# EXPERIMENTAL STUDIES TO DETERMINE THE EFFECT OF PROTEIN CHARACTERISTICS ON TRANSLOCATION OF FUSION PROTEINS IN ESCHERICHIA COLI

by

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# ABSTRACT


## EXPERIMENTAL STUDIES TO DETERMINE THE EFFECT OF PROTEIN CHARACTERISTICS ON TRANSLOCATION OF FUSION PROTEINS IN ESCHERICHIA COLI

Previous experimental work to translocate maltose binding protein-glucose isomarase fusion protein from the cytoplasmic to the periplasmic space have shown that the fusion protein mainly remained in the cytoplasm and only one per cent of the expressed fusion protein was transported to the periplasm.

In the present study, genetic engineering techniques are exploited to investigate the effect of protein size on translocation. For this purpose, two industrial enzymes of different sizes were cloned using the pMAL-p2 vector. These two enzymes are: pullulanase and putative serine protease of *Thermus Thermophilus* HB8 which are 718 and 251 amino acids long, respectively. Glucose isomease from the same organism is 395 amino acids long, thus in between pullulanase and serine protease.

The gene coding for serine protease and pullulanase from *Thermus thermophilus* HB8 (ATCC 27634) were amplified using PCR and inserted between *XmnI* and *EcoRI* site of the plasmid vector pMAL-p2. The fusion proteins MBP-serine protease and MBP-pullulanase were expressed in recombinant XL1 cells and the cellular distribution of the fusions were determined in cytoplasmic and periplasmic compartments by the appropriate enzyme assays and SDS-PAGE analysis. The experimental results showed that most of the MBP-serine protease fusion expressed was translocated to the periplasm with the highest value of 77 per cent and almost all of the MBP-pullulanase fusion expressed was translocated to the periplasm with the highest value of 99 per cent. On the other hand, the results for MBP-GI case was not satisfactory and not more than one per cent of the total protein produced was translocated to the periplasm. Therefore, it was obvious that the length of the protein to be translocated didn't have any effect on translocation.

# ÖZET

## ESCHERICHIA COLI'DA BİR FÜZYON PROTEİNİNİN TAŞINMASINA, PROTEİN KARAKTERİSTİĞİNİN ETKİSİNİN BELİRLENMESİNİN DENEYSEL ÇALIŞMALARI

Maltoza bağlanan protein-glikoz isomeraz füzyon proteinin sitoplazmik boşluktan periplazmik boşluğa taşınması üzerine yapılan önceki deneysel çalışma, füzyon proteininin sitoplazmada kaldığını ve üretilen füzyon proteininin çok az bir kısmının periplazmaya taşındığını göstermiştir.

Bu çalışmada, taşınma üzerinde protein boyutunun etkisini araştırmak için genetik mühendisliği teknikleri kullanılmıştır. Bu amaçla, farklı boyutlarda endüstriyel enzimler pMAL-p2 vektörüne klonlanmıştır. Bu çalışmada kullanılan iki enzim sırasıyla 718 ve 251 amino asit uzunluğunda olan pullulanaz ve serin proteaz enzimleridir. 395 mino asit uzunluğundaki glikoz izomeraz da pullulanaz ve serin proteazın arasındadır.

*Thermus thermophilus* HB8 (ATCC 27634) bakterisindeki serin proteaz ve pullulanaz genleri PCR kullanılarak çoğaltıldı ve pMAL-p2 vektörünün *XmnI* ve *EcoRI* bölgeleri arasına yerleştirildi. MBP-serin proteaz ve MBP-pullulanaz füzyon proteinleri recombinant XL1 hücrlerinde üretildi ve füzyonların sitoplazmik ve periplazmik kısınlardaki hücresel dağılımları gerekli enzim testleri ve SDS-PAGE ile belirlendi. Deneysel sonuçlar, MBP-serin proteaz füzyonunun yüzde 77'lik en yüksek değerle çoğunun periplazmaya taşındığını, MBP-pullulanaz füzyonunun ise yüzde 99'luk en yüksek değerle neredeyse tamamının periplazmaya taşındığını göstermiştir. Diğer taraftan, MBP-GI füzyonu için sonuçlar pek de tatmin edici değildir ve üretilen toplam proteinin yüzde birden fazlası periplazmaya taşınmamaktadır. Böylelikle, taşınacak protein boyutunun taşınma üzerine etkisi olmadığı açıktır.

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# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| Δp | proton motive force |
| ΔpH | pH gradient |
| ΔΨ | membrane potential |
| | |
| Arg | arganine |
| Asn | asparagine |
| Asp | aspartic acid |
| ATP | adenosine triposphate |
| Da | Dalton |
| DNS | dinitrosalicylic acid |
| GI | glucose isomerase |
| Gln | glutamine |
| Glu | glutamic acid |
| GTP | guanosine triphosphate |
| His | histidine |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| Lys | lysine |
| MBP | maltose binding protein |
| MBP-GI | maltose binding protein- glucose isomerase |
| OD | optical density |
| Phe | phenylalanine |
| rpm | rotation per minute |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SP | serine protease |
| SRP | signal recognition partical |
| SRRXFLK | serine-arginine-arginine-X-phenylalanine-leucine-lysine |
| TAT | twin arginine translocation |
| TEMED | N,N,N,N-tetramethylethylenediamine |
| Trp | tryptophan |
| Tyr | tyrosine |
| U | enzyme units |

# 1. INTRODUCTION

All living organisms, no matter how simple or complex, possess the ability to translocate proteins across biological membranes and into different cellular compartments. Although a range of membrane transport processes exist, the major pathway used to translocate proteins across the bacterial cytoplasmic membrane is known as the Sec pathway. Over the past two decades the Sec pathway has been studied extensively and is well characterised at genetic and biochemical levels (Stephenson, 2005).

The use of gene fusions can be used to target the protein to different cellular compartments or even to the culture medium. This has greatly facilitated the expression and subsequent purification of heterologous proteins in *E. coli* (Ford at al., 1991). Thus, construction of fusion proteins is widely used for engineering proteins. A fusion protein is constructed by fusing a naturally occuring secretory protein is fused to the amino termini of the protein under study. Secretory proteins are synthesized as preproteins containing an amino-terminal signal sequence which is used for translocation by the fusion protein. Maltose binding protein (MBP) is one periplasmic protein that uses the Sec pathway for translocation. Proteins fused to MBP with the signal sequence, responsible for initiating export through the membrane are targeted to the periplasm.

In a previous work, thermostable glucose isomerase (GI), which is a cytoplasmic protein, of *Thermus thermophilus* HB8 was cloned in vector pMAL-p2 for translocation to the periplasmic space. The GI cloned in pMAL-p2 was expressed as a maltose binding protein-glucose isomerase (MBP-GI) fusion protein which was expected to be transported to the periplasm by the signal peptide on MBP. This experimental work to translocate MBP-GI fusion protein has shown that GI was poorly translocated and the fusion protein mainly remained in the cytoplasm. No more than 1 per cent of the expressed fusion protein was transported to the periplasm (Sarıyar, 2003). In this present work, the effect of protein characteristics on translocation was investigated. For this purpose, two other industrial enzymes namely, serine protease and pullulanase from *Thermus thermophilus* HB8 were cloned into the same vector pMAL-p2 to compare and evaluate the efficiency of translocation.

A theoretical background on protein translocation across membranes is presented in Section 2. Following a brief description of serine protease, pullulanase, maltose binding protein and pMAL-p2 vector system, the materials and methods used in experimental studies conducted are described. Then, the results of the experiments conducted on the translocation of the two fusion proteins, maltose binding protein-serine protein and maltose-binding protein-pullulanase are given. The results are discussed and compared with maltose binding protein-glucose isomerase. In the final section, conclusions and recommendations are given.

# 2. PROTEIN TRANSLOCATION ACROSS MEMBRANES

The passage of protein molecules through biological membranes is a process crucial to the life of biological cells. These membranes act as semipermeable barriers to ions and macromolecules (Agarraberes and Dice, 2001). Information transfer in the living cell does not end with the biosynthesis of a polypeptide chain. The familiar central dogma of molecular biology comes with an essential small print: a polypeptide chain will only fulfil its designated function if it is correctly folded. Additionally, for a significant subset of cellular proteins (more than 30 per cent of the total proteome), folding must take place in the correct subcellular, membrane-enclosed compartment. The modern cell has developed elaborate mechanisms to deal with both the membrane translocation and the secretion of polypeptides, as well as with their subsequent folding (Economou, 1999).

Gram-negative bacteria such as *Escherichia coli* are surrounded by two membranes, the inner membrane and the outer membrane (Luirink, et al., 2005). All proteins in *Escherichia coli* are initially synthesized in the cytoplasm, then follow a pathway that depends upon their ultimate cellular destination. Many proteins destined for the periplasm are synthesized as precursors carrying an N-terminal signal sequence that directs them to the general secretion machinery at the inner membrane. After translocation and signal sequence cleavage, the newly exported mature proteins are folded and assembled in the periplasm (Miot and Betton, 2004).

The transport of a protein across the membrane may occur during its synthesis (co-translationally) or after its completion (post-translationally). In both cases, the process is initiated by a targeting phase. Figure 2.1 shows both transport mechanisms.

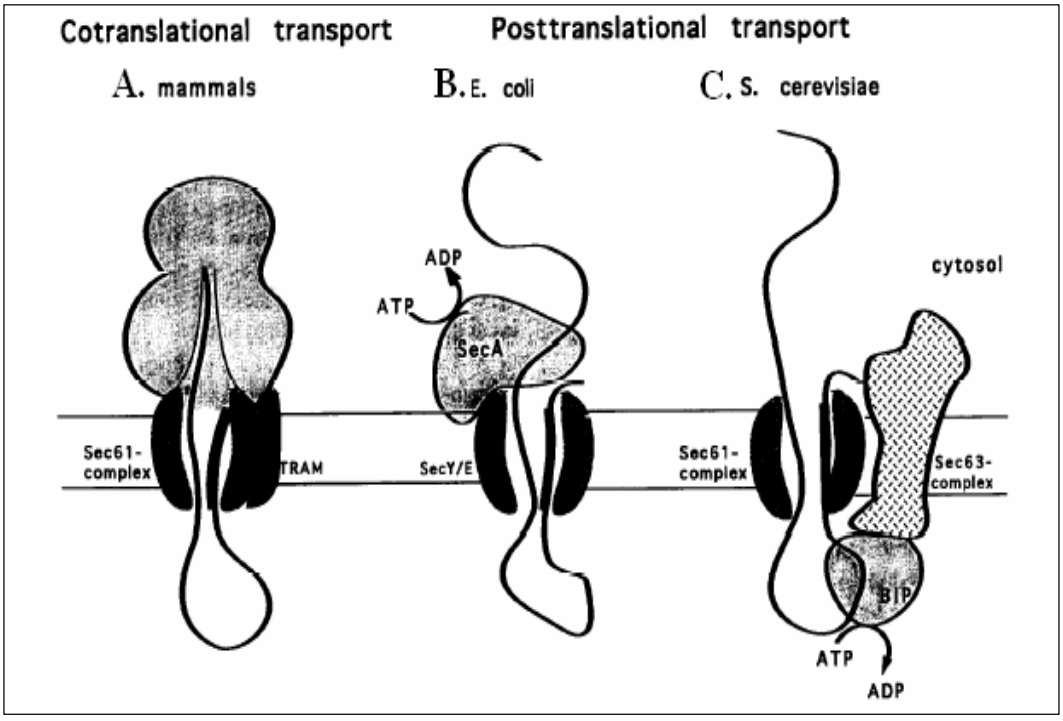



Figure 2.1. A simplified scheme for co- and post-translational translocation pathways. A. Cotranslational transport in mammals, B. Posttranslational transport in *E. coli*, C. Posttranslational transport in S. cerevisia (Jungnickel et al., 1994)

In co-translational mode translocation may start as early as the amino acid chain emerges from the ribosome and it should be completed before the protein acquires its three-dimensional structure (Davis and Tai, 1980).

In post-translational mode, translation of the amino acid chain is first completed. Translocation can take place before the proteins fold. When released from the ribosome, specific chaperones will carry the proteins to the machinery on the membrane. If the protein folds against the membrane surface then it is subsequently transferred by engulfment by the membrane (Davis and Tai, 1980). The post-translational mode of transport is best known for *E. coli.* Only two membrane components are essential: the peripheral membrane protein, SecAp, and the integral SecYp complex. SecAp is an ATPase (translocation ATPase) that accepts the polypeptide chain from the cytoplasmic chaperon, SecBp. SecYp complex consists of SecYp, SecEp and the BandI protein (Jungnickel et al., 1994).

Proteins are able to translocate across membranes in a molecule-by-molecule fashion by mechanisms that share common features. Packets of proteins can also cross membranes by the fusion of protein-filled vesicles with recipient membranes. These vesicular pathways are important for protein secretion, endocytosis, and targeting of some proteins to their appropriate organelles (Agarraberes and Dice, 2001).

### 2.1. Protein Translocation Across the Bacterial Plasma Membrane

In all organisms, genetic messages are translated primarily by cytosolic ribosomes, yet the translation products end up in various cellular locations (Mori and Ito, 2001). Gram-negative bacteria target endogenous proteins to one of five different sites: the cytoplasm, the cytoplasmic (inner) membrane, the outer membrane, the periplasm between the two membranes, and the external milieu (d'Enfert et al., 1989).

In *E. coli*, exported proteins with an ultimate destination of the periplasm and outer membrane are synthesized as precursors with a cleavable amino-terminal signal sequence. Depending on the nature of the precursors, different pathways exist for their transport across the inner membrane. (Miot and Betton, 2004)

At least four protein translocation/secretion pathways have been described in Gram-negative bacteria such as *E. coli*: the general translocation system is mediated by two molecular chaperones, SecAp and SecBp, and is therefore designated the Sec-dependent pathway. The twin arginine translocation (TAT) system does not require either Sec protein and is therefore known as the Sec-independent pathway. In addition two other pathways have been described for targeting of integral membrane proteins to the inner membrane, the signal recognition particle (SRP) system and the YidC-dependent pathway. The SRP system is responsible for targeting and co-translational insertion into the plasma membrane of inner membrane proteins. The YidC-dependent pathway is required for the insertion of some proteins, such as the coat protein of phage M13, into the plasma membrane (Agarraberes and Dice, 2001).

### 2.1.1. The Sec-dependent Pathway

A large number of bacterial proteins are active in extracytoplasmic locations. Targeting and membrane translocation of the vast majority of these secretory and membrane polypeptides is mediated by the Sec pathway. (Economou, 2001)

The Sec pathway is responsible for the export of newly synthesized outer membrane and periplasmic proteins across the plasma membrane (Figure 2.2). Proteins transported by this way have in common a signal peptide of 18-26 amino acids in their amino-terminal region. These signal peptides have three characteristic regions: a positively charged amino acid at the N-terminus, a highly hydrophobic region, and a polar region containing the signal peptidase cleavage site. The Sec translocon, defined as the minimal machinery that can accomplish protein transport across the membrane, can only export unfolded polypeptides.

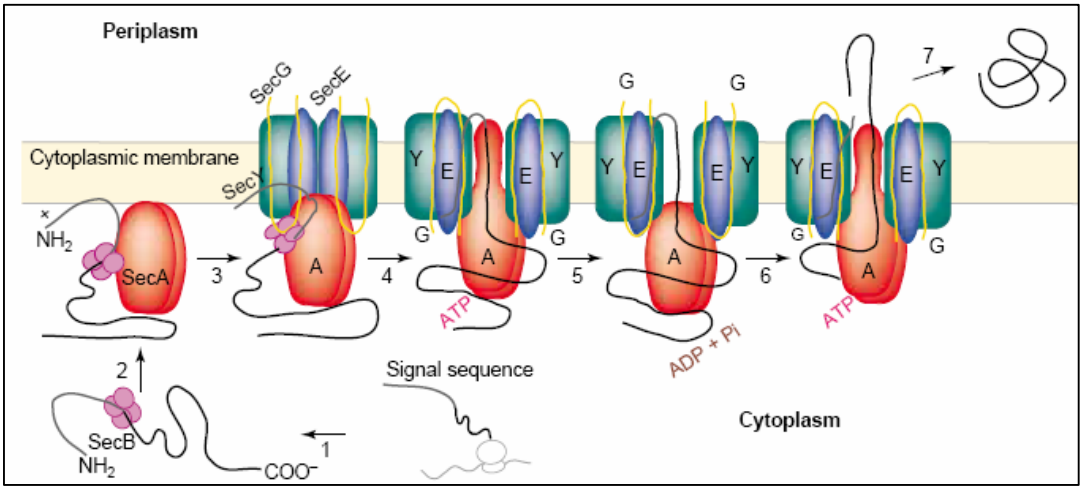



Figure 2.2. Sec factors and translocation processes (Mori and Ito, 2001)

Protein translocation by the Sec pathway is dependent upon both ATP and a proton-motive force. The proton-motive force results from a combination of membrane-potential ($\Delta\Psi$) and a pH gradient ($\Delta$pH) (Agarraberes and Dice, 2001).

The preprotein is represented by a black line, with the gray region showing the signal sequence. Steps from 1 to 3 represent targeting. A signal sequence and its immediate

carboxy-terminal region comprise an initiation domain that is recognized by the Sec machinery. SecB, the Sec-system-specific chaperone, channels the preprotein to the Sec translocation pathway and, additionally, actively targets the bound precursor to the translocase by its ability to bind SecA. The preprotein-bearing SecA then binds to the membrane, at a high-affinity SecA-binding site. SecY, SecE and SecG form a hetero-trimeric complex, SecYEG, which constitutes a pathway ('channel') for polypeptide movement. Steps 4 and 5 represent initiation. The initiation step requires ATP but not its hydrolysis. Step 6 represents continuation. Continued translocation requires cycles of ATP hydrolysis and/or proton-motive force across the membrane. Translocation is thought to occur in a step-wise fashion with a step of 20–30 amino acid residues. Step 7 represents completion. As yet, little is known about the completion process, which occurs on the periplasmic side, leading to the release and/or folding of the substrate protein into the periplasmic space (Mori and Ito, 2001).

### 2.1.2. The Twin Arginine Translocation (TAT) Pathway

The twin-arginine translocation (Tat) pathway is present in a wide variety of prokaryotes and is capable of exporting partially or fully folded proteins from the cytoplasm. Although diverse classes of proteins are transported via the Tat pathway, in most organisms it facilitates the secretion of a relatively small number of substrates compared to the Sec pathway (Dilks et al., 2005).

Proteins exported by this pathway have an unusually long signal sequence of up to 48 amino acids. The signal sequence has an invariant twin arginine motif in its amino-terminal region followed by a short hydrophobic sequence and one or more basic amino acids. A consensus twin arginine targeting motif is serine-arginine-arginine-X-phenylalanine-leucine-lysine (SRRXFLK) in which X can be any amino acid. The twin-arginine motif constitutes a 'Sec avoidance' signal. Replacement of the arginine-arginine motif by lysine residues, increasing the hydrophobicity of the middle region, or elimination of the basic amino acid in the carboxyl terminus renders this protein incapable of translocation by the TAT pathway. Any of these modifications in the signal peptide transforms the protein into a substrate for the Sec-dependent pathway (Agarraberes and Dice, 2001).

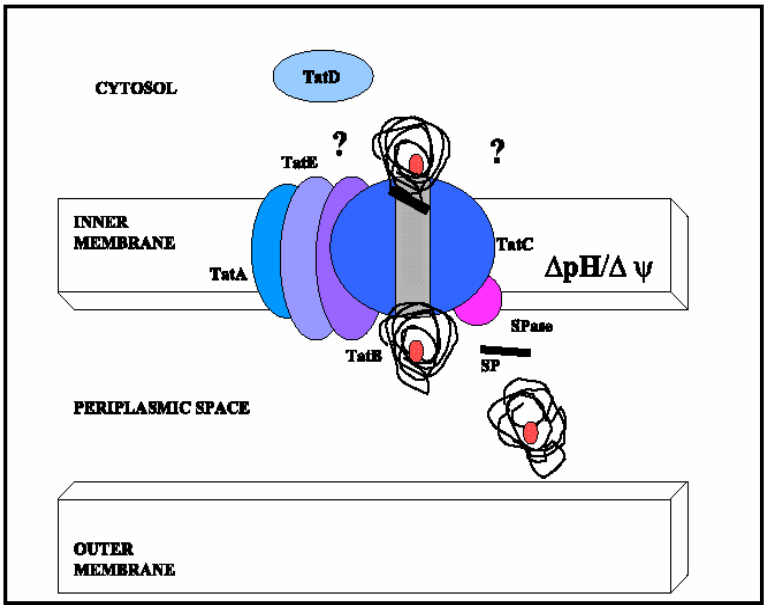



Figure 2.3. The twin arginine translocation pathway (Agarraberes and Dice, 2001)

TatA-E are proteins required for this pathway of protein translocation. ΔpH/ΔΨ indicates that a proton-motive force across the inner membrane is required for protein translocation. An unusual aspect of the TAT pathway is that proteins can be translocated across the plasma membrane in a folded state. (Agarraberes and Dice, 2001)

### 2.1.3. The Signal Recognition Particle Pathway

The SRP pathway in bacteria is involved primarily in translocation of inner membrane proteins. In addition, a small number of proteins are secreted by this pathway. A particular feature of this pathway is that insertion into the translocon takes place co-translation

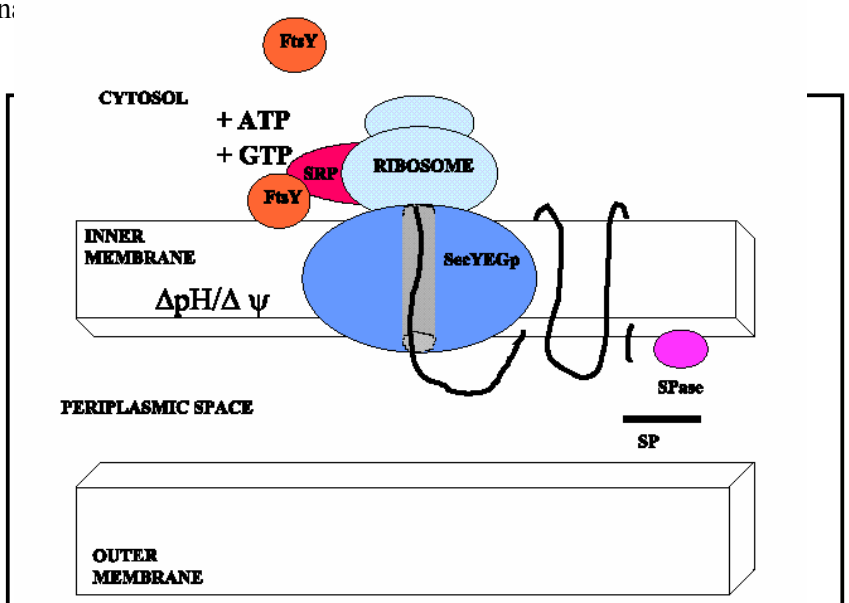


Figure 2.4. The signal recognition particle protein translocation pathway in bacteria (Agarraberes and Dice, 2001)

The SRP pathway uses the SecYEGp translocon as does the post-translational Sec pathway. Peptide bond elongation by the ribosome, together with GTP hydrolysis, pushes the protein through the translocon, and protein often integrates into the inner membrane through this pathway.

# 3. SERINE PROTEASE AND PULLULANASE FROM THERMUS THERMOPHILUS, MALTOSE BINDING PROTEIN AND pMAL-p2 VECTOR

To study protein translocation from the cytoplasm to the periplasm of *E. coli*, a system with the maltose-binding protein (*MalE* or MBP), a natural secretory protein, was used. In this section, the proteins of interest namely serine protease and pullulanase which were fused downstream from MBP to construct fusion proteins and targeted to the periplasmic compartment were described briefly, in addition to MBP and the vector pMAL-p2.

## 3.1. Serine Protease

Proteases have been widely used in industrial production for a long time, especially in the food, chemical, weaving, washing detergent and leather industries. Proteases from bacteria such as *B. licheniformis*, *B. subtilis*, *B. amyloiquefacillus*, *B. pumilus* and *B. alcaphilus* have been intensively studied previously (Pan et al., 2004). Proteases are the group of enzymes that catalyse the hydrolysis of covalent peptidic bonds. In the case of serine proteases the mechanism is based on nucleophilic attack of the targeted peptidic bond by a serine. Serine protease is an enzyme that belongs to the protease family. In mammals, serine proteases (SPs) feature a wide variety of important physiological functions, including digestion, blood coagulation, fibrinolysis, fertilization, embryonic development and immunity (Jimenez-Vega et al., 2005). The N-terminal sequence of this enzyme was analyzed and the absence of a signal sequence indicated that this protease is cytoplasmic.

Thermophiles such as *Thermus* species are known to produce highly thermostable enzymes. Serine protease from *T. thermophilus* is a polypeptide of 251 aminoacids. The nucleotide sequence for serine protease of *T. thermophilus* (location from 983,377 to 984,132 in *T. thermophilus* genomic DNA) is given below:

1
ATGCGCGGCCTCGTGCTTTCCGGAGGCGGGGCGAGGGGGTTCGCCCACAT
51
CGGCGCCCTGGAGGTCTTCGTGGAGGCGGGGCTGGACTTTGAGGTGGTGG
101
CCGGGGCGAGCATGGGGGCCATCGTGGGGGCGCTTTTCGCCGCGGGAAGA
151
AGCCCTCAGGAGATCCTGGAGATCGCCCGGAACACCCCCTGGCTCGGGAT
201
TTTGGGCTTCTCCTTTCGGGAGGCCCTCTTCTCCCGGGGGCGGCTCAGGC
251
GGTACCTCGCCGAGCACCTGCCCCAGGACTTCGCCGACCTCAAGCGCAAG
301
CTCGTGGTCACGGCGGTGGAGGTGCGGAGCGGGCGGGTGGTCTACCTCTT
351
CCAGGGCGACCTGGTGAGCGCGGTCTTGGCCTCGGCGGCCCACCCCCTCC
401
TCCTCAGGCCGGTGCGGCGGGAGGGCCTCCTCCTCTTTGACGGCGGGGTC
451
CTGGACAACCTGCCCGTGGACGCGGCCAGGTTTTTGGGGGCGGAGGAGGT
501
TGGGCCGTGGACGTGACCCCGGAGCGGAGCGTGGAAAAGCCCCCCGAGG
551
GGGTTTTGGGCCTCGCCCTCAGGGCGGTGGACCTGATGCAGCACCACCTC
601
ACGGCAAGCCGCATGGCCCTCTACGCCCCCGAGGTCTACCTGAGGCCCGA
651
GCTTGGGGAGGTCGCGGTGCAGGACTTCCTCCGCCTCGAGGAGGCGGTGG
701
AGGCGGGCAGGAAGGCGGCCAAGCGCTTTCTGGAGAGTAGAGTGGGGGAG
751
GTATGA

### 3.2. Pullulanase

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) hydrolyzes the (1-6)-a-glucosidic linkages in pullulan, which is produced by *Aureobasidium pullulan*s, and starch to form maltotriose as the final product. This enzyme was discovered by Bender and Wallenfels in *Aerobacter aerogenes*, strains of which are now classified as members of the genus Klebsiella. Pullulanase is used to elucidate the structures of polysaccharides and to produce useful materials such as maltose, amylose, and glucose by debranching starch with and without α-amylase, α- amylase, or glucoamylase. Pullulanase is normally a periplasmic protein. However, the used in this study was reported to be a cytoplasmic protein (Tomiyasu et al., 2001)

Several pullulanases have been reported for thermophilic bacteria. Especially *Thermus* is a suitable source of a thermostable pullulanase (Tomiyasu et al., 2001). Pullulanase from *T. thermophilus* HB8 is a polypeptide of 718 aminoacids. The nucleotide sequence for pullulanase of *T. thermophilus* (location from 2218 to 4374 in *T. thermophilus* genomic DNA) is given below:

1
ATGCTTCACATCAGCCGAACGTTTGCCGCCTATTTGGACGAGATGGATCA
51
AATCGTTGTGCTTGCGCCGAAATCGCTCGGCTTTGATGGAATGGCGCCGT
101
TACGCTCGTGGCGCCGAGCGGCGAGGAGATTCCGCTGTCCGTGCAGCAC
151
GTCGAGGATGTTGGGGAGACGGTGAAATATGTGTGCCGGTTTGCATCCGC
201
GTTCGAGTTTGGAGCGACATACTGGGTGCGTTCTTGCCGCGGGGAGGAGA
251
CGATGTTCAAATCGGCGCCGTTGTGCGCACTCCTGCATTTGATGATCGGT
301
TTTTCTATGATGGACCGTTAGGAGCGGAGTATCTCAAAGAACAGACGGTA

351
TTTCGCGTATGGGCGCCGACCGCCACCGCGGTTAGCGTCAAGCTGGTTCA
401
TCCGCATCTCGACGAGATCCGCTGCGTGCCGCTTGTGCGCGGCGAACGCG
451
GCGTATGGTCAGCCGTCGTCCCCGGCGATTGGGAGCGAGCGCGTTACACA
501
TATATCGCCTGCATCAACCGCGTATGGCGCGAGGCAGTGGACCCGTATGC
551
GACCGCGGTTTCGGTCAATGGCGAGTTCGGCGTCGTGATCGACTGGGAGA
601
AAACGAAGCTGGCGCCGCCCTCTTTGCCGCTTCCACCGCTCTGTTCGCCGA
651
CGGATGCCATCATTTATGAGCTGAGCATCCGCGACTTTACCAGCCACCCGG
701
ACAGCGGCGCCGTCCATAAAGGGAAGTATCTCGGGCTGGCCGAAACGAAC
751
ACGAGCGGGCCGAACGGGACGGCCACCGGGCTTTCGTATGTCAAAGAGCT
801
GGGCGTCACCCATGTGCAGCTCATGCCGTTTATGGACTTTGCGGGCGTCGA
851
TGAGCGCGACCCACAAGCGGCTTACAACTGGGGATACAATCCCCTTCATCT
901
ATATGCGCCGGAAGGGAGTTATGCGACCGATCCAGCGGATCCATACGCGC
951
GCATTGTAGAATTGAAGCAGGCGATCCACACGCTGCACGAAAATGGCTTG
1001
CGCGTCGTGATGGATGCGGTCTACAACCATGTCTATGACCGGGAGCAATC
1051
GCCGCTTGAGAAGCTCGTTCCCGGTTATTACTTCCGCTACGACGCCTATGG
1101
CCAACCGGCCAACGGCACCGGCGTCGGCAACGACATCGCTTCGGAGCGGC

1151
GGATGGCGCGCCGCTGGATCGTCGATTCGGTGGTGTTTTGGGCGAAAGAA
1201
TATGGCATTGACGGGTTCCGCTTTGATTTGATGGGCGTGCACGATATCGA
1251
GACGATGAAAGCGGTGCGCGATGCCCTCGACGCCATCGATCCGTCGATCC
1301
TTGTGTATGGGGAAGGGTGGGATTTGCCGACGCCTCTTCCACCGGAACAA
1351
AAGGCGACGATGGCCAACGCCAAGCAGCTGCCGCGCTTCGCTTATTTCAA
1401
TGACCGGTTTCGCGATGCGGTGAAAGGGAGCACCTTTCATTTGCCGGACC
1451
GTGGGTTCGCCCTCGGCAACCCAGGCGGGCGAGAACAGGTGAAGCTCGCC
1501
ATTGCCGGGAGCTTGCGAGCGCTCGGCGGGCTGTTTTGCCACCCGCGTCA
1551
GTCAATCAATTACGTCGAATGTCATGACAACCATACGTTTTGGGATAAGA
1601
TGGAGGCGGCCAACCATGATGAGCCGGAATGGCTCCGGCGAAAGCGGCAA
1651
AAGCTGGCGACGGCGATCGTTCTGTTGGCGCAAGGCATTCCGTTTTTGCA
1701
CAGCGGCCAAGAGTTTTATCGGACGAAAGGCGGCGATGGGAACAGCTACC
1751
GATCGCCGGATGCGGTCAATCAGCTGGATTGGGAGCGGAAAAGCCGCTAT
1801
GAAGACGACGTCCGCTACGTTCAAGGATTGATCGCCCTTCGCCGTGCGCA
1851
TGGCGCATTTCGCCTCGCCACGGAGGCGGAAGTGCTGCGTCATTTCACGT
1901
TTCTTGAGCCGCTGCCGCCGTCGGTCATCGCCTACCGATTGCATGATGCC

1951
GCCGTCTATGGGCCTTGGGAGGACATCATCGTCGTGCATCATAACGAGGA
2001
GAAAGAGACAGCCATTGCGCTCCCCGACGAGCGCGAGTGGGCGGTTGTAT
2051
GCGACGGACAGCGATGCGGGACAACGCCCTTTGGCCAAGCGCGCGGCATG
2101
CTTCGGCTTGACGGCATCGGCACATGGGTGCTCGTCCATCCTGCAGGGTG
2151
A

### 3.3. Maltose Binding Protein

Maltose binding protein (MBP or MalE), the *malE* gene product, serves as the periplasmic receptor for high-affinity membrane transport of maltose and maltodextrins in *E. coli*. Because of its role in the maltose transport system, export of MBP into the periplasm is essential for cells to utilize maltose as a carbon source. MBP has also been extensively used as a model of protein translocation across the cytoplasmic membrane.

MBP is synthesized as a precursor, preMBP, with an N-terminal signal sequence that is cleaved off during or shortly after translocation across the cytoplasmic membrane. Translocation requires that the nascent preMBP polypeptide chain reaches a critical molecular weight corresponding to 80 per cent of the final length, and that the precursor exists in an export-competent conformation representing a partially unfolded state. Both the signal peptide and the binding of SecB, the molecular chaperone involved in protein secretion, participate in the maintenance of this initial conformation (Betton and Hofnung, 1994).

Maltose Binding Protein (MBP) is the product of the *malE* gene of *E. coli* K12. *MalE* gene encodes the pre-protein of 396 amino acid residues, 26 of which constitute the signal peptide. The expression of MBP is controlled by *malEp*, a strong promoter that is activated by protein MalT in the presence of inducing maltose or maltodextrins and repressed by glucose. It is a binding protein specific for maltose and maltodextrins. The

binding site recognizes the αl-4 glycosidic bond, linking the glucose moieties of maltose. MBP is exported across the cytoplasmic membrane and localized in the periplasm via its signal sequence, and it is essential for the energy dependent translocation of maltose and maltodextrins through the cytoplasmic membrane (Duplay et al., 1984).

The use of MBP as a fusion partner in the expression and purification of foreign proteins in *E. coli* has many advantages. MBP and its precursor can be purified by affinity chromatography on crosslinked amylose and then the proteins can be eluted by competition with maltose. Hence, fusion proteins can be purified from cell extracts in one step with high yield and high degree of purity. The materials used are easy to prepare and inexpensive and cross-linked amylose is reusable, making large-scale purification simple and feasible. MBP fusion systems can also contain the sequence coding for the recognition site of a specific protease allowing MBP to be cleaved from the protein of interest after purification. Moreover, the purification takes place under very mild, physiological conditions, enabling the protein to maintain its full activity (Maina et al., 1988).

### 3.4. pMAL-p2

In the protein fusion and purification system, the cloned gene is inserted into a pMAL vector down-stream from the *malE* gene, which encodes maltose-binding protein (MBP). This results in the expression of an MBP-fusion protein. (Maina et al.,1988)

The technique uses the strong $P_{tac}$ promoter and the translation initiation signals of MBP to express large amounts of the fusion protein. The fusion protein is then purified by a one-step affinity purification for MBP. It is 6721 base pairs long and has an ampicilin resistant gene. There are approximately 20 copies of the vector per cell. In the large majority of the cases, fusion proteins expressed from a pMAL-p2 plasmid constitutes 1-5 per cent of the total cellular protein.

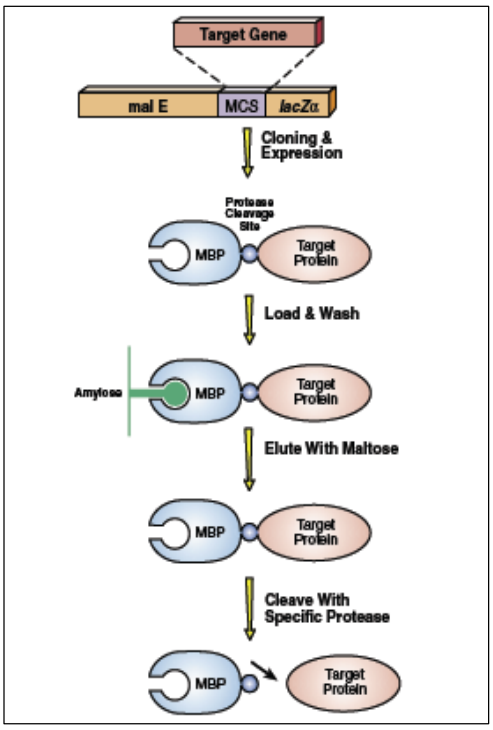



Figure 3.1. The protein fusion and purification system (Kellerman and Ferenci, 1982)

The vector expresses the *malE* gene fused to the *lacZ*α gene. Restriction sites between *malE* and *lacZ*α are available for inserting the coding sequence of interest to generate fusions with a foreign polypeptide. β-galactosidase α-fragment activity of the *malE-lacZ*α gene fusion results in blue colony formation when α-complementing hosts such as TB1 or JM109 are transformed with the pMAL-p2 vector. Insertion of a DNA fragment into the polycloning site interrupts the *malE-lacZ*α gene fusion, destroying α-fragment activity and hence allowing blue-white screening for inserts. There is a spacer sequence coding for ten asparagines residues between the *malE* gene and the polylinker. This spacer insulates MBP from the target gene increasing affinity binding to amylose resin and allows a one-step purification of the fusion protein (Kellerman and Ferenci, 1982). The vector also has a sequence coding for the recognition site of Factor Xa which cleaves after the amino acids Ile-Glu-Gly-Arg allowing the removal of the MBP fusion tail after purification. The polylinker includes an *Xmn* I site superimposed on the Factor Xa site. When this site is used for cloning, no vector-derived residues remain on the target protein upon cleavage with Factor Xa.

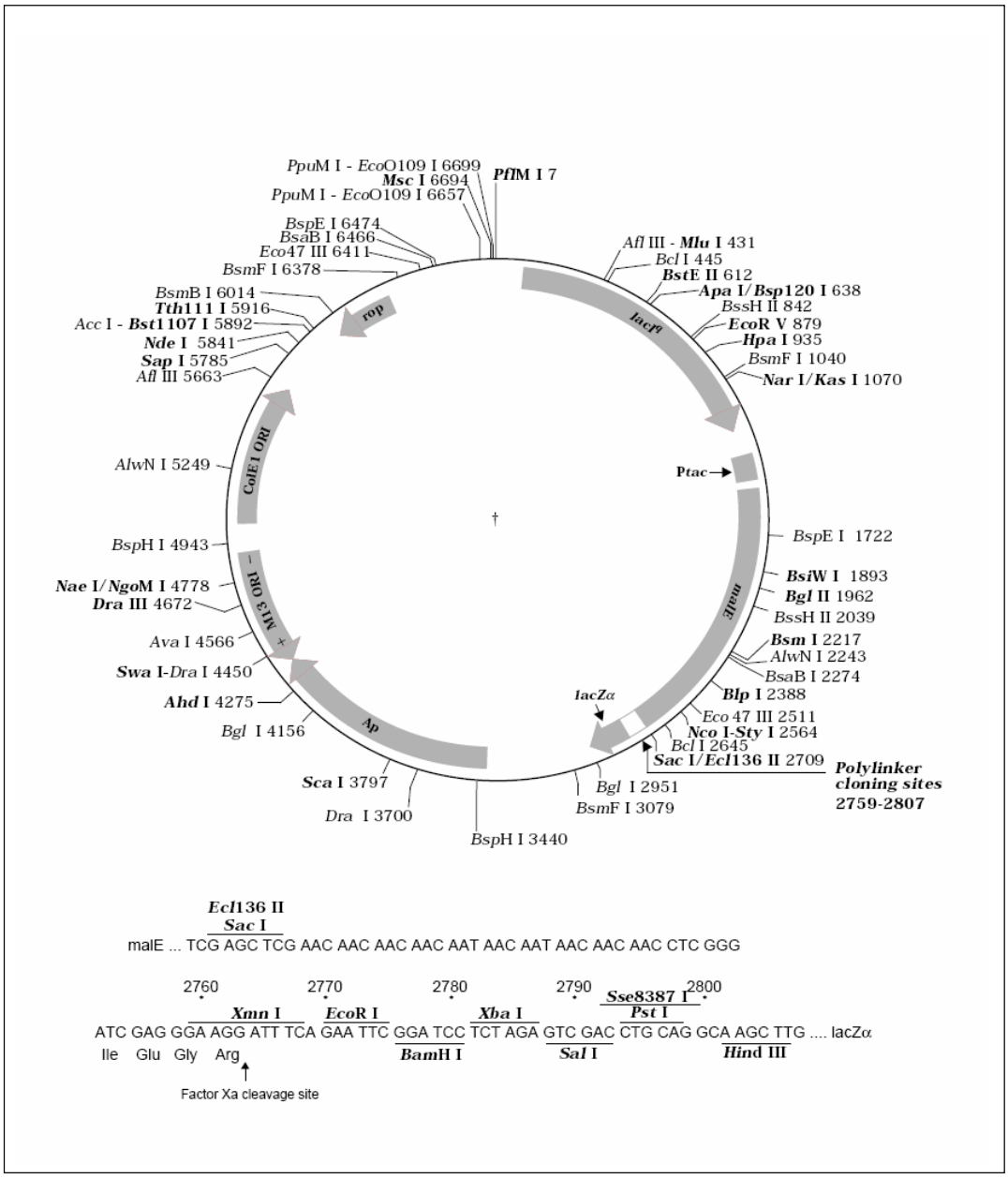



Figure 3.2. pMAL-p2 expression system

# 4. MATERIALS

## 4.1. Bacterial Strains and Plasmids

The source of serine protease and pullulanase genes, *T. thermophilus* HB8 was purchased from ATCC. *E. coli* XL1 strain was (*endA1*, *hsdR17* (*rk-, mk+*), *supE44, thi-1, λ-, recA1, gyrA96, relA1,* Δ(*lac*), [F' *proAB, lacI*$^{q}$*Z* Δ *M15, Tn10(tet*$^{r}$*)*]) was provided by TÜBİTAK, Marmara Research Center.

## 4.2. Chemicals

All chemicals and solutions used in this study were from MERCK (GERMANY) or SIGMA (USA) or DIFCO (USA) unless stated otherwise in the text. The restriction endonucleases were purchased from PROMEGA (USA) and New England Biolabs (USA).

## 4.3. Bacterial Cell Media

The media used throughout this study were LB medium. 80-100 μg/ml of ampicillin was added into either the liquid medium or the agar medium after they cooled down to 50°C following sterilization.

LB Medium

| | |
|---|---|
| Tryptone | 10 g |
| Yeast Extract | 5 g |
| NaCl | 5 g |

pH = 7.0

per liter of deionized and distilled water.

LB-Agar Medium

| | |
|---|---|
| Agar | 15-20 g |

pH = 7.0

per liter of LB Medium.

## 4.4. Buffers and Solutions

### 4.4.1. Polyacrylamide Gel Electrophoresis Buffers

| | |
|---|---|
| TEMED | N,N,N,N-tetramethylethylenediamine |
| Acrylamide-bisacrylamide mixture | (30:0.8) |
| Acrylamide | 29.2 g |
| N'N'-bis-methylene-acrylamide | 0.8 g |
| $dH_2O$ | to 100 ml. |

| Electrophoresis Separating Gel (10%) | |
|---|---|
| 1.5M Tris-HCl (pH 8.8) | 2.50 ml |
| Acrylamide/Bis(30:0.8) | 3.33 ml |
| 10%(w/v) SDS | 100 µl |
| 10%(w/v) Ammonium persulfate | 50 µl |
| TEMED | 5 µl |
| $dH_2O$ | to 10 ml. |

| Electrophoresis Stacking Gel (5%) | |
|---|---|
| 0.5M Tris-HCl (pH 6.8) | 2.5 ml |
| Acrylamide/Bis(30:0.8) | 1.7 ml |
| 10%(w/v) SDS | 100 µl |
| 10%(w/v) Ammonium persulfate | 50 µl |
| TEMED | 10 µl |
| $dH_2O$ | to 10 ml. |

| Sample Buffer | |
|---|---|
| 0.5M Tris-HCl (pH 6.8) | 1.0 ml |
| Glycerol | 0.8 ml |
| 10%(w/v) SDS | 1.6 ml |
| 2-β-mercaptoethanol | 0.4 ml |
| 0.05%(w/v) Bromophenol blue | 0.2 ml |

5X Running Buffer

| | |
|---|---|
| Tris-base | 15 g |
| Glycine | 72 g |
| SDS | 5 g |

complete to one liter with $dH_2O$.

Stain

| | |
|---|---|
| Coomassie blue R-250 | 0.1 % |
| Methanol | 40.0 % |
| Acetic acid | 10.0 % |

Destain

| | |
|---|---|
| Isopropanol | 25.0 % |
| Acetic acid | 10.0 % |

Fixing

| | |
|---|---|
| Acetic Acid | 7 % |

**4.4.2. Agarose Gel Electrophoresis Buffers**

10X Loading Buffer

2.5 mg/ml bromophenol blue

1% SDS in 1 ml glycerol.

5X TBE Buffer

445 mM Trizma base

10 mM EDTA

445 mM Boric acid (pH 8.3)

| | |
|---|---|
| Ethidium bromide | 10 mg/ml |

### 4.4.3. Buffers and Solutions for Plasmid DNA Isolation

STE Buffer
10 mM Tris-HCI (pH 8.0)
100 mM NaCl
1 mM EDTA (pH 8.0)

Solution I
25 mM Tris-HCl (pH 8.0)
50 mM D-glucose
10 mM EDTA (pH 8.0)

Solution II
0.2 M NaOH
1% SDS

Solution III

| | |
|---|---|
| 5 M Potassium Acetate | 60 ml |
| Glacial Acetic Acid | 11.5 ml |

per 100 ml of $dH_2O$

TE Buffer
10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

### 4.4.4. DNS (dinitrosalicylic acid) Solution

| | |
|---|---|
| NaOH | 2 g |
| DNS | 1 g |
| Rochelle Salt (Sodium-potasium tartarate) | 30 g |

Per 100 ml $dH_2O$

### 4.5. Kits

Plasmid Midi Kit (25), Plasmid Mini Kit (100) and Qiaquick Gel Extraction Kit (50) were purchased from QIAGEN.

### 4.6. Laboratory Equipments

| | |
|---|---|
| Autoclave | Medexport, CIS, C.W.I.S. Model MAC-601 (Eyela, Japan) |
| Balance | 80A-200M (Precisa, Switzerland)- |
| Centrifuges | SORVALL RC-5B Refrigirated Superspeed Centrifuge (DuPont, USA) |
| | Biofuge 28 RS (HERAEUS, Germany) |
| | Centrifuge 5415 C (EPPENDORF, Germany) |
| Cold Room | VWR SCIENTIFIC, VCR 422DBA (USA) |
| Deepfreezers | -80°C, (HETO, Denmark) |
| | -20°C, (BOSCH, Germany) |
| | -20°C, (ARÇELIK, Turkey) |
| Fraction Collector | Model 2110, (BIORAD, USA) |
| Freeze Drier | VR-1, (HETO, Denmark) |
| | GD-1, (HETO, Denmark) |
| Gel Dryer | Vacuum Dryer, (BIOMETRA, Germany) |
| Ice Machine | FBOC, (Akteknik, Turkey) |
| Incubators | EN500, (NÜVE, Turkey) |
| | FN500, (NÜVE, Turkey) |
| | Lab-Line Ambi-High-Low Chamber, (Lab-Line Instruments, USA) |
| Laminar Flow | HBB 2460 LaminAir, (Holten, Denmark) |
| Orbital Shakers | GFL 3032, (GFL,Germany) |
| | INNOVA 4340, Illuminated refrigerated Incubator Shaker, (New Brunswick Scientific,USA) |
| pH meters | HI 8521, (HANNA Instruments, Singapore) |

| | |
|---|---|
| | inoLab pH level 1 (WTE. Germany) |
| Power Supply | Power/Pac 300, (BIORAD, USA) |
| Pumps | Econo Pump, (BIORAD, USA) |
| | TVP 200, (Sem, Turkey) |
| Refrigerators | +4°C, (ARÇELİK, Turkey) |
| | +4°C, (SİMTEL, Turkey) |
| Sonifier | Branson Sonifier 450, (VWR Scientific, USA) |
| Spectrophotometer | Beckman Du 640 (Beckman, USA) |
| UV Monitor | EconoUV Monitor, (BIORAD, USA) |
| Vortex | ELEKTROMAG, Turkey |
| Water Baths | BM102, (NÜVE, Turkey) |
| | DT Hetotherm, (HETO, Denmark) |
| | CB 8-30e $DT_1$, (HETO, Denmark) |
| | CB 8-30e $AT_{110}$, (HETO, Denmark) |
| Water Purification Systems | MILLI-Q UF Plus, (MILLIPORE, USA) |
| | MILLI-RQ Plus, (MILLIPORE, USA) |
| | Model 2004, (GFL, Germany) |

# 5. EXPERIMENTAL METHODS

During the experiments, sterilized equipment was used. Pipetman tips, eppendorf tubes, centrifuge tubes, columns, solutions and culture media were all sterilized at 1.02 atm and 121°C for 20 minutes in an autoclave. Glassware was sterilized at 180°C for two hours in an oven.

## 5.1. Preparation of Preculture

Recombinant *E. coli* XL1 cells were kept in glycerol solutions at -80°C. Frozen glycerol cultures were used by streaking on LB agar plates for the preparation of master plates. During the experiments, subplates, produced from the master plates, were prepared to pick single colonies.

Preparation of plates, inoculation and all other experimental work that require sterile environment were carried out under sterile laminar flow.

10 ml of sterile liquid nutrient medium was inoculated with a single colony of bacteria from a slant culture. The inoculating loop was sterilized by heating in a flame and cooled on the agar plate. The preculture was incubated in an orbital shaker at 37°C overnight. This preculture was used to inoculate the working volume of the medium used.

## 5.2. Determination of Dry Cell Weight

Cells were grown overnight in 100 ml of medium in a 500 ml of flask. The cells were centrifuged and washed with distilled water for two to three times. 50 ml of the cells, resuspended in 100 ml of distilled water were dried and the dry cell weight was determined. The remaining 50 ml, with the optical density 0.4-0.6 was used to prepare samples of 0, 2, 4, 6, 8 and 10 ml. All volumes were completed to 10 ml by distilled water. $OD_{600\ nm}$ values were read in a spectrophotometer and cell dry weight for each sample was determined. Calibration chart was prepared by plotting optical density vs. cell dry weight.

The slope of this graph was used to determine the dry cell weight of a known $OD_{600nm}$ value.

### 5.3. Plasmid Isolation

Three methods were used to isolate plasmid from the transformants; namely large-scale plasmid isolation, mini-prep method for isolation of up to 20 µg of plasmid DNA, and midi-prep method for isolation of up to 100 µg of plasmid DNA.

#### 5.3.1. Large-scale Plasmid Isolation

Large scale plasmid isolation was carried out by alkaline lysis method (Maniatis et al., 1989). 500 ml LB medium containing 100µl/ml ampicillin was inoculated with 5 ml of the overnight culture and incubated for 16-20 hours at 37ºC with vigorous shaking.

Bacterial cells were harvested by centrifugation at 9000 rpm for 6 minutes at 4ºC. The pellet was resuspended in 100 ml STE buffer and collected by centrifugation at the previous conditions.

The washed bacterial pellet was resuspended in 18 ml Solution I containing 100 µg lysozyme. The cells were lysed by the addition of 40 ml of Solution II with gentle swirling. 20 ml of ice-cold Solution III was then added to the bacterial lysate to neutralize the alkali and shaken vigorously until a flocculent white precipitate formed. The precipitate contains cell debris and chromosomal DNA. After 15 minutes of incubations on ice, the lysate was centrifuged at 6000 rpm for 15 minutes at 4ºC and the supernatant was filtered into a sterile centrifuge tube.

The nucleic acids were precipitated by the addition of 0.6 volume of isopropanol was added to the supernatant, mixed, and incubated 30 minutes at room temperature. Nucleic acids were recovered by centrifugation at 10000 rpm for 15 minutes at 4ºC. The pellet was dissolved in sterile TE buffer.

DNAse free RNAse was added to this solution to a final concentration of 10 μg/ml and the mixture was incubated for one hour at room temperature.

### 5.3.2. Mini-Prep Method Plasmid Isolation

QIAGEN plasmid mini purification kit was used to obtain up to 20 μg of plasmid DNA. DNA was isolated from overnight cultures of E. coli picked from single colonies.

### 5.3.3. Midi-Prep Method Plasmid Isolation

QIAGEN plasmid midi purification kit was used to obtain up to 100 μg of plasmid DNA.

## 5.4. Determination of DNA Content

Spectrophotometric measurement and agarose gel electrophoresis were used to quantify and qualify DNA.

### 5.4.1. Spectrophotometric Measurement

DNA was diluted to ratios of 1:1000, 1:333.3 and 1:166.7 and the UV absorption of DNA was read at 260 and 280 nm using quartz cuvettes. Since 50 μg of double stranded DNA has an absorbance of 1.0 at 260 nm, the concentration of the DNA in each sample was calculated using the formula:

$$50\ \mu g/ml \times OD_{260} \times \text{Dilution Factor} = \text{Concentration in } \mu g/ml \qquad (5.1)$$

The values were averaged to obtain the concentration of the plasmid DNA. The OD260/OD280 ratio was calculated to determine the purity of the DNA samples. A ratio of 1.8 was considered to be pure whereas higher and lower values of the ratio suggested RNA and protein contamination respectively.

### 5.4.2. Agarose Gel Electrophoresis

Gels were prepared by boiling agarose in 0.5 x TBE, cooling to 50 ºC, adding ethidium bromide to a concentration of 5 µg/ml and solidifying this in the gel former. Different dilutions of DNA samples were loaded on the agarose gel, after mixing with 0.5 volume of DNA loading buffer and were run at 150 V with a standard DNA marker. Ethidium bromide intercalades DNA, and in this state fluoresces when illuminated by UV light. DNA was visualized by illuminating the gel with UV light on a transilluminator. The unknown amount was estimated by comparing the intensity of fluorescence in each sample.

## 5.5. Cloning

### 5.5.1. Cloning of Serine Protease Gene into pMAL-p2 Plasmid Vector

Genomic DNA from *T. thermophilus* was used as a template for the PCR amplification of the serine protease gene using the designed primers. The PCR reactions were carried out in a reaction volume of 25 µl containing 12.5 pmole of both forward and reverse primers, 2.5 µl of 10X buffer with 25 mM $Mg^{++}$ , 0.25 mM dNTP, 1 unit of VentR DNA polymerase and 0.2 µg of *T. thermophilus* genomic DNA.

Then the PCR conditions were optimized using melting temperatures of the primers. Melting temperature for the forward and the reverse primers were calculated as 64.8 and 59.1 °C, respectively. Initial denaturation was at 94ºC for 5 minutes. This was followed by thirty cycles of thirty seconds denaturation at 94ºC, thirty seconds of primer annealing at 55 ºC and 1.5 minutes of chain elongation at 72 ºC, ending with ten minutes of incubation at 72 ºC. The reaction was checked for amplification on an agarose gel.

### 5.5.2. Cloning of Pullulanase Gene into pMAL-p2 Plasmid Vector

The recombinant plasmid constructed by Prof. Sakai was used as a template for the PCR amplification of the pullulanase gene using the designed primers. The PCR reactions were carried out in a reaction volume of 25 µl containing 12.5 pmole of both forward and

reverse primers, 2.5 µl of 10X buffer with 25 mM $Mg^{++}$ , 0.25 mM dNTP, 1 unit of VentR DNA polymerase and 0.2 µg of pTPi61 plasmid.

Then the PCR conditions were optimized. Melting temperature for forward and reverse primers were 63.3 and 62 °C, respectively. Initial denaturation step was at 94ºC for 5 minutes. This was followed by thirty cycles of thirty seconds of denaturation at 94ºC, thirty seconds of primer annealing at 55 ºC and 1.5 minutes of chain elongation at 72 ºC, ending with ten minutes of incubation at 72 ºC. The reaction was checked for amplification on ana agarose gel.

### 5.6. Isolation of PCR Products from Low-Melting Temperature Agarose Gels

The PCR amplification product was purified from excess primers, dNTPs, DNA polymerase and unspecified products using low melting point agarose gel by QIAquick Gel Extraction Kit. DNA fragments were excised from the gel with a clean, sharp scalpel. The the protocol, povided by the manufacturer, was followed.

### 5.7. Digestion of the Inserts and The pMAL-p2 Vector with Restriction Endonucleases

After purification and quantification, the PCR product was digested with *XmnI* and *EcoRI*. The mixture was incubated at 37ºC overnight. Then the digest was cleaned up using the QIAquick Gel Extraction Kit as described by the manufacturer

The plasmid vector pMAL-p2 was also digested with *XmnI* and *EcoRI* under the same condition. Then the digest was again cleaned up using the QIAquick Gel Extraction Kit as describedby the manufacturer. The inserts and the plasmid vector were stored at -20ºC untill ligation reaction.

### 5.8. Ligation Reaction

100 ng of pMAL-p2 and 300 ng of insert were mixed in a microfuge tube and incubated at 65ºC for five minutes and were then cooled on ice. 1µl of 10 X Ligase Buffer

and $8x10^5$ U T4 DNA Ligase were added to a final volume of 10 µl. The reaction mixture was incubated at 4ºC overnight. The results of ligation were verified on agarose.

## 5.9. Transformation

$CaCl_2$ method was used to transform the recombinant plasmid into the host cell. Ten ml LB medium was inoculated with one 100 µl of overnight culture and was incubated with vigorous shaking at 37 ºC until the $OD_{600}$ reached 0.5. The culture was separated in to one ml aliquods of one ml and centrifuged at 3000 rpm for ten minutes. The pellet was dissolved in 100 µl of 100 mM $CaCl_2$ and chilled on ice for thirty minutes. One-two µl of ligation product was added, and chilled on ice for one hour. After a heat shock at 42 ºC for one minute, the mixture was chilled on ice for ten minutes. 800 µl of LB medium was added and incubated at 37ºC for one hour. Then the cells were centrifuged at 3000 rpm for five minutes and the pellet was redissolved in 100 µl of LB medium. Different dilutions of the transformed cells were spread on LB agar plates containing ampicillin and incubated at 37 ºC for 16-20 hours.

## 5.10. Selection of the Recombinant Cells

### 5.10.1. Blue-White Selection

Many vectors carry coding information for the first 146 amino acids of the β-galactosidase gene. Embedded in this coding region is the polycloning site into which insert DNA is cloned. When expressed, this 146 amino acid fragment of β-galactosidase protein is incapable of acting on the chromogenic substrate, X-gal. But when expressed in appropriate host cell which expresses the carboxyl terminal fragment of the β-galactosidase protein, these two proeşn fragments can associate to form an enzymatically active protein. This is called as α-complementation and such cells turn blue when plated on plates containing X-gal. But if the insert DNA has gotten cloned in the polycloning site, those colonies remain white.

Each colony on the LB plate with ampicillin was transferred to a new LB agar plate containing ampicillin (100 µg/ml), X-Gal (10 µg/ml), and IPTG (1 mM). The colonies

were left to grow at 37ºC overnight. The colonies that turned into blue were the ones harboring only the plasmid vector, whereas the white ones were the recombinant colonies harboring the insert and the plasmid. The white colonies were further tested and their plasmids were isolated by QIAGEN mini-prep plasmid isolation kit.

### 5.10.2. Cracking

The plasmids of the white colonies were analyzed by cracking method. Single colonies from the agar plates were picked and dissolved in 25 µl of 10 mM EDTA, 25 µl of freshly prepared 10X cracking buffer (pH 8.0) and the samples were incubated at 70 ºC for 15 minutes and then cooled down to room temperature. 1.5 µl of 4M KCl and 0.5 µl of 0.4 per cent bromophenolblue were added, and the cells were vortexed and incubated for ten minutes on ice. The samples were centrifuged at 12000 rpm for three minutes and the supernatant was checked on agarose gel.

### 5.10.3. Restriction Enzyme Analysis

Recombinant plasmid DNAs were single digested with *EcoRI* and double digested with *EcoRI* and *XmnI* to check presence and orientation of the gene of interest. Restriction enzyme analysis was carried our at 37 ºC overnight. The digestion products were checked on agarose gel and the colonies with the correct plasmid were selected.

## 5.11. Shake-flask Growth of Recombinant *E. coli* Cells and Expression of Recombinant Proteins

Recombinant *E. coli* cells were grown in LB medium supplemented with 100mg/ml ampicillin for the selection of the plasmid harbouring cells in orbital shakers at 37°C at 180 rpm. Culture volume was kept at one fifth of the flask volume for efficient aeration. Growth was monitored by measuring the optical density (OD) at 600 nm using a spectrophotometer. The chemical inducer IPTG (isopropyl-β-D-thiogalactopyranoside) [0-2.0mM] was added to the culture, since the inserts pullulanase and serine protease were cloned downstream from the *mal*E gene of *E. coli* which encodes for the periplasmic MBP. *E. coli* cells harboring the recombinant plasmids pOSP and pOPU express the fusion

proteins MBP-serine protease and MBP-pullulanase under control of the strong *tac* promoter .

Recombinant cells were incubated for 24 hours after induction. 30 ml samples were taken at random time intervals. Cell growth was measured by spectrophotometer at $OD_{600}$. For OD values higher than 0.7, the samples where diluted.

## 5.12. Detection of the Expressed Fusion Proteins

### 5.12.1. Preparation of Cell Extracts

Cytoplasmic and periplasmic fractions were prepared and the distribution of enzyme activities in these three compartments was determined.

Induced cells from samples were harvested by centrifugation at 6000 g for 20 minutes at 4$^{o}$C. The supernant was removed and the cells were resuspended in Tris-HCl buffer, pH 8.0. The periplasmic proteins were released from the precipitated cells by osmotic shock as described by Harrison et al., 1996. The harvested cells were resuspended in 0.02 culture volume of 30 mM Tris-HCl containing 20 per cent glucose. 1 mM EDTA was added to the suspension and incubated for 20 minutes at room temperature with gentle stirring. Then the cells were reharvested by centrifugation at 9000 rpm for 20 minutes at 4$^{o}$C and shocked by resuspending and gentle stirring for 20 minutes in 0.03 culture volume of ice-cold 5 mM $MgSO_4$. Cells, separated from the osmotic shock fluid, were dissolved in 50 mM Tris-HCl buffer.

The cytoplasmic cell extract was prepared by ultrasonication on ice. Insoluble proteins and membranes were removed from the lysate by centrifugation at 9000 rpm for 30 min at 4$^{o}$C. To determine the enzyme activity, the extracts from the different compartments were assayed. The presence of the fusion protein in these three compartments was shown by SDS-PAGE electrophoresis.

### 5.12.2. Determination of Serine Protease Activity

5.12.2.1. Enzyme Assay for Serine Protease Activity. 500 $\mu$l of extract containing serine protease was mixed with 2.5 ml of Tris(hydroxymethyl)-amino methane-maleate (Tris-maleate) buffer which is a solution of 0.2 M of Tris(hydroxymethyl)-amino methane and maleic acid, and 0.2 M NaOH solution supplemented with 0.6 per cent casein solution. Initially, buffers with pH values varying from six to ten were prepared to optimize the enzyme activity. Optimum activity was found in Tris-maleate buffer, pH=7.0. The reaction proceeded at 30$^{o}$C for 20 minutes and it was stopped by addition of 2.5 ml of TCA solution. After incubation at 30$^{o}$C for 30 minutes, the mixture was filtered and 2.5 ml of $Na_2CO_3$ was added into the filtrate. The mixture formed by the addition of 0.5 ml of double diluted folin reagent was incubated at room temperature for thirty minutes. During the reaction, casein was converted into tyrosine by serine protease in the sample solution, thus the color turned into blue. Finally, the change in color was observed and the absorbance at 660 nm was measured.

5.12.2.2. Measurement of Serine Protease Activity. One unit of activity was defined as the amount of enzyme which released 1$\mu$g of tyrosine per minute under the assay conditions described in Section 5.11.2.1. A calibration chart was prepared with tyrosine as the standard. Then the optical density readings at 660 nm were converted to $\mu$g of tyrosine in the reaction mixture. Knowing the amount of tyrosine formed, the serine protease activity was converted to enzyme units.

### 5.12.3. Determination of Pullulanase Activity

5.12.3.1. Enzyme Assay for Pullulanase Activity. DNS method was used to measure reducing sugar. 500 µl of extract containing pullulanase was mixed with 500 µl of pullulan solution prepared previously. The mixture was incubated at 37$^{o}$C for 3 minutes and then 3 ml of DNS solution was added. After incubation in boiling water for 10 minutes, the mixture was incubated on ice and 8 ml of distilled water was added. During the reaction, pullulan was converted into maltotriose by pullulanase and the color turned into brown. Finally, the change in color was observed and the absorbance at 540 nm was measured.

5.12.3.2 Measurement of Pullulanase Acvtivity. One unit of activity was defined as the amount of enzyme which released 1$\mu$g of maltotriose per minute under the assay conditions described in Section 5.11.3.1. A calibration chart was prepared with maltotriose as the standard. Then the optical density readings at 540 nm were converted to $\mu$g of maltotriose in the reaction mixture. Knowing the amount of malotriose formed, the pullulanase activity was converted to enzyme units.

## 5.13. SDS-Polyacrylamide Gel Electrophoresis of Proteins

Sodium dodecyl sulfate-poyacrylamide gel electrophoresis (SDS-PAGE) method separates proteins based on their molecular weights as described by Laemmli, 1970. SDS binds to hydrophobic regions of a protein, disrupting its folded structure and allowing it to exist stably in solution in an extended form.

The gel sandwich was assembled by placing the spacers between the two thin glass plates in the holder. The separating gel was prepared by mixing acrylamide-bisacrylamide, 1.5 M Tris-HCl and SDS at the specified percentage with TEMED and 10 % ammonium persulfate added last and poured carefully into the gel sandwich about two cm from the top of the front plate. The top of the separating gel was covered with water to prevent contact with air and to keep the gel surface flat. The gel was allowed to polymerize and then the water was poured off. 5 % stacking gel was prepared by mixing acrylamide-bisacrylamide, 0.5 M Tris-HCl and SDS with TEMED and 10 % ammonium persulfate added last again and was poured on top of the separating gel. The comb was carefully inserted and the stacking gel was allowed to polymerize for about half an hour. After polymerization, the comb was removed and the gel was placed in the electrophoresis chamber. Electrophoresis buffer was added to the inner and outer reservoirs and any air bubbles trapped in the wells were removed by rinsing.

The protein sample was mixed with sample buffer in an eppendorf tube and the mixture was heated at 98°C for three minutes. The sample solution and molecular weight standards were loaded to the wells and the electrode plugs were attached to the proper electrodes and the power supply was turned on to 200 V. The electrophoresis was carried out at constant voltage setting until the dye front had migrated to the bottom of the gel.

Then the chamber was disconnected from the power supply, gel plates were pried apart, the gel was removed and placed into staining solution for half an hour. The stain was then poured out and destain was added to get rid of unbound stain. After half an hour, destain was poured out and fixing solution was added to fix the proteins. The gels were then dried onto chromatography paper under vacuum for 30 minutes at $80^0$C.

# 6. RESULTS AND DISCUSSION

## 6.1. Cloning

### 6.1.1. Cloning of Serine Protease into pMAL-p2 Expression Vector

Serine protease gene was amplified using *T. thermophilus* genomic DNA as template in PCR as described in Section 5.4.1. The following forward and reverse primers were designed with *XmnI* and *EcoRI* restriction enzyme sites for amplification of the 756 nucleotide-long serine protease gene.

Forward Primer: 5'- AAGAGAAGG ATTTCTATGCGCGGCCTCGTG – 3'
*XmnI* site

Reverse Primer: 5' – ATTG AATTCTCATACCGCCCCCACTCT – 3'
*EcoRI* site

The PCR product was verfied on one per cent agarose gel. After isolation of the PCR product was isolated from low melting point agarose gel, as described in Section 5.5, the insert was purified by QIAGEN Gel Extraction Kit.

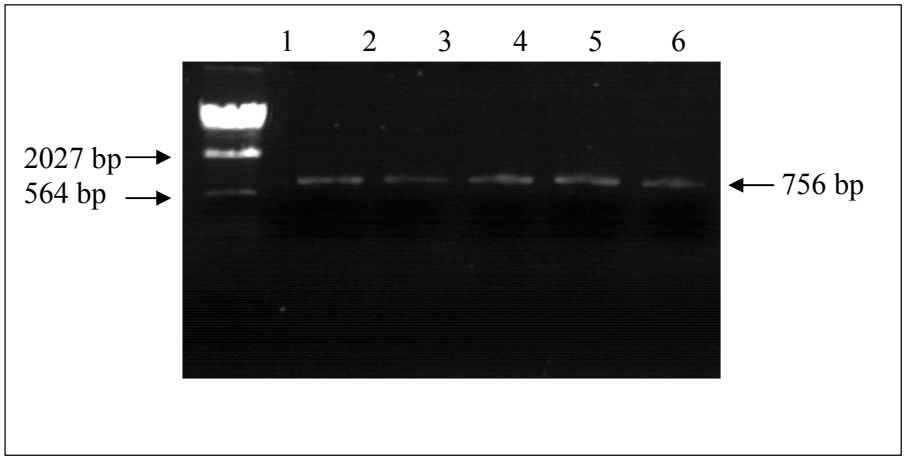



Figure 6.1. PCR amplification of serine protease. Lane 1: λDNA-HindIII marker, Lane 2-6: Serine protease PCR product

The amplified putative serine protease gene and the vector plasmid pMAL-p2 were digested with *XmnI* and *EcoRI* as described by the manufacturer, followed by ligation by T4 DNA ligase as described in Section 5.7. Thus, the ligation of 756 bp-long insert and 6721 bp-long vector pMAL-p2 resulted in the recombinant plasmid pOSP of length 7487 bp including the additional nucleotides in the primer design (Figure 6.2).

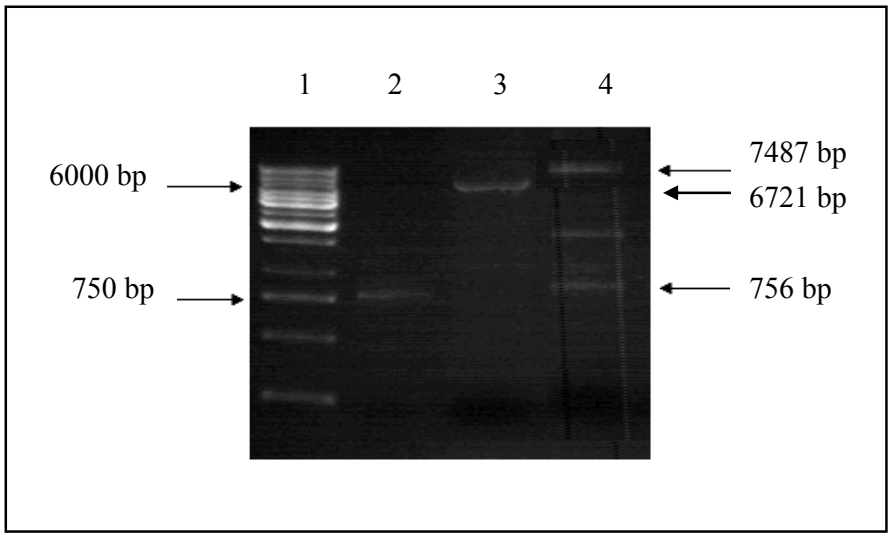



Figure 6.2. The result of ligation reaction. Lane 1: GeneRuler 1 kb DNA Ladder, Lane 2: The insert, serine protease, Lane 3: The vector, pMAL-p2, Lane 4: The ligation of the insert and the vector

The construction of the recombinant vector pOSP is described in Figure 6.3.

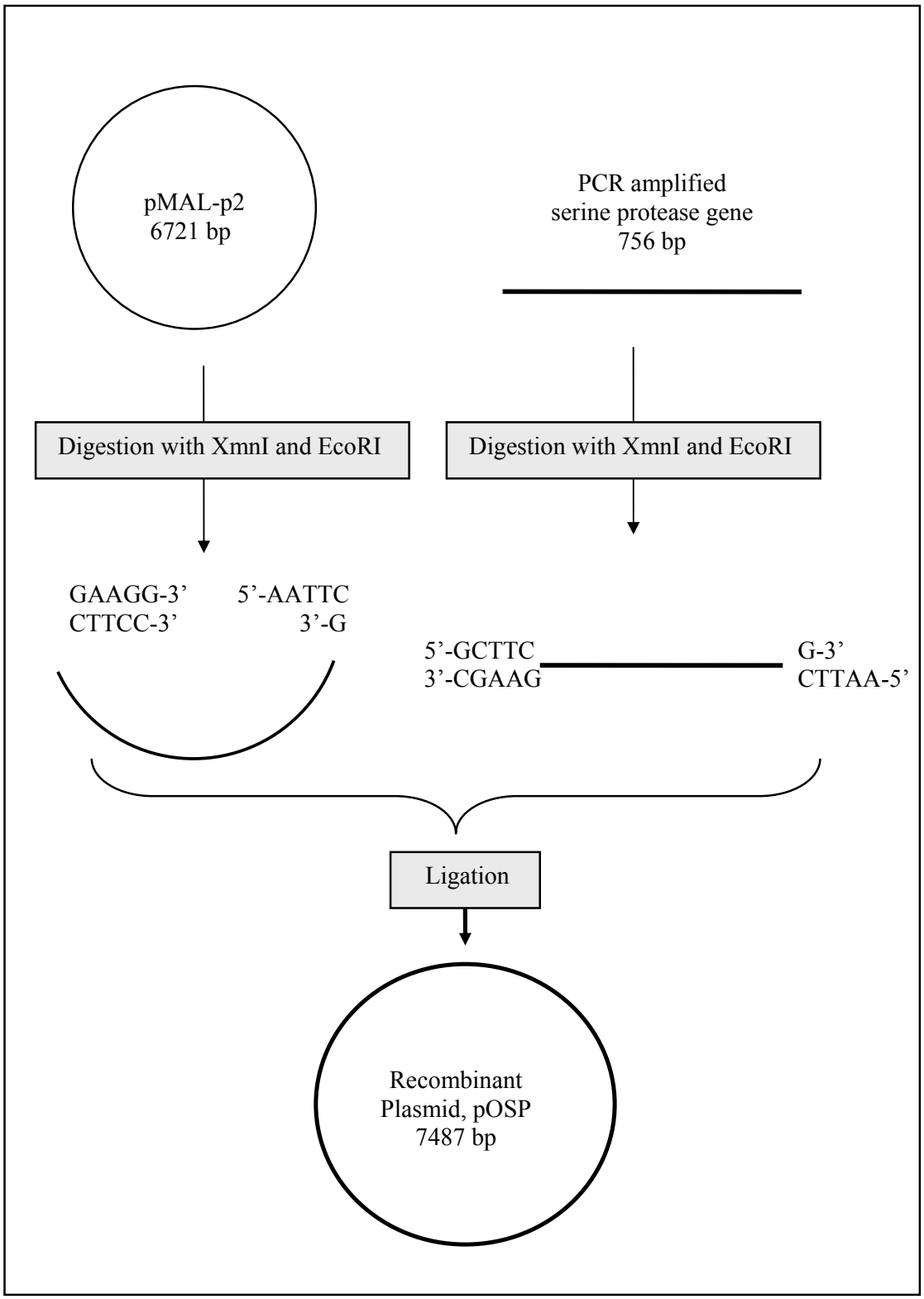



Figure 6.3. Construction of recombinant pOSP plasmid

The recombinant plasmid pOSP was transformed into *E. coli* XL1 cells. The cells were grown on LB agar plates supplemented with 100mg/ml ampicillin for the selection of the cells harboring the vector and on LB agar plates containing ampicillin (100 µg/ml), X-Gal (10 µg/ml), and IPTG (1 mM) for the selection of the cells harboring the plasmid with the insert. The white colonies formed were analyzed by cracking method for their plasmids (Figure 6.4).

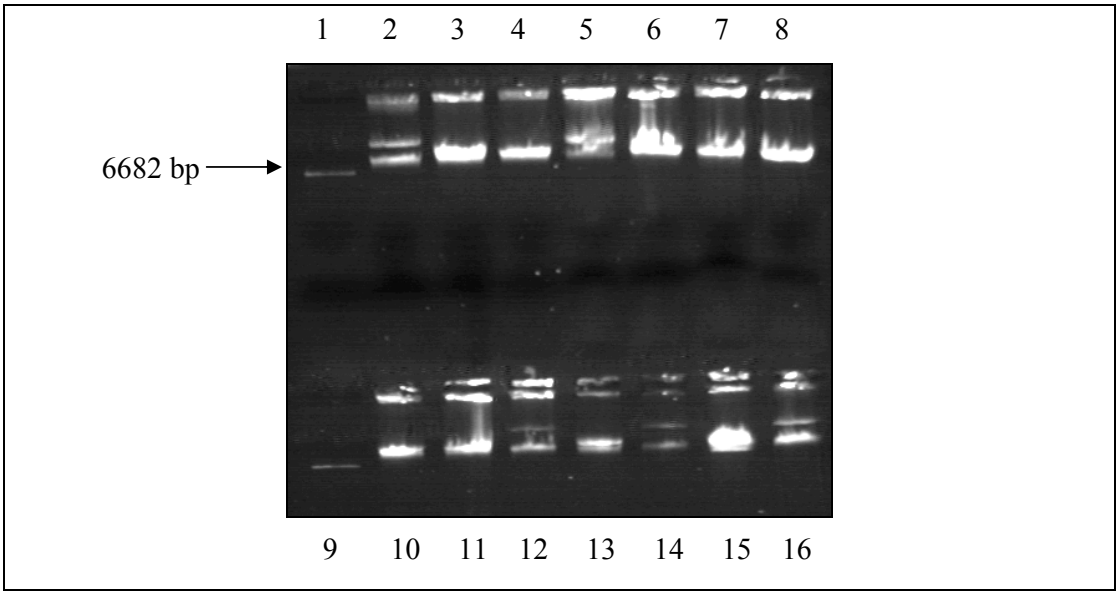



Figure 6.4. The cracking results of white colonies. Lane 1 and 9: Single digested pMAL-p2 plasmid, Lanes 2-8 and 10-16: Plasmids of various colonies obtained from cracking

The colonies harboring plasmids larger than pMAL-p2 were further analyzed by digestion by restriction enzymes. Plasmids from 30 colonies were isolated. The results of *EcoRI* digestion are shown in Figure 6.5 and the results of *EcoRI* and *XmnI* double digestion are shown in Figure 6.6. The two bands with the lengths of the insert and the plasmid were obtained after the digestion with *XmnI* and *EcoRI*. The size of the DNA fragments obtained after digestion were compared with the expected size obtained from restriction map of the recombinant plasmid.

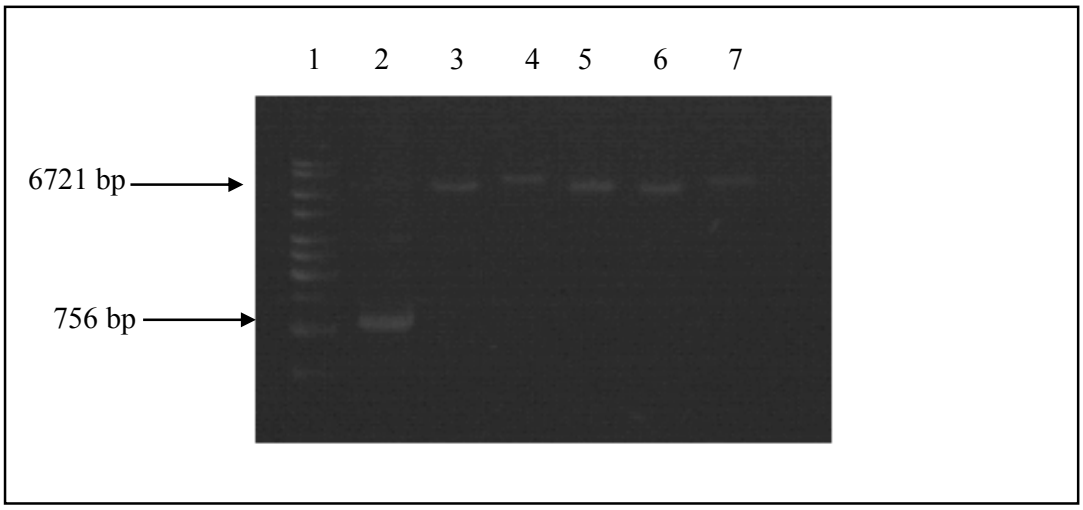



Figure 6.5. Single digested plasmids obtained from cracking. Lane 1: GeneRuler 1 kb DNA Ladder, Lane 2: The insert, serine protease, Lane 3: The vector pMAL-p2, Lane 4: $10^{th}$ colony, Lane 5: $12^{th}$ colony, Lane 6: $20^{th}$ colony, Lane 7: $27^{th}$ colony

The plasmids from the $10^{th}$ and the $27^{th}$ colonies were larger than the vector plasmid pMAL-p2 (Figure 6.5).

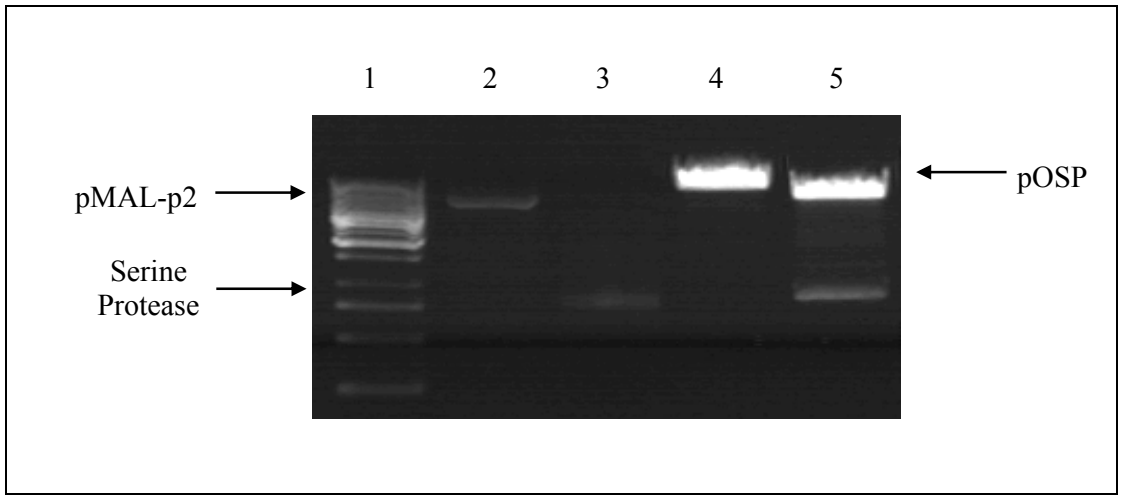



Figure 6.6. Restriction enzyme analysis of pOSP recombinant plasmid. Lane 1: GeneRuler 1 kb DNA Ladder, Lane 2: The vector pMAL-p2, Lane 3: The insert, serine protease, Lane 4: The recombinant plasmid, pOSP digested with *EcoRI*, Lane 5: The recombinant plasmid, pOSP digested with both *XmnI* and *EcoRI*

Figure 6.6 shows *EcoRI* and *EcoRI/XmnI* digest of the plasmid from colony 10. The results indicated that this plasmid had the insert and thus this was chosen for expression studies.

### 6.1.2. Cloning of Pullulanase into pMAL-p2 Expression Vector

Pullulanase gene was amplified using the recombinant plasmid pTPi61, kindly provided by Prof. Hiroshi Sakai from University of Shizuoka as the template in PCR as desribed in Section 5.5.1. The coding region of the *Thermus thermophilus* HB8 pullulanase was between the 2218$^{th}$ and 4374$^{th}$ positions of pUC119 plasmid.

The following forward and reverse primers were designed for amplification of the 2156 nucleotide-long pullulanase gene.

Forward Primer: 5'- ATAGGAAGG ATTTCTATGCTTCACATCAGCCGAACG – 3'
*XmnI* site

Reverse Primer: 5' – AGTG AATTCTCACCCTGCAGGATGGAC – 3'
*EcoRI* site

The PCR product was verified on one per cent agarose gel (Figure 6.7). After isolation of the PCR product was isolated from low melting point agarose gel, as described in Section 5.5, the insert was purified by QIAGEN Gel Extraction Kit.

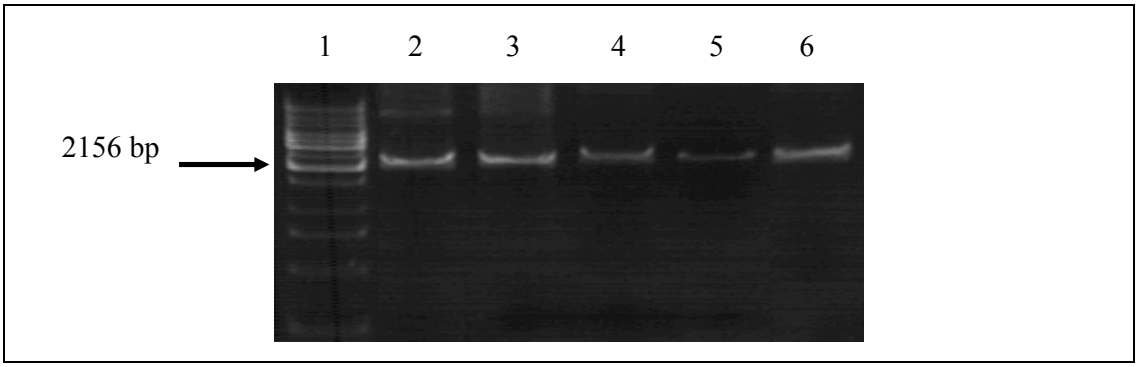



Figure 6.7. PCR amplification of pullulanase. Lane 1: GeneRuler 1 kb DNA Ladder, Lane 2-6: Pullulanase PCR product

The amplified pullulanase gene and the vector plasmid pMAL-p2 were digested with *XmnI* and *EcoRI* as described by the manufacturer, following ligation by T4 DNA ligase as described in Section 5.8. Thus, the ligation of 2156 bp-long insert and 6721 bp-long vector pMAL-p2 resulted in the recombinant plasmid pOPU of length 8887 bp including the additional nucleotides in the primer design. (Figure 6.8)

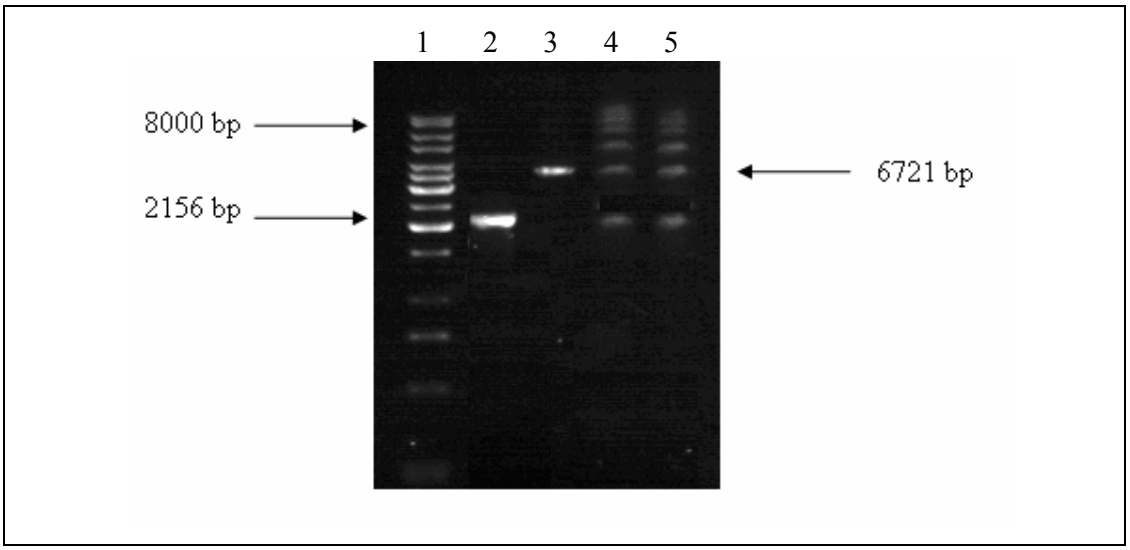



Figure 6.8. The result of ligation reaction. Lane 1: GeneRuler 1 kb DNA Ladder, Lane 2: The insert, pullulanase, Lane 3: The vector, pMAL-p2, Lane 4 and 5: The ligation of the insert and the vector

The recombinant plasmid pOPU was transformed into *E. coli* XL1 cells. The cells were grown on LB agar plates supplemented with 100mg/ml ampicillin for the selection of the cells haboring the vector and on LB agar plates containing ampicillin (100 µg/ml), X-Gal (10 µg/ml), and IPTG (1 mM) for the selection of the cells harboring the plasmid with the insert. The white colonies formed were analyzed by cracking method for their plasmids.

The colonies harboring plasmids larger than pMAL-p2 were further analyzed by digestion by restriction enzymes. Plasmids from 30 colonies were isolated. The results of *EcoRI* digestion and *EcorI*/*XmnI* double digestion are shown in figure 6.9. The two bands obtained after digestion with *EcoRI* and *XmnI* were the insert and the plasmid.

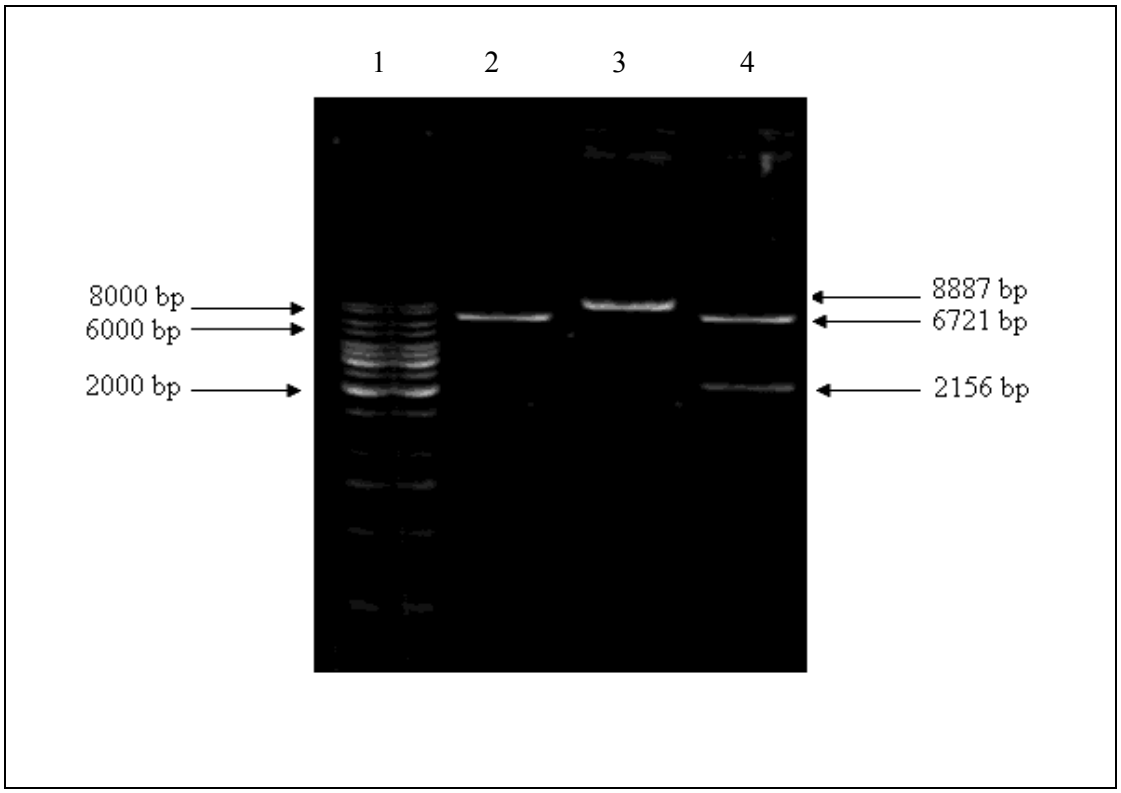



Figure 6.9. Electrophoretic analysis of the digestion of plasmid pOPU with *XmnI* and *EcoRI*. Lane 1: Gene Ruler 1 kb DNA ladder. Lane 2: plasmid pMAL-p2, Lane 3: plasmid pOPU, Lane 4: pOPU plasmid digested with *XmnI* and *EcoRI*

The construction of the recombinant vector pOPU is described in Figure 6.10.

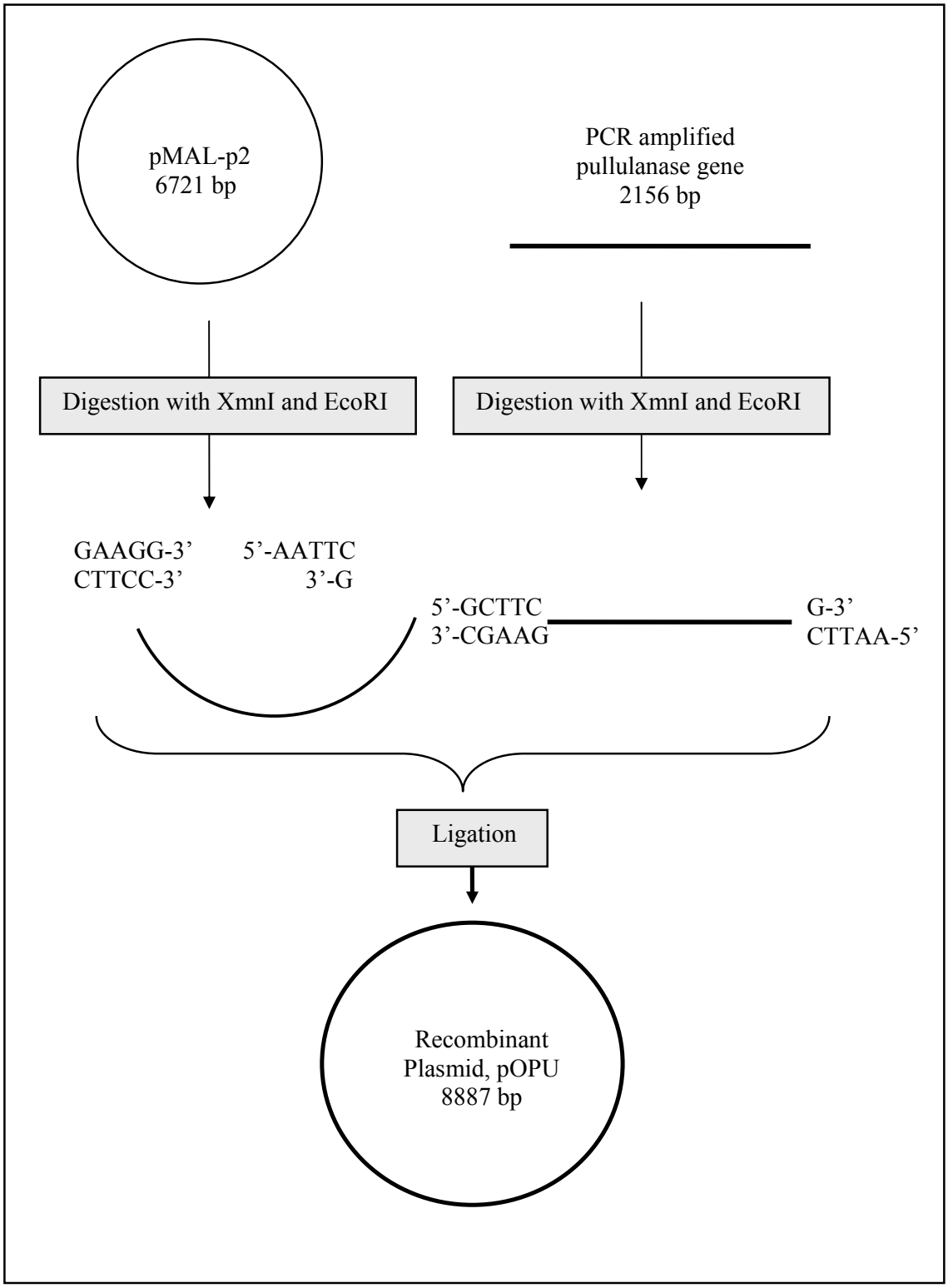



Figure 6.10. Construction of recombainant pOPU plasmid

## 6.2. Growth of Recombinant *E. coli* (pOSP) Cells and Expression of Maltose-Binding Protein-Serine Protease Fusion Protein

*E. coli* XL1(pOSP) were grown at 37°C in LB medium with ampicillin for the selection of cells harboring the recombinant plasmid and incubated for 20-25 hours after induction with 1mM IPTG at mid-exponential phase. Samples were taken in one to two hours intervals to determine the optical density of the cells at 600 nm. The dry weight of the cells were determined as described in Methods 5.2. Growth characteristics of the recombinant XL1(pOSP) cells were compared with XL1(pMAL-p2) cells which were lacking the insert encoding for serine protease. The growth curve for both XL1(pMAL-p2) and XL1(pOSP) cells are given in Figure 6.11.

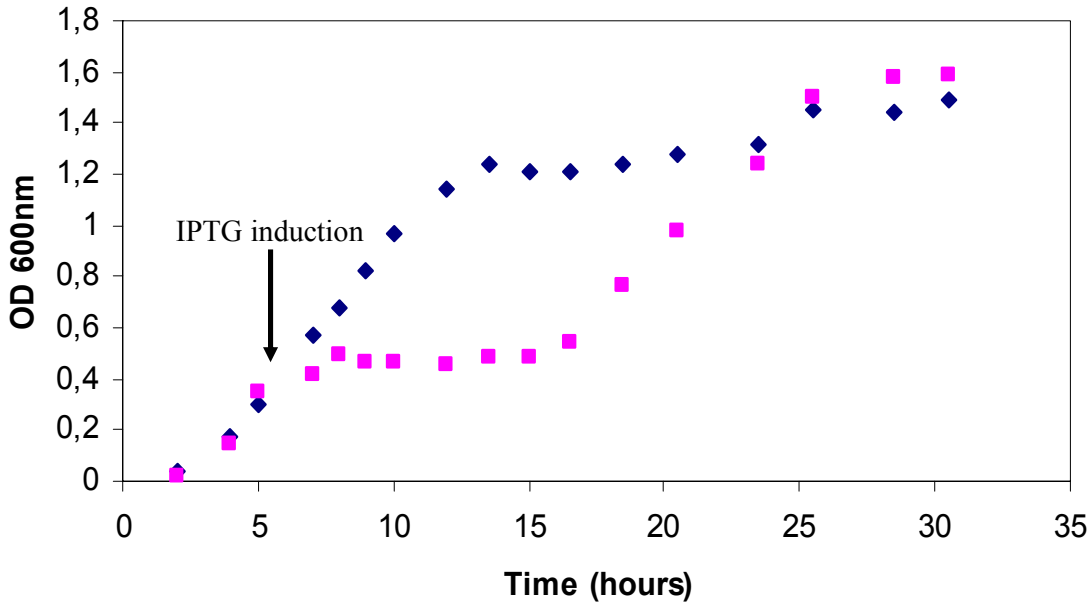



Figure 6.11. Growth curve of the recombinant XL1(pOSP) and XL1(pMAL-p2) cells, XL1(pOSP) cells (■), XL1(pMAL-p2) cells (♦)

It was observed that the growth of XL1(pOSP) cells ceased upon induction and resumed ten hours after induction with IPTG and the cells continued growing after the fifteenth hour until the cells reached the stationary phase at an $OD_{600\ nm}$ value of 1.6.

Serine protease was produced under the control of the strong *tac* promoter and the *mal*E translation initiation signals as an MBP-serine protease fusion protein from pOSP in

recombinant *E. coli* XL1 cells. Since MBP is destined to be localized in the periplasm using the Sec machinery, it was expected that the fusion protein would be translocated to the periplasm.

Cytoplasmic and periplasmic extracts were prepared as described in Section 5.12.1 to determine the amount of fusion protein in each compartment. The periplasmic and cytoplasmic extracts were analyzed for serine protease activity as described in Section 5.12.2 and the results are given in Table 6.1.

Table 6.1. Serine protease activity in different cellular compartments of XL1(pOSP) cells

| Time (hours) | Periplasm U/gDCW | Cytoplasm U/gDCW | Total U/gDCW |
|---|---|---|---|
| 10 | 76.6 | 22.3 | 98.9 |
| 15 | 105.9 | 18.9 | 124.8 |
| 20 | 22.0 | 16.2 | 38.2 |
| 25 | 1.8 | 9.0 | 10.8 |
| 30 | 0,8 | 5.3 | 6.1 |

The cellular distribution of MBP-serine protease with time is plotted in Figure 6.12.

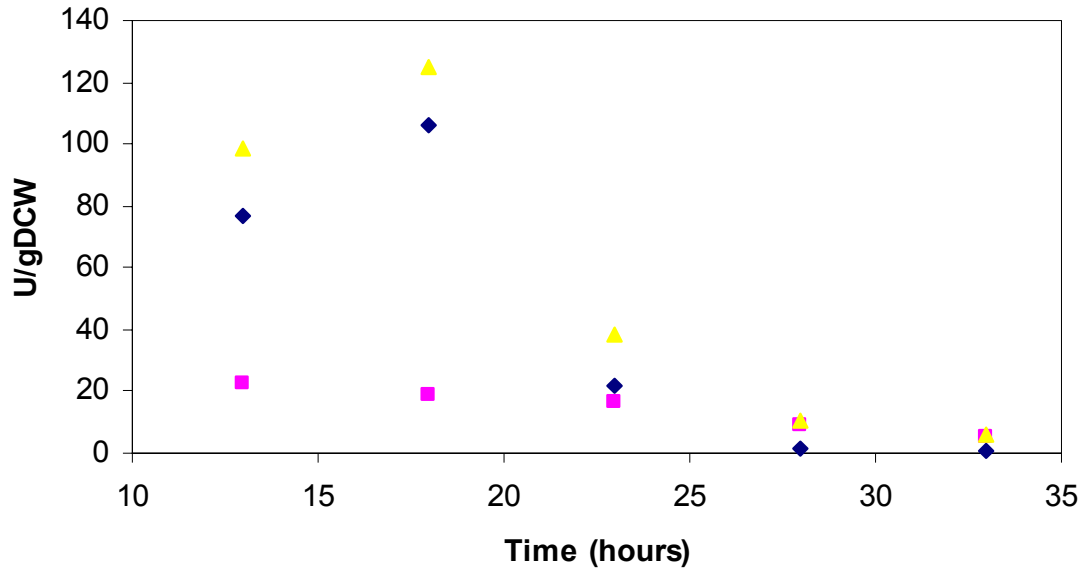
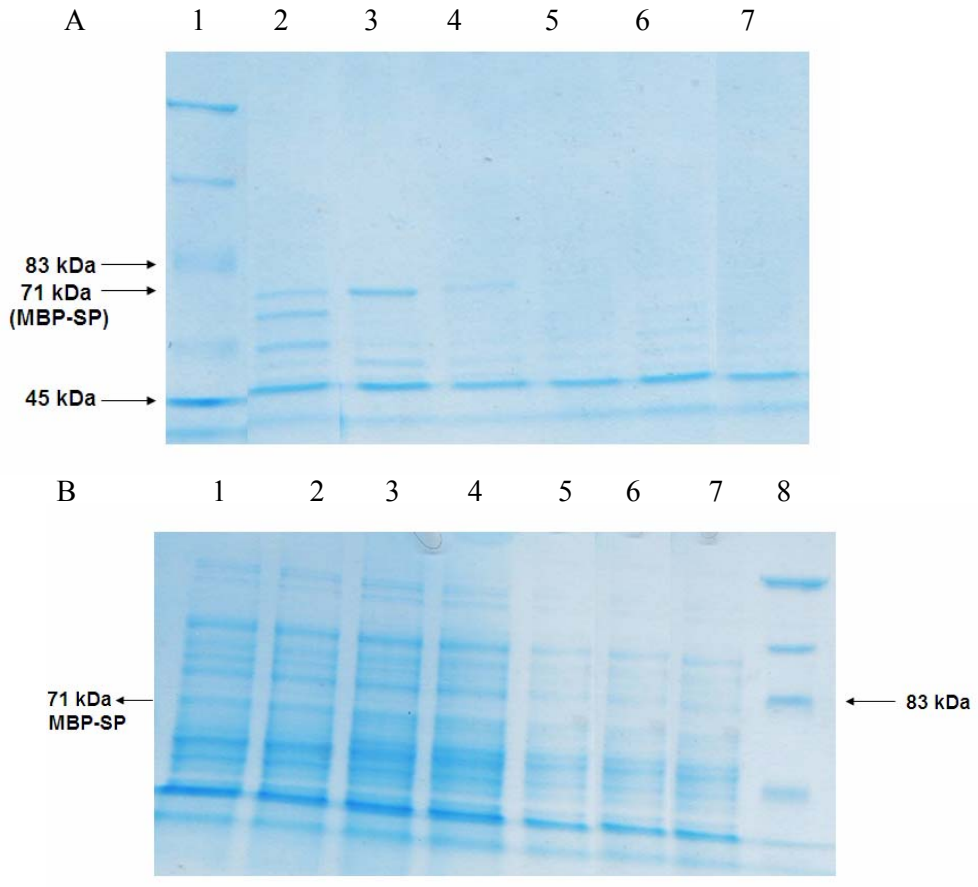



Figure 6.12. Time dependent cellular distriution of MBP-serine protease activity

(♦) data points show the periplasmic time profile, (■) data points show the cytoplasmic time profile of serine protease and (▲) data points show the time profile of total cellular serine protease.

The cellular distribution of the fusion protein was analysed on SDS-PAGE as below:




A: Periplasmic extract. Lane 1: Marker, Lane 2: Periplasmic extract five hours after the induction, Lane 3: Periplasmic extract ten hours after the induction, Lane 4: Periplasmic extract fifteen hours after the induction, Lane 5: Periplasmic extract twenty hours after induction, Lane 6-7: Periplasmic extract twenty five hours after the induction. B: Cytoplasmic extract. Lane 1 and 2: Cytoplasmic extract five hours after the induction, Lane 3-4: Cytoplasmic extract ten hours after the induction, Lane 5: Cytoplasmic extract fifteen hours after the induction, Lane 6: Cytoplasmic extract twenty hours after induction, Lane 7: Cytoplasmic extract twenty five hours after the induction, Lane 8: Marker.

Figure 6.13. SDS-PAGE analysis of time dependent cellular distribution of MBP-serine protease fusion protein

Samples were taken at five hour time intervals after induction and the distribution of the fusion protein was determined. It can be seen in Figure 6.12 that the amount of serine protease in the periplasmic space was significantly high at the fifth and tenth hour after induction. MBP-serine protease fusion protein was succesfully translocated to the periplasm for ten hours and then production dropped considerably. When Figure 6.11 and Figure 6.12 are considered simultaneously, it is obvious that the growth of cells ceased during high recombinant protein production. As the amount of fusion protein produced decreased, the cells resumed to grow which was after the tenth hour. Therefore, it can be concluded that the high rate of MBP-serine protease production affected cell growth.

There might be several possible reasons for this effect of recombinant protein expression on depression of growth. Proteases are responsible for the regulated destruction of proteins in all cells and inactivation of host defense molecules. These proteases bind protein substrates and power their mechanical denaturation (Kenniston et al., 2005). The reason for stationary growth pattern ten hours after the IPTG induction and the high amounts of serine protease activity observed at this time interval can be understood when the fact that the foreign protein, serine protease is a protein which is responsible for protein destruction and inactivation of host defense molecules. In ten hours after the induction, the recombinant protein was expressed, and serine protease probably started to destruct the host proteins and inactivated the host defense molecules. At the end of ten hours, the cells resisted this foreign protein destruction and resumed to grow.

## 6.3. Growth of Recombinant *E. coli* (pOPU) Cells and Expression of Maltose-Binding Protein-Pullulanase Fusion Protein

*E. coli* XL1(pOPU) cells were grown in LB medium with ampicillin for the selection of cells harboring the recombinant plasmid at 37$^{o}$C and incubated for 20-25 hours after induction with 1mM IPTG at late exponential phase. Samples were taken in one to two hours intervals to determine the absorbance of the cells at 600 nm. The dry weight of the cells were determined as described in Methods 5.2. Growth characteristics of the recombinant XL1(pOPU) cells were compared with XL1(pMAL-p2) cells which were lacking the insert encoding for pullulanase. The growth curve for both XL1(pMAL-p2) and XL1(pOPU) cells are given in Figure 6.14.

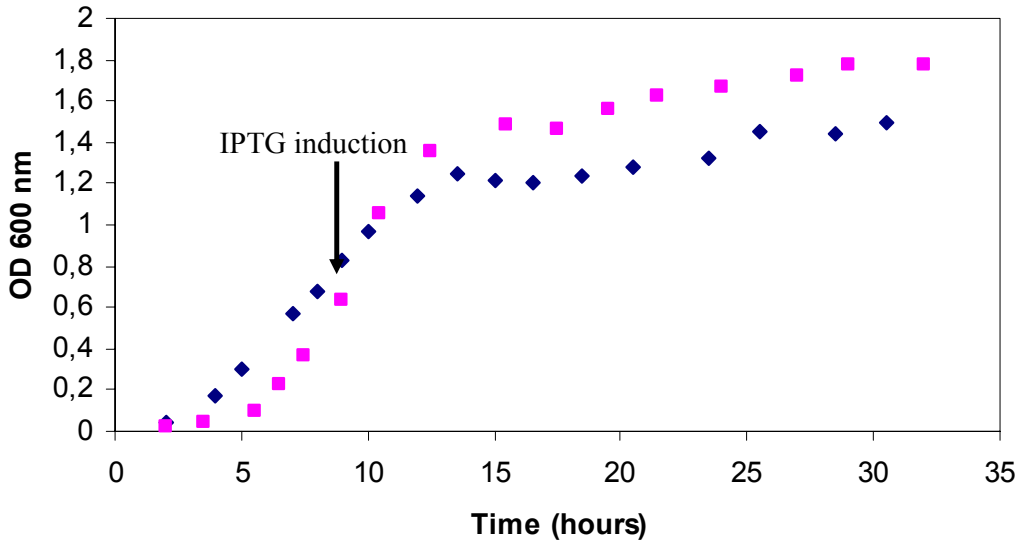



Figure 6.14. Growth curve of the recombinant XL1(pOPU) and XL1(pMAL-p2) cells. XL1(pOPU) cells (■), XL1(pMAL-p2) cells (♦)

It was observed that the growth pattern of XL1(pOPU) cells were not affected by the recombinant protein expression after induction with IPTG. Moreover, the recombinant cells grew faster after the tenth hour than XL1(pMAL-p2) cells. The XL1(pOPU) cells reached the stationary phase at an $OD_{600\ nm}$ value of 1.8 around twenty sixth hour.

Cytoplasmic and periplasmic extracts were prepared as described in Section 5.12.1 to determine the amount of fusion protein in each compartment. The periplasmic and cytoplasmic extracts were analyzed for pullulanase activity as described in Section 5.12.2 and the results are given in Table 6.2.

Table 6.2. Pullulanase activity in different cellular compartments of XL1(pOPU) cells

| Time (hours) | Periplasm U/gDCW | Cytoplasm U/gDCW | Total U/gDCW |
|---|---|---|---|
| 12 | 3225.8 | 224.1 | 3449.9 |
| 17 | 4861.3 | 181.9 | 5043.2 |
| 22 | 6416.6 | 148.1 | 6564.7 |
| 27 | 7756.2 | 115.9 | 7872.1 |
| 32 | 4560.4 | 143.8 | 4704.2 |

The cellular distribution of MBP-pullulanase with time is plotted in Figure 6.15.

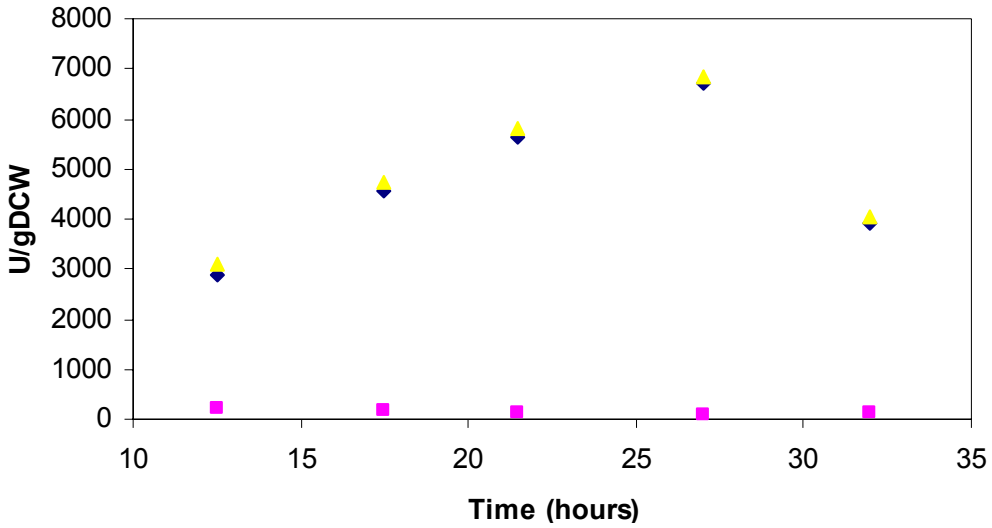
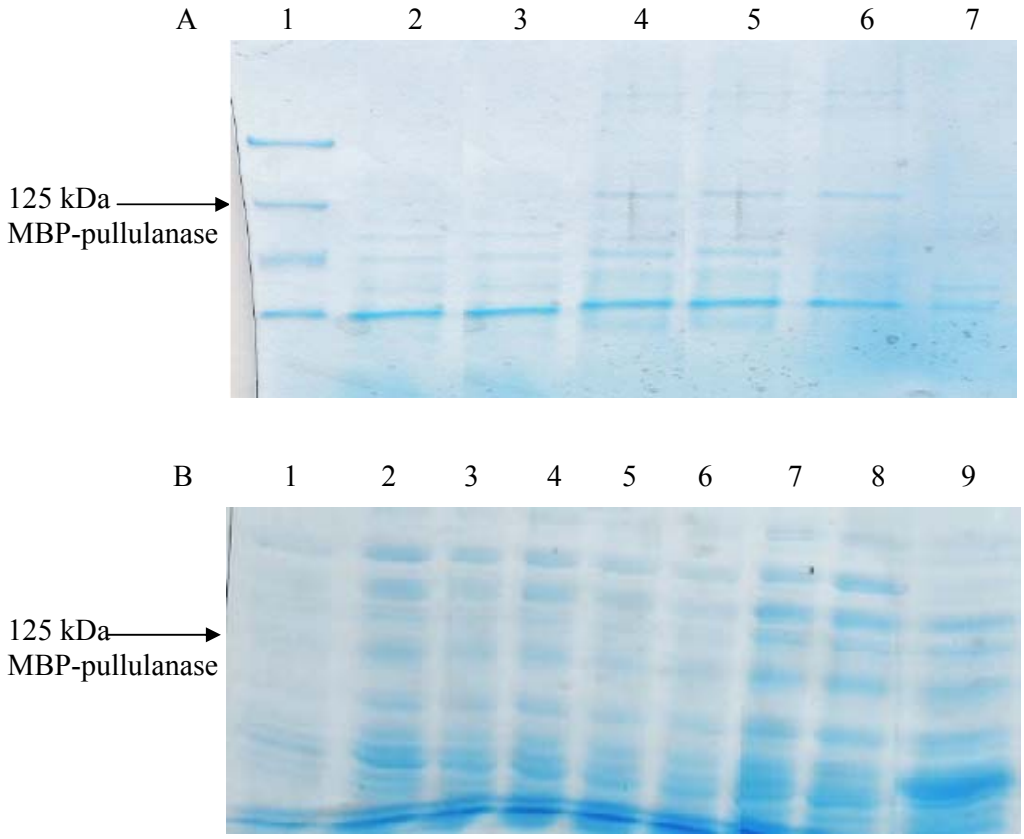



Figure 6.15. Time dependent cellular distriution of MBP-pullulanase activity

(♦) data points show the periplasmic time profile, (■) data points show the cytoplasmic time profile of pullulanase and (▲) data points show the time profile of total pullulanase.

The time dependent cellular distribution of MBP-pullulanase fusion protein was also analysed on SDS-PAGE in Figure 6.16.




A: Periplasmic extract. Lane 1: Marker, Lane 2: Periplasmic extract five hours after induction, Lane 3: Periplasmic extract ten hours after induction, Lane 4: Periplasmic extract fifteen hours after induction, Lane 5: Periplasmic extract twenty hours after induction, Lane 6: Periplasmic extract twenty five hours after induction, Lane 7: Periplasmic extract of XL1(pMAL-p2), the cells without the insert.

B: Cytoplasmic extract. Lane 1: Marker, Lane 2: Cytoplasmic extract five hours after induction, Lane 3-4: Cytoplasmic extract ten hours after induction, Lane 5-6: Cytoplasmic extract fifteen hours after induction, Lane 7-8: Cytoplasmic extract twenty hours after induction, Lane 9: Cytoplasmic extract twenty five hours after induction.

Figure 6.16. SDS-PAGE analysis of time dependent cellular distribution of MBP-pullulanase fusion protein

Samples were taken at five hours time intervals after induction and the distribution of the fusion protein was determined. In Figure 6.13, it is seen that the amount of pullulanase continuously increased up to 7756.2 U/gDCW at twenty seventh hour and then decreased. A very high percentage of MBP-pullulanase fusion was translocated to the periplasm. By the time the expression of the fusion continued, the recombinant cells also continued to grow. Therefore, it can be concluded that the expression of the fusion protein did not affect the growth pattern.

## 6.4. Comparison of Translocation Results: Relation to the SecB Binding Affinity and the Transfer Free Energy Change Through the Periplasmic Membrane

The experimental results obtained were compared with the previous results of MBP-GI fusion protein referring to B. Sarıyar's Ph.D. thesis in therms of SecB binding affinity and the transfer free energy change through the periplasmic membrane for the three enzymes, namely glucose isomerase, serine protease and pullulanase.

Firstly, the percentage of translocated proteins were given and compared. Then the characteristics of the three enzymes were compared.

Some data points and the percentage of translocated protein to that of the total protein are given in Table 6.3.

Table 6.3. Per cent of glucose isomerase transported to the periplasm

| Time (hours) | Per cent of protein transported to the periplasm |
|---|---|
| 7 | 1.379 |
| 10 | 1.075 |
| 17.5 | 0.011 |
| 21.5 | 0.009 |
| 25 | 0.010 |

The amount of enzyme in cytoplasmic and periplasmic compartments, the total protein expressed and the per cent of protein translocated to the periplasm for serine protease are given in Table 6.4.

Table 6.4. Enzyme activity of serine protease in different cellular compartments

| Time (hours) | Periplasmic (U/gDCW) | Cytoplasmic (U/gDCW) | Total (U/gDCW) | Per cent of protein translocated to the periplasm |
|---|---|---|---|---|
| 10 | 76.58 | 22.26 | 98.84 | 77.5 |
| 15 | 105.89 | 18.90 | 124.79 | 84.9 |
| 20 | 21.99 | 16.24 | 38.23 | 57.5 |
| 25 | 1.79 | 9.01 | 10.80 | 16.6 |
| 30 | 0.76 | 5.29 | 6.05 | 12.6 |

The amount of enzyme in cytoplasmic and periplasmic compartments, the total protein expressed and the per cent of protein translocated to the periplasm for pullulanase are given in Table 6.5.

Table 6.5. Enzyme activity of pullulanase in different cellular compartments

| Time (hours) | Periplasmic (U/gDCW) | Cytoplasmic (U/gDCW) | Total (U/gDCW) | Per cent of protein translocated to the periplasm |
|---|---|---|---|---|
| 12 | 2869.38 | 205.95 | 3075.33 | 93.3 |
| 17 | 4554.00 | 174.31 | 4728.31 | 96.4 |
| 22 | 5655.02 | 135.79 | 5790.81 | 97.7 |
| 27 | 6722.91 | 106.58 | 6829.49 | 98.4 |
| 32 | 3926.28 | 130.57 | 4056.85 | 96.8 |

Tables 6.3, 6.4 and 6.5 show that about 98 per cent of pullulanase, which is the longest protein among the three enzymes, was translocated to the periplasm, whereas about 85 per cent of serine protease was translocated. The translocation of glucose isomerase was very low when compared to serine protease and pullulanase.

The most important effect on translocation is the characteristic of the protein, such as its length, amino acid sequence and composition. However, these experimental results

have shown that the length is not a very significant factor as assumed. If this had been the case, the highest amount of protein in the periplasm would have been that of the serine protease and the least amount would have been that of the pullulanase. In addition, the amount of glucose isomerase translocated to the periplasm would have been in between. The next step was to study the amino acid sequence and composition of the three proteins.

The first step of translocation by the Sec pathway is the binding of the protein being translocated to the SecB bacterial chaperon. The sequence motif that is recognized by SecB is poorly understood. In the case of MBP, SecB binds to multiple fragments covering a large fraction of the primary sequence. The SecB tetramer has a binding site for positively charged peptides, the occupation of which is proposed to result in conformational changes that expose hydrophobic binding sites (Randall, 1992).

The binding of a secretory protein to SecB is highly dependent on the type of amino acid sequence. The binding motif is enriched in basic and aromatic residues, whereas acidic residues are disfavored unless localized in the neighborhood of basic residues. So, the amino acid sequences of MBP and the three proteins are examined for the acidic, aromatic, and basic residues to decide on which is more likely o bind to SecB. The polar, therefore hydrophilic acidic amino acids are aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), and glutamine (Gln). The aromatic amino acids are phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) are hydrophobic. Lastly, the polar basic amino acids are histidine (His), lysine (Lys), arginine (Arg) (Knoblauch et al., 1999).

High affinity SecB-binding regions are predicted as consecutive peptide sequences of 9 or 10 residues according to the following rules (Knoblauch et al., 1999).

(i) They contain at least 3 aromatic or basic residues.

(ii) The presence of each acidic residue in a SecB-binding region has to be compensated by another basic residue.

(iii) In the absence of aromatic residues, at least four net positive charges are required.

(iv) The positioning of charged and aromatic residues within the motif is not important, except that 2 aromatic residues In the direct neighbourhood do not contribute more to SecB affinity as compared with only one residue.

Another important point in SecB binding is that peptides with 2 aromatic residues bind SecB only if there is an additional Arg or Lys, whereas peptides with 3 aromatic residues bind in the absence of Arg or Lys. In all cases, SecB binding can be inhibited by the presence of acidic residues within the peptides.

The amino acid sequence of glucose isomerase is given below. The basic and aromatic residues which enrich the binding are highlighted in blue, and acidic residues which disfavors the binding are highlighted in pink.

MYEPKPEHRFTFGLWTVGNVGRDPFGDAVRERLDPVYVVHKLAELGAYGVNLH
DEDLIPRGTPPQERDQIVRRFKKALDETGLKVPMVTANLFSDPAFKDGAFTSPDPW
VRAYALRKSLETMDLGAELGAEIYVVWPGREGAEVEATGKARKVWDWVREALN
FMAAYAEDQGYGYRFALEPKPNEPRGDIYFATVGSMLAFIHTLDRPERFGLNPEFA
HETMAGLNFVHAVAQALDAGKLFHIDLNDQRMSRFDQDLRFGSENLKAAFFLVD
LLESSGYQGPRHFDAHALRTEDEEGVWAFARGCMRTYLILKERAEAFREDPEVKE
LLAAYYQEDPAALALLGPYSREKAEALKRAELPLEAKRRRGYALERLDQLAVEY
LLGVRG

When the amino acids of glucose isomerase are considered, the acidic residues coming one after the other and forming groups of two, three, or four can be eas,ly detected. These groups disfavor binding. On the other hand, there are basic and aromatic residues in groups of two, three or four. However, the number of these groups are relatively low when compared to the acidic residue groups. In addition, there are no basic or aromatic groups that can eliminate the undesirable effect of these acidic residues. Thus, the binding of this protein seems to be not promising.

The amino acid sequence of serine protease is given below. The basic and aromatic residues which enrich the binding are again highlighted in blue, and acidic residues which disfavors the binding are highlighted in pink.

MRGLVLSGGGARGFAHIGALEVFVEAGLDFEVVAGASMGAIVGALFAAGRSPQEI
LEIARNTPWLGILGFSFREALFSRGRLRRYLAEHLPQDFADLKRKLVVTAVEVRSG
RVVYLFQGDLVSAVLASAAHPLLLRPVRREGLLLFDGGVLDNLPVDAARFLGAEE

VWAVDVTPERSVEKPPEGVLGLALRAVDLMQHHLTASRMALYAPEVYLRPELGE
VAVQDFLRLEEAVEAGRKAAKRFLESRVGEV

In the amino acid sequence above, the acidic residues are low and appear to be single, or at most in pairs. When the basic and aromatic residues are considered, they are generally in groups of two, or three and these are in such positions that they can eliminate the negative effect of acidic residues on binding.

Below is the amino acid sequence of pullulanase. The basic and aromatic residues which enrich the binding are again highlighted in blue, and acidic residues which disfavors the binding are highlighted in pink.

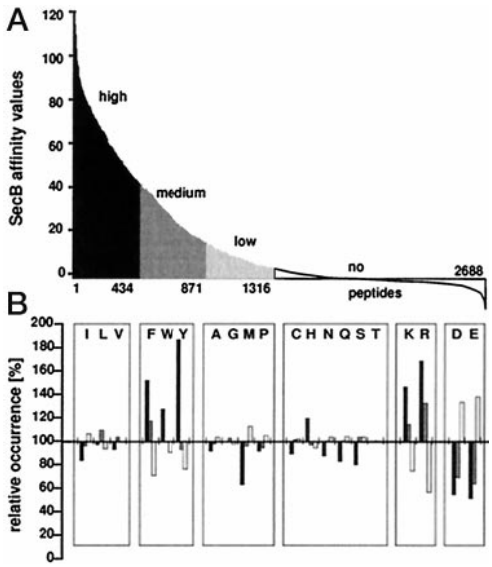
MLHISRTFAAYLDEMDQIVVLAPKSLGFDGMAPFTLVAPSGEEIPLSVQHVEDVGE
TVKYVCRFASAFEFGATYWVRSCRGEETDVQIGAVVRTPAFDDRFFYDGPLGAEY
LKEQTVFRVWAPTATAVSVKLVHPHLDEIRCVPLVRGERGVWSAVVPGDWERAR
YTYIACINRVWREAVDPYATAVSVNGEFGVVIDWEKTKLAPPSLPLPPLCSPTDAII
YELSIRDFTSHPDSGAVHKGKYLGLAETNTSGPNGTATGLSYVKELGVTHVQLMP
FMDFAGVDERDPQAAYNWGYNPLHLYAPEGSYATDPADPYARIVELKQAIHTLH
ENGLRVVMDAVYNHVYDREQSPLEKLVPGYYFRYDAYGQPANGTGVGNDIASE
RRMARRWIVDSVVFWAKEYGIDGFRFDLMGVHDIETMKAVRDALDAIDPSILVY
GEGWDLPTPLPPEQKATMANAKQLPRFAYFNDRFRDAVKGSTFHLPDRGFALGN
PGGREQVKLAIAGSLRALGGLFCHPRQSINYVECHDNHTFWDKMEAANHDEPEW
LRRKRQKLATAIVLLAQGIPFLHSGQEFYRTKGGDGNSYRSPDAVNQLDWERKSR
YEDDVRYVQGLIALRRAHGAFRLATEAEVLRHFTFLEPLPPSVIAYRLHDAAVYGP
WEDIIVVHHNEEKETAIALPDEREWAVVCDGQRCGTTPFGQARGMLRLDGIGTW
VLVHPAG

The amino acid sequence of pullulanase, as given above, has a basic and aromatic residue dominant characteristic. The acidic residues are distributed in between groups of basic and aromatic residues. The basic and aromatic groups are long, which in turn makes the binding easier. Therefore, pullulanase seems to be promising in binding.

Finally, the amino acid sequence of MBP is as follows:

MKIKTGARILALSALTTMMFSASALAKIEEGKLVIWINGDKGYNGLAEVGKKFEK
DTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDAFQ
DKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAK
GKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLV
DLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTF
KGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVN KDKPLGAVAL
KSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDE
ALKDAQTRITK

For the MBP case, the groups of basic and aromatic residues are longer than the acidic residues which are located in between the basic and aromatic ones. So, the binding capacity of MBP is high, when the characateristics of amino acid residues are analysed. In Figure 6.17, types of amino acids and their SecB affinity values are given (Knoblauch et al., 1999). According to the graph, isoleucine, leucine, and valine have high SecB affinities, phenylalanine, threonine, and tyrosine have medium affinity, alanine, glycine, methionine, and praline have low affinity, and the remaining amino acids have no SecB affinity.




A: Affinity of SecB for peptides, B: Comparison of peptides of different classes.

Figure 6.17. SecB affinities and relative occurrence of peptides (Knoblauch et al., 1999)

The previous discussion was concerned with the binding of the fusion protein to SecB which is actually an acidic protein. Although the component of fusion protein functioning in binding to SecB is MBP, the characteristics of the protein to be translocated may affect the binding motif of MBP.

Another important factor that may affect the efficiency of translocation could be the transfer through the membrane. The amino acid sequence of a protein is used to calculate the free-energy change in transferring residues 1 to 20 from the membrane interior (Stryer, 1995). The same calculations can be made for residues 2 to 21, 3 to 22, and so forth until the end of the sequence is reached. The transfer of the protein will be favorable, if the calculated result was negative. If it was positive, the transfer is unfavorable. The free-energy estimates for amino acid residues are given in Table 6.6.

Table 6.6. The free energies for the transfer of amino acid residues

| **Amino Acid Residue** | **Transfer-free Energy (kcal/mol)** | **Amino Acid Residue** | **Transfer-free Energy (kcal/mol)** |
|---|---|---|---|
| Phe | 3.7 | Ser | 0.6 |
| Met | 3.4 | Pro | -0.2 |
| Ile | 3.1 | Tyr | -0.7 |
| Leu | 2.8 | His | -3.0 |
| Val | 2.6 | Gln | -4.1 |
| Cys | 2.0 | Asn | -4.8 |
| Trp | 1.9 | Glu | -8.2 |
| Ala | 1.6 | Lys | -8.8 |
| Thr | 1.2 | Asp | -9.2 |
| Gly | 1.0 | Arg | -12.3 |

A MATLAB code to calculate the free-energy change in transferring residues. The code is given in Appenix A and the output is given in Appendix B.

The analysis of the calculated free-energy change showed that the calculated values were all negative and the smallest among the four proteins. This result shows that the transfer of glucose isomerase is highly favorable. At the very beginning, the free energy

values for serine protease was high and positive. But then the values get smaller and become negative. Since the disfavored amino acids were at the very beginning, where the fusion partners were connected, MBP eliminates this disfavored transfer through the membrane. For pullulanase, the transfer free energy values were calculated very low and negative. Therefore, the transfer from the membrane is favorable. Finally, MBP has some amino acids with high transfer free energy, but then the values become highly negative, making the transfer favorable.

# 7. CONCLUSIONS AND RECOMMENDATIONS

In previous experimental works, thermostable glucose isomerase, which is a cytoplasmic protein, of *Thermus thermophilus* HB8 was cloned in vector pMAL-p2 for translocation to the periplasmic space. The GI cloned in pMAL-p2 was expressed as a maltose binding protein-glucose isomerase fusion protein which was expected to be transported to the periplasm by the signal peptide on MBP. This experimental work have shown that the fusion protein mainly remained in the cytoplasm and very little portion of the expressed fusion protein was transported to the periplasm.

Even though the glucose isomerase protein was destined to be localized in the periplasm, the total protein expressed was found to be localized in cytoplasm. There might be several reasons for this result. One reason could be the fast folding rate of GI, so that the folding tendency interfered with the recognition of the fusion by the Sec machinery. But most probably the translocation efficiency considerably depended on the characteristics of the protein to be secreted. The characteristics may include the size of the protein, amino acid sequence and composition, the behavior of the host towards the specific protein, binding capacity to SecB, hydrophilic/hydrophobic properties, the interactions of the residues with the membrane through which the protein is about to pass, etc.

Investigating the characteristics of protein to be translocated is a good point to start with. So, cloning serine protease and pullulanase genes to the same plasmid vector pMAL-p2 as in the case of MBP-GI would give us a chance to compare the results of translocation efficiency among the three enzymes. For a good comparison, all the operating conditions were kept the same, temperature, medium composition, pH, IPTG concentration, the induction time etc. The only condition differing from the previous study was the type of strain. However, the results obtained in different types of *E. coli* strains such as TB1, XL1 and ER didn't differ much in terms of translocation efficiency. So, in this experimental study *E. coli* XL1 strain was used rather than TB1 strain.

The experimental results in this thesis showed that more than 90 per cent of pullulanase and more thn 70 per cent of serine protease were translocated to the

periplasmic space as a fusion partner with MBP. It is reported in B. Sarıyar's Ph.D. thesis that not more than 1 per cent of the total protein produced was translocated to the periplasm.

During the experiments performed in this thesis work, the operating conditions were not optimized, since the main aim was not to produce the enzymes at maximum amounts, but just to compare the cellular distribution. Although the optimization was not included in this work, it could give better results and improve the fusion protein expression.

Further studies can be performed to understand the step at which the failure in translocation occurred. The MBP-GI fusion may not fully bound to SecB, and thus can't be translocated to the periplasm, or there may be a problem during the transfer through the membrane.

# APPENDIX A: THE MATLAB CODE FOR CALCULATION OF FREE TRANSFER ENERGIES

```
Residue(700,3)=0;
Residue(:,:)=0;
protsize(4)=0;
protsize(:)=0;
for t = 1:4
   n=1
   while Aminoacids(t,n) ~= 'Z'
      protsize(t)=n;
      n=n+1;
   end
end
t=0;
for t = 1:4
   for i = 1:protsize(t)
      for j = 1:20
         if (Aminoacids(t,i) == Namecoef(j))
         Aminovalue(t,i)=Coef(j);
         j=20
         end
      end
   end
end
t=0;
i=1;
for t = 1:4
   for i = 1:(protsize(t)-19)
      tot=0;
      for j = 0:19
         tot=tot+Aminovalue(t,(j+i));
```

```
        end
        Residue(i,t)=tot;
    end
end
```

# APPENDIX B: THE OUTPUT OF THE MATLAB CODE FOR CALCULATION OF TRANSFER FREE ENERGIES

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 1 to 20 | -20,5 | 5,0 | -14,2 | 2,4 |
| 2 to 21 | -22,9 | -6,6 | -14,8 | -0,4 |
| 3 to 22 | -34,5 | 8,3 | -16,0 | 10,0 |
| 4 to 23 | -35,5 | 11,0 | -12,8 | 10,6 |
| 5 to 24 | -35,5 | 10,8 | -24,7 | 21,0 |
| 6 to 25 | -23,0 | 0,0 | -24,7 | 22,6 |
| 7 to 26 | -22,2 | -1,2 | -9,6 | 23,2 |
| 8 to 27 | -23,2 | -0,8 | -9,8 | 12,8 |
| 9 to 28 | -18,6 | 1,0 | -9,8 | 25,1 |
| 10 to 29 | -3,7 | -9,2 | -20,6 | 16,9 |
| 11 to 30 | -19,7 | -6,5 | -21,2 | 5,9 |
| 12 to 31 | -29,1 | -16,3 | -17,1 | 5,3 |
| 13 to 32 | -45,1 | -1,4 | -18,3 | -6,3 |
| 14 to 33 | -43,3 | 0,2 | -8,9 | -4,1 |
| 15 to 34 | -55,3 | -1,9 | 3,0 | -3,1 |
| 16 to 35 | -57,0 | -2,5 | 0,8 | -5,9 |
| 17 to 36 | -55,6 | 2,1 | 12,8 | -5,2 |
| 18 to 37 | -58,9 | -0,4 | 19,5 | -6,4 |
| 19 to 38 | -57,3 | 2,0 | 18,0 | -14,6 |
| 20 to 39 | -49,9 | 1,4 | 15,6 | -17,0 |
| 21 to 40 | -55,5 | 0,2 | 13,6 | -29,9 |
| 22 to 41 | -65,3 | 11,5 | 11,8 | -39,3 |
| 23 to 42 | -50,2 | 11,5 | 2,0 | -39,9 |
| 24 to 43 | -39,4 | 8,8 | -6,4 | -41,2 |
| 25 to 44 | -47,8 | 7,8 | 5,5 | -47,6 |
| 26 to 45 | -48,7 | 18,8 | 5,1 | -49,4 |
| 27 to 46 | -48,7 | 20,9 | 5,1 | -48,2 |
| 28 to 47 | -37,9 | 21,5 | 4,7 | -37,8 |
| 29 to 48 | -40,2 | 20,3 | 3,6 | -46,0 |
| 30 to 49 | -41,8 | 30,5 | 8,7 | -35,2 |
| 31 to 50 | -26,9 | 14,5 | 4,7 | -26,0 |
| 32 to 51 | -23,5 | 23,3 | 3,9 | -35,8 |
| 33 to 52 | -8,4 | 20,9 | -5,9 | -35,8 |
| 34 to 53 | -14,2 | 14,2 | -15,3 | -34,9 |
| 35 to 54 | -14,2 | 4,4 | -16,4 | -45,7 |
| 36 to 55 | -22,6 | 6,5 | -16,6 | -54,5 |
| 37 to 56 | -34,4 | 7,7 | -27,6 | -65,6 |
| 38 to 57 | -30,9 | -1,1 | -29,0 | -64,4 |
| 39 to 58 | -30,4 | -1,4 | -28,0 | -58,6 |
| 40 to 59 | -32,8 | -0,8 | -37,0 | -59,6 |
| 41 to 60 | -42,1 | -14,7 | -38,3 | -59,2 |
| 42 to 61 | -32,3 | -22,6 | -36,7 | -47,8 |
| 43 to 62 | -33,9 | -24,0 | -26,5 | -47,6 |
| 44 to 63 | -35,3 | -24,8 | -30,6 | -44,3 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 45 to 64 | -26,9 | -24,5 | -30,0 | -47,7 |
| 46 to 65 | -33,8 | -24,5 | -28,6 | -51,7 |
| 47 to 66 | -43,0 | -27,2 | -30,8 | -54,3 |
| 48 to 67 | -56,9 | -25,7 | -29,8 | -65,1 |
| 49 to 68 | -65,4 | -24,5 | -28,7 | -65,7 |
| 50 to 69 | -70,5 | -24,5 | -32,8 | -65,5 |
| 51 to 70 | -70,0 | -8,5 | -26,1 | -74,7 |
| 52 to 71 | -62,6 | -8,5 | -27,7 | -74,1 |
| 53 to 72 | -77,7 | -5,0 | -17,9 | -74,1 |
| 54 to 73 | -87,0 | -13,2 | -7,5 | -74,1 |
| 55 to 74 | -74,1 | -13,2 | -10,8 | -65,7 |
| 56 to 75 | -74,7 | -14,7 | -9,9 | -61,0 |
| 57 to 76 | -74,3 | -14,7 | 0,9 | -49,2 |
| 58 to 77 | -75,5 | -2,8 | -12,6 | -48,8 |
| 59 to 78 | -75,8 | -5,3 | -14,6 | -48,2 |
| 60 to 79 | -85,2 | -19,2 | -3,8 | -47,0 |
| 61 to 80 | -81,1 | -5,9 | -15,4 | -37,2 |
| 62 to 81 | -80,9 | -13,4 | -17,0 | -49,0 |
| 63 to 82 | -81,1 | -11,8 | -27,2 | -49,2 |
| 64 to 83 | -78,5 | -24,3 | -23,1 | -51,6 |
| 65 to 84 | -87,5 | -38,5 | -25,6 | -52,6 |
| 66 to 85 | -80,8 | -42,0 | -36,4 | -49,6 |
| 67 to 86 | -72,4 | -40,2 | -34,4 | -49,8 |
| 68 to 87 | -56,7 | -41,7 | -40,1 | -36,9 |
| 69 to 88 | -44,9 | -52,7 | -40,7 | -26,2 |
| 70 to 89 | -39,6 | -56,7 | -31,5 | -27,4 |
| 71 to 90 | -41,1 | -57,6 | -33,6 | -22,2 |
| 72 to 91 | -48,5 | -58,0 | -32,0 | -23,2 |
| 73 to 92 | -33,4 | -65,8 | -31,0 | -26,7 |
| 74 to 93 | -17,4 | -62,7 | -44,5 | -26,7 |
| 75 to 94 | -20,5 | -50,8 | -42,6 | -25,9 |
| 76 to 95 | -20,9 | -50,8 | -44,3 | -20,8 |
| 77 to 96 | -11,9 | -62,8 | -45,3 | -24,1 |
| 78 to 97 | -11,9 | -63,7 | -29,3 | -24,1 |
| 79 to 98 | -11,0 | -73,1 | -39,1 | -29,8 |
| 80 to 99 | -10,6 | -73,1 | -50,3 | -30,4 |
| 81 to 100 | -11,6 | -82,9 | -50,3 | -30,4 |
| 82 to 101 | -11,8 | -67,8 | -47,6 | -18,4 |
| 83 to 102 | -11,2 | -68,0 | -35,7 | -16,6 |
| 84 to 103 | -10,3 | -53,1 | -28,2 | -15,2 |
| 85 to 104 | -0,3 | -39,6 | -38,6 | -14,2 |
| 86 to 105 | -2,3 | -37,3 | -28,4 | -14,2 |
| 87 to 106 | -2,3 | -37,5 | -30,8 | -13,0 |
| 88 to 107 | -14,9 | -47,3 | -23,9 | -16,5 |
| 89 to 108 | -17,3 | -36,5 | -26,0 | -27,6 |
| 90 to 109 | -16,6 | -45,8 | -25,4 | -38,0 |
| 91 to 110 | -15,6 | -48,0 | -35,2 | -33,4 |
| 92 to 111 | -23,1 | -47,2 | -38,5 | -20,5 |
| 93 to 112 | -24,3 | -55,4 | -38,3 | -12,3 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 94 to 113 | -28,7 | -43,6 | -34,8 | -25,2 |
| 95 to 114 | -27,7 | -44,7 | -44,2 | -35,0 |
| 96 to 115 | -15,7 | -47,0 | -48,5 | -33,2 |
| 97 to 116 | -28,2 | -35,0 | -48,9 | -33,2 |
| 98 to 117 | -38,6 | -34,1 | -50,0 | -34,6 |
| 99 to 118 | -41,7 | -29,4 | -37,1 | -26,8 |
| 100 to 119 | -30,1 | -16,1 | -40,2 | -26,2 |
| 101 to 120 | -29,1 | -16,5 | -25,3 | -25,3 |
| 102 to 121 | -28,9 | -16,5 | -27,1 | -37,3 |
| 103 to 122 | -27,1 | -16,5 | -29,2 | -38,5 |
| 104 to 123 | -40,0 | -18,5 | -28,3 | -37,5 |
| 105 to 124 | -38,4 | -18,1 | -17,9 | -41,6 |
| 106 to 125 | -38,0 | -17,1 | -17,3 | -42,3 |
| 107 to 126 | -36,6 | -16,9 | -16,3 | -48,3 |
| 108 to 127 | -35,6 | -7,1 | -17,5 | -47,5 |
| 109 to 128 | -33,0 | -9,1 | -15,9 | -47,1 |
| 110 to 129 | -33,9 | 4,8 | -16,9 | -35,5 |
| 111 to 130 | -34,9 | 5,8 | -6,1 | -37,1 |
| 112 to 131 | -30,8 | 1,8 | -14,2 | -39,2 |
| 113 to 132 | -29,3 | 14,3 | -14,2 | -35,8 |
| 114 to 133 | -29,3 | 14,5 | -2,8 | -26,4 |
| 115 to 134 | -28,3 | 14,7 | 2,4 | -17,6 |
| 116 to 135 | -28,5 | 18,2 | 6,7 | -18,8 |
| 117 to 136 | -14,3 | 3,1 | 2,5 | -15,5 |
| 118 to 137 | -5,3 | -0,4 | 2,7 | -23,9 |
| 119 to 138 | -4,9 | 6,3 | -10,2 | -26,0 |
| 120 to 139 | -20,0 | -7,0 | -6,1 | -24,4 |
| 121 to 140 | -20,0 | -10,1 | -5,6 | -25,7 |
| 122 to 141 | -20,2 | -21,1 | -19,8 | -13,7 |
| 123 to 142 | -22,0 | -22,7 | -19,4 | -15,3 |
| 124 to 143 | -21,0 | -20,5 | -17,0 | -18,6 |
| 125 to 144 | -21,2 | -19,3 | -18,0 | -11,1 |
| 126 to 145 | -30,4 | -19,1 | -16,8 | -19,2 |
| 127 to 146 | -30,4 | -18,2 | -15,4 | -23,6 |
| 128 to 147 | -21,0 | -29,0 | -29,3 | -21,8 |
| 129 to 148 | -22,8 | -28,6 | -30,9 | -10,2 |
| 130 to 149 | -32,6 | -29,2 | -39,7 | -12,8 |
| 131 to 150 | -32,6 | -28,2 | -54,6 | -17,6 |
| 132 to 151 | -36,7 | -22,4 | -44,8 | -19,0 |
| 133 to 152 | -48,6 | -31,8 | -45,0 | -18,1 |
| 134 to 153 | -45,3 | -39,4 | -45,7 | -27,1 |
| 135 to 154 | -46,0 | -39,4 | -42,1 | -25,9 |
| 136 to 155 | -57,8 | -42,0 | -40,7 | -25,6 |
| 137 to 156 | -57,8 | -27,1 | -35,1 | -36,4 |
| 138 to 157 | -55,4 | -36,5 | -35,3 | -36,4 |
| 139 to 158 | -68,7 | -37,5 | -25,9 | -38,0 |
| 140 to 159 | -64,6 | -23,6 | -16,7 | -40,6 |
| 141 to 160 | -54,8 | -23,6 | -29,0 | -39,6 |
| 142 to 161 | -53,0 | -11,7 | -14,8 | -39,6 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 143 to 162 | -59,4 | -9,9 | -25,0 | -48,8 |
| 144 to 163 | -47,5 | -11,7 | -39,9 | -56,9 |
| 145 to 164 | -46,7 | -12,9 | -38,5 | -60,3 |
| 146 to 165 | -36,9 | -23,9 | -53,6 | -48,7 |
| 147 to 166 | -36,9 | -35,8 | -56,9 | -48,3 |
| 148 to 167 | -38,8 | -24,0 | -43,4 | -49,5 |
| 149 to 168 | -38,2 | -23,1 | -45,1 | -61,1 |
| 150 to 169 | -37,6 | -22,5 | -33,8 | -60,3 |
| 151 to 170 | -48,4 | -22,5 | -19,9 | -64,3 |
| 152 to 171 | -40,2 | -34,5 | -18,9 | -63,9 |
| 153 to 172 | -30,4 | -22,7 | -18,4 | -62,5 |
| 154 to 173 | -33,7 | -16,7 | -25,1 | -50,9 |
| 155 to 174 | -34,6 | -19,3 | -38,0 | -48,7 |
| 156 to 175 | -26,1 | -27,7 | -37,0 | -46,9 |
| 157 to 176 | -40,3 | -42,6 | -37,7 | -43,5 |
| 158 to 177 | -39,2 | -32,8 | -52,6 | -32,5 |
| 159 to 178 | -25,3 | -31,8 | -61,0 | -36,6 |
| 160 to 179 | -14,3 | -41,6 | -60,4 | -45,0 |
| 161 to 180 | -24,1 | -38,1 | -48,6 | -46,4 |
| 162 to 181 | -26,7 | -41,6 | -59,7 | -49,9 |
| 163 to 182 | -30,7 | -44,2 | -51,3 | -37,0 |
| 164 to 183 | -34,2 | -53,4 | -39,7 | -27,0 |
| 165 to 184 | -42,4 | -54,0 | -39,7 | -16,9 |
| 166 to 185 | -52,2 | -43,2 | -26,2 | -19,5 |
| 167 to 186 | -53,6 | -32,2 | -23,9 | -7,9 |
| 168 to 187 | -65,2 | -33,8 | -22,5 | -9,5 |
| 169 to 188 | -65,8 | -32,9 | -21,2 | 0,9 |
| 170 to 189 | -66,8 | -32,9 | -21,7 | 1,5 |
| 171 to 190 | -54,5 | -32,7 | -28,1 | 1,1 |
| 172 to 191 | -51,1 | -35,8 | -29,1 | 1,5 |
| 173 to 192 | -48,4 | -36,8 | -40,4 | 0,9 |
| 174 to 193 | -46,1 | -35,4 | -31,9 | -2,6 |
| 175 to 194 | -45,9 | -44,8 | -18,6 | -4,4 |
| 176 to 195 | -42,6 | -33,8 | -18,6 | -4,4 |
| 177 to 196 | -29,3 | -18,1 | -17,9 | -8,4 |
| 178 to 197 | -32,4 | -22,8 | -2,5 | -11,9 |
| 179 to 198 | -30,6 | -28,4 | -3,5 | -16,0 |
| 180 to 199 | -30,6 | -23,2 | -3,2 | -12,6 |
| 181 to 200 | -20,8 | -11,6 | -14,0 | -11,8 |
| 182 to 201 | -17,3 | -10,6 | -13,6 | -19,9 |
| 183 to 202 | -5,4 | -9,2 | -12,6 | -24,3 |
| 184 to 203 | -8,6 | -0,4 | -20,7 | -34,7 |
| 185 to 204 | -2,6 | -13,7 | -19,5 | -36,6 |
| 186 to 205 | 8,4 | -12,9 | -19,1 | -45,6 |
| 187 to 206 | -1,0 | -14,1 | -20,5 | -57,6 |
| 188 to 207 | -1,0 | -12,3 | -22,9 | -55,0 |
| 189 to 208 | -1,8 | -15,8 | -22,9 | -55,6 |
| 190 to 209 | -0,8 | -15,8 | -22,7 | -54,6 |
| 191 to 210 | -16,2 | -18,4 | -17,7 | -54,6 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 192 to 211 | -11,8 | -14,3 | -15,9 | -60,4 |
| 193 to 212 | -14,5 | -13,3 | -7,5 | -59,8 |
| 194 to 213 | -13,3 | -16,6 | -11,0 | -58,1 |
| 195 to 214 | -19,3 | -4,6 | -9,2 | -58,1 |
| 196 to 215 | -21,7 | -19,7 | -9,8 | -70,6 |
| 197 to 216 | -30,9 | -22,9 | -11,8 | -60,2 |
| 198 to 217 | -27,8 | -27,0 | -14,7 | -58,5 |
| 199 to 218 | -29,6 | -21,2 | -4,3 | -47,5 |
| 200 to 219 | -35,4 | -17,2 | -15,4 | -41,5 |
| 201 to 220 | -45,2 | -28,2 | -5,6 | -38,8 |
| 202 to 221 | -47,7 | -26,8 | 6,3 | -27,2 |
| 203 to 222 | -47,4 | -26,8 | 8,2 | -23,9 |
| 204 to 223 | -42,8 | -24,8 | 16,3 | -23,9 |
| 205 to 224 | -43,0 | -16,6 | 5,3 | -21,1 |
| 206 to 225 | -43,0 | -29,2 | 6,5 | -12,3 |
| 207 to 226 | -38,6 | -27,1 | 6,9 | -11,9 |
| 208 to 227 | -22,6 | -27,1 | 9,8 | -19,3 |
| 209 to 228 | -20,2 | -38,7 | -3,1 | -29,1 |
| 210 to 229 | -15,0 | -37,5 | -15,1 | -34,7 |
| 211 to 230 | -1,1 | -45,9 | -11,6 | -22,1 |
| 212 to 231 | -2,2 | -45,9 | -13,2 | -22,1 |
| 213 to 232 | -1,6 | -46,9 | -12,8 | -22,1 |
| 214 to 233 | -8,5 | -43,6 | -16,0 | -32,3 |
| 215 to 234 | -2,1 | -54,6 | -18,6 | -32,7 |
| 216 to 235 | 0,5 | -40,7 | -29,8 | -33,1 |
| 217 to 236 | -0,5 | -39,9 | -29,8 | -35,4 |
| 218 to 237 | -2,6 | -44,0 | -29,0 | -35,8 |
| 219 to 238 | -3,2 | -55,6 | -28,6 | -38,6 |
| 220 to 239 | -9,0 | -55,0 | -16,8 | -38,2 |
| 221 to 240 | 2,0 | -45,2 | -21,4 | -50,1 |
| 222 to 241 | 4,5 | -56,6 | -33,3 | -51,3 |
| 223 to 242 | -1,9 | -70,5 | -35,4 | -52,3 |
| 224 to 243 | -0,4 | -69,4 | -43,5 | -39,4 |
| 225 to 244 | -10,6 | -62,5 | -36,0 | -47,0 |
| 226 to 245 | -10,6 | -61,5 | -36,0 | -55,8 |
| 227 to 246 | -10,6 | -64,6 | -35,6 | -46,0 |
| 228 to 247 | -23,5 | -79,7 | -35,9 | -49,4 |
| 229 to 248 | -30,2 | -64,8 | -22,0 | -39,4 |
| 230 to 249 | -39,5 | -66,6 | -21,0 | -34,8 |
| 231 to 250 | -37,7 | -66,6 | -23,5 | -34,8 |
| 232 to 251 | -39,7 | -55,8 | -29,5 | -28,8 |
| 233 to 252 | -53,6 | 0,0 | -28,9 | -30,4 |
| 234 to 253 | -45,8 | 0,0 | -25,3 | -26,0 |
| 235 to 254 | -56,6 | 0,0 | -24,5 | -26,2 |
| 236 to 255 | -63,5 | 0,0 | -15,1 | -16,8 |
| 237 to 256 | -63,5 | 0,0 | -20,5 | -14,2 |
| 238 to 257 | -62,3 | 0,0 | -20,5 | -13,2 |
| 239 to 258 | -75,6 | 0,0 | -20,9 | -11,3 |
| 240 to 259 | -63,1 | 0,0 | -21,9 | -12,3 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 241 to 260 | -64,9 | 0,0 | -17,7 | -8,9 |
| 242 to 261 | -68,0 | 0,0 | -7,9 | -10,5 |
| 243 to 262 | -73,2 | 0,0 | -6,1 | -21,3 |
| 244 to 263 | -81,1 | 0,0 | 3,3 | -23,8 |
| 245 to 264 | -69,1 | 0,0 | 3,3 | -18,4 |
| 246 to 265 | -80,7 | 0,0 | 3,1 | -18,4 |
| 247 to 266 | -74,3 | 0,0 | -6,7 | -16,8 |
| 248 to 267 | -63,5 | 0,0 | -17,7 | -13,4 |
| 249 to 268 | -55,7 | 0,0 | -16,5 | -15,3 |
| 250 to 269 | -39,7 | 0,0 | -7,3 | -15,9 |
| 251 to 270 | -40,3 | 0,0 | -5,9 | -16,7 |
| 252 to 271 | -38,3 | 0,0 | 0,1 | -16,7 |
| 253 to 272 | -35,2 | 0,0 | -4,1 | -14,1 |
| 254 to 273 | -36,1 | 0,0 | -2,1 | -6,5 |
| 255 to 274 | -24,1 | 0,0 | -7,2 | -7,3 |
| 256 to 275 | -28,2 | 0,0 | -4,6 | -6,3 |
| 257 to 276 | -18,4 | 0,0 | 3,6 | -4,5 |
| 258 to 277 | -20,6 | 0,0 | 2,8 | -14,9 |
| 259 to 278 | -7,3 | 0,0 | 5,3 | -15,8 |
| 260 to 279 | -11,7 | 0,0 | 7,1 | -20,5 |
| 261 to 280 | -16,8 | 0,0 | -3,3 | -15,5 |
| 262 to 281 | -16,4 | 0,0 | -0,6 | -14,9 |
| 263 to 282 | -8,0 | 0,0 | -1,8 | -14,5 |
| 264 to 283 | -15,5 | 0,0 | -1,4 | -15,5 |
| 265 to 284 | -21,3 | 0,0 | 1,9 | -12,4 |
| 266 to 285 | -8,8 | 0,0 | -9,9 | -1,0 |
| 267 to 286 | -19,6 | 0,0 | -9,3 | -2,6 |
| 268 to 287 | -19,6 | 0,0 | -13,4 | 4,8 |
| 269 to 288 | -26,3 | 0,0 | -25,4 | 8,3 |
| 270 to 289 | -28,4 | 0,0 | -26,2 | 7,9 |
| 271 to 290 | -28,4 | 0,0 | -32,9 | 6,9 |
| 272 to 291 | -43,3 | 0,0 | -32,5 | 6,7 |
| 273 to 292 | -32,9 | 0,0 | -27,9 | 4,1 |
| 274 to 293 | -43,9 | 0,0 | -31,2 | -3,5 |
| 275 to 294 | -55,9 | 0,0 | -31,9 | -2,1 |
| 276 to 295 | -55,9 | 0,0 | -32,8 | -1,7 |
| 277 to 296 | -64,7 | 0,0 | -35,2 | -4,8 |
| 278 to 297 | -64,3 | 0,0 | -36,1 | 4,2 |
| 279 to 298 | -62,7 | 0,0 | -44,6 | -1,6 |
| 280 to 299 | -60,1 | 0,0 | -47,8 | -6,3 |
| 281 to 300 | -54,4 | 0,0 | -35,8 | -14,7 |
| 282 to 301 | -51,7 | 0,0 | -42,5 | -12,5 |
| 283 to 302 | -50,3 | 0,0 | -41,3 | -2,1 |
| 284 to 303 | -50,3 | 0,0 | -43,0 | -11,1 |
| 285 to 304 | -46,3 | 0,0 | -44,0 | -23,0 |
| 286 to 305 | -48,0 | 0,0 | -34,6 | -21,9 |
| 287 to 306 | -35,4 | 0,0 | -34,6 | -20,1 |
| 288 to 307 | -49,3 | 0,0 | -21,3 | -30,9 |
| 289 to 308 | -45,1 | 0,0 | -11,5 | -38,5 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 290 to 309 | -47,4 | 0,0 | -12,4 | -39,8 |
| 291 to 310 | -47,4 | 0,0 | -6,7 | -38,6 |
| 292 to 311 | -32,0 | 0,0 | -7,1 | -36,8 |
| 293 to 312 | -30,4 | 0,0 | -17,9 | -35,6 |
| 294 to 313 | -31,0 | 0,0 | -17,0 | -40,0 |
| 295 to 314 | -30,0 | 0,0 | -10,6 | -49,8 |
| 296 to 315 | -34,1 | 0,0 | -21,7 | -50,4 |
| 297 to 316 | -24,3 | 0,0 | -22,5 | -48,2 |
| 298 to 317 | -33,5 | 0,0 | -22,5 | -56,6 |
| 299 to 318 | -34,5 | 0,0 | -16,1 | -50,2 |
| 300 to 320 | -32,7 | 0,0 | -28,6 | -38,8 |
| 301 to 321 | -46,6 | 0,0 | -28,3 | -35,4 |
| 302 to 322 | -58,5 | 0,0 | -22,7 | -47,0 |
| 303 to 323 | -69,3 | 0,0 | -33,7 | -57,8 |
| 304 to 324 | -56,8 | 0,0 | -30,2 | -57,8 |
| 305 to 325 | -66,0 | 0,0 | -40,6 | -49,4 |
| 306 to 326 | -65,4 | 0,0 | -44,9 | -50,3 |
| 307 to 326 | -77,6 | 0,0 | -35,1 | -52,1 |
| 308 to 327 | -73,5 | 0,0 | -33,0 | -42,3 |
| 309 to 328 | -71,9 | 0,0 | -36,6 | -34,9 |
| 310 to 329 | -68,4 | 0,0 | -34,7 | -32,6 |
| 311 to 330 | -69,6 | 0,0 | -33,5 | -32,6 |
| 312 to 331 | -71,1 | 0,0 | -37,7 | -44,2 |
| 313 to 332 | -74,6 | 0,0 | -36,7 | -44,8 |
| 314 to 333 | -66,5 | 0,0 | -41,7 | -36,3 |
| 315 to 334 | -62,4 | 0,0 | -42,3 | -36,3 |
| 316 to 335 | -58,3 | 0,0 | -30,3 | -45,5 |
| 317 to 336 | -69,1 | 0,0 | -42,8 | -56,5 |
| 318 to 337 | -60,7 | 0,0 | -39,5 | -45,5 |
| 319 to 338 | -60,7 | 0,0 | -38,5 | -45,5 |
| 320 to 339 | -62,8 | 0,0 | -22,8 | -56,9 |
| 321 to 340 | -47,7 | 0,0 | -35,1 | -61,3 |
| 322 to 341 | -37,9 | 0,0 | -36,1 | -52,3 |
| 323 to 342 | -25,9 | 0,0 | -25,3 | -55,4 |
| 324 to 343 | -23,3 | 0,0 | -28,8 | -46,6 |
| 325 to 344 | -14,1 | 0,0 | -24,8 | -45,2 |
| 326 to 345 | -16,5 | 0,0 | -23,7 | -46,4 |
| 327 to 346 | -8,4 | 0,0 | -22,7 | -46,2 |
| 328 to 347 | 0,4 | 0,0 | -26,5 | -44,4 |
| 329 to 348 | -14,7 | 0,0 | -32,7 | -55,2 |
| 330 to 349 | -25,7 | 0,0 | -46,2 | -61,6 |
| 331 to 350 | -36,1 | 0,0 | -57,2 | -62,8 |
| 332 to 351 | -36,1 | 0,0 | -58,3 | -58,1 |
| 333 to 352 | -43,6 | 0,0 | -49,5 | -67,5 |
| 334 to 353 | -41,3 | 0,0 | -44,5 | -65,8 |
| 335 to 354 | -34,4 | 0,0 | -42,7 | -65,8 |
| 336 to 355 | -35,0 | 0,0 | -53,7 | -57,6 |
| 337 to 356 | -38,1 | 0,0 | -50,2 | -46,0 |
| 338 to 357 | -36,7 | 0,0 | -50,0 | -48,6 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 339 to 358 | -46,5 | 0,0 | -50,0 | -55,0 |
| 340 to 359 | -45,3 | 0,0 | -53,2 | -46,2 |
| 341 to 360 | -47,9 | 0,0 | -43,0 | -36,8 |
| 342 to 361 | -46,7 | 0,0 | -45,3 | -41,1 |
| 343 to 362 | -57,7 | 0,0 | -48,6 | -25,4 |
| 344 to 363 | -58,9 | 0,0 | -44,2 | -24,8 |
| 345 to 364 | -68,7 | 0,0 | -51,7 | -24,8 |
| 346 to 347 | -81,2 | 0,0 | -49,4 | -22,7 |
| 347 to 366 | -92,8 | 0,0 | -61,2 | -22,0 |
| 348 to 367 | -105,7 | 0,0 | -58,9 | -26,1 |
| 349 to 368 | -92,4 | 0,0 | -50,4 | -16,3 |
| 350 to 369 | -84,9 | 0,0 | -37,1 | -8,9 |
| 351 to 370 | -74,5 | 0,0 | -33,0 | -22,8 |
| 352 to 371 | -73,3 | 0,0 | -28,7 | -17,5 |
| 353 to 372 | -73,3 | 0,0 | -27,7 | -7,1 |
| 354 to 373 | -87,2 | 0,0 | -32,7 | -5,5 |
| 355 to 374 | -87,2 | 0,0 | -34,5 | 2,7 |
| 356 to 375 | -87,6 | 0,0 | -25,1 | -2,1 |
| 357 to 376 | -79,4 | 0,0 | -15,3 | -3,9 |
| 358 to 377 | -78,2 | 0,0 | -15,5 | -2,5 |
| 359 to 378 | -68,4 | 0,0 | -17,1 | 2,9 |
| 360 to 379 | -68,6 | 0,0 | -22,1 | 3,9 |
| 361 to 380 | -77,0 | 0,0 | -32,3 | -8,6 |
| 362 to 381 | -80,5 | 0,0 | -28,5 | -8,6 |
| 363 to 382 | -69,5 | 0,0 | -26,2 | -10,8 |
| 364 to 383 | -68,3 | 0,0 | -29,3 | -8,8 |
| 365 to 384 | -58,5 | 0,0 | -25,2 | -19,6 |
| 366 to 385 | -43,6 | 0,0 | -36,8 | -31,5 |
| 367 to 386 | -43,6 | 0,0 | -39,9 | -31,8 |
| 368 to 387 | -30,3 | 0,0 | -38,1 | -28,3 |
| 369 to 388 | 0,0 | 0,0 | -35,8 | -38,7 |
| 370 to 389 | 0,0 | 0,0 | -49,1 | -50,5 |
| 371 to 390 | 0,0 | 0,0 | -57,3 | -36,6 |
| 372 to 391 | 0,0 | 0,0 | -55,6 | -41,9 |
| 373 to 392 | 0,0 | 0,0 | -54,1 | -42,3 |
| 374 to 393 | 0,0 | 0,0 | -46,7 | -57,2 |
| 375 to 394 | 0,0 | 0,0 | -56,9 | -57,2 |
| 376 to 395 | 0,0 | 0,0 | -57,5 | -51,2 |
| 377 to 396 | 0,0 | 0,0 | -55,9 | -61,6 |
| 378 to 397 | 0,0 | 0,0 | -55,9 | 0,0 |
| 379 to 398 | 0,0 | 0,0 | -53,2 | 0,0 |
| 380 to 399 | 0,0 | 0,0 | -46,5 | 0,0 |
| 381 to 400 | 0,0 | 0,0 | -35,7 | 0,0 |
| 382 to 401 | 0,0 | 0,0 | -47,6 | 0,0 |
| 383 to 402 | 0,0 | 0,0 | -57,4 | 0,0 |
| 384 to 403 | 0,0 | 0,0 | -58,7 | 0,0 |
| 385 to 404 | 0,0 | 0,0 | -49,5 | 0,0 |
| 386 to 405 | 0,0 | 0,0 | -34,1 | 0,0 |
| 387 to 406 | 0,0 | 0,0 | -31,0 | 0,0 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 388 to 407 | 0,0 | 0,0 | -33,4 | 0,0 |
| 389 to 408 | 0,0 | 0,0 | -31,3 | 0,0 |
| 390 to 409 | 0,0 | 0,0 | -31,3 | 0,0 |
| 391 to 410 | 0,0 | 0,0 | -15,3 | 0,0 |
| 392 to 411 | 0,0 | 0,0 | -26,4 | 0,0 |
| 393 to 412 | 0,0 | 0,0 | -26,7 | 0,0 |
| 394 to 413 | 0,0 | 0,0 | -25,9 | 0,0 |
| 395 to 414 | 0,0 | 0,0 | -15,7 | 0,0 |
| 396 to 415 | 0,0 | 0,0 | -13,7 | 0,0 |
| 397 to 416 | 0,0 | 0,0 | -19,3 | 0,0 |
| 398 to 417 | 0,0 | 0,0 | -31,1 | 0,0 |
| 399 to 418 | 0,0 | 0,0 | -31,7 | 0,0 |
| 400 to 419 | 0,0 | 0,0 | -41,8 | 0,0 |
| 401 to 420 | 0,0 | 0,0 | -42,2 | 0,0 |
| 402 to 421 | 0,0 | 0,0 | -30,0 | 0,0 |
| 403 to 422 | 0,0 | 0,0 | -30,6 | 0,0 |
| 404 to 423 | 0,0 | 0,0 | -28,3 | 0,0 |
| 405 to 424 | 0,0 | 0,0 | -26,7 | 0,0 |
| 406 to 425 | 0,0 | 0,0 | -42,1 | 0,0 |
| 407 to 426 | 0,0 | 0,0 | -42,1 | 0,0 |
| 408 to 427 | 0,0 | 0,0 | -41,5 | 0,0 |
| 409 to 428 | 0,0 | 0,0 | -42,4 | 0,0 |
| 410 to 429 | 0,0 | 0,0 | -39,3 | 0,0 |
| 411 to 430 | 0,0 | 0,0 | -41,4 | 0,0 |
| 412 to 431 | 0,0 | 0,0 | -29,1 | 0,0 |
| 413 to 432 | 0,0 | 0,0 | -41,1 | 0,0 |
| 414 to 433 | 0,0 | 0,0 | -44,3 | 0,0 |
| 415 to 434 | 0,0 | 0,0 | -44,7 | 0,0 |
| 416 to 435 | 0,0 | 0,0 | -44,2 | 0,0 |
| 417 to 436 | 0,0 | 0,0 | -38,4 | 0,0 |
| 418 to 437 | 0,0 | 0,0 | -26,6 | 0,0 |
| 419 to 438 | 0,0 | 0,0 | -30,4 | 0,0 |
| 420 to 439 | 0,0 | 0,0 | -21,2 | 0,0 |
| 421 to 440 | 0,0 | 0,0 | -30,6 | 0,0 |
| 422 to 441 | 0,0 | 0,0 | -33,0 | 0,0 |
| 423 to 442 | 0,0 | 0,0 | -22,3 | 0,0 |
| 424 to 443 | 0,0 | 0,0 | -33,1 | 0,0 |
| 425 to 444 | 0,0 | 0,0 | -32,9 | 0,0 |
| 426 to 445 | 0,0 | 0,0 | -20,4 | 0,0 |
| 427 to 446 | 0,0 | 0,0 | -10,0 | 0,0 |
| 428 to 447 | 0,0 | 0,0 | -11,4 | 0,0 |
| 429 to 448 | 0,0 | 0,0 | -11,4 | 0,0 |
| 430 to 449 | 0,0 | 0,0 | -2,0 | 0,0 |
| 431 to 450 | 0,0 | 0,0 | -3,4 | 0,0 |
| 432 to 451 | 0,0 | 0,0 | -14,7 | 0,0 |
| 433 to 452 | 0,0 | 0,0 | -9,6 | 0,0 |
| 434 to 453 | 0,0 | 0,0 | -18,6 | 0,0 |
| 435 to 454 | 0,0 | 0,0 | -17,6 | 0,0 |
| 436 to 455 | 0,0 | 0,0 | -19,5 | 0,0 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 437 to 456 | 0,0 | 0,0 | -18,9 | 0,0 |
| 438 to 457 | 0,0 | 0,0 | -19,9 | 0,0 |
| 439 to 458 | 0,0 | 0,0 | -24,0 | 0,0 |
| 440 to 459 | 0,0 | 0,0 | -23,4 | 0,0 |
| 441 to 460 | 0,0 | 0,0 | -24,0 | 0,0 |
| 442 to 461 | 0,0 | 0,0 | -29,1 | 0,0 |
| 443 to 462 | 0,0 | 0,0 | -28,2 | 0,0 |
| 444 to 463 | 0,0 | 0,0 | -18,8 | 0,0 |
| 445 to 464 | 0,0 | 0,0 | -33,9 | 0,0 |
| 446 to 465 | 0,0 | 0,0 | -30,4 | 0,0 |
| 447 to 466 | 0,0 | 0,0 | -30,0 | 0,0 |
| 448 to 467 | 0,0 | 0,0 | -30,9 | 0,0 |
| 449 to 468 | 0,0 | 0,0 | -30,0 | 0,0 |
| 450 to 469 | 0,0 | 0,0 | -35,0 | 0,0 |
| 451 to 470 | 0,0 | 0,0 | -44,4 | 0,0 |
| 452 to 471 | 0,0 | 0,0 | -48,5 | 0,0 |
| 453 to 472 | 0,0 | 0,0 | -40,7 | 0,0 |
| 454 to 473 | 0,0 | 0,0 | -44,2 | 0,0 |
| 455 to 474 | 0,0 | 0,0 | -55,0 | 0,0 |
| 456 to 475 | 0,0 | 0,0 | -54,6 | 0,0 |
| 457 to 476 | 0,0 | 0,0 | -55,4 | 0,0 |
| 458 to 477 | 0,0 | 0,0 | -65,8 | 0,0 |
| 459 to 478 | 0,0 | 0,0 | -60,0 | 0,0 |
| 460 to 479 | 0,0 | 0,0 | -61,0 | 0,0 |
| 461 to 480 | 0,0 | 0,0 | -51,0 | 0,0 |
| 462 to 481 | 0,0 | 0,0 | -43,2 | 0,0 |
| 463 to 482 | 0,0 | 0,0 | -49,0 | 0,0 |
| 464 to 483 | 0,0 | 0,0 | -46,4 | 0,0 |
| 465 to 484 | 0,0 | 0,0 | -33,9 | 0,0 |
| 466 to 485 | 0,0 | 0,0 | -46,8 | 0,0 |
| 467 to 486 | 0,0 | 0,0 | -60,7 | 0,0 |
| 468 to 487 | 0,0 | 0,0 | -59,0 | 0,0 |
| 469 to 488 | 0,0 | 0,0 | -59,0 | 0,0 |
| 470 to 489 | 0,0 | 0,0 | -52,6 | 0,0 |
| 471 to 490 | 0,0 | 0,0 | -40,6 | 0,0 |
| 472 to 491 | 0,0 | 0,0 | -27,3 | 0,0 |
| 473 to 492 | 0,0 | 0,0 | -35,8 | 0,0 |
| 474 to 493 | 0,0 | 0,0 | -23,3 | 0,0 |
| 475 to 494 | 0,0 | 0,0 | -13,1 | 0,0 |
| 476 to 495 | 0,0 | 0,0 | -13,7 | 0,0 |
| 477 to 496 | 0,0 | 0,0 | -28,6 | 0,0 |
| 478 to 497 | 0,0 | 0,0 | -28,0 | 0,0 |
| 479 to 498 | 0,0 | 0,0 | -33,1 | 0,0 |
| 480 to 499 | 0,0 | 0,0 | -31,1 | 0,0 |
| 481 to 500 | 0,0 | 0,0 | -41,1 | 0,0 |
| 482 to 501 | 0,0 | 0,0 | -42,0 | 0,0 |
| 483 to 502 | 0,0 | 0,0 | -37,4 | 0,0 |
| 484 to 503 | 0,0 | 0,0 | -37,1 | 0,0 |
| 485 to 504 | 0,0 | 0,0 | -35,7 | 0,0 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 486 to 505 | 0,0 | 0,0 | -25,5 | 0,0 |
| 487 to 506 | 0,0 | 0,0 | -12,6 | 0,0 |
| 488 to 507 | 0,0 | 0,0 | -10,8 | 0,0 |
| 489 to 508 | 0,0 | 0,0 | -26,8 | 0,0 |
| 490 to 509 | 0,0 | 0,0 | -26,8 | 0,0 |
| 491 to 510 | 0,0 | 0,0 | -26,8 | 0,0 |
| 492 to 511 | 0,0 | 0,0 | -26,8 | 0,0 |
| 493 to 512 | 0,0 | 0,0 | -21,0 | 0,0 |
| 494 to 513 | 0,0 | 0,0 | -18,4 | 0,0 |
| 495 to 514 | 0,0 | 0,0 | -15,7 | 0,0 |
| 496 to 515 | 0,0 | 0,0 | -14,7 | 0,0 |
| 497 to 516 | 0,0 | 0,0 | -5,4 | 0,0 |
| 498 to 517 | 0,0 | 0,0 | 3,0 | 0,0 |
| 499 to 518 | 0,0 | 0,0 | -5,2 | 0,0 |
| 500 to 519 | 0,0 | 0,0 | -11,9 | 0,0 |
| 501 to 520 | 0,0 | 0,0 | -2,5 | 0,0 |
| 502 to 521 | 0,0 | 0,0 | -2,2 | 0,0 |
| 503 to 522 | 0,0 | 0,0 | -8,6 | 0,0 |
| 504 to 523 | 0,0 | 0,0 | -12,4 | 0,0 |
| 505 to 524 | 0,0 | 0,0 | -11,4 | 0,0 |
| 506 to 525 | 0,0 | 0,0 | -20,6 | 0,0 |
| 507 to 526 | 0,0 | 0,0 | -19,2 | 0,0 |
| 508 to 527 | 0,0 | 0,0 | -25,0 | 0,0 |
| 509 to 528 | 0,0 | 0,0 | -21,9 | 0,0 |
| 510 to 529 | 0,0 | 0,0 | -28,3 | 0,0 |
| 511 to 530 | 0,0 | 0,0 | -34,1 | 0,0 |
| 512 to 531 | 0,0 | 0,0 | -33,9 | 0,0 |
| 513 to 532 | 0,0 | 0,0 | -31,2 | 0,0 |
| 514 to 533 | 0,0 | 0,0 | -32,1 | 0,0 |
| 515 to 534 | 0,0 | 0,0 | -45,0 | 0,0 |
| 516 to 535 | 0,0 | 0,0 | -55,8 | 0,0 |
| 517 to 536 | 0,0 | 0,0 | -49,4 | 0,0 |
| 518 to 537 | 0,0 | 0,0 | -57,8 | 0,0 |
| 519 to 538 | 0,0 | 0,0 | -43,9 | 0,0 |
| 520 to 539 | 0,0 | 0,0 | -38,2 | 0,0 |
| 521 to 540 | 0,0 | 0,0 | -43,6 | 0,0 |
| 522 to 541 | 0,0 | 0,0 | -49,7 | 0,0 |
| 523 to 542 | 0,0 | 0,0 | -54,1 | 0,0 |
| 524 to 543 | 0,0 | 0,0 | -61,6 | 0,0 |
| 525 to 544 | 0,0 | 0,0 | -64,0 | 0,0 |
| 526 to 545 | 0,0 | 0,0 | -64,0 | 0,0 |
| 527 to 546 | 0,0 | 0,0 | -64,1 | 0,0 |
| 528 to 547 | 0,0 | 0,0 | -58,3 | 0,0 |
| 529 to 548 | 0,0 | 0,0 | -61,4 | 0,0 |
| 530 to 549 | 0,0 | 0,0 | -68,9 | 0,0 |
| 531 to 550 | 0,0 | 0,0 | -74,7 | 0,0 |
| 532 to 551 | 0,0 | 0,0 | -88,2 | 0,0 |
| 533 to 552 | 0,0 | 0,0 | -96,0 | 0,0 |
| 534 to 553 | 0,0 | 0,0 | -106,7 | 0,0 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 535 to 554 | 0,0 | 0,0 | -94,7 | 0,0 |
| 536 to 555 | 0,0 | 0,0 | -84,3 | 0,0 |
| 537 to 556 | 0,0 | 0,0 | -86,5 | 0,0 |
| 538 to 557 | 0,0 | 0,0 | -76,7 | 0,0 |
| 539 to 558 | 0,0 | 0,0 | -75,2 | 0,0 |
| 540 to 559 | 0,0 | 0,0 | -74,2 | 0,0 |
| 541 to 560 | 0,0 | 0,0 | -66,6 | 0,0 |
| 542 to 561 | 0,0 | 0,0 | -60,8 | 0,0 |
| 543 to 562 | 0,0 | 0,0 | -50,0 | 0,0 |
| 544 to 563 | 0,0 | 0,0 | -45,9 | 0,0 |
| 545 to 564 | 0,0 | 0,0 | -45,1 | 0,0 |
| 546 to 565 | 0,0 | 0,0 | -33,8 | 0,0 |
| 547 to 566 | 0,0 | 0,0 | -35,5 | 0,0 |
| 548 to 567 | 0,0 | 0,0 | -34,6 | 0,0 |
| 549 to 568 | 0,0 | 0,0 | -19,5 | 0,0 |
| 550 to 569 | 0,0 | 0,0 | -10,2 | 0,0 |
| 551 to 570 | 0,0 | 0,0 | -0,8 | 0,0 |
| 552 to 571 | 0,0 | 0,0 | 12,5 | 0,0 |
| 553 to 572 | 0,0 | 0,0 | 12,5 | 0,0 |
| 554 to 573 | 0,0 | 0,0 | 13,1 | 0,0 |
| 555 to 574 | 0,0 | 0,0 | 14,0 | 0,0 |
| 556 to 575 | 0,0 | 0,0 | 11,7 | 0,0 |
| 557 to 576 | 0,0 | 0,0 | -1,8 | 0,0 |
| 558 to 577 | 0,0 | 0,0 | -2,2 | 0,0 |
| 559 to 578 | 0,0 | 0,0 | -14,1 | 0,0 |
| 560 to 579 | 0,0 | 0,0 | -15,7 | 0,0 |
| 561 to 580 | 0,0 | 0,0 | -17,5 | 0,0 |
| 562 to 581 | 0,0 | 0,0 | -29,5 | 0,0 |
| 563 to 582 | 0,0 | 0,0 | -30,1 | 0,0 |
| 564 to 583 | 0,0 | 0,0 | -30,8 | 0,0 |
| 565 to 584 | 0,0 | 0,0 | -31,2 | 0,0 |
| 566 to 585 | 0,0 | 0,0 | -35,0 | 0,0 |
| 567 to 586 | 0,0 | 0,0 | -47,5 | 0,0 |
| 568 to 587 | 0,0 | 0,0 | -50,6 | 0,0 |
| 569 to 588 | 0,0 | 0,0 | -53,2 | 0,0 |
| 570 to 589 | 0,0 | 0,0 | -59,4 | 0,0 |
| 571 to 590 | 0,0 | 0,0 | -58,4 | 0,0 |
| 572 to 591 | 0,0 | 0,0 | -56,8 | 0,0 |
| 573 to 592 | 0,0 | 0,0 | -57,5 | 0,0 |
| 574 to 593 | 0,0 | 0,0 | -53,4 | 0,0 |
| 575 to 594 | 0,0 | 0,0 | -54,3 | 0,0 |
| 576 to 595 | 0,0 | 0,0 | -62,8 | 0,0 |
| 577 to 596 | 0,0 | 0,0 | -48,6 | 0,0 |
| 578 to 597 | 0,0 | 0,0 | -58,0 | 0,0 |
| 579 to 598 | 0,0 | 0,0 | -61,5 | 0,0 |
| 580 to 599 | 0,0 | 0,0 | -71,3 | 0,0 |
| 581 to 600 | 0,0 | 0,0 | -71,7 | 0,0 |
| 582 to 601 | 0,0 | 0,0 | -74,8 | 0,0 |
| 583 to 602 | 0,0 | 0,0 | -76,5 | 0,0 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 584 to 603 | 0,0 | 0,0 | -79,9 | 0,0 |
| 585 to 604 | 0,0 | 0,0 | -89,7 | 0,0 |
| 586 to 605 | 0,0 | 0,0 | -98,2 | 0,0 |
| 587 to 606 | 0,0 | 0,0 | -83,3 | 0,0 |
| 588 to 607 | 0,0 | 0,0 | -96,2 | 0,0 |
| 589 to 608 | 0,0 | 0,0 | -97,1 | 0,0 |
| 590 to 609 | 0,0 | 0,0 | -85,3 | 0,0 |
| 591 to 610 | 0,0 | 0,0 | -91,0 | 0,0 |
| 592 to 611 | 0,0 | 0,0 | -92,6 | 0,0 |
| 593 to 612 | 0,0 | 0,0 | -85,0 | 0,0 |
| 594 to 613 | 0,0 | 0,0 | -77,8 | 0,0 |
| 595 to 614 | 0,0 | 0,0 | -79,0 | 0,0 |
| 596 to 597 | 0,0 | 0,0 | -67,0 | 0,0 |
| 597 to 616 | 0,0 | 0,0 | -81,2 | 0,0 |
| 598 to 617 | 0,0 | 0,0 | -85,3 | 0,0 |
| 599 to 618 | 0,0 | 0,0 | -71,4 | 0,0 |
| 600 to 619 | 0,0 | 0,0 | -65,6 | 0,0 |
| 601 to 620 | 0,0 | 0,0 | -65,2 | 0,0 |
| 602 to 621 | 0,0 | 0,0 | -51,3 | 0,0 |
| 603 to 622 | 0,0 | 0,0 | -46,9 | 0,0 |
| 604 to 623 | 0,0 | 0,0 | -51,0 | 0,0 |
| 605 to 624 | 0,0 | 0,0 | -39,0 | 0,0 |
| 606 to 625 | 0,0 | 0,0 | -28,2 | 0,0 |
| 607 to 626 | 0,0 | 0,0 | -29,6 | 0,0 |
| 608 to 627 | 0,0 | 0,0 | -25,5 | 0,0 |
| 609 to 628 | 0,0 | 0,0 | -23,2 | 0,0 |
| 610 to 629 | 0,0 | 0,0 | -34,0 | 0,0 |
| 611 to 630 | 0,0 | 0,0 | -27,3 | 0,0 |
| 612 to 631 | 0,0 | 0,0 | -25,5 | 0,0 |
| 613 to 632 | 0,0 | 0,0 | -40,6 | 0,0 |
| 614 to 633 | 0,0 | 0,0 | -46,7 | 0,0 |
| 615 to 634 | 0,0 | 0,0 | -44,6 | 0,0 |
| 616 to 635 | 0,0 | 0,0 | -46,2 | 0,0 |
| 617 to 636 | 0,0 | 0,0 | -30,2 | 0,0 |
| 618 to 637 | 0,0 | 0,0 | -15,1 | 0,0 |
| 619 to 638 | 0,0 | 0,0 | -24,9 | 0,0 |
| 620 to 639 | 0,0 | 0,0 | -21,7 | 0,0 |
| 621 to 640 | 0,0 | 0,0 | -19,9 | 0,0 |
| 622 to 641 | 0,0 | 0,0 | -21,3 | 0,0 |
| 623 to 642 | 0,0 | 0,0 | -24,8 | 0,0 |
| 624 to 643 | 0,0 | 0,0 | -11,9 | 0,0 |
| 625 to 644 | 0,0 | 0,0 | -12,1 | 0,0 |
| 626 to 645 | 0,0 | 0,0 | -10,6 | 0,0 |
| 627 to 646 | 0,0 | 0,0 | -10,2 | 0,0 |
| 628 to 647 | 0,0 | 0,0 | -2,7 | 0,0 |
| 629 to 648 | 0,0 | 0,0 | -16,6 | 0,0 |
| 630 to 649 | 0,0 | 0,0 | -5,6 | 0,0 |
| 631 to 650 | 0,0 | 0,0 | -11,2 | 0,0 |
| 632 to 651 | 0,0 | 0,0 | -23,2 | 0,0 |

| Residues | Glucose Isomerase | Serine Protease | Pullulanase | Maltose Binding Protein |
|---|---|---|---|---|
| 633 to 652 | 0,0 | 0,0 | -9,3 | 0,0 |
| 634 to 653 | 0,0 | 0,0 | -4,7 | 0,0 |
| 635 to 654 | 0,0 | 0,0 | -5,8 | 0,0 |
| 636 to 655 | 0,0 | 0,0 | -7,7 | 0,0 |
| 637 to 656 | 0,0 | 0,0 | -10,4 | 0,0 |
| 638 to 657 | 0,0 | 0,0 | -13,0 | 0,0 |
| 639 to 658 | 0,0 | 0,0 | -2,9 | 0,0 |
| 640 to 659 | 0,0 | 0,0 | -11,3 | 0,0 |
| 641 to 660 | 0,0 | 0,0 | -23,3 | 0,0 |
| 642 to 661 | 0,0 | 0,0 | -20,4 | 0,0 |
| 643 to 662 | 0,0 | 0,0 | -17,5 | 0,0 |
| 644 to 663 | 0,0 | 0,0 | -15,5 | 0,0 |
| 645 to 664 | 0,0 | 0,0 | -15,5 | 0,0 |
| 646 to 665 | 0,0 | 0,0 | -21,6 | 0,0 |
| 647 to 666 | 0,0 | 0,0 | -26,2 | 0,0 |
| 648 to 667 | 0,0 | 0,0 | -30,3 | 0,0 |
| 649 to 668 | 0,0 | 0,0 | -26,2 | 0,0 |
| 650 to 669 | 0,0 | 0,0 | -37,2 | 0,0 |
| 651 to 670 | 0,0 | 0,0 | -43,0 | 0,0 |
| 652 to 671 | 0,0 | 0,0 | -42,0 | 0,0 |
| 653 to 672 | 0,0 | 0,0 | -42,4 | 0,0 |
| 654 to 673 | 0,0 | 0,0 | -42,4 | 0,0 |
| 655 to 674 | 0,0 | 0,0 | -41,9 | 0,0 |
| 656 to 675 | 0,0 | 0,0 | -39,6 | 0,0 |
| 657 to 676 | 0,0 | 0,0 | -37,8 | 0,0 |
| 658 to 677 | 0,0 | 0,0 | -37,8 | 0,0 |
| 659 to 678 | 0,0 | 0,0 | -48,9 | 0,0 |
| 660 to 679 | 0,0 | 0,0 | -48,9 | 0,0 |
| 661 to 680 | 0,0 | 0,0 | -52,0 | 0,0 |
| 662 to 681 | 0,0 | 0,0 | -63,3 | 0,0 |
| 663 to 682 | 0,0 | 0,0 | -64,5 | 0,0 |
| 664 to 683 | 0,0 | 0,0 | -65,5 | 0,0 |
| 665 to 684 | 0,0 | 0,0 | -65,5 | 0,0 |
| 666 to 685 | 0,0 | 0,0 | -59,9 | 0,0 |
| 667 to 686 | 0,0 | 0,0 | -54,9 | 0,0 |
| 668 to 687 | 0,0 | 0,0 | -59,3 | 0,0 |
| 669 to 688 | 0,0 | 0,0 | -50,1 | 0,0 |
| 670 to 689 | 0,0 | 0,0 | -46,0 | 0,0 |
| 671 to 690 | 0,0 | 0,0 | -49,5 | 0,0 |
| 672 to 691 | 0,0 | 0,0 | -39,3 | 0,0 |
| 673 to 692 | 0,0 | 0,0 | -39,5 | 0,0 |
| 674 to 693 | 0,0 | 0,0 | -39,9 | 0,0 |
| 675 to 694 | 0,0 | 0,0 | -41,8 | 0,0 |
| 676 to 695 | 0,0 | 0,0 | -43,2 | 0,0 |
| 677 to 696 | 0,0 | 0,0 | -42,3 | 0,0 |
| 678 to 697 | 0,0 | 0,0 | -41,5 | 0,0 |
| 679 to 698 | 0,0 | 0,0 | -36,4 | 0,0 |
| 680 to 699 | 0,0 | 0,0 | -26,6 | 0,0 |
| 681 to 700 | 0,0 | 0,0 | -26,6 | 0,0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 682 | to | 701 | 0,0 | 0,0 | -17,4 | 0,0 |
| 683 | to | 702 | 0,0 | 0,0 | -15,9 | 0,0 |
| 684 | to | 703 | 0,0 | 0,0 | -14,7 | 0,0 |
| 685 | to | 704 | 0,0 | 0,0 | -29,6 | 0,0 |
| 686 | to | 705 | 0,0 | 0,0 | -29,4 | 0,0 |
| 687 | to | 706 | 0,0 | 0,0 | -40,6 | 0,0 |
| 688 | to | 707 | 0,0 | 0,0 | -30,4 | 0,0 |
| 689 | to | 708 | 0,0 | 0,0 | -28,3 | 0,0 |
| 690 | to | 709 | 0,0 | 0,0 | -23,2 | 0,0 |
| 691 | to | 710 | 0,0 | 0,0 | -9,7 | 0,0 |
| 692 | to | 711 | 0,0 | 0,0 | -9,8 | 0,0 |
| 693 | to | 712 | 0,0 | 0,0 | -8,2 | 0,0 |
| 694 | to | 713 | 0,0 | 0,0 | -6,6 | 0,0 |
| 695 | to | 714 | 0,0 | 0,0 | -5,2 | 0,0 |
| 696 | to | 715 | 0,0 | 0,0 | -8,4 | 0,0 |
| 697 | to | 716 | 0,0 | 0,0 | -11,9 | 0,0 |
| 698 | to | 717 | 0,0 | 0,0 | -11,3 | 0,0 |
| 699 | to | 718 | 0,0 | 0,0 | -6,2 | 0,0 |
| 700 | to | 719 | 0,0 | 0,0 | 0,0 | 0,0 |

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