Chapter 3 Results

3.1 *In vitro* construction of *adiponectin*

In vitro gene construction is a simple and cost effective method for full length gene construction compared with mRNA extraction and DNA synthesis. Instead of RNA extraction from human fat tissue and cDNA preparation, full length *adiponectin* was constructed by overlapping exon 1 and exon 2 using 4 primers.

The full length *adiponectin* fragments were constructed successfully to express in *E. coli* and *P. pastoris* by overlapping exon 1 with exon 2 at annealing temperature 55°C using proof reading *Pfu* DNA polymerase. Prior to that, exon 1 was constructed successfully using two types of forward primers. The first construct of exon 1 included *adiponectin* native signal peptide (*ADPs*). This construct was made by exon 1 forward primer with signal peptide and exon 1 reverse primer with expected size 203 bp. (Figure 3.1). The second construct of exon 1 was made using exon 1 forward primer that starts from the first codon of *adiponectin* skipping signal peptide sequence (*ADPws*) and ends with exon 1 reverse primer with expected size 161 bp. In addition exon 2 was constructed using exon 2 forward and reverse primers with expected size 531 bp. After overlapping each one of the two types of exon 1 separately with exon 2 at annealing temperature 55°C the expected size of full length *ADPs* was 734 bp and *ADPws* was 692bp (Figure 3.2). To express *adiponectin* fragments in *E. coli*, the full length of *adiponectin* fragments were amplified using different primer sets that were designed for cloning and expression into *E. coli* specific vector. *ADPs* (expected size 734 bp) and *ADPws* (expected size 692 bp) were successfully amplified at annealing temperature 55°C using proof reading *Pfu* DNA polymerase (Figure 3.3). The two fragments of *adiponectin* were amplified by additional PCR using forward primer of exon 1 and

reverse primer of exon 2 using *Taq* polymerase to collect sufficient amount of PCR product for next steps of cloning (Figure 3.3).

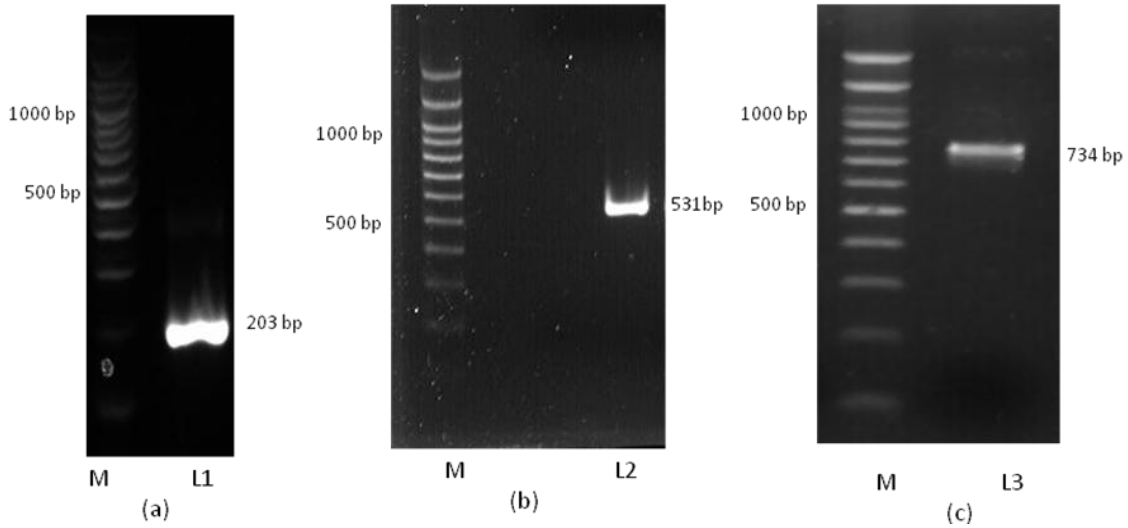


Figure 3.1: *In vitro* construction of *ADPs.* (a) PCR product of exon 1 (b) PCR product of exon 2 (c) the full lengths of *ADPs.* M: 100bp DNA marker, L1: exon 1(203 bp), L2: exon 2 (531 bp), L3: full length *ADPs* (734 bp).

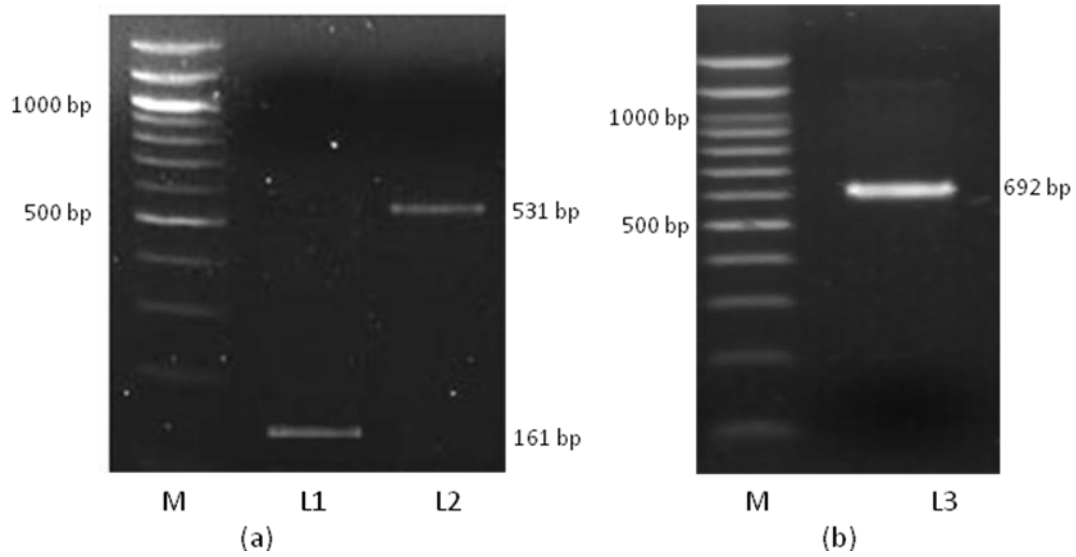


Figure 3.2: *In vitro* construction of *ADPws*. (a) PCR product of exon 1 and exon 2 (b) PCR product of full length *ADPws*. M : 100bp DNA marker, L1: exon 1(161 bp), L2: exon 2 (531 bp), L3: full length *ADPws* (692 bp).

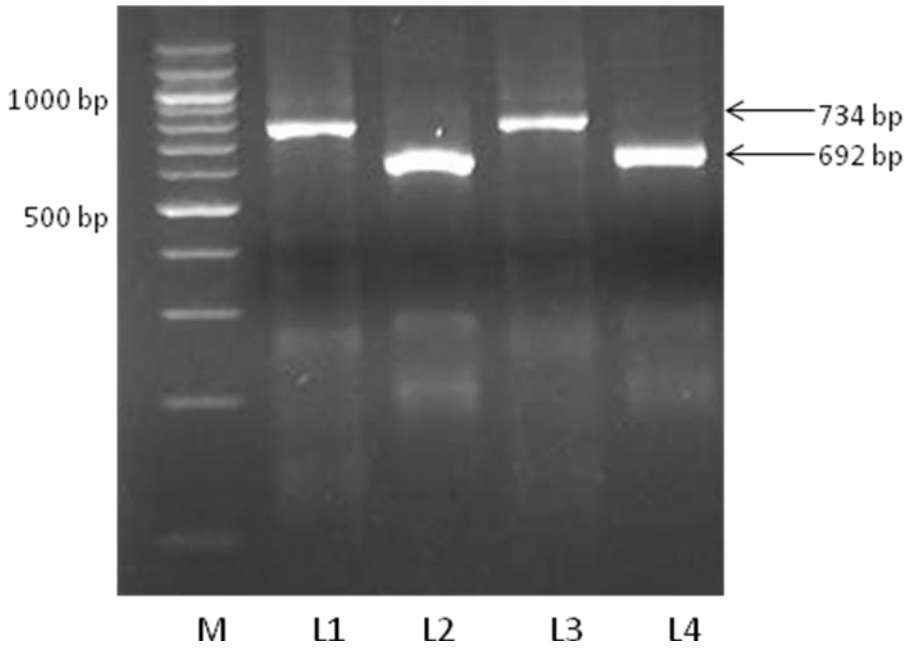


Figure 3.3: Amplification of *ADPs* and *ADPws* using different primers for cloning into *P. pastoris* and *E. coli*. M: 100bp DNA marker, L1 and L3: *ADPs* (734bp), L2 and L4: *ADPws* (692bp).

3.2. Expression of *adiponectin* gene in *E. coli*

The pMAL™ Protein Fusion and Purification System was used to express human *adiponectin* fragments in *E. coli*. Two types of plasmids are provided with the kit. The pMAL™-p4x plasmid which contains signal peptide sequence that can be used for periplasm recombinant protein secretion and the pMAL™-c4x which does not contain signal peptide sequence therefore this plasmid can be used for intracellular recombinant protein expression (Figure 3.4).

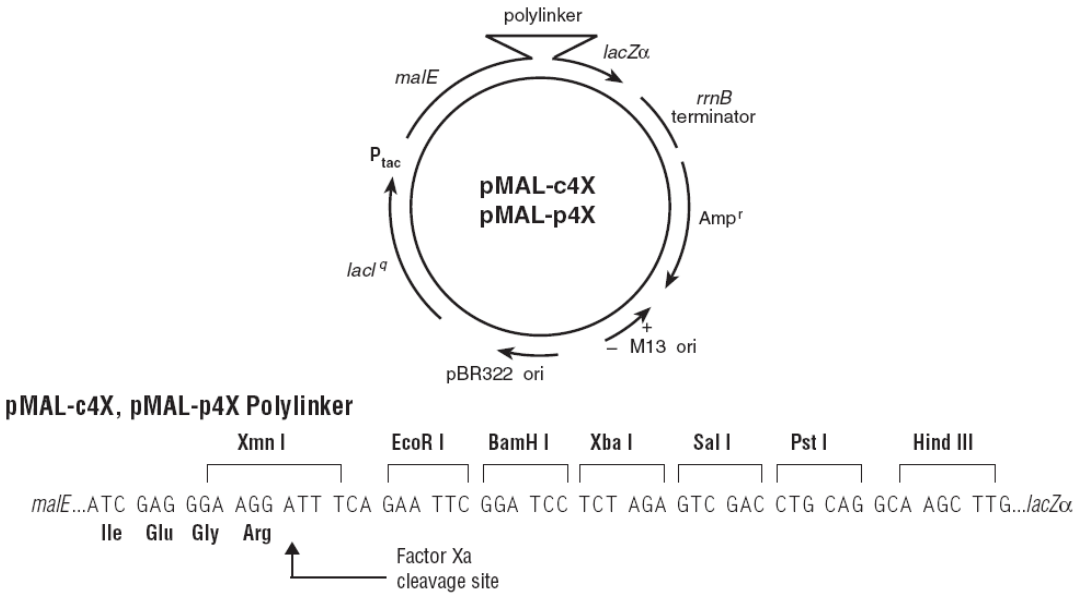


Figure 3.4: The map of pMAL™-p4x and pMAL™-c4x vectors explaining enzymes restriction sites.

3.2.1. Restriction enzyme digestion

Adiponectin fragments were purified and digested with *Hind*III to facilitate ligation into cloning vector (Figure 3.5). At same time, the pMAL™-p4x was used for extracellular secretion of recombinant protein. This vector was digested with *Xmn* I restriction enzyme which cuts at the consensus sequence 5'- GAANN ¦ NNTTC-3' to create blunt end. The second digestion of the pMAL™-p4x plasmid was with *Hind* III restriction enzymes to create sticky end (Figure 3.5).

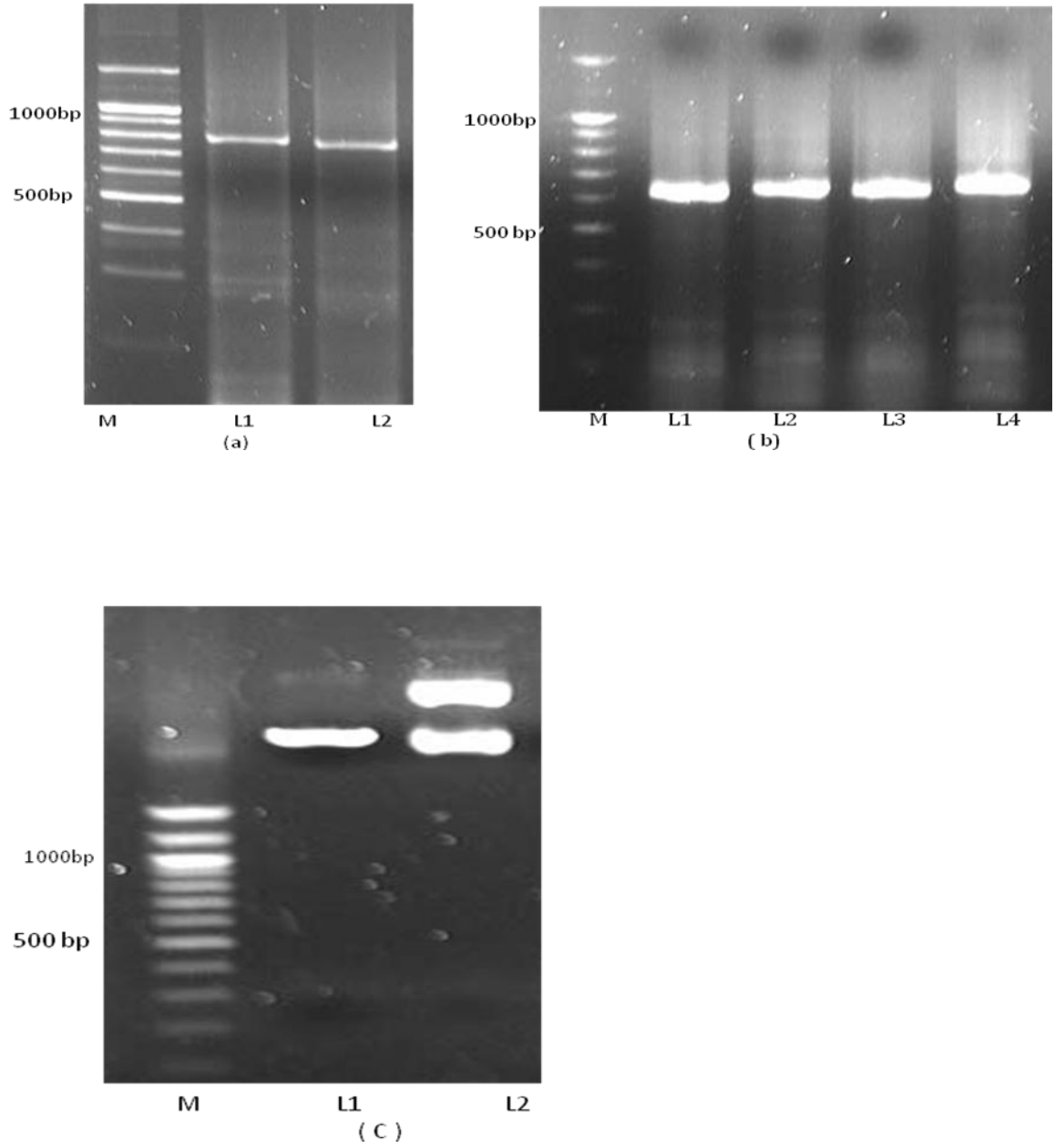


Figure 3.5: Restriction enzyme digestion for cloning into *E. coli*. (a) Digestion *ADPs* (expected size 734 bp) with *Hind*III restriction enzyme. M: DNA marker (100bp), L1: *ADPs* before digestion, L2: *ADPs* after digestion. (b) Digestion *ADPws* (expected size 692 bp) with *Hind*III restriction enzyme. M: DNA marker (100bp). L1, L2 and L3: *ADPws* after digestion. L4: *ADPws* before digestion. (c) Digestion of pMAL™p4x vector with *Xmn*I and *Hind*III restriction enzymes. M: DNA marker (100bp), L1: pMAL™p4x vector after digestion, L2: pMAL™p4x vector before digestion.

3.2.2. Selection of recombinant *E. coli* colonies

After ligation of digested *adiponectin* fragments with digested pMAL™p4x vector, the recombinant plasmid was transformed into *E. coli*. Bacterial cells were grown overnight on LB-agar-ampicillin medium without IPTG. Therefore all of the growing colonies were white colour colonies. The colonies were then transferred to the master library plate and sub-library plate contains IPTG and X-Gal to determine *Lac* phenotype. The white colonies grown on sub-library were selected for colony PCR amplification and the blue colonies were ignored. The PCR reaction was performed to confirm the presence of *adiponectin* fragments in the correct orientation in pMAL™p4x vector. Therefore the forward primer that was used in colony screening PCR was the signal peptide *mal*E primer (5´-GGTCGTCAGACTGTCGATGAAGCC- 3´) and *adiponectin* fragments specific reverse primer. After annealing *mal* E primer, the 5´ end of this primer is about 98 bases from the 5´ end of the *Xmn*I site in the plasmids (see Figure 3.4). Thus, the expected size of the colony screening PCR products were approximately 832 bp and 790 bp depending on the expected size of *ADPs* (734 bp) (Figure 3.6) and *ADPws* (692 bp) (Figure 3.7).

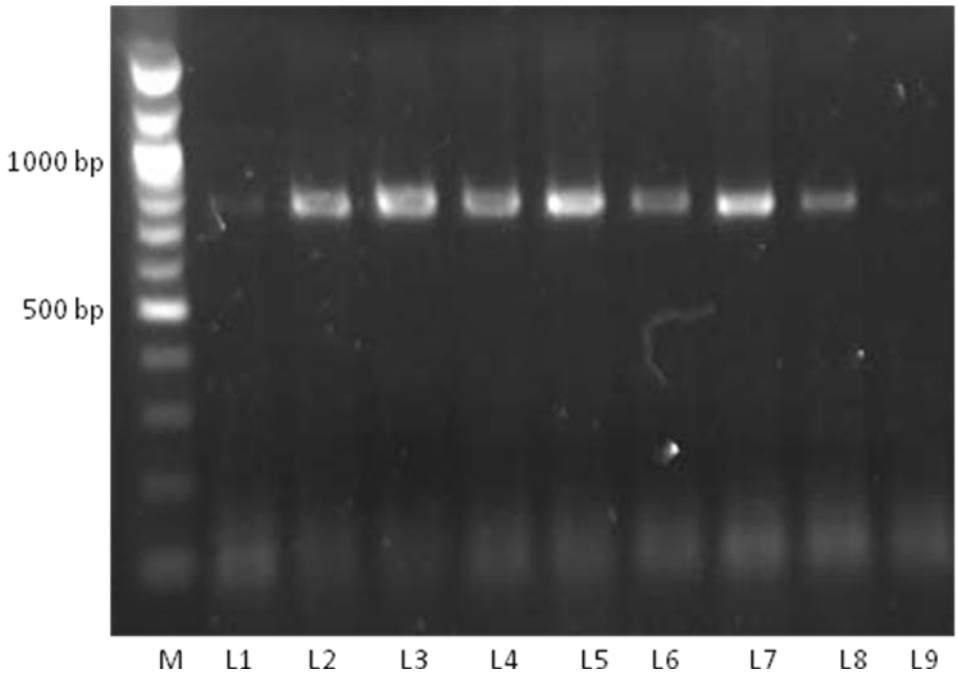


Figure 3.6: Agarose gel electrophoresis of colony screening PCR for *ADPs.* The positive colonies (L2 to L8) were selected according to the expected size (~832 bp) of PCR product using signal peptide *mal*E primer as forward primer and *adiponectin* fragments reverse primer. However the lane without expected size of PCR product (L1 and L9) stands for negative colony.

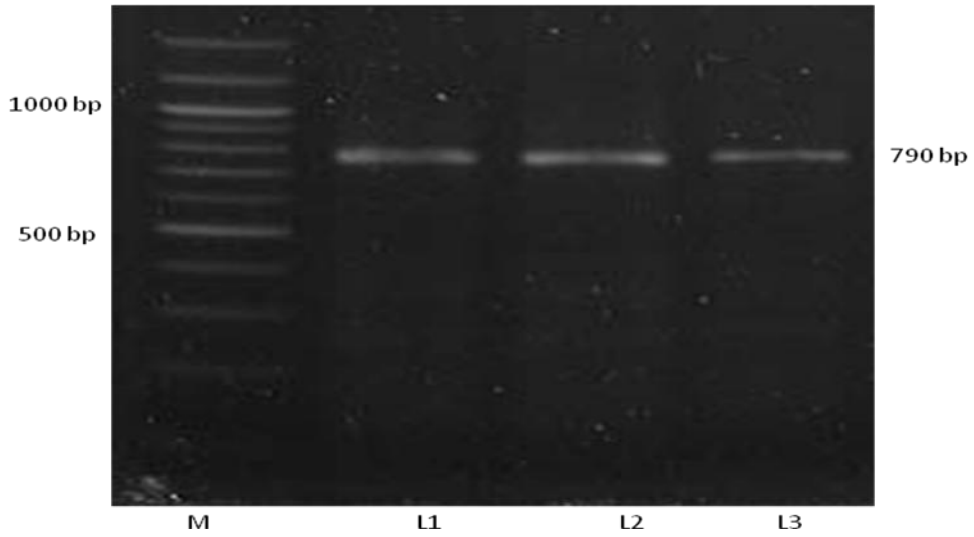


Figure 3.7: Agarose gel electrophoresis of colony screening PCR for *ADPws*. The positive colonies had been selected according to the expected size (~790 bp) of PCR product using signal peptide *mal*E primer as forward primer and *adiponectin* fragments reverse primer.

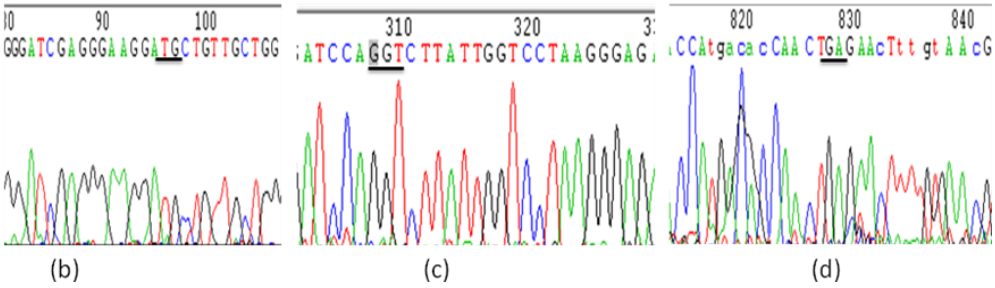
3.2.3. Sequencing analysis

ADPs construct was used to express by *E. coli* after positive colonies selection by PCR. Then, the recombinant plasmid *ADPs*-pMAL™-p4x was isolated and sequenced. The sequence was matched with *adiponectin* coding sequence in GenBank for exon 1 and exon 2 (Figure 3.7(a)). Results show that joining exon 1 and exon 2 by overlapping PCR was successfully carried out.

In the sequencing process, the forward primer was *mal*E primer (5´-GGTCGTCAGACTGTCGATGAAGCC- 3´) that anneals with *mal*E gene and the reverse primer was pUC30 (5´-CGCCAGGGTTTTCCCAGTCACGAC-3´) that anneals with *lacZα* sequence (see Figure 3.4). According to the sequencing data, *ADPs* started from ATG initiation codon (Figure 3.7 (b)) and ended by TGA stop codon (Figure 3.7(d)). In addition, the full length of *ADPs* was confirmed by GGT codon that joins exon 1 with exon 2 (Figure 3.7 (a & c)). There was no mutation detected in nucleotides sequences during gene construction and amplification *in vivo* and *in vitro*.

ATG CTG TTG CTG GGA GCT GTT CTA CTG CTA TTA GCT CTG CCC GGT CAT GAC CAG GAA ACC ACG ACT
CAA GGG CCC GGA GTC CTG CTT CCC CTG CCC AAG GGG GCC TGC ACA GGT TGG ATG GCG GGC ATC CCA
GGG CAT CCG GGC CAT AAT GGG GCC CCA GGC CGT GAT GGC AGA GAT GGC ACC CCT GGT GAG AAG GGT
GAG AAA GGA GAT CCA ***GGT*** CTT ATT GGT CCT AAG GGA GAC ATC GGT GAA ACC GGA GTA CCC GGG GCT
GAA GGT CCC CGA GGC TTT CCG GGA ATC CAA GGC AGG AAA GGA GAA CCT GGA GAA GGT GCC TAT GTA
TAC CGC TCA GCA TTC AGT GTG GGA TTG GAG ACT TAC GTT ACT ATC CCC AAC ATG CCC ATT CGC TTT
ACC AAG ATC TTC TAC AAT CAG CAA AAC CAC TAT GAT GGC TCC ACT GGT AAA TTC CAC TGC AAC ATT
CCT GGG CTG TAC TAC TTT GCC TAC CAC ATC ACA GTC TAT ATG AAG GAT GTG AAG GTC AGC CTC TTC
AAG AAG GAC AAG GCT ATG CTC TTC ACC TAT GAT CAG TAC CAG GAA AAT AAT GTG GAC CAG GCC TCC
GGC TCT GTG CTC CTG CAT CTG GAG GTG GGC GAC CAA GTC TGG CTC CAG GTG TAT GGG GAA GGA GAG
CGT AAT GGA CTC TAT GCT GAT AAT GAC AAT GAC TCC ACC TTC ACA GGC TTT CTT CTC TAC CAT GAC
ACC AAC ***TGA***

(a)



(b) (c) (d)

Figure 3.8: Sequencing data of *ADPs*. (a) The full sequence of *ADPs* starting by initiation codon ATG and ending by stop codon TGA. The red color stands for exon 1 sequence whereas the green color stands for exon 2 sequence and these exons were joined by GGT codon. Sequence chromatography showed *ADPs* start codon(ATG) (b) and GGT codon that joined exon 1 with exon 2 (c) and the stop codon TGA (d).

3.2.4. Recombinant adiponectin analysis

Following conformation of the full sequence of *ADPs*, a pilot experiment for adiponectin expression was carried out. One positive colony was used to inoculate 10ml of LB-ampicillin broth. Later, the overnight culture was used to inoculate 80ml of the medium. When the bacterial cells were at an early log phase, a sample was taken before induction. Then, IPTG was added to induce P_{tac} promoter located up stream of the *mal*E gene that encodes maltose binding protein (MBP) and *ADPs* (see Figure 3.4). Induction of P_{tac} promoter will induce the expression of *mal*E gene and *ADPs* to produce a fusion protein that consists of MBP fused with adiponectin protein. Bacterial cells samples were taken before induction and after induction with IPTG. All samples were prepared for SDS-PAGE analysis and staining by coomassie stain. It was shown that recombinant adiponectin expression was undetectable before induction with IPTG. However, the expression level varied with the time after induction. According to the SDS-PAGE and western blot analysis using anti-adiponectin antibody, the highest expression level was at 2 hours after induction (Figure 3.8 and 3.9).

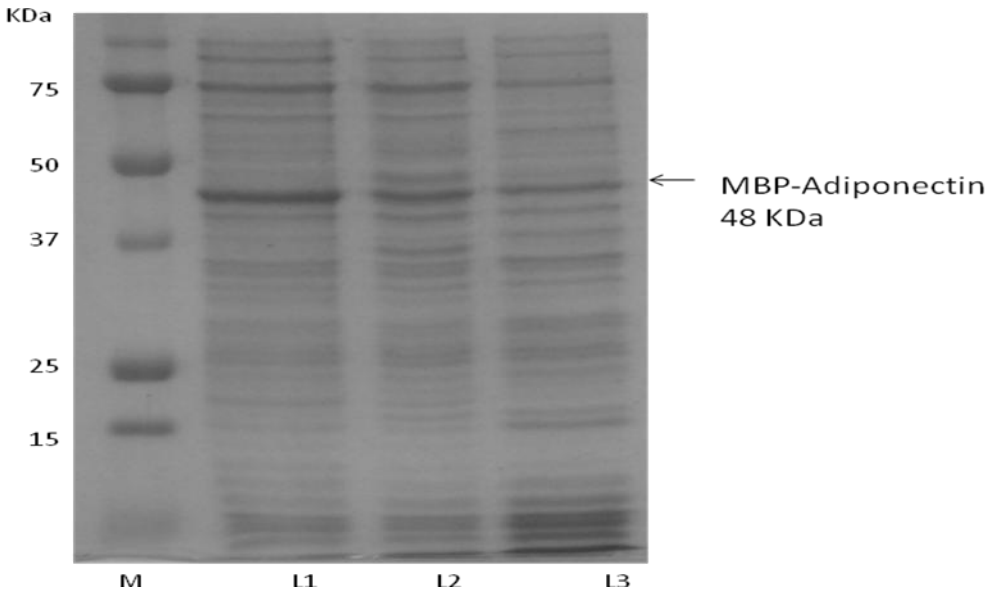


Figure 3.9: SDS-PAGE analysis of adiponectin expression before and after induction with IPTG. According to the protein marker (M) the expected size of fusion protein (MBP-adionectin) was approximately 48 kDa. The bands of fusion protein had been denser after 2 hours (L1). In addition, one hour after induction with IPTG, the fusion protein was more detectable (L2) and the band of fusion protein was faint before induction (L3).

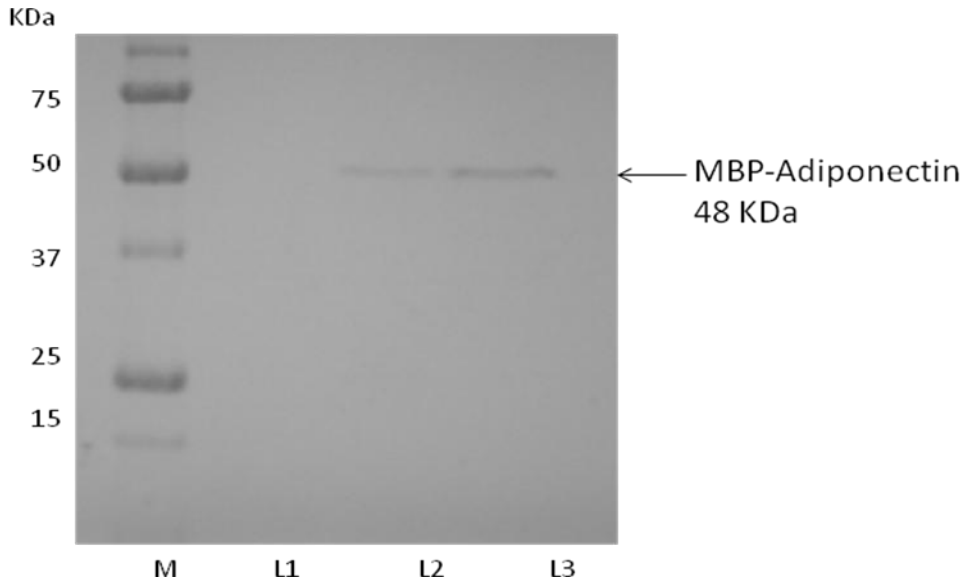


Figure 3.10: Western blot analysis of adiponectin expression before and after induction with IPTG. According to the protein marker (M) the expected size of fusion protein (MBP-adionectin) was undetectable before induction (L1) and after one hour of induction with IPTG the band of fution protein was detectable (L2). However, the bands of fusion protein had been densest at 2 hours after induction (L3).

3.2.5. Scale up expression of adiponectin by *E. coli*

Based on the previous data the culture batch was scaled up to one liter of LB rich medium. One colony of recombinant *E. coli* was used to inoculate 10ml of LB broth. Then, the overnight culture was used to inoculate one liter of LB medium. The initial OD_{600} was 0.089 when the inoculation was initially made. The culture was incubated until OD600 reached 0.72. Then, IPTG was added for induction and the culture were allowed to continue in incubation for 3 hours or until the OD_{600} was 6. The amount of 3.9g total bacterial wet cells weight was used for recombinant protein extraction and the total recombinant adiponectin recovered was 40mg/L.

3.2.6. Recombinant adiponectin purification

The cloned *ADPs* was inserted down-stream from the *mal*E of *E. coli*, that encodes maltose binding protein (MBP) resulting in the expression of an MBP fusion protein. Therefore, the amylose resin column of 25ml volume was used for fusion protein purification. The SDS-PAGE result after first purification indicated that the expected size of the fusion protein was approximately 48 kDa (Figure 3.10). As mentioned previously, maltose binding protein was linked with adiponectin protein by four amino acids (ile-glu-gly-arg). These amino acids can be recognized by Factor Xa for cutting and separating adiponectin and maltose binding protein. The expected size of adiponectin after digestion with Factor Xa was approximately 30 kDa (Figure 3.11).

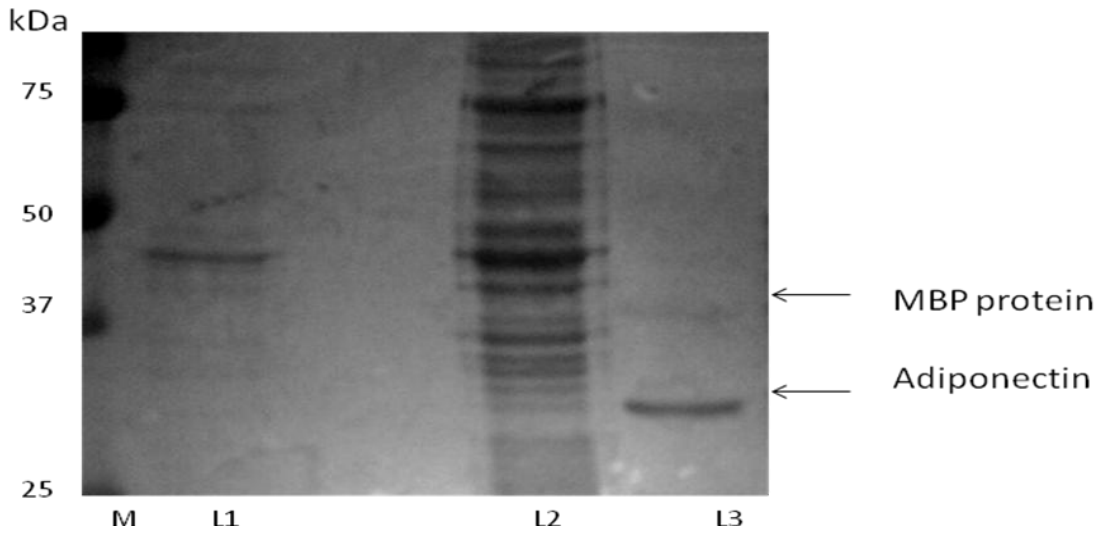


Figure 3.11: Purification by amylose resin column. According to the protein marker (M) the expected size of fusion protein was approximately 48 kDa (L1 after purification & L2 before purification). However, the expected size of adiponectin protein was approximately 30 kDa (L3). It is important to notice that the expected size of adiponectin (~30 kDa) had not appeared in the protein profile before purification and faint band of maltose binding protein (MBP protein) had appeared at expected size of 37 kDa (L3).

In order to optimize Factor Xa digestion, protein samples were incubated for 2, 4 and 8 hours with the same concentration of Factor Xa. One of crude protein sample was digested with same concentration of Factor Xa as a positive control. Initial results show that there was no clear difference between protein samples after digestion by incubation with Factor Xa for different length of time (Figure 3.12). Therefore, when protein digestion was scaled up, all protein samples were incubated with Factor Xa for two hours. In the positive control that contains crude protein digested with Factor Xa, maltose binding protein (MBP) has a higher molecular weight than adiponectin protein. This protein, like other *E. coli* native proteins totally disappeared after second purification by amylose resin column (Figure 3.12). The second purification was carried out to eliminate maltose binding protein residue. Purification was performed by passing the fusion protein cleavage product through the hydroxyapatite column to remove maltose. The eluted protein was then loaded into the amylose resin column and the flow through fractions were collected. Protein in the flow through was free of MBP protein and consists of adiponectin protein (Figure 3.12).

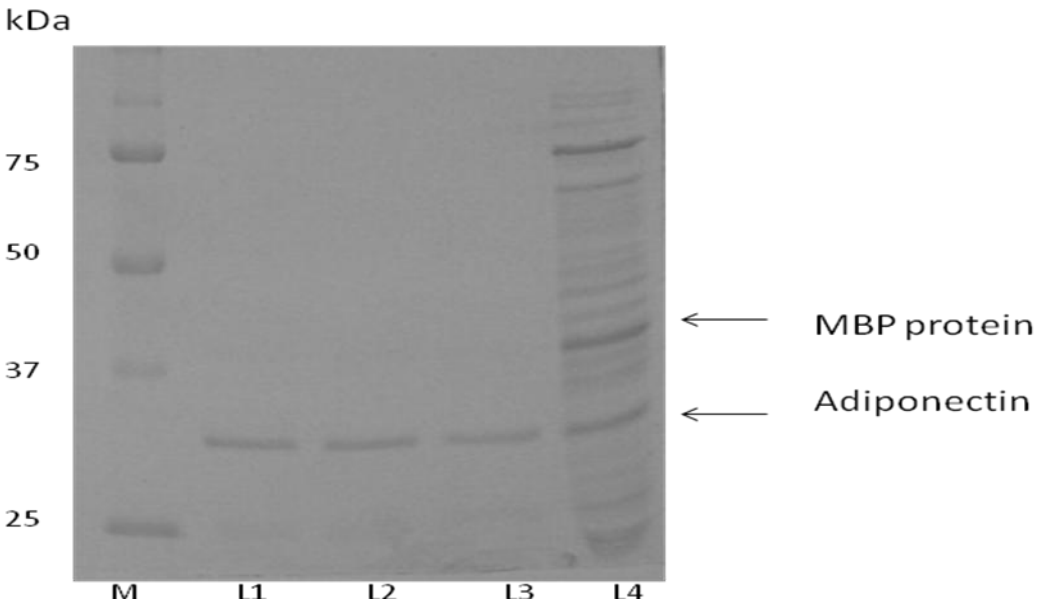


Figure 3.12: SDS-PAGE for optimization of Factor Xa digestion. Results show that there was no clear difference between protein digestion with Factor Xa for 2 hours (L1), 4 hours (L2) and 8 hours (L3). Digestion of crude protein with Factor Xa showed that the expected size of adiponectin protein is approximately 30 kDa (L4).

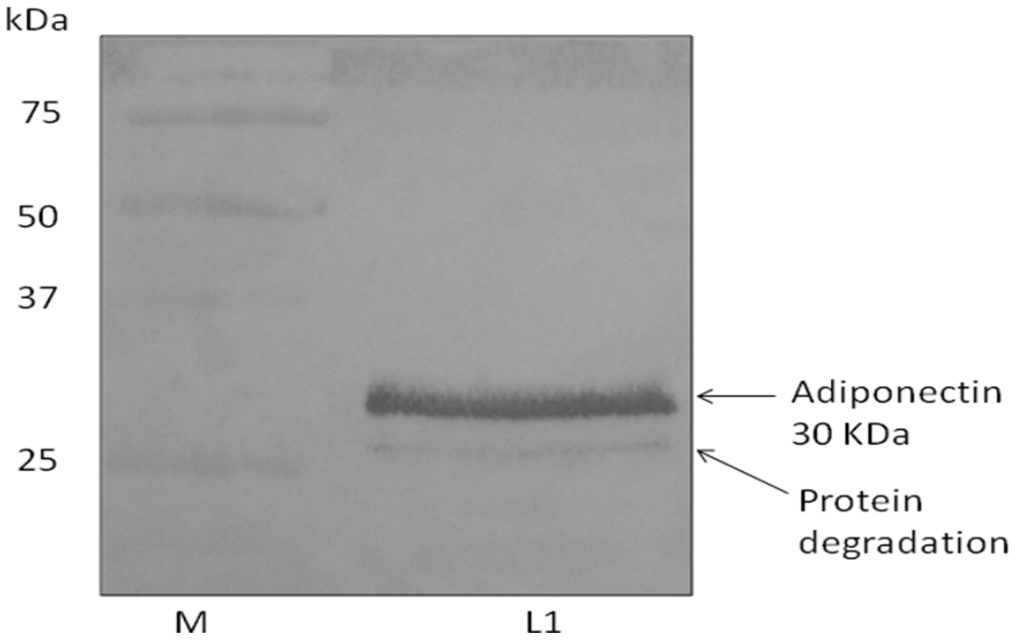


Figure 3.13: SDS-PAGE for second purification by hydroxyapatite column and amylose resin column. One band appears in the expected size of adiponectin protein (30 kDa).

3.3. Expression of *adiponectin* by *Pichia pastoris*

The yeast *P. pastoris* was used to express human *adiponectin* in eukaryotic expression system. Therefore, Easy Select ™ pichia Expression Kit was used to produce recombinant human adiponectin as extracellular secreted protein by yeast. pPICZαA plasmid contains *Saccharomyces cerevisiae* α-factor secretion signal peptide for extracellular protein secretion. *Adiponectin* sequence was cloned in frame with the sequence of α-factor secretion signal peptide (Figure 3.14).

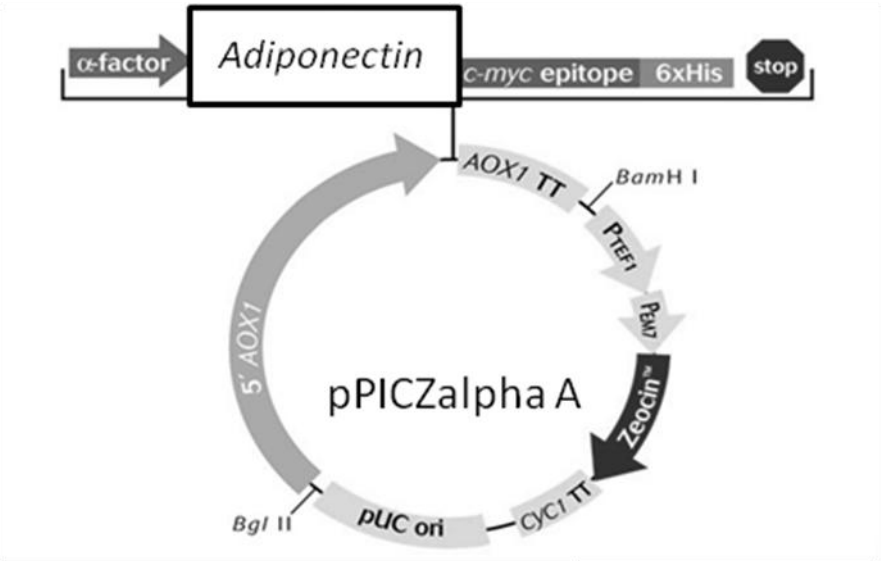


Figure 3.14: The map of pPICZα A plasmid that was used to clone the gene of interest into *P. pastoris. Adiponectin* sequence was cloned in frame with the α-factor secretion signal peptide sequence. The AOX1 promoter was induced to allow drive *adiponectin* transcription by adding methanol to the yeast culture. The presence of c-*myc* epitope can be used for antibody detection and 6xHis tag can be used for antibody detection and Nickel column purification.

3.3.1. Cloning of *adiponectin* in pGEM-T vector

Full length *adiponectin* was constructed *in vitro* by overlapping extension PCR in order to join exon 1 with exon 2. Subsequently, two fragments of *adiponectin* were constructed with (*ADPs*) and without (*ADPws*) *adiponectin* signal peptide. The expected size of *ADPs* was 734 bp and *ADPws* was 692 bp. On the other hand, the expected size of plasmid DNA fragment between M13 forward and reverse primers without gene insertion is 250 bp. Therefore, the expected size between M13 primers with each of *adiponectin* fragments was 984 bp for *ADPs* (Figure 3.16) and 942 bp for *ADPws* (Figure 3.17).

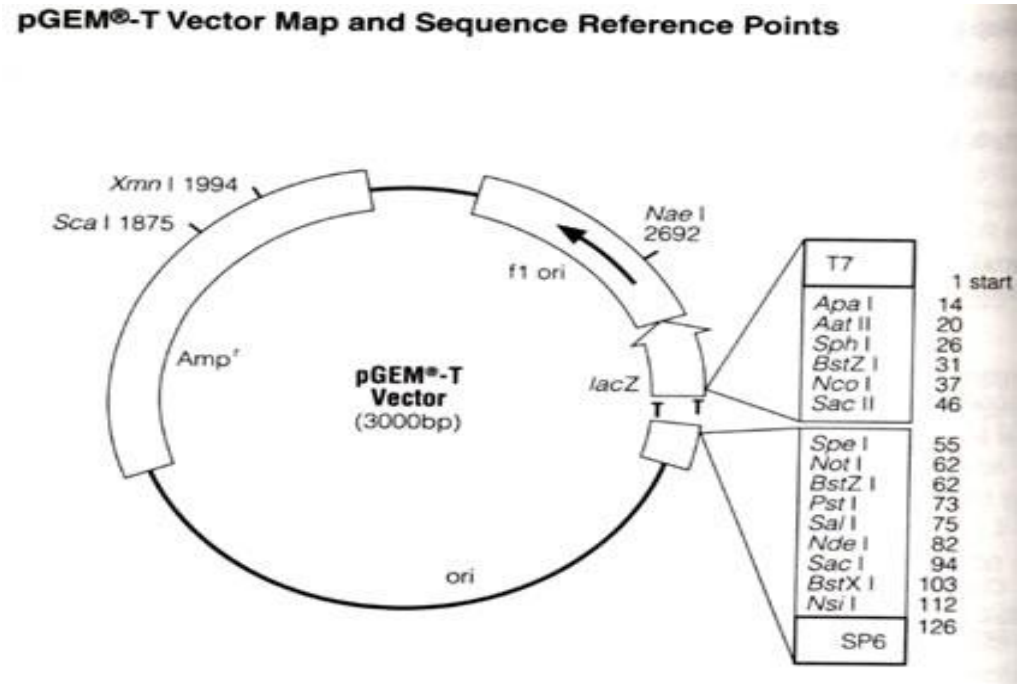


Figure 3.15: pGEMT-vector map. The plasmid contains an ampicillin resistant gene encoding a β-Lactamase. Once transformation is performed with pGEMTvector and E.coli JM109, color screening is done to identify recombinant clones. White colonies have a DNA fragment inserted while blue colonies have no insertion. The insertion disrupts the *β- galactosidase* gene and the colonies appear white.

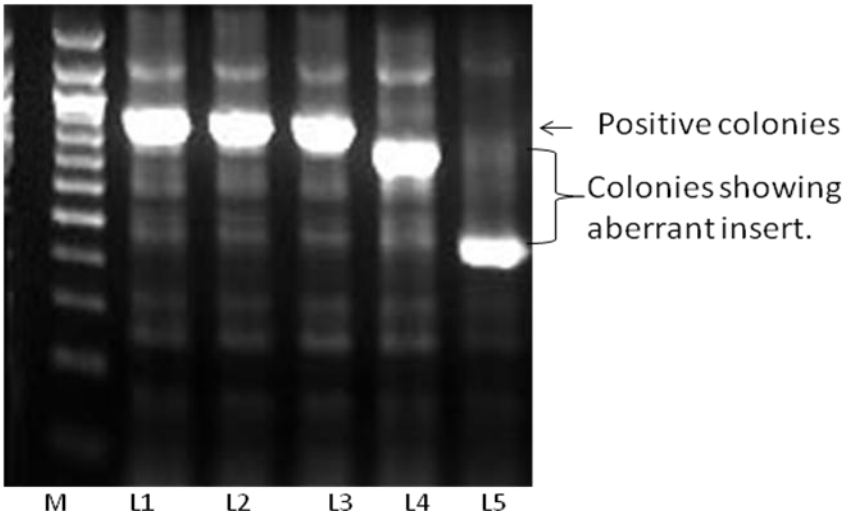


Figure 3.16: Agarose gel electrophoresis of colony selection by PCR using M13 forward and reverse primers to detect existence of *ADPws*. M: DNA marker (100bp). L1, L2 and L3: positive colonies showing the expected size between M13 primers with *ADPws* was 942 bp. L4 and L5: colonies showing aberrant insert.

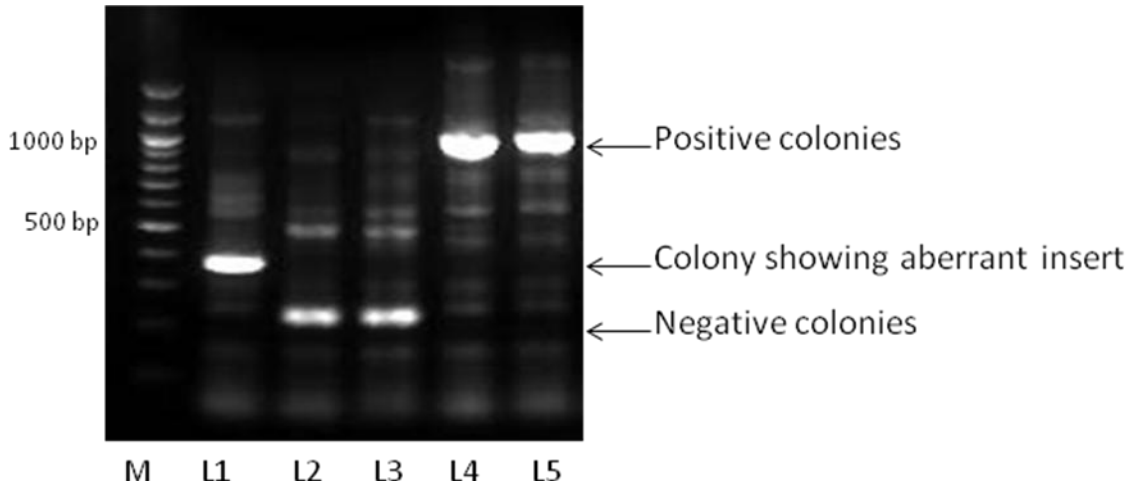


Figure 3.17: Agarose gel electrophoresis of colony selection by PCR using M13 forward and reverse primers to confirm existence of *ADPs*. M: DNA marker (100bp). L1: aberrant insert. L2 and L3: negative colonies showing expected size between M13 forward and reverse primers without gene insertion (250 bp). L4 and L5: positive colonies showing the expected size of the *ADPs* fragment was 984 bp.

3.3.2. *Eco*RI and *Not*I digestion

The bacterial colonies that contained the desired recombinant plasmid were used for plasmid isolation (Figure 3.18). The recombinant plasmid was digested with *Eco*RI and *Not*I in order to generate sticky ends of *Eco*RI and *Not*I termini. After restriction enzyme digestion, the expected size of *ADPs* was 734bp (Figure 3.19) whereas the expected size of *ADPws* was 692bp (Figure 3.20). The cloning in the pPICZαA expression vector should regard the open reading frame of *Saccharomyces cerevisiae* α-factor secretion signal peptide. The expression vector pPICZαA was isolated from *E. coli* (Top 10™) strain and also digested with *Eco*RI and *Not*I restriction enzymes (Figure 3.21).

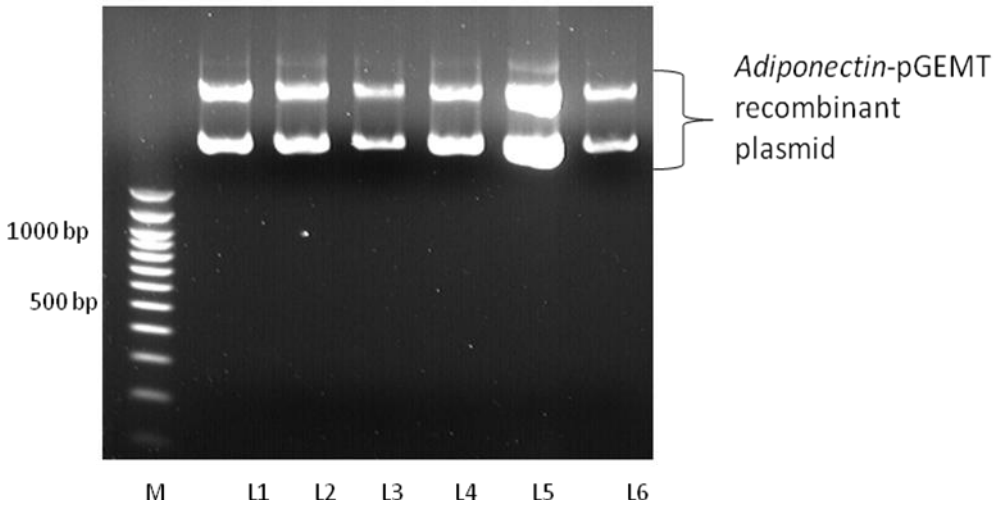


Figure 3.18: Agarose gel electrophoresis of *adiponectin*-pGEMT recombinant plasmid (L1 to L6).

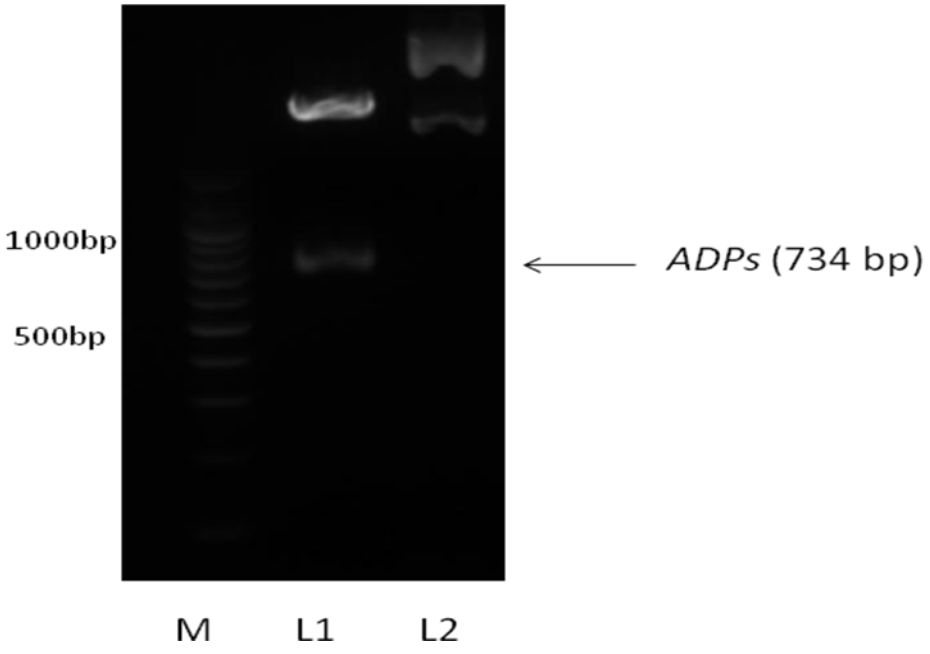


Figure 3.19: Agarose gel electrophoresis of restriction enzyme digestion to generate *ADPs* with two sticky ends. The expected size of *ADPs* after digestion was approximately 734 bp. M: DAN marker (100bp). L1: *ADPs*-pGEMT recombinant plasmid after digestion with *Eco*RI and *Not*I. L2: Control.

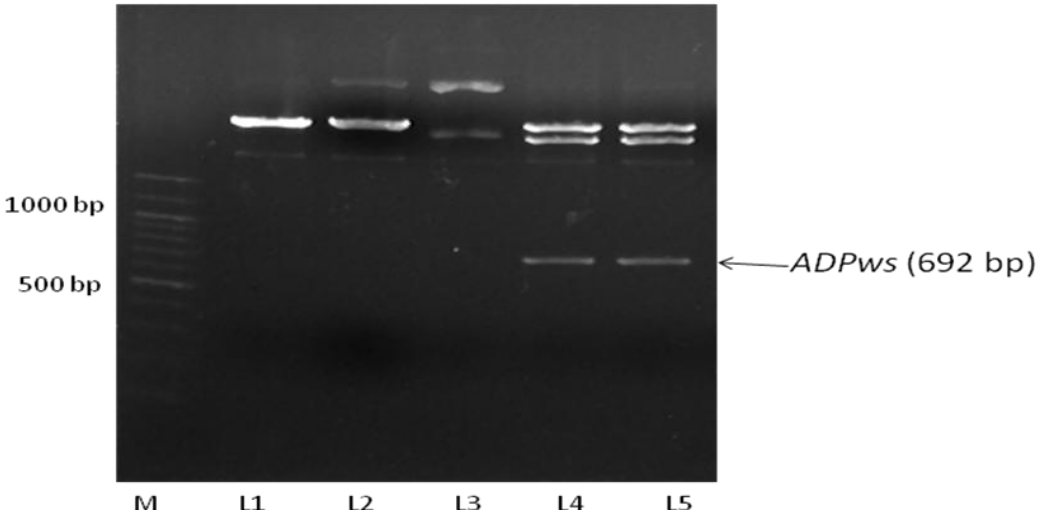


Figure 3.20: Agarose gel electrophoresis of restriction enzyme digestion to generate *ADPws* with two sticky ends. The expected size of *ADPws* after digestion was approximately 692 bp. M: DNA marker (100bp). L1: Adiponectin-pGEMT recombinant plasmid after digestion with *Eco*RI. L2: *ADPws*-pGEMT recombinant plasmid before digestion with *Eco*RI. L3: control plasmid without restriction enzyme. L4 and L5: *ADPws*-pGEMT recombinant plasmid after digestion with *Eco*RI and *Not*I.

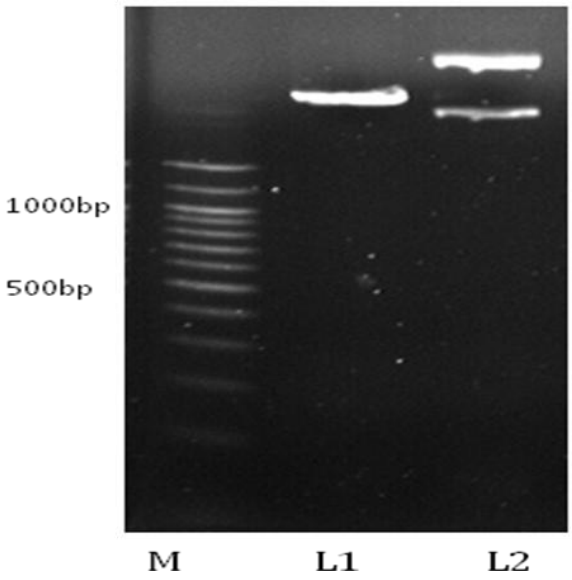


Figure 3.21: Agarose gel electrophoresis of pPICZαA plasmid digestion with *Eco*RI and *Not*I. M: DNA marker (100bp), L1: digested plasmid, L2: plasmid without digestion.

3.3.3. Cloning *adiponectin* in pPICZαA

Each one of *Adiponectin* fragments with two sticky ends and the digested pPICZαA plasmid were ligated by DNA ligase. Then, each of the recombinant plasmids (*ADPs*-pPICZαA and *ADPws*-pPICZαA) was transformed into *E. coli* (Top 10™) strain using heat shock. When bacterial cells were plated on the solid medium and grown overnight, all the gowning colonies were white color colonies because there is no *Lac* phenotype. Therefore, colony screening PCR was used to select the positive colonies that carried the recombinant plasmids. The α-factor forward primer and 3'AOX1 reverse primer was used to select positive colonies. The expected size from the beginning of α-factor priming site to 3' end of AOX1 priming site was 250bp. Therefore, the expected size of the band in the agarose gel was approximately 942bp for *ADPws* (Figure 3.22) and for *ADPs* was 984bp (Figure 3.23). However, there are two bands in same lane one of them in the expected size of the recombinant plasmid (985bp) and another one in the expected size of the plasmid without gene insertion (250bp).

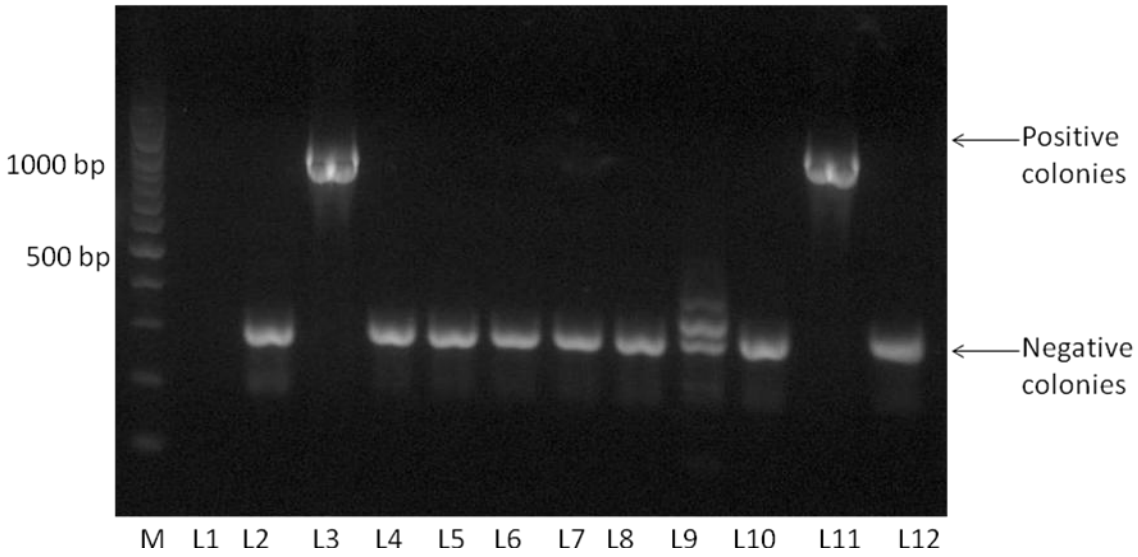


Figure 3.22: Colony screening PCR using α-factor forward primer and 3'AOX1 reverse primer to detect *ADPws*. The expected size of *ADPws* was 942bp included the expected size from the beginning of α-factor priming site to 3' end of AOX1 priming site (250bp). L1: control, L2 to L12: negative colonies except L3 and L11were positive colonies.

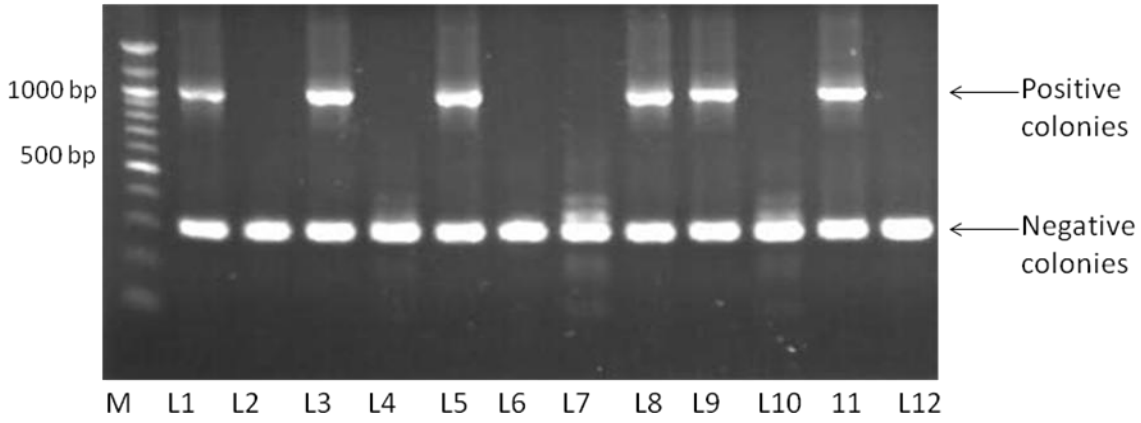


Figure 3.23: Colony screening PCR using α-factor forward primer and 3'AOX1 reverse primer to detect *ADPs.* The expected size of *ADPs* was 985bp included the expected size from the beginning of α-factor priming site to 3' end of AOX1 priming site (250bp). L1, L3, L5, L8, L9 and L11: positive colonies while L2, L4, L6, L7, L10 and L12: negative colonies.

3.3.4. Sequencing data

As mentioned previously, the coding sequence of *adiponectin* gene consists of two exons. These two exons were spliced *in vitro* by overlapping–extension PCR. Then, the two types of *adiponectin* constructs (with and without signal peptide) were amplified *in vivo* by cloning in pGEM-T vector and transformed into *E. coli*. After recombinant plasmid isolation (*adiponectin*-pGEMT) and restriction enzyme digestion, the two constructs of *adiponectin* were cloned into pPICZαA vector and also transformed into *E. coli*. It is important to confirm that the *ADPws* construct of *adiponectin* matched with the *Adiponectin* sequence obtained from GenBank under the accession number NC_000003.11 (Sherer et. al, 1995) (Figure 3.24). However, the sequencing data of *ADPs* construct was not clear enough to check with *Adiponectin* sequence in GenBank because peaks interaction (Figure 3.25).

In the sequencing process, the forward primer was α-factor primer anneals with α-factor signal peptide sequence whereas, the reverse primer was 3'AOX1 primer that anneals with the 3' end of alcohol oxidase coding gene (*AOX1*). The expected size of DNA fragment between α-factor primer annealing site and 3'AOX1 primer annealing site is approximately 250 bp that provides sufficient flanking sequence to read and check *adiponectin* sequence.

According to the sequencing data, *adiponectin* fragments ended with AAC codon which is before stop codon. The stop codon was deleted in order to join *adiponectin* sequence with the sequence of *myc*-epitope and polyhistidine tag that are important for antibody detection and affinity purification. It was also confirmed that *adiponectin* sequence was in the open reading frame with α-factor signal peptide sequence. Furthermore, no mutation in nucleotides sequences had been made during gene construction and amplification *in vivo* and *in vitro*.

(a)

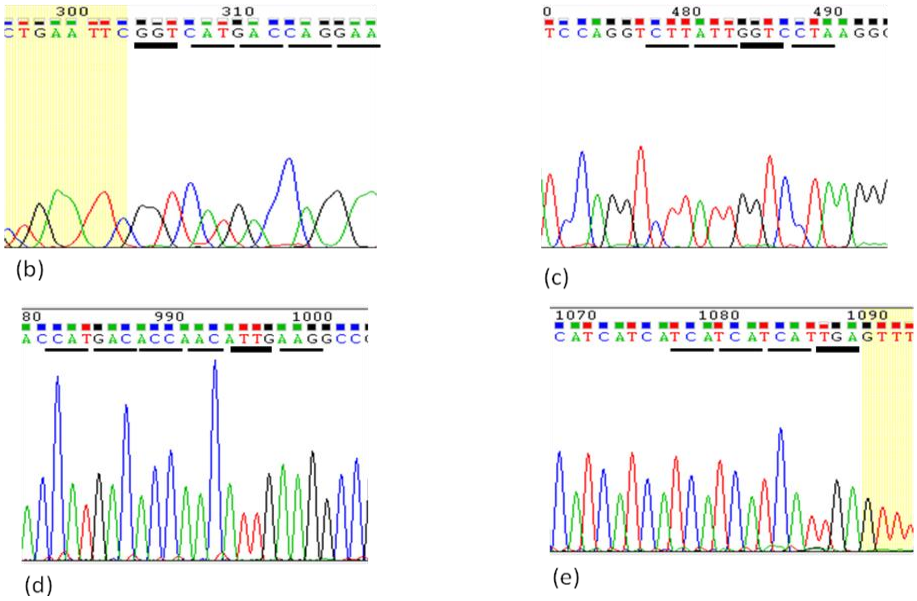


Figure 3.24: Sequencing data of *ADPws* after cloning in pPICZαA. (a) The full sequence of *adiponectin* starting with ATG codon and ending with stop codon TGA, (b) The sequence of *ADPws* starting with GGT, (c) GGT codon that joins exon 1 with exon 2, (d) Deletion of *adiponectin* stop codon TGA, and (e) 6XHis-tag sequence and stop codon TGA provided by the vector.

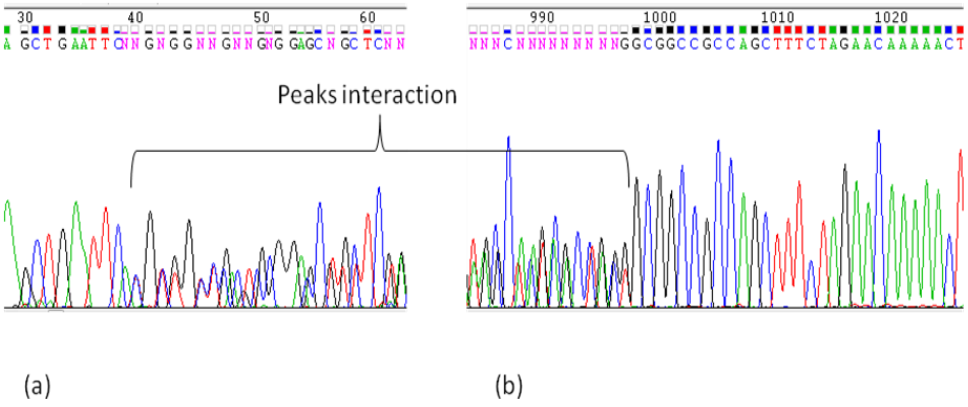


Figure 3.25: The sequence of *ADPs* after cloning in pPICZαA. The figure shows interactions of two different chromatography peaks starting with *adiponectin* sequence (a) to the end of *adiponecin* sequence (b).

3.3.5. Transformation into *Pichia pastoris*

The transformation efficiency of linear plasmid is higher than circular plasmid due to the increased ability to integrate into the *P. pastoris* genome. Therefore, recombinant plasmid pPICZαA-*adiponectin* was linearized with *Sac*I restriction enzyme at the *AOX1* locus (figure 3.26). Before digestion with *Sac*I, *adiponectin* sequence were ensured that there is no site for this restriction enzyme. Other restriction enzymes can be used to make linear plasmid such as *Pme*I and *BstX*I.

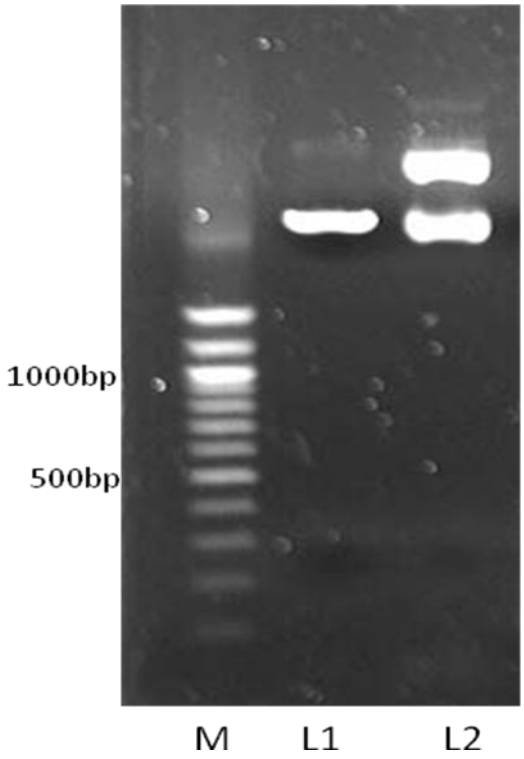


Figure 3.26: Recombinant plasmid linearization with *Sac*I restriction enzyme. L1: linear plasmid, L2: circular plasmid.

3.3.6. Colony selection

Linearized recombinant plasmid was transformed into X-33 strain of *P. pastoris* by heat shock. The transformation mixture was streaked on YPD-Zeocin™ plates and the plates were incubated at 30°C for 3 days. The colonies were then selected for screening the presence of the *adiponectin* fragments by PCR reaction using pPICZαA vector primers (α-factor forward primer and 3AOX1 reverse primer). The expected size of PCR product for self ligated vector (without insertion) was approximately 250 bp (Figure 3.27 and 3.28).

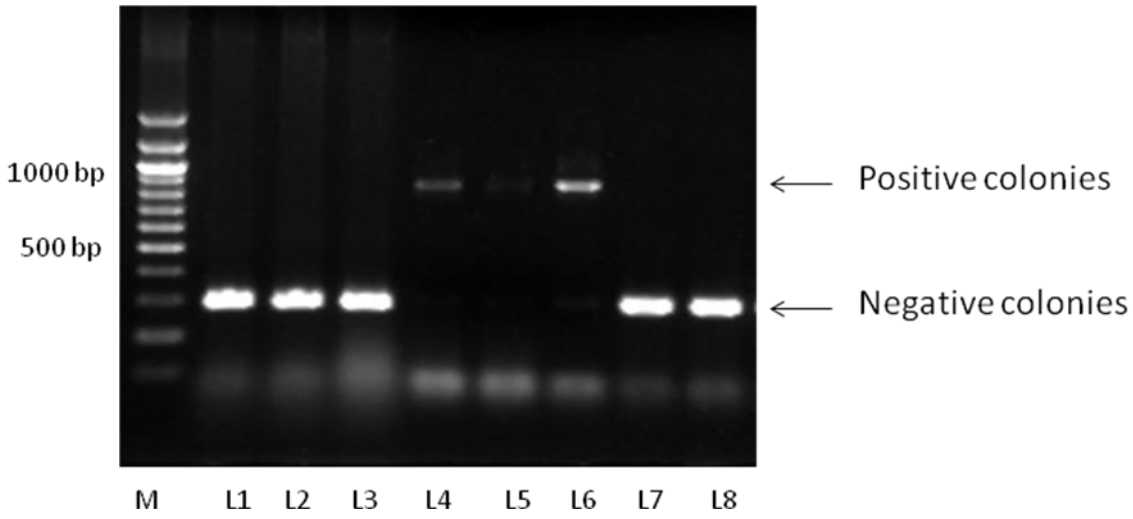


Figure 3.27: Colony PCR screening using α-factor forward primer and 3'AOX1 reverse primer to detect *ADPws* sequence into yeast colonies. L4, L5 and L6: The expected size of amplified plasmid fragment included *ADPws* was 942 bp (the positive colonies). L1, L2, L3, L7 and L8 without *ADPws* were 250 bp (negative colonies).

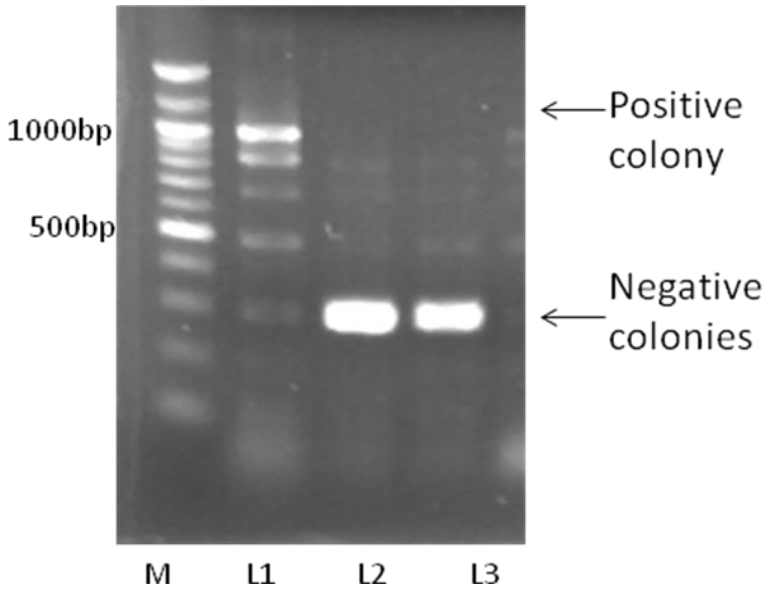
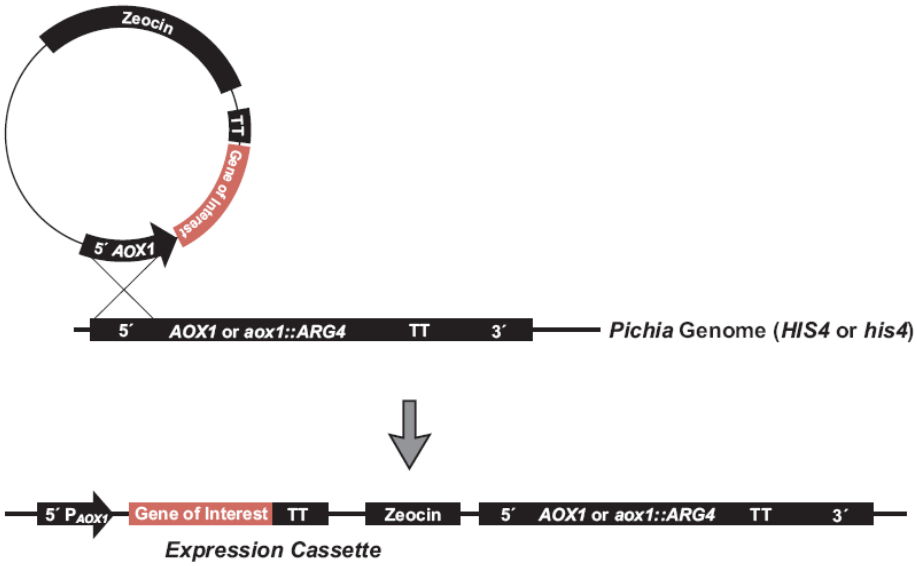


Figure 3.28: Colony screening PCR using α-factor forward primer and 3'AOX1 reveres primer to detect *ADPs* into yeast colonies. L1: the expected size of amplified plasmid fragment included *ADPs* was 985 bp (the positive colony). L2 and L3 without *ADPs* was 250 bp (negative colonies).

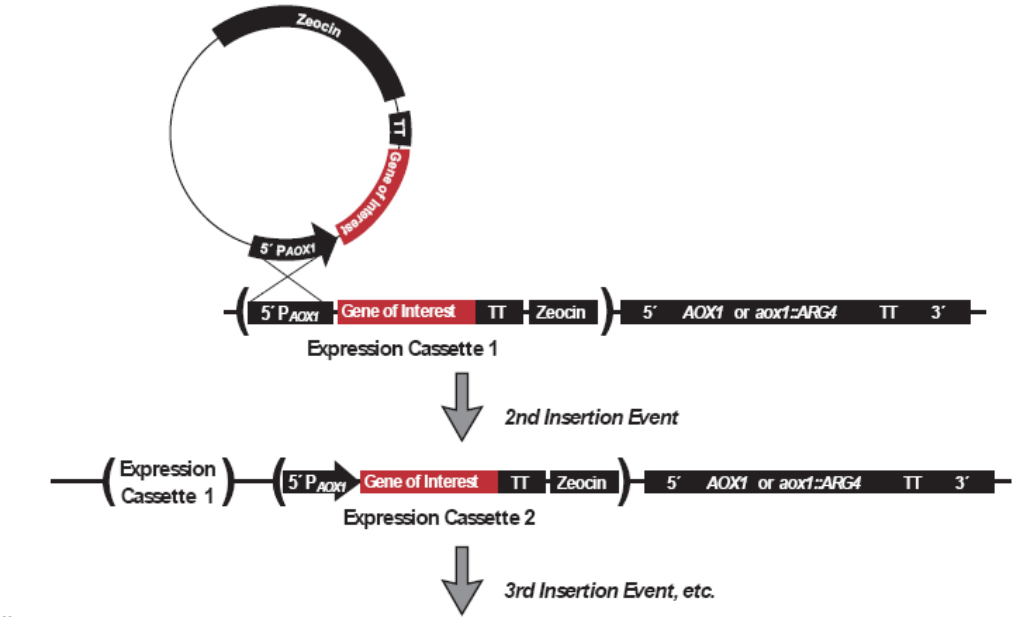
3.3.7. Selection of Mut$^+$ phenotype with high insertion copy number colonies.

The insertion of the *Adiponectin* gene construct at the *AOX1* loci takes place from a single crossover event between the loci and the *AOX1* promoter or the *AOX1* transcription termination region (TT) (figure 3.29, a). However, the presence of the *AOX1* sequence in the plasmid, it is possible that recombination will occur in the 3'*AOX1* region as well. This event will lead to the disruption of the wild type *AOX1* gene and creating Mut^s phenotype. The colonies that grow on the YPD-Zeocin™ agar and YPM-Zeocin™-agar were selected as Mut$^+$ phenotype because these colonies have an ability to use methanol as a source of carbon when methanol is available. However, the colonies that grew slowly on YPM-Zeocin™-agar and grew normally on YPD-Zeocin™-agar were not able to use methanol as a source of carbon (Mut^s phenotype). The confirmed Mut$^+$ colonies were used to detect multiple gene insertion (inserted gene copies).

Ideally, multi insertion of expression cassette (figure 3.29, b) will increase the ability of yeast colonies to resist higher concentration of the Zeocin™ antibiotic. The Mut$^+$ colonies that grew on the YPD agar-Zeocin™ plate contains 200 μg/ml as a final concentration of Zeocin™ were selected for small scale expression of recombinant adiponectin protein.



(a)



(b)

Figure 3.29: Multi insertion of expression cassette into yeast cells. (a) The result of an insertion of the plasmid 5' to the intact AOX1 locus (Mut^{+}). (b) Multiple gene insertion events at a single AOX1 locus. (Instruction manual, Invitrogen)

3.3.8. Small scale expression of adiponectin

Positive colonies were used for expression of extracellular adiponectin prorein. Six colonies of Mut^+ colonies carrying *ADPws* and five of *ADPs* were used to inoculate baffled flasks each contains 10 ml of BMGY media contain glycerol as a source of carbon. According to SDS-PAGE results, all of the yeast colonies carrying *ADPws* successfully produced recombinant adiponectin protein at expected size approximately 30 kDa. However, yeast colonies that carrying *ADPs* were not able to produce recombinant adiponectin (Figure 3.30 and 3.31).

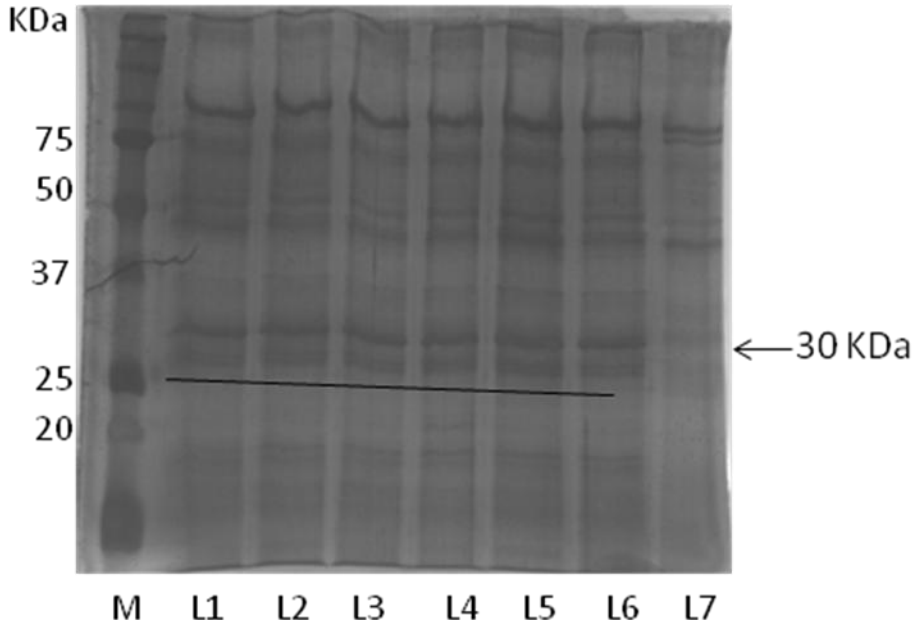


Figure 3.30: SDS- PAGE analysis of the supernatant collected from cultures inoculated with yeast colonies carrying *ADPws*. M: protein molecular weight marker. L1 to L6: protein profile includes recombinant adiponectin at expected size 30 kDa. L7: control.

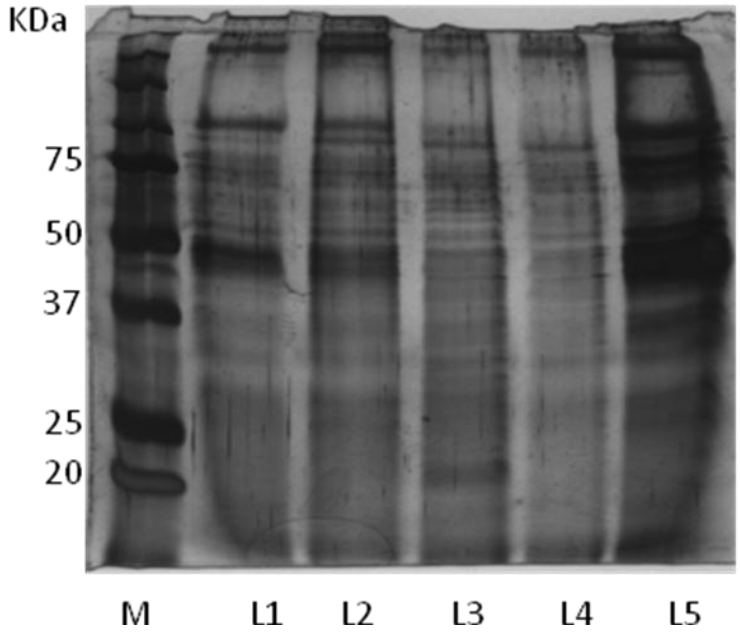


Figure 3.31: SDS- PAGE analysis of the supernatant collected from cultures inoculated with yeast colonies carrying *ADPs*. M: protein molecular weight marker. L1 to L4: protein profile did not contain recombinant adiponectin. L5: control.

3.3.9. Optimization of adiponectin expression

A time course study of adiponectin expression was performed to determine optimal methanol supply and culture harvesting. The growth phase included two stages, the first stage was performed by inoculating 10 ml BMGY media with yeast colony and grown overnight. Whereas, the second stage included transferring the cells pellet to 50ml BMGY media and grown overnight. Next, the cells pellet was transferred to 50ml of BMMY media and one flask of 50ml was containing BMGY medium as control. The time point after transferring cells pellet to induction media (BMMY) was 12 hours and one flask containing 50ml culture was harvested at each time point. The growth rate of yeast was monitored by reading the optical density at OD_{600} (Figure 3.32) and the pH culture was monitored and maintained to be 6. The SDS –PAGE results showed that the expression of adiponectin protein was detectable from 12 hours of methanol induction to 96 hours of induction at the same expected size (30kDa) (Figure 3.33). Similar results were obtained by western blot analysis using anti-adiponectin antibody which showed expected size of around 30kDa (Figure 3.34). The highest concentration of expressed protein was 111µg/ml at 60 hours from the beginning of methanol induction (table 3.1).

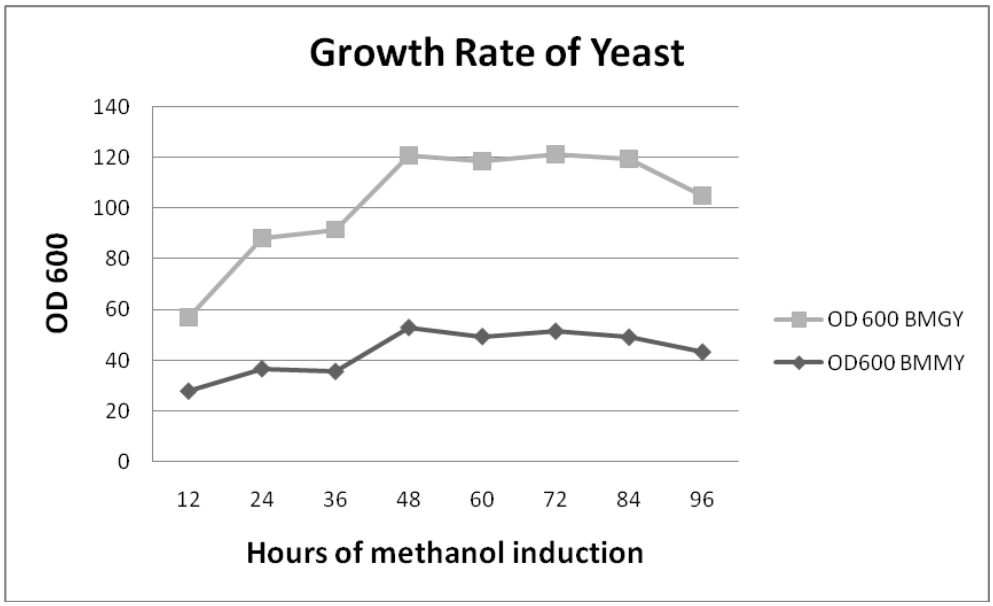


Figure 3.32: The growth rate of yeast during time course expression. The growth rate was measured by the optical density in growing media (BMGY) that was used as control and in induction media (BMMY).

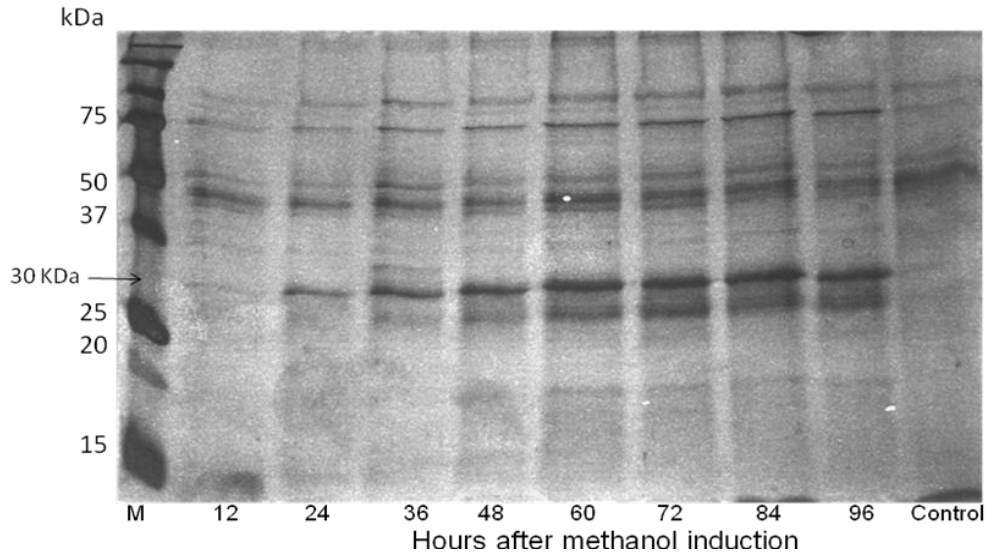


Figure 3.33: SDS-PAGE analysis of the time course for adding methanol and culture harvesting every 12 hours after methanol induction. The bands of adiponectin protein increased in intensity from 12 hours the beginning of methanol induction to 96 hours the end of induction at same expected size (30kDa) and there was no band of adiponectin expected size in conrtol.

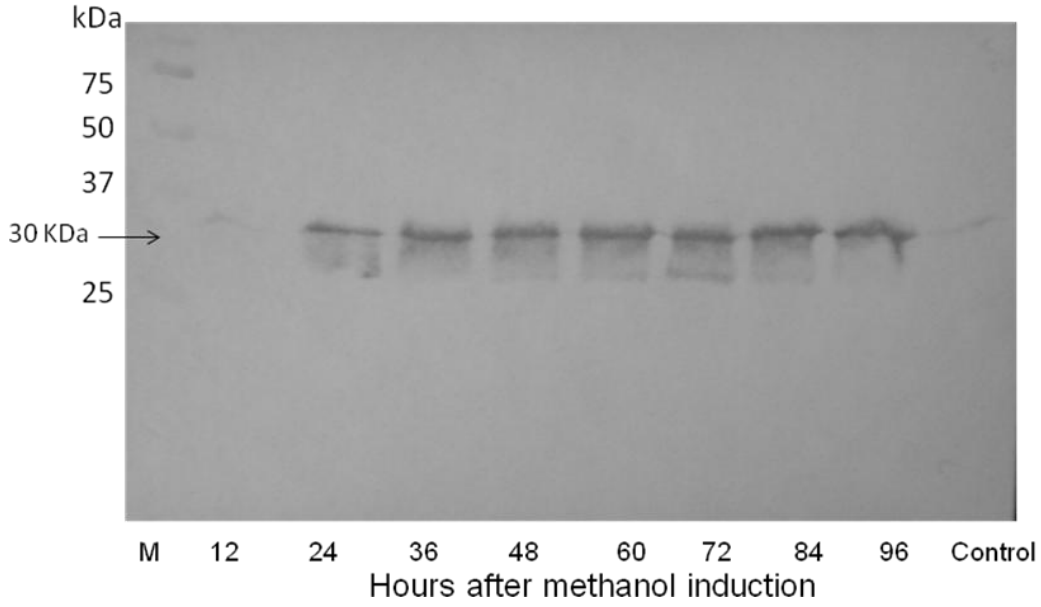


Figure 3.34: Western blot analysis of the time course for adding methanol and culture harvesting every 12 hours after induction. The bands of adiponectin protein can be detected at 12 hours after methanol induction. In addition, the bands of adiponectin protein graded in density from 12 hours the beginning of methanol induction to 96 hours the end of induction at same expected size (30kDa) and there was no band of adiponectin expected size in control.

The growth rate of yeast cells was higher in BMGY medium comparing with BMMY medium. However, the growth rate of yeast cells was exponentially increased in the BMGY and BMMY media from 12 hours until 48 hours of course time. The growth rate was at constant level in BMGY and BMMY media from 48 hours until 84 hours with decline appeared at 96 hours (figure 3.32).

Recombinant protein concentration was undetectable at 12 hours after methanol induction. However, protein concentration exponentially increased from 48 hours until 60 hours after methanol induction and there was inconstant decrease in protein concentration from 72 hours until 96 hours. The highest protein concentration (111µg/ml) was at 60 hours after methanol induction when the total cells mass was 1.6g (Table 3.1). These results were used in scale- up expression of adiponectin protein and the total amount of recombinant adiponectin was 60mg/L.

Table 3.1: The differences were observed in the optical density, total cells mass and protein concentration at different time points after methanol induction.

Methanol Induction Time (hour)	Cell density (OD_{600})	Total cells mass (g)	Protein Conc. (µg/ml)
12	27.75	1.02	--
24	36.57	1.49	22
36	35.5	1.51	25
48	52.8	1.6	93
60	49.25	1.6	111
72	51.33	1.8	78
84	49	1.62	48
96	43.15	1.78	77

3.3.10. Analysis of recombinant adiponectin protein produced by *E. coli* and *P. pastoris*

SDS-PAGE without denaturing conditions was applied to detect the oligomerization process in adiponectin protein using anti-adiponectin antibody. The expected size of adiponectin monomer was approximately 30kD for each of the two types of protein. However, the recombinant adiponectin protein which was produced by *E. coli* (E-ADP) showed less oligomerization compared with adiponectin protein produced by *P. pastoris* (P-ADP) (Figure 3.35).

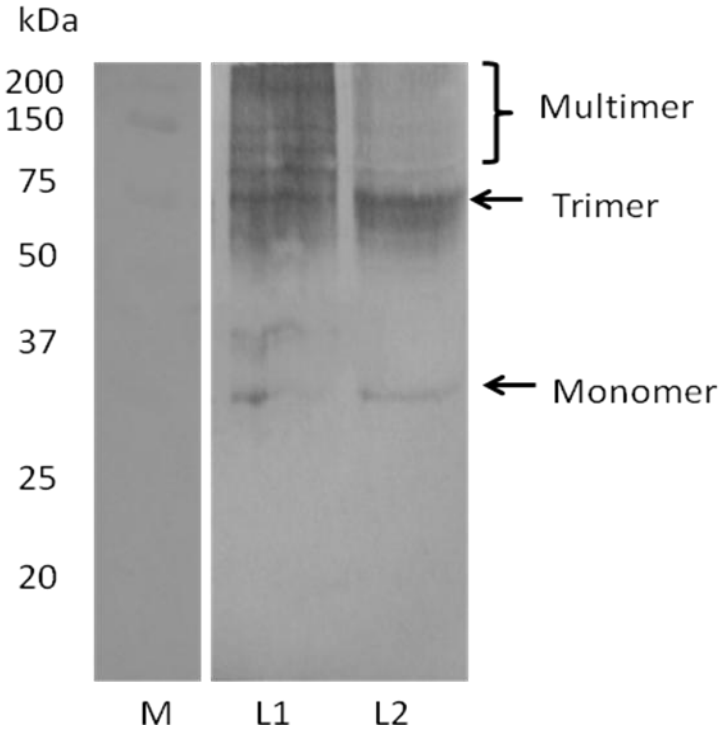


Figure 3.35: SDS-PAGE without denaturing condition showed less oligomers for E-ADP compared with P-ADP. M: Protein marker. L1: Recombinant adiponectin produced by *P. pastoris.* L2: Recombinant adiponectin produced by *E. coli.*

3.4. Biological activity assessment of recombinant adiponectin

ICR mice were used as an experimental model to assess the biological activity of recombinant adiponectin. Experimental animals were fed high sucrose/fat diet then treated with purified adiponectin protein intraperitoneally. Adiponectin bioactivity was measured by the changes in blood glucose level and lipid profile. All statistical analysis was performed using SPSS statistical software v.17.

3.4.1. Comparison of adiponectin bioactivity produced by yeast and *E. coli*

This experiment was carried out to assess the biological activity of adiponectin expressed by *P. pastoris* (P-ADP) and adiponectin expressed by *E. coli* (E-ADP). This experiment was designed to study the effect of these two types of recombinant adiponectin proteins on blood glucose level and lipid profile. P-ADP and E-ADP were significantly lowered blood glucose during the experiment period using a dosage of 1.8 mg/kg BW (Figure 3.36). However, recombinant P-ADP had a higher ability to reduce blood glucose compared with E-ADP ($p<0.05$). Additionally, the two types of recombinant adiponectin have significant effect on changing lipid profile by decreasing triglycerides levels ($p<0.01$) and increasing HDL levels ($p<0.05$) at the end of the experiment period, while there was no significant effect on total cholesterol and LDL levels (Figure 3.37). Biological activity of P-ADP and E-ADP was approximately similar as there are no significant differences between them.

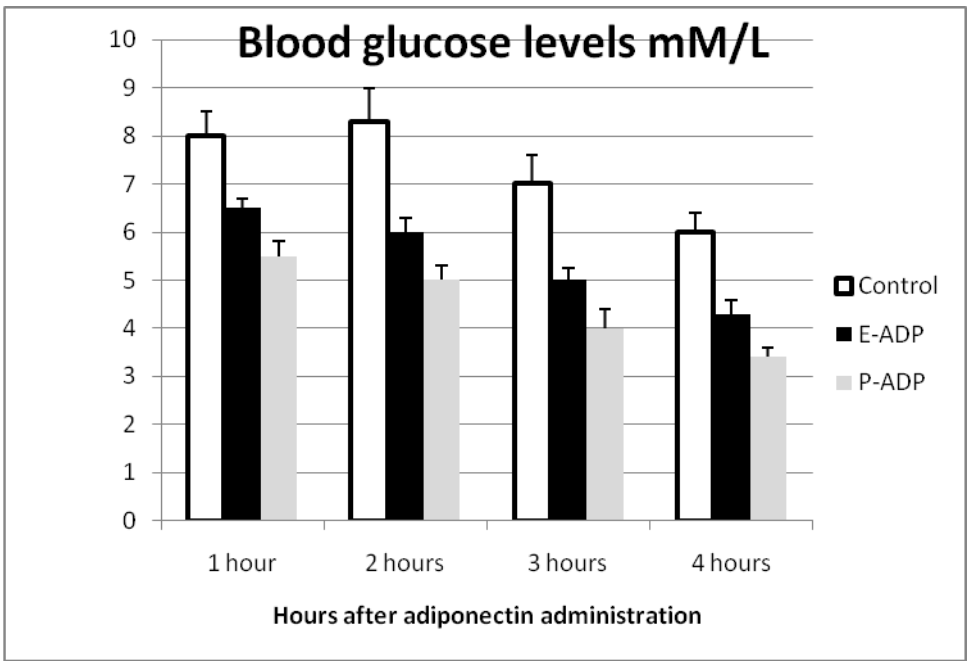


Figure 3.36: Effect of P-ADP and E-ADP on the levels of blood glucose. P-ADP and E-ADP significantly lowered blood glucose (the *p* value of *t-test* < 0.01) and there was significant difference (the *p* value of *t-test* < 0.05) between P-ADP and E-ADP in lowering blood glucose. (*Data was calculated by mean ± SEM).*

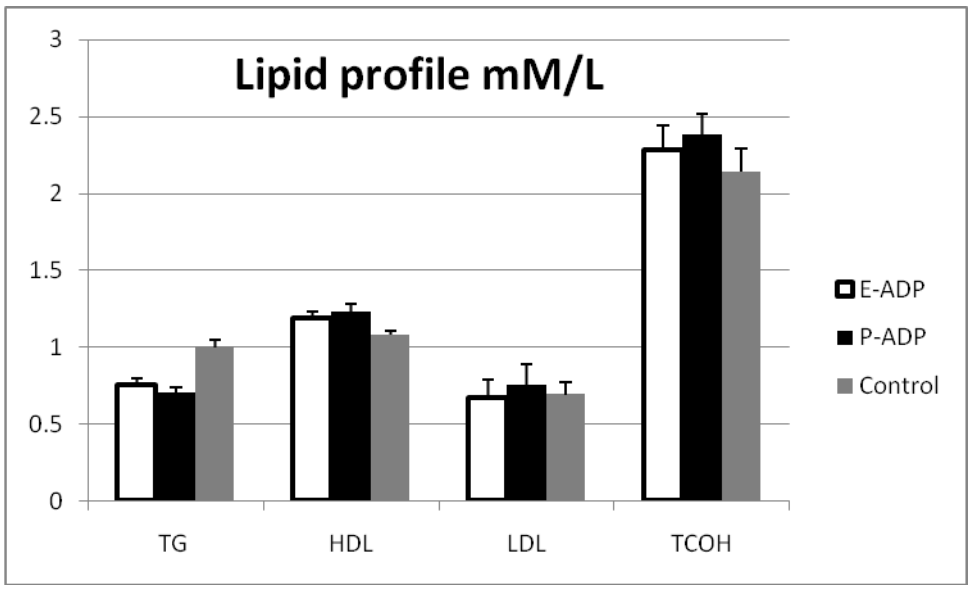


Figure 3.37: Effect of P-ADP and E-ADP on lipid profile. The two type of protein significantly (the *t-test p* value < 0.01) lowered the triglyceride levels whereas these proteins also caused significant increase (the *t-test p* value < 0.05) of HDL levels. There were no significant effects on total cholestrol levels or the LDL levels . Also, there were no significant differences between P-ADP and E-ADP. (*Data was calculated by mean ± SEM).*

3.4.2. Effect of the high and low doses of P-ADP on blood glucose and lipid profile

To investigate the physiological role and the effective dose of P-ADP on blood glucose and lipids, we injected mice that were fed a high-fat /sucrose diet with saline as a control group and treatment groups injected with recombinant adiponectin at 1.25mg/kg BW (2X 0.625mg/kg BW) as low dose and 2.5mg/kg BW (2x 1.25mg/kg BW) as high dose. The first injection was at feeding time and the second injection was after one hour. Blood glucose measured at each hour for four hours and blood lipids measured after four hours are recorded. The results showed that high dose treatment significantly lowered blood glucose levels throughout the four hours after injection, whereas the low dose lowered blood glucose levels significantly just after each injection (Figure 3.38). The low and high doses of P-ADP significantly reduced triglyceride levels and increased high density lipoprotein levels after four hours of injection and there was no significant between the high and low doses (Figure 3.39).

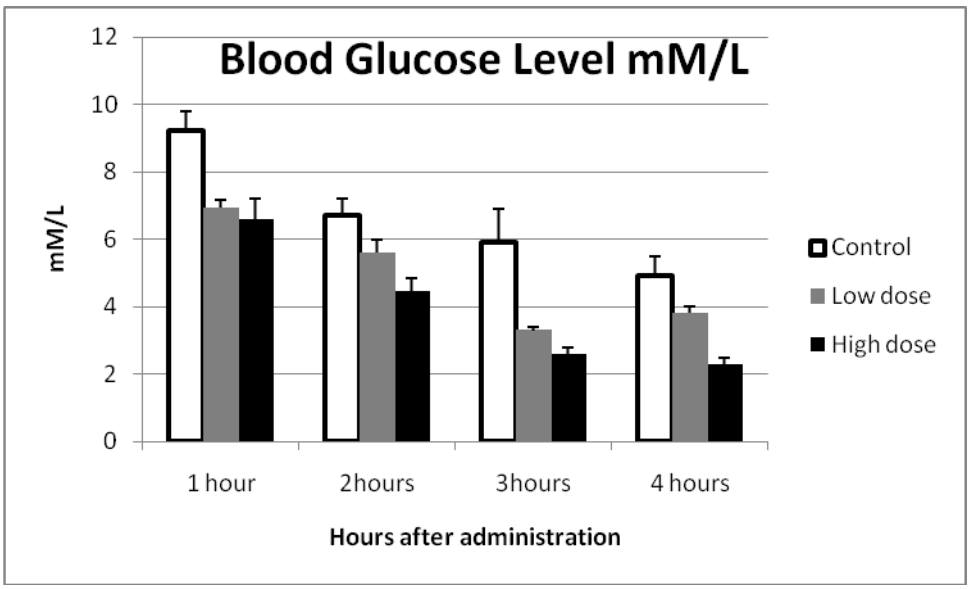


Figure 3.38: Effect of the high and low doses of P-ADP on blood glucose levels. The difference between low dose and control was significant ($p<0.05$) and the difference high dose and control was very significant ($p<0.01$). In addition, the difference between low dose and high dose was significant ($p<0.05$). (*Data was calculated by mean ± SEM).*

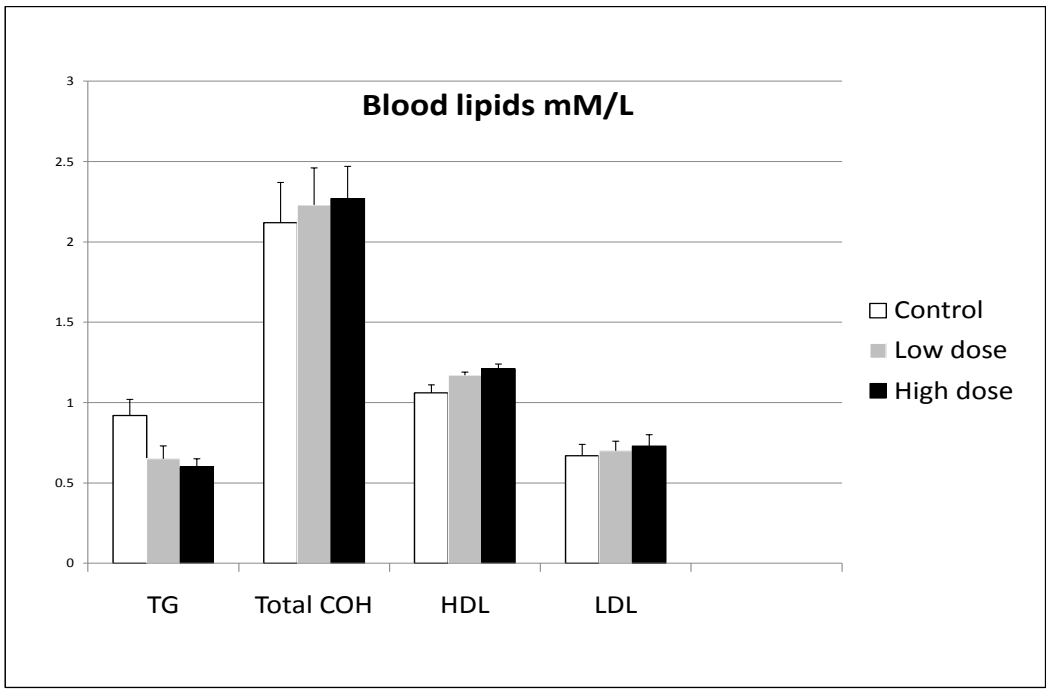


Figure 3.39: Effect of the high and low doses of P-ADP on blood lipid profile. P-ADP significantly decreased triglycerides TG (p<0.01) and increased high density lipoprotein HDL (p<0.05). There are no significant changes in total cholesterol TCOH and low density lipoprotein LDL between treatment groups and control and there are no significant differences between low and high doses (p>0.05). *(Data was calculated by mean ± SEM).*

3.4.3. Oral glucose tolerant test

The ability of recombinant P-ADP in lowering blood glucose had been evaluated by oral glucose tolerance test. The overnight fasted animals were received an oral glucose challenges (2g/kg BW) and injected 2.5mg/kg of P-ADP. Blood glucose levels were significantly lowered at all hours of experiment (Figure 3.40).

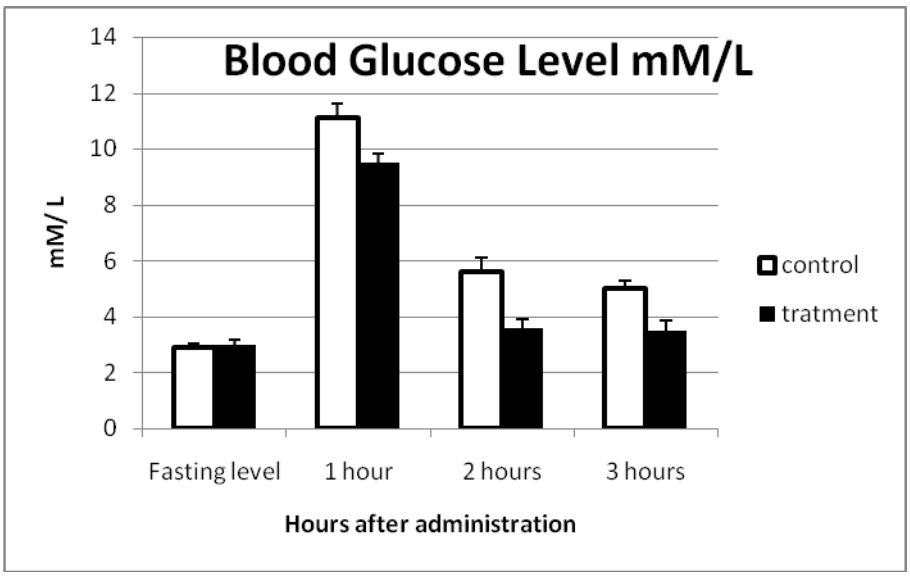


Figure 3.40: Oral glucose tolerance test. The results showed the difference between control and treatment at 1, 2 and 3 hours is statistically very significant (p<0.01). *(Data was calculated by mean ± SEM).*

3.5. Effect of P-ADP administration on the expression of glucagon, insulin and leptin receptors.

This experiment was carried out to determine the possible mechanisms of P-ADP activity in lowering blood glucose and changing lipid profile. *β-actin* gene was selected as an internal control, used to normalize the signal value of each sample so that the differences between samples are the result of a real biological difference and not because of inconsistent loading.

3.5.1. Total RNA extraction and DNase treatment

Total RNA was extracted from liver, skeletal muscle, fat tissue and kidney for treatment group and control group of mice (Figure 3.41). After DNase treatment to remove any contamination with genomic DNA (Figure 3.42), cDNA was prepared by reverse transcriptase reaction using random hexamers primer.

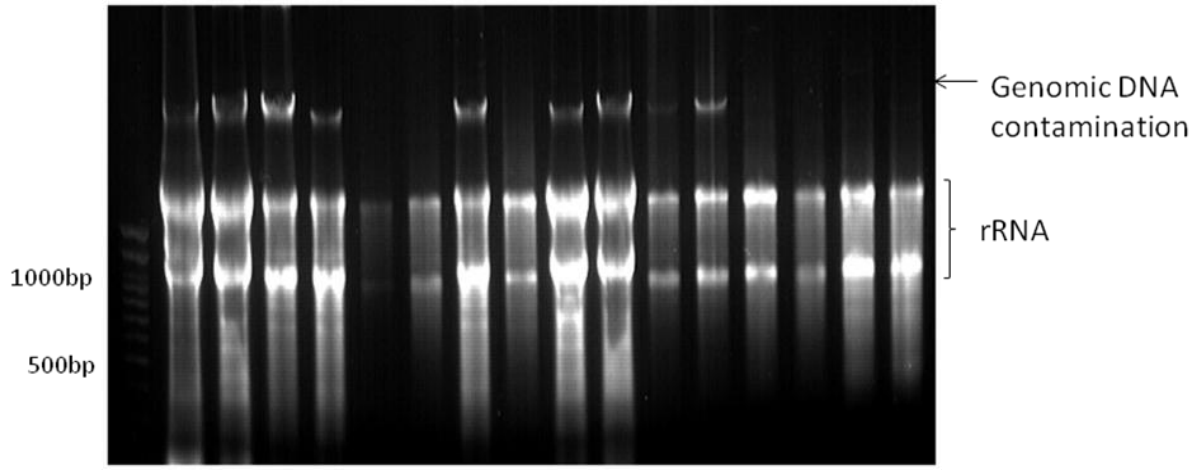


Figure 3.41: Total RNA extraction by TRIzol™ method. The RNA was extracted from liver, skeletal muscle, fat tissue and kidney for treatment group and control group of mice. The upper bands stand for genomic DNA contamination and the other double bands stand for ribosomal RNA.

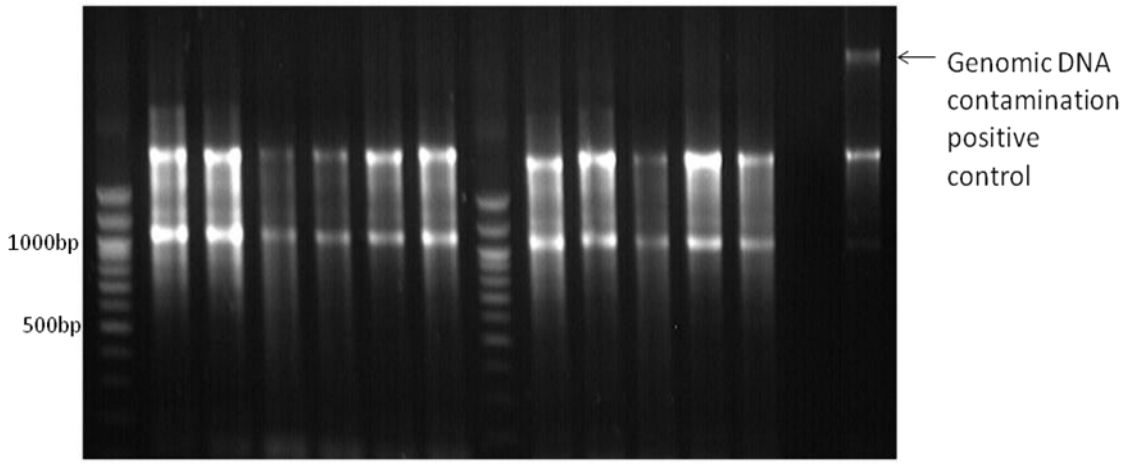


Figure 3.42: Total RNA after DNase treatment. The treatment removed genomic DNA contamination that were apparent in the positive control.

3.5.2. RT-PCR

RT-PCR was used as a quantitative analysis method to estimate mRNA levels of glucagon, insulin and leptin receptors genes. Two step RT-PCR was used that includes reveres transcription reaction and PCR reaction. As the reaction cycle progresses, RT-PCR instrument monitors and records the increase in fluorescence over time by using SYBR® Green I as the reporter fluorophore.

3.5.2.1. Expression of glucagon receptors

Gene expression of glucagon receptors was studied in the liver, abdominal fat tissue and kidney tissue for animal biological groups (treatment and control groups). Each biological group contain four animals (four samples) and ΔCt value for each sample was calculated and plotted in the figure 3.43. The mean ΔCt value for each biological group was calculated and statistically analysed by *t-test* rather than plotted in figure 3.44. In addition, relative quantification value (RQ) was calculated after normalize mean ΔCt for each group with one of control groups (fat tissue control group) (Table 3.3). The statistical analysis of RQ values by *t-test* indicated that there were significant differences between liver treatment and control ($p<0.05$) as well as kidney treatment and control ($p<0.01$). However, there was no significant difference between fat tissue treatment and control ($p>0.05$) (Figure 3.45). RT-PCR results indicated that animal treatment with P-ADP caused down regulation of glucagon receptors in the liver and kidney whereas there was no clear effect due to P-ADP treatment on glucagon receptors in the abdominal fat tissue.

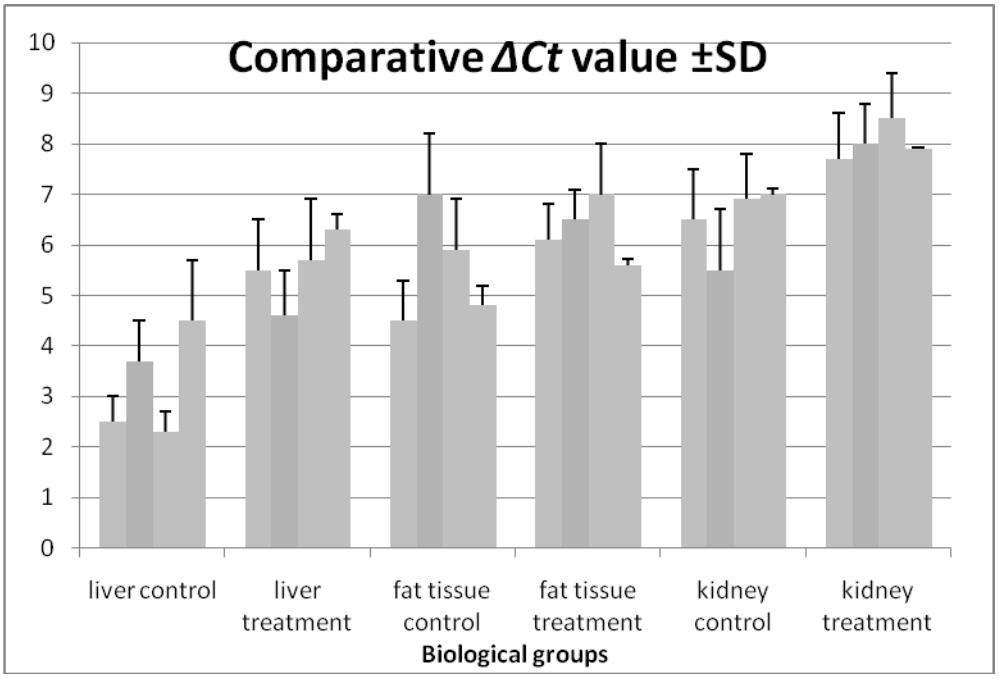


Figure 3.43: Expression of glucagon receptors: The comparative *ΔCt* value for each of biological group's samples. Each four sample represent biological group and control group such as liver, fat tissue, kidney. In addition, one sample consists of four target gene replicates and four endogenous gene replicates. (*Data was calculated by mean ± SD).*

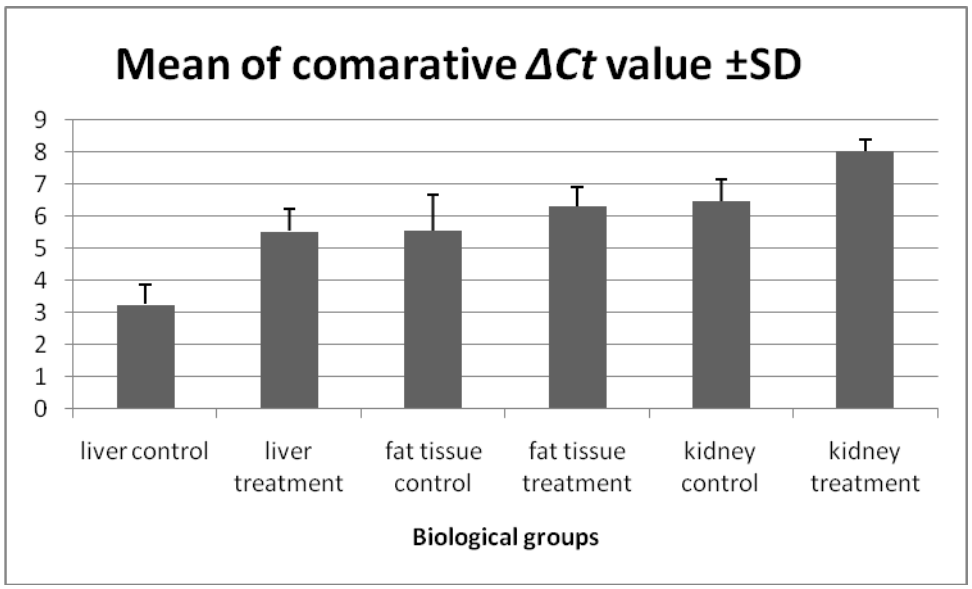


Figure 3.44: Expression of glucagon receptors: The mean of biological group's *ΔCt* value for liver, abdominal fat tissue and kidney for treatment and control groups. The statistical analysis test (*t-test*) indicated that there is significant difference between liver control and treatment ($p<0.05$) and kidney control and treatment ($p<0.01$). However, there is no significant difference between abdominal fat tissue control and treatment ($p>0.05$). *(Data was calculated by mean ± SD).*

Table 3.2: The results of real time PCR and the results of statistical analysis for mean *ΔCt* values and relative quantification values (RQ) using *t-test* statistical analysis.

Biological group name	**Animals number**	**Mean *ΔCt***	**Mean *ΔΔCt***	***stdev***	***SEM***	***ΔCt* *P* value**	**RQ value**	***stdev***	***SEM***	**RQ *P* value**
Liver control	4	3.25	-2.32	0.60	0.30	0.020∗	4.99	2.4	1.20	0.023∗
Liver treatment	4	5.53	-0.32	0.70	0.35	0.020∗	1.25	0.64	0.32	0.023∗
Fat tissue control	4	5.55	0.00	1.13	0.57	0.287 (N.S.)	1.00	0.78	0.39	0.631 (N.S.)
Fat tissue treatment	4	6.30	0.75	0.59	0.30	0.287 (N.S.)	0.59	0.24	0.12	0.631 (N.S.)
Kidney control	4	6.48	0.93	0.65	0.34	0.006∗∗	0.52	0.23	0.11	0.005∗∗
Kidney treatment	4	8.03	2.48	0.34	0.17	0.006∗∗	0.18	0.04	0.02	0.005∗∗

∗ The result is significant ($p<0.05$).

∗∗ The result is significant ($p<0.01$).

N.S. The result is not significant ($p> 0.05$).

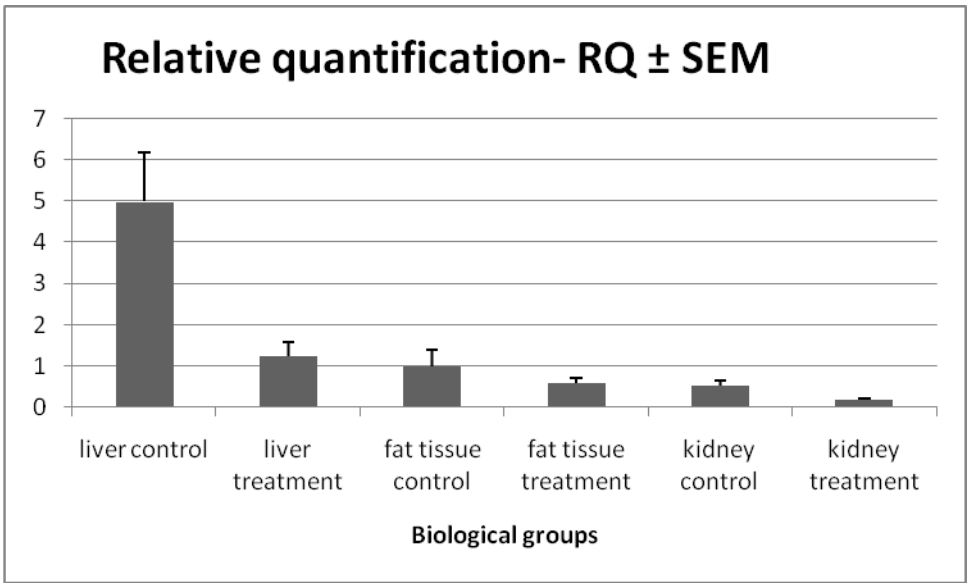


Figure 3.45: Expression of glucagon receptors: Relative quantification differences between treatment and control groups. The differences of RQ values between treatment and control group were statistically significant for liver ($p<0.05$) and kidney tissues ($p<0.01$) and there is no significant difference between fat tissue treatment and control ($p>0.05$). (*Data was calculated by mean ± SEM).*

3.5.2.2. Expression of insulin receptors

The expression of insulin receptors gene was studied in the liver, abdominal fat tissue, skeletal muscles and kidney tissue for animal biological groups (treatment and control groups). As mentioned previously (see section 3.5.2.4) each biological group contain four samples for four animals and *ΔCt* value for each sample was computed and plotted in the figure 3.46. The mean *ΔCt* value for each biological group was calculated and statistically analysed by *t-test* and then plotted in figure 3.47. In addition, relative quantification value (RQ) was calculated after normalize mean *ΔCt* for each group with one of control groups (fat tissue control group) (Figure 3.48). Statistical analysis of RQ values by *t-test* indicated that there were no significant differences between all of treatment groups and control groups ($p>0.05$) (Table 3.4). Real time PCR results indicated that there was no effect of treatment with adiponectin on the regulation of insulin receptors in the liver, abdominal fat tissue, skeletal muscle and kidney.

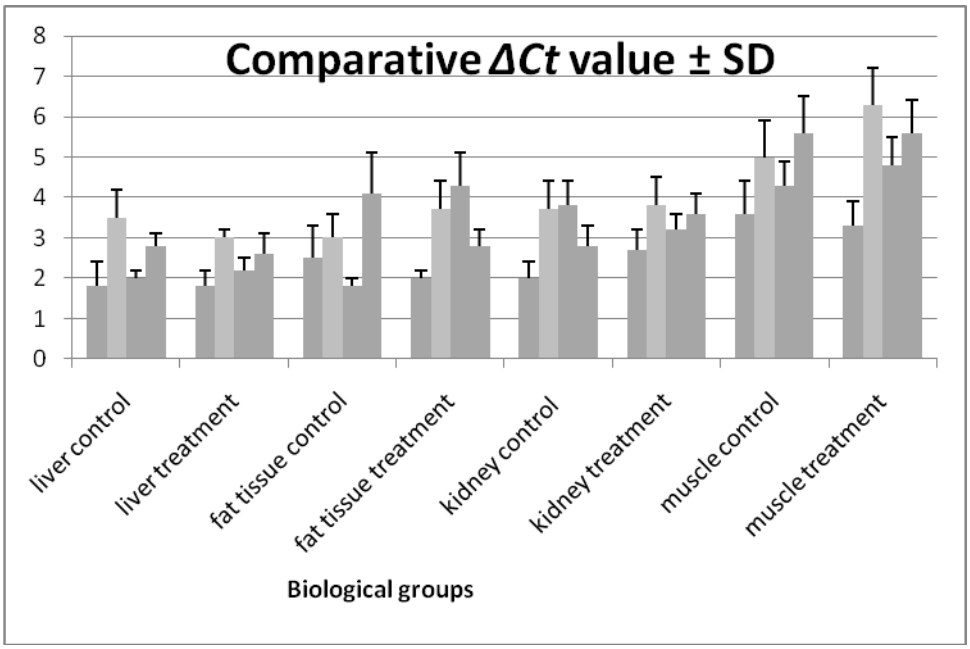


Figure 3.46: Expression of insulin receptors: The comparative *ΔCt* value of the biological groups that contain control or treatment groups. Each group consists of four samples such as liver, fat tissue, skeletal muscle and kidney. Each sample was presented as four target gene replicates and four endogenous gene replicates. *(Data was calculated by mean ± SD).*

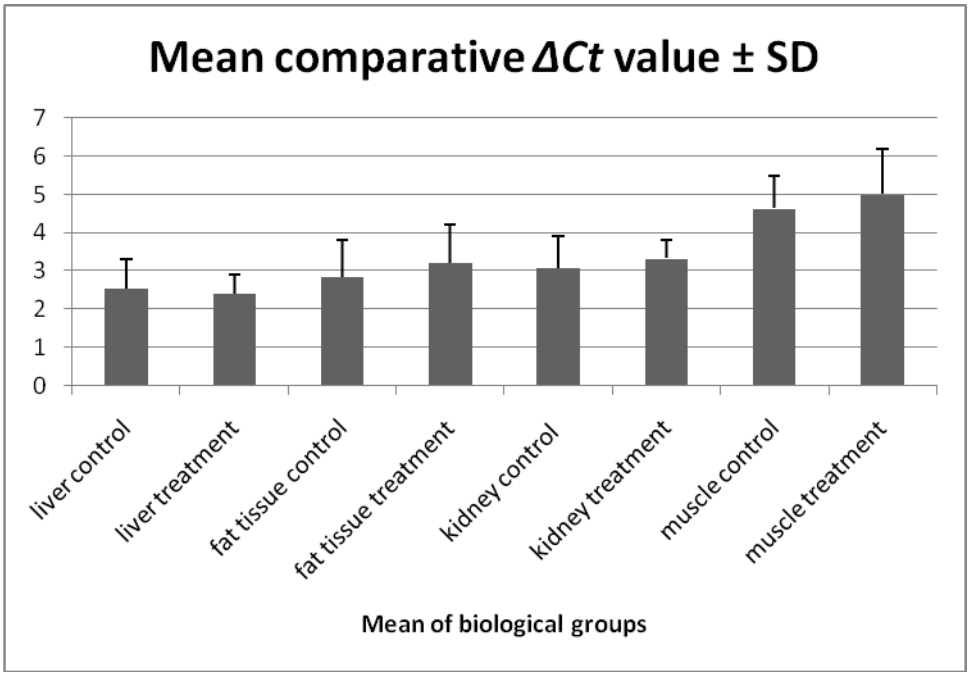


Figure 3.47: Expression of insulin receptors: The mean of biological group's *ΔCt* value for liver, abdominal fat tissue, skeletal muscle and kidney for treatment and control groups. The statistical analysis test (*t-test*) indicated that there are no significant differences between all of biological groups ($p>0.05$). (*Data was calculated by mean ± SD).*

Table 3.3: The results of real time PCR and the results of statistical analysis for mean *ΔCt* values and relative quantification values (RQ) using *t-test* statistical analysis.

Biological group name	**Animals number**	**Mean *ΔCt***	**Mean *ΔΔCt***	***stdev***	***SEM***	***ΔCt* *P* value**	**RQ value**	***stdev***	***SEM***	**RQ *P* value**
Liver control	4	2.53	-0.32	0.78	0.39	0.798 (N.S.)	1.24	0.60	0.33	0.7786 (N.S.)
Liver treatment	4	2.40	-0.45	0.52	0.26	0.798 (N.S.)	1.36	0.48	0.24	0.7786 (N.S.)
Fat tissue control	4	2.85	0.00	0.96	0.48	0.634 (N.S.)	1.00	0.66	0.33	0.7878 (N.S.)
Fat tissue treatment	4	3.21	0.36	1.00	0.50	0.634 (N.S.)	0.78	0.54	0.27	0.7878 (N.S.)
Kidney control	4	3.07	0.22	0.84	0.42	0.626 (N.S.)	0.86	0.50	0.25	0.3846 (N.S.)
Kidney treatment	4	3.33	0.48	0.49	0.24	0.626 (N.S.)	0.72	0.24	0.12	0.3846 (N.S.)
Muscle control	4	4.63	1.78	0.86	0.43	0.646 (N.S.)	0.29	0.17	0.09	0.6713 (N.S.)
Muscle treatment	4	5.00	2.15	1.28	0.64	0.646 (N.S.)	0.23	0.20	0.10	0.6713 (N.S.)

N.S. The result is not significant ($p > 0.05$).

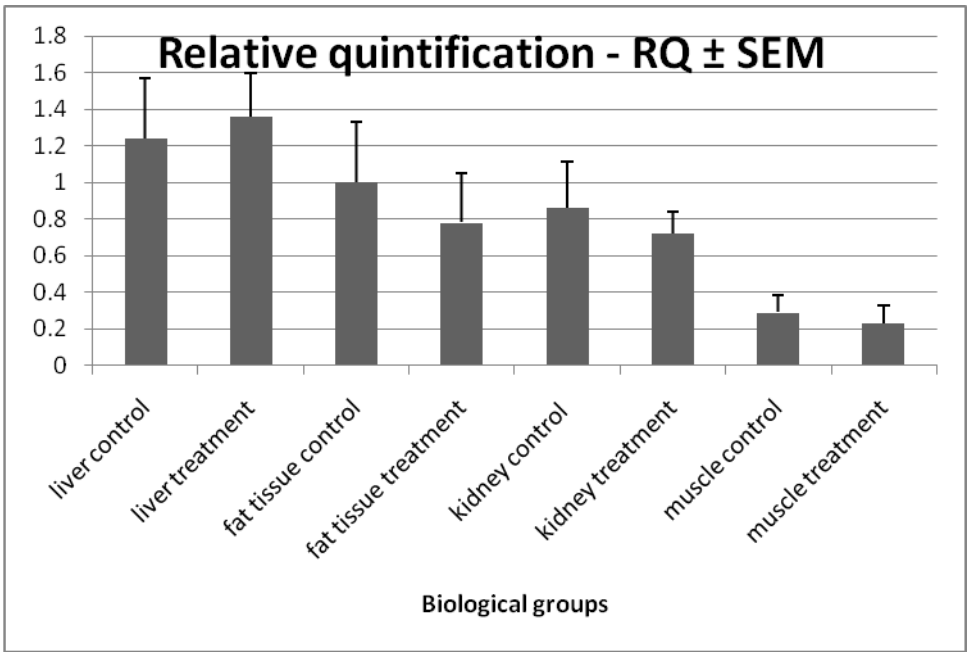


Figure 3.48: Expression of insulin receptors: Relative quantification differences between treatment and control groups. The differences of RQ values between treatment and control group were statistically not significant for all treatment and control groups (p>0.05). (*Data was calculated by mean ± SEM).*

2.5.2.3. Expression of leptin receptors

In order to study the effect of P-ADP administration on leptin receptors, the expression of leptin receptors gene was studied in the liver, abdominal fat tissue and kidney tissue for animal biological groups (treatment and control groups). The *ΔCt* value for each sample was calculated and plotted in the figure 3.49 according to the biological groups. The mean *ΔCt* value for each biological group was calculated and statistically analysed by *t-test* rather than plotted in figure 3.50. In addition, relative quantification value (RQ) was calculated after normalize mean *ΔCt* for each group with one of control groups (fat tissue control group) (Figure 3.51). The statistical analysis of RQ values by *t-test* indicated that there were significant differences between treatment group and control groups of liver, abdominal fat tissue ($p<0.05$) and kidney ($p<0.01$) (Table 3.5). RT-PCR results indicated that there was significant effect of the treatment with P-ADP on the up-regulation of leptin receptors in the liver, abdominal fat tissue and kidney.

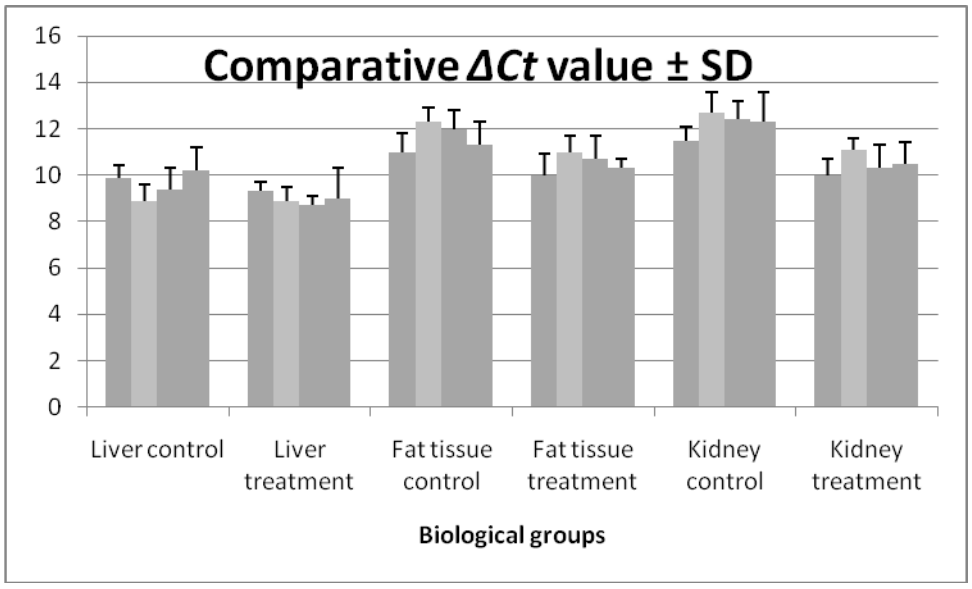


Figure 3.49: Expression of leptin receptors: The comparative *ΔCt* value of the biological groups that contain control or treatment groups. Each sample was presented as four target gene replicates and four endogenous gene replicates. *(Data was calculated by mean ± SD).*

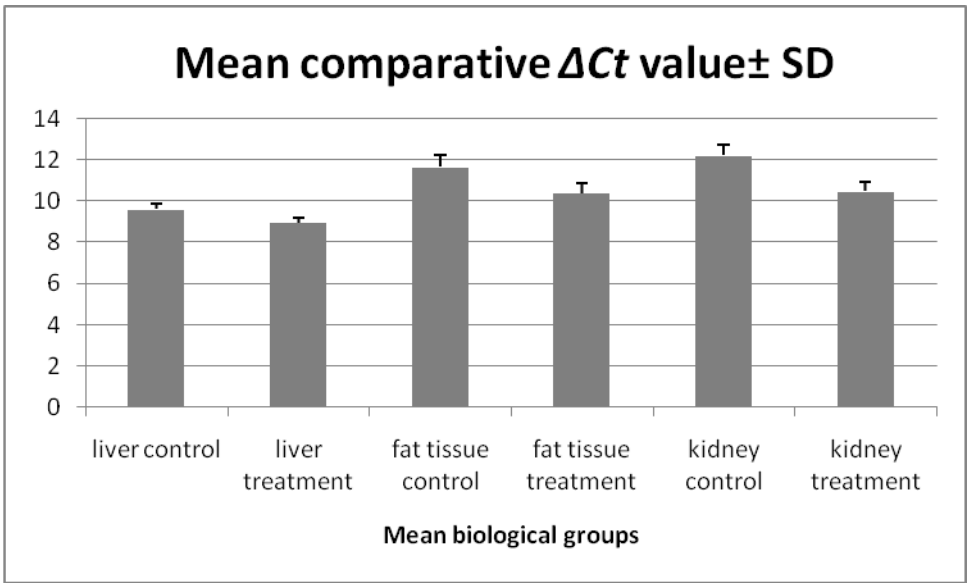


Figure 3.50: Expression of leptin receptors: The mean of biological group's *ΔCt* value for liver, abdominal fat tissue and kidney for treatment and control groups. The statistical analysis test (*t-test*) showed significant differences between control and treatment groups for liver, fat tissue ($p<0.05$) and kidney ($p<0.01$). (*Data was calculated by mean ± SD).*

Table 3.4: The results of real time PCR and the results of statistical analysis for mean *ΔCt* values and relative quantification values (RQ) using *t-test* statistical analysis.

Biological group name	**Animals number**	**Mean *ΔCt***	**Mean *ΔΔCt***	***stdev***	***SEM***	***ΔCt* *P* value**	**RQ value**	***stdev***	***SEM***	**RQ *P* value**
Liver control	4	9.60	-2.05	0.26	0.13	0.013∗	4.14	0.74	0.37	0.0141∗
Liver treatment	4	8.97	-2.68	0.25	0.13	0.013∗	6.41	1.10	0.55	0.0141∗
Fat tissue control	4	11.65	0.00	0.61	0.30	0.021∗	1.00	0.42	0.21	0.0206∗
Fat tissue treatment	4	10.5	-1.15	0.44	0.22	0.021∗	2.22	0.67	0.33	.0.0206∗
Kidney control	4	12.22	0.57	0.51	0.25	0.002∗∗	0.67	0.23	0.11	0.0054∗∗
Kidney treatment	4	10.47	-1.18	0.46	0.23	0.002∗∗	2.27	0.72	0.36	0.0054∗∗

∗ The result is significant ($p<0.05$).

∗∗ The result is significant ($p<0.01$).

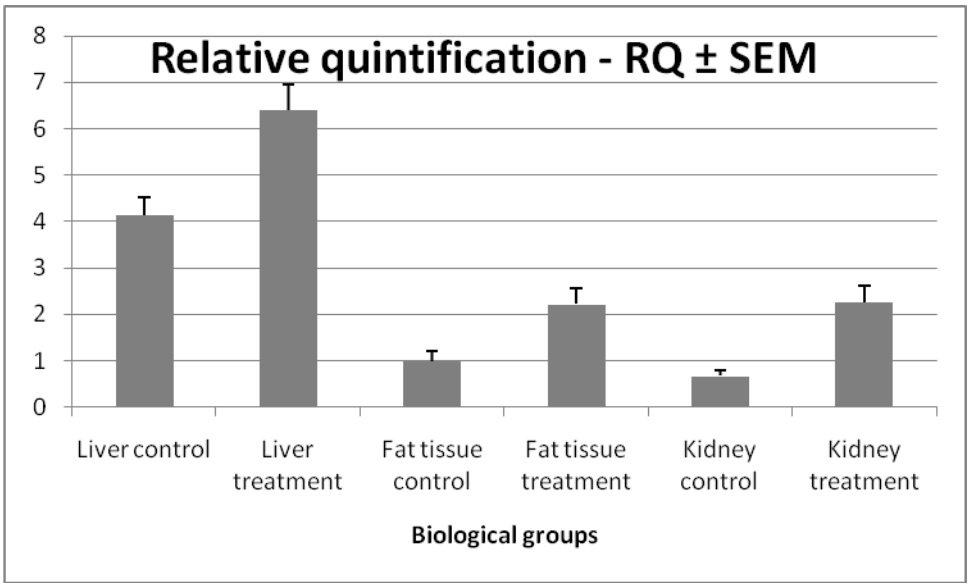


Figure 3.51: Expression of leptin receptors: Relative quantification differences between treatment and control groups. The differences of RQ values between treatment and control group were statistically significant for liver and abdominal fat tissue ($p<0.05$) and kidney ($p<0.01$). (*Data was calculated by mean ± SEM).*