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Investigation of the effect of different culture conditions on recombinant protein production

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Abstract: After the COVID-19 pandemic, vaccine production technologies have become the focus of attention of researchers. As a matter of fact, recombinant protein-based antigen production, which is one of them, has taken its place in the first place. Proteins obtained by recombinant DNA technology are used in many industrial areas, especially vaccine applications, due to their reliability. Therefore, it is very important to produce targeted recombinant proteins in large quantities. This study, for the high amounts production of Omp25 protein, which is used as a vaccine candidate against brucellosis, in laboratory conditions, is aimed to reveal the effects of conditions that are the pre-culturing process, inoculation in LB or TB media, denatured or native purification, culturing with/without IPTG. All the results were analyzed by SDS-PAGE, confirmed Western Blot, and the total protein amounts were measured Bradford method. According to the results, Omp25 protein could not be obtained under native purification conditions in both cultures without induction, but it was observed under denatured conditions. This result can be explained that the protein in the cell is either misfolded or incorporated into the membrane. The amount of protein appears to be much higher in the presence of the inducer in both media inoculated with the starter pre-culture compared to the overnight pre-culture; 8.79 mg and 39.4 mg from 1 L culture, respectively. Additionally, as expected, the addition of IPTG increased the amount of protein, approximately one-and-a-half-fold for LB and about three-fold for TB. Finally, it was observed that TB medium provided higher protein production than LB, which can be explained by the presence of glycerol and high yeast extract in the medium. Although our study contains results that will attract the attention of vaccine industry, it should be kept in mind that all process should always be optimized depending on the structure of the targeted protein and thus the production amount can be further increased.

Keywords: Recombinant protein; protein amount; culture condition; Omp25

1 Introduction

The popularity of recombinant protein-based vaccine production, which is among the biotechnology topics, has increased even more after the COVID-19 pandemic (Hortsch and Weuster-Botz 2011; Hu et al. 2022; Huleani et al. 2022). The main purpose of the recombinant protein industry, which acts in many different fields such as health (vaccine, diagnosis, treatment), environment, food, and agriculture, is to obtain protein in high quantity and efficiency without reducing its quality. Due to the different structural properties of recombinant proteins, it is necessary to establish specific optimization conditions for each protein. For this, first of all, the appropriate expression system must be determined. Different heterologous hosts have been used to produce the recombinant protein efficiently. However, current research contains with the *E. coli* host, which is the best-known and most widely used (Anné et al. 2012; Packiam et al. 2020). Recombinant protein production in *E. coli* began in 1977 with somatostatin for the first time. Nowadays, protein production studies have accelerated actively after the COVID-19 pandemic in 2019 (Jiang et al. 2020, Overton 2014, Yao et al. 2022). After host selection, the growth medium formulation for high protein production should be determined, which usually results in a preference for those containing yeast extract and hydrolyzed protein (Danquah and Forde 2007). In addition, a carbon-energy source (glucose, glycerol, or other sugars) and a nitrogen source are needed for medium formulations. Therefore, Luria Bertani (LB) and Terrific Broth (TB) are chosen as the most suitable media for recombinant protein production (Coskun et al. 2022, Thongbhubate et al. 2021). Another important parameter is the identification of the inducer. Although isopropyl β-d-1-thiolgalactopyranoside (IPTG) is frequently used, it should be noted that the inducer is chosen in conjunction with the

designed vector. Apart from these, modification of the target DNA and vector, determination of the host according to the protein structure (*E. coli* BL21(DE3), BL21(DE3) pLysS, Rosetta™ (DE3) and derivatives, SHuffle® T7 Express and derivatives, CyDisCo, BL21-AI, Tuner (DE3) and derivatives, Lemo21 (DE3), SixPack, Mutant56 (DE3)), optimization of culture parameters such as temperature, time, bacterial density are other factors that directly affect the amount of recombinant protein (Hemamalini et al. 2020; Papaneophytou and Kontopidis 2014; Rosano and Ceccarelli 2014). In addition to high production, high recovery of recombinant proteins is also important in the efficiency of production. Therefore, different parameters such as resin type, solvent, pH, buffer system, protein structure and ionic strength should be considered in the purification optimization of recombinant proteins (Coskun et al. 2022).

The Omp25 protein, chosen as a representative model for effective protein production in this investigation, constitutes a hydrophobic and antigenic protein located in the outer membrane of *Brucella abortus*. This protein holds significant promise as a candidate molecule for vaccine research. Brucellosis is an infectious zoonotic illness caused by the *Brucella abortus* and *B. melitensis*, also termed Maltese fever, Mediterranean fever, and fluctuating fever (Yumuk and O'callaghan 2012). Brucellosis affects the gastrointestinal, muscle/joint, neurological, cardiovascular, pulmonary, genitourinary, and hematological systems, as well as the skin, ophthalmic, and endocrinological systems (Çelebi and Hacımustafaoğlu 2004).

Based on this information, in this study, the high and efficient production of the rOmp25 protein was evaluated by various factors, which include the preculture procedure, LB or TB media usage, denatured or native purification, and IPTG induction.

2 Materials and Method

2.1 Bacterial strains

B. abortus strain, endemic to the Erzurum Region of Turkey, was grown for three days at 37 °C in blood agar medium under microaerophilic (10% CO_2) conditions (Baily et al. 1992). This strain exists in the culture collection of the Microbiology Laboratory at the Department of Molecular Biology and Genetics, Yıldız Technical University.

2.2 Amplification, Cloning and Transformation

Genomic DNA of bacteria was isolated using the GeneDireX genomic DNA isolation kit (Catalog number: NA023-0100). The forward primer (5'-CACCCGCACTCTTAAGTCTCTCGTAAT-3') and the reverse primer (5'-GAACTTGTAGCCGATGCC-3') were used to amplify the Omp25 gene. Phusion High-Fidelity DNA Polymerase (Thermo Scientific - F530L) was used to create amplicons. PCR conditions consisted of an initial denaturation at 98 °C for 30 seconds; followed by 30 cycles at 98 °C, 10 s; 61 °C, 30 s; 72 °C, 30 s, and a final extension of 72 °C, 10 min. The Omp25 gene was cloned into the PET102/D-TOPO® expression vector containing thioredoxin, which increases protein solubility. After cloning positive plasmids were transformed into *E. coli* BL21 by the heat shock method (Froger and Hall 2007), the confirmation was performed by colony PCR and sequence analysis (Clustal Omega) (Atabey et al. 2021).

2.3 The optimization of protein expression with different parameters

The flow chart in Figure 1 depicts various factors used to enhance the quantity of recombinant Omp25 protein. These factors include the incubation time of *E. coli* BL21 cells in the LB medium for pre-culture, the choice of LB or TB as the main culture medium, and with/without IPTG culture conditions.

Both overnight and starter pre-cultures containing different incubation times of *E. coli* BL21 were established using distinct methods in the LB medium. The overnight culture involved incubating a single colony in 5 mL of LB medium within a shaking oven at 37 °C and 200 rpm for one night. The second method, for the starter culture, required cultivating it in 10 mL of LB medium at 37 °C and 200 rpm until the absorbance reached OD_{600} 0.5–0.6 A. Subsequently, the culture was divided into Eppendorf tubes, subjected to centrifugation at maximum speed for 5 minutes, the supernatant was discarded, and the resulting pellet was stored at -20 °C. (Research 1998).

LB and TB mediums with different contents were evaluated separately as the main medium. Briefly, LB medium was prepared with ampicillin (0.1 g/L), tryptone (10.0 g/L), yeast extract (5.0 g/L), NaCl (5 g/L). Also, TB medium contained ampicillin (0.1 g/L), tryptone (12.0 g/L), yeast extract (24.0 g/L), glycerol (5 g/L), K_2HPO_4 (12.54 g/L), and KH_2PO_4 (2.31 g/L) (Mühlmann et al. 2017, Zhang et al. 2019). The overnight pre-culture (1:100, v/v) or starter pre-culture (2:100, v/v) was introduced into the prepared LB or TB medium.These cultures were incubated in a shaking incubator at 37 °C 200 rpm until 0.5-0.7 $_{OD600}$ and 1-1.5$_A$ were reached for LB and TB media, respectively. Afterthat, 0.5 mM IPTG was added to the only determining cultures and the cultures incubated in a shaking incubator at 22 °C and 200 rpm for 24 hours. At the end of time, they were centrifuged at 6000 rpm for 15 minutes at 4 °C, and the pelets were stored at -80 °C.

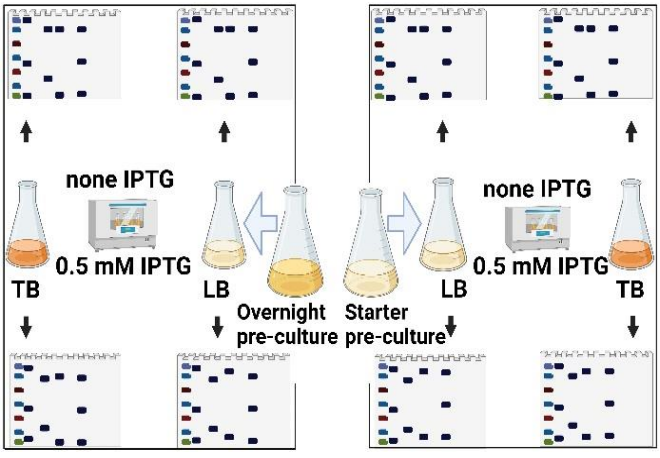

Fig. 1. Flow chart showing optimization of all experimental conditions in this study. (Figure created with BioRender.com)

2.4 Protein purification

Protein purification was carried out by affinity chromatography and rOmp25 binding to Ni-NTA resin by using the His tag located at the N-terminus of the protein was eluted by competing with imidazole. Briefly, the cells removed from -80 °C were kept at room temperature until thawed. After adding 4 ml of lysis buffer, the tubes were sonicated at 65% power for 3 minutes (10 sec. pulse, 20 sec. pause) (Zhang et al. 2018), and centrifuged at 14000 g for 20 minutes at 4 °C (Beckman Allegra X-30 -C650 rotor). For purification at native condition, the supernatant was loaded into spin columns containing HisPur™ Ni-NTA resin by mixing an equal volume of equilibration buffer (0.68 g/L imidazole, 17.55 g/L NaCl, 0.454 g/L NaH_2PO_4, 2.754 g/L Na_2HPO_4, pH 7.4). After the loaded colon was incubated at 4 °C about one hour, it was centrifuged at 700 g for 2 minutes at 4 °C and added the wash buffer (1.702 g/L imidazole, 17.55 g/L NaCl, 0.454 g/L NaH_2PO_4, 2.754 g/L Na_2HPO_4, pH 7.4). After this process is repeated three times, the purification stage was finalized by adding elution buffer (17.7 g/L imidazole, 17.55 g/L NaCl, 0.454 g/L NaH_2PO_4, 2.754 g/L Na_2HPO_4, pH 7.4) to the colon. To achieve denatured conditions for protein purification from the pellet, 8 M urea (480 g/L) was introduced into the solutions. Protein fractions were collected in 1 mL of elution buffer. (Cloeckaert et al. 1996a, Cloeckaert et al. 1996b).

2.5 Protein analysis

The purified proteins were analyzed by SDS-PAGE for their molecular size, then confirmed by Western blotting and the protein amount was determined using the Bradford test. Briefly, the proteins in the elution buffer were boiled at 96 °C for 5 min after mixing with 5X loading buffer containing 5% (v/v) β-mercaptoethanol and glycerol. Denatured samples were loaded in equal volumes (20 µl) on 12% SDS-PAGE gels. After electrophoresis, the gels were stained with Coomassie Blue (Naseri et al. 2022).

For western blotting, rOmp25 was transferred to a nitrocellulose membrane for 30 minutes at 25 V/1A using the Trans-Blot® Semi-Dry system. The membrane was blocked with a 5% milk solution in PBS-Tween-20. After blocking, the membrane was incubated with the primary His-probe antibody (H-3): sc-8036 (1:250) at 4 °C overnight. At the end of the period, the membrane was washed three times with PBS-Tween 20 and incubated for 2 hours in 5% milk powder (1:5000) HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody (Invitrogen, MA, ABD). After washing in PBS Tween-20, they were treated with 3,3-diaminobenzidine (DAB)/H_2O_2 for a few minutes to observe the protein band (Tiwari et al. 2011, Woodward et al. 1985).

In the Bradford method, the eluant was added to the microplate containing 250 µl of 1x dye reagent, and the plate was incubated for at least 5 minutes in the dark at room temperature. Then, the microplate was measured in triplicate at 595 nm using a spectrophotometer. (Khramtsov et al. 2021).

3 Results and Discussion

3.1 Amplification, Cloning and Transformation

Omp25 (642 bp) was successfully cloned into the expression vector pET102/D-TOPO. The Omp25 gene sequence was aligned with other Omp25 sequences reported using BLAST and Clustal Omega for homology searches. The Omp25 gene, which was taken from *B. abortus* biovar 3 (Erzurum, Turkey), was found to be identical to gene ID: 3787431, (accession no. AM040264). It also showed higher sequence similarity to other *Brucella spp.* (data not shown).

3.2 Determining optimum parameters for protein expression

The optimization conditions in this study were overnight or previously prepared starter culture as beginner culture, culturing with 0.5 mM IPTG or non-IPTG, and LB or TB medium usage, protein purification in native or denaturing conditions. Firstly, The fusion rOmp25 (41 kDa) protein was successfully produced by purification under denatured conditions from expression cultures (LB-TB) separately initiated with overnight and starter pre-cultures, with IPTG or without IPTG.

In Figure 2, the parameters applied in LB and TB media are shown comparatively by SDS-PAGE analysis. In Figure 2, the characteristic band profile (Lines A.1–6, B.1–4) of the rOmp25 protein obtained only in denatured conditions rather than in native conditions without IPTG (A-B. Lines 7–10) is clearly shown. The band density observed as a result of the comparison of the parameters applied for the denatured Omp25 protein is noteworthy (A. Lines 11–17, B. Lines 12–19).

Optimization parameters for overexpression of the rOmp25 protein are shown in Figure 3, which were confirmed by western blot after SDS-PAGE analysis. The band profile of the denatured rOmp25 protein is more intensely observed in starter pre-culture rather than overnight pre-culture, and in TB compared to LB. As shown in Figures 2–3, the band intensities of rOmp25 match the Bradford results. In addition, it was shown that the starter pre-culture reached a high protein amount (3.94 mg/mL) at TB medium with 0.5 mM IPTG (Table 1). In Figure 4, the effect of different absorbance values on the amount of protein is illustrated with SDS-PAGE images.

In the literature, the amount of rOmp25 produced in LB medium with overnight starter culture was found to be 50 µg/mL (Ahmed et al. 2015) and 220 µg/mL. (Yousefi et al. 2016) In our study, the fact that the protein amount increased to 0.965 mg/ml for LB and 3.2 mg/ml for TB in a single elution tube with the starter pre-culture application indicates the success in optimization.

The rDME-E protein purified under denaturing conditions was produced in amounts of 10.37 mg/L, 16.53 mg/L, 17.31 mg/L and 21.65 mg/L in LB, TB, SB and TY media, respectively, after the addition of 0.5 mM IPTG and overnight preculture (Tripathi et al. 2009). In our study, the fact that higher protein (39.4 mg/L) was produced in TB medium with starter pre-culture emphasises the importance of beginner culture.

It is known that the use of IPTG as an inducing agent increases protein yield. This confirms that saturation of the Sec-translocon capacity is directly related to the production efficiency of membrane proteins, as stated in the study by Zhe Zhang et al., and that saturation cannot be reached in the absence of the inducer. (Zhang et al. 2015).

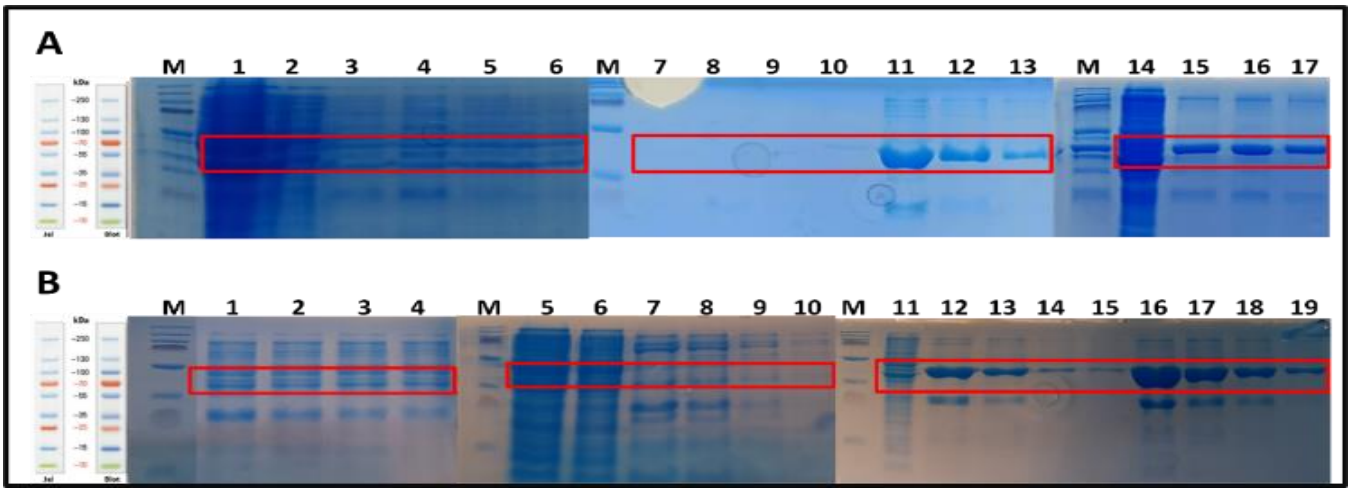

Fig. 2. SDS-PAGE images illustrating the outcomes of the evaluated parameters for LB (A) and TB medium (B).
A) Line M: Marker; Lines 1-6: The rOmp25 proteins were extracted following the conditions given in Table 1 parameter 7; flow-through, wash, and elution fractions, respectively; Lines 7–10: the elution fractions purified under native conditions after overexpression using overnight pre-culture and without IPTG, elution fractions 1-4, respectively (not rOmp25); Lines 11-13: The rOmp25 proteins were isolated following the procedure outlined in Table 1, parameter 2; elution fractions 1-3 respectively; Lines: 14-17: rOmp25 protein extracted from cells under Table I, parameter 1; cell crude extract, elution fractions 1-3, respectively.
B) Line M: Marker; Lines 1-4: The rOmp25 proteins were extracted as described in Table 1 parameter 5; elution fractions 1-4, respectively; Lines 5-10: Elution fractions purified under native conditions after overexpression using overnight pre-culture and without IPTG (not-rOmp25), respectively, flow-through, wash, elution fractions; Lines 11-15: The condition of the fourth parameter product rOmp25, as described in Table 1 parameter 3, is cell crude extract, elution fractions 1-4, respectively; Lines 16-19: Elution fractions obtained in the 4th parameter conditions in Table 1, elution fractions 1-4, respectively.

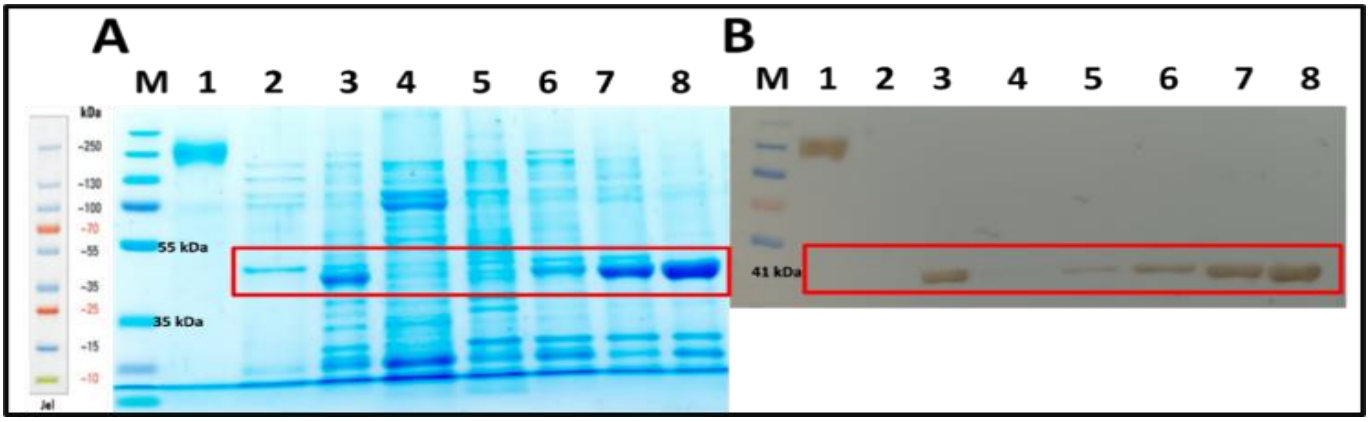

Fig. 3. Western blot image with SDS-PAGE analysis containing all parameters. A-B) Line M: Marker; Line 1: Commercial protein; Line 2: rOmp25 not obtainable under native conditions (overnight pre-culture-LB); Line 3: rOmp25 (Parameter 7th, Table 1); Line 4: rOmp25 not obtainable under native conditions (overnight pre-culture-TB); Line5: rOmp25 (Parameter 5th, Table 1); Line 6: rOmp25 (Parameter 1st, Table 1); Line 7: rOmp25 (Parameter 2nd, Table 1); Line 8: rOmp25 Parameter 3rd, Table 1).

Figure 4 shows the effect of bacterial absorbance value on protein yield during addition of inducing agent in different environments. Expression results in TB medium supplemented with IPTG after starter pre-culture indicate the highest amount of protein. When not induced, the amount of protein appears to be reduced by about half. This situation is similar for LB. In addition, both protein content and protein yield increase in TB, which is rich in yeast extract and phosphate salts, depending on the increase in cell density (Tripathi et al. 2008).

Table 1 Comparative total protein of purified denaturing conditions rOmp25 protein in culture

Parameters	Overnight/Starter pre-culture	IPTG (mM)	OD_{600} (A)	Medium	Total Protein Amount (mg/L)
1	Overnight	0.5	0.58	LB	3.05
2	Starter	0.5	0.6	LB	8.79
3	Overnight	0.5	1.1	TB	21.8
4	Starter	0.5	1.1	TB	39.4
5	Overnight	-	1	TB	9.8
6	Starter	-	1	TB	14.16
7	Overnight	-	0.58	LB	2.2
8	Starter	-	0.59	LB	5.9

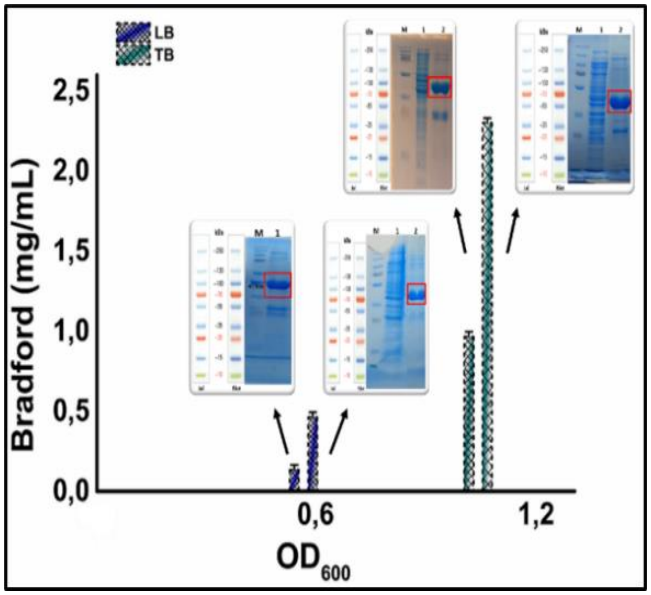

Fig. 4. Demonstration of the effect of bacterial growth and pre-culture conditions on protein yield with SDS-PAGE images.

*Short peak; The effect of LB (0.5-0.6A) and TB (1-1.5A) values on overnight pre-culture protein yield,
*Long peak; The effect of LB (0.5-0.6A) and TB (1-1.5A) values on starter pre-culture protein yield.

It is stated in the literature that a single dose of rOmp25 used for vaccine purposes is administered to experimental animals in the range of 0.25 - 40 µg (Goel and Bhatnagar 2012, Ma et al. 2015, Goel et al. 2013, Yousefi et al. 2018) and Therefore, the protein amounts obtained after the optimization in this study provide an advantage to vaccine research in terms of time, cost and workload. In addition, it is also important that the amount of rOmp25 produced in our study meets the desired protein amount (5 mg/ml) (Goel et al 2013) for different strategies in nanotechnological methods, which is the last point reached by vaccination technologies.

Conclusion

Consequently, rOmp25, known as the outer membrane protein, was produced in high amount in the presence of inducer in TB mother medium started with starter preculture, and its recovery was much more efficient under denaturation conditions. Moreover, in protein expression induced by overnight preculture, TB medium produced much higher rOmp25 than LB. Finally, another critical factor that significantly increased the amount of protein was the presence of IPTG (isopropyl β-d -1-thiolgalactopyranoside) in the expression medium.

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Authors' contributions: İA: Investigation, methodology, formal analysis, writing-original draft, writing-review& editing, visualization. TÖ: Investigation, writing-original draft, review&editing, supervision.

Conflict of interest disclosure:

The authors of this study declare that they have no conflict of interest.

References

Anné J, Maldonado B, Van Impe J, Van Mellaert L, Bernaerts K (2012) Recombinant protein production and streptomycetes. J of biotec 158:159-167. doi: 10.1016/j.jbiotec.2011.06.028

Atabey T, Acar T, Derman S, Ordu E, Erdemir A, Taşlı P N, Gür G K, Şahin F, Güllüce M, Arasoğlu T (2021) In Vitro Evaluation of Immunogenicity of Recombinant OMP25 Protein Obtained from Endemic Brucella abortus Biovar 3 as Vaccine Candidate Molecule Against Animal Brucellosis. Protein and Pept Let 28:1138-147. doi:https://doi:10.2174/0929866528666210615104334

Ahmed IM, Khairani-Bejo S, Hassan L, Bahaman AR, Omar RA (2015) Serological diagnostic potential of recombinant outer membrane proteins (rOMPs) from Brucella melitensis in mouse model using indirect enzyme-linked immunosorbent assay. BMC Vet Res 11:275 https://doi.org/10.1186/s12917-015-0587-2

Baily G, Krahn J, Drasar B, Stoker N (1992) Detection of Brucella melitensis and Brucella abortus by DNA amplification. J Trop Med Hyg 95:271-275.

Cloeckaert A, Verger J-M, Grayon M, Zygmunt M S, Grépinet O (1996a) Nucleotide sequence and expression of the gene encoding the major 25-kilodalton outer membrane protein of Brucella ovis: evidence for antigenic shift, compared with other Brucella species, due to a deletion in the gene. Infect immun 64:2047-2055. doi: 10.1128/iai.64.6.2047-2055.1996

Cloeckaert A, Zygmunt M, Bezard G, Dubray G (1996b) Purification and antigenic analysis of the major 25-kilodalton outer membrane protein of Brucella abortus. Res Microbiol 147:225-235. doi: https://doi.org/10.1016/0923-2508(96)81383-0

Coskun KA, Yurekli N, Abay EC, Tutar, M., Al, M, Tutar, Y. (2022). Structure-and Design-Based Difficulties in Recombinant Protein Purification in Bacterial Expression. Protein Detection. 3.

Çelebi S, Hacimustafaoğlu M, (2004). Brusellozis. Güncel Pediatri, 2(1), 39-43.

Danquah M K, Forde GM (2007) Growth medium selection and its economic impact on plasmid DNA production. J Biosci Bioeng 104:490-497. doi: 10.1263/jbb.104.490

Froger A, Hall JE (2007) Transformation of plasmid DNA into E. coli using the heat shock method. J Vis Exp 6:e253. doi: 10.3791/253

Goel D, Bhatnagar R, (2012). Intradermal immunization with outer membrane protein 25 protects Balb/c mice from virulent B. abortus 544. Mol Immun 51: 159 -168. doi:10.1016/j.molimm.2012.02.126

Goel D, Rajendran V, Ghosh PC, Bhatnagar R, (2013). Cell mediated immune response after challenge in Omp25 liposome immunized mice contributes to protection against virulent Brucella abortus 544. Vaccine, 31(8), 1231–1237. doi:10.1016/j.vaccine.2012.12.04

Hemamalini N, Ezhilmathi S, Mercy AA (2020) Recombinant protein expression optimization in Escherichia coli: A review. Indian J Anim Res 54:653-660. doi: 10.18805/ijar.B-3808

Hortsch R, Weuster-Botz D (2011) Growth and recombinant protein expression with Escherichia coli in different batch cultivation media. App microbiol biotechnol 90:69-76. doi: 10.1007/s00253-010-3036-y

Hu Z, Chen J-P, Xu J-C, Chen Z-Y, Qu R, Zhang L, Yao W, Wu J, Yang H, Lowrie DB (2022) A two-dose optimum for recombinant S1 protein-based COVID-19 vaccination. Virol J 566:56-59. doi: 10.1016/j.virol.2021.11.011

Huleani S, Roberts MR, Beales L, Papaioannou EH (2022) Escherichia coli as an antibody expression host for the production of diagnostic proteins: significance and expression. Crit Rev Biotechnol 42:756-773. https://doi.org/10.1080/07388551.2021.1967871

Jiang H, O'Callaghan D, Ding J-B (2020) Brucellosis in China: history, progress and challenge. Infect Dis Poverty 9:101-104. doi: 10.1186/s40249-020-00673-8

Khramtsov P, Kalashnikova T, Bochkova M, Kropaneva M, Timganova V, Zamorina S, Rayev M (2021) Measuring the concentration of protein nanoparticles synthesized by desolvation method: Comparison of Bradford assay, BCA assay, hydrolysis/UV spectroscopy and gravimetric analysis. Int J Pharm 599:120422. https://doi.org/10.1016/j.ijpharm.2021.120422

Ma QL, Liu AC, Ma XJ, Wang YB, Hou YT, Wang ZH (2015). Brucella outer membrane protein Omp25 induces microglial cells in vitro to secrete inflammatory cytokines and inhibit apoptosis. Int J Clin Exp Med. 15;8(10):17530-5. PMID: 26770344; PMCID: PMC4694244.

Mühlmann M, Forsten E, Noack S, Büchs J (2017) Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures. Microb Cell Factories 16:1-12. https://doi.org/10.1186/s12934-017-0832-4

Naseri N, Mirian M, Mofid MR (2022) Expression of recombinant insulin-like growth factor-binding protein-3 receptor in mammalian cell line and prokaryotic (Escherichia coli) expression systems. Adv Biomed Res 11:19. doi: 10.4103/abr.abr_197_20

Overton TW (2014) Recombinant protein production in bacterial hosts. Drug Discov Today 19:590-601. doi: 10.1016/j.drudis.2013.11.008

Packiam KAR, Ramanan RN, Ooi CW, Krishnaswamy L, Tey BT (2020) Stepwise optimization of recombinant protein production in *Escherichia coli* utilizing computational and experimental approaches. Appl Microbiol Biotechnol 104:3253-3266. doi.org/10.1007/s00253-020-10454-w

Papaneophytou CP, Kontopidis G (2014) Statistical approaches to maximize recombinant protein expression in Escherichia coli: a general review. Protein Expr Purif 94:22-32. doi: 10.1016/j.pep.2013.10.016

Ratnaningsih E, Sukandar SI, Putri RM, Kadja GT, Wenten IG (2022) Optimization of haloacid dehalogenase production by recombinant E. coli BL21 (DE3)/pET-hakp1 containing haloacid dehalogenase gene from Klebsiella pneumoniae ITB1 using Response Surface Methodology (RSM). Heliyon e11546. doi: 10.1016/j.heliyon.2022.e11546

Research PW (1998) E. Coli Culture for Protein Expression. In: Investigating the Biochemistry & Cellular Physiology of NHE1EST. [online] Available at. https://home.sandiego.edu/~josephprovost/E.%20Coli%20protein%20expression%20protocol.pdf Accesed: 08 March 2022

Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in Escherichia coli: advances and challenges. Front Microbiol 5:172. https://doi.org/10.3389/fmicb.2014.00172

Thongbhubate K, Irie K, Sakai Y, Itoh A, Suzuki H (2021) Improvement of putrescine production through the arginine decarboxylase pathway in Escherichia coli K-12. AMB Express 11:1-13. https://doi.org/10.1186/s13568-021-01330-5

Tiwari AK, Kumar S, Pal V, Bhardwaj B, Rai GP (2011) Evaluation of the recombinant 10-kilodalton immunodominant region of the BP26 protein of Brucella abortus for specific diagnosis of bovine brucellosis. Clin Vaccine Immunol 18:1760-1764. doi: 10.1128/CVI.05159-11

Tripathi NK, Babu JP, Shrivastva A, Parida M, Jana AM, Rao PL (2008) Production and characterization of recombinant dengue virus type 4 envelope domain III protein. J Biotechnol 134:278-286. doi: 10.1016/j.jbiotec.2008.02.001

Tripathi N K, Shrivastva A, Biswal KC, Rao, PVL (2009). METHODS: Optimization of culture medium for production of recombinant dengue protein in Escherichia coli. Indust. Biotech. 5(3), 179–183. doi:10.1089/ind.2009.3.179

Woodward M, Young Jr W, Bloodgood R (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J Immunol Methods 78:143-153. doi: 10.1016/0022-1759(85)90337-0

Yao M, Guo X, Wu X, Bai Q, Sun M, Yin D (2022) Evaluation of the Combined Use of Major Outer Membrane Proteins in the Serodiagnosis of Brucellosis. Infect Drug Resist 4093-4100. doi: 10.2147/IDR.S372411

Yousefi S, Abbassi-Daloii T, Sekhavati MH, & Tahmoorespur M, (2018). Evaluation of immune responses induced by polymeric OMP25-BLS Brucella antigen. Microb Pathog 115: 50–56. doi:10.1016/j.micpath.2017.12.045

Yousefi S, Tahmoorespur M, Sekhavati MH, (2016) Cloning, expression and molecular analysis ofIranian Brucella melitensis Omp25 gene for designing a subunit vaccine, Res pharm 11(5) 412.doi: 10.4103/1735-5362.192493

Yumuk Z, O'Callaghan D (2012). Brucellosis in Turkey—an overview. International J Infect Dis 16(4): e228-e235

Zhang B, Gu H, Yang Y, Bai H, Zhao C, Si M, Su T, Shen X (2019) Molecular mechanisms of AhpC in resistance to oxidative stress in Burkholderia thailandensis. Front Microbiol 10:1483. https://doi.org/10.3389/fmicb.2019.01483

Zhang W, Lu J, Zhang S, Liu L, Pang X, Lv J (2018) Development an effective system to expression recombinant protein in E. coli via comparison and optimization of signal peptides: expression of Pseudomonas fluorescens BJ-10 thermostable lipase as case study. Microb Cell Factories 17:1-12. https://doi.org/10.1186/s12934-018-0894-y

Zhang Z, Kuipers G, Niemiec Ł, Baumgarten T, Slotboom DJ, de Gier J-W, Hjelm A (2015) High-level production of membrane proteins in E. coli BL21 (DE3) by omitting the inducer IPTG. Microb Cell Factories 14:1-11. https://doi.org/10.1186/s12934-015-0328-z