INSIGHTS INTO THE PROTEIN PROFILES OF STRESS TOLERANT AND MESOPHILIC YEAST BY PROTEOMICS APPROACH: A COMPARATIVE STUDY

B. Bhima, T. Anjana Devi[1] M. Sudhakara Reddy, Y. Ramana Reddy[2] and L. Venkateswar Rao*

*Department of Microbiology, University College of Science, Osmania University, Hyderabad, A.P. 500 007, INDIA.
[1]Centre for Chemical Biology, Indian Institute of Chemical Technology, Tarnaka, Hyderabad, A.P.
[2]Department of Animal Nutrition, College of Veterinary Science, Sri Venkateshwara Veterinary University, Rajendra Nagar, Hyderabad, A.P.

E-mail: vrlinga@gmail.com

ABSTRACT: Proteins of the stress tolerant and mesophilic yeast were extracted using optimized protein extraction method and estimated by *Bradford* method. Immobilized pH gradient (IPG) strips were rehydrated with known concentrations of protein samples. Rehydrated IPG strips were run in isoelectric focusing (IEF) to separate the proteins on the basis of their pH gradient. 2DE gels were run, stained and image of the stress tolerant yeast was compared with the gel image of mesophilic yeast. The image analysis using the image master software resulted in the identification of differentially expressed spots in stress tolerant yeast. Among the differentially expressed spots, six were selected and characterized by MALDI-TOF as Enolase, Fructose bisphosphate aldolase, Alcohol dehydrogenase, 30KDa HSP, HSP70 and HSP90.

KEY WORDS: Stress tolerant yeast, 2D gel electrophoresis, Differentially expressed proteins, MALDI-TOF MS analysis.

1

INTRODUCTION

Most of the differential proteome studies on *Saccharomyces cerevisiae* were performed on cells cultured in batch mode, *i.e.* in shake flasks or in reactors. In terms of microbial physiology, such batch cultivation is relatively poorly controlled, because the composition of the growth medium and consequently, the growth rate changes continuously (Gancedo 1998). The high concentration of carbon source in the culture, which is essential for this type of cultures, may lead to carbon catabolite repression (Gancedo 1998). Proteomic technologies are powerful tools for examining alterations or study of protein profiles of an organism/system (Porubleva and Chitnis 2000). Several studies have also shown that mRNA levels do not correlate well with protein expression levels, hence the study of the whole dynamic proteome of an organism has gained elevated significance (Griffin *et al*. 2002) in this era. Earlier studies on fungi included study of proteins such as *HXT5* expression in *Saccharomyces cerevisiae* which is determined by growth rates (Verwaal *et al*. 2002), proteome analysis of yeast response to various nutrient limitations (Annemieke Kolkman *et al*. 2006).

Much of the studies have also concentrated on *Aspergillus* and *Candida* species due to their role in human health and disease. These include study of proteins associated with the conidial surface of *A.fumigatus* which are potent vaccine candidates or allergens (Asif *et al*. 2006). Kakiuchi *et al.* (2007) reported that they have identified proteins by mass spectrometry, which have diverse biochemical functions and cellular roles, including cell signaling, metabolism, and cell cycle regulation. Importantly, there are a number of protein subsets that are involved in the regulation of yeast physiology through a variety of cell signaling pathways, including stress-induced transcription, cell division, and chitin synthesis. Differential proteomic analysis of thermotolerant and thermosensitive strains of *Propionibacterium freudenreichii* by 2D gel electrophoresis revealed distinct but overlapping cell responses to heat stress (Anastasiou Rania *et al.,* 2006). In the present study, proteins which are differentially expressed and contributing to stress tolerance were characterized in stress tolerant yeast *Saccharomyces cerevisiae* OBV-9, using 2-dimensional gel electrophoresis and MALDI-TOF analysis.

MATERIALS AND METHODS

Protein expression studies were carried out by comparing the protein profiles of stress tolerant yeast *Saccharomyces cerevisiae* OBV-9 with that of mesophilic yeast, *Saccharomyces cerevisiae* MTCC-1813. Differentially expressed proteins in stress tolerant yeast were characterized by MALDI-TOF mass spectrometry.

Optimized protein extraction for 2D gel electrophoresis

Crude protein extracts of the yeast were prepared by rapidly homogenizing approximately 500 mg of the yeast pellet (washed with phosphate buffered saline) using a mortar and pestle in liquid nitrogen and 1 ml protein extraction buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, 2.5 mM PMSF, 0.1 per cent Triton-X-100. The homogenate was centrifuged at 12,000 rpm for 10 minutes. The supernatant was collected and protein was estimated. In the final method, collected supernatant was precipitated with 100 per cent TCA (final concentration of 10 per cent) and kept overnight at –20˚C for precipitation. The protein pellet was recovered by centrifugation at 12,000rpm for 10min at 4˚C, washed three times with cold acetone, lyophilized and stored at –70˚C. Protein pellet was dissolved in rehydration buffer containing 8M Urea, 2 per cent CHAPS, 50 mM DTT, 0.5 per cent biolyte or 1X ready strip buffer (Ampholyte), tinge of bromophenol blue.

Protein estimation

The protein concentration of yeast protein extracts was determined using the Bradford protein assay (Sigma) according to the manufacturer's instructions and using BSA as a standard. BSA standard was made by diluting the stock of 10mg/ml to a final concentration of 1mg/ml. Clean, autoclaved glass tubes were taken, labeled and added 0,1 ,2, 3, 4, 5, 6, 7, 8, 9, 10 µg BSA respectively. The volume in each tube was made to 800 µl with double distilled water. Two hundred µl of Bradford reagent was added to these tubes to make up to a final volume of 1ml and vortexed for few seconds. The same process was repeated for unknown samples. Each unknown was taken in duplicates or triplicates. The tubes were incubated at room temperature for 5 minutes (in dark) and the samples were read at 595 nm using spectrophotometer (*Systronics* Pvt. Ltd.) against a reagent blank. A standard curve was plotted and protein concentration of the unknown samples was estimated from the standard curve.

Two dimensional gel electrophoresis or 2D-PAGE

Rehydration of the IPG strip

About 600µg of protein sample in 185µl (for 11cm gel) of rehydration buffer was then used for 2D PAGE. The protein sample was pipetted as a line along the back edge of a channel in a rehydration tray and care was taken to prevent air bubbles. The coversheet from the ready strip IPG strip (Biorad, 11cm and pH 5-8) was peeled off using forceps. The strip was placed gently with the gel side down onto the sample and overlaid with 2 ml of mineral oil to prevent evaporation during the rehydration process. Rehydration was kept overnight on a level bench for 16 hours. This was followed by IEF (Isoelectric focussing/focussing in the first dimension).

Isoelectric focusing (IEF)

For this a clean, dry IEF focusing tray was taken and paper wicks were placed at both ends of the channels covering the wire electrodes. These were made wet with 8µl of nano pure water. The mineral oil was drained from the rehydration tray. Using forceps each strip from the rehydration tray was carefully transferred to the IEF tray with the gel side down. Each IPG strip was covered with 2ml of mineral oil and air bubbles were removed. The focusing tray was placed in the IEF cell and covered. The following program was selected for IEF run and IEF was started.
Step 1 - 500V, 30 minutes, slow
Step 2 - 8000V, 2.30 hours, linear
Step 3 - 8000V- 40,000V, 1 hour, rapid
Step 4 - 500V for holding, 5 hours to prevent diffusion.

Equilibration and SDS PAGE

After the IEF run, strips were equilibrated for 15 minutes in a buffer containing 6 M urea, 0.375 M Tris HCl, pH 8.8, 2 per cent SDS, 20 per cent glycerol, 2 per cent (w/v) DTT and followed by equilibration for another 15min in a buffer containing 6M urea, 0.375M Tris HCl, pH 8.8, 2 per cent SDS, 20 per cent glycerol, 2.5 per cent (w/v) Iodoacetamide. The equilibrated IPG strips were then loaded on horizontal SDS PAGE gels containing 12 per cent w/v resolving gel and 4 per cent stacking gel w/v. Electrophoresis was carried out in an in-house electrophoresis tank at 15 mA/gel.

Staining of the gels

The gels were stained by fast coomassie staining method. After electrophoresis the gel was put in a microwave proof plastic container containing an appropriate amount of reagent A (enough to cover the gel, 0.05g coomassie brilliant blue R-250 in 25ml Isopropanol, 10ml acetic acid, and 65ml deionized water) and heated in microwave oven on fullpower until the solution started boiling(~2min). The gel was cooled at room temperature for 5min with gentle shaking. Reagent A was discarded and the gel was rinsed briefly with deionized water. At this stage bands with more than 100ng of proteins can be visualized despite the blue background. Reagent B (0.005g coomassie brilliant blue R-250 in 10ml Isopropanol, 10 ml acetic acid, and 80 ml deionized water) was added and the gel was heated in microwave oven on full power until the solution started boiling. (~1min20sec). Hot reagent B was discarded and the gel was rinsed with deionized water. After this step, bands containing more than 50ng of proteins can be visualized. Reagent C (0.002g coomassie brilliant blue R-250 in 10ml acetic acid and 90 ml de ionized water) was added and the gel was heated in microwave oven on full power until the solution started boiling (~1min20sec). Hot reagent C was discarded and the gel was rinsed with de ionized water. After this step, bands containing more than 25ng of proteins can be visualized.

Destaining was performed using Reagent D (10 per cent acetic acid i.e. 10 ml acetic acid mixed with 90 ml de ionized water). For this, reagent D was added and the gel was heated in microwave oven on full power until the solution started boiling. (~1min20sec). The gel was cooled at room temperature for 5 minutes. At this stage, bands containing 5 ng of proteins or more can be visualized. The de staining step was repeated. The gel was finally stored in 1 per cent acetic acid.

Image analysis

The 2D gels were immediately scanned and acquired using Fluor-S-multiimager (Biorad, USA). Image Master™ 2D Platinum v7.0 software (GE health care life sciences) was used to analyze the gel images and identify the differentially expressed protein spots in stress tolerant yeast *Saccharomyces cerevisiae* OBV-9 compared to the mesophilic yeast, *Saccharomyces cerevisiae* MTCC-1813, as described in the user's manual of GE health care life sciences.

In-gel trypsin digestion of the protein spots

Protein spots of interest were excised from the fast coomassie stained gels and subjected to in-gel trypsin digestion according to the procedure of Applied Biosystems (Applied Biosystems, Foster city, USA). The gel pieces were destained by washing three times with 50 per cent ACN/25 mM NH_4HCO_3, pH 8.0 for 15 min and were then soaked in 100 per cent ACN for 5 minutes to dehydrate the gels. ACN was removed and gel slices were dried in speed vac for 20-30min. Trypsin was added (Sigma, proteomics grade, modified trypsin 10-15ng/ml in 25 mM NH_4HCO_3, pH 8.0) and incubated at 37 ºC for 16 hours. This was followed by extraction of peptides using 50 per cent ACN, 0.3 per cent TFA for 30-60 minutes with gentle agitation. The supernatant was lyophilized and stored at –70ºC.

Protein identification and database search

The samples were dissolved in a solution consisting of 50 per cent ACN and 0.1 per cent TFA in ultrapure water. The sample (0.5 ul) was mixed with an equal volume of the matrix solution (5mg/ml cyano-4-hydroxy cinnamic acid dissolved in the same solution as above) and then loaded on to the matrix assisted laser desorption ionization (MALDI) sample target plate and dried in air. Mass spectrometric (MS) analysis was performed on a MALDI TOF mass spectrometer. Before each analysis, the instrument was calibrated with the calibration mixture. Database searching was performed using the mascot program (Matrix science, London, UK). Search criteria were, NCBI database, taxonomy-Saccharomyces cerevisiae, parent ion mass tolerance at 50 ppm, carbamidomethylation of cysteine (fixed modification), methionine oxidation (variable modification) and maximum one missed cleavage site.

RESULTS

Characterization of differentially expressed proteins of stress tolerant yeast

Proteome map of thermotolerant and mesophilic yeast

The proteome map of stress tolerant yeast *Saccharomyces cerevisiae* OBV-9 and mesophilic yeast, *Saccharomyces cerevisiae* MTCC-1813 resulted in ≈350 visible reproducible protein spots on the fast coomassie stained 2D gels in the range of pH 5-8 on a 11cm 12 % SDS PAGE gel.

Identification of differentially expressed proteins in stress tolerant yeast

A 2D gel image of the stress tolerant yeast was compared with the gel image of mesophilic yeast. The image analysis using the image master software resulted in the identification of spots expressed differentially (Figure 1; Table 2). These spots were marked. Spots that showed consistent position on different gels were considered to be the same proteins. Six differentially expressed proteins were picked up for identification by MALDI- TOF MS analysis (Figure 2). A comparision of the intensities of these spots of the mesophilic and stress tolerant yeast are summarized in Table 1.

Table 1. Comparison of the protein spot intensities of the identified spots

S. No.	Spot ID	Spot intensity of Mesophilic yeast	Spot intensity of Stress tolerant yeast
1	30	0.05	0.11
2	75	0.04	0.05
3	92	0.09	0.45
4	144	0.14	0.75
5	186	0.03	0.17
6	274	0.14	0.26

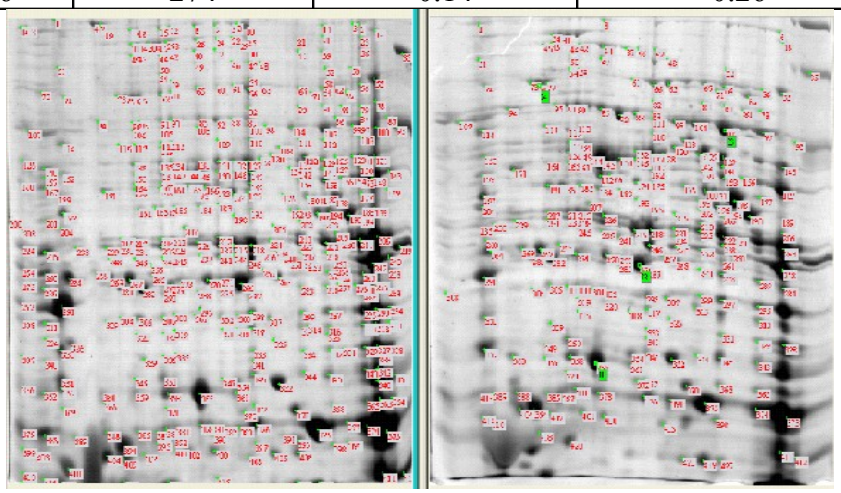

Figure 1. 2D gel Images of (A) mesophilic and (B) stress tolerant yeast showing protein spots with spot ID s for determination of differentially expressed proteins by Image analysis

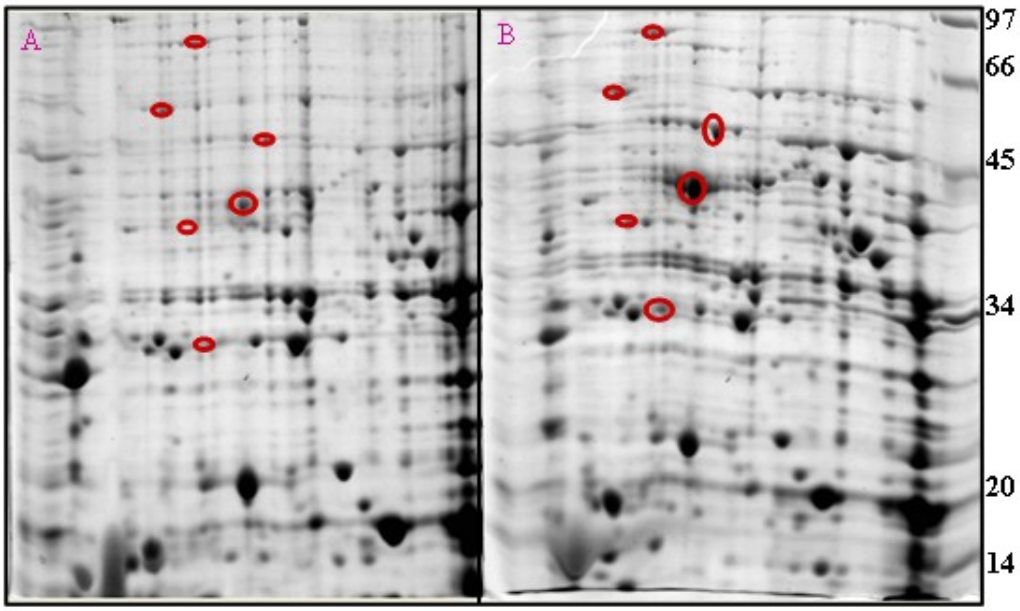

Figure 2. 2D gel images of the (A) mesophilic and (B) stress tolerant yeast showing differential expression of proteins. The differentially expressed proteins are marked with red circles in the 2D PAGE

Table 2. Number of proteins that are differentially expressed in stress tolerant yeast compared to mesophilic yeast

S. No.	Status of protein	No. of spots
1	Up regulated	206
2	Down regulated	57
3	Present	86
4	Absent	0
5	Total spots	349

These proteins were identified as Enolase (spot1), Fructose bisphosphate aldolase (spot2), Alcohol dehydrogenase (spot3), 30KDa HSP (spot4), HSP70 (spot5) and HSP90 (spot6) (Figure 3 and Table 3).

Table 3. List of proteins identified in the yeast proteome by mass spectrometry

Spot no	Protein name	Observed pI	Observed M.Wt	Uniprot Id	Number of peptides matched	Peptide sequence	Function
1	Enolase	5.8	47,000	P00925	5	GNPTVEVELTTEK LGANAILGVSMAAAR AVDDFLLSLDGTANK SGETEDTFIADLVVGLR TAGIQIVADDLTVTNPAR	Glycolysis
2	Fructose bisphosphate aldolase	5.48	40,400	P14540	7	AAQDALLLR EAAYYQQGAR ATPEQVSDYTLK DRATPEQVSDYTLK ATPEQVSDYTLKLLHR LASIGLENTEANRQAYR TVVSIPNGPSELAVKEAAWGLAR	Glycolysis
3	Alcohol dehydrogenase	6.3	40300	P00330	10	DIVGAVLK IGDYAGIK EALDFFAR ANELLINVK GVIFYESHGK SISIVGSYVGNR SIGGEVFIDFTK ANGTTVLVGMPAAK VVGLSTLPEIYEK VLGIDGGEGKEELFR	Fermentation
4	HSP30	5.2	26000	P15992	6	LLGEGGLRGYAPR KDIDIEYHQNK EVARPNNYAGALYDPR NQILVSGEIPSTLNEESK SVAVPVDILDHDNNYELKVVVPGVK VITLPDYPGVDADNIKADYANGVLTLTVPK	Chaperone
5	HSP70	5.0	65000	P10591	7	TKDNNLLGK MVAEAEKFK SNKITITNDK DAGTIAGLNVLR LEQADKDTVTK MKETAESYLGAK EDIEKMVAEAEK	Chaperone
6	HSP90	5.1	85000	P15108	8	APFDLFESK RVDEGGAQDK FYSAFAKNIK DSSMSSYMSSK VDEGGAQDKTVK AVEKSPFLDALK RVDEGGAQDK RAPFDLFESK	Chaperone

DISCUSSION

The detection of proteins in the gel also depends on the staining technique employed. There are several techniques, which differ in terms of the type of dye binding to the protein, sensitivity and other parameters (Westermeier 2001). These include staining with coomassie R250, Coomassie G250, fast coomassie staining and silver staining. By using the Fast coomassie staining method we were able to observe maximum number of spots in the gel and also this method was compatible with further mass spectrometry (MALDI TOF MS) for protein identification. Earlier studies also report that its sensitivity is comparable to that of silver staining.

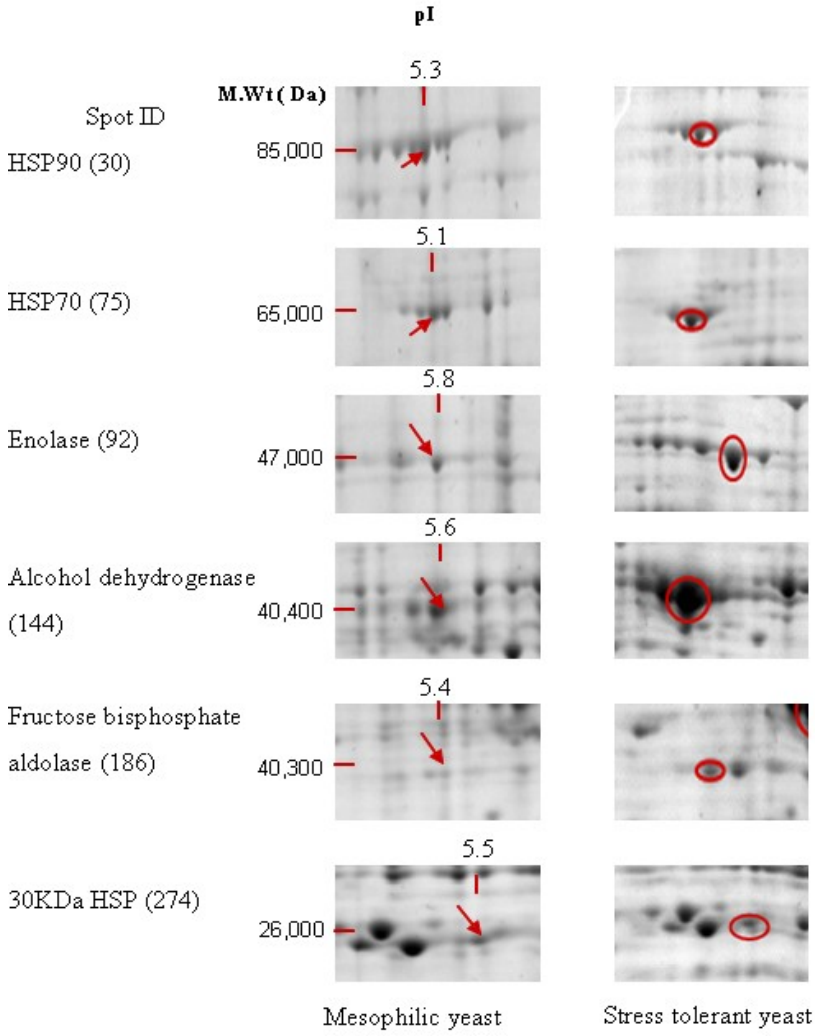

Figure 3. Differentially expressed protein spots identified in stress tolerant yeast *Saccharomyces cerevisiae* OBV-9

It is also noteworthy that in our studies we observe an increased level of HSP expression in the stress tolerant yeast compared to the mesophilic yeast which is in accordance with the earlier reports (Anastasiou Rania *et al.,* 2006; **Hem Shukla** 2006) and explains the stress tolerance of the selected yeast strain. The study also gives important clue of indirect possible implication of the glycolytic enzymes in conferring thermotolerance.

Here we report some of the functions of these differentially expressed proteins.

Spot number 1 was identified as Enolase. Enolase (EC4.2.1.11) is an enzyme of the glycolytic pathway catalyzing the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate in the presence of Mg^{2+} ions.

Spot number 2 was identified as fructose bisphosphate aldolase. This is another key enzyme involved in the glycolytic pathway.

Spot number 3 was identified as alcohol dehydrogenase which plays a key role in ethanol production in yeast fermentations. Spot numbers 4, 5, and 6 were identified as HSPs 30, 70 and 90 KDa respectively. These heat shock proteins levels are known to increase as a mechanism of heat and other resistance when yeast strains are grown in a medium containing glucose as sole carbon source.

SUMMARY

A 2DE gel image of the stress tolerant yeast was compared with the gel image of mesophilic yeast. The image analysis using the image master software resulted in the identification of spots expressed differentially in stress tolerant yeast. Among the differentially expressed spots, six were selected and characterized by MALDI-TOF as Enolase, Fructose bisphosphate aldolase, Alcohol dehydrogenase, 30KDa HSP, HSP70 and HSP90.

ACKNOWLEDGEMENTS

The authors acknowledge the financial assistance received from the Department of Biotechnology (DBT), Ministry of Science and Technology, New Delhi, India to carry out this research work.

REFERENCES

Anastasiou Rania, Leverrier Pauline, Krestas Ioannis, Rouault Annette, Kalantzopoulos George,Boyaval Patrick, Tsakalidou Effie and Jan Gwénaël. 2006. Changes in protein synthesis during thermal adaptation of Propionibacterium freudenreichii subsp. Shermanii. International journal of food microbiology. 108: 301-314.

Annemieke Kolkman, Pascale Daran-Lapujade, Asier Fullaondo, Maurien, M. A., Olsthoorn, Jack T.Pronk, Monique Slijper and Albert, J. R. Heck. 2006. Proteome analysis of yeast response to various nutrient limitations. Molecular Systems Biology. 2: 0026.

Asif, A.R., Oellerich, M., Amstrong, V.W., Riemenschneider, B., Monod, M., and Reichard, U. 2006. Proteome of conidial surface associated proteins of Aspergillus fumigatus reflecting potential vaccine candidates and allergens. J. Proteome Res. 4 : 954-962.

Gancedo, J.M. 1998. Yeast Carbon Catabolite Repression. Microbiology and Molecular Biology Reviews. 62: 334-361.

Griffin, T.J., Gygi, S.P., Ideker, T., Rist, B., Eng, J., Hood, L., and Aebersold, R. 2002. Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. Mol. Cell Proteomics. 1 : 323-333.

Hem, D. Shukla. 2006. Proteomic analysis of acidic chaperones and stress proteins in extreme halophile Halobacterium NRC-1: A comparative proteomic approach to study heat shock response.Proteome Science. 4: 6.

Kakiuchi, K., Yamauchi, Y., Taoka, M., Iwago, M., Fujita, T., Ito, T., Song, S.Y., Sakai, A., Isobe, T., Ichimura, T. 2007. Proteomic analysis of in vivo 14-3-3 interactions in the yeast Saccharomyces cerevisiae. Biochemistry. 3(46): 7781-7792.

Porubleva L, Chitnis PR. 2000. Proteomics: a powerful tool in the post-genomic era. Indian J Biochem Biophys. 37(6): 360-368.

Verwaal, R., Paalman, J. W., Hogenkamp, A., Verkleij, A. J., Verrips, C. T. and Boonstra, J. 2002.HXT5 expression is determined by growth rates in Saccharomyces cerevisiae. Yeast. 19: 1029-1038.

Westermeier, R. 2001. Electrophoresis in practice. 3rd edition. Weinheim, Wiley-VCH, 332.