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PRELIMINARY PROTEIN PROFILING OF COPPER AND ZINC TREATED *LACTOBACILLUS RHAMNOSUS*

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ABSTRACT

Toxic metals are a class of elements with no biological role but with extreme toxicity. Humans, plants and also beneficial microbes suffers from metal toxicity. Probiotic bacteria are very beneficial to humans and animals due to their antipathogenic potential. Probiotic bacteria have become anintegral elements to everyday healthy living. The aim of this study was to determine the preliminary protein profiling of Zinc and Copper influenced *Lactobacillus rhamnosus* MTCC 1408 by using the techniques SDS-PAGE and 2-D PAGE. The results shown that the protein profiles of Copper stressed proteins showed significant difference when compared to the Zinc stressed proteins.

Key words: Metal toxicity, Protein profiling, SDS-PAGE, 2-D PAGE.

INTRODUCTION

Nowadays metal toxicity confers major part of pollutants to environment. This toxicity directly or indirectly involved in human health problems by distributing in water sources and also causes disturbances in food chains (Islam E et al. 2007). Metal ions can also be toxic even at relatively low concentration due to the formation of reactive oxygen species (ROS), which is one of the prime mechanisms of metal-induced stress. These reactive oxygen species causes oxidative damage which leads to chronic inflammatory diseases which are initiator of cancer. The characteristic health disturbances caused by metal toxicity are inflammation, premature ageing and neurological disorders etc (Varsha Mudgal et al. 2010). Metals exert their toxicity not only on humans, but also on plants and microbes. The main mechanisms involved in metal toxicity caused are, disruptions of metabolic processes, competitions with nutrient trace element and metal-binding transporter and storage systems (Bertin G, 2006; Houston MC, 2007). Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) i.e., non pathogenic. Due to their non pathogenic nature LAB are widely used in food industry. In food industry, LAB are often exposed to metal ions induced stress (Jasna Mrvcic *et al.,* 2012). The interactions between LAB and metal ions are very poorly investigated. Because of this reason, we investigated the influence of metal toxicity on *Lactobacillus rhamnosus* by using MIC, PAGE and 2-D PAGE.

MATERIALS AND METHODS

1. CULTURE COLLECTION

The bacterial sample i.e., lyophilized *Lactobacillus rhamnosus* (MTCC 1408) was purchased from IMTECH, Chandigarh.

2. CULTIVATION OF BACTERIAL STRAINS

The lyophilized culture was activated by dissolved in 0.85% saline. *L.rhamnnosus* strains were cultivated in the presence of de Man Rogosa Sharpe (MRS) medium (De man JC et al. 1960) which is specific for *Lactobacillus* species. The plates were incubated 24 hrs at 37^0C under aerobic conditions. Then the plates were stored at 4^0C.

3. EFFECT OF METAL IONS ON GROWTH INHIBITION

The bacterial cells were grown in 5 ml of MRS broth (Peptone 10gm/ L Beef extract 10gm/L, Yeast extract 5gm/L, K2HPO4 1g/L, $NaCH_3CO_2.3H_2O$ 1g/L, Tri ammonium citrate 2g/L, $MgSO_4.7H_2O$ 0.2g/L, $MnSO_4.4H_2O$ 0.05g/L, Glucose 50g/L, Tween1ml/L, Glycerol 12% pH 6.5) supplemented with 100 g/L ampicillin and incubated at 37ºC, for overnight. Then 50 µl of overnight cultures were transferred into 5 ml of MRS broth and grown until OD reached 0.5. Then the cells were adjusted to equal OD 0.05 in MRS broth 200 mg/L ampicillin supplemented MRS broth. Aliquots of cell (100 µl) were added to 100 µl of MRS broth containing various concentrations of copper sulphate and zinc chloride were then added. These metals were used in order to prefer biosorption of metal on the cell surface to its intracellular uptake (Kotrba P et al. 1999). Then the samples were further incubated at 37ºC with for 30 hours. The growth rate was determined by taking the absorbance at 540 nm. The concentrations of metal ions those gave rise approximately 50% growth inhibition were selected for further experiments.

4. PREPARATION OF PROTEIN SAMPLES FOR ONE DIMENSIONAL ELECTROPHORESIS

Bacterial cells were removed from the 40 mM copper influenced, 60 mM zinc influenced culture by centrifugation (5,500 x g, 10 min, 4°C). The cells were suspended in 3ml lysis buffer ((Sambrook JE et al. 1989) and sonicated for 5 min at 45 Hz with an interval of 30s. The cell lysate was subjected to centrifugation at 10,000 rpm for 10 min. The supernatant was treated as protein sample. The concentration of protein obtained after protein extraction was determined by Bradford protein assay [Bradford M. 1976].

5. ONE DIMENSIONAL GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE (12%) was carried out in 1.0 mm thick discontinuous gel by method of Laemmlie 1989 at 40 mA constant current vertical slab gel electrophoresis assembly (Bio-Rad) Prepared 10 ml of 12% resolving gel and allowed it to polymerize for 20-30 min. Onto it, added 1 ml of 5% stacking gel, placed a 15-well comb and allowed it to polymerize. Protein samples (15µg) were mixed with the protein loading dye and loaded into the wells. Protein molecular weight marker was added in a well along side for reference.

6. PREPARATION OF PROTEIN SAMPLES FOR TWO DIMENSIONAL GEL ELECTROPHORESIS

At first cells were grown at 37ºC for overnight in 5 ml MRS broth supplemented with ampicillin .Cells were sub cultured and further incubated at 37ºC for 6 hours to mid-exponential phase. Cells were inoculated in 50 ml MRS/Amp and incubated at 37ºC for 4 hours. Copper and Zinc metal solutions were then added to the cultures to obtain the final concentration of 40 mM of Copper and Zinc, Then the cells were again incubated for another 12 hours. Preparation of crude protein extracts was performed as previously described (Sambrook JE. 1989). cells were collected, washed, and resuspended in Tris buffer. The suspension was then mixed with 250 µl of lysis solution (7 M urea, 2 M thiourea, 4% CHAPS; freshly prepared by supplementation with 10 mg/ml dithiothreitol (DTT) and 10 µl/ml protease inhibitor cocktail). Cells were disrupted on ice by sonic disintegration by using sonifier. The whole cell lysates was subjected to centrifugation centrifugation at 15,000 rpm for 60 min at room temperature. Bradford's method was used for quantification of protein amounts using bovine serum albumin as a standard. The protein solution was finally mixed with 1 M acrylamide (at 1:10 of total volume) and stored at room temperature for 10 min.

7. TWO DIMENSIONAL GEL ELECTROPHORESIS

Two-dimensional gel electrophoresis was carried out using 2-D Electrophoresis System (Panpumthong P and Vattanaviboon P, 2006). Protein samples (300µg) were applied to an immobilized pH gradient (IPG) strip (11 cm, pH 3-10) using a passive rehydration method (16 h of rehydration at 20°C). The strips were then transferred to an isoelectric focusing (IEF) cell. IEF was performed by applying a voltage of 250 V for 30 minutes Slow, Ramping to 8,000 V over 2 hours-Linear, and holding to 8, 000 V until 40 KVh was reached. Prior to second dimension, the gel strips were equilibrated for 15 min twice in equilibration buffers. The second dimension was performed using 12% SDS-PAGE at 80 V. The gels were stained using the fast-coomassie staining method. At least two independent runs were made

for each sample to ensure the accuracy of analyses. The 2D gel maps were analyzed. The quantity of each spot in a gel was normalized as a percentage of the total quantity of all spots in that gel and evaluated in terms of optical density (OD).

RESULTS AND DISCUSSION

1. GROWTH INHIBITION OF E. COLI CELLS BY METAL IONS

In our previous study, effect of metal ions (copper and zinc) on the growth inhibition of *L.rhamnosus* MTCC 1408 was investigated (Sreevani S et al. 2013). These ions exerted approximately 50% inhibition at the concentrations of 40 mM. These inhibitory concentrations were selected for further analysis.

2. PRELIMINARY PROTEIN PROFIING OF THE CONTROL AND METAL STRESSED L.RHAMNOSUS PROTEIN SAMPLES BY ONE DIMENSIONAL GEL ELECTROPHORESIS

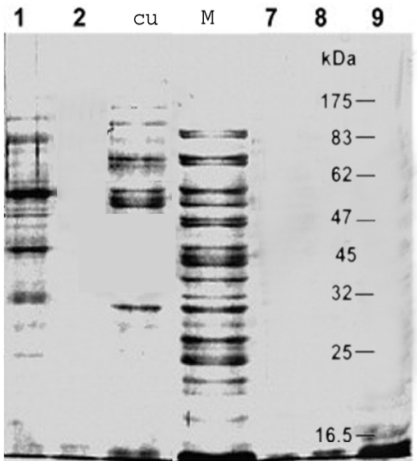

Figure 1
SDS-PAGE analysis of total proteome of L. rhamnosus MTCC1408

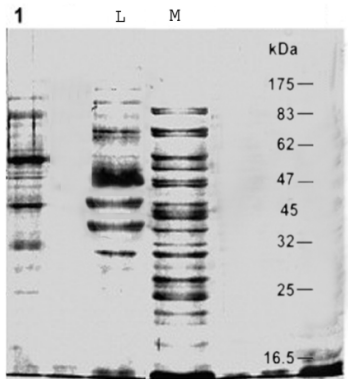

Figure 2
SDS PAGE analysis of Copper and Zinc stressed L.rhamnosus MTCC 1408 proteins

For preliminary analysis of the total proteins of the control and metal stressed states of *L.rhamnosus* MTCC 1408, the total proteome was isolated from the *L.rhamnosus* MTCC 1408 and subjected to 1D SDS-PAGE analysis. The comparison of the 1D protein profiles of the 2 samples showed differences in the levels of expression of protein(s) (bands) of Copper and Zinc stressed *L.rhamnosus* MTCC 1408 proteins (Figure 1 & Figure 2).

However there is much difference in the band intensity was observed in Copper treated protein profiles in comparison with control Compared to Zinc treated proteins. Since 1D gel or an SDS PAGE separates the proteins only on the basis of Molecular weight. That's why we planned to perform two dimensional electrophoresis to reduce the complexity and investigate the protein profiles (separation based on both Molecular weight and pI) of these samples. 2-DE of these 2 samples was performed.

TWO DIMENSIONAL GEL ELECTROPHORESIS OF THE METAL STRESSED L.RHAMNOSUS MTCC 1408 PROTEIN SAMPLES

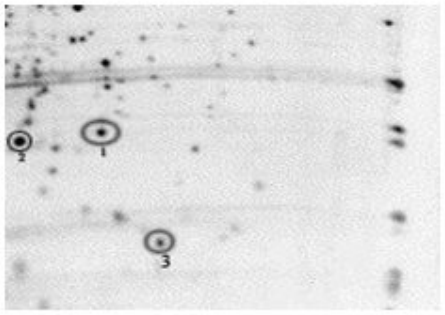

Figure 3: Control without any treatment

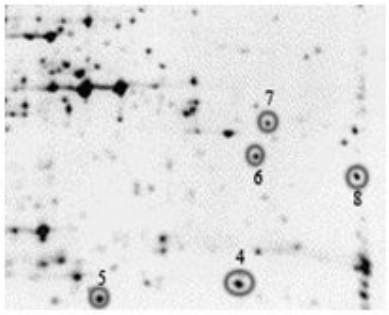

Figure 4: Differentially expressed spots of Proteins treated with Copper

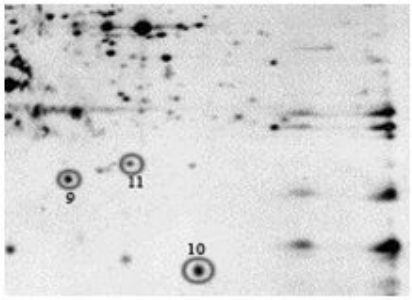

Figure 5: Differentially expressed spots of proteins treated with Zinc

In an effort to gain insight into the molecular mechanisms underlying the metal stressed proteins, we performed a comparative analysis of the *L.rhamnosus* MTCC1408 protein expression profiles of control and Copper and Zinc treated samples of *L.rhamnosus* MTCC 1408 using two-dimensional gel electrophoresis. Proteins extracted and estimated as described in materials and methods for all the chosen samples, were separated in the first dimension by isoelectric focusing on strip gel with pI range 4-7 and in second dimension using 12.0% SDS–PAGE and stained with fast coomassie staining method. The conditions used for profiling 2-D gel allowed access to a total of 100 proteins. Control sample was chosen as a reference for comparison with the gels of metal stressed samples. Comparison of the gels resulted in the identification of differentially expressed protein spots between A) control and Copper stressed proteins and B) 3 differentially expressed protein spots between control and Zinc stressed proteins shown in Figures 3, 4 & 5. For the initial identification and annotation of the protein spots, all the visual spots were marked in the control sample. Our future prospect will reveal the final outcome of the study which includes gel trypsin digestion and Mass spectroscopy.

CONCLUSION

Although some trace metals are important and essential elements, at high concentrations most of the trace metals can be proved as toxic to humans, plants and microbes. We have established one dimensional and two dimensional gel electrophoresis of specific metal treated proteins. From the present study we conclude that more Copper stressed proteins were different compared to control than Zinc stressed proteins. By using these results we will be find out, which proteins are differentially expressed with the help of mass spectroscopy. The understanding of bacterial metal toxicity system has been useful for both environmental sciences and medicine.

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