CRUDE PECTINOLYTIC ENZYMES PRODUCTION IN FED-BATCH SHAKE FLASK CULTIVATION

A Thesis Submitted to
the Graduate School of İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
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May, 2023
İZMİR

ACKNOWLEDGMENTS

First of all, I would like to thank my esteemed advisor, Assoc.Prof.Dr. Sibel Uzuner. Her influence on my personal and academic development is enormous.

I would also like to thank Assoc.Prof.Dr. Hümeyra Taşkent Sezgin. She read my studies with great devotion and interest, and her contributions supported my academic development.

"Basic Principles of Bioprocess Engineering" is one of the postgraduate courses taught by Assoc.Prof.Dr. Ali Oğuz Büyükkileci, laid the foundation for my bioprocess studies and became one of the lines determining my academic framework. I would like to thank Assoc.Prof.Dr. Ali Oğuz Büyükkileci.

Prof. Dr. Yusuf Baran and Prof. Dr. Onur Güneşer are two scientists who motivated me during my graduate education. They have always stood by me with their motivating words and I owe my success to these two names.

I would like to thank Assoc.Prof.Dr. Sırma Yeğin. She contributed a lot to my thesis and my academic perspective. She shared important information for my further studies and thesis.

I would like to thank ABDİBİO Biotechnology Facility and Biotechnology and Bioengineering Application, Research Center (BİYOMER) and IZTECH Council of Scientific Research (2022IYTE-1-0005) for funding and support during my thesis work.

I would also like to thank my close friends, whom I call "Caniko", who were with me mentally during my thesis work.

Finally, I would like to thank my sisters Ayşegül Esen Çetin and Sümeyye Esen, my parents Bekir - Ayşe Esen for their support.

ABSTRACT

CRUDE PECTINOLYTIC ENZYMES PRODUCTION IN FED-BATCH SHAKE FLASK CULTIVATION

The use of waste in the production of enzymes, which is one of the products with high added value, is one of the right strategies to reduce the production cost of the product and sustainability movement.

In this study, the production of polygalacturonase (PGase) and pectin lyase (PLase) enzymes from *Bacillus subtilis* ATCC 6633 in fed batch submerged fermentation, the conditions and composition of the fermentation medium and the effects of pretreatment methods (thermal, thermo-chemical, microwave assisted dilute acid (MW-DA)) on the conversion of fermentable sugar from black carrot pulp were investigated.

The MW-DA was chosen the best with higher fermentable sugar content (FSC). The three different powers (300, 600, 850 W) and 3 different treatment time (30, 60, 90 s) were examined by Taguchi design. The highest FSC was found at 300 Watt for 30 seconds. MW-DA followed by ES produced the most fermentable sugar (0.493 g/g, 87.3% conversion). The amount of fermentable sugar was enhanced from 15.8% to 87.3% when MW-DA treatment is combined with enzymatic saccharification (ES). Yeast extract, whey and pea protein were examined as nitrogen sources. According to the enzyme activity results obtained, the fermentation medium was modified with pea protein. Certain concentrations (2.5%, 5%, 10%, 15%) were fed to the fermentation medium. The highest PGase activity was determined at the 15% feed concentration and 72^{th} hours (164.34±2.26 U/L) whereas the highest PLase activity was obtained at 72^{th} hours (188.22±1.72 U/L) at 5% feed concentration.

Keywords: *Polygalacturonase, Pectin Lyase, Bioconversion, Waste Management, Fed Batch Fermentation, Microwave Pretreatment*

ÖZET

BESLEMELİ-KESİKLİ ÇALKALAMALI SİSTEMDE HAM PEKTİNOLİTİK ENZİMLERİN ÜRETİMİ

Katma değeri yüksek ürünlerden biri olan enzimlerin üretiminde atıkların kullanılması, ürünün üretim maliyetinin düşürülmesi ve sürdürülebilirlik hareketi için doğru stratejilerden biridir.

Bu çalışmada, *Bacillus subtilis* ATCC 6633'ten poligalakturonaz ve pektinliyaz enzimlerinin kesikli beslemeli batık fermantasyonda üretimi ve fermantasyon ortamının koşulları, bileşimi ve uygulanan önişlem yöntemlerinin (termal, termo-kimyasal, mikrodalga destekli seyreltik asitli) siyah havuç posasından indirgen şekere dönüşümü üzerine etkileri incelenmiştir.

Mikrodalga destekli seyreltik asit yöntemi fermente edilebilir şeker (FEŞ) miktarında etkili sonuçlar vermiştir. Taguchi dizaynında 3 farklı güç (300, 600, 850 W) ve 3 farklı süre (30, 60, 90 s) seçilerek tasarlanmıştır. Dizayn sonucu en iyi indirgen şeker miktarını veren güç ve süre 300 Watt ve 30 saniyedir. MW-DA ve ardından ES, en fazla fermente edilebilen şekeri üretmiştir (0.493 g/g, %87.3 dönüşüm). MW-DA işlemi enzimatik sakarifikasyon (ES) ile birleştirildiğinde FSC %15,8'den %87,3'e yükselmiştir. Isıl ön işlemine kıyasla, MW ön işlemi daha az enerji kullanmıştır. Kokteyl enzimlerinin üretiminde protein kaynağı olarak maya ekstraktı, peynir altı suyu proteini ve bezelye proteini denenmiştir. Elde edilen enzim aktivite sonuçlarına göre fermantasyon ortamı bezelye proteini kullanılarak modifiye edilmiştir. Belirli konsantrasyonlarda (%2.5, %5, %10, %15) fermantasyon ortamı substrat ile beslenmiştir. Yapılan çalışmalar sonucunda en yüksek poligalakturonaz (PGaz) aktivitesi %15'lik besleme konsantrasyonunda 72. saatte (164.34±2.26 U/L) elde edilmiştir. En yüksek pektinliyaz (PLaz) aktivitesi, %5 besleme konsantrasyonunda 72. saatte (188.22±1.72 U/L) elde edilmiştir. Taze substrat beslemesi enzim aktivitesini ve fermentasyon süresini arttırarak daha çok ürün elde edilmesini sağlamıştır.

Anahtar Kelimeler: *Poligalakturonaz, Pektinliyaz, Biyodönüşüm, Atık yönetimi, Kesikli Besleme Fermantasyon, Mikrodalga Önişleme*

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LIST OF ABBREVIATIONS

BCP	Black carrot pomace
DNS	3,5-dinitrosalicylic acid
ES	Enzymatic saccharification
FSC	Fermentable sugar content
g	Gram
h	Hour
HMF	Hydroxymethylfurfural
HPLC	High pressure liquid chromatography
J	Joule
kDa	Kilodalton
kJ	Kilojoule
L	Liter
min	Minute
mL	Milliliter
MW-DA	Microwave assisted diluted acid
PGase	Polygalacturonase
PLase	Pectinlyase
s	Second
SmF	Submerged fermentation
SSF	Solid state fermentation
T	Thermal
TC	Thermo-chemical
U	Unit

CHAPTER 1

INTRODUCTION

Due to the gradual development of enzyme technology, the diversity of usage areas and the high economic value of products, various researchers in the field of industrial enzymes in biotechnology are gaining even more importance (Kıran et al., 2006). On the other hand, the development of the bioeconomy is supported by the use of enzymes and industrial biotechnology, as the bioeconomy encompasses the production and use of renewable resources to make value-added products, processes and services (OECD, 2018).

Enzymes constitute an 8 billion dollar industry in the world. The five big companies control the enzyme market on a global scale. Turkey, on the other hand, imports about 150 million dollars of industrial enzymes every year. Today, it is seen that commercial enzymes are insufficient to meet the industrial needs. It is estimated that the enzyme market value will be 7 billion dollars in 2023 (Liu and Smith, 2020). As the enzyme market grows globally, the research of new enzymes and the development of new low-cost fermentation methods will gain importance and rate. Enzymes used in the food and beverage industry constitute the largest portion of the industrial enzyme market, approximately 50%. Enzymes such as pectinase, cellulase and xylanase are used in many areas such as wastewater removal, fruit juice clarification, energy production and the production of chemicals and additives (Ali et al., 2013). Pectinolytic enzymes (consisting of polygalacturonase, pectin lyase and pectin methyl esterase), which make up 25% of food enzyme production, are a group of enzymes that can degrade pectic compounds by different mechanisms (Alkorta et al., 1998). Pectin-degrading enzymes (generally called pectinases) increase fruit juice yield by softening tissues especially during pressing fruit in the food industry (Acar and Gökmen, 2005), facilitating filtration in fruit juice production and increasing efficiency, obtaining galacturonic acid, which is the starting material for vitamin C synthesis, in wine industry. It is used for new applications in the extraction of oils, the preparation of pigments and cellulose fibers, the production of oligosaccharides as functional foodstuffs in coffee and tea fermentation (Patil et al., 2006). In addition, pectinolytic enzymes used for clarification are also used to prevent condensed juices from gelling on the shelf. In addition, it is used to remove the mucilage

sheath, 3⁄4 of which consists of pectin, around the coffee bean, thus helping to reduce the fermentation time (Doğan and Taşkın, 2021).

In bioprocess systems, many factors such as the correct selection of the raw material and microorganism to be used in fermentation, the type of fermentation, and the fermentation environment conditions are effective.

For industrial enzymes, 30-40% of the product cost is the cost of the culture medium. Low cost substrates are used to reduce the media cost. In recent years, the use of various food-agricultural industry wastes as substrates has rapidly become popular in order to reduce the cost of enzyme production. Among them, wheat bran, soybean, sugar beet, potato, sorghum, coffee pulp, wheat and rice stalks, corn, sugarcane pulp, various fruit pulps, wastes from oil processing industries and many different regional wastes remaining from vegetable processing are being tested (Doğan and Taşkın, 2021). Wheat bran, sugar beet pulp and corn husk are among the most researched raw materials. On the other hand, it has been reported that the enzyme produced in liquid fermentation media is easily affected by different carbon and nitrogen sources, regardless of the microorganism used (Hoondal et al., 2002). It is seen that some of the carbon or nitrogen sources mentioned are substances containing small amounts of pectin such as orange peel, wheat bran or beet pulp, while others are lignocellulosic substances without pectin or pure substances such as glucose, cellulose. The most important information from this is that different amounts of enzymes can be produced from different raw materials. However, the use of lignocellulosic biomass as a raw material has some limitations, such as low yield and high hydrolysis process cost, in terms of feasibility and economics.

The main source of industrial enzymes is microorganisms, 50% of them are fungi and yeast, 35% are bacteria, the remaining 15% are plant or animal sources (Anisa and Girish, 2014). The microorganisms of bacterial and fungal origin are used for pectinase enzyme production and many agricultural wastes are investigated as carbon source (Table 1 and Table 2). Pectinases (enzymes that break down pectin) play an important role in the food industry, especially during the pressing of fruit juices, by softening the tissues, reducing the viscosity, increasing the yield of fruit juice, liquefying the pulp, facilitating filtration and clarification. (Kashyap et al., 2001; Kareem and Adebowale, 2007; Chaudhri and Suneetha, 2012; Makky and Yusoff, 2015). Various processes are applied to different products such as softening of fruits and vegetables, pureeing and paste (Sreenath et al., 1994; Demir et al., 2000; Tochi et al., 2009). The pectin lyase enzyme provides juice clarification without destroying the volatile ester components that give the

fruit juice a specific fruit smell (Targano and Pelosof, 1994). On the other hand, there is very limited information in the literature about bacterial pectin lyase production (Demir et al., 2011, 2014; Gopinanth and Suneetha, 2012; Kaur and Gupta, 2017).

Table 1. Different carbon sources used for pectinolytic enzymes production by bacterial species (Source: Samreen et al., 2019)

Microorganism	Substrate	Product
Aeromonas salmonicida	-	Pectinase
Bacillus sp.	Wheat bran	Pectinase
Bacillus pumulis	-	Thermostable pectinase
Bacillus subtilis	Wheat bran	Thermostable pectinase
Bacillus subtilis	Wheat bran	Alkaline pectinase
Bacillus subtilis	Pectin	Pectinase
Bacillus sphaericus	Citrus pectin	Polygalacturonase
Bacillus subtilis	Date syrup	Pectinase
Bacillus sp.	Cassava waste	Pectinase
Bacillus firmus	Pectin	Pectinase
Erwinia cartonora	Pectin	Pectinase
Bacillus pumulis	Agricultural waste	Exo-pectinase
Bacillus licheniformis	Apple pectin	Pectinase
Bacillus subtilis	Hazelnut shell hydrolysate	Pectinase
Bacillus subtilis	Pectin	Pectinase
Bacillus pumulis	Citrus limetta peel	Pectinase
Bacillus licheniformis	Pectin	Polygalacturonase
Bacillus pumulis	Wheat bran	Pectinase
Bacillus lsonorensis	Pectin	Pectinase
Chryseobacterium indologenes	Citrus pectin	Pectinase

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cont. of Table 1.

Entereobacter tabaci	-	Pectinase
Bacillus subtilis	Pectin	Pectinase

Table 2. Different carbon sources used for pectinolytic enzymes production by fungal species (Source: Samreen et al., 2019)

Microorganism	Substrate	Product
Aspergillus niger	Apple	Pectinase
Aspergillus niger	Citrus	Pectin methyl esterase
Penicillium occitanis	Citrus pectin	Pectinase
Penicillium occitanis	Citrus pectin	Pectin esterase
Penicillium frequentans	Wheat bran	Pectinase
Aspergillus foetidus	Soy and wheat bran	Pectinase
Aspergillus niger	-	Ectopectinase
Aspergillus niger	Wheat	Pectinase
Penicillium viridicatum	Orange bagasse and wheat bran	Pectinase
Penicillium viridicatum	Wheat bran	Pectinase
Thermoascus aurantiacus	Wheat bran	Pectinase
Thermoascus aurantiacus	Sugar bagaesse	Pectinase
Thermoascus aurantiacus	Orange waste	Pectinase
Aspergillus niger	Citrus peel	Pectinase
Aspergillus niger	Sugar beet pulp	Pectinase
Aspergillus awamori	Grape pomace	Pectinase
Penicillium decumbens	Wheat bran	Pectinase
Aspergillus fumigates	What flour	Pectinase
Aspergillus heteromorphus	Orange peel	Pectin methyl esterase
Fomes sclerodermeus	Soy and wheat bran	Polygalacturonase

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cont. of Table 2.

Mucor circinelloides	Pectin methyl ester	Polygalacturonase
Penicillium chrysogenum	Sucrose	Pectinase
Aspergillus foetidus	Mango peel	Pectinase
Rhizomucor	Pectin	Pectinase
Rhodotorula glutinis	Citrus pectin	Pectinase
Aspergillus foetidus	Mango peel	Pectinase
Aspergillus oryzae	Lemon peel	Pectinlyase
Aspergillus sojae	Wheat bran	Polygalacturonase
Trichoderma viridae	Orange peel	Pectinase
Aspergillus niger	Banana peel	Pectinase
Aspergillus niger	Orange waste peel	Pectinase
Aspergillus oryzae	Citrus waste	Pectinase
Aspergillus terreus	Banana peel	Pectinase
Aspergillus niger	Orange pomace	Pectinase
Aspergillus niger	Orange peel	Pectinase

Bacteria are widely used in enzyme production. Bacterial enzymes have some advantages such as faster growth in submerged culture fermentation compared to the enzymes of mold origin. The fact that *Bacillus* species is easier to adapt to changes in growth conditions enables better enzyme production (Jahan et al., 2017). *B.subtilis* and *B.licheniformis* from *Bacillus* species producing polygalacturonase enzyme meet 50% of the total enzyme production (Schallmet et al., 2004). The production potential of pectinase group enzymes polygalacturonase (Uzuner and Cekmecelioglu, 2015) and pectin lyase (Kaur and Gupta, 2017) from agricultural wastes using *B.subtilis* has been demonstrated in the literature.

With the co-production of enzymes, enzyme cocktails are synergistically advantageous in the degradation of multiple carbohydrate polymers such as cellulose, hemicellulose and pectin. On the other hand, information about the simultaneous production of two different pectinolytic enzymes has found a limited number of research

opportunities in the literature. By producing multiple (cocktail) enzymes and using the plant cell during hydrolysis, a more effective degradation efficiency and lower cost enzyme usage will be in question. However, the production of polygalacturonase and pectin lyase from food wastes such as black carrot pomace by simultaneous fed-batch deep culture fermentation has not been found in the literature.

Some operational feeding strategies such as batch, fed-batch have been developed to increase fermentation efficiency. Fed-batch fermentation has some advantages over continuous and batch fermentation systems. Since it does not have sufficient nutrient medium at the beginning of fermentation, fed batch fermentation strategies have positive effects on the growth of the microorganism (Astolfi et al., 2011). This cultivation strategy is advantageous for various microorganisms such as *Escherichia coli* (Lee, 1996), *Saccharomyces cerevisiae* (George et al., 1993), *B. subtilis* (Öztürk et al., 2016) and *Pichia pastoris* (Looser et al., 2014). The fed batch fermentation strategy has generally been carried out in bioreactors for enzyme production (Zou et al., 2014; El Enshasy et al., 2018). In a study, the gene of *Aspergillus kawachii* mold was transferred into *S. cerevisiae* and polygalacturonase enzyme production was carried out in batch and semi-batch culture medium (Rojas et al., 2010). The semi-batch fermentation system used in this study was carried out in a large-scale bioreactor system. Studies on enzyme production using the fed-batch flask system are limited in the literature. The fed-batch strategy was used for the production of mannanase enzyme using *Penicillium occitanis* and it was reported that the enzyme yield was 10 times higher than the batch shake system (Blibech et al., 2011). In addition, the design of the fed-batch shaking flask system will be developed in this project, since the installation costs of the equipment providing process control in the bioreactor are high and the high efficiency in the fed-batch shaking flask system compared to the bioreactor system.

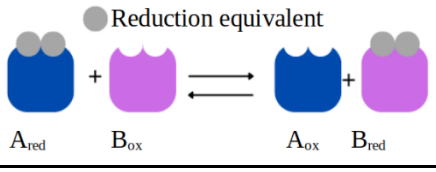
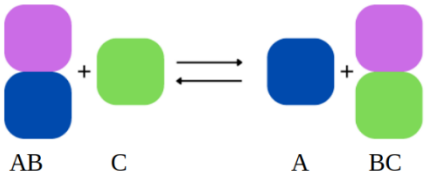
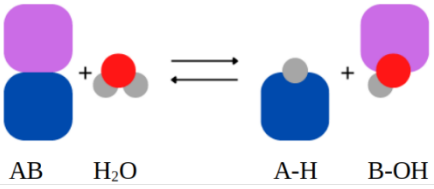
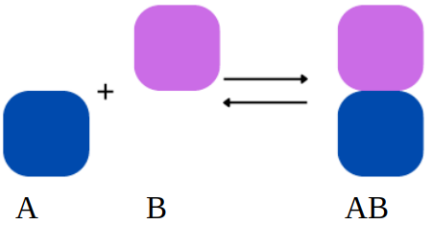
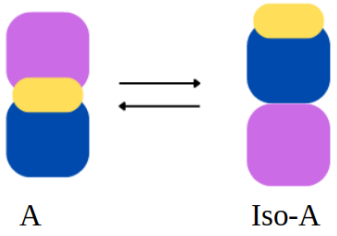
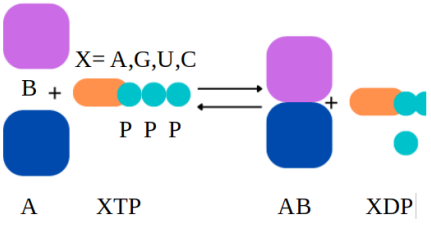
1.1 Enzymes

Enzymes are organic substances that act in the cell (in vivo) and outside the cell (in vitro) conditions without undergoing any changes, and that catalyze biochemical reactions in the protein structure (Aehle, 2007).

Enzymes are catalysts of organisms. All of the chemical transformations in the organism, called metabolism, occur with the presence of enzymes. Substances transformed by the enzyme are called substrates. According to a 1961 report by the

Enzyme Commission, enzymes are classified into six categories based on the sort of reaction they catalyze. These are; oxidoreductases, transferases, hydrolases, lyases, isomerases, lygases (Aehle, 2007) (Table 3).

Table 3. Classification of Enzyme

Name	Explain	Mechanism
Oxidoreductases	This group enzymes is generally used redox reactoion.	
Transferases	This group enzymes is transferred to specific group for example; phosphate, acyl, methyl etc. substances.	
Hydrolases	This group of enzymes breaks the bond formed by carbon with oxygen, nitrogen, or another carbon with the help of water.	
Lyases	This group of enzymes breaks the bond of carbon with oxygen, nitrogen and other carbon atoms and causes double bonds to form.	
Isomerases	This group of enzymes provides the reorder of geometry and structure in a compound.	
Lygases	This group of enzymes functions in the reaction of two molecules joined by the breakdown of a P-O-P bond or ATP in a triphosphate nucleotide with water.	

1.2 Pectinolytic (Pectinase) Enzymes

Pectolytic enzymes are a group of enzymes that are capable of breaking down pectin, a complex carbohydrate found in the cell walls of plants (Barman, 1969). Two common types of pectolytic enzymes are polygalacturonase (PGase) and pectinlyase (PLase).

Pectin esterases, depolymerizing enzymes (hydrolases, lyases), and protopectinases are the three types of pectolytic enzymes. Additionally, depending on the reaction type and substrate they affect, such as: (1) the preferred substrate being pectin, pectic acid, or oligo D-galacturonate; They can also be divided into groups based on how they function, such as whether they (1) work by trans elimination or hydrolysis, (2) liquefy or depolymerize molecules, or (3) cleave randomly or end-directedly (exo- or conjugate enzymes). To create soluble pectin, protopectinases break down protopectin. Polygalacturonic acid is produced when methoxyl and acetyl residues from pectin are removed by esterase (pectin methyl esterases and pectin acetyl esterases). According to whether transelimination (pectin lyases and pectate lyases) or hydrolysis (polygalacturonases) are at the terminal end of the random or exo-motion pattern, depolymerases catalyze glycosidic -(1-4) bonds between galacturonic residues (Alkorta et al., 1998; Shet et al., 2018; Anand et al., 2020).

PGase breaks down pectin by hydrolyzing the alpha-1,4-galacturonosyl linkages that hold the pectin molecules together. This results in the degradation of pectin and the softening of fruits and vegetables (Barman, 1969). PGase enzymes are commonly used in the food industry to soften fruits and vegetables, as well as to improve juice and wine production.

PLase is another type of pectolytic enzyme that breaks down pectin by hydrolyzing the alpha-1,4-galacturonosyl linkages and alpha-1,2-rhamnosyl linkages. PLase enzymes have been found to have a higher degree of specificity than PGase, breaking down the pectin in a more controlled manner. (Barman,1969)

Both PGase and PLase enzymes are commonly used in food industry, especially in the juice and wine production, as well as in the processing of fruits and vegetables. They are also used in other industries.

According to Tapre and Jain (2014), the processing of mashes, juice extraction, and clarity are necessary for pectinase application in the fruit and vegetable business. Pectinases help to reduce viscosity, clarify juice, and macerate vegetables in addition to

reducing the duration of fermentation (Ramadan, 2019). During the processing of citrus juice, this enzyme also helps to remove turbidity and stabilize fruit juice (Sandri et al., 2011). To clear juices, pectinases are mixed with other enzymes such amylases, cellulases, and hemicellulases (Sharma et al., 2018).

Pectinases are widely used in the winemaking process to enhance wine quality, promote the extraction process that provides filtration, and ultimately enhance color and aroma (Mojsov et al., 2015). Wines treated with enzymes require less time for filtration than wines treated using conventional techniques (Jayani et al., 2005).

It is common practice to extract various vegetable oils from rapeseed (canola), coconut, sunflower, palm, kernel, and olive using organic solvents like hexane, which has the potential to cause cancer (Anand et al., 2020). Pectinases are alkaline by nature, therefore using them to process oil makes it easier to extract oil in an aqueous phase. Higher stability and organoleptic (polyphenolic and vitamin E) content increase oil production (Kashyap et al., 2001).

Due to the usage of chlorine-containing bleaching agents that seriously harm the environment, the use of different enzymes such pectinases, xylanases, and ligninases are growing in the paper and pulp sectors (Bajpai, 2018). According to Viikari et al. (2001), the presence of pectinases causes the galacturonic acid polymers to depolymerize, which lowers the cationic demand of pectin solutions and peroxide bleaching permeate and eliminates retention issues in pulp bleaching.

De-inking, which removes ink particles from the fiber surface and separates them from the fiber slurry by washing and flotation processes, is a step in the recycling of waste paper from printing machines (Lasheva et al., 2013). De-ink removal by enzymes is urgently needed since traditional de-inking procedures utilize significant amounts of environmentally risky chemicals. Due to its great efficiency, ability to save energy, and ability to be environmentally benign, enzymatic de-inking (pectinases, hemicellulases, cellulases, and lignolytics) has a wide range of uses.

The textile industry now uses fewer chemicals and does less environmental harm thanks to the employment of enzymes like pectinases and other hemicellulosic enzymes (amylases, lipases, cellulase , etc.) to remove unwanted compounds.

The use of pectinase and its derivatives as prebiotic/functional foods has gained interest recently, and it has been hypothesized that the enzymatic production of pectin and pectin-derived oligosaccharides will strengthen the host immune system (Anand et al., 2020).

1.3 Production of Enzymes

1.3.1 Submerged Fermentation

Submerged fermentation (SmF) is a type of fermentation process in which microorganisms are grown in a liquid medium that is completely submerged in the culture vessel. This method of fermentation is widely used in industrial biotechnology for the production of various products including enzymes, antibiotics, amino acids, and organic acids. The microorganisms used in SmF can be bacteria, yeasts, or fungi, depending on the desired end product.

One of the key advantages of SmF is that it allows for a high cell density and high productivity, as the microorganisms are able to access all the nutrients in the liquid medium. Additionally, SmF is easy to scale up, as the process can be easily controlled and monitored. Furthermore, it is relatively simple and inexpensive to implement when compared to other fermentation techniques, such as solid-state fermentation (Martinez Medina et al., 2019)

However, there are also some disadvantages associated with SmF. One of the main drawbacks is that oxygen transfer can be a limiting factor, as the microorganisms are submerged in the liquid medium, which can lead to low oxygen levels and reduced growth and productivity. (Hixson and Gaden, 1950) To overcome this limitation, various aeration and agitation strategies have been developed such as sparging, bubbling, and mechanical agitation.

Another important aspect of SmF is the medium composition, which plays a vital role in the growth and productivity of the microorganisms. The medium must contain all the necessary nutrients such as carbon, nitrogen, and minerals as well as vitamins and trace elements to support the growth of the microorganisms (Lekha and Losane, 1997).

1.3.2 Solid State Fermentation

Solid state fermentation (SSF) is a microbial cultivation technique that involves the use of a solid substrate without or with limited moisture content to produce various metabolites, including enzymes. Enzymes produced through SSF have numerous applications in various fields, such as food processing, textile, paper, and pharmaceutical industries (Rodriquez-Couto and Sanroman, 2006).

SSF has several advantages over liquid-state fermentation, including low-cost production, low energy requirements, and reduced wastewater production. One of the major advantages of SSF is its ability to produce high enzyme titers due to the high substrate-to-microorganism ratio. Moreover, SSF has a low risk of contamination, as the solid substrate provides a protective barrier against microbial contamination (Davies and Demain, 1999).

Substrate type, moisture content, pH, temperature, and incubation time are several factors that affect enzyme production in SSF. The optimization of these factors can lead to the production of high-quality enzymes with desirable characteristics (Durand, 2003).

1.3.3 Carbon Source

Carbon source is one of the important elements in bioprocess systems. Microorganisms need energy for many biochemical reactions, especially growth. The carbon source in the medium is should meet the need. High sugar content, ease of use and cheapness are the features sought in a suitable carbon source for fermentation processes. In Table 4, some carbon sources and microorganisms used for the production of pectinase group enzymes studied in the literature are represented.

Table 4. Carbon sources used in pectinase production

Carbon Source	Microorganism	Enzymes	References
Algal biomass	*Bacillus licheniformis*	Pectinase	Pervez et al. (2017)
Sunflower head	*Aspergillus niger*	Pectinase	Patidar et al. (2016)
Wheat bran	*Aspergillus giganteus, Aspergillus sojae.*	Polygalacturonase	Demir and Tari, (2014), Heerd et al., (2012), Anand et al. (2017), Ortiz et al., (2017)
Rice husk and rice bran	*Aspergillus fumigatus*	Polygalacturonase	Wong et al., (2017), Tai et al., (2014)
Orange Peel	*Bacillus stearothermophilus, Bacillus cereus*	Pectinase	Torimiro and Okonji (2013)

1.3.3.1 Black Carrot Pomace

Black carrots are a type of vegetable originating from Central Asia, whose origin dates back 3000 years. Today, their cultivation and consumption are mostly in eastern countries such as Turkey, Far East, Pakistan and India (Sucheta et al., 2020). The global production of carrots (including black carrots) has been gradually increasing over the years, according to the FAO. With a growth rate of 10.3%, production climbed from 40.2 million tons in 2015 to 44.8 million tons in 2019 (FAOSTAT, 2019). Although black carrots are mainly used as a food coloring, they are a rich source of anthocyanin and pectin. Turkey is the world's leading black carrot producer today. Their production is increasing every year on the basis of tonnage due to the increasing demand in the natural food colorant industry and the functional food industry due to their rich bioactive content. Between the years 2005-2010 in Turkey, the production yield increased from 18,000 tons to 46,000 tons. In 2013, 14,000 tons of black carrot concentrate, with a dry matter content of approximately 60%, was produced in Turkey and exported as a natural food colorant to countries such as Italy, Denmark, France, Japan and China (Meyed, 2011; Kumar et al., 2019).

1.3.3.2 Black Carrot Pomace Composition

Black carrot pomace (BCP) is a byproduct of the juice and food industry, obtained after the extraction of juice from black carrots. It is rich in various bioactive compounds such as anthocyanins, flavonoids, and phenolic acids. Sharma et al. (2018), found to be the total phenolic and anthocyanin content of BCP approximately 69.5 mg GAE/g dry weight and 34.5 mg/g dry weight, respectively. In another study, El-Ghonemy et al. (2019), found that BCP contains high levels of antioxidants, including beta-carotene and lutein. The study also reported that pomace is a good source of dietary fiber, with a total dietary fiber content of around 21.3%.

Black carrot pomace is a rich source of bioactive compounds and has high antioxidant and dietary fiber content, making it a valuable ingredient for functional food and nutraceutical applications. The pomace of carrots contains a lot of sugar. Carrot pomace contains a range of biopolymers, including cellulose (10-28%), hemicellulose (5-20%), and pectin (2-8%), according to a study by Andres et al. (2022). It is also claimed

that the other primary ingredients of carrot pomace are free sugars from carrots, such as sucrose (14.3-47.2%), glucose (7.9-30.44%), and fructose (5.4-14.2%).

1.3.3.3 Usage of Black Carrot Pomace

Black carrot pomace is a by-product of the juice and food industry, obtained after the extraction of juice from black carrots. Due to its high antioxidant and dietary fiber content, it has potential uses in various industries, such as food, cosmetics, and nutraceuticals. In the food industry, black carrot pomace can be used as a natural colorant and source of antioxidants in various food products such as cakes, jams, jellies, and ice cream. The black carrot pomace can be used as a natural colorant in the food industry due to its high anthocyanin content (Sharma et al., 2018). In the cosmetic industry, the high antioxidant content of BCP makes it a potential ingredient in skin care products, such as creams, lotions, and serums. Park et al. (2019), found that a cream containing BCP extract improved the skin's moisture level and reduced wrinkles. In the nutraceutical industry, black carrot pomace can be used as a dietary supplement due to its high antioxidant and dietary fiber content. Also Park et al.(2019), found that BCP has a high antioxidant activity, making it a potential ingredient in dietary supplements.

1.3.4 Nitrogen Source

Microorganisms need a nitrogen source to grow and produce products, so nitrogen source is very important in bioprocess systems. In the literature, many organic and inorganic nitrogen sources have been examined in the fermentation medium for enzyme production (Table 5).

Table 5. Nitrogen sources used in pectinase group enzyme production

Nitrogen Source	Microorganism	Enzyme	References
Casein, peptone, yeast extract, urea NH_4Cl, $(NH_4)_2MoO_4$, H_2NaO_4P, KNO_3, $NaNO_3$	*Aspergillus niger*	Exo-polygalacturonase, endo-polygalacturonase, endo-pectinlyase, endo-pectin methyl esterase	Begum and Munjam (2021)
Casein, peptone, yeast extract, urea, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3	*Bacillus subtilis*	Pectinase	Oumer and Abate (2018)

Pea is one of the most important leguminous crops over the world. Pea protein is a relatively new type of plant proteins and it becomes more and more popular due to its availability and low cost. It has been used as a functional ingredient in global food industry. Pea contains 20-25 % protein and includes for major classes (globulin, albumin, prolamin, glutelin), in which globulin and albumin are major storage proteins in pea seeds (Lu et al., 2019) (Table 6).

Table 6. Classification of pea protein (Source: Lu et al., 2019)

Class	Content (%)	Protein
Globulin	55-65	Legumin, vicilin, convicilin
Albumin	18-25	Albumin
Prolamin	4-5	Prolamin
Glutelin	3-4	Glutelin

1.3.5 Cultivation Type

1.3.5.1 Batch Type Cultivation

Batch type cultivation is a type of cultivation process in which microorganisms are grown in a closed system for a specific period of time, known as the cultivation period. The cultivation process is carried out in a single batch and once the cultivation period is complete, the culture is harvested and the process is terminated (Ingledew and Lin, 2011).

Batch cultivation is the most commonly used cultivation method in industrial biotechnology for the production of various products such as enzymes, antibiotics, amino acids, and organic acids. The process is relatively simple and inexpensive to implement when compared to other cultivation methods such as continuous cultivation (Ingledew and Lin, 2011).

One of the key advantages of batch cultivation is that it allows for easy control of the cultivation conditions such as temperature, pH, and nutrient levels. Additionally, the process can be easily scaled up by increasing the volume of the culture vessel (Stanbury et al., 2016).

However, there are also some disadvantages associated with batch cultivation. One of the main drawbacks is that it is not as efficient as continuous cultivation, as the

microorganisms are grown in a closed system and cannot be replenished with fresh nutrients. Additionally, the process can be time-consuming and costly, as the culture must be harvested and the process terminated after each batch (Stanbury et al., 2016).

1.3.5.2 Fed-Batch Type Cultivation

Fed-batch cultivation, also known as fed-batch fermentation, is a type of cultivation process in which the growth of microorganisms is controlled by the addition of nutrients over time. The cultivation process is carried out in a closed system and the nutrient addition is performed in a controlled manner to ensure optimal growth conditions for the microorganisms (Bengelsdorf et. al, 2018).

Fed-batch cultivation is commonly used in the industrial production of various products such as enzymes, antibiotics, and amino acids. This method offers several advantages compared to other cultivation methods such as batch cultivation and continuous cultivation. For example, fed-batch cultivation allows for better control over the cultivation conditions, including temperature, pH, and nutrient levels, and results in higher productivity and yields compared to batch cultivation (Amarasekara, 2014). Another advantage of fed-batch cultivation is that it allows for the control of growth rate, enabling the cultivation process to be optimized for specific products. Additionally, the method is less wasteful than continuous cultivation, as the nutrient addition can be controlled to ensure that only the necessary amount of nutrients are added, reducing the amount of waste generated (Pang et al., 2022).

1.3.5.3 Continuos Type Cultivation

Continuous type cultivation refers to a process of fermenting a substrate in a continuous manner over an extended period of time. This type of cultivation is often used in industrial applications for the production of various products, including ethanol, organic acids, and enzymes. Continuous cultivation has several advantages over batch fermentation, including higher productivity, better process control, and lower operational costs (Li et al., 2014)

Continuous cultivation is based on the principle of maintaining a steady-state culture by continuously adding fresh substrate and removing fermented product. The fermentation process occurs in a bioreactor, which is a closed vessel that provides a

controlled environment for microbial growth and product formation. The bioreactor can be operated in different modes, including chemostat, turbidostat, and perfusion (Matteau et al., 2015).

In a chemostat, the flow rate of the substrate is kept constant, and the microbial population is regulated by adjusting the dilution rate. In a turbidostat, the microbial population is regulated by adjusting the flow rate of the substrate based on the optical density of the culture. In a perfusion mode, a small amount of culture is removed and replaced with fresh medium at regular intervals (Matteau et al., 2015; Mozdzierz et al., 2015).

1.4 Usage of Microorganisms for Pectinase Production

The utilization of microorganisms for the production of pectinlyase and polygalacturonase enzymes has become increasingly important in recent years due to their extensive applications in various industries, including food and beverage processing, biorefining, and agriculture.

Several species of bacteria and fungi have been found to produce pectinlyase and polygalacturonase enzymes Table 7, including:

Table 7. Microorganisms used for Pectinlyase and Polygalacturonase production (Source: Haile and Ayele, 2022)

Enzyme	Microorganism
Pectinlyase	*A. niger*
	A. flavus
	A. japonicus
	B. subtilis
	R. arrhizus
Polygalacturonase	*P. expansum*
	A. niger
	B. subtilis
	R. stolonifer

1.4.1 *Bacillus* species

Bacillus species are well-known microorganisms that have been widely used for the production of various enzymes, including pectinlyase and polygalacturonase (Table 8).

Bacillus species constitute a total of 50% of the enzyme market. Selected strains of *Bacillus* that produce and secrete large amounts of extracellular enzymes (20-25 g/L) have placed them among the most important industrial enzyme producers (Kavuthodi and Sebastian, 2018).

Bacillus species are commonly used bacteria for various reasons in the field of industrial microbiology. The reasons use of this group; they are enviromental friendly, easy to grow and their nutritional requirements are not too high. Furthermore, several characteristic of *Bacillus* strains have been reported that make them superior in industrial biotechnology, including their high growth rate leading to short fermentation cycle times, their ability to secrete proteins into the extracellular envrioment, and their abilitiy to adapt to changing enviromental and nutritional conditions. Also, many researchers have used *Bacillus* species because this genus includes strains that can grow on inexpensive substrates such as agricultural waste (Kavuthodi and Sebastian, 2018).

Bacillus species as *B. subtilis* and *B. licheniformis* have the Food and Drug Administration's GRAS (generally recognized as safe) status (Kavuthodi and Sebastian, 2018).

Bacillus subtilis (Figure 1), a well-known species of *Bacillus*, has been reported to produce pectinlyase enzyme. *B. subtilis* is known for its ability to produce a range of enzymes, including proteases, amylases, and cellulases, making it an attractive host for the production of various industrial enzymes (Yin et al., 2019).

In a study by Yin et al. (2019), *B. subtilis* was found to produce high levels of pectinlyase enzyme when grown in a pectin-rich medium. The study also showed that the enzyme produced by *B. subtilis* was highly active and exhibited good stability, making it a promising candidate for industrial applications.

Table 8. List of *Bacillus* species for pectinase production (Source: Kavuthodi and Sebastian, 2018)

Bacillus species	Type of Pectinase	Characteristic	
		pH	T (^{o}C)
B. subtilis	Pectinase	8	50
	Exo-polygalacturonase	5	60
B. subtilis KSM-P358	Exo-polygalacturonase	8	55
B. subtilis EFRL 01	Polygalacturonase	8	45
B. subtilis C4	Polygalacturonase	9	60
B. subtilis BKDS1	Polygalacturonase, Pectinlyase, Pectatelyase	8	40
B. subtilis AD11	Pectinase	8.42	30
B. halodurans M29	Pectinase	10	80
B. subtilis Btk27	Pectinase	7.5	50
Bacillus sp. ZGL14	Pectinase	8.6	50
Bacillus sp. ZJ1407	Pectinase	5	37
B. pumulis	Exo-pectinase	8	30
B. pumulis (NRRL B-212)	Exo-pectinase	8	30
B. licheniformis KIBGE IB-21	Polygalacturonase	7	37
		8-10	45
B. licheniformis SHG 10	Polygalacturonase	8	37.8
B. licheniformis KIBGE IB-3	Polygalacturonase	7	37
B. stearothermophilus	Pectinase	7.5	60
B. cereus	Pectinase	8.5	37
B. cereus	Pectinase	8	50
B. sphaericus (MTCC 7542)	Polygalacturonase	6.8	30
B. clasuii	Pectinlyase	10	60
B. firmus	Polygalacturonase	7	50
B. mojavensis 14	Pectinase	8	60

1.5 Aim of Study

In order to expand the market potential, new biopolymers with high added value should be brought to the market. For this reason, it is important to minimize production costs by developing new production technologies. With this project, agricultural waste is used as a carbon source to develop commercial-targeted fermentation technologies for the production of high value-added products such as cocktail enzymes. In addition to this situation, in order to reduce foreign dependency in the food industry, it is aimed to develop/produce commonly used and generally imported food auxiliaries in a competitive and sustainable way in terms of technology and cost, without posing a risk in terms of food safety. With this project, it is aimed to find solutions to two main problems encountered in enzyme production, such as cost and low efficiency.

The aim is to model the production by producing cocktail pectinolytic enzymes containing polygalacturonase and pectin lyase enzymes with the fed batch fermentation technique instead of the batch fermentation technique used in enzyme production.

The goal of this study was to enhance pectinase blend production using black carrot pomace as a carbon source by batch and fed-batch fermentation and to determine culture conditions for maximal polygalacturonase and pectin lyase production. The objectives supporting our goal are;

1. To analyze potential of black carrot pomace as substrate

2. To discover importance of nitrogen sources during enzyme synthesis

3. To show correlation between fermentable sugar content, biomass, protein content and pectinase production at maximum conditions

The novelties of this study;

1. To introduce a new carbon source to the literature by using black carrot pomace, which is an industrial waste and never used as a carbon source before, in enzyme production.

2. To use of waste peas as a nitrogen source that is not commercially acceptable in size and color and damaged during processing, to investigate of effect of enzyme production and to introduce a new organic nitrogen source to the literature by using pea protein

3. To obtain the highest amount of sugar that can be obtained by spending less energy and time without creating toxic substances with microwave, which is an innovative method and set a optimal conditions of microwave pretreatment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Black Carrot Pomace

The black carrot pomace (BCP) was kindly provided by Goknur Gıda A.Ş in Nigde, a province of Turkey. After drying in a convection oven at 70 °C for 4 h, BCP which is the residue from black carrot concentrate production were stored in plastic bags at room temperature until use.

2.1.2 Microorganism, Growth and Fermentation Media

Bacillus subtilis ATCC 6633 was kindly provided by the Hıfzısıhha Institute, Turkey. Stock cultures of *B.subtilis* was grown on nutrient agar 37^{o}C and maintained at 4^{o}C.

B. subtilis was maintained on modified media containing, yeast extract (1 g/L), glucose (10 g/L), pectin (2 g/L), $MgSO_4$ (0.4 g/L), K_2HPO_4 (0.4 g/L), and KH_2PO_4 (0.2 g/L) at incubated 35^{o}C for 14 h with agitation 120 rpm (Kapoor and Kuhad, 2002). Stock cultures of *B.subtilis* was prepared with 40% glycerol-distiled water and store -80^{o}C for long term storage.

The media of batch submerged fermentation were prepared in 500 mL Erlenmeyer flasks with the 200 mL working volume. Medium was consisting of black carrot pomace hydrolyzate, pea protein powder (30 g/L), $MgSO_4.7H_2O$ (1 g/L), K_2HPO_4(0.4 g/L). Each flask was inoculated with $3x10^7$ CFU/100 mL of inoculum and incubated at 35^{o}C, 120 rpm and 96 h.

The media of fed-batch submerged fermentation were prepared in 500 mL Erlenmeyer flasks with the 100 mL working volume. Medium was consisting of black carrot pomace hydrolyzate, pea protein powder (15 g/L), $MgSO_4.7H_2O$ (0.5g/L), K_2HPO_4(0.2g/L). Each flask was inoculated with $3x10^7$ CFU/100 mL of inoculum and incubated at 35^{o}C, 120 rpm and 72h.

2.1.3 Chemicals and Enzymes

All chemicals and enzymes used in this study are listed in Appendix A

2.1.4 Buffers and Solutions

Buffers and solutions used throughout the study, along with their preparation, are listed in Appendix B.

2.2 Methods

2.2.1 Analytical Methods

2.2.1.1 Chemical Analysis

General composition analysis (moisture, ash, fat, sugar profile) contents of BCP were determined as described in the following sections.

2.2.1.1.1 Moisture Content

Moisture content was calculated according to the Standard Official Methods of Analysis of the AOAC (1984). Approximately 1 g of ground sample was placed in a pre-weighed glass dish and dried in an oven set at 105°C until it reached a constant weight. Analysis was done in 4 replicates. The moisture content of the sample was determined using the following formula:

$$Moisture\ \%\ (wb) = \frac{(W_2\ - W_1)}{W_2}$$

where;

W_2 = Weight of wet sample (g)

W_1 = Weight of dry sample (g)

2.2.1.1.2 Ash Content

Total ash content of ground samples was calculated by incineration, as described by AOAC (1984). Approximately 1 g dried sample put on crucibles and combusted at 575°C for 24 h until constant weight was reached. The content of ash was then determined using the formula:

$$Ash\ \%\ (db) = \frac{(W_{C2} - W_{C1})}{(W_S - T)}$$

where ;

W_{C2} = Weight of initial crucible (g)

W_{C1} = Weight of final crucible (g)

W_S = Weight of sample (g)

T = Total solids (%)

2.2.1.1.3 Fat Content

The total fat in ground black carrot pomace was determined according to the Soxhlet method (Hara & Radin, 1978). Approximately five grams of oven dried sample was weighed and placed in a Soxhlet apparatus filled with 300 mL of hexane and heated for 3 h. The hexane and oil mixture was placed into 40°C vacuum-oven to dry. The content of fat was determined using the formula:

$$Fat\ \% = \frac{(W_2 - W_1)}{W_S} \times 100$$

where;

W_2 = Weight of fat and balloon (g)

W_1 = Weight of balloon (g)

W_S = Weight of sample (g)

2.2.1.1.4 Total Nitrogen and Crude Protein Content

Crude protein content of black carrot pomace was determined by Bradley et al. (1992). Approximately 0.2 grams of sample was weighed into the Kjeldahl tube and 25 mL of sulfuric acid (d=1.82 g/mL) was added to it. After adding Kjeldahl burning tablet to the tubes, the samples were incinerated at 380°C in the wet burning unit. Distillation was carried out in the steam distillation unit by adding 40 mL of distilled water and 2% boric acid to the cooled Kjeldahl tube after combustion. The ammonium borate obtained as a result of distillation was titrated with 0.098 N HCl until the solution color turned light pink. Total nitrogen and crude nitrogen protein amount were calculated according to the specified formulas.

$$Total\ Nitrogen\ \% = \left(\frac{(V_2 - V_1) \times N \times 0.014}{W_S}\right) \times 100$$

$$Crude\ Protein\ \% = Total\ Protein\ \% \times 6.25$$

where;

V_2 = Volume of HCl spent in titration (mL)

V_1 = Volume of HCl spent for blank (mL)

N = The normality of HCl solution used in the titration (mL)

W_S = Weight of sample (g)

0.014 = Milliequivalent weight of nitrogen

2.2.1.1.5 Sugar Profile and Inhibitory Compounds

The identification and quantification of sugars and inhibitors such as hydroxymethyl furfural (HMF) of untreated and MW-DA pretreated were performed in HPLC system (Prostar, Varian, CA, USA) equipped with an RI detector for HMF and sugars using Metacarb 87H column (300 mm X 7.8 mm, varian, S/N: 05517112, Varian) and the operation conditions were 35°C with 0.08 NH_2SO_4 as the eluent flowing at 0.5 mL / min (Xie et al., 2011).

2.2.1.1.6 Compositional Analysis

The untreated dried black carrot pomace samples were subjected to composition analysis by applying two-step acid hydrolysis (Uzuner and Çekmecelioğlu, 2015) . Dried black carrot pomace sample (0.3 g) was mixed with 3 mL of 72% H_2SO_4 solution and kept at room temperature for 1 hour. Then, 84 mL of distilled water was added to the sample and autoclaved at 121°C for 15 minutes. The liquid part and the solid part were separated from each other by centrifugation at 10,000 rpm for 10 minutes. After centrifugation, the pH of the samples was adjusted to 5.0. The amount of fermentable sugar in the pretreated samples was calculated using the DNS method.

2.2.2 Total Fermentable Sugar Analysis

Total fermentable sugars were determined spectrophotometric method using dinitrosalicycilic (DNS) acid reagent (Miller, 1959). DNS reagent was prepared and used as specified in Appendix C.

Fermentable sugars were assessed using glucose as a standard (refer to Appendix D). Fermentable sugar was dissolved in distilled water to create six different concentrations (0.15, 0.2, 0.4, 0.6, 0.8, and 1.0 g/L). A mixture of 3 mL of diluted samples and 3 mL of DNS reagent was heated for 15 minutes at 90°C in a test tube, and then 1.0 mL of 40% Rochelle salt (prepared in Appendix B) was added while the solution was still hot. The tubes were cooled and their absorbance was measured at 575 nm using a spectrophotometer. A blank consisting of 3 mL of distilled water, DNS, and Rochelle salt solution was also prepared. The measurements were duplicated.

2.2.3 Polygalacturonase Assay

Polygalacturonase (PGase) activity was measured by determining the release of reducing groups from polygalacturonic acid using the DNS reagent assay (Miller, 1959), and expressed as galacturonic acid with galacturonic acid monohydrate as the standard (refer to Appendix E). To assess PGase activity, 0.5 mL of enzymatic extract and 0.5 mL of polygalacturonic acid solution (1% w/v polygalacturonic acid in 0.05 M phosphate buffer at pH 7.0) were mixed. This mixture (0.3 mL) and distilled water (2.7 mL) was

transferred to each parallel tube. DNS reagent (3 mL) was added to each paralel tube. Every tube incubated at 50°C for 30 minutes (Kapoor et al., 2000). The 40 %Rochelle salt (1 mL) was added. Every tube incubated at 50°C for 30 minutes (Kapoor et al., 2000). The absorbance at 575 nm was measured. The blank was prepared in the same way, except for the crude enzyme. The enzymatic activity was defined as one unit (U) if it released one μmol of galacturonic acid per minute under the assay conditions.

2.2.4 Pectinlyase Assay

Pectinlyase (PLase) activity was determined by the colored derivative formation method (Nedjma et al., 2001). The activity determination is based on measuring the absorption of unsaturated uronic ester derivatives formed as a result of pectinlyase activity with thiobarbutyric acid at 550 nm. The following procedure was followed in the determination process. 0.25 mL enzyme solution was added to 0.25 mL substrate solution (1.5% pectin, ED 93%) and incubated for 10 minutes at 37°C. Then, 50 μL of 1 N NaOH was added to the 0.5 mL reaction mixture and incubated in a water bath at 80°C for 5 minutes and then cooled. After adding 0.6 mL of 1 N HCl and 0.5 mL of 0.04 M thiobarbutyric acid solution to the cooled reaction mixture, it was incubated a second time at 80°C for 5 minutes and cooled. The optical density of the resulting colored derivative was read at 550 nm against the blank. The sample prepared by adding the buffer containing the distilled water instead of the enzyme solution was used as a blank. One enzyme unit (U) was determined as the amount of enzyme that forms 1 nmol of product in 1 minute.

2.2.5 Determination of Cell Density

The density of cells was determined by measuring the turbidity at a wavelength of 600 nm. The growth curves of *B. subtilis* is presented in Appendix F.

2.2.6 Determination of Protein Content

The total protein content of the samples was determined according to the method of Lowry et al. (Lowry et al., 1951), using bovine serum albumin (BSA) as the protein standard. The Lowry solution was prepared as detailed in Appendix G. A 0.5 mL sample was mixed with 0.7 mL of Lowry solution, incubated in the dark at room temperature for 20 minutes, and subsequently, 0.1 mL of Folin's reagent was added. After mixing, the solution was left in the dark for 30 minutes at room temperature, and the absorbance of each sample was measured at 750 nm against a blank.

2.3 Pretreatment Methods

2.3.1 Thermal Method

The steps of the thermal (T) pretreatment carried out in an autoclave was shown in Figure 1. First, the black carrot pomace was dried at 70°C for 4 hours. Five g of dried black carrot pomace was weighed and 100 mL of distilled water was added to it. It was heat pretreated in an autoclave at 121°C for 15 minutes. The black carrot pomace hydrolyzate coming out of the autoclave was adjusted to pH 5 with 10% NaOH. 100 U/g Viscozyme L (arabanase, cellulase, beta-glucanase, hemicellulase and xylanase) was applied for the enzymatic hydrolysis step. Enzyme applied hydrolysates were subjected to a shaking incubator at 120 rpm for 24 h at 50°C. The amount of fermentable sugar was measured by the DNS method with the help of a spectrophotometer.

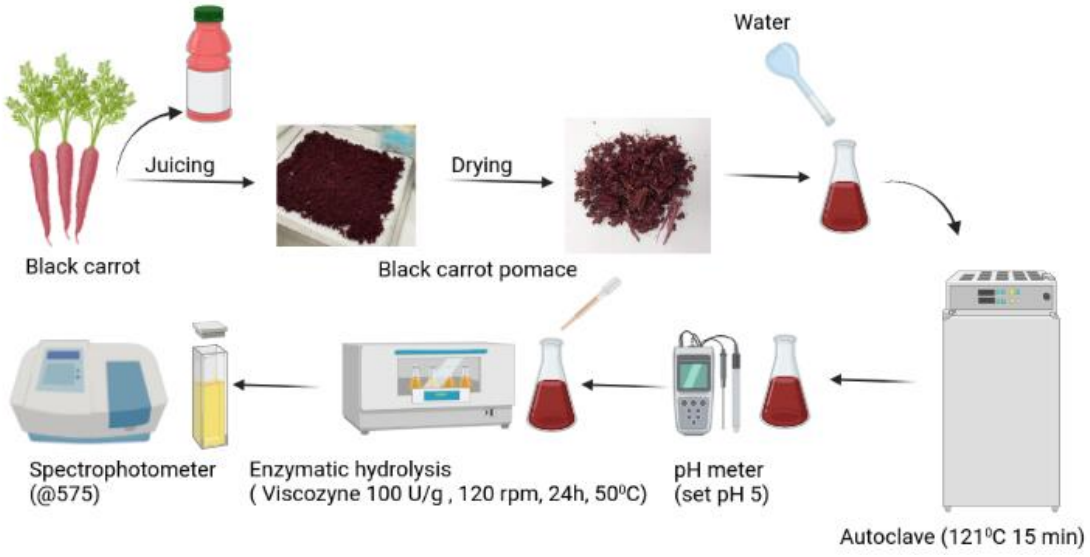


Figure 1. Schema of thermal pretreatment

2.3.2 Thermo-chemical Method

The steps of the thermo-chemical (TC) pretreatment carried out in an autoclave was shown in Figure 2. First, the black carrot pomace was dried at 70°C for 4 hours. Five g of dried black carrot pomace was weighed and 2% diluted acid (v/v) (H_2SO_4) solutions was added to it. It was heat pretreated in an autoclave at 121°C for 15 minutes. The black carrot pomace hydrolyzate coming out of the autoclave was adjusted to pH 5 with 10% NaOH. 100 U/g Viscozyme L was applied for the enzymatic hydrolysis step. Enzyme applied hydrolysates were subjected to a shaking incubator at 120 rpm for 24 h at 50°C. The amount of fermentable sugar was measured by the DNS method with the help of a spectrophotometer.

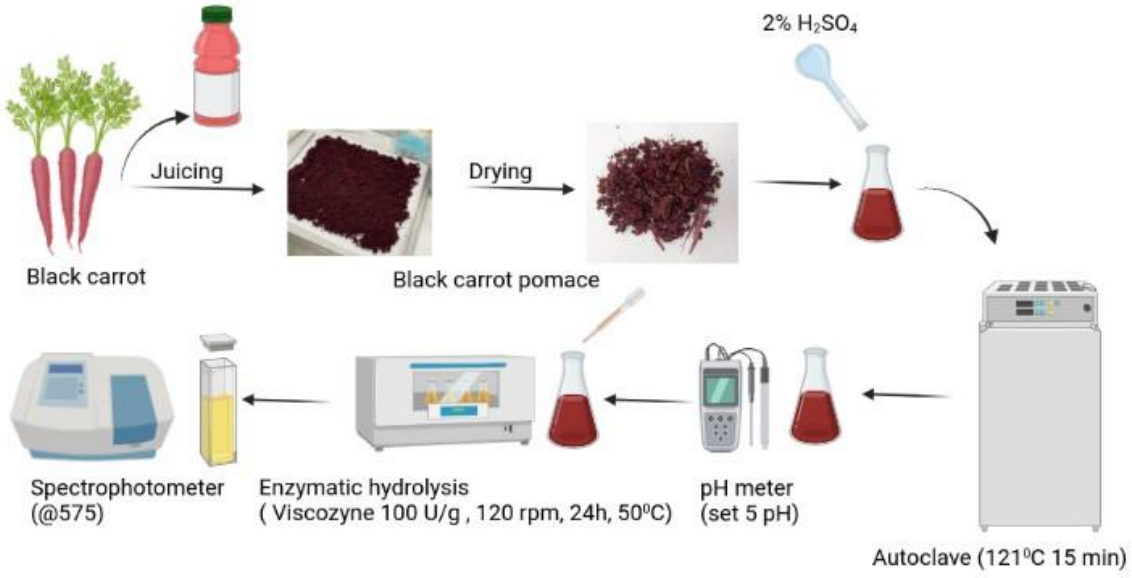


Figure 2. Schema of thermo-chemical pretreatment

2.3.3 Microwave Assisted Dilute Acid (MW-DA) Pretreatment

The steps of the microwave with diluted acid (MW-DA) pretreatment carried out in an microwave (Samsung -MS23F300EEK) was shown in Figure 3. First, the black carrot pomace was dried at 70°C for 4 hours. Five g of dried black carrot pomace was weighed and 2% diluted acid (v/v) (H_2SO_4) solutions (1:20 solid/liquid ratio) was added to it. Pretreatment was carried out at three different levels; power (X_1 ; 300, 600 and 850 W) and pretreatment time (X_2 ; 30, 60 and 90 s) (Table 10). The black carrot pomace hydrolyzate, which came out of the microwave application, was adjusted to pH 5 with

10% NaOH. 100 U/g Viscozyme L was applied for the enzymatic hydrolysis step. Enzyme applied hydrolysates were subjected to a shaking incubator at 120 rpm for 24 h at 50°C. The amount of fermentable sugar was measured by the DNS method with the help of a spectrophotometer.

Figure 3. Schema of microwave assisted diluted acid pretreatment

The following calculations were made to determine the fermentable sugar content, yield and energy consumption.

$$Fermentable\ Sugar\ Content\ (FSC)\ \left(\frac{mg}{g}\right) = \frac{M \times (84\ mL) \times DF}{m}$$

M = fermentable sugar concentration (g/L)

m = dry weight of pretreated biomass (g)

DF = dilution factor

Fermentable sugar yield (%w/w) for each pretreatment method was calculated as follows:

$$Yield\ \% = \left(\frac{a}{b}\right) \times 100$$

a = amount of fermentable sugar in hydrolyzate obtain from pretreatment of dry biomass (g/g)

b = amount of fermentable sugar in untreated dry biomass (g/g)

Energy consumption was calculated as a formula (Lu et al., 2011):

$$Energy\ consumption\ \left(\frac{KJ}{g}\right) = \frac{P \times t}{g}$$

P = microwave or autoclave power (KW)

t = time (s)

g = fermentable sugar content in pretreatment (g)

2.4 Enzymatic Hydrolysis

Five gram of dried black carrot pomace was added to 100 mL water and 100 mL 2% (v/v) H_2SO_4 solution, separately. The samples was autoclaved at 121°C for 15 minutes. The hyrolyzates was adjusted pH 5 with acid (10% HCl) and basis (10% NaOH) solutions. The samples were separately hyrolyzed using Viscozyme L and Cellulase from *Trichoderma reesei* enzymes at 50°C and 120 rpm for 48 hours with different enzyme loadings (25 U/g, 50 U/g, 100 U/g) in 250 mL bottles (solid liquid ratio 1/20 (w/v)) (Table 9). After enzymatic hydrolysis, the samples were heated at 100°C for 15 minutes to inactivate the enzymes. The amount of fermentable sugar was calculated using the DNS method.

Table 9. The plan of enzymatic hydrolysis

Code	Solvent type	Enzyme name	Enzyme loading (U/g)
1	Water	Viscozyme L	50
2	2% H_2SO_4	Viscozyme L	50
3	2% H_2SO_4	Viscozyme L	100
4	2% H_2SO_4	Cellulase	25
5	2% H_2SO_4	Cellulase	50
6	Water	Cellulase	50

2.5 Enzyme Production by Submerged Fermentation (SmF)

The mechanism of the submerged fermentation (SmF) process for the production of cocktail enzyme is schematized in Figure 4. A modified medium was prepared from the growth medium for batch fermentation. In this medium, black carrot pomace hydrolyzate was used instead of glucose in the growth medium, and pea protein was used instead of yeast extract in the growth medium a 500 mL shake flask containing 200 mL of fermentation medium. Before inoculation, the bottles and medium were autoclaved at 121^{o}C for 15 minutes to make them suitable for fermentation. After the sterilization step, the fermentation medium was inoculated with 1 mL of bacterial culture containing 3x10^{7} CFU/mL, incubated at 35^{o}C in a 120 rpm shaking incubator. After fermentation, they were centrifuged at 10,000 rpm for 15 minutes and the enzyme activity was measured according to the method described in sections 2.2.3 and section 2.2.4.

A modified medium was prepared from the growth medium for fed batch fermentation. In this medium, black carrot pomace hydrolyzate was used instead of glucose in the growth medium, and pea protein was used instead of yeast extract in the growth medium a 500 mL shaker bottle containing 100 mL of fermentation medium. Before inoculation, the bottles and medium were autoclaved at 121^{o}C for 15 minutes to make them suitable for fermentation. After the sterilization step, the fermentation medium was inoculated with 1 mL of bacterial culture containing 3x10^{7} CFU/mL, incubated at 35^{o}C in a 120 rpm shaking incubator. Feeding was done with 5% amount of feeding of carbon source at the 54th hour of the process. After fermentation, they were centrifuged at 10,000 rpm for 15 minutes and the enzyme activation was measured according to the method described in sections 2.2.3 and section 2.2.4.

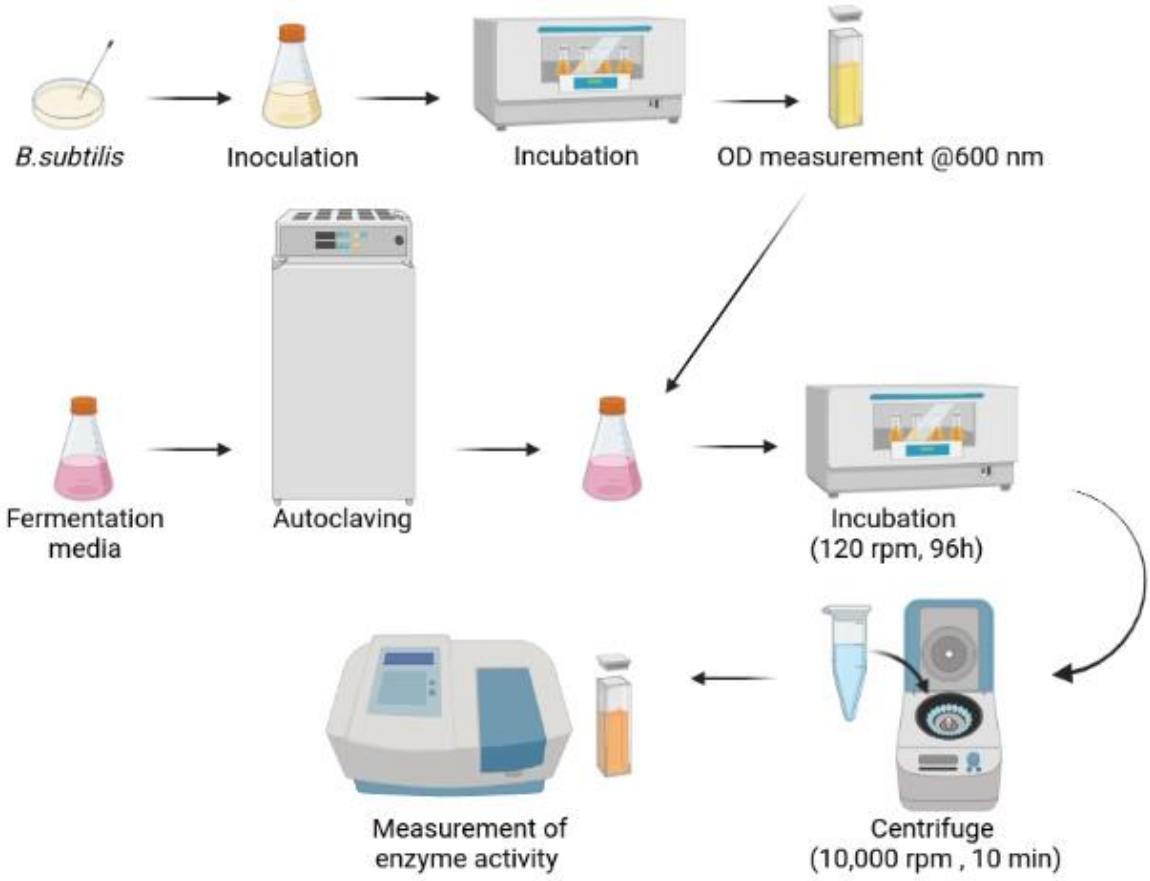


Figure 4. General diagram for cocktail enzyme production

2.6 SDS-PAGE Analysis

Crude cocktail enzyme samples were used by Biotechnology and Bioengineering Applications and Research Centre (BİYOMER) , Izmir, Turkey for determination of molecular weight by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) method according to Laemmli, (1970). Electrophoresis system including 12% separating and 5% stacking gel. A pre-stained protein molecular weight marker (Thermo Scientific, USA) with the range of 15–100 kDa was used as the size standard in the protein electrophoresis. After the sample (10 µL) and protein marker (50 µL) were loaded on the gel and run on the gel under 200 Volt for 50 minutes. Staining of the gel was applied by the silver staining technique.

2.7 SEM Analysis

SEM analysis was conducted in İzmir Institute of Technology Center for Materials Research (FEI Quanta 250 FEG) in order to view and compare the changes of the physical structure of untreated, microwave with diluted acid treated and, enzymatic hydrolysis of black carrot pomace, operating at the electron beam energy of 10.00 kV.

2.8 Statistical Analysis

All the experiments were carried out in triplicate. Statistical analyses were conducted to test the significant mean differences in MW-DA pretreatment in terms of fermentable sugar content as a function of power, and pretreatment time. The orthogonal arrays for control factors are shown in Table 10. There were 18 experimental runs with 2 factors and 3 levels generated by MINITAB 16.0 (Minitab Inc., State College, PA, USA). The levels of two variables were determined through preliminary experiments. The taguchi design was constructed by using two predictors with three levels, in each of which; power (300, 600 and 850 W), and pretreatment time (t; 30, 60 and 90 s) are used (Table 10). The results of the L9 (2^3) three-level Taguchi design are shown in Table 10. The quality performance was evaluated at 'nominal the best'. All the statistical analyses were performed by using MINITAB 16.0 (Minitab Inc., State College, PA, USA). Analysis of Variance (ANOVA) and regression models were performed at a 95% confidence interval ($p < 0.05$) to define the significant terms of the predictive model. Variance analysis was performed to determine statistically significant effects of the two predictors ($p < 0.05$). Multiple comparisons were made by using Tukey's test.

Table 10. The factors of Taguchi design

Factor	Levels		
Pretreatment time (sec)	30	60	90
Power (W)	300	600	850

CHAPTER 3

RESULT AND DISCUSSION

3.1 Composition of Raw Material

Black carrot pomace contained 5.17±0.07 % ash, 8.63±0.19 % moisture, 4.83±0.23 % protein, 1.15±0.01% fat (dry basis), and 0.565±0.001 g fermentable sugar / g dry biomass, showing its potential as a source of sugars such as glucose, xylose, arabinose. These results agree well with those of Elik (2021) who studied hot-air-assisted radio frequency drying of BCP. The chemical composition of hazelnut shells varies with geographic location, season, harvesting practice, as well as analysis procedures.

3.2 Sugar Profile and Inhibitory Compounds of Untreated and Pretreated Black Carrot Pomace

Glucose, fructose, arabinose were found as 0.750±0.050 g/L, 0.004±0.000 g/L, 0.350±0.020 g/L, respectively in raw biomass. No xylose was detected in both raw material and pretreated sample. After MW-DA pretreatment and sequential enzymatic hydrolysis of black carrot pomace, glucose, fructose, arabinose amounts were found as 2.33±0.04 g/L, 1.50±0.01 g /L, 1.42±0.11 g/L, respectively. Some inhibitors such as furfural, and hydroxymethyl furfural (HMF) which inhibit the fermentation are formed during pretreatment of biomass (Karunanithy & Muthukumarappan 2011). At power, pretreatment time, and acid concentration of 300 W, 30 s, and 2% (v/v) H_2SO_4, respectively, no HMF and furfural were detected in the hydrolyzate.

3.3 Effect of Enzyme Type, Enzyme Load and Hydrolysis Time on The Enzymatic Hydrolysis

Fermentable sugar content after enzymatic hydrolysis was determined under the different enzyme loading (25, 50, and 100 U/g) at solid loadings of 1/20, 2% (v/v) H_2SO_4 with pretreatment time of 24 h and 48 h. Results of fermentable sugar under the different enzyme loading and solvent type were shown in Table 11. It was obtained that pretreated BCP with water did not have any significant effect on enzymatic saccharification with

Viscozyme L and Cellulase from *Trichoderma reesei* (Table 11, Code 1&6). The fermentable sugar content showed an increasing trend for pretreated samples for 24 h compared to the hydrolysis time of 48 h. In order to understand the effectiveness of the enzymes used, Viscozyme L and Cellulase from *Trichoderma reesei* were compared by keeping the solvent type and enzyme loading amount the same (Table 11, Code 2 & 5). As a result of this comparison, Viscozyme L (10.67±0.01 g/L) was found to be more effective than the Cellulase from *Trichoderma reesei* (4.11±0.04 g/L) enzyme in terms of the amount of sugar obtained for 24 h. The best results of fermentable sugar with 100 U/g of enzyme load were observed for Viscozyme L. It was observed that the highest fermentable sugar was obtained as 10.67±0.01 g/L for 24 h under the enzyme loading of 100 U/g dry substrate at 2% (v/v) H_2SO_4 solvent type (Table 11).

Table 11. Effects on different solvent type, enzyme type and loading on fermentable sugar content

Code	Solvent type	Enzyme name	Enzyme loading (U/g)	Fermentable Sugar (g/L)	
				24 h	48 h
1	Water	Viscozyme L	50	Nd	Nd
2	2% H_2SO_4	Viscozyme L	50	5.23 ± 0.01^{bA}	4.98 ± 0.01^{Bb}
3	2% H_2SO_4	Viscozyme L	100	10.67 ± 0.01^{aA}	8.76 ± 0.09^{Ab}
4	2% H_2SO_4	Cellulase	25	3.99 ± 0.03^{dA}	2.31 ± 0.03^{Db}
5	2% H_2SO_4	Cellulase	50	4.11 ± 0.04^{cA}	3.99 ± 0.09^{Cb}
6	Water	Cellulase	50	Nd	Nd

nd: not detected

3.4 Effect of Different Pretreatment Methods

The effectiveness of thermal, thermo-chemical and microwave pretreatments on the conversion of black carrot pomace to fermentable sugar content with the minimum sugar loss was assessed. Three different pretreatment strategies such as thermal (T), thermo-chemical (TC), and MW-DA combined with and without enzymatic hydrolysis are represented in Figure 5. The MW-DA pretreatment resulted in the highest fermentable

sugar content (0.089±0.004 g/g), whereas fermentable sugar content of 0.039±0.000 and 0.077±0.001 g/g were obtained from thermal and thermo-chemical pretreatment, respectively (Figure 5). This result indicates that different pretreatment methods have a partial effect without enzymatic hydrolysis. After enzymatic hydrolysis, fermentable sugar content increased from 6.9 to 37% and 13.6 to 40% for thermal and thermo-chemical pretreatment, respectively. The fermentable sugar yield increased from 15.8% to 87.3% when the MW-DA treatment was combined with enzymatic hydrolysis. This result indicates that enzymatic hydrolysis is a necessary step in fermentable sugar or a different name is fermentable sugar production. Among all pretreatment strategies, MW-DA followed by enzymatic hydrolysis gave the maximum fermentable sugar production (0.493 g /g, 87.3% conversion) (Figure 5).

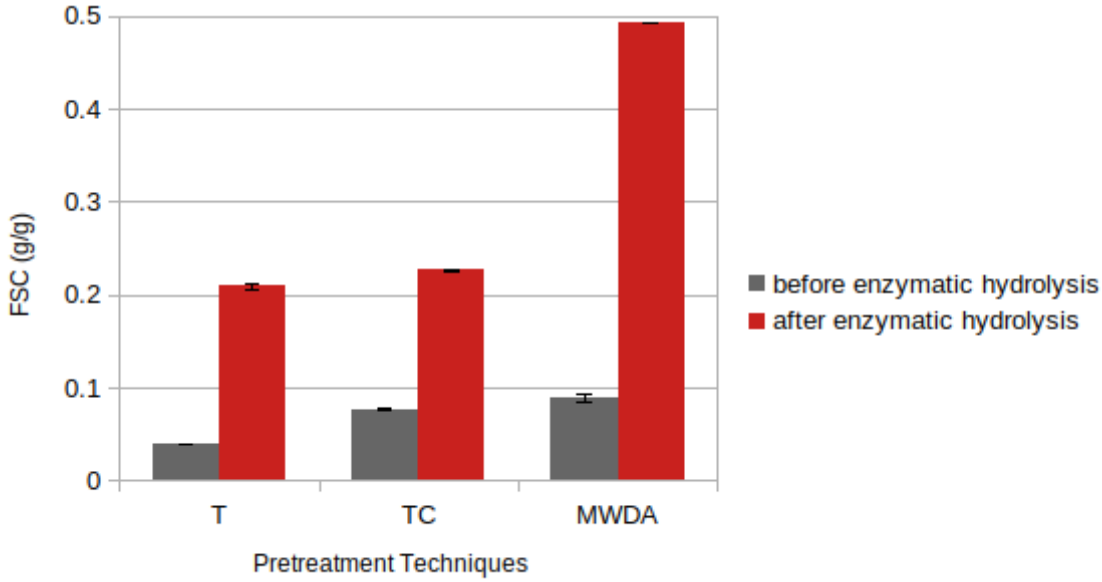


Figure 5. Fermentable sugar content under the different pretreatment methods

3.5 Effect of MW-DA on Fermentable Sugar Before and After Enzymatic Saccharification

The effects of microwave power and pretreatment time on the pretreatment and enzymatic hydrolysis conditions of black carrot pomace were examined using Taguchi design. The fermentable sugar content of untreated black carrot pomace was 0.565±0.001g/g biomass (Table 12). Fermentable sugar content from various pretreated samples without enzymatic hydrolysis ranged at 0.089-0.117 g/g. Overall, 0.339 to 0.515 g/g fermentable sugar content was obtained when black carrot pomace was treated with

MW-DA and enzymatic hydrolysis depending on pretreatment conditions shown in Table 12. The highest amount of fermentable sugar after MW-DA pretreatment followed by enzymatic hydrolysis (0.515±0.002 g/g) was obtained at MW power of 600 for 90 s with 22.69% enzymatic conversion (Table 12, Code 6). These fermentable sugar content recovery results with the microwave pretreatment were in accordance with other studies (Ethaib, S. et. al, 2020), which showed high fermentable sugar content recovery at a low microwave power (100 W to 150 W). A decrease in the fermentable sugar content recovery was observed when the power was increased to 600 and 850 W (Table 12) (except MW-6&7). Maximum enzymatic conversion was found as 32.51% at MW power of 600 W for 30s (Table 12).

Table 12. Comparison of fermentable sugar content under MW-DA w/o ES and w/ES

Code	Power (W)	Time (s)	Fermentable Sugar Content (g/g)		Enzymatic Conversion (%)
			MW-DA w/o ES	MW-DA w/ ES	
MW-1	300	30	0.089±0.004	0.493±0.000	18.09
MW-2	300	60	0.113±0.005	0.484±0.004	23.44
MW-3	300	90	0.103±0.001	0.453±0.003	22.75
MW-4	600	30	0.110±0.000	0.339±0.001	32.51
MW-5	600	60	0.117±0.001	0.395±0.006	29.73
MW-6	600	90	0.117±0.004	0.515±0.002	22.69
MW-7	850	30	0.106±0.003	0.502±0.000	21.17
MW-8	850	60	0.108±0.003	0.439±0.006	24.57
MW-9	850	90	0.117±0.001	0.451±0.008	25.96

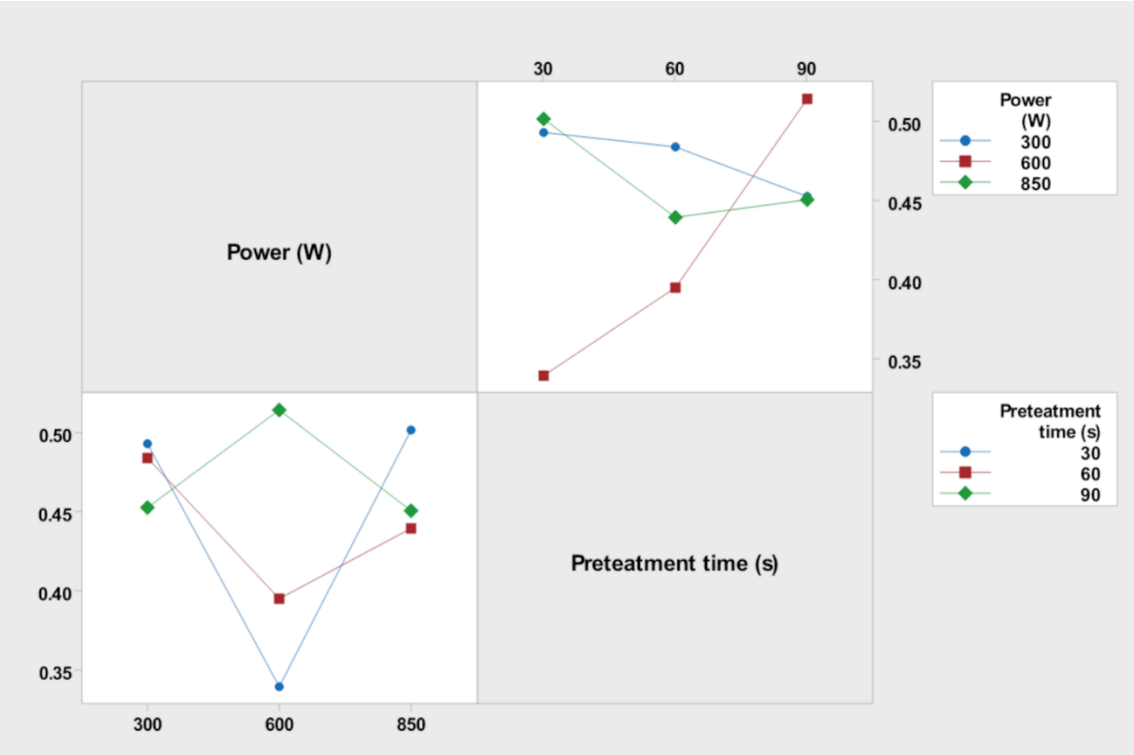


Figure 6. Interaction plot for fermentable sugar content mean at 3 levels

Figure 6 illustrates the interaction plot for fermentable sugar content. The interaction of MW power against pretreatment time as it affects the fermentable sugar content is shown in Figure 6. The microwave power of 600 W results in a significant enhancement between 30 and 90 s (Figure 6). The pretreatment time of 90s results the significant improvement in fermentable sugar content at power of 300-600 W, while beyond 600 W, the fermentable sugar content decreased (Figure 6). The maximum fermentable sugar content was attained at 600 W for 90s, while the minimum fermentable sugar content was determined at 600 W for 30 s (Figure 6).

It is important to know the amount of energy consumed during pretreatment to obtain the maximum fermentable sugar content. Table 13 represents the amount of energy consumed in each pretreatment with the amount of energy produced from the released sugars during hydrolysis stage. The lowest energy consumption (18.3 KJ/g) to release 1 g of fermentable sugar content was achieved with MW-DA at 300 W for 30 s. The highest energy consumption (8,612 KJ/g) was achieved with thermal pretreatment at 121^{o}C for 15 min. Although the higher amount of energy is applied to reach higher temperatures in autoclave, the fermentable sugar content did not increase with this pretreatment (Table 13). The fermentable sugar content obtained is not increasing linearly or proportionally to the energy consumed at higher MW powers (600 and 850W) (Table 13).

Table 13. Energy consumption and fermentable sugar content values during MW-DA and thermal pretreatments of BCP followed by enzymatic saccharification

Sample Name	Power (W)	Time (s)	Fermenatble Sugar Content (g/g)	Energy (KJ)	Energy Consumption (KJ/g)
Untreated	0	0	0.565±0.001	0	0
MW-1	300	30	0.493 ± 0.00^{bc}	9	18.3^{i}
MW-2	300	60	0.484 ± 0.00^{c}	18	37.2^{h}
MW-3	300	90	0.453 ± 0.003^{d}	27	59.6^{e}
MW-4	600	30	0.339 ± 0.001^{f}	18	53.1^{f}
MW-5	600	60	0.396 ± 0.006^{e}	36	91.2^{d}
MW-6	600	90	0.515 ± 0.002^{a}	54	104.9^{c}
MW-7	850	30	0.502 ± 0.000^{ab}	25.5	50.9^{g}
MW-8	850	60	0.439 ± 0.006^{d}	51	116.3^{b}
MW-9	850	90	0.451 ± 0.008^{d}	76.5	166.8^{a}
Thermal-T	-	900	0.209±0.004	1800	8,612

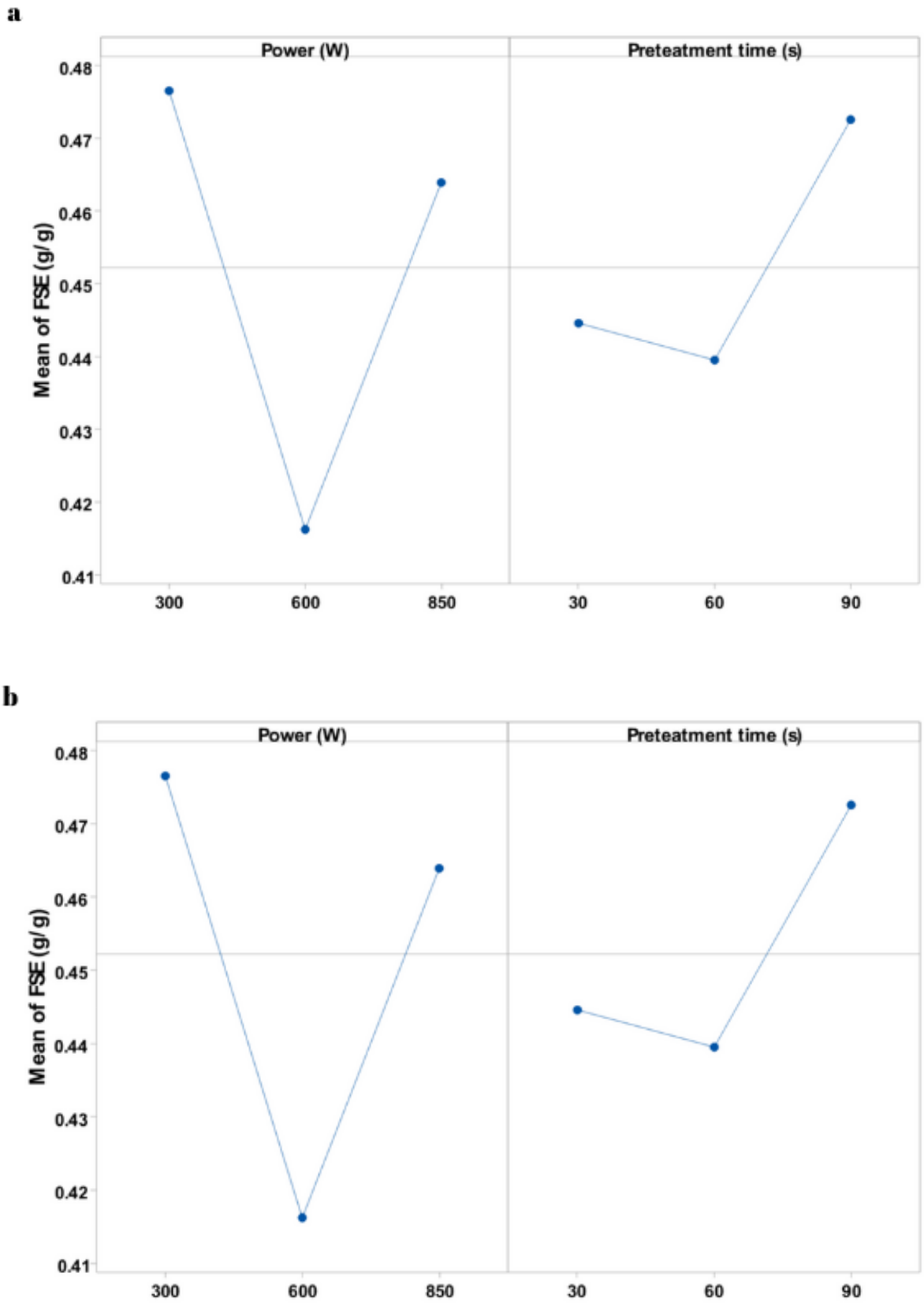


Figure 7. Main effects plot for fermentable sugar content (FSC) and S/N ratio

The main effect plot (data means) in Figure 7 confirmed the profiles of each parameter as regards to fermentable sugar content and energy consumption. When the power and pretreatment time increased from 300 to 600 W and from 30 to 60 s, respectively, there is a decline in fermentable sugar content (Figure 7). The optimum mean fermentable sugar content is attined at 300 W and 90 s (Figure 7a). An increase in power and pretreatment time led to an increase in the energy consumption. Therefore, the minimum energy consumption is realized at 300W and 30 s (Figure 7b). Valorization of black carrot pomace may be feasible using combination of microwave pretreatment and enzymatic hydrolysis.

The variations in the mean signal to noise (S/N) ratio and fermentable sugar content for all factors are presented in Table 14. The values at level 1, 2, and 3 for power and pretreatment time are presented in Table 14. Corresponding delta value, ranking of the parameters was made depicting power and pretreatment time to be most significant for fermentable sugar content and S/N ratio (Table 14). The order of importance of the factors for fermentable sugar content is power and pretreatment time.

Table 14. S/N ratio and FSC at different level of two factors and delta values

	Level	Power (W)	Pretreatment time (s)
S/N ratio	1	49.02	58.93
	2	52.23	45.11
	3	42.23	48.31
	Delta	10.00	13.82
	Rank	2	1
Fermentable Sugar Content	1	0.4766	0.4446
	2	0.4162	0.4395
	3	0.4639	0.4726
	Delta	0.0603	0.0331
	Rank	1	2

3.6 SEM Analysis

The changes in surface morphology of BCP after MW-DA pretreatment and enzymatic saccharification were investigated using SEM. SEM images for black carrot pomace untreated, microwave assisted acid pretreated and microwave with enzyme-hydrolyzed samples are shown in Figure 8. It was observed that untreated black carrot pomace sample showed regular and recessed cellular structures and undamaged surfaces (Figure 8a). The rigid surface was damaged with MW-DA treatment (Figure 8b). A more porous and heterogeneous sugar-like structures were observed with MW-DA pretreated black carrot pomace (Figure 8b). Based on Rajeswari et al., (2020) the similar morphological changes was observed in the MW treated aloe vera leaf rind. The structure of black carrot pomace cell wall was lost in the hemicellulose matrix to enhance enzyme access to cellulose and fermentable sugar content during the MW-DA pretreatment. The fiber structures and some solid residues were observed after enzymatic saccharification (Figure 8c).

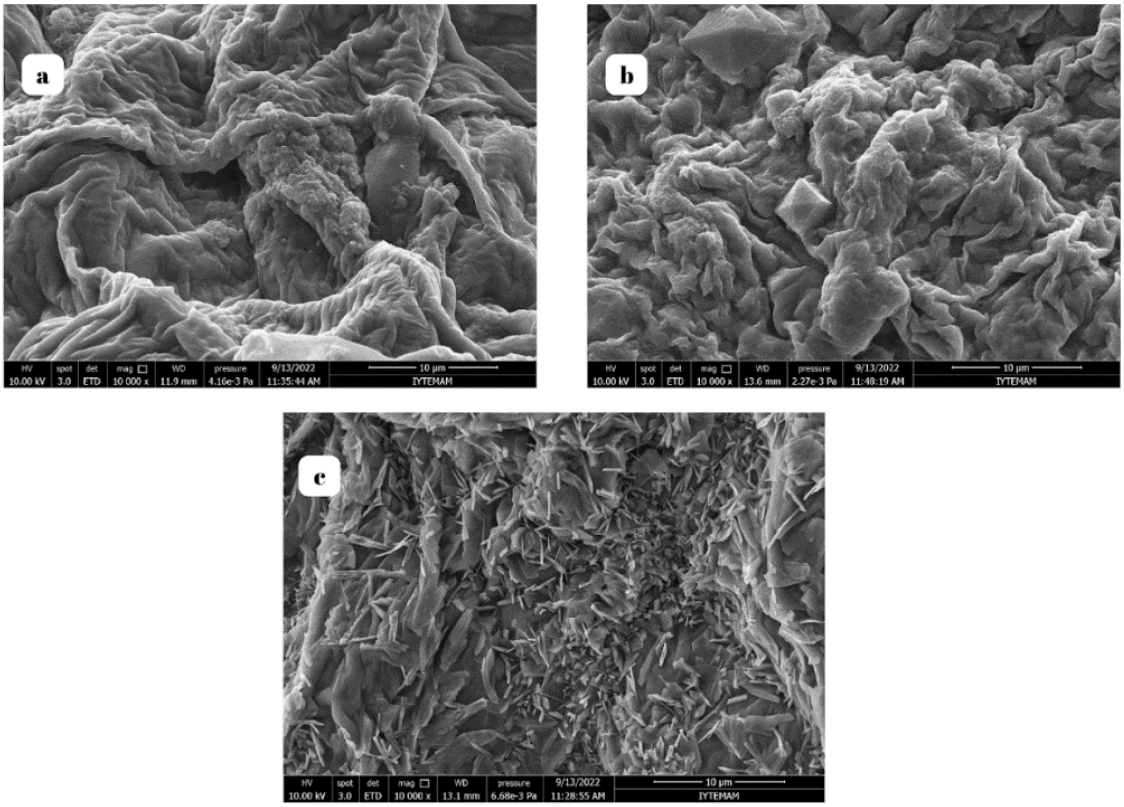


Figure 8. SEM images. a) untretated b) microwave with diluted acid pretreatment c) microwave with diluted acid pretratment + enzymatic hydrolysis

3.7 Comparison of Fermentable Sugar Content with Previous Studies

Binod et al.(2012) investigated that fermentable sugar production with a microwave-dilute acid strategy using sugarcane bagasse. The fermentable sugar yield was found as 0.091 g/g under 1% sulfuric acid concentration at 600 W for 4 min. Germec et al.(2017) investigated the effet of MW-DA conditions on fermentable sugar production from different biomass such as wheat bran, barley husk, oat husk, and rye bran. The maximum fermentable sugar was found to be 49.65 g/L (0.83 g/g) from wheat bran at 700 W. The fermentable sugar content (0.493 g/g) from this study after MW-DA pretreatment was higher than what was reported by Binod et al. (2012) and Ethaib et al. (2020) investigated the effect of three types of MW pretreatments followed by enzymatic saccharification of sago palm bark on total fermentable sugar. The maximum total fermentable sugar (0.386 g/g) was obtained after enzymatic saccharification at 5% solid/liquid ratio and 0.05 M H_2SO_4 under 440 W for 15 min. Zhu et al. (2015) determined pretreated *Miscanthus* biomass conversion (75.3%) under 0.2 M H_2SO_4 for 20 min at 300 W MW power and saccharified using Celluclast and Novozyme 188. The fermentable sugar yield (87.3%) from this study after MW-DA followed by enzymatic saccharification was higher than what was reported by Zhu et al.(2015) and Ethaib et al.(2020).

3.8 Evaluation of Key Variables Affecting Enzyme Production

3.8.1 Effect of Different Organic Nitrogen Source of Polygalacturonase (PGase) and Pectinlyase (PLase) Activity

The cultivation was started as a batch mode for 48 h. To determine the best inducing nitrogen source, different organic nitrogen sources such as yeast extract (0.5 g), whey protein (0.5 g) and pea protein (0.5 g), are shown in Figure 9. The Glucose, pH, fermentation time, temperature, inoculum volume (%v/v), magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4] were kept at a constant, enzyme activities were measured to determine the effect different organic nitrogen source on PGase and PLase production

The highest PGase production was obtained with pea protein (20.5±0.52 U/L). The highest Plase production was obtained with pea protein (46.44±3.45 U/L). Figure 8

clearly is showed that the pea protein (20.5±0.52 U/L, 46.44±3.45 U/L) was more effective than other tried organic nitrogen sources (yeast extract (19.38±0.32 U/L,22± 3.56 U/L), whey (14.67±0.37 U/L,8.55±1.72 U/L)) in the production of PGase and PLase. OD is directly proportional to enzyme production activity.

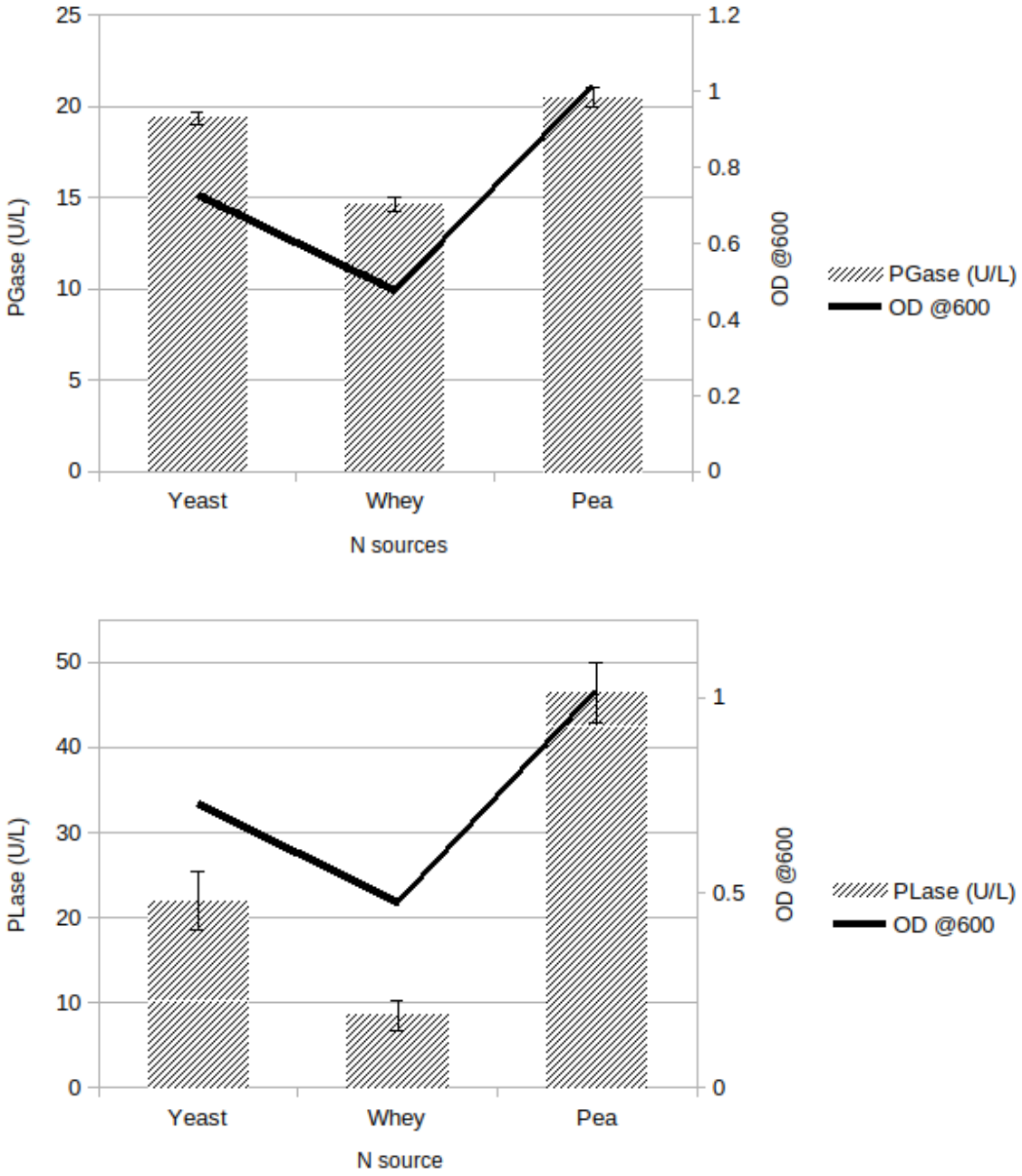


Figure 9. Effect of different organic nitrogen sources on PGase and PLase

Swain and Ray (2010) studied on PGase enzyme production by using different organic and inorganic nitrogen sources (yeast extract, beef extract, peptone, urea, casein,

NH_4Cl, $(NH_4)SO_4$, CH_3COONH_4) of *B.subtilis* CM5. In this study, peptone provided the highest PGase production compared to yeast extract and other nitrogen sources. Oumer and Sabate (2018) compared submerged fermentation and solid state fermentation for the production of polygalacturonase from *B.subtilis* strain Btk 27 in their study. The effects of different nitrogen sources (yeast extract, casein, glycine, peptone, urea, NH_4Cl, NH_4NO_3, NH_4SO_4) under submerged fermentation conditions were investigated. The combination of yeast extract and casein using in PGase production showed the highest polygalacturonase activity (67.7±4.7 U/mL). In the study of Kaur and Gupta (2017), pectinlyase was produced by *B.subtilis* SAV 21 using different nitrogen sources such as yeast extract, peptone, beef extract and ammonium salts. The yeast extract was determined as the best nitrogen source in pectinlyase production (Kaur and Gupta, 2017).

3.8.2 Effect of The Different Amount of Nitrogen Source on PGase and PLase Activity

The cultivation was started as a batch mode for 48 h. Pea protein as a nitrogen source was added at a different content (0.25, 0.75, 1.5, 2 g). Black carrot pomace hydrolysate, pH, fermentation time, temperature, inoculum volume (%v/v), magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4] were kept at a constant, enzyme activities were measured to determine the effect of the different amount of nitrogen source on PGase and PLase production (Figure 10).

The highest PGase activity (109.43±0.97 U/L) was obtained by adding 1.5 g of pea protein . The highest PLase activity (101.85±2.82 U/L) was obtained by adding 2 g of pea protein. The amount of 1.5 g pea protein can be preferred from a statistical and industrial point of view.

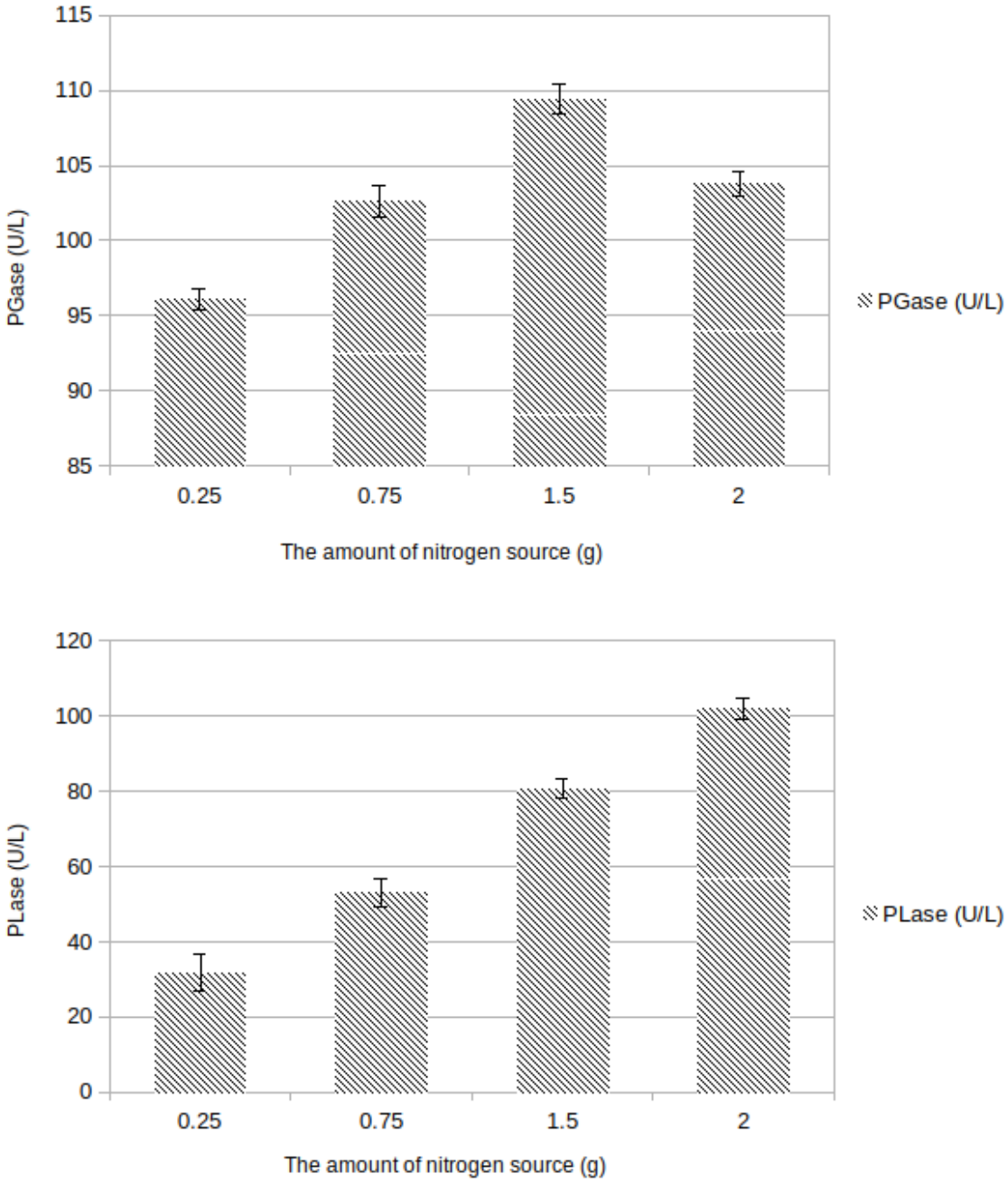


Figure 10. Effect of the different amount of nitrogen source on PGase and PLase activity

Nitrogen and carbon are most important component of bioprosess medium for microorganism growth and production of value added product (Ulhiza et al., 2018, Xiao et al.,2018). Excessive addition of nitrogen source can reduce enzyme production.

3.8.3 Effect of Fermentation Time on PGase and PLase Activity

Black carrot pomace hydrolyzate, pea protein, pH, fermentation time, temperature, inoculum volume (%v/v), $MgSO_4$, and K_2HPO_4 were kept at a constant, enzyme activities were measured at the end of 12, 24, 36, 48, 60, 72, 84 and 96 h to determine the effect of time on PGase and PLase production (Figure 11).

The results indicated that the highest enzyme production of PGase (139.33±0.48 U/L) and PLase (140.96±6.15 U/L) was achieved after 60 h at 35° C, pH 7.0 and with the agitation of 120 rpm. OD and enzyme activity showed same trend.

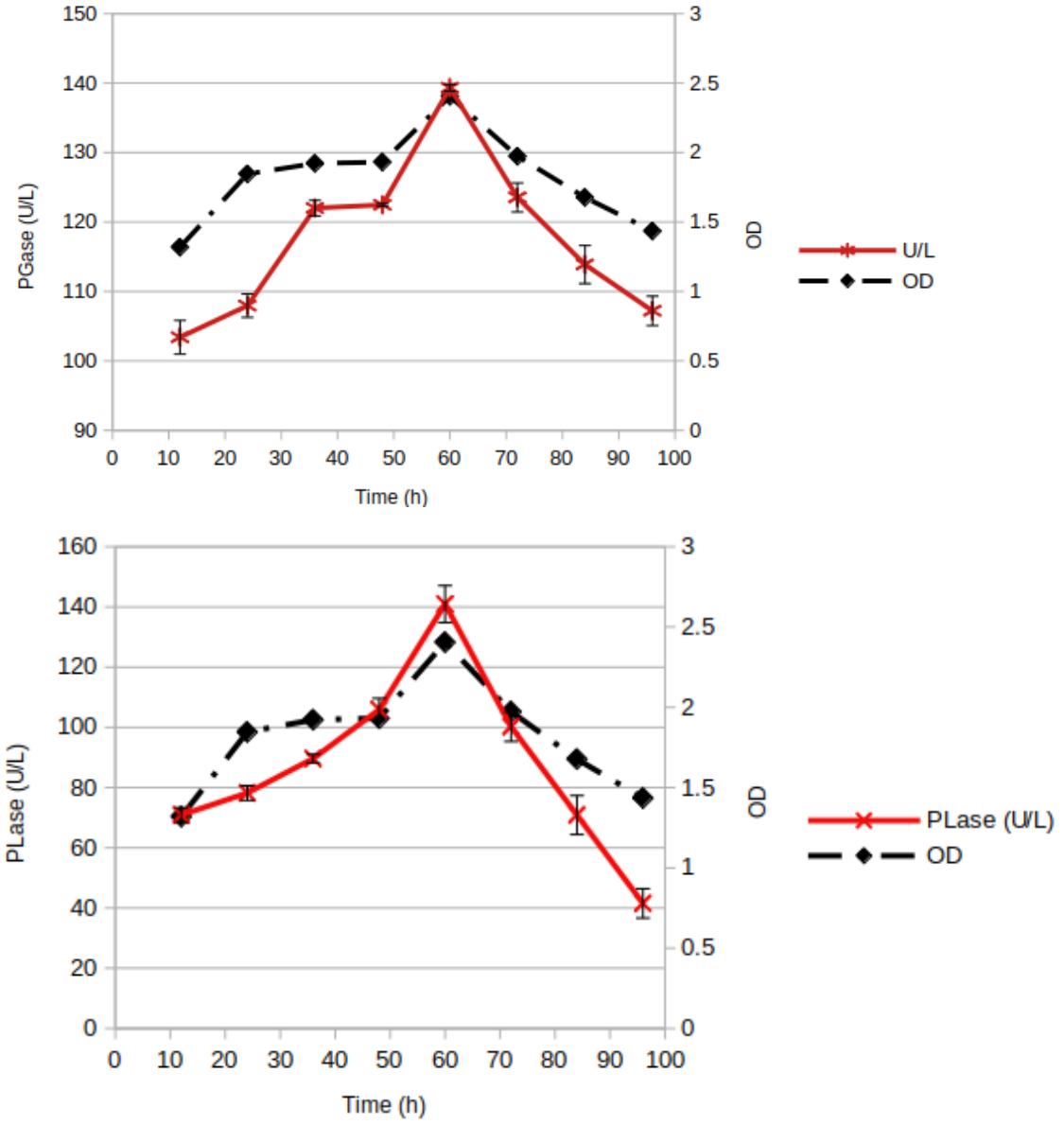


Figure 11. Change in PGase and PLase activity depending on fermentation time.

According to Alqahtani et al. (2022) studied PGase production from *Bacillus subtilis* 15A-B92. The highest PGase activity was obtained at the 36th hour under pH 4.5 and 40^{o}C conditions. Another study, Esmail et al. (2013), was found the maximum PLase production from *B.subtilis* RSY7 at pH 8.5 and 40^{o}C for 72th hour.

3.8.4 Effect of Different Amount of Feeding of Carbon Source of PGase and PLase Activity

The cultivation was started as a batch mode for 96h. At 54th h, and before entering the stationary growth phase, black carrot pomace hydrolysate as a carbon source was added at a different amount of feeding of carbon source (2.5%, 5%, 10%, 15%). Pea protein, pH, fermentation time, temperature, inoculum volume (%v/v), $MgSO_4$, K_2HPO_4 were kept at a constant, enzyme activities were measured to determine the effect of feed concentration on PGase and PLase production (Figure 12).

The highest PGase activity (164.34±2.26 U/L) occurred at the 15% amount of feeding of carbon source at 72th hours. The highest PLase activity (188.22±1.72 U/L) occurred at the 5% amount of feeding of carbon source at 72th hours. The addition of fresh substrate to the system, regardless of the concentration, increased the fermentation time and enzyme activity. Since no statistically significant difference was observed in terms of PGase activities at 15% amount of feeding of carbon source (164.34±2.26 U/L) and 5% amount of feeding of carbon source (162.3±2.28 U/L), the study was continued with 5% amount of feeding of carbon source.

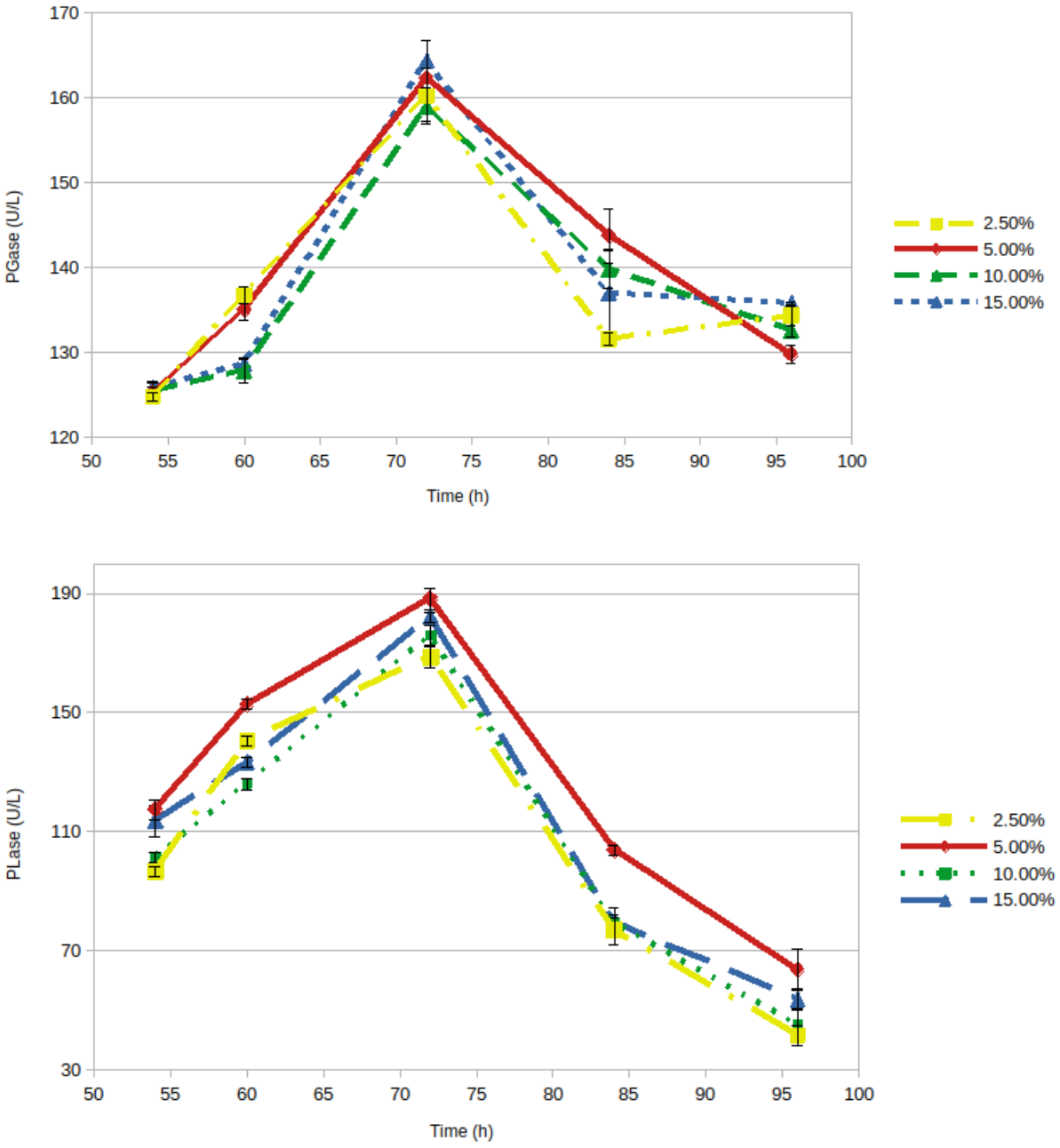


Figure 12. Changes in enzyme activities depending on the different amount of feeding of carbon source and time

3.8.5 Effect of 5% Amount of Feeding of Carbon Source on Amount of Total Protein with PGase and Plase

The cultivation was started as a batch mode for 96h. At 54th h, and before entering the stationary growth phase, black carrot pomace hydrolysate as a carbon source was added at 5% amount of feeding of carbon source. Pea protein, pH, fermentation time,

temperature, inoculum volume (%v/v), magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4] were kept at a constant.

Enzyme activities and total protein content were measured to determine the effect of 5% amount of feeding of carbon source on amount of total protein (Figure 13). The highest total protein content (52.25±0.059 mg/L) occurred at 72th hours with PGase activity and PLase activity at 5% amount of feeding of carbon source.

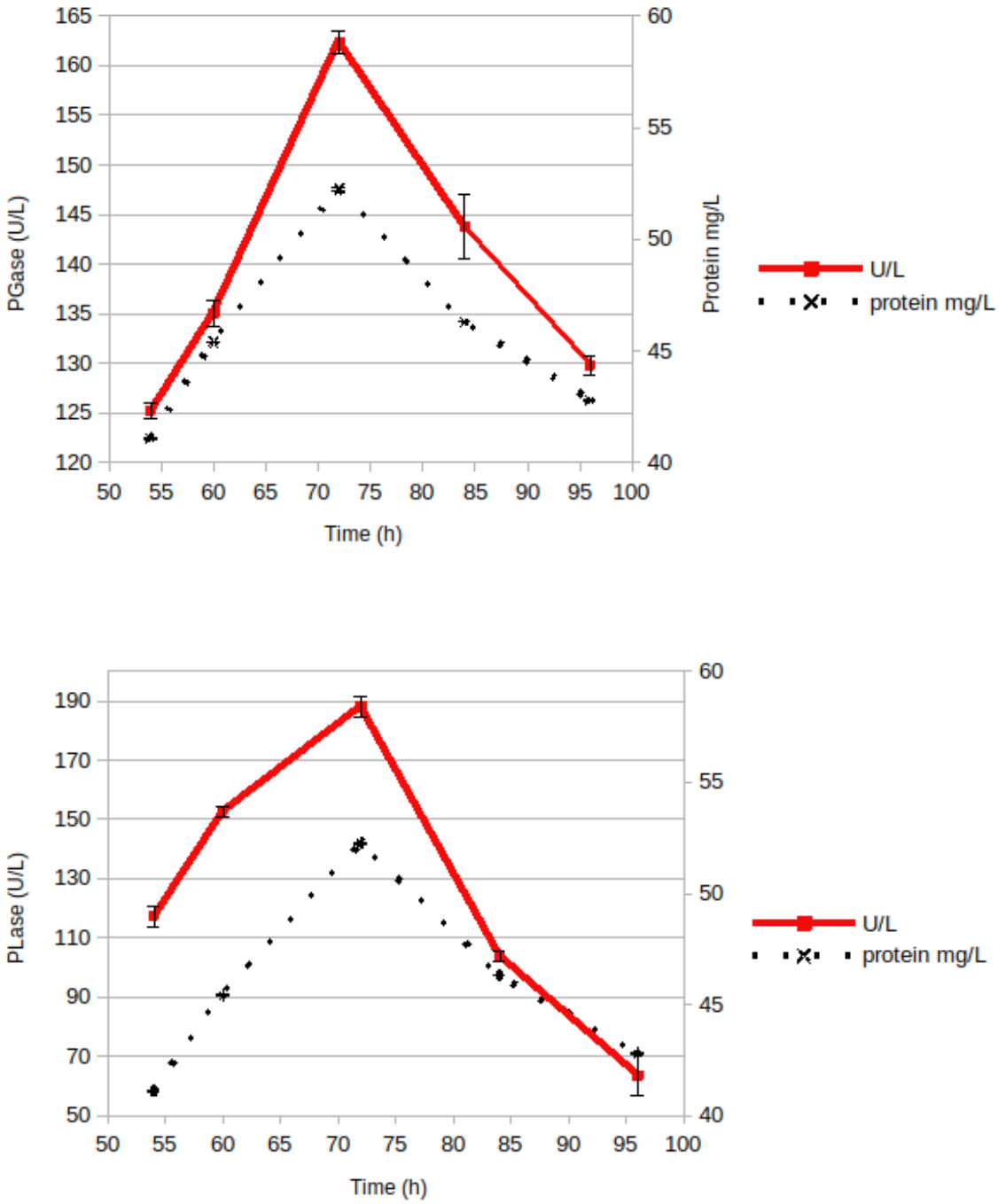


Figure 13. Relationship between PGase and protein content – PLase and protein content

Zou et al. (2014) in their study on the production of alkaline polygalacturonate lyase from *B.subtilis* in the fed-batch system, determined that the enzyme activity and the total protein amount were directly proportional. The following decrease could be due to; the pause of protein secretion because of the fast growth of cells, the proteins because of the degradation of over time.

3.8.6 Effect of 5% Amount of Feeding of Carbon Source on Fermentable Sugar Content with PGase and PLase Activity

The cultivation was started as a batch mode for 96h. At 54^{th} h, and before entering the stationary growth phase, BCP hydrolysate as a carbon source was added at 5% feed concentration. Pea protein, pH, fermentation time, temperature, inoculum volume (%v/v), magnesium sulphate [$MgSO_4$], and dipotassium hydrogen phosphate [K_2HPO_4] were kept at a constant. Enzyme activities and fermentable sugar content were measured to determine the effect of 5% amount of feeding of carbon source on fermentable sugar content (Figure 14).

FSC were measured before and after feeding of fresh substrate. In the first hour of feeding, the FSC and enzyme activity increased. Depending on the time, the FSC decreased with the continuation of the enzyme activity.

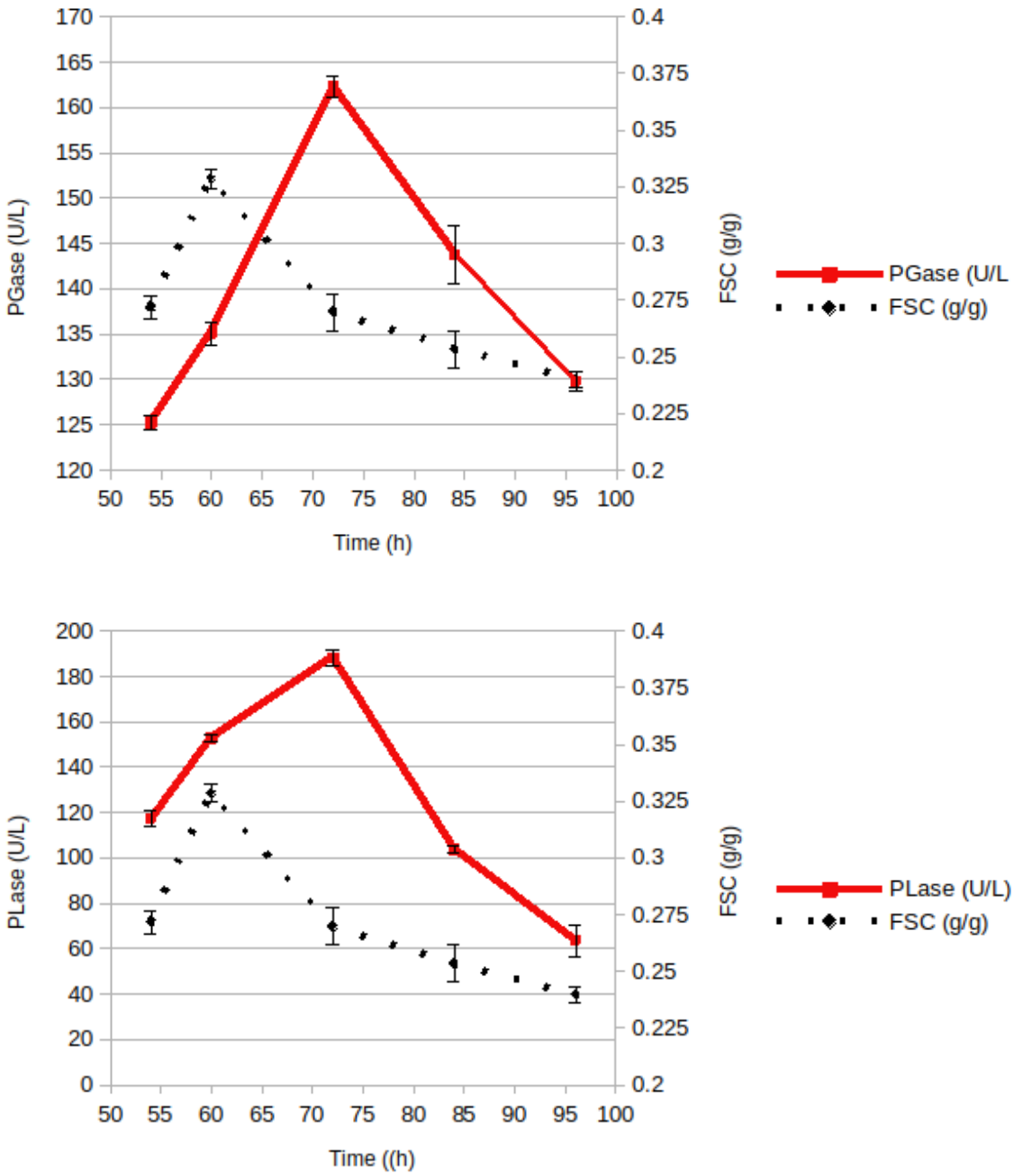


Figure 14. Relationship between PGase activity and FSC at 5% amount of feeding of carbon source and PGase activity and FSC at 5% amount of feeding of carbon source.

3.9 SDS-PAGE Analysis

The sample taken at the 72th hour of the enzyme activity at fed batch was centrifuged. It was subjected to SDS-PAGE analysis. Sample was stained with silver nitrate (Figure 15). Two bands with molecular weights of 38.52 kDa and 26.86 kDa,

respectively, were seen in the resulting image. Since polygalacturonase and pectinlyase are enzymes that can be obtained from many different microorganisms, there are differences in the molecular weight of these enzymes reported in the literature. Karahalil et al. (2017) obtained polygalacturonase from *Aspergillus soaje* in a study they conducted, and stated that the molecular weight of the polygalacturonase enzyme they obtained was in the range of 40-50 kDa. Swain and Ray (2010) found the molecular weight of the enzyme in the range of 45-60 kDa in their study on the production of polygalacturonase using *Bacillus subtilis* CM5. Rahman et al. (2019) studied the production of polygalacturonase enzyme from *Bacillus paralicheniformis* CBS32 and reported the molecular weight of the enzyme they obtained as 110 kDa. Alqahtani et al. (2022) found the molecular weight of the polygalacturonase enzyme, which they produced from *Bacillus subtilis* 15A-B92, to be 14.41 kDa in a study they conducted.

Bai et al. (2012) produced pectin lyase from *Bacillus clausii* in a study they conducted, and reported the molecular weight of the enzyme obtained in the relevant study as 35kDa. Yadav et al. (2017) purified an alkaline pectin lyase from *Fusarium oxysporum* MTCC 1755. It was found the molecular weight of the purified enzyme was determined by was determined to be approximately 31 ± 01 kDa. Sinitsyna et al. (2007) found the molecular weight of the extracellular pectin lyase enzyme obtained from *Penicillium canescens* as 38 kDA in their study.

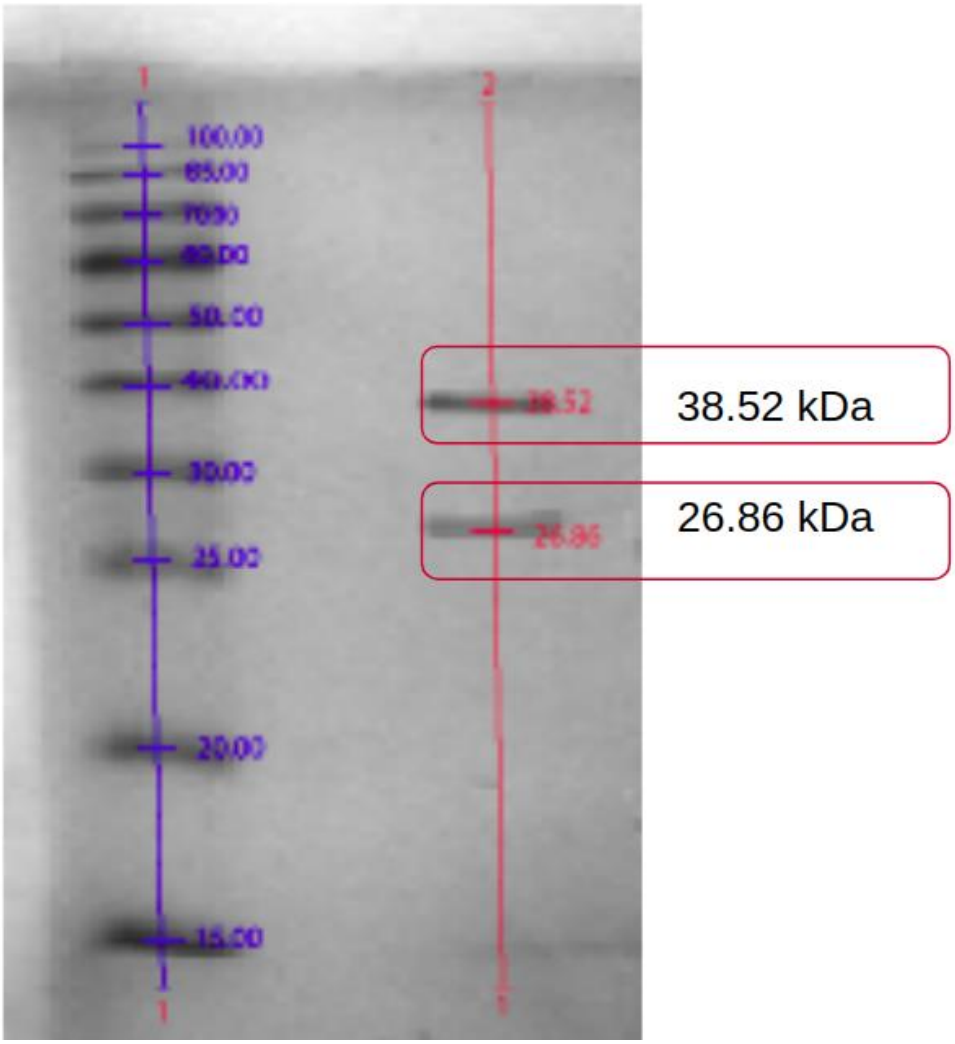


Figure 15. SDS-PAGE image. Blue is a standard and red is sample.

CHAPTER 4

CONCLUSION

This study showed that BCP can be used as an alternative source of carbon in pectinolytic enzyme production from *Bacillus subtilis* by submerged fermentation. For this purpose, in order to increase the production of pectinolytic enzymes, pretreatment methods such as thermal, thermochemical and microwave assisted dilute acid, which is an innovative technology, were used to obtain fermentable sugar, and fed-batch cultivation was tried as the cultivation type.

Microwave assisted dilute acid pretreatment method gave the best results according to the fermentable sugar amounts obtained from the three different pretreatment methods used in the study and the amount of energy consumed. When microwave assisted dilute acid pretreatment (18.3 KJ/g) is compared with thermal method (8.612 KJ/g) , the energy consumption is very low by microwave asssisted dilute acid pretreatment. The maximum FSC obtained from MW-DA pretreatment was obtained as 0.493 g/g (87.3% conversion yield) with 300 W for 30s followed by enzymatic saccharification step. MW-DA pretreatment sequential with enzymatic saccharification has provided a high amount of FSC in a short period of time and energy consumption with minimum formation of inhibitors. The combined microwave assisted dilute acid and enzymatic hydrolysis was also found more effective than microwave assisted dilute acid pretreatment alone. The results indicated that BCP is an preferable source of fermentable sugars and BCP for source of production of various value added products.

Black carrot pomace and pea protein, an process waste of who fruit and vegetable industry, was shown to may use as an appropriate carbon and nitrogen source for the production of pectinolytic (pectinase) enzymes by *Bacillus subtilis.*

When batch type cultivation (PGase; 139.33±0.48 U/L, PLase; 140.96±6.15 U/L) is compared with fed batch cultivation (PGase; 164.34±2.26 U/L, PLase; 162.3 ±2.28 U/L), the amount of enzyme produced by fed batch cultivation increased both of them.

This study has been a pioneering study in terms of adaptability of pea protein as a plant-based protein sources to bioprocess systems. In the lead of this study, there is a need for studies on the adaptability of other plant-based proteins (soy, lentil, chickpea, bean etc.) to bioprocess systems. The effects of pea protein on cell growth and increasing enzyme activity should be investigated. In addition, the stability, kinetic studies, scale up studies and clarification studies of the enzymes whose activities were measured in this study should be made and brought to the literature.

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APPENDICES

APPENDIX A

Table A.1 Chemicals with supplier information.

Chemicals	Information
Bovine serum albümine	Sigma-Aldrich
Cellulase from *Trichoderma reesei*	Sigma-Aldrich
Citrus Pectin	Sigma-Aldrich
$CuSO_4.5H_2O$	Merck
D-glucose	Merck
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
DNS (3,5-dinitrosalicylic acid)	Sigma-Aldrich
Folin-ciocalteu	Sigma-Aldrich
Galacturonic acid monohydrate	Sigma-Aldrich
Hexane	Merck
Hydrocloric acid	Merck
Magnesium sulphate ($MgSO_4.7H_2O$)	Merck
Nutrient Agar	Merck
Pea protein	Roquette
Phenol	Merck
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck
Sodium phosphate dibasic	Merck
Sodium phosphate monobasic	Merck
Sodium hydroxide	Merck
Sodium potassium tartarate	Merck
Sulphuric acid	Merck
Thiobarbutiric acid	Sigma-Aldrich
Viscozyme L	Sigma-Aldrich
Whey protein	Hardline
Yeast extract	Merck

APPENDIX B

Buffer and Solition

Composition of Phosphate Buffer (pH 7)

Stock solutions:

A: 0.1 M solution of sodium phosphate monobasic

B: 0.1 M solution of sodium phosphate dibasic

39 mL of solution A and 61 ml of solution B are mixed in 200 mL total volume to adjust the pH 7.0.

40% Rochelle Salt

40 g sodium potassium tartarate add to 100 mL distilled water.

0.04 M Thiobarbutyric Acid Solution

0.23 g thiobarbutyric acid add to 40 mL distilled water.

APPENDIX C

Table C.1 Composition of DNS reagent

Chemicals	Amount (g/100 mL)
DNS	1
Phenol	0.2
Rochelle salt	40
Sodium hydroxide	1

APPENDIX D

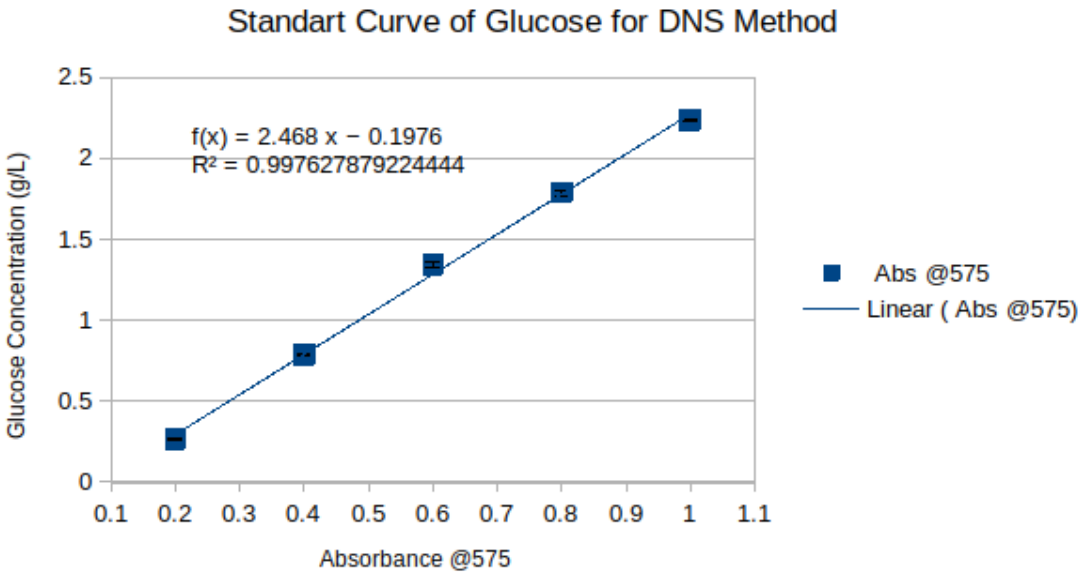

Figure D.1 The standard curve for glucose concentration by DNS method**.**

The total fermentable sugar concentration was calculated as below:

$$The\ total\ fermentable\ sugar\ concentration\ \left(\frac{g}{L}\right)$$
$$= \left(\frac{absorbance + 0.1976}{2.468}\right) \times dilution\ factor$$

APPENDIX E

Standart Curve of Polygalacturonase Acitivity

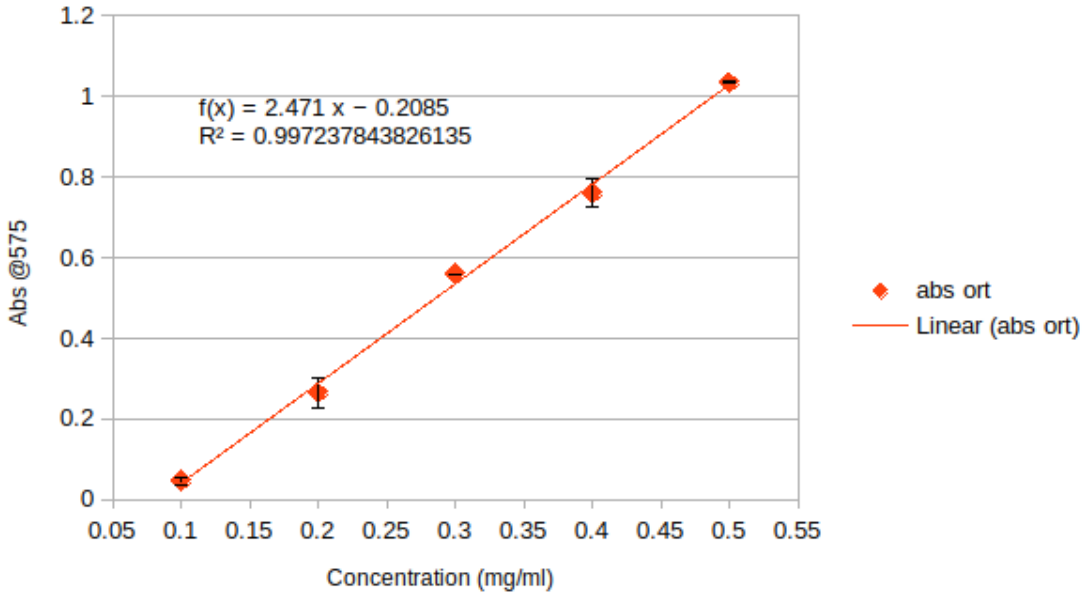

Figure E.1 D-Galacturonic acid standard curve for determination of polygalacturonase activity

$$\frac{U}{L} = Absorbance \times F \times \frac{1}{incubation\ time} \times \frac{1}{212.12} \times R$$

F= a factor to convert absorbance to g of galacturonic acid

Incubation time= time of incubation of the enzyme with a substrate (30 min)

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

DF=Dilution factor

R_v=Amount of enzyme in the reaction mixture (ml)

APPENDIX F

Growt Curve of Microorganism

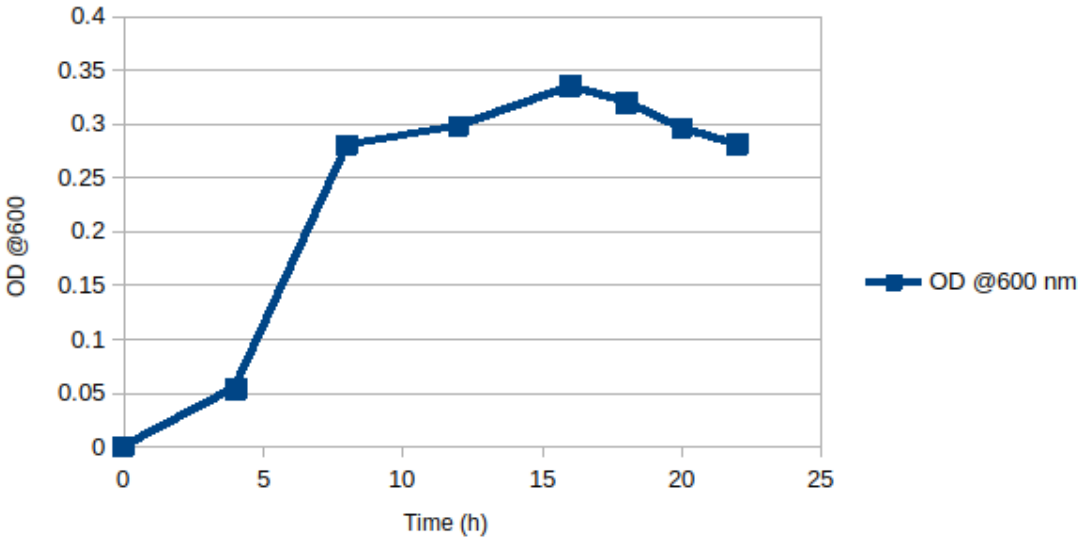

Figure F.1 Growth curve of *B.subtilis*

APPENDIX G

Lowry Protein Assay

Solutions

A

(alkaline solution, for 500 ml)

2.8598 g NaOH

14.3084 g Na_2CO_3

B

(for 100 ml)

1.4232 g $CuSO_45H_2O$

C

(for 100 ml)

2.85299 g Na_2Tartarate.2(H_2O)

Lowry solution

(fresh:0.7 mL/sample)

A + B + C with a ratio (v/v) of (100:1:1)

Folin Reagent

(instant fresh, 0.1 ml/sample)

5 mL of 2N Folin Ciocalteu Phenol Reagent + 6 mL distilled water. This solution light-sensitive. So it should be prepared at the last 5 min of the first sample incubation and kept in an amber container.

BSA Standard

1 mg BSA in 1 mL of water

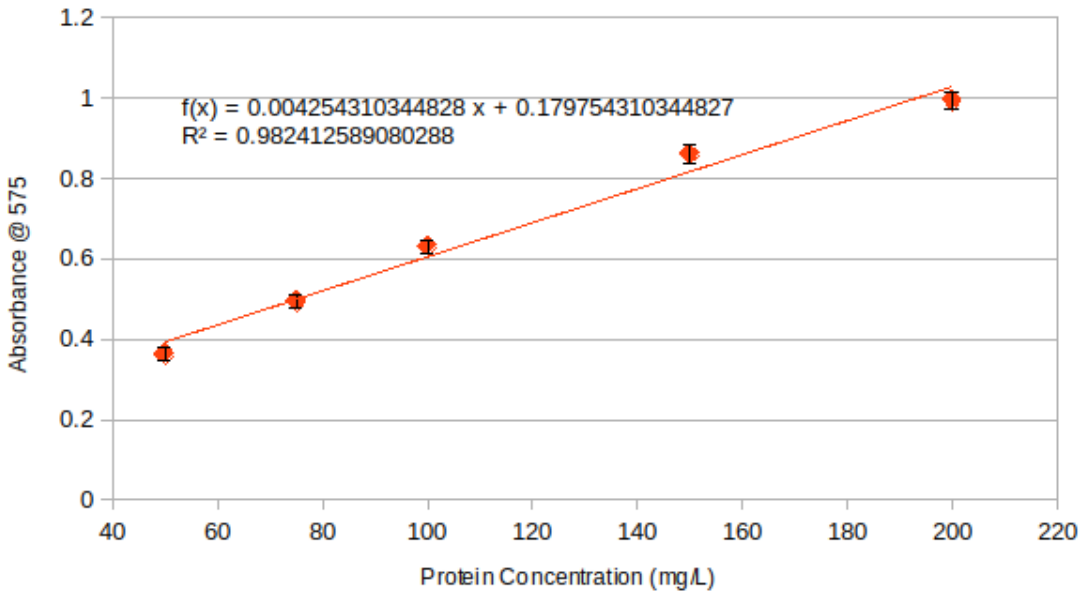

Figure G.1 BSA standard curve used in Lowry Method for the determination of total protein concentration