UTILIZATION OF WINE WASTE FOR FERMENTATIVE PROCESSES

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ABSTRACT

UTILIZATION OF WINE WASTE FOR FERMENTATIVE PROCESSES

Grape pomace is generally considered as most valuable waste of winemaking process. Two different grape types of Syrah (red) and Muscat (white) were collected in the middle of the harvest season after pressing steps of both red and white wine making process Carbohydrate content of grape pomace was hydrolysed by enzymatic and acid hydrolysis. After screening possible fermentable sugars of grape pomace lactic acid fermentation were performed from grape pomace suspension and liquid extract phase.

Lactic acid fermentation by *Lactobacillus casei* showed that grape pomace can be used as a substrate for lactic acid production. Different solid loadings and yeast extract concentrations effect the lactic acid production yield from grape pomace.

Enzymatic hydrolysis was performed to hydrolyse pectin, cellulose and hemicellulose of grape pomace. Commercial pectinase, cellulase and β-glucosidase were supplemented into grape pomace suspensions at different concentrations. Maximum hydrolysed glucose and xylose from extracted solid phase of grape pomace were calculated as 8.93 ± 0.21 and 4.52 ± 0.11 % of total solid. Furthermore, acid hydrolysis showed that two stages acid hydrolysis is more efficient in releasing glucose from extracted solid phase of grape pomace but dilute acid hydrolysis is also more efficiency on hydrolysis of xylose and arabinose.

Exo-polygalacturonase production from grape pomace was conducted using different filamentus fungi, namely *Aspergillus sojae, Rhizopus oryzae* and *Aspergillus niger* but no significant enzyme activity was obtained.

Maximum 84 % of fermentable sugar in dry grape pomace was converted to lactic acid by *L. casei*. Effect of yeast extract researches designated that commercial yeast (bakers' yeast) can be used as nitrogen source instead of yeast extract and 10 g/l of yeast extract was the most suitable concentration for lactic acid production from grape pomace by *L.casei*. This study showed the potential of the grape pomace for fermentative processes.

ÖZET

ŞARAP ATIKLARININ FERMANTASYON SÜREÇLERİNDE DEĞERLENDİRİLMESİ

Üzüm posası şarap atıklarının en değerlisi olarak kabul edilir. iki farklı çeşit olan Syrah (kırmızı) ve Muscat (beyaz) üzümleri kırmızı ve beyaz şarap yapım işlemlerinin pres aşamasından sonra toplanmıştır. Üzüm posasının karbonhidrat içeriği enzimatik ve asidik hidrolizlerle incelenmiştir. Fermantasyon sırasında kullanılabilecek şeker içeriğinin belirlenmesi sonrasında, üzüm posasından laktik asit üretimi araştırıldı. Kurutulmuş ve öğütülmüş üzüm posasının ekstraksiyonu sonucunda elde edilen sıvı kısım da mikroorganizma için besin maddesi olarak kullanıldı.

Enzimatik hidroliz işlemi üzüm posasının pektin, selüloz ve hemiselüloz kısımlarını parçalamak amacıyla ticari enzimlerle incelendi. Pektinaz, selüllaz ve β-glikozidaz enzimleri değişik hacimlerde üzüm posası süspansiyonuna eklenmiştir. 500 µl pektinaz, 500 µl selülaz ve 50 µl β-glikozidaz ile yapılan hidroliz sonrasında yapılan analizlerde sıvıya geçen glikoz ve ksiloz yüzdesinin toplam katının en çok % 8.93 ± 0.21 ve 4.52 ± 0.11 ini oluşturduğu ölçülmüştür. Ayrıca asit hidrolizi sonuçlarına gore 2 aşamalı asit hidrolizinin ekstrakte edilmiş katı kısımdan glikoz ayrıştırmada seyreltik asit hidrolizine göre daha verimli olduğunu göstermiştir. Ancak seyreltik asit hidrolizi de 2 aşamalı asit hidrolizine göre ksiloz ve arabinozu daha verimli ayrıştırabildiği görülmüştür.

Aspergillus sojae mutant and *Aspergillus sojae WT*, *Rhizopus oryzae*, *Aspergillus niger* gibi birçok mikroorganizma ile üzüm posasından exo-polygalacturonase üretimi çalışılmıştır. Ancak yapılan analiz sonuçlarına göre dikkat çekici bir aktivite görülememiştir.

En dikkat çekici sonuçlar laktik asit üretiminden elde edildi. Üzüm posasındaki basit şeker formlarının % 84 ünün *Lactobacillus casei* tarafından laktik aside dönüştürülebildiği görülmüştür. Maya özütü ve ticari pasta mayalarının üzüm posasından laktik asit üretimine etkisi incelendi ve 10 g/l değerinin en uygun maya özütü değeri olduğu, ayrıca ticari pasta mayalarının da maya özütü yerine kullanılabileceği sonucuna varılabilmektedir. Bu çalışma potansiyel olarak üzüm posasının fermantasyon işlemlerinde değerlendirilebileceğini belirtmiştir.

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CHAPTER 1

INTRODUCTION

Wine making process is one of the most historical applications of biotechnology. The earliest known wine production may have been in region of Iran as long ago as 6000 BC. Wine has been around for thousand years and from ancient times to modern times has been enjoyed by many folks. After centuries, this process without any knowledge combined with technological development and became a larger industrial area. Therefore the requirement of raw material also increased.

In terms of organic chemistry, wine is a complex mixture of a large number of compounds including carbohydrates, alcohols, aldehydes, esters, acids, proteins and vitamins. It is also home to a number of polyhydroxy aromatic compounds, such as tannins, anthocyanins and flavonols, which contribute hugely to colour and taste. The basic raw material for a wine fermentation is a fermentable sugar, such as fructose or sucrose, rather than the less soluble, non-fermentable starch, which is the raw material for most beers (Hornsey 2007).

Generally wine is produced from grapes, honey, grains, rice and sugarcane. Depending on the cultivation conditions of the region, one of these ingredients can be fermented up to ethanol which is the most desirable chemical compound in alcoholic beverages. Conversion of sugar to ethanol finishes with having a liquid phase that contains ethanol. Starting with solid phase to obtain alcoholic phase generally needs separation, discharging and sedimentation steps. In winemaking process, it is possible to have stalks, pulp, skin and lees. Most of them can be called as a waste for wineries but reducing sugar, cellulose, hemicellulose and pectin content shows us that can be called as a substrate for different biotechnological pathways.

Discharging of winery waste to soil is a different concern for environment. According to recent studies, germination properties of soil are inhibited by discharging of winery wastes because of the biological oxygen demand (BOD), carbon and phenolic compounds. Grape pomace is the major waste generated in the winemaking process and the utilization of its components, such as skins, pulp, stalks and seeds, have an important environmental impact in waste reduction and permit the production of added

value products (Bail et al., 2008; Spigno et al., 2008; Ping et al., 2011a,b; Prozil et al., 2012b) Generally grape pomace is used as fertilizer, animal feed or extraction raw material of seed oil and polyphenols. Limited need of these compounds cannot be a solution for waste treatment of viticulture. Also, most of the winery owners and winemakers surprise with the disasters on grape-vines after discharging winery wastes nearby the vineyards as a fertilizer because of the lack of knowledge. On the other hand, utilization of winery waste is promising in a light of new biotechnological applications. Reducing sugar content can be extracted from red or white grape pomace. Also, complex carbohydrates (cellulose, hemicellulose and pectin) participate in grape pomace can be hydrolyzed up to reducing sugar by different methods as extraction, acid hydrolyses and enzymatic hydrolyses.

Different types of grapes were used to compare red and white grape pomace. Red wine making process starts with alcoholic fermentation of grape skin and pulp together without pressing. White wine making process starts with the pressing and then liquid phase is processed to alcoholic fermentation.

The main purpose of this study is to assess the carbohydrate content of grape pomace and develop a profitable method for conversion of grape pomace into cheap nutrients for fermentation media. According to this purpose lignocellulose composition of grape pomace is investigated and required pre-treatments were applied in order to obtain more monosaccharide from cellulose, hemicellulose and pectin content of grape pomace. After all analyses, it is obviously seen that winery waste still contained monosaccharide on it that can be extracted by hot water extraction. Also, lignocellulose composition of winery waste needs pre-treatments for utilizing as a carbon source, but the results of these steps can be profitable with very controlled processes.

CHAPTER 2

GLOBALWINE and GRAPE PRODUCTIONS

2.1. Global Wine Production

Wine production is one of the biggest alcoholic beverage industry since was discovered. Palaeolithic man was probably the first to become familiar with wine, purely by the accidental 'spoilage' of stored or over-ripe grapes. Wine may, of course, have been the result of unsuccessful attempts to store grape juice, which is a particularly unstable beverage (Hornsey 2007). Of course when was discovered it did not seem so complicated but, after evolution of a science showed us that it is more than we know. Still its chemistry is not completely understood.

After centuries, wine had been produced all over the world. Countries from different continents are now in a competition of wine production. France, Italy, Spain, United States and Argentina are major wine manufacturers around the world. Generally wine making process is similar but grape types, soil characteristics, weather conditions during season, geographical positions and cultural practices make wines different from each other.

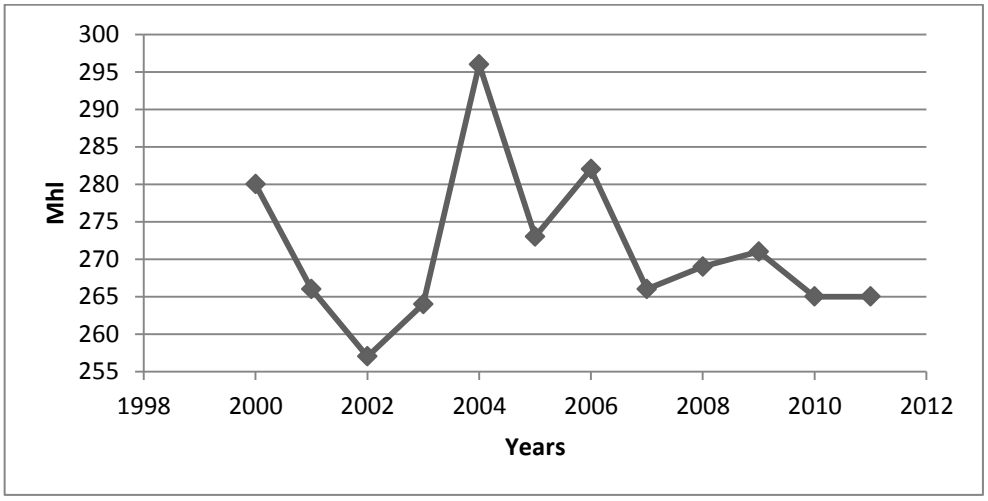

Figure 2.1 World production of wine
(Source: OIV 2011 report)

According to International Organisation of Vine and Wine reports (OIV) world production of wine has decreased about 15Mhl in 2011 that 2000.

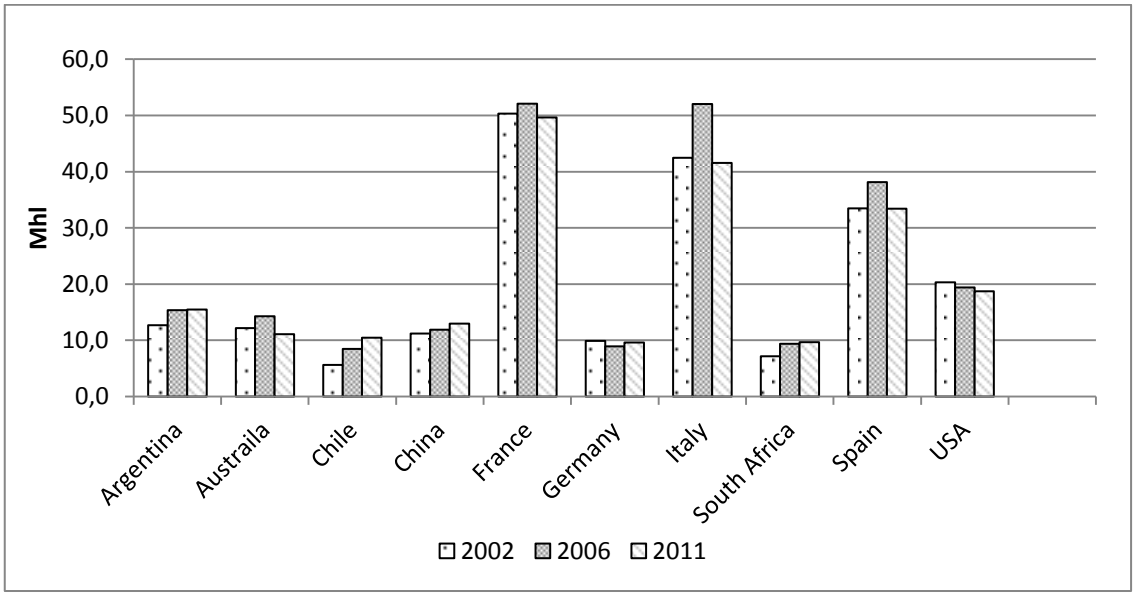

Figure 2.2 Top 10 wine producers around the world
(Source: OIV 2011 report)

As it can be seen from Figure 2.2 France, Italy and Spain are one of the most important winemaker countries. Total wine produced from these three countries is more than %50 of world's total production. Also, it is possible to understand that five major wine producer countries have decreased their production in five years period possibly because of the economic crisis around the world and the raising taxes in most of these countries from alcoholic beverages.

Table 2.1 Wine production amount (million hl)
(Source: OIV 2011 report)

	2003	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
Argentina	13.2	15.5	15.2	15.4	15.0	14.7	12.1	16.3	15.5
Australia	10.8	14.7	14.3	14.3	9.6	12.4	11.7	11.3	11.1
Chile	6.7	6.3	7.9	8.4	8.2	8.7	10.1	8.8	10.5
China	11.6	11.7	11.8	11.9	12.5	12.6	12.8	13.0	13.0
France	46.4	57.4	52.1	52.1	45.7	42.7	46.3	45.7	49.6
Germany	8.2	10.0	9.2	8.9	10.3	10.0	9.2	6.9	9.6
Italy	41.8	49.9	50.6	52.0	46.0	47.0	47.3	48.5	41.6
S. Africa	8.9	9.3	8.4	9.4	9.8	10.2	10.0	9.3	9.7
Spain	41.8	43.0	37.8	38.1	34.8	36.2	35.2	35.2	33.4
USA	19.5	20.1	22.9	19.4	19.9	19.3	22.0	20.9	18.7

The top five wine producers in the world in 2011 were France (49.6 million hl), Italy (41.6 million hl), Spain (33.4 million hl), the United States (18.7million hl) and Argentina (15.5 million hl). Italy surpassed France as the largest wine producer in the world in 2008. Poor weather conditions (mild winter, late spring frost and excessive humidity in spring and summer) and decreased land under vine are largely responsible for the drop in production in France. By contrast, the good weather conditions that prevailed in Italy helped vineyard yields improve after having fallen significantly in 2007 (FAO report 2011). But after in three years Italian wine production decreased about 15% and France took the first stage in global wine production with 15% rate of increase.

2.2. Global Grape Production

Grapevine is the most valuable horticultural crop in the world. The majority of the fruit is processed into wine, but significant portions of the worldwide crop are destined for fresh consumption, dried into raisins, processed into non-alcoholic juice, and distilled into spirits. Significant grape acreage exists on all continents of the globe, save for Antarctica. Worldwide estimates are that approximately 8 million hectares are currently planted to grapevine and more than 60 million metric tons of fruit are produced annually (FAO production statistics) (Owens 2008).

Grapes are grown more than 80 countries of the world with different purposes. Asian acreage generally serves as table grapes and raisins. Leading countries for production of table grapes and raisins are China, Turkey and Iran (OIV report 2011). As it can be understood from Table 2.1 these countries do not produce significant wine than European and American wine producers even they produce approximately quarter of the total world grape. Spain, France and Italy have greatest grape production for wine making and they produce approximately 130 mhl wine with 25 m tones of grape (OIV report 2011).

The fruit, a berry, is essentially an independent biochemical factory. It is primarily composed of water, sugars, amino acids, minerals, and micronutrients. The berry has the ability to synthesize other berry flavor and aroma components that define a particular berry or wine character. The berry is a commercial source of tartaric acid and is also rich in malic acid. Cultivation is easiest in a Mediterranean type climate with

hot dry summers and cool rainy winters, however grapevines are grown throughout the world's temperate climates.(Riaz, Doligez et al. 2007)

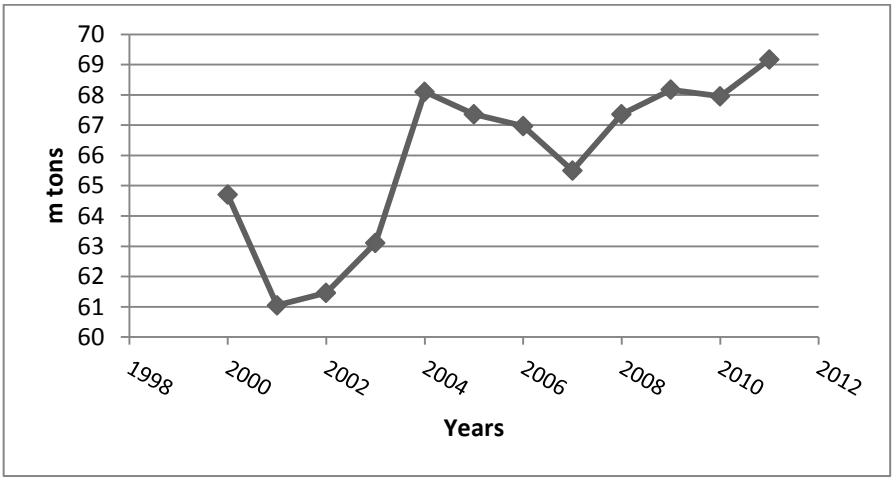

Figure 2.3 World production of grape
(Source: OIV 2011 report)

According to the OIV Statistical report on vitiviniculture 2011 European vineyards are in first stage with 2.85 mha, Asian vineyards and American vineyards are following with 1.36 mha and 1.16 mha.

Grape is also consumed as a table grape (fresh consumption) and a raisin. 22 m tons of grapes are consumed as fresh consumption by China, India, Turkey, Iran and Italy which are the major table grape producers around the world. Also, 12 m tons of grapes are consumed as raisins by Turkey, USA, Iran, Chile and South Africa which are the major raisins producers around the world (OIV report 2011).

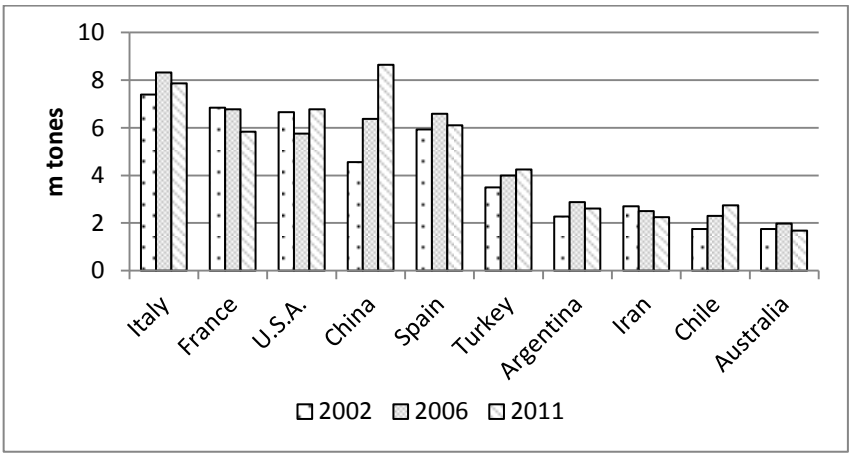

Figure 2.4 Top 10 grape producers around the world
(Source: OIV 2011 report)

CHAPTER 3

WINE MAKING PROCESS

Wine is an ancient drink that has been an important part of human societies for literally thousands of years. From its origins in ancient Greece, wine culture and the art of wine making spread throughout the ancient Mediterranean, Europe, and China. Today, wine is consumed on every continent in the world, and mainly produced in Europe, the Americas, South Africa, Australia, and New Zealand. The process of wine making has evolved throughout the centuries, and today there are thousands of wineries producing hundreds of varieties of wines.

Wine making process (Vinification) is basically a biotechnological process that transforms sugar in grape into ethanol. Yeast and appropriate fermentation conditions can provide this process happen. But in wine making process generally transformation of ethanol is not enough to obtain qualified or drinkable wine. There are lots of wine making techniques in order to combine aromatic compounds and alcohol. Most qualified wines are in balance of acidity, sugar, alcohol and phenolic compounds. There is no easy way to obtain this balance and wine making techniques are based on different biotechnological, chemical and physical methods. Enology is often defines as the science of winemaking, but in practice it combines the science, technology and engineering of the process. It is combination of interdisciplinary knowledge and principles (from chemistry, biochemistry, microbiology, chemical engineering and nutrition) which we consider to be the essence of enology (Boulton, Singleton et al. 1996).

Wine is classified in three major categories: table wines, sparkling wines, and fortified wines. Table wines, also called still or natural wines, are consumed mostly with food, they tend to compliment the meal. Sparkling wines, for example champagne is distinguishable by its effervescence and is drunk for the most part on festive occasions such as weddings, birthdays, and during the holidays. Fortified wines, such as sherry or vermouth are most commonly drunk before or after meals and it is also frequently used in cooking.

Table wines are further classified by color as red, rose and white. Three of them basically have same production methods but they have some differences. Red wine production has a maceration step which takes 15-25 days with a skin contact that provides extraction of phenolic compounds into liquid phase before pressing.

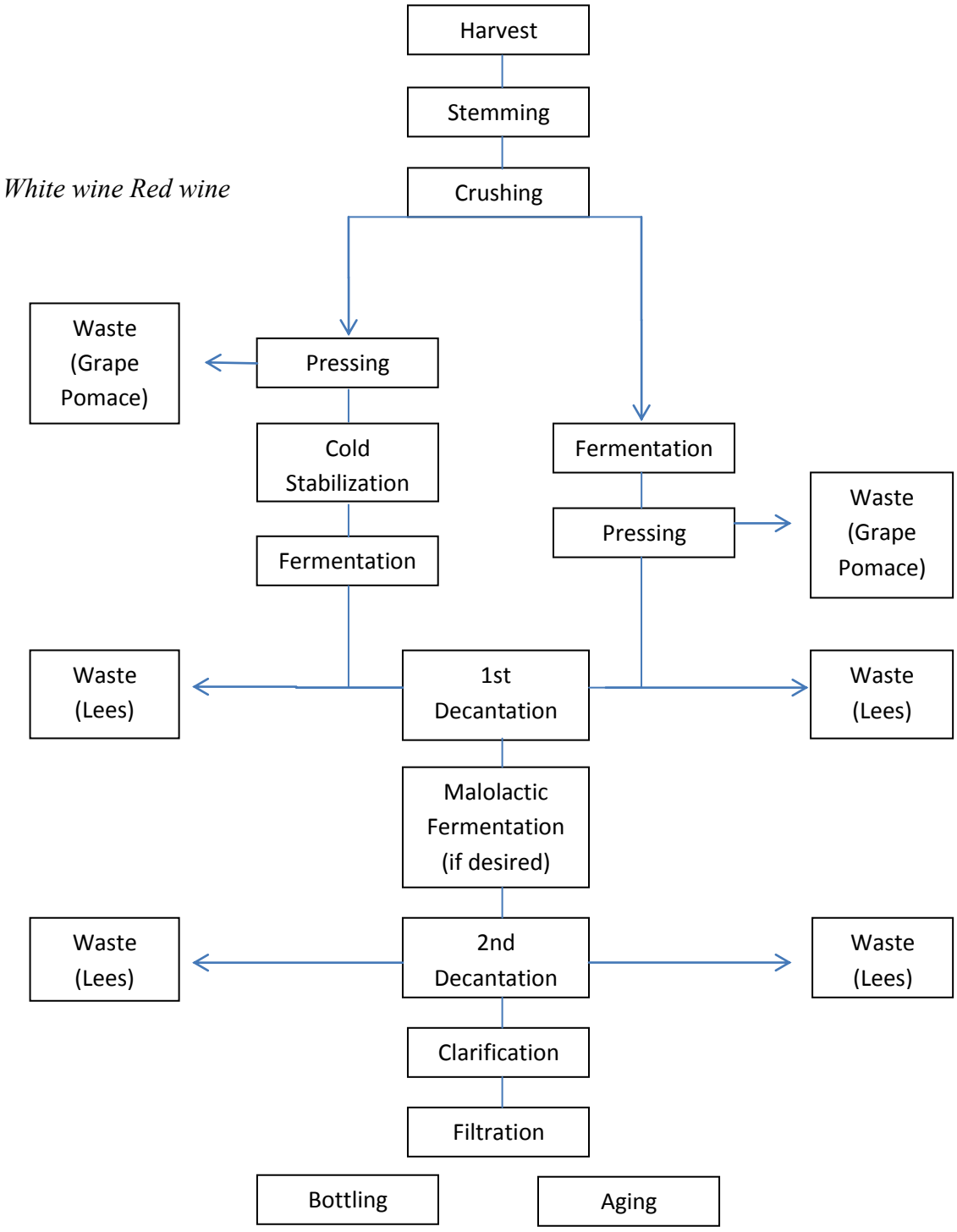

Figure 3.1. Winemaking flow chart
(Source: Arvanitoyannis et al. 2006)

3.1. Major Process Steps of Wine Making

3.1.1. Crushing and Destemming

Crushing is employed to cause berry breakage and juice release from the grapes, and ordinarily 100% of berries will be broken. It is the beginning of the juice, skin, pulp and seed contact that will influence the extent of extraction from these grape components. A secondary aspect of crushing process is the elimination of the stems from the juice and skins and the isolation and collection of them to disposal. Stems are often shredded and dispersed throughout the vineyard, dumped as solid waste or incinerated. Under some conditions partial stem removal or addition of some stems back to the must is practiced. However complete removal is generally sought (Boulton, Singleton et al. 1996).

3.1.2. Fermentation

The next major step is the fermentation, in which the fermentable sugars (glucose and fructose) present in the grape juice (including any added sugar) are converted by yeasts into ethanol (ethyl alcohol) and carbon dioxide, with the generation of heat. To an extend that depends on the temperature; the fermentation also produces many of the aromatic characteristics of the finished wine. The fermentation is usually carried out in large, closed stainless-steel tanks, which are temperature controlled so as to lower the fermentation temperature as appropriate.

Yeasts are unicellular microorganisms that are classified taxonomically as fungi. Yeasts have several commercial applications, and they are used also for beer brewing, baking and biomass production. Yeasts used in winemaking generally belong to the *Saccharomyces* genus, the most important species of which, *cerevisiae*, has some unique characteristics- perhaps one of the most useful ones being its tolerance to ethanol (up to 15% v/v), a very toxic compound for most other microorganisms (Clarke R.J. 2007).

Red wines are fermented between 18-35°C in the presence of the skins for 3–6 days, depending on the intensity of color (anthocyanin) and dry flavor (tannins) desired. The partially fermented must is then decanted and pressed from the skins, and a

secondary slower fermentation carried out to the extent required (Hocking 2005). Temperatures required for white wine fermentations are generally lower (rarely above 20°C) than those used for red wines, so that there is some survival of fruity esters. Hence, temperature control during white wine fermentation is much more critical. Chaptalisation is practiced by some white winemakers, but not as frequently as is necessary for red wine production. Many white wines are not fermented out to complete dryness (i.e. they contain residual sugar), and this is best achieved by halting the fermentation, by either rapid chilling or yeast removal. After fermentation is deemed to be complete, the wine- maker has to decide whether extended lees contact and malolactic fermentation are required(Mendes, Prozil et al. 2013).

3.1.3. Pressing

Pressing the grape mass (pomace) occurs after the free-run wine has been removed from the fermentation vat, and takes place when the winemaker decrees that the required amounts of color, flavor and tannin have been extracted. The timing can vary from 2 days to 3 weeks post-fermentation, according to wine style. Some wineries consistently leave the wine in prolonged contact with skins (and, sometimes, seeds and stalks) after fermentation has been completed, usually for a period of 2 or 3 weeks. This practice, which was at one time a characteristic of Bordeaux wines, is called ‘extended maceration’, and can often have a pronounced effect on the wine, increasing phenolic content and diminishing color. There is also some evidence that wines produced in this way have a better ageing capability (Hornsey 2007).

White wine production starts with a juice extraction by pressing immediately after crushing and draining of the grapes. Part of the juice runs out of the crushed grapes (free run juice) without added pressure and is followed by immediate pressing. Sometimes white grapes are not crushed, but immediately pressed to minimize extraction of compounds from the skins, seeds or stalks. The fermentation is carried out on the must or grape juice without the skin or pomace (Vincenzi, Marangon et al. 2011).

Grape pomace obtained from pressing step will need to be removed and taken from winery in order to avoid the microbial growth in place. The most common means by which this is done is the use of belt or screw conveyors. These are often fixed in place, but in small wineries can be portable and moved into place as needed. In larger

wineries, it is more usual to transfer pomace by a series of interconnecting screw conveyors that feed a group of presses and have a common dumping system (Boulton, Singleton et al. 1996).

3.2. Winery Wastes

Winemaking process generates different types of solid or liquid wastes. They can be characterized by high content of suspended solids or biodegradable compounds. After different winemaking steps sediments from recursive clarification steps, plant remains after de-stemming step, pomace from pressing, lees and seed can be obtained. The wastewater generated from decantation steps consist of dead yeasts, grape pulp, seeds and lees. Despite Spain is not the major wine producer, has a biggest role in wastewater produced from wineries with 2 million m^3wastewater every year (Bustamante, Paredes et al. 2005).

As mentioned in Figure 2.5 pressing and decantation steps are the main steps that winery waste obtained from winemaking process. More than 25% of wine waste is produced at these steps(Arvanitoyannis, Ladas et al. 2006). Every 25-30 kg of 100 kg grape end up after vinification process as stems, seed, lees and pomace. Stems and seed are also waste generated but, grape pomace and lees are most valuable by-products of winemaking by the meaning of media or substrate for microbial activities. Different names can be given those major wastes of winemaking considering to their physical and chemical characteristics. Some of the definitions are given below including concerning steps.

3.2.1 Lees

The definition of wine lees given by EEC regulation No. 337/79 states that ‘‘wine lees is the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product”. Lees generally is disposed as wastewater from wineries. After different decantation steps lees generally settle at the bottom of the tanks or barrels when the supernatant wine separated from the lees.

Table 3.1Concentration of Organic Compounds in Less (g/l)
(Source:Bustos et al. 2004)

Lees	glucose	ethanol	lactic acid	acetic acid
Lees from pressed grape	0.4 ± 0.1	61.9 ± 1.9	4.3 ± 0.4	1.5 ± 0.2
White lees. first decanting step	1.4 ± 0.2	80.9 ± 3.5	5.0 ± 0	2.4 ± 0.3
White lees, second decanting step	0 ± 0	55.9 ± 0.8	5.2 ± 1	1.6 ± 0.2
Red lees, first decanting step	0.1± 0.1	74.5 ± 2.2	3.3 ± 0.2	1.3 ± 0.1
Red lees, second decanting step	0 ± 0	63.5 ± 1.5	11.4 ± 0.8	6.6 ± 0.5

Wine lees are generally 5% (v/v) of total wine produced at the end of the process (Moldes, Vázquez et al. 2008). According to the wine making plan, lees obtained steps can be multiplied. Because of winemaking is a biotechnological method, organic compounds concentrations of lees vary up to decantation steps. It is also possible to recover 4-8 L of 96° ethanol, 8-12 kg of calcium tartrate and 8-10 kg of protein that has 2.4-4.0 kg of crude protein form from 100 kg of fresh lees (Solanes et al. 1988).

3.2.2. Grape pomace

Generally grape pomace defines as solid residue after juice and wine making processes. Grape pomace is also a fibrous material that consists of processed skins, seeds and stems. Wine making processes for white and red grapes are different from each other. Figure 3.1showed that red grape pomace generated after pressing step when is 2-3 weeks after fermentation starts. But white grape pomace is directly racked to press with pumps without skin contact in white wine making process.

Table 3.2 Chemical composition of grape pomace (GP)
(Source:Zheng et al. 2012)

Component	Red GP (wt% ,dry basis)	White GP (wt% ,dry basis)
cellulose	14.5	9.2
hemicellulose	10.3	4
pectin	5.4	5.7
lignin	17.2	11.6
protein	14.5	7
water soluble sugars	2.7	49.1
total C	48.2	44.3

The carbohydrate composition of grape pomace is a potential source of fermentable sugars that can be utilized in different fermentation processes. As it is clear from Table 3.2 that grape pomace has large amount of cellulose, hemicellulose, pectin and lignin content. Grape pomace consists of four major polysaccharides groups. Cellulose consists of glucose subunits; hemicellulose consists of glucose, xylan, mannose and arabinose subunits. Starch also consists of glucose subunits but it serves as an energy source in plants while other complex carbohydrates are not in use. Pectin consists of D-galactronic acid subunits. (Korkie, Janse et al. 2002)

CHAPTER 4

FOOD and AGRICULTURAL WASTES

For developed countries industrialization has a key role on maintaining the economic and environmental system for the modern citizen life circle. Because of overpopulation all around the world, faster and more efficient systems have taken place in order to meet energy, food and technological demands of humanity. Industrial production contributes goods, services and jobs but it is also major reason of pollution and waste.

According to the United Nation's future projections world global population will increase about 9.5 billion people by 2050. Population rates will be different than each other depends on geographical and economic reasons that Europe's population will be decline, Africa will be double and India will reach up to the population of China by 2030. Looking ahead it is not hard to imagine problems that world will face with. Population and consumption growth will be the main reasons for risk of hunger (Godfray, Crute et al. 2010). In order to feed all world population food production should increase about 30-50% (Smil 2005). That means requirement of more supplies in order to meet the demand of energy and food will occur. Considering the reaction of nature to extra 3 billion people, increasing interests on different technologies and improvements will peak.

Today about 4 billion metric tons of food produced for human consumption per a year. Due to poor harvesting, processing activities and consumer wastages about 30-50% of food never reaches to human consumption. Furthermore there is also large amount of wastewater, fertilizers and lands have been lost for production that amount of food. Producing food that will not be consumed also determines unnecessary CO_2 emission which is the major effect of the global warming (Global Food Waste not Want not).

Comparatively waste generated from agricultural process is generates in more concentrated manner which also can collected or utilized easier than consumer wastes of food. Problems associated with such waste generally include;(Lin, Pfaltzgraff et al. 2013)

- High chemical oxygen demand (COD) and biological oxygen demand (BOD)

- Varying pH and the chemical composition due to seasonal changes in food industry
- High risks of microbial contaminations
- High accumulation rate leads the disposal problems.

4.1. Key Facts about Food Waste

- UK household waste is about 6.7 million tons of food every year. Around one third of 21.7 million tons of food is purchased by UK government.
- Nearly half of food (46%) thrown away is in fresh, raw or minimally processed. 27% of total food cooked or prepared for human consumption and 20% of ready-to eat food thrown away.
- 1.2 million tons of food thrown away in its package material either opened or unopened.
- Most of the starch based food thrown away. 45000 tons of rice, 33000 tons of pasta and 105000 tons of potato thrown away in UK every year (WRAP 2008).
- 13-15% weight of rice is lost during post-harvest activities in Asia (Grolleaud 2002).
- 20% of total fruits and 30% of total vegetables produced in Egypt is lost after harvesting (Blond 1984).
- If all the wasted food could have been eaten, the benefit would be equal to take 1 of 5 cars from traffic (http://england.lovefoodhatewaste.com/content/facts-about-food-waste-1).

Waste generally defines in different formal and research papers. Food waste occurs at different stage of food supply chain. According to these different production and consumption steps, waste can be defined separately. Most of the agricultural wastes can be used as a substrate for microbial productions and nutrition values can be separated for food additives. Also food crops can be used to meet human vital requirements directly and can be diverted into feeding livestock, different by-products and biodiesel. There are some different waste definitions described in separate research areas as;

- Edible materials intended for human consumption that are discharged, lost, degraded or consumed by pests (FAO 1981).
- As definition1 but including agricultural materials that used for animal feeding or by-products of food processing activities (Stuart 2009).
- As definition 1 and 2 but including the interval between energy consumption of food per capita and energy consumption of food needed per capita (Smil 2004).

4.2. Agricultural Wastes

Agriculture and industry have been traditionally viewed as two different sectors in terms of their characteristics and role in economic growth. Agriculture has a key role in civilization for human being and also after thousands of years it is still indispensable need for humanity. Instead of having to hunt and gather food, early humans learned to grow their food and life became easier for them to generate. Along the development path, increasing population stimulated the development of industrialization and after centuries agriculture needs industry in order to meet the increasing food demand (FAO The State of Food and Agriculture 1997 Part 3). Over population, global warming and scarcity of fossil sources forced the industrialization to development very fast and competitive with itself. Global over population became major reason to overcome energy requirements. Our society faced a mortal problem that has never been faced before. These circumstances drive the industry to find new ways like waste treat management, recycling systems, renewable sources(Lin, Pfaltzgraff et al. 2013).

Agricultural wastes are generated during food processing from animal derived or plant derived products for human consumption. Globally 140 billion metric tons of biomass is generated in a year. Most of this amount is used as animal feed or burned. When considering sugar, protein, carbon and mineral content it is hard to describe as a waste. The presence of carbon source in these wastes provides suitable conditions reuse these valuable compounds in other processes. Table 4.1 indicates some of the major agricultural waste types with volumes generated per year from different geographical locations around the world (Mussatto et al. 2012).

Table 4.1‘Food supply chain waste’ mapping.
(Source: Pfaltzgraff et al. 2013)

Waste type	Volume / year (metric tons)	Geographical location
Olive mill residue	30.000.000	Mediterranean basin
Waste vegetable oil	50.000-100.000	U.K.
Food waste	89.000.000	E.U.-27
Sugarcane bagasse	194.620.000	Brazil
Grape pomace	15.000.000	USA
Corn residue	42.000.000	Brazil
Apple pomace	4.000.000	Global
Rice straw	731.000.000	Global
Barley straw	58.000.000	Global
Citrus fruit processing	15.6000.000	Global

4.2.1. Extent of Agricultural Wastes

Roughly one-third of food produced for human consumption is lost or sorted out which is about 1.3 billion ton per year (FAOSTAT 2012). Food weight reduced from harvesting in farm to final consumer due to different reasons and effected by technological, geological and social difficulties. Beside these reason industrial development decreased the accessibility to food products for most of the people around the world. According to the data from FAO Statistical Yearbook 2012 food availability has reduced to 2790 kcal/person/day from 2200kcal/person/day in 60 years. The need of avoid waste accumulation and find new renewable sources for increasing energy demand forced industry and science to improve energy production from .

Waste is major issue over the world. Most of the industries have their own residues to dispose to open areas, seas, rivers. This accumulation is a reason of finding new technologies to utilize most promising, less complex agro-industrial wastes as substrates. Food and Agricultural Organization of the United Nation distinguished five system boundaries in plant-derived commodities that effect food supply chain(Gustavsson 2011).

- **Agricultural production**; loses due to mechanical damage or harvesting processes.

- **Post-harvest, handling and storage**; including losses due to spillage and degradation during handling, storage and transportation between farm and distribution.
- **Processing**; losses and wastes obtain after processing steps or sorted out parts which are not suitable for production.
- **Distribution**; including wastes and losses in market system while transporting
- **Consumption**; including domestic residue and losses during consumption by consumers at household level.

Processing operations can be categorized as plant-derived and animal-derived.In a light of data from AWARENES report plant-derived waste has a higher proposition (%63) than animal waste. Food production can be classified into two major steps: pre-consumer and post-consumer. Pre- consumer part includes agricultural wastes, post-harvest and processing. Post-consumer part represents distribution and consumption parts. Pre-consumer division has a higher proportion when considering improved food production industry. Figure 4.1 indicates kg of food losses and wastes at consumption and pre-consumption stages per capita in different regions.

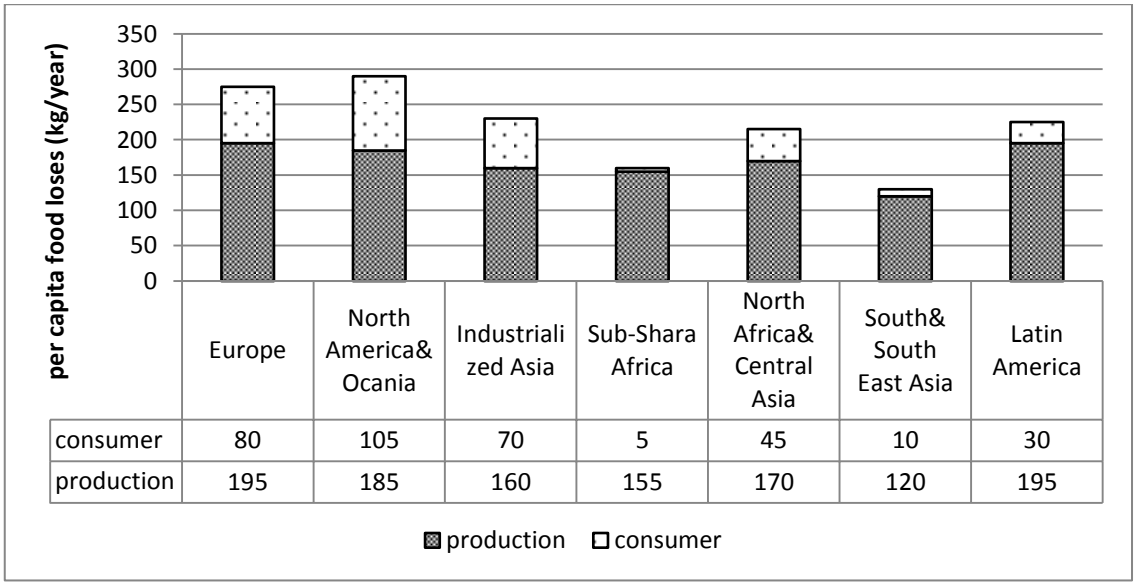

Figure 4.1 Per capita food losses and wastes (kg/year)
(Source:Gustavsson 2011)

Figure 4.1shows that per capita food losses and waste in North America & Oceania and Europe is about 270-290 kg/year. Waste generated per capita at

consumption step is also about 180-190 kg/year which is more than production losses of South Asia. Latin America and Europe production wastes per capita are close to each other with 190-195 kg/year. Industrialized Asia and Latin America have same amount of food losses and wastes but in term of production losses Latin America generate more than Industrialized Asia. As can be seen from the graph, industrialized regions produce consumption losses per capita than developing or undeveloped regions. Also it is possible to expound as industrialized regions generates more losses at both consumption and production stages that undeveloped or developing regions. One of the dominant crops in South Asia is rice and harvesting, post-harvesting and handling processes generate large amount of food waste because of technological or economical defects. Figure 4.1 also tells us the percentage of edible parts of wasted or discharged food produced for human consumption.

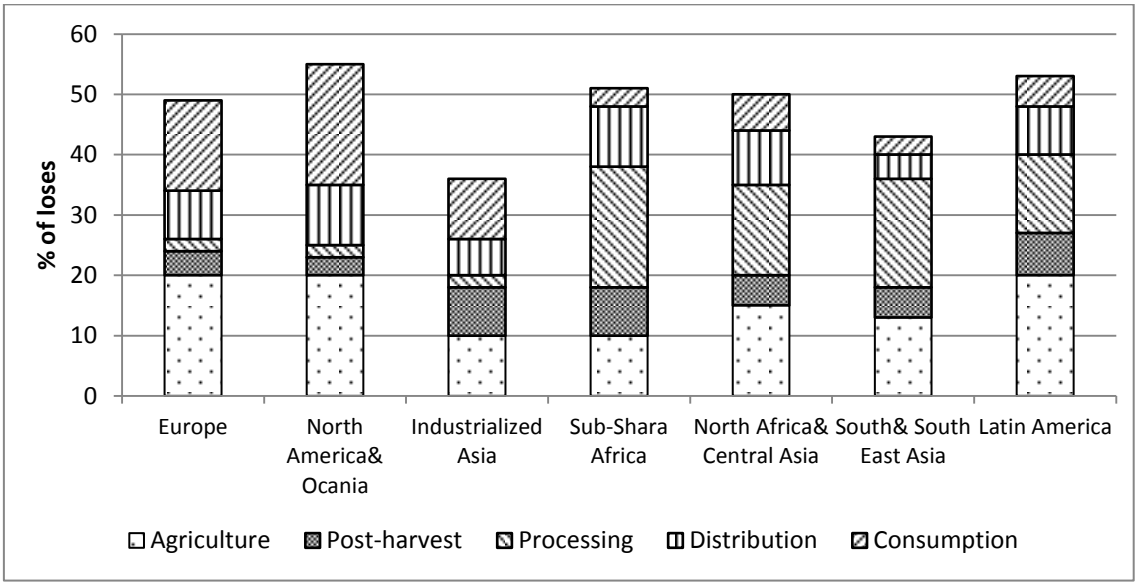

Figure 4.2 Percentage of production losses and wastes for fruit and vegetable derived production in different regions (Source: Gustavsson 2011)

In the *fruit and vegetable* group, that is the dominant food losses and wastes all around the world, harvesting effects and agricultural losses have a great importance considering all food wastes and losses. Figure 4.2 indicates that processing step generates big amount of losses for fruit and vegetable derived production in Africa, Asia and Latin America. In all three industrialized regions (Europe, North America and Industrialized Asia) processing step does not have same importance as in non-industrialized region but agricultural wastes and consumption losses are at the first stage of food losses.

In the *oil crops and pulses* commodity group, sunflower seed and rape seed are the major crop supplies in Europe, while soybeans are the major crop supply in North America and Oceania and Industrialized Asia. Losses in industrialized and undeveloped regions are relatively large during agricultural production, contributing waste percentages between 6 and 12% during harvest.

In the *roots and tubers* group, potato (sweet potato in China) is the major crop supply in industrialized and undeveloped regions. Results indicate that main losses in production processes occur at agricultural activities. Technological advantages can limit these production losses but waste treatment and clean technology has not still been widely acknowledged.

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CHAPTER 5

MATERIALS and METHODS

5.1. Materials

Grape pomace (GP) was collected from Urla Winery in İzmir in September 2012 and kept at -80°C. Two different types of GP Muscat and Syrah as white and red grape varieties, respectively, were used in this study. GP was dried in the drying oven at 60°C for 24 hours. Dry GP was milled with small kitchen grinder and undesired materials such as seeds and stem were separated from GP.

Chemicals are given below that are used for all analyses in this study.

Table 5.1 Chemicals and their producers

NO	CHEMICAL	CODE
1	Ammonium molybdatetetrahydrate	Sigma 31402
2	Ammonium sulfate	Sigma 31119
3	Bacteriologycal Agar	BD 214010
4	Calcium carbonate	Sigma 12010
5	Carboxymethyl cellulose (CMC)	Aldrich 41928
6	Cobalt(II)chloride hexahydrate	Riedel-De Haën 12914
7	Copper(II)chloride dihydrate	Sigma 12848
8	Copper(II)sulfate pentahydrate	Sigma 12849
9	D-(+)-Glucose monohydrate	Sigma 16301
10	D-(+)-Galacturonic acid	Fluka 48280
11	Ethanol 96%	Merck 1.00971
12	Glycerol	Sigma G5516
13	Iron(II)sulfate heptahydrate	Riedel-De Haën 12354
14	Magnesium sulfate heptahydrate	Sigma 63140
15	Malt extract	Riedel-De Haën 13255
16	Manganese(II)sulfate monohydrate	Pakmaya Kemalpaşa

(cont. on nextpage)

Table5.1. (cont.)

17	Molasses	BD 211677
18	Peptone	Sigma P3850
19	Polygalacturonic acid	AppliChem A3871
20	Potassium hydroxide	Sigma 04243
21	Potassium phosphate monobasic	Merck 1.10130
22	Potato dextrose agar	BD 254920
23	Potato dextrose broth	Merck 1.08087
24	Potassium sodium tartrate tetrahydrate	Sigma 25022
25	Sodium acetate trihydrate	Sigma A6756
26	Sodium arsenate dibasic heptahydrate	Sigma 31437
27	Sodium bicarbonate	Sigma 13418
28	Sodium carbonate	Aldrich 419311
29	Sodium carboxymethyl cellulose (CMC)	Riedel-De Haën 13423
30	Sodium chloride	Panreac 141687
31	Sodium hydroxide	Fluka 71507
32	Sodium dihydrogen phosphate monohydrate	Riedel-De Haën 04272
33	Sodium phosphate dibasic dihydrate	Sigma 13464
34	Sodium sulfate	Sigma 15487
35	Sulfuric acid 98%	Sigma 13256
36	Baker's yeast	Pakmaya
37	Yeast extract	Merck 1.03753
38	Pectinase	Novozymes KRN05630
39	Cellulase	Novozymes CCN03125
40	B-glucosidase	Novozymes DCN00216

5.2. Sugar Content of Grape Pomace

Sugar composition of GP was determined by extracting with water and two stages acid hydrolysis. Residual sugars on GP were extracted by water extraction while complex carbohydrates (cellulose, hemicellulose and pectin) were hydrolyzed by two stages acid hydrolysis.

5.2.1. Water Extraction

Transferring residual sugar into liquid phase was done by water extraction. GP was added into distilled water at 80 °C for 1 hour. After extraction, liquid phase (extract) was separated from solid phase which is called extracted grape pomace (ex-GP) by filtration under vacuum. Ex-GP was also washed two times while filtering in order to eliminate the residual sugar on ex-GP. Extract was kept at -20 °C until required. Ex-GP was dried in oven at 60 °C for 24 hours and kept in a closed bag. Ex-GP of Muscat and Syrah were named in this study as M ex-GP and S ex-GP, respectively.

5.2.2. Hydrolysis Medium

GP and ex-GP samples were hydrolyzed using different H_2SO_4 concentrations in same experimental period. For acid hydrolysis 10 % (w/v) of GP suspensions were hydrolyzed with 12M H_2SO_4 for 3 hours at 20 °C, then followed by 0.8M H_2SO_4 for 4 hours at 100 °C,(Zhou and Ingram 2000) which can be called two stages acid hydrolyses. Hydrolyses was done in 10 ml test tubes or 30 ml bottles.

5.3. Exo-polygalactronase Production

5.3.1. Microorganisms

Aspergillus sojae ATCC20235, *Aspergillus sojae* (UV mutated), *Rhizopus oryzae* and *Aspergillus niger* were used in order to produce exo-polygalactronase enzyme from grape pomace. The fungal strains were kindly provided by Prof. Dr. CananTarı.

5.3.2. Spore propagation

Stock cultures of these strains were prepared in 20 % glycerol water and stored at -80° C. The propagation of the cultures was done on YME agar slant medium containing, malt extract (10 g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l),

incubated at 30° C until well sporulation (1 week). The spore suspensions used as inoculum were obtained on molasses agar slants containing glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l), $FeSO_4.7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4.5H_2O$ (12 mg/l), $MnSO_4.H_2O$ (15 mg/l) and agar (20 g/l), after the pre-activation step performed on YME agar using the stock cultures. The incubation temperature and time for each of the steps were 30°C and one week, respectively. The harvesting of the spores from the slants was done using 5 ml of Tween80-water (% 0.2). The spore suspension was collected in a sterile falcon tube and stored at 4°C until the actual study. The initial spore counts and viability counts were recorded.

5.3.3. Production medium

Required amount of samples were autoclaved at 121°C for 15min to obtain sterile substrate for enzyme production.

As a liquid substrate, GP was extracted in water at 80°C for 1h. Solid part was separated by filter paper under vacuum. Extract (liquid phase) was stored at -20°C until required. Liquid extract for enzyme production were also autoclaved at 121°C for 15min to obtain sterile substrate for enzyme production. As nutrients for preparation of medium, 5 g/l $(NH_4)_2SO_4$, 2 g/l K_2HPO_4, 1 g/l $MgSO_4$ were added to solid state and submerged fermentations media.

Liquid cultures were conducted in 250 ml flask with 70 ml working volume. The incubation temperature and time for each of the steps were 30 °C and one week, respectively. Spore concentration was 1 $x10^6$ spore / ml.

5.4. Enzymatic Hydrolysis

Procurement and pre-processes of GP for enzymatic hydrolysis was the same as mentioned in Section 5.2.1. Enzymatic hydrolysis was applied to ex-GP. Extraction parameters were 80°C and 1 hour. After extraction supernatant liquid was separated and solid phase was washed with pure water in order to remove soluble sugar remained on the solid phase, considering the accuracy of experiments.

Enzymatic hydrolysis applied on extracted GP with 5% solid loadings. Sodium acetate buffer solution (pH 4.8) was used to stabilize medium pH for enzymatic activity. Pectinase, cellulose and β-glucosidase were used as commercial enzymes. Also penicillin was used instead of autoclave not to effect enzymes activities. Different enzyme concentrations, temperature (30°C and 45°C) and hydrolysis time (48 and 124 h) were applied.

5.5. Lactic Acid Production

5.5.1. Microorganism

The bacterium, *Lactobacillus casei* NRRL B-441, was kindly provided by United States Department of Agriculture, National Center for Agricultural Utilization Research. The bacterium was supplied in lyophilized form and activated in the propagation medium.

5.5.2. Culture Propagation

The activation of *L. casei* cultures were done on MRS agar using stock cultures that is kept at -80 °C. MRS agar composition is peptone from casein 10.0 g/L; Meat extract 10.0 g/L; yeast extract 4.0 g/L; D(+) glucose 20.0 g/L; K_2HPO_4 2.0 g/L; Tween 80 1.0 g/L; di-ammonium hydrogen citrate 2.0 g/L; sodium acetate 5.0 g/L; $MgSO_4$ 0.2 g/L; $MnSO_4$ 0.04 g/L; agar-agar 14.0 g/L. After sterilization of MRS agar at 121°C and 15 min. in autoclave(Hirayama autoclave) 10 % (v/v) *L. casei* stock culture inoculated and incubated at 37 °C for 24 hours. 50% (w/w) glycerol was added in order to avoid breaking down the cell integrity while keeping at -80 °C.

Same parameters were employed while propagation of *L.casei* culture in MRS broth. 24 hour old fresh cultures were used as the inocula for the fermentations.

5.5.3. Fermentation Medium for Lactic Acid Production

GP was prepared for lactic acid production by *L.casei* as mentioned in Section 5.2.1.

Fermentation medium was composed of yeast extract 5-15 g/l; K_2HPO_4 0.5 g/l; $MgSO_4$ 0.2 g/l; $MnSO_4$ 0.05 g/l and 5-10% GP (w/v) or 5% of extracted GP (v/v). Extraction process was the same as mentioned in Section 5.2.1.

Fermentations were carried out in 250 ml flasks with 70 ml working volume in a temperature controlled flask shaker at 37 °C and 1 g. Flasks were inoculated with 2-3 ml of MRS cultures that had been incubated at 37 °C for 24 hours. The tops of the flasks were covered with aluminum foil. In order to investigate the individual sugar concentration, mineral solutions, GP or extract and yeast extract were sterilized separately and reconstituted after the sterilization or the medium was sterilized as a whole. $CaCO_3$ powder was sterilized separately in both cases and added before the inoculation (1g $CaCO_3$for each flask). Sterilization temperature and time were 121 °C and 15 min, respectively.

5.6. Analytical Methods

5.6.1. Water Extraction

Samples were taken after extraction and centrifuged at 3024 g. Supernatants were diluted at least 30 times. Dilutions were done with mobile phase used in the HPLC analysis (5 mM H_2SO_4).

Glucose, fructose, xylose, arabinose an galacturonic acid contents were measured by HPLC (Perkin Elmer, USA) with Aminex HPX-87H column (Biorad Laboratories, USA) operated at 65 °C using a refractive index detector. 5 mM H_2S0_4 was used as a mobile phase at a flow rate 0.6 ml/min.

Standard curve was done at four different sugar concentrations for all sugars (0.125, 0.25, 0.5 and 1g/l).

5.6.2. Two Stages Acid Hydrolysis

Samples were taken just after hydrolysis and $CaCO_3$ powder was added in order to decrease the acidity. After neutralization, samples were centrifuged at 3024 g and supernatants were diluted at least 5 times. Dilutions were done with mobile phase used in the HPLC analysis (5 mM H_2SO_4).

Glucose, fructose, xylose, arabinose an galacturonic acid contents were measured by HPLC (Perkin Elmer, USA) with Aminex HPX-87H column (Biorad Laboratories, USA) operated at 65 °C using a refractive index detector. 5 mM H_2SO_4 was used as a mobile phase with a flow rate 0.6 ml/min.

Concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, were calculated by using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or 162/180) for C-6 sugars (glucose, galactose, and mannose) (Sluiter, Hames et al. 2008)

$$C_{anhydro} = C_{corr} \times \text{Anhydro correction}$$
$$C_{hemicellulose} = C_{xylan+arabinan} \times (132/150)$$
$$C_{cellulose} = C_{glucose} \times (162/180)$$

5.6.3. Exo-polygalactronase Production

Exo-polygalacturonase (exo-PG) activity was assayed according to the procedure given by Panda et al. (1999) by using 2.4 g/l of polygalacturonic acid as substrate (pH 6.6) at 26 °C. The amount of substrate and enzymes used were 0.4 and 0.086 ml respectively. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. In this study, one unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions. Galacturonic acid was used as standard for the calibration curve of PG activity. Calibration curve was prepared using 50, 100, 200, 300, 400, 500 μl of the stock solution containing 500 nmol galacturonic acid in a total volume of 500 μl. Enzyme activity was calculated according to following equation:

$$Activity(U/ml) = (mg\ of\ galactronic\ acid\ /212.12) \times (1/20) \times (1/0.086)$$

Where, 212.12 is the molecular weight of galacturonic acid (mg/mole), 20 is the reaction time (min.) and 0.086 is the amount of enzyme in the reaction mixture (ml). Activity was measured as U/ml of mixture.

5.6.4. Enzymatic Hydrolysis

Samples were taken after hydrolysis and centrifuged at 3024 g and supernatants were diluted at least 10 times. Dilutions were done with mobile phase used in the HPLC analysis (5 mM H_2SO_4).

Glucose, fructose, xylose, arabinose and galactronic acid contents were measured by HPLC (Perkin Elmer, USA) using Aminex HPX-87H column (Biorad Laboratories, USA) operated at 65 °C and a refractive index detector. 5 mM H_2S0_4 was used as the mobile phase at a flow rate of 0.6 ml/min.

5.6.5. Lactic Acid Production

Samples were taken at different time intervals centrifuged at 3024 g and supernatants were kept at -20 °C until required. Samples were diluted at least 30 times in order to decrease the sugar and lactic acid concentration below 1 g/l for HPLC analysis. Dilutions were done with the mobile phase used in the HPLC analysis (5 mM H_2S0_4).

Glucose, fructose and lactic acid contents were measured by HPLC (Perkin Elmer, USA) using Aminex HPX-87H column (Biorad Laboratories, USA) operated at 65 °C using a refractive index detector. 5 mM H_2SO_4 was used as a mobile phase at a flow rate of 0.6 ml/min.

CHAPTER 6

RESULTS and DISCUSSION

6.1. Sugar Content of Grape Pomace

Characterization of sugar content of GP is one of the main steps in this study. Soluble sugars left in GP can be extracted by liquid extraction. Also with acid hydrolysis pectin, cellulose and hemicellulose content of GP can be hydrolyzed to corresponding monosaccharides. Different solid loadings and temperature value were used in extraction methods. Considering microbial spoilage and carbohydrate degradation, samples were kept at -80°C or dried. All analyses in this study were performed on dry base (db) in order to have accurate results to discuss.

Moisture was measured as 62.0 ± 3.14 % of GP. Dry GP was first extracted in distilled water at 80 °C for 24 hours. Extract was analyzed in order to obtain residual sugar of GP. Ex-GP was then hydrolyzed by $H_2SO_4$to measure the cellulose and hemicellulose content of GP. Water soluble extractives of Muscat and Syrah include 36.40 ± 2.10 (18.70 ± 1.15% glucose and 17.70 ± 1.05% fructose) and 34.60 ± 2.45% (17.80 ±1.35% glucose and 16.80 ±1.10% fructose) residual sugars, respectively. After two stages acid hydrolysis cellulose was calculated based on the equations mentioned in Section 5.6.2 for Muscat and Syrah as 10.64 ± 0.10 and 10.04 ± 0.15%. Also hemicellulose was calculated according to the same equations for Muscat and Syrah as3.41 ± 0.10 and 4.01 ± 0.10%, respectively.

Water extraction method was mentioned in Seciton 5.6.1 and the residual sugar results were given for Muscat and Syrah in Table 6.1

Table 6.1 Sugar Content of GP (% db).

Components	Muscat	Syrah
Cellulose	16.36 ± 0.10	15.54 ± 0.15
Hemicellulose	4.27 ± 0.10	3.19 ± 0.10
Residual sugars	36.40 ± 2.10	34.60 ± 2.45

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6.1.1. Two Stages Acid Hydrolysis

Acid hydrolysis of GP involves dilute and concentrated acid treatments to break down the rigid structure of lignocellulosic plant-derived materials. Most common used chemical for acid hydrolysis of lignocellulosic materials is sulphuric acid. Sulphuric acid is generally used to remove hemicellulose and can be a part of fractionating the components of lignocellulosic materials (Brodeur, Yau et al. 2011).

Red and white GP and ex-GP were kept in 12 M H_2SO_4at 10 % (w/v) solid loadings for 3 hours at 20 °C, followed by in 1 M H_2SO_4 for 4 hours at 100 °C(Valiente, Arrigoni et al. 1995).

Two stages acid hydrolysis was performed in order to characterize the sugar composition of red and white GP and ex-GP. Cost of high concentrated acid treatment on biomass and need for recovery limit the process of released sugars through concentrated acid hydrolysis. Another drawback is effect of high acid concentration may lead to hydroxymethyl furfural (HMF) and furfural formation due to degradation of complex polysaccharides (Taherzadeh, Gustafsson et al. 2000).

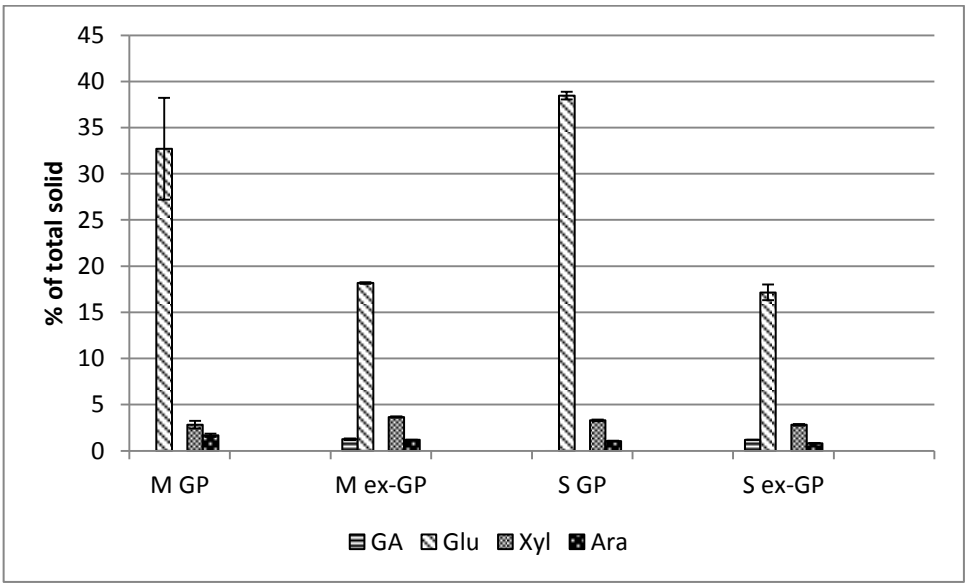

Figure 6.1 Yield of 2 stages acid hydrolysis (GA; galacturonic acid Glu; glucose, Fru; fructose, Xyl; xylose, Ara; arabinose, M GP; Muscat grape pomace, S GP; Syrah grape pomace, M ex-GP; Muscat extracted grape pomace, S ex-GP; Syrah extracted grape pomace)

Table 6.2 Concentrations of monosaccharides after 2 stages acid hydrolysis (g/l).

	GA	Glucose	Fructose	Xylose	Arabinose
Muscat GP	0	2.73 ± 0.46	0	0.24 ± 0.04	0.14± 0
Muscat ex-GP	0.11± 0	1.52± 0	0	0.31± 0	0.10± 0
Syrah GP	0	3.21 ± 0.04	0	0.28± 0	0.09± 0
Syrah ex-GP	0.1± 0	1.43 ± 0.07	0	0.24± 0	0.07± 0

Two stages acid hydrolysis did not hydrolyze fructose from all four substrates. Table 6.2 indicates that glucose was the main monosaccharide hydrolyzed from all four types of substrates. Also it is possible to say according to the Table 6.2 that glucose concentrations of GP and ex-GP of red and white grapes showed some similarities. Glucose content of ex-GP was found to be approximately 50% that of GP. Xylose concentrations of four substrates were measured to be similar. Maximum xylose concentration was measured as 0.31 g/l for M ex-GP and minimum xylose concentration was measured as 0.24 g/l for S ex-GP.

In a previous study same parameters were applied in order to hydrolyze fermentable sugars from complex polysaccharides of red and white GP which results were significantly different from our data. Chemical analysis results of sugars (% w/w dried pomace) released by 2 stages acid hydrolysis were given for glucose and fructose as 3.56 and 0.32(Korkie, Janse et al. 2002) Hydrolysis parameters were kept same except solid loadings of GP which was applied as 10 % instead of 15 %. Figure 6.1 indicates that glucose yields of 2 stages acid hydrolysis were measured significantly different from Korkie and Janse (2002).

6.2. Enzymatic Hydrolysis

GP consists of four major polysaccharides which are cellulose, hemicellulose, starch and pectin. The polysaccharides in GP should be degraded to monosaccharide in order to be utilized as a substrate for fermentation processes. Wine making yeast *Saccharomyces cerevisiae* is able to ferment monosaccharide into ethanol but is not able to degrade complex carbohydrates to monosaccharide. Because of this, GP which is obtained from wine making process consists of polysaccharides. This set of experiments aimed to degrade polysaccharides in GP to monosaccharide with commercial enzymes which were cellulose, β-glucosidase and pectinase. Water soluble sugars in GP were

extracted before applying enzymatic hydrolysis in order to have accurate results for GP composition.

Hydrolysis process was applied on ex-GP of Muscat and Syrah which still consists of all polysaccharides. Different enzyme concentrations, temperature and retention times were used to hydrolyze the solid residue of GP. For two sets of experiments solid loading was kept as 5 % (w/v). For Set 1, supplemented volume of cellulose and pectinase were 100µl but β-glucosidase was 50 µl into 20 ml of total working volume at 37 °C and 2 days of hydrolysis. For Set 2, supplemented volume of cellulose and pectinase were 500µl but β-glucosidase was 50 µl into 20 ml of total working volume at 45 °C and 5 days of hydrolysis. At both sets shaking speed was kept as 1 g. In order to prevent microbial spoilage, 1 mg of penicillin was added to hydrolysis media. The reason of penicillin usage is important factor for enzyme activity and eliminating the extraction effect of autoclave on substrate.

After water extraction of 10 % dry GP at 80 °C for one hour, solid phase was separated and filtered under vacuum. Solid phase was washed two times with distilled water to wash out the residual sugar. Thereafter, solid phase was dried in drying oven at 60 °C for 24 h. Preliminary experiments that were done in our laboratory demonstrated that residual sugars still exist on extract even washing 2 times. Because of this enzyme-free flasks with same solid loadings were also analyzed as control groups. All the results are given below were calculated with considering the control groups. Muscat (white) and Syrah (red) were hydrolyzed with three different commercial enzymes namely cellulase, pectinase and β-glucosidase.

The main carbohydrates after enzymatic hydrolysis were glucose, xylose, fructose and arabinose. As it was mentioned before cellulose (consisting of glucose subunits), hemicellulose (consisting of glucose, arabinose, xylose, mannose and galactose), pectin (consisting of GA subunits) exist in GP. Table 6.1 indicates that highest concentration after enzymatic hydrolysis was glucose with 3.79 ± 0.05g/l for Syrah and 3.18 ± 0.50 g/l for Muscat. It is also possible to say that cellulase was the most effective enzyme in hydrolysis process. Cellulose component of red GP is the most dominant complex carbohydrate after lignin. Chemical compositions of red and white GP as determined by Zheng and Lee (2012) are given in Table 6.4.

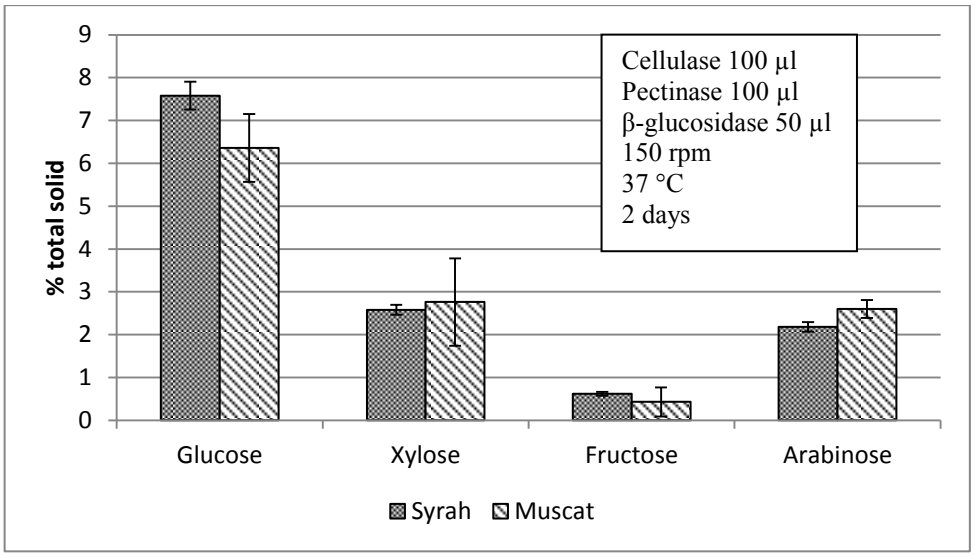

Figure 6.2 Enzymatic hydrolysis results of Set 1

Figure 6.2 shows some similarities with Table 6.4 in the meaning of hydrolyzed monosaccharide. Table 6.4 indicated that cellulose content of red and white GP were 14.5 and 9.2 %. Figure 6.2 also shows that glucose content of red GP was higher than white GP with 7.58 ± 0.11 and 6.36 ± 1.01 % of total solid. Zheng and Lee studied on characterization of GP. In our study enzymatic hydrolysis were done for sugar hydrolysis in order to investigate the possible fermentable sugars. Comparison of two different data can give us a clue about the effectiveness of enzyme hydrolysis.

Table 6.3 Sugar Released (g/l) during Set 1 from Syrah and Muscat.

Sugars	Syrah	Muscat
Glucose	3.79 ± 0.05	3.18 ± 0.50
Xylose	1.29 ± 0.02	1.38 ± 0.17
Fructose	0.31 ± 0.05	0.22 ± 0.10
Arabinose	1.10 ± 0.01	1.30 ± 0.10
Total	6.49 ± 0.13	6.08 ± 0.42

There is one incompatibility between Figure 6.2 and Table 6.4 which is based on hemicellulose hydrolysis. In our experiment results xylose and arabinose content of red GP is slightly lower than xylose and arabinose content of white GP. As it is mentioned before hemicellulose consists of glucose, arabinose, xylose, mannose and galactose. The yield determination of hemicellulose hydrolysis can be done according to the equations mentioned in Section 5.6.2.

Table 6.4 Chemical composition of grape pomace (GP)
(Source:Zheng et al. 2012)

Chemical component	Red GP (wt, % dry basis)	White GP (wt, % dry basis)
Cellulose	14.5	9.2
Hemicellulose	10.3	4
Pectin	5.4	5.7
Lignin	17.2	11.6
Protein	14.5	7
WSC	2.7	49.1

WSC; water soluble carbohydrate

Zheng and Lee (2012) demonstrated that hemicellulose content of red GP is higher than white GP which means xylose and arabinose concentrations of red GP can be more than white GP. In our experiment xylose and arabinose were calculated as 2.58 ± 0.04 and 2.18 ± 0.01 % of total red GP solid. Also xylose and arabinose were calculated as 2.76 ± 0.33 and 2.60 ± 0.20 % of total white GP solid. It is possible to say that according to Table 6.4enzyme amount used in our experiment may be not enough to hydrolyze total hemicellulose content of red GP. There also may be another reason for this circumstance that phenolic compound of red GP might have limited the activity on red GP.

β-glucosidase was supplemented in flasks as 50 µl which was the lowest amount of enzymes used in hydrolysis process. HPLC analyses showed us that addition of high β-glucosidase in hydrolysis process caused complicated HPLC data. In order to have accurate and clean data from hydrolyses process β-glucosidase was supplemented as low as possible.

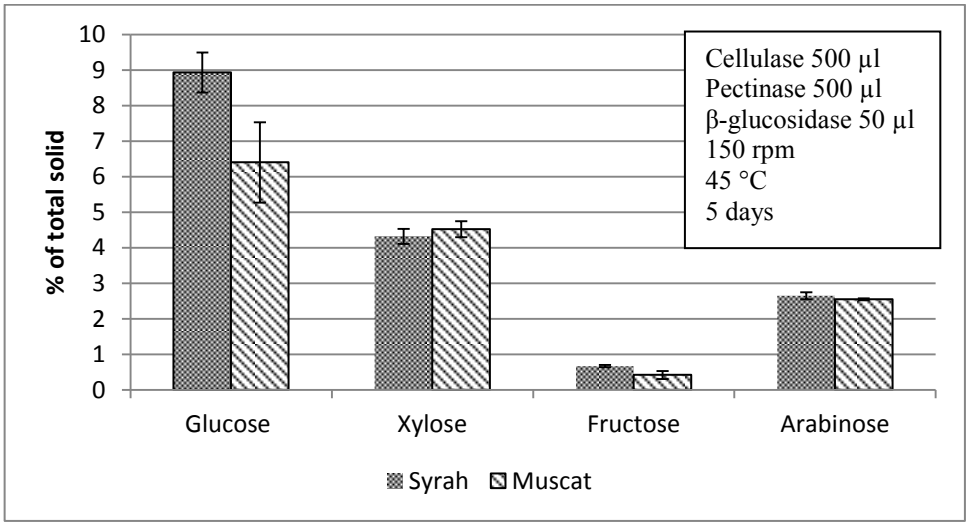

Figure 6.3 Enzymatic hydrolysis results of Set 2.

Table 6.5Sugar Released (g/l) during Set 2 from Syrah and Muscat.

Sugars	Syrah	Muscat
Glucose	4.47 ± 0.10	3.20 ± 0.11
Xylose	2.16 ± 0.03	2.26 ± 0.05
Fructose	0.34 ± 0.05	0.21 ± 0.03
Arabinose	1.33 ± 0.03	1.28 ± 0.03
Total	8.30 ± 0.21	6.95 ± 0.22

In second enzymatic hydrolysis run (Set 2) amounts of cellulose, pectinase and β-glucosidase added to 20 ml of hydrolysis medium were 500, 500 and 50 µl respectively. Temperature was kept at 45 °C and shaking speed of incubator was 1 g for 5 days. Sodium acetate was used the buffer solution to keep the pH at 4.8.

As seen in Figure 6.3, glucose was the most abundant compound in both red and white ex-GP in Set 2. Xylose, arabinose and fructose followed glucose. Cellulose was the main hydrolyzed complex polysaccharide by cellulose. In Set 1 hydrolyzed glucose was 3.79 ± 0.05 g/l from Syrah ex-GP. However increased cellulose amount increased the hydrolyzed glucose from red ex-GP, but not for white ex-GP.

Glucose concentrations showed the biggest difference between red and white ex-GP with 4.47 ± 0.11 and 3.20 ± 0.11 g/l. Most of the concentrations of the other monosaccharides were similar close to each other. Figure6.3 also indicates that yield of xylose and arabinose hydrolysis for red ex-GP were calculated as 4.32 ± 0.03 and 2.65 ± 0.07 % of total solid. For white ex-GP yield values of xylose and arabinose were calculated as 4.52 ± 0.11 and 2.55 ± 0.07. These values can be considered as low for substrate for fermentative processes. Total sugar results showed us that maximum 16.60 % of ex-GP could have been hydrolyzed (Figure 6.3) by three different commercial enzymes.

Comparison of yield values obtained in the two sets of enzymatic hydrolysis was not realistic because of using different parameters. However, it was possible to say that Set 2 was more effective on hydrolysis of hemicellulose. Extended hydrolysis time and/or higher temperature might have positively affected the xylose and arabinose hydrolysis. Also higher amount of enzyme supplementation may have been the reason to observe increased fermentable sugar hydrolysis from red and white ex-GP. According to Figure6.2 and Figure 6.3 xylose showed the maximum increase in yield of hydrolysis as 39 and 41%for red and white ex-GP respectively.

. 6.3. Dilute Acid Hydrolysis

Two stages acid hydrolysis results lead to improve released sugar concentrations from GP and ex-GP of red and white wine. As mentioned before second step of two stages acid hydrolysis was done using 1 M H_2SO_4 for 4 hours at 100 °C. In this part of the study the second stage of the two stages hydrolysis was applied alone. In other words, GP was exposed to dilute acid hydrolysis.

Table 6.6 Concentrations of monosaccharide after dilute acid hydrolysis (g/l).

	GA	Glucose	Fructose	Xylose	Arabinose
Muscat GP	0	2.94 ± 0.15	0	0.34 ± 0.04	0.14± 0
Muscat ex-GP	0.06± 0	0.46± 0	0	0.38± 0	0.24± 0
Syrah GP	0	3.08 ± 0.04	0	0.15 ± 0.07	0.15± 0
Syrah ex-GP	0.05± 0	0.40 ± 0.06	0	0.37± 0	0.22± 0

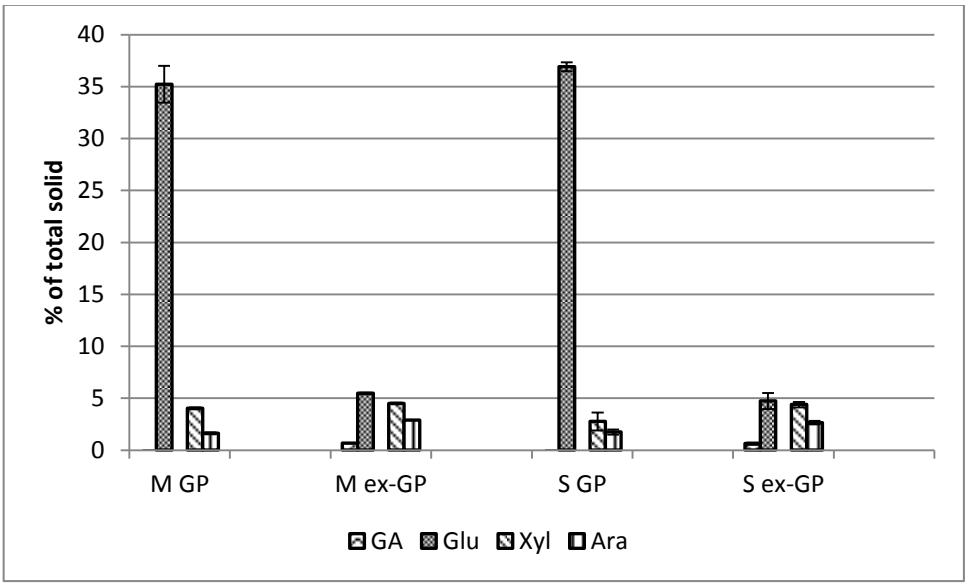

Figure 6.4 Yield of dilute acid hydrolysis (GA; galacturonic acid Glu; glucose, Fru; fructose, Xyl; xylose, Ara; arabinose, M GP; Muscat grape pomace, S GP; Syrah grape pomace, M ex-GP; Muscat extracted grape pomace, S ex-GP; Syrah extracted grape pomace).

According to the Table 6.5 GA was just hydrolyzed from ex-GP of Syrah and Muscat. GA concentrations of ex-Syrah and ex-Muscat were measured as 0.06 and 0.05 g/l that were calculated in Figure 6.4 as 0.67 and 0.62 % of total solid. It is also possible to say that pectin was hydrolyzed under the hydrolysis conditions from S ex-GP and

Mex-GP but no GA was obtained in the hydrolyzates from M GP and S GP. Glucose concentrations of ex-GP and GP were also measured different from each other both in Muscat and Syrah. Glucose concentrations of four types of substrates are given in Table 6.6. Hydrolyzed glucose yields of M ex-GP and S ex-GP were calculated in Figure 6.4 as 35.2 and 36.9 % of total solid. The maximum hydrolyzed component after dilute acid hydrolysis in all types of substrates was glucose.

Xylose and arabinose concentrations of four types of substrates were low in order to utilize as a substrate for fermentation processes. Maximum xylose concentration was measured in M ex-GP as 0.38 g/l which corresponds to 4.5 % of total solid. S ex-GP also showed similar result from xylose concentration as 0.37 g/l that corresponds to 4.38 % of total solid. Arabinose concentrations were demonstrated that hydrolysis of ex-GP with dilute acid was more efficient than GP of Syrah and Muscat. Arabinose concentrations of M ex-GP and S ex-GP were measured as 0.24 and 0.22 g/l Table 6.6 that corresponds to 2.88 and 2.64 % of total solid (Figure 6.4).

Two stages acid hydrolysis was applied to characterize the possible fermentable sugar content of GP and ex-GP as mentioned before. Comparison of 2 stages and dilute acid hydrolysis do not display the efficiency of hydrolysis processes in order to investigate the possibility of GP usage as a substrate for fermentative processes. As it was mentioned before two stages acid hydrolysis was done to show sugar composition of GP. Comparison of two different process parameters for scientific studies may be useful. Glucose concentrations released by 2 stages and dilute acid hydrolysis from GP were similar but it is possible to say that 2 stages acid hydrolysis is more efficient than dilute acid hydrolysis in hydrolysis of ex-GP. GA concentrations hydrolyzed by 2 stages and dilute acid hydrolysis did not reach significant level in both GP and ex-GP. Fructose did not appear in both 2 stages and dilute acid hydrolysate, which was also predicted. Xylose and arabinose concentrations measured in dilute acid hydrolysates were more than in 2 stages acid hydrolysates of both GP and ex-GP.

6.4. Exo-polygalacturonase Production from Grape Pomace

Pectinases consist of different group of enzymes which can degrade the pectin. Most of these enzymes are generally used in clarification or extraction of fruit juices. Although several types of enzymes can be found, exo-polygalacturonases (exo-PG) are the most widely used and studied ones which represent the 25 % of total industrial enzymes sales (Díaz, de Ory et al. 2012). Agricultural wastes are generally used as substrates to prduce these enzymes by solid state fermentation (SSF) and submerged fermentation (SmF). Most of the agricultural wastes alone are not sufficient to support production of pectolytic enzymes but with supplementation of nitrogen and organiz salts can be utilized as substrates.

As discussed before GP has a potential residual sugar content which may provide the microbial growth with supplementation of different salts. In field of enzyme production several agro industrial wastes used as substrates e.g., corn, rice, sugar cane, wheat, banana waste, potato, tea, coccus, apple and citrus fruits (Botella, Ory et al. 2005). Increasing interest of utilizing agro industrial wastes leads to investigation of the GP as a substrate for exo-PG enzyme production.

GP and GP extractswere used as substrate to produce exo-PG by *Aspergillus niger*, *Rhizopus oryzae*, *Aspergillus sojae* (mutant type), *Aspergillus sojae WT* (wild type). SSF and SmF were carried out to investigate the exo-PG production behavior of different microorganisms on GP and extracted liquid phase of GP. Units of enzyme activity were given as U/ml and U/gds (g dry solid) for SSF and SmF, respectively. Samples were taken at every 24 h of incubation and maximum enzyme activity results were reported.

Table 6.7 shows the enzyme activities obtained by different microorganisms, substrate types, fermentation types and parameters. Maximum exo-PG activity was measured as 2.99 U/ml by *A. sojae mutant* in SmF from GP of Muscat.

Extract of Syrah and Muscat did not show any significant enzyme activity as a substrate. Also according to the Table 6.7 most of the exo-PG activities were below 1.0 U/ml. It is also possible to say that using GP as a substrate for exo-PG production was more efficient than using extract. After some preliminary experiments and literature survey on the behavior of microorganisms on GP, *A.sojae mutant* and *A.sojae WT* were inoculated in extract of GP. It can be obviously seen that, extract of GP was not a

suitable substrate for enzyme production by these microorganisms. *A. sojae mutant* showed maximum activity of 0.521 U/ml from Syrah and 0.315 U/ml from Muscat with liquid extract as a substrate.

Comparison of SSF and SmF by four different microorganisms indicates that SmF is more promising than SSF even just *A. sojae mutant* and *A. sojae* showed exo-PG activity higher than 1.0 U/ml.

According to the Table 6.6 designated that GP and ex. phase of GP may not provide exo-PG without any supplementation of extra carbon sources.

Table 6.7 Enzyme activity results by different microorganisms

Type of m.o	Substrate	Fermentation	Parameters	Results
A.sojae mutant	GP(Muscat)	SSF	6days/ 30°C	no activity
R.oryzae	GP(Muscat)		6days/ 30°C	0.56 U/gds
A.niger	GP(Muscat)		6days/ 30°C	0.43 U/gds
A.sojae WT	GP(Muscat)		6days/ 30°C	no activity
A.sojae mutant	GP (Muscat)	SmF	6days/ 30°C/250rpm	1.79 U/ml
	GP (Syrah)			0.1 U/ml
A.sojae WT	GP (Muscat)		6days/ 30°C/250rpm	2.99 U/ml
	GP (Syrah)			0.87 U/ml
A.sojae mutant	GP (Syrah)		3days/ 30°C/250rpm	2.7 U/ml
A.niger	GP (Syrah)		3days/ 30°C/250rpm	0.9 U/ml
A.sojae mutant	extract (Muscat)		6days/ 30°C/250rpm	0.315 U/ml
	extract (Syrah)			0.521 U/ml
A.sojae WT	extract (Muscat)		6days/ 30°C/250rpm	no activity
	extract (Syrah)			no activity
A.niger	GP (Muscat)		6days/ 30°C/250rpm	no activity
	GP (Syrah)			no activity
R.oryzae	GP (Muscat)		6days/ 30°C/250rpm	no activity
	GP (Syrah)			no activity

6.5. Lactic Acid Production

6.5.1. Lactic Acid Production from Grape Pomace

Previous studies in our laboratory and preliminary experiments in this study gave a hint about maximum lactic acid production time. In order to investigate the maximum lactic acid production times, glucose (20 g/l) and fructose (20 g/l) mixture; 10 g/l fructose solutions and 10 % Muscat and Syrah dry GP suspensions were used.

Figure 6.5 and 6.6 represent the use of commercial glucose-fructose and fructose solutions as substrate for lactic acid production.

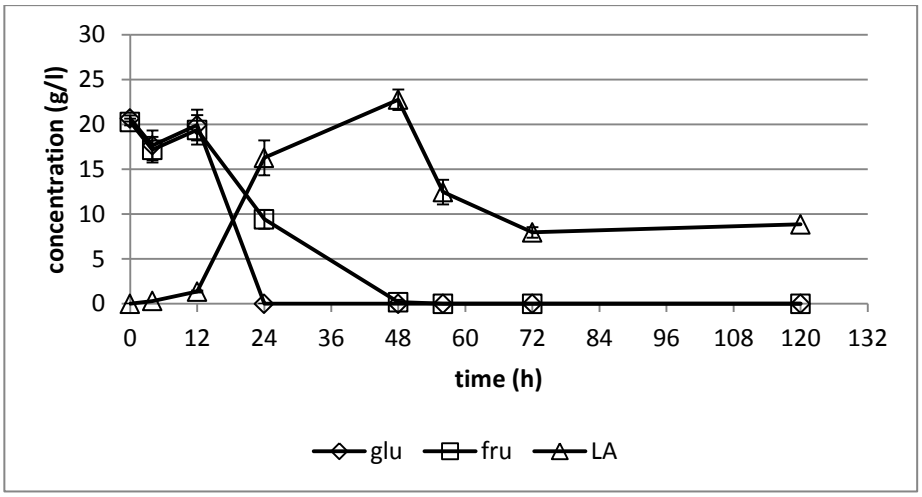

Figure 6.5 Kinetics of lactic acid production and sugar consumption in glucose (20 g/l) and fructose (20 g/l) mixture.

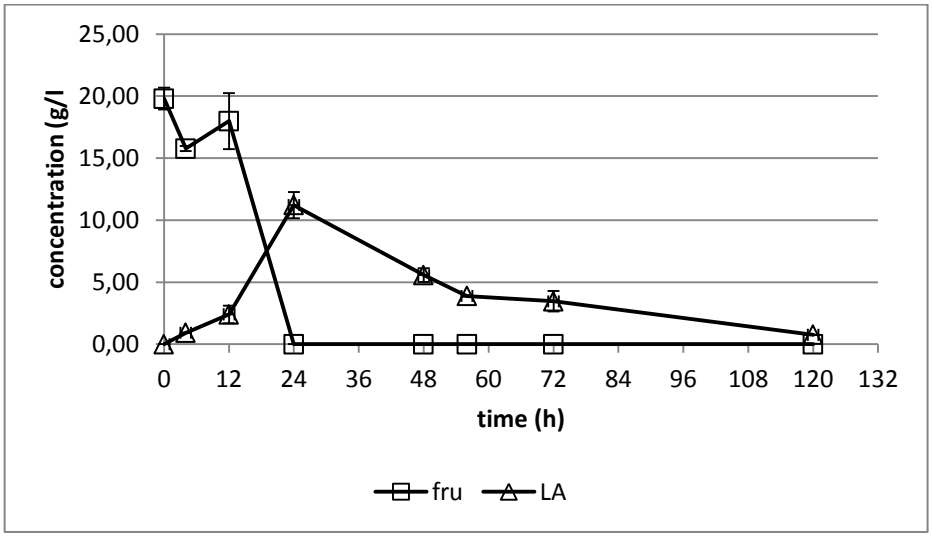

Figure 6.6 Kinetics of lactic acid production and sugar consumption in fructose (20 g/l)

Figure 6.7 and 6.8 represent Muscat and Syrah dry GP as substrate for lactic acid production. Same medium compositions, temperature, shaking speed, inoculum level were used for four different substrates. As it can be seen from Figure 6.5 and Figure 6.6 maximum lactic acid production times for glucose-fructose solution and fructose solution were 48h and 24h with maximum lactic acid production. Figure 6.7 and 6.8 indicate that maximum lactic acid production times for Muscat and Syrah dry GP are 72h with maximum lactic acid levels of 33.3 and 27.45 g/l.

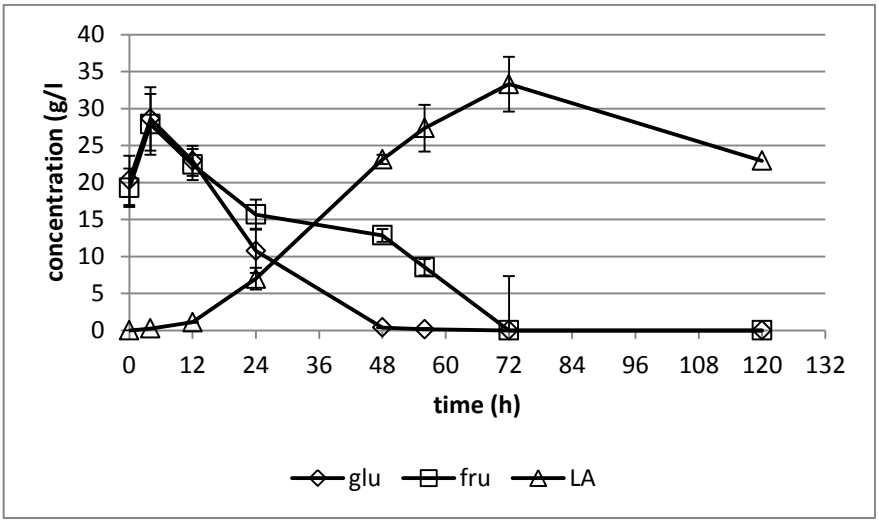

Figure 6.7 Kinetics of lactic acid production and sugar consumption in Muscat GP (10 % solid loading).

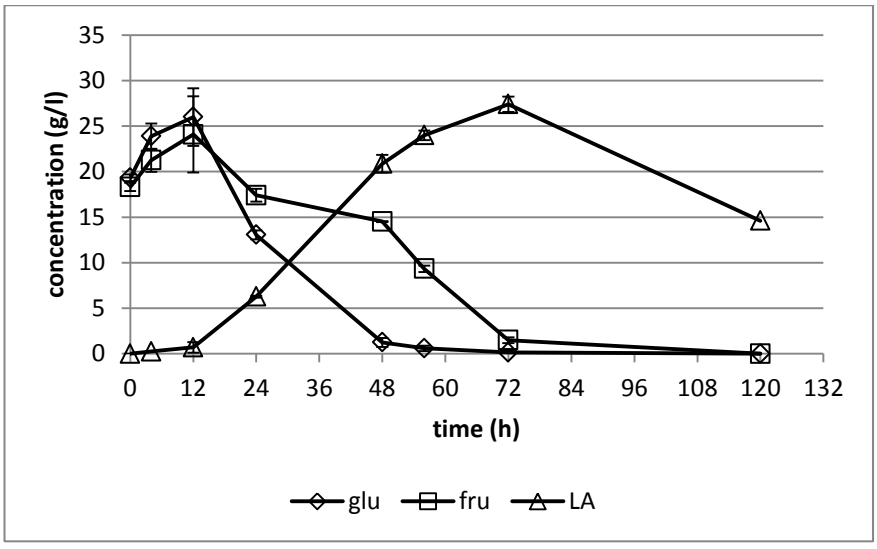

Figure 6.8 Kinetics of lactic acid production and sugar consumption in Syrah GP (10 % solid loading).

Table 6.8 Yield, production and consumption rates of lactic acid production

	S-initial (g/l)		P-max. (g/l)	Yield (P/S)	Total sugar consumption rate (g/l·h)	Overall Production rate (g/l·h)
	glucose	fructose	LA			
glu-fru	20	20	22.74	0.57	0.83	0.47
fru	0	20	11.21	0.56	0.83	0.47
muscat	20.3	19.3	33.3	0.84	0.55	0.46
syrah	19.3	18.3	27.45	0.73	0.52	0.38

As it can be seen clearly from Figure 6.5, 6.7 and 6.8glucose consumption by *L.casei* were faster than fructose consumption when glucose and fructose were present together in the medium. Behavior of *L. casei* was similar within complex and defined media as indicated in Figure 6.7 and 6.5. Glucose consumption started initially but fructose is also consumed at a slower consumption rate. Experiments showed that maximum lactic acid production and substrate consumption time limits can be different. But in all experiments *L. casei* consumed glucose faster than fructose.

Table 6.8shows yield, consumption rate and productivity values. Consumption rates for glucose-fructose solution (glu-fru) were the same as fructose solution (fru) (0.83 g/l·h). Muscat and Syrah consumption rates were calculated as0.55 and 0.52 g/l·h which were very close to each other and different from glu-fru and fructose solutions. In a parallel with consumption rates, production rates in glu-fru and fructose were higher than Syrah with 0.47 g/l·h. Production rate of Muscat (0.46g/l·h)was also calculated more than Syrah(0.38 g/l·h). As it is mentioned in Section 3.1, red GP is obtained after fermentation and white GP is obtained before fermentation. This can be a clear hint for residual sugar concentrations for two GP however, with same solid loading rate, inlet sugar concentrations were so close to each other with 39.6 g/l for Muscat and 37.6 g/l for Syrah dry GP. Zheng et al. (2013) mentioned ‘fermented grape pomace’ (Fe GP) and ‘fresh grape pomace’ (Fr GP) has more or less same amount of chemical compounds except water soluble carbohydrate. They indicated that red grape pomace (Fe GP) and white grape pomace (Fr GP) have 2.7 and 49.1 % water soluble carbohydrate on dry basis. Based on these data, ethanol and lactic acid concentrations after fermentation with different homofermentative and heterofermentative lactic acid bacteria strains of red and white GP were significantly different from each other (Zheng, Lee et al. 2012)

6.5.2. Utilization of GP Extract for Lactic Acid Fermentation

Extraction process is based on transferring the water soluble carbohydrates into liquid phase with water at high temperature. In some preliminary experiment done in our laboratory indicated that most efficient extraction condition for GP was at 80 °C and 1 hour. Cost of preliminary process is an important criterion for large volume productions. In order to investigate the feasible substrate amount, different solid loadings were tried (10% and 15%). Extraction parameters were adjusted as 80 °C for 1 h considering the feasibility to industrial area.

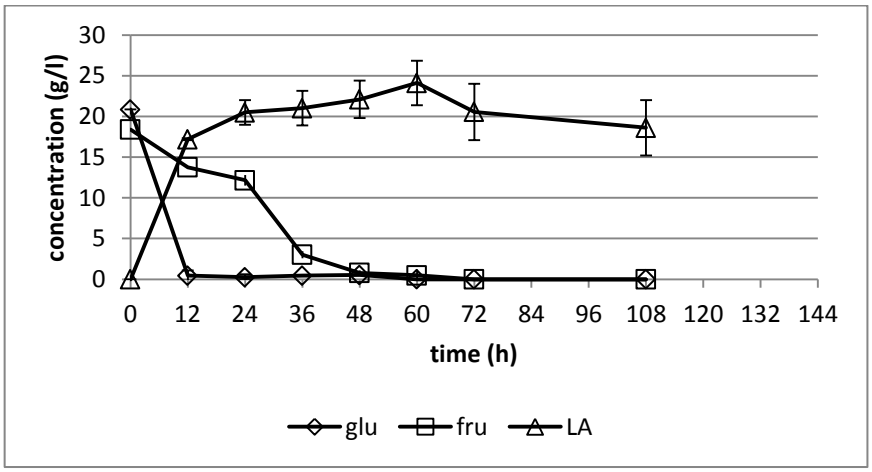

Figure 6.9 Kinetics of lactic acid production and sugar consumption in Muscat extract (10% solid loading in extraction)

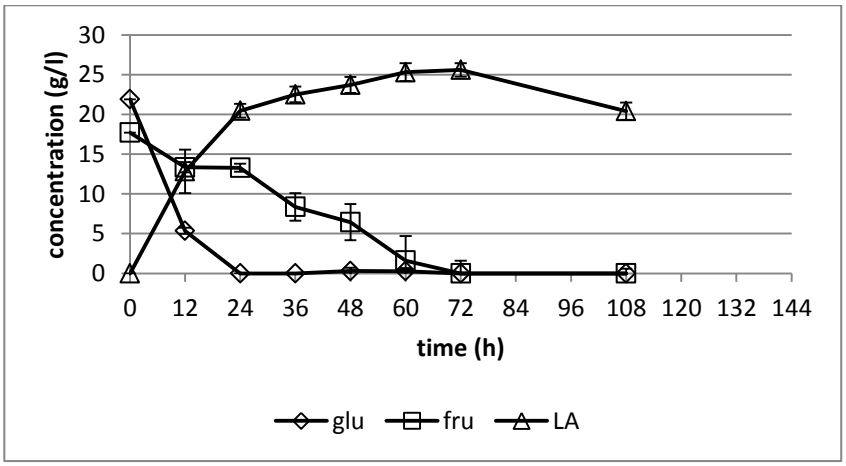

Figure 6.10 Kinetics of lactic acid production and sugar consumption in Syrah extract (10% solid loading in extraction)

After studying utilization of dry GP by lactic acid fermentation, extracted reducing sugar were examined as a substrate for lactic acid production by *L. casei.* Two

different solid loading parameters were set for extraction of GP as 10 % and 15 %.Culture conditions were as used in dry GP utilization runs. Salts and concentrations were also same in order to compare the productivity of dry GP and extracted liquid phase.

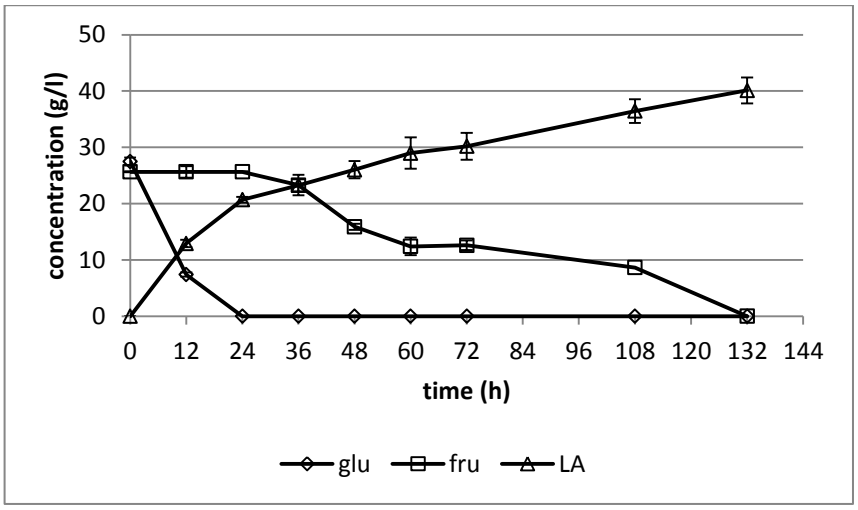

Figure 6.11 Kinetics of lactic acid production and sugar consumption in Muscat extract (15% solid loading in extraction)

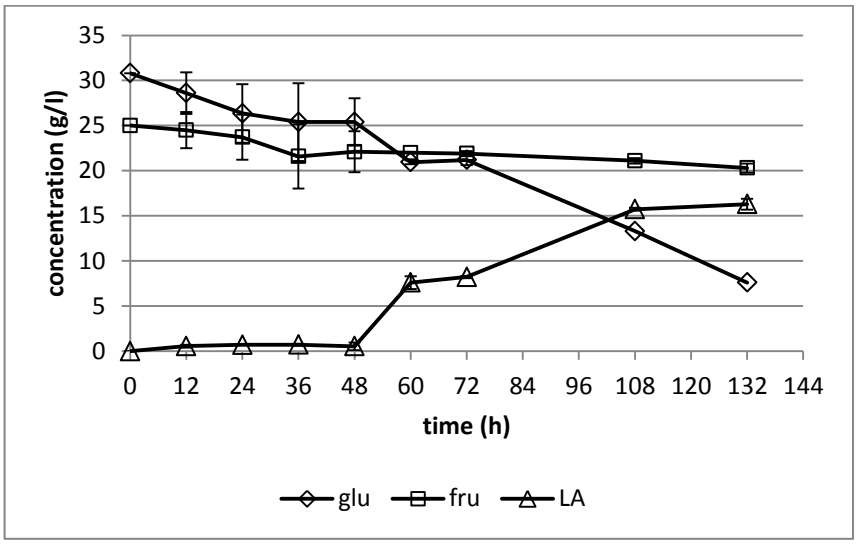

Figure 6.12 Kinetics of lactic acid production and sugar consumption in Syrah extract (15% solid loading in extraction)

Figure 6.9 and 6.10 represent the lactic acid production in extracts of 10 % Muscat and 10 % Syrah dry GP. Figure 6.11 and 6.12 represent the lactic acid production from extracted liquid phase of 15 % Muscat and Syrah dry GP. It is possible to say that lactic acid concentration and fermentation time increased when sugar concentration increased. Maximum lactic acid concentrations in extracts of 10 %

Muscat, 10 % Syrah were measured as 24.11 and 25.6 g/l, respectively. Same as in dry GP experiments, glucose consumption started first at a high rate. After glucose was depleted, fructose consumption rate slightly increased. Maximum lactic acid productions were reached in 60 h and 72 h in 10% Muscat and 10% Syrah extracts respectively. Maximum lactic acid concentrations were obtained later compared to previous experiment set. This circumstance can be possible because of the inhibition effect of extracted polyphenols from GP on lactic acid production.

Figure 6.11 showed that increased substrate concentrations could affect positively the lactic acid production. Initial glucose and fructose concentrations were measured as 27.50 and 27.65 g/l for 15 % Muscat which are higher than 10 % Muscat extracted phase. High initial residual sugar concentration did not cause any inhibition on lactic acid production, but considering the time to reach maximum lactic acid concentration (40.3 g/l), production process took 132 h. As it was also mentioned before, reaching to 25.6 g/l lactic acid concentration took 60 h with 10 % Muscat extracted liquid phase. After consumption of initial glucose, production rate of lactic acid slightly decreased in all experiments. The reason of decreasing rate of lactic acid production may have been the accumulation of fermentation byproducts or increased extraction rate of polyphenols and condensed tannins which may have acted as inhibitors of further biotechnological transformation.

Lactic acid production behavior is different in 15 % Syrah extract. Figure 6.12 indicates that after 132 h of lactic acid fermentation there was still reducing sugar in the fermentation medium. Initial glucose and fructose concentrations were 30.8 and 25.0 g/l which were similar to 15 % Muscat extract however, after 132 h of fermentation 7.6 g/l glucose and 20.3 g/l fructose existed with 16.3 g/l lactic acid. Comparison of 10 % Muscat and 10 % Syrah showed that consumption times of glucose were different from each other (12 vs. 24 h). Also according to Figure 6.9 and 6.10 fructose consumption times were different for 10 % Muscat and 10% Syrah (60 vs. 72 h). These differences were larger in 15 % of Syrah and Muscat GP extracts used as substrate for lactic acid production. Red GP and white GP total phenolic compounds are different from each other. In a previous study, total phenolic compounds of red and white GP were measured as 21.4-26.7 and 11.6-15.8 mg GAE/ g DM (Deng, Penner et al. 2011). This could be the reason for observing different lactic acid production and the consumption times in red and white GP. Higher amount of phenolic compounds could have been

extracted when high amount of GP was used and this may have inhibited the lactic acid production from GP by *L. casei*.

Table 6.9 Concentration, yield and rate values of Muscat (white) and Syrah (red) GP extracts.

	S-initial (g/l)		P-max. (g/l)	Yield (P/S)	Total sugar consumption rate (g/l·h)	Overall Production rate (g/l·h)
	glucose	fructose	LA			
M 10%	20.8 ± 0.2	18.4 ± 0.1	24.11 ± 2.74	0.61 ± 0.05	0.60 ± 0.03	0.40 ± 0.05
S 10%	21.9 ± 0.2	17.7 ± 0.2	25.6 ± 0.84	0.64 ± 0.02	0.55 ± 0.02	0.35 ± 0.03
M 15%	27.5 ± 0.3	25.6 ± 0.5	40.13 ± 2.3	0.75 ± 0.04	0.40 ± 0.02	0.30 ± 0.03
S 15%	30.8 ± 0.3	25.0 ± 0.2	n/a	n/a	n/a	n/a

Product yield values of four substrates showed that 15 % Muscat extract is the most efficient one. Product yield of 10% Muscat and 10% Syrah were calculated close to each other as 0.61 and 0.64. Because of having different maximum lactic acid production time limits, consumption and production rates of 15 % Muscat extract has the lowest value. As it is mentioned before fermentation with 15 % Syrah extract was not completed in 132, therefore comparison with 15 % Muscat could not be done. Comparing the lactic acid productions in 10% red and white GP, red GP was found to be a more efficient substrate than white GP in terms of yield.

Considering the economic value of fermentation in industrial scale, longer fermentation times may be a problem. Through having close maximum lactic acid concentrations, shorter fermentation process can be preferred in industrial area. The largest increase in lactic acid concentration was 0 to 17.85 g/l for 10% Muscat in 12 h. this value represent 70% of total lactic acid produced from 10 % Muscat. 10 % Syrah and 15 % Muscat produced 12.84 and 12.93 g/l lactic in same first 12 h period of fermentation which corresponded to 50 % and 32 % of total lactic acid produced. Overall production rates of 10 % Muscat, 10 % Syrah and 15 % Muscat were 0.40; 0.35 and 0.30g/l·h, respectively (Table 6.9). Considering the production in the first 12 h, production rates were 1.43; 1.07 and 1.0g/l·h

6.5.3. Effect of Yeast Extract Concentration in Lactic Acid Fermentation

Yeast extract is the one of the most important and expensive complex ingredient used in lactic acid fermentation by lactic acid bacteria. It is generally used for growth of the bacteria by providing nitrogen, vitamins and co-factors (Yue, Yu et al. 2012). Yeast extract concentration that was used in the previous experiments was kept as 10 g/l. Considering the cost of lactic acid production by *L. casei,* the effect of yeast extract concentration was investigated. In order to compare the effect of different yeast extract concentrations, same type of substrate was used for all experiments. 5-10-15 g/l yeast extract concentrations were used to produce lactic acid from 10 % (w/v) Muscat GP.

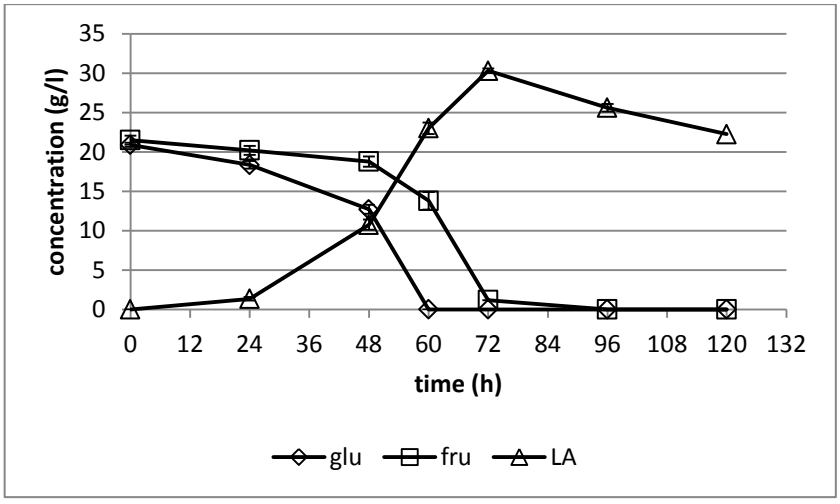

Figure 6.13 Fermentation with 10 g/l Yeast extract

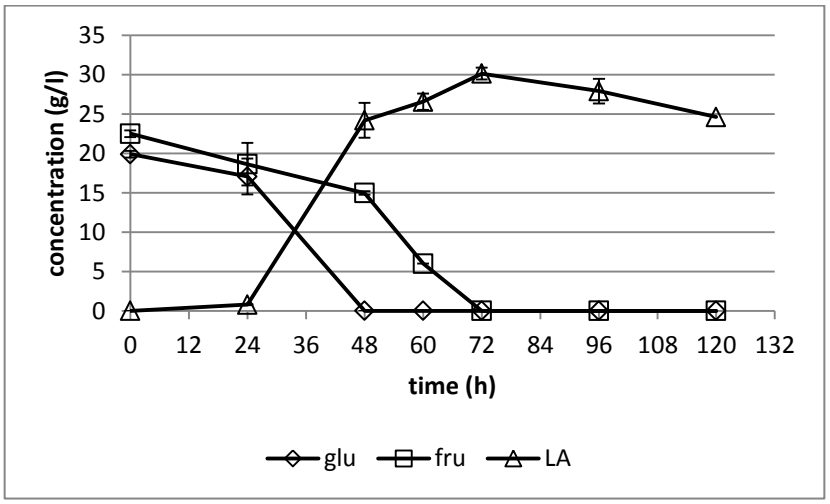

Figure 6.14 Fermentation with 15 g/l Yeast extract

As it was mentioned before fermentation process required nitrogen source in order to develop cell maintenance. Experiments were carried out to determine yeast extract concentration which was sufficient for lactic acid production from GP by *L. casei*. Figure 2.9, 2.10 and 2.11 represent lactic acid production from 10% (w/v) Muscat pomace by *L. casei* with yeast extract concentrations as 5, 10 and 15 g/l, respectively. 5 g/l yeast extract concentration was not enough for lactic acid production from GP. Initial glucose and fructose concentrations were measured as 20.8 and 20.6 g/l. After 120 h of fermentation there were no decrease on carbohydrate concentrations and there was no increase on lactic acid concentration. It was concluded that 5 g/l yeast extract was not enough to sustain growth, thus lactic acid production in GP.

Figure 6.13 and 6.14 represent similar behavior on lactic acid production however in fermentation with 15 g/l yeast extract all glucose was consumed after 48 h which was 12 h earlier than in fermentation with 10 g/l yeast extract. In fermentation with 10 g/l yeast extract there was still 12.7 g/l glucose in fermentation medium after 48h. At this time point lactic acid concentrations were 24.18 and 10.75 g/l with 15 and 10 g/l yeast extract, respectively (Figure 6.14 and 6.13). Using 15 g/l yeast extract lactic acid production rate was maximum between 48-60 hours of fermentation where the consumption rate of glucose was maximum as well (Figure 6.14). 10 g/l yeast extract resulted in similar behavior, but in different fermentation time periods (Figure 6.13). Maximum consumption rate of glucose and production of lactic acid took place between 24-48 hours of fermentation.

Table 6.10 Effect of yeast extract concentration on yield, production and consumption rates in lactic acid fermentation

	S-initial (g/l)		**P-max. (g/l)**	**Yield (P/S)**	**Consumption rate (g/l·h)**	**Production rate (g/l·h)**
YE	**glucose**	**fructose**	**LA**			
5 g/l	20.8 ± 0	20.6 ± 0	0	0	0	0
10 g/l	20.9 ± 0.3	21.5 ± 0.6	30.29 ± 0.7	0.72 ± 0.03	0.58 ± 0.04	0.42 ± 0.03
15 g/l	19.9 ± 0.4	22.5 ± 0.4	30.12 ± 1.0	0.71 ± 0.02	0.58 ± 0.03	0.42 ± 0.04

As a general view of lactic acid production from 10 and 15 g/l yeast extract concentration indicates that final lactic acid concentrations were so close each other (30.29 and 30.12 g/l). Reaching to these amounts of lactic acid concentrations took 72 hours of fermentation for both yeast extract concentrations. On the other hand, at high

yeast extract concentration 80 % of total lactic acid was produced in 48 hour however, at the same point 36 % of total lactic acid was produced at the lower concentration.

Starting point of this experiment set was to minimize the economic cost of lactic acid fermentation from GP by *L. casei*. According to the Table 6.10, yields of fermentations with 10 and 15 g/l yeast extract were so close to each other as 0.72 and 0.71. In a laboratory scale fermentation minimum yeast extract concentration may be more economic when reaching same amount of lactic acid with the same initial carbohydrate concentrations, but for an industrial scale less 24 hours of fermentation can be a big economic advantage even would not produce 100 % of theoretical lactic acid. Heating of fermentation tanks for industrial process to 37 °C and mixing at 1 g has a big economic problem comparing with laboratory scale experiments. According to this assumption, working volume for lactic acid production with GP by *L. casei* is an important parameter in order to designate the initial yeast extract concentration.

6.5.4. Fed-Batch System for Lactic Acid Production

After having promising results of lactic acid production from GP by *L. casei*, addition of more substrate to fermentation medium was tried in order to increase final lactic acid concentration. In the previous experiments 30.45 ±3.1 g/l lactic acid could have been produced from 10% (w/v) dry GP. For all analyses that discussed below 7 g dry GP was added to 60 ml of distilled water to reach 70 ml working volume. In this experiment, 7 or 3.5 g dry GP was also added after the glucose and fructose consumed. Aim of this experiment was to investigate the lactic acid production behavior with additional substrate but after analyzing the results of additional GP showed significant difference from previous analyses.

Two sets of experiments both started with 7 g dry Muscat pomace added into 60 ml distilled water in order to reach 10 % dry GP suspension. After 72 hours of lactic acid fermentation 3.5 and 7 g of dry Muscat pomace were added to each flasks and fermentations continued for 168 h. Experiment sets that 3.5 and 7 g dry Muscat pomace were supplemented in fermentation media were named as Case 1 and Case 2, respectively.

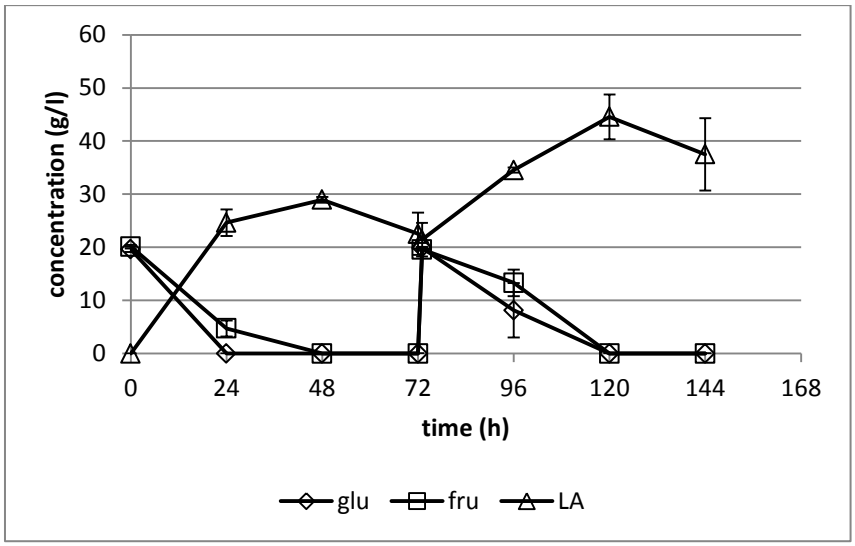

Figure 6.15 Case 1 lactic acid fermentation (3.5 g dry Muscat GP addition)

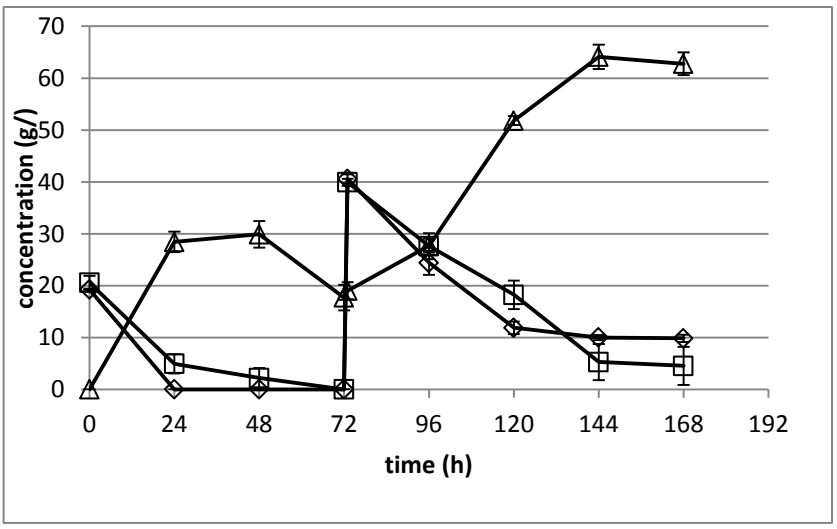

Figure 6.16 Case 2 lactic acid fermentation (7 g dry Muscat GP addition)

Figure 6.15 and 6.16 showed similar lactic acid production trends until 72 hours which time fresh GP was added. Initial glucose and fructose concentrations were measured for Case 1and Case 2 lactic acid fermentations as 19.28±0.17 g/l glucose and 20.57±0.33 g/l fructose ; 19.61±0.5 g/l glucose and 20.13±0.33 g/l fructose. Maximum lactic acid concentration for first phase of Case 1 and Case 2 fermentations were measured as 28.95±0.9 and 29.92±1.52 g/l at 48 h.

According to previous experiment results maximum lactic acid concentrations were measured for 10 % dry gape pomace suspension as 60-72 hours of fermentation. Because of this reason addition of GP was applied at 72 hours of fermentation. However, in this set lower lactic acid was observed at 72 h compared to 48 h. Lactic

acid concentrations were measured for Case 1 and Case 2 as 17.71±2.47 and 22.55±3.96 g/l at 72 hours of fermentations. 3.5 and 7 g dry Muscat pomace were added into each three flasks and after 15 min of mixing glucose and fructose concentrations were measured. Expected glucose and fructose values were similar or small increased with initial glucose and fructose concentrations but, sugar analyses from samples taken after pomace addition for Case 1 and Case 2 showed significant differences. Sugar analyses at 73 hours (which means after pomace addition) were measured as 40.59±0.87 g/l glucose and 39.93±0.66 g/l fructose for Case 1; 19.91±0.19 g/l glucose and 19.58±0.2 g/l fructose for Case 2 fermentation.

As it is observed in previous experiments, rate of glucose consumption was higher than fructose consumption. Also same consumption behavior was observed in the first stage (before pomace addition) of lactic acid fermentations for both Case 1 and Case 2. However, at the second stage (after pomace addition) of fermentations fructose consumptions were same or faster than glucose consumption by *L. casei*. Glucose and fructose were depleted at the same time (120 hour) (Figure 6.15In Case 2 that fructose was consumed more than glucose at the second stage of the fermentation (Figure 6.16).

Final lactic acid concentrations in Case 1 and Case 2 were measured as 44.55±4.2 and 64.3±2.34 g/l. In Case 1 fermentation all glucose and fructose were consumed in 120 h and the maximum lactic acid concentration was observed at this point of fermentation (Figure 6.15). In Case 2 fermentation process was stopped at 144 h while glucose and fructose were still present in fermentation medium with concentrations of 10.01±0.5 and 5.28±3.49 g/l (Figure 6.16). This can be due to high lactic acid concentration which may have inhibited the growth. It is also demonstrated that optimum pH value for lactic acid production from *L. casei* is between 5.5 and 6.5 (Büyükkileci and Harsa 2004). During all analyses pH value was kept at this point by addition of $CaCO_3$.

Table 6.11 Yield calculations of Case 1 and Case 2 fermentations.

		S-initial (g/l)		**P-max (g/l)**	**Yield (P/S)**
		glucose	**fructose**	**LA**	
Case 1	**1st phase**	19.6 ± 0.5	20.1 ± 0.3	29.9 ± 2.6	0.75 ± 0.07
	2nd phase	19.9 ± 0.2	19.6 ± 0.2	20.0 ± 4.7	0.55 ± 0.03
Case 2	**1st phase**	19.3 ± 0.2	20.6 ± 1.3	28.9 ± 0.5	0.73 ± 0.03
	2nd phase	40.6 ± 0.9	39.9 ± 0.7	46.7 ± 4.7	0.57± 0.01

Table 6.12 Yield (g/g) calculations of Case 1 and Case 2 fermentations.

		S-initial (g)	P-max (g)	Yield (P/S)
		GP	LA	
Case 1	**1st phase**	7,0	2.02 ± 0.04	0.29
	2nd phase	3.5	1.40 ± 0.33	0.40 ± 0.04
Case 2	**1st phase**	7.0	2.09 ± 0.03	0.30
	2nd phase	7.0	3.27 ± 0.33	0.47 ± 0.04

Table 6.11 showed the yield values of two cases of fermentations with two different phases considering the concentrations of initial sugar and final lactic acid. Yield values in first phases were calculated as more than second phases of Case 1 and Case 2. In Case 1, yield values for first and second phases were calculated as 0.75 ± 0.07 and 0.55 ± 0.03 which had a big difference from each other. This circumstance was predictable through accumulation of fermentation metabolites and depletion of salts in fermentation media. There may have been other reasons for the differences as increased lactic acid concentration and viscosity of fermentation media. Table 6.12 indicates that yield values for first phase was lower than second phase of Case 1.Yield values showed in Table 6.12 were calculated considering the initial sugar amount (g) and final lactic acid amount (g). It is possible to translate that substrate addition to fermentation medium increased the lactic acid production. In Case 12.02 ± 0.04 g of lactic acid was produced from 7 g of dry GP at first phase. Later with addition of 3.5 g of dry GP was able to produce1.40 ± 0.33 g of lactic acid at second phase of fermentation. Extraction performance of GP was the determining factor for this circumstance. Extracted sugar values for first phase of Case 1 were given in Table 6.11 as 19.6 ± 0.5 g/l glucose and 20.1 ± 0.3 g/l fructose which were extracted from 7 g of dry GP. After end of 72 hours of fermentation, 3.5 g of dry GP was extracted to 19.9 ± 0.2 g/l glucose and 19.6 ± 0.2 g/l fructose when the lactic acid concentration was 22.55 ± 3.9 g/l.

For Case 2, yield values showed same behavior as Case 1. As it is shown in Table 6.11 yield values based on initial sugar and final lactic acid concentrations for the first and second phases were calculated as 0.73 ± 0.03 and 0.57 ± 0.01. Same reasons which were mentioned above for Case 1 were valid for the difference in yield values for Case 2. Starting sugar concentrations for first phase were calculated as 19.3 ± 0.2 g/l glucose and 20.6 ± 1.3 g/l fructose. After 72 h of fermentation same amount of GP (7 g) was added into fermentation medium which was extracted to 40.6 ± 0.9 g/l glucose and 39.9 ± 0.7 g/l fructose when the lactic acid concentration was 17.71 ± 2.47 g/l. Same

extraction performance which was mentioned for Case 1 was observed for Case 2 too. Increased initial sugar concentration also increased the yield value of the second phase. Table 6.12 indicates that yield values for the first and second phase of Case 2 were measured as0.30 and 0.47 ± 0.04.

Table 6.12 indicates that more substrate addition increased the yield of lactic acid production. Even with unfermented sugar content (9.84 ± 0.7 g/l glucose and 4.55 ± 3.6 g/l fructose) in fermentation medium of Case 2 showed higher yield than Case 1. Yield values for second stages of Case 1 and Case 2 according to the Table 6.12 were measured as 0.40 ± 0.04 and 0.47 ± 0.04. These data can be a good reference in order toGP usage for industrial processes. As a general view GP is a cheap agricultural source. Therefore more substrate usage may be possible for larger working volume processes. But the purification process should be applied to obtain pure lactic acid. Also with an additional GP fermentation took more 48-72 hours after the first phase of lactic acid fermentation. Considering the economic cost of process more fermentation time may lead to have inefficient fermentation process. Working with larger volume needs larger process area and heating of fermentation tanks in a larger area may be more complex than laboratory scale fermentation.

6.5.5. Use of Commercial Yeast as Nitrogen Source

Yeast extract is generally required for the most of the microorganisms and cell growth in fermentation processes. Yeast extract is rich in different vitamins, amino acids and other growth stimulating compounds. Using of individual amino acids to maintain cell structure is more expensive than yeast extract usage. But still for most of the fermentation processes yeast extract is the most expensive compound (Hakobyan, Gabrielyan et al. 2012). Therefore, investment of different nitrogen sources for fermentation processes is necessary in order to decrease the experimental cost. Commercial yeast (CY) (*Saccharomyces cerevisiae*) which is used for bakery products with a commercial name Pakmaya was used instead of yeast extract for lactic acid fermentation from GP by *L.casei.*

Whole package of baker's yeast (42 g) was first dried in oven for 24 hours at 60 °C. 12 g of dry baker's yeast (which means 72 % moisture) supplemented into 250 ml flask with distilled water to reach 120 ml total working volume (10 % w/v solid

loading). pH value was adjusted between 5-7 to obtain accurate autolysis. Shaking speed was kept at 100 rpm at 50 °C for 48 hours. After incubation total volume of suspension was centrifuged and supernatant liquid was separated in order to supplement to fermentation flasks as nitrogen source instead of yeast extract. 10 ml or 25 ml of suspensions were supplemented into 250 ml of flasks with same salt and GP concentrations. Solid loading was kept same as previous experiments as 10 % and inoculation volume was also kept same as 2 ml of *L. casei*. Fermentations with 10 ml and 25 ml of baker's yeast suspensions were named as Case1 and Case 2 fermentations in order to simplify the writing.

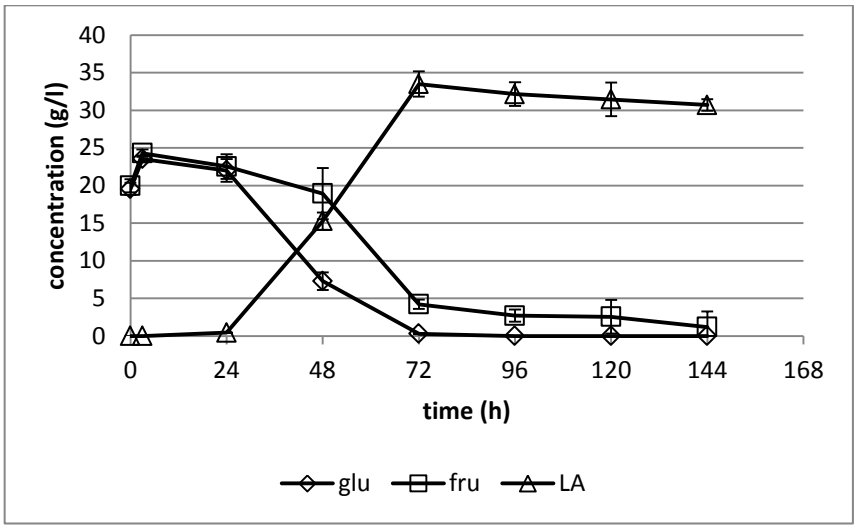

Figure 6.17.Lactic acid fermentation results with 10 ml baker yeast suspension (Case1).

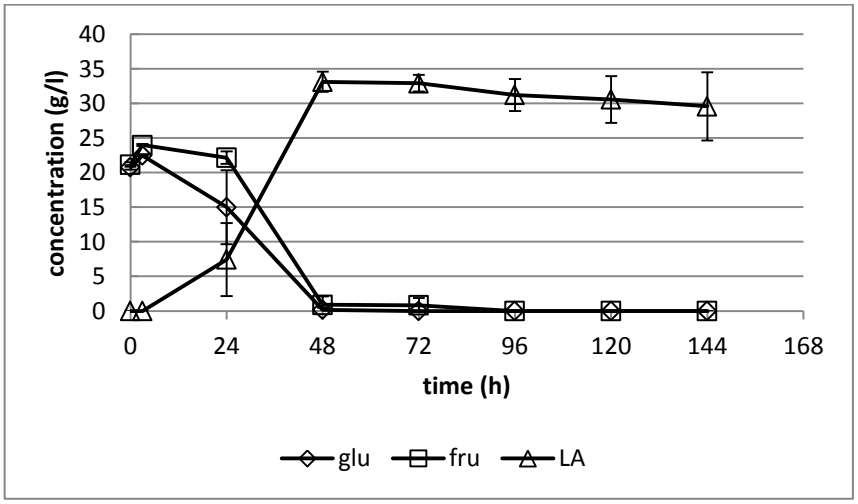

Figure 6.18.Lactic acid fermentation results with 25 ml baker yeast suspension (Case 2).

Lactic acid fermentation results with commercial baker's yeast extract from Muscat GP were shown in Figure 6.17 and 6.18 represent lactic acid fermentation results with 10 ml and 25 ml of baker's yeast suspensions from Muscat GP, respectively.

Figure 6.17 indicates that maximum lactic acid concentration was obtained at 72 h of fermentation for Case 1. Maximum lactic acid concentration was measured as 33.49 ± 1.68 g/l. It is also possible to say that lactic acid production started between 24-48 hours of fermentation and reached maximum amount at 72 hours. Initial glucose and fructose concentrations were measured as 19.55 ± 0.81 and 19.95 ± 0.86 g/l.

Figure 6.18 indicates that maximum lactic acid concentration was obtained at 48 hours that was 24 hour earlier than Figure 6.17. Maximum lactic acid concentration was measured as 33.11 ± 1.46 g/l that is similar with Case 1 fermentation. Lactic acid production started between 24-40 hours and reached the maximum level at this time limit.

Table 6.13 Consumption rate, productivity and yield calculations of different set of lactic acid fermentations.

	S- initial (g/l)		**P-max (g/l)**	**Consumption rate (g/l·h)**	**Productivity (g/l·h)**	**Yield**
	glucose	**fructose**	**LA**			
Case 1	19.55 ± 0.81	19.95 ± 0.86	33.49 ± 1.69	0.55 ± 0.03	0.47 ± 0.02	0.84
Case 2	20.75 ± 0.32	21.16 ± 0.32	33.11 ± 1.46	0.87 ± 0.01	0.69 ± 0.03	0.79

Table 6.13 indicates that major differences of Case 1 and Case 2 fermentations are consumption rate and productivity which are connected with maximum lactic acid production time or consumption of initial substrate time. Initial sugar concentrations for two cases were measured close to each other. Supplementation with different baker's yeast suspension volumes generally effected on maximum lactic acid production time limit and starting time of lactic acid production from GP.

According to the previous experiments (Section6.5.1, 6.5.2, 6.5.3 and 6.5.4) which were conducted in the presence of analytical grade yeast extract powder with different concentrations showed us that lactic acid concentrations generally sharply decreased just after reaching the maximum lactic acid concentration level. 10-25 % of total lactic acid was lost in next 24-48 h. Lactic acid concentration did not decrease as

sharply as fermentation processes. Stability of lactic acid may be achieved longer with CY than YE.

Yield values for Case 1 and Case 2 were calculated as 0.84 and 0.79. Comparing with the previous experiments baker's yeast usage increased the yield value of lactic acid production from GP. As mentioned before 10 ml and 25 ml of baker's yeast suspensions were used to produce lactic acid from GP. 10 ml of 10 % (w/v) solid loading represents 1 g of dry commercial yeast and 25 ml of 10 % (w/v) solid loading represents 2.5 g of dry commercial yeast. Yield values due to consumption of CY and maximum lactic acid production indicates the efficiency of commercial yeast usage as nitrogen source for lactic acid production from GP. In Section 6.5.3 which investigated the effect of yeast extract concentration on lactic acid production designates the yeast extract on lactic acid production. 5-10 and 15 g/l YE concentrations had been supplemented into fermentation flasks with the same conditions with fermentation processes that maintained by commercial yeast suspensions.

Table 6.14 Comparison of different nitrogen sources

	N source (g/l)	**P-max (g/l)**	**Yield**
		LA	**g LA/g dry N source**
Case 1	14	33.49	2.34
Case 2	35	33.11	0.93
10 g/l YE	10	30.29	3.03
15 g/l YE	15	30.12	2.0

YE; yeast extract

According to the Table 6.10 in Section6.5.3 maximum lactic acid concentrations obtained from 10 and 15 g/l analytical YE concentrations were measure as 30.29 ± 0.72 and 30.12 ± 1.0 g/l which mean in 70 ml of total volume as 2.12 and 2.10 g lactic acid. Experiment results of Case 1 and Case 2 ended up with 33.49 ± 1.69 and 33.11 ± 1.46 g/l lactic acid concentrations which mean in 70 ml of total volume as 2.34 and 2.32 g lactic acid. Comparison of these two individual experiments designates that increased nitrogen source may not increase the lactic acid amount per g of nitrogen source. It was also hard to compare two different experiments in a small range of nitrogen source concentrations but, CY usage has a high yield value which is close to YE usage. Considering the cost of experiments CY usage as nitrogen source instead of YE may be promising for lactic acid production from GP. CY which can be purchased

from most markets and much cheaper than YE but considering the autolysis process which is necessary to utilize CY as nitrogen source may increase the general cost of experiment. Heating up to 50 °C for 2 days is an expensive process that requires electricity and time. Development of autolysis process may provide the utilization of CY as nitrogen source for fermentation processes.

CHAPTER 7

CONCLUSION

Chemical composition of GP shows us that there was still residual sugar in GP after pressing and even after ethanol fermentation. Glucose and fructose concentrations that were measured before lactic acid fermentation processes designates that utilization of GP for fermentative processes is an efficient way to decrease the cost of fermentation processes and environmental pollution. According to the literature most of the studies about utilization of agricultural wastes include supplementation of extra carbon source in order to reach satisfied amount of products. Utilization of GP in this study demonstrated that GP can be used as a substrate individually for lactic acid production by *L. casei.*

Hydrolysis results of GP give us a clue about the requirement of optimization process in order to obtain fermentable sugar as possible as far as possible. In addition of obtaining no promising results from hydrolysis processes in this study, causing more acidic waste to obtain fermentable sugar is also a different consider for environment.

As a general view to this study GP is a carbon source that is generally disposed to open areas by wineries. Also lees that include dead yeast and other fermentative compounds is thrown away as a waste water. As mentioned before phenolic compound of these wastes inhibit the germination properties of soil. In order to prevent soil pollution waste management systems may be designed to wineries to refine and collect.

As general purposes extraction of phenolic compounds from GP may increase the yield of fermentation processes. Wineries may also design fermentation process in order to utilize GP and lees. Seeds, stems and lees may be investigated increasingly because of possibility of conveniently separation in wine making process.

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