BACTERIAL CELLULASE PRODUCTION USING GRAPE POMACE HYDROLYSATE AND SYNTHETIC SUGAR AS SOLE CARBON SOURCES BY SHAKE-FLASK SUBMERGED FERMENTATION

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ABSTRACT

BACTERIAL CELLULASE PRODUCTION USING GRAPE POMACE HYDROLYSATE AND SYNTHETIC SUGAR AS SOLE CARBON SOURCES BY SHAKE-FLASK SUBMERGED FERMENTATION

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Grape pomace is the major waste in the wine industry and it consists of high moisture content and residual sugars, which make it susceptible to rapid microbial spoilage. Thus, it needs to be disposed of with care to eliminate both environmental and health problems. The carbohydrate fraction of grape pomace is a fibrous material, and is an additional source of fermentable sugars to produce biofuel and hydrolytic enzymes.

In this study, grape pomace hydrolysate was used for cellulase production with *Bacillus subtilis* Natto DSM 17766 at 37 °C and 130 rpm using batch fermentation method. Cellulase production was optimized for solid loading of grape pomace, pH, and fermentation period by Box-Behnken response surface method. Furthermore, cellulase was produced by *Bacillus subtilis* NRRL B-4219 using monosaccharides which were mixed to obtain similar sugar content with grape pomace. Cellulase activity from both carbon sources were assayed by the DNS method using filter paper as substrate.

The maximum cellulase activity was obtained as 0.48 IU/mL from experiments conducted by synthetic sugar, which has the monosaccharide combination of glucose, fructose, xylose, arabinose, mannose, and galactose. In contrast, the highest cellulase activity using grape pomace was achieved at 0.196 IU/mL with 12.5% of solid loading at pH 7.0 on 5th day. The quadratic response surface model predicted an optimal cellulase activity of 0.178 IU/mL with 15% solid loading and pH 6.0 on 7th day. These results indicate that grape pomace is a potential carbon source for bacterial cellulase production.

Keywords: Cellulase, Grape Pomace, *Bacillus subtilis*, Submerged Fermentation, DNS Method, Filter Paper

ÖZ

KARBON KAYNAĞI OLARAK ÜZÜM POSASI HİDROLİZATI VE SENTETİK ŞEKER KULLANILARAK BATIK KÜLTÜR FERMANTASYONLA BAKTERİYEL SELÜLAZ ÜRETİMİ

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Şarap endüstrisinin temel atık maddesi olan üzüm posası yüksek miktarda nem ve artık şeker içerdiğinden dolayı hızlı mikrobiyal bozulmaya yatkındır. Bu yüzden, üzüm posası çevresel problemlere neden olmayacak şekilde atılmalıdır. Üzüm posasının karbonhidrat yapısı lifli madde olup biyoyakıt ve hidrolitik enzimlerin üretimi için ek bir mayalanabilir şeker kaynağıdır.

Bu çalışmada, üzüm posası hidrolizatından *Bacillus subtilis* Natto DSM 17766 ile 37 °C ve 130 rpm'de kesikli fermantasyon yöntemi kullanılarak selülaz üretildi. Selülaz üretimi Box-Behnken yüzey tepki yöntemiyle üzüm posası yüklemesi, pH, ve fermantasyon periyodu için optimize edildi. Ayrıca, üzüm posasının şeker içeriğini oluşturan benzer monosakkaritler kullanılarak *Bacillus subtilis* NRRL B-4219 ile selülaz üretildi. İki karbon kaynağından da elde edilen selülaz aktiviteleri substrat olarak filtre kağıdı kullanılarak DNS yöntemi ile analiz edildi.

Sentetik şeker kullanılarak gerçekleştirilen deneylerde en yüksek selülaz aktivitesi glikoz, fruktoz, ksiloz, arabinoz, mannoz, ve galaktoz monosakkaritlerinin

bileşiminde 0.48 IU/mL olarak elde edildi. Buna karşın, üzüm posasından elde edilen en yüksek selülaz aktivitesine 0.196 IU/mL olarak %12.5 katı yüklemesiyle pH 7.0'de 5. günde ulaşılmıştır. İkinci derece yüzey tepki modeli en yüksek selülaz aktivitesini %15 katı yüklemesiyle pH 6.0'da 7. günde 0.178 IU/mL olarak tahmin etmiştir. Bu sonuçlar üzüm posasının bakteriyel selülaz üretimi için potansiyel karbon kaynağı olduğunu göstermektedir.

Anahtar Kelimeler: Selülaz, Üzüm Posası, *Bacillus subtilis*, Batık Kültür Fermantasyonu, DNS Yöntemi, Filtre Kağıdı

To the ones that have meant so much to me, especially my beloved family

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CHAPTER 1

INTRODUCTION

Cellulose is a polysaccharide mostly found in plant biomass, and typically remains after various agricultural and food processes. It is also produced by several species of algae and some species of bacteria (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011). Cellulose contains ringed glucose molecules, which are linearly chained by β-1,4 linkages and released by the action of celullase enzyme with oligosaccharides.

Cellulase is an enzyme that attacks to the cellulose chain to hydrolyze oligosaccharides and polysaccharides to glucose monomers. It can be produced by microbial fermentation of synthetic sugar complex or lignocellulosic waste materials.

Grape pomace is one of the lignocellulosic waste materials generated by the wine industry. After fermentation of must, pomace is formed including skins, seeds, and stems. The grape pomace is an important source of phenolics and cellulosic biocompounds, but mostly discarded or under-utilized causing environmental problems.

Utilization of grape pomace is gaining importance to overcome environmental and economic impacts. In the literature, there are several studies reporting use of grape pomace to produce phenolic compounds, especially antioxidants and antimicrobials to enrich products in the food industry. Also, enrichment with fiber is applied to cereal

products. While growth of the spoilage and pathogenic microorganisms is inhibited due to the antimicrobial effect of grape pomace, the probiotics are protected by grape pomace against external factors. Furthermore, grape pomace is a source of natural food coloring. In this study, grape pomace was used for cellulase production by via *Bacillus subtilis* Natto DSM 17766.

This study also aims at increasing sustainability to the wine industry and utilizing grape pomace for cost-effective cellulase production, which is currently expensive due to use of pure substrates. By this way, the discarded waste of wine industry would be reduced, and environmental effects would be eliminated. Besides, an inexpensive substrate would be provided to the enzyme production industry.

The cellulase production was carried out after optimizing growth conditions of *Bacillus subtilis*. The synthetic monosaccharides were used to produce cellulase by *Bacillus subtilis* NRRL B-4219 in comparison to sugar substrates obtained from grape pomace (GP). The GP trials were undertaken at different conditions (pH, solid loading, and incubation time) designed by response surface method.

The current literature on grape pomace utilization and cellulase production by using different microorganisms from different waste materials are reviewed in chapter 2 for better understanding. Also, the factors that affect the cellulase production were discussed. In the light of literature review, cellulase production was carried out and optimal conditions were examined by Box-Behnken design, which were verified experimentally and by analysis of variance (ANOVA) to report reliable results.

To conclude, the effect of the independent variables (solid loading, pH, and time) on cellulase activity were analyzed. Also, the maximum and minimum cellulase activities

obtained from synthetic sugar and grape pomace were compared (Chapter 4). In addition, the recommendations are made to possibly increase the cellulase activity in future studies (Chapter 5).

CHAPTER 2

LITERATURE REVIEW

2.1. Grape pomace

Grape is mostly grown and consumed as fruit all over the world due to importance on human health, vinification process and economic aspects (Sousa et al., 2014). While nearly 50% of the grown grapes are used by the wine industry, one third is produced as table grape to consume freshly, and the rest is stored as dried, grape juice or musts. In addition to these products, there is a variety of processed forms of grapes such as vinegar, jam, grape seed extract and oil (FAO, 2016).

The agriculture of grape increases year by year because of usage on these manufacturing areas (FAO & USDA Foreign Agricultural Service, 2019). According to the statistics of global fruit production (FAO, 2019), by variety, the grape is in fourth place with 74.28 million metric tons. While 37% of the world production of grape is in Europe, Asia produces 34% of total world grapes, and 19% is produced in America. China is the leading grape producer with 13.7 million tons in 2017 (USDA Foreign Agricultural Service, 2018). Although China has the highest grape production, Spain has the most extensive vineyards. According to the research conducted by OIV (2018), Europe is using grape mostly in viniculture. The report of OIV shows that 73.3 million tons of grapes are produced all over the world and 52% of the total amount is used for wine production in 2017.

In the wine industry, processing of grape produces high amounts of grape pomace, which comprises skins, seeds, and stems. After fermentation and extraction of grapes, the generated residues are used for composting or discarded in the land field as wastes, which bring about environmental issues (Rondeau, Gambier, Jolibert, & Brosse, 2013). The high content of organic matter in grape pomace causes pollution problems when the pomace is discarded to open areas (Prozil, Evtuguin, & Lopes, 2012). As an organic solid waste, grape pomace emits methane gas, which is one of the most harmful greenhouse gases to influence climate change 20 times more than carbon dioxide. Thus, grape pomace needs to be biotransformed (Khatiwada, Ahmed, Sohag, Islam, & Azad, 2016).

The nutritional value of grape pomace is deficient to be an animal feed. Therefore, only 3% of residues are processed as animal feed (Brenes, Viveros, Chamorro, & Arija, 2016). As a result of fermentation, 13.5% to 14.5% of the total volume of grapes crushed are obtained as residues. It may reach up to 20% of total volume depending on the harvested grape conditions (Sousa et al., 2014). The amounts of residues also depend on grape species, and equipment used and process applied for pressing (Dwyer, Hosseinian, & Rod, 2014). Some researches show that 20-30% of the grape weight is obtained as grape pomace (Beres et al., 2017; Teixeira, Mateus, De Freitas, & Oliveira, 2018; J. Yu & Ahmedna, 2013). According to study conducted by García-Lomillo and González-SanJosé (2017), 75% of 63 million tons produced grape was used for wine processing and 20% of this grape, approximately 10 million tons, turned into grape pomace. This enormous amounts of grape pomace have severe consequences on the environment such as surface and groundwater pollution, unpleasant odor, oxygen reduction in soil (Beres et al., 2017). In recent studies, grape pomace is used for obtaining value added products because of sustainability and its significant amount of bioactive compound content.

2.1.1. Composition of grape pomace

The main components of grape pomace are water, proteins, carbohydrates, lipids, vitamins, and minerals. In addition to these components, it is composed of fiber, vitamin C, and phenolic compounds, which have significant biological properties. The composition and quality of grape pomace depends upon type of grape, stage of ripeness, fertilization conditions, harvesting and processing conditions, location and climate (Beres et al., 2017; Pujol et al., 2013; Rondeau et al., 2013; Sousa et al., 2014; Teixeira et al., 2018).

The dried grape pomace contains 8.49% protein, 29.2% carbohydrate, 8.16% lipid, 3.92% pectin, 8.91% fructose, 7.95% glucose and 46.17% total dietary fiber of dry basis (Table 2.1). Moreover, 26.25 mg ascorbic acid and 131 mg anthocyanin are found in 100 g of dried grape pomace. Also, mineral analysis showed that there are high concentrations of iron, potassium, zinc, manganese, and calcium in grape pomace flour (Sousa et al., 2014). Notably, the total sugar amount varies with wine type. Grape pomace from white wine making process has higher sugar content because skins and seeds do not go through fermentation. A study performed by Dwyer, Hosseinian, and Rod (2014) investigated the average total soluble solid content of grape pomace obtained from red and white wine processes. The total soluble solid content for grape pomace from white wine was reported as 78.15%, while value for red wine is 26.03%.

Table 2.1. *Typical composition of grape pomace* (Sousa et al., 2014)

Physicochemical Composition (% dry basis)	*Results (Mean ± SD)*
Protein	8.49 ± 0.02
Total Lipids	8.16 ± 0.01
Ash	4.65 ± 0.05
Carbohydrate	29.20
Pectin	3.92 ± 0.02
Fructose	8.91 ± 0.08
Glucose	7.95 ± 0.07
Total Dietary Fiber	46.17 ± 0.80

Rondeau et al. (2013) studied monosaccharide composition of eight different grape pomaces in addition to general composition and reported significant amount of glucose and xylose. For instance, 100 g of pomace obtained from grapes grown in Alsace, was composed of 21.54 g glucose, 4.59 g xylose, 4.13 g galacturonic acid, 2.66 g mannose, 2.04 g galactose, and 1.49 g arabinose. On the other hand, 100 g of pomace from Val de Loire contains 9.85 g glucose, 1.49 g xylose, 3.05 g galacturonic acid, 1.97 g mannose, 1.41 g galactose, and 0.97 g arabinose. The monosaccharide composition of other types of pomace is given in Table 2.2. Furthermore, Deng, Penner, and Zhao (2011) reported that grape pomace contains mostly glucose as neutral sugar in the analyzed five types of grape pomaces. Additionally, the fibrous fraction of grape pomace contains cellulose, hemicellulose, starch, and pectin. After hydrolysis of these polysaccharides, fermentable sugars are obtained as mono and disaccharides (Korkie, Janse, & Viljoen-Bloom, 2002). These polysaccharides from grape pomace also provide eco-friendly supporting products like biodegradable packaging material (Deng et al., 2011).

Table 2.2. *Monosaccharide compositions of grape pomaces (Deng et al., 2011)*

Monosaccharides (% dry matter)	*Cabernet Sauvignon*	*Merlot*	*Muller Thurgau*
Glucose	7.66 ± 0.54	9.54 ± 0.56	4.79 ± 0.64
Xylose	1.97 ± 0.12	2.17 ± 0.12	0.93 ± 0.09
Galactose	1.04 ± 0.05	1.17 ± 0.09	0.79 ± 0.09
Arabinose	0.83 ± 0.07	0.88 ± 0.07	0.5 ± 0.06
Mannose	1.53 ± 0.1	1.63 ± 0.08	0.54 ± 0.06

In another study (Beres et al., 2017), the chemical composition of grape pomace concerning different varieties such as Mario Mucato, Merlot, Pinot Noir, Cabernet Sauvignon was determined and Mario Mucato gave the highest soluble sugar content with 77.53%, whereas Merlot had the lowest content with 1.34%. Mario Mucato was the most moderate in total dietary fiber content with 17.3% which was around 50% for other grape pomaces. Besides, Yildirim et al. (2005) studied antioxidant activity and phenolic content of different types of grapes and reported the highest antioxidant activity and phenolic content in Cabernet Sauvignon and Merlot varieties.

The composition of wine pomace is affected by the conditions and type of wine-making process (García-Lomillo & González-SanJosé, 2017). Dietary fiber concentration was reported as 43-75%. Also, grape pomace contains significant concentration (4-14% in dry matter) of tartrates such as potassium bitartrate and calcium tartrate as minerals. Also, potassium, phosphorus, sulfur, magnesium, and calcium were detected in wine pomace.

As grape pomace composition is influenced by several conditions such as climate, grape type, location of grape growth, harvesting, and fermentation process, application

areas of grape pomace could differ by the composition of grape waste. Also, rich carbohydrate content of grape pomace makes it valuable resource for biorefinery.

2.1.2. Utilization of grape pomace

In recent years, the wine industry is concerned about the utilization of grape pomace. There are some studies conducted by Teles et al. (2019), Deng and Zhao (2011), Botella et al. (2005) to benefit from grape pomace, which is usually discarded to open areas. While 3% of grape pomace obtained from wine processing in the world is turned into animal feed, the rest is used for composting, producing high added value food products, and discarded as waste material. Additionally, it is used in cosmetic, and pharmaceutical industries and as supplements for sustainable practices such as dietary fiber and polyphenols, biosurfactants, grape seed oil, and antioxidants (Beres et al., 2017). Grape pomace is restricted in animal feed because of the high content of lignified fiber and tannin component, which affects animal nutrition negatively (Brenes et al., 2016).

Fresh grape pomace can go through composting, which is performed due to the aerobic microbial decomposition of natural elements like carbon and nitrogen and used in vineyards. Nitrogen content increases after composting and nitrogen is beneficial in the soil for vineyard growth. Although nitrogen need is provided by grape pomace compost in vineyards, the pomace is inadequate to supply minerals for land (Dwyer et al., 2014).

Some researches working on the bioactive composition of grape pomace show that obtaining biosurfactants, which have a significant role in food processing application as emulsifiers is possible (Rivera, Moldes, Torrado, & Domínguez, 2007).

Biosurfactants that are produced with the usage of grape pomace have lower toxicity than synthetic biosurfactants. Another positive aspect of creating natural biosurfactants is the higher biodegradability than synthetics (Dwyer et al., 2014).

In regard to another research (Beres et al., 2017), grape pomace extract (GPE) and flour (GPF) are a good source to prevent lipid oxidation resulting from antioxidant ability and content of dietary fibers. GPE and GPF are effective antioxidant sources for meat products. According to research conducted by García-Lomillo & González-SanJosé (2017), GPE and GPF can be used to inhibit lipid oxidation in a wide range of meat products. They show distinctive antioxidant activities under different storage conditions. They also have antioxidant effects on dairy products like yogurt and cheese. When grape pomace is compared with isolated phenolic compounds, the antioxidant activities of grape pomace products are higher.

Since grape pomace includes pectin, celluloses, and sugars, it can be used for edible film formation. Additionally, natural pigments, flavors and polyphenols in grape pomace have a positive effect on film formation (Deng & Zhao, 2011). The grape pomace extract and flour are beneficial with edible chitosan films to extend the shelf life of food (Beres et al., 2017). Grape seed extracts enhance the chitosan film capability (García-Lomillo & González-SanJosé, 2017).

Moreover, wine pomace inhibits the compounds that have a toxicological effect due to high-temperature processes. These compounds are produced because of Maillard reaction which occurs between free amino groups and carbonyl compounds. Grape pomace helps to decrease acrylamide which is formed in cooked starchy food like potatoes and cereal products throughout Maillard reaction (Beres et al., 2017; García-Lomillo & González-SanJosé, 2017).

Furthermore, grape seed obtained from pomace can be extracted to provide grape seed oil which is a source of lipophilic compounds for pharmaceutical, cosmetic, food, and biodiesel industries. The grape seed oil has biological significance due to phytosterols, tocopherols, tocotrienols, flavonoids, phenolic acids, and carotenoids contents (Beres et al., 2017). Furthermore, it has a high smoke point and enhances the nutritional properties of meat products. Hence, it provides a decrease in animal fat contents (García-Lomillo & González-SanJosé, 2017).

Recently, grape pomace has been used as carbon source to produce enzymes as there is growing interest in enzyme production from waste material. In the study conducted by Díaz, Ory, Caro and Blandino (2009), xylanase, exo-polygalacturonase, and CMCase were produced using grape pomace and orange peel mixtures by *Aspargillus awamori*. Also, Botella et al. (2005) reported cellulase, xylanase and pectinase enzymes using grape pomace by *Aspergillus awamori*. In addition, a cocktail of hydrolytic enzymes were investigated by *Aspergillus niger* in another study using grape pomace and wheat bran mixtures as substrates (Teles et al., 2019). Xylanase, CMCase, polygalacturonase, β-glucosidase, and tannase productions were reported in the study of Teles et al. (2019).

In the literature, cellulase production using grape pomace was generally obtained by fungi and solid state fermentation method was applied. On the contrary, in this study, *Bacillus subtilis* was used instead of fungi to produce cellulase using grape pomace as carbon source. Furthermore, submerged fermentation method was applied for producing cellulase.

2.2. Cellulase

Cellulose which is the primary component of plant biomass is produced as a result of photosynthetic reaction. Therefore, several lignocellulosic waste sources provide cellulose as by-product from industrial and agricultural processes (Sadhu & Maiti, 2013). Khatiwada et al. (2016) carried out research about the utilization of solid waste produced in Bangladesh. The authors claimed that 80% of the organic solid waste consists of cellulose, which can be a source for cellulase production. In addition, cellulose is the most abundant polysaccharide that is found on the earth (Abou-Taleb, Mashhoor, Nasr, Sharaf, & Hoda, 2009; Bayer, Chanzyt, Lamed, & Shoham, 1998; Behera, Sethi, Mishra, Dutta, & Thatoi, 2017; Decker, Adney, Jennings, Vinzant, & Himmel, 2003). It is composed of hemicellulose and lignin. Glucose units are linearly bound by β-1,4 linkages and thus form the cellulose component (Khatiwada et al., 2016). The chemical structure of cellulose is given in Figure 2.1. In order to obtain monomeric units of cellulose, a catalyst called cellulase is needed to perform the hydrolysis process (Behera et al., 2017).

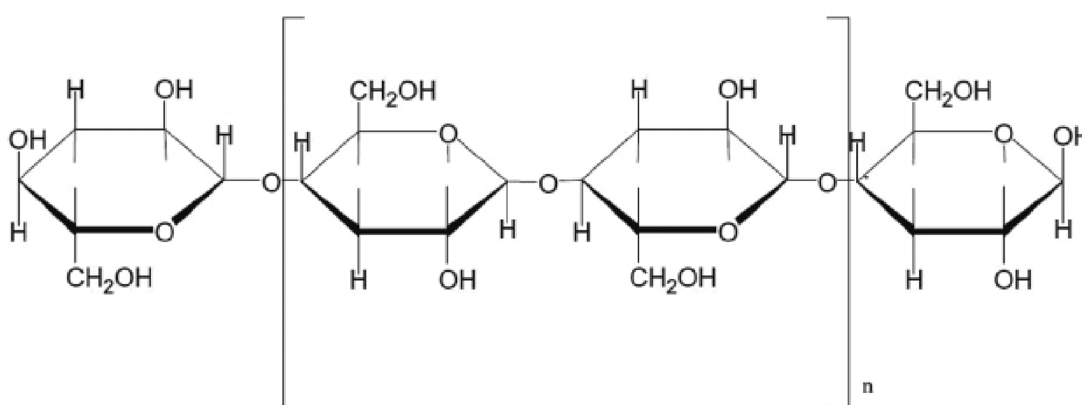

Figure 2.1. Chemical structure of cellulose (Thakur, Thakur, & Gupta, 2014)

Cellulases attacks the β-1,4 linkages in cellulose chain for hydrolysis (Bayer et al., 1998; Zhang & Zhang, 2013). According to Bayer et al. (1998), multiple enzymes are required to hydrolyze cellulose entirely because it is resistant to microbial degradation due to its crystalline structure. These enzymes are mainly categorized as endoglucanases, exoglucanases, and β-glucosidases with respect to their amino acid sequences and crystal structures.

Endoglucanases cleave internal β-1,4 bonds and produce new ends randomly. They bind to cellulose with their cleft shaped active sites and produce glucose, soluble cellodextrin, insoluble cellulose fragment or cellobiose by breaking the cellulose chain. Exoglucanases generate glucose or cellobiose as main products as a result of cleavage at non-reducing end of the cellulose chain. However, they have tunnel shape active sites (Bayer et al., 1998). Moreover, exoglucanases are mainly separated into two due to working processively from reducing end or non-reducing end (Orji et al., 2016). Endoglucanases and exoglucanases may have carbohydrate binding modules, whereas β-glucosidases do not include them. β-glucosidases generate glucose by attacking the nonreducing glucose unit in soluble cellodextrins and cellobiose with their pocket-shaped active site. They are inactive against the insoluble cellulose chains (Zhang & Zhang, 2013).

Cellulase breaks down the cellulose and hemicellulose to free sugars. Large amount of enzyme is required to hydrolyze cellulose completely. 25 kg cellulase is necessary to release most of the sugars from 1 ton cellulose (Carroll & Somerville, 2009). In another study, the required cellulase activity to hydrolyze cellulose is given as 10 000 FPU. In other words, 14-20 g enzyme protein is needed to hydrolyze 1 kg cellulose (Doppelbauer, Esterbauer, Steiner, Lafferty, & Steinmüller, 1987).

There is an increasing demand for cellulase in several industries such as textile, pulp and paper, food, and detergent industries (Abou-Taleb et al., 2009). Especially in the food industry, it is used for starch processing, grain alcohol fermentation, malting and brewing, and fruit and vegetable juice processing. Moreover, it is used in agriculture to prevent plant pathogen and disease (Behera et al., 2017). Also, it increases the nutritional quality and the digestibility of animal feed (Sadhu & Maiti, 2013). However, the cost of cellulase is the main problem that limits the usage of the enzyme (Vyas, Putatunda, Singh, & Vyas, 2016; Zhang & Zhang, 2013). Although the production cost of cellulase is high, the cellulase market expands because of bioethanol and biobased products formation from cellulosic materials (Sadhu & Maiti, 2013). Nowadays, utilization of the agricultural wastes which are produced abundantly and cause environmental pollution provides low-cost cellulose source for cellulase production (Lugani, Singla, & Sooch, 2015; Vyas et al., 2016).

The cellulase enzyme is secreted by several organisms like fungi, bacteria, protozoans, plants, and animals. Mostly, fungi and bacteria, which can be aerobic, anaerobic, mesophilic, or thermophilic synthesize cellulase during growth on cellulosic substances. According to Zhang and Zhang (2013), generally, aerobic microorganisms secrete a group of individual cellulases. These cellulases have a carbohydrate binding module in their structure. On the other hand, anaerobic microorganisms produce cellulosomes, which are large multienzyme complexes containing some enzymes that have the carbohydrate binding module.

Although bacteria have a higher growth rate and shorter generation time than fungi, it is not extensively used for cellulase production (Abou-Taleb et al., 2009; Khatiwada et al., 2016). In addition, bacteria are genetically varied and have high adaptability. They are highly amendable against genetic manipulation (Lugani et al., 2015). Thus, cellulase production can be achieved by bacteria too.

In the literature, there are several studies about producing cellulase by utilizing agricultural or industrial waste. Some of them were performed by fungi, such as *Trichoderma reesei* and *Aspergillus niger,* which were most commonly preferred microorganisms. In general, solid state fermentation method is applied when fungi are used for cellulase production. According to the research conducted by Abdullah and Greetham (2016)**,** municipal solid waste was used to obtain cellulase by *Aspergillus niger* and *Trichoderma reesei*. A cost-effective cellulase production carried out due to the utilization of solid waste, which is cheap and formed abundantly. The study showed that fungal cells need a longer time to grow and produce cellulase as 168 h. Thus, the length of incubation time may be a problem for developing the process of commercial cellulase production.

In another study, cellulase was produced by *Aspergillus niger* from coconut water and waste paper (Sharma, Sharma, & Kuila, 2016)**.** Moreover, Sun et al. (2010) studied on cellulase production by *Trichoderma sp*. from apple pomace. Also, Abo-State, Swelim, Hammad, and Gannam (2010) carried out a research about some critical factors that affect the cellulase production by *Aspergillus terreus* Mam-F23 and *Aspergillus flavus* Mam-F35 from wheat straw. According to these studies, the length of incubation time was accepted as a limiting factor to produce cost-effective cellulase.

Furthermore, a study performed by Mrudula and Murugammal (2011) investigated the production of cellulase by *Aspergillus niger* from coir waste. In addition, the production of hydrolytic enzymes like xylanase, exo-polygalacturonase, and carboxymethylcellulase by *Aspergillus awamori* from a mixture of grape pomace and orange peels was studied by Diaz et al. (2009).

In recent times, bacteria is studied as a potential culture for enzyme production because of its positive aspects that were mentioned before. Notably, the most significant advantage of bacteria over fungi is the high growth rate. *Cellulomonas*, *Cellvibrio*, *Pseudomonas sp*., *Bacillus sp*., and *Micrococcus* are some of the bacteria that have cellulolytic property. Sethi et al. (2013)**,** reported optimization of cellulase production from different bacteria isolated from soil, namely, *Pseudomonas fluorescens*, *Bacillus subtilis*, *E.coli*, and *Serratia marcescens. Pseudomonas fluorescens* was reported to be the highest cellulase producer compared to others.

According to a study conducted by Maki, Leung, and Qin (2009), in addition to having high growth rate property of bacteria, cellulases, which are produced by bacteria are more complex and found in multi-enzyme complexes. These forms increase the function and synergy of cellulase. Bacteria resistant to environmental stresses; therefore, they have a wide variety of habitat. Additionally, they produce celluloytic strains that are stable under harsh conditions. Also, these strains produce enzymes that can survive under extreme conditions. *Paenibacillus sp*. B39, *Paenibacillus campinasensis* BL11, *Bacillus subtilis* DR, *Brevibacillus sp*. JXL, *Bacillus agaradhaerens* JAM-KU023, *Cellulomonas flavigena*, and *Terendinibacter turnerae* T7902 were examined as an example of novel bacteria that produce cellulase.

In regard to another research (Islam & Roy, 2018), cellulase-producing bacteria in molasses were isolated and used to produce cellulase. These bacteria were identified as *Paenibacillus sp*., *Bacillus sp*., and *Aeromonas sp*. after isolated from molasses. Various optimized conditions were found for growth of different strains.

Ladeira et al. (2015) also researched on cellulase production by thermophilic *Bacillus sp*. SMIA-2 using the cellulosic waste materials sugarcane bagasse and corn steep

liquor. As stated in the study, numerous extracellular polysaccharide hydrolyzing enzymes including cellulase can be achieved by *Bacillus sp*. The reason for preferring fungi to *Bacillus sp*. for production of cellulase is that some *Bacillus* cellulases cannot hydrolyze the crystalline structure of cellulose.

The cellulase yields not only depend on the types of microorganism but also depend on culture conditions. According to Abou-Taleb et al. (2009) and Lugani et al. (2015), cellulase production is based on some nutritional and environmental factors such as inoculum size, pH value, temperature, presence of inducers, carbon source, nitrogen source, aeration, growth, etc. which are discussed in the following section and Table 2.3 shows cellulase activities obtained by different bacteria under different conditions.

Table 2.3. *Bacterial cellulase activity and production conditions*

Bacteria	*Cellulase Activity (IU/mL)*	*Production Conditions*
Bacillus sp. Y3	6.84 IU/mL	37 °C, pH 7.0, for 96 h (Lugani et al., 2015)
Bacillus licheniformis MVS1	0.542 IU/mL	50 °C, pH 6.5, for 60 h (Acharya & Chaudhary, 2012)
Bacillus sp.	20 IU/mL	45 °C, pH 7.5, for 48 h (Verma, Verma, & Kushwaha, 2012)
Bacillus sp. MTCC10046	6.41 IU/mg protein	37 °C, pH 7.0, for 8 days (Sadhu, Ghosh, Aditya, & Maiti, 2014)
Bacillus sp. BSS3	104.68 IU/mL	37 °C, pH 9.0, for 6 h (Sreedevi, Sajith, & Benjamin, 2013)
Bacillus sp. C1AC55.07	0.366 IU/mL	32 °C, for 54 h (Dias et al., 2014)
Bacillus subtilis	3205 IU/mg	50 °C, pH 6.0, for 10 h (Chan & Au, 1987)
Bacillus subtilis	31.87 IU/mL	30 °C, pH 7.0, for 72 h (Bai et al., 2012)
Bacillus pumilis EB3	0.011 IU/mL	60 °C, pH 6.0, for 24 h (Ariffin, Abdullah, Kalsom, Shirai, & Hassan, 2006)
Bacillus sp. FME2	45 IU/mL	30 °C, for 6 days (Kumar et al., 2008)
Bacillus subtilis AS3	0.75 IU/mL	39 °C, pH 7.2, for 48 h (Deka et al., 2013)
Bacillus sp. SMIA-2	0.29 IU/mL	50 °C, pH 8.0, for 168 h (Ladeira et al., 2015)

2.2.1. The factors that affect the production of cellulase

There are various factors that affect cellulase production. These factors are pH, temperature, source of carbon and nitrogen, solid load, aeration etc. For instance, Khatiwada et al. (2016) worked on cellulase from municipal solid wastes and rice straw wastes by isolated three different bacteria, which were *Bacillus sp.*, *Pseudomonas sp.*, and *Serratia sp*. The effects of pH and temperature on cellulase activity and stability were studied. The research concludes that the optimum temperature for production by *Bacillus sp*. and *Pseudomonas sp*. was found at 37 °C, whereas the highest cellulase yield was obtained at 35 °C for the production performed by *Serratia sp*. In addition, optimum initial pH value for all bacteria species was found as 7.

In another study, the aeration effect was studied (Abou-Taleb et al., 2009). 0, 50, 100, 150, and 200 rpm of shaking rates were experimented and the maximum cellulase activity was obtained as 2.97 IU/mL at 150-200 rpm for *B. alcalophilus* S39 and *B. amyloliquefaciens* $C2_3$. Also, same agitation speed values were used in the study conducted by Sreedevi et al. (2013) and 150 rpm gave the highest cellulase activity.

A study performed by Sreedevi, Sajith, and Benjamin (2013) investigated cellulase producing bacteria from the wood-yards. In this study, optimization was performed to analyze the effects of pH, temperature, carboxymethylcellulose (CMC) concentration and agitation. CMC was used as a fermentation substrate. When the molecular characterization of wood-yards was performed, *Achromobacter xylosoxidans* BSS4, *Bacillus sp.* BSS3, and *Pseudomonas sp.* BSS2 were identified. In conclusion, the maximum cellulase activity was 91.28 IU/mL at pH 8.0 and 12 h incubation by using *Pseudomonas sp.* BSS2, whereas *Achromobacter xylosoxidans* BSS4 has the highest

activity as 68.37 IU/mL at pH 7.0 after 6 h incubation. In contrast, *Bacillus sp.* BSS3 performed the maximum activity as 104.68 IU/mL at pH 9 after 6 h incubation. Optimum temperature and agitation were found respectively 37 °C and 150 rpm for all three cultures that are used in the study. Additionally, the optimum substrate concentration for *Bacillus sp*. BSS3 and *Pseudomonas sp.* BSS2 was analyzed as 1%, while it is obtained as 0.5% for *Achromobacter xylosoxidans* BSS4.

Vyas, Putatunda, Singh, and Vyas (2016) reported cellulase production by *Bacillus subtilis* M1 using groundnut shell as a source of cellulose. According to the research, the pretreatment of groundnut shell is necessary to expand the surface area of cellulose and decrease its crystallinity. As a consequence of the fermentation, the medium which was prepared with pretreated groundnut shell had higher cellulase activity than the medium developed with untreated groundnut shell. Furthermore, the influence of substrate concentration on cellulase production was studied. As a result, the activity of endoglucanase increases gradually up to 2% substrate concentration. The highest endoglucanase activity was found as 0.564 IU/mL. However, 1% of substrate concentration provided the maximum exoglucanase activity as 0.090 IU/mL.

In another research, the optimization of cellulase production from newly isolated *Bacillus sp.* Y3 was studied (Lugani et al., 2015). The factors that affect the cellulase production were chosen to study like carbon source, nitrogen source, temperature, pH, inoculum concentration, and incubation time. As a result of the study, the maximum cellulase activity was observed at 37 °C as respectively 4.17 and 4.37 IU/mL for filter paper assay and carboxymethylcellulase assays. In another study conducted by Khatiwada et al. (2016), the optimum pH value was obtained as 7. The maximum cellulase activities were found as respectively 4.22 and 4.76 IU/mL as a result of FPase and CMCase assays. In addition, the maximum FPase and CMCase activities were

observed 4.89 and 5.36 IU/mL respectively when 2% (v/v) of inoculum concentration was used. The optimum incubation time was found 96 h.

In the light of these studies, the optimum pH and temperature for cellulase production were examined as 7.0 and 37 °C. In addition, *Bacillus subtilis* was found as the most effective bacteria for cellulase production (Lugani et al., 2015; Verma et al., 2012). Furthermore, CMC and lignocellulosic materials were found more effective than other synthetic sugar components to produce cellulase. In the study conducted by Lugani et al. (2015), different carbon sources were used to produce cellulase. Wheat bran, rice bran, lactose, glucose, and CMC with 1% solid load were the experimented carbon sources at 37 °C for 72 h and the highest cellulase activity was obtained from CMC as 3.74 IU/mL. Wheat bran and rice bran also had high activities in comparison glucose and lactose.

2.3. *Bacillus subtilis*

Bacillus subtilis is a Gram-positive, facultative anaerobic, and rod shape bacterium. *Bacillus subtilis* produces spores under extreme conditions (Aizawa, 2014). *Bacillus subtilis* can sense the oxygen concentration and adapt their metabolism correspondingly (Wang et al., 2008). *Bacillus subtilis* is examined by numerous studies since it can generate varieties of significant biotechnological substances. Some of the *Bacillus* strains are the most significant industrial enzyme producer in terms of producing a high amount of extracellular enzymes. There are several studies that showed that *Bacillus subtilis* can produce many enzymes such as amylases, proteases, lipases, xylanases, pectinases, and cellulases (Barros, Simiqueli, De Andrade, & Pastore, 2013; Blanco, Durive, Pérez, Montes, & Guerra, 2016; Chan & Au, 1987;

Nawawi, Mohamad, Tahir, & Saad, 2017; Vijayalakshmi, Ranjitha, & Devi Rajeswari, 2013).

Bacillus subtilis (natto) Takahashi is used for preparing a traditional Japanese food called natto. It is a fermented soybean product. When it is grown in sucrose and L-glutamate containing medium, the production of poly ϒ-glutamic acid and levan is observed. One of the most important activities of *Bacillus subtilis* (natto) is producing proteases, amylases, and cellulases. The conditions at 37 °C and pH 7.0 is convenient for optimum growth (Shieh, Thi, & Shih, 2009).

2.4. Cellulase assay

Enzyme assays are categorized in two as continuous assays and discontinuous assays. Continuous assays monitor the changes in substrates or products continuously. They are principally performed by spectroscopic techniques, for instance, electronic ultraviolet-visible absorption and fluorescence emission (Harris & Keshwani, 2009). The simplest way of continuous assay is to observe the change in absorbance. Another continuous assay is a coupled continuous method. Usually, other enzymes are involved in the process to obtain the final product. The main advantage of this method is that product inhibition is prevented by the elimination of product (Scopes, 2002). Ultraviolet-visible absorption spectroscopy is a suitable method in order to determine concentration changes. However, concentration range and the number of enzyme-substrate complexes and products limit the method (Harris & Keshwani, 2009).

On the other hand, discontinuous assays, also called stopped assays, monitor the changes in the concentration of substrate and product. The enzymatic reaction is stopped at a specific time to analyze product formation. In this method, the enzyme is

denatured by the addition of strong acid, alkali, or inhibitor; or heat treatment to stop the reaction and measure the activity (Scopes, 2002).

There are several assays that measure cellulase activity such as viscosimetric assay, cellulose azure assay, and filter paper degrading assay (Ghose, 1987). Most assays are accomplished with soluble cellulose derivatives like carboxymethylcellulose, which is easily degradable. The cellulase activity is often detected by CMC-agar plate method. CMC, which is the substrate on agar plate observed with respect to precipitation of undigested ones. The advantage of this method is that it can be applied to large numbers of samples and results can be obtained simultaneously. In spite of extensive usage of this method, the main problem is its low specificity and producing halos around independent substrates (Johnsen & Krause, 2014).

Most of the assays analyze cellulase activity by measuring reducing sugar or total sugar discharged in solution. While the dinitrosalicylic acid (DNS) method and the Somogyi-Nelson method are used for analyzing reducing sugar, the phenol H_2SO_4 and the anthrone H_2SO_4 methods analyze total sugar in solution (Wood & Bhat, 1988).

Some assays establish existence of the cellulase components, β-glucosidase, endoglucanase, and exoglucanase. *p*-nitrophenyl β-D-1,4-glucopyranoside (*p*NPG) method is applied for the analysis of β-glucosidase activity. The other assay recommended by IUPAC for analyzing β-glucosidase is cellobiose assay. Endoglucanase also called CMCase assay is recommended by IUPAC for analyzing endoglucanase activity. DNS method is applied to measure reducing end. Lastly, exoglucanase activity is estimated by Avicel substrate that has the highest accessibility. Also, amorphous cellulose can be used as a substrate (Zhang, Hong, & Ye, 2018).

The most common method for measuring the cellulase activity, filter paper assay (FPA), is a very simple and useful method that does not require complicated equipment. It is recommended by the International Union of Pure and Applied Chemistry (IUPAC). Total cellulase formation is established by FPA (Zhang et al., 2018). As a substrate, 50 mg (1x6 cm) Whatman No. 1. filter paper is used. Reducing sugar is estimated by DNS method to carry out the filter paper assay. The cellulase activity is measured by determining the released reducing sugar from filter paper (Decker, Adney, Jennings, Vinzant, & Himmel, 2003). Quantitative spectrophotometric is carried out with DNS to quantify the activity (Johnsen & Krause, 2014). Although FPA is used worldwide, the problems about this method are labor intensiveness, low throughput and consuming a high amount of substrate, enzyme, and chemicals (Xiao, Storms, & Tsang, 2004; Yu et al., 2016). In addition, size, shape, and improper folding of filter paper can bring about errors in the analysis (Decker et al., 2003). In contrast to its disadvantages, the filter paper assay is easily found and inexpensive. Also, it provides reliable results (Xiao et al., 2004).

2.5. Submerged fermentation

Fermentation is a process that converts complex substances into simple components by several microorganisms like fungi or bacteria (Sadhu & Maiti, 2013). Mainly, alcohol and carbon dioxide are released during fermentation. Also, there are secondary metabolites that are formed as additional compounds. Various antibiotics, peptides, enzymes, and growth factors are examples of secondary metabolites and also called bioactive compounds due to biological activity. These bioactive compounds are produced by two techniques as solid state fermentation and submerged fermentation, which are designed for industrial level production (Subramaniyam & Vimala, 2012). These techniques are preferred regarding the type of used substrate and microorganism

and environmental conditions. Both of them were studied for cellulase production (Abdullah, Greetham, Pensupa, Tucker, & Du, 2016).

Solid state fermentation uses solid wastes as substrate (Sadhu & Maiti, 2013). It is carried out very slowly, so it requires a long fermentation period. In addition, solid state fermentation is performed with low water activity. Thus, fungi are the most convenient microorganism for it (Sadhu & Maiti, 2013; Subramaniyam & Vimala, 2012). On the other hand, submerged fermentation or liquid fermentation requires high moisture content. Therefore, bacteria are the most suitable microorganisms for submerged fermentation. They use liquid substrates like molasses and broths (Subramaniyam & Vimala, 2012). This method takes place quicker than solid state fermentation. Also, produced enzymes by submerged fermentation can be purified easily (Sadhu & Maiti, 2013). Since the genetically modified microorganisms can be utilized, submerged fermentation is the most used method for industrial enzyme production (Sreedevi et al., 2013). Moreover, the cultivation parameters and environmental conditions can be controlled easily during submerged fermentation (Hashemi, Mousavi, Razavi, & Shojaosadati, 2013). Another advantage of submerged fermentation is that it requires less number of space (Sharma et al., 2016).

2.6. Objectives of the study

Grape pomace is the main waste material in the wine industry. It is not convenient for animal feed due to its inadequate nutrition level. Therefore, industries prefer to discard grape pomace to open areas. This causes serious environmental problems. This waste material is an important source for bioprocessing in economic way while reducing the environmental effects.

Cellulase is the main enzyme required to hydrolyze cellulose and used in the several industries. Thus, there is an increasing demand for cellulase. Nowadays, production of cellulase is carried out by using the cellulosic waste materials from agriculture and food industry. While this approach reduces cost, it decreases the waste material that causes pollution too.

There are several methods that are suggested for cellulase synthesis such as submerged and solid state fermentation. The efficiency of production depends on the method used and microorganisms preferred. Submerged fermentation is more effective for cellulase production by bacteria. Also, *Bacillus subtilis* is a promising microorganism that produces enzymes like proteases, amylases, and cellulases using different waste materials as stated in section 2.3.

In this thesis, the main objective is to produce cellulase enzyme using synthetic sugars and grape pomace as sole carbon sources and to confirm bacterial production of cellulase at a minimal cost. For this purpose, *Bacillus subtilis* NRRL B-4219 and *Bacillus subtilis* Natto DSM 17766 were used to carry out fermentation at 37 °C, and 130 rpm. Hence, the growth characteristics of *Bacillus subtilis* were investigated and optimum conditions were determined.

Five different synthetic sugar media were experimented for cellulase production in comparison to the lignocellulosic grape pomace medium. The synthetic sugar content was kept at 2%. Temperature, agitation speed, and pH were held constant at 37 °C, 130 rpm, and pH 7.0.

The experiments containing grape pomace was designed and optimized using response surface methodology with three independent variables, pH (5.0-9.0), solid loading (5-20%), and incubation time (3-7 days). The fermentation temperature and agitation speed were kept constant at 37 °C, and 130 rpm.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Raw materials

Grape pomace, a waste material from vinification process, was supplied by Kavaklıdere Wines Co. (Ankara, Turkey). Grape pomace was dried in a laboratory scale tray dryer (Eksis Endustriyel Kurutma Sistemleri, Isparta, Turkey) at 70 °C for 24 h with 1.2 m/s of air blowing and trays rotating at 6 rpm. The dried pomace was then milled through a 1 mm sieve by a laboratory type grinding mill (Fritsch Industriestrasse 8, D-55743 Idar-Oberstein, Tüv-Cert, Germany) and stored in zip-lock bags until use. The grape pomace flour was stored at room temperature.

Bacillus subtilis Natto DSM 17766 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and *Bacillus subtilis* NRRL B-4219 (Agricultural Research Service (ARS) Culture Collection, Northern Regional Research Laboratory, Peoria, Illinois, USA) were used for cellulase production. Bacteria were activated in nutrient broth. The activated microorganisms were mixed with 50% glycerol-water and stored in 1 mL eppendorf tubes at -80 °C for long term use (Revco Elite Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA). Some culture was also kept at -20 °C for short term use.

3.1.2. Chemicals

The chemicals used in this study to prepare growth and fermentation media and to carry out enzymatic analysis were analytical grade. The used chemicals are listed in Table A.1 in Appendix A.

3.1.3. Buffers and solutions

Citrate buffer and DNS solution were used for measuring enzymatic activity and reducing sugar concentration. Their preparations are shown in Appendix B.

3.1.4. Growth medium

The growth medium for activation of bacteria and inoculum preparation was composed of 1 g/L KH_2PO_4; 0.05 g/L $CaCl_2.2H_2O$; 1.145 g/L K_2HPO_4; 0.4 g/L $MgSO_4.7H_2O$; 0.00125 g/L $FeSO_4.7H_2O$; 10 g/L glucose and 2 g/L yeast dissolved in distilled water. The pH of growth media was adjusted to 7.0 by 10 M NaOH and 10 M H_2SO_4 and sterilized at 121 °C for 15 min in an autoclave (Tomy SX-700E, Tomy Kogyo Co., Tokyo, Japan).

3.2. Methods

3.2.1. Dilute acid pretreatment and preparation of grape pomace hydrolysate

Dilute acid pretreatment method was used to release sugar from grape pomace. Grape pomace flour was weighed as 5 g, 12.5 g and 20 g. Different solid loads of grape pomace flour was pretreated with dilute sulfuric acid at concentration of 3% (w/v). Acid treatment was performed at 121 °C for 15 min in an autoclave. After acid hydrolysis, the liquid, which has sugar released from grape pomace, was obtained by suction filtration (Vac Torr 75, GCA/Precision Scientific, Chicago, Illinois & MVP 6, Woosung Vacuum Co., Ltd., Incheon, Korea). The pH of obtained filtrates were then adjusted to 5.0, 7.0 and 9.0 using 10 M NaOH and 10 M H_2SO_4. When the pH adjustments were carried out, salt production occurred in the filtrate due to acid-base reaction. Therefore, in order to remove undesirable salt from the liquid media, centrifugation was performed at 20 °C, 19620 x *g* for 12 min (Sigma 2-16PK, SciQuip Ltd., UK).

3.2.2. Fermentation media preparation

3.2.2.1. Fermentation medium includes grape pomace as a carbon source

Minerals viz. 1 g/L KH_2PO_4; 0.05 g/L $CaCl_2.2H_2O$; 1.145 g/L K_2HPO_4; 0.4 g/L $MgSO_4.7H_2O$; 0.00125 g/L $FeSO_4.7H_2O$; and 2 g/L yeast extract were dissolved in 100 mL centrifuged liquid to form media for growth and fermentation in the 500 mL flasks (Dias et al., 2014). The produced fermentation media were sterilized at 121 °C

for 15 min in the autoclave (Tomy SX-700E, Tomy Kogyo Co., Tokyo, Japan). Figure 3.1 shows the process for preparation of fermentation medium using grape pomace.

3.2.2.2. Fermentation medium includes synthetic sugar as a carbon source

Synthetic sugars of different components as shown in Table 3.1 were mixed with chemical nutrients to prepare the synthetic fermentation media. Into 100 mL of fermentation media prepared in the 500 mL flasks, 2% sugar was added. Then all flasks were autoclaved at 121 °C for 15 min.

Table 3.1. *Monosaccharide content that used in synthetic sugar media*

Flask Number	*Monosaccharide Content*
F1	Glucose + Fructose
F2	Glucose + Fructose + Xylose
F3	Glucose + Fructose + Xylose + Arabinose
F4	Glucose + Fructose + Xylose + Arabinose + Mannose
F5	Glucose + Fructose + Xylose + Arabinose + Mannose + Galactose

3.2.3. Biomass assay

Bacillus subtilis Natto DSM 17766 and *Bacillus subtilis* NRRL B-4219 were grown at 37 °C, 130 rpm for 24 h and used as starter cultures for cellulase production by submerged fermentation. Optical density of bacterial culture was measured at 600 nm using a spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan).

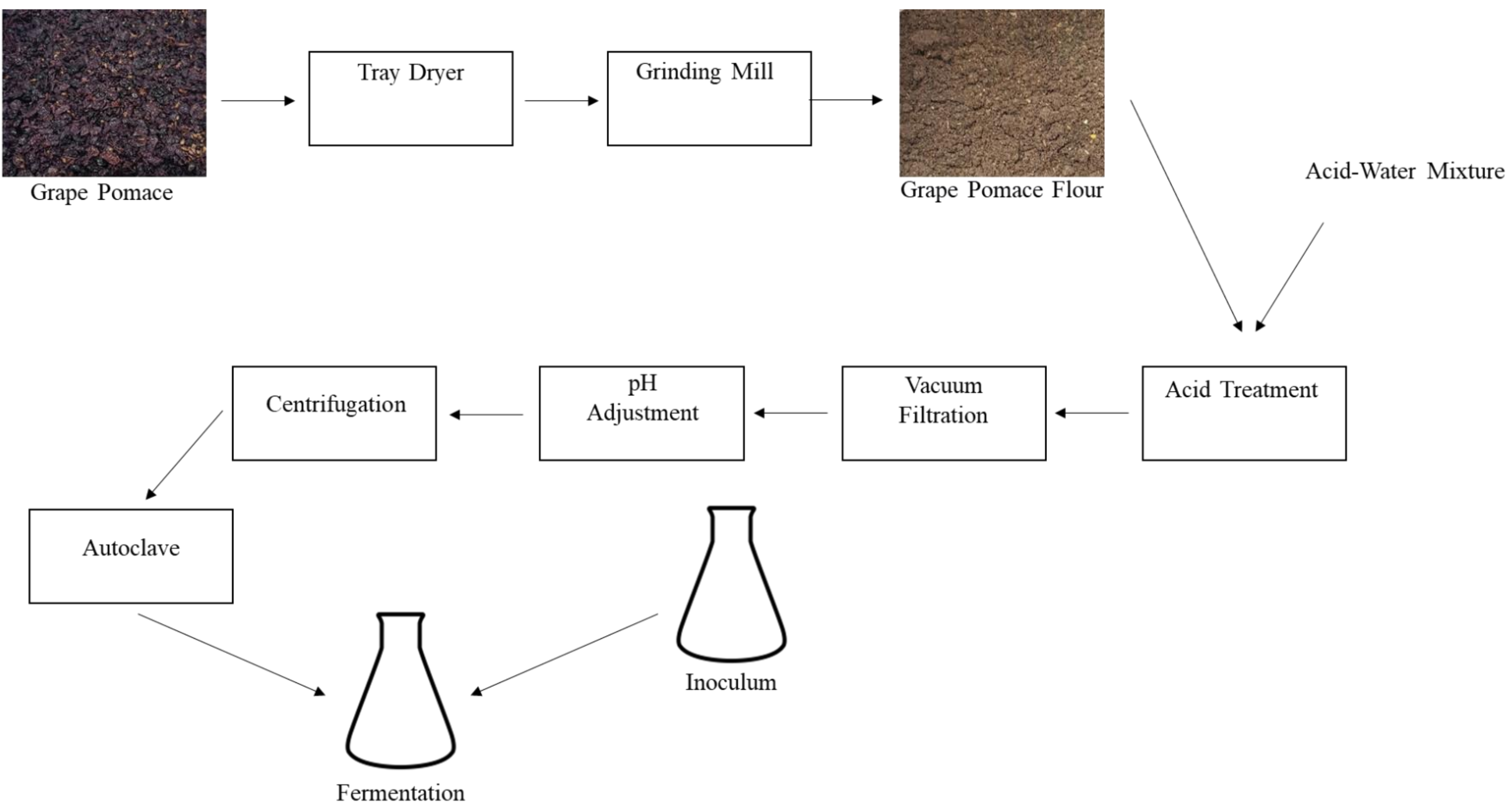

Figure 3.1. Overview of the processes carried out for cellulase production by submerged fermentation

3.2.4. Reducing sugar analysis

Reducing sugar analysis was performed to measure initial and final fermentable sugar concentration in all fermentation media. Samples were centrifuged to obtain cell free media (MPW-15 Mini Centrifuge, MPW Med. Instruments Co., Warsaw, Poland) which were diluted 60 times with distilled water. The DNS method was used to measure reducing sugar (Miller, 1959). The DNS solution for reducing sugar analysis is given in Appendix B.

For DNS analysis, 3 mL diluted sample were placed to tubes and 3 mL DNS solution was added to each tube. Then, the tubes were put into water bath at 100 °C for 15 min. After boiling, 1 mL of Rochelle salt solution was added to each tube and samples were cooled to ambient temperature. The absorbance of samples was measured by a spectrophotometer at wavelength of 575 nm. The absorbance values were translated into glucose concentration using standard curve prepared for 0-1 g/L of glucose (Appendix D).

3.2.5. Cellulase assay

The cellulase assay was carried out by the DNS method using filter paper as substrate (Ghose, 1987; Promega, 2018; Stoll & Blanchard, 1990; Wood & Bhat, 1988). The DNS solution for enzyme analysis is given in Appendix B. The samples were centrifuged to obtain the enzyme supernatant, which was diluted 15 times with citrate buffer. Whatman No.1 filter paper stripes (1.0 x 6.0 cm) were folded and placed into glass tubes. Then, 0.5 mL of diluted samples were taken into tubes and 1 mL of citrate buffer was added to each tube. The tubes were incubated at 50 °C for 1 h to allow enzymatic reaction to occur. After incubation, 3 mL of DNS solution was added to

each tube and boiled for 15 min in water bath to terminate the reaction. The tubes were cooled to ambient temperature and absorbance values were measured by a spectrophotometer at 575 nm wavelength.

The same procedure was simultaneously applied to spectro zero, enzyme blank and substrate blank tubes. Spectro zero tube contained only citrate buffer. Enzyme blank tube contained citrate buffer and filter paper to obtain the effect of filter paper. Lastly, substrate blank tubes contained buffer and diluted sample only. The difference in absorbance values was used for calculation of enzyme activity.

For the calculation, the standard curve equation was used to convert the absorbance values into glucose concentration. The standard curve and related equation for enzyme analysis is given in Appendix D. Cellulase activity was calculated using Equation (1). One unit (U) of activity is defined as the amount of enzyme that releases 1 μmole reducing sugar from filter paper per mL per min (Kumar et al., 2008; Ladeira et al., 2015).

$$Filter\ Paper\ Activity\ \left({}^{IU}/_{mL}\right) = \frac{(GCD)\times(DR)}{0.18\times 60} \qquad (1)$$

where GCD is the glucose concentration difference calculated by finding glucose concentrations in the sample, in the enzyme blank and in the substrate blank using standard curve and DR is dilution rate. The molecular weight of glucose was taken as 0.18 mg/μmol and the incubation time was 60 min (Adney & Baker, 2008).

3.2.6. Statistical methods

Response surface methodology is an efficient method to study the impacts of the factors and to reach optimum levels of variables for desired response (Deka et al., 2013). Box-Behnken design of response surface methodology was employed for cellulase production using grape pomace as sole carbon source.

3.2.6.1. Response surface methodology

Response surface methodology (RSM) is usually applied to experiments with multiple variables to obtain effective results. RSM quantifies the relation between the variables and their influence on product yield, and helps to find optimum conditions. A large number of variables can be designed in a short time by determining upper and lower limits (Vanaja & Shobha Rani, 2007). This method has been used for cellulase production in several studies (Nargotra, Vaid, & Bajaj, 2016). In this study, the experiments performed with grape pomace were designed with Box-Behnken design of response surface methodology in Minitab 16 software (Minitab Inc., State College, PA, USA).

3.2.6.2. Box-Behnken design

Box-Behnken (BB) is a rotatable or nearly rotatable useful method for second order response surface models. BB design requires at least three levels for each factor (Tekindal, Bayrak, Ozkaya, & Genc, 2012). It can be adapted to full quadratic model, which analyzes the impacts of variables (Das & Dewanjee, 2018). It can also be used for response surface optimization.

In this study, Box-Behnken was used for improving the experimental design with three independent varibles, which were pH, solid loading, and incubation time. Their low and high level values are given in Table 3.2. These three variables were designed into 15 runs with two replications by Minitab 16 software and ANOVA was performed. Experimental results were adapted to the quadratic equation (2).

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (2)$$

where a's represent the regression coefficients, Y is the predicted response (cellulase activity) and X_1, X_2, and X_3 symbolize independent variables as shown in Table 3.2 in which the range and levels (-1, 0, +1) of these three variables are also given. Center point values (0) give the mostly reported conditions for cellulase production in the literature. Therefore, the low (-1) and high (+1) levels were determined corresponding to center point values (Deka et al., 2013).The experimental design matrix is given in Table 3.3.

Table 3.2. *Range of variables used for cellulase optimization*

Variables		*Low Level (-1)*	*Center (0)*	*High Level (+1)*
pH	X_1	5.0	7.0	9.0
Solid Load (% w/v)	X_2	5	12.5	20
Incubation Time (day)	X_3	3	5	7

Table 3.3. *Box-Behnken experimental design for cellulase optimization (coded factors)*

Run Order	*pH*	*Solid Loading (% w/v)*	*Incubation Time (day)*
1	+1	0	+1
2	0	-1	-1
3	+1	+1	0
4	0	+1	-1
5	-1	-1	0
6	0	0	0
7	0	+1	+1
8	+1	0	-1
9	-1	+1	0
10	0	-1	+1
11	+1	-1	0
12	-1	0	+1
13	-1	0	-1
14	0	0	0
15	0	0	0

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Grape pomace properties during drying

Grape pomace was obtained from the wine-producing factory after grapes were pressed. Grape pomace was dried at 70 °C for 24 h with the airflow 1.2 m/s in a laboratory-scale tray dryer, whose trays were rotated at 6 rpm (Martins, Roberto, Blumberg, Chen, & Macedo, 2016).

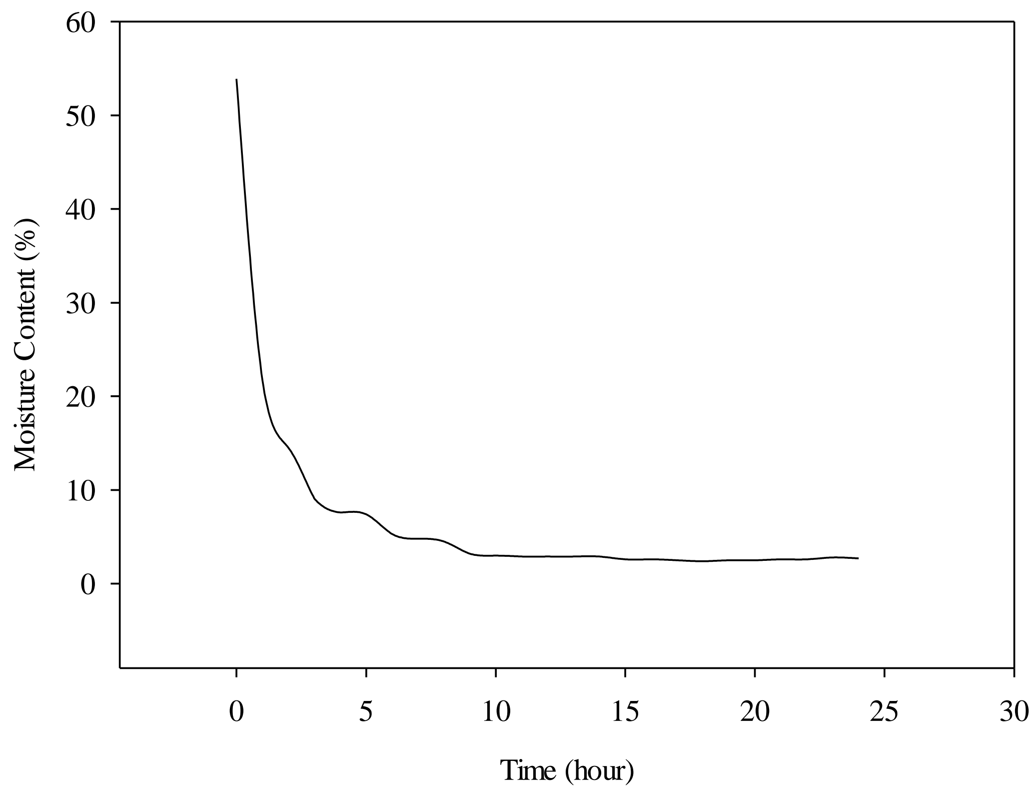

Figure 4.1. Moisture content change of grape pomace in tray dryer

Initially, the moisture content of the grape pomace was measured as 53.9% and the final moisture content was recorded as 3.0% (Figure 4.1). The weight was also measured as 7150 g at day 0 and when the drying was completed, 4409 g of dried grape pomace was obtained at 70 °C (Figure 4.2).

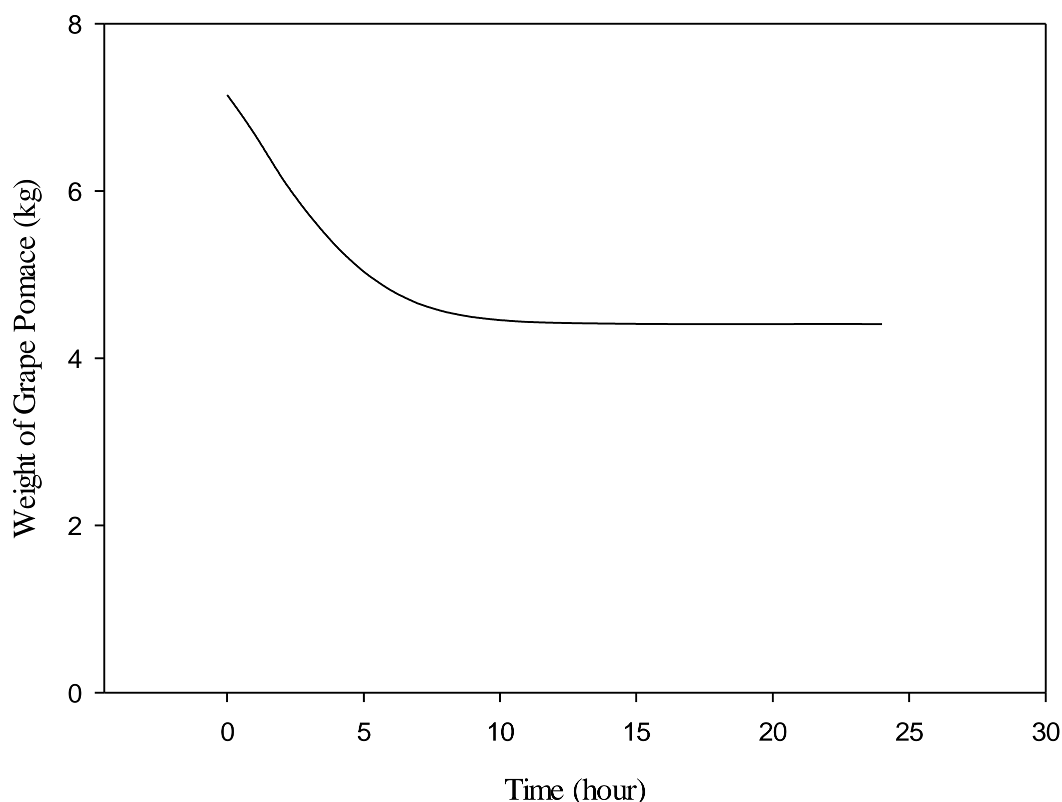

Figure 4.2. Weight change of grape pomace during drying

4.2. Sugar released by dilute acid pretreatment

In this study, dilute acid pretreatment was conducted to obtain sugar monomers for microbial growth and fermentation (Brodeur et al., 2011). Pretreatment makes cellulose more vulnerable to enzymatic degradation (Ravindran & Jaiswal, 2016).

Pretreatment was applied to grape pomace by 3% (w/v) H_2SO_4 at 121 °C for 15 min. According to Uzuner (2014), the optimum amount of reducing sugar released was conducted by 3% of acid concentration. Also, quick trials were performed to compare the reducing sugar released from 3 and 5% acid solutions. As a result of these trials, the most suitable acid concentration was found as 3%. Table 4.1 shows the effect of acid concentration on reducing sugar release.

Table 4.1. *Effect of acid concentration on release of reducing sugar*

Solid Loading (% w/v)	*Acid Concentration (% w/v)*	*Reducing Sugar Concentration (g/L)*
5%	3%	10.75
5%	3%	9.77
5%	5%	8.12
5%	5%	8.11

Different amounts of grape pomace were treated, and different amount of sugar was obtained from each (Table 4.2). According to the results, as solid loading increases, the reducing sugar amount released by dilute acid treatment increased.

Table 4.2. *Approximate reducing sugar concentration after acid treatment with 3% acid concentration*

Solid Loading (% w/v)	*Reducing Sugar Concentration (g/L)*
5%	10.26 ± 0.69
10%	18.35 ± 0.82
12.5%	19.49 ± 0.57
20%	22.87 ± 0.43

4.3. Growth of *Bacillus subtilis* Natto DSM 17766 and *Bacillus subtilis* NRRL B-4219

Bacillus subtilis Natto DSM 17766 and *Bacillus subtilis* NRRL B-4219 were grown under the conditions given in Table 4.3. The growth curve of *Bacillus subtilis* Natto DSM 17766 and *Bacillus subtilis* NRRL B-4219 are shown in Appendix C. The growth was monitored until the optical density reached 1.00. At the end of the 24 h, the bacteria reached the expected optical density. All the experiments were conducted at constant optical density after 24 h inoculum growth (Vyas et al., 2016).

Bacillus subtilis NRRL B-4219 was used for cellulase production using synthetic sugars. Both strains were then tried for cellulase production using grape pomace. Therefore, 10 g of grape pomace was pretreated by 3% sulfuric acid and pH was adjusted to 7.0 and 2% inoculum were added to sterilized media in flasks. *Bacillus subtilis* Natto DSM 17766 gave better results with grape pomace resulting in maximal cellulase activity of 0.13 IU/mL at 4th day (Figure 4.3). Therefore, all fermentation media prepared with grape pomace hydrolysate were inoculated by *Bacillus subtilis* Natto DSM 17766.

Table 4.3. *The conditions for growing of Bacillus subtilis Natto DSM 17766 and Bacillus subtilis NRRL B-4219*

Parameters	*Values*
Temperature (°C)	37
pH	7.0
Agitation speed (rpm)	130
Growth media volume (mL)	100
Glucose concentration (g/L)	10
Inoculum volume (% v/v)	1

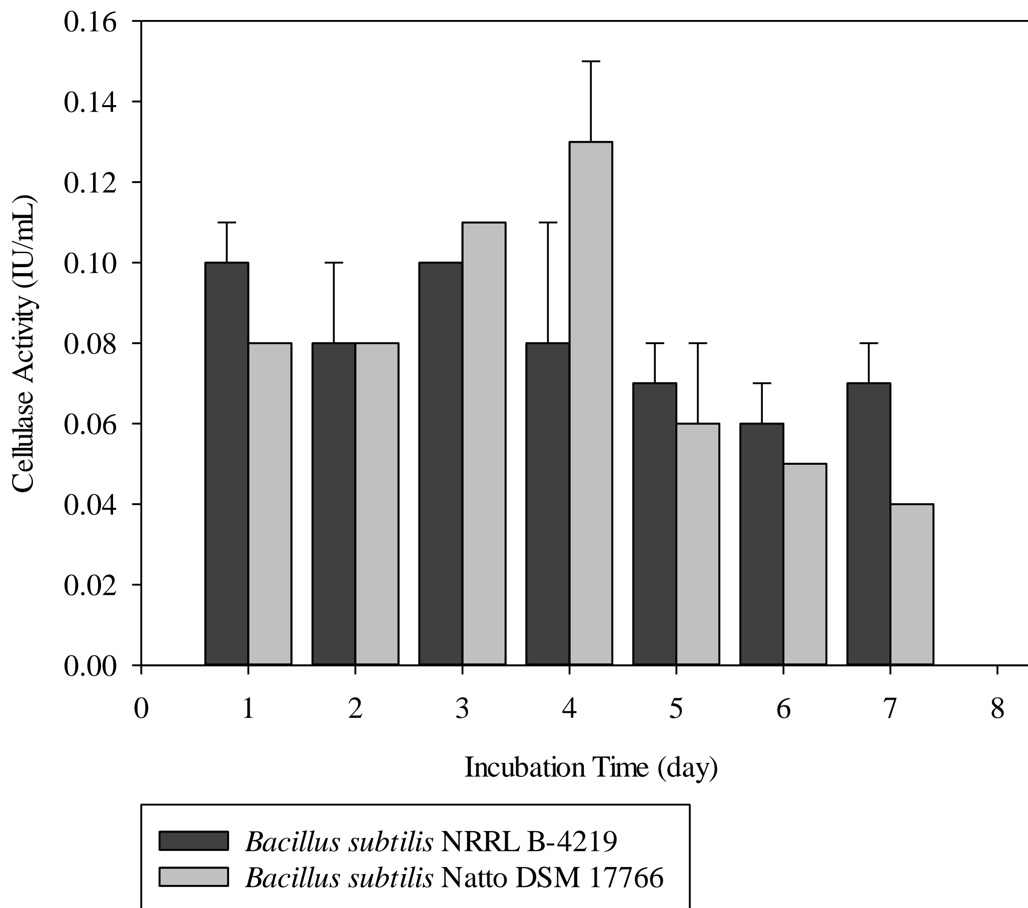

Figure 4.3. Cellulase obtained from 10 g of grape pomace hydrolysate by *Bacillus subtilis* NRRL B-4219 and *Bacillus subtilis* Natto DSM 17766

4.4. Cellulase production with synthetic sugar as sole carbon source

Fermentation media with varying synthetic sugar compositions were used as carbon source to produce cellulase for 96 h (Figure 4.4). These results revealed that cellulase was affected by composition and time. These compositions were produced to mimic the composition of grape pomace (Deng et al., 2011). The fermentation medium containing only glucose was considered as control (FC), which indicated the least

cellulase production (0.06 IU/mL). In the literature, glucose was also reported to be the least effective carbon source for cellulase production (Bai et al., 2012). Therefore, glucose was not used alone in the fermentation as carbon source.

The maximum cellulase activities were obtained after 48 hours fermentation for each sugar composition except medium F3, which gave maximum activity after 24 h. The highest enzyme activity was obtained in medium F5 as 0.48 IU/mL. Thus, mannose and galactose showed an increasing effect on cellulase activity.

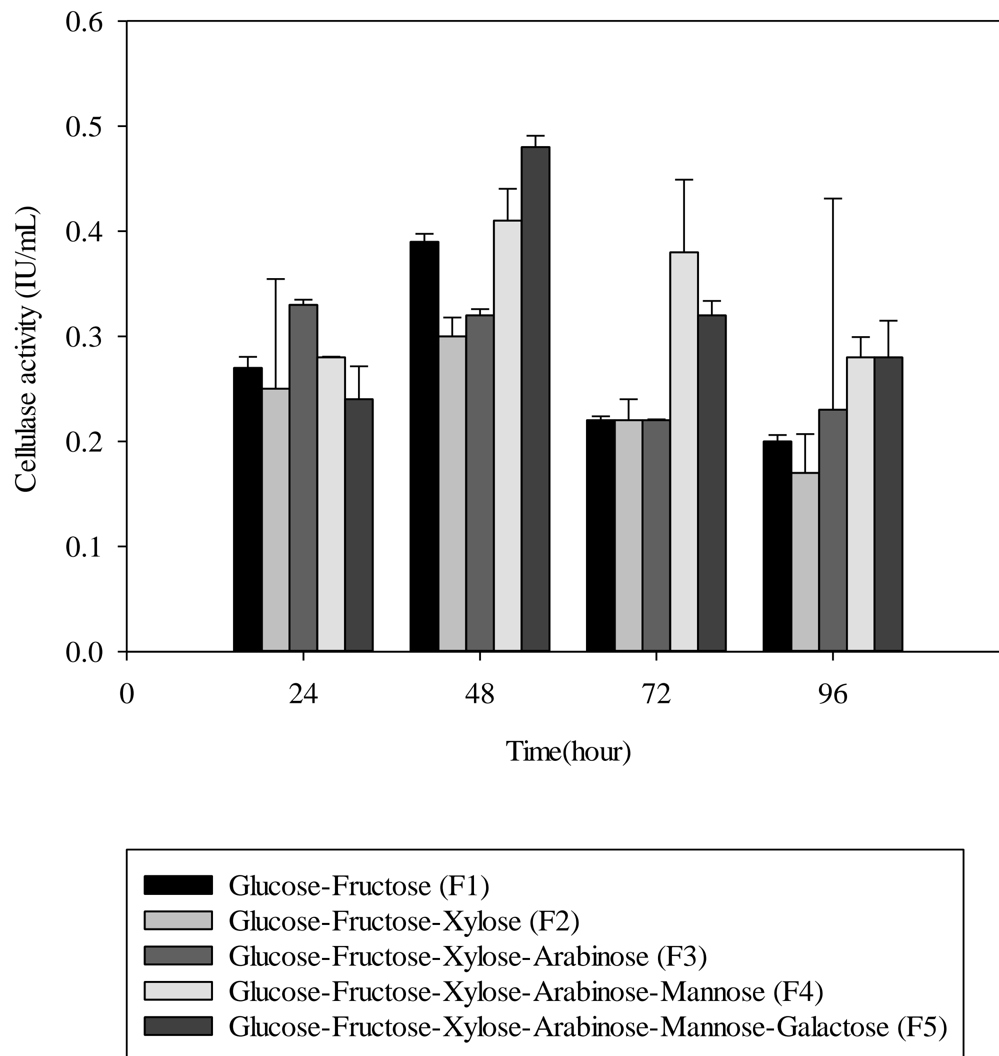

Figure 4.4. Cellulase produced with different synthetic sugar composition

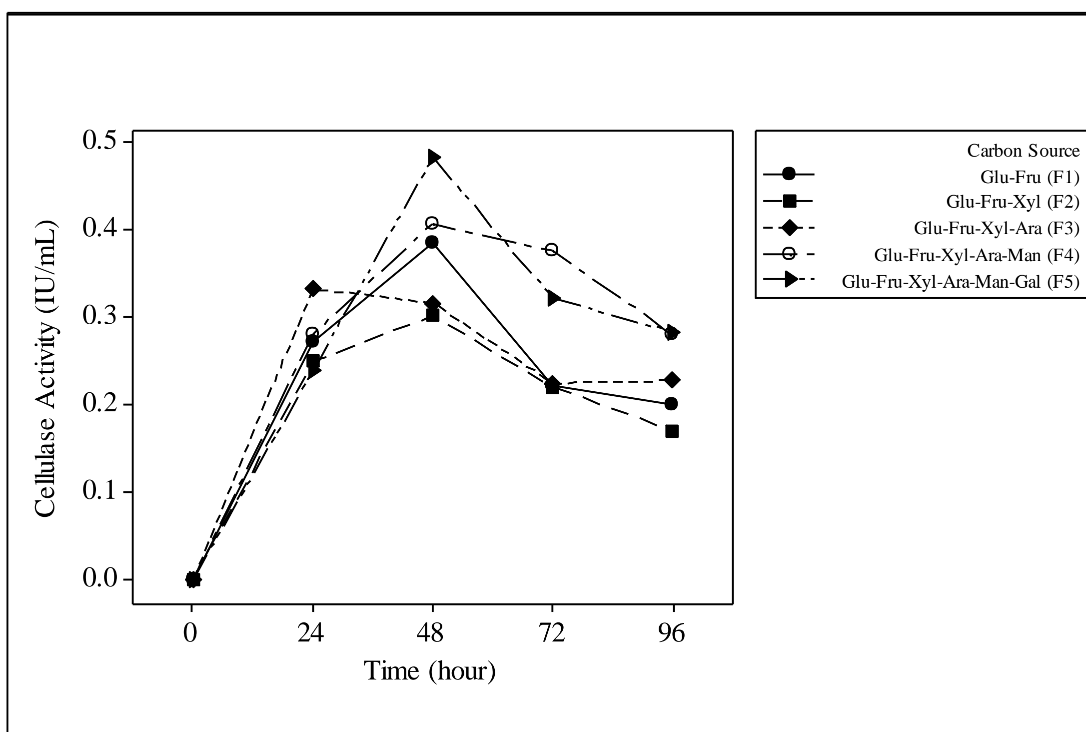

Figure 4.5. Interaction effects of carbon source and time on the cellulase activity

Two-way ANOVA was applied to the results of the cellulase activity produced using synthetic sugar composition at 95% confidence level. The ANOVA table and the regressions are given in Table 4.4. As a result of the two-way ANOVA, time was found strongly significant at p=0.000. Also, the carbon source was highly significant at p=0.006. Thus, simple main effects analysis showed that time had higher effect on cellulase activity than carbon source. However, the interaction of two terms was found insignificant at p=0.125. Therefore, the effects of carbon source and time on cellulase activity were independent and can be analyzed separately. In addition, the coefficient of determination (R^2) was recorded as 0.9355.

Table 4.4. *ANOVA for cellulase activity obtained from synthetic sugar by Bacillus subtilis NRRL B-4219*

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
Time	4	0.790037	0.790037	0.197509	79.30	0.000
Carbon Source	4	0.046823	0.046823	0.011706	4.70	0.006
Time * Carbon Source	16	0.066030	0.066030	0.004127	1.66	0.125
Error	25	0.062270	0.062270	0.002491		
Total	49	0.965160				
S=0.04991		R-Sq=93.55%		R-Sq(adj)=87.35%		

Figure 4.5 indicates the cellulase production with respect to carbon source and incubation time. The maximum cellulase was obtained in medium F5 as 0.48 IU/mL after 48 h incubation. Moreover, Figure 4.6 shows the effects of the variables on cellulase individually. As shown in the graph, 48 h of incubation time gives the highest cellulase production. After 48 h, the amount of cellulase declines. In addition, when the carbon source gets diverse, the cellulase activity increases. The least activity was observed with medium F2 as 0.17 IU/mL after 96 h fermentation. Mannose and galactose have increasing impact on the cellulase production as depicted in Figure 4.6.

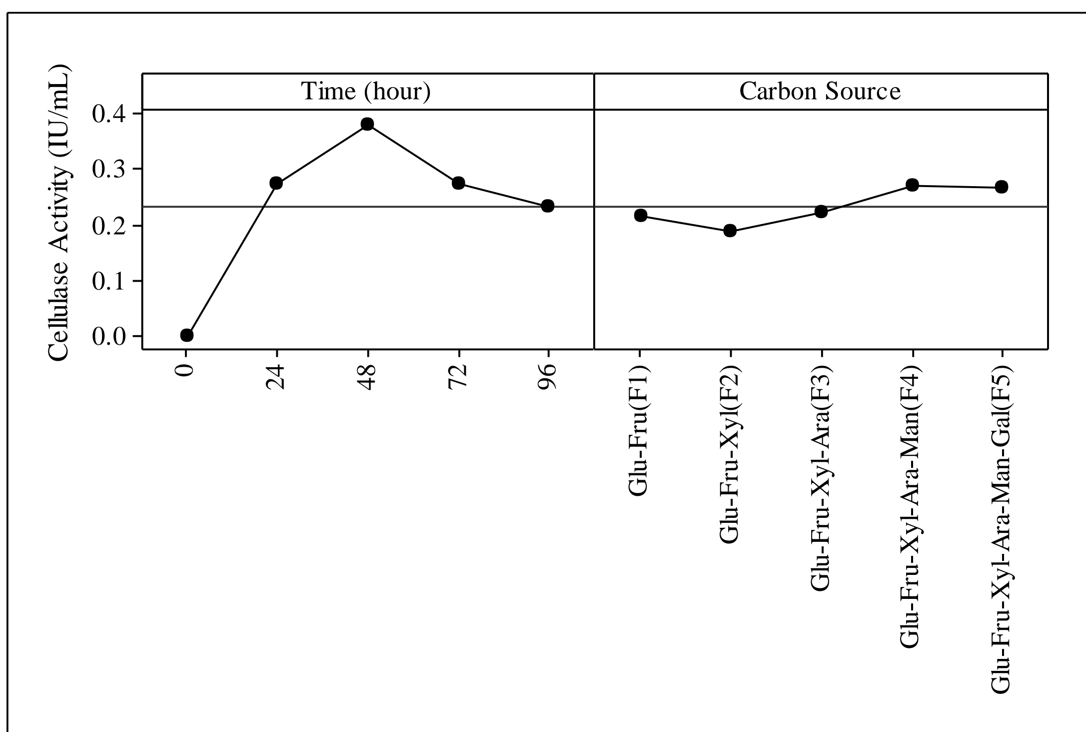

Figure 4.6. Plots of factor effects for cellulase production in synthetic sugar media

In contrast, Chan and Au (1987) found the highest cellulase activity using L-arabinose as 1693.5 IU/mg cell, which was followed by D(+) fructose as 1127.5 IU/mg cell activity. D(+) galactose yielded activity of 1078.6 IU/mg cell. The lowest activity was found as 716.2 IU/mg cell with D(+) glucose. Different carbon sources were compared with respect to their CMCase activity at pH 6.0, 50 °C in the study of Chan and Au (1987) and the highest enzymatic activity was obtained as 1693.5 IU/ mg cell.

In another study carried out by Bai et al. (2012), the optimum incubation time for growth of *Bacillus subtilis* was obtained as 72 h and the maximum cellulase activity was found as 30.33 IU/mL. The optimum temperature and pH were also found as 30 °C and 7.0, respectively. When the effect of different carbon sources was tested,

glucose was found as the worst carbon source for the cellulase production, whereas lactose gave the highest cellulase activity (23.96 IU/mL).

In contrast, Sethi et al. (2013) reported the maximum cellulase activity by *Bacillus subtilis* using glucose compared to maltose, fructose, lactose, sucrose and dextrose after 24 h incubation. Fructose and lactose also showed high cellulase activities. On the other hand, in the study conducted by Sadhu et al. (2014), glucose was not a good carbon source for cellulase production compared to fructose and galactose.

4.4.1. Reducing sugar analysis after fermentation performed with synthetic sugar

In contrast to experiments with grape pomace, the samples were withdrawn every 24 h during 4 days in these experiments. Reducing sugar and cellulase activity analysis were performed at the end of the 4 days. The withdrawn samples were kept at -20 °C in the eppendorf tubes until the analysis were performed. Initially 20 g/L monosaccharide composition was added to each flask. The reducing sugar analysis applied to samples were taken at the first day and the reducing sugar concentrations were measured approximately 20 g/L.

Figure 4.7 indicates the change in the reducing sugar concentration with respect to time and the carbon source used by *Bacillus subtilis* NRRL B-4219 for fermentation. The reducing sugar concentration in the experiment containing glucose and fructose as carbon source decreases with respect to time more than others. Reducing sugar concentration decreases mostly to 17.5 g/L. There is very little decrease in reducing sugar concentration. The least used reducing sugar content includes glucose, fructose and xylose.

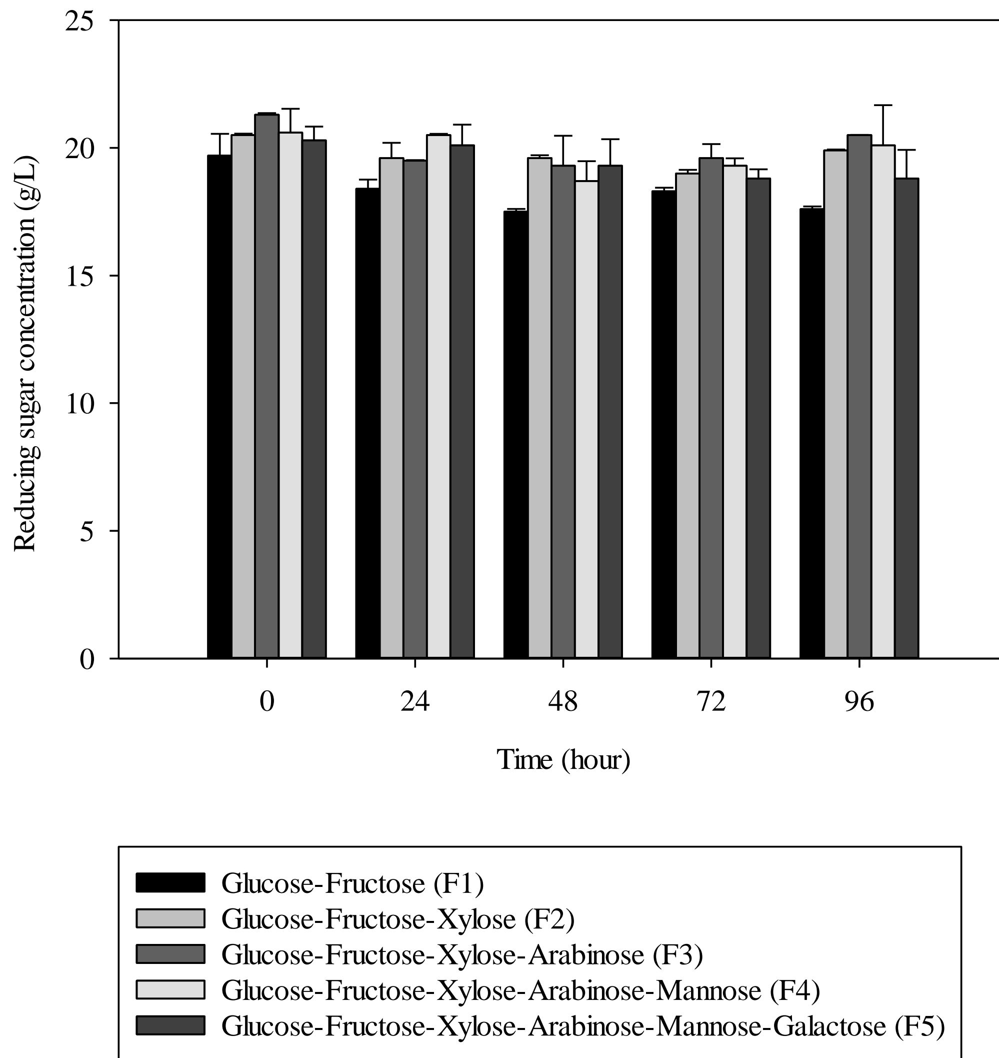

Figure 4.7. Reducing sugar concentration of the experiments conducted with five different synthetic sugar composition

4.5. Analysis of fermentation conducted with grape pomace as carbon source

Cellulase was produced in the experiments designed by Box-Behnken method since less number of experimental runs are obtained when compared to central composite design and full factorial designs. Totally, 30 experiments were generated including two replicates and the results are given in the following sections.

4.5.1. Cellulase production using grape pomace

In the literature, total cellulase production was generally evaluated by filter paper assay. When the literature was searched for temperature range, the most convenient temperature for *Bacillus subtilis* growth was found as 37 ºC (Shieh et al., 2009). Therefore, the temperature of fermentation and growth was kept constant and adjusted to 37 ºC. The inoculum size was also kept constant and chosen as 2% (v/v) for each fermentation medium.

The results of Box-Behnken response surface optimization for cellulase production are given in Table 4.5. The maximum cellulase production was estimated as 0.196 IU/mL at pH 7.0 using 12.5% solid loading after 5 days of fermentation. The initial reducing sugar concentration of this sample was found as 12.56 g/L. After fermentation, there were 5.71 g/L reducing sugar left. Moreover, the minimum cellulase activity was observed as 0.045 IU/mL at pH 9.0 from 5% solid loading after 5 days of fermentation. The initial reducing sugar concentration of this sample was measured as 5.33 g/L and the final concentration was 1.78 g/L.

Table 4.5. *Cellulase activity of the fermentation media prepared with grape pomace*

Run Order	*pH*	*Solid Loading (% w/v)*	*Incubation Time (day)*	*Cellulase Activity (IU/mL)*
1	9	12.5	7	0.093
2	7	5	3	0.079
3	9	20	5	0.144
4	7	20	3	0.070
5	5	5	5	0.085
6	9	20	5	0.162
7	7	12.5	5	0.153
8	7	20	7	0.152
9	5	5	5	0.102
10	7	20	7	0.177
11	9	12.5	3	0.123
12	7	5	3	0.142
13	5	20	5	0.063
14	7	5	7	0.067
15	9	5	5	0.070
16	9	12.5	7	0.107
17	7	12.5	5	0.196
18	9	12.5	3	0.151
19	7	20	3	0.108
20	5	12.5	7	0.166
21	5	12.5	3	0.124
22	5	12.5	3	0.138
23	7	5	7	0.103
24	7	12.5	5	0.114
25	7	12.5	5	0.171
26	7	12.5	5	0.155
27	5	20	5	0.123
28	7	12.5	5	0.176
29	5	12.5	7	0.192
30	9	5	5	0.045

When the run 12 and 14 were compared, although run 12 had lower initial reducing sugar concentration than run 14, it had higher cellulase activity. However, comparison between run 13 and 14 showed that although the initial reducing sugar concentrations were far from each other, they had close cellulase activity. Therefore, the correlation between initial reducing sugar concentration and cellulase activity was not obtained. The differences between treatments could be observed because of the filter paper assay which has inadequate sensitivity and reproducibility (Xiao et al., 2004). Also, the interactions between the independent variables had effects on cellulase activity due to Box-Behnken design of response surface model.

4.5.2. Box-Behnken response surface optimization of the cellulase production

In this section, Box-Behnken design of response surface model was analyzed and the optimum conditions for cellulase production using grape pomace by *Bacillus subtilis* Natto DSM 17766 were determined. Three process variables namely solid loading of grape pomace, pH of the fermentation media, and incubation time were tested to acquire optimum conditions. The maximum cellulase activity was observed at run order 17 as 0.196 IU/mL. The minimum cellulase activity was observed at run order 30 as 0.045 IU/mL.

Analysis of variance (ANOVA) was generated by Minitab 16 software and the results are given in Appendix E. The quadratic equation (3), which describes cellulase yield as a function of X_1 (pH), X_2 (solid load), and X_3 (incubation time) and their interactions was obtained.

$$Y = -0.1929 + 0.0689X_1 + 0.0021X_2 + 0.0340X_3 + 0.0016X_1X_2 - 0.0053X_1X_3 + 0.0017X_2X_3 - 0.0047X_1^2 - 0.0008X_2^2 - 0.0014X_3^2 \quad (3)$$

The results from ANOVA revealed that the linear terms were insignificant ($p>0.05$) while the interactions were found significant ($p<0.05$). The regression coefficient terms for square of solid load were obtained highly significant at $p=0.000$. The other coefficients were found insignificant ($p>0.05$) on cellulase activity and the R^2 was obtained as 0.7219. Also, the p value of lack-of-fit was found as 0.217, which meant to be insignificant. Therefore, the model moderately fit to the data. When the insignificant terms were neglected, the new R^2 was found as 0.6683. Therefore, all the terms were taken into consideration.

The three dimensional response surface plots were generated to show the effect of time and pH (Figure 4.8), pH and solid load (Figure 4.9), and time and solid load (Figure 4.10) on cellulase production while maintaining the third variable constant at the mid value.

Figure 4.8 indicates the relation between pH and incubation time for cellulase production at constant solid load. The cellulase activity increases with increasing pH and incubation time. As the pH reaches mid value (pH 7.0), the cellulase activity inclines. Bai et al. (2012) obtained similar observation for pH and incubation time on cellulase activity from *Bacillus subtilis*. Also, Orji et al. (2016) studied the effects of pH and incubation time on cellulase activity and similar results were acquired. On the other hand, Vaid and Bajaj (2017) reported that while pH has small effect, incubation time has the maximum effect on cellulase activity. In contrast, in this study, pH has the highest effect on cellulase activity with the coefficient of 0.0689 when the

coefficient terms are compared. Incubation time follows pH with 0.0340. Solid load has the least impact according to its coefficient of 0.0021.

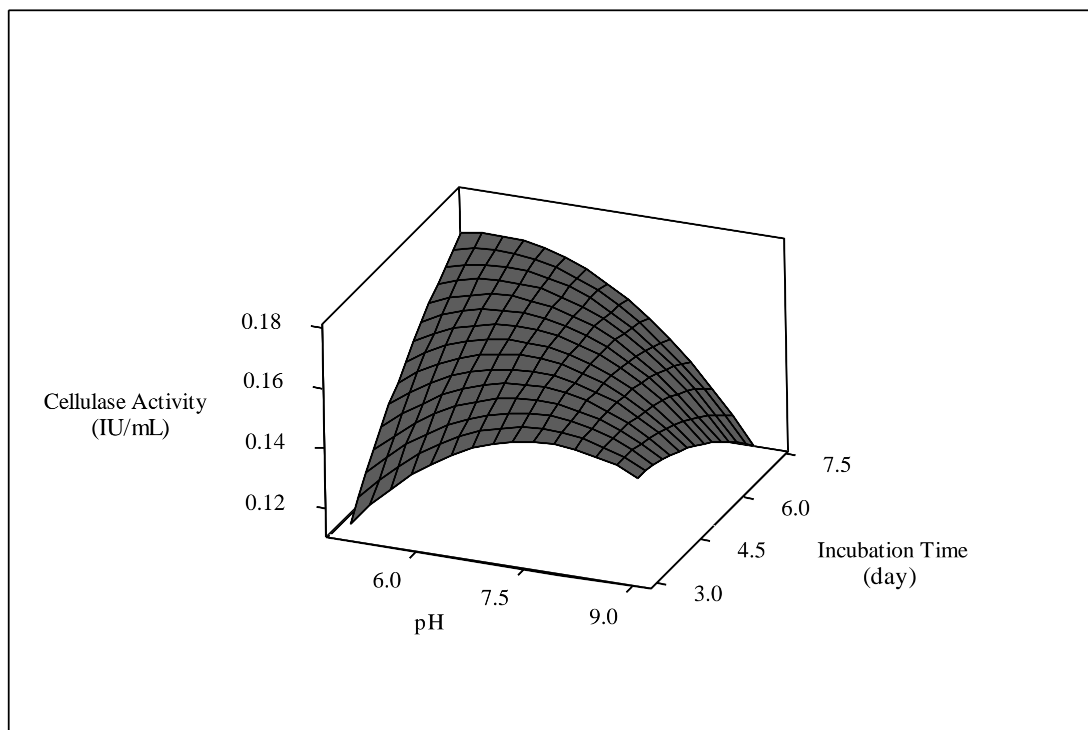

Figure 4.8. Response surface plot of cellulase activity with respect to incubation time and pH

Figure 4.9 depicts the relation between solid load and pH for cellulase activity at constant incubation time. Although the cellulase activity increases with the pH beyond the mid value of the solid load, a sharp descent of the cellulase activity is examined. Moreover, higher solid load inhibits the cellulase activity due to inhibition on the growth of microorganism. Verma et al. (2012) reported that when pH and substrate concentration increased until the mid values, the cellulase activity increased. However, there was a decline after the mid points. In contrast, Sethi et al. (2013) showed that the type of the carbon source affects cellulase activity differently. When fructose was used as carbon source, the cellulase activity increases until 2% of fructose

concentration and decreases with the increasing substrate concentration. However, the amount of lactose concentration shows increasing impact on cellulase activity up to 5% of lactose.

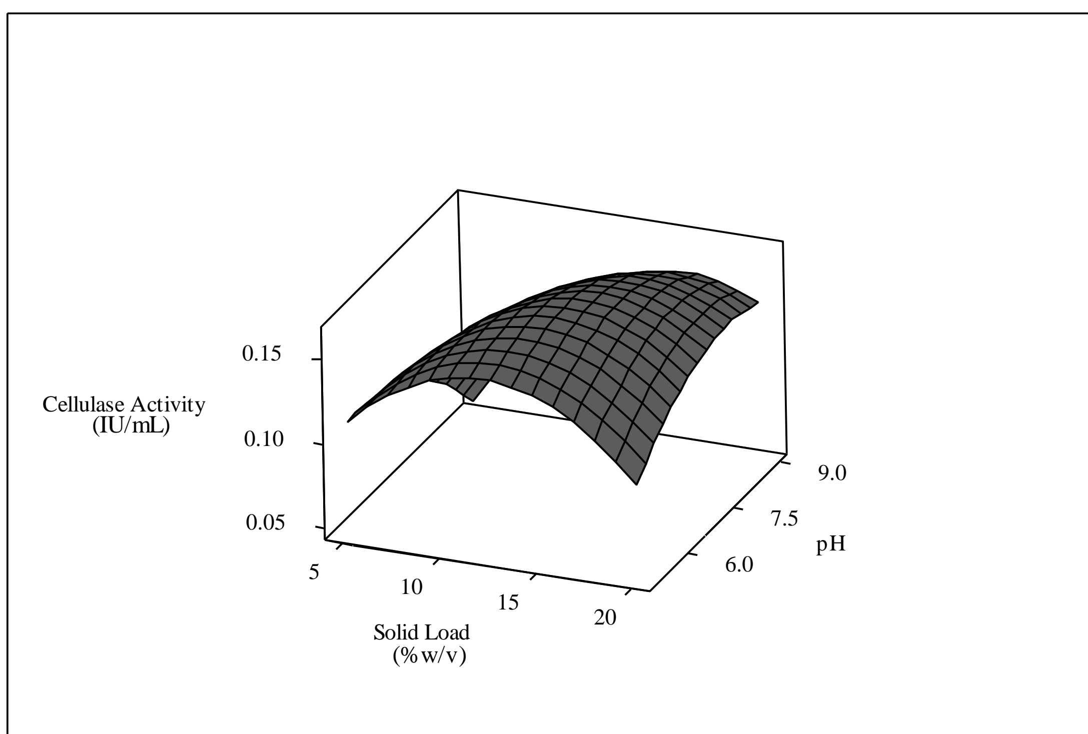

Figure 4.9. Response surface plot of cellulase activity with respect to pH and solid load

Figure 4.10 represents the relation between solid load and incubation time on cellulase activity at constant pH. The cellulase activity arises with increasing incubation time until the solid load reaches the center value. However, the activity increases with time as the solid load increases. Also, the activity increases with increasing solid load but after some point solid load inhibits the cellulase activity. Therefore, a decline in the activity is observed. Furthermore, a rise in both solid load and incubation time shows increasing effect on cellulase activity.

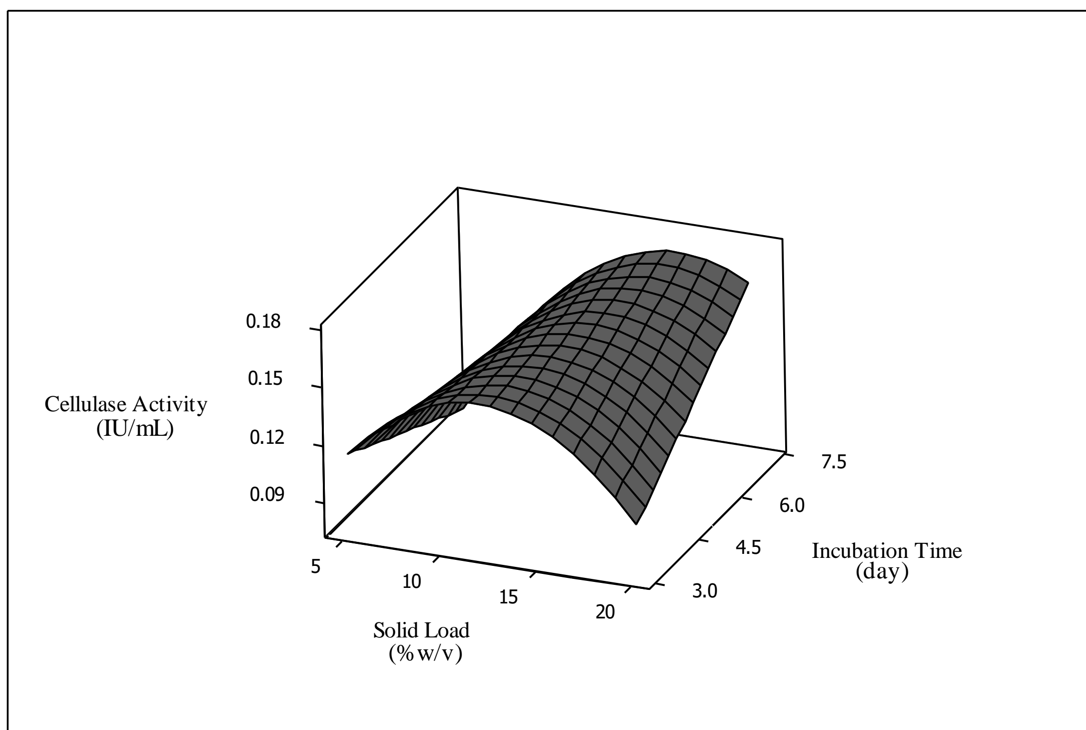

Figure 4.10. Response surface plot of cellulase activity with respect to incubation time and solid load

According to results of the Box-Behnken optimization, the process variables were shown as insignificant and thus they did not affect the cellulase activity as expected since the results were low and close to each other. According to ANOVA and the regression tables shown in Appendix E, the Box-Behnken model did not fit to the results very well, but was able to show some trend present in the data. Therefore, main effects graphs and interaction graphs were plotted for better understanding the effects of the variables.

As shown in Figure 4.11, main effects plot for cellulase activity was generated to observe the effects of the three independent variables individually. The maximum cellulase acitivity observed with 12.5% solid load and the maximum activity can be observed that it is higher than 0.14 IU/mL. In addition, the enzyme activity has the

maximum value at pH 7.0 compared to pH 5.0 and pH 9.0 as observed between 0.12 and 0.14 in Figure 4.11. Also, enzyme activity increases as incubation time increases.

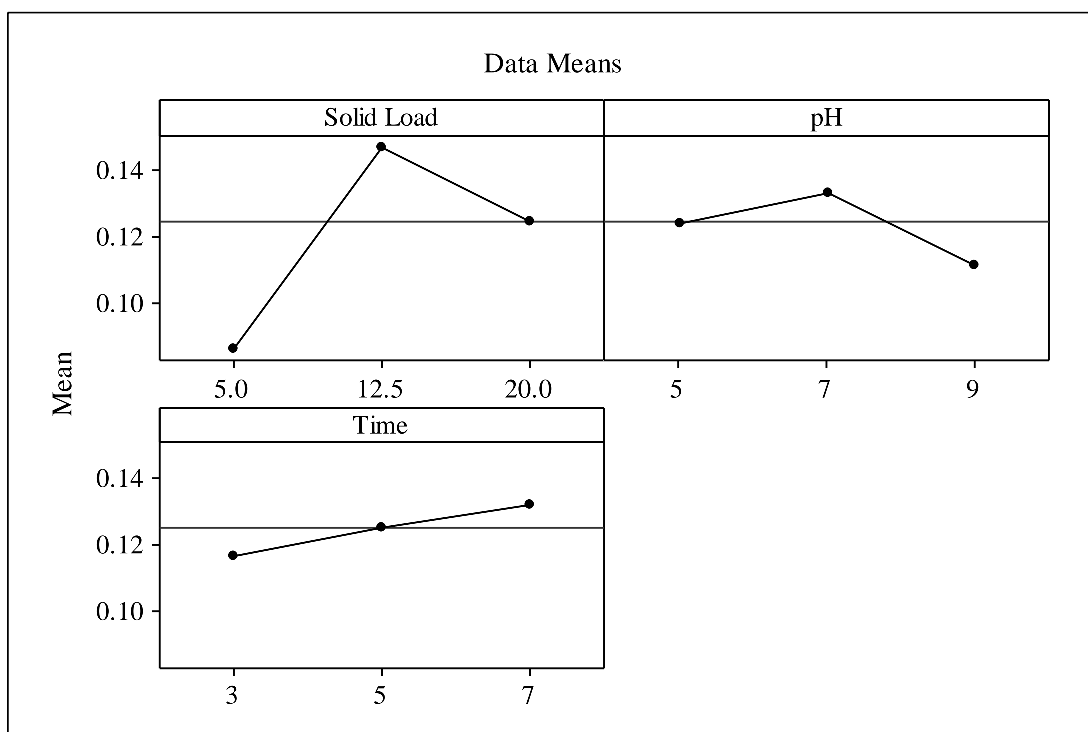

Figure 4.11. Main effects plot for cellulase activity

Furthermore, the interactions between parameters are displayed in Figure 4.12. When the interaction between solid load and pH was analyzed, the maximum activity was higher than 0.15 IU/mL at pH 7.0 with 12.5% solid load. The interaction plot between solid load and incubation time gave the maximum activity at 7th day in the 20% solid load. On the other hand, the pH and incubation time interaction gave different results. It showed that the maximum cellulase activity was obtained at pH 5.0 at 7th day. When the pH 7.0 was checked for activity, at 5th day cellulase activity was higher. However, it is less than the activity found at pH 5.0. Chan and Au (1987) reported that maximum cellulase activity with *Bacillus subtilis* was obtained at slightly acidic pH. In contrast,

Nargotra et al. (2016) showed that better enzyme activity can be obtained at pH range of 8.0 to 10.0.

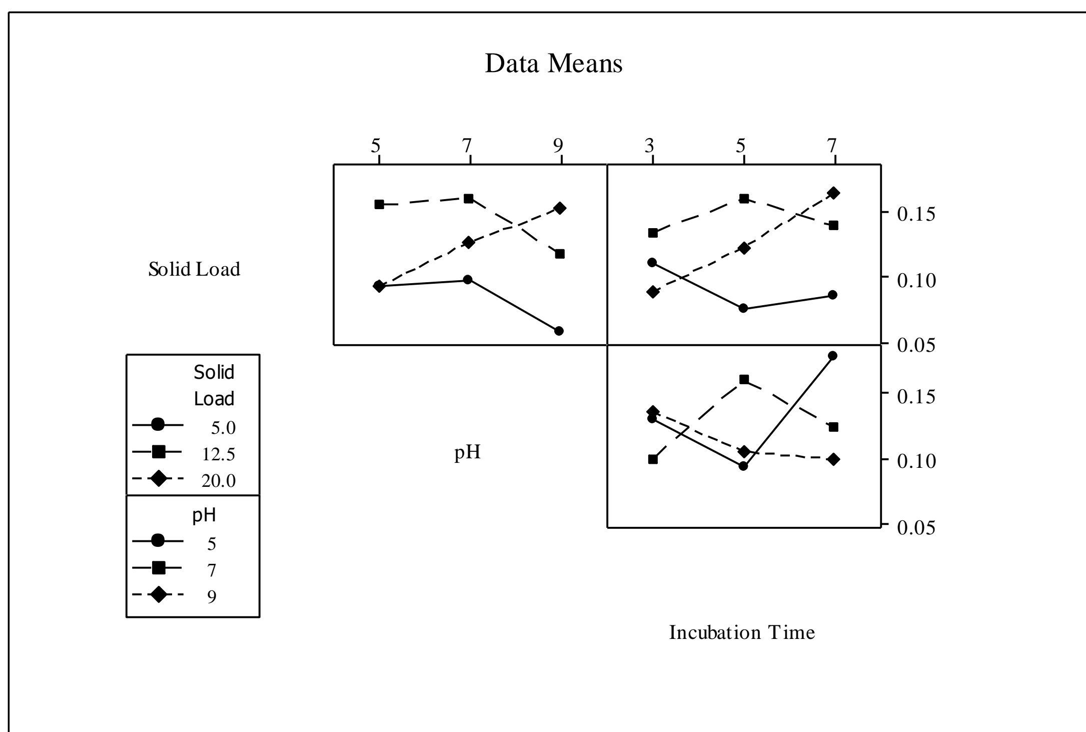

Figure 4.12. Interaction plot for cellulase activity

The optimum conditions were found as 15% (w/v) of solid loading, 6.0 pH and 7 days of incubation time with 0.92 of the composite desirability as shown in Figure E.1 and Table 4.6. At optimum conditions, the predicted cellulase activity was 0.178 IU/mL. In order to check the predicted cellulase activity, verification experiments were conducted. Two replicates were performed. The results of verification experiments confirmed the predicted results giving 0.176 ± 0.013 IU/mL of activity. Also, in the study carried out by Chan and Au (1987), effects of carbohydrates, pH and cultivation temperature were studied and the optimum pH was obtained as 6.0.

Table 4.6. *Optimum conditions for cellulase production*

Variables (units)	*Values*
pH	6.0
Solid Loading % (w/v)	15
Incubation Time (day)	7

According to the study conducted with *Bacillus sp*. Y3 by Lugani et al. (2015), the maximum filter paper activity was obtained at 37 ºC, pH 7.0, and 120 rpm as 6.84 IU/mL after 96 h. Also, the optimum inoculum concentration was reported as 2% (v/v). On the other hand, the study carried out by Khatiwada et al. (2016) found the optimum cellulase production (0.27 IU/mL) after 24 h of cultivation at 37 ºC, pH 7.0 with 5% (v/v) inoculum size. Furthermore, Kumar et al. (2008) studied different substrates and the highest FPase activity was reported as 45 IU/mL at 30 ºC, 120 rpm with 0.5% (v/v) inoculum for 24 h incubation of *Bacillus sp.* FME 2. Also, in the study of Ariffin et al. (2006), the maximum cellulase activity by *Bacillus pumilis* EB3 was obtained after 24 h as 0.011 IU/mL.

Moreover, Acharya and Chaudhary (2012) showed that FPase was produced better at a little acidic range of pH (pH 6.5) and at neutral pH from two different *Bacillus sp*. On the other hand, in the study conducted by Nargotra et al. (2016), the optimal pH and the optimal temperature for cellulase production by *Bacillus subtilis* SV1 were obtained as pH 10.0 and 45 °C, respectively.

Padilha et al. (2015) examined 10 g/L sugarcane bagasse with *Bacillus* sp. C1AC5507 for cellulase production at 37 °C and the highest cellulase activity was found 0.38 IU/mL at 72 h of incubation.

In this thesis, the cellulase activity obtained using grape pomace was found lower than several studies. However, the result was higher than the study conducted by Ariffin et al. (2006) with 0.011 IU/mL. Also, close results were obtained with the study carried out by Acharya and Chaudhary (2012), Khatiwada et al. (2016), Sethi et al. (2013), and Dias et al. (2014) with the range of 0.018-0.505 IU/mL, 0.17-0.35 IU/mL, 0.05-0.4 IU/mL, and 0.25-0.34 IU/mL, respectively.

4.5.3. Reducing sugar analysis after fermentation performed with grape pomace

Reducing sugar concentrations were measured by the DNS method. First, the standard curve was constituted to translate absorbance values measured by the spectrophotometry at 575 nm into glucose concentration. The standard curve and the related equation are given in Appendix D. For reducing sugar analysis, samples were taken twice at the beginning and end of the fermentation. The initial and final reducing sugar concentrations were obtained for each treatment.

The Box-Behnken designed experiments and their initial and final reducing sugar concentrations are given in Table 4.7. According to the results, reducing sugar consumption increased when incubation time increased independent of pH. When the reducing sugar concentrations were compared after 3 and 7 days incubation, higher sugar consumption was observed at 7th day. In addition, solid load did not affect the reducing sugar consumption. Moreover, reducing sugar consumption slightly increased as pH increased. However, incubation time seems to have the highest effect on reducing sugar consumption (Table 4.7).

Table 4.7. *Initial and final reducing sugar (RS) concentrations of the fermentation media prepared with grape pomace*

Run Order	*pH*	*Solid Loading (% w/v)*	*Time (day)*	*Initial RS Conc. (g/L)*	*Final RS Conc. (g/L)*
1	9	12.5	7	11.28	3.54
2	7	5	3	5.58	1.53
3	9	20	5	18.08	9.16
4	7	20	3	19.98	17.45
5	5	5	5	5.78	1.57
6	9	20	5	16.21	9.09
7	7	12.5	5	12.61	5.41
8	7	20	7	18.63	10.85
9	5	5	5	6.16	2.24
10	7	20	7	18.56	7.61
11	9	12.5	3	11.38	7.07
12	7	5	3	5.66	1.59
13	5	20	5	19.61	22.05
14	7	5	7	6.02	2.18
15	9	5	5	5.47	2.40
16	9	12.5	7	11.62	3.63
17	7	12.5	5	12.56	5.71
18	9	12.5	3	12.06	6.90
19	7	20	3	18.75	16.83
20	5	12.5	7	14.25	7.27
21	5	12.5	3	13.91	11.52
22	5	12.5	3	13.68	12.30
23	7	5	7	6.05	2.06
24	7	12.5	5	12.74	6.01
25	7	12.5	5	12.58	5.46
26	7	12.5	5	13.49	5.98
27	5	20	5	20.37	22.65
28	7	12.5	5	12.70	5.86
29	5	12.5	7	12.91	7.67
30	9	5	5	5.33	1.78

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

In the current study, the cellulase production was investigated with respect to carbon sources, which were categorized as synthetic sugar complex and grape pomace, and culture conditions (pH, solid load, and incubation time) by *Bacillus subtilis* strains. Two *Bacillus* strains were compared for cellulase production using grape pomace at 37 °C, pH 7.0, and 130 rpm. *Bacillus subtilis* Natto DSM 17766 showed higher cellulase. Thus, it was used for cellulase production using grape pomace in other trials.

The results of the experiments conducted with synthetic sugar showed that when the diversity of the sugar increases, the cellulase activity increases. The highest cellulase activity was observed in the mixture of glucose, fructose, xylose, arabinose, mannose, and galactose (F5) as 0.48 IU/mL. This composition is the closest one to lignocellulosic material.

The Box-Behnken design of response surface methodology was applied to determine optimal values of solid load, pH, and incubation time that yield highest cellulase production. As a result, the maximum cellulase activity was found with 12.5% solid load at pH 7 after 5 days incubation as 0.196 IU/mL at constant temperature of 37 °C and agitation speed of 130 rpm, where the reducing sugar utilization was computed as 55%. The optimum conditions predicted by Box-Behnken design were 15% solid loading, pH 6.0 and 7 days of incubation time. The predicted cellulase production (0.178 IU/mL) was very close to experimental counterpart (0.176 ± 0.013 IU/mL).

The cellulase activity obtained from grape pomace was lower than that of synthetic sugar. However, when the reducing sugar amount was compared, the initial reducing sugar concentration of synthetic sugar was higher than grape pomace. The highest cellulase activity was obtained in synthetic sugar medium as 0.48 IU/mL with 20.31 g/L initial reducing sugar content. On the other hand, the maximum cellulase activity from grape pomace was obtained as 0.196 IU/mL with 12.56 g/L initial reducing sugar content. These results confirm that the cellulase activity is affected by the concentration of reducing sugar.

The initial reducing sugar concentration did not affect the cellulase production directly which was carried out with grape pomace. Other independent variables and their interactions showed higher effect on cellulase production. They gave different cellulase activities with respect to interactions of the independent variables.

In future studies, cellulase production can be targeted at shorter times. Even though the cellulase activity from grape pomace was low, grape pomace will be utilized for cellulase production in combination with other lignocellulosic wastes. The fed-batch fermentation method can be tested to increase the activity. Finally, detoxification can be employed to grape pomace hydrolysate to decrease possible inhibitory substances.

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APPENDICES

A. CHEMICALS LIST

Table A.1. *Table of chemicals used for experiments*

Chemical	*Supplier*
3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$)	Sigma-Aldrich
Buffer solution (citric acid/sodium hydroxide/hydrogen chloride, pH=4.0)	Merck
Buffer solution (di-sodium hydrogen phosphate/potassium dihydrogen phosphate, pH=7.0)	Merck
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Merck
Carboxymethylcellulose sodium salt ($C_{28}H_{30}Na_8O_{27}$)	Merck
Citric acid monohydrate ($C_6H_8O_7.H_2O$)	Merck
D(-)-fructose ($C_6H_{12}O_6$)	Merck
D(+)-galactose ($C_6H_{12}O_6$)	Merck
D(+)-glucose monohydrate ($C_6H_{12}O_6.H_2O$)	Merck
D(+)-mannose ($C_6H_{12}O_6$)	Merck
D(+)-xylose ($C_5H_{10}O_5$)	Merck
Di-potassium hydrogen phosphate (K_2HPO_4)	Merck
Glycerol ($C_3H_8O_3$)	Merck
Granulated yeast extract	Merck
Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$)	Merck
L(+)-arabinose ($C_5H_{10}O_5$)	Merck
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck
Nutrient broth	Merck
Phenol (C_6H_5OH)	Merck

Table A.1. *Table of chemicals used for experiments (continued)*

Chemical	*Supplier*
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Potassium sodium tartrate tetrahydrate (Rochelle salt, $C_4H_4KNaO_6.4H_2O$)	Merck
Powder sodium hydroxide ($NaOH$)	Sigma-Aldrich
Sodium hydroxide anhydrous pellets ($NaOH$)	Carlo Erba Reagent
Sodium sulfite (Na_2SO_3)	Merck
Sulfuric acid 95-97% (H_2SO_4)	Merck
Tri-sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$)	Merck

B. BUFFERS AND SOLUTIONS

Citrate buffer solution – pH 4.8

4.21 g citric acid monohydrate (0.1 M) and 5.89 g tri-sodium citrate dihydrate (0.1 M) were dissolved in 200 mL distilled water separately. Then, they were mixed in a flask in the ratio of 2:3. The obtained citrate buffer should be at pH 4.8.

DNS solution for reducing sugar analysis

1 g sodium hydroxide, 1 g 3,5-dinitrosalicylic acid, 0.2 g phenol, and 0.05 g sodium sulfite were dissolved in 100 mL distilled water in an opaque flask. 40 g Rochelle salt was dissolved in 100 mL distilled water in a separate opaque flask. DNS solution and Rochelle salt were not mixed each other for reducing sugar analysis. The solution was prepared freshly for each day.

DNS solution for the cellulase analysis

1 g sodium hydroxide, 1 g 3,5-dinitrosalicylic acid, 0.2 g phenol, 0.05 g sodium sulfite, and 25.6 g Rochelle salt were dissolved in 100 mL distilled water in an opaque flask. For cellulase activity analysis, the DNS solution contains Rochelle salt in it. The solution was prepared freshly for each day.

C. GROWTH CURVES

Growth curve of *Bacillus subtilis* NRRL B-4219

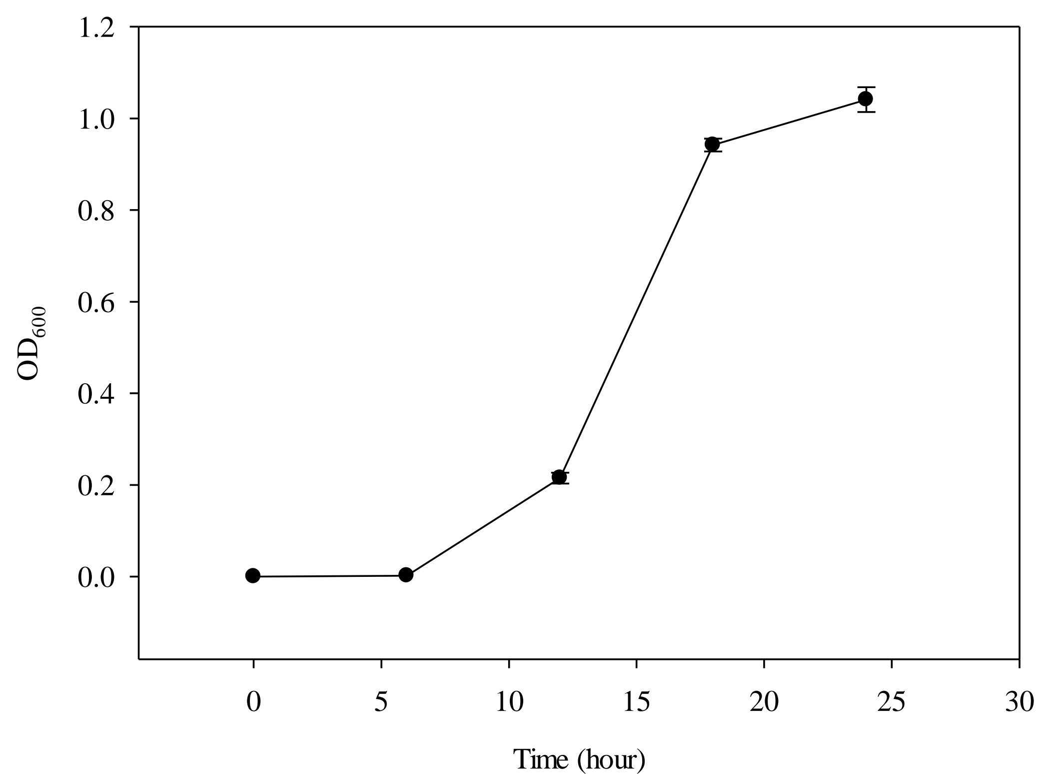

Figure C.1. Growth curve of *Bacillus subtilis* NRRL B-4219

Growth curve of *Bacillus subtilis* Natto DSM 17766

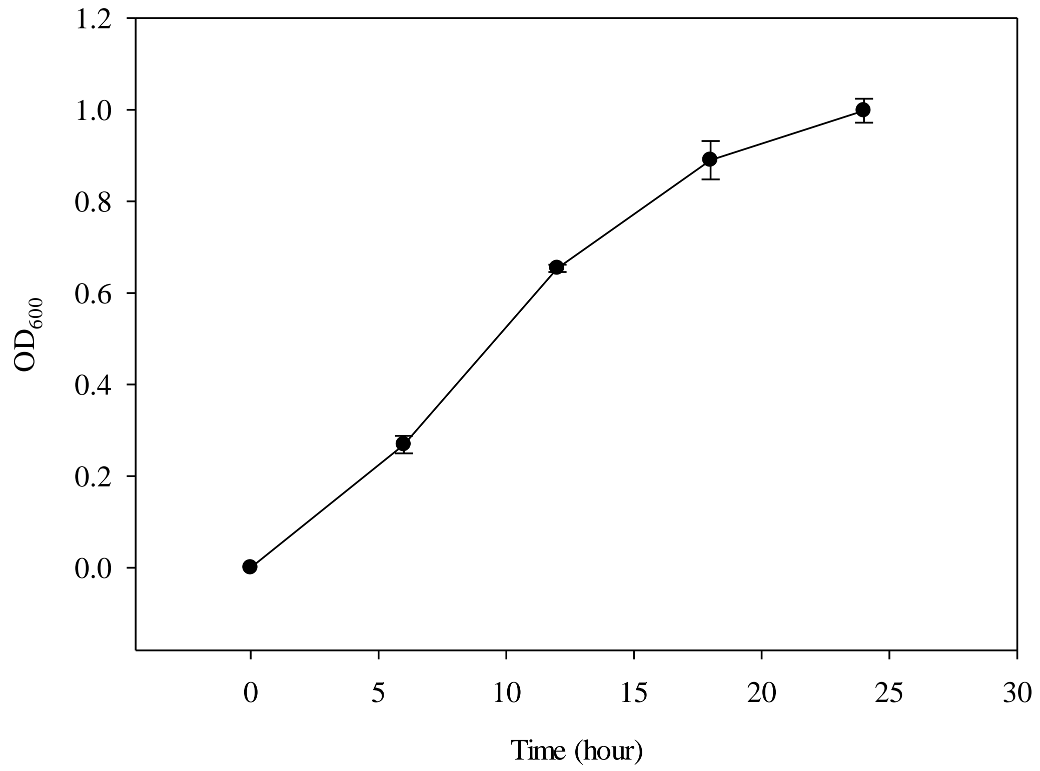

Figure C.2. Growth curve of *Bacillus subtilis* Natto DSM 17766

D. STANDARD CURVES

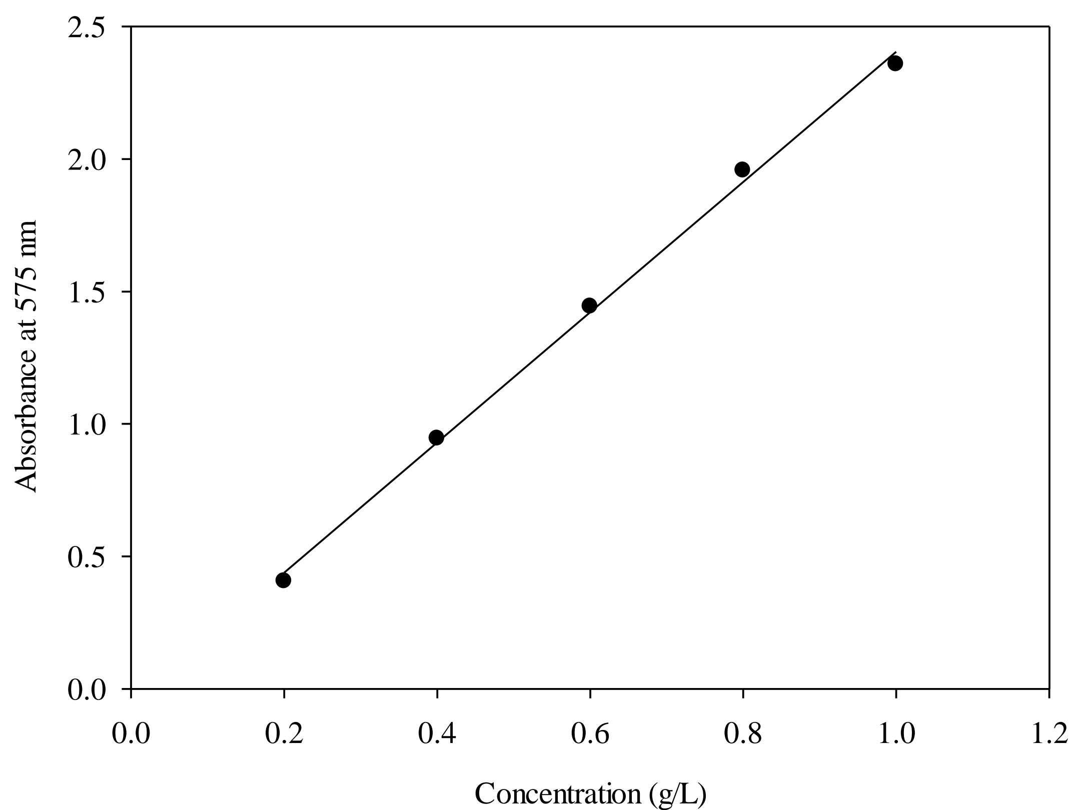

Figure D.1. Standard curve for reducing sugar analysis

The equation, used for conversion of absorbance values to concentration;

$$y = 2.4581x - 0.0531$$

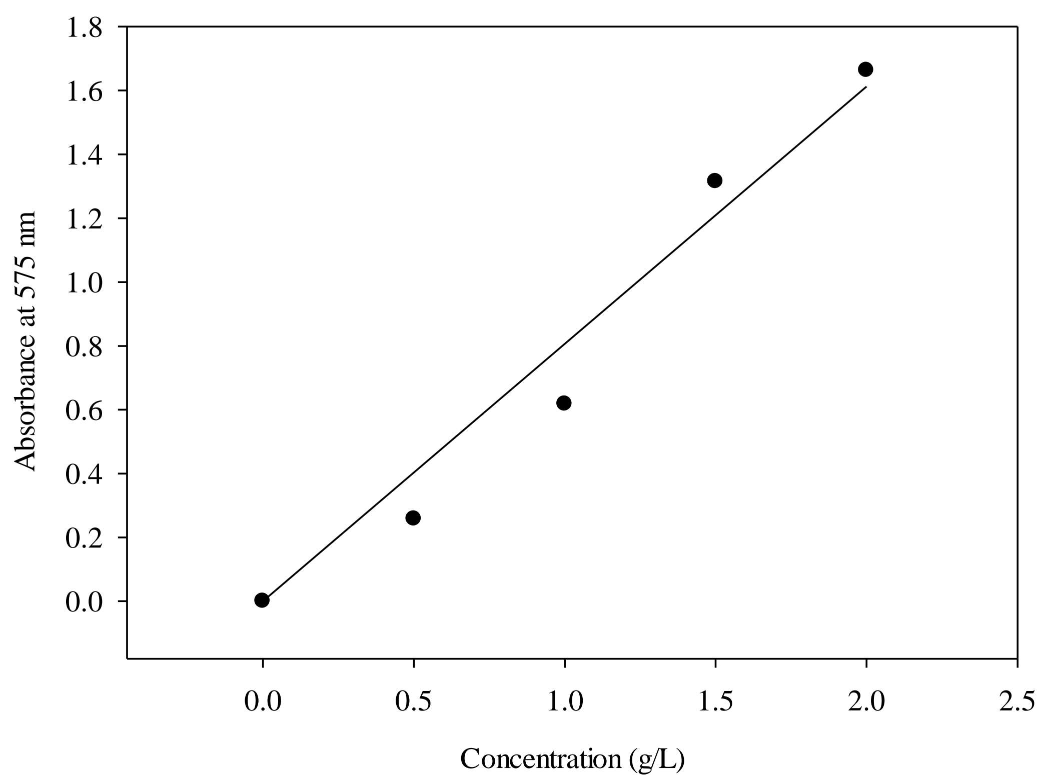

Figure D.2. Standard curve for cellulase assay

The equation, used for conversion of absorbance values to concentration;

$$y = 0.8061x$$

E. STATISTICAL ANALYSES

Table E.1. *Two-way ANOVA and Tukey's comparison test with 95% confidence level for determination of cellulase activity obtained from synthetic sugar by Bacillus subtilis NRRL B-4219*

General Linear Model: Cellulase Activity versus Time (hour); Carbon Source

Factor	Type	Levels	Values
Time (hour)	fixed	5	0; 24; 48; 72; 96
Carbon Source	fixed	5	F1; F2; F3; F4; F5

Carbon Source Values

F1; Glucose-Fructose
F2; Glucose-Fructose-Xylose
F3; Glucose-Fructose-Xylose-Arabinose
F4; Glucose-Fructose-Xylose-Arabinose-Mannose
F5; Glucose-Fructose-Xylose-Arabinose-Mannose-Galactose

Analysis of Variance for Cellulase Activity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time (hour)	4	0.790037	0.790037	0.197509	79.30	0.000
Carbon Source	4	0.046823	0.046823	0.011706	4.70	0.006
Time (hour)*Carbon Source	16	0.066030	0.066030	0.004127	1.66	0.125
Error	25	0.062270	0.062270	0.002491		
Total	49	0.965160				

S = 0.04991   R-Sq = 93.55%   R-Sq(adj) = 87.35%

Unusual Observations for Cellulase Activity

Obs	Cellulase Activity	Fit	SE Fit	Residual	St Resid
7	0.175743	0.249659	0.035290	-0.073916	-2.09 R
15	0.085632	0.227777	0.035290	-0.142145	-4.03 R
32	0.323574	0.249659	0.035290	0.073916	2.09 R
40	0.369922	0.227777	0.035290	0.142145	4.03 R

R denotes an observation with a large standardized residual.

Table E.1. *Two-way ANOVA and Tukey's comparison test with 95% confidence level for determination of cellulase activity obtained from synthetic sugar by Bacillus subtilis NRRL B-4219 (continued)*

Grouping Information Using Tukey Method and 95.0% Confidence

Time (hour)	N	Mean	Grouping
48	10	0.4	A
24	10	0.3	B
72	10	0.3	B
96	10	0.2	B
0	10	-0.0	C

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Carbon Source	N	Mean	Grouping
F4	10	0.3	A
F5	10	0.3	A
F3	10	0.2	A B
F1	10	0.2	A B
F2	10	0.2	B

Means that do not share a letter are significantly different.

Table E.2. *Response surface design analyses with full quadratic model at 95% confidence level for determination of cellulase activity obtained from grape pomace hydrolysate by Bacillus subtilis Natto DSM 17766*

Response Surface Regression: Cellulase Activity versus Solid Load; pH; Time

The analysis was done using uncoded units.

Estimated Regression Coefficients for Cellulase Activity

Term	Coef	SE Coef	T	P
Constant	-0.192872	0.173737	-1.110	0.280
Solid Load	0.002095	0.006937	0.302	0.766
pH	0.068918	0.036946	1.865	0.077
Time	0.033979	0.030480	1.115	0.278
Solid Load*Solid Load	-0.000767	0.000173	-4.436	0.000
pH*pH	-0.004661	0.002430	-1.918	0.069
Time*Time	-0.001382	0.002430	-0.569	0.576
Solid Load*pH	0.001596	0.000623	2.563	0.019
Solid Load*Time	0.001688	0.000623	2.711	0.013
pH*Time	-0.005347	0.002335	-2.290	0.033

S = 0.0264133 PRESS = 0.0313717
R-Sq = 72.19% R-Sq(pred) = 37.48% R-Sq(adj) = 59.68%

Analysis of Variance for Cellulase Activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	0.036225	0.036225	0.004025	5.77	0.001
Linear	3	0.007369	0.002790	0.000930	1.33	0.292
Solid Load	1	0.005806	0.000064	0.000064	0.09	0.766
pH	1	0.000623	0.002428	0.002428	3.48	0.077
Time	1	0.000939	0.000867	0.000867	1.24	0.278
Square	3	0.015486	0.015486	0.005162	7.40	0.002
Solid Load*Solid Load	1	0.012794	0.013731	0.013731	19.68	0.000
pH*pH	1	0.002466	0.002567	0.002567	3.68	0.069
Time*Time	1	0.000226	0.000226	0.000226	0.32	0.576
Interaction	3	0.013371	0.013371	0.004457	6.39	0.003
Solid Load*pH	1	0.004584	0.004584	0.004584	6.57	0.019
Solid Load*Time	1	0.005128	0.005128	0.005128	7.35	0.013
pH*Time	1	0.003659	0.003659	0.003659	5.24	0.033
Residual Error	20	0.013953	0.013953	0.000698		
Lack-of-Fit	3	0.003138	0.003138	0.001046	1.64	0.217
Pure Error	17	0.010815	0.010815	0.000636		
Total	29	0.050178				

Table E.3. *Response surface optimization analyses for determination of the optimum conditions and result of cellulase activity obtained from grape pomace hydrolysate by Bacillus subtilis Natto DSM 17766*

Response Optimization

```
Parameters

                    Goal    Lower  Target  Upper  Weight  Import
Cellulase Activity  Target   0,05    0,19    0,2       1       1


Global Solution

Solid Load        =   15,3030
pH                =   6,01010
Incubation Time   =         7


Predicted Responses

Cellulase Activity   =   0,178282  ,   desirability =   0,916300


Composite Desirability = 0,916300
```

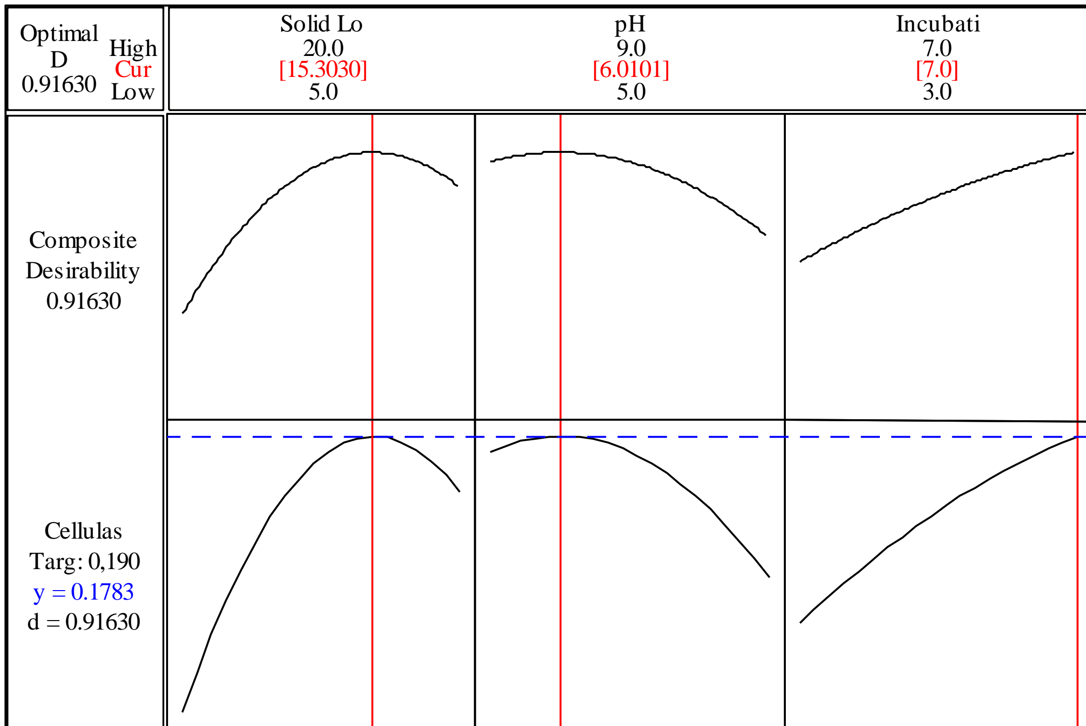

Figure E.1. Optimization plot for cellulase activity