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Ph.D. in Food Engineering

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REPUBLIC OF TURKEY

GAZİANTEP UNIVERSITY

GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES

BIO-REFINERY OF PISTACHIO PRODUCTION BY-PRODUCTS

Ph.D. THESIS

IN

FOOD ENGINEERING

BY

HATİCE NEVAL ÖZBEK

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in

Food Engineering

Gaziantep University

Supervisor

Prof. Dr. Fahrettin GÖĞÜŞ

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Hatice Neval ÖZBEK

ABSTRACT

BIO-REFINERY OF PISTACHIO PRODUCTION BY-PRODUCTS

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The objective of this thesis was to perform bio-refinery of pistachio production by-products, pistachio hull (PH) and shell (PS). For this purpose, microwave-assisted solvent extraction (MASE) of polar and non-polar compounds from PH was performed. The results showed that the pistachio hull extracts were rich in bioactive compounds such as phenolics, antioxidants and tocopherols. Also, MASE has been shown to be advantageous in extracting bioactive compounds with higher yield, antioxidant activity, and reduced extraction time and solvent consumption. Alkali pre-treatment of PS was carried out by using microwave and/or ultrasound-assisted methods to fractionate PS into hemicellulose solid precipitate and cellulose rich solid phase. The pre-treatment conditions were optimized to obtain maximum hemicellulose and cellulose recoveries and the optimum pre-treatment conditions were determined as ultrasound power of 640 W, microwave power of 248 W and NaOH concentration of 1.58 N for sequential ultrasound-microwave-assisted alkaline pre-treatment (SUMAAP). Under these conditions, the hemicellulose and cellulose recoveries were 66.14% and 90.78%, respectively. SUMAAP increased the hemicellulose recovery with a significant reduction in processing time and amounts of chemical used for the fractionation of PS into hemicellulose and cellulose. Finally, enzymatic hydrolysis of the pre-treated cellulose-rich fractions was performed to produce fermentable sugars and the highest glucose yield of 95.78 mol% obtained after SUMAAP.

Key Words: Pistachio hull, Pistachio shell, Microwave-assisted solvent extraction, Alkali pre-treatment, Enzymatic hydrolysis

ÖZET

ANTEP FISTIĞI ÜRETİM ATIKLARININ BİYORAFİNASYONU

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Bu tezin amacı, antep fıstığı üretim atıkları, fıstık yumuşak kabuğu ve sert kabuğunun biyorafinasyonunu gerçekleştirmektir. Bu amaçla, fıstık yumuşak kabuğundan polar ve polar olmayan bileşiklerin mikrodalga destekli çözgen ekstraksiyonu (MASE) gerçekleştirilmiştir. Sonuçlar, fıstık kabuğu ekstraktlarının fenolikler, antioksidanlar ve tokoferoller gibi biyoaktif bileşikler açısından zengin olduğunu göstermiştir. Ayrıca, MASE'nin biyoaktif bileşiklerin ekstraksiyonunda daha yüksek verim ile antioksidan aktivite sağlayarak ve ekstraksiyon süresi ile çözgen tüketimini azaltarak avantajlı olduğu görülmüştür. Fıstık sert kabuğunun alkali ön işlemi, hemiselüloz katı çökeltisi ve selülozca zengin katı faz elde etmek üzere mikrodalga ve/veya ultrason destekli yöntemler kullanılarak gerçekleştirilmiştir. Ön işlem koşulları, maksimum hemiselüloz ve selüloz geri kazanımları elde etmek üzere optimize edilmiş ve optimum koşullar ultrason sonrası mikrodalga destekli alkali ön işlemi (SUMAAP) için 640 W ultrason gücü, 248 W mikrodalga gücü ve 1.58 N NaOH konsantrasyonu olarak belirlenmiştir. Bu koşullarda hemiselüloz ve selüloz geri kazanımları sırasıyla %66.14 ve %90.78 idi. SUMAAP, hemiselüloz kazanımını arttırmış ve ön işlem süresi ile kullanılan kimyasal miktarlarını önemli ölçüde azaltarak fıstık sert kabuğundan hemiselüloz ve selülozun ayrılmasını sağlamıştır. Son olarak, fermente olabilir şekerler üretmek için selülozca zengin fraksiyonların enzimatik hidrolizi gerçekleştirilmiş ve %95.78 ile en yüksek glikoz verimi SUMAAP'den sonra elde edilmiştir.

Anahtar Kelimeler: Fıstık yumuşak kabuğu, Fıstık sert kabuğu, Mikrodalga destekli çözgen ekstraksiyonu, Alkali ön işlemi, Enzimatik hidroliz

"Dedicated to my lovely daughter and my beloved husband"

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AcOS	Acetyl-oligosaccharides
ANOVA	Analysis of variance
BBD	Box-Behnken design
CAP	Conventional alkaline pre-treatment
CCFD	Central composite face-centered design
CSE	Conventional solvent extraction
GlcOS	Gluco-oligosaccharides
HPLC	High performance liquid chromatography
MAAP	Microwave-assisted alkaline pre-treatment
MASE	Microwave-assisted solvent extraction
PBD	Plackett-Burman design
PH	Pistachio hull
PHE	Pistachio hull extract
PS	Pistachio shell
RSM	Response surface methodology
SEM	Scanning electron microscopy
SUMAAP	Sequential ultrasound-microwave-assisted alkaline pre-treatment
TPC	Total phenolic content
UAAP	Ultrasound-assisted alkaline pre-treatment
XOS	Xylo-oligosaccharides

CHAPTER 1

INTRODUCTION

The interest for biologically active compounds from plants has increased in recent years because of their health-promoting activities and possible protection against several diseases (Garavand et al., 2017; Olas et al., 2018). The main protective effect of these plant metabolites has been attributed to the presence of phenolic compounds. Recently, using fruit and vegetable processing residues has been attracting interest by researchers as sources of bioactive compounds (Moreira et al., 2017). Phytochemicals derived from agro-industrial wastes can be used as natural antioxidants and functional food ingredients in place of their synthetic equivalents (Tumbas Šaponjac et al., 2016). Pistachio hull (PH) is one of the by-product of the industrial post-harvest processing of pistachios and accumulated in large volumes (Çelik and Demirer, 2015; Grace et al., 2016). If not processed further, this by-product can lead to environmental pollution and waste management problems. PH is a good source for protein, fat, minerals and vitamins. It is also rich in phenolic components, antioxidants and essential oil (Chahed et al., 2008; Goli et al., 2005; Ozel et al., 2004). It has been shown that PH extracts have antioxidant, antimicrobial, antiflammatory, cytoprotective and antimutagenic properties (Barreca et al., 2016; Rajaei et al., 2010). Due to its beneficial effects, PH can be an alternative source of bioactive compounds (Kilic et al., 2016). In this way, using hull as a source of biologically active compounds will increase the value of pistachio production and provide valorisation of a by-product.

The extraction of bioactive components from solid materials is generally performed using traditional methods such as maceration and Soxhlet extraction (Kua et al., 2015). However, these methods take long time and require considerable amounts of solvents (Vieira et al., 2017). In recent years, different modern extraction techniques have been replacing traditional methods such as enzyme-assisted extraction, ultrasound-assisted solvent extraction (UASE) and microwave-assisted solvent extraction (MASE) (Li et al., 2017). Among these techniques, MASE is an effective

extraction method with several advantages such as reduced solvent, time and energy consumption (Maeng et al., 2017). Several process variables (power, time, solvent composition, etc.) could influence the extraction efficiency during MASE. It is essential to investigate the effect of these variables and their interactions since they do not only effect the extraction, but also interact with each other (Li et al., 2017). The response surface methodology (RSM), that allows the optimization of all variables simultaneously and prediction of most efficient conditions, has been recently used to optimize the extraction conditions of phenolics from various plants (Filip et al., 2017; Vieira et al., 2017; Zekovic et al., 2017).

Considering the increase in world energy demand, environmental and economic issues linked to fossil fuels, researchers focus on finding alternative sustainable sources and technologies to produce fuels and chemicals (Islam et al., 2017). One of the ways to provide a sustainable supply chain is the use of renewable lignocellulosic biomass as a potential source for the generation of fuels and other bio-based products (Galia et al., 2015). Lignocellulosic materials, such as agricultural residues and food industry wastes, are the most attractive and abundant renewable biomass sources available in nature (Hassan et al., 2018; Kamalini et al., 2018). Pistachio shell (PS) is one of the example of lignocellulosic biomass which is accumulated in large quantities. Therefore, the valorisation of PS is critical for the sustainable treatment of such large-scale wastes.

The structural complexity of lignocellulosic biomass and their natural recalcitrance to hydrolysis cause inefficient conversion to fermentable sugars and other value-added products (Galia et al., 2015). Thus, several pre-treatment methods were developed to overcome these problems. Among pre-treatment methods, alkaline pre-treatment has received high attention over the years because of its many desirable features. The most important advantages of alkaline pre-treatment are possibility to operate under milder conditions and avoid the specific reactor requirement for severe conditions (Hilares et al., 2018). However, the main disadvantage of alkali pre-treatment is longer residence time which is generally recorded in terms of hours or days (Xu and Huang, 2014). Combination of alkaline pre-treatment with other techniques including ultrasound and microwave has been employed to improve

alkaline pre-treatment for different types of biomass (da Silva et al., 2016; Jin et al., 2016; Kan et al., 2018).

The ultrasound process can enhance the efficiency of hydrolysis by modifying the physical and chemical structure of biomass resulting from cavitation forces (Wang et al., 2017). It is stated that the most important features of ultrasound assisted alkali pre-treatment were the reduction in treatment time, temperature and alkali requirement (Subhedar and Gogate, 2014; Velmurugan and Muthukumar, 2012). In addition, microwave treatment is another promising process because of its thermal and non-thermal effects in an aqueous medium. Microwave irradiation provides more direct, rapid and uniform heating by direct interaction between heated material and electromagnetic field (Vani et al., 2012). It is reported that the microwave-alkali pre-treatment decreased hemicellulose and lignin content, increased the crystallinity index and surface area of the pre-treated biomass and provided higher sugar yield compared to conventional heating (Jin et al., 2016; Kan et al., 2018).

The aim of this thesis was to perform bio-refinery of pistachio processing by-products which are PH and PS to obtain bioactive compounds, cellulose rich fraction, hemicellulose solid precipitate and subsequently fermentable sugars. In detail, bioactive components were extracted from PH by MASE method and the extraction parameters such as microwave power, extraction time and solvent to sample ratio were optimized using RSM. MASE was compared with the conventional extraction methods by considering the extraction yield, extraction time, total phenolic content (TPC) and antioxidant activity of extracts, etc. Also, it was aimed to fractionate PS into hemicellulose solid precipitate and cellulose rich solid residue by using microwave and/or ultrasound-assisted alkali pre-treatment techniques. Pre-treatment conditions were investigated and optimized using RSM. The compositional analyses were carried out on the solid and liquid fractions obtained from the pre-treatments. Furthermore, the pre-treated solids were subjected to enzymatic hydrolysis to evaluate the pre-treatment efficiency and produce fermentable sugar for further valorisation to value added products.

CHAPTER 2

LITERATURE REVIEW

2.1 Pistachio Nut (*Pistacia vera* L.)

The pistachio nut (*Pistacia vera* L.) is one of the most popular tree nuts in the world. It is widely grown in saline, dry and hot regions of the Middle East, Mediterranean countries and United States (Maskan and Karatas, 1998). *Pistacia vera* L. is the only genus of more than ten in *Pistacia* species accepted by the consumer as an edible nut and has commercial value (Kashaninejad et al., 2006; Öztürk et al., 2010). Turkey is one of the main producers of pistachio nuts in the world. With 85,000 tonnes annual pistachio nut production in 2019/20, Turkey comes after USA and Iran (Table 2.1) (USDA, 2020).

Figure 2.1 Pistachio nut (*Pistacia vera* L.)

Pistachio nuts are widely consumed because of their health benefits, sensorial and nutritional characteristics (Abdolshahi et al., 2015; Grace et al., 2016). Pistachio kernels are a good source of fat (50–60%) and more than 80% of this fat is unsaturated fatty acids such as oleic, linoleic, and linolenic acids that are essential for human diet (Kashaninejad et al., 2006). Pistachios also contain proteins, carbohydrates, fibers, minerals, vitamins, and antioxidant phytochemicals like polyphenols and anthocianes (Penci et al., 2013). The amount of these components depends on the nut variety and harvesting maturity. Since pistachio nuts have lower fat content and higher protein, carbohydrate and potassium content, they are better than other nuts by considering its nutritional value (Kashaninejad and Tabil, 2011).

Protein content of pistachio kernels ranges from 19 to 31% and the globulin, albumin, glutelin and prolamin are the proteins detected in pistachio (Catalan et al., 2017; Shokraii and Esen, 1988). Pistachio kernels contain glucose, fructose, sucrose, maltose, isomaltose, cellobiose, raffinose and stachyose as free sugars (Kashani and Valadon, 1984). Many different phytochemicals are present in pistachio nut and skin. These phytochemicals include essential oils, anthocyanins, flavonoids, phytosterols and phytostanols, carotenoids, resveratrol, polyphenols, organic acids and fatty acids (Kashaninejad and Tabil, 2011). They protect cells from oxidative damage by binding metal ions, scavenging free radicals and degrading peroxides. Pistachios also contribute to maintaining a healthy and diverse gut microbiota as a source of fiber (Catalan et al., 2017). Furthermore, pistachio kernels are good source for minerals such as K, P, Ca, Mg and S (Harmankaya et al., 2014). Pistachio nut is an attractive and healthy food source by considering its nutrients and potentially protective phytochemicals.

Table 2.1 Major world pistachio producers' production quantity in metric tons, in shell basis, 2014-2020 (USDA, 2020)

Production	**2014/15**	**2015/16**	**2016/17**	**2017/18**	**2018/19**	**2019/20**
United States	233,147	122,470	406,646	272,292	447,696	331,538
Iran	230,000	210,000	153,000	225,000	52,000	205,000
Turkey	85,000	130,000	155,000	80,000	210,000	85,000
Syria	29,000	35,000	58,000	56,500	60,000	55,000
European Union	13,000	13,800	8,400	15,000	10,610	17,530
Other	0	0	0	0	0	0
Total	590,147	511,270	781,046	648,792	780,306	694,068

Pistachios are mainly consumed as a snack food after drying and roasting with or without salt as in-shell nut (Shakerardekani et al., 2011). In addition, a certain amount of pistachios is marketed as green kernels and used as an ingredient in pastries, ice creams, baked products, candies, sausages and desserts (Ling et al., 2016; Shakerardekani et al., 2011). Pistachios can be added to various food products to enhance their nutritional value, color and flavor (Kashaninejad and Tabil, 2011). In addition, pistachios are used to produce pistachio kernel oil. This oil is used as a salad dressing or gourmet oil, as well as in cosmetics and therapeutic products (Ling et al., 2016). Moreover, pistachio butter, a semi-solid paste, is produced from a limited amount of pistachio by adding some flavorings and sweeteners after grinding and roasting the pistachio kernels. This nutritious product rich in lipids, proteins,

carbohydrates and vitamins, can be used in different food products such as ice cream, candies and cakes (Taghizadeh and Razavi, 2009).

2.1.1 Pistachio Production By-products

The main processes conducted when pistachios arrive at the processing plant are: (a) dehulling, for separation of the soft hull from nuts; (b) trash and blank separation, for removing blank pistachios and trashes such as small branches and remaining leaves; (c) separation of unpeeled pistachios, for removing unpeeled and immature nuts; (d) washing, by spraying water at high pressures on the pistachios to clean the nuts; (e) drying, to reduce moisture content of nuts from 37-40% to the appropriate level; (f) split nuts separation, for separation of split nuts from non-split ones; (g) salting; (h) roasting; and (i) packaging (Kashaninejad et al., 2006). During the dehulling process, PH surrounding the nut shell is removed as a waste (Figure 2.2.a). As the pistachio contains 18% hull, the hull is the major by-product of pistachio processing (Demiral et al., 2009). Kilic et al. (2016) have been reported that this by-product constitutes nearly 3% of the total pistachio production. If this by-product is not processed further, it may cause environmental pollution and waste management problems. PH is often mixed with soil and less commonly used as feed by local livestock farmers (Grace et al., 2016). A small part of hull is also used as herbal medicine and in human foods (mainly as pistachio hull jam) (Chahed et al., 2007).

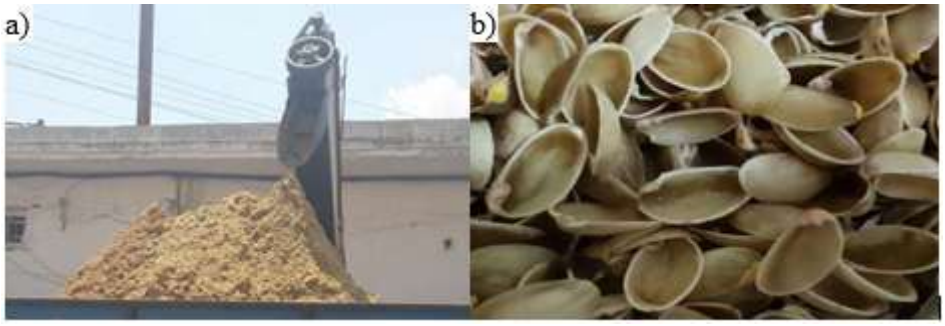

Figure 2.2 Pistachio processing by-products: hull (a) and shell (b)

A significant portion of pistachios is marketed as green kernels in Turkey. The pistachio shells are separated from the kernels and then the kernels are steam-sterilized before being sold (Ak and Açar, 1998). During this process, considering the shell/pistachio ratio (~ 45%), a large amount of pistachio shell arises as an important by-product of the pistachio production (Figure 2.2.b) (Acikalin et al., 2012). Normally, this by-product is used as a boiler fuel or in landfills

(Vijayalakshmi et al., 2011). But the combustion is not an efficient way for the evaluation of this by-product, considering its low heating value. However, promising results can be achieved by their conversion into solid, liquid and gaseous products for use as energy sources and/or raw materials for organic synthesis (Acikalin et al., 2012).

2.1.2 Chemical Composition of Pistachio Hull and Shell

PH is a rich source for protein, fat, minerals and vitamins. Also, it is a good source for antioxidants, phenolic components and essential oil (Goli et al., 2005; Ozel et al., 2004). Differences in the pistachios cultivars, harvesting, cultivation practices, kernel maturity and the dehulling process affect the nutritional value of PH (Bagheripour et al., 2008). The crude protein, acid detergent fiber, neutral detergent fiber, ether extract and ash contents of PH have been reported as 113.0 g/kg, 187.0 g/kg, 262.0 g/kg, 78.0 g/kg and 150.8 g/kg on dry matter, respectively (Bakhshizadeh et al., 2014). Grace et al. (2016) reported that 100 g of dried PH contains 3.19 g anacardic acids, 1.50 g fatty acids and 0.19 g phytosterols as major non-polar components. The carotenoid, chlorophyll and tocopherol contents were also reported as 4.93 mg, 10.27 mg and 8.83 mg per 100 g dried hull (Grace et al., 2016). The extracted essential oil yield from PH was reported in the range of 0.14 to 0.53% on dry weight basis (Chahed et al., 2007). The main components identified in the PH essential oil are α-pinene, α-terpinolene, limonene, 4-carene and δ-3-carene (Chahed et al., 2007; Ozel et al., 2004; Smeriglio et al., 2017). Total phenolic content of PH is higher than many edible fruits and vegetables that are considered to be rich in phenolic components according to the results of previously published studies (Lin and Tang, 2007; Seifzadeh et al., 2019).

Pistachio shell mainly contains cellulose, hemicellulose and lignin. Kasiri and Fathi (2018) reported that pistachio shells contain 38.1% cellulose, 31.4% hemicellulose and 25.6% lignin. Domínguez et al. (2012) reported the cellulose, hemicellulose, lignin and extractives content of pistachio shells as 15.2%, 38.5%, 29.4% and 17.0%, respectively. On the other hand, these values were reported as 53.98%, 20.10% and 25.25% and 0.67%, respectively, by Okutucu et al. (2011). Changes in the chemical composition of the shells can be attributed to the variations in pistachio subspecies, climate, geographical conditions, sample preparation and analysis method (Kasiri

and Fathi, 2018). Protein and ash content of pistachio shells were reported as 0.97% and 0.47% of dried mass, respectively (Özbek et al., 2018).

2.2 Phenolic Compounds

Phenolic components are one of the biggest and widely distributed classes of plant secondary metabolites. They are usually responsible for the defence mechanism of plants. In addition, they have many important roles such as growth and reproduction, pollination, colouring for camouflage and protection against pathogens and predators. Also, they are responsible for the colour and sensory characteristics of fruits and vegetables. Phenolic compounds have a common chemical structure containing an aromatic ring with one or more hydroxyl groups and range from simple phenolic molecules to highly polymerised compounds (Balasundram et al., 2006; Lin et al., 2016). In recent years, phenolic compounds have attracted the attention of researchers as they can reduce the risk of many diseases, especially due to their antioxidant properties. Antioxidant activities of phenolics are carried out through various mechanisms such as reduction or scavenging of reactive oxygen species, chelation of transition metal ions and inhibition of enzymes involved in oxidative stress (Cheynier, 2012).The major groups of phenolic components include flavonoids, phenolic acids, tannins, stilbenes and lignans (Cong-Cong et al., 2017).

Flavonoids are the largest group of plant phenolics. They have low molecular weight and they consist of 15 carbon atoms (Balasundram et al., 2006). Flavonols, flavones, flavanones, isoflavones, flavanonols, and anthocyanins are the subgroups of flavonoids (Cong-Cong et al., 2017). They are important components of the human diet and the most potent antioxidants from plants (Giada, 2013). It has been reported that the flavonoids have various health benefits such as anti-inflammatory, antimicrobial, antiallergic, antitumour and anticancer activity (Brglez Mojzer et al., 2016; Pereira et al., 2009).

Phenolic acids are divided into two subgroups: hydroxybenzoic acids and hydroxycinnamic acids. The hydroxybenzoic acids are the simple phenolic acids found in nature and include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids. The hydroxycinnamic acids are aromatic compounds and the most common hydroxycinnamic acids found in nature are *p*-coumaric, caffeic, ferulic and sinapic acids (Balasundram et al., 2006). One-third of the phenolic compounds in the

human's diet may be phenolic acids and it is known that these compounds have high antioxidant activity (Giada, 2013). It has been reported that phenolic acids, including caffeic, sinapic, syringic, protocatechuic, ferulic and 3,4-dihydroxyphenylacetic acid, which are especially found in virgin olive oil, reduce the proliferation of breast and prostate cancer cells (Brglez Mojzer et al., 2016).

Tannins are phenolic components with relatively high molecular weight and they have the ability to make a strong complex with carbohydrates and proteins (Montes-Ávila et al., 2017). Hydrolysable tannins and condensed tannins are the two subgroups of tannins. Condensed tannins are compounds formed by the linkage of flavonoid units. They are also called as "proanthochocyanidins" because they can be hydrolyzed to anthocyanins when treated with strong acids. Hydrolysable tannins are heterogenous polymers consisting phenolic acids, especially gallic acid and simple sugars. These metabolites can be hydrolyzed more easily with acids, bases and enzymes (Özeker, 1999). Tannins, similar to other phenolics, have several biological effects including accelerating blood clotting, reducing blood pressure, decreasing serum lipid level and produce liver necrosis (Ozcan et al., 2014). On the other hand, they can form complexes with proteins, starch and digestive enzymes and influence the utilization of vitamins and minerals. Therefore, they are considered as anti-nutrients. Considering everything, it is not recommended to take large quantities of tannins, as they can have carcinogenic and anti-nutritional activities, and therefore carry a risk of negative health effects (Brglez Mojzer et al., 2016).

Stilbenes are widely distributed in plants and they are mainly act as phytoalexins and growth regulators. Pre-existing stilbenes can protect plant tissues from the attack of fungi, insects and other organisms (Lattanzio et al., 2006). The main representative compound for stilbenes is resveratrol. It has been reported that the stilbenes have antioxidant and anti-inflammatory properties with a potential to prevent from different diseases associated with oxidative stress and cancer (Cong-Cong et al., 2017).

Lignans are a large group of plant phenolics and they can be found in a wide range of plants (Cong-Cong et al., 2017). It has been reported that lignans have antioxidant activity and they can inhibit or delay the growth of skin, breast, colon and lung cancer cell which can be attributed to their estrogenic and antiestrogenic activities

(Brglez Mojzer et al., 2016). They are also known to have cytostatic and antimitotic properties in addition to their several physiological effects including anti-inflammatory, antihypertensive and anticancer activity (Pereira et al., 2009; Brglez Mojzer et al., 2016). Major sources of lignans are flaxseed meal and flour. Also, soybeans, whole grains, fruits and vegetables can contain varying amounts of lignans (Brglez Mojzer et al., 2016).

2.2.1 Extraction Methods of Phenolic Compounds

Extraction procedure is the first step to identify and/or quantify the phenolic compounds. Phenolics have different physical and chemical properties considering their various structures and these properties are very important in the extraction processes. In the literature, there is no consensus on a single and effective standard extraction method for phenolics considering their different characteristics. Different extraction methods have been reported to extract phenolics including Soxhlet extraction, heat reflux extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical CO_2 extraction, enzyme-assisted extraction, etc. (Aires, 2017). Despite this diversity, all extraction methods have some common goals such as: extraction of target compounds from complex plant material, increasing the selectivity of extraction method, increasing the concentration of target compounds, conversion of bioactive components into a more suitable form for detection and separation and providing a powerful and repeatable process which is independent from the changes in the sample matrix (Smith, 2003).

2.2.1.1 Conventional Extraction Methods

Phenolic compounds can be extracted from samples by different conventional extraction methods. Most of these methods are based on the extraction power of the solvent used and the application of heat and/or mixing (Azmir et al., 2013). Despite several disadvantages, conventional techniques are still the most commonly used extraction processes. The reason for this is that these methods are easy to use, efficient and have wide applicability (Brglez Mojzer et al., 2016). Maceration and Soxhlet extraction are the most commonly used conventional methods for the extraction of phenolics.

Maceration has a simple procedure consist of following steps: (a) sample grinding into small particles to increase the surface area to ensure efficient mixing with solvent, (b) soaking of sample in an appropriate solvent normally under room temperature with continuous or occasional agitation, (c) separation of solid residue from liquid fraction by filtration, clarification or decantation. Agitation applied in maceration process increase the extraction yield by increasing the diffusion and removing the concentrated solution from the sample surface to increase the contact of new solvent with the sample. This method is very simple to use, but it has some disadvantages, such as being time consuming and requiring a large amount of solvent (Aires, 2017).

Soxhlet extractor was mainly designed to extract lipid, but now its use is not just limited for this. Soxhlet extraction method has been widely used for extraction of valuable bioactive components of various natural sources. It is still used as a reference method to compare newly developed extraction methods. In Soxhlet extraction, the dried and powdered sample is placed in a thimble and the thimble is then placed in an extraction chamber located on top of a collecting flask. After addition of the solvent, a reflux condenser is placed on top of the extraction chamber and the system is heated. The solvent condenses after reaching a certain temperature level. The solution in the extraction chamber is aspirated by a siphon after reaching an overflow level and unloaded back to collecting flask. This solution carries extracted solutes to the bulk liquid. The solute remains in the collecting flask and solvent returns to the solid sample. This process occurs many times until the extraction is completed (Azmir et al., 2013). Soxhlet extraction takes less time and consumes less solvent compared to the maceration (Aires, 2017). However, it is reported that Soxhlet method should be handled carefully, as excessive temperature, which is always close to the boiling point, can destroy or modify some thermo labile phenolics (Seidel, 2012).

An efficient extraction by using conventional methods mainly depends on solvent selection. The most important factor to consider when choosing a solvent is the polarity of the targeted component. Molecular affinity between solvent and sample, mass transfer, co-solvent use, environmental safety, toxicity and financial feasibility are the other factors which should be considered while selecting a solvent for

phenolic extraction (Azmir et al., 2013). Water, ethanol, methanol, acetone, hexane, chloroform and ethylacetate are the most commonly used solvents for phenolic extraction (Aires, 2017; Fernández-Agulló et al., 2013; Liu et al., 2009).

2.2.1.2 Non-Conventional Extraction Methods

The main problems associated with the conventional extraction methods are long processing time, cost and high purity solvent requirement, need to evaporate large amounts of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds. New and promising extraction methods have been developed to overcome these problems in the last years. Some of non-conventional methods are ultrasound-assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and pressurized liquid extraction.

In ultrasound-assisted extraction (UAE), bubbles are formed by high frequency sound waves. The bubbles formed can no longer absorb the energy and collapse, so "cavitation" takes place. This collapse causes a change in temperature and pressure in the bubble, thereby energy is produced for chemical reactions. Extremely high temperatures and pressures (nearly 5000 °C and 1000 bar) have been measured. Due to the cavitation effect, plant cell walls can be penetrated, which allows easier access to the cell. Also, ultrasound can increase extraction by causing swelling of plant material (Wijngaard et al., 2012). The major advantages of UAE technique have been reported as its simplicity, ease of use, low cost, high efficiency, low organic solvent consumption and reduced process time (Jahromi, 2019). Mazza et al. (2019) carried out the extraction of phenolics from from Syrah grape skin using UAE and optimized the extraction conditions of ultrasound power, citric acid concentration and solid to liquid ratio. Under optimum conditions, they extracted 59% of the quantified phenolic compounds in just 3 min. They have reported that UAE is an effective method when compared to the conventional extraction by improving the phenolic extraction. Furthermore, UAE has been used to extract phenolic components from various samples such as areca husk (Chen et al., 2014), flax seeds (Corbin et al., 2015), grapefruit leaves (Ciğeroğlu et al., 2018) and sunflower seed cake (Zardo et al., 2019).

Supercritical fluid extraction (SFE) is one of the methods that has become popular in last years for extraction of phenolics. Unlike normal solvent extraction, in SFE,

solvents are used in their supercritical state. Supercritical CO_2 is the most commonly used supercritical fluid. It is non-toxic, non-flammable and requires a minimum amount of solvent. Extraction with CO_2 is fast, selective, does not require additional cleaning and can be performed with a small amount of sample. However, high molecular weight compounds cannot be extracted by supercritical CO_2 since they are lipophilic and nonpolar. Extraction efficiency of these components with CO_2 can be improved by using co-solvents (Oroian and Escriche, 2015). SFE technique is rapid, selective, requires a small amount of solvent or no solvent. Also, a small amount of phenolics and thermolabile components can be extracted using this method (Jahromi, 2019). SFE has been applied to extract phenolic components of different materials such as olive leaves (Le Floch et al., 1998), grape seeds (Murga et al., 2000), elder berry and grape marc varieties (Vatai et al., 2009), Guaraná (*Paullinia cupana*) seeds (Marques et al., 2016) and myrtle leaves and berries (Pereira et al., 2016).

Pressurized liquid extraction (PLE) also known as pressurized fluid extraction, accelerated fluid extraction, enhanced solvent extraction and high pressure solvent extraction. PLE is based on applying high pressure to keep the solvent liquid beyond its normal boiling point which also facilitates the extraction. Small amounts of solvents are used in PLE because of the combination of high pressure and temperatures which also provide faster extraction. In addition, PLE is as a potential alternative method to SFE to extract polar components (Azmir et al., 2013). PLE has been used successfully for the extraction of bioactive components of different materials as soybeans (Rostagno et al., 2004), spinach (Howard and Pandjaitan, 2008), parsley (*Petroselinum crispum*) flakes (Luthria, 2008) and Anatolia propolis (Erdogan et al., 2011).

Enzyme-assisted extraction (EAE) method is based on the degradation of cell walls by enzymes and thus the release of bioactive compounds. By using enzymes, the effect of solvent treatment and the extraction efficiency can be increased or the amount of solvent required for extraction can be reduced. Cellulose, hemicelluloses, pectin and proteins are the compounds that mainly form the primary cell wall of plants. Furthermore, phenolic compounds are bound to cell wall polysaccharides. For that reason, cellulases, hemicellulases, pectinases and other enzymes may be used to hydrolyze cell wall components, break down complex internal storage materials,

enabling the release of intracellular phenolics (Oroian and Escriche, 2015). Gómez-García et al. (2012) studied the extraction of phenolics from grape wastes by using different commercial enzymes and they obtained a strong increase in the extraction yield. Similar observations have been reported in other studies, which concluded that EAE can be considered as an alternative technique for the extraction of bound polyphenols (Papillo et al., 2014; Sumczynski et al., 2016).

Microwave-assisted extraction (MAE) is a method in which microwave energy is used to facilitate separation of analytes from the sample into the solvent (Dai and Mumper, 2010). Microwaves are electromagnetic waves with frequencies in the range of 300 MHz to 300 GHz. They generates heat due to the induction of molecular movements that cause the cell wall rapture and release of active substances inside the cell. Polar solvents such as water, which have a higher dielectric constant than non-polar solvents, can absorb high energy, thereby increasing the extraction rate and efficiency of phenolic components. There are two MAE systems to extract phenolics, which are closed and open vessels. While extraction is carried out at high pressure and temperature in closed vessel system, extraction of phenolics is performed at atmospheric pressure in open vessel system (Jahromi, 2019). Microwave power, process time, solubility, dielectric constant and solvent property are the main physical parameters important for MAE. The solvent property is an important parameter as solvents with a high dielectric constant can absorb more microwave energy. Methanol, ethanol and water are the most commonly used solvents for extraction of phenolics from plants (Cong-Cong et al., 2017). Reduction in extraction time and solvent consumption are the main advantages of MAE compared to conventional extraction methods (Dai and Mumper, 2010).

MAE has been largely applied to extract of phenolic components. Dahmoune et al. (2015) carried out the MAE of phenolics from *Myrtus communis* leaves and compared this method with UAE and conventional solvent extraction. Their results showed that tannins, total flavonoids and antioxidant activities of MAE extracts were higher than the extracts obtained by other methods. They also indicated that MAE is advantageous for the extraction of bioactive compounds from plants by consuming less extraction solvent and saving time. In other study performed by Alara et al. (2018), phenolics were extracted from *Vernonia cinerea* leaves by MAE. Their

results showed that MAE can recover higher phenolics with higher antioxidant and anti-diabetic activities compared to liquid-liquid extraction method. Zhao et al. (2018) and Radojković et al. (2018) optimized MAE conditions for the extraction of phenolics from the fruit of *Melastoma sanguineum* and *Morus nigra* (mulberry) leaves, respectively. Moreover, they compared MAE with conventional methods. Their results indicated that MAE is more effective and produce richer extracts in terms of TPC and antioxidant activities, simultaneously decreasing solvent consumption and extraction time. In addition, MAE has been successfully carried out for the extraction of phenolics from other samples as licorice root (Karami et al., 2015), cherry laurel leaf and fruit (Karabegović et al., 2014), olive leaf (Taamalli et al., 2012) and green coffee beans (Upadhyay et al., 2012). However, MAE has been less used for the extraction of polymeric polyphenols such as tannins and anthocyanins. Because polyphenols which have higher number of hydroxyl-type substituents (long-chains) and those sensitive to higher temperatures (e.g., anthocyanins) may be degraded under MAE conditions (Aires, 2017).

2.2.2 Pistachio Hull as a Source of Phenolic Compounds

Recently, PH has caught up the interest of researchers by considering its natural phenolics and antioxidants. It has been shown that pistachio hull extracts (PHE) have antioxidant, antimicrobial, antiflammatory, cytoprotective and antimutagenic properties (Barreca et al., 2016; Rajaei et al., 2010). Investigations have also showed that the antioxidant effect of PHE was not different from the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which makes it a good alternative to synthetic equivalents (Goli et al., 2005; Vahabzadeh et al., 2004). Due to its beneficial effects, PH can be an alternative source of bioactive compounds (Kilic et al., 2016). In this way, using hull as a source of biologically active compounds will increase the value of pistachio production and provide valorisation of a by-product (Sonmezdag et al., 2017).

Goli et al. (2005) carried out the extraction of phenolic compounds from pistachio hull by solvent extraction and UAE using three different solvents (water, methanol and ethyl acetate). They also compare these two methods with SFE. Their findings indicated that the PHE obtained by solvent extraction with water or methanol have high phenolic content. Furthermore, they studied the effects of water and methanol

extracts on the stability of soybean oil during heating at 60 °C. The results indicated that the extracts were effective in retarding oil deterioration and had similar activity with synthetic antioxidants BHA and BHT.

Rajaei et al. (2010) studied antioxidant, anti-microbial and anti-mutagenicity activities of PHE (crude and purified). Antioxidant activity of PHE was compared with that of BHT and TBHQ (tert-butylhydroquinone) which are the commonly used synthetic antioxidants in food industry. The results indicated that PHE have high antioxidant potential when compared to synthetic antioxidants. The antimicrobial activity of crude and purified PHE was evaluated against Gram-positive and Gram-negative bacteria, and fungi. The results indicated that Gram-positive bacteria were the most sensitive organism. Moreover, anti-mutagenicity test results showed that phenolic components of PHE have anti-mutagenicity activity against direct mutagen of 2-nitrofluorene.

Barreca et al. (2016) studied the extraction of ripe PH using ethanol and methanol. They characterized the phenolic composition, antioxidant and cytoprotective activity of PHE. They identified 20 and 17 phenolic components in the methanol and ethanol extract, respectively. The most abundant phenolics were found as gallic acid, 4-hydroxybenzoic acid, protocatechuic acid, naringin, eriodictyol-7-O-glucoside, isorhamnetin-7-O-glucoside, quercetin-3-O-rutinoside, isorhamnetin-3-O-glucoside and catechin. Their results showed that methanol extract had higher scavenging and cytoprotective activity on lymphocytes, lipid peroxidation and protein degradation.

Grace et al. (2016) examined the phytochemical composition of PH. They obtained a polar extract containing quercetin-3-*O*-glucoside, quercetin, myricetin and luteolin flavonoids. It has been reported that the polar extract consists of gallotannins and other phenolic compounds esterified with a gallic acid moiety. Their results also showed that polar extract has the ability to inhibit the release of nitric oxide and reactive oxygen species in raw macrophage cells.

In previous work done by Kilic et al. (2016), antioxidant, enzyme inhibitory and biological activities of methanol extracts obtained from immature and mature pistachio hulls were evaluated and phytochemical compositions of the PHE were investigated. It was found that the extract obtained from immature hull had higher

amounts of phenolic and flavonoid compounds than that of obtained from mature one. It is also stated that immature hull extracts showed remarkable antioxidant and enzyme inhibitory activity.

Garavand et al. (2017) analysed and compared the phytochemical content and radical scavenging activity of PHE obtained using water, ethanol, and butanol. Water was selected as a best solvent. They also compared the phytochemicals profile of PHE obtained using ultrasound and microwave either during extraction or post-extraction processes. Their results showed that UAE and MAE increased the amounts of phenolic compounds. However, application of ultrasound and microwave as a post-extraction process caused the degradation of phenolic compounds. They also reported for the first time that PHE contain phloroglucinol and sinapic acid.

Ghandahari Yazdi et al. (2019) optimized the EAE conditions of phenolics from PH using pectinase, cellulase, and tannase enzymes. They also investigated the effect of enzymatic extraction on the antioxidant activity of the extracts and determined the phenolic profile. Their results indicated that all of the studied enzymes significantly improved the extraction yield when compared to the solvent extraction. Extracted compounds by EAE had higher antioxidant activities compared to extract obtained by the solvent extraction. In addition, their findings indicated that phenolic profile of extracts significantly affected by enzyme type, where the pectinase and cellulase enzymes increased the amount of phloroglucinol and decreased the amount of gallic acid.

Seifzadeh et al. (2019) investigated the concentration of polyphenols in the aqueous PHE using a two-stage membrane process and characterized the concentrated polyphenols. They identified at least 34 polyphenolic compounds in which eight of them were reported for first time in PH. The most abundant compounds found in the extracts were reported as galloylshikimic acids, gallic acid, theogallin, galloyl-*O*-hexoside, pyrogallol and quercetin-*O*-hexoside.

The results of literature studies indicated that PH is a good source of valuable compounds with potential usage in different industries such as food, cosmetic and drug industries. Commercial applications of PH are expected to expand in the coming years. In recent years, many efforts have been made for identification and

extraction of bioactive components from PH and to test their biological activities. In addition to their health-promoting effects, recovery of bioactive components of PH can also be beneficial from environmental and economic perspectives.

2.3 Lignocellulosic Biomass

Petroleum has been a vital resource for the production of fuels, synthetic polymers and many chemical products with numerous applications. Rapidly depleted oil reserves and over-exploitation of fossil fuels with increased energy consumption are of greatest concern globally. These concerns have adverse effects not only on the economy but also on the environment and raise issues such as global warming and air pollution (Xu and Sun, 2016). Increasing global fuel demand and environmental and economic problems caused by fossil fuel use has led scientists to search for alternative, sustainable, environmentally friendly resources and technologies to produce various fuels and chemicals (Islam et al., 2017; Zhang and Wu, 2015). Among the various renewable resources, there exists an intense focus on the lignocellulosic biomass to produce biofuels and other bio-products.

Biomass can be defined as all material that was or is a part of a living organism. However, the term generally limited to include plants or plant-derived materials (Hames, 2009). Since cellulose, hemicellulose and lignin are the largest components of plants, plant-derived biomass is known as lignocellulosic biomass. Lignocellulosic biomass can be fractionated into cellulose, hemicellulose and lignin, which are their main components after various physical, chemical or biological stages. These compounds can be used to obtain value-added products which can be used in place of fossil fuels, polymers and various other chemicals. Cellulose is converted to its monomer, glucose, after enzymatic hydrolysis. Ethanol or other bio-based chemicals such as lactic acid and succinic acid can be produced by the fermentation of glucose. Hemicellulose is used as a source to produce important materials such as thickeners, adhesives and coatings (Erdemir, 2015). In addition, by breaking down the hemicellulose into its monomers and subsequently fermentation of these monomers, xylitol, ethanol and organic acids can be produced from hemicellulose (Gürbüz et al., 2012; Saha, 2003). Lignin which is the third compound of lignocellulosic biomass, can be a good source to produce polymers, aromatic chemicals and various fuels (Azadi et al., 2013). Using renewable lignocellulosic biomass as a potential source to

produce fuels and other bio-based products is one of the ways to ensure a sustainable supply chain (Zhao et al., 2017).

2.3.1 Structure of Lignocellulosic Biomass

Lignocellulosic biomass mainly consists of three polymers: cellulose, hemicellulose and lignin with smaller amounts of ash, pectin, protein and extractives (Bajpai, 2016). Generally, lignocellulosic biomass contains 40-60% cellulose, 20-40% hemicellulose and 10-25% of lignin. The composition of lignocellulosic biomass varies based on the biomass source, age, growth stage and growing conditions (Sharma et al., 2019). Composition of lignocellulosic biomass plays an important role for the performance of biomass conversion into valuable compounds (Nashiruddin et al., 2020). Lignocellulosic biomass structure is shown in Figure 2.3 (Rubin, 2008).

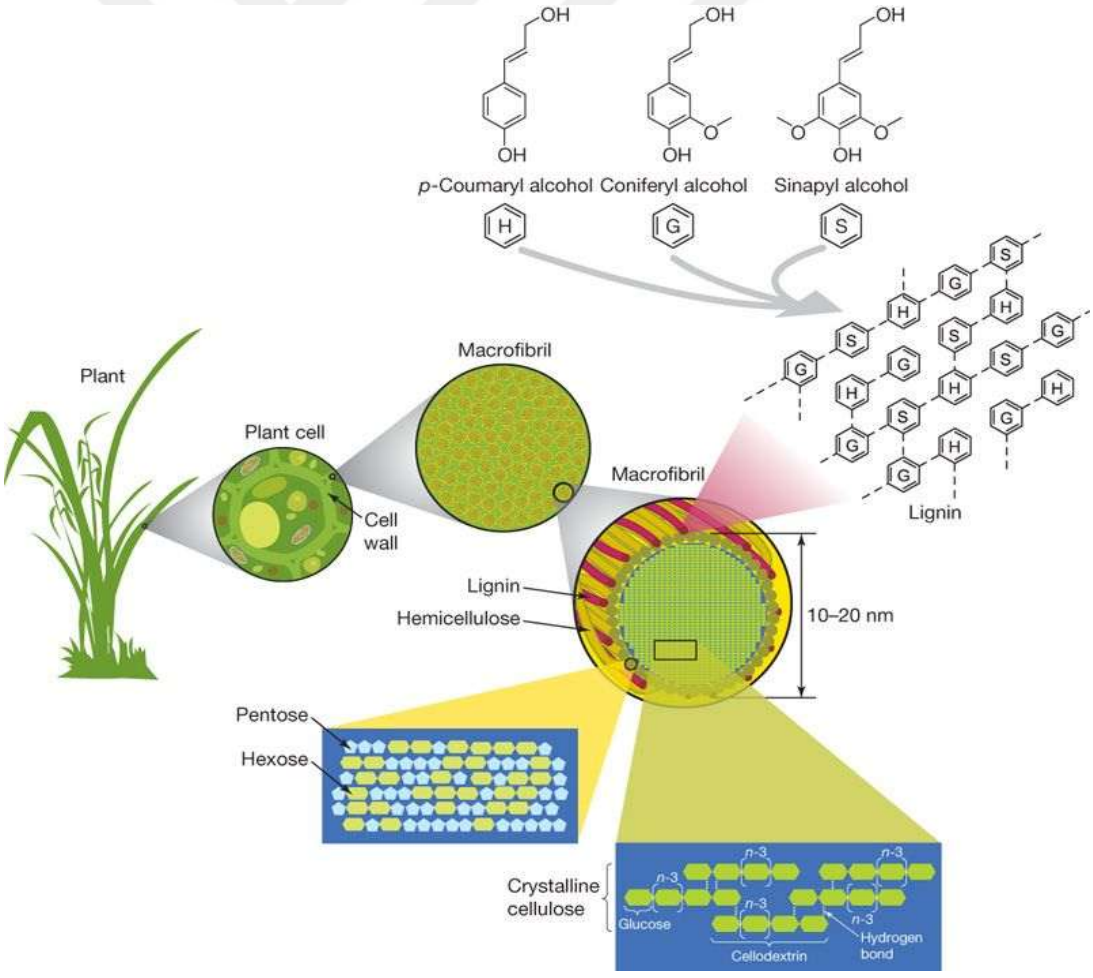

Figure 2.3 Structure of lignocellulosic biomass (Rubin, 2008)

Cellulose is a polymer formed by D-glucose subunits tightly linked with β-1, 4-glycoside bonds. Long polysaccharide chains are unbranched and arranged in parallel to form cellulose microfibrils (Sharma et al., 2019). Cellulose is one of the main compounds of the plant cell wall and the most abundant source of organic components available in nature. Cellulose molecules are linked by hydrogen bonds and have different orientations which result different crystallinity levels. Crystallinity is an important factor for cellulose biodegradation and generally higher crystallinity makes cellulose biodegradation difficult. It is known that the hydrogen bonds hold the crystalline cellulose structure and make it insoluble. The cellulose characteristics cause difficulties in biodegradation or digestion of this component (Koupaie et al., 2019).

Hemicellulose is the second most abundant polymer of lignocellulosic biomass. It is derived from different sugars and uronic acids. The sugars include pentoses (xylose, rhamnose, arabinose) and hexoses (glucose, mannose, galactose). Hemicellulose is either a homopolymer or a heteropolymer with short branches, which are connected by β-1,4-glycoside bonds, and occasionally β-1,3-glycoside bonds. Their molecular weight is lower compared to cellulose and they have branches with short side chains which are easily hydrolysed. Hemicelluloses are the most thermo-chemically sensitive components of lignocellulosic biomasses (Bajpai, 2016). The hemicellulose content and its chemical structure can be varied with different plants and their parts. Xylan is the most abundant hemicellulose in nature, whose backbone is usually highly substituted with arabinose, glucuronic acid, acetyl groups or xylose. Because of their branched structure, hemicelluloses are likely to be in amorphous form in plants (Sun et al., 2016).

Lignin is a complex and large compound consisting of phenylpropane units linked in a three-dimensional structure. *p*-hydroxyphenyl alcohol, coniferyl alcohol, and sinapyl alcohol are the main monomers of lignin. Cellulose and hemicellulose are linked each other with lignin, which acts as cement material, thereby a rigid three-dimensional structure of the plant cell wall is formed. Lignin is an optically inert compound and insoluble in water which make it the most resistant component of lignocellulosic biomass to the biological and chemical degradation (Koupaie et al., 2019). Also, it has been reported that the high lignin content in lignocellulosic

materials makes their biological and chemical degradation difficult (Zheng et al., 2014).

2.3.2 Pistachio Shell as a Lignocellulosic Biomass

Pistachio shell, one of the by-products of pistachio nut processing, is an example of lignocellulosic biomass generated in significant quantities. It mainly contains cellulose, hemicellulose and lignin. Pistachio shell can be converted into energy, fuels and chemicals using different techniques. There are many literature studies on the evaluation of pistachio shell (Acikalin et al., 2012; Balasundar et al., 2019; Donat and Erden, 2017; Sadeghian-Abadi et al., 2019). Various methods, especially combustion, gasification, liquefaction and pyrolysis, have been developed for the thermochemical conversion of pistachio shells. It is stated that liquid products produced with these methods are similar to existing petroleum-based fuels and can be used directly for the production of electricity and energy production. Also, it is reported that the produced bio-oil can be used for extracting and synthesizing various chemicals since it contains different valuable chemicals (Acikalin et al., 2012; Apaydin-Varol et al., 2007; Okutucu et al., 2011).

Acikalin et al. (2012) carried out the pyrolysis of pistachio shell in a well-swept fixed bed reactor. They also studied the pyrolysis conditions and analysed the products. Their results indicated that the liquid product was a highly oxygenated complex mixture with 18 identified compounds and had a higher heating value. Their solid product was a valuable solid fuel with energy content higher than subbituminous coal. Faramarzi et al. (2015) prepared activated carbon from the pistachio shell which can be used to heavy metal adsorption. Karaca and Bektas (2017) performed direct liquefaction of pistachio shells to produce biofuel and investigate the effects of different experimental factors on direct liquefaciton yields of shells. Their results showed that the both oil product and residue samples were more valuable than pistachio shells as energy sources and the oil product has an energy content very close to conventional petroleum fuels.

Hydrolysis of pistachio shells has been also stated in the literature. Cho et al. (2002) performed enzymatic hydrolysis of agricultural wastes including pistachio shells to produce D-xylose after milling or steam explosion pre-treatment methods using the mixed crude enzymes. Their results showed that steam explosion pre-treatment was

more effective than milling process for enzymatic hydrolysis. They obtained 36 g D-xylose from 100 g pistachio shells when an enzyme mixture containing 3,000 U xylanase and 33 U β-xyosidase activities per g of substrate was applied after milling process (Cho et al., 2002).

Domínguez et al. (2012) carried out xylitol production from pistachio shell by acid hydrolysis and fermentation. They studied the effect of acid concentration (1-3%) and hydrolysis time (15-45 min) on acid hydrolysis of pistachio shell at 130 °C and optimized the hydrolysis conditions to obtain maximum hemicellulose hydrolysis with the minimum cellulose degradation. Optimum conditions were found as 2.9% of acid concentration and 25.3 minutes of hydrolysis time. Finally, with the fermentation of sugars obtained after hydrolysis of pistachio shells under optimum conditions, they produced xylitol, which is used as a sweetener in foods (Domínguez et al., 2012).

Subhedar et al. (2018) performed fermentable sugar production from pistachio shell. They investigated the effect of ultrasound-assisted alkali pre-treatment on delignification and enzymatic hydrolysis of pistachio shell and compared this method with conventional alkali pre-treatment. The results showed that the ultrasound-assisted method increased lignin removal from 38% to 78.9% compared to the conventional method. In addition, ultrasound-assisted alkali pre-treatment significantly increased the fermentable sugar yield obtained by enzymatic hydrolysis of pre-treated solids (Subhedar et al., 2018).

2.3.3 Pre-treatment of Lignocellulosic Biomass

Lignocellulosic biomass can be used as a source for the production of several valuable compounds after enzymatic and microbial/chemical processes. But, its recalcitrant structure limits the access of hydrolytic enzymes to cellulose and hemicellulose. A suitable pre-treatment process is required to overcome the natural recalcitrance of biomass and increase the hydrolysis efficiency. Pre-treatment is a critical step in the processing of lignocellulosic biomass. Because it has a major effect on final yield and also contributes significantly to overall costs (Moreno and Olsson, 2017). A pre-treatment process aimed to remove hemicellulose and lignin, reduce the cellulose crystallinity and increase the porosity and specific surface area of lignocellulosic biomass (Loow et al., 2016). There are different pre-treatment

techniques and they are mainly classified as physical, chemical, physicochemical and biological methods. It is not possible to define the best pre-treatment technique. Because, each pre-treatment method has its own advantages and disadvantages. The selection of pre-treatment method depends on several factors such as biomass type, target product, economy and environmental impact (Moreno and Olsson, 2017; Sharma et al., 2019).

Physical pre-treatments are generally the first step of pre-treatment methods. The main purpose of physical pre-treatment is to reduce the size of the biomass and break the physical structure (Amin et al., 2017). Mechanical comminution and extrusion are included in physical pre-treatment. In mechanical comminution, the particle size of lignocellulosic biomass is reduced by grinding and/or chipping (ranging from µm to mm). After these processes, different milling methods can be used to obtain fine biomass powder. The particle size and the crystallinity of lignocellulosic biomass can be reduced and consequently its enzymatic hydrolysis can be improved by milling. However, the industrial application of mechanical comminution methods is limited because of the long processing time and relatively high energy consumption (Sun et al., 2016). In extrusion, the lignocellulosic structure is modified by promoting defibrillation and shortening of fibres. The available surface area for the hydrolytic enzymes is increased and crystallinity and degree of polymerization are reduced. Chemical or biological catalysts can be integrated to the extrusion. This results in a water swelling effect and more effective biomass fractionation which increase the sugar yields in the subsequent saccharification step. Unlike mechanical methods that require high energy, extrusion is a promising pre-treatment method due to its adaptability to process modifications and its versatility for the use of various raw materials (Moreno and Olsson, 2017).

The chemical pre-treatment is one of the most widely used method for the lignocellulosic biomass degradation. Chemical methods include alkaline pre-treatment, acid pre-treatment, oxidative delignification, organosolv process and ionic liquid (ILs) pre-treatment (Lee et al., 2014). In acid pre-treatment, acids are used to break linkages in hemicellulose and cellulose which makes the cellulose more accessible to enzymes by increasing the porosity. Inorganic acids (sulfuric, nitric, hydrochloric and phosphoric acids) and organic acids (formic, acetic and propionic

acid) can be used in acid pre-treatment. Biomass is treated with dilute or concentrated acid. In concentrated acid pre-treatment, an acid concentration of higher than 30% is used at low temperature. Since the concentrated acids are toxic and corrosive, the equipment to be used must be corrosion resistant. In dilute acid pre-treatment, the acid concentration is below 10% and the process is carried out at high temperature (Fersiz, 2018). Alkaline pre-treatment is the most widely used technique for separation of lignin and hemicellulose from lignocellulosic samples. Ester bonds between hemicellulose and lignin can be easily broken down in alkaline medium. Also, ether linkages between hemicellulose and lignin can be degraded at relatively high temperatures. Cleavage of these linkages significantly increases the hemicelluloses and lignin dissolution, which also increases the accessibility of enzymes to cellulose. NaOH, KOH, Ca $(OH)_2$, Na_2CO_3 and NH_4OH are the alkaline reagents used for the pre-treatment of lignocellulosic biomass (Sun et al., 2016).

In oxidative delignification, oxidation agents such as organic peroxide (H_2O_2, $C_2H_4O_3$), ozone, oxygen or air are used for delignification of biomass. These agents attacks and break down the ring structure of lignin and catalyse the delignification. Oxidation agents are often used to enhance the effects of alkaline pre-treatments. Oxidative delignification is an effective treatment for oxidizing the part of hemicellulose polymer to carboxylic acid compounds such as acetic, formic and oxalic acids. This method is suitable to extract cellulose since the oxidation agents are more effective on lignin, partially on hemicellulose and cellulose is hardly decomposed under moderate conditions (Lee et al., 2014). Organosolv pre-treatment is carried out for lignin removal and hydrolyzation of hemicellulosic sugars by using organic or aqueous solvents. Also, acids, bases or salts can be used as catalysts. Methanol, ethanol, acetone and ethylene glycol are the common solvents used in organosolv pre-treatment. Solvents should be recovered after pre-treatment to reutilize them and avoid the inhibition of these compounds on hydrolytic enzymes and fermentative microorganisms. Although organosolv pre-treatment can be potentially applied to any material with high lignin removal and saccharification yields, it requires high capital costs and increases the formation of degradation products (Moreno and Olsson, 2017). One of the remarkable pre-treatments in recent years is the use of ionic liquids (ILs). ILs are salts that have melting points usually below 100 ° C. They consist large organic cations and small inorganic anions that are

liquid at relatively low temperatures. They can dissolve cellulose, hemicellulose and lignin under moderate conditions without degradation of chain structure. ILs are thermally and chemically stabile, non-flammable, have wide liquid temperature ranges and good dissolving properties for different types of biomass (Lee et al., 2014; Mäki-Arvela et al., 2010).

Physicochemical pre-treatments include methods which combines physical and chemical effects to enhance the biomass hydrolysability. Steam explosion, ammonia fiber explosion (AFEX), wet oxidation, microwave, ultrasound, autohydrolysis and CO_2 explosion are the most widely applied physicochemical pre-treatments among others. Autohydrolysis is also called as hydrothermal pre-treatment in which the biomass is treated with pressurized water. The main advantage of the method is it's relatively low cost, mainly due to the absence of an external chemical or catalyst. The process is carried out similar to the dilute acid treatment and the hydronium (H_3O^+) ion formed during the process is used as a catalyst (Nitsos et al., 2013). In steam explosion method, saturated steam at high pressure is used to treat biomass and after a period of time, the pressure is released rapidly, which causes an explosive decompression of the lignocellulosic biomass. Steam explosion is generally performed using the temperatures in the range of 160 to 260 °C that correspond to a pressure of 0.69-4.83 MPa for several seconds to a few minutes before the biomass is exposed to atmospheric pressure (Hu and Ragauskas, 2012). Similar to the steam explosion, AFEX is carried out at high pressures. The biomass is treated with liquid ammonia at moderate temperatures (below 100 °C) and high pressures (above 3 MPa) for about 10 to 60 minutes. After pre-treatment, the pressure is reduced and the ammonia is recycled. AFEX increases the water holding capacity and digestibility of the material (Galbe and Zacchi, 2007). Wet oxidation is carried out at high temperatures (170–200 °C) and pressures (10-15 bar) by using oxygen or air as a catalyst. It provides 60-70% lignin removal by promoting lignin oxidation and degradation of linkages in lignin-carbohydrate complexes. In CO_2 explosion method, compressed CO_2 is used at temperatures above its critical point. This method is used for delignification of biomass and to increase its accessibility. The main advantages of this method are that CO_2 is non-toxic, non-flammable and its recovery is easy (Moreno and Olsson, 2017). The ultrasound process can enhance the efficiency of hydrolysis by modifying the physical and chemical structure of biomass

resulting from cavitation forces (Wang et al., 2017). Ultrasound produces cavitation effect which creates high temperature and pressure in an aqueous system and accelerates the formation of free radicals cleaving the bonds between lignin and hemicellulose (da Silva et al., 2016). In addition, microwave pre-treatment is another promising process due to its thermal and non-thermal effects in an aqueous medium. Microwave irradiation provides more direct, rapid and uniform heating by direct interaction between heated material and electromagnetic field (Vani et al., 2012). The formed electromagnetic field helps to speed up the destruction of the crystal structure and changes the molecular structure of biomass (Keshwani and Cheng, 2010).

Biological pre-treatment based on the biomass degradation by using bacteria, fungi and enzymes. They change or degrade the lignocellulosic structure extracellularly by secreting hydrolytic enzymes such as hydrolases and ligninolytic enzymes that depolymerize lignin. Therefore, the cell wall structure is opened up which also allows subsequent biopolymer hydrolysis. In biological pre-treatment, cellulose and hemicellulose are generally hydrolyzed to monomeric sugars by cellulolytic and hemicellulolytic microorganisms (Sharma et al., 2019). Unlike other pre-treatment technologies, the biological pre-treatment method is defined as environmentally-friendly treatment because it does not require chemicals, pressurized and corrosion resistant reactors and reduces energy requirements. However, the long processing times and the expensive operating costs are the disadvantages of the biological pre-treatment method (Sindhu et al., 2016).

2.3.3.1 Alkaline Pre-treatment of Lignocellulosic Biomass

Alkaline pre-treatment methods have been investigated to increase the enzymatic digestibility of the lignocellulosic materials by using different reagents. The most commonly used alkali reagents are sodium hydroxide, ammonia and calcium hydroxide. Alkali pre-treatment is performed at low temperature and pressure and can even be completed under ambient conditions. The reagents used in alkaline pre-treatment have less corrosive effect compared to acids such as sulfuric acid, and the process is performed under modarate conditions. In this kind of methods, there is no need to expensive materials and special designs to deal with corrosion and harsh reaction conditions. Recovery and reuse of chemical reagents are also possible in

some alkaline pre-treatment techniques (Kim et al., 2016). Time is the main drawback of this method; the reaction may take hours or even days to complete. Temperature is an important parameter affecting the performance of alkali pre-treatment and pre-treatment time may be reduced by increasing the temperature (Xu and Sun, 2016).

Alkali pre-treatment causes swelling modifying the structure of lignocellulosic materials. After this process, the internal surface area of biomass is increased and its polymerization degree and cellulose crystallinity are decreased. During alkaline pre-treatment, the major reactions are dissolution of lignin and hemicellulose and saponification (de-esterification) of intermolecular ester bonds. These reactions change the physical properties of biomass which include increase in porosity and decrease in crystallinity, thereby expanding the accessible surface area for enzymes (Kim et al., 2016; Xu and Sun, 2016).

Alkali pre-treatment has been applied to different types of biomass samples. Nath Barman et al. (2014) studied the effect of mild alkali pre-treatment on the structure of reed straw by using NaOH at different concentration (1% to 2.5%) at 105 °C for 10 min. Then, they carried out enzymatic hydrolysis of the pre-treated solids for the production of fermentable sugars. They stated that pre-treatment with 2% NaOH provided 76.6% of cellulose recovery with the 56.4% and 69.9% of hemicellulose and lignin removal, respectively. The reducing sugar yield reached to 81.2% after enzymatic hydrolysis of the pre-treated biomass at 50 °C for 72 h (Nath Barman et al., 2014).

Ballesteros et al. (2015) extracted the polysaccharides from spent coffee grounds (SCG) using an alkaline pre-treatment with NaOH at 25 °C. They also determined the chemical composition, antioxidant and antimicrobial activities of the extracted polysaccharides. Their results indicated that the alkali pre-treatment using 4 M NaOH is an efficient process to extract polysaccharides from SCG and the obtained polysaccharides were thermostable with good antioxidant and antimicrobial activities (Ballesteros et al., 2015).

Zhang et al. (2015) combined extrusion with alkali pre-treatment to increase the production of methane from rice straw. Also, they examined the physical and

chemical structural changes in biomass after pre-treatment. They reported the optimum conditions as an alkali loading of 3% at 35 °C for 48 h. Under these conditions, methane production was 54% higher and the energy recovery efficiency was increased from 38.9% to 59.9% when compared to control sample pre-treated by milling. The authors attributed these increases to the changes in the physical and chemical structure and chemical composition of rice straw as a result of the synergistic effects of combining extrusion and alkaline pre-treatment (Zhang et al., 2015).

Safari et al. (2017) performed dilute alkali pre-treatment of softwood pine to produce ethanol and biogas. They carried out alkali pre-treatment by using 0–2% (w/v) NaOH at 100–180 °C for 1-5 h and optimized the pre-treatment conditions. The liquid fraction obtained after pre-treatment was used to produce biogas by anaerobic digestion and the pre-treated solid fraction was subjected to enzymatic hydrolysis and fermentation to produce ethanol. The results showed that alkali pre-treatment significantly increased the ethanol yield to a maximum 78% while the yield was only 6% for the untreated wood. In addition, the authors successfully produced biogas from the hemicellulosic liquid fraction. Moreover, their economic evaluation showed that the pre-treatment using 1% NaOH at 180 °C for 2 and 5 h had lower manufacturing costs compared to the untreated wood (Safari et al., 2017).

Shetty et al. (2017) carried out the alkali pre-treatment to increase the biogas production from rice straw. They aimed to minimize the alkali consumption and eliminate heating of biomass sample during pre-treatment. Their results showed that NaOH pre-treatment significantly enhanced the biogas production and the highest methane yield was obtained after treatment with 1% NaOH at ambient temperature for three hours. Methane yield enhanced by 34% compared to the milled rice straw without alkali pre-treatment. These findings indicated that the alkali pre-treatment performed using lower NaOH concentration at ambient temperature can make this method more economical compared to the previous reports (Shetty et al., 2017).

Alkali pre-treatment is a time consuming process and has a large amount of alkali requirement, despite its high delignification efficiency. For the reduction of alkali consumption and/or reduce the pre-treatment time, a suitable physical or chemical approach is required during alkaline pre-treatment of the lignocellulosic biomass (Xu

and Sun, 2016). The biomass is soaked in basic solution, such as sodium hydroxide, and then heated for a certain time in alkaline pre-treatment. Conventional heating methods are generally used to heat the biomass. These methods have some disadvantages such as slow heat transfer, long processing time, excessive energy consumption and low efficiency by causing various degradation reactions (Jin et al., 2016). These disadvantages have led researchers to search for alternative methods for the pre-treatment of biomass, which increases reaction efficiency while shortening the processing time. Microwave and ultrasound assisted pre-treatment methods are among the fast, efficient and modern methods developed in recent years and the efficiency of alkaline pre-treatment can be enhanced by applying ultrasound and microwave irradiation (Li, H. et al., 2016; Subhedar and Gogate, 2014).

Microwaves are an electromagnetic radiation with wavelengths ranging from 1mm to 1 m and their frequency ranges from 300 MHz to 300 GHz on the electromagnetic spectrum. The heating mechanism of the microwave is different from conventional heating. In conventional heating, heat is transferred from the outer surface of the material to the interior through conduction and convection. Therefore, while the interior of the material is still cold, overheating may occur on the surface (Hassan et al., 2018). In contrast, heat is generated by direct interaction between heated material and the formed electromagnetic field by ionic conduction and dipole rotation (Aguilar-Reynosa et al., 2017b; Jin et al., 2016). Microwave heating has several advantages such as providing fast and homogeneous heating, reducing processing time, saving energy and reducing the formation of by-products during reactions when compared to the conventional heating methods (Aguilar-Reynosa et al., 2017b). The heating effect of microwave irradiation is also called as thermal effect. Thermal effect increases the temperature and subsequently pressure which disturbs lignin and cell walls. In addition to the thermal effects of microwave application, it is stated that non-thermal effects are also effective in the pre-treatment of biomass. Non-thermal effects occur when microwaves force dipoles to align with the oscillating electric field and cause degradation of hydrogen bonds. The degredation of hydrogen bonds can lead to destruction of cell walls, disintegration of cellulose chains and breakdown of the crystal structure of cellulose molecules, and thus enhanced hydrolysis (Bundhoo, 2018). The dielectric properties of biomass, that represent the material's ability to store electromagnetic energy and convert this energy into heat, have

significant effect on the performance of microwaves. Despite its low microwave absorption, the presence of relatively high moisture and inorganic substances can increase the absorption capacity of biomass (Li, J. et al., 2016).

Ultrasound generates high temperature, pressure and extreme shear forces (cavitation effect) in a liquid medium. With the cavitation effect, water molecules decompose into free radicals, which contribute to the hydrolysis of biomass by cleavage of the linkages in the lignin and xylan networks (Wu et al., 2017). Moreover, ultrasound can disrupt α-O-4 and β-O-4 linkages in lignin which leads to the degradation of structural polysaccharides and lignin with the formation of small cavitation bubbles. During the application of ultrasound, cavitation bubbles form, grow to a certain critical size, then become unstable and collapse violently. Meanwhile, the pressure and temperature reach up to 1800 atm and 2000-5000 K, respectively (Hassan et al., 2018). Furthermore, it is stated that using ultrasonic waves with a proper intensity can increase the permeability of cell membranes and promote the catalytic activity of enzymes (Wang et al., 2017). Therefore, ultrasound is one of the methods effectively applied for the pre-treatment of lignocellulosic materials.

Combination of alkaline pre-treatment with microwave and ultrasound has been applied widely to complement each other. Jin et al. (2016) carried out microwave-assisted alkaline pre-treatment of catalpa sawdust to increase enzymatic digestibility. They pre-treated the biomass using microwave-water, -NaOH, and -$Ca(OH)_2$. In addition, they determined the changes in physicochemical properties of biomass after pre-treatment. Their findings indicated that the enzymatic hydrolysis of biomass was enhanced by $Ca(OH)_2$ pre-treatment assisted with microwave. Compositional and structural changes in catalpa sawdust were obtained in both water and alkali treatments. However, the crystallinity and enzymatic digestibility were improved by only microwave-assisted alkaline pre-treatment. They obtained a maximum reducing sugar yield of 402.73 mg/g after microwave-$Ca(OH)_2$ pre-treatment (Jin et al., 2016).

Microwave-assisted alkaline pre-treatment of cassava stem was performed and the pre-treatment conditions were optimized to increase sugar recovery by Kamalini et al. (2018). They evaluated the effect of reaction time, NaOH concentration, solid to liquid ratio and microwave frequency on sugar yield. Under optimum conditions (reaction time of 116.4 s, NaOH concentration of 3.21%, solid to liquid ratio of

1:62.07 g/mL and microwave frequency of 719.86 Hz), maximum total reducing sugar and xylose yields were 43.60 µg/mL and 91.71 µg/mL, respectively. They stated that the NaOH pre-treatment combined with microwave irradiation is an effective method to produce fermentable sugars within less time (Kamalini et al., 2018).

Harahap et al. (2019) optimized the microwave-assisted alkali delignification conditions of oil palm empty fruit bunch by response surface methodology. They evaluated microwave power (280-840 W), NaOH concentration (1-3% w/v), and reaction time (3-9 min) to enhance lignin removal. They obtained maximum lignin removal of 88.1% under optimum pre-treatment conditions (microwave power of 832.9 W, NaOH concentration of 2.7% (w/v) and reaction time of 8.9 min). In addition, they evaluated the efficiency of pre-treatment using FTIR and SEM analysis which indicated that the pre-treatment changed the chemical and morphological structure of biomass. The authors reported that the microwave-assisted alkaline pre-treatment could effectively remove lignin in a relatively short time by using low alkaline concentration (Harahap et al., 2019).

Ultrasound-assisted alkaline pre-treatment was carried out to produce fermentable sugars from sugarcane bagasse by Velmurugan and Muthukumar (2012) and they investigated the effect of particle size, liquid-solid ratio, NaOH concentration, temperature and sonication time on lignin removal and reducing sugar yield. They reported the optimum pre-treatment conditions as 0.27 mm particle size, 25 mL/g liquid-solid ratio, 2.89% (w/v) NaOH concentration, 70.15 °C temperature and 47.42 min pre-treatment time and they reached 92.11% reducing sugar yield under these conditions. Also, they stated that the significant reduction in pre-treatment time and temperature with enhanced efficiency is the most attractive characteristics of ultrasound-assisted alkali pre-treatment (Velmurugan and Muthukumar, 2012).

SriBala et al. (2016) examined the morphological and structural changes occur during ultrasound-assisted alkaline pre-treatment of cellulose. Further, they evaluate the pre-treatment efficiency on glucose production by enzymatic hydrolysis. Their findings indicated that the ultrasound-assisted alkaline pre-treatment significantly decrease particle size of cellulose and reduced the processing time and the required

NaOH concentration to reach a low crystallinity of cellulose, while increasing the glucose yield 2.5 times compared to the untreated biomass (SriBala et al., 2016).

Ultrasound-assisted alkali (US-NaOH) pre-treatment of *Crotalaria juncea* was performed by Manasa et al. (2018) to enhance enzymatic saccharification. They treated the biomass with NaOH and US-NaOH at 121 °C. Their results showed that cellulose content was higher (67%) for US-NaOH pre-treated biomass when compared to the NaOH pre-treated biomass (55%). Also, the crystallinity index of sample was improved from 39% to 53% after US-NaOH pre-treatment which indicated that lignin was removed and cellulose content of samples increased. In addition, the authors stated that the US-NaOH pre-treatment significantly increased the enzymatic accessibility of cellulose to release reducing sugars (Manasa et al., 2018).

Many studies similar to the examples given above are also available in the literature (Gabhane et al., 2014; Kuittinen et al., 2016; Lai and Idris, 2016; Wu et al., 2017). As a common result of all these studies, it has been revealed that the microwave and ultrasound-assisted pre-treatments, which reduce the processing time and energy requirement, are good alternatives to classical acid or alkali applications performed at high temperature and pressure.

2.4 Hydrolysis of Lignocellulosic Biomass

A process called as hydrolysis is performed to convert the carbohydrate polymers in lignocellulosic biomass to the simple sugars before fermentation. There are several processes for the hydrolysis of lignocellulosic biomass. Among these methods, chemical and enzymatic methods have proven to be more successful. Cellulose and hemicellulose are converted to bioethanol by hydrolysis and fermentation processes, while lignin separated as a by-product at the end of the hydrolysis (Adıgüzel, 2013).

In chemical hydrolysis, biomass is treated with a chemical for a period of time at a certain temperature for the production of fermentable sugars from cellulose and hemicellulose. Acids are usually applied in chemical hydrolysis. Sulfuric and hydrochloric acid are widely used catalysts for hydrolysis of lignocellulosic biomass. Dilute or concentrated acids can be used in the chemical hydrolysis process. Higher sugar yield is obtained from concentrated acid process when compared to the dilute

acid process. Another important advantage of concentrated acid process is its application at low temperatures (e.g. 40°C). However, acid concentration is very high in concentrated acid process (e.g. 30-70%). So, the main disadvantages of this method are being extremely corrosive, requirement of corrosion resistant reactors, having energy demanding acid recovery process and high investment and maintenance costs (Taherzadeh and Karimi, 2007). Acid concentration between 2 to 5% is usually used in dilute acid hydrolysis, which is advantageous than concentrated acid process. However, this method is performed at high temperatures to obtain acceptable cellulose conversion rates. The high temperature increases the formation of toxic compounds from hemicellulosic sugars which reduce fermentable sugar concentration, inhibit the yeasts and cause low ethanol yield in the subsequent fermentation step (Verardi et al., 2012).

Enzymatic hydrolysis is performed using highly specific cellulase enzymes, and hydrolysis products are usually glucose-containing reducing sugars. This process is carried out at pH 4.8 and 45-50^{o}C, which is the optimum for cellulase enzyme, so that more moderate conditions are used compared to chemical hydrolysis. Enzymatic hydrolysis is advantageous than chemical hydrolysis, because this method does not cause corrosion problems. But, the process time, which is a few minutes in chemical hydrolysis, increases to several days in enzymatic hydrolysis. In addition, the end products of enzymatic hydrolysis inhibit the enzyme and negatively affect hydrolysis as long as they are not removed from the medium immediately after formation (Binod et al., 2011). There are three steps in the enzymatic hydrolysis of cellulose. These steps are adsorption of cellulase to the cellulose surface, cellulose hydrolysis to glucose and desorption of cellulase. The presence of non-cellulose components such as lignin and hemicellulose in the environment and the high crystallinity of cellulose prevent the adsorption of cellulase. Enzymatic hydrolysis process can be affected by substrate concentration, enzyme activity and reaction conditions (temperature, pH, etc.) (Sun and Cheng, 2002).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Folin-Ciocalteau phenol reagent, gallic acid, α-, β-, γ- and δ-tocopherols standards were provided by Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), sodium carbonate, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), citric acid, 3,5-dinitrosalicylic acid, sodium hydroxide, glucose, xylose, arabinose, furfural, acetic acid, hexane, ethanol, methanol, 2-propanol and other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA.). All solvents and reagents were analytical or chromatographic grade. The Cellic Ctec2 enzyme mixture used for enzymatic hydrolysis was kindly provided by Novozymes (Beijing, China).

3.1.2 Raw Materials

Mature healthy pistachio nuts were harvested from a village nearby Gaziantep, Turkey in September. The hulls of the collected nuts were removed and dried in an alpha 1-4 LD plus freeze drier (Christ, Osterode am Harz, Germany). The hulls were ground and the fraction that was sieved using a 250-mesh sieve was stored at -20 °C in airtight bags until being used. The final moisture content of the samples was less than 3% (w/w on wet basis). Pistachio shell was kindly supplied from Altin Fistik Gida Mad. San. Ve Tic. Ltd. Şti., Gaziantep, Turkey. Pistachio shell was ground using a knife mill and the particles smaller than 425 μm were kept in sealed bag at room temperature until further use.

3.2 Methods

3.2.1 Extraction of Non-polar Compounds from Pistachio Hull and Characterization of Extracts

3.2.1.1 Extraction Methods

3.2.1.1.1 Soxhlet Extraction

Dried PH sample (10 g) was extracted with 220 mL hexane for 8 h on a hot plate by using a Soxhlet apparatus. At the end of extraction, the solvent was evaporated at 40 °C using a rotary evaporator (Heidolph Instrument GmbH & Co. KG, Schwabach, Germany) and the extract was stored at -20 °C until analysis.

3.2.1.1.2 Microwave-assisted Solvent Extraction (MASE)

Dried PH sample (1.5 g) and different amount of hexane were placed into a 35 mL vessel. The mixture was stirred at high level under a closed system, using a microwave reactor (CEM Corporation, USA). The extraction was performed in dynamic mode. Synergy software was used to set the microwave extraction conditions. The extraction variables which were liquid to solid ratio (5:1-20:1 v/w), microwave power (170-280 W) and irradiation time (5-17.5 min) were studied to obtain maximum extraction yield (%). After extraction process, the mixture was centrifuged at 6000 rpm for 15 min and upper solution was collected. Solvent was evaporated at 40 °C using a rotary evaporator and the extract was stored at -20 °C prior to analysis.

3.2.1.2 Preparation of Cold Pressed Pistachio Oil

Pistachio oil was extracted from pistachio kernel by cold pressing to compare its fatty acid composition with that of hexane extracts of hull. The dry pistachio kernels were pressed at room temperature with a manual cold press (YP 0420, Ceselsan Makina, Giresun Turkey) and stored at 4 °C in a refrigerator before the analysis.

3.2.1.3 Chemical Composition of Pistachio Hull

Moisture, ash and protein contents of PH were analyzed by the standard methods of ASTM E1756-01 (ASTM, 2001), ASTM E1755-01 (ASTM, 2003) and AOAC 984.13 (AOAC., 1990), respectively. Non-polar extract was obtained by Soxhlet

method. Carbohydrate content was calculated by subtracting others components from 100.

3.2.1.4 Characterization of Non-polar Extracts

3.2.1.4.1 Total Phenolic Content (TPC)

The TPC of non-polar extracts was analysed according to the Folin-Ciocalteu colorimetric method as described by Fuentes et al. (2012) at a wavelength of 765 nm using Perkin Elmer Lambda 25 UV/Vis spectrophotometer (Connecticut, USA). TPC of extracts was calculated using a gallic acid calibration curve and reported as gallic acid equivalent per gram dry weight of sample (mg GAE/g dw).

3.2.1.4.2 Antioxidant Activity

The antioxidant activity of the PHEs were evaluated in terms of radical scavenging ability, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to procedure described by Kalantzakis et al. (2006). Briefly, 1 mL of ethyl acetate solution of extract at different concentrations (0.05-25 mg/mL) was mixed with 4 mL freshly prepared DPPH solution (0.1mM) in ethyl acetate. The mixture was shaken vigorously and incubated in the dark for 30 min at room temperature. At the end of the incubation period, the absorbance value of the solution monitored at 515 nm using a Perkin Elmer Lambda 25 UV/Vis spectrophotometer (Connecticut, USA). Antiradical action of extracts was determined from the difference in absorbance with or without sample (control). The percent inhibition was calculated using the following formula:

$$Percent\ Inhibition\ (I\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100 \qquad (3.1)$$

IC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained from the graph of percent inhibition versus concentration of samples. Trolox was used for comparison.

3.2.1.4.3 Tocopherol Content

Individual tocopherols of non-polar extracts were quantified by HPLC following the ISO 9936 standard method (ISO, 2016). Briefly, extract was dissolved in hexane and a 10 µL aliquot was analyzed by HPLC using a silica column and eluting with hexane:2-propanol (99.5:0.5 v/v) at a flow rate of 1 mL/min. A fluorescence detector

was set at 290 nm excitation wavelength and 330 nm emission wavelength. The tocopherols were identified by comparing the retention times with standards of α-, β-, γ- and δ-tocopherols.

3.2.1.4.4 Fatty Acid Composition

The fatty acid compositions of pistachio oil obtained from pistachio kernel by cold pressing and non-polar extracts were determined after converting to fatty acid methyl esters (FAME) prior to GC analysis according to procedure described by Çiftçi et al. (2009) with some modifications. After methylation, the fatty acid composition was determined by using a 7890A gas chromatography system (Agilent Technologies, USA) equipped with a flame ionization detector (FID), a split/splitless injector (operated with a split ratio of 1:50), and a capillary column HP-88 (88% Cianopropylaryl 100 m × 0.250 mm ID × 0.20 μm). Helium was used as the carrier gas. The injection temperature was 250 °C and injection volume was 1μL. Firstly, the oven temperature was held at 120 °C for 1 min. Then, it was set to increase of 10 °C/min until 175 °C, held for 10 min, followed by increase of 5 °C/min up to 210 °C, held for 5 min. Finally, the increase in temperature of 5 °C/min up to 230 °C was applied and held for 5 min. The detector was set at 260 °C with 350 mL/min air flow, 35 mL/min hydrogen flow, and 15 mL/min helium makeup flow. The fatty acids were identified from retention times based on comparison with fatty acid standards.

3.2.2 Extraction of Polar Compounds from Pistachio Hull and Characterization of Extracts

3.2.2.1 Extraction Procedures

3.2.2.1.1 Microwave-assisted Solvent Extraction (MASE)

The MASE process was performed in a closed vessel pressurized microwave reactor working at 0-300 W microwave output with 0-2.07 × 103 kPa pressure control (Discover SP, CEM, Matthews, NC, USA). The system was equipped with an infrared temperature sensor, an electromagnetic stirring and simultaneous air cooling. Thirty experimental MASE runs on different microwave power, time, ethanol concentration and solvent to sample ratio were performed. In each experimental run, 1 g of dried and powdered hull was mixed with aqueous ethanol in a 35-mL microwave quartz vial. After extraction, the reaction mixture was quickly cooled.

After that, the mixture was centrifuged at 6000 rpm for 15min (Hettich -EBA 20, Andreas Hettich GmbH & Co. KG, Germany) and the supernatant was collected. Subsequently, the supernatant was concentrated at 40 °C under vacuum using a rotary vacuum evaporator (Heidolph Instrument GmbH & Co. KG, Schwabach, Germany). Finally, concentrated extract was freeze dried in an alpha 1-4 LD plus freeze drier (Christ, Osterode am Harz, Germany) and dry extracts were kept at -20 °C until being use. The extraction yield was reported as g extract per 100 g of dry hull.

3.2.2.1.2 Conventional Solvent Extraction (CSE)

CSE was applied using the method described by Dahmoune et al. (2015). The ground sample (1 g) was extracted with 50 mL of 50% ethanol (v/v) for 2 h at 60 °C in a rotary incubator (New Brunswick Scientific, Nova 40, Edison, NJ, USA). At the end of the extraction, the dry extract was obtained as described in previous section.

3.2.2.2 Experimental Design and Optimization by Response Surface Methodology (RSM)

A two level, central composite face-centered design (CCFD) with four factors was performed for the evaluation of extraction factors on the response and to optimize extraction conditions. The independent factors were microwave power (X_1, 80-140 W), extraction time (X_2, 1-5 min), solvent to sample ratio (X_3, 8:1-20:1 v/w) and ethanol concentration (X_4, 30-70%). The limits of variables were selected by considering the values obtained in pre-experiments. The response was the TPC of extracts. The design was consisted of 30 experimental runs with six replicates at the centre point (Table 4.4). The extraction conditions were optimized to get the highest TPC.

3.2.2.3 Compositional Analysis of Pistachio Hull, Hull Extract and Residue after Extraction

3.2.2.3.1 Moisture, Protein and Ash Content

Moisture, ash and protein contents of samples were analysed by the standard methods of ASTM E1756-01 (ASTM, 2001), ASTM E1755-01 (ASTM, 2003) and AOAC 984.13 (AOAC., 1990), respectively.

3.2.2.3.2 Extraction of Non-polar Compounds

Non-polar fraction from samples was extracted with *n*-hexane for 8 h on a hot plate by using a Soxhlet apparatus. At the end of the extraction, solvent was removed at 40 °C using a rotary vacuum evaporator and the percentage of non-polar fraction was determined using the following formula:

$$Np_{fr}\ (\%) = \left(\frac{m(extract)}{m(dry\ hull)}\right) \times 100 \quad (3.2)$$

3.2.2.3.3 Pectin Content

Conventional pectin extraction from samples was performed according to Bagherian et al. (2011) and the pectin content was determined by using following formula:

$$Pectin\ content\ (\%) = \left(\frac{Dried\ pectin\ (g)}{Dry\ hull(g)}\right) \times 100 \quad (3.3)$$

3.2.2.3.4 Total Sugar Content

Total sugars from each sample were extracted with water for 30 min. After centrifugation, the upper solution was collected and analyzed using 3,5-dinitrosalicylic acid (DNS) method as reported by (Miller, 1959). Briefly, 1 mL of samples was taken to the test tubes and the hydrolysis was made with 0.05 mL of concentrated HCl for 10 min at 90 °C. Then, the solution was neutralized with 0.15 mL of 5 N KOH and 3 mL of DNS solution was added. The tubes were put in a boiling water bath. After 5 min, they were cooled to room temperature and distilled water was added to each tube to volume of 10 mL. Absorbance measurements at 540 nm were made with a spectrophotometer (Optima SP 3000 Nano, Tokyo, Japan). Glucose was used for the preparation of calibration curve and the results were given as g/100 g dry weight (dw).

3.2.2.3.5 Acidity

Titratable acidity of samples was determined according to standard method of AOAC 942.15 (AOAC, 2000). Results are reported as g of citric acid/100 g dry weight (dw).

3.2.2.3.6 Fiber Analysis

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) contents of samples were determined according to ANKOM Method 13 (ANKOM Technology, 2003), Method 12 (ANKOM Technology, 2017) and Method

8 (ANKOM Technology, 2016), respectively, by using an ANKOM 2000 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA).

3.2.2.3.7 Total Phenolic Content

TPC of PH extracts was determined using Folin-Ciocalteu method and the procedure reported by Fernández-Agulló et al. (2013). TPC of extracts was calculated using a gallic acid calibration curve and reported as gallic acid equivalent (mg GAE/g dry hull).

3.2.2.3.8 Antioxidant Activity Analysis

The antioxidant activity of the PHEs were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity according to procedure reported by Azaizeh et al. (2012). Briefly: A 0.1 mL of PHEs at different concentrations (250-2000 ppm) was mixed with 3.9 mL of methanolic DPPH solution (50 ppm). The solutions were mixed vigorously and kept at 37 °C for 30 min. At the end of the time, the absorbance values of the solutions monitored against pure methanol (blank) at 517 nm using a UV-Vis spectrophotometer (Optima SP 3000 Nano, Tokyo, Japan). The control consisted of methanol instead of the hull extract. Trolox was used for comparison. The scavenging activity (%) was determined using the formula:

$$Scavenging\ activity\ (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100 \qquad (3.4)$$

The scavenging ability of the different extracts was presented as IC_{50}, which is the effective concentration at which DPPH radicals were scavenged by 50%. IC_{50} was found from the graph of scavenging activity (%) versus sample concentrations. Low IC_{50} value shows stronger ability of the extract to act as DPPH scavenger (Ahmad et al., 2014).

3.2.3 Fractionation of Cellulose, Hemicellulose and Lignin from Pistachio Shell

3.2.3.1 Compositional Analysis of Pistachio Shell

3.2.3.1.1 Determination of Moisture, Ash, Protein and Extractive Material

The moisture, ash and extractable material contents of PS were determined according to NREL/TP-510-42621 (Sluiter, A, Hames, B, Hyman, D, et al., 2008), NREL/TP-

510-42622 (Sluiter, Amie et al., 2008) and NREL/TP-510-42619 (Sluiter, A, Ruiz, R, et al., 2008). Protein content was determined by Kjeldahl method according to AOAC 984.13 standard method using the N×6.25 conversion factor (AOAC, 2006).

3.2.3.1.2 Determination of Acid-Soluble and Acid-Insoluble Lignin

The acid-soluble and insoluble lignin contents of pistachio shell were determined according to NREL/TP-510-42618 (Sluiter, A, Hames, B, Ruiz, R, et al., 2008). According to this method, 0.3 g of shell was weighed into test tube and 3 mL of 72% H_2SO_4 was added. The tube was placed in a water bath at 30 ° C and incubated for 1 hour. The sample was stirred every five to ten minutes using a stir rod without removing the sample from the bath. Then, the acid concentration was diluted to 4% by adding 84 mL of deionized water and the mixture was autoclaved at 121 ° C for 1 hour. At the end of the period, the remaining mixture was cooled and filtered through sinter glass filter crucible. The crucible and the residue were dried at 105 ° C until a constant weight and then placed in a muffle furnace at 575 °C for 24 h. The change in the mass before and after the combustion process was calculated as the amount of acid-insoluble lignin.

In order to calculate the amount of acid-soluble lignin, the absorbance of the hydrolysis liquor was adjusted into the range of 0.7-1.0 by diluting the solution with distilled water and the dilution factor was recorded. The absorbance of the sample was measured at 320 nm using a UV-Vis spectrophotometer (Optima SP 3000 Nano, Tokyo, Japan). The amount of acid-soluble lignin was calculated using the following equation:

$$Acid\ soluble\ lignin\ (\%) = \frac{UV_{abs} \times V_{filtrate} \times Dilution}{\varepsilon \times Weight\ of\ sample} \times 100 \qquad (3.5)$$

where UV_{abs}, $V_{filtrate}$ and ε are UV-Vis absorbance of the sample at specified wavelength, volume of the filtrate (mL) and absorptivity constant of biomass at specific wavelength (L/g.cm), respectively.

3.2.3.1.3 Determination of Glucan, Xylan, Arabinan and Acetyl Groups Content

Monomeric sugars (glucose, xylose and arabinose) and acetic acid contents in pistachio shell acid hydrolysates were analysed according to NREL/TP-510-42618 (Sluiter, A, Hames, B, Ruiz, R, et al., 2008) using a Shimadzu SCL-10A vp HPLC

system (Kyoto, Japan) and a Bio-Rad Aminex HPX-87H column (Hercules, CA, USA) at 60 °C. 5 mM H_2SO_4 was used as a mobile phase at a flow rate of 0.6 mL/min. A refractive index detector (Shimadzu RID-10A) was used to analyse sugars and organic acid contents of samples. Quantitative analysis was performed by external calibration of the components. The amount of cellulose (as glucan), xylan, arabinan and acetyl groups of pistachio shell was determined using the following equations (Browning, 1967):

$$Glucan\ (\%) = F \times \frac{100}{1005} \times \frac{162}{180} \times \frac{Glc \times W_{sol}}{S} \quad (3.6)$$

$$Xylan\ (\%) = F \times \frac{100}{1005} \times \frac{132}{150} \times \frac{Xyl \times W_{sol}}{S} \quad (3.7)$$

$$Arabinan\ (\%) = F \times \frac{100}{1005} \times \frac{132}{150} \times \frac{Ara \times W_{sol}}{S} \quad (3.8)$$

$$Acetyl\ groups\ (\%) = F \times \frac{100}{1005} \times \frac{60}{61} \times \frac{Ac \times W_{sol}}{S} \quad (3.9)$$

where F is correction factor; W_{sol} is the mass of the solution; S is the mass of the dry sample; Glc, Xyl, Ara and Ac represent the glucose, xylose, arabinose and acetic acid concentrations (g/L) present in the hydrolysate, respectively.

3.2.3.2 Alkaline Pre-treatment of Pistachio Shell

Three different alkali pre-treatment methods, which are microwave-assisted, ultrasound-assisted and sequential combination of ultrasound with microwave were carried out for fractionation of pistachio shell using NaOH. In addition, conventional alkali pre-treatment was performed as a control.

3.2.3.2.1 Conventional Alkaline Pre-treatment (CAP)

CAP of PS was carried out as explained previously by Singh et al. (2017) with some modifications. The raw material (10 g) was mixed with 100 mL of NaOH solution at different concentrations (0.1, 0.5, 1, 1.5 and 2 N). The pre-treatment was carried out at 35 °C and 150 rpm for 16 h in a rotary incubator (New Brunswick Scientific, Nova 40, Edison, NJ, USA). Vacuum filtration was applied to separate the solid and liquid fractions after pre-treatment. The solid fraction was washed with distilled water until neutral pH, dried at 50 °C for 24 h and stored at room temperature until used for compositional analysis. The cellulose recovery from the remaining solid phase was calculated using the formula below:

$$Cellulose\ recovery\ (\%) = \frac{Weight\ of\ cellulose\ in\ dry\ pretreated\ biomass}{Weight\ of\ cellulose\ in\ dry\ biomass} \times 100 \quad (3.10)$$

The liquid phase was centrifuged at 5000 rpm for 15 min. The supernatant was collected and its pH was adjusted to 5 by using glacial acetic acid. 1.5 times the volume of ethanol (95%) was added to this solution for precipitation of hemicellulose. This mixture was stored at 4 °C overnight. The precipitates formed were separated by centrifugation, washed twice with distilled water and dried at 50 ° C. The remaining filtrate (black liquor) contains dissolved lignin. Hemicellulose recovery was calculated by the following formula:

$$Hemicellulose\ recovery\ (\%) = \frac{Dry\ weight\ of\ extracted\ hemicellulose}{Weight\ of\ hemicellulose\ in\ dry\ biomass} \times 100 \quad (3.11)$$

3.2.3.2.2 Microwave-assisted Alkali Pre-treatment (MAAP)

MAAP was performed in a closed-vessel microwave reaction system (Discover SP, CEM, Matthews, NC, USA) working at 0-300 W microwave output with a frequency at 2455 MHz. The reactor had an infrared temperature sensor, an electromagnetic stirring and simultaneous air cooling. 1 g of milled PS was mixed with sodium hydroxide solution of desired concentration at 5% solid loading in a 35 mL glass vial. The vial was sealed with a silicon cap, placed in microwave system and irradiated for a set time and power as determined in the experimental design. After pre-treatment, the reaction mixture was subjected to the same processes described in section 3.2.3.2.1.

The factors affecting the MAAP of PS were evaluated using a three factor, three level Box-Behnken design (BBD). The independent variables were selected as microwave power (X_1, 100-300 W), NaOH concentration (X_2, 0.1-2 N) and pre-treatment time (X_3, 1-5 min). The design was consisted of 17 experimental runs including five replicates at the central point (Table 4.8). The response values were selected as hemicellulose recovery (%) from the liquid phase and cellulose recovery (%) in the solid phase. Pre-treatment conditions were optimized for maximum hemicellulose recovery (%) in the liquid fraction and the highest cellulose recovery (%) in the solid fraction using the response surface methodology.

3.2.3.2.3 Ultrasound-assisted Alkaline Pre-treatment (UAAP)

UAAP was performed using an ultrasonic water bath (Sonorex Digiplus DL 255 H, Bandelin, Heinrichstraße, Berlin, Germany) operated at 35 kHz. The maximum

ultrasonic power of the system was 640 W which is adjustable from 20-100%. The ultrasonic bath was connected to a water circulation system (C200-H13, Labo Makina San. ve Tic. A.Ş., Istanbul, Turkey) to adjust and maintain the temperature during the pre-treatment. One gram of shell was immersed in 20 mL alkaline solution of desired concentration in a glass vial. Then, the mixture was placed in the ultrasonic bath and UAAP was performed under different conditions determined in the experimental design. The solid and liquid phase obtained after the pre-treatments were subjected to the same processes described in section 3.2.3.2.1.

A four factor, three level BBD was performed to evaluate the effect of pre-treatment parameters on the responses. The independent variables were ultrasound power (Y_1, 128-640 W), pre-treatment time (Y_2, 30-90 min), NaOH concentration (Y_3, 0.1-2 N) and temperature (Y_4, 45-95 °C). The design was consisted of 29 experimental runs including five replicates at the central point (Table 4.11). The response values were selected as hemicellulose recovery (%) from the liquid phase and cellulose recovery (%) in the solid phase. Pre-treatment conditions were optimized for maximum hemicellulose recovery (%) in the liquid fraction and the highest cellulose recovery (%) in the solid fraction using the response surface methodology.

3.2.3.2.4 Sequential Ultrasound-microwave-assisted Alkaline Pre-treatment (SUMAAP)

For SUMAAP, PS was first pre-treated by using ultrasound as described in section 3.2.3.2.3. Then, the ultrasound pre-treated mixture was allowed to cool down to 25 °C and transferred to a microwave reactor working at 0-300 W microwave output (Discover SP, CEM, Matthews, NC, USA). The experiments were performed under different conditions determined in the experimental design. After the pre-treatment, the solid and liquid phases were subjected to the same processes described in section 3.2.3.2.1.

The optimization of parameters affecting the SUMAAP was carried out in two stages. Initially, six variables were screened using a Plackett-Burman design (PBD) to identify the parameters, which significantly influenced pre-treatment. For PBD, ultrasound power (UP, 128-640 W), ultrasound temperature (UT, 45-95 °C), ultrasound time (Ut, 30-90 min), microwave power (MP, 100-300 W), microwave

time (Mt, 1-5 min) and NaOH concentration (NC, 0.1-2 N) were selected as the independent variables. Each variable was set at two levels, a high level and a low level and the experimental design consisting of 12 experimental runs is given in Table 4.14. According to PBD, UP, MP and NC were found to be the most important parameters affecting SUMAAP. In the second step, the selected independent variables of UP (128-640 W), MP (100-300 W), NC (0.1-2 N) was investigated using BBD. The design was consisted of 17 experimental points including five replicates at the central point (Table 4.16). The UT, Ut and Mt, which were found to be insignificant as a result of PBD, were kept constant in all experiments as 95 ° C, 30 min and 2.5 min, respectively.

3.2.3.3 Compositional Analysis of Solid and Liquid Fractions Obtained after Pre-treatments

The solid fractions obtained after the pre-treatment was dried at 105 °C and their moisture content was reduced to less than 10%. 0.1 g of samples were weighed into test tubes and 1 mL of 72% H_2SO_4 was added to each tubes. The tubes were kept in a 30 °C water bath for 1 h. Then the acid content in each tube was diluted to 4% by adding deionized water and the mixtures were autoclaved at 121 °C for 1 hour. At the end of the period, the mixtures were cooled and filtered through sinter glass filter crucible. The cellulose, hemicellulose and lignin content of the shell residues were determined as described in sections 3.2.3.1.2 and 3.2.3.1.3.

The monomeric sugar and organic acid contents of the liquid fractions were determined using the HPLC system as described in section 3.2.3.1.3.

3.2.3.3.1 Analysis of Furfural and HMF

Furfural and HMF analyses were performed using the HPLC system and an UV/Vis detector (Shimadzu SPD-20A) at a wavelength of 280 nm as described in section 3.2.3.1.3.

3.2.3.3.2 Analysis of Oligosaccharides

Post-hydrolysis of the liquid phase was carried out according to the method described by Duarte et al. (2004) to determine the amount of oligosaccharides in the liquid fractions. The sugar content of post-hydrolysis liquors was analysed using the

HPLC system as described in section 3.2.3.1.3. The concentration of oligosaccharides in the liquid fractions was calculated from the increase of monosaccharides' concentration after post-hydrolysis procedure (Duarte et al., 2004).

3.2.3.4 Enzymatic Hydrolysis of Pre-treated Solids

Enzymatic hydrolysis of raw PS and pre-treated solids were performed using a solid loading of 15% (w/v) in 5 mL total volume containing sterile 50 mM citrate buffer (pH 4.8). The enzyme loading was 20 FPU/g glucan and the hydrolysis reactions were performed in 25 mL flasks in shaker incubator (New Brunswick Scientific, Nova 40, USA) at 50 °C and 200 rpm. 10 µL of sodium azide (20 g/L) was added to the reaction mixture to prevent microbial contamination. Aliquots were collected at 3, 6, 9, 24, 48 and 72 h and placed in a boiling water bath for 5 min to deactivate the enzymes. Then, the enzymatic liquors were filtered and analysed by HPLC as mentioned in section 3.2.3.1.3. The glucan to glucose yield was calculated from the amount of released glucose (g/L) by using the following equation:

$$Glucan\ to\ glucose\ yield\ (\%) = \frac{\left(\frac{162}{180}\right)\times[glucose]\times V}{glucan\ content} \times 100 \qquad (3.12)$$

where [glucose] is the glucose concentration in the enzymatic hydrolysate in g/L, V is the volume of the enzymatic solution (L), glucan content is the amount of glucan in native or pre-treated samples (% of dried weight) prior to enzymatic hydrolysis.

3.2.3.4.1 Determination of Enzyme Activity

The total cellulase activity of Cellic Ctec2 enzyme mixture was determined before the application of enzyme according to NREL/TP-510-42628 (Adney and Baker, 1996). 50 mg of Whatman No: 1 filter paper was weighed into test tubes. 0.5 mL of enzyme solution prepared in different concentrations and 1 mL of citrate buffer (pH = 4.8) were added to each tubes. The solutions were incubated at 50 °C for 60 min. At the end of the incubation period, 3 ml of DNS solution was added to each tube and the tubes were kept in a boiling water bath for 5 min. The tubes were then kept in a cold water bath for 1 min and cooled. 0.2 mL of the reaction mixture was taken into the spectrophotometer cuvettes and diluted with 2.5 mL of water. The absorbance of the mixture was measured at a wavelength of 540 nm using a spectrophotometer (Optima SP 3000 Nano, Tokyo, Japan). The glucose concentration was determined

using the glucose calibration curve. Then, a graph showing the amount of released glucose against enzyme concentration was drawn on semi-logarithmic paper and the enzyme concentration corresponding to 2 mg of glucose was determined. Enzyme activity was calculated in terms of filter paper unit (FPU) using the following equation:

$$Filter\ paper\ activity\ (FPU/mL) = \frac{0{,}37}{[enzyme] releasing\ 2\ mg\ glucose} \qquad (3.13)$$

3.2.3.4.2 Investigation of Enzymatic Hydrolysis Data Using Fractal Kinetic Model

Enzymatic hydrolysis data were analysed using the fractal kinetic model proposed by Kopelman which is described by the following equation (Kopelman, 1988):

$$P(t) = S_0[1 - exp(-k.t^{1-h}) \qquad (3.14)$$

where P(t), S_0, k, h and t are the concentration of product (g/L), initial concentration of glucan (g/L), time dependent rate coefficient, fractal exponent, and the reaction time (h), respectively.

3.2.4 Scanning Electron Microscopy (SEM) Analysis

After extraction of polar and non-polar compounds, to investigate the effect different extraction methods (Soxhlet, CSE and microwave-assisted extractions) on the surface morphology of samples, the solid residues after extractions were collected and analyzed with SEM. Samples were mounted on aluminium SEM stubs and coated with gold:palladium in an Emitech SC7620 (Kent, UK) sputter coater and imaged with JSM-6390LV (JEOL Ltd., Japan). The SEM photographs were taken at 20 kV and a magnification of 300×.

After alkaline pre-treatments, PS and pre-treated solids were characterized with Zeiss GeminiSem300 (Carl Zeiss Ltd. Co., Germany) scanning electron microscope to evaluate the effect of pre-treatments on the surface morphologies of samples. Samples were prepared as described above. The SEM images were observed at 10 kV and a magnification of 300×.

3.2.5 Statistical Analysis

All experiments were carried out three times. The results were averaged and expressed as means ± standard deviations. The experimental data obtained after extraction procedures were statistically analysed by independent samples *t*-test at the significance level of $p < 0.05$ using the SPSS statistical software, version 21.0 (SPSS Inc., Chicago, IL, USA). RSM was performed for experimental design, regression and graphical analysis and to optimize extraction and pre-treatment parameters, using Design Expert v. 7.0 (Stat--Ease, Inc., Minneapolis, MN, USA). The significance level of 95% was used for all analysis. The analysis of variance (ANOVA) was used to determine the significance of the independent variables and their interactions, the adequacy of the developed model and statistical significance of the regression coefficients. One-Sample *t*-Test was used after the optimization process to test the reliability of the predicted and experimental values. Hemicellulose and cellulose recoveries obtained by CAP, MAAP, UAAP and SUMAAP processes were statistically analysed by SPSS 22.0 (SPSS Inc., Chicago, IL, USA) applying the One Way ANOVA and Duncan multiple comparison test was used to compare the experimental results. Sigma Plot 12.0 (Systat Software Inc., Erkrath, Germany) was used to analyze the enzymatic hydrolysis data.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Microwave-assisted Extraction of Non-polar Compounds from Pistachio Hull and Characterization of Extracts

4.1.1 Chemical composition of Pistachio Hull

Some chemical characteristics of PH are given in Table 4.1. The moisture content was 70.81 ± 0.77% (wb). The contents of ash, protein and non-polar extract were 11.40 ± 0.41, 8.54 ± 0.37 and 9.50 ± 0.16 % on the dry basis, respectively. Non-polar extract composed of oil, lipophilic vitamins, phenolic compounds, phytosterols and fatty acids (Grace et al., 2016). The results obtained are similar to those reported by Moghaddam et al. (2009) except for the protein content. This could be related to differences in the pistachio varieties, growing practices, nut maturity and the dehulling method as stated by Bakhshizadeh et al. (2014).

Table 4.1 Some chemical characteristics of pistachio hull

Compound	Pistachio hull
Moisture content (%wb)[a]	70.81 ± 0.77 [b]
Ash (%db)[c]	11.40 ± 0.41
Protein (%db)	8.54 ± 0.37
Non-polar extract (%db)	9.50 ± 0.16
Total carbohydrates (%db)	70.56 ± 0.89

[a] wet basis, [b]each value in the table represented as the mean ± standard deviation of triplicate analyses, [c] dry basis

4.1.2 Extraction of Non-polar Components

Non-polar extracts from PH were obtained by using MASE method. Soxhlet extraction, which is a conventional and accepted process for the extraction of solid samples, was also performed as a control (De Castro and Garcıa-Ayuso, 1998). The extraction yield for Soxhlet method was 9.50 ± 0.16% (db).

MASE parameters including liquid to solid ratio, power and extraction time were investigated to obtain maximum extraction yield (%). As for liquid to solid ratio,

Figure 4.1 shows that the extraction yield increased with the increasing liquid to solid ratio from 5:1 to 15:1 (v/w). Furthermore, with the increase in liquid to solid ratio from 15:1 to 20:1 (v/w), a slight decrease was observed in extraction yield. This was probably because larger volume of solvent will absorb more microwave energy and insufficient microwave energy may be available for breaking the cell walls and releasing the target constituents (Liu et al., 2014). Hence, 15:1 (v/w) of liquid to solid ratio was selected for further experiments.

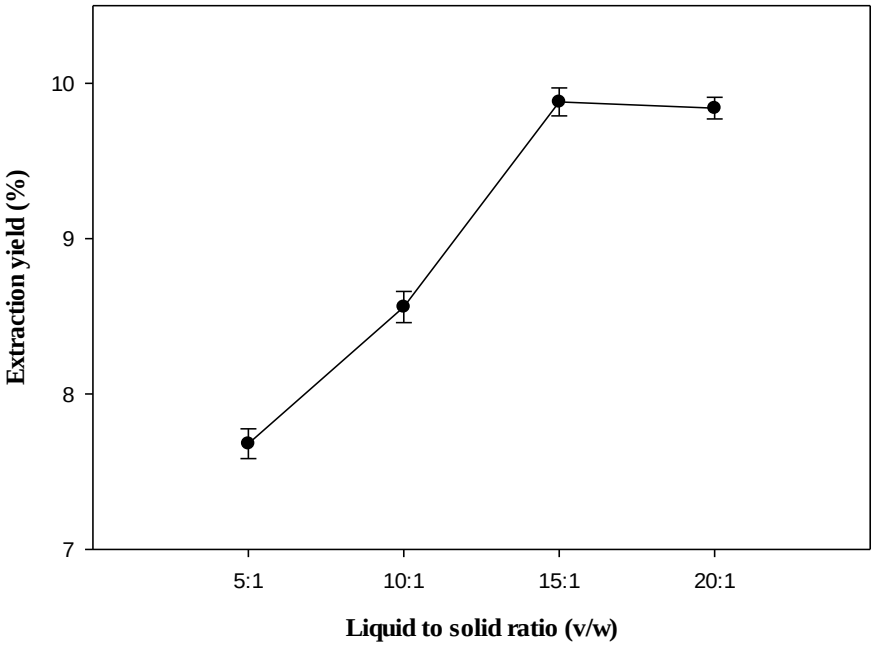

Figure 4.1 The effect of liquid to solid ratio on the extraction yield (%). Extractions were performed at 275 W for 15 min

Figure 4.2 shows the effect of power on the extraction yield. The extraction yield increased from 8.10 to 9.78% with the increasing microwave power from 170 to 250 W. This can be explained by the rapid heat generation inside the sample with the microwave energy absorption and subsequently higher pressure gradient formation inside the sample when higher microwave power is applied as reported by Ranitha et al. (2014). However, the extraction was almost completed at 250 W and after this point a further increase in yield was not observed. Based on these findings, microwave power of 250 W was selected for further extractions.

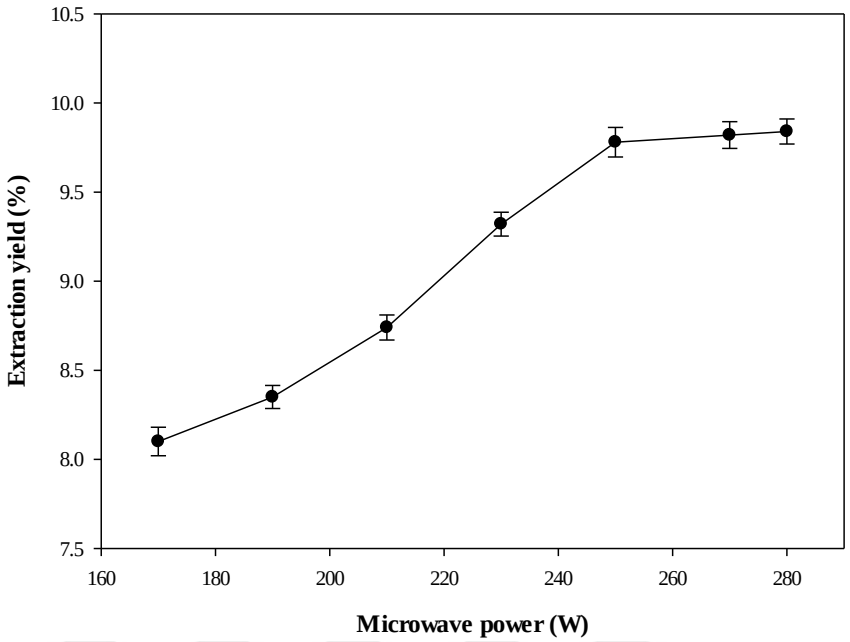

Figure 4.2 The effect of microwave power on the extraction yield (%). Extractions were carried out for 15 min and at a liquid to solid ratio of 15:1 (v/w)

Figure 4.3 shows the effect of extraction time on the extraction yield. The extraction yield increased from 8.53 to 9.81% by increasing extraction time from 5 to 12.5 min. With further increase in time above 12.5 min the extraction yield remained almost constant. A similar trend in the relation between extraction yield and time has also been observed in other studies (Kittiphoom and Sutasinee, 2015). According to these results, 12.5 min was chosen as the extraction time. Rajaei et al. (2010) also studied the MASE of PH using a household microwave oven and found optimum extraction time as 45 min. Shorter extraction time obtained in this study could be running the extraction under a high pressure compared to the atmospheric pressure in Rajaei et al.'s study. In addition, the MASE using a closed-vessel microwave system and Soxhlet extraction methods were compared in this study and MASE had the higher extraction efficiency with reduced extraction time and less solvent consumption. Based on these results, MASE can be a good alternative to Soxhlet method.

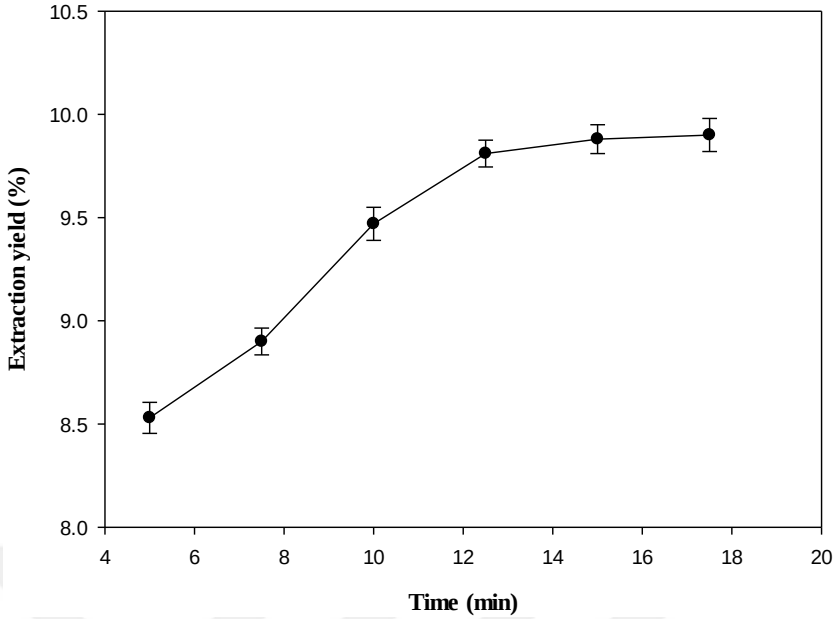

Figure 4.3 The effect of time on the extraction yield (%). Extractions were performed under 250 W and at a liquid to solid ratio of 15:1 (v/w)

4.1.3 Characterization of Non-polar Extracts

Total phenolic content (TPC), antioxidant activity and tocopherol content of PHEs were investigated and the results are given in Table 4.2.

TPC of extract obtained by MASE was higher (33.37 mg GAE/g dw) than Soxhlet extract (24.84 mg GAE/g dw). TPC of extracts obtained using the two mentioned methods was significantly different ($p < 0.05$) from each other. During MASE process, the extraction temperature did not exceed 60 °C and the time was 12.5 min. However, the traditional Soxhlet method involved long extraction time (8 h) and exposure to high temperatures that may cause thermal degradation of phenolic components. Goli et al. (2005) studied the TPC of PHEs which was extracted by water, methanol and ethyl acetate. They have reported that the water and methanol extracts had high phenolic content (32.0–34.0 mg TAE/g dry weight of sample). However, there is no study in the literature about the TPC of non-polar extracts from PH.

The IC_{50} values of extracts obtained by Soxhlet and MASE methods were 2.58 and 2.47 mg extract/mL, respectively. The difference between IC_{50} values of extracts was not statistically significant ($p > 0.05$). However, all the extracts showed lower

antioxidant activity than that of trolox (IC_{50} value for trolox was 0.021 mg/mL). Valavanidis et al. (2004) have reported that IC_{50} values of extra virgin olive oil, corn oil, sunflower oil and soybean oil were 11.00, 15.00, 14.00 and 10.00 mg/mL, respectively. Thus, non-polar extracts of PH might actually possess better antioxidant activity when compared with commercial edible oils.

Table 4.2 Total phenolic content, antioxidant activity and tocopherol content of extracts obtained by Soxhlet and MASE methods

Properties		**Soxhlet extraction**	**MASE**
Phenolic content (mg GAE[a]/g dw)		24.84±0.32[b]	33.37±0.59
Total antioxidant activity (DPPH assay, IC_{50}, mg extract/ml)		2.58±0.15	2.47±0.18
Tocopherol content (mg/kg extract)	α-tocopherol	466.44±3.73	567.65±2.53
	γ-tocopherol	23.94±1.03	14.69±0.47
	δ-tocopherol	268.47±1.38	264.49±1.22
	Total	758.85±1.47	846.84±1.05

[a] GAE: gallic acid equivalent.
[b] Each value in the table represents the mean ± standard deviation of triplicate analyses.

Tocopherols are one of the minor components of vegetable oils and have antioxidant properties which make them essential nutrient for human health (Kiralan et al., 2014). Tocopherol content of PHEs were determined (Table 4.2) and α-tocopherol was the major tocopherol (466.44 and 567.65 mg/kg extract for the Soxhlet and MASE methods, respectively) while γ-tocopherol was the rare tocopherol (23.94 and 14.69 mg/kg extract for the Soxhlet and MASE methods, respectively) found in extracts. It can be reported that the concentration of tocopherol in non-polar extracts was affected by the extraction method. MASE resulted in significantly higher tocopherol concentration (846.84 mg/kg extract) than that of Soxhlet extraction method (758.85 mg/kg extract) (p <0.05). This can be due to the effect of microwave irradiation that causes damage on hull cell membrane and allows increased release of tocopherols which also enhance their amount in the extract (Azadmard-Damirchi et al., 2010). Grace et al. (2016) obtained non-polar extract by extracting the PH with dichloromethane and analyzed the tocopherol content of the extract. They reported that the α-, γ- and δ-tocopherol contents of non-polar extracts were 140, 790 and 50 mg/kg extract, respectively. The differences in tocopherol composition are thought to be due to the differences in solvent type, extraction method, pistachio cultivars and

growing practices. Gliszczynska-Swiglo et al. (2007) analyzed the tocopherol content in edible plant oils and reported that the refined corn and soybean oils have the highest tocopherol content (829 mg/kg) while refined grapeseed and extra virgin olive oils have the lower tocopherol content (121 and 177 mg/kg, respectively). Özrenk et al. (2012) have also demonstrated that pistachios contained 1.36–26.93 mg/kg of α-tocopherol, 36.17-170 mg/ kg of γ-tocopherol and 0.45–2.61 mg/kg of δ-tocopherol.

Table 4.3 Fatty acid compositions (g/100 g fatty acid) of pistachio kernel oil and oil in hexane extracts obtained by Soxhlet and MASE methods

	Fatty acid composition (g / 100 g fatty acid)		
Fatty acid	**Pistachio kernel oil**	**Soxhlet extraction**	**MASE**
Palmitic (C16:0)	8.80±0.02[a]	15.90±0.25	16.40±0.58
Palmitoleic (C16:1)	0.61±0.01	nd[b]	nd
Heptadecenoic (C17:1)	0.07±0.01	nd	nd
Stearic (C18:0)	1.85±0.01	4.36±0.28	5.78±0.16
Elaidic (C18:1 trans)	nd	4.10±0.26	3.21±0.04
Oleic (C18:1 cis)	70.36±0.01	42.01±1.17	48.46±1.54
Linoelaidic (C18:2 trans)	nd	4.37±0.36	nd
Linoleic (C18:2 cis)	17.05±0.01	20.90±0.69	19.01±0.51
γ-linolenic (C18:3 *n*-6)	0.17±0.02	nd	nd
α-linolenic (C18:3 *n*-3)	0.22±0.03	9.69±0.50	7.14±0.27
Eicosenoic (C20:1)	0.43±0.01	nd	nd
Eicosatrienoic (C20:3 *n*-6)	1.13±0.03	nd	nd

[a] Each value in the table represents the mean ± standard deviation of triplicate analyses.
[b] Not detected

The fatty acid composition of pistachio kernel oil and non-polar extracts are given in Table 4.3. The results showed that the oleic and linoleic acids were the major unsaturated fatty acids, while the palmitic acid was the main saturated fatty acid present in non-polar extracts. These findings were similar to the results of Ghaffari et al. (2014) who determined the fatty acid composition of pistachio by-products. Although *trans* fatty acids were found in both extracts, their level in Soxhlet extract was higher than the extract obtained by MASE. Pérez-Serradilla et al. (2007) reported that formation of *trans* fatty acids can be due to thermally induced *cis-trans* isomerization. In MASE, the extraction time was much shorter and extraction was conducted at lower temperatures than those applied in Soxhlet method.

Similar to the non-polar extracts of PH, the major saturated fatty acid of cold pressed pistachio kernel oil was palmitic acid while the major unsaturated fatty acids were oleic and linoleic acids. Kirbaslar et al. (2012) have also reported similar results. Pistachio kernel oil had higher oleic acid concentration but lower palmitic, stearic and α-linolenic acid contents compared to hull extracts. *Trans* fatty acids were not detected in kernel oil. This could be due to the fact that kernel oil was extracted by cold press and not exposed to temperature.

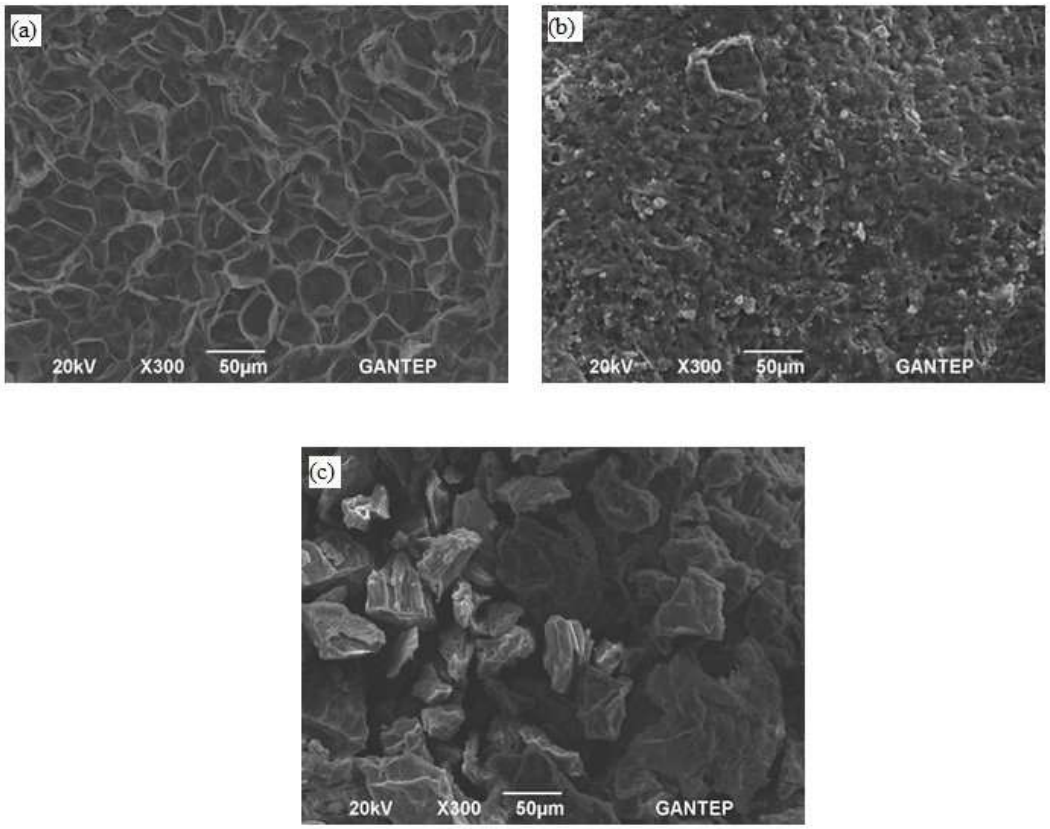

Figure 4.4 Scanning electron microscope images of pistachio hull (a) before extraction (b) after extraction by Soxhlet (c) after microwave assisted extraction

4.1.4 Scanning Electron Microscopy (SEM) Analysis

The untreated PH and PH residues after Soxhlet and MASE methods were examined by SEM for structural analyses to investigate the effect of different extraction techniques (Figure 4.4). After Soxhlet extraction, few slight ruptures were detected on the surface of the hull sample when compared to the untreated hull (Figure 4.4 (b)). In Soxhlet method, a heated solvent slowly diffuses into the solid matrix,

dissolves and extracts the components that cause little destruction on sample microstructure. After MASE, obvious changes on the surface morphology were observed (Figure 4.4 (c)). These changes suggested that microwave treatment played an important role in breaking up plant cell walls. Physical cell structure of sample is affected from microwave irradiation owing to the sudden temperature rise and internal pressure increase due to high vapor pressure inside the plant cells (Dahmoune et al., 2015).

4.2 Optimization of Microwave-assisted Extraction of Polar Compounds from Pistachio Hull

4.2.1 The Central Composite Face-centered Design (CCFD) and Optimization

4.2.1.1 Model Fitting

RSM was employed with CCFD to evaluate the effects of microwave power, extraction time, solvent to sample ratio and ethanol concentration on TPC of the PH. The design matrices for the CCFD with the predicted and the actual experimental results of the responses have been presented in Table 4.4. Multiple regression analysis was carried out to analyse the actual data. A quadratic polynomial regression model equation (Equation (4.1)) was created, that demonstrates the relationship between TPC and the four variables:

$$TPC = -9.23 - 0.03X_1 + 2.02X_2 + 4.70X_3 + 0.55X_4 - 0.03X_1X_2 + 0.0044X_1X_3 + 0.0014X_1X_4 + 0.13X_2X_3 + 0.0014X_2X_4 + 0.0016X_3X_4 + 0.0002X_1^2 - 0.009X_2^2 - 0.16X_3^2 - 0.0065X_4^2 \quad (4.1)$$

ANOVA was applied for determination of the significance of each variable for the model fitted (Table 4.5). The F-value of 14.09 and low *p*-value (< 0.0001) indicated that the generated model was significant. A good correlation between response and the independent factors was achieved with the determination coefficient value (R^2) of 0.9293. In addition, low coefficient of variance (CV) value (<10%) indicated the good reproducibility of investigated design. Furthermore, the model validity was verified by Lack of Fit Test, where *p*-value higher than 0.05 indicates that the applied model could fit accurately with the experimental results (Quanhong and Caili, 2005). The *p*-value for this test was insignificant with the value of 0.3576. With this result,

it can be concluded that the applied model ensures proper representation of experimental results.

Table 4.4 A four factorial face-centred central composite design generated for MASE with actual and predicted response values

Run	Factors				Response	
	X_1 (W)	X_2 (min)	X_3 (v/w)	X_4 (%)	TPC (mg GAE/dw)	
					Actual value	Predicted value
1	80	1	8	30	37.00	35.62
2	140	1	8	30	38.92	39.95
3	80	5	8	30	40.15	40.30
4	140	5	8	30	36.05	38.57
5	80	1	20	30	44.61	45.73
6	140	1	20	30	54.25	53.28
7	80	5	20	30	57.02	56.88
8	140	5	20	30	58.90	58.36
9	80	1	8	70	35.60	36.65
10	140	1	8	70	43.88	44.39
11	80	5	8	70	40.22	41.56
12	140	5	8	70	43.84	43.23
13	80	1	20	70	49.71	47.55
14	140	1	20	70	58.14	58.50
15	80	5	20	70	59.44	58.92
16	140	5	20	70	62.05	63.80
17	80	3	14	50	53.11	53.63
18	140	3	14	50	62.29	58.24
19	110	1	14	50	52.82	53.26
20	110	5	14	50	62.22	58.25
21	110	3	8	50	46.98	42.39
22	110	3	20	50	56.67	57.73
23	110	3	14	30	53.31	51.49
24	110	3	14	70	56.45	54.73
25	110	3	14	50	56.96	55.72
26	110	3	14	50	55.99	55.72
27	110	3	14	50	52.23	55.72
28	110	3	14	50	55.87	55.72
29	110	3	14	50	52.37	55.72
30	110	3	14	50	50.31	55.72

X_1: Microwave Power; X_2: Extraction time; X_3: Solvent to sample ratio; X_4: Ethanol concentration

4.2.1.2 Effect of MASE Process Parameters on TPC

Effect of extraction factors (microwave power, extraction time, solvent to sample ratio and ethanol concentration) on TPC of PH was studied and the ANOVA results of estimating model are shown in Table 4.5. The microwave power, extraction time, solvent to sample ratio and ethanol concentration were statistically significant (*p*

<0.05) factors on TPC. In addition, regression coefficients were compared with each other for a better understanding of the relative importance of each significant factor. According to the regression coefficients, the solvent to sample ratio was the most important factor while the effects of power and time were almost same. The effect of interaction terms was statistically insignificant on TPC.

Table 4.5 ANOVA results for the fitted polynomial quadratic model

Source	TPC					
	Sum of squares	**df**	**Mean square**	**F value**	***p*-value**	**Coefficient**
Model	1827.54	14	130.54	14.09	< 0.0001	
Linear						
X_1	95.57	1	95.57	10.32	0.0058[a]	2.30
X_2	112.31	1	112.31	12.12	0.0033[a]	2.50
X_3	1059.79	1	1059.79	114.39	<0.0001[a]	7.67
X_4	47.17	1	47.17	5.09	0.0394[a]	1.62
Interactive						
X_1X_2	36.77	1	36.77	3.97	0.0649[b]	-1.52
X_1X_3	10.32	1	10.32	1.11	0.3079[b]	0.80
X_1X_4	11.58	1	11.58	1.25	0.2812[b]	0.85
X_2X_3	41.76	1	41.76	4.51	0.0508[b]	1.62
X_2X_4	0.049	1	0.049	0.0052	0.9429[b]	0.055
X_3X_4	0.61	1	0.61	0.066	0.8007[b]	0.20
Quadratic						
X_1^2	0.12	1	0.12	0.013	0.9114[b]	0.21
X_2^2	0.0036	1	0.0036	0.0003	0.9845[b]	0.037
X_3^2	82.96	1	82.96	8.95	0.0091[a]	-5.66
X_4^2	17.60	1	17.60	1.90	0.1884[b]	-2.61
Lack of fit	103.32	10	10.33	1.45	0.3576[b]	
R^2	0.9293					
Adj R^2	0.8634					
CV	5.98					

[a] significant at p <0.05; [b] not significant at p >0.05.

The TPC varied from 35.60 to 62.29 mg GAE/g dry hull. The highest TPC was obtained at run 18 in which the extraction time, solvent to sample ratio, ethanol concentration and microwave power were 3 minutes, 14:1 v/w, 50 % and 140 W, respectively. Three dimensional response surface plots (Figure 4.5) show the relationships between the response (TPC) and independent factors. According to Figure 4.5a, with the increasing extraction time, the amount of phenolic compounds extracted from PH also increased. As it is seen in Table 4.5, the microwave power has a significant (p <0.05) positive linear effect on TPC which means the increasing microwave power enhance the TPC (Figure 4.5a, b, c). This may be explained by the

easier penetration of solvent into the plant structure with increasing power that provides the cell rupture and enhances the extraction (Mendes et al., 2016). More phenolic yield was obtained at the longer extraction time and higher solvent to sample ratio (Figure 4.5d). The response value increased with the increasing solvent to sample ratio, but when the ratio exceeded nearly 18:1 (v/w), the TPC value began to decrease (Figure 4.5f).

Although the ethanol concentration had a slight effect on TPC, the TPC value increased by increasing the ethanol concentration up to 56% and then decreased at higher ethanol concentration (Figure 4.5e). Maeng et al. (2017) and Filip et al. (2017) also reported similar results that 50% ethanol solution was the most suitable solvent for the MASE of phenolics from *Coriolus versicolor* mushroom and *Ocimum basilicum*. In this work, binary solution of water and ethanol was used as a solvent. Ethanol was chosen for its nontoxic and food grade properties (Zekovic et al., 2017). For the extraction of phenolics, mixture of ethanol with water is more effective than ethanol alone or water alone. Because the extraction depends on the polarity of solvent and the extracted components (Filip et al., 2017). In addition, using only water as an extraction solvent causes higher amounts of impurities in the extract (Maeng et al., 2017).

4.2.1.3 Optimization of MASE Conditions and Model Verification

Extraction conditions were optimised to get highest TPC using Design Expert v. 7.0 software (Stat-Ease, Inc.) and the optimal conditions were found as: microwave power of 140 W, extraction time of 4.5 min, solvent to sample ratio of 19:1 (v/w) and ethanol concentration of 56%. The predicted TPC was 63.47 mg GAE/g dry hull under the optimum conditions. Three extractions were done at the optimum conditions for the verification of the predicted results. The result was 62.24 ± 0.92 mg GAE/ g dry hull. The predicted and actual results were in good agreement.

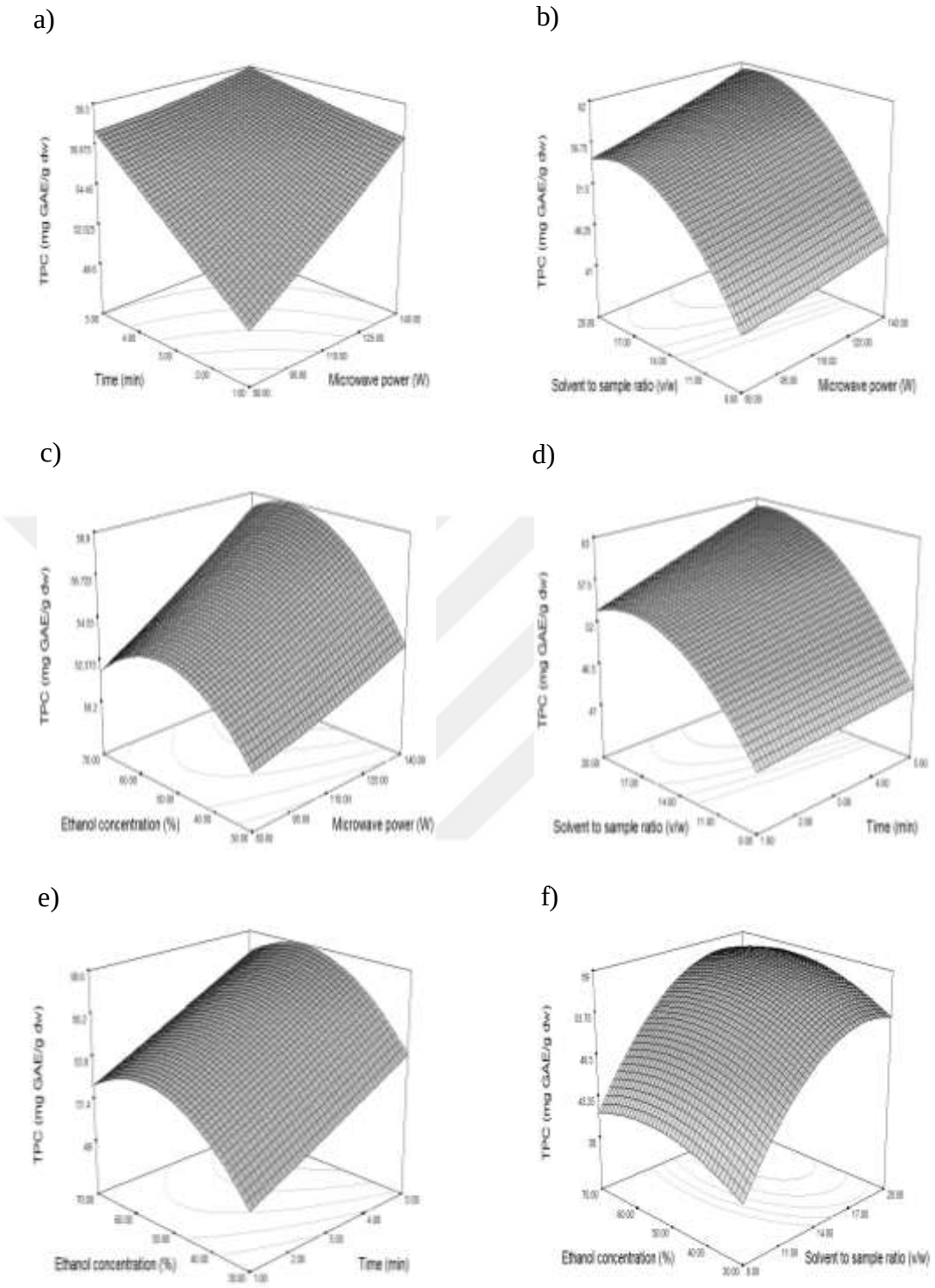

Figure 4.5 Response surface plots for TPC as a function of: (a) time and microwave power, (b) solvent-to-sample ratio and microwave power, (c) ethanol concentration and microwave power, (d) solvent-to-sample ratio and time, (e) ethanol concentration and time, (f) ethanol concentration and solvent-to-sample ratio. (The value of missing independent variable in each plot was kept at the center point.)

4.2.2 Comparison of Extraction Methods

The efficiency of MASE was compared with CSE and the results are summarized in Table 4.6. The MASE method enhanced the extraction and gave a higher yield of 51.67% while the yield was 42.85% for CSE. Moreover, the extraction time was significantly reduced by MASE process when compared to the CSE (4.5 min vs. 2 h). This faster and efficient extraction of polar compounds from PH could be described by the mechanism of microwave irradiation that causes molecular motion via ionic conduction and dipole rotation. As a result of the resistance towards this electrophoretic migration of ions, a rapid and uniform heating could be obtained in solution. Eventually, the rapid increase in temperature could provide the destruction of cellular matrix which also supports the release of plant components (Leone et al., 2014; Li et al., 2017). Similar results were obtained in previous studies on extraction of polyphenols from apple tree wood and natural antioxidants from exotic Gordonia axillaris fruit (Li et al., 2017; Moreira et al., 2017).

TPC and antioxidant activity of PHEs obtained by MASE and CSE methods were also compared. Extracts recovered by MASE showed higher TPC (62.24 mg GAE/g dry hull) than the extract (48.35 mg GAE/g dry hull) obtained by CSE. The difference between the TPC of extracts obtained by MASE and CSE was significant (p <0.05). The closed vessel MASE combines the advantage of microwave-assisted and pressurised solvent extraction methods. Bayramoglu et al. (2008) showed that the solid matrix had a higher internal pressure in the MASE which enhanced the extraction and thus the MASE provided a higher phenolic content. The results of this study are comparable to the results reported in previous studies (Grace et al., 2016; Rajaei et al., 2010). Rajaei et al. (2010) determined the TPC of PHEs extracted with different solvents (water, methanol acetone etc.) and reported that the water extracts have the highest phenolic content (49.3 mg GAE/g sample). Kilic et al. (2016) obtained 33.65 mg GAE in gram hull extract obtained by maceration with methanol during 8 h. Grace et al. (2016) reported the TPC of PHE as 43.43 mg of GAE per gram dry hull obtained by maceration with 80% acidified methanol. The variations in TPC can be related to the genetic backgrounds, growing practices of the pistachio and the extraction procedure (applied extraction method, time, temperature and solvent type) (Barreca et al., 2016; Filip et al., 2017; Roostaee et al., 2017).

Table 4.6 Comparison of MASE with CSE

Extraction methods[a]	Ethanol concentration (%)	Time	Extraction yield (%)[b]	TPC (mg GAE/g dry hull)	IC_{50} (mg extract/ml)
MASE	56	4.5 min	51.67±1.71	62.24±0.92	1.06 ± 0.11
CSE	50	2 h	42.85±1.37	48.35±1.37	1.59 ± 0.13

[a] All comparisons between MASE and CSE were significant for all analysis, with $p < 0.05$.
[b] g extract/100 g dry hull

Antioxidant activities of the extracts obtained by CSE and MASE methods were determined by DPPH radical scavenging ability and presented as IC_{50}. The IC_{50} values of extracts were 1.59 and 1.06 mg extract/mL for CSE and MASE, respectively, and the extraction methods had statistically significant effect (p <0.05) on the antioxidant activity of extracts. However, both extracts showed lower antioxidant activity than that of trolox (IC_{50} value for trolox was 0.28 mg/mL). Differences in TPC depending on the extraction method might be the reason of this significant difference in the IC_{50} values. Consequently, the results indicated that MASE is better and more effective than CSE with higher extraction yield, TPC and antioxidant activity as previously stated in literature (Maeng et al., 2017). The antioxidant potential of PHEs obtained using different solvents and methods have been studied by other researchers. Rajaei et al. (2010) have demonstrated that the water extract of hull showed a strong DPPH activity with the IC_{50} value of 1.94 µg/mL extract. The IC_{50} value of water extract of PH was obtained as 10.00 µg/mL extract by Roostaee et al. (2017). Kilic et al. (2016) have also reported that the DPPH antioxidant activity of pistachio hull methanol extract was 137.16 ± 3.50 mg TE/g extract.

4.2.3 Composition of Pistachio Hull, Pistachio Hull Extract and the Residue after Extraction

Some chemical properties of the hull, extract and the residue were determined and the results are given in Table 4.7. The moisture content of untreated hull, extract and residue was 71.05, 0.82 and 2.92% (wb), respectively. The ash, protein, non-polar fraction and pectin content of PH were 12.56, 8.26, 9.50 and 9.62% on the dry basis, respectively. Non-polar fraction is composed of resins, essential oils, triglycerides, diglycerides, monoglycerides and fatty acids. Mohammadi Moghaddam et al. (2009) have reported that the ash, protein, fat and pectin content of PH were 13.1, 13.1, 9.67

and 5.75%, respectively. Bagheripour et al. (2008), who determined the chemical composition of pistachio by-products from de-hulling process after different treatments (freeze-drying, sun-drying and ensiling), stated that the freeze-dried pistachio by-products contains 9.16% protein, 32.13% NDF, 21.00% ADF and 5.66% lignin. Bakhshizadeh et al. (2014) evaluated the chemical properties and the nutritional value of pistachio epicarp and reported that it contains 11.30% crude protein, 7.80% ether extract, 15.08% ash, 18.70% ADF and 26.20% NDF. As stated by Bakhshizadeh et al. (2014), the variations in the chemical composition of PH may be due to the differences in the pistachio cultivars, growing practices, maturity of the kernel and the de-hulling technique.

According to the literature survey, different investigations have been performed for the extraction of bioactive components of PH. However, all of these studies have mainly focused on the TPC and antioxidant activity of the extract. The experimental results of this study indicated that the most of the sugar, protein, acid and ash in the hull were extracted together with phenolic compounds during the extraction process. These results indicate that the extract must be purified according to the intended use.

Table 4.7 Chemical composition of pistachio hull, pistachio hull extract and the residue after extraction

Compound	Pistachio hull	Pistachio hull extract	Residue after extraction
Moisture content (%wb)	71.05 ± 2.44	0.82 ± 0.09	2.92 ± 0.25
Ash (%db)	12.56 ± 0.24	12.85 ± 0.19	6.63 ± 0.15
Protein (%db)	8.26 ± 0.13	12.49 ± 0.18	4.83 ± 0.15
Non polar fraction (%db)	9.50 ± 0.16	14.26 ± 0.78	5.62 ± 0.45
Pectin (%db)	9.62 ± 0.84	5.69 ± 0.52	12.21 ± 0.89
ADF (%db)	15.70 ± 0.75	-	30.85 ± 0.87
NDF (%db)	17.08 ± 0.81	-	33.43 ± 0.94
ADL (%db)	7.87 ± 0.33	-	18.07 ± 0.64
Sugar (%db)	13.80 ± 0.37	22.26 ± 0.96	6.67 ± 0.48
Acidity (%db)	7.48 ± 0.57	12.66 ± 0.69	3.04 ± 0.43

wb:wet basis; db:dry basis

4.2.4 Scanning Electron Microscopy (SEM) Analysis

The structural differences of the untreated PH and PH residues (after CSE and MASE) were analysed by SEM (Figure 4.6). As shown in Figure 4.6(a), untreated hull has spherical cell structure, possessing an unbroken cell wall. The cell structure was partially damaged and few slight ruptures on the sample surface after CSE were observed (Figure 4.6(b)). After MASE, obvious changes on the surface morphology were observed (Figure 4.6(c)) and most of the cell walls and membranes of PH were totally destroyed. These changes suggested that microwave treatment played an important role for breaking up plant cell walls and its non-thermal effects enhance the extraction. Indeed, when the cell structure is more severely damaged, more components will be released from the cells. It has been also stated that the physical cell structure is affected during microwave treatment because of the sudden increase in temperature and internal pressure (Dahmoune et al., 2015; Lou et al., 2010).

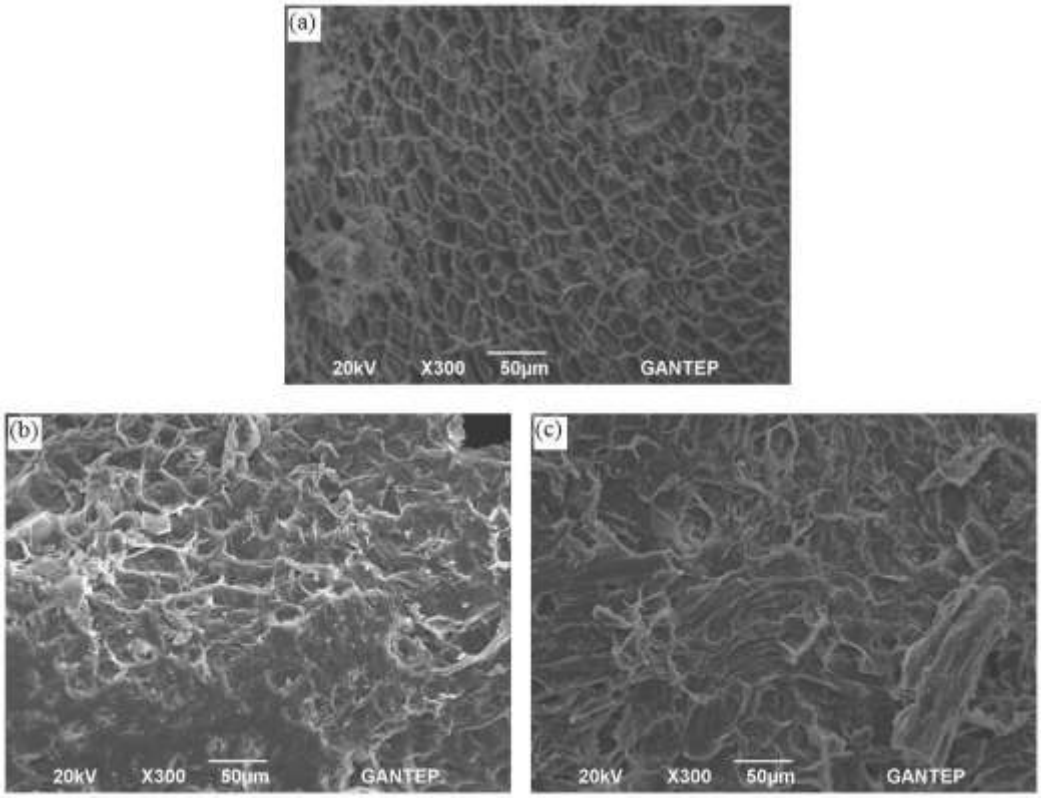

Figure 4.6. SEM images of pistachio hull samples: (a) untreated pistachio hull, (b) solid residue after CSE and (c) solid residue after MASE (MASE was performed under optimum conditions.)

4.3 Fractionation of Cellulose, Hemicellulose and Lignin from Pistachio Shell by using Ultrasound and/or Microwave-Assisted Pretreatment Methods

4.3.1 Composition of Pistachio Shell

The moisture, ash, protein and extractable material contents of PS were 1.90±0.08%, 0.53±0.04%, 0.95±0.01%, and 4.03±0.12%, respectively. The amounts of cellulose (as glucan), xylan, acetyl groups, acid soluble lignin and acid insoluble lignin in PS were 25.15±0.88%, 35.04±1.12%, 8.24±0.72%, 1.84±0.12% and 24.11±1.29% on dry basis, respectively. Arabinan was not detected in PS which was also reported by Domínguez et al. (2012).

4.3.2 Microwave-assisted Alkali Pre-treatment (MAAP) for Fractionation of Pistachio Shell

4.3.2.1 Model Fitting

BBD was used to analyse the effect of the pre-treatment factors on the MAAP of PS for the hemicellulose and cellulose recoveries. The observed experimental and predicted response values are given in Table 4.8. Statistical modelling of MAAP was performed by multiple regression analysis and the quadratic model was found as a best fitting model for the experimental data. The insignificant factors and interaction terms were eliminated from the model by using backward elimination. Quadratic polynomial models for the recovery of hemicellulose and cellulose were given in equations (4.2) and (4.3), respectively, in terms of coded factors:

$$Hemicellulose\ Recovery\ (\%) = +39.95 + 2.54 \times X_1 + 24.44 \times X_2 + 0.98 \times X_3 + 1.52 \times X_1 \times X_2 + 1.02 \times X_2 \times X_3 - 6.84 \times X_1^2 - 4.31 \times X_2^2 - 7.72 \times X_3^2 \quad (4.2)$$

$$Cellulose\ Recovery\ (\%) = +93.21 - 0.56 \times X_1 - 2.59 \times X_2 - 0.40 \times X_3 - 0.70 \times X_1^2 + 2.45 \times X_2^2 \quad (4.3)$$

ANOVA was applied for determination of the significance of each variable for the fitted models. Table 4.9 and Table 4.10 show the ANOVA results for hemicellulose and cellulose recovery, respectively. The significant p values (<0.0001) and the insignificant lack of fit tests showed that the produced quadratic models were well adapted to the responses with the coefficient of determination values of 0.9985 and 0.9606 for hemicellulose and cellulose recoveries, respectively. Microwave power, NaOH concentration and pre-treatment time were statistically significant ($p < 0.05$)

factors on the hemicellulose recovery. Moreover, interactive effect between microwave power and NaOH concentration (X_1*X_2) and quadratic effect of variables (X_1^2, X_2^2, X_3^2) were statistically significant model terms. The most significant factor was NaOH concentration for the extraction of hemicellulose from PS by considering its higher F value (4710.94) and low *p*-value (< 0.0001). For cellulose recovery, microwave power, NaOH concentration and their quadratic effects (X_1^2, X_2^2) were statistically significant ($p < 0.05$) model terms.

Table 4.8 Box-Behnken design generated for MAAP of PS with the observed experimental and predicted values

Run no	X_1 (W)	X_2 (N)	X_3 (min)	Hemicellulose recovery (%)		Cellulose recovery (%)	
				Exp	Pre	Exp	Pre
1	100	0.10	3	3.78±0.15 [a]	3.34	98.44±1.46	98.35
2	300	0.10	3	5.91±0.28	5.37	97.29±1.27	97.16
3	100	2.00	3	48.64±1.16	49.18	92.99±0.82	93.12
4	300	2.00	3	56.85±2.08	57.30	91.97±0.63	92.06
5	100	1.05	1	22.68±0.86	22.03	93.05±1.05	93.07
6	300	1.05	1	27.34±1.03	26.79	92.28±0.92	92.34
7	100	1.05	5	23.12±0.97	23.67	92.72±1.12	92.66
8	300	1.05	5	28.41±1.24	29.06	91.16±0.74	91.14
9	200	0.10	1	2.42±0.17	3.51	98.16±1.52	98.23
10	200	2.00	1	50.25±1.86	50.36	93.62±0.49	93.48
11	200	0.10	5	3.53±0.23	3.42	97.71±1.16	97.85
12	200	2.00	5	55.45±2.21	54.36	92.33±0.36	92.26
13	200	1.05	3	39.61±1.32	39.95	93.05±0.94	93.38
14	200	1.05	3	41.14±1.84	39.95	92.54±1.18	93.38
15	200	1.05	3	39.46±1.57	39.95	94.60±0.75	93.38
16	200	1.05	3	38.95±1.76	39.95	93.24±1.18	93.38
17	200	1.05	3	40.58±1.24	39.95	93.48±0.67	93.38

X_1: Microwave power (W); X_2: NaOH concentration (N); X_3: Pre-treatment time (min); [a] Average of three replicates; Exp: Experimental; Pre: Predicted.

4.3.2.2 Effect of MAAP Parameters on Hemicellulose and Cellulose Recovery

Hemicellulose recovery varied from 2.42±0.17% to 56.85±2.08% after MAAP (Table 4.8). The hemicellulose yield achieved in this thesis was comparable with the results of Singh et al. (2017) and Mihiretu et al. (2017) who carried out the microwave-assisted alkaline and hot water extraction, respectively, for hemicellulose extraction. The highest recovery was obtained from the run no 4 where microwave power, NaOH concentration and time were 300 W, 2 N and 3 min, respectively.

Table 4.9 ANOVA results for hemicellulose recovery after MAAP

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Coefficient
Model	5436.34	8	679.54	669.73	< 0.0001 [a]	
X_1	51.46	1	51.46	50.72	< 0.0001 [a]	2.54
X_2	4779.98	1	4779.98	4710.94	< 0.0001 [a]	24.44
X_3	7.64	1	7.64	7.53	0.0253 [a]	0.98
X_1*X_2	9.24	1	9.24	9.11	0.0166 [a]	1.52
X_2*X_3	4.18	1	4.18	4.12	0.0768 [b]	1.02
X_1^2	196.93	1	196.93	194.09	< 0.0001 [a]	-6.84
X_2^2	78.36	1	78.36	77.23	< 0.0001 [a]	-4.31
X_3^2	251.04	1	251.04	247.41	< 0.0001 [a]	-7.72
Lack of fit	4.95	4	1.24	1.56	0.3382 [b]	

R^2=0.9985, Adj R^2= 0.9970, Pred R^2=0.9880
[a] Significant at $p < 0.05$; [b] Not significant at $p > 0.05$.

Table 4.10 ANOVA results for cellulose recovery after MAAP

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Coefficient
Model	84,02	5	16,80	53,71	< 0.0001 [a]	
X_1	2,53	1	2,53	8,09	0.0160 [a]	-0,56
X_2	53,51	1	53,51	171,02	< 0,0001 [a]	-2,59
X_3	1,27	1	1,27	4,07	0.0689 [b]	-0,40
X_1^2	2,08	1	2,08	6,65	0.0257 [a]	-0,70
X_2^2	25,35	1	25,35	81,03	< 0,0001 [a]	2,45
Lack of fit	1,11	7	0,16	0,27	0,9359 [b]	

R^2=0.9606, Adj R^2= 0.9428, Pred R^2=0.9255
[a] Significant at $p < 0.05$; [b] Not significant at $p > 0.05$.

Figure 4.7 represents the three-dimensional response surface graphs that represent the change in hemicellulose recovery as a function of independent factors. It is seen that the hemicellulose recovery increased with the increasing NaOH concentration (Figure 4.7 a). This can be explained by the cleavage of the ester and/or ether bonds between hemicellulose and lignin in the presence of alkaline solution that favour the hemicellulose release (Kim et al., 2016). The increase in microwave power and pre-treatment time was also increased hemicellulose recovery. It has been stated that microwave irradiation can cause a physical explosion effect within the microfibers, resulting the fragmentation of the recalcitrant biomass structure. Moreover, the created electromagnetic field by microwaves causes physicochemical effects which accelerate the disintegration of the crystal structure (Vani et al., 2012). However, microwave power higher than 220 W and pre-treatment time longer than 3 min had led to a decrease in hemicellulose recovery (Figure 4.7 b, c). A similar trend was obtained by Ethaib et al. (2017) and Vani et al. (2012) who performed microwave-

assisted pre-treatment of sago palm bark and cotton plant residue, respectively. Increasing microwave power and pre-treatment time also increases the temperature which leads to high localized overheating and consequently causes the breakdown of hemicellulose into its monomeric sugars or by-products (Ethaib et al., 2017; Vani et al., 2012).

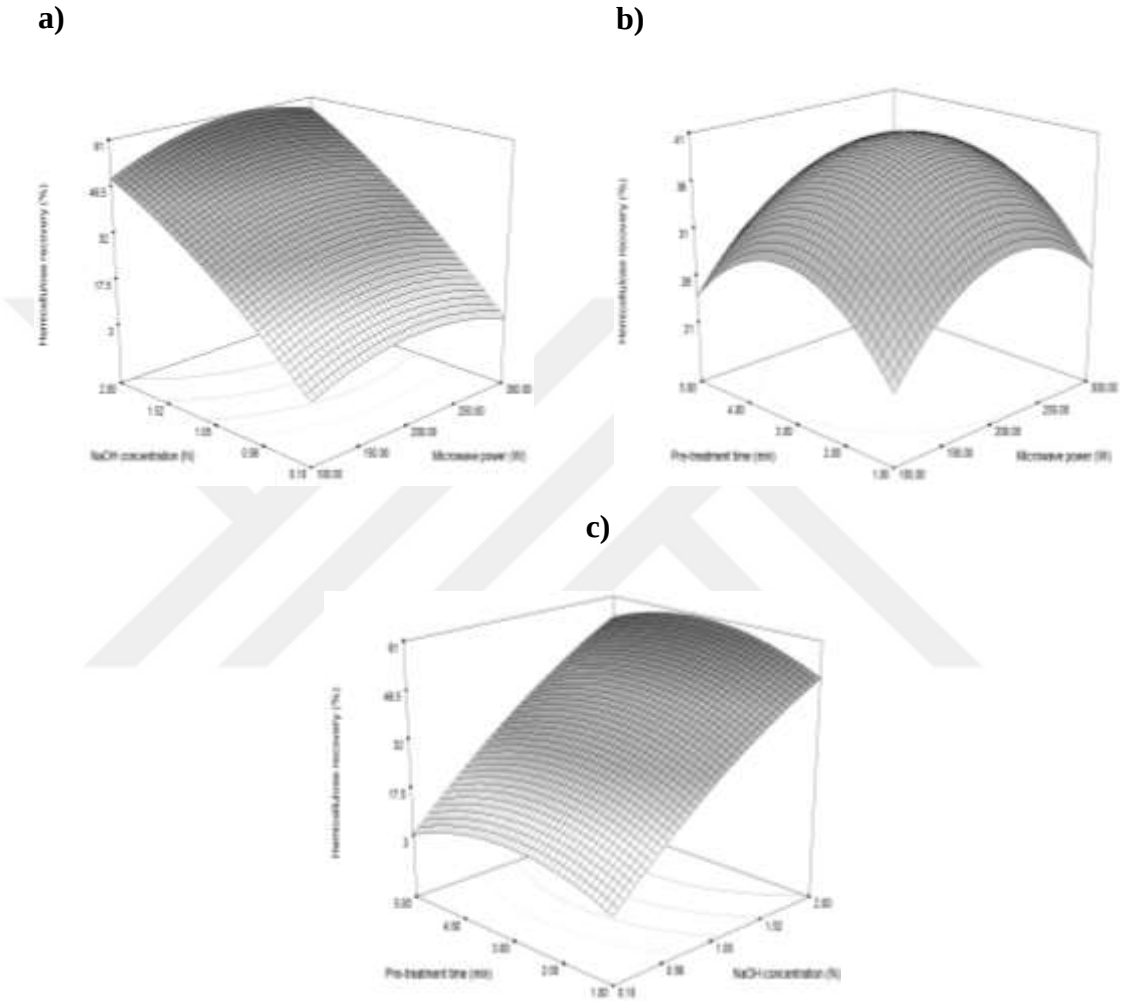

Figure 4.7 Response surface plots for hemicellulose recovery (%) as a function of: a) NaOH concentration and microwave power, b) pre-treatment time and microwave power, c) pre-treatment time and NaOH concentration (The value of missing independent variable was kept at the centre point in each plot.)

After MAAP of PS, the cellulose recovery varied between 91.16±0.74 - 98.44±1.46% which indicated that the most of the cellulose was preserved in the solid fractions (Table 4.8). Wan et al. (2011) and Chen et al. (2013) who performed the alkaline pre-treatment of soybean straw and corn stover, respectively, reported

higher cellulose recovery in the solid fractions that is comparable with the results of this study. The high percentage of cellulose in the solid fraction after alkali pre-treatment may be elucidated by the high crystallinity of cellulose and its low reactivity to alkali (Chen et al., 2013).

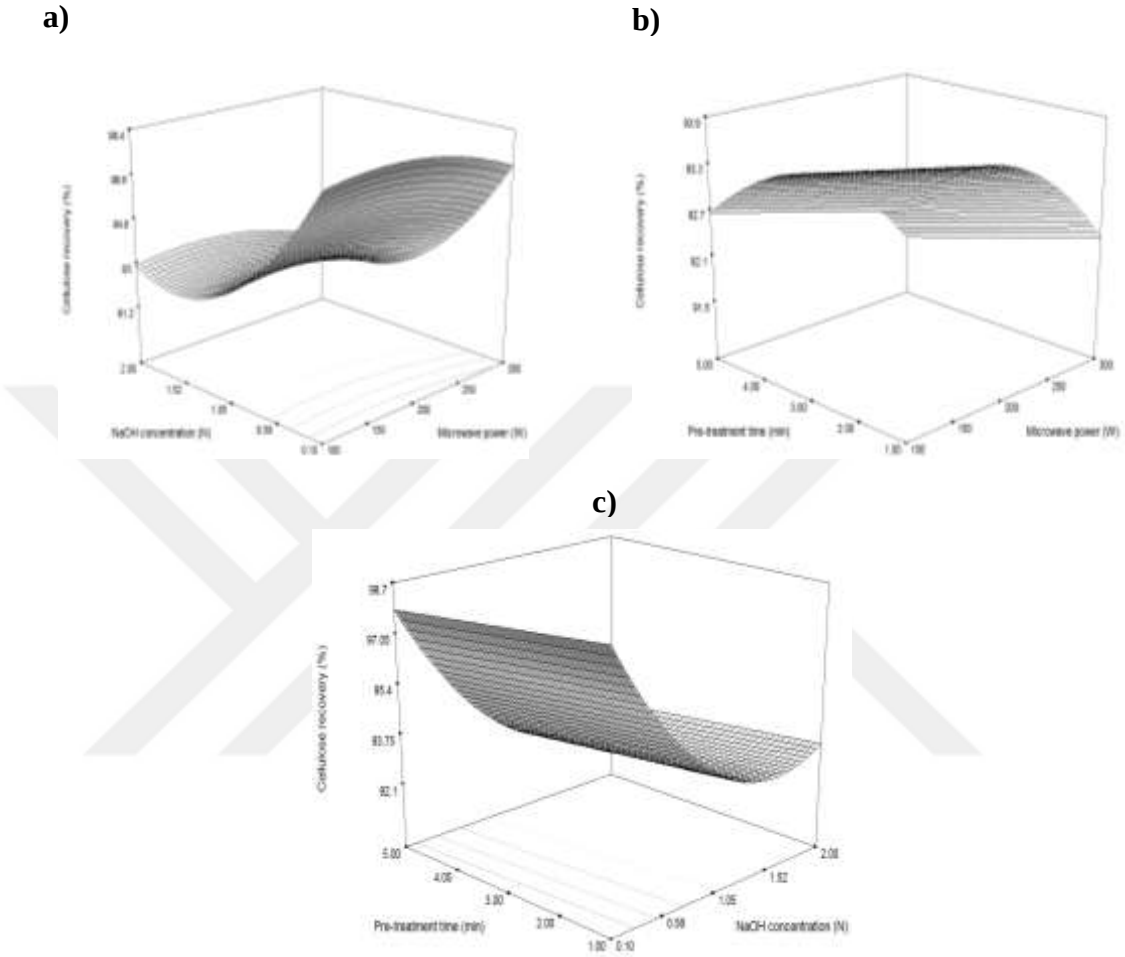

Figure 4.8 Response surface plots for cellulose recovery (%) as a function of: a) NaOH concentration and microwave power, b) pre-treatment time and microwave power, c) pre-treatment time and NaOH concentration (The value of missing independent variable was kept at the centre point in each plot.)

Figure 4.8 shows the 3D response surface graphs for cellulose recovery. It is seen that the cellulose recovery decreased with increasing NaOH concentration (Figure 4.8 a). The reason for the decreasing cellulose yield can be attributed the fact that high NaOH concentration cause degradation of cellulose besides hemicellulose and lignin and the degraded cellulose passed to the liquid fraction. However, increasing NaOH concentration after 1.52 N caused a slight increase in cellulose recovery.

Increasing the microwave power up to about 160 W led to a slight increase in cellulose recovery, but exceeding this value caused a decrease on the response value (Figure 4.8 b). This may be explained by the rapid increase in temperature and internal pressure as a result of increasing power which also damage the cellulosic structure (Li et al., 2014). Although the effect of pre-treatment time was statistically insignificant, increase in pre-treatment time caused a slight decrease in cellulose recovery (Figure 4.8 c).

4.3.2.3 Optimization of MAAP Conditions and Model Verification

MAAP conditions were optimized to obtain maximum hemicellulose recovery (%) from the liquid phase and the highest cellulose recovery (%) in the solid phase. The optimum conditions were selected as microwave power of 224 W, NaOH concentration of 1.96 N and pre-treatment time of 2.63 min. The predicted hemicellulose and cellulose recoveries under these conditions were 59.30% and 93.11%, respectively. The predicted results by the model verified by performing three parallel experiments under the proposed conditions and the hemicellulose and cellulose recoveries were found as 58.35±1.14% and 92.46±0.93%, respectively. Also, applied One-Sample *t*-test showed that there was no statistically significant difference between the values suggested by the program and the experimental results (p >0.05) which was also indicated the reliability of the pre-treatment conditions obtained by RSM.

4.3.3 Ultrasound-assisted Alkali Pre-treatment (UAAP) for Fractionation of Pistachio Shell

4.3.3.1 Model Fitting

The effects of the pre-treatment parameters on the UAAP of PS were investigated using BBD. The observed experimental and predicted response values are given in Table 4.11. Statistical modelling of UAAP was performed by multiple regression analysis and the quadratic model was found as a best fitting model for the experimental data. Insignificant factors and interaction terms were eliminated from the model by backward elimination. Quadratic polynomial models for the recovery of hemicellulose and cellulose were given in equations (4.4) and (4.5), respectively, in terms of coded factors:

$$Hemicellulose\ Recovery\ (\%) = +35.86 + 2.74 \times Y_1 + 3.58 \times Y_2 + 24.06 \times Y_3 + 2.04 \times Y_4 - 2.50 \times Y_1 \times Y_2 + 3.46 \times Y_1 \times Y_4 - 2.22 \times Y_2 \times Y_4 - 1.66 \times Y_3 \times Y_4 - 1.37 \times Y_1^2 + 2.77 \times Y_2^2 - 7.29 \times Y_3^2 \quad (4.4)$$

$$Cellulose\ Recovery\ (\%) = +94.34 - 0.24 \times Y_1 - 0.68 \times Y_2 - 2.11 \times Y_3 - 0.29 \times Y_4 - 0.42 \times Y_1 \times Y_2 + 0.39 \times Y_1 \times Y_4 + 1.43 \times Y_3 \times Y_4 + 1.82 \times Y_1^2 + 0.81 \times Y_2^2 + 0.37 \times Y_3^2 + 0.27 \times Y_4^2 \quad (4.5)$$

Table 4.11 Box-Behnken design generated for UAAP of PS with the observed experimental and predicted values

Run no	Y_1 (W)	Y_2 (min)	Y_3 (N)	Y_4 (°C)	Hemicellulose recovery (%)		Cellulose recovery (%)	
					Exp	Pre	Exp	Pre
1	128	30	1.05	70	25.73±1.84[a]	28.21	97.64±0.85	97.47
2	640	30	1.05	70	37.76±1.27	38.70	97.98±0.68	97.84
3	128	90	1.05	70	41.22±1.96	40.39	96.95±0.97	96.95
4	640	90	1.05	70	43.23±1.42	40.85	95.60±1.14	95.63
5	384	60	0.10	45	1.45±0.16	1.25	99.07±1.75	98.81
6	384	60	2.00	45	53.76±1.98	52.68	91.35±0.96	91.73
7	384	60	0.10	95	7.45±0.75	8.64	95.89±1.37	95.37
8	384	60	2.00	95	53.14±2.08	53.45	93.88±0.73	94.00
9	128	60	1.05	45	33.78±1.63	33.60	97.58±1.42	97.35
10	640	60	1.05	45	32.78±1.37	32.16	96.28±1.27	96.09
11	128	60	1.05	95	29.31±1.42	30.77	95.73±1.64	95.98
12	640	60	1.05	95	42.13±1.83	43.15	96.00±0.79	96.30
13	384	30	0.10	70	3.74±0.24	3.20	98.47±0.85	98.56
14	384	90	0.10	70	9.48±0.83	10.93	96.38±1.09	96.70
15	384	30	2.00	70	52.49±1.98	51.88	94.09±0.86	93.84
16	384	90	2.00	70	57.11±2.56	58.48	93.00±0.74	92.97
17	128	60	0.10	70	2.2±0.53	0.26	98.45±1.58	98.68
18	640	60	0.10	70	5.54±0.89	5.58	98.45±1.79	98.60
19	128	60	2.00	70	49.21±1.32	48.23	94.92±0.96	94.85
20	640	60	2.00	70	52.85±2.34	53.85	94.13±0.63	93.98
21	384	30	1.05	45	30.84±1.43	31.22	96.05±0.94	96.40
22	384	90	1.05	45	41.12±1.86	42.83	95.06±1.37	95.02
23	384	30	1.05	95	42.39±2.12	39.74	95.69±0.89	95.81
24	384	90	1.05	95	43.78±1.76	42.46	94.72±1.61	94.45
25	384	60	1.05	70	36.59±1.61	35.33	94.53±1.15	94.34
26	384	60	1.05	70	34.44±1.83	35.33	93.65±1.03	94.34
27	384	60	1.05	70	34.23±1.24	35.33	94.39±1.34	94.34
28	384	60	1.05	70	35.29±1.87	35.33	94.72±0.92	94.34
29	384	60	1.05	70	36.12±1.56	35.33	94.41±1.34	94.34

Y_1: Ultrasound power; Y_2: Pre-treatment time; Y_3: NaOH concentration; Y_4: Temperature; [a] Average of three replicates; Exp: Experimental; Pre: Predicted.

Table 4.12 ANOVA results for hemicellulose recovery after UAAP

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Coefficient
Model	7810.84	11	710.08	238.45	< 0.0001 [a]	
Y_1	89.87	1	89.87	30.18	< 0.0001 [a]	2.74
Y_2	154.01	1	154.01	51.72	< 0.0001 [a]	3.58
Y_3	6945.64	1	6945.64	2332.42	< 0.0001 [a]	24.06
Y_4	49.90	1	49.90	16.76	0.0008 [a]	2.04
Y_1*Y_2	25.10	1	25.10	8.43	0.0099 [a]	-2.50
Y_1*Y_4	47.75	1	47.75	16.03	0.0009 [a]	3.46
Y_2*Y_4	19.76	1	19.76	6.63	0.0196 [a]	-2.22
Y_3*Y_4	10.96	1	10.96	3.68	0.0721 [b]	-1.66
Y_1^2	12.66	1	12.66	4.25	0.0548 [b]	-1.37
Y_2^2	51.68	1	51.68	17.36	0.0006 [a]	2.77
Y_3^2	357.22	1	357.22	119.96	< 0.0001 [a]	-7.29
Lack of fit	46.41	13	3.57	3.39	0.1241 [b]	

R^2=0.9936, Adj R^2= 0.9894, Pred R^2=0.9744

[a] Significant at $p < 0.05$; [b] Not significant at $p > 0.05$.

Table 4.13 ANOVA results for cellulose recovery after UAAP

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Coefficient
Model	93.40	11	8.49	61.68	< 0.0001 [a]	
Y_1	0.67	1	0.67	4.85	0.0418 [a]	-0.24
Y_2	5.62	1	5.62	40.80	< 0.0001 [a]	-0.68
Y_3	53.51	1	53.51	388.69	< 0.0001 [a]	-2.11
Y_4	1.01	1	1.01	7.33	0.0149 [a]	-0.29
Y_1*Y_2	0.71	1	0.71	5.19	0.0360 [a]	-0.42
Y_1*Y_4	0.62	1	0.62	4.48	0.0494 [a]	0.39
Y_3*Y_4	8.15	1	8.15	59.21	< 0.0001 [a]	1.43
Y_1^2	21.50	1	21.50	156.14	< 0.0001 [a]	1.82
Y_2^2	4.26	1	4.26	30.95	< 0.0001 [a]	0.81
Y_3^2	0.87	1	0.87	6.33	0.0222 [a]	0.37
Y_4^2	0.47	1	0.47	3.41	0.0821 [b]	0.27
Lack of fit	1.68	13	0.13	0.78	0.6749 [b]	

R^2=0.9756, Adj R^2= 0.9597, Pred R^2=0.9209

[a] Significant at $p < 0.05$; [b] Not significant at $p > 0.05$.

Table 4.12 and Table 4.13 show the ANOVA results for hemicellulose and cellulose recovery, respectively. The significant *p* values (<0.0001) showed that the produced quadratic models were well adapted to the responses with the coefficient of determination values of 0.9936 and 0.9756 for hemicellulose and cellulose recoveries, respectively. In addition, the *p* values for lack of fit tests were found as 0.1241 and 0.6749. These values indicated that the quadratic models created for the responses are sufficiently compatible with the experimental data. Regression coefficients were compared with each other for a better understanding of the relative

importance of each significant factor. NaOH concentration was the most important factor for both hemicellulose and cellulose recoveries and followed by pre-treatment time. Negative coefficient values of linear terms for cellulose recovery indicated that the increase in pre-treatment conditions caused a decrease in cellulose recovery. In contrast, an inverse trend was obtained for hemicellulose recovery.

4.3.3.2 Effect of UAAP Parameters on Hemicellulose and Cellulose Recovery

Hemicellulose recovery obtained by UAAP of PS varied between 1.45±0.16% and 57.11±2.56% (Table 4.11). The highest recovery was obtained from run 16 where ultrasound power, time, NaOH concentration and temperature were 384 W, 90 min, 2 N and 70 °C, respectively. Figure 4.9 shows the 3D response surface graphs that represent the individual and interactive effects of independent variables on hemicellulose recovery. It is seen that the hemicellulose recovery increased with the increasing NaOH concentration (Figure 4.9 b, d, f). This can be explained by the cleavage of the ester and/or ether bonds between hemicellulose and lignin in the presence of alkaline solution that favor the hemicellulose release (Farhat et al., 2017).The increase in pre-treatment time also increased the yield and a similar trend was reported by Fu et al. (2006) who performed hemicellulose extraction from apple pomace by ultrasound assisted alkali process. The interaction effect of time with power and temperature was statistically significant on hemicellulose recovery. The yield increased from 25.73 to 37.76% at the end of 30 min with the increasing power from 128 to 640 W. However, the hemicellulose recovery just increased from 41.22 to 43.23% with the increasing power when the pre-treatment was performed at 90 min (Figure 4.9 a). Similar observation was obtained for temperature. For the pre-treatment time of 30 min, increasing temperature from 45 to 95 °C resulted in a significant increase in the yield (30.84 to 42.39%). The increase in temperature at the end of 90 min did not affect the yield significantly (Figure 4.9 e). Ultrasound power and temperature were other factors that have significant positive effect on hemicellulose yield. The increasing trend in hemicellulose yield with respect to power and temperature may be due to the formation of more amount of free radical at higher temperature and power by breaking the bonds between lignin and xylan (Ramadoss and Muthukumar, 2016; Wu et al., 2017). The increase in power at low temperatures did not significantly affect hemicellulose yield, but the increase in power at high temperatures increased the yield significantly (Figure 4.9 c).

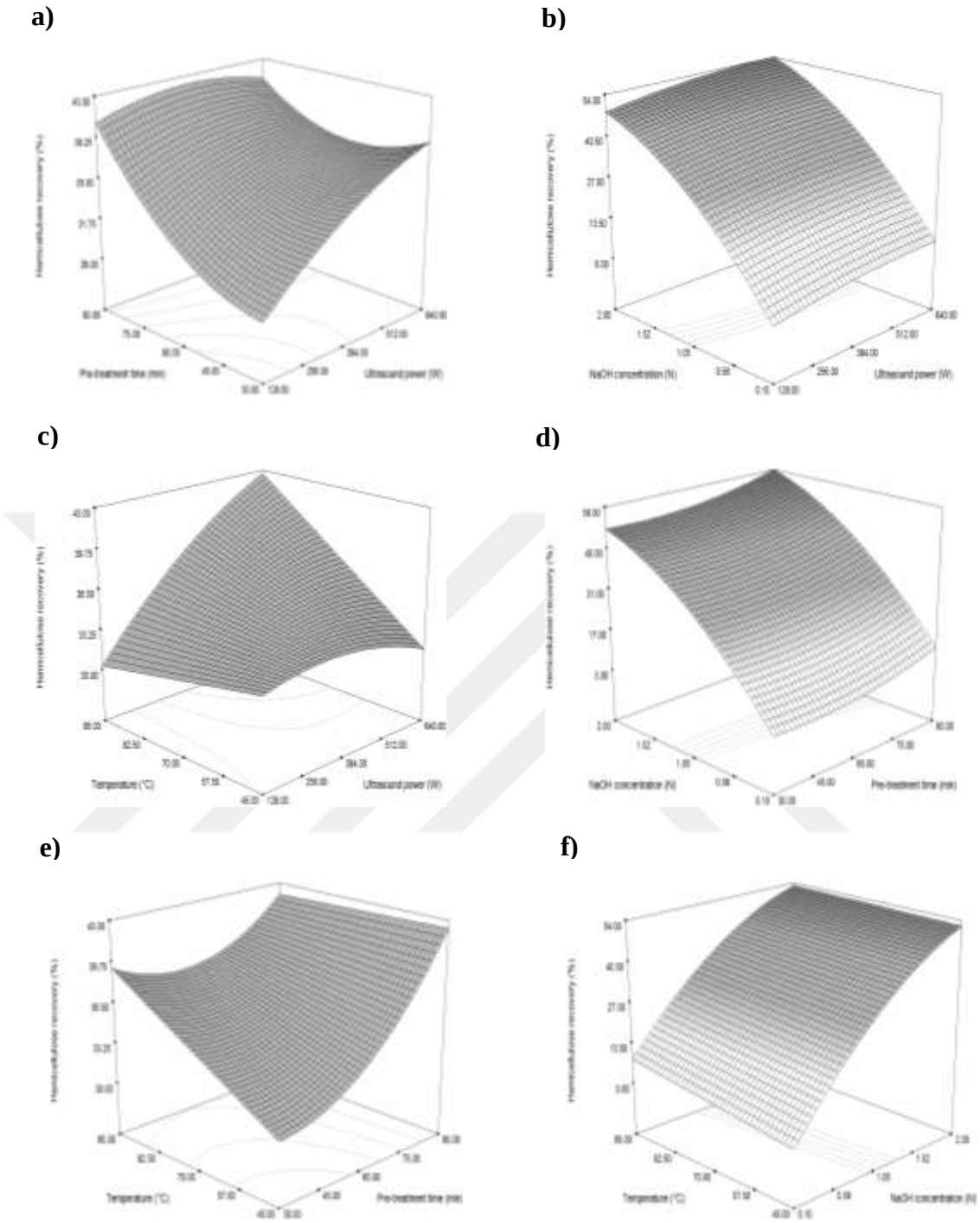

Figure 4.9 Response surface plots for hemicellulose recovery (%) as a function of: a) pre-treatment time and ultrasound power, b) NaOH concentration and ultrasound power, c) temperature and ultrasound power, d) NaOH concentration and pre-treatment time, e) temperature and pre-treatment time, f) temperature and NaOH concentration (The value of missing independent variable in each plot was kept at the centre point.)

After UAAP of pistachio shell, the cellulose recovery in the solid fraction varied between 91.35±0.96 - 99.07±1.75% (Table 4.11). The high percentage of cellulose recovery in the solid phase after alkali pre-treatment can be due to the high crystallinity of cellulose and its low reactivity to alkali (Chen et al., 2013). Figure 4.10 shows the 3D response surface graphs for cellulose recovery. It is seen that the cellulose recovery decreased with increasing NaOH concentration (Figure 4.10 b, d). Decreasing cellulose yield can be explained by the fact that high NaOH concentration cause degradation of cellulose as well as hemicellulose and lignin and the degraded cellulose passed to the liquid fraction. The cellulose recovery decreased with longer pre-treatment times, however pre-treatment for longer than 75 min led to a small increase in the response (Figure 4.10 a, d, e). The interaction effect of temperature with NaOH concentration and power was statistically significant on cellulose recovery. The yield decreased from 99.07 to 91.33% at 45 °C with the increasing NaOH concentration from 0.1 to 2 N. However, the decrease in cellulose recovery was just 2.01% (from 95.89 to 93.88%) with the increasing NaOH concentration when the pre-treatment was performed at 95 °C (Figure 4.10 f). Increase in power from 128 to 640 W caused a decrease in cellulose recovery at 45 °C but did not affect the yield at 95 °C (Figure 4.10 c). Selective extraction of hemicellulose without degrading cellulose obtained in this study showed that the UAAP is a preferred process, as previously mentioned by other researchers in the literature (Manasa et al., 2018; Ramadoss and Muthukumar, 2014; Velmurugan and Muthukumar, 2012).

4.3.3.3 Optimization of UAAP Conditions and Model Verification

UAAP conditions were optimized to achieve maximum hemicellulose recovery (%) in the liquid phase and the highest cellulose recovery (%) in the solid fraction. At optimal conditions (ultrasound power of 640 W, pre-treatment time of 87.44 min, NaOH concentration of 1.96 N and temperature of 93 °C), the predicted hemicellulose and cellulose recoveries were 58.94% and 95.39%, respectively. The experimental verification confirmed these values (57.67±1.34% and 94.05±0.79% for hemicellulose and cellulose recoveries, respectively). Applied One-Sample *t*-Test showed that there was no statistically significant difference between the values suggested by the program and the experimental results ($p>0.05$) which was also showed the reliability of the pre-treatment conditions obtained by RSM.

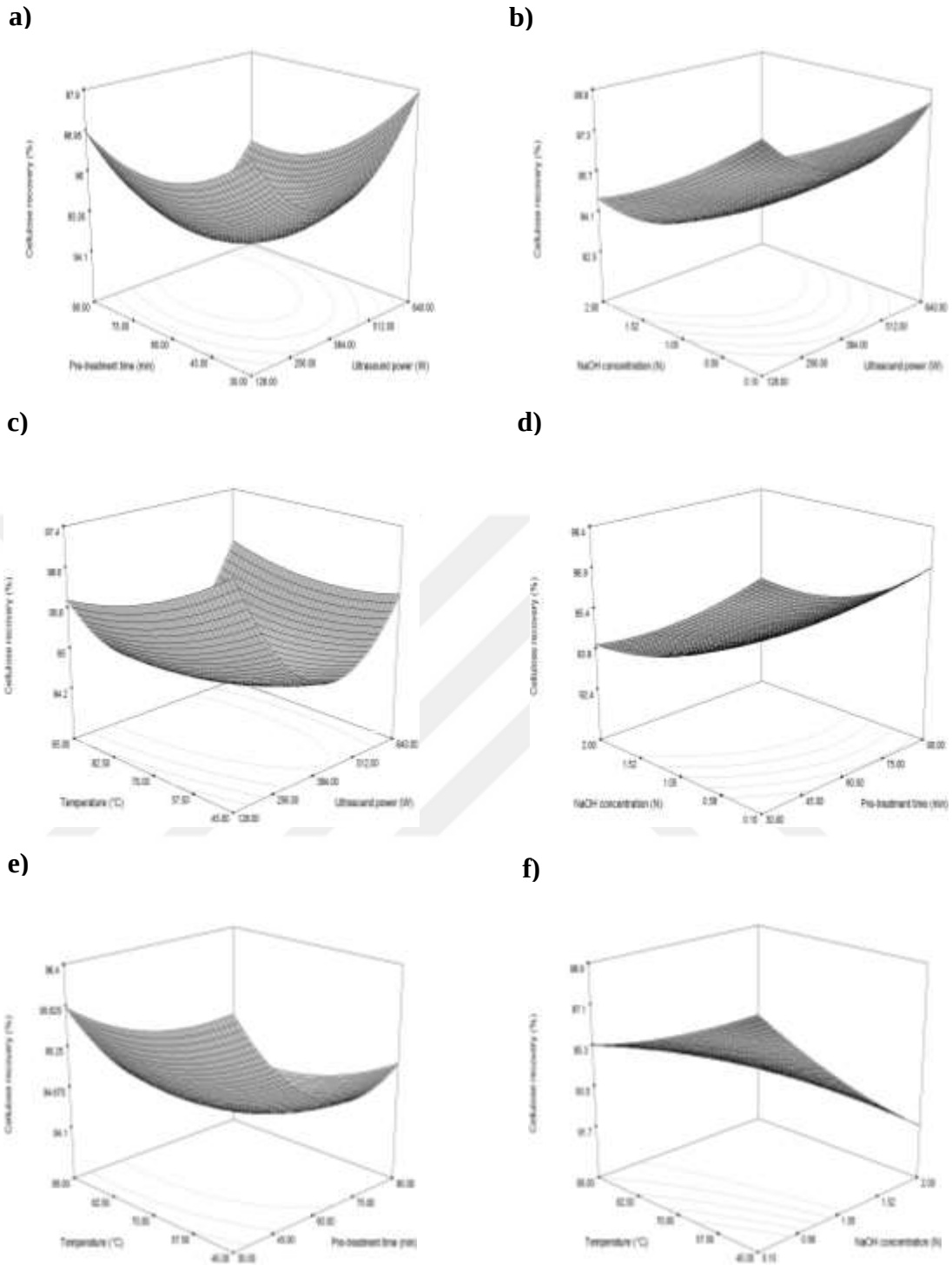

Figure 4.10 Response surface plots for cellulose recovery (%) as a function of: a) pre-treatment time and ultrasound power, b) NaOH concentration and ultrasound power, c) temperature and ultrasound power, d) NaOH concentration and pre-treatment time, e) temperature and pre-treatment time, f) temperature and NaOH concentration (The value of missing independent variable in each plot was kept at the centre point.)

4.3.4 Sequential Ultrasound-microwave-assisted Alkaline Pre-treatment (SUMAAP) for Fractionation of Pistachio Shell

Optimization of factors affecting SUMAAP was performed in two stages. Firstly, the most important factors affecting the pretreatment among the six variables were determined using the Plackett-Burman design (PBD). Then, the effects of these important factors affecting SUMAAP were investigated using BBD.

4.3.4.1 Plackett-Burman Design (PBD)

PBD is an effective mathematical methodology used for the identification of the most significant factors in any process. It suggests a quick screening procedure and mathematically calculates the importance of multiple parameters in one experiment; this saves time (Singh et al., 2011). Preliminary experiments were carried out to identify significant factors with PBD. The effects of different parameters (UP, UT, Ut, MP, Mt and NC) were evaluated on the basis of hemicellulose and cellulose recoveries after the SUMAAP and the observed experimental and predicted values are given in Table 4.14.

ANOVA was performed for testing the significance of the developed model and the results are given in Table 4.15. The *p* values of the models for hemicellulose and cellulose recoveries implied that the generated models are significant ($p < 0.05$). The most significant parameters affecting the SUMAAP for both hemicellulose and cellulose recoveries were found as UP, MP and NC. As shown in Table 4.15, NC had the highest contribution (67.05%) followed by MP (20.77%), UP (6.10%), Ut (2.32%), Mt (0.95%) and UT (0.87%) on hemicellulose recovery. For cellulose recovery, the contribution of independent variables was aligned as NC (60.87%), MP (22.94%), UP (9.52%), UT (2.25%), Mt (1.69%) and Ut (1.07%). Furthermore, the variables of NC, MP and UP had a positive effect on hemicellulose recovery while they presented negative effect on cellulose recovery as showed by Pareto charts in Figure 4.11. According to the results obtained from PBD, the NC, MP and UP were selected for further study by BBD to optimize the SUMAAP.

Table 4.14 Plackett-Burman design generated for SUMAAP of PS with the observed experimental and predicted values

Run no	UP (W)	UT (°C)	Ut (min)	MP (W)	Mt (min)	NC (N)	Hemicellulose recovery (%)		Cellulose recovery (%)	
							Experimental	Predicted	Experimental	Predicted
1	640	95	30	300	5	2.00	67.04±2.24[a]	66.60	88.82±0.86	89.20
2	128	95	90	100	5	2.00	35.19±1.56	37.06	94.44±1.07	94.22
3	640	45	90	300	1	2.00	65.56±1.87	61.48	90.77±0.78	91.36
4	128	95	30	300	5	0.10	31.92±1.45	29.78	95.17±0.93	95.33
5	128	45	90	100	5	2.00	35.71±1.21	33.84	94.85±1.25	95.07
6	128	45	30	300	1	2.00	53.43±1.99	58.21	93.11±1.16	92.52
7	640	45	30	100	5	0.10	19.87±0.88	19.35	97.04±0.97	97.14
8	640	95	30	100	1	2.00	54.48±1.64	54.22	93.02±0.73	92.63
9	640	95	90	100	1	0.10	18.46±1.06	20.66	97.65±1.24	97.61
10	128	95	90	300	1	0.10	29.10±1.63	27.87	96.55±1.16	96.65
11	640	45	90	300	5	0.10	26.72±1.42	29.83	95.67±0.67	95.02
12	128	45	30	100	1	0.10	15.59±0.76	14.18	99.28±1.32	99.61

UP: ultrasound power; UT: ultrasound temperature; Ut: ultrasound time; MP: microwave power; Mt: microwave time; NC: NaOH concentration; [a] Average of three replicates.

Table 4.15 ANOVA results for Plackett-Burman design generated for SUMAAP

Source	Hemicellulose recovery			Cellulose recovery		
	Cont.	F value	*p*-value	Cont.	F value	*p*-value
Model[a]		42.02	0.0004[b]		46.76	0.0003[b]
UP	6.10	15.68	0.0107[b]	9.52	27.19	0.0034[b]
UT	0.87	2.23	0.1955[c]	2.25	6.43	0.0522[c]
Ut	2.32	5.97	0.0584[c]	1.07	3.04	0.1415[c]
MP	20.77	53.40	0.0008[b]	22.94	65.52	0.0005[b]
Mt	0.95	2.43	0.1794[c]	1.69	4.82	0.0796[c]
NC	67.05	172.42	< 0.0001[b]	60.78	173.56	< 0.0001[b]

UP: Ultrasound power; UT: Ultrasound temperature; Ut: Ultrasound time; MP: Microwave power; Mt: Microwave time; NC: NaOH concentration; Cont: Contribution.

[a] The coefficients of determination (R^2) of the models were 0.9806 and 0.9825 for hemicellulose and cellulose recoveries, respectively.

[b] Significant at $p < 0.05$; [c] Not significant at $p > 0.05$.

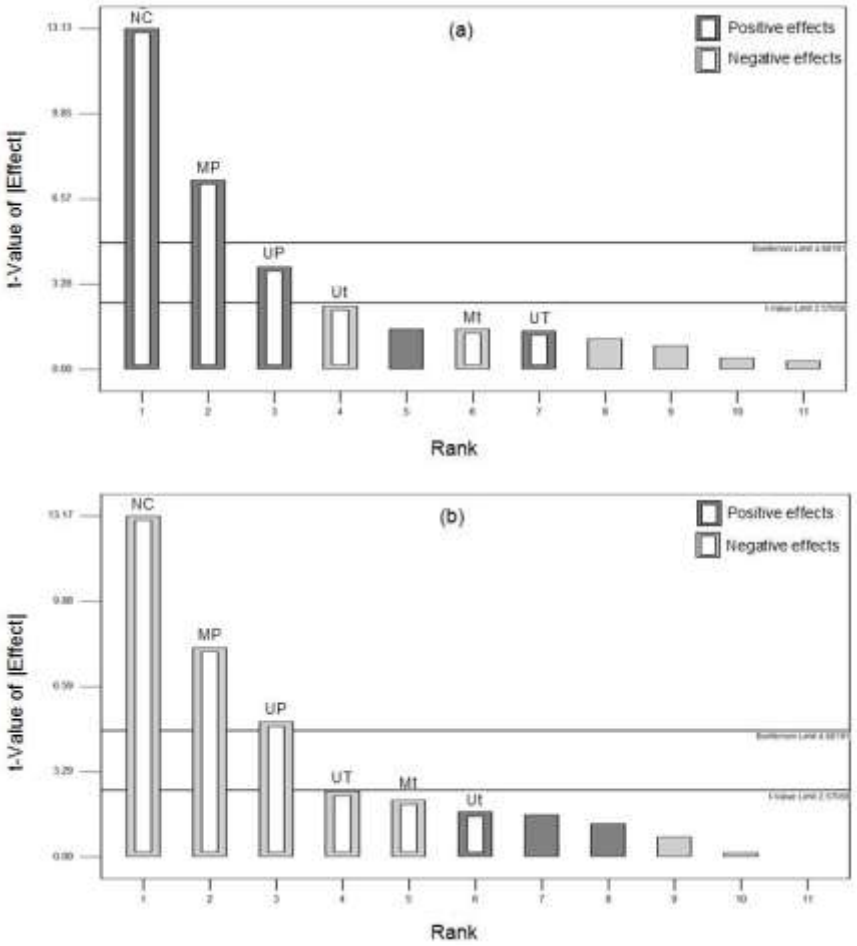

Figure 4.11 Pareto charts from Plackett-Burman design a) showing the significant parameters for hemicellulose recovery, b) showing the significant parameters for cellulose recovery (UP: ultrasound power; UT: ultrasound temperature; Ut: ultrasound time; MP: microwave power; Mt: microwave time; NC: NaOH concentration)

4.3.4.2 Box–Behnken Experimental Design

BBD was applied to determine the effect of NC, MP and UP on the responses. The UT, Ut and Mt, which were found to be insignificant as a result of PBD, were kept constant in all experiments as 95 ° C, 30 min and 2.5 min, respectively. The experimental and predicted responses are presented in Table 4.16. Multiple regression analysis was carried out for the statistical modelling of SUMAAP and the quadratic model was selected as a best fitting model for the experimental data. Insignificant factors and interaction terms were eliminated from the model by backward elimination. Quadratic polynomial models for the recovery of hemicellulose and cellulose were given in equations (4.6) and (4.7), respectively, in terms of coded factors:

$$Hemicellulose\ recovery\ (\%) = +54.27 + 2.77 \times UP + 3.20 \times MP + 22.77 \times NC - 14.54 \times NC^2 \quad (4.6)$$

$$Cellulose\ recovery\ (\%) = 95.27 - 0.36 \times UP - 0.70 \times MP - 4.06 \times NC - 1.30 \times NC^2 \quad (4.7)$$

Table 4.16 Box-Behnken design generated for SUMAAP of PS with the observed experimental and predicted values

Run no	UP (W)	MP (W)	NC (N)	Hemicellulose recovery (%)		Cellulose recovery (%)	
				Exp	Pre	Exp	Pre
1	128	100	1.05	46.65±1.64 [a]	47.23	96.41±1.17	96.33
2	640	100	1.05	52.77±2.34	52.81	95.89±0.86	95.58
3	128	300	1.05	53.7±1.87	53.67	94.58±0.97	94.89
4	640	300	1.05	59.76±2.27	59.18	94.12±1.46	94.20
5	128	200	0.10	14.86±0.96	14.67	98.25±1.65	98.33
6	640	200	0.10	18.74±0.78	19.10	97.73±0.91	98.04
7	128	200	2.00	59.45±1.98	59.10	90.96±0.77	90.65
8	640	200	2.00	65.57±2.66	65.76	89.57±1.03	89.49
9	384	100	0.10	13.98±0.84	13.59	98.49±1.29	98.49
10	384	300	0.10	20.26±1.26	20.49	97.62±0.64	97.23
11	384	100	2.00	59.85±1.57	59.63	90.14±0.59	90.53
12	384	300	2.00	65.15±2.24	65.54	88.97±0.76	88.97
13	384	200	1.05	53.5±1.73	55.11	95.31±1.35	95.28
14	384	200	1.05	55.85±1.38	55.11	95.19±1.20	95.28
15	384	200	1.05	57.15±1.76	55.11	95.70±0.99	95.28
16	384	200	1.05	56.74±1.25	55.11	94.75±1.07	95.28
17	384	200	1.05	52.29±1.62	55.11	95.45±1.34	95.28

UP: ultrasound power; MP: microwave power; NC: NaOH concentration; [a] Average of three replicates; Exp: Experimental; Pre: Predicted.

Table 4.17 and Table 4.18 show the ANOVA results for the fitted model for hemicellulose and cellulose recoveries, respectively. The low *p* values (<0.0001) and the insignificant lack of fit tests indicated that the produced quadratic models well describes the responses. The R^2 values for hemicellulose and cellulose recoveries were 0.9945 and 0.9888, respectively, that imply a high correlation between the experimental and the predicted values. In addition, p <0.05 values for model terms showed that UP, MP and NC have significant effect on the responses.

Table 4.17 ANOVA results for hemicellulose recovery after SUMAAP

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Coefficient
Model	5187.04	4	1296.76	541.01	< 0.0001	
UP	61.49	1	61.49	25.66	0.0003	2.77
MP	82.05	1	82.05	34.23	< 0.0001	3.20
NC	4148.69	1	4148.69	1730.82	< 0.0001	22.77
NC^2	894.81	1	894.81	373.31	< 0.0001	-14.54
Lack of fit	10.85	8	1.36	0.30	0.9292	

R^2=0.9945, Adj R^2= 0.9926, Pred R^2=0.9913

Table 4.18 ANOVA results for cellulose recovery after SUMAAP

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Coefficient
Model	143.81	4	35.95	264.60	< 0.0001	
UP	1.04	1	1.04	7.68	0.0169	-0.36
MP	3.98	1	3.98	29.26	0.0002	-0.70
NC	131.63	1	131.63	968.75	< 0.0001	-4.06
NC^2	7.16	1	7.16	52.71	< 0.0001	-1.30
Lack of fit	1.14	8	0.14	1.15	0.4794	

R^2=0.9888, Adj R^2= 0.9851, Pred R^2=0.9759

4.3.4.3 Effect of SUMAAP Parameters on Hemicellulose and Cellulose Recovery

Hemicellulose recovery obtained by SUMAAP of pistachio shell varied between 13.98±0.84% and 65.57±2.66% (Table 4.16). Figure 4.12 shows the 3D surface plots for hemicellulose recovery. Hemicellulose yield increased with increasing ultrasound and microwave powers (Figure 4.12 a). It has been stated that microwave irradiation can cause a physical explosion effect within the microfibers, resulting the fragmentation of the recalcitrant biomass structure. Moreover, the created electromagnetic field by microwaves causes physicochemical effects that accelerate the disintegration of the crystalline regions (Vani et al., 2012). On the other hand, increasing ultrasound power creates high temperature, pressure and cavitation effect which accelerates the formation of free radicals breaking the bonds between lignin

and xylan (Wu et al., 2017). These might be the reasons of increased hemicellulose yield with the increasing ultrasound and microwave powers. NC was the most important parameter for hemicellulose recovery. The yield increased with the increasing NC as is evident from Figure 4.12 b and c. This can be explained by the cleavage of the ester and/or ether bonds between hemicellulose and lignin in the presence of alkaline solution as stated before (Farhat et al., 2017).

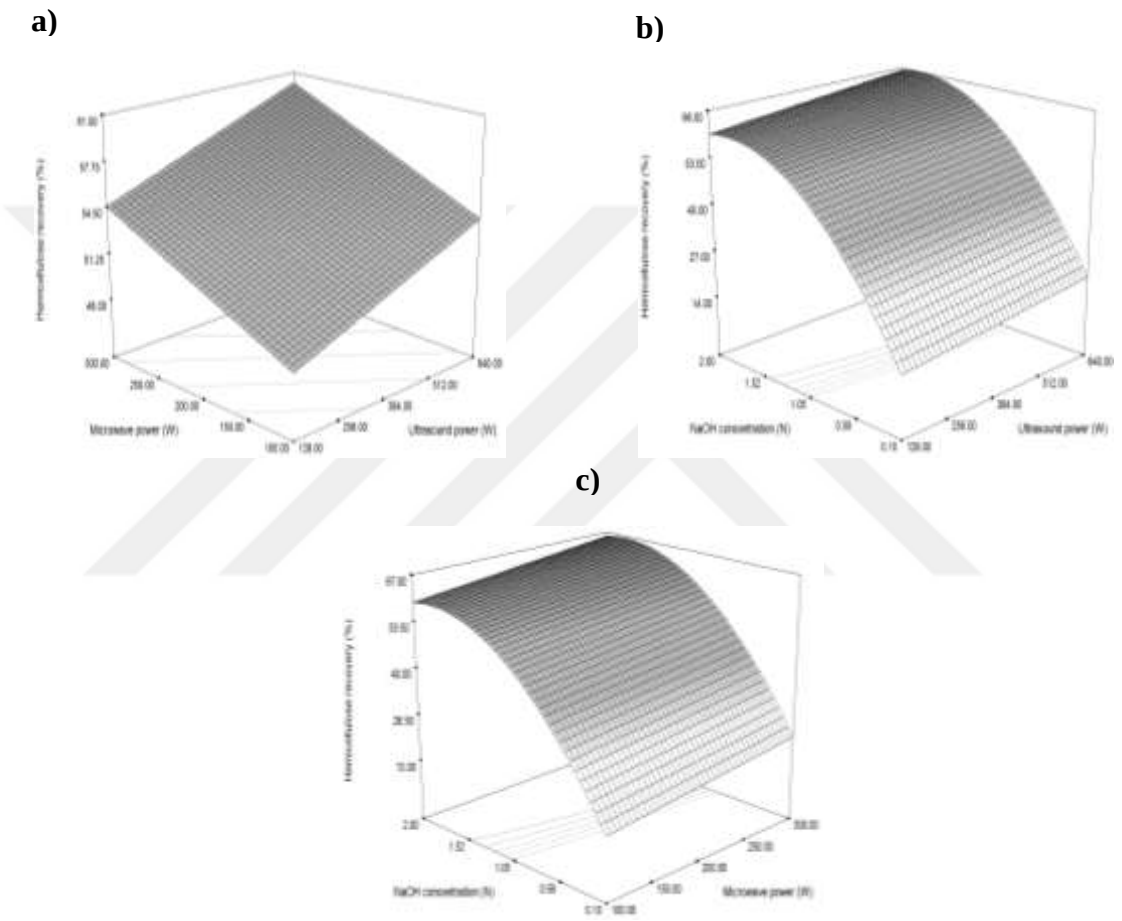

Figure 4.12 Three dimensional surface plots for hemicellulose recovery (%) after SUMAAP showing interactive effects of: a) microwave power and ultrasound power, b) NaOH concentration and ultrasound power, c) NaOH concentration and microwave power (The value of missing independent variable in each plot was kept at the centre point.)

After SUMAAP of pistachio shell, the cellulose recovery in the solid fraction varied between 88.97±0.76 - 98.49±1.29% (Table 4.16). Figure 4.13 shows the 3D response surface plots for cellulose recovery. Cellulose yield decreased with the increase in

ultrasound and microwave power (Figure 4.13 a). The cell structure of biomass can be damaged by microwave irradiation that causes a sudden increase in temperature and internal pressure (Li et al., 2014). Also, the ultrasound waves break the biomass structure mainly due the formation and explosion of bubbles which lead to the formation of high temperature and pressure (Ravindran et al., 2017). These might be the reasons of cellulose degradation which resulting the decrease in cellulose yields at higher microwave and ultrasound power. The lower cellulose yields upon higher NC may be explained by the degradation of cellulose into its oligomeric and monomeric forms at high NC (Figure 4.13 b, c).

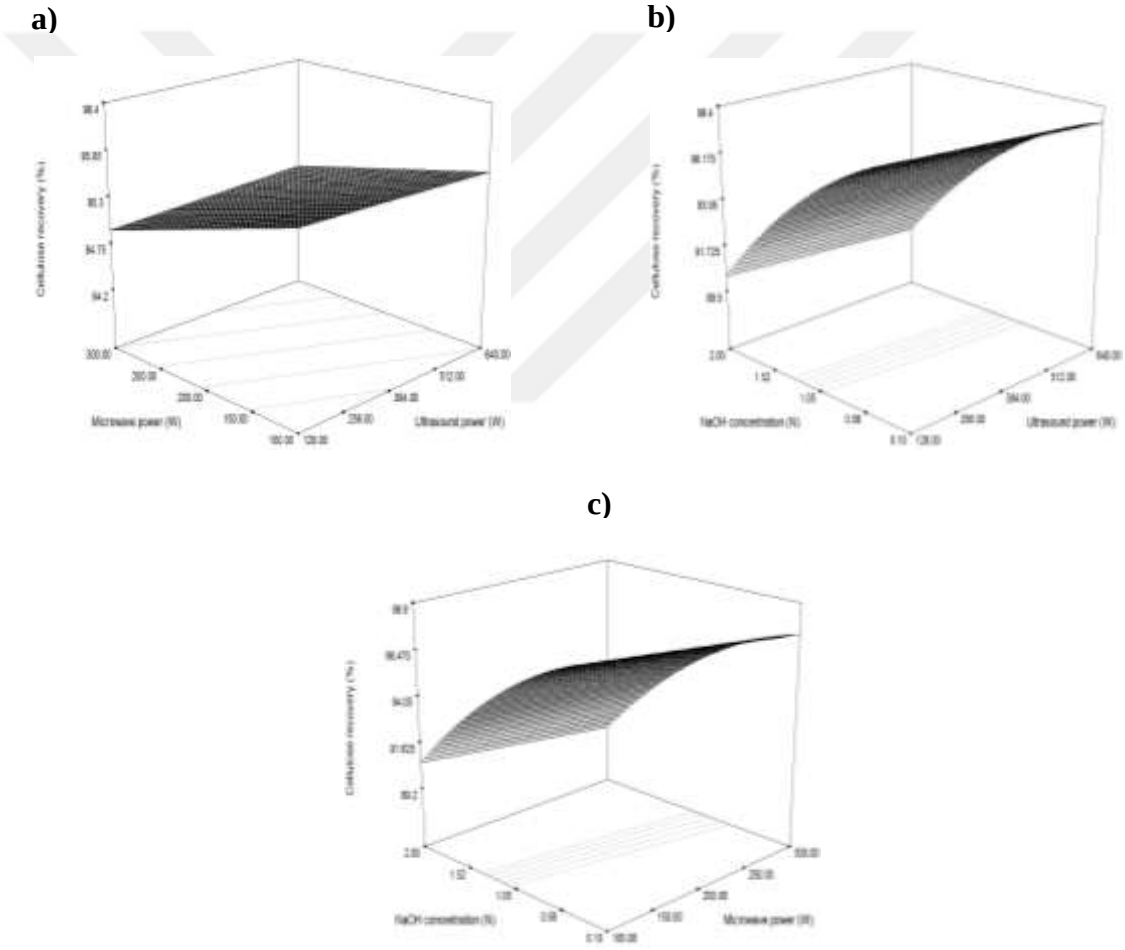

Figure 4.13 Three dimensional surface plots for cellulose recovery (%) after SUMAAP showing interactive effects of: a) microwave power and ultrasound power, b) NaOH concentration and ultrasound power, c) NaOH concentration and microwave power (The value of missing independent variable in each plot was kept at the centre point.)

The sequential application of microwave and ultrasound during pre-treatment of wheat straw was reported by Bussemaker et al. (2013). In their study, the ultrasound was combined with microwave pre-heating and this method was compared with the microwave pre-treatment. They have reported that ultrasound increased the purity of the solid residue and altered the physical structure. But, the microwave pre-treatment was more efficient for lignin degradation which is desirable for subsequent cellulose and hemicellulose hydrolysis (Bussemaker et al., 2013). In this study, unlike the literature, the factors affecting SUMAAP were studied in detail and the microwave was applied as a separate pre-treatment step after ultrasound treatment not only as a pre-heating process. Furthermore, the sequential use of ultrasound and microwave was studied for different applications. Liew et al. (2016) performed sequential ultrasound-microwave assisted extraction (UMAE) of pectin from pomelo peel using citric acid and optimized the extraction parameters (pH, ultrasound time, microwave power, microwave time) using the Box-Behnken design. They reported optimum UMAE conditions as pH of 1.80, 27.52 min of sonication followed by 6.40 min microwave irradiation at 643.44 W. They also compared UMAE with microwave-ultrasound assisted extraction (MUAE), ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE) methods. They reported the pectin yields as 36.33, 30.50, 27.65 and 14.25, respectively, for UMAE, MUAE, MAE and UAE. Their results indicated that UMAE method is more efficient than other methods for extraction of pectin. In another study carried out by Gole and Gogate (2013), esterification and transesterification reactions were performed in order to produce biodiesel from Nagchampa oil using sequential combination of microwave and ultrasound. They also compared this method with individual approaches of microwave, ultrasound and conventional method. Their results showed that the sequential use of ultrasound and microwave in the esterification reaction reduced reaction time, methanol requirement and catalyst concentration compared with other methods. Similarly, the sequential use of ultrasound and microwave in the transesterification reaction reduced the reaction time and methanol requirement. In the light of information obtained from the literature and this study, the sequential use of ultrasound and microwave reduced the process time and the amount of chemical used, thus saving energy.

4.3.4.4 Optimization of SUMAAP Conditions and Model Verification

SUMAAP was optimized to obtain maximum hemicellulose recovery (%) in the liquid phase and the highest cellulose recovery (%) in the solid fraction. At optimal conditions of 640 W ultrasound power, 248 W microwave power and 1.58 N NaOH concentration, the predicted hemicellulose and cellulose recoveries were 66.57% and 91.92%, respectively. The experimental verification confirmed these values (66.14±1.02% and 90.78±1.12% for hemicellulose and cellulose recoveries, respectively). Also, applied One-Sample t-Test indicated that there was no statistically significant difference between the values suggested by the program and the experimental results (p >0.05).

4.3.5 CAP of PS and Its Comparison with MAAP, UAAP and SUMAAP

CAP of PS was carried out by using different alkali concentrations (0.1, 0.5, 1, 1.5 and 2 N) and the obtained hemicellulose and cellulose recoveries are shown in Figure 4.14. Hemicellulose recovery ranged from 2.86±0.85 to 16.28 ±0.83%, while cellulose yield ranged from 97.78±0.13 to 98.70±0.21%. Hemicellulose recovery increased with the increasing NaOH concentration, while there was no significant change in cellulose recovery.

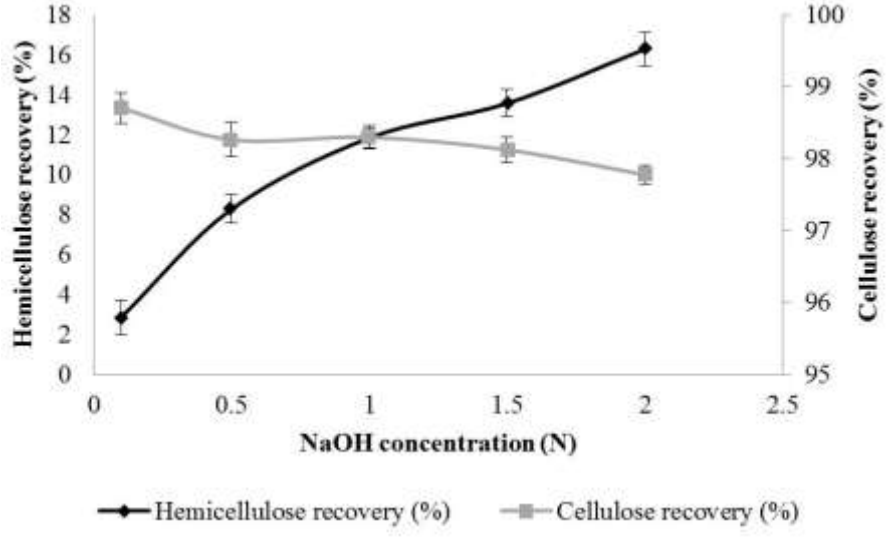

Figure 4.14 Hemicellulose and cellulose recoveries (%) obtained after CAP of PS

The MAAP, UAAP and SUMAAP performed under optimum conditions were compared with the CAP. The pre-treatment conditions and the results obtained for each pre-treatment are given in Table 4.19. Hemicellulose recovery increased by 3.5

times after MAAP and this yield was achieved in a very short time (2.63 min) compared to the CAP. Zhu et al. (2006) reported the similar results that the alkali pre-treatment assisted by microwave provided higher hemicellulose and lignin removal from wheat straw in a shorter time when compared to the CAP. This observation can be explained by the differences between microwave and conventional heating methods. In conventional heating, heat energy is transferred through conduction and convection from the outer surface of the material (Bundhoo, 2018). On the contrary, heat is generated internally through direct interaction between electromagnetic field and the heated material in microwave heating that provides volumetric and rapid heating (Puligundla et al., 2016). In addition to the thermal effects of microwave application, it is known that the non-thermal effects of it cause a physical explosion effect within the microfibers which accelerate the destruction of the recalcitrant structure (Vani et al., 2012). These can be the reasons of increased hemicellulose yield by the application of microwave in a shorter time.

When the UAAP was compared with CAP, it was seen that the hemicellulose recovery increased by 3.5 times. The UAAP time was 87.44 min and relatively low compared to the CAP time (16 h). Subhedar et al. (2018) performed delignification and subsequent hydrolysis of three different lignocellulosic biomasses, including pistachio shell to produce fermentable sugars using ultrasonic irradiation. They applied only alkaline pre-treatment in which the NaOH concentration and time were 1.75 N and 7 h, respectively. Also, ultrasound assisted alkaline pre-treatment was performed using 1 N NaOH for 70 min. Their results showed that the raw materials pre-treated with ultrasound assisted alkaline pre-treatment showed better delignification when compared to only alkaline pre-treatment. This finding indicated that the use of ultrasonic irradiation increased the yield of target compound and reduced the processing time as demonstrated in the present study.

The hemicellulose recovery increased to 66.14% after SUMAAP. This result indicated that the sequential use of ultrasound and microwave techniques increased the hemicellulose yield and reduced the used NC compared to the CAP, MAAP and UAAP. During ultrasound application, fractures occur on the biomass tissue to release the desired component by the effect of ultrasonic waves. This effect provides a greater contact area between the extraction solvent and the target compounds.

Subsequently, with the rapid heating of the microwave applied, the biomass tissue is further broken down, allowing the targeted compounds to pass into the solvent (Liew et al., 2016). This information given in the literature explains the increase in hemicellulose yield by sequential use of ultrasound and microwave. In addition, the statistical analysis indicated that there were statistically significant differences between the cellulose and hemicellulose recoveries obtained from CAP, MAAP, UAAP and SUMAAP (p <0.05).

Table 4.19 Comparison of different alkaline pre-treatment methods

Parameters	CAP	MAAP	UAAP	SUMAAP
Ultrasound power (W)	-	-	640	640
Microwave power (W)	-	224	-	248
Time	16 h	2.63 min	87.44 min	32.5 min
Temperature (°C)	35	142	93	95
NaOH concentration (N)	2	1.96	1.96	1.58
Hemicellulose recovery (%)	16.28 ± 0.83^{a}	58.35 ± 1.14^{b}	57.67 ± 1.34^{b}	66.14 ± 1.02^{c}
Cellulose recovery (%)	97.78 ± 0.13^{a}	92.46 ± 0.93^{b}	94.05 ± 0.79^{c}	90.78 ± 1.12^{d}

The different letters (a, b, etc.) in each line indicate statistical difference (p <0.05).

4.3.6 Composition of Solid and Liquid Fractions Obtained after Pre-treatments

4.3.6.1 Composition of Solid and Liquid Fractions Obtained after MAAP

The composition of solid and liquid fractions obtained after MAAP is shown in Table 4.20 and Table 4.21, respectively. The solid recovery was 55.04 to 85.78% after MAAP of PS. Increase in the pre-treatment severity by increasing NaOH concentration, microwave power and pre-treatment time resulted in lower solid recovery. Hemicellulose and lignin removal after pre-treatment can be explained as the reason for the low solid recovery which is also stated by Kim and Han (2012) after alkaline pre-treatment of rice straw. The cellulose content of the treated solids varied from 28.78 to 42.19% while the initial cellulose content of PS was only 25.15%. This finding confirms that the hemicellulose and lignin were effectively removed after MAAP. Similar results were already stated by other researchers for different types of biomasses after MAAP (Kamalini et al., 2018; Singh et al., 2014). Hemicellulose and lignin contents of pre-treated solids were between 31.31-52.37% and 15.35-26.86%, respectively. Decreasing amount of hemicellulose and lignin after pre-treatment indicates that the MAAP is capable to remove these components. In addition, reduction in lignin and hemicellulose amount in the pre-treated solids can be attributed to the effect of NaOH which causes hemicellulose and lignin

dissolution by enhancing the cleavage of lignin-hemicellulose linkages (Kim et al., 2016).

Table 4.20 Composition of solid fractions obtained from microwave-assisted alkaline pre-treatment

Run no	Solid recovery (%)	Composition of solid fraction (%)		
		Cellulose	Hemicellulose	Lignin
1	83.90	29.51	49.34	20.73
2	76.80	31.86	52.37	15.35
3	60.97	38.36	35.13	26.12
4	57.03	40.56	32.45	26.52
5	73.02	32.05	45.30	22.34
6	71.61	32.41	42.92	24.18
7	70.73	32.97	46.28	20.36
8	64.69	35.44	46.61	17.25
9	85.78	28.78	48.85	22.01
10	58.69	40.12	35.85	23.72
11	81.75	30.06	50.63	18.97
12	55.04	42.19	33.81	23.69
13	64.88	36.07	40.99	22.55
14	63.16	36.85	41.22	21.75
15	67.55	35.22	39.58	24.59
16	65.34	35.89	41.03	22.56
17	66.48	36.15	40.56	23.12
OP	57.56	40.40	31.31	26.86

OP-microwave-assisted alkaline pre-treatment performed under optimum conditions

Xylo-oligosaccharides (XOS) were the main compounds in the liquid fractions. XOS concentrations varied between 0.52 to 12.55 g/L based on the severity of the pre-treatment conditions. XOS concentration found in this research was comparable with the data presented in the literature as the reported XOS concentration is generally below 16 g/L (Aguilar-Reynosa et al., 2017a; Galia et al., 2015; Toscan et al., 2017). The highest XOS concentration was obtained after MAAP performed under optimum conditions (OP). XOS concentration in liquid fractions increased with increasing NaOH concentration, microwave power and pre-treatment time. But, it is important to note here that the increase in these factors after a certain point leads to the depolymerisation of XOS into its monomeric form of xylose and further degradation of this sugar to furfural and formic acid. For example, in run no 3 and 4, while NaOH concentration and time were the same, increasing power from 100 to 300 W increased the XOS content from 10.46 to 12.22 g/L. In addition, increasing

microwave power caused the formation of xylose, furfural and formic acid in the liquid phase. Similarly, while the other pre-treatment factors were the same in run no 9 and 10, increasing NaOH concentration from 0.1 to 2 N cause a significant increase in the amount of XOS (from 0.52 to 10.80 g/L), but also increase the formation of xylose and by-products. The highest acetic acid concentration (3.49 g/L) was under the level of 6 g/L, which inhibits the cellulase activity and ethanol production (Velmurugan and Muthukumar, 2012). Since NaOH was primarily effective on the lignin and hemicellulose, the concentration of GlcOS and glucose did not exceed 1.11 g/L and 0.91 g/L, respectively. HMF and levulinic acid were not detected in the liquid phases.

Table 4.21 Composition of liquid fractions obtained from microwave-assisted alkaline pre-treatment

Run no	Composition of liquid fraction (g/L)							
	Glucose	**Xylose**	**Acetic acid**	**Formic acid**	**Furfural**	**XOS**	**GlcOS**	**AcOS**
1	-	0.11	0.24	-	-	0.81	0.20	0.15
2	-	0.24	0.49	-	-	1.27	0.34	0.24
3	0.63	1.92	3.16	-	0.17	10.46	0.88	1.95
4	0.91	2.76	3.49	0.41	0.35	12.22	1.01	2.27
5	-	0.16	2.38	-	-	4.88	0.87	0.91
6	0.22	0.64	2.57	-	-	5.88	0.97	1.09
7	0.15	0.51	2.86	-	-	4.97	0.92	0.92
8	0.56	1.73	3.34	0.14	0.29	6.11	1.11	1.14
9	-	0.18	0.34		-	0.52	0.23	0.10
10	0.65	1.97	3.11	0.11	0.24	10.80	0.80	2.01
11	-	0.22	0.57	-	-	0.76	0.29	0.14
12	0.87	2.48	3.42	0.34	0.31	11.82	0.96	2.22
13	0.22	0.68	2.87	-	0.13	8.52	0.87	1.58
14	0.18	0.59	2.79	-	0.11	8.85	0.94	1.65
15	0.25	0.66	2.86	-	0.15	8.48	0.68	1.58
16	0.21	0.57	2.76	-	0.12	8.37	0.85	1.56
17	0.19	0.61	2.95	-	0.19	8.94	0.77	1.60
OP	0.87	2.62	3.47	0.38	0.30	12.55	0.95	2.33

XOS-xylo-oligosaccharides; GlcOS-gluco-oligosaccharides; AcOS-acetyl oligosaccharides; OP-microwave-assisted alkaline pre-treatment performed under optimum conditions

The obtained results in this research are comparable with the results reported in the literature. Aguilar-Reynosa et al. (2017a) carried out autohydrolysis of corn stover using microwave and conduction-convection heating methods to produce bioethanol.

They obtained solid recovery values between 48.23 to 74.02% and reported that the glucan content in pre-treated solids reached to the 64.67% after microwave-assisted pre-treatment that was only 31.89% in raw biomass. Maximum XOS, GlcOS, AcOS, xylose, acetic acid, formic acid and furfural concentrations were 13.71, 2.15, 1.90, 0.60, 2.45, 1.49 and 0.62 g/L in the hydrolysate. In addition, they stated that when compared to conventional heating, microwave heating method provides higher oligosaccharide yield in a shorter time, while reducing the formation of acetic acid and furfural. Mihiretu et al. (2017) studied the hemicellulose extraction from aspenwood sawdust and sugarcane trash by using single-step microwave-assisted hot water extraction method. They extracted about two third of the original xylan in aspenwood and over half of that in sugarcane trash. They also reported that the severe extraction conditions caused the formation of degradation products such as furfural in the liquid fraction. Santos et al. (2018), who performed the autohydrolysis of *Pinus radiata* wood for hemicellulose extraction using conventional heating, stated that the amount of cellulose, which is 43.8% in raw biomass, increased up to 56%. While the maximum amount of oligosaccharides in the liquid phase was 12.7 g/L, the concentration of pentoses and hexoses was found to be 4.8 and 8 g/L, respectively. In addition, it was reported that the concentration of degradation products increased with increasing temperature and time and the highest acetic acid, formic acid, furfural and HMF concentrations were found as 2.66, 1.46, 1.74 and 3.57 g/L, respectively. The amount of degradation products formed in this study is lower than the values reported by Santos et al. (2018), which can be explained by the difference in heating methods. Besides that, high temperature and long-time used in their study explains this situation. The main advantage of pre-treatment combined with microwave is less by-product formation which is also stated in literature (Aguilar-Reynosa et al., 2017a; Li et al., 2012).

4.3.6.2 Composition of Solid and Liquid Fractions Obtained after UAAP

The composition of solid and liquid fractions obtained after UAAP is presented in Table 4.22 and Table 4.23, respectively. The solid recovery varied from 55.28 to 83.30 % depending on the severity of the pre-treatment conditions. The lower solid recovery values were attributed to the removal of hemicellulose and lignin that is also stated by Kim and Han (2012), who performed the alkaline pre-treatment of rice straw. The pre-treated solids had higher cellulose content ranging from 29.91% to

42.31% than that of native PS. Increase in the cellulose percentage evidently showed that hemicellulose and lignin were effectively removed after pre-treatment. Similar observations were already reported in the literature for different biomasses after ultrasound assisted alkaline pre-treatment (Goshadrou et al., 2011; Wang et al., 2018). Hemicellulose and lignin contents of solids were between 31.36%-51.79% and 16.68%-29.34%, respectively. NaOH causes dissolution of hemicellulose and lignin by enhancing the cleavage of lignin-hemicellulose linkages which explained the reduction of lignin and hemicellulose in the pre-treated solid (Kim et al., 2016).

Table 4.22 Composition of solid fractions obtained after ultrasound-assisted alkaline pre-treatment

Run no	Solid recovery (%)	Composition of solid fraction (%)		
		Cellulose	Hemicellulose	Lignin
1	71.95	34.13	44.37	21.10
2	68.24	36.11	39.19	24.21
3	68.26	35.72	37.06	26.82
4	68.40	35.15	35.75	28.71
5	83.30	29.91	50.79	18.70
6	60.51	37.97	32.77	28.75
7	79.37	30.39	50.26	19.06
8	60.48	39.04	33.24	27.27
9	69.56	35.28	41.09	23.34
10	69.32	34.93	41.85	23.01
11	67.27	35.79	45.24	18.51
12	65.61	36.80	38.02	24.87
13	79.81	31.03	51.79	16.78
14	78.54	30.86	49.57	19.15
15	57.53	41.13	35.33	23.04
16	55.28	42.31	33.27	24.03
17	82.10	30.16	51.04	18.14
18	78.91	31.38	51.45	16.68
19	58.44	40.85	37.31	21.38
20	57.25	41.35	35.37	22.81
21	72.02	33.54	41.25	24.75
22	70.13	34.09	36.03	29.34
23	64.23	37.47	38.51	23.56
24	61.83	38.53	39.14	22.03
25	69.72	34.10	39.05	26.29
26	68.87	34.20	41.09	24.32
27	69.07	34.37	41.00	24.21
28	69.03	34.51	40.26	24.72
29	69.14	34.34	39.68	25.42
OP-1	57.61	41.06	31.36	27.07

OP-1-Ultrasound-assisted alkaline pre-treatment under optimum conditions

In the liquid phase, XOS were the main components and its concentration varied from 0.31 to 12.40 g/L. The highest amount of XOS was obtained after the pre-treatment performed under optimum conditions (OP-1). The amount of XOS increased with increasing NaOH concentration, ultrasound power, temperature and time. However, after a certain point, the increase in these factors leads to the conversion of XOS into its monomeric form of xylose and further degradation of this sugar to furfural and formic acid. For example, in run no 1 and 2, while the other conditions were the same, increasing power from 128 to 640 W increased the XOS content from 5.53 to 8.12 g/L. But this increase also caused an increase in the amount of xylose, furfural and formic acid. Similarly, increasing time from 30 to 90 min did not change significantly the amount of XOS, but increased the formation of xylose and by-products in run no 23 and 24. The maximum acetyl-oligosaccharide (AcOS), gluco-oligosaccharide (GlcOS), glucose and xylose concentrations were 2.31 g/L, 1.09 g/L, 1.41 g/L and 2.88 g/L, respectively. Also acetic acid, formic acid and furfural concentrations were maximum 3.39 g/L, 0.47 g/L and 0.51 g/L, respectively. In addition, HMF and levulinic acid were not detected in liquid phases. Velmurugan and Muthukumar (2012) optimized ultrasound-assisted NaOH pretreatment conditions to produce fermentable sugars from sugarcane bagasse and compared this method with the NaOH pretreatment performed in an autoclave. They reported that the solid recovery ranged between 72.14 to 89.12% depending on the pre-treatment conditions. Their results showed that the ultrasound-assisted NaOH pretreatment did not affect the cellulose in biomass and more than 97% of the initial cellulose remained in the solid residue. In the liquid phase obtained after ultrasound pre-treatment performed under optimum conditions, the amount of glucose, xylose, arabinose and acetic acid was reported as 0.13, 1.60, 0.47 and 0.36 g/L, respectively, while the furfural was not detected. These values were 0.28, 4.16, 0.64 and 0.53 g/L in the liquid fraction obtained from the pretreatment performed in the autoclave and it is stated that the liquid phase contains 0.12 g/L furfural. The main advantage of alkaline pre-treatment combined with ultrasound is less by-product formation which is also stated in literature (Ramadoss and Muthukumar, 2014; Velmurugan and Muthukumar, 2012).

Table 4.23 Composition of liquid fractions obtained after ultrasound-assisted alkaline pre-treatment

Run no	Composition of liquid fraction (g/L)							
	Glucose	Xylose	Acetic acid	Formic acid	Furfural	XOS	GlcOS	AcOS
1	0.42	0.64	2.11	-	-	5.53	0.30	1.03
2	0.49	0.88	2.34	0.14	0.26	8.12	0.25	1.51
3	0.56	1.09	2.87	-	0.17	8.86	0.38	1.65
4	0.64	1.25	2.85	0.16	0.31	9.29	0.55	1.73
5	-	0.22	0.31	-	-	0.31	0.12	0.06
6	0.97	2.39	3.29	0.27	0.37	11.56	1.09	2.15
7	0.11	0.15	1.07	-	-	1.60	0.52	0.30
8	1.08	2.57	3.31	0.18	0.31	11.43	0.77	2.13
9	0.46	0.94	2.51	-	0.13	7.26	0.30	1.35
10	0.58	1.11	2.65	-	0.18	7.05	0.47	1.31
11	0.57	0.83	2.53	-	0.10	6.30	0.54	1.17
12	0.69	1.07	2.86	0.19	0.29	9.06	0.50	1.69
13	-	0.13	0.74	-	-	0.80	0.19	0.15
14	0.07	0.32	1.16	-	-	2.04	0.46	0.38
15	0.96	2.44	3.21	0.21	0.34	11.29	0.74	2.10
16	1.24	2.88	3.37	0.39	0.42	12.28	0.88	2.28
17	-	0.17	0.44	-	-	0.47	0.19	0.09
18	0.22	0.58	0.93	-	-	1.19	0.20	0.22
19	1.05	2.69	3.18	0.25	0.39	10.58	0.64	1.97
20	1.13	2.63	3.21	0.33	0.46	11.36	0.74	2.11
21	0.41	0.85	1.94	-	0.13	6.63	0.50	1.23
22	0.69	1.42	2.81	0.24	0.27	8.84	0.62	1.64
23	0.49	1.10	2.73	0.17	0.32	9.11	0.54	1.70
24	0.76	1.36	2.89	0.12	0.29	9.41	0.66	1.75
25	0.38	0.65	2.68	-	0.14	7.87	0.69	1.46
26	0.43	0.69	2.75	-	0.25	7.40	0.80	1.38
27	0.35	0.71	2.81	-	0.19	7.36	0.71	1.37
28	0.41	0.73	2.64	-	0.26	7.59	0.66	1.41
29	0.36	0.67	2.79	-	0.22	7.77	0.70	1.44
OP-1	1.41	2.74	3.39	0.47	0.51	12.40	0.75	2.31

XOS-xylo-oligosaccharides; GlcOS-gluco-oligosaccharides; AcOS-acetyl-oligosaccharides; OP-1-Ultrasound-assisted alkaline pre-treatment under optimum conditions

4.3.6.3 Composition of Solid and Liquid Fractions Obtained after SUMAAP

The composition of solid and liquid fractions obtained after SUMAAP is given in Table 4.24 and Table 4.25, respectively. The solid recovery varied between 48.95% and 76.55% based on the pre-treatment conditions. The cellulose content of pre-treated solids reached 46.74 %, while the initial cellulose content of PS was only 25.15%. This finding confirms that the SUMAAP nearly doubles the glucan content in the pre-treated solid by the removal of hemicellulose and lignin. Hemicellulose and lignin contents in treated samples varied between 26.84% to 48.98% and 16.78% to 31.08%, respectively.

Table 4.24 Composition of solid fractions obtained after sequential ultrasound-microwave-assisted alkaline pre-treatment

Run no	Solid recovery (%)	Composition of solid fraction (%)		
		Cellulose	Hemicellulose	Lignin
1	63.36	38.27	36.15	25.09
2	60.38	39.94	32.96	26.18
3	61.50	38.68	32.11	28.52
4	59.16	40.01	29.29	29.63
5	76.55	32.28	47.82	19.01
6	74.98	32.78	46.37	20.19
7	48.95	46.74	35.64	17.27
8	49.48	45.53	29.77	24.34
9	74.86	33.09	48.98	16.78
10	73.82	33.26	46.06	19.35
11	50.44	44.94	33.65	20.16
12	49.05	45.62	30.56	23.34
13	60.61	39.55	32.63	26.47
14	61.96	38.64	30.42	29.65
15	60.72	39.64	30.28	29.23
16	61.75	38.59	29.82	31.08
17	60.85	39.45	33.24	26.49
OP-2	54.21	42.11	26.84	30.86

OP-2-Sequential ultrasound-microwave-assisted alkaline pre-treatment performed under optimum conditions

When the composition of liquid fractions from SUMAAP was analysed, it was seen that the XOS were the main component and the highest XOS concentration of 14.22 g/L was obtained from pre-treatment performed under optimum conditions (OP-2). Concentration of XOS reported in this study was comparable with the data presented in the literature. The typical range of XOS concentrations reported in literature for lignocellulosic biomasses such as elephant grass, olive tree prunings and Arundo donax, is normally below 16 g/L (Cara et al., 2012; Galia et al., 2015; Toscan et al., 2017). The amount of XOS increased with increasing NaOH concentration, microwave power and ultrasound power. However, as in the UAAP, after a certain point, the increase in these factors leads to the conversion of XOS into its monomeric form of xylose and further degradation of this sugar to furfural and formic acid. For example, in run no 6 and 8, while the microwave and ultrasound powers were the same, increasing NaOH concentration from 0.1 N to 2 N increased the amount of XOS from 4.03 to 14.10 g/L. But this increase also caused an increase in the amount of xylose, furfural and formic acid. Similarly, while the other conditions were same

in run no 11 and 12, increasing microwave power from 100 W to 300 W increased the XOS concentration from 12.87 to 14.01 g/L and also inceased the formation of xylose and by-products. The maximum AcOS and acetic acid concentrations were 2.62 and 4.24 g/L, respectively. Since NaOH was primarily effective on the lignin and hemicellulose, the concentration of GlcOS and glucose did not exceed 1.39 g/L and 1.49 g/L, respectively. HMF and levulinic acid were not detected in the liquid phases.

Table 4.25 Composition of liquid fractions obtained after sequential ultrasound-microwave-assisted alkaline pre-treatment

Run no	Composition of liquid fraction (g/L)							
	Glucose	**Xylose**	**Acetic acid**	**Formic acid**	**Furfural**	**XOS**	**GlcOS**	**AcOS**
1	0.71	1.86	2.47	0.27	0.44	10.03	0.45	1.87
2	0.79	1.97	2.69	0.25	0.48	11.35	0.52	2.11
3	0.87	2.14	3.21	0.71	0.65	11.55	0.68	2.15
4	0.93	2.63	3.49	0.64	0.71	12.85	0.74	2.39
5	0.45	1.12	1.15	0.12	0.24	3.19	0.22	0.59
6	0.53	1.39	1.21	0.07	0.27	4.03	0.29	0.75
7	1.12	3.14	3.84	0.54	0.61	12.78	1.14	2.38
8	1.28	3.08	4.07	0.72	0.68	14.10	1.31	2.62
9	0.41	1.22	0.94	0.09	0.20	3.01	0.19	0.56
10	0.65	1.57	1.39	0.19	0.31	4.36	0.30	0.81
11	0.97	2.78	3.62	0.43	0.59	12.87	1.24	2.39
12	1.49	3.27	4.24	0.92	0.75	14.01	1.39	2.61
13	0.75	1.91	3.51	0.23	0.48	11.50	0.59	2.14
14	0.88	1.85	3.44	0.19	0.35	12.01	0.61	2.23
15	0.85	2.17	3.32	0.26	0.45	12.29	0.54	2.29
16	0.77	2.09	3.56	0.21	0.37	12.20	0.66	2.27
17	0.81	1.96	3.49	0.17	0.41	11.24	0.57	2.09
OP-2	1.25	2.96	4.16	0.83	0.61	14.22	1.16	2.65

XOS-xylo-oligosaccharides; GlcOS-gluco-oligosaccharides; AcOS-acetyl oligosaccharides; OP-2-Sequential ultrasound-microwave-assisted alkaline pre-treatment performed under optimum conditions

4.3.7 Enzymatic Hydrolysis of Pre-treated Biomass

4.3.7.1 Determination of Enzyme Activity

The cellulase activity of the enzyme was determined according to NREL/TP-510-42628 (Adney and Baker, 1996). According to this method, the absorbance of the glucose solutions prepared at different concentrations was measured at 540 nm using a spectrophotometer and the standard curve given in Figure 4.15 was drawn. Then,

the absorbance of solutions including glucose which was released by the enzyme solutions prepared at different concentrations was measured and the amount of glucose was determined using the glucose standard curve. After that, a graph showing the amount of glucose versus enzyme concentration was drawn on the semi-logarithmic graph paper and is given in Figure 4.16. Finally, using this graph, the enzyme concentration corresponding to the amount of 2 mg of glucose was determined and enzyme activity was found to be 192.06 FPU/mL using equation 3.13. In all enzymatic hydrolysis experiments, the amount of enzymes used was calculated based on this data.

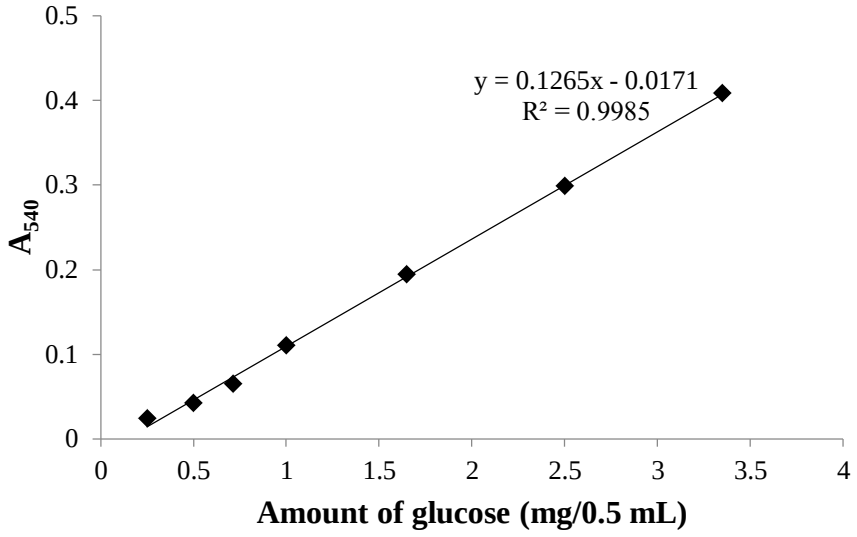

Figure 4.15 Standard curve prepared for glucose solutions

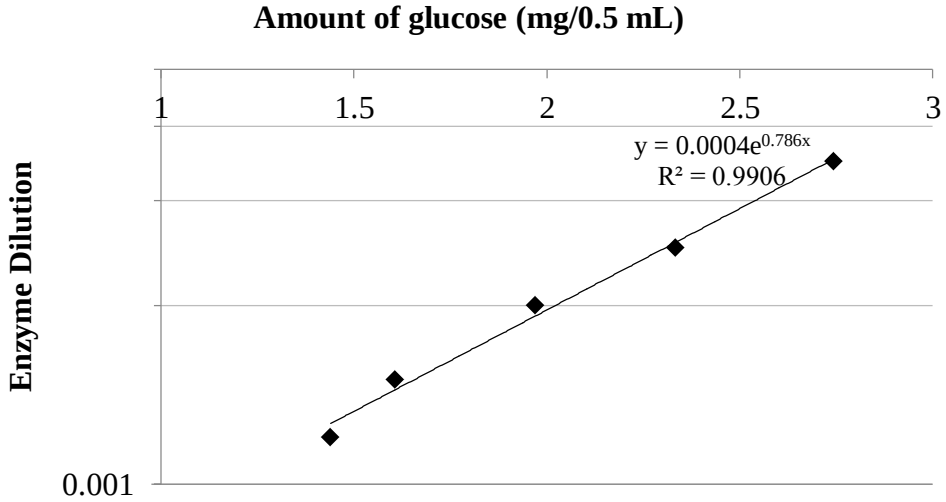

Figure 4.16 Graph showing the amount of glucose versus enzyme dilution

4.3.7.2 Enzymatic Hydrolysis of Pre-treated Solids

Pre-treated solids after MAAP, UAAP and SUMAAP were enriched in cellulose by hemicellulose and lignin removal. For the valorisation of the pre-treated solids, enzymatic hydrolysis of glucan was performed to produce fermentable sugar. Glucan to glucose yield obtained from 72 h enzymatic hydrolysis of pre-treated solids after MAAP, UAAP and SUMAAP performed under different conditions are given in Figure 4.17, Figure 4.18 and Figure 4.19, respectively. In addition, enzymatic hydrolysis yields for untreated PS, solid residue after CAP, pre-treated solids after MAAP, UAAP and SUMAAP performed under optimum conditions are shown in Figure 4.20.

Depending on the MAAP conditions, glucan to glucose yields varied from 37.17 to 78.93 mol%. The concentration of NaOH was the most important parameter on glucose yield and followed by microwave power. Glucose yield increased with increasing NaOH concentration, microwave power and pre-treatment time. For raw PS, the glucose yield was only 11.29 mol% (Figure 4.20). The glucose yield increased to 41.97 mol% after CAP which indicated the effect of alkaline treatment to enhance the enzymatic digestibility of lignocellulosic material (Wang et al., 2017). The highest glucose yield of 82.67 mol% obtained from the solid after MAAP performed under optimum conditions (microwave power of 224 W, NaOH concentration of 1.96 N and pre-treatment time of 2.63 min). This result showed that alkaline treatment combined with microwave irradiation significantly increased the enzymatic hydrolysis yield. When compared to the conventional heating, microwave irradiation provides volumetric and selective heating of biomass that facilitates the degradation of recalcitrant structures more efficiently (Chittibabu et al., 2014). Increasing enzymatic hydrolysis efficiency can be explained by the removal of hemicellulose and lignin after pre-treatment since these compounds are the main barrier for the efficient hydrolysis of cellulose by enzymes (Jin et al., 2016). By the removal of hemicellulose and lignin, the biomass is enriched in cellulose and also become more open-structured that make the solid residue more accessible for enzymes (Mihiretu et al., 2017). Increasing enzymatic hydrolysis yield after MAAP have been already reported by other researchers for different types of biomasses (Boonsombuti et al., 2013; Chittibabu et al., 2014; Jin et al., 2016; Keshwani and Cheng, 2010). In a study carried out by Mihiretu et al. (2017), it was reported that the

glucose yields obtained after enzymatic hydrolysis of aspenwood and sugar cane trash increased from 18.85 to 80% (w/w) and 19 to 75% (w/w) after MAAP, respectively. Similarly, after MAAP of switchgrass and costal bermudagrass, the glucose yield increased from 30 to 82% and 9 to 87%, respectively (Keshwani and Cheng, 2010).

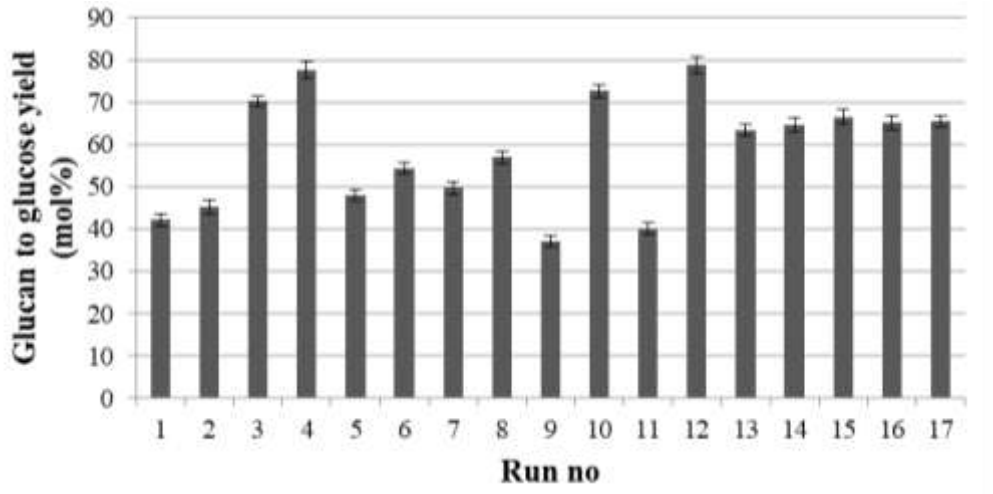

Figure 4.17 Glucan to glucose yield obtained from 72 h enzymatic hydrolysis of microwave-assisted alkali pre-treated solids

The glucan to glucose yields after the enzymatic hydrolysis of the solids from UAAP ranged between 39.89-81.07 mol% depending on the pre-treatment conditions. Enzymatic hydrolysis yield obtained from the pre-treated solids significantly increased by increasing alkali concentration which may be due to the high cellulose and low hemicellulose and lignin contents of solids pre-treated at higher alkali concentration. For example, glucose yield enhanced from 50.90 mol% to 79.85 mol% by increasing the alkali concentration from 0.1 to 2 N after the enzymatic hydrolysis of solids pre-treated under the conditions where ultrasound power, pre-treatment time and temperature were 384 W, 60 min and 95 °C, respectively. The highest glucose yield of 84.86 mol% obtained from the solid after UAAP performed under optimum conditions (ultrasound power of 640 W, pre-treatment time of 87.44 min, NaOH concentration of 1.96 N and temperature of 93 °C). This result showed that alkaline treatment combined with ultrasound significantly increased the enzymatic hydrolysis of biomass. This might be due to the removal of hemicellulose and lignin and the changes in crystal structure of the biomass, emerging after UAAP. Increasing enzymatic hydrolysis yield after ultrasound application was also reported

by other researchers for different biomass samples (da Silva et al., 2016; Gu et al., 2019; Wang et al., 2017). Wang et al. (2017) stated that the amount of reducing sugar obtained after the enzymatic hydrolysis of grass clipping after ultrasound-assisted calcium hydroxide pre-treatment increased 3.5-fold compared to untreated biomass. Gu et al. (2019) stated that enzymatic hydrolysis yield of the dried distillers' grains increased to 65% after UAAP while this value was 40% for the untreated biomass.

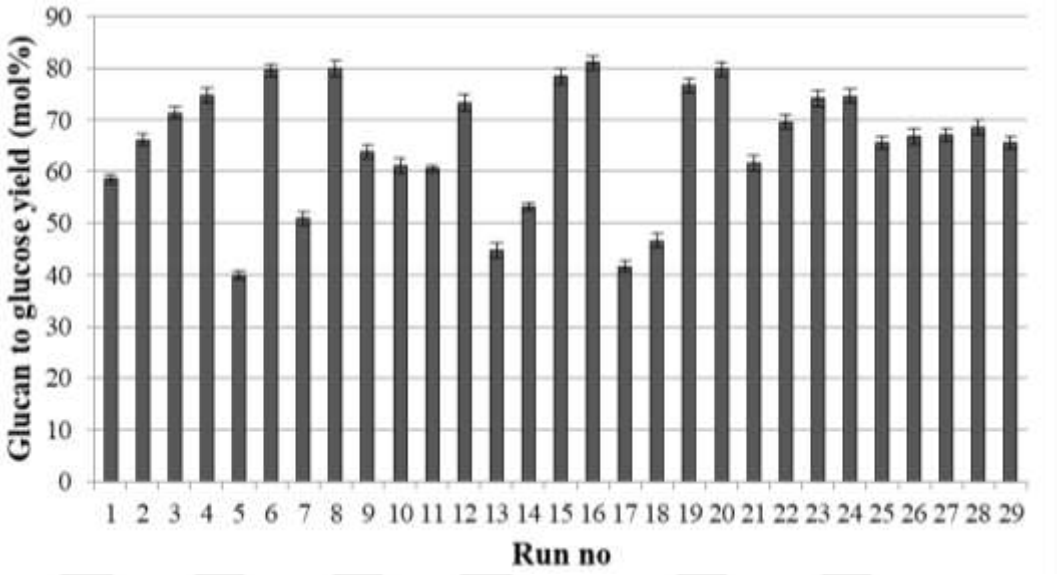

Figure 4.18 Glucan to glucose yield obtained from 72 h enzymatic hydrolysis of ultrasound-assisted alkali pre-treated solids

The glucan to glucose yields obtained after the enzymatic hydrolysis of the solids from SUMAAP varied from 53.14 to 93.82 mol% depending on the pre-treatment conditions. Enzymatic hydrolysis yield obtained from the pre-treated solids significantly improved with increasing alkali concentration. For example, for run 5 and run 7, where UP and MP were 128 W and 200 W, respectively, increasing NC from 0.1 to 2 N increased the enzymatic hydrolysis efficiency from 53.14 mol% to 84.00 mol%. The highest glucose yield of 95.78 mol% obtained from the solid after SUMAAP performed under optimum conditions (640 W ultrasound power, 248 W microwave power and 1.58 N NaOH concentration). On the other hand, it was stated in the sections 4.3.2.2, 4.3.3.2 and 4.3.4.3 that the cellulose recovery in pre-treated solids decreased with increasing NC. This was explained by the degradation of cellulose as well as hemicellulose and lignin at higher NC. Considering this situation, it is not desired to increase NC during pre-treatment. SUMAAP was advantageous because the application of microwave and ultrasound reduced the used NC and

increased the enzymatic hydrolysis efficiency. In addition, the hydrolysis yields were higher for the solids pre-treated at higher UP and MP which may be due to the destruction of crystal structure of the biomass by the application of ultrasound and microwave.

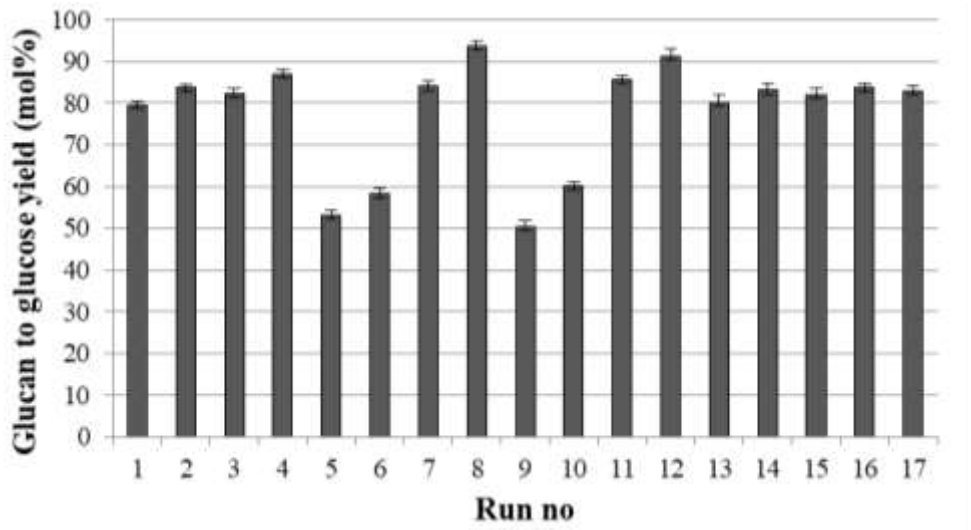

Figure 4.19 Glucan to glucose yield obtained from 72 h enzymatic hydrolysis of pre-treated solids after SUMAAP

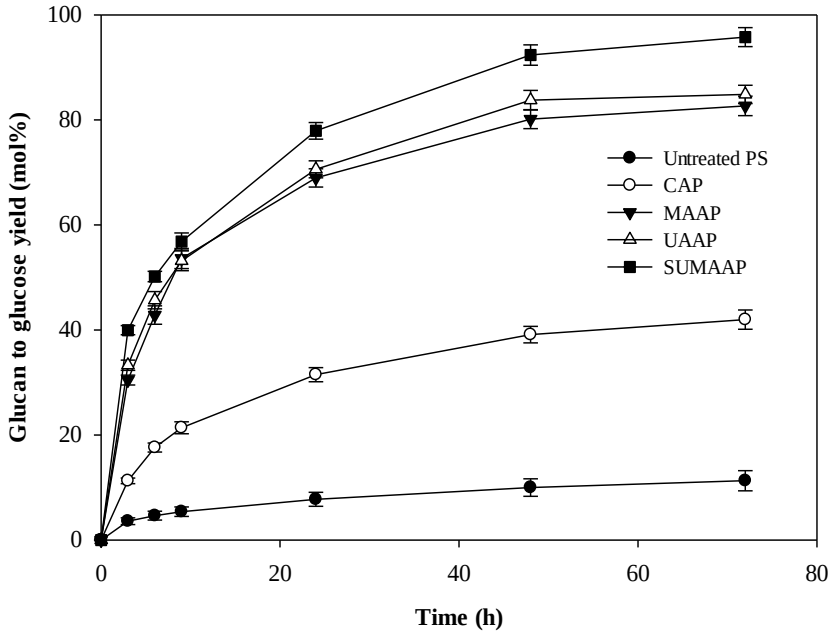

Figure 4. 20 Glucan to glucose yield obtained from 72 h enzymatic hydrolysis of PS and pre-treated solids after CAP, MAAP, UAAP and SUMAAP (in CAP, the NaOH concentration was 2 N; MAAP, UAAP and SUMAAP performed under optimum conditions)

4.3.7.3 Investigation of Enzymatic Hydrolysis Data Using Fractal Kinetic Model

The hydrolysis efficiency of cellulose depending on time are given in Table 4.26, Table 4.27 and Table 4.28 for MAAP, UAAP and SUMAAP, respectively. In addition, enzymatic hydrolysis data for the pre-treated solid after CAP and untreated PS is shown in Table 4.28.

Table 4.26 The enzymatic hydrolysis yield as glucan to glucose (mol%) for pre-treated solids after MAAP

Time (h)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	12.42±0.51	12.55±0.73	23.28±1.12	27.85±0.97	15.73±0.48	19.10±1.14
6	20.22±0.67	18.86±1.08	33.73±0.84	36.98±1.15	20.85±0.84	25.35±0.68
9	23.09±1.14	23.43±0.85	42.91±1.23	44.41±1.26	25.28±1.14	30.94±1.43
24	30.59±0.82	34.98±1.17	60.09±1.74	63.86±1.86	37.03±1.45	41.23±0.99
48	39.83±1.56	43.24±0.97	68.69±1.45	75.19±1.47	46.27±1.16	52.69±1.38
72	42.10±1.42	45.22±1.63	70.21±1.25	77.56±1.92	47.98±1.32	54.30±1.41
Time (h)	**Run 7**	**Run 8**	**Run 9**	**Run 10**	**Run 11**	**Run 12**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	16.21±0.78	20.05±0.98	11.48±0.86	25.50±1.16	12.75±1.48	27.39±1.63
6	21.77±1.37	25.61±0.64	18.43±0.98	34.55±1.21	19.68±0.76	39.64±1.04
9	26.92±1.54	34.71±1.31	20.90±1.69	41.25±0.96	22.88±1.37	50.16±1.51
24	38.53±1.56	47.13±0.85	30.25±0.93	58.06±1.24	29.90±1.56	65.15±1.43
48	47.07±1.67	55.49±1.24	36.28±1.86	69.58±1.74	38.22±1.25	76.24±1.14
72	49.71±1.51	56.95±1.55	37.17±1.41	72.66±1.56	40.08±1.38	78.93±1.97
Time (h)	**Run 13**	**Run 14**	**Run 15**	**Run 16**	**Run 17**	**OP**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	24.94±1.42	24.80±1.21	26.64±0.97	25.65±1.21	26.16±1.08	30.46±0.95
6	32.39±1.27	33.12±1.34	35.24±1.16	33.17±1.38	34.00±1.14	42.84±1.75
9	36.84±1.06	37.67±1.24	37.36±1.44	38.97±1.33	37.65±1.17	53.61±1.91
24	53.13±0.97	53.04±1.15	54.13±1.63	53.85±1.21	53.57±0.79	68.96±1.73
48	61.46±1.35	62.80±0.96	65.08±1.65	64.26±0.92	64.62±1.47	80.15±1.81
72	63.26±1.34	64.55±1.64	66.47±1.79	65.10±1.62	65.45±1.36	82.67±1.86

OP:MAAP performed under optimum conditions

Table 4.27 The enzymatic hydrolysis yield as glucan to glucose (mol%) for pre-treated solids after UAAP

Time (h)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	14.10±0.62	21.06±0.87	22.84±1.14	23.96±1.23	12.66±1.47	27.15±1.35
6	28.93±0.84	32.48±1.21	35.25±0.96	36.16±1.07	18.49±0.88	39.61±1.24
9	35.28±1.34	40.16±1.76	43.98±1.24	43.97±1.51	22.22±1.63	49.70±1.76
24	42.73±0.93	52.53±1.52	56.60±1.27	59.08±1.43	30.51±1.76	65.06±1.31
48	58.16±0.64	65.32±0.78	70.15±0.92	71.52±1.67	37.20±1.34	77.55±1.56
72	58.58±0.88	66.12±1.16	71.39±1.10	74.69±1.43	39.89±0.86	79.63±1.17
Time (h)	**Run 7**	**Run 8**	**Run 9**	**Run 10**	**Run 11**	**Run 12**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	16.53±1.46	28.44±0.83	21.26±0.79	19.38±1.34	18.14±1.61	23.99±0.94
6	22.98±1.13	40.50±1.27	31.70±1.31	28.93±1.16	28.92±1.21	33.24±0.87
9	27.22±0.98	47.87±1.41	39.55±1.44	36.22±0.76	34.26±1.73	44.51±1.05
24	40.39±1.67	65.14±1.73	50.14±0.87	46.75±1.26	46.56±1.58	54.23±1.65
48	47.55±1.10	78.67±1.63	60.71±1.57	59.20±1.21	58.85±1.33	70.15±1.64
72	50.90±1.20	79.85±1.49	63.82±1.29	61.16±1.48	60.53±0.54	73.33±1.67
Time (h)	**Run 13**	**Run 14**	**Run 15**	**Run 16**	**Run 17**	**Run 18**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	14.76±0.57	18.37±1.06	27.74±1.21	29.44±1.64	12.49±1.13	16.78±1.46
6	20.17±0.79	23.24±1.04	40.79±1.23	40.09±1.37	19.52±0.98	19.55±1.16
9	24.04±1.59	29.38±1.13	49.75±0.97	45.69±1.73	23.53±1.67	26.31±1.24
24	33.94±0.79	42.79±1.76	70.87±1.34	62.84±1.63	30.43±1.27	34.57±1.14
48	42.15±0.92	50.59±1.63	75.55±1.71	78.01±1.34	38.53±1.21	43.30±1.57
72	44.78±1.52	53.11±0.70	78.35±1.50	81.07±1.36	41.56±1.14	46.60±1.30
Time (h)	**Run 19**	**Run 20**	**Run 21**	**Run 22**	**Run 23**	**Run 24**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	26.39±1.37	28.50±1.11	20.04±0.96	21.80±1.17	24.97±1.34	23.44±1.43
6	36.12±1.31	39.73±1.28	30.28±1.43	32.43±1.57	35.44±1.08	36.43±1.40
9	44.65±1.75	46.49±1.56	35.02±1.37	40.70±0.64	43.88±1.54	44.02±0.89
24	60.02±0.89	62.74±1.64	47.46±1.32	51.34±1.43	57.23±0.86	57.46±1.04
48	72.78±1.62	76.92±1.71	58.03±0.75	65.40±0.99	71.58±1.24	70.48±1.39
72	76.67±1.36	79.90±1.40	61.58±1.42	69.62±1.36	74.18±1.57	74.55±1.34
Time (h)	**Run 25**	**Run 26**	**Run 27**	**Run 28**	**Run 29**	**OP-1**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	21.93±1.51	22.80±0.93	22.31±1.16	23.29±1.25	22.94±1.38	33.28±0.97
6	32.42±1.34	33.60±1.28	34.38±1.51	35.08±1.63	33.07±0.95	45.67±1.64
9	40.59±1.35	41.17±1.71	42.15±0.83	42.07±1.61	41.03±1.54	53.17±1.88
24	51.98±0.92	50.30±1.71	50.69±0.98	51.01±1.35	51.84±1.28	70.59±1.62
48	63.33±1.46	64.86±1.62	65.40±1.14	66.30±1.49	63.25±1.73	83.76±1.86
72	65.61±1.18	66.82±1.54	67.07±1.32	68.52±1.48	65.52±1.28	84.86±1.74

OP-1:UAAP performed under optimum conditions

Table 4.28 The enzymatic hydrolysis yield as glucan to glucose (mol%) for pre-treated solids after SUMAAP, CAP and for the untreated biomass

Time (h)	Run 1	Run 2	Run 3	Run 4	Run 5
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	22.78±0.57	25.18±1.12	27.08±1.35	33.59±1.048	14.47±0.64
6	36.95±1.24	38.60±0.87	38.62±1.30	46.38±1.17	21.79±1.23
9	46.72±0.92	49.68±1.56	46.20±0.87	57.40±1.47	26.09±1.04
24	65.55±1.38	64.75±1.67	67.31±1.33	69.24±1.42	40.95±1.58
48	75.62±1.63	81.23±1.81	80.82±1.43	84.04±1.34	52.04±1.68
72	79.64±0.68	83.70±0.76	82.23±1.30	86.94±1.33	53.14±1.16
Time (h)	**Run 6**	**Run 7**	**Run 8**	**Run 9**	**Run 10**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	16.32±1.31	31.54±1.47	36.76±1.13	13.33±0.82	17.75±1.48
6	24.96±1.14	42.74±0.84	47.62±1.34	21.17±1.06	25.37±1.18
9	30.52±1.35	50.43±1.33	55.00±1.64	28.25±0.86	31.95±0.97
24	42.64±1.47	70.73±1.73	76.76±1.02	36.38±1.17	45.60±1.36
48	55.60±1.84	83.46±0.99	90.95±1.83	48.73±1.40	57.35±1.14
72	58.24±1.27	84.00±1.40	93.82±0.99	50.25±1.69	60.16±0.78
Time (h)	**Run 11**	**Run 12**	**Run 13**	**Run 14**	**Run 15**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	32.12±1.27	35.36±1.53	28.99±0.97	26.55±1.43	28.32±1.16
6	43.11±1.37	45.79±1.51	40.64±1.23	38.41±1.45	39.85±0.95
9	53.80±0.91	54.62±1.48	49.48±1.26	47.40±1.62	48.72±1.14
24	71.53±1.12	77.59±1.83	66.56±1.05	65.46±0.93	64.98±1.48
48	84.76±1.61	88.83±1.37	78.49±1.63	80.27±1.44	79.20±1.07
72	85.65±0.84	91.22±1.97	80.15±1.82	83.09±1.70	81.98±1.50
Time (h)	**Run 16**	**Run 17**	**OP-2**	**CAP**	**Raw PS**
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	27.66±0.94	26.32±1.37	37.96±0.87	11.27±0.53	3.58±0.64
6	38.16±1.20	37.78±1.47	49.17±0.99	17.61±0.86	4.64±0.85
9	46.43±1.57	45.89±1.36	56.84±1.63	21.37±1.14	5.40±0.91
24	66.61±1.12	64.12±1.36	77.93±1.58	31.49±1.35	7.76±1.34
48	80.04±1.81	79.14±1.45	92.36±1.94	39.12±1.58	9.99±1.67
72	83.74±1.16	82.86±1.34	95.78±1.81	41.97±1.84	11.29±1.92

OP-2:SUMAAP performed under optimum conditions; CAP: Conventional alkaline pre-treatment performed using 2 N NaOH

The experimental data of enzymatic hydrolysis were analysed using the fractal kinetic model proposed by Kopelman (1988). Fractal kinetic parameters of k and h were calculated and presented in Table 4.29 and Table 4.30 with the R^2 values. The R^2 values were very high and in general were superior to 0.99 which confirmed the ability of fractal kinetic model to fit the experimental data. The fractal exponent, h,

indicates the accessibility of enzymes to substrate and its value changes from 0 to 1 (Wojtusik et al., 2016). Also, h shows the multiple effects of the pre-treatment on the lignocellulosic structure and low h values indicate more accessible substrates for enzymes which may be due to the pre-treatment (Aguiar et al., 2013). The h values for pre-treated samples were found to be lower compared to the raw PS. The highest h value of 0.6262 was found for the raw PS with a glucan conversion of only 11.29 mol%. The h value decreased after CAP, MAAP, UAAP and SUMAAP and the lowest h values for each pre-treatment method were found as 0.5632, 0.5079, 0.4977 and 0.4101, respectively. This is the evidence that the pre-treatment methods make biomass more accessible by enzymes. Also, h values generally decreased due to the increase in pre-treatment conditions. For example, the h value of 0.6237 was obtained after the enzymatic hydrolysis of pre-treated solid by MAAP, where the pre-treatment condition were 200 W, 0.1 N NaOH and 1 min (run no:9). The h value decreased and calculated as 0.5028 after the hydrolysis of the solid phase obtained from run no 4, where the pre-treatment conditions were 300 W, 2 N NaOH and 3 min. A similar trend was observed for the pre-treated solids after UAAP and SUMAAP. When the h values obtained after the different pre-treatment methods were compared, it was seen that the lowest h value was obtained after SUMAAP. h values obtained after MAAP and UAAP were found close to each other. This result indicated that the sequential combination of ultrasound with microwave makes biomass more accessible by enzymes. This situation was also proved by the high glucose yield obtained after SUMAAP.

Rate coefficient, k, is related to the binding capacity between substrate and enzyme that also directly related to the reaction rate. Increasing k value indicates the increase in the overall reaction rate achieved during enzymatic hydrolysis (Liu et al., 2016; Wojtusik et al., 2016). The pre-treatment methods resulted in relatively high k values (0.0886, 0.2365, 0.2465 and 0.2580 h^{-1} for CAP, MAAP, UAAP and SUMAAP, respectively), while the raw PS had a very low k value of 0.0245 h^{-1}. Also, it was seen that the k values generally increased by increasing the pre-treatment conditions. These findings proved that the CAP, MAAP, UAAP and SUMAAP make biomass more accessible by enzymes as a result of their effects on the biomass structure mentioned before.

Table 4.29 Fractal kinetic parameters of the enzymatic hydrolysis data obtained for pre-treated solids after MAAP and SUMAAP

Run no	k (h^{-1})	h	R^2	Run no	k (h^{-1})	h	R^2
MAAP 1	0.1034	0.6007	0.9910	**SUMAAP 1**	0.1750	0.4587	0.9903
MAAP 2	0.0974	0.5574	0.9892	**SUMAAP 2**	0.1787	0.4369	0.9939
MAAP 3	0.1868	0.5349	0.9861	**SUMAAP 3**	0.1817	0.4446	0.9960
MAAP 4	0.1957	0.5028	0.9959	**SUMAAP 4**	0.2580	0.5046	0.9950
MAAP 5	0.1141	0.5765	0.9932	**SUMAAP 5**	0.1050	0.5164	0.9888
MAAP 6	0.1439	0.5901	0.9946	**SUMAAP 6**	0.1214	0.5242	0.9939
MAAP 7	0.1210	0.5805	0.9942	**SUMAAP 7**	0.2196	0.4733	0.9963
MAAP 8	0.1571	0.5869	0.9860	**SUMAAP 8**	0.2271	0.4101	0.9988
MAAP 9	0.1003	0.6237	0.9837	**SUMAAP 9**	0.1081	0.5475	0.9858
MAAP 10	0.1855	0.5290	0.9969	**SUMAAP 10**	0.1291	0.5250	0.9943
MAAP 11	0.1068	0.6226	0.9914	**SUMAAP 11**	0.2258	0.4673	0.9962
MAAP 12	0.2163	0.5156	0.9923	**SUMAAP 12**	0.2233	0.4173	0.9984
MAAP 13	0.1933	0.5973	0.9932	**SUMAAP 13**	0.2183	0.5065	0.9950
MAAP 14	0.1929	0.5893	0.9948	**SUMAAP 14**	0.1808	0.4454	0.9973
MAAP 15	0.2014	0.5883	0.9945	**SUMAAP 15**	0.2028	0.4832	0.9972
MAAP 16	0.1984	0.5899	0.9938	**SUMAAP 16**	0.1807	0.4428	0.9985
MAAP 17	0.1981	0.5888	0.9944	**SUMAAP 17**	0.1757	0.4457	0.9984
OP	0.2365	0.5079	0.9941	**OP-2**	0.2506	0.4232	0.9976

OP: MAAP performed under optimum conditions; OP-2:SUMAAP performed under optimum conditions

Table 4.30 Fractal kinetic parameters of the enzymatic hydrolysis data obtained for PS and pre-treated solids after CAP and UAAP

Run no	k (h^{-1})	h	R^2	Run no	k (h^{-1})	h	R^2
UAAP 1	0.1367	0.5439	0.9711	**UAAP 16**	0.2041	0.4977	0.9985
UAAP 2	0.1739	0.5501	0.9884	**UAAP 17**	0.1045	0.6092	0.9920
UAAP 3	0.1864	0.5324	0.9893	**UAAP 18**	0.1191	0.6041	0.9948
UAAP 4	0.1856	0.5142	0.9944	**UAAP 19**	0.1915	0.5124	0.9975
UAAP 5	0.1025	0.6152	0.9936	**UAAP 20**	0.2060	0.5057	0.9981
UAAP 6	0.2090	0.5013	0.9932	**UAAP 21**	0.1613	0.5723	0.9949
UAAP 7	0.1261	0.5829	0.9933	**UAAP 22**	0.1689	0.5342	0.9938
UAAP 8	0.2103	0.5007	0.9957	**UAAP 23**	0.1872	0.5217	0.9953
UAAP 9	0.1796	0.5808	0.9906	**UAAP 24**	0.1854	0.5197	0.9941
UAAP 10	0.1565	0.5633	0.9909	**UAAP 25**	0.1822	0.5694	0.9905
UAAP 11	0.1475	0.5516	0.9905	**UAAP 26**	0.1859	0.5701	0.9908
UAAP 12	0.1791	0.5210	0.9913	**UAAP 27**	0.1887	0.5706	0.9876
UAAP 13	0.1111	0.5973	0.9960	**UAAP 28**	0.1908	0.5668	0.9904
UAAP 14	0.1353	0.5818	0.9916	**UAAP 29**	0.1908	0.5822	0.9916
UAAP 15	0.2252	0.5184	0.9837	**OP-1**	0.2465	0.4996	0.9973
CAP	0.0886	0.5632	0.9920	**Raw PS**	0.0245	0.6262	0.9997

OP-1: UAAP performed under optimum conditions; CAP: Conventional alkaline pre-treatment performed using 2 N NaOH

4.3.8 Investigation of Morphology Changes after Pre-treatments by SEM

The surface morphologies of PS and the pre-treated solids after CAP, MAAP, UAAP and SUMAAP were investigated using SEM for evaluation of different pre-treatment methods and the SEM images are shown in Figure 4.21. Significant morphological differences were obtained between raw PS and pre-treated solids. Raw PS had a rigid, smooth and continuous surface structure (Figure 4.21 a). The surface of the sample was deteriorated after CAP and had rough and broken structure (Figure 4.21 b). These changes can be explained by the effect of alkali on the biomass which causes an increase in porosity and surface area by breaking the lignin-hemicellulose complex (Singh et al., 2017).

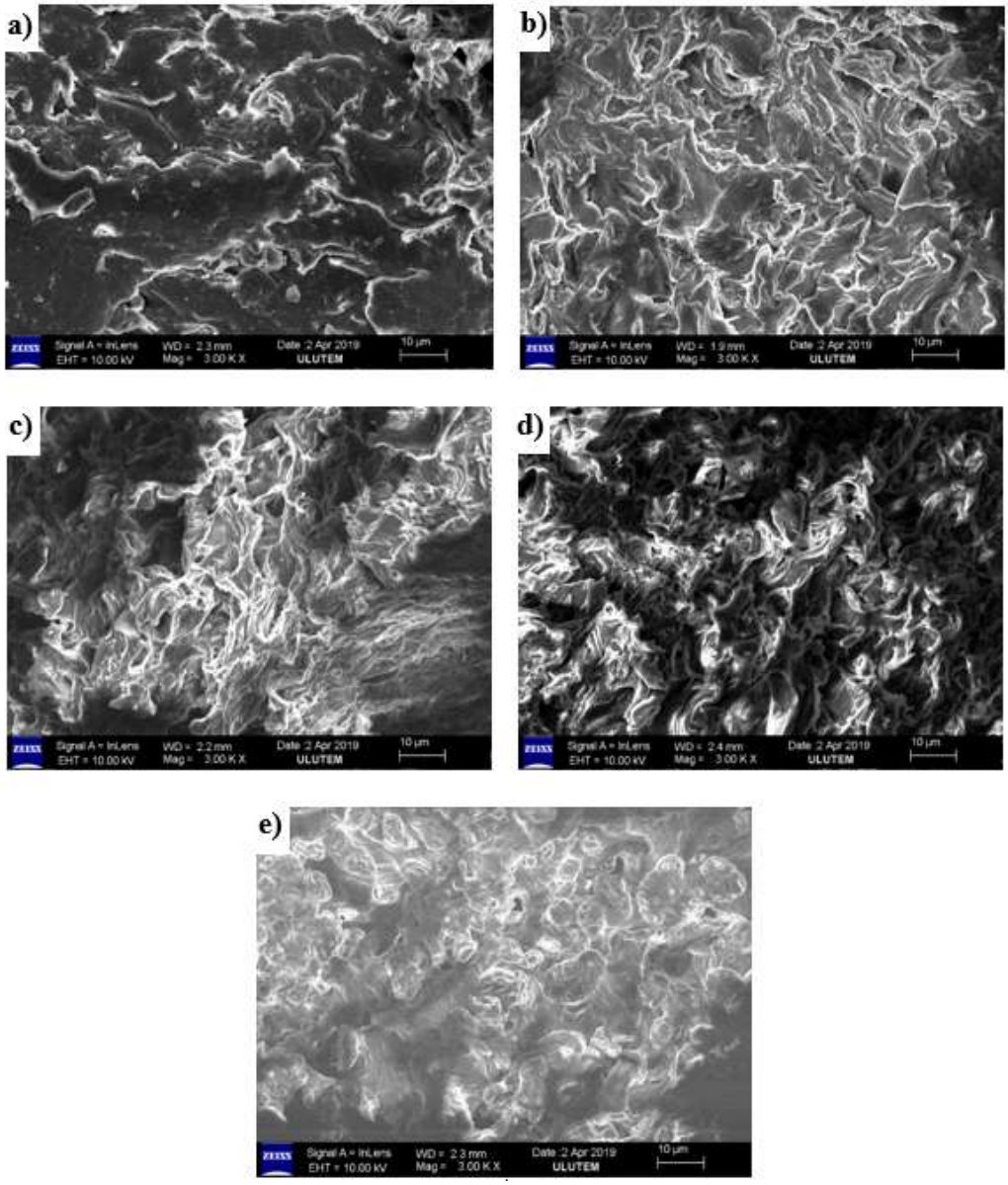

Figure 4.21 SEM images of pistachio shell before and after pre-treatment: untreated pistachio shell (a) pistachio shell after pre-treatment with CAP (b), MAAP (c), UAAP (d) and SUMAAP (e). (The solids was obtained from pre-treatments performed under optimum conditions for MAAP, UAAP and SUMAAP. For CAP, the NaOH concentration was 2 N.)

After MAAP and UAAP, biomass was significantly deformed and had highly unstructured rough surface (Figure 4.21 c, d). The application of microwave irradiation and ultrasound together with alkali caused an extensive deformation of the structure. This observation can be explained by the synergistic effect of alkali (NaOH) with microwave and ultrasound. The presence of alkali solution causes the cleavage of the ester and/or ether bonds between hemicellulose and lignin (Kim et

al., 2016). The cell structure of biomass can be damaged by the rapid increase in temperature and internal pressure as a result of microwave irradiation (Li et al., 2014). The shock waves and the free radicals generated by cavitation effect resulting from ultrasonication can cleave the pistachio shell which causing disintegration of the lignocellulosic structure (Wang et al., 2016). Similar observations have been reported after MAAP and UAAP of different lignocellulosic biomasses (Kan et al., 2018; Sudha et al., 2017; Wang et al., 2017; Wu et al., 2017). The most severe deterioration was observed after SUMAAP (Figure 4.21 e). The mechanical (cavitation) effect of ultrasound breaks down the cell wall to form pores and the sudden increase in temperature and pressure during microwave application damages the cell structure (Li et al., 2014; Manasa et al., 2018). The increase in enzymatic hydrolysis yields after pre-treatments may also be explained by the morphological changes that increase the enzyme accessibility to cellulose.

CHAPTER 5

CONCLUSIONS

In this thesis, it was aimed to perform bio-refinery of pistachio production by-products which are PH and PS. A strategy consisting of four steps was proposed to reach the stated bio-refinery process. In the first step, MASE of non-polar compounds from PH was performed. MASE conditions were chosen as liquid to solid ratio of 15:1 (v/w), power of 250 W and time of 12.5 min to obtain the maximum extraction yield (%). Under these conditions, higher extraction yield (%) and extract with higher antioxidant activity, total phenolic and tocopherol contents were obtained by MASE when compared to the traditional Soxhlet method. SEM observation of the extraction residues suggested that microwave irradiation destroyed the plant tissue, which probably enhanced the release of chemical substances into the solvents.

Second step was the extraction of phenolic compounds from PH using MASE with green solvents, water and ethanol. Optimization of MASE factors (microwave power, extraction time, solvent to sample ratio and ethanol concentration) was performed by RSM to enhance the TPC. The results indicate that the all parameters were important for MASE of phenolics from PH. Optimum conditions were found as 140 W microwave power, 4.5 min extraction time, 19:1 (v/w) solvent to sample ratio and 56% ethanol concentration to get maximum TPC (62.24 mg GAE/g dry hull). The comparison between the MASE and CSE methods showed MASE is advantageous in extracting phenolics with higher yield, higher antioxidant activity, reduced extraction time and solvent consumption. The compositional analysis showed that PHE contains sugar, protein, acid, pectin and ash in addition to phenolic compounds.

In the third step, alkali pre-treatment of PS was carried out by using microwave and/or ultrasound-assisted methods to fractionate PS into hemicellulose solid precipitate and cellulose rich solid residue. The pre-treatment conditions (microwave or ultrasound power, temperature, time, NaOH concentration) were investigated and optimized by using RSM to obtain maximum hemicellulose recovery in the liquid

phase and maximum cellulose recovery in the solid phase. Optimum pre-treatment conditions were determined as mirowave power of 224 W, NaOH concentration of 1.96 N and time of 2.63 min for MAAP; ultrasound power of 98%, time of 87.44 min, NaOH concentration of 1.96 N and temperature of 93 °C for UAAP; ultrasound power of 640 W, microwave power of 248 W and NaOH concentration of 1.58 N for SUMAAP. Under these optimum conditions, the hemicellulose and cellulose recoveries were found as 58.35% and 92.46% for MAAP, 57.67% and 94.05% for UAAP and 66.14% and 90.78% for SUMAAP. Also, CAP was performed at 35 °C for 16 hours using 2 N NaOH to compare with these three methods and the hemicellulose and cellulose recoveries were found as 16.28% and 98.70%, respectively. These results showed that MAAP, UAAP and SUMAAP increased the hemicellulose recovery with a significant reduction in processing time when compared to CAP. The compositional analysis of the solid and liquid phases obtained from the pre-treatments indicated that the XOS were the main components in the liquid phases while the solid phases were enriched with cellulose.

In the final step of the thesis, enzymatic hydrolysis of the pre-treated cellulose-rich solid fractions was performed to produce fermentable sugars. The enzymatic hydrolysis efficiency was significantly increased for solids pre-treated with CAP, MAAP, UAAP and SUMAAP as compared to the native biomass. The glucose yield after SUMAAP was 95.78 mol% which was significantly higher than the values of 11.29, 41.97, 82.67 and 84.86 mol% obtained for untreated PS, CAP, MAAP and UAAP, respectively.

The results of this thesis indicated that PH is a good source of value-added compounds with potential usage in different industries such as food, cosmetic and drug industries. For the extraction of bio-active compounds, MASE is a better and environmental friendly technique than CSE and Soxhlet methods. In addition, this thesis showed that the combination of alkaline pre-treatment with ultrasound or microwave irradiation and especially their sequential use is a reliable and effective process which reduced the time and amounts of chemical used to save energy for the fractionation of pistachio shell into its valuable compounds, hemicellulose and cellulose.

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EDUCATION

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Ph.D.	Gaziantep University, Food Engineering Department	
M.Sc.	Gaziantep University, Food Engineering Department	2014
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ACADEMIC/WORK EXPERIENCES

Year	Place	Enrollment
September 2012-Present	Gaziantep University, Food Engineering Department	Research Asst.
January 2016-February 2016	Participation in the project COST Action: TD1203, "Extracting of phenolic compounds from Pistacia as antioxidant products using DPPH-assay guided approach" Institute of Applied Research, The Galilee Society, Shefa Amr, Israel.	Visiting Researcher
May 2017- July 2017	Execution of a project entitled "Bioproducts from pistachio shells" Laboratório Nacional de Energia e Geologia (LNEG), Unidade de Bioenergia, Lisbon, Portugal.	Visiting Researcher

PROJECTS

2018- Present	Pilot Scale Dried Apricot Production with Combined Solar and Radio Frequency System and Optimum Process Design: Pretreatment (sulfuring/extract application), Drying and Storage	TUBITAK 1003
2018 - 2019	Effect of Ultrasound and/or Microwave-Assisted Pretreatments on the Extraction of Lignin, Cellulose and Hemicellulose from Pistachio Shell	TUBITAK 1002
2013 - 2015	Production of modified lipid with incorporation of hydrophobic and essential amino acids into refined olive pomace oil and its characterization	TUBITAK 1001

PUBLICATIONS

Papers Published in International indexed Journals

1) **Özbek, H. N.**, Koçak Yanık, D., Fadıloğlu, S., Göğüş, F. (2020). Ultrasound-assisted alkaline pre-treatment and its sequential combination with microwave for fractionation of pistachio shell. Renewable Energy, 157, 637-646.
2) **Özbek, H. N.**, Halahlih, F., Göğüş, F., Koçak Yanık, D., Azaizeh, H. (2020). Pistachio (Pistacia vera L.) hull as a potential source of phenolic compounds: Evaluation of ethanol–water binary solvent extraction on antioxidant activity and phenolic content of pistachio hull extracts. Waste and Biomass Valorization, 11(5), 2101-2110.
3) **Özbek, H. N.**, Koçak Yanık, D., Fadıloğlu, S., Göğüş, F. (2020). Optimization of microwave-assisted extraction of bioactive compounds from pistachio (Pistacia vera L.) hull. Separation Science and Technology, 55(2), 289-299.
4) **Özbek, H. N.**, Fockink, D., Koçak Yanık, D., Göğüş, F., Lukasik, R. (2018). The green biorefinery concept for the valorisation of pistachio shell by high-pressure CO_2 /H_2O system. Journal of Cleaner Production, 196, 842-851.
5) **Özbek, H. N.**, Koçak Yanık, D., Fadıloğlu, S., Keskin Çavdar, H., Göğüş, F. (2018). Microwave-assisted extraction of non-polar compounds from pistachio hull and characterization of extracts. Grasas y Aceites, 69(3), 260.

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