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UMA CEPA DE *E. COLI* BL21(DE3) ROSETTA GENETICAMENTE MODIFICADA/PET-28A(+): EXPRESSÃO OTIMIZADA DA PULULANASE RECOMBINANTE TIPO I DE *METABACILLUS INDICUS*

A GENETICALLY MODIFIED *E. COLI* BL21(DE3) ROSETTA STRAIN/PET-28A(+): OPTIMIZED EXPRESSION OF RECOMBINANT TYPE I PULLULANASE FROM *METABACILLUS INDICUS*

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RESUMO:

Introdução: A pululanase é comumente utilizada na hidrólise das ligações glicosídicas α-1,6 no amido e no pullulan. O nível de produção da pululanase tipo I ainda não atende à demanda para aplicações industriais a partir de micro-organismos selvagens e sistemas de expressão heterólogos. **Objetivos:** Clonar e expressar o gene da pululanase tipo I de *Metabacillus indicus* LGM 22858 em células *E. coli* BL21(DE3) Rosetta. Em seguida, as condições de expressão para a pululanase recombinante podem ser otimizadas investigando os efeitos de diferentes concentrações de IPTG, vários meios de crescimento e diferentes tempos de indução. Finalmente, deve-se determinar o tempo de incubação ideal para o ensaio da enzima pululanase. **Métodos:** Foram investigadas diferentes concentrações do indutor IPTG: 0,3, 0,5, 0,7 e 1 mM. Foi estudado o efeito do uso de vários meios de produção (por exemplo, *LB*, *TB*, *2xTY*, *M9*, *5xLB* e *SB*). A influência do tempo de indução também foi testada com 8, 20 e 36 h de indução. Além disso, foi testado o tempo de incubação ideal para o ensaio enzimático: 5, 10, 15, 20, 25 e 30 min. **Resultados:** O nível máximo de atividade Pull 2,55±0,18 U/mg foi alcançado com 0,3 mM de IPTG. O LB foi o meio de crescimento mais apropriado, com 0,96 ± 0,11 U/mg. A análise por SDS-PAGE revelou os mesmos resultados. O melhor tempo de indução foi alcançado após 20 h de indução 1,08±0,02 U/mg. O tempo de incubação ideal foi de 5 min para obter 11,57±0,37 U/mL. **Discussão:** O nível preliminar de atividade da pululanase obtido foi submetido a uma estratégia de otimização para alcançar as condições ideais que favorecessem o nível máximo de atividade da pululanase. **Conclusões:** Os resultados sustentam a pululanase tipo I recombinante de *Metabacillus indicus*, que foi superexpressa em células *E. coli* BL21(DE3) Rosetta.

Palavras-chave: *E. coli BL21(DE3); Expressão otimizada; Pululanase tipo I; Metabacillus indicus.*

ABSTRACT:

Background: Pullulanase is commonly utilized when hydrolyzing the α-1,6 glycosidic bonds in starch and pullulan. The production level of pullulanase type I is still not meeting the demand for industrial applications from wild-type microorganisms and heterologous expression systems. **Aims:** To clone and express the type I pullulanase gene from *Metabacillus indicus* LGM 22858 in *E. coli* BL21(DE3) Rosetta cells. Then, the expression conditions for recombinant pullulanase can be optimized by investigating the effects of different IPTG concentrations, various growth media, and different induction times. Finally, the optimal incubation time for the pullulanase enzyme assay must be determined. **Methods:** Different concentrations of the induction of the inducer IPTG were investigated:0.3, 0.5, 0.7, and 1 mM. The effect of using various production media (e.g., *LB*, *TB*, *2xTY*, *M9*, *5xLB*, and *SB* was studied. The influence of induction time was also tried 8, 20, and 36 h of induction. Moreover, the optimal incubation time for enzyme assay was tested: 5, 10, 15, 20, 25 and 30 min. **Results:** The

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ultimate level of Pull activity 2.55±0.18 U/mg was achieved at 0.3 mM IPTG. LB was the most appropriate growth medium, with 0.96 ± 0.11 U/mg. SDS-PAGE analysis revealed the same results. The best induction time was realized after 20 h of induction 1.08±0.02 U/mg. The optimal incubation time was 5 min to realize 11.57±0.37 U/mL. **Discussion:** The preliminary level of attained pullulanase activity was subjected to an optimization strategy to attain the optimal conditions encouraging the ultimate level of pullulanase activity. **Conclusions:** Results underpin the recombinant type I pullulanase of *Metabacillus indicus*, which was overexpressed in E.coli BL21(DE3) Rosetta cells.

Keywords: *E. coli BL21(DE3); Optimized expression; Type I pullulanase; Metabacillus indicus.*

1. INTRODUCTION:

Amylolytic enzymes, or simply amylases, are a group of starch-degrading enzymes that include α -amylases (EC 3.2.1.1), glucoamylases (EC 3.2.1.3), β-amylases (EC 3.2.1.2.), and debranching enzymes (e.g., pullulanases (EC 3.2.1.41)) (Djekrif *et al.*, 2016). This amylolytic complex (i.e., endoamylases, exoamylases, and debranching enzymes) for the degradation of starch is used widely in industry.

Starch is a complex polymer of amylose (a linear chain molecule of α-1,4-linked glucans) and amylopectin (a branched glucose polymer with linear α-1,4-linked glucans branched with α-1,6 linkages). Endoamylases and exoamylases, for the depolymerization of starch into oligosaccharides and smaller sugars, have primary affinity for α-1,4-glucosidic linkages. However, debranching enzymes (e.g., pullulanases) catalyze the hydrolysis of α-1,6-glucosidic bonds in amylopectin, pullulan, and related polymers (Miao *et al.*, 2018).

Amylolytic enzymes can avoid chemical issues and improve cost-effectiveness processes, resulting in their potential use in several industrial applications, including detergency, fermentation, food formulations, pharmaceuticals, paper, biofuels, and textile fields. α -amylases and pullulanases are two of the most common and important types of industrial amylases (Belay *et al.*, 2021; Hammami *et al.*, 2018).

Microorganisms are considered robust cell factories for the production of amylases and pullulanases. In this context, microbial cells offer numerous advantages over ortholog producers from plants and animals; of paramount importance are rapid growth, great biochemical diversity, regular availability, and ease of genetic manipulation (with a likelihood of increased production, enzyme excretion, and modified performance characteristics) (Farias *et al.*, 2021). Nevertheless, wild-type microorganisms normally produce amylolytic enzymes at low levels, which are not enough to meet the demand for biotechnological applications. Molecular cloning of the corresponding genes and their overexpression in heterologous hosts is a possible approach to circumvent this problem (Hii *et al.*, 2012b). Moreover, novel and putative amylases/pullulanases DNA sequences of microbial origin published recently in the DNA Databases have addressed the indispensable need to study these genes encoding amylolytic enzymes in the lab aiming to define candidate amylolytic enzymes with industrially promising properties (Reddy *et al.*, 2017).

In the light of fulfilling enzyme market demands from amylases and/or pullulanases, the discovery of such novel enzymes with robust characteristics, and enhancing their yield from the producer strains, the present study is an attempt to overexpress recombinant type I pullulanase of bacterial origin in the genetically modified *E.coli*.

2. MATERIALS AND METHODS:

2.1. Materials, Equipment, and reagents used in this research:

2.1.1. Bacterial strains and plasmids:

- *E. coli* BL21 (DE3) Rosetta strain
- pET-28a(+) vector with *pulL* gene insert (pET-28a(+)/*pulL* construct).

2.1.2. Growth media:

- Luria-Bertani (LB) broth and agar
- Terrific Broth (TB)
- Two Times Tryptone Yeast medium (2xTY)
- Minimal Salt (M9) medium
- Five Times Luria Bertani broth (5xLB)
- Super Broth (SB)

2.1.3. Reagents:

- Kanamycin
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)

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- Calcium chloride (CaCl2)
- Glycerol
- Tris-HCl buffer
- Sodium chloride (NaCl)
- Lysozyme
- Pullulan substrate
- 3,5-Dinitrosalicylic acid (DNS) reagent
- D-glucose standard

2.1.4. Enzymes:

- HindIII and XhoI restriction enzymes

2.1.5. Equipment:

- Incubator shaker
- UV-Visible spectrophotometer
- Microcentrifuge
- Sonicator
- Agarose gel electrophoresis apparatus
- UV transilluminator
- SDS-PAGE apparatus
- Water bath
- PCR thermocycler

2.1.6. Other materials:

- PCR primers (T7 promoter and terminator)
- DNA ladder
- Protein ladder
- Eppendorf tubes
- Micropipettes and tips
- Erlenmeyer flasks
- Petri dishes
- Glass beads

2.2. Methods

2.2.1. Bacterial strains and expression vector

Escherichia coli BL21 (DE3) Rosetta (Promega Co., USA) was utilized as the cloning and expression host.

The open reading frame (ORF) encoding the pullulanase type I gene from *Metabacillus indicus* LMG 22,858, spanning c1647736-1649868 in the whole genome with the accession number JGVU02000002.1, was retrieved from GenBank database.

The retrieved nucleotide sequence of the pullulanase type I gene (2133 bp) was chemically synthesized by GenScript Biotech®. Co., USA (U495WHA200-2) using the Clone EZ (Ligation independent cloning (LIC) method) and cloned on the expression vector pET-28a (+) Figure 1. The newly synthesized construct was designated pET-28a (+)/*Pull* (Al-Mamoori *et al*., 2023).

2.2.2. Confirming the presence of pullulanase open reading frame on pET-28a (+)/pulL

The lyophilized construct pET-28a (+)/*pulL* was dissolved in 20 µL nuclease-free water. Then, proving the existence of the pullulanase (pulL) open reading frame on the pET-28a (+)/pulL construct was done by conducting two experiments: PCR using the universal primer set T7 promoter/T7 terminator of the universal pET-28 a (+) vector and double restriction digestion of the plasmid construct using XohI and HindIII.

2.2.2.1 PCR using T7 promoter/T7 terminator primer set

The pullulanase open reading frame harbored on the pET-28a (+)/pulL construct was amplified using the universal primer set of the vector T7 promoter/T7 terminator. Briefly, the PCR reaction mixture and the PCR conditions were performed according to the recipe settled in Table 1. After PCR termination, an aliquot of the expected PCR product was checked by running it on 1% (w/v) agarose gel electrophoresis. The PCR product was visualized on a UV-transilluminator (Cleaver Scientific CO., UK).

2.2.2.2 Double restriction digestion using XhoI and HindIII

The pET-28a (+)/pulL construct was digested by the double restriction enzymes XohI and HindIII. The recipe for the restriction digestion reaction is displayed in Table 2. The restriction digestion reaction was incubated overnight at 37 °C in a water bath (Raypa, Spain).

After completion of the reaction, the digestion pattern was visualized using 1% (w/v) agarose gel electrophoresis. However, the expected DNA fragments (5.469 Kbp and 1.737 Kbp) resulting from the enzymatic digestion of the pET-28a (+)/pulL construct were visualized under a UV-transilluminator.

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Table 2. *Recipe of restriction digestion of pET-28a (+)/pulL construct by HindIII and XhoI*

Reaction component	Volume (µL)
pET-28a (+)/*pulL* construct:	3.0
HindIII (20 U/ µL):	2.0
XohI (20 U/ µL):	2.0
CutSmart Buffer (10X)®:	2.5
Nuclease-free water:	15.5
Total volume:	25.0

2.2.3. Preparation of E. coli BL21 (DE3) Rosetta chemically competent cells

The competent cells of *E. coli* BL21 (DE3) Rosetta were prepared according to a previously reported protocol (Sambrook *et al.*, 1989) using $CaCl_2$.

Concisely, 100 mL of LB in a 250 mL Erlenmeyer flask was inoculated with 1 mL of an overnight *E. coli* Rosetta strain seed culture. The inoculated broth was incubated at 37 °C for 2-3 h with an agitation speed of 200 rpm (Brunswick Incubator Shaker, USA) until the optical density of the culture reached 0.2-0.4 at 600 nm (UV-Visible Spectrophotometer, Shmidizu, Japan).

Then, the growth of the bacterial strain was halted by putting the culture on ice for 30 min. Cells were harvested by centrifugation at a microcentrifuge (Hettich MIKRO 120, Germany) at 4,500 rpm for 20 min at 4 °C. The bacterial pellet was suspended further in 20 mL of cold 0.1 M $CaCl_2$ and was retained on ice for 30 min.

The bacterial cells were re-harvested at 4,500 rpm by centrifugation at 4 °C for 20 min. After that, bacterial cells were suspended in 2 mL of cold 0.1 M $CaCl_2$. This bacterial suspension is normally designated as "chemically competent *E. coli* cells" at this step.

Long-term storage of the chemically prepared competent *E. coli* cells was achieved by dispensing in Eppendorf tubes; each tube harbored 100 µL of the bacterial suspension and 100 µL of 100% (v/v) glycerol. Lastly, the Eppendorf tubes containing the glycerol stock of the competent (*E. coli*) cells were kept at -80 °C.

2.2.4. Transformation of E. coli BL21 (DE3) Rosetta chemically competent cells

The chemically competent *E. coli* cells, dispensed in an Eppendorf tube (200 µL), were mixed with 2-3 µL (50 ng) of pET-28a (+)/pulL.

The mixture was retained for 40 min on ice. After that, the cells were subjected to heat shock for 45 sec at 42 °C in a water bath, then immediately immersing the Eppendorf tube for 5 min in ice. (800 µL) LB broth was added to the Eppendorf tube with further incubation at 37 °C for 1.5 h at 180 rpm.

After the termination of the incubation time, the 1 mL culture was spread on the surface of LB agar plates containing kanamycin (34 µg/mL) as a selectable marker using sterilized glass beads.

Then, the inoculated agar plates were incubated at 37 °C in a static incubator (JSR, Korea) for 24 h.

2.2.5. DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed following a previously reported procedure (Sambrook *et al.*, 1989).

2.2.6. induction and expression of recombinant pullulanase (pulL)

Two Milliliters of a seed culture (16 h) of the recombinant *E. coli* BL21 (DE3) Rosetta strain harboring the recombinant construct pET-28a (+)/pulL were used to inoculate 200 mL of LB in a 500 mL Erlenmeyer flask supplemented with a final concentration of 34 µg/mL of kanamycin.

The incubation was conducted to inoculate broth for 3 h at 37 °C with 180 rpm minimum agitation speed. When the optical density (OD) of the culture reached 0.6-0.8 at 600 nm, isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1.0 mM was added. The induced culture was incubated at room temperature at 180 rpm for another 18 h.

After the indicated time, cells were harvested by centrifugation for 20 min (at 4 oC) at 8,000 rpm. Then, resuspended cell pellets were conducted in 5 mL of lysis buffer (50 mg/mL lysozyme, 50 mM Tris-HCl, pH 7.6, and 300 mM NaCl).

A previous technique was applied to break down the induced cells (Abady *et al.*, 2022). Briefly, incubation of the suspended cells was achieved at 37 °C for 30 min with continuous gentle shaking at 100 rpm. After that, sonication of the cell suspension was performed for 8 cycles at

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14.000 Hz; each cycle: 30 sec at (14.000 Hz) with an interval of 1 min on ice between cycles.

Centrifugation was carried out at 10,000 rpm at 4 °C for 15 min to remove cell debris. The clear supernatant was transferred to new Eppendorf tubes. The pullulanase activity was assayed in the clear supernatant fraction. Both soluble and insoluble fractions were kept at -20 °C for further processing.

2.2.7. Optimized production of recombinant Pull

The production of the recombinantly expressed *Pull* was optimized through a four-step optimization strategy. The three experiments included in the optimization of expressed *Pull* were the effect of induction temperature, different concentrations of the inducer IPTG, using different growth media, and induction time.

2.2.7.1 Effect of induction temperature

An overnight seed culture of the recombinant *E. coli* BL21(DE3) Rosetta cells harboring the pET-28a (+)/pulL was used to inoculate three LB broths, each 100 mL in a 500 mL Erlenmeyer flask. One mL of the overnight seed culture was used to inoculate each broth. The three inoculated broths were incubated at 37 °C at 180 rpm until the optical density of each culture reached 0.6-0.8 at 600 nm.

After that, the inducer IPTG was added to each culture at a final concentration of 1.0 mM. Then, the three cultures were incubated further for 18 h at three different induction temperatures: room temperature, 30 °C, and 37 °C. After 18 h of induction, the harvested cells were broken by sonication, and the Pull activity was performed. The specific activity was determined at each induction temperature.

2.2.7.2 Effect of inducer concentration

Four cultures of recombinant *E. coli* BL21(DE3) Rosetta cells harboring the pET-28a (+)/*pulL* were prepared as mentioned above until the optical density of the culture reached 0.6-0.8 at 600 nm. Then, the IPTG as the inducer was added to the four cultures at four tested concentrations:0.3, 0.5, 0.7, and 1.0 mM. The induction and cell breakage were performed as mentioned above, and the enzyme assay was carried out. The specific activity was determined at each inducer concentration.

2.2.7.3 effect of induction time

Three cultures were prepared, as mentioned in the section on induction and expression of the recombinant enzyme. When the IPTG was added as an inducer, one culture was further incubated for 8 h post-induction, one culture was further incubated for 20 h post-induction, and the last culture was further incubated for 36 h post-induction. The bacterial cells were harvested from the three cultures after 8, 20, and 36 h post-induction. The harvested bacterial pellets were processed as mentioned above. The *Pull* activity was estimated at each induction time, and the specific activity was determined at each induction time.

2.2.7.4 effect of growth media

The following growth media were tried to assess their effects towards an enhanced level of Pull expression: LB, TB, SB, 2XTY, 5XLB, and M9. The induction of *Pull* from the recombinant *E. coli* BL21 (DE3) Rosetta strain / pET-28a (+)/pulL in the six cultures was conducted. Twenty hours post-induction, unless otherwise stated, bacterial cells were harvested from each culture, and the bacterial pellets were subjected to cell breakage by sonication, as mentioned above. The activity of the *Pull* was measured. Additionally, the specific activity was determined for each growth media.

2.2.8. Pullulanase assay

The pullulanase assay was carried out according to a procedure previously reported with a slight modification (Wang *et al.*, 1997). The D-glucose standard curve is shown in Figure 2. One unit of pullulanase activity is defined as the amount of enzyme that catalyzes the release of 1 µmole D-glucose from pullulan per minute at 40 °C.

2.2.9 Localization of the recombination Pull

The *Pull* activity was checked in two fractions of the cells: the cell-free supernatant (CFS) and the cytoplasmic fraction. An induced culture of recombinant *E. coli* Rosetta cells harboring the recombinant construct pET-28a (+)/pulL was prepared as mentioned above in section induction and expression of the recombinant enzyme. After 20 h of induction, the bacterial pellets were harvested by centrifugation at 10.000 rpm for 5 min. The cell-free supernatant was checked for Pull activity. The bacterial pellets were subjected to sonication, as mentioned above. The activity of *Pull* was checked in the

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crude soluble *fraction* (cytoplasmic fraction) obtained after centrifugation.

2.2.10 Effect of incubation time

The effect of incubation time on Pull activity was determined. Pull activity was assayed as mentioned above using pullulan as the substrate at different incubation times of 5, 10, 15, 20, 25, and 30 min.

2.2.11 Prediction of Quaternary Structure

Using the online program Expasy was conducive to predicting the primary structure of PulL, which is localized on the server (https://web.expasy.org/translate/).

The server (https://swissmodel.expasy.org/) was used to predict the quaternary structure of PulL using the online program SWISS_MODEL.

2.2.12 Statistical analysis

Statistical analysis was conducted according to the overlapping rules for SE (standard error) bars stated by Cumming *et al.* (2007).

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1 Validation of pET-28a (+)/pulL construct

The pET-28a (+)/pulL construct, synthesized by GenScript Co., was validated by two approaches: restriction double digestion by XohI/ HindIII and PCR amplification of the pulL insert using a set of universal primer (T7 promoter/T7 terminator). The pET-28a (+)/pulL construct with two conformational forms is illustrated in Figure 3. The pulL insert (2312 bp) amplified by PCR using the universal primer set T7 promoter/T7 terminator is shown in Figure 4. The banding pattern of the DNA fragments 5469 and 1737 bp, derived from double restriction digestion of the pET-28a (+) /pulL construct with XohI/HindIII, was illustrated in Figure 5.

3.1.2 Recombinant expression of pulL in E. coli BL21 (DE3) Rosetta strain

3.1.2.1 Preliminary expression of recombinant Pull: pilot experiment

The recombinant *Pull* was successfully expressed in 1 mM IPTG-induced *E. coli* BL21 (DE3) Rosetta cells after 18 h of induction at 37 °C. The attained activity level was 0.96± 0.11 U/mg (Figure 6). This preliminary level of attained pullulanase activity was subjected to an optimization strategy to attain the optimal conditions encouraging the ultimate level of pullulanase activity. Moreover, the crude soluble protein fraction of the uninduced recombinant *E. coli* cells showed no Pull activity.

3.1.2.2 Localization of Pull in the Recombinant E. coli cells

The cell-free supernatant of the induced *E. coli* cells displayed no Pull activity (Table 3). However, the crude soluble protein fraction (cytoplasmic fraction) of the induced *E. coli* cells exhibited appreciable levels of *Pull* (15 U/mL), as demonstrated in Table 3.

Table 3. *Distribution of Pull in the recombinant induced E. coli cells*

Cell fraction	Pull activity (U/mL)
Cell-free supernatant (extracellular fraction)	0.000
Cytoplasmic fraction (crude soluble proteins fraction)	15.00±0.21

3.1.3 Optimizing the level of Pull activity

3.1.3.1 Effect of using different growth media

The effect of different growth media (LB, TB, SB, 2XTY,5XLB, and M9) on the level of recombinant *Pull* was studied, as shown in Figures 7 and Table 4. The cultivation conditions were 37 °C, 1 mM IPTG, and an 18 h induction. As shown in Table 4, LB was found to be the most appropriate growth medium, favoring the highest level of *Pull* (0.96 ± 0.11 U/mg) when compared to the activity levels of *Pull* attained upon using the other growth media, SB, 2XTY,5XLB, and M9. Similarly, SDS-PAGE analysis revealed that LB was the most proper growth medium, triggering the highest levels of Pull activity.

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Table 4. *Levels of Pull expressed in recombinant E. coli cells in different growth media.*

Growth medium	Pull specific activity (U/mg)
LB	0.96± 0.11
TB	0.14 ± 0.01
SB	0.10 ± 0.02
2XTY	0.03 ± 0.00
5XLB	0.13 ± 0.01
M9	0.00 ± 0.00
30	2.03±0.13
37	1.67±0.03

Values are the mean of three readings ±SE.

3.1.3.2 effect of induction time

The influence of induction time on the level of the recombinantly expressed Pull in induced recombinant *E. coli* cells was tested by using three induction times: 8, 20, and 36 h. The induction conditions were LB growth medium, 37 ˚C, and 1 mM IPTG. Data revealed that the best induction time was realized after 20 h of induction (Table 5 and Figure 8), with appreciable levels of Pull of 1.08±0.02 U/mg.

Table 5. *Levels of Pull activity in three induced cultures for three different induction times (8, 20, and 36 h)*

Induction time (h)	Pull specific activity (U/mg)
8	0.96±0.04
20	1.08±0.02
36	0.9±0.00

3.1.3.3 Effect of Induction temperature

Table 6 displayed the effect of using different induction temperatures: room temperature, 30 °C, and 37 °C on the level of Pull from the IPTG-induced recombinant *E. coli* BL21 (DE3) Rosetta cells. The induction conditions were LB as a growth medium, 1 mM IPTG, and a 20 h induction. The highest significant level of Pull activity (2.08±0.09 U/mg) was realized at room temperature when compared to the other levels attained using 30 °C and 37 °C as the induction temperatures. Moreover, the crude cell lysate of the induced recombinant *E. coli* BL21 (DE3) Rosetta cells was analyzed through SDS-PAGE, as shown in Figure 9. No significant increase in the level of the recombinantly expressed Pull was noticed on SDS-PAGE upon using the three tested induction temperatures: room temperature, 30 °C, and 37 °C.

Table 6. *Levels of Pull activity in three induced cultures under three induction temperatures (room temp., 30 °C, and 37 °C)*

Induction temp. (ºC)	Pull specific activity (U/mg)
Room temperature	2.08±0.09
30	2.03±0.13
37	1.67±0.03

3.1.3.4 effect of inducer

The effect of using different IPTG concentrations (0.3, 0.5, 0.7, and 1 mM) on the level of recombinantly expressed Pull is shown in Table 7. The induction conditions were LB as a growth medium, induction at room temperature, and a 20 h induction. The ultimate level of Pull activity (2.55±0.18 U/mg) was achieved when the recombinant *E. coli* cells were induced at 0.3 mM IPTG when compared to the Pull levels obtained at 0.5, 0.7, and 1 mM IPTG. Conversely, analysis of the crude cell lysate of the recombinant *E. coli* cells induced at the four tested IPTG concentrations on SDS-PAGE (Figure 10) did not reveal any significant differences in the yield of Pull as a protein.

Table 7. *Levels of Pull in four cultures of recombinant E. coli cells induced at four IPTG concentrations (0.3, 0.5, 0.7, and 1 mM)*

IPTG (mM)	Pull specific activity (U/mg)
0.3	2.55±0.18
0.5	2.35±0.05
0.7	1.38±0.01
1.0	1.33±0.01

3.1.5 Optimal incubation time

The optimal incubation time for attaining the ultimate Pull activity was studied through different incubation times (5-30 min) (Figure 11). Data revealed that the optimal incubation time was 5 min to realize 11.57±0.37 U/mL. A significant

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reduction in PulI activity was noticed when the incubation time was extended beyond 5 min.

3.1.6 PulI amino acid sequence: Primary structure

The translated nucleotide sequence of PulI (2133 bp) had a protein sequence of 710 amino acid residues, as depicted in Figure 12. An analysis of the amino acid sequence in its linear form (primary structure) by Signal IP 6.0 revealed that it lacked a signal peptide.

3.1.7 Multimerization Status of PulI

SWISS_MODEL predicted the quaternary structure of PulI. The predicted quaternary structure indicated its multimerization status in the monomeric form (Figure 13).

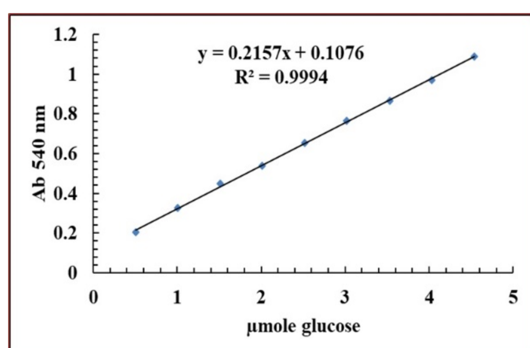

Figure 2. The *D-glucose standard curve was established using the DNS reagent (Miller, 1959).*

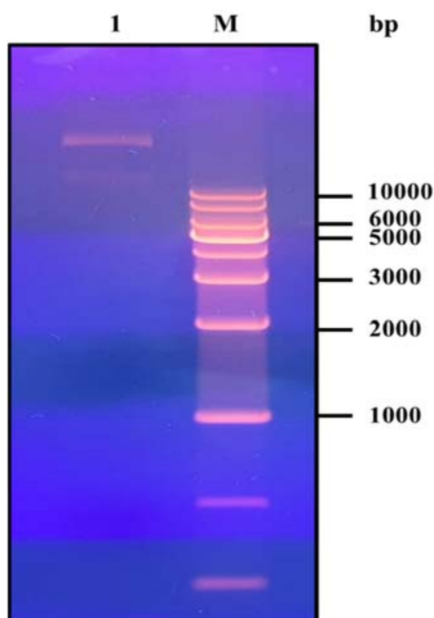

Figure 3. *1% agarose gel electrophoresis showing two conformational forms of the pET-28a (+)/pulL construct. Lane M: 1 kbp DNA ladder. Lane 2: pET-28a (+)/pulL construct.*

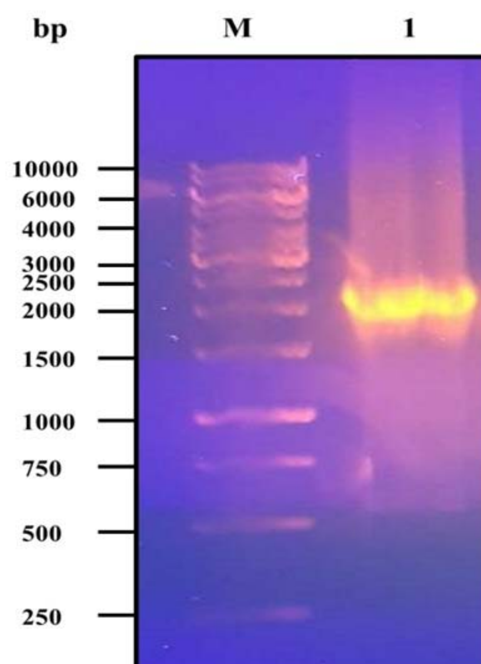

Figure 4. *1% agarose gel electrophoresis illustrating PCR amplification of the pulL insert by PCR using a set of universal primers (T7 promoter and T7 terminator). Lane M: 1 kbp DNA ladder. Lane 2: pulL insert of 2312 bp.*

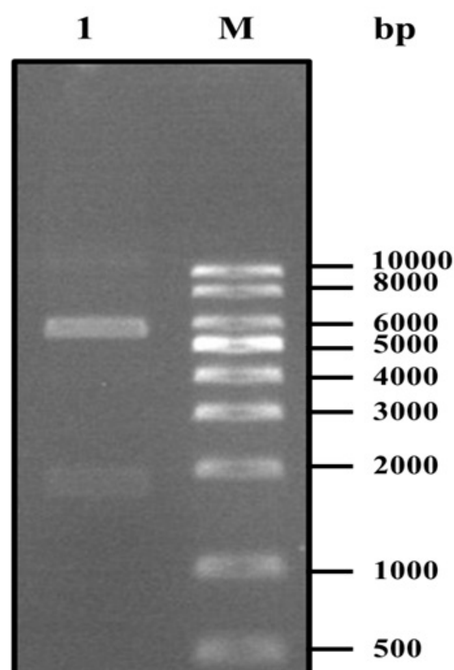

Figure 5. *1% agarose gel electrophoresis showing double restriction digestion of the pET-28a (+)/pulL construct with XohI and HindIII. Lane M: 1 Kbp DNA ladder. Lane 1: cut pET-28a (+)/pulL construct cut with XohI/HindIII.*

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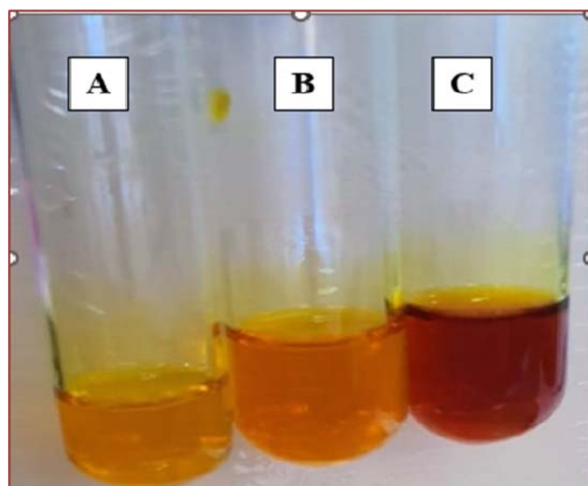

Figure 6. *Pullulanase assay of Pull using DNS reagent is used to estimate the reduction of sugars from pullulan as the substrate. A: Blank, B: control: as a test, but DNS reagent was added prior to enzyme addition, and C: test.*

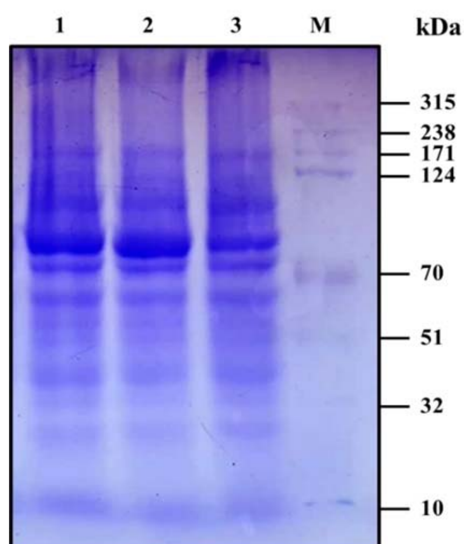

Figure 8. *12% SDS-PAGE showing the expressed Pull using three induction times. Lanes 1-3: soluble crude cell lysate from induced recombinant E. coli cells, 8, 20, and 36 h post-induction, respectively. Lane M: Protein ladder.*

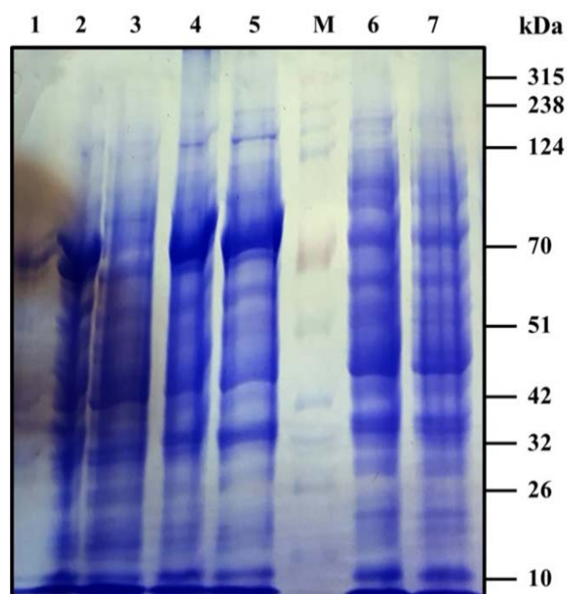

Figure 9. *12% SDS-PAGE showing crude cell lysate proteins of recombinant E. coli cells induced under three different induction temperatures. Lane M: Protein Ladder. Lanes 1, 4, 6: soluble proteins of crude cell lysate of recombinant E. coli cells induced at 30 °C, room temperature, and 37 °C, respectively. Lanes 2, 5, 7: insoluble proteins of crude cell lysate of recombinant E. coli cells induced at 30 °C, room temperature, and 37 °C, respectively. Lane 3: soluble proteins of crude cell lysate of uninduced recombinant E. coli cells.*

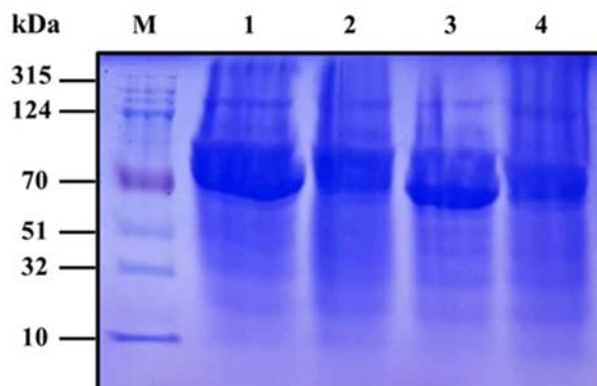

Figure 10. *12% SDS-PAGE showing the levels of recombinantly expressed Pull in recombinant E. coli cells induced at four IPTG concentrations. Lane M: Protein Ladder. Lanes 1-4: soluble crude cell lysate of recombinant E. coli cells induced at four IPTG concentrations of 0.3, 0.5, 0.7, and 1.0 mM, respectively.*

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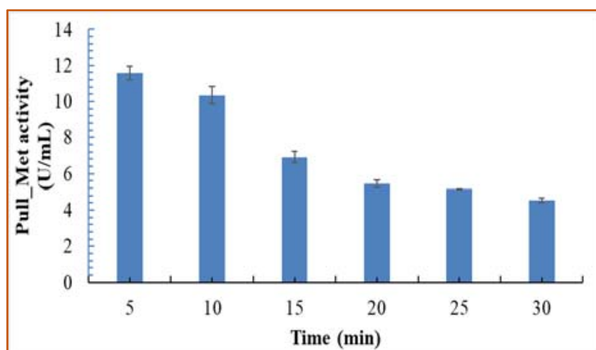

Figure 11. *Effect of incubation time in Pull assay. Values are the mean of three readings ±SE.*

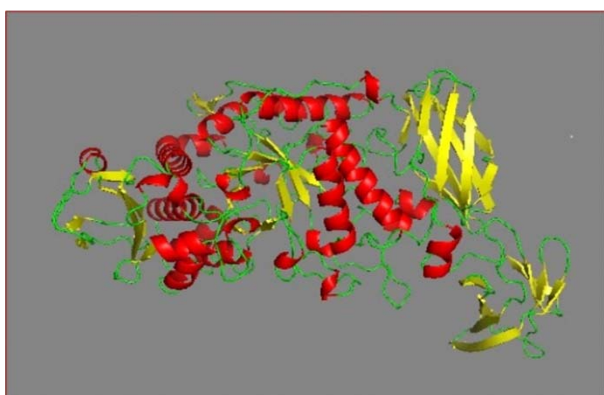

Figure 13. *The predicted quaternary structure of Pull, generated by SWISS-MODEL, indicated its multimerization in the monomeric form.*

3.2. Discussion

The debranching enzyme pullulanase is the most significant biocatalyst for industrial applications. Such enzymes with unique features (e.g., cold-adeptness, detergent stability, organic solvents stability, metal ions stability) are increasingly in demand to fit into the rigorous industrial processes with hard conditions (Naik *et al.*, 2023; Sharma *et al.*, 2021).

Despite pullulanase enzymes being available from wild-type bacteria, they face significant obstacles to commercialization and industrialization: multistep purification protocols, dramatically low productivity, and possible hazards inherent in handling pathogenic pullulanase producers (Hii *et al.*, 2012a; Singh *et al.*, 2022). Typically, the cloning and expression of pullulanase using the heterologous host *E. coli*, a species generally regarded as safe (GRAS), is considered the gold standard solution for avoiding these obstacles.

Recently, great attention has been paid to the cloning and expression of pullulanase from a wide range of bacteria (Bertoldo *et al.*, 1999; Erden-Karaoğlan *et al.*, 2019; Messaoud *et al.*, 2002; Sun *et al.*, 2011), especially after the publish a comprehensive workflow for microbial genome sequencing by the NCBI. Nevertheless, wild-type producers are still considered a venture in its infancy to the cloning of pullulanase-encoding genes. In addition, tracing novel pullulanase to cope with the demands of the worldwide enzyme market has become an urgent necessity. A thorough, in-depth survey review of the literature revealed that pullulanases from the genus Metabacillus had not been discovered yet in either their native or recombinant form (Al-Mamoori *et al.*, 2023). This addressed the crucial need to reveal the characteristics of pullulanases from such a new genus, namely Metabacillus. In this light, the current study was the first to proceed with the cloning, optimized expression, and in silico molecular characterization of recombinant type I pullulanase from *Metabacillus indicus* LGM 22858 in *E.coli* BL21(DE3) Rosetta cell for the first time ever.

The full-length pullulanase type I ORF from *M. indicus* was successfully cloned, consisting of 2133 bp, with 710 deduced amino acid residues; the cloned gene was designated pulL, then its nucleotide sequence was consigned in GenBank under the accession number OP585545.1. As well, the pET-28a (+)/pulL construct was validated by two approaches: restriction double digestion by XohI/ HindIII and PCR amplification of the pulL insert using a set of universal primer (T7 promoter and T7 terminator), Recombinant systems harboring the *M. indicus* pullulanase gene pulL were constructed with T7 promoter. The successful expression of pullulanase type I with lac operator regulation provides an effectual way to achieve enhancement of expression stability and, therefore, high-level production of the target protein in recombinant *E. coli* (Nie *et al.*, 2013; Zhang *et al.*, 2023).

Heterologous expression in the *E. coli* microbial cell factory is one of the well-proven strategies for producing recombinant proteins on a small and large scale because of the various advantages that *E. coli* offers like easy growth, cost-effective industrial production, a short life cycle, and ease of manipulation (Pouresmaeil & Azizi-Dargahlou, 2023).

A review of the literature reported various strategies for obtaining soluble, properly folded, active protein species, including genetic manipulation of the target and modifying the

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culture conditions (temperature, inducer, and growth rate) (Bhatwa *et al*., 2021).

The recombinant Pull was successfully expressed in (1 mM) IPTG-induced *E. coli* BL21 (DE3) Rosetta cells. This preliminary level of attained pullulanase activity was subjected to an optimization strategy to attain the optimal conditions, encouraging the ultimate level of pullulanase activity. The effect of different growth media (LB, TB, SB, 2XTY, 5XLB, and M9) on the level of recombinant Pull was studied. LB was found to be the most appropriate growth medium, favoring the highest level of Pull (0.96 ± 0.11 U/mg) when compared to the activity levels of Pull attained upon using the other growth media, SB, 2XTY, 5XLB, and M9. Similarly, SDS-PAGE analysis revealed that LB was the most proper growth medium, triggering the highest levels of Pull activity. The potential effects on the cellular physiology of the recombinant *E. coli* (DE3) cells of various media have been determined. Consequently, the nature and the folding status of the expressed recombinant enzymes from these strains are probably affected by the differences in cellular physiology among different *E. coli* (DE3) cells growing on different types of media (Abady *et al*., 2022). Remarkably, in future studies, a further development in pull activity from the recombinant Rosetta strain could likely be achieved by scaling up the process in a laboratory-scale fermenter.

An appreciable enzyme activity of Pull was realized at low induction temperatures (i.e., room temperature). Reduced induction temperatures are reportedly one of the proven strategies to decrease the possibility of inclusion bodies that may form from recombinant proteins (Bhatwa *et al*., 2021; Zalai *et al*., 2020). At most, the hydrophobic interactions that would cause inclusion bodies to form are encouraged at a higher induction temperature of 37 °C (de Groot & Ventura, 2006). Reportedly, repressed T7-based expression systems such as pET-28a(+) reduced inclusion body formation while increasing soluble protein yield at low induction temperatures (Mahmoud *et al*., 2021).In addition, increases the solubility of the recombinant protein at the expense of its yield as a result of low induction temperatures.

In the pET system with the strong T7 promoter and high inducer concentrations, product yields can exceed 50% of the total cytoplasmic proteins; however, this leads to increased misfolding and inclusion body(IB) formation in *E. coli* (Nag *et al*., 2022). IPTG induces T7 RNA polymerase and protein production in *E. coli*. The ultimate level of Pull activity (2.55±0.18 U/mg) was achieved when the recombinant *E. coli* cells were induced at 0.3 mM IPTG when compared to the Pull levels obtained at 0.5, 0.7, and 1 mM IPTG.

A review of the literature reported various strategies for obtaining soluble, properly folded, active protein species, including genetic manipulation of the target and modifying the culture conditions (temperature, inducer, and growth rate) (Bhatwa *et al*., 2021).

Previous findings disagreed with our finding about the concentration of IPTG, stating that using high concentrations of IPTG does increase the yield of recombinant protein at the expense of its solubility (Nag *et al*., 2022). due to the strong affinity of T7 promoter and lac repressor at low IPTG concentration (0.3 mM), pull production during cultivation also occurred slowly, enhancing the solubility of Pull. One potential explanation for this increased pull solubility at low IPTG concentration is its slow production of increased solubility. As a rule, the optimal concentration of IPTG required for obtaining solubilized recombinant protein is variable from one protein to another.

The optimal incubation time for attaining the ultimate Pull activity was determined through different incubation times (5-30 min) (Figure 11). The maximum production of Pull was observed at 5 min of incubation (11.57±0.37 U/mL). A reduction in pull activity was noticed when the incubation time was extended beyond 5 min. This finding is similar to studies carried out by Bertoldo *et al*. (2004), who reported the same trend during the production of pullulanase in Anaerobranca gottschalkii.

As regards the multimerization status (quaternary structure) of Pull, the predicted data derived from SWISS-MODEL was in good agreement with the experimental data derived from native–PAGE (monomeric subunit). Furthermore, the majority of previously reported pullulanases type I (e.g., *Exiguobacterium acetylicum* and *L amylophillus* GV6) (Dakhmouche Djekrif *et al*., 2021; Qiao *et al*., 2015), except for pullulanases from F. pennivorans and Geobacillus thermoleovorans US105 (Kang *et al*., 2011; Zouari Ayadi *et al*., 2008), which have a dimeric structure, and it was in a good agreement with the quaternary structure of *Pull*.

4. CONCLUSIONS

In this study, we obtained a novel pullulanase gene from the strain *Metabacillus indicus*, which was functional overexpressed in *E.*

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coli BL21(DE3) Rosetta cells through induction expression strategy optimization. To the best of our knowledge, this is the first attempt to express recombinant type I pullulanase from the *M. indicus* and to optimize its production for possible scale-up.

The developed strategy might help future large-scale production of pullulanase and efficient production of other recombinant proteins with engineered *E. coli*.

5. DECLARATIONS

5.1. Study Limitations

5.1.1. Experimental Variables: The study investigates various factors affecting pullulanase expression, such as different induction concentrations, growth media, and incubation times. However, additional variables or conditions could not be explored, which might impact the enzyme's activity levels.

5.1.2. Generalizability: The findings are specific to this study's experimental conditions and methodologies. The generalizability of the results to different strains, expression systems, or environmental conditions may be limited.

5.2. Acknowledgements

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5.3. Funding source

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5.4. Competing Interests

The authors have no conflict of interest related to this publication.

5.5. Open Access

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6. HUMAN AND ANIMAL-RELATED STUDIES

6.1. Ethical Approval

The study protocol, subject information, and consent form were reviewed and approved by a local Ethics Committee according to document number 001CSE, dated May 14, 2024, to get this approval..

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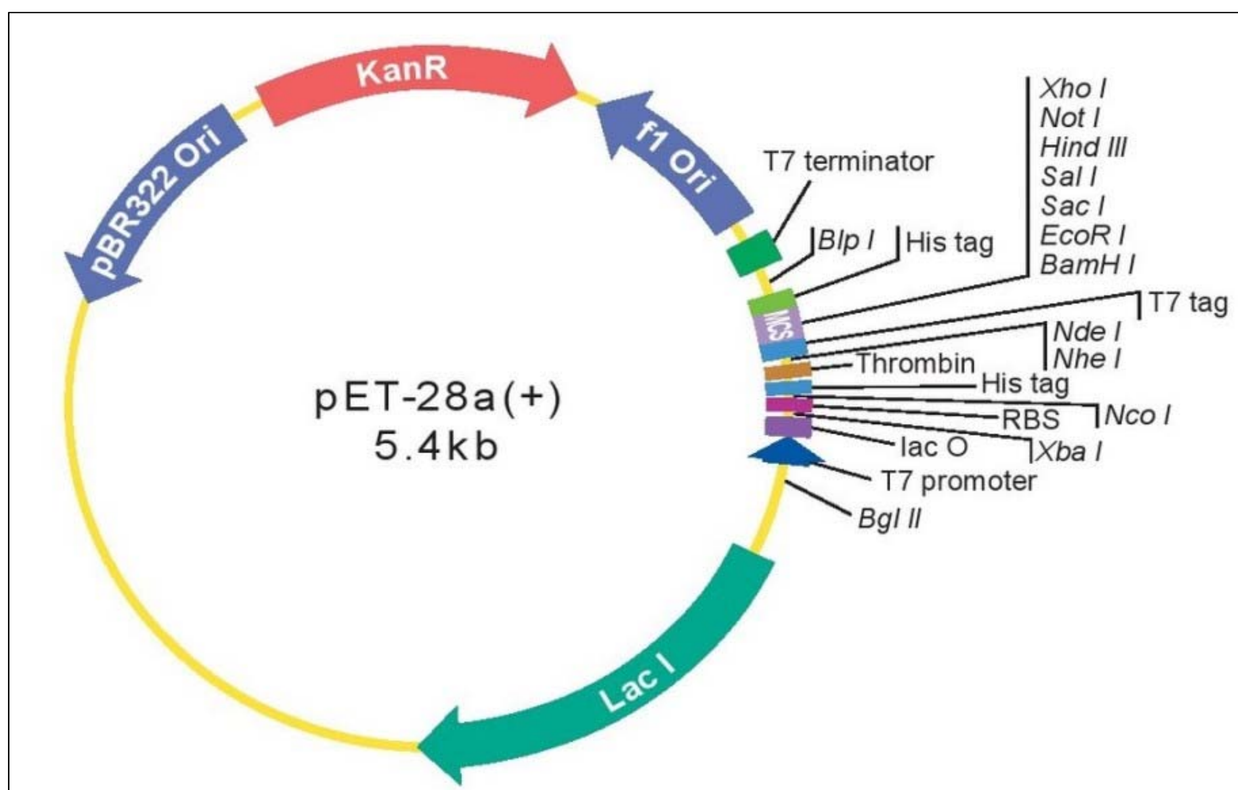

Figure 1. *The map of pET-28a (+) expression vector.*

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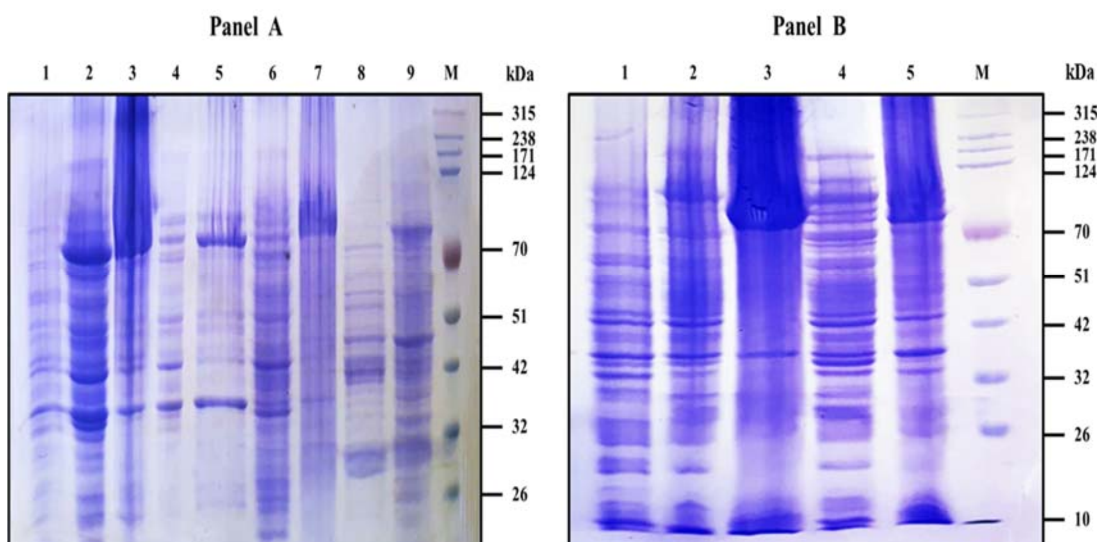

Figure 7. *12% SDS-PAGE showing the effect of using different growth media on the levels of Pull activity. Panel A: Lane M: Protein ladder. Lane 1: uninduced. Lanes 2, 4, 6, 8: soluble crude cell lysate of induced recombinant E. coli cells cultivated in LB, M9, 5XLB, and 2XTY, respectively. Lanes 3, 5, 7, 9: insoluble crude cell lysate of induced recombinant E. coli cells cultivated in LB, M9, 5XLB, and 2XTY, respectively. Panel B: Lane M: Protein ladder. Lane 1: uninduced recombinant E. coli cells. Lanes 2 and 4: soluble crude cell lysate of induced recombinant E. coli cells cultivated in TB and SB, respectively. Lanes 3 and 5: insoluble crude cell lysate of induced recombinant E. coli cells cultivated in TB and SB, respectively.*

```
MLTVQRAYHA YLDEMNLITI LIPAEMQGDG KRTFYLLDGE TRTELKCERI ETIENRVKIQ

        70         80         90        100        110        120
CRFDPSVKPF GKLYEICDDL NRKTDLQMGA VIRTDLFDDY FYYGENDLGA VCGEESTVLK

       130        140        150        160        170        180
VWAPTAIEVK VKLLFPDVNK IETMPMTLGE KGIWMIELQG NFDSVFYTYL VCVNLIWNEA

       190        200        210        220        230        240
VDPYAKAVSI NGEYGVITDL KKTAVLRKKP QPFEKLTDAI IYEAHIRDFS IHPDSGIKQK

       250        260        270        280        290        300
GKYAAFNEKE TKIAGGLSSG LSYLQELGVT HLELLPFNDF EGVDEQDTSA EYNWGYNPLH

       310        320        330        340        350        360
FNAPEGSYSA KPDDPAERIR ELKSAIQSIH ESGIRVIMDV VYNHVYIRET SSFEKIVQGY

       370        380        390        400        410        420
YFRHDHNGLP SDGTGVGNDI ASERKMARKF IVDSVSYWLS EYDVDGFRFD LMGILDVETM

       430        440        450        460        470        480
NEVHASCMRI KPDLLILGEG WDLNTPLAYE KKAIIANAGK MPGISFFNDQ FRDVIKGSTF

       490        500        510        520        530        540
NLYDKGFIFG HTSNPDLLAR VMTGSIAHFT SPAQSINYVE SHDNHTLWDK MEVSNPHEAH

       550        560        570        580        590        600
EDRRMRQKLA AGMVILAQGI PFFHSGQEFY RTKKGVENSY KSPDDINQLD WNEREKYAED

       610        620        630        640        650        660
VKYVKHLIAL RKSHGAFRFA ASDEISRHYR VLKNDGGVYA YALMDVKDYG PWRHIAVIHC

       670        680        690        700        710
SHRNGAVIPL PAGGEWEIAS SPSMHKNHHD QKADSQITAN EIGTLVLFQK
```


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Figure 12. *Primary structure of Pull. Amino acid residues in the rectangle indicate the signatures conserved motif YNWGYNP of pullulanase type I.*

Table 1. *PCR recipe and PCR conditions for proving the presence of pullulanase open reading frame on pET-28a (+)/pulL using T7promoter/T7 terminator primer set*

PCR COMPONENT	**V. (µL)**	**PCR CONDITIONS**
PLASMID DNA (50 NG):	5.0	Initial denaturation (1 cycle):
T7 PROMOTER PRIMER (0.5µM):	2.0	95℃, 5 min.
T7 TERMINATOR PRIMER (0.5µM):	2.0	Amplification(30cycles):
MYTAQ™ MIX (2X) (MASTER MIX):	25.0	Each cycle has 3 segments:
NUCLEASE-FREE WATER:	16.0	
TOTAL VOLUME:	50.0	Denaturation: 94℃, 1 min.
		Annealing: 55℃, 1 min.
		Extension: 72℃, 1.8 min.
		Final extension (1 cycle):
		72°C, 10 min.

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