid	9.65	4.5	364
Serine	6.80	1.3	110
Histidine	2.80	0.1	65
Glycine	3.55	0.6	97
Threonine	5.10	0.4	106
Arginine	3.20	0.1	179
Alanine	10.55	0.7	124
Proline	5.80	-	93
Tyrosine	1.70	-	219
Valine	9.05	0.3	112
Methionine	1.04	0.1	106

Cysteine	0.80	-	28
Isoleucine	3.80	0.2	82
Leucine	9.30	0.3	139
Phenylalanine	3.10	0.9	111
Lysine	4.20	0.2	126
Tryptophan	0.95	-	-

Source: (Gomis et al., 1990; Ackermann et al., 1992; Mattila et al., 2002; Ayaz et al., 2007).

2.2.2.2 Potential health benefits

Potential health benefits of carob kibble are related to its high content of phenolic compounds, D-pinitol and dietary fibre. Carob phenolics have been proven to possess strong antioxidant property, and to be able to regulate gene expression (Kumazawa et al., 2002; Makris & Kefalas, 2004). Health benefits of carob phenolic contents are further detailed and discussed in the Section 2.4 Carob phenolics. According to Ruiz-Roso et al. (2010) and Zunft et al. (2003), carob kibble can be a promising source of food ingredients to control body weight and regulate blood lipid level in obese persons. It has been demonstrated to significantly reduce the content of low-density lipoprotein (LDL) cholesterol in hypercholesteraemic patients. In addition, carob dietary fibre is believed to be able to improve the outcomes of colon cancer and type 2 diabetics' patient treatment. Finally, carob D-pinitol also has antihyperglycemia, antihyperlipidemia and anti-inflammation properties (Bates et al., 2000; Singh et al., 2001; Geethan & Prince, 2008). These properties of carob D-pinitol are detailed and discussed in Section 2.3 carob D-pinitol.

2.2.2.3 Current use of carob kibble

Carob kibble for the production of syrup

Carob kibble is the main raw material for production of carob syrup which can be utilised as a sugar substitute in food formulations. For example, it is used as a sweetener in hot and cold drinks, in meat and fish marinade and as a topping for desserts and over pancake and ice-cream (Carobs Austrlia). By comparison to other fruit-made drinks, such as grape juice, carob juice has comparable sensory properties, but a higher content of total polyphenols (19.8 mg /gdw versus 6.2 mg /g in grape juice) (Adebowale et al., 2008). Moreover, due to its high sugar content (sucrose, glucose, and fructose), carob syrup can also be used for mannitol production (Carvalheiro et al., 2011).

Carob kibble as a source of animal feed and bioethanol production

Due to its high content of sugar, carob kibble has been mainly used for the production of animal feed. In recent years, the kibble has become an economical source of material for ethanol production. Fermentation of carob extract using *S. cerevisiae* is one of the common methods for producing ethanol (Turhan et al., 2010b; Mazaheri et al., 2012; Saharkhiz et al., 2013). According to Saharkhiz et al. (2013), carob pod treated with *Zymomonas mobilis* can produce a significant ethanol yield of 0.3 - 0.42 g ethanol/g initial sugars. As ethanol is an environmentally friendly and renewable energy source, the use of carob kibble as a raw material for this industry is projected to continue increasing (Turhan et al., 2010b; Ercan et al., 2013; Demirci, 2016).

2.3 Carob D-pinitol

2.3.1 Molecular characteristics

D-pinitol is an alcohol sugar, highly soluble in water and found in a large number of plants (Kim et al., 2005b; Poongothai & Sripathi, 2013). Currently, D-pinitol has been found in 291 species of plants, belong to 9 families, of which 227 are in the *Leguminosae* family. The full name of D-pinitol is the 3-O-methyl ether of D-chiro-inositol. Its chemical structure is cyclic with carbon atoms present in the ring, which is different from the common sugar structure where oxygen is included in the ring (Phillips et al., 1982).

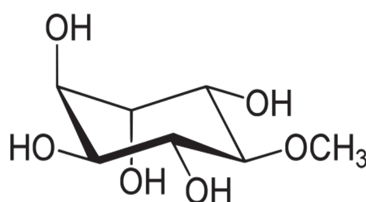


Figure 2.2 Chemical structure of D-pinitol

Synonyms: 1-methoxy-2, 3, 4, 5, 6-pentahydroxy cyclohexane D-3-O-methyl-chiro-inositol
Molecular Formula: $C_7H_{14}O_6$
Molecular Weight: 194.2

Source: (Garland et al., 2009)

2.3.2 Natural sources

Plant D-pinitol was first isolated from some *Pinaceae* species (e.g *Pinus lambertiana*) in the late 1940s (Lindstedt, 1949; Gottlieb & Brauns, 1951). Since then, D-pinitol has been found in a variety of plant species. *Leguminosae* species, such as carob, soy bean and pea, are the richest sources of plant D-pinitol (Table 2.6).

Soybean is one of the richest source of D-pinitol, with a content of 13.0 - 30.4 mg/gdw of tissue (Streeter et al., 2001; Kim et al., 2005b; Chen et al., 2014). D-pinitol was found abundant in the root nodules of soybean and therefore believed to be associated with soybean's ability to adapt to dry condition (Phillips et al., 1982; Streeter et al., 2001). In soybean seeds, D-pinitol ranges from 2.3 - 27.9 mg/gdw depending on the stage of growth and seed tissue. Its content in seed cotyledon, coat, and embryonic axes is 27.9 mg/gdw, 20.9 mg/gdw and 17.6 mg/gdw, respectively (Kuo et al., 1997). In a recent investigation of 12 different soybean cultivars conducted by Chen et al. (2014), D-pinitol content accounted for 3.04 % of dry weight of soybean tissues, equivalent to 30.4 mg/gdw. The investigation also showed that the D-pinitol content of soybean was the highest in leaves (3.01 %), followed by stems (1.6 %), pods (0.4 %) and beans (0.28 %). This pattern of D-pinitol distribution was also reported by a previous study, which found that the gradient of D-pinitol accumulation in the soybean plant was two- to three-fold from the bottom to the top of the plant. During plant development, the concentration of D-pinitol was consistently higher in the uppermost leaves (Streeter et al., 2001). The content of plant D-pinitol highly depends on cultivars and climates (Streeter, 2001; Kim et al., 2005b; Chen et al., 2014).

Other *Leguminosae* species also contain significant amount of D-pinitol (Table 2.6). Orange, lemon and lime have a much lower content of D-pinitol. An investigation of 14 different Korean plants showed that D-pinitol content ranged from 0.2 - 40 mg/gdw with carob pods, *Bougainvillea* and soybean containing a significant amount (Kim et al., 2005b). Some species of bean, such as mung bean and black gram, and peas, such as chickpea and pigeon pea, showed a significant amount of D-pinitol at

18.8 - 27.9 mg/gdw (Ford, 1984; Oliveira et al., 2004; Lahuta et al., 2005; Seo et al., 2011).

Among the *Leguminosae* species, carob plants have the highest content of D-pinitol. On average, wild and cultivated Turkish carob pod contains 37.8 and 42.6 mg/gdw D-pinitol, respectively (Turhan, 2014), meanwhile its content in soybean, one of the leading source of plant D-pinitol, was 13.0 - 30.4 mg/gdw of tissue (Streeter, 2001; Kim et al., 2005b; Chen et al., 2014). In a previous study, (Kim et al., 2005b) also reported a similar content (40 mg/gdw) of D-pinitol in carob. In Spanish carob, (Chafer & Berna, 2014) an even higher content of D-pinitol at approximately 60 mg/gdw has been reported. According to Turhan (2014), there was a high correlation between the content of carob D-pinitol and glucose. This correlation was apparent in both wild and cultivated species. In comparison to other plants, carob appears to be the least expensive, but richest source of plant D-pinitol (Turhan, 2011; Chafer & Berna, 2014; Tetik & Yüksel, 2014; Turhan, 2014).

Table 2.5. D-pinitol content in some plants

Plant	D-Pinitol content (mg/gdw)	Reference
Carob	38.7 - 60.0	(Yatmaz et al., 2012; Chafer & Berna, 2014; Turhan, 2014)
Soybean	13.0 - 30.4	(Streeter, 2001; Kim et al., 2005b; Chen et al., 2014)
Beans	18.8 - 27.9	(Ford, 1984; Oliveira et al., 2004)
Orange, lemon, lime	1.57; 0.61; 0.19	(Kim et al., 2005b)

2.3.3 Health benefits

Diabetes mellitus is currently considered as the most common metabolic and endocrine disorder. It is associated with a significant increase in symptoms of the

metabolic syndrome, such as total cholesterols, hypertriglyceridemia and hyperglycaemia (Garber, 2002). The management of diabetes mellitus targets the control of blood glucose and lipid metabolism as well as reduction of oxidative stress (Garber, 2002; Geethan & Prince, 2008).

Recently, D-pinitol has received considerable attention due to its potential health benefits, such as anti-inflammatory, anti-hyperlipidemic and anti-hyperglycemic activities. In an animal experiment, D-pinitol extracted from *Abies pindrow* leaves presented a significant anti-inflammatory effect at a dose of 10 mg/kg body (Singh et al., 2001). Osteoclasts (OCs) are a type of multi-nucleus cells that degrades the bone matrix. Problems with the differentiation of these cells, such as an increase in bone resorption, may cause bone-related diseases such as osteoporosis and bone inflammation (Pierce et al., 1991; Boyce et al., 2009). D-chiro-inositol, mostly D-pinitol, significantly inhibited the formation of OCs by reducing the expression of OC marker genes (Yu et al., 2012). D-pinitol is therefore suggested to be a potential candidate to treat and prevent bone inflammation and osteoclastogenesis in diabetes mellitus (Yu et al., 2012). D-pinitol was also suggested to have the potential to develop into an anti-inflammatory drug due to its anti-inflammatory effectiveness on acute and subacute rats (Kim et al., 2005a). This study also proved a significant improvement of the activity D-pinitol if it was combined with glucosamine.

Anti-hyperlipidemic activity is another property of D-pinitol. Both animal and human trials showed that D-pinitol could also decrease total cholesterol, low-density lipoprotein (LDL) cholesterol, the low- to high-density lipoprotein (HDL) cholesterol ratio, and blood pressure (Geethan & Prince, 2008; Saikia & Lama, 2011; Asuzu & Nwaehujor, 2013). Geethan and Prince (2008) showed that *Bougainvillea*

spectabilis-derived D-pinitol showed a positive result in regulation of the level of blood glucose and lipids in streptozotocin (STZ)-induced diabetic rats. When administered at a dose of 100 mg/kg body weight, the blood glucose level of these rats decreased significantly, while there was no change in the blood glucose level of normal rats. Similar effects were also observed for D-pinitol extracted from soybean and soy whey (Kim et al., 2005b; Saikia & Lama, 2011; Asuzu & Nwaehujor, 2013). Carob pod has been reported as a rich source of D-pinitol with approximate 4 % of total dry weight (Turhan, 2011; Chafer & Berna, 2014; Tetik & Yüksel, 2014; Turhan, 2014). Having an identical chemical structure to soybean D-pinitol, carob D-pinitol has also been reported to possess anti-diabetic type 2 properties (Kim et al., 2005b). In two human studies, Kang et al., (2006) and Kim et al. (2005b) showed a great benefit of soybean D-pinitol to a reduction in blood glucose in type 2 diabetes patients.

In addition, plant D-pinitol has also demonstrated to considerably reduce triglycerides, free fatty acids and phospholipids in serum, liver, kidney and heart in the disease rats after D-pinitol treatments (Geethan & Prince, 2008; Saikia & Lama, 2011; Asuzu & Nwaehujor, 2013). D-pinitol was highly effective in controlling total cholesterol, LDL-cholesterol and the LDL/HDL-cholesterol ratio (Geethan & Prince, 2008; Saikia & Lama, 2011). D-chiro-inositol is a component of an inositol phosphoglycan, which is a mediator in the insulin action (Richard Jr et al., 1996). In the body, plant D-pinitol could be converted into D-chiro-inositol and mimic the ability of insulin. This could, in turn, prevent glucose from being released from the liver and stimulate glucose uptake into tissues such as muscle and fat.

Bates et al. (2000) reported that D-pinitol exerts an insulin-like effect on the control of blood glucose level in diabetic-induced rats. This effect might come from the interaction of D-pinitol with a cellular signal that connects insulin and glucose transport (Bates et al., 2000; Kim et al., 2007). Another study reported D-pinitol acting as an insulin sensitiser (Oliveira et al., 2004; Do et al., 2008; Kim et al., 2011a). The increase in the risk of type 2 diabetes and other related diseases, such as cardiovascular and obesity, could be caused by insulin resistance (Saltiel & Kahn, 2001). One of the reasons leading to this insulin problem is an excess of adipose tissue. It is suggested that D-pinitol does not possess any negative effect on adipocyte differentiation, adipogenesis, and lipid accumulation. More importantly, it enhances the process of adipogenesis in 3T3-L1 preadipocytes by acting as an insulin sensitiser (Do et al., 2008). In a similar way to its derivatives as myo-inositol, D-pinitol is able to stimulate translocation of glucose transporter 4 to the plasma membrane in the skeletal muscle, which could prevent diabetes mellitus (Dang et al., 2010). D-pinitol has been reported to benefit not only disease-induced subjects, the effectiveness of D-pinitol on the decrease in hyperglycaemia and circulation in insulin level in healthy subjects was observed (Hernández-Mijares et al., 2013).

Antioxidant activity of plant D-pinitol extract *in vitro* models have been demonstrated (Asuzu & Nwaehujor, 2013; Rengarajan et al., 2014b). By contrast, an animal study showed the antioxidant potential of soybean D-pinitol in protection of pancreatic tissue from free radical-mediated oxidative stress in streptozotocin-induced diabetic rats (Sivakumar & Subramanian, 2009).

D-pinitol has been reported to possess anti-cancer activity. The Michigan cancer foundation-7 (MCF-7) cell line is a breast cell line isolated from metastatic breast

patients (Horwitz et al., 1975; Levenson & Jordan, 1997). D-pinitol showed the potential to induce apoptosis in the MCF-7 cell line. The proliferation of MCF-7 cells in a concentration-dependent manner was significantly inhibited by D-pinitol, moreover, the expression of other related proteins was regulated. D-pinitol may have potential as an anti-breast cancer drug (Rengarajan et al., 2014a).

2.3.4 Method for D-pinitol extraction from plants

Generally, ethanol and water have been used as solvents to isolate D-pinitol from plants before purification. Sharma et al. (2014) used a chromatography column with silica gel to recover a significant amount of D-pinitol from ethanolic plant extracts, obtaining up to 37 mg/gdw D-pinitol from a *Argyrolobium roseum* leaf extract. D-pinitol was purified from the root bark of *Tamarindus indica* Linn using a chromatography method with a silica gel column after extraction with ethanol (Jain et al., 2007). In studies of leguminous plants, water was used to extract D-pinitol, followed by purification and separation using chromatography. Kim et al. (2005b) purified D-pinitol from various plants using water as a solvent followed by carbon activated chromatography. Among the samples tested, carob pod had the highest content of D-pinitol, at 40 mg/gdw, followed by *Bougainvillea* and soy whey with 20 mg/gdw, and soybean with 2.59 - 17.4 mg/gdw. These authors also mentioned a limitation in the application of D-pinitol because of its relatively high price, despite its obvious health benefits.

Carob pod has been reported as a rich and cheap source of natural D-pinitol (Turhan, 2011). Common methods of aqueous extraction of D-pinitol from carob kibble have several drawbacks, such as high cost, but also low yield because of interfering

compounds. In addition, D-pinitol product from aqueous extraction usually has low bioactivity, mainly due to the presence of high levels of other sugars which interfere with the bioactivity of D-pinitol (Turhan, 2011; McDonald IV et al., 2012; Ruiz-Aceituno et al., 2012). Ion-exchange resins can be used to produce D-pinitol with 90 % purity, but this approach is costly and time consuming (Camero & Merino, 2004). Development of a commercially viable technique of extracting D-pinitol by selective removal of interfering sugars is therefore of great interest to industry (Ruiz-Aceituno et al., 2012; Rodríguez-Sánchez et al., 2013).

2.4 Carob phenolics

2.4.1 Benefits of natural phenolics

The imbalance between the generation of free radicals and the antioxidant defenses of human organs could cause oxidative stress. This may result in damage to the DNA, lipid and proteins, which have link to many diseases including cancers, diabetes and metabolic disorders (Gülçin, 2005; Kadiiska et al., 2005). Both synthetic and natural antioxidants may protect humans from stress-related diseases.

Two of the most prevalent synthetic antioxidants commonly used in food are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). At the recommended dose, these compounds are believed to have significant anti-mutagenic and anti-carcinogenic properties (Shahidi, 2000). However, there is increasing evidence that synthetic antioxidants exert considerable side effects, such as lipid alteration, cell proliferation and even carcinogenicity (Sun & Fukuhara, 1997; Botterweck et al., 2000). For example, both BHA and BHT were reported to cause weight gain in some organs, such as liver, lungs, and adrenals (Witschi et al., 1989).

This is a cause for concern since their long-term ingestion may cause the acute toxicity of various chemicals due to their modifications (Lobo et al., 2010).

Dietary antioxidants from plant-based products such as phenolics have received increasing attention for their health benefits (Kris-Etherton et al., 2002). Phenolics can be found in a variety of different plants (e.g. vegetables, fruits, cereals, nuts and legumes). The level of phenolic compounds in a specific plant can vary greatly depending on their formation and cultivation conditions. Some phenolic compounds, such as flavonoids, can be abundant in leaves, but present at low levels in the roots (Bravo, 1998).

According to Quideau et al. (2011), the health benefits of plant phenolics are related to their antioxidant properties, which is the most remarkable characteristics of these compounds. These properties have crucial implications for the prevention of oxidative stress-related chronic conditions such as cardiovascular and neurodegenerative diseases and aging (Duthie & Brown, 1994; Dillard & German, 2000; Yang et al., 2001; Tsao & Akhtar, 2005). For this reason, American and European dietary guidelines recommend five servings of fruits and vegetables per day to maintain a healthy diet (Quideau et al., 2011).

There are numerous studies which report health benefits of plant polyphenols. An extensive review by Yuan (2013) showed that green tea polyphenols can reduce the risk of oral digestive tract cancers. For example, Hoang et al. (2016) reported an inverse association between habitual green tea drinking and the risk of prostate cancer. Plant polyphenols are also beneficial in terms of prevention of cardiovascular diseases. According to Quinones et al. (2013), polyphenols have protective effects

against oxidation of low-density lipoproteins, inflammation and apoptotic processes in the vascular endothelium, which are risk factors for cardiovascular diseases. Regular drinking of green tea is also associated with a lower risk of ischemic stroke.

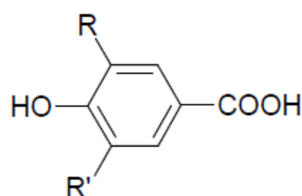
2.4.2 Main groups of natural phenolics

Plant phenolics are the most common natural antioxidants found in daily foods. There are over 8,000 phenolic compounds found in plants. The major groups of these compounds are phenolic acids, flavonoids, and tannins (Kabera et al., 2014; Goulas et al., 2016).

2.4.2.1 Phenolic acids

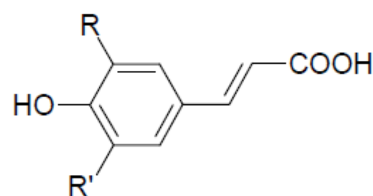
Phenolic acids are divided into two groups, namely benzoic and cinnamic acids, with respect to their $C_1 – C_6$ and $C_3 – C_6$ backbones (Fig. 2.4). The benzoic group includes common acids such as gallic acid, syringic acid, protocatechuic acid and vanillic acid, while the cinnamic group includes ferulic acid, β-coumaric, caffeic acid and sinapic acid (Tsao, 2010). They are often found in free forms in fruits and vegetables, and in complex forms in grains, seeds and hull. These bound phenolics can also be hydrolysed into free forms with alkali. Natural sources of these acids are legumes (e.g soybean, pea and bean), fruits (e.g strawberry and raspberry) and vegetables (e.g cucumber and carrot) (Chong et al., 2001; Xu & Chang, 2008a; Russell et al., 2009). In addition to strong antioxidant activity, these phenolics exhibit various potential benefits for human health such as inhibition of HIV, HSV1-2 and fungal growth (Li et al., 2005; Kratz et al., 2008; Kaur et al., 2009; Seo et al., 2013).

Benzoic acid derivatives



R = R' = H; *p*-hydroxybenzoic acid
R = OH, R' = H; protocatechuic acid
R = OCH_3, R' = H; vanillic acid
R = R' = OH; gallic acid
R = R' = OCH_3; syringic acid

Cinnamic acid derivatives



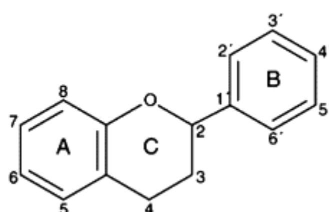
R = R' = H; *p*-coumaric acid
R = OH, R' = H; caffeic acid
R = OCH_3, R' = H; ferulic acid
R = R' = OCH_3; sinapic acid

Source: (Pereira et al., 2009; Tsao, 2010)

Figure 2.3 Chemical structures of phenolic acids

2.4.2.2 Flavonoids

Flavonoids are the largest group of plant phenolics. The chemical structure of flavonoids differs with respect to heterocyclic oxygen rings around the structure, but generally include a C_6–C_3–C_6 carbon skeleton. The nature of the flavonoid structure determines their levels of hydroxylation and methylation, and their bioactivities (Fig. 2.5). In plants, most flavonoids exist as glycosides. The main flavonoid groups are isoflavones, flavonols, flavanols, flavones, flavanones and anthocyanidins.

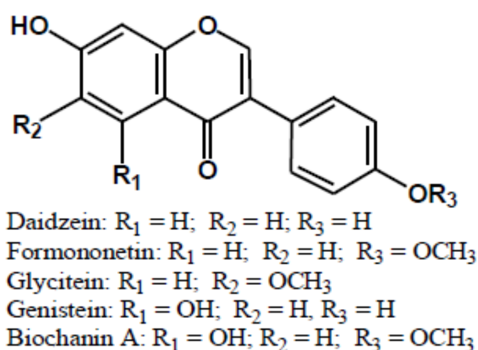


Source: (Yilmaz, 2006; Tsao, 2010)

Figure 2.4 Basic monomeric structure of flavonoids

The typical chemical structure of isoflavones is presented in Fig. 2.6. The major members of the isoflavonoid group include daidzein, genistein, glycitein, malonyldaidzin and malonylgenistin. Isofalavones can be found in both plant and

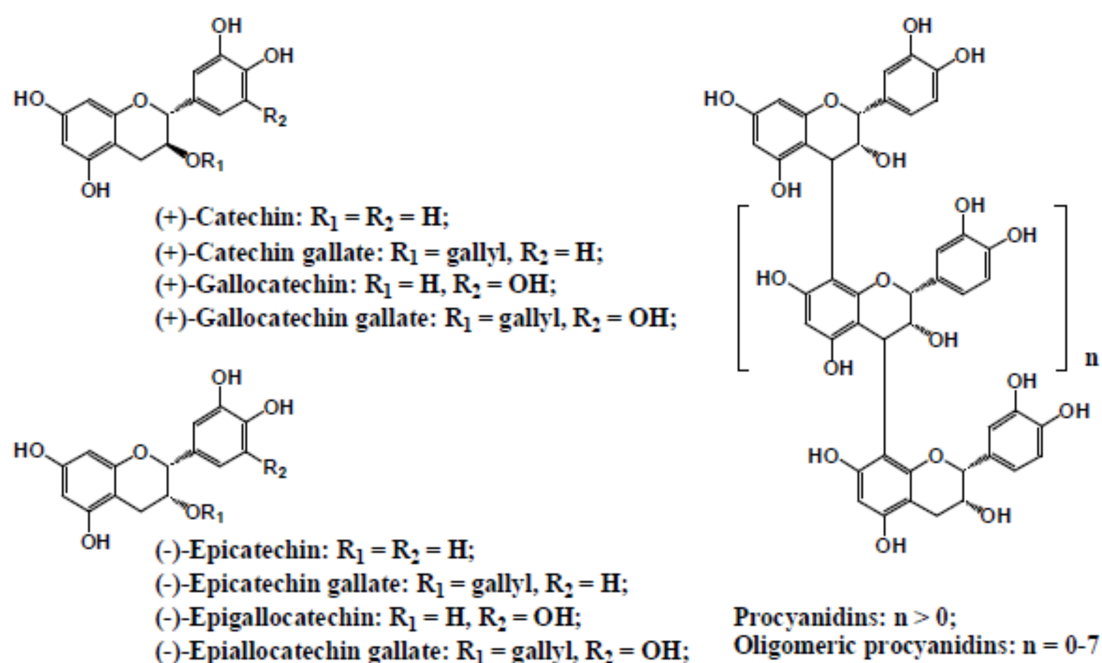
animals sources. For example, total content of isoflavone in soybean and pea is between 1.02 - 1.53 mg/gdw and 0.3 - 0.65 mg/gdw, respectively (Šibul et al., 2016), while other sources include animal meats (beef 1 - 3 µg/100gdw; chicken 2 - 4 µg/100gdw), prawn 3 µg/100gdw and tuna 5 µg/100gdw (Kariyil, 2010).



(Tsao, 2010)

Figure 2.5 Chemical structure of Isoflavones

Flavonols and flavanols are two of the most common subgroup of flavonoids in plants, which share similar general chemical structures (Fig. 2.7). Flavonols include quercetin, kaempferol and myricetin, whereas, flavanols are catechin, epicatechin, epicatechin gallate and epigallocatechin-3-gallate. Catechin makes up 6 – 24 % and up to 60 - 90 % of total flavonoids in black and green tea, respectively (Cabrera et al., 2003; Higdon & Frei, 2003). Other rich natural sources of catechin are fruits such as strawberry and cherry and legumes, such as bean and lentil. The total catechin in chocolate is four times higher than tea (Lee et al., 2003; Peterson et al., 2005). Natural flavonoids also possess strong antioxidant and anticancer properties (Kim et al., 2003; Oikawa et al., 2003; Sang et al., 2003; Schroeder et al., 2003).



Source: (Yilmaz, 2006; Tsao, 2010)

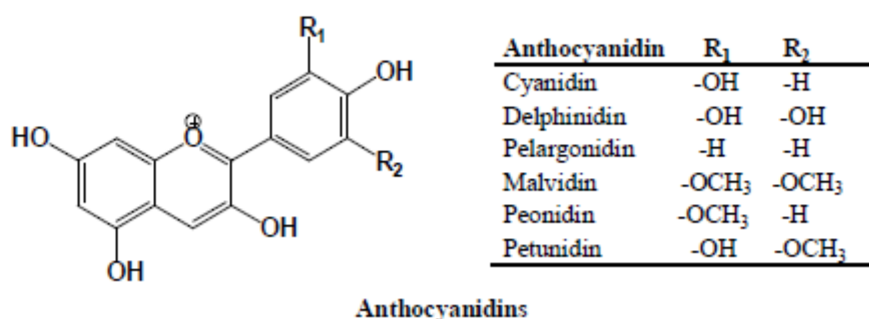
Figure 2.6 Chemical structures of Flavanols

2.4.2.3 Tannins

Anthocyanidin

The content of total anthocyanidins *(*ACN) varies significantly in fruit and vegetable sources (Suda et al., 2005; Wu et al., 2006; Koponen et al., 2007). Berry fruits are a rich source of ACN with 1480 mg/100g fresh weight in chokeberry, 1375 mg/100g fresh weight in elderberry, 687 mg/100g fresh weight in black raspberry and 245 mg/100g fresh weight blackberry (Zhang et al., 2004; Wu et al., 2006; Yue & Xu, 2008). Some other fruits such as apple (1.3 - 12.3 mg/100g fresh weight), grape (26.7 - 120.1 mg/100g fresh weight) and plum fruits 19.0-124.5 mg/100g fresh weight have a relatively low amount of ACN (Wu et al., 2006; Koponen et al., 2007). Vegetables are considered as significant sources of ACN, but at different levels. Red bean has only 6.7 mg ACN, while black bean, red radish and red cabbage contains

44.5, 100.1 and 322 mg/100g fresh weight respectively (Suda et al., 2005; Wu et al., 2006). Antioxidant activity is a typical property of anthocyanidins. Pomegranate extract has all three anthocyanidin compounds, including cyanidin, delphinidin and pelargonidin and exhibited free radical scavenging (Noda et al., 2002). Anthocyanidins have been reported to possess α-glucosidase inhibitory activity and increase insulin secretion which may be useful in diabetic treatments, (Jayaprakasam et al., 2005; Nizamutdinova et al., 2009; Kumar et al., 2011; Sancho & Pastore, 2012). ACNs have also been found to have anti-cancer activity, such as ability to inhibit the growth of tumour cells (Seeram et al., 2003; Zhang et al., 2005; Thomasset et al., 2009).



Anthocyanidin	R_1	R_2
Cyanidin	-OH	-H
Delphinidin	-OH	-OH
Pelargonidin	-H	-H
Malvidin	$-OCH_3$	$-OCH_3$
Peonidin	$-OCH_3$	-H
Petunidin	-OH	$-OCH_3$

(Tsao, 2010)

Figure 2.7 Chemical structure of anthocyanidin

Proanthocyanidins

Proanthocyanidins are also known as condensed tannins, and are formed from a polymerisation of catechin and epicatechin (Kähkönen & Heinonen, 2003). Natural sources of proanthocyanidins include grape seed (Bagchi et al., 2003; Sano et al., 2003), lentils (Dueñas et al., 2003), fruits (Tsao et al., 2003), chololate (Counet & Collin, 2003) and cacao (Hatano et al., 2002). Proanthocyanidinins with 2 - 7 unit

oligomers are increasingly considered as strong antioxidants, immunomodulators and bacterial inhibitors (Bagchi et al., 1997; Bagchi et al., 2000; Yousef et al., 2009).

Table 2.6 Major groups of plant phenolic compounds, their key bioactivity and natural resource

	Compound	Activity	Natural resource	Reference
Phenolic acids	**Benzoic acids:** Protocatechuic acid, Vanillic acid	antioxidant, anti-HIV, anti-HSV1,2, anti-fungal, anti-cancer	Tobacco, rose flower, strawberry, raspberry, soybean, bean, pea	(Chong et al., 2001; Xu & Chang, 2008a; Russell et al., 2009); (Li et al., 2005; Kratz et al., 2008; Kaur et al., 2009; Seo et al., 2013)
	Cinnamic acids: Ferulic acid, β-coumaric, Caffeic acid, Sinapic acid	Antioxidant, antimicrobial, anti-carcinogenic	Apple, strawberry, red raspberry, potato, tomato, carrot, cucumber	(Mattila & Kumpulainen, 2002; Ding et al., 2007); (Foti et al., 1996; Proestos et al., 2006)
Flavonoids	**Isoflavones:** Daidzein, malonyldaidzin, malonylgenistin, Daizein, genistein, glycitein, formononetin	Antioxidant, anti-carcinogenic, antidiabetic, antimicrobial, prevention of cardiovascular diseases	Legumes (soybean, bean, red clover flower	(Nakamura et al., 2001; Krenn et al., 2002; Luthria et al., 2007); (Mezei et al., 2003; Sarkar & Li, 2003; Yamamoto et al., 2003; Dasgupta et al., 2008; Choi et al., 2010)
	Flavonols: quercetin, kaempferol and myricetin, rutin	Antioxidant, anti-carcinogenic, anti-inflammatory, prevention of cardiovascular diseases	Vegetables (onion, tomato, broad bean, chives), fruits (apple, cranberry, bilberry), teas and soybean	(Sladkovský et al., 2001; Aherne & O'Brien, 2002; Erlund, 2004); (Ackland et al., 2005; Nöthlings et al., 2007)
	Flavanols: catechin, epicatechin, epicatechin gallate and epigallocatechin-3-gallate	Antioxidant activity, antimicrobial, antimutagenic, antitoxic, anticarcinogenic	Black and green teas, strawberry and cherry, bean and lentils	(Cabrera et al., 2003; Higdon & Frei, 2003; Peterson et al., 2005) (Kawase et al., 2000; Bais et al., 2002; Kim et al., 2003; Geetha et al., 2004; Muthuswamy & Rupasinghe, 2007)

Tannins	**Hydrolysable tannins:** gallotannins, elligatannins and complex tannins	Anti-diarrhea, adiuretic against stomach and duodenal tumours and anti-inflammatory		(Howell et al., 2005) (Hatano et al., 2002; Luximon-Ramma et al., 2002; Counet & Collin, 2003; Howell & Vorsa, 2003; Gonçalves et al., 2005; Wang et al., 2012a)
	Condensed tannins: Proanthocyanidins	Antioxidant, anti-diabetic enzymes, Anti-biological anti-bacterial, anti-inflammatory	Grape, apple, cranberry, lentils, chocolate and cacao	
	Anthocyanidins	Antioxidant, anti-diabetics and anti-carcinogenic	Fruits chokeberry, black raspberry and apple; and vegetables (red bean and cabbage)	(Suda et al., 2005; Wu et al., 2006; Koponen et al., 2007).

2.4.3. Carob phenolics

Phenolics can be found in various part of the carob tree. In carob pods, the total phenolic content accounts for 0.19 - 24.8 % the weight of the dried material (Kumazawa et al., 2002; Ouzounidou et al., 2012; Vekiari et al., 2012; Roseiro et al., 2013a). On average, each kg carob pod contains 4,142 mg extractable polyphenolic compounds, comprising more than 40 individual ones. The phenolic content of carob kibble is highly dependent on types of cultivar, region of origin and type of solvent used for extraction. Total phenolic content in carob pod harvested from the Greece ranged 100 - 248 mg/gdw, while 1.9 – 13.5 mg/gdw was found in Turkey (Kumazawa et al., 2002; Turhan, 2011; Vekiari et al., 2011; Ouzounidou et al., 2012; Vekiari et al., 2012). Carob trees growing in Portugal contained between 18 mg/gdw and 20.4 mg/gdw total phenolics in a pod (Custódio et al., 2011; Roseiro et al.,

2013b; Almanasrah et al., 2015; Custódio et al., 2015). Acetone (70%) was identified as the best solvent for extracting phenolic compounds in carob pod compared to water, ethanol, methanol and hexane (Avallone et al., 1997; Balaban, 2004; Papagiannopoulos et al., 2004; Roseiro et al., 2013a; Benchikh & Louailèche, 2014).

Carob leaf contains higher content of polyphenols which make up from 31 to 680 mg/gdw (Custódio et al., 2009; Hsouna et al., 2011; Custódio et al., 2015; Hsouna et al., 2015). Custodio et al. (2009) reported that the total leaf phenolic content ranged from 31.0 - 39.4 mg/gdw, depending on the gender of the carob trees. Hsouna et al. (2015) showed that carob leaf contained from 91 to 680 mg/gdw total polyphenols, of which, flavonoid content contributed 21 - 193 mg/gdw, followed by tannins (21.07 mg/gdw). In a similar way to carob pod, the phenolic content in the leaf varied greatly depending on the solvent used for extraction (Hsouna et al., 2015).

Eldahshan (2011) reported 8 major phenolic compounds extracted from Egyptian carob leaves, namely gallic acid, quercetin, afzelin, quercitrin, catechin, kaempferol, and isoquercetin. According to Corsi et al. (2002), the concentration of gallic acid and catechin in leaves reached 6.28mg/g. Leaf extracts of Moroccan carob contained from 0.45 to 2.64 g/L GAE (gallic acid equivalent), depending on extraction methods. El Hajaji et al. (2010) reported that ethyl acetate was the best solvent for polyphenol extraction from carob leaves, followed by ethyl ether and dicholoromethane. Phenolic content of leaves varies across cultivars and carob genders. Custódio et al. (2009) isolated phenolic compounds from 6 different carob cultivars and showed that the total phenolic content ranged from 16.4 - 39.4 mg/gdw. Similarly, flavonoid content ranged from 2.1 - 13.4 mg/gdw.

Carob seed contains the lowest amount of phenolics as compared to the pod, leaf, and wood. The total phenolic content in the seed is about 2.5 % of the dry weight (Sebai et al., 2013; Albertos et al., 2015).

Generally, the total phenolic content in carob wood was significantly lower than that in carob pod and leaf. The content ranged from 0.5 - 4.8 % the dry sample weight, differing from extract solvents, genders and geography (Balaban, 2004; El Hajaji et al., 2011; Custódio et al., 2013; Custódio et al., 2015). For example, methanol extract resulted in 15.4 mg/gdw from carob wood, while water extract resulted in only 4.8 mg/gdw (Balaban, 2004; Custódio et al., 2013). Hajaji et al., (2011) found 0.76 mg/mL total phenolic content in wood of spontaneous male trees, while only 0.54 mg/mL in that of spontaneous female trees. Unlike carob leaves, where flavonoids are more abundant, carob wood contains much lower content of flavonoid (Custódio et al., 2007; Sebai et al., 2013; Albertos et al., 2015). Tannins account for a major proportion of total phenolics in the carob wood (Balaban, 2004; Custódio et al., 2013; Custódio et al., 2015).

The major phenolic compounds in carob wood are condensed tannins. Balaban (2004) reported that heartwood of carob contained 50.4 mg total phenolics, 14.8 mg gallic acid and 10.27 mg proanthocyanidins per g dried matter. Meanwhile, the phenolic contents of sapwood were much lower (i.e. total phenols: 4.28 mg/gdw, gallic acid 1.7 mg/gdw: and proanthocyanidins: not determined) (Balaban, 2004).

Table 2.7 Phenolic contents in different carob parts compared to other plants

	Total content (mg/gdw)			
Source	Phenolics	Flavonoids	Tannins	References
Carob pod	190 - 248	0.1 – 3	0.1 - 95	(Kawase et al., 2000; Makris & Kefalas, 2004; Albertos et al., 2015; Custódio et al., 2015; Rached et al., 2016)
Carob leaf	31 - 680	6 – 193	0.3 - 24	(Custódio et al., 2009; Hsouna et al., 2011; Hsouna et al., 2015)
Carob seed	0.1 - 17	0.1 - 15	0.1 - 13	(Avallone et al., 1997; Sebai et al., 2013; Durazzo et al., 2014; Albertos et al., 2015)
Carob wood	5 - 48	0.1 - 0.5	1 - 10	(Balaban, 2004; El Hajaji et al., 2011; Custódio et al., 2013);
Soybean	1.06 - 6.69	0.32 - 3.43	0.88 - 7.31	(Xu & Chang, 2008b; Kumar et al., 2010)
Peas	1.2 - 2.5		0.03 - 1.71	(Amarowicz & Pegg, 2008; Han & Baik, 2008)
Beans	12.3 - 16.9 17.0 - 23.8		0.22 - 1.2	(Djordjevic et al., 2011; Luo et al., 2015);
Apple	0.62 - 3.28	1.0 - 1.2		(Bravo, 1998; Vrhovsek et al., 2004; Balasundram et al., 2006; Loncaric et al., 2014)
Grape	0.50 - 6.87		0.95 - 10.6	(Bravo, 1998; Balasundram et al., 2006; Liang et al., 2014);
Strawberry	0.38 - 2.90			(Bravo, 1998; Balasundram et al., 2006)
Wheat	16.2 - 50			(Bravo, 1998; Luo et al., 2015)
Cereals	18.2 – 52			
Oats	8.7 - 15.4			
Sorghum	0.1 - 1.7			

2.5 Fermentation

2.5.1. Definition

Fermentation may be defined as a metabolic process during which microorganisms such as bacteria, yeasts and fungi convert complex substrates into simple useful compounds (Subramaniyam & Vimala, 2012). Some good examples of the use of fermentation methods are the production of antibiotics (Saykhedkar & Singhal, 2004; Maragkoudakis et al., 2009) and enzymes (Pandey et al., 1999; Sandhya et al., 2005; Vintila et al., 2009; Souza, 2010).

There are several types of fermentation protocols that can be employed, depending on the specific product and substrate of interest. These include solid state fermentation (SSF), submerged state fermentation (SmF) and liquid fermentation (LF). SSF is a bioprocess using solid substrates with a low content of moisture (Pandey et al., 2000; Pandey, 2003). Moisture in the substrate is only needed to support growth and development of the host organisms to a small extent. The substrates used in the process tend to be agricultural crops and agro-industrial residues. The selection of suitable microorganisms is an important aspect of SSF. While bacteria require high moisture for their growth, fungi and yeast are used for SSF because of the low water requirement for their activities (Pandey, 2003; Hölker et al., 2004). The advantages of the method are its cost effectiveness and environmental-friendly because of the lower energy requirement, lower wastewater production and simple resolution of solid wastes (Durand & Blachere, 1997; Pandey, 2003). SSF usually requires a long treatment time, and the process is labour-intensive with a high risk of contamination, resulting in high production costs. In contrast,

SmF and LF require a liquid medium. SmF is the cultivation of microorganisms in solid submerged medium, into which desired products (e.g enzymes) are released (Jackson et al., 1997; Mascarin et al., 2015). SmF is best suited for yeast-like blastopores and bacteria that required high moisture content for their growth and are easily purified and harvested (Jackson et al., 2003; Subramaniyam & Vimala, 2012). In addition, SmF requires a short fermentation time from several hours or few days, and is able to be scaled up. LF is a process of substrate extract or media broth fermentation. The advantages of this liquid process are to produce pure products and time saving (Rodríguez-Sánchez et al., 2013). This approach, however, requires an initial process of extracting a liquid fraction from substrate and wastewater treatment which increases the cost of production.

2.5.2. Current application of fermentation to carob

Carob fermentations have been used in production of industrial products such as biofuel, acids, yogurt and sugars (Sánchez-Segado et al., 2012; Germec et al., 2015). Carob pod contains high level of fermentable sugars such as sucrose, glucose and fructose. Carob pod extract is used as a feedstock for *S. cerevisiae* and *Zymomonas mobilis* for production of ethanol (Turhan et al., 2010b; Vaheed et al., 2011; Mazaheri et al., 2012; Demirci, 2016). This technique has also been optimised and improvements of equipment made, enhancing productivity and reducing the cost of the final products (Turhan et al., 2010b; Germec et al., 2015; Rodrigues et al., 2016). In addition, SmF of carob kibble with *Actinobacillus succinogenes, Rhizopus oryzae* and *Aspergillus niger* have been studied to produce various acids such as succinic acid, lactic acid and citric acid, respectively (Carvalho et al., 2014, 2016; Sosa et al., 2016). While lactose in yoghurt is a nutrient it may also be responsible for some

ingestion and absorption-related disorders (Wilt et al., 2010). The addition of carob flour to yogurt leads to the production of a low lactose yogurt (Atasoy, 2009; Moreira et al., 2016). Carob pod is also utilised as a fermentable source of carbohydrates for producing other products such as sugars (e.g. fructose, dextran and mannitol) (Santos et al., 2005; Carvalheiro et al., 2011), pullulan (Roukas & Biliaderis, 1995) and protein (Smail et al., 1995).

Table 2.8. Current application of fermentation in carob

Source	Microorganism	Product	Reference
Carob pod extract	*Actinobacillus succinogenes* 130Z	Succinic acid	(Carvalho et al., 2014, 2016; Sosa et al., 2016)
	Saccharomyces cerevisiae, *Zymomonas mobilis*	Ethanol	(Turhan et al., 2010b; Vaheed et al., 2011; Mazaheri et al., 2012; Sánchez-Segado et al., 2012; Germec et al., 2015; Demirci, 2016)
	Rhizopus oryzae	Lactic acid	(Bulut et al., 2004; Turhan et al., 2010a)
	Aspergillus niger	Protein	(Smail et al., 1995)
		Citric acid	(Roukas, 1998a; Roukas, 1998b; Roukas, 1999; Pramod & Lingappa, 2008)
		Fructose; dextran	(Santos et al., 2005)
	Leuconostoc mesenteroides NRRL B51	Pullulan	(Roukas & Biliaderis, 1995)
Carob pod flour	*Kluyveromyces lactis*	Low lactose yogurt	(Atasoy, 2009; Moreira et al., 2016)
Carob syrup	*Lactic acid bacteria*	Mannitol	(Carvalheiro et al., 2011)

2.5.3. Advantages of fermentation in food processing and technology

Fermentation has been a common method to remove unwanted components such as soluble low molecular weight carbohydrates in food. *Zymomonas mobilis* consumes over 90 % of the initial amount of sugars (glucose, fructose and sucrose) in LF but

only 68 % of these sugars in SSF (Vaheed et al., 2011; Mazaheri et al., 2012; Saharkhiz et al., 2013). *Aspergillus niger* removed approximately 40 – 64 % of the initial sugars in carob pods during the ethanol production (Roukas, 1994, 1999; Lingappa et al., 2007). *S. cerevisiae* is a generally recognised as safe organism and has been widely used in food processing. The specificity of *S. cerevisiae* for removal of mono- and disaccharide by-products in carbohydrate preparations by fermentation has been studied. The yeast consumed common monosaccharides (D-glucose, D-fructose, D-mannose, and D-galactose), and disaccharides (maltose, sucrose, and turanose) completely. In addition, D-glucuronic acid, D-ribose, lactose and cellobiose were partially consumed, while it had no effect on melibiose (Yoon et al., 2003). A study reported that *S. cerevisiae* consumed from 90 to 95 % of the initial sugars in a liquid material preparation (Lima-Costa et al., 2012).

Fermentation can also be applied as a simple and economical approach for the production and improvement of bioactive compounds from agricultural by-products and agro-industrial residues (Oliveira et al., 2010; Schmidt & Furlong, 2012). There is evidence showing that fermentation can result in the alteration of polyphenol content and bioactivity of plants. Previous studies showed a significant increase in TPC in plant-derived food products, such as legumes, by microbial treatment (Dueñas et al., 2005; Đorđević et al., 2010; Juan & Chou, 2010; Xiao et al., 2014). Razak et al. (2015) reported that total phenolic content and antioxidant activity of rice bran significantly improved after fermentation. Juan et al. (2010) demonstrated that both the content of total phenolics and flavonoids, and the associated antioxidant activity of fermented black soybean were much higher than those in original samples. Diabetic enzyme inhibitory activity of plant-derived products were also

found to be improved after fermentation (Chen et al., 2007; Zhu et al., 2011; Wang et al., 2012b; Watawana et al., 2016). Chen et al., (2007) showed a significant increase in anti-α-glucosidase activity of aqueous extracts of soybean and soybean-based food after fungal treatments. Many plant phenolics showed inhibitory activity against diabetic enzymes such as α-glucosidase and α-amylase (Ranilla et al., 2010; Kumkrai et al., 2015; Zhang et al., 2015).

2.6 Response surface methodology

Response Surface Methodology (RSM) is a group of mathematical and statistical approaches entailing the fit of an equation to a data set (Bezerra et al., 2008). Box and Wilson initially developed this method in the early 1950's (Anderson & Whitcomb, 2000). The method has been used for analytical optimisation of the performance of a system or production process when there is interaction of multiple factors with respect to effects on interested outcome(s). Statistical models, resulting from this method, allows not only the identification of an optimal experiment condition, but also prediction of the optimal outcome(s).

There are several advantages of RSM compared to conventional optimisation methods. Traditional methods investigate the effect of one factor at a time, while keeping the other factors constant, with respect to the outcome response. This technique is also known as "one variable/factor at a time" (Bezerra et al., 2008). This method is not able to assess interactions among or combined effects of multiple variables on the outcome(s) of interest. Furthermore, traditional optimisation methods are time consuming and cost-ineffective because they require large number of experiments. In contrast, RSM reduces significantly the number of experimental

runs. More importantly, it allows assessment of interactions between or combined effects of multiple factors on responded outcome(s).

There are two commonly used RSM models. The linear function is the simplest one, and represents the relationship between the independent variables and outcome through a first-order polynomial function as indicated below:

$$y = \beta 0 \sum_{i=1}^{k} \beta i X i + \varepsilon$$

(Bezerra et al., 2008)

where y is the response of interest, x_i is the variable i, k is the number of variables, β_0 and β_i are the constant and the coefficients, respectively; and ε is the statistical error.

The other one is a higher degree polynomial function called a second-order model which is used when the response shows a curvature or when additional/interaction effect(s) occur among independent variables.

Basically, a procedure for RSM application includes 6 key steps:

1) Identification of factors (independent variables) and responses (dependent variables)

Variables to be included in an RSM model, such as pH, temperature and chemical concentration, are identified through review of literature and/or preliminary work or personal experience. Each variable/factor will include a range of values where each level will lead to an experimental run.

2) Selection of experimental design

An experimental design is defined as a specific set of experiments consisting of different combinations of the variables to obtain the response. The choice of a suitable design is crucial due to a large influence on the efficiency and accuracy and cost saving of the RSM.

3) Screening of independent variables

A screening study is necessary to reduce/remove the number of parameters that do not show major impacts on the responses on a RSM model (Bezerra et al., 2008). Factorial 2^k designs may be used in screening independent variables because of their efficiency and economical aspect.

4) Mathematical and statistical treatment

The data collected from each experiment point of the selected design is statistically analysed, which is required to fit a mathematical equation. The equation allows prediction of the behaviour of the responses with respect to the levels of the values studied.

5) Evaluation of the model's fitness

Model fitness was evaluated using ANOVA. The F and p values of 'Lack of fit' describe the quality of fitness. For example, a non-significance of 'Lack of fit' ($p > 0.05$) shows the selected model is fitted.

6) Confirmation of the optimal conditions

Normally, statistical analyses will result in an optimal RSM model which suggests a set of experimental conditions which allow an optimal outcome(s) to be achieved. However, this set of conditions can only be considered suggestion. In order to confirm whether or not the suggested parameters are optimal, it is necessary to conduct an experiment using parameters suggested by RSM method. If the outcome(s) is close to the RSM predicted response, the suggested parameters can be seen as optimal (Bezerra et al., 2008).

A central composite design (CCD) is the most commonly used design in RSM. The polynomial model describes the interaction among the all independent variables in the experiment. A two-level factorial (2^k) design, centre points and axial points are components that constitute CCD. The settings of CCD are the factorial 2^k maximum and minimum, a centre point and axial points.

The factorial (2^k) is given two levels at maximum and minimum points coded -1 and 1, presenting the low and high values of each independent variable.

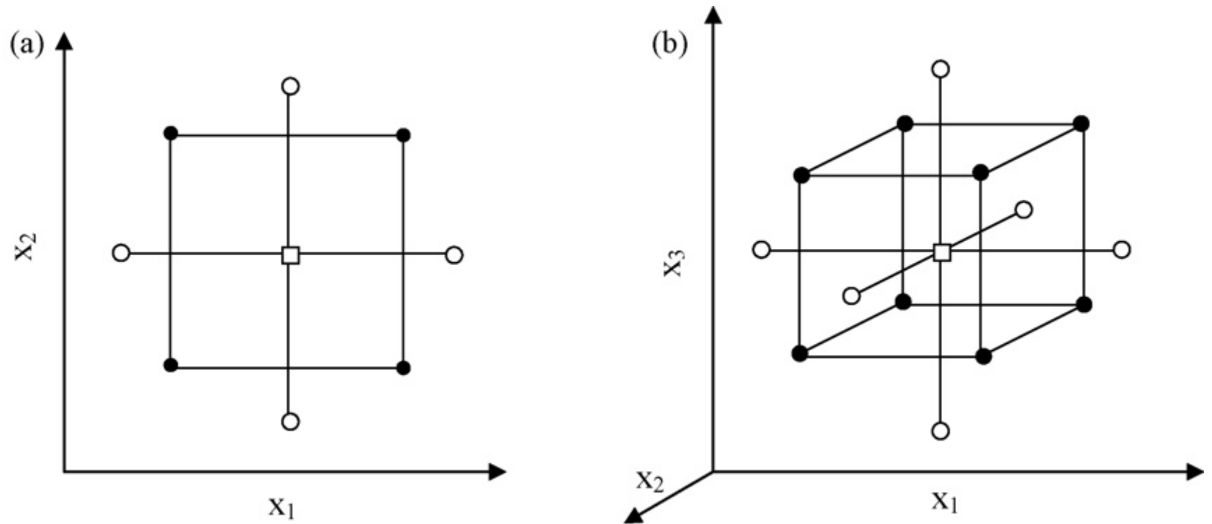
The centre point is where the supposed optimal conditions occur, which is the mean of the low and high values in the factorial. The model ensures accuracy because the centre point is replicated several times.

The axial points coded (-α, α) are middle values in centre of a formed cube. The values of α are calculated by the equation$\alpha = 2^{(k-p)/4}$, and highly depend on the number of variables (k) selected. For example, the number of variables are 2, 3 and 4, so the values of α are 1.41, 1.68 and 2, respectively. Therefore, each independent variable is examined at five levels: -α, -1, 0, 1, +α. The requirement of number of experiments is according to $N = k^2 + 2k + c_p$. where N is number of runs, k is number of independent variables, and c_p is number of replicate of the centre point.

The independent and dependent variables are fitted as the given equation containing quadratic term:

$$y = \beta 0 + \sum_{i=1}^{k} \beta ixi + \sum_{i=1}^{k} \beta iix^2 i + \sum_{1\le i\le j}^{k} \beta ijxixj + \varepsilon$$

Where β_{ii}; $\beta_{ij,}$ are coefficients of the interaction and quadratic respectively.



where: (a) k=2, (b) k=3

Figure 2.8 Examples of central composite design

Table 2.10 presents an optimisation of variables using CCD. The number of experimental combination of variables and the values of factorial, axial and centre points are coded. Clearly, CCD generated nine combinations in terms of two variables studied in which an equal division of 8 for factorial design and axial points (a). As one variable added, the number of the interacted groups of three in the factorial design is 8, while less than two groups are recorded in axial (b).

Table 2.9. The number of combinations of variables in CCD

(a)	X_1	X_2
Factorial design	-1	-1
	1	-1
	-1	1
	1	1
Axial points	-α	0
	α	0
	0	-α
	0	α
Centre point	0	0

(b)	X_1	X_2	X_3
Factorial design	-1	-1	-1
	1	-1	-1
	-1	1	-1
	1	1	-1
	-1	-1	1
	1	-1	1
	-1	1	1
	1	1	1
Axial points	-α	0	0
	α	0	0
	0	-α	0
	0	α	0
	0	0	-α
	0	0	α
Centre point	0	0	0

Notes: (a) two variables and (b) three variables

CCD allows an investigator to run a minimum number of required experiments, so is a time and cost-effective technique. Each of variables is examined at up to five levels and the centre point is repeated several times, so the model is accurate. CCD experiments can be carried out in blocks and run sequentially. CCD provides more comprehensive information on the effects of variables on the responses, in addition to overall errors.

2.7 Summary

Carob kibble is a large by-product source of carob seed production. This is also a rich source of D-pinitol, antioxidants and alpha α-glucosidase inhibitors. However, it has been mainly used for animal feed and bioethanol production. Fermentation is an effective approach to improve bioactive compounds in plant-based food. However, the application of this approach to enhance bioactive compounds in carob kibble remains limited. It is suggested that fermentation can improve health and commercial values of carob kibble.

Chapter 3 Development of a fermentation process, and establishment of the initial pH and time parameters for removing unwanted sugars and increasing D-pinitol content in carob kibble

Some of the materials in this chapter have been presented in the following conference:

Thi, HV, Vijay, J, Zhongxiang, F & Gary, D. (2016). Concentration of D-pinitol from carob kibble using submerged fermentation by Saccharomyces cerevisiae. *18th International Conference on Food Engineering*, Singapore, September 08-09, 2016, Oral presentation.

3.1 Introduction

Of the leading sources of plant D-pinitol, carob is the most cost-effective for D-pinitol production. On average, carob pod contains 38.7 - 60 mg/gdw D-pinitol (Yatmaz et al., 2012; Chafer & Berna, 2014; Turhan, 2014), while soybean, another rich source of D-pinitol, contains 13.0 - 30.4 mg/gdw. In other plants such as bean, orange and lemon the D-pinitol content is much lower (Streeter, 2001; Kim et al., 2005b; Chen et al., 2014).

D-pinitol has a number of health benefits, such as anti-inflammatory, anti-hyperlipidemic and anti-hyperglycemic effects (Bates et al., 2000; Geethan & Prince, 2008). Singh et al. (2001) reported an anti-inflammatory effect of D-pinitol extracted from *Abies pindrow* leaves on carrageenin-induced rats. This compound can also exhibit plasma glucose control activity in type II diabetic patients by increasing insulin sensitivity (Kim et al., 2004; Geethan & Prince, 2008). Kim et al. (2005b) compared the antihyperglycemic effect of carob and soy D-pinitol in an *in vivo* model. The authors found that D-pinitol from both plants had significant effects on diabetic rats, and that their antihyperglycemic effect was not different from one to another. The authors also demonstrated that carob D-pinitol has an identical chemical structure to soybean D-pinitol, which can explain their identical bioactivity. Experiments indicated that D-pinitol can decrease total cholesterol, low-density lipoprotein cholesterol, low-density lipoprotein cholesterol to high-density lipoprotein cholesterol ratio and blood pressure in rats (Kim et al., 2004; Geethan & Prince, 2008).

There have been a number of attempts to obtain and purify D-pinitol from carob pod. These methods focus on collecting D-pinitol from liquid extracts of carob materials. As mentioned previously, carob pod also contains a very high content of sugars (mainly sucrose, glucose and fructose), and there is a positive correlation between glucose and D-pinitol content in carob pod (Turhan, 2014). Both D-pinitol and low molecular weight sugars are highly soluble, and therefore, they are extracted together from carob kibble. Consequently, the purification of carob D-pinitol require greater cost. For example, partition chromatography with ion-exchange resins has been used to purify this compound (Camero & Merino, 2004). However, these methods not only produced low yields, but are also expensive (Camero & Merino, 2004; Turhan, 2011; Chafer & Berna, 2014). Furthermore, the co-extracted sugars may also prevent D-pinitol in the liquid extract of carob pod from fully exhibiting its bioactivity. The separation of carob D-pinitol from these interfering sugars is the main drawback for the production of D-pinitol from carob kibble (Tetik & Yüksel, 2014).

A fermentation approach is a promising solution for the extraction of carob D-pinitol thanks to the ability of some microorganisms to selectively remove unwanted sugars from carob materials. Rodríguez-Sánchez et al. (2013) demonstrated that monosaccharides, disaccharides and oligosaccharides in carob pod extracts could be significantly reduced by liquid fermentation with *Saccharomyces cerevisiae* (*S. cerevisiae*) without any negative effect on inositols, a group of derivatives of D-pinitol. This ability of *S. cerevisiae* was also effective in selective removal of unwanted carbohydrate in mulberry extracts while preserving the inositol content (Rodríguez-Sánchez et al., 2013). *Lactobacillus plantarum (L. plantarum),* a lactic acid bacterium, has also been commonly used in food fermentations (Schnürer &

Magnusson, 2005; Ammor et al., 2006). Recently, this bacteria has been reported to enhance both the antioxidant capacity and inhibitory enzyme activity in several plants, such as black olives, soybean and wheat (Dueñas et al., 2005; Pistarino et al., 2013).

To date, to the best of our knowledge, the only fermentation approach to obtain carob D-pinitol has been through the use of a liquid extract. No studies on purification of D-pinitol using a submerged fermentation (SmF) of carob kibble with *S. cerevisiae* have been reported. In addition, there have also been no publications which investigate the effect of lactic acid bacteria, in particular, *L. plantarum,* on the enrichment of D-pinitol in plant extract and its activity.

The aim of this study was to improve D-pinitol content and associated bioactivity in carob kibble by selectively removing sugars using submerged fermentation approach. The specific objectives included:

1) To evaluate the capabilities of *S. cerevisiae* and *L. plantarum* to selectively remove carob sugars, in order to select the best candidate for a submerged fermentation of carob kibble.
2) To evaluate the effect of initial pH and fermentation time on the contents of sugars removed and D-pinitol in carob kibble
3) To examine the antioxidant and anti α-glucosidase activity of the fermented carob extract.

3.2 Materials and methods

3.2.1 Carob kibble and preparation

Carob kibble was purchased from Australian Carobs Pty Ltd (South Australia, Australia). The kibble was ground into pieces using a grinder (Retsch grindomix GM 200, Haan, Germany) and passed through a 2-mm sieve. The ground kibble was stored at 4°C prior to experiments.

3.2.2 Microorganisms and activation

Saccharomyces cerevisiae type II purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) (1 g) was activated in 125 mL of a broth medium (yeast extract 10 g/L, peptone 10 g/L, glucose 10 g/L and sodium chloride 5 g/L, pH 6.2 ± 0.2). The inoculum was incubated in an orbital water bath shaker (OLS200 Grant, VWR international, Pty Ltd.) at 150 rpm and 30°C till reaching at 3 x 10^8 CFU (Colony-Forming Unit)/mL measured by the plate count method as described in 3.2.4.

Lactobacillus plantarum was purchased from the Department of Agriculture and Food (Western Australia, Australia). The bacterial culture was activated in MRS **(**Man, Rogosa, Sharpe**)** broth in a digital series incubator (Contherm Scientific Ltd, New Zealand) at 35°C till reaching at 5 x 10^9 CFU/mL as measured by the same method of count plate as mentioned in 3.2.4.

3.2.3 Submerged fermentation

To select the best candidate for removing sugars in carob kibble:

The fermentation was carried out in 500-mL Erlenmeyer flasks at original carob pH (5.0) and 35^0C over 70 h. A medium including carob kibble and water at 1:4 (w/v) was sterilised in an autoclave (Siltex Pty. Ltd, Australia) at 121°C for 15 min. After cooling to room temperature, the medium was separately inoculated with 10 % (v/v) *S. cerevisiae* or 10 % (v/v) *L. plantarum*, or mixture of 5 % (v/v) *S. cerevisiae* and 5 % (v/v) *L. plantarum* (Mohanty et al., 2009). These treatments were incubated at 35°C under shaking at 150 rpm for 70 h. All experiments were performed in triplicates. A sample was taken every 5 h to determine the microbial population, Brix and pH values. The samples were stored at -18 °C for later analysis.

To evaluate the effect of initial pH and time on the fermentation process with *S. cerevisiae*:

A similar fermentation process to that above was performed, but using a range of pHs from 5.0 – 7.0, at 30^0C over 50 h only. The pHs of the substrate at 5.0, 5.5, 6.0, 6.5 and 7.0 was adjusted using 1 M sodium hydroxide or 1 M hydrochloric acid. The medium was inoculated with 10 % (v/v) *S. cerevisiae,* then incubated at 30^0C under shaking condition at 150 rpm for 50 h. Samples consisting of solid substrate and liquid were withdrawn every 5 h after extensive mixing. The solutions of kibble alone and yeast alone were used as fermentation controls. The kibble incubated with medium after the removal of the yeast by centrifuging was used to examine production of enzymes during the activation period. All samples were stored at -18^0C for later determination of D-pinitol content, total carbohydrate content antioxidant and anti α-glucosidase activity.

3.2.4 Microbiological counts, Brix and pH analysis

The viable cell counts were determined using the plate count method described by Jackson et al. (2000). Briefly, a series of dilutions was made by adding 1 mL of the fermented solution into a test tube of 9 mL sterile physiological saline (0.85 %, w/v). *S. cerevisiae* was counted on YEP (yeast extract peptone) agar adjusted at pH 6.2 ± 0.2, and then incubated in a digital series incubator (Contherm Scientific Ltd, New Zealand) at 30°C for 3 days. *L. plantarum,* on the other hand, was counted on MRS agar adjusted at pH 6.2 ± 0.2, incubated at 35°C in the incubator for 3 - 5 days. Viable colonies were expressed as CFU/mL. Changes in pH of the carob solutions were measured at room temperature using a pH meter (Thermo Fisher Scientific, Germany). The Brix value was tested using a digital refractometer (Kruss, Germany).

3.2.5 Preparation of D-pinitol extracts

Extraction of D-pinitol was achieved using the ultrasonic method described by Nedim Tetik (2014) with some modifications. The carob sample was diluted with water (1:1, w/v) in 50 mL capped test tubes, was then homogenized at no.2 speed for 60 s using a digital homogenizer (Ultra-Turrax T25, Germany). The mixture was placed into an ultrasonic water bath (DSA100-GL2, China) at 65°C with an ultrasonic power of 110 W for 1 h, then centrifuged at 7,000 rpm (5810 R, Eppendorf, Hamburg, Germany) for 15 min. This procedure was repeated three times for each sample. The combined supernatant was diluted with distilled water and then filtered through 0.45 μm membrane before levels of various components were determined.

3.2.6 Determination of D-pinitol and sugars

Levels of D-pinitol, glucose, fructose and sucrose were determined using high performance liquid chromatography (HPLC) (Turhan, 2011, 2014). An RSpak DC-613 column (6.0 id x 150 mm, Shodex, Singapore) was equipped with a guard column (RSpakDC-G 4.6 id – 10 mm, Shodex, Singapore) and D-pinitol and individual sugars were detected by an evaporative light scattering detector (ELSD). Ultra-pure water and acetonitrile (30: 70, v/v) was used as the mobile phase and allowed to flow at a rate of 1.4 mL/min. The column was heated to 70°C and a sample volume of 20 µL was injected into the chromatography system using an autosampler (Shimadzu, SIL-20A, Kyoto, Japan). Concentrations of D-pinitol, glucose, fructose and sucrose were calculated using standard calibrations obtained from injection of standard solutions of D-pinitol, glucose, fructose and sucrose prepared in distilled water at concentrations from 25 - 200 µg/mL. Each sample was analysed in duplicate.

3.2.7 Determination of total carbohydrates

Total carbohydrates were analysed by the phenol-sulphuric acid method (Nielsen, 2003) with minor modification. The extract (1.5 mL) had 1 mL 2% (v/v) phenol solution added to it, and subsequently, addition of 2.5 ml sulphuric acid (98%) before leaving to stand at room temperature for 25 min in the dark. The absorbance was measured at 490 nm. Total carbohydrate content was calculated using a standard curve for glucose at a concentration range of 50 - 200 µg/mL. Each sample was analysed in duplicate.

3.2.8 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Radical scavenging activity of the carob extracts was determined as described by Gungor et al. (2013) with some modification. The extracts, 15 h fermented and un-fermented samples were used at the same concentration of 250 μg/mL. Ascorbic acid (AC) and butylated hydroxyanisole (BHA) were used at 40 μg/mL, while the D-pinitol standard was used at a range of 160 – 1,000 μg/mL. A volume of 20 μL sample was added to DPPH (280 μL at 60 μM in methanol 98 %). The mixtures were incubated in the dark at room temperature for 30 min. Absorbance was read at 517 nm using a plate reader (BioTek, USA). The same volume of water replaced the sample as a control, while methanol 98 % instead of DPPH was used to replace the sample as a control. Free radical scavenging activity was expressed as the percentage of inhibition based on equation 3.1:

$$\% \text{ Inhibition} = [(Ac-Ae)/Ac] \times 100 \qquad (3.1)$$

Ac and Ae are the absorbance values of the control and sample, respectively.

3.2.9 *In vitro* α-glucosidase inhibitory assay

The D-pinitol extract was assessed for α-glucosidase inhibitory activity using the procedure of Kazeem, Ogunbiyi and Ashafa (2013). The assay was carried out by using a plate reader (BioTek, USA). The extracts, 15 h fermented and un-fermented samples were used at 50 μg/mL. D-pinitol and acarbose (Acb) standards were used at a similar range of concentration as above (40 - 500) for D-pinitol and at two concentrations of 50 and 100 μg/mL for Acb. A mixture of the sample (50 μL) and 100 μL α-glucosidase solution (0.25 unit/mL) was incubated at 25 °C for 10 min.

The substrate solution (50 μL) of 25 mM p-nitrophenylglucopyranoside (pNPG) in 20 mM phosphate buffer (pH 6.9) was added to them. The reaction mixture of enzyme and substrate was incubated at 25 °C for 5 min before being stopped by adding 200 μL of 0.1 M Na_2CO_3. The absorbance was measured at 405 nm. The buffer was used in place of the extract as control in the same procedure. The percentage of inhibition was calculated using equation 3.1 given.

3.2.10 Statistical analysis

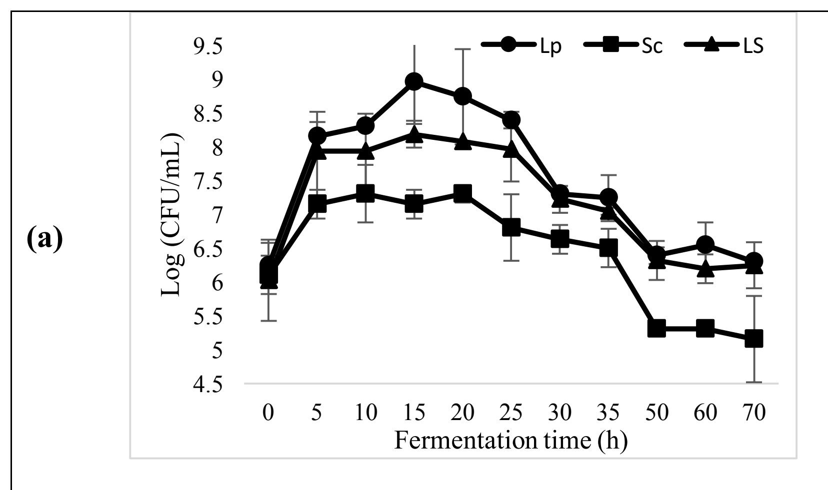
Data was reported as the average of three different individual experiments ± SD (standard deviation). Analysis of variance was used to compare the means among different pH-treated groups, and among different points of time of fermentation. A significance level of $p < 0.05$ was used. Microsoft Office Excel 2010 (Microsoft, Washington) was used for all data analysis.

3.3 Results and discussion

3.3.1 Microbial density, Brix and pH

The microbiological counts, Brix and pH in the submerged fermentations with *S. cerevisiae* (Sc) or *L. plantarum* (Lp) or *S. cerevisiae* and *L. plantarum* (LS) were examined (Fig. 3.1). As expected, bacteria grew more quickly than yeast. The highest viable cell counts of Sc (2×10^7 CFU/mL) was reached at 20 h, while Lp and LS reached 1×10^9 and 1×10^8 CFU/mL at 15 h. As the period of fermentation increased the microbial population decreased, a trend noted in all three treatments. The values of pH were closely corresponding to the growth performance of the strains. A decline in pH was more noticeable during the period of microbial population increase up to 15 h and then slowed down later during the fermentation,

particularly in the treatment with Lp. This could be due to the accumulation of lactic acid, produced by Lp during the fermentation (Fu & Mathews, 1999). Brix degree is a unit used in the direct measurement of the concentration of water-soluble substrates (Ball, 2006). The Brix value of the kibble solution (0 h) was ~ 14 %, recorded in all three treatments. This level significantly decreased to 8.0 % in both Sc and LS at 15 h and 20 h, respectively, then remained constant until at the end of the process. In contrast, there was no significant difference in the brix values observed in the Lp treatment over the 70-h process. It could be because Lp did not contribute to a decrease in dissolved materials, such as sugars, over the fermentation time. In addition, the total carbohydrate content of kibble at 0 h and at 70 h were not significantly different ($p > 0.5$, data not shown). For these reasons, Sc was selected to study the effect of pH and fermentation time on content of D-pinitol and related bioactivity in carob kibble.



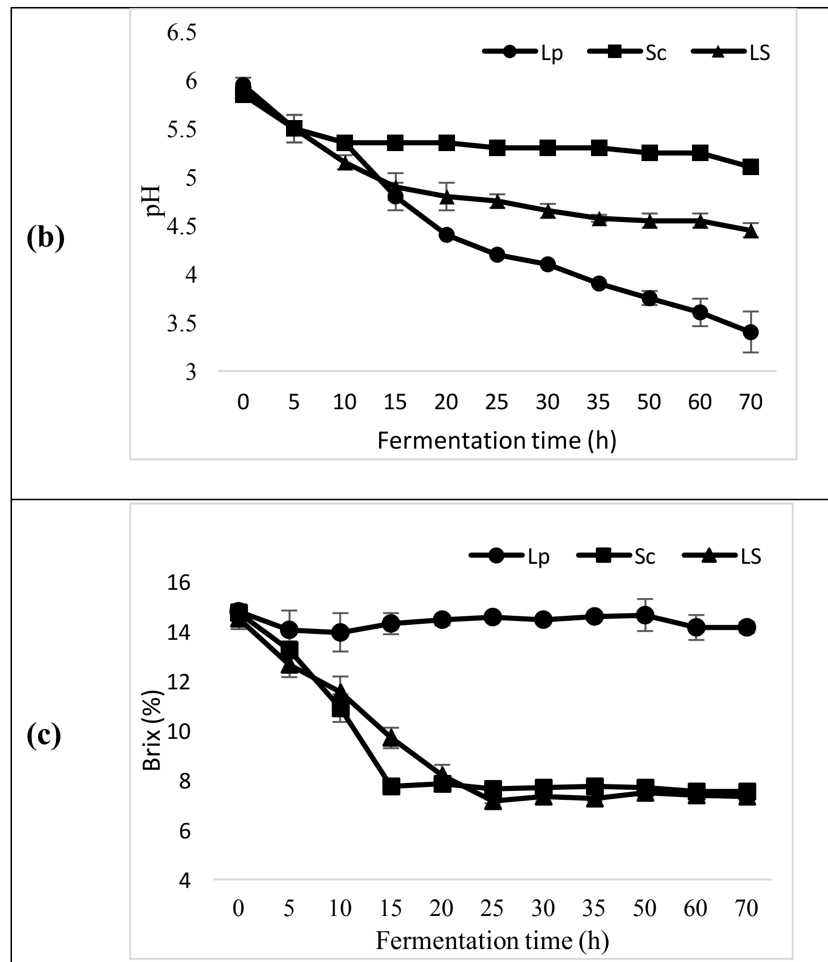


Figure 3.1 Cell density, pH and Brix values of carob solutions during the fermentation

Carob kibble fermented with *Saccharomyces cerevisiae* (Sc), *Lactobacillus plantarum* (Lp) and a combination of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (LS). *(a)* cell density, *(b)* pH and *(c)* Brix. Each value is expressed as mean with standard deviation of triplicates. *CFU:* Colony Forming Unit.

3.3.2 Total carbohydrate content

Table 3.1 shows the changes in the content of total carbohydrates in carob kibble during the fermentation at different initial medium pHs. The unfermented carob

kibble in the present study contained approximately 698 mg/gdw of total carbohydrates which is a similar amount (620 mg/g) to that previously reported by Turhan (2014). The total carbohydrates were reduced by nearly 70 % in the first 15 h of fermentation but there was no further significant reduction after 20 h. The consumption of total carbohydrates by *S. cerevisiae* was lower than that reported (90 - 95 %) in the production of citric acid from liquid fermentation of carob extract using *Zymomonas mobilis* (Hossein et al., 2011; Mazaheri et al., 2012). It was, however, higher than that reported (60 – 65 %) for the production of ethanol from solid carob pod substrate fermented with *Asperilus niger* (Roukas, 1999). The amount of carbohydrate consumed is highly dependent on the types of microorganism and fermentation conditions. Of the three main fermentation approaches (solid state fermentation, submerged fermentation and liquid extract fermentation) for carob, liquid extract fermentation produces the highest concentration of D-pinitol with respect to removal of unwanted sugar (Rodríguez-Sánchez et al., 2013). This approach, however, requires an initial process of extracting a liquid fraction from carob pods that increases the cost of production. Solid state fermentations, on the other hand, allow the retention of a high level of D-pinitol in the final product without using an extra extraction process. A disadvantage of existing solid-state fermentation is that only low levels of carbohydrate content are removed. For example only 60 – 65 % of the carbohydrate content of carob kibble was removed by *Asperilus niger* (Roukas, 1999; K Lingappa, 2007). In the present work, *S. cerevisiae* only consumed 45 – 50 % total carbohydrates in carob kibble after 7 days of solid state fermentation with 60 – 80 % moisture (data not shown). Comparatively, the fermentation appears to be an effective option for production of D-pinitol, since it can remove up to 70 % of total carbohydrates in the final product

while retaining a high level of D-pinitol (Table 3.1). This study suggested that 15 h of fermentation is the optimal time for *S. cerevisiae* to remove the highest amount of carbohydrate in carob kibble.

The effect of initial pHs (5.0 – 7.0) on the removal of total carbohydrate content in carob kibble was examined. The results of our study showed that the substrate pHs (5.0 – 7.0) had no significant effect on the reduction in total carbohydrate content during the fermentation. At the same fermentation time (15 h), approximately 70 % of the carbohydrate was removed. This occurred at all pHs tested (Table 3.1). The finding was supported by other studies (Mohanty et al., 2009; Saharkhiz et al., 2013).

Table 3.1 Total carbohydrate content in carob kibble fermented with *S. cerevisiae* at initial pHs of 5.0 (original carob), 5.5, 6.0, 6.5 and 7.0 over 50 h

	Total carbohydrates (mg/gdw)					
	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	Overall[†]
Un-fermented [a]	655 ± 43	717 ± 80	680 ± 76	752 ± 55	685 ± 48	698 ± 37
Fermented (h) [b]						
0	677 ± 45	704 ± 59	659 ± 41	702 ± 10	669 ± 24	682 ± 20
5	580 ± 83	598 ± 69	629 ± 106	644 ± 48	593 ± 69	609 ± 27
10	553 ± 46	505 ± 48	576 ± 131	527 ± 88	552 ± 78	543 ± 27
15	241 ± 22	296 ± 65	322 ± 79	311 ± 40	294 ± 66	293 ± 31
20	234 ± 19	215 ± 26	239 ± 76	287 ± 18	244 ± 59	244 ± 27
25	229 ± 9	226 ± 25	264 ± 14	231 ± 16	226 ± 28	235 ± 16
30	220 ± 16	208 ± 22	213 ± 60	227 ± 12	216 ± 29	217 ± 70
50	208 ± 7	209 ± 11	233 ± 17	221 ± 8	216 ± 17	217 ± 10

Each value is presented as mean ± SD (standard derivation) of three replicates. Values in same row were not significantly different ($p > 0.05$). In each column, [b] values gradually and significantly decrease (p for trend < 0.05), from 0 h to 50 h, and is significantly different from [a] value ($p < 0.05$). [†] Mean ± SD of total carbohydrate content at pH 5.0, 5.5, 6.0, 6.5, and 7.0. gdw; gram dry weight.

3.3.2 D-pinitol content

D-pinitol is a dominant alcohol sugar in plants of the legume family. In this study, the carob kibble contained 42.9 mg/gdw of D-pinitol (Table 3.2), which is higher than that in other legume products such as dried soy (20 mg/g) and soybean (5 mg/g), and similar to that in other sources of carob pods (42.6 mg/g) (Yatmaz et al., 2012; Turhan, 2014). Turhan (2014) reported that carob pods could contain a range of D-pinitol concentrations from 42.6 to 84.6 mg/g of D-pinitol depending on the type (cultivar) and geographic origin of carobs.

Figure 3.2 shows the change in content of D-pinitol (peak1), sucrose (peak 2) and glucose + fructose (peak 3) during the fermentation. As showed in the Figure 3.2(a), the three peaks did not change in size between 0 and 50 h because the sample contained carob kibble only, meaning there was no change in their contents. In contrast, when treated with *S. cerevisiae,* sucrose (peak 2) and glucose + fructose (peak 3) were catalyzed and consumed by the yeast during the fermentation. Meanwhile, there was slight change in the size of peak 1. Specifically, the size of peak 1 slightly increased between 0 and 5 h then slightly decreased until 15 h, then remained unchanged to 50 h of fermentation (Figure 3.2b). The temporary increase in the size of the peak 1 could be due to a monosaccharide which was produced

during the first 5 hours of fermentation, and has similar molecular weight to D-pinitol. That monosaccharide was then consumed by *S. cerevisiae* between 5 and 15 h, which explained the size of the peak 1 remained unchanged after 15 h of fermentation. This explanation is supported by the Figure 3.2(a and b) which shows a stable content of D-pinitol, sucrose, and glucose + fructose when the samples were untreated or treated with activated medium only (yeast removed).

The content of D-pinitol significantly increased from 42.9 to approximately 71.8 mg (67.4 % of increase) by dry weight after the fermentation (Table 3.2). Importantly, the content of D-pinitol was stable for 50 h of fermentation, suggesting that *S. cerevisiae* did not consume the D-pinitol. This finding has been supported by other studies (Yatmaz et al., 2012; Rodríguez-Sánchez et al., 2013).

The substrate pHs (5.0 – 7.0) also showed no negative effect on D-pinitol content during the fermentation. Overall, this study suggested that *S. cerevisiae* could be used under a relatively wide range of pH conditions (pH 5.0 - 7.0) to ferment carob kibble for the purpose of D-pinitol increase. The original pH of the carob kibble (pH 5.0) was used for other parts of this study.

Table 3.2 D-pinitol content in carob kibble fermented with *S. cerevisiae* at initial pHs of 5.0 (original carob), 5.5, 6.0, 6.5 and 7.0 over 50 h

	D-pinitol content (mg/gdw)					
	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	Overall†
Un-fermented [a]	43.1 ± 1.7	41.8 ± 0.7	43.1 ±2.2	43.4 ±1.5	43.0 ± 1.8	42.9 ± 0.6
Fermented (h) [b]						
0	70.1 ± 6.1	68.3 ± 8.1	67.8 ± 2.9	78.1 ± 5.3	69.1 ± 3.8	70.7 ± 4.2
5	74.5 ± 3.9	71.9 ± 7.2	78.7 ± 5.3	76.3 ± 8.4	73.5 ± 4.2	75 ± 2.6
10	78.1 ± 5.5	71.5 ± 7.0	80.9 ± 6.4	75.9 ± 1.4	72.8 ± 8.5	75.8 ± 3.8
15	73.6 ± 2.6	70.9 ± 2.3	69.0 ± 4.7	73.6 ± 2.8	71.8 ± 4.2	71.8 ± 1.9
20	70.8 ± 1.2	68.3 ± 2.2	71.0 ± 3.3	68.1 ± 4.1	69.0 ± 3.1	69.4 ± 1.4
25	71.3 ± 2.5	68.8 ± 2.9	70.6 ± 3.2	70.2 ± 1.9	71.9 ± 2.0	70.6 ± 1.2
30	71.1 ± 3.1	70.1 ± 2.2	69.9 ± 1.8	68.3 ± 1.6	71.9 ± 1.4	70.3 ± 1.4
50	69.6 ± 2.2	70.0 ± 0.7	70.5 ± 2.9	69.5 ± 1.8	70.4 ± 1.8	70 ± 0.50

Each value is presented as mean ± SD (standard deviation) of three experiments. Values in the same row are not significantly different ($p > 0.05$). In each column, [b] values, from 0 h to 50 h, are not statistically different ($p > 0.05$), but significantly different from [a] value ($p < 0.05$). †Mean ± SD of D-pinitol content at pH 5.0, 5.5, 6.0, 6.5 and 7.0. gdw: gram dry weight.

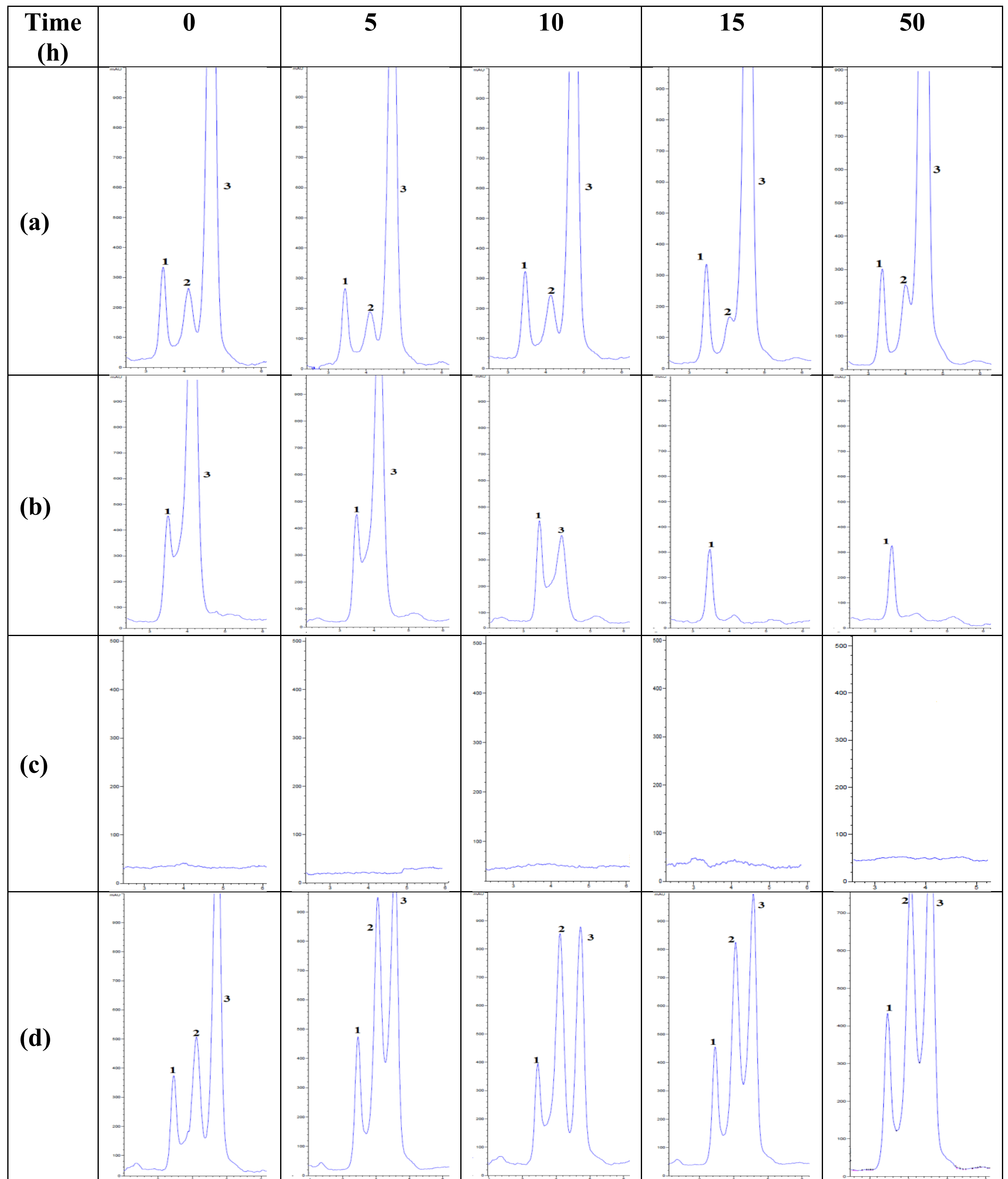


Figure 3.2 HPLC chromatogram of carob kibble fermented at pH 5.0

(a): carob kibble alone, *(b)*: carob kibble and yeast, *(c):* yeast alone, and *(d):* carob kibble and activated medium after removing yeast, *1, 2* and *3*: peaks of D-pinitol, glucose and fructose, and sucrose respectively.

3.3.3 Sucrose, fructose and glucose contents

The sucrose, fructose and glucose contents of the fermented carob kibble are shown in Figure 3.3. Initially carob kibble had 374 mg/gdw sucrose and 117 mg/gdw glucose and fructose, and these were totally removed after 15 hours of fermentation. The sucrose content in the carob was reduced by 90% after 5 hours of fermentation, while the glucose and fructose content were substantially increased. This could be due to the hydrolysis of sucrose into glucose and fructose by the action of the extracellular enzyme (β-fructosidase) produced by *S. cerevisiae* (Sainz-Polo et al., 2013). While sucrose was eliminated after 10 h of the fermentation, the complete removal of glucose and fructose was observed after 15 h. This behaviour was also previously observed by other authors (Rodríguez-Sánchez et al., 2013) during the yeast fermentation of legume extracts.

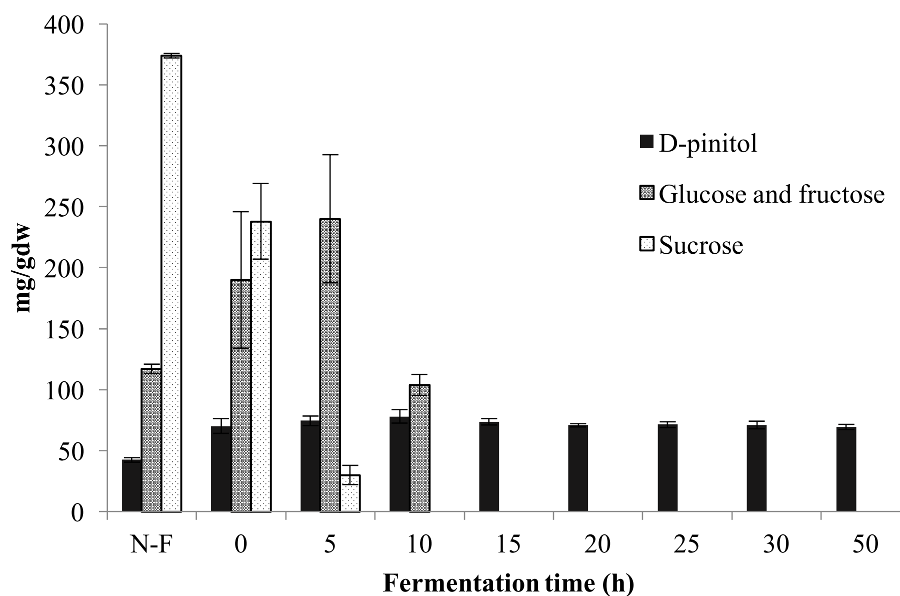


Figure 3.3 D-pinitol and sugar contents in carob kibble fermented at pH 5.0

Each value is expressed as mean ± standard deviation (SD) of three experiments. gdw: gram dry weight. *N-F:* non-fermentation.

3.3.4 *In vitro* bioactivity of D-pinitol extract

Bioactivities of D-pinitol extract and pure D-pinitol, including α-glucosidase and antioxidant effects were examined in present study. A range of pure D-pinitol concentrations (160 - 1,000 μg/mL for the antioxidant assay and 40 – 500 μg/mL for the enzyme assay) was used. The results showed that pure D-pinitol did not have any activities of antioxidant and inhibitory enzyme, although its contents were concentrated. The results showed that pure D-pinitol did not show any DPPH radical scavenging activity or inhibition of α-glucosidase enzyme, although the concentrations used were high.

Antioxidant activity of D-pinitol established by DPPH radical scavenging, superoxide scavenging, nitric oxide scavenging, and ferric reducing power has been reported (Asuzu & Nwaehujor, 2013; Rengarajan et al., 2014b). These authors demonstrated that D-pinitol (standard grade) is not only a DPPH radical scavenger at IC_{50} 290 μg/mL, but also a significant inhibitor of superoxide and nitric oxide radicals at the concentrations of 360 and 390 μg/mL, respectively. These findings were not, however, supported by the results in the present study. No antioxidant activity for pure D-pinitol was observed, even when the level of D-pinitol was increased to 1,000 μg/mL (Figure 3.4). However, 7.9 and 13% inhibition of DPPH radical scavenging was observed in the carob extracts both before and after fermentation, respectively. Water soluble phenolics present in carob kibble, such as gallic acids, may be involved in this effect. A study by Sivakumar and Subramanian (2009) found that plant D-pinitol possessed an antioxidant activity *in vivo*, specifically soybean D-pinitol is capable of protecting pancreatic tissue from free radical-mediated oxidative stress in streptozotocin-induced diabetic rats.

Anti-hyperglycemia activity of D-pinitol standard and D-pinitol extracted from plants, such as soybean, have been reported for *in vivo* models (Bates, Jones, & Bailey, 2000; Geethan & Prince, 2008) but no study has determined their activity *in vitro*. The present study showed D-pinitol grade has no α -glucosidase inhibitory activity outside living organism (Figure 3.4). Interestingly, a similar α-glucosidase inhibition by 90 % was recorded for the unfermented and fermented extracts. However, Acb only inhibited α-glucosidase by 29.6 % at the same concentration of 50 µg/mL, which slightly increased to 37 % at the double concentration (100 µg/mL). This suggested that carob kibble contains one or more water-soluble compounds (not D-pinitol) that produce α-glucosidase inhibitory activity in the *in vitro* model. These compounds were not affected by the fermentation. In addition, previous studies reported phenolic compounds such as flavonoids and gallic acids have enzyme inhibition activity (Christhudas et al., 2013; Xiao et al., 2013; Zhang et al., 2015). Further study should therefore be done to identify the unknown inhibitors in carob kibble.

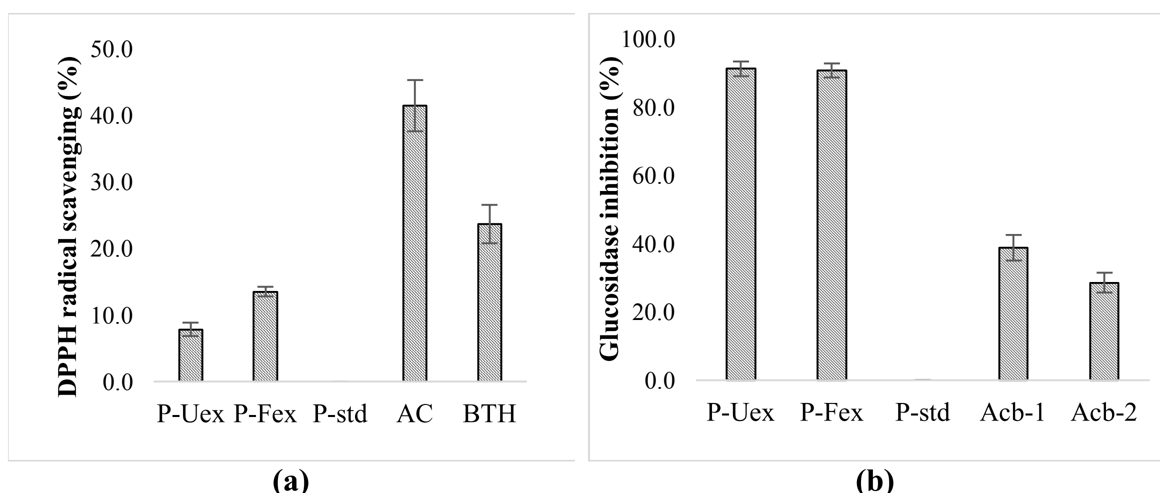


Figure 3.4 Bioactivity of the carob kibble extracts vs authentic compounds.

(a) Antioxidant activity and *(b)* α-glucosidase inhibition activity; P-Uex: the unfermented extract, P-Fex: the 15 h fermented extract, P-std: D-pinitol standard, Acb-1: acarbose at 100 µg/mL and Acb-2: acarbose at 50 µg/mL, AC: ascorbic acid, BTH: butylated hydroxyanisole. The value is expressed as mean ± standard deviation (SD) of three replicates.

3.4 Conclusion

This work showed that *S. cerevisiae* was the most suitable of the microorganism tested to concentrate D-pinitol and selectively remove the interfering carbohydrates. There was up to 70 % total carbohydrates removed, largely glucose, fructose and sucrose. The results also showed that 15 h of fermentation was sufficient to achieve a maximum D-pinitol content. The study also established that a range of pHs from 5.0 to 7.0 had no significant effect on removal of total carbohydrates and retaining D-pinitol content during SmF with *S. cerevisiae*. Finally, D-pinitol has no α-glucosidase inhibition and antioxidant activity in *in vitro* models. This approach has the potential to be used for production of D-pinitol and other bioactive components from plant sources with high concentrations of carbohydrates.

Chapter 4 Development of a fermentation process for increasing the total phenolic content and associated bioactivity in carob kibble

Some works in this chapter have been presented in the following conference:

Thi. HV, Vijay, J, Zhongxiang, F & Gary, D. (2016). Evaluation of the capabilities of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* in improvement of total phenolic content and antioxidant activity in carob kibble. *18th International Conference on Food Science Research, Technology and Innovation*, Sydney, Australia, Oral presentation.

4.1 Introduction

Carob kibble is known to contain high levels of phenolic compounds (Kumazawa et al., 2002; Roseiro et al., 2013a). The major phenolic groups found in carob kibble include phenolic acids, flavonol-glycosides, hydrolysable and condensed tannins (Papagiannopoulos et al., 2004). Their contribution to the total phenolic content can vary depending on countries of origin and cultivars of the carob tree. Among the most common carob phenolics, gallic acid, catechin, epicatechin, myricetin rhamnoside, eriodictyol glycoside, quercetin glycoside and quercetin rhamnoside are the major ones (Avallone et al., 1997; Corsi et al., 2002; Papagiannopoulos et al., 2004). For instance, Ayaz et al. (2007) reported that the Turkish carob kibble contains about 3.3 mg/gdw gallic acid, and was the most abundant phenolic compound.

As previously studied, carob phenolics possess strong antioxidant property. Kumazawa et al. (2002) reported that carob phenolic extract exhibited a stronger ability to scavenge DPPH free radicals than did pure compounds, such as epicatechin, epicatechin gallate and procyanidin B2. Custódio et al. (2015) reported the ability of carob phenolic extract to significantly inhibit α-glucosidase and α-amylase in *in vitro* models. Although carob kibble is a rich source of bioactive compounds, it is used mainly for animal feed and bioethanol production. This could be explained by the high content of sugars, and tannins which are anti-nutrient factors in the carob kibble (Custódio et al., 2015). To date, there is limited information about the exploitation of the bioactive properties of carob kibble.

There is evidence showing that fermentation can not only remove unwanted sugars but also, more interestingly, alter the polyphenolic composition of plant substrates and potentially their bioactivity. The consumption of sugars by fermenting microorganisms is detailed and discussed in the introduction Section, Chapter 3. In addition, Razak et al. (2015) reported that total phenolic content and antioxidant activity of rice bran significantly increased after fermentation. Juan et al. (2010) demonstrated that both the content of total phenolics and flavonoids, and the antioxidant activity of fermented black soybean were much higher than in the original black soybean. Similarly, alteration in enzyme inhibition (e.g. anti-α-glucosidase activity) of plant-based products was also reported (Chen et al., 2007; Zhu et al., 2011; Wang et al., 2012b; Watawana et al., 2016). To date, no information on the effect of fermentation on carob total phenolics and their activities has been reported.

Saccharomyces cerevisiae (*S. cerevisiae*) and *Lactobacillus plantarum* (*L. plantarum*) are food grade microorganisms. *S. cerevisiae* is able to remove sugars and improve the content and properties of polyphenols in several other plants (Đorđević et al., 2010; Rashad et al., 2013), but its impact on carob phenolic compounds remains unknown. Although the removal of water soluble components including the main sugars by *L. plantarum* is less effective than *S. cerevisiae*, this bacterial species is able to enhance content of bioactive polyphenols in some plants such as cowpea (Xiao et al., 2015). *L. plantarum* significantly improved both antioxidant capacity and inhibitory enzyme activity in several plants such as black olives, soybean, wheat and purple artichoke (Dueñas et al., 2005; Pistarino et al., 2013; Wang et al., 2016). Moreover, tanninase produced by *L. plantarum* is believed

to be capable of hydrolysing tannin into simple and active compounds (Nishitani & Osawa, 2003; Nishitani et al., 2004; Vaquero et al., 2004). Tannin is both abundant and an anti-nutrient in carob kibble. There is no existing practical method to reduce this compound thereby improving other bioactive compounds. If *L. plantarum* tanninase is effective on carob tannin, this could represent a potential approach for making use of carob kibble as a source of polyphenolics rather than as by-product. The aim of the work was to select a candidate for a submerged fermentation of carob kibble in order to increase the phenolic content and bioactivity. The specific objective was to evaluate the capability of *S. cerevisiae* and *L. plantarum* to improve the content of phenolic compounds, and their antioxidant and anti α-glucosidase capacity, in carob kibble.

4.2 Materials and methods

4.2.1 Preparation of carob kibble

Carob kibble was prepared as described in Section 3.2.1, Chapter 3.

4.2.2 Microorganisms

Activation cultures and medium preparation were described as in Section 3.2.2, Chapter 3.

4.2.3 Submerged fermentation

The fermentation was carried out as described in Section 3.2.3, Chapter 3. Samples taken were analysed for total phenolic content, antioxidant and anti α-glucosidase activity.

4.2.4 Preparation of phenolic extracts

Extraction of phenolics from carob kibble followed the method of Benchikh and Louaileche (2014) with slight modification. A mixture of the sample and acetone (70 %) or water (1:5 w/v) was homogenized at speed 2 for 60 s using a digital homogenizer (Ultra-Turrax T25, Germany). The mixture was placed in a water bath (OLS200 Grant, VWR international, Pty Ltd.) at 70^0C for 90 min and vortexed at 30 min interval. The supernatant was collected using a centrifuge at 6,000 rpm (5810 R, Eppendorf, Hamburg, Germany) for 12 min. The procedure was repeated three times. The combination of supernatant was kept at 4^0C for next analysis.

4.2.5 Total phenolic content

Total phenolic content (TPC) was determined by the Folin-Ciocalteu colorimetric method according to Roseiro et al. (2013a). The acetone and water extracts (500 μL) were mixed with Folin-Ciocalteu reagent 0.2N (2.5 mL) and sodium carbonate 7.5 % (2 mL). The mixture was allowed to stand in the dark, at room temperature for 40 min. Absorbance was measured at 765 nm in a spectrophotometer (UV-1800 UV-VIS, Shimadzu, Japan). TPC was expressed as mg gallic acid equivalents per 1g dry weight (mg GA/gdw). Acetone (70 %) and water, instead of the sample extracts, were used as blanks. Each sample was tested in duplicate.

4.2.6 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity of the solvent and water extracts were determined using DPPH assay as described in Section 3.2.8.

4.2.7 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging activity

Antioxidant activity of the solvent and water extracts were also examined using ABTS assay (Thaipong et al., 2006). The stock solutions of 5 mM potassium persulfate and 15 mM ABTS were prepared. The working solution of $ABTS^+$ (7.5 mM) was made by mixing the two stock solutions in a 1:1 (v/v) ratio and then allowing to stand in the dark at room temperature for 12 - 15 h. The $ABTS^+$ solution was diluted with methanol (80 %) to an absorbance of 0.700 ± 0.02 at 734 nm using a plate reader (BioTek, USA). The acetone and water extracts, and pure compounds such as ascobic acid, BTH (50 μL), were added to the working solution of $ABTS^+$ (200 μL). The mixture was mixed and incubated for 6 min and the absorbance was read at 734 nm in the plate reader. Methanol (80 %) and water in place of the sample extracts were used as controls. Each sample was tested in duplicate. The percentage scavenging of ABTS radical was calculated as given in equation (3.1) in Section 3.2.9.

4.2.8 *In vitro* α-glucosidase inhibitory

The activity of the carob phenolic extracts against α-glucosidase was determined as described in the method described in Section 3.2.9.

4.2.9 Statistical analysis

The measurements were presented as mean ± standard deviation (SD) and percentage of change/inhibition. Differences in means among treatment groups were tested using analysis of variance (ANOVA). A significance was considered when level of p

< 0.05. Microsoft Office Excel 2010 (Microsoft, Washington) was used for all data analysis.

4.3 Results and discussion

4.3.1 Total phenolic content

TPC in carob kibble during the submerged fermentations with *S. cerevisiae* (Sc) or L. *plantarum* (Lp) or their combination (LS) was determined in this study. The phenolic compounds were extracted with two solvents, water and acetone (70 %), and the results are presented in Figure 4.1. A significant increase in TPC was observed for the Sc and LS treatments at 15 and 30 h, respectively, followed by a substantial decrease at the end of the process ($p < 0.05$). For example, the TPC in acetone and water extracts from the Sc fermentation increased by 75.7 and 64.1 % at 15 h as compared to TPC at 0 h, respectively. Similarly, there was a 64.6 and 36.9 % increase in TPC in acetone and water extracts at 20 and 30 h for the LS, respectively. In contrast, the Lp fermentation showed no change in TPC in acetone extract ($p > 0.05$), while there was a noticeable decrease in these compounds in the water extract throughout the process ($p < 0.05$). Lp was reported to improve TPC in soy whey and cowpeas, but not in some other plants such as durum wheat (Dueñas et al., 2005; Xiao et al., 2015; Ferri et al., 2016). This could be due to the negative effect of these substrates on this Lp metabolism, which could result in the different changes in TPC. The results of the present study suggested the use of Lp for carob fermentation with the purpose of increasing TPC is not effective.

As the duration of fermentation increased to 70 h, a reduction of TPC was observed for all three fermentation treatments, which were approximately 19.7 mg/gdw and

5.8 mg/gdw in Sc acetone and water extracts, respectively. A study on *Lactobacillus. acidophilus*, a species related to Lp, also showed a similar reduction in TPC in pear fruit extracts during fermentation (Ankolekar et al., 2012). This may be because of the degradation of soluble and active mono-phenolics by the organisms used (McCue & Shetty, 2005). These findings also are in accordance with the previous studies of Apostolidis et al. (2007) and Kwon, Apostolidis and Shetty (2006).

The present study identified Sc as a potential organism for improving total phenolics in carob kibble. At the same points in the fermentation, the TPC of Sc fermented samples was higher than that of Lp samples. These finding are supported by previous studies indicating that Sc fermentation significantly improved the TPC in plant materials such as buckwheat barley, wheat and rye (Đorđević et al., 2010; Pistarino et al., 2013). The benefits of a mixed culture fermentation using yeast and a lactic acid bacteria for improving polyphenol compounds have been studied (Đorđević et al., 2010; Pistarino et al., 2013). In the present study, at a ratio of Sc and Lp of 1:1 (v/v), the TPC increased to the greatest amount at 40.2 mg/gdw at 30 h. This improvement was equivalent to that in the carob treated with Sc, but at 15 h only.

The current study indicated the advantages of fermentation to improve TPC in carob kibble. At 24.3 mg/gdw total phenolics in the carob kibble (at 0 h), this content increased to 42.8 mg/gdw after the submerged fermentation with Sc ($p < 0.05$). Previous studies showed a significant increase in TPC in plant-derived food products, such as legumes, by microbial treatments (Dueñas et al., 2005; Đorđević et al., 2010; Juan & Chou, 2010; Xiao et al., 2014). This increase might be explained by the release of phenolic compounds bound to the insoluble fibre during fermentation (Chandrasekara & Shahidi, 2012). Another explanation by Lee et al.

(2008b) is that the production of β-glucosidase by the organism during the microbial treatment improves TPC. The results of our study were also supported by previous reports on the advantages of fermentation in increasing TPC in plants (Pistarino et al., 2013; Adetuyi & Ibrahim, 2014; Razak et al., 2015). Overall, the Sc alone was the most effective to improve TPC in carob kibble as compared to the Lp and their mixed cultures. The optimal incubation period for the yeast fermentation was 15 h.

The nature and effectiveness of solvents in extraction of phenolic compounds in carob kibble was also studied. In the present study, acetone (70 %) was more efficient than water in extracting phenolic compounds, as shown by TPC at 42.8 and 25.8 mg/gdw in the acetone and water extracts, respectively in the Sc samples at 15 h. This result was in agreement with previous reports indicating among the solvents of the same concentration (70 %) including acetone, ethanol, methanol and water, acetone was the best for extracting phenolic compounds from carob pods (Benchikh & Louailèche, 2014). In the work of Makris and Kefalas (2004), TPC in carob pod acetone (80 %) extracts was the highest as compared to other solvents including methanol (80 %) and acetonitrile (80 %) extracts. However, the study of Sebai et al. (2013) showed the opposite effect in the same carob pod sample extracted by pure sovlents. The authors found total polyphenols (13 mg/gdw) in acetone extract lower than that in other extracts such as water (287 mg/gdw), methanol (84 mg/gdw), ethanol (223 mg/gdw) and hexan (25 mg/gdw). This difference could be because these solvents were used pure (100 %) which may be less efficient than a binary solvent system (60 – 80 % in water). This suggestion was supported by studies of Avallone et al. (1997), Makris and Kefalas (2004), Sebai et al. (2013) and Yassine and Hayette (2014), who found that the use of aqueous forms of acetone (70 %) and

methanol (70 - 80 %) was better than their use in absolute forms (100 %). In comparative studies on extraction of phenolic compounds from other plants, Chew et al. (2011) and Xu & Chang (2007) also showed a binary-solvent systerm was more efficient than a mono-solvent one. This could be because water enhances penetration of solvent into solid particles (Gertenbach, 2002; Zhang et al., 2007; Luthria, 2012). This easier penetration results in improvement of the efficiency of extraction of these compounds.

The present study suggested that most of the phenolic compounds in carob kibble dissolved in organic solvents particularly in acetone (70 %) better than in water. The Sc fermentation alone was the most suitable for improvement of TPC in carob kibble as compared to the Lp and their mixed culture fermentation. The optimal incubation period in for the Sc fermentation was 15 h.

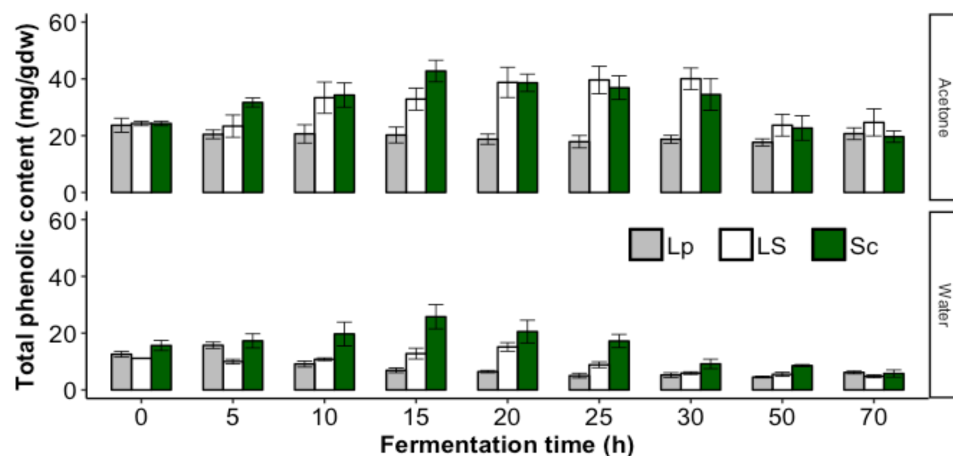


Figure 4.1 Total phenolic content

The carob kibble was fermented with *Saccharomyces cerevisiae* (Sc), *Lactobacillus plantarum* (Lp) and a mixed culture of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (LS). Error bars represent standard

deviations with respect to the mean values of three replicates. mgGA/gdw: milligram gallic acid per gram dry weight.

4.3.2 Antioxidant activity

Carob kibble contains a high level of phenolic compounds at 19.2 %, contributing a strong antioxidant effect including radical scavenging and metal reduction (Kumazawa et al., 2002; Makris & Kefalas, 2004). In the present study, the antioxidant activity of carob kibble subjected to three fermentation treatments with Lp, Sc and LS were determined in both acetone and water extracts using two commonly used assays (ABTS and DPPH).

For the ABTS method, the Sc and LS resulted in a significant increase in radical scavenging activity for both extracts ($p < 0.05$). Carob kibble fermented with Lp alone showed no apparent difference during the fermentation in the acetone extract, but showed a progressive decrease in the water fraction (Figure 4.2). The acetone extract of carob kibble subjected to Sc fermentation had highest ABTS radical scavenging activity at just over 63.9 % at 15 h and then declined to 38.3 % at 70 h. This trend was similar to LS in both the acetone and water fractions, where the highest capacity was 61.0 % at 25 h. By contrast, the case of Lp was not different in the acetone samples throughout the process, while the clearest decrease in this activity from 28.5 % to just under 19.0 % was observed in the water. The scavenging activity of the organic and water extracts against the ABTS radical was different. In general, the ABTS scavenging activity in the acetone was significantly higher than in the water and Sc showed the highest activity.

As the DPPH scavenging ability of carob extract was weaker than ABTS, the concentration of TPC in the water extract was doubled (Figure 4.3). In general, although the water extract was used at a double concentration, its inhibition was weak, at under 10 % DPPH absorbance, for both Lp and LS treatments. This inhibition was however the highest at 15 % in the Sc water at 15 h. In acetone extracts, the highest activity was 50.7 and 47.7 % at 15 and 25 h for Sc and LS, respectively ($p < 0.05$). They then slightly decreased to 38.6 and 34.6 % by the end of the process. In contrast, there was a slight fluctuation in antioxidant capacity of Lp extract throughout the process.

Work reporting an increase in antioxidant activity of plant and plant-based foods after fermentation has been recently published. It suggests that bioactive constituents as antioxidants can change positively during fermentation by the metabolic activity of microbes. Enzymes such as amylase, β-glucosidase, invertase, phenolic acid decarboxylases and tanninase released from plants and organisms could contribute to catalysis of the free and active phenolics and antioxidants prior to the extraction process (McCue & Shetty, 2003; Kim et al., 2011b). The microorganisms present during fermentation may result in a degree of modification of bioactive compounds and their bioactivity in plant materials. The antioxidant capacities of black bean fermented with fungi including *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus sojae*, *Rhizopus azygosporus* and *Rhizopus* sp. No. 2 were different with the extracts from the *Aspergillus awamori* fermentation showing the greatest antioxidant activity (Lee et al., 2008a). Similarly, Moore et al. (2007) reported that while Fleischmann RapidRise yeast and Hodgson Mill active-dry yeast increased, Fleischmann active-dry yeast decreased the antioxidant capacity of Lakin wheat bran, by both ABTS and

DPPH assays. This indicated that the yeast preparations themselves might have different radical scavenging capacities. In the present study, the radical scavenging effect increased in the Sc fermented carob after 15 h, while the Lp fermented decreased until the end of the process (Fig. 4.2 and 4.3). This result is supported by research of Othman et al. (2009) who indicated that Lp decreased ABTS scavenging inhibition in three samples of green and varicoloured and black olives. An explanation for these results could be that the low pH, produced by Lp, may have resulted in a negative effect on the activity and growth.

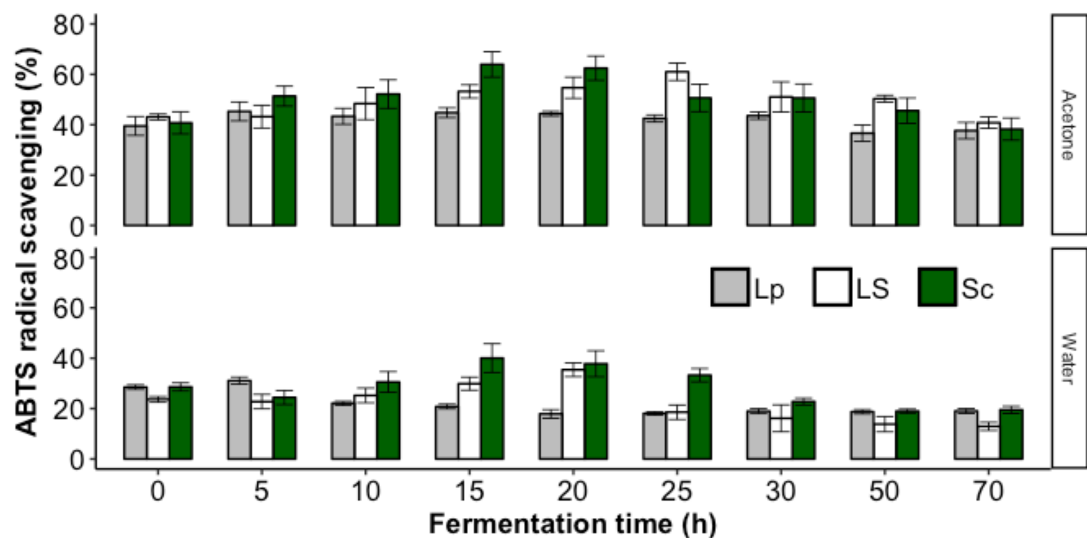


Figure 4.2 ABTS radical scavenge activity

The carob kibble was fermented with *Saccharomyces cerevisiae* (Sc), *Lactobacillus plantarum* (Lp) and a mixed culture of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (LS). Error bars represent standard deviations with respect to the mean values of three replicates.

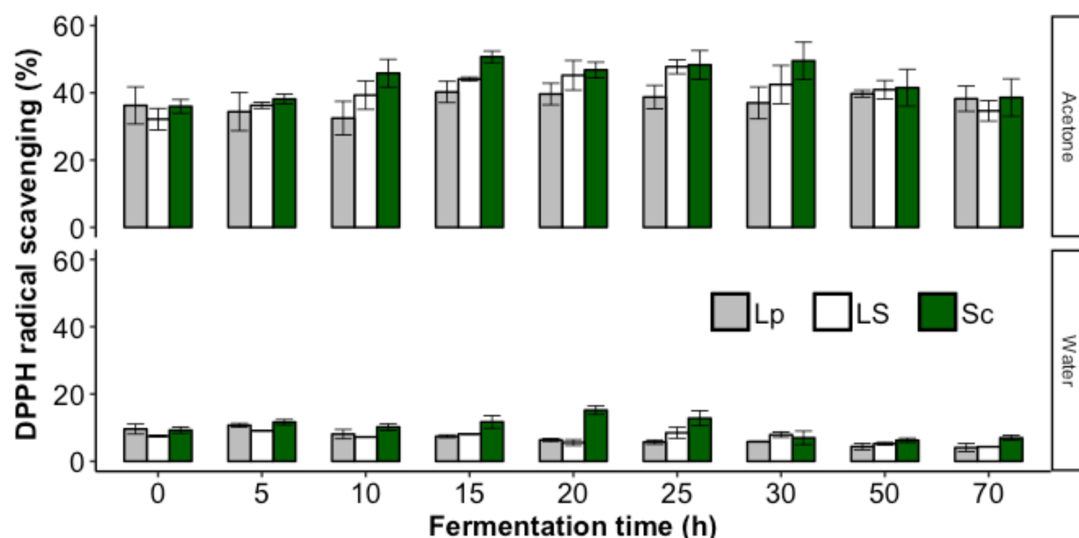


Figure 4.3 DPPH radical scavenge activity

The carob kibble was fermented with *Saccharomyces cerevisiae* (Sc), *Lactobacillus plantarum* (Lp) and a mixed culture of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (LS). Error bars represent standard deviations with respect to the mean values of three replicates.

4.3.3 Correlation between TPC and antioxidant activity

The correlations (R^2) between total phenolic content and antioxidant activity of carob kibble fermented with Lp, Sc and LS were examined in the study. A strong radical scavenging activity corresponded to a high TPC in the carob extracts, and the samples showing low inhibition capacity exhibited relatively low TPC. The carob fermented with Sc showed the highest TPC and the highest scavenging of both ($ABTS^+$) and ($DPPH^+$) (Figure 4.1, 4.2 and 4.3). For example, the relationship between ABTS scavenging activity and TPC, Sc showed a close relationship at an R^2 = 0.82769 for acetone and 0.84248 for water extracts. It also showed a similar relationship in the water for the Lp and LS treatments (R^2 = 0.95378 and 0.96774,

respectively). These interactions were, on the other hand, poorer in acetone extracts for LS ($R^2 = 0.60691$), while no correlation was observed in Lp ($R^2 = 0.08956$). In DPPH, the Sc TPC showed the highest linear correlation for DPPH in the acetone extract (Figure 4.4), where $R^2 = 0.62131$. In contrast, although this relationship was apparently high in Lp water extracts ($R^2 = 0.85441$), no significant correlation was observed in acetone extract ($R^2 = 0.24192$) for DPPH. Previous studies reported that phenolic compounds are major antioxidant constituent in plants (Dorman et al., 2004; Surveswaran et al., 2007; Piluzza & Bullitta, 2011). This is in accordance with the results of our Sc and LS fermentations. However, the different relationship between TPC and related activity may result from many factors. For example, not all phenolics show antioxidant activity, many non-phenolics exhibit antioxidant activity and reaction conditions such as pH and temperature directly affect the activity.

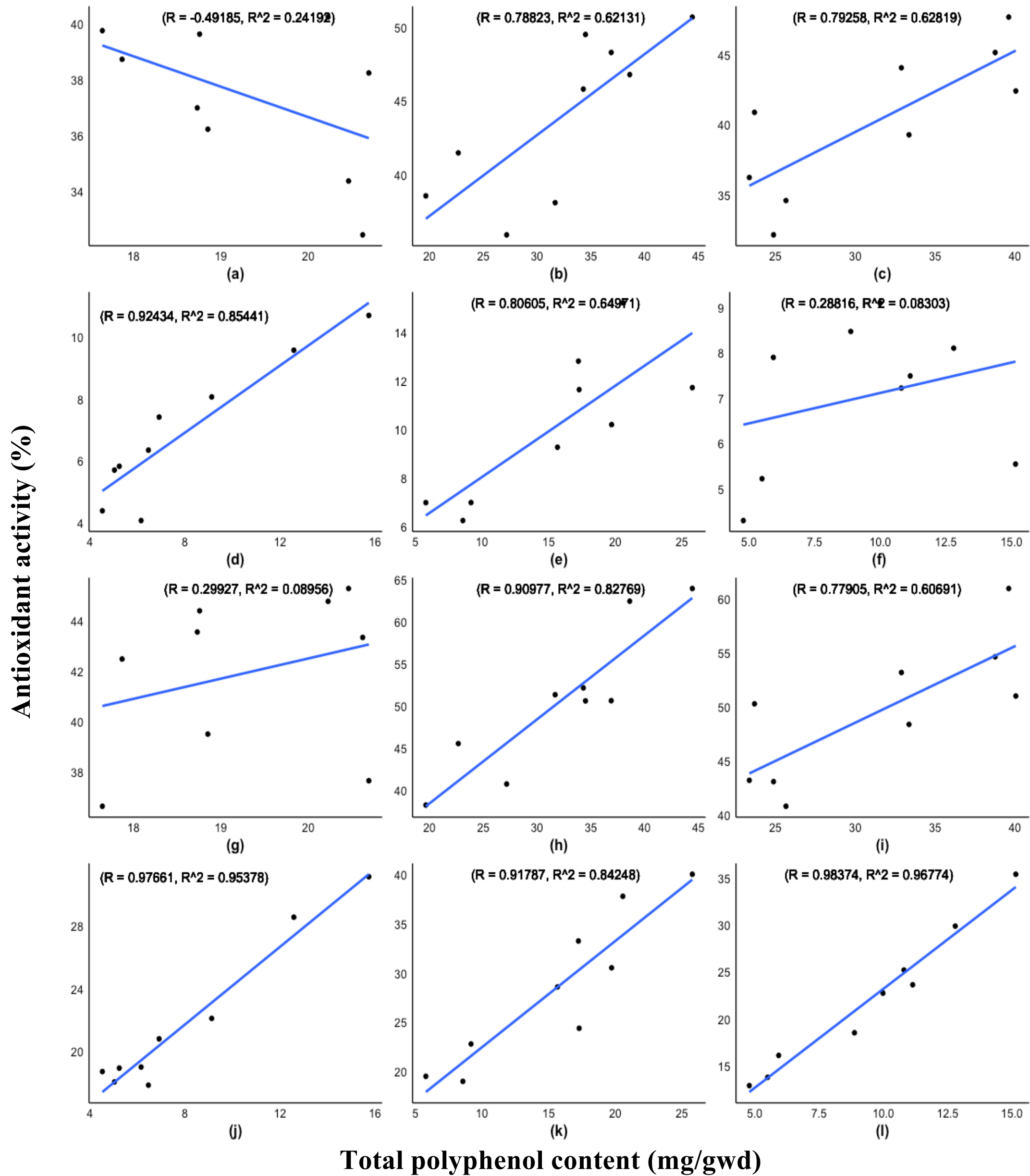


Figure 4.4 Correlation between TPC and antioxidant activity

DPPH: (a): Lp acetone, (b): Sc acetone, (c): LS acetone, (d) Lp water, (e) Sc water and (f) LS water extracts; ABTS: (g): Lp acetone, (h): Sc acetone, (i) LS acetone, (j) Lp water, (k) Sc water and (l) LS water extracts.

4.3.4 Anti α-glucosidase activity

Inhibitory activity against α-glucosidase could be an indicator of ability of a compound to prevent and manage diabetic type 2 disease (van de Laar, 2008). Natural α-glucosidase inhibitors are of interest because of their minimal side effects. In the present study, Australian carob kibble was identified as a potential source of α-glucosidase inhibitors. The results showed that the unfermented carob kibble inhibited up to approximately 80 and 90 % activity of α-glucosidase at 50 µg/mL in acetone and water extracts, respectively. This inhibition was higher than Acb, a drug used as a positive control. In comparison, Custodio et al. (2015) reported that carob leave extracts showed the highest inhibition ability (97 %), followed by stem bark (65 %), and that both had higher activity than the positive controls, Acb and glucobay (35 and 51 %, respectively).

The changes in this activity of carob kibble against α-glucosidase during the fermentations using Sc, Lp and LS were examined. There was a switch in changes in the enzyme inhibition during fermentations using Sc and LS, while Lp showed a down ward trend during the fermentation (Figure 4.5). Specifically, the enzyme inhibition of Sc and LS remained almost unchanged at about 75 % (for acetone extract) and 90 % (for water extract) until the 25th h of incubation. The inhibition activity of Sc and LS, then, dropped to 48.7 % ($p < 0.05$) and 55.5 % ($p < 0.05$), respectively, in acetone extract, by 70 h. The decrease in enzyme inhibition was even greater in water extract, namely to 13.6 % for Sc, and 15.9 % for LS by 70 h. As reported in previous studies, many plant phenolic compounds provide diabetic enzyme inhibitory ability (Ranilla et al., 2010; Kumkrai et al., 2015; Zhang et al., 2015). As the duration of fermentation increased, formation of these phenolics was

reported (Adetuyi & Ibrahim, 2014; Hur et al., 2014), which resulted in reduced bioactivity of active phenolics, such as antioxidant and enzyme inhibition activities (Apostolidis et al., 2006; Adetuyi & Ibrahim, 2014).

Water extracts showed a markedly higher inhibition than the acetone extracts during the first 20 h for all three fermentation treatments ($p < 0.05$). Specifically, the inhibition of α-glucosidase activity of acetone extract was about 10 % lower compared to that of water extracts. This ability was considerably stronger than Acb which was 30.3 % at the same concentration. Interestingly, it was shown in Figure 4.1 that TPC in acetone extract was higher than in water. This could be explained by the presence of non-phenolic compound(s) that are highly water soluble having strong enzyme inhibitory activity. Many non-phenolic compounds such as alkaloids (coptisine, jatrorrhizine and palmatine), sulphonium sulphates (salacinol, kotalanol and salaprinol) and sugars (deoxynojirimycin) that inhibit α-glucosidase and α-amylase have been found (Akaki et al., 2014; Zhou et al., 2014; Boisson et al., 2015; Morikawa et al., 2015). D-pinitol which is a predominant alcohol sugar in carob kibble significantly reduces blood glucose in *in vivo* models (Bates et al., 2000; Kim et al., 2005b; Kang et al., 2006). This study strongly indicated that D-pinitol has no α-glucosidase inhibition activity in *in vitro* models (Figure 3.4). It may therefore be that an unknown α-glucosidase inhibitor(s) is present in carob kibble. Further study is required to establish if this is the case.

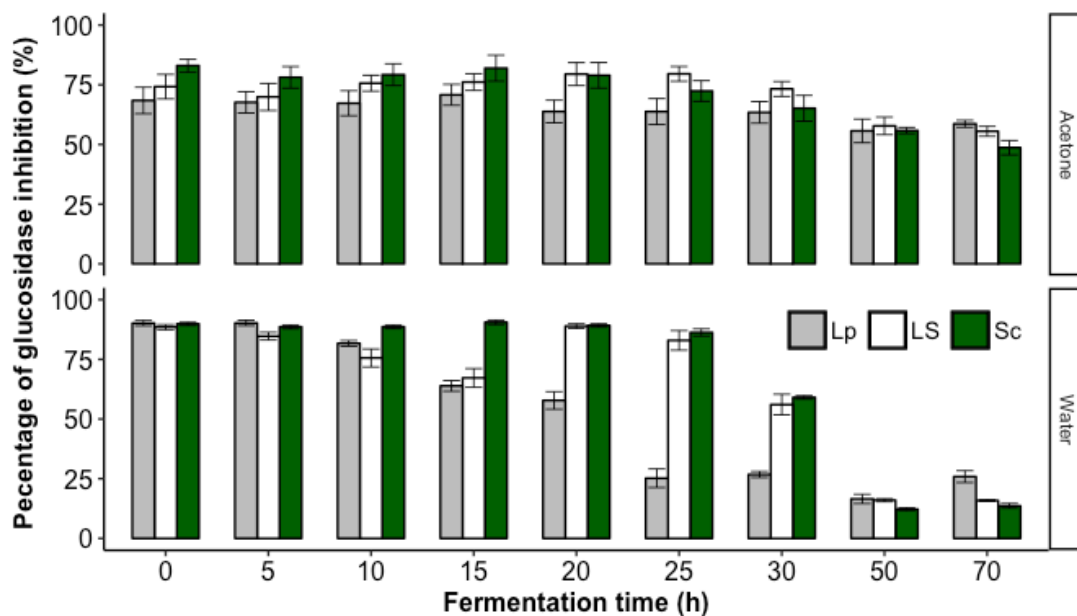


Figure 4.5 Anti α-glucosidase activity

Carob kibble fermented with *Saccharomyces cerevisiae* (Sc), *Lactobacillus plantarum* (Lp) and a mixed culture of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (LS). Error bars represent standard deviations with respect to the mean values of three different replicates.

4.3.4 Correlation between TPC and anti α-glucosidase activity

The correlation between TPC and anti α-glucosidase in the carob kibble fermented with Lp, Sc and LS was determined in the present study. A moderately high correlation between TPC and the antienzyme activity was shown in water extracts, namely R^2 = 0.75584, R^2 = 0.72959 and R^2 = 0.63012 for Lp, Sc and LS treatments, respectively. These relationships were however weaker in acetone extracts, particularly in Lp (R^2 = 0.18544) (Figure 4.6). TPC has been shown to be highly correlated with inhibitory activity against α-glucosidase in plants (Mai et al., 2007; Kwon et al., 2008; Yao et al., 2009; Johnson et al., 2011). It is therefore expected that the sample that exhibited stronger anti enzyme activity contained higher concentration of total phenolic compounds. Among the three treatments studied, Sc

had the highest association between TPC and this activity for both water and acetone extracts with a R^2 = 0.72959 and R^2 = 0.51544, respectively. In contrast, a relatively poor relationship was observed for acetone extracts in the case of Lp (R^2 = 0.18544) although these extracts contained a high level of TPC. This indicates that phenolic compounds present in the Lp extracts exhibited a weak inhibition of α-glucosidase. An explanation may be because of the low pH in the Lp-fermented samples negatively influenced glucosidase inhibitory activity of phenolics. It may also be due to the presence of non-phenolic α-glucosidase inhibitors in the carob water extracts.

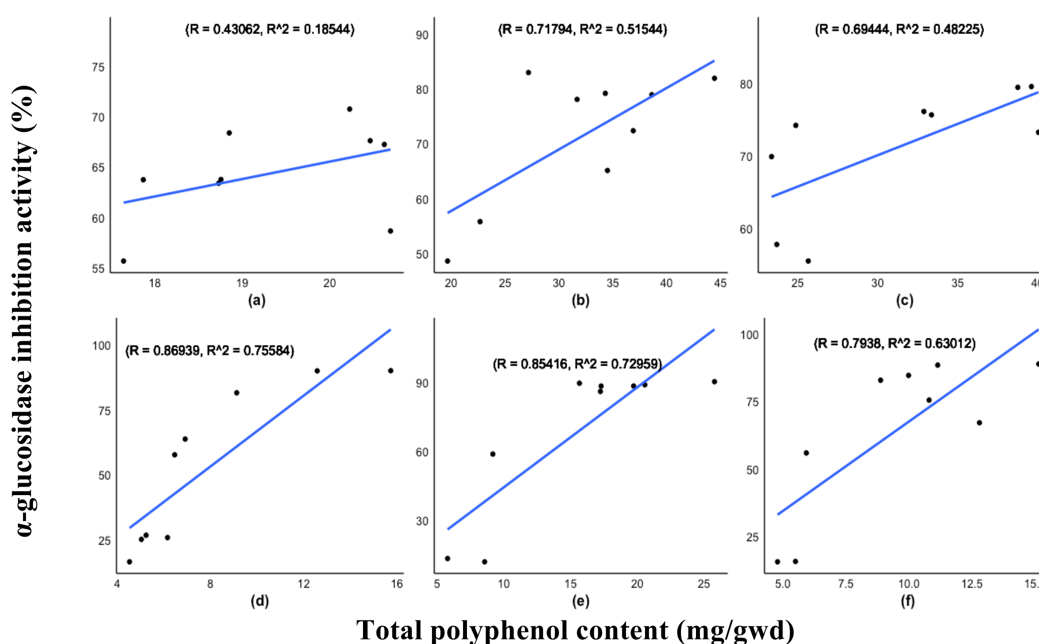


Figure 4.6 Correlation between TPC and α-glucosidase inhibition activity

(a): Lp acetone, (b); Sc acetone, (c): LS acetone, (d): Lp water, (e): Sc water and (f): LS water extracts

4.4 Conclusion

This work showed that Sc is a more appropriate candidate than Lp for improvement of TPC and antioxidant activity in carob kibble. TPC in acetone extracts is

significant higher than water extracts. Similarly, the acetone extracts displayed a stronger antioxidant capacity than water for both the ABTS and DPPH assays. Correlation between TPC and antioxidant activity in the fermented kibble was relatively high for both DPPH and ABTS. Importantly, it was found that carob kibble extracts strongly inhibited α-glucosidase by 90 % at 50 μgdw/mL, in both acetone and water extracts. The results from enzyme assay suggested that there may be unknown α-glucosidase inhibitor(s) in the carob extracts.

Chapter 5 Establishment of the initial pH and time parameters for increasing the total phenolic content and associated bioactivity in carob kibble

Some works of this chapter have been presented in the following conference:

Thi, HV, Haelee, F, Thi, HTN, & Gary, D. (2016). Effect of initial ph and fermentation duration on total phenolic content and antioxidant activity of carob kibble fermented with *saccharomyces cerevisiae*. *18th International Conference on Food Science Research, Technology and Innovation*, Sydney, Australia, Poster presentation.

5.1 Introduction

Fermentation is increasingly considered as a useful tool to improve the levels of bioactive compounds and their activity in plant-based foods. Fermentation conditions such as organisms and substrates significantly influence biochemical changes during the process (Hur et al., 2014). pH is one of the key fermentation parameters affecting the fermentation. This factor is highly correlated with growth of microbial organism, results in changes in chemical structures of substrate components and stability of bioactive compounds. For example, the growth of *Saccharomyces cerevisiae* (*S. cerevisi*ae) is negatively affected under an initial acidic pH (Casey et al., 2010; Liu et al., 2015). Catechins, one of the main groups of plant phenolic antioxidants, are highly stable in acidic solutions, but unstable in alkaline solutions (Chu & Chen, 2006). The radical scavenging capacity of fermented plant extracts increases with increasing pH of the medium because of changes in the content and structure of the phenolic antioxidants (Muzolf et al., 2008). Fermentation time is another important factor also affecting the contents and activity of fermented products. Longer fermentation time causes the polymerization of simple and active compounds, leading to a decrease in their content and activity of the final product. Identification of optimal fermentation conditions are therefore crucial for achieving the greatest content and activity of the bioactive compounds.

Study results presented in Chapter 4 show that *S. cerevisiae* alone is more beneficial than *L. plantarum* to improve total phenolic content and bioactivity in carob kibble. The aims of the work presented in this chapter was to determine effect of initial pH and fermentation time on the total phenolic, total condensed tannin and total flavonoid contents in carob kibble during submerged fermentation with *S. cerevisiae*.

Antioxidant activity and diabetic enzyme inhibition activity were also investigated. The purpose of this study is to establish the optimal initial pH of substrate and fermentation time for achieving the highest content of total phenolics and bioactivity of the fermented carob.

5.2 Materials and Methods

5.2.1 Preparation of carob kibble

Carob kibble was prepared as described in Section 3.2.1.

5.2.2 Microorganisms

Saccharomyces cerevisiae activation and substrate preparation were described in Section 3.2.2.

5.2.3 Submerged fermentation

The fermentation was carried out as described in Section 3.2.3 for evaluation of the effect of initial pH and time on the fermentation process with the selected microorganism (*S. cerevisiae*) with some changes. A range of pHs (5.5, 6.0, 6.5, 7.0 and 7.5) was investigated. A sample was withdrawn every 5 h for chemical analyses. All samples were freeze dried, then kept at -18 °C for later analysis.

5.2.4 Preparation of phenolic extract

Phenolic compounds were extracted from carob kibble as described in Section 4.2.5. It was found in Chapter 4 that acetone (70 %) was more effective than water in extracting phenolic compounds from carob kibble. The acetone extract was therefore

used to determine the contents of total phenolics, total condensed tannins, total flavonoids and antioxidant activity. The water extract was used to examine the enzyme inhibitory activity in this section.

5.2.5 Total phenolic content (TPC)

TPC in the fermented acetone extract was determined as described in Section 4.2.6.

5.2.6 Total condensed tannin content (TCT)

TCT in the fermented acetone extract was determined by using the Vanillin method (Kumazawa et al., 2002) with minor changes. This extract diluted with methanol 80 % (2 mL) was added to 4 mL of vanillin (1 % in 7M H_2SO_4) in a test tube. The mixture was incubated in the dark, at room temperature for exactly 15 min. Absorbance was measured at 500 nm using a spectrophotometer (UV-1800 UV-VIS, Shimadzu, Japan). The sample was replaced with methanol 80 % as a blank. TCT was calculated as the amount of catechin hydrate (mg CE/gdw) from a calibration curve. All analysis was carried out in duplicate.

5.2.7 Total flavonoid content (TFC)

TFC in the fermented acetone extract was analysed by using the colorimetric method of Juan and Chou (2010) with some changes. The extract diluted with methanol (80 %) (2 mL) was added to $NaNO_2$ 5 % (150 µL) and $AlCl_3$ 10 % (150 µL). After mixing with NaOH 1M (1 mL), the mixture was immediately added to 1.7 mL H_2O. Absorbance was measured at 510 nm by a spectrophotometer (UV-1800 UV-VIS, Shimadzu, Japan). TFC was calculated as rutin equivalent from a calibration curve

(mg RT/gdw). The same amount of methanol (80 %) instead of extract sample was used as a blank. All analysis was conducted in duplicate.

5.2.8 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity of the fermented acetone extract was determined using DPPH assay as described in Section 3.2.8.

5.2.9 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging activity

ABTS assay was also used to determine antioxidant activity in the fermented acetone extract. This method was described in Section 4.2.8.

5.2.10 *In vitro* α-glucosidase inhibitory

The inhibitory α-glucosidase activity of the fermented carob kibble was performed as the procedure described as in Section 3.2.9. The activity of carob kibble was tested only for the water extract.

5.2.11 *In vitro* α-amylase inhibitory assay

The water extract was assessed for α-amylase inhibitory activity by using the procedure of Kwon et al. (2008). The extract (200 µL) was added to 200 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 6 µM sodium chloride) containing α-amylase solution (0.5 mg/mL), which was incubated at 25°C for 10 min. The same amount of sodium phosphate buffer as above containing 1 % starch solution as substrate (200 µL) was then added. The mixture was again incubated at 25°C for 10 min. The reaction was stopped by adding 400 µL dinitrosalicylic acid colour reagent

and then placed in boiling water for 5 min. The mixture was cooled down to room temperature before being added to 9 mL water. The sodium phosphate buffer and the pure compounds gallic acid (GA), ascorbic acid (AC), butylated hydroxyanisole (BHA), epicatechine (EC), rutin (RT) and catechin (CT), instead of sample extract, were used as negative and positive controls, respectively. The mixture was measured for absorbance at 540 nm. The percentage of inhibition was calculated as given in equation 3.1.

5.2.12 Statistical analysis

The measurements were presented as mean ± standard deviation (SD) and percentage of change/inhibition. Differences in means among treatment groups were tested using analysis of variance (ANOVA). A significance was considered when level of $p< 0.05$. Microsoft Office Excel 2010 (Microsoft, Washington) was used for all data analysis.

5.3 Results and discussion

5.3.1 TPC, TCT and TFC

In this chapter, only acetone (70 %) was used for extracting the phenolics in carob kibble because it was demonstrated to be more effective than water. The changes in TPC, TCT and TFC in the fermented kibble at the initial pHs studied were determined.

As shown in Figure 5.1, TPC increased slightly and reached a peak at 15 h before decreasing to a level lower than that at the starting point ($p < 0.05$). These trends were observed at most of pHs tested. For example, at pH 5.5 there was an increase in

TPC from 27.1 mg/gdw at 0 h to 41.6 mg/gdw at 15 h. This content, on the other hand, was higher ($p < 0.05$) at 47.6 and 54.2 mg/gdw at the same points of time at pH 7.0 and 7.5, respectively. As the time of fermentation increased to 50 h, the level of total phenolics declined significantly to approximately 22.0 mg/gdw and 27.1 mg/gdw at pH 5.5 and 7.5, respectively ($p < 0.05$). A duration of 15 h was sufficient for reaching the highest TPC. These findings were supported by previous reports on the advantages of fermentation to TPC in plants (Adetuyi & Ibrahim, 2014; Hur et al., 2014; Razak et al., 2015). Most of the phenolic compounds in plant exist naturally in combined form with sugars which may reduce their bioactivity (Vattem & Shetty, 2003). This complex is hydrolysed into free, soluble and simpler forms by hydrolytic enzymes such as β-glucosidase produced by organisms during fermentation (Vattem & Shetty, 2003; Vattem et al., 2004). An increase in bioactive free phenolic compounds was observed during the exponential phase of treatment with *S. cerevisiae* over the first 15 h. During the stationary phase from 20 to 50 h, a decline of TPC was observed which was probably because of polymerization, diffusion and oxidation of the released active polyphenol forms (Bhanja et al., 2009). Phenolic significantly decreased as the fermentation period increased (Figure 5.1) that is in agreement with a large number of previous studies (McCue & Shetty, 2003; Ajila et al., 2012; Adetuyi & Ibrahim, 2014).

The initial pH of the substrate was characterised as a factor that affected TPC during the fermentation process. A range of adjusted pH from 5.5 to 7.5 was considered. As shown in Figure 5.1, an initial substrate pH 7.5 was the most effective for the release of the TPC in carob kibble ($p < 0.05$). For example, the TPC was improved by 53.5 % at pH 5.5, but was approximately 66.3 % at pH 7.5 after 15 h. Similar findings

were observed at most points of time through the process. The production of hydrolytic enzymes, which is responsible for release of free polyphenols, by the microbial organism may be more effective in alkali than acidic pH environment.

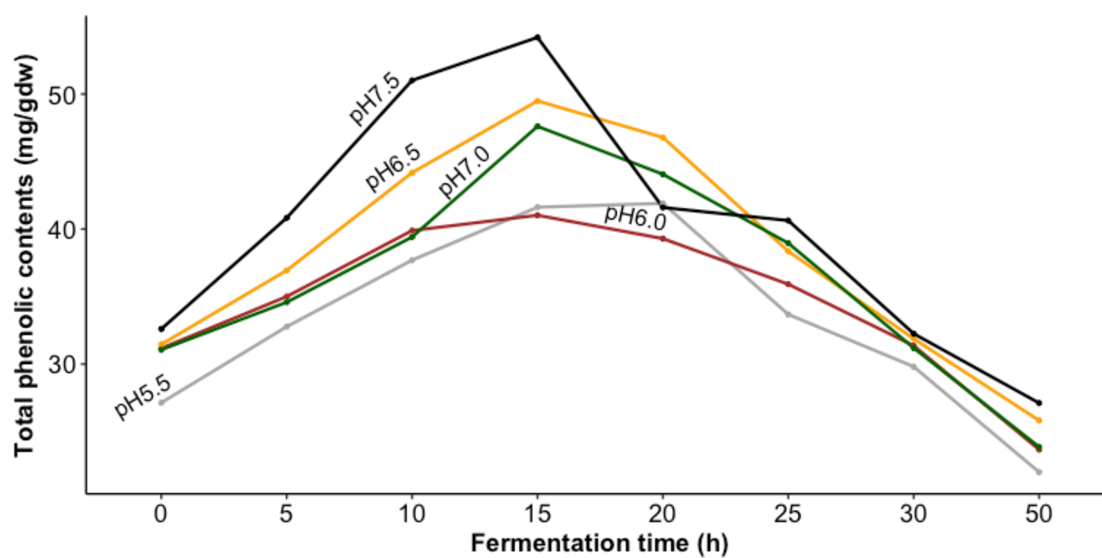


Figure 5.1 Effect of substrate pH on total phenolic content (TPC)

The carob kibble was fermented at pH 5.5; 6.0; 6.5; 7.0 and 7.5. The data is presented as mean of three experimental replicates. TPC is expressed as milligram of gallic acid per gram dry weigh sample (mg GA/gdw).

Carob kibble contains high amounts of tannins, mostly as condensed tannin with levels ranging from 4.4 to 43.7 mg/gdw depending on carob tree cultivars, climatic region and solvents of extraction (Kumazawa et al., 2002; Makris & Kefalas, 2004; Custódio et al., 2011). In the present study, the TCT in carob kibble was determined during the treatment with *S. cerevisiae*. The TCT appeared to slightly increase during the first 15 h, albeit not statistically significant ($p > 0.05$). However, the contents of these phenolic compounds were decreased sharply between 15 h and 50 h (Figure 5.2). For example, the TCT in the pH 7.5 sample increased from about 27.0 to 35

mg/gdw between 0 h and 15 h, but then decreased sharply to about 13 mg/gdw at 50 h. This apparent reduction in the content of TCT after 15 h showed a negative effect of fermentation time ($p < 0.05$). Similar patterns were also observed for all other the tested pHs. These results suggested that optimal fermentation time for achieving the highest TCT was 15 h, and that changes in TCT during the fermentation may not be affected by initial substrate pH.

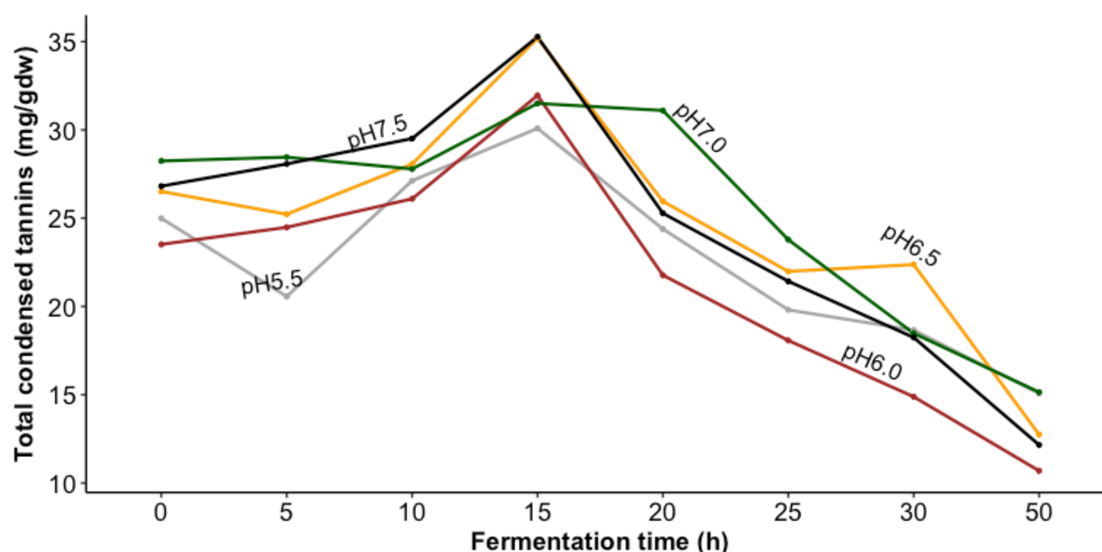


Figure 5.2 Effect of substrate pH on total condensed tannins content (TCT)
The carob kibble was fermented at pH 5.5; 6.0; 6.5; 7.0 and 7.5. The data is presented as mean of three experimental replicates. TCT is expressed as milligram of catechin hydrate per gram dry weigh sample (mg CE/ gdw).

The change in TFC in carob kibble during submerged fermentation with *S. cerevisiae* is shown in Fig. 5.3. In the present study, carob kibble contained 27.0 mg/gdw of total flavonoids, while previous findings reported a wide range from 0.5 to 17.0 mg/gdw (Kumazawa et al., 2002; Juan & Chou, 2010; Custódio et al., 2011; Benchikh & Louailèche, 2014). The Australian carob kibble may therefore be a good source of nature flavonoids. Interestingly, this content increased to the highest level

at 48.9 mg/gdw at pH 7.0 after 15 h ($p < 0.05$). As incubation time increased, TFC decreased significantly ($p < 0.05$) and this trend was similar in other pHs studied. As observed at the same point of time, there was no difference in TFC from all the tested pHs. This suggested that carob substrate at a range of initial pH 5.5-7.5 has no significant effect on release of free forms of flavonoids from carob kibble by *S. cerevisiae*. These findings showed that duration of treatment had a strong effect on the release of total flavonoids, and is supported by previous work (Adetuyi & Ibrahim, 2014). In the present study, 15 h was the optimal time for the release of TFC. This result further confirmed that microbiological treatment could improve an already relatively high level of total flavonoid in plant. The total flavonoid content increased by 45 to 90 % in plants such as black bean, wheat and okra after fermentation, depending on conditions of treatment and organism used (Moktan et al., 2008; Juan & Chou, 2010; Yao et al., 2010).

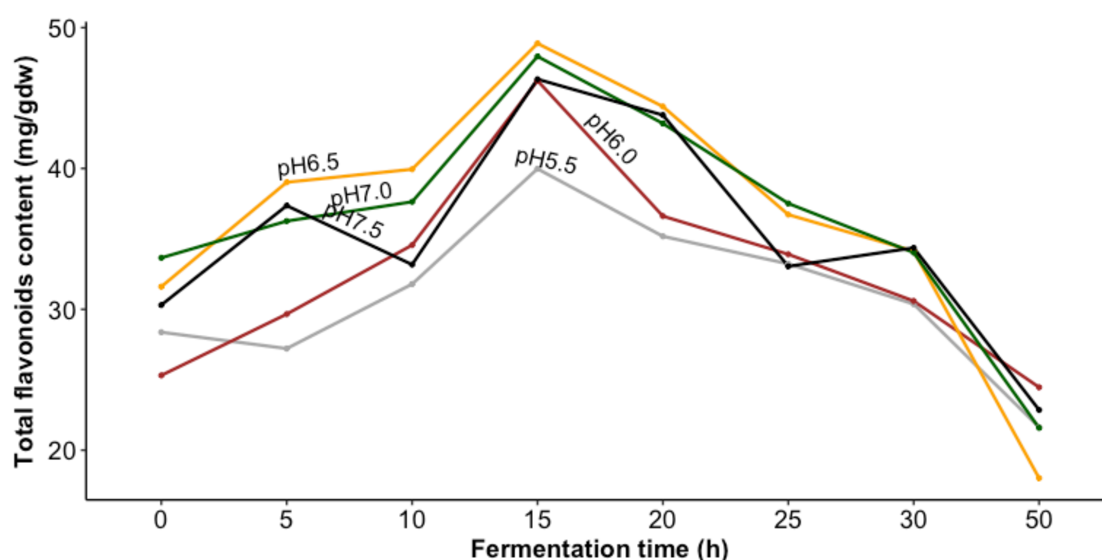


Figure 5.3 Effect of substrate pH on total flavonoid content (TFC)

The carob kibble was fermented at initial pH 5.5; 6.0; 6.5; 7.0 and 7.5. The data is presented as mean of three experimental replicates. TFC content is expressed as milligram of rutin per gram dry weigh sample (mg RT/gdw).

5.3.2 Antioxidant activity

Antioxidant activity can be attributable to one or many different antioxidant compounds through distinct mechanisms against oxidizing agents. A single method of examining this activity cannot fully examine the oxidant capacity of a complex. DPPH and ABTS are two of the most common methods for determination of antioxidant activity. In the DPPH assay, a donor of the free radical 2,2-diphenyl-1-picrylhydrazyl accepts both electron and hydrogen, but is more selective for H-donors (Je et al., 2009). On the other hand, ABTS is poorly selective for H-atom donors, so that it is only able to react with electron donors.

The antioxidant activity of carob kibble increased at 15 h, then decreased at the end of fermentation period at 50 h ($p < 0.05$), which were observed in both DPPH and ABTS assays. The results for the DPPH radical-scavenging activity are shown in Figure 5.4. At a start pH of 7.5, the antioxidant activity improved to 66.2 % at 15 h before significantly decreasing to 42.7 at 50 h. This was lower than that of the untreated sample, which was 48.5 % (data not shown). As also shown in Figure 5.4, the ABTS scavenging activity increased to 78.5 and 67.4 % at pH 7.5 and 5.5 at 15 h before significantly decreasing to 45.5 and 49.7 % at 50 h as compared to 0 h, respectively. Gallic acid, a strong and dominant antioxidant, exists in both free and bound forms in carob kibble (Kumazawa et al., 2002; Makris & Kefalas, 2004; Bernardo-Gil et al., 2011; Roseiro et al., 2013a). The release of gallic acid and other active compounds from the bound compounds through the fermentation process may be due to the activity of β-glucosidase (McCue & Shetty, 2003; Tapati Bhanja, 2009) from *S. cerevisiae*. As a result, the antioxidant activity of the kibble significantly increased after microbial fermentation. The advantages of microbial fermentation

shown in the present work agree with other studies on legume family plants (Lee et al., 2008a; Juan & Chou, 2010; Yao et al., 2010; Razak et al., 2015). The prolonged treatment may cause the decrease in this activity due to loss of antioxidant compounds in samples by oxidation and diffusion (Sakihama et al., 2002; Bhanja et al., 2009; Ajila et al., 2012; Adetuyi & Ibrahim, 2014). Regarding these results, 15 h was the optimal length of time for the fermentation of carob kibble with *S. cerevisiae*.

In order to examine effect of initial pH on the free radical scavenging activity of carob kibble, initial substrate pHs of 7.5 and 5.5 were chosen. For DPPH, although the fermented carob reached the highest activity at 60.6 % for pH 5.5, but at 66.2 % for 7.5, the difference was not significant ($p > 0.05$). This observation was recorded at most points of time through the process. In contrast, the ABTS assay showed the highest activity reached at 78.5 % of scavenging for pH 7.5, which was higher than the 67.4 % scavenging for pH 5.5 ($p < 0.05$). As incubation prolonged, the activity decreased at all tested pHs by the end of the process for both DPPH and ABTS, but was not different ($p > 0.05$). It suggests initial pH 7.5 has a positive effect on the increase in antioxidant activity of carob kibble fermented with *S. cerevisiae*.

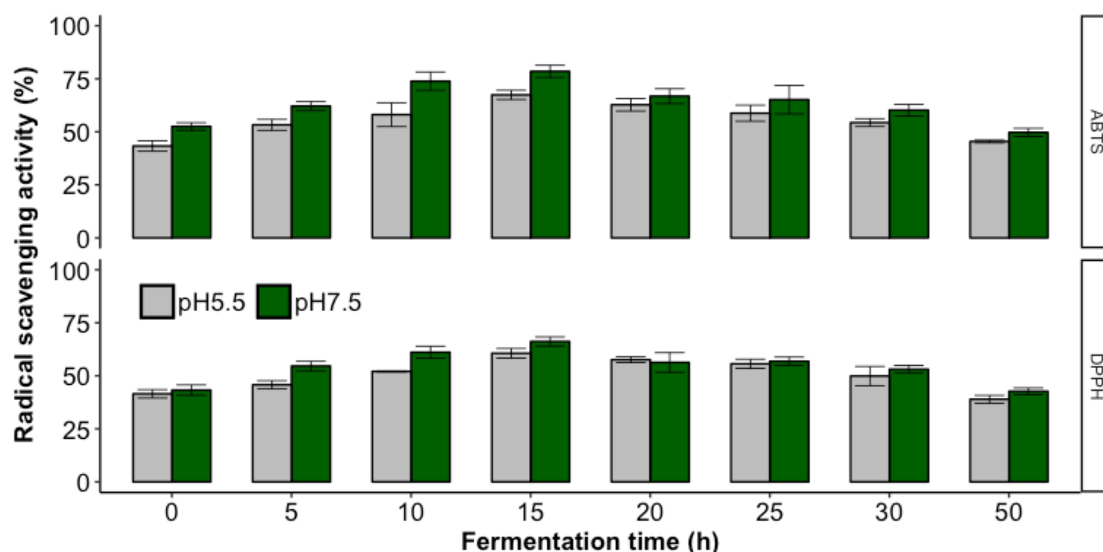


Figure 5.4 Effect of substrate pH on antioxidant capacity

The carob kibble was fermented at initial pH 5.5 and 7.5. ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity at a concentration of carob powder at 250 µg/mL, Values are expressed as mean ± SD (standard deviation) (n = 3) at each point of time.

The antioxidant activity of the fermented samples was compared with those of AC, BTH and well-known polyphenol compounds such as GA, EC, RT and CT. The sample of fermented kibble at pH 7.5 and at 15 h was used for both DPPH and ABTS assays. Three concentrations of 0.5, 0.7 and 1 mg dw kibble/mL were evaluated, while positive controls were used at 40 µg/mL under the same condition. As shown in Figure 5.1, GA showed the highest activity at 90 % inhibition of DPPH and inhibited ABTS absorbance almost completely. In contrast, RT showed the lowest activity at ~ 20% inhibition for both assays. By contrast, the fermented samples inhibited 58.6 and 71.1 % for DPPH and ABTS at 500 µg/mL, respectively. As the concentration of the kibble increased to 1 mg/mL, its activity increased to close to those of GA, shown in both methods. As presented in Figure 1, the fermented carob

had a TPC of 54.2 mg/gdw. It could therefore be suggested that the fermented carob exhibited a strong radical scavenging activity if the concentration of TPC was the same as the positive controls. These findings were in agreement with a study reported by Kumazawa et al. (2002), who showed antioxidant activity of carob pod extract was significantly higher than those of GA, EC and CE. These results combined with the findings of present study indicate that submerged fermentation with *S. cerevisiae* significantly improved antioxidant activity in carob kibble at 15 h.

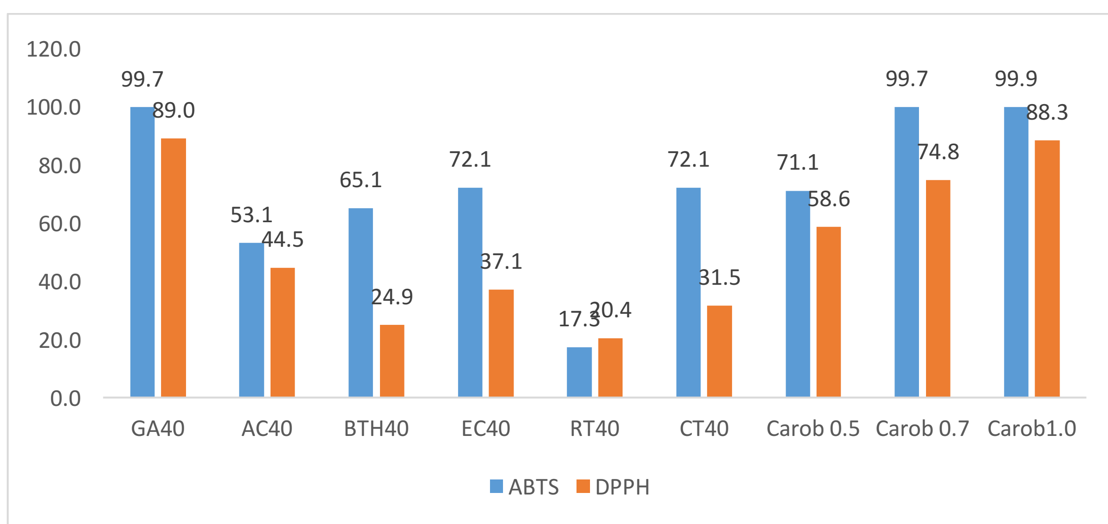


Figure 5.5 Antioxidant activity of the fermented carob vs several pure compounds

ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl)

5.3.3 Effect of sugars on antioxidant activity

Carob kibble is rich in sugars (60 – 75 %) consisting mainly of sucrose, fructose and glucose (Ayaz et al., 2007; Khlifa et al., 2013). The influence of these sugars on the DPPH and ABTS scavenging activity in carob was investigated (Figure 5.6). In our study, levels of sugars were 374 mg/gdw sucrose, and 117 mg/gdw fructose and

glucose, and were almost completely reduced at 15 h of fermentation (Figure 3.3, Chapter 3). As previously reported, the ratio of fructose to glucose in carob pod was approximately 2:1 (Biner et al., 2007; Turhan, 2014). A combination of sucrose, fructose and glucose (9:2:1 respectively) was independently added to all positive controls and 15 h fermented sample at the same level (1:1). The DPPH results showed that the antioxidant activity had been negatively affected by these sugars ($p < 0.05$) for AC and BAH, but positively affected EC and RT. This activity in GA, CT and carob samples, on the other hand, was not significantly changed ($p > 0.05$). For the ABTS assay, these sugars strongly inhibited the scavenging activity of BAH, EC and RT ($p < 0.05$), but not that of the carob kibble ($p < 0.05$). It was concluded that the sugars at similar ratio as in carob kibble did not have a significant effect on the antioxidant activity.

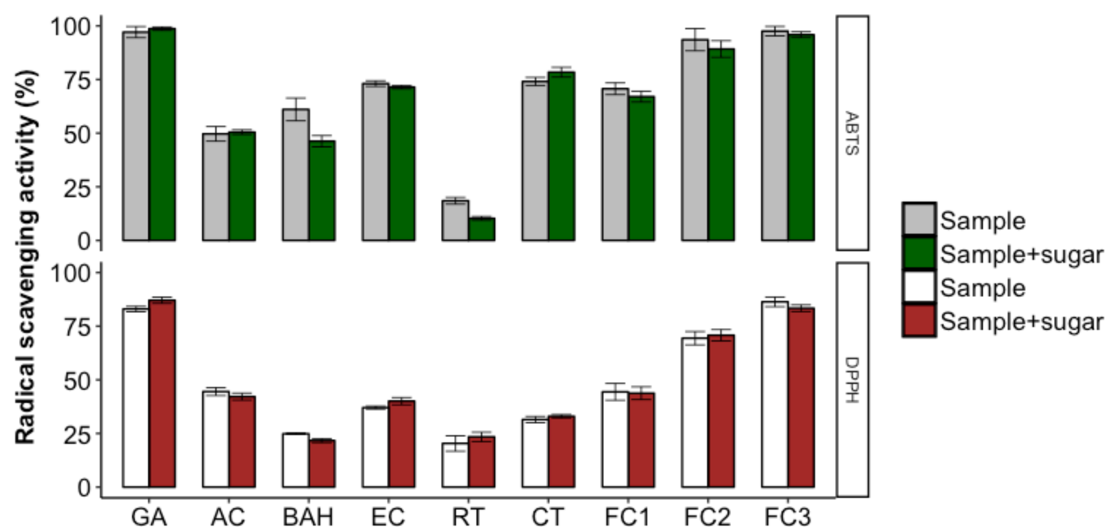


Figure 5.6 Effect of sugars on antioxidant activity

The standard compounds including GA (gallic acid), AC (ascorbic acid), BAH (butylated hydroxyanisole), EC (epicatechin), RT (rutin) and CT (catechin) were used at the same concentration at 40 µg/mL. The fermented carob extract (FC1, FC2 and FC3) were used at the three concentrations of 500, 700 and 1,000 µg/mL, respectively. These samples were added a combination of sucrose, fructose and glucose at (1:1 w/w). ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl). Each value is expressed as mean ± SD (standard deviation) (n=3) at each point of time.

5.3.4 Correlation between TPC, TCT and TFC and antioxidant activity

The correlation between the content of TPC, TCT and TFC and antioxidant activity in carob kibble was evaluated for pH 5.5 samples in the present study. In general, both ABTS and DPPH scavenging activities were highly correlated with TPC, TCT and TFC. For example, the relationship between TFC and antioxidant activity was the closest for both ABTS and DPPH, where R^2 = 0.9809 and 0.9533, respectively. Although the contribution of TCT to this activity was weaker, values of R^2 were still high at 0.7501 and 0.6916 for ABTS and DPPH, respectively. This demonstrated that phenolic compounds are the major contributors to antioxidant activity in the fermented carob, especially TCT and TFC. In addition, while there are likely to be condensed tannins and flavonoids involved, there are other non-phenolic compounds that can contribute to the antioxidant activity of the fermented carob. Non-phenolic compounds such as carotenoid in plants are antioxidants. The antioxidant activity of a mixture is not only dependent on concentration of antioxidants, but also on the interaction among these compounds. Consequently, the sample with similar concentration of total phenolics could be different in their antioxidant activity (Amarowicz et al., 2000; Soong & Barlow, 2004).

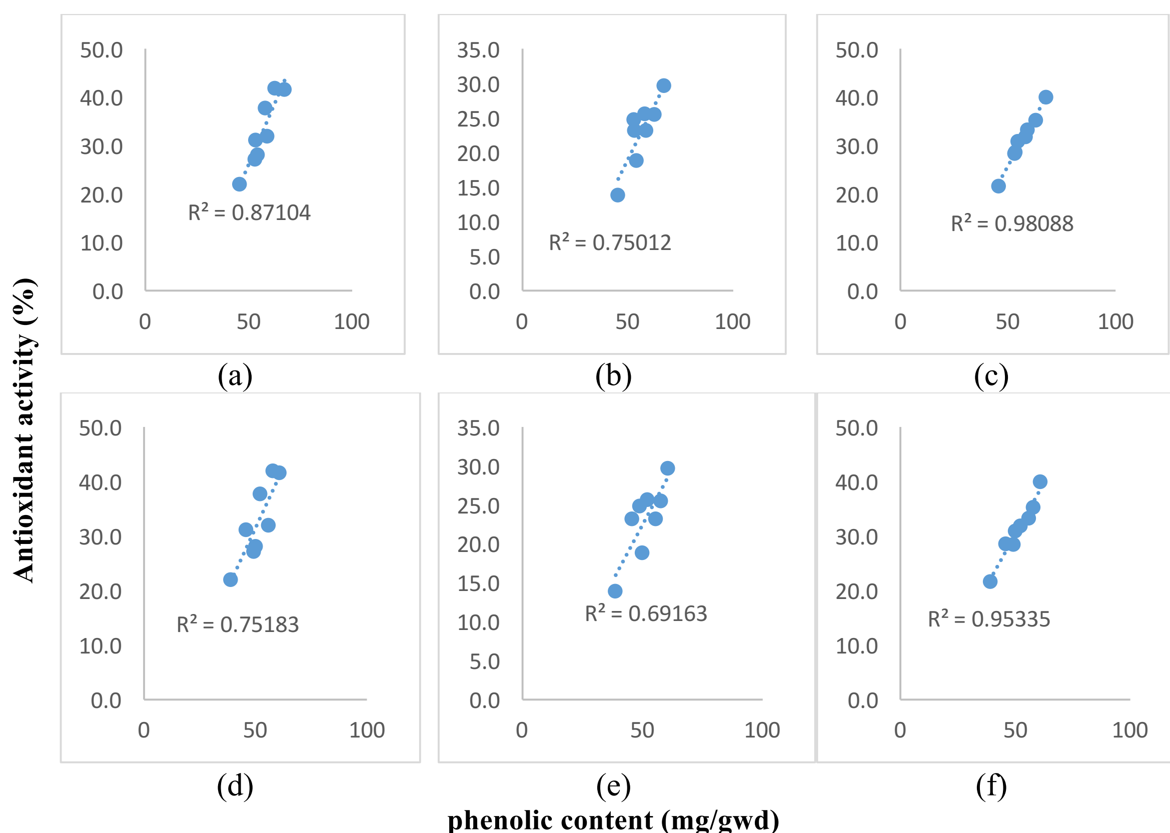


Figure 5.7 Correlation between TPC, TCT and TFC and antioxidant activity (a): TPC and ABTS, (b): TCT and ABTS, (c): TFC and ABTS, (d): TPC and DPPH, (e): TCT and DPPH and (f): TFC and DPPH

5.3.5 Enzyme inhibition activity

In this section, the α-amylase and α-glucosidase inhibitory activity of the carob fermented with *S. cerevisiae*, was determined. Initial substrate pH at 5.5 and 7.5, and the water extract only were selected. As indicated in Figure 5.8, the carob exhibited activity against α – glucosidase to a significantly greater extent than α-amylase, and

both were more effective than Acb under the same conditions. For example, the carob extract (at 0 h) decreased α–glucosidase activity by 80 % at 50 µg/mL, which was considerably lower than a reduction of 46.4 % for α - amylase at 1.25 mg/mL. Similarly, the fermented carob extracts inhibited α–glucosidase and α-amylase activity at 86.4 and 58.9 %, respectively, which was the greatest inhibition achieved by the carob as a result of fermentation. Custodio et al., (2015) indicated that carob leave extract had inhibitory activity (97 %) to α - glucosidase greater than that (74 %) to α-amylase at the same concentration of 1 mg/mL. In contrast, germ flour extracts inhibited α-amylase activity more strongly than α-glucosidase at the same concentration (Custódio et al., 2015).

Figure 5.8 showed that fermentation duration impacted on these inhibitory activity, but initial pH of the substrates (5.5 – 7.5 did not. For α–glucosidase, inhibition by carob was not significant for the first 20 h ($p < 0.05$), but a significant decrease was apparent by the end of the process. A similar result was apparent in the α-amylase assay, where a fluctuation of around 41.9 % and 58.9 % was recorded over the first 30 h, and then a slight decrease to less than 40 % was observed at pH 5.5. Johnson et al. (2011) showed that the α-glucosidase inhibitory capacity was maintained at any time point during the fermentation of blueberry extract with *Saccharomyces bayanus*. The activity of the blueberry extracts against α-amylase, however, decreased from 103 % to 92 % over the time of the experiment (Johnson et al., 2011). Initial pH had no effect on the inhibitory properties of either of the enzymes with inhibition at the same point of time not significant ($p > 0.05$).

These findings suggested that the carob kibble fermented with *S. cerevisiae* inhibited α-glucosidase activity more effectively than α-amylase. Initial medium pH (5.5 - 7.5) has an insignificant effect on both α-glucosidase and α-amylase inhibitory capacity.

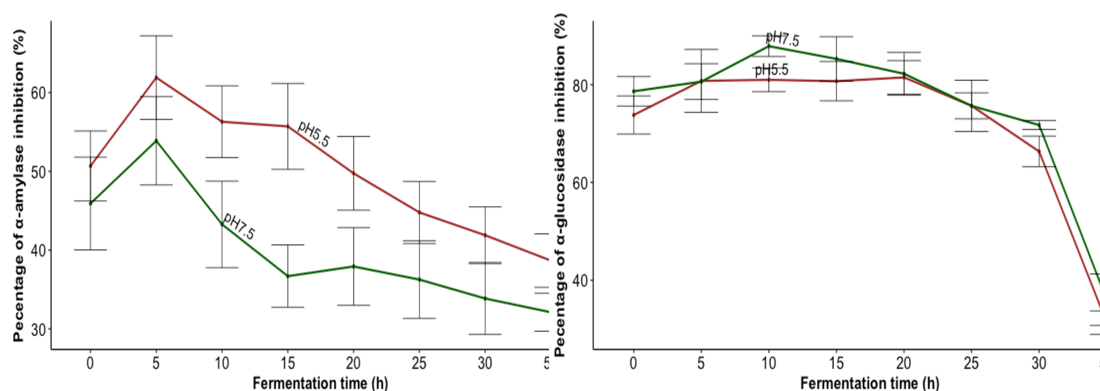


Figure 5.8 Effect of substrate pH on anti-enzyme activity

The carob kibble fermented at initial pH 5.5 and 7.5. (a) α-amylase inhibition at carob powder concentration of 1.25 mg/mL, (b) α-glucosidase inhibition at carob powder concentration of 50 µg/mL. Each value is expressed as mean ± SD (standard deviation (n=3) at each point of time.

5.3.6 Effect of sugars on antienzyme activity of the carob extracts

As presented in Chapter 3, the carob kibble used in this study contained ~ 70 % total carbohydrates, mostly sugars such as sucrose, fructose and glucose (Figure 3.5 Chapter 3). The effect of these sugars on the α-glucosidase and α-amylase inhibitory activity was also examined in the present study. A mixture of sucrose, fructose and glucose (9:2:1 respectively), which is similar ratio found in carob kibble, was added to the 15 h fermented sample, with the sugars almost completely removed, at the ratio of 1:1. Figure 5.9 shows that the sugars reduced the inhibitory activity of RT, Acb against α-glucosidase and RT against α-amylase ($p < 0.05$). For example, the α-amylase inhibitory activity of Acb reduced from 37.7 % to less than 10 %. In

contrast, the antienzyme capacity of carob against both α-amylase and α-glucosidase was not affected by these sugars ($p > 0.05$).

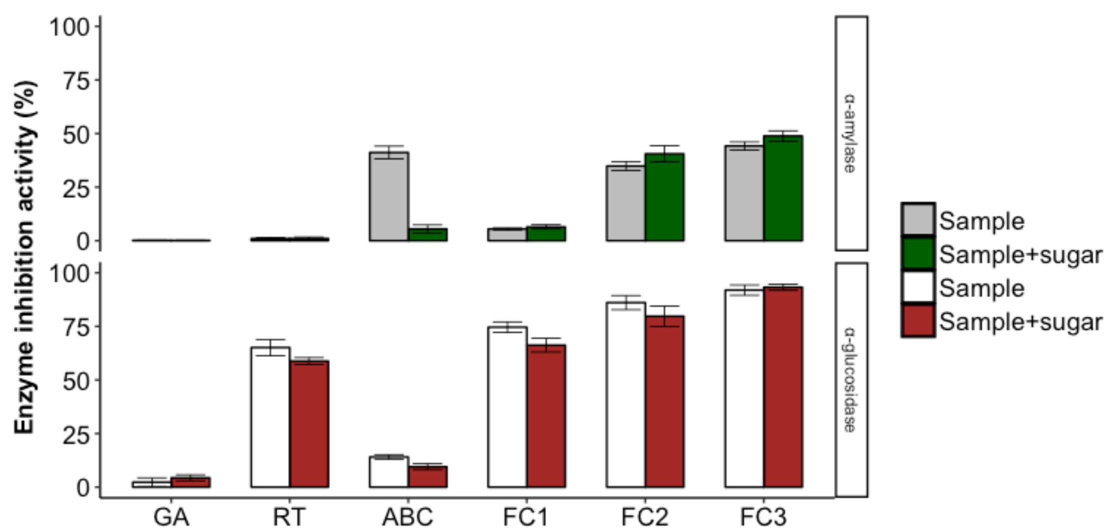


Figure 5.9 Effect of sugars on antienzyme activity

The well-known compounds including GA (gallic acid), RT (rutin) and ABC (acarbose) were used at the same concentration at 40 µg/mL for α-glucosidase and 1.25 mg/gdw α-amylase. The fermented carob extract (FC1, FC2 and FC3) were used at the three concentrations of 50, 70 and 100 µg/mL, respectively in α-glucosidase assay, and at 1.25, 2.50 and 5.00 mg/mL, respectively in α-amylase assay. All samples were added a combination of sucrose, fructose and glucose at (1:1 w/w). Each value was expressed as mean ± SD deviation (n=3) at each point of time.

5.4 Conclusion

The submerged fermentation of carob kibble using *S. cerevisiae* can improve TPC and antioxidant activity, while it had no effect on the activity against α-amylase and

α-glucosidase. At pH 5.5 and 7.5 a significant effect on TPC and antioxidant activity was apparent, with pH 7.5 showing the more effective. TCT and TFC did not differ among the test pHs. The duration of fermentation was also an important parameter, which was optimal at 15 h. This study also examined the effect of sugars (70 % in carob kibble) on the antioxidant and antienzyme activity of the fermented carob and found that the carob sugars had no effect on these activities.

Chapter 6 Optimization of fermentation conditions using response surface methodology to enhance the D-pinitol content and antioxidant activity in carob kibble

6.1 Introduction

In Chapter 3, submerged fermentation (SmF) with *Saccharomyces cerevisiae (S. cerevisiae)* was reported to improve both D-pinitol content and antioxidant activity (AA) in carob kibble. The content of D-pinitol increased by over 67.4 % from 42.9 mg/gdw to 71.8 mg/gdw after 15 h of fermentation. *S. cerevisiae* removed up to 70 % total carbohydrates in carob kibble without any negative effect on D-pinitol. The pH of the initial fermentation medium appeared not to affect consumption of sugars and concentration of D-pinitol. In contrast, initial pH did affect antioxidant capacity of the kibble. Accordingly, the initial pH of 7.5 resulted in the highest level of AA, compared to the other tested pHs which ranged from 5.5 to 7.0. The optimal duration of fermentation was established at 15 h for both D-pinitol and AA.

The conditions during fermentation are crucial for achieving optimal outcomes. According to the literature review (Chapter 2), the major factors for fermentation of carob, with *S. cerevisiae,* were inoculum concentration, temperature, amount of substrate, agitation, duration and initial medium pH of fermentation (Lima-Costa et al., 2012; Rodríguez-Sánchez et al., 2013; Ruiz-Aceituno et al., 2013; Saharkhiz et al., 2013). There may be interaction among these factors with respect to the outcomes of interest during the fermentation process. However, apart from effects of pH and fermentation duration investigated in Chapter 3, it remains unknown about effect of the other factors on the content of D-pinitol and AA of fermented carob kibble.

Response surface methodology (RSM) is an advanced technique to determine design factor settings to optimise the response of a process (Bezerra et al., 2008). As

described in Chapter 2, RSM is of a greater advantage than conventional methods with respect to optimisation of a process. Specifically, RSM can evaluate both main and interacting effects of the factors of interest with respect to outcome(s), whereas conventional methods usually evaluate effect of tested factors individually. Furthermore, with the use of central composite design (CCD), RSM optimisation is a cost-effective approach thanks to a requirement for fewer experimental runs as compared to other approaches (Bezerra et al., 2008).

The aim of this work was to use RSM with a CCD to determine optimal levels of these parameters to achieve the highest D-pinitol content and AA in a submerged fermentation of carob kibble with *S. cerevisiae*. The fermentation parameters used were inoculum concentration, temperature, amount of substrate and agitation rate.

6.2 Material and methods

6.2.1 Preparation of carob kibble

Carob kibble was prepared as described in Section 3.2.1.

6.2.2 Microorganism

Saccharomyces cerevisiae was purchased and activated as described in Section 3.2.2.

6.2.3 Experimental design using response surface method

As determined in Chapter 3, the content of D-pinitol and AA of fermented carob kibble reached the highest level at 15h of fermentation and with an initial medium pH of 7.5 (AA only). Therefore, only the four other parameters, namely inoculum amount (v/v

%), temperature (C), amount of substrate (gram) and agitation (rpm) were included in the RSM models for evaluation.

Review of the literature found the effective ranges of the mentioned fermenting parameters for *S. cerevisiae* were large (Chapter 2). For example, (Rodríguez-Sánchez et al., 2013) reported that *S. cerevisiae* can work effectively at 25 - 37°C. In the present research project, the levels of inoculum concentrations, carob substrate and agitation were set between 8 - 20 %, 15 - 39 g and 50 - 200 rpm, respectively. Employing CCD approach, actual levels of independent variables were generated (Table 6.1). Each variable had a maximum and minimum value which were coded as -1 and 1. In order to ensure accuracy of the response surface curves, low and high values outside of the range of min and maximum values were added, and coded as –α and α. The robustness of the model was also ensured by using multiple centre points (0). Given the suggested parameter levels (Table 6.1), a set of 30 experimental runs was generated using the software Design Expert (Stat-Ease Inc, USA). Each run consisted of a combination of four independent variables at different levels (Table 6.2).

Table 6.1 Coded variables and levels used in the CCD design

Factor code	Independent factors	Units	-α	-1	0	1	+α
A	Inoculum	%	8	11	14	17	20
B	Fermentation temperature	^{o}C	25	28	31	34	37
C	Amount of carob kibble	g	15	21	27	33	39
D	Agitation rate	rpm	50	87.5	125	162.5	200

6.2.4 Preparation of extractions

The extractions of D-pinitol and phenolic compounds were carried out as described in Sections 3.2.6 and Section 4.2.5, respectively.

6.2.5 Submerged ffermentation

All experiments were carried out in duplicate at initial pH 7.5 for 15 h, and strictly followed the generated designs (Table 6.2).

6.2.6 Determination of D-pinitol

The content of D-pinitol in fermented carob kibble was carried out as described in Section 3.2.7.

6.2.7 Antioxidant activity

AA of the fermented kibble was followed as presented in Section 3.2.8.

6.2.8 Optimisation verification

The statistical analyses resulted in a set of optimal values of fermentation parameters for producing the highest D-pinitol content and AA level from carob kibble under the available conditions. The analysis also generated a model which predicts the outcomes of fermentation using suggested optimal parameters. Based on the suggested optimal values of fermentation parameters, a verification experiment was run. The experimental results (D-pinitol content and AA level) were then compared to the predicted ones for verification.

6.2.9 Statistical analysis

The experimental designs (30 runs) and corresponding responses (D-pinitol content and AA) were analysed by the Design Expert (dx10) software. A multiple regression model was used to analyse the data. F ratio, correlation coefficient (R2) and lack of fit were used to determine if the model was adequate. These analyses were considered significant when $P < 0.05$ (Montgomery, 2012). The predicted values of D-pinitol content and AA (from the RSM model) were compared to the actual ones from the verification experiments at a significance level of $P < 0.01$.

6.3 Results and discussion

6.3.1 Effect of inoculum, temperature, amount of carob kibble and agitation on D- pinitol level

The results of 30 experimental runs designed by RSM showed a large variation in the contents of D-pinitol in the fermented carob kibble, ranging from 70.9 - 132.6 mg/gdw (Table 6.2). A linear model was selected for describing the combined effects of inoculum, temperature, amount of carob kibble and agitation on the level of D-pinitol (Equation 6.1). This model and its terms are significant because the values of F and p are 23.38 and < 0.001 respectively (Table 6.3). The "Lack of Fit F-value" of 3.18 and p = 0.1014 indicates the model was adequate. The suitability of model is determined by the maximum values of R-Squared, Adjusted R-Squared and Predicted R-Squared and minimum value of PRESS. In this case, these values are 0.7891, 0.7553, 0.6817 and 2591.71, respectively (Table 6.3). The "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this model, a ratio of 19.071 indicates

an adequate signal. The "Pred R-Squared" of 0.6817 is in reasonable agreement with the "Adj R-Squared" of 0.7553, the difference is less than 0.2 (Table 6.3)

Equation 6.1 $Y_{D\text{-pinitol}} = 99.5 + 1.69*A - 0.16*B - 16.13*C + 2.13*D$

Where A = inoculum (%), B = temperature (^{o}C), C = amount of carob kibble (g) and D = agitation (rpm)

Table 6.2 Levels of independent variables and corresponding experimental results of D-pinitol and antioxidant activity

	Independent factors				Dependent responses	
Run	A	B	C	D	D-pinitol (mg/gdw)	Antioxidant activity (%)
1	20	31	27	125	110.5	61.87
2	17	34	33	87.5	82.24	53.21
3	14	31	27	50	100.4	58.95
4	14	31	27	125	103.8	56.10
5	14	31	39	125	70.94	43.29
6	14	31	27	125	100.1	55.16
7	14	37	27	125	85.94	50.78
8	17	28	21	87.5	105.1	61.19
9	17	34	33	162.5	80.59	53.11
10	17	28	33	162.5	87.54	49.02
11	14	31	15	125	117.5	68.19
12	11	28	21	162.5	122.3	64.30
13	17	28	33	87.5	85.20	48.35
14	11	28	21	87.5	120.1	50.49
15	11	28	33	162.5	76.92	36.85
16	11	34	33	162.5	83.03	46.89
17	11	28	33	87.5	86.58	47.28
18	17	28	21	162.5	125.5	61.09
19	14	31	27	200	98.68	56.71
20	14	31	27	125	110.2	49.61
21	8	31	27	125	100.3	38.42
22	11	34	33	87.5	72.69	42.51
23	14	25	27	125	80.56	49.81
24	14	31	27	125	98.27	56.32
25	11	34	21	87.5	109.5	48.05
26	17	34	21	87.5	113.3	55.06

27	17	34	21	162.5	132.6	46.30
28	11	34	21	162.5	120.6	57.88
29	14	31	27	125	97.35	48.74
30	14	31	27	125	106.3	46.82

Table 6.3 Model summary statistics

Source	**D-pinitol**	**Antioxidant activity**
Selected model	Linear	Linear
F-value	23.38	8.27
p-value	<0.0001	0.0002
Lack of fit		
F-value	3.18	2.29
p-value	0.1014	0.1825
R-Squared	0.7891	0.5696
Adj R-Squared	0.7553	0.5007
Pred R-Squared	0.6817	0.3539
Adeq Precision	19.071	10.050
PRESS	2591.71	962.65
Std. Dev.	8.29	5.02
Mean	99.48	51.94
C.V. %	8.33	9.75

The effect of these independent variables on D-pinitol level during the fermentation and the corresponding regression coefficient in the selected model were presented in Table 6.4. As can be clearly seen only the parameter of amount of substrate had a significant effect on the D-pinitol content ($p < 0.05$), while no influence of other parameters, namely, inoculum, temperature and agitation rate ($p > 0.05$) was apparent.

Inoculum was one of the parameters used to evaluate the effect of submerged fermentation with *S. cerevisiae* on enrichment of D-pinitol in carob kibble. The ANOVA results show that the amount of inoculum (8 – 20 %) had no significant effect on the content of D-pinitol in carob kibble during fermentation ($p = 0.3261$).

Temperature highly influences the growth and development of microorganisms, which may result in effect on D-pinitol level during the treatment process. In the present study, fermentation temperature, another parameter, was investigated. A range of temperatures from 25 - 37^0C which are suitable for *S. cerevisiae* growth have been reported (Turhan et al., 2010b; Ruiz-Aceituno et al., 2013; Saharkhiz et al., 2013). As with inoculum, temperature was not significantly associated with D-pinitol level during fermentation ($p = 0.9244$). Although temperature was important for development of yeast and production of final products, the results of this study could be because the temperature selected was outside the points that affected the content of D-pinitol in carob kibble fermented with *S. cerevisiae*. However, the range of temperatures fit well with the peak biological activity of the yeast and it may be that a specific optimal temperature is not identifiable. Figure 6.1 (a, c and d) confirmed that temperature did not interact with the other parameters studied.

Another parameter investigated in this study was amount of substrate. The amount of solid substrate in *S. cerevisiae* submerged fermentation was reported to have effect on quantity and quality of final products (Saharkhiz et al., 2013). An amount of carob sample from 15 - 39 gram was used as solid substrate for all runs. The model of this study showed this amount of carob kibble had a significant effect on D-pinitol level ($p < 0.0001$) (Table 6.4). In addition, this association was negative (CE = - 16.13) (Table 6.4). Figure 6.1 (b, d and e) confirmed the negative relationship between amount sample and response, in which D-pinitol level increased as amount of the fermented carob decreased. As previously reported, carob contained high level of tannins, mostly condensed tannin with a range from 4.4 to 43.7 mg/gdw depending on carob tree cultivars and climate regions and solvents of extract (Kumazawa et al., 2002; Makris

& Kefalas, 2004; Custódio et al., 2011). This study found that total condensed tannin increased from 20 % after the *S. cerevisiae*-treated process (Fig. 5.2). Tannins are potentially antimicrobial agents which could inhibit the growth and development of microrganisms (Rauha et al., 2000; Min et al., 2008; Colak et al., 2010). The increase in the level of tannins could inhibit the growth and development of *S. cerevisiae*, therefore negatively affecting D-pinitol level.

The last parameter considered in this study was agitation rate. Because *S. cerevisae* is an aerobic organism, rate of agitation during the incubation was considered. An agitation rate from 50 - 200 rpm was used as presented in Table 6.1. As can be seen in the Table 6.4, agitation rate did not significnatly affect D-pinitol content (p = 0.2199).

Table 6.4 ANOVA for response surface linear model and repression coefficients

Source	D-Pinitol			Antioxidant activity		
	Coefficient Estimate (CE)	F value	p-value Prob > F	Coefficient Estimate (CE)	F value	p-value Prob > F
Model	-	23.38	< 0.0001	-	8.27	0.0002
Intercept	99.48	-	-	51.94	-	-
A-Inoculum	1.69	1.00	0.3261	3.17	9.38	0.0052
B-Fermentation temperature	-0.16	0.91	0.9244	-1.15	1.24	0.2765
C-Amount of carob kibble	-16.13	90.92	< 0.0001	-4.71	20.72	0.0001
D-Agitation rate	2.13	1.58	0.2199	1.37	1.75	0.1975
Lack of fit	-	3.18	0.1014	-	2.29	0.1825

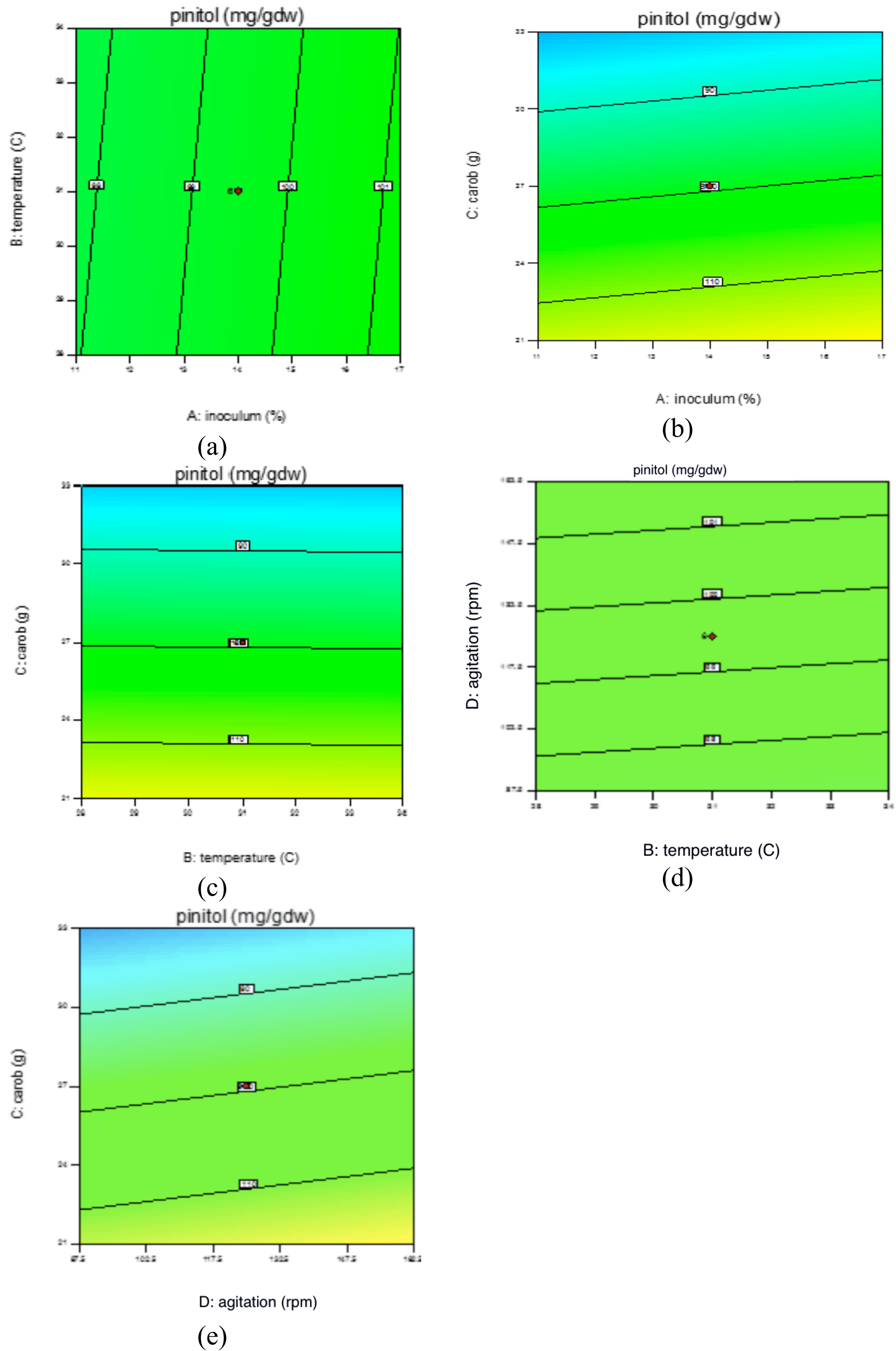


Figure 6.1 Contour plots illustrating the interactive effects inoculum, temperature, amount of kibble and agitation on D-pinitol

6.3.2 Effect of inoculum, temperature, amount of carob kibble and agitation on antioxidant activity

In a similar way to D-pinitol, phenolic antioxidants are other important bioactive compounds in carob pod (Kumazawa et al., 2002; Makris & Kefalas, 2004; El Hajaji et al., 2011; Roseiro et al., 2013a). These compounds, for example, can inhibit over 60 % the free radical used at 25µg/mL of carob pod crude polyphenols (Kumazawa et al., 2002).

The results in Chapter 5 showed that *S. cerevisiae* significantly increased antioxidant activity in carob kibble after fermentation (Fig. 5.5). This chapter determined the optimal fermenting conditions for the highest antioxidant activity of carob kibble. As presented in Table 6.2, antioxidant activity of the fermented carob ranged from 38.4 to 68.2 % inhibition of the free radical used. A linear model was also selected for describing the interactive effects of inoculum, temperature, amount of carob kibble and agitation on the level of this activity (Equation 6.2). This model is significant because the values of F and p are 8.27 and 0.0002 (less than 0.05), respectively (Table 6.4). The "Lack of Fit" showing the pure error is not significant due to an F value of 2.29 and $p = 0.1825$ ($p > 0.05$) (Table 6.3). The fit model is required, so that non-significant “Lack of Fit” is needed. The suitability of model is determined by the maximum values of R-Squared, Adjusted R-Squared and Predicted R-Squared, and minimum value of PRESS. Although the value of R-Squared ($R^2 = 0.5760$) is lower than that of remaining models, the values of Adjusted R-Squared and Predicted R-Squared and PRESS, 0.5007, 0.3539 and 962.65 were the best. "Adeq Precision" which measures the signal to noise ratio was 10.050 (greater than 4), indicating the

adequate model. The "Pred R-Squared" of 0.3539 is in reasonable agreement with the "Adj R-Squared" of 0.5007, the difference is less than 0.2 (Table 6.3).

Equation 6.2 $$Y_{antioxidant\ activity} = 51.9 + 3.17*A - 1.15*B - 4.71*C + 1.37*D$$

where A = inoculum (%), B = temperature (^oC), C = amount of carob kibble (gram) and D = agitation (rpm)

Table 6.4 presents the effect of these independent parameters and their combinations on the antioxidant activity of the fermented carob kibble. The two parameters of the amount of inoculum and amount of substrate had a significant effect on this activity ($p < 0.05$). In constrast, temperature and agitation rate had no influence on this activity during fermentation ($p > 0.05$). A positive relationship between inoculum level and antioxidant activity was observed ($p = 0.0052$, CE = 3.17) (Table 6.4). This relationship was again confirmed in Figure 2 (a, b and d). However, Table 6.4 combined with Figure 6.2 (c, d and f) showed that the association between amount of substrate and antioxidant acitivity was statistically negative ($p = 0.0001$, CE = - 4.71). Figure 6.2 d illustrates the combined significant effect of inoculum and substrate amount ($p < 0.05$), where as inoculum of yeast increased conrresponding amount of kibble decreased, its antioxidant capacity increased.

As demonstrated in previous studies, production of catalytic enzymes by microbials results in improvement of release of simple and active phenolics in plants (Moore et al., 2007; Đorđević et al., 2010). Due to this antioxidant activity in the samples increased as amount of inoculum increased. However, if tannin is concentrated in the medium a negative effect on the growth and development of yeast can be observed. This may result in a negative effect on antioxidant activity in the extract.

Both temperature and agitation rate showed no effect on antioxidant activity. The range of temperatures (25 - 39°C) is the most suitable with the peak biological activity of the yeast. This study may however be that a specific optimal temperature is not identifiable for the changes in antioxidant activity in the fermented carob. It could be thus concluded that there was no difference in antioxidant activity in carob kibble during the 15 h of fermentation at temperature from 25 – 39^{0}C.

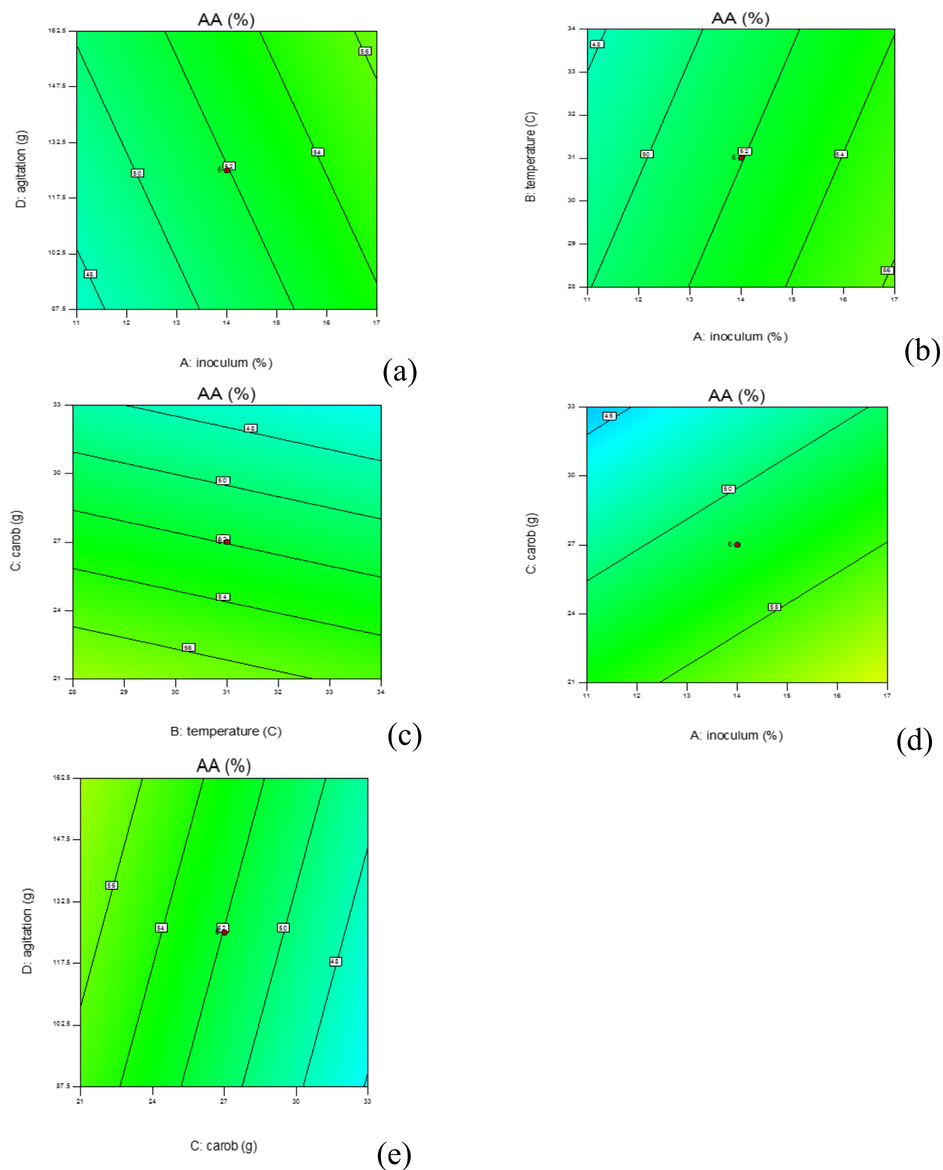


Figure 6.2 Contour plots illustrating the interactive effects inoculum, temperature, amount of kibble and agitation on antioxidant activity

6.3.3 Verification of the RSM model

The values of independent variables predicted from RSM models to show the maximum D-pinitol level and maximum antioxidant activity in the fermented carob samples were: A 17 %, B 28^0C, C 21 g, D 162.5 rpm. The responses achieved 115.7 mg/gdw of D-pinitol and 60.9 % inhibition of antioxidant acitivty.

Actual experiments showed 96.2 mg/gdw of D-pinitol and 71.5 % antioxidant activity of the fermented carob kibble under the conditions suggested. The experimental results were not significantly different from that of the predicted values ($p > 0.05$).

6.4 Conclusion

RSM suggested fermentation conditions for some parameters to produce a carob product containing the highest D-pinitol content and antioxidant activity. Although three of the four parameters selected, namely inoculum size, temperature and agitate rate showed no significant effect and interaction, D-pinitol content increased from 71.8 to 96.2 mg/gdw as compared to the non-RSM fermentation ($p < 0.05$). Antioxidant activity of the carob fermented under the conditions suggested by the RSM was not different at 71.5 %, compared to 66.2% in the non-RSM fermented carob. Importantly, RSM improved a yield resulting in a carob product rich in both D-pinitol and with a high antioxidant activity.

Chapter 7 General conclusions and future recommendations

7.1 General conclusions

The present study aimed to develop a biological approach to improve the contents of bioactive compounds and bioactivity in carob kibble. Specifically, the aims were to develop a submerged fermentation method to enrich D-pinitol content, TPC and to increase associated bioactivity in the fermented carob kibble.

With respect to this work, a number of conclusions can be made:

The submerged fermentation that was developed is a simple and effective technique to increase D-pinitol in carob kibble. *S. cerevisiae* is a more appropriate organism than *L. plantarum* to remove unwanted water-soluble compounds in the carob solution. The *S. cerevisiae* fermentation performed at 30^0C and 15 h was optimal in achieving the greatest D-pinitol content and removing the most sugar. A range of initial substrate pHs (5.0 – 7.0) did not significantly influence the fermentation process. The content of D-pinitol increased by 67.4 % in the fermented product. Total carbohydrate (70 %) was removed as a result of the fermentation.

The effect of fermentation on TPC and their related activity in carob kibble has not been previously reported. The submerged fermentation using *S. cerevisiae* was found to significantly improve the TPC and antioxidant activity in carob kibble. In contrast, *L. plantarum* did not influence these outcomes of the fermentation. The *S. cerevisiae* fermentation was carried out at the original carob pH of 5.0 and was optimal at 15 h fermentation time. Although the combined culture of *S. cerevisiae* and *L. plantarum* also showed a similar increase in TPC and associated bioactivity, this was achieved at 20 h of fermentation. In addition, the carob kibble was identified as an important source of natural α – glucosidase inhibitors. The fermentation using either *S.*

cerevisiae or *L. plantarum* did not show an effect on anti α – glucosidase activity in carob kibble.

Initial pHs (5.5 - 7.5) of the *S. cerevisiae* fermentation significantly affected TPC and antioxidant activity, but not TCT and TFC in the carob extracts. A pH of 7.5 was the most effective in improving the TPC and antioxidant activity in the carob kibble. Similarly, anti α-glucosidase activity did not change over the fermentation at a range pH 5.5 - 7.7. These results were similar in a α- amylase assay. Time is a key factor in the fermentation process. Our research identified the optimal time for the *S. cerevisiae* fermentation at 15 h, at which the fermented kibble reached the highest TPC and its bioactivity.

In order to target a carob product rich in D-pinitol content and strong antioxidant activity, the response surface methodology was used to optimise the submerged fermentation with *S. cerevisiae*. Of the four fermentation parameters, inoculum size and amount of carob kibble were shown to have interactions affecting the process. The content of D-pinitol in the fermented carob kibble improved dramatically, but DPPH free radical scavenging activity increased slightly.

7.2 Future recommendations

The submerged fermentation using *S. cerevisiae* is a practical solution to improve nutritional value and health benefits of carob kibble. In the present study, the technique was carried out on a laboratory scale using 60 g carob kibble in a 500-mL flask. This tool is also considered to be cheap, simple and environmentally friendly. Scaling up the technique into a pilot or commercial plant requires further work and improvements of yields may also be possible in this process. A larger scale may result in a commercially viable carob product and economic advantages to carob growers.

Since carob kibble is rich in fermentable sugars such as sucrose (32 %), fructose (12 %) and glucose (%), it will be a good substrate for the development and growth of many organisms. Therefore, identification of other microbial strains that are able to consume interfering compounds in D-pinitol and more interestingly improve D-pinitol content in carob kibble are feasible.

Carob kibble contains a high level of dietary fibre at 75 %, consisting of mostly insoluble components. Plant dietary fibre is reported to be positively affected by fermentation. Further study should investigate changes in carob fibre compounds such as pectin, hemi-cellulose, cellulose and lignin during fermentation. Several functional properties such as water-holding capacity, bile acid-binding capacity, glucose absorption capacity and cation-exchange capacity of the carob fibre should be considered.

Plant α-glucosidase and α-amylase inhibitors are of interest because of their negligible side effect in humans. One or more α-glucosidase inhibitors present in the

Australian carob kibble have still not been identified. They are soluble in both acetone 70 % and preferentially in water, and are not phenolic compounds. The submerged fermentation with *S. cerevisiae* and *L. plantarum* have no effect on the inhibitory capacity of carob kibble against α-glucosidase over the first 30 h, but the activity decreased significantly as the incubation time increased. A further study should be undertaken to identify these compounds and their characteristics. Carob kibble will be an important plant α-glucosidase inhibitor source for food and pharmaceutical industry.

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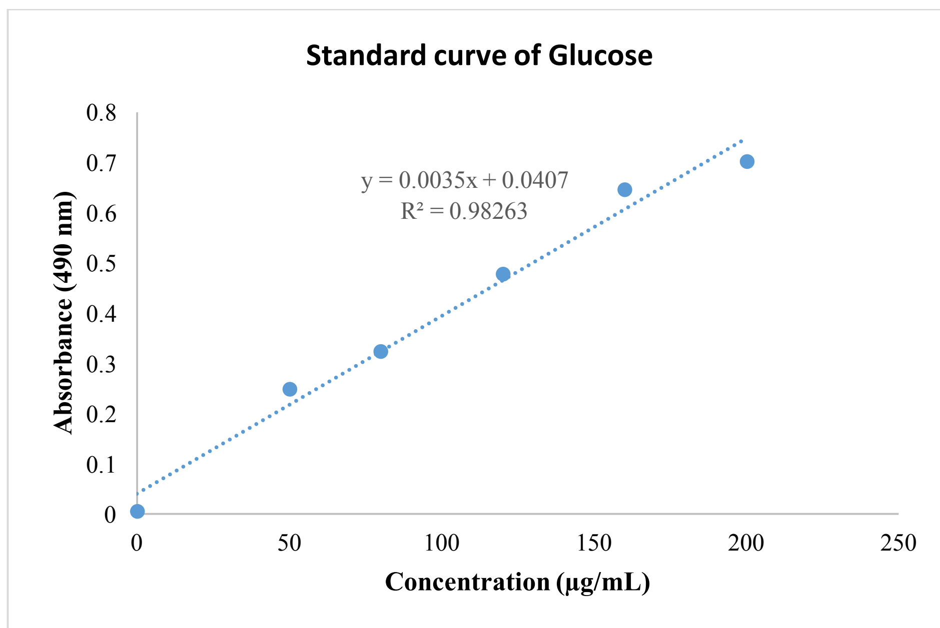
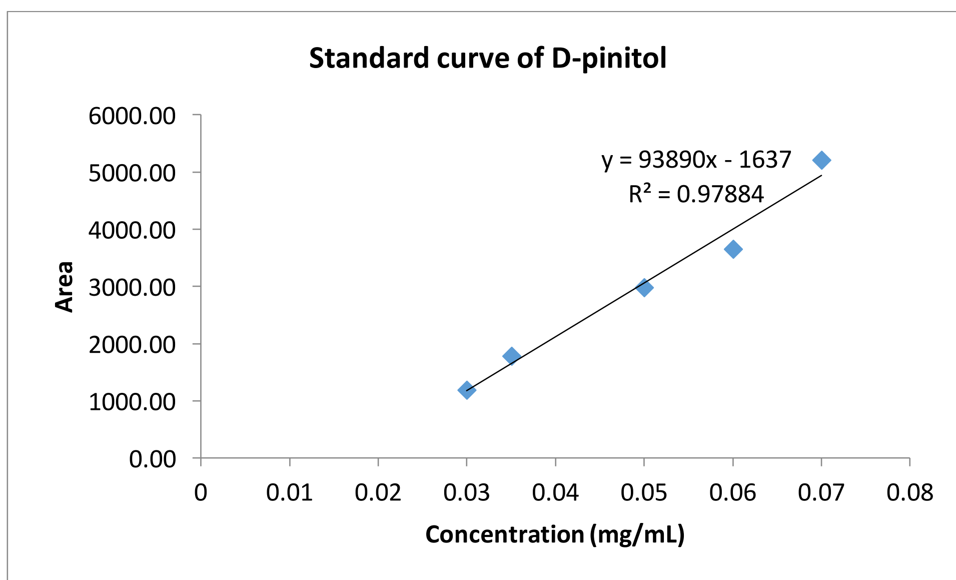
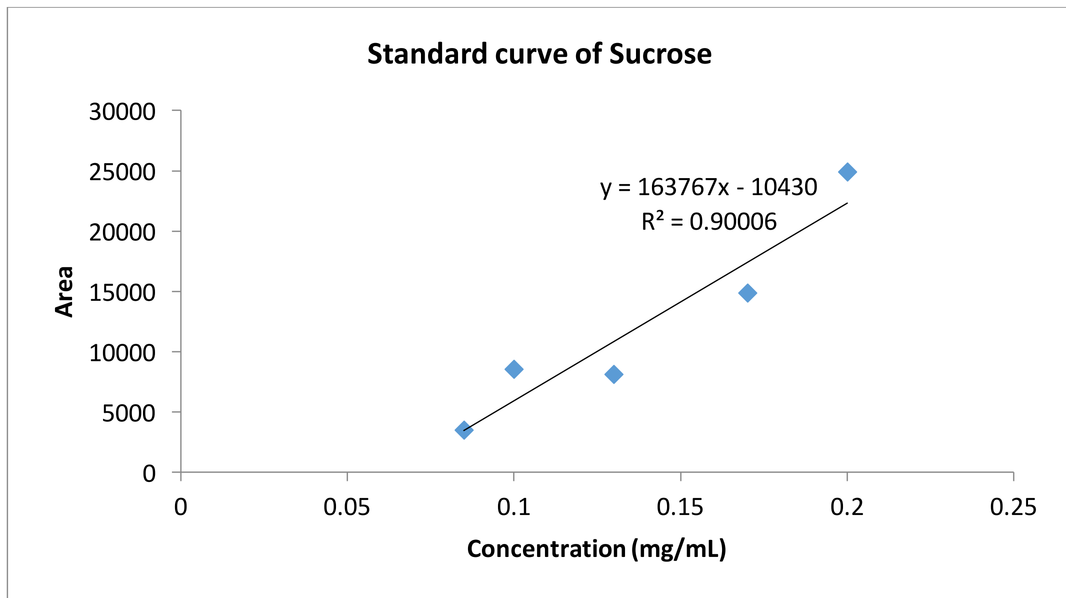
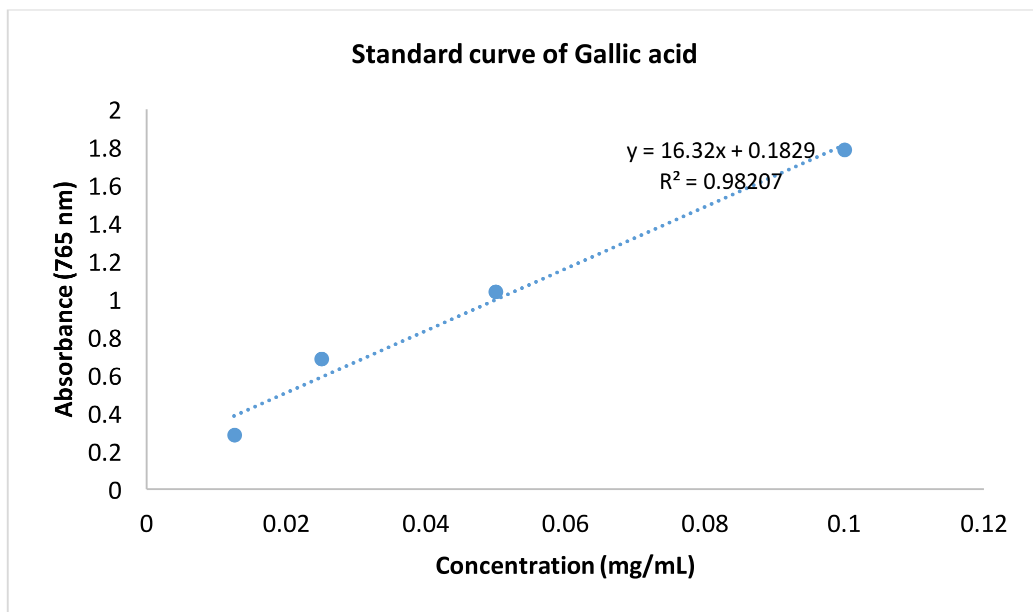
Appendices

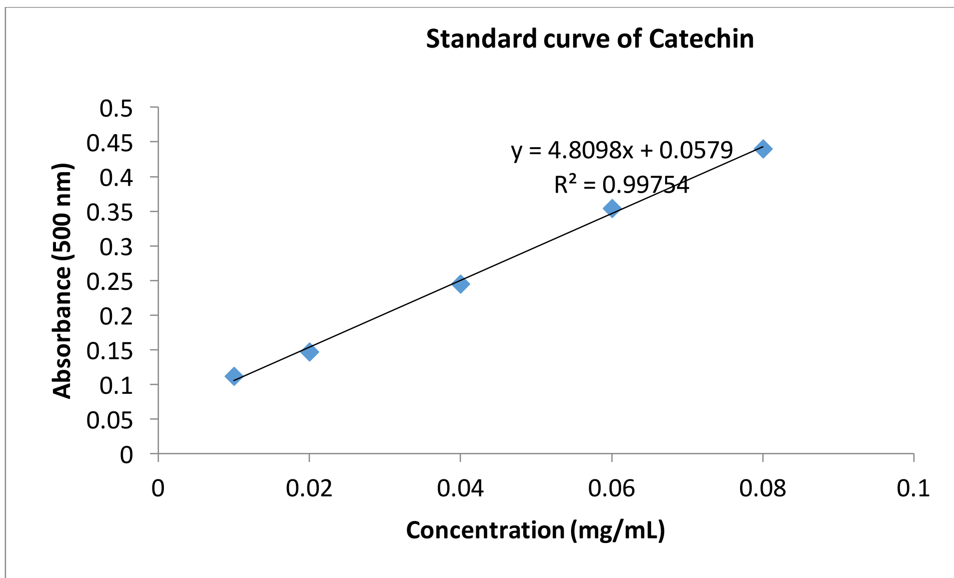
Appendix 1 Standard curves

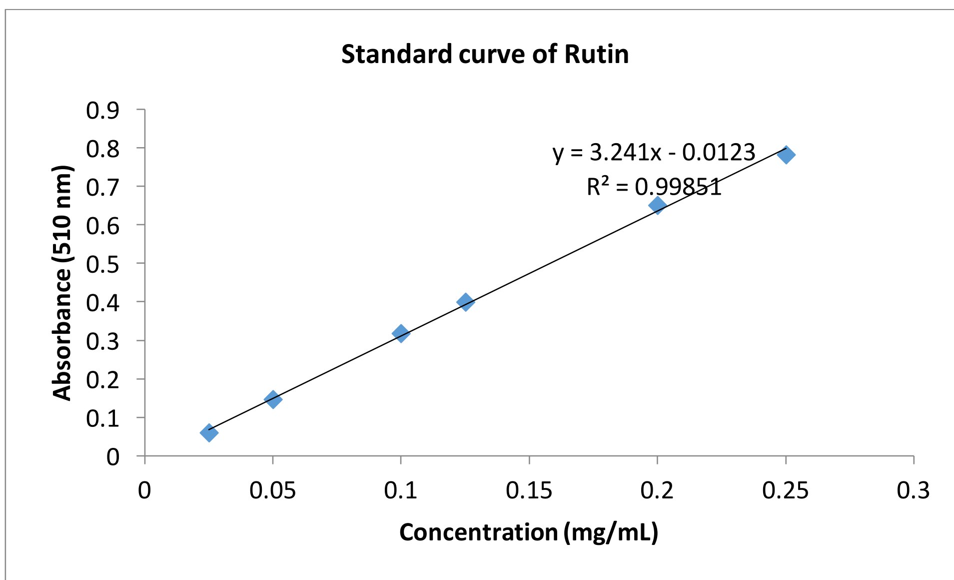




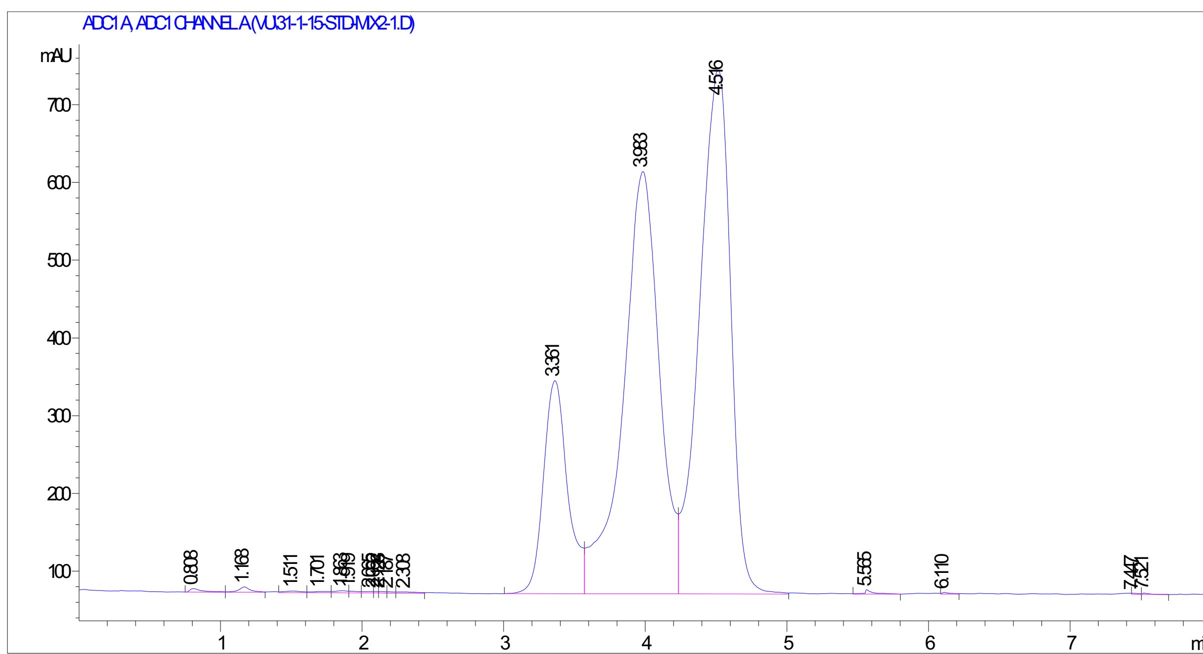

Standard curve of Sucrose
30000
25000
20000
15000
10000
5000
0
Area
y = 163767x - 10430
R² = 0.90006
0
0.05
0.1
0.15
0.2
0.25
Concentration (mg/mL)


Standard curve of Gallic acid
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (765 nm)
y = 16.32x + 0.1829
R² = 0.98207
0
0.02
0.04
0.06
0.08
0.1
0.12
Concentration (mg/mL)


Standard curve of Catechin
y = 4.8098x + 0.0579
R² = 0.99754
Absorbance (500 nm)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.02
0.04
0.06
0.08
0.1
Concentration (mg/mL)

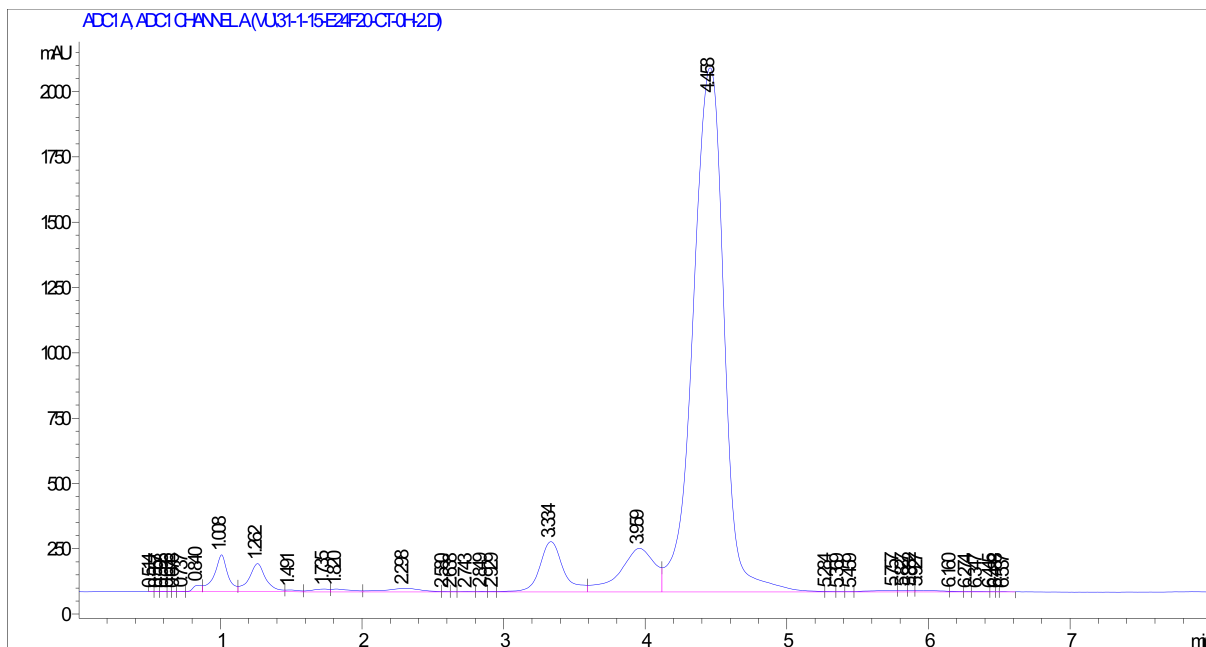

Standard curve of Rutin
y = 3.241x - 0.0123
R² = 0.99851
Absorbance (510 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.05
0.1
0.15
0.2
0.25
0.3
Concentration (mg/mL)

Appendix 2 HPLC chromatogram of D-pinitol and sugars

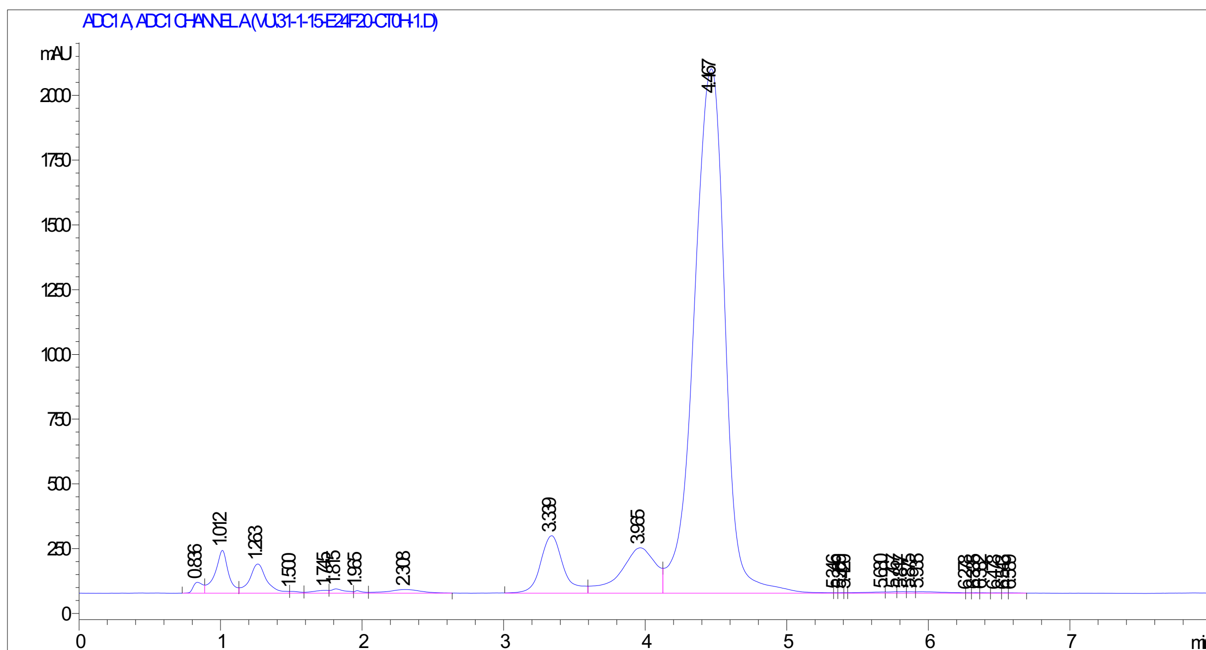


HPLC chromatogram of D-pinitol, glucose and fructose, and sucrose

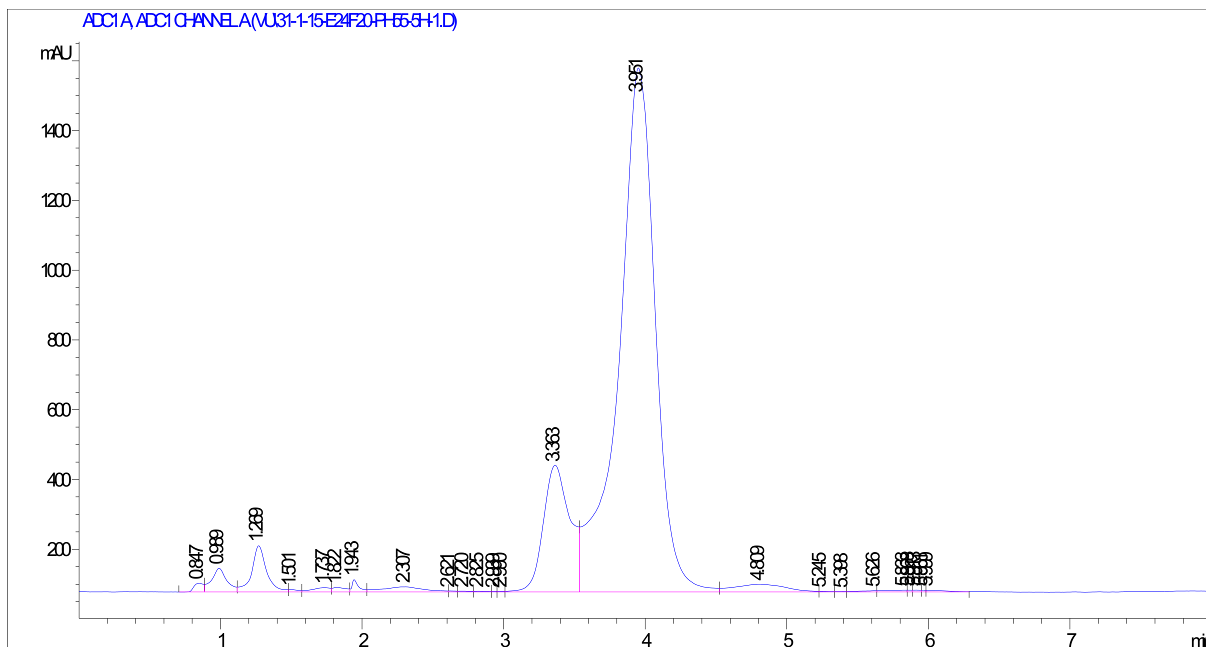
Peaks 3.361: D-pinitol, 3.938: glucose and fructose, and 4.316: sucrose



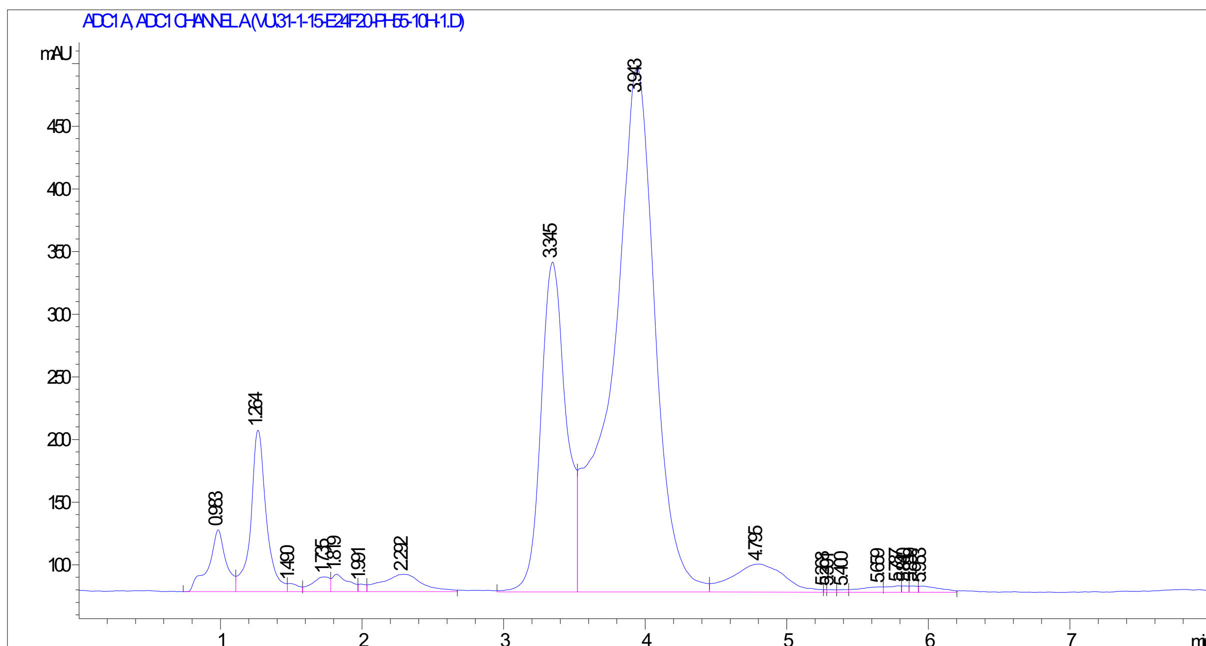
HPLC chromatogram of the unfermented kibble



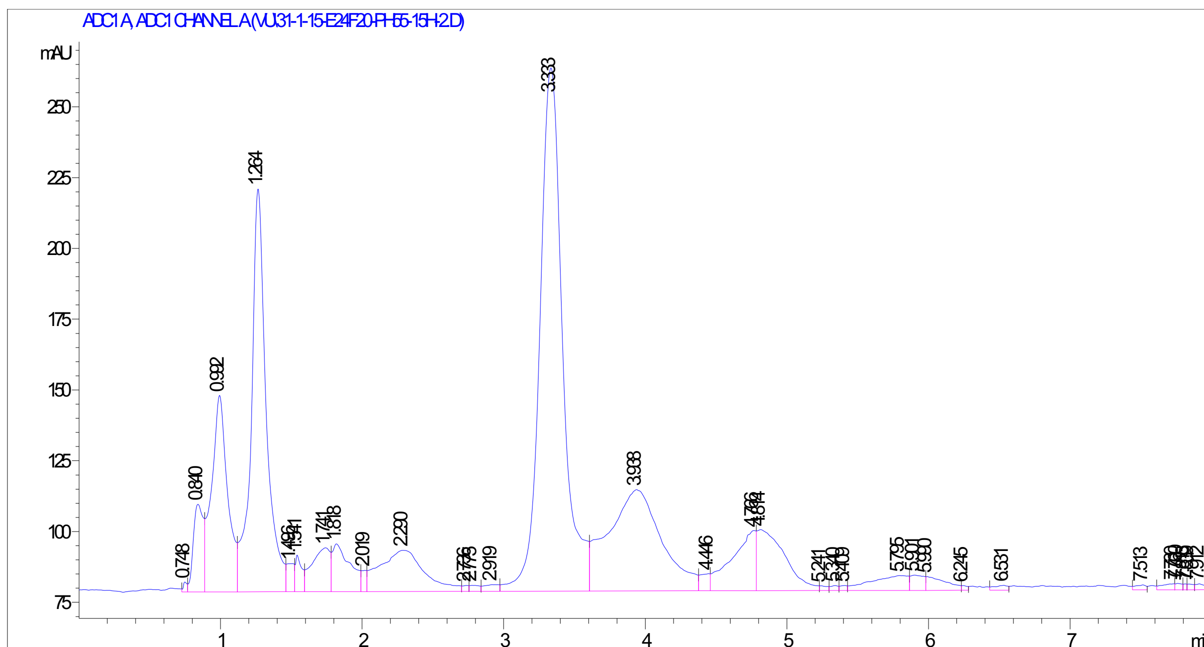
HPLC chromatogram of the fermented kibble at 0 h



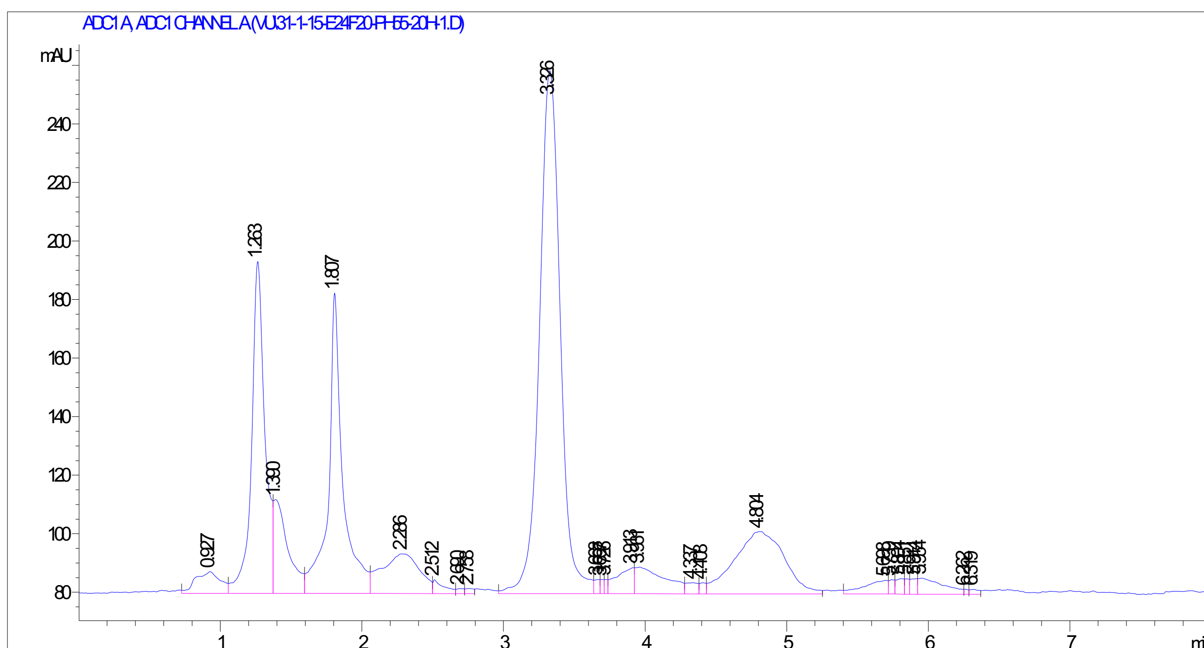
HPLC chromatogram of the fermented kibble at 5 h



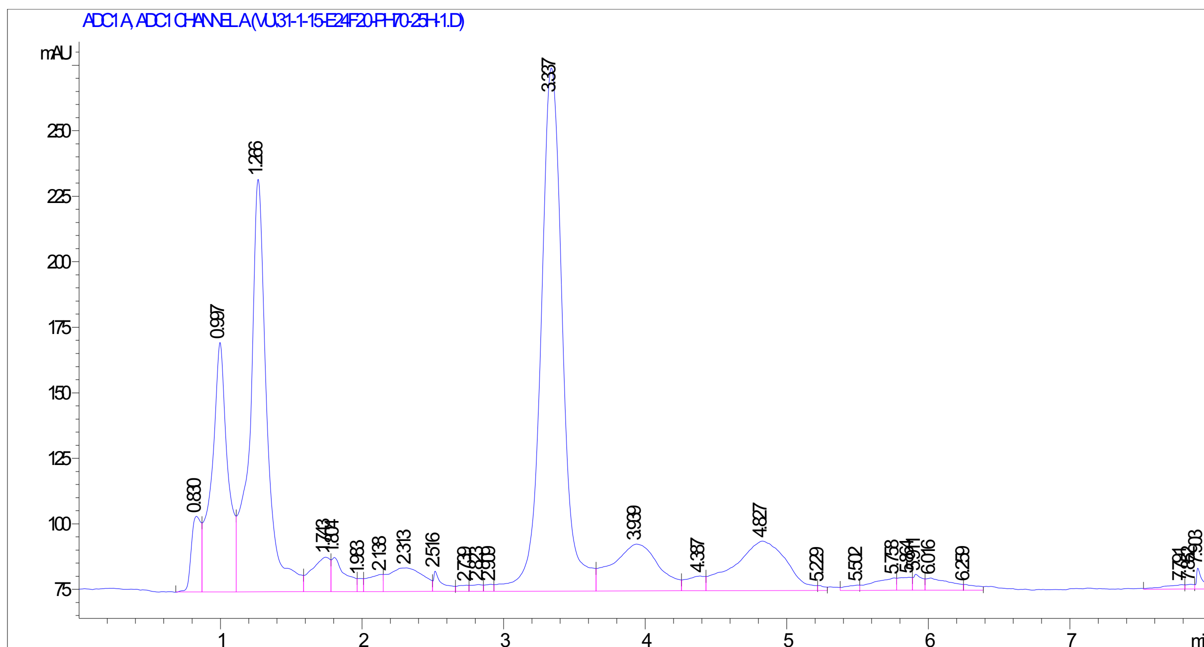
HPLC chromatogram of the fermented kibble at 10 h



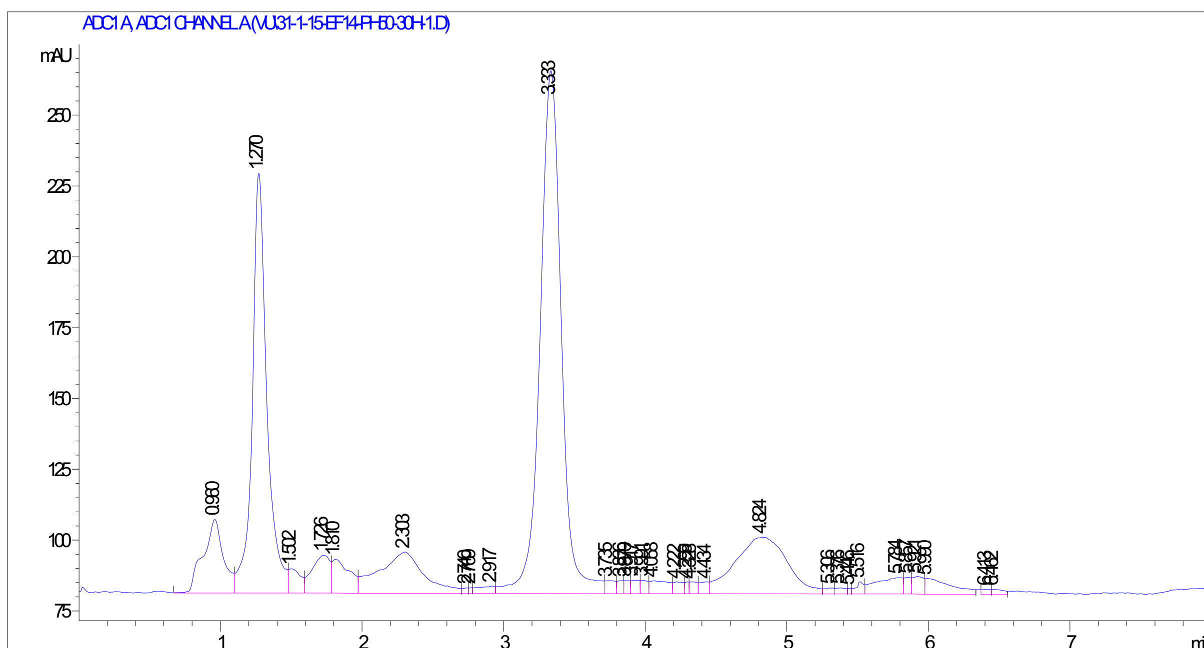
HPLC chromatogram of the fermented kibble at 15 h



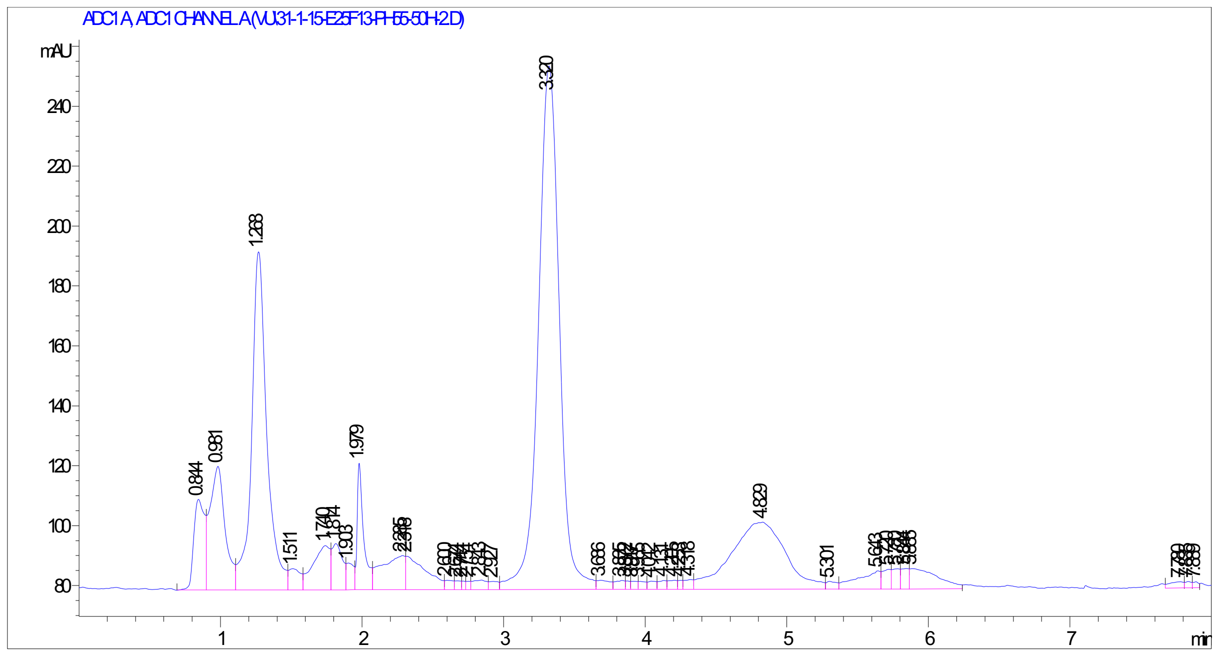
HPLC chromatogram of the fermented kibble at 20 h



HPLC chromatogram of the fermented kibble at 25 h



HPLC chromatogram of the fermented kibble at 30 h



HPLC chromatogram of the fermented kibble at 50 h

Appendix 3 Predicted value of D-pinitol regarding models

Final Equation in Terms of Coded Factors:

pinitol =
+99.48
+1.69 * A
-0.16 * B
-16.13 * C
+2.13 * D

Final Equation in Terms of Actual Factors:

Pinitol =
+158.74828
+0.56492 * inoculum
-0.054070 * temperature
-2.68861 * carob
+0.056762 * agitation

AA-Final Equation in Terms of Coded Factors:

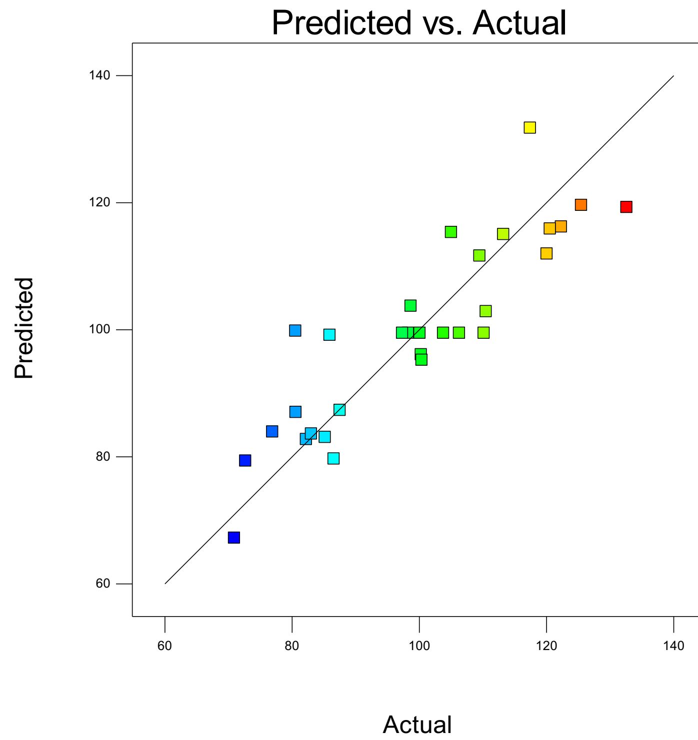
AA =
+51.94
+3.17 * A
-1.15 * B
-4.71 * C
+1.37 * D

Final Equation in Terms of Actual Factors:

AA =
+65.67034
+1.05523 * inoculum
-0.38338 * temperature
-0.78432 * carob
+0.036498 * agitation

Design-Expert® Software
pinitol

Color points by value of
pinitol:
132.622
70.9357



Design-Expert® Software
AA

Color points by value of
AA:
68.1907
38.4241