Journal of Cell and Molecular Research (2019) 10 (2), 90-96 DOI: 10.22067/jcmr.v10i2.73401

Research Article

Prefractionation in Proteome Profile Analysis of ANXC4 Gene Mutant in *Aspergillus Fumigatus*

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Received 9 June 2018 Accepted 8 March 2019

Abstract

Aspergillus fumigatus is one of the pathogenic filamentous fungi that could cause opportunistic infection, allergy and poisoning. Since, genetic sequencing of some of these fungi has been completed, identifying the protein profile of these fungus cells is necessary. One of these genes is the Annexin family. Annexin C4 is a new member of fungal annexins. This study investigates the effect of ANXC4 mutant gene in proteome profile of *Aspergillus Fumigatus*. Moreover, in order to enhance the power of protein complex separation, we used an optimized prefractionation method. Using reverse phase-high performance liquid chromatography eight fractions were separated. Then to confirm the protein concentration, each fraction was tested by SDS-PAGE. Protein profile of these fractions was analyzed using 2-DE, and Image Master software. Among the proteins identified statistically, two emerging proteins were observed. The results show that the expression of ANXC4 could affect the expression of some proteins in *A.Fumigatus*. To accurately identify these proteins, further experiments are needed including Mass spectrometry analysis.

***Keywords*:** *Aspergillus Fumigatus*, RP-HPLC, 2-DE, Prefractionation, Proteome profile

Introduction

The genus *Aspergillus* contains nearly 200 species, only approximately 2 dozen of which are known to cause human disease; primarily *A. fumigatus, A. flavus, A. niger, A. terreus,* and *A. nidulans,* with each species at times causing unique clinical infections (W.J. Steinbach 2018). *A. Fumigatus*is an opportunistic filamentous fungus that causes prevalent infections in diseases such as tuberculosis, asthma and cystic fibrosis colonization of this fungus leads to respiratory tract destruction (Dagenais and Keller, 2009). Immunosuppression is the primary factor for the opportunistic infections that are generally called aspergillosis (Debeaupuis *et al.*, 1997)(Latgé, 2001). Secretion of extracellular proteins such as toxins and enzyme play a significant role in pathogenesis of this fungus. The need to study *A. fumigatus'* pathogenesis side mechanisms and its similar strains, and the necessity to develop disease prevention and treatment methods, have been the causes of getting more attention to genetic studies of these strains (Ronning *et al.*, 2005). Given the fact that, genetic sequencing of some fungi has been completed, identifying the protein profile of these fungus cells is necessary. One in particular that is at the center of our study is the Annexin family that is made up of more than a thousand members (KamandKhalaj et al. 2015).

Annexins are multifunctional proteins that bind to phospholipid membranes in a calcium-dependent manner (Maria Maryam 2019). They are expressed in most strains and branches of eukaryotes. These proteins have different functions in the cell, for example, participation in organization of exocytosis and endocytosis performance of the cell membrane, membrane integration and regulation of calcium channels. Annexins have been divided into five major families (A, B, C, D and E). Fungal annexins belong to group C. This gene is structurally different from other annexins (Gerke and Moss, 2002)(Moss and Morgan, 2004). In the past, *ANXC3.1* and *ANXC3.2* have been surveyed. Khalaj*et al.* (2004) identified and introduced *ANXC4*, as a new member of this family. At first these genes were identified by bioinformatics and sequencing, and then they were ascertained in the lab (Khalaj *et al.*, 2004). Disruption of *ANXC4* gene didn't show any particular phonotypical changes under different growth conditions, but in Khalaj*et al*. (2011) study, many considerable changes of protein expression were observed in *ANXC4* mutant and then with proteomics studies it was determined

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that these proteins were often involved in responding to oxidative stress (Khalaj *et al.*, 2011).
Understanding the genome and its polymorphisms is a way to comprehend gene function in cellular processes. Despite the importance of the genomics studies, cellular mechanism of cell function in different situations cannot be predicted based on its gene sequence. To apprehend the dynamic and varied processes of cells, including those actions that interfere with the development of disease, we must study the proteins that contribute to these advancements by proteomics method (Anderson, *et al.*, 2000)(Van Eyk, 2001). If these changes are exclusive to a particular condition of a disease, it is possible that they would be used as a biomarker for diseases (Srinivas *et al.*, 2002)(Govorun and Archakov, 2002).The three main parts of proteomics include, the analysis of complex protein compounds, quantification of analyzed compounds and identification of analyzed compounds. There are different methods to reach these aims. Today, the most successful technology is the combination of 2-DE[1] and mass spectrometry (Karpievitch *et al.*, 2010).
2-DE is used to analyze complex proteins before mass spectrometry, but analysis with this method alone has its limitations, including the fact that most of the proteins involved in diseases or drug targets, are of low-abundant proteins. To observe these proteins, it is necessary to increase their amount in the sample (Neverova and Van Eyk, 2005)(Karpievitch *et al.*, 2010). Prefractionation of the protein complex is an efficient way to increase the power of separation. There has been a considerable amount of effort for the creation and development of prefractionation methods as tools, to enrich the level of low abundant proteins in the sample. Included in these methods are chromatography, electrophoretic and fractionation by centrifuge (Srinivas *et al.*, 2002)(Badock *et al.*, 2001)(Righetti *et al.*, 2003).
RP-HPLC[2] is a technique that along with other methods of purification of macromolecules, is used for the analysis and purification of biomolecules (Righetti *et al.*, 2005). In Vaziri*et al*. (2006) the efficiency of RP-HPLC prefractionation on baby hamster kidney cells infected by rabies virus was assessed. It was then determined that prefractionation of cell extract successfully increases the power of protein separation (Vaziri *et al.*, 2006).

In the present research, a combination of two methods including 2-DE and RP-HPLC were used, to compare the expression profiles of *ANXC4* gene in two mutant and wild samples. Through a comparative study the efficiency of prefractionation in the separation of protein complex was investigated.

Materials and Methods

Culture of microorganisms

Aspergillus fumigatus fungus strain AF293 was cultivated in the biotechnology department of Pasteur Institute of Iran, and annexin gene *ANXC4* was knocked out in it. Creation of this mutant strain, called 'treat 18', was repeated three times. The wild strain, as the control group, was called 27 I, II, III. Samples were taken from these two strains and they were lysed.

RP-HPLC

The mutant and wild lysed samples were prepared for fractionation. A microtube containing 300 µl of sample was centrifuged at 14,000 g for 15 min. To analyze the proteins based on hydrophobicity properties, 70 µl of centrifuged sample was injected onto NucleosilC8 HPLC 4.6 mm column (Knaver, Germany) that was equilibrated with 100% buffer A (Ultra pure water and 0.1 TFA%). The UV-visible detector (Pharmacia LKB, Sweden) was set at 220 and 280 nm. From minute 5 to 90, fractions were collected on 10 min intervals. Fractions were then dried in a vacuum refrigerated concentrator (Krist, Germany), and stored in a freezer at -20°C. At the end, 8 fractions were collected from each sample.
The optimized method used, is based on step gradient concentration of buffer B (5% acetonitrile containing 0.1% TFA) and buffer A, which is shown in Table 1. The method used in HPLC step is as follows: first detection wavelength λ1 220 nm, second detection wavelength λ2 280 nm, flow rate 0.5 ml/min. In each run approximately 70 µg was injected onto the column. (Knaver, Germany).

SDS-PAGE

Dried fractions from the RP-HPLC step were dissolved in 30 µl of loading buffer and were boiled for 10 min in 90°Cin heater block. After cooling down, the samples, and the molecular weight marker were injected into gel wells. The gel electrophoresis was run at 100 V, and gels were stained using Coomassie brilliant blue R-250 (Bio Rad, USA).

[1] Two dimensional electrophoresis
[2]Reverse phase high-performance liquid chromatography

Table 1. Optimized method used for protein prefractionation

	Buffer A (%)	Buffer B (%)
Step 1	100	0
Step 2	75	25
Step 3	73	27
Step 4	70	30
Step 5	65	35
Step 6	50	50
Step 7	40	60
Step 8	10	90
Step 9	100	0

2-DE

First dimension: A non-linear immobilized pH gradient 4-7 IPG 7 cm ReadyStrip, was used. Each strip was placed into a tray channel. Samples were loaded on the strips, which were soaked in hydrating buffer, and after an hour were covered with mineral oil and kept at room temperature for 16 hours. Based on concentration, 35 µg of each sample was collected, then with the aid of hydrating buffer, containing 0.0072 g DTT, each sample's volume was increased to 125 ml. All solutions were centrifuged for 15 min with 14000×g at 4˚c. Supernatant of each solution was separately poured into trays. Then 16 IPG strips were discharged and placed on an IEF cell based on a time schedule shown in Table 2.

Table 2. Time schedule for the first dimension of electrophoresis (IEF)

Step	Ramping Mode	Maximum Voltage(V)	Time (min)	Volt – Hour
1	Linear	250	20	-
2	Linear	4000	120	10000 – 14000
3	Rapid	4000	300	10000 – 14000

IPG strip preparation for the second dimension: Strips were placed in equilibration buffer in two steps, so that the SDS contained in the buffer would get loaded on sample proteins. On first step, to revive protein disulfide bonds, for each 7 cm strip, 0.06 mg of 2% DTT was added to 3 ml of equilibration buffer. On second step, 70 ml iodoacetamide was added (the same ratios). This step was done for the alkylation of the thiol group contained in the sample. 2-DE and SDS-PAGE tests were repeated three times for each fraction 4, 5 and 6 of the control and treat samples. Electrophoresis of reduced and alkylated samples was carried out using 7cm 12% SDS-PAGE gels (Mini-PROTEAN 3, Bio Rad) and finally was stained by silver nitrate EBT (Bio Rad, USA).

Image Analysis

For the analysis and enumeration of present protein stains ImageMaster software was used. First the gels were scanned using GS_800 Calibrated Densitometer (Bio Rad, USA). With the use of the obtained images and ImageMaster 2D software, spot detection matching and editing steps took place, and finally the gels were analyzed. Then, we made a report about all the spots that had changed expression.

Results

Fractionation of proteins using RP-HPLC

The obtained chromatogram from repeated analytical tests, using RP-HPLC, could be observed in figures 1 and 2.In Figure 1 the increased amount of acetonitrile buffer, of RP-HPLC of lysed *A. fumigatus* proteins is analyzed. This analysis was based on hydrophobicity. Fractions that were more hydrophobic were used for SDS-PAGE. In Figure 2, the graphs from RP-HPLC of the control and treat samples, at 220 nm wavelengths were compared. Proteomes of *A. fumigatus'* cells were analyzed in eight fractions. F: Fraction, A: Absorption at unit

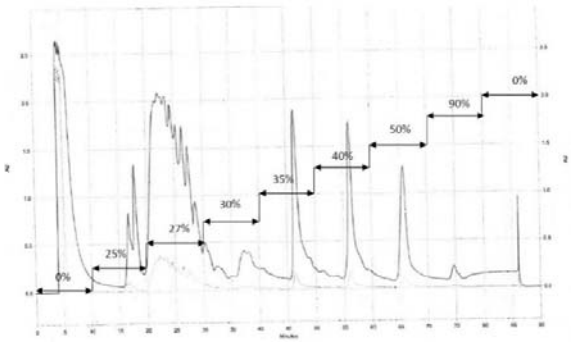

Figure 1.RP-HPLC chromatogram control

Results from SDS-PAGE method

After the RP-HPLC operation, each of the obtained fractions were analyzed using SDS-PAGE

method. The results are shown in Figure 3. The expression profile of the 8 fractions after chromatography was compared (A: Treat, B: Control). Analysis was done using 12% SDS-PAGE gels that confirm the RP-HPLC results.

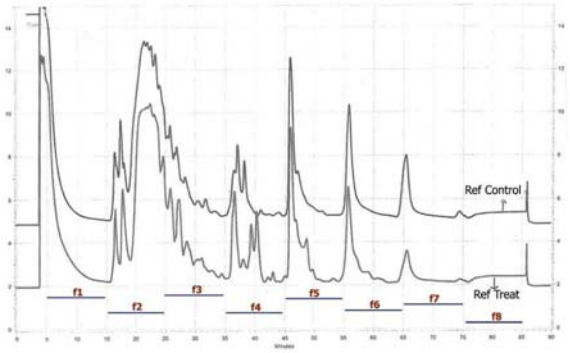

Figure 2. RP-HPLC chromatogram control and treat

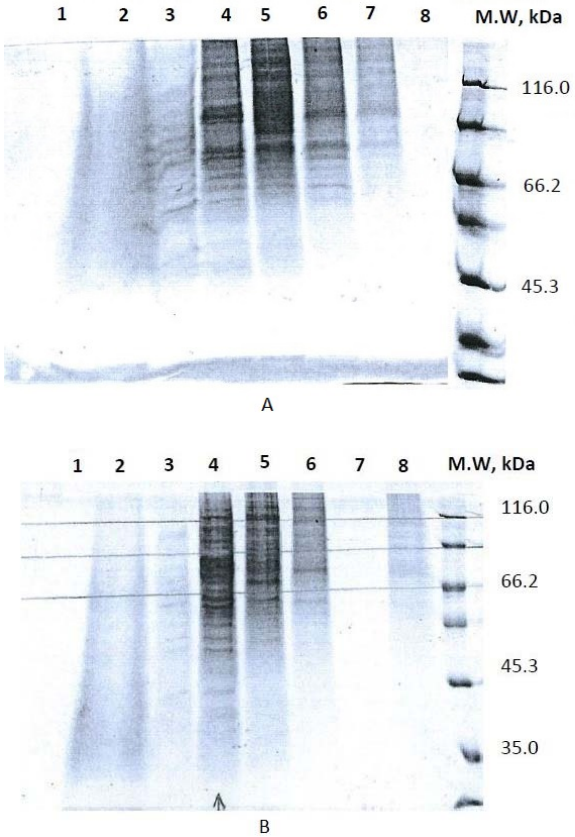

Figure 3. SDS-PAGE gel

2-DE Gel

After the fractionation and assessment of the sample concentration with the use of SDS-PAGE, fractions 4, 5, and 6 from control and mutant samples were selected and for each of them a two dimensional gel were used. The protein stains from 2-DE analysis are shown in Figure 4. 2-DE gels for the treat fractions 4, 5 and 6 are compared to the control samples using 12% large 2-DE gels.

Image analysis

With respect to the considered confidence level of this study (95%), based on the calculated degrees of freedom, the minimum t value was considered to be 2.77. Therefore, changes in which the t value was higher than 2.77 were considered statistically significant. The data from Image Master software was obtained with 5% confidence level consideration. In these settings, changed proteins in fractions 4 to 6 were assessed for increase or decrease. Table 3 contains the data that compare the changed protein stains in control and treat groups. CV: Coefficient variation, Fold: change of expression level, t: Average of three treatments, c: Average of three controls, F: Fraction

Discussion

Proteomics is an efficient technique for detection and identification of existing proteins in *Aspergillus fumigatus.* It is one of the pertinent methods to identify significant features of this fungus and classify its protein profile. In this study, to assess the expression profile of annexin gene ANXC4, and its changes as a result of a knockout, we used a prefractionation based RP-HPLC method. In order to compare the chromatograms, they were superimposed onto each other. The results of this comparison have been shown in Figure 3.2. Therefore, it was determined that this method has a satisfactory performance and it is replicable.

For the general analysis of the quality of fractionation for each fraction, SDS-PAGE was performed. In light of the SDS-PAGE results shown in Figure 3.3, it was determined that fractions 4 to 6 contain protein samples. Comparison of the peaks obtained from RP-HPLC with gels from SDS-PAGE shows that, firstly initial fractions, especially fractions 1, 2 and 3 that were from minute 5 to 35, contain very high amount of salt, so much that it has impaired the SDS-PAGE gel. Secondly, taking into account the protein content and the existing protein bands in each fraction, it was determined that, with respect to quality and quantity, SDS-PAGE gel is consistent with the acquired peaks from RP-HPLC. With the satisfactory results from the power of RP-HPLC in

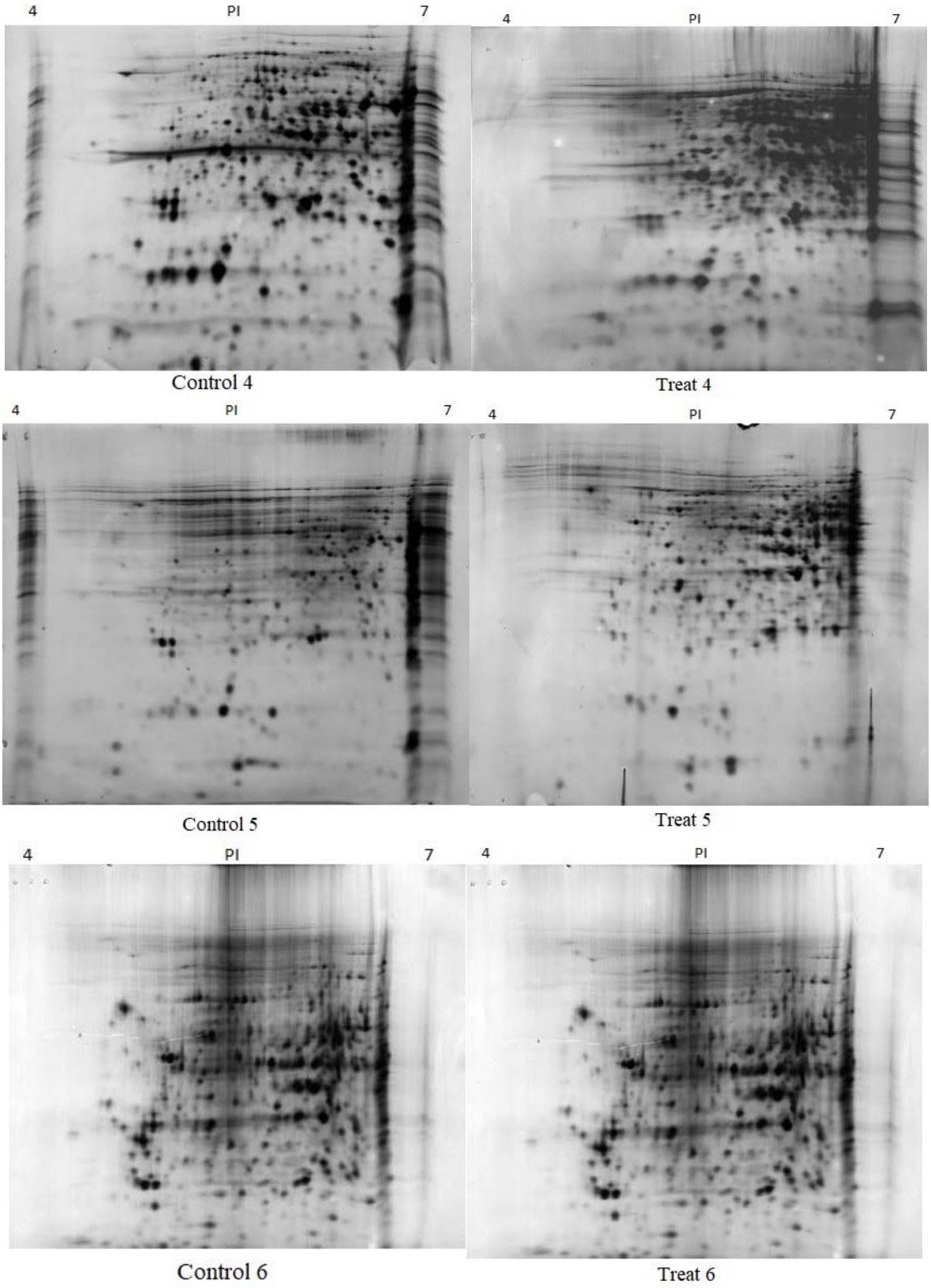

Figure 4. 2-DE gels for control and treat fractions 4, 5, 6

Table 3. Statistical analysis of observed protein stains from2-DE gel

Match ID	c (vol %)	t (vol %)	Coefficient Variation c	Coefficient Variation t	t -student	Fold change c/t
970 F4	0.368655	-	15.422	-	3.86304	-
1036 F4	1.010225	0.566525	9.851612	7.870546	7.05	1.783196
1272 F5	1.20446	0.57773	8.154575	5.015742	10.2693	2.021693
1174 F5	0.321039	0.10402	25.36911	41.45974	3.76044	3.086297
1055 F5	0.901037	0.602063	10.5398	21.6898	3.20709	1.496584
1044 F5	0.322710	0.230281	16.18483	10.65181	2.77666	1.401378
924 F5	0.468550	0.335923	9.25208	4.882869	6.3104	1.552839
1153 F5	1.051612	0.616247	8.723319	13.09232	6.17248	1.706478
1036 F5	1.103101	0.872303	9.539745	3.871478	3.61706	1.264585
1176 F5	0.960174	0.540951	13.8282	6.784911	5.27109	1.774973
1354 F6	0.724183	0.331232	22.28373	23.02627	3.07	1.804723
1496 F6	0.443131	0.159491	22.16693	22.14439	4.70641	2.778406
1517 F6	0.441796	-	9.851612	-	2.91	-

protein analysis, the two-dimensional electrophoresis was performed afterward. As SDS-PAGE gel compared with fraction 5 has several protein bands, while the gels obtained from 2-DE compared with SDS-Page have much higher analytical power. For analysis and enumeration of protein stains, ImageMaster software was used. With the attained results from the software, the number of changed proteins in fractions 4 to 6 was assessed for increase or decrease of expression. Among the 1654 identified proteins in these fractions, and in view of the considered t-test, 11 proteins in treat group had decreased expression, and two emerged proteins were encountered.

Although 2-DE is a powerful system to analyze proteins, it is unable to analyze low abundant, very small and very large proteins. In prefraction RP-HPLC proteins were separated based on hydrophobicity. In light of the other added protein characteristics in this analysis before 2-DE, this technique could be an efficient method used to increase the power of analysis and separation of complex protein mixtures in *Aspergillus Fumigatus* proteome. Therefore, the analysis and fractionation prior to doing 2-DE caused increased accuracy in identifying changes in protein profile expression of *ANXC4* gene. In fact, with this method, another dimension is added to the analysis of proteins. In order to accurately identify proteins with changed expression, the use of mass spectroscopy methods is necessary. With the results of the present study, we can identify the proteins involved in genomes of *Aspergillus Fumigatus* more precisely, and benefit from it in future research projects.

Acknowledgements

Authors acknowledge Dr. Vahid Khalajin biotechnology department of Pasteur Institute of Iran, for Giving samples of mutant strain of *Aspergillus fumigatus*. The authors also express their gratitude to Dr. Behrouz Vaziri for their valuable advices.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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