Department of BSBE
Indian Institute Of Technology Guwahati

Dr. Sanjukta Patra

BT 207
Genetic Engineering
Recombinant production of cellulase – A case study
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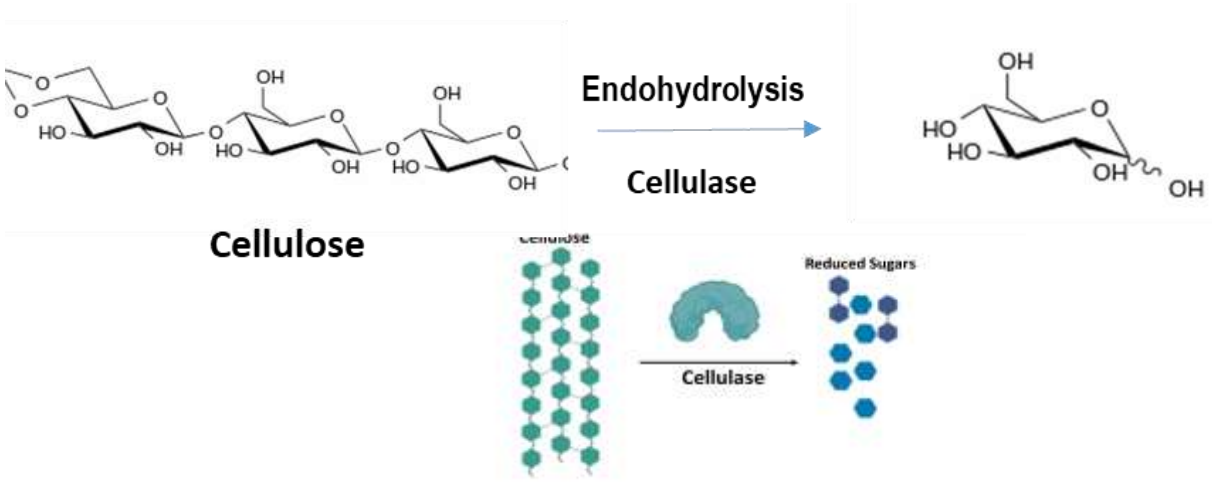
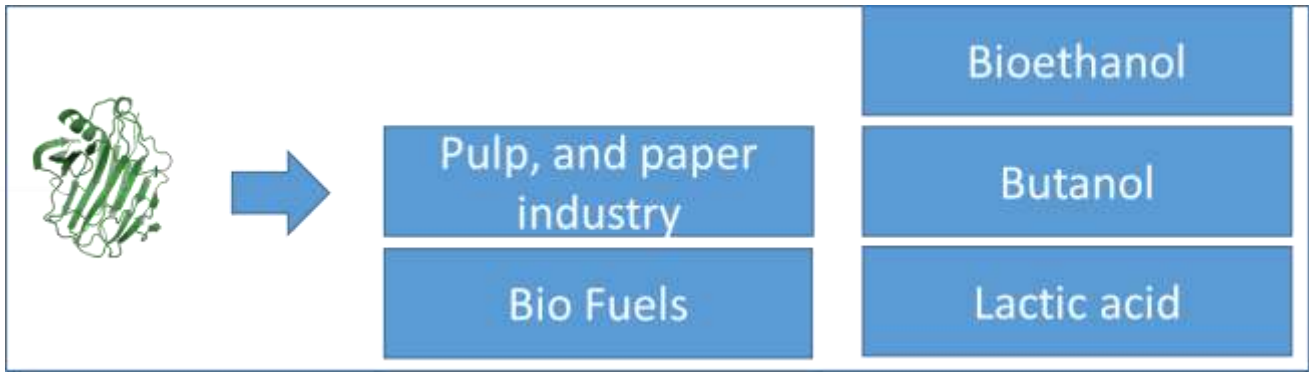
Recombinant production of cellulase

- Cellulose and hemicelluloses make up the majority of plant cell walls, and mixed linked 1, 3-1, 4-glucans make up the majority of cereal cell walls.
- Cellulose and hemicelluloses make up the majority of plant cell walls, and mixed linked 1, 3-1, 4-glucans make up the majority of cereal cell walls. As a substitute for petroleum fuels, the bioconversion of lignocellulosic feedstock to bioethanol has garnered interest on a global scale. Many technical advancements are required for commercialising this method .
- One of the main drawbacks of lignocellulosic-base bioethanol technologies is the high cost of the enzymes required to convert the cellulose component into fermentable sugars. Enzymes from the glycosyl hydrolase family hydrolyze oligosaccharides and polysaccharides to breakdown cellulose.





Why from *Bacillus subtilis?*

- β-glucosidases from several species of bacteria have been identified, characterized and applied in various biotechnological applications.
- There is always a need to search for novel β-glucosidases that withstand high temperature and remain efficiently active in presence of high glucose and organic solvents.
- The enzyme belonged to the third group of β-glucosidases exhibiting broad substrate specificity, hydrolyzing both aryl glycosides and natural glycosides.

Gene Name	bglS
Gene Size	726bp
Source Organism	*Bacillus subtilis*
Strain No	168
EC no	EC:3.2.1.73

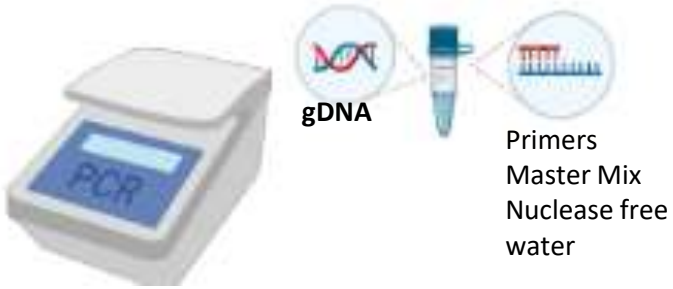
Methods

***B. subtilis* MTCC(168) is Cultured and DNA Extraction**

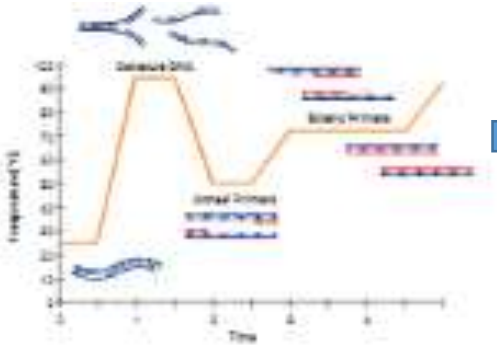
Properties	Forward Primer	Reverse Primer
Sequence	*ATT*AAGCTTCAAACAGGTGGATCG	*CCG*CTCGAGTTATTTTTTTGTATAGC
Primer length	24	26
GC Content	41.7	38.5
Tm (Melting Temp)	55%	57%

Primer Designing for BglS (B-glucanase) gene

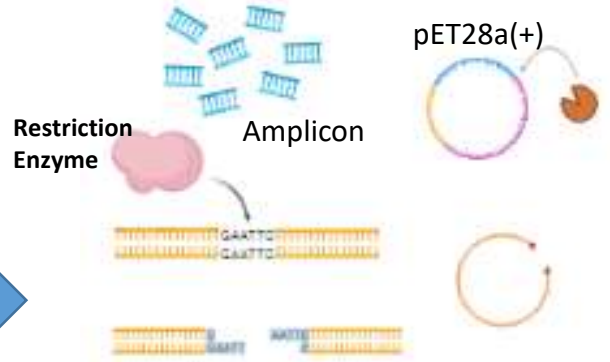
Red Colour: Restriction sites for Hind III (F) and Xho (R)
***Italic*:** Cushioning sequence



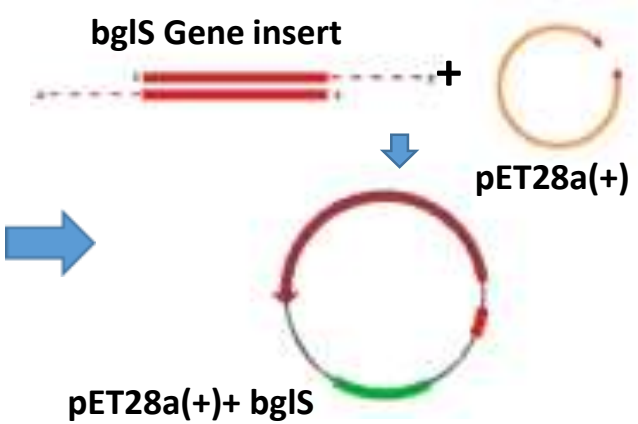
BglS gene Amplification from gDNA using PCR



pET28a(+) Plasmid Extraction



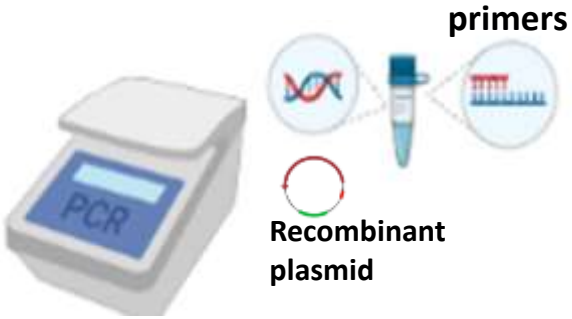
Restriction Digestion of PCR amplicon and Vector backbone using Hind III and Xho I



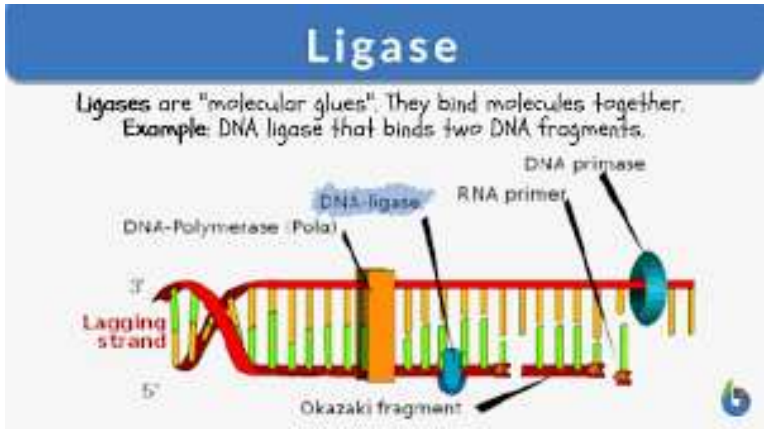
Ligation of the Digested vector and gene insert by T4 DNA Ligase Overnight at 4°C

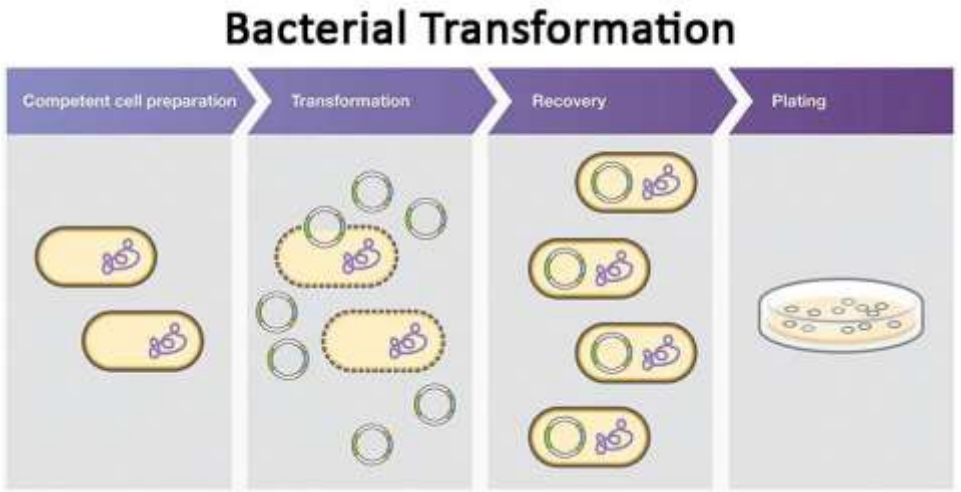
Transformation of competent *E .coli* DH 5α cells with Ligated product(pET28a+bglS) by heat-Shock and Cacl2 method

Screening of colonies using Mobility difference of plasmid in Agarose Gel electrophoresis



Screening of clones in transformed cells by PCR and Restriction Digestion





DH5-Alpha Cells are *E. coli* cells engineered by American biologist Douglas Hanahan to maximize transformation efficie ncy. They are defined by three mutations: recA1, endA1 which help plasmid insertion and lacZΔM15 which enables blue white screening.

What is **pET28a(+)?**

pET 28a is both a cloning vector and an expression vector.

What is the difference pET28a and pET28a(+)?

- pET28a(+) and pET28a are between plasmids commonly used in molecular biology for cloning and protein expression. The difference between the two lies in the presence or absence of an additional DNA sequence in the pET28a(+) plasmid.
- The "+" sign in pET28a(+) refers to the presence of a DNA sequence called the polylinker or multiple cloning site (MCS) in the plasmid. The polylinker allows for the insertion of DNA fragments into the plasmid at multiple sites, thus increasing the flexibility of the cloning process. pET28a(+) has 13 unique restriction sites, while pET28a has only 10 unique restriction sites.

Why are you using *E .coli* DH 5α cells? –

Using *E .coli* DH 5α cells for replication of pET28a(+) containing your gene of interest (cellulase).

E coli DH5-α Strain, application and advantages:

- DH5-Alpha Cells are *E. coli* cells engineered by

American biologist Douglas Hanahan to maximize transformation efficiency.

- They are defined by three mutations:
- recA1, endA1 which help plasmid insertion and lacZΔM15 which enables blue white screening.

the recA1 mutation is a single point mutation that replaces glycine 160 of the recA polypeptide with an aspartic acid residue in order to disable the activity of the recombinases and inactivate homologous recombination.

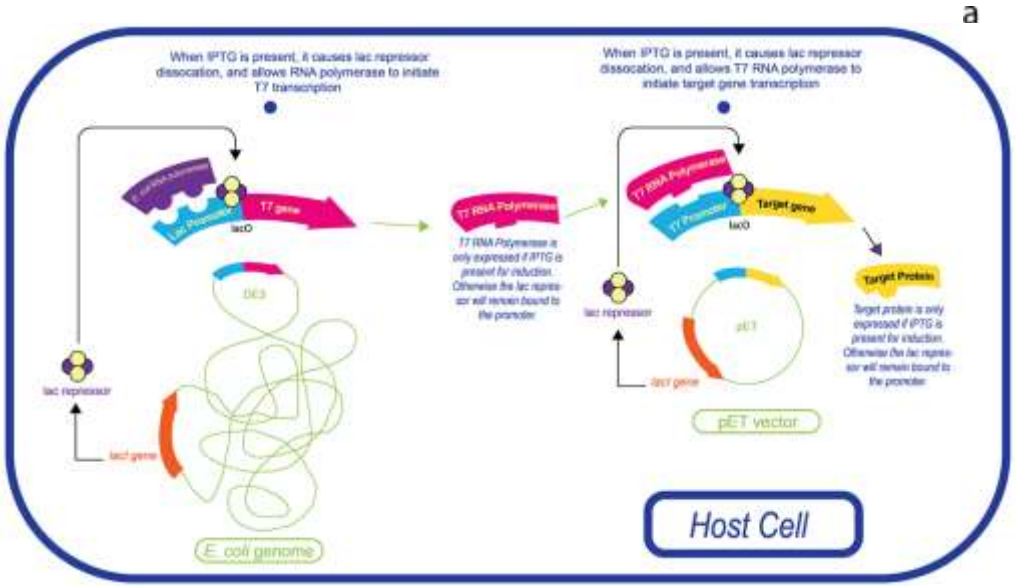
The endA1 mutation inactivates an intracellular endonuclease to prevent it from degrading the inserted plasmid

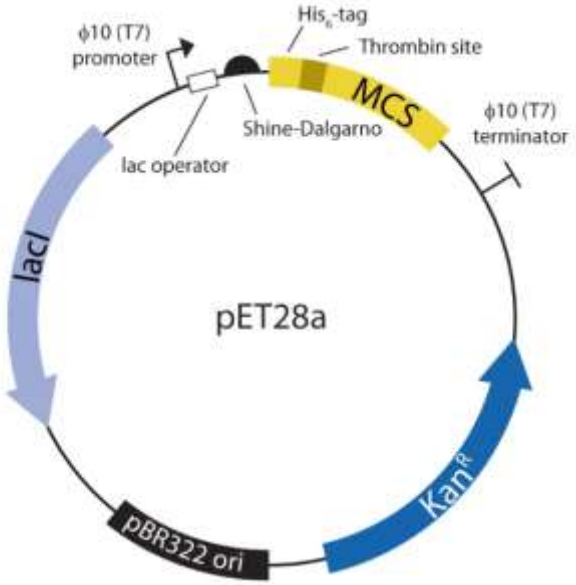
The cells are competent and often used with calcium chloride transformation to insert the desired plasmid

pET 28 Expression vectors

What is an expression vector?

The vector contains the necessary regulatory elements, including a promoter, terminator, and enhancer sequences, that enable the expression of the GOI in the host cell





The pET series of expression plasmids are widely used for recombinant protein production in *Escherichia coli*. The genetic modules controlling transcription and translation in these plasmids were first described in the 1980s.

It contains the T7 promoter and an adjacent *lac* operator sequence that is included to suppress uninduced expression[5]. Translation initiation is mediated by a Shine–Dalgarno (SD) sequence originating from the major capsid protein of T7 (*gene 10* protein). In a typical experiment, the coding sequence to be expressed is cloned downstream of, and in frame with, the coding sequence for a poly-histidine purification tag (His_6).

What is an expression vector?

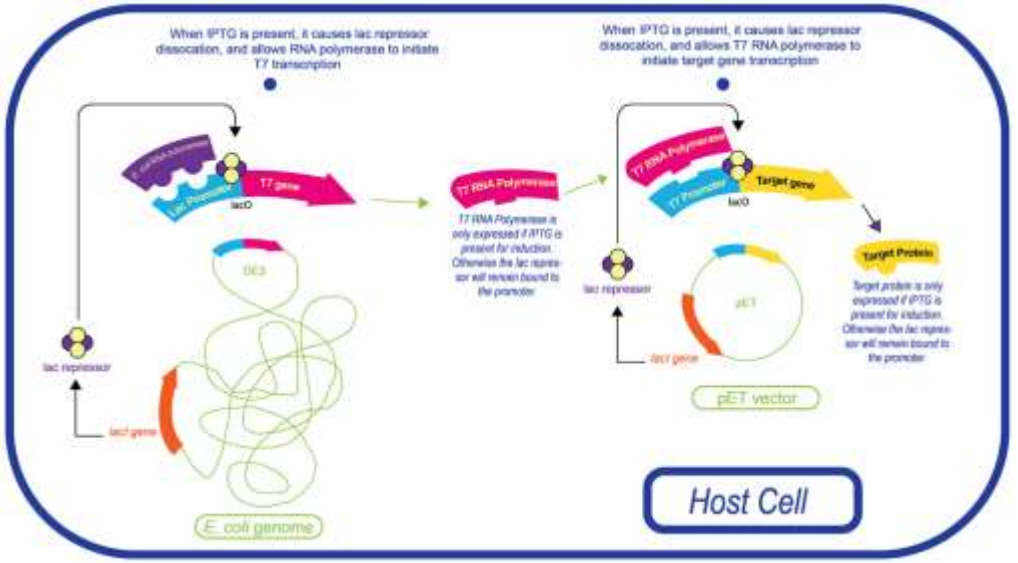
The vector contains the necessary regulatory elements, including a promoter, terminator, and enhancer sequences, that enable the expression of the GOI in the host cell

What is an expression host?

An expression vector is a type of plasmid or viral vector used to express a specific gene of interest (GOI) in a host cell

Why E.coli Dh5 alpha is not an expression host?

It contains natural protease which will make it unstable for recombinant protein production and it does not contain T7 RNA polymerase specific for the T7 promoter present in Expression vectors like pET 28



Why *E coli* BL 21 and BL 21 (DE3) Expression Host?

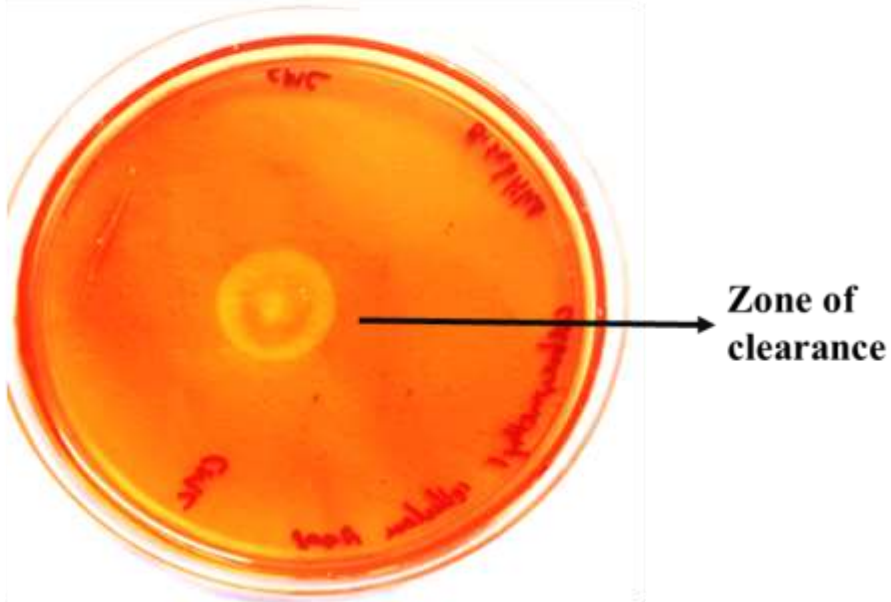
***E coli* BL 21 and BL 21 (DE3) Expression Host :**

Both strains are B strains and thus both are deficient in Lon protease (cytoplasm) and OmpT protease (outer membrane). B strains are generally preferred for recombinant protein expression.

The DE3 designation means that respective strains contain the λDE3 lysogen that carries the gene for T7 RNA polymerase under control of the lacUV5 promoter. IPTG is required to maximally inducing expression of the T7 RNA polymerase in order to express recombinant genes cloned downstream of a T7 promoter. BL21(DE3) is suitable for expression from a T7 or T7-lac promoter or promoters recognized by the *E.coli* RNA polymerase: e.g. lac, tac, trc, ParaBAD, PrhaBAD and also the T5 promoter.

Note that BL21 does not carry the gene for T7 RNA polymerase and thus is only suitable for expression from promoters recognized by the *E.coli* RNA polymerase: e.g. lac, tac, trc, ParaBAD, PrhaBAD and also the T5 promoter.

I. Screening for cellulase Enzyme and Primer Design for cellulose gene



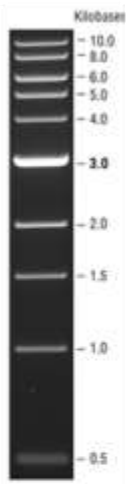
Cellulase Assay of *B. subtilis* grown in CMC agar with Congo-Red

Parameters	Value
DNA Concentration	42ng
Primer Conecntration	0.4 µM
Annealing Temperature	50.7 °C
No of Cycles	30

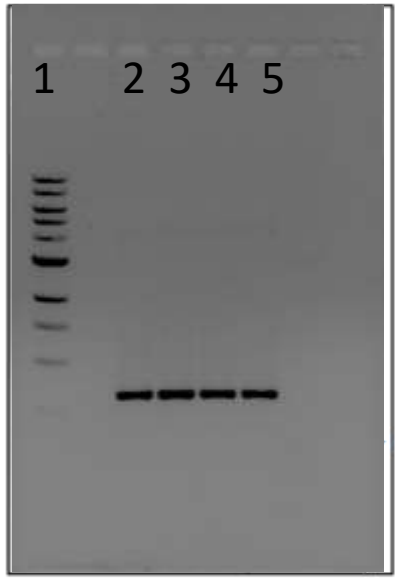
Table1 Optimal Conditions for PCR

Figure 1. Cellulase assay for utilization of CMC substrate (Carobxy Methyl Cellulose) using Congo-red and optimized conditions for amplification of bglS (cellulase) gene by PCR (Table 1).

II. PCR amplification of Cellulase gene and cloning of bglS(Cellulase gene)

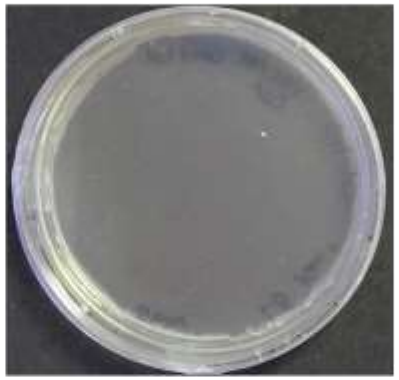


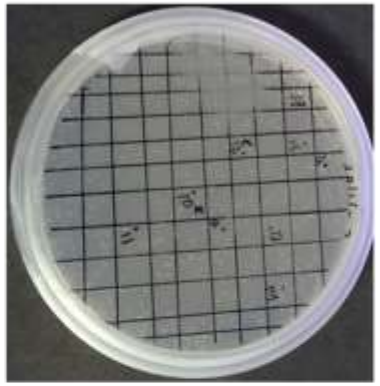
1kb ladder



Amplified bglS gene (0.7kbp)

PCR amplification of bglS from *B. subtilis*.
Lane 1-Marker, Lane(2-5)-gradient annealing temperature (50.1°C,50.7°C,51.8°C,53.7°C)

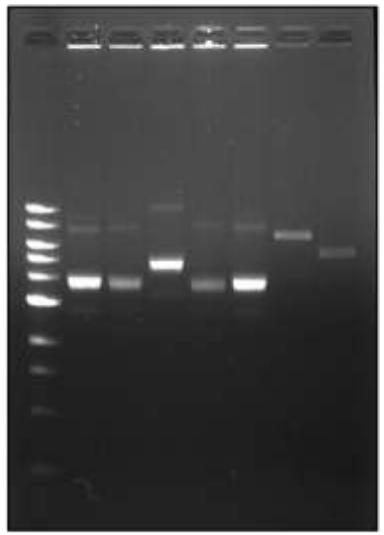


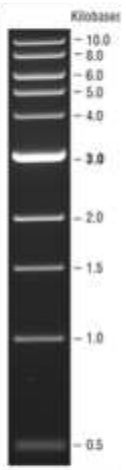


E. coli DH 5α colonies grown on LBA Kanamycin after transformation with pET28a+bglS ligated product (1:5, Insert to vector)

Figure 2.PCR amplification of bglS gene from gDNA of *B. subtilis* gradient annealing temperature

II. Screening of bglS(Cellulase) positive clone and Transformation for BL 21





1kb ladder

B. Lane(1) Marker, Lane(2,3,4,5,6)undigested plasmids screening for mobility difference , Lane 7 pET28a+bglS single digest ,Lane 8 Naked pET 28a single Digest

Marker

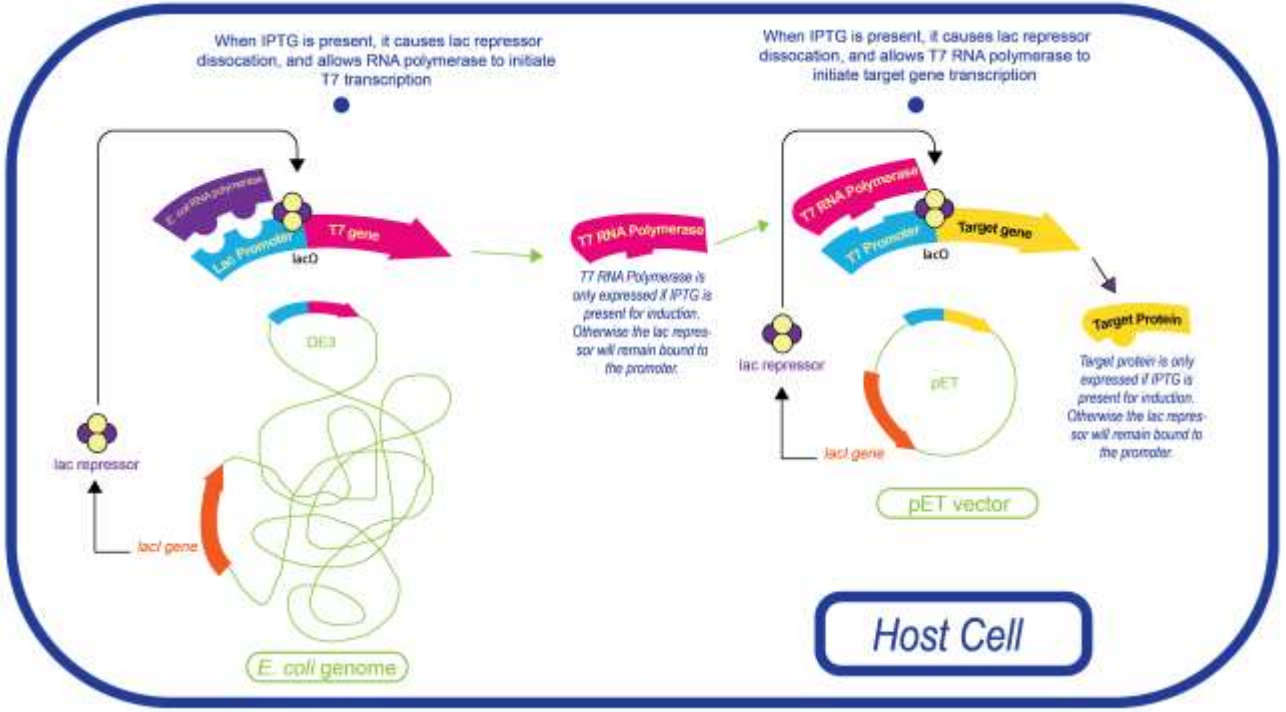
Amplified gene

A. Screening of Positive clones in *E.coli* BL 21 by PCR using bglS gene primer from isolated plasmid

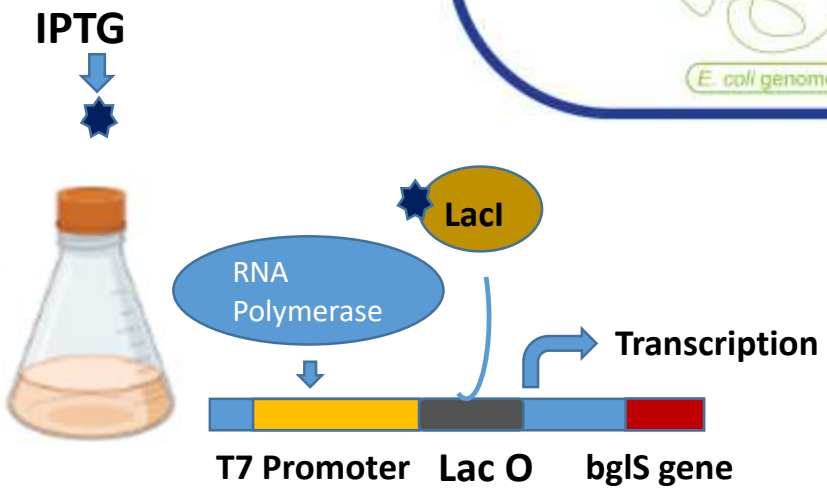
Figure 3. (A) Signal restriction enzyme digest using HindIII and Mobility difference. (B) Screening for Positive clones by PCR using primers of bglS gene and isolated plasmid as DNA template.

Expression of Recombinant gene in BL 21

Escherichia coli BL21 and BL21(DE3) it lack in lon and ompT proteases and it harbors a prophage DE3 derived from a bacteriophage λ, which carries the T7 RNA polymerase gene under the control of the *lac*UV5 promoter.



Transformation of competent *E .coli* BL 21 cells with Ligated product(*pET28a+bglS*) by heat-Shock method

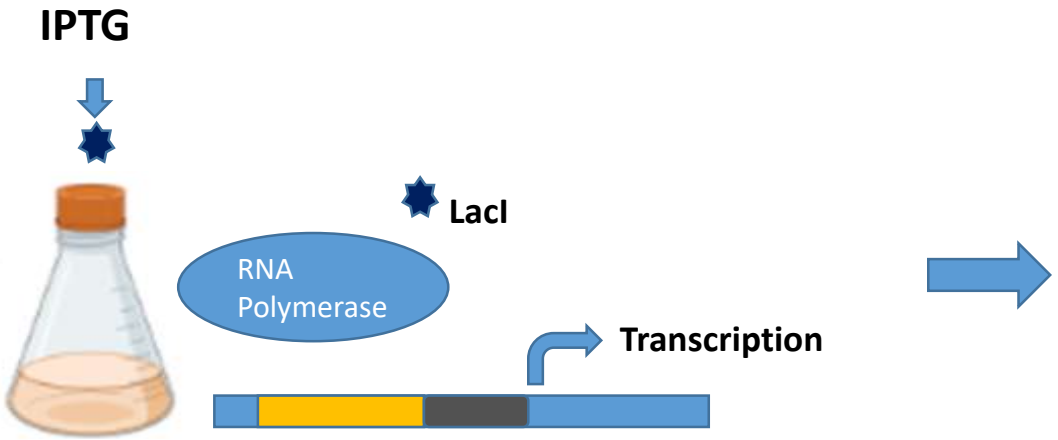


Cellulase(bglS) Expression in LB kanamycin broth, 1mM IPTG , at 37°C with 230 rpm shaking for 4 hrs

Recombinant protein purification

The DNA sequence specifying a string of six to nine histidine residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6xHis or poly-His-tag fused to its N- or C-terminus.

Expressed His-tagged proteins can be purified and detected easily because the string of histidine residues binds to several types of immobilized metal ions, including nickel, cobalt and copper, under specific buffer conditions. In addition, anti-His-tag antibodies are commercially available for use in assay methods involving His-tagged proteins. In either case, the tag provides a means of specifically purifying or detecting the recombinant protein without a protein-specific antibody or probe.



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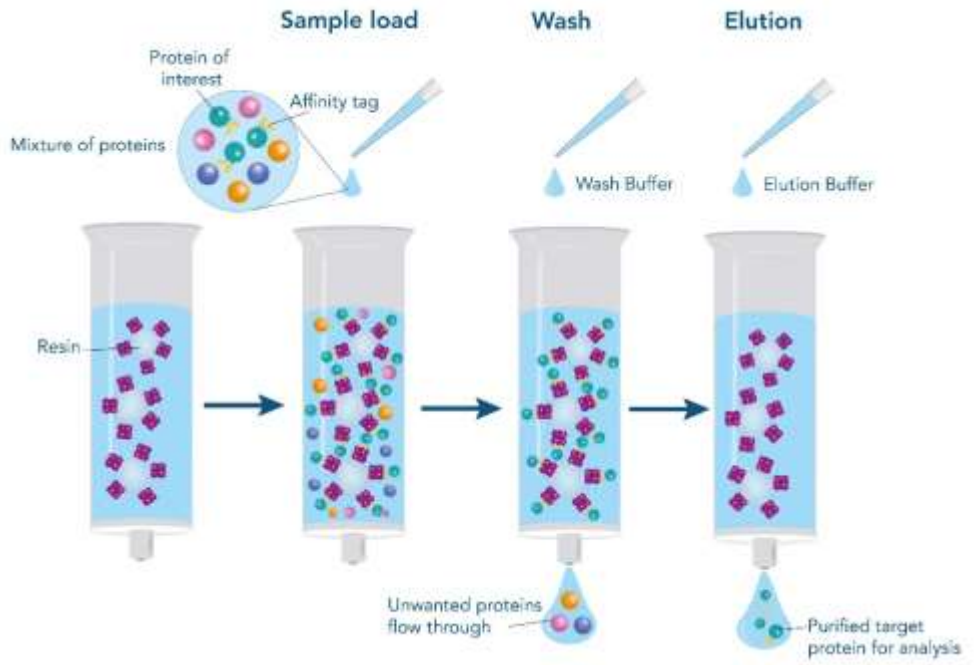
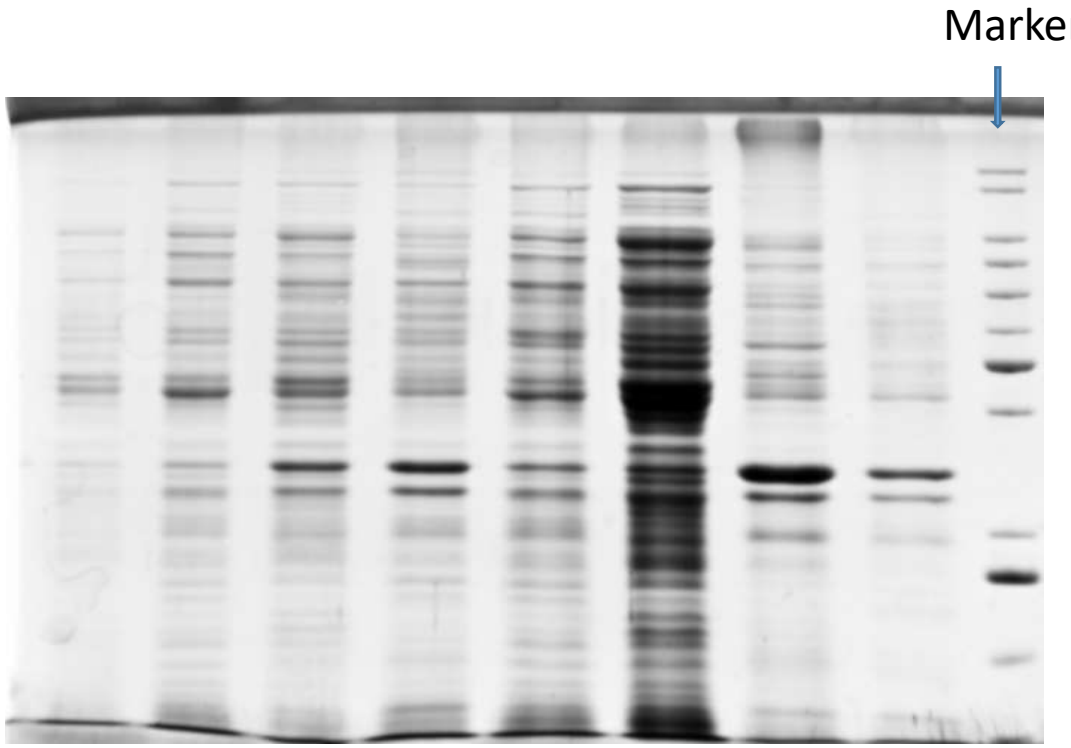


Ni-His Affinity Purification

Protein Expression



Lane 1 : PBS(Soluble control, Lane 2: S 1mM, Lane 3: pellet Control, L4: pellet 1mM,) Imidazole (Lane 5: soluble control, L6: Soluble 1mM IPTG, Lane 7: pellet Control , Lane8: Pellet 1mM IPTG) ,Lane 9: Marker