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GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF THE BIOTECHNOLOGY

UTILIZATION POTENTIAL OF AGRO-INDUSTRIAL BY-PRODUCTS AND WASTE SOURCES: LACCASE PRODUCTION IN BIOREACTOR WITH K*omagataella pastoris*

BUSE ÇALOĞLU
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
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THESIS SUPERVISOR

ASSOC. PROF. DR. BARIŞ BİNAY

GEBZE

2023

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

TARIMSAL ENDÜSTRİYEL YAN ÜRÜNLERİN VE ATIK KAYNAKLARININ KULLANIM POTANSİYELİ: *Komagataella pastoris* İLE BİYOREAKTÖRDE LAKKAZ ÜRETİMİ

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DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

GEBZE
2023

GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

GTÜ Biyoteknoloji Enstitüsü Yönetim Kurulu'nun 17/07/2023 tarih ve 2023/14 sayılı kararıyla oluşturulan jüri tarafından 28/07/2023 tarihinde tez savunma sınavı yapılan Buse ÇALOĞLU'nun tez çalışması Biyoteknoloji Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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…… tarih ve ……. sayılı kararı.

İMZA/MÜHÜR

SUMMARY

Circular bioeconomy is a model applied to bioprocesses. The assurance of a circular bioeconomy model dependent on the billion metric tons output of agricultural by-products and wastes every year made valorization of waste materials a desirable strategy. With *Komagataella pastoris* fermentation serving as a significant platform for the manufacture of value-added bioproducts, particularly recombinant proteins, research is moving quickly to apply the model to these processes.

In the scope of this study, the synthesis of *Madurella mycetomatis* laccase in the *K. pastoris* X33 expression system was examined using solid dextrose and liquid dextrose from an agricultural processing plant and watermelon peel from a market. Fermentation with glycerol/methanol was taken as a reference study and studies showed that the laccase activity was about 2-fold and 1.30-fold higher for the solid dextrose and liquid dextrose fermentation strategies, respectively.

Although the development stage produced the lowest cell density, watermelon peel hydrolysate was used to produce the highest laccase activity. The influence of carbon sources stimulating the creation of recombinant proteins in *K. pastoris* was confirmed by using one-way ANOVA analysis. These findings demonstrated bioprocess viability which was created to valorize by-products and wastes in *K. pastoris* cultivations.

Keywords: Agro-industrial wastes, *Komagataella pastoris*, laccase enzyme, bioprocess development, circular bioeconomy

ÖZET

Yıllık, milyar metrik ton tarımsal-endüstriyel yan ürün ve atık üretimine bağlı olarak, biyoproseslerde atık malzemelerin değerlendirilmesi, döngüsel biyoekonomi modeli ile çekici bir yaklaşım olmaktadır. *Komagataella pastoris* fermantasyonunun, özellikle rekombinant proteinler olmak üzere katma değerli biyolojik ürünler üretmek için etkili bir platform olmasıyla birlikte, üretim verimliliğini artırarak döngüsel biyoekonomi modelini bu süreçlere uygulama çalışmaları hız kazanmaktadır.

Bu çalışmada, yerel bir mısır işleme endüstrisinden alınan katı ve sıvı dekstroz ve yerel bir pazardan alınan karpuz kabuğu, biyoreaktörde *K. pastoris* X33 konak hücresinden *Madurella mycetomatis* lakkaz üretimi için değerlendirilmiştir. Kontrol çalışması olarak yapılan gliserol/metanol içeren fermantasyona göre alternatif kaynak kullanımı ile katı dekstroz kullanımından 41 g DCW/L ile yaklaşık 2 kat ve sıvı dekstroz kullanımından 50 g DCW/L ile lakkaz aktivitesinde yaklaşık 1,30 kat artış tespit edilmiştir. Karpuz kabuğu hidrolizatı kullanımında maksimum lakkaz aktivitesi elde edilirken, büyüme evresinde en düşük hücre yoğunluğu gözlemlenmiştir. Tek yönlü ANOVA analizi ile *K. pastoris* ekspresyon sisteminde heterolog protein üretimini teşvik eden karbon kaynaklarının etkisini doğrulanmıştır. Bu sonuçlar göz önüne alındığında *K. pastoris* fermantasyonunda alternatif karbon kaynakları olarak katı/sıvı dekstroz ve karpuz kabuğu hidrolizatının değerlendirilmesi için tasarlanan biyoproseslerin gelecekteki ölçeklendirme için uygunluğunu kanıtlanmıştır.

Anahtar kelimeler: Tarımsal endüstriyel atıklar, *Komagataella pastoris*, lakkaz enzimi, biyoproses geliştirme, döngüsel biyoekonomi

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Barış BİNAY for the continuous support of my MSc study, and his patience, motivation, and immense knowledge.

Besides my supervisor, I would like to thank Kübra LAÇIN, Berin Yılmazer Aktar, Mehmet Mervan Çakar, and all my friends from GERC Lab at Gebze Technical University. I would like to express my endless thanks to Gebze Technical University Gebze Enzyme Recognition Center (GERC) for the infrastructure opportunities they have provided for me to carry out my studies.

In the end, I would like to express appreciation to my beloved family, İnci ÇALOĞLU, Nedim ÇALOĞLU, and Tuğçe ÇALOĞLU for their love and endless support. There aren't enough words to express how grateful I am to them. They taught me the importance of having principles and values which helped me to become the individual I am today.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
aa	:	Amino acid
BMGY	:	Buffered Glycerol Complex Medium
DMP	:	2,6–dimethoxyphenol
DNA	:	Deoxyribonucleic acid
DO	:	Dissolved Oxygen
*Eco*RI	:	*Escherichia coli* RY13 I
Ferm	:	Fermentation
GERC	:	Gebze Enzyme Recognition Center
k_{cat}	:	Turnover number
kDa	:	Kilo Dalton
K_m	:	Michaelis–Menten constant
LD	:	Liquid Dextrose
M	:	Molar
mM	:	Millimolar
*Mm*Lac	:	Laccase gene encoding from *Madurella mycetomatis*
min	:	Minute
mL	:	Milliliter
NCBI	:	National Center for Biotechnology Information Database
*Pme*I	:	*Pseudomonas mendocina* I
rpm	:	Rounds per minute

RPP	:	Recombinant Protein Production
*Sal*I	:	*Streptomyces albus* I
sec	:	Second
SD	:	Solid Dextrose
U	:	Units
UniProt	:	Universal Protein Resource
V	:	Volt
YNB	:	Yeast Nitrogen Base
YPD	:	Yeast Nitrogen Dextrose
$Y_{x/s}$	:	Biomass yield from substrate
WPH	:	Watermelon Peel Hydrolysate
μg	:	Microgram
μL	:	Microliter
μ_{max}	:	Maximum specific growth rate

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1. INTRODUCTION

1.1 The Aim and Contribution

This thesis primarily addresses two issues: assessing the possibility of use of wastes and by-products from the agro-industrial sector, and enhancing the small-scale expression yield of recombinant laccase.

The circular bioeconomy is a fascinating economic model that fights resource depletion by promoting sustainable production (Muscat et al., 2021). Agricultural sector, which removes wastes having nutrient-rich content, makes it the most common utilizing field for circular bioeconomy applications. These features of the agricultural industry provided the studies by integrating the industry to bioprocesses and wastes became the most favored as raw materials (Awasthi et al., 2022).

Application of the circular bioeconomy is required depending on the high media costs in industrial-scale bioprocesses, particularly in the heterologous protein production from a methylotrophic yeast; *Komagataella pastoris* (*K. pastoris*, also known as *Pichia pastoris*), that has ability to benefit from methanol as carbon and energy source (Ata et al., 2021; Yadav et al., 2011). It is currently among the most researched hosts in recombinant protein production (RPP) (Ergün et al., 2021).

The major objective is to develop bioprocess models using wastes in accordance with the circular bioeconomy model. According to searches, there are more than 100 million tons of watermelons produced worldwide each year (Jiang et al., 2022). Starch Manufacturers Association of Turkey (NISAD) reported its predictions with starch sugar and maize starch exportation of about 431,568 tons and 198,241, respectively in 2021 (Starch Industry Association (NISAD), 2021). These productions generated a significant amount of trash and byproducts, which drew attention to the bioprocesses development. In the scope of the thesis, evaluation of solid/liquid dextrose as agro-industrial by-products and watermelon peel as agro-industrial waste as a carbon source instead of glycerol were performed in order to determine the suitability of by-products and wastes for *K. pastoris* cultivation during growth phase and laccase enzyme expression in a bioreactor.

2. LITERATURE SURVEY

2.1 Circular Bioeconomy

A circular bioeconomy is a new economic paradigm that replaces the large range of non-renewable, fossil-based products now in use by emphasizing the utilization of renewable natural capital and emphasizing waste minimization.

The linear economy model was identified as the take-make-dispose paradigm. Moving away from a linear economy and toward a circular bioeconomy contribute to the construction of a sustainable bioeconomy with the principles of recycling, reuse, remanufacturing, and maintenance of waste biorefineries (Figure 2.1) (Leong et al., 2021).

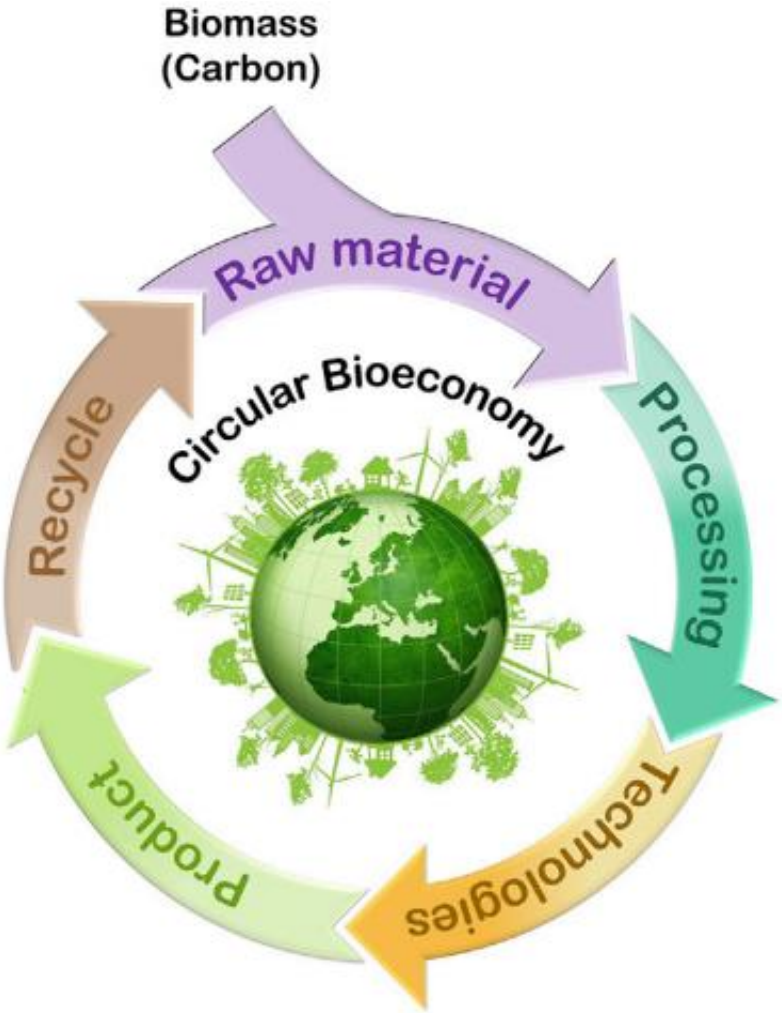

Figure 2.1: General schematic representation of circular bioeconomy model using biomass (Caetano et al., 2022).

According to circular bioeconomy design, the method differs from existing systems because it uses materials for as long as feasible and adopts methods that reduce emissions. The circular way by using technical enhancements and scientific methods is commonly used design for industrial productions to provide more sustainable materials and regeneration (D'Amato, Veijonaho, and Toppinen, 2020; Stegmann, Londo, and Junginger, 2020; Carus and Dammer, 2018;).

Bioprocess focuses on value-added bioproduct production in a way of renewable and environmentally friendly biodegradability, representing superior waste stream management. Municipal solid and liquid waste are regarded as waste-to-treasure and valorization of them contributes to sustainable bioproduct formation in bioprocesses. Depending on the sustainable and affordable production feedstock, waste biorefinery bioprocesses on RPP, polymers, and bioenergy also addresses the energy and environmental security challenges with an economic and environmental platform (Leong et al. 2021).

2.2 *Komagataella pastoris*

A methylotrophic yeast, *Komagataella pastoris* utilize methanol as a carbon and energy source, in the family Ascomycetes. Guilliermond initially discovered *K. pastoris* in the exudates of a chestnut tree in France, and it was given the scientific name *Zygosaccharomyces pastoris* (Ata et al., 2021; Zahrl et al., 2017).

When it was discovered, *Komagataella pastoris* was first used in single-cell proteins productions as feedstock owing to its capacity to reach high cell densities in the cultivations in simple medium including methanol as a carbon source. Methanol production from methane is quite affordable in the late 1960s and depending on this conversion, single-cell protein production from methanol by utilizing *K. pastoris* was regarded as economically viable. The creation of foreign proteins by using The Pichia expression system was constructed by Salk Institute Biotechnology/Industrial Associates (SIBIA) and the expression system was made available in 1993 by Phillips Petroleum Company. After the availability of it in academic platforms, it has gained attention gradually with their easy allowance to genetic modification (Figure 2.2) (Robinson, 2014).

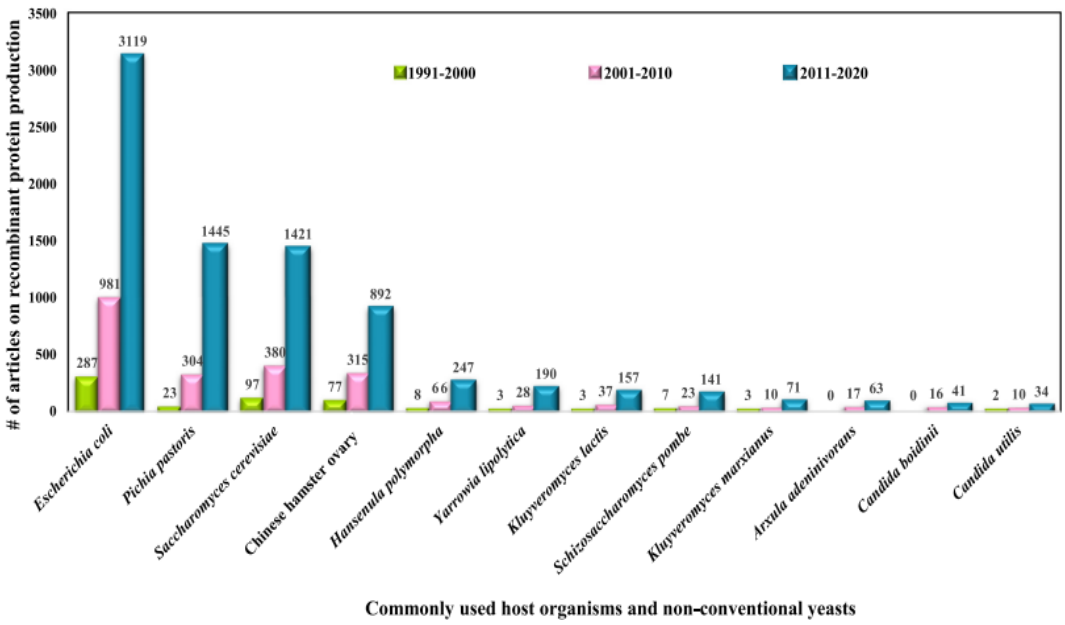

Figure 2.2: Number of articles for recombinant protein production from different host cells according to three different periods (Ergün et al. 2021).

Genetic manipulation of *K. pastoris* is made easier by the physiological control of mating. It is easiest to isolate and characterize mutants since it is most stable in their vegetative haploid condition. Y-11430 strain is wild type strain and X33, KM71, GS115, SMD1168H are some of the strains used for recombinant protein production under the promoters like strong promoters of alcohol oxidase (pAOX), The glyceraldehyde-3-phosphate dehydrogenase (pGAP) (Karbalaei, Rezaee, and Farsiani, 2020).

AOX, a necessary enzyme for metabolizing methanol, is produced by *K. pastoris*. Methanol conversion to formaldehyde is the first stage in the metabolism of methanol catalyzing by AOX and the process produces hydrogen peroxide. This reaction happens inside the peroxisome, a specialized membrane-bound organelle. When *K. pastoris* uses methanol, there is a noticeable multiplication of peroxisomes for the removal of the harmful hydrogen peroxide from the rest of the cell. Additionally, AOX has a low oxygen affinity. Alcohol oxidase activity is encoded by the *AOX1* and *AOX2* genes. The *AOX1* promoter is carefully controlled, methanol induces it, but under other circumstances, such as carbon deprivation, it remains suppressed. Particularly suited for the regulated production of foreign genes is this promoter (Robinson, 2014).

There are other inducible and constitutive promoters, such as the glutathione-dependent formaldehyde dehydrogenase gene (pFLD1) (Baerends et al. 2002), and glyceraldehyde-3-phosphate dehydrogenase gene (pGAP) (Türkanoğlu Özçelik, Yılmaz, and Inan 2019). pFLD1 is independently inducible by methylamine and methanol, and pGAP have also been used for RPP in *K. pastoris* (Yılmaz, and Inan, 2019; Wang et al., 2019; Baerends et al., 2002).

K. pastoris expression has advantages for RPP with strong similarities to cutting-edge eukaryotic expression systems like CHO cell lines that are one of its benefits and reasonably priced, contain co-translational, posttranslational processing, and relatively quick expression durations. Furthermore, *K. pastoris* is an appropriate microorganism for the extracellular expression of recombinant proteins straight into the culture medium's supernatant. *K. pastoris* synthesis endogenous protein restrictedly and owing to this restricted synthesis, recombinant protein purification is simple. Additionally, ability of easy scalability of *K. pastoris* is important depending on the utilization of industrial bioreactors to manufacture desired proteins in big quantities (Robinson, 2014).

Any recombinant gene expression in *K. pastoris* can be achieved through three steps: vector selection and relevant gene cloning to vector; transformation of the vector into the genome of *K. pastoris*; and (c) trials with the strains to test the expression of the recombinant integrated gene (Figure 2.3). According to the EasySelect^{TM} Pichia Expression Kit (Invitrogen), the cloned vector must first be linearized treating with restriction enzymes (SacI, PmeI, and BstX). The competent cells should next be electroporated with linear DNA. Through the crossover recombination event, the entering gene is incorporated into the cell genome (Karbalaei, Rezaee, and Farsiani, 2020).

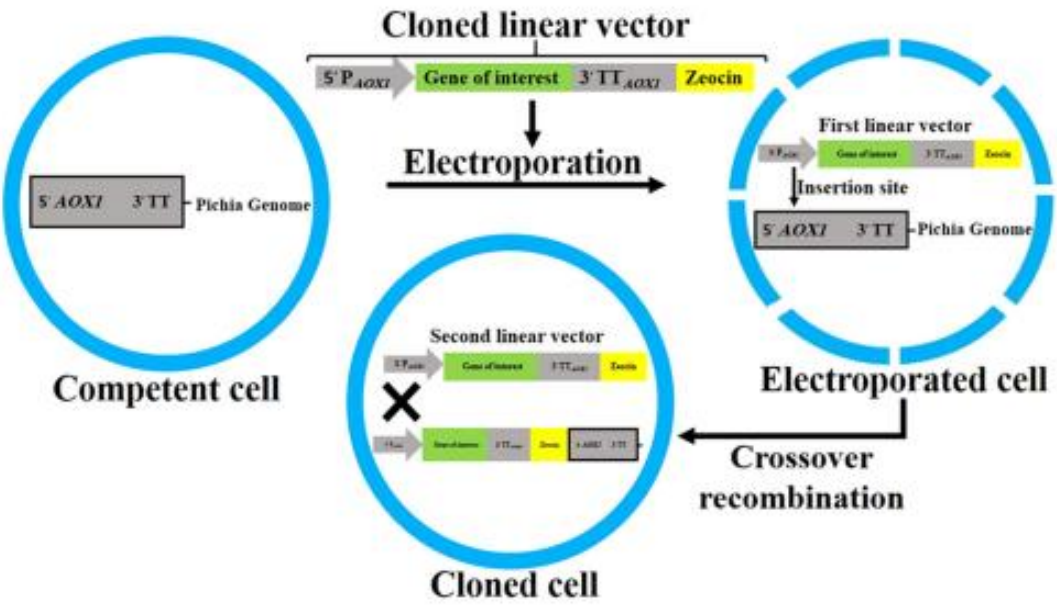

Figure 2.3: Schematic view of *K. pastoris* transformation process steps (Karbalaei, Rezaee, and Farsiani, 2020).

In the studies for *K. pastoris* expression after the transformation procedure, methanol, glucose, and glycerol are the common carbon sources for *K. pastoris* fermentations. Nevertheless, some studies exhibit other utilizable and non-utilizable sources for *K. pastoris* cultivations (Figure 2.4) (Ergün et al., 2022).

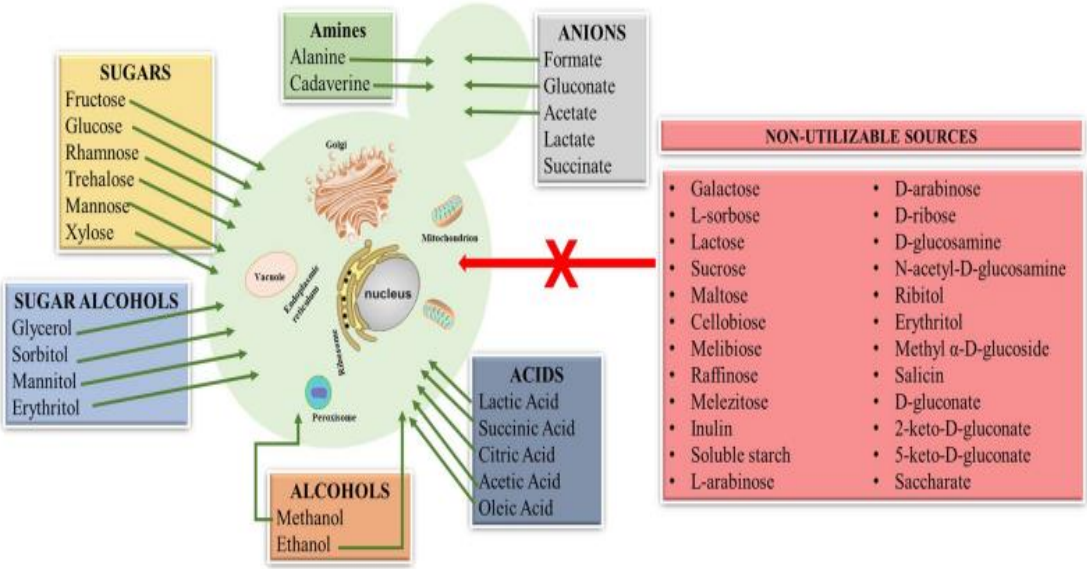

Figure 2.4: Non-utilizable and utilizable sources of *K. pastoris* (Ergün et al., 2022).

2.3 Bioprocess Development

Fermentation plants are an example of a bioprocess plant that uses microorganisms and/or enzymes. These plants have many traits with chemical plants. Depending on it, it would be advantageous to use a engineering scientific approach to the design of various plants involving biological systems, taking into account the variations in the physical properties of certain materials (Basile, 2013).

Preparation, manufacturing, and purification are the three main steps that bioprocesses can be divided into. While bioreaction kinetics, oxygen transfer, and operational strategy are crucial to the production stage, as well as separation procedures for product purification, preparation typically involves nutrient medium and equipment sterilization. But in addition to these three phases' optimization, a biocatalyst that can effectively use substrate and produce the required product with little to no byproducts is also essential to a successful bioprocess (Clarke, 2013).

Numerous chemical and biological reactions take place throughout bioprocesses. Reactants, their type of usage, compositions, and also products' composition must be understood in order to successfully build a reactor. For example, it is necessary to calculate the production, disappearance, and mass balance of specific components to model behavior in a bioreactor including biochemical reactions (Basile, 2013).

Utilization of industrial fermentation processes has reached common applications in the manufacturing of chemicals, pharmaceuticals, vaccines, and other products (Gernaey, 2015). Chemical engineers use the phrase "industrial fermentation" to refer to processes that utilize a chemical change brought about by a living thing or an enzyme, particularly bacteria, yeasts, molds, or fungus, that results in the production of a particular product (Mazzeo and Piemonte, 2020).

2.4 Recombinant DNA Technology

Recombinant DNA technology is a method that provides to change a genetic material of a host organism to gain an organism new traits and entails inserting DNA fragments with acceptable gene sequences.

A gene was cloned to a suitable vector according to the chosen host organism. In the manipulation process of the genome, the addition of one or more new genes and also regulatory elements or recombination of existing genes and regulatory elements to reduce or prevent the expression of endogenous genes can take place. The first recombinant DNA (rDNA) molecules were produced in 1973 and numerous health-improving items including therapeutic agents, hormones, vaccinations, and diagnostic technologies have been created since the mid-1980s (Khan et al., 2016).

Recombinant DNA technology can be utilized for a broad range of industrial purposes such as applications in agriculture including enhanced nutrition and disease resistance in animals as well as increased productivity, disease resistance in plants and expanded habitats. Production of substances like hormones, cytokines, blood proteins, and novel vaccinations are all application examples in human health areas. Increased manufacture of already available antibiotics as well as the creation of new antibiotics are further uses in the realm of human health. Other pharmacological applications include the improvement of chemotherapeutic drugs, disease diagnosis, and genetic problem treatment. Recombinant DNA technology may potentially be utilized to develop new fuel generation methods and address issues with the biodegradation of household and commercial trash (Jones and Fayerman, 1987).

2.5 Enzymes

Enzymes, which have a backbone with N- and C-termini and an active site, are biological catalysts that speed up biochemical reactions. It is acknowledged that Wilhelm Kuhne coined the term "enzyme" in 1877 to describe a biological catalyst. Today, all enzyme names end with the "-ase" suffix, as suggested by Émile Duclaux in 1898. A notable exception to this general norm pertains to certain proteolytic enzymes (O’Connor, Adams, and Fairman, 2010).

Enzymes are produced by microorganisms, animals, or vegetables. Enzymes are not consumed and changed in chemical reactions, additionally, there is no change in the equilibrium of a reaction. They attend to different metabolic reactions leading to the creation and oxidation of organic compounds in cells, digestion, muscle contraction, and cell respiration; these events are made possible by the catalytic function of enzymes by decreasing the activation energy of the reaction contributing

to reaction rate. Reaction rate is the significance of an enzyme for the survival of life. The pace at which enzymes catalyze processes is influenced by a variety of factors such as concentration of the substrate and the enzyme, pH, temperature, reaction time, reaction products, enzyme inhibitors and activators, radiation, pressure, and light, increasing or reducing their activity (Harvey and Ferrier, 2011; Nelson and Cox, 2001).

The International Association of Biochemistry and Molecular Biology (IUBMB) divided the enzymes into six primary classes, each of which contained subgroups according to the reactions they catalyze. Enzyme codes are made up of four numbers separated by dots, and the first number identifies the six primary enzyme groups of the enzyme while the other digits append more and more specificity (Table 2.1). For example, laccase (EC 1.10.3.2) belongs to an oxidoreductase (EC 1) class that uses diphenols and related substances as donors (EC 1.10) with oxygen as acceptor (EC 1.10.3) (Harvey and Ferrier, 2011).

Table 2.1: Enzyme classification according to the International Association of Biochemistry and Molecular Biology.

Class	Name	Enzyme Code	Catalyzed Reaction
1	Oxidoreductases	EC 1.X.X.X	Electron transfer
2	Transferases	EC 2.X.X.X	Functional groups transfer
3	Hydrolyses	EC 3.X.X.X	Breaking of bonds with water
4	Lyses	EC 4.X.X.X	Breaking of C-C, C-O, C-N, and other bonds, often forming a double bond
5	Isomerases	EC 5.X.X.X	Group exchanges within a molecule
6	Ligases	EC 6.X.X.X	Bond formation by ATP hydrolysis

With the advancements in the field of recombinant DNA technology, enzymes that have been utilized in the manufacturing of cheese, vinegar, alcoholic beverages,

and similar goods since the early times have become a focus point for scientific investigations and industrial uses. Enzymes are utilized nowadays in a wide range of industries, including the food business and the medical sector for both diagnosis and therapy (Chandra et al., 2020; KC et al., 2020; Naveed et al., 2021; Reetz, 2013; Wu et al., 2021).

The industrial enzymes market was estimated to be worth $10,807.10 million globally in 2030 with a growth of 6.27% according to the Global Industrial Enzymes Market Anticipated to Reach CAGR (S. Singh, 2022). Currently, 4000 enzymes have been found from roughly 200 different microbial sources, and 75% of these are hydrolytic enzymes with more than 70% of all enzyme sales going to carbohydrases, proteases, and lipases, these three enzyme types dominate the market. Making enzymes that are appropriate for industrial use and lowering the price at which they are produced is of utmost importance (Li et al., 2012).

2.5.1 Oxidoreductases

Peroxidase, reductase, dehydrogenase, oxidase, oxygenase, and hydroxylase are only a few of the various enzymes that make up oxidoreductase (Figure 2.5). It is one of the enzymes present in carrots, the widely grown root crop with an anaerobic and aerobic metabolism. Typical sugars, such as glucose, sucrose, etc., can be transformed into other beneficial compounds using oxidoreductase. However, the majority of the existing methods for processing food include the use of heat, which renders most enzymes inactive, particularly the oxidoreductases (Ramli et al., 2022).

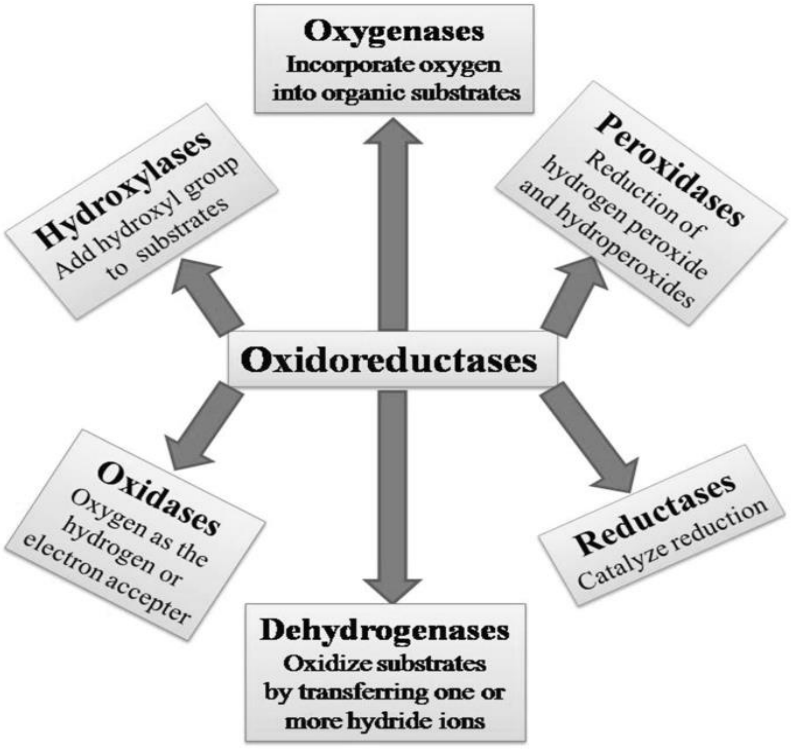

Figure 2.5: Oxidoreductase enzymes and their reaction type (Younus, 2019).

2.5.2 Laccases

Laccase (EC 1.10.3.2) was first found in a plant *Rhus vernicifera* resin in 1883 by Hikorokuro Yoshida. The first study of laccase was done by Gabriel Bertrand for metal analysis of the laccase enzyme. Laccase is a family of multicopper-containing oxidase class of enzymes and catalyzes the oxidation of a broad range of substrates; phenols, non-phenols, amines, anilines and aromatic thiols, and other aromatic compounds combined with the reduction of molecular oxygen to water (Giardina et al., 2010; Thurston, 1994; Yoshida, 1883).

The popularity of the enzyme increased in the 1990s to degrade a wide range of complicated contaminants which are constructively similar to lignin and can be oxidized by fungal laccase (Giardina et al., 2010). Laccase enzymes use molecular oxygen to catalyze different free radical reactions according to their structural properties and reaction. During the reaction, while one molecule of oxygen and four substrate molecules are oxidized, two H_2O molecules are produced, and hydrogen peroxide formation is not observed. The reaction catalyzed by laccase can be seen in Figure 2.6 (Viikari et al., 2009).

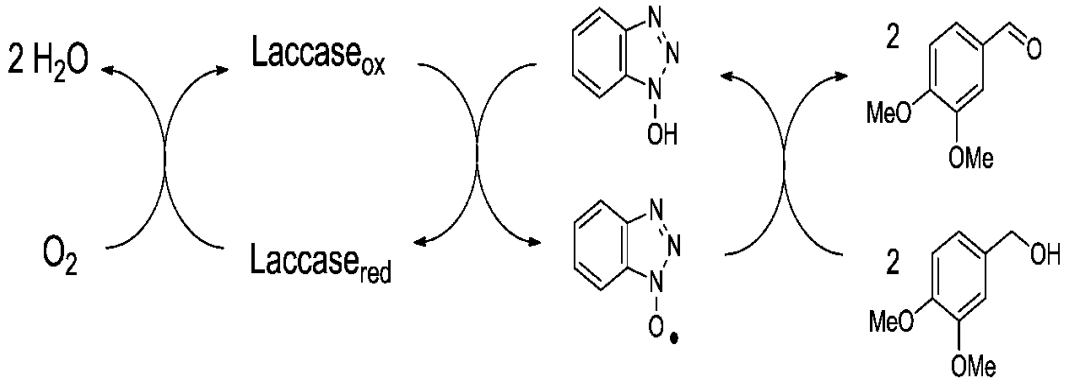

Figure 2.6: Reaction scheme catalyzed by laccases (Woolridge, 2014).

Laccases, have a molecular weight in the range of 50 -130 kDa, and belong to the multicopper oxidases superfamily which is a family of enzymes made up of several different proteins with different substrate specificities and biological functions (Rivera-Hoyos et al., 2013). Multicopper oxidases contain three spectroscopically different copper centers: type I, type II, and type III. They consist of 2 or more homologous domains (Figure 2.7). Therefore, laccases are considered to exist as monomeric, homotetrameric, heterodimeric, or multimeric glycoproteins with copper atoms as part of the cupredoxin superfamily. Therefore, laccases are considered to exist as monomeric, homotetrameric, heterodimeric, or multimeric glycoproteins with copper atoms as part of cupredoxin superfamily. The existence of cupredoxin-like domains provides all multicopper oxidases to reduce oxygen to water without generating harmful by-products. Laccases catalyze the oxidation of a wide range of substrates, and besides, like other oxidoreductases, laccase can perform the catalytic process using molecular oxygen as the sole co-substrate instead of hydrogen peroxide, producing only water as the final byproduct, which is the reason why they are considered as a "green tool”. These multicopper oxidases can be found in bacterial, plants, and fungi. The physiological behavior may be altered based on the source. Most of the biotechnologically beneficial laccases originated from fungi. However, laccases were also identified in plants, insects, and bacteria (Agrawal, Chaturvedi, and Verma, 2018; Moreno et al., 2020).

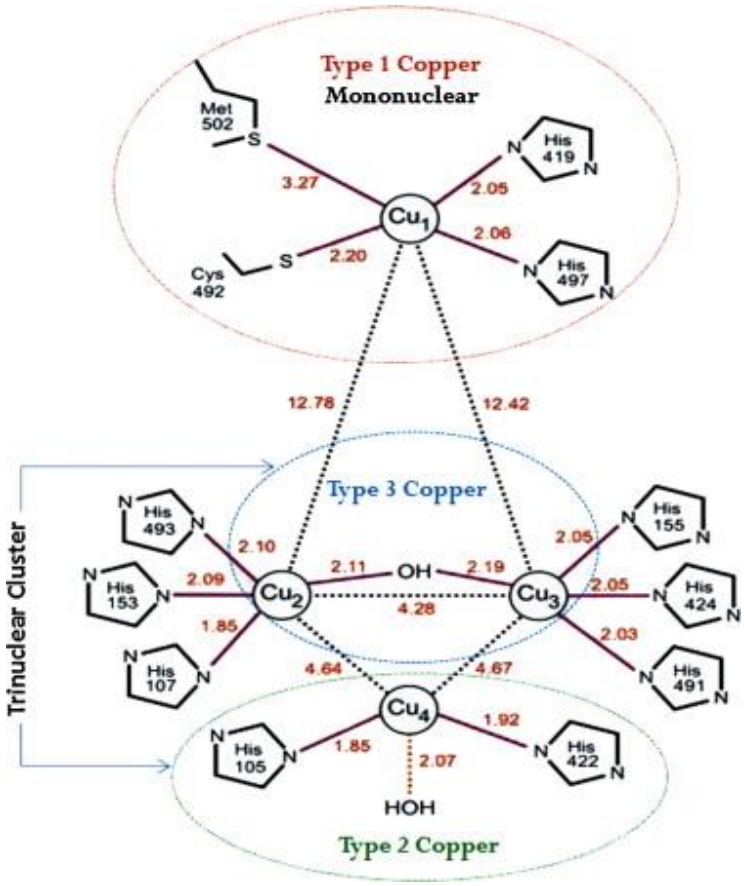

Figure 2.7: Copper coordination centers of laccase together with all pertinent ligands' interatomic distances (Dwivedi et al., 2011).

Laccases from bacteria, plants, and insects typically have low redox potential, whereas laccases from fungi have high redox potential. There are certain conserved domains connected to laccases with low, medium, and high redox potential. Despite the fact that the homology of the laccase amino acid sequence varies between organisms, the catalytic region is very conserved. Laccases are involved in a variety of cellular functions in these organisms, including both anabolic and catabolic events (Janusz et al., 2020).

Fruit stem development, color synthesis, sporulation, and plant disease are all impacted by fungal laccases. One of the fungal laccases, white rotten *Basidiomycetes*, plays a role in the effective breakdown of lignin, cellulose, and hemicellulose into carbon dioxide. The fungal laccases in this group have been investigated and characterized the most (Baldrian 2006). Bacterial laccases perform a variety of tasks, including morphogenesis processes and the manufacture of pigments like melanin and brown spore pigment. They also play a role in spore production and copper homeostasis (Santhanam et al. 2011; Strong and Claus 2011).

2.5.3. Industrial Applications of Laccases

Laccases are widely used in many biotechnological applications which are textile industry for the decolorization of dyes, bioremediation, pulp paper processing, food processing, biosensor technology, and organic synthesis, due to their broad range of substrate specificity Figure 2.8 (Kudanga et al. 2011; Patel et al. 2019). Laccase enzymes are commonly used in other fields such as biofuels and organic synthesis (Kudanga and Le Roes-Hill 2014; Kunamneni et al. 2008; Zheng et al. 2008).

Figure 2.8: Industrial application areas of laccases (Akram et al. 2022).

Laccase enzymes are among the enzymes whose production gains importance with the increase in industrial areas where they are used. The textile industry is one of the sectors where laccase enzyme is used the most. In this sector, laccases are mainly used in dye conversion and biosynthesis, waste treatment, and dye bleaching applications in the textile industry (Abadulla et al. 2000; S Rodríguez Couto et al. 2004; Susana Rodríguez Couto and Sanromán 2005).

With the use of laccase enzyme in these applications, significant savings have been achieved in chemical, energy, and water use. Laccases are used to improve or

change the color appearance of food or beverages in the food industry. By taking part in the removal of phenolic compounds that cause turbidity in the colors of beverages, fruit juice, beer, and wine, it increases the clearness of colors and mixing and structural properties of the dough (Cantarelli et al. 1989; Selinheimo et al. 2006). While in biofuel applications, laccase enzymes are used in the biodegradation pre-treatment of lignin (delignification) and are very economical (Rico et al. 2015) in biosensor applications, these enzymes are available in sensors used to detect phenol compounds (Rodríguez-Delgado et al. 2015). In the paper industry, the laccase enzyme plays a role in removing lipophilic cores that cause pitch accumulation, providing different properties to pulp by developing lignocellulosic fibers and making toxic substances resulting from polymerization/depolymerization reactions non-toxic (Fillat, Colom, and Vidal 2010; G. Singh and Arya 2019).

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Laboratory Devices

For experimental study, laboratory devices, which are utilized are specified in Table 3.1.

Table 3.1: Laboratory Devices and their brand list.

Device	Brand
Autoclave	BioTek
Biosafety Cabinet	N-BioTek
Centrifuge	Beckman Coulter
Centrifuge	Hettich
Deep freezers (-80 °C)	Arctiko
Incubator	Nüve
Mini Centrifuge	Sigma
Magnetic Stirrer	WiseStir
Micropipettes	Axygen
Micropipettes	ISOLAB
Microplate Reader	BioTek-ELx808
Microplate Reader	BioTek-EpocH2
pH Meter	Hanna
Refrigerator	Vestel
Scientific Balance	Shimadzu
Shaking Incubator	N-BioTek
Vortex	BioTek
Bench-Top Bioreactor (3L)	Applikon

3.1.2. Strains, Chemicals and Kits

Unless otherwise stated, all compounds have been provided by Sigma-Aldrich (St. Louis, Missouri, USA). Solid/liquid dextrose and watermelon peel were supplied by the local market and Pendik Starch Company, respectively.

Madurella mycetomatis laccase *was transformed K. pastoris* X33 strain with p*PICZαA* vector and all the characterization studies were performed by Tülek et al. (Tülek et al. 2021). Gebze Enzyme Recognition Center supported the study with recombinant *K. pastoris* X33 for laccase expression.

3.1.3. Growth Medium

For the experimental study, media utilized are specified in Table 3.2.

Table 3.2: Growth medium and their compositions.

Medium	**Content (for 1 L)**	
	Component	Amount
YPD	Peptone	20 g
	Yeast extract	10 g
	Dextrose	20 g
	100 µg/mL Zeocin (if required)	1 mL
BMGY	Peptone	20 g
	Yeast extract	10 g
	Potassium phosphate buffer (1 M, pH 6.0)	100 mL
	10X YNB	100 mL
	500 X Biotin	2 mL

Table 3.3: Continued.

	40X glycerol	100 mL
BMGY (solid dextrose)	Peptone	20 g
	Yeast extract	10 g
	Potassium phosphate buffer (1 M, pH 6.0)	100 mL
	10X YNB	100 mL
	500 X Biotin	2 mL
	Solid dextrose (different concentrations)	100 mL
BMGY (liquid dextrose)	Peptone	20 g
	Yeast extract	10 g
	Potassium phosphate buffer (1 M, pH 6.0)	100 mL
	10X YNB	100 mL
	500 X Biotin	2 mL
	Solid dextrose (different concentrations)	100 mL
BMGY (WPH)	Peptone	20 g
	Yeast extract	10 g
	Potassium phosphate buffer (1 M, pH 6.0)	100 mL
	10X YNB	100 mL
	500 X Biotin	2 mL
	WPH (different concentrations)	According to concentration

3.1.4. Buffers and Solutions

For the experimental study, buffers and solutions which are utilized are specified in Table 3.3.

Table 3.3: Buffers, solutions and their compositions.

Buffers and solutions	**Content**	
	Component	Amount
Zeocine solution (100 mg/mL)	Zeocine powder	1 g
	HEPES buffer (5 g/L, pH: 7.2)	10 mL
Sodium acetate buffer (0.1 M, pH 5.0, 1 L)	Sodium acetate	5.772 g
	Acetic acid	1.778 g
Biotin (500X)	Biotin	40 mg
	Distilled water	200 mL
YNB (10X)	YNB	134 g
	Distilled water	1 L
Methanol (10X)	Methanol	50 mL
	Distilled water	950 mL
Glycerol (40X)	Glycerol	400 mL
	Distilled water	600 mL
Potassium phosphate buffer (1 M, pH 6.0)	di-Potassium hydrogen phosphate	2.405 g
	Potassium di-hydrogen phosphate	11.73 g

3.2 Methods

3.2.1. Preparation of Glycerol Stock

5 mL YPD broth including zeocine (100 µg/mL) was inoculated with recombinant *K. pastoris* X33. The mixture was then incubated for an overnight period at 30 °C and 200 rpm. 250 µL overnight culture and 250 µL of 50% (v/v) sterile glycerol solution were combined to generate a stock. The stocks were stored at – 80 °C.

3.2.2. Strain Reactivation

K. pastoris X33/pPICZA-*Mm*Lac glycerol stock was cultivated on a YPD agar including zeocin (100 µg/mL). The plate was incubated at 30 °C and 2 days. 50 mL falcon tube containing 10 mL of YPD broth was inoculated to reactivate the cells. Incubation of pre-culture was achieved at 30 °C and 220 rpm (until OD_{630} equals 1).

3.2.3. Watermelon Peel Pre-treatment

A modified version of the Martiniano et al. (Martiniano et al. 2020) technique was applied for the pre-treatment. Watermelon peels were divided into pieces of 2 cm^2 and drying process was at 100 °C and 2 days. Boiling process for 1 h was performed for 1:5 (w/v) combination containing watermelon peels (dried) and sulphuric acid (1.0 % v/v). Final liquid was filtrated through Whatman paper, then NaOH solution (6.5M) was used to bring the pH increase to 6.0.

After being autoclaved for 15 minutes at 121 °C, the resultant solution was centrifuged for 30 minutes at 10,000 rpm using an Allegra X-30 Benchtop Centrifuge from Beckman CoulterTM in Brea, California, USA. The hydrolysate (watermelon peel hydrolysate, or WPH) served as a carbon source for *K. pastoris* growth. The dried form of watermelon peel's nutritional breakdown is as follows: 17.23% of protein, 1.05 % of fats, 83.9% of carbohydrates, and 2.55% of ash. The composition of foods includes pectin, free sugar, cellulose, and other carbohydrates (Méndez et al. 2021).

3.2.4. Identification of Optimal Concentrations for Carbon Sources

A range of concentrations were used to determine the best WPH, solid, and liquid dextrose concentrations for *K. pastoris* development. For WPH concentration optimization, 5% and 20 % (v/v) were studied and 1, 2, 3, 4 and 5% (w/v)) of solid and liquid dextrose were evaluated. *K. pastoris* were fermented in 20 mL of BMGY. Liquid dextrose, solid dextrose, and WPH were used as carbon sources in separate trials.

The culture was inoculated into the cultivations at a rate of 10% (v/v) from pre-culture. The amples were taken every two hours up to ten hours and 18th hour.

Optical density (OD_{630}) measurements were made in triplicate for each sample using an ELx808 Elisa Microplate Reader from Agilent Technologies in Santa Clara, California, to determine the cell density. Media without cells was utilized as a blank.

3.2.5. *K. pastoris* Fermentation in Bioreactor with By-Products and Waste

K. pastoris fermentation includes three primary fermentation stages; glycerol batch, glycerol fed-batch, and methanol fed-batch for *K. pastoris* growth and production under *AOX1* promoter in the bioreactor. The experiment used appropriate amounts of WPH, solid, and liquid dextrose separately in place of glycerol for the first two stages of the fermentation. With a few minor modifications, the technique followed the InvitrogenTM Pichia fermentation process parameters (Invitrogen Corporation 2002). The fermentation temperature was arranged to 30 °C and 18 °C for growth and the induction phase, respectively. Dissolved oxygen (DO) was controlled with 1 vol of air per volume of fermentation broth per minute (vvm) airflow and 500-1000 rpm agitation in a cascade mode to provide 30% or higher dissolved oxygen. 3 M KOH and 3 M H_3PO_4 were used as acid and base to keep pH at 6.0 in the fermentation broth. Additionally, Table 3.4 presents designed fermentation techniques based on the carbon sources employed during the growth and induction stages.

Table 3.4: Carbon sources of media according to phases of different fermentation strategies.

Ferm. Strategy Codes	**Utilized Carbon Sources**		
	Phase I	**Phase II**	**Phase III**
Ferm. 1	Glycerol	Glycerol	Methanol
Ferm. 2	Solid dextrose	Solid dextrose	Methanol
Ferm. 3	Liquid dextrose	Liquid dextrose	Methanol
Ferm. 4	WPH	WPH	Methanol

150 mL of the fermentation inoculum was incubated for 18 hours at 30 °C and 220 rpm. For bioreactor cultivations, 1.5 L BMGY was used as a fermentation medium in a 3 L bioreactor (Applikon, Schiedam, The Netherlands). BMGY was

prepared with the optimal concentrations of solid/liquid dextrose, watermelon peel hydrolysate, and 4% glycerol, individually, in accordance with the fermentation approach. Batch phase (Phase I) came to an end when a DO spike was noted, and carbon source feeding (5% glycerol or other at their optimal) was started for phase II at a period of 4 hours with a feeding rate of 16.0 mL/(L.h). End of Phase II, four hours was waited to consumption of present carbon source and temperature was arranged as 18 °C for expression stage. Consumption of carbon source that is present in first two phase is important to avoid repressing effect of them on the promoter. Then, the induction phase was initiated with a methanol feeding of 1.00 mL/(L.h) and the methanol feeding rate was increased exponentially to 3.00 mL/(L.h). During phase III, methanol feeding solution included 0.2 mM Cu_2SO_4 at final concentration. The fermentation conditions were the same for all carbon sources. Samples were obtained every 24 hours during the phase changes and during the induction phase.

The pellet was separated by centrifugation at the conclusion of Phase III to collect the supernatant, and it was then kept at 4 °C for further analysis to determine the enzyme activity, cell concentration (g DCW/L), and calculation of biomass yield from the substrate ($Y_{X/S}$).

3.2.6. Statistical Analysis to Evaluate Activity Change Affected by Utilization of Alternative Carbon Sources in *K. pastoris* Fermentation

To statistically analyze the change in laccase activity brought on by the fermentation processes, Tukey's Test and One-way ANOVA were utilized in GraphPad Prism (GraphPad software, La Jolla, USA).

3.2.7. Determination Dry Cell Weight

1 mL samples were filled in dried Eppendorf tubes. Samples were centrifuged at 15,000 rpm and 4 °C for 10 min. For 24 hours, the pellets were held at 80 °C. To calculate dry cell weight in 1 mL of the culture, the Eppendorf tubes' final weights were measured.

3.2.8. Enzyme Activity Assay

To ascertain extracellular laccase activity, substrate, 2,6-Dimethoxyphenol (2,6-DMP, $\varepsilon 468 = 49.600\ M^{-1}.cm^{-1}$) was used and an activity assay was carried out measuring the absorbance change at 468 nm with the culture supernatants as enzyme source using a Microplate spectrophotometer (Epoch 2; Agilent Technologies, Santa Clara, California, USA). Kinetic testing was conducted with culture supernatant (20 µL), substrate (2,6-DMP; 10 µL of 12 mM) and acetate buffer pH 5.0 (170 µL of 100 mM) for 30 minutes. One unit was defined as the quantity of enzyme required to convert 1 mol of 2,6-DMP into the product every minute.

3.2.9. Calculation of $Y_{X/S}$

Biomass yield from substrate ($Y_{x/s}$) was calculated for agro-industrial by-products; solid/liquid dextrose, given by Eq. (3.1):

$$Y_{X/S} = \text{g biomass produced / g substrate consumed} \quad (3.1)$$

4. RESULTS AND DISCUSSION

4.1. Growth profile of recombinant *K. pastoris* X33 in BMGY at shake-flask level

K. pastoris have two cultivation stage for recombinant protein production processes: growth stage and induction stage. Glycerol is the most commonly used carbon source for growth stage of *K. pastoris* cultivation, which can be referenced to control the growth profile of *K. pastoris* in different carbon sources.

As a control study, *K. pastoris* was grown in BMGY including 4% glycerol (maximum concentration according to Invitrogen manual (Invitrogen Corporation 2002)) and cell density was observed as optical density at certain times of 18 h cultivation period (Figure 4.1).

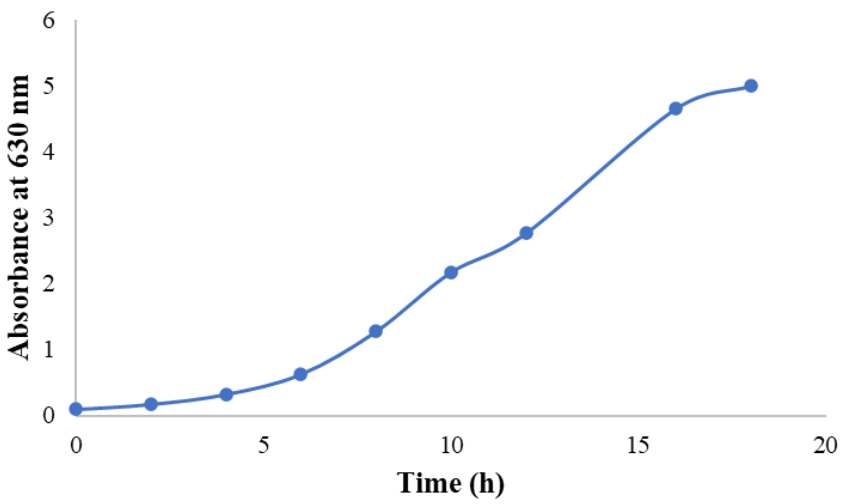

Figure 4.1: Cell density profile of recombinant *K. pastoris* X33 in BMGY at shake-flask level for 18h.

K. pastoris X33 cultivation in 4% glycerol medium showed that lag phase took approximately 4-6 hours. It may have resulted in a higher concentration of carbon source in medium. Exponential growth phase was determined between 6-16 hours and after 16 h, stationary phase started. Maximum specific growth rate was calculated from exponential growth phase and it was determined as 0.401 h^{-1}. At the end of cultivation, absorbance at 630 nm was observed as 4.998 ± 0.542.

4.2. Growth profile of recombinant *K. pastoris* X33 in BMGY including solid dextrose as a carbon source at shake-flask level

Carbon sources concentration should be optimized in medium in order to obtain efficient growth of a microorganism. Solid dextrose is an industrial by-product and it contains impurities in their inclusion. In the scope of solid dextrose concentration optimization for recombinant *K. pastoris* X33 growth, 5 different concentrations of solid dextrose were used in BMGY instead of glycerol. Growth profile, final optical density at 630 nm and maximum specific growth rate was determined (Figure 4.2).

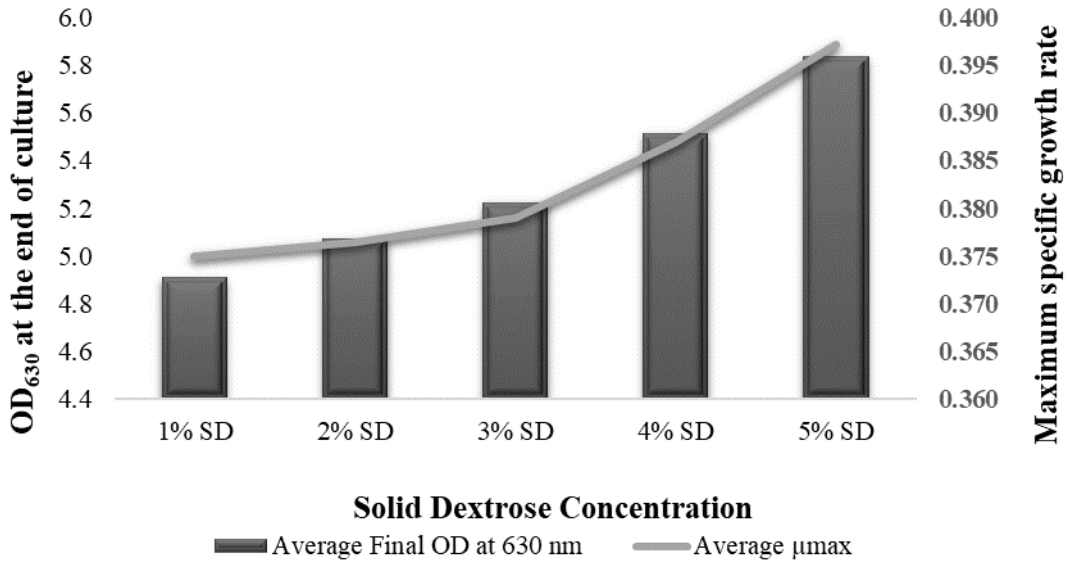

Figure 4.2: Cell density and maximum specific growth rate measurement results in BMGY including 5 different concentrations solid dextrose instead of glycerol at shake-flask level for 18h.

According to results from the cultivation measurements and calculations, cultivation in increasing concentration of solid dextrose resulted in higher maximum specific growth rate and cell density at the end of cultivation. 5% solid dextrose inclusion in medium was observed as optimum with 5.834 absorbance at 630 nm and 0.397 h^{-1} maximum specific growth rate for the growth stage of recombinant *K. pastoris* X33.

4.3. Growth profile of recombinant *K. pastoris* X33 in BMGY including liquid dextrose as a carbon source at shake-flask level

Liquid dextrose is also an industrial by-product obtained from a starch company. The form of dextrose depends on the obtained process of the company. Solid and liquid dextrose are same, however, they have different impurities and concentrations because of the stage of the process that is obtained. Like solid dextrose study, liquid dextrose was also studied with 5 different concentrations and growth profile, cell density as absorbance and maximum specific growth rate of recombinant *K. pastoris* X33 in medium including different concentrations of liquid dextrose was observed (Figure 4.3).

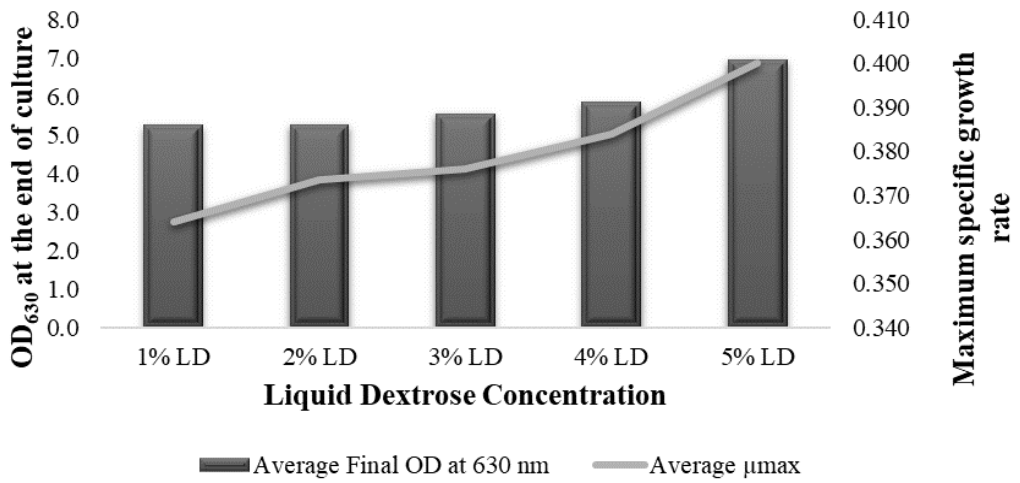

Figure 4.3: Cell density and maximum specific growth rate measurement results of recombinant *K. pastoris* X33 in BMGY including 5 different concentrations of liquid dextrose instead of glycerol at shake-flask level for 18h.

Similarly, 5% concentration of liquid dextrose was determined as optimum by obtaining higher cell density and maximum specific growth rate when 5 different concentration utilization results were evaluated. OD at 630 nm was determined as 6.959 and maximum specific growth rate was 0.400 in 5% liquid dextrose study. 5% liquid dextrose inclusion in medium provided the highest cell density among the optimization and control studies.

4.4. Growth profile of recombinant *K. pastoris* X33 in BMGY including WPH as a carbon source at shake-flask level

Watermelon peel is a waste obtained from agricultural industry or domestic waste. It contains soluble and insoluble carbon in its inclusion. The acidic pre-treatment process was applied to increase soluble carbon and hydrolysate was used in medium as a carbon source. 2 different concentrations were studied to evaluate the effect of WPH in recombinant *K. pastoris* X33 growth.

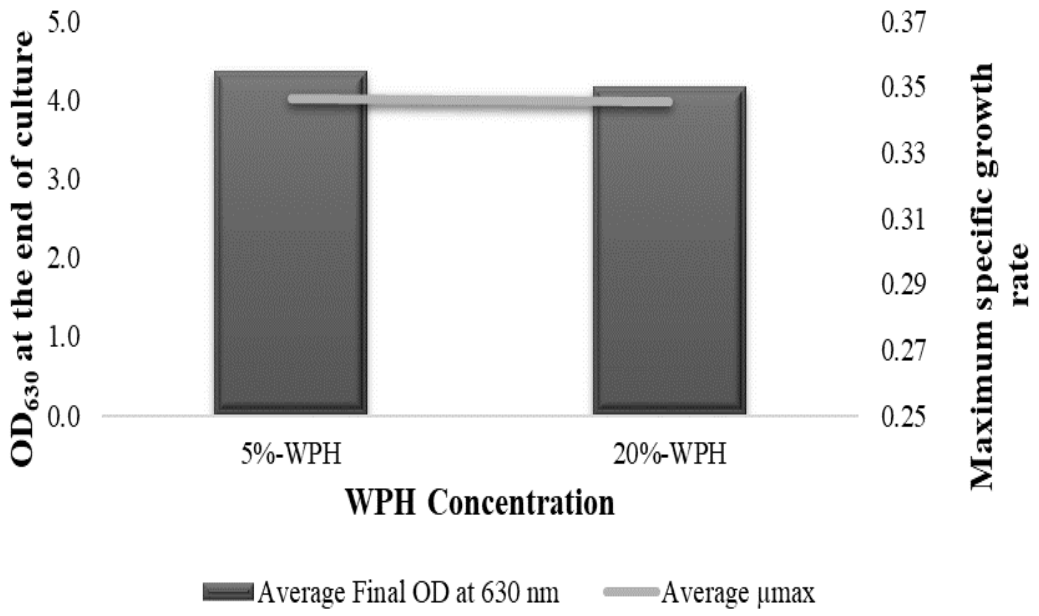

Figure 4.4: Cell density and maximum specific growth rate measurement results of recombinant *K. pastoris* X33 in BMGY including 5 different concentrations of WPH instead of glycerol at shake-flask level for 18h.

Cell density as OD_{630} and maximum specific growth rate results was indicated in Figure 4.4. There is no significant difference in OD_{630} results and maximum specific growth rate calculations for the cultivation with the two different concentrations of WPH. Higher concentrations of WPH may also cause an inhibiting effect on growth although it includes higher concentrations of soluble carbon depending on toxic inclusion resulting from the pre-treatment process of watermelon peel.

4.5. Cell growth of high-cell density fermentation with low-cost substrates

In all phases of the recombinant *K. pastoris* production, cell densities were observed using four distinct fermentation techniques, and *K. pastoris* cell density profiles are shown in Figure 4.5.

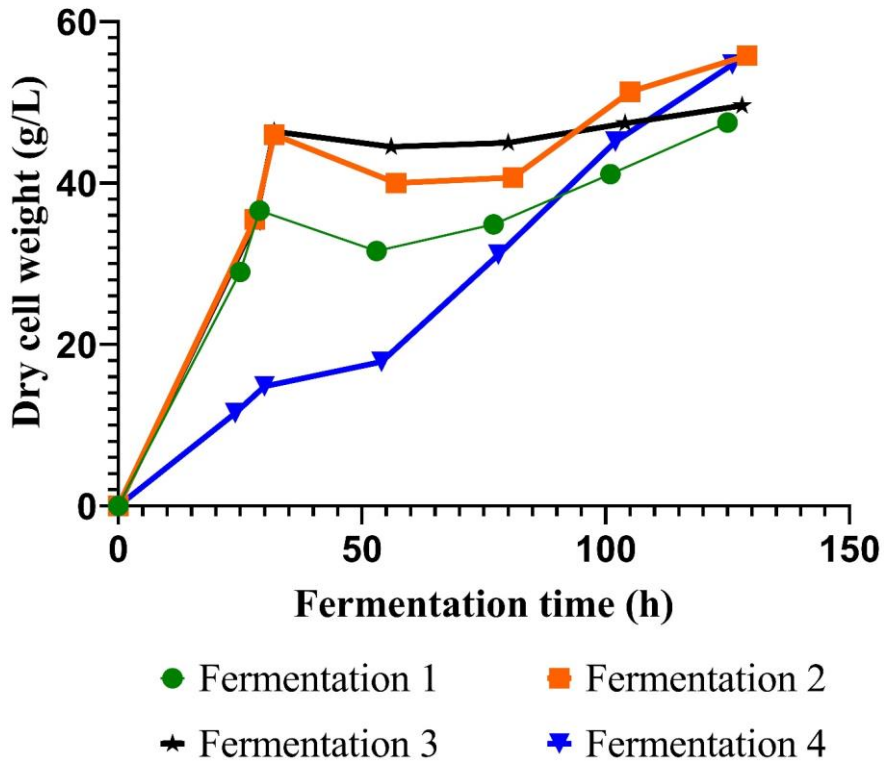

Figure 4.5. Profiles for DCW of recombinant *K. pastoris* X33 in three fermentation phases with identical fermentation conditions during the process for *Mm*Lac expression under the *AOX1* promoter in the bioreactor. The standard deviation was found to be very low.

The batch cultivation with liquid dextrose as one of the other carbon sources produced the highest cell density (35.1 g DCW/L), and this cultivation produced results that were comparable to those obtained with the addition of glycerol providing 35.5 g DCW/L. When the process using solid dextrose reached 29.0 g DCW/L in 25 hours, which was slightly lower growth than liquid dextrose and glycerol, 24 h cultivation with WPH resulted in 11.5 g DCW/L. In all of the study's strategies, the batch cultivation period was almost the same and although dextrose in its solid and liquid forms has the same amount of sugar, different fermentation techniques led to varying cell densities.

The contaminants included in by-products derived from the corn starch industry may have had a detrimental impact on *K. pastoris* development. Glucose was used for *K. pastoris* cell growth (Beiroti et al., 2019) and other carbohydrates, containing a combination of fructose, glucose and oligosaccharides that are waste, was also studied for the generation of recombinant proteins (Gao et al., 2021a). Watermelon peel hydrolysate which contains both fermentable and non-fermentable sugars, has the lowest cell density. Lower cell density development during cultivation may be caused by pre-treatment procedure efficiency and hazardous chemical synthesis (Méndez et al. 2021; Zhou, Zhou, and Zhang, 2021)**.** Wheat bran hydrolysate was used in a work by Zhou et al. (Zhou, Zhou, and Zhang, 2021) to produce heterologous xylanase B from the *K. pastoris* expression system. This study examined the effectiveness of BMGY and *K. pastoris* in valorizing agro-industrial waste and promoting cell proliferation. The pre-treatment for wheat bran was the same as that used to obtain WPH. Even though the hazardous chemicals created during the chemical pre-treatment process were removed by the detoxification process, the results from the usage of wheat bran hydrolysate in this study by BMGY revealed reduced cell density.

Although higher concentration of carbon source was present in Fermentation 3, $Y_{x/s}$ results of it were determined as lower than the first fermentation strategy for phase I because nearly equal cell development profiles were seen in both. The growth for solid dextrose fermentation was the lowest with 0.581 g DCW/g substrate. Phase III has lower Yx/s values in accordance with the use of methanol to express protein rather than biomass production (Duman et al., 2020)**.** Because WPH contains both fermentable and non-fermentable components, it was challenging to compute the Yx/s required for WPH fermentation.

A similar condition was identified at the conclusion of the fed-batch procedure by identifying the fermentation with liquid dextrose that produced the maximum cell density (46.4 g DCW/L). Calculations made for Fermentation 1's dry cell weight and Yx/s (Table 2) are equivalent to those made for the Duman et al. (Duman et al., 2020) investigation. This validates the repeatable outcomes. Except for Fermentation 4, which showed reduced cell concentration, a modest drop in dry cell weight was seen at the start of the induction. The temperature drop from 30 °C to 18 °C affects the decrease in cell concentration (Duman et al., 2020; Shaked and Whitesides, 1980)**.**

Despite the fact that the methods had varying cell densities at the end of phase II, similar cell density profiles were found after 4 days of induction. These findings demonstrated that liquid dextrose, an agricultural by-product, can function as a comparable carbon source to glycerol in the *K. pastoris* high cell density fermentation, producing an identical amount of biomass. Alternative sources include solid dextrose and WPH, but these are less effective than liquid dextrose and commercial glycerol.

Table 4.1: $Y_{x/s}$ values for each of the three cultivation phases for fermentation techniques.

Ferm. Strategy Codes	**$Y_{x/s}$ (g/g)**		
	Phase I	**Phase II**	**Phase III**
Ferm. 1	0.882 ± 0.014	0.197 ± 0.028	0.050 ± 0.015
Ferm. 2	0.580 ± 0.027	0.152 ± 0.025	0.032 ±0.007
Ferm. 3	0.711 ±0.040	0.223 ± 0.045	0.026 ± 0.04
Ferm. 4	-	-	0.175 ± 0.009

4.6. Results of the laccase activity measurements from various high-cell density fermentation techniques using inexpensive substrates

The cultivation conditions for *Mm*Lac expression from *K. pastoris* X33 were improved at the shake-flask level in the work carried out by Tulek et al. (Tülek et al., 2021), and specific activity was established. The purpose of this study is to determine how the usage of alternate carbon sources during the growth stage affects the laccase activity generated by pAOX1. Utilizing culture supernatant as an enzyme source, activity experiments were carried out. In contrast to the control study, no adverse influence on laccase production was seen in the specified bioprocess models. Except for Fermentation 4, which expresses the use of WPH as a carbon source in the growth stage (Figure 4.6), other fermentation techniques produced results that were comparable.

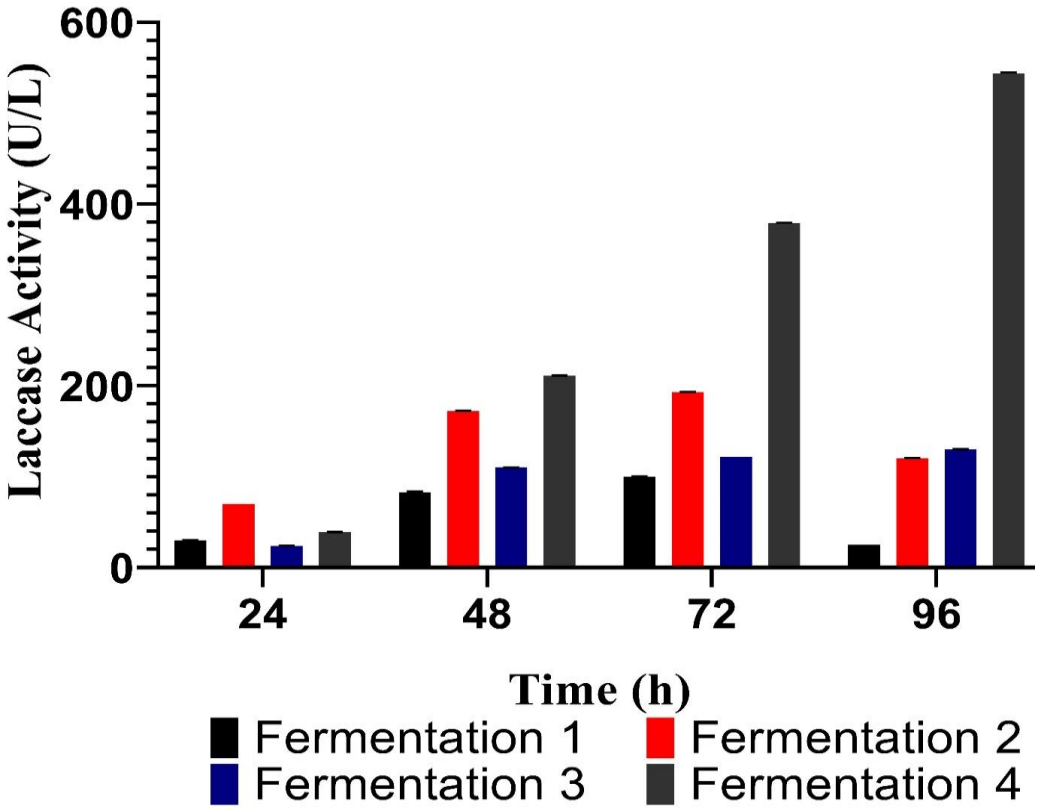

Figure 4.6. Laccase relative activity based on control study (glycerol/methanol) throughout 4 days of induction for different fermentation strategies.

According to fermentation methodologies, enzyme activity rose from the day of induction until 72 hours later. After 72 hours, enzyme activity in Fermentations 1 and 2 started to decline, whilst other fermentation techniques reached their peak activity at 96 hours. Despite the fact that dextrose is a repressive carbon source, Fermentation 1 (control study) revealed increases in liquid and solid dextrose of 1.30 and 1.93 folds at 96 and 72 hours, respectively. According to a study by Wahida et al. (Wahida et al., 2021), *K. pastoris* expression system cultivation in sago bioethanol liquid waste with glycerol, glucose, and lactic acid produced 0.00159 U/mL laccase activity under optimal circumstances, contributing a 1.5-fold increase. This outcome is comparable to those of liquid and solid dextrose that were valued for laccase formation.

When the results investigated, the maximum activity was found to be 20.8 U/L at 96 hours in Fermentation 4. Even though this fermentation technique produced the lowest cell density at the conclusion of the growth stage, the highest laccase activity was seen. By separating it from other potential carbon sources, its 17.23% protein inclusion can be attributed to these increases in activity (Méndez et al., 2021).

Similar to WPH, detoxified WPH was utilized as the growing medium for recombinant *K. pastoris*, and xylanase B activity under pAOX1 was found to be 90.9% of BMGY in the hydrolysate without any additional growth or maintenance support (Zhou, Zhou, and Zhang, 2021). While wheat bran hydrolysate met all of the cells' needs in the previously published investigation, this study's discovery of increased enzyme activity may have been caused by the use of a complex medium that contained a lot of protein and the trace elements needed for protein expression.

Based on the findings, alternate waste carbon sources for *K. pastoris* fermentations and laccase synthesis from *K. pastoris* may be possible. They are appealing choices because they support the circular economy paradigm and may be used in labs to produce vast quantities of recombinant proteins using *K. pastoris* expression systems. In addition to these, this work is unique since it uses waste watermelon peel, solid and liquid dextrose, and agro-industrial by-products as carbon sources for recombinant *K. pastoris* fermentation (Table 4.2).

Table 4.2: Effects on the synthesis of recombinant proteins under pAOX1 of using various carbon and nitrogen sources for the fermentation of *K. pastoris*.

Source	**Product**	**Result**	**Ref.**
Solid dextrose (by-product)	Laccase	+	This study
Liquid dextrose (by-product)	Laccase	+	This study
Watermelon peel	Laccase	+	This study
Crude glycerol, Soybean hydrolysate and Rice bran	Xylanase	+	(Cahyati, Hudiyono, and Helianti, 2021)
Miscellaneous waste carbohydrates of high fructose syrup	Endo-β-1,3-glucanase	+	(Gao et al., 2021)
Crude glycerol	Lipase	+	(Tian et al., 2021)
Sago bioethanol liquid waste	Laccase	+	(Wahida et al., 2021)

Table 4.2: Continued.

Wheat bran hydrolysate	Xylanase B	~	(Zhou, Zhou, and Zhang, 2021)
Maize meal Bean pulp	Xylanase	+ 	(Sun et al., 2020)
Wheat bran	Xylanase	+	(Lee, 2018)
Crude glycerol	β-Mannanase	+	(Luo et al., 2018)
Gluconate	Human interferon gamma	+	(Prabhu and Veeranki, 2018)
Formate Sorbitol	Lipase B	+ +	(Jayachandran, Athiyaman, and Sankaranarayanan, 2017)
Wheat bran Corncob Cottonseed hull Corn bran	Xylanase	+ - + +	(Shang et al., 2017)
Corn processing wastewater	Lipase	+	(Yan et al., 2017)
Lactic acid Sorbitol Acetate	Angiostatin	+ + -	(Xie et al., 2003)
Alanine Sorbitol Mannitol Trehalose Glucose Ethanol Acetate	β-galactosidase	+ + + + - - -	(Inan and Meagher, 2001)

positive effect: +, negative effect: -, equivalence: ~

4.7. One-way ANOVA analysis

The greatest enzyme activity achieved from the researched fermentation techniques was statistically evaluated. When comparing the control study with other methods, one-way ANOVA analysis demonstrated that the difference in enzyme activity was considered to be statistically significant. The findings of the Tukey's Test show that there is a substantial difference between the fermentations, with the exception of the control study's combination of Fermentation 3 and Fermentation 1. According to the control study, enzyme activity in Fermentation 3 was not significantly different, but when by-product valorization is taken into account, getting the same production amount from the by-product with the conventional approach may be preferred for the applications. Fermentation 3 also produced a high cell density, despite the fact that laccase activity was not noticeably elevated. When cell density formation data for *K. pastoris* were analyzed, it can be applied to other investigations.

Table 4.3: One-way analysis of ANOVA and post-hoc (Tukey's Test) test for the effects of different strategies benefiting from by-products and waste valorization on enzyme activity.

ANOVA Results					
Comparison	**SS**	**DF**	**MS**	**F**	**Sig.**
Treatment (between strategies)	555.3	3	185.1	842.3	P<0.0001
Residual (within strategies)	1.8	8	0.2		
Total	557.1	11			

Table 4.3: Continued.

Post-Hoc Test Results					
Compared strategy	**Mean 1**	**Mean 2**	**q**	**DF**	**Adjusted P Value**
Ferm 1 - Ferm 2	3.83	7.41	13.22	8	<0.0001
Ferm 1 - Ferm 3	3.83	4.96	4.19	8	0.0701
Ferm 1- Ferm 4	3.83	20.82	62.79	8	<0.0001
Ferm 2 - Ferm 3	7.41	4.96	9.03	8	0.0010
Ferm 2 - Ferm 4	7.41	20.82	49.57	8	<0.0001
Ferm 3 - Ferm 4	4.96	20.82	58.60	8	<0.0001

5. CONCLUSION

By examining the effects on growth and the production of heterologous proteins, this study assesses the value-added of agro-industrial wastes and by-products as substitute carbon sources in the bioprocess development for recombinant *K. pastoris*. This analysis evaluates the inexpensive carbon sources for their utilization potential in manufacturing benefiting from the circular bioeconomy. Among the other inexpensive substrates, strategies showed that an agro-industrial by-product; liquid dextrose resulted in the best growth for the *K. pastoris* cultivation. The highest activity was obtained from fermentation with WPH. The study's preliminary results demonstrated that strategies in this study scope can be a promising substitute for using waste materials, watermelon peel, by-products of the corn starch industry, as well as solid and liquid dextrose. This will help in the bioprocess developments for heterologous protein production in *K. pastoris* for subsequent studies.

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BIOGRAPHY

Buse completed her undergraduate studies at the Department of Bioengineering, Marmara University and graduated in second place. After graduating from a bachelor's degree, Buse started a job in VSY Biotechnology as a quality assurance assistant specialist. After one year working, she started Biotechnology MSc. program at the Gebze Technical University under the supervision of Assoc. Prof. Barış Binay. She conducted her MSc studies at Gebze Enzyme Recognition Center (GERC).

APPENDICES

Appendix A: Publications related to the thesis

Çaloğlu, B., Binay, B. 2023. "Utilization potential of agro-industrial by-products and waste sources: Laccase production in bioreactor with *Pichia pastoris*". Biochemical Engineering Journal, 193, 108854.

Caloglu, B., Binay, B., "Utilization Potential of Agri-industrial By-products and Waste Sources for Recombinant protein Production in Bioreactor with *Pichia pastoris* Protein Expression System: Laccase Enzyme", 3rd Enzyme&Bioprocess Days, Turkey (9-11 September 2022).