Glucose transporters are integral membrane proteins (IMP) and facilitate entry of sugars into mammalian cells. Its natural abundance is low and therefore, has to be overexpressed in eukaryotic or prokaryotic recombinant system followed by purification. Escherichia coli are preferred system since they are cost effective, genetically manipulable and can be grown to large biomass in very short period of time (Peti and Page, 2007; Robinson and Loll, 2011).

GLUT4, being an integral membrane protein, faces a challenge of expression screening methods to get purified protein in enough quantities in a homogeneous form for structural and functional studies. Finding optimal conditions for overexpression of IMP is not straightforward and usually requires laborious screening procedures involving different strains and culture/induction regimes. Many membrane proteins are overexpressed and form inclusion bodies. Their refolding into functional proteins is often not successful. To avoid inclusion body formation, accumulation of overexpressed membrane proteins is directed towards the cytoplasmic membrane. This can be achieved by optimization of bacterial growth conditions to slower down the transcription/translation rates of target membrane protein and ensure that the Sec translocon integrates the overexpressed proteins in the cytoplasmic membrane (Wagner et., al, 2007, Wagner et., al, 2008).

The E. coli, BL21(DE3) and its pLysS harboring strain BL21(DE3)pLys are widely used protein production systems. pLysS plasmid encodes for T7RNA polymerase inhibitor T7LyS, which provides stringent control on T7RNA polymerase synthesis hence protein production (Studier, 1991). Two mutant derivatives of BL21(DE3), the C41(DE3) and C43(DE3) also called Walker strains, have less powerful lacUV5 promoter which results in much lower amounts of T7 RNA polymerase production and hence stringent control over protein production (Miroux and Walker, 1996). pLysS harboring C41(DE3)pLysS and C43(DE3)pLysS strains are also being used for protein production (Sánchez-Arreola P et. al., 2013) and may show further stringency in expression optimization. Another strain, Lemo21(DE3) is a T7 strain containing plasmid pLemo, a pACYC184 derivative carrying the lysY gene, is designed for the expression of challenging proteins including integral membrane proteins. In Lemo21(DE3), T7 inhibitor T7 lys ozyme modulates T7 RNA polymerase activity. The expression of T7 lysozyme is controlled by well titratable rhamnose promoter (Wagner et al., 2008). Subsequent slower transcription/translation rates of the target membrane protein ensure integration of the overexpressed proteins in the cytoplasmic membrane in all above strains.

No systematic and high-throughput-compatible method is available for the optimization of expression in E. coli, therefore, optimization of different
concentrations of inducer, different growth temperature and time interval in different bacterial strains need to be carried out for different membrane proteins. An attempt was made to overexpress GLUT4 and other membrane proteins in C43(CD3) cells by fusion of short hydrophilic bacterial proteins, YaiN(α) and YbeL(β) at the ends of the GLUT4 and constructed all possible eight fusion protein expression cassettes in pET28a(+) vector (Leviatan et al., 2010). These proteins serve as facilitating factors for expression and purification. GLUT4 was expressed only in α-GLUT and β-GLUT-β form and rest of the forms did not show expression as proved by western blotting. They did not attempt to optimize the induction and other growth conditions for optimum expression. Alisio and Mueckler also made an attempt by generating mutant (aglyc o forms) and truncated form of GLUT1 and GLUT4 and expressed them in P. pastoris (Alisio and Mueckler, 2010).. They used an expensive and difficult yeast system to work with but could not purify wild type GLUT proteins using this system.

In the present proposal we plan to use simple and inexpensive system of E. coli for optimization of protein expression. Six different strains of E. coli, BL21(DE3)pLysS, [C43(DE3) and C41(DE3)], their pLysS harbouring strains [C43(DE3)pLysS and C41(DE3)pLysS] and Lemo21(DE3) will be used for expression optimization. The camel GLUT4 is cloned in pET28a(+) vector and protein expression is confirmed by Western botting. GLUT4 containing pET28a(+) vector will be transformed into above mentioned six E. coli strains. Optimization of expression will be carried out at different temperatures for different period of time in different concentrations of IPTG for each strain except Lemo21(De3) where rhamnose will be used as inducer. In future, further studies on the GLUT4 will be carried out and GLUT4 will purified by affinity chromatography using Ni-NTA agarose beads and by gel filtration chromatography to develop crystals for X-ray crystallography studies to determine its structure. Structural determination of the membrane proteins will lead to the development and design of drugs for diabetic and cancer patients with high efficacy and specificity with least undesirable side effects.

Methodology (max 300 words)

Competent cells preparation of different E. coli strains:
Streak cells (BL21(DE3)pLys, C41(DE3)pLysS, C43(DE3)pLysS and Lemo21(DE3)) on LB plate+34 mg/ml chloramphenicol and C41(DE3) and C43(DE3) on plain LB plates. Grow cells at 37oC overnight. Pick single colony and place in 10 mL LB media (+antibiotic selection if necessary) and grown overnight at 37oC. Transfer 1 ml overnight grown culture into 100 mL LB media in 500 L flask and allow to grow at 37oC (150 rpm), until OD600= 0.4-0.5(~2-3 hours). Transfer culture into two 50 mL tubes and place on ice for 30 mins. Centrifuge at 4oC for 10 mins at 3,000 g and discard supernatant. Resuspend cells in 25 mL of cold 0.1 M CaCl2 and keep on ice for 30 mins. Centrifuged again at 4oC for 10 mins at 3,000 g and discard supernatant. Resuspend cells (by pipetting) in 2 mL cold 0.1M CaCl2 containing 15% glycerol. Transfer 150 mL into (1.5 mL) Ependorff tubes placed on ice. Freeze the cells in chilled Ethanol and stored at -80oC and use for up to ~6 months.
Transformation of GLUT4-pET28a in different E. coli strains:
Transformation of GLUT4-pET28a vector into chemical competent E. coli cells will be done by heat shock method. Briefly, chemically competent BL21(DE3)pLysS, C41(DE3), C43(DE3), C41(DE3)pLysS, C43(DE3)pLysS and Lemo21(DE3) E. coli cells will be thawed and mixed with 50ng GLUT4-pET28a vector. After incubation on ice for 30 min, heat shock will be given at 42°C for 40 seconds in water bath. Tubes will be transferred to ice and 1 ml of 37°C warmed SOC media (without antibiotic) will be added and incubated for 1 hour at 37°C. 100-150 µl of the resulting culture will be spread on LB plates containing 50µg/ml kanamycin for C41(DE3), C43(DE3) and 50µg/ml kanamycin plus 35µg/ml chloramphenicol for BL21(DE3)pLysS, C41(DE3)pLysS, C43(DE3)pLysS and Lemo21(DE3)S, and grown overnight at 37°C.

Optimization of Expression of CdGLUT4 in E. coli: Colonies (2 per strain) will be picked and placed in 50 mL LB with 50µg/ml kanamycin and/or 35µg/ml chloramphenicol. The cultures will be grown overnight at 37 °C in a shaker incubator. Next morning one of the grown cultures from each strain will be selected for optimization of CdGLUT4 expression. Fresh LB media (10ml) in individual 50 ml tubes will be inoculated and grown at 37 °C with antibiotics until the cultures reached the OD600 of ~0.4 to 0.5. The culture will be cooled down to respective temperatures of induction (18, 25, 30, 37 °C) and induced with different concentrations of IPTG (10, 25, 50, 100, 200, 500, 750 and 1000 µM) except Lemo21(DE3) culture. One uninduced and one non transformed control will be collected as shown above. An aliquot of 1 ml uninduced cultures will be withdrawn at 0 time followed by collection (1ml) of induced aliquots at different time intervals (1, 2, 3, 5, 18 and 24 h). Cells will be harvested by centrifugation at 4,500g for 15 min at 4 °C and will be processed for analysis of recombinant CdGLUT4 protein expression or stored at −80°C for future analysis.

Lemo21(DE3) induction: L-rhamnose induction of Lemo21(DE3) cultures will be standardized instead of IPTG. Fresh cultures will be grown at 30 °C with different amounts of L-rhamnose (50, 100, 250, 500, 750, 1,000 and 2,000 µM) until OD600 reaches 0.4–0.5. The cultures will be set to different temperature (18, 25, 30, 37 °C) and induction will be carried out with IPTG (400 µM). Culture will be grown at respective temperatures and aliquots will be withdrawn at different time intervals (0, 1, 2, 3, 5, 18 and 24 h). Cells will be harvested by centrifugation at 4,500g for 15 min at 4°C and will be processed for analysis of recombinant CdGLUT4 protein expression or stored at −80°C for future analysis.

Sample preparation for CdGLUT4 expression analysis: Cell pellet will be resuspended in 100 µl of resuspension buffer [20 mM phosphate pH 7.4, 300 mM NaCl, 0.5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 1 mg/ml lysozyme, protease inhibitor cocktail and 10 units/ml benzonase]. The suspension will be incubated on ice for 30 min and sonicated with intermittent cooling between successive bursts. The resulting lysate will be centrifuged at 10,000g for 30 min at 4 °C. The supernatant containing soluble proteins and fragmented membranes will be
subjected to ultracentrifugation at 150,000 g for 45 min at 4 °C to separate the membrane and soluble protein fractions. The pellet containing membrane fractions will be resuspended in cold resuspension buffer (without lysozyme, PI cocktail and benzonase) and protein concentration will be determined using BCA protein assay kit. The concentrations of the fractions will be adjusted to 10 mg/ml with PBS.

SDS-PAGE and Western blotting:
Samples will be mixed with 4× sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 20% β-Mercaptoethanol, 40% Glycerol and 0.04% Bromophenol Blue) and will be loaded onto two different precasted SDS-PAGE gels. Proteins in one set of gels will be stained with Coomassie followed by destaining and another set of gels will be electrophoresed onto nitrocellulose membranes to analyze the level of CdGLUT4 expression in membrane fractions by Western blotting. The transferred protein will be checked by Ponceaus S. The membrane will be destained, and blocked with 3% BSA in TBS-T buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.05% Tween20 (v/v)) for 1.5 h at room temperature. The membranes will be incubated with a primary antibody (anti-His) dissolved in TBS-T buffer containing 1% BSA. Antigen and primary antibody complexes will be visualized upon binding with a secondary antibody conjugated with HRP using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Luminescence will be recorded on exposure to X-ray film or using CCD camera. Intensities of the CdGLUT4 bands in both SDS-PAGE and Western blot will be compared and analyzed for each culture using the Image J software.

| The project is applicable within (months) | 12 |