T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOMOLECULAR SCIENCES

RECOMBINANT PRODUCTION AND CHARACTERIZATION OF NEW BIFIDOBACTERIAL GLYCOSIDASES

MASTER OF SCIENCE THESIS

BERFİN SUCU

Thesis supervisor:

Assoc. Prof. Dr. SERCAN KARAV

ÇANAKKALE – 2021

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ÇANAKKALE – 2021

ETHICAL STATEMENT

Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü Tez Yazım Kuralları'na uygun olarak hazırladığım bu tez çalışmasında; tez içinde sunduğum verileri, bilgileri ve dokümanları akademik ve etik kurallar çerçevesinde elde ettiğimi, tüm bilgi, belge, değerlendirme ve sonuçları bilimsel etik ve ahlak kurallarına uygun olarak sunduğumu, tez çalışmasında yararlandığım eserlerin tümüne uygun atıfta bulunarak kaynak gösterdiğimi, kullanılan verilerde herhangi bir değişiklik yapmadığımı, bu tezde sunduğum çalışmanın özgün olduğunu, bildirir, aksi bir durumda aleyhime doğabilecek tüm hak kayıplarını kabullendiğimi taahhüt ve beyan ederim.

In this thesis study that I prepared following the Thesis Writing Rules of School of Graduate Studies of Çanakkale Onsekiz Mart University; I declare that I have obtained the data, information, and documents I presented in the thesis within the framework of academic and ethical rules, I have presented all information, documents, evaluations, and results following scientific ethics and ethical rules, I cited all the works that I used in my thesis study by making appropriate reference, I did not make any changes in the data used and that the study I presented in this thesis is original. Otherwise, I undertake and declare that I accept all loss of rights that may arise against me.

…….

Berfin SUCU

28/06/2021

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Berfin SUCU

Canakkale, June 2021

ÖZET

YENİ BİFİDOBAKTERİ GLİKOSİDAZLARININ REKOMBİNANT OLARAK ÜRETİLMESİ VE KARAKTERİZASYONU

Berfin SUCU
Çanakkale Onsekiz Mart Üniversitesi
Lisansüstü Eğitim Enstitüsü
Biyomoleküler Bilimler Anabilim Dalı Yüksek Lisans Tezi
Danışman: Doç. Dr. Sercan KARAV
28/06/2021, 93

Protein glikozilasyonu, protein katlanması, stabilitesi ve enzimatik koruma dahil olmak üzere protein fonksiyonu gibi önemli rollerle ilişkilendirilmiş yaygın bir translasyon sonrası modifikasyondur. Glikoproteinler (laktoferrin, immünoglobulinler gibi), protein ve glikan ikilisinden oluşmaktadır. Bakteriler, glikoproteinleri sindirebilecek gerekli enzimleri içermediğinden onları karbon kaynağı olarak kullanamaz. Bundan dolayı bakteriler üzerinde gözlemlenen gelişimin karbon kaynağı glikanlardır. Glikanların glikoproteinlerden ayrılıp hücre içine alınabilmesi için ise önce bakterilerin glikosidazlarının bu glikanları kesmeleri gerekmektedir. Glikoproteinlere bağlanan *N*-glikanlar, konakçı tarafından sindirilemez ve bazı yararlı bakteriler tarafından tüketildikleri kalın bağırsağa ulaşır. Bu nedenle, bağırsak mikrobiyomundaki yararlı mikroorganizmaları seçici bir şekilde uyarabilen yeni nesil prebiyotik bileşikler olarak kabul edilirler. Bifidobakteriler, *N*-glikan salınımı için benzersiz bir genomik yeteneğe sahiptir.

Bu tez kapsamında, glikoproteinlerden *N*-glikan salınımı için farklı organizmalardan (bebekler, tavşanlar, tavuklar ve yaban arıları) izole edilen Bifidobakteri suşlarından yeni glikosidazların rekombinant üretimi amaçlanmıştır. İlk olarak, *Bifidobacterium longum subsp. infantis* ATCC 15697'den izole edilen endo-*β*-*N*-asetilglukozaminidaz enziminin amino asit dizisine benzerlikleri açısından farklı Bifidobakteri suşlarından yeni glikosidazlar seçilmiştir. Bu enzimlere genetiksel olarak farklı füzyon etiketler (N-His, SUMO, C-His) eklenmiştir ve enzimleri kodlayan genlerin klonlaması için *E. cloni* 10G hücrelerinde in vivo klonlama tekniği kullanılmıştır. Hedef glikanları değiştirilen enzimler batch methodu ile

yüksek verimde üretilmiş ve saflaştırılmıştır. Ardından saflaştırılan enzimlerin model protein RNase B üzerindeki aktiviteleri SDS-PAGE analiziyle test edilmiştir ve aktivite gösteren beş enzimin optimum reaksiyon koşulları ve kinetik parametreleri belirlenmiştir.

Anahtar sözcükler: Bifidobakteriler, Glikosidazlar, Glikoproteinler, Glikanlar

ABSTRACT

RECOMBINANT PRODUCTION AND CHARACTERIZATION OF NEW BIFIDOBACTERIAL GLYCOSIDASES

Berfin SUCU
Canakkale Onsekiz Mart University
School of Graduate Studies
Master of Science Thesis in Biomolecular Sciences
Advisor: Assoc. Prof. Dr. Sercan KARAV
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Protein glycosylation is a common post-translational modification. It has been associated with important roles such as protein function including protein folding, stability, and enzymatic protection. Glycoproteins (lactoferrin, immunoglobulins, etc.) are composed of protein and glycan structures. Bacteria cannot use them as a carbon source because they do not contain the necessary enzymes that can digest proteins. Therefore, the carbon source of growth observed in bacteria is glycans. For glycans to be released from glycoproteins and taken into the cell, glycosidases of bacteria must first cleave these glycans. *N*-glycans attached to glycoproteins are indigestible by the host and reach the large intestine, where they are consumed by certain beneficial bacteria. Therefore, they are considered next-generation prebiotic compounds that can selectively stimulate beneficial bacteria in the gut microbiome. Bifidobacteria possess a unique genomic capability for *N*-glycan cleavage.

Within the scope of this thesis, the recombinant production of novel glycosidases from Bifidobacterial strains isolated from different organisms (infants, rabbit, chicken, bumblebee) for *N*-glycan release from glycoproteins was aimed. Firstly, novel glycosidases from different Bifidobacterium strains were selected in terms of their similarity to the amino acid sequence of a reference enzyme, endo-*β*-*N*-acetylglucosaminidase. This enzyme was isolated from a microbe in the infant gut, *Bifidobacterium longum subsp. infantis* ATCC 15697. Different fusion tags (N-His, SUMO, C-His) were genetically added to these enzymes, and *in vivo* cloning technique was used *E. cloni* 10G cells to clone the genes encoding the enzymes. All enzymes were produced and purified in high efficiency by the

batch method. The activities of the purified enzymes on RNase B protein were then tested by SDS-PAGE analysis. Finally, the optimum reaction conditions and kinetic parameters of five enzymes active on the protein were determined.

Keywords: Bifidobacteria, Glycosidases, Glycoproteins, Glycans

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SYMBOLS AND ABBREVIATIONS

Asn	Asparagine
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
e.g.	Exempli Gratia
et al.	Friends
GalNAc	*N*-acetylgalactosamine
HCl	Hydrochloric acid
HexNAc	*N*-acetylglucosamine
HMO	Human milk oligosaccharide
LB	Luria-Bertani
L-cys (L-cystein)	(R)-2-Amino-3-mercaptopropionic acid
LPO	Lactoperoxidase
MRS	De Man-Ragosa-Sharpe
N-glycan	*N*-linked glycan
O-glycan	*O*-linked glycan
NaCl	Sodium chloride
Ni	Nickel
O.D	Optical density
PCR	Polymerase Chain Reaction
pH	Power of hydrogen
PNGase	Peptide-*N*-Glycosidase
Rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
subsp.	Subspecies
TBE	Tris-Boric acid-EDTA
Thr	Threonine
g, G	Gram
M	Molar
L	Liter
Kg	Kilogram
Mg	Miligram

Mm	Millimeter
mL	Milliliter
µg	Microgram
µm	Micrometer
µM	Micromolar
µL	Microliter
ng	Nanogram
kDa	Kilodalton
kV	Kilovolt
min	Minute
v	Volume
w	Weight
U	Unit
%	Percent
°C	Degrees Celsius
PBS	Phosphate-buffered saline
m^2	Square meters
h	Hour/hours
s	Second/seconds
V	Volts

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CHAPTER 1
INTRODUCTION

1.1. Intestinal Microbiota

The human gastrointestinal tract (GI) is one of the biggest environments between antigens, environmental factors of the human body, and the host (Bengmark, 1998: 2). A community of bacteria, eukarya, and archaea, also known as "gut microbiota," colonizes the GI tract and it has developed together over the years to establish a mutually, complex but beneficial relationship with the host (Backhed et al., 2005; Neish, 2009). These microorganisms constitute 90% as cell numbers and 99% as gene numbers in our body. More than 1000 species can colonize in the intestinal microbiota (Turnbaugh et al., 2006). The diversity may vary between individuals and is subject to lifelong changes by endogenous and exogenous factors. A person's sex, age, level of antibiotic use, mode of birth, geographic location, and feeding types are also highly effective on microbiota (Conlon and Bird, 2015). One-third of the intestinal microbiome is known to be common to most people. It is also known that two-thirds are specific to each individual. In other words, the microbiota in the intestines is thought to be an individual identity card.

The intestinal microbiota has many different functions in most of the body functions and healthy living. Food digestion, regulation of the immune system, production and absorption of certain vitamins, prevention of inflammation, and their contribution to the fulfillment of brain functions are the most important of these functions (Vael and Desager, 2009). The association of microbiota problems with various diseases has been discussed by many researchers. These health problems include asthma, autism, cancer, celiac disease, diabetes, food allergy, obesity, and weight gain, and various skin and intestinal diseases (Carding et al., 2015). It has been emphasized that abnormal changes in human intestinal microbiota, which is an important research subject of recent years, are associated with low-grade inflammatory bowel disease, diabetes, and obesity (Turnbaugh et al., 2006) (Figure 1).

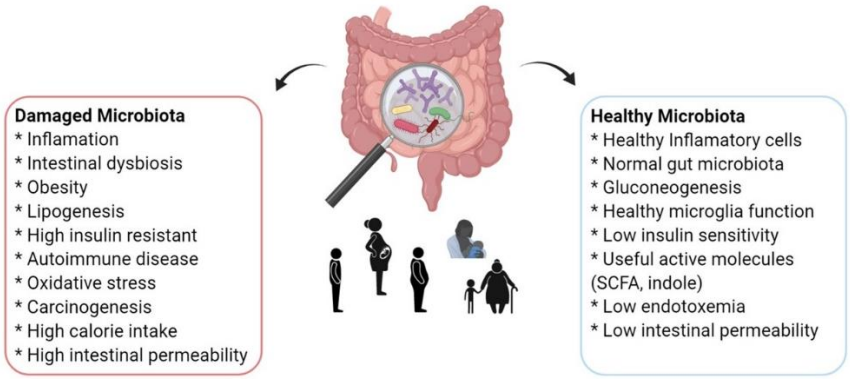


Figure 1. Effects of damaged and healthy microflora on our body.

Infants are sterile until the moment of birth and their microbiome is rapidly shaped by bacteria from the mother and the environment during birth (Nicholson et al., 2005). In the early stages of infant development, the two main phyla Proteobacteria and Actinobacteria dominate the microbiota, usually of low diversity (Backhed et al., 2005). In the first year of life, microbial diversity begins to increase. Thus, the composition of the microbiota takes the form of a distinct microbial profile similar to the microbiota of adults and changes over time specific to each infant (Palmer et al., 2007). The microbiota composition, variety, and functional abilities of an approximately 2.5-year-old baby are similar to that of the adult microbiota (Koenig et al., 2011; Rodríguez et al., 2015). Although the composition is relatively stable in adulthood, the microbial population in individuals over 65 years of age changes with an increased abundance of Clostridium and Bacteroidetes phylum (Dethlefsen and Relman, 2011).

While viruses, fungi, and other microorganisms are also present in the GI tract, the majority of microbiota are bacteria (Villanueva-Millán et al., 2015). A commonly found normal healthy human flora is subdivided into six classes of bacteria. These are Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia (Lozupone et al., 2012). Among these classes, Firmicutes and Bacteroidetes suppress 90 percent of the intestinal microbiota. When examined on class levels, bacteria have been identified as *Bifidobacterium, Bacteroides, Lactobacillus, Eubacterium, Clostridium, Escherichia, Ruminococcus, Fusobacterium, Peptococcus, Peptostreptococcus, Enterobacter, Enterococcus, Proteus, and Klebsiella* (Suau et al., 1999). These microorganisms compete for survival in the intestinal microbiota. The most important

strategy they use in this struggle is to be able to colonize by using nutrients in the environment with different genomic equipment.

1.2. Bifidobacteria

Members of the genus Bifidobacterium, which provide benefits to their hosts, are one of the first microbes to colonize the human GI tract. Bifidobacteria with health-promoting properties have been included as active ingredients in many functional foods. (O'Callaghan and van Sinderen, 2016). Bifidobacteria, first isolated and identified by Tissier in 1899-1900, are similar to lactobacilli due to their morphological and physiological characteristics. Therefore, they have been classified as members of the genus lactobacilli for the greater part of the 20th century and have subsequently been described as a different genus (Turroni et al., 2011). These microorganisms called *Bacillus bifidus*, which are morphologically rod-shaped, non-gaseous (gas-free), and show anaerobic properties, were found in the feces of infants fed with breast milk (Soccol et al., 2010; Turroni et al., 2011). Due to the presence of fructose-6-phosphate phosphoketolase (F6PPK) enzyme in a new species bifidobacteria and the lack of secretion of glucose-6-phosphatase dehydrogenase and aldolase enzymes found in lactobacilli, they are classified as bifidobacteria (Martinez et al., 2013). Since then, bifidobacteria that occur in different ecological niches such as the insect gut, oral cavity, and sewage, have been isolated from the GI tract of various mammals and water kefir (Klijn et al., 2005; Laureys et al., 2016; Ventura et al., 2007).

Actinobacteria, Bacteroidetes, Firmicutes are the most abundant phylum in human intestinal microbiota. The dominant phyla in adults are Firmicutes and Bacteroidetes, and the dominant phyla in breast-fed infants are Actinobacteria. *Bifidobacterium longum subsp. infantis* and *Bifidobacterium breve*, which belong to the phylum Actinobacteria and can reach higher levels than the majority of the total bacterial population, have been shown to be predominantly found in infant feces. But *Bifidobacterium adolescentis* is characteristically only found in the feces of adults while *Bifidobacterium longum* and *Bifidobacterium bifidum* have been isolated from both the feces of adults and infants (Hidalgo-cantabrana et al., 2017; Ventura, 2001).

HMO Metabolism

Infant gut Bifidobacteria is thought to metabolize human milk oligosaccharides (HMOs) (Figure 2). Specific bifidobacteria, typically isolated from infants, can capture HMO and use the structure as a sole carbon source. Bacteria with a genome adapted for HMO metabolism are *B. bifidum* and *B. infantis* (Duranti et al., 2015; D A Sela et al., 2008). Bacteria with a genome adapted for HMO metabolism are *B. bifidum* and *B. infantis*. However, other infant gut bifidobacterial species, *B. breve* and *B. longum*, encode different HMO catabolic enzymes. They exhibit more strain-specific phenotypic variation and can degrade certain HMOs (LoCascio et al., 2009). It has been shown that these strains can also utilize carbohydrates released by other bifidobacteria such as *B. bifidum*.

Some adult-type bifidobacteria such as *B. animalis* and *B. adolescentis* do not degrade HMO species. It is thought that this could be due to the absence of a gene found in infant-type bifidobacteria (*B. infantis*), in these species (David A. Sela and Mills, 2010). *B. infantis* possesses a unique glycan metabolism strategy. The Bifidobacterium species can release glycan structures of the glycoproteins using an enzyme, endo-*β*-*N*-acetylglucosaminidase (EndoBI-1), and take intact glycans into the cells. This feature of *B. infantis* reduces the potential for cross-feeding of pathogens since the hydrolysis of glycans into monosaccharides takes place inside the cell (Chaplin et al., 2015; Egan et al., 2014).

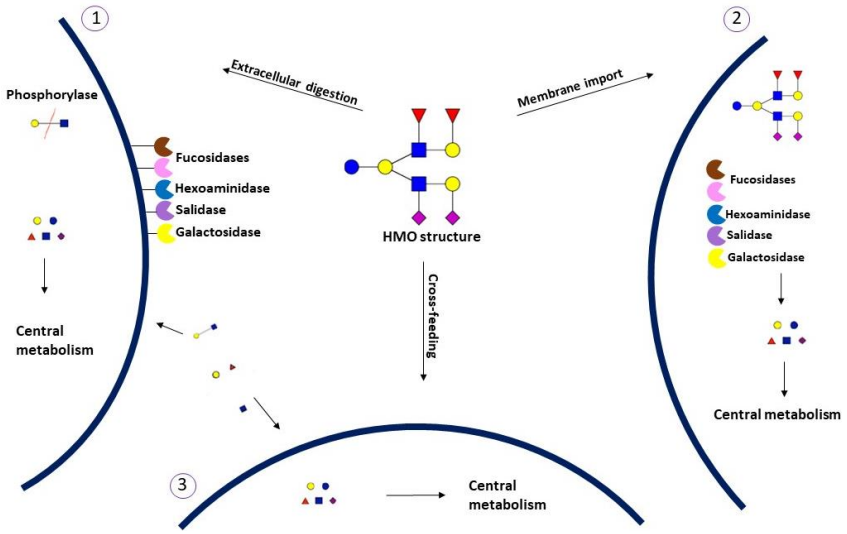


Figure 2: There are possible strategies for the utilization of human milk oligosaccharides (HMOs) by Bifidobacteria (blue squares represent GlcNAc; red triangles, galactose; purple diamonds, fucose; yellow circles, blue circles, glucose).

In, Figure 2, 1) Some *Bifidobacterium* species, such as *B. bifidum*, cleave the linkages found in HMOs with extracellular glycosyl hydrolases and can use these structures. 2) *B. infantis* can release glycans from the glycoproteins by EndoBI-1 and take the intact glycans into the cells. 3) *B. breve* utilizes HMO monosaccharides cleaved by extracellular glycosidases of other gut bifidobacteria because they cannot degrade HMO species.

1.3. Glycoproteins

Glycoproteins can be defined as covalently attached carbohydrate-containing proteins. Glycoproteins are present in all living organisms, both soluble and insoluble forms, in different biological functions and properties. The carbohydrate moiety of glycoproteins varies between 1% and 80% depending on the molecule (Varki et al., 2009). Glycoproteins have many functions such as the immune system, reproduction, hormones, cells, and organism protection (Eylar, 1966). Milk contains mostly *N*-glycosylated whey proteins and a high proportion of O-glycosylated casein. Whey is high in glycoprotein content and is a byproduct of cheese-making. Whey consists of protein and lactose and mostly water (Mollea et al., 2013). Whey mostly contains glycoproteins such as lactoferrin, immunoglobulins, α-lactalbumin, osteopontin, transferrin, and glycomacropeptide (Walstra et al., 2005). Lactoferrin, a multifunctional whey glycoprotein, is found in milk, saliva, mucus, salvia (Levay and Viljoen, 1995; Steijns, 2001). The lactoferrin concentration of the milk of various mammals may vary according to species and individuals (Hirai et al., 1990). These proteins differ in their glycan profiles. The differences in the biological functions of lactoferrins are believed to be due to differences in these glycan profiles. Lactoferrin has biological functions such as strong iron-binding ability, bifidogenic, anti-inflammatory (Kane et al., 2003), and antimicrobial (Qiu et al., 1998). It also has effects such as antioxidant effects, anticarcinogenic (Bezault et al., 1994), and immunomodulatory (Legrand et al., 2006). The most interesting of all these biological functions of lactoferrin is its antimicrobial activity. Iron is an essential nutrient source for all microorganisms. The antimicrobial effect of lactoferrin is thought to be due to the depletion of iron. Lactoferrin has also been shown to protect the intestine mucosal lining from pathogens (Barboza et al., 2012), and its direct interaction with pathogens causes cell lysis. The major role of glycans in this mechanism (as signaling agents) is considered (Antonini et al., 2015). While this protein promotes the growth of Bifidobacterium and Lactobacillus, it kills pathogens with its antimicrobial effect.

The glycan profiles of lactoferrin isolated from different mammalian milks were compared with each other (Chung, 1984) and it was shown that they have very similar amino acid sequences but their glycan content is almost completely different from each other. In a supportive study, 16, 18, and 6 specific glycan structures of human, goat, and cow lactoferrin have been identified respectively (Le Parc et al., 2014).

In another similar study, the glycan profile of recombinantly produced human lactoferrin in transgenic cows has been shown to be quite different from that of human and cow lactoferrin (Parc et al., 2017). It is thought that the biological activity of lactoferrin and similar glycoproteins vary in different mammals due to differences in glycan profiles.

1.4. Glycans and Their Roles

Glycosylation is the important and most common protein post-translational modification. Glycosylation is the covalent attachment of carbohydrate molecules (glycans) to the surface of the protein and is the most common chemical modification that occurs naturally in protein (Solá and Griebenow, 2010: 11). It is thought that the biological activity of many therapeutic glycoproteins may be dependent on their glycosylation state (Mamedov and Yusibov, 2011).

Glycans are present in nature free and attached to proteins, peptides, and lipids by *N*- or *O*- glycosidic bonds. In milk, free glycans, also known as oligosaccharides, are shaped by *N*-acetylglucosamine (GlcNAc), sialic acids, and fucoses (Varki et al., 2009). Human milk also includes abundant free oligosaccharides. Infants cannot digest human milk oligosaccharides (HMOs) due to glycosidase deficiency. However, in the GI tract, HMOs have been shown to be preferably consumed by various Bifidobacteria strains (LoCascio et al., 2009). Glycans are also found in conjugated forms as *N*-glycans and *O*-glycans that are attached to proteins, peptides, or lipids by glycosidic bonds. *O*-glycans are the most common type of mucin-type glycans.

They are usually attached to hydroxyl in a serine/threonine of proteins via *N*-acetylgalactosamine (Steen et al., 1998). *O*-glycan structures contain eight different cores, while *N*-glycans contain only one core consisting of two HexNAc and three mannose

residues. *N*-glycans are attached to asparagine residues in Asn-X-Ser/Thr of the polypeptide chain via HexNAc (X can be referred to as any amino acid except proline). Monosaccharides such as fucose and sialic acid are added to this common structure by glycosyltransferase and glycosidase enzymes, thus producing different glycans (Ohtsubo and Marth, 2006). According to this modification of the common structure, *N*-glycans exist as complex, hybrid, and high mannose glycans (Figure 3).

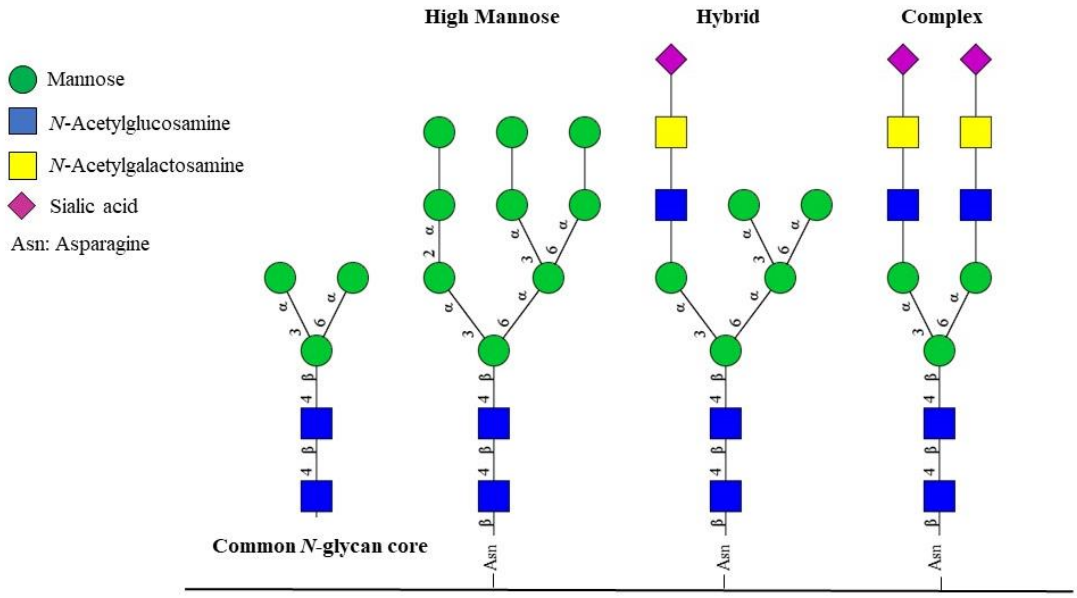


Figure 3. The common core structure and different types of *N*-glycan (The figure was prepared by using GlycoWorkbench software (v2.1)).

Glycans, which are important in various cellular mechanisms related to health and disease, play a role in cell-cell and cell-microorganism communication, folding, and stabilization of proteins. In studies on the glycan role in some functions such as receptor activation and cell adhesion (Barboza et al., 2012), the glycoprotein structure has been shown to protect the host against various viral and microbial attacks. It was also found that sialic acid present in glycans prevents the binding of pathogens to epithelial cells in the intestine, prevents diarrhea-causing rotavirus infection in infants, and reduces the binding of leukocytes to endothelial cells (Bode, 2006).

Sialic acid in glycans has been also shown to be very important in brain development and the development of the learning abilities of infants (Morgan and Winick, 1980). The *N*-glycan molecule acts as a selective prebiotic by stimulating the growth of specific probiotics such as *B. infantis* found in the gut (Karav et al., 2015a). Characterization of glycans by release from proteins and the biggest problem in large-scale production is the limited number of deglycosylation techniques used currently. An optimal technique is currently not available, as chemical and enzymatic methods used to release glycans from glycoproteins have many disadvantages.

1.5. Glycan Release Strategies from Glycoproteins

Glycans should be released from glycoproteins to be able to characterize them, examine their effects in different studies, and understand their contribution to the functions of glycoproteins. Various chemical and enzymatic methods are used to release glycans from glycoproteins. However, these biological and physicochemical properties of glycans are not fully understood due to the limitations of these methods (Le Parc et al., 2014; Royle et al., 2008).

Chemical Strategies for Release of Glycans: Chemical methods are widely used because of their easy applicability, low cost, and active on many substrates (Sojar and Bahl, 1987). Deglycosylation processes first started with the application of chemical methods such as β-elimination and hydrazination used to release the glycan moieties from glycoproteins. However, the biggest problem of these methods is that the remaining sodiums after the reaction inhibit the analysis of glycan in the mass spectrometer, the used chemicals have a carcinogenic effect (Choudhary and Hansen, 1998), and the loss of the natural structure of the glycans and the remaining polypeptide moieties due to substrate denaturation requirement (Patel et al., 1993). In the β-elimination method, glycans are released from glycoproteins under alkaline conditions (Dwek et al., 1993). The released glycan structure can be disrupted as a result of the reactions known as the peeling action during β-elimination. To avoid this limitation, reducing agents such as sodium borohydride are used to protect the chemical structure of the released glycans (Carlson, 1968; Turyan et al., 2014). Besides, glycan loss is observed during β-elimination of the high salt used during the reaction (Turyan et al., 2014).

The hydrazination technique is a method by which the release of *N*- and *O*-glycan can be manipulated by changing the conditions of reaction such as temperature (Patel et al., 1993). Therefore, these chemical methods cannot be used in the food and health sector, and as they alter the structure of glycoproteins, limiting the relationship of their biological functions with glycans.

Enzymatic Strategies for Release of Glycans: On the other hand, to avoid these problems, different enzymes such as peptidyl-*N*-glycosidases (PNGases) were used to release *N*-glycan from glycoproteins, but not enough glycosylation was achieved due to the lack of an active enzyme on all the glycan species (Altmann et al., 1995). Commercially available PNGase F derived from *Flavobacterium* (Triguero et al., 2010), cleaves glycans attached to asparagine to hydrolyze the amide side chain (Nuck et al., 1990). If there is fucose in the glycan core linked by a 1,3 bond to *N*-acetylglucosamine, this fucose bound to the *N*-glycan co-structure limits the activity of these enzymes and thus prevents the release of glycans (Tretter et al., 1991). Besides, the form or structure of the glycoprotein is another limitation of the activity of PNGase F on *N*-glycans. The accessibility of enzymes to glycans can be increased by denaturing glycoproteins. For this, high heat and detergent use is required. Under these harsh conditions, the structure of isolated glycans and remaining polypeptide structures can be degraded. The analysis of glycans in mass spectrometry is also adversely affected by detergent contamination. Various enzymes called endoglycosidase F1, F2, and F3, which do not require substrate denaturation, have been discovered, but the use of these enzymes has remained very limited due to their activity on a limited number of glycan species. These enzymes are found to able to cut especially multiple-antennary glycans at extremely low levels (Table 1) (Trimble and Tarentino, 1991).

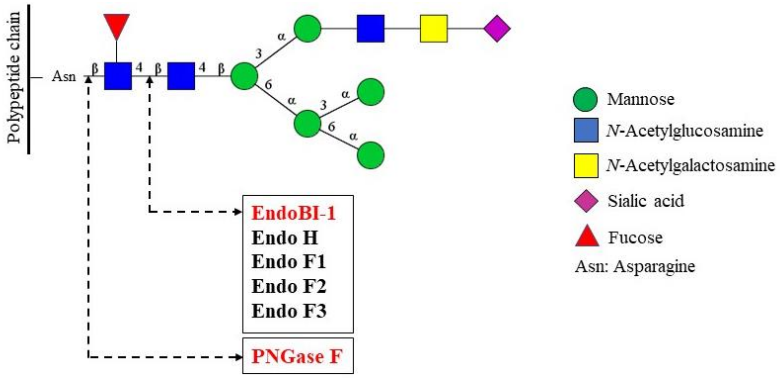


Figure 4. Activity of EndoBI-1, PNGase F, and other endoglycosidases (endo H, F1, F2, F3) on different regions of glycans.

A novel *N*-glycosidase EndoBI-1 was firstly isolated from *B. infantis* found in the infant's gut. EndoBI-1 is accepted as an alternative strategy to all these limitations. This enzyme has been shown to be effective at very low pH and high temperatures and to release all *N*-glycan cores without any protein denaturation (Karav et al., 2015a; Karav et al., 2015b). EndoBI-1 and PNGase F enzymes are shown the activity at different regions of the core of *N*-glycan, releasing *N*-glycan structures from different regions (Figure 4). EndoBI-1 can release all *N*-glycan types by acting on *N, N'*-diacetylchitobiosyl moiety located in the core of *N*-glycan. Additionally, unlike PNGase F, this enzyme is not affected by fucosylation (Garrido et al., 2012).

Table 1

Activities of EndoBI-1 and other glycosidases on different types of glycan (Karav et al., 2017)

Endoglycosidase	Native (not denatured)	Core fucosylation	High mannose	Hybrid	Bi-Ant.	Tri-Ant.	Tetra-Ant.
PNGase F	-	-	+	+	+	+	+
EndoBI-1	+	+	+	+	+	+	+
Endo H	+/-	+	+	+	-	-	-
Endo F1	+/-	+	+	+/-	-	-	-
Endo F2	+/-	+	+	-	+	-	-
Endo F3	+/-	+	-	-	+	+	-

+: high active, -: little activity or inactive, +/-: less activity, Ant.: Antennary

1.6. Importance of Specific Glycan Release

Lactoferrin-like glycoproteins such as immunoglobulin, fetuin, and casein contain very different glycan structures. The contribution of these glycans to these glycoproteins is not yet understood. The association of these glycans based on species is very important in terms of improving the biological activities of these glycoproteins. Besides, it has been found that some glycan types have very specific biological activities. Among these, free glycans containing sialic acid have attracted the attention of researchers. It has been shown that these structures have important effects on the development of the brain and learning skills in infants (Wang, 2009). These compounds, which are given to infants in very high amounts by breast milk, have also been shown to inhibit pathogen binding by imitating oligosaccharides on the cell surface that pathogens use to bind (Wang et al., 2007).

In a study, it has been shown that some of the glycans (4Hexo-3*N*-acetylglucosamine-1 Fucose and 3Hexo-3*N*-acetylglucosamine) released from proteins of milk with EndoBI-1, contribute to the development of *B. infantis* at a high level and can be utilized as a sole carbon source (Karav et al., 2016). In another study, the release of glycans from lactoferrin was performed to understand whether specific glycan types contribute to the antimicrobial function of lactoferrin using novel forms of recombinantly produced EndoBI-1. The different forms of lactoferrin were formed by treatment with the fusion-tagged enzymes (free, genetically attached Glutathione-S-transferase and polyhistidine). After analysis and confirmation of the released glycan structures, it has been proven that sialylated glycan structures play a major role in the antimicrobial activity of lactoferrin on *E. coli* (Karav, 2018). Therefore, understanding the biological activity of each glycan species will enable these functions to be used in many areas such as the health sector, the pharmaceutical industry, and athletic support products.

1.7. Release of Different Glycan Species by Manipulation of Enzyme Activity

EndoBI-1 has been shown to release all *N*-glycans from different milk glycoproteins. Most glycan release strategies require substrate denaturation. This enzyme allows the glycans and remaining polypeptide moieties to be produced in their native forms without the need for this process. Thus, the biological functions of glycan structures can be examined

and investigated using proteins in a native state. It can also be shown as the only strategy by which different glycan species can be released (Karav et al., 2015a; Karav et al., 2015b). In this context, a study published in the journal *Biotechnology Progress* showed that EndoBI-1 tends to release different glycan species by changing reaction conditions. For example, predominantly neutral glycans can be isolated when pH 7, 65 °C for 130 min and 1/1500 enzyme/protein ratio are used as the reaction conditions. However, the isolation of acidic glycans can be possible, when the reaction is performed at pH 5.3, 40 °C for 360 min, and 1/500 enzyme/protein ratio (Parc et al., 2015). However, although this strategy is effective in the release of different glycan species, it resulted in low enzyme efficiency as the conditions for the enzyme's glycan release were not optimal. It is suggested that enzyme specificity can also be achieved with the help of fusion tags which alter the target substrate of the enzyme.

Fusion tags designed primarily to produce high purity recombinant proteins are expected to increase the solubility of proteins providing higher production yields. These fusion tags are emphasized to must be easily applicable while allowing the enzyme to retain its activity (Karav, Talak, et al., 2017). In a previous study, different fusion tags were genetically added to the EndoBI-1 enzyme, and it was investigated that these enzymes are effective on which glycan types. Primarily, optimum reaction conditions of the enzyme in the three different forms (free, GST-tagged, and 6xHis-tagged) were investigated and it was found that the three forms showed the highest activity at similar pH and temperature. However, considering the total amount of glycan release, free and His-tagged enzyme was found to release glycan more efficiently compared to the GST-tagged enzyme form. When looking at the glycan profiles released by the three forms of EndoBI-1, it was found that the GST-tagged enzyme released a very high number of neutral glycans, while free and His-tagged enzymes released the glycans containing sialic acid (Karav, 2018). In line with all these studies, it is thought that it is possible to selectively release glycans by using different tags and changing reaction conditions. It is believed that it is possible to release specific glycan species by adding new enzymes to these two approaches.

1.8. Aim of The Study

The partial glycan release from glycoproteins is required to understand whether or what glycans contribute to the biological function of therapeutic proteins. Thus, it will be possible to investigate how the biological functions of proteins are affected after the removal of specific glycans. This study aims to detect and discover novel glycosidases with similar properties and unique specificities isolated from different *Bifidobacterium* species found in the feces of different organisms (healthy humans, bee, rabbit, chicken microbiome) by bioinformatics studies and to produce these enzymes recombinantly with *in vivo* molecular cloning system. Besides, it is aimed to manipulate the activities of the enzymes using different techniques such as genetically attached fusion tags to the enzymes, and to release the desired types of glycans from glycoproteins. Novel glycosidases will be recombinantly produced in the correct gene sequence and high purity, and enzymes that have never been studied before in this field will be brought to the field of glycobiology. The bioactive *N*-glycan release from glycoproteins by using recombinantly produced enzymes containing fusion tags would be a promising novel tool. There is no previously designed approach for specific glycan production in this field. Thus, this thesis will contribute to filling a large gap in the field of glycobiology in the literature.

In the scope of the proposed thesis;

a- The novel glycosidases were selected from various Bifidobacterial species in the feces of the different organisms.
b- *In vivo* cloning system (N-His, SUMO, C-His) was used for the molecular cloning of these novel enzymes.
c- All His-tagged enzymes were produced by batch purification method using Nickel-resins column affinity chromatography.
d- The activity of purified enzymes was determined on a model protein, RNase B.
e- Finally, the selected five enzymes that showed activity on RNase B, were characterized by determining the optimum reaction conditions and the kinetic parameters.

CHAPTER 2
PREVIOUS STUDIES

The biomolecules of mammalian milk, which have been developed for many years, have not yet been defined by scientists. In addition, breastfed infants, which are known to be highly beneficial, have been shown to have a lower risk of some diseases such as eczema or diarrhea than formula-fed infants (Hamosh, 2001; Holman et al., 2006). The glycosylation extent in milk proteins thought to have beneficial effects is an interesting milk characteristic. More importantly, the glycans of milk are not digested by infants, but they encourage beneficial bacterial growth. Thus, these glycans protect infants from any pathogens and stimulate a healthy environment of the intestine (Karav et al., 2016; Marcobal and Sonnenburg, 2012).

Milk glycoproteins have different functions that shaped the infant microbiome development. They also contribute to various cellular processes such as signaling and adhesion. These proteins have been shown to have many biological activities. For example, lactoferrin, one of the milk proteins, protects the cells of the intestine against bacterial and viral infections and stimulates immune cells. In addition, other studies have shown that milk proteins have bifidogenic effects (Actor et al., 2009; Jenssen and Hancock, 2009; van der Strate et al., 2001). When lactoferrin and its glycan profiles isolated from different mammalian milk are compared with each other (Chung, 1984), it has been shown that lactoferrins have very similar amino acid sequences and the glycan content is almost completely different from each other. Therefore, it is considered that glycans play important role in glycoprotein biological activity. At this point, a study showed that recombinant human lactoferrin found in transgenic cows has different glycan structures and profile and biological roles than lactoferrin of native humans and cows, although they had similar sequences of the amino acid (Parc et al., 2017).

Many methods have been examined and used to study milk protein glycosylation and to understand whether and how *N*-glycans contribute to the glycoproteins' biological properties. Therefore, various chemical and enzymatic methods are used for the release of glycans from glycoproteins. However, these biological and physicochemical properties of glycans are not fully understood due to the limitations of these methods (Le Parc et al., 2014; Royle et al., 2008).

As an alternative strategy to all these limitations, a novel enzyme EndoBI-1 has been firstly isolated from *B. infantis* found in the infant's gut. This enzyme has been shown to act on the core of all *N*-glycan types (Parc et al., 2015). *B. infantis* releases and utilizes all *N*-glycan structures using this enzyme from glycoproteins such as milk protein lactoferrin. In a study, the release of glycan by EndoBI-1 from the colostrum of bovine was performed using various parameters such as enzyme/protein ratio, pH, temperature, and reaction time. As a result of the study, This enzyme released high amounts of glycans from milk glycoproteins at the medium values of temperature (~52°C), lower pH values, higher enzyme/protein ratios, and longer reaction times. EndoBI-1 has also been shown to show higher activity on natural proteins and retain its activity at high temperatures (Karav et al., 2015a). In another study, the EndoBI-1 kinetic parameters were determined on concentrated whey (bovine colostrum) and ribonuclease B (RNase B) of bovine lactoferrin. At the end of this study, EndoBI-1 exhibited a very strong enzyme-substrate affinity on RNase B and the maximum reaction rate on concentrated whey (Karav et al., 2015b). In another study with the EndoBI-1 activity, different glycan types were shown to be released in the different reaction conditions. For example, predominantly neutral glycans can be isolated when pH 7, 65 °C for 130 min and 1/1500 enzyme/protein ratio are used as the reaction conditions. However, the isolation of acidic glycans can be possible, when the reaction is performed at pH 5.3, 40 °C for 360 min, and 1/500 enzyme/protein ratio (Parc et al., 2015).

In a previous study, different fusion tags were genetically added to the EndoBI-1 enzyme and it was investigated that these enzymes are effective on which glycan types. Primarily, optimum reaction conditions of three enzyme forms (GST-tagged, free, and 6-his-tagged) were investigated and it was found that the three forms showed the highest activity at similar pH and temperature. However, considering the total amount of glycan release, free and His-tagged enzyme was found to release glycan more efficiently compared to the GST-tagged enzyme form. When looking at the glycan profiles released by the three forms of EndoBI-1, it was found that the GST-tagged enzyme released a very high number of neutral glycans, while free and His-tagged enzymes released the glycans containing sialic acid (Karav, 2018). In line with all these studies, it is thought that it is possible to selectively release glycans by using different tags and changing reaction conditions. It is believed that it is possible to release specific glycan species by adding novel enzymes to these approaches.

In this study, novel enzymes with a sequence similar to the amino acid sequence of EndoBI-1, isolated from five different organisms, will be produced. Until this study, *in vivo* cloning, manipulation of activity using fusion tag, control of activity on model proteins, and kinetic characterization were not performed for these enzymes, which we used in this study. Recombinantly produced these enzymes would be a promising novel tool to release bioactive *N*-glycans from glycoprotein-rich substrates.

CHAPTER 3
MATERIAL METHOD

3.1. Materials

3.1.1. Chemicals, Kits, Culture Media, and Essential Items

In Table 2, the chemicals, kits, and essential items for given studies were listed.

Table 2

List of chemicals, kits, culture media, and essential items

Name of Materials	Codes	Items
Anaerocult A	113829.0001	Anaerobic conditions for Bifidobacteria
Anaerotest	115112.0001	Anaerobic conditions for Bifidobacteria
Anaerobic jar	116387	Anaerobic conditions for Bifidobacteria
L-Cysteine	C7352	Amino acid in culture media
EconoTaq PLUS 2X Master Mix	30035-1	Amplification of target genes
Safe-Red Loading Dye	G108-R	Gel electrophoresis (DNA)
1 kb Plus DNA Ladder	D011-500	Gel electrophoresis (DNA)
Agarose	16500-500	Gel electrophoresis (DNA), bacterial culture media
Boric Acid	BOR001.500	Gel electrophoresis (DNA)
EDTA	EDT001.500	Gel electrophoresis (DNA)
Kanamycin Monosulfate	K-120-5	Antibiotic in bacterial culture media
Expresso Rhamnose Cloning and Expression System Kit, N-His	49011-1	Cloning Kit
Expresso Rhamnose Cloning and Expression System Kit, SUMO	49013-1	Cloning Kit

Table 2 (continue)

Expresso Rhamnose Cloning and Expression System Kit, C-His	49012-1	Cloning Kit
Qubit Protein Assay Kit	Q33211	Measurement of protein concentration
Qubit™ dsDNA BR Assay Kit	Q32850	Measurement of DNA concentration
Glycerol Solution	15524-1L-R	Preparation of glycerol stock
L-Rhamnose monohydrate	83650	Induction of protein expression
2X Laemmli Sample Buffer	TGS10	SDS-Page analysis
SureCast 40% (w/v) Acrylamide	HC2040	SDS-Page analysis
SureCast APS	HC2005	SDS-Page analysis
SureCast TEMED	HC2006	SDS-Page analysis
10X Running Buffer	TGS10	SDS-Page analysis
Tris	TRS001.1	SDS-Page analysis, DNA gel electrophoresis, lysis buffer
10% SDS	S100	SDS-Page analysis
PageRuler Plus Prestained Protein Ladder	26619	SDS-Page analysis
Acetic acid	27225	SDS-Page analysis
Methanol	947.046.2500	SDS-Page analysis
Coomassie Brilliant Blue (R-250)	0472	SDS-Page analysis
Imidazole	56750	Cell lysis
NaCl	31434-5Kg-R	Cell lysis
Sodium Phosphate Monobasic Anhydrous	0571-1Kg	Sodium phosphate buffer for cell lysis
Sodium Phosphate Dibasic Dihydrate	04272-1Kg	Sodium phosphate buffer for cell lysis
HisPur™ Ni-NTA Resin	88222	Production of enzymes
10-kDa-cut-off centrifugal filter	UFC9010	Concentrating enzymes
Ethanol	920.027.2501	Protein precipitation
LB Broth, Miller (Luria-Bertani)	J106-2KG	Bacterial culture media
De Man, Rogosa, and Sharpe (MRS)	1106610500	Bacterial culture media

3.1.2. Substrates

Recombinantly production of novel glycosidases which have been tagged with three different fusion tags in this study was carried out firstly using *Bifidobacterium longum subsp. infantis* ATCC 15697 strain and different Bacterial strains provided from The Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG). RNase B protein (Code, P07817S) used in the determination of enzyme activity was supplied from Biolabs. Lactoferrin from bovine colostrum (Code, L4765-50MG), and IGG from bovine serum (Code, I5506-50MG) used in the enzyme kinetics were supplied from Sigma-Aldrich.

3.1.3. Laboratory Equipment Used in Molecular Analysis

All laboratory equipment required for the thesis studies in the research lab of the Molecular Biology and Genetics Department at the Canakkale Onsekiz Mart University (COMU) was used and maintained according to the user's manuals. The devices used in this study are a sensitive scales (Shimadzu), an autoclave (Nüve), incubator (Indem Nüve EN 400), fume cupboard (Setra), -80 °C freezer (Operon), Millipore pure water system, Gel Imaging System (Vilber Lourmat, France), horizontal electrophoresis and power supply (Thermo), Vertical electrophoresis system (BioRad), Thermal Cycler (BioRad), NanoQuant DNA concentration meter (Tecan Infinite® M200 PRO), -20 °C freezers (Bosch), refrigerator (Bosch), cooling centrifuge (Hettich), heated stirrer (Wisd), mini centrifuge (CF-5 Wisd), Vibra Cell/ Sonicator (Sonics), Shaker (Benchmark), vortex (Vortex Genie 2), dry block heater (BSH1002 Benchmark), Qubit™ 3 Fluorometer (Invitrogen), Centrifuge (Beckman Allegra X-15R), Vertical Electrophoresis System (Thermo), Shaker (Benchmark-Incu Shaker 10LR), Shaker (Major Science Waver Shaker), microwave (Bosch), pH meter (IsoLab), thermometer (IsoLab), and ice machine (izmak).

3.2. Methods

3.2.1. Detection of Novel Bifidobacterial N-Glycosidases and Phylogenetic Relations According to Amino Acid Sequences

Firstly, the study was carried out to determine novel *N*-glycosidases in the different Bifidobacterial strains and to select different sequences closely and distantly related to the amino acid sequence of EndoBI-1 referenced by showing phylogenetic relationships between these sequences.

Using the current reference sequence (ACJ53522.1 *Bifidobacterium longum* subsp. *infantis* ATCC 15697), homology sequences were determined by searching protein-protein BLAST-based (NCBI) in The Belgian Co-ordinated Collections of Micro-organisms (LMG) and NCBI gene banks. The 9 sequences were selected from the glycosyl hydrolase family considering similarity percentages. Multiple sequence alignments for 10 samples containing the amino acid sequences of the EndoBI-1 with these selected sequences and the current reference sequence were extracted using the MUSCLE alignment method. Then, using this

data set, the Neighbor-Joining (NJ) tree based on the distance relationship was constructed. For phylogenetic relationship states, the analysis based on the Maximum Likelihood (ML) algorithm was made by taking into consideration WAG, which was determined as the most suitable model for this data set. While creating branching models, 1000 bootstrap applications were performed. Multiple sequencing, model determination, and MEGAX program were used for tree construction analyzes (Kumar et al., 2018). The Neighbor-joining (NJ) dendrogram is shown in Figure 5 and the Maximum Likelihood phylogram is shown in Figure 6. Both tree topologies provided consistent groupings. These strains are shown with GenBank access numbers on the trees.

Bootstrap values are shown on nodes. 70 and above of Bootstrap values were evaluated as indicating significantly different branches in terms of genetics/phylogenetics. Thus, in the study, 5 different sequences that are both closely and distantly related in terms of genetics/phylogenetics with the EndoBI-1 were taken into consideration. These sequences have been used in ongoing studies. The information about Bifidobacterial strains and target genes used in the thesis are shown in Table 3.

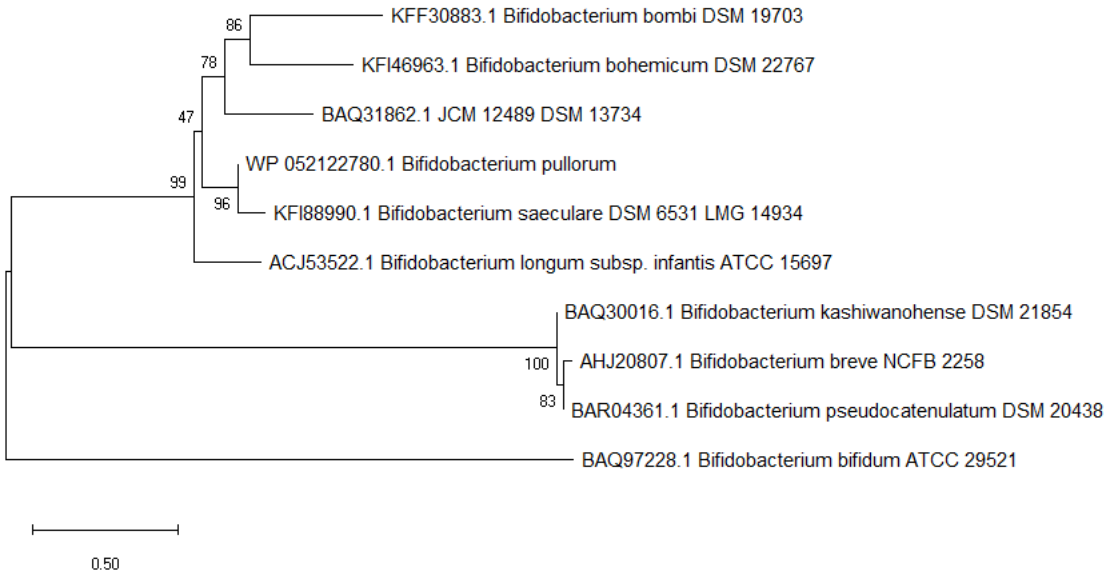


Figure 5. The Neighbor-Joining tree obtained using amino acid sequences of EndoBI-1 and different proteins encoding glycosyl hydrolase, which shows similarities in the different levels with the EndoBI-1 amino acid sequence. Bootstrap values are shown on nodes. The used strains were given with GenBank access numbers.

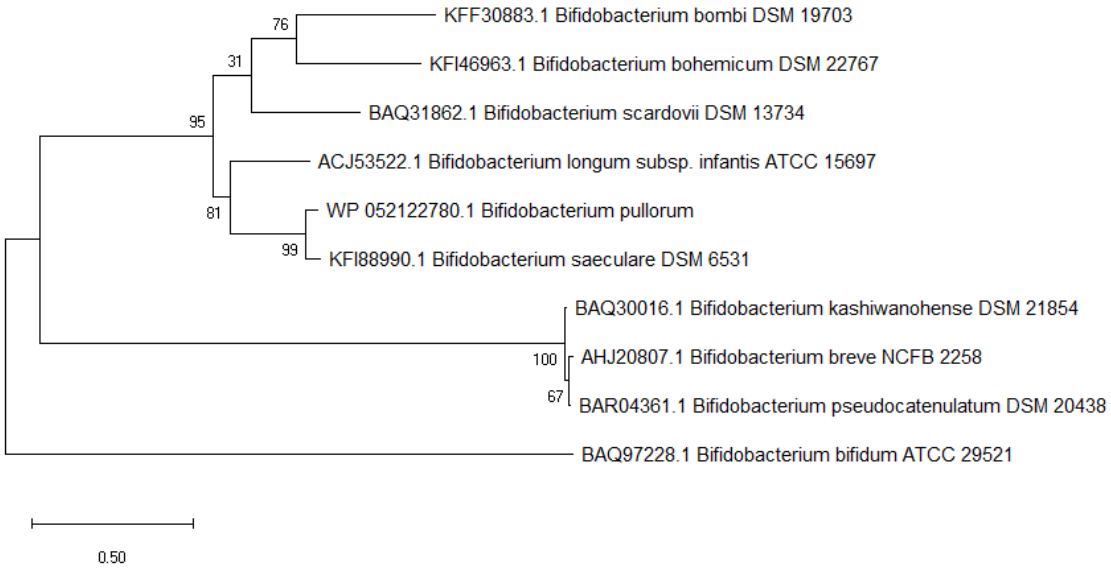


Figure 6. The Maximum Likelihood tree obtained using amino acid sequences of EndoBI-1 and different proteins encoding glycosyl hydrolase, which shows similarities in the different levels with the EndoBI-1 amino acid sequence. Bootstrap values are shown on nodes. The used strains were given with GenBank access numbers.

Table 3

Information about bacterial strains and target genes used in the thesis

Bacterial Strains	**Organisms**	**Accession Number**	**Enzyme Codes**
B. pullorum	Chicken feces	WP052122780.1	OU11_RS07620
B. saeculare	Rabbit feces	KFI88990.1	BSAE_0444
B. kashiwanohense	Child feces	BAQ30016.1	BBKW_1881
B. pseudocatenulatum	Infant feces	BAR04361.1	BBPC_1683
B. bohemicum	Bumblebee digestive tract	KFI46963.1	BBOH_0438

3.2.2. Anaerobic Growth Conditions of Bifidobacterial Species

100 mL 60% Glycerol and 500 mL de Man-Rogosa-Sharpe (MRS)+cysteine liquid medium were prepared before preparing stock culture from bacteria. Glass ampoules containing lyophilized bacterial cells (freeze-dried cultures) were broken by marking from the cotton part as recommended by the company in a sterile environment. 1 mL MRS+cysteine liquid medium was added to the lyophilized cells in the broken ampoules and dissolved by gently mixing with the pipette tip.

The modified MRS medium with L-cysteine (L-Cys) (0.05%) was used as the anaerobic medium. L-Cys that is an essential nitrogen source for bacteria, was added into the MRS medium to reduce the redox potential and provided a better anaerobic environment for the growth of bifidobacteria. 2.5 liters of Anaerobic jar (Merck) and Anaerocult A (Merck) were used to keep the internal oxygen, and Anaerotest (Merck) was used to show that internal oxygen is depleted (Figure 7).



Figure 7. Anaerobic jar, Anaerocult A and Anaerotest A used to create an anaerobic environment.

The inoculation was started by adding 700 µL from each cell to 15 mL falcon tubes containing 7 mL MRS+cys medium. The cells were cultured at 37 °C for 48 hours (Figure 8). Then this process was repeated to form a subculture from each cell. The stock cultures were prepared with a concentration of 15% glycerol (1.5 mL liquid culture + 0.5 mL 60% glycerol) from each culture after 24 h.



Figure 8. The anaerobic system used for the growth of Bifidobacteria

3.2.3. Molecular Cloning of Bifidobacterial Genes

Expresso Rhamnose Cloning and Expression System (Lucigen) kit with three different fusion tags (N-His, SUMO, C-His) was used for the molecular cloning (Figure 9). This kit provides faster, and more reliable results compared to other conventional cloning protocols. In this system, also known as the *in vivo* cloning system, cloning can be performed by mixing the amplified gene region and vector (provided with the kit) with competent cells without the need for enzymatic ligation. The linear vector in this system contains 18 nucleotide long sticky ends at both tips. Primers required for the gene amplification can be designed according to these ends and thus a strong binding can be provided. After cloning, the production of protein is increased by using rhamnose, and the purification of protein is carried out by binding to NTA-Ni resin containing columns with the 6xHistidine tag in three different vectors (pRham™ N-His, pRham C-His, and pRham™ N-His SUMO).

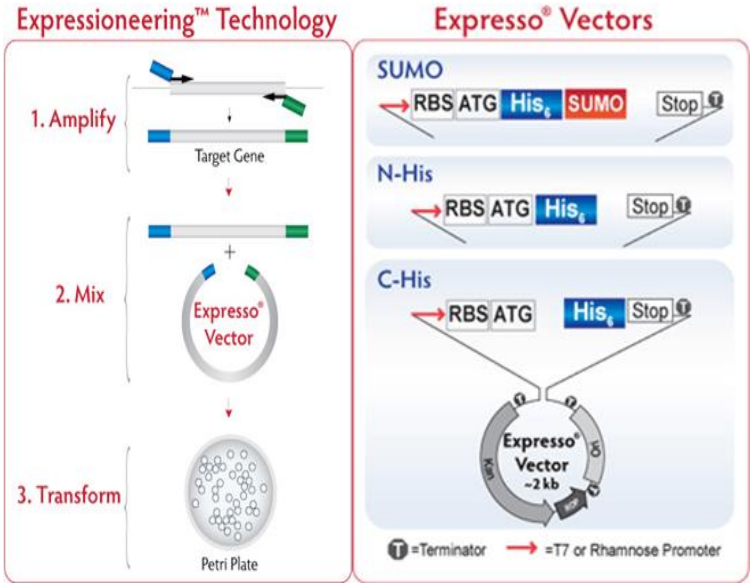


Figure 9. Molecular cloning strategy and vectors

3.2.4. Primer Design of Target Genes with Different Fusion Tags

Primers were designed to start with the sequences of the sticky ends for each fusion tag and then continue with the sequences specific to the gene of interest. In this context, the nucleotide sequences described in the kit and shown below belong to these sticky ends, and the parts are shown as XXX refer to the respective target gene sequence (Figure 10).

. Fusion to an N-terminal 6xHis tag (pRham™ N-His Kan Vector):

Forward primer (defined vector sequence includes 6 His codons):

5'-CAT CAT CAC CAC CAT CAC XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8

Reverse primer (defined vector sequence includes Stop anticodon):

5'-GTG GCG GCC GCT CTA TTA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6}

(XXX_n - XXX_{n-6} represents the sequence **complementary** to the last 7 codons of the target coding region).

. Fusion to a C-terminal 6xHis tag (pRham C-His Kan Vector):

Forward primer (defined vector sequence includes Start codon):

5'-GAA GGA GAT ATA CAT ATG XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8

(XXX_2-XXX_8 represents codons 2 through 8 of the target coding region).

Reverse primer (defined vector sequence includes 6 His anticodons):

5'-GTG ATG GTG GTG ATG ATG XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6}

(XXX_n - XXX_{n-6} represents the sequence **complementary** to the last 7 codons of the target coding region).

Fusiın to N-terminal tag SUMO Kan Vector

Forward primer (defined sequence includes the last 6 codons of SUMO):

5'- CGC GAA CAG ATT GGA GGT XXX_2 XXX_3 XXX_4 XXX_5 XXX_6 XXX_7 XXX_8

(XXX_2-XXX_8 represents codons 2 through 8 of the target coding region).

Reverse primer (vector sequence includes Stop anticodon):

5'-GTG GCG GCC GCT CTA TTA XXX_n XXX_{n-1} XXX_{n-2} XXX_{n-3} XXX_{n-4} XXX_{n-5} XXX_{n-6}

XXX_n - XXX_{n-6} represents the **reverse complement** of the sequence of the last 7 codons of

Figure 10. Primer design for the amplification of target genes with different fusion tags

Primer sets designed to produce five different genes with three different fusion tags depending on this strategy are given in Tables 4, 5, and 6. The orange sequence is the part required for binding to the vector, while the blue sequence is the sequence necessary for the amplification of the target gene.

Table 4

Designed primers for pRham™ N-His vector for molecular cloning

Primer	Base Sequence (5'-3')
BBKW_1881F	CAT CAT CAC CAC CAT CAC TTTCCAGGAAGGCGTTTTTCC
BBKW_1881R	GTG GCG GCC GCT CTA TTA TAGCACCGGTCCGATGATGCG
BBPC_1681F	CAT CAT CAC CAC CAT CAC ATGAAACCCCTGTTTCCAGGA
BBPC_1681R	GTG GCG GCC GCT CTA TTA TAGCACCGGTCCGATGATGCG
OU11_RS07620F	CAT CAT CAC CAC CAT CAC GGCGCAAGCCATGTGTTTCCC
OU11_RS07620R	GTG GCG GCC GCT CTA TTA AGCACGGCCTTGTTGACCTCG
BBOH_0438F	CAT CAT CAC CAC CAT CAC TCATCGACTCATGCGTCTTCG
BBOH_0438R	GTG GCG GCC GCT CTA TTA ATGATGAATAGGAACTTGCAT
BSAE_0444F	CAT CAT CAC CAC CAT CAC TCAGACCTTCCTCCGCTGCCA
BSAE_0444R	GTG GCG GCC GCT CTA TTA ATGGTGTACTACGCGCGTGGC

Table 5

Designed primers for pRham™ N-His SUMO vector for molecular cloning

Primer	Base Sequence (5'-3')
BBKW_1881F	CGC GAA CAG ATT GGA GGT TTTCCAGGAAGGCGTTTTTCC
BBKW_1881R	GTG GCG GCC GCT CTA TTA TAGCACCGGTCCGATGATGCG
BBPC_1681F	CGC GAA CAG ATT GGA GGT ATGAAACCCCTGTTTCCAGGA
BBPC_1681R	GTG GCG GCC GCT CTA TTA TAGCACCGGTCCGATGATGCG
OU11_RS07620F	CGC GAA CAG ATT GGA GGT GGCGCAAGCCATGTGTTTCCC
OU11_RS07620R	GTG GCG GCC GCT CTA TTA AGCACGGCCTTGTTGACCTCG
BBOH_0438F	CGC GAA CAG ATT GGA GGT TCATCGACTCATGCGTCTTCG
BBOH_0438R	GTG GCG GCC GCT CTA TTA ATGATGAATAGGAACTTGCAT
BSAE_0444F	CGC GAA CAG ATT GGA GGT TCAGACCTTCCTCCGCTGCCA
BSAE_0444R	GTG GCG GCC GCT CTA TTA ATGGTGTACTACGCGCGTGGC

Table 6

Designed primers for pRham C-His vector for molecular cloning

Primer	Base Sequence (5'-3')
BBKW_1881F	GAA GGA GAT ATA CAT ATG TTTCCAGGAAGGCGTTTTTCC
BBKW_1881R	GTG ATG GTG GTG ATG ATG TAGCACCGGTCCGATGATGCG
BBPC_1681F	GAA GGA GAT ATA CAT ATG ATGAAACCCCTGTTTCCAGGA
BBPC_1681R	GTG ATG GTG GTG ATG ATG TAGCACCGGTCCGATGATGCG
OU11_RS07620F	GAA GGA GAT ATA CAT ATG GGCGCAAGCCATGTGTTTCCC
OU11_RS07620R	GTG ATG GTG GTG ATG ATG AGCACGGCCTTGTTGACCTCG
BBOH_0438F	GAA GGA GAT ATA CAT ATG TCATCGACTCATGCGTCTTCG
BBOH_0438R	GTG ATG GTG GTG ATG ATG ATGATGAATAGGAACTTGCAT
BSAE_0444F	GAA GGA GAT ATA CAT ATG TCAGACCTTCCTCCGCTGCCA
BSAE_0444R	GTG ATG GTG GTG ATG ATG ATGGTGTACTACGCGCGTGGC

The concentrations of the primers (oligomers) were obtained as 100 µM by adding sterile water in the amounts determined by the company. 10 µM new stocks were prepared from these stocks to be used in PCR amplification of the target genes.

3.2.5. PCR Amplification of Targeted Genes by Three Vector Primer Sets (pRham N-His, pRham C-His, and pRham N-His SUMO)

The target genes were amplified with the three different tagged primer sets. As a positive control, the *Blon_2468* gene (EndoBI-1) encoding by the *B. infantis* strain used in our Career Project 117z132 was used. The amplification was carried out in 50 µL of the final volume containing 2 µL of template DNA (bacterial cells), 1 µL forward and reverse primer at 0.2 µM, 25 µL PCR master mix, 21 µL distilled water (DNase/RNase-free) in all tubes. After the mixture was prepared, the PCR tubes were put to the Bio-Rad thermal cycler. As the PCR cycle, the process was first carried out as 1 cycle: 95 °C 5 min (first denaturation step) for the release of genomic DNA, 40 cycles: 95 °C 30 s (initial denaturation), 60 °C 30 s (annealing step), 72 °C 1 min (elongation step), 1 cycle: 72 °C 10 min (final extension).

Agarose Gel Electrophoresis

The control of PCR products was performed by gel electrophoresis method using SafeRed loading dye by mixing with the PCR products and DNA ladder at a ratio of 1:5 (5 µL PCR product + 1 µL SafeRed and 5 µL 1 kb DNA ladder + 1 µL SafeRed) and run on 1% agarose gel at 100 V for 60 min with 1X TBE buffer. PCR products were visualized under a gel documentation system ST4 1100 (Vilber Lourmat, France). The PCR product concentrations were measured by using a Qubit 3 Fluorometer with a dsDNA assay kit before the molecular cloning step. The concentrations should be in the range required for molecular cloning (25-100 ng / µL).

3.2.6. Preparation of Growth Lysogeny Broth (LB) Media

Preparation of LB Agar Medium for Molecular Cloning: 12.5 g of LB and 6 g of agarose were dissolved in 500 mL of dH2O and autoclaved (sterilization at 121 °C for 20 min). 15 mg of kanamycin (30 µg/mL) was dissolved with 1 mL of dH2O and store at -20 °C. After the autoclaving, 1 mL of kanamycin was added into the bottle containing the sterile

500 mL of LB agar medium. ~25 mL of LB agar kanamycin media was poured into each plate in the laboratory cabinet.

Preparation of LB Medium for Colony PCR: 10 g of LB was dissolved with 400 mL of the dH2O in a bottle and autoclaved (sterilization at 121 °C for 20 min). 12 mg of kanamycin (30 µg/mL) was dissolved with 1 mL of dH2O and put at -20 °C. After autoclaving, 1 mL of kanamycin was added into the bottle containing the sterile 400 mL of LB medium and store at 4 °C.

3.2.7. Heat Shock Transformation of His-tagged PCR Products and pRham Vectors into *E. cloni* 10G Cells

The Recovery Medium provided with the kit (the medium used for rapid healing of cells after transformation) was taken from -80 ºC to room temperature before cloning. *E. cloni* 10G cells at -80 ºC thawed completely on wet ice. The amounts of His-tagged PCR products (N-His, SUMO, C-His) for each strain in Tables 7, 8, 9 (25-100 ng) were mixed with 2 µL of pRham™ Vector DNA and added to 40 µL of *E. cloni* 10G cell. These mixtures were transferred to 15 mL falcon tubes. All tubes were put on ice for 30 min. The thermal shock was then performed in a 42 °C water bath for 45 seconds to insertion of the vector and PCR product into the cell. These mixtures were quickly put on ice for 2 min to close the cell pores. 960 µL of recovery medium which allowing rapid repair of the cells was put into each tube. They are incubated at 37 °C for 1 hour at 250 rpm, and these cells were put to the centrifuge at 4000 rpm for 10 minutes. The different phases were then formed. The supernatant part was discarded and the formed pellet part was dissolved and homogenized 100 µL recovery medium using a pipette, then cultured in LB agar kanamycin plates. For the negative control, only *E. cloni* 10G cells were cultured on LB agar kanamycin plates. All plates were put in the 37 °C incubator overnight.

Table 7

Products and quantities used for the transformation of N-His tagged proteins

Enzyme Codes	PCR products	pRham Vector DNA	*E. cloni* 10G cell
OU11_RS07620	3	2	40
BSAE_0444	2	2	40
BBKW_1881	3	2	40
BBPC_1683	3	2	40
BBOH_0438	3	2	40

Table 8

Products and quantities used for the transformation of SUMO tagged proteins

Enzyme Codes	PCR products	pRham Vector DNA	*E. cloni* 10G cell
OU11_RS07620	3	2	40
BSAE_0444	3	2	40
BBKW_1881	2	2	40
BBPC_1683	2	2	40
BBOH_0438	2	2	40

Table 9

Products and quantities (μL) used for the transformation of C-His tagged proteins

Enzyme Codes	PCR products	pRham Vector DNA	*E. cloni* 10G cell
OU11_RS07620	3	2	40
BSAE_0444	3	2	40
BBKW_1881	3	2	40
BBPC_1683	3	2	40
BBOH_0438	3	2	40

3.2.8. Screening of Transformants by Colony PCR

To confirm all transformants carrying the recombinant genes, colonies were randomly selected. The target genes were then amplified by PCR using the sequencing primers supplied with the cloning kit. If the gene of interest cannot be inserted into the vector, there will be no region where these primers can multiply, so there is no band in the gel. In this way, successful transformants can be distinguished. Firstly, one part of each colony was

transferred to a PCR tube for PCR amplification, and the other part was inoculated into a falcon tube (15 mL) with 5 mL LB+kanamycin liquid medium (Figure 11). All falcon tubes were incubated at 250 rpm 37 °C overnight in a shaking incubator.

All PCR process was performed in the same manner as in step 3.2.5. In this step, unlike the other step, the sequencing primers supplied with the kit were used instead of the oligomer primers (Table 10).

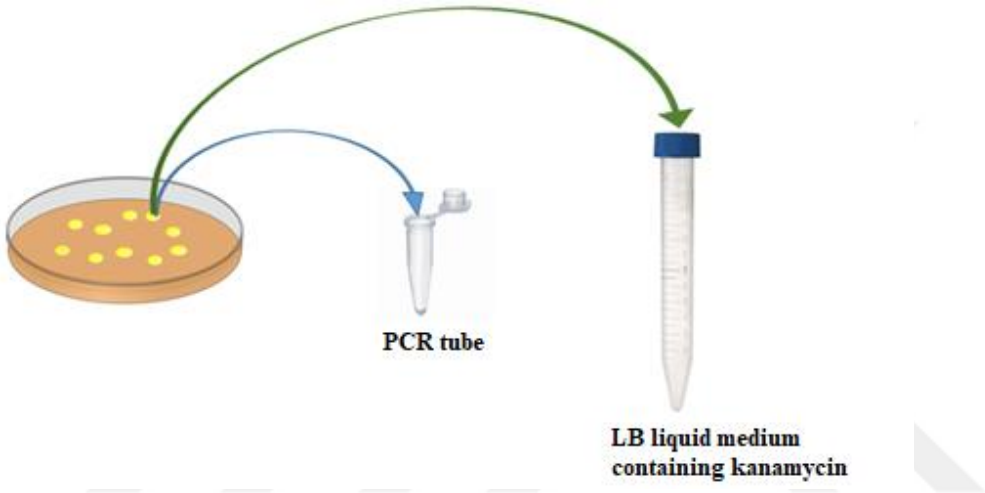


Figure 11. General scheme of Colony PCR

Table 10

List of sequencing primers supplied with the kits used for gene amplification in Colony PCR.

Kits	Primer Code	Base Sequence (5'-3')
N-His	pRham Forward	GCTTTTTAGACTGGTCGTAGGGAG
	pETite Reverse	CTCAAGACCCGTTTAGAGGC
N-His SUMO	SUMO Forward	ATTCAAGCTGATCAGACCCCTGAA
	pETite Reverse	CTCAAGACCCGTTTAGAGGC
C-His	pRham Forward	GCTTTTTAGACTGGTCGTAGGGAG
	pETite Reverse	CTCAAGACCCGTTTAGAGGC

Agarose Gel Electrophoresis for The Control of PCR Products

The control of PCR products was performed by gel electrophoresis method using SafeRed loading dye by mixing with the PCR products and DNA ladder at a ratio of 1:5 (5

µL PCR product + 1 µL SafeRed and 5 µL 1 kb DNA ladder + 1 µL SafeRed) and run on 1% agarose gel at 100 V for 60 min with 1X TBE buffer. The PCR products were visualized under a gel documentation system ST4 1100 (Vilber Lourmat, France). To prepare 15% glycerol stocks of the successful transformants, 500 µL of 60% glycerol and 1500 µL of the liquid culture of the successful transformants were mixed in the cryotubes. These stocks were stored at -80 °C.

3.2.9. Sequencing of Related Genes After Cloning

The verification of the target gene sequences in the genomic DNA and the sequencing results of the related genes after cloning were first analyzed using the Geneious Prime 2020.2 program, and the reading qualities of the sequences were evaluated. Regions with low reading quality in sequencing results were trimmed appropriately, resulting in a more accurate and clean result. The correct and clean sequences obtained with 4 different primers (5 different primers for the enzymes coded BBOH_0438) were combined and the appropriate and complete sequences of the related genes and target genes were obtained. All these sequences were blasted using NCBI Nucleotide Blast with appropriate reference sequences taken from the NCBI database, and their similarity percentages were detailed and reported. Besides, multiple alignment analyses (Geneious Alignment) of the target gene and the related gene sequence results with genomic DNA sequence in terms of both nucleotide and amino acid were performed in the Geneious Prime 2020.2 program.

3.2.10. Protein Production and Purification

In general, protein production was primarily carried out on a small scale (8 mL). It was then produced in large quantities using 250 mL of medium.

L-rhamnose Induction of Protein Expression: A preculture was prepared with 1 L of LB+kanamycin liquid medium. 8 mL of LB medium was put in 50 mL falcon tubes for each strain. One of the tubes containing only the liquid medium was used as a negative control. For 20% L-rhamnose as stock; 0.5 g of L-rhamnose was dissolved with 2.5 mL dH_2O. Stored at -20 °C until use it. 10 µL of the bacterial stocks were added into the falcon tubes containing 8 mL liquid media (vortex them gently). They were then cultured at 37 °C overnight in an incubator (shaking). 250 mL of LB liquid medium were poured into a

sterilized 2 L Erlenmeyer flask. After overnight, 2.5 mL of the liquid culture were inoculated to a 2 L flask containing LB liquid medium at a ratio of 1:100 between the flask and the medium and incubated rpm at 37 °C for 4 hours at 150 rpm in the shaking incubator. Cells' optical density was measured at 600 nm (OD600) by a spectrophotometer. When the optical density of the cells was reached to 0.5-0.6, 2.5 mL of 20% rhamnose (final concentration is 0.2%) were put into the 250 mL LB culture and incubated overnight at 250 rpm and 37 °C in the incubator. After overnight, the liquid culture was transferred into the 5 x 50 mL falcon tubes. They were then put into the centrifuge for 15 minutes at 4000 rpm and 4 °C. Two phases were formed as supernatant and pellet and the supernatant phase was discarded. The pellet was stored at -20°C until the purification.

Cell Lysis of *E. cloni* 10G containing His-tagged Enzymes

The pellets from -80°C were dissolved in 5 mL of dH_2O and centrifuged for 15 minutes at 4000 rpm and 4 °C. After the formed supernatant part was discarded, the formed pellet was homogenized with 6300 µL of lysis buffer and 63 µL protease inhibitor (1:100 ratio) for a 50 mL culture pellet. The samples were waited for incubation on ice for 30 minutes, vortex in each 10 min. After incubation, the mixture with bacteria was homogenized for the second time with a sonicator. The pulse mode of a sonicator was set as cycle on 10 s and cycle off 59 s, and the amplitude as 37%. After the sonication process (6 pulses for 10 s with 1 min cooling), the samples were put into the centrifuge for 45 minutes at 4000 rpm and 4 °C. All supernatant parts were transferred in a falcon tube and centrifuged again. Then the formed supernatant parts were collected in a falcon tube and taken 100 µL of the sample for SDS-PAGE. The concentrations of the cell lysates were measured by using a Qubit 3 Fluorometer.

Purification of His-tagged Enzymes by Batch Method

1 mL of Ni-NTA resin was added into a falcon tube for each protein and centrifuged for 2 min at 700 × *g* and formed supernatant parts were discarded. Then 2 mL (twice the resin volume) of the equilibration buffer were added into all tubes and mixed well until the resin was fully suspended. All tubes were centrifuged for 2 min at 700 × *g*, and all

supernatant parts were discarded. The protein extracts and equilibration buffer were mixed in a ratio of 1:1 in a falcon tube for each protein.

The mixtures were added to the falcon tubes containing resin to bind the proteins to resin and put on a shaker at 150 rpm during 30 min. All tubes were put into the centrifuge for 2 minutes at 700 × *g*, and formed supernatants were discarded. The resin parts were washed with 5 mL wash buffer and centrifuged for 2 minutes at 700 x *g*. The washing step was repeated until the concentrations of the supernatant decrease to the baseline and the potential pollution was removed by washing. Then 1 mL of elution buffer was added into all tubes to elute bound His-tagged proteins. The elution step was repeated three times. All tubes were put into the centrifuge for 2 minutes at 700 x *g*, and 100 µL from formed supernatant parts were taken for SDS-PAGE analysis. The concentrations of each supernatant were measured by using a Qubit 3 Fluorometer. All purified proteins were collected and concentrated in the Amicon tube (10-kDa cut-off). The concentration of protein was measured with the Qubit 3 fluorometer using a Qubit protein test kit and 100 µL of the purified enzymes were taken for SDS-PAGE analysis. All purified enzymes were put into new eppendorf tubes and stored them at -80 °C.

SDS-PAGE Analysis of Purified Proteins

SDS-PAGE analysis was performed by using a BioRad protein gel system to determine the purity of the enzymes. 4% stacking and 12% separating gel were prepared for the analysis of protein bands. To compare the molecular weight of proteins, a protein ladder was used. 2X Laemmli sample buffer was added into the samples in a ratio of 1:1. The samples were then incubated for 5 minutes at 95 °C to denature proteins. The mixtures were homogenized with a vortex. After the incubation, The 1X running buffer was used for the running of proteins in the gel. The samples and the protein ladder were loaded into the gel. The proteins were run firstly at 80 V, and the current was raised to 120 V when the proteins were moved from the stacking gel to the resolving gel. Proteins separated according to their molecular weight were stained at 55 rpm for 30 min with Coomassie Brilliant Blue. A destaining solution (50% dH2O, 10% Glacial Acetic Acid, 40% Methanol) was applied for 3 h to ensure clarity of the bands and was changed at intervals.

3.2.11. Determination of Purified Enzyme Activities on RNase B by SDS-PAGE Analysis

For the preparation of 0.2 M sodium phosphate buffer (pH 5), 35.60 g $Na_2HPO_4 \cdot 2H_2O$, 23.99 g $NaH_2PO_4 \cdot H_2O$ were dissolved separately with dH_2O, and each stock solution volume was adjusted to 1 L in a glass bottle. 50 mL of buffer was prepared by mixing 38.5 mL of the stock $Na_2HPO_4 \cdot 2H_2O$ and 11.5 mL of the stock $NaH_2PO_4 \cdot H_2O$ and the pH was adjusted to 5.

6 µL of dH_2O and 1 µL of RNase B (5 mg/mL) were put into all sample tubes and control tubes, and the proteins were denatured for 5 minutes at 95 °C. 1 µL of sodium phosphate buffer were added into all tubes on ice (a ratio of 1:10 between the buffer and the total volume). 2 µL of purified enzymes and dH_2O were added into the sample tubes and the control tubes, respectively. All reactions were incubated for 1 hour at 37 °C. All samples were mixed with 2X Laemmli sample buffer in a ratio of 1:1. All samples were incubated for 10 minutes at 70 °C. All tubes were then put for 2 min on ice, and the activity of all enzymes on RNase B was controlled by SDS-PAGE analysis.

3.2.12. Preparation of Proteins for Enzyme Kinetics

In addition to Lactoferrin (Lf) and Immunoglobulin G (IgG) pure proteins selected for use in enzyme kinetics, whey proteins and soy proteins were firstly cleaned from compounds that could adversely affect *N*-glycan quantification.

Preparation of Whey Protein Isolate: 150 mg of whey powder was dissolved with 10 mL of dH_2O. To increase the dissolution efficiency, it was kept in a bath of water (60 °C) and vortexed. The dissolved sample was incubated at 4 °C for 2 hours. The sample was then put into the centrifuge for 20 minutes at 4000 rpm and 4 °C to separate the formed fat part of whey. 40 mL of cold ethanol at a ratio of 1:4 (v/v) was added into the supernatant part formed after centrifugation and incubated them for 1 hour at -20 °C. The sample was then centrifuged, and the formed pellet part was dissolved 10 mL of dH_2O. The process of ethanol precipitation was applied twice. The final pellet part was dissolved in 10 mL of dH_2O and

concentrated with a 50 mL 10-kDa-cut-off centrifuge filter. It was ensured that these impurities were removed with the phenol sulfuric acid total carbohydrate test used in the quantification of glycans. Qubit Fluorometer 3.0 was used for the determination of the protein concentrations obtained. The prepared protein isolate was stored at -20 °C until it was used.

Preparation of Soy Protein Isolate: 150 mg of soy powder was dissolved with 10 mL of dH_2O (15 mg/mL). The solution was kept in a bath of water (60 °C) for 1 hour and vortexed intermittently. The pH value of the prepared sample was adjusted to 8.2 with 2N NaOH. The sample waited for 2 hours at room temperature in a shaker incubator. The sample was then centrifuged for 15 minutes at 4 °C and 4000 rpm. The formed supernatant part was transferred into a new tube and the pH was adjusted with 2M HCl to reach pH 4.8. The sample was then put into the centrifuge for 2 min at 4000 rpm and 4 °C. The formed pellet part was dissolved 10 mL of dH_2O and the pH was adjusted again to 8.2 with 2N NaOH. The prepared protein isolate was stored at -20 °C until it was used.

3.2.13. Determination of Optimum Reaction Conditions of Purified Enzymes

Optimization experiments for 5 successful enzymes whose activation was tested on RNase B protein were carried out under 3 different pH and temperature conditions. The combination of pH 5, 6, 7 and temperature 24, 30, 37 °C conditions was tried for each of the C1, C2, C3, N3, and N5 enzymes (Table 11). Within the scope of optimization experiments, 2 mL of whey protein, 5 mg of IgG, and 5 mg of Lf protein mixture were used as substrate. For each reaction, 15 µL of the substrate, 2.5 µL sodium phosphate buffer, 0.5 µL the enzyme, and 6.5 µL dH_2O were used. Each sample and control was used with duplicates, and 0.5 µL of dH_2O was used instead of the enzyme as a control. Firstly, the proteins were denatured at 50 °C for 10 min. Then sodium phosphate buffer and enzyme were added into the denatured proteins and incubated at the required temperature conditions overnight. The amount of glycan obtained as a result of the reaction was measured calorimetrically and the obtained glycan concentration was determined using the absorbance values obtained.

Table 11

Optimization condition values tested on enzymes

Enzyme Codes	pH	Temperature (°C)
C1, C2, C3, N3, N5	5	24
	5	30
	5	37
	6	24
	6	30
	6	37
	7	24
	7	30
	7	37

After incubation was completed under appropriate pH and temperature conditions, the amounts of released glycan in the sample contents were determined by the phenol sulfuric acid method. For this analysis, 25 µL of the sample, 25 µL of phenol (5%, m/v), and 125 µL of sulfuric acid were mixed using a 96 microplate, respectively, and incubated for 30 minutes at 35 rpm, room temperature, and dark condition. The OD was then measured at 485 nm. 1 mg/mL of the stock glucose solution was prepared as standard and was diluted to the concentrations 0.8, 0.6, 0.4, 0.2, 0.1 mg/mL. NCSS12 Statistical Software program was used to evaluate and plot the results. A three-dimensional (3D) surface plot was preferred for the evaluation and visualization of the results of the study conducted to see the impacts of different pH, temperature combinations on each enzyme activity.

3.2.14. Determination of Kinetic Parameters of Enzymes

For the kinetic characterization of recombinantly produced enzymes, the substrate mixture (whey, IgG, Lf, and soy protein) with eight different concentrations determined in the range of 0.9-7.2 mg/mL was reacted with these enzymes under optimum conditions for 20 min. The amounts of released glycans were determined calorimetrically, and the reaction rate (v) for each substrate concentration was calculated based on these glycan concentrations. Then, the kinetic parameters (Maximum velocity ($Vmax$), the constant of Michaelis Menten (Km), Turnover number ($kcat$), and $kcat/km$) were determined by considering the slope of the initial part of the reaction.

In addition to non-linear regression, Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf methods, which are linearized plotting with linear regression analysis, were used to reduce the margin of error and potential deviation in the determination of kinetic parameters (Hanes, 1932; Lineweaver and Burk, 1934; Woolf, 1931). Graphpad Prism 6.0 program was used for the determination of kinetic parameters, non-linear and linear regression calculations, and graphing of the obtained results.

CHAPTER 4
RESEARCH FINDINGS

4.1. Anaerobic Growth of Bifidobacterial Species

In the study of Bacterial growth, one of 5 cells, *B. pullorum subsp. pullorum* strain could not be grown. The stock culture of this strain could not be prepared, but the cells that were added at first by centrifuging the liquid medium of this strain were collected and kept at -80°C. Since only the genomic DNA of these cells will be used in the continuation of the thesis, this problem encountered at this stage did not show a restrictive feature in the realization of thesis studies.

4.2. PCR Amplification of The Target Genes by The Designed Primers

Targeted genes were amplified by PCR as previously mentioned. DNA gel electrophoresis was performed on all PCR products for the visualization to control the concentration and the purity of the inserts (Figure 12). The gel images showed that each amplification was successfully completed as there was only one clear band for each insert. Thus, the study for the reproduction of the target 5 genes with 3 different primers was completed with 100% success. Each PCR amplicon was measured by a Qubit 3 Fluorometer to determine the concentration of the PCR product (Table 12).

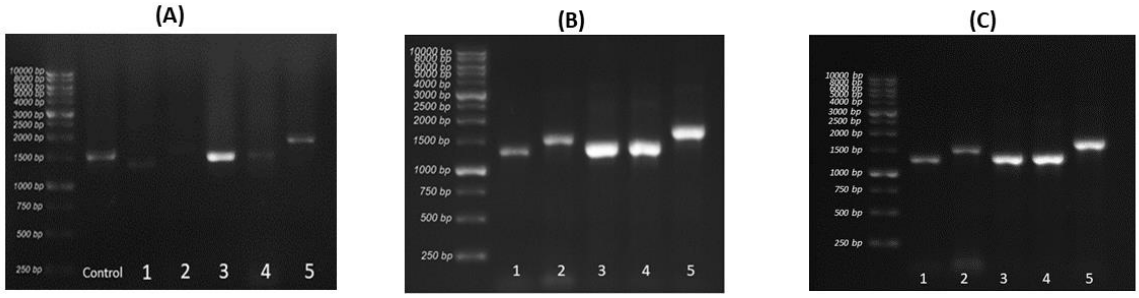


Figure 12. PCR amplification of targeted genes by three different vector primer sets. (A) pRham N-His (B) pRham C-His (C) pRham N-His SUMO. Lanes 1-5 represents OU11_RS07620, BSAE_0444, BBKW_1881, BBPC_1683 and BBOH_0438 amplification, respectively.

Table 12

Measurements of concentration of PCR amplicons by Qubit (ng/µL)

Samples	N-His	SUMO	C-His	Final Volume
B. pullorum subsp. pullorum (OU11_RS07620)	9	14.2	10.76	50 µL
B. pullorum subsp. saeculare (BSAE_0444)	24	12.66	11.74	50 µL
B. kashiwanohense (BBKW_1881)	14.6	35.9	20.6	50 µL
B. pseudocatenulatum (BBPC_1683)	20	35.3	21.9	50 µL
B. bohemicum (BBOH_0438)	30.8	32.5	10.4	50 µL

4.3. Heat Shock Transformation of His-Tagged PCR Amplicons and pRhamTM Vectors into E. cloni 10G Cells

The transformation of His-tagged PCR amplicons and pRham™ vectors (pRham™ N-His Kan, pRham™ C-His Kan, and pRham™ N-His SUMO Kan) into *E. cloni* 10G cells were performed by the heat shock transformation method. Firstly, 1-3 µL of the PCR products and 2 µL of pRham vectors were added into 40 µL of *E. cloni* 10G cells. The mixtures were stirred gently with a pipet tip, transferred into 15 mL falcon tubes. All tubes were put on ice for 30 min. The thermal shock was then performed in a 42 °C water bath for 45 seconds to insertion of the vector and PCR product into the cell. These mixtures were quickly put on ice for 2 min to close the cell pores. 960 µL of recovery medium which allowing rapid repair of the cells was put into each tube. They are incubated at 37 °C for 1 hour at 250 rpm, and these cells were put to the centrifuge at 4000 rpm for 10 minutes. The different phases were then formed. The supernatant part was discarded and the formed pellet part was dissolved and homogenized 100 µL recovery medium using a pipette, then cultured in LB agar kanamycin plates. For the negative control, only *E. cloni* 10G cells were cultured on LB agar kanamycin plates. All plates were put in the 37 °C incubator overnight. Since these negative cells do not contain antibiotic-resistant vectors, no improvement should be observed. In this way, contaminations that could adversely affect the experiment and the stability of antibiotics were also tested. After the transformations, 20-60 colonies were obtained as expected (Figures 13 and 14). The growth of these cells on a medium containing LB agar kanamycin medium was indicated that the kanamycin-resistant vectors were successfully transferred to the cells.

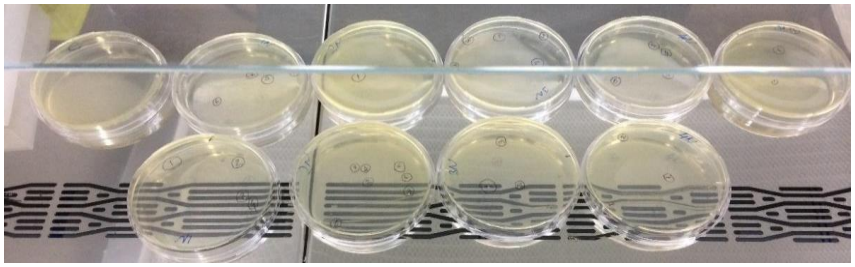


Figure 13. Colonies after cloning and transformation of N-His tagged target genes and vectors. 20-60 colonies were obtained as expected on LB agar kanamycin plates.

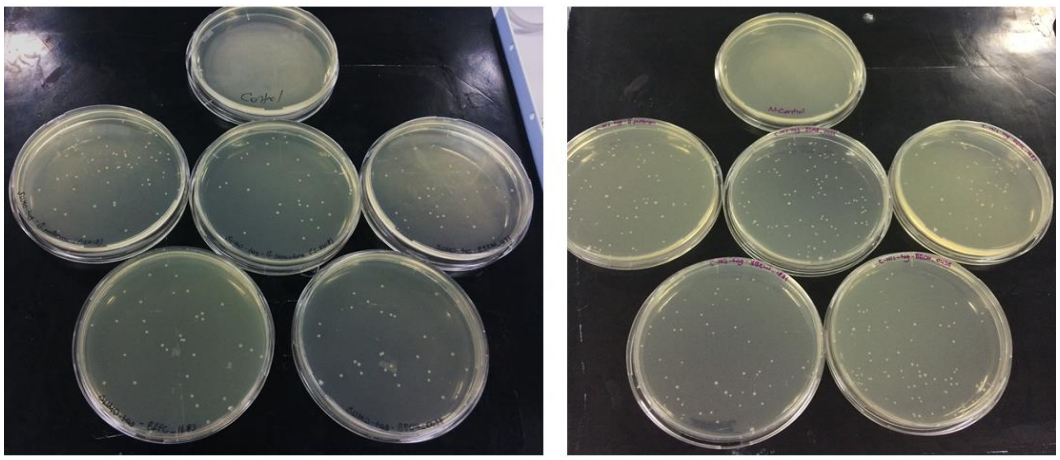


Figure 14. Colonies after cloning and transformation of SUMO and C-His tagged target genes and vectors. 20-60 colonies were obtained as expected on LB agar kanamycin plates.

4.4. Confirmation of Transformants by Colony PCR

Successful transformants were confirmed by the colony PCR method. Firstly, one part of each colony was transferred into a PCR tube for PCR amplification and the other part was inoculated to 5 mL LB+kanamycin medium in a falcon tube (15 mL) to be used in case of positive results. PCR amplification was performed using 1 µL of forward and reverse primer (sequencing primers provided by the manufacturer), 25 µL master mix, 23 µL of dH_2O. The program was set as previously mentioned. 5 µL of PCR products mixed with SafeRed at a ratio of 1:1, were run on 1% agarose gel for 70 min at 100 volts and visualized with the gel documentation system.

4.4.1. Screening of Transformants for N-His tagged PCR products by Colony PCR

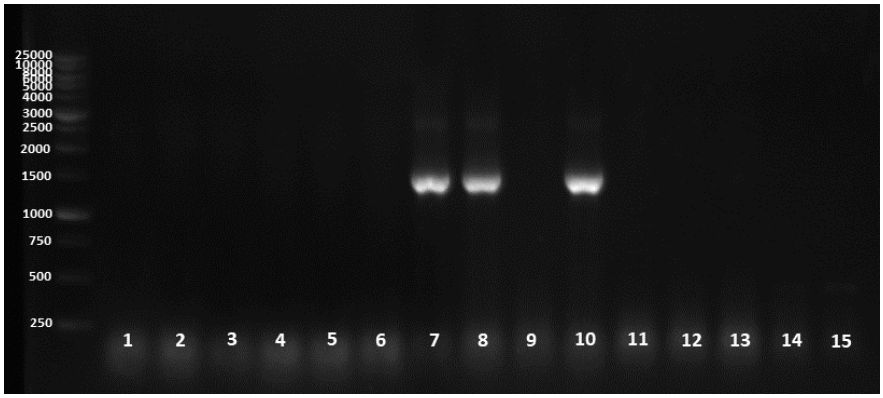


Figure 15. Gel image of N-His tagged target genes obtained after Colony PCR. Lanes 1-5, 6-10, 11-15 represents OU11_RS07620, BSAE_0444, and BBKW_1881 amplifications, respectively.

N-His tagged PCR products after colony PCR were visualized by gel electrophoresis. When the gel image in Figure 15 was examined, it was observed that the three colonies (lanes 7, 8, and 10) were only successful in the transformation of the target gene OU11_RS07620. The colony PCR was repeated by increasing the number of colonies tested.

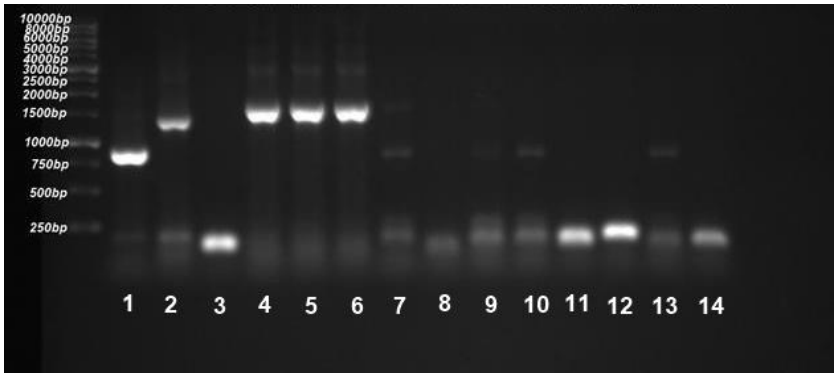


Figure 16. Gel image of N-His tagged target genes obtained after Colony PCR. Lanes 1-3, 4-6, 7-9, 10-12, and 13-14 represents OU11_RS07620, BSAE_0444, BBKW_1881, BBPC_1683, and BBOH_0438 amplifications, respectively.

When the gel image of repeated amplification in Figure 16 was examined, it was observed that one colony (Lane 2) was successful in the transformation of the target gene OU11_RS07620, and three colonies (Lanes 4, 5, and 6) were successful in the transformation of the target gene BSAE_0444. Positive transformants were determined and 15% glycerol stocks were prepared. After this stage, cloning was repeated for unsuccessful cells, and then positive transformants were obtained for BBKW_1881, BBPC_1683, and BBOH_0438 (Figures 17, 18, and 19). When the gel image of amplification in Figure 17 was examined after repeated cloning, it was observed that 5 colonies (Lanes 11, 12, 13, 15, and 17) were successful in the transformation of the target gene BBPC_1683. In Figure 18, lanes 3 and 4 were successful transformants and the base difference between these two bands is related to the primer sets used. To eliminate a problem caused by primers, both target gene-specific primer sets (oligomers) and universal sequencing primer sets in the plasmid were used. Since universal sequencing primer sets replicate a larger region, there is a difference of approximately 200 bases between the two PCR products.

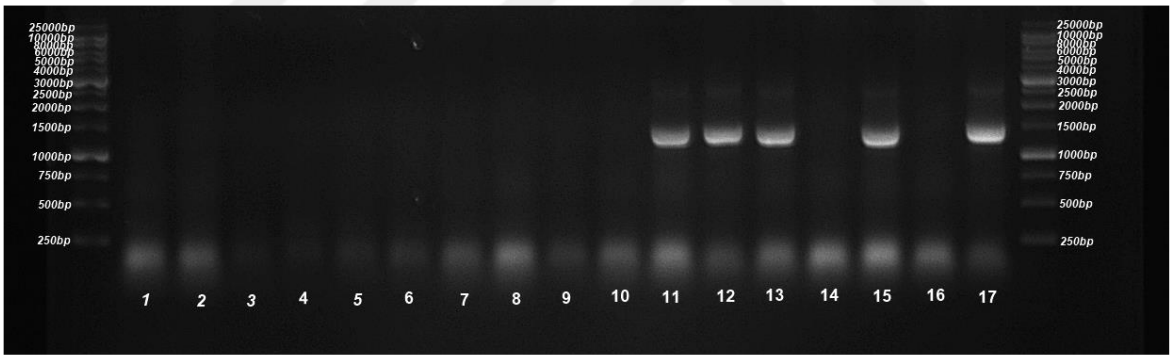


Figure 17. Gel image of N-His tagged target genes obtained after Colony PCR. Lanes 1-10, 11-17 represents BBKW_1881 and BBPC_1683 amplifications, respectively.

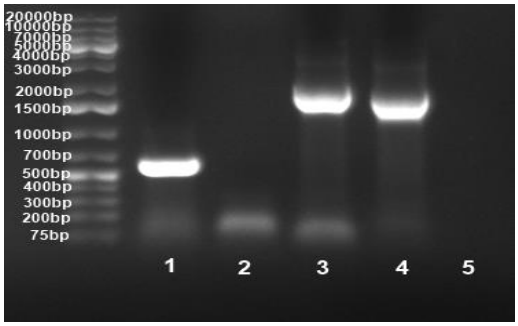


Figure 18. Gel image of N-His tagged BBOH_0438 gene obtained after Colony PCR (Lane 1-5 colonies).

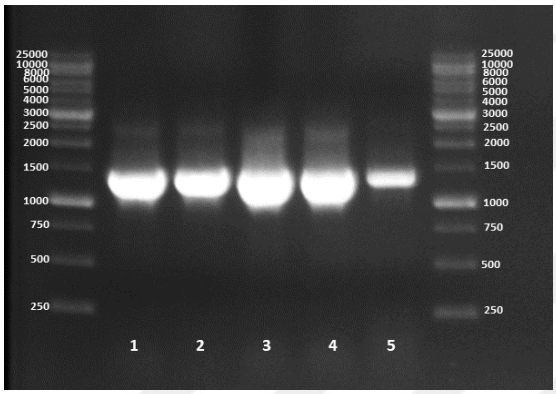


Figure 19. Gel image of N-His tagged BBKW_1881 gene obtained after Colony PCR (Lanes 1-5 colonies).

Consequently, positive transformants for all N-His tagged genes were separated from the unsuccessful colonies. When the gel images were examined, it was observed that almost all colonies were successful in the transformation of the target genes. 15% glycerol stocks were prepared from the liquid cultures of the successful colonies.

4.4.2. Screening of Transformants for N-His SUMO tagged PCR products by Colony PCR

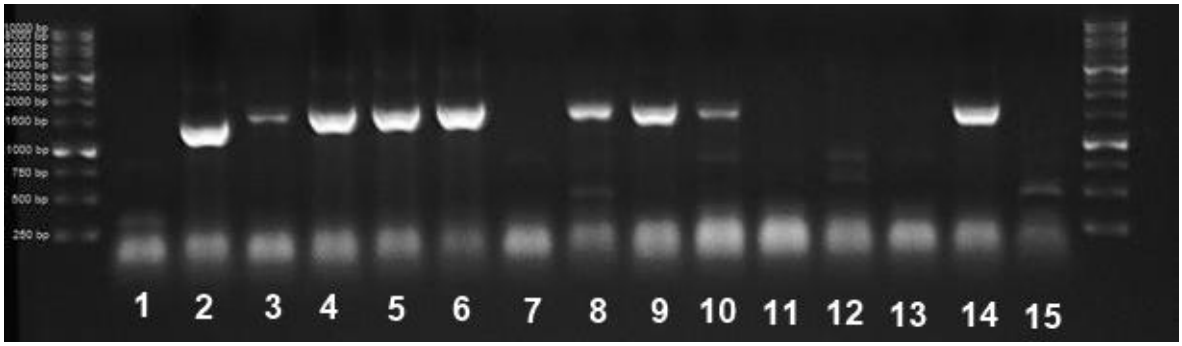


Figure 20. Gel image of N-His SUMO tagged target genes obtained after Colony PCR. Lanes 1-3, 4-6, 7-9, 10-12, and 13-15 represents OU11_RS07620, BSAE_0444, BBKW_1881, BBPC_1683, and BBOH_0438 amplifications, respectively.

N-His SUMO tagged PCR products after colony PCR were visualized by gel electrophoresis. When the gel image was examined, it was observed that one colony (Lane 2), three colonies (Lanes 4, 5, and 6), and one colony (Lane 14) were successful in the transformation of the target genes OU11_RS07620, BSAE_0444, and BBOH_0438, respectively (Figure 20).

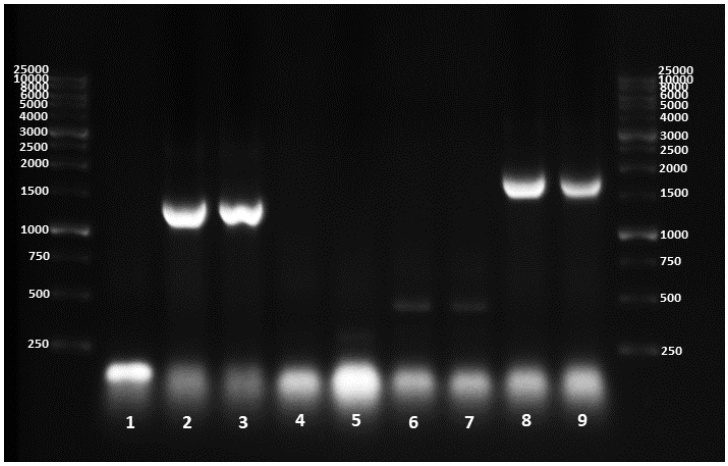


Figure 21. Gel image of SUMO tagged target genes obtained after Colony PCR. Lanes 1-3, 4-6, and 7-9 represents OU11_RS07620, BBPC_1683, and BBOH_0438 amplifications, respectively.

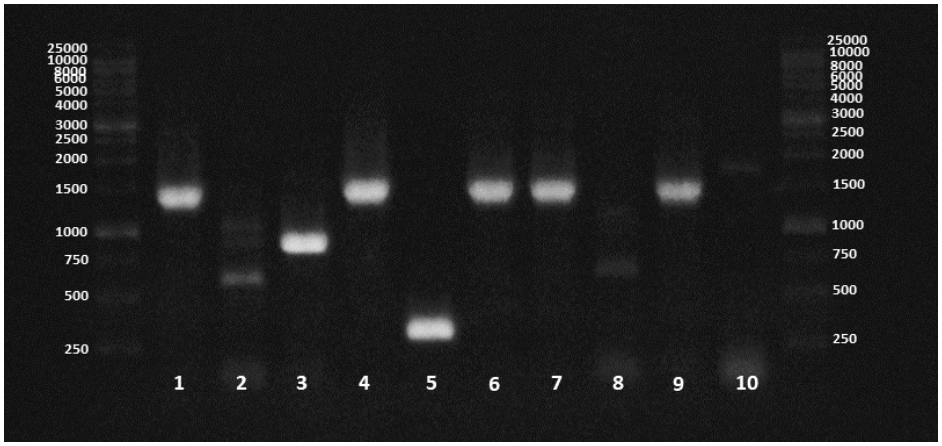


Figure 22. Gel image of SUMO tagged re-cloned genes obtained after Colony PCR. Lanes 1-5 and 6-10 represents BBKW_1881 and BBPC_1683 amplifications, respectively.

The colony PCR was repeated to extra stocks of OU11_RS07620, BBPC_1683, and BBOH_0438 target genes (Figure 21). When the gel image of amplification was examined, it was observed that 2 colonies (Lanes 2, 3) and 2 colonies (Lanes 8, 9) were successful in the transformation of the target gene OU11_RS07620 and BBOH_0438, respectively. Positive transformants were determined, and 15% glycerol stocks were prepared. The cloning step was also repeated for SUMO tagged BBKW_1881 and BBPC_1683 genes (Figure 22). When the gel image of amplification was examined, it was observed that 2 colonies (Lanes 1, 4) and 3 colonies (Lanes 6, 7, 9) were successful in the transformation of the target gene BBKW_1881 and BBPC_1683, respectively. Consequently, positive transformants for all SUMO tagged genes were separated from the unsuccessful colonies.

4.4.3. Screening of Transformants for C-His tagged PCR products by Colony PCR

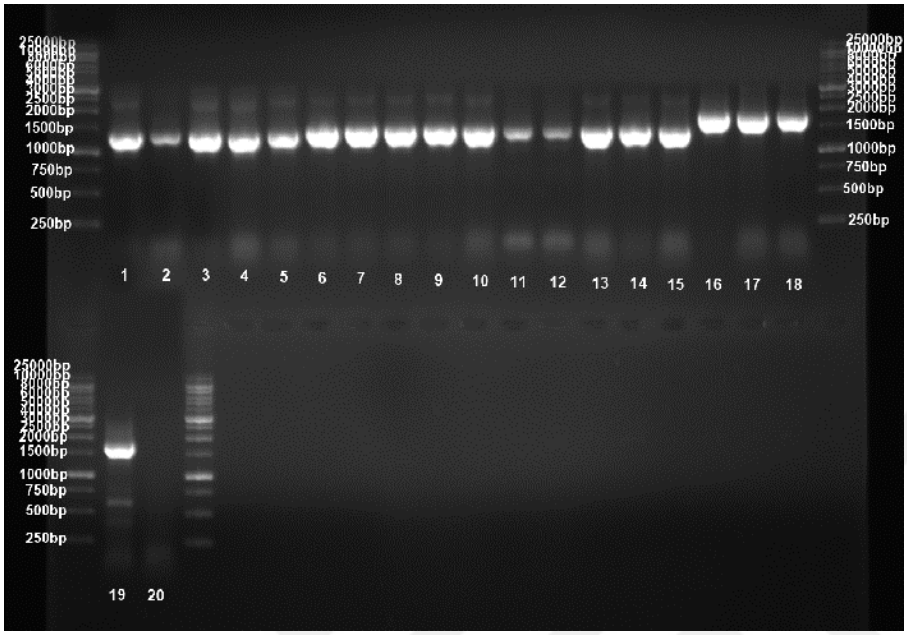


Figure 23. Gel image of C-His tagged target genes obtained after Colony PCR. Lanes 1-5, 6-10, 11-15, and 16-20 represents OU11_RS07620, BBKW_1881, BBPC_1683, and BBOH_0438 amplifications, respectively.

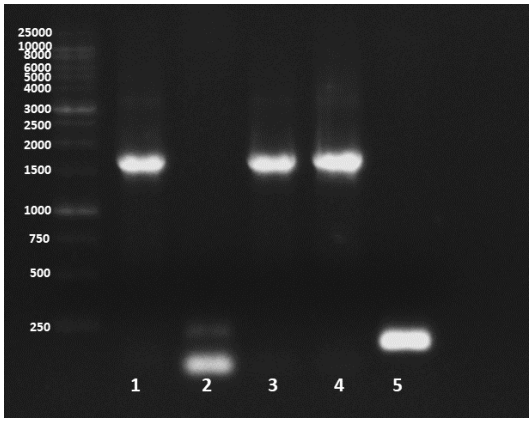


Figure 24. Gel image of C-His tagged BSAE_0444 obtained after Colony PCR (Lanes 1-5).

C-His tagged PCR products after colony PCR were visualized by gel electrophoresis. When the gel image was examined, it was observed that all colonies were successful in the transformation of the SUMO tagged target genes OU11_RS07620, BBKW_1881, BBPC_1683, and BBOH_0438, respectively (Figure 23).

C-His tagged BSAE_0444 PCR products after colony PCR were visualized by gel electrophoresis. When the gel image was examined, it was observed that 3 colonies (Lanes 1, 3, 4) were successful in the transformation of the target gene BSAE_0444 (Figure 24). Thus, successful transformants for each target gene were distinguished from the unsuccessful colonies. According to these results, 15% glycerol stocks were prepared from liquid cultures obtained from these successful colonies.

In this thesis, bands with expected base lengths for the five target genes with the three different fusion tags were obtained (Table 13). The cloning processes for 15 enzymes have been completed.

Table 13

Information about numbers of base-pair and kDa values of target genes.

Locus Tag of Target Genes	**Number of Base-Pair**	**kDa values**
OU11_RS07620	1226	44
BSAE_0444	2811	52.5
BBKW_1881	1302	50
BBPC_1683	1314	47
BBOH_0438	1650	62

4.5. Verification of Target Gene Sequences in Genomic DNA

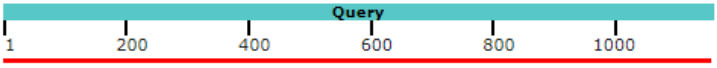
All PCR products were sequenced to confirm the genes of interest. Sequencing data for each gene were read with a total of 4 different primers (5 different primers for the enzymes coded BBOH_0438). The sequences obtained from these readings were compared with the genomic DNA sequence to check whether there was any mutation. Any nucleotide or amino acid changes were not detected in the analysis of 5 targeted genes (OU11_RS07620, BSAE_0444, BBKW_1881, BBPC_1684, and BBOH_0438) and genomic DNA sequences of these genes. As a result of the sequencing of the target gene 2, nucleotide changes were observed at the end of the sequence due to the reading quality problem. Similarity percentages for all these analyzes are reported as follows.

Target Gene-Genomic DNA Sequence Blast and Alignment Analysis:

Target Gene 1 / OU11_RS07620

As a result of the analysis comparing the sequencing result obtained with 4 different primers for the target gene 1 with the genomic DNA sequence, the similarity percentage was determined as 100%.

Distribution of the top 1 Blast Hits on 1 subject sequences



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Query  661   TCGACTACGTGGCCTACCAGCAGTACGGCTCCACATCGACCCGCACCGCCGGCGCCGTCG  720
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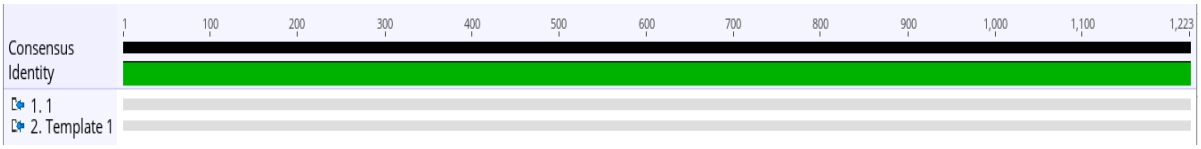
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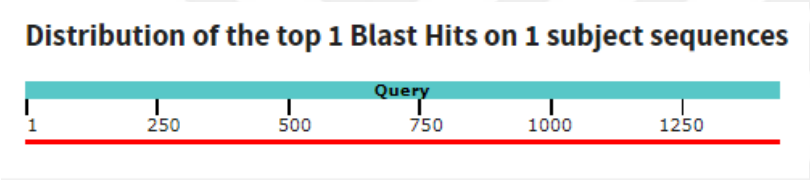
As a result of the alignment performed using Geneious Prime 2020.2 program for target gene 1 and genomic DNA sequence, 100% similarity was determined.

	1	Template 1
1		100%
Template 1	100%	



Target Gene 2 / BSAE_0444

As a result of the analysis comparing the sequencing result obtained with 4 different primers for the target gene 1 with the genomic DNA sequence, the similarity percentage was determined as 99.86%.



Score	Expect	Identities	Gaps	Strand
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Query  421  TCACCTCGTACAGGTTCGTGCCCTGCTCCACGGCCGCACGCGCGTCGTCCGCGCTCACGG  480
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Sbjct  450  TCACCTCGTACAGGTTCGTGCCCTGCTCCACGGCCGCACGCGCGTCGTCCGCGCTCACGG  509

Query  481  CGGCGTCACGCACGGTGGACTGGCGCAGCGACATGCGGTCGATGTAGTGGTTGGCCGCGG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  510  CGGCGTCACGCACGGTGGACTGGCGCAGCGACATGCGGTCGATGTAGTGGTTGGCCGCGG  569

Query  541  CCTTCGCCCGATCCAGCGGCATGCCCTGGGACTCGGCGATGGCGGTCTTGGTCCACAGCA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  570  CCTTCGCCCGATCCAGCGGCATGCCCTGGGACTCGGCGATGGCGGTCTTGGTCCACAGCA  629

Query  601  GGTTCGACGGGACGATGCGGGTCAGATCCTCCTCGTAGTTGCGGCCGTCGCGGTCGAGCG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  630  GGTTCGACGGGACGATGCGGGTCAGATCCTCCTCGTAGTTGCGGCCGTCGCGGTCGAGCG  689

Query  661  CGTACACGAACATGCCACCAAGCTTGTGCTCGTTGACGTACGAGGCGACCTGGTAGAAAT  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  690  CGTACACGAACATGCCACCAAGCTTGTGCTCGTTGACGTACGAGGCGACCTGGTAGAAAT  749

Query  721  TGCTCTCCTCGTACGGCTCCGAGGCGTCATACCAGCGGTTGTTCATGTCGCCTTCCTCCG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  750  TGCTCTCCTCGTACGGCTCCGAGGCGTCATACCAGCGGTTGTTCATGTCGCCTTCCTCC-  808
```

```
Query  781   GGGAGGGTCAGACCGGGCACAAACTCGCTGCCGATGTGCGGGGCGTAGTCGCCGAACGCG  840
             |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  809   GGGAAGGTCAGACCGGGCACAAACTCGCTGCCGATGTGCGGGGCGTAGTCGCCGAACGCG  868

Query  841   TTATCGGTGCGCTCGGCGTTCGAGCCGTACTGCTGGTAGGCCACGTAGTCGAAGCAGTCC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  869   TTATCGGTGCGCTCGGCGTTCGAGCCGTACTGCTGGTAGGCCACGTAGTCGAAGCAGTCC  928

Query  901   GCCACGTTCCAGAACGGCGCCAGATCGGACGCGTTGGTGTCATACAGGAACATCGTGCCG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  929   GCCACGTTCCAGAACGGCGCCAGATCGGACGCGTTGGTGTCATACAGGAACATCGTGCCG  988

Query  961   TCCGGGTTGTCGGACTTGGGGCCGATGTGCTTGGACAGGGCGCGAATCACGTTATCGGAG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  989   TCCGGGTTGTCGGACTTGGGGCCGATGTGCTTGGACAGGGCGCGAATCACGTTATCGGAG  1048

Query  1021  ACGGCGACGTCGGCCTCATCCGGATGCGTTTCCATATCGATATCCAGGCCGTCCAAGCCG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1049  ACGGCGACGTCGGCCTCATCCGGATGCGTTTCCATATCGATATCCAGGCCGTCCAAGCCG  1108

Query  1081  ACCGAGGTCATGTACTCGTCGATGACCTGCAAGGCGTACTCGTCGTAGTCGGCCTCGGTG  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1109  ACCGAGGTCATGTACTCGTCGATGACCTGCAAGGCGTACTCGTCGTAGTCGGCCTCGGTG  1168

Query  1141  GCCTCCTGCTCGGTCAACCCCTTGGCCTCGATGAAGTCGCGGAACCCGTTGACGATCACA  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1169  GCCTCCTGCTCGGTCAACCCCTTGGCCTCGATGAAGTCGCGGAACCCGTTGACGATCACA  1228

Query  1201  CCCGTGTAGTCCACACCGCGCACGAGCTTGATGCCGCGCGAATGCAGATACGGGGCGTAG  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1229  CCCGTGTAGTCCACACCGCGCACGAGCTTGATGCCGCGCGAATGCAGATACGGGGCGTAG  1288

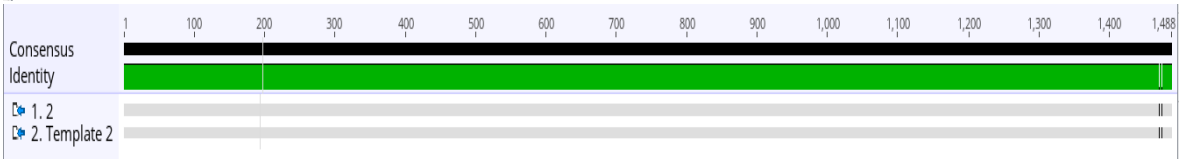
Query  1261  TCGGACTTCAGCTTGTCGTAATACGGCTGGGCCAGCTCCTCCTGACCCGACGGAACATAA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1289  TCGGACTTCAGCTTGTCGTAATACGGCTGGGCCAGCTCCTCCTGACCCGACGGAACATAA  1348

Query  1321  CTGAACACGTTGACCACATCCACGCCATACGGGATGTCATACATCGAAATCCAGTTCTCA  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1349  CTGAACACGTTGACCACATCCACGCCATACGGGATGTCATACATCGAAATCCAGTTCTCA  1408

Query  1381  TCCGGCAGATCCGTATTCACACCCTTCATCGTCACATCACGCCACGCGCG  1430
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1409  TCCGGCAGATCCGTATTCACACCCTTCATCGTCACATCACGCCACGCGCG  1458
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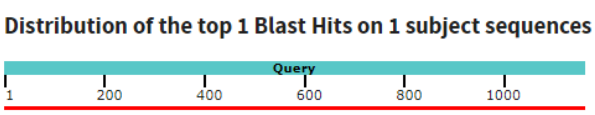
As a result of the alignment performed using Geneious Prime 2020.2 program for target gene 1 and genomic DNA sequence, 99.798% similarity was determined. A few nucleotide changes were detected at the end of the target gene sequence due to poor reading quality.

	2	Template 2
2		99.798%
Template 2	99.798%	



Target Gene 3 / BBKW_1881

As a result of the analysis comparing the sequencing result obtained with 4 different primers for the target gene 1 with the genomic DNA sequence, the similarity percentage was determined as 100%.



Score	Expect	Identities	Gaps	Strand
2124 bits(1150)	0.0	1150/1150(100%)	0/1150(0%)	Plus/Plus

```
Query  1     TCGCGCTCGTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56    TCGCGCTCGTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGA  115

Query  61    AATTGCCGGAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  116   AATTGCCGGAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGT  175

Query  121   TCGAATCCAAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  176   TCGAATCCAAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCG  235

Query  181   ACGGCTGCCAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  236   ACGGCTGCCAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTG  295

Query  241   ATCTTGACAGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  296   ATCTTGACAGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCG  355

Query  301   GCGGGCAGAACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  356   GCGGGCAGAACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACG  415

Query  361   CATACCAGCAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  416   CATACCAGCAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACA  475

Query  421   CGAATCTCGACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  476   CGAATCTCGACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCA  535

Query  481   TCGCCAATGAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  536   TCGCCAATGAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCA  595

Query  541   GCCTGACGCTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  596   GCCTGACGCTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATG  655

Query  601   CGTTTCTGGATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  656   CGTTTCTGGATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACG  715

Query  661   TGGCATCCACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  716   TGGCATCCACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACG  775

Query  721   CGCAATACAAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  776   CGCAATACAAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGG  835

Query  781   AACTGCTGGGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  836   AACTGCTGGGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGC  895

Query  841   TCGACAATGCGCGCGGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCA  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  896   TCGACAATGCGCGCGGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCA  955

Query  901   TGTGGTCGTTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  956   TGTGGTCGTTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCA  1015

Query  961   AAACGTTCTGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1016  AAACGTTCTGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAG  1075

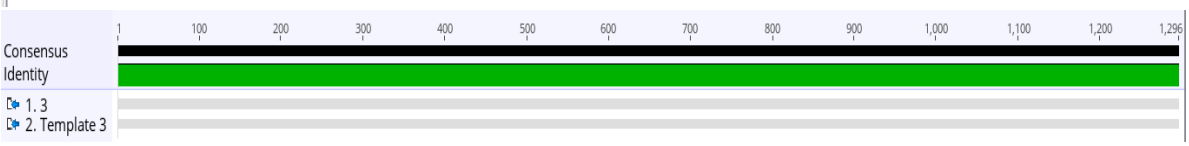
Query  1021  GATTCCGAGGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTC  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1076  GATTCCGAGGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTC  1135

Query  1081  AGCAGGCCTACCCCACATGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCT  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1136  AGCAGGCCTACCCCACATGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCT  1195

Query  1141  GGAACGGCAA  1150
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Sbjct  1196  GGAACGGCAA  1205
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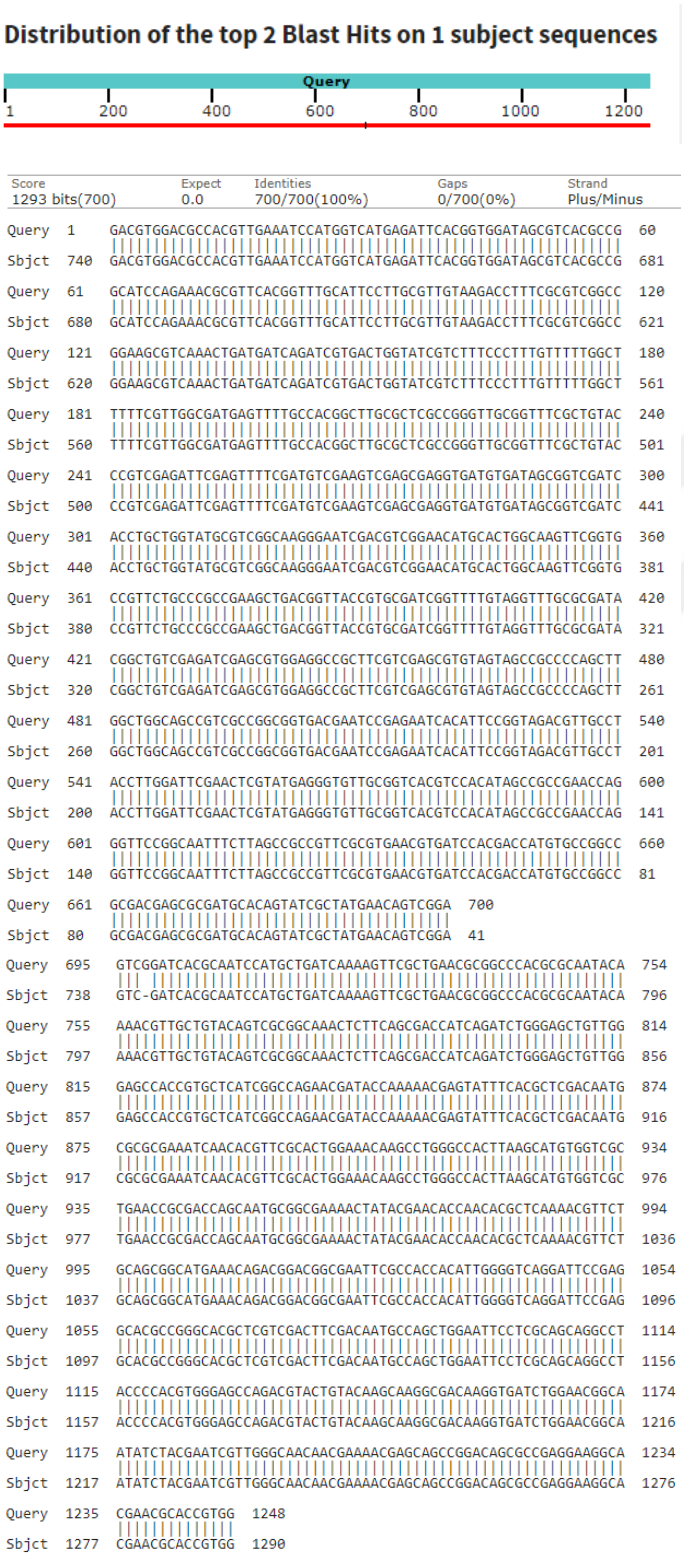
As a result of the alignment performed using Geneious Prime 2020.2 program for target gene 3 and genomic DNA sequence, 100% similarity was determined.

	3	Template 3
3		100%
Template 3	100%	



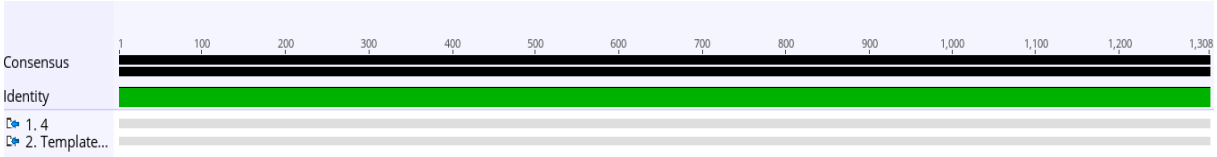
Target Gene 4 / BBPC_1684

As a result of the analysis comparing the sequencing result obtained with 4 different primers for the target gene 1 with the genomic DNA sequence, the similarity percentage was determined as 100%.



As a result of the alignment performed using Geneious Prime 2020.2 program for target gene 4 and genomic DNA sequence, 100% similarity was determined.

	4	Template 4
4		100%
Template 4	100%	



Target Gene 5 / BBOH_0438

As a result of the analysis comparing the sequencing result obtained with 5 different primers for the target gene 5 with the genomic DNA sequence, the similarity percentage was determined as 100%.



Score	Expect	Identities	Gaps	Strand
3005 bits(1627)	0.0	1627/1627(100%)	0/1627(0%)	Plus/Plus

```
Query  1    ATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGCAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    ATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGCAA  62

Query  61   CAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATTCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   CAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATTCT  122

Query  121  TCGAGGTCGAAGCAGTGGCAGGGTCATGAGCATTAAGCGATGTGCACAACGTCGTACCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  TCGAGGTCGAAGCAGTGGCAGGGTCATGAGCATTAAGCGATGTGCACAACGTCGTACCCG  182

Query  181  GTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGCAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  GTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGCAT  242

Query  241  CACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGCTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  CACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGCTG  302

Query  301  AATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGACAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  AATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGACAT  362

Query  361  TCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTCCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  TCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTCCA  422

Query  421  GTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCCCA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  GTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCCCA  482

Query  481  GCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGACT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  GCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGACT  542

Query  541  TCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGACT  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  543  TCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGACT  602

Query  601  TGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGAAG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  603  TGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGAAG  662

Query  661  GCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  663  GCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATACA  722

Query  721  GGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACTGC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  723  GGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACTGC  782

Query  781  TCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAACG  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  783  TCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAACG  842

Query  841  TCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGAAG  900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  843  TCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGAAG  902
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Query  901   CCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACTGA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  903   CCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACTGA  962

Query  961   CGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGTCATATAATAGCATTGTGCCATTGT  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  963   CGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGTCATATAATAGCATTGTGCCATTGT  1022

Query  1021  TCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCTTGATCT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1023  TCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCTTGATCT  1082

Query  1081  GCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACGCATCCA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1083  GCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACGCATCCA  1142

Query  1141  TCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTTCGGCAC  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1143  TCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTTCGGCAC  1202

Query  1201  TGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGACACCAC  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1203  TGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGACACCAC  1262

Query  1261  GGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGGCCTGAG  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1263  GGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGGCCTGAG  1322

Query  1321  CGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGGGGATGT  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1323  CGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGGGGATGT  1382

Query  1381  CATTCATCGTGATCCAGTTGTCATCGGTCAACGAGGTGTTCACTCCTTTCATGGTTTTGT  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1383  CATTCATCGTGATCCAGTTGTCATCGGTCAACGAGGTGTTCACTCCTTTCATGGTTTTGT  1442

Query  1441  CGCGCCAGGCACGGTAATATACCATGAAATGTCGCTTCTGGGTAGCAGTTTGCTGCTGCT  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1443  CGCGCCAGGCACGGTAATATACCATGAAATGTCGCTTCTGGGTAGCAGTTTGCTGCTGCT  1502

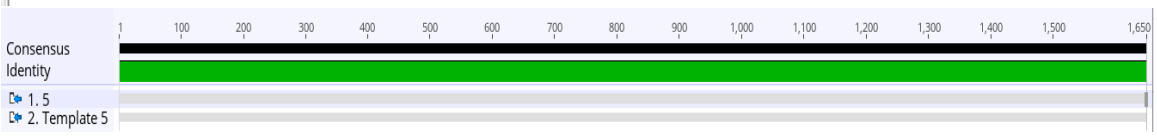
Query  1501  CGGACAAGGCGGATGGTTCCGACTGGCCAATGGCGCCAGCGGCCGGCGCAAACACCAGCA  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1503  CGGACAAGGCGGATGGTTCCGACTGGCCAATGGCGCCAGCGGCCGGCGCAAACACCAGCA  1562

Query  1561  TGGTCGCAGCGATCACGGCAATCGCCACAGCCGAACATAATGGTCGCAGTCTCTCGGAAA  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1563  TGGTCGCAGCGATCACGGCAATCGCCACAGCCGAACATAATGGTCGCAGTCTCTCGGAAA  1622

Query  1621  CGTCGAT  1627
             |||||||
Sbjct  1623  CGTCGAT  1629
```

As a result of the alignment performed using Geneious Prime 2020.2 program for target gene 4 and genomic DNA sequence, 100% similarity was determined.

	5	Template 5
5		100%
Template 5	100%	



4.6. Verification of Related Gene Sequences After Cloning

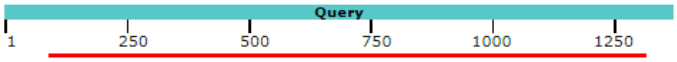
Some nucleotide changes were detected in the analysis of sequencing results of 14 related gene sequences (5 N-His, 5 SUMO, 4 C-His tagged) with Genomic DNA sequences. In particular, the similarity percentages of target gene 5 (BBOH_0438) were found to be less than the others due to low reading quality. Similarity percentages of analyzes performed using Geneious Prime 2020.2 program and NCBI Nucleotide Blast are reported as follows. These changes in percentages of similarity can reduce the activity of the produced enzymes, or block them altogether, even if they do not interfere with protein production. These changes will be considered after controlling the activities of the proteins. Since the C-his tagged BSAE_0444 enzyme was cloned after DNA sequencing, there is no sequence data for this cloning.

Related Gene-Genomic DNA Sequence Blast and Alignment Analysis:

N1 / OU11_RS07620

As a result of the analysis comparing the sequencing result obtained with 4 different primers for N-His tagged recombinant enzyme (OU11_RS07620) with the genomic DNA sequence, the similarity percentage was determined as 99.92%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2254 bits(1220)	0.0	1222/1223(99%)	0/1223(0%)	Plus/Plus

```
Query  90    GGCGCAAGCCATGTGTTTCCCCAGGGCGGAACGACGGTGAAACGTCGTATCGCCGCCATC  149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4     GGCGCAAGCCATGTGTTTCCCCAGGGCGGAACGACGGTGAAACGTCGTATCGCCGCCATC  63

Query  150   GCCGCGGGCATCGCCACCGCGGCGATGGCTCTCGGCGGCGTCGTCGCGCCGCCCGCCACG  209
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GCCGCGGGCATCGCCACCGCGGCGATGGCTCTCGGCGGCGTCGTCGCGCCGCCCGCCACG  123

Query  210   GCATATGCCGACGAGCCCGCAGCCGCGGACGTCAGCAGTGTCACGAACCCGAACAACCGT  269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GCATATGCCGACGAGCCCGCAGCCGCGGACGTCAGCAGTGTCACGAACCCGAACAACCGT  183

Query  270   CATTTCATGGTGTACTACCGTGCGTGGCGTGATGTGACGATGAAGGGTGTGAATACCGAT  329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   CATTTCATGGTGTACTACCGTGCGTGGCGTGATGTGACGATGAAGGGTGTGAATACCGAT  243

Query  330   CTGCCGGATGAGAACTGGATTTCGATGTACGACATCCCGTATGGCGTGGATGTGGTGAAC  389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   CTGCCGGATGAGAACTGGATTTCGATGTACGACATCCCGTATGGCGTGGATGTGGTGAAC  303

Query  390   GTGTTCAGTTATGTGCCGGCGGGTCAGGAGGCGGCGGCCCAGCCGTTCTATGACAAGCTC  449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   GTGTTCAGTTATGTGCCGGCGGGTCAGGAGGCGGCGGCCCAGCCGTTCTATGACAAGCTC  363

Query  450   AAGGCCGAGTATGCCCCGTATCTGCATTCGCGTGGCATCAAGCTCGTGCGCGGCATCAAC  509
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   AAGGCCGAGTATGCCCCGTATCTGCATTCGCGTGGCATCAAGCTCGTGCGCGGCATCAAC  423

Query  510   TACGAACAGGTCACCGTCGAAGGCTTCCGCGACTACATGGCCGATCTGGGCAAGACGGCC  569
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Sbjct  424   TACGAACAGGTCACCGTCGAAGGCTTCCGCGACTACATGGCCGATCTGGGCAAGACGGCC  483

Query  570   GATGAGGCCACCGAGGCCGACTATGATGCCTATGCGCTTGAGATCATCGACGAGTACATG  629
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   GATGAGGCCACCGAGGCCGACTATGATGCCTATGCGCTTGAGATCATCGACGAGTACATG  543

Query  630   ACCTCGGTCGGCCTGGACGGTCTGGACATCGATATGGAGACCTATCCGACCGACGCGGAT  689
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   ACCTCGGTCGGCCTGGACGGTCTGGACATCGATATGGAGACCTATCCGACCGACGCGGAT  603

Query  690   GTGGCGATTTCCGATAACGTGATCCGCGCCCTGTCCAAGCACATCGGCCCCAAGTCGGAC  749
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604   GTGGCGATTTCCGATAACGTGATCCGCGCCCTGTCCAAGCACATCGGCCCCAAGTCGGAC  663

Query  750   GATCCGGATGGCACGATGTTCCTGTACGACACCAACGGCAGCAACGCCAAGCCGTTCGCG  809
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   GATCCGGATGGCACGATGTTCCTGTACGACACCAACGGCAGCAACACCAAGCCGTTCGCG  723

Query  810   AACGTGGCGGACTGCTTCGACTACGTGGCCTACCAGCAGTACGGCTCCACATCGACCCGC  869
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   AACGTGGCGGACTGCTTCGACTACGTGGCCTACCAGCAGTACGGCTCCACATCGACCCGC  783

Query  870   ACCGCCGGCGCCGTCGCGGACTACTCGCCGTACATCGGCGCCGACAAGTTCGTGCCCGGC  929
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   ACCGCCGGCGCCGTCGCGGACTACTCGCCGTACATCGGCGCCGACAAGTTCGTGCCCGGC  843

Query  930   CTGGCCTTCCCCGAGGAAGGCGACCACAACCGTTGGTATGACGCCACGGAGCCGTATACG  989
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  844   CTGGCCTTCCCCGAGGAAGGCGACCACAACCGTTGGTATGACGCCACGGAGCCGTATACG  903

Query  990   GACAGCCACATCTACGACATCGCGTCGTATGTGCGTGACAACGGCCTCGGCGGCATGTTC  1049
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904   GACAGCCACATCTACGACATCGCGTCGTATGTGCGTGACAACGGCCTCGGCGGCATGTTC  963

Query  1050  CTGTACGCGCTTGACCGCGACGGCCGCACCTACGAGGCCGAGGACTGGAGCCACATCGTC  1109
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  964   CTGTACGCGCTTGACCGCGACGGCCGCACCTACGAGGCCGAGGACTGGAGCCACATCGTC  1023

Query  1110  CCGTCGAACCTGCTGTGGACCAAGACCGCCATCGCTGAGTCGCAGGACATGACGATGGAG  1169
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1024  CCGTCGAACCTGCTGTGGACCAAGACCGCCATCGCTGAGTCGCAGGACATGACGATGGAG  1083

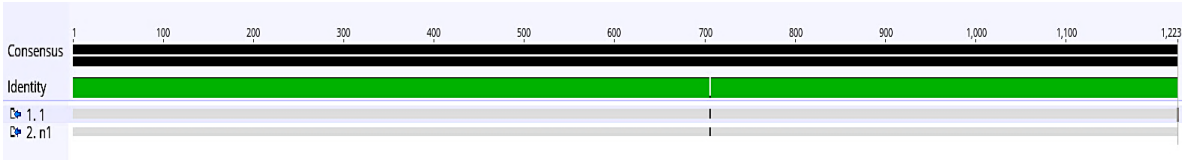
Query  1170  CAGGCCAAGGCCGCCGCGAACCACTACATCGAGCGCATGTCGCTGACCGAGGAAGGGGCT  1229
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Sbjct  1084  CAGGCCAAGGCCGCCGCGAACCACTACATCGAGCGCATGTCGCTGACCGAGGAAGGGGCT  1143

Query  1230  GCCGGCGTGGCCCTGAACGCCGAGGACGCGCTCGAAGCCGTGGAACAGGCCACGAACCTC  1289
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  GCCGGCGTGGCCCTGAACGCCGAGGACGCGCTCGAAGCCGTGGAACAGGCCACGAACCTC  1203

Query  1290  TACGAGGTCAACAAGGCCGTGCT  1312
             |||||||||||||||||||||||
Sbjct  1204  TACGAGGTCAACAAGGCCGTGCT  1226
```

As a result of the alignment performed using the Geneious Prime program for N1 and genomic DNA sequences, 99.918% similarity was found.

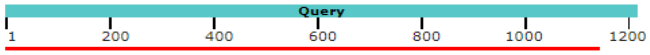
	1	n1
1		99.918%
n1	99.918%	



C1 / OU11_RS07620

As a result of the analysis comparing the sequencing result obtained with 4 different primers for C-His tagged recombinant enzyme (OU11_RS07620) with the genomic DNA sequence, the similarity percentage was determined as 99.74%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2078 bits(1125)	0.0	1131/1134(99%)	0/1134(0%)	Plus/Plus

```
Query  1     TCCCGGCGGCGTCGTCGCGCCGCCCGCCACGGCATATGCCGACGAGCCCGCAGCCGCGGG  60
             ||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93    TCTCGGCGGCGTCGTCGCGCCGCCCGCCACGGCATATGCCGACGAGCCCGCAGCCGCGGA  152

Query  61    CGTCAGCAGTGTCACGAACCCGAACAACCGTCATTTCATGGTGTACTACCGTGCGTGGCG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153   CGTCAGCAGTGTCACGAACCCGAACAACCGTCATTTCATGGTGTACTACCGTGCGTGGCG  212

Query  121   TGATGTGACGATGAAGGGTGTGAATACCGATCTGCCGGATGAGAACTGGATTTCGATGTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213   TGATGTGACGATGAAGGGTGTGAATACCGATCTGCCGGATGAGAACTGGATTTCGATGTA  272

Query  181   CGACATCCCGTATGGCGTGGATGTGGTGAACGTGTTCAGTTATGTGCCGGCGGGTCAGGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273   CGACATCCCGTATGGCGTGGATGTGGTGAACGTGTTCAGTTATGTGCCGGCGGGTCAGGA  332

Query  241   GGCGGCGGCCCAGCCGTTCTATGACAAGCTCAAGGCCGAGTATGCCCCGTATCTGCATTC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  333   GGCGGCGGCCCAGCCGTTCTATGACAAGCTCAAGGCCGAGTATGCCCCGTATCTGCATTC  392

Query  301   GCGTGGCATCAAGCTCGTGCGCGGCATCAACTACGAACAGGTCACCGTCGAAGGCTTCCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  393   GCGTGGCATCAAGCTCGTGCGCGGCATCAACTACGAACAGGTCACCGTCGAAGGCTTCCG  452

Query  361   CGACTACATGGCCGATCTGGGCAAGACGGCCGATGAGGCCACCGAGGCCGACTATGATGC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453   CGACTACATGGCCGATCTGGGCAAGACGGCCGATGAGGCCACCGAGGCCGACTATGATGC  512

Query  421   CTATGCGCTTGAGATCATCGACGAGTACATGACCTCGGTCGGCCTGGACGGTCTGGACAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513   CTATGCGCTTGAGATCATCGACGAGTACATGACCTCGGTCGGCCTGGACGGTCTGGACAT  572

Query  481   CGATATGGAGACCTATCCGACCGACGCGGATGTGGCGATTTCCGATAACGTGATCCGCGC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573   CGATATGGAGACCTATCCGACCGACGCGGATGTGGCGATTTCCGATAACGTGATCCGCGC  632

Query  541   CCTGTCCAAGCACATCGGCCCCAAGTCGGACGATCCGGATGGCACGATGTTCCTGTACGA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  633   CCTGTCCAAGCACATCGGCCCCAAGTCGGACGATCCGGATGGCACGATGTTCCTGTACGA  692

Query  601   CACCAACGGCAGCAACACCAAGCCGTTCGCGAACGTGGCGGACTGCTTCGACTACGTGGC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693   CACCAACGGCAGCAACACCAAGCCGTTCGCGAACGTGGCGGACTGCTTCGACTACGTGGC  752

Query  661   CTACCAGCAGTGCGGCTCCACATCGACCCGCACCGCCGGCGCCGTCGCGGACTACTCGCC  720
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753   CTACCAGCAGTACGGCTCCACATCGACCCGCACCGCCGGCGCCGTCGCGGACTACTCGCC  812

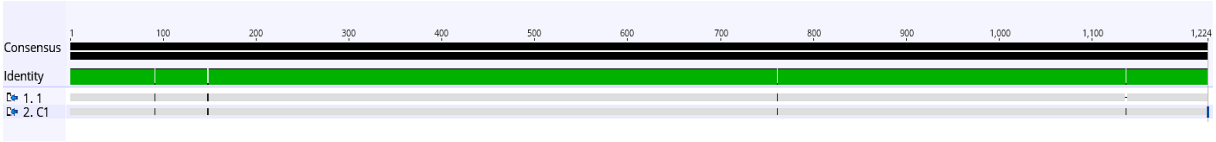
Query  721   GTACATCGGCGCCGACAAGTTCGTGCCCGGCCTGGCCTTCCCCGAGGAAGGCGACCACAA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  813   GTACATCGGCGCCGACAAGTTCGTGCCCGGCCTGGCCTTCCCCGAGGAAGGCGACCACAA  872

Query  781   CCGTTGGTATGACGCCACGGAGCCGTATACGGACAGCCACATCTACGACATCGCGTCGTA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  873   CCGTTGGTATGACGCCACGGAGCCGTATACGGACAGCCACATCTACGACATCGCGTCGTA  932

Query  841   TGTGCGTGACAACGGCCTCGGCGGCATGTTCCTGTACGCGCTTGACCGCGACGGCCGCAC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  933   TGTGCGTGACAACGGCCTCGGCGGCATGTTCCTGTACGCGCTTGACCGCGACGGCCGCAC  992
```

As a result of the alignment performed using the Geneious Prime program for C1 and genomic DNA sequences, 99.673% similarity was found.

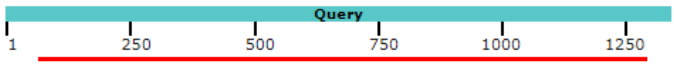
	1	C1
1		99.673%
C1	99.673%	



S1 / OU11_RS07620

As a result of the analysis comparing the sequencing result obtained with 4 different primers for SUMO tagged recombinant enzyme (OU11_RS07620) with the genomic DNA sequence, the similarity percentage was determined as 100%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2259 bits(1223)	0.0	1223/1223(100%)	0/1223(0%)	Plus/Plus

```
Query  65    GGCGCAAGCCATGTGTTTCCCCAGGGCGGAACGACGGTGAAACGTCGTATCGCCGCCATC  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4     GGCGCAAGCCATGTGTTTCCCCAGGGCGGAACGACGGTGAAACGTCGTATCGCCGCCATC  63

Query  125   GCCGCGGGCATCGCCACCGCGGCGATGGCTCTCGGCGGCGTCGTCGCGCCGCCCGCCACG  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GCCGCGGGCATCGCCACCGCGGCGATGGCTCTCGGCGGCGTCGTCGCGCCGCCCGCCACG  123

Query  185   GCATATGCCGACGAGCCCGCAGCCGCGGACGTCAGCAGTGTCACGAACCCGAACAACCGT  244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GCATATGCCGACGAGCCCGCAGCCGCGGACGTCAGCAGTGTCACGAACCCGAACAACCGT  183

Query  245   CATTTCATGGTGTACTACCGTGCGTGGCGTGATGTGACGATGAAGGGTGTGAATACCGAT  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   CATTTCATGGTGTACTACCGTGCGTGGCGTGATGTGACGATGAAGGGTGTGAATACCGAT  243

Query  305   CTGCCGGATGAGAACTGGATTTCGATGTACGACATCCCGTATGGCGTGGATGTGGTGAAC  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   CTGCCGGATGAGAACTGGATTTCGATGTACGACATCCCGTATGGCGTGGATGTGGTGAAC  303

Query  365   GTGTTCAGTTATGTGCCGGCGGGTCAGGAGGCGGCGGCCCAGCCGTTCTATGACAAGCTC  424
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   GTGTTCAGTTATGTGCCGGCGGGTCAGGAGGCGGCGGCCCAGCCGTTCTATGACAAGCTC  363

Query  425   AAGGCCGAGTATGCCCCGTATCTGCATTCGCGTGGCATCAAGCTCGTGCGCGGCATCAAC  484
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   AAGGCCGAGTATGCCCCGTATCTGCATTCGCGTGGCATCAAGCTCGTGCGCGGCATCAAC  423

Query  485   TACGAACAGGTCACCGTCGAAGGCTTCCGCGACTACATGGCCGATCTGGGCAAGACGGCC  544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424   TACGAACAGGTCACCGTCGAAGGCTTCCGCGACTACATGGCCGATCTGGGCAAGACGGCC  483

Query  545   GATGAGGCCACCGAGGCCGACTATGATGCCTATGCGCTTGAGATCATCGACGAGTACATG  604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   GATGAGGCCACCGAGGCCGACTATGATGCCTATGCGCTTGAGATCATCGACGAGTACATG  543

Query  605   ACCTCGGTCGGCCTGGACGGTCTGGACATCGATATGGAGACCTATCCGACCGACGCGGAT  664
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   ACCTCGGTCGGCCTGGACGGTCTGGACATCGATATGGAGACCTATCCGACCGACGCGGAT  603

Query  665   GTGGCGATTTCCGATAACGTGATCCGCGCCCTGTCCAAGCACATCGGCCCCAAGTCGGAC  724
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604   GTGGCGATTTCCGATAACGTGATCCGCGCCCTGTCCAAGCACATCGGCCCCAAGTCGGAC  663

Query  725   GATCCGGATGGCACGATGTTCCTGTACGACACCAACGGCAGCAACACCAAGCCGTTCGCG  784
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   GATCCGGATGGCACGATGTTCCTGTACGACACCAACGGCAGCAACACCAAGCCGTTCGCG  723

Query  785   AACGTGGCGGACTGCTTCGACTACGTGGCCTACCAGCAGTACGGCTCCACATCGACCCGC  844
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   AACGTGGCGGACTGCTTCGACTACGTGGCCTACCAGCAGTACGGCTCCACATCGACCCGC  783

Query  845   ACCGCCGGCGCCGTCGCGGACTACTCGCCGTACATCGGCGCCGACAAGTTCGTGCCCGGC  904
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   ACCGCCGGCGCCGTCGCGGACTACTCGCCGTACATCGGCGCCGACAAGTTCGTGCCCGGC  843

Query  905   CTGGCCTTCCCCGAGGAAGGCGACCACAACCGTTGGTATGACGCCACGGAGCCGTATACG  964
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  844   CTGGCCTTCCCCGAGGAAGGCGACCACAACCGTTGGTATGACGCCACGGAGCCGTATACG  903
```

```
Query  965   GACAGCCACATCTACGACATCGCGTCGTATGTGCGTGACAACGGCCTCGGCGGCATGTTC  1024
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904   GACAGCCACATCTACGACATCGCGTCGTATGTGCGTGACAACGGCCTCGGCGGCATGTTC  963

Query  1025  CTGTACGCGCTTGACCGCGACGGCCGCACCTACGAGGCCGAGGACTGGAGCCACATCGTC  1084
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  964   CTGTACGCGCTTGACCGCGACGGCCGCACCTACGAGGCCGAGGACTGGAGCCACATCGTC  1023

Query  1085  CCGTCGAACCTGCTGTGGACCAAGACCGCCATCGCTGAGTCGCAGGACATGACGATGGAG  1144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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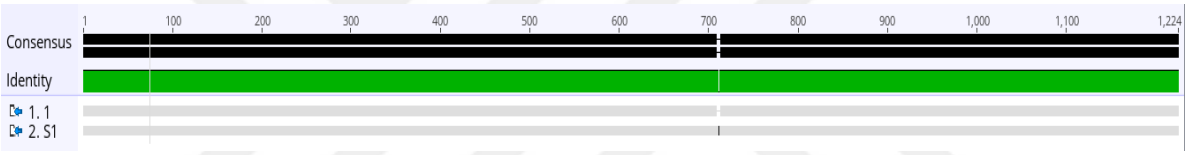
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084  CAGGCCAAGGCCGCCGCGAACCACTACATCGAGCGCATGTCGCTGACCGAGGAAGGGGCT  1143

Query  1205  GCCGGCGTGGCCCTGAACGCCGAGGACGCGCTCGAAGCCGTGGAACAGGCCACGAACCTC  1264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  GCCGGCGTGGCCCTGAACGCCGAGGACGCGCTCGAAGCCGTGGAACAGGCCACGAACCTC  1203

Query  1265  TACGAGGTCAACAAGGCCGTGCT  1287
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Sbjct  1204  TACGAGGTCAACAAGGCCGTGCT  1226
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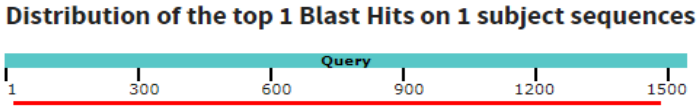
As a result of the alignment performed using the Geneious Prime program for S1 and genomic DNA sequences, 99.918% similarity was found.

	1	S1
1		99.918%
S1	99.918%	



N2 / BSAE_0444

As a result of the analysis comparing the sequencing result obtained with 4 different primers for N-His tagged recombinant enzyme (BSAE_0444) with the genomic DNA sequence, the similarity percentage was determined as 99.73%.



Score	Expect	Identities	Gaps	Strand
2691 bits(1457)	0.0	1466/1470(99%)	1/1470(0%)	Plus/Plus

```
Query  20    TCAGACCTTCCTCCGCTGCCAGACCGTCAGGCCGATGCCGGCGGCGGCGAACGCCACGAC  79
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TCAGACCTTCCTCCGCTGCCAGACCGTCAGGCCGATGCCGGCGGCGGCGAACGCCACGAC  60

Query  80    CAGGACAGTCATGACGGCGACCGCCGAACCCGTTGCGACGAGCGAATCGTCGGCGGGATT  139
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    CAGGACAGTCATGACGGCGACCGCCGAACCCGTTGCGACGAGCGAATCGTCGGCGGGATT  120

Query  140   CTGGGTTCCGGCATCGTCGTGTTTCCCGGAATCGCCTTCACCCGGGGCTTCGGTCTTGCC  199
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CTGGGTTCCGGCATCGTCGTGTTTCCCGGAATCGCCTTCACCCGGGGCTTCGGTCTTGCC  180

Query  200   TTCAAGGGCGGCCTTAATGCCGGCGGCCCAGGATTCGACCTGCTCGGCCGTGTAGGTCTT  259
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TTCAAGGGCGGCCTTAATGCCGGCGGCCCAGGATTCGACCTGCTCGGCCGTGTAGGTCTT  240

Query  260   GCCGGTCAGGCCATCGATCGCGGCGTCGCGGGCCGCGCGGACGGCGGCCTTGATATCGGC  319
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GCCGGTCAGGCCATCGATCGCGGCGTCGCGGGCCGCGCGGACGGCGGCCTTGATATCGGC  300

Query  320   GCTGGTGGCGTCGGCCTCCAGCACCGTGCCCGCCTCGGCGATCAGTTCCTCCAACTCGGC  379
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GCTGGTGGCGTCGGCCTCCAGCACCGTGCCCGCCTCGGCGATCAGTTCCTCCAACTCGGC  360

Query  380   GGTGTCGATGTCCAGCAACCCAGCCTCCAGGGTCGGATCGTAGGTGTTGCTGAACCCCTC  439
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  361   GGTGTCGATGTCCAGCAACCCAGCCTCCAGGGTCGGATCGTAGGTGTTGCTGAACCCTTC  420

Query  440   ACCATAGTCGCCGCCGAGCACGGCCTTGTTCACCTCGTACAGGTTCGTGCCCTGCTCCAC  499
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Sbjct  421   ACCATAGTCGCCGCCGAGCACGGCCTTGTTCACCTCGTACAGGTTCGTGCCCTGCTCCAC  480

Query  500   GGCCGCACGCGCGTCGTCCGCGCTCACGGCGGCGTCACGCACGGTGGACTGGCGCAGCGA  559
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GGCCGCACGCGCGTCGTCCGCGCTCACGGCGGCGTCACGCACGGTGGACTGGCGCAGCGA  540

Query  560   CATGCGGTCGATGTAGTGGTTGGCCGCGGCCTTCGCCCGATCCAGCGGCATGCCCTGGGA  619
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   CATGCGGTCGATGTAGTGGTTGGCCGCGGCCTTCGCCCGATCCAGCGGCATGCCCTGGGA  600

Query  620   CTCGGCGATGGCGGTCTTGGTCCACAGCAGGTTCGACGGGACGATGCGGGTCAGATCCTC  679
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   CTCGGCGATGGCGGTCTTGGTCCACAGCAGGTTCGACGGGACGATGCGGGTCAGATCCTC  660

Query  680   CTCGTAGTTGCGGCCGTCGCGGTCGAGCGCGTACACGAACATGCCACCAAGCTTGTGCTC  739
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   CTCGTAGTTGCGGCCGTCGCGGTCGAGCGCGTACACGAACATGCCACCAAGCTTGTGCTC  720

Query  740   GTTGACGTACGAGGCGACCTGGTAGAAATTGCTCTCCTCGTACGGCTCCGAGGCGTCATA  799
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   GTTGACGTACGAGGCGACCTGGTAGAAATTGCTCTCCTCGTACGGCTCCGAGGCGTCATA  780

Query  800   CCAGCGGTTGTTCATGTCGCCTTCCTCCGGGAAGGTCAGACCGGGCACAAACTCGCTGCC  859
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   CCAGCGGTTGTTCATGTCGCCTTCCTCCGGGAAGGTCAGACCGGGCACAAACTCGCTGCC  840

Query  860   GATGTGCGGGGCGTAGTCGCCGAACGCGTTATCGGTGCGCTCGGCGTTCGAGCCGTACTG  919
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   GATGTGCGGGGCGTAGTCGCCGAACGCGTTATCGGTGCGCTCGGCGTTCGAGCCGTACTG  900

Query  920   CTGGTAGGCCACGTAGTCGAAGCAGTCCGCCACGTTCCAGAACGGCGCCAGATCGGACGC  979
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   CTGGTAGGCCACGTAGTCGAAGCAGTCCGCCACGTTCCAGAACGGCGCCAGATCGGACGC  960

Query  980   GTTGGTGTCATACAGGAACATCGTGCCGTCCGGGTTGTCGGACTTGGGGCCGATGTGCTT  1039
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   GTTGGTGTCATACAGGAACATCGTGCCGTCCGGGTTGTCGGACTTGGGGCCGATGTGCTT  1020

Query  1040  GGACAGGGCGCGAATCACGTTATCGGAGACGGCGACGTCGGCCTCATCCGGATGCGTTTC  1099
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  GGACAGGGCGCGAATCACGTTATCGGAGACGGCGACGTCGGCCTCATCCGGATGCGTTTC  1080

Query  1100  CATATCGATATCCAGGCCGTCCAAGCCGACCGAGGTCATGTACTCGTCGATGACCTGCAA  1159
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  CATATCGATATCCAGGCCGTCCAAGCCGACCGAGGTCATGTACTCGTCGATGACCTGCAA  1140

Query  1160  GGCGTACTCGTCGTAGTCGGCCTCGGTGGCCTCCTGCTCGGTCAACCCCTTGGCCTCGAT  1219
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  GGCGTACTCGTCGTAGTCGGCCTCGGTGGCCTCCTGCTCGGTCAACCCCTTGGCCTCGAT  1200

Query  1220  GAAGTCGCGGAACCCGTTGACGATCACACCCGTGTAGTCCACACCGCGCACGAGCTTGAT  1279
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  GAAGTCGCGGAACCCGTTGACGATCACACCCGTGTAGTCCACACCGCGCACGAGCTTGAT  1260

Query  1280  GCCGCGCGAATGCAGATACGGGGCGTAGTCGGACTTCGGCTTGTCGTAATACGGCTGGGC  1339
             |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  1261  GCCGCGCGAATGCAGATACGGGGCGTAGTCGGACTTCAGCTTGTCGTAATACGGCTGGGC  1320

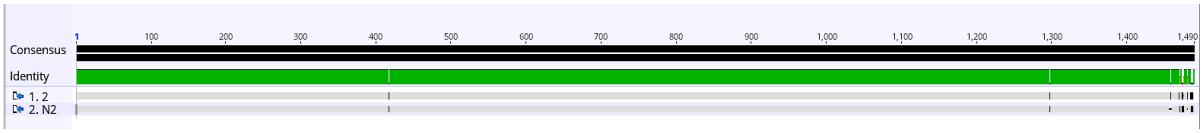
Query  1340  CAGCTCCTCCTGACCCGACGGAACATAACTGAACACGTTGACCACATCCTCGCCATACGG  1399
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1321  CAGCTCCTCCTGACCCGACGGAACATAACTGAACACGTTGACCACATCCACGCCATACGG  1380

Query  1400  GATGTCATACATCGAAATCCAGTTCTCATCCGGCAGATCCGTATTCACACCCTTCATCGT  1459
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1381  GATGTCATACATCGAAATCCAGTTCTCATCCGGCAGATCCGTATTCACACCCTTCATCGT  1440

Query  1460  CACATCACGCCACGCGCG-TAGTACACCAT  1488
             ||||||||||||||||| |||||||||||
Sbjct  1441  CACATCACGCCACGCGCGATAGTACACCAT  1470
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As a result of the alignment performed using the Geneious Prime program for N2 and genomic DNA sequences, 99.261% similarity was found.

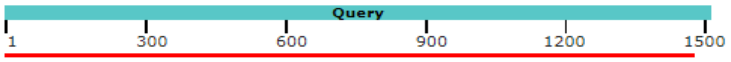
	2	N2
2		99.261%
N2	99.261%	



S2 / BSAE_0444

As a result of the analysis comparing the sequencing result obtained with 4 different primers for SUMO tagged recombinant enzyme (BSAE_0444) with the genomic DNA sequence, the similarity percentage was determined as 99.73%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2686 bits(1454)	0.0	1463/1467(99%)	2/1467(0%)	Plus/Plus

```
Query  1     GACCTTCCTCCGCTGCCAGACCGTCAGGCCGATGCCGGCGGCGGCGAACGCCACGACCAG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4     GACCTTCCTCCGCTGCCAGACCGTCAGGCCGATGCCGGCGGCGGCGAACGCCACGACCAG  63

Query  61    GACAGTCATGACGGCGACCGCCGAACCCGTTGCGACGAGCGAATCGTCGGCGGGATTCTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GACAGTCATGACGGCGACCGCCGAACCCGTTGCGACGAGCGAATCGTCGGCGGGATTCTG  123

Query  121   GGTTCCGGCATCGTCGTGTTTCCCGGAATCGCCTTCACCCGGGGCTTCGGTCTTGCCTTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GGTTCCGGCATCGTCGTGTTTCCCGGAATCGCCTTCACCCGGGGCTTCGGTCTTGCCTTC  183

Query  181   AAGGGCGGCCTTAATGCCGGCGGCCCAGGATTCGACCTGCTCGGCCGTGTAGGTCTTGCC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   AAGGGCGGCCTTAATGCCGGCGGCCCAGGATTCGACCTGCTCGGCCGTGTAGGTCTTGCC  243

Query  241   GGTCAGGCCATCGATCGCGGCGTCGCGGGCCGCGCGGACGGCGGCCTTGATATCGGCGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   GGTCAGGCCATCGATCGCGGCGTCGCGGGCCGCGCGGACGGCGGCCTTGATATCGGCGCT  303

Query  301   GGTGGCGTCGGCCTCCAGCACCGTGCCCGCCTCGGCGATCAGTTCCTCCAACTCGGCGGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   GGTGGCGTCGGCCTCCAGCACCGTGCCCGCCTCGGCGATCAGTTCCTCCAACTCGGCGGT  363

Query  361   GTCGATGTCCAGCAACCCAGCCTCCAGGGTCGGATCGTAGGTGTTGCTGAACCCTTCACC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   GTCGATGTCCAGCAACCCAGCCTCCAGGGTCGGATCGTAGGTGTTGCTGAACCCTTCACC  423

Query  421   ATAGTCGCCGCCGAGCACGGCCTTGTTCACCTCGTACAGGTTCGTGCCCTGCTCCACGGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424   ATAGTCGCCGCCGAGCACGGCCTTGTTCACCTCGTACAGGTTCGTGCCCTGCTCCACGGC  483

Query  481   CGCACGCGCGTCGTCCGCGCTCACGGCGGCGTCACGCACGGTGGACTGGCGCAGCGACAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   CGCACGCGCGTCGTCCGCGCTCACGGCGGCGTCACGCACGGTGGACTGGCGCAGCGACAT  543

Query  541   GCGGTCGATGTAGTGGTTGGCCGCGGCCTTCGCCCGATCCAGCGGCATGCCCTGGGACTC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   GCGGTCGATGTAGTGGTTGGCCGCGGCCTTCGCCCGATCCAGCGGCATGCCCTGGGACTC  603

Query  601   GGCGATGGCGGTCTTGGTCCACAGCAGGTTCGACGGGACGATGCGGGTCAAATCCTCCTC  660
             |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  604   GGCGATGGCGGTCTTGGTCCACAGCAGGTTCGACGGGACGATGCGGGTCAGATCCTCCTC  663

Query  661   GTAGTTGCGGCCGTCGCGGTCGAGCGCGTACACGAACATGCCACCAAGCTTGTGCTCGTT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   GTAGTTGCGGCCGTCGCGGTCGAGCGCGTACACGAACATGCCACCAAGCTTGTGCTCGTT  723

Query  721   GACGTACGAGGCGACCTGGTAGAAATTGCTCTCCTCGTACGGCTCCGAGGCGTCATACCA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   GACGTACGAGGCGACCTGGTAGAAATTGCTCTCCTCGTACGGCTCCGAGGCGTCATACCA  783

Query  781   GCGGTTGTTCATGTCGCCTTCCTCCGGGAAGGTCAGACCGGGCACAAACTCGCTGCCGAT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   GCGGTTGTTCATGTCGCCTTCCTCCGGGAAGGTCAGACCGGGCACAAACTCGCTGCCGAT  843

Query  841   GTGCGGGGCGTAGTCGCCGAACGCG-TATCGGTGCGCTCGGCGTTCGAGCCGTACTGCTG  899
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  844   GTGCGGGGCGTAGTCGCCGAACGCGTTATCGGTGCGCTCGGCGTTCGAGCCGTACTGCTG  903

Query  900   GTAGGCCACGTAGTCGAAGCAGTCCGCCACGTTCCAGAACGGCGCCAGATCGGACGCGTT  959
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904   GTAGGCCACGTAGTCGAAGCAGTCCGCCACGTTCCAGAACGGCGCCAGATCGGACGCGTT  963

Query  960   GGTGTCATACAGGAACATCGTGCCGTCCGGGTTGTCGGACTTGGGGCCGATGTGCTTGGA  1019
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  964   GGTGTCATACAGGAACATCGTGCCGTCCGGGTTGTCGGACTTGGGGCCGATGTGCTTGGA  1023

Query  1020  CAGGGCGCGAATCACGTTATCGGAGACGGCGACGTCGGCCTCATCCGGATGCGTTTCCAT  1079
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1024  CAGGGCGCGAATCACGTTATCGGAGACGGCGACGTCGGCCTCATCCGGATGCGTTTCCAT  1083

Query  1080  ATCGATATCCAGGCCGTCCAAGCCGACCGAGGTCATGTACTCGTCGATGACCTGCAAGGC  1139
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084  ATCGATATCCAGGCCGTCCAAGCCGACCGAGGTCATGTACTCGTCGATGACCTGCAAGGC  1143

Query  1140  GTACTCGTCGTAGTCGGCCTCGGTGGCCTCCTGCTCGGTCAACCCCTTGGCCTCGATGAA  1199
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  GTACTCGTCGTAGTCGGCCTCGGTGGCCTCCTGCTCGGTCAACCCCTTGGCCTCGATGAA  1203

Query  1200  GTCGCGGAACCCGTTGACGATCACACCCGTGTAGTCCACACCGCGCACGGGCTTGATGCC  1259
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1204  GTCGCGGAACCCGTTGACGATCACACCCGTGTAGTCCACACCGCGCACGAGCTTGATGCC  1263

Query  1260  GCGCGAATGCAGATACGGGGCGTAGTCGGACTTCAGCTTGTCGTAATACGGCTGGGCCAG  1319
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1264  GCGCGAATGCAGATACGGGGCGTAGTCGGACTTCAGCTTGTCGTAATACGGCTGGGCCAG  1323

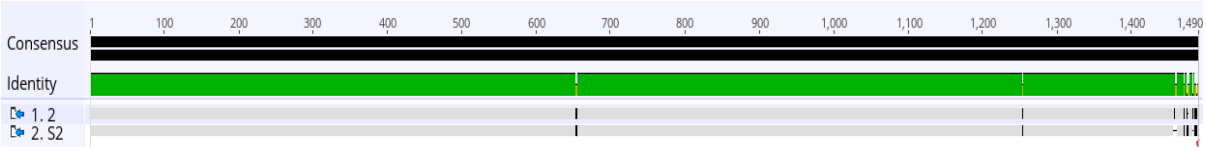
Query  1320  CTCCTCCTGACCCGACGGAACATAACTGAACACGTTGACCACATCCACGCCATACGGGAT  1379
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Sbjct  1324  CTCCTCCTGACCCGACGGAACATAACTGAACACGTTGACCACATCCACGCCATACGGGAT  1383

Query  1380  GTCATACATCGAAATCCAGTTCTCATCCGGCAGATCCGTATTCACACCCTTCATCGTCAC  1439
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1384  GTCATACATCGAAATCCAGTTCTCATCCGGCAGATCCGTATTCACACCCTTCATCGTCAC  1443

Query  1440  ATCACGCCACGCGCG-TAGTACACCAT  1465
             ||||||||||||||| ||||||||||
Sbjct  1444  ATCACGCCACGCGCGATAGTACACCAT  1470
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As a result of the alignment performed using the Geneious Prime program for S2 and genomic DNA sequences, 99.261% similarity was found.

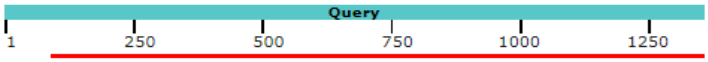
	2	S2
2		99.261%
S2	99.261%	



N3 / BBKW_1881

As a result of the analysis comparing the sequencing result obtained with 4 different primers for N-His tagged recombinant enzyme (BBKW_1881) with the genomic DNA sequence, the similarity percentage was determined as 99.84%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2331 bits(1262)	0.0	1266/1268(99%)	0/1268(0%)	Plus/Plus

```
Query  90    TTTCCAGGAAGGCGTTTTTCCTTCCTCCGATTGTTCATAGCAATACTATGCATCGCGCTC  149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4     TTTCCAGGAAGGCGTTTTTCCTTCCTCCGATTGTTCATAGCAATACTATGCATCGCGCTC  63

Query  150   GTCGCGGCCGGCGCATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCG  209
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCG  123

Query  210   GAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGTTCGAATCC  269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGTTCGAATCC  183

Query  270   AAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCGACGGCTGC  329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   AAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCGACGGCTGC  243

Query  330   CAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTGATCTTGAC  389
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Sbjct  244   CAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTGATCTTGAC  303

Query  390   AGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCGGCGGGCAG  449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   AGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCGGCGGGCAG  363

Query  450   AACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACGCATACCAG  509
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   AACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACGCATACCAG  423

Query  510   CAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACACGAATCTC  569
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Sbjct  424   CAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACACGAATCTC  483

Query  570   GACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCATCGCCAAT  629
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Sbjct  484   GACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCATCGCCAAT  543

Query  630   GAAAAAGCGTAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCAGCCTGACG  689
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Sbjct  544   GAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCAGCCTGACG  603

Query  690   CTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATGCGTTTCTG  749
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Sbjct  604   CTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATGCGTTTCTG  663

Query  750   GATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCATCC  809
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   GATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCATCC  723

Query  810   ACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACGCGCAATAC  869
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   ACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACGCGCAATAC  783

Query  870   AAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGGAACTGCTG  929
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   AAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGGAACTGCTG  843
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Query  930   GGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGCTCGACAAT  989
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  844   GGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGCTCGACAAT  903

Query  990   GCGCGCGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCATGTGGTCG  1049
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904   GCGCGCGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCATGTGGTCG  963

Query  1050  TTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCAAAACGTTC  1109
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  964   TTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCAAAACGTTC  1023

Query  1110  TGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAGGATTCCGA  1169
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1024  TGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAGGATTCCGA  1083

Query  1170  GGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTCAGCAGGCC  1229
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Sbjct  1084  GGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTCAGCAGGCC  1143

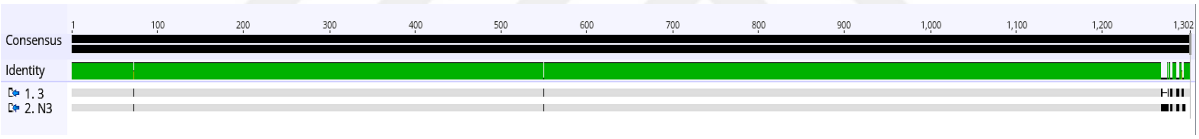
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  TACCCCACATGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACGGC  1203

Query  1290  AATATCTACGAATCGCTGGGCAACAACGAAAACGAGCAGCCAGACAGCGCCGAGGAAGGC  1349
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1204  AATATCTACGAATCGCTGGGCAACAACGAAAACGAGCAGCCAGACAGCGCCGAGGAAGGC  1263

Query  1350  ACCAACGC  1357
             ||||||||
Sbjct  1264  ACCAACGC  1271
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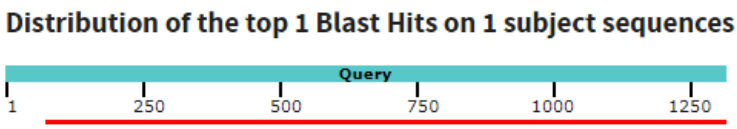
As a result of the alignment performed using the Geneious Prime program for N3 and genomic DNA sequences, 98.534% similarity was found.

	3	N3
3		98.534%
N3	98.534%	



C3 / BBKW_1881

As a result of the analysis comparing the sequencing result obtained with 4 different primers for C-His tagged recombinant enzyme (BBKW_1881) with the genomic DNA sequence, the similarity percentage was determined as 99.92%.



Score	Expect	Identities	Gaps	Strand
2270 bits(1229)	0.0	1231/1232(99%)	0/1232(0%)	Plus/Plus

```
Query  75    TGTTTCCAGGAAGGCGTTTTTCCTTCCTCCGATTGTTCATAGCAATACTATGCATCGCGC  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2     TGTTTCCAGGAAGGCGTTTTTCCTTCCTCCGATTGTTCATAGCAATACTATGCATCGCGC  61

Query  135   TCGTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGC  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62    TCGTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGC  121

Query  195   CGGAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGTTCGAAT  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122   CGGAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGTTCGAAT  181

Query  255   CCAAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCGACGGCT  314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182   CCAAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCGACGGCT  241

Query  315   GCCAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTGATCTTG  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242   GCCAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTGATCTTG  301

Query  375   ACAGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCGGCGGGC  434
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302   ACAGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCGGCGGGC  361

Query  435   AGAACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACGCATACC  494
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362   AGAACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACGCATACC  421

Query  495   AGCAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACACGAATC  554
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422   AGCAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACACGAATC  481

Query  555   TCGACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCATCGCCA  614
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482   TCGACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCATCGCCA  541

Query  615   ATGAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCAGCCTGA  674
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542   ATGAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCAGCCTGA  601

Query  675   CGCTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATGCGTTTC  734
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602   CGCTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATGCGTTTC  661

Query  735   TGGATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCAT  794
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662   TGGATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCAT  721

Query  795   CCACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACGCGCAAT  854
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722   CCACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACGCGCAAT  781

Query  855   ACAAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGGAACTGC  914
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782   ACAAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGGAACTGC  841

Query  915   TGGGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGCTCGACA  974
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   TGGGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGCTCGACA  901

Query  975   ATGCGCGCGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCATGTGGT  1034
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   ATGCGCGCGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCATGTGGT  961

Query  1035  CGTTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCAAAACGT  1094
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  962   CGTTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCAAAACGT  1021

Query  1095  TCTGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAGGATTCC  1154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1022  TCTGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAGGATTCC  1081

Query  1155  GAGGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCACAGCAGG  1214
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  1082  GAGGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTCAGCAGG  1141

Query  1215  CCTACCCCACATGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACG  1274
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1142  CCTACCCCACATGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACG  1201

Query  1275  GCAATATCTACGAATCGCTGGGCAACAACGAA  1306
             ||||||||||||||||||||||||||||||||
Sbjct  1202  GCAATATCTACGAATCGCTGGGCAACAACGAA  1233
```

As a result of the alignment performed using the Geneious Prime program for C3 and genomic DNA sequences, 99.462% similarity was found.

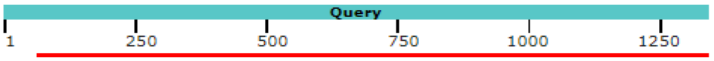
	3	C3
3		99.462%
C3	99.462%	



S3 / BBKW_1881

As a result of the analysis comparing the sequencing result obtained with 4 different primers for SUMO tagged recombinant enzyme (BBKW_1881) with the genomic DNA sequence, the similarity percentage was determined as 99.92%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2344 bits(1269)	0.0	1271/1272(99%)	0/1272(0%)	Plus/Plus

```
Query  65    TTTCCAGGAAGGCGTTTTTCCTTCCTCCGATTGTTCATAGCAATACTATGCATCGCGCTC  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4     TTTCCAGGAAGGCGTTTTTCCTTCCTCCGATTGTTCATAGCAATACTATGCATCGCGCTC  63

Query  125   GTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCG  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCG  123

Query  185   GAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGTTCGAATCC  244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GAGCCCTGGTTCGGCGGCTATGTGGACGTGACCACAACCCCCTCATACGAGTTCGAATCC  183

Query  245   AAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCGACGGCTGC  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   AAGGTGGGCAATGTCTACCGGAATGTGATTCTCGGATTCGTCACTGCCGGCGACGGCTGC  243

Query  305   CAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTGATCTTGAC  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   CAGCCAAGCTGGGGCGGCTATTACACGCTTGATGAAGCGGCCTCCACACTTGATCTTGAC  303

Query  365   AGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCGGCGGGCAG  424
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   AGTCGCATCGCGCAAACCTACAAAACCGACCGCACGGTAACCGTCAGCTTCGGCGGGCAG  363

Query  425   AACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACGCATACCAG  484
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   AACGGCACCGAACTGGCCGCCGCATGCACCGACGTCGACGCACTTGCCAACGCATACCAG  423

Query  485   CAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACACGAATCTC  544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424   CAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGATATCGAAAACACGAATCTC  483

Query  545   GACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCATCGCCAAT  604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   GACGGCTACAGTGAAACCGCAACCCGGCGAGCGCAAGCCGTGGTAAAACTCATCGCCAAT  543

Query  605   GAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCAGCCTGACG  664
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   GAAAAAGCGAAAAACAAAGGAAAAGACGACACCAGTCACGATCTGATCATCAGCCTGACG  603

Query  665   CTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATGCGTTTCTG  724
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604   CTTCCAGCCGACGCGAAAGGCCTTACAGCGCAAGGAATGCAAACCGTGAATGCGTTTCTG  663

Query  725   GATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCATCC  784
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   GATGCTGGCGTGACACTCTCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCATCC  723

Query  785   ACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACGCGCAATAC  844
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   ACGTCGATCACACAATCCACCCTGATTAAAAGCTCATTGAACGCGGCCCACGCGCAATAC  783

Query  845   AAAACCTTACTGCACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGGAACTGCTG  904
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   AAAACCTTACTGTACAGTCGCGGCAGACTCTTCAGCGACCATCAGATCTGGGAACTGCTG  843

Query  905   GGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGCTCGACAAT  964
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  844   GGAGCCACCGTGCTCATCGGCCAGAATGACACCAAAAACGAGTATTTCACGCTCGACAAT  903

Query  965   GCGCGCGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCATGTGGTCG  1024
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904   GCGCGCGAGATCAACACGTTCGCGCTGGAAACAAGCCTGGGACACTTAAGCATGTGGTCG  963

Query  1025  TTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCAAAACGTTC  1084
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  964   TTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACAAACACGCTCAAAACGTTC  1023

Query  1085  TGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAGGATTCCGA  1144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1024  TGCAGCGGCAGGAAACAGACGGACGGCGAATTCGCCACCACTCTGGGATCAGGATTCCGA  1083

Query  1145  GGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTCAGCAGGCC  1204
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084  GGCACACCGGGCACGCTCGTCGATTTCGACAACACAAGCTGGAATTCCTCTCAGCAGGCC  1143

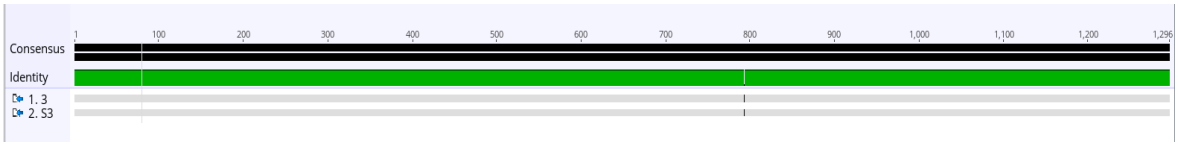
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  TACCCCACATGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACGGC  1203

Query  1265  AATATCTACGAATCGCTGGGCAACAACGAAAACGAGCAGCCAGACAGCGCCGAGGAAGGC  1324
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1204  AATATCTACGAATCGCTGGGCAACAACGAAAACGAGCAGCCAGACAGCGCCGAGGAAGGC  1263

Query  1325  ACCAACGCACCG  1336
             ||||||||||||
Sbjct  1264  ACCAACGCACCG  1275
```

As a result of the alignment performed using the Geneious Prime program for S3 and genomic DNA sequences, 99.923% similarity was found.

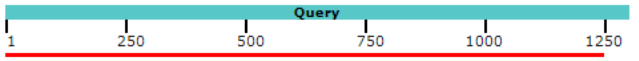
	3	S3
3		99.923%
S3	99.923%	



N4 / BBPC_1683

As a result of the analysis comparing the sequencing result obtained with 4 different primers for N-His tagged recombinant enzyme (BBPC_1683) with the genomic DNA sequence, the similarity percentage was determined as 100%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2287 bits(1238)	0.0	1238/1238(100%)	0/1238(0%)	Plus/Plus

```
Query  1    TCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   TCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCGG  136

Query  61   AACCCTGGTTCGGCGGCTATGTGGACGTGACCGCAACACCCTCATACGAGTTCGAATCCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  AACCCTGGTTCGGCGGCTATGTGGACGTGACCGCAACACCCTCATACGAGTTCGAATCCA  196

Query  121  AGGTAGGCAACGTCTACCGGAATGTGATTCTCGGATTCGTCACCGCCGGCGACGGCTGCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  AGGTAGGCAACGTCTACCGGAATGTGATTCTCGGATTCGTCACCGCCGGCGACGGCTGCC  256

Query  181  AGCCAAGCTGGGGCGGCTACTACACGCTCGACGAAGCGGCCTCCACGCTCGATCTCGACA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  AGCCAAGCTGGGGCGGCTACTACACGCTCGACGAAGCGGCCTCCACGCTCGATCTCGACA  316

Query  241  GCCGTATCGCGCAAACCTACAAAACCGATCGCACGGTAACCGTCAGCTTCGGCGGGCAGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  GCCGTATCGCGCAAACCTACAAAACCGATCGCACGGTAACCGTCAGCTTCGGCGGGCAGA  376

Query  301  ACGGCACCGAACTTGCCAGTGCATGTTCCGACGTCGATTCCCTTGCCGACGCATACCAGC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  ACGGCACCGAACTTGCCAGTGCATGTTCCGACGTCGATTCCCTTGCCGACGCATACCAGC  436

Query  361  AGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGACATCGAAAACTCGAATCTCG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  AGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGACATCGAAAACTCGAATCTCG  496

Query  421  ACGGGTACAGCGAAACCGCAACCCGGCGAGCGCAAGCCGTGGCAAAACTCATCGCCAACG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  497  ACGGGTACAGCGAAACCGCAACCCGGCGAGCGCAAGCCGTGGCAAAACTCATCGCCAACG  556

Query  481  AAAAAGCCAAAAACAAAGGGAAAGACGATACCAGTCACGATCTGATCATCAGTTTGACGC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  557  AAAAAGCCAAAAACAAAGGGAAAGACGATACCAGTCACGATCTGATCATCAGTTTGACGC  616

Query  541  TTCCGGCCGACGCGAAAGGTCTTACAACGCAAGGAATGCAAACCGTGAACGCGTTTCTGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  617  TTCCGGCCGACGCGAAAGGTCTTACAACGCAAGGAATGCAAACCGTGAACGCGTTTCTGG  676

Query  601  ATGCCGGCGTGACGCTATCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCGTCCA  660
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Sbjct  677  ATGCCGGCGTGACGCTATCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCGTCCA  736

Query  661  CGTCGATCACGCAATCCATGCTGATCAAAAGTTCGCTGAACGCGGCCCACGCGCAATACA  720
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Sbjct  737  CGTCGATCACGCAATCCATGCTGATCAAAAGTTCGCTGAACGCGGCCCACGCGCAATACA  796

Query  721  AAACGTTGCTGTACAGTCGCGGCAAACTCTTCAGCGACCATCAGATCTGGGAGCTGTTGG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  797  AAACGTTGCTGTACAGTCGCGGCAAACTCTTCAGCGACCATCAGATCTGGGAGCTGTTGG  856

Query  781  GAGCCACCGTGCTCATCGGCCAGAACGATACCAAAAACGAGTATTTCACGCTCGACAATG  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  857  GAGCCACCGTGCTCATCGGCCAGAACGATACCAAAAACGAGTATTTCACGCTCGACAATG  916
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Query  841   CGCGCGAAATCAACACGTTCGCACTGGAAACAAGCCTGGGCCACTTAAGCATGTGGTCGC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  917   CGCGCGAAATCAACACGTTCGCACTGGAAACAAGCCTGGGCCACTTAAGCATGTGGTCGC  976

Query  901   TGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACCAACACGCTCAAAACGTTCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  977   TGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACCAACACGCTCAAAACGTTCT  1036

Query  961   GCAGCGGCATGAAACAGACGGACGGCGAATTCGCCACCACATTGGGGTCAGGATTCCGAG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1037  GCAGCGGCATGAAACAGACGGACGGCGAATTCGCCACCACATTGGGGTCAGGATTCCGAG  1096

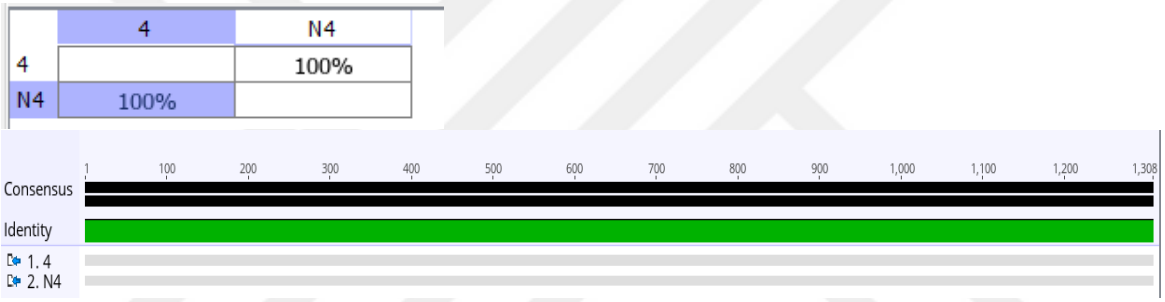
Query  1021  GCACGCCGGGCACGCTCGTCGACTTCGACAATGCCAGCTGGAATTCCTCGCAGCAGGCCT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1097  GCACGCCGGGCACGCTCGTCGACTTCGACAATGCCAGCTGGAATTCCTCGCAGCAGGCCT  1156

Query  1081  ACCCCACGTGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACGGCA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1157  ACCCCACGTGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACGGCA  1216

Query  1141  ATATCTACGAATCGTTGGGCAACAACGAAAACGAGCAGCCGGACAGCGCCGAGGAAGGCA  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1217  ATATCTACGAATCGTTGGGCAACAACGAAAACGAGCAGCCGGACAGCGCCGAGGAAGGCA  1276

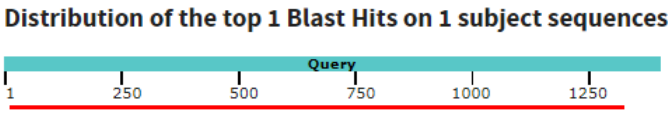
Query  1201  CGAACGCACCGTGGCGCATCATCGGACCGGTGCTATAA  1238
             ||||||||||||||||||||||||||||||||||||||
Sbjct  1277  CGAACGCACCGTGGCGCATCATCGGACCGGTGCTATAA  1314
```

As a result of the alignment performed using the Geneious Prime program for N4 and genomic DNA sequences, 100% similarity was found.



C4 / BBPC_1683

As a result of the analysis comparing the sequencing result obtained with 4 different primers for C-His tagged recombinant enzyme (BBPC_1683) with the genomic DNA sequence, the similarity percentage was determined as 99.77%.



Score	Expect	Identities	Gaps	Strand
2405 bits(1302)	0.0	1309/1312(99%)	1/1312(0%)	Plus/Plus

```
Query  15    ATGATGAAACCCCTGTTTTCCAGGAAGACGATTTTCCTTCCTCCGACTGTTCATAGCGAT  74
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGATGAAACCCCTG-TTTCCAGGAAGACGATTTTCCTTCCTCCGACTGTTCATAGCGAT  59

Query  75    ACTGTGCATCGCGCTCGTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGC  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60    ACTGTGCATCGCGCTCGTCGCGGCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGC  119

Query  135   GGCTAAGAAATTGCCGGAACCCTGGTTCGGCGGCTATGTGGACGTGACCGCAACACCCTC  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120   GGCTAAGAAATTGCCGGAACCCTGGTTCGGCGGCTATGTGGACGTGACCGCAACACCCTC  179

Query  195   ATACGAGTTCGAATCCAAGGTAGGCAACGTCTACCGGAATGTGATTCTCGGATTCGTCAC  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180   ATACGAGTTCGAATCCAAGGTAGGCAACGTCTACCGGAATGTGATTCTCGGATTCGTCAC  239

Query  255   CGCCGGCGACGGCTGCCAGCCAAGCTGGGGCGGCTACTACACGCTCGACGAAGCGGCCTC  314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240   CGCCGGCGACGGCTGCCAGCCAAGCTGGGGCGGCTACTACACGCTCGACGAAGCGGCCTC  299

Query  315   CACGCTCGATCTCGACAGCCGTATCGCGCAAACCTACAAAACCGATCGCACGGTAACCGT  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300   CACGCTCGATCTCGACAGCCGTATCGCGCAAACCTACAAAACCGATCGCACGGTAACCGT  359

Query  375   CAGCTTCGGCGGGCAGAACGGCACCGAACTTGCCAGTGCATGTTCCGACGTCGATTCCCT  434
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360   CAGCTTCGGCGGGCAGAACGGCACCGAACTTGCCAGTGCATGTTCCGACGTCGATTCCCT  419

Query  435   TGCCGACGCATACCAGCAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGACAT  494
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420   TGCCGACGCATACCAGCAGGTGATCGACCGCTATCACATCACCTCGCTCGACTTCGACAT  479

Query  495   CGAAAACTCGAATCTCGACGGGTACAGCGAAACCGCAACCCGGCGAGCGCAAGCCGTGGC  554
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480   CGAAAACTCGAATCTCGACGGGTACAGCGAAACCGCAACCCGGCGAGCGCAAGCCGTGGC  539

Query  555   AAAACTCATCGCCAACGAAAAAGCCAAAAACAAAGGGAAAGATGATACCAGTCACGATCT  614
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  540   AAAACTCATCGCCAACGAAAAAGCCAAAAACAAAGGGAAAGACGATACCAGTCACGATCT  599

Query  615   GATCATCAGTTTGACGCTTCCGGCCGACGCGAAAGGTCTTACAACGCAAGGAATGCAAAC  674
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  600   GATCATCAGTTTGACGCTTCCGGCCGACGCGAAAGGTCTTACAACGCAAGGAATGCAAAC  659

Query  675   CGTGAACGCGTTTCTGGATGCCGGCGTGACGCTATCCACCGTGAATCTCATGACCATGGA  734
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660   CGTGAACGCGTTTCTGGATGCCGGCGTGACGCTATCCACCGTGAATCTCATGACCATGGA  719

Query  735   TTTCAACGTGGCGTCCACGTCGATCACGCAATCCATGCTGATCAAAAGTTCGCTGAACGC  794
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  720   TTTCAACGTGGCGTCCACGTCGATCACGCAATCCATGCTGATCAAAAGTTCGCTGAACGC  779

Query  795   GGCCCACGCGCAATACAAAACGTTGCTGTACAGTCGCGGCAAACTCTTCAGCGACCATCA  854
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  780   GGCCCACGCGCAATACAAAACGTTGCTGTACAGTCGCGGCAAACTCTTCAGCGACCATCA  839

Query  855   GATCTGGGAGCTGTTGGGAGCCACCGTGCTCATCGGCCAGAACGATACCAAAAACGAGTA  914
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  840   GATCTGGGAGCTGTTGGGAGCCACCGTGCTCATCGGCCAGAACGATACCAAAAACGAGTA  899

Query  915   TTTCACGCTCGACAATGCGCGCGAAATCAACACGTTCGCACTGGAAACAAGCCTGGGCCA  974
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  900   TTTCACGCTCGACAATGCGCGCGAAATCAACACGTTCGCACTGGAAACAAGCCTGGGCCA  959

Query  975   CTTAAGCATGTGGTCGCTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACCAA  1034
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  960   CTTAAGCATGTGGTCGCTGAACCGCGACCAGCAATGCGGCGAAAACTATACGAACACCAA  1019

Query  1035  CACGCTCAAAACGTTCTGCAGCGGCATGAAACAGACGGACGGCGAATTCGCCACCACATT  1094
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1020  CACGCTCAAAACGTTCTGCAGCGGCATGAAACAGACGGACGGCGAATTCGCCACCACATT  1079

Query  1095  GGGGTCAGGATTCCGGGGCACGCCGGGCACGCTCGTCGACTTCGACAATGCCAGCTGGAA  1154
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1080  GGGGTCAGGATTCCGAGGCACGCCGGGCACGCTCGTCGACTTCGACAATGCCAGCTGGAA  1139

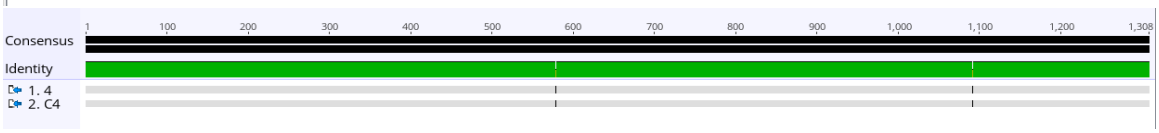
Query  1155  TTCCTCGCAGCAGGCCTACCCCACGTGGGAGCCAGACGTACTGTACAAGCAAGGCGACAA  1214
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1140  TTCCTCGCAGCAGGCCTACCCCACGTGGGAGCCAGACGTACTGTACAAGCAAGGCGACAA  1199

Query  1215  GGTGATCTGGAACGGCAATATCTACGAATCGTTGGGCAACAACGAAAACGAGCAGCCGGA  1274
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1200  GGTGATCTGGAACGGCAATATCTACGAATCGTTGGGCAACAACGAAAACGAGCAGCCGGA  1259

Query  1275  CAGCGCCGAGGAAGGCACGAACGCACCGTGGCGCATCATCGGACCGGTGCTA  1326
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1260  CAGCGCCGAGGAAGGCACGAACGCACCGTGGCGCATCATCGGACCGGTGCTA  1311
```

As a result of the alignment performed using the Geneious Prime program for C4 and genomic DNA sequences, 99.847% similarity was found.

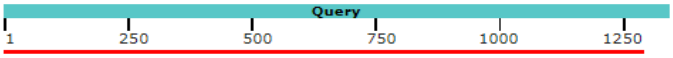
	4	C4
4		99.847%
C4	99.847%	



S4 / BBPC_1683

As a result of the analysis comparing the sequencing result obtained with 4 different primers for SUMO tagged recombinant enzyme (BBPC_1683) with the genomic DNA sequence, the similarity percentage was determined as 99.85%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
2377 bits(1287)	0.0	1291/1293(99%)	0/1293(0%)	Plus/Plus

```
Query  1     GGAAGACGATTTTCCTTCCTCCGACTGTTCATAGCGATACTGTGCATCGCGCTCGTCGCG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  22    GGAAGACGATTTTCCTTCCTCCGACTGTTCATAGCGATACTGTGCATCGCGCTCGTCGCG  81

Query  61    GCCGGCACATGGTCGTGGATCACGTTCACGCGAACAGCGGCTAAGAAATTGCCGGAACCC  120
             ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  82    GCCGGCACATGGTCGTGGATCACGTTCACGCGAACGGCGGCTAAGAAATTGCCGGAACCC  141

Query  121   TGGTTCGGCGGCTATGTGGACGTGACCGCAACACCCTCATACGAGTTCGAATCCAAGGTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  142   TGGTTCGGCGGCTATGTGGACGTGACCGCAACACCCTCATACGAGTTCGAATCCAAGGTA  201

Query  181   GGCAACGTCTACCGGAATGTGATTCTCGGATTCGTCACCGCCGGCGACGGCTGCCAGCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  202   GGCAACGTCTACCGGAATGTGATTCTCGGATTCGTCACCGCCGGCGACGGCTGCCAGCCA  261

Query  241   AGCTGGGGCGGCTACTACACGCTCGACGAAGCGGCCTCCACGCTCGATCTCGACAGCCGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262   AGCTGGGGCGGCTACTACACGCTCGACGAAGCGGCCTCCACGCTCGATCTCGACAGCCGT  321

Query  301   ATCGCGCAAACCTACAAAACCGATCGCACGGTAACCGTCAGCTTCGGCGGGCAGAACGGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322   ATCGCGCAAACCTACAAAACCGATCGCACGGTAACCGTCAGCTTCGGCGGGCAGAACGGC  381

Query  361   ACCGAACTTGCCAGTGCATGTTCCGACGTCGATTCCCTTGCCGACGCATACCAGCAGGTG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382   ACCGAACTTGCCAGTGCATGTTCCGACGTCGATTCCCTTGCCGACGCATACCAGCAGGTG  441

Query  421   ATCGACCGCTATCACATCACCTCGCTCGACTTCGACATCGAAAACTCGAATCTCGACGGG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442   ATCGACCGCTATCACATCACCTCGCTCGACTTCGACATCGAAAACTCGAATCTCGACGGG  501

Query  481   TACAGCGAAACCGCAACCCGGCGAGCGCAAGCCGTGGCAAAACTCATCGCCAACGAAAAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  502   TACAGCGAAACCGCAACCCGGCGAGCGCAAGCCGTGGCAAAACTCATCGCCAACGAAAAA  561

Query  541   GCCAAAAACAAAGGGAAAGACGATACCAGTCACGATCTGATCATCAGTTTGACGCTTCCG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  562   GCCAAAAACAAAGGGAAAGACGATACCAGTCACGATCTGATCATCAGTTTGACGCTTCCG  621

Query  601   GCCGACGCGAAAGGTCTTACAACGCAAGGAATGCAAACCGTGAACGCGTTTCTGGATGCC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  622   GCCGACGCGAAAGGTCTTACAACGCAAGGAATGCAAACCGTGAACGCGTTTCTGGATGCC  681

Query  661   GGCGTGACGCTATCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCGTCCACGTCG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  682   GGCGTGACGCTATCCACCGTGAATCTCATGACCATGGATTTCAACGTGGCGTCCACGTCG  741

Query  721   ATCACGCAATCCATGCTGATCAAAAGTTCGCTGAGCGCGGCCCACGCGCAATACAAAACG  780
             ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  742   ATCACGCAATCCATGCTGATCAAAAGTTCGCTGAACGCGGCCCACGCGCAATACAAAACG  801

Query  781   TTGCTGTACAGTCGCGGCAAACTCTTCAGCGACCATCAGATCTGGGAGCTGTTGGGAGCC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  802   TTGCTGTACAGTCGCGGCAAACTCTTCAGCGACCATCAGATCTGGGAGCTGTTGGGAGCC  861

Query  841   ACCGTGCTCATCGGCCAGAACGATACCAAAAACGAGTATTTCACGCTCGACAATGCGCGC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  862   ACCGTGCTCATCGGCCAGAACGATACCAAAAACGAGTATTTCACGCTCGACAATGCGCGC  921

Query  901   GAAATCAACACGTTCGCACTGGAAACAAGCCTGGGCCACTTAAGCATGTGGTCGCTGAAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  922   GAAATCAACACGTTCGCACTGGAAACAAGCCTGGGCCACTTAAGCATGTGGTCGCTGAAC  981

Query  961   CGCGACCAGCAATGCGGCGAAAACTATACGAACACCAACACGCTCAAAACGTTCTGCAGC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  982   CGCGACCAGCAATGCGGCGAAAACTATACGAACACCAACACGCTCAAAACGTTCTGCAGC  1041

Query  1021  GGCATGAAACAGACGGACGGCGAATTCGCCACCACATTGGGGTCAGGATTCCGAGGCACG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1042  GGCATGAAACAGACGGACGGCGAATTCGCCACCACATTGGGGTCAGGATTCCGAGGCACG  1101

Query  1081  CCGGGCACGCTCGTCGACTTCGACAATGCCAGCTGGAATTCCTCGCAGCAGGCCTACCCC  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1102  CCGGGCACGCTCGTCGACTTCGACAATGCCAGCTGGAATTCCTCGCAGCAGGCCTACCCC  1161

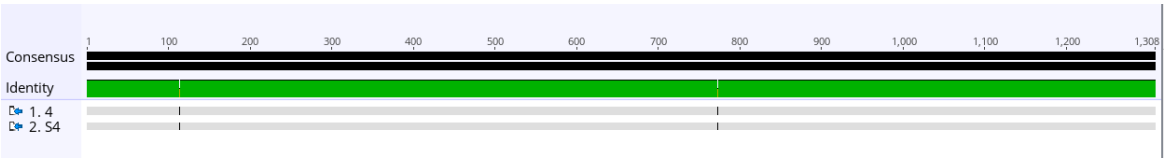
Query  1141  ACGTGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACGGCAATATC  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1162  ACGTGGGAGCCAGACGTACTGTACAAGCAAGGCGACAAGGTGATCTGGAACGGCAATATC  1221

Query  1201  TACGAATCGTTGGGCAACAACGAAAACGAGCAGCCGGACAGCGCCGAGGAAGGCACGAAC  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1222  TACGAATCGTTGGGCAACAACGAAAACGAGCAGCCGGACAGCGCCGAGGAAGGCACGAAC  1281

Query  1261  GCACCGTGGCGCATCATCGGACCGGTGCTATAA  1293
             ||||||||||||||||||||||||||||||||
Sbjct  1282  GCACCGTGGCGCATCATCGGACCGGTGCTATAA  1314
```

As a result of the alignment performed using the Geneious Prime program for S4 and genomic DNA sequences, 99.847% similarity was found.

	4	S4
4		99.847%
S4	99.847%	



N5 / BBOH_0438

As a result of the analysis comparing the sequencing result obtained with 5 different primers for N-His tagged recombinant enzyme (BBOH_0438) with the genomic DNA sequence, the similarity percentage was determined as 99.64%.



Score	Expect	Identities	Gaps	Strand
3013 bits(1631)	0.0	1644/1650(99%)	2/1650(0%)	Plus/Plus

```
Query  90    TCATCGACTCATGCGTCTTCACAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGC  149
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1     TCATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGC  60

Query  150   AACAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATT  209
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    AACAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATT  120

Query  210   CTTCGAGGTCGAAGCAGTGGCAGGGTCATGAGCATTAAGCGATGTGCACAACGTCGTACC  269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CTTCGAGGTCGAAGCAGTGGCAGGGTCATGAGCATTAAGCGATGTGCACAACGTCGTACC  180

Query  270   CGGTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGC  329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CGGTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGC  240

Query  330   ATCACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGC  389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   ATCACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGC  300

Query  390   TGAATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGAC  449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   TGAATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGAC  360

Query  450   ATTCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTC  509
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   ATTCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTC  420

Query  510   CAGTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCC  569
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Sbjct  421   CAGTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCC  480

Query  570   CAGCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGA  629
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Sbjct  481   CAGCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGA  540

Query  630   CTTCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGA  689
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   CTTCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGA  600

Query  690   CTTGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGA  749
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   CTTGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGA  660

Query  750   AGGCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATA  809
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Sbjct  661   AGGCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATA  720

Query  810   CAGGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACT  869
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   CAGGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACT  780

Query  870   GCTCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAA  929
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   GCTCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAA  840

Query  930   CGTCAGA-CAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGA  988
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Sbjct  841   CGTCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGA  900
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Query  989   AGCCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACT  1048
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   AGCCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACT  960

Query  1049  GACGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGTCATATAATAGCATTGCGCCATT  1108
             ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  961   GACGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGTCATATAATAGCATTGTGCCATT  1020

Query  1109  GTTCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCTTGAT  1168
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  GTTCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCTTGAT  1080

Query  1169  CTGCTCGGTGGTGGGACGAGTCTCCATGTCAATAT-CAGACCATCGAGTCCCCACGCATC  1227
             ||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||
Sbjct  1081  CTGCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACGCATC  1140

Query  1228  CATCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTTCGGC  1287
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  CATCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTTCGGC  1200

Query  1288  ACTGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGACGCC  1347
             |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  1201  ACTGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGACACC  1260

Query  1348  ACGGGCATGTAGGTTTGGGGCGTAATCACTTTTCAGCTTGTCAAAGAACGGCTGGGCCTG  1407
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1261  ACGGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGGCCTG  1320

Query  1408  AGCGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGGGGAT  1467
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Sbjct  1321  AGCGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGGGGAT  1380

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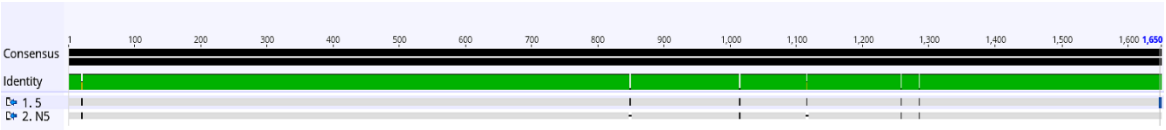
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Sbjct  1501  CTCGGACAAGGCGGATGGTTCCGACTGGCCAATGGCGCCAGCGGCCGGCGCAAACACCAG  1560

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Sbjct  1561  CATGGTCGCAGCGATCACGGCAATCGCCACAGCCGAACATAATGGTCGCAGTCTCTCGGA  1620

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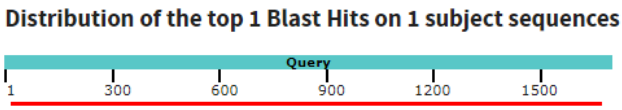
As a result of the alignment performed using the Geneious Prime program for N5 and genomic DNA sequences, 99.636% similarity was found.

	5	N5
5		99.636%
N5	99.636%	



C5 / BBOH_0438

As a result of the analysis comparing the sequencing result obtained with 5 different primers for C-His tagged recombinant enzyme (BBOH_0438) with the genomic DNA sequence, the similarity percentage was determined as 98.37%.



Score	Expect	Identities	Gaps	Strand
2896 bits(1568)	0.0	1627/1654(98%)	9/1654(0%)	Plus/Plus

```
Query  19    TCATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGC  78
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TCATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGC  60

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Sbjct  61    AACAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATT  120

Query  139   CTTCGAGGTCGAAGCAGTGGCAGGGTCATGAGCATTAAGCGATGTGCACAACGTCGTACC  198
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Sbjct  181   CGGTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGC  240

Query  259   ATCACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGC  318
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Sbjct  241   ATCACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGC  300

Query  319   TGAATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGAC  378
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Sbjct  301   TGAATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGAC  360

Query  379   ATTCAGAATCGAATCTGCCTTGTCCATCAACGCCAATAAGCCGTTCAAATCAACACTTTC  438
             ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  361   ATTCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTC  420

Query  439   CAGTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATACCCGCCGGTGTAATCGGGCCC  498
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Sbjct  421   CAGTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCC  480

Query  499   CAGCAACGCCTTGTTAGCCTCGTAGGTATTGGTCGCAGACGAGACCGCATCACCGACCGA  558
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Sbjct  481   CAGCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGA  540

Query  559   CTTCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGA  618
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Sbjct  541   CTTCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGA  600

Query  619   CTTGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGA  678
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Sbjct  601   CTTGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGA  660

Query  679   AGGCAAGATATGGTTCAAATCCGGGTCACTATACGTACGCCCGTCACGATCAGGAGCATA  738
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Sbjct  661   AGGCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATA  720

Query  739   CAGGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACT  798
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Sbjct  721   CAGGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACT  780

Query  799   GCTCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAA  858
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Sbjct  781   GCTCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAA  840

Query  859   CGTCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGA  918
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Sbjct  841   CGTCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGA  900

Query  919   AGCCGTACGCGCCGCGTCGTCACCATATTACTGATAGGCCACGTAGTCGAAACAGTCACT  978
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Sbjct  901   AGCCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACT  960

Query  979   GACGTTGCGTAAAGGACGAAGGGCGC-AAC--AAGCGTCCATTGCAT-ATAGCATTGTGC  1034
             |||||||||||||||||||||||||| ||| |  |  | || || |  || ||||||||||||
Sbjct  961   GACGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGT-CA-T--ATAATAGCATTGTGC  1016

Query  1035  CATTGTTCACTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCT  1094
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1017  CATTGTTCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCT  1076

Query  1095  TGATCTGCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACG  1154
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Sbjct  1077  TGATCTGCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACG  1136

Query  1155  CATCCATCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTT  1214
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Sbjct  1137  CATCCATCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTT  1196

Query  1215  CGGCACTGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGA  1274
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Sbjct  1197  CGGCACTGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGA  1256

Query  1275  CACCACGGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGG  1334
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Sbjct  1257  CACCACGGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGG  1316

Query  1335  CCTGAGCGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGG  1394
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Query  1455  TTTTGTCGCGCCAGGCACGGTAATATACCATGAAATGTCGCTTCTGGGTAGCAGTTTGCT  1514
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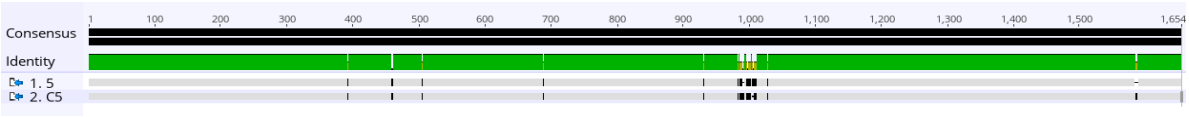
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Sbjct  1497  GCTGCTCGGACAAGGCGGATGGTTCCGACTGGCCAATGGCGCCAGCGGCCGGCGCAAACA  1556

Query  1575  CCAGCATGGTCGCAGCGATCACGGCAGTCGCCACAGACGAAAATAATGGTGGCAGTATCT  1634
             |||||||||||||||||||||||||||| ||||||||| |||| |||||||| ||||| |||
Sbjct  1557  CCAGCATGGTCGCAGCGATCACGGCAATCGCCACAGCCGAACATAATGGTCGCAGTCTCT  1616

Query  1635  CGGAAACGTCGATA-GCAAGTTCGTATTCATCAT  1667
             ||||||||||||||| |||||||| ||||||||||
Sbjct  1617  CGGAAACGTCGATATGCAAGTTCCTATTCATCAT  1650
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As a result of the alignment performed using the Geneious Prime program for C5 and genomic DNA sequences, 98.609% similarity was found.

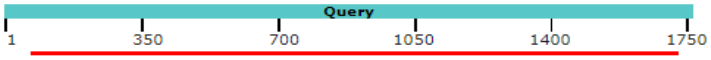
	5	C5
5		98.609%
C5	98.609%	



S5 / BBOH_0438

As a result of the analysis comparing the sequencing result obtained with 5 different primers for SUMO tagged recombinant enzyme (BBOH_0438) with the genomic DNA sequence, the similarity percentage was determined as 99.88%.

Distribution of the top 1 Blast Hits on 1 subject sequences



Score	Expect	Identities	Gaps	Strand
3037 bits(1644)	0.0	1648/1650(99%)	0/1650(0%)	Plus/Plus

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Query  69    TCATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGC  128
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Sbjct  1     TCATCGACTCATGCGTCTTCGCAACATGAGCAACGTCCCAGATCCAGATATCAACCCGGC  60

Query  129   AACAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATT  188
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Sbjct  61    AACAGCCGCAAACAACCCAACCACGGAAACACCCGTCTTTGCCAAGCCTTGTCCTTGATT  120

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Sbjct  121   CTTCGAGGTCGAAGCAGTGGCAGGGTCATGAGCATTAAGCGATGTGCACAACGTCGTACC  180

Query  249   CGGTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGC  308
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Sbjct  181   CGGTTGTTTGGCGTCCTTTACCGGTGCGGGAGCATATGGCTTTAACGCTTTAGTCAGTGC  240

Query  309   ATCACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGC  368
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Sbjct  241   ATCACTCCACTGAGCGACCTGAACCTGCGCATAGCTCTTGGCAGCAATCCCCTGAGCCGC  300

Query  369   TGAATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGAC  428
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Sbjct  301   TGAATCCCTTGCAACCCGCAGCGCTGCAAATTCTTCACTGCTCGTTTTGACAGTTGCGAC  360

Query  429   ATTCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTC  488
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Sbjct  361   ATTCAGAATCGAATCTGCCTTGTCCATCAACGTCAATAAGCCGTTCAAATCAACACTTTC  420

Query  489   CAGTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCC  548
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Sbjct  421   CAGTTGGCGTTCTAGTGTTGGATCGTAGATCTTGCTATATCCGCCGGTGTAATCGGGCCC  480

Query  549   CAGCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGA  608
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Sbjct  481   CAGCAACGCCTTGTTAGCCTCGTAGATATTGGTCGCAGACGAGACCGCATCACCGACCGA  540

Query  609   CTTCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGA  668
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Sbjct  541   CTTCGGCGCAGGACTGCTATATGACATTCTCAACAAATAGTGGTTTGCCGCTGCTTTTGA  600

Query  669   CTTGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGA  728
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Sbjct  601   CTTGGCAAGGCTCATGCCTTGGCTCTGCGCAACGGCAGTTTTGGTCCACAACATCGTCGA  660

Query  729   AGGCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATA  788
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Sbjct  661   AGGCAAGATATGGTTCAAATCCGGGTCACCATACGTACGCCCGTCACGATCAGGAGCATA  720

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Sbjct  721   CAGGAACATACCACCTAGACCATGATCTCTGACATATGAGGACACAGAATTGATATGACT  780

Query  849   GCTCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAA  908
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Sbjct  781   GCTCGTATAAGGTTCCACGGCATCATACCAACGATTATTCTTGTCCTGTTCCTCGGGGAA  840

Query  909   CGTCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGA  968
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Sbjct  841   CGTCAGACCAGGAACGAACCGGTCCCTACCGATCAGATCCTGATAGTCGTTCAATGCCGA  900
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Query  969   AGCCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACT  1028
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Sbjct  901   AGCCGTACGCGCCGCGTCGTCACCATATTGCTGATAGGCCACGTAGTCGAAACAGTCACT  960

Query  1029  GACGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGTCATATAATAGCATTGTGCCATT  1088
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   GACGTTGCGTAAAGGACGAAGGTCGCTTCCGTTGGTGTCATATAATAGCATTGTGCCATT  1020

Query  1089  GTTCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCTTGAT  1148
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Sbjct  1021  GTTCGCTTTGGGGCCGACCAACGTCGAAAGCGCTTTGATCACGCCATCGGAGAGCTTGAT  1080

Query  1149  CTGCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACGCATC  1208
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Sbjct  1081  CTGCTCGGTGGTGGGACGAGTCTCCATGTCAATATCCAGACCATCGAGTCCCCACGCATC  1140

Query  1209  CATCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTTCGGC  1268
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  CATCAGCTCGCTTTTCAGCTGCTTGGCATAGTCGAGATATTCGGTCTCGGTGGGTTCGGC  1200

Query  1269  ACTGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGACACC  1328
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  ACTGGCATGAGGAACACGCAACAGTTTTCCATAGTCGAAACCTCGCACCAGCTTGACACC  1260

Query  1329  ACGGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGGCCTG  1388
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Sbjct  1261  ACGGGCATGTAGGTTTGGGGCGTAGTCACTTTTCAGCTTGTCAAAGAACGGCTGGGCCTG  1320

Query  1389  AGCGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGGGGAT  1448
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Sbjct  1321  AGCGTCCTGACCACAGGGCACATAGCTGAACACATTGACGACATCGACTCCGTAGGGGAT  1380

Query  1449  GTCATTCATCGTGATCCAGTTGTCATCGGTCAACGAGGTGTTCACTCCTTTCATGGTTTT  1508
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Sbjct  1381  GTCATTCATCGTGATCCAGTTGTCATCGGTCAACGAGGTGTTCACTCCTTTCATGGTTTT  1440

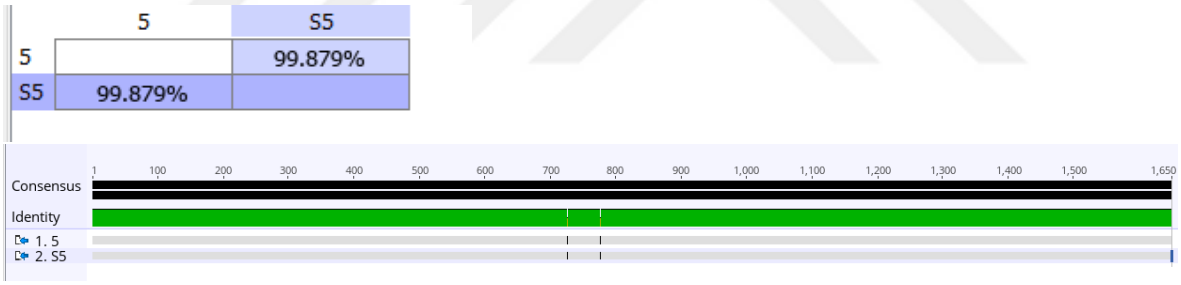
Query  1509  GTCGCGCCAGGCACGGTAATATACCATGAAATGTCGCTTCTGGGTAGCAGTTTGCTGCTG  1568
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Sbjct  1441  GTCGCGCCAGGCACGGTAATATACCATGAAATGTCGCTTCTGGGTAGCAGTTTGCTGCTG  1500

Query  1569  CTCGGACAAGGCGGATGGTTCCGACTGGCCAATGGCGCCAGCGGCCGGCGCAAACACCAG  1628
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1501  CTCGGACAAGGCGGATGGTTCCGACTGGCCAATGGCGCCAGCGGCCGGCGCAAACACCAG  1560

Query  1629  CATGGTCGCAGCGATCACGGCAATCGCCACAGCCGAACATAATGGTCGCAGTCTCTCGGA  1688
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1561  CATGGTCGCAGCGATCACGGCAATCGCCACAGCCGAACATAATGGTCGCAGTCTCTCGGA  1620

Query  1689  AACGTCGATATGCAAGTTCCTATTCATCAT  1718
             ||||||||||||||||||||||||||||||
Sbjct  1621  AACGTCGATATGCAAGTTCCTATTCATCAT  1650
```

As a result of the alignment performed using the Geneious Prime program for S5 and genomic DNA sequences, 99.879% similarity was found.



4.7. Protein Production and Purification

The production of all enzymes was initially carried out on a small scale (8 mL), then it was produced in large quantities using 250 mL of medium. Firstly, protein production was initiated with 1% inoculation of LB+kanamycin medium, allowing the *E. cloni* 10G optical density to reach approximately 0.5-0.6. L-rhamnose at 0.2% final concentration was added for the induction of protein expression. After overnight incubation, the bacteria pellets were collected by centrifugation. The cell lysis method was used to break the cell membranes to release DNA, RNA, or protein from the induced cells. The homogenization of the cells was performed by adding lysis buffer and protease inhibitor. The sonication method was also used for secondary homogenization. Then centrifugation was used to remove cell debris from lysed cells.

Protein purification was performed using the batch method. Firstly, an equilibration buffer was added into both the Ni-NTA resin and the proteins to ensure that the 6xHis-tagged proteins interact effectively with the resin. Ni-NTA resin was added to 6xHis-tagged proteins and incubated to attach the proteins to the resin. 6xHis tagged proteins bound to nickel resins, while other natural proteins flowed through the system. The mixture containing the proteins attached to the resin was washed with wash buffer for the removal of potential pollution by repeated centrifugation processes. The proteins were separated from the resin with elution buffer containing high imidazole. The purified proteins were concentrated with an Amicon tube. The list of purified enzyme codes is listed in Table 14.

Table 14

Codes of N-His tagged, SUMO tagged and C-His tagged purified enzymes

Enzymes	**N-His**	**SUMO**	**C-His**
OU11_RS07620	N1	S1	C1
BSAE_0444	N2	S2	C2
BBKW_1881	N3	S3	C3
BBPC_1683	N4	S4	C4
BBOH_0438	N5	S5	C5

According to SDS-PAGE analysis results of each enzyme, it was seen that the enzymes produced were at the expected kDa value and at least 95 percent purity. It has been reported that the His-tag causes an increase of 3-5 kDa in the molecular weight of proteins, and the His-SUMO tag causes an increase of approximately 18 kDa. The expected values were determined as a result of the theoretical molecular weights of each enzyme (Table 13). None of the changes detected in DNA sequencing (step 5) caused any stop codon formation. Consequently, all targeted proteins were successfully produced and purified in high yield by the batch purification method (Figure 25).

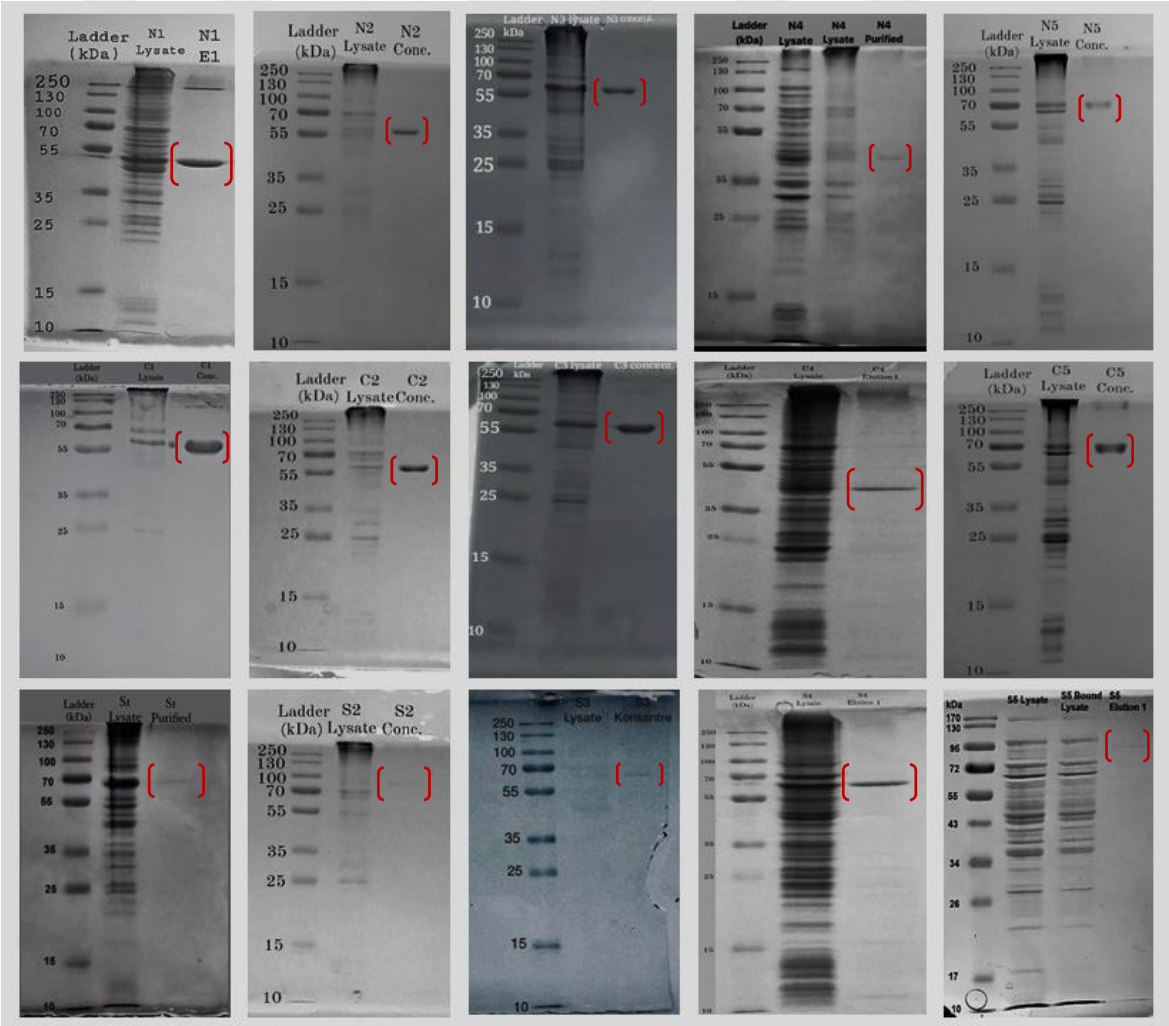


Figure 25. SDS-PAGE analysis of all purified enzymes. N-His tagged, C-His tagged, and SUMO tagged enzymes were successfully purified with high yield by the batch purification method.

4.8. Determination of Purified Enzyme Activities on RNase B by SDS-PAGE Analysis

The activities of purified enzymes were controlled on RNase B that is 17 kDa *N*-glycosylated protein. The molecular weight of RNase B decreases from 17 kDa to 14 kDa as a result of deglycosylation. Since this difference is a change that can be distinguished by SDS-PAGE analysis, it is a quick strategy to visually demonstrate the activation of the enzymes produced. 5 enzymes successfully demonstrated activity on the glycoprotein for 1 hour 30 min at 37 °C reaction conditions. The molecular weight of the deglycosylated protein was 14-15 kDa by the purified enzymes. After deglycosylation of RNase B by the enzymes C1, C2, C3, N3, N5, the deglycosylated protein was visualized at 14-15 kDa in Figure 26.

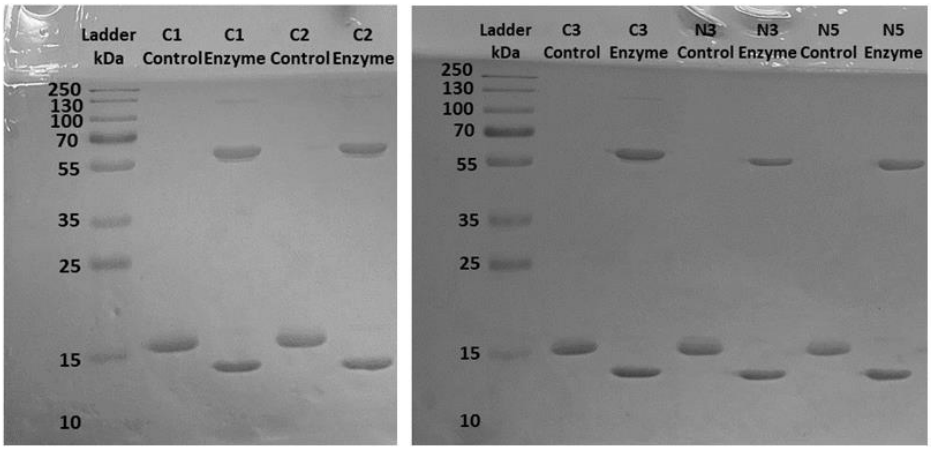


Figure 26. Determination of Activity of Purified Enzymes on Model Protein RNase B by SDS-Page analysis; C1, C2, C3, N3, N5 (C, N refer to C-His-tagged, N-His-tagged enzymes respectively).

4.9. Determination of Optimum Reaction Conditions of Enzymes

The protein mixture used as substrate was prepared by adding 5 mg of immunoglobulin and 5 mg of lactoferrin proteins to 2 mL whey protein isolate. The amount of glycan in the substrate content was increased by adding highly glycosylated immunoglobulin and lactoferrin proteins to whey protein isolate. When the results obtained are examined; in general, it has been observed that the glycan concentration obtained as a result of the reaction was decreased inversely as the pH was increased, regardless of the temperature.

However, enzymes were more effective at pH 5 and 6 than at pH 7. When the results of the reaction performed at pH 5 and 6 were compared, it was seen that the enzymes tested at pH 5 showed optimum activity and it was evaluated as the optimum reaction condition. The results were evaluated as combinations of pH and temperature and enzymes were observed to be more effective on the substrate at 30 °C and 37 °C than on 24 °C. It was concluded that especially N3 enzyme showed optimum activity at pH 5 and 37 °C. NCSS12 Statistical Software program was used to evaluate and plot the results. A three-dimensional (3D) surface plot was chosen to evaluate and visualize the results of the study conducted to see the effect of different pH, temperature combinations on each enzyme activity. 3D surface graphs of 5 enzymes are listed below (Figures 27-31).

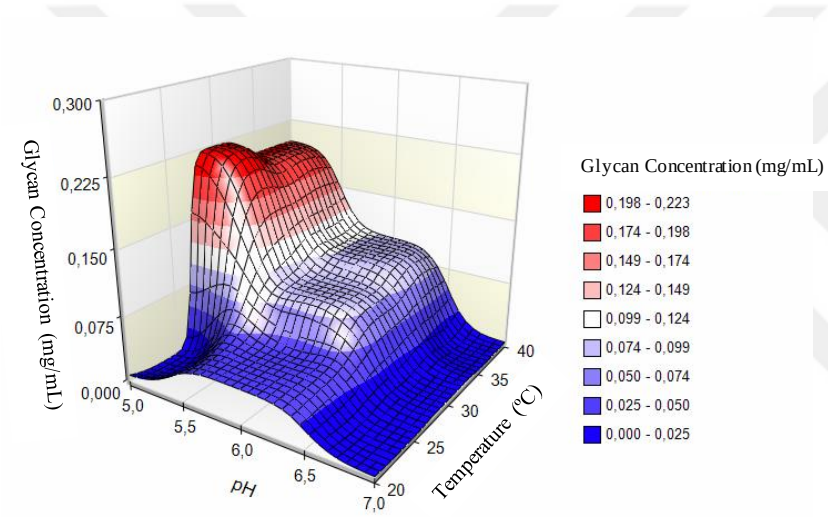


Figure 27. A 3D surface graph of C1 enzyme

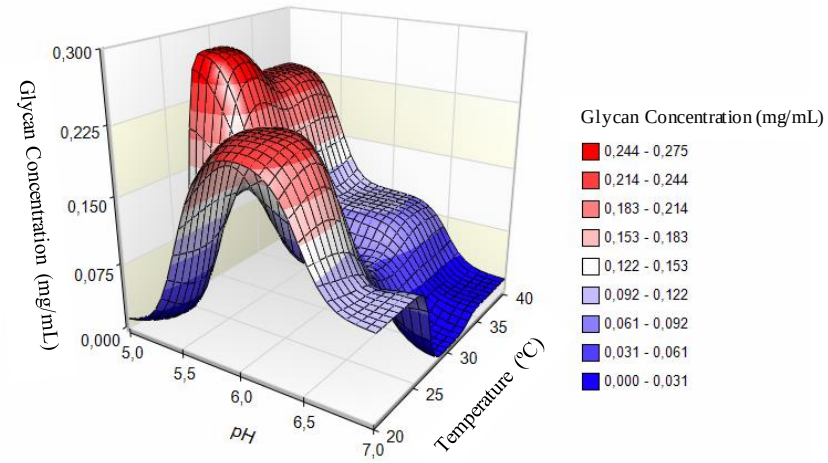


Figure 28. A 3D surface graph of C2 enzyme

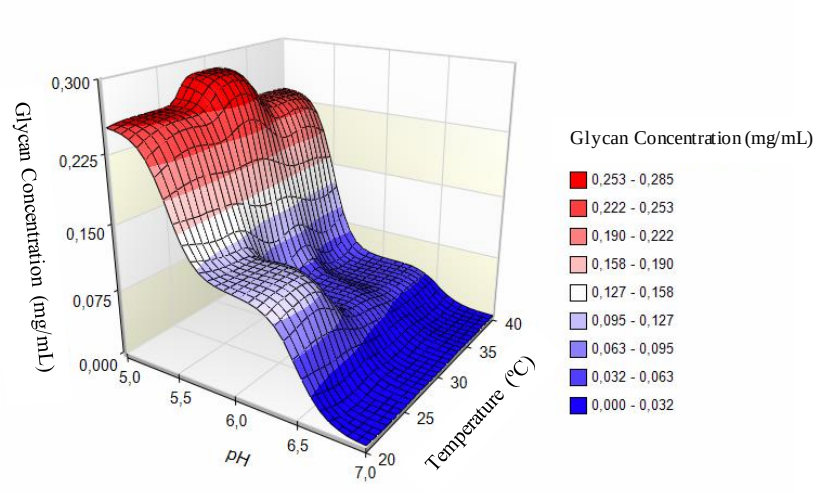


Figure 29. A 3D surface graph of C3 enzyme

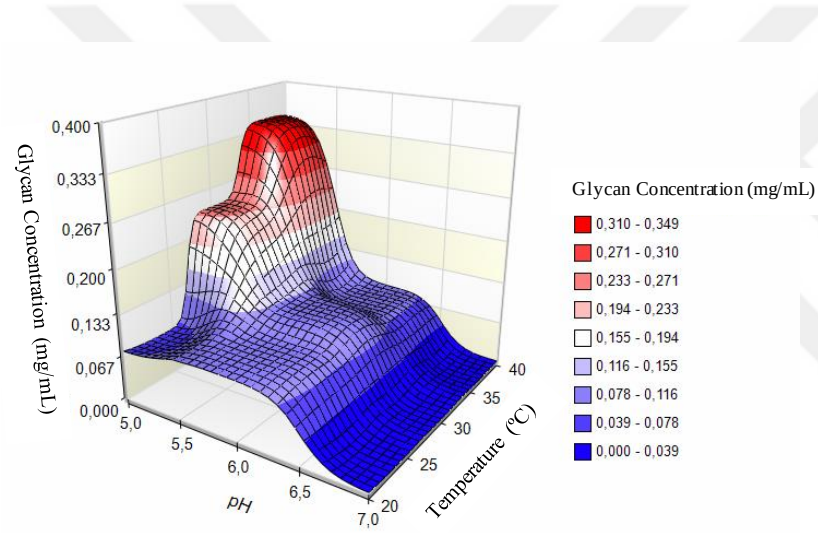


Figure 30. A 3D surface graph of N3 enzyme

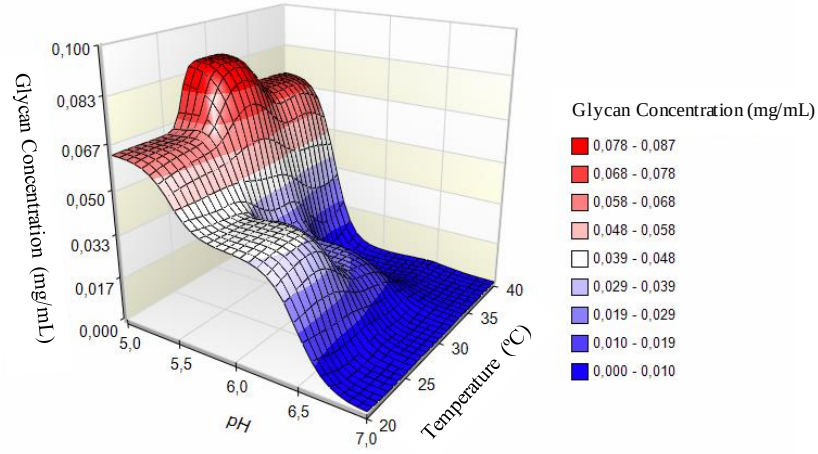


Figure 31. A 3D surface graph of N5 enzyme

4.10. Determination of Kinetic Parameters of Enzymes

All enzyme kinetic parameters (*Vmax*, *Km*, *Kcat/Km*, and *Kcat*) were calculated considering the slope of the initial part of the reaction. In addition to non-linear regression, Lineweaver-Burk, Hanes-Woolf, and Eadie-Hofstee methods, which are linearized plotting, were calculated in Graph Prism 6.0 program to reduce the margin of error in the determination of the kinetic parameters (Figure 32).

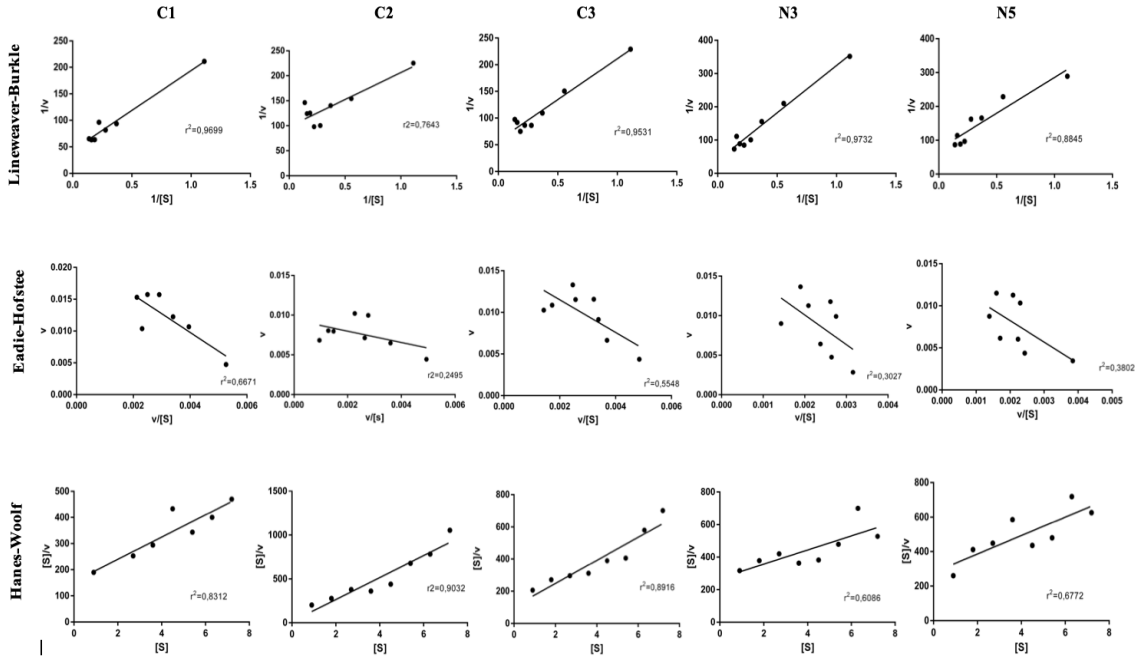


Figure 32. Lineweaver-Burke, Hanes-Woolf, and Eadie-Hofstee linearized plotting techniques to estimate the kinetic parameters of all enzymes

When the results are examined, the *Vmax* (mg/mL×min) and *Km* (mg/mL) values obtained from the non-linear regression analysis and plotting based on the Michaelis-Menten model were determined. The *Vmax* and *Km* values for each enzyme were determined as a result of the calculations made on the Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf linearized plotting technique. *Kcat* values of 0.916, 0.404, 0.688, 1.008, and 0.540 min^{-1} and *Kcat/Km* values of 0.266, 0.371, 0.261, 0.140, and 0.189 mL/(mg x min^{-1}) were calculated for C1, C2, C3, N3 and N5 enzymes, respectively. *Km* and *Vmax* values of C1, C2, C3, N3, and N5 estimated from these linear and non-linear plotting techniques are shown in Table 15.

Table 15

Kinetic parameters and equations (*Km*, *v*, nonlinear equation, linear equations) of 5 different enzymes (Values plotted on the x and y-axis).

	Michaelis-Menten	**Lineweaver–Burke**	**Eadie–Hofstee**	**Hanes–Woolf**
Equations of Kinetic characterization	$v = \frac{Vmax\,[S]}{Km + [S]}$	$\frac{1}{v} = \left(\frac{Km}{Vmax}\right)\frac{1}{[S]} + \frac{1}{Vmax}$	$v = Km\frac{v}{[S]} + Vmax$	$\frac{[S]}{v} = \frac{1}{Vmax}[S] + \frac{Km}{Vmax}$
x	*S*	*1/S*	*v/S*	*S*
y	*v*	*1/v*	*v*	*S/v*
C1				
Vmax	2.37 x 10^{-2}	2.29 x 10^{-2}	2.17 x 10^{-2}	2.35 x 10^{-2}
Km	3.62	3.44	2.94	3.63
C2				
Vmax	9.67 x 10^{-3}	1.01 x 10^{-2}	9.40 x 10^{-3}	7.99 x 10^{-3}
Km	0.79	1.09	0.71	3.63
C3				
Vmax	1.53 x 10^{-2}	1.72 x 10^{-2}	1.54 x 10^{-2}	1.40 x 10^{-2}
Km	1.84	2.64	1.96	1.47
N3				
Vmax	2.25 x 10^{-2}	2.52 x 10^{-2}	1.76 x 10^{-2}	2.30 x 10^{-2}
Km	5.67	7.18	3.75	6.2
N5				
Vmax	2.09 x 10^{-2}	1.35 x 10^{-2}	1.34 x 10^{-2}	1.87 x 10^{-2}
Km	6.21	2.85	2.56	5.22

CHAPTER 5
RESULT AND RECOMMENDATIONS

At the beginning of the study, target genes were determined using bioinformatics tools. While selecting these genes, attention was paid to their similarity with the amino acid sequence of EndoBI-1 as well as different Bifidobacteria origin isolated from the feces of different organisms. Therefore, new glycosidases with similar properties and unique specificities were determined by bioinformatics studies as intended, and these enzymes were produced recombinantly by *in vivo* molecular cloning system. Recombinant enzyme activities were tested on RNase B, and then optimum reaction conditions and kinetic parameters for the selected five enzymes were determined.

The *in vivo* cloning system kit used in the molecular cloning of target genes provides fast and reliable results compared to other traditional cloning protocols. In this system, also known as *in vivo* cloning, cloning can be performed by mixing the amplified gene with the cell and the vector (provided with the kit) without the need for enzymatic ligation. The linear vector in this system contains 18 nucleotide-long sticky ends at both ends and these sequences are not complementary to each other. Owing to this feature, the vector completely prevents the risk of self-ligation or inverted insertion of sequences. Primers required for the amplification of the target gene are designed according to these ends for strong binding. The pRham vectors provided in cloning kits contain amino or carboxyl-terminal 6xHis affinity tags. These vectors facilitate immediate cloning of target genes with these tags, which enable the purification of proteins fastly and easily in the native or denaturing conditions.

Although the transformation success efficiency of this cloning system is above 95%, one of the few disadvantages is the competent cells, which cannot be produced in typical laboratory conditions. The linearized vector containing competent cells is also only provided by only the manufacturer. Thus, the storage and their application should be performed carefully. I have observed that heat shock transformation should be applied in 15 mL falcon tubes compared to 0.2 mL PCR tubes, which are commonly preferred by conventional molecular cloning methods. After the heat shock transformation, the Colony PCR was used to confirm successfully transformed cells. When the gel images of amplification were examined, positive transformants for all tagged genes were separated from the unsuccessful

colonies and obtained. All PCR products were then sequenced to confirm the genes of interest. Sequencing data for each gene were read with a total of 4 different primers (5 different primers for the enzymes coded BBOH_0438). In particular, the similarity percentages of target gene 5 (BBOH_0438) were found to be less than the others due to low reading quality. None of the changes detected in DNA sequencing caused any stop codon formation. Consequently, all targeted proteins were successfully produced and purified in high yield by the batch purification method.

After molecular cloning, protein production is increased by using L-rhamnose and its purification is performed by binding to nickel columns with 6xHistidine tag in three different vectors. To improve the purification efficiency, a SUMO tag has been tested in this study. SUMO is known to improve the solubility of proteins and this enhances the recovery of purified protein (Butt et al., 2005). However, no significant increases in recovery were observed when comparing the SUMO tag with the N- and C-Histidine tags.

Protein purification is a critical step for obtaining enzymes in high purity. Impurities recovered with the purified protein might mislead downstream experiments, efficiency calculations, or activity assays, in the case of enzymes. Successful purification efficiency is often considered to be 95% or more based on visual observation of DNA gel electrophoresis. Although the same process can be applied for structurally similar proteins, each step of the purification process should be optimized for higher protein production efficiency and purity. One of the important steps in this process is the application of a washing buffer to remove unwanted proteins and other compounds. The concentration of washing buffer can contain 10 to 50 mM imidazole. While a low concentration of imidazole can cause impurities in enzyme solution, a high concentration of imidazole might diminish the recovery of proteins from the column. Therefore, at least 3 different concentrations of imidazole should be tested for the highest efficiency of protein production with the highest purity. All enzymes were produced in high purity and yield by batch purification method.

The activities of purified enzymes were tested on model glycoprotein RNase B. Only 5 of the 15 enzymes (C1, C2, C3, N3, N5) successfully demonstrated activity on the glycoprotein. It was determined by both phenol sulfuric acid test and gel mobility test that 5 SUMO-tagged enzymes did not show activity on RNase B. In addition, serious yield problems were encountered in the production and purification of these enzymes. Although

they are obtained in pure form, it has been observed that it is not possible to produce high amounts of pure in laboratory conditions.

The optimum reaction conditions of recombinant enzymes showed an activity on RNase B were determined using glycoprotein-rich substrate containing whey, immunoglobulin G (IgG) and lactoferrin (Lf). The amount of glycan obtained as a result of the reaction was measured calorimetrically and the obtained glycan concentration was determined using the absorbance values obtained. When the results obtained are examined; in general, it has been observed that the glycan concentration obtained as a result of the reaction was decreased inversely as the pH was increased, regardless of the temperature. In general, it was concluded that all five enzymes showed optimum activity at pH 5 at temperatures of 30-37 °C.

For the kinetic parameters (K_m and V_{max}) of recombinant enzymes, a protein coctail with whey, IgG, Lf, and soy proteins were used as a substrate. Firstly, the substrate mixture with eight different concentrations, determined in the range of 0.9-7.2 mg/mL, was reacted with five enzymes under optimum conditions and the amounts of glycans released after 20 minutes were determined calorimetrically. In addition to Michaelis-Menten non-linear regression, Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf linearized plotting techniques were used. Graphpad Prism 6.0 program was used for the determination of kinetic parameters, non-linear and linear regression calculations, and graphing of the obtained results.

As a result, enzymes encoded by Bifidobacteria obtained from different organisms were produced recombinantly and purified in high yield, their activities were tested on the model protein, and the optimum reaction conditions and kinetic parameters of the five enzymes that showed activity on the protein substrates were determined.

REFERENCES

Actor, J., Hwang, S.-A., and Kruzel, M. (2009). Lactoferrin as a Natural Immune Modulator. *Current Pharmaceutical Design*. https://doi.org/10.2174/138161209788453202

Altmann, F., Schweiszer, S., and Weber, C. (1995). Kinetic comparison of peptide: N-glycosidases F and A reveals several differences in substrate specificity. *Glycoconjugate Journal*, *12*(1), 84–93.

Antonini, T. N., Van Horn Kerne, V., Axelrad, M. E., Karaviti, L. P., and Schwartz, D. D. (2015). Neurocognitive profile of a young adolescent with DK phocomelia/von Voss phocomelia/von Voss Cherstvoy syndrome. *American Journal of Medical Genetics, Part A*. https://doi.org/10.1002/ajmg.a.37039

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-Bacterial Mutualism in the Human Intestine. *Science*, *307*(5717), 1915–1920. https://doi.org/10.1126/science.1104816

Barboza, M., Pinzon, J., Wickramasinghe, S., Froehlich, J. W., Moeller, I., Smilowitz, J. T., Ruhaak, L. R., Huang, J., Lönnerdal, B., German, J. B., Medrano, J. F., Weimer, B. C., and Lebrilla, C. B. (2012). Glycosylation of human milk lactoferrin exhibits dynamic changes during early lactation enhancing its role in pathogenic bacteria-host interactions. *Molecular and Cellular Proteomics*. https://doi.org/10.1074/mcp.M111.015248

Bengmark, S. (1998). Ecological control of the gastrointestinal tract. The role of probiotic flora. *Gut*, *42*(1), 2–7. https://doi.org/10.1136/gut.42.1.2

Bezault, J. A., Bhimani, R., Wiprovnick, J., and Furmanski, P. (1994). Human Lactoferrin Inhibits Growth of Solid Tumors and Development of Experimental Metastases in Mice. *Cancer Research*, *54*(9), 2310–2312.

Bode, L. (2006). Recent advances on structure, metabolism, and function of human milk oligosaccharides. *The Journal of Nutrition*, *136*(8), 2127–2130.

Butt, T. R., Edavettal, S. C., Hall, J. P., and Mattern, M. R. (2005). SUMO fusion technology for difficult-to-express proteins. In *Protein Expression and Purification*. https://doi.org/10.1016/j.pep.2005.03.016

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., and Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microbial Ecology in Health and Disease*, *26*(1), 40–43. https://doi.org/10.3402/mehd.v26.26191

Carlson, D. M. (1968). Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. *The Journal of Biological Chemistry*, *243*(3), 616–626. http://www.ncbi.nlm.nih.gov/pubmed/5637714

Chaplin, A. V., Efimov, B. A., Smeianov, V. V., Kafarskaia, L. I., Pikina, A. P., and Shkoporov, A. N. (2015). Intraspecies Genomic Diversity and Long-Term Persistence of Bifidobacterium longum. *PLOS ONE*, *10*(8), e0135658. https://doi.org/10.1371/journal.pone.0135658

Choudhary, G., and Hansen, H. (1998). Human health perspective of environmental exposure to hydrazines: A review. *Chemosphere*, *37*(5), 801–843.

Chung, M. (1984). Structure and Function of Transferrin. *Biochemical Education*, *12*(4), 146–154.

Conlon, M. A., and Bird, A. R. (2015). The impact of diet and lifestyle on gut microbiota and human health. *Nutrients*, *7*(1), 17–44. https://doi.org/10.3390/nu7010017

Dethlefsen, L., and Relman, D. A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(SUPPL. 1), 4554–4561. https://doi.org/10.1073/pnas.1000087107

Duranti, S., Milani, C., Lugli, G. A., Turroni, F., Mancabelli, L., Sanchez, B., Ferrario, C., Viappiani, A., Mangifesta, M., Mancino, W., Gueimonde, M., Margolles, A., van Sinderen, D., and Ventura, M. (2015). Insights from genomes of representatives of the human gut commensal B ifidobacterium bifidum. *Environmental Microbiology*, *17*(7), 2515–2531. https://doi.org/10.1111/1462-2920.12743

Dwek, R. A., Edge, C. J., Harvey, D. J., Wormald, M. R., and Parekh, R. B. (1993). Analysis of glycoprotein-associated oligosaccharides. In *Annual Review of Biochemistry*. https://doi.org/10.1146/annurev.bi.62.070193.000433

Egan, M., Motherway, M. O., Kilcoyne, M., Kane, M., Joshi, L., Ventura, M., and van Sinderen, D. (2014). Cross-feeding by Bifidobacterium breve UCC2003 during co-

cultivation with Bifidobacterium bifidum PRL2010 in a mucin-based medium. *BMC Microbiology*, *14*(1), 282.

Eylar, E. H. (1966). On the biological role of glycoproteins. *Journal of Theoretical Biology*, *10*(1), 89–113. https://doi.org/10.1016/0022-5193(66)90179-2

Garrido, D., Nwosu, C., Ruiz-Moyano, S., Aldredge, D., German, J. B., Lebrilla, C. B., and Mills, D. A. (2012). Endo-beta-N-acetylglucosaminidases from infant gut-associated bifidobacteria release complex N-glycans from human milk glycoproteins. *Molecular and Cellular Proteomics : MCP*, *11*(9), 775–785. https://doi.org/10.1074/mcp.M112.018119

Hamosh, M. (2001). Bioactive factors in human milk. *Pediatric Clinics of North America*. https://doi.org/10.1016/S0031-3955(05)70286-8

Hanes, C. S. (1932). Studies on plant amylases: The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *The Biochemical Journal*, *26*(5), 1406–1421. http://www.ncbi.nlm.nih.gov/pubmed/16744959%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1261052

Hidalgo-cantabrana, C., Delgado, S., Ruiz, L., Ruas-madiedo, P., Sánchez, B., and Margolles, A. (2017). *Bifidobacteria and Their Health-Promoting Effects*. *May 2018*, 1–19. https://doi.org/10.1128/microbiolspec.BAD-0010-2016.Correspondence

Hirai, Y., Kawakata, N., Satoh, K., Ikeda, Y., Hisayasu, S., Orimo, H., and Yoshino, Y. (1990). Concentrations of Lactoferrin and Iron in Human Milk at Different Stages of Lactation. *Journal of Nutritional Science and Vitaminology*. https://doi.org/10.3177/jnsv.36.531

Holman, R. C., Stoll, B. J., Curns, A. T., Yorita, K. L., Steiner, C. A., and Schonberger, L. B. (2006). Necrotising enterocolitis hospitalisations among neonates in the United States. *Paediatric and Perinatal Epidemiology*. https://doi.org/10.1111/j.1365-3016.2006.00756.x

Jenssen, H., and Hancock, R. (2009). Antimicrobial properties of lactoferrin. *Biochimie*, *91*(1), 19–29. https://doi.org/10.1016/j.biochi.2008.05.015

Kane, S. V, Sandborn, W. J., Rufo, P. A., Zholudev, A., Boone, J., Lyerly, D., Camilleri,

M., and Hanauer, S. B. (2003). Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. *The American Journal of Gastroenterology*, *98*(6), 1309–1314.

Karav, S. (2018). Selective deglycosylation of lactoferrin to understand glycans' contribution to antimicrobial activity of lactoferrin. *Cellular and Molecular Biology*. https://doi.org/10.14715/cmb/2018.64.9.8

Karav, S., Bell, J. M. L. N. D. M., Parc, A. Le, Liu, Y., Mills, D. A., Block, D. E., and Barile, D. (2015). Characterizing the release of bioactive N- glycans from dairy products by a novel endo- β - N- acetylglucosaminidase. *Biotechnology Progress*, *31*(5), 1331–1339. https://doi.org/10.1002/btpr.2135

Karav, S., German, J. B., Rouquié, C., Le Parc, A., and Barile, D. (2017). Studying lactoferrin N-glycosylation. In *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms18040870

Karav, S., Le Parc, A., Leite Nobrega de Moura Bell, J. M., Frese, S. A., Kirmiz, N., Block, D. E., Barile, D., and Mills, D. A. (2016). Oligosaccharides Released from Milk Glycoproteins Are Selective Growth Substrates for Infant-Associated Bifidobacteria. *Applied and Environmental Microbiology*, *82*(12), 3622–3630. https://doi.org/10.1128/AEM.00547-16

Karav, S., Parc, A. Le, Moura Bell, J. M. L. N. de, Rouquié, C., Mills, D. A., Barile, D., and Block, D. E. (2015). Kinetic characterization of a novel endo-β-N-acetylglucosaminidase on concentrated bovine colostrum whey to release bioactive glycans. *Enzyme and Microbial Technology*, *77*, 46–53. https://doi.org/10.1016/j.enzmictec.2015.05.007

Karav, S., Talak, E., Tuncer, M., and Ozleyen, A. (2017). The Effect of Fusion Tags on Enzyme Specificity and Protein Purification Efficiency. *International Journal of Agriculture Innovations and Research*, *6*(3), 2319–1473.

Klijn, A., Mercenier, A., and Ariogon, F. (2005). Lessons from the genomes of bifidobacteria. *FEMS Microbiology Reviews*, *29*(3), 491–509. https://doi.org/10.1016/j.femsre.2005.04.010

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T., and Ley, R. E. (2011). Succession of microbial consortia in the developing

infant gut microbiome. *Proceedings of the National Academy of Sciences*, *108*(Supplement_1), 4578–4585. https://doi.org/10.1073/pnas.1000081107

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Laureys, D., Cnockaert, M., De Vuyst, L., and Vandamme, P. (2016). Bifidobacterium aquikefiri sp. nov., isolated from water kefir. *International Journal of Systematic and Evolutionary Microbiology*, *66*(3), 1281–1286. https://doi.org/10.1099/ijsem.0.000877

Le Parc, A., Dallas, D. C., Duaut, S., Leonil, J., Martin, P., and Barile, D. (2014). Characterization of goat milk lactoferrin N-glycans and comparison with the N-glycomes of human and bovine milk. *Electrophoresis*, *35*(11), 1560–1570. https://doi.org/10.1002/elps.201300619

Legrand, D., Elass, E., Carpentier, M., and Mazurier, J. (2006). Interactions of lactoferrin with cells involved in immune function. *Biochemistry and Cell Biology*, *84*(3), 282–290. https://doi.org/10.1139/O06-045

Levay, P. F., and Viljoen, M. (1995). Lactoferrin: A general review. In *Haematologica*.

Lineweaver, H., and Burk, D. (1934). The Determination of Enzyme Dissociation Constants. *Journal of the American Chemical Society*, *56*(3), 658–666. https://doi.org/10.1021/ja01318a036

LoCascio, R. G., Niñonuevo, M. R., Kronewitter, S. R., Freeman, S. L., German, J. B., Lebrilla, C. B., and Mills, D. A. (2009). A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. *Microbial Biotechnology*. https://doi.org/10.1111/j.1751-7915.2008.00072.x

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230. https://doi.org/10.1038/nature11550

Mamedov, T., and Yusibov, V. (2011). Green algae Chlamydomonas reinhardtii possess endogenous sialylated N-glycans. *FEBS Open Bio*, *1*(1), 15–22. https://doi.org/10.1016/j.fob.2011.10.003

Marcobal, A., and Sonnenburg, J. L. (2012). Human milk oligosaccharide consumption by intestinal microbiota. *Clinical Microbiology and Infection, 18*(s4), 12–15. https://doi.org/10.1111/j.1469-0691.2012.03863.x

Martinez, F. A. C., Balciunas, E. M., Converti, A., Cotter, P. D., and de Souza Oliveira, R. P. (2013). Bacteriocin production by Bifidobacterium spp. A review. *Biotechnology Advances*, *31*(4), 482–488. https://doi.org/10.1016/j.biotechadv.2013.01.010

Mollea, C., Marmo, L., and Bosco, F. (2013). Valorisation of Cheese Whey, a By-Product from the Dairy Industry. In *Food Industry*. InTech. https://doi.org/10.5772/53159

Morgan, B. L. G., and Winick, M. (1980). Effects of Environmental Stimulation on Brain N-Acetylneuraminic Acid Content and Behavior. *The Journal of Nutrition*, *110*(3), 425–432. https://doi.org/10.1093/jn/110.3.425

Neish, A. S. (2009). Microbes in Gastrointestinal Health and Disease. In *Gastroenterology*. https://doi.org/10.1053/j.gastro.2008.10.080

Nicholson, J. K., Holmes, E., and Wilson, I. D. (2005). Gut microorganisms, mammalian metabolism and personalized health care. *Nature Reviews Microbiology*, *3*(5), 431–438. https://doi.org/10.1038/nrmicro1152

Nuck, R., Zimmermann, M., Sauvageot, D., Josić, D., and Reutter, W. (1990). Optimized deglycosylation of glycoproteins by peptide-N4-(N-acetyl-β-glucosaminyl)-asparagine amidase from Flavobacterium meningosepticum. *Glycoconjugate Journal*. https://doi.org/10.1007/BF01073372

O'Callaghan, A., and van Sinderen, D. (2016). Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00925

Ohtsubo, K., and Marth, J. D. (2006). Glycosylation in Cellular Mechanisms of Health and Disease. *Cell*, *126*(5), 855–867. https://doi.org/10.1016/j.cell.2006.08.019

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLoS Biology, 5*(7), 1556–1573. https://doi.org/10.1371/journal.pbio.0050177

Parc, A. Le, Karav, S., Bell, J. M. L. N. D. M., Frese, S. A., Liu, Y., Mills, D. A., Block, D. E., and Barile, D. (2015). A novel endo-β-N-acetylglucosaminidase releases

specific N-glycans depending on different reaction conditions. *Biotechnology Progress*. https://doi.org/10.1002/btpr.2133

Parc, A. Le, Karav, S., Rouquié, C., Maga, E. A., Bunyatratchata, A., and Barile, D. (2017). Characterization of recombinant human lactoferrin N-glycans expressed in the milk of transgenic cows. *PLOS ONE*, *12*(2), e0171477. https://doi.org/10.1371/journal.pone.0171477

Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Parekh, R., and Jaques, A. (1993). Use of hydrazine to release in intact and unreduced form both N-and O-linked oligosaccharides from glycoproteins. *Biochemistry*, *32*(2), 679–693.

Qiu, J., Hendrixson, D. R., Baker, E. N., Murphy, T. F., St. Geme, J. W., and Plaut, A. G. (1998). Human milk lactoferrin inactivates two putative colonization factors expressed by Haemophilus influenzae. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(21), 12641–12646. https://doi.org/10.1073/pnas.95.21.12641

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M. C., Marchesi, J. R., and Collado, M. C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease*, *26*, 1–17. https://doi.org/10.3402/mehd.v26.26050

Royle, L., Matthews, E., Corfield, A., Berry, M., Rudd, P. M., Dwek, R. A., and Carrington, S. D. (2008). Glycan structures of ocular surface mucins in man, rabbit and dog display species differences. *Glycoconjugate Journal*. https://doi.org/10.1007/s10719-008-9136-6

Sela, D A, Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., Lapidus, A., Rokhsar, D. S., Lebrilla, C. B., and German, J. B. (2008). The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. *Proceedings of the National Academy of Sciences*, *105*(48), 18964–18969.

Sela, David A., and Mills, D. A. (2010). Nursing our microbiota: Molecular linkages between bifidobacteria and milk oligosaccharides. In *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2010.03.008

Soccol, C. R., Vandenberghe, L. P. de S., Spier, M. R., Medeiros, A. B. P., Yamaguishi, C. T., De Dea Lindner, J., Pandey, A., and Thomaz-Soccol, V. (2010). The potential of probiotics: A review. *Food Technology and Biotechnology*, *48*(4), 413–434.

Sojar, H. T., and Bahl, O. P. (1987). A chemical method for the deglycosylation of proteins. *Archives of Biochemistry and Biophysics*, *259*(1), 52–57. https://doi.org/10.1016/0003-9861(87)90469-3

Solá, R. J., and Griebenow, K. (2010). Glycosylation of Therapeutic Proteins. *BioDrugs*, *24*(1), 9–21. https://doi.org/10.2165/11530550-000000000-00000

Steen, P. Van den, Rudd, P. M., Dwek, R. A., and Opdenakker, G. (1998). Concepts and Principles of O-Linked Glycosylation. *Critical Reviews in Biochemistry and Molecular Biology*, *33*(3), 151–208. https://doi.org/10.1080/10409239891204198

Steijns, J. M. (2001). Milk ingredients as nutraceuticals. *International Journal of Dairy Technology*. https://doi.org/10.1046/j.1364-727x.2001.00019.x

Suau, A., Gibson, G. R., Collins, M. D., Dore, J., Bonnet, R., Sutren, M., Godon, J. J., and Doré, J. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology*, *65*(11), 4799–4807. http://www.ncbi.nlm.nih.gov/pubmed/10543789%5Cnhttp://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieveanddb=PubMedanddopt=Citationandlist_uids=10543789

Tretter, V., Altmann, F., and Marz, L. (1991). Peptide-N4-(N-acetyl-β-glucosaminyl)asparagine amidase F cannot release glycans with fucose attached α1 → 3 to the asparagine-linked N-acetylglucosamine residue. *European Journal of Biochemistry*, *199*(3), 647–652. https://doi.org/10.1111/j.1432-1033.1991.tb16166.x

Triguero, A., Cabrera, G., Royle, L., Harvey, D. J., Rudd, P. M., Dwek, R. A., Bardor, M., Lerouge, P., and Cremata, J. A. (2010). Chemical and enzymatic N-glycan release comparison for N-glycan profiling of monoclonal antibodies expressed in plants. *Analytical Biochemistry*. https://doi.org/10.1016/j.ab.2010.01.027

Trimble, R. B., and Tarentino, A. L. (1991). Identification of distinct endoglycosidase (endo) activities in Flavobacterium meningosepticum: endo F1, endo F2, and endo F3. Endo F1 and endo H hydrolyze only high mannose and hybrid glycans. *The Journal of*

Biological Chemistry, *266*(3), 1646–1651. http://www.ncbi.nlm.nih.gov/pubmed/1899092

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, *444*(7122), 1027–1031. https://doi.org/10.1038/nature05414

Turroni, F., van Sinderen, D., and Ventura, M. (2011). Genomics and ecological overview of the genus Bifidobacterium. *International Journal of Food Microbiology*, *149*(1), 37–44. https://doi.org/10.1016/j.ijfoodmicro.2010.12.010

Turyan, I., Hronowski, X., Sosic, Z., and Lyubarskaya, Y. (2014). Comparison of two approaches for quantitative O-linked glycan analysis used in characterization of recombinant proteins. *Analytical Biochemistry*, *446*, 28–36. https://doi.org/10.1016/j.ab.2013.10.019

Vael, C., and Desager, K. (2009). The importance of the development of the intestinal microbiota in infancy. *Current Opinion in Pediatrics*, *21*(6), 794–800. https://doi.org/10.1097/MOP.0b013e328332351b

van der Strate, B. W. ., Beljaars, L., Molema, G., Harmsen, M. ., and Meijer, D. K. . (2001). Antiviral activities of lactoferrin. *Antiviral Research*, *52*(3), 225–239. https://doi.org/10.1016/S0166-3542(01)00195-4

Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Marth, J. D., Bertozzi, C. R., Hart, G. W., and Etzler, M. E. (2009). Symbol nomenclature for glycan representation. *Proteomics*, *9*(24), 5398–5399. https://doi.org/10.1002/pmic.200900708

Ventura, M. (2001). Molecular microbial analysis of Bifidobacterium isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Microbiology Ecology*, *36*(2–3), 113–121. https://doi.org/10.1016/S0168-6496(01)00123-4

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., and van Sinderen, D. (2007). Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum. *Microbiology and Molecular Biology Reviews*, *71*(3), 495–548. https://doi.org/10.1128/MMBR.00005-07

Villanueva-Millán, M. J., Pérez-Matute, P., and Oteo, J. A. (2015). Gut microbiota: a key player in health and disease. A review focused on obesity. *Journal of Physiology and Biochemistry*, *71*(3), 509–525. https://doi.org/10.1007/s13105-015-0390-3

Walstra, P., Walstra, P., Wouters, J. T. M., and Geurts, T. J. (2005). Dairy Science and Technology. In *Dairy Science and Technology*. https://doi.org/10.1201/9781420028010

Wang, B. (2009). Sialic acid is an essential nutrient for brain development and cognition. *Annual Review of Nutrition*, *29*, 177–222.

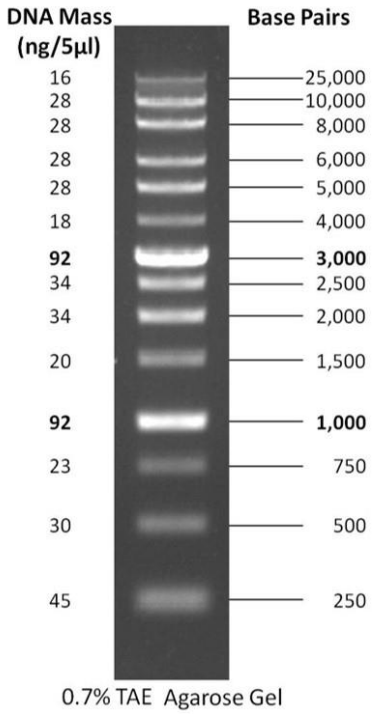
Wang, B., Yu, B., Karim, M., Hu, H., Sun, Y., McGreevy, P., Petocz, P., Held, S., and Brand-Miller, J. (2007). Dietary sialic acid supplementation improves learning and memory in piglets. *American Journal of Clinical Nutrition*, *85*(2), 561–569. https://doi.org/10.1093/ajcn/85.2.561

Woolf, B. (1931). The addition compound theory of enzyme action. *Biochemical Journal*, *25*(1), 342–348. https://doi.org/10.1042/bj0250342

APPENDICES

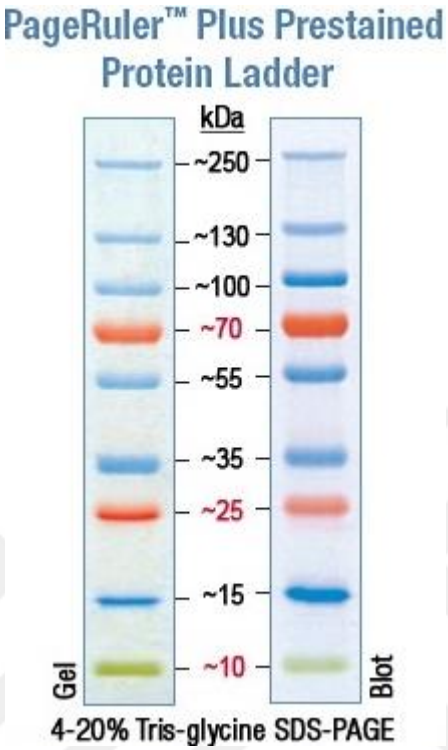
APPENDIX 1

DNA LADDER USED AS MARKER



APPENDIX 2

PROTEIN LADDER USED AS MARKER



APPENDIX 3

BUFFERS AND CONTENTS USED IN PROTEIN PURIFICATION

	Lysis Buffer pH 8.0	**Equilibration Buffer pH 7.4**	**Wash Buffer pH7.4**	**Elution Buffer pH 7.4**
Contents				
NaCl	200 mM	300 mM	300 mM	300 mM
Imidazole	1 mM	10 mM	25 mM	250 mM
NaH_2PO_4	-	20 mM	20 mM	20 mM
SDS	1%	-	-	-
Tris-HCl	50 mM	-	-	-