Cloning and over expression studies of ovine somatotropin cDNA of Kajli (sheep breed) in a prokaryotic system

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Abstract: Genetic studies including the quest, cloning and expression of genes encoding proteins responsible for various vital physiological processes and beneficial characteristics of economic perspective have made the biotechnology research progressively auspicious. Due to its great zootechnical and industrial importance somatotropin gene have been cloned from various animal species. Current study was designed to clone mature GH cDNA of a sheep breed Kajli and carry out over expression studies of cloned GH cDNA in a suitable prokaryotic expression system. Sheep GH cDNA was cloned in T/A vector with signal peptide and confirmed by nested PCR and restriction digestion. The gene was then ligated in pLEX expression vector and restricted plasmids showed a fragment insert of ~600bps. Restriction analysis confirmed positive clones, were induced for protein expression analysis. The pET vectors have an IPTG inducible strong T7 promoter and *E. coli* expression strain of BL21 (DE3) pLysS contains DNA fragment from T7 phage which harbors RNA polymerase. Therefore, for expressing recombinant proteins, cells were induced with various IPTG concentrations to optimize expression levels. Cells were induced with different IPTG concentrations (0.1 mM to 0.8 mM) followed by SDS-PAGE. Results indicated maximum expression level of oGH at 5

hrs after induction of cells with 0.3 mM IPTG concentration with a molecular weight of 22 kDa. As for as cellular localization of protein is concerned accumulation of expressed oGH is observed in inclusion bodies. The successful expression of the cloned GH cDNA of sheep confirms the functional viability of the clone.

Key words: Cloning; over expression; cDNA; ovine somatotropin; Kajli sheep, prokaryotic system

1. Introduction

Pakistan is an agricultural country, whose agricultural products are considered to be the backbone of the economy. The utilization of agricultural products and livestock has great importance not only to meet the necessities of people residing in Pakistan but all over the world (Ahmed, S., 2017). The contribution of Pakistan on the agricultural basis to GDP is about 21% with the increment of about 2.7%, annually (Ahmed, V. and Javed, A., 2016; Ajayi, A.O., 2018). An imperative sub-sector of agriculture is livestock, which has contributed the value addition of approximately 56% to the agricultural sector and around 11 percent to GDP (Azam, A. and Shafique, M., 2017; Baumgard, L.H., *et al.* 2017). Sheep is an important ruminant animal, kept as livestock in Pakistan (Bilal, M.Q., 2008). It has made a substantial proportion of the meat share (1170000 tons), Lamb skin (3.08 million), and milk (36000 tons) production (Capillary, A., 2000). The increase in the human population and modern living style has made people more dependent on junk food due to which the demand for meat and milk is rapidly increasing.

To meet the rapidly increasing demand for meat and milk, traditional strategies like cross-breeding, animal food management strategies and appropriate livestock are chosen to handle but still, the demand is not fulfilled (Chiaradia, E. *et al.* 2012). Therefore, one of the modern technologies is recombinant DNA technology has resulted in breakthroughs

in livestock and food biotechnology. The rDNA technology has empowered people by providing different paths to satisfy the need and demand for meat and milk. Moreover, it has also increased the dairy industry to increase production of recombinant bovine somatotropin (rbST) (Choi, T.J. and Geletu, T.T., 2018). In the early 1908s, the U.S. FDA approved recombinant bovine somatotropin with the advent of biotechnology techniques in the agriculture sector. Somatotropin being homeorhetic control has been involved in the regulation of Nutrient partitioning. Introducing somatotropin to dairy animals, the milk synthesizing process will efficiently increase milk production by the uptake of glucose to the mammary epithelial cells through a passive, facilitative process (Farooq, U., 2016; Giadinis, N.D., *et al.* 2015). Therefore, the expression of the genes of glucose transporters which are primarily present in the mammary glands (epithelial cells), skeletal muscles, and in omental fat of respective animals, is regulated by this hormone (Khalid, S., *et al.* 2008). The large-scale dairy industry follows milk output per feed source input, to enhance the production of milk which has prime importance in reducing the consumption of natural resources with minimal feed production expenditures. Moreover, a reduction in animal waste will have a positive impact on the environment. During transitions and early lactation periods, lower doses of rbST will improve dry matter intake and provoke healthy alternations in concentrations of non-esterified fatty acids, insulin-like growth factor-1, somatotropin, insulin, calcium, and glucose as a result leading to improved milk production (Khan, M.S., 2008; Kvidera, S.K. *et al.* 2017). The expression of the somatotropin gene has a pivotal role in milk and meat production and can be cloned from various species such as cow, buffalo, sheep, panda, fish, and goat. Therefore, based on the beneficial aspects of the hormone (somatotropin) following strategy was designed to clone the mature GH cDNA of a sheep breed Kajli where the prokaryotic expression

system was used to study the gene expression of the sheep breed Kajli (Mishra, A.K. *et al.* 2016; Naz, S. and Khan, N.P., 2018).

2. Materials and methods

2.1. 2.1. T/A cloning vector used for Cloning and Ligation of oGH cDNA

This research strategy was proposed to study the gene expression of GH cDNA of a sheep breed Kajli in a suitable prokaryotic expression system and was accredited by the ethical committee of PMAS-Arid Agriculture University Rawalpindi (AAUR), Pakistan. T/A vector with a single peptide was used for the cloning of sheep (ovine) GH cDNA (oGH cDNA), the proposed and performed methodology was testified by Khalid and coworkers (O'Boyle, N.J. *et al.* 2012). The purified product of PCR was ligated by using the manufacturer's protocol (InsT/Aclone^{TM} PCR cloning kit by the Fermentas # K1214) in a T/A cloning vector. The composition of the ligated mixture comprised of 3 µl PEG 400 solution, 6 µl of PCR product, 3 µl of ligase buffer (10X), 1 µl T4 DNA ligase, 3 µl InsT/A clone^{TM} Vector, and the required amount of deionized water to make the total volume of 30 µl. Afterward, the prepared mixture was incubated at 22°C in the water bath overnight (Okazaki, S., 2006). The chemically competent cells i.e. B10 and BL21 (DE3) pLysS had been prepared. After incubation, screening was performed on the positively cloned plates. From the prepared culture media, an isolated single colony was picked up and 3mL of amp^{+} LB broth was used for inoculation. Afterward, incubated at 37^{o}C on a shaking incubator. For the confirmation of recombinant plasmid miniprep was performed.

2.2. 2.2 Analysis of restricted products and their purification

Restriction analysis was conducted, to ensure the presence of oGH cDNA in the prepared recombinant plasmid. The enzymes used for restricting the plasmid were *Hind*III and

*Nde*I followed by electrophoresis, where 1.2% of Agarose gel was used. The DNA fragments depicting oGH cDNA were restricted in a T/A cloning vector by using the enzymes that have been purified with a DNA gel extraction kit (Novagen Canada). The prokaryotic expression vector pET23b$^+$ Novagen (USA) was used for the ligation of a purified restricted fragment of oGH cDNA. The extracted plasmid from already grown liquid culture through restriction analysis ensures the presence of recombinant plasmid (Peel, C.J. and Bauman, D.E., 1987; Qureshi, M.A., 2010).

2.3. 2.3 Protein expression studies

To study the gene expression, the positive clones obtained from restriction analysis were used. For the preparation of the starter culture, the host strain BL21 (DE3) pLysS, having a pET23b$^+$ vector was used by inoculating a single colony in LB broth of 3 mL containing chloramphenicol and ampicillin. Overnight incubation was provided to the inoculated culture, 200 rpm at 37°C. Afterward, 500 µL of overnight grown culture was taken and poured into the 50 mL of LB broth containing ampicillin, again incubated at 37°C on a shaking incubator until the OD_{600} reached ~0.5. Several different tubes were used to induce the prepared culture with different concentrations of IPTG i.e. 0.1 mM to 0.8 mM respectively where cells that were not induced were used as control group. The induced tubes were incubated at continuous shaking at 200 rpm and 37°C. For the optimization of induction time, the induced culture and uninduced culture as control were taken out at different intervals of time as 0 hr, 2 hrs, 3hrs, 4 hrs, and 5 hrs respectively. For the collection of induced cells from the culture, centrifugation was performed at 12000 rpm for 20 minutes. Pellets obtained after discarding the supernatant were stored 80 at -80^{o}C till further use. To determine the localization of the expressed protein inside the cell, the cells were induced at 24 ^{o}C, 30 ^{o}C, and 37 ^{o}C respectively. SDS-PAGE was performed to

electrophorese the proteins, by using 15% of polyacrylamide gel for 3 hours at 250V. Lastly, a dot blot was performed to evaluate the expression of desired proteins and for the optimization of antibody concentration.

3. Results

3.1. 3.1 Renewal of the Thermus aquaticus clones & amplification by PCR amplification

The renewal of full-length pTZ57R oGH clones of cDNA Kajli (sheep breed) took place, during the extraction of plasmid having a single peptide (Figure 1a). Specifically designed forward and reverse primers were used for the removal of the signal peptide by PCR and the gene is amplified without a stop codon. (Figure 1b). After successful purification, the agarose gel based eluted product of PCR containing oGH cDNA is shown in the figure 1c.

3.2. Cloning of the oGH cDNA in the T/A cloning vector & the restriction analysis

Successfully obtaining the PCR product in purified form, ligation was done in a pTZ57R cloning vector. Afterward, the ligated product was transformed into a chemically competitive strain of *E.coli* B10. LB agar plates containing antibiotic ampicillin were used to plate the chemically competitive cells and incubated at 37°C overnight. The colonies obtained the next day were isolated and cultured in 2-5ml of LB liquid medium already containing 50 µg/ml ampicillin, incubated on a shaker incubator at 37°C overnight. From the overnight-grown culture, the plasmid was extracted. To confirm the presence of extracted plasmid, gel electrophoresis was performed. Therefore, successful cloning is shown in (Figure 2a) having an insert of 3459 bp in the plasmid. Afterward, restriction enzymes *Nde* I and *Hind III* were used for the restriction analysis of extracted plasmid. Interestingly, the targeted sites for restriction analysis used by restriction

enzymes were already present in primers, used for PCR amplification. For the confirmation of a positive clone having oGH cDNA, gel electrophoresis was performed which shows a fragment of the insert of ~ 600 bps (Figure 2b) as a confirmation. Afterward, the restricted insert was purified as shown in Figure 2c.

3.3. Revitalization of the pET23b & prokaryotic expression vector; purification, ligation & transformation

For the sub-cloning of Ovine GH (oGH) into the pET23b$^+$ expression vector, a strong T7 promotor was used. Afterward, it was transformed into a strain of *E.coli* BL21 (DE3). The glycerol stocks of the plasmid expression vectors pET23b and cGH clone containing gene, were used to get the pET23b+ vector minus cGH for cloning of oGH cDNA. For cloning of culture, 3 mL of LB was used in an appropriate antibiotic. The recombinant plasmid was isolated from the culture that was grown overnight where 1 % agarose gel was used for confirmation of the presence of recombinant plasmid (Figure 2d). Afterward, restriction enzymes i.e. *HindIII and NdeI* were used to restrict the pET plasmid having cloned cGH (Figure 2e). The plasmid after the restriction was eluted out to clone oGH cDNA in pET23b+, and gel electrophoresis was performed for confirmation (Figure 2f). The pET23b+ vector after restriction and purification was used to ligate the purified oGH cDNA. The recombinant plasmid vectors were used to transform the expression strains, derived from BL21 (DE3) pLysS. Single colonies were picked from transformed bacterial cells, antibiotic- supplemented LB broth was used for inoculation. An insert of oGH (pEToGH) present in the recombinant plasmid was isolated from the transformed cells of the BL21 (DE3) pLysS and then gel electrophoresis was performed (Figure 2g). The size of pET23b+ was increased with transformation. For confirmation

of insert, restriction analysis was performed on isolated recombinant plasmids by *NdeI* and *HindIII* enzyme as depicted in Figure 2h.

3.4. Analysis of Protein Expression

After restriction analysis, the obtained positive clones were further induced for the analysis of protein expression. A promoter (T7) that is inducible by IPTG was present on the pET vectors. RNA polymerase was obtained from DNA fragments of the T7 phage from the BL21 (DE3) pLysS's *E. coli* expression strain. To optimally enhance the expression levels and to study the expression of recombinant proteins, the different amounts of IPTG were used. For maximal presence of cells in the logarithmic phase of growth, the BL21 (DE) pLysS which is the expression strain and harboring the pEToGH was grown in Amp^+ and Cam^+ complemented LB broth till OD_{600} becomes 0.5. The cells that were induced with different IPTG concentrations (0.1 mM to 0.8 mM) were followed by SDS-PAGE. The results showed a maximum level of expression of oGH at a 0.3 mM concentration. The molecular weight of oGH observed, was to be 22 kDa and the relevant results are shown in Figure 3a. After the determination of optimal IPTG concentration, the level of expression was maintained in relevance to the time where the Figure 3b demonstrates the expression results of SDS-PAGE of oGH with the time gradient. After 5 hours, the maximal expression levels of the cells induction with 0.3 mM of the IPTG quantity were observed. The discontinuous sonication of cell pellets was performed, to ensure the cellular localization of the protein. As a result of localization and sonication, the proteins obtained were divided into various groups, i.e., total cytoplasmic proteins (TCP) or dissolved proteins, **s**upernatant and inclusion bodies (IB), pellet. The accumulation of expressed oGH in the inclusion bodies is represented in figure 3c. Consequently, for the confirmation of proteins that were expressed with IPTG

concentration and time gradient optimized samples, dot blot analysis was performed (Figure 3d).

4. Discussion

The current research and proposed methodology mentioned above, to study gene expression of ovine growth hormone (oGH) has been carried out in Pakistan for the first time and the sheep breed used was Kajli, in a prokaryotic system. Livestock has prime importance in the agricultural sector of Pakistan. Among several other animals in Pakistan, sheep are considered to be a vital domesticated animal for people running small-scale businesses. Indigenous varieties of sheep breeds are present in Pakistan that can be categorized based on their properties i.e. the thin tail and fat tail breeds (Peel, C.J. and Bauman, D.E., 1987; Raza, S.A., 2012). The major meat and milk source are domestic ovine species, predominantly present in the country's rural areas (Rehman, A. *et al.* 2017). The milk obtained from sheep is considered to be the important raw source for the manufacturing of cheese on a larger scale (Salin, S. *et al.* 2017). A yield of coarse-type wool is obtained from sheep breeds that are present in Pakistan. However, the growth of sheep mostly took place in assorted flocks where all breeds are present collectively (Sarwar, M., 2002). The significance of the sheep breeds is that they can fit well in the diverse socio-economic environment. Moreover, sheep also have the importance of being a central sacrificial animal. Recent research has reported that due to the expanding human population the day-to-day need for meat and milk has increased tremendously. Historic studies mentioned that man is trying to meet the need and demand for milk and meat for its survival by using conventional and biotechnology-based methods which include cross-breeding, animal feed management, and proper livestock management. Therefore, growth hormones have a massive impact on the dairy industry and can now be produced by

biotechnology methods (Salin, S. *et al.* 2017; Shakweer, W.M.E.S. and Abd EL-Rahman, H.H., 2020). To increase lactation and performance, the exogenous, non-glycosylated polypeptide GH has been given in laboratories to animals as well as to mammals (Sundarraj, A.A. *et al.* 2018). For a substantial increase in milk production farmers are using GH in goats, pigs, cows, and sheep manufactured by the procedure mentioned in the above study (Tufail, M.S. *et al.* 2021). Therefore, the pET expression system was used to study the cDNA of GH for milk and meat production in a breed of sheep i.e., Kajli, which was expressed in bacteria. It was reported in different other studies that the bacterial system can be used for the expression of growth hormones (Yousef, M.I., 2005; Yunyun, L., 2008).

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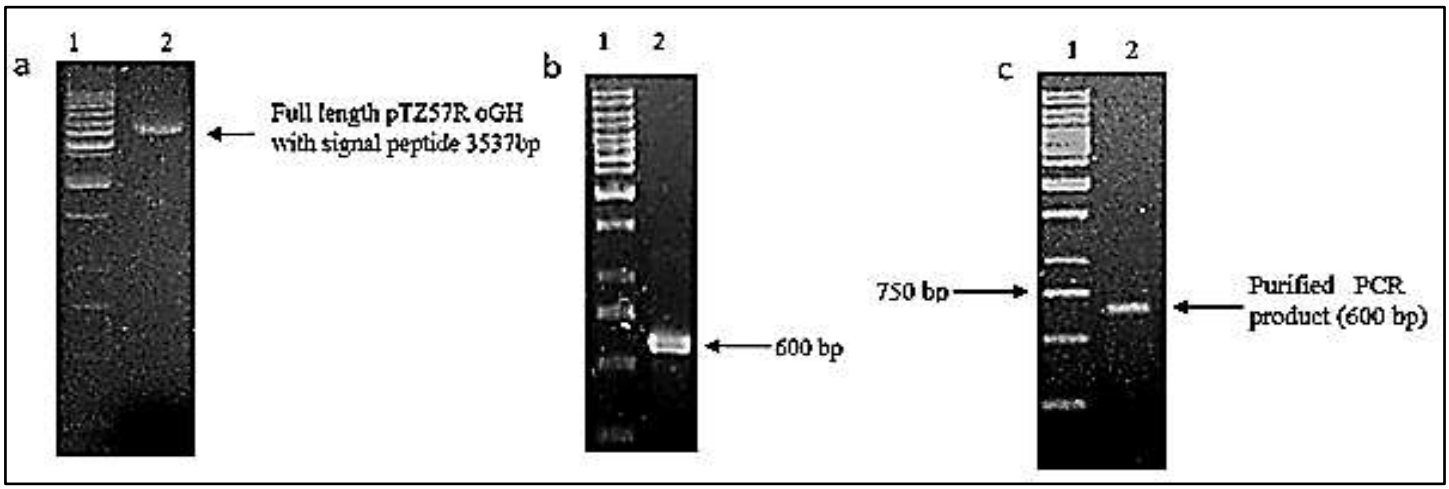


Figure 1. **A**: Miniprep of pTZ57R oGH of sheep breed Kajli with signal peptide. Lane 1 GeneRuler^{TM} 1 kb DNA size marker. Lane 2 contains pTZ57R oGH Kajli sheep breed clone with signal peptide; **B**: cDNA of oGH without signal peptide (600bp); **C**: gel eluted fragment to confirm PCR product after gel purification

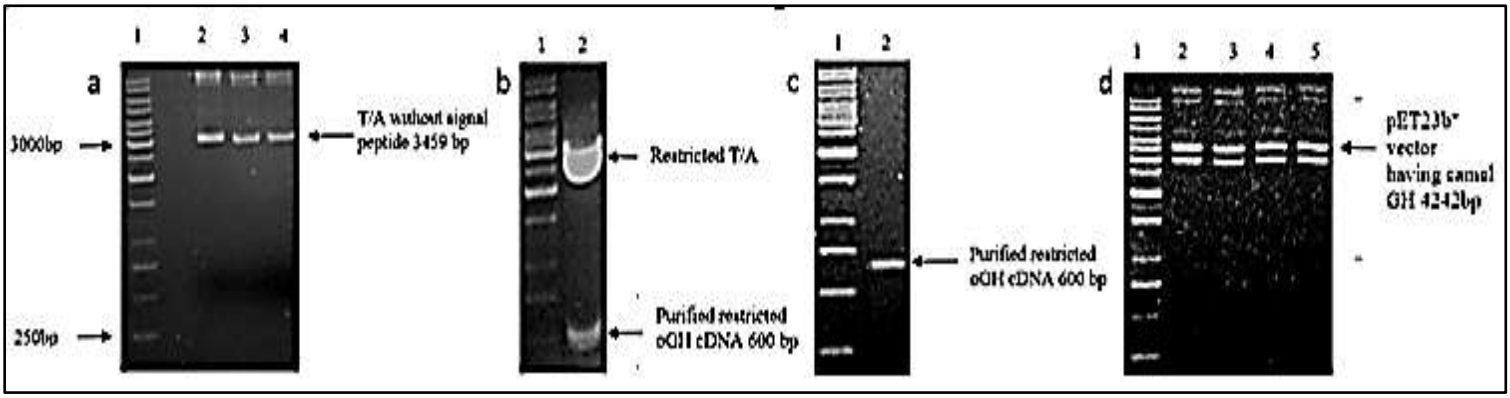
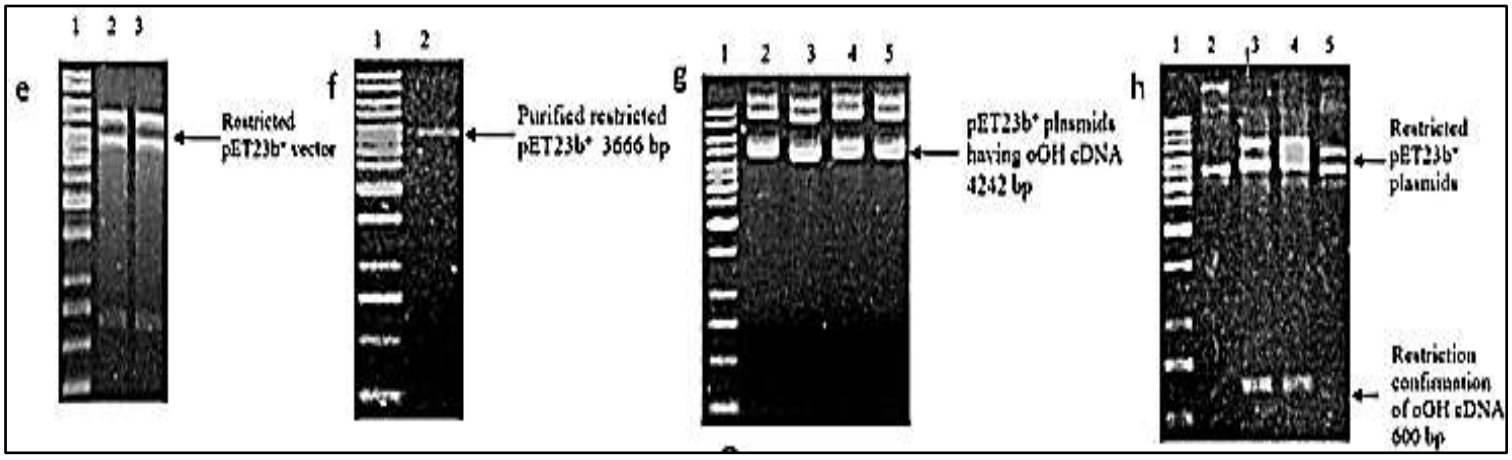


Figure 2. a: Miniprep of T/A clones of sheep breed Kajli without signal peptide; **b**: Restriction confirmation of the positive T/A oGH cDNA clones with the restriction enzymes *Nde*I *and HindIII*; **c**: Purified restricted oGH cDNA; **d**: Miniprep of pET23b$^+$ clones having camel GH

Figure 2. e: Miniprep of plasmid pET23b$^+$ clone restricted with *NdeI* and *HindIII*; **f**: Purified



restricted pET23b$^+$ plasmid; **g**: Miniprep of pET23b$^+$ plasmid having oGH cDNA; **h**: Restriction confirmations of the positive pET23b$^+$ clones having oGH cDNA with the restriction enzymes *NdeI and HindIII*

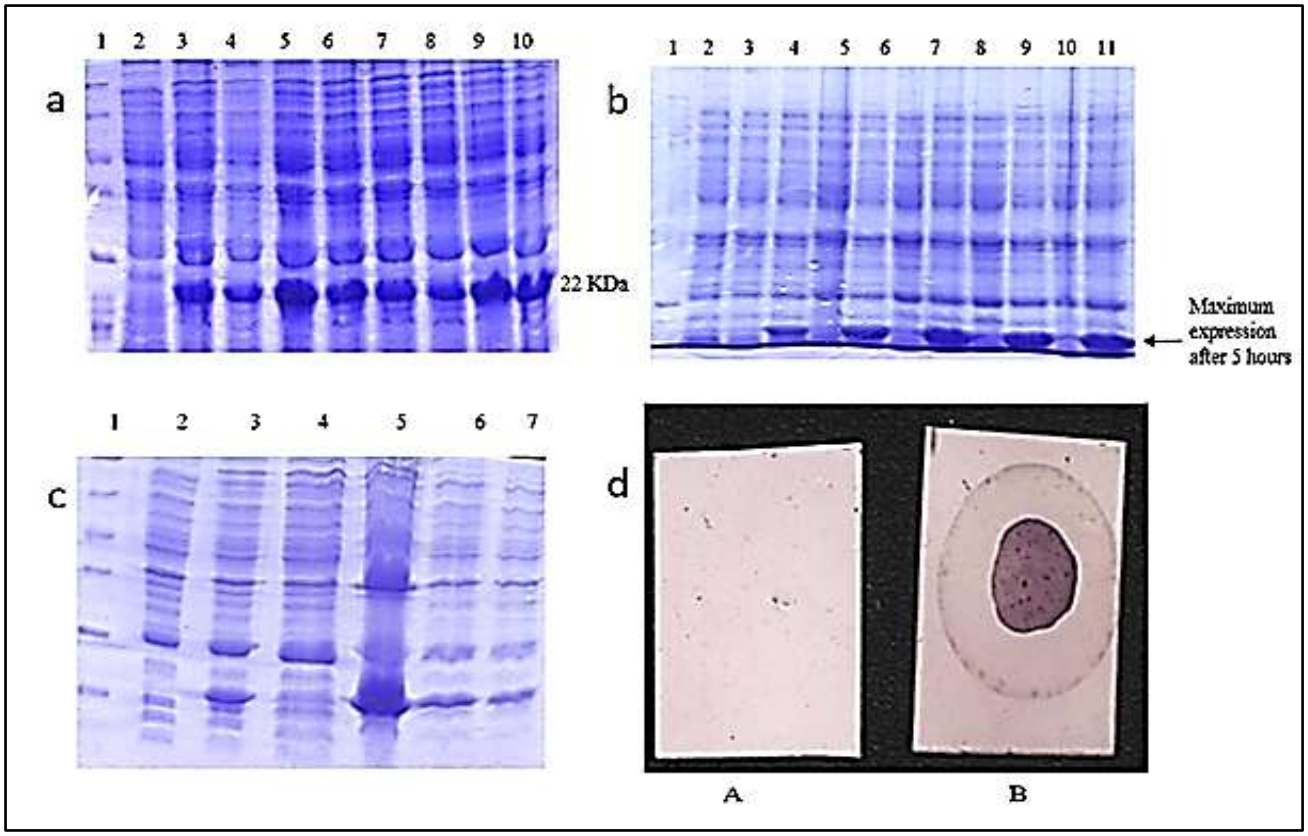


Figure 3. **a**: 15% SDS-PAGE showing the level of expression when induced with various concentrations of IPTG. Lane 1: Fermentas Protein Marker (Sm0431) Lane 2: uninduced BL21 (DE3) pLysS cells having pET oGH, Lane 3-10: Induction with 0.1 – 0.8 mM IPTG; **b**: 15% SDS-PAGE showing the level of expression with respect to time at 0.3 mM IPTG concentration. Lane 1: Marker Lane 2: BL21 (DE3) pLysS cells having pET oGH at 0 hr, Lane 3,5,7,9,11: uninduced cells at 1hr to 5 hr s, while Lane 4,6,8,10,12: induce cells at 1 hr to 5 hrs; **c**: 15% SDS-PAGE showing cellular localization of oGH at 37 ^{o}C. Lane 1: Marker Lane 2: uninduced BL21 (DE3) pLysS cells having pET oGH, Lane 3: induced BL21 (DE3) pLysS cells having pET oGH, Lane 4: TCP after sonification, Lane 5: IB after sonification. Lane 6: wash 1 and Lane 7: wash 2; **d**: Dot blot analysis of oGH A: Negative control B: Cells induced at 0.3 mM IPTG concentration after 5 hrs of induction.