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Isolation and Identification of Antimicrobial Protein from *Saccharomyces cerevisiae* and its Efficacy Against the Human Pathogens

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ABSTRACT

Antimicrobial peptides (AMPs) are an important component of the natural defences of most living organisms against invading pathogens. In the present study marine yeast (*Saccharomyces cerevisiae*) was cultured in Yeast Malt Agar medium followed by protein extraction and quantification by using sonication method and Bradford assay, respectively. The isolated protein was then subjected for native PAGE and the four distinct bands were excised and tested for antibacterial activity against five bacterial human pathogens viz. *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholera*, *Salmonella typhi* and *Shigella flexneri*. Of the four protein bands, only one band exhibited antibacterial activity which was identified to have a molecular weight of 60 KDa. The peptides analysis was made through using the MASCOT search engine, using MALDI-TOF/MS result revealed that the peptides machinhg 51%, 10 peptides sequence was mached 60 KDa heat shock protein. the protein retrieve uniprot database total primary sequence 341 amino acids. The secondary structure of the identified protein indicated that the amino acids was mainly composed of helical form (49.85%) followed by the Coil structure (24.63%) and strand (14.08%). The identification of HPS60 heat shock proteins from marine yeast provides further evidence that this protein may play a pivotal role against a wide range of human pathogens.

Key words: Antimicrobial peptides, *Saccharomyces cerevisiae*, native PAGE, MALDI-TOF/MS, HPS60

INTRODUCTION

As cells grow, divide and respond to their environment, they must synthesize lipids to meet metabolic demands while also ensuring the correct balance of a wide array of structurally and functionally diverse lipid species. At the same time, cells need to maintain the correct distribution of lipids within the membrane bilayers of different organelles and the plasma membrane. Regulatory mechanisms that ensure proper levels of some lipid species including sterols (Brown and Goldstein, 2009; Hampton and Garza, 2009; Burg *et al*., 2008). The proteomics is the study of proteomes, to find new proteins or protein variants, elucidate protein networks and to correlate protein expression and modification. The contemporary development in protein extraction,

purification and identification methods has significantly advanced our ability to address an increasing number of biological questions using proteomic approaches (Sheoran *et al*., 2009). Mass spectrometry became a viable technique in protein analysis as a result of the development of two new ionization methods, MALDI (Matrix-assisted laser desorption ionisation) (Karas *et al*., 1987; Karas and Hillenkamp, 1988) and electrospray (Fenn *et al*., 1989). The MALDI-TOF/MS (Matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry) is most frequently used for protein analysis for its sensitivity, speed, ease-of-use and flexibility.

Multidrug resistant clinical bacteria pose a serious problem which demands new antibiotics and antimicrobial proteins against wide spectrum of drug resistant bacteria. It is thus becoming increasingly important to identify antimicrobial compounds with novel modes of action for which the bacteria are unable to mount a quick response and to build resistance. Perhaps the most promising among these novel compounds is a family of antibacterial peptides originally isolated from insects (Bulet *et al*., 1993; Mackintosh *et al*., 1998). The mechanism of this antibacterial action by the peptide is not clearly known. However, a direct correlation between antibiotic effect and membrane disruption has been found for other antimicrobial peptides derived from mammals and insects (Cociancich *et al*., 1993). Such antibacterial peptides of insect and mammalian origin that lyse bacterial membranes are potentially toxic to eukaryotic cells and are, therefore, unsuitable as a systemic drug. The high conservation of metabolic and regulatory mechanisms between eukaryotes has contributed to the wide-spread use of yeast as a model system for diverse biological studies (Sherman, 2002). The present study aimed to analyse the amino acid composition, peptide structure and identification of the yeast proteins responsible for potent antibacterial activity.

MATERIAL AND METHODS

Culture condition and extraction of intracellular protein: Cells of *S. cerevisiae* (Accession:JN387604) were grown in Yeast Malt Agar medium consisted of glucose (5 g), peptone (5 g), yeast extract (3 g), malt extract (3 g), prepared in 1 L of 50% seawater. After 72 h of incubation at room temperature, the cells were harvested by centrifugation and washed twice with 50 mM-Tris-HCl buffer (pH 7.5) and the cells were sonicated (Bandelin sonoplus, Berlin) for 10 min. After sonication the cell-free filtrate was recovered by centrifugation at 3000 rpm for 10 min. Cell-free filtrate was treated with 2 volumes of acetone and allowed to precipitate at 4°C overnight. The precipitated material was recovered by centrifugation at 3000 rpm for 30 min and dialyzed (1 KDa) against distilled water. Protein quantification was performed using the Bradford assay (Bio-Rad, Richmond, CA, USA). Transfer to an Eppendorf tube containing about 300-400 µL of glass beads on ice.

Elution native protein: The four native bands Vortex at top speed at 4°C for 5 min in cold room. band separate the extract from the glass beads, place each tube on top of another Eppendorf and poke a hole in the top and bottom of the tube using an 18-gauge needle. Give the tandem tubes a quick spin in the microfuge. The extract will now be in the bottom tube and the glass beads in the top tube. The extract is ready for heating collect the protein sample.

Antibacterial activity of bioactive protein: To identify the bioactive protein, native-PAGE (without the detergent SDS) was run for the protein extracted from *S. cerevisiae* (Laemmli, 1970). The elution protein was tested for antibacterial activity. Bacterial human pathogens (*E. coli*, *Staphylococcus aureus*, *Vibrio cholera*, *Salmonella typhi* and *Shigella flexneri*) were swabbed on

Muller Hinton agar plates. The 40 μL (1 mg mL^{-1}) of the protein sample was poured into the wells and the plates were incubated at 37°C for 24 h. The inhibitory activity of the bioactive protein against pathogens was assessed based on the zone of inhibition around the wells.

MALDI-TOF-MS (MS analysis): Electrophoretically resolved protein spots were excised from the gel, washed successively with 25 mM NH_4HCO_3, pH 8.0 and 50% acetonitrile in 25 mM NH_4HCO_3 (pH 8.0). After a final wash with triple distilled water, dehydration in a vacuum dryer was performed. "In gel," tryptic digestion was performed for 5 h at 37°C in 5 μL of trypsin solutions (10 ng μL trypsin in 5 mM NH_4HCO_3, 5% acetonitrile (pH 8.0).

Peptide mass fingerprinting: One microlitre of digested supernatant was spotted onto the MALDI sample probe on top of a dried 2 μL mixture made of saturated α-cyano-4-hydroxy-trans-cinnamic acid (5 mg) solubilized in 40% (v/v) acetone, 50% (v/v) acetonitrile, 9.9% (v/v) water and 0.1% (w/v) trifluoroacetic acid (TFA) in water. From this mix, 1 μL was deposited onto the MALDI target. MALDI-TOF mass spectra of peptide mixtures were obtained using Ultraflex mass spectrometer (laser beam 150 (337 nm, 50 Hz, N2 laser, Bruker Daltonics, Germany). Monoisotopic peptide masses were analysed with a MALDI-TOF/MS/MS mass spectrometer in positive mode. Background ions from trypsin autolysis and contamination by keratins were removed from mass lists.

Data base search and phylogenetic analysis: Protein identification was performed in uniprot database using the MASCOT search engine using GPS explorer v3.5 software. The amino acid sequence of the 60 KDa protein was analysed by using BLASTP (Basic Local Alignment Search Tool) (Altschul *et al*., 1997). Sequences were aligned by clustal W Multiple Sequence Alignment Program (Thompson *et al*., 1994). Phylogenetic analyses were carried out by using the Maximum Likelihood method with 1000 bootstrap in MEGA 5.0 software (Tamura *et al*., 2011). The prediction of protein secondary structure of yeast protein was predicted based on its primary structure. The secondary structure prediction was made by using SOPMA (Self Optimized Predicted Method with Alignment) (Geourjon and Deleage, 1995) and 3D Tertiary structure was generated by GENE3D.

RESULTS

Native PAGE for protein from *S. cerevisiae*: To identify the functional protein of *Saccharomyces cerevisiae*, native-PAGE without using the detergent SDS was run for the yeast protein extract. The result was shown in Fig. 1. Four distinct bands with molecular weights of 25, 40, 60 and 70 KDa and one native-page control were detected on the native the gel was excised carefully using sterile razor in to four different protein bands (Table 1).

Antibacterial activity of native PAGE protein bands: The four distinct bands were tested for inhibition activity on nutrient agar plate swabbed with five strains (Entero toxigenic *E. coli*, *Staphylococcus aureus*, *Vibrio cholera*, *Salmonella typhi*, *Shigella flexneri*). Of the four protein bands, only one band exhibited antibacterial activity as evident by five human pathogenic bacteria distinct clearing zone around the band the control is zone not present (Fig. 2). This band was identified to have a molecular weight of 60 KDa. This protein was used for further analysis in MALDI-TOF/MS/MS.

MALDI-TOF/MS/MS analysis of bioactive protein: The 60 KDa protein of potent antibacterial activity against five human pathogenic bacteria was taken after digesting it with

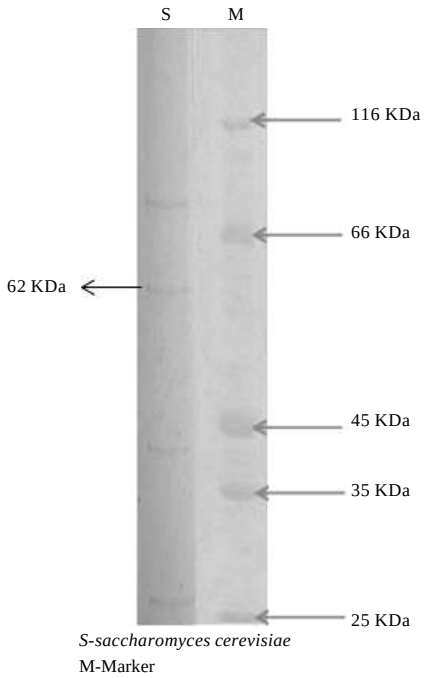

Fig. 1: Native PAGE showing four distinct protein bands of *Saccharomyces cerevisiae*

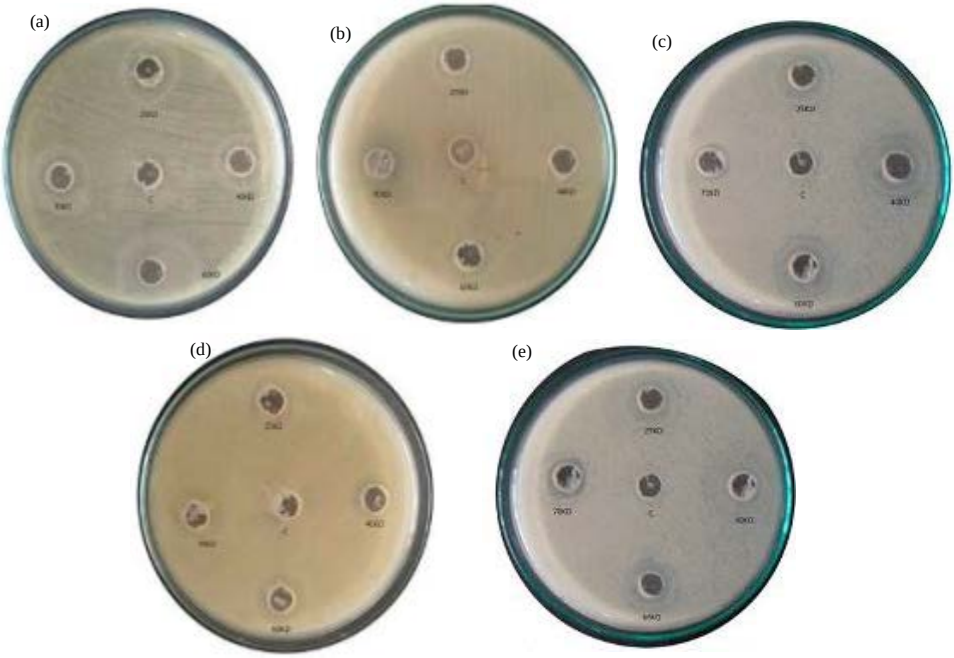

Fig. 2(a-e): Inhibition zone around the *Saccharomyces cerevisiae* 60 KDa protein, (a) Entero toxigeneic *E. coli*, (b) *Shigella flexneri*, (c) *Vibrio cholera*, (d) *Staphylococcus aureus* and (e) *Salmonella typhi*

Table 1: Antibacterial activity of four proteins with native page control of *Saccharomyces cerevisiae*

	Zone of inhibition (mm)				
Bacterial pathogens	25 KDa	40 KDa	60 KDa	70 KDa	Native gel control (mm)
E. coli	22	15	40	20	-
Staphylococcus aureus	8	12	22	10	-
Vibrio cholera	18	28	24	-	-
Salmonella typhi	20	7	25	10	-
Shigella flexneri	12	8	-	-	-

Table 2: Computed secondary structure elements of the *HSP60 Saccharomyces cerevisiae* protein

Alpha helix (Hh)	56.01%
310 helix (Gg)	0.00%
Pi helix (Ii)	0.00%
Beta bridge (Bb)	0.00%
Extended strand (Ee)	14.96%
Beta turn (Tt)	0.00%
Bend region (Ss)	0.00%
Random coil (Cc)	19.06%
Ambiguous states	0.00%
Other states	0.00%

trypsin in MALDI-TOF/MS. From the spectral data, 18 peaks were observed at 1457.949, 1642.135, 1795.100, 1807.239, 1810.208, 1852.208, 1864.225, 2284.592, 2619.109, 2707.652, 3061.875, 3310.875, 3324.857 and 3338.881. There was a prominent peak at 1810.482 (Fig. 3). The peak values, amino acid composition for each peak was detected by MASCOT search engine.

Data analysis for predicting protein structure of yeast protein

Primary structure of protein: MALDI-TOF/MS/MS data of 60 KD protein was analysed through using the MASCOT search engine maching ten peptides. The protein was retrieve uniprot database of 341 amino acids sequence as given below:

FLIGMTQGLICLITRKMVSLAEATRNAEEAGLDLVEISPNAEPPVCKVMDYGKEMAHQDIGIDLLNRVKTDLEDIATCESFPRRCLK
VIEFLSANKKEITTSEEIAQVATISANGDSHVGKLLASAMEKVGKEGVITIREGRTLEDELEVTEGMRFDRGFISPYFITDPKSSKVEF
EKPLLLLSEKKISSIQDILPALEISNQSRRPLLIIAEDVDGEALAACILNKLRGQVKVCAVKAPGFGDNRKNTIGDIAVLTGGTVFTEE
LDLKPEQCTIENLGSCDSITVTKEDTVILNGSGPKEAIQERIEQIKGSIDITTTNSYEKEKLQERLAKLSGGVAVI

Secondary structure of protein: Based on the amino acid sequence, the secondary structure was predicted by SOPMA the yeast protein indicated that the amino acids were mostly arranged in the forms of helix, coil and strand. However, helical form was more prevalent with 56.01% than other two forms. Coil structure was 19.06% and strand was 14.96% (Table 2) (Fig. 4). The 60 Ka heat shock protein were predicated tertiary structure (Fig. 5).

Phylogenetic analysis of yeast protein: The phylogenetic tree reveals the evolutionary history of the yeast protein as given in Fig. 6 the protein was identified related to other heat shock proteins of 60 KDa molecular weight.

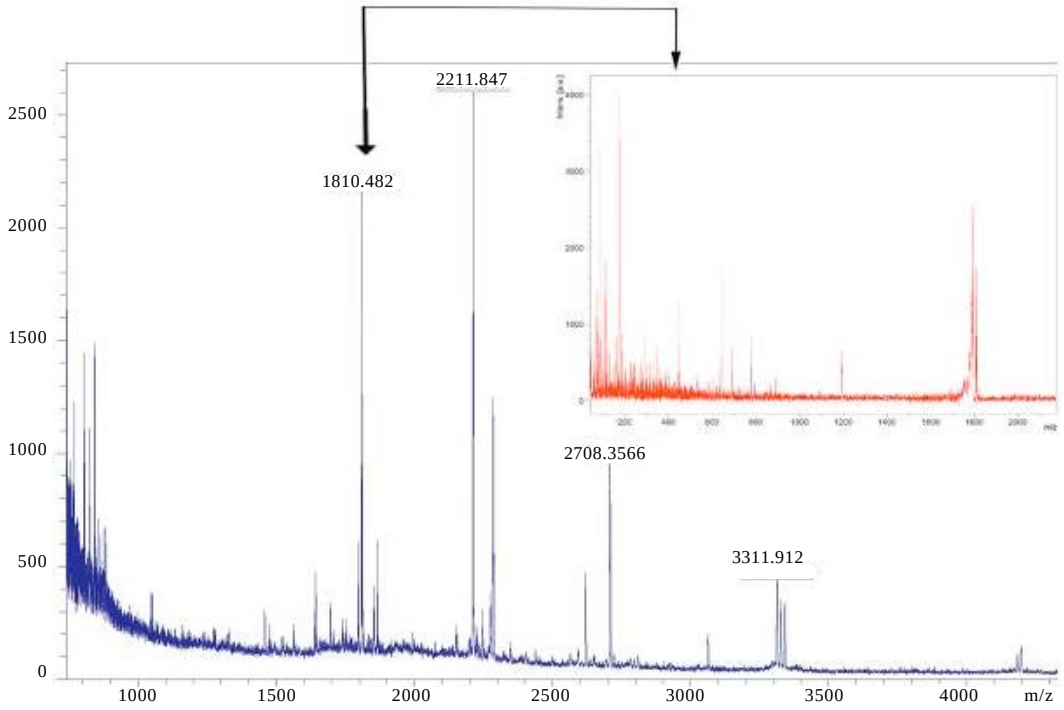

Fig. 3: MALDI-TOF/MMS spectra for 60 KDa protein isolated from *Saccharomyces cerevisiae*

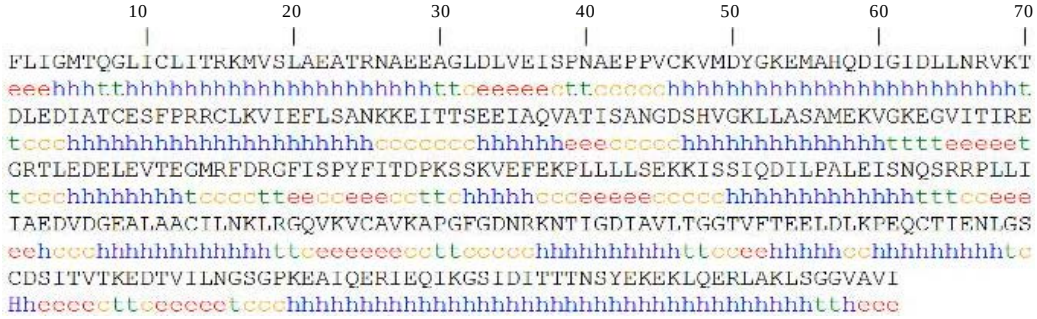

Fig. 4: Secondary structure of *Saccharomyces cerevisiae* protein

DISCUSSION

One of the most serious emerging health concerns today is the appearance and spread of antibiotic-resistant bacterial strains (Tomasz, 1994). The present study reports an antibacterial peptide with a molecular weight of 60 KDa from marine yeasts. Amino acid sequence analysis provides important insight into the structure of proteins which in turn greatly facilitates the understanding of its biochemical and cellular function (Nagano, 1973; Chou and Fasman, 1974). Computational methods are used in predicting protein structure based only on sequence information. Regarding the secondary structure of protein, globular proteins are typically composed of two basic secondary structure types, the α-helix and the β-strand which are easily distinguishable because of their regular (periodic) character. Other types of secondary structures

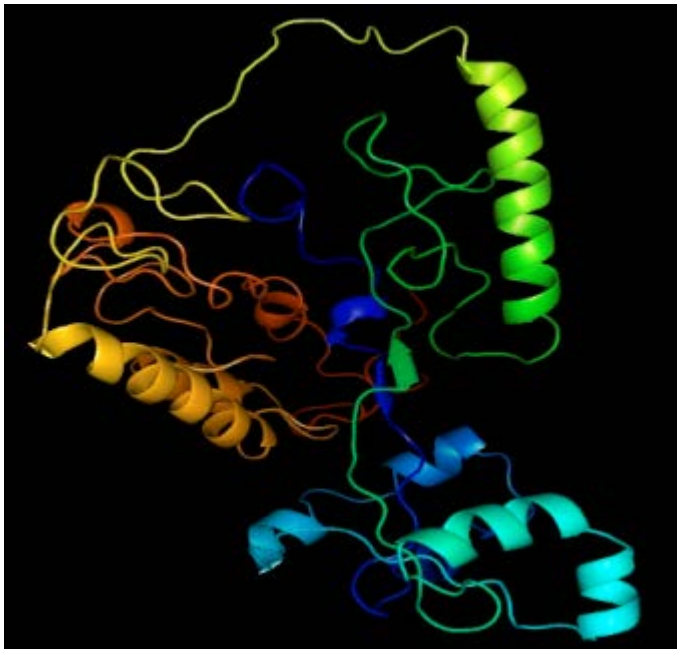

Fig. 5: Tertiary (three-dimensional) structure of 60 KDa *Saccharomyces cerevisiae* protein

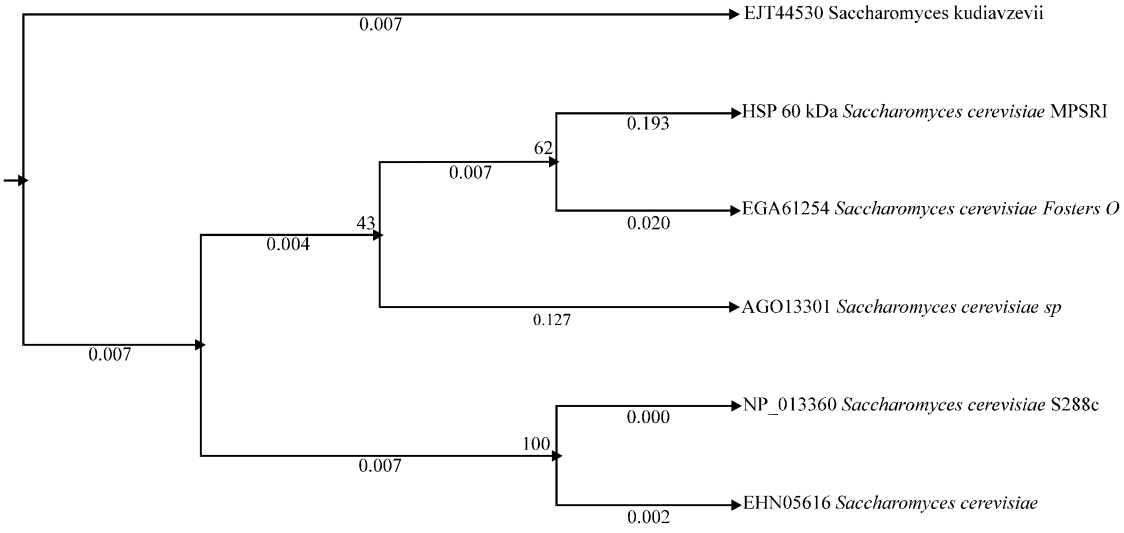

Fig. 6: Phylogentic tree showing relationship of 60 KDa *Saccharomyces cerevisiae* protein with other similar proteins

such as different turns, bends, bridges and non-α helices are less frequent and more difficult to observe and classify. The non-α structures are often referred to as coil or loop. In the present study, yeast protein of 60 KDa was found to have more helix (49.85%) than others: 65% coil (19.06%) and strand (14.96%) (Table 2). The majority of secondary structure prediction methods are aimed

at predicting only these three classes of local structure: Coil, helix and strand. In general, globular proteins have more coil structure of 50% than others: 30% α-helix, 20% β-strand (Garnier *et al.*, 1978).

The yeast protein of the present study related to other heat shock proteins of 60 KDa molecular weight as evident by phylogenetic analysis. The Heat Shock Proteins (HSPs) are synthesized rapidly at cellular level to react with deleterious stress conditions (Lindquist, 1986). These HSPs are reported to protect the organism from cell protein damage due to exposure to a wide variety of stressors, including elevated temperature (Lindquist, 1986; Sanders, 1993), oxidative stress (Lindquist, 1986), increased UV irradiation (Sanders, 1993), heavy metals (Sanders, 1993), xenobiotics (Sanders, 1993) and other pollutants (Lindquist, 1986; Wiens and Servedio, 1998). The yeast protein showed potent antibacterial activity against human pathogens. These antibacterial peptides could have act as inhibitors of enzymes produced by the bacteria either by serving as a pseudo-substrate or by tight binding to the active site eliminating the accessibility of the native substrate (Andreu and Rivas, 1998). Further molecular and bioinformatics studies on this novel yeast protein will help to understand the possible mechanism of action and to develop antibiotic peptides against the drug resistant pathogens.

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