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Affinity Based Proteomic Profiling of Yeast in Response to Phenobaritone.

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ABSTRACT

In the last two decades the field of proteomics has been grown enormously. This is ensued because of the world's attention is shifted from genome projects to proteome projects for understand the biological processes on the protein level. The study of proteins (proteome) is important because the dynamics of all living beings is lies in the biochemical structure and function of proteins. It is more complex than analysis of genomes. Most of the current proteomic techniques are unable to separate each and every protein of an organism and which only focused on the analysis of high abundance proteins. Therefore in the present study we have tried to overcome these problems using combinatorial approach. In briefly, we have grown yeast *Saccharomyces cerevisiae* on YPD media under control and phenobarbitone treated growth conditions. These grown cells were harvested, homogenized and isolated cytosolic proteins. These proteins were further seperated using affinity chromatographic and gel electrophoresis. The obtained results were clearly showed that this approach can be used to detect, fractionate and purify targeted families of low-abundance or extremely acidic or basic proteins from the complex organisms. It can also be used for prefractionation of proteins before 2-DE or MS. Hence it is simple, reliable, low-cost and superb tool in proteome analyses.

Keywords: Affinity chromatography, cytosolic proteome, phenobarbitone, prefractionation, proteomic profiling, yeast etc.

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INTRODUCTION

In the last two decades there is rapid development in the technologies of genomics and proteomics to understand the biological processes of an organism at molecular level such as genes, proteins or transcripts. Only the analysis of genes (genome) and transcripts (transcriptome) are not only adequate to understand the biological processes but also analysis of proteins (proteome) is important because the dynamics of all living beings lies in the biochemical structure and function of proteins [1,2]. Therefore, the systematic study of all proteins expressed by genome at given time and at a given condition and provides the complete information about the biological processes of healthy and disease conditions. These proteins are actual workhorses of biological cells and they are involved in all most all biochemical reactions of life. Ahead to that, if we identify a proteins involved in metabolic pathways with their connection with neighboring pathways than we can understand whole system of an organism (i.e. system biology). In eukaryotic organisms the study of all these proteins is challenging job because of complexity of eukaryotic cell. The study of proteins with their subcellular organelles is challenge ahead to researchers [3-5]. That means the number of proteins present inside a cell are more in numbers than genes that is because of an alternate splicing. These proteins can be change according to growth of development and environmental factors [6]. So, study of all these proteins of a complex organism is required to understand the biological processes of healthy or diseased condition. Such type of study is largely depends on advanced techniques that start from two-dimensional gel electrophoresis (2-DE) followed by mass spectroscopy (MS) [7].

Most of the present techniques are unable to detect, separate and identify very high, very low-abundance, extremely basic and extremely acidic proteins involved in vital biological processes [8]. Hence, there is a need to develop more proficient techniques to analyses complex proteomes. At present major focus of proteomic studies is on subcellular level to unravel novel concept in cell biology leading to a better understanding of complex cellular processes. This type of approach can overcome the limitations of present proteomic technologies and helpful in the reduction of complexity of complex proteome into simple proteomes and to study of the diverse properties of proteins such as expression, structure, function and interactions of diseased conditions [9-12]. Therefore, there is need to develop new technologies or use combinatorial approaches as well as uses simple, reliable, low-cost and superb tools to study above mentioned problems of proteome analyses.

Currently, many efforts have been made to enhance the resolution, identification and characterization of low abundance proteins using various methods such as fractionation, isolation, labeling, purification etc. The present techniques used for the fractionation and purification of proteins need further development to fractionate, detect and purify all such proteins before 2DE or MS [7, 13-14]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been the most frequently used classical method to fractionate proteins from complex samples [15]. This gel-based method is also unable to resolve very high, very low-abundance, extremely basic and extremely acidic proteins at a time. Another disadvantage of gel-based methods is narrow dynamic range and limited sensitivity. Thus, liquid-based methods can be the best choice to supplement the gel-based approaches. Various combinations of chromatographic techniques with gel-based methods can be good approach to improve the level of protein fractionation, detection and purification of proteins [16-17].

In the present study we have applied simple, cost effective and combination of routinely applicable techniques such as chromatography and electrophoresis for the isolation and purification of yeast proteins [18]. In brief, the yeast *Saccharomyces cerevisiae* (NCIM No. 3090) cells were grown on YPD (1% yeast extract, 2% peptone and 2% dextrose sugar) media. Some of the cells were exposed to protein inducer such as Phenobarbitone with control [19]. These grown cells were homogenized, fractionated and isolated cytosol and their proteins [20]. The isolated cytosolic proteins were directly fractionated by 10 different MIMETIC ligand (A6XL) affinity chromatographic columns on the basis of their affinity to immobilized ligands [21-23]. 2 ml of solubilized cytosolic proteins of control and altered conditions were loaded on pre-equilibrated columns (25 mM sodium phosphate buffer, pH 6.8) one after another and all adsorbed proteins were eluted with elution buffer containing 2M sodium chloride as per procedure given by company. The columns effluent was monitored and total protein content of pooled fractions was estimated by Lowry's methods [24]. The results obtained from AC clearly showed that the protein pattern of altered condition is changed due to the effect of phenobarbitone as compare to control. The collected fractions of AC were further resolved by SDS-PAGE. The results obtained from both the techniques shows that the complexity of protein has been reduced on the basis

of their affinities [18]. These induced proteins can be further identify and characterize by MS and followed by bioinformatics databases. One of the advantages of this approach is that it helps to resolve particular families of low and high abundant proteins from the population of proteins in complex samples. So this combinatorial approach is simple, reliable, low-cost and superb tools and can be used for pre-fractionation before 2-DE or MS and it can be used an alternative to 2-DE as well as reduction of complexity of proteins from complex proteome [25-28].

MATERIALS AND METHODS

Materials

Standard laboratory chemicals used in this work were obtained from Sigma (St. Louis, MD), Ranbaxy (A-3, Phase-1, New Delhi), SRL (Mumbai, India). The yeast *Saccharomyces cerevisiae* (NCIM No.3090, ATCC No. 9763, NCYC No. 87) was purchased from National Chemical Laboratory, Pune (MS), India. MIMETIC legend A6XL adsorbents with PIKSI^{TM} test kit were from Affinity chromatography Ltd., Freeport, Ballasalla, Isle of Man, British Isles (UK). All chemicals used in this work were of analytical grade.

Cell Growth and cell Disruption

Yeast *Saccharomyces cerevisiae* (Strain No.3090) was grown aerobically on YPD (1% yeast extract, 2% peptone and 2% dextrose) media at 28°c with occasional shaking. The cells were grown in identical culture flasks and out of them few were exposed to classical protein inducer such as Phenobarbitone. After 48 hours of fermentation, the cells were harvested using centrifugation (Centrifuge R-14) at 8000 rpm at 4°C for 20 minutes and then suspended in 25 mM sodium phosphate buffer (pH 6.8) containing 0.25M sucrose [20].

The above harvested cells were lyesed by sonication (Sonifier Branson-450) in cold condition to avoid denaturation of enzymes or proteins. 50% duty cycle, 5 (micro tip limit) output control and 20-20 seconds shocks were given up to 5 minutes for cell lysis. Seven strokes were given to open the tough cell wall of *S. cerevisiae*. The resultant lysate was used for protein profiling of yeast. The same procedure was used for altered samples [18-19].

Isolation of yeast cytosolic proteins

The above resultant lysate was centrifuged (Centrifuge R-14) at 8000 rpm at 4°C for 20 minutes for the removal of cell debris. These cell debris contained contaminants, nutrient particles, organelles etc. The resultant supernatant was collected and contained all cytosolic proteins or enzymes and targeted biomolecules and used as a sample for further analysis [29-32]. The protein content of this fraction was measured by Lowry protein assay [24]. The same procedure was used for altered samples.

Affinity Chromatography

The proteins of above cytosolic fraction were directly purified by affinity chromatography (AC) on the basis of their affinity to various immobilized ligands [33-34]. Pre-packed integrated unit of PIKSI kit containing 10 different properties MIMETIC ligand (A6XL) columns (1.5 cm diameter and 2.4 cm height) were used for purification of cytosolic proteins (Affinity chromatography Ltd., Freeport, Ballasalla, Isle of Man, British Isles). These columns were equilibrated with the several volumes of equilibration buffer such as 25 mM sodium phosphate buffer, pH 6.8, in which sample were prepared. After the equilibration, 2 ml of solubilized proteins (3.097 mg/ml of control and 4.373 mg/ml of altered) of yeast extract were loaded carefully on pre-equilibrated column and followed by 3 ml of equilibration buffer. Total non-adsorbed and all adsorbed proteins were eluted with equilibration buffer and elution buffer containing 2M sodium chloride respectively. The entire procedure was conducted at room temperature. The flow rate of each column was already adjusted at 10-30 ml/h/cm and 5 ml fractions were collected from each column [35-36]. The column effluent was monitored and total protein content of pooled fractions was estimated by Lowry's assay. Bovine serum albumin (BSA) was used as a standard [24]. All columns were washed several times with preservative and stored for several weeks at 4ºC. The same procedure and column was used for respective altered samples.

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Acetone Precipitation

Those fractions having significant change in control and altered condition have been selected for further study. One volume of selected fraction and three volume of ice-cold acetone were mixed together and kept for two hours on ice bath. After incubation, the precipitated proteins were collected by centrifugation at 2000 rpm for 10 minutes at 4˚c [18]. These proteins were then dissolved in distilled water and used for further analysis with SDS-PAGE electrophoresis. Same procedure was used for remaining fractions.

SDS-PAGE

The above purified fractions by chromatography were further purified on electrophoresis on the basis of charge and mass. The equal volume of above concentrated proteins and sample buffer containing DTT, bromophenol blue and glycerol were mixed and heated in boiling water bath for 2 minutes. These samples were cooled at room temperature and then 100µl samples were loaded on 12% SDS-PAGE gels. Electrophoresis was performed on slabs (10cm×8cm×1mm) for approximately 8 hours at 20 mA. Gels were stained overnight with 0.1% Coommassie Brilliant Blue G-250 in methanol, acetic acid and water (4:1:5) and destained with the same solution except Coommassie Brilliant Blue G-250 [18].

Statistical analysis

The growths of cell and protein concentration of both the conditions were determined identically. The mean and standard deviation of each five experiments were determined statistically.

RESULTS

Effect of phenobarbitone on growth and protein profile of yeasts:

In the present study we have selected yeast *Saccharomyces cerevisiae* (Strain No. 3090) as a model organism and cultured on YPD media. Some of the cells of yeast grown under normal condition and some were grown in phenobarbitone treated growth condition as described previously in the material and method. After 48 hours of fermentation, these cells were harvested, weighted, sonicated and isolated cytosolic proteins. The protein content of isolated cytosolic fractions of both conditions was measured by Lowry's method [24]. The obtained results were clearly showed that the protein content and cell number of altered condition is increased as compare to control and it is shown in **fig.1**.

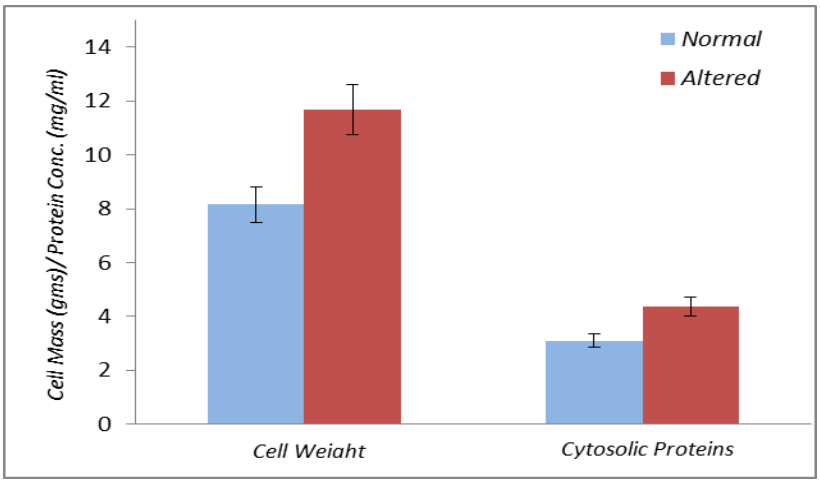

Fig. 1: The effect of phenobarbitone on the growth and protein content of yeasts.

Use of affinity chromatography for the fractionation of yeast cytosolic proteins:

Above solubilized cytosolic proteins were further fractionated by affinity chromatography on the basis of interaction of proteins with ligands. Two milliliters of above protein sample was loaded on each pre-

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equilibrated column and 5 ml fractions were collected from each column with pre-adjusted 10-30 ml/h/cm flow rate. The first fraction contained all non-adsorbed proteins and remaining fractions contained adsorbed proteins. These adsorbed proteins have been eluted by increasing concentration of NaCl in equilibrium buffer. Each fraction was monitored and then total protein content was measured by Lowry's method [24]. The obtained results were showed in the **table no. 1** and **table no. 2**. The same procedure was used for altered samples. The obtained results from both the conditions from different columns clearly indicate that some of the protein families of altered growth condition has been over expressed as compare to control and it is showed in **figures 2-11**.

Table 1: Detail of affinity chromatographically purified cytosolic proteins at control condition.

Column numbers	Loaded sample mg/2 ml	I Fraction mg/5ml	Adsorbed proteins (mg)	% of adsorbed Proteins	% of non-absorbed proteins	II Fraction Mg/5ml	Adsorbed proteins (mg)	III Fraction Mg/5ml	IV Fraction Mg/5ml	Remaining Proteins
I0020	6.602	5.03	1.572	32.81	76.18	1.105	0.467	-.01	0	0.467
II0030	6.602	4.96	1.642	24.87	75.12	0.945	0.697	-0.01	0	0.449
III0050	6.602	4.805	1.797	27.21	72.78	0.535	1.262	-0.025	0	1.262
IV0070	6.602	4.725	1.877	28.43	71.56	0.77	1.107	0.094	0	1.0125
V0090	6.602	4.455	2.147	32.52	67.47	1.055	1.092	0.02	0	1.072
VI0013	6.602	4.23	2.372	35.92	64.07	0.48	1.892	0.03	0	1.862
VII0040	6.602	4.695	1.907	28.88	71.11	0.42	1.487	-0.021	0	1.487
VIII0060	6.602	4.53	2.072	31.38	68.61	0.75	1.322	-0.000	0	1.322
IX0080	6.602	4.1	2.502	37.89	62.10	0.82	1.682	0.995	0	0.687
X0100	6.602	5.195	1.407	21.31	78.68	0.955	0.452	0.245	0	0.207

Table 2: Details of affinity chromatographically purified cytosolic proteins at altered condition.

Column numbers	Loaded sample mg/2ml	I Fraction mg/5ml	Adsorbed proteins (mg)	% of adsorbed Proteins	% of non-absorbed proteins	II Fraction Mg/5ml	Adsorbed proteins (mg)	III Fraction Mg/5ml	IV Fraction Mg/5ml	Remaining proteins
I0020	8.746	4.42	4.326	49.47	50.53	1.715	2.611	-0.17	-ve	2.611
II0030	8.746	5.08	3.666	41.92	58.08	2.00	1.666	-ve	-ve	1.666
III0050	8.746	5.475	3.271	37.40	62.60	1.49	1.781	0.115	-ve	1.666
IV0070	8.746	5.22	3.526	40.32	59.68	1.10	2.426	-0.19	-ve	2.426
V0090	8.746	5.54	3.206	36.66	63.34	1.76	1.466	-ve	0.145	1.301
VI0013	8.746	4.795	3.951	45.18	54.82	1.755	2.196	-ve	-ve	2.196
VII0040	8.746	4.01	4.736	54.16	45.84	1.105	3.631	-ve	-ve	3.631
VIII0060	8.746	5.14	3.606	41.24	58.76	1.155	2.451	-ve	0.515	1.936
IX0080	8.746	5.41	3.336	38.15	61.85	0.99	2.346	-ve	-ve	2.346
X0100	8.746	5.31	3.436	39.29	60.71	1.26	2.176	-ve	0.08	2.096

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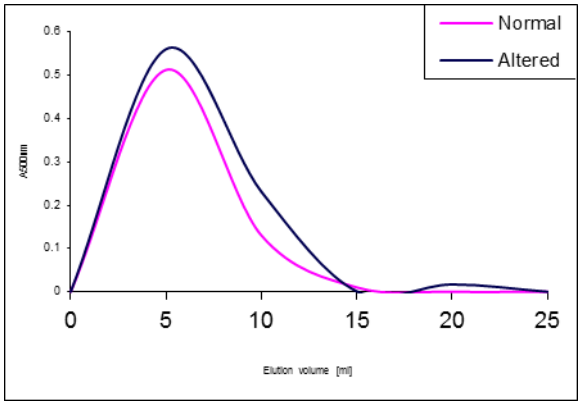

Fig. 2: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0020 MIMETIC RED™ A6XL.

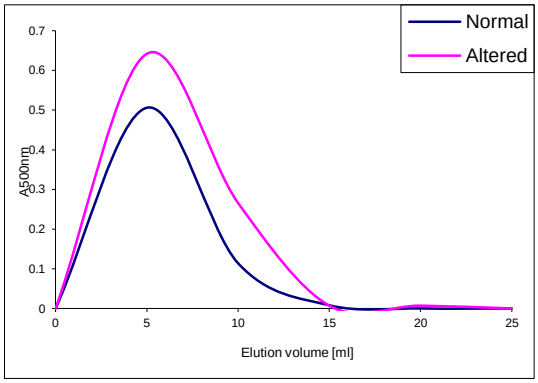

Fig. 3: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0030 MIMETIC ORANGE™ A6XL.

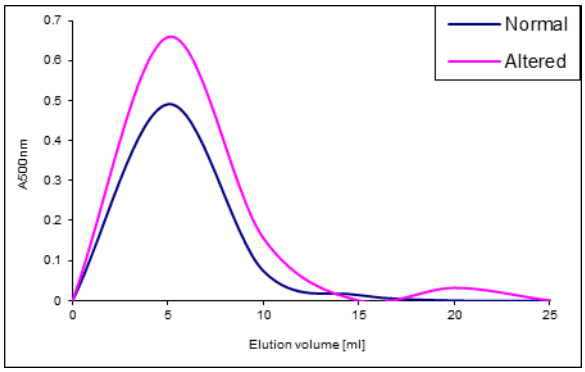

Fig. 4: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0050 MIMETIC ORANGE™ 2 A6XL.

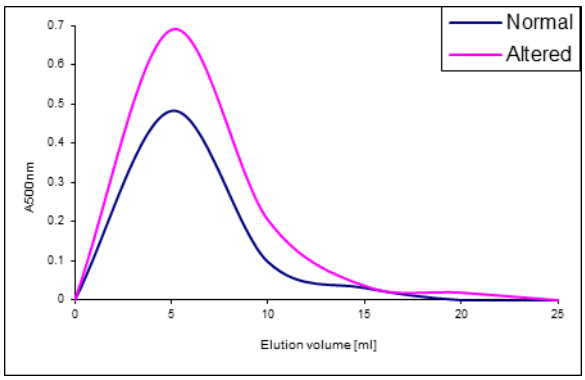

Fig. 5: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0070 MIMETIC YELLOW2 A6XL.

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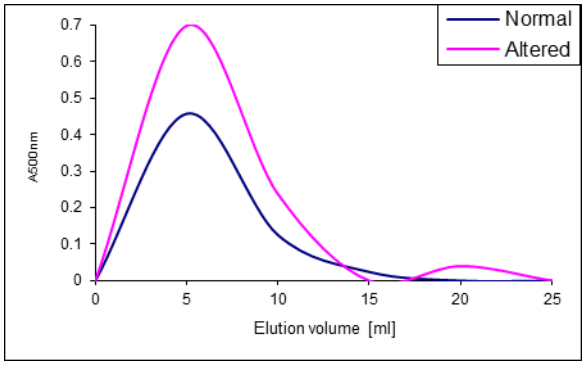

Fig. 6: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0090 MIMETIC BLUE™ A6XL.

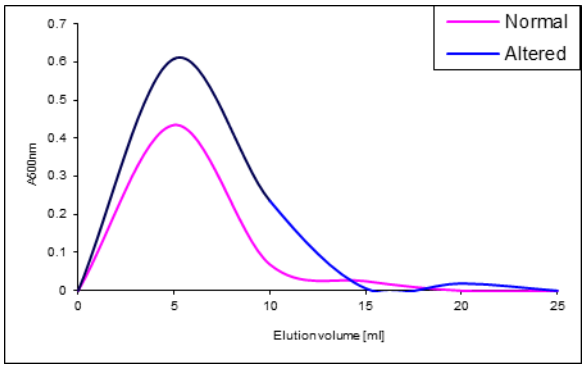

Fig. 7: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0013 MIMETIC RED™ 3A6XL.

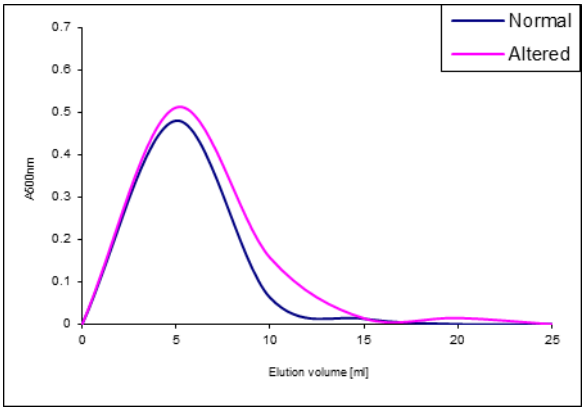

Fig. 8: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0040 MIMETIC ORANGE™ A6XL.

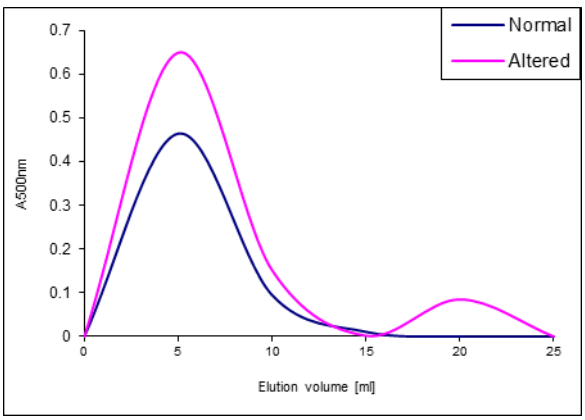

Fig. 9: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0060 MIMETIC YELLOW™ A6XL.

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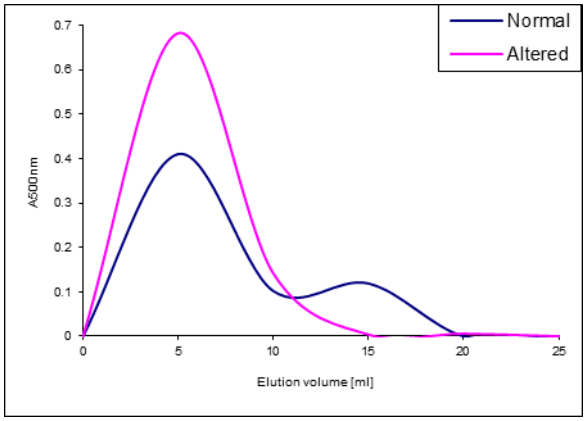

Fig. 10: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0080 MIMETIC GREEN™1 A6XL.

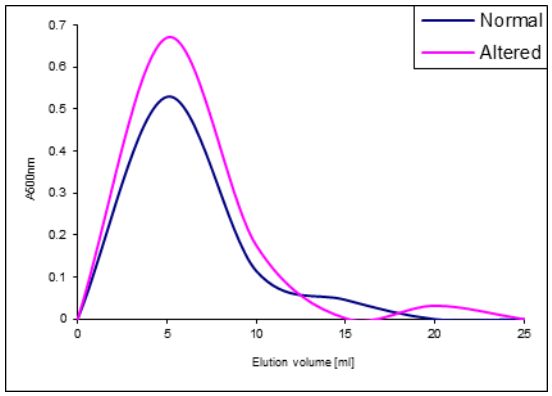

Fig. 11: Affinity chromatographic protein profile of yeast cytosol by affinity column: 0100 MIMETIC BLUE 2 A6XL.

Use of SDS-PAGE for further analysis of yeast cytosolic proteins:

Above chromatographically fractionated proteins were further purified by SDS-PAGE on the basis of charge and mass. Those fractions of control and altered condition showing significant changes in their protein contents they were selected concentrated and resolved on 12% SDS-PAGE. A typical protein band pattern of each pooled fraction of both conditions was obtained after staining and destaining and it is shown in **fig. 12** and **fig. 13**. The results obtained from one dimensional electrophoresis (1-DE) again showed that the protein profile of altered condition contains more proteins than control. The futuristic study will includes identification and characterization of altered proteins of cytosol. For this the above electrophoretically separated proteins can be excise, destain and fragmented by proteolytic enzymes and further analyze with matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) followed by bioinformatics tools such as various software, database and worldwide webs [13, 17, 25].

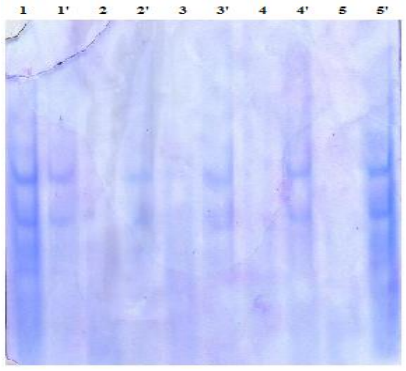

Fig. 12: 1-DE of affinity chromatographically separated yeast cytosolic proteins of control (1-5) and altered (1'-5') conditions. Proteins have been resolved on 12.5 % SDS-PAGE.

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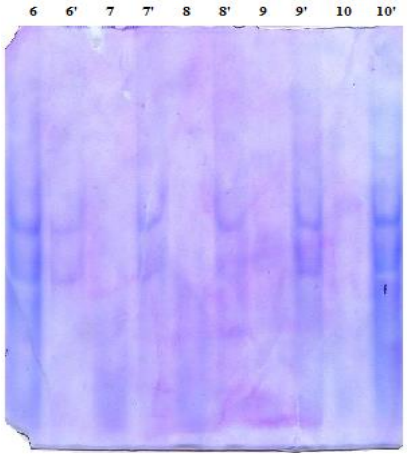

Fig. 13: 1-DE of affinity chromatographically separated yeast cytosolic proteins of control (6-10) and altered (6'-10') conditions. Proteins have been resolved on 12.5 % SDS-PAGE.

DISCUSION

Saccharomyces cerevisiae has been the subject of a wide variety of proteomic analysis because of its availability of complete genome sequence and its 30% genes are homologous with human genes [19]. The understanding of these proteins is more complex and technically challenging job, however yeast can be viewed as a window to study human's proteins and it was one of the goal of this study using yeast as a model organism. A variety of approaches have been applied for the fractionation, detection, quantification, isolation, purification and characterization of entire proteins expressed by its genome in a given conditions. Currently there are several many techniques have been developed to achieve this goal but these techniques have their own limitations and unable to resolve low abundance or very high abundance or extremely basic and acidic proteins or analyses complex proteomes. Keeping this in mind we tried to detect low abundance proteins and fractionation of families of proteins as well as we have tried to reduce the complexity of yeast proteome. To achieve this goal we have used simple, reliable, low cost and routine applicable techniques such as affinity chromatography and electrophoresis for the fractionation, isolation, purification and characterization of proteins. The results obtained from above mentioned combinatorial approach is successfully fractionated, detected and quantified families of yeast cytosolic proteins of both the conditions. The obtained results clearly shown that a lot of changes have appeared in protein pattern of altered as compare to control due the effect of phenobarbitone. These altered proteins may be extremely resourceful to interpret various growth conditions and overall metabolic patterns of proteins as well as genetic alteration such as gene deletion or over expression in yeast cytosolic proteome. This changed protein pattern representing that the genetic makeup or some metabolic pathways are activated by this drug. Therefore, the quality of data obtained will be quite helpful to analyze selectively and also as syndrome of reactions. The proteome of organisms varies with the nature of the state of development, disease or in response to some drugs. One of the advantages of this approach is that it helps to resolve all such proteins from the population of proteins in complex samples. The numbers of advanced technologies are of little help to resolve these proteins from protein samples. AC separates each protein on the basis of affinity and SDS-PAGE further separates proteins on the basis of charge and mass. Therefore, this can be one of the important approach in the proteomic research for the pre-fractionation of proteins before 2-DE or MS.

CONCLUSION

There is convening evidence that this type of combinatorial approach can be used for fractionation, detection, quantification and purification of various families of proteins and it can be used for pre-fractionation of proteins prior 2-DE or MS. This an approach can overcome the limitations of 2-DE as well as helpful in the reduction of complexity of complex proteome into simple proteomes. Thus, it is simple, reliable, low-cost and superb tool for pre-fractionation and proteome analysis.

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