BACTERIAL POLYGALACTURONASE PRODUCTION USING APPLE POMACE BY SUBMERGED FERMENTATION

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ABSTRACT

BACTERIAL POLYGALACTURONASE PRODUCTION USING APPLE POMACE BY SUBMERGED FERMENTATION

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Over the past decade, there has been a growing interest in the waste management sector, primarily due to the increasing environmental threat posed by waste disposal. Scientific research in recent years has focused on finding solutions to this issue by processing agricultural lignocellulosic residues into valuable products.

Industries across various sectors, including but not limited to food production, heavily rely on enzymes. Nevertheless, the high cost of producing enzymes at an industrial scale remains a notable obstacle. As a result, there is a rising inclination towards using lignocellulosic materials to produce enzymes, with the aim of both cost reduction and minimizing environmental impact.

In this study, it was aimed to enhance polygalacturonase (PGase) production using apple pomace by fed-batch submerged fermentation. *Bacillus subtilis* NRRL B-4219 was employed as PGase producer and all fermentation experiments were carried out at 30 ^{o}C and 130 rpm. Box-Behnken experimental design of Response Surface

Methodology was used for optimization of parameters, namely additional substrate amount, addition time, and fermentation period.

The highest result (12.27 U/mL) was obtained when the fermentation lasted three days and 1 g/L pectin was added at the second day of fermentation. Analysis of the results revealed optimum process to be with no additional substrate and three days of fermentation; however, the results support addition of 1 g/L pectin on day 2. Overall, the present study proves apple pomace as a cost-effective alternative for bacterial PGase production.

Keywords: Apple pomace, *Bacillus subtilis*, polygalacturonase production, optimization, submerged fermentation

ÖZ

ELMA POSASINDAN SIVI KÜLTÜR FERMANTASYONU YÖNTEMİYLE BAKTERİYAL POLİGALAKTURONAZ ÜRETİMİ

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Son on yıl boyunca, atık yönetimi sektörüne artan bir ilgi gözlemlenmiştir, bunun temel nedeni atık bertarafının çevresel tehdidinin artmasıdır. Son yıllarda bilimsel araştırmalar, tarımsal lignoselülozik artıkları değerli ürünlere dönüştürerek bu soruna çözümler bulmaya odaklanmıştır.

Gıda üretimi gibi çeşitli sektörlerde, enzimlerden önemli ölçüde yararlanılmaktadır. Ancak, endüstriyel ölçekte enzim üretiminin yüksek maliyeti önemli bir engeldir. Bu nedenle, hem maliyeti düşürmeye hem de çevresel etkiyi azaltmaya yönelik lignoselülozik malzemelerin enzim üretiminde kullanımına artan bir eğilim vardır.

Bu çalışmada, beslemeli sıvı kültür fermantasyonu yöntemiyle elma posasından poligalakturonaz (PGaz) üretiminin artırılması amaçlanmıştır. *Bacillus subtilis* NRRL B-4219 PGaz üreticisi olarak kullanılmış ve tüm fermantasyon deneyleri 30°C ve 130 rpm koşullarında gerçekleştirilmiştir. Parametrelerin, yani ek substrat

miktarı, eklenme zamanı ve fermantasyon süresinin optimizasyonu için Cevap Yüzeyi Yöntemi'nin Box-Behnken deneysel tasarımı kullanılmıştır.

En yüksek sonuç (12.27 U/mL), fermantasyon üç gün devam ettiğinde ve ikinci gününde 1 g/L pektin eklenmesi durumunda elde edilmiştir. Sonuçların analizi, optimum sürecin ek substrat kullanılmadan üç günlük fermantasyon olduğunu ortaya çıkarmıştır; ancak sonuçlar ikinci günde 1 g/L pektin eklenmesini desteklemektedir. Genel olarak, bu çalışma, elma posasının bakteriyel PGaz üretimi açısından maliyet etkin bir alternatif olduğunu kanıtlamaktadır.

Anahtar Kelimeler: Elma posası, *Bacillus subtilis,* poligalakturonaz üretimi, optimizasyon, sıvı kültür fermantasyonu

To my family

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CHAPTER 1

INTRODUCTION

Enzymes, as biological catalysts, accelerate biochemical reactions in living organisms. They have a rich history of utilization in the food industry, spanning thousands of years, and have proven valuable in various sectors like textiles, paper manufacturing, and biofuel production (Robinson, 2015). Enzymes are known for their efficiency and specificity, performing well-defined reactions repeatedly. They operate optimally under specific pH and temperature conditions, and being derived from nature, they are sustainable and biodegradable. This eco-friendly nature makes them an appealing alternative to chemical additives in the food industry.

In the food industry, enzymes find extensive application, playing diverse roles in enhancing food quality, texture, and taste, while also reducing processing time and costs. These enzymes can be sourced from plants or produced through microbial fermentation and subsequent purification (Kermasha & Eskin, 2020).

Pectinases are responsible for breaking down pectic substances and find applications in various fields, including textiles, food processing, and pectic wastewater treatment. Their main function involves the degradation of pectin, a complex polysaccharide found in the cell walls of plant cells, which contributes to the structural integrity of fruits. As essential enzymes, pectinases are gaining increasing significance in both industrial and biotechnological settings (Jayani et al., 2005).

Apple pomace (AP), a pectin-rich lignocellulosic waste generated by apple-processing industries during the production of apple juice, jam, and jelly, has gained popularity in recent years. With apple fruit being extensively cultivated worldwide, substantial amounts (roughly 5-7 million tons) of AP are generated annually to be treated (Zhang et al., 2021); this surplus poses serious health risks and environmental concerns.

Discovering different approaches to utilize apple pomace instead of simply disposing of it directly shows significant potential in reducing pollution and health risks while also saving money (Vendruscolo et al., 2008). Given that it is quite rich in dietary fibers such as pectin (5.50%–11.70%), lignin (15.30%–23.50%), cellulose (7.20%–43.60%), and hemicellulose (4.26%–24.40%), AP can serve as a valuable resource to create a diverse range of value-added products, including enzymes, organic acids, protein-rich animal feeds, edible mushrooms, ethanol, aroma compounds, natural antioxidants, and edible fibers. These products find applications in various industries such as food, feed, cosmetics, and pharmaceuticals (Bhushan et al., 2008).

Hence, this study aims to optimize the bacterial pectinase, specifically polygalacturonase, production by submerged fermentation using apple pomace as carbon source and *Bacillus subtilis* NRRL B-4219 as the producer at the laboratory scale. The objective is twofold: to provide effective waste management and the cost-effective production of enzymes through an environmentally friendly approach. The focus is on optimizing the quantity of added substrate, the timing of its addition, and the duration of fermentation, rather than adjusting the physical conditions such as pH, inoculation volume, temperature, and agitation speed, in order to enhance the enzyme yield. Box-Behnken experimental design of Response Surface Methodology (RSM) was used to carry out the optimization.

Key points to understand in the literature, including but not limited to apple pomace, pectinases, and RSM are first reviewed in Chapter 2.

Specific details about the methods and materials concerning the experiments that were done in the present study are described in Chapter 3.

Finally, the experimental results and several examples from the literature to further support or disclaim the interpretations regarding the experiments are discussed and the key results are concluded while recommendations for further studies are offered in Chapters 4&5.

CHAPTER 2

LITERATURE REVIEW

2.1 Food processing wastes and their importance

The term “food processing waste” refers to the leftovers, residues, and waste treatment by-products that arise throughout the various stages of food production, processing, distribution, and consumption. These waste materials, in both liquid and solid form, can originate from activities such as commercial food manufacturing or packaging processes (Van Dyk et al., 2013). Around 30% of the raw materials utilized in the food processing industries end up as waste instead of being transformed into products with added value (Tamer and Çopur, 2014). Food processing wastes can be generated by several processing methods such as membrane processes (Routray & Orsat, 2019), packaging and storage, food preparation and cooking, food processing and manufacturing, fruit and vegetable processing (Bayram et al., 2021), animal slaughtering and processing (Narasimmalu & Ramasamy, 2020), food trimming and peeling, solvent extraction, chromatographic separations, and centrifugation.

Food waste and by-products pose a significant issue, resulting in adverse environmental, economic, and social consequences. A global objective is to decrease food waste, but effectively utilizing these waste materials as raw materials or food additives can bring economic advantages to the industry, contribute to addressing nutritional concerns, promote beneficial health effects, and mitigate environmental problems associated with waste management (Torres-León et al., 2018). The conversion of these food wastes and by-products into value-added products holds great significance, encompassing not only economic considerations but also social

and environmental benefits (Ben-Othman et al., 2020; Singh et al., 2019). The utilization of food waste from industrial food processing yields numerous economic, social, and environmental advantages, including the production of value-added products across various applications like organic fertilizers, animal feed, biofuels, and electricity (Bayram et al., 2021). Valorizing food by-products into edible materials is one of the most promising ways to reduce food waste and efficiently utilize solid vegetable waste, resulting in reduced costs for investments and raw materials (Laufenberg et al., 2003). Reducing food waste brings significant environmental and economic advantages, and a highly promising strategy to accomplish this can be through the utilization of food by-products into consumable materials (Socas-Rodríguez et al., 2021).

Producing edible materials is not the only silver lining of food processing wastes; non-edible value-added products are also possible to obtain from lignocellulosic wastes, in fact preferable over edible products (Mirabella et al., 2014). Biocatalysis and various other methods can convert food processing waste into value-added products that are not intended for consumption. Enzymes, which are natural catalysts responsible for facilitating biochemical reactions essential for living organisms, can be employed to convert food waste into valuable products (Karmee, 2023; Patel et al., 2016). Enzymes can be immobilized and employed to transform food waste streams into profitable sources of revenue. Utilizing enzymes in food waste biorefinery shows great potential in reducing the environmental repercussions of waste while also generating economic benefits for the industry (Andler & Goddard, 2018). Through biocatalytic techniques, food waste can also be utilized to produce bioethanol, which is an additional form of non-edible value-added product. Bioethanol finds diverse applications, including but not limited to fuel for transportation, heating, and electricity generation (Manikandan et al., 2023). Table 2.1 summarizes some food processing wastes and value-added products that may be obtained using them.

Table 2.1 Examples of wastes generated from several food processing steps.

Type of food processing	Generated waste	Potential value-added products	References
Packaging and storage	Food scraps and leftovers, paper packaging wastes, expired or spoiled food products	Compost, animal feed, biofuels, organic fertilizers	Bayram et al. (2021), Routray & Orsat (2019), Xiong et al. (2019)
Fruit and vegetable processing	Spoiled fruits and vegetables, leaves, peels, skins, rinds, pulp, cores, seeds, pomace, stems, pits, twigs	Bioplastics, animal feed, packaging, nutraceuticals, organic fertilizers, biofuels, flavoring agents, waste-induced nanoparticles	Ganesh et al. (2022), H. Kumar et al. (2020), Laufenberg et al. (2003), Ueda et al. (2022)
Food processing and manufacturing	Dairy processing waste (whey), bakery waste, meat processing and slaughtering waste	Animal feed, biofuels, biogas, bioplastics, biofertilizers	Chavan et al. (2022), Gunjal (2019)
Food preparation and cooking	Food scraps and leftovers, bones and meat trimmings, coffee grounds, tea leaves, cooking oil and grease	Compost, animal feed, biofuels, organic fertilizers, soap, biodiesel	Dattatraya Saratale et al. (2020), del Pozo et al. (2021), Gunjal (2019)

2.2 Apple pomace

Considering the worldwide consumption of fresh fruits, apple fruit ranks fourth – first three being grapes, oranges, and bananas (Lyu et al., 2020) – given that its high nutritional value and favorable taste (Zhang et al., 2021). In 2013, global consumption of apples per capita was over 9 kg according to the reports, which has been even higher over the years. In 2017, global annual production of apples reached 83.1 million metric tons with an increase rate of 48% over the last twenty years according to FAO. China accounted for 41.4 million metric tons of apple production since Asia is the primary source for apples and being responsible for 65.4% of worldwide apple production (Lyu et al., 2020). Consumption of apple fruit has been associated with prevention of certain diseases such as lung cancer, cerebrovascular disease, obstructive pulmonary disease, and coronary disease (Younis & Ahmad, 2015).

Some of the apple fruits are not suitable for consuming freshly due to being deformed or misshaped; in that case, apple is further processed to produce jelly or juice (Vendruscolo et al., 2008). Consequently, apple is proven to be an important source for food processing industry in addition to being a preferable fresh fruit for direct consumption. Of all the apples that are produced all over the world, around 70% is consumed as fresh fruit and 25-30% is processed to several food products (Shalini & Gupta, 2010). Apple juice concentrates and apple jams are two examples of some of the processed apple products popular in the food industry. Nevertheless, processing of the apple fruit generates large amounts of biodegradable waste called apple pomace (AP), whose improper disposal may potentially create a serious environmental problem that needs to be resolved urgently due to increasingly developing processing industry and the amount of apple processed.

After processing of apples, approximately 25-30% of the original fruit remains as apple pomace, which makes nearly 5-7 million tons of AP annually when calculated (Zhang et al., 2021). Due to its high moist nature and subsequently being bulky, apple pomace is vulnerable in terms of microbial decomposition; therefore, proper and rapid disposal is a great necessity in order to prevent environmental pollution and eliminate public health hazards. However, bulky nature of apple pomace makes its transportation financially – e.g., $10 million annual disposal fee has been reported in the United States (Shalini & Gupta, 2010) – and logistically difficult (Bhushan et al., 2008), thus direct disposal to nearby landfills remains the most popular method. Concerning this problem, several successful attempts have been made to convert this crisis to our advantage by utilizing apple pomace to obtain value-added products that have commercial value as well (Lyu et al., 2020). Hence, finding various methods to utilize apple pomace instead of its direct disposal is a promising way to eliminate pollution and health risks, and to save money as well (Bhushan et al., 2008).

The use of AP offers significant benefits in addressing this problem and making a positive impact on the economy (Zhang et al., 2021). AP can be used to generate a variety of value-added products, such as enzymes, organic acids, protein-rich animal feeds, edible mushrooms, ethanol, aroma compounds, natural antioxidants, and edible fibers. These products can be used in a variety of fields, including food, feed, cosmetics, and pharmaceuticals. (Vendruscolo et al., 2008).

2.2.1 Composition of apple pomace

Apple pomace contains a variety of dietary fibers, antioxidants, and insoluble sugars (e.g., lignin, cellulose, hemicellulose). While the water content is high, AP is mainly composed of soft tissue, stem, calyx, core, seed, and peel. The amount of simple sugar content (e.g., sucrose, fructose, glucose) and other components (e.g., proteins, vitamins, minerals) may vary with respect to the type of apple and methods used

during processing, including but not limited to the period of processing (Vendruscolo et al., 2008).

Dietary fibers make up approximately 35-60% of apple pomace, which include both soluble (14.6%) and insoluble (36.5%) fibers. Pectin, lignin, cellulose, and hemicellulose are the main components of dietary fibers; their approximate percentage of presence are 5.5-11.7%, 15.3-23.5%, 7.2-43.6%, 4.26-24.4%, respectively (Bhushan et al., 2008). A summary of physical and chemical composition of apple pomace is given in Table 2.2 and Table 2.3.

Table 2.2 Physical composition of apple pomace (Bhushan et al., 2008; Zhang et al., 2021).

Components	**%**
Apple skin/flesh	95.0
Seeds	2.0-4.0
Stems	1.0
Ash	1.5-2.95
Moisture	3.9-10.8

Table 2.3 Chemical composition of apple pomace (Bhushan et al., 2008).

Components	% (Dry weight basis)
Protein	2.94 – 5.67
Fiber	
Insoluble fiber (36.5%)	4.7 – 51.1
Soluble fiber (14.6%)	
Fat	1.2 – 3.9
Pectin	3.5 – 14.32
Cellulose	7.20 – 43.60
Total carbohydrate	
Glucose (19.5 – 19.7%)	
Fructose (48.3%)	48.0
Saccharose (3.8 – 5.8%)	
Xylose, mannose, galactose (1.2 – 4.4%)	
Arabinose, rhamnose (6.0 – 7.9%)	

2.2.1.1 Pectin

Pectin is a valuable component of apple pomace in which it is found abundantly, approximately 3.50-14.32% (Joshi & Attri, 2006), and it can be used in various ways in the food industry in addition to many other fields. This indicates that utilization of apple pomace might be employed in order to produce pectin, in fact, it is one of the most reasonable ways to take advantage of this apple processing waste in terms of utilization. Pectin is a dietary fiber that has been suggested to have many advantages to human health, including causing apoptosis of cancer cells in certain types of cancer. In addition to these advantages, pectin is also used in many ways in food industry, such as gelling agent in fruit jams and marmalades (Min et al., 2010).

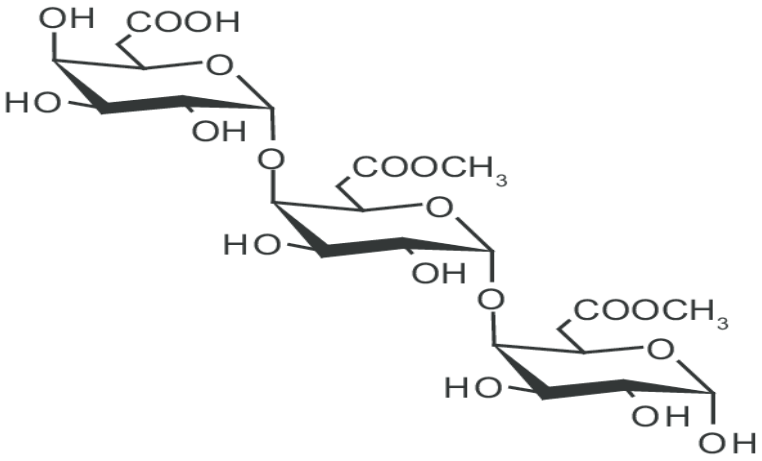

Figure 2.1 Structure of pectin (Ali et al., 2015).

The main component of pectin is D-Galacturonic acid, which is classified as a uronic acid with an acid group attached to aldose sugar (Fig. 2.1). Pectin forms a gel in the presence of bivalent cations (since it becomes water-insoluble) and also in low pH if the sugar concentration is high; hence it is widely used in food industry as a gel-forming agent during production of marmalades and jams as they are supposed to have a jelly-like structure. Processing of some agricultural materials – such as apple, sugar beet, and orange – yields side products that are rich in pectin, processing of which is relatively expensive due to the fact that drying is required in order to prevent decomposing of such residues.

It is possible to hydrolyze pectin to D-Galacturonic acid by treating the source material with sulfuric acid; nonetheless, this process may result is decomposition along with the hydrolysis. A more efficient, environment-friendly, and suitable process would be hydrolyzing pectin with enzymes, more specifically pectinases (Richard & Hilditch, 2009).

2.2.1.2 Cellulose and hemicellulose

Alongside with pectin, apple pomace is also abundant in cellulose, contributing to a complex chemical and physical composition together with lignin and hemicellulose (Ma et al., 2019). It is reported that AP has cellulose content of 127.9 g/kg and hemicellulose content of 7.2 – 43.6 g/kg on a dry weight basis (Lyu et al., 2020). The cellulose and hemicellulose derived from apple pomace have multiple applications, including the production of composites, water treatment, and serving as a biofuel source (Gowman et al., 2019; Sudha, 2011).

Cellulose, a fibrous carbohydrate present in all plants, serves as the main structural element in plant cell walls. Its primary application lies in the production of paper and paper-related items. Furthermore, cellulose shows potential for utilization in composites and water treatment processes (Etale et al., 2023).

Hemicelluloses, which are polysaccharides similar to cellulose, make up approximately 20-35% of the biomass in terrestrial plants. Unlike cellulose, hemicellulose lacks significant physical strength and exhibits an amorphous nature; it can be easily broken down by weak acids or bases, as well as certain enzymes (Zoghlami & Paës, 2019).

Utilization of cellulose and hemicellulose can increase the yield of fermentable sugars, which is essential for producing specific microbial enzymes, by acid hydrolysis as the present acid increases the number of hydrogen ions, causing an increase in hydrolysis reaction rate (Kuvvet, 2016). Hence, the cellulose and hemicellulose found in apple pomace can be used as a substrate for the production of pectinases (Perussello et al., 2017). Pectin, on the other hand, hinders the

enzymatic hydrolysis of cellulose and hemicelluloses present in the cell wall of apples by cellulases and hemicellulases (Ben-Shalom, 1986).

2.3 Enzymes

Enzymes are proteins that act as biological catalysts, speeding up biochemical reactions in living organisms. Their application in the food industry has a long history spanning thousands of years, and they have also found utility in diverse sectors like textiles, paper manufacturing, and biofuel production (Robinson, 2015). Enzymes exhibit high efficiency and specificity, often executing a singular, well-defined reaction repeatedly. They tend to have optimal pH and temperature conditions and are sustainable and biodegradable since they are generally derived from nature. This makes them an attractive alternative to chemical processing in the food industry. Enzymes find extensive application in the food industry, serving diverse functions such as enhancing the quality, texture, and taste of food, while also reducing processing time and expenses. Enzymes can be obtained from plants or produced through microbial fermentation and subsequent purification (Kermasha & Eskin, 2020).

Enzyme engineering, a cutting-edge technology, combines principles from enzymology and chemical technology, offering a solution to overcome inherent limitations associated with numerous chemical processes across multiple industries (Victorino da Silva Amatto et al., 2022). The employment of enzymes in the food industry offers numerous advantages, including the enhancement of nutritional value, texture, and taste of food items, alongside the reduction in processing time and expenses (Motta et al., 2023; S. Wu et al., 2021). Enzymes are also utilized for extracting juice from fruits, clarifying wine, and enhancing the texture and flavor of processed foods (Patel et al., 2016). Furthermore, the utilization of enzymes in food waste biorefinery show potential as a means to decrease the environmental

consequences of waste while concurrently generating economic benefits for the industry (Karmee, 2023). Table 2.4 shows some of the enzymes used in the food industry.

Table 2.4 Enzymes used in the food industry (Raveendran et al., 2018).

Enzyme	Purpose	Food products
Amylase	Breaks down starch into sugars	Bread, beer, breakfast cereals, fruit juices
Protease	Breaks down proteins into amino acids	Meat tenderizers, cheese, soy sauce
Lipase	Breaks down fats into fatty acids	Cheese, butter, margarine, salad dressings
Pectinase	Breaks down pectin in fruits and vegetables	Fruit juices, jams, jellies, wine
Lactase	Breaks down lactose in milk	Dairy products for people with lactose intolerance
Glucose oxidase	Oxidizes glucose to produce gluconic acid	Bakery products, canned fruits, dried fruits
Catalase	Converts hydrogen peroxide into water and oxygen	Wine, beer, juices

Use of enzymes for the utilization of apple pomace is suggested to be preferrable due to their catalytic properties and ability to be produced by a variety of microbes (e.g., bacteria, fungi, yeasts), plants, and animals. When they are employed during the process of catalysis of apple pomace to fermentable sugars instead of using

hazardous materials, such as certain acids, they can be advantageous by reducing the cost and duration of the process, being more environment-friendly and less toxic, and decreasing the energy and work needed for the process (Ozzeybek & Cekmecelioglu, 2022). In the literature, enzyme production and purification has been widely studied. It is obvious that enzymes of fungal origin (e.g., *Aspergillus* sp.) take longer time to produce and purify, compared to those with bacterial origin (e.g., *Bacillus* sp.) since usually shorter period of fermentation is required with bacteria (Uzuner & Cekmecelioglu, 2021).

2.3.1 Pectinases

Among numerous uses of apple pomace, probably the most important one is the enzyme production, mainly pectic enzymes or pectinases. Pectic enzymes are responsible for degrading pectic substances and can be applied in many fields such as textile sector and food processing as well as in pectic wastewater treatment. Fermentation process, both submerged fermentation (SmF) and solid-state fermentation (SSF), of apple pomace with fungi or bacteria can produce these handy pectic enzymes (Kumar Joshi et al., 2006; Vendruscolo et al., 2008).

Pectinases' primary duty is to degrade complex polysaccharides called pectin, which is located in the cell wall of plant cells and mainly responsible in a structural manner such as rigidity of the fruit. Classification of pectinases may be observed in three classes: pectinesterases, depolymerizing enzymes (hydrolases, lyases), and protopectinases, based on certain criteria: (1) their substrate preference, which may be pectin, pectic acid, or oligo-D-galacturonate; (2) the type of action they perform, whether it's trans-elimination or hydrolysis; and (3) the manner in which they cleave, which can be either random (liquefying or depolymerizing enzymes) or 'endwise' (saccharifying enzymes) (Shet et al., 2018) (Fig. 2.2).

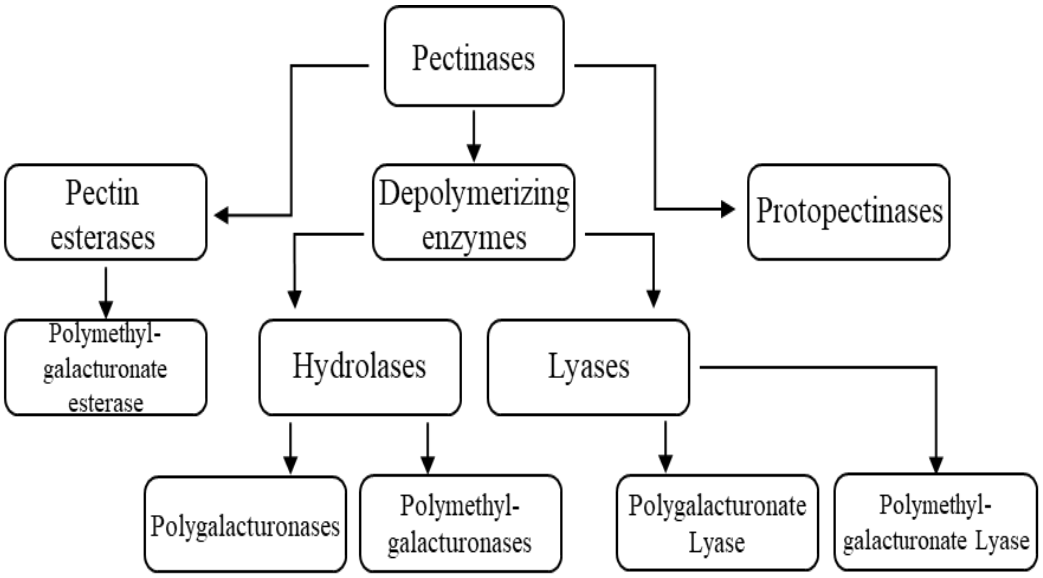

Figure 2.2 Schematics of pectinase classification.

Pectinases are important enzymes increasingly employed in industry as well as for biotechnological purposes (Kashyap et al., 2001; Sakai et al., 1993). In the literature, successful production of microbial pectinases with the help of fermentation process using substrates that are rich in pectin has been reported (Abdul Sattar Qureshi, 2012; Rehman et al., 2012). Given that they have been gradually popular over the years since their first known application in 1930 by Kertesz, commercially produced pectinases currently lead the industrial market among other commercially produced enzymes. Industrial enzymes can be produced by various sources, with microorganisms leading the way by being accountable for 85% of industrial enzyme production (50% yeasts and fungi, 35% bacteria); plant or animal production of industrial enzymes only makes 15% of the overall enzyme production. Many microorganisms of several kinds are responsible for pectinase production (Garg et al., 2016).

As it is mentioned above, Kertesz reportedly used pectinases with the purpose of clarifying apple juice in 1930, which makes it the first known application of pectinases commercially (Garg et al., 2016). Since then, they are employed with other purposes due to the important role they have in various processes such as pulp liquefaction (hence clarification), maceration (paste and puree production), reducing viscosity, and removal of peels (Shet et al., 2018).

Pectinases are generally used in processing of several fruits and vegetables like citrus, banana, and papaya as they contribute to juice stabilization and higher yield. When a certain fruit is treated with pectinase, phenolic content is released from the skin of the fruit. This content has a significant advantage regarding health maintenance since some of the components are antioxidants and can contribute to prevention of certain diseases and cancers, especially heart problems (Garg et al., 2016).

Another application of pectinases is the biorefinery field; they are used in waste management and obtaining value-added products from lignocellulosic materials. Pectinases help convert the agricultural wastes resulting from fruit processing to fermentable sugars, which can be used to produce biofuel and many other value-added products with the assist of certain microorganisms (Kashyap et al., 2001). In order for microbial pectinases to be commercially produced and applied widely in the industrial sector and various other fields, a great knowledge about the properties of these enzymes is necessary and should be studied thoroughly.

One of the challenges of enzyme production is maintaining the stability and enzyme activity, on which many studies have been conducted. These studies can improve understanding of the function and structure of the enzymes, as well as shedding a light on how to design and produce enzymes, especially pectinases. Temperature,

pH, activators, and inhibitors have been reported to affect the pectinase stability, while many studies focused on the effect of physical parameters (temperature and pH). It is claimed that keeping one of the parameters constant and changing the other one has altered the stability. Furthermore, stability was also altered when the interaction of both parameters with each other was studied (Gummadi & Panda, 2003).

2.3.1.1 Polygalacturonase

Polygalacturonase (PGase) is an enzyme classified under the pectinase group that functions by breaking down pectin through the hydrolysis of O-glycosyl bonds within pectin's polygalacturonan network (Rehman et al., 2012). Polygalacturonic acid (or pectin) serves as the substrate for PGase. The enzymatic reaction of PGase involves the hydrolysis of the α-1,4 glycosidic bonds connecting galacturonic acid residues (Richard & Hilditch, 2009). The reaction can be represented as follows (also visualized in Fig. 2.3):

(1,4-α-D-galacturonosyl)n+m + H_2O = (1,4-α-D-galacturonosyl)n + (1,4-α-D-galacturonosyl)m (BRENDA:EC3.2.1.15)

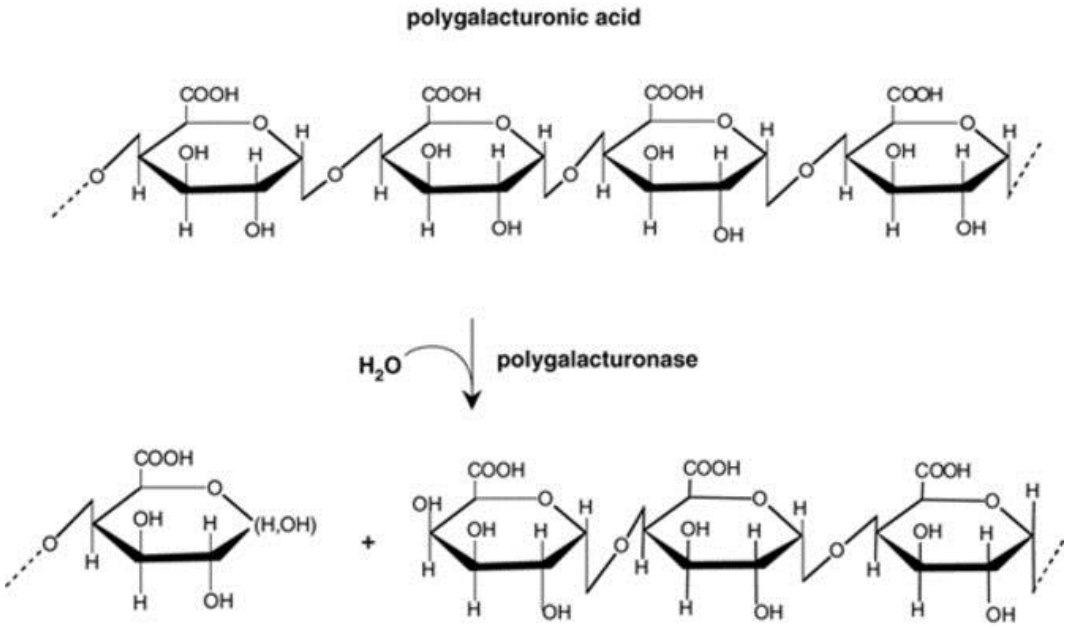

Figure 2.3 Reaction mechanism of polygalacturonase (Kongruang & Penner, 2003).

Polygalacturonase is classified as an endo-acting enzyme, meaning it cleaves the internal glycosidic bonds within the polygalacturonan chain. Its role is significant in fruit ripening, where it contributes to the softening and sweetening process by breaking down the pectin network found in plant cell walls (Niture, 2008). Interestingly, phytopathogens also utilize PGase to weaken the pectin network, enabling them to release digestive enzymes into the host plant for nutrient acquisition (D'Ovidio et al., 2004).

Polygalacturonase is categorized as a depolymerase due to its role in the depolymerization process. The enzyme is extensively studied within the pectinase enzyme family, which plays a significant role in the food processing industry and the interactions between plants and fungi, both in terms of functionality, technical applications, and biological implications (Haile & Ayele, 2022; Rehman, 2023). The

versatile applications of polygalacturonase span various industries, including food processing, textiles, paper production, and biofuel manufacturing. It can be employed to extract juice from fruits, clarify wine, and enhance the texture and flavor of processed food products (Ramya & Pulicherla, 2015).

Polygalacturonase exhibits diverse biochemical properties and mechanisms of action, which vary depending on the microbial source from which it is derived. In general, most PGase enzymes exhibit optimal hydrolysis rates within a pH range of 3.5 to 5.5 and thrive at temperatures between 30 to 50°C. To enhance PGase production, agro-waste pectin can be utilized as a carbon source (Satapathy et al., 2020). The enzyme can be produced by a variety of microorganisms, including *Bacillus subtilis*, *Aspergillus giganteus*, and various fungi (Ramya & Pulicherla, 2015). Table 2.5 reviews several bacterial and fungal sources of PGase production.

Table 2.5 Microbial polygalacturonase producers of bacterial and fungal origin along with the optimum working temperatures and pH values for PGase (if available).

PGase producer	**Opt. temperature (°C)**	**Opt. pH**	**Source**
Agrobacterium vitis	-	-	Vordtriede & Yoder (2008)
Aspergillus aculeatus ATCC16872	50	5	Abdulrachman et al. (2017)
Aspergillus alliaceus	-	-	Sreenath et al. (1986)

Table 2.5 (continued)

Aspergillus carbonarius	-	-	Nakkeeran et al. (2010)
Aspergillus fumigatus MTCC 2584	30	10	Anand et al. (2016)
Aspergillus japonicus	30-50	4-5	Semenova et al. (2003)
Aspergillus kawachii	-	2-3	Esquivel & Voget (2004)
Aspergillus luchuensis IFO 4308	-	5	Voget et al. (2006)
Aspergillus niger	-	-	Woosley et al. (2006)
Aspergillus niger SA6	40	4.5	Buga et al. (2010)
Aspergillus niger SC323	50	5	Zhou et al. (2015)
Aspergillus niger T0005/007-2	50	-	Henrique Hendges et al. (2011)
Aspergillus sojae	50	3.5-5	Fratebianchi et al. (2017)
Aspergillus sp. N12	45-50	5	Mendes De Freitas et al. (2006)
Aspergillus tubingensis	60	5	Tai et al. (2013)
Bacillus paralicheniformis CBS3	50-60	9.1	Khan et al. (2018)

Table 2.5 (continued)

Bacillus pumilus	-	-	Deuel & Stutz (2006)
Bacillus subtilis C4	60	9	Kusuma & Reddy (2014)
Kluyveromyces fragilis	55	5-7	Sakai et al. (1984)
Kluyveromyces marxianus	55	4.5	Serrat et al. (2002)
Kluyveromyces marxianus CCEBI 2011	-	-	Serrat et al. (2004)
Kluyveromyces marxianus CECT1043	55	4.5	Sieiro et al. (2009)
Penicillium sp.	40	3.5	Yuan et al. (2011)
Saccharomyces cerevisiae BY4742	45	4.5	Eschstruth & Divol (2011)
Saccharomyces cerevisiae L2323	-	-	Louw et al. (2010)

Table 2.5 shows only a small portion of PGase producers while in fact there are many more, possibly even more microorganisms are yet to be studied or discovered. According to enzyme database BRENDA, most studies used *Aspergillus* species to produce PGase (EC3.2.1.15), especially *Aspergillus niger* strains were used. Several studies also purified and immobilized the enzyme for further experiments (Fratebianchi et al., 2017; Khan et al., 2018; Kusuma & Reddy, 2014). Studies researching microbial PGase production often studied the temperature and pH at

which the enzyme can work optimally. The results differ for each study, yet it is possible to determine a specific range for the optimum conditions: the optimum temperature is between 30-60 ^{o}C and the optimum pH is between 2-10 according to studies listed in Table 2.5. However, the range for pH is too wide since it can also depend on the temperature. While the optimum conditions differ for each microorganism and their strains, it is important to optimize the enzyme production conditions in terms of physical conditions like pH, temperature, and agitation speed.

2.4 Optimization of enzyme production

Enzymes are sensitive to both physical and chemical factors such as pH, temperature, carbon source and available substrate amount since there is a possible risk of inhibition by excess amounts; therefore optimization of enzyme production is a crucial step in the industrial production of microbial enzymes by fermentation. It is really important to set the parameters in the most suitable way while carrying out the enzyme production; there are several strategies which can help achieving this goal, such as optimization of fermentation and enhancing the enzyme activity.

Fermentation optimization is a highly effective technique that can swiftly enhance titers, yields, and productivities; and it involves optimizing various parameters such as pH, temperature, aeration rate, and agitation speed (Deng et al., 2016). By fine-tuning these fermentation conditions, it becomes possible to produce microbial enzymes with the desired properties. Achieving cost-effectiveness in fermentation requires the utilization of inexpensive substrates, alongside the optimization of crucial parameters like pH, temperature, and aeration rate (Niyonzima et al., 2020). Maintaning these parameters at a constant level may require the use of bioreactors, especially for the aeration rate. Process duration is another crucial factor for the enzyme yield. Extended durations in processes can result in increased yields, but they can also lead to enzyme deactivation. Hence, optimizing the duration of the

process is crucial to attain the maximum yield while ensuring enzyme stability is not compromised (Kabir & Ju, 2023).

In addition to optimizing the reaction conditions, techniques such as protein engineering, directed evolution, and site-directed mutagenesis can be employed to enhance enzyme activity. In the pursuit of enhancing enzyme performance for food and feed applications, significant attention should be given to enhancing thermal stability. This objective can be accomplished through approaches like protein engineering, immobilization, and the incorporation of additives. (Fernandes, 2010).

In this manner, optimization of the procedure is required for the maximum yield during enzyme production, which bears the necessity of using certain methods or statistical softwares as an aid to this goal. Response surface methodology (RSM) and central composite design (CCD) are frequently employed statistical techniques for the optimization of microbial enzyme production, both of which are useful for optimizing the medium components or physical parameters of fermentation (Kanmani et al., 2013; Talluri et al., 2019). Table 2.6 gives several examples of statistical optimization methods.

Table 2.6 Examples of statistical optimization methods for enzyme production in the literature.

Optimization method	**Enzyme of interest**	**Reference**
Taguchi method	Laccase	Chenthamarakshan et al. (2017)
Plackett-Burman design (PBD)	Alkaline protease	Reddy et al. (2008)

Table 2.6 (continued)

Box-Behnken design	Keratinase	Awad et al. (2011)
Artificial neural networks (ANN)	Lipase	Sales de Menezes et al. (2021)
Extreme vertices mixture design (EVMD)	Cellulase, Pectinase	Ozzeybek & Cekmecelioglu (2022)

The Taguchi method relies on the principle of signal-to-noise ratio, which quantifies the distinction between the desired process output (signal) and unwanted variation (noise). A higher signal-to-noise ratio indicates a more consistent process output. To design experiments, the Taguchi method utilizes orthogonal arrays, which are collections of experimental runs aimed at minimizing the number of required runs while estimating the effects of various factors (Malhotra & Chapadgaonkar, 2020).

The Plackett-Burman design is a specific two-level variant of fractional factorial design that operates under the assumption of complete neglect of interactions, allowing the calculation of main effects using a reduced number of experiments (Das & Dewanjee, 2018). Plackett-Burman designs offer high efficiency in terms of the required number of experimental runs. For instance, a 100-run Plackett-Burman design can effectively screen up to 99 factors. This efficiency arises from the design's orthogonality, meaning that the effects of different factors are not entangled or confounded with one another (Humbird & Fei, 2016).

A Box-Behnken design is an experimental design employed to construct a quadratic model for a response surface, in which multiple parameters can be optimized simultaneously. In this model, the main effects of factors and their interactions are

included in the optimization (Ferreira et al., 2007). Box-Behnken design was employed for this study as well.

Artificial neural networks (ANN) are computational structures that draw inspiration from the functioning of living beings' nervous systems. They find application in problem-solving and machine learning (Abiodun et al., 2018). Employing ANN allows for the optimization of enzyme production by forecasting enzyme behavior under various conditions (Baş et al., 2007). ANN can also predict the enzyme activity through using electronic and geometrical characteristics of substrates (Szaleniec, 2012).

Extreme vertices mixture design is a specific type of mixture design employed in experiments involving mixtures, especially when the response can be represented by a linear model. These designs are also known as constrained mixture designs. Extreme vertices designs focus on a subset or smaller region within the simplex, which is essential when the mixture components are restricted by certain constraints (Snee & Marquardt, 1974).

2.5 Aim of the study

Food processing industries produce significant amounts of solid and liquid sludge wastes. Improper management of such waste not only leads to substantial losses of water, land, fertilizer, energy, and labor but also results in the wastage of potentially valuable bioactive compounds found in seeds, skin, rind, and pomace (Choudhury et al., 2020; Gołębiewska et al., 2022).

Particularly, apple processing industries generate substantial quantities of solid residues comprising a mixture of apple skin, pulp, and seeds (Dhillon et al., 2013).

Apple pomace, in particular, is a valuable resource containing carbohydrates, pectin, crude fiber, and minerals, making it a nutritious and beneficial ingredient. This byproduct can be utilized in various industrial sectors, including food, feed, and pharmaceuticals (Shalini & Gupta, 2010).

Pectinases are enzymes that degrade pectin, a complex polysaccharide present in plant cell walls. In the food industry, pectinases find extensive application for multiple purposes, including but not limited to enhancing food quality, texture, and flavor, as well as reducing processing time and expenses (Patel et al., 2016).

To obtain microbial enzymes with desired characteristics, it is necessary to optimize fermentation conditions. This involves utilizing cost-effective substrates and fine-tuning fermentation parameters such as pH, temperature, and aeration rate. To achieve cost-effectiveness, various statistical tools can be employed, including response surface methodology, central composite design, Taguchi method, artificial neural networks, Plackett-Burman design, and Box-Behnken design, all of which aid in optimizing enzyme production. This optimization process is crucial in attaining high yields, cost reduction, and enhancing the performance of biocatalysts for food and feed applications (Niyonzima et al., 2020).

This study aims at optimization of polygalacturonase production using Box-Behnken Response Surface Methodology. Fermentation was carried out in a fed-batch manner and the objective was to fix the fed-batch conditions rather than the fermentation conditions. In other words, it was aimed to optimize added substrate amount, addition time, and fermentation period rather than the physical conditions like pH, inoculation volume, temperature, and agitation speed to increase the enzyme yield.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

Apple pomace (AP) was acquired from Göknur Foodstuff Company in Ankara, Turkey; essentially as company's residual waste resulting from the apple processing carried out in order to produce apple concentrate. Before further handling, AP was dried using a tray drier (Eksis Industrial Dryer Systems, Isparta, Turkey) for 6 hours at 60 ^{o}C. Dried AP was ground to particles of 1 mm using a laboratory-scale grinding mill (FRITSCH Industries. D - 55743 Idar-Oberstein, Germany). After drying and grinding, AP was stored at room temperature to be used in fermentation experiments.

3.1.2 Activation and storage of microorganisms

Bacillus subtilis NRRL B-4219 was used to produce pectinase using apple pomace as it is known as pectinase producer (Uzuner & Cekmecelioglu, 2021); it was kindly provided by Agricultural Research Service (ARS) Culture Collection, Northern Regional Research Laboratory, Peoria, Illinois, USA. Nutrient broth (Merck, Turkey) was used to activate bacteria and prepared as 50 mL in 250 mL Erlenmeyer flasks according to the instructions on the box (8.0 g/L). Activated bacteria were stocked in 1.5 mL Eppendorf tubes with 25% glycerol solution in aceptic conditions and stored at -80 ^{o}C (Revco Elite Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA) for long term preservation (Korsten & Cook, 1996).

Frozen cells were reactivated in 50 mL nutrient broth in 250 mL Erlenmeyer flasks, at 30 ^{o}C and 130 rpm in a shaking incubator (INFORS AG CH-4103, Bottmingen, Switzerland). Nutrient broth was sterilized before use at 121 ^{o}C for 15 minutes with an autoclave (Tomy SX-700E, Tomy Kogyo Co., Tokyo, Japan). Cell growth was monitored by measuring the optical density at 600 nm wavelength (OD_{600nm}) with a spectrophotometer (UV-1280 – UV-VIS Spectrophotometer, Shimadzu Corp., Kyoto, Japan). Incubation period for reactivation was 18 hours for *Bacillus subtilis.*

3.1.3 Polygalacturonase production media

Mainly apple pomace hydrolysate was used for production of pectinase (more specifically, polygalacturonase); however, synthetic medium was also used for comparison of sugar consumption of bacteria. Cell growth, total reducing sugar content, and enzyme activity were usually measured every 24 h during fermentation period by spectrophotometric methods. Analyses were detailed in section 3.2.

3.1.3.1 Apple pomace hydrolysate

Acid pretreatment method was applied for the preparation of apple pomace hydrolysate; the method is detailed in section 3.2.1. The hydrolysate was placed to 500 mL Erlenmeyer flasks with a working volume of 100 mL and the flasks were sterilized using autoclave at 121 ^{o}C for 15 min. No chemicals added to the fermentation media, except for pectin in certain experiments.

3.1.3.2 Synthetic media

Synthetic fermentation medium was prepared with a working volume of 100 mL in 500 mL Erlenmeyer flasks consisting of different amounts of glucose in each flask while keeping other chemicals constant. Components of the media are listed in Table 3.1 (Kuvvet, 2016). Flasks were sterilized using autoclave at 121 ^{o}C for 15 min.

Table 3.1 Components of synthetic fermentation media (Kuvvet, 2016).

Components	Amount (g/L)
Glucose	5.0, 10.0, 20.0, 30.0
Pectin	2.0
Yeast extract	1.0
K_2HPO_4	0.4
$MgSO_4$	0.4
KH_2PO_4	0.2

3.1.4 Buffers and solutions

DNS solution and phosphate buffer were used in analyses for measuring pectinase activity and reducing sugar content. 1% polygalacturonic acid (PGA) solution were used as substrate in enzyme activity analysis. Content and preparation of all the solutions and buffers are detailed in Appendix A.

3.1.5 Chemicals

All the chemicals used in this study were analytical grade and acquired from Merck and Sigma-Aldrich in Turkey (Appendix B).

3.2 Methods

3.2.1 Apple pomace hydrolysate preparation with acid pretreatment

Acid pretreatment method was applied to obtain fermentable sugars from apple pomace (Van Dyk et al., 2013). 0.5 g, 1 g, 3 g, and 5 g of dried and ground apple pomace were treated with 2% (w/v) dilute H_2SO_4 and autoclaved at 121 ^oC for 15

min for hydrolysis of apple pomace. Solid parts were removed by vacuum filtration (TW-1A Vacuum Pump, Star Machine LLC., Baltimore, USA) after the hydrolysis and pH of the resulting hydrolysate was adjusted to 5.0 (Nur Indah Koni et al., 2017) using 10 M H_2SO_4 and 10 M NaOH (Tuhanioğlu, 2021). The hydrolysate was then centrifuged one time at 8000xg for 10 min at 4 ^oC to get rid of the pellet formed by the pH adjustment, consisting of mainly salt (NF 1200R Multi-purpose Bench-Top Centrifuge, NÜVE Sanayi Malzemeleri İmalat ve Ticaret A.Ş, Ankara, Turkey) (Kurt, 2019).

3.2.2 Polygalacturonase production

After the acid pretreatment of apple pomace and sterilization of the resulting fermentation media, the flasks of 100 mL medium each were then inoculated in aseptic conditions with 2% (v/v) *Bacillus subtilis* to start the fermentation process. Fermentation media were kept in a shaking incubator at 30 ^oC (Hours et al., 1988) and 130 rpm during the fermentation process. Same inoculation steps and fermentation conditions were applied for synthetic media as well. Praparing of the fermentation media and the subsequent inoculation are visualized in Fig. 3.1.

3.2.3 Substrate addition

In this study, pectin was added to apple pomace hydrolysate media before or during the fermentation; it was added in both solid and liquid forms separately. However, due to pectin forming a gel-like structure when mixed with water, solid form was preferred since it was easier to handle and concentration adjustment being more straightforward.

Pectin was sterilized with an autoclave at 121 ^oC for 15 min before use in both liquid and solid forms; addition took place in aseptic conditions. Solid pectin was added at

t=0, t=1, and t=2 (days) of the fermentation period and samples were collected regularly in accordance with the nature of the experiment to monitor the change in enzyme activity.

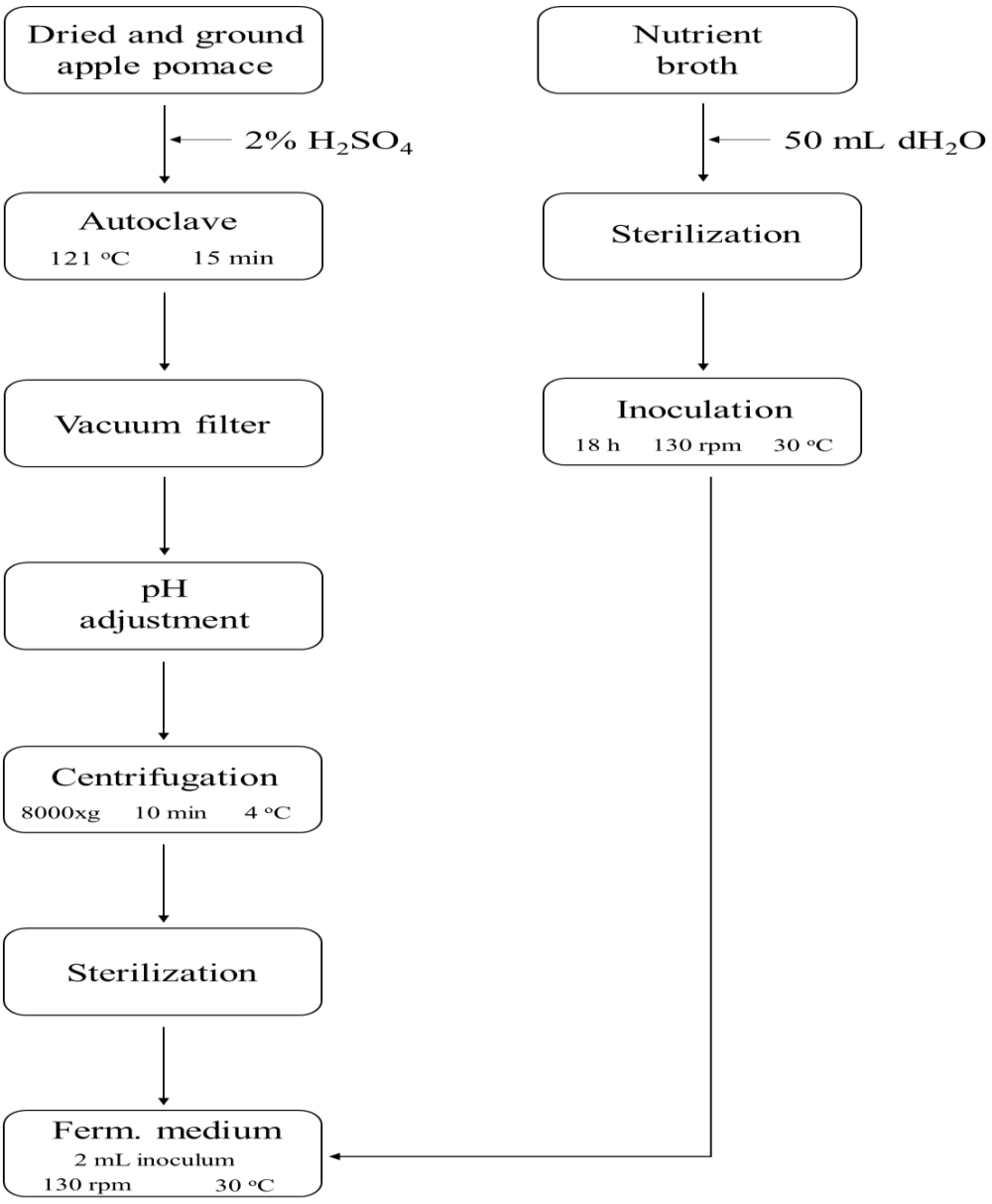

Figure 3.1 Schematic visualization of apple pomace hydrolysate preparation and inoculation.

3.2.4 Cell growth

Optical density was measured to determine cell growth. 1 mL of the growth medium was taken after 18 h of growth. The sample was placed in a 1.5 mL Eppendorf tube and centrifuged for 10 min at 8000xg using a bench-top micro-centrifuge (MPW-15 Mini Centrifuge, MPW Med. Instruments Co., Warsaw, Poland). The supernatant was decanted, and distilled water was added up to 1 mL and centrifuged for 10 min again. This was repeated one more time so that the cells were washed twice. Finally, the cells were resuspended and placed into spectrophotometer cuvettes with 10x dilution. Optical density was measured at 600 nm wavelength in a spectrophotometer using distilled water as spectro-zero.

3.2.5 Total reducing sugar analysis

DNS method was used to analyze total reducing sugar content in the media before, during, and after the fermentation process (Miller, 1959). Total reducing sugar was monitored every 24 h during fermentation period. 1 mL sample was collected from each flask containing fermentation media and centrifuged for 10 min in 1.5 mL Eppendorf tubes. Supernatant of the samples was diluted to a final volume of 3 mL in test tubes and mixed with 3 mL of DNS solution. The tubes were placed in a water bath (SBD-313 Water Bath, Şimşek Laborteknik Ltd. Şti., Ankara, Turkey) at 95 ^{o}C along with a blank tube containing no sample – only distilled water and DNS solution, prepared at the same volume as the samples – and kept there for 15 min. After they were removed from the water bath, the tubes were left to cool down in tap water for 10 min so that the already visible color change would be clearer, and they can be handled safely. After the assay was finalized, absorbance values of the samples were measured at 575 nm wavelength using a spectrophotometer versus the blank tube as spectro-zero. The absorbance values were converted to reducing sugar concentrations using a standard curve previously prepared with various glucose concentrations ranging from 0 to 2 g/L (Appendix C).

3.2.6 Pectinase assay

Polygalacturonase (PGase) activity was monitored every 24 h, occasionally every 2-6 h, during the fermentation period; DNS method was used for this assay as well. Collected samples of 1 mL were centrifuged and supernatant was diluted to 3 mL with phosphate buffer. For this experiment, two sets of test tubes were prepared containing 0.5 mL of the diluted samples; one set was mixed with 0.5 mL polygalacturonic acid (PGA) while other was mixed with 0.5 mL phosphate buffer, the latter being substrate-blank (enzyme + no substrate) samples. Two additional tubes were prepared simultaneously: an enzyme-blank (no enzyme + substrate) sample containing PGA and phosphate buffer, and a spectro-zero (containing only phosphate buffer) against which the other tubes were measured in the spectrophotometer. All the samples and blanks, final volume of 1 mL each, were incubated in a water bath (WiseBath, Wisd Laboratory Instruments) at 50 ^{o}C for 30 min. After incubation, 3 mL DNS solution was immediately added to all the tubes, and they were directly placed in a 95 ^{o}C water bath for 15 min. After cooling down, the absorbance values were measured at 575 nm wavelength with a spectrophotometer. The results were then translated into enzyme activity by using an equation (created with differences between blanks and actual samples) and a standard curve prepared with different concentrations of D-Galacturonic acid ranging from 0 to 1 g/L. Pectinase activity is expressed by "units per milliliter (U/mL)", as one unit represents the amount of enzyme that releases 1 micromole of D-galacturonic acid per minute by catalysis. The aforementioned equation and the standard curve are given in Appendix D.

3.2.7 Optimization of polygalacturonase production

Optimization of fermentation process is crucial for the enzyme production. When there is more than one variable that affect the product, using statistical methods is necessary for the most accurate and precise results, along with statistical tools as

certain software are useful for rapidly designing a large number of variables by establishing upper and lower limits (Vanaja & Rani, 2007).

3.2.7.1 Response Surface Methodology and Box-Behnken Design

Response Surface Methodology (RSM) allows for the measurement of the relationship between different variables and their impact on product yield, which can aid in identifying optimal conditions (Kurt, 2019). Several studies have used RSM for optimizing the fermentation conditions. In this study, experiments were designed with Box-Behnken design of RSM with the help of Minitab 13 software (Minitab Inc., State College, PA, USA). Minimum and maximum values of three independent parameters (fermentation period, additional substrate concentration, addition time) were determined and 15 runs were designed by Minitab with two replicates (total of 30 runs). Analysis of Variance (ANOVA) test was carried out by Minitab and full-quadratic equation (1) was formed to quantify the relations of all the parameters with each other, where Y is polygalacturonase activity, X_1 is additional substrate concentration, X_2 is addition time, and X_3 is fermentation period. Extreme levels of each parameter and experimental design are given in Tables 3.2 and 3.3.

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (1)$$

Table 3.2 Range of parameters used for optimization of PGase production.

	Parameter	**Min (-1)**	**Center (0)**	**Max (+1)**
X_1	Add. substrate concentration (g/L)	0	1	2
X_2	Addition time (day)	0	1	2
X_3	Fermentation period (day)	1	2	3

Table 3.3 Box-Behnken experimental design for optimization of PGase production.

Run Order	Add. substrate concentration (g/L)	Addition time (day)	Fermentation period (day)
1	1	0	1
2	2	1	3
3	2	1	1
4	0	1	3
5	0	0	2
6	0	0	2
7	0	1	3
8	1	0	3
9	0	2	2
10	1	2	1
11	1	1	2
12	1	1	2
13	0	1	1
14	2	0	2
15	0	2	2
16	1	1	2
17	2	1	1
18	2	2	2
19	1	0	3
20	1	1	2
21	1	1	2
22	2	2	2
23	1	1	2
24	1	2	1
25	1	2	3
26	2	1	3
27	2	0	2
28	0	1	1
29	1	0	1
30	1	2	3

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth of *Bacillus subtilis* NRRL B-4219

Nutrient broth was used as growth medium for *Bacillus subtilis* NRRL B-4219 and growth was monitored for 24 h since death phase was observed after 24 h in a previous study (Younis et al., 2010). Samples were analyzed to determine the optical density at 600 nm wavelength every 3 hours during the 24 h. Growth medium was incubated at conditions determined in accordance with the previous studies (Table 4.1) (Ozzeybek & Cekmecelioglu, 2022). Maximum growth was observed after 18 h as 0.8 OD_{600nm}; as a result, all the experiments were conducted with an inoculum of constant optical density (~1.0 OD_{600nm}) after 18 h of growth (Fig. 4.1).

Table 4.1 Growth conditions of *Bacillus subtilis* NRRL B-4219.

Parameters (unit)	**Values**
Growth medium volume (mL)	50
Inoculum volume (v/v%)	1
Temperature (^oC)	30
Agitation speed (rpm)	130

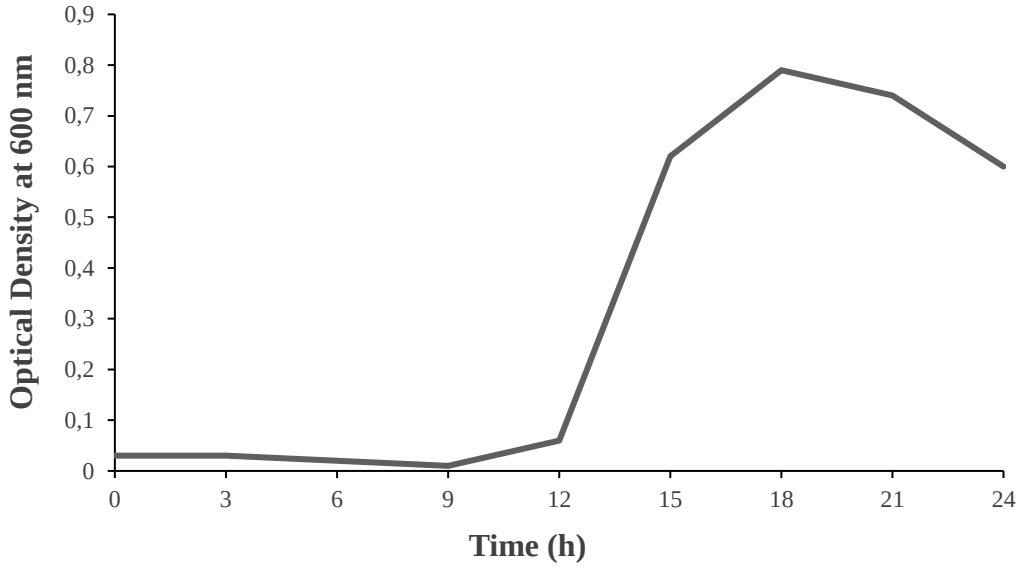

Figure 4.1 Growth curve of *Bacillus subtilis* NRRL B-4219.

The growth curve of *Bacillus subtilis* changes under different conditions and with different media, however, the general doubling time for the microorganism is found to be 20 minutes between 30-37 ^{o}C in some studies (Errington & van der Aart, 2020).

4.2 Initial screening experiments

Initial screening tests were performed in order to determine the most effective solid load, to compare apple pomace hydrolysate with synthetic media, and to fix the fermentation period.

4.2.1 Determination of fermentation period

In order to determine the fermentation period, 2 identical flasks (replicates) were prepared by acid hydrolysis of 10 g/L apple pomace concentration and observed for 7 days by collecting samples every day. Samples were then analyzed to calculate the pectinase production (enzyme activity) for each day. According to the results, enzyme activity was the highest during the first and second day of fermentation and

started to decline gradually after the fourth day (Fig. 4.2). Taking this into consideration, all the experiments were carried out for 3 days as it seemed to be the optimal fermentation period for pectinase production by *Bacillus subtilis.*

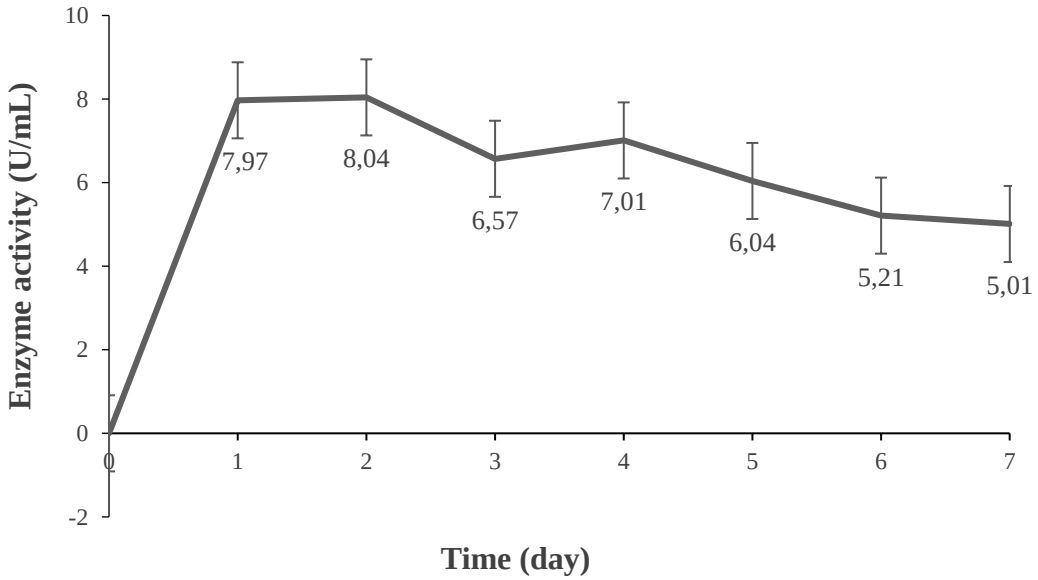

Figure 4.2 Daily pectinase production activity of *Bacillus subtilis* NRRL B-4219.

While the most suitable fermentation period for maximum enzyme activity changes with fermentation conditions and some other factors (e.g., strain of the microorganism), it is generally estimated to be between 24 h and 96 h when the literature is reviewed (Abdul Sattar Qureshi, 2012; Alqahtani et al., 2022; Kumar Joshi et al., 2006; Kuvvet et al., 2019).

4.2.2 Determination of solid load

Optimal solid load was determined by comparing the enzyme activity of different concentration of solid loads, that is, 5 g/L, 10 g/L, 30 g/L, 50 g/L. All the samples

were prepared with two replicates and all the fermentation media were prepared with 100 mL working volume in a 500 mL Erlenmeyer flasks. pH was adjusted to 5 for all the fermentation media and they were incubated at constant conditions (30 ºC with an agitation speed of 130 rpm) (Errington & van der Aart, 2020; Nur Indah Koni et al., 2017). Samples were analyzed on the second day of the fermentation period and enzyme activity was measured as it is detailed in section 3.2.

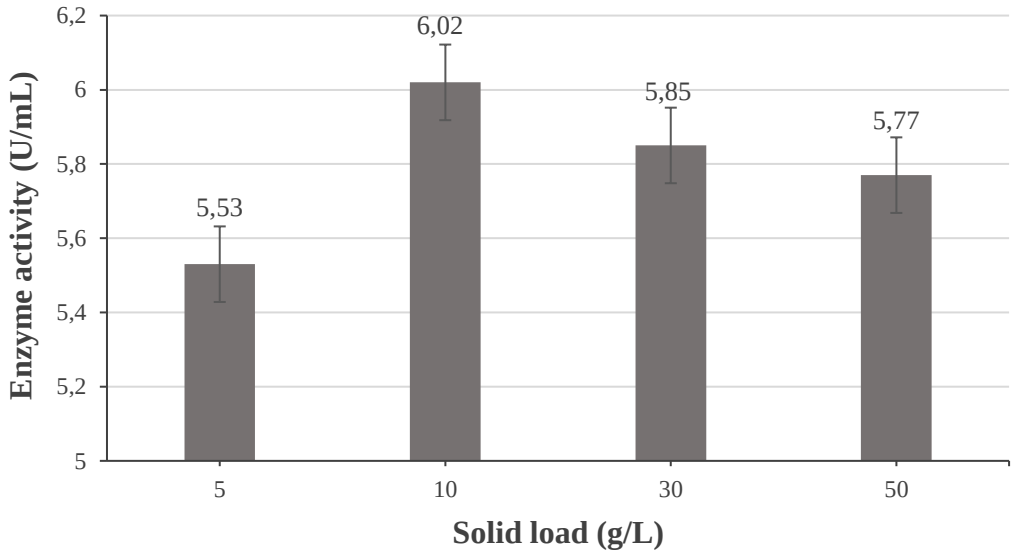

Figure 4.3 Enzyme activity of *Bacillus subtilis* for different concentrations of apple pomace (results are averages of two replicates).

According to test results, although the measurements of all the samples were close to each other, 10 g/L solid load was proven to have the highest enzyme activity at the second day (Fig. 4.3), even though it is generally expected to have higher enzyme activity when the initial substrate amount is higher. While having no solid information in the literature about why this is the case, most likely explanation would

be enzyme inhibition due to excessive amount of substrate present, known as catabolite repression (Görke & Stülke, 2008). Therefore, all the experiments were conducted with 10 g/L apple pomace concentration due to these results and also the fact that it is easier to handle lesser concentrations of apple pomace during submerged fermentation.

4.2.3 Comparison of apple pomace hydrolysate with synthetic media

One of the main objectives of this study is to take advantage of the apple processing wastes in order to produce value-added products, in this case, enzymes. To achieve this, it is necessary to find out whether fermentable sugars obtained by hydrolyzing the apple pomace are instead as advantageous and beneficiary as the synthetic sugar (glucose). If this is actually the case, producing PGase from apple pomace would be cheaper and quicker with apple pomace as the carbon source. In order to prove this, two sets of experiments were designed where apple pomace hydrolysates were used as the fermentation medium in one of the sets and synthetic media were used in the other set. The former was prepared with different concentrations of AP (5 g/L, 10 g/L, 30 g/L, 50 g/L) and the latter was prepared with different concentrations of glucose (5 g/L, 10 g/L, 20 g/L, 30 g/L) which are roughly equivalent of the total reducing sugar content of the individual AP concentrations when they were hydrolyzed with sulfuric acid. Each set was prepared with two replicates for every concentration of AP and glucose, and results were visualized with the average of the replicates. Total reducing sugar content of each concentration of AP was measured after acid hydrolysis and before they were inoculated with *Bacillus subtilis* to start the fermentation (Table 4.2); synthetic media were prepared accordingly with different concentrations of glucose as it was previously described in Table 3.1. Samples were taken at the second day of fermentation and analyses were done as they are detailed in section 3.2.

Table 4.2 Total reducing sugar content of various concentrations of apple pomace after acid hydrolysis and before the fermentation.

Concentration (g/L)	Total reducing sugar (g/L)
5	5.34
10	10.73
30	22.35
50	34.71

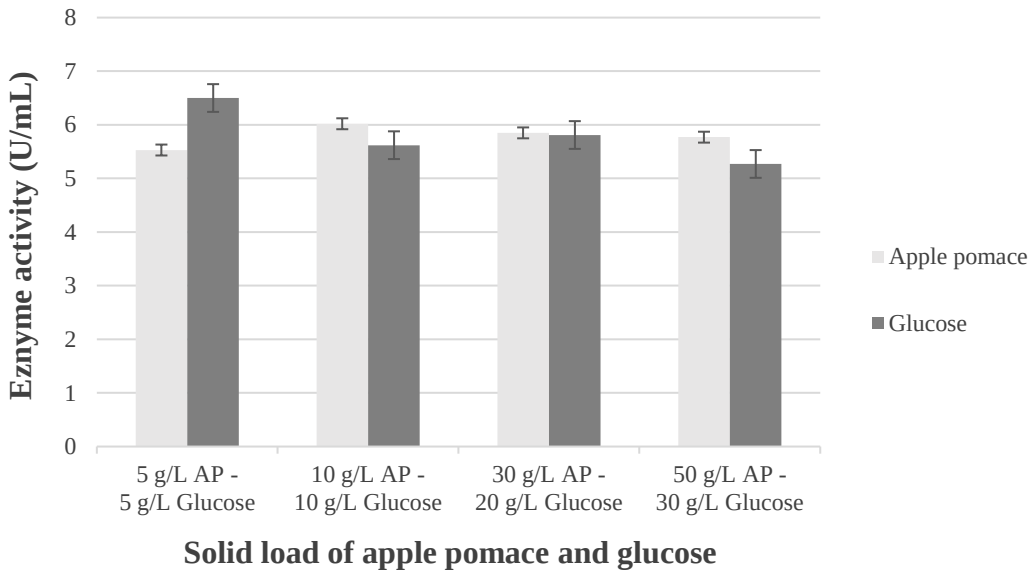

Figure 4.4 Comparison of enzyme activities between various concentrations of apple pomace hydrolysates and synthetic media with corresponding concentrations of glucose after two days of fermentation with *Bacillus subtilis*.

As in Fig. 4.4., there is no significant difference of enzyme activity between the apple pomace hydrolysates and synthetic media after two days of fermentation with *Bacillus subtilis,* while the enzyme activity is slightly higher with the 10, 30, 50 g/L AP hydrolysates than their corresponding synthetic media. According to these results, apple pomace can be proven as a cheaper alternative to synthetic sugar as it is possible to successfully produce polygalacturonase using *Bacillus subtilis*.

4.2.3.1 Enzyme kinetics comparison for apple pomace hydrolysate and synthetic media

To further support the claim of apple pomace can be used effectively for fermentable sugar source instead of synthetic sugars, enzyme kinetics of both AP hydrolysates and synthetic media were examined with Lineweaver-Burk plots (Lineweaver & Burk, 1934).

$$V = \frac{V_{max}\,[S]}{K_m + [S]} \tag{2}$$

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}\,[S]}\right) + \frac{1}{V_{max}} \tag{3}$$

While calculating and assessing the kinetic properties of the enzyme produced by *Bacillus subtilis*, Michaelis – Menten equation (2) was employed. The Michaelis – Menten equation is a mathematical model that describes the rate of enzymatic reactions by establishing a relationship between the reaction rate (V) and the concentration of a substrate ([S]). It is made up of two parameters: the maximum reaction rate (V_{max}) and the Michaelis constant (K_m). V_{max} refers to the highest attainable velocity in an enzyme-catalyzed reaction when the enzyme is fully saturated with its substrate and represents the enzyme's catalytic capability. The V_{max}

value is directly related to the enzyme concentration; as the concentration of the enzyme increases, the V_{max} of the reaction also increases. K_m, known as the Michaelis constant, signifies the substrate concentration at which the enzyme reaction rate reaches half of its maximum velocity and is used to gauge the enzyme's affinity for the substrate. A lower Km value suggests a stronger enzyme-substrate affinity, while a higher Km value indicates a weaker enzymatic attraction to the substrate (Roskoski, 2015).

The Michaelis–Menten equation is a valuable tool for understanding the kinetics of enzymatic reactions. It can be used to determine the V_{max} and K_m values for a particular enzyme, which can then be used to predict how the reaction rate will change in response to changes in substrate concentration (Cho & Lim, 2018).

Accurately determining the values of K_m and V_{max} from the nonlinear curve of interactive Michaelis-Menten kinetics is challenging. However, this nonlinearity can be transformed into a linear plot by taking the reciprocal of both sides of the Michaelis-Menten equation, giving the Lineweaver-Burk plot, which is a linear plot of 1/V versus 1/[S] where the y-intercept corresponds to $1/V_{max}$ and the x-intercept corresponds to $1/K_m$ (Kermasha & Eskin, 2020). 1/V and 1/[S] values are calculated using the Equation (3). The corresponding plots are given in Fig. 4.5 and Fig. 4.6 where "[S]" represents the solid load of apple pomace (g/L), "V" represents the enzyme activity (μmol min^{-1} mL^{-1}).

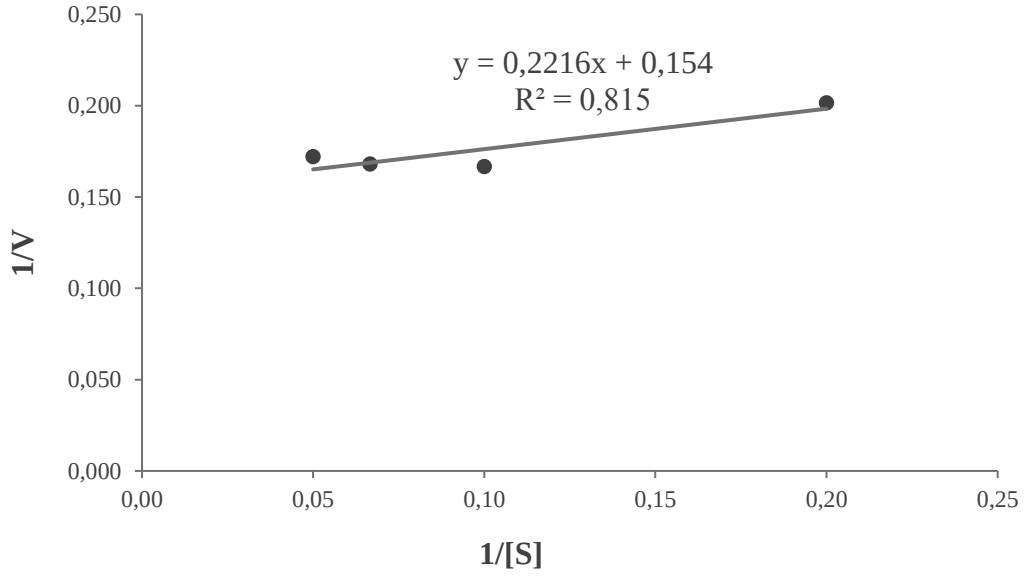

Figure 4.5 Lineweaver-Burk plot of pectinase production with apple pomace hydrolysates.

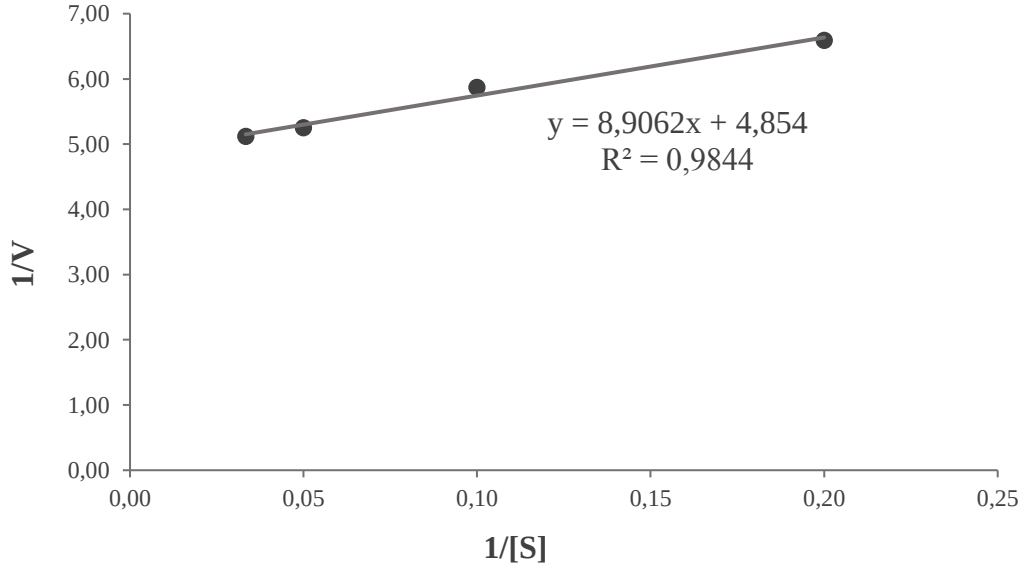

Figure 4.6 Lineweaver-Burk plot of pectinase production with synthetic media.

Table 4.3 K_m – V_{max} and 1/ K_m – 1/ V_{max} values of the apple pomace hydrolysates and synthetic media.

Medium	**K_m (mg/mL)**	**V_{max} (µmol min^{-1} mL^{-1})**	**1/ K_m (mL/mg)**	**1/ V_{max} (mL min $µmol^{-1}$)**
Apple pomace	1.44	6.49	0.695	0.154
Synthetic	1.83	0.206	0.545	4.85

As it can be seen in Table 4.3, K_m value of apple pomace hydrolysates for pectinase production is lower than that of synthetic media and V_{max} value is much higher, which means that enzyme binds more tightly to the substrate (pectin) in apple pomace hydrolysate than it does in pectin-containing synthetic media. Hence, reaction is catalyzed at a faster rate with apple pomace medium, meaning that it can be preferred over synthetic media for pectinase production. K_m values for pectinase production with apple pomace ranges from 0.01 mg/mL to 3 mg/mL in the literature; related K_m value of this experiment falls within the range. A few study results are listed in Table 4.4.

Table 4.4 K_m and V_{max} values for pectinase production with apple pomace in the literature.

Study	**K_m (mg/mL)**	**V_{max} (unit)**
Alqahtani et al. (2022)	1.72	1609 U/g
Meena et al. (2015)	2.43	-
Mutlu et al. (1999)	0.011	0.0276 mg ml^{-1} min^{-1}
Denès et al. (2000)	0.098	3.86 mmol min^{-1} ml^{-1}

4.3 Experiments with additional substrate

Before moving on to the optimization step, experiments with additional substrate were carried out, in which different concentrations of substrate (pectin) were added to apple pomace hydrolysates before or during the fermentation. Time of addition varied between t=0 and t=48 (h); pectin was added both in solid form (pectin powder) and in liquid form (dissolved in distilled water) and the experiment was done with two replicates. Samples were generally taken every 2 h for 6 h after addition, Table 4.5 shows the results after 6 h. One setback of using the liquid form was the difficulty to prepare and handle the pectin solution due to pectin easily forming a gel-like structure when dissolved, hence solid form was used in most of the experiments including the optimization runs.

Another issue was to sterilize the pectin and maintaining the sterilization while adding it to the flasks. In liquid form it was not a major problem; pectin was sterilized in a separate flask at 121 ^{o}C for 15 min in an autoclave and injectors were used while transferring to the fermentation flasks. However, in solid form, autoclaving was relatively harder since keeping the powder dry in an autoclave required additional precautions, such as protecting it from the moisture. In this case, the powder was placed into a small test tube and the lid was closed tightly; the test tube was then covered completely with several layers of aluminum foil before placing it into the autoclave. Fast cooling feature of the autoclave was used after the sterilization period was complete in order to remove the powder from the autoclave relatively earlier. When it came to transfer the solid pectin to the fermentation flasks, the powder in the test tube was simply decanted into the flasks near a Bunsen burner, which were previously autoclaved with magnetic stirrer bars.

One of the concerns of this experiment was whether the pectin would be decomposed when exposed to high temperatures and high pressure during autoclaving. In one

study, it is stated that pectin in solid form is more stable compared to its liquid form, that is, pectin solution. However, pectin still can be affected by high temperatures in low-pH or high-pH environments (Einhorn-Stoll et al., 2019). Yet, in other studies, it was found that about 70% of pectin would be decomposed between 210 – 260 ^{o}C, with the quickest reaction rate at 250 ^{o}C (Fisher et al., 2002; Wu et al., 2019). Moreover, samples were tested for pectin decomposition by measuring total reducing group content before and after autoclaving the media containing pectin at 121 ^{o}C for 15 min; slight increase was observed but it was not significant, which was consistent with the literature. Hence, standard autoclave procedure was continued as the decomposition of pectin was negligible.

Table 4.5 Substrate (pectin) addition before or during fermentation, with replicates.

Pectin amount (g/L)	Addition time (h)	Enzyme activity (U/mL)
0.1	24	7.97 ± 0.02
0.2	24	7.36 ± 0.05
0.3	24	7.15 ± 0.31
10	0	9.19 ± 0.87
30	48	8.40 ± 0.06

Results are averages of two replicates; ± stands for standard deviation.

According to Table 4.5, the highest enzyme activity was observed when the substrate was added at the beginning of the fermentation (t=0) while the enzyme activity slightly increased or stayed the same with the addition of substrate regardless of the addition time and amount. The reason why the range between pectin amounts is immensely broad is that they were chosen completely random in accordance with

how possible they were to handle due to pectin forming gel-like structure when mixed with water. While 0.1, 0.2, and 0.3 g/L pectin was added in liquid form, 10 and 30 g/L pectin was added in solid form.

Even though it was thought that higher amounts could be added when it was in solid form, this time other problems occurred. For instance, it was challenging to stir the fermentation medium as it took a lot of time for high amount of pectin to dissolve; the flasks needed to stay out of the incubator for a considerably long time which could have disrupted the fermentation period and thus the results. If the flasks were placed back in the incubator without dissolving the pectin completely, the results could have been unreliable as the aimed amount had not merged with the fermentation medium. In order to address all these issues and difficulties, it was decided that lesser amounts of pectin should be used in solid form for the optimization of enzyme production (section 4.4).

4.4 Optimization of polygalacturonase production with Response Surface Methodology

Box-Behnken design of Response Surface Methodology (RSM) was used in this study in order to optimize the pectinase production. The growth temperature and pH were kept constant at 30 ^{o}C and 5 respectively, as they were the optimum conditions for *Bacillus subtilis* (Errington & van der Aart, 2020; Nur Indah Koni et al., 2017). Some of the fermentation experiments were done in a fed-batch manner, i.e., the substrate was added during the ongoing fermentation.

Optimization parameters were determined as fermentation period, additional substrate (pectin) concentration, and addition time as per the experiments done with additional substrates; 30 runs of experiments were constructed by Minitab 13 using Box-Behnken experimental design feature. Pectinase assay was performed for each

individual run to determine the enzyme activity, which is an assay that uses polygalacturonic acid as the substrate for samples (details were given in section 3.2.4). The range of parameters and the Box-Behnken experimental design for polygalacturonase production had been previously given in Tables 3.2 and 3.3, respectively.

During the optimization experiments, total reducing sugar content of the runs was not measured as it was deemed irrelevant for the purpose of optimization. In the previous experiments, total reducing sugar content of 10 g/L apple pomace before and after various days of fermentation was measured and it was found that *Bacillus subtilis* never used all the fermentable sugar in any of the experiments. Hence, it was decided that it was unnecessary to waste resources and it was best to solely focus on the enzyme activity for the sake of the experiment.

Regarding the 30 runs Minitab has designed, there are some runs that could not be executed due to being physically impossible. For instance, in the run 10 and 24, Box-Behnken design required the substrate (pectin) addition to be done at the second day of fermentation; however, the fermentation period of the runs was supposed be one day. These types of runs, including the runs that have substrate addition on the day they were supposed to be analyzed as well, have been excluded from the total experiment since they were not carried out. These runs are indicated as “not applicable (NA)” in the cells that normally would have shown their enzyme activity in Table 4.6.

Table 4.6 Polygalacturonase activity results of Box-Behnken experimental design.

Run Order (Replicates)	Pectin concentration (g/L)	Addition time (day)	Fermentation period (day)	Enzyme activity (U/mL)
1-29	1	0	1	7.23±0.09
2-26	2	1	3	12.06±0.03
3-17	2	1	1	NA
4-7	0	1	3	12.02±0.04
5-6	0	0	2	11.24±0.03
8-19	0	0	2	11.81±0.01
9-15	0	1	3	11.25±0.11
10-24	1	0	3	NA
11-16	0	2	2	8.93±0.08
12-20	1	2	1	9.21±0.14
13-28	1	1	2	6.99±0.08
14-27	1	1	2	10.41±0.01
18-22	0	1	1	NA
21-23	2	0	2	9.54±0.04
25-30	0	2	2	12.26±0.02

Results are averages of two replicates; ± stands for standard deviation.

The highest enzyme activity was observed in the run 25 and its replicate (run 30) as 12.27 U/mL and 12.24 U/mL respectively, in which the fermentation period was three days and 1 g/L pectin was added at the second day of fermentation. The runs whose enzyme activity was just as much (12.04 U/mL and 12.05 U/mL) were run 2 and run 7, along with their individual replicates (run 26 and run 4, respectively).

4.4.1 Interaction between variables and response optimization with Box-Behnken design

In order to examine the results obtained by Response Surface Methodology and analyze the interaction between independent variables, Analysis of Variance (ANOVA) test was carried out in addition to response optimization of Box-Behnken design. In this section, it is aimed to determine the optimum conditions for *Bacillus subtilis* to produce polygalacturonase using apple pomace according to the enzyme activity results. The data was converted to log(U/mL) so that it would fit the model better; the quadratic equation (4) was generated accordingly.

$$logY = 0.670 - 0.083X_1 - 0.093X_2 + 0.265X_3 + 0.015X_1X_2 - 0.005X_1X_3 + 0.040X_1^2 + 0.045X_2^2 - 0.038X_3^2 \quad (4)$$

ANOVA was executed by Minitab 13 software and the quadratic equation (4) was formed describing the pectinase yield as a function of X_1 (pectin concentration), X_2 (addition time), and X_3 (fermentation period). The interaction of these variables was also analyzed by Minitab. According to the ANOVA results, the interaction between X_1 and X_2 was significant ($p < 0.05$) while interaction between X_1 and X_3 was insignificant ($p > 0.05$). All the other interactions, including the squares, were found highly significant at $p = 0.000$. The R^2 value was calculated as 0.992 and the p value of lack-of-fit was insignificant as it was found to be 0.065 ($p > 0.05$). The results mean that the data excellently fit to the model. Details of the ANOVA test are given in Appendix E.

To further analyze the interactions, 3D response surface plots were created to show the interactions between addition time, fermentation period, and pectin concentration

on polygalacturonase production. The third variable was held constant at the mid-value for each plot (Figures 4.7, 4.8, and 4.9).

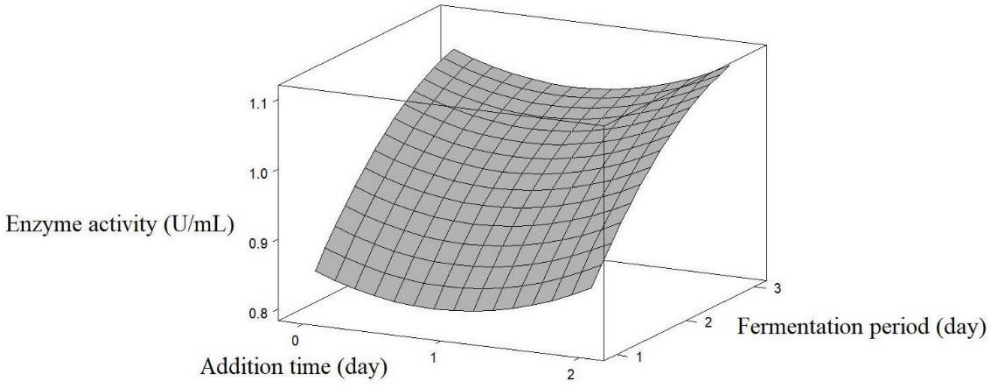

Figure 4.7 Response surface plot of enzyme activity with respect to addition time and fermentation period.

Figure 4.7 indicates that the relationship between the timing of addition and the duration of fermentation is significant. This implies that the impact of when the addition is made relies heavily on the length of the fermentation period; in other words, polygalacturonase production is expected to be higher when pectin addition is done relatively later if the fermentation period is longer. For instance, when the addition is done after 24 hours from the time fermentation started instead of 12 hours, polygalacturonase production will be substantially more pronounced if the fermentation period is also prolonged by 12 hours. Since fermentation period has a positive effect on PGase production, although not being linear, it is expected to have

higher enzyme activity if both the time of addition and fermentation period increase; this can be observed in the runs 11 and 25 (Table 4.6).

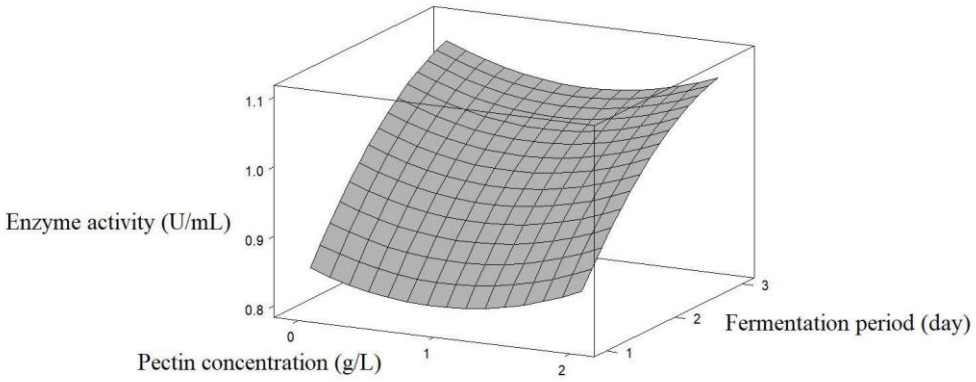

Figure 4.8 Response surface plot of enzyme activity with respect to pectin concentration and fermentation period.

The interaction between pectin concentration and fermentation period (Fig. 4.8) resembles the interaction between addition time and fermentation period (Fig. 4.7), although the former not being as strong as the latter. According to Figure 4.8, there is a correlation between the two parameters; the enzyme activity is expected to be higher if pectin concentration increases and fermentation becomes longer at the same time.

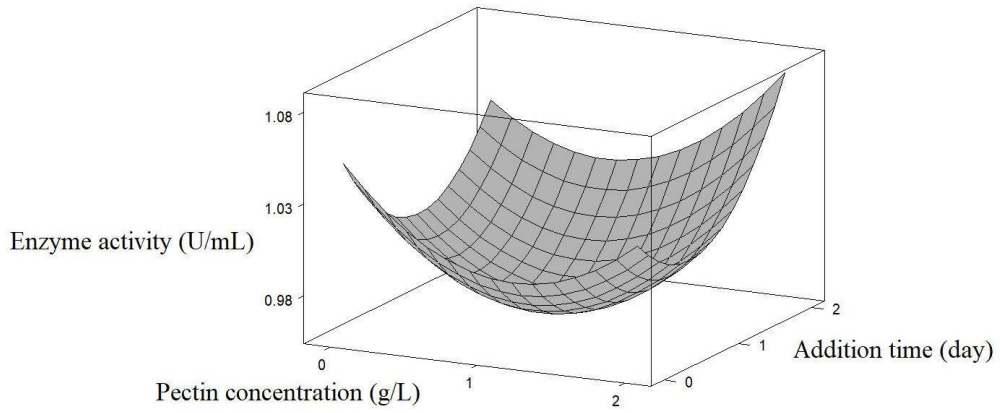

Figure 4.9 Response surface plot of enzyme activity with respect to pectin concentration and addition time.

According to Figure 4.9, the relationship between pectin concentration and addition time notably strong. Consequently, the impact of pectin concentration on enzyme activity relies significantly on the timing of the addition. On the other hand, the response surface plot also indicates that pectin concentration has a negative effect on the enzyme activity even though its impact is relatively weak, meaning that the rate of increase in enzyme activity tends to drop when the pectin concentration increase.

Aside from the ideal conditions, Figure 4.9 demonstrates that a set of conditions exists wherein the polygalacturonase yield falls within roughly 10% of the optimal yield. This indicates that there is some flexibility in the fermentation conditions and achieving a high yield of polygalacturonase does not necessitate excessive precision.

Overall, all three of the response surface plots are not enough by themselves to determine the optimum conditions as they indicate that optimal range is very extensive, meaning that optimal points may not exist withing the Box-Behnken experimental design. In order to address this issue, response optimizer feature of Minitab software was used. Optimum conditions were revealed to be as pectin concentration: 0 g/L, addition time: 0 day (before fermentation started), and fermentation period: 3 days with a desirability (D) of 1.0 (Fig. 4.10). This combination is not directly included in the Box-Behnken design, yet there are runs that have essentially the same conditions (Table 4.6): runs 4 and 7 have 0 g/L pectin concentration with addition on day 1, and 3 days of fermentation; since there is no pectin addition, addition on day 1 actually means nothing, which causes the runs to have the same setting with the optimum conditions that were calculated by response optimizer.

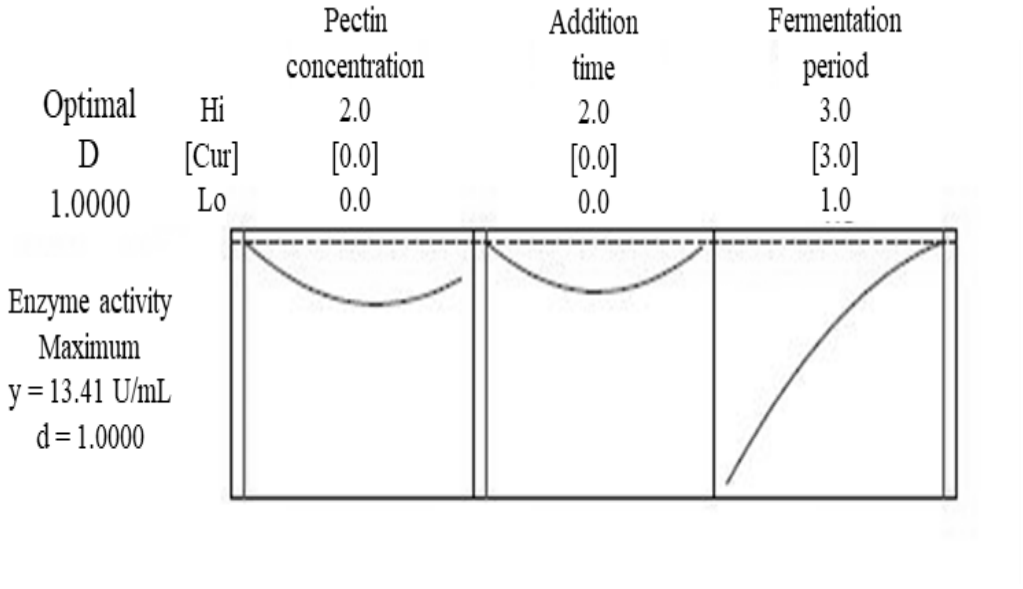

Figure 4.10 Response optimization of Box-Behnken experimental design.

While the optimum fermentation duration being three days was predicted according to all trials performed before optimization, optimum additional substrate amount being 0 was not expected. Despite it brings some financial advantages, it does not comply with the expected advantages of fed-batch fermentation method (Shariat Panahi et al., 2023; Yang & Sha, n.d.).

However, there are runs in the design that have higher enzyme activity than the ones that have the optimum settings. Runs 25 and 30 (1 g/L pectin, addition on day 2, 3 days of fermentation) showed a PGase activity of 12.27 U/mL and 12.24 U/mL respectively, whereas runs 4 and 7 (0 g/L pectin, addition on day 1, 3 days of fermentation) showed that of 11.99 U/mL and 12.05 U/mL respectively. Overall, the enzyme activity results of Box-Behnken experimental design indicate that fed-batch fermentation with pectin offers enhanced PGase activity in contrast to the results of the response optimizer.

When the results of the Box-Behnken experimental design are compared with the similar studies that used food processing wastes to produce polygalacturonase, it is obvious that overall production of PGase increased with this study. Uzuner (2014) found a maximum PGase activity of 5.60 U/mL using hazelnut shell with *Bacillus subtilis*, Kuvvet (2016) found 11.40 U/mL using apple pomace with co-culture of *B. pumilus* & *B. subtilis*, and Özzeybek (2022) found 8.27 U/mL using a mixture of orange peel, hazelnut shell, apple pomace with *B. subtilis*. The details of the fermentation conditions and the raw material of interest of the studies are given in Table 4.7.

Table 4.7 Maximum PGase activities of previous studies and comparison with the present study.

Variable (unit)	**The present study**	**Uzuner (2014)**	**Kuvvet (2016)**	**Özzeybek (2022)**
Raw material	Apple pomace	Hazelnut shell	Apple pomace	Orange peel, hazelnut shell, apple pomace
Solid load (%w/v)	1	5	15	30, 50, 20
Culture	*B. subtilis*	*B. subtilis*	*B.pumilus* / *B. subtilis*	*B. subtilis*
Additional substrate (g/L)	1	-	-	-
Ferm. period (day)	3	3	1	3
PGase activity (U/mL)	**12.27**	**5.60**	**11.40**	**8.27**

This experiment is done with using pectin as the additional substrate; apple pomace was not used directly due to logistical concerns (e.g., handling issues since it does not dissolve in the medium). Therefore, it is unclear whether using apple pomace, which also contains pectin, instead of sole pectin as additional substrate would be more effective in increasing the enzyme activity during fed-batch fermentation. Moreover, there are very few studies that tested the fed-batch submerged fermentation with additional substrates, if exist; further studies in this context may be useful to understand the nature of optimizing polygalacturonase production.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The present study investigated the polygalacturonase (PGase) production using apple pomace (AP) with submerged fermentation by *Bacillus subtilis* NRRL B-4219 as the PGase producer and optimization of fed-batch fermentation process using the Box-Behnken experimental design of Response Surface Methodology.

Firstly, the suitability of the AP as the carbon source was tested by making comparison with synthetic equivalent as the carbon source (glucose). The pectinase activity of AP hydrolysates was either slightly higher or roughly the same as that of synthetic media. Further comparison was made by using the Michaelis-Menten kinetics; K_m and V_{max} values of both sets were calculated so that the affinity of enzyme-substrate complex could be compared. Polygalacturonase showed greater affinity to the substrate in the AP hydrolysates than in the synthetic media as the K_m value was lower. Therefore, apple pomace has the potential as a cost-effective alternative for bacterial enzyme production by fermentation.

Secondly, according to optimization of the fed-batch fermentation with Response Surface Methodology, the highest polygalacturonase activity (12.27 U/mL) was observed when 1 g/L pectin was added to the fermentation medium at the second day within three-day fermentation.

Further studies are necessary for the optimization of culture conditions in a fed-batch manner. In addition, the following studies may be carried out:

- Instead of using pectin for added substrate, use of other substrates, such as direct apple pomace, should be investigated for the optimization.
    - If apple pomace were to be used as the additional substrate, whether it needs to be hydrolyzed before addition should be investigated since this might be an optimization experiment by itself.
- The optimization step can be executed at a larger scale, e.g., bioreactors may be employed for the experiment.
- A wider range of pectin concentration should be determined, which bears the necessity of finding more convenient ways to handle pectin.

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APPENDICES

A. BUFFERS AND SOLUTIONS

Table A.1 DNS reagent for total reducing sugar analysis and pectinase assay.

Component	Amount (g/L)
Sodium hydroxide	10.0
Dinitrosalicylic acid	10.0
Phenol	2.0
Sodium sulfite	0.5
Potassium sodium tartrate tetrahydrate (Rochelle salt)	268.0

Table A.2 Components of phosphate buffer (pH=7).

Component (0.2 M solutions)	Amount (g/L)
Sodium phosphate monobasic (A)	27.8
Sodium phosphate dibasic (B)	35.6

Preparation of phosphate buffer: Solutions A and B are mixed with 39:61 ratio (100 mL) and diluted with 100 mL of distilled water (total volume of 200 mL).

Preparation of 1% PGA solution: 0.5 g of PGA is dissolved in 50 mL phosphate buffer.

B. LIST OF CHEMICALS AND PROVIDERS

Table B.1 Chemicals used in the experiments and their brands.

Chemicals	**Brands**
3-5-Dinitrosalicylic acid	Sigma-Aldrich
D(+)-Glucose	Merck
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
D-Galacturonic acid	Sigma-Aldrich
Glycerol	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	Merck
Nutrient broth	Merck
Pectin	Sigma-Aldrich
Phenol	Merck
Polygalacturonic acid	Sigma-Aldrich
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck
Potassium sodium tartrate tetrahydrate ($C_4H_4KNaO_6.4H_2O$)	Merck
Sodium hydroxide 97%, powder (NaOH)	Sigma-Aldrich
Sodium hydroxide, pellets (NaOH)	Merck
Sodium phosphate dibasic ($Na_2HPO_4.7H_2O$)	Merck
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	Merck
Sodium sulfite (Na_2SO_3)	Merck
Sulfuric acid 95-97% (H_2SO_4)	Merck
Yeast extract	Merck

C. STANDARD CURVE FOR TOTAL REDUCING SUGAR

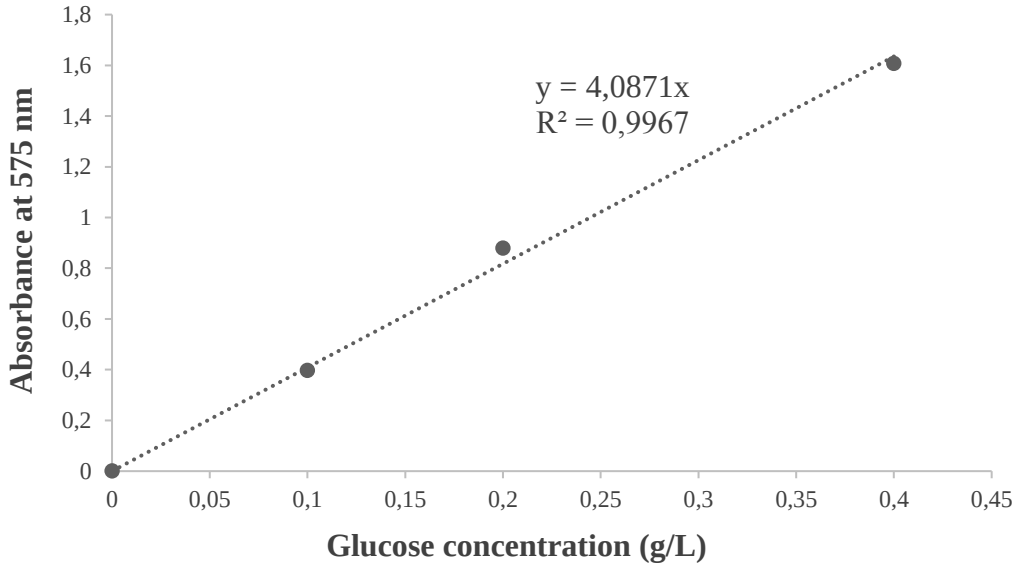

Figure C.1 Standard curve of DNS analysis for total reducing sugar.

Total reducing sugar content is calculated using the following equation:

$$\text{Total reducing sugar concentration } \left(\frac{\text{g}}{\text{L}}\right) = \left(\frac{\text{Absorbance}}{4.0871}\right) * \text{Dilution factor} \qquad (5)$$

D. STANDARD CURVE FOR ENZYME ACTIVITY

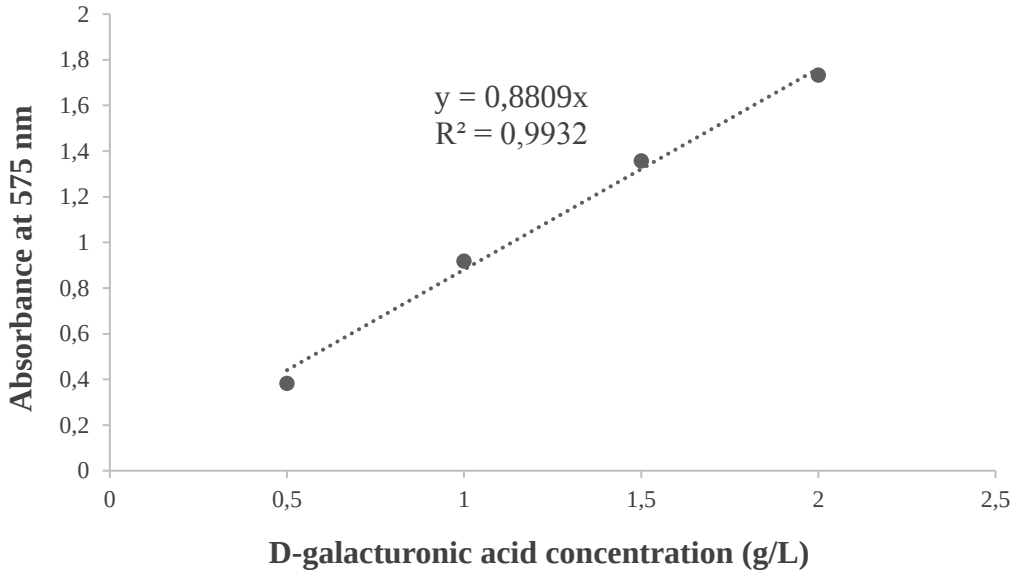

Figure D.1 Standard curve for pectinase assay.

Pectinase activity is calculated using the following equation:

$$\frac{\text{IU}}{\text{mL}} = \text{C} * \left(\frac{1}{\text{incubation time}}\right) * \left(\frac{1}{212.12}\right) \quad (6)$$

C = ((Absorbance*F)$_{\text{sample}}$ – (Absorbance*F)$_{\text{substrate blank}}$) * Dilution factor – (Absorbance*F)$_{\text{enzyme blank}}$

Incubation time = 30 minutes

F = Conversion factor with which absorbance is adapted to grams of D-galacturonic acid by standard curve

1/212.12 = conversion from grams to moles of D-galacturonic acid

E. REGRESSION COEFFICIENTS AND ANOVA RESULTS FOR POLYGALACTURONASE PRODUCTION

Table E.1 Estimated regression coefficients for enzyme activity.

Term	Coef	SE Coef	T	P
Constant	0.67000	0.017536	38.208	0.000
Pectin concentration	-0.08250	0.012475	-6.613	0.000
Addition time	-0.09250	0.010155	-9.109	0.000
Fermentation period	0.26500	0.017482	15.158	0.000
Pectin c.*Pectin c.	0.04000	0.005123	7.807	0.000
Add. time*Add. time	0.04500	0.004330	10.392	0.000
Ferm. per.*Ferm. per.	-0.03750	0.004330	-8.660	0.000
Pectin c.*Add. Time	0.01500	0.006124	2.449	0.027
Pectin c.*Ferm. period	-0.00500	0.006124	-0.816	0.427

S = 0.009487 R^2 = 99.2% R^2 (adj) = 98.7%

Table E.2 Analysis of variance for enzyme activity.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	8	0.159346	0.159346	0.019918	221.31	0.000
Linear	3	0.134717	0.027890	0.009297	103.30	0.000
Square	3	0.024069	0.021860	0.007287	80.96	0.000
Interaction	2	0.000560	0.000560	0.000280	3.11	0.074
Residual Error	15	0.001350	0.001350	0.000090		
Lack-of-Fit	1	0.000300	0.000300	0.000300	4.00	0.065
Pure Error	14	0.001050	0.001050	0.000075		
Total	23	0.160696				