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GEBZE TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EXPRESSION AND BIOCHEMICAL CHARACTERIZATION OF HYPSIBIUS DUJARDINI EPOXIDE HYDROLASE ENZYME IN PICHIA PASTORIS

HAKAN ONUR

A THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

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ASSOC. PROF. DR. BARIŞ BİNAY

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

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YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

GEBZE
2022

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

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 16/06/2022 tarih ve 2022/29 sayılı kararıyla oluşturulan jüri tarafından 28/06/2022 tarihinde tez savunma sınavı yapılan Hakan ONUR'un tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

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ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun ……./……/….. tarih ve ……../….. sayılı kararı.

SUMMARY

Epoxide hydrolases are enzymes that hydrolyse epoxides to their diols and degrade oxirane derivatives. Epoxide hydrolases has been isolated from many organisms such as fungi, plants, bacteria, and insects. The factors that directly affect the reaction efficiency of epoxide hydrolases obtained from different sources are their thermal stability, pH tolerance, and redox potentials. The epoxide hydrolases plays an important role in the detoxification processes of xenobiotics and takes part in the metabolism of some biologically important molecules. Biotechnologically, epoxide hydrolases with high enantioselectivity are useful biocatalysts for producing optically active epoxides and diols that act as chiral building blocks in the synthesis of bioactive drugs.

In this study, the epoxide hydrolase gene (*hd*eh) of *Hypsibius dujardini*, an extremophile organism, was successfully transferred to the heterologous expression system *Pichia pastoris* GS115 host cell through pPIC9K construct. After transformation, the integration of the transformed putative *hd*eh gene into the *Pichia pastoris* genome was confirmed by colony PCR. Recombinant *Hd*EH was purified. While the kinetic values of the actively obtained *Hd*EH enzyme were calculated as Km: 0.981 mM and kcat: $9.23x10^3$ s^{-1}, the catalytic efficiency of the enzyme (kcat/Km) was determined as $9.4x10^3$ M^{-1} s^{-1}. The enzyme's optimum pH and temperature values were determined as 8.0 and 20 °C, the best storage stability being determined as +4. Due to the good catalytic efficiency of the *Hd*EH enzyme, testing against different diols, its racemic preferences, improving the catalytic efficiency with enzyme engineering studies, and high production in bioprocess create the potential for future studies.

Key Words: Epoxide hydrolase, *Hypsibius dujardini*, *Pichia pastoris* GS115, Recombinant enzyme production

ÖZET

Epoksit hidrolazlar epoksitleri diollerine hidrolize eden ve oksiran türevlerini parçalayan enzimlerdir. Epoksit hidrolaz enzimi mantar, bitki, bakteri, böcek gibi birçok organizmadan izole edilmiştir. Farklı kaynaklardan elde edilen epoksit hidrolaz enzimlerinin reaksiyon verimlerine doğrudan etki eden faktörler termal kararlılıkları, pH toleransları ve redoks potansiyelleridir. Epoksit hidrolaz enzimi ksenobiyotiklerin detoksifikasyon süreçlerinde önemli rol oynarlar, biyolojik olarak önemli bazı moleküllerin metabolizmasında yer alırlar. Biyoteknolojik olarak, yüksek enantiyoseçiciliğe sahip epoksit hidrolaz enzimleri biyoaktif ilaçların sentezinde kiral yapı taşları olarak görev alan optik olarak aktif epoksitlerin ve diollerin üretimi için yararlı biyokatalizörlerdir.

Bu tez çalışmasında, ekstremofil bir organizma olan *Hypsibius dujardini*' nin epoksit hidrolaz geni (*hd*eh), pPIC9K yapısı aracılığıyla heterolog ekspresyon sistemi *Pichia pastoris* GS115 konak hücresine başarıyla aktarılmıştır. Dönüşümden sonra, dönüştürülmüş varsayılan *hd*eh geninin *Pichia pastoris* genomuna entegrasyonu, koloni PCR'si ile doğrulandı. Rekombinant *Hd*EH saflaştırıldı. Aktif olarak elde edilen *Hd*EH enziminin kinetik değerleri Km: 0.981 mM ve kcat: 9.23x103 s-1 olarak hesaplanırken, enzimin katalitik verimi (kcat/Km) 9.4x103 M-1 s-1 olarak belirlendi. Enzimin optimum pH ve sıcaklık değerleri sırasıyla 8.0 ve 20 °C olarak belirlenmiş; En iyi depolama stabilitesi +4 olarak belirlendi. *Hd*EH enziminin iyi katalitik verimi nedeniyle farklı diollere karşı test edilmesi, rasemik tercihleri, enzim mühendisliği çalışmaları ile katalitik veriminin iyileştirilmesi ve biyoproseste yüksek üretim, gelecekteki çalışmalar için potansiyel oluşturmaktadır.

Anahtar Kelimeler: Epoksit hidrolaz, *Hypsibius dujardini*, *Pichia pastoris* GS115, Rekombinant enzim üretimi

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms	:	**Explanations**
µg	:	Microgram
µL	:	Microliter
°C	:	Celcius Degree
aa	:	Amino acid
BLAST	:	Basic Local Alignment Search Tool
BMGY	:	Buffered Glycerol Complex Medium
BMMY	:	Buffered Methanol Complex Medium
bp	:	Base pair
BRENDA	:	Braunschweig Enzyme Database
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
EcoRI	:	*Escherichia coli* RY13 I
IMAC	:	Immobilized Metal Affinity Chromatography
kcat	:	Turnover number
kDa	:	Kilo Dalton
Km	:	Michaelis–Menten constant
LB	:	Luria–Bertani
M	:	Molar
MD	:	Minimal Dextrose
mM	:	Millimolar
min	:	Minute
mL	:	Milliliter
*hd*eh	:	*Hypsibius dujardini* epoxide hydrolase gene
*Hd*EH	:	*Hypsibius dujardini* epoxide hydrolase enzyme
ng	:	Nanogram
nm	:	Nanometer
NCBI	:	National Center for Biotechnology Information Database
Ni^{2+}–NTA	:	Nickel–Nitrilotriacetic Acid

PCR	:	Polymerase chain reaction
PmeI	:	Pseudomonas mendocina I
pNBP	:	4-(4-Nitrobenzyl) pyridine
RNA	:	Ribonucleic acid
rpm	:	Rounds per minute
SalI	:	Streptomyces albus I
SDS–PAGE	:	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SOC	:	Super Optimal Broth
TAE	:	Tris–Acetate–EDTA
TEMED		Tetramethylethylenediamine
U	:	Units
UniProt	:	Universal Protein Resource
V	:	Volt
YNB	:	Yeast Nitrogen Base
YPD	:	Yeast Nitrogen Dextrose
YPDS	:	Yeast Nitrogen Dextrose Sorbitol

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1. INTRODUCTION

1.1. Aim and Contribution of Thesis

The thesis study presented the expression of the *Hypsibius dujardini* epoxide hydrolase (*hd*eh) gene in *Pichia pastoris*, which is the heterologous expression system, and the biochemical characterization of the obtained enzyme was carried out using recombinant DNA technology. The purely produced *Hypsibius dujardini* epoxide hydrolase (*Hd*EH) enzyme was modeled computationally and its catalytic region was analyzed.

Epoxide hydrolases enzymes are catalase the reaction that hydrolyze epoxides to their diols. Due to these properties, they are important enzymes for industrial use. The production of enzymes that are used on an industrial scale is common in *Pichia pastoris*, a methylotrophic yeast, due to its features such as production in uncomplicated media, reaching high cell densities, the need for enzymes (such as alcohol oxidase) necessary for methanol use, and having strong and controllable promoters. Due to the increasing demand for enzymes with versatile application potential such as epoxide hydrolase; Identification, biochemical characterization, and production of these enzymes from new sources are gaining importance day by day.

Briefly mentioned the aim, contribution, and content of the thesis in the first chapter. In the second chapter, basic information about enzymes is given, structure, functions and importance of epoxide hydrolase enzymes are emphasized. In which expression system the enzyme is produced, the reasons are explained. The third chapter gives information about the materials and experiments used in the thesis. In the fourth and last chapter, the results obtained and the interpretations of these results are explained under the relevant headings.

2. LITERATURE REVIEW

2.1. Enzymes as Catalysts

Enzymes are protein structure-based biological catalysts that accelerate many kinds of biochemical reactions. Enzymes are significant main blocks of life, so can be obtained from all living organisms. They are not consumed during the reaction and remain unchanged at the end of the reaction. Important vital activities of the organism, such as the production and destruction of organic substances are the result of metabolic reactions, and these reactions are possible by the catalytic effect of enzymes. Enzyme activity and reaction rate depend on many factors (substrate concentration, enzyme concentration, pH, temperature, reaction time, reaction products, enzyme inhibitors and activators, radiation, pressure, and light). All known enzymes have been classified by the International Union of Biochemistry and Molecular Biology (IUBMB) into 6 main groups, including many subgroups, according to the type of reaction they catalyze, with the help of a code consisting of four different digits. Enzyme code numbers consist of four digits separated by dots, the first digit indicates which of the six main enzyme groups the enzyme belongs to, the second digit indicates the chemical structure or functional group it acts on, the third digit the acceptor, and the fourth digit the sequence number of the enzyme in a particular class (Table 2.1) [1], [2]. Since ancient times, enzymes have been used unconsciously in daily life. For example, the production of cheese, yogurt, vinegar, and alcoholic beverage. Now, thanks to developments in recombinant DNA technology and protein engineering, have become a focal point for scientific studies and industrial applications [3], [4].

Table 2.1: Enzymes classification table.

Class	The group name of enzymes	Reaction Catalyzed
EC 1	Oxidoreductases	Catalase the oxidation-reduction reactions
EC 2	Transferase	Catalase the transfer of functional groups
EC 3	Hydrolase	Catalase the hydrolysis reactions
EC 4	Lyases	Catalase the group elimination (forming double bonds)
EC 5	Isomerase	Catalase the Isomerization reactions
EC 6	Ligases	Catalase the Bond formation coupled with a triphosphate cleavage

It is of great importance to make enzymes used in many fields suitable for industrial use or to reduce the cost of the product produced by enzymes. About 4000 enzymes are known from about 200 microbial sources. 75% of known enzymes are hydrolytic enzymes [5].

2.2. Epoxide Hydrolases

2.2.1. Epoxide Hydrolase mechanism and sources

Epoxide hydrolases (EC 3.3.2.3) include a group of enzymes that hydrolyze (catalyze the addition of water) oxirane ring-containing compounds (epoxides) and consequently produce diols [6]. In nature, organisms are exposed to their own metabolically produced epoxides or epoxide-containing compounds from external sources. Epoxides are organic tricyclic ring compounds containing polarized carbon-oxygen bonds. The epoxide (oxirane) ring structure is highly unstable in aqueous media. The labile tricyclic ring structure exhibits nucleophilic activity with irreversible toxic effects on critical cell components such as DNA, amino acids or purines. This activity may cause mutagenesis and carcinogenesis. [7]. Epoxides can be produced by metabolic pathways in organisms, for example, in the human lungs, the epoxide is produced as a result of the oxidation of toxic substances (xenobiotics) inhaled by the cytochrome P-450 monooxygenase enzyme [8]. In mammals, hydrolysis of epoxides prevents genotoxicity [9], [10]. The epoxide hydrolase enzyme plays a vital role in the discovery of new drugs that can be used in balancing blood pressure, relieving inflammation, preventing cancer and many other diseases [11]. Epoxide-containing compounds are signal-regulating molecules. However, it may react with other cell components and cause chemical changes when not controlled [12]. Therefore, regulating epoxide levels is very important. For the removal of epoxides, the enzyme of epoxide hydrolase catalyzes the epoxide ring, water-soluble and easy to remove producing diols (Figure 2.1) [13], [14].

There are two different families of epoxide hydrolases. Most epoxide hydrolases are members of the α/β hydrolases enzyme group, which consists of eight β-strands linked by α-helices [15]. The other group, limonene epoxide hydrolases (LEHs), different from the α/β epoxide hydrolase family, has a unique catalytic mechanism and active site, has little sequence similarity, is homodimeric, and is a small family with

few enzymes. LEHs have a unique structure consisting of four α-helices structured on a highly curved six-strand β-sheet. There are differences in the catalytic mechanisms of the two epoxide hydrolase enzyme families. α/β epoxide hydrolases have the Asp-His-Glu/Asp catalytic triad in their catalytic site and use a two-step reaction mechanism (Figure 2.1) [16],[17]. The epoxide ring forms a covalent substrate-enzyme complex by the nucleophilic action of the aspartate residue. A tyrosine residue donates a proton to the epoxide oxygen, while the histidine residue of the catalytic triad acts as a base, activating the water molecule attacking the alkyl-enzyme intermediate at the active site [18]. The second aspartate residue of the catalytic triad interacts with the histidine residue, favoring the activation of water in the active site [19]. Activation of the water molecule creates a tetrahedral intermediate molecule. In an H-G-X-P motif, which has a conserved region and negative charge in the enzyme structure, the nitrogen of the X residue is stabilized by a single H-bond interaction. The resulting tetrahedral intermediate decomposes to release the diol product [20], [21]. Unlike α/β EH enzymes, LEH enzymes catalyze the conversion of epoxide to diol with a one-step mechanism [22] (Figure 2.1b).

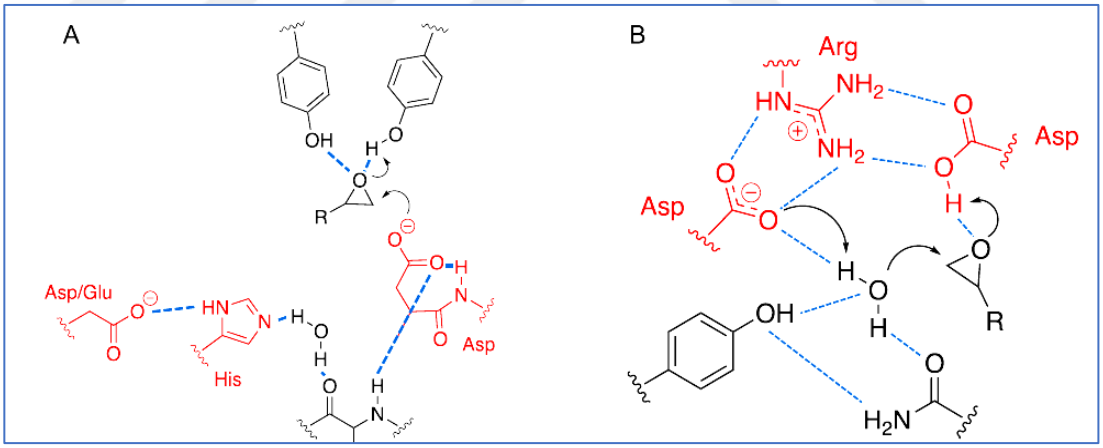

Figure 2.1: Schematic representation of two different forms of enzymatic epoxide hydrolysis. a) The 2-step mechanism of α/β EHs propagating through a substrate-enzyme intermediate. b) 1-stage mechanism of LEHs. Catalytic triplet residues of each enzyme type are shown in red and H-bonds in blue. Additional residues involved in the orientation of substrates and water in stabilizing catalytic intermediates are shown in black.

Epoxide hydrolase enzymes are found in many different organisms but have only been studied in very limited numbers at the molecular level. The role of these enzymes differs from organism to organism. Generally, epoxide hydrolases are involved in

regulation of signal molecules, catabolism pathway regulation and detoxification. EHs are involved in the catabolism of natural, epichlorohydrin (environmental pollution agent) industrial carbon sources such as tartaric acid or limonene. In plants, EHs are involved in pathogen defense, stress responses, and cuticle formation. Juvenile hormone catabolism is an example of plant defense. EHs are very important industrially due to their potential use in chiral molecule synthesis [23], [24]. Recombinant heterologous expression systems are generally used today due to low enzyme expression and yield when the organism itself is used as the main source for epoxide hydrolase enzymes. Bacteria, yeast, and virus expression systems have been widely used for this purpose (Table 2.2).

Table 2.2: Some sources of epoxide hydrolase obtained from different organisms and produced in different expression systems.

Species	**Organism**	**Host**	**Source**
Sphingomonas sp.	Bacteria	*E.coli*	[6]
A. mediolanus	Bacteria	*E.coli*	[22]
Streptomyces sp.	Bacteria	*E. coli*	[54]
X. dendrorhous	Fungus	*E.coli*	[55]
B. napus	Bitki	*P. pastoris*	[57]
Bombyx mori	Insect	*Baculovirus*	[59]
R. mucilaginosa	Fungus	*Y. lipolytica*	[60]
Nocardia sp.	Bacteria	-	[61]
Manduca sexta	Insect	*Baculovirus*	[62]
A. thaliana	Bitki	*P. pastoris*	[63]
A. brasiliensis CCT	Fungus	*E.coli*	[64]
A. gambiae	Insect	*Baculovirus*	[65]
Karenia brevis	Bacteria	-	[66]
T. reesei	Fungus	*E.coli*	[67]
Rhodococcus ruber	Bacteria	*E. coli*	[68]
Homo sapiens	Mammal	*E.coli*	[69]
M. tuberculosis	Bacteria	*E.coli*	[70]
M.rosenbergii	Insect	*E.coli*	[71]

Many new α/β epoxy hydrolases and LEHs from nature continue to be defined and added to the literature. These enzymes have been produced and characterized by many different living things such as mammals, bacteria, plants, and insects. In some studies, using genome mining, six new α/β EHs with broad substrate selectivity were found [25]. They discovered two enantioselective α/β EHs from DNA isolated from the organism obtained from a biofilter used in the gas outlet containing styrene. These enzymes, exhibited opposite enantioselectivity, preferentially hydrolyzing epoxides in the (S)- or (R)-configuration, respectively [26]. In addition, α/β epoxy hydrolase (CH65-EH) is the most thermotolerant EH disclosed to date [27]. These examples demonstrate that nature continues to be a fertile resource for the discovery of new epoxy hydrolases.

2.2.2. Application areas of Epoxide Hydrolases

Enantiopure epoxides and diols are valuable chiral synthetic intermediates in the industry (especially chemical and pharmaceutical). Their preparation by conventional chemical means causes low catalytic efficiency. In addition, these methods require the use of expensive and toxic metal catalysts. Epoxide hydrolases, which hydrolyze epoxides to diols, provide these chiral intermediates to be environmentally friendly, sustainable, cost-effective, independent of expensive co-factors, with high enantiomeric and regioselectivity. These properties make epoxide hydrolases very interesting for bio-industrial applications [28]. For example, the (S)-enantiomer of α-methyl styrene oxide was used for the synthesis of (S)-ibuprofen [29]. Epoxide hydrolases are also sold by companies to be used in commercial applications [30]. Different biotransformation reactions and wide substrate selectivity provided by epoxide hydrolases make these enzymes valuable in biotechnology. Also, tolerance to organic solvents is important as epoxide solubility can be a limiting factor. Epoxide hydrolases have given successful results even in the presence of solvents that reach very high concentrations in the reaction [31], [32], [33].

2.2.3. *Hypsibius dujardini*

Hypsibius dujardini, also known as water bear, is a freshwater Tardigrade species found in algae and sediments of rivers, streams and lakes (Figure 2.2) [36].

Hypsibius dujardini is of a cosmopolitan nature, found in many different environments around the world. They have been found to survive in many different environments. It is because they are the hardiest organisms in the world, which places these organisms in the category of extremophiles. They have been found to survive at extreme conditions (high and low temperatures, air deprivation, starvation, dehydration, radiation, high and low pressures, even in space) [34], [35].

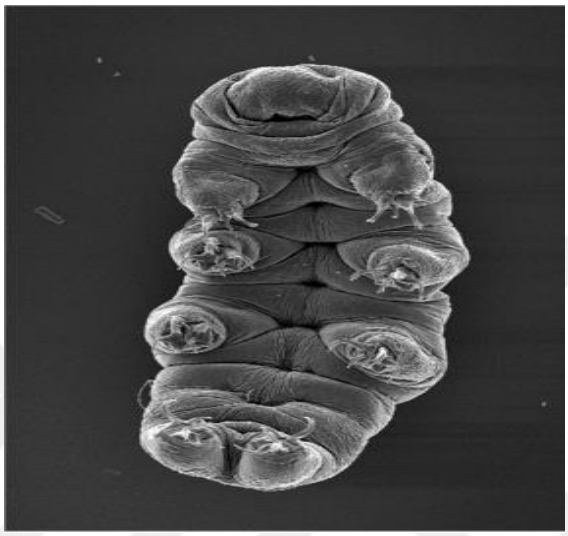

Figure 2.2: *Hypsibius dujardini* scanning electron micrograph.

The ability of *Hypsibius dujardini* to survive is due to its ability to undergo cryptobiosis (anhydrobiosis) and the environmental stress response induced by desiccation when conditions become unfavorable. Anhydrobiosis induces desiccation when they become ametabolic and dehydrated to survive extreme environmental stressors until conditions become suitable again [35]. Although the organism has many unique innovative features, no industrial enzymes from *Hypsibius dujardini* have been reported. This is the first study to report an enzyme isolated from *Hypsibius dujardini* for industrial application. The *Hypsibius dujardini* genome, which was reported in 2016, was chosen as the gene source in the project [36]. (DDBJ/EMBL/GenBank accession numbers: MTYJ00000000.1, BioProject PRJNA360553).

2.3. Recombinant DNA Technology

Proteins are macromolecules that are composed of building blocks called amino acids that come together in accordance with the genetic code and are produced by all

living forms in their own metabolism. Proteins play a significant role in all cell life activities [37]. Some proteins are specialized to have different activities. For example, they increase the rate of biocatalyst reactions [38].

In the 1970s, most enzymes were traditionally produced from plant and animal sources. However, in recent years, high-cost, low-level enzyme production could not meet expectations. In the 1990s, microbial enzymes came to the fore and were produced on an industrial scale [37]. Today; With the help of recombinant DNA technology, enzymes and the processes in which these enzymes work can be designed in accordance with the needs of the user. The first stage in the production of recombinant protein; After the desired DNA is obtained, this protein is replicated in the selected production system. Proteins; can be produced in bacteria, yeast, mold, mammalian cell culture or in plants, insects, transgenic plants and animals. Choosing the right expression system for recombinant protein production; Factors such as protein quality, functions, and production rate are also affected [38].

Table 2.3: Characteristics of host organisms that are highly preferred in recombinant protein production.

	E. coli	*P. pastoris*	CHO cell
Cost of growth medium	Cheap	Cheap	Expensive
The complexity of growth medium	Simple	Simple	Complex
Doubling time	20-30 min	1-2 h	~24 h
Expression level	High	Low to high	Low to moderate
Glycosylation & phosphorylation & acetylation and Extracellular expression	No	Yes	Yes

The cheapest, easiest and fastest protein production is carried out from *Escherichia coli*. However, *Escherichia coli* can't produce massive proteins, and this host system cannot be used in productions that require posttranslational modifications. For this, *Saccharomyces cerevisiae* and *Pichia pastoris* yeasts are mostly used in recombinant protein expression. Yeasts are host systems that can synthesize large amounts of heterologous proteins at a low cost, produce large proteins, remove signal sequences, and perform glycosylation [39], [40].

2.3.1. *Pichia pastoris* expression systems

Different high-scale production strategies are being developed to produce economically viable recombinant proteins using high-throughput expression systems [40]. *Pichia pastoris* has become one of the promising host-vector systems in the production of recombinant genes compared to other eukaryotic and prokaryotic expression hosts. There are advantages to using *Pichia pastoris* over another host cell. I) It can use methanol as a carbon and energy source and is economical for both basic research and industrial processes. II) It can reach high cell densities in a short time. III) Well-characterized expression vectors are easy to genetically manipulate, have many distinct promoters, and can integrate vector DNA into the cell genome by homologous recombination procedures. IV) It can produce a higher amount of target protein at a lower cost than other host eukaryotes, and it can also secrete the produced protein. V) It can perform post-translational modifications of proteins (signal sequence processing, disulfide bridge formation, O- and N-linked glycosylations). These advantages make *Pichia pastoris* more preferred than other expression hosts [37], [41], [42].

Pichia pastoris, a methylotrophic yeast, was first described by Guilliermond in 1919; spherical or oval-shaped, singly or in pairs, and highly variable morphology. It was introduced more than 50 years ago by Philips Petroleum Company as an animal feed additive as it achieves high cell density using methanol as a carbon source. The rise in oil prices in the 1970s affected the sustainability of methanol-based production [39]. Phillips Petroleum licensed its patent on the *Pichia pastoris* protein expression system to Invitrogen. With the success of the *Pichia pastoris* strain in protein production, developed the first-generation expression system for efficient recombinant protein expression (Figure 2.4) [40], [43]. Its applications are now extended to the fields of synthetic biology and whole-cell biotransformation.

Various genetic and physiological factors determine the efficiency of the *Pichia pastoris* recombinant protein production system. These factors are codon optimization of the heterologous gene, gene copy number, promoters, the efficiency of transcription and translation processes, secretion signals, optimization of production conditions (temperature, pH, carbon source, production volume, feeding profile), type of protein production (intracellular, extracellular or cell surface) protein folding and secretion [44], [45]. Studies have indicated that an increase in gene copy numbers can

significantly increase productivity. Since episomal vectors developed for *Pichia pastoris* are not stable, they are integrated into the genome [46]. The integrant thus formed can remain stable even in the absence of selective pressure. Expression vector in general; promoter sequence (AOX1, GAP, FLD1, PEX8, YPT1, etc.), single or multiple cloning site, transcription termination sequence-poly A tail, origin of replication, selection marker (ADE1, ARG4, G418, HIS4, URA3, Zeor , Ampr) and secretion signal (PHO1, SUC2, pGKL, PHA-E, α-factor) [41]. Many secretion signals are used in the *Pichia pastoris* expression system. However, the α-factor pre-pro secretion signal, which includes the pre-region containing a 19 amino acid signal peptide and the pro region consisting of 60 amino acids, from S. cerevisiae is the most widely used. In *Pichia pastoris*, the α-pre-pro sequence is cut mainly by the product of the KEX2 gene (prohormone-endoprotease) and the product of the STE13 (dipeptidyl amino peptidase) gene [45], [47].

There are three types of *Pichia pastoris* host strains that differ in their ability to handle methanol. Strains that are wild type (natural phenotype); While methanol use has a positive phenotype (Mut+), there are strains in the methanol use slow phenotype (MutS) due to deletions in the AOX1 gene, and strains that cannot use methanol (Mut-) due to deletions in both AOX1 and AOX2 genes. Although deletion of AOX genes provides an advantage in large-scale production as it requires less methanol than wild-type strains, there are studies showing that it is better than wild-type strains in recombinant protein production [37], [41], [42], [45], [48].

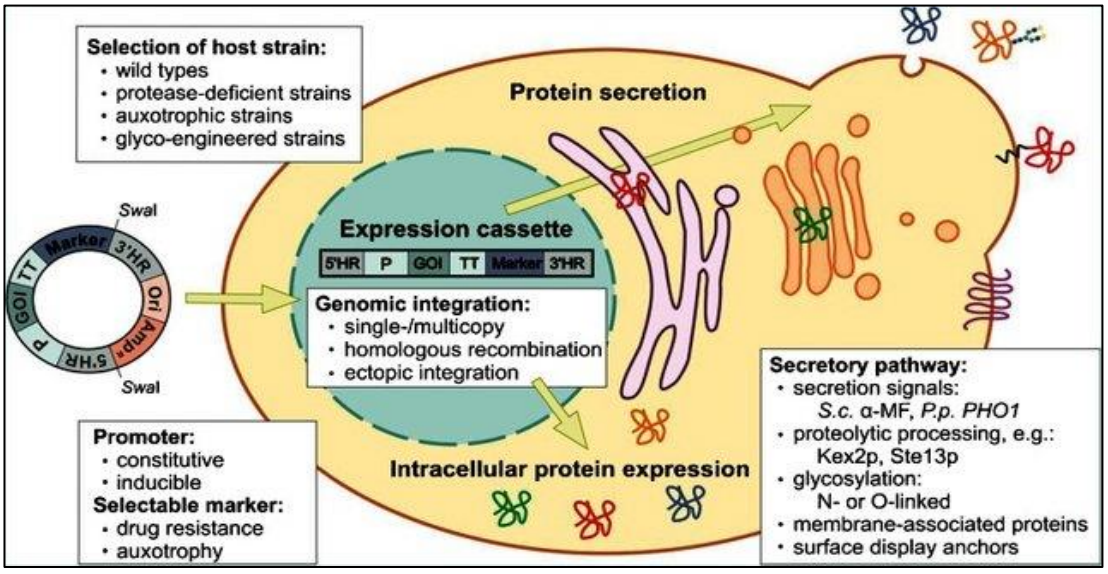

Figure 2.3: General considerations for heterologous gene expression in *Pichia. pastoris.*

The methanol-inducible alcohol oxidase 1 (AOX1) promoter, involved in methanol metabolism, is widely used for Pichia pastoris recombinant protein production system. Alcohol oxidase is the first enzyme of the methanol metabolic pathway that catalyzes the oxidation of methanol to formaldehyde. In the *Pichia pastoris* genome, there are two alcohol oxidase genes (AOX1 and AOX2) that encode the alcohol oxidase enzyme. the AOX1 gene; is responsible for about 90% of alcohol oxidase activity in the cell and has a strong and highly regulated promoter (pAOX1), while the AOX2 gene is responsible for about 10% of alcohol oxidase activity. The AOX1 promoter, which plays a role in methanol metabolism, is strongly induced by methanol and suppressed in the presence of glucose, glycerol and ethanol [40], [45], [48].

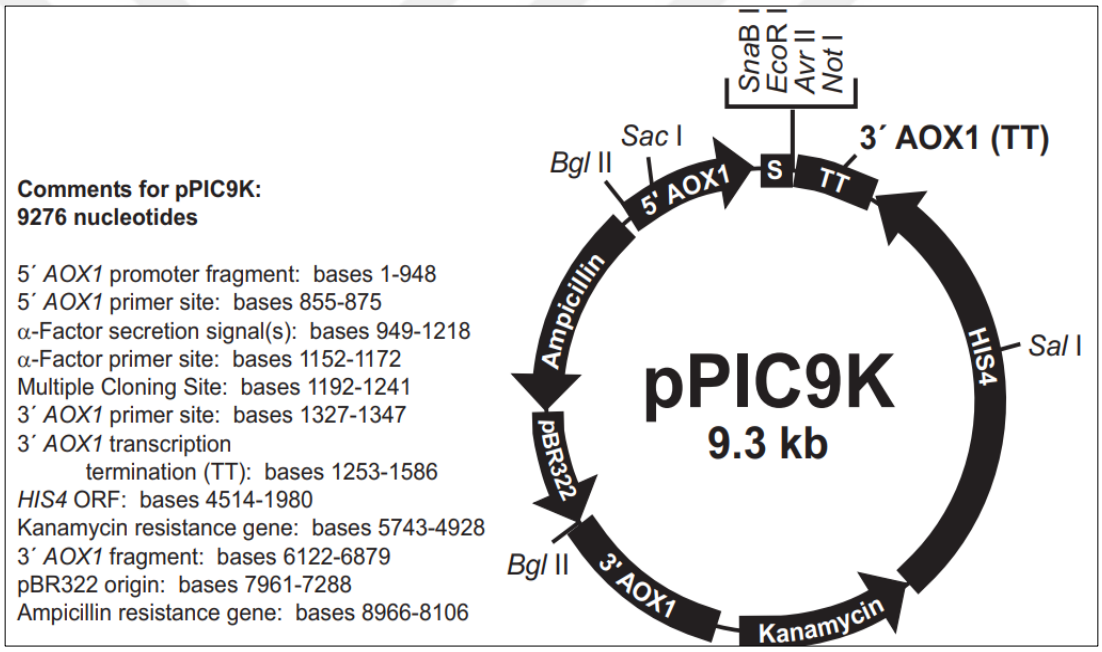

Figure 2.4: The figure below shows the map of pPIC9K.

One of the most important factors affecting the production of recombinant protein for *Pichia pastoris* yeast is the selection of the promoter in the expression vector. In order to increase the success of heterologous protein production by using the *Pichia pastoris* expression system more effectively and efficiently, studies are continuing on the development of existing promoters or the discovery of alternative promoters [43].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Enzymes, Chemicals, Strains, Equipment, and Kits

Enzymes, Chemicals, Strains, Equipment, and Kits used in this study are given in Table 3.1.

Table 3.1: Enzymes, Chemicals, Strains, Equipment, and Kits.

	Material	**Trademark**
Enzymes	Restriction enzyme (SacI), Taq DNA polymerase	Nebbiolabs
Chemicals	Acetic acid	Merck
	Agar	Biolife
	Agarose	Sigma
	Ammonium persulfate	Fisher BioReagents
	Anti·His Antibodies, BSA-free	Qiagen
	Biotin	Sigma
	DMSO	Sigma
	Dextrose	Fisher BioReagents
	DNA ladder	1 kb, Thermo
	EDTA	Merck
	Ethanol	Merck
	Glycerol	Sigma
	Imidazole	Alfa Aesar
	LB-Agar	Formedium
	Methanol	Merck
	Peptone	Fischer Scientific
	pNBP	Acros Organics
	Potassium carbonate	Isolab
	Potassium phosphate (KH_2PO_4 & K_2HPO_4)	Multicell
	SDS-PAGE Gel Stain	Generon
	SDS-PAGE Gels and Running Buffer	CBS Scientific
	Sodium Chloride	Fisher Scientific
	Styrene oxide	Acros Organics
	TEMED	Fisher Scientific
	Tryptone	LabM
	Tris-Base	Formedium
	Yeast extract	Biolife
	Yeast Nitrogen Base	BD

Table 3.1: Continuation.

Strains	*P. pastoris* GS115 and pPIC9K vector	Invitrogen
	Escherichia coli TOP10	NEB
Equipment	Immobilon-P Membrane	Merc
	Concentrators	Sartorius
	HisTrap Column	GE
	Syringe Filters	Sartorius
Kits	Plasmid Miniprep	Qiagen
	PCR Clean-up	Macherey-Nagel
	Western Blotting Kit	Life Technologies
	BCA Assay Kit	Serva

3.1.2. Mediums and Buffers

Mediums used in this study are given in Table 3.2 and Table 3.3.

Table 3.2: Mediums.

	Yeast extract	Peptone	10X Dextrose	Tryptone	NaCl	Agar	Note
LB (Luria–Bertani) Medium (1 L) Autoclave	5 g			10 g	5 g	15 g (for LB agar)	
YPD (Yeast Extract Peptone Dextrose) (1 L) Autoclave	10 g	20 g	10 g			20 g (for YPD agar	182.2 g sorbitol (for YPDS)

Table 3.3: Mediums.

Ingredient / Medium	Yeast extract	Peptone	1 M potassium phosphate buffer (pH 6.0)	10 X Glyserol (%10)	10 X Methanol (%5)	10 X Yeast nitrogen base (134 g dissolved in 1 L)	500X Biotin (8 mg dissolved in 50 mL)
BMGY- BMMY (Buffered Glycerol-Methanol Complex Medium) (1 L)	10 g	20 g	100 mL	100 mL (for BMGY)	100 mL (for BMMY)	100 mL	2 mL
	Add 700 mL distiled water. Autoclave		Autoclave	Autoclave	Filter Sterilize (0.22 µm)	Filter Sterilize (0.22 µm)	Filter Sterilize (0.22 µm)

- Proteins purification from the supernatant was done by using HisTrap protein purification columns. Purification buffers are given below:

  - Buffer A

    - 2.76 g $NaH_2PO4.H_2O$ (20 mM), 29.22 g NaCl (500 mM), and 2.04 g imidazole (30 mM) were dissolved in dH_2O and the solution volume was made up to 1 L. Finally, the pH was fixed to 7.4 using NaOH.

  - Buffer B

    - 2.76 g $NaH_2PO4.H_2O$ (20 mM), 29.22 g NaCl (500 mM), and 34.04 g imidazole (500 mM) were dissolved in dH_2O and solution volume was made up to 1 L. Finally, the pH was fixed to 7.4 using NaOH.

  - Buffer 100 mM imidazole (250 mL)

    - 212.5 mL Buffer A and 37.50 mL Buffer B were mixed.

  - Buffer 200 mM imidazole (250 mL)

    - 160 mL Buffer A and 90 mL Buffer B were mixed.

  - Buffer 400 mM imidazole (250 mL)

    - 52.5 mL Buffer A and 197.5 mL Buffer B were mixed

- The pH effect on enzyme activity was measured with buffers of different pH. Buffers are given below.

  - Potassium phosphate buffer (1 M, pH: 6.0)

    - A solution of 1 molar K_2HPO_4 and 1 molar KH_2PO_4 was prepared and mixed. Finally, the pH was fixed to 6 using pH adjustments for K_2HPO_4 as a base and KH_2PO_4 as an acid.

  - Sodium Phosphate Buffer Solution (50 mM)

    - 7.085 g Na_2HPO_4 and 6.9 g $NaH_2PO_4.H_2O$ (50 mM) were dissolved in dH_2O and the final volumes were completed to 1 L. pH adjustments for NaPi buffer were done by using Na_2HPO_4 as a base and $NaH_2PO_4.H_2O$ as an acid.

  - Citrate Buffer (50 mM)

    - 0.734 g $Na_3C_6H_5O_7$ and 0.48 g $C_6H_8O_7$ were dissolved in ddH_2O. pH adjustments were done by using $Na_3C_6H_5O_7$ as a base and $C_6H_8O_7$ as an acid.

- Tris–HCl Buffer Solution (50 mM)
  - 6.05 g $C_4H_{11}NO_3$ were dissolved in ddH_2O. pH adjustments were done by using HCl (50 mM).
- Sodium bicarbonate / Sodium hydroxide buffer (50 mM):
  - Prepare 400 mL of distilled water in a suitable container. Add 2.11 g of Sodium bicarbonate. pH adjustments were done by using 1M NaOH. Add distilled water until the volume is 500 mL.

- Solutions Used for Agarose Gel Electrophoresis:
  - 50X TAE Buffer (2 L)
    - Measure 484 g Tris, 114.2 mL glacial acetic acid, 200 mL 0.5 M EDTA (pH: 8), and complete to 2 L with the final volume ddH_2O. 0.8% Agarose gel (40 ml): 0.32 g agarose, 3 µL Serva DNA Stain Clear-G, 40 mL 1X TAE.

- Solutions Used for SDS-PAGE:
  - 4X Sample Buffer (pH: 6.8)
    - 4% SDS, 20% glycerol, 10% 2- mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl.
  - 10X SDS Running Buffer (2 L)
    - 303 g of Tris, 1440 g of glycine, and 100 g of SDS are dissolved in ddH_2O and solution volume was made up to 2 L.
  - 30% Acrylamide-Bisacrylamide Solution (pH: 7.0)
    - 145 g of acrylamide and 5 g of N, N'-Methylenebisacrylamide is dissolved in ddH_2O and solution volume was made up to 500 mL, storage at 4 °C.
  - Coomassie Staining Solution (2 Liter)
    - 2 g Coommasie Blue (R-250) 900 mL methanol, 200 mL glacial acetic acid, and 900 mL ddH_2O.
  - Destaining Solution
    - 200 mL methanol, 500 mL glacial acetic acid, and 250 mL ddH_2O.

- SOC (1 L) medium
    - 2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, glucose is added final concentration of 20 mM were measured medium completed to 1 liter with ddH_2O.

- Antibiotic solutions:
    - Ampicillin Solution
        - 100 mg/mL concentration of ampicillin is dissolved in distilled water, passed through a 0.22 μm filter, divided into 1 mL aliquots, and stored at -20 °C.
    - Geneticin solution
        - 100 mg/mL concentration of Geneticin is dissolved in distilled water and filtered through a 0.22 μm filter, divided into 1 mL aliquots and stored at -20°C.

- Western blotting solutions
    - 10X PBS Solution:
        - Dissolve 160 g NaCl, 4 g KCl, 28.8 g Na_2HPO_4, 4.8 g KH_2PO_4 in ddH_2O. After the pH is adjusted to 7.4, the volume is made up to 2 L with ddH_2O. It is sterilized by autoclaving at 121°C for 30 minutes and stored at room temperature.
    - 1X TPBS Solution
        - It is prepared by adding 0.1% Tween-20 to 1X PBS solution.
    - Blocking Buffer
        - It is prepared by adding 1% Bovine Serum Albumin (BSA) to 1X PBS solution.

3.1.3. Laboratory Equipment

Used laboratory equipment in this study is given in Table 3.4.

Table 3.4: Laboratory equipment.

Material	**Trademark**
Magnetic Stirrer	WiseStir
Biosafety cabinet (class 2)	Faster
Autoclave	BioTek
Centrifuge	Beckman Coulter
Deep freezers (-80 °C)	Arctiko
Electrophoresis Power Supply	Bio-Rad
Western blot system	Bio-Rad
Micropipettes	Axygen
Microplate Reader	Tecan
pH Meter	Hanna
SDS-PAGE Electrophoresis System	Bio-Rad
Thermal Cycler	Benchmark
Vortex	Thermomac
Water-Bath	Stuart
Shaking Incubator	Nüve
Sonicator	Bandelin

3.2. Methods

3.2.1. Bioinformatic Analysis

The unique EH gene to be used in the project study was determined using the National Center for Biotechnology Information (NCBI, 2019) and The Universal Protein Resource (UniProt, 2019) databases. A tardigrade of the Eutardigrade clade, the *Hypsibius dujardini* EH gene (*hd*eh) (UniProt accession number A0A1W0X158, NCBI accession number OQV21190.1) has been identified. It was determined that the related *hd*eh gene (446 amino acids) had not been studied before by searching the NCBI BLAST interface and literature [35], [36]. In BLAST and alignment applications with other previously studied, academically pioneering, and valuable sequences from the Protein Data Bank, the sequence similarity ratio and conserved region similarities with other epoxide hydrolases were examined and the results showed that the *hd*eh gene to be studied has academic value and industrial potential. Signal peptide content of the relevant gene (1-24 amino acids) with Signal IP 4.1 (Petersen, 2011) software, glycosylation sites with NetOGlyc 1.0 (Steentoft et al., 2013) software, domain features such as ligand binding, with PyMOL software (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

Since there is no commercial stock of the organism with the related gene, the cDNA of *Hypsibius dujardini* was obtained by referring to the previous study of Koutsovoulos et al. that elucidated the *Hypsibius dujardini* genome [49]. The fusion structure of the designed *hd*eh gene and the pPIC9K vector is shown in Figure 3.1. Accordingly, the natural signal sequence in the *hd*eh gene was removed using PCR, and 6xHistag was added to the 5' region. The 5' region of the pPIC9K vector contains the MF-α extracellular secretion signal sequence [50]. From the cDNA, the *hd*eh gene is designed to amplify the region that does not contain the first 24 amino acids (the signal sequence in the gene's structure has been removed) (Figure 3.1).

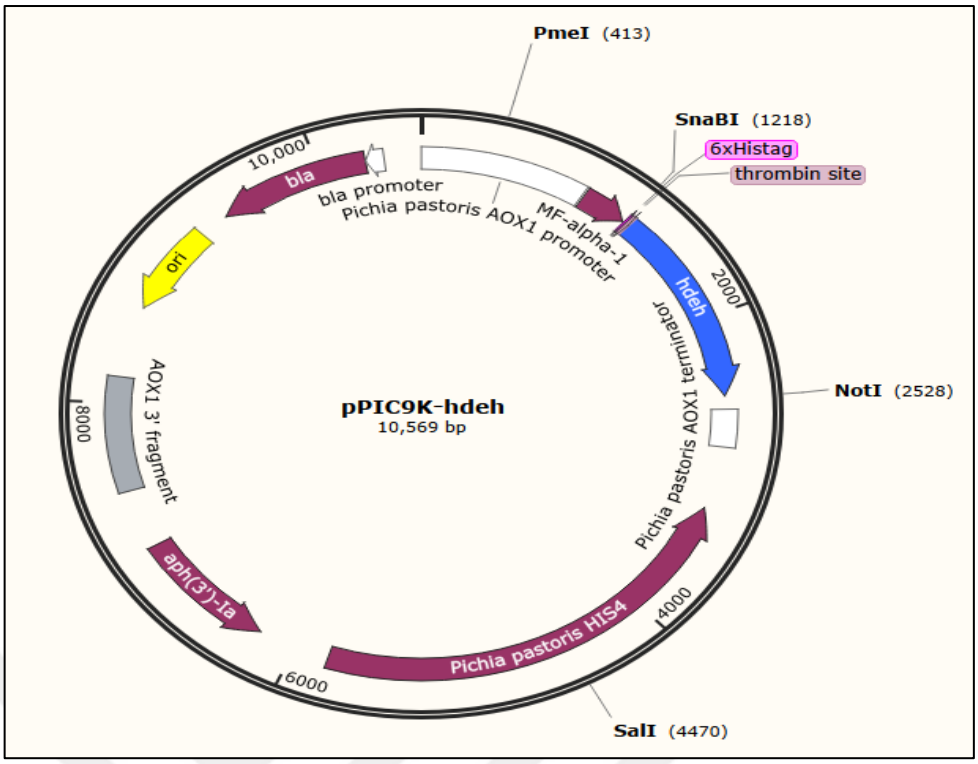

Figure 3.1: pPIC9K-*hd*eh vector construct.

In the project, the *Pichia pastoris* GS115 expression system, which is a methylotrophic yeast that is widely used in the production of recombinant protein today, was used as the host organism. The reason for choosing this expression system is the selection of the *Hypsibius dujardini* EH gene, which is a eukaryotic creature, and its ability to perform post-translational modifications such as proteolytic processes, disulfide bridge formation, and glucosylation included in the *Pichia pastoris* expression system for the production of this enzyme. *Pichia pastoris* GS115 strain cannot grow in a histidine-free medium because the gene required for histidine biosynthesis is silenced, and this situation provides colony selection in a selective medium (MD: Minimal Dextrose medium) by the transformation of the vector carrying this gene required for histidine biosynthesis.

3.2.2. Genetic Transformation

3.2.2.1 Bacterial Transformation

5 µl of ligation mixture was added to 100 µl of potent *Escherichia coli* TOP10 cells, which were capable of chemical transformation by the thawed $CaCl_2$ method and

incubated on ice for 30 minutes. Then, the cells, which were exposed to heat shock at 42 °C for 45 seconds, were kept on ice for five (5) minutes and cooled, after which 950 µL of SOC medium was added and incubated at 37 °C at 150 rpm for 80 minutes. Transformant cells seeded on LB plates containing the appropriate antibiotic (100 µg/ml ampicillin) in the transferred plasmid will be incubated overnight at 37 °C. After incubation, 5-10 colonies were selected from the colonies formed on the plates and incubated in 10 ml liquid LB (100 µg/mL ampicillin) medium at 37 °C overnight at 180 rpm. After the incubation period, the isolation of the recombinant plasmid was performed using the Miniprep method. The recombinant plasmids obtained were prepared for transforming into competent *Pichia pastoris* cells by cutting with *Sna*B1 restriction endonuclease.

3.2.2.2 Yeast Transformation

At the end of the cut reaction with *Sna*B1, the restriction enzyme was treated for 20 minutes at 95 °C for inhibition of restriction enzyme inhibition. Recombinant plasmids digest and linear with PmeI restriction enzyme were first treated with phenol/chloroform isoamyl alcohol (CIA) for use in electroporation. With this method, restriction enzymes are removed. Ethanol precipitation was used to concentrate linear recombinant plasmids. The Phenol chloroform extraction and ethanol precipitation method used is summarized below:

- For the 300 µL sample, 150 µL CIA and 150 µL phenol were added. Centrifuged for 5 min at 11.000×g. The supernatant was carefully collected with the pipette. Added 150 µL CIA and 150 µL phenol to the supernatant. Centrifuged for 5 min at 11.000×g. The supernatant was carefully collected with the pipette. Added 300 µL CIA to the supernatant. Centrifuged for 5 min at 11.000×g. The supernatant was carefully collected with the pipette. Added 1/10 volume of sodium acetate (3 M, pH 5.2). Added 2.5–3.0X volume (calculated after addition of sodium acetate) of at least 95% ethanol. Incubated at room temperature or on ice for at least 15 minutes. Centrifuged at 13.000×g for 30 minutes at –4 °C. Discarded supernatant and added 70% ethanol. Centrifuged again for 15 minutes and discarded supernatant. The pellet was dissolved in ddH_2O.

A modified protocol was used to generate competent yeast cells. Single colony *Pichia pastoris* GS115 is inoculated as 2x1 cm on a YPD agar medium plate. It is incubated for 18 hours at 30 °C. Cells from the YPD agar medium plate are transferred to Eppendorf containing 1 mL of YPD broth. Cells are dissolved by making a vortex. Centrifuge at room temperature for 3 minutes at 1500×g and discard the supernatant. 1 mL sterile ddH_2O is added to the pellet and dissolved by pipette. The supernatant is discarded by centrifugation at 1500×g for 3 minutes at room temperature. This step is repeated 3 times. Add 1 mL of the following solution (Table 3.5) to the pellet and incubate at 100 rpm for 20 minutes at 30 °C. The supernatant is discarded by centrifugation at 1500×g for 3 minutes at room temperature.

Table 3.5: Competent cell solutions.

Contents	Amounts
HEPES-NaOH (pH: 8.0)	40 µL (1 M stock)
DTT	40 µL (1 M stock)
LiCl	100 µL (1 M stock)
Sorbitol	600 µL (1 M stock)
ddH_2O	220 µL

1 mL of cold 1M sorbitol is added to the pellet and the pellet is dissolved by pipette. The supernatant is discarded by centrifugation at 1500×g for 3 minutes at 4 °C. This step is repeated for 750, 500, 250, and 100 µL sorbitol (1 M), respectively. At the last stage, 70 µL of 1 M sorbitol is added to the pellet and dissolved by pipette. The competent cells obtained are used in electroporation. A linearized 5 µg plasmid mixed with 80 µL competent *Pichia pastoris* GS115 was placed in a cold 0.2 mm electroporation cuvette and kept on ice for 5 minutes. Electroporation was performed at 1500 V, 25 µF, and 200 Ω. Cold 1 M sorbitol was added into the electroporation cuvette and taken into 15 mL falcon. Cells are incubated for 2 hours at 100 RPM at 30 °C. After incubation, cells were spread on a YPDS medium containing different concentrations of geneticin (500, 1000, and 2000 µg/ml). The plates were incubated for 3-10 days at 30 °C until colonies were seen.

3.2.3. Polymerase Chain Reaction (PCR)

The *hde*h gene in the transformed recombinant plasmid became integrated into the chromosome by homologous recombination. The presence of the *hde*h gene in the

chromosome was checked by colony PCR using AOX1 promoter and terminator primers in Table 10. To obtain the plasmid DNA required for colony PCR, the colonies taken with the help of a sterile toothpick were placed in Eppendorf in 0.02 M NaOH and kept at 95 °C for 15 minutes. The resulting intracellular extract was used as a template DNA source. The presence of the *hde*h gene in the *Pichia pastoris* genome was carried out under colony PCR conditions (Table 3.6 and Table 3.7) with the PCR reaction established using the AOX1 promoter and terminator primers in Table 3.8. PCR products were run on a 1% agarose gel and the presence of fragments of the expected size was checked.

Table 3.6: Primers.

Primers	Sequence
*hdeh*_F1	5'- CTGGTGCCGCGCGGCAGCGGAGTGCTCCCGGAAGCA-3'
*hdeh*_F2 (*Sna*BI)	5'-*TACGTA*CACCATCATCATCATCATCTGGTGCCGCGCGGCAGCG -3'
*hdeh*_R (*Not*I)	5'- *GCGGCCGC*TTAATCTCTTGACAAAACATTTTTGG -3'

Table 3.7: PCR solutions.

PCR Component	***Amount (µL)***
ddH_2O	27
5' MF-α forward primer (10 pmol/µL)	1
3' AOX1 reverse primer (10 pmol/µL)	1
dNTP Mix (25 mM)	1
$MgCl_2$ (25 mM)	5
Taq DNA Polimeraz (5 U/µL)	0.25
10X PCR buffer	5

Table 3.8: PCR cycling parameters.

Cycle	Temperature (°C)	Time (min)
1	95	1
35	94	1
	58	1
	72	1:30
1	72	10

3.2.4. Expression of the *hd*eh Gene in *P. pastoris* GS115 Cells

It was incubated in a 10 mL BMGY medium in a 50 mL flask at 30 °C at 250 rpm until OD_{600} 2-6 (approximately 18-20 hours). The cells obtained after incubation will be harvested by centrifuging for five (5) minutes at 3000xg at room temperature and then the cell precipitate obtained is left to incubate at 30 °C in a 500 mL notched flask containing 100 mL of BMMY. Inducing at 24-hour intervals with a final methanol concentration of 0.5%, 1%, and 2%, incubation was continued for 8 days, and samples were taken for SDS-PAGE every 12 hours. To determine the expression level, 1 mL sample taken into Eppendorf at specified periods was centrifuged at 13.000xg for five (5) minutes at 4 °C, and the supernatant and precipitate were analyzed by SDS-PAGE. For optimization of extracellular epoxide hydrolase production conditions, the induction time (48, 72, 96, 120, 144, 168, and 192 hours), temperature (18, 22, 26 and 30 °C), and daily methanol concentration addition (0.5%), 1% and 2%) were tested and the epoxide hydrolase production level was optimized.

Epoxide hydrolase protein was expressed extracellularly and His-taq tagged in *Pichia pastoris* GS115 cells. Supernatants of recombinant *Pichia pastoris* GS115 cultures in BMMY were harvested after 5 (optimal induction day) days by centrifugation (11000xg, 4 ºC, 60 min) and filtered through a 0.45 μm filter. It was then purified with HisTrap column equilibrated with Buffer A. The column was washed with 5 mL of Buffer A and *Hd*EH was eluted with different concentrations of imidazole (100, 200, and 400 mM). To control the purity, the supernatant, washing, and elution samples obtained during the whole process and the fractions containing pure protein were analyzed by SDS-PAGE. Proteins on the SDS-PAGE gel were transferred to the membrane for Western blot analysis, where the expressed His-tagged *Hd*EH protein was identified by anti-His antibody using a commercially available Western blotting kit. Fractions containing pure protein were combined and concentrated with Amicon Millipore centrifugal concentrators. The concentration of purified *Hd*EH was determined with the BCA Assay Kit. Purified and concentrated was stored at 4 °C until used in further analyses.

3.2.5. Biochemical Analysis of *Hd*EH

Assuming that all of the purified and concentrated recombinant epoxide hydrolase is active, the optimum temperature and pH for its biochemical characterization and storage stability were determined.

3.2.5.1.Epoxide Hydrolase Activity Assay

Epoxide hydrolase activity Cedrone et al. [51] according to the protocol established and modified. Accordingly, the following protocol was applied;
All experiment steps run in 1.5 mL Eppendorf tube and triplicate.

- 200 µL concentred enzyme was added
- 200 µL substrate solution was added (20 mM racemic styrene oxide dissolved in 50 mM potassium phosphate buffer pH 7 and containing 10% v/v DMSO) (incubation 30 min 30 °C)
- 200 µL 100 mM pNBP [4-5(p-nitrobenzyl)pyridine] (in DMSO) was added (incubation 20 min 80 °C)
- 400 µL Ethanol was added.
- 200 µL 1 M K_2CO_3 was added
- 5 min. 15000 rpm centrifuge. Dispense 3 replicates of supernatant into a 200 µL 96 well plate. Rapidly read OD_{570} nm

3.2.5.2.Effect of pH and Temperature on Activity

Different buffers were tested to determine the optimal pH values of the *Hd*EH enzyme. Activity measurements were performed by adding 200 µL of the concentrated enzyme to the reaction solutions containing the styrene oxide substrate. In activity measurements, pH was measured between 5 - 11. Citrate buffer (pH 5, 6), Sodium Phosphate Buffer (pH 6, 6.5, 7, 7.5 and 8), Tris buffer (pH 7, 8 and 9), and Bicarbonate buffer (pH 10, 11) were used for activity measurements.

To test the effect of temperature on enzyme activity, enzyme activity measurements were measured by adding 200 µL of the concentrated enzyme to the reaction solution at the optimum pH determined for the styrene oxide substrate at different temperatures (10-70 °C) in increments of 10 °C.

3.2.5.3. Storage Stability

To determine the storage stability of *Hd*EH enzyme activity, the enzyme activity was determined at certain time intervals by storing the epoxide hydrolase enzyme at different temperatures (4, 25, -20, and -80 °C). Enzyme activity was determined by adding 200 µL of the concentrated enzyme to the reaction solution containing 20 mM styrene oxide at optimum temperature and pH once a week and then for 8 weeks. Results are expressed as relative percent changes in epoxide hydrolase activity versus time.

3.2.5.4. Kinetic Assay

Kinetic studies were performed in multi-well microplates in sets of three, with measurements at 570 nm with the addition of a fixed amount of 200 µL of concentrated *Hd*EH enzyme to reaction solutions containing varying concentrations of 0.125 - 3 mM of each substrate. The obtained results were adapted to the Michaelis-Menten kinetic model; The k_{cat} and K_M values were calculated for each substrate with GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) [52].

3.2.6. Homology Modelling and Molecular Docking of Epoxide Hydrolase

The 3D homology model of the *Hd*EH enzyme was created via Swiss-Model (https://swissmodel.expasy.org/). The amino acid sequence of *Hd*EH and structurally characterized epoxide hydrolases from the Research Collaboratory for Structural Bioinformatics Protein Database (RCSB PDB) Bombyx mori-PDB ID: 4QLA; *Aspergillus niger*-PDB ID: 3G02 and 1QO7 aligned with the Clustal Omega program. Protein secondary structure was predicted with the ESPript 3 program [58]. The published structure of *Bombyx mori* epoxide hydrolase (PDB ID: 4QLA) was used as a template for homology modeling. The pentaethyleneglycol substrate (PubChem CID 62551) was embedded in the *Hd*EH binding site via the AutoDock platform [53]. Illustrations were made using PyMOL (Molecular Graphics System, Version 2.0 Schrödinger, LLC).

4. RESULTS AND DISCUSSIONS

4.1. Bioinformatics Analysis and vector design of *hd*eh gene

Bioinformatics analysis of the *hd*eh gene; The GenBank accession numbers used in the project work: OQV21190.1 and 1338 bp and the nucleotide sequence of the putative *hd*eh gene are given in Figure 4.1 and the amino acid sequence and functional regions of *Hd*EH are given in Figure 4.2. The amino acid sequence of the hypothetical *Hypsibius dujardini Hd*EH enzyme was compared with the epoxide hydrolases whose structure was elucidated before, and the presence of conserved amino acids in their catalytic and functional regions was detected (Figure 4.3). The total molecular weight (Mw) of the hypothetical *Hd*EH enzyme with 6xHis tag was determined as 48.97 kDa and its isoelectric point pI: 9.02.

```
5′ATGACTGCGTCCTGGAAAGGATCTTCCCGTTGCCAGACATCAAGACGGTCTTCCCGTTCGG
CGATGAAGAGTGGAGTGCTCCCGGAAGCAAACCGGCAAAAGCGGACGATTGCAGTCGTGCCTT
GGAAGTTGCAGGTGTCTAACAAAGATTTGGAGGATTTGAAGGATCGTCTACGGAACACTCGAT
ATGGAACAGCCATGCCTGGACAGAACTTTGTTTATGGATTTCCGGCGGATCAGCTGAAAAAGG
TTGTGGATTACTGGTTGAACAAGTATGACTGGAAGAAGCAGGAAGCTCTGATCAACCAGTACG
ACCACTTCAAGACCAACATCGAAGGACTCAACATCCACTTTGTTCGAGTTCAGCCGGCAAAGT
CCACGGACCCTAAACGGAATGTCCGTGTCACTCCGATCATCCTCCTTCACGGCTGGCCTGGAT
CCTTTTTCGAGTTGTACAAGCTCATCCCGTTGCTGACCACTCCGCAACAGATATCTGGGAATG
ACTACGCGGCTTTTGAAGTTATCATCCCATCGCTACCGGGATACGGATTCTCGGATGCAGCGC
AGAAGACAGGCCTCAATCCGCCGCATATTGCGAGAATCTTCAACAAATTGATGACCCGTCTCA
AGCTCATTAAATACTTTGTTCACGGCGGTGACTGGGGAAGTGCGGTAGGAAGATCCCTGGCTA
TAATGTACCCTGACTGTGTTTTGGGTGTCGATCTGACGTTGTACGGTATGGCCGTTCCTCCGG
GTCCCTCTTTCATAAAAAATATCCTCTATCTCACCATGCCGCGGTTCCTGTTTAACGTCTCCG
AACCACATAAGCTCCTTCCCACCAATTTCTTCTCGCTACTGCGGGAATCGGGTTACATGCACA
TACAAGCTACAAAGCCTGATACTGTCGGTGCCGCTCTGTTGGATAGTCCGGCTGGTCTTGCAG
CTTACATCCTGGAGAAGTTCTCAACCTGGACCAACGTGGACAACATCAACAAGCCGGACGGAG
CTCTTTCTAAGTACTTCTCCCTGGACGATCTCCTGACTAACGTCATGGTCTACTGGGTTTCTG
GAAGTATCATCACCTCGCAGCGACTGTATAAGGAAACATTCTCGGCGGATCCAAATGACGGGT
TTCCGGTGTTGGTGCCCAGTCTGCTGTCGGATTTCCGTCATGAAATACCCGGTGCAAACAGTC
CTGATGAATTCGTCAGGCCTTATTACAAAGACCTCATCACCATCTCTAAGCACAAATCCGGCG
GTCATTTCCCAGCCATGGAATTGCCCATTGAACTTGCCGCGGACATTTGGGCGTTGGCCAAAA
ATGTTTTGTCAAGAGATTAA-3′
```

Figure 4.1: Nucleotide sequence (1338 bp) of the putative *hd*eh gene.

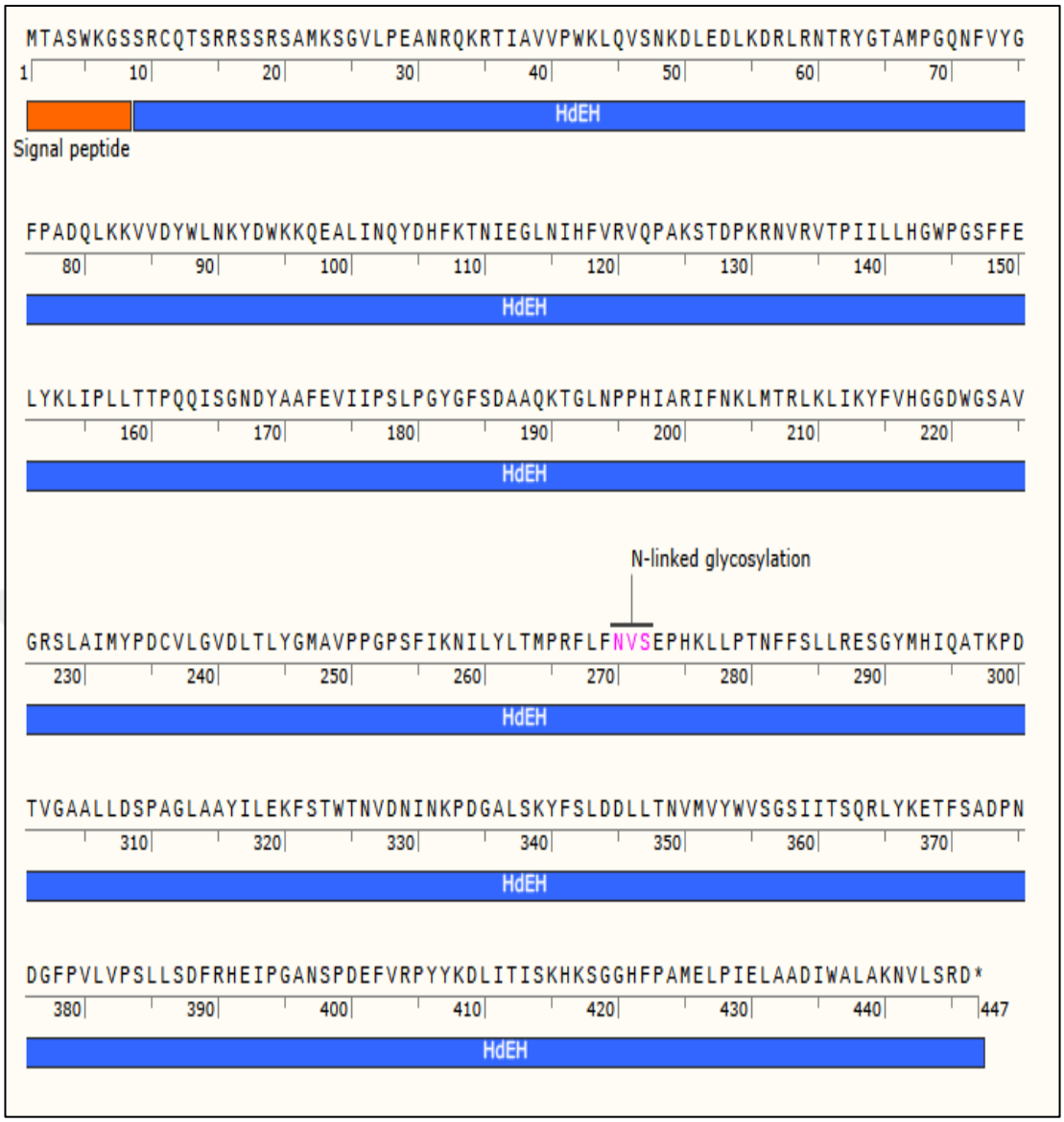

Figure 4.2: Amino acid sequence of putative *Hd*EH. The orange region shows the native signal sequence, the blue region shows the mature *Hd*EH protein, while the pink color N-glycosylation region (Asn-X-Ser/Thr) is shown.

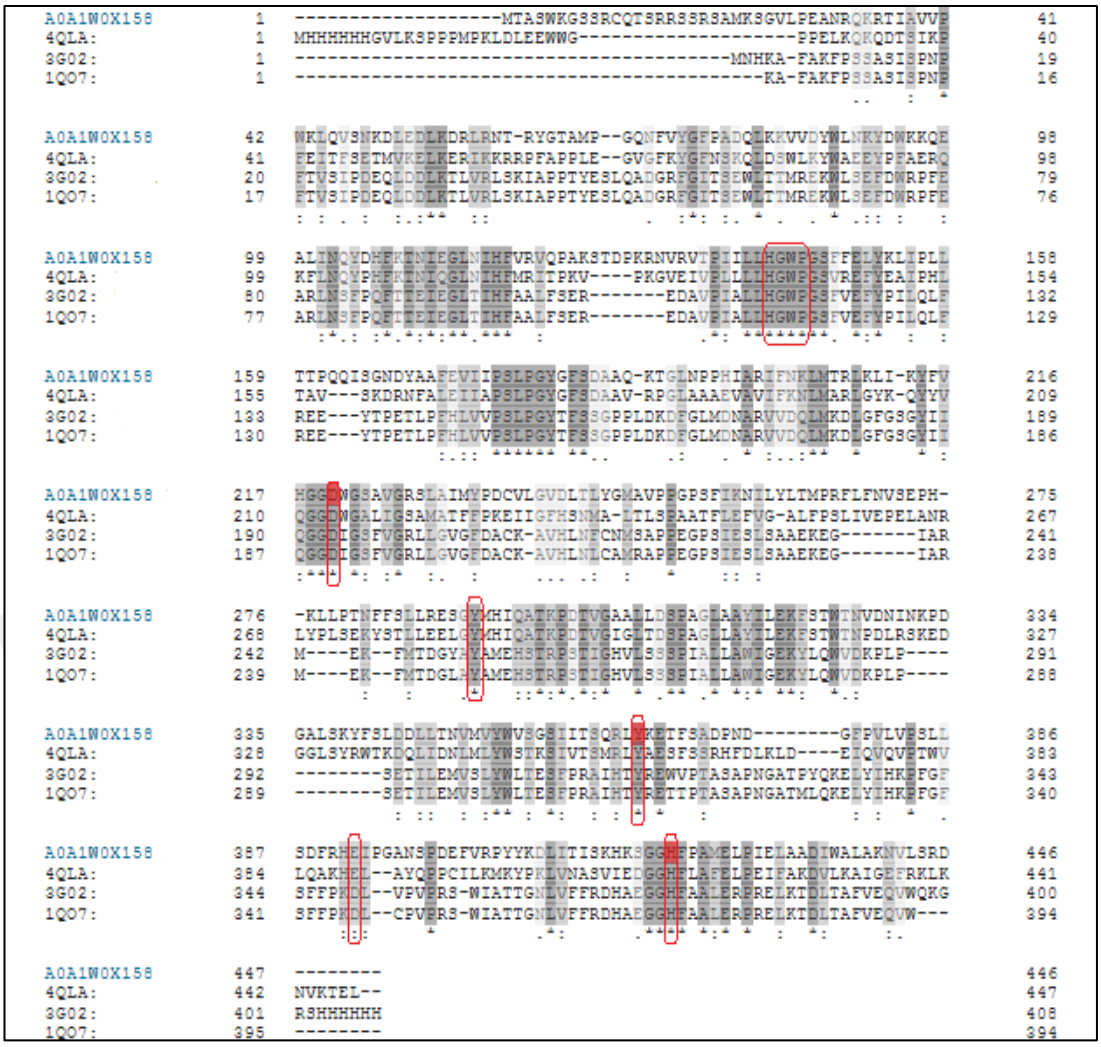

Figure 4.3: Alignment sequence analysis of *Hd*EH enzyme. Conserved regions of the putative *Hd*EH enzyme are shown with red markers (HGWP motif, Asp220, Try291, Try366, Glu403, and His430) and similarity ratios are shown with gray and shades. Uniprot accession number: A0A1W0X158-*Hypibius dujardini*. Protein Data Bank (PDB) accession numbers: 4QLA-*Bombyx mori*, 3G02, and 1QO7-*Aspergillus niger*.

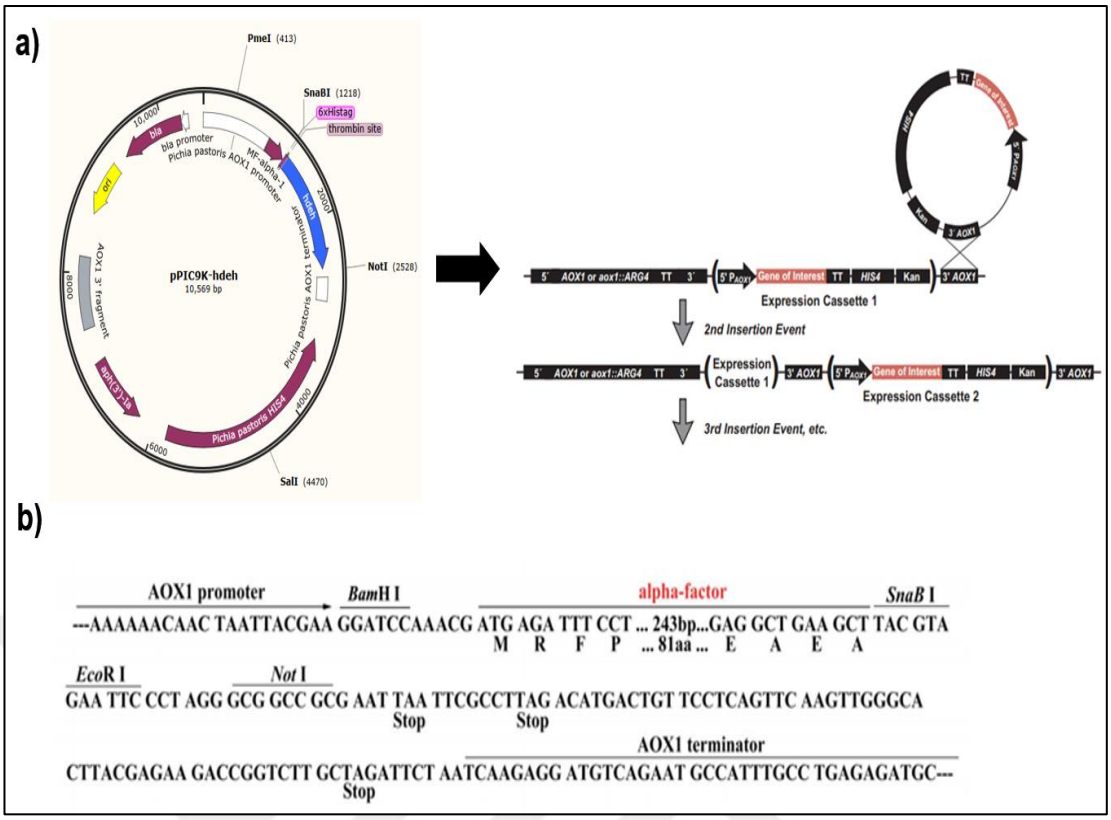

Figure 4.4: Homologous recombination process for pPIC9K-*hde*h (a) and multiclonal region map of pPIC9K plasmid (b).

Vector design of pPIC9K- *hde*h: In the studies, the pPIC9K vector was used in accordance with *Pichia pastoris* GS115 host cells (Figure 4.4). Thanks to its kanamycin and ampicillin antibiotic resistance genes, this vector has enabled the selection of recombinant colonies for the propagation of the vector in the bacterial host (*Escherichia coli* TOP10). The kanamycin resistance gene also enabled the selection of recombinant yeast colonies in a medium containing (increasing concentration) geneticin as a result of transformation into *Pichia pastoris* GS115 host cells. The pPIC9K vector provided its selectivity in histidine-free medium (MD-H: Histidine-Free minimal dextrose medium) in *Pichia pastoris* GS115 host cells silenced with the *HIS*4 gene it contained. Colony selection was carried out in yeast cells thanks to these selectivity methods. The pPIC9K vector used was commercially designed to integrate *Pichia pastoris* GS115 cells into the yeast chromosome by homologous recombination from the AOX region. Thus, the integration of the *hde*h gene into the chromosome was carried out. It contains the MF-α (mating factor-alpha) signal sequence that will enable the protein to be produced in the pPIC9K vector to be secreted out of the cell in host yeast cells. Through this signal sequence (pre-pro peptide), the related polypeptide

chain is processed and secreted out of the cell with modifications in the Endoplasmic reticulum and Golgi apparatus, respectively. After these processes take place, the cut regions in the signal sequence are cut by the enzymes (Kex2, Ste13) in the secretion system of the organism and are not included in the structure of the enzyme [42],[54]. After the pPIC9K vector is transferred to the host *Pichia pastoris* GS115 cells, it integrates into the yeast chromosome as a result of homologous recombination from its AOX1 (Alcohol oxidase 1) regions (Figure 4.5).

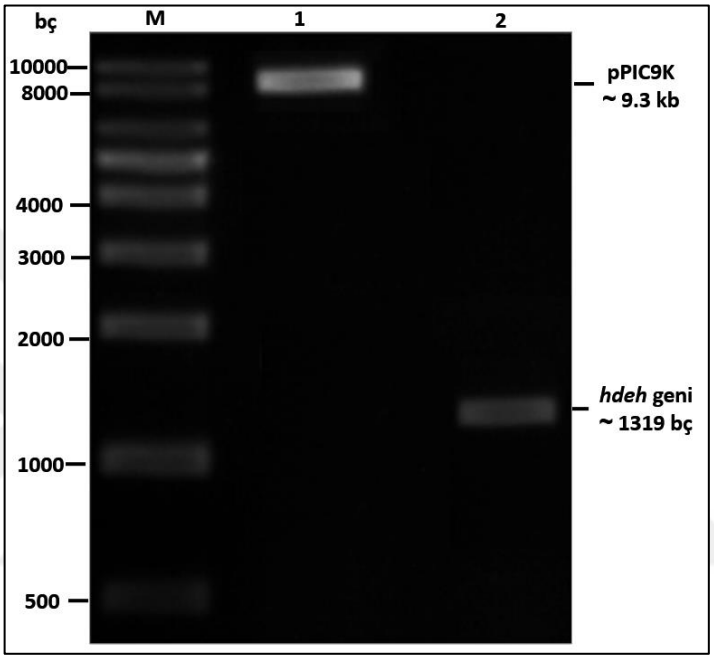

Figure 4.5: *hd*eh gene fragments amplified by PCR from *Hypsibius dujardini* cDNA. M: Marker, 1: Negative control (pPIC9K), 2 and 3: *hd*eh gene fragments ~1319 bp long.

Hypsibius dujardini cDNA obtained from Koutsovoulos et al. [49] was amplified by PCR and visualized on an agarose gel. The putative *hd*eh PCR product and the pPIC9K plasmid were prepared for ligation by digesting with SnaBI and NotI restriction enzymes. The appearance of the *hd*eh gene fragment, which does not contain pPIC9K and its native signal peptide, in 1% agarose gel after cutting by the SnaBI and NotI enzyme pair is shown. Accordingly, bands of expected base size were detected as single bands for the plasmid and *hd*eh gene (Figure 4.6).

4.2. Transformation of Plasmid into *E. coli*

Recombinant plasmids obtained after the ligation reaction were transformed into E. *Escherichia coli* TOP 10 cells by heat shock. Recombinant cells were seeded on LB agar plates containing 100 µg/mL ampicillin and 50 µg/mL kanamycin. *Escherichia coli* TOP 10 without recombinant plasmid was used as a control group (Figure 4.8.). Cells grown on the plate were used as a source of a recombinant plasmid containing the *hd*eh gene construct for further studies.

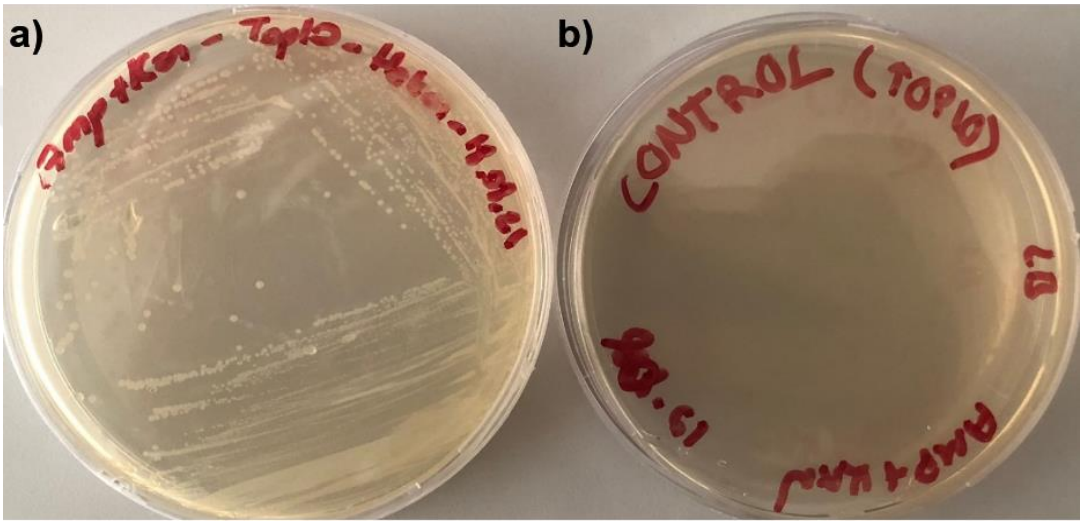

Figure 4.6: *Escherichia coli* TOP10 cells grown on LB agar plates containing ampicillin and kanamycin after transformation. a) *Escherichia coli* TOP10 cells transformed with pPIC9K-*hd*eh plasmid b) *Escherichia coli* TOP10 cells without plasmid (control).

4.3. Transformation of Plasmid into *Pichia pastoris*

4.3.1. Linearization of Plasmid and Electroporation

After the transformation of the linearized recombinant plasmids to *Pichia pastoris* GS115 cells by electroporation, they were spread as 100 µL in a YPDS agar medium containing different concentrations of geneticin (0.25, 0.5, 1, 1.5, 2 mg/mL), and colony growth was followed for 5 days. Colony development was not observed in the control group during this period, while colonies containing the putative *hd*eh gene structure were observed (Figure 4.7).

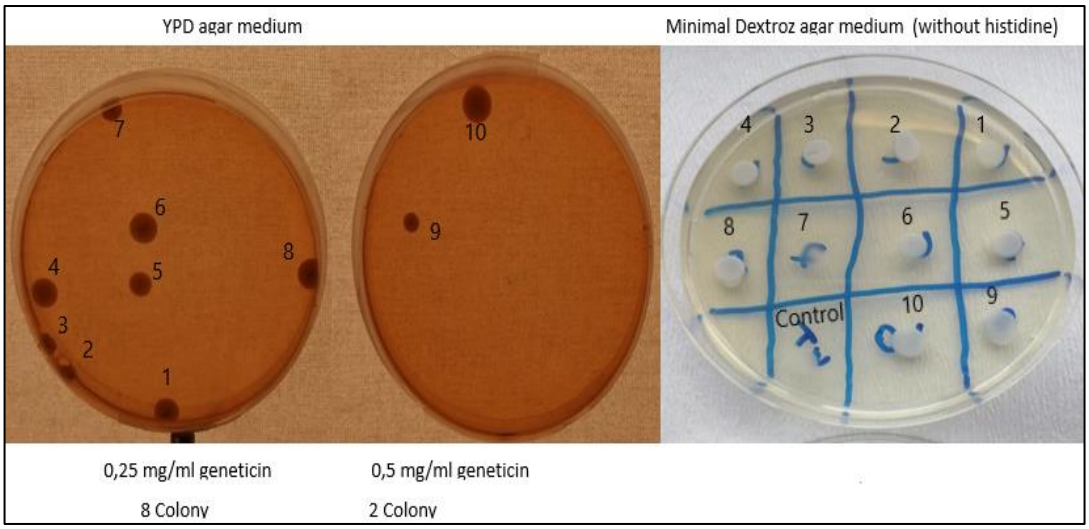

Figure 4.7: Colonies growing on YPDS agar and Minimal Dextrose Agar plate after transformation. Recombinant *Pichia pastoris* GS115 cells, *Pichia pastoris* GS115 colonies grown on histidine-free Minimal Dextrose Agar (Wild-type *Pichia pastoris* GS115 strains were used as controls).

Colonies were seen only on plates containing 0.25 (8 colonies), 0.5 (2 colonies) mg/mL geneticin after transformation. Growing colonies were also cultivated on Minimal dextrose agar medium (without histidine) to identify His^+ transformant colonies. Because the pPIC9K plasmid contains the His4 gene, recombinant colonies will be able to grow (Figure 4.7).

4.3.2. Selection of Transformed Cells

The presence of putative *hde*h gene construct in *Pichia pastoris* GS115 colonies was determined by colony PCR. For the pPIC9K-*hde*h gene construct, MF-α forward and AOX1 terminator reverse primers were used. The results obtained after PCR were visualized on 1% agarose gel (Figure 4.8). Based on these results, the integration of recombinant plasmids with transforming *Pichia pastoris* GS115 and colonies was shown to contain the putative *hde*h gene sequence.

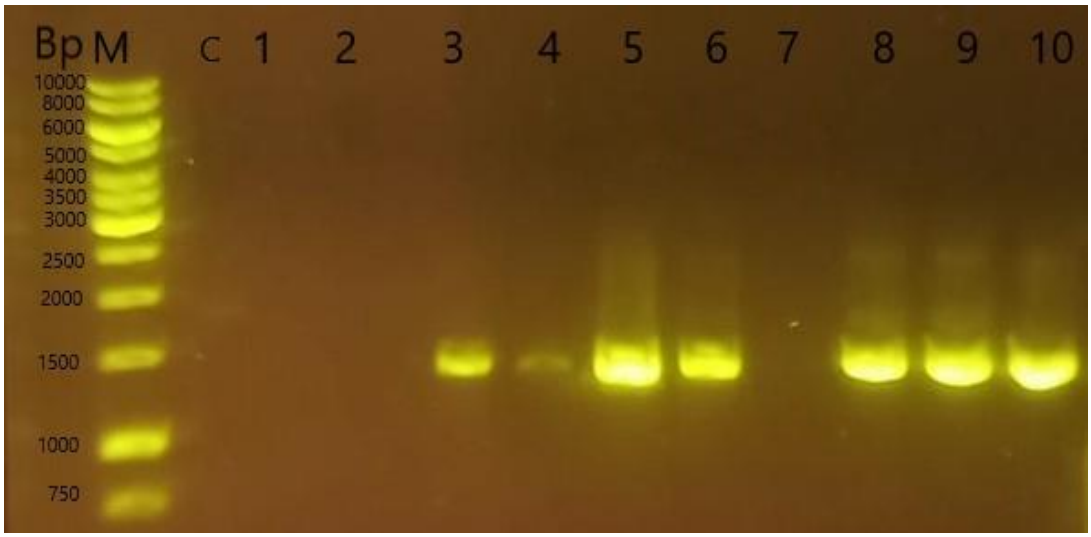

Figure 4.8: Colony PCR results. M: Marker, C: Control (wild type *Pichia pastoris* GS115). 3-4-5-6-8-9-10: recombinant colonies containing the *hd*eh construct (~1500 bp).1, 2, and 7: *P. Pichia pastoris* GS115 colonies without the *hd*eh gene construct.

4.4. Determination of Optimum Expression Conditions

Effect of methanol concentration on *hd*eh gene expression in recombinant *Pichia pastoris* GS115 cells; ın order to determine the effect of methanol concentration on extracellular *hd*EH production conditions, 3 different methanol concentrations (0.5%, 1 and 2%) were tested. Induction conditions for the best recombinant *Pichia pastoris* GS115 were determined at 1% methanol concentration (Fig. 4.9). The effect of methanol concentration on *hd*eh gene expression was determined as 1.0%>0.5%>2%.

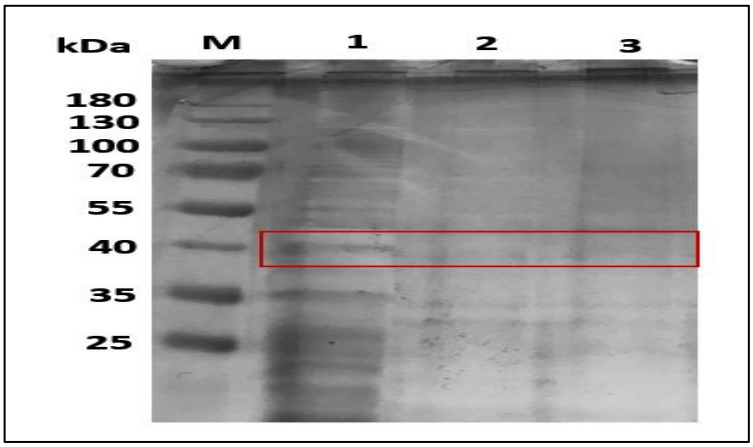

Figure 4.9: SDS-PAGE image of the effect of methanol concentration on hdeh expression. M: Marker, wells 1, 2, and 3 show *hd*eh expression at methanol concentrations of 1%, 0.5%, and 2%, respectively.

As a result of the SDS-PAGE images obtained from the samples taken every day, band intensity an increase was observed arithmetically until the 5th day. It was observed that the band intensity started to decrease after the 5th day. Considering the SDS-PAGE bands, the 5th-day bands have a higher intensity than the bands of the other days. Therefore, the best induction time was determined as the 5th day (Figure 4.10).

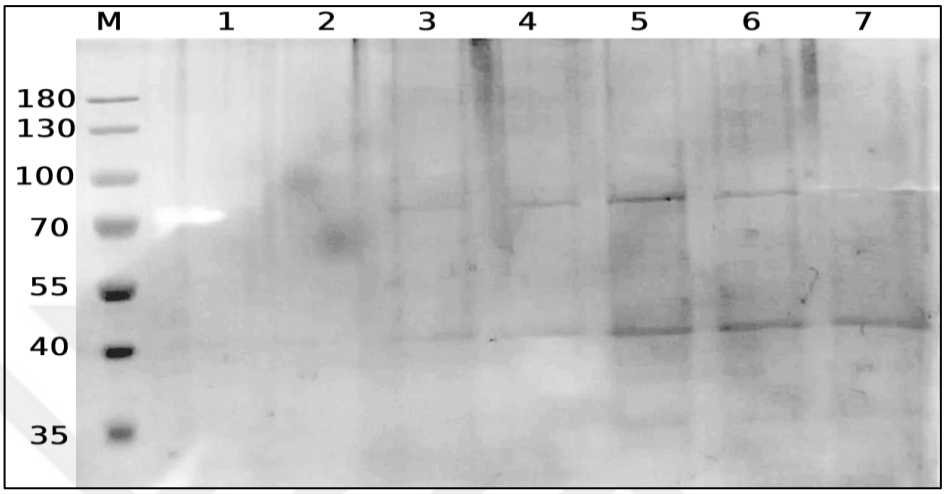

Figure 4.10: SDS-PAGE images at 30 °C and %1 methanol induction of C10 (GS115-pPIC9K- *hd*eh) and control (*Pichia pastoris* GS115). M: Marker. Line 1-7: 10X concentre supernatant samples taken for 7 days uploaded day by day.

Effect of temperature on the growth of recombinant *Pichia pastoris* GS115 cells: Increasing concentration of geneticin (0.25-2 mg/mL) was used to determine the multiplex transformant to be used in *hd*eh expression. Accordingly, growth was observed only on the YPD agar medium at a concentration of 0.5 mg/mL. Furthermore, the highly expressed transformant growing on Minimal Dextrose Agar plate (without histidine) was selected for *hd*eh expression experiments. The effect of different temperatures (18, 22, 26, 30 °C) on cell growth was determined (Figure 4.11). Based on the data obtained, the highest cell growth was obtained at 18 °C at the end of the 5th day (Figure 4.10).

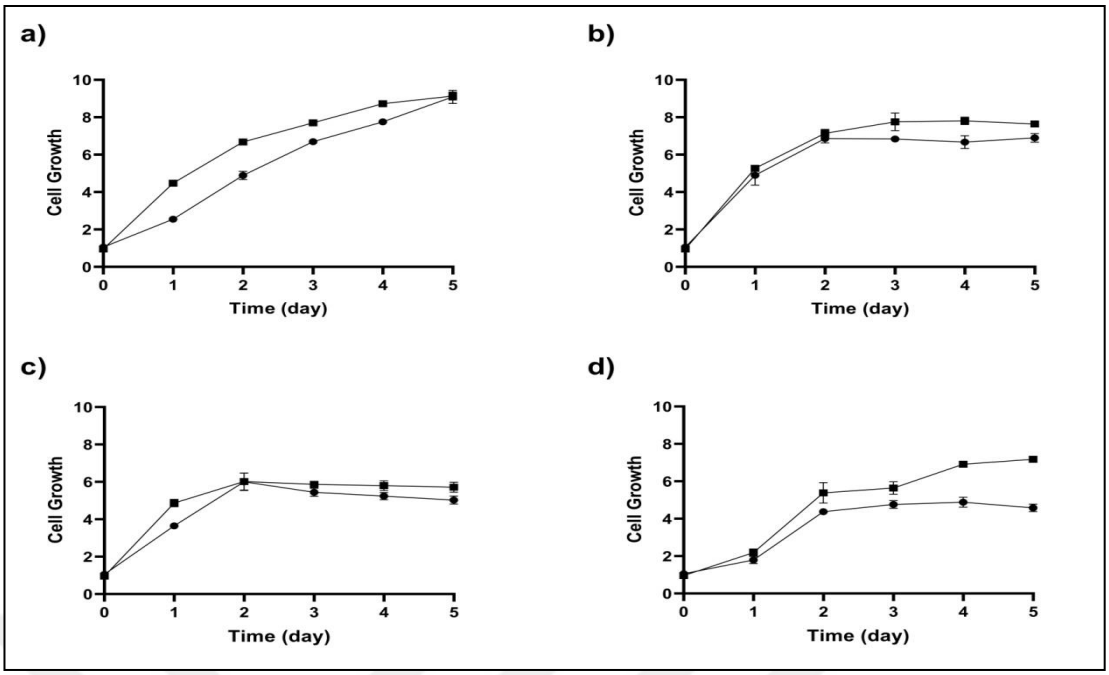

Figure 4.11: Cell growth graph of the effect of temperature on recombinant (■) and wild-type (●) *P. Pichia pastoris* GS115. a) 18 °C, b) 22 °C, c) 26 °C, d) 30 °C.

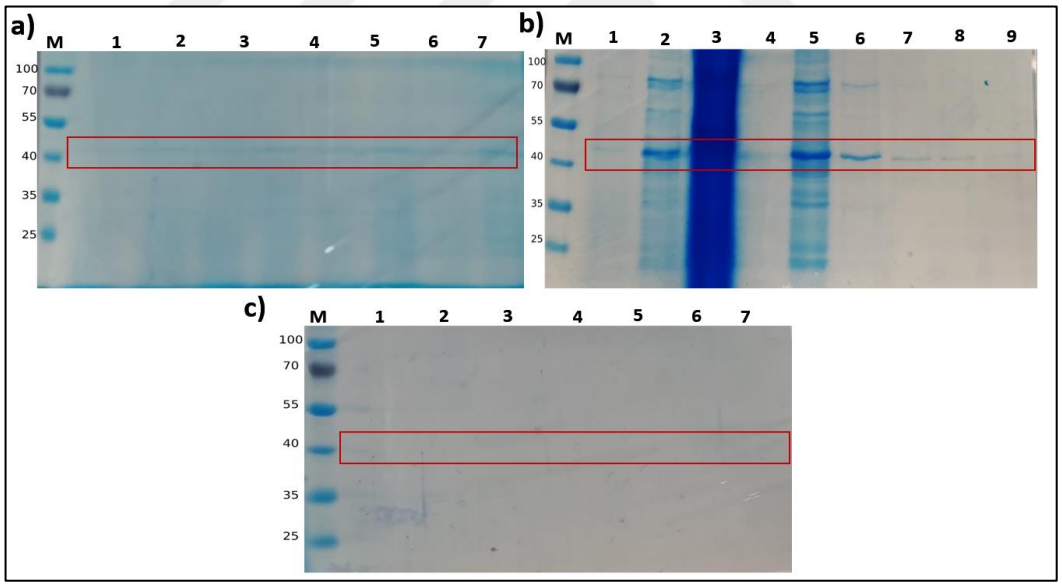

Figure 4.12: *hd*eh expression and purification results in 1% methanol induction at 18 °C. a) 1-3-5-7: supernatant *hd*eh expression level in wild-type *Pichia pastoris* GS115 cells (days 1-2-3-4, respectively); 2-4-6-8: Supernatant *hd*eh expression level in recombinant *Pichia pastoris* GS115 cells (days 1-2-3-4, respectively). b) Purification fractions of supernatant *Hd*EH in wild-type and recombinant *Pichia pastoris* GS115 cells. 1: Control supernatant, 2: Recombinant cell supernatant, 3: Recombinant 5th day 10X concentrated supernatant, 4: Concentrator thrash, 5: Flow-through fraction. 6: Pre-column wash (Buffer A 30 mM concentration fraction). 7-8-9: 100 mM imidazole concentration fraction. c) 1-2-3: 200 mM imidazole concentration fraction, 4-5-6: 400 mM imidazole concentration fraction, 7: Buffer B (500 mM imidazole concentration) fraction. M: Marker.

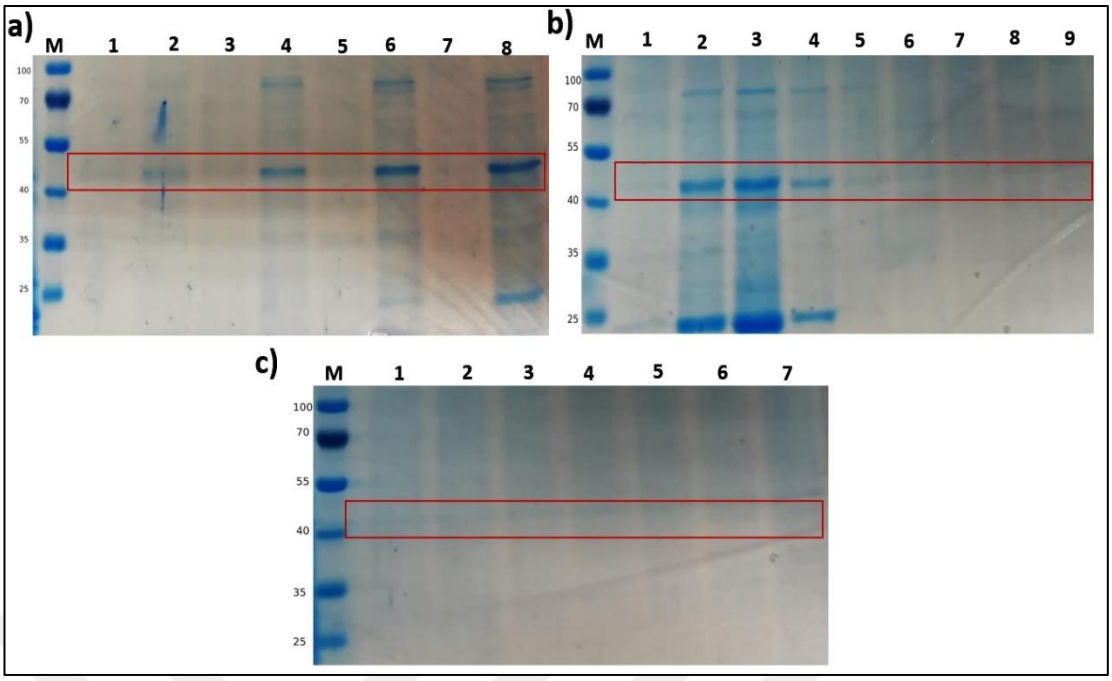

Figure 4.13: *hd*eh expression and purification results in 1% methanol induction at 22 °C. a) 1-3-5-7: supernatant *hd*eh expression level in wild-type *Pichia pastoris* GS115 cells (days 1-2-3-4, respectively); 2-4-6-8: Supernatant *hd*eh expression level in recombinant *Pichia pastoris* GS115 cells (days 1-2-3-4, respectively). b) Purification fractions of supernatant *Hd*EH in wild-type and recombinant *Pichia pastoris* GS115 cells. 1: control supernatant. 2: Recombinant 5th day 10X concentrated supernatant 3: Flow-through fraction. 4: Pre-column washing (Buffer A 30 Mm imidazole concentration fraction). 5-6-7-8-9: 100 mM imidazole concentration fraction. c) 1-2-3: 200 mM imidazole concentration fraction, 4-5-6: 400 mM imidazole concentration fraction, 7: Buffer B (500 mM imidazole concentration) fraction. M: Marker.

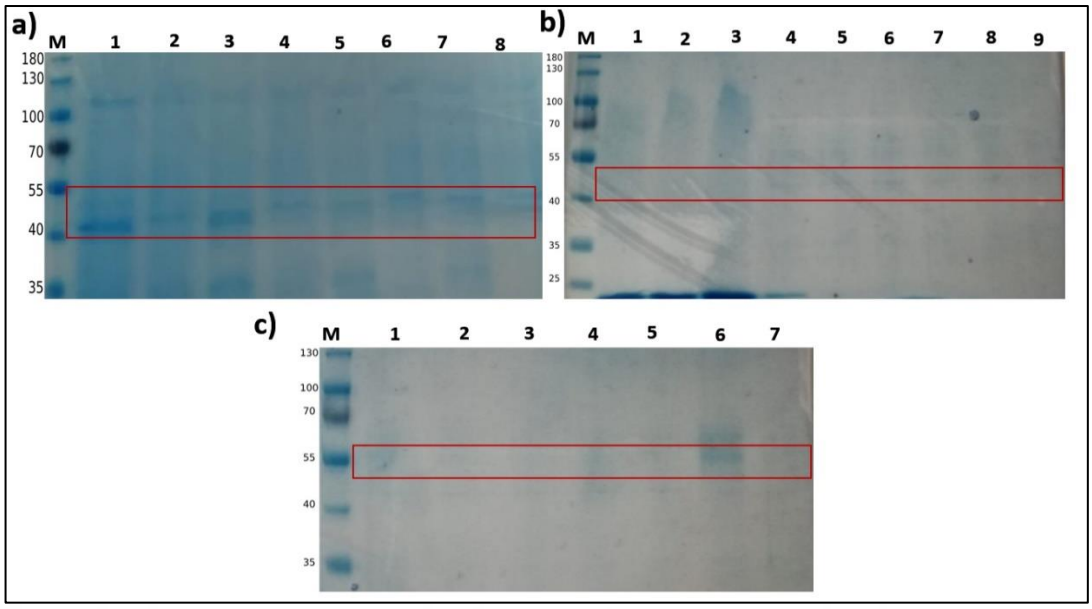

Figure 4.14: *hd*eh expression and purification results in 1% methanol induction at 26 °C. a) 1-3-5-7: Supernatant *hd*eh expression level in recombinant *Pichia pastoris* GS115 cells (day 4-3-2-1, respectively); 2-4-6-8: supernatant *hd*eh expression level in wild-type *Pichia pastoris* GS115 cells (day 4-3-2-1, respectively). b) Purification fractions of supernatant *Hd*EH in wild-type and recombinant *Pichia pastoris* GS115 cells. 1: Control supernatant. 2: Recombinant supernatant, 3: Flow-through fraction, 4: Pre-column washes (Buffer A 30 Mm imidazole concentration fraction). 5-6-7-8-9: 100 mM imidazole concentration fraction. c) 1-2-3: 200 mM imidazole concentration fraction, 4-5-6: 400 mM imidazole concentration fraction, 7: Buffer B (500 mM imidazole concentration) fraction.

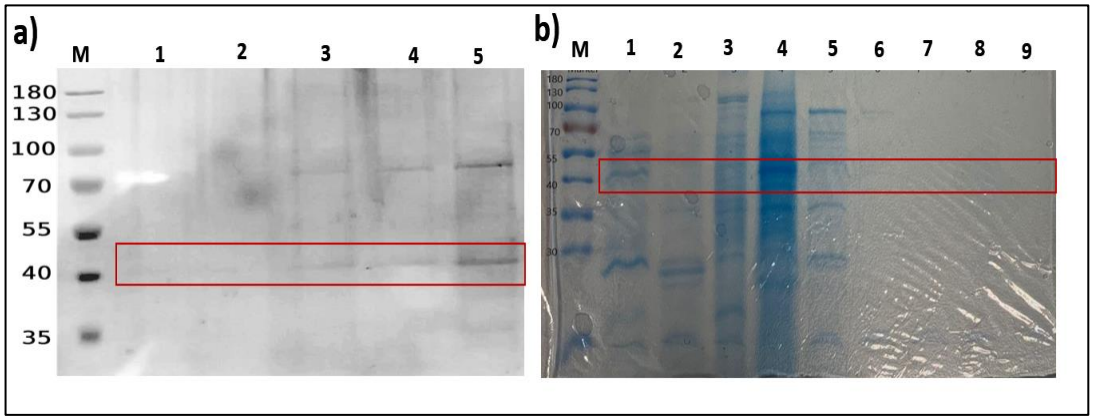

Figure 4.15: *hd*eh expression and purification results in 1% methanol induction at 30 °C a) Supernatant *hd*eh expression level in recombinant *Pichia pastoris* GS115 cells. 1-5: 10X concentrated supernatant samples taken over 5 days. b) Purification fractions of supernatant *Hd*EH in wild-type and recombinant *Pichia pastoris* GS115 cells. 1: Recombinant 10X concentrate supernatant, 2: Concentrate 10X control supernatant, 3: Pellet control samples, 4: Recombinant pellet samples, 5: Flow-through fraction, 6: Pre-column wash (Buffer A 30 Mm imidazole concentration fraction). 7: 100 mM imidazole concentration, 8: 200 mM imidazole concentration, 9: 400 mM imidazole concentration.

In summary, OD_{600} spectrophotometer measurements were taken from the samples taken daily during the Expression experiments, and SDS-PAGE analysis was performed. According to the analysis results, the optimum temperature was determined as 18 °C and the optimum methanol induction rate was determined as 1%. As a result of the SDS-PAGE images obtained from the samples taken every day, the best induction time was determined as 5 days (Figures 4.9 - 4.15).

The presence of the enzyme was checked by Western blot (with His-tag antibody) technique using His-tag in the structure of the purified *Hd*EH enzyme (Figure 4.16).

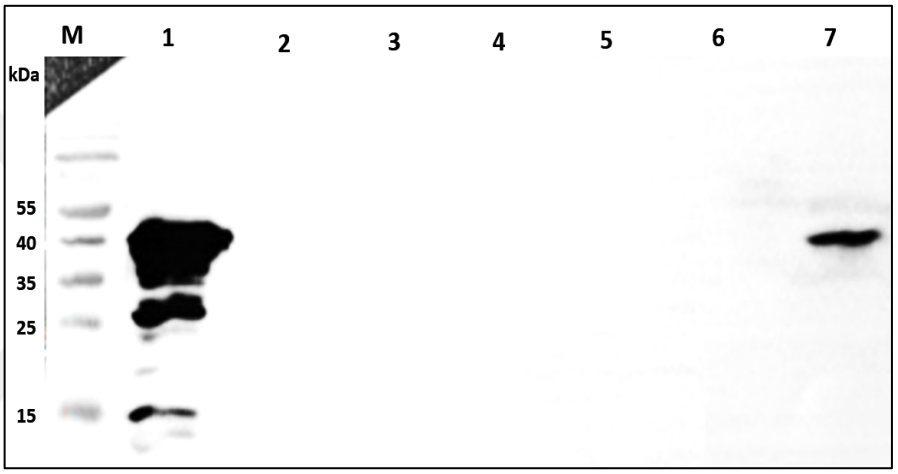

Figure 4.16: Imaging results of Western Blot gel for *Hd*EH enzyme with ECL Substrate Western blot technique. 1: His-tag positive control (*Chaetomium thermophilum* Formate dehydrogenase), 2-3: 10X control supernatant, 4-5: Control pellet, 6: Recombinant pellet, 7: Recombinant *Hd*EH purification result.

According to the western blot results, the recombinant *Hd*EH was displayed as ~45 kDa (Figure 4.16).

4.5. Biochemical Characterization Analysis

4.5.1. Effect of pH, Temperature on Activity

Determination of optimum pH: The optimum pH of the recombinant epoxide hydrolase enzyme was determined as 8 versus styrene oxide. While the activity of the recombinant enzyme in acidic conditions was lower than in basic and neutral

environments, it increased with the increase in pH and reached the highest level (pH: 8) at basic pHs; At pH: 10, the activity decreased below 20%. While the enzyme showed more than 70% activity between pH 7 and 8.5, it did not show activity above pH 10 (Figure 4.17).

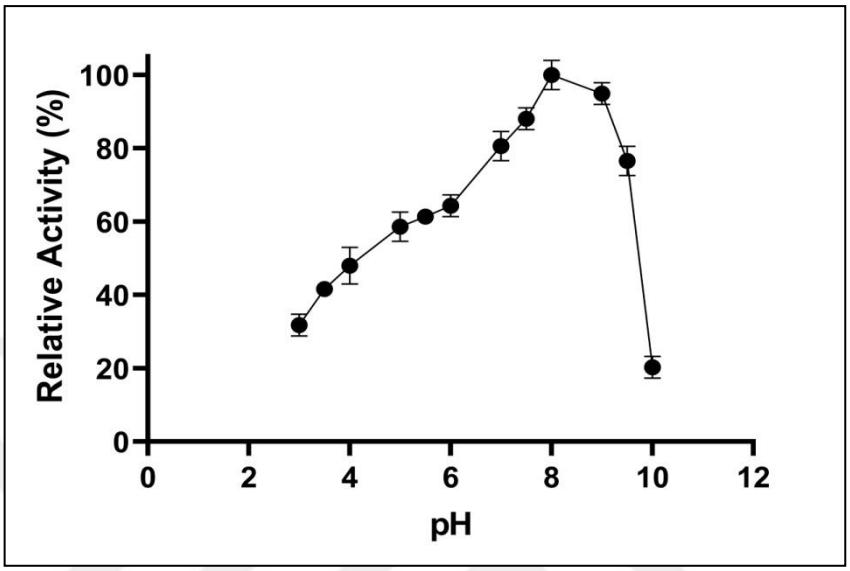

Figure 4.17: Effect of pH on *Hd*EH enzyme activity.

Determination of the optimum temperature: The optimum temperature value of the recombinant *Hd*EH enzyme against the styrene oxide substrate was determined as 20 °C (Figure 4.18). However, the enzyme activity decreased with the increase in temperature. After 60°C, the enzyme lost more than 80% of its activity (Figure 4.18).

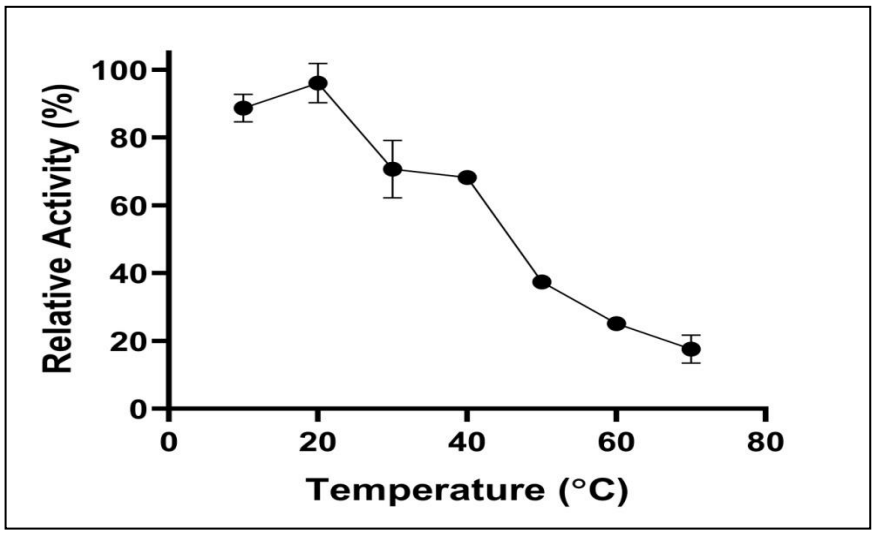

Figure 4.18: Effect of temperature on *Hd*EH enzyme activity.

4.5.2. Storage Stability Assay

Determination of storage stability on the activity of *Hd*EH enzyme: The effect of storage stability on the activity of purified *Hd*EH enzyme is shown in Figure 4.21. It was determined that the storage stability of the *Hd*EH enzyme at +4, +25, and -80 °C was higher than -20 °C. The optimum storage conditions of the enzyme were determined as +4 °C (Figure 4.19).

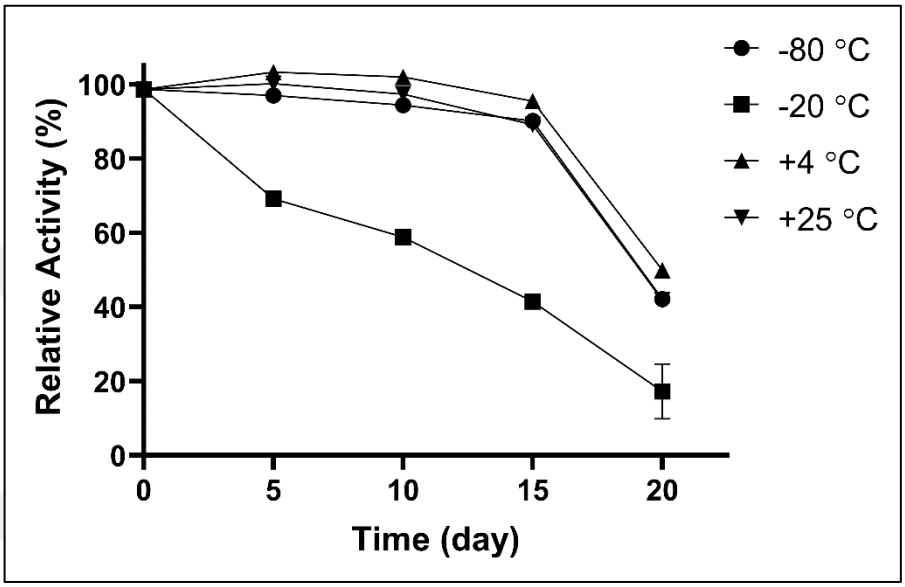

Figure 4.19: Time-dependent storage stability of *Hd*EH enzyme at different temperatures.

4.5.3. Enzyme Activity Determination and Kinetic Parameters

The kinetic study was performed against the styrene oxide substrate at varying concentrations and measurements were made in the spectrophotometer at OD_{570}. At the end of the study, K_m, kcat, and kcat/Km values were calculated using the "GraphPad" program (Figure 4.20). After Ni-NTA chromatography, the *Hd*EH was purified 20.9-fold. The specific activity of *Hd*EH and protein concentration of the Ni^{2+}-NTA was determined as 1004 U/mg protein and 30.6 mg/L *Hd*EH and k_{cat}: $9.23x10^3$ s^{-1}.

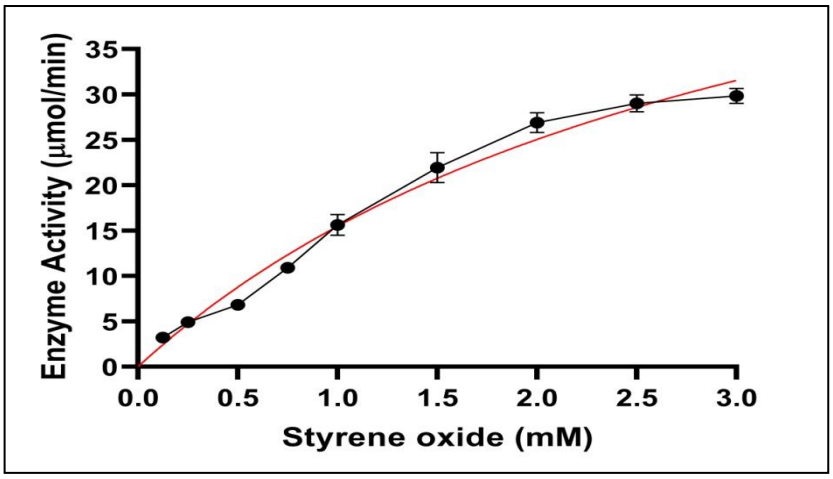

Figure 4.20: Michaelis-Menten plot of the styrene oxide substrate of the *Hd*EH enzyme. K_M: 0.981 mM.

When the catalytic activity performance (kcat/Km) of the *Hd*EH enzyme is compared with the literature, it is observed that the efficiency for styrene oxide is relatively higher than most (Table 4.1). This indicates that the enzyme is a potential candidate for industrial areas. Since there are more inhibitor studies of this enzyme in the literature than characterization studies [47–50], the publication on epoxide hydrolase and kinetic properties to be compared is limited. In addition, epoxide hydrolase enzymes are generally used in industry to synthesize enantiomers of chiral molecules. The applications of these enzymes in different industrial areas such as environmental pollution are quite limited and this creates a potential for future studies.

Table 4.1: Comparison of the activity performance of *Hd*EH enzyme with other enzymes in the literature.

Organism	Host organism	k_{cat}/K_M [M^{-1} s^{-1}]	Source
Hypsibius dujardini	*P. pastoris*	$9.4x10^3$	In this study
Manduca sexta	*Baculovirus*	29×10^4	[6]
Agrobacterium radiobacter AD1	-	70×10^2(1R) 4.2 $x10^2$(1S)	[56]
Bombyx Mori	*Baculovirus*	77×10^3	[57]
Aspergillus niger M200	*E. coli TOP10*	34×10^{-2}	[22]
Aspergillus niger SQ-6	*E. coli*	17×10^2	[58]
Heliothis virescens	Sf-9 insect cells	2.2×10^3	[59]

4.6. Homology Modelling and Molecular Docking of *Hd*EH

Homology modeling results showed that the *Hd*EH enzyme is dimeric (Figure 4.21) and resembles a typical a/b hydrolase enzyme. The active site of *Hd*EH contains the conserved catalytic triplet, amino acid residues Asp213, His416, and Glu389. Similar to the Bombyx mori epoxide hydrolase enzyme, the active site pocket of *Hd*EH contains two tyrosine residues (Tyr284 and Tyr359) that stabilize and donate protons to the oxygen atom of the epoxide ring. The conserved HGWP motif structure (between amino acids 138-141) involved in the release of the diol product from epoxide hydrolases is also found in the *Hd*EH structure (Figure 4.22) [60]. Docking analysis showed that the substrate (pentaethyleneglycol) binding site of the *Hd*EH enzyme is formed by amino acid residues Ser280, Met292, His293, Thr297, Phe370, Ser371. Many of these amino acids are also present in the enzyme *Bombyx mori* (PDB ID: 4QLA), which is used as a template for modeling *Hd*EH (Fig. 4.24) [24],[57].

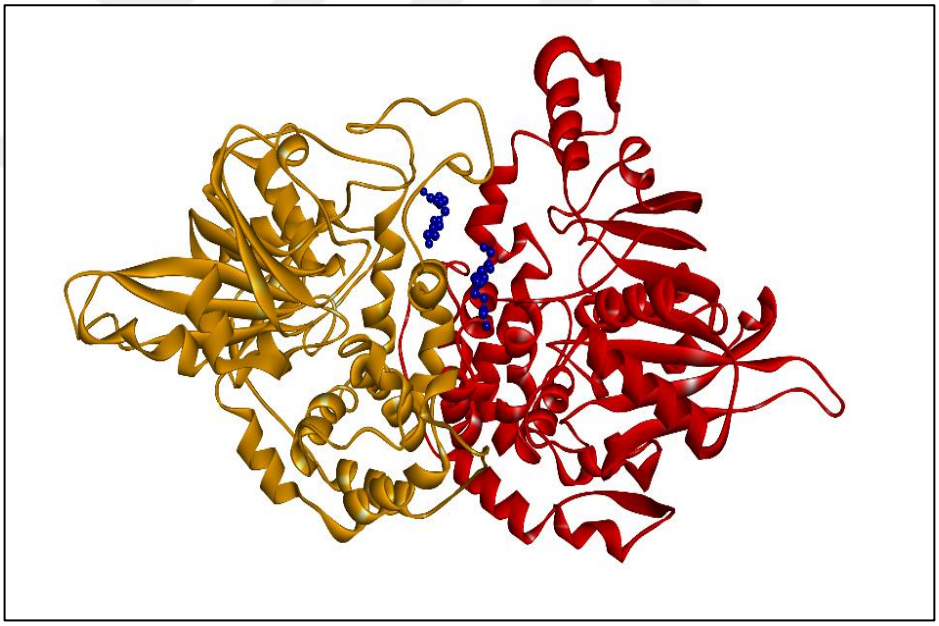

Figure 4.21: 3D dimeric structure of *Hd*EH enzyme according to homology modeling. Red and yellow regions indicate *Hd*EH monomers, and blue regions indicate substrates (pentamethylene glycol) bound by docking analysis.

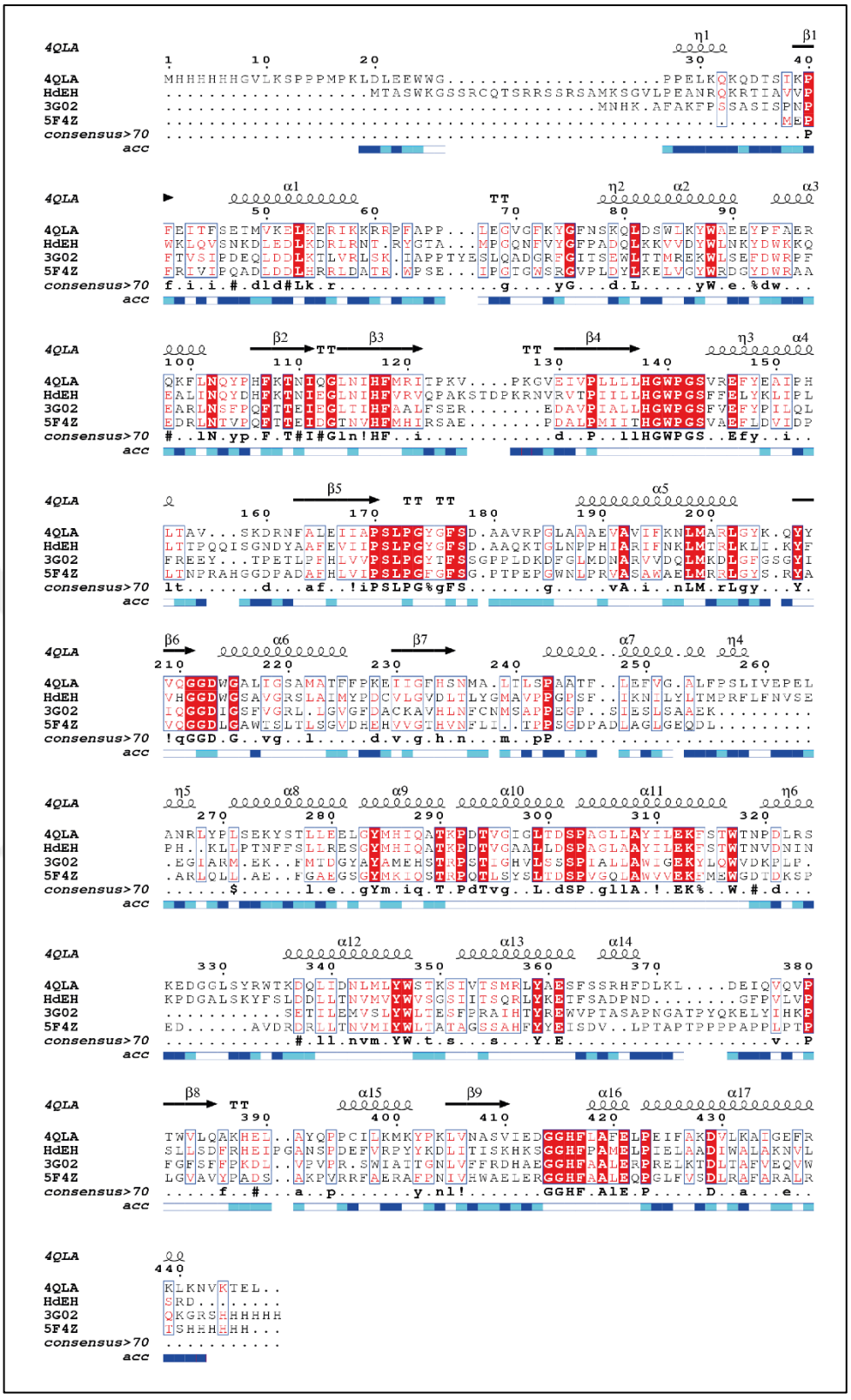

Figure 4.22: Amino acid sequence alignment of selected epoxide hydrolase enzymes. The secondary structure shown above the primary sequence is based on the atomic structure of *Bombyx mori*. (PDB ID: 4QLA). RCSB PDB entries used for this alignment*: Aspergillus niger* (PDB ID: 3G02) and *Streptomyces carzinostaticus* (PDB ID: 5F4Z). Active site residues are marked with black arrows. Helices are indicated by η, β-turns TT and α-turns TTT.

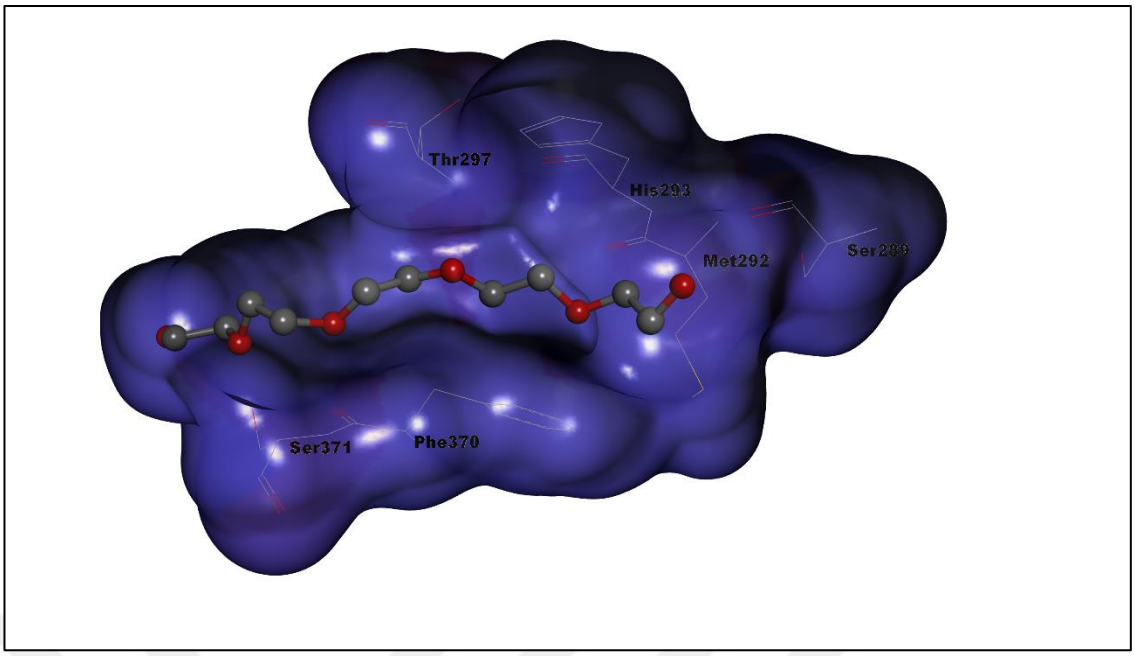

Figure 4.23: Substrate (pentamethylene glycol, PubChem CID62551) binding site of *Hd*EH enzyme as a result of docking analysis. The amino acid residues are shown as bars, the substrate as sphere bars in red-grey, and the 3D structure of the binding pocket in blue.

5. CONCLUSION

Epoxide hydrolases have great potential for versatile industrial applications, studied structurally and biochemically in many organisms. Within the scope of the project, a new epoxide hydrolase enzyme from *Hypsibius dujardini* was cloned into methylotrophic yeast *Pichia pastoris*, the host of heterologous expression, and highly purified and biochemical characterization was performed. The low epoxide hydrolase expression efficiency in natural and genetically modified hosts is a disadvantage for industrial-scale applications.

The expression of the *Hypsibius dujardini* epoxide gene, which was determined as the aim of the project, in heterologous expression system *Pichia pastoris* systems, purification in high amounts, and obtaining a new epoxide hydrolase enzyme with biochemical and kinetic characterization shows that the project achieved its purpose.

As a result of this study, a new epoxide hydrolase enzyme was obtained which was characterized experimentally and computationally. It is expected that the obtained enzyme will contribute to both the literature and epoxide hydrolase-based industrial applications with its properties. At the end of the project, new project ideas aiming at redesigning, immobilizing and producing in a bioreactor with techniques that will include protein engineering studies to be applied for different purposes related to the enzyme were also revealed at the end of the project.

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APPENDICES

Publications related with the thesis

Aslan E. S., Onur H., Tülek A., Binay B., (2021), ‘’ Expression and Biochemical Characterization of *Hypsibius dujardini* Epoxide Hydrolase (*Hd*EH) Enzyme in *Pichia pastoris*’’, 3th Eurasia Biochemical Approaches & Technologıes (EBAT) Congress ’’, 75, Antalya Turkey, 4-7 November 2021

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