FORMULATION OF FOOD PROCESSING WASTES FOR CO-PRODUCTION OF BACTERIAL PECTINASE AND CELLULASE ENZYMES

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MEHLİKA ÖZZEYBEK

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Submitted by **MEHLİKA ÖZZEYBEK** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Serpil Şahin
Head of Department, **Food Engineering** _____

Prof. Dr. Deniz Çekmecelioğlu
Supervisor, **Food Engineering, METU** _____

Examining Committee Members:

Prof. Dr. Alev Bayındırlı
Food Engineering, METU _____

Prof. Dr. Deniz Çekmecelioğlu
Food Engineering, METU _____

Prof. Dr. Hami Alpas
Food Engineering, METU _____

Assoc. Prof. Dr. Sibel Uzuner
Food Engineering, Izmir Instıtute of Technology _____

Assoc. Prof. Dr. Yeşim Soyer
Food Engineering, METU _____

Date: 16.05.2022

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Mehlika Özzeybek

Signature :

ABSTRACT

FORMULATION OF FOOD PROCESSING WASTES FOR CO-PRODUCTION OF BACTERIAL PECTINASE AND CELLULASE ENZYMES

Özzeybek, Mehlika
Master of Science, Department of Food Engineering
Supervisor: Prof. Dr. Deniz Çekmecelioğlu

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Production of enzymes has always been the focus of research with its ever-increasing application area, yet it is an expensive process due to the need for pure carbon sources to carry out the production. Exploring new ways to reduce cost while maximizing enzyme production is an ongoing battle, even in today's opportunities.

In this research, a mixture of food wastes was valorized and bacterial pectinase and cellulase were co-produced. Optimum proportions of the hazelnut shells, orange peel, and apple pomace in the fermentation medium were determined by the Extreme Vertices Mixture Design (EVMD) method for the purpose of enhancing the pectinase and cellulase production by *Bacillus subtilis*. Enzyme activities from the mixed medium were compared with a single feedstock medium.

The fermentation medium was prepared by pretreating the mixture of feedstocks with acid and then fortifying with minerals. *Bacillus subtilis* was grown in the fermentation

medium at 30^{o}C and pH 7 for 72 h. Optimum proportions for the feedstocks were found to be 50% hazelnut shells, 30% orange peel, and 20% apple pomace.

Pectinase and cellulase activity resulted from the optimum mixture properties were 8.27 U/mL, and 0.5 U/mL, respectively.

It was also observed that pectinase and cellulase activity resulted from only hazelnut shells medium were 5.95 U/mL and 0.49 U/mL respectively, which indicates that pectinase production was increased by 40% while cellulase change was insignificant.

Furthermore, it was observed that pectinase production from hazelnut shells medium was higher than apple pomace and orange peel medium.

This research proved that pectinase production from a single feedstock fermentation medium could be enhanced by applying a mixture of food wastes.

Keywords: Mixture design, cellulase, polygalacturonase, *Bacillus subtilis*, single feedstock, mixed feedstock

ÖZ

BAKTERİYEL PEKTİNAZ VE SELÜLAZ ENZİMLERİNİN ÜRETİMİ İÇİN GIDA ATIKLARI KARIŞIMININ FORMÜLASYONU

Özzeybek, Mehlika
Yüksek Lisans, Gıda Mühendisliği
Tez yöneticisi: Prof. Dr. Deniz Çekmecelioğlu

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Enzimlerin üretimi, giderek artan uygulama alanları nedeniyle her zaman araştırmaların odak noktası olmuştur ancak üretimde saf karbon kaynaklarına ihtiyaç duyulması nedeniyle de pahalı bir süreçtir. Enzimi maksimum üretirken maliyeti düşürmenin yeni yollarını keşfetmek ve denemek, günümüzün fırsatlarında bile devam eden bir uğraştır.

Bu araştırmada, gıda atıklarının karışımı değerlendirilmiş ve bakteriyel pektinaz ve selülaz enzimleri birlikte üretilmiştir. Pektinaz ve selülaz üretimini arttırmak amacıyla, fındık kabuğu, portakal kabuğu ve elma posasının fermantasyon ortamındaki optimum oranları Uç Nokta Karma Tasarım (UNKT) yöntemi ile saptanmıştır. Atıkların karışımından elde edilen enzim aktiviteleri, çalışmanın amacı doğrultusunda tekli hammaddelerle üretilenlerle karşılaştırılmıştır.

Fermantasyon ortamı, atıkların karışımının asitle ön işleme tabi tutulmasıyla hazırlandı ve ardından minerallerle takviye edildi. Daha sonra *Bacillus subtilis*, 30°C'de 72 saat pH 7'deki fermentasyon ortamında çoğaltıldı. Ön deneyler sonucunda tasarlanan

hammaddeler için optimum oranlar ve sonuçlar %50 fındık kabuğu, %30 portakal kabuğu ve %20 elma posasıdır.

Optimum karışım oranlarıyla hazırlanan fermentasyon ortamından elde edilen pektinaz ve selülaz aktiviteleri sırasıyla 8.27 U/mL ve 0.5 U/mL.

Sadece fındık kabukları içeren ortamdan üretilen pektinaz ve selülaz aktivitelerin ise sırasıyla 5.95 U/mL ve 0.49 U/mL olduğu gözlenmiştir. Sonuçlara göre pektinaz üretiminin %40 arttığı, selülaz değişiminin ise önemsiz olduğu gözlemlenmiştir.

Ayrıca sadece fındık kabukları içeren ortamda pektinaz üretiminin elma posası ve portakal kabuğu içerenlere göre daha yüksek olduğu gözlemlenmiştir.

Bu araştırma, tek çeşit karbon kaynağı içeren fermentasyon ortamında pektinaz üretiminin, gıda atığı karışımları uygulanarak artırılabileceğini kanıtlamıştır.

Anahtar kelimeler: Karışım formülasyonu, selülaz, poligalakturonaz, *Bacillus subtilis*, tek çeşit atık, karışım atıklar

To my dearest parents and little brother…

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CHAPTER 1

INTRODUCTION

Enzymes could be of different origins such as microbial, plant, or animal, and are essential catalysts for many processes. Enzymes are also important for process efficiency, quality, and cost while supporting the environment (Li et al., 2012).

Although there are many important enzymes, pectinase and cellulase are two of the most important multi-purpose enzymes that have a wide range of applications for different industries. For instance, pectinases are used in the juice industry as well as tea and coffee, alcoholic beverages, and oil extraction (Kashyap et al., 2001; Sandri et al., 2011). Cellulases, on the other hand, are employed by the food, textile, waste treatment, and feed industries (Choi et al., 2015; Singh et al., 2016).

The purpose of research on enzymes is to find ways to reduce the cost and search for inexpensive carbon sources while enhancing enzyme production. This can be achieved by several approaches such as utilizing wastes throughout the process, making it valuable for both the environment and the industries in terms of cost and sustainability, using strains that have high yield capacity, and optimizing different parameters during fermentation (Ozatay S., 2020).

In recent years, lignocellulosic wastes have been used as an inexpensive and environmentally friendly carbon source for enzyme production. Since they are composed of different cellulosic structures, which induce enzyme production and are easy to access, they are considered valuable materials for many industries. Utilizing food and agricultural wastes is still not fully implemented in every industry, which further causes problems for the environment and economical loss. In Chapter 2, enzyme production by utilizing various materials is mentioned along with pectinase and cellulase particularly, production and classification were reviewed by using different microbial sources.

In the literature, there is no research on co-production of pectinase and cellulase enzymes using a mixture of feedstocks with *Bacillus subtilis* by implementing the EVMD method. This study has a new and alternative approach for the enzyme industry and waste management together, hence it will be one of the leading studies in the industry.

This study supports the idea that the co-production of enzymes can be enhanced by using mixtures of feedstocks rather than using a single feedstock by optimizing the ratio of each material in the mixture. In addition, co-production of enzymes also had benefits from an economic and production point of view. In Chapter 3, pretreatment methods, fermentation conditions, experimental design, and optimization of mixture formulation to increase enzyme yield were examined.

In the results section, Chapter 4, enzyme production from single feedstocks and mixtures were concluded. Validation results were presented for the pectinase and cellulase production and compared with the literature to come to an overall conclusion.

CHAPTER 2

LITERATURE REVIEW

Food and agricultural industries generate waste materials underutilized and causing environmental pollution and economic burden. In Turkey, according to Waste Report released in 2018, food waste was amounted to 26 million tons which is more than 15% of the income (Tekiner et al., 2021). The United States Environmental Protection Agency reported the amount of waste in 2021 to be 2 billion tons, and this rate is expected to increase by 70% by 2050 (Swain et al., 2022).

Upon developments and research on bio-industries, these wastes started to be considered an alternative carbon source for the fermentation processes. In this way, the cost of enzyme production is lowered by using inexpensive carbon sources instead of pure ones.

Each material may have different physical and chemical characteristics which further affects enzyme yields. Lignocellulosic wastes are comprised of cellulose, pectin, hemicellulose, and lignin along with proteins, lipids, and minerals. They have significant importance due to their easy accessibility, low cost and environmentally friendly attributes (Maki et al., 2009). The utilization of wastes according to their chemical compositions and characteristics is important for the enzyme production and growth of microorganisms (Laothanachareon et al., 2022).

2.1. Pectin

Pectin is considered one of the complex polysaccharides with a backbone made up of galacturonic acid, rhamnose units, fucose, arabinose, xylose, and galactose that are linked by α-1,4- glycosidic bonds in the side chain (Figure 2.1). Pectin is found in the cell walls of fruits and vegetables and the middle lamella of ground plants (Chen et al., 2015). It has a wide range of applications in different industries from the food and pharmaceutical industry to biotechnology industries (Freitas et al., 2020). Pectin content can vary in different fruits and vegetables (Table 2.1). The fruits containing the highest pectin belong to citrus family such as lemons and oranges, which have a pectin content between 12.4% and 28.0% on a dry basis (Jayani et al., 2005).

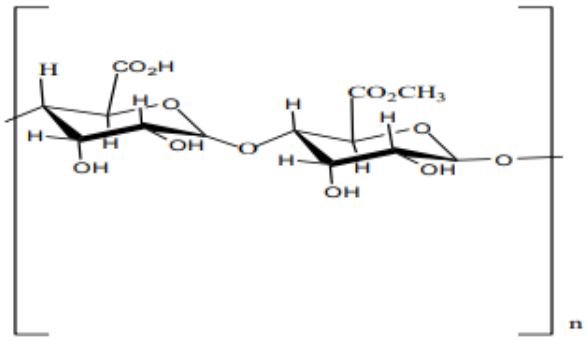

Figure 2.1 Pectin representation (Jabarah et al., 2012).

Table 2.1 Percentages of pectin in various fruits and veggies.

Food	%Pectin content	Reference
Apple (Fresh)	0.5-1.6	Jayani et al. (2005)
Banana (Fresh)	0.44-1.02	Wade et al. (1992)
Orange Pulp (Dry matter)	12.4-28.0	Jayani et al. (2005)
Carrot	0.63-1.01	Kawabata & Sawayama (1973)
Tomato (Dry matter)	2.4-4.6	Jayani et al. (2005)
Blackberries	0.40-1.19	Jayani et al. (2005)

There are two main classes of pectin, which depend on the methyl esterification degree; high (HMP) and low-methoxyl pectin (LMP). Pectin has different functional properties such as emulsifying capacity, water holding capacity, or rheological and inhibitory characteristics that allow it to be used for different purposes. HMP and LMP are very important tools and are used for many processes (Table 2.2).

According to recent research, pectin is consumed at 45,000 tons and the market is predicted to grow at a rate of 5-6% (Marić et al., 2018). Pectin is mainly produced by the citrus family such as orange peel or lemon peel approximately 85.5% followed by apple pomace with 14% and sugar pulp with 5%. In addition to main sources, alternative by-products are also studied in great amounts ever since pectin utilization become prevalent in different industries (Muñoz-Almagro et al., 2021).

There are different extraction methods from conventional ones to new emerging alternative methods such as the combination of enzymatic extraction, ultrasound, and microwaves (Marić et al., 2018).

Table 2.2 Commercial & alternative pectin sources.

Sources	**% Pectin extraction**	**Reference**
Apple pomace (Commercial)	15-20/7-23 (HMP)	Rao, M., & Lopes da Silva, J. (2006)
Citrus family (Commercial)	30-35/24 (HMP)	Rao, M., & Lopes da Silva, J. (2006)
Sugar beet pulp (Commercial)	13-19 (LMP)	Pacheco et al. (2019)
Banana peel (Alternative)	2-22 (HMP)	Oliveira et al. (2016)
Chicory root (Alternative)	12 (LMP)	Zhang et al. (2020)
Rapeseed cake (Alternative)	2-6 (LMP)	Jeong et al. (2013)
Pumpkin (Alternative)	7-22 (HMP)	Ptichkina et al. (2008)
Pomegranate peel (Alternative)	3-20 (HMP)	Talekar et al. (2019)
Soy hull (Alternative)	18-28 (HMP)	Kalapathy & Proctor (2001)

Table 2.2 (continued)

Sweet potato (Alternative)	5-10 (LMP)	Zhang & Mu (2011)
Chickpea	8 (LMP)	Urias-Orona et al. (2010)
Carrot peel	8-22 (LMP)	Khubber et al. (2020)
Coffee pulp	15 (LMP)	Reichembach & de Oliveira Petkowicz (2020)
Fig skin	9-14 (LMP)	Gharibzahedi et al. (2019)
Melon peel	13-28 (LMP)	Raji et al. (2017)
Plum pomace	20-39 (HMP)	Kosmala et al. (2013)
Sunflower head	8-13 (LMP)	Muñoz-Almagro et al. (2020)

As mentioned before, pectin has different application areas (Table 2.3) from food industries to textile, medicine, and drugs with different purposes (Freitas et al., 2021).

Table 2.3 Pectin usage by different industries (Dranca & Oroian, 2018).

Application	Information
Emulsifying agent	For low-fat, low-salt product development as a fat substitute, to improve quality
Edible coatings	To extend shelf life by mixing with other materials c to its biodegradable, biocompatible, and renewable nature (Green packaging)
Health benefits	Bioactive compounds, antitumor properties, immunity enhancement, prevention of tumor growth
Drug delivery	Encapsulating agent
Therapeutical agents	Inhibition of cancer cell growth and supporting the growth of apoptotic cells
Dietary fiber in products	To improve digestibility
Nanoparticle manufacturing	
Gelling agents	Jams/Jellies
Stabilizers	Milk & Juice industry

2.2. Cellulose

Cellulose is composed of D-glucopyranose units linked by β-1,4 –glucosidic linkages as main units. Anhydroglucose units are comprised of D-glucopyranose units, which further compose cellulose when two of them are linked and more of them together are called anhydrocellobiose (Fig. 2.2) (Nechyporchuk et al., 2016).

Cellulose originated from plants, bacteria, wood, algae, and tunicate (Seddiqi et al., 2021). However, according to Klemm et al. (2011), due to cost and availability, the most used sources for cellulose were wood and plant. Cellulose is considered a non-soluble and fibrous polysaccharide found in the cell walls of plants to increase strength (Brigham, 2018). It has been used in many industries such as wood, cosmetics, clothes, and pharmaceutical (Table 2.5&Table 2.6) (Trache et al., 2016).

The report released in 2021 indicated that the cellulose market was estimated to be 506.8 million dollars in 2020 and expected to increase by 8.1% and reach 980.9 million dollars at the end of 2028 (Swain et al., 2022).

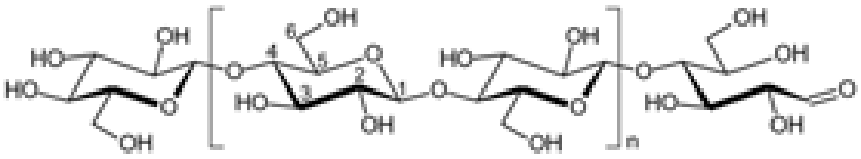

Figure 2.2 Cellulose (Nechyporchuk et al., 2016).

Plant cellulose and bacterial cellulose are different from each other by their physicochemical characteristics, which further cause the bacterial cellulose to be purer, lack lignin, and hemicellulose, more hydrophilic and mechanically strong (Tsouko et al., 2015). Cellulose sources could be natural or synthetic (Lavanya et al., 2011) (Table 2.4).

Table 2.4 Different cellulose sources and cellulose percentages.

Sources	**% Cellulose**
Cotton, kapok (Seed Fibers)	90-95
Sisal, agave, fique (Leaf fibers)	33
Hemp, ramie, jute, kenaf, vine (Skin/Bast fibers)	33
Coconut (Fruit fibers)	30-50
Corn cob	45
Softwood	40-44
Hardwood	43-47
Flax	71

Table 2.5 Application areas of bacterial cellulose (Urbina et al., 2021).

Sources	Areas
Apple pomace	Food packaging (Films based on antioxidants)
Coconut water	Dressing of wound
Apple pomace	Water purification
Molasses	Food packaging
Watermelon peel	Ingredient
Sugarcane straw	Films (Electronic devices)
Banana	Composites

Table 2.6 Cellulose and derivates usage in different areas.

Applications	References
Tissue Engineering (Regeneration, films) from micro/nano crystalline cellulose	Petreus et al. (2014)

Table 2.6 (continued)

Biosensor/Wound dressing (Biocellulose)	Mohanpuria et al. (2007)
Product weaving (Biocellulose)	Yamanaka et al. (2011)
Tissue Engineering/Micro & Nano fibrils	Courtenay et al. (2017)

2.3. Enzymes

Enzymes are protein-structured catalysts that can increase the rate of reactions 10^6 to 10^{12} factors by decreasing the activation energy. For centuries, enzymes are used for different purposes.

In the food industry, enzymes have been generally used as processing aid and ingredient production in beverages, dairy, baking, etc. (Olempska-beer et al., 2006). Fermentable sugars can be acquired from agricultural wastes in copious amounts such as beet, potato, sugar, and starch. Different enzymes can be employed to convert polysaccharides into sugars. In addition, cell walls are broken down to release intracellular carbohydrates from the cell matrix, which is called the liquefaction process (Kashyap et al., 2001).

There are various sources of enzymes such as microorganisms, plants, or animals. Microorganisms have advantages over other sources in terms of stability, process control, optimization, and cost (Ejaz et al., 2021). Microbial enzymes have different applications from textiles, detergents to animal foods and biofuels (Table 2.7) (Adrio & Demain, 2014; Singh, et al., 2016).

Table 2.7 Microbial enzyme kinds and functionality.

Sources	**Enzyme**	**Functionality**
Bacillus subtilis, A. oryzae	Proteinase	Cheese ripening and debittering
Aspergillus niger	Catalase	Cheese production
A. oryzae, Aspergillus niger	Lipase	Cheese production
Aspergillus niger	Xylanase/Lipase/Glucose oxidase	Dough stability, conditioning& Strength
Streptoverticillium sp., streptomyces sp	Transglutaminase	Dough Strength
Aspergillus sp., Bacillus sp.	Amylase	Flour adjustment, bread hardness

Table 2.7 (continued)

Bacillus subtilis, Aspergillus oryzae, Penicillium funiculosum	Pectinase	Depectinization, clarification
Bacillus subtilis, Aspergillus niger, Trichoderma atroviride	Cellulase	Liquefaction
Aspergillus niger	Naringinase	Debittering
Aspergillus niger, A. oryzae	Limoninase	Debittering
Lactobacillus brevis, L. plantarum	Aminopeptidases	Breakdown of proteins
Aspergillus sp., Bacillus sp.	Xylanase	Bleaching
Bacillus subtilis	Laccase	Bleaching, Delignification
Bacillus subtilis	Protease	Biofilm production
Aspergillus niger	β-glucanase	Better functioning of digestive systems
Aspergillus sp., Bacillus subtilis	Amylase	Fiber breakdown

Table 2.7 (continued)

Aspergillus oryzae, Candida tropicalis	Lipase	Oil deprivation
Fusarium solani f. pisi	Cutinase	Plastic degradation
Pseudomonas sp., Rhodococcus sp.	Oxygenase	Contaminant removal
Trametes versicolor	Laccase	Waste management
Rhodococcus sp.	Nitrile hydratase	Waste degradation
Bacillus, Aspergillus	α-Amylase	Hydrolysis of starch
Aspergillus niger, A. flavus, Bacillus subtilis	Protease	Dead skin extraction
Bacillus subtilis, Trametes versicolor	Laccase	Hair coloring

2.3.1 Pectinases

Pectic substances are hydrolyzed by enzymes called pectinases. They have a wide range of applications in the food industry and approximately 25% of sales belong to them mostly in the textile and juice industries in addition to the biotechnology industry. There are different types of pectic substances according to backbone chain modification and named as pectin, pectic acid, protopectin, and pectinic acid (Bemiller, 1986).

There are three types of pectinases, which are named as pectinesterases, depolymerizing enzymes, and protopectinase. The reason is due to substrate differences, the act of pectinase, and location of cleavage (Kashyap et al., 2001).

Pectinesterases play a role in the cleavage of the methoxyl group, while depolymerizing enzymes break down the α-1,4 bond by hydrolysis and lastly protopectinases act on protopectin to liberate the water-soluble pectin (Alkorta et al., 1998).

Glycosidic bonds are hydrolyzed by polymethygaacturonases (PMG) and polygalacturonases (PG). While PMG hydrolytically breaks down α-1,4 linkages, PG also catalyzes the cleavage of the α-1,4 linkages in pectic acid. They both have endo and exo types, which are differentiated for where the cleavage occurs. Endo-PMG randomly acts on the α-1,4 bond on the pectin, exo-PMG acts consecutively on the non-reducing part of the pectin. Endo-PG randomly hydrolyzes the α-1,4 glycosidic bond while exo-PG consecutively breaks down the linkages (Kashyap et al., 2001).

Polymethyl galacturonate (PMGL) and polygalacturonate (PGL) lyases also cleave α-1,4 bonds by trans-elimination and the resulting molecule called galacturonide. PMGL cleaves pectin while PGL cleaves α-1,4 glycosidic bond in pectic acid. They both have endo and exo types according to cleavage type as random or sequentially. Lastly, protopectinases have two types acidic and alkaline pectinases (Kashyap et al., 2001).

2.3.1.1 Applications of pectinases

Pectinases are one of the oldest enzymes that have been used throughout history. Commercially the first usage was in the 1930s for the fruit juices and wine industry (Kashyap et al., 2001). There have been increasing application areas of pectinases from paper, textile, and juice to the biotechnology industry. One of the most used areas among them is the juice industry. To reduce cloudiness and increase sweetness, pectinases are used. In addition, the textile industry also benefits from pectinase to increase the quality and degumming process of fiber crops (Ahlawat et al., 2009). As for the fermentation process of tea and coffee, extraction of oils, also wastewater treatment is other applications of pectinases. Novel approaches are also emerging in recent years such as pectinase usage for the production of functional ingredients (Kuvvet et al., 2019).

Removal of non-cellulosic materials by implementing an alternative method called bioscuring is done by enzymes to make the surface favor water. Since the hydrophobic properties of cotton are caused by pectins, using pectinolytic enzymes degrades waxes and reduces the use of chemicals which makes the process environment friendly (Klug-Santner et al., 2006). According to Klug Santner et al. (2006), 80% of pectin was removed from cotton by using *Bacillus pumilus* BK2, which produced endo-pectate lyase.

To help the extraction of oil from vegetable cells, enzyme application is used since it does not involve toxic chemicals such as hexane. Instead, pectin enzymes degrade the cell wall and increase the extraction of diverse kinds of oils (Kashyap et al., 2001).

Furthermore, pectinases are an active ingredient in the maceration process, and the enzymes specific for that purpose are called macerases. The result of the maceration process is a pulpy structure base, which is further used in pulpy nectars, yogurts, juices, puddings, and baby foods. Transformation of cells into intact cells by enzymes is the purpose of the maceration process according to Bock et al. (1983).

Pectinases work on the middle lamella of plants. One of the important parts of this process is the inactivation of endogenous pectinase since exogenous pectinase breaks down the pectin and other dietary components. In addition, avoiding leaking starch molecules from the cell structure is also crucial.

2.3.1.2 Pectinase production

Pectinase sources can vary from bacteria, fungi, and yeast to plants (Kashyap et al., 2000). Acidic pectinases are mostly produced by fungi, while alkaline pectinases are produced by bacteria. Examples of them can be seen in Table 2.8 and Table 2.9. They both have similar applications, however they could differ by their nature.

Acid pectinases are often applied to clarification, pectin removal, extraction, production of puree, and pastes by maceration. Alkaline pectinases are on the other hand used for pectin removal from wastewater, fermentation of tea and coffee, paper pulp treatment, and textile fibers (Pedrolli et al., 2009).

Pectinases produced by microbial sources are advantageous over animal or plant-sourced ones. According to Chaudhri (2012), microbial sourced pectinases are easier and faster to produce, less harmful to the environment and gene manipulation is also easier.

Mostly used commercial pectinase sources mentioned by Jayani et al. (2005) are fungi such as *Aspergillus japonicus*, *Fusarium oxysporum*, *Neurospora crassa*, and *Alternaria mali*.

Table 2.8 Fungal pectinases.

Source	Type of Pectinase	Opt. pH activity	Opt. Temp(°C)	Reference
Aspergillus niger CH4	Acidic pectinase(Endo&exo-pectinase)	4.5-6.0	<50	Acuña-Argüelles et al. (1995)
Sclerotium rolfsii	Acidic pectinase(Endo-PG)	3.55	55	Channe& Shewale (1995)
Rhizoctonia solani	Acidic pectinase(Endo-PG)	4.8	50	Marcus et al. (1986)
Mucor pusilus	PG	5.0	40	Al-Obaidi, Z. S., Aziz, G. M., & Al-Bakir (1987)
Penicillium italicum	-	8.0	50	Mojsov et al. (2012)
Aspergillus fumigatus	-	3-9	65	Mojsov et al. (2012)
Streptomyces sp.QG-11-3	-	3-9	60	Mojsov et al. (2012)
Amycolata sp.	-	10	70	Mojsov et al. (2012)

Table 2.9 Bacterial pectinases.

Source	Type of Pectinase	Opt. pH activity	Opt. Temp (°C)	Reference
Bacillus sp. R K9	PGL	10.0	30-40	Kubra et al. (2018)
Bacillus sp. NT-33	PG	10.5	75	Kubra et al. (2018)
Bacillus polymyxa	PG	8.4-9.4	45	Kubra et al. (2018)
Bacillus pumilis	PATE	8.0-8.5	60	Kubra et al. (2018)

Commercial pectinases are mostly produced by *Aspergillus* species in the form of pectin methyl esterases, polygalacturonases, and pectin lyases (Table 2.10) (Pedrolli et al., 2009).

Table 2.10 Commercial pectinases (Pedrolli et al., 2009).

Commercial Names	Opt. pH activity	Opt. Temp(°C)	Pattern
Pectinase CCM	4.0/6.0	50/40	PG/PL
Grindamyl 3PA	4.0	55	PL
Pectinex 3XL	4.7/5.0-6.5	50/35	PG/PL
Rapidase C80	4.0/6.0	55/40-45	PG/PL

Pectinase production by microorganisms starts with screening and isolation of the microorganisms followed by growth on a medium then inoculation and fermentation step and lastly enzyme purification performed.

There are two fermentation methods to produce pectinase, which are solid-state fermentation and submerged fermentation. The submerged fermentation method is more suitable for bacterial pectinases while solid-state fermentation is mostly used to produce fungal pectinases (Pedrolli et al., 2009). The reason for that is due to the water requirement differences of fungi and bacteria. Fungi need less water activity to grow than bacteria. Submerged fermentation is easier to apply in the industry since solid-state fermentation cause problems during the purification step. In addition, scale-up is easier with the SmF method and enzyme recovery is higher than with the SSF method (Kapoor et al., 2002). During fermentation, control of the experiment at a large scale is easier when the SmF method is applied. In the literature, there are studies on higher pectinase production using the SmF method rather than the SSF method (Kuvvet et al., 2019).

In the literature, not only sources but also the efficacy of fermentation methods are compared. Kumar et al. (2011) studied cellulase and pectinase production together by implementing the solid-state method and submerged method for fermentation by using *Aspergillus niger* NCIM 548. They investigated the effect of pH, time, and the concentration of carbon sources on enzyme yield for both fermentation methods. As a result, pectinase was produced at 25.23 U/g, and cellulase was at 5.54 U/g in SmF whereas in the SSF method the results were higher as 179.83 U/g for pectinase and 10.81 U/g for cellulase, respectively. It indicates that a solid environment is more suitable for the enzymes produced by *Aspergillus niger*. In addition, pectinase production by *Bacillus* species is also investigated and showed encouraging results by the submerged fermentation method.

Nawawi et al. (2017) reported pectinase activity of 62.17 U/mL produced by *Bacillus subtilis* ADI1 in a medium supported with 2% (w/v) rice bran at 30°C for 72 h fermentation. Sharma and Satyanarayana (2006) reported optimum pectinase production by *Bacillus pumilus* by the submerged method as 34-41 fold and could be applied in the fiber industry to reduce the usage of alkali components. Kashyap et al. (2000) stated that *Bacillus sp.* DT7 produced pectinase at 53 U/mL by submerged fermentation method. However, there are also studies on bacterial pectinase production by solid-state fermentation method and results are also high as in the submerged fermentation method. Oumer and Abate (2018) investigated pectinase from *Bacillus subtilis* Btk 27, and the yield was found as 1272 U/g after optimization of fermentation conditions. In another study conducted by Nadaroğlu et al. (2010), *Bacillus pumilus* was used to produce pectin lyase by solid-state fermentation method.

Taking into consideration the studies mentioned above, the pectinase enzyme is produced by both bacteria and fungi, and this production can be enhanced by different methods. However, adding extra artificial sources and minerals to the fermentation medium can increase enzyme yield but also increase the cost, leading to the need to find low-cost carbon sources and easy-to-apply industrial methods.

2.3.2 Cellulases

Cellulases are produced by a wide range of microorganisms such as bacteria, fungi, and actinomycetes. Cellulases degrade cellulose by working on the β-1,4-glycosidic linkages (Acharya et al., 2012). Cellulases produced by bacteria have advantages over fungal ones since it is easier to control the growth of bacteria than fungi.

There are three groups of cellulase enzymes differentiated as endo, exo-glucanes, and β-glucosidases. End of cellulose chains are paired by exoglucanase while endoglucanase acts randomly, and glucosidases attack on specific parts of cellobiose disaccharides, and at the end glucose is released. Anaerobic bacteria and fungi have different working systems. Cellulases have been used for more than 30 years in different industries and research is still ongoing. Examples of applied industries are textile, biomass, pulp and paper, winemaking, animal feed, laundry, and more commonly food.

2.3.2.2 Application of cellulases

Cellulase usage in the pulp and paper industry has been favored environmentally and economically since it saves energy between 20 to 40% and reduces the pulp amount. Without enzyme usage, immense waste materials are disposed off from the paper process, which further causes serious environmental problems.

Cellulase usage in the industry can also be expanded by other enzymes, which improve the paper quality even more and decrease the stiffness and defibrillation. Cellulases are often combined with hemicellulases for hydrolyzing and deinking processes, which further helps reduce the usage of chemicals, increasing fiber quality in terms of strength, brightness, and cleanliness. During the paper production process, colloidal substances can cause drainage in mills, which could be decreased by cellulase treatment (Kuhad et al., 2011).

In the textile industry, cellulase usage is preferred over traditional methods. Before cellulase treatment, jeans were processed with amylase enzyme, which causes fiber damage, rapid machine decay, low productivity, and environmental pollution.

Usage of acidic cellulases increases softness while increasing the water absorption capability of fiber and the result is a cleaner structure. When cellulase is paired with endoglucanases, the biopolishing process will be more successful in terms of color, appearance, and sense of fabric (Kuhad et al., 2011).

The bioethanol industry is another application area of cellulase, gaining popularity in recent years. Usage of lignocellulosic materials and turning them into higher-value products by enzymatic process decreases production cost and promotes a clean environment. By optimizing the enzyme production, the cost of ethanol production from lignocellulosic materials can be lowered. More studies are conducted for developing better functioning cellulase (Sukumaran et al., 2005).

One of the benefits of using enzymes in the process is to decrease the rancidity while increasing the antioxidant level and vitamin E in the oil. In addition, the process will be more environmentally friendly by increasing waste quality. The reuse of enzymes could be another effective way to reduce the cost. Recombinant microorganisms are also used for the enzyme industry to increase efficiency for the microorganisms that cannot be suitable for large-scale production (Olempska-Beer et al., 2006).

2.3.2.3 Cellulase production

Cellulases can be produced by microorganisms that are anaerobic, thermophilic, aerobic, and mesophilic. The most famous ones are *Aspergillus, Clostridium, Trichoderma, Cellulomonas.* Bacteria is a more suitable source for cellulase production than fungi due to their fast-growing ability and as a result higher yield of enzyme production. Other advantages are the higher synergy and functionality of the enzyme which is more complex than the fungal ones and the wide variety of strains that produce more resistant and stable enzymes also valuable for the bioconversion industry (Maki et al., 2009).

There are solid state and submerged fermentation techniques for cellulase production which can be preferred according to source and conditions (Table 2.11). Due to cost problems, the submerged fermentation technique was favored, and commercially cellulase mostly produced by *Aspergillus* and *Trichoderma* (Zhang & Zhang, 2013). There are end product and catabolic repression factors, which affect the submerged fermentation process and have economic importance. SSF has also advantages such as low energy consumption and low cost (Sadhu et al., 2013).

Table 2.11 Fermentation types for cellulase production (Sadhu et al., 2013).

Bacteria	**Opt. Temp (°C)**	**Opt. pH**	**Fermentation Type**	**Type of substrate**
Bacillus subtilis	37.0	7.0	SmF	CMC
Bacillus cereus	-	-	SSF	Palm Kernel Cake
Clostridium thermocellum	-	-	SmF & SSF	Cellulose and paper pulp
Bacillus sp. NZ	50.0	9-10	SSF	Bagasse agricultural resides

2.3.3 Pectinase and cellulase co-production

The co-production of enzymes is advantageous in many aspects. As a solution to industries' biggest issue ''cost'', they have the potential to reduce the cost while enhancing efficiency due to the synergistic effect between them. The co-production of enzymes will bring great convenience to different industries such as beverage, bioethanol etc. (Wang et al., 2014).

In the literature, there are few studies on the co-production of enzymes. Generally, conditions and factors were searched and optimized in the literature.

In one study, co-production of pectinase, xylanase, and cellulase by *Bacillus subtilis* ABDR01 was investigated. Production of enzymes was enhanced by performing ultrasonic irradiation at a low level on bacteria to increase the permeability of the cell wall which further enables to get more nutrients inside and increase productivity (Yadav et al., 2020).

Zehra et al. (2020) studied pectinase and xylanase production together from banana peels by using *Aspergillus fumigatus* MS16. Interaction of factors that were investigated and influenced the production of enzymes were temperature, presence of mineral salt in the medium, time, SmF or SSF, and pH. As a result, it was reported that mineral salt enhanced the production, and the optimum temperature range was between 30 and 35 °C under submerged fermentation. The concentration of carbon source in the medium was also a crucial factor to produce pectinase and xylanase. The higher the concentration, the more pectinase was produced, and the opposite works for xylanase. Its optimum concentration was found as 0.25%. It is also discovered that the applied fermentation temperature on SSF was higher than on SmF.

Furthermore, *Aspergillus niger* NCIM 548 was used to produce cellulase and pectinase in both solid-state and submerged fermentation conditions (Kumar et al., 2011). Maximum enzyme production was found at 1.64 U/mL for SmF and cellulase was found at 0.36 U/mL. Fermentation conditions were set as 126 h by using 65 g/L as a carbon source at pH 4.6. Results were changed when SSF is applied (Kumar et al., 2011).

In another study, the co-production of cellulase, pectinase, and xylanase was investigated by isolated *Aspergillus SP.55SZ* in solid-state fermentation. It was found that orange peel is the most effective carbon source among apple, wheat straw, banana peel, mandarin, and banana pseudo stem for the co-production of enzymes. It was also shown that using pectinase and cellulase together enhances the yield of juice extraction by 100% (Kashyap et al., 2000).

As a result, it is advantageous to co-produce enzymes and more research is needed to find the optimum conditions that enhance the yield and quality of the processes. This study was designed over this idea and utilization of food and agricultural wastes makes it one of the leading studies on cost reduction and waste management field. Besides, none of the studies implemented EVMD method to optimize the fermentation medium proportions.

2.4 Optimization of enzyme production

There are important parameters that needed to be considered to optimize enzyme production, which include medium formulation, growth and fermentation conditions, and type of enzyme and sources. In the literature, many experiments were carried out accordingly to get maximum yield. For instance, it is important to find suitable carbon sources for the fermentation as well as optimum growth conditions for the source of microorganisms. For this study, proportions of mixtures were optimized for maximum co-production of enzymes. To achieve that, the EVMD method was implemented.

The mixture of feedstocks was first treated with an acid solution before fermentation and *Bacillus subtilis* was grown under various conditions.

2.4.1 Medium formulation

It is important to carry out enzyme production under the optimum conditions for the maximal yield. In the literature, there are studies on different medium formulations. In one study, pectinase was produced by using *Bacillus subtilis* EFRL 01 in a medium in which composition was optimized. The investigated parameters were the carbon source, nitrogen source, yeast extract, pH, and temperature. After several experiments, 15 g/L of date syrup used as a carbon source along with 7.5 g/L of yeast extract at 45 °C for 48 h with a pH of 8 were found as optimum conditions for pectinase production (Chandrasekaran & Bahkali, 2013).

Different carbon sources are investigated due to cost issues. In the literature, Ghazala et al. (2015) stated that carrot waste could be a potential carbon source for pectinase production as it is rich in pectin. The Box-Behnken RSM method were applied for the optimization of incubation time, carrot peel powder, inoculum size, and NH_4Cl. The optimum formulation was found as 6.5% carrot peel with 0.3% NH_4Cl and 3% inoculum level for the maximum enzyme production.

A nitrogen source is also important for enzyme production. Rathnan et al. (2013) used the carboxyl methylcellulose (CMC) broth together with paper waste for cellulase production. They supplemented the fermentation medium with glycine, yeast extract, peptone, and malt extract. As a result, it was reported that 0.5% nitrogen amount is optimum at 40 °C for 72 h incubation for the maximum cellulase production (Rathnan et al., 2013).

2.4.2 Culture condition

It is important to optimize the culture conditions to improve enzyme production as Pathania and his friends (2016) reported. Optimization of xylanase, pectinase, and cellulase was performed by implementing central composite design (CCD). To optimize and investigate interactions of variables and conditions, RSM is a preferable method. In this study, incubation period, temperature, and moisture were chosen as parameters. After 20 experiments were performed, optimum conditions were found as 7 days of incubation, 1:3.5 moisture ratio, and 30 °C (Pathania et al., 2016). Kuvvet et al. (2019) also studied pectinase production and reported that 30 °C at pH 9.0 with 15% solid load was the most optimum condition for co-production of *Bacillus subtilis* and *Bacillus pumilis.*

2.4.3 Extreme Vertices Mixture Design method

Response surface methodology (RSM) is designed to explain and optimize the relationship between the variables by using mathematical and statistical tools. It collects data responses and creates a relationship between them (Tanyildizi et al., 2005). It is the most preferred tool for the optimization of variables. However, the Extreme Vertices Mixture Method (EVMD) is one of the promising methods for mixture formulation. It is specially designed for mixture formulations and enables to reach the results with fewer experiments.

In the literature, some studies implemented EVMD in their experimental designs. In one study, they applied the EVMD method to understand the relationship between different components on each other (Rispoli et al., 2007). They investigated different parameters of cutinase enzyme production such as starch, glucose, magnesium sulfate, etc.

Papadaki et al. (2020) used the mixture design to find the optimum percentages of the feedstock to achieve the highest yield of polygalacturonase, protease, amylase, and cellulase. Optimum proportions were found as 15% white pomace, 15% red grape pomace, and 70% wheat bran.

2.5 Feedstocks

The utilization of lignocellulosic wastes is becoming more important due to depleting resources and increasing waste generation. Disposing valuable wastes into the environment not only cause pollution but also loss of valuable materials (e.g. bioactive substances). Conversion of these wastes into high-value products and metabolites could be an important acquisition of biomass (Vendruscolo et al., 2008).

Each waste comprises a different amount of chemical compositions such as cellulose, lignin, pectin, and hemicellulose, which further affects the type of enzyme and the growing ability of microorganisms. High amount of cellulose and pectin in the medium, leads to relevant production of enzymes like cellulase and pectinase thus it is more advantageous to use different types of lignocellulosic wastes together rather than alone.

2.5.1 Apple pomace

Apple is abundantly grown and processed into assorted products over the centuries. Apple generated about 87.2 million tons in 2019 according to the FAO report and 70% of the apple was consumed as fresh while the rest were utilized by industries (FAO, 2021). However, 30% of the apple came out as waste (Tuhanioğlu, A. 2021). During processing and disposal, important environmental problems can occur. Thus, it encourages researchers to study apple pomace and promising results are obtained in different areas

such as the production of enzymes, animal feeds, which is also limited due to insufficient protein and other nutrition, organic acids, fibers, ethanol, and aroma compounds (Devrajan et al., 2004; Favela-Torres et al., 2006; Shojaosadati & Babaeipour, 2002).

Apple pomace is composed of a mixture of core, stem, peel, seed, soft tissue, and calyx. As can be seen in Table 2.12, apple mainly consists of water, simple sugars such as fructose, glucose, sucrose, and also insoluble parts such as cellulose, lignin, and hemicellulose, other parts are vitamins, minerals, and proteins.

Apple pomace is considered rich in fiber content, which is pectin, cellulose, gums, hemicellulose, and lignin. The extraction of these valuable contents has been studied by many researchers. As Figuerola et al. (2005) reported in their study, consuming dietary fibers in daily life is important as it is linked to being healthy and preventing lots of illnesses such as heart diseases, cancer, diabetes, or diverticulitis. Apple pomace also had high polyphenols approximately 31% to 51% (Schaefer et al., 2006).

One of the important utilizing areas for apple pomace is enzyme production mostly for pectin degrading enzymes since apple pomace contains a high amount of pectin. In different industries such as textile, degumming, food, or wastewaters, enzyme production is applied by using microorganisms (Favela-Torres et al., 2006). Apple pomace is also used for the following applications: ethanol production, aroma compounds, organic acid production, heteropolysaccharides, biopolymers, baker’s yeast, and edible mushrooms (Vendruscolo et al., 2008).

Kuvvet et al. (2019) reported pectinase using apple pomace waste at 15% solid load by using co-culture of microorganisms and obtained 11.25 IU/mL pectinase.

Since apple pomace is not enough by itself, other food wastes with a high amount of pectin were studied for enzyme production in the literature. Martin et al. (2010) used a mixture of orange bagasse and wheat bran for the fermentation and found pectinase at 13.6 U/mL

in submerged fermentation. In another study, Silva et al. (2002) used a mixture of mango, banana, and sugar cane for polygalacturonase and pectin lyase activity and found out that the enzymes were produced higher than when they were applied alone.

Table 2.12 Apple pomace components (Magyar et al., 2016).

Components	**%**
Pectin	14.4
Cellulose	21.0
Hemicellulose	11.1
Lignin	24.7
Glucan	21.1
Xylan	3.7
Galactan	3.0
Arabinan	4.4
Ash	2.2
Fructose	19.2
Sucrose	1.0
Ethanol	0.9

2.5.2 Orange peel

Orange juice is considered one of the most preferred beverages over others for centuries (Martín et al., 2010). Most citrus fruits are used in the process of juice or other industries such as marmalade (Embaby et al., 2014). According to Wilkins et al. (2007), 50 to 60% is generated as waste from processing and accumulates in enormous amounts. It further causes an environmental problem along with the need to search for other alternative solutions. Using wastes as raw materials for high-value components is an important way of decreasing costs. Orange peel waste chemical components are given in Table 2.13.

For the polygalacturonase enzyme, according to Embaby et al. (2014), orange peel waste was found to be the best carbon source among six wastes, namely, pomegranate, wheat bran, lemon, artichoke peel, and banana. With the help of response surface methodology (RSM), Plackett-Burman (PB) and one variable at a time (OVAT) were the tools that they had been used. Fermentation time was found as 48 h to get maximum enzyme production at a pH level of 8.0 and 37.8 °C. Polygalacturonase activity was found as 2.69 μg/min*mg.

Table 2.13 Orange peel chemical components (Tsouko et al., 2020).

Components	**%**
Pectin	20-22
Cellulose	18-20
Hemicellulose	14-16
Lignin	5-7
Ash	3-3.5
Protein	6-7

2.5.3 Hazelnut shell

Hazelnut is one of the most important lignocellulosic substances used for different purposes such as biofuel, enzyme, or chemical production, which also has an increasing trend over the last centuries due to its high availability and low cost. Hazelnut shell is left out from hazelnut production and is valuable for its composition as mostly 34.64% lignin followed by 28.2% hemicellulose and 24.2% cellulose (Uzuner & Cekmecelioglu, 2014). High percentages of hemicellulose and cellulose makes hazelnut shell is a potential carbon source for enzyme production, organic acids, ethanol, etc.

Uzuner and Cekmecelioglu (2014) used hazelnut shells for pectinase production since hazelnuts are mainly produced in Turkey and exported to the world. According to Köksal et al. (2006), hazelnut production in Turkey covers 60-80% of the market and annual production is between 250,000 and 400,000 tons.

2.6 Optimization of co-enzyme production

Increasing the efficiency of enzyme production while decreasing the cost is the purpose of the fermentation processes. To decrease the cost of the production, the type of microorganisms, carbon sources, fermentation medium composition, and conditions are key factors to be considered.

There are numerous studies conducted on pectinase and cellulase production separately and few together. Generally, the synthetic medium is used for the fermentation process instead of cost-effective alternatives. To be cost-effective, the carbon sources must be abundant, renewable, and have all the necessary nutrients for the microorganisms to grow.

When nutrients are absent in the fermentation medium, additional supplement support will be necessary to avoid enzyme yield to decrease.

There is numerous research on optimization of carbon sources for enzyme production along with nutrient types, nitrogen sources, and fermentation types for the most efficient and low-cost production. Using lignocellulosic wastes for enzyme production is one way to reduce to cost of the process and different types are given in Table 2.14. To reduce the cost, a mixture of apple pomace, hazelnut shell, and orange peel was selected for this study.

In the literature, mostly RSM is used for optimization of fermentation process. It saves time and helps the cost of the experiments.

Extreme vertices mixture design (EVMD) is on the other hand specially devised for the formulation of mixtures and optimization is also achieved with few experiments. For instance, to evaluate different variables on enzyme production, the EVMD method was used by Rispoli and Shah (2007) who investigated the relationship between variables and the product. Since it is better for mixture design, optimization has been done by the EVMD method.

Table 2.14 Different enzyme production using food waste.

Enzyme type	**Food waste**	**Reference**
Pectinase	Tomato waste	Catalkaya et al. (2019)
Cellulase	Coir waste	Mrudula et al. (2011)
Amylase	Sago bagasse	Kumar et al. (2019)
Xylanase	Oil palm frond bagasse	Mazlan et al. (2020)

2.7 Aim of the study

Pectinases are one of the multi-purpose enzymes applied for different purposes as well as cellulases. While pectinases break downs the pectin, cellulases target oligosaccharides. They both have broad applications such as pectinases can enhance the juice quality by clarification and extraction of pectin structures while cellulases, on the other hand, are an important tool in waste management and the paper industry. (Ozzeybek & Cekmecelioglu, 2022).

Waste management has become more popular due to the immense increase in the human population and consequently the number of industries. The utilization of waste is advantageous for both decreasing the cost and increasing environmental safety. Wastes are generally made from fruits, dairy products, vegetables, and bakeries. The amount of waste has been expected to increase from 278 to 416 million tons by 2025 (Mohanty et al., 2022). For this study, apple pomace, orange peel, and hazelnut shell were selected.

To optimize the process parameters, there are many methods studied by researchers. RSM for instance, one of the common methods was used for optimizing the independent parameters. However, for mixture design formulation, the EVMD method is more suitable since it creates fewer experiments with more dependent parameters.

In this study, the aim was to optimize the proportions of apple pomace, orange peels, and hazelnut shells by the Extreme Vertices Mixture Design (EVMD) method to increase the co-production of bacterial pectinase and cellulase enzymes. Thus, to achieve this aim following objectives are stated;

1. Dilute acid pretreatment of apple pomace, orange peel, and hazelnut shells mixtures
2. Co-production of enzymes with single feedstock
3. Optimization of co-production of pectinase and cellulase by EVMD method

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Lignocellulosic biomass and chemicals

Hazelnut shell was provided by a plant from Ordu while orange peel was provided from local stores in Ankara, and apple pomace was supplied by Elite Naturel Fruit Juice Company located in Ankara, Turkey. Orange peel and apple pomace were dried at 70 °C for 24 h in an oven (Oven ST-120, Şimşek Laborteknik Ltd. Şti). Afterward, they were grounded by using a grinding mill which has a 1 mm sieve and stored at room temperature for further usage (FRITSCH Industries.55743 Idar-Oberstein, Germany).

3.1.2 Microorganisms and growth medium

Bacillus subtilis NRRL B-4219 was procured from Northem Regional Research Laboratory 32 (NRRL), Peoria, Illinois, USA.

Growth medium was prepared to activate the bacteria and the composition is listed in Table 3.1. The growth medium was prepared in a 100 mL Erlenmeyer flask at 50 mL and then autoclaved for 15 min at 121 ℃ for sterilization. Bacteria were grown at 35 ℃ and 120 rpm for 24 h. (Kuvvet et al., 2019).

Table 3.1 Composition of growth medium (Kuvvet et al., 2019).

Components	**Amount (g/100 mL)**
Yeast extract	0.1
Glucose	1
Pectin	0.2
$MgSO_4$	0.04
K_2HPO_4	0.04
KH_2PO_4	0.02

The chemicals used for the analysis are given in Appendix A.

3.1.3 Buffers and solutions

In Appendix B, all the solutions and buffers for this experiment are listed.

3.1.4 Acid pretreatment

The mixture of apple pomace, orange peel, and hazelnut shell was treated with sulfuric acid at 3% (w/w) in an autoclave for 15 min at 121.1 °C. After cooling down, the slurry was filtered, pH was adjusted to 7.0 with 10% NaOH solution, and then centrifuged to eliminate solid particles before the addition of minerals. The solid load was kept constant at 10 g/100 mL throughout the study.

3.1.5 Co-enzyme production

The fermentation medium was composed of mixture of hazelnut shell, apple pomace, and orange peel hydrolyzates. It was also enriched with $MgSO_4 7H_2O$ (0.04 g/100 mL), yeast extract (0.1 g/100 mL), K_2HPO_4 (0.04 g/100 mL) (Kapoor et. al., 2002). The solid load of the fermentation medium was 10 g/100 mL. *Bacillus subtilis* was added into autoclaved fermentation medium as 1 mL OD_{600}=1.2. Fermentation conditions were 30 °C and at 130 rpm for 72 h. At specific periods, aliquots of samples were taken out aseptically for further analysis.

3.2 Analytical methods

3.2.1 Optical density

To measure the optical density (OD) at 600 nm of *Bacillus subtilis*, the spectrophotometer was used (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan). Samples were taken from the growth medium and centrifuged to obtain biomass for which was washed twice for 5 min with deionized water. OD was measured against water as a blank solution.

3.2.2 Total reducing sugar

During the fermentation process, the production of enzymes and the growth of bacteria are highly related to total reducing sugar content. It is measured by the dinitrosalicycilic acid (DNS) method with some modifications (Miller, 1959). By this method, free carbonyl groups are measured as equivalent to glucose. In the procedure followed, samples taken from the fermentation medium were centrifuged and the supernatant part was diluted to 3

mL as the final volume and mixed with 3 mL of DNS solution and placed in a 95℃ water bath for 15 min to develop color (Appendix C). Lastly, tubes were cooled down and absorbance was measured by using a spectrophotometer at 575 nm against blank solution, which was also prepared with deionized water and DNS solution. Results were interpreted according to the standard curve, which was also prepared with different glucose concentrations between 0 to 1.0 g/L (Figure C.1).

3.2.3 Pectinase assay

Pectinase activity was measured by the DNS method and expressed in terms of polygalacturonase activity. Diluted samples of 0.5 mL were mixed with polygalacturonic acid (1%) in phosphate buffer at pH 7.0. Simultaneously, enzyme control solution, blank and sample control solutions were prepared. All the tubes were incubated at 50 °C for 30 min. Following incubation, 3 mL of DNS solution was added into tubes and immediately placed into a water bath at 90 °C for 15 min for the color to develop. Lastly, absorbance was measured at 575 nm after cooling them and the results were translated by a standard curve, which was also prepared by following the same procedure mentioned above (Appendix D). The only difference is that as a sample D-galacturonic acid was used in different concentrations between 0 to 1.5 mg/mL. One unit of pectinase is defined as amount of enzyme released one micromole of galacturonic acid per minute under assay conditions.

3.2.4 Cellulase assay

Cellulase activity is expressed as equivalent to micromole of glucose released per minute under assay conditions. To measure cellulase activity, samples were centrifugated and diluted as needed. For incubation, 0.5 mL of samples were taken, and filter paper was used for substrate with citrate buffer. Tubes were incubated at 50 °C for one hour and

simultaneously enzyme and substrate control and blank were also prepared and incubated. Following incubation, 3 mL of DNS solution was added into each tube, and all were placed into a water bath at 90 °C for 15 min. After cooling, absorbances were measured at 575 nm by using a spectrophotometer and the results were converted into glucose (mg) by a standard curve prepared by using different concentrations of glucose between 0 to 2 mg/mL (Appendix E).

3.2.5 Extreme vertices mixture design (EVMD)

EVMD is a statistical design method for the formulation of mixtures. In this study, orange peel, hazelnut shell, and apple pomace proportions were designed by the EVMD method. The range of proportions was decided as a minimum of 20% to a maximum of 60% for each feedstock (Table 3.2). As a result, 7 design points were obtained for the optimum co-enzyme production. All the experiments were replicated, and the results were expressed as mean values. As can be seen in Figure 3.1 which is a simplex plot representation, the triangular system shows the whole mixture as 1. The vertices show a fraction of a single component. The edges indicate two components. The points within the triangle indicate 3- component mixtures. The experimental design for this study can be seen in Table 3.3 and Figure 3.1.

The experimental data is fit to a quadratic equation as followed;

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{3} b_{ij} X_i X_j \qquad (1)$$

Where Y represents enzyme activity (U/mL), b represents regression coefficients and X's are denotations for percentages of apple pomace, hazelnut shells, and orange peel.

Significant model terms and coefficients were calculated by Minitab 16.0 (State College, USA). According to the results of seven experiments, the optimum percentages of each feedstock were chosen for the maximum co-enzyme production.

Table 3.2 Maximum and minimum percentages of each feedstock.

Waste Material type	**-1**	**0**	**+1**
Hazelnut Shells	20	40	60
Orange Peel	20	30	40
Apple Pomace	20	30	40

Table 3.3. Experimental design of co-production of enzymes.

Experiment No	Hazelnut Shells (%)	Orange Peel (%)	Apple Pomace (%)
1	46.7	26.7	26.7
2	40	20	40
3	43.3	33.3	23.3
4	60	20	20
5	40	40	20
6	43.3	23.3	33.3
7	53.3	23.3	23.3

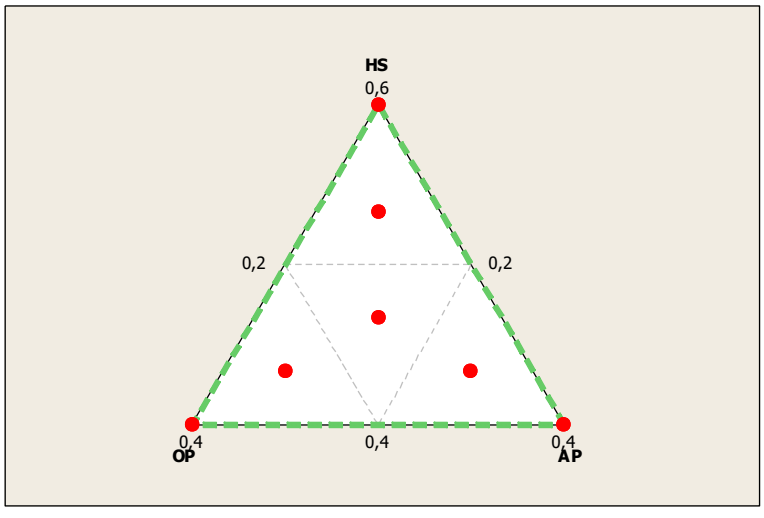

Figure 3.1 Simplex plot illustration of hazelnut shell, orange peel, and orange pomace.

CHAPTER 4

RESULTS AND DISCUSSION

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4.1 Pretreatment methods for the production of reducing sugar

All the raw materials were first dried and then grounded into a 1 mm particle size which is one of the principal factors for the efficiency of acid treatment. As Uzuner and Cekmecelioglu (2014) reported, a small particle size means a larger surface area for interaction to happen, and molecules can collide easily with each other so that the efficiency of acid penetration will get higher.

Three different concentrations of sulfuric acid (1%, 2%, 3% w/w) were tested for the efficiency of the acid pretreatment. Apple pomace, orange peel, and hazelnut shell were treated with sulfuric acid at 121 °C for 15 min in an autoclave, and the fermentable sugar amount released from the mixture was measured by using the DNS method. In Fig. 4.1, average reducing sugar contents are given. As acid concentration is increased from 1% to 3%, reducing sugars released from 20.9 g/L to 33.1 g/L and yield is increased from 0.209 g/g to 0.331 g/g.

Results also agreed well to the literature. For instance, Ayala et al. (2021) reported orange peel reducing sugar as 24.585 g/L while in apple pomace was found as 16.16 g/L by Evcan et al. (2015) and hazelnut shell was studied by Uzuner and Cekmecelioglu (2014) and reducing sugar content was found as 19.2 g/L.

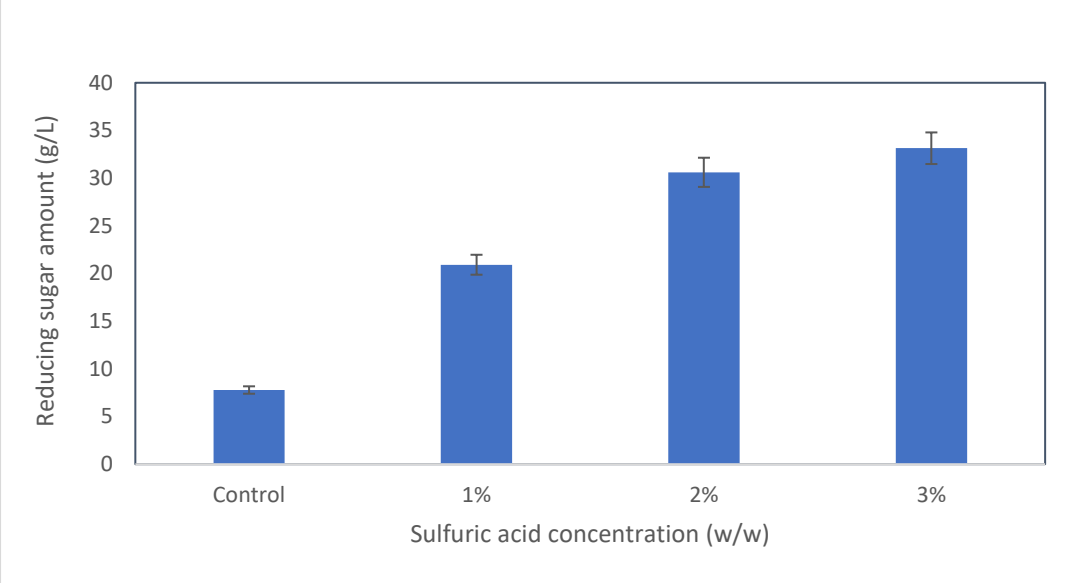


Figure 4.1 Comparison of different acid concentrations on reducing sugar amount (121 °C, 15 min).

Lignocellulosic waste materials are useful substances that can be converted into high-value end products, which make them important for many processes. However, because of their high cellulose and hemicellulose contents, it is not easy to convert them into their constituent sugars for further usage. In the literature, there are numerous studies on pretreatment methods that make them easily accessible and increase the rate of hydrolysis (Kumar et al, 2009). In general, pretreatment methods have been used for degrading cellulose, removal of lignin, and increasing porosity. As Lu et al. (2007) reported, acid hydrolysis could improve the enzyme yield by disrupting the structure and increasing the permeability. They studied different acid concentrations (2, 4 and 6% sulfuric acid) to attain the maximum yield of glucose and xylose from corn stover along with other parameters. As a result, they found that by using 2% sulfuric acid achieved 77% of xylose yield along with 8.4% of glucose yield. The reason of 2% gives a higher yield of xylose and glucose than 6% could be explained by furfural formation from the

degradation process. Increasing acid solution concentration is better for fermentation however, after reaching optimum concentration, byproducts could inhibit the further fermentation process.

For this study, a mixture of lignocellulosic materials was used which makes pretreatments necessary to have maximum enzyme production. However, in the literature, there are few studies on enzyme production from a mixture of feedstocks. Kuvvet et al. (2019) studied the valorization of apple pomace for pectinase production and tested two pretreatment methods, which are one-step acid hydrolysis and two-step hydrolysis. According to their results, one-step acid hydrolysis was more efficient for reducing sugar concentration. By applying two-step acid hydrolysis, the result was 19.32 g/L while 20.26 g/L for one-step acid hydrolysis. In addition, Uzuner and Cekmecelioglu (2014) studied hazelnut shells for pectinase production and applied acid hydrolysis as a pretreatment at 3.42 % concentration and found the maximum reducing sugar. Since there is no exact mixture combination of apple pomace, hazelnut shell, and orange peel, reducing sugar content cannot be compared. However, the percentage of acid concentration is compatible with the literature and chosen as 3% sulfuric acid at 121℃ for 15 min (Rocha et al., 2008).

4.2 Single feedstock for co-enzyme production

Cellulase and pectinase produced using apple pomace, orange peel, and hazelnut shells were measured, and the results are given in Table 4.1. As can be seen from the table, the highest cellulase activity was observed in the hazelnut shell medium, which is likely to be proportional to the cellulose content. Hazelnut shell has the highest cellulose content of 24.20 ± 0.99 % followed by apple pomace and orange peel. It is also speculated by the literature that enzyme activity is highly affected by the chemical composition of agricultural food wastes (Laothanachareon et al., 2022). In addition, Olsson et al. (2003) reported that the higher the cellulose content, the higher the endoglucanase production.

Pham et al. (1998) also supported these results by finding more hemicellulase production linked with the hemicellulose content of the substrate in which microorganisms are grown.

Table 4.1 Results for pectinase and cellulase activity of each material.

Type of waste material	**Pectinase Activity (U/mL)**	**Cellulase Activity (U/mL)**
Hazelnut Shells	5.93±0.19	0.49±0.15
Orange Peel	4.77±0.19	0.42±0.01
Apple Pomace	5.23±0.25	0.43±0.04

[1]Results are averages of two replicates; ± stands for standard deviation

It can also be observed that the pectinase amount is also higher in hazelnut shells containing medium than apple pomace and orange peel-containing ones. Nevertheless, the results were insignificant ($p<0.05$). In another study, pectinase production was higher in a non-pectic medium than pectin comprising one (Piccoli-Valle et al., 2001). According to our study, pectinase activity was affected by total carbon content, which is mainly composed of cellulose and hemicellulose also called holocellulose. It explains the high pectinase amount in hazelnut shells, which lack pectin but higher in cellulose and hemicellulose than apple pomace and orange peel. Also, other studies support our findings, such as Uzuner and Cekmecelioglu (2014) reported that pectinase activity of 5.60 U/mL by *Bacillus subtilis* in a medium containing hazelnut shells only. In another study, by using a mixed culture of *Bacillus subtilis* and *Bacillus pumilis*, Kuvvet et al.

(2019) found that pectinase production was improved to 11.48±0.51 U/mL in an apple pomace containing fermentation medium. Lastly, Kumar et al. (2016) measured polygalacturonase activity as 12.66 U/mL by *Bacillus subtilis* with an orange peel-containing medium. These results showed that enzyme type and activity are highly dependent on chemical composition and carbon content.

4.3 Optimization of mixture components by EVMD method

The purpose of implementing the mixture design method was to determine the proportions of apple pomace, orange peel, and hazelnut shells in the submerged fermentation medium to increase the co-production of pectinase and cellulase by *Bacillus subtilis.*

Experiments were designed according to the EVMD method and the activity of pectinase and cellulase are shown in Table 4.2 for each combination. The highest pectinase activity was found as 7.50 U/mL in Run 5 containing 40% hazelnut shell, 40% orange peel, and 20% apple pomace. The highest cellulase activity was found as 0.46 U/mL in two different combinations which are both high in hazelnut shells. It can be observed that both pectinase and cellulase production was increased when hazelnut shells and orange peel are high in the fermentation mixture. This could be explained by the high amount of pectin and cellulose in total.

Cellulase activity was highest with hazelnut shells dominated mixtures. It can be explained by the high cellulose and hemicellulose amount present in the mixture. As long as the hazelnut shells amount is high in the fermentation medium, the apple pomace or orange peel amount does not have a drastic change in enzyme production. Uzuner and Cekmecelioğlu (2014) also found that the high cellulose content of hazelnut shells encourages cellulase production more than apple pomace and orange peel (Orozco et al., 2014; Gowman et al., 2019). These experiments are also a demonstration of how fermentation medium composition affects enzyme production.

Table 4.2 Enzyme activity results for each experiment.

Experiment No	Hazelnut Shells (%)	Orange Peel (%)	Apple Pomace (%)	Pectinase A.(U/mL)	Cellulase A.(U/mL)
1	46.7	26.7	26.7	5.77±0.01	0.46±0.012
2	40.0	20.0	40.0	5.97±0.06	0.43±0.008
3	43.3	33.3	23.3	7.41±0.09	0.46±0.014
4	60.0	20.0	20.0	5.71±0.30	0.40±0.014
5	40.0	40.0	20.0	7.50±0.07	0.42±0.005
6	43.3	23.3	33.3	4.76±0.36	0.41±0.00
7	53.3	23.3	23.3	4.80±0.21	0.42±0.006

[a] Results are the averages of two replicates; ± denotes standard deviation.

As a result of regression analysis, two polynomial equations to represent pectinase and cellulase production as a function of hazelnut shells, orange peel, and apple pomace are developed and shown in equations 1-2.

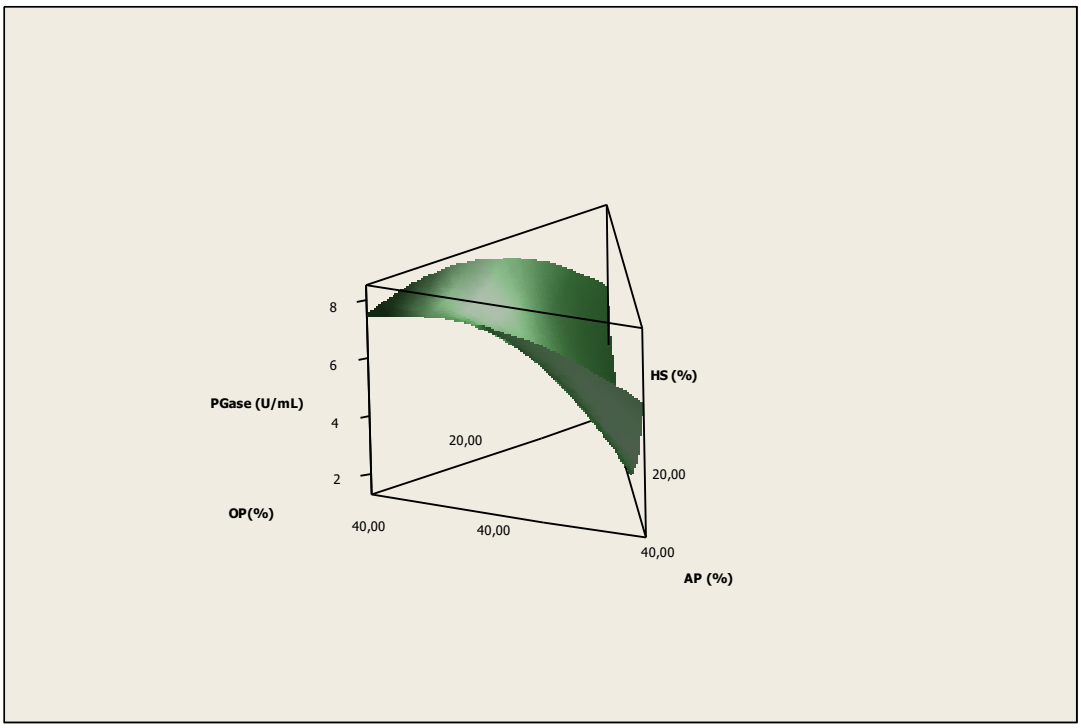
$$Y_{pectinase} = 1.37X_1 + 0.40X_2 - 0.82X_3 - 0.04X_1X_2 + 0.009X_1X_3 + 0.01X_2X_3 \quad (1)$$

and

$$Y_{cellulase} = 0.027X_1 + 0.0003X_2 - 0.04X_3 - 0.00059X_1X_2 + 0.0003X_1X_3 + 0.0009X_2X_3 \quad (2)$$

where $Y_{pectinase}$ and $Y_{cellulase}$ correspond to enzyme activity while X_1, X_2, and X_3 are denotations of apple pomace, hazelnut shells, and orange peels, correspondingly.

The R^2 is 0.95 for pectinase and 0.735 for cellulase model. As stated by ANOVA for pectinase activity, the interaction between hazelnut shells and others demonstrated significant effects ($p<0.05$). Nevertheless, it is not the case for apple pomace and orange peel since the interaction between them was not significant according to ANOVA ($p>0.05$). For cellulase activity, on the other hand, hazelnut shells and orange peel interactions were significant ($p<0.05$), and the other interactions were found insignificant ($p>0.05$). Experimental and predicted pectinase and cellulase activity are plotted and compared in Figures 4.2 (a) and (b).



(a) Pectinase activity (U/mL).

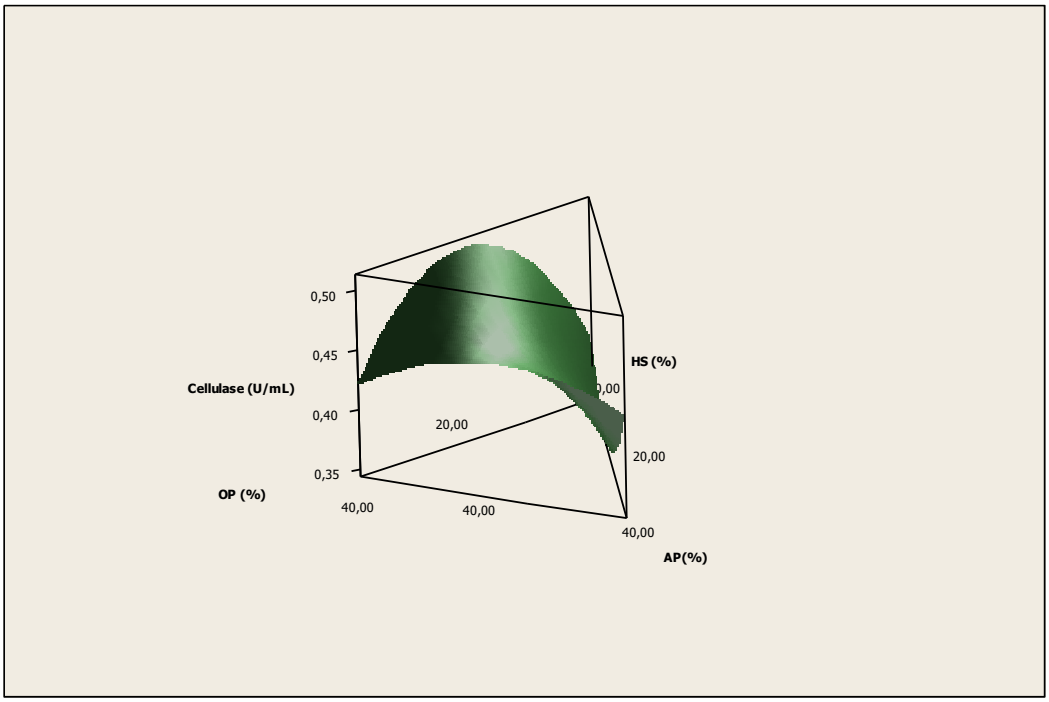


Figure 4.2 Pectinase (a) and cellulase (b) activities U/mL with respect to feedstocks.

The EVMD method has 3D response surface plots for the demonstration of the interaction between hazelnut shells, orange peel, and apple pomace. As seen in Figure 4.2 (a), hazelnut shells and orange peel have a significant positive impact on pectinase activity. Conversely, pectinase production is decreased with hazelnut shells and apple pomace combinations. In Figure 4.2 (b), cellulase activity increases when hazelnut shells and orange peel are used and decreases with apple pomace replaced with orange peel.

The predicted values for the maximum production of pectinase and cellulase were 8.17 U/mL and 0.5 U/mL, respectively. These results were achieved with 50% hazelnut shells, 30% orange peel, and 20% apple pomace. For verification, the predicted proportions were tested, and the activities of the enzymes were found as 8.27 U/mL for pectinase and 0.47 U/mL for cellulase. These experimental values were close to predicted ones therefore it was confirmed that the developed model was successful. The results were also supported by the literature. For instance, Ali et al. (2010) reported that pectinase production was 3.33 U/mL using orange waste. In addition, pectinase activity was found as 3.4 U/mL by Tepe and Dursun (2014) using a sugar beet pulp-containing medium. Cellulase was on the other hand studied by Kumar et al. (2011) and found as 0.36 U/mL by using agro-industrial wastes as carbon sources.

The studies on co-production of enzymes have recently been reported. Amadi et al. (2022) studied optimization of xylanase, pectinase, and cellulase by *Bacillus* sp. using citrus peel as a carbon source. As a result, they reported cellulase activity at 2.563 ± 0.082 U/gds, pectinase activity at 2.910 ± 0.097 U/gds and, lastly xylanase activity at 2.253 ± 0.101 U/gds. However, due to the lack of literature on the co-production of enzymes from feedstocks mixtures, these results could not directly compare with the reported findings. Still, co-enzyme production results using a single feedstock in the fermentation medium are similar to the literature as previously discussed. Pectinase production can be different for various combinations of nutrient components in the medium or the fermentation conditions (Martin et al., 2004; Jahan et al., 2017). In addition, Cekmecelioglu and Demirci (2020) indicated that the co-production of enzymes has advantages such as

increasing hydrolysis performance by enhancing binding capability and synergistic effect on the production. Çelik et al. (2019) also studied the co-production of xylanase, pectinase, and cellulase using wet orange peel as a substrate. *Aspergillus fumigatus* 55SZ was used in their study by solid state fermentation and tested different substrate combinations for enzyme production and reported orange peel as the best substrate giving pectinase activity of 33.55 ± 0.16 U/g, cellulase activity of 13.99 ± 0.411 U/g, and xylanase of 18.82 ± 0.43 U/g.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

In this study, food processing wastes were examined both individually and, in mixed form, to enhance the co-production of bacterial pectinase and cellulase by implementing the Extreme Vertices Mixture Design method. The results prove that the combination of wastes in the fermentation medium improves enzyme production compared to the single feedstock in the medium. It revealed that the activity of pectinase and cellulase enzymes worked well with the mixture of food wastes. In addition, for optimizing the proportions of each feedstock to increase enzyme production, the EVMD method was found to be successful.

According to the EVMD method, the optimum mixture was suggested as 50% hazelnut shells, 30% orange peel, and 20% apple pomace, which gave 8.27 U/mL of pectinase and 0.47 U/mL of cellulase activities.

For further study, investigation of solid load range for possibly higher co-production of enzymes is needed. In addition, to make the enzymes industrially available, they can be produced on a large scale and kinetic models can be developed. Moreover, the co-production of more than two enzymes can be investigated and utilized for biomass hydrolysis.

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APPENDICES

APPENDIX A

Table A.1 Chemicals and supplier information.

CHEMICALS	INFORMATION
Bovine serum albumine	Sigma-Aldrich
Citrus Pectin	Sigma-Aldrich
D-Glucose	Merck
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
3-5-Dinitrosalcylic acid	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	Merck
Nutrient agar	Merck
Phenol	Merck
Polygalacturonic acid	Merck
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck
Rochelle salt	Merck
Sodium phosphate dibasic	Merck
Sodium phosphate monobasic	Merck
Sulfuric acid	Merck
Sodium hydroxide	Merck
Sodium sulfite	Merck
Yeast extract	Merck

APPENDIX B

Table B.1 DNS reagent composition.

Component	**Amount (g/100 mL)**
Dinitrosalicylic acid	1.0
Phenol	0.2
Sodium sulfite	0.05
Sodium hydroxide	1.0
Rochelle salt	20.0

Phosphate Buffer Preparation

Table B.2 Phosphate buffer composition (pH 7).

Component	Amount (g/100 mL)
A	0.1 M solution of sodium phosphate monobasic
B	0.1 M solution of sodium phosphate dibasic

Preparation: pH is adjusted to 7 by solution A and solution B mixed at 39:61 ratio and made up with water to 200 mL total volume.

Citrate Buffer Preparation

Table B.3 Citrate buffer composition (pH 4.8).

Component	Amount (g/100 mL)
A	0.1 M solution of citric acid monohydrate (4.21 g/200 mL)
B	0.1 M solution of trisodium citrate dihydrate (5.89 mL/200 mL)

Preparation: Solution A (40 mL) was mixed with 60 mL of solution B to reach pH 4.8.

APPENDIX C

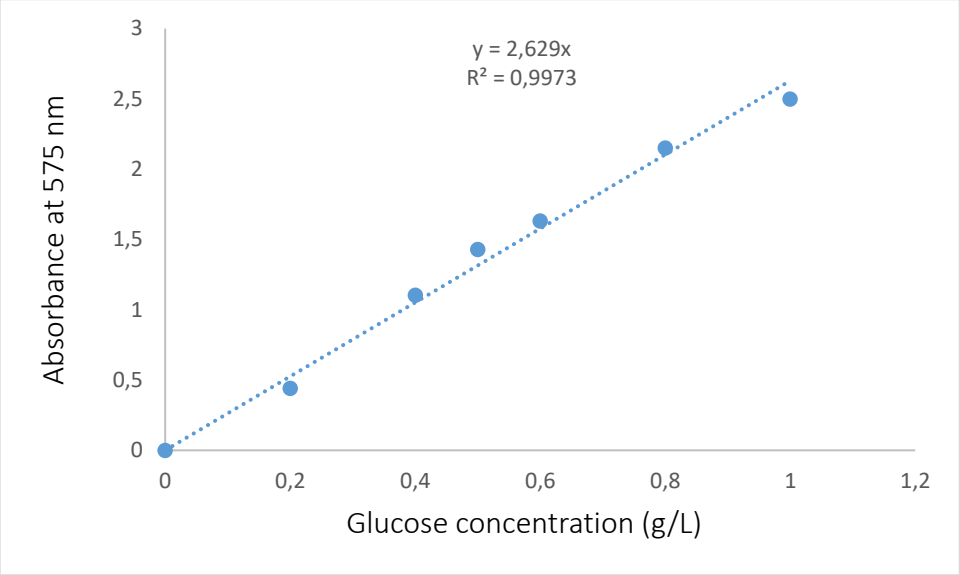

Figure C.1 The standard curve for glucose concentration by DNS method.

The total reducing sugar content is calculated by the following equation;

$$\text{Total reducing sugar concentration } \left(\frac{g}{l}\right) = \left(\frac{Absorbance}{2.629}\right) * Dilution\ rate$$

APPENDIX D

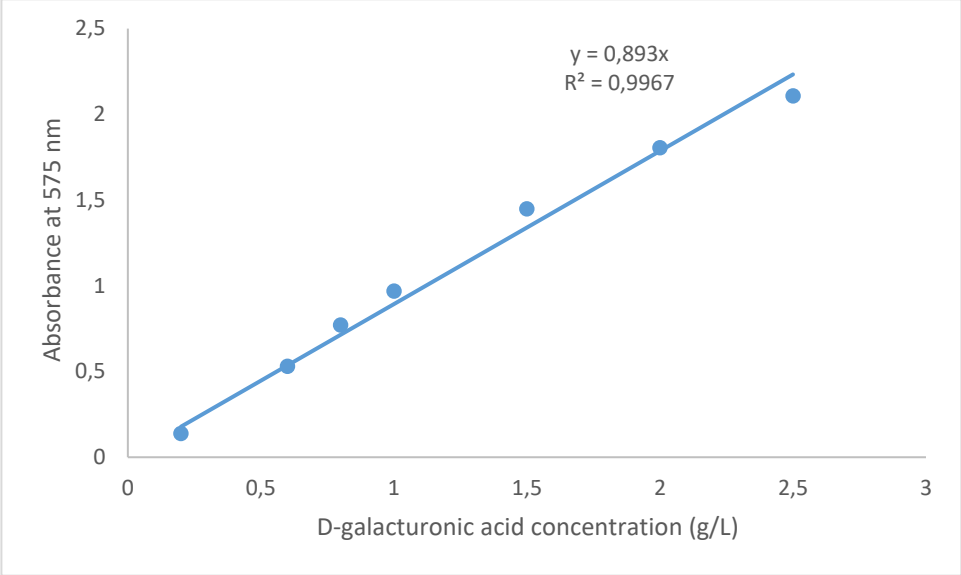

Figure D.1 The standard curve for pectinase by DNS method.

Pectinase activity was measured by following equation;

$$\frac{U}{l} = C * \left(\frac{1}{\text{incubation time}}\right) * \left(\frac{1}{212.12}\right) \quad (1)$$

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

$\mathbf{F} = conversion\ factor\ \ from\ absorbance\ to\ galacturonic\ acid\ (g)\ by\ standard\ curve$

$\boldsymbol{Incubation\ time} = 30\ minutes$

$\frac{\mathbf{1}}{\mathbf{212.12}}$ = conversion from *grams* to moles of galacturonic acid

APPENDIX E

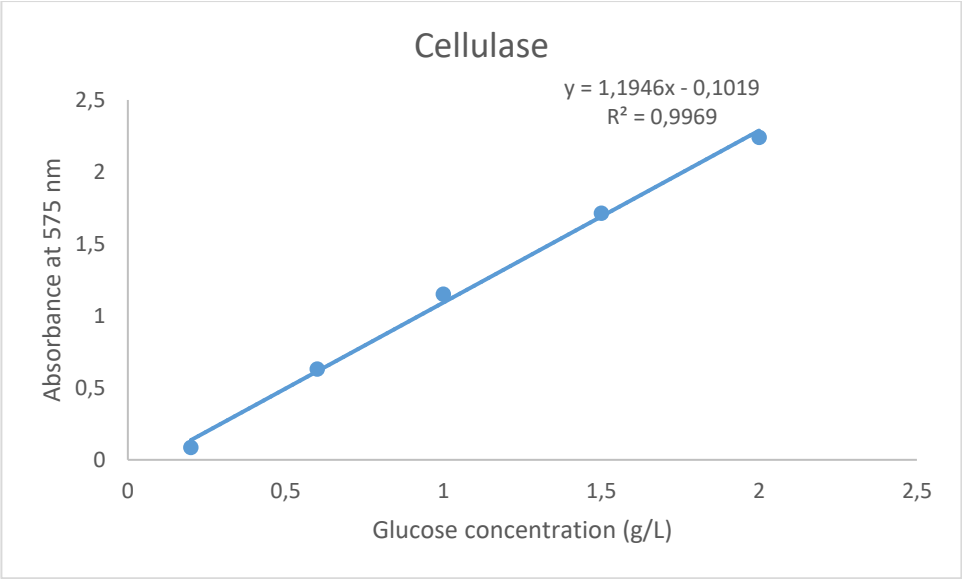

Figure E.1 The standard curve for cellulase by DNS method.

Cellulase activity was expressed as below;

$$\frac{\mathrm{U}}{\mathrm{mL}} = \mathrm{C} * \left(\frac{1}{\text{incubation time}}\right) * \left(\frac{1}{0.18}\right) \quad (2)$$

$$\mathrm{C} = \left((\text{Absorbance} * \mathrm{F})_{\text{sample}} - (\text{Absorbance} * \mathrm{F})_{\text{substrate}}\right) * \text{Dilution factor} - (\text{Absorbance} * \mathrm{F})_{\text{enzyme blank}}$$

$\mathbf{F} = conversion\ factor\ \ from\ absorbance\ to\ glucose\ by\ standard\ curve$

$\boldsymbol{Incubation\ time} = 60\ min$

$\frac{\mathbf{1}}{\mathbf{0.18}}$ = conversion from $rams$ to moles of glucose

APPENDIX F

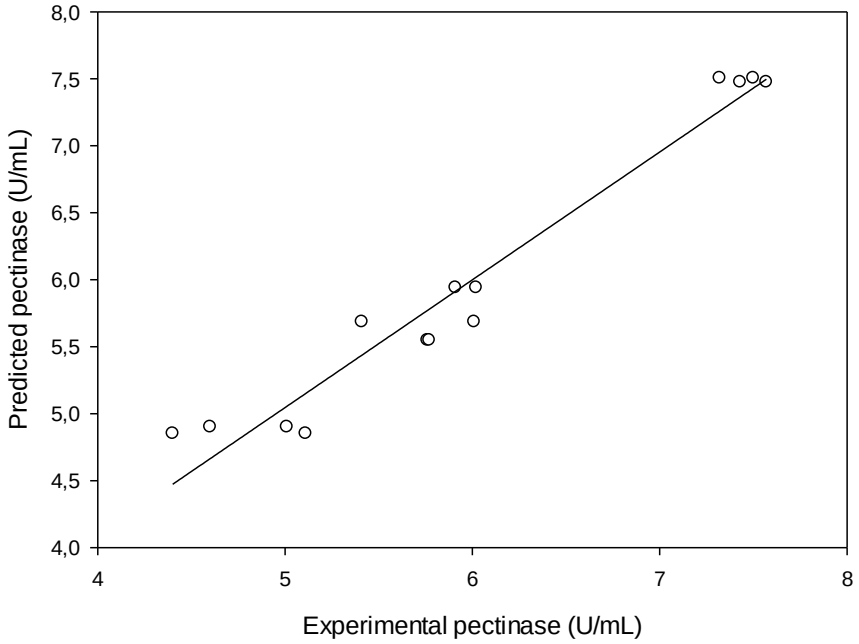

Figure F.1 Predicted vs. experimental pectinase activities (U/mL).

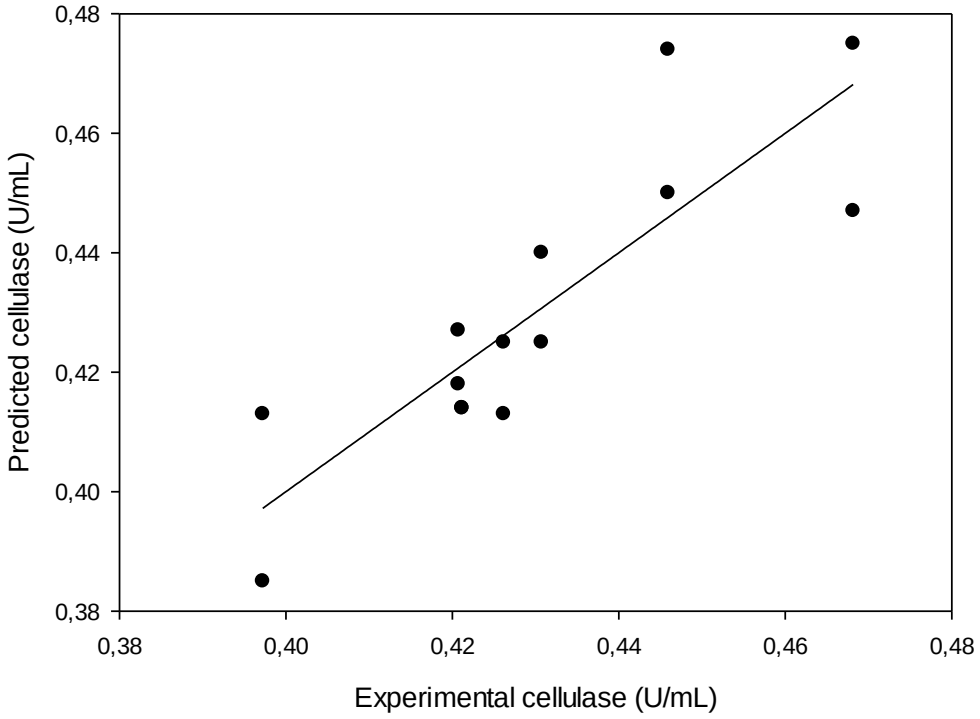

Figure F.2 Predicted vs. experimental cellulase activities (U/mL).

APPENDIX G

Table G.1 ANOVA analysis for pectinase.

Source	**Coefficient**	**P**
Regression		0.000
Linear		0.002
Quadratic		0.001
Haz*Or	145.0	0.040
Haz*Ap	-421.2	0.000
Or*Ap	91.8	0.159

Table G.2 ANOVA analysis for cellulase.

Source	**Coefficient**	**P**
Regression		0.031
Linear		0.056
Quadratic		0.026
Haz*Or	9.602	0.019
Haz*Ap	-5.998	0.104
Or*Ap	3.077	0.374