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Full Length Research Paper

Subcloning and expression of human alpha-fetoprotein gene in *Pichia pastoris*

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Alpha-fetoprotein protein (AFP) is naturally found in fetal serum while production and appearance after birth is indicative of presence of malignant tumors. Therefore, by measuring this protein during fetal period and after birth, it is possible to diagnose abnormalities and tumors in fetus or the newborn. The objective of this study was to produce and purify AFP protein using DNA recombinant technology to apply in diagnostic kit preparations. In this study *Pichia pastoris* as metylotrophic yeast was used for AFP production. After construction of recombinant plasmid, pS1-AFP, electroporation and lithium chloride techniques were used for transferring to susceptible cells. The quantity and quality of the produced protein were checked by SDS-PAGE and ELISA methods. Selection of transformed mutant strains of Mut^S and culturing them in glycerol media (YPG) up to OD_{600}=6 and their transfer to methanol media (YPM) with augmentation of methanol to 1% final concentration resulted in inducing protein production in auxotrophic media lacking histidine. This protein could be useful in monoclonal antibody production and in diagnostic kit preparations.

Key words: Alpha-fetoprotein, *Pichia pastoris*, cloning, expression.

INTRODUCTION

Alpha-fetoprotein protein (AFP) is a major serum protein synthesized during fetal life by fetal liver and yolk sac (Abelev, 2001). After birth it disappears from the circulation and its reappearance of AFP in adult serum often signals pathological conditions, particularly hepatocarcinomas and teratocarcinomas (Filmus et al., 2004). AFP blood level change is an important marker for liver tumors that is widely used in clinical practice. Therefore, the study of the molecular and cellular mechanisms participating in regulation of the oncoembryonal protein, AFP, is an important task (Yin et al., 2003).

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The AFP gene belongs to albumin gene family (Gabant et al., 2002). All of them are synthesized in liver and secreted into blood serum, providing delivery of their bound ligands to different tissues. Albumin and AFP genes are located on chromosome 5 of mouse (Yang et al., 1990), 14 of rat (Remmers et al., 1993), and on the long arm of chromosome 4 of human (4q11-q13) (Song et al., 1999). They indicate that the closely related AFP and albumin genes are constituted by 15 exons containing about 2 kb of DNA and 14 introns with approximately 20 kb. With investigation of cDNA obtain from human Alpha-fetoprotein mRNA, three important parts are recognized: 44 non-coding nucleotides at the 5´ end, 1830 coding nucleotides and 155 non-coding nucleotides at the 3´ end (Peyton et al., 2000).

All the products of AFP gene transcription can be translated. The 2.1 kb nmRNA corresponds to

polypeptides weighing 68 and 70 kD (Karamova et al., 2003), that was shown like a single band by electrophoresis. The amino acid sequence was deduced from the nucleotide sequence, which revealed 19 amino acids in the signal sequence and 590 amino acids in mature AFP and with 4% carbohydrate residues. It is a secretory protein and it is linked by disulfide bridges make tube structures that have especial places for ligands linking (Morinaga et al., 1983).

On various experimental models, it has been shown that the expression is regulated mainly on the transcriptional level, the AFP gene having a 7 kb regulatory region upstream. Within this region, a tissue-specific promoter, three independent enhancers and a silencer (that is at least partially responsible for AFP gene expression decrease in adult liver) have been defined. Studies on the correlation of the methylation level of the AFP gene or its regulatory region and the gene expression level have given contradictory results (Hasse et al., 1994).

The use of DNA recombinant technology method for producing human alpha-fetoprotein was started by Giuliani in prokaryotes (Giuliani et al., 1989). But because of some difficulties in prokaryote expression system (such as non-production of AFP protein complete structure and inclusion bodies), the eukaryote expression system is preferred. One of the eukaryote that has been used to produce foreign proteins such as AFP is yeast. Methylotrophic yeasts are widely used as organisms for the production of recombinant heterogonous proteins (Cereghino et al., 2000). As unicellular eukaryotic organisms they combine the advantages of simplicity of molecular genetic manipulation with the ability to introduce post-translational eukaryotic modifications into protein products or foreign proteins (Minic et al., 2005). The use of strong and tightly regulated methanol-inducible promoters allows for controlled induction of recombinant gene expression and the generation of high yields of the foreign proteins. In *Pichia pastoris*, alcohol oxidase (AOX) is the first enzyme in the methanol utilization pathway and encoding by two genes, AOX1 and AOX2. Regulated expression can be obtained with the AOX1 promoter. The use of the AOX1 promoter has yielded expression levels that were either very high (on methanol) or very low (on glucose or other carbon sources) (Waterham et al., 1997). *P. pastoris* has been shown to be a suitable host for the industrial production of hetrologous glycoproteins (Sreekrishna et al., 1997). In this paper we report a high level expression of human AFP in *P. pastoris*.

MATERIALS AND METHODS

Strains and growth conditions

P. pastoris strains GS115 (His4) was provided by the Invitrogen company. Strain GS115 is the original His-deficient mutant strain (GS115/ His^-). The AOX1 gene disruptant (His4, aox1: HIS4) derived from GS115 was constructed by replacing the AOX1 gene with the HIS4 gene, and was used to make the diploid strain. *P. pastoris* strain (GS115/ -galactosidase) is provided as a His^+ Mut^+ intracellular expression control. Growth of strain during Mut^+ expression provides a positive control for expression conditions. *P. pastoris* strain (GS115/Albumin) is provided as a His^+ Mut^s intracellular expression control (Invitrogen, San, Diego).

Yeast strains were grown at 30°C either in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone or krypton, 1% (w/v) dextrose] , YPM medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.5% (v/v) methanol] , YPG medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1% (v/v) glycerol] , or minimal media containing 1.34% (w/v) yeast nitrogen base without amino acids and with ammonium sulfate, 0.5% (w/v) supplemented with one of the following: 0.5% (v/v) methanol (YNM medium); 1% (w/v) dextrose (YND medium).

Sub cloning

Escherichia coli strain JM109 (Invitrogen, San, Diego) was used in all cloning procedures involving plasmid propagation. Polymerase chain reaction (PCR), plasmid isolation, restriction analysis, cloning techniques, transformation of *E. coli* and isolation of genomic DNA were performed according to standard protocols (van Passel et al., 2004). Taq 2000 DNA polymerase (Taq Stratagene. La Jolla, CA) was used for all PCR reactions. PCR was conducted in 30 cycles of 1 min at 94°C (2 min for the first cycle), of 1 min at 55°C, and of 1 min at 72°C (7 min for the last cycle).

Yeast cells were transformed by electroporation and lithium chloride (Clemson et al., 2003). This procedure was adapted from the method of Beacker and Guarente (1991) . A 50- ml culture of GS115, a His4 auxotophic strain of *P. pastoris* (Gould et al, .1992), was grown in YPD at 30°C with good aeration to an OD_{600} of 1-2. The culture was supplemented with 1 ml of 1.0 M-Na + -HEPES, pH 8.0 and 1 ml of 1.0 M- DTT, and incubation was continued for an additional 15 min. The cells were transferred to a chilled 50-ml Falcon tube, centrifuged for 3 min at 2000 g in a tabletop centrifuge at 4°C, and resuspended in 50 ml ice-cold double-distilled H_2O. This H_2O wash step was repeated, followed by a wash with 20 ml cold 1.0 M-sorbitol. Finally, the cells were resuspended in 200 µl cold 1.0 M-sorbitol. A 40-µl aliquot of yeast cells was mixed with 0.1-1 µg DNA in 5 µl of a low-salt solution. This mixture was transferred to a chilled electroporation cuvette (0.2 cm gap) and pulsed with a Bio-Rad Gene Pulser set at 1.5 KV , 25 µF, 200 ohms (time constant ~ 5 ms). The cell suspension was immediately diluted with 1 ml of cold 1.0 M-sorbitol and transferred to a 1.5- ml centrifuge tube. After centrifugation for 1 min at 2000 g in a microfuge at room temperature, the upper 800 µl of liquid was removed and the cells were gently resuspended in the remaining volume. Finally, the cells were spread on YND - His^- plates supplemented with 1.0 M-sorbitol, and the plates were incubated at 30°C for 2-3 days until colonies appeared.

A 50- ml culture of GS115 in YPD at 30°C preparated for lithium chloride transformation method. After washing with sterile water, sterile TE buffer (10 mM Tris- HCl, pH 7.4, 1 mM EDTA, pH 8.0) , sterile TE buffer/LiCl (0.1 mM LiCl ,10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and incubate the tube at 30°C for one hour. For each transformation sample, the following were added to a sterile 12 75 mm polypropylene tube: 0.1 to 20 g of transforming DNA and 0.1 ml of competent GS115 cells. After incubating the tube at 30°C for 30 min, 0.7 ml of 40% polyethylene glycol (PEG-3350) in TE buffer/LiCl was added and vortexed briefly to mix. The samples were heat shocked at 37°C for 5 min and spread on selective plates such as YNM medium. Incubation of the plates was for 2-3 days at 30°C. Colonies were picked and screen for Mut^s and Mut^+ phenotypes.

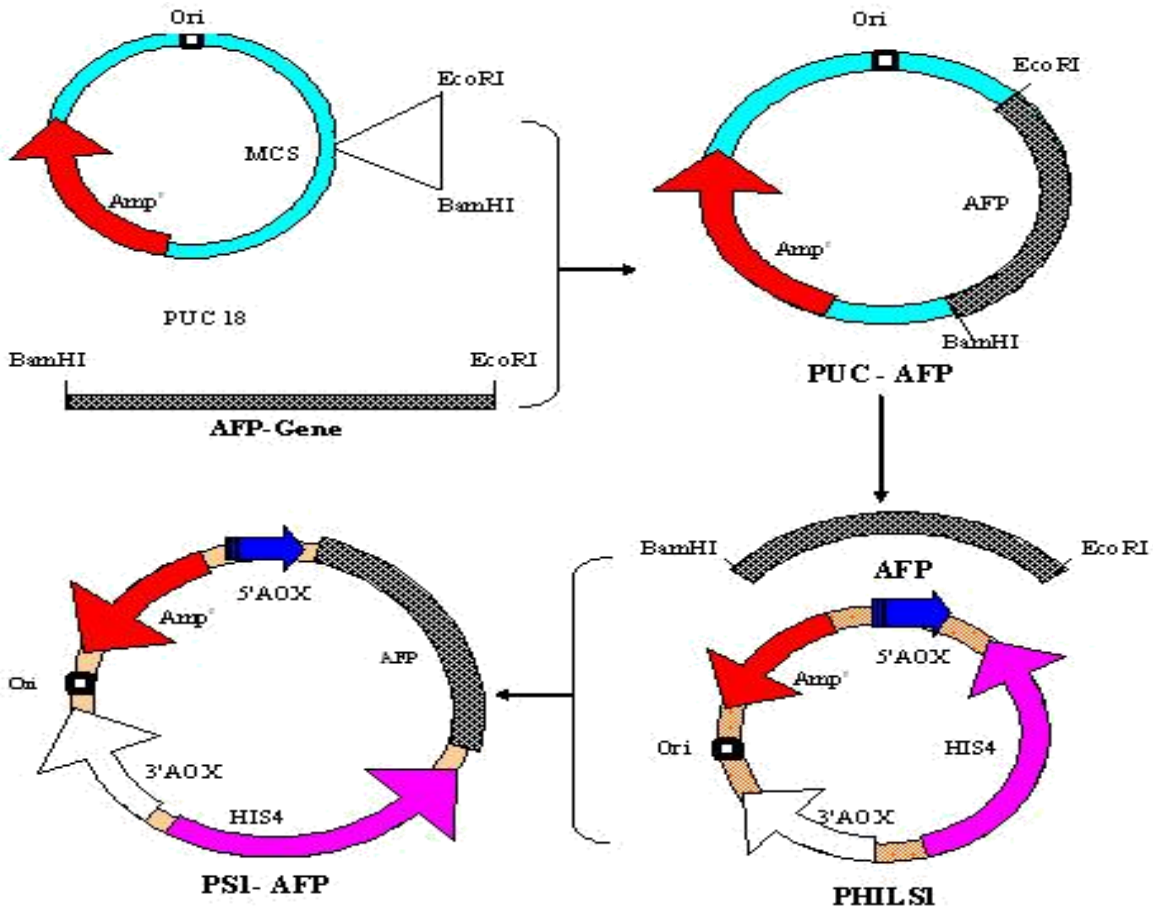

Figure 1. Constraction of expression vectors. PUC 18 and AFP cDNA were digested with EcoRI and BamHI and subsequently was isolated by preparative agarose gel electrophoresis and ligated to give pUC-AFP. pHIL-S1, a plasmid vector that for expression AFP cDNA in yeast. The 1.8 kb AFP cDNA fragment from pUC-AFP was inserted at the same sites (EcoRI and BamHI) of pHIL-S1 to create Ps1-AFP.

For screening for Mut^S and Mut^+ transformants, GS115 cells with Bgl II and Sal I – linearized pHIL-S1 constructs favor recombination at the AOX1 and HIS4 locus. Most of the transformants should be Mut^+; however, with presence of the AOX1 sequences in plasmid, there is a chance that recombination will occur at the AOX1 locus, disrupting the wild-type AOX1 gene and creating $His^+ Mut^S$ transformants. Displacement of the AOX1 structural gene occurs at a frequency of 5-35% of His^+ transformants. By patching or replica-plating on minimal dextrose (YND) versus minimal methanol (YNM) plates, Mut^S and Mut^+ transformants can be readily distinguished. Because Mut^S transformants are not producing alcohol oxidase (the product of the AOX1 gene), they cannot efficiency metabolize methanol as a carbon source and therefore grow poorly on YNM medium. This slow gene has been disrupted ($His^+ Mut^S$) from His^+ transformants with an intact AOX1 gene ($His^+ Mut^+$) (Invitrogen, San, Diego).

Subcloning of the AFP gene and construction of the yeast expression vector pHIL-S1

The subcloning strategy is depicted in Figure 1. The 1779 bp fragment of AFP gene, were amplified using the primers 5'-GCCGGAATTCACACTGCATAGAAATGAATATGG-3' (F-AFP; upstream primer including an EcoRI site), 5'-CGCGCCTAGGAATTTGAGGGTTTCGTCGTGCTC-3' (R-AFP; downstream primer including a BamHI site). After phenol/chloroform extraction and ethanol precipitation, the amplified products were subjected to agarose gel electrophoresis and extracted from the gel. The 1779 bp fragment of AFP gene and cloning vector (PUC 18) digested with EcoRI and BamHI, and then ligated to generate the recombinant plasmid (PUC18-AFP). After preparation, plasmid was transformed under heat shock (42°C) and calcium chloride for 90 s into *E. coli* (Invitrogen, Carlsbad, CA). Extraction and purification of subcloned plasmid were done using SDS (1%), NaOH (0.02 N), acetate sodium (3M) (Clemson et al., 2003).

PHIL-S1 is an *E. coli*/*P. pastoris* shuttle vector capable of expressing the *P. pastoris* AOX1 gene. Ps1-AFP was constructed as follows: First, the AFP fragment was digested with EcoR I and BamH I enzymes of plasmid PUC18-AFP. This resulted in a fragment with an EcoRI site at the 5' terminus and a BamHI site at the 3' terminus. Second, expression vector pHIL-S1 as well as digested with EcoRI and BamHI. Subsequently, the AFP fragment was inserted into the same sites to create Ps1-AFP. The plasmid was linearlized within the AOX1 promoter region by digestion with BglII and SalI prior to transformation into *P. pastoris*.

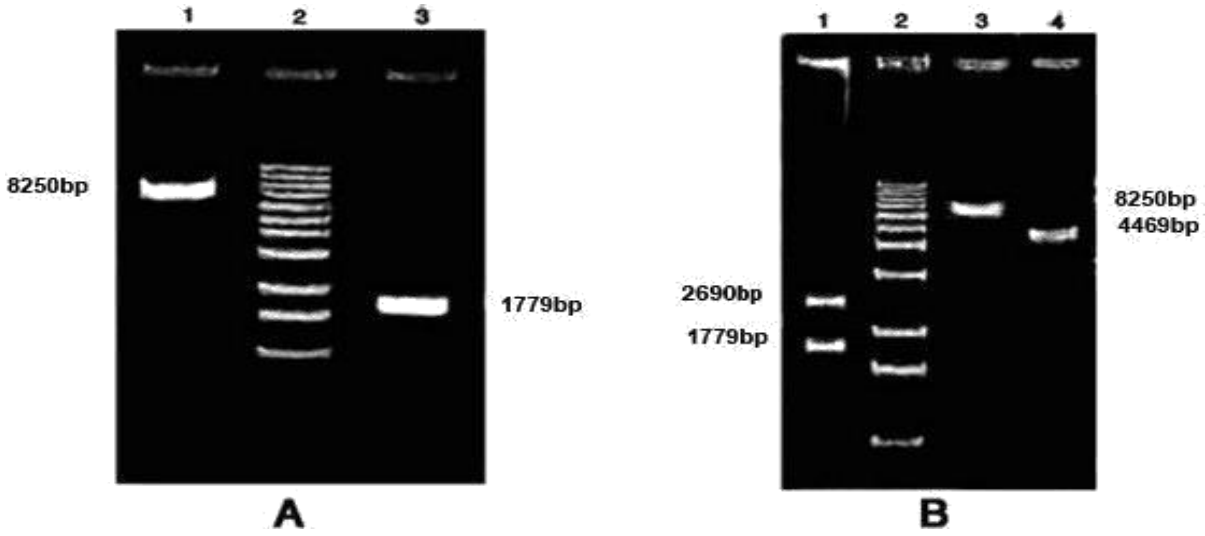

Figure 2. Analyses of AFP genes by agarose gel electrophoresis. **(A)** Agarose gel (1%) stained by ethidium bromide after electrophoresis. Lane 1, pHIL-S1 digested with EcoRI and BamHI; Lane 2, size marker (1 kb); and lane 3, AFP fragment digested with EcoRI and BamHI. **(B)** Lane 1, pUC-AFP digested with EcoRI and BamHI; lane 2, size marker (1 kb); lane 3, pHIL-S1 before digestion; and lane 4, pUC-AFP.

Expression of AFP cDNA in yeast

The expression vector (Ps1-AFP) was BglII digested and the expression cassette transformed in to histidine-requiring auxotroph (His^-) GS115 *P. pastoris* strain which acquired a His^+ phenotype following transformation. The transformed cells were analyzed by screening *P. pastoris* strains (GS115/ His^+ Mut^+ and GS115/ His^+ Mut^S) on YNM medium , to check for correct integration by replacement of the *P. pastoris* host AOX1 gene by expression cassette of the Ps1-AFP and to assure stable expression of a single integrant.

P. pastoris cells expressing AFP were grown in shake flask with 50 ml of YPG medium (10 g yeast extract, 20 g peptone, and 2% (w/v) of glycerol) at 30°C until OD_{600} of 5 – 10. Then they were harvested, washed once with YP (glycerol free YPG medium), resuspended in YP at approx. OD_{600} 5 and incubated at 30°C for 3 to 4 days with methanol to induce expression. The culture media from methanol induced transformants were tested to assess the percentage of AFP expression by densitometric scanning of the coomassie brilliant blue stained protein bands fractioned by SDS-PAGE using a LKB laser densitometer and also determined by ELISA. Temperature was maintained at 30°C. Cell yields were estimated by the wet cell pellet weight. Inoculations for the bioreactor runs were grown in a 1 L Erlenmeyer flask containing 500 ml of YPG medium. Bioreactor cultures grown in the batch mode were propagated in YPG medium until the available glycerol was exhausted, then methanol was added to the culture for 96 h at a rate of 2.5 ml - 1 L for batch mode bioprocess.

Yeast cells were washed once in disruption buffer (50 mM sodium phosphate, 5 mM EDTA, 10% sucrose, 0.3 M NaCl, 1 mM 2-mercaptoethanol, pH 7) and resuspended in the same buffer at a concentration of 350 g/l. The cells were disrupted for 14 min on a bead mill (Dynomill) equipped with a cold water cooling jacket at a flow rate of 20 ml/min. The pellet with 1 liter of washing buffer (50 mM sodium phosphate, 5 mM EDTA, 0.5% Triton X-100, 0.3 M NaCl, 1 mM 2-mercaptoethanol, pH 7), was centrifuged at 9000 g for 20 min and subjected to another three washings with a buffer containing 50 mM sodium phosphate, 5 mM EDTA, 0.5% Triton X-100, 0.3 mM NaCl, 1 mM 2-mercaptoethanol, pH 7.

Preparation of crude yeast lysates

Yeast cells were washed with sterile ice-cold water and harvested by centrifugation. The cell pellets were resuspended in an equal volume of 50 mM Tris-HCl, pH 7 with 0.2 mM PMSF and 5µg/ml leupeptin as protease inhibitors. 1 ml of the suspension was mixed with 500 µl of acid-washed glass beads and vortexed for 5 min. Cell debris was pelleted for 20 s at maximal speed in an Eppendorf centrifuge and the clear supernatant representing the crude cell lysate was used for biochemical analysis.

RESULTS AND DISCUSSION

Subcloning of the AFP gene and construction of the yeast expression vector pHIL- S1

AFP gene fragment which has restriction point of EcoRI and BamHI were inserted in polyclonal site (PCS) in pUC18 plasmid. JM109 competent cells were used for transformation and cultured in LB media containing AMP. In addition some colony as a positive control was obtained. Using alkaline procedure, recombinant plasmids of PUC-AFP from transformed cells were isolated and then restriction enzymes (Bam HI, EcoRI) were applied for further clarification and the fragments with length of 1779 bp and 2665 bp were ran on agarose gel electrophoresis (Figure 2A). The colonies which contain plasmids, pHIL-S1 and pUC-AFP, were cultured in LB media in large scale. Expression vector, pHIL-S1, was linearized by using restriction enzymes as mentioned above and confirmed on 1% gel agarose electrophoresis (Figure 2B). Recombinant plasmid ps1-AFP was constructed by T4 DNA ligase enzyme. For propagation

Table 1. Screening of transformed cells with pS1-AFP by YPD, MD, MM media. Positive marks shows high growth of transformed cells.

Number of transforming colony	YPD	MD	MM	Number of transforming colony	YPD	MD	MM
	+ +	+ +	+ +		+ ++	+ + +	+ +
2	+ ++	+ +	+ +	18	+ +	+ +	+ +
3	_++	+ +	-	19	+ +	+ +	+ +
4	+ +	+ + +	+ +	20	+ +	+ +	+ +
5	++	+ +	+	21	+ + +	+ + +	+ + +
6	+ ++	+ + +	+ +	22	+ +	+ +	+ +
7	+ ++	+ +	+ +	23	+ +	+ +	+ +
8	_	_	_	24	+ ++	+ + +	+ +
9	+ + +	+ + +	+ + +	25	++	+ +	+ +
10	++	+ +	+	26	+ +	+ +	+ +
11	+++	+ +	-	27	+ ++	+ + +	+ +
12	+ ++	+ +	+ +	28	+ ++	+ + +	+ +
13	+ +	+ + +	+ +	29	+ + +	+ +	+ + +
14	+ +	+ +	+ +	30	+ +	+ +	+ +
15	+ +	+ +	+ +	Control Mut^S	+++	+ + +	-
16	++	++	++	Control Mut+	++	+++	+++

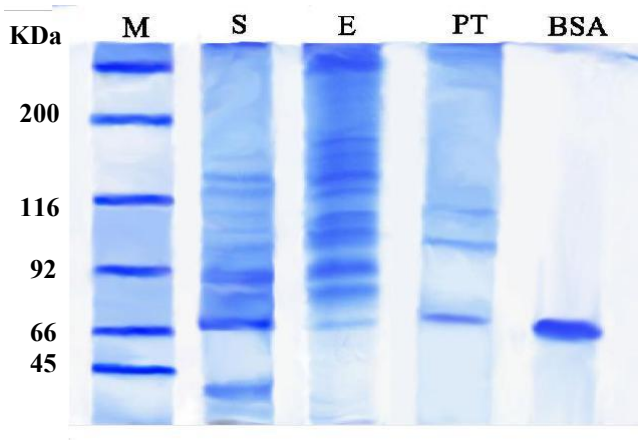

Figure 3. Analyses of expressed AFPs by 12% SDS-polyacrylamide gel electrophoresis. Coomassie blue-stained showing total cell extracts from GS115 *P. pastoris* strain induced with methanol, in each lane were loaded 100 g of total protein. Apparent molecular weight for human AFP is 69 kDa. **M**, molecular weight marker; **S**, cell extract; **E**, before induction; **PT**, secreted proteins after induction; **BSA**, bovine serum albumin (positive control).

of recombinant plasmid, JM109 strain was transformed by heat shot method and then cultured in LB media containing Apicillin. Before transforming in yeast cells for protein production, restriction mapping was carried out by using restriction enzymes such as BamHI, ECoRI and pVU II. For creating a stable recombinant, homologous regions between pS1-AFP and yeast genome were applied. For this purpose, above mentioned plasmid was cut by using BgIII enzyme and of this cut two different fragments were obtained. The fragment with 2870 bp had f1 ori and Amp^r region and the fragment with 7190 bp which contain AFP gene insert had 5' Aox1, HIS4, and 3' AOX1. The big fragment was isolated from gel and transformed into GS115/HIS strain. Using lithium chloride method and transporation methods 5 and 100 colonies were obtained, respectively. Two recombinant strains, His$^+$ Mut$^+$ and His$^+$ Mut^S based on big fragment insertion became linear in AOX1 region of yeast genome. From selected colonies, a few of them were recognized as His+Muts phenotype (Table 1). Based on screening on culture media (YPD, MD, and MM) mutants form like Mut$^+$ were recognized in plates 3, 11, and 25 in comparison to controls and other colonies were determined as Mut$^+$. For exploring AFP gene linking in yeast genome, PCR was done by using special primers.

Expression of AFP cDNA in yeast

Based on using pHIL-S1 plasmid and acid phosphatase secretory signals before AFP gene in yeast genome, it is expected that the produced protein should be secreted. Therefore, after methanol induction at 0, 24, 48, 72, 96, 120 h, induced cell suspensions were centrifuged and then electrophoresis was carried out on 12% SDS-PAGE and then stained by comassie blue (Figure 3). The obtained fragment on lane PT is indication of this secretory protein. For evaluation of AFP production, immunoassay procedure (ELISA) was used and AFP

protein concentration was measured within and outside the cell and the concentration were 10 and 2 ug/ml, respectively.

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Conclusion

Alpha-fetoprotein could be evaluated by immunological methods in fetal serum and in normal individual. Production of specific antibodies against AFP has diagnostic value in different condition particularly tumoral and non-tumoral disorders. Initially, for the production of recombinant AFP protein, prokaryotic systems such as *E. coli* were used (Nishi et al., 1988) . However, AFP is a eukaryotic protein and has specific characteristics which prokaryotic systems cannot process efficiency. Therefore, production in a simple eukaryotic system (yeast) is required (Yamamoto et al., 1995). At Iranian Pasteur Institute, AFP gene was constructed under phosphoglycerate kinase promoter in *S. cerevisiae* and for each ml of cultured media 1 ug recombinant AFP protein was obtained which had similar physicochemical characteristic to native one (Shahbazzadeh et al., 1995). However, for increasing productivity and stability of AFP protein, methylotroph *P. pastoris* yeast is needed. We recommend the use of a fermenter for increasing of the product ratio. The expressed AFP could be used for diagnostic applications.

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