NOVEL CELLULASE ENZYME PRODUCTION TOWARDS BIOFUELS SECTOR BY RECOMBINANT BACTERIUM *Escherichia coli*

BİYOYAKIT SEKTÖRÜNE YÖNELİK YENİ NESİL SELÜLAZ ENZİMLERİNİN REKOMBİNANT *Escherichia coli* BAKTERİSİNDE ÜRETİMİ

ZEHRA YILDIRIM

ASST. PROF. DR. EDA ÇELİK AKDUR
Supervisor

Submitted to Graduate School of Science and Engineering of Hacettepe University
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This work named **"Novel Cellulase Enzyme Production Towards Biofuels Sector by Recombinant Bacterium *Escherichia coli*"** by **ZEHRA YILDIRIM** has been approved as a thesis of Degree of **MASTER OF PHILOSOPHY IN BIOENGINEERING** by the below mentioned Examining Committee Members.

Prof. Dr. Hülya YAVUZ ERSAN ....
Head

Asst. Prof. Dr. Eda ÇELİK AKDUR ....
Supervisor

Asst. Prof. Dr. Urartu Özgür Şafak ŞEKER ....
Member

This thesis has been approved as a thesis for the Degree of **MASTER OF PHILOSOPHY IN BIOENGINEERING** by Board of Directors of the Institute for Graduate School of Science and Engineering.

Prof. Dr. Fatma SEVİN DÜZ
Director of the Institute of
Graduate School of Science and Engineering

to my family…

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ABSTRACT

NOVEL CELLULASE ENZYME PRODUCTION TOWARDS BIOFUELS SECTOR BY RECOMBINANT BACTERIUM *Escherichia coli*

Zehra YILDIRIM

Master of Philosophy, Department of Bioengineering

Supervisor: Asst. Prof. Dr. Eda ÇELİK AKDUR

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Low cost, pH- and thermo-stable cellulase enzymes are important factors for commercially viable production of bioethanol, which is a renewable source of energy. Instead of food raw materials such as corn and sugar cane, ethanol obtained from cellulosic biomass by endoglucanase type of cellulase will reduce the production costs. *Escherichia coli* bacteria are one of the most preferred hosts for the production of recombinant proteins and the presence of glycosylation patterns in bacteria have been discovered recently, a simple protein glycosylation locus (pgl) found in the bacterium *Campylobacter jejuni* was transferred to *E. coli*, which gave the capability of bacterial protein glycosylation to this industrially important host. While cellulase enzymes produced in eukaryotic cells reveal the positive effects of glycosylation, glycosylated cellulase production in bacteria is not

found in the literature. On the other hand, enzymes produced in bacterial systems are known to be more economical compared to eukaryotic cells.

In this study, pET28a-ssDsbA-Cel5A-Flag-6xHis, pET28a-ssYebF-Cel5A-6xHis, pTrc99A-ssDsbA-Cel5A-Flag-6xHis, and pTrc99A-ssYebF-Cel5A-6xHis, plasmids were designed and sixteen new production systems were created by transforming these new plasmids with or without pMAF_pglB and/or pPglΔB, into *E. coli* CLM24 or *E. coli* BL21 (DE3). The designed plasmids were constructed using standard genetic engineering techniques and the sequence of the plasmids were confirmed with restriction digestion and DNA sequencing analysis. Glycosylated and wild-type cellulase enzymes (Cel5A) were analyzed and confirmed by SDS-PAGE and Western Blotting methods.

Thereafter, in order to increase the cellulase production efficiency, bioprocess parameters; such as (i) growth media (ii) inducer concentration (iii) carbon source and additional supplements at growth phase (iv) $OD_{induction}$, (v) induction temprature, (vi) sampling time, (vii) cell lyses conditions were optimized and enzyme activity assay was modified. The bioprocess was fully developed and as a result; activity of four different systems that produce cellulase were increased as; intracellular cellulase 21-fold, up to 0.219 IU/mL; extracellular cellulase 1.8-fold, up to 0.302 IU/mL, glycosylated intracellular cellulase 68-fold, up to 1.02 IU/mL and glycosylated extracellular cellulase 3-fold, up to 0.782 IU/mL, which is 6-fold higher compared to a recently reported non-glycosylated cellulase. Enzyme kinetic studies revealed similar values for K_m (ca. 3.2 mM) for both glycosylated and non-glycosylated enzyme, which suggested glycosylation does not affect the enzyme active site.

The recombinant cellulase enzyme is extremely important for overcoming the bottlenecks in the biofuels and energy sector and with the development of bioprocess for cellulase production, this study has the potential to reduce the cost of overall bioethanol production, if improved further.

Key Words: Biofuel, Cellulase, Recombinant *E. coli*, Glycosylation, Glycoprotein

ÖZET

BİYOYAKIT SEKTÖRÜNE YÖNELİK YENİ NESİL SELÜLAZ ENZİMLERİNİN REKOMBİNANT *Escherichia coli* BAKTERİSİNDE ÜRETİMİ

Zehra YILDIRIM

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Yenilenebilir bir enerji kaynağı olan biyoetanolün üretim prosesinde yer alan selülaz enzimlerinin, pH-stabil, termo-stabil ve ekonomik olması, ticari verimlilik açısından önem teşkil etmektedir. Mısır ve şeker kamışı gibi gıda hammaddeleri yerine selülozik biyokütleden biyoetanol eldesinde kullanılan endoglukanaz tipi selülazların üretim maliyetlerinin düşürülmesine yönelik geliştirilen proseste, konakçı hücrenin seçimi son derece önemlidir. *E. coli* bakterisi, rekombinant protein üretiminde en çok tercih edilen mikroorganizmaların başında gelmektedir ve glikozilasyon mekanizmalarının bakterilerde varlığı yakın zamanda keşfedilmiş, *Campylobacter jejuni* bakterisinde bulunan basit bir protein glikozilasyon gen bölgesi (pgl), *E. coli* bakterisine aktarılarak, endüstriyel önem teşkil eden bu bakteriye proteinleri glikozilleme yeteneği kazandırmıştır.

Çalışmamızda, pET28a-ssDsbA-Cel5A-Flag-6xHis, pET28a-ssYebF-Cel5A-6xHis, pTrc99A-ssDsbA-Cel5A-Flag-6xHis, ve pTrc99A-ssYebF-Cel5A-6xHis plazmidleri tasarlanmış ve bu yeni plazmidlerin yanı sıra, pMAF_pglB ve/veya pPglΔB plazmidlerini de içeren onaltı yeni sistem geliştirilmiştir. Tasarlanan plazmidler

standart genetik mühendisliği teknikleri kullanılarak sentezlendikten sonra restriksiyon analizi ve DNA dizi analizi yönlemleri ile doğrulanmıştır. Doğal ve glikozillenmiş selülaz enzimleri (Cel5A) SDS-PAGE ve Western Blot analizleri ile doğrulanmıştır. Daha sonra selülaz üretiminin arttırılması amacıyla, (i) besi ortamı (ii) indükleyici konsantrasyonları, (iii) karbon kaynağı, (iv) OD_{ind} (v) indükleme sonrası ortam sıcaklığı, (vi) örnekleme zamanı, (vii) hücre parçalama koşulları gibi biyoproses koşulları optimize edilmiş ve DNS aktivite metodu modifiye edilmiştir. Biyoproses tam olarak geliştirilmiş ve sonuç olarak üretilen dört farklı selülazın aktiviteleri; hücre-içi doğal tip 21-kat (0.219 IU/mL'e); hücre-dışı doğal tip, 1.8-kat (0.302 IU/mL'e); glikozillenmiş hücre-içi tip, 68-kat (1.02 IU/mL'e); hücre-dışı doğal tip 3-kat (0.782 IU/mL'e) arttırılmıştır ve bu değer yakın zamanda rapor edilen glikozillenmemiş selülaza göre 6-kat daha yüksektir. Glikozillenmiş ve glikozillenmemiş enzimlerin her ikisi için de enzim kinetiği çalışmalarında yaklaşık aynı K_m değeri elde edilmiş (3.2 mM), glikozilasyonun enzim aktif bölgesini etkilemediği düşünülmüştür.

Rekombinant selülaz enzimi, biyoyakıtlar ve enerji sektöründeki darboğazı gidermesi açısından son derece önemlidir ve bu çalışma, daha da geliştirildiği takdirde, ticari ölçekli biyoetanol üretim prosesinde kritik öneme sahip olan selülaz enziminin üretim maliyetini düşürme potansiyeline sahiptir.

Anahtar Kelimeler: Biyoyakıt, Selülaz, Rekombinant *E. coli*, Glikolizasyon, Glikoprotein

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I am sure that I cannot find any appropriate words to express my love and gratitude to my husband Kadir. He is always with me only to encourage, cheer up and love.

This M.Sc. is dedicated to my mother for her never-ending support and caring even as I followed my ambitions. When I have to cope with any difficulties, talking to her and even thinking of her is enough for me. I hope I make her proud at the end.

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LIST OF ABBREVIATIONS

Amp	: Ampicillin
Bp	: Base pair
BSA	: Bovine serum albumine
CIAP	: Calf intestinal alkaline phosphate
Cm	: Chloramphenicol
dH_2O	: Distilled water
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
DMSO	: Dimethyl sulfoxide
EB	: Elution Buffer
EDTA	: Ethylenediaminetetraacetic acid
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
Kan	: Kanamycin
Kb	: Kilo base
LB-Broth	: Luria Bertani broth
OD	: Optical density
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
Pgl	: Protein glycosylation locus
RT	: Room temperature
SDS-PAGE	: Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SOB	: Super optimal broth
SOC	: SOB with added glucose
T	: Tween

TBE	**:** Tris borat EDTA
TB	**:** Terrific Broth
Tp	**:** Trimethoprim
wt	**:** Wild-type

1 INTRODUCTION

Energy need is rapidly increasing with the increase in world population and developments in technology while the fossil fuel reserves are diminishing. Bioethanol is commonly used for transportation energy because of its potential use as a renewable alternative to fossil fuels [1]. The amount of produced biofuel was 70.79 Million-Ton Equivalent of Petroleum (MTEP) in 2013 worldwide [2].

By 2017, the consumption of biomass for biofuel production is projected to be 103 million tons [1]. Issues related to the current feed stocks of bioethanol i.e., corn and cane sugars, due to their parallel consumption, have led to a broad search for an alternative fuel source [3]. Generating the biofuels from abundant feedstocks such as lignocellulose and cellulosic waste avoids many of the problems associated with the current grain-based biofuels.

Cellulose can be degraded into simpler sugars by chemical (concentrated acid hydrolysis, cadoxen, and ionic liquids) and enzyme mediated transformations [4] (Taherzadeh and Karimi, 2007). Non-enzyme based hydrolysis of cellulose are generally more complex, non-specific and yields less sugar [5]. The enzymatic alternative, using cellulases, avoids the use of strong acids and results in a cleaner stream of sugars for fermentation and fewer by-products [6]. Obtaining bioethanol by enzyme-based process from cellulosic biomass involves numerous steps, among which the enzymatic conversion of the polymer to individual sugar units has been the main focus of the biotechnology industry (Figure 1).

Technical enzymes are valued at just over $1 billion in 2010 and the global market for industrial enzymes is expected to reach $4.4 billion by 2015. The highest sales of technical enzymes occurred in the leather market, followed by the cellulases and hemicellulases used in the bioethanol market [6].

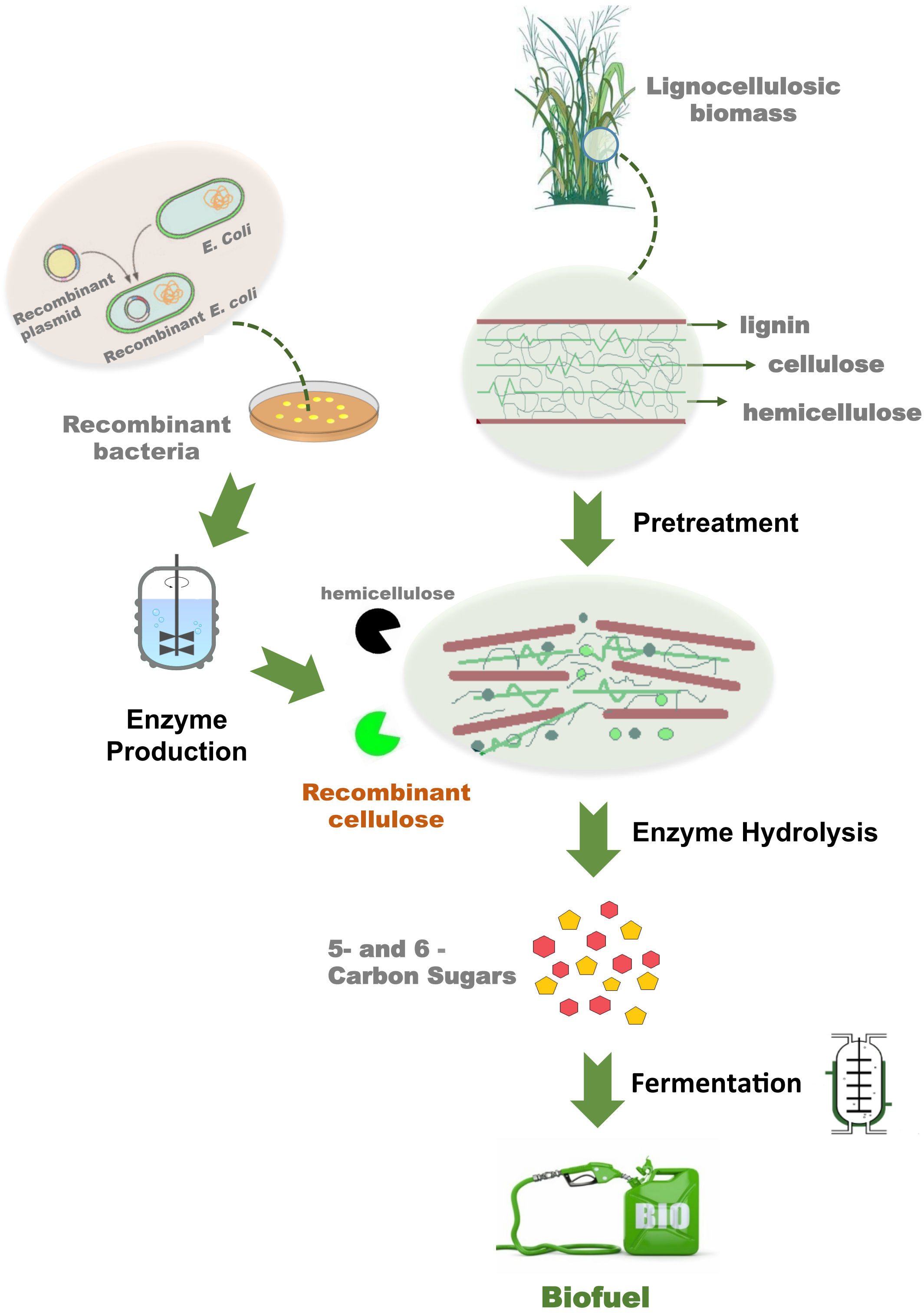

Figure 1.1. Schematics of bioethanol production process.

Cellulase enzyme complex catalyzes cellulose degradation and comprises of three different enzymes: cellobiohydrolases (exoglucanase) (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and *β*-glucosidases (BGL) (EC 3.2.1.21) which acts synergistically for complete hydrolysis of cellulose [7]. Cellulases are used for the critical step of cellulosic biomass conversion to sugar, which is then used in fermentation processes to produce bioethanol.

Commercially, in order to satisfy the market demand for cellulases, fungal microorganisms (*Hypocrea jecorina*, *Aspergillus niger* and *Fusarium sp.*) or bacteria (*Cellulomonas sp.*, *Bacillus sp.*) are being employed under state-of-the-art production technologies using inexpensive raw materials [5]. The utilization of low-cost carbohydrates, strain improvement, and gene manipulations has been alternatively aimed at reducing the cost of cellulases production [8]. However, the high cost of cellulase enzymes still remains a challenge, which accounts for approximately 40-50% of the total ethanol production cost [5, 9]. The high cost of cellulases has created a bottleneck, resulting in an uneconomic production process. Improvement of cellulase expression has the potential to change the nature of the biofuel (bioethanol + biodiesel) industry [10].

Escherichia coli bacterium is one of the most preferred hosts for the production of recombinant proteins. Moreover, the presence of glycosylation patterns in bacteria have been discovered recently, a simple protein glycosylation locus (pgl) found in the bacterium *Campylobacter jejuni* was transferred to *E. coli*, which earned bacterial protein glycosylation capabilities to this industrially important host [11]. Thus in industry, glycoprotein production in *E. coli* is still a very new concept. Considering the studies in the literature and industry about cellulase enzymes, mostly filamentous fungi and yeast (*P. pastoris*, etc.) were preferred as host cells, mainly because of the post-translational changes, particularly glycosylation (addition of glycans, sugar groups to proteins). These glycosylated recombinant enzymes show higher durability with broader pH and temperature ranges; and they can bind both crystalline and amorphous nature of the cellulosic substrate with greater affinity. Glycosylation blocks enzymes to form aggregation, which is also important in biofuel production. Thus, cellulase enzymes produced in eukaryotic cells reveal the positive effects of glycosylation, but glycosylated cellulase production in bacteria is not found in the literature. On the other hand, enzymes produced in bacterial systems are known to be more economical compared to eukaryotic cells.

The presence of glycosylation patterns in bacteria have been discovered recently, and in industry, glycoprotein production in *E. coli* is still a very new concept. Glycosylated cellulase production in bacteria is not found in the literature. On the other hand, enzymes produced in bacterial systems are known to be more economical compared to eukaryotic cells. In this project cellulase enzyme has selected as a model-protein to research bacterial glycosylation.

In this M.Sc. thesis study, endoglucanase (EC 3.2.1.4) gene was derived from a *Fusarium sp.* cloned into an expression vector (pET28a) and transferred into recombinant *E. coli* strain, which has glycosylation genes (laboratory stock). The plasmid was verified by restriction analysis and DNA sequencing and glycosylated cellulase production was performed in shake flask bioreactors. SDS-PAGE and Western blotting was used to analyze the generated recombinant enzyme, and enzyme activity was tested by a modified DNS assay. The bioprocess was fully developed by the optimization of production conditions, such as temperature, concentration of the carbon source, inducer concentration and induction period.

A key advantage of our approach is that, this study is the first step towards the production of recombinant glycosylated cellulase enzyme in a well-known industrial host cell (*E. coli* bacteria). Thus, the significance of the research lies in overcoming the current bottlenecks in biofuel industry; this technology, if improved further, has the potential to reduce the cost of overall bioethanol production significantly.

2 LITERATURE SURVEY

2.1. Biofuels from Biomass

Bioethanol is commonly used for transportation energy because of its potential use as a renewable alternative to fossil fuels [1]. Each year U.S. could use nearly 200 million dry tons of lignocellulosic biomass to produce 16 billion gallons of ethanol [12]. Lignocellulosic biomass has vast potential to contribute to worldwide energy, chemical, and material demands in a renewable, sustainable method. Ethanol is a valuable blend stock for light-duty vehicles in the transportation area; however, there are important issues with its large-scale use containing malfunctions associated with hygroscopy, blending limits with gasoline, and the need for a new distribution system beyond petroleum derived fuels. Thus, third-generation biofuels are now undergoing focused research and development with the goal of cost effective production of infrastructure-compatible fuels from lignocellulosic biomass including fuels to fulfill demands in the jet fuel, diesel, maritime sectors and gasoline [13].

Biofuels (bioethanol, biodiesel, etc.) production from lignocellulosic biomass comprising cellulose complex is the most important application and accounts for maximum exploitation of enzymes in industrial processes [7]. Sugarcane-based ethanol used in Brazil and starch-based ethanol used in U.S. are also developed industries, nonetheless both are contend with food consumptions and thus lignocellulosic biomass is gaining industrial recognition. Companies such as Abengoa, BP-Verenium, Coskata, Dupond-Daniska and Poet are endeavoring to commercialize cellulase for cellulosic ethanol in the next decade [12].

Most current process configurations that result ethanol yields, are around 0.21 g ethanol/g dry cellulosic feedstock. Theoretically, it can be increased up to 0.27 g ethanol/g biomass (83 gal/ton) using less complicated methods [12].

Cellulose can be degraded into ethanol by following two ways:

1. Cellulolysis (cellulose hydrolysis) processes are based on the use of enzymes to breakdown complex cellulose into simple sugars. Steps to produce ethanol through biological method are followed as; (i) pretreatment of lignocellulosic material, (ii) cellulolysis to break down the molecules into sugars, (iii) separation of the sugar solution from the remaining ingredients, (iv) fermentation and distillation

to yield around 95% pure alcohol. Furthermore, dehydration by molecular filters can carry the ethanol concentration up to 99.5% [14].

2. Gasification method converts the lignocellulosic raw material into vaporous carbon monoxide and hydrogen and these gases can be used to produce ethanol by chemical catalysis or fermentation. This process does not rely on chemical decomposition; instead, the carbon in the raw material is transformed into synthesis gas. The hydrogen, carbon dioxide and carbon monoxide may then be fed into a special kind of fermenter. Instead of using yeast for fermentation, this process uses *Clostridium ljungdahlii* bacteria [15]. This microorganism will consume carbon monoxide, carbon dioxide and hydrogen to produce ethanol and water [16].

2.2. Enzymes

2.2.1. General Characteristics

Enzymes are protein and/or RNA molecules that act as catalysts of biological processes. When enzymes are compared to chemical catalysts, the foremost functional characteristics are their high efficiency, specificity and capacity for regulation. Moreover, generally they result in more environmentally friendly processes. They accelerate and speed up reactions by forming transition state complexes with their substrate, which reduce the activation energy of the reaction [17].

2.2.2. Classification of Enzymes

There are more than 3000 different known enzymes of which approximately 160 are used commercially. These enzymes can alternate chemicals or processes, which have safety or environmental problems [6].

Enzymes have been classified into six categories (E.C.1 Oxidoreductases, E.C.2 Transferases, E.C.3 Hydrolases, E.C.4 Lyases, E.C.5 Isomerases and E.C.6 Ligases) according to the type of reaction they catalyze. Each of the main classes is further divided by the individual reactions and the nature of substrates involved [18]. Each enzyme is then assigned an EC (Enzyme Commission) four-digit classification number and an identifying systematic name.

E.C.3 group, “hydrolases”, catalyzes the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Glycoside hydrolases (EC 3.2.1. -) are an extensive group, which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. An organization of glycoside hydrolases in families based on amino acid sequence resemblances has been offered a few years ago.

The cellulase used in this study is EC 3.2.1.4, which catalyzes the hydrolysis of β-1,4-glycosidic bonds of cellulose polymers of four or more glucose units.

2.2.3. Enzyme Activity

In order to get consistent values for each enzyme, *International Unit,* IU described as the activity of an enzyme which, catalyzes the conversion of 1 μmol of substrate per minute, under standard conditions [17].

In this study, 1 unit of cellulase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute [3].

2.2.4. Enzyme Kinetics

The kinetics of many biological reactions are either zero-order, first-order, or a combination of these called Michaelis-Menten kinetics. Michealis-Menten equation effectively represents the kinetics of most enzyme reactions. If the conventional symbols of Michealis-Menten equation are adopted for biological reactions and the reactant is called as *substrate*, it can be written in the form:

$$\upsilon = \frac{\upsilon_{\max} s}{K_m + s} \tag{2-1}$$

where υ is the volumetric rate of reaction and s is the substrate concentration, υ_{max} (mol $m^{-3}s^{-1}$) is the maximum rate of reaction, and K_m (mol m^{-3}) is the Michealis constant. υ_{max} is a volumetric rate that is proportional to the amount of active enzyme present. K_m is a measure of the affinity of an enzyme for a particular substrate, at which $\upsilon = \upsilon_{max}/2$. A low K_m value represents a high affinity and a high K_m a low.

One of the important features of Michaelis-Menten kinetics is that the catalyst becomes saturated at high substrate concentrations. Figure 2.1 shows the form of Eq. (2.1); the reaction rate υ does not increase indefinitely with substrate concentration but approaches a limit, υ_{max}. When $\upsilon = \upsilon_{max}$, all enzymes are bounded to substrate in the form of enzyme-substrate complex affinity [19].

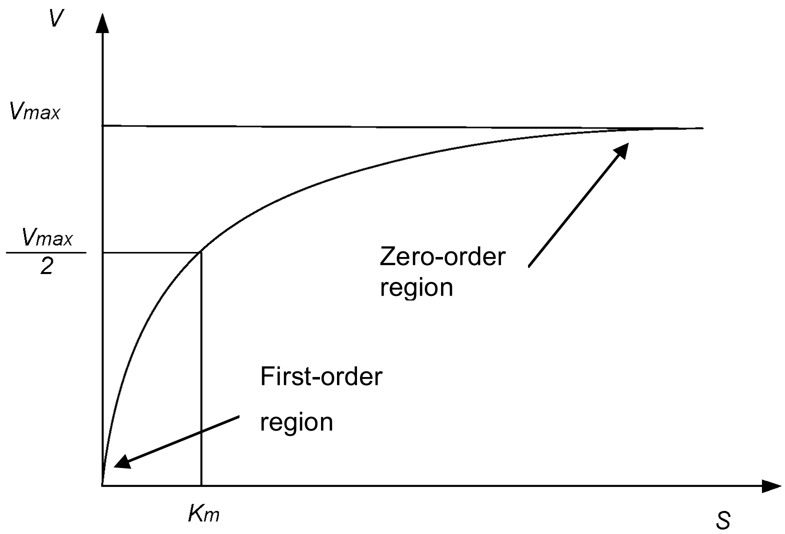

Figure 2.1. Michaelis-Menten plot

2.3. Cellulase

Cellulose is the core carbohydrate in lignocellulosic materials and also the most abundant and renewable biopolymer on earth (Figure 2.2). Chemically, it is a simple homopolymer, which consists of up to 1000 β-1,4-linked anhydroglucopyranoside units. Despite its potential as a renewable source of energy, cellulose gained attention only after the cellulose degrading enzymes or "cellulases" had been identified [20]. However, it is a challenging substrate for enzymes owing to its physical properties [17].

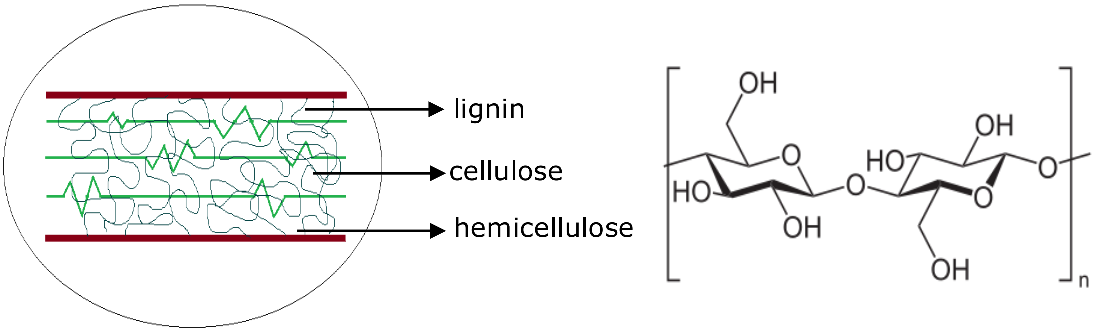

Figure 2.2. [1] Lignocellulose and [2] Cellulose molecule.

As a result of studies for over two decades, cellulase reaction mechanisms have been exposed at the molecular level for many enzyme components [17] . Cellulases hydrolyze oligosaccharides and/or polysaccharides into glucose [5, 21]. The efficient enzymatic degradation of lignocellulosic material involves a set of different cellulase enzymes; cellobiohydrolases, endoglucanases and β - glucosidases (Figure 2.3).

Endoglucanase (EC 3.2.1.4) randomly hydrolyzes internal β-1,4-glycosidic bonds of cellulose. It solubilizes cellulose polymers and provides a substrate for exo-β,1-4-glucosidase by cutting cellulose chains. Cellobiohydrolases (EC 3.2.1.91) characteristically cleaves off the glucose-β-1,4 dimer, cellobiose, by attacking the non-reducing ends of cellulose chains. β-glucosidase (EC 3.2.1.21) hydrolyzes cellobiose (a dimer of glucose linked by a β-1,4 glycosidic bond) into two molecules of glucose [22]. Unless endoglucanases are present to offer sufficient substrate, the activity will be low because, this enzyme only attacks the ends of chains [23].

Current efforts are towards searching for novel and superior cellulase producing strains and their development by traditional methods, such as physical and chemical mutagenesis [24, 25]; designing of novel and effective cellulases by adopting protein re-engineering techniques [26, 27]; development of recombinant strains for improved cellulase titers [25]; and development of authentic measurement assay of cellulases [28]; developing low cost medium [29-32], and increased secretion of cellulases to the extracellular medium [3].

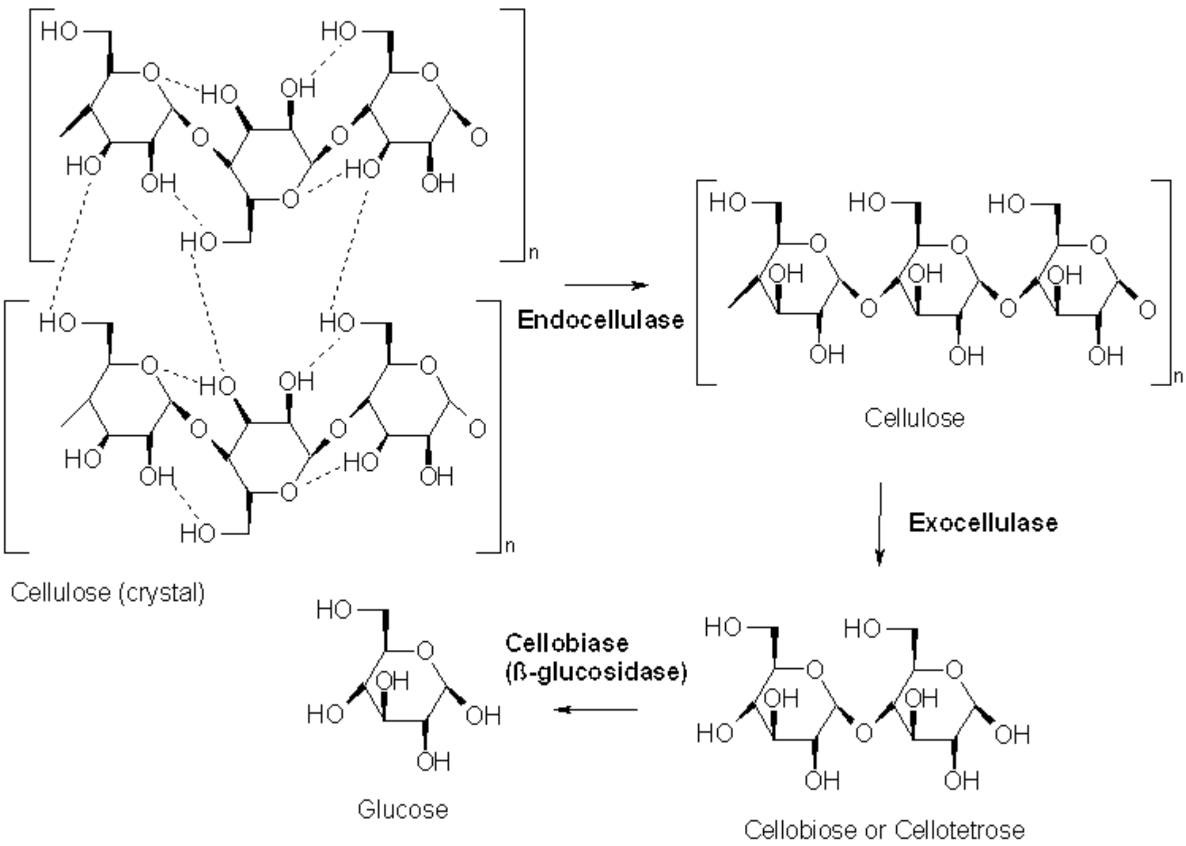

Figure 2.3. Digestion mechanism of cellulases [33].

Knowledge of how promoters affect recombinant enzyme production has significantly advanced in recent years, and is being applied to cellulase production systems [34]. Methods to express important recombinant cellulases and other glycosyl hydrolase (GH) enzymes are under serious investigation (Table 2.1). Increased thermostability is one of the most desired property from the cellulase engineering viewpoint [35]. Extensive saccharification reactions are carried out at high temperatures; therefore, thermostable enzymes are vital to maximize hydrolysis product yield [36].

Table 2.1. Cellulase expression and activity levels produced in bacteria, yeast and fungi

Expression microorganism	Cellulase/s expressed/Gene	Native organism	Enzyme Yields (U/ml) or (U/g)	Reference
Bacterial Recombinant Expression Systems				
E. coli	Endoglucanase	*Paenibacillus sp.* MTCC5639	0.15 U ml^{-1}	[3]
E. coli	β-glucosidase		2.2 U ml^{-1}	[3]
E. coli	Endoglucanase Cel5A	*metagenomic library prepared from vermicompos (of uncultured microorganisms)*	615 U μmol^{-1}	[36]
Saccharomyces cerevisiae	*Cel9A, Cel9G, Cel9I*	*Thermobifida fusca, Clostridium cellulolyticum, C. thermocellum*	600 U μmol^{-1}, 500 U mg⁻1, 400 U $mg^{-1,}$	[27]
E. coli BL21; *E. coli* Rosetta	Unidentified cellulases	Archael derived	138 U	[37]
E. coli BL21 (DE3)	1. BglA 2. Bgl-cohesin chimera	*Clostridium thermocellum*	-	[38]
E. coli BL21	CelStrep	*Streptomyces sp.* G12	600 U mg⁻1	[39]
E. coli		*Fusarium oxysporum*	20.26 μM reducing sg rel./min	[28]
Trichoderma viride	Cellulase Onozuka	*Trichoderma viride*	0.0242U/mL immobilized: 0.9712 U/mL	[40]
Fungi Expression Systems				
Trichoderma reesei RUT-C30	Cellulase	*Trichoderma reesei RUT-C30*	18.5 U ml^{-1}	[41]
Aspergillus niger	Endoglucanase	*Aspergillus niger*	21.8 U g^{-1}	[42]
Stachybotrys microspora	Endoglucanase	*Stachybotrys microspora*	0.49 U/ml	[43]
Yeast Recombinant Expression Systems				
S. cerevisiae strain	Endoglucanase	*S. cerevisiae* strain, CICC 1445	7.5 FPU/g	[44]
Saccharomyces cerevisiae YRH400	CTec2 and CTec3	Novozymes North America	147 and 217 (FPU)/mL	[45]
Saccharomyces pastorianus CMBS-33		*Trichoderma reesei*	75.45 U^{BGLI}	[46]

The largest enzyme supplier, Novozymes, achieved a 40% decrease in cellulase enzyme costs by supplying enzymes at cost of US $0.5 per gallon of ethanol produced. Although this amount looks attractive, in order to support an economical and robust biorefinery industry, a more decrease is still essential to rich US$0.10/gallon [47].

The endoglucanases that can stand high temperatures gain reasonably more attention [1] and Cel5A has the highest catalytic efficiency among the endoglucanases [48].

Studies to produce endoglucanases and improve enzyme activity are improving recombinant strains [49, 50], site directed mutations [51-53], enzyme immobilization [54], direct ethanol production [55], metabolic engineering of host cell [56-58], and isolation of new strains [59].

2.3.1. Cellulase Gene from *Fusarium sp.*

Compared to *T. reesei, Fusarium graminearum* (teleomorph = *Gibberella zeae*) contains many additional cellulases, hemicellulases, pectinases, carbohydrate binding modules (CBM), carbohydrate esterases, and polysaccharide lyases [28, 60]. As well as having a greater total number of Cell Wall Degrading Enzymes (CWDEs) as expected from genomic analysis, *F. graminearum* also possesses CWDE from several families of glycosyl hydrolases that are not represented in *T. reesei* genome [28, 60-62]. Thus, the genome of the phytopathogen *F. graminearum* has double the number of genes encoding cellulases and xylanases as *T. reesei* [63].

2.4. Determination of Cellulase Activity

Endogluconase assays displayed as;

1. Endogluconase Assay Using Carboxymethylcellulose (CMC)/DNS

2. Endogluconase Assay Using CMC/Bicinchoninic Acid (BCA)

3. Endogluconase Assay Using CMC/Viscosity

4. Semiquantitative Endogluconase Assay Based on Dye Release

    - Microbe-Secreted Endogluconase Assay on Agar Medium

    - Endogluconase Assay on Agarose Gel

- Endogluconase Assay on Polyacrylamide Gel [64]

In this study the soluble cellulase substrate, carboxymethylcellulose (CMC), was used to determine cellulase activity, as it has been extensively preferred to characterize enzyme activity of endoglucanases because of the easiness to measure the catalysis product (glucose) by reducing sugar assay. CMC is specific for endo-acting cellulases, since its structure has been engineered to decrystallize cellulose and create amorphous sites that are best for endoglucanase action. CMC plays an important role for activity assays in order to screen for superior cellulase enzymes that are essential for cellulosic ethanol conversion. As a result of the improved understanding of the mechanism of cellulose depolymerization, exocellulases are exposed to be dominant in the degradation of crystalline (e.g. Avicel) and non-soluble (e.g. CMC) cellulose [28, 29, 35, 65, 66].

2.5. Bioprocess Parameters in Enzyme Production

Any process involving the transformation of raw materials (biological or non-biological) into products by microorganisms, animal or plant cell cultures, or by materials (e.g. enzymes, organelles) derived from them, may be termed as a “bioprocess”.

2.5.1. Host Organism Selection and Engineering

In bioprocesses, the choice of the host cell is extremely important in order to develop a more economical production. Considering the studies in literature and industry about cellulase enzymes, mostly filamentous fungi (*Aspergillus niger*, etc.) and yeast (*Pichia pastoris*, etc.) were preferred as host cells, mainly because of the post-translational changes, particularly glycosylation (addition of glycans, sugar groups to proteins). Cellulase enzymes produced in eukaryotic cells reveal the positive effects of glycosylation, but glycosylated cellulase production in bacteria is not found in the literature. On the other hand, enzymes produced in bacterial systems are known to be more economical compared to those produced in eukaryotic cells. However, the presence of glycosylation patterns in bacteria have been discovered recently [67], a simple protein glycosylation locus (pgl) found in the bacterium *Campylobacter jejuni* was transferred to *E. coli* [11], which earned bacterial protein glycosylation capabilities to this industrially important host.

2.5.1.1. *Escherichia coli*

In addition to be the most preferred hosts for the production of recombinant proteins, *E. coli* also has been a desired microorganism for scientists in the biofuel area because of its simplicity for genetic manipulation [68, 69]. Even though, secreting proteins into the extracellular medium has been a challenging task, *E. coli* could efficiently secrete recombinant proteins, such as cellulases [33]. *E. coli* is naturally capable of converting sugars into ethanol by hetero-fermentative process and under anaerobic conditions [70]. A consolidated bioprocessing approach could be applied where the same organism could hydrolyze the biomass and produce biofuel [33]. In this regard, a few groups attempt to produce biofuel by *E. coli* [71, 72], but there is a need for a systematic analysis of this methodology [3].

Glycosylation blocks enzymes to form aggregation, which is also important in biofuel production. In industry, glycoprotein production in *E. coli* is still a very new process. Glycosylated recombinant enzymes show higher durability in broader pH and temperature ranges [48]; and they can bind both crystalline and amorphous nature of the cellulosic substrate with greater affinity.

2.5.1.2. Secretion of Protein and Post-Translational Modifications

Before being functional in different body cells, most of the proteins that are translated from mRNA experience chemical modifications. These modifications are named as post-translational modifications, which have a critical role in generating the heterogeneity in proteins and also support utilizing identical proteins for different cellular functions in different cell types.

The amino terminal sequences are removed by proteolytic cleavage when the proteins cross the membranes and target the proteins in order to transport them to their actual point of action in the cell. Protein-post translational modifications may occur in several ways as listed below:

1. Processing of signal peptide
2. Folding
3. Disulfide bond formation
4. Certain types of lipid addition
5. *O*- and *N*-linked glycosylation

Glycosylation of Proteins

Glycosylation is one of the major ways in which proteins are post-translationally modified and involves linking of oligosaccharides via glycosidic bonds to form a glycan that is covalently attached to a biomolecule, such as a protein or a lipid, and in different forms in eukaryotic and prokaryotic cells (Fig 2.4). Glycoproteins play critical roles in cellular processes such as protein sorting, immune recognition, receptor binding, inflammation, and pathogenicity. Over 50% of eukaryotic proteins are predicted to be glycosylated [73]. Glycosylation in proteins results in addition of a glycosyl group to either asparagine, hydroxylysine, serine, or threonine [74]. Carbohydrates in the form of asparagine-linked (N-linked) or serine/threonine-linked (O-linked) oligosaccharides are major structural components of many cell surface and secreted proteins.

Glycosylation can occur at several amino acid residues, most commonly through asparagine (*N*-linked) and serine or threonine (*O*-linked) [75]. Although there are currently no established rules for predicting the effect of glycosylation on protein folding or function, empirical evidence reveals numerous roles for this protein modification. For instance, glycans can effect folding, stability, molecular interactions, and quality control, as reviewed by Baker et al. [76]. The importance of glycosylation is further illuminated by the fact that about 70% of therapeutic proteins that are either approved by European and US regulatory agencies or are in clinical and preclinical development are glycoproteins [77]. For this reason, besides cellulases, many other genes have been expressed in yeasts, which have the ability to express glycosylated active proteins [78-83].

On the other hand, glycoprotein production in *E. coli* system is under serious investigation. For instance, Srichaisupakit et al. constructed the expression plasmids with the different genetic organizations, and use the *E. coli* B-strain derivative, BL21(DE3) strain as the protein expression host to produce humanized glycoproteins in *E. coli* [84].

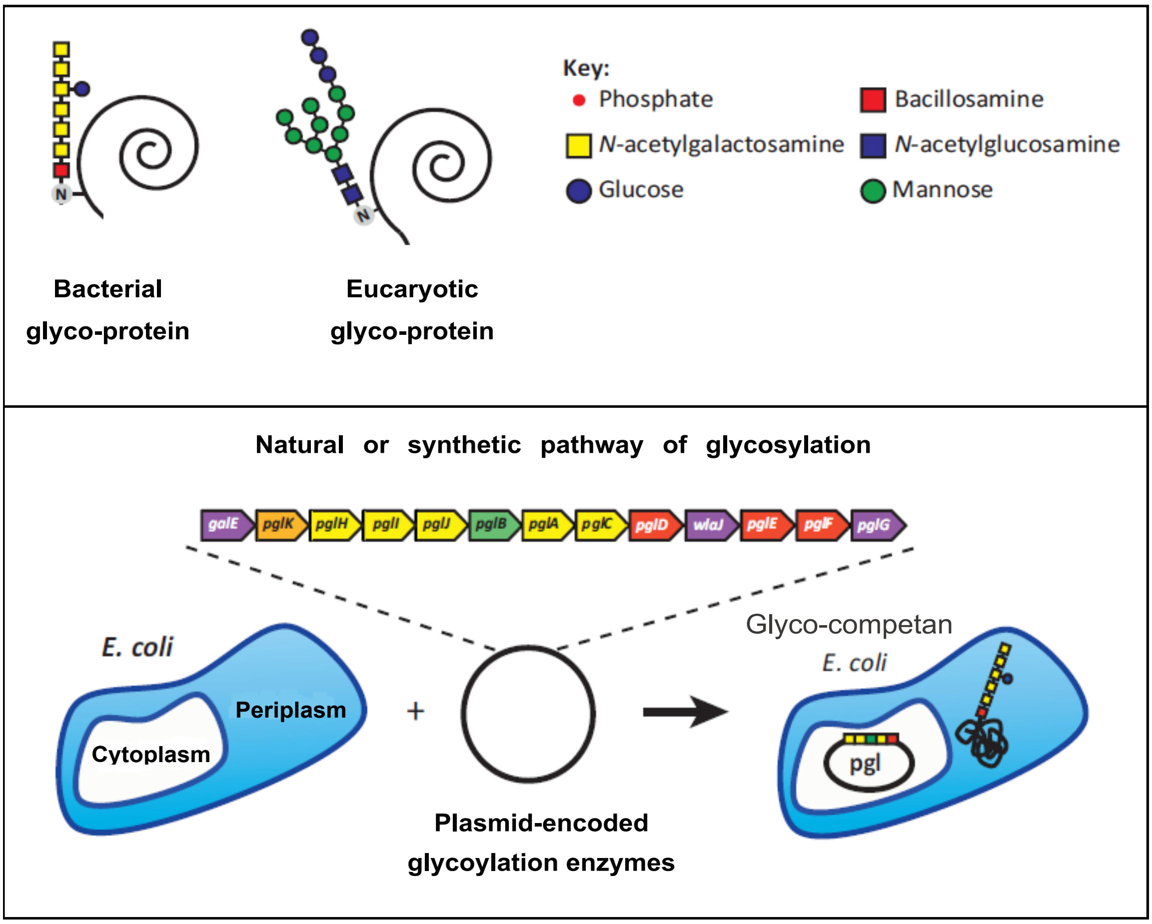

Figure 2.4. [1] Basic differences of bacterial and eukaryotic glycol-proteins, [2] Functional transfer of the *Campylobacter jejuni* protein glycosylation pathway on plasmid pACYC-pgl (pgl) into *E. coli* (Modified from Baker et al. [76]).

Effect of Glycosylation on Enzymes

Glycosylated proteins reveal outstanding results compared to the native proteins. Masarova et al., (2001) [80] reported that "The pH- and thermostability of glycosylated penicillin G acylase expressed in yeast were improved remarkably in comparison to the native enzyme". Most of these carbohydrate-active enzymes (cellulase etc.) derived from filamentous fungi contain *N*-linked and *O*-linked glycosylation. The efficiency and heterogeneity of glycosylation depends on growth conditions, expression host, and the presence of glycan trimming enzymes in the secretome and all of these factors have influence on enzyme activity [85].

Qin et al., (2008) [48], reported that recombinant Cel5A (endoglucanase II) has greater features than native Cel5A. With the increasing extent of enzyme glycosylation, their pH range of activity become wider and they become more resistant to higher temperatures. Jeoh et al., (2008) [86], expressed *Trichoderma reesei* Cel7A in *Aspergillus niger* which resulted in a 6-fold increase in the *N*-glycans on the catalytic domain (CD). This led to reduced activity on crystalline and amorphous cellulose, and recombinant enzyme has higher binding affinity to the substrate.

Glycans increase the overall stability of glycoproteins [87]. Glycosylated recombinant enzymes show higher durability with broader pH and temperature ranges; and they can bind both crystalline and amorphous nature of the cellulosic substrate with greater affinity [85, 88]. There are several reports where glycosylation has been shown to either reduce or prevent protein aggregation [89]. Prevention of enzyme aggregation is particularly relevant because biofuels processes are run at high solids loadings to minimize cost [85].

2.5.1.3. Genetic Engineering of the Microorganism: Techniques and Methodology

Development of the bioprocess for recombinant protein production starts with the genetic manipulation of the host organism, if necessary. Along with the nucleic acid sequencing, techniques for manipulating DNA *in vitro* and *in vivo* (in the test tube and in living systems) have produced outstanding advantages in biochemistry, cell biology, and genetics. Purifying specific DNA sequences and preparing them in sufficient amount for researches is now possible with this recombinant DNA technology.

The steps used to obtain and amplify a segment of foreign DNA to recombinant DNA molecule, also schematically shown in Figure 2.5, are:

1. A fragment of DNA is generated by a restriction enzyme, PCR, or chemical synthesis.
2. The fragment is combined into a vector, which contains the sequences necessary to direct DNA replication.
3. The vector, with the DNA of interest, is transformed into cells.
4. Recombinant vector is replicated in cells.
5. Cells containing the desired DNA are selected.

Cloning refers to the production of multiple identical organisms derived from a single ancestor. The term clone refers to the collection of cells that contain the recombinant vector or to the DNA itself. Cloned DNA can be purified and sequenced. Moreover, the host can produce great amounts of the RNA and protein encoded by the gene, if which is flanked by the appropriately positioned regulatory sequences for RNA and protein synthesis [90].

Cloning Vectors Carrying Foreign DNA

A variety of small, autonomously replicating DNA molecules are used as cloning vectors. Plasmids are circular DNA molecules that 1 to 200 kb length and found in bacteria or yeast cells. Plasmids can be considered as molecular parasites, however in many cases hosts benefit from them by their functions, such as resistance to antibiotics, that the host absences.

Some types of plasmids present in one or few copies per cell and replicate only when the bacterial chromosome replicates. On the other hand, the cloning plasmids are characteristically present in numerous copies per cell and can be induced to replicate until the cell contains two or three thousand copies. The plasmids that have been constructed for laboratory use are relatively small, replicate easily, carry genes specifying resistance to one or more antibiotics, and contain a number of conveniently located restriction endonuclease sites into which foreign DNA can be inserted [90].

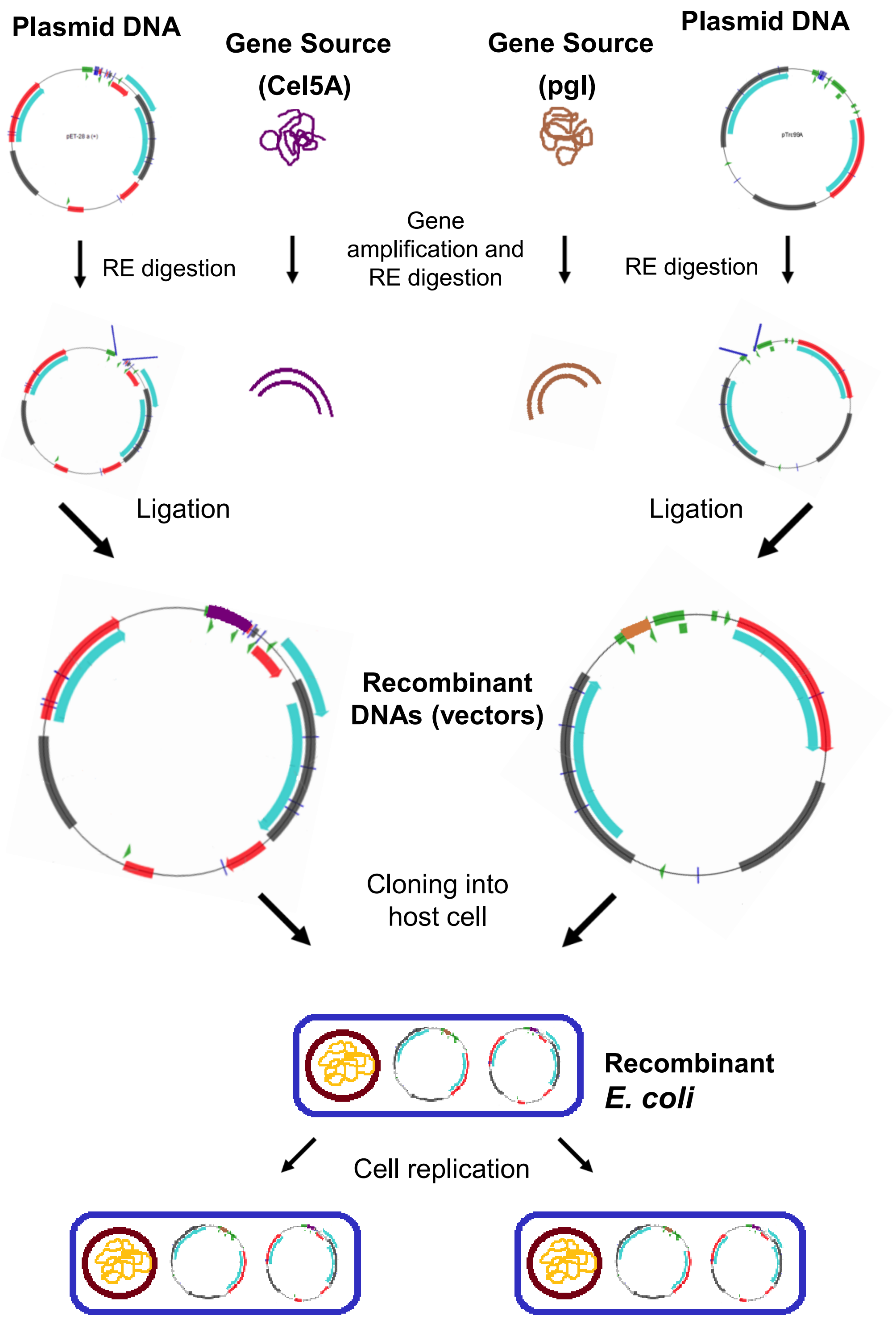

Figure 2.5. Schematic diagram illustrating the basic concept of genetic engineering using two vectors and the genes of interest.

2.5.2. Inducers

An inducer is a molecule that starts gene expression and it can bind to repressors or activators. In this study IPTG was used to induce pTrc99A vector and L-arabinose was used to induce pPglΔB and pMAF_pglB vectors.

IPTG (Isopropyl β-D-1-thiogalactopyranoside)

IPTG is a molecular biology reagent, which is a mimic of allolactose. Lactose metabolite triggers the transcription of the *lac* operon, thus it is used to induce protein expression where the gene is under the control of the lac operator.

Despite allolactose, IPTG cannot be metabolized by *E. coli* and makes it suitable for in vivo studies. Its concentration remains constant and the rate of expression of lac p/o-controlled genes is not a variable in the experimentation. IPTG can be used as a sterile, filtered 1000 mM solution, generally added by 1:1000 dilution into bacterial culture for induction at logarithmic growth phage [91, 92]. Optimum IPTG concentration for cell culture is critical, since it is the trigger of recombinant protein expression. Gupta et al. reported that 20 µM IPTG concentration gave the maximum enzyme activity out of 0, 2, 20 and 200 µM for pET28a vector in *E. coli* BLR(DE3) [3], on the other hand Telke et al. induced same vector in *E. coli* BL21(DE3) with 0.2 mM IPTG [36].

IPTG does not need permease to enter the cell and is not cleaved by β-galactosidase. Consequently it is transported efficiently even the lacY gene is functional. Moreover, its concentration doesn’t vary during the course of an experiment, since cells don’t metabolize IPTG.

L-Arabinose

The bacterium *Escherichia coli* can use L-arabinose as a source of carbon and energy, which is a five-carbon sugar and a constituent of some plant cell walls.

For biosynthetic reasons, most saccharides are almost always more abundant in nature as the “D”-form, or structurally analogous to D-glyceraldehyde. Conversely, L-arabinose is actually more common than D-arabinose in nature and is found in nature as a component of biopolymers such as hemicellulose and pectin. The L-arabinose operon is an extremely important operon in bioengineering.

The L-arabinose operon (ara operon), is a gene sequence that express enzymes needed for the catabolism of arabinose to xylulose-5-phosphate, an intermediate of the pentose phosphate pathway. It has both positive and negative regulation and found in *E. coli* [93].

2.5.3. Temperature

Temperature is a well-known and –controlled factor in bioprocesses that are monitored and controlled usually in a tight range of 0–121°C. Temperature may affect both the growth rate and the product; yet the optimum temperature for growth and product formation may be different and desired to be kept constant at its optimum value. If temperature is increased over the optimum value, the maintenance necessities of cells increase. The yield coefficient is also affected by temperature [94]. Generally, protein production is performed at 30°C after induction, as reported by Boock et al. and Fisher et al. [92, 95], however some other groups as Gupta et al. and Telke et al. produce cellulase enzyme at 37°C and 22°C, respectively [3, 36].

2.5.4. Oxygen Transfer Rate

Oxygen is an important regulator parameter for all aerobic bioprocesses and can have influence on culture performance. Transfer of oxygen into the microbial cell in aerobic fermentation processes affects product formation by influencing metabolic pathways and changing metabolic fluxes [96]. In this study regular Erlenmeyer flask was compared to baffled Erlenmeyer flask since baffles increase aeration. Moreover shaking speed of the incubator was investigated in order to detect the effect of oxygen transfer rate.

3 MATERIAL AND METHODS

3.1. Buffers and stock Solutions

All buffers and stock solutions listed in Appendix A were prepared with distilled water. The sterilization of solutions was performed either by autoclaving at 121°C for 20 min or by filter sterilization through 0.22 µm filters (Sartorius AG, Gottingen, Germany).

3.2. Strains, Plasmids and Maintenance of Microorganisms

E. coli strain DH5α *(F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1)* was used for cloning , while strain, *E. coli* CLM24 [97] and *E. coli* BL21(DE3) (Invitrogen) was used for enzyme production. *E. coli* strains were grown in Luria Bertani (LB) medium at 37°C. Culture medium was supplemented with 20 g/L glucose (growth phase), and 0.1 mM IPTG (production phase) or 0.02 mM IPTG (optimized production), and 0.2 % L-arabinose (production phase) or 0.4 % L-arabinose (optimized production), with the appropriate antibiotics at the following concentrations: 100 µg/mL Ampicillin (Amp), 20 µg/mL Chloramphenicol (Cm), 50 µg/mL Kanamycin (Kan) and 50 µg/mL Trimethoprim (Tp).

For long-term storage of microorganisms, *E. coli* strains were suspended in 1 mL LB medium containing 15% glycerol and stored at -80°C. The compositions and preparation protocols of all the media used are given in Appendix B.

The strains and plasmids used in this study are summarized in Table 3.1. The sequences and schematic representation of the plasmids are given in Appendix C.

Table 3.1. Strains and plasmids used in the study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
E. coli DH5α	F⁻ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
E. coli BL21(DE3)	*F-ompT hsdSB (rB-, mB-) galdcmrne131 (DE3)*	Invitrogen
E. coli CLM24	W3110, Δ*waaL*	[97]
Plasmids		
pET28a	Cloning vector, arabinose-inducible, Amp^r	[95]
pTrc99-ssDsbA-AcrA-4x- Flag-6His	Cloning vector, arabinose-inducible, Amp^r	[95]
pPglΔB	pMW07 containing the C. jejuni pgl locus without pglB	[98]
pMAF_pglB	pMAF containing the C. jejuni pgl locus	[99]
pET28a-*fg*Cel5A	pET28a containing Cel5A	[92]
pTRC99A- ssDsbA-Cel5A-Flag-6xHis	Cloning vector, arabinose-inducible, mp^r	This study
pTRC99A- ssYebF-Cel5A- 6xHis	Cloning vector, arabinose-inducible, Amp^r	This study
pET28a-ssDsbA-Cel5A-Flag-6xHis	Cloning vector, arabinose-inducible, Kan^r	This study
pET28a-ssYebF-Cel5A- 6xHis	Cloning vector, arabinose-inducible, Kan^r	This study

3.3. Genetic Engineering Techniques

Molecular genetics methods were performed according to standard protocols and schematized in Figure 3.1 [100].

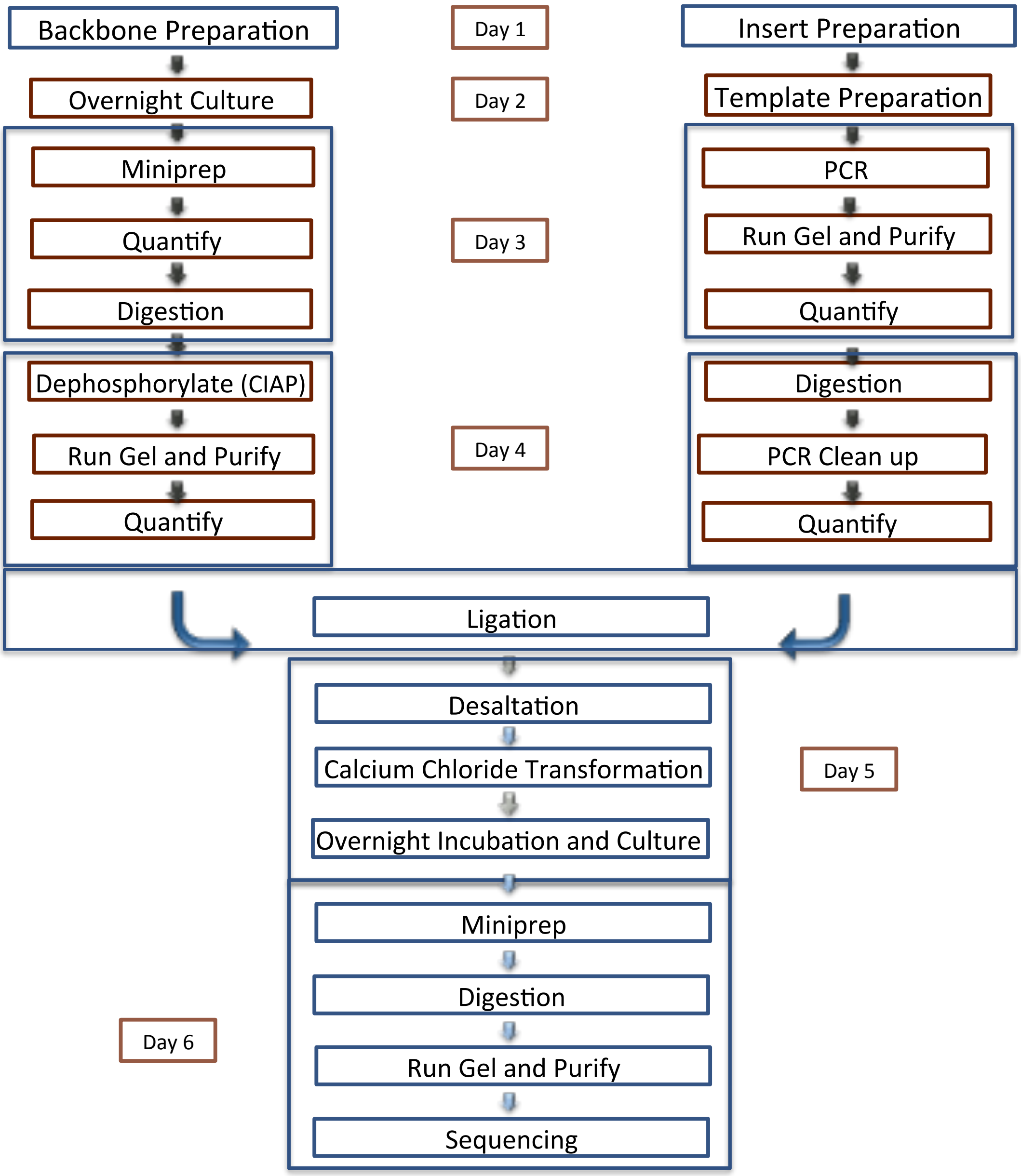

Figure 3.1. Schematic representation of the general cloning procedure

3.3.1. Plasmid DNA Isolation from *E. coli*

Plasmid DNA from *E. coli* cells was extracted using Mini-Prep Kit, (QiaGen, Valencia, CA, USA) according to manufacturer's instructions. For this purpose, a single colony from selective solid medium was inoculated into 5 mL selective liquid medium and grown overnight (16 hours) at 37˚C and 150 rpm. At the end of the protocol, 50 µL of elution buffer (EB) was used to elute DNA from spin column and the extracted plasmid was stored at -20˚C.

3.3.2. Agarose Gel Electrophoresis

Gel electrophoresis was used to separate and visualize DNA strands. 1 g of agarose was dissolved in 100 mL 1xTBE and heated until boiling point in a microwave oven. After cooling to approximately 50°C, 0.5 µg mL^{-1} of ethidium bromide was added and the gel was poured into a gel tray. An appropriate comb was inserted and the gel was allowed to cool. The gel tank was filled with 1xTBE buffer. DNA samples mixed with loading buffer were loaded into the wells, together with the DNA ladder (Appendix D) for size estimation. Electrophoresis was performed at 100 V for 45-60 min. The bands were visualized under UV illumination and photographed.

3.3.3. DNA Extraction from Agarose Gels

In the case where a single band of DNA is to be purified from agarose gel after electrophoresis, Gel Extraction Kit (Qiagen, Valencia, CA, USA) was used according to manufacturer's instructions. The DNA fragment as visualized above UV illuminating board, was excised from the gel with a clean, sharp scalpel and weighed in a microcentrifuge tube, to be approximately 400 mg of gel. At the end of the protocol, 50 µL of EB was used to elute DNA from QIAquick spin column (Qiagen) and the extracted DNA was stored at -20˚C till further use.

3.3.4. Primer Design

The primers used in this study are given in Table 3.2. The sequences for restriction enzyme recognition sites are underlined. Extra nucleotides were added to the 5' end of restriction enzyme recognition sites. The priming sites for sequence verification are given in Appendix F. Formation of primer dimmer and self-complementation were controlled by the program Oligo 2.0.

Table 3.2. Primers used in this study and their sequences

#	Name	Sequence (5'-> 3')
P1	XbaI-*Fg*Cel5A-For	atcatatctagacaatccagcgcctgggctc
P2	SalI-*Fg*Cel5A-Rev	atcatagtcgacgacataggttttcagcagg
P3	HindIII-6xHis-Flag-Rev	attcataagcttttagtgatgatgatgatgatgtcc
P4	NdeI-ssDsbA-For	atcatacatatgaaaaagatttggctgg
P5	EcoRI-ssYebF-For	atatacggatccgaattcgagctcgagaaaaac

3.3.5. Polymerase Chain Reaction (PCR)

The amplification of DNA was carried out by PCR. The reaction mixture, prepared on ice, contained the following:

10x PCR Reaction buffer (with Mg2+)	: 5 μL
dNTP (10 mM stock)	: 1 μL
Forward primer 5μM stock	: 3 μL
Reverse primer 5μM stock	: 3 μL
Template DNA	: 100 ng (1-3 μL)
Taq DNA polymerase (1U μL-1)	: 1 μL
Sterile dH2O	: to 50 μL

A commonly used, representative PCR program is given in Figure 3.2,

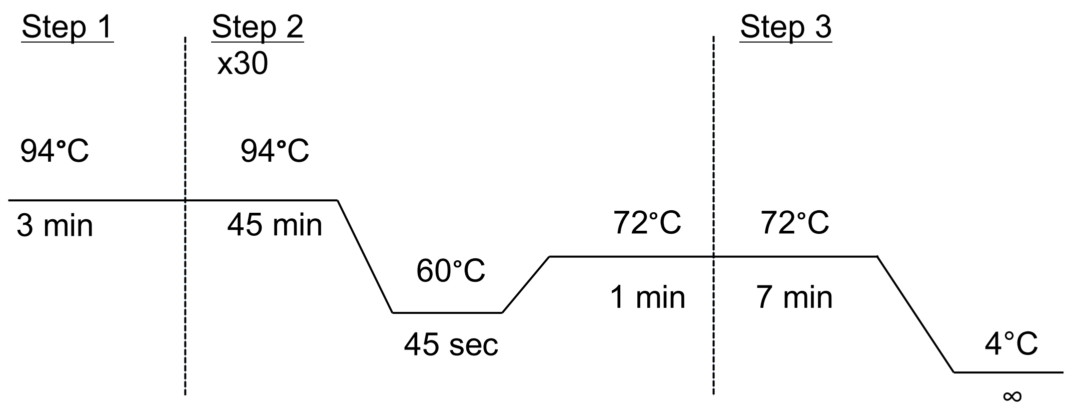

Figure 3.2. PCR reaction parameters

After each PCR reaction, 8 µl DNA sample were mixed with loading buffer were loaded into the wells, together with the DNA ladder (Appendix D) for size estimation. The bands were visualized under UV illumination and photographed.

3.3.5.1 DNA Purification after PCR

Size estimation followed by removal of primers, nucleotides, polymerases and salts, which performed by QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions. The purified DNA was eluted with 50 µL of EB from QIAquick spin columns.

3.3.6. Digestion of DNA Using Restriction Endonucleases

Restriction enzyme digestion reaction mixture prepared in microcentrifuge tube, contained:

DNA fragment	: 0.1 to 5 µg
10x Reaction buffer (supplied with the enzyme)	: 2 µL
Restriction enzyme	: up to 10 U/µg DNA
Sterile dH2O	: to 20 µL

The samples were incubated at 37˚C water-bath for minimum 2 h and thereafter run on agarose gel.

3.3.6.1 DNA Purification after Digestion

After restriction endonuclease digestion, removal of primers less than 100 bases, enzymes, salts and unincorporated nucleotides was achieved by QiaGen Purification Kit (QiaGen, Valencia, CA, USA), according to manufacturer's instructions. At the end of the protocol, purified DNA was eluted with 50 µL of EB from the spin column.

3.3.7. Ligation

20 µL first Ligation reaction mixture as an example contained the following:

10X ligation buffer	: 2 µL
Insert DNA (1518 kb)	: 10.8 µL (82 ng)
Double digested vector DNA (5344 kb)	: 1 µL (100 ng)
T4 DNA ligase	: 1 µL
Sterile dH2O	: 5,2 µL

The amount of insert DNA to be added to the reaction mixture was calculated such that insert:vector ratio of 1:3 was achieved. A control reaction was also set up, containing all the reagents listed above except the insert. The reaction was carried out for 16h in 16˚C water-bath equipped with cooler. The ligation product was desalted and stored at -20˚C till required and 5 μL was used for *E. coli* transformation.

3.4. Transformation of *E. coli*

For propagation of plasmids, or the ligation mixture, *E. coli* DH5α chemically competent cells were used. One 50 μL vial was thawed on ice for 5 min. 5 μL of the plasmid was added and gently mixed by tapping. The vial was incubated on ice for 30 min, then in 42˚C water-bath for exactly 90 sec and immediately placed on ice for 2 min. 250 μL of pre-warmed SOB medium (Appendix B) was added to the vial and the vial was incubated at 37˚C for exactly 45 min. with shaking. 50-200 μL of transformation mixture was spread on prewarmed LB agar containing the appropriate antibiotics and incubated at 37˚C overnight.

3.4.1. DNA Sequencing

The sequence of the constructed plasmid was sequenced using sequencing primers. 1μg of purified plasmid DNA was mixed with 1 μL of 4 μM sequencing primer and the final volume was adjusted to 18 μL with sterile dH_2O. Samples were run by the Sentegen Biotech, Ankara.

The provided data was analyzed using the freeware ApE v.2.0.45 (http://biologylabs.utah.edu/jorgensen/wayned/ape/) and BLAST freeware (http://blast.ncbi.nlm.nih.gov)

3.4.2. Recombinant Protein Production

Protein production was carried out in batch cultures using laboratory scale air filtered shake bioreactors. Production started with transform plasmids into *E. coli* BL21 or CLM24 producing cellulase enzyme from glycerol stocks was streaked onto LB Agar solid medium, containing proper antibiotics, incubated for 16 h at 37°C then a single colony was inoculated into 5 mL LB media with proper antibiotics and shaken at 150 rpm, 37°C overnight. Next day, cells were subcultured into 25 mL LB-Broth containing 20 g/L glucose, glucose and proper antibiotics, such that the initial OD_{600}=0.06 and shaken 2-4 hours @ 37°C up to OD_{600}=0.3-0.6.

To calculate inoculation volume; ex: for 25 ml production, where DR=5, OD_i= 0.06,

$$\frac{25+V}{V} = \frac{OD \times 5}{0.06} \qquad (3\text{-}1)$$

Afterwards, the temperature dropped to 30°C and cell culture was induced with 0.1 M IPTG (1:1000) and %20 L-Arabinose (10:1000) (if using glycosylation related vectors) and cultivated for 5 to 45 hours at 30°C, 150-250 rpm. Cells producing intracellular protein were harvested by centrifugation at 2000-5000 g for 5-10 min and resuspended in PBS buffer than sonicated 2 times (%2 cycles on 30 sec on, 2 min off). To obtain extracellular protein, cell culture was spun down at 13000 g for 10 min at 4ºC and the supernatant was kept for further analysis.

3.5. Analysis

3.5.1. Cell Concentration

Cell concentration was measured using a UV-Vis Spectrophotometer at 600 nm and 1 mL sample taken from liquid medium was diluted with dH_2O to read OD_{600} in the range 0.1 – 0.8.

3.5.2. DNA Concentration

DNA concentration was measured using a Thermo Scientific, NanoDrop 1000, US with 2 µL sample. $OD_{260/280}$ and $OD_{260/230}$ rates were also analyzed.

3.5.3. Total Protein Concentration

Total protein concentration was determined spectrophotometrically using Bradford assay (Bradford, 1976). 20 µL of sample was mixed with 1 mL of Bradford reagent (Sigma), incubated at room temperature for 5 min and the absorbance was read at 595 nm by UV-spectrophotometer. The calibration curve was obtained using BSA in the concentration range of 0-2 mg mL^{-1} (Appendix E).

3.5.4. Enzyme Activity Assay

DNS Assay was used to measure cellulose activity [28] at 540 nm with UV-Vis spectrophotometer. Hydrolysis reactions were carried out in 96-well flat-bottom plates (Corning). Each well contained 15 µL sample or standard than 15 µL of %2 CMC substrate in 0.1 M sodium acetate buffer pH 5.5 were added. After preparing the hydrolysis reactions, plates were sealed with aluminum film and incubated at 50 °C for 20 min. The reaction were stopped by freezing at -20 °C for 2 min. After cooling 20 µL of mixture was taken into 96-well PCR tubes and 40 µL DNS reagent were added. The mixture were boiled at 95 °C for 5 minutes and than cooled at -20 °C for 2 min. 50 µL of mixture were added to 200 µL of dH_2O in 96-well plate and read at 540 nm (EZ Read, Biochrom). The method is given Appendix G.

3.5.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970) [101]. This standard method of protein analysis for rough determination of the size of the polypeptides was carried out in polyacrylamide gels under conditions that ensure dissociation of proteins into their individual polypeptide subunits and that minimize aggregation. The strong anionic detergent SDS (sodium dodecyl sulfate) was used in combination with a reducing agent (2-mercaptoethanol) and boiled to dissociate the proteins for 5 min, before they are loaded onto the gel. 20-50 µL of the samples and 1-5 µL protein standard were run simultaneously at 40 mA of constant voltage.

3.5.6. Western Blotting

Western blot analysis was used to detect the presence of Cel5A in cell lysate or extracellular medium, by using His-Tag specific antibody. Glycosylation was detected by anti-glycan antibody (kindly provided by M. Aebi, ETH, Zurich). After the samples were run on SDS-PAGE gel, the gel was sandwiched between membrane, blotting paper, sponges and the blotting cassette. The membrane (PVDF) used, had been pre-wet with methanol and equilibrated with the Transfer Buffer. Blotting was performed electrophoretically (BioRad), for 3 h at 50 V. The blotted membrane was washed with PBS-T 2 times, than blocking was performed with Blocking Reagent, BioRad for o/n, at 4°C. After 2 washes with PBS-T, the membrane was incubated 1 h at RT on shaker platform, in primary antibody diluted 1:1000 with PBS-T-BSA. After 3 washes with PBS-T, the membrane was incubated for 1 h at room temperature, in secondary antibody diluted 1:2500 with PBS-T. After 2 washes, the presence of proteins was visualized by peroxidase-based immunocytochemical procedure using an Opti-4CN colorimetric kit (Biorad). Detailed protocol is given in Appendix G.

4 RESULTS AND DISCUSSION

4.1. Design of Plasmids

The research program is schematically summarized in Figure 3.1, for the production of new plasmids. As part of this study; four new plasmids have been developed. These new plasmids; pTRC99-ssDsbA-Cel5A-Flag-6xHis, pTRC99-ssYebF-Cel5A-6xHis, pET28a-ssDsbA-Cel5A-Flag-6xHis, pET28a-ssYebF-Cel5A-6xHis are schematically summarized in Figure 4.1.

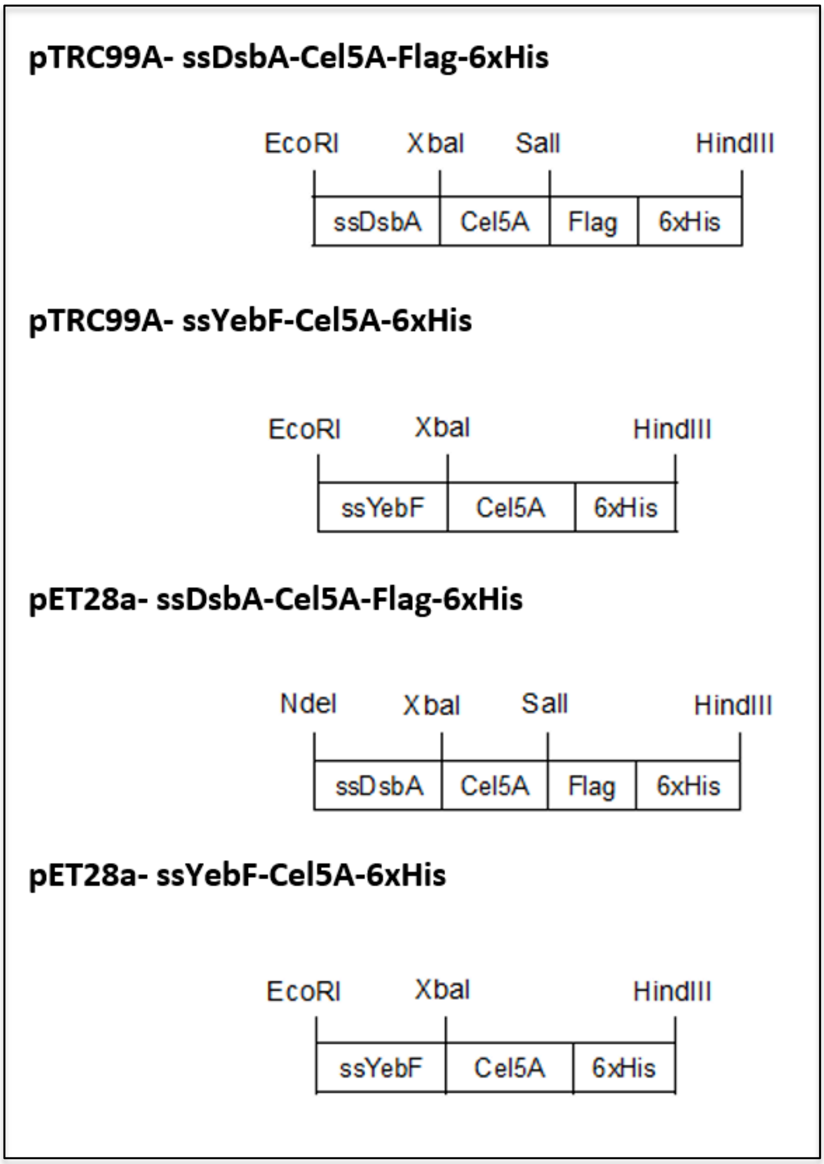

Figure 4.1. Schematic representation of new plasmids

4.1.1. Propagation and Purification of pET28a and pTrc99A

The pTrc99A-ssDsbA and pTrc99A-ssYebF plasmids were obtained from Fisher et al., [95] and pET28a plasmid and *fgCel5A* insert gene cassette was obtained from pET28a-fgCel5A [92] and inserted into the vectors after PCR.

4.1.2. PCR Amplification of Gene Cassettes

Forward and reverse primers given in Table 3.2 were designed to amplify the gene cassettes in PCR, to obtain the plasmids given in Fig 4.1, and paired with the template and other primers, with properties given in Table 4.1. The expected product sizes are also displayed in Table 4.2.

Table 4.1. Thermodynamic properties of primers

Name	T_m (˚C)	Product Size (bp)
P1	64	31
P2	60	31
P3	57	36
P4	50	28
P5	60	33

Table 4.2. PCR components and product description

PCR	Template	Forward Primer	Reverse Primer	Product Size	Reference
PCR 1	pET28a-*fg*Cel5A	P1	P2	1143 bp	[92]
PCR 2	pET22b-*fg*Cel5A-Flag-6xHis	P1	P3	1122 bp	[92]
PCR 3	pTRC99a-ssDsbA-*fg*Cel5A-Flag-6xHis	P4	P3	1242 bp	This study
PCR 4	pTRC99a-YebF-*fg*Cel5A-6xHis	P5	P3	1518 bp	This study

These expected PCR product sizes were controlled by agarose gel, run at 90 V and visualized under UV light as seen from the agarose gel photos (Fig 4.2). Then, the DNA fragments were excised from the gel (Qiagen Gel Purification Kit) and purified from the other gel components. DNA concentration was determined with NanoDrop (The Thermo Scientific NanoDrop 1000) and stored at 4°C until used further in digestion reactions.

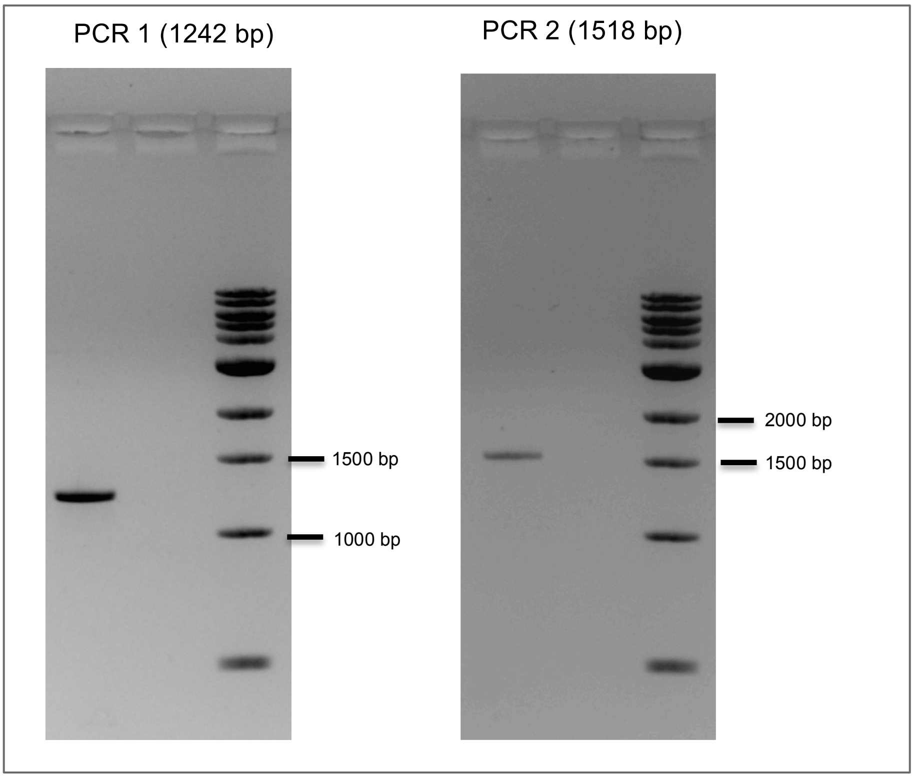

Figure 4.2. Agarose gel electrophoresis results of amplified Cel5A gene cassettes, M: 1 kb DNA molecular weight marker (NEB, MA).

4.1.3. Digestion and Ligation Reactions

The backbone plasmids were isolated from DH5α strain. The restriction digestion enzymes, given in Table 4.3, had been selected with criteria to cut the template DNA with correct restriction sites matching them. Calf-intestinal alkaline phosphatase enzyme (CIAP) (NEB, MA) was used after restriction digestion of vector DNA to remove 5'-phosphates groups to prevent recircularization of linearized vector DNA, before ligation. pET28a backbone was digested with EcoRI-HF, HindIII-HF and with both enzymes. Than the expected fragment sizes for pET28a backbone were controlled by agarose gel run at 90 V and visualized under UV light as seen from the agarose gel photos (Fig 4.3). The size confirmed band (Lane 4: 5344 bp, digested with both enzymes) was then purified from gel (Qiagen Gel Purification Kit). Vector and gene cassette's concentrations were determined with NanoDrop.

Table 4.3. Digestion Reactions

Digestions	**Template**	**Forward RE**	**Reverse RE**	**Restriction Size**
D1	pTrc99-ssDsbA-AcrA-4x-Flag-6His	XbaI	SalI	4251 bp
D2	pET28a-*fg*Cel5A	XbaI	SalI	1110 bp
D3	pTrc99A-ssYebF-GT	XbaI	*Hind*III	4494 bp
D4	pET28a-*fg*Cel5A-6xHis-Flag	XbaI	*Hind*III	1170 bp
D5	pET28a	*Nde*I	*Hind*III	5299 bp
D6	pTrc99A-ssDsbA-Cel5A-Flag-6xHis	*Nde*I	*Hind*III	1230 bp
D7	pET28a	*Eco*RI	*Hind*III	5344 bp
D8	pTrc99A-ssYebF-Cel5A-6xHis	*Eco*RI	*Hind*III	1506 bp

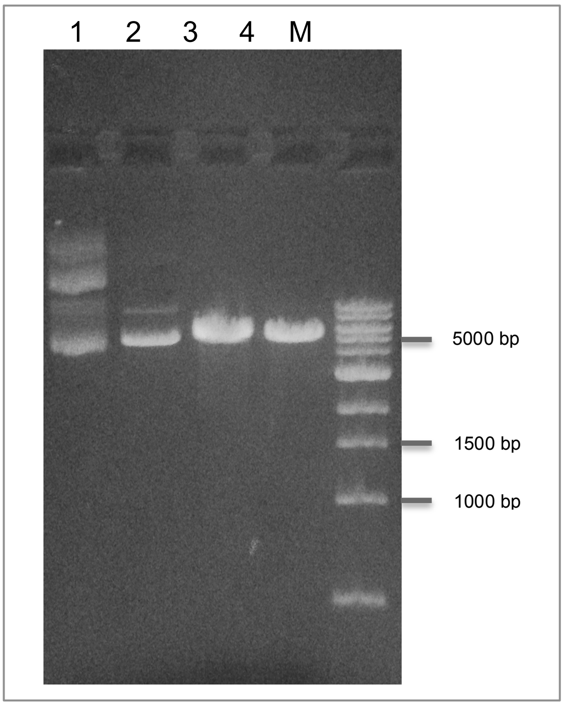

Figure 4.3. Agarose gel electrophoresis of pET28a backbone 1: undigested, 2: digested with EcoRI-HF, 3: digested with HinIII-HF, and 4 (D7): digested with EcoRI-HF and HinIII-HF, (5344 bp), M: 1 kb DNA Ladder, NEB.

Digestion products were than ligated by T4 DNA ligase with various combinations. The ligated pieces of digestion products and the products of the ligation reactions with putative plasmids named as pTRC99-ssDsbA-Cel5A-Flag-6xHis, pTRC99-ssYebF-Cel5A-6xHis, pET28a-ssDsbA-Cel5A-Flag-6xHis, pET28a-ssYebF-Cel5A-6xHis, as well as their final sizes are given in Table 4.4. The ligation products were controlled after the transformation step.

Table 4.4. Ligation products

Ligation #	**L1**	**L2**	**L3**	**L4**
Digestions used in ligation	D1-D2	D3-D4	D5-D6	D7-D8
Product	pTRC99-ssDsbA-Cel5A-Flag-6xHis	pTRC99-ssYebF-Cel5A-6xHis	pET28a-ssDsbA-Cel5A-Flag-6xHis	pET28a-ssYebF-Cel5A-6xHis
Product Size	5361 bp	5625 bp	6529 bp	6850 bp

4.1.4. Transformation of *E. coli*

E. coli DH5α strain were transformed using $CaCl_2$ transformation with the desalted ligation products and the transformed cells were spread onto LB-ampicillin and LB-kanamycin agar plates, for overnight growth at 37°C. Negative controls for transformation reaction included only the backbone plasmid but not the insert. As negative controls, non-transformed cells were also included. Colonies from selective plates were grown in LB-ampicillin and LB-kanamycin cultures medium overnight, and plasmids were isolated the next day. Backbone only plates compared to those including insert contained at least 5-fold less colonies indicating that colonies on selective plates carried the insert. Moreover, non-transformed control cells yielded no colonies on selective plates as expected.

4.1.5. Restriction Digestion and DNA Sequencing to Select the True Transformants

The plasmids were purified from transformants and digested again with restriction digestion enzymes. The digestion products were run on agarose gel for confirmation of the size and consecutively visualization under UV light (Fig. 4.4). All colonies picked potentially had the gene of interest, as proven by the size of the digestion product at the expected size.

Thereafter, the plasmids with correct digestion products (pTRC99-ssDsbA-Cel5A-Flag-6xHis, pTRC99-ssYebF-Cel5A-6xHis, pET28a-ssDsbA-Cel5A-Flag-6xHis, pET28a-ssYebF-Cel5A-6xHis) were all sequenced for confirmation (Sentegen Biotech, Ankara) and a sequence report is given as a sample in Appendix C.

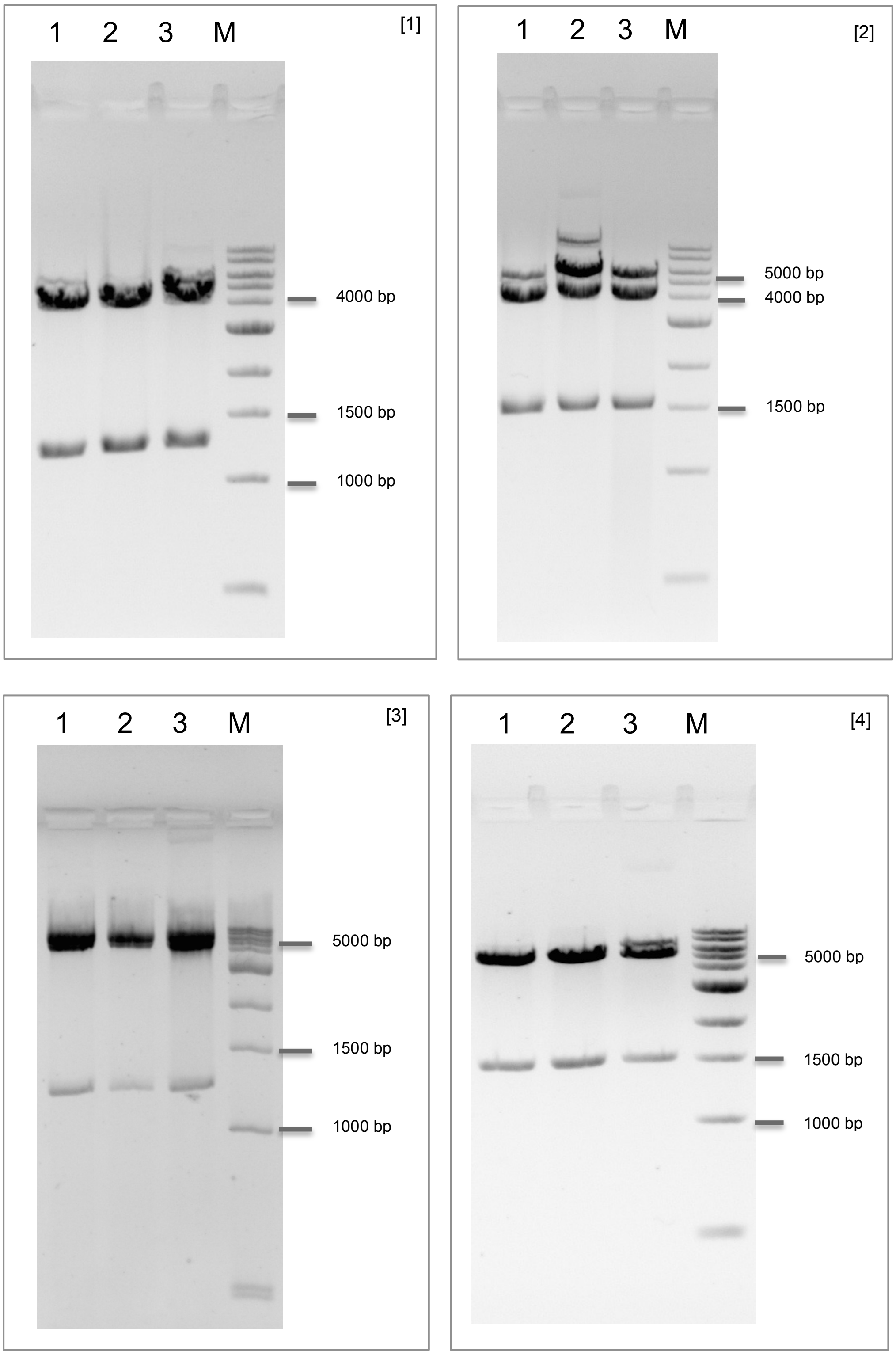

Figure 4.4. Agarose gel electrophoresis of digested recombinant plasmids after ligation. [1] 1-2-3; L1 (4251-1110 bp), [2] 1-2-3; L2 (4119-1506 bp), [3] 1-2-3; L3 (5299-1230 bp), [4] 1-2-3; L4 (5344-1506 bp), M: 1 kb DNA Ladder, NEB.

4.1.6. Design of Recombinant Cel5A Production Systems

E. coli CLM24 and *E. coli* BL21(DE3) strains were used as hosts. Table 4.5 displays the recombinant systems containing different combinations of plasmids.

Table 4.5. Recombinant systems used in the study.

System No	Host Cell	Plasmid I	Plasmid II	Plasmid III
1	*E. coli* BL21 (DE3)	pET28a-ssDsbA-Cel5A-Flag-6xHis	-	-
2	*E. coli* BL21 (DE3)	pET28a- ssYebF-Cel5A-6xHis	-	-
3	*E. coli* CLM24	pTrc99A- ssDsbA-Cel5A-Flag-6xHis	-	-
4	*E. coli* CLM24	pTrc99A-ssYebF-Cel5A-6xHis	-	-
5	*E. coli* BL21 (DE3)	pET28a	-	-
6	*E. coli* CLM24	-	pPglΔB	pMAF_pglB
7	*E. coli* CLM24	pTrc99A	-	-
8	*E. coli* CLM24	pTrc99A	pPglΔB	-
9	*E. coli* CLM24	pTrc99A	-	pMAF_pglB
10	*E. coli* CLM24	pTrc99A	pPglΔB	pMAF_pglB
11	*E. coli* CLM24	pTrc99A- ssDsbA-Cel5A-Flag-6xHis	pPglΔB	-
12	*E. coli* CLM24	pTrc99A- ssDsbA-Cel5A-Flag-6xHis	-	pMAF_pglB
13	*E. coli* CLM24	pTrc99A- ssDsbA-Cel5A-Flag-6xHis	pPglΔB	pMAF_pglB
14	*E. coli* CLM24	pTrc99A-ssYebF-Cel5A-6xHis	pPglΔB	-
15	*E. coli* CLM24	pTrc99A-ssYebF-Cel5A-6xHis	-	pMAF_pglB
16	*E. coli* CLM24	pTrc99A-ssYebF-Cel5A-6xHis	pPglΔB	pMAF_pglB

The systems 1, 2, 3 and 4 include only the Cel5A encoding plasmid, where systems 1 and 3 produce the intracellular enzyme and systems 2 and 4 produce the extracellular enzyme. pTrc99A vector was chosen after comparing to pET28a by enzyme activity as explained in section 4.1.7. Subsequently, systems 5, 6, 7, 8, 9, 10, 11, 12, 14 and 15 were designed as different combinations of negative controls for Cel5A and glycosylated Cel5A. System 13 involves pTrc99A-ssDsbA-Cel5A-Flag-6xHis, pPglΔB and pMAF_pglB vectors and expresses intracellular glycosylated cellulase enzyme. System 16 involves pTrc99A-ssYebF-Cel5A-6xHis, pPglΔB and pMAF_pglB vectors and expresses extracellular glycosylated cellulase enzyme.

4.1.7. Cell Growth Rates and Activities

After designing the systems, firstly, cell growth of systems 1-2-5 and 3-4-7 were compared in groups. There weren't any significant difference on cell growth rate between the systems (Fig 4.4; Fig 4.5.1 and Fig 4.6.1), which shows that Cel5A production, which started after induction at t=3h, does not affect the cell growth.

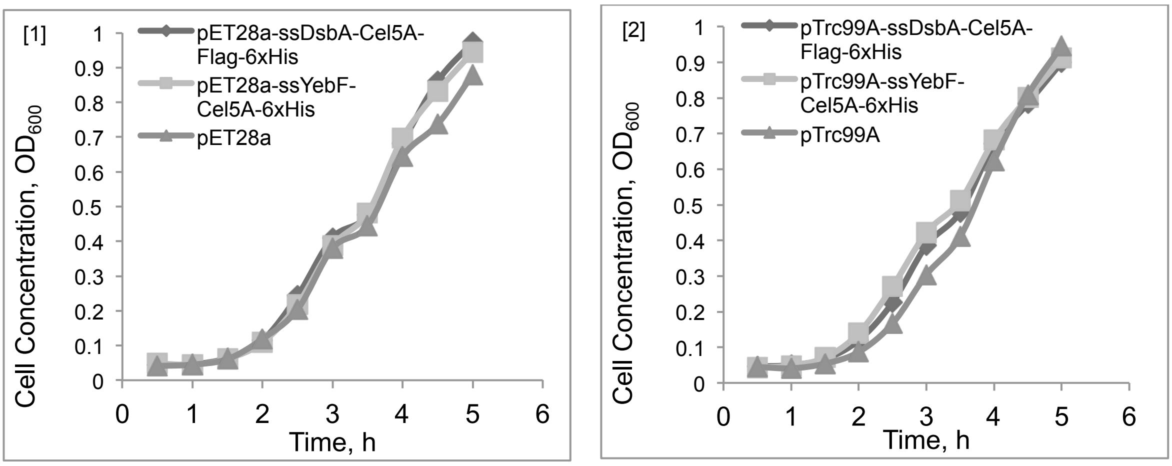

Figure 4.4. Growth curves of new systems containing [1] pET28a and [2] pTRc99A vectors.

Enzymes produced by *E. coli* CLM24 containing pTrc99A-ssDsbA-Cel5A-Flag-6xHis (Lane 2) or pTrc99A-ssYebF-Cel5A-Flag-6xHis (Lanes 4, 5) and *E. coli* BL21(DE3) containing, pET28a-ssDsbA-Cel5A-Flag-6xHis plasmids, were confirmed by SDS-PAGE and the bands were detected as expected (Fig 4.5). *E. coli* CLM24 containing pTrc99A-ssDsbA-Cel5A-Flag-6xHis plasmid expressed more cellulase than *E. coli* BL21(DE3) containing pET28a-ssDsbA-Cel5A-Flag-

6xHis according to relatively thicker band on SDS-PAGE. Moreover, the systems designed with *E. coli* CLM24 containing pTrc99A plasmid, expressed more r-protein and showed more enzymatic activity than *E. coli* BL21(DE3) containing pET28a plasmid (Fig 4.6). Therefore, *E. coli* CLM24 strain containing pTrc99A plasmid was chosen for further experiments.

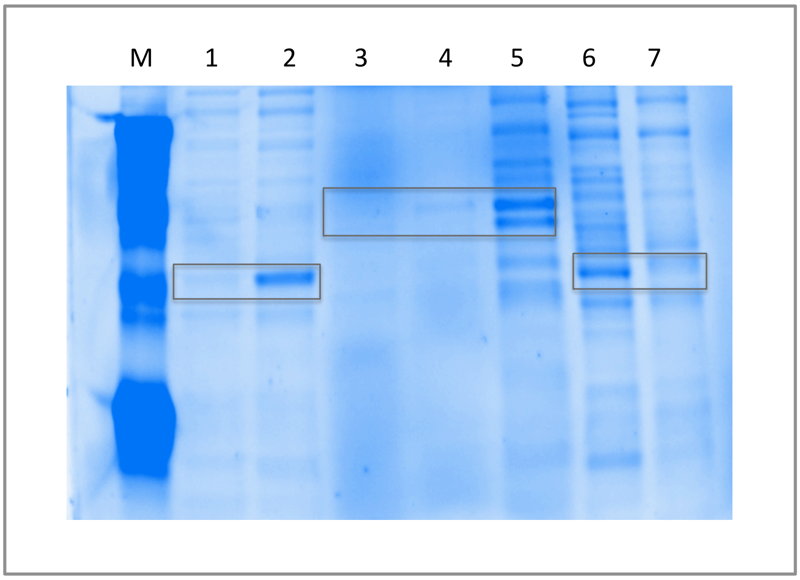

Figure 4.5. M: Protein standard, Bio-Rad, Hercules, CA., [1] pTrc99A plasmid at t=5 h, [2] pTrc99A-ssDsbA-Cel5A-Flag-6xHis plasmid at t=5 h, [3] empty pTrc99A plasmid at t=28 h, [4] pTrc99A-ssYebF-Cel5A-6xHis plasmid at t=28 h, [5] pTrc99A-ssYebF-Cel5A-6xHis plasmid at t=28 h, [6] pET28a-ssDsbA-Cel5A-Flag-6xHis plasmid at t=21 h, [7] empty pET28a plasmid at t=21 h.

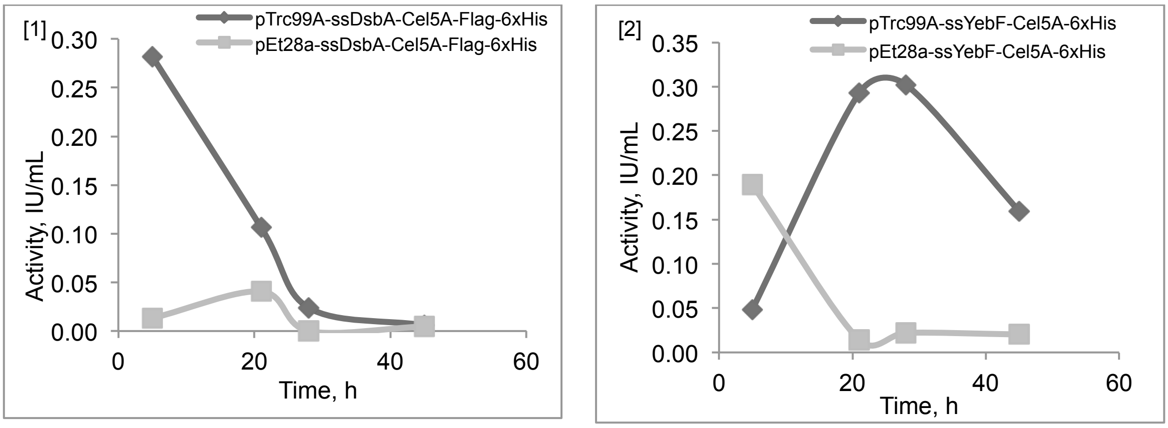

Figure 4.6. Comparison of activity of [1] intracellular enzyme produced by *E. coli* CLM24 containing pTrc99A-ssDsbA-Cel5A-Flag-6xHis plasmid and *E. coli* BL21(DE3) containing pTrc99A-ssDsbA-Cel5A-Flag-6xHis plasmid. [2] extracellular enzyme produced by *E. coli* CLM24 containing pTrc99A-ssYebF-Cel5A -6xHis plasmid and *E. coli* BL21(DE3) containing pTrc99A-ssYebF-Cel5A -6xHis plasmid.

E. coli CLM24 contaning pTrc99A-ssDsbA-Cel5A-Flag-6xHis and pTrc99A-ssYebF-Cel5A-6xHis vectors were further compared to pTrc99A vector by enzyme activity (Fig 4.7.2 and Fig 4.8.2*). E. coli* CLM24 containing pTrc99A plasmid as a negative control, did not give any activity as expected and the ones with pTrc99A-ssDsbA-Cel5A-Flag-6xHis or pTrc99A-ssYebF-Cel5A-6xHis plasmids showed 0.22 IU/mL and 0.083 IU/mL cellulase activity, respectively.

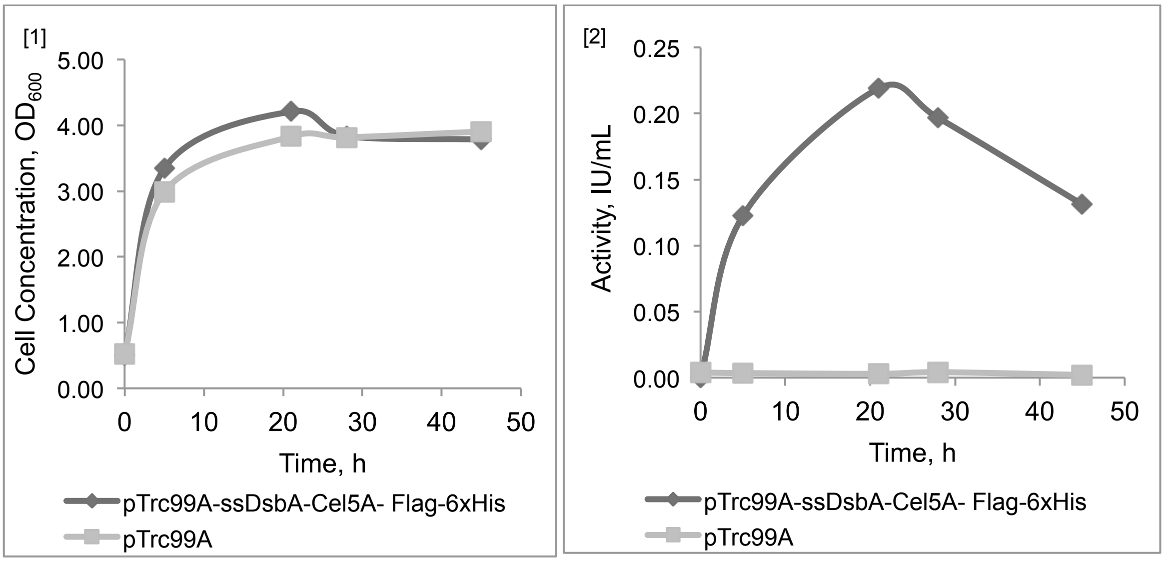

Figure 4.7. [1] Cell growth of recombinant *E. coli* bacteria and [2] Comparison of activity of enzyme produced by *E. coli* CLM24 containing empty pTrc99A or pTrc99A-ssDsbA-Cel5A-Flag-6xHis plasmids.

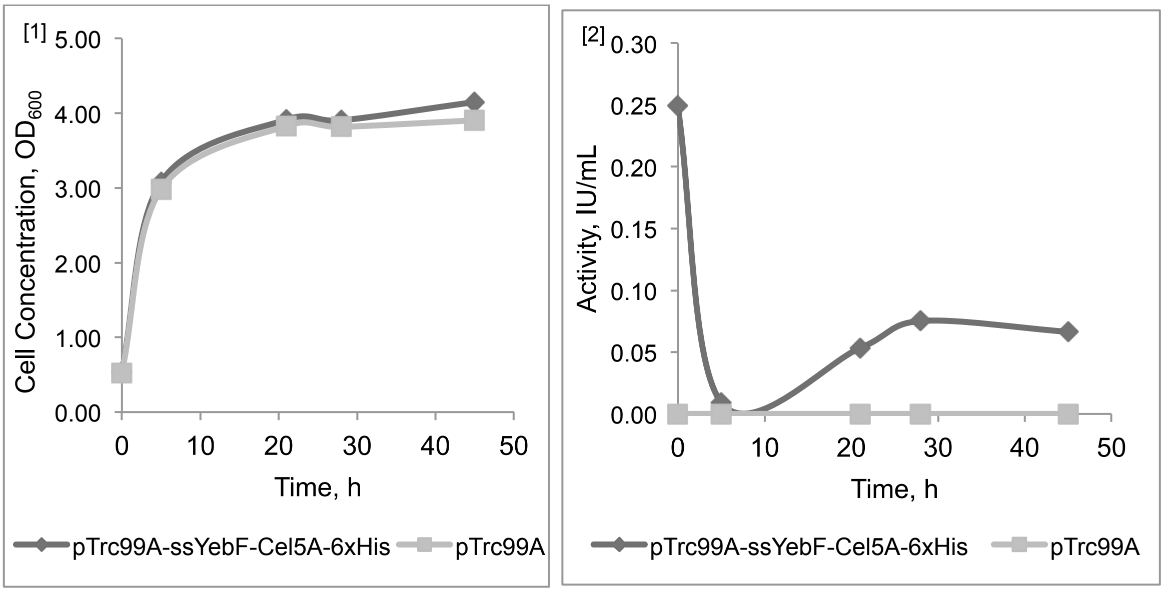

Figure 4.8. [1] Cell growth of recombinant *E. coli* bacteria and [2] Comparison of activity of enzyme produced by *E. coli* CLM24 containing empty pTrc99A and pTrc99A-ssYebF-Cel5A-6xHis plasmids.

4.2. Optimization of Enzyme Activity Assay

Cellulase activity was measured by a common method, 3,5-dinitrosalisilik acid DNS assay which is based on reducing sugar released per minute [102] (Miller,1959). Hydrolysis reactions were carried out in 96-well flat-bottom plates according to method modified from King et al. [28]. In this study, the assay conditions (i.e. sonication conditions, sample dilution ratio, CMC concentration, incubation temperature and cooling temperature after reaction) were optimized.

4.2.1. Effect of Cell Dilution Ratio Before Sonication on Enzyme Activity

After enzyme production the sample was diluted (using PBS for intracellular enzyme and LB broth for extracellular enzyme) in order to increase the amount of protein obtained from cells. Two-fold diluted cell culture before sonication gave the optimum activity (Fig 4.9) and the sample volume was set as V_{sample}=100-200 µL.

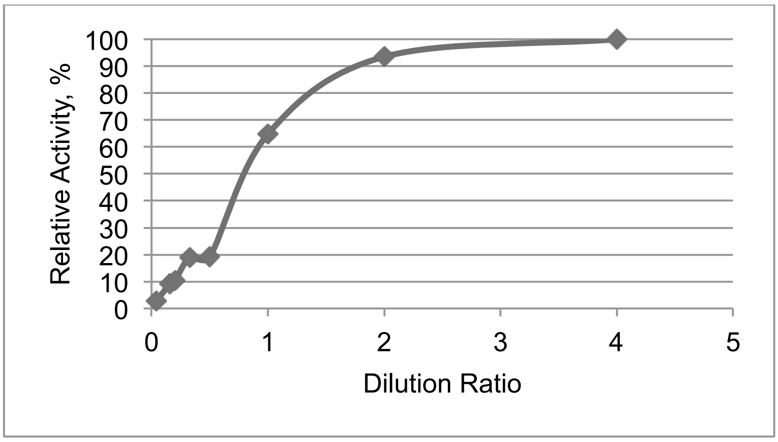

Figure 4.9. Comparison of centrifuged and concentrated cell pellets of intracellular enzyme system #3 at 20h; in PBS; 1.5mL medium's cell pellet in 0.3mL PBS, 1.5mL medium's cell pellet in 0.5mL PBS, 1.5mL medium's cell pellet in 0.75mL PBS, 6.25mL medium's cell in 1 PBS, 12.5mL medium's in 0.5 PBS, and diluted cell pellet in PBS; 1-, 2-, 4-fold by DNS Assay (50°C, 30 min).

4.2.2. Effect of Sonication on Enzyme Activity

Sonication process, where high frequency energy is applied to culture samples to agitate and disrupt the cell membranes, was used for cell lysis. It was performed using an ultrasonic probe. The first step of optimization experiments was to obtain maximum enzyme from cells, thus sonication conditions were investigated. Maximum activity was seen as 0.105 IU/mL, at 2 times sonication with 2-fold diluted cell culture (Fig 4.10). After 2nd time, the heat might have effected the proteins and cause denaturation, although cells were kept on ice.

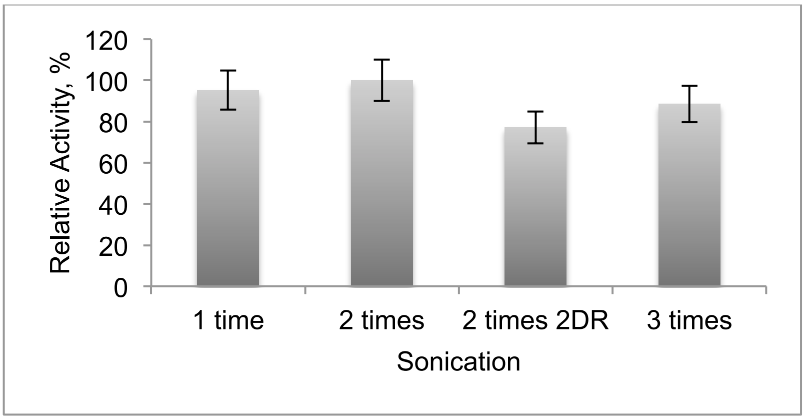

Figure 4.10. Comparison of sonicated cells with 2% cycle, 30 sec, 100% power, with 1 time, 2 times, 2 times with 2 fold diluted enzyme solution (after sonication), 3 times sonication.

4.2.3. Effect of CMC Concentration on Enzyme Activity

Different dilution ratios of extracellular enzyme solution and CMC concentration were tested simultaneously in order to determine the maximum possibility of occurrence of enzyme-substrate complex. Maximum activity obtained with 2% CMC, at 2-5-fold diluted enzyme solution after sonication (Fig 4.11).

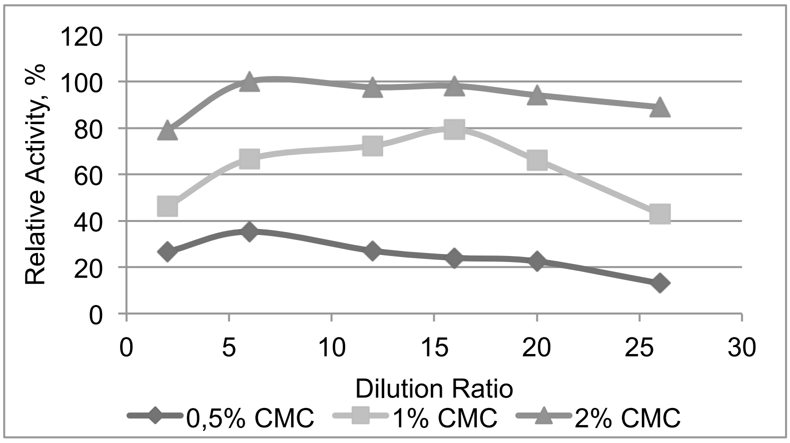

Figure 4.11. Variation of enzyme activity with solution dilution ratio (2, 5, 12, 16, 20, 26-fold) for different CMC concentrations (% 0.5, 1 and 2).

4.2.4. Effect of Incubation Temperature on Enzyme Activity

Temperature variations directly affect enzymes. As the incubation temperature increases, the activity will increase; however, after certain temperatures (depends on individual enzyme) enzymes will be denatured and the reaction rate will decrease, then eventually stop. In order to determine the optimum incubation temperature, 40, 50, 60, 65, 70 and 75°C have been screened (Fig 4.12). The enzymes remain active trough wide temperature ranges and 50°C was chosen as optimum.

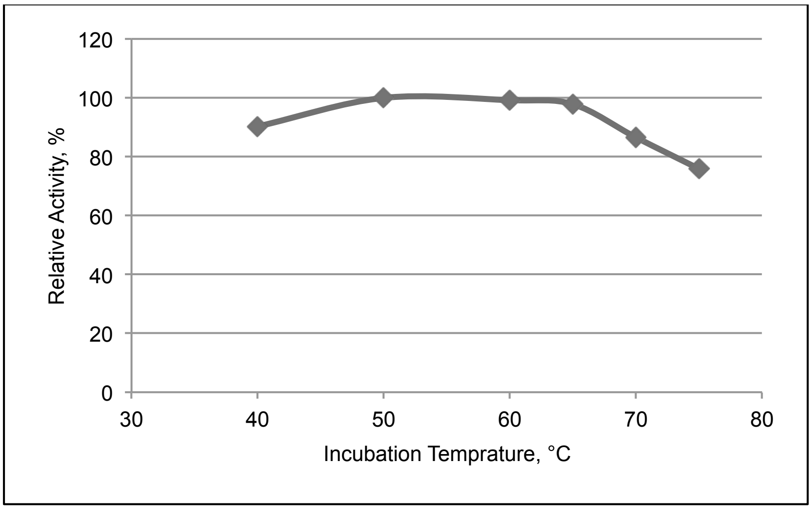

Figure 4.12. Enzyme-substrate incubation temperature (40, 50, 60, 65, 70, 75°C) screening based on activity. Enzyme activities were normalized based on the highest activity obtained (50°C).

4.2.5. Effect of Enzyme-Substrate Reaction Cooling Temperature

Right after the incubation at 50°C, the reaction is stopped by cooling the reaction mixture. 96-well flat-bottom plates were cooled at +4°C and -20°C for an hour and another one was cooled only for 2 min on ice. All three conditions were boiled with DNS Acid at 95°C for 5 min. There was no significant difference between cooling temperatures after reactions, prior to DNS assay (Fig 4.13), and -20°C was chosen, for the ease of handling large number of samples.

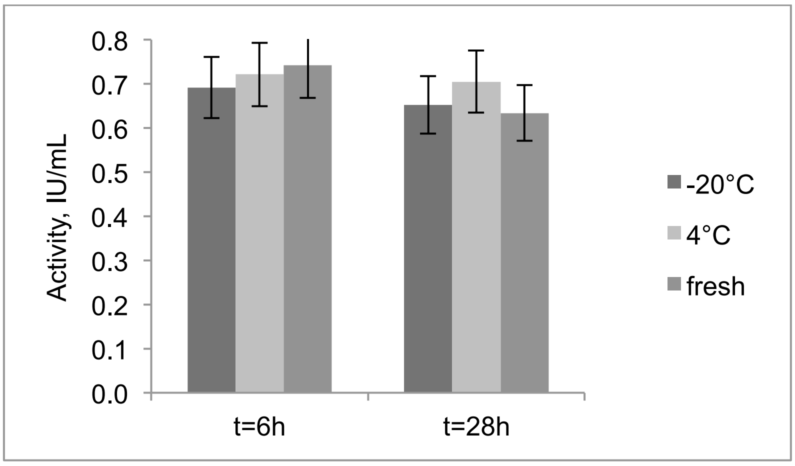

Figure 4.13. Comparison of activities at t=6h and t=8h hours of a sample for reaction hydrolases, (enzyme and CMC substrate incubated at 50°C for 30 min. and cooled at ice for 2 min.) stored at -20°C, +4°C for an hour or used freshly in DNS assay.

4.2.6. Effect of Centrifugation on Obtaining Cells from Culture

Cell culture was centrifuged and resuspended in PBS solution before sonication step. In order to determine the effect of speed and time, cell culture was spun at 2000 g for 5 min and 5000 g for 10 min. Six-fold higher activity was obtained by centrifugation of cell culture at 2000g for 5 min (Fig 4.14). Long and high-speed centrifugation had negative effect on activity, most likely due to lysis of cells and loss of protein.

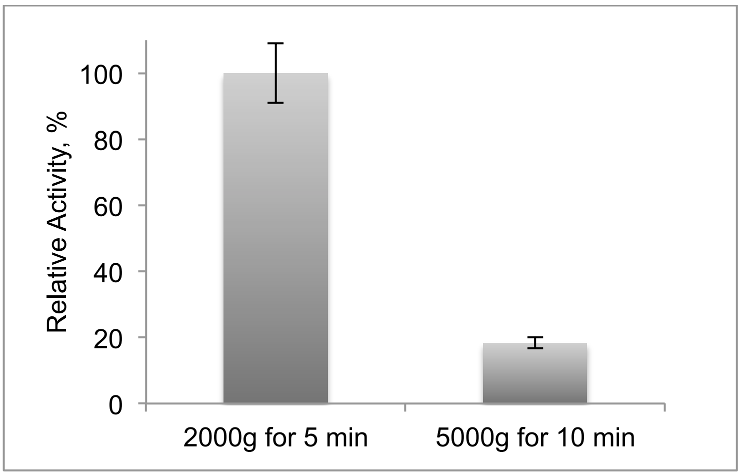

Figure 4.14. Comparison of cell culture centrifugation conditions, 2000g for 5 min and 5000g for 10 min, by enzyme activity.

4.3. Bioprocess Optimization

The bioprocess was developed by the optimization of generated recombinant enzyme production conditions such as inducer concentration, induction period, medium design, concentration of the carbon source, culture volume and incubation conditions.

4.3.1. Effect of Inducers on Cell Growth and Enzyme Activity

IPTG, Isopropyl β-D-1-thiogalactopyranoside, and L-arabinose were used as inducers of lac operon and P_{BAD} promoter respectively. IPTG induces pTrc99A and pET28a plasmids, which express the recombinant enzyme, and L-arabinose triggers pPglΔB and pMAF-pgl plasmids, which produce glycans and attach them respectively. In initial experiments, glycosylated extracellular cellulase (#16; with pTrc99A-ssYebF-Cel5A-6xHis, pPglΔB, pMAF_pglB) showed activity 0,782 IU/mL, whereas un-induced conditions and controls (#10; with pTrc99A, pPglΔB, pMAF_pglB) did not show activity as expected (Fig 4.15.2).

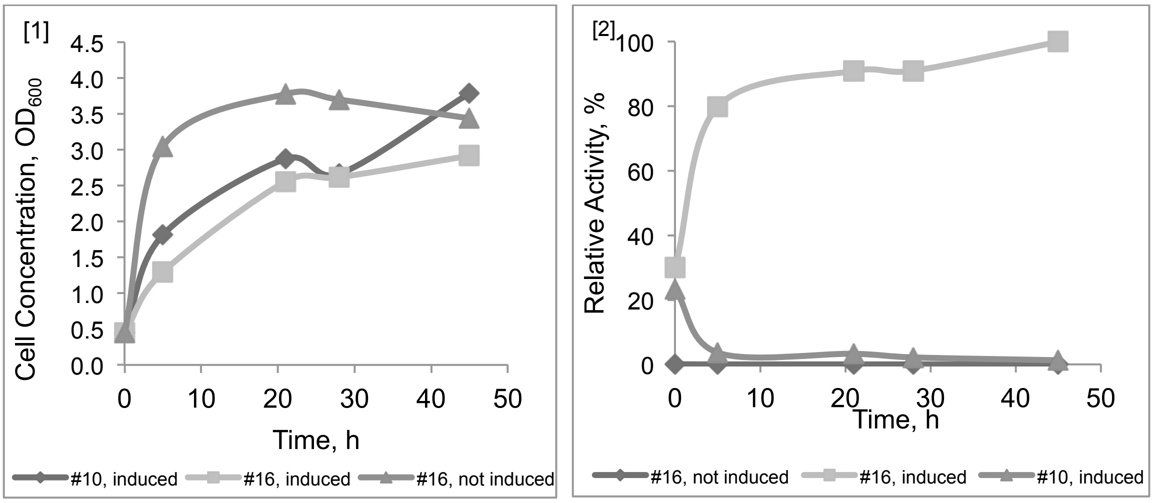

Figure 4.15. Effect of induction with L-arabinose and IPTG inducers on [1] cell growth and [2] enzyme activity for #16 (pTrc99A-ssYebF-Cel5A-6xHis, pPglΔB, pMAF_pglB) compared to uninduced #16 and #10 (pTrc99A, pPglΔB, pMAF_pglB).

Glycosylated intracellular (#13; with pTrc99A-ssDsbA-Cel5A-Flag-6xHis, pPglΔB, pMAF_pglB) enzymes showed activity, where un-induced condition and control (#10; with pTrc99A, pPglΔB, pMAF_pglB) remain as nearly zero (Fig 4.16.2). SDS-PAGE analyse of glycosylated intracellular cellulase supported the activity assay results (Fig 4.17).

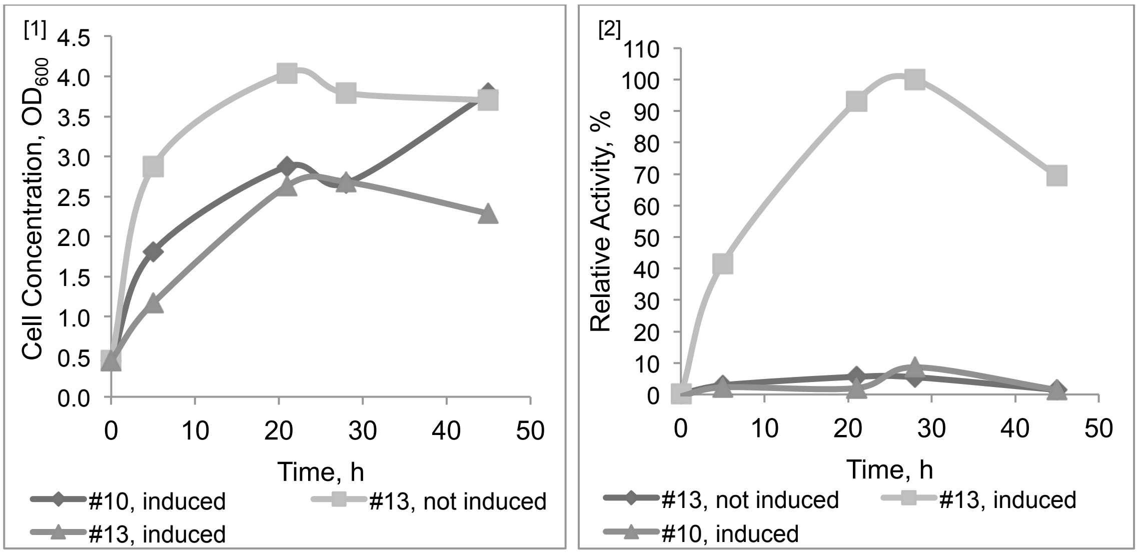

Figure 4.16. Effect of induction with L-arabinose and IPTG inducers on [1] cell growth and [2] intracellular enzyme activity for #13 (pTrc99A-ssDsbA-Cel5A-Flag-6xHis, pPglΔB, pMAF_pglB) compare to un-induced #13 and #10 (pTrc99A, pPglΔB, pMAF_pglB).

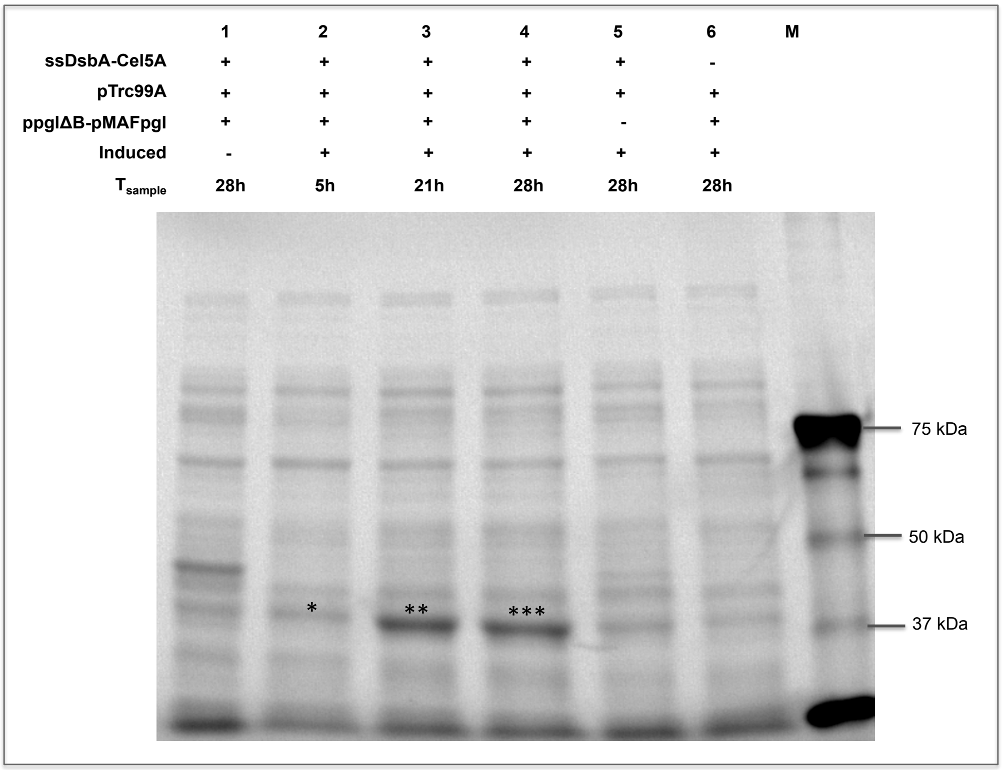

Figure 4.17. SDS-PAGE analysis of glycosylated intracellular cellulase (Lane 2, 3 and 4) by sampling hours, compared to non-induced glycosylated intracellular cellulase (Lane 1) and wild-type (Lane 5) and empty plasmid (Lane 6).

After SDS-PAGE, Western Blotting was performed to distinguish the proteins further, by using anti-His antibody (Fig 4. 18). The presence of endogluconase was visualized by Opti-4CN colorimetric kit (Biorad).

The presence of recombinant cellulase (expressed from pTrc99A-ssDsbA-Cel5A, pMAF_pglB, pPglΔB; 3-plasmid system) bands was observed at t=5-21-45h for; (Lane 2, 3 and 4) and at t=5h from the single plasmid system (pTrc99A-ssDsbA-Cel5A only) (Lane 5). As seen in Figure 4.18, protein bands gets thicker through incubation time course, where there was no band at non-induced control at t=45h (Lane 1). Glycosylated cellulase displays thicker band than the wild-type (lane 5) and negative control (Lane 6) was clear.

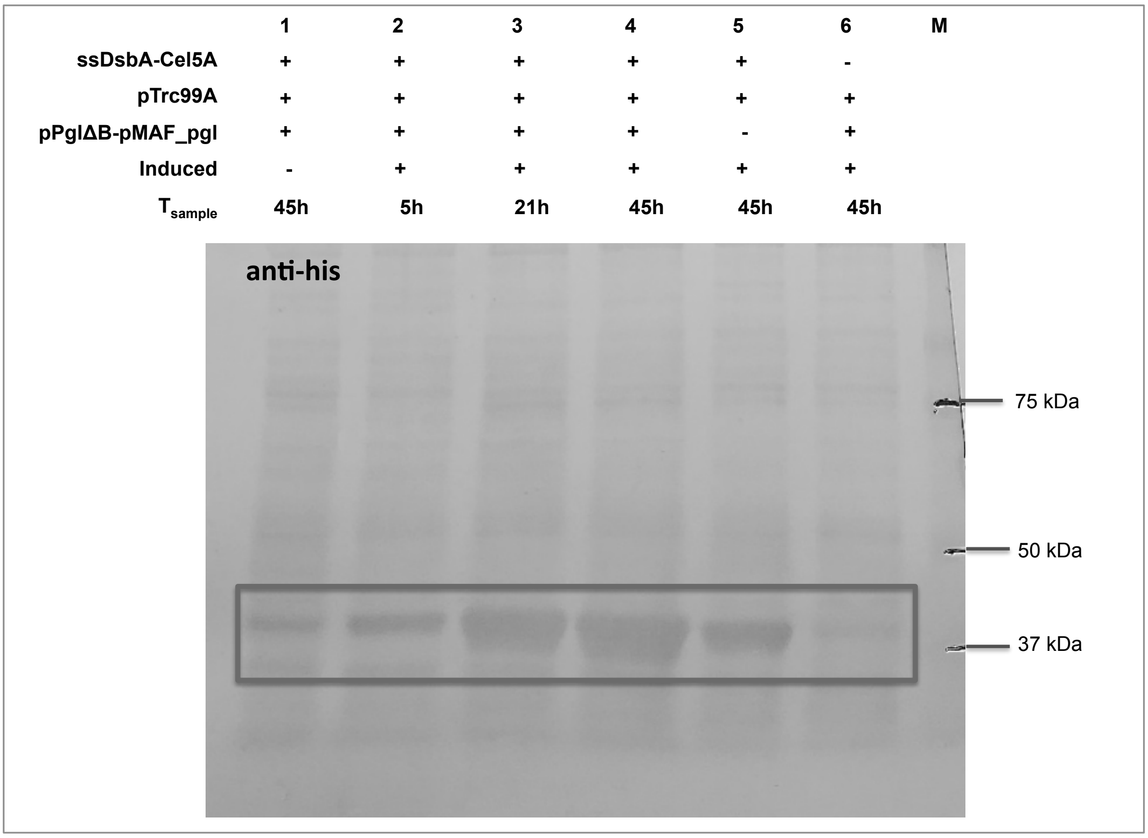

Figure 4.18. Western blot analysis of glycosylated intracellular endogluconase produced from *E. coli* CLM24 carrying pTrc99A-ssDsbA-Cel5A with or without pPglΔB and pMAF_pglB plasmids. The samples from sonicated cell culture were loaded based on cell concentration.

Previous results pointed to need for further experiments on inducers and therefore various combinations of IPTG and L-arabinose were investigated. Maximum activity was achieved at 0.02 mM IPTG and 0.4 % L-Arabinose concentrations and cell growth was not affected significantly (Fig 4.19), which is the same IPTG concentration as reported by Gupta et al. [3].

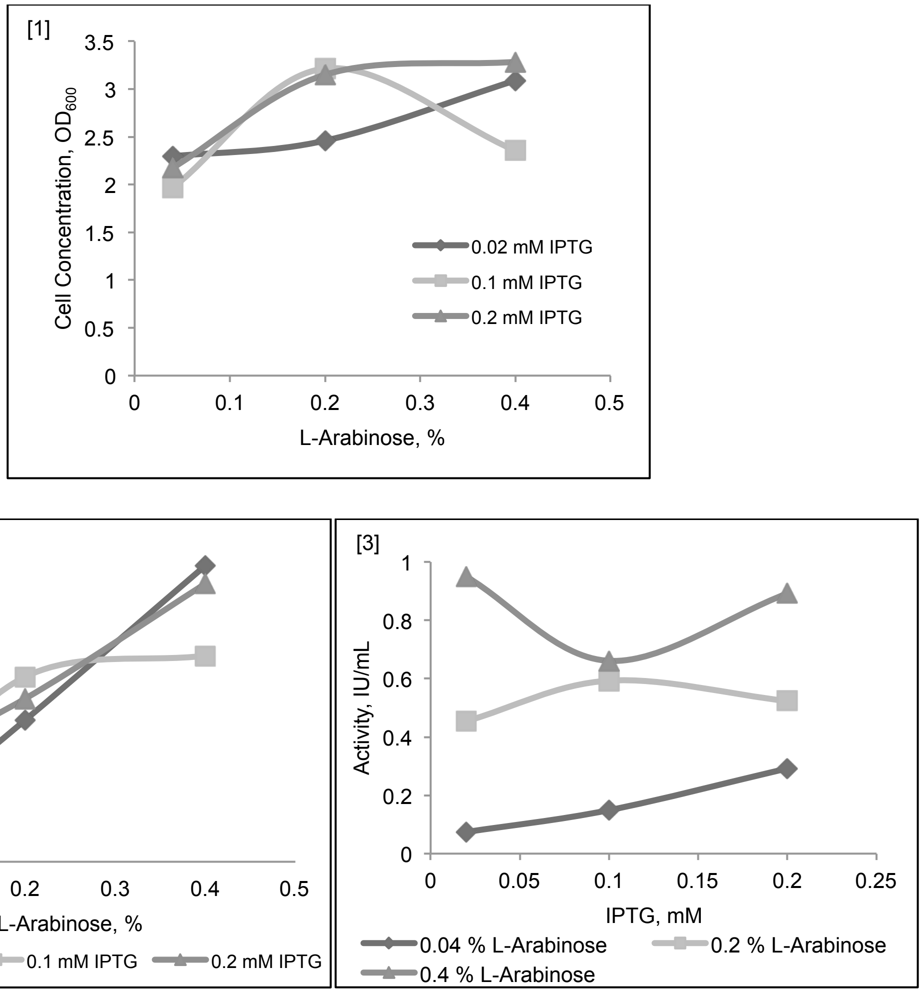

Figure 4.19. Effect of inducers on recombinant extracellular [1] bacteria growth and enzyme activity, at various [2] L-arabinose and [3] IPTG concentrations.

Previously optimized induction conditions for the extracellular enzyme also gave an extended production period for the intracellular enzyme (i.e. at 0.02 mM IPTG and 0.4% L-Arabinose) (Fig 4.20). Inducer concentrations were further anlysed by various inducer concentration and comparison to non-induced glycosylated intracellular cellulase. The results of enzyme activity assay suggest that the increment of IPTG concentration had a negative effect (Fig 4.21).

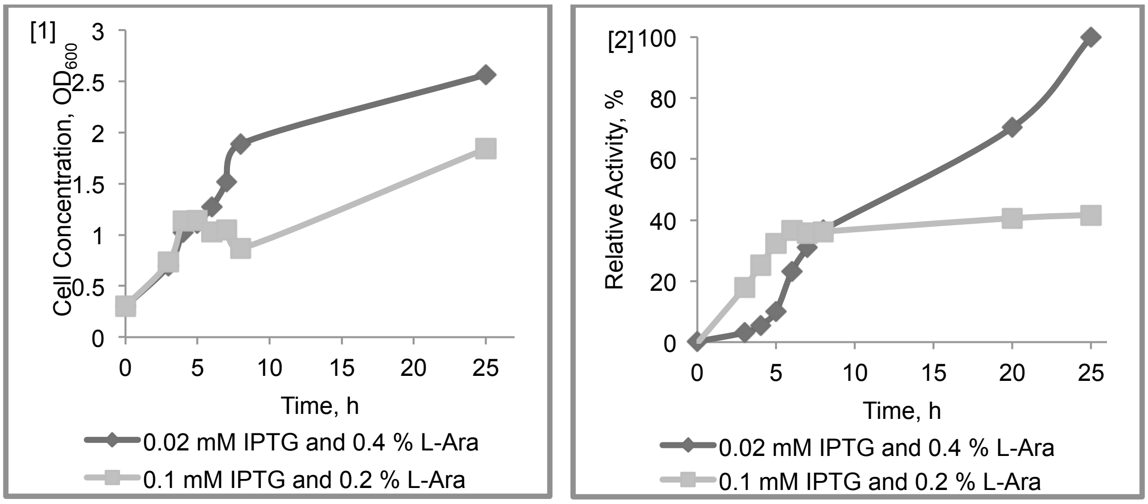

Figure 4.20. Comparison of [1] cell growth profile and [2] enzyme activity profile for the recombinant intracellular enzyme for different inducer concentrations.

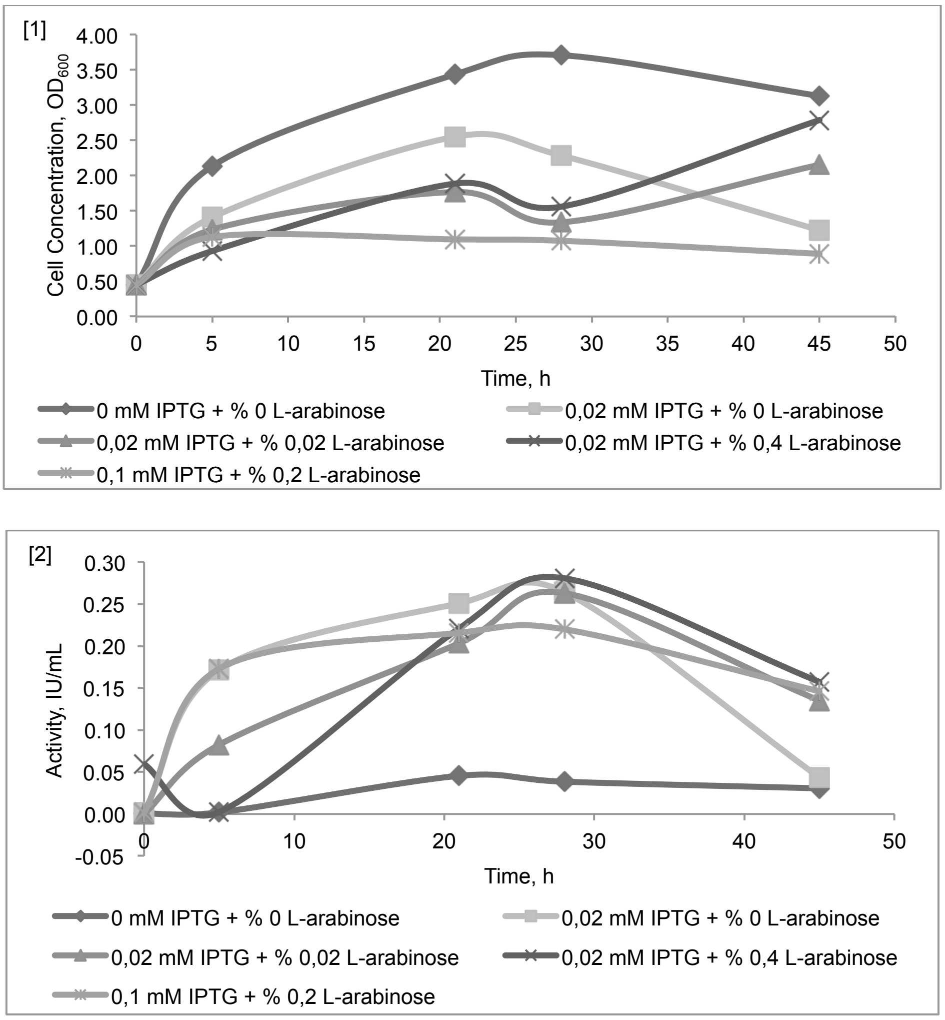

Figure 4.21. Comparison of intracellular enzyme produced in various concentration of inducers for [1] cell growth and [2] enzyme activity.

4.3.2. Effect of $OD_{Induction}$ on Cell Growth and Enzyme Activity

Cell concentration at induction phage is an important factor for protein production, which was measured by optical density (OD). In this context, wide range of $OD_{induction}$ was compared by enzyme activity assay and the maximum activity was achieved at OD_{600} =0.3 (Fig 4.22).

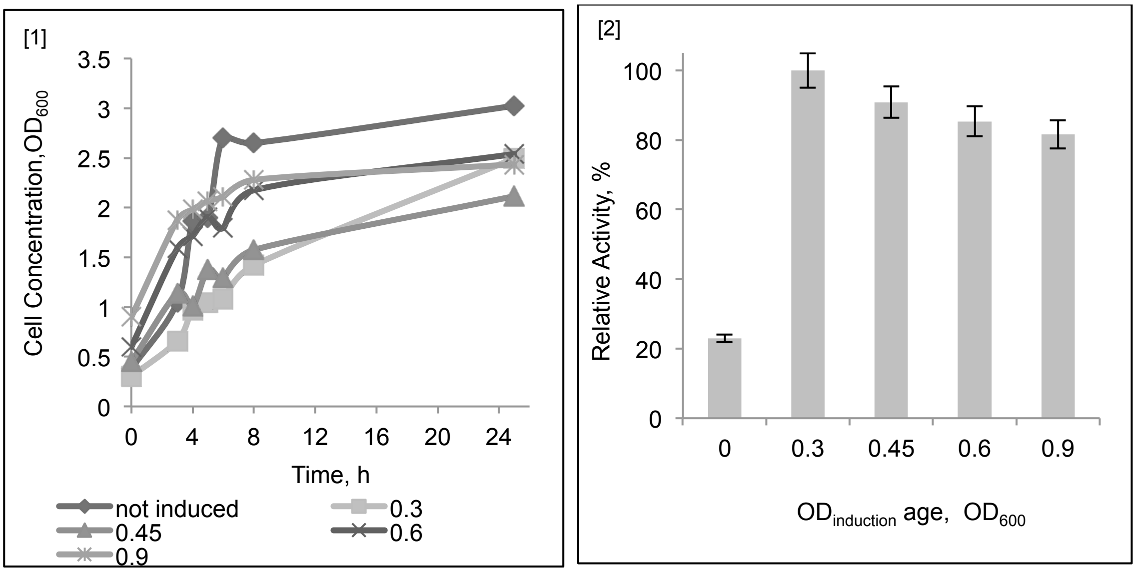

Figure 4.22. [1] Cell growth of recombinant bacteria with time for variation of OD_i, [2] Enzyme activities of cells induced at OD_{600} 0.3, 0.45, 0.6 and 0.9 A.

4.3.3. Effect of Additional Amp and IPTG at 3th hour on Cell Growth and Enzyme Activity

In order to ensure that the recombinant cells carry the plasmids designed by the antibiotics resistant, additional Ampicillin (Amp) was added to the production environment at t=3h. It appeared that the amount of antibiotic remains sufficient in the medium during production. Similar results were obtained for the IPTG added to the medium at t=3h (Fig 4.23). Supplementing the medium with IPTG or Amp at t=3h did not affect significantly. This shows that there is no need for a fed-batch strategy for the inducer.

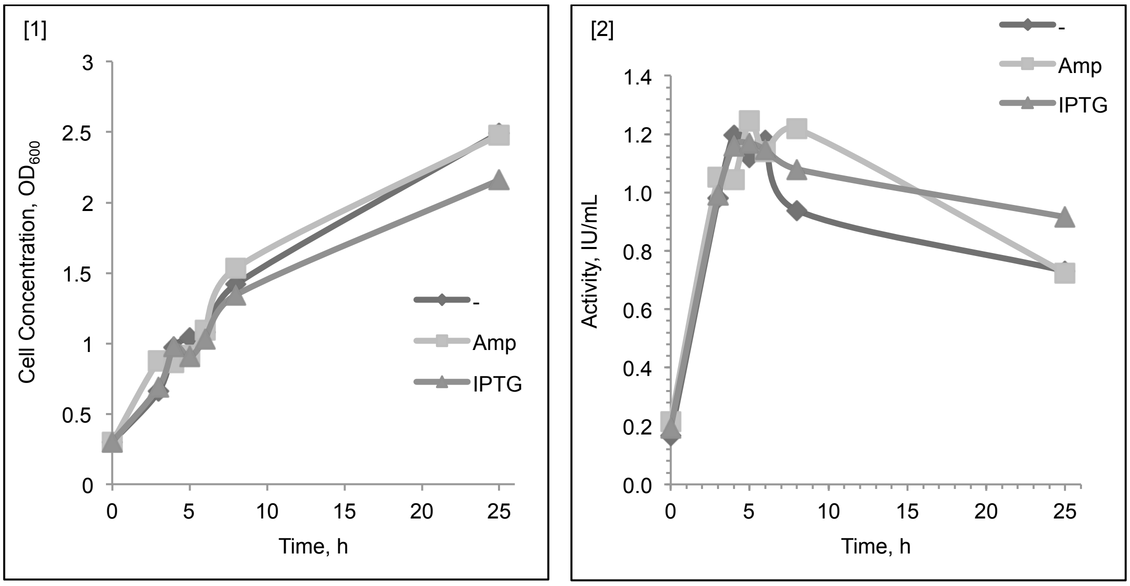

Figure 4.23. [1] Cell growth of extracellular enzyme [2] Comparison of enzyme activities with time for additional Amp and IPTG after 3 hours from induction.

4.3.4. Effect of Additional Mg^{2+} to Media on Cell Growth and Enzyme Activity

Despite the positive influence of Mg^{+2} for the cell growth (Fig 4.24), the unfavorable effects were observed in the extracellular enzyme activity with the addition of Mg^{2+}. On the other hand, intracellular enzyme activity increased and the optimal activity was obtained at 0.4 mM as 0.9 IU/mL, at t=45h (Fig 4.25). This situation is anticipated by virtue of the fact that the Mg^{+2} blocks the enzyme transfer by binding to the cell wall and therefore prevents the enzyme loss.

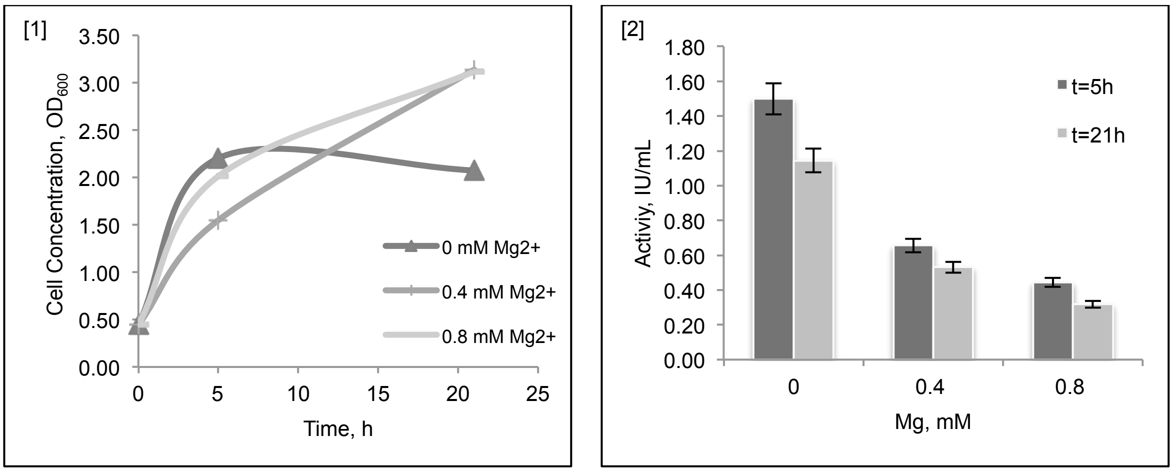

Figure 4.24. Contribution of the Mg^{2+} to [1] recombinant cell growth and [2] extracellular enzyme activity.

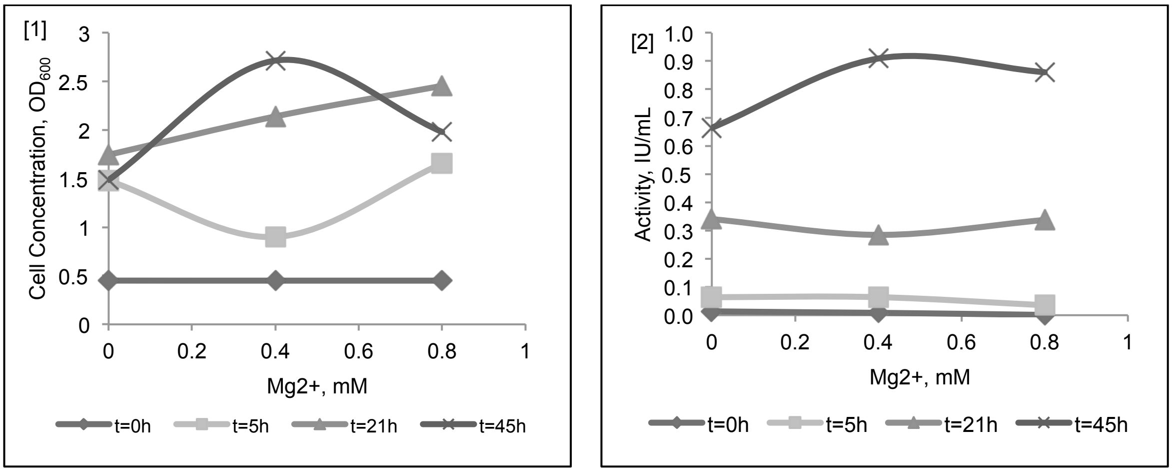

Figure 4.25. Contribution of the Mg2+ to [1] recombinant cell growth and [2] intracellular enzyme activity.

4.3.5. Effect of Additional Glucose at Growth Phase

In order to provide a rapid cell growth, glucose was added to the pre-induction culture as a carbon source, in the 1-15 g/L range; however, the excess glucose that remains in the medium after the induction process, is known to block the promoters. The increase in the glucose concentration affected both the cell production rate and extracellular enzyme activity (Fig 4.26). The higher activity value at the beginning arose from the signal of the glucose by DNS method. The optimum initial concentration was detected as 2 g/L for extracellular cellulase.

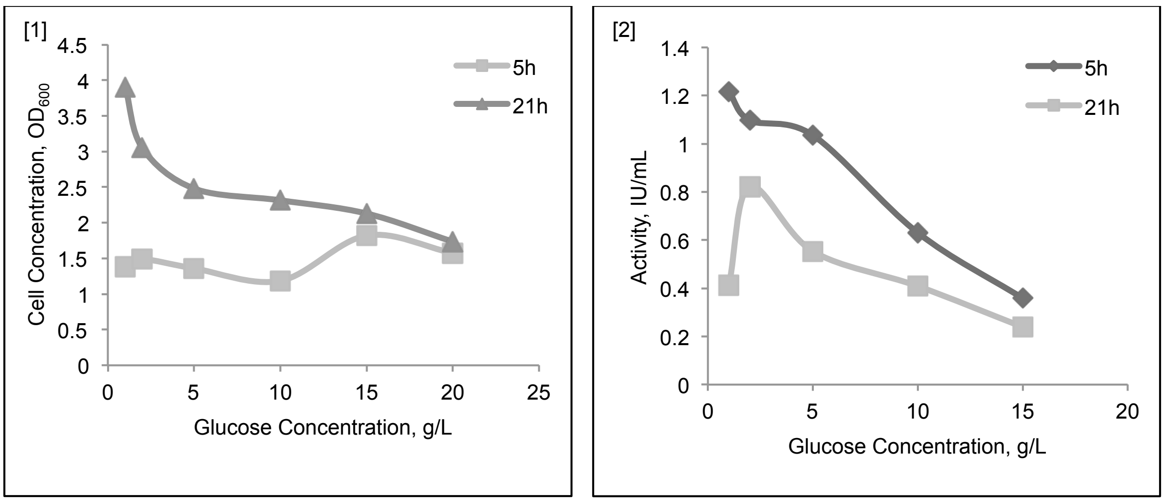

Figure 4.26. Contribution of glucose at [1] growth phase to recombinant cell growth and [2] extracellular enzyme activity.

Although an increase in activity was related to the glucose concentration in the early samples, the highest activity was obtained with 4 g/L initial concentration at t=45h and the activity was conserved as the glucose concentration increased. The optimum initial concentration was chosen as 4 g/L for intracellular enzyme production (Fig 4.27).

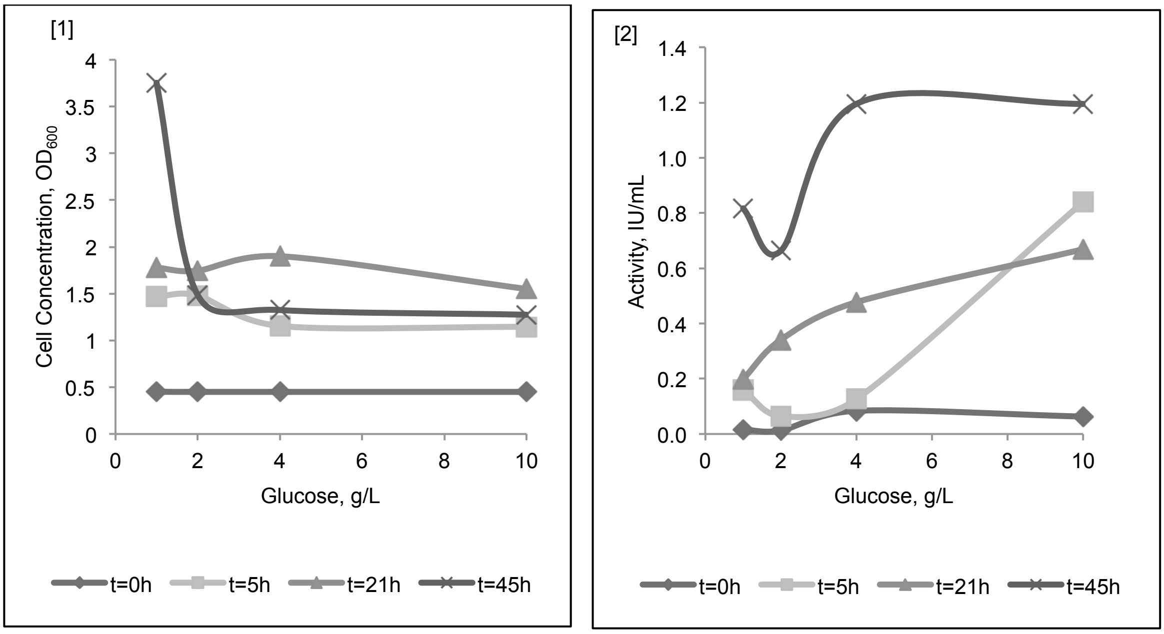

Figure 4.27. Contribution of glucose (C_G=1-2-4-10 g/L) at [1] growth phase to recombinant cell growth and [2] intracellular enzyme activity.

4.3.6. Comparison of Media Volumes in Luria Bertani Medium

5 mL and 25 mL cell culture volumes were tested by enzyme activity in order to reduce the culture volume for large number of conditions to be tested simultaneously. There was no significant difference on cell growth between 5 mL and 25 mL medium, however activity was higher by 1.4-fold in 5 mL medium (Fig 4.28).

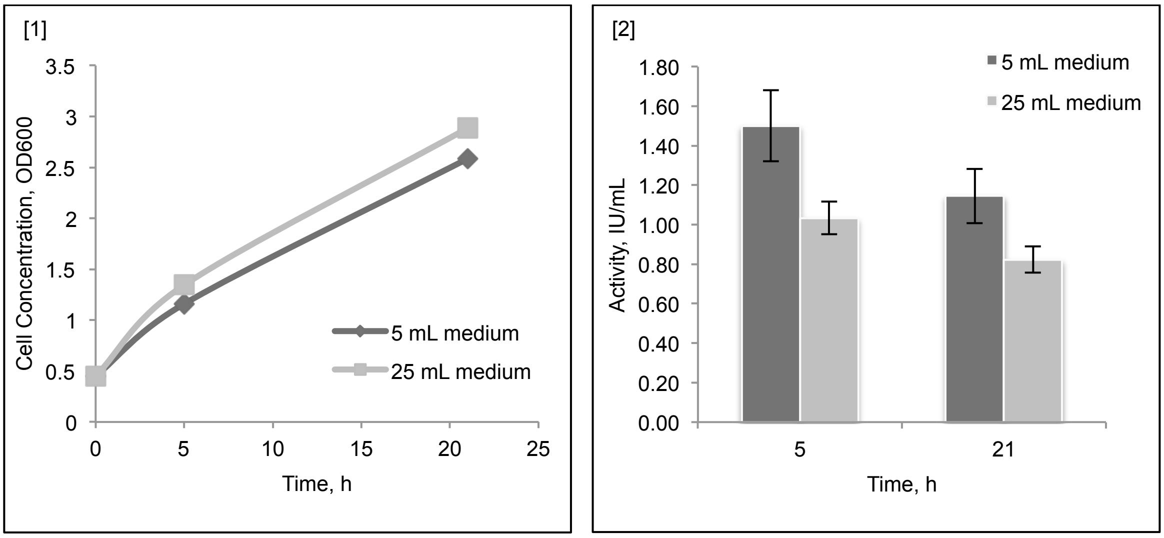

Figure 4.28. Comparison of media volumes by [1] cell growth and [2] activity of enzymes produced by *E. coli* CLM24 expressing extracellular cellulase in 5mL and 25mL culture volume.

4.3.7. Comparison of Luria Bertani (LB) and Terrific Broth (TB) Media

The standard growth medium Luria Bertani (LB) was compared to Terrific Broth (TB) medium for recombinant protein production. Despite the negative influence at extracellular enzyme expression (decreased 6.5-fold) (Fig 4.29), TB media had increased the intracellular enzyme expression 1.5-fold (Fig 4.30).

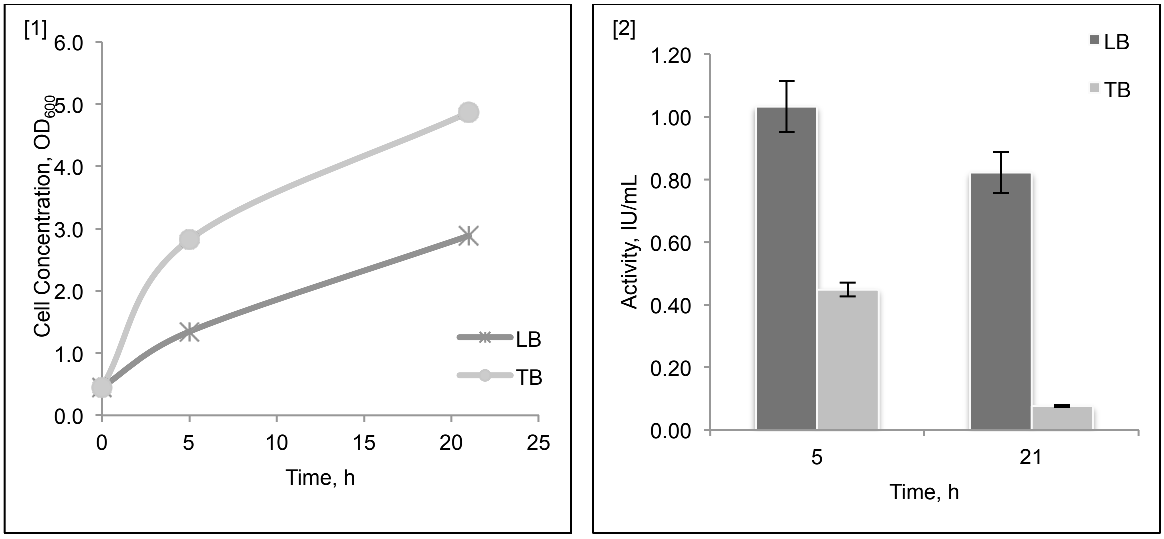

Figure 4.29. [1] Cell growth [2] Comparison of Luria Bertani and Terrific Broth media with extracellular enzyme activity.

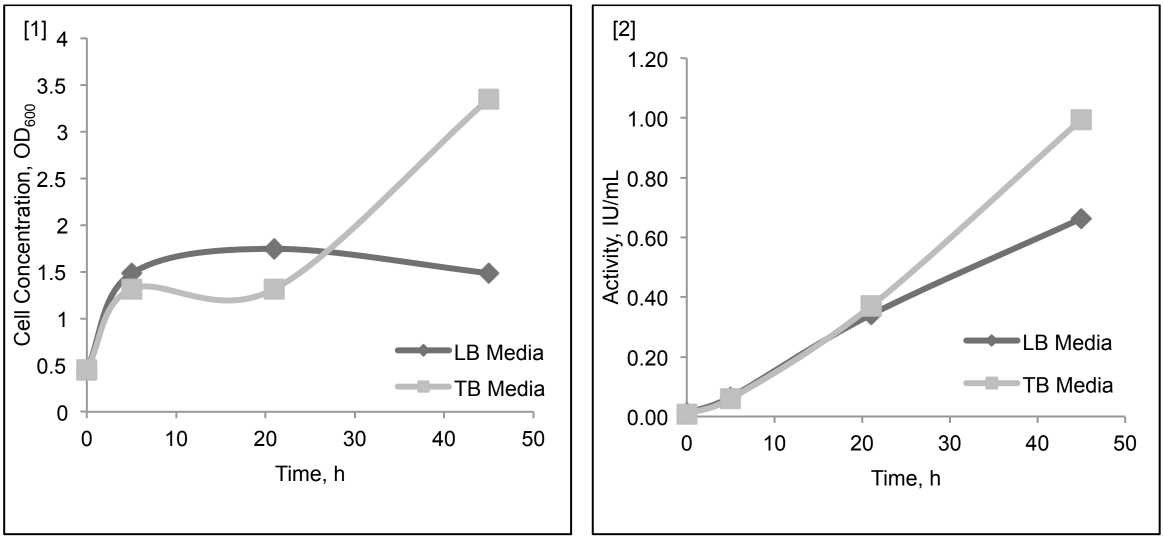

Figure 4.30. [1] Cell growth [2] Comparison of Luria Bertani and Terrific Broth media with intracellular enzyme activity.

4.3.8. Effect of Shaking Speed after Induction on Cell Growth and Enzyme Activity

Various shaking speed rates were investigated in order to detect the effect of oxygen transfer rate. Optimum shaking speed was found as 150 rpm (Fig 4.31), which matches with the results of Gupta et al. [3].

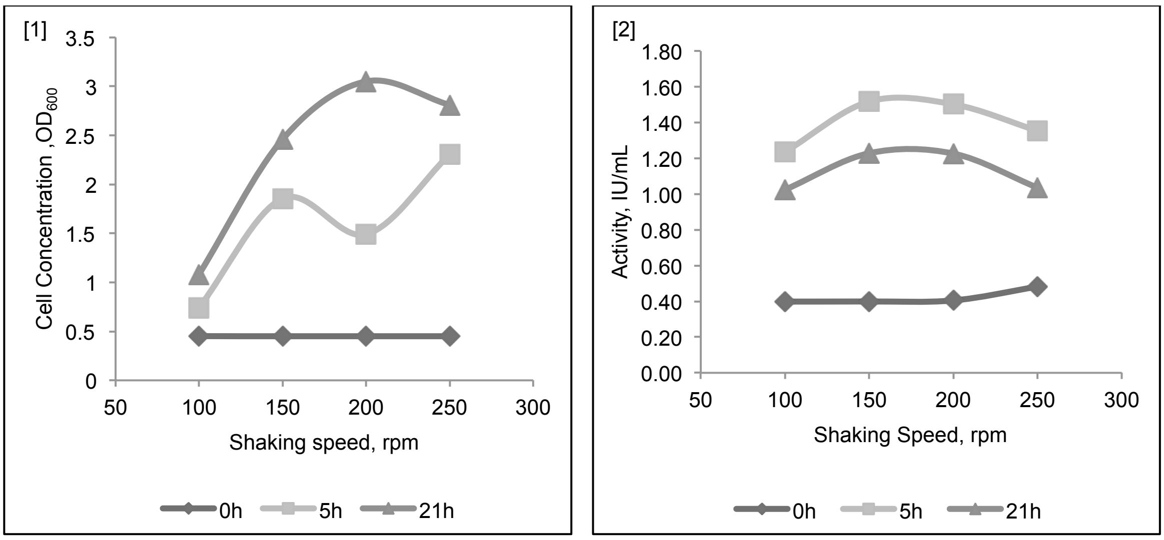

Figure 4.31. [1] Cell growth of recombinant bacteria for different speeds of shaking and [2] Variation of activity with shaking speeds for samples from different hours.

4.3.9. Effect of Incubation Temperature on Cell Growth and Enzyme Activity

After reaching certain concentration, cells are induced and the incubation temperature is dropped. In order to determine the optimum temperature, 25, 30 and 33°C were screened. Optimum protein production was performed at 30°C. The higher activity at 25°C at t=21h is most likely due to the better conditions for proteins, where proteolytic activity is potentially lower (Fig 4.32).

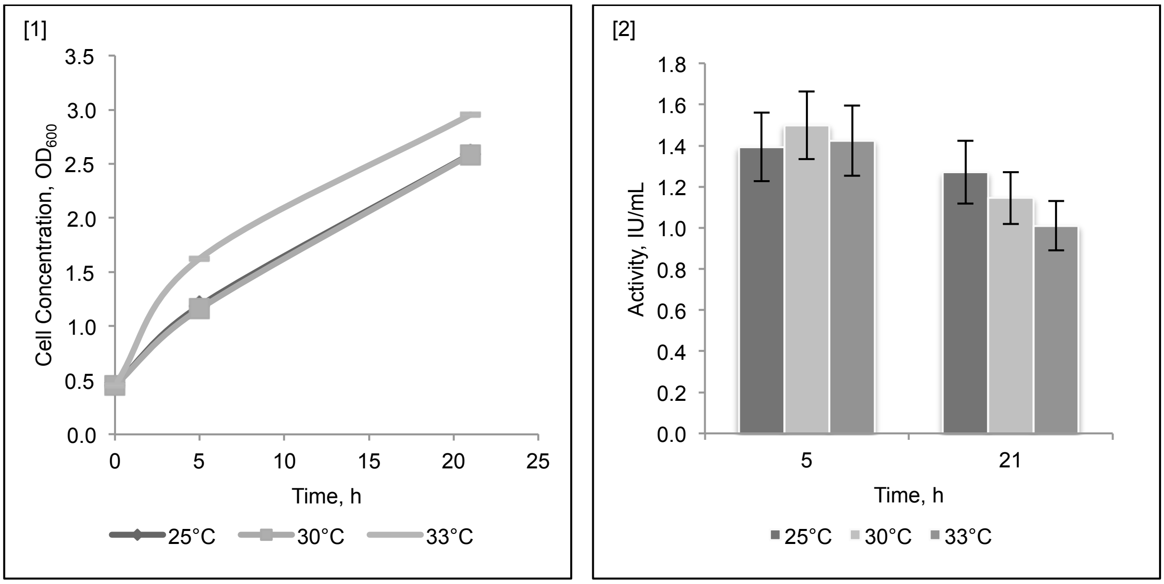

Figure 4.32. Comparison of different incubation temperatures after induction by [1] cell growth of recombinant *E. coli* CLM24 containing pTrc99A-ssYebF-Cel5A-6xHis, pPglΔB, pMAF_pglB and [2] extracellular cellulase activity .

4.3.10. Comparison of Baffled and Regular Erlenmeyer Flask

Regular Erlenmeyer flask was compared to baffled Erlenmeyer flask since baffles increase aeration. Cells grown in baffled flask expressed 1.3-fold more enzyme than the regular flask (Fig 4.33.2).

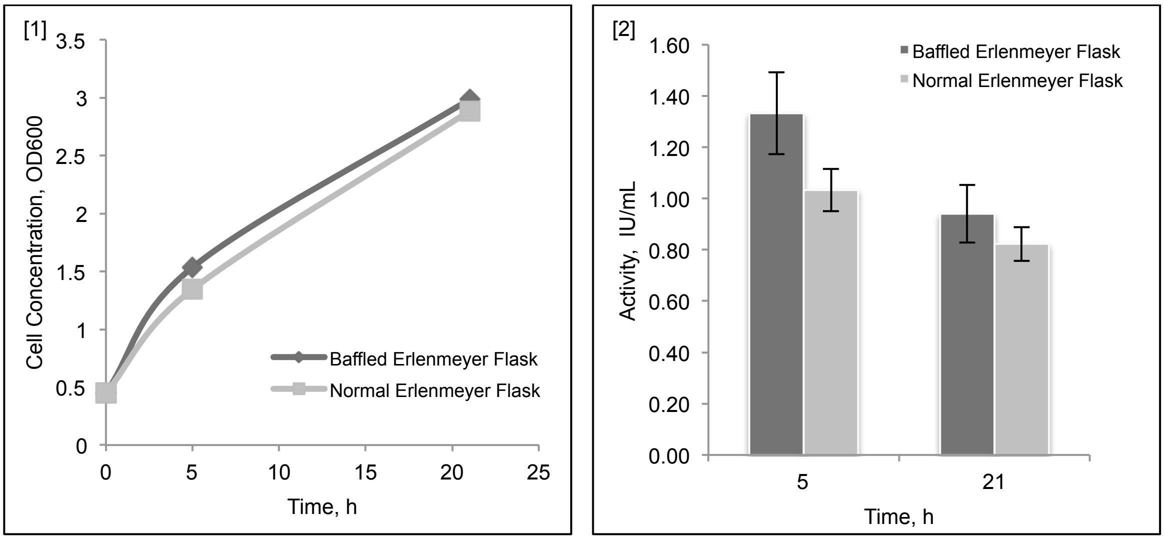

Figure 4.33. Comparison of different incubation temperature after induction; [1] Cell growth of recombinant bacteria [2] Enzyme activity.

4.3.11. Determination of Enzyme Kinetic Parameters

The enzyme kinetic parameter K_m was determined by linearized Lineweaver-Burke plot with varying substrate concentrations, as 3.25 mM for the glycosylated Cel5A and 3.24 mM for the wild-type Cel5A, which were within the range reported previously for cellulases (0.67 – 79.4 mM) [103, 104] and Vmax was determined as 100.0 µM/min for both glycosylated and wild-type Cel5A (Fig 4. 34).

The Michaelis-Menten constant, K_m, is the substrate concentration, which permits the enzyme to reach half value of maximal reaction rate, Vmax. In enzyme kinetics reaction, K_m value characterizes how the reaction rate increases with substrate availability. So, lower K_m value indicates high substrate affinity because low substrate levels are needed to reach the certain reaction rate. The K_m value for cellulase enzyme depends on the substrate and the other conditions such as pH and temperature. Vmax determines the turnover number of an enzyme, which is the number of molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated by substrate.

The results suggest that glycosylation does not affect the active site of the enzyme, yet, activity results reveal that glycosylated enzymes show more activity compared to the wild-type.

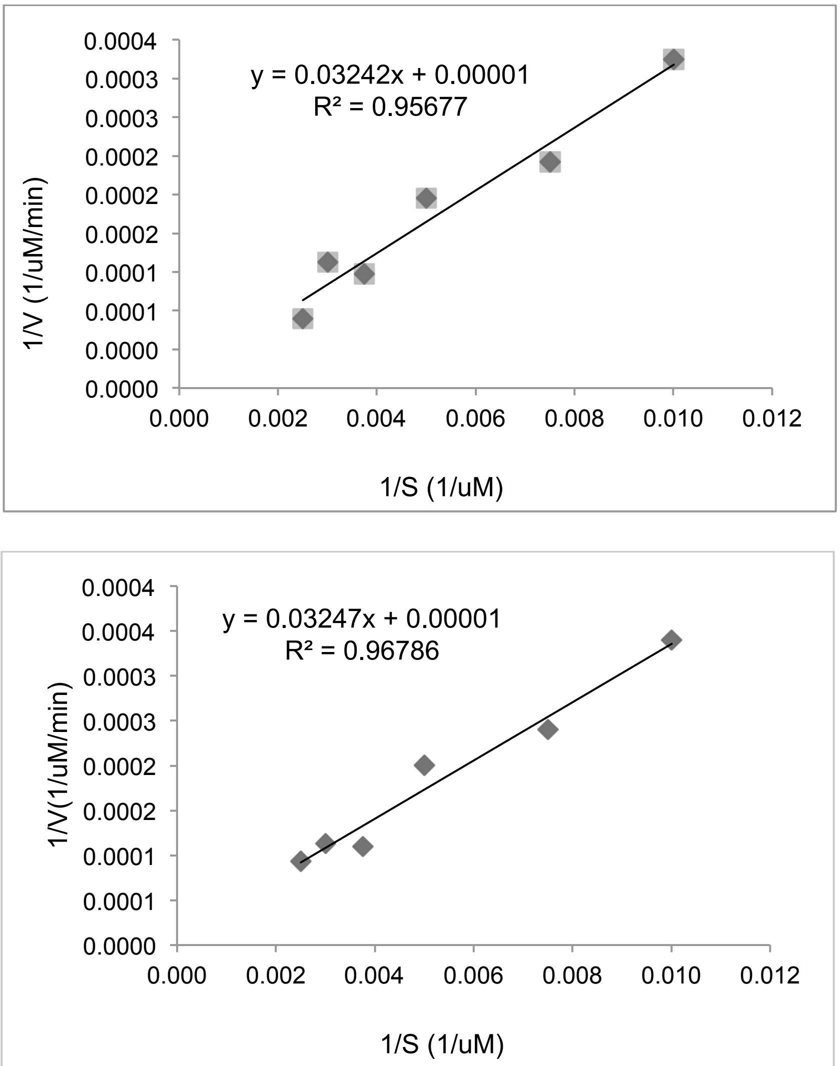

Figure 4.34. Lineweaver-Burk plot for [1] intracellular wild-type cellulase and [2] glycosylated cellulase.

4.3.12. Comparison Glycosylated and Wild-type Cellulase

After optimization of bioprocess, all new systems were compared to investigate the effect of glycosylation on enzyme activity. Glycosylated intracellular and extracellular cellulase activities were higher than the native types (Fig 4.35.2, Fig 4.36.2). However, cell growths were affected by anibiotics, therefore systems including glycosylation plasmids had relatively less cell concentrations (Fig 4.35.1 and Fig 4.36.1). More importantly, glycosylated intracellular enzyme activity was 1.5-fold higher and retained its activity up to t=45h and glycosylated extracellular enzyme activity was 27-fold higher at t=21h and retained its activity up to t=45h whereas, wild type enzyme almost lost its activity at t=28h.

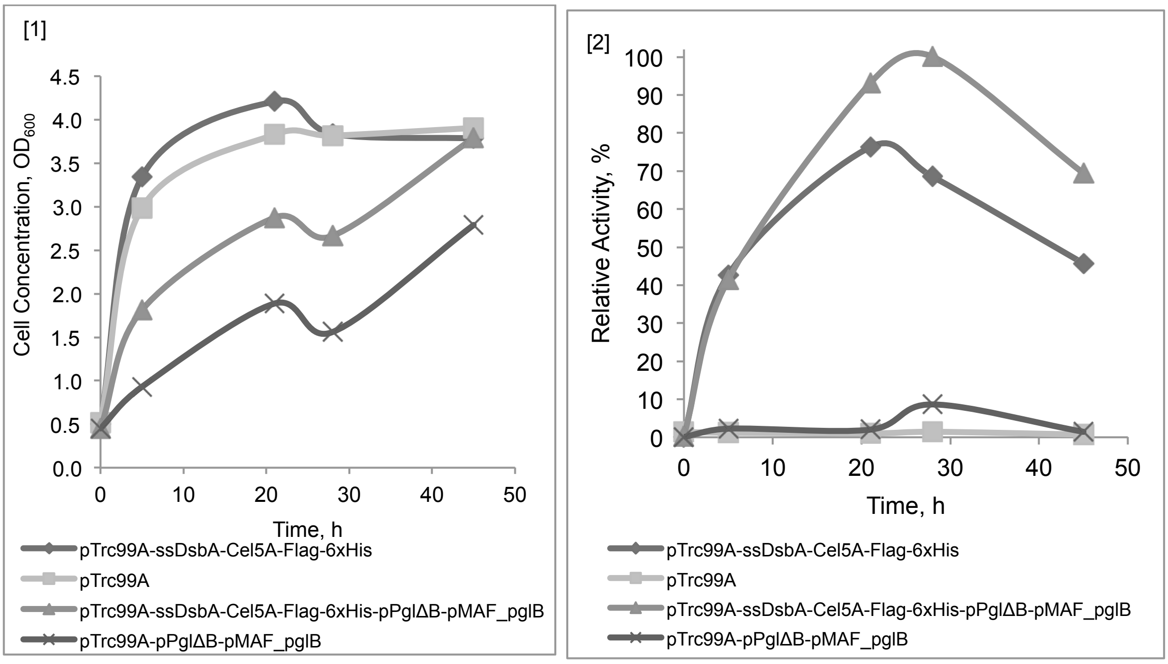

Figure 4.35. Comparison of *E. coli* CLM24 expressing glycosylated intracellular and wild type intracellular cellulase and their negative controls by [1] cell growth of recombinant cells and [2] enzyme activity.

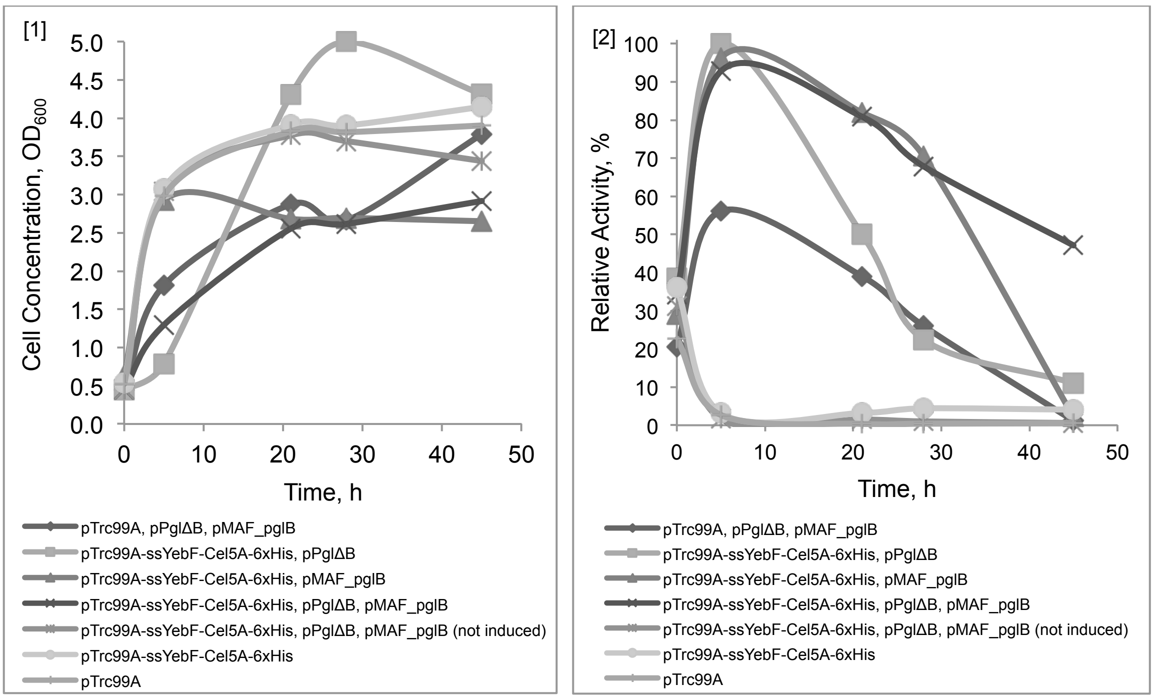

Figure 4.36. Comparison of *E. coli* CLM24 expressing glycosylated extracellular and wild type extracellular cellulase and their negative controls by [1] cell growth of recombinant cells and [2] enzyme activity.

Western Blotting was performed to distinguish the proteins by using anti-His and anti-glycan (anti-Hept) antibodies. After the samples were run on SDS-PAGE gel, blotting was performed electrophoretically (BioRad), for 3 h at 50 V. The presence of endogluconase was visualized by Opti-4CN colorimetric kit (Biorad).

The presence of glycosylated and wild type extracellular proteins were observed at t=45h for *E. coli* CLM24 containing pTrc99A-ssDsbA-Cel5A-Flag-6xHis, pMAF_pglB, pPglΔB (Lane 1), pTrc99A-ssDsbA-Cel5A-Flag-6xHis (Lane 2) by anti-His respectively, and as a control pTrc99A (Lane 3) was clear. Glycosylated extracellular cellulase (Lane 1) displayed thicker band with anti-His compared to wild type (Lane 2), where there was no band at negative control (Lane 3). Moreover, glycosylated cellulase (Lane 4) gave an immunoreactive band with anti-Hept, where wild type and control lanes had no band (Lane 5,6) (Fig 4. 37).

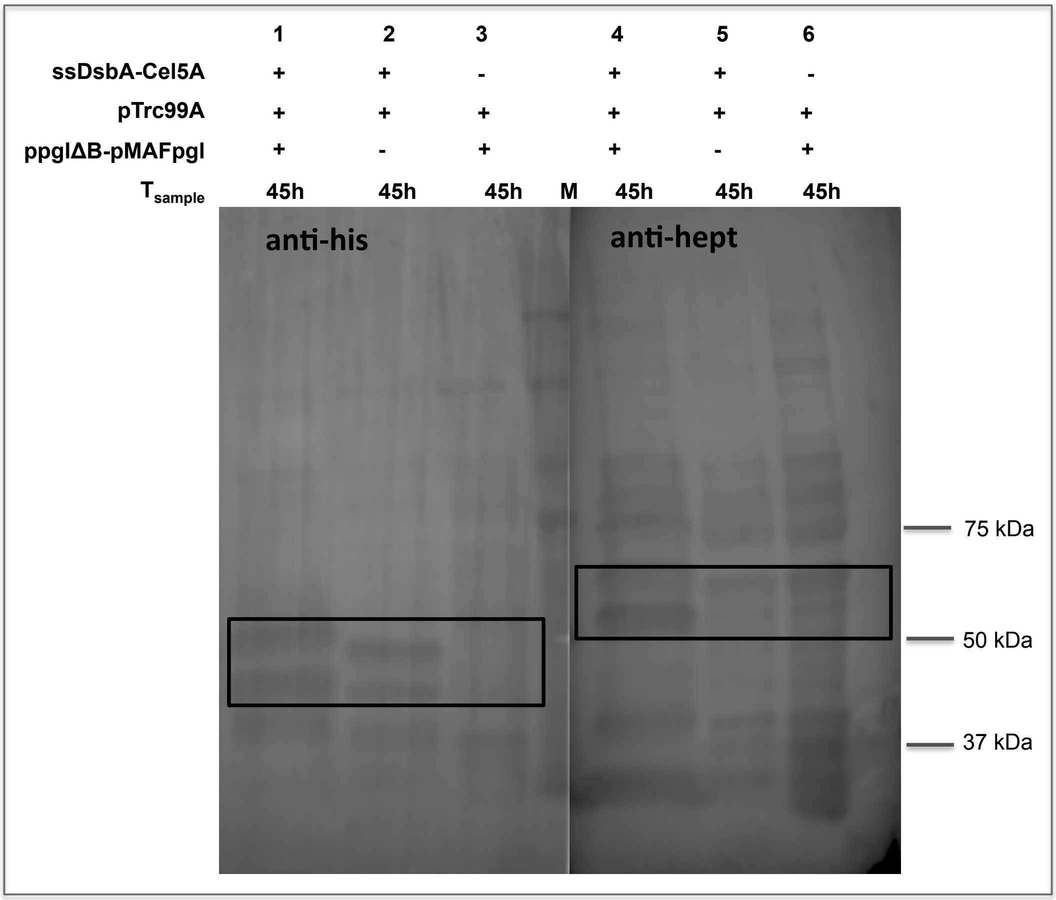

Figure 4.37. Analysis of the glycosylated extracellular Cel5A and wild type Cel5A produced *E. coli* CLM24 containing pTrc99A plasmid. 1, overexpressed glycosylated extracellular Cel5A; 2, wild type Cel5A and 3, empty pTrc99A with anti-His. 4, overexpressed glycosylated extracellular Cel5A; 5, wild type Cel5A and 6, empty pTrc99A with anti-Hept. Samples were optimized by OD_{600}.

4.3.13. Comparison of Enzymes Produced Initially and After Optimizations

After optimizations extracellular cellulase was increased by 1.77-fold, intracellular cellulase was increased by 21-fold (Fig 4.38), glycosylated intracellular cellulase was increased by 68-fold and glycosylated extracellular cellulase was increased by 3-fold (Fig 4.39).

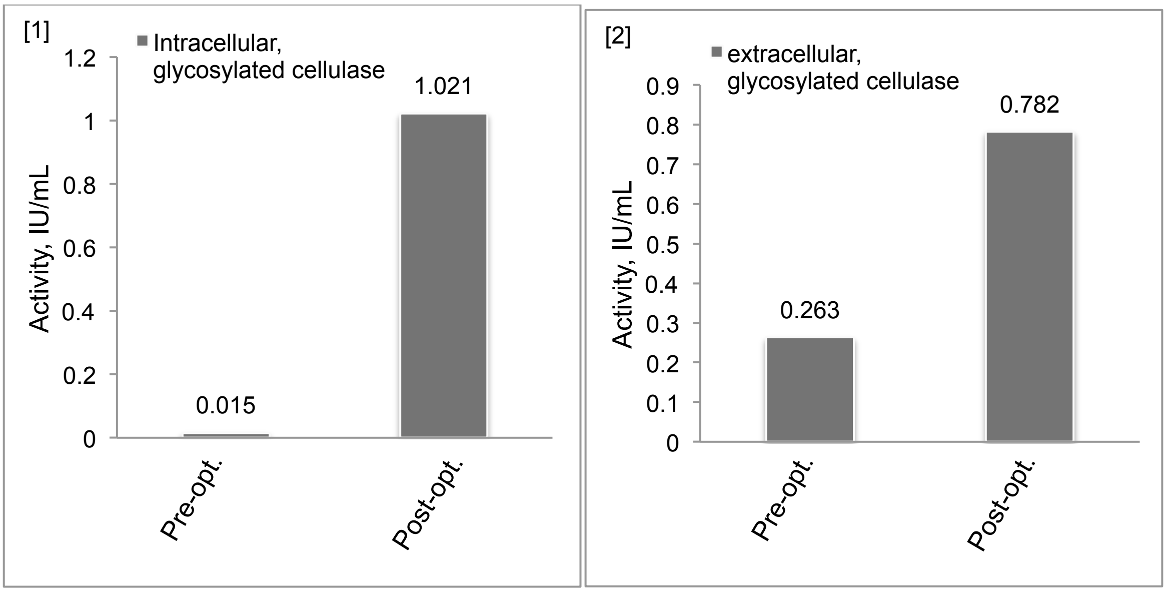

Figure 4.38. Glycosylated [1] Intracellular enzyme and [2] extracellular enzyme produced in optimized process compared to pre-optimization.

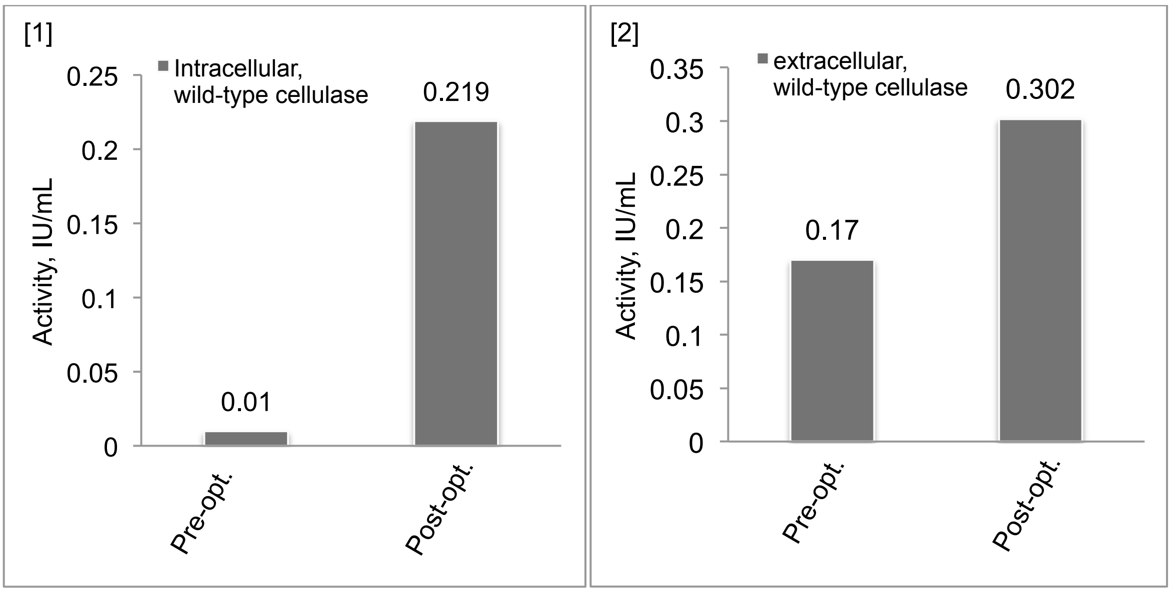

Figure 4.39. [1] intracellular and [2] extracellular enzyme produced in optimized process compared topre-optimization.

5 CONCLUSION

In order to satisfy the market demand for cellulases, novel glycosylated Cel5A was expressed by genetically engineered *E. coli* CLM24 and *E. coli* BLM21 (DE3) strains for the first time in literature, since the high cost of cellulase enzymes still remains a challenge. In this regard, pET28a-ssDsbA-Cel5A-Flag-6xHis, pET28a-ssYebF-Cel5A-6xHis, pTrc99A-ssDsbA-Cel5A-Flag-6xHis, and pTrc99A-ssYebF-Cel5A-6xHis, plasmids were designed and sixteen new systems were created by using these new plasmids with or without pMAF_pglB and/or pPglΔB. *E. coli* CLM24 strain containing pTrc99A plasmid was chosen over *E. coli* BLM21 (DE3) containing pET28a, due to its higher glycoprotein production capacity.

Expression of glycosylated and wild type cellulase enzymes were analyzed and confirmed by SDS-PAGE and Western Blotting methods prior to optimizations. Moreover kinetic parameters were investigated in order to determine the effect of glycosylation on active site of the enzyme.

Enzyme activity assay was optimized and performed as; 2-fold diluted cell suspension before sonication; 2 times sonication; 2-5-fold diluted enzyme solution after sonication used in DNS assay; 2% CMC substrate; $T_{inc.}$= 50°C; t_{inc}=20 min; $T_{cooling}$= - 20 °C after enzyme-substrate reaction.

Bioprocess was developed by the optimization of product conditions as 0.02 mM IPTG and 0.4% L-arabinose for induction; $OD_{induction}$ at OD_{600} = 0.3-0.45; 2 g/L initial glucose concentration; addition of 0.4 mM Mg^{2+} at growth phase for intracellular enzyme production; T= 30°C after induction; shaking speed 150 rpm after induction; sampling at t=5, 21, 28, 45 h.

After optimizations, activity of four different systems that produce cellulase were increased as; intracellular cellulase 21-fold, up to 0.219 IU/mL; extracellular cellulase 1.8-fold, up to 0.302 IU/mL, glycosylated intracellular cellulase 68-fold, up to 1.02 IU/mL and glycosylated extracellular cellulase 3-fold, up to 0.782 IU/mL, which is 6-fold higher compared to a recently reported study [3].

The recombinant glycosylated cellulase enzymes and the bioprosses developed in this study are important for overcoming the bottlenecks in the biofuels and energy sector.

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