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Research Article

Cloning and Expression of Recombinant Human Insulin Gene in *Pichia pastoris*

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ABSTRACT

The main goal of the current study was cloning and expression of the human insulin gene in *Pichia pastoris* expression system, using genetic engineering techniques and its treatment application. Total RNA was purified from fresh normal human pancreatic tissue. RNA of good quality was chosen to obtain a first single strand cDNA. Human preproinsulin gene was amplified from cDNA strand, by using two sets of specific primers contain *EcoR1* and *Not1* restriction sites. The amplified preproinsulin gene fragment was double digested with *EcoRI* and *Not 1* restriction enzymes, then inserted into *pPIC9K* expression vector. The new *pPIC9K-hpi* constructive expression vector was transformed by the heat-shock method into the *E.coli* DH5α competent cells. pPic9k *–hpi*, which was propagated in the positive transformant *E. coli* cells, was isolated from cells and then linearised by restriction enzyme *Sal*I, then transformed into *Pichia pastoris* GS115 using electroporation method. Genomic DNA of His+ transformants cell was extracted and used as a template for PCR analysis. The results showed, that the pPic9k – *hpi* was successfully integrated into the *P. pastoris* genome, for selected *His+* transformants clones on the anticipated band at 330 bp, which is corresponded to the theoretical molecular size of the human insulin gene. To follow the insulin expression in transformans, Tricine–SDS gel electrophoresis and Western blot analysis were conducted. The results showed a successful expression of recombinant protein was detected by the presence of a single major band with about (5.8 KDa) on the gel. These bands correspond well with the size of human insulin with the theoretical molecular weight (5.8 KDa).

Keywords*: Recombinant Human insulin, Pichia Pastoris, cloning, Tricine SDS- page.*

INTRODUCTION

The use of modern recombinant DNA technology permits the synthesis and purification of human proteins in heterologous cell systems, which are potentially useful for many medical applications. Many proteins such as, human insulin cannot be obtained from their natural sources in suitable quantities, because of their low abundance or difficulty of purification by conventional methods from human tissue samples, organs or cell lines[1]. Recombinant DNA technology enabled the production of large quantities of protein drugs as cost-efficient sources ; these proteins perform the same function as naturally occurring proteins in body[2]. Proteins started being used as pharmaceuticals in the 1920s, with insulin extracted from pig pancreas. In the early 1980s, human insulin was prepared in recombinant bacteria and this protein, which proved of better quality than the conventional insulin, recombinant human insulin is almost exact to natural human insulin, and has completely replaced animal insulin because animal insulin is a slower rate of absorption into the bloodstream[3]. It causes Immunological complications, allergy has been particularly common, with local symptoms still occurring in ~5% of patients, it is less effective, and it is expensive to produce[4]. The human insulin gene (*INS)* is located on the short arm of chromosome (11p15.5), which is the homologous to chromosomes 1 and 7 in mice and rats respectively[5]. This gene contains three expressed sequences (exons) separated by two intervening sequences (introns) or IVS[6]. This gene encodes a 1430-nucleotide insulin messenger RNA precursor, which contains two intervening sequences of 179 and 786 nucleotides that are excised from the precursor to generate the insulin messenger RNA molecule. The insulin messenger RNA directs the synthesis of the insulin precursor protein, pre-proinsulin .This manuscript is concerned with the chromosome localization of the human insulin gene. The three exons code for the signal peptide, the B-chain, C-peptide, and the A-chain of the insulin molecule. Human insulin is naturally occurring polypeptide hormone, with a molecular weight of about 5,800 KD, it is secreted by the beta cells of the islets of Langerhans from the pancreas in response to increased levels of glucose in the blood, as well as to the parasympathetic nervous system and other stimuli[7]. Mature insulin consists of 51 amino acid residues organized in two polypeptide chains A and B chains interconnected via two disulfide bridges chain, A chain consists of 21 residues with an extra disulphide bond between A6 and A11, B chain consists of 30 amino acids[8]. Insulin structure is highly conserved among vertebrates, which are showed by its high degree of

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homology. Porcine insulin has a single amino acid variation from the human variety (alanine substituting threonine in position B30), while bovine insulin has three amino acid variations) alanine instead of threonine in positions A8 and B30 and valine instead of leucine in position A10[9].

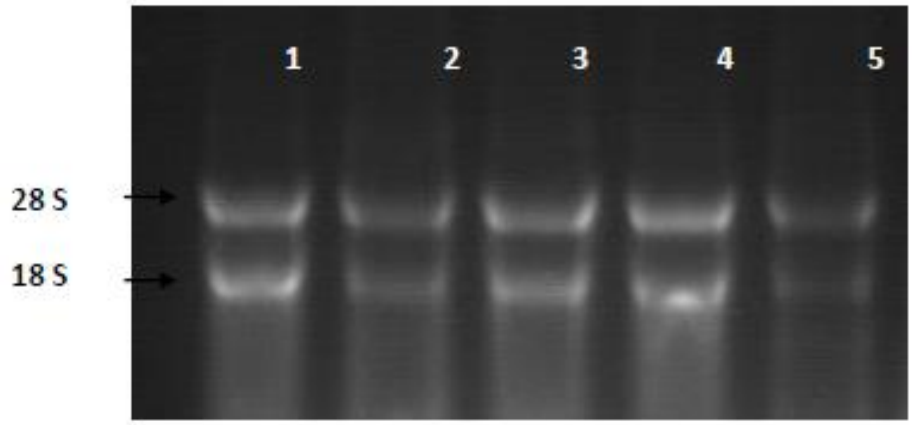

Figure 1: Agarose gel electrophoresis for total RNA isolated from pancreatic tissue was fractionated on 1% agarose .lane 1,2,3,4 and 5 the bands of 28S and 18S rRNA.

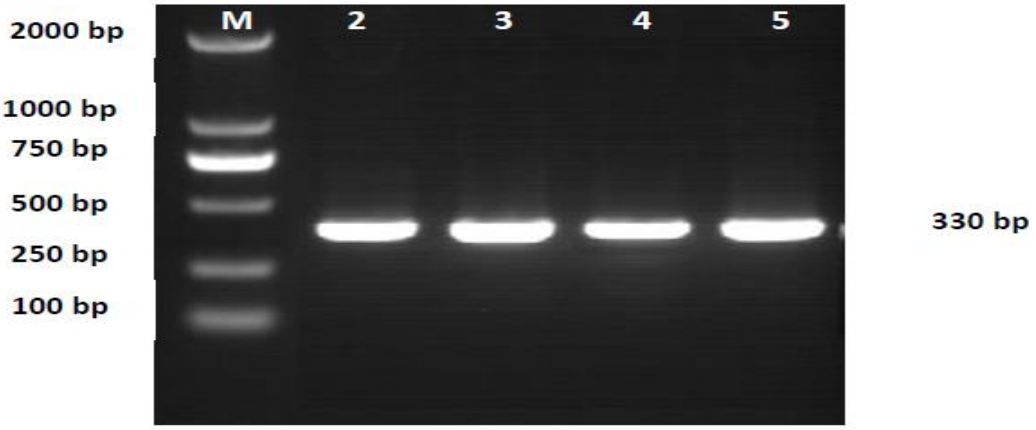

Figure 2: Amplification of human preproinsulin gene by RT-PCR. The products were fractionated on 1.5 % agarose gel electrophoresis visualized under U.V light after staining with Ethidum bromide. Lane M: DNA marker 2000 bp. molecular weight standard (2000, 1000, 750, 500, 250, and 100) Lane 2-5: preproinsulin gene.

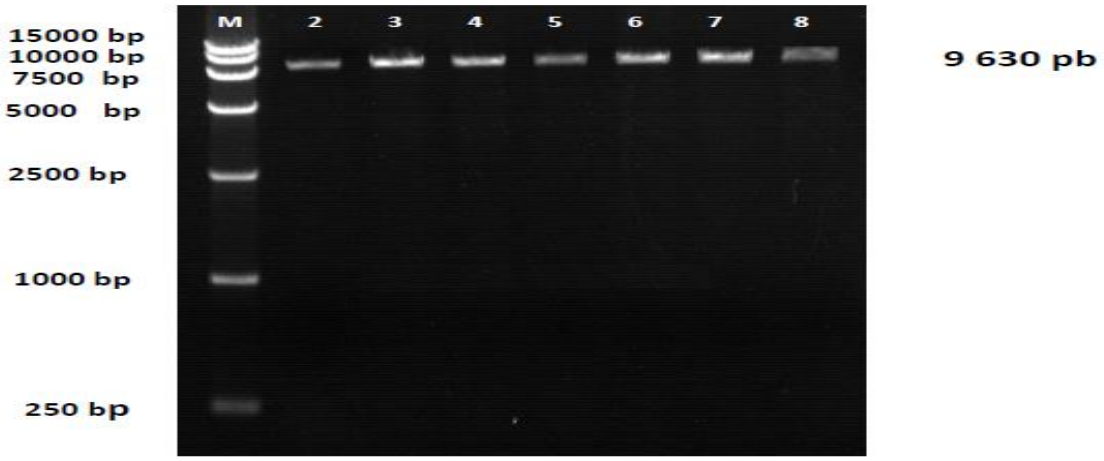

Figure 3: Agarose gel electrophoresis of constructive expression vector *pPIC9K - hpi* fractionated on 1% agarose gel electrophoresi Lane M: DNA marker 15000 bp. molecular weight standard (15000, 10000, 7500, 5000, 2500, 1000, 250). Lanes 2,3,4,5,6 7 and 8: the size of *pPIC9K P. pastoris* expression vector which containing the PCRproduct amplified preproinsulin gene.

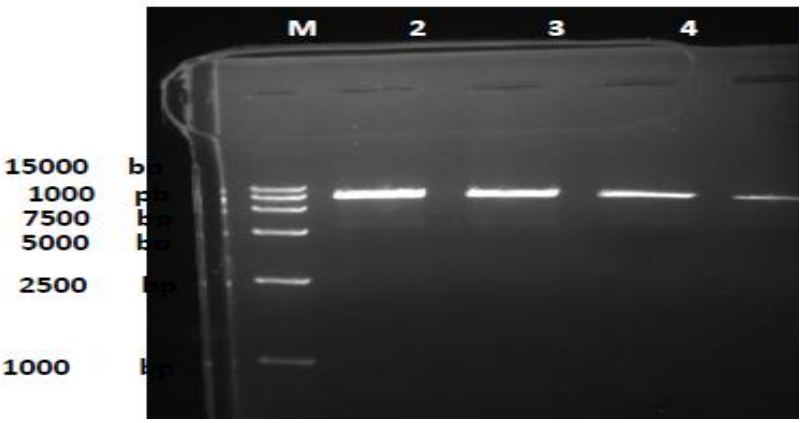

Figure 3: Agarose gel electrophoresis of Linearised *hpi - pPic9k* Plasmid with *Sal*I . DNA was fractionated on 1% agarose gel electrophoresi Lane M: DNA marker 15000 bp. molecular weight standard (15000, 10000, 7500, 5000 , 2500 , 1000, 250) . Lanes 2, 3, 4 and 5: band of Linearised *pPIC9K*.

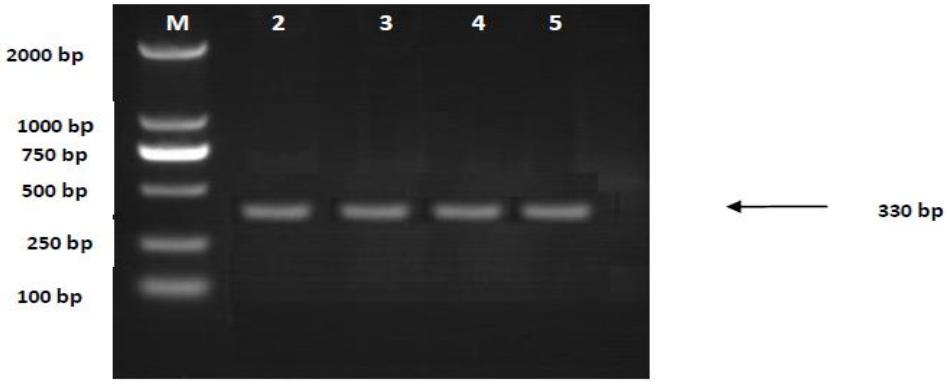

Figure 3.12: Agarose gel electrophoresis of DNA isolated from *His+* transformants grown on higher concentrations of geneticin G418 antibiotic, DNA was fractionated on 1.5 % agarose gel electrophoresis visualized under U.V light after staining with Ethidum bromide .Lane M: DNA marker 2000 bp molecular weight standard (2000, 1000,750, 500, 250, 100)Lane 2-5 : *His+* selected transformants .

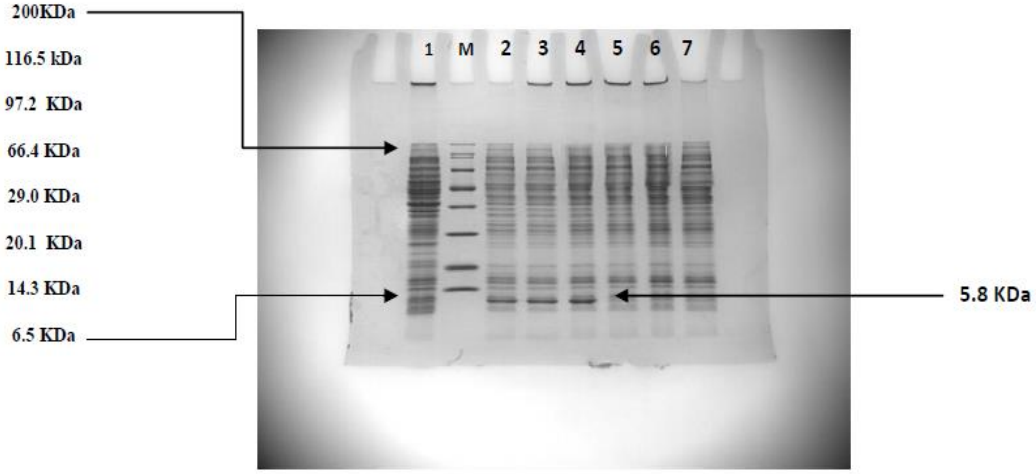

Figure 3.13: Tricine–SDS–PAGE analysis of the supernatant from fermentation of recombinant *P. pastoris* .Samples were collected at 24 h intervals after methanol induction, analysed by 12% Tricine-SDS-PAGE reducing gel and subsequently stained with Coomassie brilliant blue R-250. Lane 1 = Uninduced *Pichia pastoris* GS115 Lane M = Standard molecular weight protein marker (200, 116.5, 97.2, 66.4, 29.0, 20.1, 14.3, and 6.5 KD). Lane 2 = Induced recombinant *Pichia pastoris* GS115 (24 hrs) Lane 3 = Induced recombinant *Pichia pastoris* GS115 (48 hrs) Lane 4 = Induced recombinant *Pichia pastoris* GS115 (72 hrs) Lane 5= Uninduced recombinant *Pichia pastoris* GS115 Lane 6 = Uninduced recombinant *Pichia pastoris* GS115 Lane 7= Uninduced recombinant *Pichia pastoris* GS115

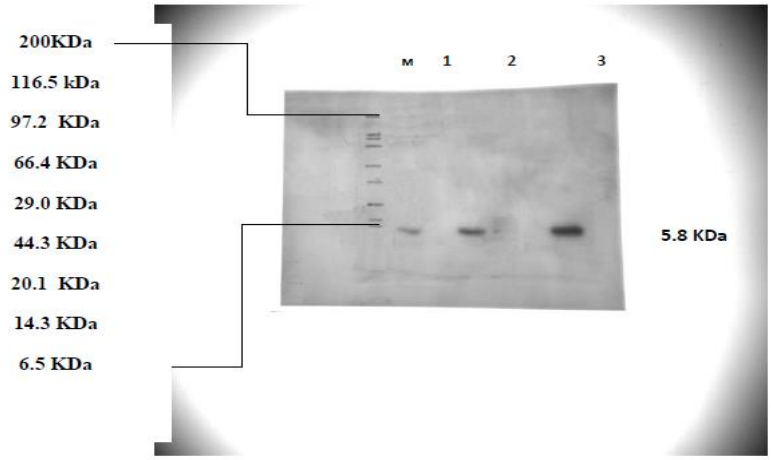

Figure 3 .14: Western blot analysis of separated Protein from recombinant *P. pastoris* Gs115.
Lane M: Standard molecular weight protein marker (200, 116.5, 97.2, 66.4, 29.0, 20.1, 14.3, and 6.5 KD).
Lane 1: human insulin protein expressed in recombinant strain GS115/ in 24 hrs, was about 5800 kDa, which was consistent with the theoretical molecular weight of insulin protein.
Lane 2: human insulin protein expressed in recombinant strain GS115/ in 48 hrs, was about 5800 kDa, which was consistent with the theoretical molecular weight of insulin protein.
Lane 3: human insulin protein expressed in recombinant strain GS115/ in 72 hrs, was about 5800 kDa, which was consistent with the theoretical molecular weight of insulin protein

MATERIALS AND METHODS

Isolation of Total Cellular RNA from Human pancreatic tissue

Normal human pancreatic tissue of a brain patient was provided from the Cancer center for organ recovery and education resource (C.O.R.E). Fudan medical university, the organ was obtained from deceased healthy multi-organ donor with the ages of 38 who consented to research use. The sample was immediately frozen in liquid nitrogen and then kept at -80˚C, total RNA was extracted by trizol method according to Sambrook & Russell 2011, using total RNA Qiagene RNeasy extraction mini kit according to manufacturer's protocol.

Concentration and purity of eluted RNA were determined by measuring their absorbance at 260 (A_{260}) and 280 (A_{280}) using DU-640 nucleic acid and protein analyzer (Backmen,USA). Total pancreatic RNA integrity was evaluated by agarose gel electrophoresis, the quality of the isolated total RNA was assayed by visualization of discrete 28S, 18S and 5S rRNAs. Through 0.8% agarose gel, according to the[10] RNA Samples of good quality were chosen for reverse transcription reaction (cDNA synthesis), by using reverse transcription system kit **(**Promega, USA) according to the manufacturer's instructions.

PCR amplification of human preproinsulin gene

The amplification of the human propreinsulin gene was carried out by using the first-strand cDNA as a template using non - proofreading thermostable DNA polymerase, Nova *Taq*. DNA polymerase, and two sets of specific primers were used the forward primer was 5' TAC GAATTC ATG GCC CTG TGG ATG CGC CTG -3'and the reverse primer was 5' -CAT GCGGCC GTT GCA GTA GTT CTC CAG CTG -3', Primers contain *EcoR1* and *Notl* restriction sites on the 5`ends for amplification of human preproinsulin fragment.

Construction of human preproinsulin into expression vector

The modified *pPIC9K* expression vector used for ligation of *hpi* gene was provided from (invitrogene). The vector employed for this study containing the *a*-factor secretion signal directed release of the recombinant protein into the culture supernatant media, and has an N-terminal flag-tag for immunodetection and a stretch of ten consecutive histidine residues as an affinity handle. The vector and the PCR amplified gene double restricted with *EcoRI* and *Not 1* enzymes. Both the restricted products were cleaned up using (*Axygen*) gel clean up kit. The product obtained after cleanup is checked on 1 % agarose gel for sizes, and ligation reactions was carried out by the *hpi* into the *EcoRI and Not*I sites of pPIC9K to produce the expression plasmid, in which the *hip* gene inserted in front of under methanol-inducible alcohol oxidase (*AOX1*) promoter and is under its control. The gene insertions at the *Eco*RI site of *pPIC9K* were used to generate an in-frame protein fusion of the inserted DNA with the *Saccharomyces cerevisiae* α-mating factor signal sequence to enable the secretion of recombinant protein.

Transformation of competent cells with pPic9k

In the present work, the strain of *E.coli* DH5α competent cells were transformed by the heat-shock method as described by[11]. Expression vector, pPic9k-*hpi* which was propagated in the positive transformant *E. coli*, was isolated from cells and linearised using the restriction enzyme *SalI* prior to the transformation process to increase integration through recombination events. *Pichia pastoris* GS115 was used for the transformation procedure with recombinant plasmid by the electroporation method as described by[12]. This strain

has a mutation in histidinol dehydrogenase (his4) that prevents it from synthesizing histidine[13].

Screening and expression of Recombinant Protein in *P. pastoris* strains

The genomic DNA was extracted from His+ transformants and used as template for PCR analysis to confirm whether or not the *P. pastoris* transformants contain *pPic9k- hpi* plasmid expression vector according to Linder 1996.

The reaction cycle was carried out under the following conditions, started at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min and a final extension at 72˚C for 5 min. PCR products were purified by using PCR purification Kit, (Axygen, china). Successful PCR amplification was confirmed by electrophoresis on a 1.0% agarose gel[14].

Recombinant protein expression was assessed by performing small scale expression studies using a single colony able to grow on MD plates without histidine supplement. Tricine - Sodium dodecyl sulfate polyacrylamide gel electrophoresis was employed to follow the insulin expression in cultures, as described by Schagger and von[15], after that Western blot analysis was performed according to the procedure described by Bjerrum[16].

RESULTS AND DISCUSSION

Purification of total RNA from human pancreatic tissue

Extraction of total RNA pancreatic cells was prepared by homogenization of normal human pancreatic tissue of a brain death healthy multiorgan donor patient. These cells represent the specific cells type which expresses a characteristic set of gene (that is preproinsulin genes) and mRNA preparation from a particular tissue usually contains some specific sequence of higher abundance. Total RNA was extracted directly from the cells using trizol method which recommended by Sambrook & Russel, (2001), and purification of RNA was done using a phenol /chlorophorm method. Concentration and purity of RNA was estimated as 4.91 µg/µl and 1.87 respectively, which considered within acceptable limit indicating RNA free of protein and other impurities. Results shown in figure (1) indicate the presence of two major sharp bands represented the bands of 28S and 18S rRNA related to preproinsulin genes synthesis.

Preproinsulin genes synthesis

The mRNA isolated from pancreatic cells was used as a template and converted to (cDNA) using RT-PCR reaction and then preproinsulin gene was amplified using specific primers. Primers were used containing the sequence to the prime synthesis of sense and antisense strands as well as sequence which contain *EcoRI* and *NotI* restriction sites to be converted latter by these enzymes to sticky ends for cloning of the gene. The results shown in figure 2 indicated a presence of PCR product of 330 bp when an electrophoresis on 1.5 % agarose gel and corresponded with a DNA ladder marker which gives good indicator and confirm that the PCR reaction is carried out correctly

Construction of human preproinsulin into expression vector

To generate a cloning vector that contains a desired insert, genes to be cloned and backbone vector were digested with the same restriction endonucleases. Therefore the PCR product was double digested with *EcoRI* and *Not 1* sites which cuts in the primers part as long as it contains the restriction sites and no internal sites presents within the sequence of the gene. The gene was inserted in the correct direction, following the multiple cloning sites for unidirectional insertion which are in front of and under the control of methanol-inducible alcohol oxidase (*AOX1*) promoter, and in the correct reading frame. Result shown in Figure (3) indicated the presence of a sharp band of about 9630 bp, which represent the size of *pPIC9K*.

Transformation of Pichia pastoris

pPic9k -hpi expression vector, which propagated in the positive *E. coli* transformants was isolated and linearised using *Sal*I prior to the transformation process to increase integration into the *P.pastoris* genome through recombination events and according to the strategy of insertion in an expression cassette. Results shown in Figure (3) indicate the presence of band of about 9630 pb as compared to a 1 kb DNA ladder. Linearisation of hpi - *pPic9k* facilitated integration of the plasmid with the cell chromosome and prevents disruption of gene of interest during integration. Moreover the number of clones we get would be higher with linearized plasmid[17].

PCR analysis of Pichia Pastoris integrants

Genomic DNA was isolated from *His+* transformants and used as template for PCR reaction in order to confirm integrants of *hpi* gene using homologous recombination events. Results shown in Figure (3.12) indicate the presence of intense band of 330 bp which is referred to *hpi* gene multicopies. PCR amplify DNA in a manner depending on starting material (copies of DNA)which increased proportionally with initial number .

Appearance of this band was expected as long as the recombinant plasmid, it was successfully integrate in the genome through heterologous recombination between sequences of vectors and homologous sequences in *AOX1* locus of *P. pastoris GS115* and the sequence was amplified by using *5'AOX1* and *3'AOX1* primers which anneal to the 5' and 3' *AOXI* sequence in Mut+ clones, where endogenous *AOXI* is present along with inserted recombinant DNA, These primers amplify *AOX1* promoter region of the *P. pastoris* genome which is considered a strong inducible promoter that induced the gene inserted within the *5'-AOX1*and *3'-AOX1*primming sites by the use of methanol along with the insert[18].

Recombinant Protein expression in P. pastoris strains

In the present study, tricine- sodium dodecyl sulfate polyacrylamide gel electrophoresis was employed to follow the insulin expression in cultures. Samples from small scale methanol inducible culture were subjected to 12 % Tricine-SDS- page analysis. Results shown in Figure (3.13) indicate the successful expression of recombinant protein as indicated by the presence of a single major band with about 5.800 KDa this band

correspond well with the size of human insulin with the theoretical molecular weight of 5.800 kDa as compared with the result of the control negative *P. pastoris* competent cells free of *pPIC9K-hpi* plasmid.

The Tricine-SDS–PAGE revealed the successful production and secretion of the recombinant human insulin into the culture supernatants under the induction of methanol which increased gradually with time as compared to plasmid free samples lane .6, 7, 8, in fig (3.13). In conclusion the high level of expression can be attributed to two factors; first, human insulin gene was successfully integration downstream of methanol-induced alcohol oxidase promoter into the genome of *P. pastoris* and under its control. Most *P. pastoris* expression systems use the methanol-induced alcohol oxidase (*AOX1*) promoter for the expression of the heterologous gene upon induction by methanol. Second, target gene was cloned in frame and downstream of the α-factor signal sequence in order to allow secretion of the recombinant protein into the surrounding medium to facilitate harvesting and purification[19]. Moreover, result of tricine-SDS–PAGE were coincided with the similar results of expression patterns of western blot analysis that shown in fig. (3. 14) which confirms the presence of the expressed recombinant protein according to the size of protein which was secreted in the medium and developed on the SDS page gel. Hence western blot analysis could be taken as an evidence for successfully expression of the recombinant protein.

Results were shown in figure (3.14) indicate the presence of band of approximately 5.800 kDa which mimic the calculated band of human insulin -protein (5 .800 kDa). It can be concluded, that recombinant protein had been successfully expressed and subjected to the efficient proteolytic cleavage in *P. pastoris* of fusion proteins based on the alpha-factor prepro-leader in the *P. pastoris* cells and secreted in the medium and developed on the SDS page gel, as well as detection on western blot membrane[20].

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