Characterization of ribosomes from moderate halophile *Halomonas eurihalina*- structural and proteomic studies

A thesis submitted to the University of Hyderabad for the award of a Ph.D. degree in Biochemistry

By

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DECLARATION

I, **G. Usha Kumari**, hereby declare that this thesis entitled **"Characterization of ribosomes from moderate halophile *Halomonas eurihalina*- structural and proteomic studies"** submitted by me under the guidance and supervision of **Prof. T. Suryanarayana** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date:

Name: G. Usha Kumari

Signature of the student:

Regd. No. 06LBPH01

CERTIFICATE

This is to certify that this thesis entitled **"Characterization of ribosomes from moderate halophile *Halomonas eurihalina*- structural and proteomic studies"** is a record of bona fide work done by Ms. G. Usha Kumari, a research scholar for Ph.D. programme in Biochemistry Department, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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Contents of the thesis

Abbreviations

Introduction 1

Materials and methods 25

Results

Chapter 1: Growth phase and growth media dependent variation of ribosomal proteins in *H. eurihalina* and their comparison with ribosomal proteins from mesophiles 39

Chapter 2: Structural studies on ribosomes from logarithmic and stationary phase *H. eurihalina* 50

Chapter 3: Proteomic studies of ribosomal proteins from *H. eurihalina* 83

Chapter 4: Partial purification and identification of S1 from ribosomes and low molecular weight, strong poly (U) binding proteins from S-100 of *H. eurihalina* 184

Discussion 204

Consolidated summary 218

References 225

ABBREVIATIONS

A_{260} or A_{280}	Absorbance at 260 or 280 nanometres
ACN	Acetonitrile
Bis-Tris	Bis [2-hydroxyethyl] imino tris [hydroxyl methyl] methane
BLAST	Basic local alignment search tool
CHCA	α-cyano-4-hydroxycinnamic acid
DEAE-cellulose	Diethyl amino ethyl-cellulose
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
g/RCF	Relative centrifugal force
g, mg, µg	Gram(s), milligram(s), microgram(s)
h	Hour(s)
kDa	Kilo Dalton
L, ml, µL	Litre(s), millilitre(s), microlitre(s)
MALDI	Matrix-assisted laser desorption/ionization
min	Minutes
Mr	Molecular weight
M, mM	Molar, millomolar
MS/MS	Tandem mass spectrometry
PMF	Peptide mass fingerprint
Poly (U)	Poly uridylic acid
rpm	Revolutions per minute
S	Svedberg unit
SDS	Sodium dodecyl sulphate
TCA	Trichloro acetic acid
TEMED	N, N, N', N'-tetramethyl-ethylene diamine
TFA	Trifluoroacetic acid
Tris	Tris [hydroxyl methyl] amino methane
V	Volts

Halophiles-an introduction:

Halophiles are salt-loving organisms that inhabit hypersaline environments. Halophiles are found in each of the three domains: Archaea, Bacteria, and Eucarya (Oren, 2002a).

Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Halophiles are found distributed all over the world in hyper saline environments, many in natural hypersaline brines in arid, coastal and even deep sea locations as well as in artificial salterns used to mine salts from the sea. Their novel characteristics and capacity for large-scale culturing make halophiles potentially valuable for biotechnology. Although salts are required for all life forms, halophiles are distinguished by their requirement of hypersaline conditions for growth. (DasSarma and Arora, 2001).

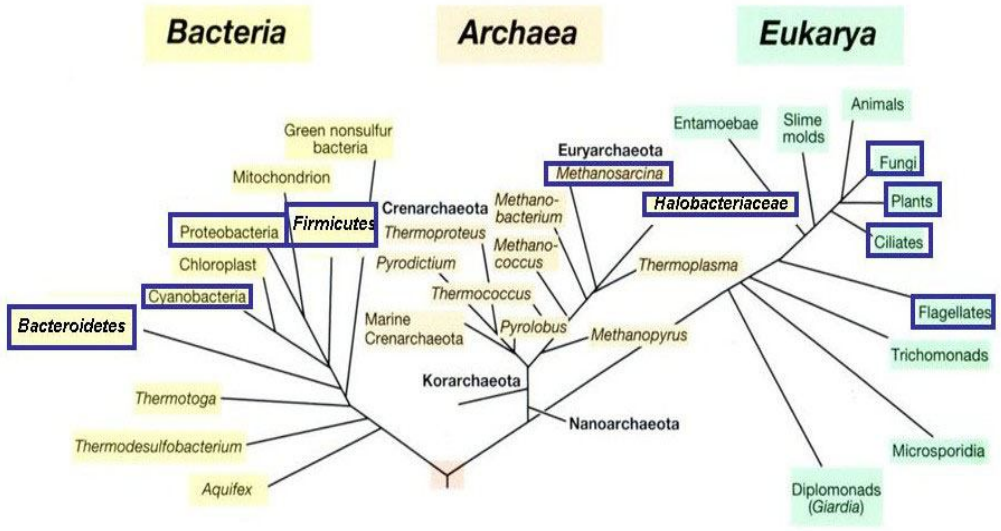
Categories of halophilic microorganisms (Kushner, 1985)

Category	**Salt concentration (M)**	
	Range	**Optimum**
Non-halophile	0-1.0	<0.2
Slight halophile	0.2-2.0	0.2-0.5
Moderate halophile	0.4-3.4	0.5-2.0
Borderline extreme halophile	1.4-4.0	2.0-3.0
Extreme halophile	2.0-5.2	>3.0
Halotolerant	0->1.0	<0.2
Haloversatile	0->3.0	0.2-0.5

Distribution of halophiles:

Halophiles and nonhalophilic relatives are often found together in the phylogenetic tree, and many genera, families and orders have representatives with greatly different salt requirement and tolerance. Within the small subunit rRNA gene sequence-based tree of life we find three groups of prokaryotes that are both phylogenetically and physiologically coherent and consist entirely or almost entirely of halophiles. Within the *Euryarchaeota* we encounter the order *Halobacteriales* with a single family, the *Halobacteriaceae* (Oren, 2006a). In the bacterial kingdom, the family *Halomonadaceae* (class *Gammaproteobacteria*, order *Oceanospirillales*) predominantly contains halophiles (Arahal and Ventosa, 2006). Members of the *Halobacteriaceae* and the *Halomonadaceae* are aerobic heterotrophs, some of which have a limited potential for anaerobic growth. The third phylogenetically coherent group contains the anaerobic fermentative bacteria of the order *Halanaerobiales* (*Firmicutes*, families *Halanaerobiaceae* and *Halobacteroidaceae*) (Oren, 2006b).

Extremophiles, such as thermophilic or halophilic microorganisms, can survive under conditions where most “conventional” organisms cannot (Madigan *et al.*,., 1999, Ventosa *et al.*,., 1998). Halophilic microorganisms living in high-salt environments may be classified into extremely halophilic archaea and moderately halophilic eubacteria (Kushner, 1985). Extremely halophilic archaea, which are adapted to survive in environments with extremely high concentrations of salt such as the Dead Sea, are unable to survive and tend to lyse at salt concentrations below 2.5 M. Moderately halophilic bacteria are defined as a group of microorganisms which show the ability to grow over a wide range of salinities: optimally at 0.5~2.5 M, but sometimes even up to close to saturated NaCl (Ventosa *et al.*, 1998).



The universal phylogenetic tree of life as based on small subunit rRNA gene sequences, and the distribution of halophilic microorganisms within the tree. Groups marked with boxes contain at least one halophilic representative (eg: the *Bacteroidetes*, of which *Salinibacter ruber* is the sole halophilic member described to date); others such as the *Halobacteriale* consist entirely of halophiles.

Kushner and Kamekura (1988) defined moderate halophiles as those microorganisms that grow optimally at NaCl concentrations between 0.5 and 2.5 M. These bacteria have a specific requirement for Na^+ ions, which cannot be replaced by other cations or nonionizable solutes. In hypersaline habitats both heterotrophic and phototrophic moderately halophilic eubacteria have been found. The first group is constituted by a large variety of bacteria, including Gram-positive and Gram-negative species (Ventosa, 1988).

Halomonas:

The genus *Halomonas* includes straight or Gram negative rods that require high NaCl for growth, motile or non-motile, catalase positive and aerobic. They are highly versatile in terms of their ability to successfully grow in a variety of temperature and pH conditions. This versatility led to *Halomonas* species being used as a substitute for the utilization of the starch-derived raw materials (Quillaguaman *et al.*, 2005). Some of the fatty acids in

Halomonas species include C (18:1) omega7C, C (16:0) & C (19:0) cyclo omega 8C. They are also traditionally extreme halophiles with full motile capabilities because they are either polarly or laterally flagellated. Some *Halomonas* species have been demonstrated to perform denitrification to gain energy through the processing of nitrate to nitrogen.

The members of the genus *Halomonas* were usually found in water sources with high salinity levels such as the Dead Sea and even the frigid waters of Antarctica. They can also inhabit the deep sea sediment, deep sea waters affected by hydrothermal plumes and hydrothermal vent fluids (Okamoto *et al.*, 2004).

Halomonas eurihalina (Volcaniella eurihalina):

Halomonas eurihalina is a moderate halophile belonging to the family *Halomonadaceae* which belongs to γ-class of Proteobacteria. *Volcaniella,* named for B. Elazari-Volcani, the microbiologist who first described halophilic microorganisms from the Dead Sea. It was proposed by Franzmann *et al.*, (Franzmann *et al.*, 1988) based on the results obtained with 16S rDNA cataloguing technique to accommodate the moderately halophilic and marine bacteria of the genera *Halomonas* and *Deleya*.

More recently, a study based on the comparison of 16S rDNA sequences from several moderately halophilic bacteria concluded that *Volcaniella eurihalina* should be reclassified as *Halomonas eurihalina* (Mellado *et al.*, 1995).

The organisms are nonmotile, Gram-negative short rods that are oxidase negative; they are aerobic with a strictly respiratory type of metabolism; they are moderate halophiles, optimal growth occurs at a total salt concentration of 7.5% (wt/vol), and they exhibit a strongly euryhaline character; and they have a specific requirement for Na^+ ions (sodium can be

supplied as NaCl, Na_2SO_4, or NaBr). The minimum NaCl concentration required is 1.5% (wt/vol). The guanine-plus-cytosine content of the DNA is 59.1 to 65.7 mol%. This organism was isolated from hypersaline habitats, including saline soils and salt ponds, and from seawater (Quesada *et al.*, 1990).

They show cream pigmentation. They are negative for acid production from arabinose, glucose, lactose, trehalose and mannitol. These organisms show hydrolysis of gelatin, esculin, tween80, DNA but not casein and starch. They are positive for H_2S production and phosphatase production. They show nitrate reduction but not nitrite reduction. Organic osmotic solutes such as glycine betaine, ectoine, glutamate are present within these bacteria. *Halomonas eurihalina* F2-7 produces large amounts of an extracellular polyanionic polysaccharide (Quesada *et al.*, 1993). The polymer, consisting of 42% carbohydrates (mostly hexoses) and 15% protein, is a potent emulsifying agent, exhibiting pseudoplastic behavior. It forms gels of high viscosity at acid pH. In view of these properties, the polysaccharide (EPS V2-7) may find broad applications in pharmaceuticals, in the food industry, and in biodegradation processes (Calvo *et al.*, 1995). Having a high content in sulfate groups, this polysaccharide was shown to have remarkable in vitro immunomodulating activity, enhancing the proliferative effect of human lymphocytes as a response to the presence of the anti-CD3 monoclonal antibody in blood (Pérez *et al.*, 1997).

Adaptations to high and changing salt concentrations:

High osmolarity in hypersaline conditions can be deleterious to cells since water is lost to the external medium until osmotic equilibrium is achieved (Galinski, 1993). To be able to live at high salt concentrations, halophilic and halotolerant microorganisms must maintain a cytoplasm that is osmotically isotonic with the outside medium. Two different strategies have been used to achieve this osmotic equilibrium. The first involves accumulation of molar concentrations of KCl. This strategy requires adaptation of the intracellular

enzymatic machinery, as proteins should maintain their proper conformation and activity at near-saturating salt concentrations. The proteome of such organisms is highly acidic, and most proteins denature when suspended in low salt. Such microorganisms generally cannot survive in low salt media. The second strategy is to exclude salt from the cytoplasm and to synthesize and/or accumulate organic 'compatible' solutes that do not interfere with enzymatic activity. Few adaptations of the cells' proteome are needed and organisms using the 'organic-solutes-in strategy' often adapt to a surprisingly broad salt concentration range. Most halophilic bacteria, but also the halophilic methanogenic archaea use such organic solutes (Oren, 2008).

Compatible solutes are polar, highly soluble molecules, most of them being either uncharged or zwitterionic at physiological pH (Reed, 1986). They belong to several categories: Polyols (glycerol, arabitol, mannitol, erythritol), sugars (sucrose, trehalose) and heterosides (glucosylglycerol), Betaines (trimethylammonium compounds) and thetines (dimethylsulfonium compounds), Amino acids (proline, glutamate, glutamine and derivatives), Glutamine amide derivatives (Nα-carbamoylglutamine amide; Nα-acetyl-glutaminylglutamine amide), N-acetylated diamino acids (Nδ-acetylornithine, Nε-acetyllysine), Ectoines (ectoine, β-hydroxyectoine) (Oren, 2002b).

The 'high-salt-in strategy' is not limited to the *Halobacteriaceae*. The *Halanaerobiales* (*Firmicutes*) also accumulate salt rather than organic solutes. A third, phylogenetically unrelated organism accumulates KCl (Oren, 2008).

Biomolecule profile of halophiles:

Research into the physiology and biochemistry of the extreme halophiles has revealed that their basic life processes are similar to those of non-halophilic bacteria except for a salt requirement (Flannery, 1956; Brown, 1964, 1976; Larsen, 1967; Kushner, 1968, 1971; Lanyi, 1974).

Halophiles produce a large variety of stable and unique biomolecules that may be useful for practical applications. Pigmented halophilic archaea and micro-algae absorb light-energy in saltern ponds, thereby raising the water temperature, increasing the rate of evaporation and hastening the deposition of salt. Halophiles posses many hydrolytic enzymes such as DNases, lipases, amylases, gelatinases and proteases capable of functioning under conditions that lead to precipitation or denaturation of most proteins. Halophilic proteins compete effectively with salts for hydration, a property that may result in resistance to other low water activity such as in the presence of organic solvents. Some of the commercial applications include use of beta carotene as health food additive isolated from carotenoid rich *Dunaliella* strains (Ben-Amotz and Avron, 1989) and use of ectoine from moderately halophilic bacteria as enzyme protectant and as a moisturizer in the cosmetic industry (Galinski and Louis, 1999).

Proteins of halobacteria are either resistant to high salt concentrations or require salts for activity. As a group, they contain an excess ratio of acidic to basic amino acids, a feature likely to be required for activity at high salinity. This characteristic is shared with proteins from some halophilic bacteria. Surface negative charges are thought to be important for solvation of halophilic proteins, and to prevent the denaturation, aggregation and precipitation that usually results when nonhalophilic proteins are exposed to high salt concentrations. Halobacteria produce large quantities of red-orange carotenoids. Carotenoids have been shown to be necessary for stimulating an active photo repair system for repair of thymine dimers resulting from ultraviolet radiation. The most abundant carotenoids are C-50 bacterioruberins, although smaller amounts of biosynthetic intermediates such as β-carotene and lycopene are also present. Retinal is produced by oxidative cleavage of β-carotene, a step that requires molecular oxygen. Several retinal proteins, in addition to bacteriorhodopsin, are also produced by halobacteria, including halorhodopsin, which is an inwardly directed light-driven chloride pump, and two sensory rhodopsins, which mediate the photo tactic response (swimming

towards green light and away from blue and ultraviolet light) (DasSarma and Arora, 2001).

Perhaps a better illustration of the extent of biochemical adaptation to high ionic concentrations is the protein synthesis system. Such a system is composed of an organelle, the ribosome, and a multitude of enzymes, co-factors, and RNA molecules. The effect of salts on the function of proteins is important and also on protein-protein, protein-RNA, and RNA-RNA interactions.

Ribosomes:

Ribosomes are cytoplasmic organelles composed of RNA and protein and are sites of protein synthesis found in all living cells. Ribosomes were first described as small particulate components of the cytoplasm by Palade (Palade, 1955). Upon isolation, they were shown to contain 60% ribosomal RNA (rRNA) and 40% protein. Ribosomes from eubacteria, archaea and eukaryotes morphologically and biochemically differ although they perform same function (Lake *et al.*, 1982, Lake, 1983). Ribosomes from eubacteria and archaea are of 70 S type and those from eukaryotes are of 80 S type. Ribosomes are also found in the mitochondria and chloroplasts of eukaryotic cells. They are always smaller than the 80 S cytoplasmic ribosomes, and are comparable to prokaryotic ribosomes in both size and sensitivity to antibiotics, although the sedimentation values vary somewhat in different phyla. The genetic code is the same in all living organisms with some exceptions in case of some codons (eg: mitochondria, *Mycoplasma* etc.) and it has been demonstrated that eukaryotic ribosomes are able to translate bacterial mRNAs correctly.

Eukaryotic ribosomes are much larger than prokaryotic ones and have more number of proteins which are different from prokaryotes. Mitochondrial and chloroplast ribosomes resemble those in bacteria. In summary, there is little structural but considerably functional homology between prokaryotic and

eukaryotic ribosomes. Several studies on archaeal ribosomes particularly ribosomes from thermophilic and methanogenic archaea indicated close similarity of these archaeal ribosomal protein to eukaryotic ribosomal protein (Hill *et al.,* 1990). Cells devote considerable effort to the production of these essential organelles. For example, an *E. coli* cell contains approximately 15,000 ribosomes, each one with a molecular weight of about three million Daltons. Ribosomes therefore represent 25% of the total mass of these bacterial cells.

Structure:

Ribosomes are tiny particles, about 200A° and are composed of both proteins and RNA; in fact it has approximately 37-62% RNA, and the rest are made up of proteins. The ribosomal RNA (rRNA) in eukaryotes, is produced in the nucleolus, a prominent globular structure in the nucleus. Thus, the proteins are gene products of themselves, and one ribosome is made up of dozens of genes. Eukaryotic ribosomes fall into two categories; those that are free in cytoplasm and those that are bound to endoplasmic reticulum. The two kinds of ribosomes play similar roles in translating mRNA to produce proteins. The ribosomes in the cytoplasm synthesize non-secretory proteins, while the bound ribosomes are involved in the synthesis of secretory proteins.

Lake's view:

Ribosomes from organisms within eubacteria, archaeabacteria and eukaryotic lineages, each have different three-dimensional structures. Sulfur dependent archaeal ribosomes are structurally different from the ribosomes found in eubacteria, eukaryotes and other archaea and constitute a fourth type of ribosome structure (Lake *et al.,* 1982, 1984).

All the four-ribosome types share a common structural core; in addition all show their own independent structures (Lake *et al.,* 1982, 1983). The top of

the small subunit is called head and the bottom is called the base. Platform protrudes from the base to the right, which is separated from the head by the cleft.

Small subunits from archaea, sulfur dependent archaea and eukaryotes contain a structure that resembles a duckbill, the archaeal bill. Only a small bill is present in eubacterial ribosomes. It extends from the head of the subunit and estimated to be of size with that of L7/L12 stalk of bacteria. This bill may have a function in factor related steps, as it is located near factor binding sites.

Bacterial 50 S subunit shows a large projection in the center, known as central protuberance. The long projections on the right and left are called stalk and ridge respectively. The space between the ridge and the central protuberance is called the valley. When the 30 S and 50 S subunits nestle together, the 30 S platform extends into the valley of the 50 S subunit.

In prokaryotes, the 70 S ribosomes dissociate into large subunit i.e., 50 S and small subunit i.e., 30 S. The 50 S has two types of rRNA - a 23 S and a 5 S. It also has 32 different proteins. It has a particle weight of $1.65x10^6$ Da. On the other hand; the 30 S contains a single 16 S rRNA plus, 21 different types of proteins and has a particle weight of $0.9x10^6$ Da. Eukaryotes have 80 S ribosomes with 60 S and 40 S subunits. Most of the ribosomal proteins (prokaryotic and eukaryotic) in both the subunits are basic proteins except a few (examples: S1, S6, L7/L12) are acidic proteins (Subramanian, 1980). Ribosomes from halophilic archaea contain more number of acidic proteins (Bayley, 1966a), whereas methanogenic archaea also contain acidic proteins but less acidic compared to halophiles. The ribosomes of sulfur dependent bacteria contain more number of basic proteins. All the proteins can be separated by 2D-gel electrophoresis into distinct spots.

The essentially complete atomic structures of an archaeal 50 S subunit from *Haloarcula marismortui* at 2.4 Aº resolution (Ban *et al.,* 2000) and a

bacterial 30 S subunit from *Thermus thermophilus* at 3.3 Aº resolution (Schluenzen *et al.,* 2000) and at 3.05 Aº (Wimberly *et al.,* 2000) published in 2000 were the basis for the phasing and/or molecular interpretation of every subsequent structure of the ribosome or its subunits. Such structures include low-resolution structures of the *Thermus thermophilus* 70 S ribosome at 5.5 Aº resolution by crystallography (Yusupov *et al.,* 2001) or cryoelectron microscopy (Gao *et al.,* 2003), the structure of a bacterial 50 S subunit at 3.1 Aº resolution from *Deinococcus radiodurans* (Harms *et al.,* 2001) and more recent high-resolution structures of the 70 S ribosomes (Schuwirth *et al.*, 2005; Selmer *et al.,* 2006;). Finally, mobile elements of the 50 S subunit such as the L1 or L7/L12 stalks that are partly or completely disordered in most high-resolution structures of the ribosome or the 50 S subunit have been solved in isolation (Nikulin *et al.,* 2003; Diaconu *et al.*, 2005;). In addition to structural studies, increasingly sophisticated biochemical methods such as single-molecule studies will help to dissect the various steps of complicated processes.

Halophilic ribosomes:

The basic structure and functioning of the halophilic protein synthesis system closely resembles that of the non-halophiles (Bayley, 1976). The complete ribosome is a 70 S particle consisting of a 50 S subunit and a 30 S subunit (Bayley and Kushner, 1964). The 50 S subunit contains a 23 S and 5 S RNA molecules and the 30 S subunit contains a 16 S molecule (Visentin *et al.,* 1972). The ribosome is composed of 60% RNA and 40% protein, and has a Mg^{2+}/phosphorous molar ratio of 0.3. The number of proteins in each subunit is also the same as the non-halophile, *E. coli*, with 34 proteins in the 50 S subunit and 21 proteins in the 30 S subunit (Storm and Visentin, 1973).

The difference between halophilic and non-halophilic ribosomes was first shown by Bayley and Kushner's (1964) demonstration that the halophilic ribosomes required high (3-4 M) KCl concentrations to remain in the

associated (monomer) form. In 2 M NaCl or KCl, the ribosomes of non-halophiles lose not only their monomer association but also their structure. The ribosomes of the extreme halophiles lost their structure when placed under conditions which favor the stability of non-halophilic ribosomes (Bayley and Kushner, 1964; Bayley, 1966a, 1966b; Visentin *et al.,* 1972). Bayley and Griffiths (1968a) showed that the entire system necessary for *in vitro* protein synthesis required 3.8 M KCl, 1 M NaCl, 0.4 M NH_4Cl and 0.04 M $MgCl_2$ for optimum incorporation of amino acids. A similar system from the non halophile, *E. coli,* has an optimum salt concentration of 0.16 M KCl or NH_4Cl and 7-15mM $MgCl_2$ (Conway, 1964). The amino acyl synthetases of the halophile require 3.8 M KCl for activity (Griffiths and Bayley, 1969).

Since the difference between the halophilic and non-halophilic protein synthesizing system is not at the gross structural level, nor is it in the mechanism in which the protein synthesis occurs (Bayley, 1976), the difference probably occurs in the protein and RNA components of the system. The ribosomal RNA (rRNA) of the halophilic ribosomes showed no unusual properties. The C+G content is only slightly higher than that of the non-halophile, *E. coli* (Visentin *et al.,* 1972). However, distinct differences in the ribosomal proteins of halophiles and non-halophiles were shown. Bayley and Kushner (1964) observed that the ribosomal proteins released from the ribosomes suspended in low K^+ concentrations were acidic. These proteins could also be reassociated on the ribosomes by restoring the KCl concentration (Bayley, 1966b). Bayley (1966a) investigated this further and found that most of the ribosomal proteins were acidic, having isoelectric point around 3.9. The base/acid amino acid ratio of total ribosomal proteins was 0.49, which is far below that of *E. coli* ribosomal proteins (0.99). Visentin *et al.,* (1972) selectively removed the ribosomal proteins from *H. cutirubrum* ribosomes with different salt washings and found that the more basic proteins were more difficult to remove. The ribosomal proteins first removed by low K^+ presumably contain the external proteins, those on the surface, which encounter the high ionic environment. This protein fraction is also the most acidic.

However, Falkenberg *et al.,* (1976) showed that the ribosomal proteins from a moderate halophile are slightly more acidic than those of *E. coli* but much less acidic than those from the extreme halophile, *H. cutirubrum* as judged by their electrophoretic mobility on polyacrylamide gels and their amino acid composition. The electrophoretic profile on polyacrylamide gels of the ribosomes from moderate halophile is similar whether the cells are grown in 0.5M NaCl or 4.25 M NaCl.

Storm and Visentin (1973) developed a procedure for the simultaneous separation and identification of the halophilic ribosomal proteins. Using this procedure, Storm *et al.,* (1975) studied effects of ions on protein-RNA interactions of the 50 S subunit. They postulated that the ribosomal proteins of the extreme halophiles are significantly different from those of the non-halophiles, but retain certain conserved portions that may be important for their functions in protein synthesis and/or in the interaction with the rRNA. Studies on the sequence homologies between purified *H. cutirubrum* ribosomal proteins and equivalent ribosomal proteins from other species of bacteria have been performed (Chow *et al.,* 1972; Yaguchi *et al.,* 1973; Visentin *et al.,* 1974; Oda *et al.,* 1974; Duggleby *et al.,* 1975).

Ribosomal subunit association:

The primary events of initiation of protein synthesis in prokaryotes are the dissociation of the 70 S ribosomes into subunits and the formation of the pre initiation complex with the 30 S ribosomal subunit, fMet tRNA and mRNA. This 30 S initiation complex reassociates with 50 S subunit to yield activated ribosomes. Repeated association and dissociation of the ribosomal particles is essential in the living cell. In *in vitro,* variety of factors such as monovalent and divalent cations, polyamines, antibiotics and nonionic detergents affects the association of ribosomal subunits. The magnesium ion dependent association reaction obeys a true equilibrium as shown by Zitomer

and Flaks (1972) in case of *E. coli* ribosomes. Ribosomal subunit association studies have established the role of ribosomal subunits in the initiation of protein synthesis (Nomura *et al.,* 1967; Guthrie *et al.,* 1968; Ghosh and Khorana *et al.,* 1967; and several others).

However, an alternative pathway for translation initiation on leaderless mRNAs with 70 S ribosomes has been put forwarded by Balakin *et al.,* (1992a). They have shown that 70 S ribosomes form a translation initiation complex at the 5′ terminal start codon of leaderless λ *c*I mRNA. Moll *et al.,* (2004) have demonstrated the proficiency of dedicated 70 S ribosomes in *in vitro* translation of leaderless mRNAs and also that a natural leaderless mRNA can be translated with cross linked 70 S wild-type ribosomes.

Goss and Harrigan (1986) in their studies have found that spermidine in low concentrations reduces the aggregation of ribosomes. Spermidine is a powerful promoter of subunit association (Gorisch *et al.,* 1976). Polyamines cannot be substituted entirely for Mg^{2+} (Weiss and Morris, 1970 and 1973). Introduction of 1 mM spermidine results in three-fold fall of the need of Mg^{2+} for half association. In *E. coli*, initiation factor IF3, serves as an allosteric effector to regulate the subunit equilibrium (Goss *et al.,* 1980). Rabbit reticulocyte initiation factor eIF3 and wheat germ initiation factor eIF6, both have been reported to have ribosomal dissociation activity. The incubation of ribosomal subunits with IF3 reduced the Mg^{2+} induced association rate by a factor of five. Ribosomal subunit association was studied using static techniques such as ultra centrifugation (Noll and Noll, 1976) and light scattering experiments (Zitomer and Flaks, 1972).

Role of polyamines:

The naturally occurring polyamines such as spermine, spermidine and putrescine stabilize the secondary structure of RNA against thermal

denaturation. Polyamines stimulate the polypeptide synthesis (Watanabe *et al.*, 1981; Igarashi *et al.*, 1974, 1982).

Polyamines also stimulate the assembly of 30 S subunit (Echandi *et al.*, 1975; Igarashi *et al.*, 1979) and increase the fidelity of protein synthesis (Ito and Igarashi, 1986). Polyamines cause a structural change of relatively unstable double stranded rRNA. Spermidine was found to stimulate Met-tRNA binding to 40 S ribosomal subunits. Igarashi *et al.*, (1982) have reported that polypeptide synthetic activity of 30 S ribosomal subunits prepared in the presence of spermidine was at least 4 times greater than that of subunits prepared in its absence. Polyamines influence the various stages of protein synthesis by influencing the secondary structures of mRNA, tRNA and rRNA. Some polyamines like putrescine even act as inhibitors of protein synthesis (Cammarano *et al.*, 1982; Friedman and Oshima, 1989).

Polyamines have a stimulatory effect and can increase the maximal rate of protein synthesis with an optimal concentration of magnesium. Lowering of optimum concentration of magnesium required, for *in vitro* protein synthesis and aminoacylation in a cell free system of *Bacillus*, in the presence of spermine has been reported by Takeda *et al.*, (1969a and b). Friedman (1985) demonstrated that addition of 3 mM spermine results in extensive association of ribosomal subunits at lower magnesium concentrations and showed that polypeptide-synthesizing activity was four fold more in the presence of 10 mM Mg^{2+} and 3 mM spermine than in the presence of only Mg^{2+} (30 mM).

Effect of RNase A on the structure of ribosomes:

Pancreatic RNase is an endonuclease. It specifically hydrolyses RNA after C and U residues and produces mono and or oligonucleotides terminating with pyrimidine nucleoside 3´ P. It is active under wide range of reaction conditions and stable even at boiling temperatures. RNase disrupts the 3

dimensional structure of the ribosome. The enzymatic degradation of RNA in the ribosomes has proved to be an important approach to the elucidation of ribosomal structure. Tal (1969) used RNase degradation of ribosomes and the hyper chromic effect obtained by heating a ribosomal suspension as a measure to study the stability of ribosomes. Santer and Smith (1966) have reported the mild digestion of *E. coli* ribosomes with pancreatic RNase A. The nuclease-digested ribosomes were almost inactive in protein synthesis (Cahn, 1970). Delihas (1970) has studied the effect of ribonuclease action on *E. coli* ribosomes.

Removal of Mg^{2+} from the ribosomes by EDTA greatly increases the sensitivity of their rRNA to ribonuclease (Pinder and Gratzer, 1972). Ghysen (1970) have reported that ribosomal stability and conformation is dependent on the monovalent to divalent cation concentrations. The RNase A enzyme activity is inhibited by low concentrations of polyamines and this activity can be reversed by the higher concentrations of polyamines.

The extent of degradation of RNA in situ depends in general on the physicochemical state of the particle, as determined by the composition of the incubation medium, where the presence of magnesium ions has a critical role (Cox, 1969).

Effect of various agents on the stability and conformation of ribosomes:

Both mono and divalent cations are effective in stabilizing the RNA structure. Magnesium associates with the folded form of RNA in both site specific and delocalized, nonspecific manner (Laing *et al.,* 1994). Magnesium binds to RNA 50-fold more tightly than the other alkaline earth metal ions under the same conditions (Burkhman and Draper, 1997).

Magnesium promotes folding of RNA into compact structure and results in protein binding (Xing *et al.,* 1996). Divalent cations like magnesium

effectively reduce the repulsions and stabilize the folded structure of the most RNA molecules. Thus, magnesium binding is strongly coupled to tertiary structure formation of the most RNAs and favors the folding reaction (Misra *et al.,* 1998). Ammonium ion is more effective than K^+ in stabilizing the tertiary structure of rRNA as it has the ability to stabilize the RNA by forming four hydrogen bonds (Wang *et al.,* 1993; Lu and Draper, 1994).

Studies based on differential scanning micro calorimetry and dielectric spectroscopy by Blasi *et al.,* (2000) suggested that RNA structural domains exposed to the solvent play a fundamental role in the stability of the 3D structure of the ribosomal particle.

EtBr is a phenanthridine dye that specifically binds with nucleic acids. The binding of dyes to nucleic acids depends on the conformation of the nucleic acid. EtBr binds with the double stranded regions of the rRNA in the ribosome. Using RNase I as a probe, Suryanarayana and Burma (1975) have studied effect of intercalating agents like EtBr and acridine orange on the conformation of the ribosomes and showed that these agents unfold the ribosome structure. Similar unfolding effect was seen by thiol reacting reagents (Suryanarayana, 1998), whereas polyamines tighten the structure of ribosome as indicated by RNase I sensitivity (Suryanarayana and Datta, 1978). The assembly of 30 S ribosomal subunit was never complete with the 16 S rRNA, when it was treated with EtBr due to alterations in the structure of rRNA (Bollen *et al.,* 1970).

Growth cycle coupled variation of ribosomes:

During the growth transition of bacteria from exponential to stationary phase, the expression of growth-related changes is mostly turned off and instead a set of genes required for stationary-phase survival is switched on (Hengge-Arions, 1993; Ishihama, 1997; Ishihama, 1999;). For this drastic change in gene expression pattern, structural and functional modulations take

place on both transcriptional and translational apparatuses (Ishihama, 1997; Ishihama, 1999). Growth phase coupled variation of ribosomal profile is reported in *E. coli* by Wada *et al.,* (2000). They observed that ribosomal modulation factor (RMF), a small basic protein, expresses transcriptionally in the stationary phase of *Escherichia coli* cells and binds to 50 S ribosomal subunits. The RMF bound 70 S ribosomes dimerize to form 100 S particles that have no translational activity (Wada, 1998).

Ribosomal protein S1:

Initially, S1 was found as a component of *E. coli* 30 S ribosomal subunit (Kurland *et al.,* 1969; Nomura *et al.,* 1969; Traut *et al.,* 1969; Kaltschmidt and Wittmann, 1970). Subsequently it has been established that S1 is a component of 70 S ribosome performing essential function in protein synthesis (Van Knippenberg *et al.,* 1974; Van Duin and Van Knippenberg, 1974; Suryanarayana and Subramanian, 1983). The S1 protein has been independently isolated as i (interference) factor (Groner *et al.,* 1972a) and subunit I or α of Qβ replicase (Kamen *et al.,* 1972; Groner *et al.,* 1972 b). The i factor was shown to inhibit translation of synthetic and natural mRNA (Groner *et al.,* 1972 c; Miller *et al.,* 1974b). Subunit I prepared from Qβ replicase, i factor isolated from 1 M NH_4Cl ribosomal wash, and S1 prepared from 30 S particles were shown to be identical (Wahba *et al.,* 1974; Inouye *et al.,* 1974).

Purification of S1:

Protein S1 as an interference factor was purified from high salt ribosomal wash (Groner *et al.,* 1972a, b; Miller *et al.,* 1974a; Jay and Kaempfer, 1974 & 1975). Tal *et al.,* (1972) isolated S1 by exposing the 70 S ribosomes to low ionic strength buffers. S1 was selectively removed from the 30 S subunit at 1 mM concentration of Tris buffer. Protein S1 was also purified directly from the nucleic acid free crude cell extracts by poly (C)-cellulose chromatography (Carmichael, 1975). Suryanarayana and Subramanian (1979

& 1983) purified S1 in high yield by affinity chromatography of ribosomes on poly (U)-sepharose.

Physical properties of S1:

The physical constants of S1 have been determined in several laboratories (Subramanian, 1984). S1 is the largest (Mr 61,159) and the longest (23 nm) of all the ribosomal proteins. Its large radius of gyration (7 nm) and intrinsic viscosity (9.8 ml g^{-1}) as well as low sedimentation coefficient (3.2 S) and diffusion constant (4.5×10^{-7} Cm^2 S^{-1}) indicated a very asymmetric elongated shape (Giri and Subramanian, 1977). Small angle X-ray scattering analysis revealed that protein S1 in solution behaves like a rod shaped molecule (Laughrea and Moore, 1977; Labischinski and Subramanian, 1979). The iso-electric point (pI) of the protein is 4.5. S1 shows anomalously low electrophoretic mobility (Subramanian, 1983). Wittmann (1974) showed that the molecular weight of S1 from small subunit of the *Escherichia coli* 70 S ribosome is 68 kDa. (Wittmann, 1974). It is interesting that that S1 (23 nm) is slightly longer than the biggest dimension of the ribosome (22 nm), which may be important in the function of the protein.

Primary structure of S1:

The structural gene of S1 (rps A) has been mapped at 20 min on the *E. coli* K-12 chromosome (Ono *et al.,* 1979). Kitakawa *et al.,* (1980) isolated transducing λ phage carrying *rps A*. Both, amino acid sequencing of the protein S1 from *E. coli* strain MRE 600 is a single chain of 557 amino acid residues.

Interaction of S1 with 30 S ribosomal subunit:

S1 is loosely associated with the 30 S subunit. It is partially lost from the ribosomes during the isolation procedures. Furthermore, S1 dissociates from 30 S at 1 mM Tris concentration (Tal *et al.,* 1972). S1 binds to 30 S

subunit mostly through protein-protein interactions (Boni *et al.,* 1982). Protein S1 binding site on the ribosome is reported to be near the proteins S2, S10 and S18 (Boliaeu *et al.,* 1981). S1 can bind to 30 S subunits subjected to mild nucleolytic cleavage but not to 30 S subunits subjected to mild proteolysis indicating that S1 binds to ribosome through protein-protein interactions (Boni *et al.,* 1982). It has been suggested that a pyrimidine rich region upstream of the Shine–Dalgarno (SD) sequence of mRNA interacts with protein S1 and serves as one of the ribosome recognition sites (Boni *et al.,* 1991). Protein S1 has been reported to be necessary for translation initiation (Suryanarayana and Subramanian, 1983; Tzareva *et al.,* 1994) and also for translation elongation (Potapov and Subramanian, 1992).

S1-like proteins in other organisms:

S1-like protein is not present in ribosomes from *Bacillus stearothermophilus* (Isono and Isono, 1976). *Bacillus subtilis* also does not have a protein showing structural homology with *E. coli* S1 (Higo *et al.,* 1982). Muralikrishna and Suryanarayana (1985) made comparison of several Gram-negative bacteria and Gram-positive eubacteria for the presence of S1 using several criteria. They observed that most Gram-negative bacteria (all enterobacteria) contained protein S1 while most Gram-positive tested with the exception of *Micrococcus luteus* did not contain protein S1. Yamada (1982) isolated a ribosomal protein from *Mycobacterium smegmatis* which binds to poly (U). However, this protein was found to be non homologous to *E. coli* protein S1 either immunologically or functionally. Schneir and Faist (1985) compared the structural gene *rps A* coding for protein S1 and its preceding regulatory region (having four promoters) from *E. coli* with other bacterial species using southern blotting. Their results indicated high degree of homology in S1 structural genes of *E. coli* and other Gram-negative but not Gram-positive bacteria.

Muralikrishna and Suryanarayana (1987a, b) purified S1 from Gram-negative nitrogen fixing bacterium *Azotobacter vinelandii* (Av-S1) and Gram-positve Micrococcus luteus (Ml-S1) by employing poly (U)-sepharose chromatography. Hahn *et al.,* (1988) identified S1- like protein in spinach chloroplast ribosomes by means of immunoblotting technique. Ravi and Suryanarayana (1994) have isolated S1 like protein from the ribosomes of thermophilic and halophilic archaea. This study indicated differential conservation of structural domain of S1 in archaea. Protein S1 is from T*hermus thermophilus* is detected, identified and overproduced in *E. coli* (Shiryaev *et al.*, 2002).

S1 in bacteria and translation specificity:

Stallcup *et al.,* (1974 & 1976) have reported that ribosomes from Gram-positive bacteria could not translate mRNA from Gram-negative bacteria while the ribosomes from Gram-negative bacteria translated mRNA from both types of bacteria. This difference in translation has been attributed to the weak Shine-Dalgarno interaction operating between Gram-positive ribosomes and Gram-negative messenger RNA (McLaughin *et al.,* 1981a, b; Murray and Rabinowitz, 1982). The Gram-positive appear to possess strong Shine-Dalgarno regions. Higo *et al.,* (1982) suggested that the absence of S1 from bacterial ribosomes may account for species-specific translation.

Roberts and Rabinowitz (1989) studied translation activity of *E. coli* S1 on *B. subtilis* ribosomes. S1 is active on *B. subtilis* ribosomes in translation of poly (U) but that is unable to overcome the characteristic inability to translate *E. coli* mRNA. The removal of S1 from *E. coli* ribosomes causes them to become translationally specific to preferentially translate messengers having strong SD regions. In summary there appears to be an evolutionary correlation between the presence of S1, the type of Shine and Dalgarno sequence on mRNA and the translational specificity exhibited by bacteria (Roberts and Rabinowitz, 1989). Detailed analysis on the occurrence of S1 in Gram-positive

bacteria by Rabinowitz and co-workers had led them to suggest that, translational specificity of the ribosomes from Gram-positive bacteria is correlated with low genomic G+C content and lack of ribosomal protein S1. The Gram-positive organisms with high G+C content have S1-like proteins on their ribosomes and are translationally non-specific (Farwell and Rabinowitz, 1991). Balakin *et al.,* (1992b) showed that the mRNA with an extended Shine-Dalgarno sequence is translated independent of S1.

Scope and objectives of the present investigation

Till date, extensive work has been carried out on ribosomes of mesophiles, extreme halophiles and extreme thermophiles. On perusal of literature it was understood that in spite of the availability of large amount of data on ribosomes among all three domains of life, namely Archaea, Prokaryotes and Eukaryotes, ribosomes from moderate halophiles were poorly characterized. Hence, **Characterization of ribosomes from moderate halophile, *Halomonas eurihalina*** was chosen as the main objective for the present study.

H. eurihalina was chosen as the model organism by virtue of its strong euryhaline nature which allows the strain to grow in a wide range of salt concentrations varying from moderate to extreme halophilic conditions. Thus allowing us to study the variation of ribosomes, if any, depending on the salt concentration. Present study deals with the characterization of ribosomes from moderate halophile, *H. eurihalina.*

In the present investigation studies were carried out on growth cycle and growth media dependent variation of ribosomal proteins and their comparison with ribosomal proteins from mesophiles. There are no reports available on the growth cycle coupled variation of ribosomes in moderate halophiles. Our studies showed that some proteins were missing in stationary phase and many proteins decreased in concentration where as few proteins showed increase. These studies on growth cycle coupled variation of ribosomes, might provide an insight into the translation regulation in stationary phase growth of moderate halophiles.

Effect of magnesium ion concentration on moderately halophilic ribosomal subunit dissociation was studied using several methods. These studies showed that magnesium ion dependent ribosomal subunit dissociation is absent in *H. eurihalina*.

The structural studies of ribosomes in terms of RNase A sensitivity under different experimental conditions and comparison with mesophilic ribosomes were carried out. The stability of ribosomes to protein unfolding reagents was studied using urea. Studies indicated that ribosomes of moderate halophile are structurally more compact and showed much more resistance to different treatments. These studies also showed that logarithmic phase ribosomes are more stable in comparison to stationary phase ribosomes. These studies help in understanding the stability of moderately halophilic ribosomes under different environmental stress conditions.

We also extended our studies to carry out proteomic studies of *H. eurihalina* ribosomes by identifying many of the ribosomal proteins by MALDI-TOF MS analyses. These studies showed that majority of the ribosomal proteins of moderate halophile are homologous to ribosomal proteins of gram positive and gram negative eubacteria and none of them were found to be homologous to ribosomal proteins of extreme halophilic archaea. It was also found that S3 is homologous to *Euryarchaeota* members. Relatedness of ribosomal proteins of *H. eurihalina* was compared to ribosomal proteins from other domains of life by multiple sequence alignment. These studies showed that the peptides identified in *H. eurihalina* are highly conserved among other bacterial ribosomal proteins. These studies provided identity of the ribosomal proteins showing growth cycle coupled variation and thus their role can be understood. This information may provide valuable information on the variations in the stability of logarithmic and stationary phase ribosomes. These studies may also provide useful information on the phylogenetic relatedness of *H. eurihalina* to organisms from other domains of life.

Homolog of ribosomal protein S1 was partially purified by poly (U) affinity chromatography and identified by MALDI-TOF MS analyses. During our studies on S1, we have observed the presence of two low molecular weight, strong poly (U) binding proteins in S-100 and they were identified as cold shock proteins.

Materials:

N,N'-Methylene bis acrylamide was obtained from Merck chemical company, Germany. Acrylamide, Glycine, Urea were obtained from Merck (India) chemical company, Mumbai. Tris, Bis-Tris, Spermidine trihydrochloride were obtained from USB chemicals, USA. SDS was obtained from Serva chemical company, Germany. Alumina, Agarose, Potassium glutamate, Potassium acetate, MES, Coomassie blue, Ethidium bromide, Sucrose (RNase free), Spermine, Poly (U), Pancreatic RNase A were obtained from Sigma-Aldrich company, USA. Poly (U)-Sepharose 4B, VivaspinMWCO20 were obtained from GE healthcare, Sweeden. DEAE-Cellulose, Cellulose, Whatman filter papers were obtained from Whatman International Limited, England. Molecular weight markers were obtained from Fermentas Molecular Biology Tools, Canada. DNase I was obtained from Bangalore Genei, India. Nitrocellulose filters (0.45 micron) were obtained from Millipore company, India. Bromophenol blue, Glycerol, Basic fuchsin, Sodium chloride, Potassium chloride, Calcium chloride, Sodium bromide, Sodium hydrogen carbonate, Glucose, Ammonium chloride, Ammonium sulphate, Phenol, Hydrochloric acid, Sodium acetate were obtained from Fisher Scientific, India. Magnesium chloride, Magnesium sulphate, Peptone, Yeast extract, Methanol, Agar-Agar, Acetone, Ammonium per sulphate and TEMED were obtained from Hi-media chemicals, Mumbai. Glacial acetic acid, Magnesium acetate, Sepharose-4B and 2-mercaptoethanol were obtained from SRL chemical company.

Methods:

Bacterial strains:

Halomonas eurihalina strain DSM 5720 was obtained from DSMZ, Braunschweig, Germany. *Escherichia coli* strain D10 (RNase I $^{-}$) was a gift from Dr. R. Sir Deshmukh, Center for Cellular and Molecular biology, India.

Bacterial growth:

Halomonas eurihalina was grown at 30 °C with vigorous aeration in MH medium containing 6% NaCl, 1.5% $MgCl_2$, 0.74% $MgSO_4$, 0.027% $CaCl_2$, 0.15% KCl, 0.0045% $NaHCO_3$, 0.0019% NaBr, 0.5% peptone, 1% yeast extract, 0.1% glucose. pH was adjusted to 7.2 with 2N NaOH (Quesada *et al* 1983). The cultures were chilled and harvested at 4 ºC after 12 h and 24 h of growth for logarithmic and stationary phase cells respectively and pellets were frozen at -80 °C.

E. coli strain D10 (RNase I^{-}) was grown in Luria broth containing 1% tryptone, 1% sodium chloride, 0.5% yeast extract at 37 °C with vigorous aeration. The mid logarithmic cultures were chilled and harvested. The cell pellet obtained was stored at -80 °C.

Growth of *H. eurihalina* in increasing salt (NaCl) concentrations:

H. eurihalina was grown in media containing different concentrations of NaCl (5%, 6%, 10%, 15% and 20%). The concentration of all other ingredients was same as that of the regularly used media and the growth was monitored by measuring the absorbance at 660 nm. Bacteria were grown at 30 °C with vigorous agitation until they reached stationary phase.

Isolation of ribosomes by ultracentrifugation:

Ribosomes were isolated from *E. coli* according to Minks *et al* (1978). The same procedure was followed to isolate ribosomes from *H. eurihalina* logarithmic and stationary phase cells using buffer conditions mentioned below. All operations were carried out at 4 °C. Cells were ground with double the weight of alumina in the presence of DNase (RNase free, 3 µg/g of cells) until soft and sticky and extracted with buffer (3 ml/gm of cells) containing 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 10 mM magnesium chloride and 7 mM 2-mercaptoethanol ($TKM_{10}Me$) for *E. coli* and for *H. eurihalina*, a buffer containing 20 mM Tris-Cl (pH 7.6), 250 mM ammonium chloride, 20 mM magnesium acetate and 7 mM 2-mercaptoethanol ($TNM_{20}Me$) was used. The suspension was centrifuged at 10,000 g to remove the cell debris and alumina and the supernatant was centrifuged at 30,000 g for 30 min to obtain S-30 extract. The S-30 extract was centrifuged in Beckman Ti 70 rotor at 1,00,000 g for 4 h to pellet ribosomes. The upper two thirds of the supernatant (S-100) was collected and the *E. coli* and *H. eurihalina* ribosomal pellets were suspended in small volume of $TKM_{10}Me$ and $TNM_{20}Me$ buffers respectively and processed for further purification or stored frozen at -80 °C until further purification.

Ammonium chloride wash of ribosomes:

Ribosomes were suspended in their respective ribosomal buffers containing 0.5 M or 1 M ammonium chloride and left at 4 ºC overnight. The ribosomal suspensions in 1 M ammonium chloride were pelleted by ultra centrifugation in Beckman Ti 80 rotor at 1,50,000 g for 3 h 30 min at 4 ºC. The pellets thus obtained were dissolved in respective ribosome buffers and then analyzed by SDS-PAGE. Ribosomal proteins were extracted from ammonium chloride washed ribosomes and analyzed by two-dimensional gel electrophoresis.

Ultra centrifugation of ribosomes on 10% sucrose cushion:

Ribosomes were layered on top of 10% sucrose cushions prepared in respective ribosomal buffers for *E. coli* and *H. eurihalina* ribosomes (logarithmic and stationary phases). These ribosomes were subjected to ultra centrifugation in Beckman Ti 80 rotor at 150,000 g for 3 h 30 min at 4 ºC. The pellets thus obtained were dissolved in respective ribosomal buffers and stored in small aliquots at -80 ºC.

Extraction of ribosomal proteins from ribosomes:

Extraction of ribosomal proteins was carried out according to the method of Hardy *et al* (1969). Two volumes of 100% glacial acetic acid containing 0.05 M magnesium acetate were added to one volume of ribosomes (10 mg/ml) and left on ice for 45-60 min with constant stirring. Then the suspension was centrifuged at 12,000 rpm for 20 min. Supernatant was collected and the pellet was re extracted with 0.5 volumes of 67% glacial acetic acid containing 0.1 M magnesium acetate. Supernatant was collected after the centrifugation to remove the precipitated RNA. Both the supernatants containing ribosomal proteins were precipitated with 5 volumes of ice-cold acetone and left overnight at -20 °C. The ribosomal proteins were pelleted at 12,000 rpm for 10 min. The pellet was dissolved in 8 M urea and 7 mM 2-mercaptoethanol and stored frozen at -80 °C. The extracted proteins were analyzed by SDS-PAGE and 2D gel electrophoresis.

SDS-poly acrylamide gel electrophoresis:

SDS-PAGE was performed using 18% gels as described by Thomas and Kornberg (1975). The ratio of acrylamide to bisacrylamide was 30:0.3. The 18% separating gel was in 0.75 M Tris-Cl pH 8.8, 0.002 M sodium EDTA and 0.1% SDS and the stacking gel was of 5% polyacrylamide in 0.08 M Tris-Cl pH 6.8, 0.002 M sodium EDTA and 0.1% SDS. Protein samples were treated

with 0.1% SDS and 1% 2-mercaptoehanol at 65 ºC for 15 min and layered on the slots of slab gels. Electrophoresis was carried out at 90 V for 4 h for mini gels and for 10 h for standard gels. Electrode buffer used was 0.05 M Tris, 0.38 M glycine and 0.1% SDS.

Staining of poly acrylamide gels:

Coomassie blue staining:

Gels were fixed for 1 h in 45:45:10 methanol : water : glacial acetic acid and then stained with coomassie blue R-250 (0.15% in 45% methanol, 45% water and 10% glacial acetic acid acid) for 1 h and destained with 5% methanol and 7.5% glacial acetic acid in water.

Two-dimensional gel electrophoresis:

Two-dimensional gel electrophoresis of ribosomal proteins was performed according to the method of Geyl *et al* (1981). Protein samples were taken in 6 M urea, 10 mM DTT and 10 mM Bis-Tris acetic acid. Basic fuchsin was used as a marker dye. 150 µg of ribosomal protein samples were loaded on to the first dimensional 4% polyacrylamide gels containing 0.0568 M Bis-Tris acetic acid (pH 5.0), 6 M urea, 6.5 mM EDTA and 0.1% bis acrylamide. 0.01 M Bis-Tris acetic acid (pH 4.0) was used as upper electrode buffer and 0.089 M potassium acetate (pH 5.0) was used as lower electrode buffer. The electrophoresis was done at 110 V for 5-6 h. After the run, the gels were removed and placed on top of two dimensional slab gels containing 18.6% polyacrylamide and 0.48% methylene bis acrylamide, 1% 5 N KOH and 6 M urea pH 4.5. Electrophoresis in the second dimension was performed, at 10 °C with 80 V, using electrode buffer containing 0.28 M Glycine (pH 4.5), until tracking dye reached the bottom of the gel for 18 h. After electrophoresis the gels were stained in 0.15% coomassie brilliant blue R-250 in 50% methanol

and 7.5% acetic acid. The gels were then destained in a destaining solution containing 5% methanol and 7.5% acetic acid.

In the case of acidic proteins (cold shock proteins), gel electrophoresis was carried out according to system I of Madjar *et al*, 1979. Protein samples were taken in 6 M urea, 10 mM DTT and 20 mM Bis-Tris acetic acid and 5 mM MES. Bromophenol blue was used as marker dye. 30 µg of ribosomal protein sample were loaded on to the first dimensional 4% polyacrylamide gels containing 0.0568 M Bis Tris-acetic acid (pH 5.0), 6 M urea, 6.5 mM EDTA and 0.1% bis acrylamide. 0.02 M Bis-Tris acetic acid and 5 mM MES (pH 7.0) was used as upper electrode buffer and 0.028 M Bis-Tris acetic acid (pH 6.0) was used as lower electrode buffer. The electrophoresis was done at 110 V for 5-6 h. acidic. Electrophoresis in the second dimension was performed according to Metz and Bogorad (1974). After the run, the gels were removed and placed on top of two dimensional slab gels containing 10% polyacrylamide and 0.5% methylene bis acrylamide and 0.14 M Bis-Tris acetic acid pH 6.75. Electrophoresis in the second dimension was performed at 10 °C with 50 V, using upper electrode buffer containing 0.07 M Bis-Tris acetic acid, 0.07 M MES, 0.2% SDS and 0.000015% thioglycolic acid and lower electrode buffer containing 0.028 M Bis-Tris acetic acid (pH 6.75), until tracking dye reached the bottom of the gel for 4 h. After electrophoresis the gels were stained in 0.15% coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid. The gels were then destained in a destaining solution containing 5% methanol and 7.5% acetic acid.

Isolation of rRNA from ribosomes:

Equal volumes of saturated phenol was added to ribosomes (75 A_{260} units/ml) in $TNM_{20}Me$ buffer and vigorously shaken for 30 min. The mixture was centrifuged at 10,000 rpm for 20 min. The upper aqueous layer was carefully taken out and the lower phenol phase was reextracted with equal volumes of buffer. Both the aqueous layers were pooled and the RNA was

precipitated with 1/10 volume of 20% sodium acetate pH 5.5 and 2 volumes of absolute alcohol. All the operations were carried out at 4 °C. It was left overnight at -20 °C. The precipitated RNA was pelleted by centrifugation at 12,000 rpm for 20 min at 4 °C. The RNA pellet was dissolved in 10 mM sodium acetate and stored frozen at -80 °C. In case of *E. coli* D10 (RNase I$^-$) $TKM_{10}Me$ buffer was used.

Agarose gel electrophoresis:

Ribosomal RNA was analyzed on 1% agarose gels. The electrophoresis was carried out in a buffer containing 89 mM Tris-Cl, 89 mM boric acid, 2.5 mM EDTA (pH 8.3) at 150 V. After electrophoresis the gels were stained in 300 mM sodium acetate containing 0.02% methylene blue. The gels were destained in streile double distilled water.

Structural studies of *Halomonas eurihalina* ribosomes

Sucrose density gradient centrifugation:

a. On 5-20% gradients:

Undialysed *H. eurihalina* logarithmic and stationary phase ribosomes (ribosomes obtained after pelleting from S-30 in 20 mM magnesium acetate buffer) were layered carefully layered on the top of 5-20% linear sucrose gradients prepared in ribosomal buffer and centrifuged for 1 h 30 min at 40,000 rpm in SW 50.1 rotor. The gradient was fractionated into 1 ml fractions. The absorbance of the fractions was measured at 260 nm and 280 nm after appropriate dilution with respective buffers. *E. coli* ribosomes were dialysed against $T_{20}K_{50}M_{0.2}Me_7$ buffer. Undialysed ribosomes (in 10 mM magnesium acetate buffer) layered on sucrose density gradients were taken as controls. 10 A_{260} units of ribosomes were layered on top of each gradient tube.

b. On 10-30% gradients:

Logarithmic and stationary phase ribosomes were dialyzed against different buffer conditions (20 mM Tris-Cl pH 7.6, 0.2 mM magnesium acetate, 7 mM 2-mercaptoethanol, 500 mM salt) each for one sample for 24 h. The different salts used were ammonium chloride, potassium chloride, potassium glutamate and sodium chloride. 10 A_{260} units of dialyzed ribosomes were layered carefully on the top of 10-30% linear sucrose gradients prepared in respective buffers and centrifuged for 1 h 30 min at 40,000 rpm in SW 50.1 rotor. Fractions were collected from top of the gradient and the absorbance of the fractions was measured at 260 nm and 280 nm after appropriate dilution with the same buffer. Undialysed ribosomes layered on sucrose density gradients were taken as controls.

Same procedure was carried out with undialyzed S-30 obtained from *H. eurihalina* logarithmic, stationary and *E. coli* cells. 10-30% gradients were used for *H. eurihaina* S-30 and 5-20% gradients were used for *E. coli* S-30.

Hydrophobic interaction chromatography:

Hydrophobic interaction chromatography was performed according to Krillov *et al* (1978). Logarithmic and stationary phase 70 S ribosomes of *H. eurihalina* were subjected to Sepharose-4B column chromatography separately after equilibration in buffer A (20 mM Tris-Cl (pH 7.6), 1.5 M $(NH_4)_2SO_4$, 0.2 mM magnesium acetate, and 7 mM 2-mercaptoethanol). Ribosomes (150 A_{260} units) dialyzed against same buffer were applied on 60 ml of Sepharose-4B column. The ribosomes were solubilized differentially by a reverse salt gradient elution with buffer A, buffer B (20 mM Tris-Cl, pH 7.6, 0.05 M $(NH_4)_2SO_4$, 0.2 mM magnesium acetate, 7 mM 2-mercaptoethanol), respectively at the flow rate of 15-20 ml/h. The column was eluted with 130 ml gradient buffer. The absorbance of the fractions was measured at 260 nm. The

fractions corresponding to different peaks were pooled separately and stored at -80 °C. The proteins in the fractions were analyzed by SDS-PAGE.

RNase A degradation studies of *H. eurihalina* ribosomes:

The degradation of *H. eurihalina* logarithmic and stationary phase ribosomes by RNase A was carried out in a 1 ml spectrophotometer cuvette containing 20 mM Tris-Cl pH 7.6, magnesium acetate (concentration as indicated in figures), 250 mM ammonium chloride (salt and concentrations varied as indicated in figures) and 1 A_{260} unit of ribosomes. In case of *E. coli*, 10 mM Tris-Cl pH 7.6, magnesium acetate (as indicated in figures) 50 mM potassium chloride and 1 A_{260} unit of ribosomes were used. Concentration as indicated in figures (for *H. eurihalina* logarithmic and stationary phase ribosomes) or 0.3 μg (for *E. coli* ribosomes) of RNase A was used in each case. Reaction mixture without the enzyme was used to adjust the initial absorbance of the reaction mixture to zero. Absorbance of the mixture was measured continuously until degradation reaches a plateau. The assay was performed at room temperature (25 °C). The percent increase was calculated on the basis of the initial A_{260} reading of the reaction mixture.

Effect of different salts and their concentrations on RNase A sensitivity of *H. eurihalina* ribosomes:

Ribosomes isolated from both logarithmic and stationary phase cells of *H. eurihalina* were dialyzed against the buffer containing 20 mM Tris-Cl pH 7.6, 0.1 mM magnesium acetate, 7 mM 2-mercaptoethanol and varying concentration of salt (as indicated in figures). Different concentrations of RNase A as indicated in figures were added. The salts used were ammonium chloride, potassium chloride, potassium glutamate and sodium chloride.

Treatment of ribosomes with spermine or spermidine:

Treatment of ribosomes (logarithmic and stationary phases) was carried out as described by Weiss and Morris (1973) with some modifications. *E. coli* ribosomes (70 A_{260}/ml) were dialyzed against the buffer containing 10 mM Tris-Cl pH 7.6, 0.1 mM magnesium acetate, 60 mM potassium chloride, 7 mM 2-mercaptoethanol and 0.5 mM spermine or 1 mM spermidine overnight. *H. eurihalina* logarithmic and stationary phase ribosomes were dialysed against a buffer containing 20 mM Tris-Cl pH 7.6, 20 mM magnesium acetate, 250 mM ammonium chloride and 7 mM 2-mercaptoethanol. Samples of ribosomal preparations dialyzed against the respective buffers but without spermine or spermidine were taken as respective controls.

Treatment of ribosomes with Ethidium bromide:

Treatment of ribosomes with Ethidium bromide was carried out according to Stevens and Pascoe (1972) with some modifications. 1.5 mM ethidium bromide was added to the *E. coli* ribosomes in a buffer containing 10 mM Tris-Cl pH 7.6, 0.1 mM magnesium acetate, 300 mM potassium chloride and 7 mM 2-mercaptoethanol and the solution was incubated at 37 ºC for 1 h. For *H. eurihalina* logarithmic and stationary phase ribosomes a buffer containing 20 mM Tris-Cl pH 7.6, 0.1 mM magnesium acetate, 300 mM ammonium chloride and 7 mM 2-mercaptoethanol was used. Excess amount of EtBr was removed by dialyzing the treated ribosomes against respective buffers without ethidium bromide. Samples of ribosomal preparation dialyzed against the same buffers but without EtBr were taken as controls.

Ribosome unfolding studies with urea:

Ribosomes of *H. eurihalina* (logarithmic and stationary phases) were incubated in $TNM_{20}Me$ buffer containing 2 M, 4 M, 6 M and 8 M urea for 15 min at room temperature. Absorption spectra of the treated ribosomes were

recorded from 220 to 300 nm. Absorption spectra of ribosomes incubated in $TNM_{20}Me$ was taken as control. In case of *E. coli*, ribosomes were incubated in $TKM_{10}Me$ buffer containing 2 M, 4 M, 6 M and 8 M urea for 15 min at room temperature for obtaining the spectra. Ribosomes incubated in $TKM_{10}Me$ buffer were used for recording *E. coli* control absorption spectrum.

Light scattering studies:

Effect of magnesium ion concentration on ribosomal dissociation:

Ribosomal subunit dissociation studies were carried according to Zitomer and Flaks (1972) with few modifications, in Jasco-777 spectrofluorimeter. The excitation and emission band widths were set to 5 nm. The excitation and emission wavelengths were set at 400 nm. The intensity of the light scattered by the particles was read at an angle of 90 degrees. All measurements were made at room temperature. A total volume of 1 ml sample was placed in cuvette. Each experiment was carried out for more than three times in spectrofluorimetric studies.

To determine the dissociation of 70 S logarithmic or 70 S stationary phase ribosomes, ribosomes from a stock (250-300 A_{260}/ml) were diluted into 1 ml of buffer containing 20 mM Tris-Cl pH 7.6, 250 mM ammonium chloride, 7 mM 2-mercaptoethanol and the final concentration of magnesium varied from 30 mM to 1 mM.

Final concentration of ribosomes in each reaction mixture was 2 A_{260}/ml, 1.6 A_{260}/ml for logarithmic and stationary phase ribosomes respectively. The reaction mixtures were allowed to equilibrate for 10 min at room temperature and relative light scattering was determined. For *E. coli* ribosomes the buffer contained 20 mM Tris-Cl pH 7.6, 30 mM ammonium chloride and 7 mM 2-mercaptoethanol and magnesium concentration as mentioned above. Final concentration of *E. coli* ribosomes in each reaction

mixture was 4 A_{260}/ml. A graph was plotted against the relative scattering and magnesium concentration.

Purification of S1 protein:

S1 protein from *H. eurihalina* has been purified by taking advantage of its property of strong binding to poly pyrimidines. This involves affinity chromatography of ribosomes on poly (U)-sepharose column. 300 A_{260} units of *Halomonas eurihalina* ribosomes were passed through a 5 ml poly (U)-sepharose column equilibrated with 20 mM Tris-Cl pH 7.6, 250 mM ammonium chloride, 20 mM magnesium acetate, 7 mM 2-mercaptoethanol. The column was washed with 20 ml of buffer C (20 mM Tris-Cl pH 7.6, 1 M ammonium chloride, 20 mM magnesium acetate and 7 mM 2-mercaptoethanol) to recover ribosomes devoid of S1. S1 bound to the column was eluted with 15 ml of buffer D (buffer C + 7 M urea). Absorbance of the fractions was measured at 280 nm. Fractions containing S1 were pooled and stored at -80 °C. Molecular weight of the S1 protein was determined by SDS-PAGE analysis.

Coupling of poly (U) to activated cellulose:

Poly (U) was coupled to activated cellulose according to Carmichael (1975). For each column, 2 grams of ethanol-washed whatman CF-11 cellulose were mixed with 20 mg of RNA, in 20 ml of solution containing 7 ml of 100% ethanol and 18 ml of buffer (0.05 M Tris-Cl, pH 6.85, 0.1 M NaCl and 0.001 M EDTA) and allowed to stand at room temperature for 1 h. The slurry was then diluted with 500 ml of cold (-20 ºC) ethanol and filtered through Whatman no.1 filter paper using Buchner funnel. The powder was allowed to dry at room temperature and then suspended in 25 ml of 100% ethanol containing 0.5 ml of 1 M magnesium acetate. This suspension, in an uncovered petridish, was irradiated for 20 min at a distance of 10 cm from two general electric 15-watt germicidal bulbs and then diluted with 250 ml of cold ethanol and collected by filtration as before. The poly (U)-cellulose was stored until use as a dry powder

below 4 ºC. Poly (U)-cellulose (2 g) was swollen in $TNM_{20}Me$ buffer at 4 ºC and packed into a small column (1.2 × 6 cm). The column was washed with the same buffer until the A_{260} of the effluent was less than 0.05. In general, about 10 mg of protein may be bound to a column to which 10 mg of RNA have been coupled.

Poly (U)-cellulose column chromatography of S-100:

S-100 was loaded on poly (U)-cellulose column that was previously equilibrated with $TNM_{20}Me$ buffer. The column was eluted with a buffer containing 20 mM Tris-Cl pH 7.6, 20 mM magnesium acetate, 1 M ammonium chloride and 7 mM 2-mercaptoethanol (buffer C). Then the column was eluted with buffer E (buffer C + 8 M urea). Absorbance of the fractions was recorded at 280 nm. Peak fractions were analyzed by SDS-PAGE, pooled and stored at -80 ºC.

Proteomic analyses: in-gel digestion and mass spectrometry (MS)

In-gel digestion and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis was conducted with a MALDI-TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Bremen, Germany) according to the method described by Shevchenko et al. (1996) with slight modifications. Coomassie-stained protein spots were manually excised from three reproducible gels. The excised gel pieces were destained with 100 µL of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for five times. Thereafter, the gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56 ºC for 1 h. This is followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2 ºC), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac concentrator and rehydrated in 20 µL of 25 mM NH_4HCO_3 solution containing 12.5 ng μL^{-1} trypsin (sequencing grade, Promega, Wisconsin, USA). The above mixture was incubated on ice

for 10 min and kept overnight for digestion at 37 ºC. After digestion, a short spin for 10 min was given and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 µL of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled together and dried using speed vac and were reconstituted in 5 µL of 1:1 ACN and 1% TFA. 2 µL of the above sample was mixed with 2 µL of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µL was spotted on target plate.

Protein identification: peptide mass fingerprinting and MS/MS analysis

Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (www.matrixscience.com) employing Biotools software (Bruker Daltonics).The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss-Prot database. The taxonomic categories searched were eubacteria, archaea (archaeobacteria) and eukaryotes. The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT probability analysis (P\0.05), only significant hits were accepted for protein identification.

Computational analyses:

BLAST (National Center for Biotechnology Information) was used for homologous sequence searches. Among the first 100 hits, 10 hits were chosen. Even though several species of same genera showed homology to the search sequence, only one species was chosen as representative for a genus, thus encompassing the widest possible taxonomic and habitat ranges. FASTA format sequences were obtained for the select hits by using BLAST (National Center for Biotechnology Information). ClustalX2 (www.clustal.org) was used to generate multiple sequence alignments.

Growth of *Halomonas eurihalina*:

H. eurihalina was grown as described in the methods. Fig: 1 shows the growth curve of *H. eurihalina*. It can be observed that *H. eurihalina* had a generation time of 2 h and entered stationary phase after 16 h of growth. For our studies cultures were grown for 12 h and 24 h representing mid logarithmic phase and stationary phase cells respectively. Cell pellets were stored frozen at -80º C.

Isolation of ribosomes from *H. eurihalina*:

Ribosomes were isolated as described in methods. In case of *E. coli* ribosomes, the non-ribosomal proteins bound to ribosomes were usually removed by incubating crude ribosomes in a buffer containing 1 M ammonium chloride (1 M NH_4Cl washing) overnight at 4º C clarified by low speed centrifugation at 15,000g for 30 min and pelleted at 100,000g for 4h. The same method could not be used in case of *H. eurihalina* ribosomes as ammonium chloride wash resulted in abnormal electrophoretic mobility pattern of the ribosomal proteins. Fig: 4A & B shows the two-dimensional gel electrophoretic analysis of ribosomal proteins extracted from crude ribosomes and 1 M ammonium chloride washed ribosomes. The 1 M NH_4Cl washed ribosomes had fewer number of ribosomal proteins which appeared as streaks instead of spots.

These results indicate the possibility of strong association of chloride ions to the ribosomal proteins of *H. eurihalina* (logarithmic and stationary phases) and thus affecting their electrophoretic mobility pattern.

Ultra centrifugation of ribosomes on 10% sucrose cushion:

Purification of ribosomes, to release loosely associated non ribosomal proteins was achieved by ultra centrifugation of ribosomes on 10% sucrose

cushions (both *E. coli* and *H. eurihalina* ribosomes in respective buffers). The purity of the ribosomes was checked by A_{260} to A_{280} ratio and by electrophoresis.

SDS-PAGE analysis of ribosomes and extracted ribosomal proteins from *H. eurihalina*:

The ribosomes thus obtained were analyzed by SDS-PAGE as described in methods. Proteins were extracted from the ribosomes by acetic acid extraction as described in the methods. Several high molecular weight proteins were removed by following this procedure. Electrophoretic mobility pattern of ribosomes and ribosomal proteins from *H. eurihalina* were compared with electrophoretic mobility pattern of ribosomes and ribosomal proteins of *E. coli* (Fig: 2A and 2B). SDS gel pattern indicated that almost all the proteins were extracted from the ribosomes by acetic acid extraction method.

Agarose gel electrophoresis of rRNA from *H. eurihalina*:

Ribosomal RNA was extracted from the ribosomes of *E. coli* D10 (RNase I^-), logarithmic and stationary phase ribosomes of *H. eurihalina* as described in the methods and agarose gel electrophoresis was performed. Ribosomal RNA extracted from both the species showed similar RNA profile (Fig: 3). These results indicate that rRNA species of *H. eurihalina* correspond to 23 S, 16 S and 5 S rRNA as in *E. coli.*

Comparative analysis of mesophilic and moderately halophilic ribosomal proteins by two- dimensional gel electrophoresis:

The ribosomal proteins extracted from sucrose cushion purified *E. coli* and *H. eurihalina* ribosomes were analyzed by two- dimensional gel electrophoresis (Fig 5A & B). Electrophoretic mobility pattern indicates that the majority of moderately halophilic ribosomal proteins are basic, as in the

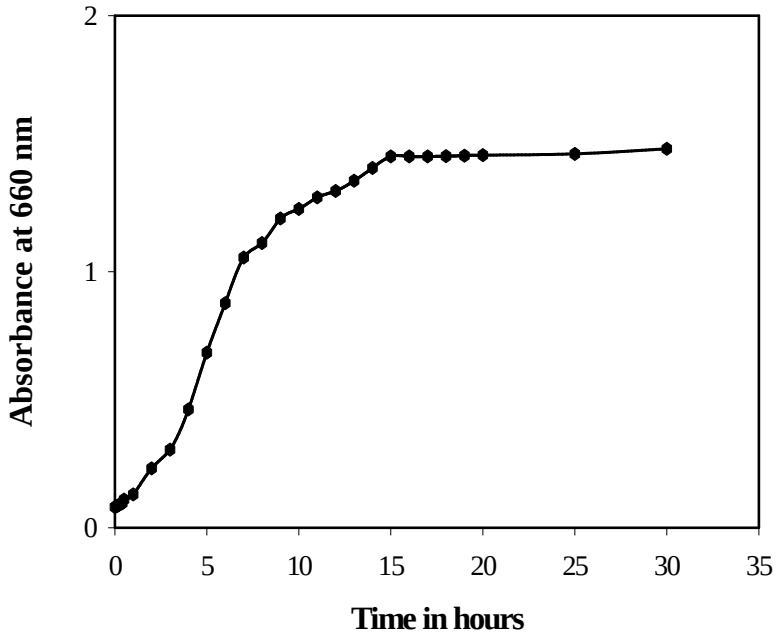


Fig: 1. Growth curve of *H. eurihalina* DSM 5720

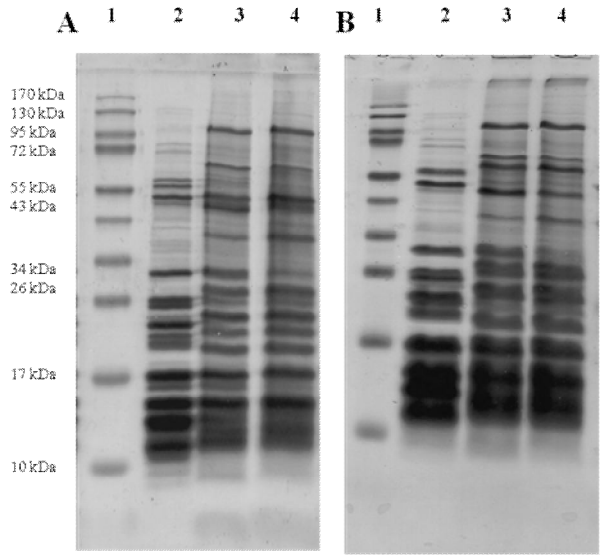


Fig: 2. Gel electrophoretic analysis of ribosomes and ribosomal proteins. A. SDS-PAGE analysis of ribosomes. B. SDS-PAGE analysis of proteins extracted from ribosomes. Lane 1: Molecular weight marker, lane 2: *E. coli*, lane 3: *H. eurihalina* logarithmic phase, lane 4: *H. eurihalina* stationary phase.

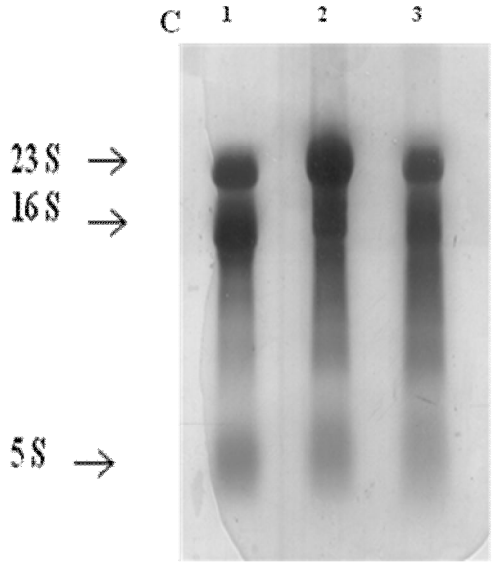


Fig: 3. Agarose gel electrophoresis of rRNA. Lane 1: *E. coli* rRNA, lane 2: *H. eurihalina* logarithmic phase rRNA, lane 3: *H. eurihalina* stationary phase rRNA.

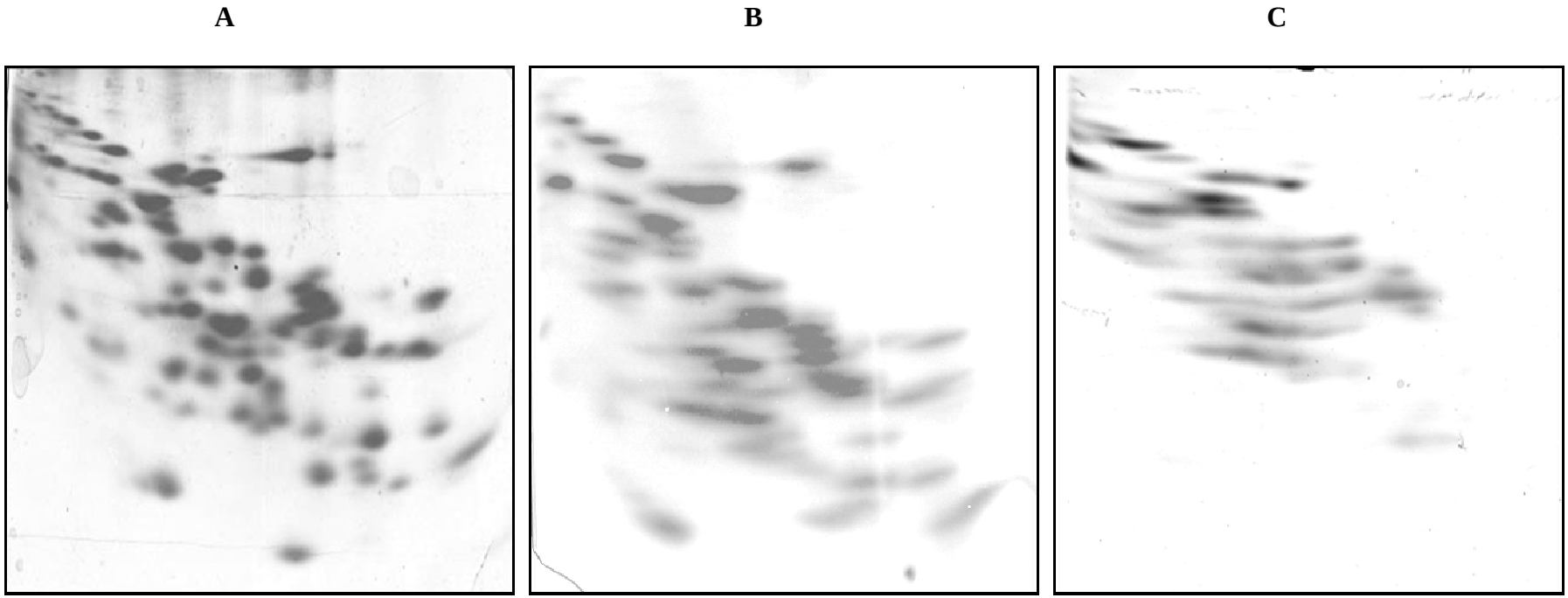


Fig: 4. Two-dimensional gel electrophoresis of *H. eurihalina* ribosomal proteins. A. Crude ribosomal proteins. B. 1M ammonium chloride washed ribosomal proteins. C. Extracted ribosomal proteins treated with 1 M ammonium chloride and dialyzed against 6 M urea.

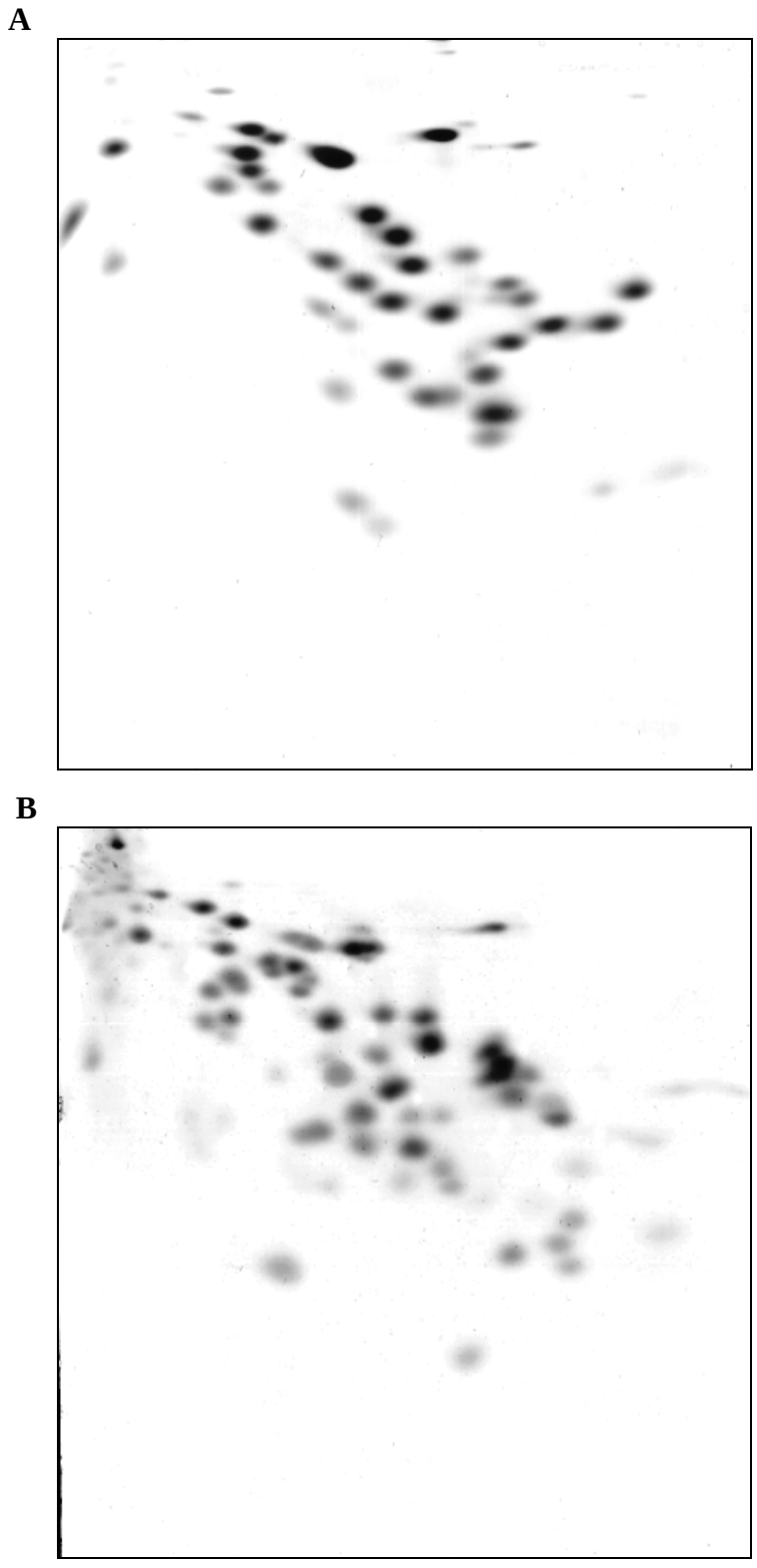


Fig: 5. Two-dimensional gel electrophoresis of ribosomal proteins extracted from sucrose cushion purified ribosomes. A. *E. coli* ribosomal proteins. B. *H. eurihalina* ribosomal proteins.

case of *E. coli*. The results also show that the nature of ribosomal proteins of moderate halophile *H. eurihalina* is unlike ribosomal proteins from extremely halophilic archaea. Number of spots observed in two- dimensional gel of *H. eurihalina* ribosomal proteins was more, indicating the possibility of presence of more number of ribosomal proteins in *H. eurihalina*, as compared to *E. coli*. Some of the proteins may also correspond to non ribosomal proteins tightly associated with ribosomes such as translation factors.

Comparative analysis of logarithmic and stationary phase ribosomal proteins of *H. eurihalina*:

Growth phase dependent variation in ribosomal proteins was studied by extracting ribosomal proteins from ribosomes isolated from equal amounts of logarithmic and stationary phase cell pellets. The ribosomal proteins extracted from sucrose cushion purified ribosomes of both logarithmic and stationary phase bacteria grown under moderately halophilic conditions were compared in terms of SDS-PAGE and also by two-dimensional gel electrophoresis (Fig: 6A & B).

The results showed that some of the spots were missing in ribosomal protein profile of stationary phase ribosomes. Some of the spots showed substantial decrease in concentration where as some spots showed increase in protein concentration in stationary phase gels in comparison to logarithmic phase cells. Some of these ribosomal proteins were identified (see Chapter 3).

Growth of *H. eurihalina* in media containing increasing NaCl concentrations:

Although *H. eurihalina* is a moderate halophile, it has been reported that the organism can grow in the presence of wide range of NaCl concentration including extreme halophilic conditions. Experiments were carried out to observe any variation in ribosomal protein composition in *H. eurihalina* grow at moderate and extreme halophilic conditions.

H. eurihalina was grown in media containing varying concentrations of sodium chloride viz., 5%, 6%, 10%, 15% and 20% and growth curves were presented in Fig: 7.

The results show that growth rate was not affected up to 10% NaCl concentration. Decline in growth rate was observed in 15% NaCl concentration. In 20% NaCl containing media, very long lag phase up to 20 hours was observed before logarithmic phase and the stationary phase was reached after 40 hours.

Comparison of ribosomal proteins isolated from *H. eurihalina* grown in moderate and extreme halophilic conditions:

The ribosomal proteins extracted from logarithmic phase ribosomes isolated from bacteria grown under moderate halophilic conditions (6% NaCl containing media) were compared with ribosomal proteins extracted from bacteria grown under extreme halophilic conditions (20% NaCl containing media) both by SDS-PAGE (Fig: 8) and two-dimensional gel electrophoresis (Fig: 9A & B).

The results show that the electrophoretic profile of ribosomal proteins is similar whether the cells were grown in moderately halophilic conditions or extremely halophilic conditions. These results also show that ribosomal proteins of moderate halophiles were basic in nature even under extremely halophilic conditions of growth unlike extremely halophilic archaea.

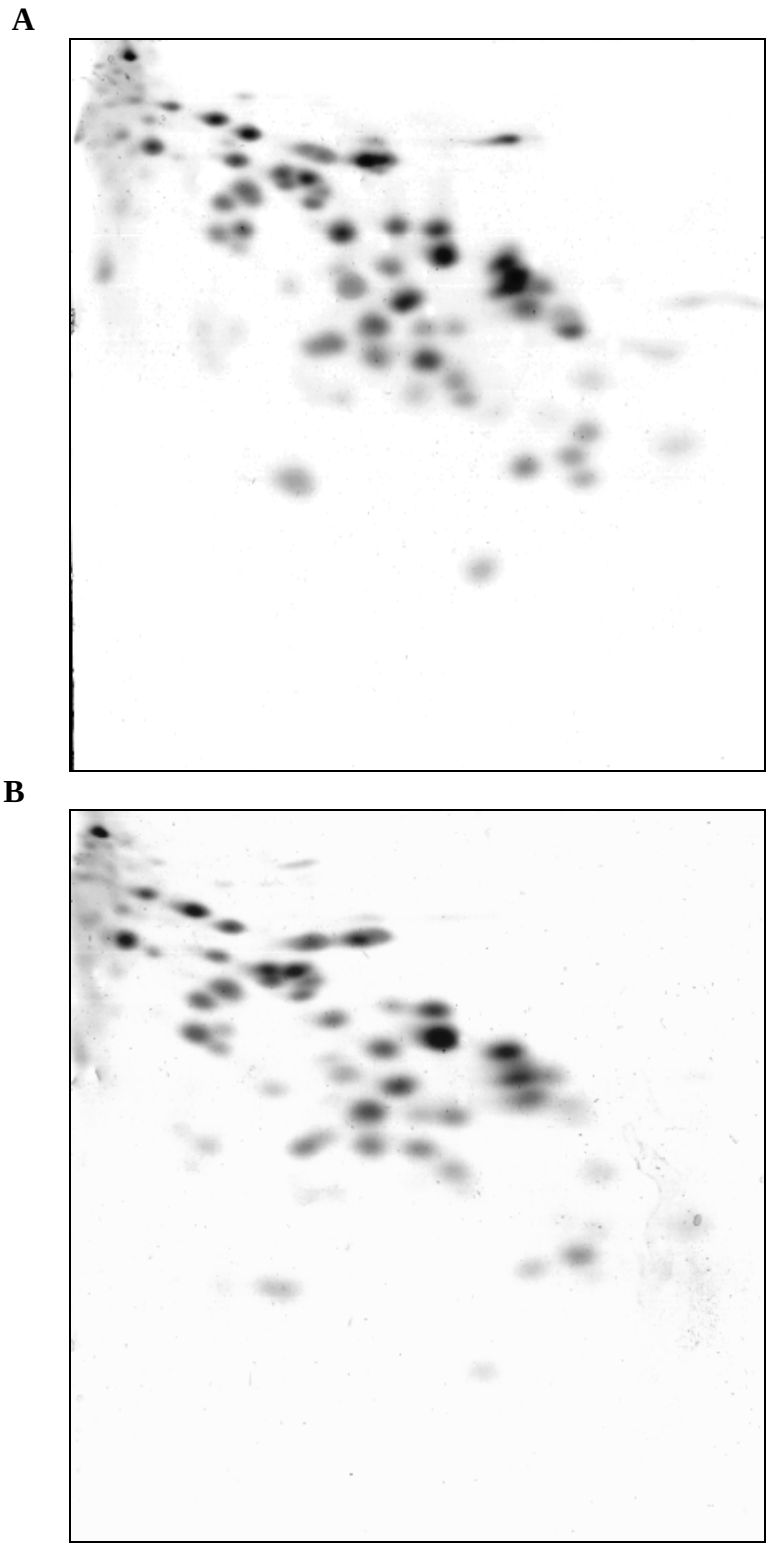


Fig: 6. Two-dimensional gel electrophoresis of ribosomal proteins extracted from sucrose cushion purified ribosomes. A. *H. eurihalina* logarithmic phase. B. *H. eurihalina* stationary phase.

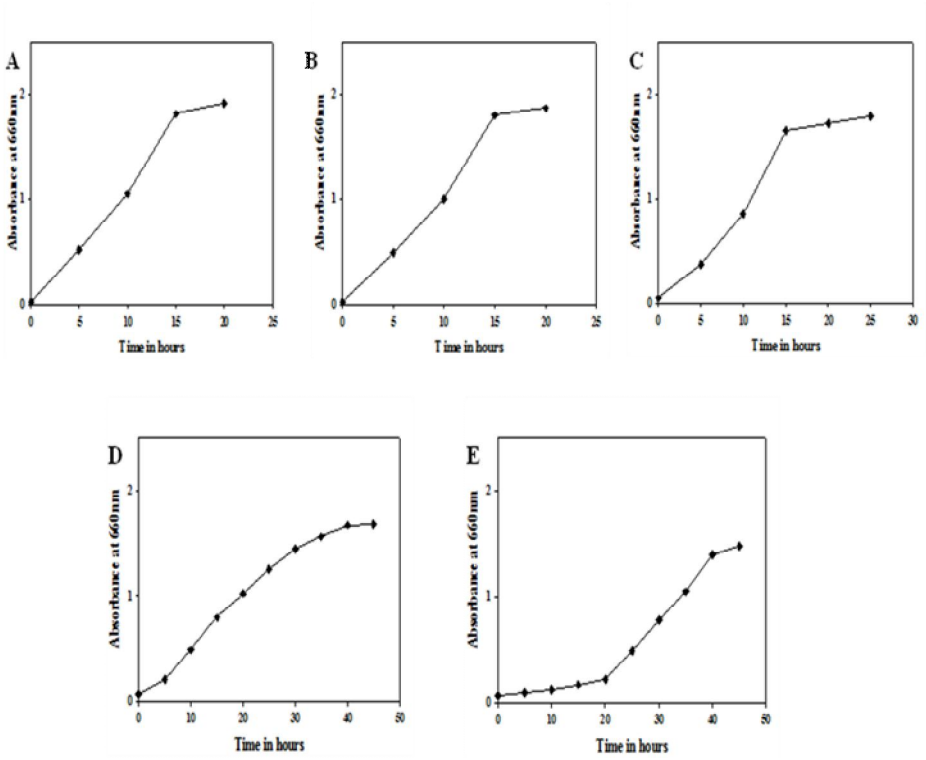


Fig: 7. Growth curves of *Halomonas eurihalina* DSM 5720 grown in different NaCl concentrations. A. 5%, B. 6%, C. 10%, D. 15% and E. 20% NaCl.

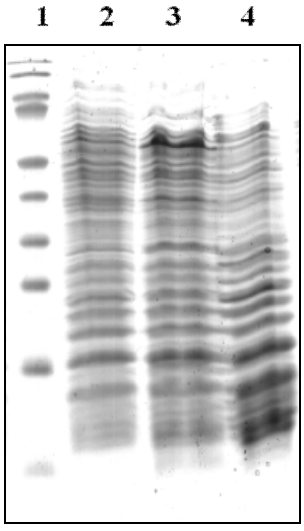


Fig: 8. SDS-PAGE analysis of *H. eurihalina* ribosomal proteins. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosomal proteins, lane 3: stationary phase ribosomal proteins, lane 4: ribosomal proteins extracted from bacteria grown in 20% NaCl containing media.

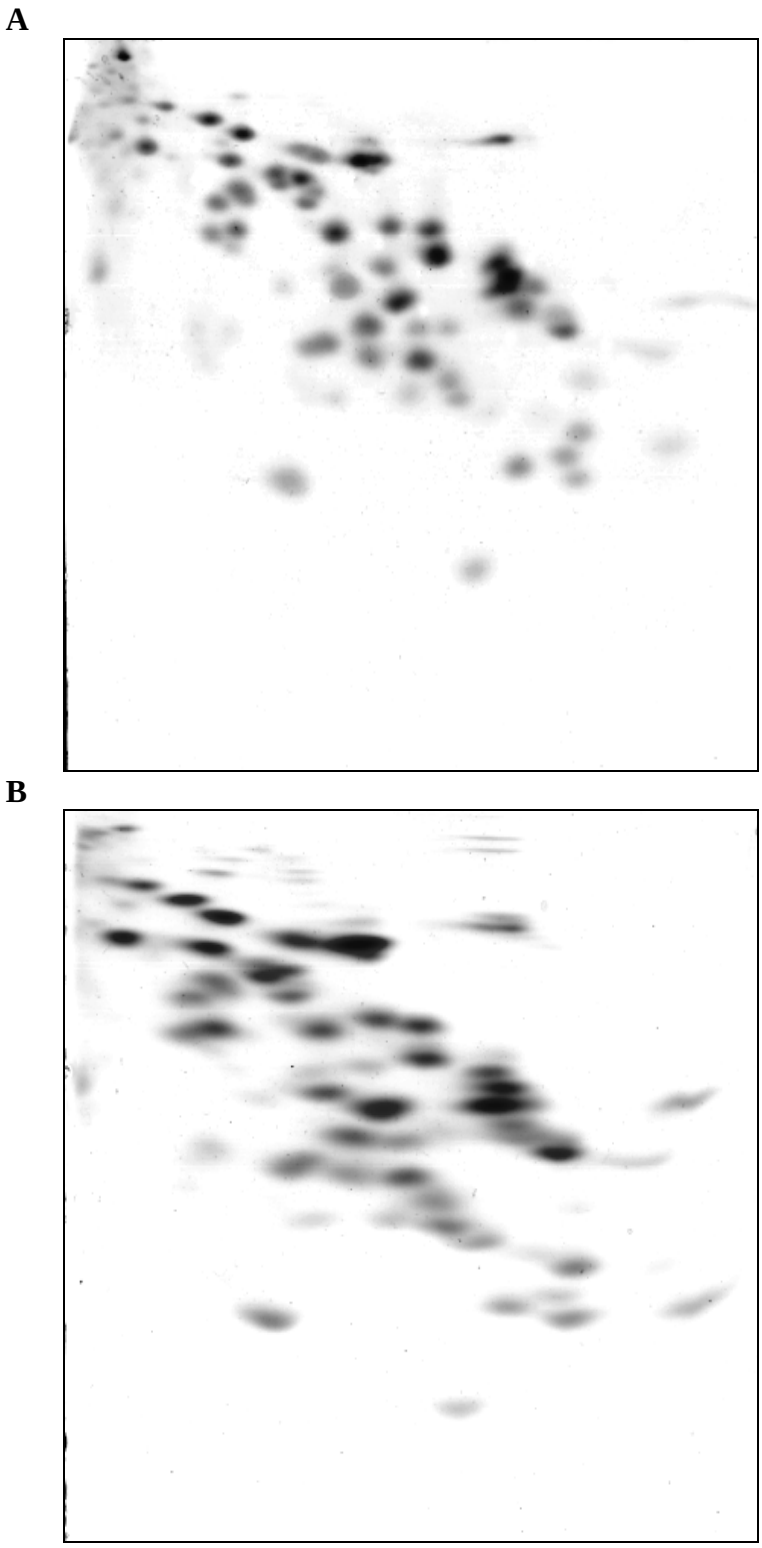


Fig: 9. Two-dimensional gel electrophoresis of *H. eurihalina* ribosomal proteins extracted from sucrose cushion purified ribosomes. A. Moderately halophilic conditions. B. Extremely halophilic conditions.

Structural studies on halophilic ribosomes

Ribosome is a complex of rRNA and proteins whose flexible conformation is formed and stabilized by mutual interactions among its components, which is influenced by specific monovalent and bivalent cations. Thus, certain changes in the ionic environment which do not change the overall structural properties of the ribosome do nevertheless change something in the conformation of the component macromolecules, which may have drastic effects on structure of the ribosome leading to variation in its functional activity. It is further known that magnesium ions play central role in stabilizing the structure of ribosome and optimum concentration of magnesium ion is required to maintain the subunits in associated monomer form, structural conformation and for the functional activity of the ribosome.

Structural studies of halophilic ribosomes were carried out by two different sets of experiments. First set of experiments were carried out to determine the ionic (magnesium ion) concentration required for the dissociation of ribosomes into subunits. Second set of experiments include studies on structural changes in ribosomes induced by agents like salts, polyamines and intercalating agents using RNase A as a probe.

Magnesium ion dependent subunit dissociation

In order to observe the effect of reduced magnesium ion concentration on *H. eurihalina* ribosomes, sucrose density gradient centrifugation studies, hydrophobic interaction chromatography studies and light scattering studies were carried out.

i. Sucrose density gradient centrifugation:

Attempts were made to separate ribosomal subunits by sucrose density gradient centrifugation, the most widely and generally used method for separation of subunits. In spite of applying different conditions, none of them resulted in dissociation of subunits.

a. On 5-20% gradients:

Centrifugation was carried out as described in methods. The sucrose density gradient centrifugation profiles of dialyzed (0.2 mM magnesium acetate) and undialyzed (10 mM magnesium acetate) *E. coli* ribosomes were shown in Fig: 10A & B. It can be observed that ribosomes dialyzed against low magnesium ion concentration resulted in dissociation of ribosomes represented by two peaks (Fig: 10A), in comparison to undialyzed ribosomes (Fig: 10B). The sucrose density gradient profiles of undialyzed (20 mM magnesium acetate) *H. eurihalina* ribosomes were given in Fig: 10C & D. *H. eurihalina* logarithmic phase ribosomes sedimented faster in comparison to stationary phase and *E. coli* ribosomes. These results show that *H. eurihalina* logarithmic phase ribosomes are more compact in comparison to *E. coli* ribosomes. *H. eurihalina* stationary phase ribosomes are less compact than logarithmic phase ribosomes and sedimentation pattern is similar to *E. coli* ribosomes on sucrose density gradients. Thus 10-30% gradients were used for sucrose density gradient studies of *H. eurihalina* ribosomes.

b. On 10-30% gradients:

Sucrose density gradient profiles of undialyzed (20 mM magnesium aetate) logarithmic and stationary phase ribosomes and respective SDS-PAGE analysis representing 70 S ribosomes were shown in Fig: 11A, B & C. Sucrose density gradient profiles and respective SDS-PAGE analysis of logarithmic and stationary phase ribosomes dialyzed against low magnesium ion concentration (0.2 mM) and salt (as indicated in figures) as mentioned in methods were given

in Fig: 12, 13, 14 & 15 (A, B & C). High salt concentration was used as it was known to favor subunit dissociation. It can be observed that in presence of salts 70 S ribosomal particles are stripped off of some proteins and hence showed lesser sedimentation rate compared to undialyzed 70 S ribosomes. Significant decrease in sedimentation rate was observed in presence of potassium glutamate in comparison with normal conditions indicating that more number of proteins was stripped off in presence of potassium glutamate followed by sodium chloride and ammonium chloride. In presence of potassium chloride much decrease in sedimentation rate was not observed. In spite of using several conditions the ribosome failed to dissociate as analyzed by sucrose density gradient ultracentrifugation.

As the ribosomes did not dissociate in presence of low magnesium concentration, sucrose density gradient centrifugation of S-30 was carried out to observe the presence of native ribosomal subunits in *H. eurihalina* extracts. The sucrose density gradient profile of undialyzed *E. coli* S-30 showed the presence of 30 S, 50 S native ribosomal subunits along with 70 S ribosomes in the cell lysate (Fig: 17). Sucrose density gradient profiles of undialyzed logarithmic and stationary phase S-30 and their respective SDS-PAGE analysis were given in Fig: 16A, B & C. Undialyzed S-30 sucrose density gradient profiles showed the absence of 30 S and 50 S native ribosomal subunits with only the presence of 70 S ribosome peaks in both logarithmic and stationary phase cell lysates. It is possible that in *H. eurihalina,* ribosomes may be existing completely in undissociated 70 S forms in the cell lysates.

H. eurihalina logarithmic and stationary phase ribosomes failed to dissociate even under very low magnesium ion concentration viz., 0.2 mM. These results show that ribosomal subunit dissociation is magnesium concentration independent in the case of *H. eurihalina* and that the subunits are held together tightly.

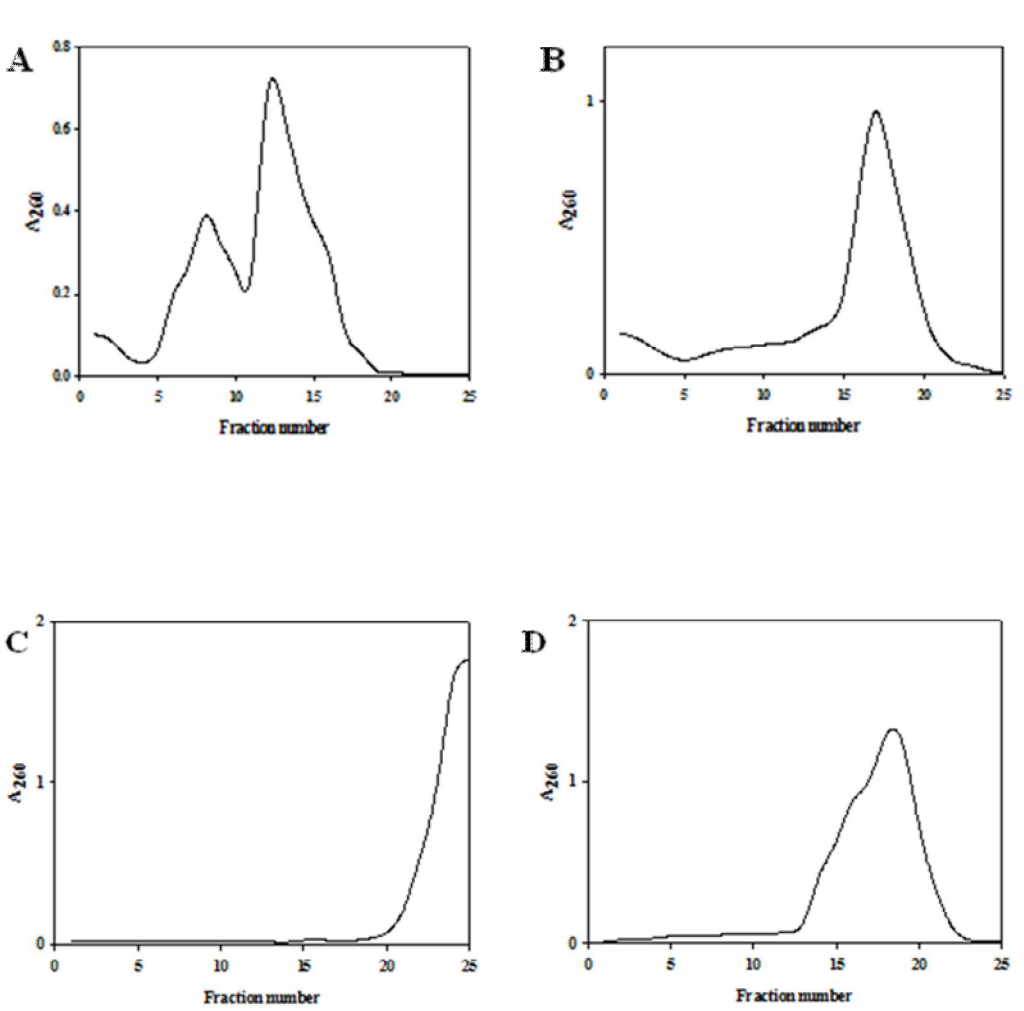


Fig: 10. Sucrose density gradient profiles of ribosomes on 5-10% gradients. A. Dialyzed *E. coli* ribosomes showing subunit dissociation. B. Undialyzed *E. coli* ribosomes. C. Undialyzed *H. eurihalina* logarithmic phase ribosomes. D. Undialyzed *H. eurihalina* stationary phase ribosomes.

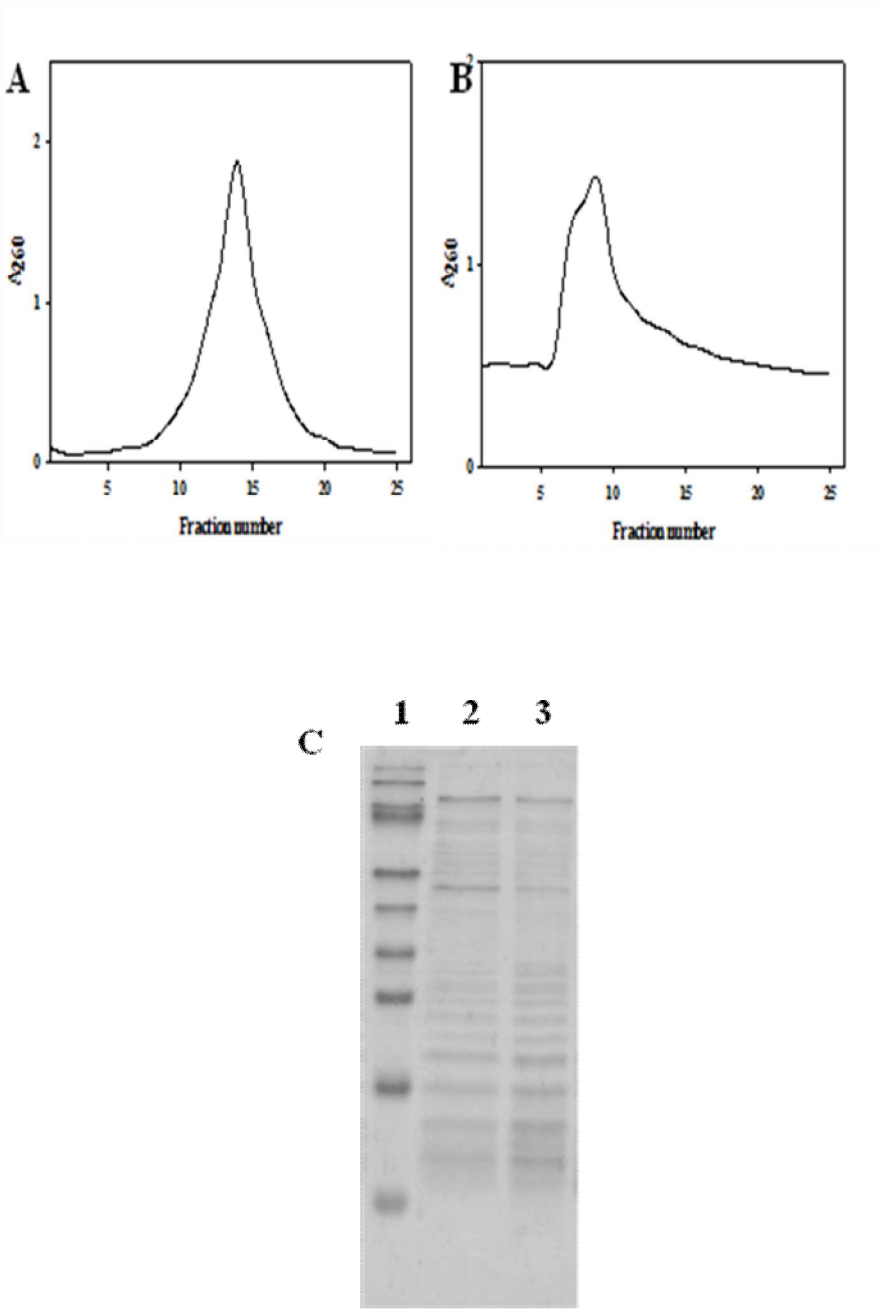


Fig: 11. Sucrose density gradient profiles of undialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2-3: Logarithmic phase ribosomes, lane 4-5: Stationary phase ribosomes.

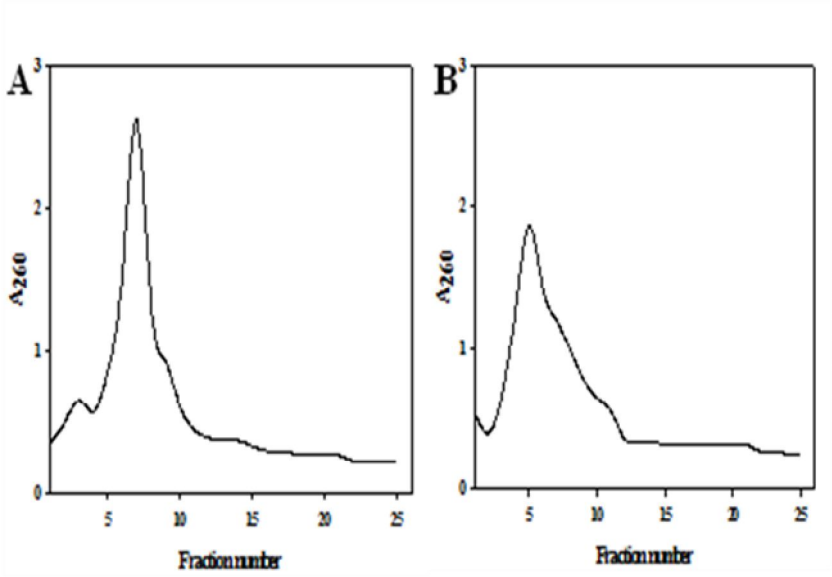


Fig: 12. Sucrose density gradient profiles of 500 mM ammonium chloride dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2 and lane 3: logarithmic phase ribosomes, lane 4 and lane 5: Stationary phase ribosomes.

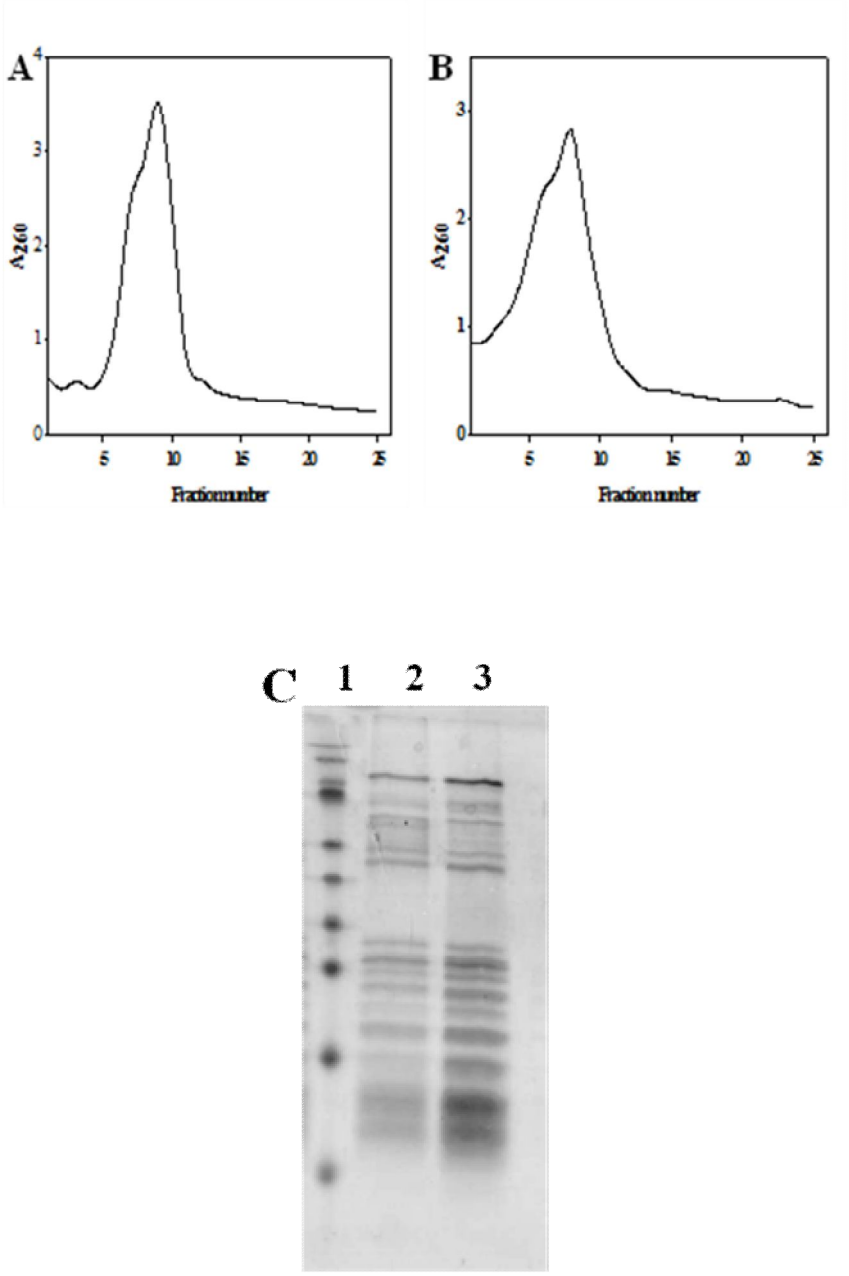


Fig: 13. Sucrose density gradient profiles of 500 mM potassium chloride dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosome and lane 3: Stationary phase ribosomes.

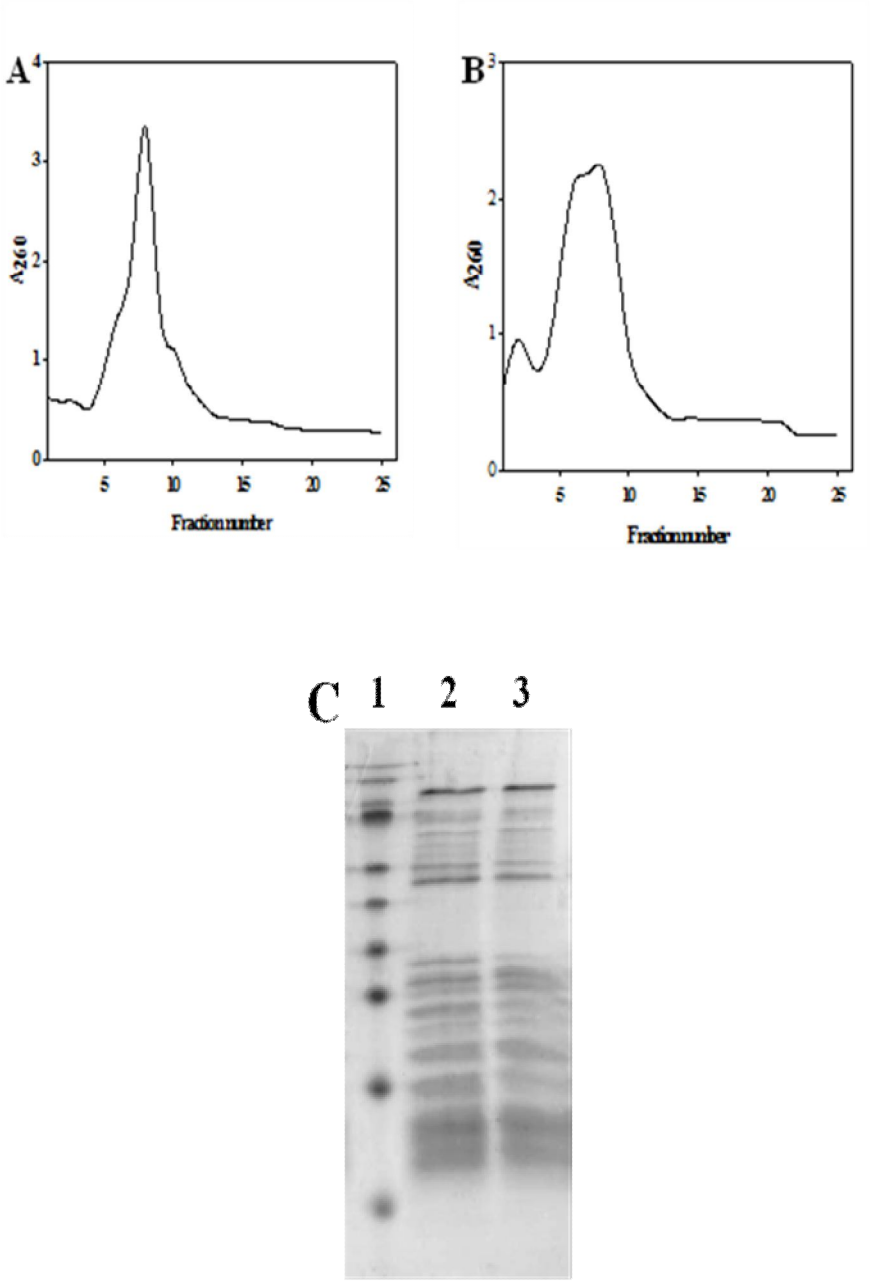


Fig: 14. Sucrose density gradient profiles of 500 mM sodium chloride dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosomes and lane 3: Stationary phase ribosomes.

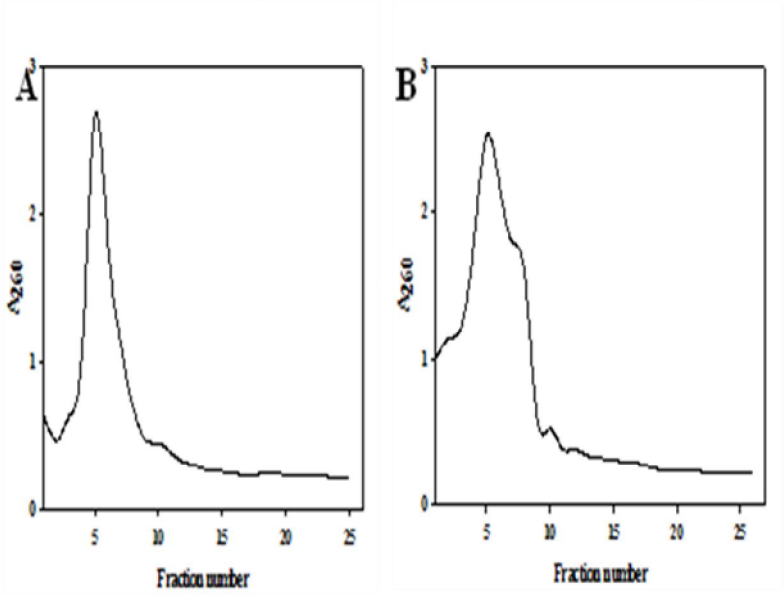


Fig: 15. Sucrose density gradient profiles of 500 mM potassium glutamate dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosomes and lane 3: Stationary phase ribosomes.

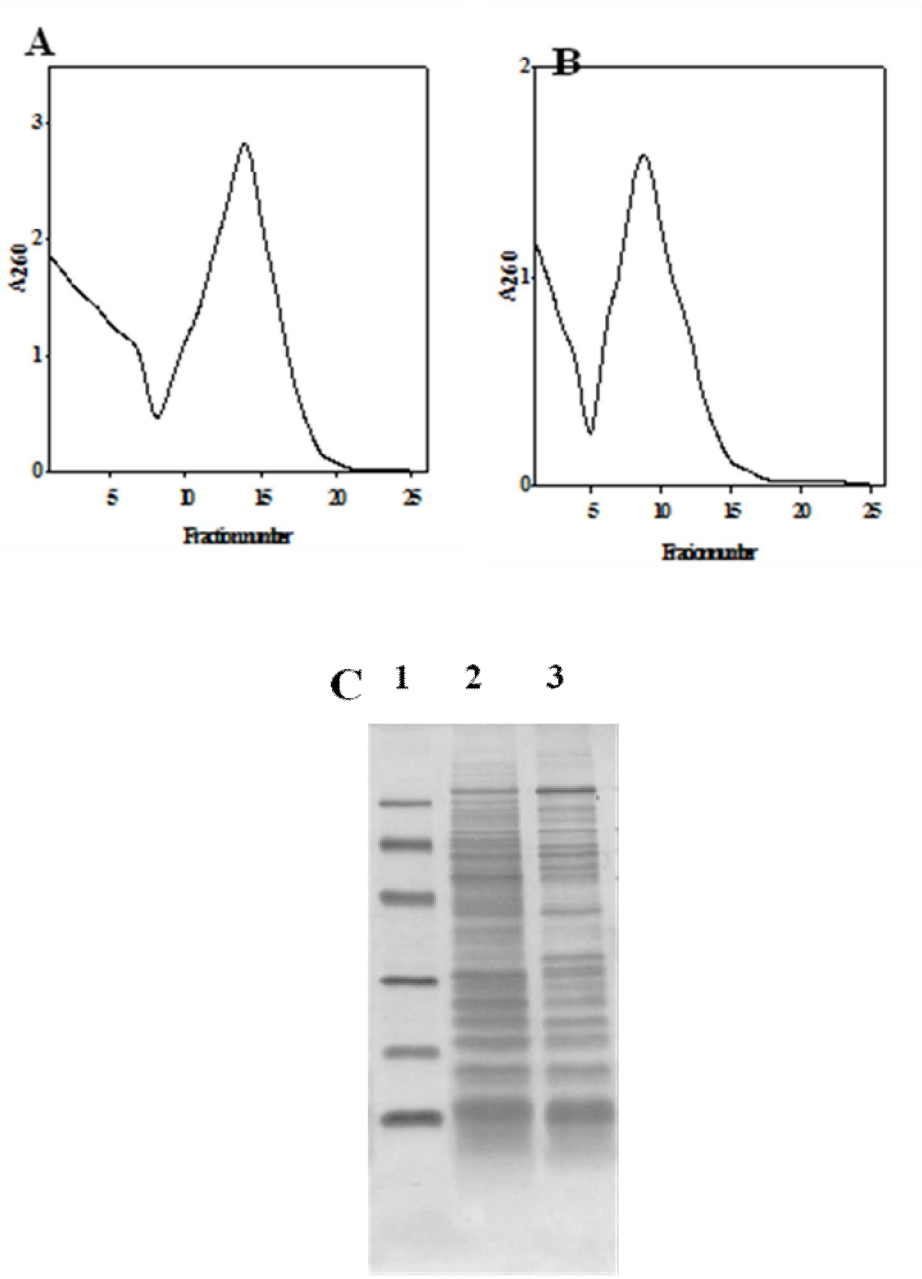


Fig: 16. Sucrose density gradient profiles of undialyzed *H. eurihalina* S-30 on 10-30% gradients. A. Logarithmic phase S-30. B. Stationary phase S-30. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase S-30 and lane 3: Stationary phase S-30.

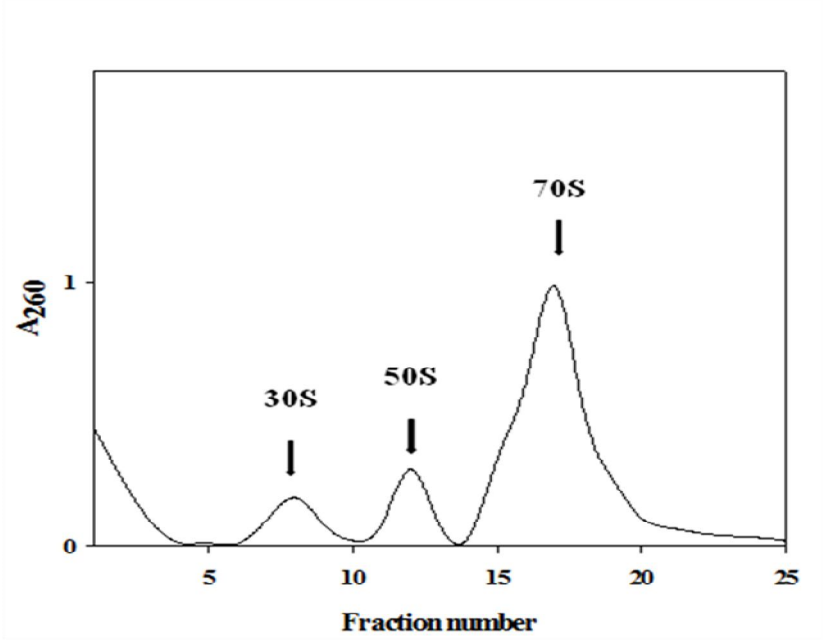


Fig: 17. Sucrose density gradient profiles of undialyzed *E. coli* S-30 on 5-20% gradients showing native ribosomal subunits and 70 S ribosomal peaks.

ii. Hydrophobic interaction chromatography for the dissociation of ribosomes:

Hydrophobic interaction chromatography involves the treatment of ribosomes with high salt and subsequent chromatography on sepharose column using reverse salt gradient. This method has been successfully used to separate ribosomal subunits from *E. coli* (Krillov, 1978). Same procedure was followed in the case of *H. eurihalina* ribosomes. Logarithmic and stationary phase *H. eurihalina* ribosomes were eluted on Sepharose-4B column using reverse salt gradient as described in methods. The Sepharose-4B reverse salt gradient elution profiles of *H. eurihalina* ribosomes were shown in Fig: 18A & 19A. Logarithmic phase ribosomal elution profile showed a very small peak A and a broad peak B. Stationary phase ribosomal elution profile showed a sharp peak A and a broad peak B which may represent heterogeneous unfolded ribosomal particles. SDS-PAGE analysis of peak fractions was shown in Fig: 18B & 19B. Despite the presence of different peaks in the elution profiles, SDS-PAGE analysis clearly shows the undissociated ribosomal band pattern.

These results show that even hydrophobic interaction chromatography of ribosomes with reverse salt gradient does not result in dissociation of ribosomes indicating the strong association of ribosomal subunits in *H. eurihalina*. This procedure was found to be not applicable for *H. eurihalina* ribosomes.

Light scattering studies:

Light scattering studies have been employed to study ribosomal subunit association and dissociation of 70 S ribosomes to subunits. Light scattering studies were carried to observe the presence of magnesium dependent subunit dissociation in *H. eurihalina* ribosomes. These studies were carried out according to Zitomer and Flaks (1972) with few modifications. Maximum scattering was taken as 100% 70 S formation.

The dependence of light scattering on the increasing concentration of ribosomes was checked as shown in Fig: 20. Relative scattering was found to be linear up to the concentration of 6 A_{260}/ml, 3.2 A_{260}/ml and 2.5 A_{260}/ml respectively for *E. coli, H. eurihalina* logarithmic and stationary phase ribosomes. In subsequent studies, concentration of ribosomes used was 4 A_{260}/ml, 2 A_{260}/ml and 1.6 A_{260}/ml for *E. coli, H. eurihalina* logarithmic and stationary phase ribosomes respectively.

The relative light scattering obtained for *E. coli* ribosomes was shown in Fig: 21A. Decrease in the amount of light scattered indicate dissociation of ribosomes into subunits. Fig: 21B & C show the relative light scattering obtained for *H. eurihalina* logarithmic and stationary phase ribosomes. Even at 1 mM magnesium ion concentration, decrease in the amount of light scattered was not observed (both logarithmic and stationary phase ribosomes). These results indicate that *H. eurihalina* ribosomes (logarithmic and stationary phase) failed to dissociate at low magnesium concentrations. This data further supports the results obtained by sucrose density gradient studies and hydrophobic interaction chromatography. *H. eurihalina* ribosomes do not dissociate into subunits under variety of conditions.

Structural studies using different probes

Conformation of moderate halophilic ribosomes, was studied using specific probes such as pancreatic RNase A (specifically acts on single stranded RNA). The stability of halophilic ribosomes was studied in presence of different salts in varying concentrations and their resistance to urea treatment. The stability of moderate halophilic ribosomes was compared with that of the mesophilic (*E. coli*) ribosomes under similar conditions.

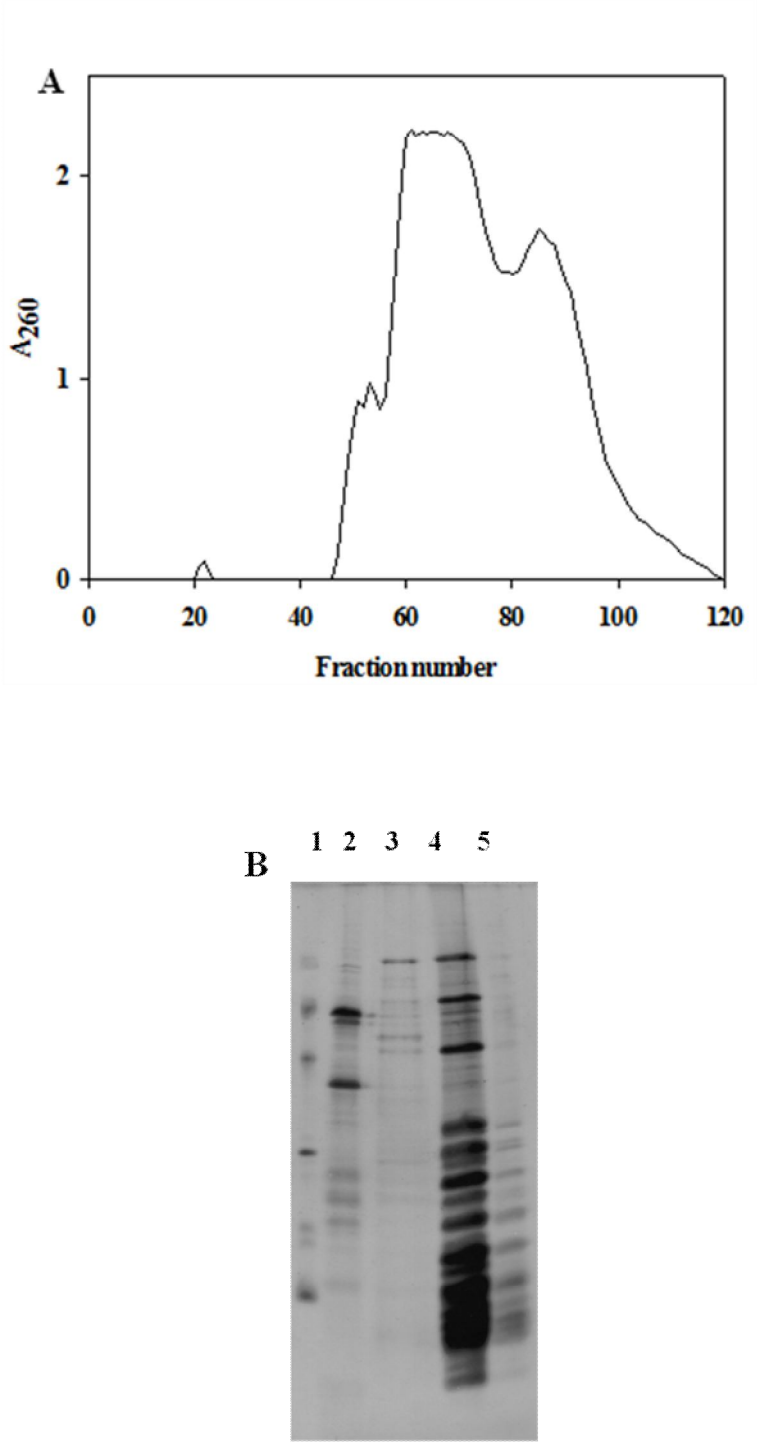
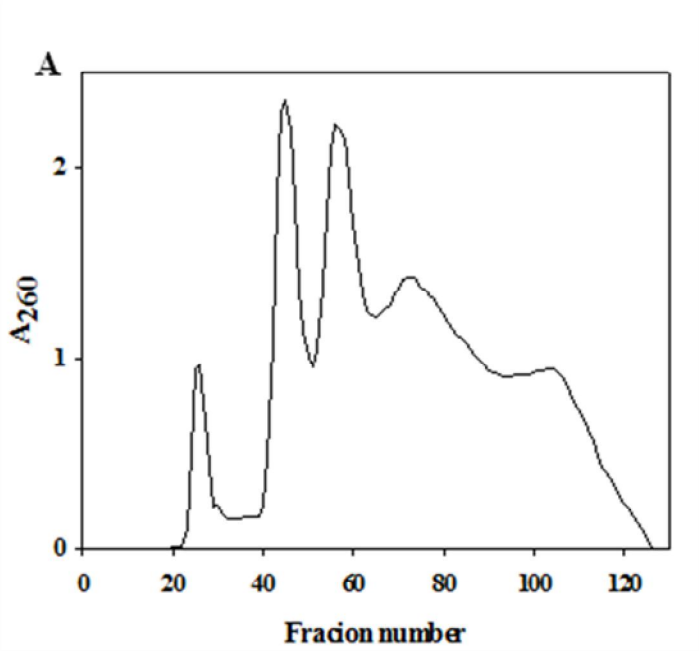


Fig: 18. Reverse salt gradient elution of *H. eurihalina* logarithmic phase ribosomes. A. Elution profile. B. Lane 1: Molecular weight marker, lanes 2-5: fractions 22, 55, 62 and 85 respectively.



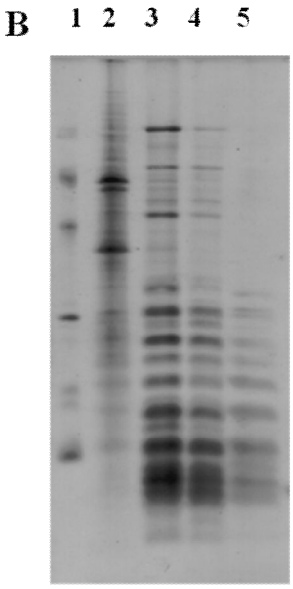


Fig: 19. Reverse salt gradient elution of *H. eurihalina* stationary phase ribosomes. A. Elution profile. B. Lane 1: Molecular weight marker, lanes 2-5: fractions 26, 45, 57 and 72 respectively.

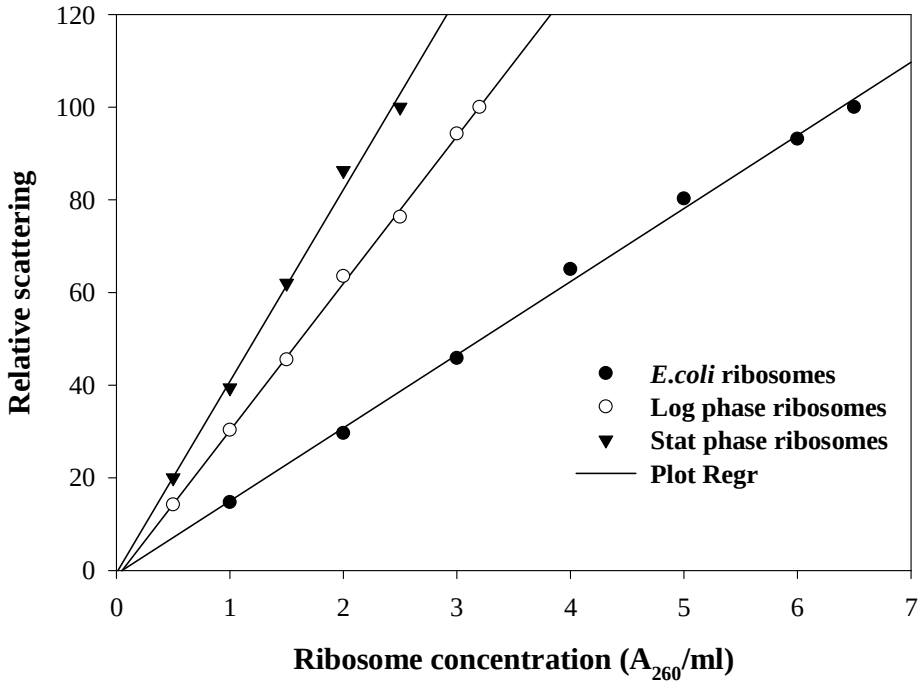


Fig: 20. Intensity of light scattering as a function of ribosome concentration. Light scattering was measured as described in methods section.

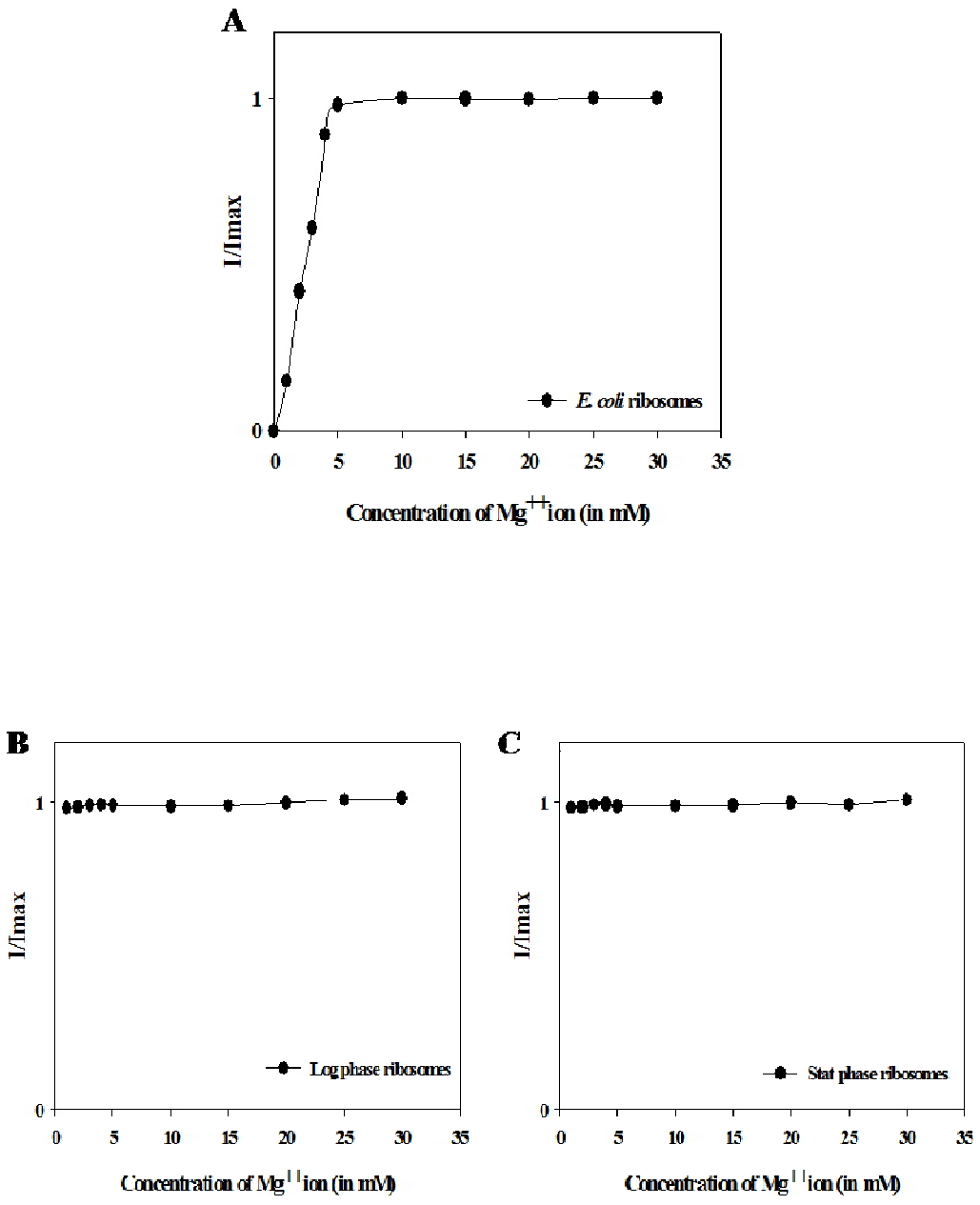


Fig: 21. Effect of magnesium on the subunit dissociation equilibrium of ribosomes. A. *E. coli* ribosomes, B. *H. eurihalina* logarithmic phase ribosomes, C. *H. eurihalina* stationary phase ribosomes.

Measurement of the degradation of ribosomes by RNase A using Spectrophotometry:

Low magnesium concentrations were used in all these experiments at room temperature, as this causes large change in the conformation of the treated and untreated ribosomes. The change in the conformation (unfolding) was detected by their susceptibility to RNase A.

RNase A hydrolyzes the single stranded regions of r-RNA that are available for its action in the ribosome. RNase A degradation causes increase in the absorbance of the ribosomal solution (hyperchromicity) which was measured in a spectrophotometer.

Measurement of the degradation of ribosomes in the presence of RNase A was done as described in methods. The measurement was made immediately after adding the enzyme until degradation reaches a plateau (Fig: 22A, B & C).

These results show that *E. coli* ribosomes required 0.3 µg of RNase A to reach maximum amount of degradation. However, *H. eurihalina* logarithmic phase ribosomes required 26 fold more concentration of RNase A i.e., 8 µg to reach maximum amount of degradation, whereas, stationary phase ribosomes required 10 fold more i.e., 4 µg to reach maximum amount of degradation.

However, the maximum hydrolysis obtained for *H. eurihalina* logarithmic and stationary phase ribosomes was less compared to maximum hydrolysis obtained for *E. coli* ribosomes. These results show that moderately halophilic ribosomes are much more stable to RNase A treatment in comparison to *E. coli* ribosomes. *H. eurihalina* stationary phase ribosomes are more susceptible to hydrolysis by RNase A than the logarithmic phase ribosomes, suggesting stationary phase ribosomes are more unfolded. This may be due to missing of some primary rRNA binding proteins (eg: L2).

Effect of magnesium on the structure of ribosomes:

The degradation of ribosomes was measured in UV-Visible spectrophotometer, at different magnesium concentrations, by the increase in absorbance at 260 nm (or the hyperchromicity obtained) in the presence of RNase A. As seen from the Fig: 23A, B & C degradation of ribosomes was more at low magnesium concentrations i.e., 0.1 mM magnesium concentration. The degradation was less at higher magnesium ion concentration in case of both *E. coli* and *H. eurihalina* (logarithmic and stationary phases) ribosomes. However, it should be noted that in these experiments also *E. coli* ribosomes were hydrolyzed with many fold less concentration of RNase A.

These results indicate that moderate halophilic ribosomes are more compact and more resistant to degradation by RNase A at low magnesium concentration compared to mesophilic ribosomes.

Effect of different salts and their concentrations on RNase A sensitivity of *H. eurihalina* ribosomes:

These experiments were carried out to observe the effect of salts on moderately halophilic ribosomes. *H. eurihalina* logarithmic and stationary phase ribosomes were dialyzed against varying concentrations of different salts and their susceptibility to RNase A was studied.

A control experiment was carried out with *E. coli* ribosomes and all the salts used in the experiment, to observe the effect of high salt concentration on RNase A activity (Fig: 24A). These results show that even at highest salt concentrations (0.5 M) used in the following experiments, none of the salts showed any inhibitory effect on enzymatic activity.

Effect of ammonium chloride:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M ammonium chloride were given in Fig: 24B, C & D respectively. Both logarithmic and stationary phase ribosomes showed less susceptibility in presence of 0.25 M and 0.5 M ammonium chloride compared to 0.1 M ammonium chloride.

Effect of potassium glutamate:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M potassium glutamate were shown in Fig: 25A, B & C respectively. Both logarithmic and stationary phase ribosomes showed less susceptibility at 0.25 M potassium glutamate concentration compared to 0.5M and 0.1 M potassium glutamate. The ribosomes in low and high salt concentrations reached the same level of degradation with less concentration of RNase A in comparison to ribosomes in 0.25 M salt. The optimal concentration of potassium glutamate that minimizes the hydrolysis by RNase A is around 0.25 M concentration.

Effect of sodium chloride:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M sodium chloride were shown in Fig: 26A, B & C respectively. Both logarithmic and stationary phase ribosomes showed maximum degradation with less concentration of RNase A. Protection against RNase A degradation was not observed at any salt concentration. These results show that sodium chloride has an unfolding effect on the ribosomes.

Effect of potassium chloride:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M potassium chloride were shown in Fig: 27A, B & C respectively. Both logarithmic and stationary phase ribosomes showed less susceptibility in presence of potassium chloride. Protection was maximum at 0.25 M potassium chloride concentration followed by 0.5 M compared to 0.1 M potassium chloride.

Among the four different salts tested, potassium chloride was more effective in stabilizing the structure of the ribosomes followed by ammonium chloride. Both *H. eurihalina* logarithmic and stationary phase ribosomes showed less susceptibility to RNase A in presence of optimum concentration (0.25 M) of potassium chloride. It should be mentioned here that in moderate halophiles, the dominant intracellular monovalent cation is K^+.

In presence of 0.25 M potassium glutamate ribosomes required more concentration of RNase A to reach maximum degradation compared to 0.1 M and 0.5 M potassium glutamate. Sodium chloride is the least effective among the four salts used, thus resulted in maximum degradation of both logarithmic and stationary phase ribosomes with less concentration of RNase A.

Effect of polyamines on the structure of ribosomes:

Polyamines play important role in maintaining the structure of nucleic acid and nucleoprotein complexes. We studied the effect of polyamines on the structure of ribosomes using RNase A as a probe. Ribosomes treated with polyamines showed less susceptibility to RNase A compared to untreated ribosomes (Fig: 28 and 29 A, B & C). However, polyamine treatment showed more protective effect on *E. coli* ribosomes in comparison to halophilic ribosomes.

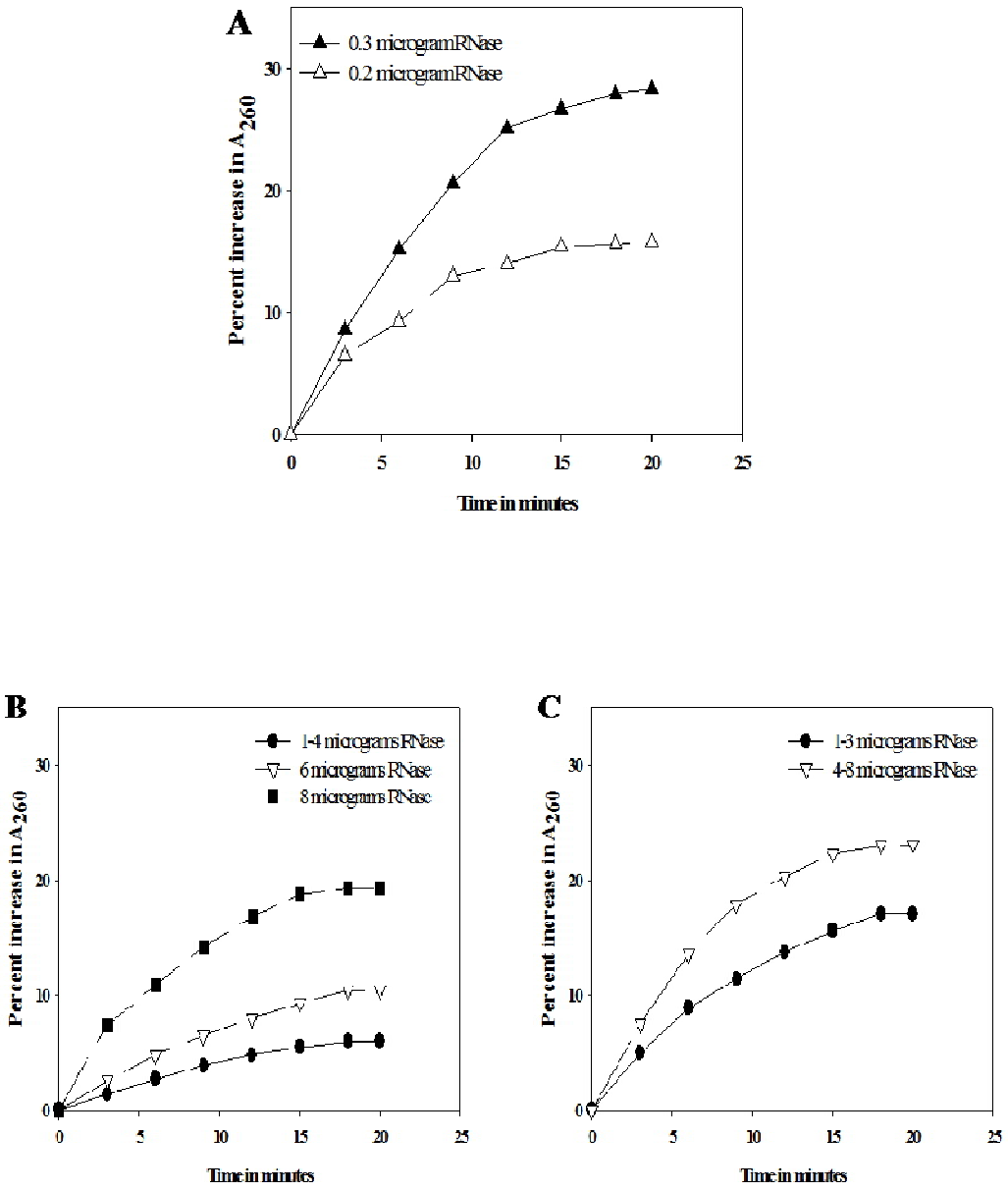


Fig: 22. Degradation profiles of ribosomes in the presence of different concentrations of RNase A at 0.1 mM Mg^{2+} concentration. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase. C. *H. eurihalina* stationary phase ribosomes.

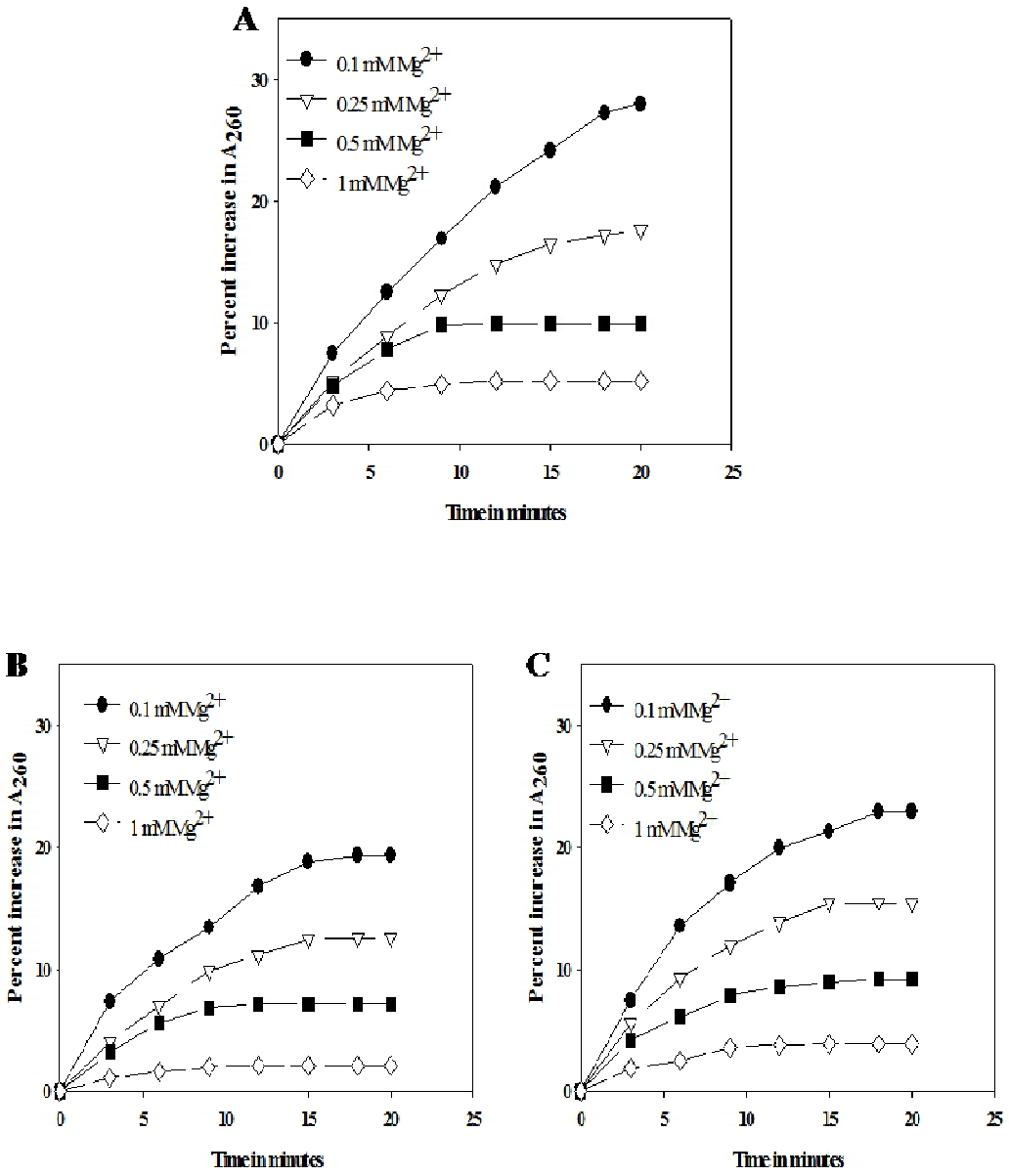


Fig: 23. Degradation profiles of ribosomes at different magnesium concentrations in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase. C. *H. eurihalina* stationary phase ribosomes.

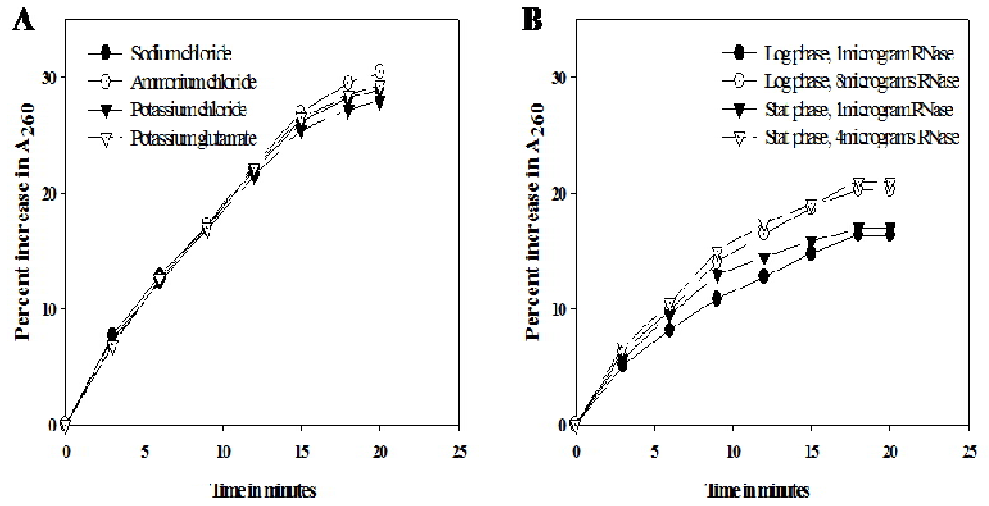


Fig: 24A. Control experiment carried out with *E. coli* ribosomes in presence of different salts.

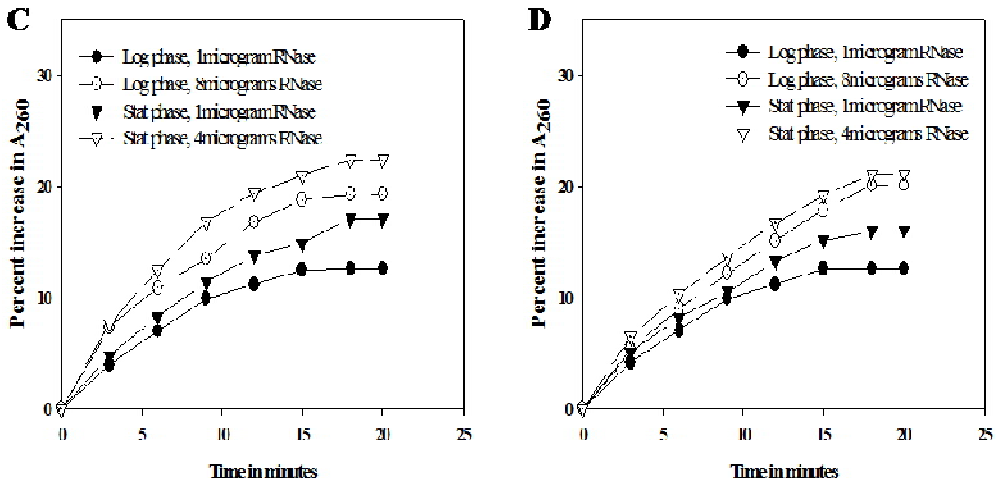


Fig: 24. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different ammonium chloride concentrations in the presence of RNase A. B. 0.1 M, C. 0.25 M and D. 0.5 M ammonium chloride.

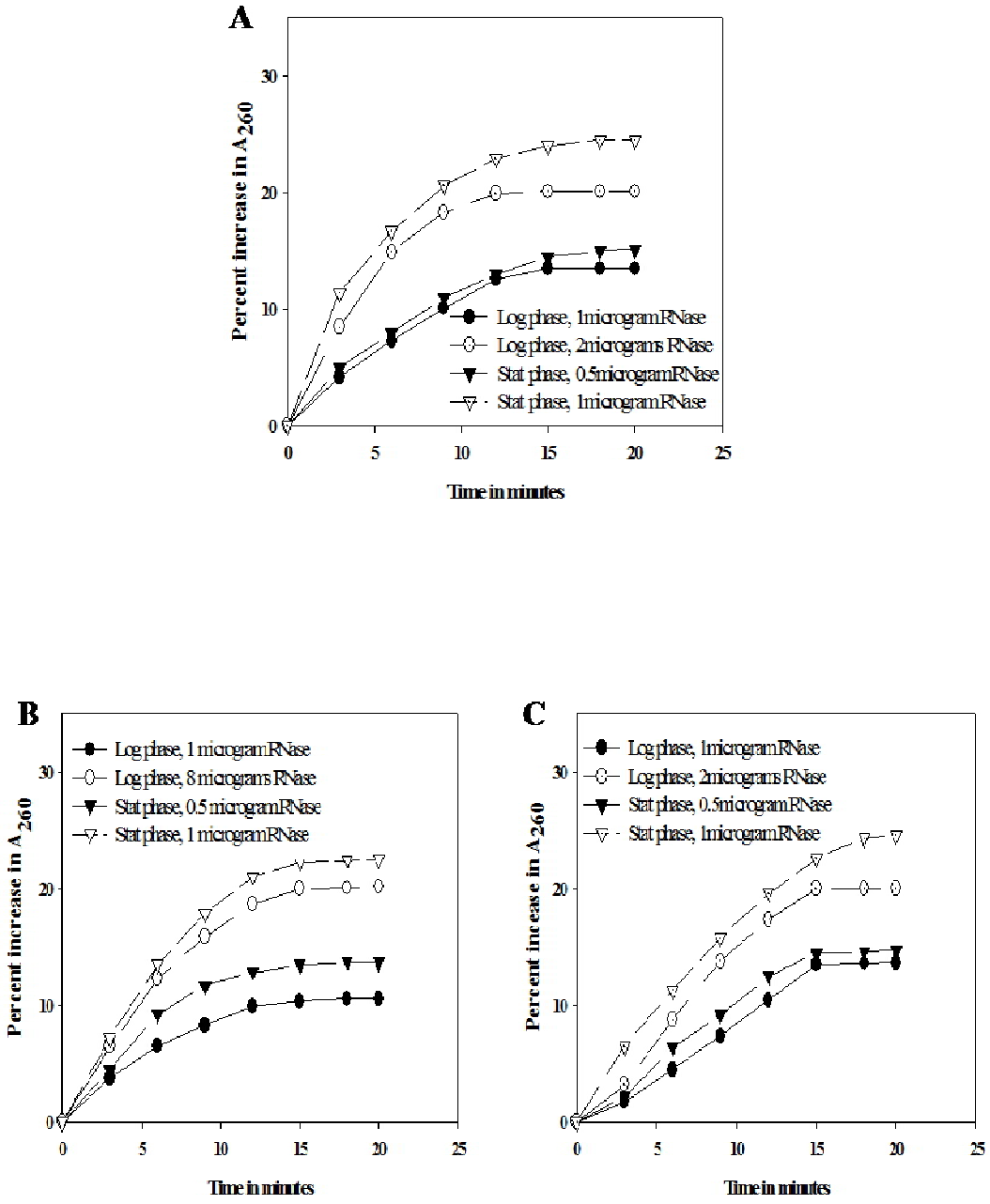


Fig: 25. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different potassium glutamate concentrations in the presence of RNase A. A. 0.1 M, B. 0.25 M and C. 0.5 M potassium glutamate.

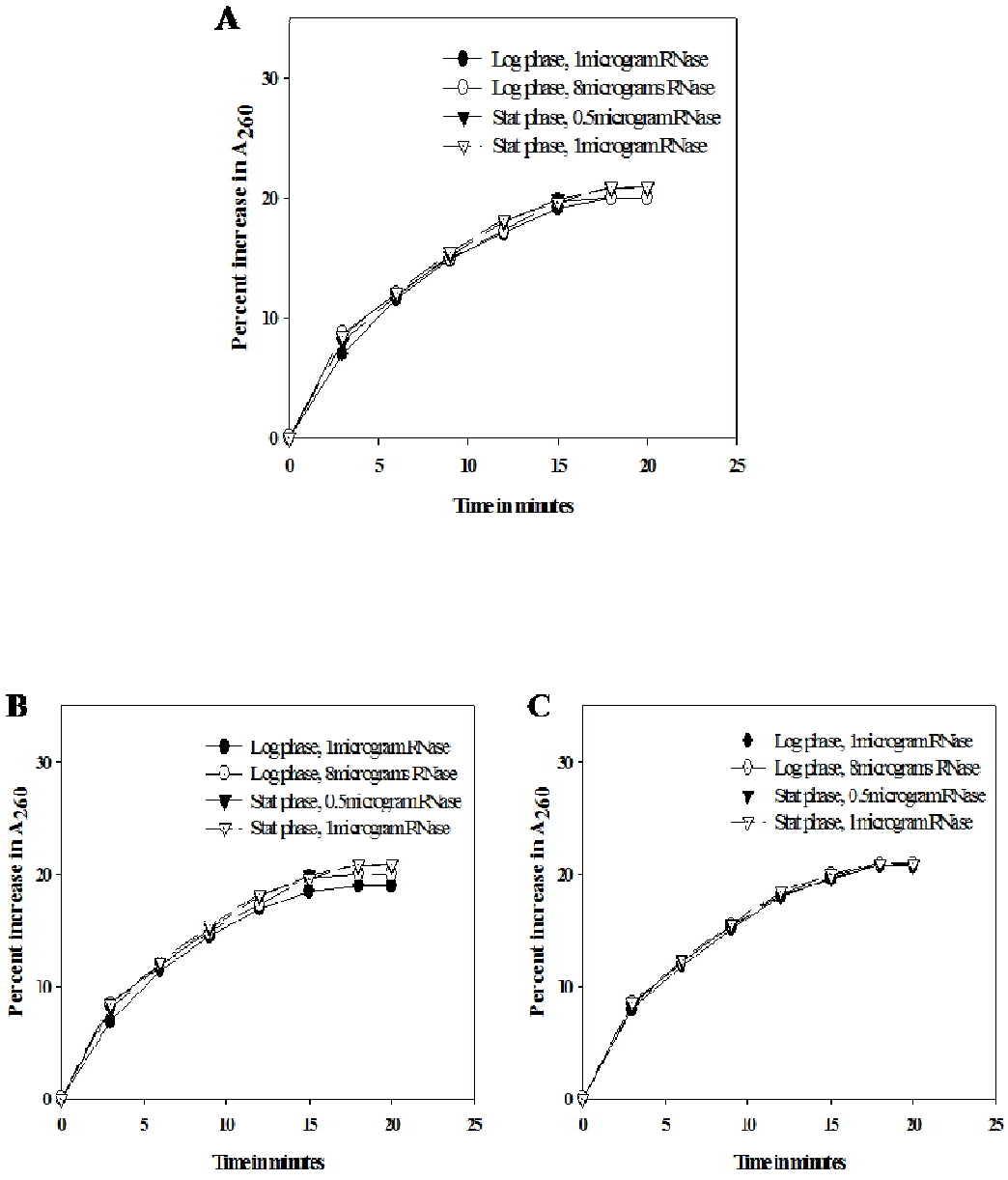


Fig: 26. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different sodium chloride concentrations in the presence of RNase A. A. 0.1 M, B. 0.25 M and C. 0.5 M sodium chloride.

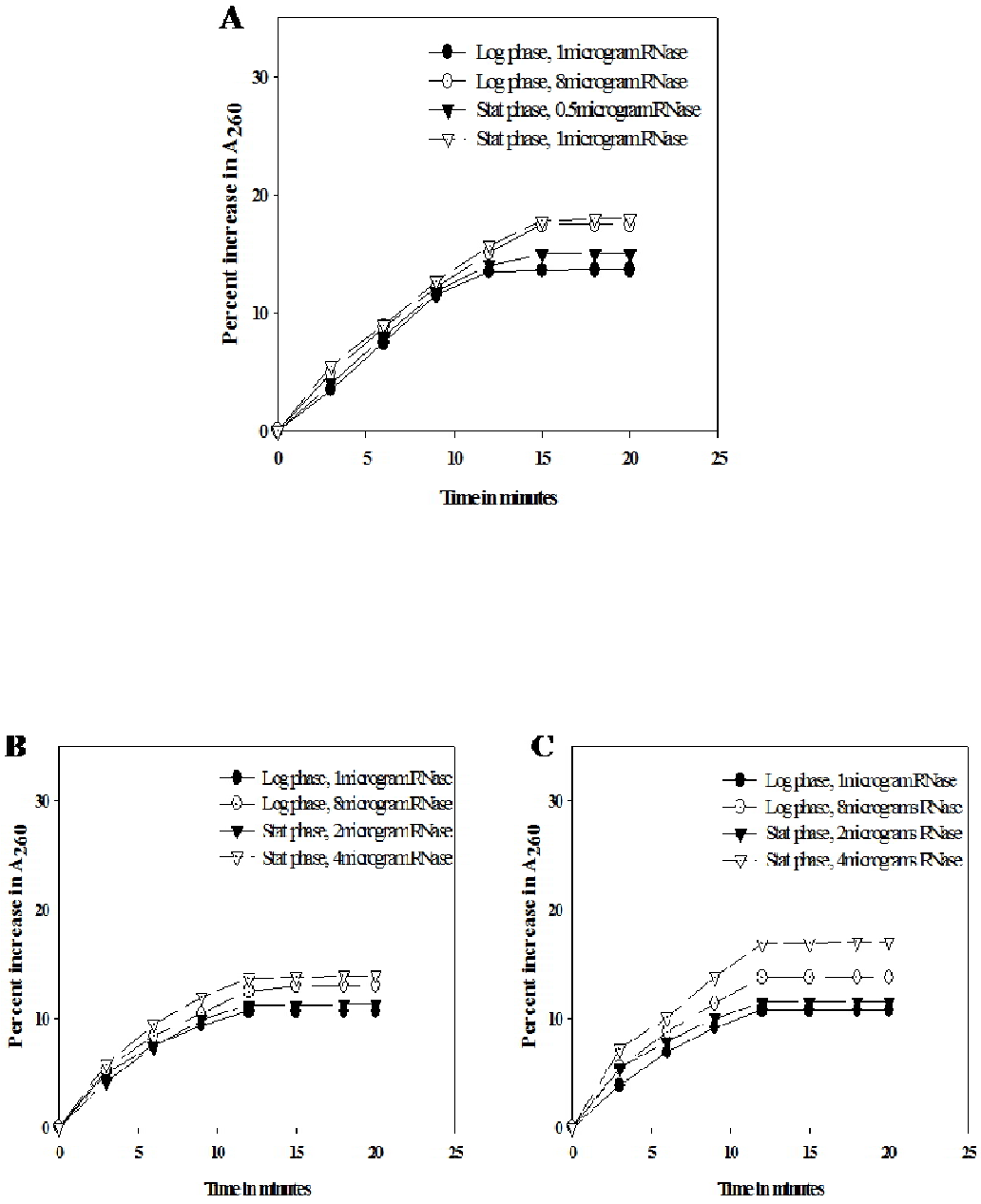


Fig: 27. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different potassium chloride concentrations in the presence of RNase A. A. 0.1 M, B. 0.25 M and C. 0.5 M potassium chloride.

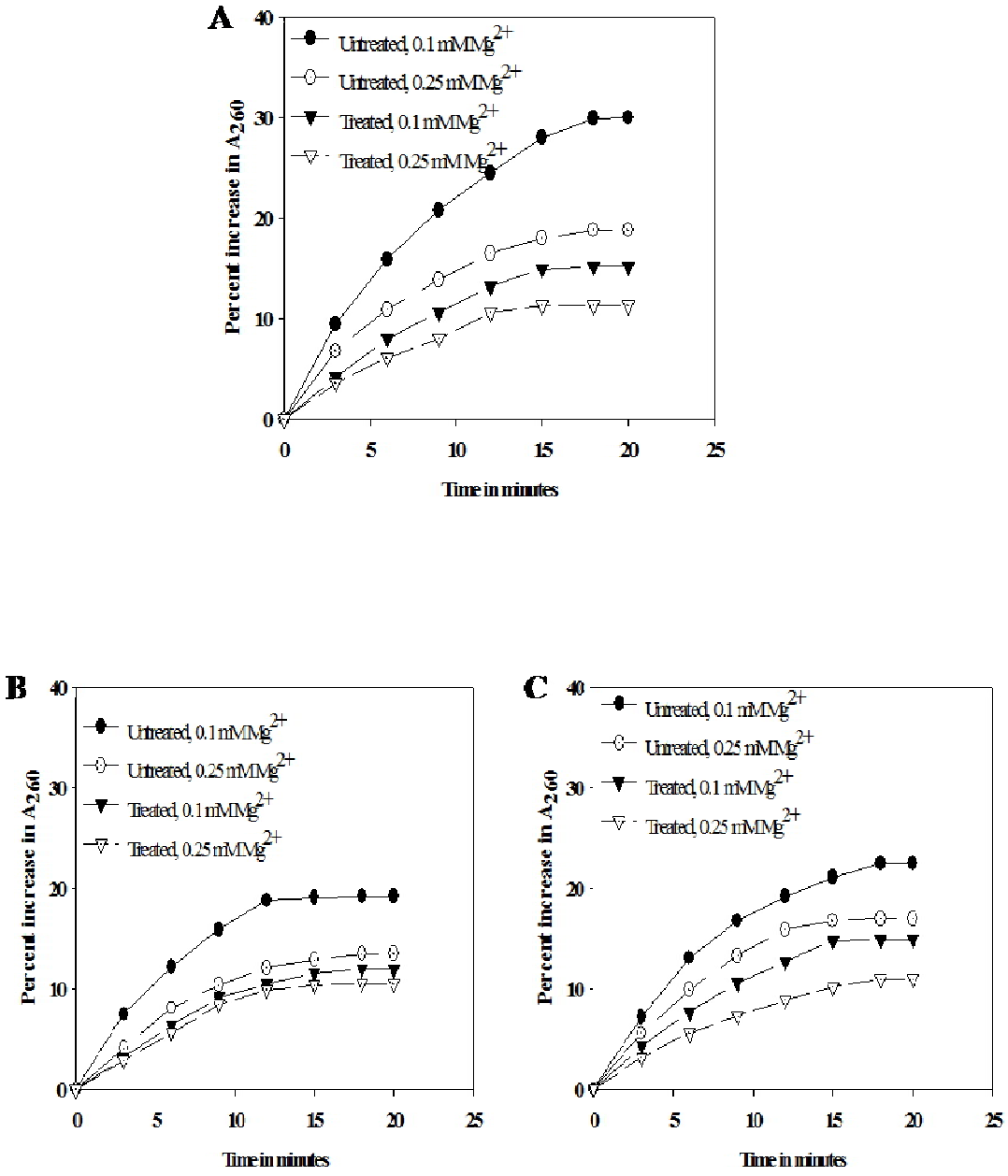


Fig: 28. Degradation profiles of spermine treated ribosomes in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase ribosomes. C. *H. eurihalina* stationary phase ribosomes.

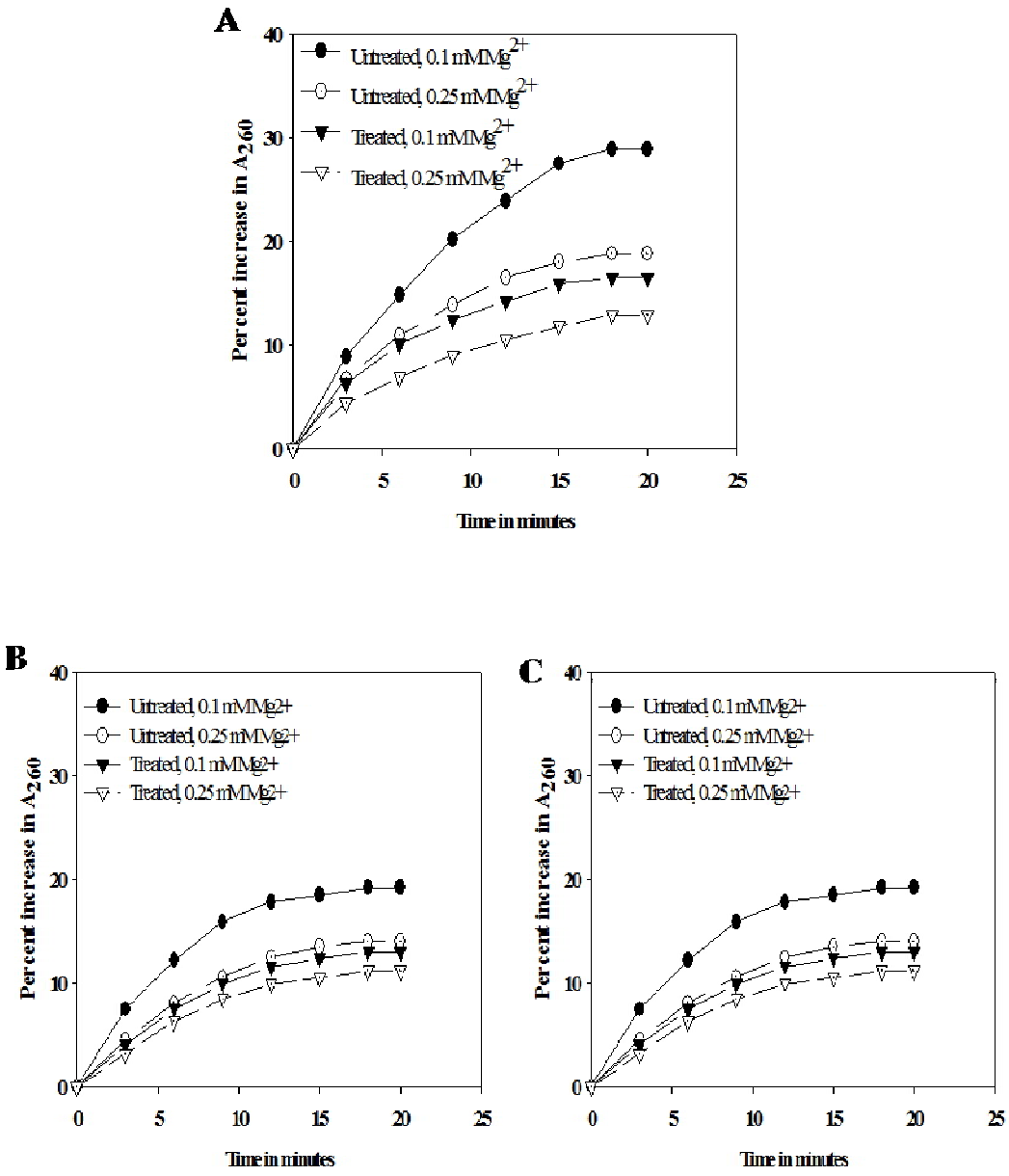


Fig: 29. Degradation profiles of spermidine treated ribosomes in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase ribosomes. C. *H. eurihalina* stationary phase ribosomes.

Spermine was more effective in stabilizing the ribosome structure compared to spermidine as the concentration of spermidine required to offer same extent of protection was double the concentration of spermine. These results also show that although polyamine treatment resulted in marginal increase of resistance towards RNase A treatment, halophilic ribosomes are inherently resistant to RNase A treatment.

Effect of EtBr on the structure of ribosomes:

Accessibility of rRNA to intercalating agents and the effect of such agents on the structure of ribosomes were also studied. EtBr treatmet of *H. eurihalina* ribosomes was carried out to see the effect of intercalating agents on halophilic ribosomes in comparison to mesophilic ribosomes. The degradation profiles of EtBr treated *E. coli*, *H. eurihalina* logarithmic and stationary phase ribosomes were given in Fig: 30A, B & C respectively. Results show that EtBr treated ribosomes were little more susceptible to RNase A activity compared to untreated ribosomes. *H. eurihalina* ribosomes treated with EtBr were more resistant to RNase A activity in comparison to the similarly treated *E. coli* ribosomes (Fig: 30A, B & C). *H. eurihalina* stationary phase ribosomes treated with EtBr also showed equal resistance to RNase A activity on par with logarithmic phase ribosomes.

These results show that *H. eurihalina* ribosomes were more resistant to intercalation and the distortions caused by EtBr when compared to *E. coli* ribosomes. Alternatively, these results also indicate that *H. eurihalina* ribosomes are organized in such a way that their rRNA was not available for intercalations by EtBr.

Ribosome unfolding studies with urea:

We have investigated the effects of urea on the structural organization of *E. coli* and *H. eurihalina* ribosomes, which would provide some indication on the mechanism of the stability of ribosomes.

The unfolding of ribosome structure on addition of urea was detected by the increase in the absorbance of the solution. Ribosomes were treated with different concentrations of urea as described in methods. Fig: 31A shows the absorption spectrum of *E. coli* and *H. eurihalina* logarithmic and stationary phase ribosomes in respective normal buffers, recorded from 220 to 300 nm at room temperature.

Absorption spectra were obtained at increasing concentration of urea, 2 M (Fig: 31B), 4 M (Fig: 31C), 6 M (Fig: 31D) and 8 M (Fig: 31E). At lower urea concentrations (2 M and 4 M) there was not much difference in the absorption spectra of *E. coli* and *H. eurihalina* ribosomes. However, at these urea concentrations increased absorption was seen at around 230 nm in the case of *H. eurihalina* ribosomes.

Striking differences in the unfolding of ribosomes by urea was observed at 6 M urea. At this concentration, *H. eurihalina* logarithmic phase ribosomes were unaffected by urea where as *E. coli* ribosomes unfolded completely. The stationary phase ribosomes also unfolded at this urea concentration. At 8 M urea concentration *H. eurhalina* logarithmic phase ribosomes were marginally less unfolded than *E. coli* ribosomes.

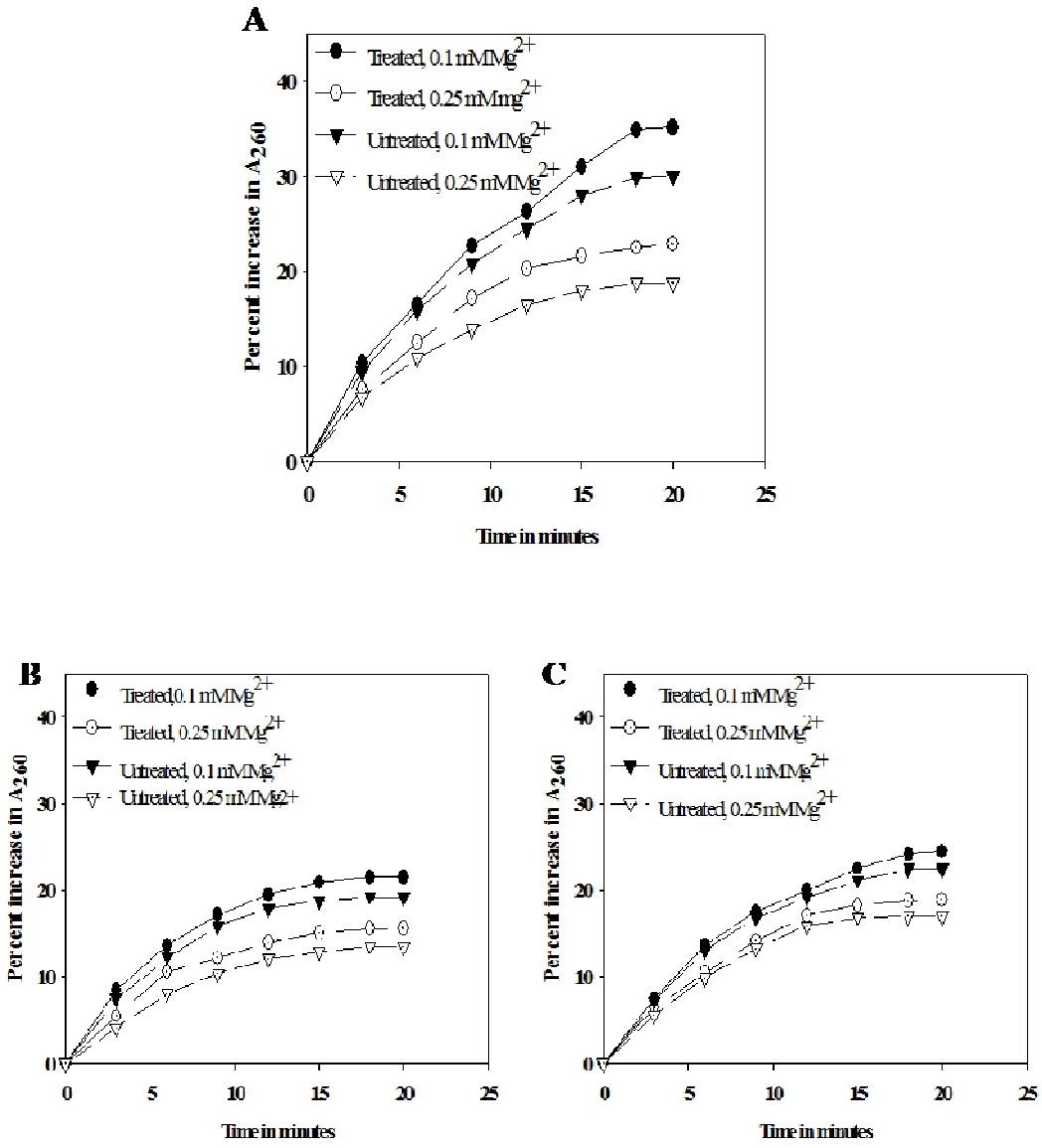


Fig: 30. Degradation profiles of EtBr treated ribosomes in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase ribosomes. C. *H. eurihalina* stationary phase ribosomes.

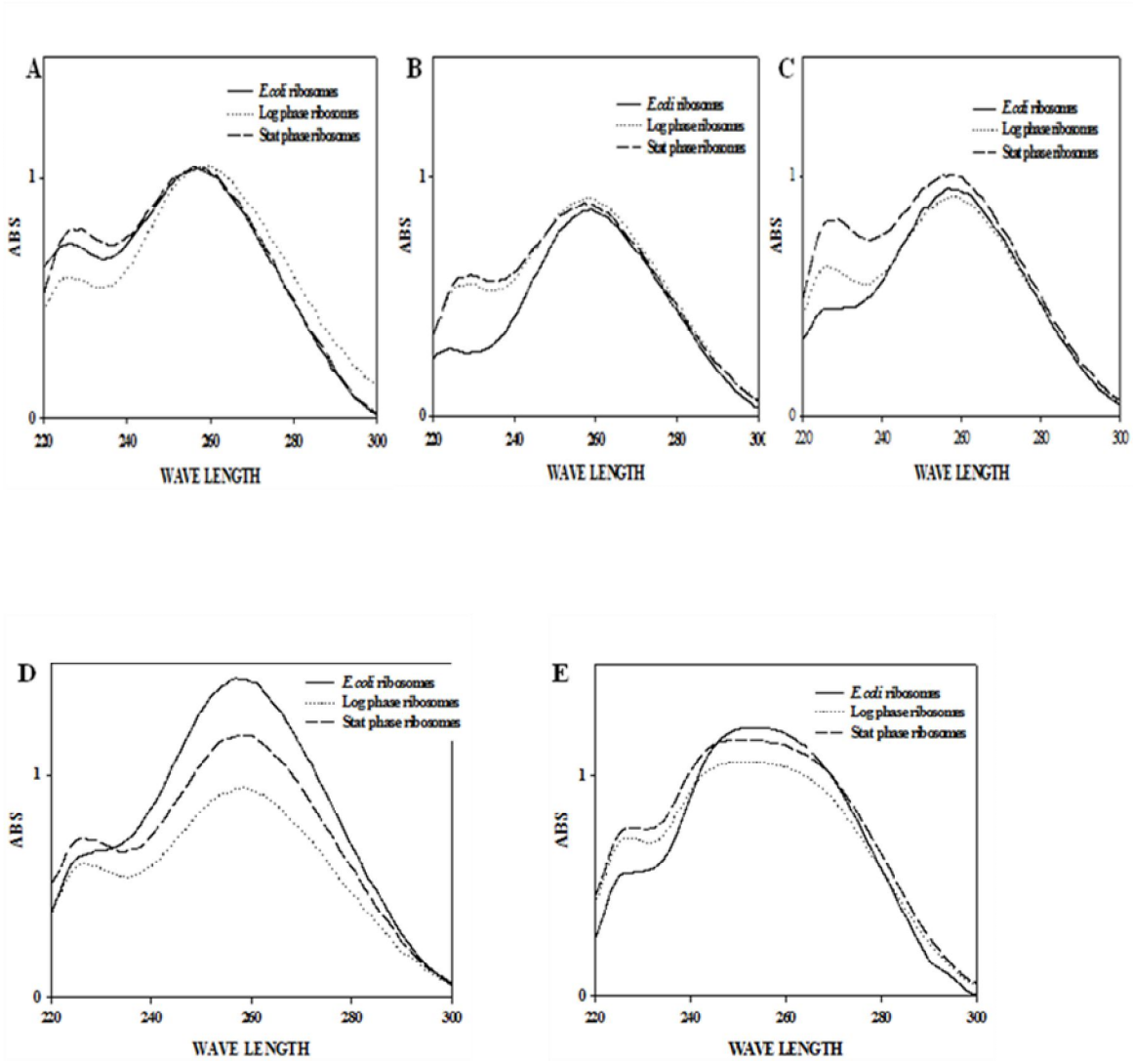


Fig: 31. Absorption spectra of *E. coli, H. eurihalina* logarithmic and stationary phase ribosomes treated with different concentrations of urea. A. Ribosomes in respective normal buffers (without urea). B. 2 M urea treated ribosomes. C. 4 M urea treated ribosomes. D. 6 M urea treated ribosomes. E. 8 M urea treated ribosomes.

Structural studies on halophilic ribosomes

Ribosome is a complex of rRNA and proteins whose flexible conformation is formed and stabilized by mutual interactions among its components, which is influenced by specific monovalent and bivalent cations. Thus, certain changes in the ionic environment which do not change the overall structural properties of the ribosome do nevertheless change something in the conformation of the component macromolecules, which may have drastic effects on structure of the ribosome leading to variation in its functional activity. It is further known that magnesium ions play central role in stabilizing the structure of ribosome and optimum concentration of magnesium ion is required to maintain the subunits in associated monomer form, structural conformation and for the functional activity of the ribosome.

Structural studies of halophilic ribosomes were carried out by two different sets of experiments. First set of experiments were carried out to determine the ionic (magnesium ion) concentration required for the dissociation of ribosomes into subunits. Second set of experiments include studies on structural changes in ribosomes induced by agents like salts, polyamines and intercalating agents using RNase A as a probe.

Magnesium ion dependent subunit dissociation

In order to observe the effect of reduced magnesium ion concentration on *H. eurihalina* ribosomes, sucrose density gradient centrifugation studies, hydrophobic interaction chromatography studies and light scattering studies were carried out.

i. Sucrose density gradient centrifugation:

Attempts were made to separate ribosomal subunits by sucrose density gradient centrifugation, the most widely and generally used method for separation of subunits. In spite of applying different conditions, none of them resulted in dissociation of subunits.

a. On 5-20% gradients:

Centrifugation was carried out as described in methods. The sucrose density gradient centrifugation profiles of dialyzed (0.2 mM magnesium acetate) and undialyzed (10 mM magnesium acetate) *E. coli* ribosomes were shown in Fig: 10A & B. It can be observed that ribosomes dialyzed against low magnesium ion concentration resulted in dissociation of ribosomes represented by two peaks (Fig: 10A), in comparison to undialyzed ribosomes (Fig: 10B). The sucrose density gradient profiles of undialyzed (20 mM magnesium acetate) *H. eurihalina* ribosomes were given in Fig: 10C & D. *H. eurihalina* logarithmic phase ribosomes sedimented faster in comparison to stationary phase and *E. coli* ribosomes. These results show that *H. eurihalina* logarithmic phase ribosomes are more compact in comparison to *E. coli* ribosomes. *H. eurihalina* stationary phase ribosomes are less compact than logarithmic phase ribosomes and sedimentation pattern is similar to *E. coli* ribosomes on sucrose density gradients. Thus 10-30% gradients were used for sucrose density gradient studies of *H. eurihalina* ribosomes.

b. On 10-30% gradients:

Sucrose density gradient profiles of undialyzed (20 mM magnesium aetate) logarithmic and stationary phase ribosomes and respective SDS-PAGE analysis representing 70 S ribosomes were shown in Fig: 11A, B & C. Sucrose density gradient profiles and respective SDS-PAGE analysis of logarithmic and stationary phase ribosomes dialyzed against low magnesium ion concentration (0.2 mM) and salt (as indicated in figures) as mentioned in methods were given

in Fig: 12, 13, 14 & 15 (A, B & C). High salt concentration was used as it was known to favor subunit dissociation. It can be observed that in presence of salts 70 S ribosomal particles are stripped off of some proteins and hence showed lesser sedimentation rate compared to undialyzed 70 S ribosomes. Significant decrease in sedimentation rate was observed in presence of potassium glutamate in comparison with normal conditions indicating that more number of proteins was stripped off in presence of potassium glutamate followed by sodium chloride and ammonium chloride. In presence of potassium chloride much decrease in sedimentation rate was not observed. In spite of using several conditions the ribosome failed to dissociate as analyzed by sucrose density gradient ultracentrifugation.

As the ribosomes did not dissociate in presence of low magnesium concentration, sucrose density gradient centrifugation of S-30 was carried out to observe the presence of native ribosomal subunits in *H. eurihalina* extracts. The sucrose density gradient profile of undialyzed *E. coli* S-30 showed the presence of 30 S, 50 S native ribosomal subunits along with 70 S ribosomes in the cell lysate (Fig: 17). Sucrose density gradient profiles of undialyzed logarithmic and stationary phase S-30 and their respective SDS-PAGE analysis were given in Fig: 16A, B & C. Undialyzed S-30 sucrose density gradient profiles showed the absence of 30 S and 50 S native ribosomal subunits with only the presence of 70 S ribosome peaks in both logarithmic and stationary phase cell lysates. It is possible that in *H. eurihalina,* ribosomes may be existing completely in undissociated 70 S forms in the cell lysates.

H. eurihalina logarithmic and stationary phase ribosomes failed to dissociate even under very low magnesium ion concentration viz., 0.2 mM. These results show that ribosomal subunit dissociation is magnesium concentration independent in the case of *H. eurihalina* and that the subunits are held together tightly.

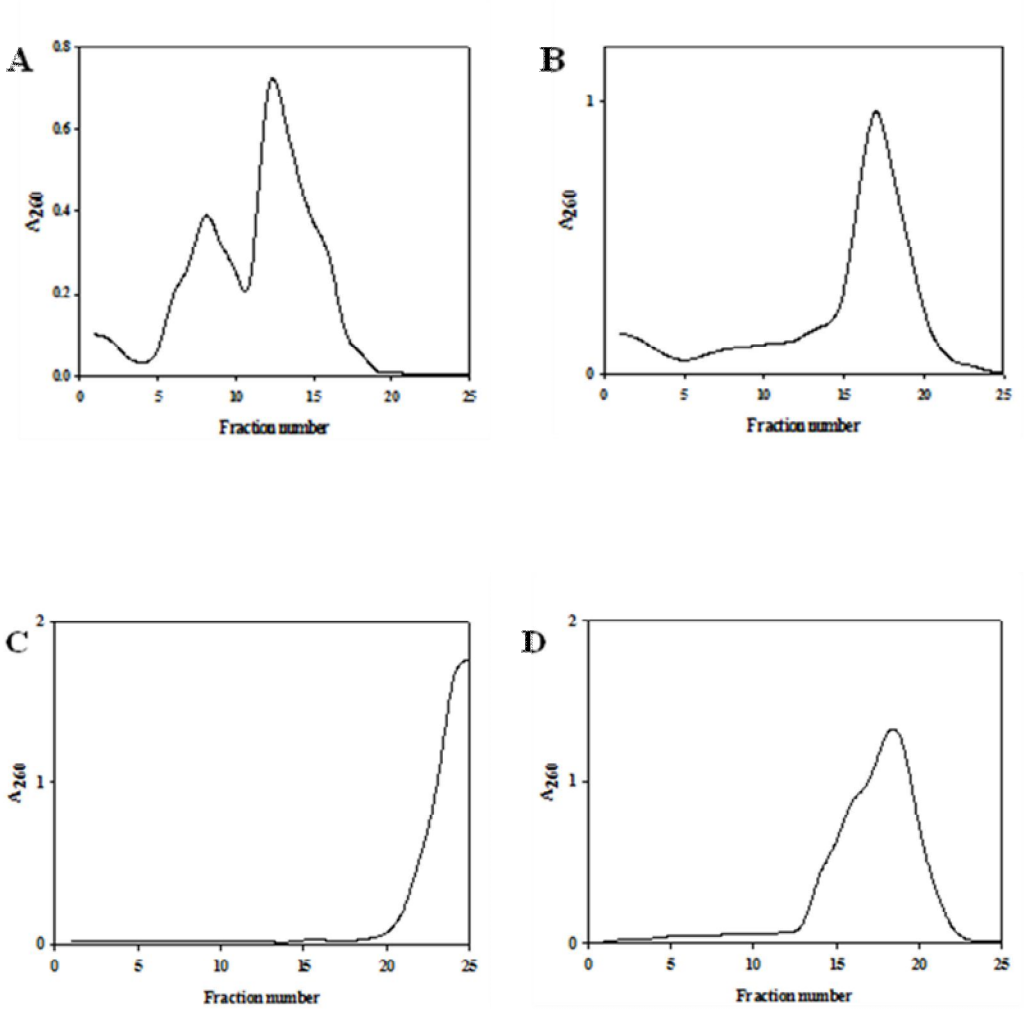


Fig: 10. Sucrose density gradient profiles of ribosomes on 5-10% gradients. A. Dialyzed *E. coli* ribosomes showing subunit dissociation. B. Undialyzed *E. coli* ribosomes. C. Undialyzed *H. eurihalina* logarithmic phase ribosomes. D. Undialyzed *H. eurihalina* stationary phase ribosomes.

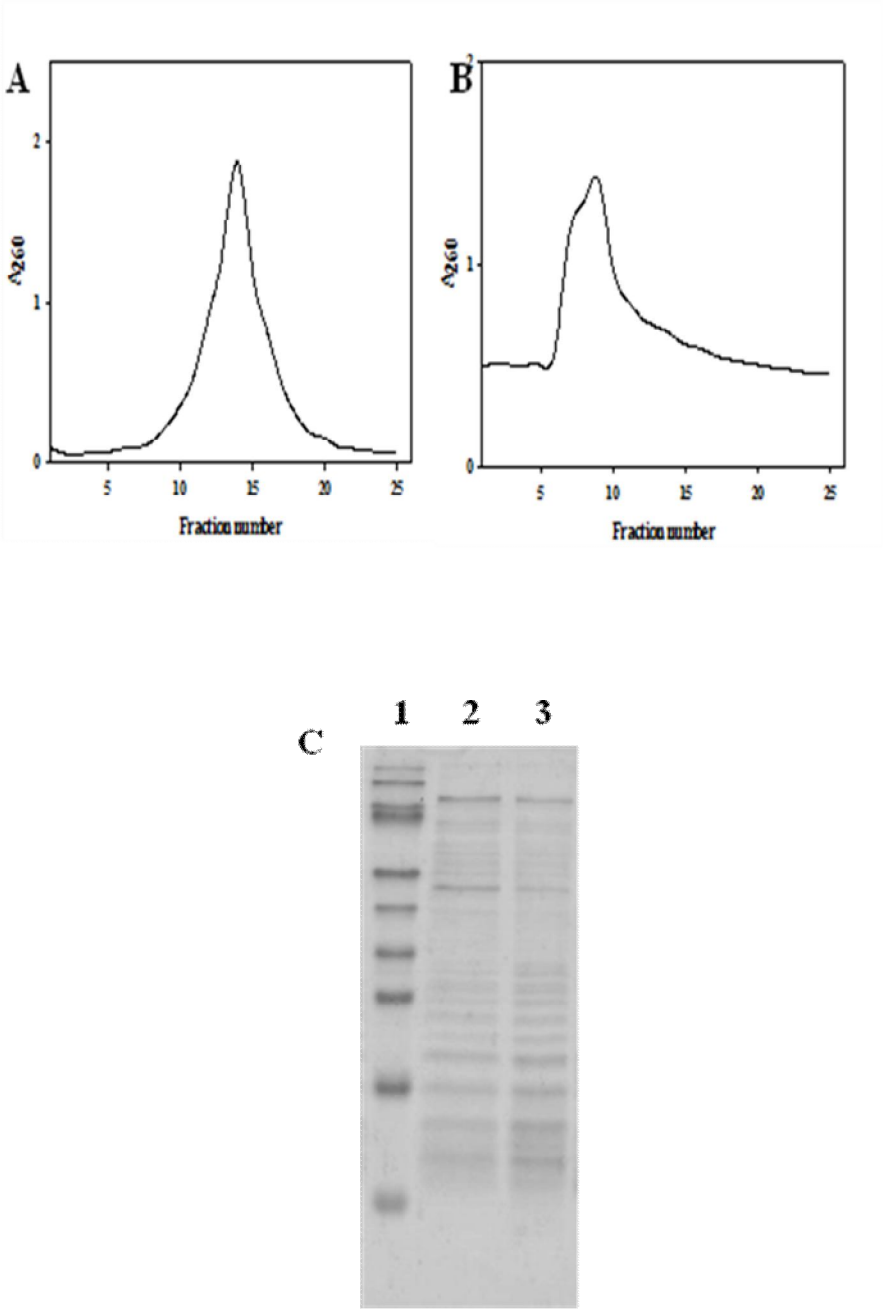


Fig: 11. Sucrose density gradient profiles of undialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2-3: Logarithmic phase ribosomes, lane 4-5: Stationary phase ribosomes.

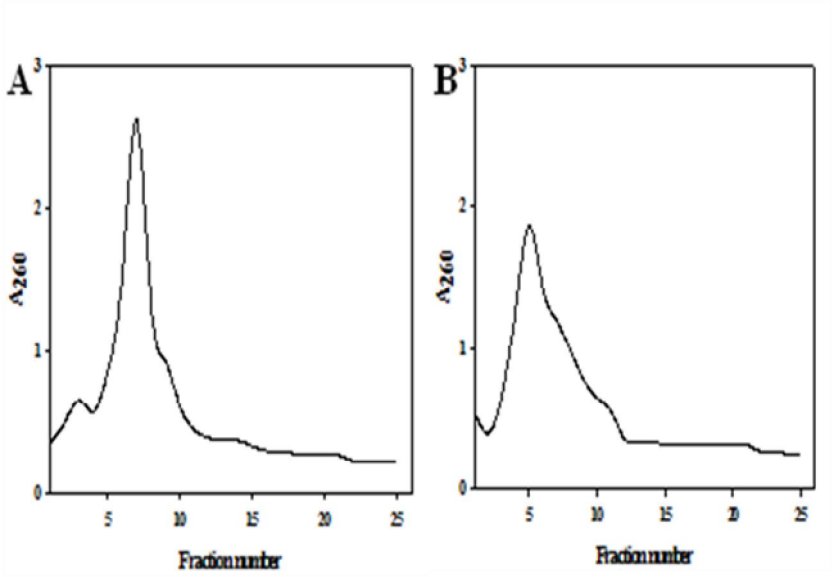


Fig: 12. Sucrose density gradient profiles of 500 mM ammonium chloride dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2 and lane 3: logarithmic phase ribosomes, lane 4 and lane 5: Stationary phase ribosomes.

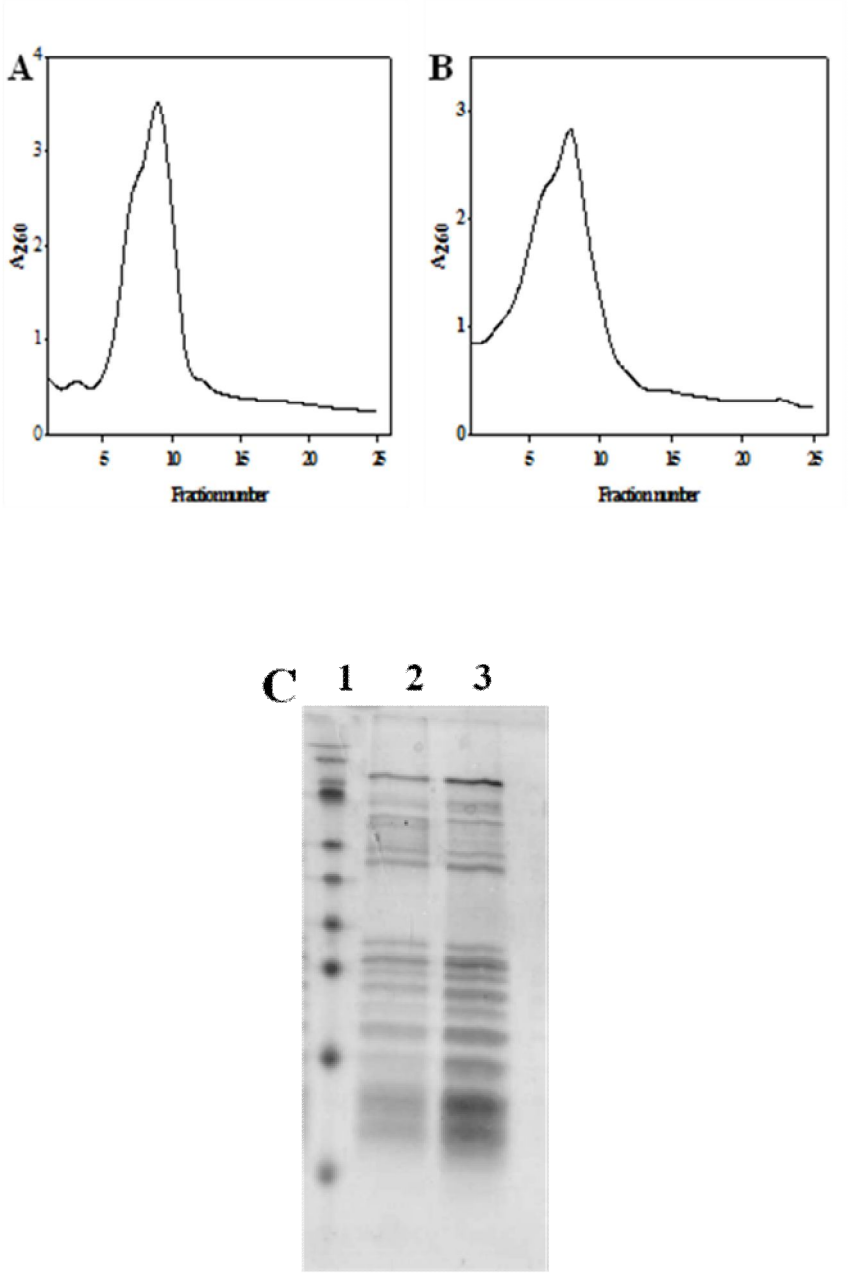


Fig: 13. Sucrose density gradient profiles of 500 mM potassium chloride dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosome and lane 3: Stationary phase ribosomes.

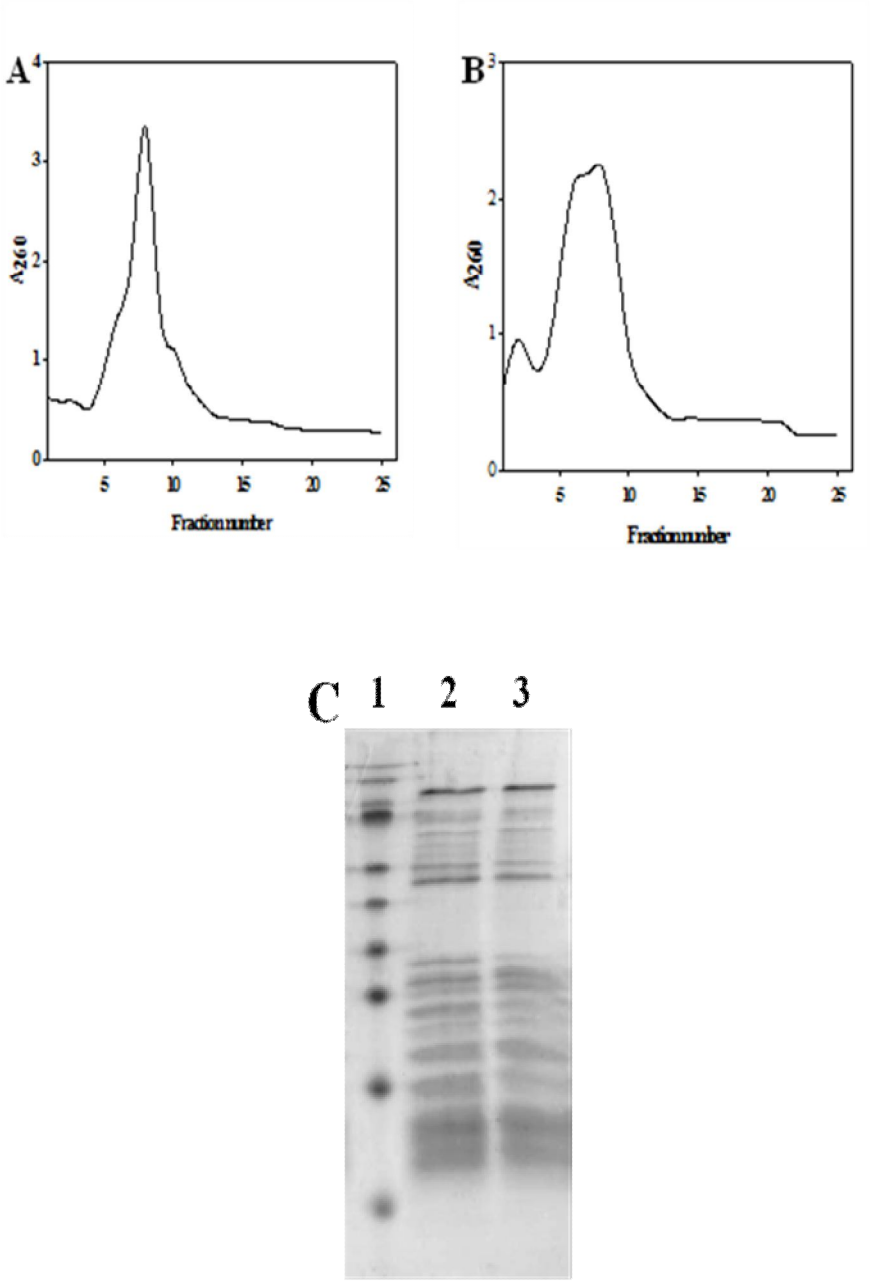


Fig: 14. Sucrose density gradient profiles of 500 mM sodium chloride dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosomes and lane 3: Stationary phase ribosomes.

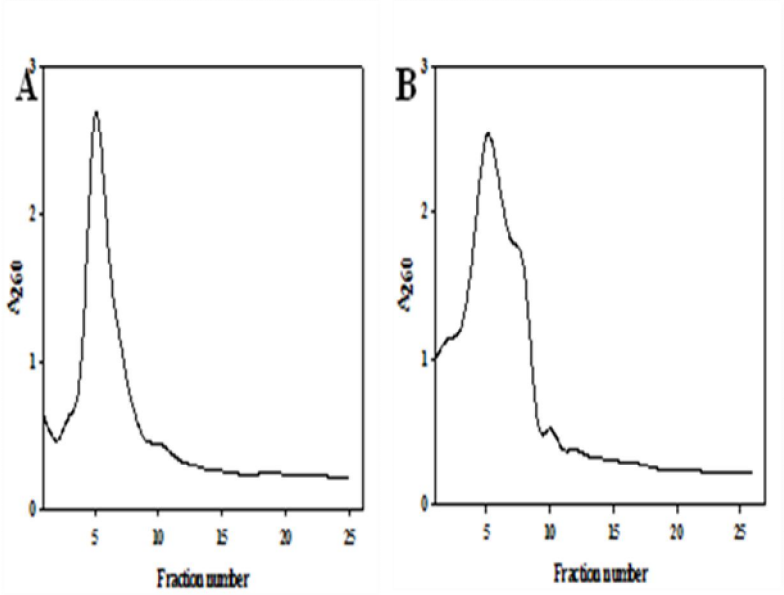


Fig: 15. Sucrose density gradient profiles of 500 mM potassium glutamate dialyzed *H. eurihalina* ribosomes on 10-30% gradients. A. Logarithmic phase ribosomes. B. Stationary phase ribosomes. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase ribosomes and lane 3: Stationary phase ribosomes.

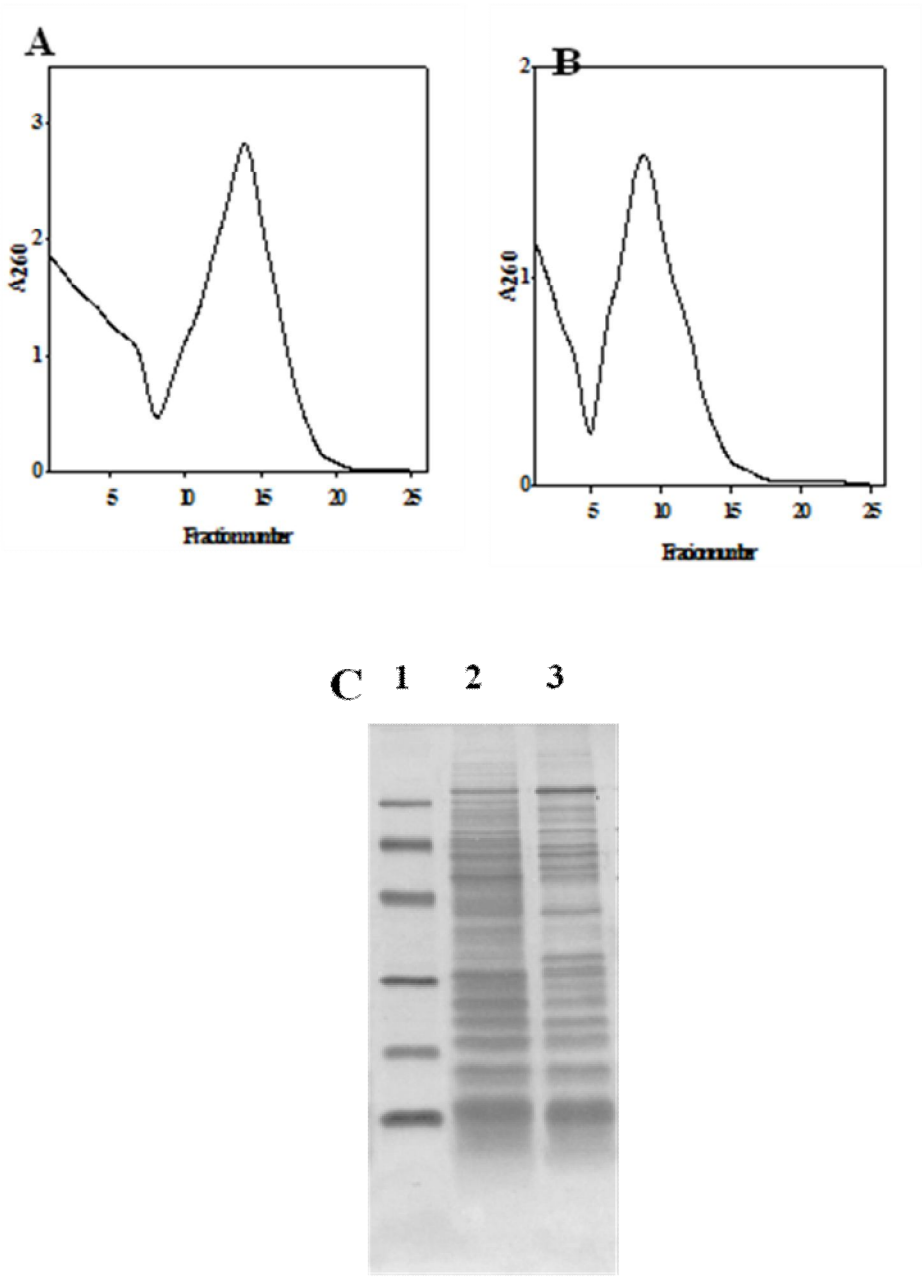


Fig: 16. Sucrose density gradient profiles of undialyzed *H. eurihalina* S-30 on 10-30% gradients. A. Logarithmic phase S-30. B. Stationary phase S-30. C. SDS-PAGE analysis of peak fractions. Lane 1: Molecular weight marker, lane 2: logarithmic phase S-30 and lane 3: Stationary phase S-30.

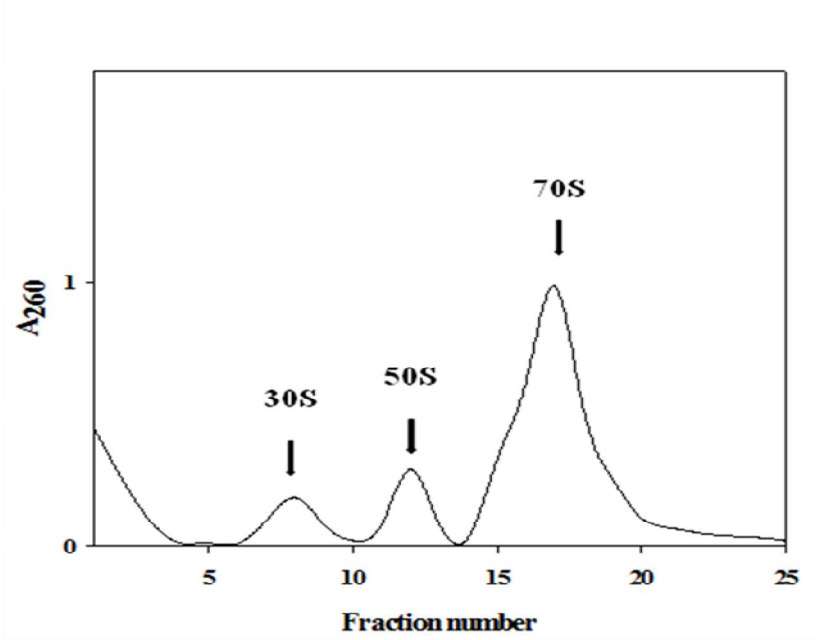


Fig: 17. Sucrose density gradient profiles of undialyzed *E. coli* S-30 on 5-20% gradients showing native ribosomal subunits and 70 S ribosomal peaks.

ii. Hydrophobic interaction chromatography for the dissociation of ribosomes:

Hydrophobic interaction chromatography involves the treatment of ribosomes with high salt and subsequent chromatography on sepharose column using reverse salt gradient. This method has been successfully used to separate ribosomal subunits from *E. coli* (Krillov, 1978). Same procedure was followed in the case of *H. eurihalina* ribosomes. Logarithmic and stationary phase *H. eurihalina* ribosomes were eluted on Sepharose-4B column using reverse salt gradient as described in methods. The Sepharose-4B reverse salt gradient elution profiles of *H. eurihalina* ribosomes were shown in Fig: 18A & 19A. Logarithmic phase ribosomal elution profile showed a very small peak A and a broad peak B. Stationary phase ribosomal elution profile showed a sharp peak A and a broad peak B which may represent heterogeneous unfolded ribosomal particles. SDS-PAGE analysis of peak fractions was shown in Fig: 18B & 19B. Despite the presence of different peaks in the elution profiles, SDS-PAGE analysis clearly shows the undissociated ribosomal band pattern.

These results show that even hydrophobic interaction chromatography of ribosomes with reverse salt gradient does not result in dissociation of ribosomes indicating the strong association of ribosomal subunits in *H. eurihalina*. This procedure was found to be not applicable for *H. eurihalina* ribosomes.

Light scattering studies:

Light scattering studies have been employed to study ribosomal subunit association and dissociation of 70 S ribosomes to subunits. Light scattering studies were carried to observe the presence of magnesium dependent subunit dissociation in *H. eurihalina* ribosomes. These studies were carried out according to Zitomer and Flaks (1972) with few modifications. Maximum scattering was taken as 100% 70 S formation.

The dependence of light scattering on the increasing concentration of ribosomes was checked as shown in Fig: 20. Relative scattering was found to be linear up to the concentration of 6 A_{260}/ml, 3.2 A_{260}/ml and 2.5 A_{260}/ml respectively for *E. coli, H. eurihalina* logarithmic and stationary phase ribosomes. In subsequent studies, concentration of ribosomes used was 4 A_{260}/ml, 2 A_{260}/ml and 1.6 A_{260}/ml for *E. coli, H. eurihalina* logarithmic and stationary phase ribosomes respectively.

The relative light scattering obtained for *E. coli* ribosomes was shown in Fig: 21A. Decrease in the amount of light scattered indicate dissociation of ribosomes into subunits. Fig: 21B & C show the relative light scattering obtained for *H. eurihalina* logarithmic and stationary phase ribosomes. Even at 1 mM magnesium ion concentration, decrease in the amount of light scattered was not observed (both logarithmic and stationary phase ribosomes). These results indicate that *H. eurihalina* ribosomes (logarithmic and stationary phase) failed to dissociate at low magnesium concentrations. This data further supports the results obtained by sucrose density gradient studies and hydrophobic interaction chromatography. *H. eurihalina* ribosomes do not dissociate into subunits under variety of conditions.

Structural studies using different probes

Conformation of moderate halophilic ribosomes, was studied using specific probes such as pancreatic RNase A (specifically acts on single stranded RNA). The stability of halophilic ribosomes was studied in presence of different salts in varying concentrations and their resistance to urea treatment. The stability of moderate halophilic ribosomes was compared with that of the mesophilic (*E. coli*) ribosomes under similar conditions.

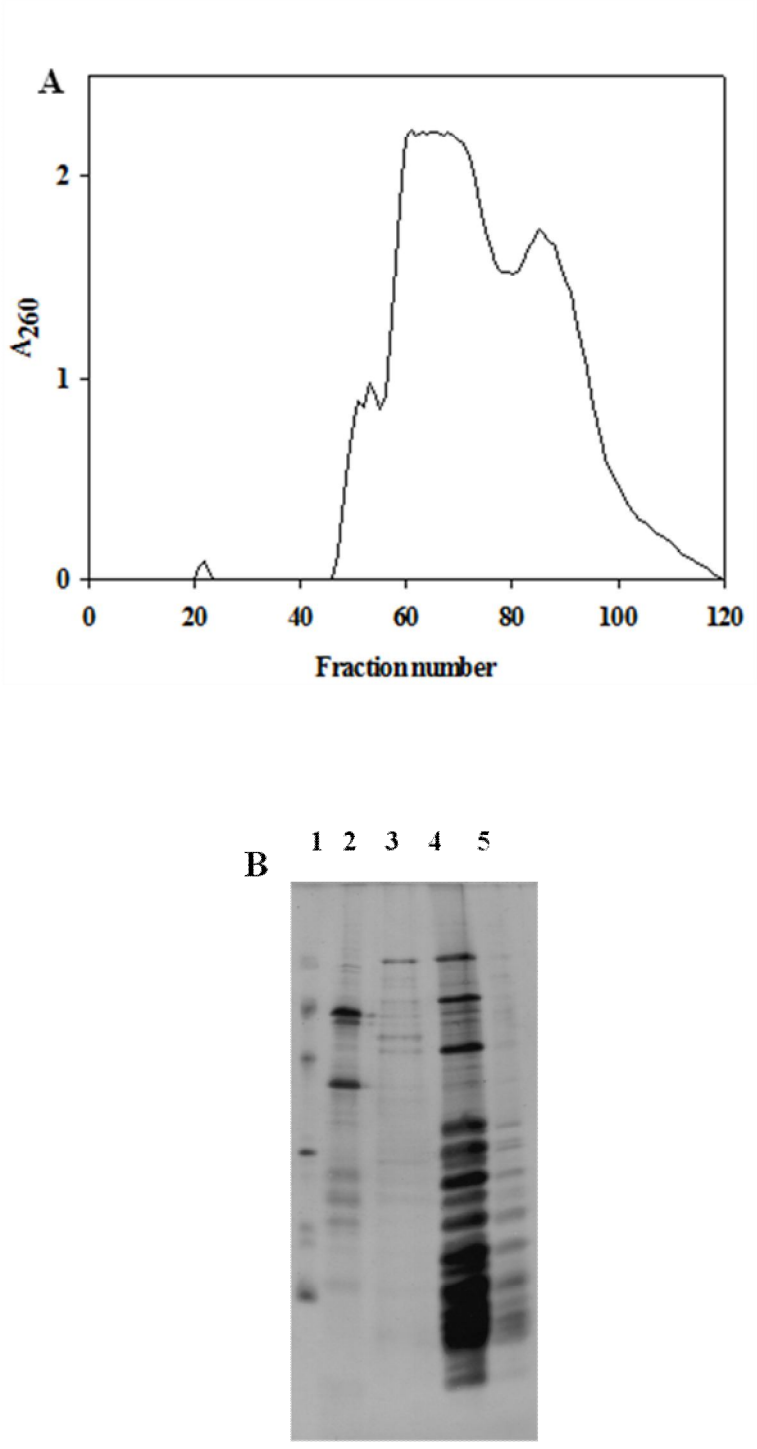
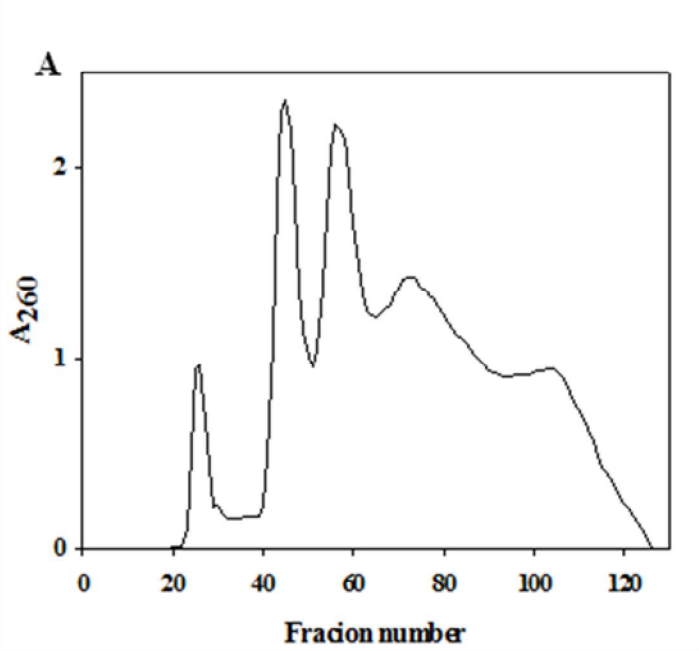


Fig: 18. Reverse salt gradient elution of *H. eurihalina* logarithmic phase ribosomes. A. Elution profile. B. Lane 1: Molecular weight marker, lanes 2-5: fractions 22, 55, 62 and 85 respectively.



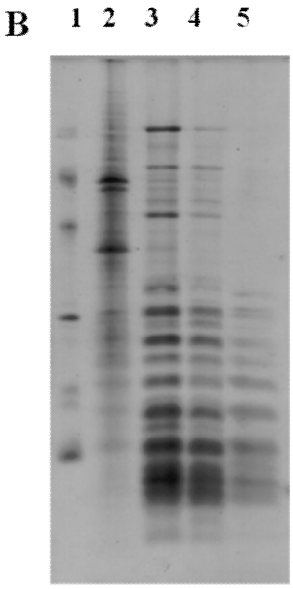


Fig: 19. Reverse salt gradient elution of *H. eurihalina* stationary phase ribosomes. A. Elution profile. B. Lane 1: Molecular weight marker, lanes 2-5: fractions 26, 45, 57 and 72 respectively.

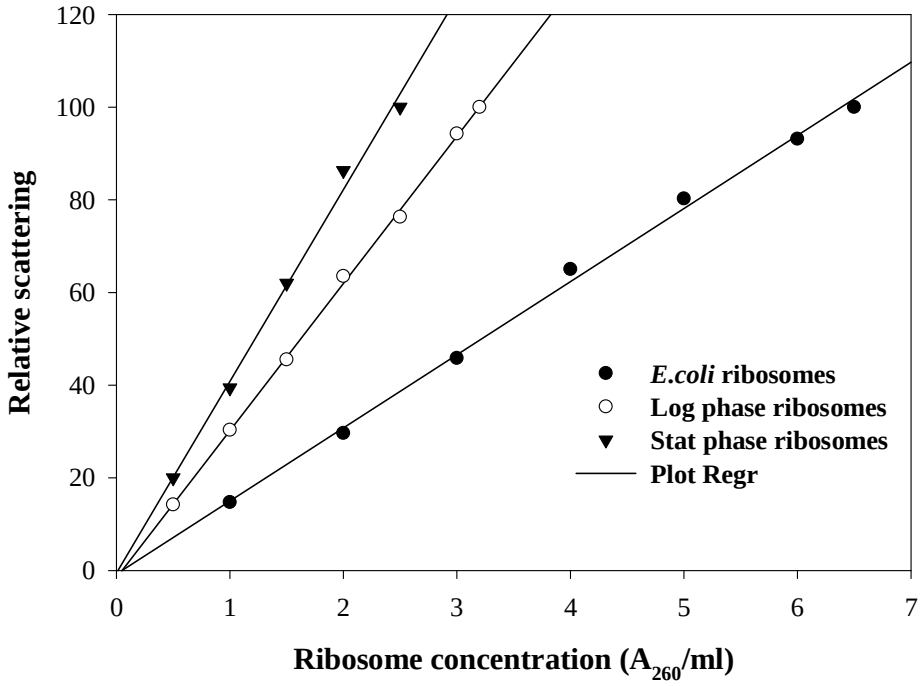


Fig: 20. Intensity of light scattering as a function of ribosome concentration. Light scattering was measured as described in methods section.

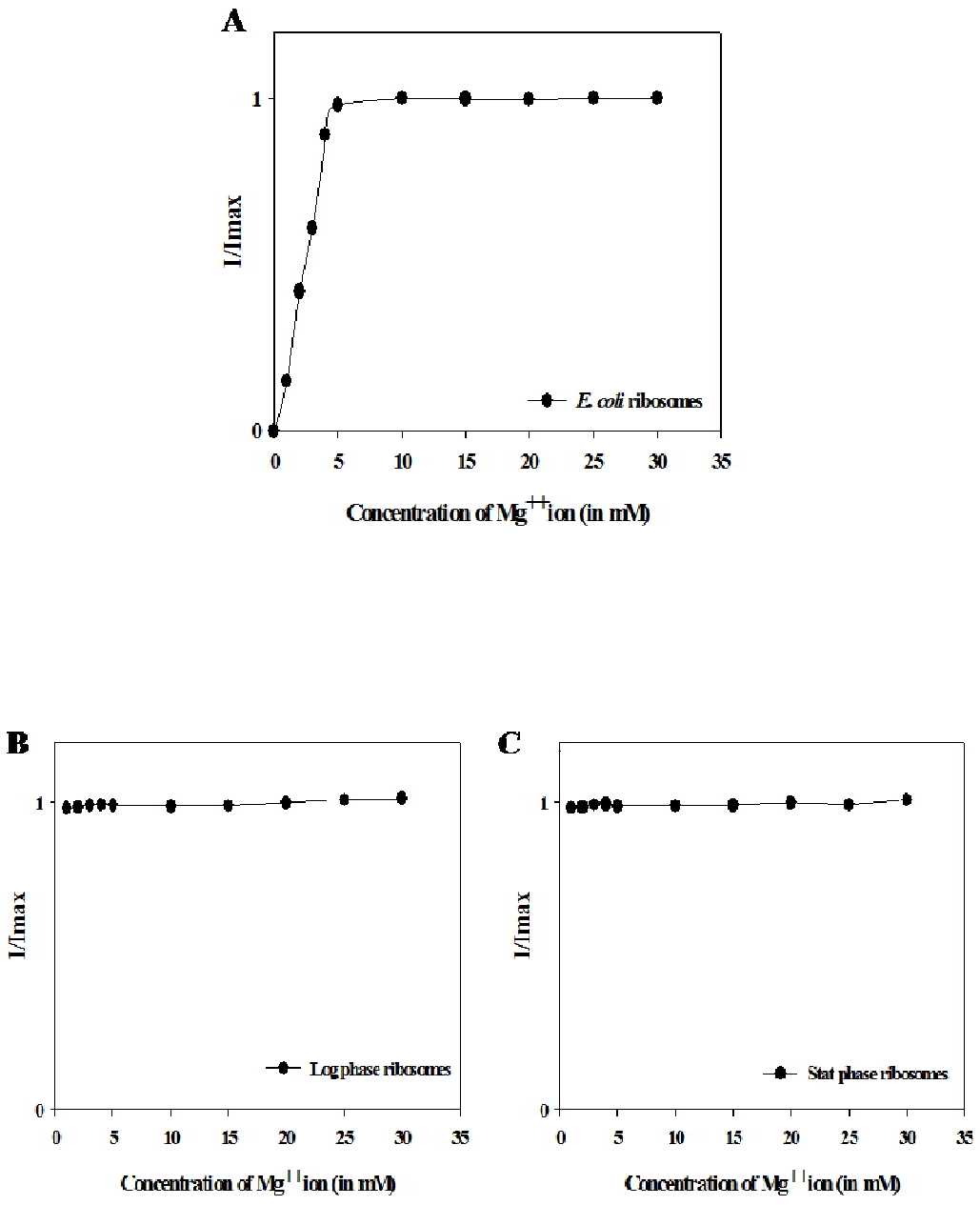


Fig: 21. Effect of magnesium on the subunit dissociation equilibrium of ribosomes. A. *E. coli* ribosomes, B. *H. eurihalina* logarithmic phase ribosomes, C. *H. eurihalina* stationary phase ribosomes.

Measurement of the degradation of ribosomes by RNase A using Spectrophotometry:

Low magnesium concentrations were used in all these experiments at room temperature, as this causes large change in the conformation of the treated and untreated ribosomes. The change in the conformation (unfolding) was detected by their susceptibility to RNase A.

RNase A hydrolyzes the single stranded regions of r-RNA that are available for its action in the ribosome. RNase A degradation causes increase in the absorbance of the ribosomal solution (hyperchromicity) which was measured in a spectrophotometer.

Measurement of the degradation of ribosomes in the presence of RNase A was done as described in methods. The measurement was made immediately after adding the enzyme until degradation reaches a plateau (Fig: 22A, B & C).

These results show that *E. coli* ribosomes required 0.3 µg of RNase A to reach maximum amount of degradation. However, *H. eurihalina* logarithmic phase ribosomes required 26 fold more concentration of RNase A i.e., 8 µg to reach maximum amount of degradation, whereas, stationary phase ribosomes required 10 fold more i.e., 4 µg to reach maximum amount of degradation.

However, the maximum hydrolysis obtained for *H. eurihalina* logarithmic and stationary phase ribosomes was less compared to maximum hydrolysis obtained for *E. coli* ribosomes. These results show that moderately halophilic ribosomes are much more stable to RNase A treatment in comparison to *E. coli* ribosomes. *H. eurihalina* stationary phase ribosomes are more susceptible to hydrolysis by RNase A than the logarithmic phase ribosomes, suggesting stationary phase ribosomes are more unfolded. This may be due to missing of some primary rRNA binding proteins (eg: L2).

Effect of magnesium on the structure of ribosomes:

The degradation of ribosomes was measured in UV-Visible spectrophotometer, at different magnesium concentrations, by the increase in absorbance at 260 nm (or the hyperchromicity obtained) in the presence of RNase A. As seen from the Fig: 23A, B & C degradation of ribosomes was more at low magnesium concentrations i.e., 0.1 mM magnesium concentration. The degradation was less at higher magnesium ion concentration in case of both *E. coli* and *H. eurihalina* (logarithmic and stationary phases) ribosomes. However, it should be noted that in these experiments also *E. coli* ribosomes were hydrolyzed with many fold less concentration of RNase A.

These results indicate that moderate halophilic ribosomes are more compact and more resistant to degradation by RNase A at low magnesium concentration compared to mesophilic ribosomes.

Effect of different salts and their concentrations on RNase A sensitivity of *H. eurihalina* ribosomes:

These experiments were carried out to observe the effect of salts on moderately halophilic ribosomes. *H. eurihalina* logarithmic and stationary phase ribosomes were dialyzed against varying concentrations of different salts and their susceptibility to RNase A was studied.

A control experiment was carried out with *E. coli* ribosomes and all the salts used in the experiment, to observe the effect of high salt concentration on RNase A activity (Fig: 24A). These results show that even at highest salt concentrations (0.5 M) used in the following experiments, none of the salts showed any inhibitory effect on enzymatic activity.

Effect of ammonium chloride:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M ammonium chloride were given in Fig: 24B, C & D respectively. Both logarithmic and stationary phase ribosomes showed less susceptibility in presence of 0.25 M and 0.5 M ammonium chloride compared to 0.1 M ammonium chloride.

Effect of potassium glutamate:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M potassium glutamate were shown in Fig: 25A, B & C respectively. Both logarithmic and stationary phase ribosomes showed less susceptibility at 0.25 M potassium glutamate concentration compared to 0.5M and 0.1 M potassium glutamate. The ribosomes in low and high salt concentrations reached the same level of degradation with less concentration of RNase A in comparison to ribosomes in 0.25 M salt. The optimal concentration of potassium glutamate that minimizes the hydrolysis by RNase A is around 0.25 M concentration.

Effect of sodium chloride:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M sodium chloride were shown in Fig: 26A, B & C respectively. Both logarithmic and stationary phase ribosomes showed maximum degradation with less concentration of RNase A. Protection against RNase A degradation was not observed at any salt concentration. These results show that sodium chloride has an unfolding effect on the ribosomes.

Effect of potassium chloride:

The degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes in the presence of buffers containing 0.1 M, 0.25 M and 0.5 M potassium chloride were shown in Fig: 27A, B & C respectively. Both logarithmic and stationary phase ribosomes showed less susceptibility in presence of potassium chloride. Protection was maximum at 0.25 M potassium chloride concentration followed by 0.5 M compared to 0.1 M potassium chloride.

Among the four different salts tested, potassium chloride was more effective in stabilizing the structure of the ribosomes followed by ammonium chloride. Both *H. eurihalina* logarithmic and stationary phase ribosomes showed less susceptibility to RNase A in presence of optimum concentration (0.25 M) of potassium chloride. It should be mentioned here that in moderate halophiles, the dominant intracellular monovalent cation is K^+.

In presence of 0.25 M potassium glutamate ribosomes required more concentration of RNase A to reach maximum degradation compared to 0.1 M and 0.5 M potassium glutamate. Sodium chloride is the least effective among the four salts used, thus resulted in maximum degradation of both logarithmic and stationary phase ribosomes with less concentration of RNase A.

Effect of polyamines on the structure of ribosomes:

Polyamines play important role in maintaining the structure of nucleic acid and nucleoprotein complexes. We studied the effect of polyamines on the structure of ribosomes using RNase A as a probe. Ribosomes treated with polyamines showed less susceptibility to RNase A compared to untreated ribosomes (Fig: 28 and 29 A, B & C). However, polyamine treatment showed more protective effect on *E. coli* ribosomes in comparison to halophilic ribosomes.

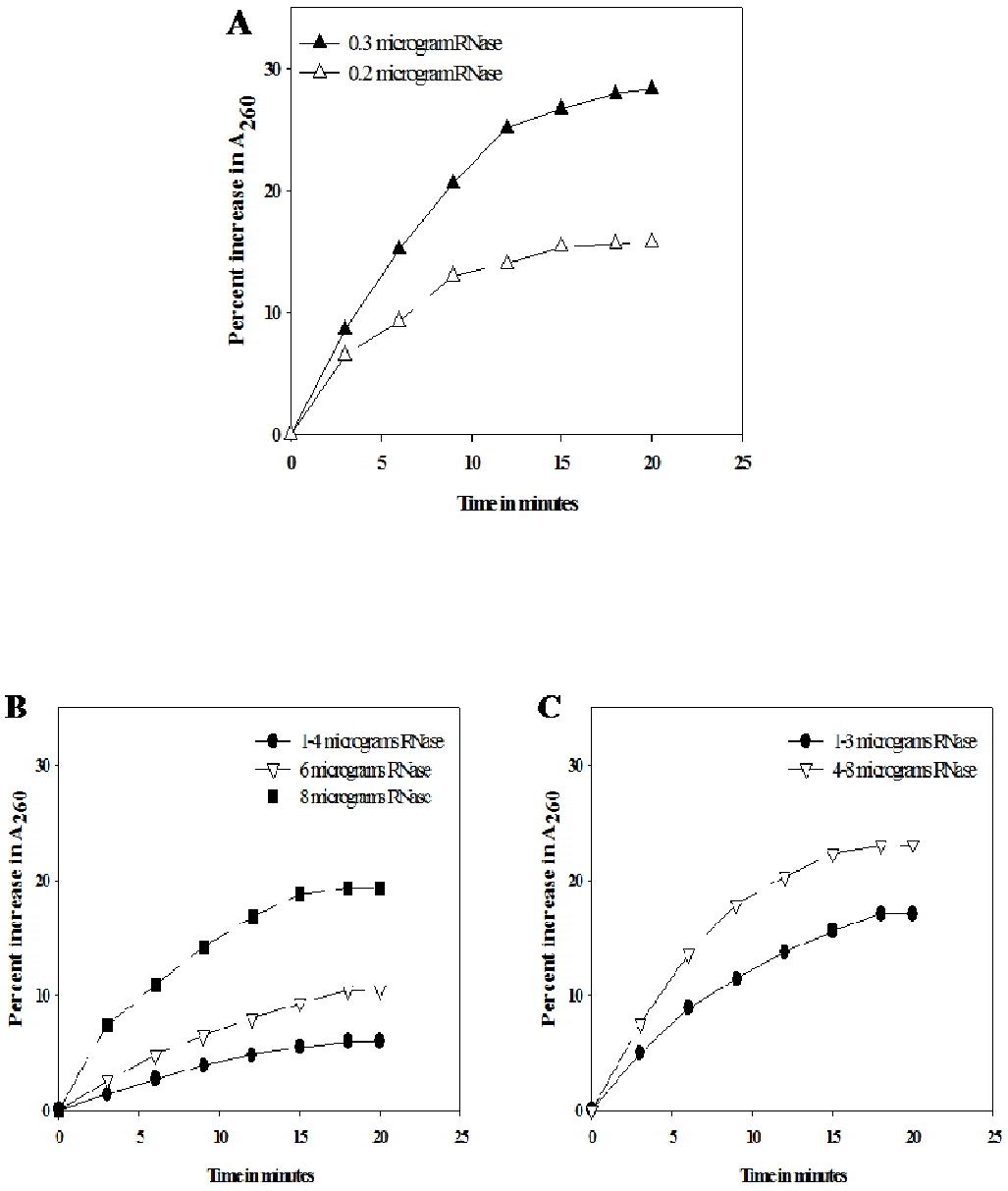


Fig: 22. Degradation profiles of ribosomes in the presence of different concentrations of RNase A at 0.1 mM Mg^{2+} concentration. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase. C. *H. eurihalina* stationary phase ribosomes.

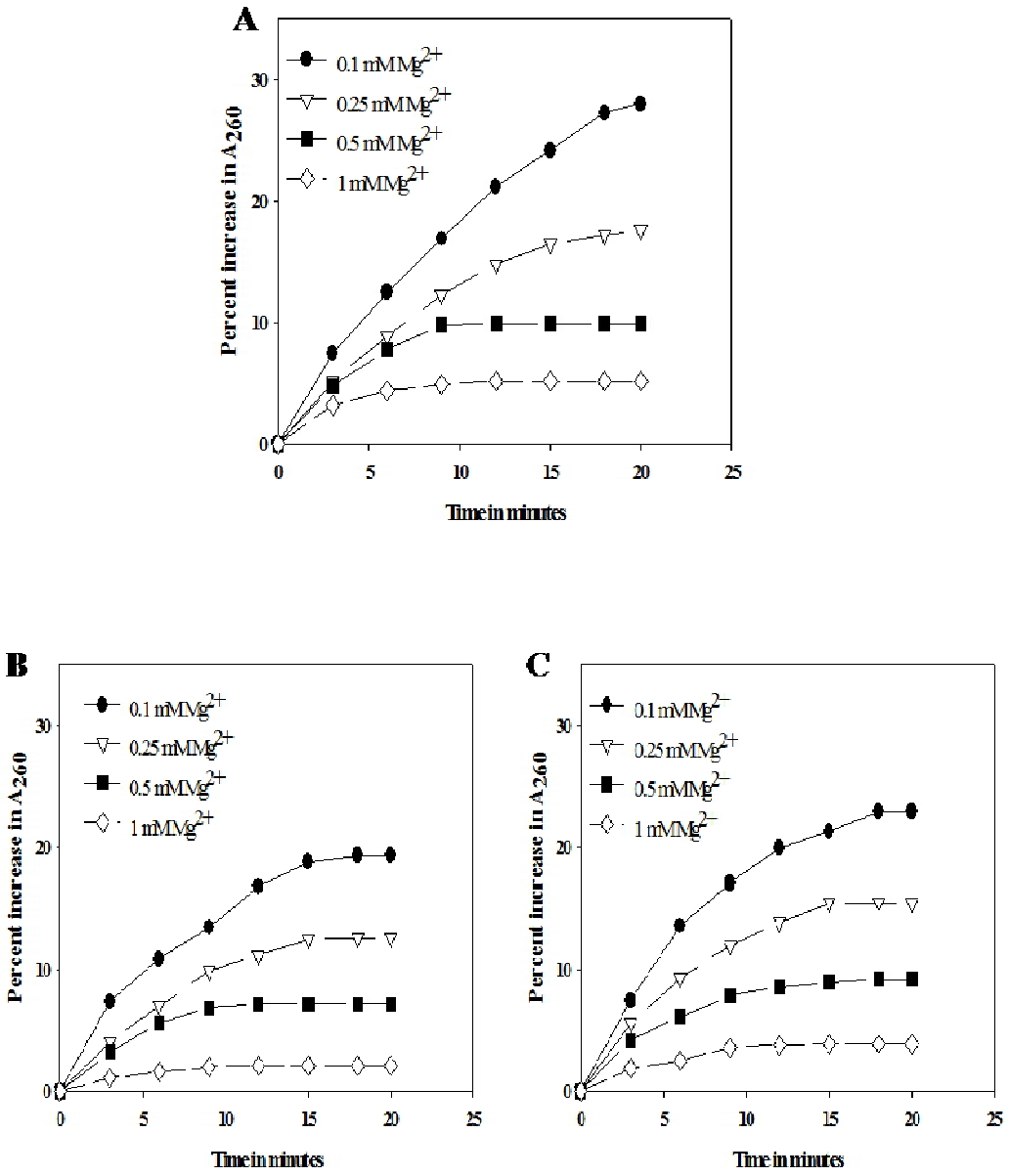


Fig: 23. Degradation profiles of ribosomes at different magnesium concentrations in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase. C. *H. eurihalina* stationary phase ribosomes.

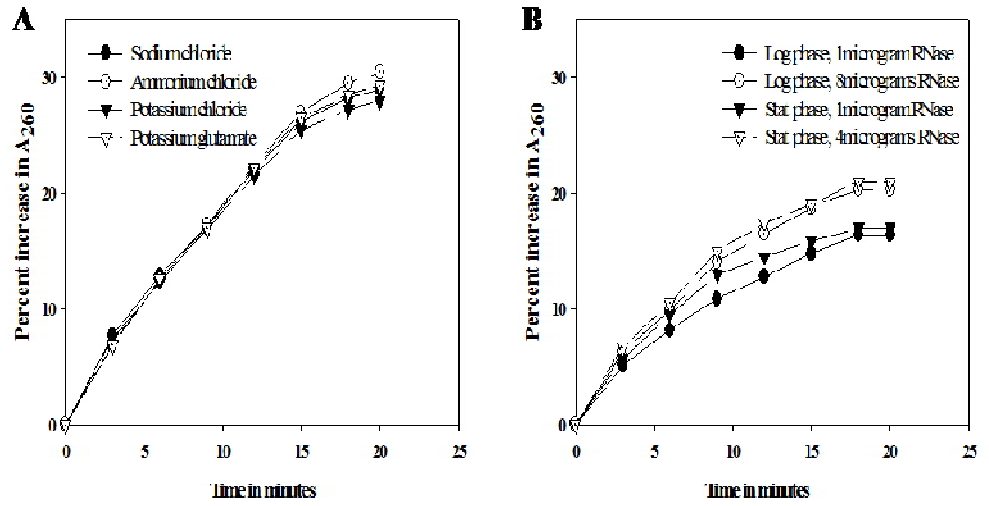


Fig: 24A. Control experiment carried out with *E. coli* ribosomes in presence of different salts.

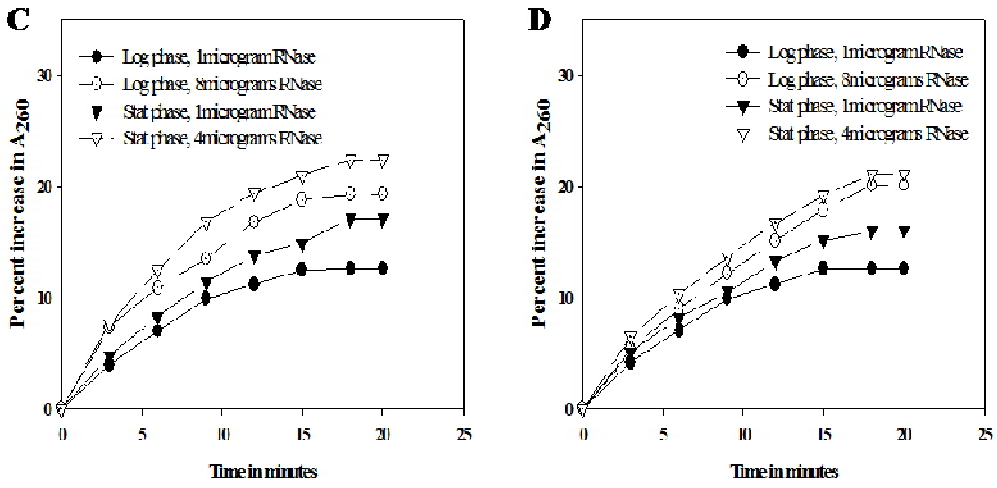


Fig: 24. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different ammonium chloride concentrations in the presence of RNase A. B. 0.1 M, C. 0.25 M and D. 0.5 M ammonium chloride.

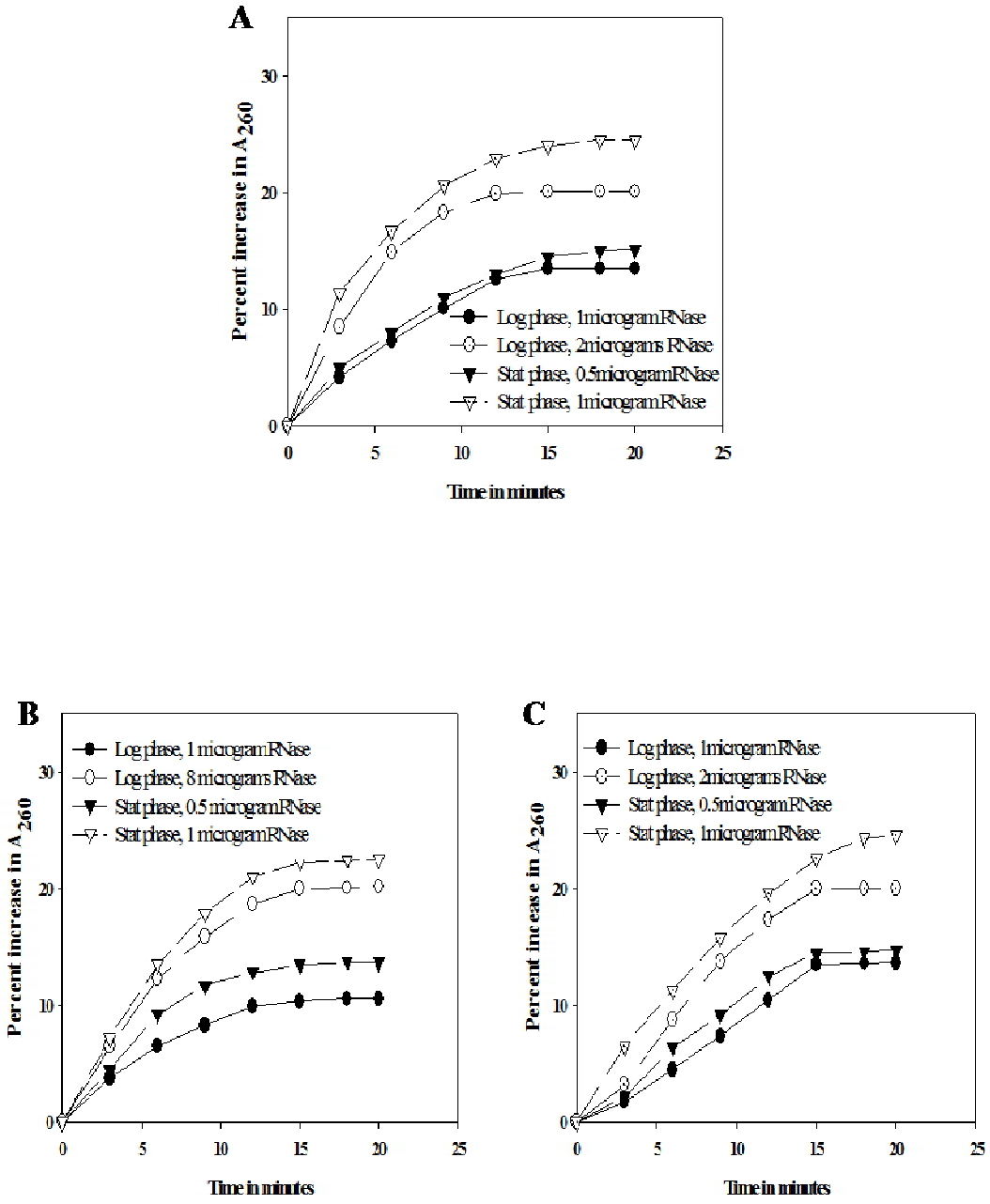


Fig: 25. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different potassium glutamate concentrations in the presence of RNase A. A. 0.1 M, B. 0.25 M and C. 0.5 M potassium glutamate.

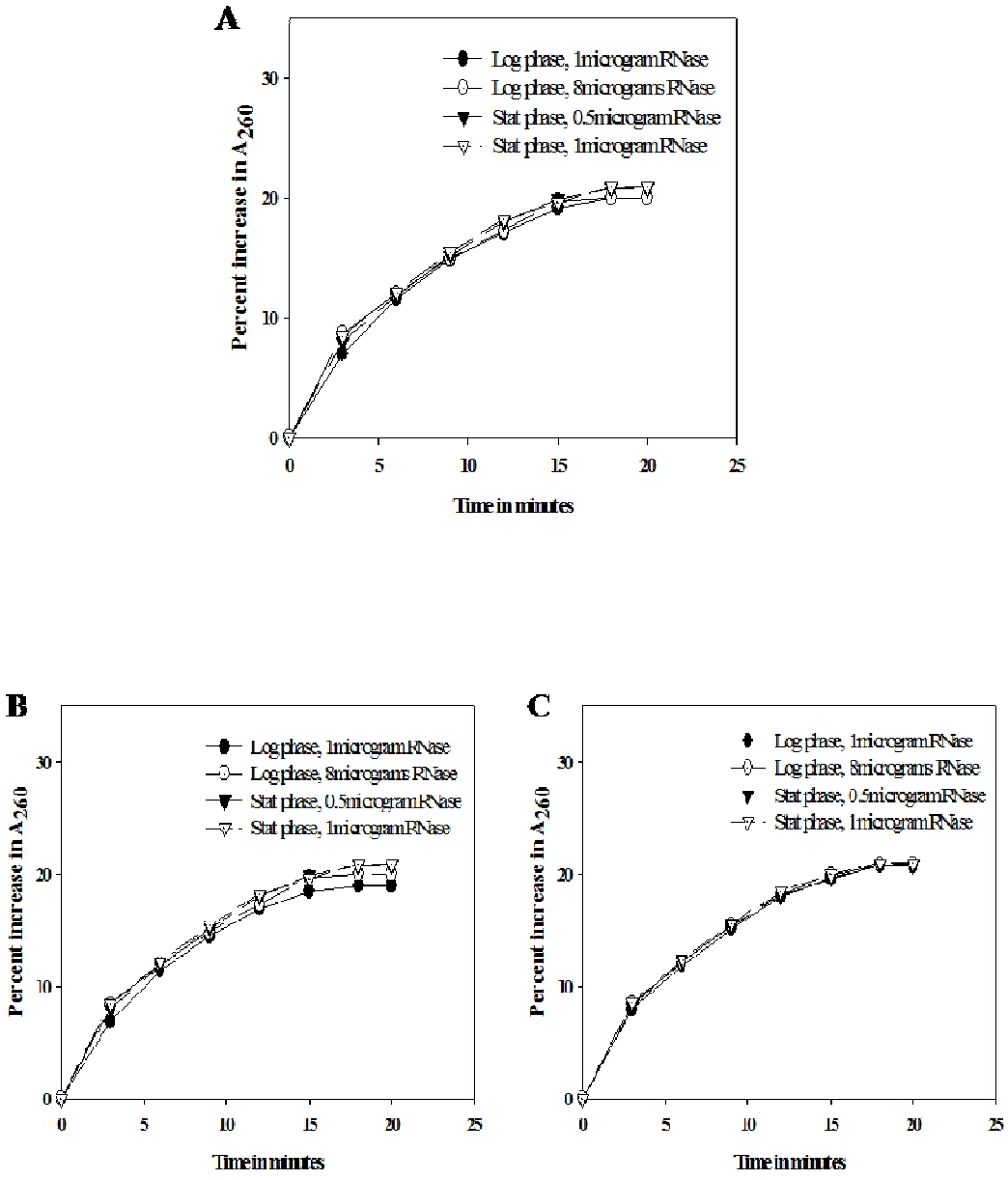


Fig: 26. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different sodium chloride concentrations in the presence of RNase A. A. 0.1 M, B. 0.25 M and C. 0.5 M sodium chloride.

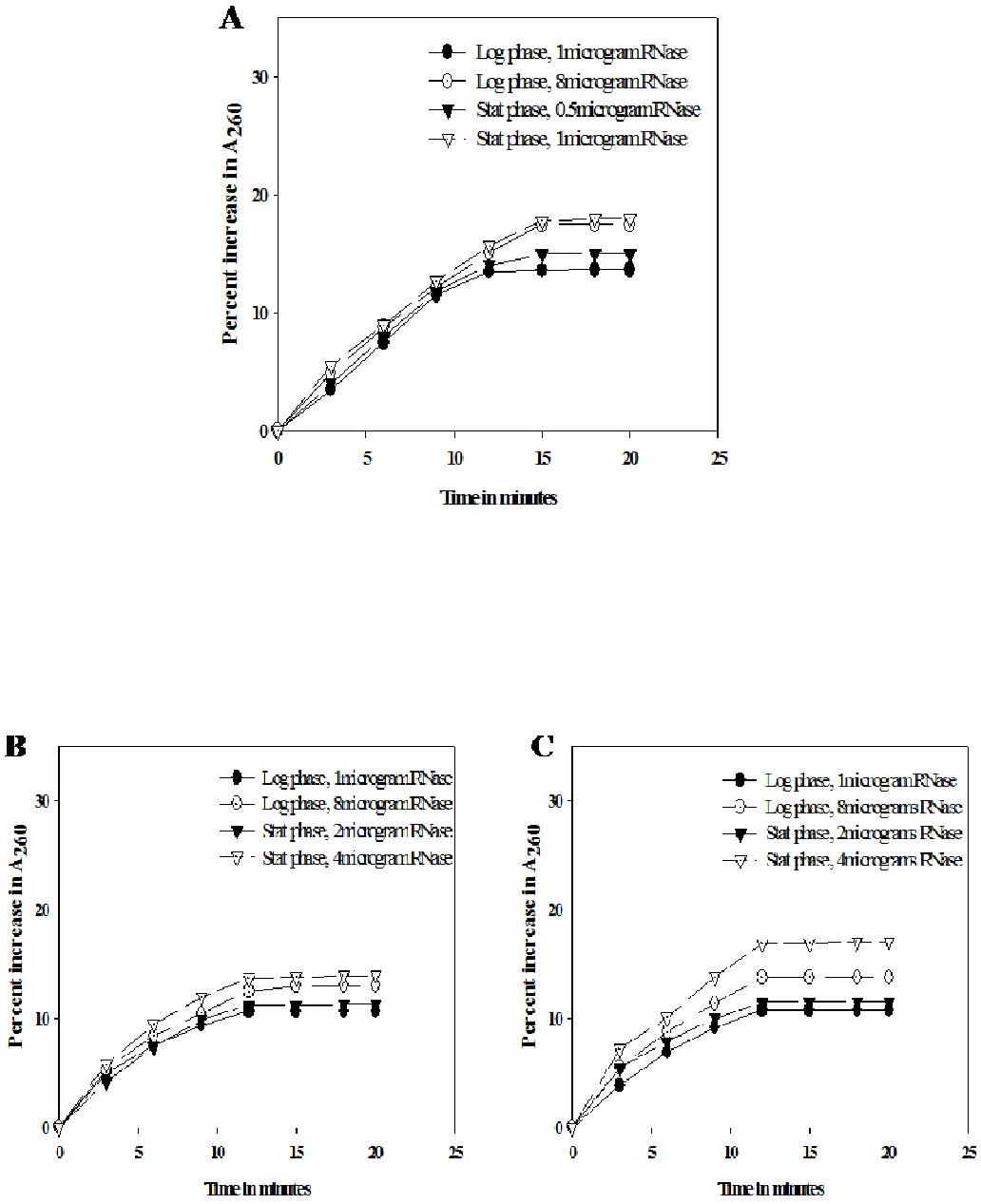


Fig: 27. Degradation profiles of *H. eurihalina* logarithmic and stationary phase ribosomes at different potassium chloride concentrations in the presence of RNase A. A. 0.1 M, B. 0.25 M and C. 0.5 M potassium chloride.

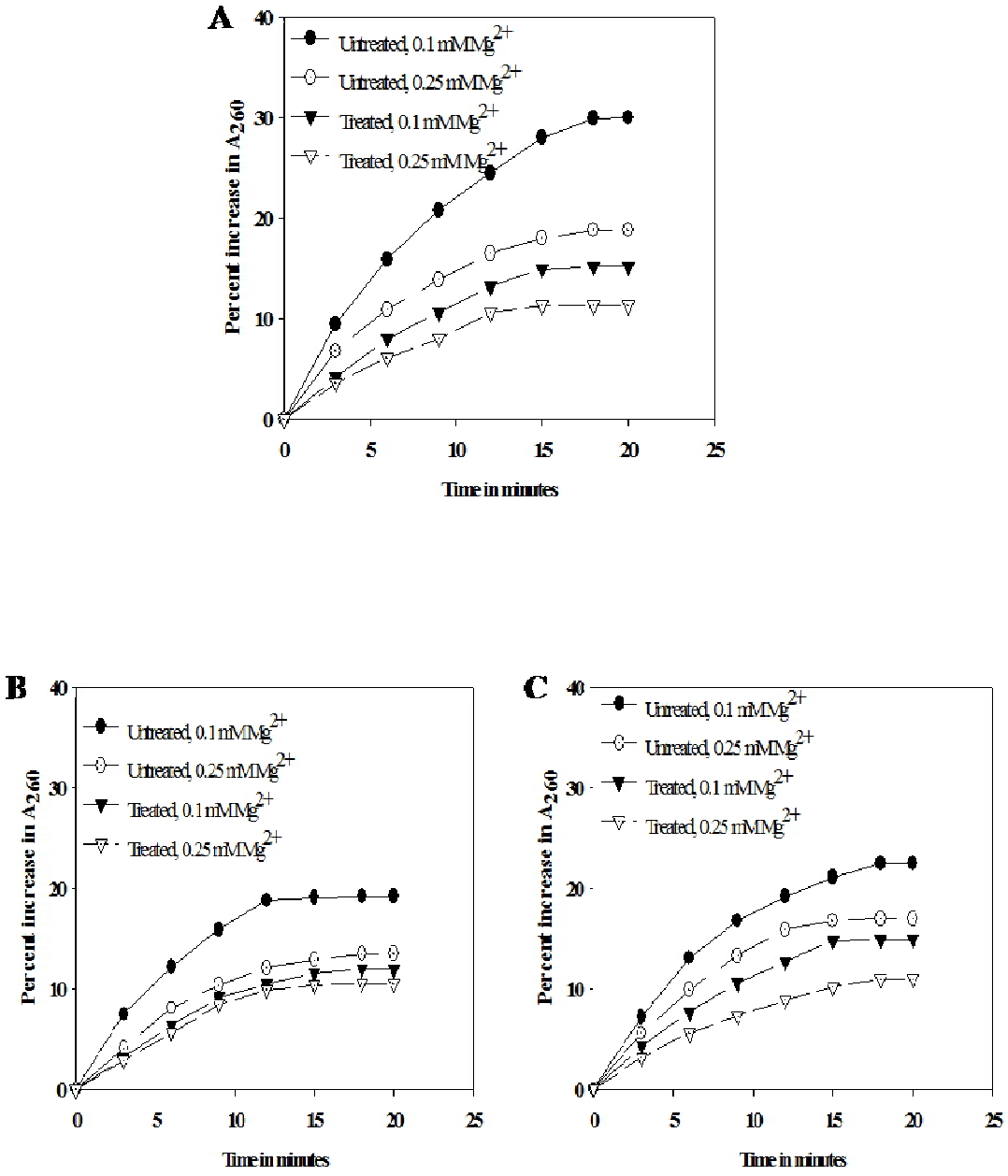


Fig: 28. Degradation profiles of spermine treated ribosomes in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase ribosomes. C. *H. eurihalina* stationary phase ribosomes.

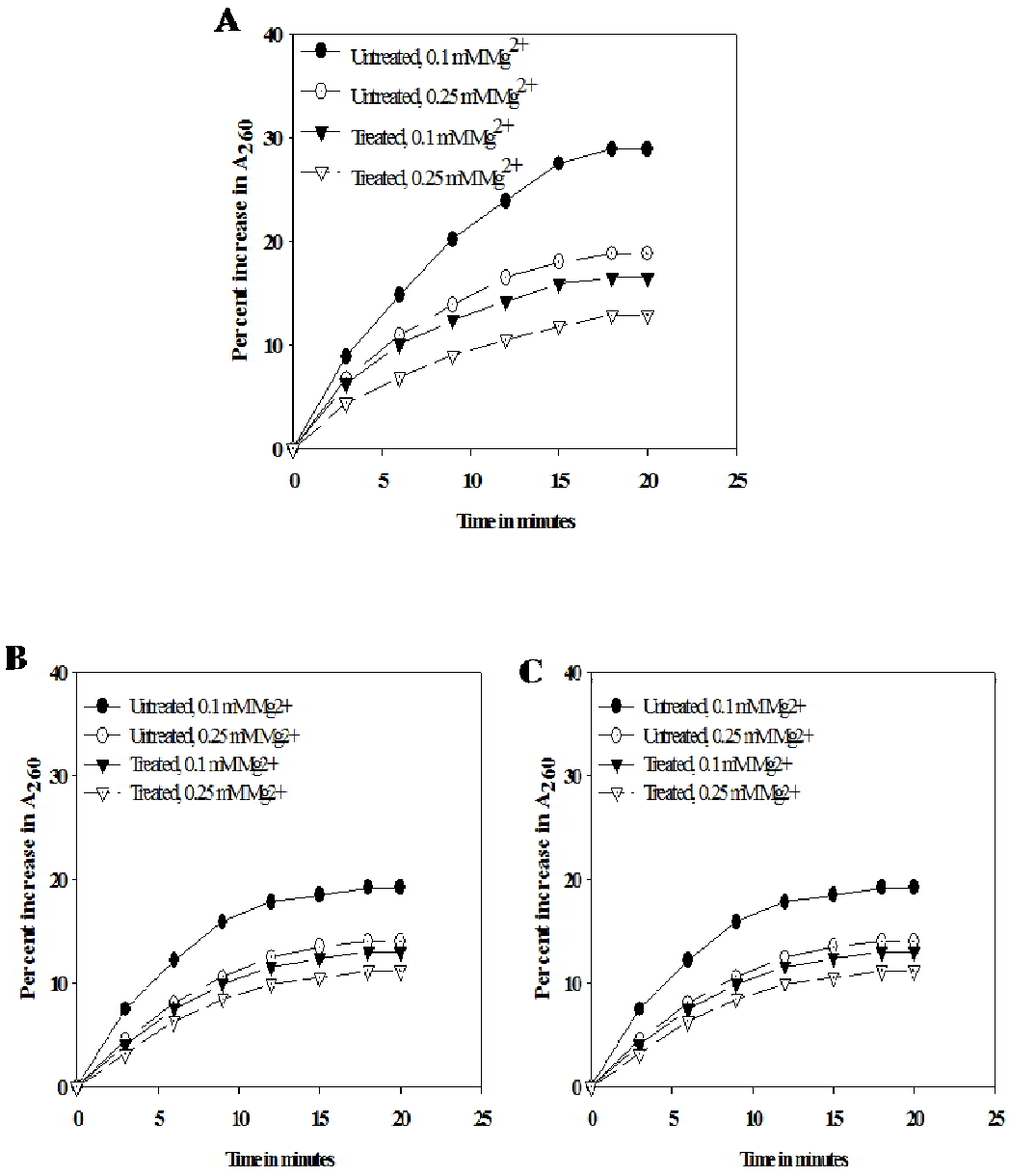


Fig: 29. Degradation profiles of spermidine treated ribosomes in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase ribosomes. C. *H. eurihalina* stationary phase ribosomes.

Spermine was more effective in stabilizing the ribosome structure compared to spermidine as the concentration of spermidine required to offer same extent of protection was double the concentration of spermine. These results also show that although polyamine treatment resulted in marginal increase of resistance towards RNase A treatment, halophilic ribosomes are inherently resistant to RNase A treatment.

Effect of EtBr on the structure of ribosomes:

Accessibility of rRNA to intercalating agents and the effect of such agents on the structure of ribosomes were also studied. EtBr treatmet of *H. eurihalina* ribosomes was carried out to see the effect of intercalating agents on halophilic ribosomes in comparison to mesophilic ribosomes. The degradation profiles of EtBr treated *E. coli*, *H. eurihalina* logarithmic and stationary phase ribosomes were given in Fig: 30A, B & C respectively. Results show that EtBr treated ribosomes were little more susceptible to RNase A activity compared to untreated ribosomes. *H. eurihalina* ribosomes treated with EtBr were more resistant to RNase A activity in comparison to the similarly treated *E. coli* ribosomes (Fig: 30A, B & C). *H. eurihalina* stationary phase ribosomes treated with EtBr also showed equal resistance to RNase A activity on par with logarithmic phase ribosomes.

These results show that *H. eurihalina* ribosomes were more resistant to intercalation and the distortions caused by EtBr when compared to *E. coli* ribosomes. Alternatively, these results also indicate that *H. eurihalina* ribosomes are organized in such a way that their rRNA was not available for intercalations by EtBr.

Ribosome unfolding studies with urea:

We have investigated the effects of urea on the structural organization of *E. coli* and *H. eurihalina* ribosomes, which would provide some indication on the mechanism of the stability of ribosomes.

The unfolding of ribosome structure on addition of urea was detected by the increase in the absorbance of the solution. Ribosomes were treated with different concentrations of urea as described in methods. Fig: 31A shows the absorption spectrum of *E. coli* and *H. eurihalina* logarithmic and stationary phase ribosomes in respective normal buffers, recorded from 220 to 300 nm at room temperature.

Absorption spectra were obtained at increasing concentration of urea, 2 M (Fig: 31B), 4 M (Fig: 31C), 6 M (Fig: 31D) and 8 M (Fig: 31E). At lower urea concentrations (2 M and 4 M) there was not much difference in the absorption spectra of *E. coli* and *H. eurihalina* ribosomes. However, at these urea concentrations increased absorption was seen at around 230 nm in the case of *H. eurihalina* ribosomes.

Striking differences in the unfolding of ribosomes by urea was observed at 6 M urea. At this concentration, *H. eurihalina* logarithmic phase ribosomes were unaffected by urea where as *E. coli* ribosomes unfolded completely. The stationary phase ribosomes also unfolded at this urea concentration. At 8 M urea concentration *H. eurhalina* logarithmic phase ribosomes were marginally less unfolded than *E. coli* ribosomes.

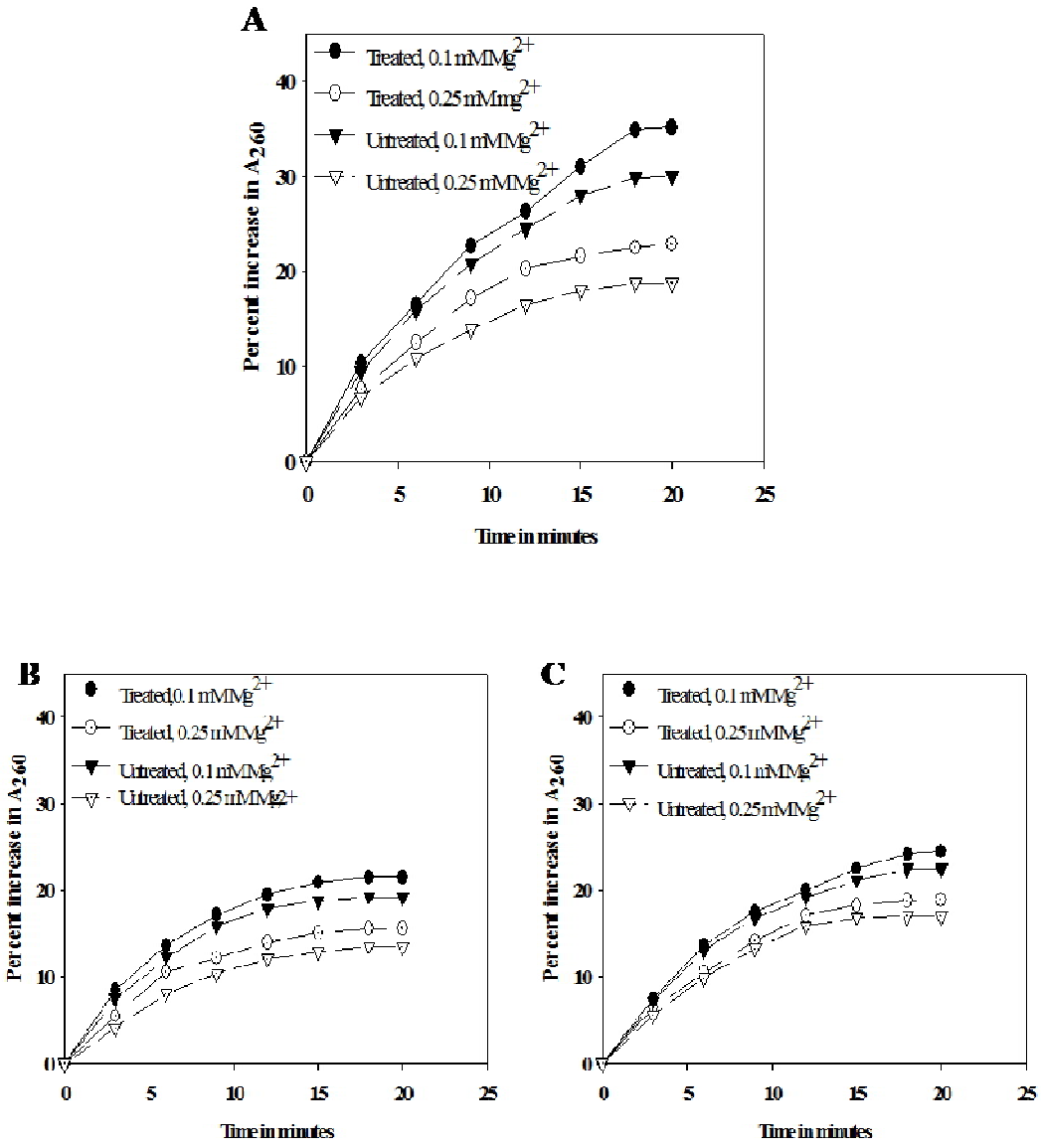


Fig: 30. Degradation profiles of EtBr treated ribosomes in the presence of RNase A. A. *E. coli* ribosomes. B. *H. eurihalina* logarithmic phase ribosomes. C. *H. eurihalina* stationary phase ribosomes.

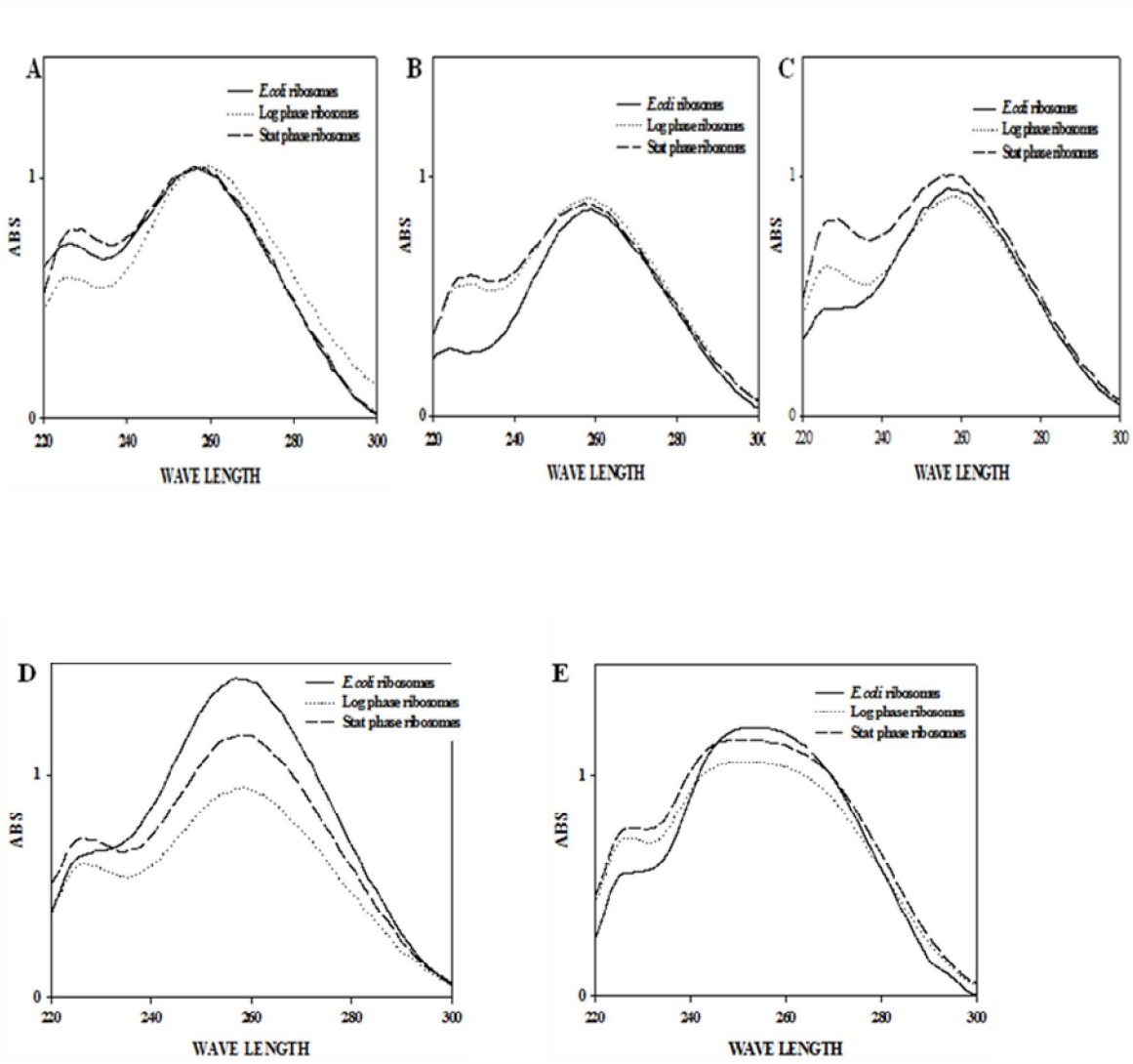


Fig: 31. Absorption spectra of *E. coli, H. eurihalina* logarithmic and stationary phase ribosomes treated with different concentrations of urea. A. Ribosomes in respective normal buffers (without urea). B. 2 M urea treated ribosomes. C. 4 M urea treated ribosomes. D. 6 M urea treated ribosomes. E. 8 M urea treated ribosomes.

Proteomic studies of ribosomal proteins

We have carried out proteomic studies of *H. eurihalina* ribosomal proteins to identify their identity as described in methods section. We wanted to perform proteomic studies of *H. eurihalina* ribosomal proteins after two-dimensional gel electrophoresis of 30 S and 50 S ribosomal proteins separately. However, we were unable to do that way as we failed to find conditions for the dissociation of 70 S ribosomes. Hence we performed proteomic studies after two- dimensional gel electrophoresis of 70 S proteins.

The data generated was used to search prokaryotic, eukaryotic and archaeal domains in Swiss-Prot and NCBInr databases using MASCOT search software. The identified peptide sequences were used to search non-redundant proteins sequences database for homologous protein sequences using BLAST. The FASTA-format protein sequences thus obtained were used to carry out multiple sequence alignments using ClustalX2.1.

Numbering of ribosomal proteins:

For carrying out proteomic studies, numbering of ribosomal proteins was given. Numbers were given for ribosomal spots based on their respective electrophoretic mobilities on two dimensional gels. The numbering of logarithmic and stationary phase ribosomal proteins was given in Fig: 32 A & B respectively.

The results showed that in logarithmic phase gels 65 spots were present. In stationary phase gels, some of the spots were found to be missing. The missed spots were 4, 5, 9, 14, 38, 44, 48, 56 and 58. Spots 7, 15, 24, 26, 27, 39, 40, 42, 49, 53, 57, 59, 61, 63, 64 and 65 have shown substantial decrease in protein concentration. Some of the spots like 1, 2, 3, 8, 16, 17, 23, 25, 28, 30 and 45 have shown increase in protein concentration.

Identification of ribosomal proteins by MALDI-TOF MS analyses:

From *H. eurihalina* ribosomes, apart from S1 protein (Chapter: 4), 26 proteins were identified including homologs of bacterial S2, S3, S5, S7, S8, S12, S15, S19, L1, L2, L5, L6, L11, L13, L14, L17, L19, L22, L27, L30, L31. Some proteins like L1, L27, S7, S8 and S15 were present in either two isoforms or truncated forms and both forms showed homology to same protein. Two forms of same protein were labelled as a and b forms (eg: L1a and L1b, Fig: 33A & B). Some spots did not resolve completely into discrete spots and hence could not be picked with precision for MALDI-TOF MS analyses. These spots include 9-11, 12-14 and 32-37 which did not show properly defined margins. For rest of the spots good spectrum was not obtained.

Two- dimensional gel of logarithmic phase ribosomal proteins along with identified ribosomal protein homologs was given in Fig: 33A. Proteins which showed similar identity were encircled together in red. It should also be noted that all of them were in closer proximity to the proteins showing similar identity. Some spots did not show homology to any protein in the databases, even though a good spectrum was obtained. They were marked with a question mark symbol. These results indicate at the possible presence of ribosomal proteins or proteins strongly associated to ribosomes, unique to *H. eurihalina*. Two- dimensional gel of stationary phase ribosomal proteins along with spot identities were given in Fig: 33B. Missed spots were encircled in red. For identified spots missing in stationary phase, red font was used. Identified missing spots in stationary phase include L2, S12 and L19. Among missing spots, spot 5 did not show homology to any protein even though a good spectrum was obtained.

L2 is a primary rRNA binding protein of 50 S ribosomal subunit. It is required for association of 30 S and 50 S subunits to form 70 S ribosome, tRNA binding to A and P sites (Diedrich, 2000). L2 is so highly conserved

that archaebacterial and even human isoforms of the protein can be functionally reconstituted in *E. coli* ribosomes (Uhlein *et al*., 1998).

S12 is a vital component of the A-site of the 30S ribosomal subunit. It is involved in both tRNA selection and resistance to streptomycin (Allen and Noller, 1989; Funatsu and Wittmann, 1972). Mutations in *rpsL* coding for ribosomal protein S12 are known to affect ribosomal accuracy to various extents, resulting in error-restrictive or non-restrictive S12 alterations (Kurland *et al*., 1996). It is also shown that S12 functions as a control element along with S13 for rRNA and tRNA driven movements of translocation and thus maintaining translational accuracy (Cukras *et al*., 2003).

Ribosomal protein L19 belongs to L19E family of proteins. It was found to be expressed 5 fold higher in malignant human cell lines in comparison to their benign counterparts and is a prognostic marker for human prostate cancer (Bee *et al*, 2006).

These results show that many of the ribosomal proteins exhibit growth phase coupled variation in *H. eurihalina*. These results correlate to the earlier findings in the present study i.e., slow sedimentation rate on sucrose density gradients and higher susceptibility of stationary phase ribosomes to RNase A and urea in comparison to logarithmic phase ribosomes. This may be due to absence of some of the ribosomal proteins and resulting in completely folded or partially unfolded ribosomal particles thus making the stationary phase ribosomes more susceptible to RNase A activity. In view of the functions of the proteins L2 and S12, which were lacking totally in stationary phase, it is possible that stationary phase ribosomes are functionally inactive. This may be the mechanism adapted by the organism to control translation during stationary phase.

Ribosomal proteins which showed growth cycle coupled variation along with their identities were given in Table: 1. Among the 65 proteins, 35 proteins showed growth phase dependent variation.

Spots that showed significant decrease in stationary phase gels were identified as L5 (15), S7a (24), L13 (26), L17 (40), L22 (42), S15b (53), S19 (59), L27b (63) and L30 (65). Spots 39 and 64 did not show homology to any protein even after obtaining good spectrum. Good spectrum was not obtained for spots 7, 27, 49, 57 and 61.

Ribosomal protein L5 binds to 5 S rRNA to form stable 5 S ribonucleoprotein particle complex in eukaryotes. S7 is a primary rRNA binding protein of 30 S subunit. It binds and organizes the folding of 3' domain of 16 S rRNA and enables the subsequential binding of other ribosomal proteins to form head of the 30 S subunit (White *et al*., 2000). L13 is known to be one of the early assembly proteins of large subunit. Ribosomal protein L17 constitutes part of the E (exit) site of ribosome. In mammals, ribosomal protein L22 binds heparin in brain and is known as heparin binding protein HBp 15 (Fujita *et al*., 1994). S15 is a primary rRNA binding protein. S19 is a secondary rRNA binding protein. N-terminus of L27 extends into peptidyl tranferase center (Selmer, 2006). Ribosomal protein L30 is a component of the machinery involved in translational recoding of UGA as selenocysteine in eukaryotes (Chavatte *et al*., 2005).

These results show that the spots which decreased in stationary phase have vital role in ribosomal assembly and function.

Spots S2 (2), L1a (3), S3 (8), L6 (16), S8a (23), S7b (25), S8b (28) and S15a (45) were identified among those spots which showed increase in protein concentration in stationary phase in comparison to logarithmic phase. Spot 30 did not show homology to any protein even after obtaining good spectrum. Good spectrum was not obtained for spots 1 and 17.

Ribosomal protein S2 is essential for the binding of S1 to 30 S subunit and S2 is involved in the formation of initiation complex. Ribosomal protein L1 is a primary rRNA binding protein (Zimmermann, 1980). L6 is located at the aminoacyl t-RNA binding site of peptidyl transferase center and is known to bind 23 S rRNA directly. S7 and S8 are primary rRNA binding proteins and S8 participates in the translational regulation of ribosomal protein expression (Kalurachchi, 1997).

These results show that both forms of S8 were increased in stationary phase ribosomes along with S2, S3, L1a, L6, S7b and S15a. It should also be noted that among the two different forms of S7 and S15 identified one form increased in concentration while other form decreased for both the proteins during stationary phase.

All the identified ribosomal proteins along with their identities and molecular masses were given in Table: 2. The organisms represented in the table showed highest homology to the peptides.

These results showed that the molecular masses of all the identified ribosomal proteins were within the range of 30 kDa–6 kDa. Although the search was performed among all three domains of life, most of the ribosomal proteins were homologous to ribosomal proteins of other bacteria. Homology was found with ribosomal proteins of bacteria belonging to phyla *Firmicutes* (eg: L1, L2, L5, L6, L11, L13, L17, L22, L27 & S8, S19), *Proteobacteria* (eg: S2, S5, S7, S8, S12, S15, S15, L1, L14, L31), *Actinobacteria* (eg: L19, L27 & L30), *Euryarchaeota* (eg: S3) and *Cyanobacteria* (eg: S7).

These observations show that ribosomal proteins of moderate halophile are homologous to ribosomal proteins of eubacteria distributed among different phyla and kingdoms. It should be noted that majority of the proteins were found to be homologous to ribosomal proteins of organisms from *Firmicutes*,

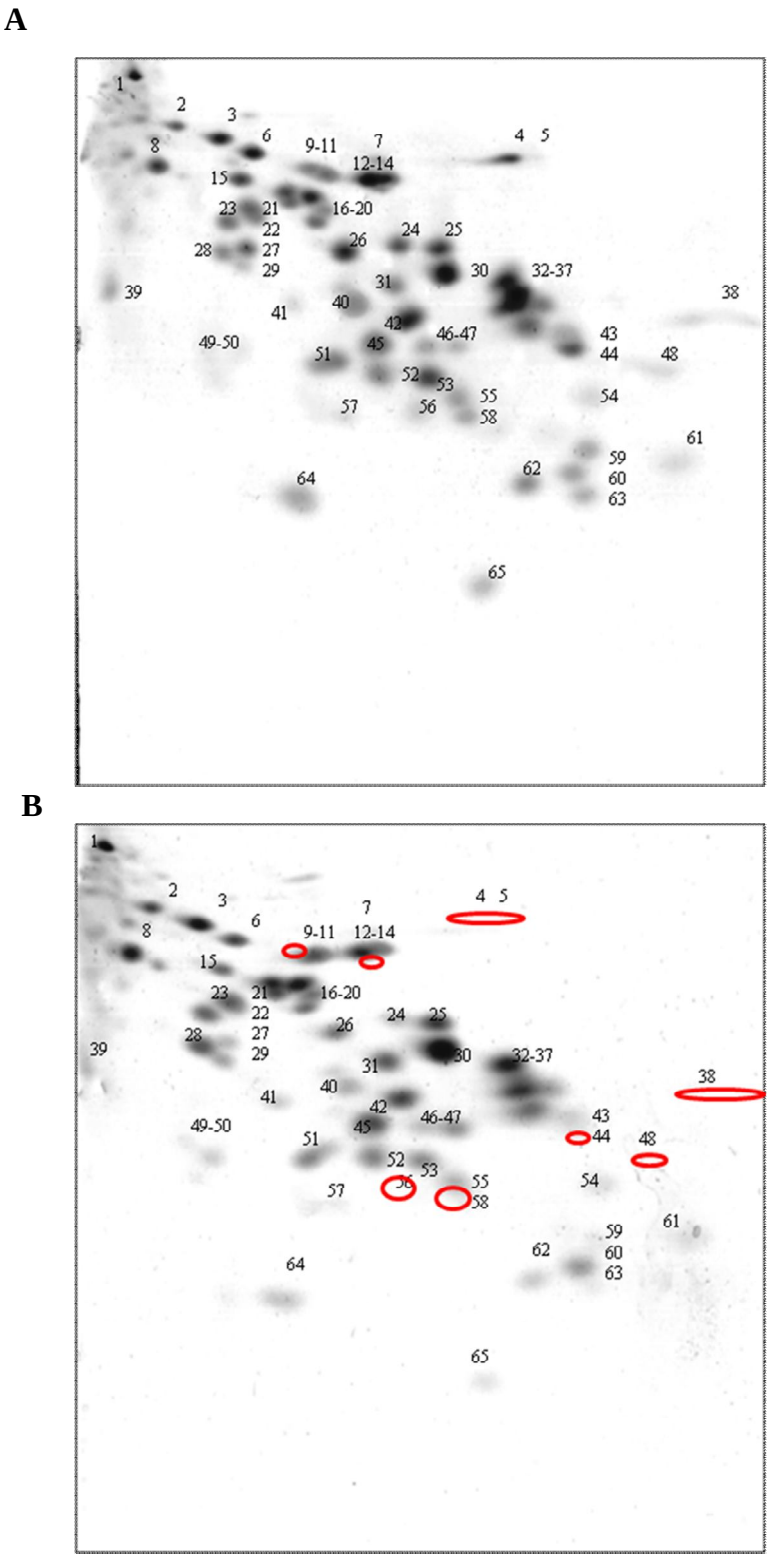


Fig: 32. Numbering of ribosomal proteins. A. Log phase ribosomal proteins. B. Stationary phase ribosomal proteins (red circles indicate the position of missing spots).

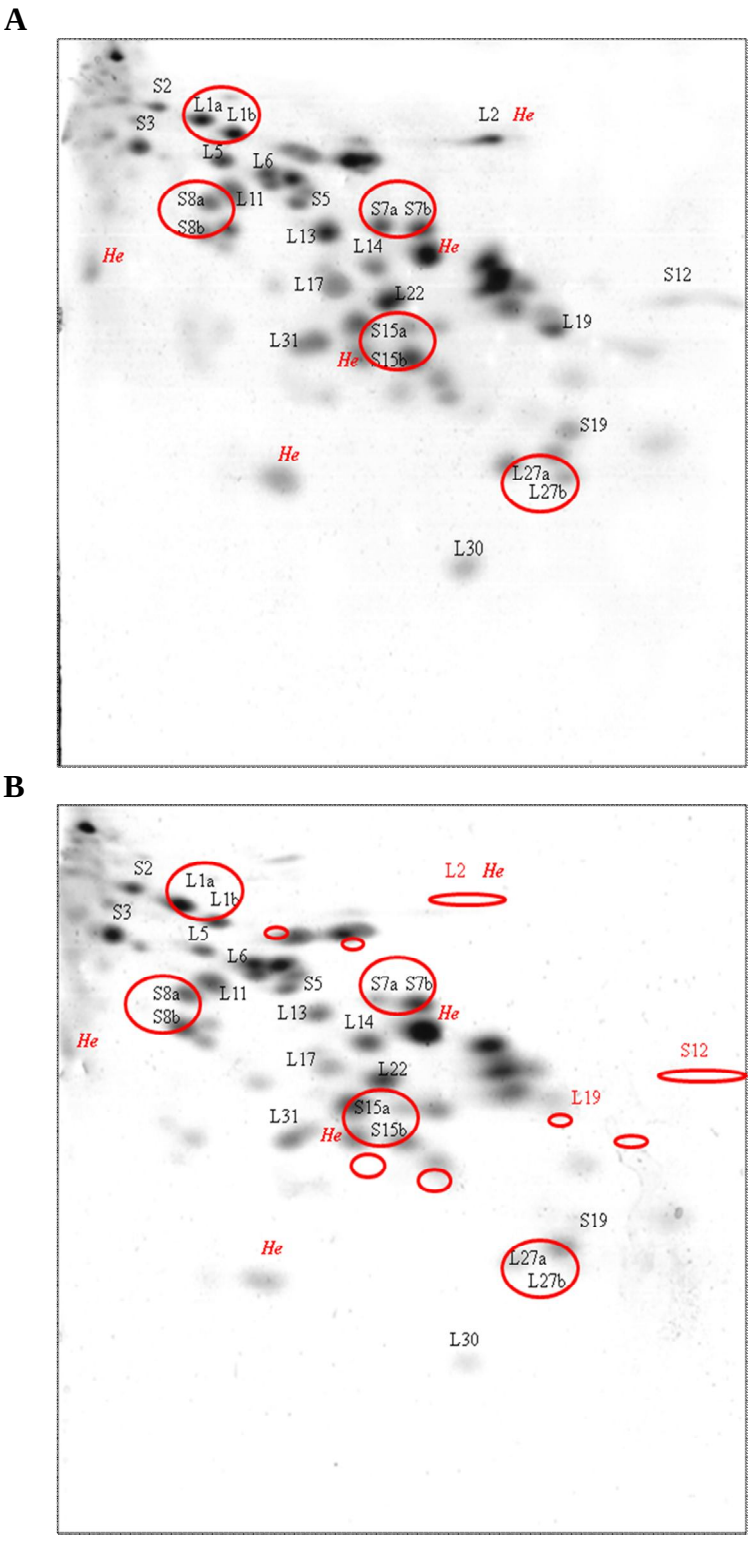


Fig: 33. Identification of ribosomal proteins by MALDI-TOF MS analyses.

A. Log phase ribosomal proteins. B. Stationary phase ribosomal proteins.

Identities were given at their respective positions. Missing spots were encircled in red, identified missing proteins were highlighted in red font. Unique proteins were labelled as *He*. Proteins showing similar identity were encircled in red.

Spot number	Identity	Logarithmic phase	Stationary phase
1	**?**	**+**	↑
2	**S2**	**+**	↑
3	**L1a**	**+**	↑
4	**L2**	**+**	-
5	***He***	**+**	-
7	**?**	**+**	↓
8	**S3**	**+**	↑
9	**?**	**+**	-
14	**?**	**+**	-
15	**L5**	**+**	↓
16	**L6**	**+**	↑
17	**?**	**+**	↑
23	**S8a**	**+**	↑
24	**S7a**	**+**	↓
25	**S7b**	**+**	↑
26	**L13**	**+**	↓
27	**?**	**+**	↓
28	**S8b**	**+**	↑
30	***He***	**+**	↑
38	**S12**	**+**	-
39	***He***	**+**	↓
40	**L17**	**+**	↓
42	**L22**	**+**	↓
44	**L19**	**+**	-
45	**S15a**	**+**	↑
48	**?**	**+**	-
49	**?**	**+**	↓
53	**S15b**	**+**	↓
56	**?**	**+**	-
57	**?**	**+**	↓
58	**?**	**+**	-
59	**S19**	**+**	↓
61	**?**	**+**	↓
63	**L27b**	**+**	↓
64	***He***	**+**	↓
65	**L30**	**+**	↓

Table: 1. Growth phase dependent variation of ribosomal proteins in *H. eurihalina*. For identified spots, spot identity was given. ?, unidentified spot; *He*, no homology to any protein; ↓, decrease in concentration; ↑, increase in concentration.

Spot number	Identity	Species	Molecular weight
2	**S2**	***Acinetobacter***	**27.5 kDa**
3	**L1a**	***Psychrobacter***	**24.6 kDa**
4	**L2**	***Staphylococcus***	**30.3 kDa**
5	***He***	**---**	**---**
6	**L1b**	***Staphylococcus***	**25.0 kDa**
8	**S3**	***Thermococcus***	**23.3 kDa**
15	**L5**	***Aerococcus***	**20.2 kDa**
16	**L6**	***Staphylococcus***	**19.6 kDa**
20	**S5**	***Moorella***	**17.1 kDa**
22	**L11**	***Staphylococcus***	**14.9 kDa**
23	**S8a**	***Xanthomonas***	**14.3 kDa**
24	**S7a**	***Synechococcus***	**17.7 kDa**
25	**S7b**	***Acinetobacter***	**17.6 kDa**
26	**L13**	***Bacillus***	**16.3 kDa**
28	**S8b**	***Staphylococcus***	**14.7 kDa**
30	***He***	**---**	**---**
31	**L14**	***Psychrobacter***	**13.4 kDa**
38	**S12**	***Acidovorax***	**13.9 kDa**
39	***He***	**---**	**---**
40	**L17**	***Staphylococcus***	**13.7 kDa**
42	**L22**	***Macrococcus***	**13.6 kDa**
44	**L19**	***Bifidobacterium***	**13.6 kDa**
45	**S15a**	***Sphingopyxis***	**10.3 kDa**
51	**L31**	***Psychrobacter***	**10.6 kDa**
52	***He***	**---**	**---**
53	**S15b**	***Psychrobacter***	**10.1 kDa**
59	**S19**	***Natranaerobius***	**10.7 kDa**
62	**L27a**	***Anoxybacillus***	**10.5 kDa**
63	**L27 b**	***Bifidobacterium***	**8.9 kDa**
64	***He***	**---**	**---**
65	**L30**	***Kineococcus***	**6.7 kDa**

Table: 2. Identification of ribosomal proteins by MALDI-TOF MS analyses.

He, spots with no homologous hits.

Peptide sequence	m/z	Spot no	Protein ID
R.DLLQAGAHFGHQTR.F	1551.01	2	RS2
K.NLGIPVIGIVDTNSNPDNVDYVIPGNDDAIR.A	3281.07	2	RS2
K.MGPYIFGAR.N	1027.62	2	RS2
R.VVGQLGTILGPR.G	1209.71	3	RL1
K.ESIDIAVNLGVDPR.K	1497.76	3	RL1
K.FKESIDIAVNLGVDPR.K	1772.92	3	RL1
K.AGNMDFDVVIAAPDAMR.V	1824.79	3	RL1
R.SANIALLVYADGEKR.Y	1619.83	4	RL2
R.GSVMNPNDHPHGGGEGR.A	1733.60	4	RL2
K.ETSVANFDASVEVAFR.L	1741.84	6	RL1
K.VIIFAASPGYVIGRGGR.R	1733.13	8	RS3
R.MYEFFDKLVTVSLPR.V	1861.16	15	RL5
R.YQGEYVR.R	914.44	16	RL6
K.DLVLNVGYSHPVEIK.A	1682.89	16	RL6
R.NLEIVGIGYR.A	1133.65	16	RL6
K.VLLKPAAPGTGVIAGGPVR.A	1773.06	20	RS5
R.TQEQAGLIIPVEISVYEDR.S	2160.17	22	RL11
K.SPPAAFLLR.K	971.58	22	RL11
K.YFEGRPVIETLKR.F	1608.12	23	RS8
R.ILYSAFDLIQER.T	1468.00	24	RS7
R.VGGSNYQVPVEVRPERR.T	1942.36	24	RS7
K.AFSHYRF.	927.61	24	RS7
K.VLNGLGIALVSTSEGVVTDKEAR.K	2328.88	26	RS8
K.SVEYIEDDKQGVIR.M	1651.22	26	RS8
R.FDDNAAVLLNQN KAPIATR.I	2071.72	31	RL14
M.PTINQLVR.Q	940.45	38	RS12
R.RGDGAESVIIELV.	1358.10	40	RL17
K.LFNDLGPR.F	931.77	40	RL17
R.LVLDLIR.G	841.81	42	RL22
K.DIHPNYQEVLFHDTNADVFFLTR.S	2792.36	51	RL31
K.HVPVFITEDMVGHKLGEFAPTR.T	2496.16	59	RS19
R.ADGQFVTGGSILYR.Q	1484.13	62	RL27
K.RADGQFVTGGSILYR.Q	1640.32	62	RL27
K.VTQINSGIGR.K	1044.96	65	RL30

Table: 3. Matching peptide sequences along with m/z values of *H. eurihalina* ribosomal proteins.

Proteobacteria and *Actinobacteria*. Only one ribosomal protein each was found to be homologous to organisms from phyla *Euryarchaeota* (S3) and *Cyanobacteria* (S7). These results show that most of the ribosomal proteins are homologous to ribosomal proteins of eubacteria, only one was homologous to ribosomal protein of euryarchaeota and none of them were found to be homologous to ribosomal proteins of eukaryotes.

Matching peptide sequences of the proteins along with their identities were given in Table: 3. Peptides with fewer score for whole peptide match were not presented in the table. Proteins only with PMF score, without peptide match were also not included in the table.

However, significant matches were considered and given in the data presented under Fig: 34 (spot2-spot65). Spectra obtained for the all the spots along with the most significant hits were presented in detail under Fig: 34 (spot2-spot65). For those spots with good spectra and no significant hits, only spectra were given.

Multiple sequence alignments were performed for the peptide sequences obtained by MALDI-TOF MS analyses and BLAST search generated protein sequences using ClustalX2. Results were given under Fig: 35A-U.

S2 peptide (m/z 1551.01) was of 16 amino acids and was found to be homologous to N-terminal end 34-48 amino acids of S2 protein from other eubacteria (Fig: 35Ai). 33 amino acid peptide (m/z 3281.07) was found to be homologous to 182-214 sequence of S2 protein (Fig: 35Aii). Both the peptides showed extensive homology to different species of *Acinetobacter*.

L1a peptide (m/z 1497.76) was of 16 amino acid residues and was found to be homologous to 39-54 position of L1 (Fig: 35Bi). Peptide (m/z 1772.92) was of 18 amino acid residues and showed homology to 37-54

position (Fig: 35Bii). 19 amino acid residue peptide (m/z 1824.79) was homologous to 105-123 position (Fig: 35Biii). L1a peptide (m/z 1209.71) was of 14 amino acids and showed homology to 122-135 residues of L1 protein from other eubacteria (Fig: 35Biv). These peptides showed extensively homology to *Psychrobacter* species.

L2 peptide (m/z 1619.75) was of 17 amino acid residues and showed homology to 89-105 position (Fig: 35Ci). 19 amino acid peptide (m/z 1733.60) was found to be homologous to 225-243 position (Fig: 35Cii). This peptide was found to be highly conserved among all the genera. Both peptides showed extensive homology to *Staphylococcus* species.

L1b peptide (m/z 1741.84) was of 18 amino acid residues and showed homology to 45-62 position of L1 of other eubacteria (Fig:35D). The peptide showed extensive homology to *Staphylococcus* species.

S3 peptide (m/z 1733.13) was of 19 amino acid residues and showed homology to 66-82 position of S3. This peptide showed extensive homology to *Thermococcus* and *Pyrococcus* species of *Euryarchaeota* (Fig: 35E). The peptide also showed homology to other eubacteria like *Clostridium* species.

L5 peptide (m/z 1861.16) was of 17 amino acid residues and showed homology to 96-112 position (Fig: 35F). The peptide showed extensive homology to *Streptococcus* species.

L6 peptide (m/z 1682.89) was of 17 amino acids and showed homology to 102-118 position. The peptide showed extensive homology to *Streptococcus* species. It also showed homology to *Synechococcus*, a unicellular cyanobacterium (Fig: 35G).

S5 peptide (m/z 1773.06) was of 21 amino acid residues and showed homology to 99-119 position. The peptide showed extensive homology to *Thermincola* species (Fig: 35H).

L11 peptide (m/z 2160.17) was of 21 amino acid residues and showed homology to 68-88 position. The peptide showed extensive homology to *Staphylococcus* species (Fig: 35I).

S8a peptide (m/z 1608.12) was of 15 amino acid residues and showed homology to 66-80 position. The peptide showed extensive homology to *Xanthomonas* species (Fig: 35J).

S7a peptide (m/z 1468.00) was of 14 amino acid residues and showed homology to 41-54 position. The peptide showed extensive homology to *Synechococcus* species (Fig: 35Ki). Peptide (m/z 1942.36) was of 19 amino acid residues and showed homology to 79-107 position of S7 protein. The peptide showed extensive homology to *Streptococcus* species (Fig: 35Kii).

S8b peptide (m/z 2328.88) was of 25 amino acid residues and showed homology to 97-121 position (Fig: 35Li). Peptide (m/z 1651.22) was of 16 amino acid residues and showed homology to 47-62 position of S8 position (Fig: 35Lii). Both the peptides showed extensive homology to *Staphylococcus* species.

L14 peptide (m/z 2071.72) was of 21 amino acid residues and showed homology to 88-108 position. The peptide showed extensive homology to *Psychrobacter* species. The peptide also showed homology to *Pichia pastoris* (Fig: 35M).

L17 peptide (m/z 1358.10) was of 14 amino acid residues and showed homology to 114-127 position. The peptide showed extensive homology to *Staphylococcus* species (Fig: 35N).

L22 peptide (m/z 1349.08) was of 14 amino acid residues and showed homology to 77-90 position. The peptide showed extensive homology to *Staphylococcus* species (Fig: 35O).

L19 PMF sequence was of 33 amino acid residues and showed homology to 38-66 position. The peptide showed extensive homology to *Bifidobacterium* species (Fig: 35P).

S15 PMF sequence was of 25 amino acid residues and showed homology to 45-69 position. The peptide showed extensive homology to *Sphingopyxis* species (Fig: 35Q).

L31 peptide (m/z 2792.36) was of 25 amino acid residues and showed homology to 3-27 position. The peptide showed extensive homology to *Psychrobacter* species (Fig: 35R).

S19 peptide (m/z 2496.16) was of 24 amino acid residues and showed homology to 56-79 position. The peptide showed extensive homology to *Phascolarctobacterium* species (Fig: 35S).

L27a peptide (m/z 1484.13) was of 16 amino acids and showed homology to 36-51 position (Fig: 35Ti). Peptide (m/z 1640.32) was of 17 amino acids and showed homology to 35-51 position (Fig: 35Tii). Both the peptides showed extensive homology to *Listeria* species

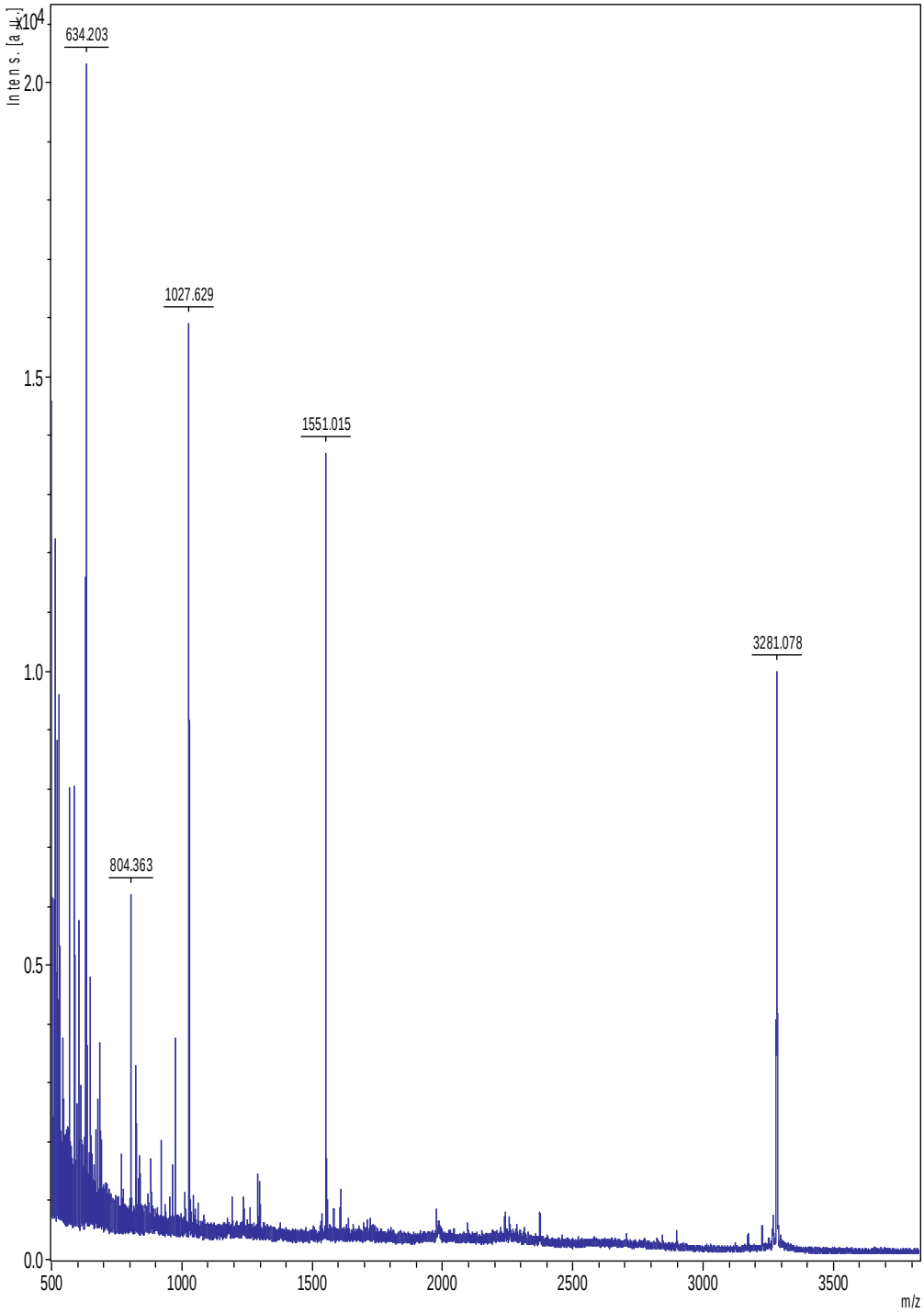
L27b peptide (m/z 1529.93) was of 17 amino acids and showed homology to 24-40 position. Peptides showed extensive homology to *Parascardovia* species (Fig: 35U).

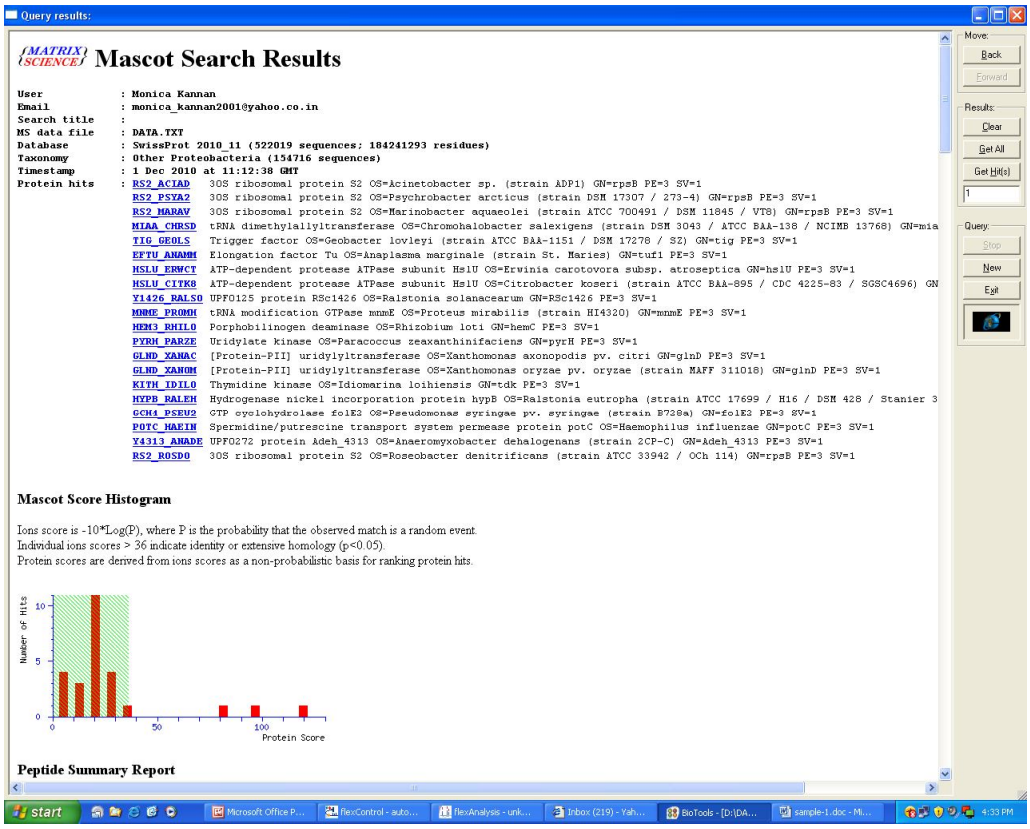
The above results on multiple sequence alignment indicate strong homology of majority of ribosomal proteins of *H. eurihalina* to eubacteria

(both gram positive and gram negative), although the results showed greater similarity to gram positive species. None of the ribosomal proteins showed similarity to extreme halophilic archaeal ribosomal proteins.

Fig: 34. In-gel digestion of ribosomal proteins

Spot: 2





RS2_ACIAD **Mass:** 27507 **Score:** 120 **Matches:** 2(2) **Sequences:** 2(2)

30S ribosomal protein S2 OS=Acinetobacter sp. (strain ADP1) GN=rpsB PE=3 SV=1

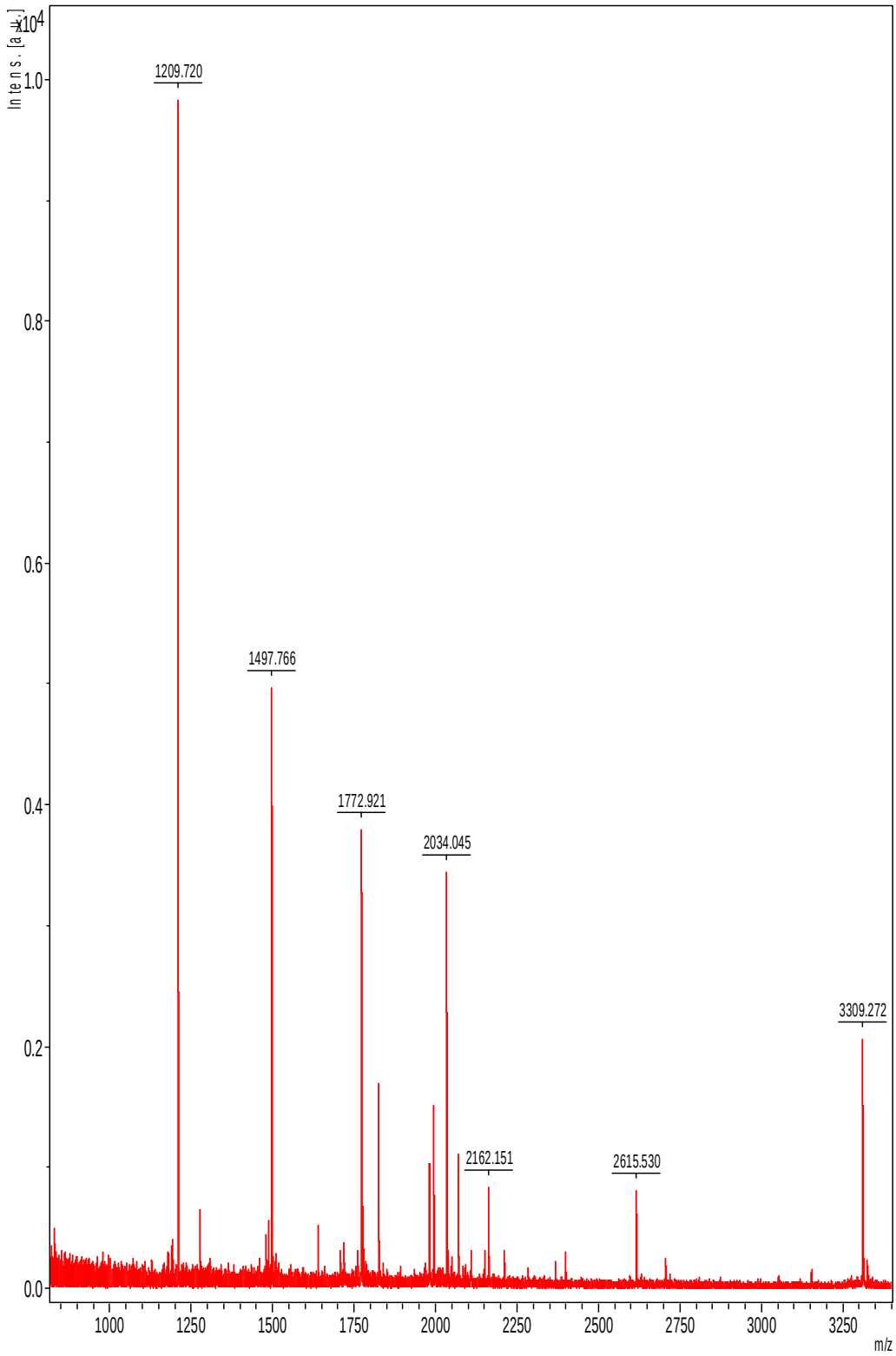
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1551.0149	1550.0076	1549.7750	150	0	81	1.4e-06	1		R.DLLQAGAHFGHQTR.F
3281.0785	3280.0712	3278.6419	436	0	38	0.028	1	U	K.NLGIPVIGIVDTNSNPD NVDYVIPGNDDAIR.A

RS2_PSYA2 **Mass:** 28866 **Score:** 94 **Matches:** 2(1) **Sequences:** 2(1)

30S ribosomal protein S2 OS=Psychrobacter arcticus (strain DSM 17307 / 273-4) GN=rpsB PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1027.6287	1026.6214	1026.4957	123	0	13	12	1	U	K.MGPYIFGAR.N + Oxidation (M)
1551.0149	1550.0076	1549.7750	150	0	81	1.4e-06	1		R.DLLQAGAHFGHQTR.F

Spot 3: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2034.045
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1250
1500
1750
2000
2250
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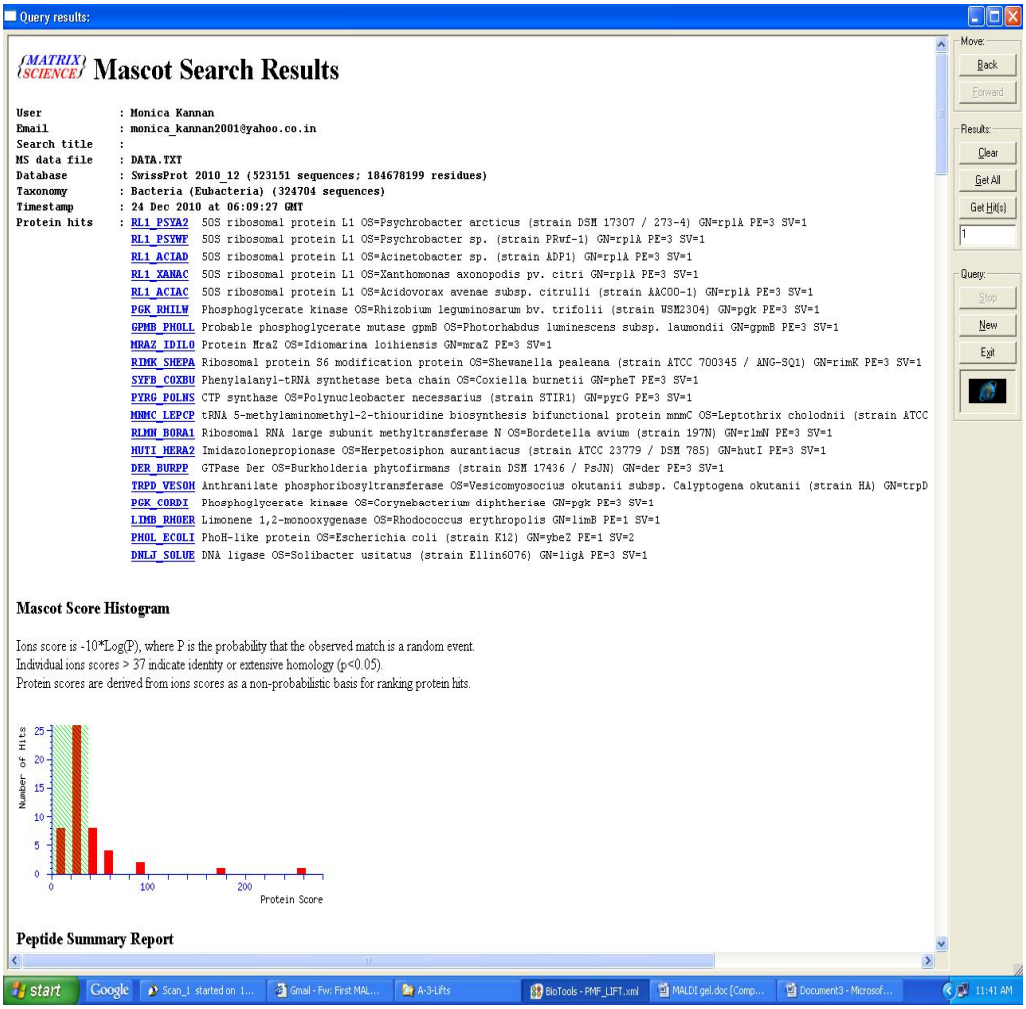
2750
3000
3250
m/z



RL1_PSYA2 **Mass:** 24696 **Score:** 257 **Matches:** 4(4) **Sequences:** 4(4)

50S ribosomal protein L1 OS=Psychrobacter arcticus
(strain DSM 17307 / 273-4) GN=rplA PE=3 SV=1

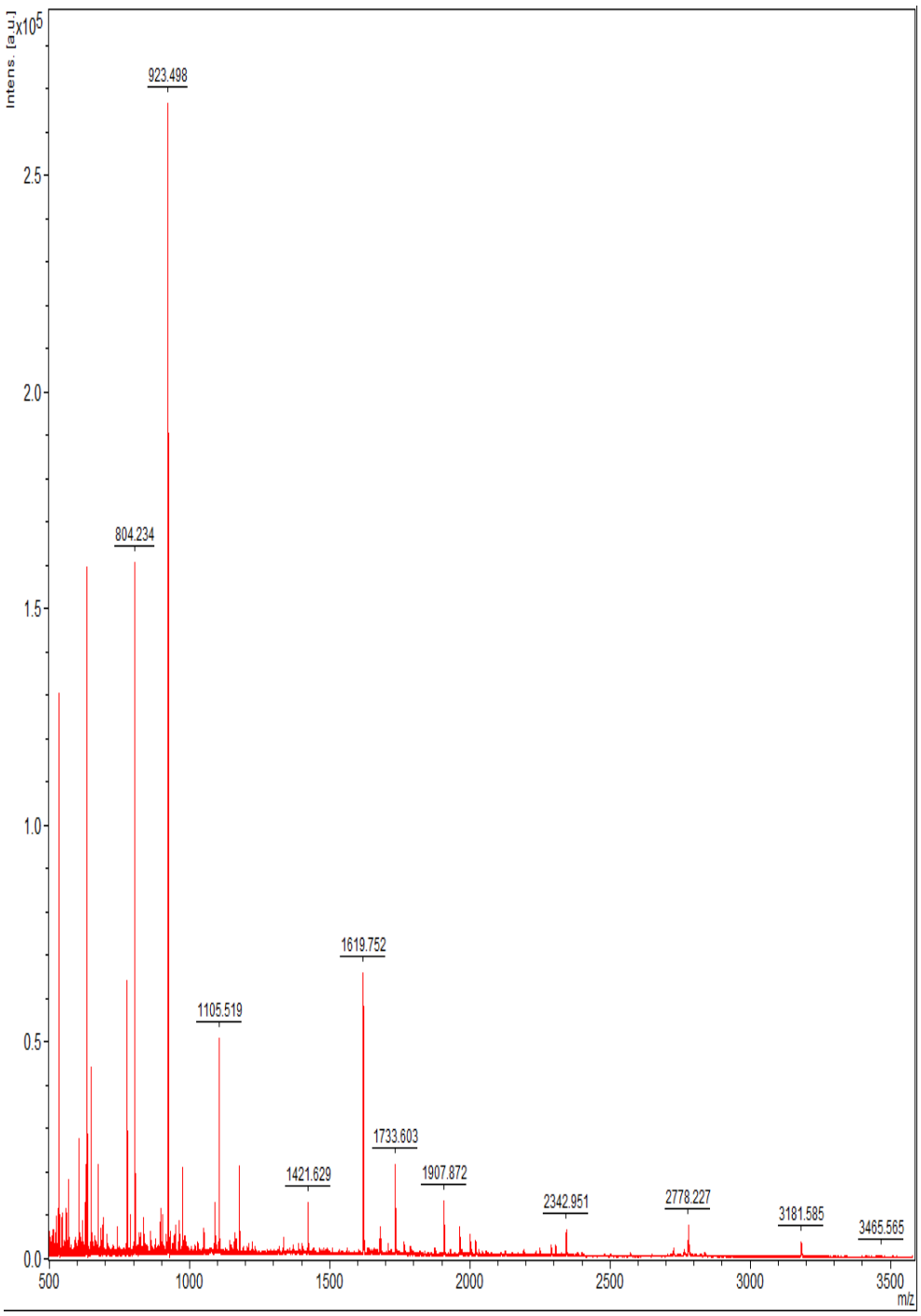
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1209.7199	1208.7126	1208.7241	-9.54	0	98	4.7e-08	1		R.VVGQLGTILGPR.G
1497.7661	1496.7588	1496.7835	-16.46	0	69	4.8e-05	1	U	K.ESIDIAVNLGVDPR.K
1772.9207	1771.9135	1771.9468	-18.83	1	45	0.012	1	U	K.FKESIDIAVNLGVDPR.K
1824.7936	1823.7863	1823.8182	-17.48	0	45	0.0089	1	U	K.AGNMDFDVVIAAPDAMR.V + 2 Oxidation (M)

Proteins matching the same set of peptides:

RL1_PSYCK **Mass:** 24670 **Score:** 257 **Matches:** 4(4) **Sequences:** 4(4)

50S ribosomal protein L1 OS=Psychrobacter cryohalolentis
(strain K5) GN=rplA PE=3 SV=1

Spot: 4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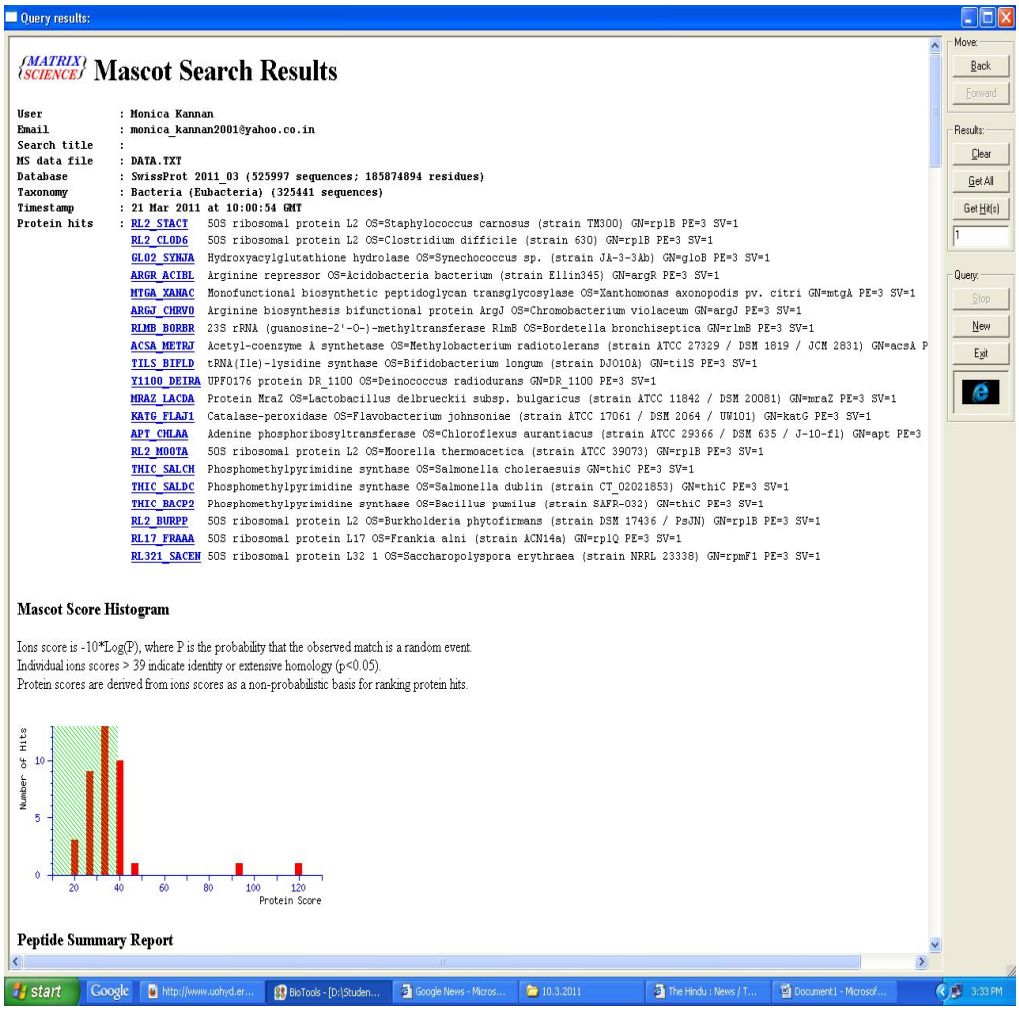
1907.872
2342.951
2778.227
3181.585
3465.565
500
1000
1500
2000
2500
3000
3500
m/z



RL2_STACT **Mass:** 30302 **Score:** 120 **Matches:** 2(2) **Sequences:** 2(2)

50S ribosomal protein L2 OS=Staphylococcus carnosus (strain TM300) GN=rplB PE=3 SV=1

Observed Mr (expt) Mr (calc) ppm Miss Score Expect Rank Unique Peptide

1619.8332 1618.8260 1618.8678 -25.86 1 95 1e-07 1 U R.SANIALLVYADGEKR.Y

1733.6027 1732.5954 1732.7336 79.74 0 56 0.00083 1 R.GSVMNPNDHPHGGGEGR.A + Oxidation (M)

Proteins matching the same set of peptides:

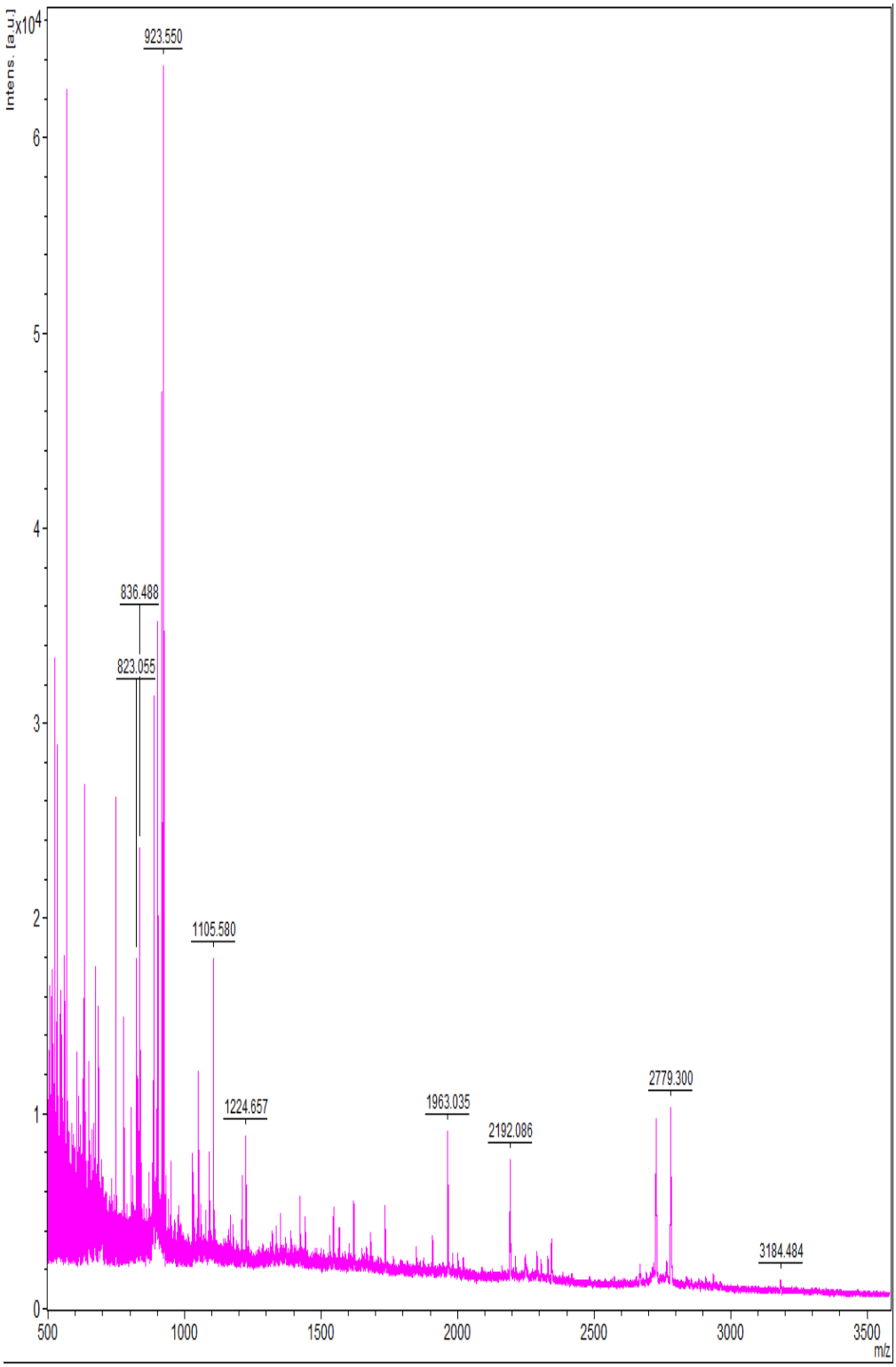
RL2_STAEQ **Mass:** 30252 **Score:** 120 **Matches:** 2(2) **Sequences:** 2(2)

50S ribosomal protein L2 OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=rplB PE=3 SV=1

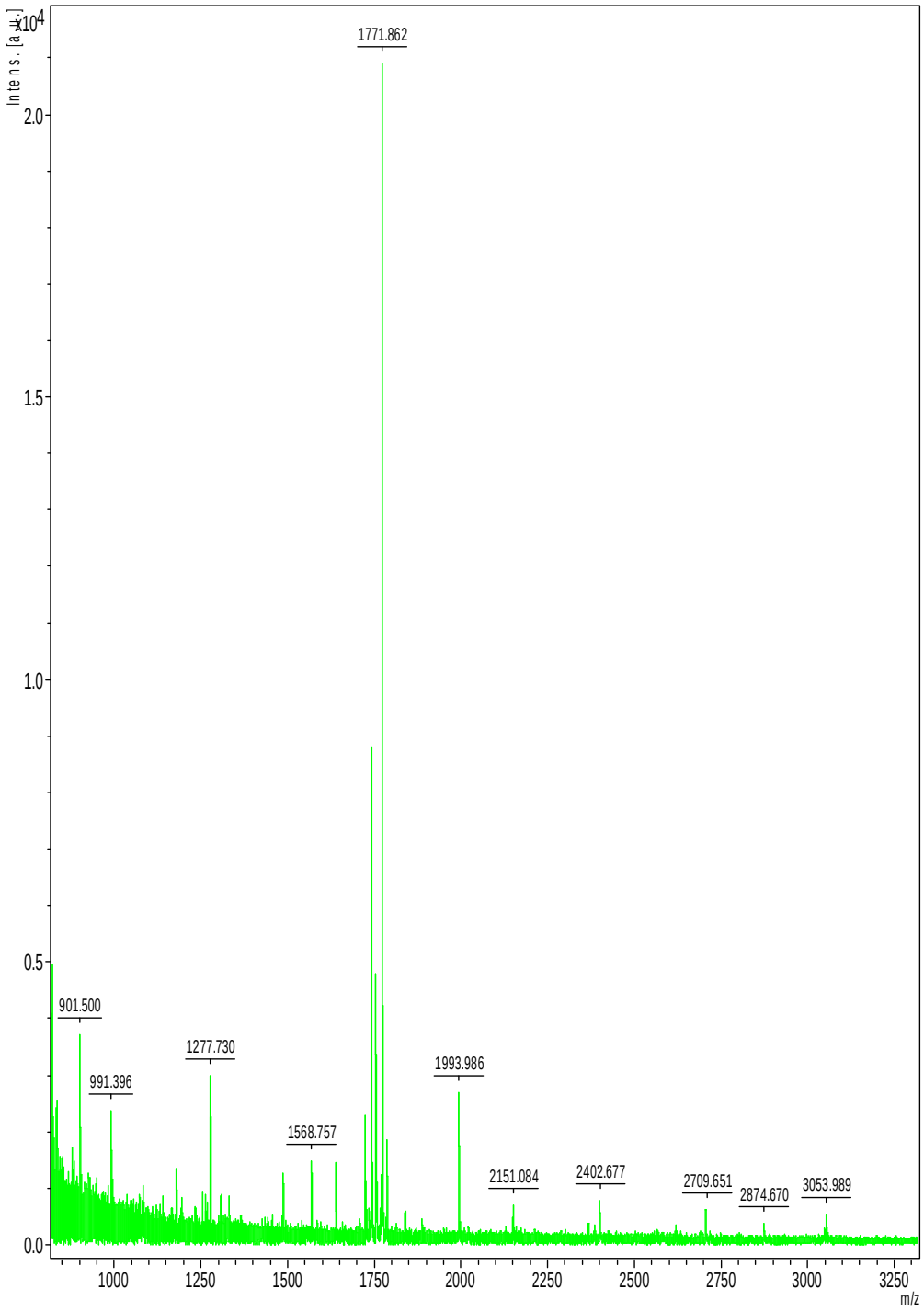
RL2_STAES **Mass:** 30252 **Score:** 120 **Matches:** 2(2) **Sequences:** 2(2)

50S ribosomal protein L2 OS=Staphylococcus epidermidis (strain ATCC 12228) GN=rplB PE=3 SV=1

Spot: 5


Intens. [a.u.]
x10^4
6
5
4
3
2
1
0
923.550
836.488
823.055
1105.580
1224.657
1963.035
2192.086
2779.300
3184.484
500
1000
1500
2000
2500
3000
3500
m/z

Spot: 6


Intens. [a.u.]
x10^4
2.0
1.5
1.0
0.5
0.0
1771.862
901.500
991.396
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1568.757
1993.986
2151.084
2402.677
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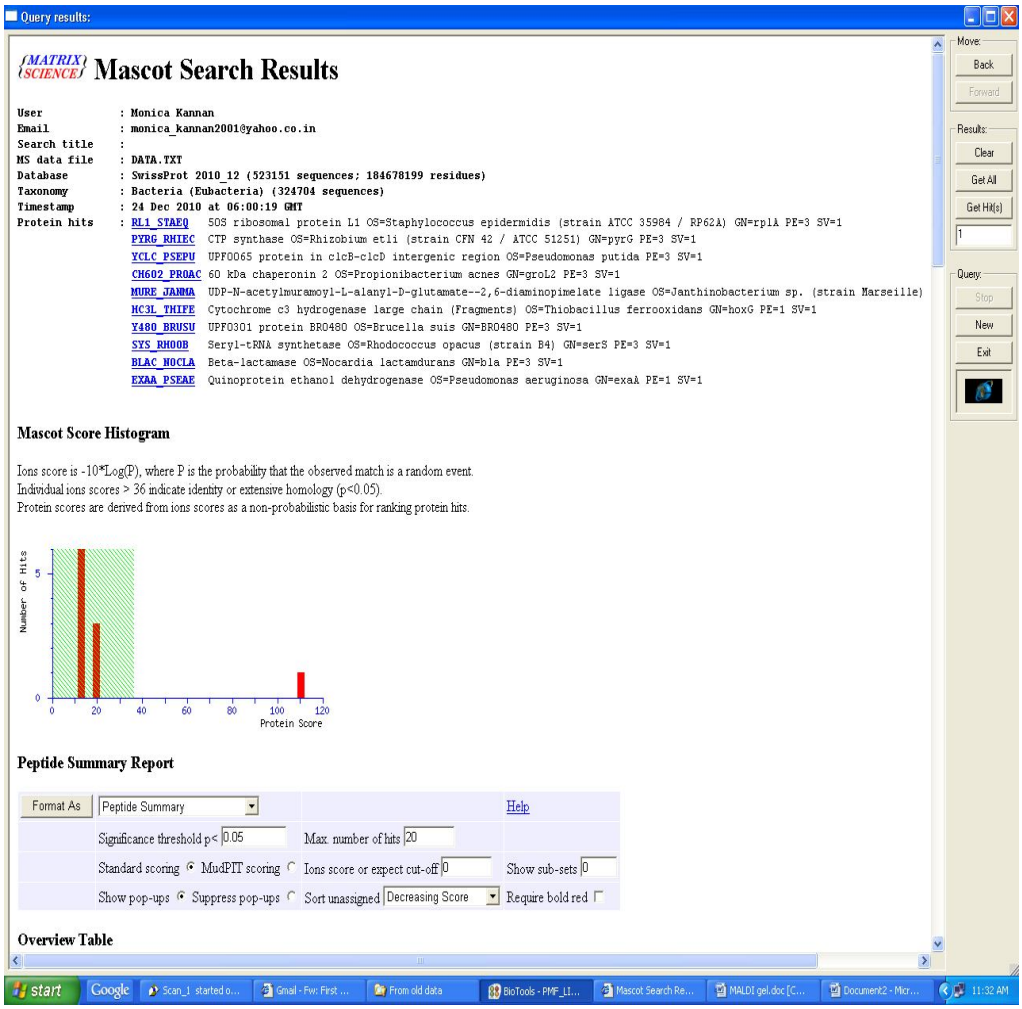
1750
2000
2250
2500
2750
3000
3250
m/z



RL1_STAEQ **Mass:** 25012 **Score:** 110 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L1 OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=rplA PE=3 SV=1

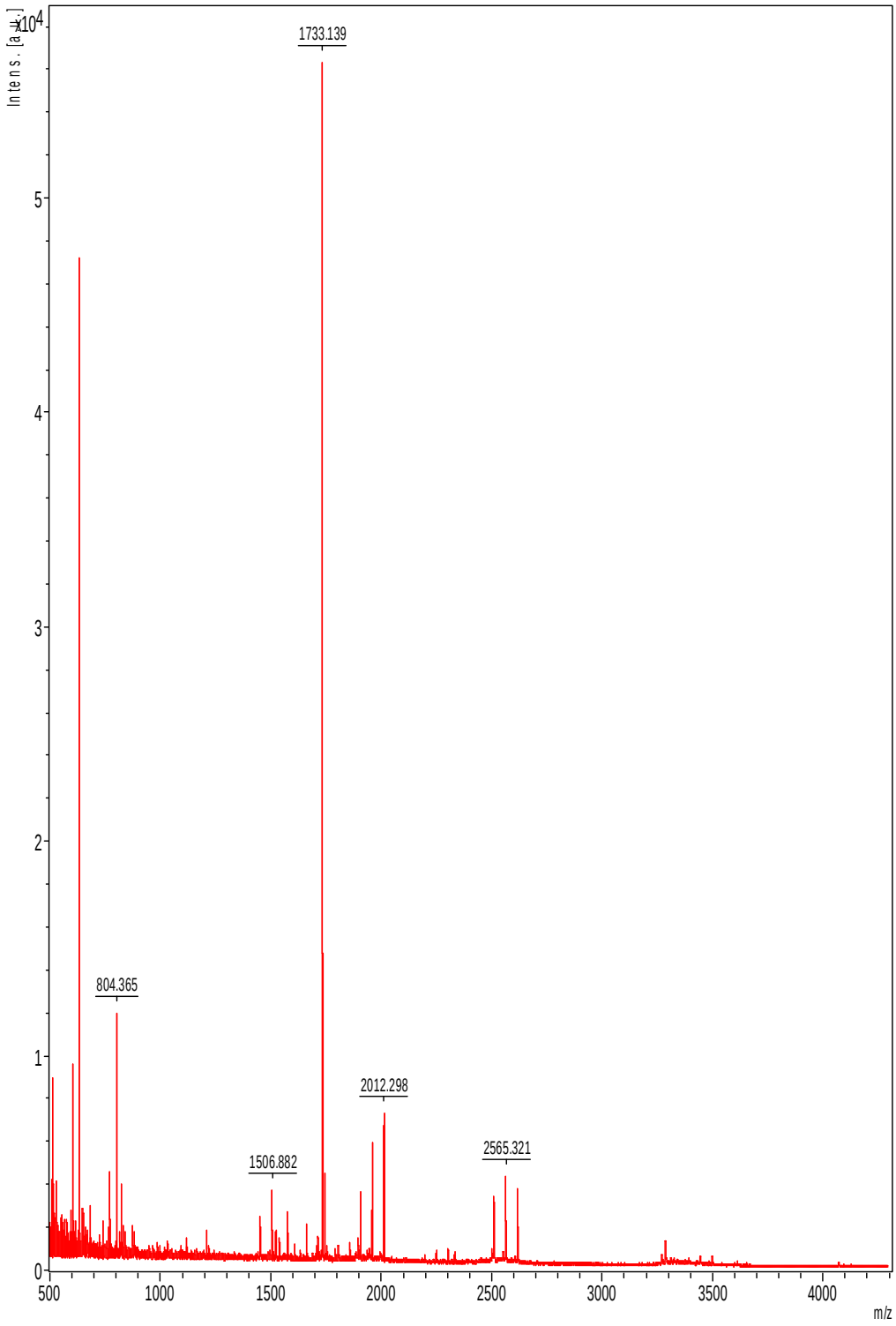
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1741.8414	1740.8342	1740.8319	1.31	0	110	2.3e-09	1	U	K.ETSVANFDASVEVAFR.L

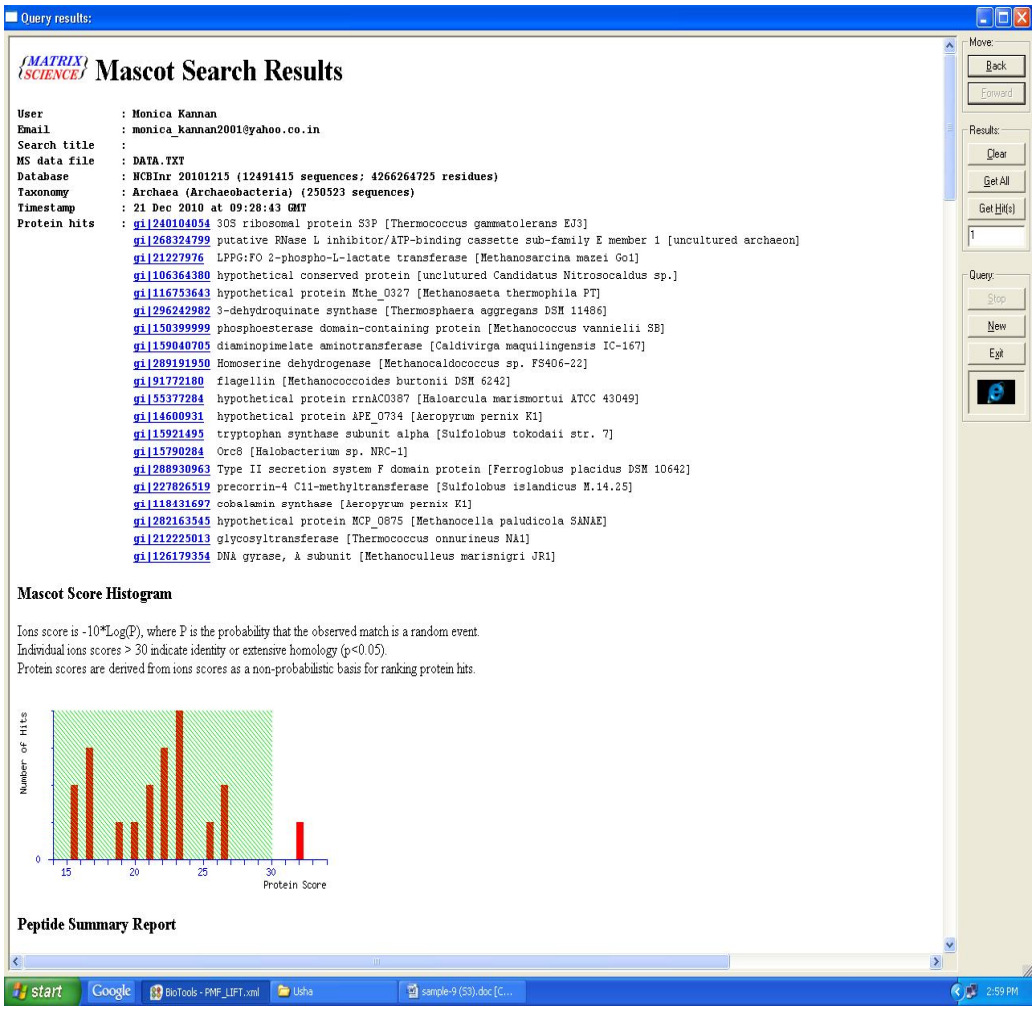
Proteins matching the same set of peptides:

RL1_STAES **Mass:** 25012 **Score:** 110 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L1 OS=Staphylococcus epidermidis (strain ATCC 12228) GN=rplA PE=3 SV=1

Spot: 8





gi|240104054 **Mass:** 23310 **Score:** 32 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S3P [Thermococcus gammatolerans EJ3]

Observed Mr (expt) Mr (calc) ppm Miss Score Expect Rank Unique Peptide

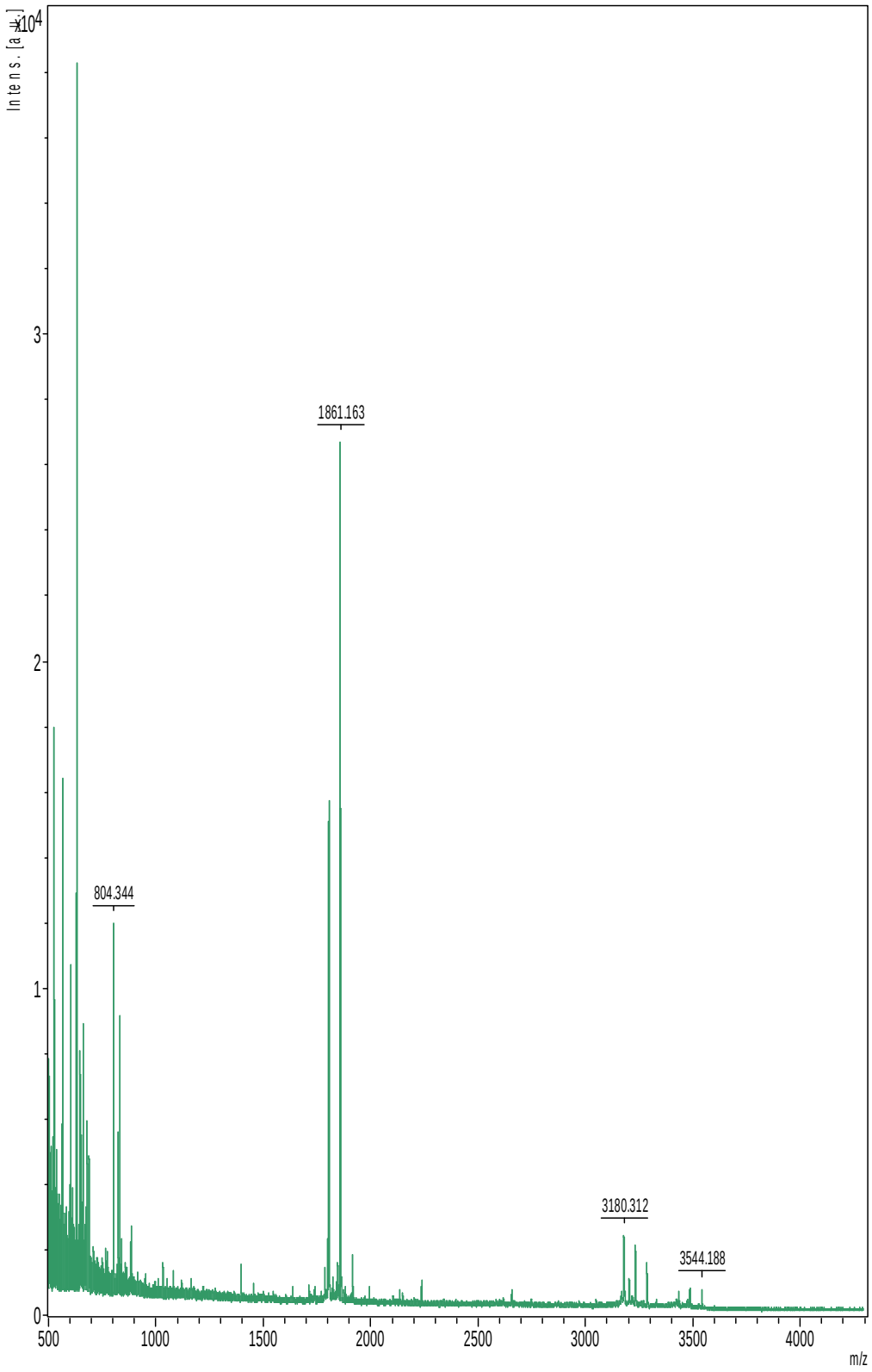
1733.1389 1732.1316 1731.9784 88.4 1 32 0.033 1 U K.VIIFAASPGYVIGRGGR.R

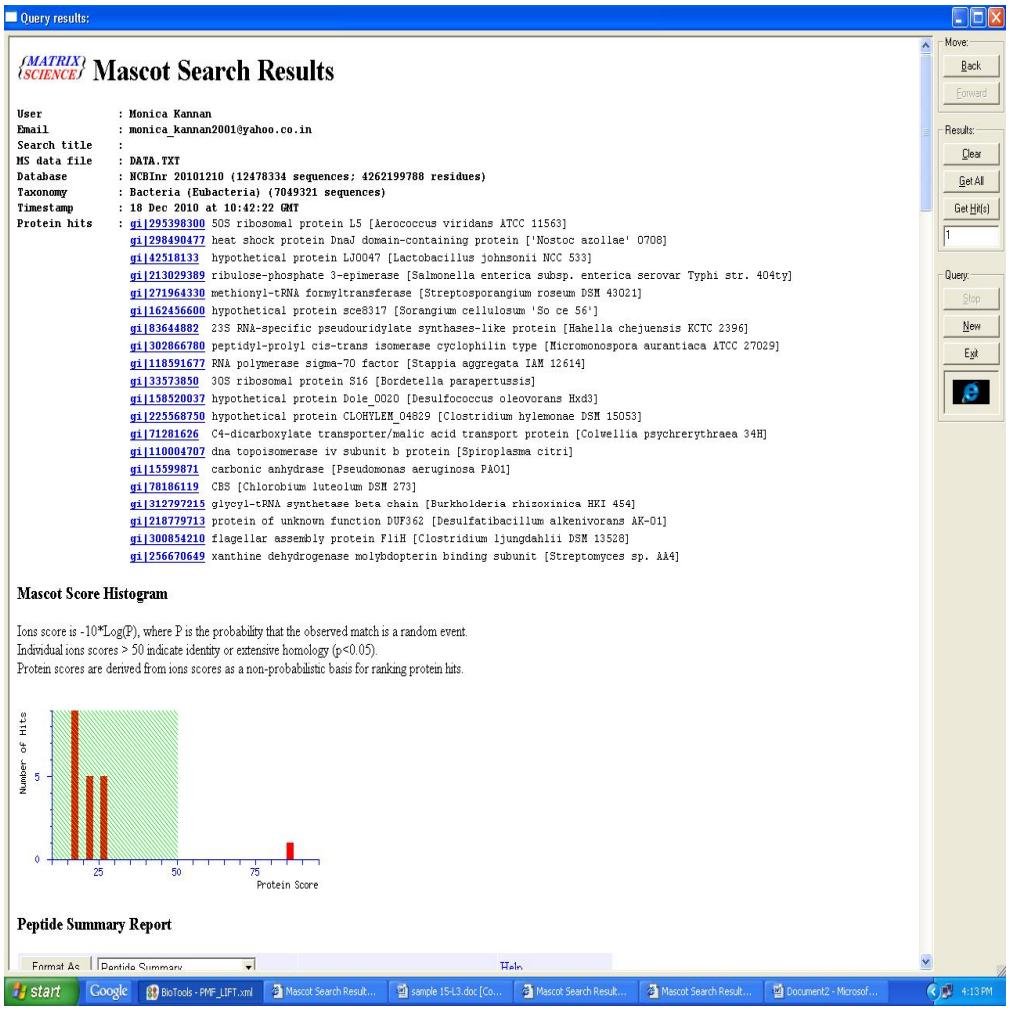
Proteins matching the same set of peptides:

gi|254172991 **Mass:** 23368 **Score:** 32 **Matches:** 1(1) **Sequences:** 1(1)

ribosomal protein S3 [Thermococcus sp. AM4]

Spot: 15


x10^4
Intens. [a.u.]
3
2
1
0
1861.163
804.344
3180.312
3544.188
500
1000
1500
2000
2500
3000
3500
4000
m/z

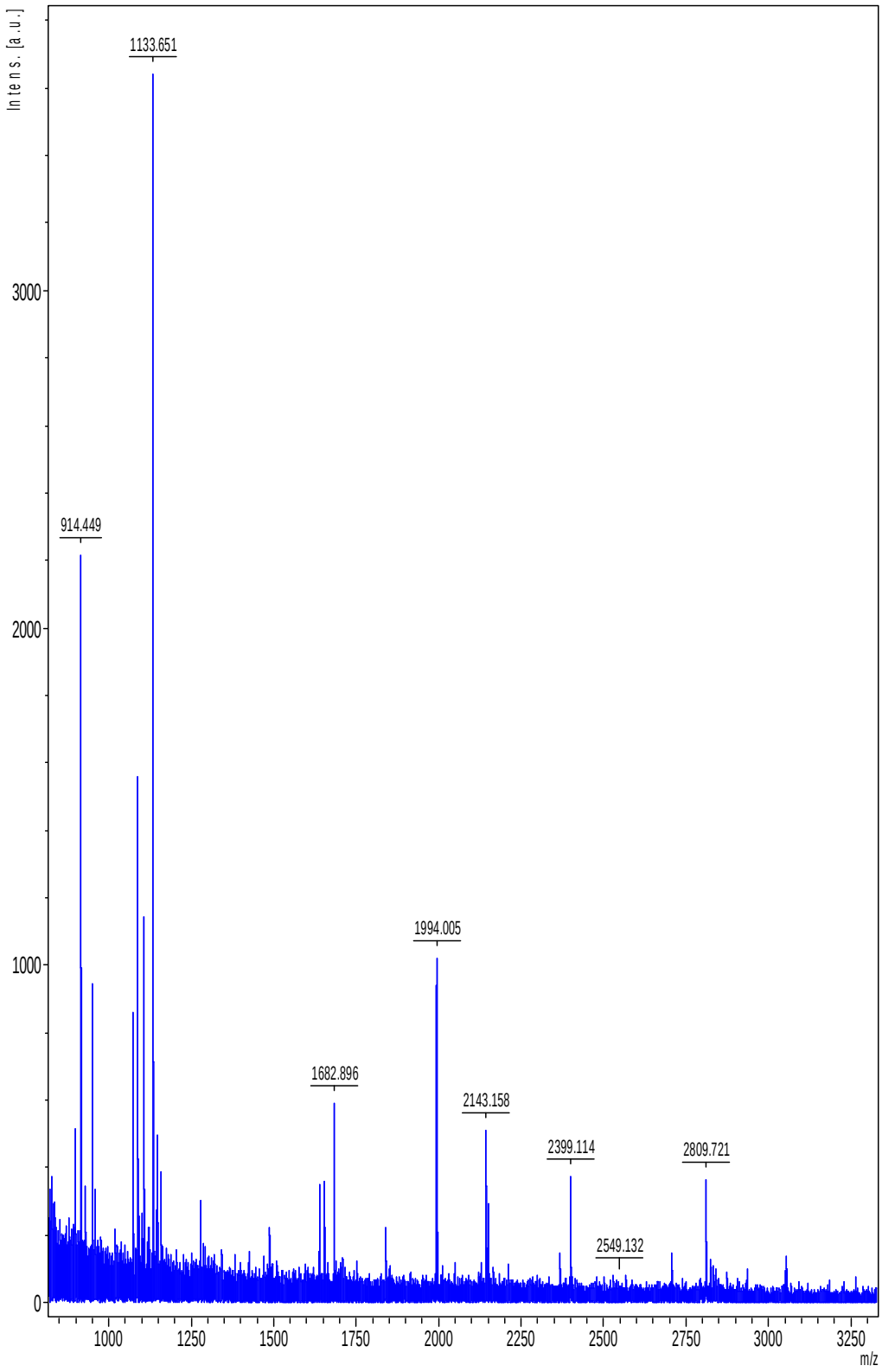


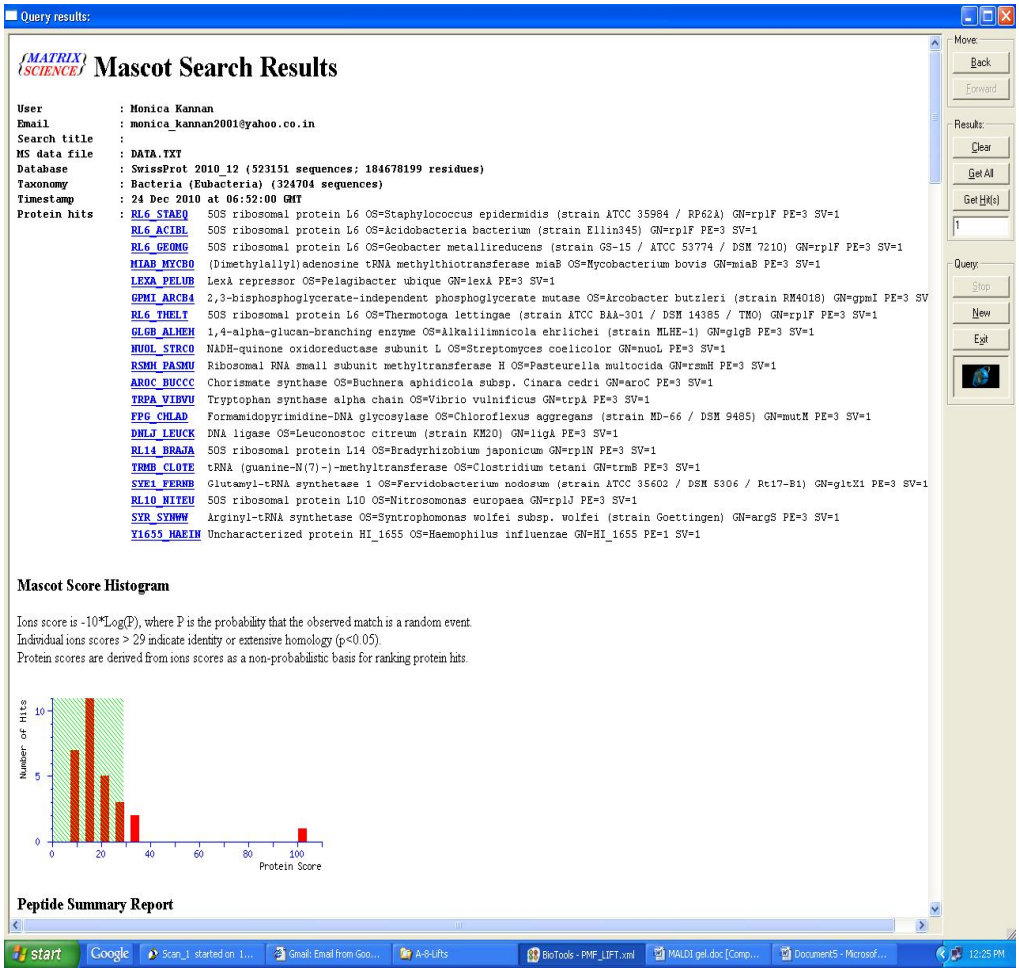
gi|295398300 **Mass:** 20225 **Score:** 86 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L5 [Aerococcus viridans ATCC 11563]

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1861.1627	1860.1554	1859.9492	111	1	86	8.9e-06	1	U	R.MYEFFDKLVTVSLPR.V + Oxidation (M)

Spot: 16


Intens. [a.u.]
1133.651
914.449
1994.005
1682.896
2143.158
2399.114
2809.721
2549.132
3000
2000
1000
0
1000
1250
1500
1750
2000
2250
2500
2750
3000
3250
m/z



RL6_STAEQ **Mass:** 19652 **Score:** 102 **Matches:** 2(2) **Sequences:** 2(2)

50S ribosomal protein L6 OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=rplF PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
914.4490	913.4417	913.4294	13.5	0	32	0.037	1	U	R.YQGEYVR.R
1682.89601681.88871681.9039	-9.04	0	70	3.9e-06	1	U	K.DLVLNVGYSHPVEIK.A		

Proteins matching the same set of peptides:

RL6_STAES **Mass:** 19652 **Score:** 102 **Matches:** 2(2) **Sequences:** 2(2)

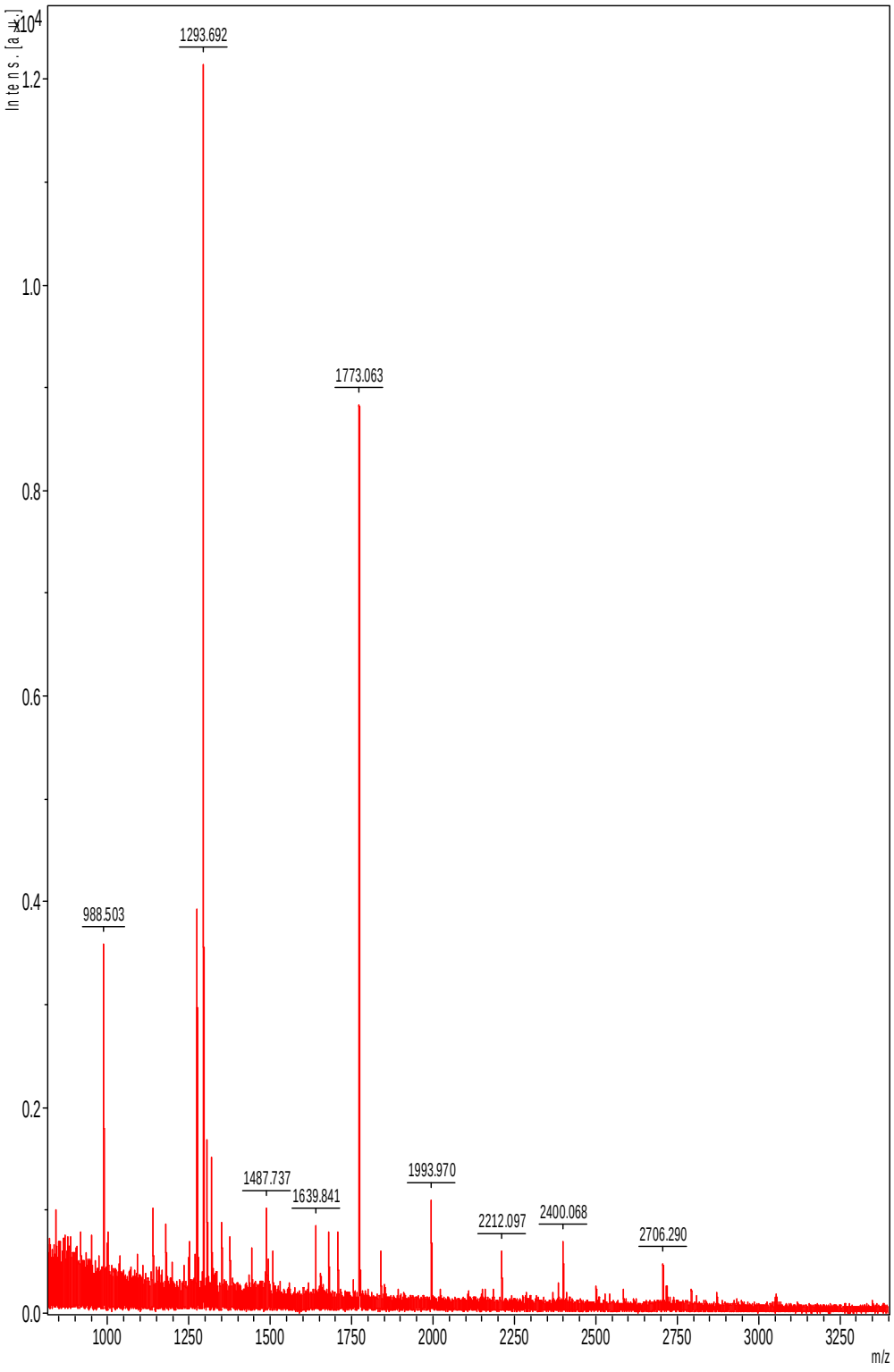
50S ribosomal protein L6 OS=Staphylococcus epidermidis (strain ATCC 12 GN=rplF PE=3 SV=1

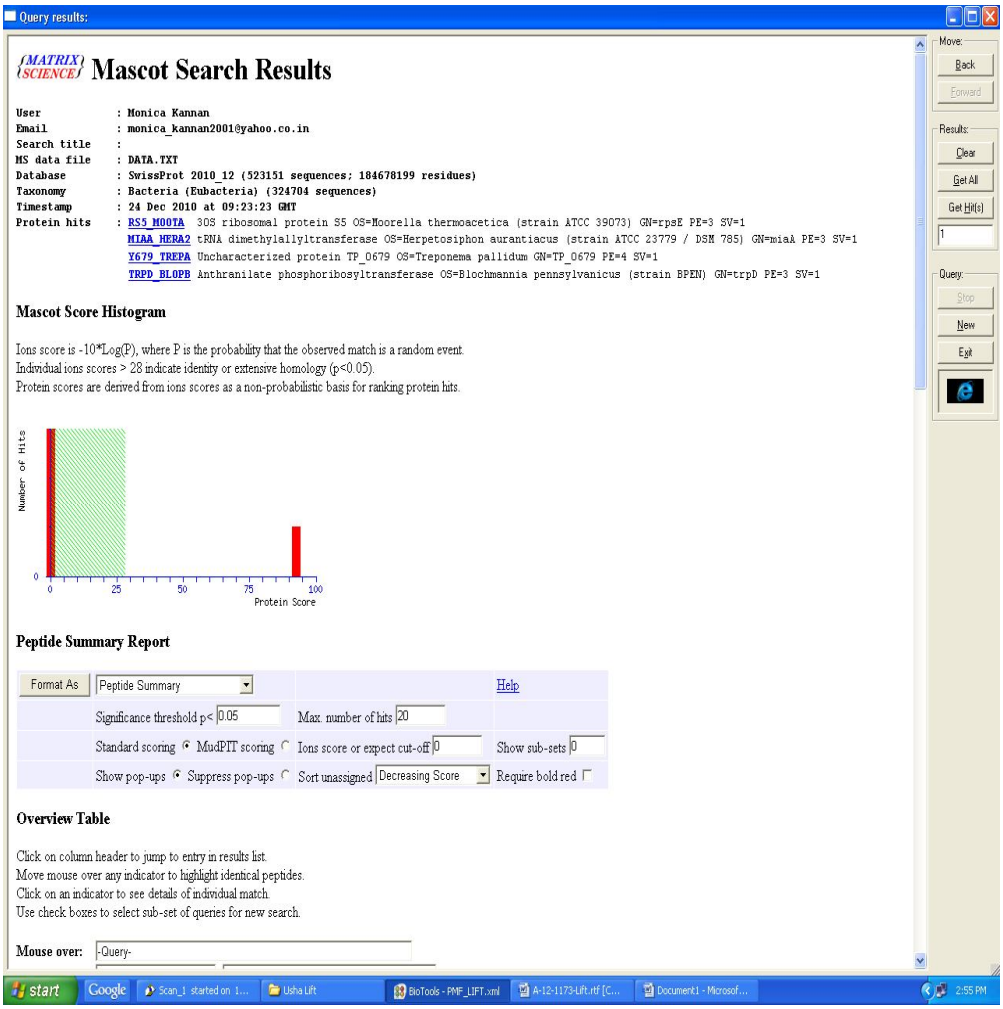
RL6_ACIBL **Mass:** 19064 **Score:** 36 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L6 OS=Acidobacteria bacterium (strain Ellin345) PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1133.6513	1132.6441	1132.6240	17.7	0	36	0.0095	1	U	R.NLEIVGIGYR.A

Spot: 20


Intens. [a.u.]
x10^4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1293.692
1773.063
988.503
1487.737
1639.841
1993.970
2212.097
2400.068
2706.290
1000
1250
1500
1750
2000
2250
2500
2750
3000
3250
m/z



RS5_MOOTA **Mass:** 17131 **Score:** 93 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S5 OS=Moorella thermoacetica(strain ATCC 39073) GN=rpsE PE=3 SV=1

Observed Mr (expt) Mr (calc) ppm Miss Score Expect Rank Unique Peptide

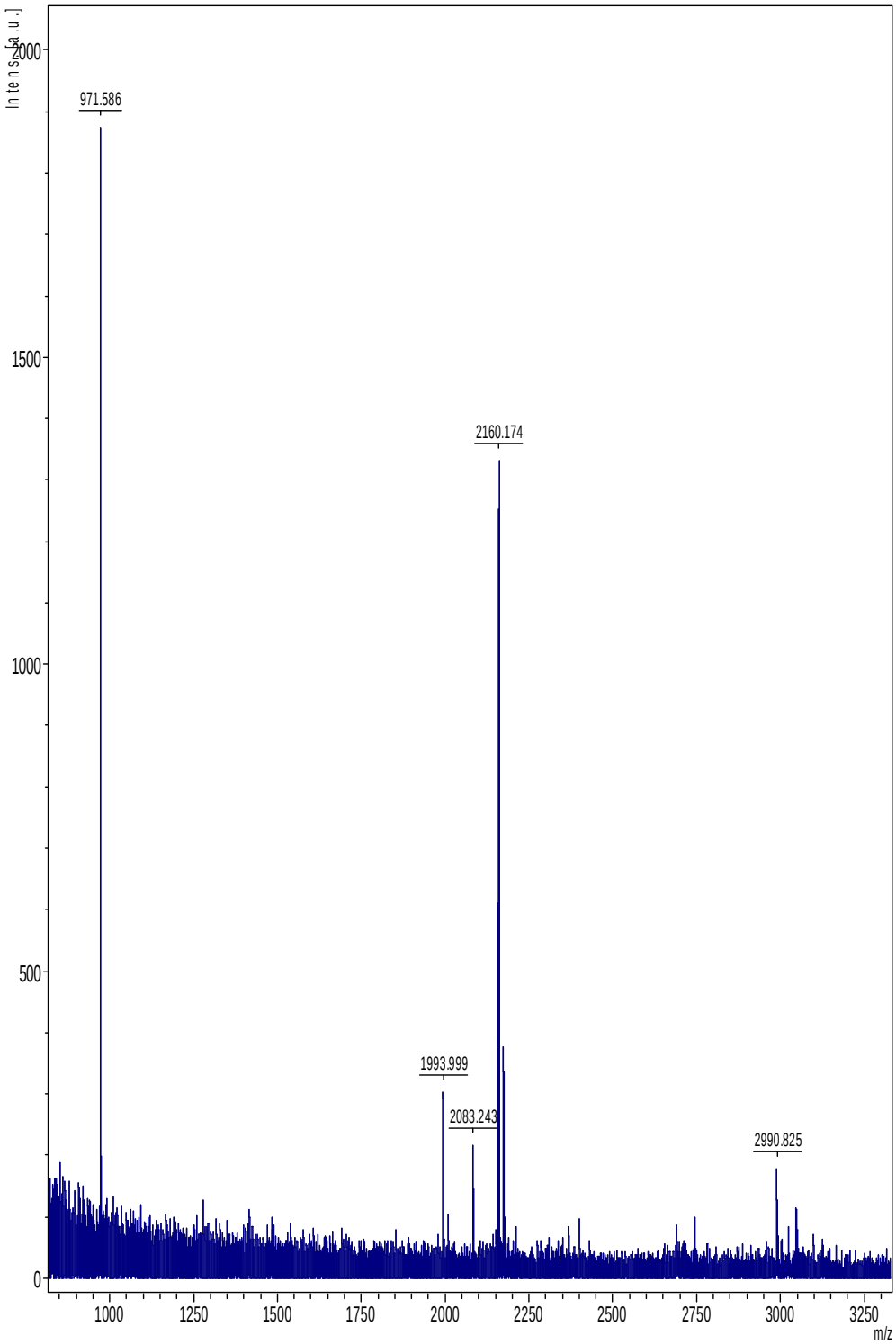
1773.0632 1772.05591772.0672 -6.38 0 93 5.6e-10 1 U K.VLLKPAAPGTGVIAGGPVR.A

Proteins matching the same set of peptides:

RS5_PELTS **Mass:** 17258 **Score:** 93 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S5 OS=Pelotomaculum thermopropionicum (strain DSM 13744 / JCM 10971 / SI) GN=rpsE PE=3 SV=1

Spot: 22


[In te n s. [a. u .]
2000
1500
1000
500
0
971.586
2160.174
1993.999
2083.243
2990.825
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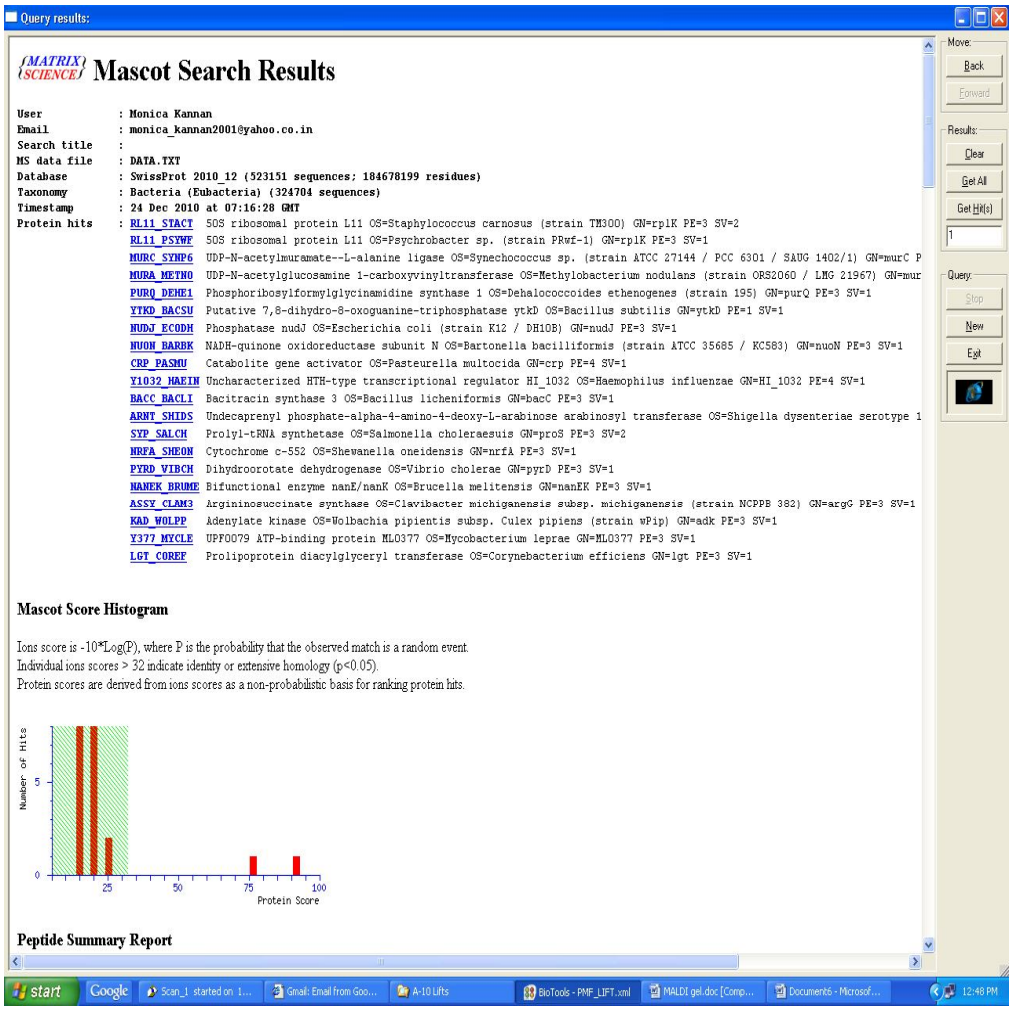
1750
2000
2250
2500
2750
3000
3250
m/z



RL11_STACT **Mass:** 14948 **Score:** 92 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L11 OS=Staphylococcus carnosus (strain TM300) GN=rplK PE=3 SV=2

Observed Mr (expt) Mr (calc) ppm Miss Score Expect Rank Unique Peptide

2160.1745 2159.1672 2159.1110 26.0 0 92 3.2e08 1 U R.TQEQAGLIIPVEISVYEDR.S

Proteins matching the same set of peptides:

RL11_STAEQ **Mass:** 14965 **Score:** 92 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L11 OS=Staphylococcus epidermidis (strain ATCC 35984 / RP62A) GN=rplK PE=3 SV=1

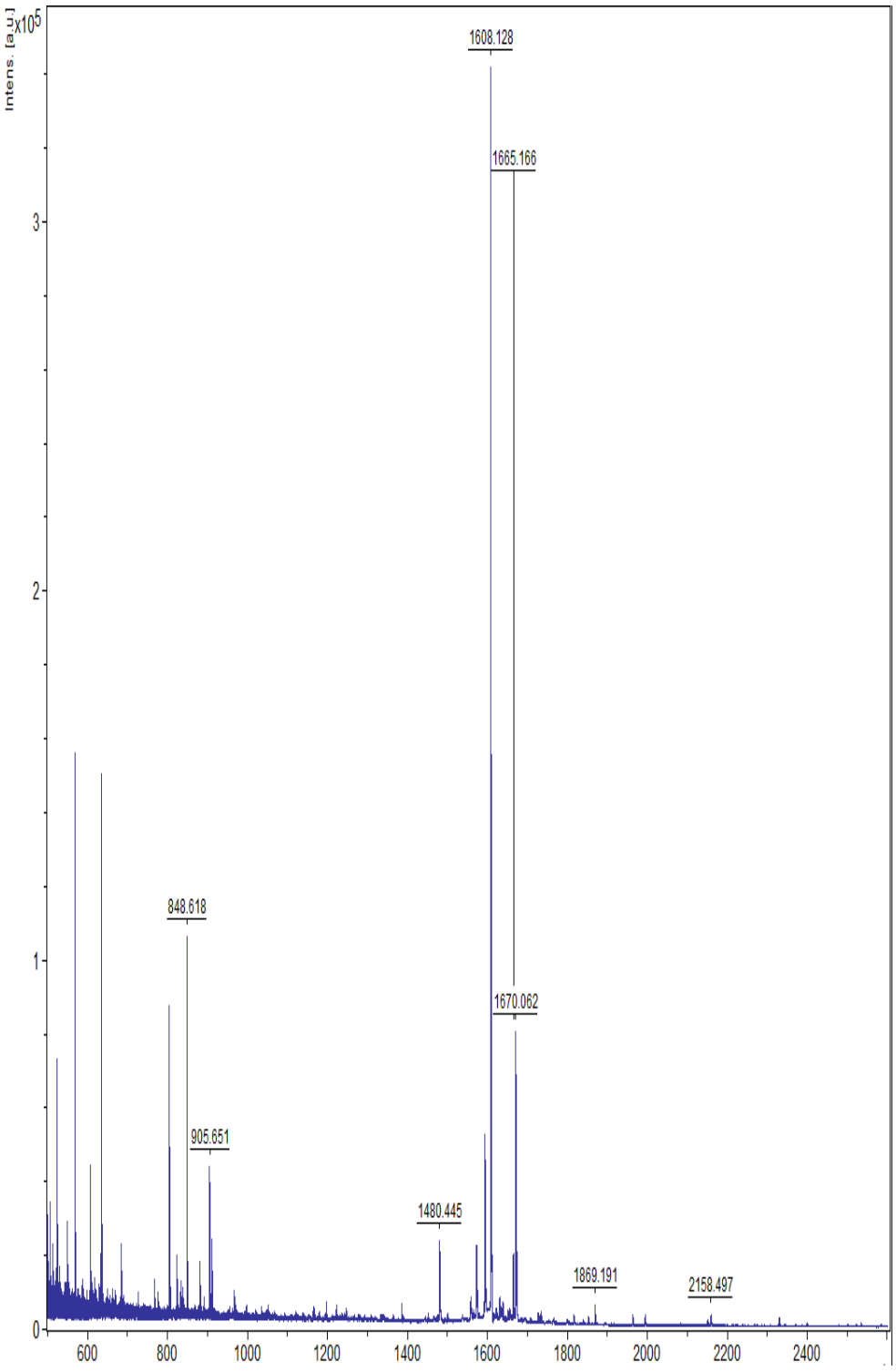
RL11_PSYWF **Mass:** 14876 **Score:** 76 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L11 OS=Psychrobacter sp. (strain PRwf-1) GN=rplK PE=3 SV=1

Observed Mr (expt) Mr (calc) ppm Miss Score Expect Rank Unique Peptide

971.5861 970.5789 970.5600 19.5 0 76 2.9e-06 1 U K.SPPAAFLLR.K

Spot: 23


Intens. [a.u.]
x10^5
3
2
1
0
1608.128
1665.166
1670.062
848.618
905.651
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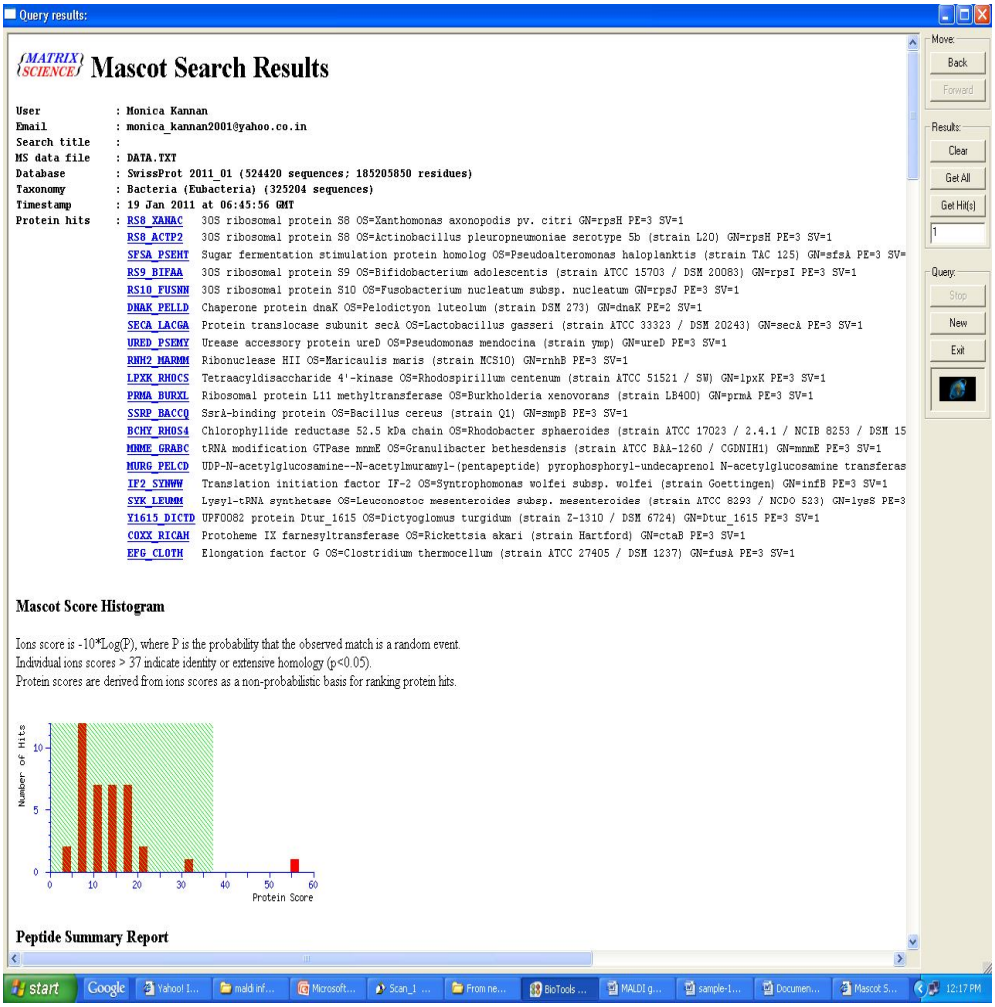
800
1000
1200
1400
1600
1800
2000
2200
2400



RS8_XANAC **Mass:** 14323 **Score:** 56 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S8 OS=Xanthomonas axonopodis pv. citri GN=rpsH PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1608.1283	**1607.1210**	**1606.8831**	**148**	**1**	**56**	**0.00042**	**1**	**U**	**K.YFEGRPVIETLKR.F**

Proteins matching the same set of peptides:

RS8_XANC5 **Mass:** 14353 **Score:** 56 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S8 OS=Xanthomonas campestris pv. vesicatoria (strain 85-10) GN=rpsH PE=3 SV=1

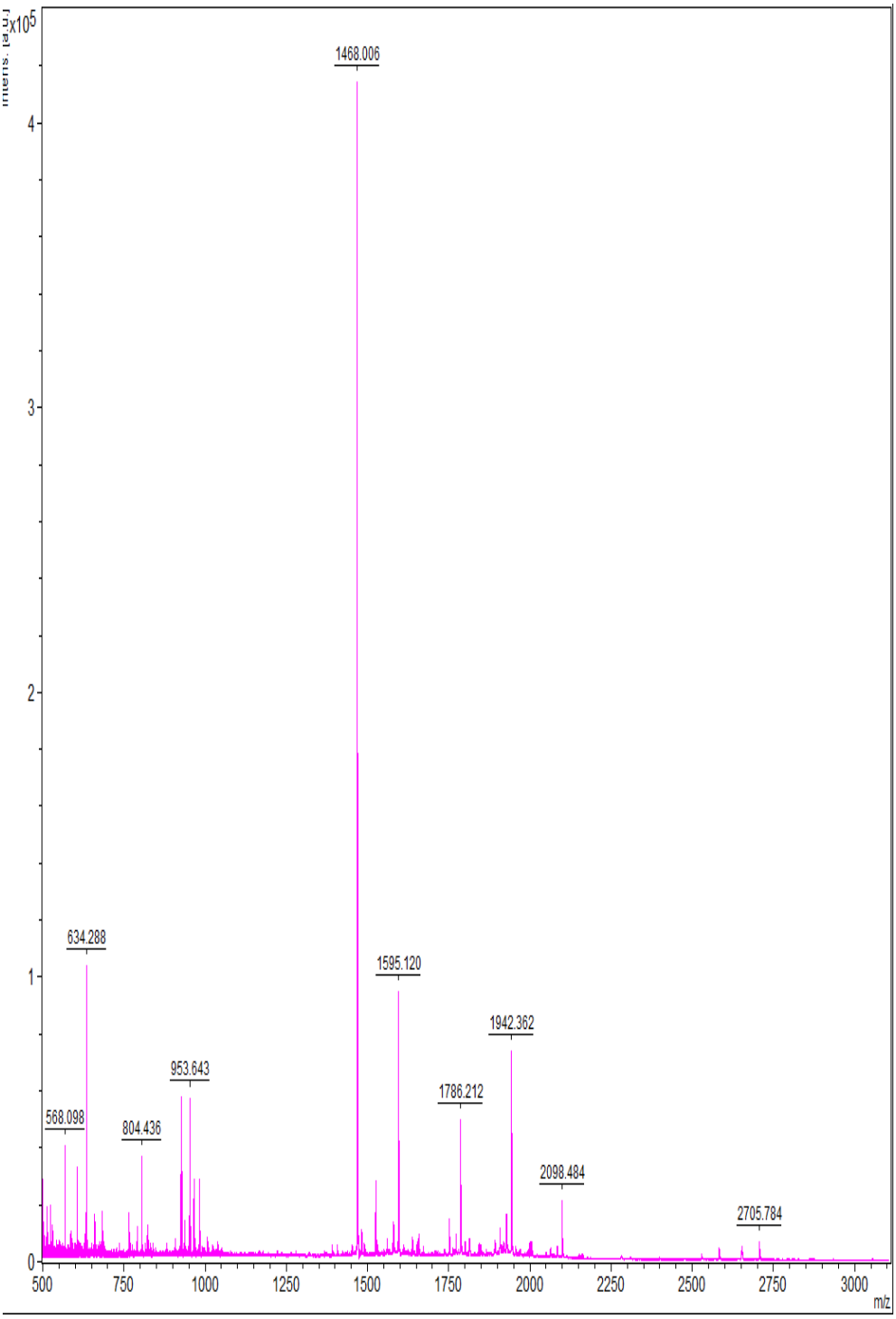
RS8_XANC8 **Mass:** 14312 **Score:** 56 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S8 OS=Xanthomonas campestris pv. campestris (strain 8004) GN=rpsH PE=3 SV=1

RS8_XANCB **Mass:** 14312 **Score:** 56 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S8 OS=Xanthomonas campestris pv. campestris (strain B100) GN=rpsH PE=3 SV=1

Spot: 24


Intens. [a.u.]
x10^5
4
3
2
1
0
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634.288
1595.120
1942.362
953.643
1786.212
568.098
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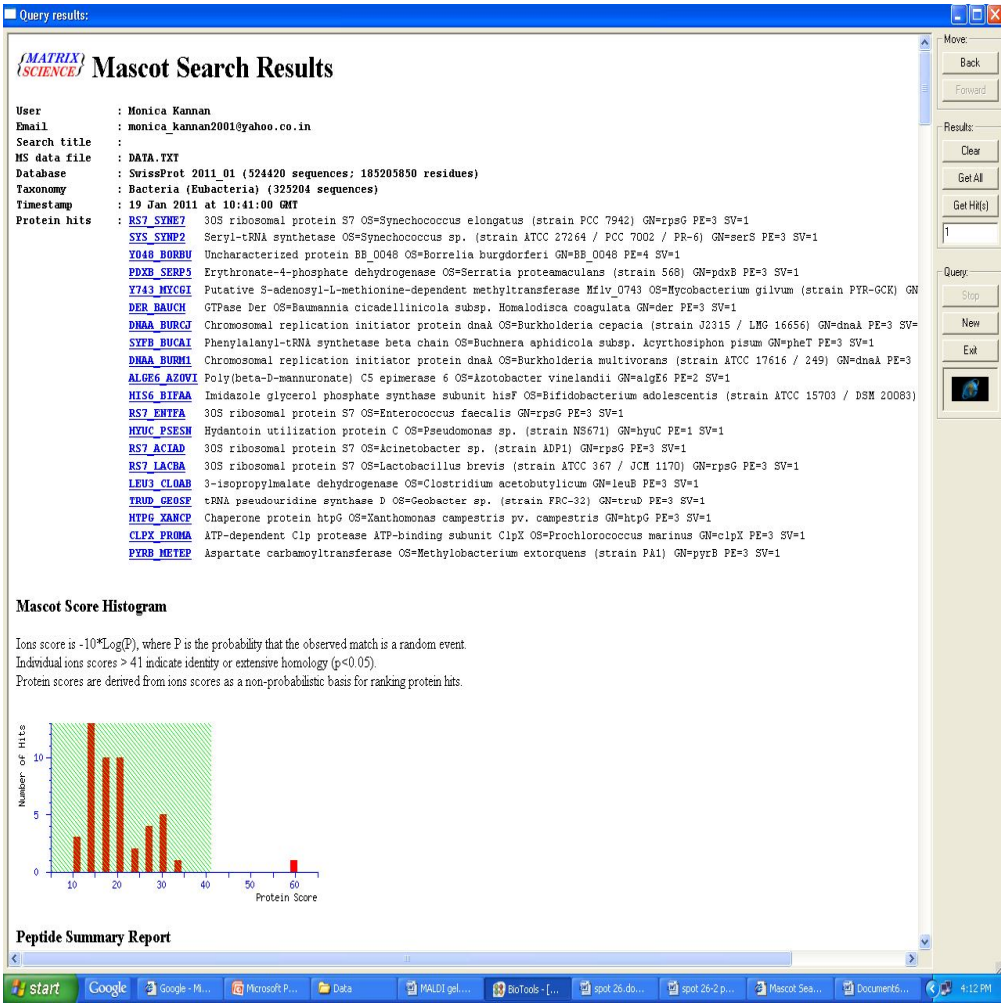
1500
1750
2000
2250
2500
2750
3000
m/z



RS7_SYNE7 **Mass:** 17745 **Score:** 60 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S7 OS=Synechococcus elongatus (strain PCC 7942) GN=rpsG PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1468.0057	1466.9984	1466.7769	151	0	60	0.00066	1	U	R.ILYSAFDLIQER.T

RS7_ENTFA **Mass:** 17822 **Score:** 23 **Matches:** 1(0) **Sequences:** 1(0)

30S ribosomal protein S7 OS=Enterococcus faecalis GN=rpsG PE=3 SV=1

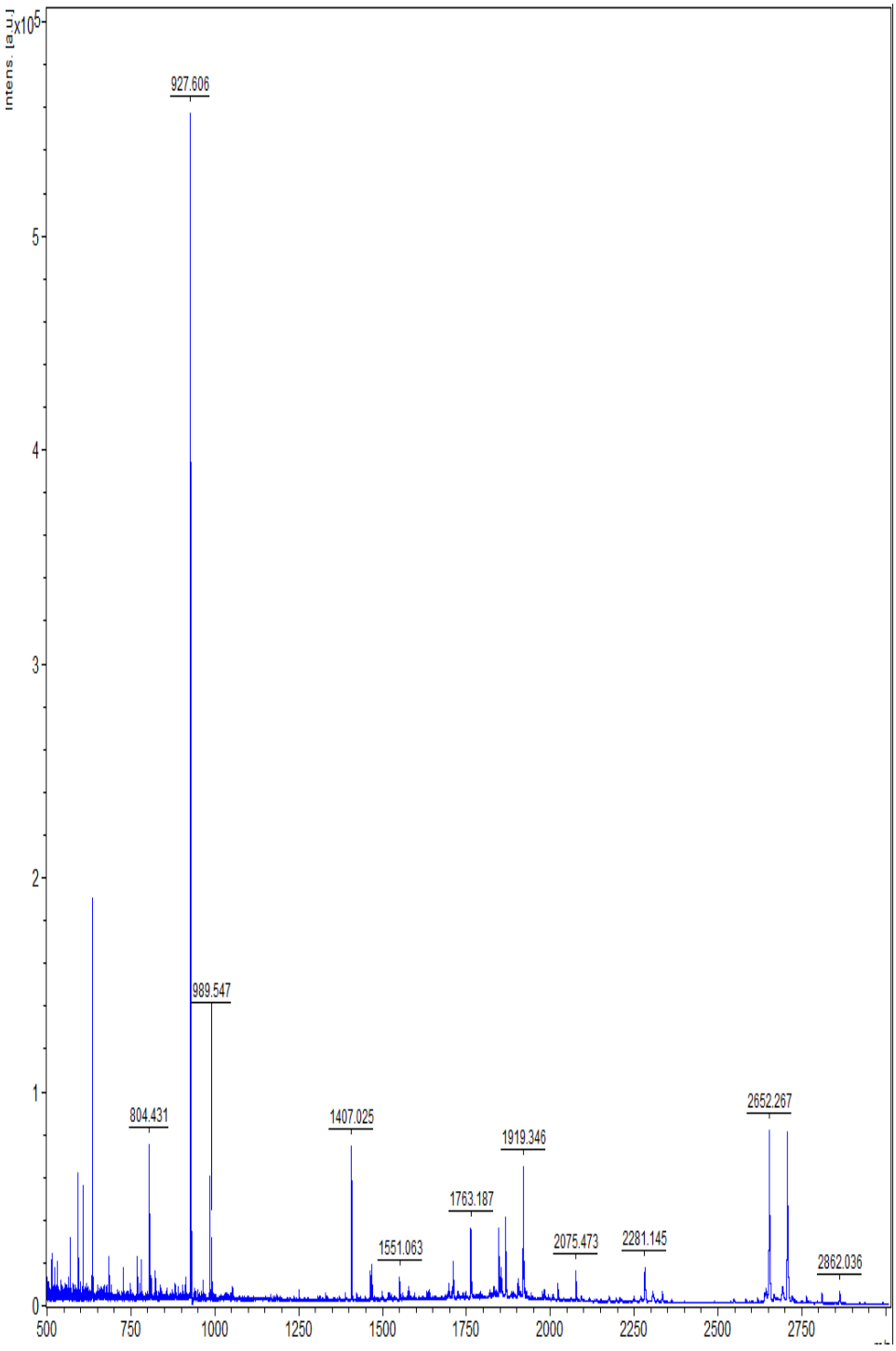
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1942.3618	1941.3545	1941.0181	173	1	25	3.3	1	U	R.VGGSNYQVPVEVRPERR.T

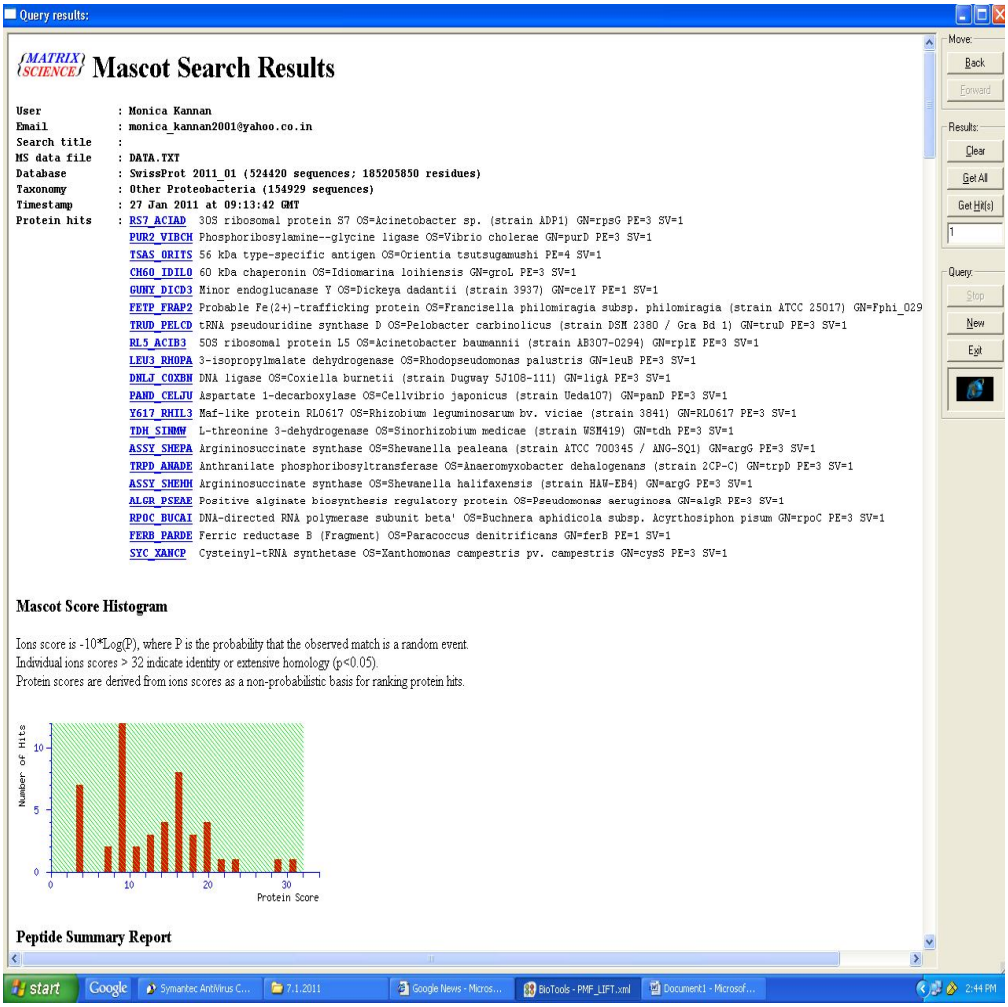
RS7_ACIAD **Mass:** 17614 **Score:** 22 **Matches:** 1(0) **Sequences:** 1(0)

30S ribosomal protein S7 OS=Acinetobacter sp. (strain ADP1) GN=rpsG PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
927.6134	926.6062	926.4399	179	1	22	3.5	1	U	K.AFSHYRF.-

Spot: 25


intens. [a.u.]
x10^5
927.606
989.547
804.431
1407.025
1551.063
1763.187
1919.346
2075.473
2281.145
2652.267
2862.036
0
1
2
3
4
5
500
750
1000
1250
1500
1750
2000
2250
2500
2750



RS7_ACIAD **Mass:** 17614 **Score:** 31 **Matches:** 1(0) **Sequences:** 1(0)

30S ribosomal protein S7 OS=Acinetobacter sp. (strain ADP1) GN=rpsG PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
927.6061	926.5989	926.4399	172	1	31	0.16	1	U	K.AFSHYRF.-

Proteins matching the same set of peptides:

RS7_ACIB3 **Mass:** 17688 **Score:** 31 **Matches:** 1(0) **Sequences:** 1(0)

30S ribosomal protein S7 OS=Acinetobacter baumannii (strain AB307-0294) GN=rpsG PE=3 SV=1

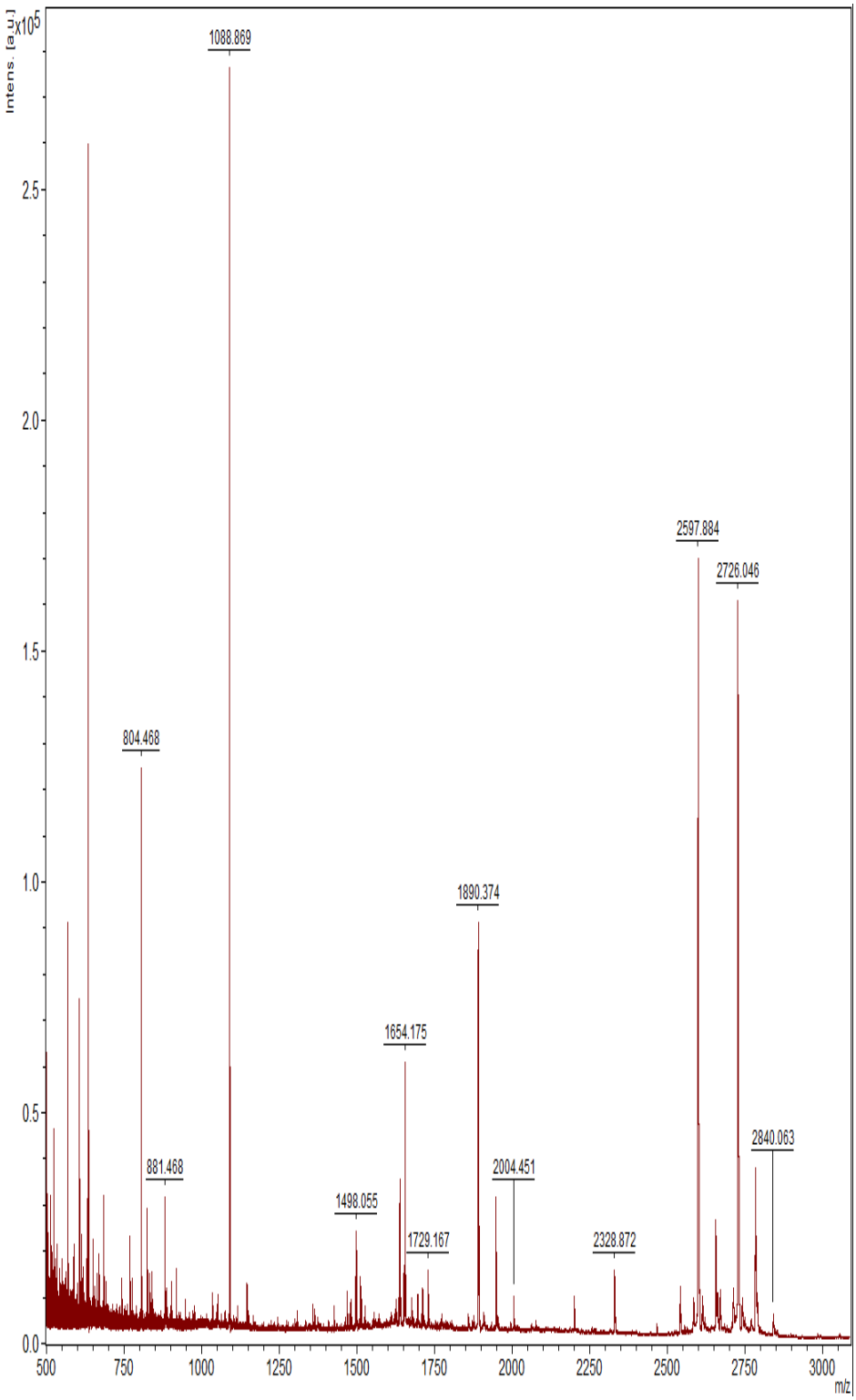
RS7_ACIB5 **Mass:** 17688 **Score:** 31 **Matches:** 1(0) **Sequences:** 1(0)

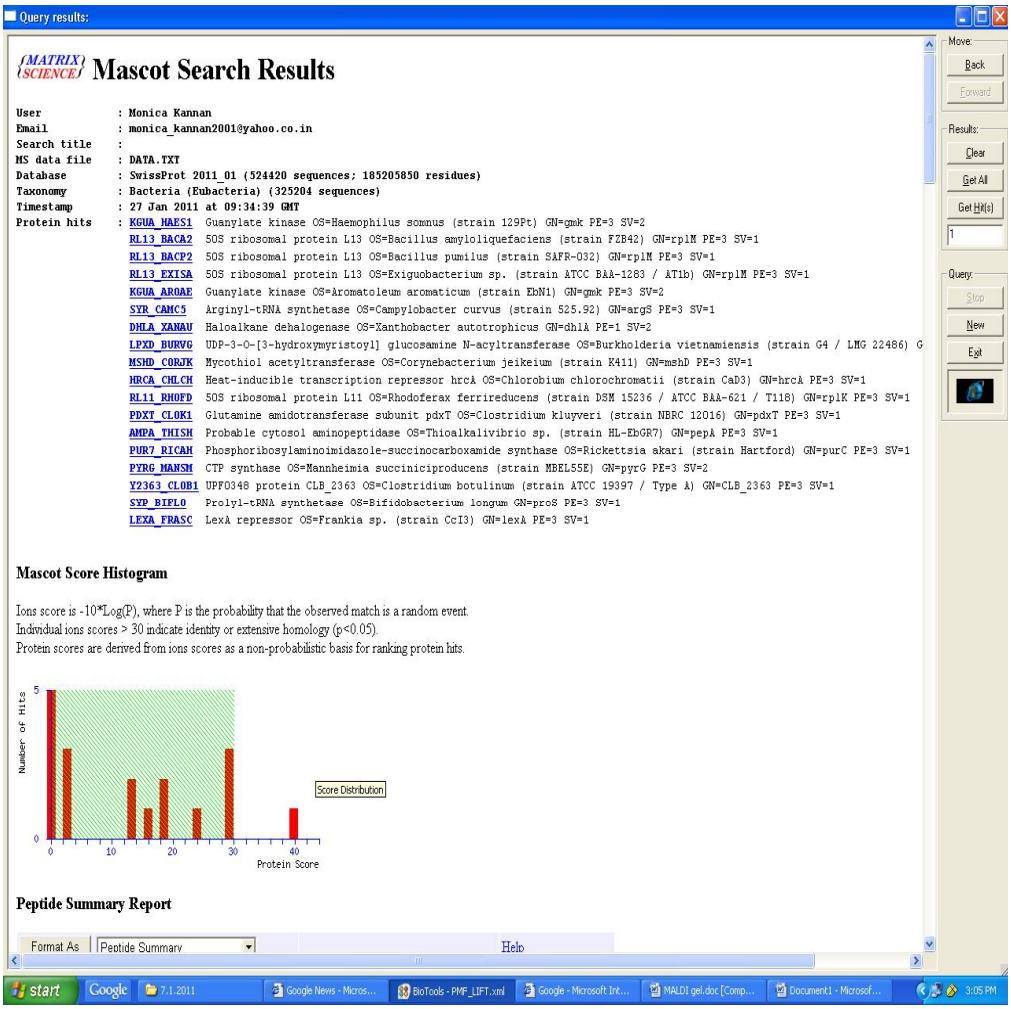
30S ribosomal protein S7 OS=Acinetobacter baumannii (strain AB0057) GN=rpsG PE=3 SV=1

RS7_ACIBS **Mass:** 17688 **Score:** 31 **Matches:** 1(0) **Sequences:** 1(0)

30S ribosomal protein S7 OS=Acinetobacter baumannii (strain SDF) GN=rpsG PE=3 SV=1

Spot: 26


Intens. [a.u.]
x10^5
2.5
2.0
1.5
1.0
0.5
0.0
1088.869
804.468
881.468
1498.055
1654.175
1729.167
1890.374
2004.451
2328.872
2597.884
2726.046
2840.063
500
750
1000
1250
1500
1750
2000
2250
2500
2750
3000
m/z



RL13_BACA2 **Mass:** 16395 **Score:** 30 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L13 OS=Bacillus amyloliquefaciens (strain FZB42) GN=rplM PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1088.8687	1087.8615	1087.6237	219	0	30	0.014	2	U	R.LSTEVASILR.G

Proteins matching the same set of peptides:

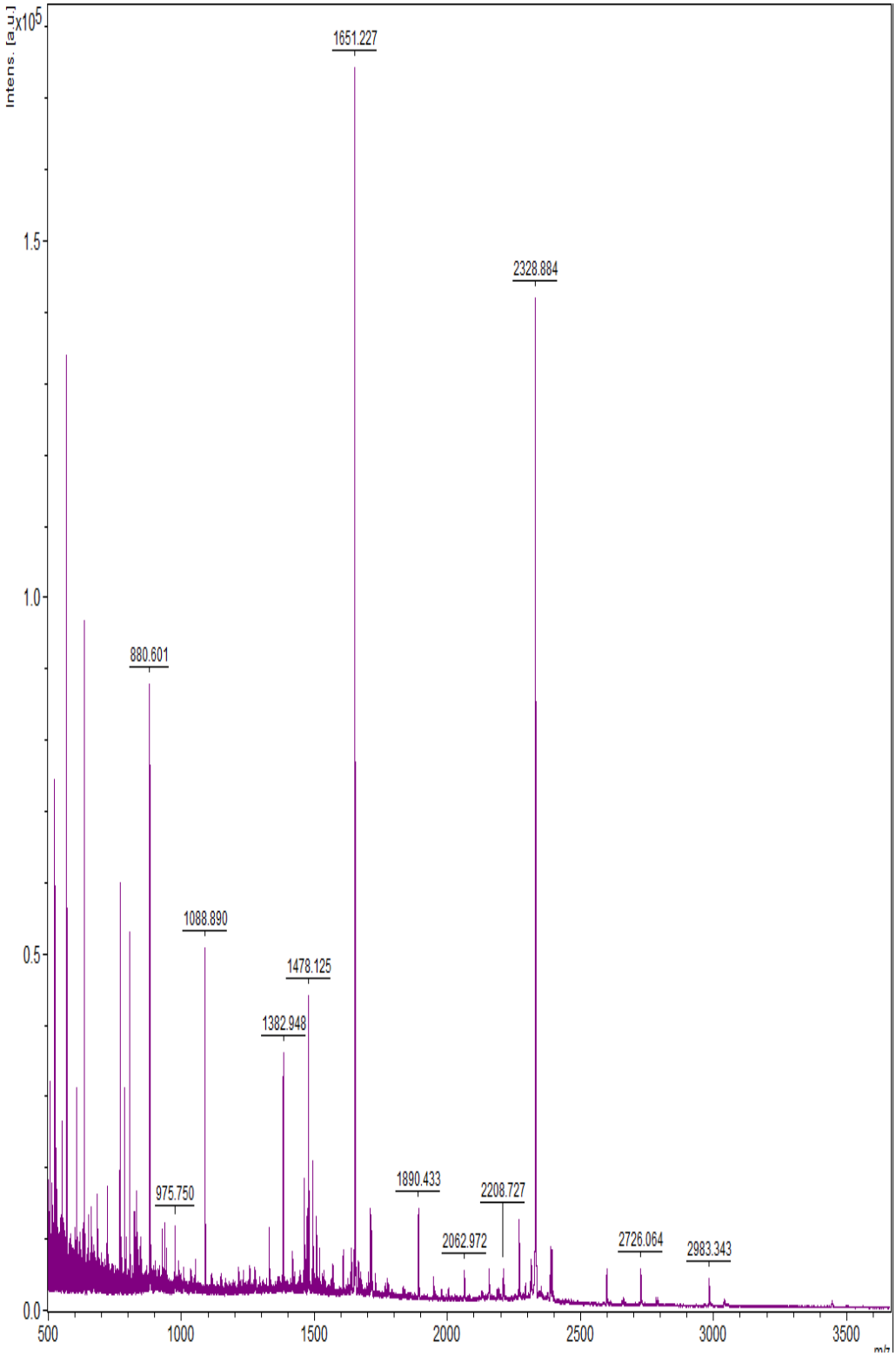
RL13_BACC2 **Mass:** 16448 **Score:** 30 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L13 OS=Bacillus cereus (strain G9842) GN=rplM PE=3 SV=1

RL13_BACLD **Mass:** 16407 **Score:** 30 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L13 OS=Bacillus licheniformis (strain DSM 13 / ATCC 14580) GN=rplM PE=3 SV=1

Spot: 28


Intens. [a.u.]
x10^5
1.5
1.0
0.5
0.0
1651.227
2328.884
880.601
1088.890
1478.125
1382.948
975.750
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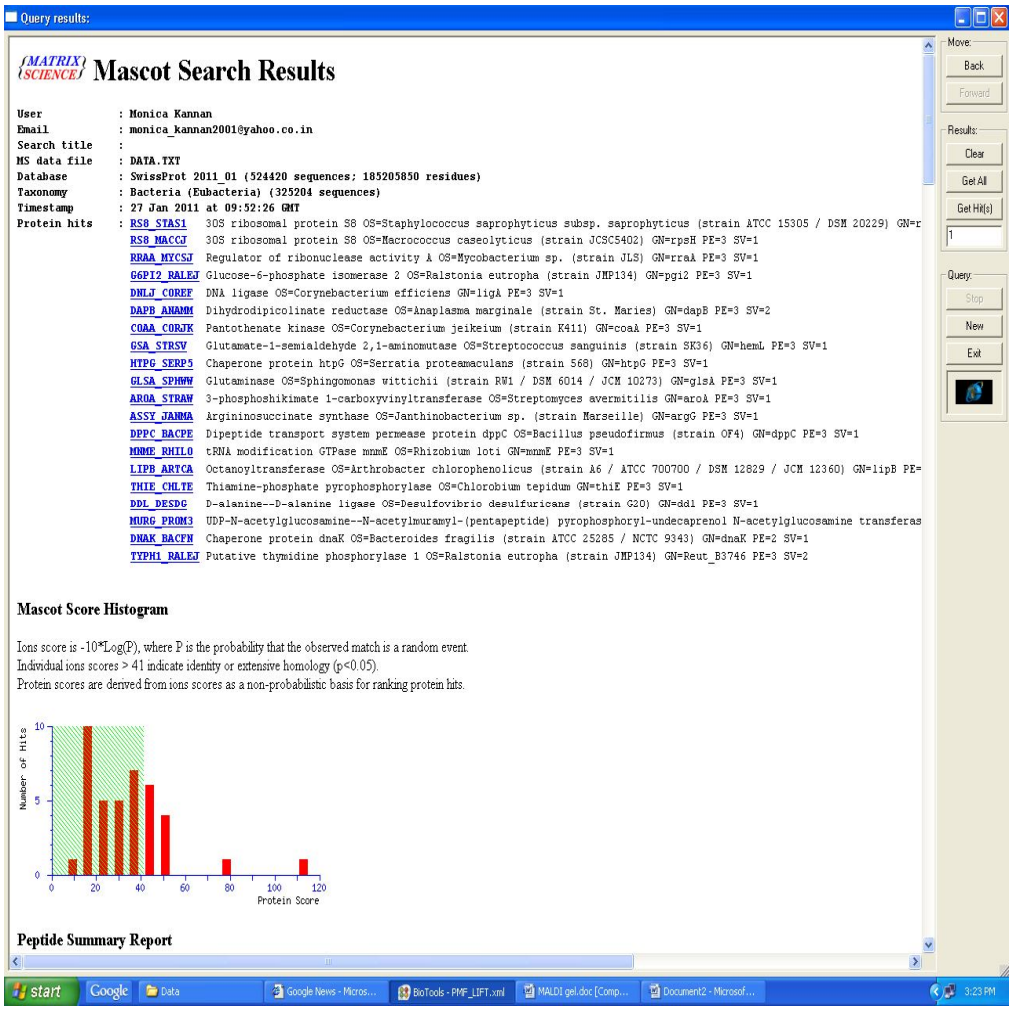
2000
2500
3000
3500
m/z



RS8_STAS1 **Mass:** 14797 **Score:** 113 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S8 OS=Staphylococcus saprophyticus subsp. saprophyticus (strain ATCC 15305 / DSM 20229) GN=rpsH PE=3 SV=1

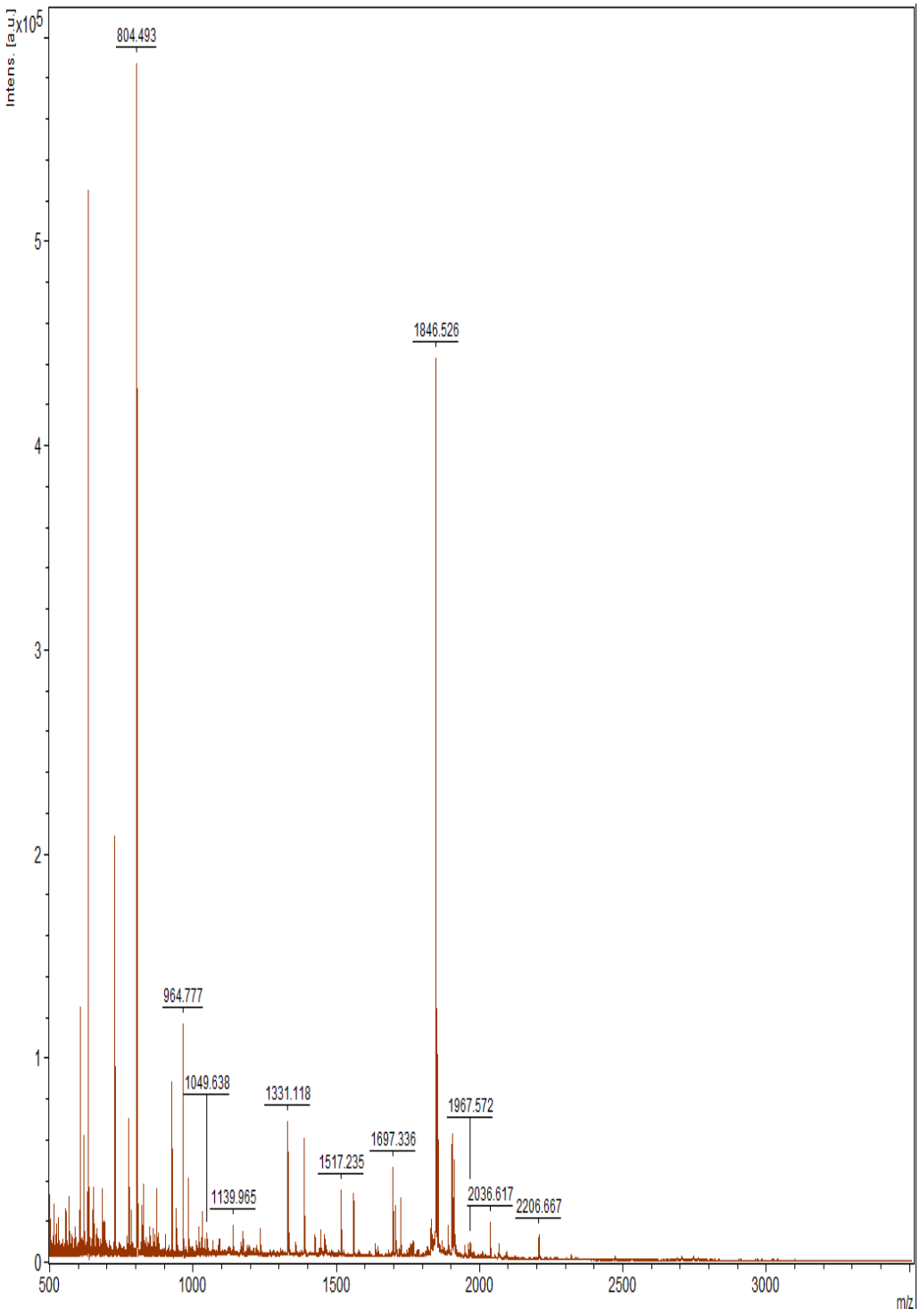
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2328.8835	2327.8763	2327.2696	261	1	113	2.9e09	1	U	K.VLNGLGIALVSTSEGVVTDKEAR.K

RS8_MACCJ **Mass:** 14834 **Score:** 76 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S8 OS=Macrococcus caseolyticus (strain JCSC5402) GN=rpsH PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1651.2274	1650.2201	1649.8260	239	1	76	1.9e-05	1	U	K.SVEYIEDDKQGVIR.M

Spot: 30


Intens. [a.u.]
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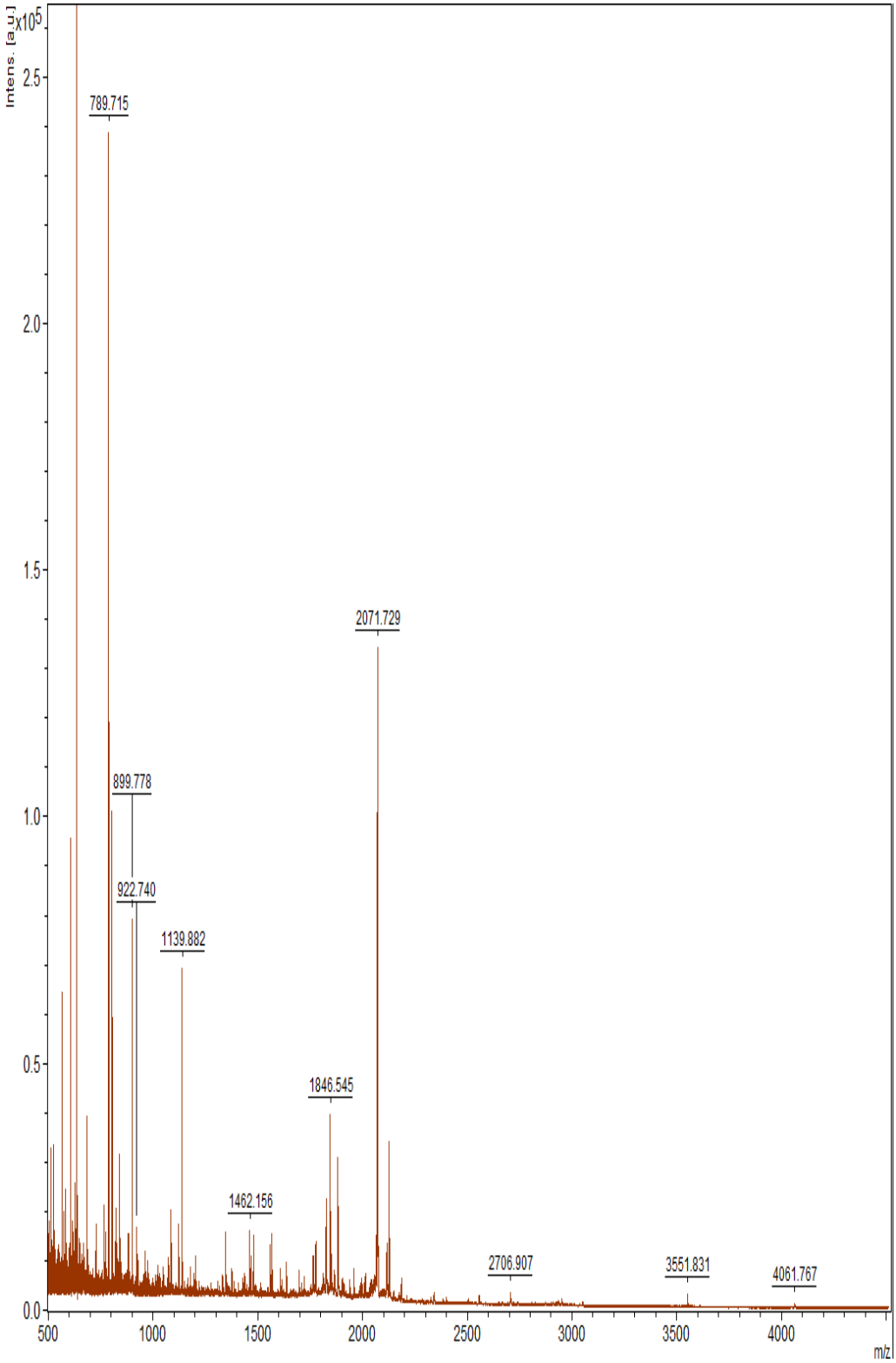
804.493
1846.526
964.777
1049.638
1331.118
1967.572
1697.336
1517.235
1139.965
2036.617
2206.667
0
1
2
3
4
5
500
1000
1500
2000
2500
3000
m/z

Spot: 31


Intens. [a.u.]
x10^5
2.5
2.0
1.5
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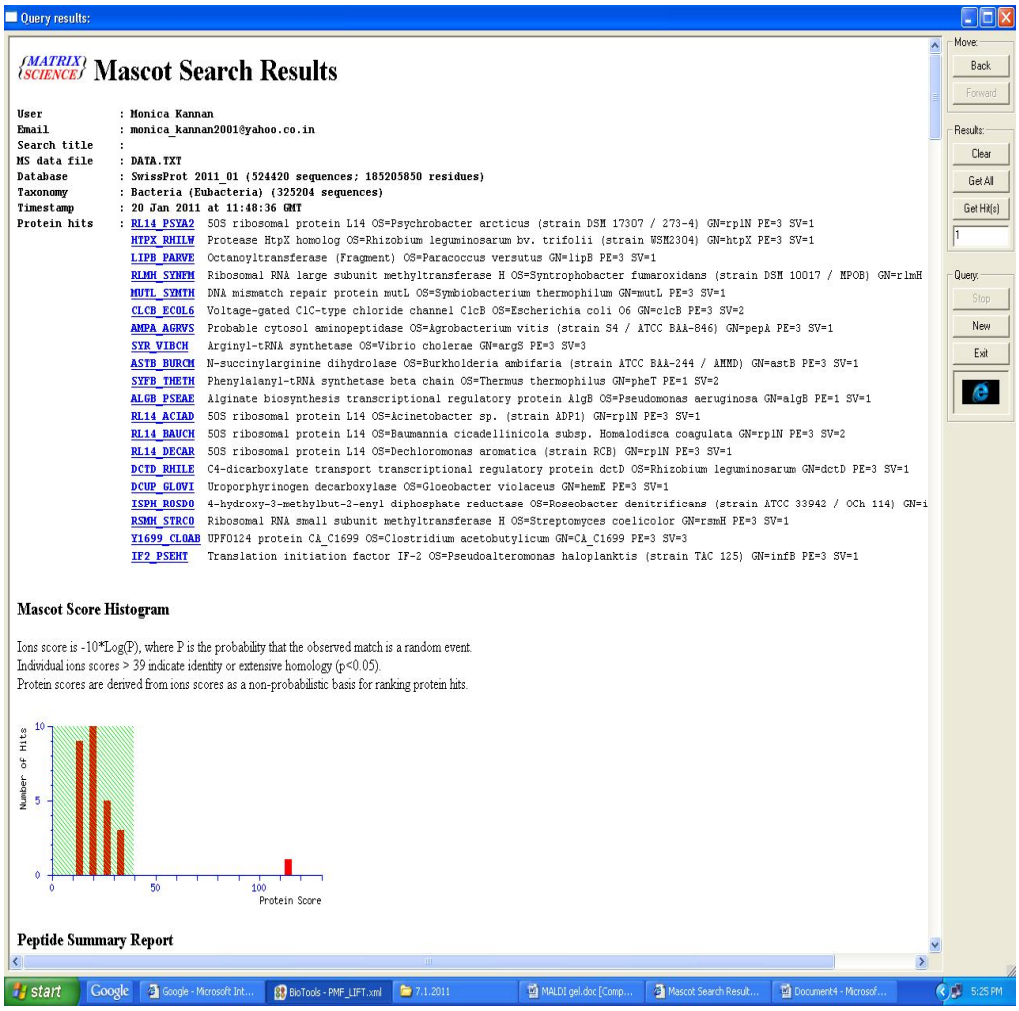
0.5
0.0
789.715
899.778
922.740
1139.882
1462.156
1846.545
2071.729
2706.907
3551.831
4061.767
500
1000
1500
2000
2500
3000
3500
4000
m/z



RL14_PSYA2 **Mass:** 13443 **Score:** 114 **Matches:** 2(1) **Sequences:** 2(1)

50S ribosomal protein L14 OS=Psychrobacter arcticus (strain DSM 17307 / 273-4) GN=rplN PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
899.7779	**898.7706**	**898.4985**	**303**	**0**	**21**	**3**	**2**	**U**	**R.RPDGSVLR.F**
2071.7293	**2070.7220**	**2070.0858**	**307**	**1**	**93**	**2.3e-07**	**1**	**U**	**R.FDDNAAVLLNQN KAPIATR.I**

Proteins matching the same set of peptides:

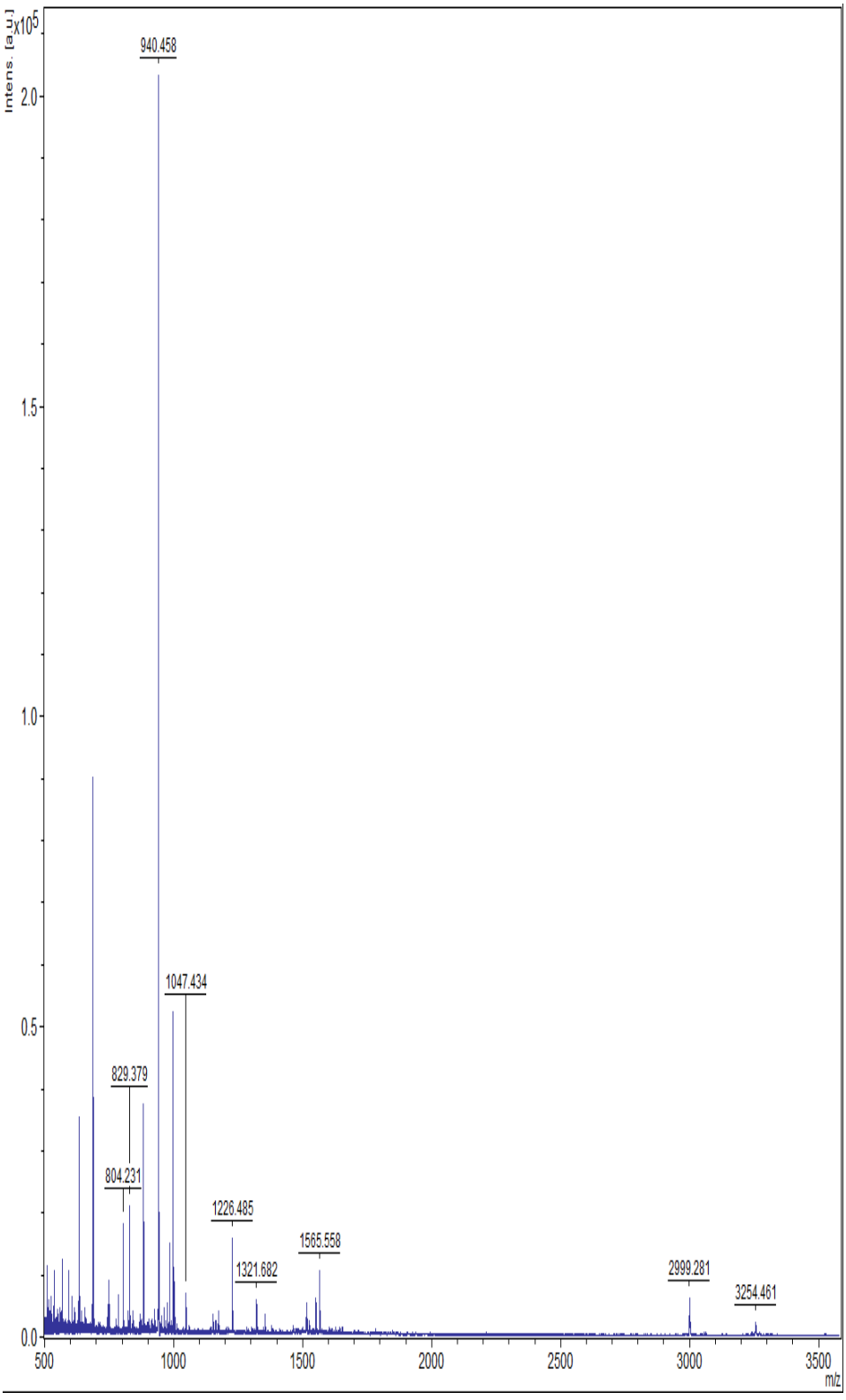
RL14_PSYCK **Mass:** 13443 **Score:** 114 **Matches:** 2(1) **Sequences:** 2(1)

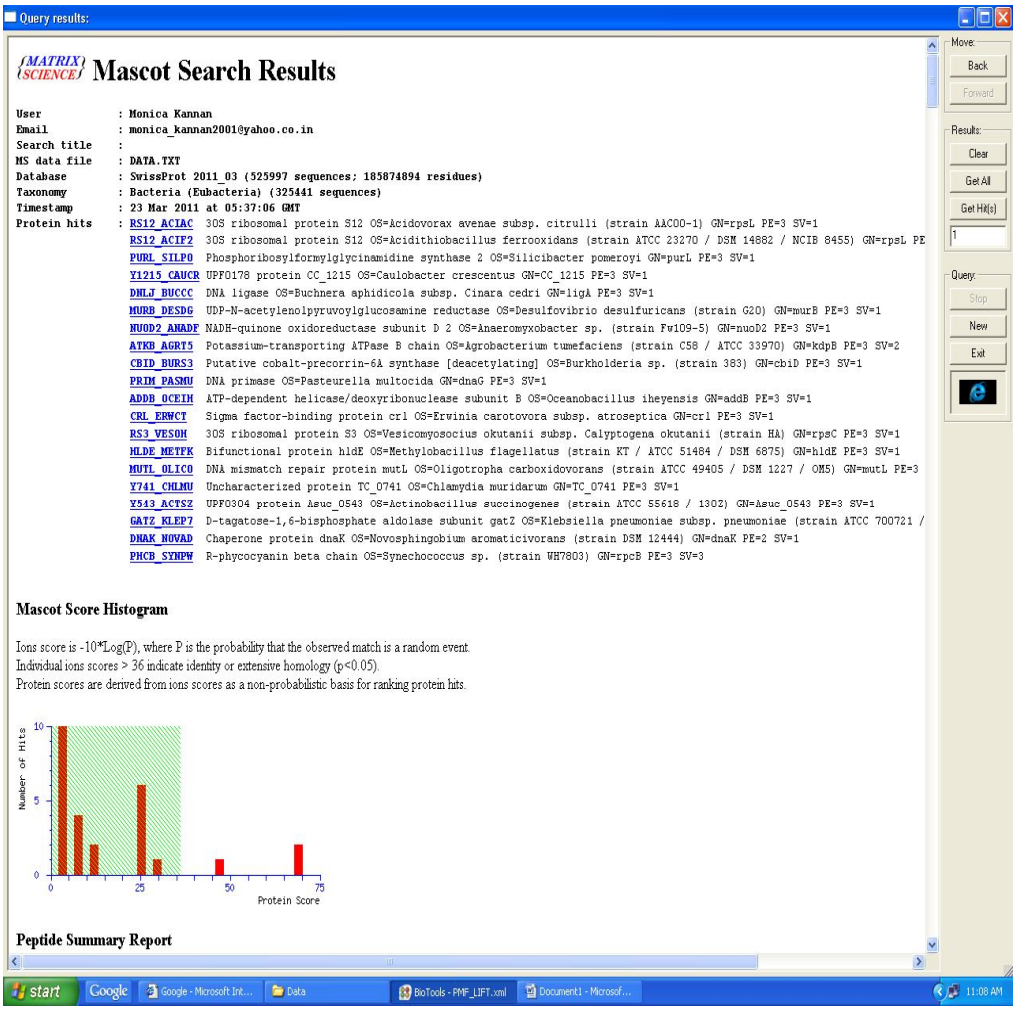
50S ribosomal protein L14 OS=Psychrobacter cryohalolentis (strain K5) GN=rplN PE=3 SV=1

RL14_PSYWF **Mass:** 13427 **Score:** 114 **Matches:** 2(1) **Sequences:** 2(1)

50S ribosomal protein L14 OS=Psychrobacter sp. (strain PRwf-1) GN=rplN PE=3 SV=1

Spot: 38


Intens. [a.u.]
x10^5
2.0
1.5
1.0
0.5
0.0
940.458
1047.434
829.379
804.231
1226.485
1565.558
1321.682
2999.281
3254.461
500
1000
1500
2000
2500
3000
3500
m/z



RS12_ACIAC **Mass:** 13969 **Score:** 69 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S12 OS=Acidovorax avenae subsp. citrulli (strain AAC00-1) GN=rpsL PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
940.4580	939.4507	939.5502	-105.83	0	69	2.8e-05	1	U	M.PTINQLVR.Q

Proteins matching the same set of peptides:

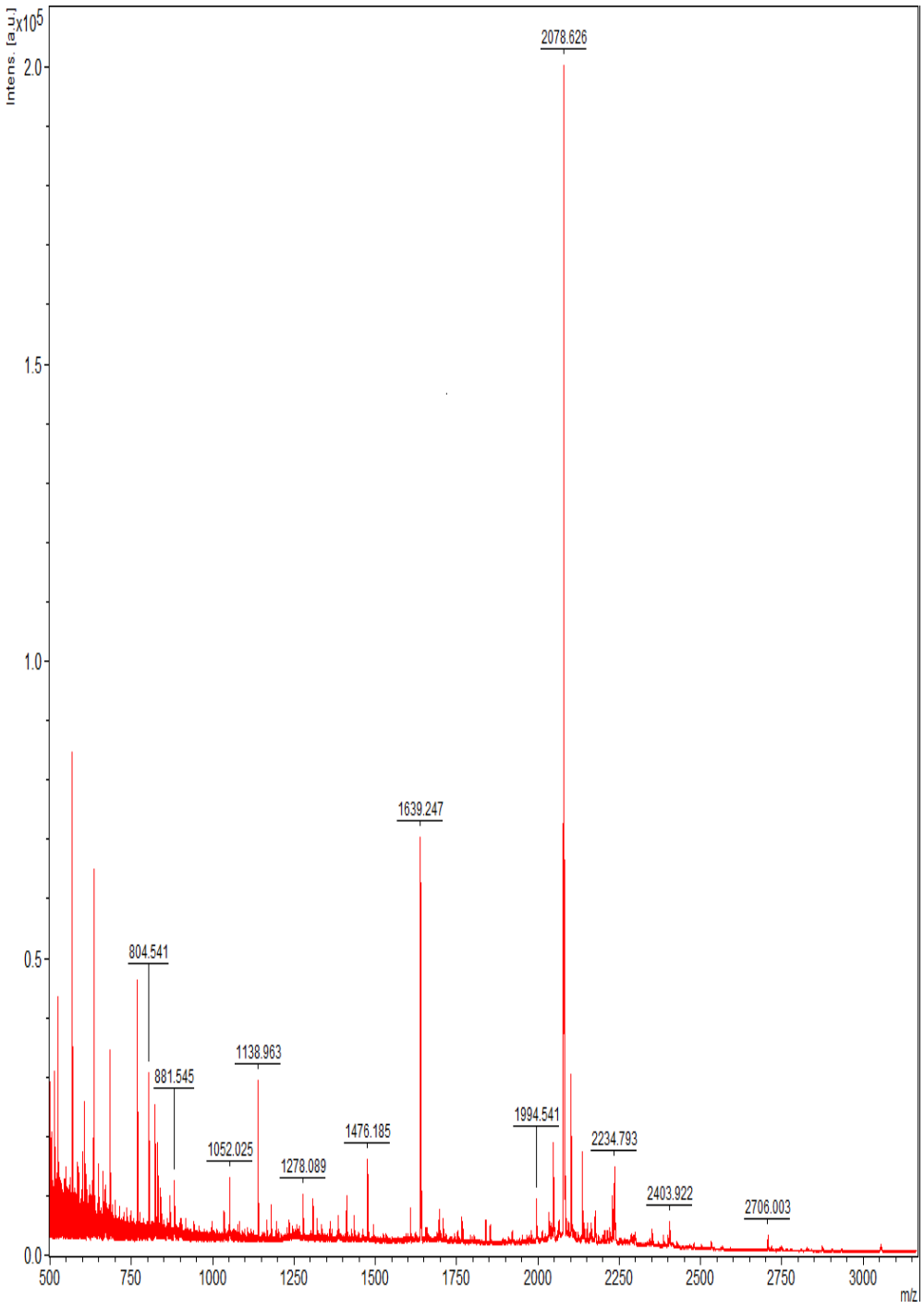
RS12_ACIET **Mass:** 14011 **Score:** 69 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S12 OS=Acidovorax ebreus (strain TPSY) GN=rpsL PE=3 SV=1

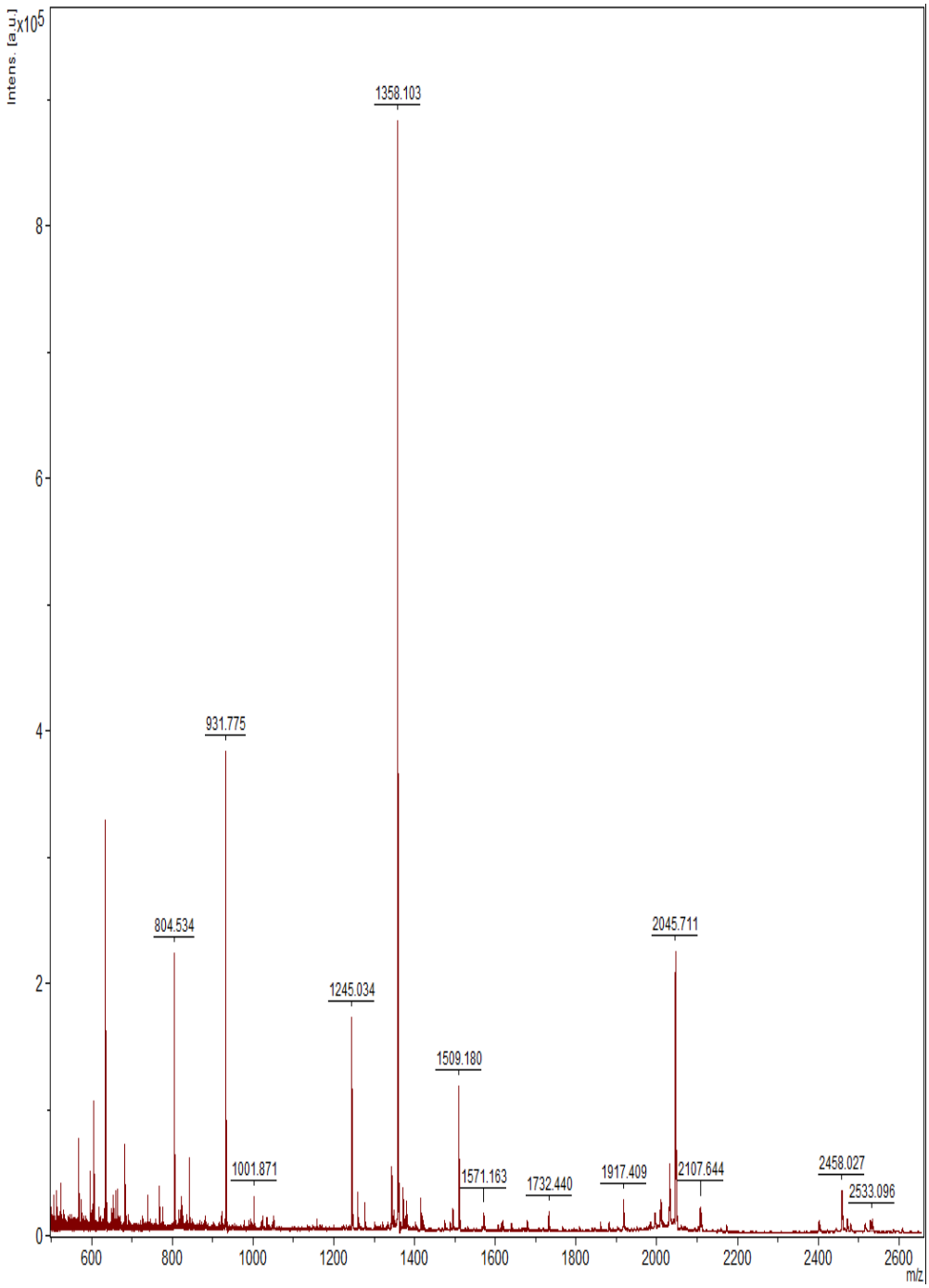
RS12_ACISJ **Mass:** 14011 **Score:** 69 **Matches:** 1(1) **Sequences:** 1(1)

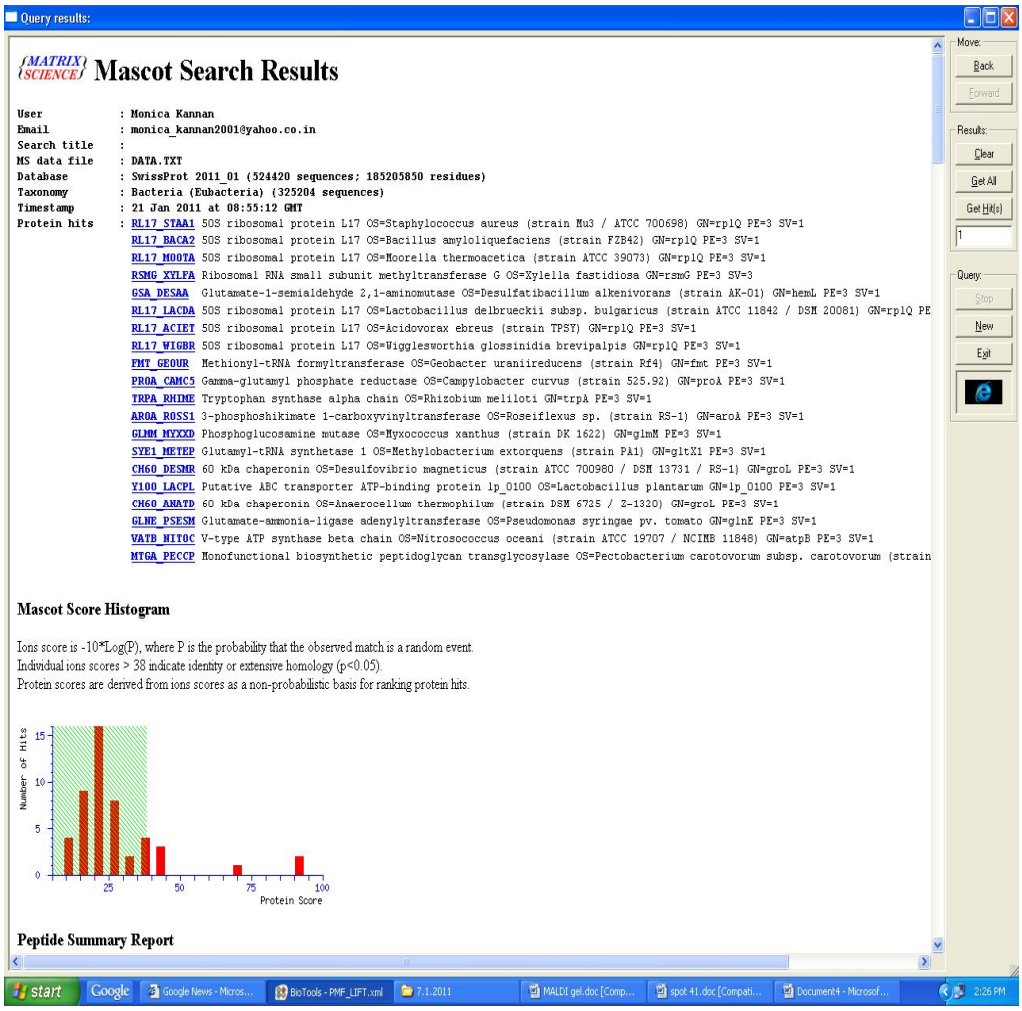
30S ribosomal protein S12 OS=Acidovorax sp. (strain JS42) GN=rpsL PE=3 SV=1

Spot: 39


Intens. [a.u.]
x10^5
2.0
1.5
1.0
0.5
0.0
2078.626
1639.247
804.541
1138.963
881.545
1994.541
2234.793
1476.185
1052.025
1278.089
2403.922
2706.003
500
750
1000
1250
1500
1750
2000
2250
2500
2750
3000
m/z

Spot: 40


Intens. [a.u.]
x10^5
8
6
4
2
0
1358.103
931.775
804.534
1245.034
1509.180
2045.711
1001.871
1571.163
1732.440
1917.409
2107.644
2458.027
2533.096
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
m/z



RL17_STAA1 **Mass:** 13739 **Score:** 92 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L17 OS=Staphylococcus aureus (strain Mu3 / ATCC 700698) GN=rplQ PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1358.1028	1357.0955	1356.7249	273	1	92	2.1e-07	1	U	R.RGDGAESVIIELV.-

Proteins matching the same set of peptides:

RL17_STAA2 **Mass:** 13739 **Score:** 92 **Matches:** 1(1) **Sequences:** 1(1)

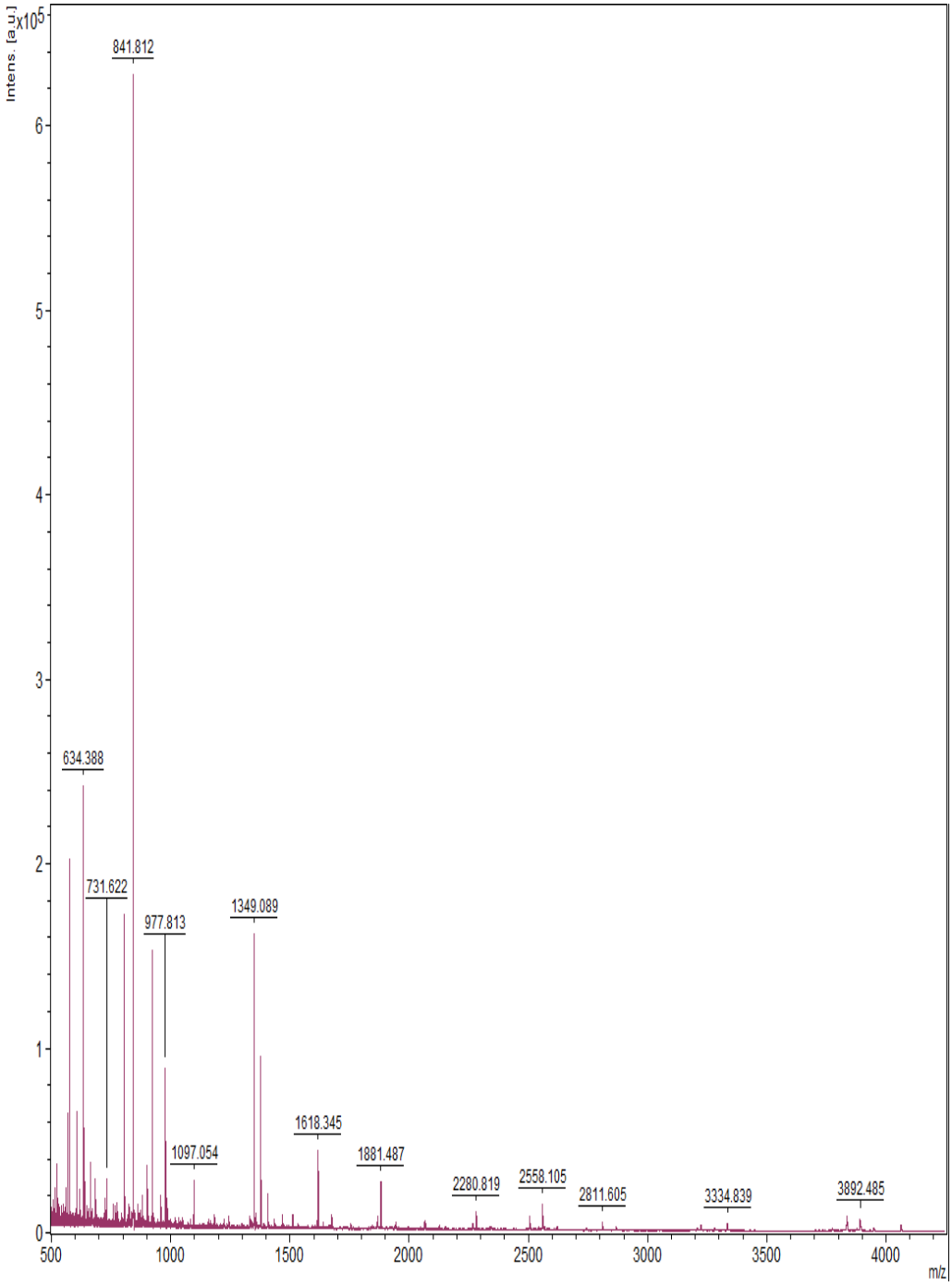
50S ribosomal protein L17 OS=Staphylococcus aureus (strain JH1) GN=rplQ PE=3 SV=1

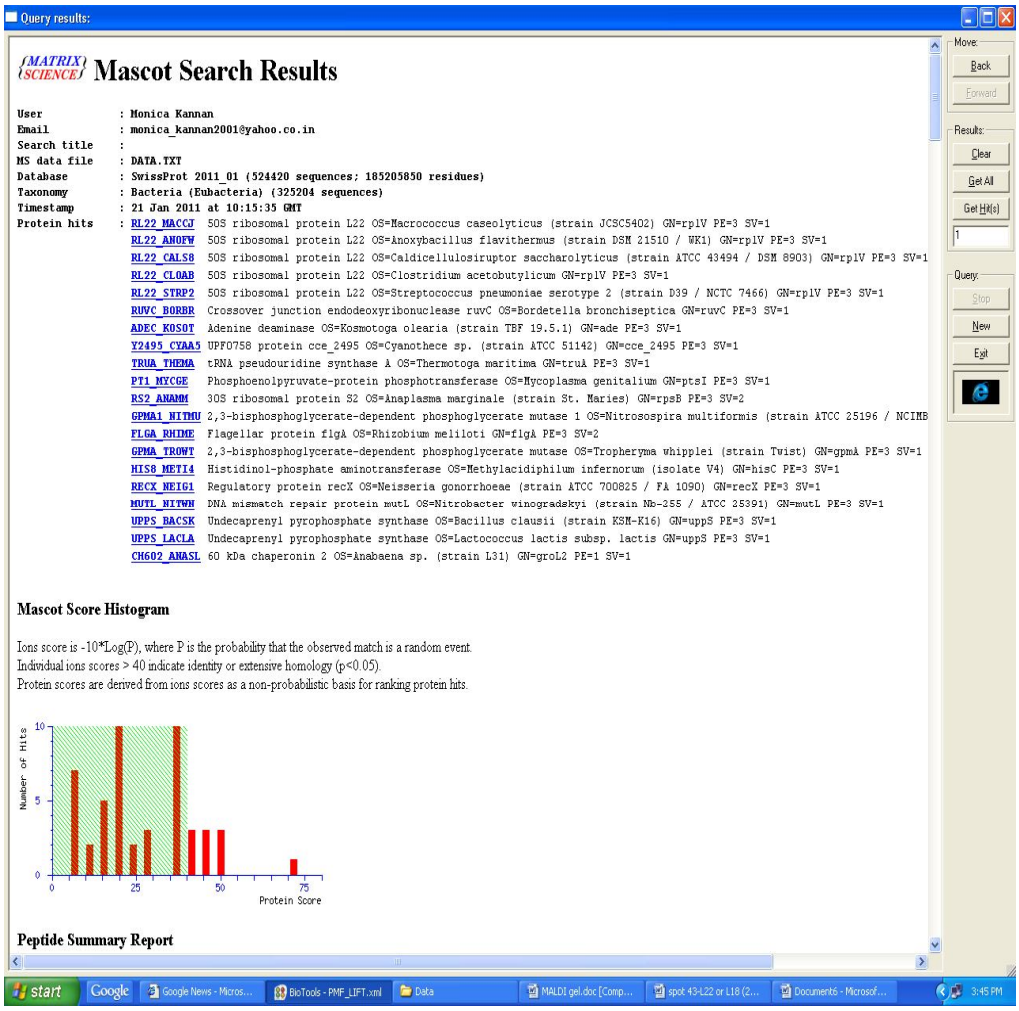
RL17_ACIET Mass: 14751 Score: 39 Matches: 1(1) Sequences: 1(1)

50S ribosomal protein L17 OS=Acidovorax ebreus (strain TPSY) GN=rplQ PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
931.7750	930.7677	930.4923	296	0	39	0.055	1	U	K.LFNDLGPR.F

Spot: 42


Intens. [a.u.]
x10^5
6
5
4
3
2
1
0
841.812
634.388
731.622
977.813
1349.089
1618.345
1097.054
1881.487
2280.819
2558.105
2811.605
3334.839
3892.485
500
1000
1500
2000
2500
3000
3500
4000
m/z



RL22_MACCJ **Mass:** 13654 **Score:** 72 **Matches:** 2(1) **Sequences:** 2(1)

50S ribosomal protein L22 OS=Macrococcus caseolyticus (strain JCSC5402) GN=rplV PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
841.8124	840.8051	840.5433	312	0	48	0.0058	1	U	R.LVLDLIR.G
1349.0890	1348.0817	1347.6782	299	1	23	3.9	2	U	K.EAYANEGPTLKR.F

Proteins matching the same set of peptides:

RL22_STAA1 **Mass:** 12827 **Score:** 72 **Matches:** 2(1) **Sequences:** 2(1)

50S ribosomal protein L22 OS=Staphylococcus aureus (strain Mu3 / ATCC 700698) GN=rplV PE=3 SV=1

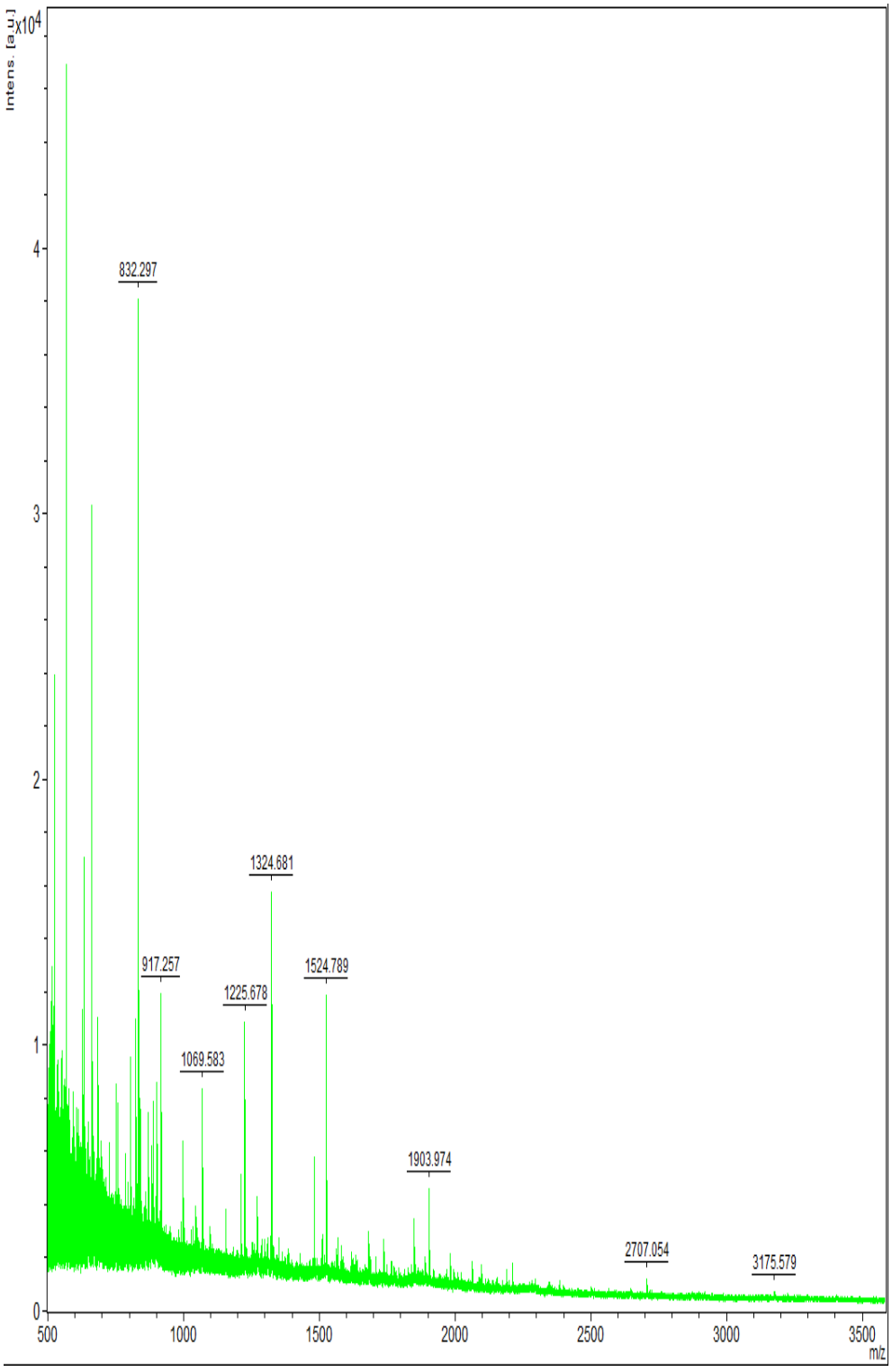
RL22_STAA2 **Mass:** 12827 **Score:** 72 **Matches:** 2(1) **Sequences:** 2(1)

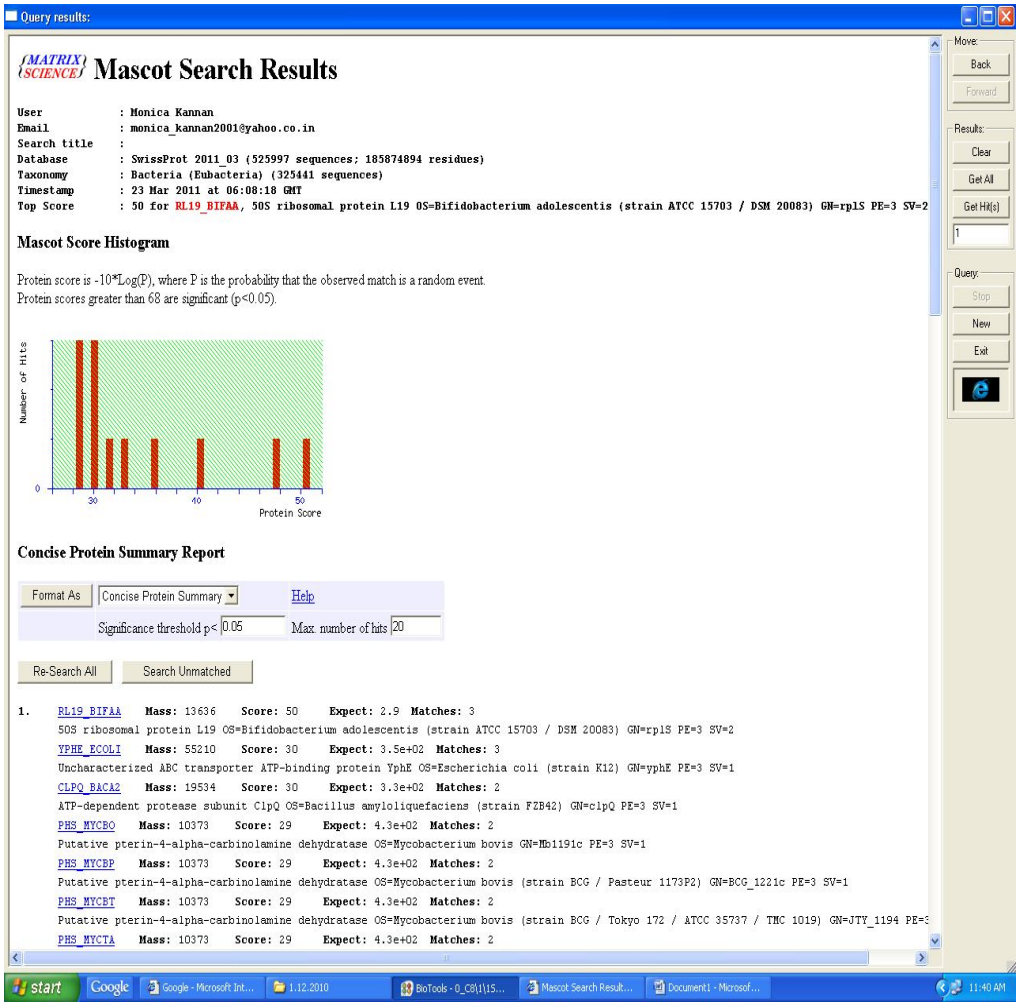
50S ribosomal protein L22 OS=Staphylococcus aureus (strain JH1) GN=rplV PE=3 SV=1

RL22_STAA3 Mass: 12827 Score: 72 Matches: 2(1) Sequences: 2(1)

50S ribosomal protein L22 OS=Staphylococcus aureus (strain USA300) GN=rplV PE=3 SV=1

Spot: 44


Intens. [a.u.]
x10^4
4
3
2
1
0
832.297
917.257
1069.583
1225.678
1324.681
1524.789
1903.974
2707.054
3175.579
500
1000
1500
2000
2500
3000
3500
m/z



RL19_BIFAA **Mass:** 13636 **Score:** 50 **Expect:** 2.9 **Matches:** 3

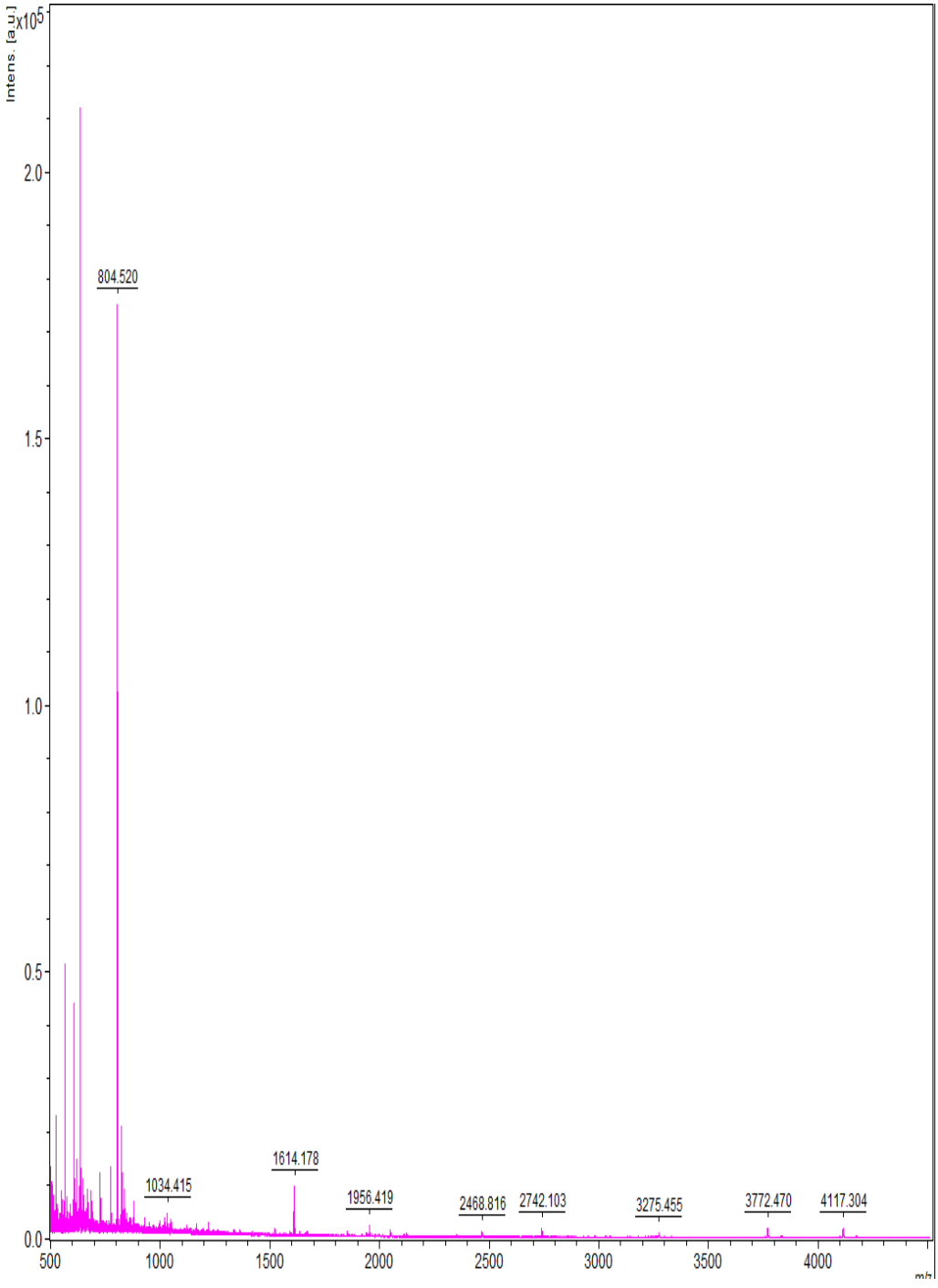
50S ribosomal protein L19 OS=Bifidobacterium adolescentis (strain ATCC 15703 / DSM 20083) GN=rplS PE=3 SV=2

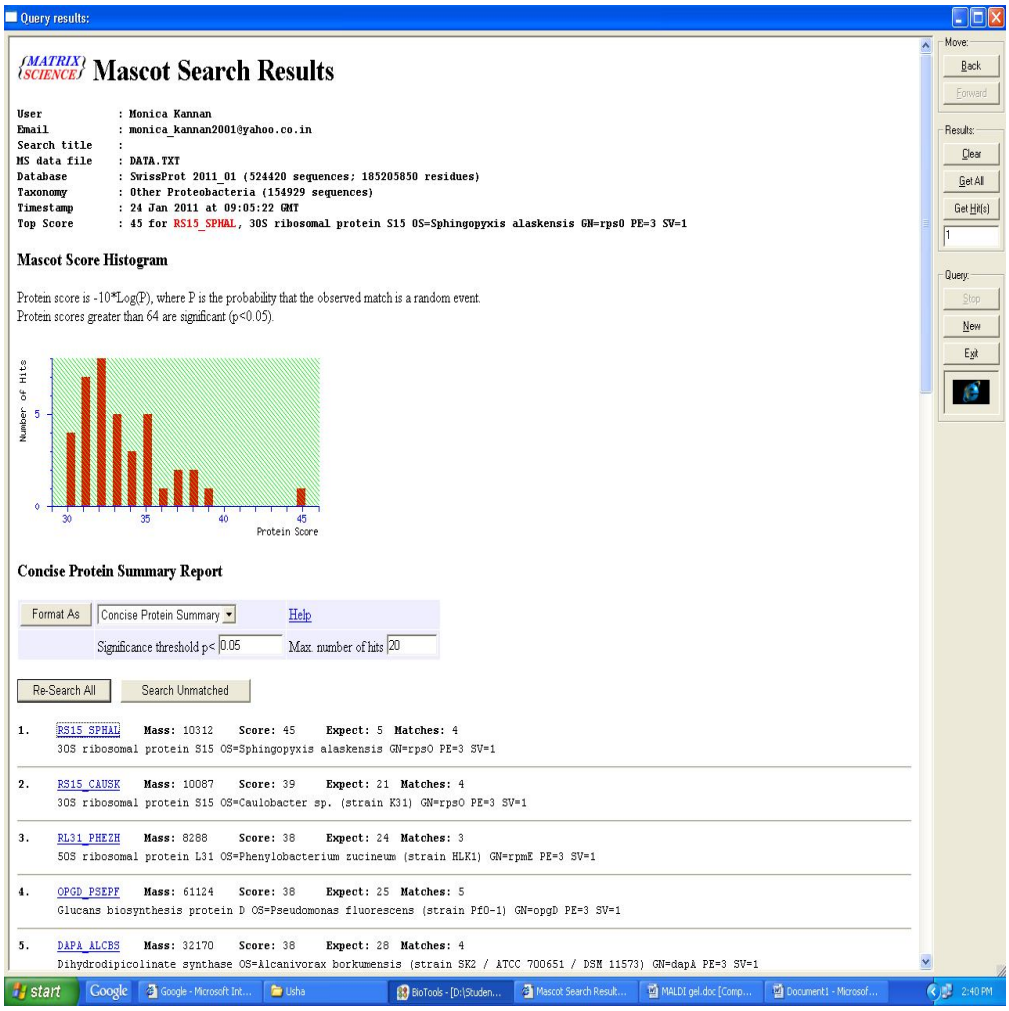
Sequence Coverage: **38%**

Matched peptides shown in **Bold Red**

```
     1 MVNAIEAFDA KHMKPAEEIP AFRPGDTVEV NVKIKEGNNS RIQAFTGVVI
    51 ARQGGGVRET FVVRKISFGT GVERRFPLHS PAIDSIKVVR KGRVRRAKLY
   101 YLRNLRGKAA RIVERRDNSE K
```

Spot: 45


Intens. [a.u.]
x10^5
2.0
1.5
1.0
0.5
0.0
804.520
1614.178
1034.415
1956.419
2468.816
2742.103
3275.455
3772.470
4117.304
500
1000
1500
2000
2500
3000
3500
4000
m/z



RS15_SPHAL **Mass:** 10312 **Score:** 45 **Expect:** 5 **Matches:** 4

30S ribosomal protein S15 OS=Sphingopyxis alaskensis GN=rpsO PE=3 SV=1

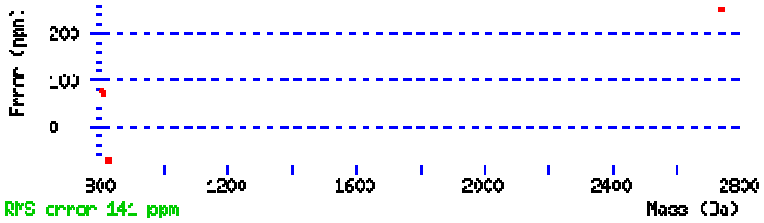
Match to: **RS15_SPHAL** Score: **45** Expect: **5**

30S ribosomal protein S15 OS=Sphingopyxis alaskensis GN=rpsO PE=3 SV=1

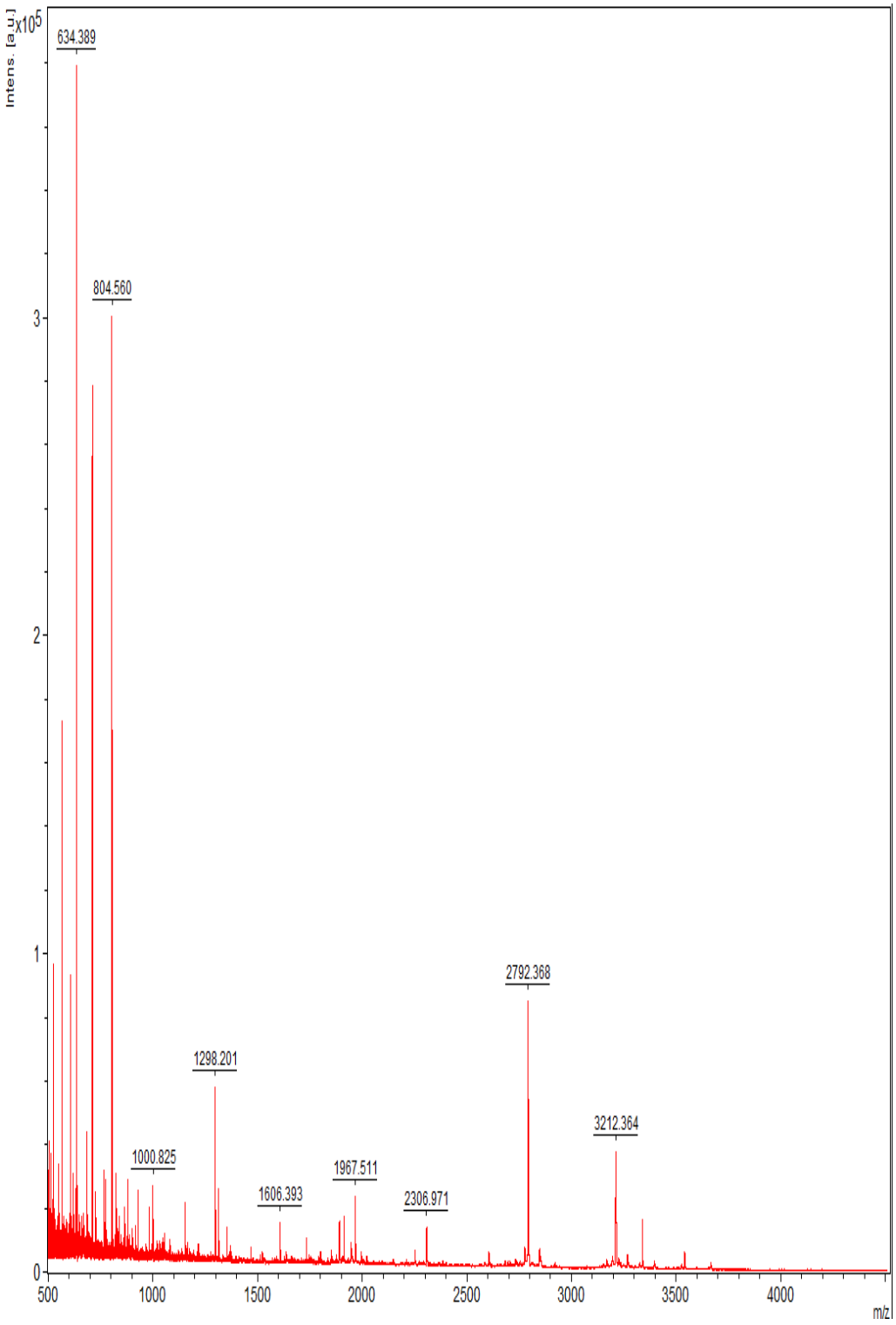
Sequence Coverage: **44%**

Matched peptides shown in **Bold Red**

1 **MSITAERK**AE VIKDNARDK**G DTGSPEVQVA ILTDRINTLT EHFK**THRKDN
51 HSRRGLLMMV NKRRSLLDYL RKKDEGR**YQA LIAK**LGLRK



Spot: 51


Intens. [a.u.]
x10^5
634.389
804.560
1298.201
1000.825
1606.393
1967.511
2306.971
2792.368
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0
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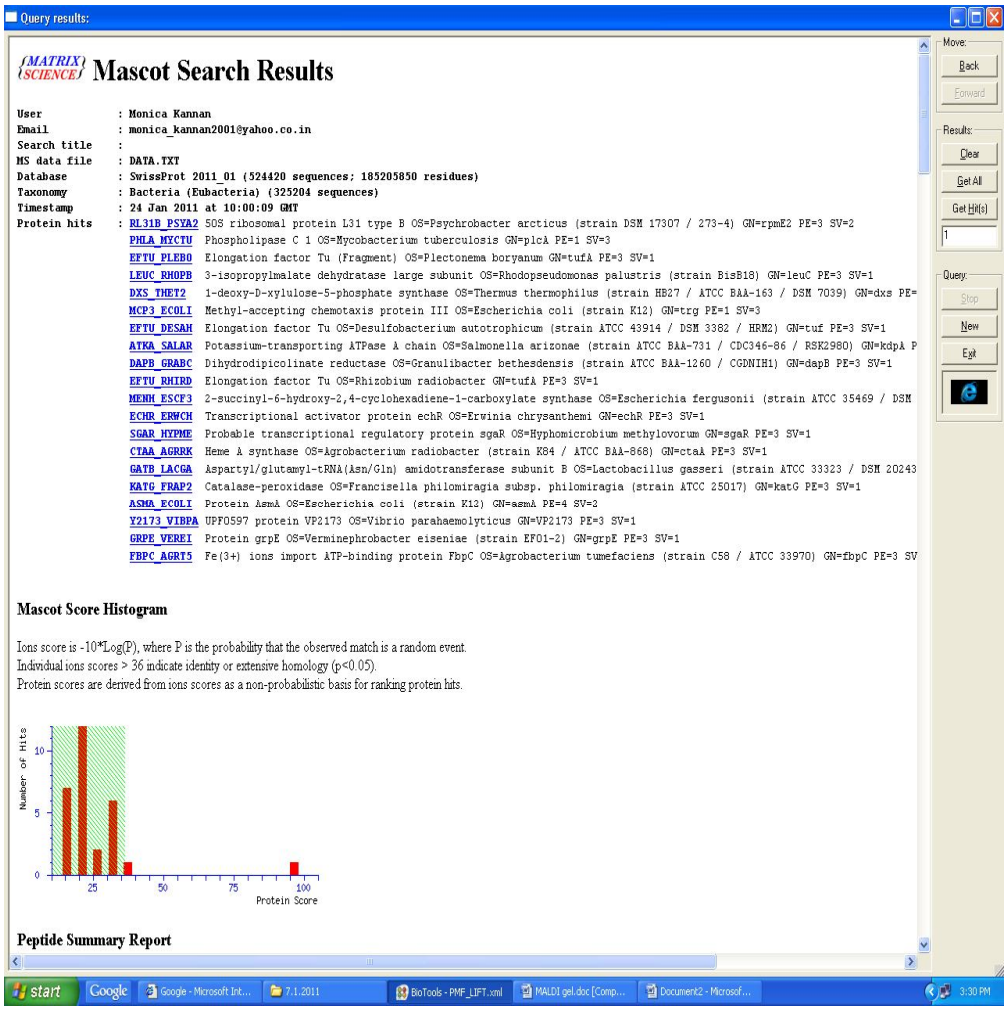
3
500
1000
1500
2000
2500
3000
3500
4000
m/z



RL31B_PSYA2 **Mass:** 10629 **Score:** 96 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L31 type B OS=Psychrobacter arcticus (strain DSM 17307 / 273-4) GN=rpmE2 PE=3 SV=2

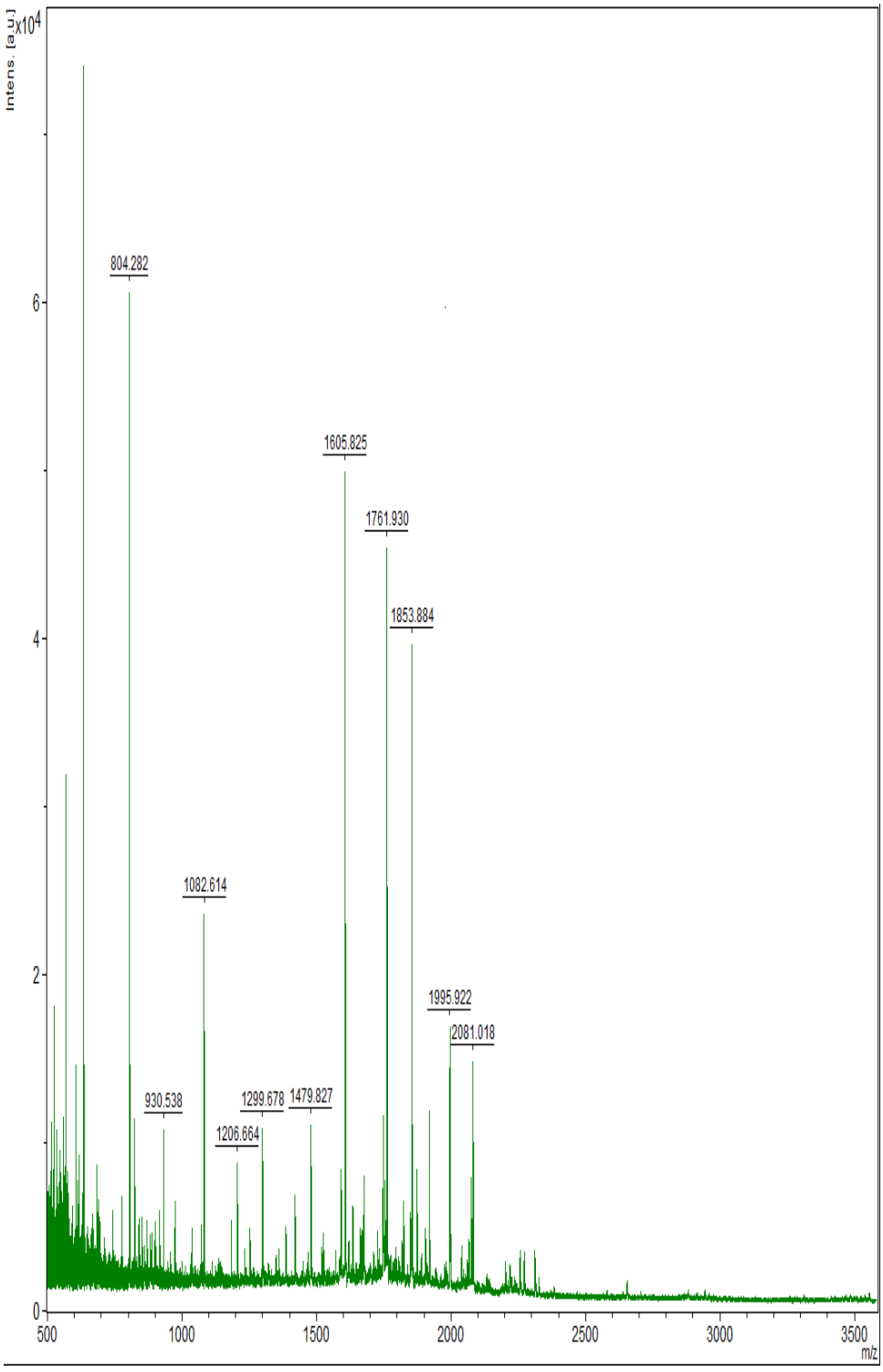
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2792.3684	2791.3611	2790.3402	366	0	96	4.3e-08	1	U	K.DIHPNYQEVLFHDTNADVFFLTR.S

Proteins matching the same set of peptides:

RL31B_PSYCK **Mass:** 10599 **Score:** 96 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L31 type B OS=Psychrobacter cryohalolentis (strain K5) GN=rpmE2 PE=3 SV=1

Spot: 52


Intens. [a.u.]
x10^4
6
4
2
0
804.282
1605.825
1761.930
1853.884
1082.614
1995.922
2081.018
930.538
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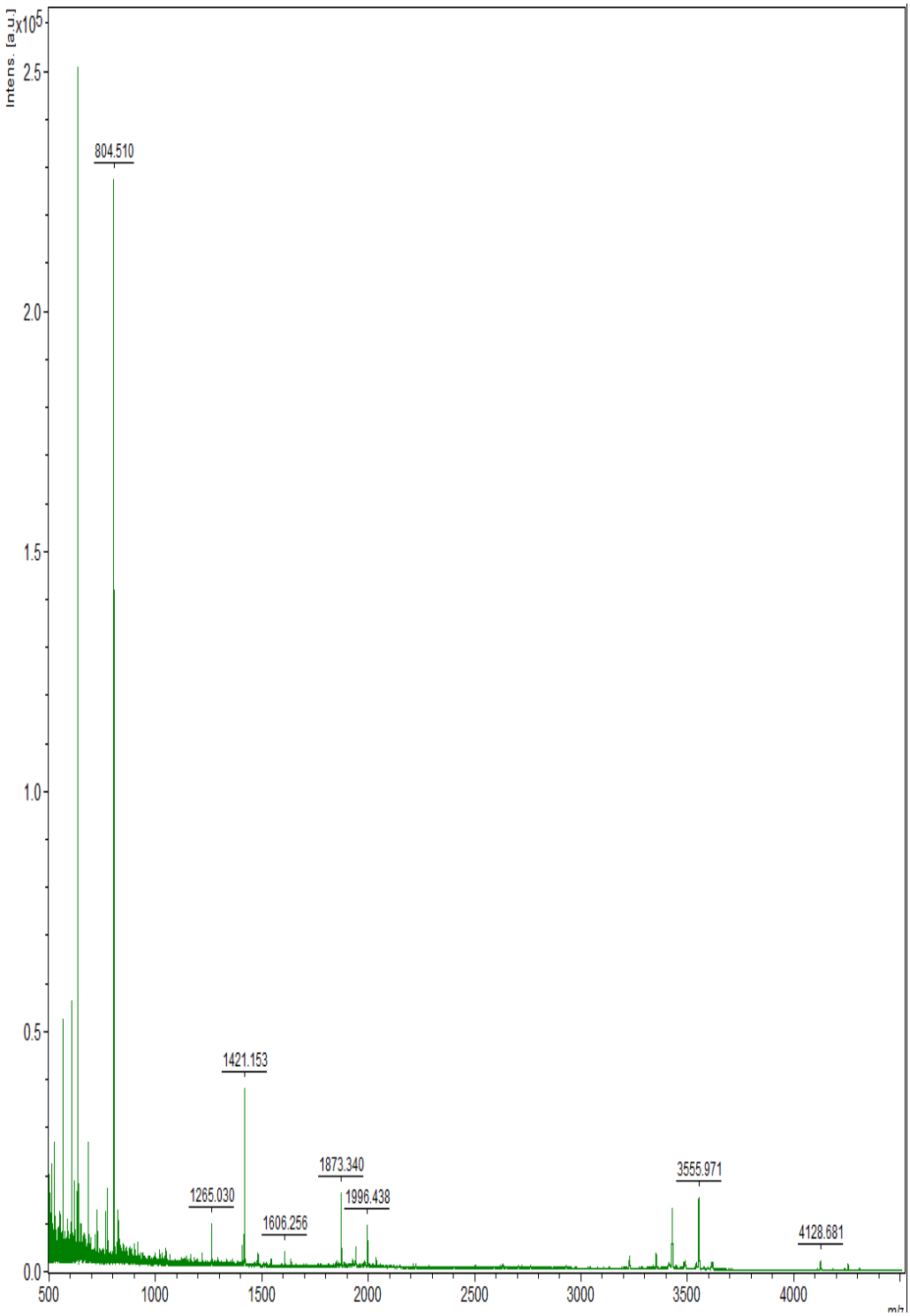
1479.827
1206.664
500
1000
1500
2000
2500
3000
3500
m/z

Spot: 53


Intens. [a.u.]
x10^5
2.5
2.0
1.5
1.0
0.5
0.0
804.510
1421.153
1873.340
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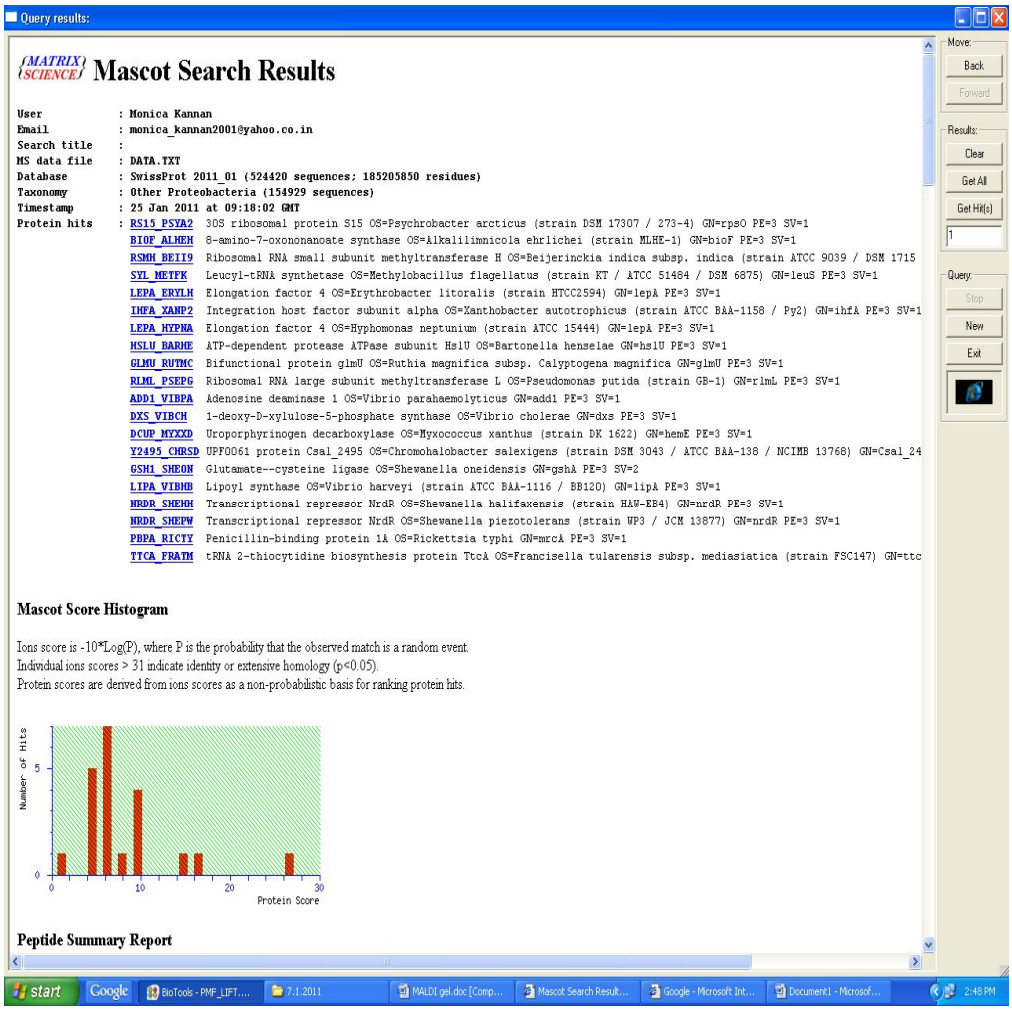
500
1000
1500
2000
2500
3000
3500
4000
m/z



RS15_PSYA2 **Mass:** 10179 **Score:** 27 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S15 OS=Psychrobacter arcticus (strain DSM 17307 / 273-4) GN=rpsO PE=3 SV=1

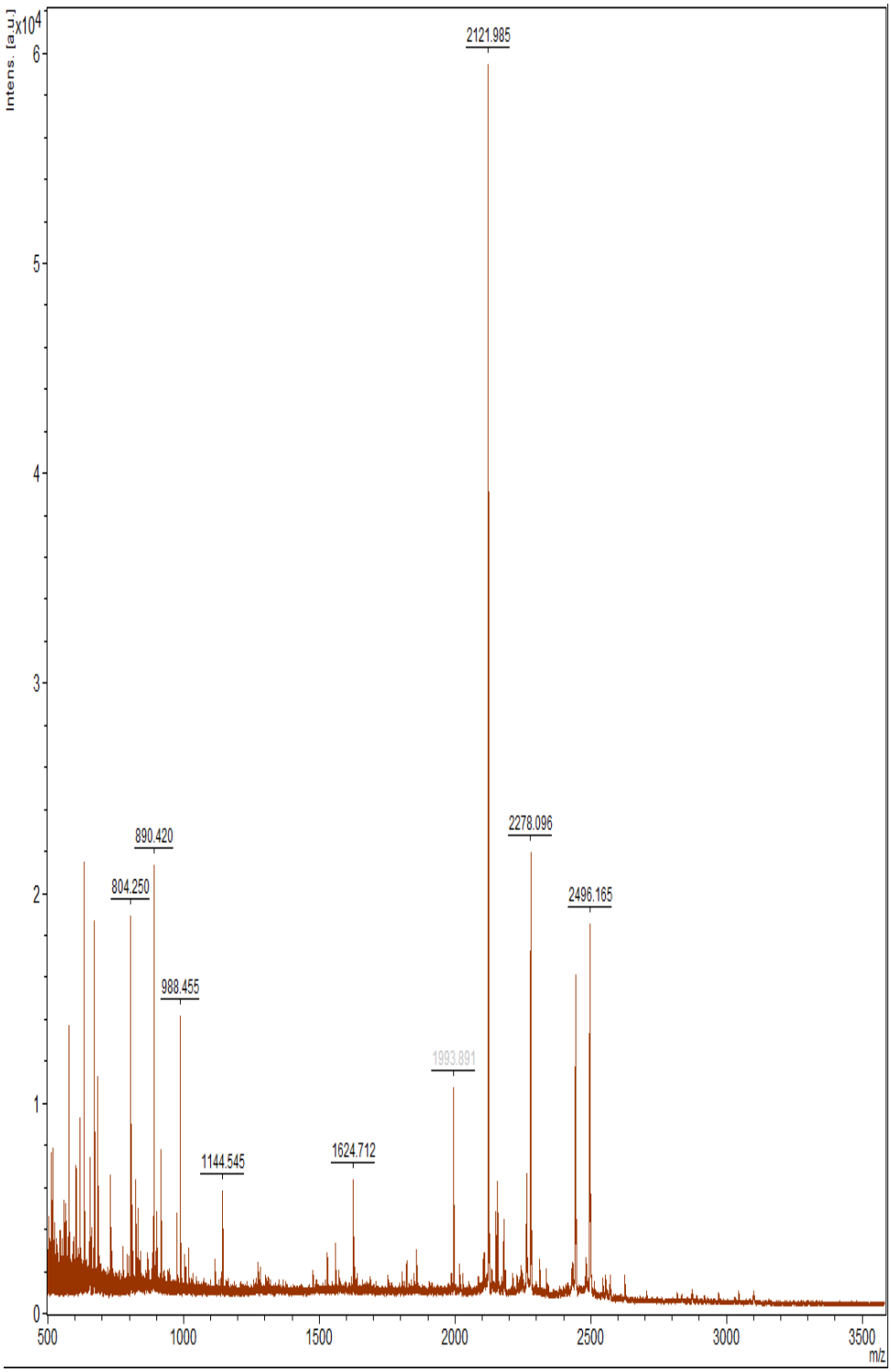
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1421.1529	1420.1456	1419.8198	229	1	27	0.022	1	U	R.YTTLISQLGLRR.-

Proteins matching the same set of peptides:

RS15_PSYCK **Mass:** 10149 **Score:** 27 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S15 OS=Psychrobacter cryohalolentis (strain K5) GN=rpsO PE=3 SV=1

Spot: 59


Intens. [a.u.]
x10^4
6
5
4
3
2
1
0
2121.985
2278.096
890.420
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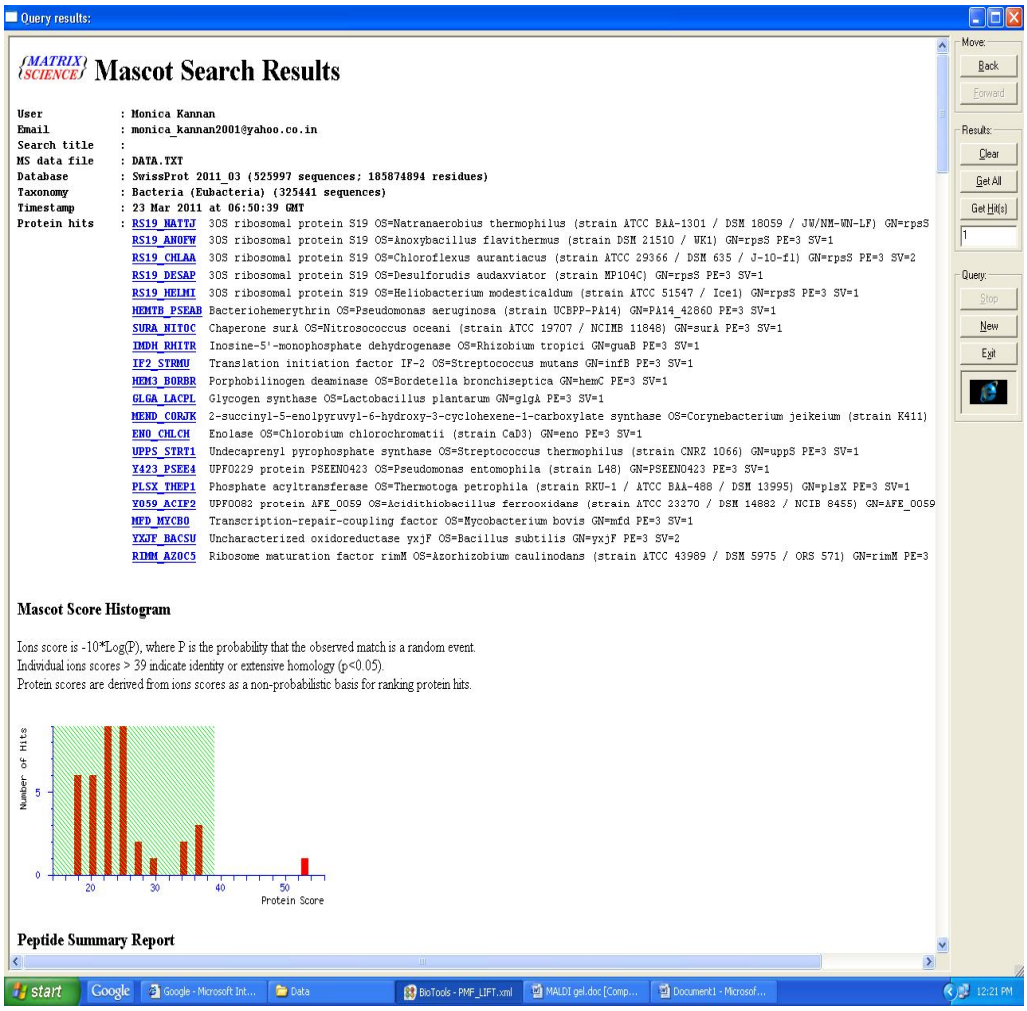
500
1000
1500
2000
2500
3000
3500
m/z

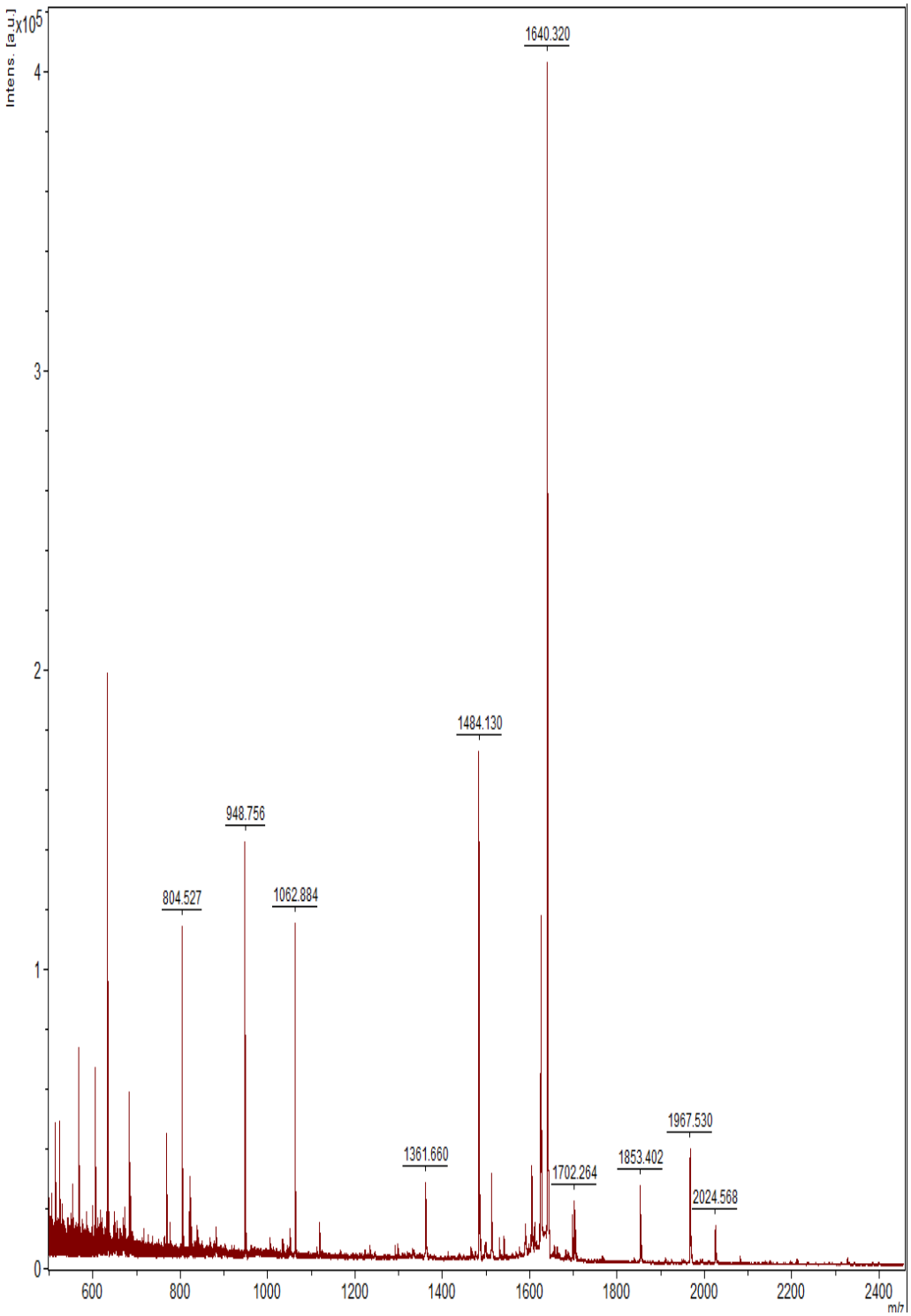


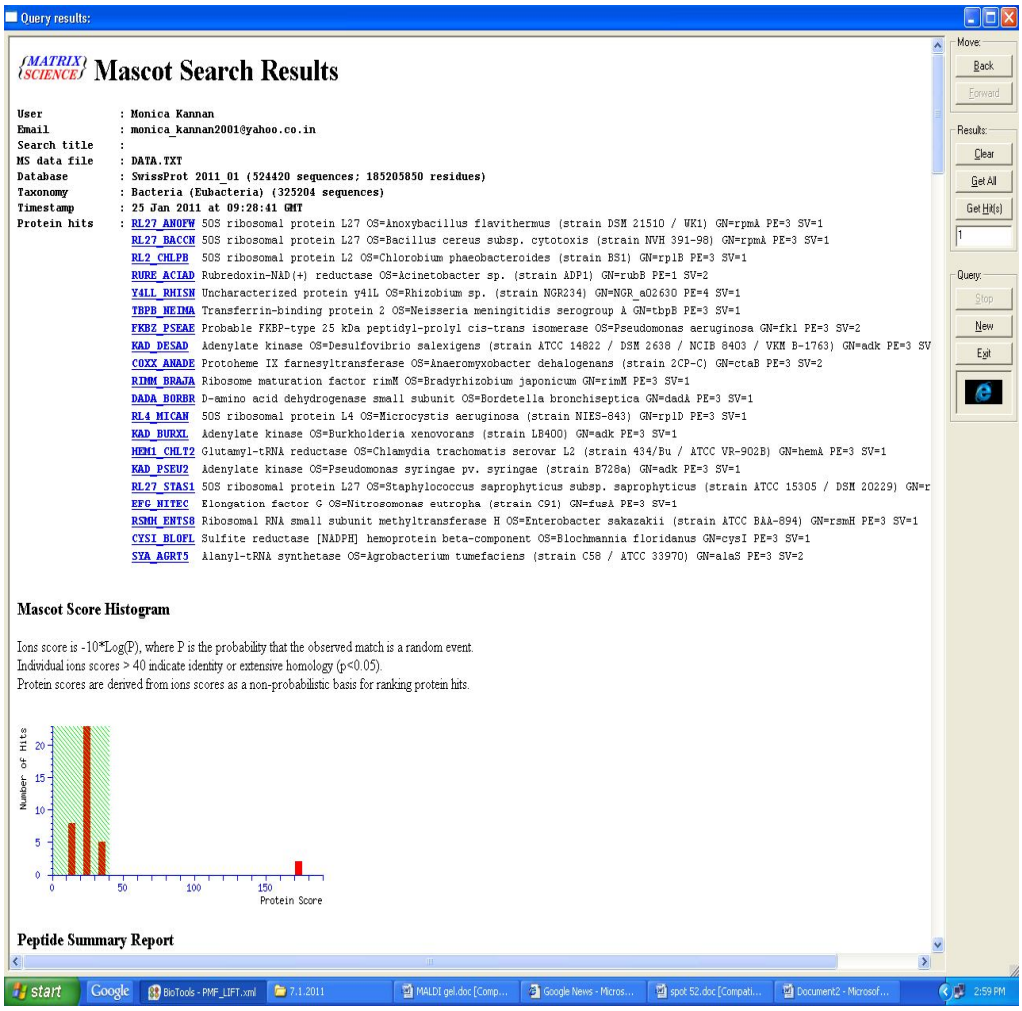
RS19_NATTJ **Mass:** 10763 **Score:** 53 **Matches:** 1(1) **Sequences:** 1(1)

30S ribosomal protein S19 OS=Natranaerobius thermophilus (strain ATCC BAA-1301 / DSM 18059 / JW/NM-WN-LF) GN=rpsS PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2496.1654	2495.1581	2495.2631	-42.08	1	53	0.0022	1	U	K.HVPVFITEDMVGHKLGEFAPTR.T + Oxidation (M)

Spot: 62


Intens. [a.u.]
x10^5
4
3
2
1
0
1640.320
1484.130
948.756
804.527
1062.884
1361.660
1702.264
1853.402
1967.530
2024.568
600
800
1000
1200
1400
1600
1800
2000
2200
2400
m/z



RL27_ANOFW **Mass:** 10588 **Score:** 173 **Matches:** 2(2) **Sequences:** 2(2)

50S ribosomal protein L27 OS=Anoxybacillus flavithermus (strain DSM 21510 / WK1) GN=rpmA PE=3 SV=1

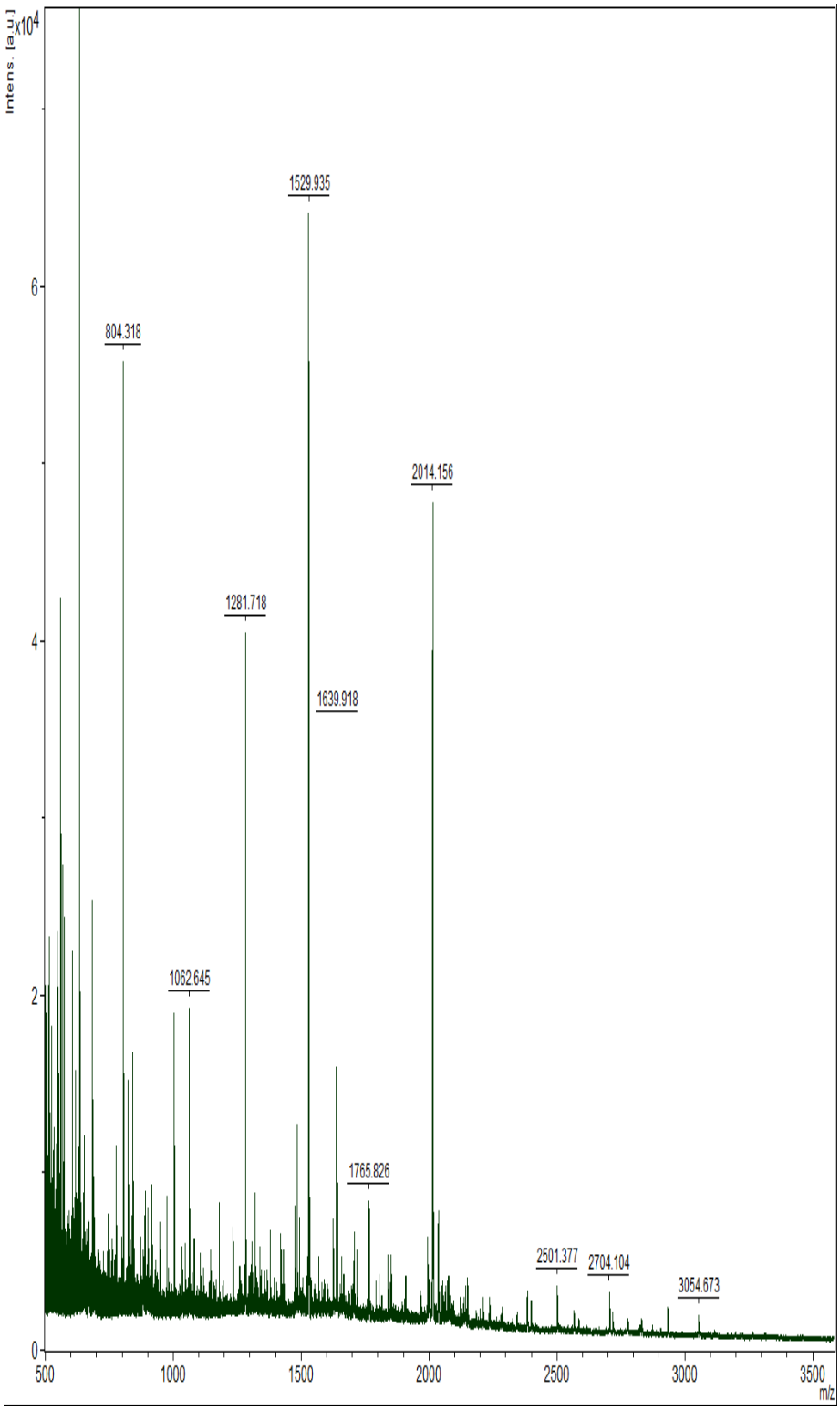
Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1484.1303	1483.1230	1482.7467	254	0	66	0.00011	1	U	R.ADGQFVTGGSILYR.Q
1640.3200	1639.3127	1638.8478	284	1	107	1e-08	1	U	K.RADGQFVTGGSILYR.Q

RL27_BACCN Mass: 10529 Score: 173 Matches: 2(2) Sequences: 2(2)

50S ribosomal protein L27 OS=Bacillus cereus subsp. cytotoxis (strain NVH 391-98) GN=rpmA PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1484.1303	1483.1230	1482.7137	276	0	66	0.00011	1	U	R.ADGQMVTGGSILYR.Q +Oxidation (M)
1640.3200	1639.3127	1638.8148	304	1	107	1e-08	1	U	K.RADGQMVTGGSILYR.Q + Oxidation (M)

Spot: 63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6
4
2
0
804.318
1529.935
2014.156
1281.718
1639.918
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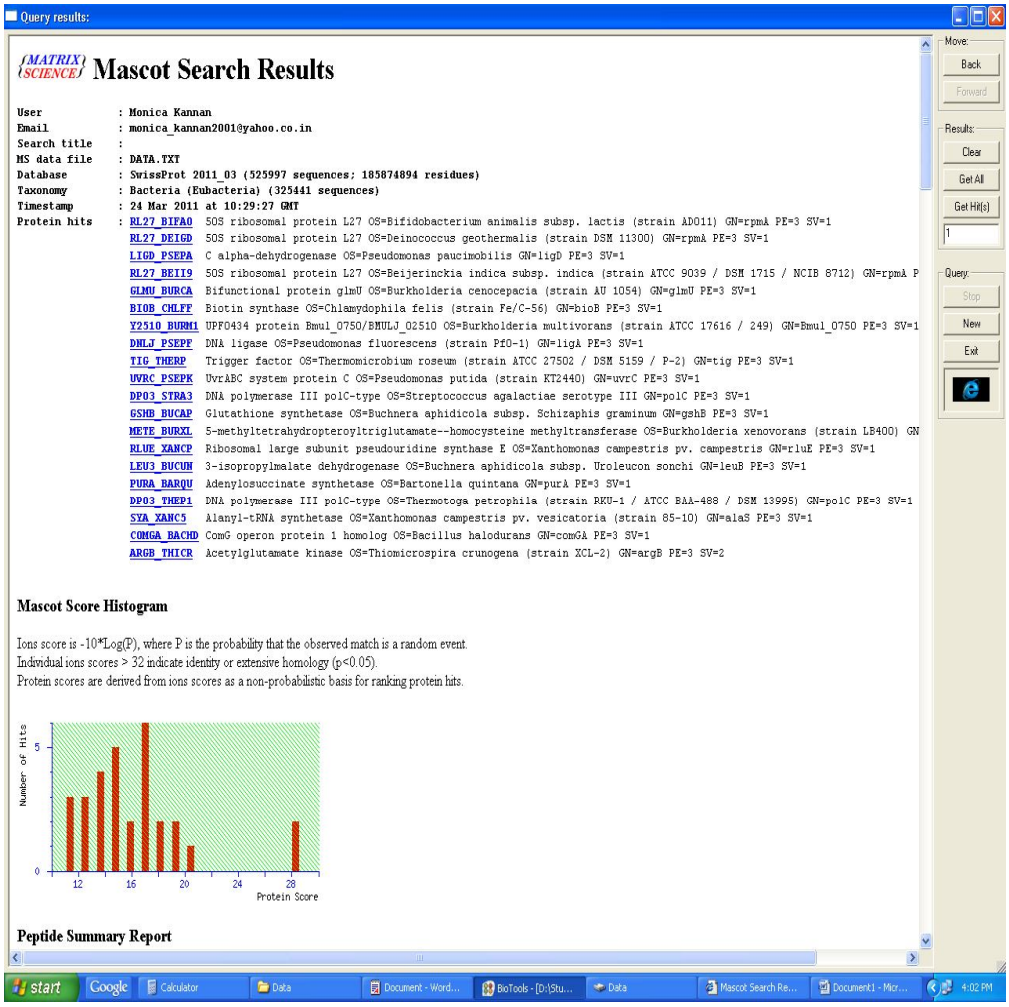
2704.104
3054.673
500
1000
1500
2000
2500
3000
3500
m/z



RL27_BIFA0 **Mass:** 8901 **Score:** 28 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L27 OS=Bifidobacterium animalis subsp. lactis (strain AD011) GN=rpmA PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1529.9352	1528.9279	1528.8726	36.2	1	28	0.051	1	U	K.KFGGEAVVAGNIIVR.Q

Proteins matching the same set of peptides:

RL27_BIFAA **Mass:** 8890 **Score:** 28 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L27 OS=Bifidobacterium adolescentis (strain ATCC 15703 / DSM 20083) GN=rpmA PE=3 SV=1

RL27_BIFLD **Mass:** 8812 **Score:** 28 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L27 OS=Bifidobacterium longum (strain DJO10A) GN=rpmA PE=3 SV=1

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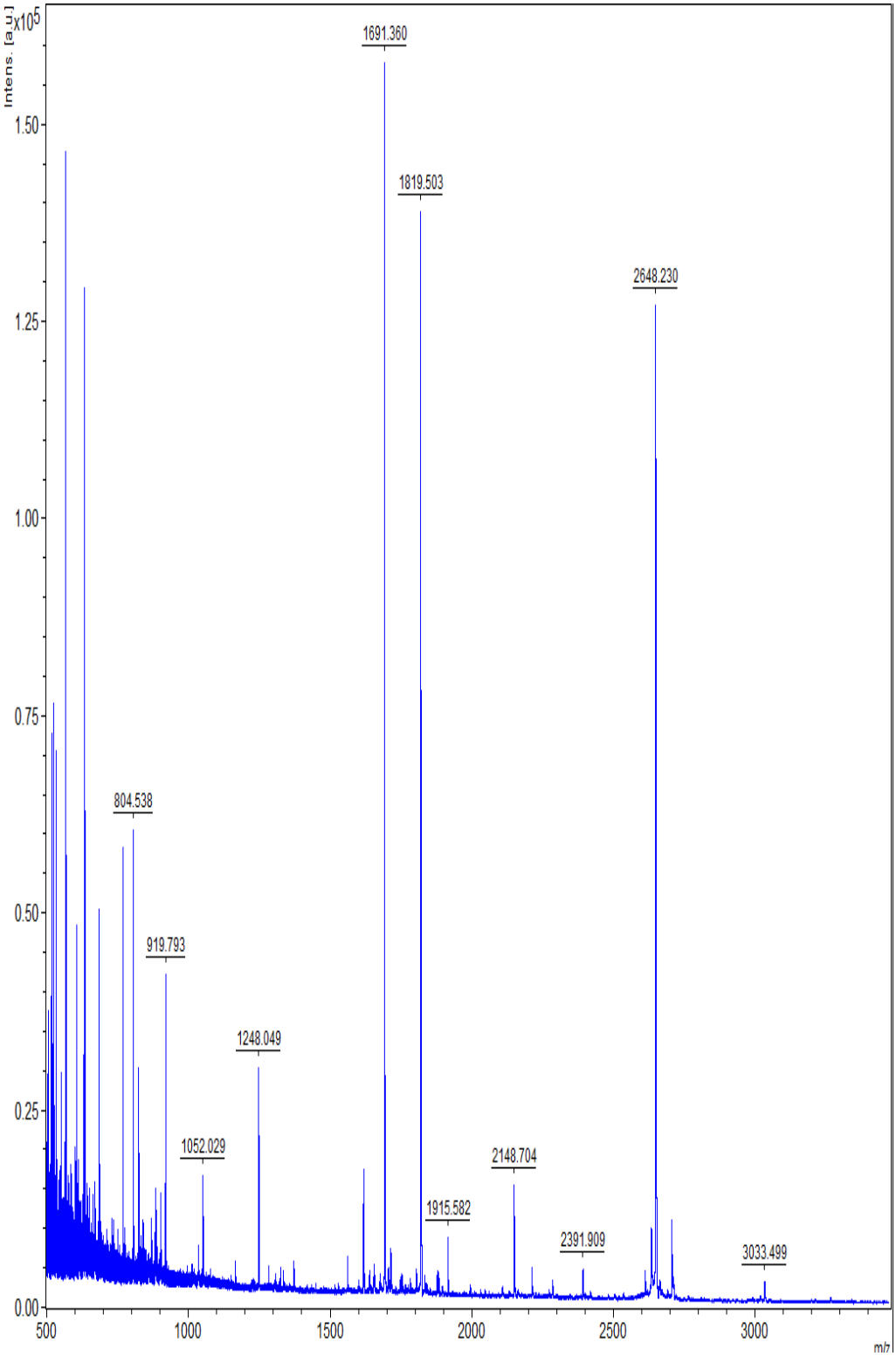
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500
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2000
2500
3000
m/z
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1819.503
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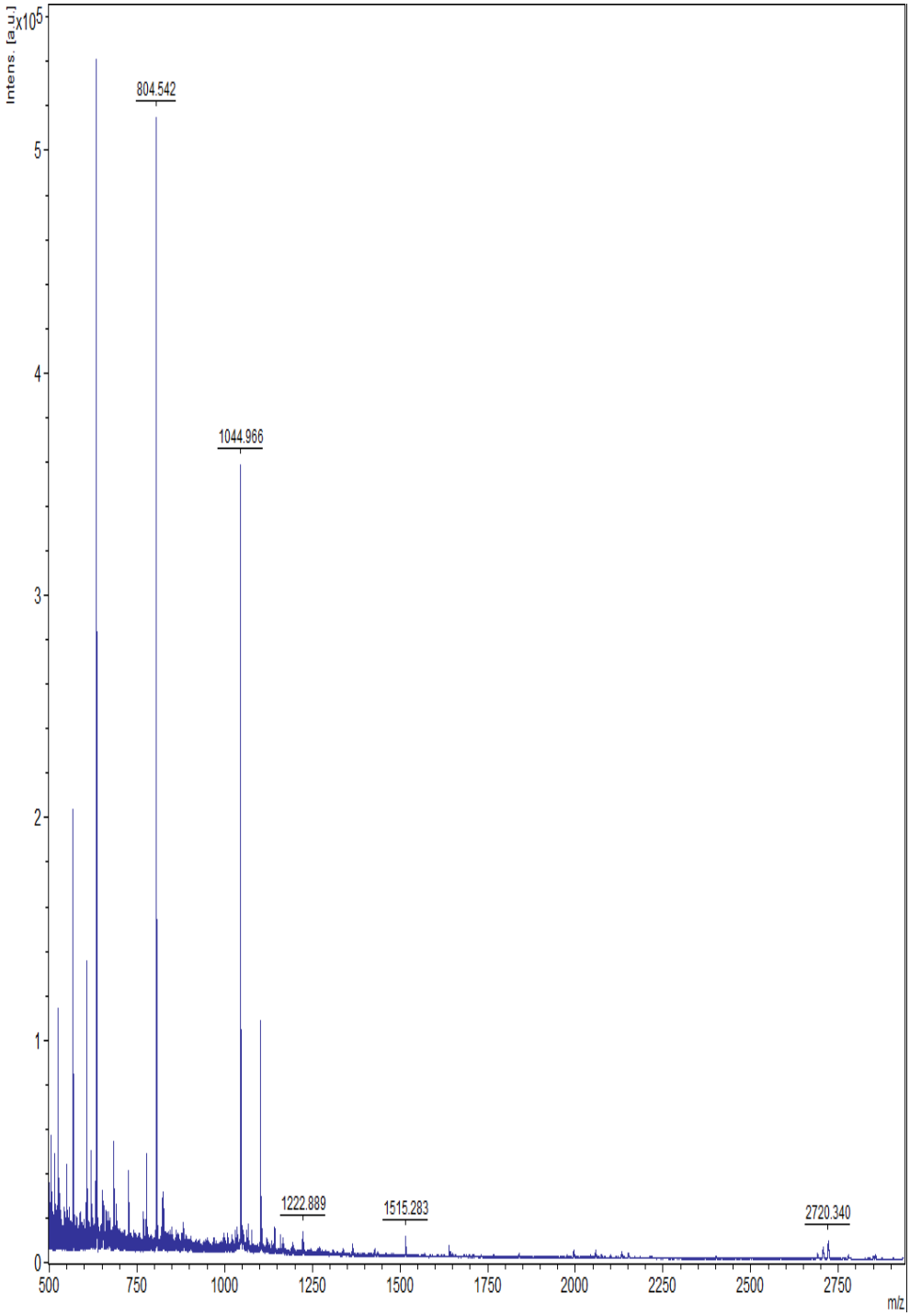
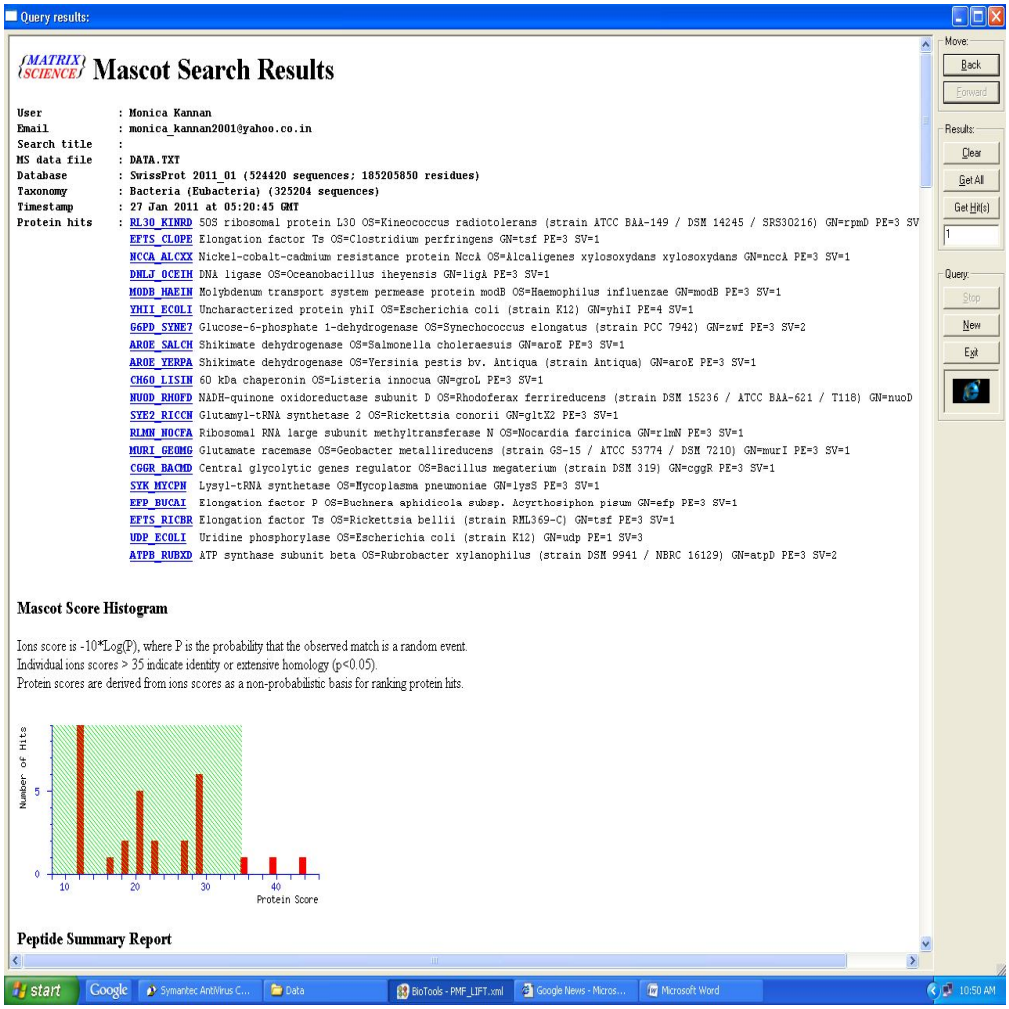




RL30_KINRD **Mass:** 6741 **Score:** 44 **Matches:** 1(1) **Sequences:** 1(1)

50S ribosomal protein L30 OS=Kineococcus radiotolerans (strain ATCC BAA-149 / DSM 14245 / SRS30216) GN=rpmD PE=3 SV=1

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1044.9664	1043.9592	1043.5724	371	0	44	0.0033	1	U	K.VTQINSGIGR.K

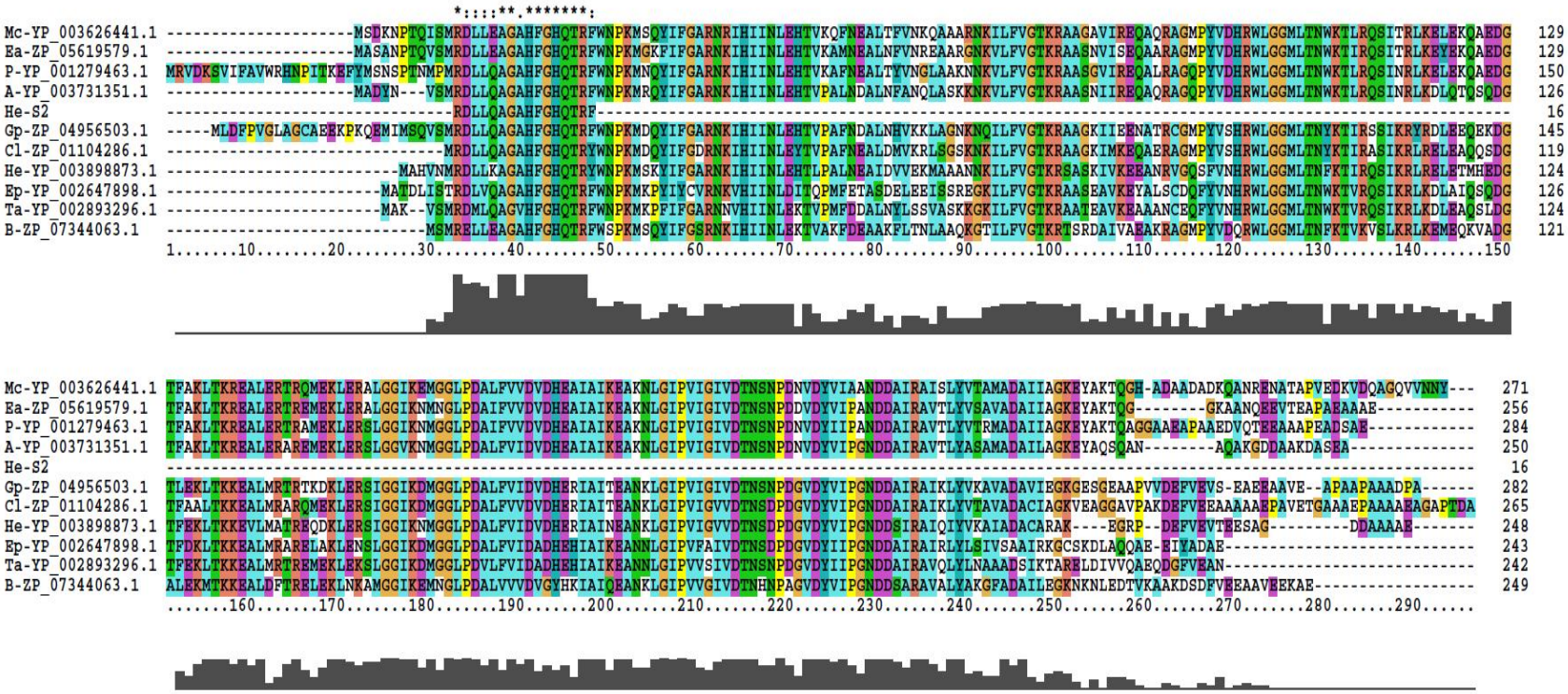


Fig: 35Ai. Multiple sequence alignment for peptide (m/z 1551.01) of ribosomal protein S2. Labels were given in top to bottom order. Mc, *Moraxella catarrhalis* RH4; Ea, *Enhydrobacter aerosaccus*; P, *Psychrobacter* sp. PRwf-1; A, *Acinetobacter* sp. DR1; He, *Halomonas eurihalina* DSM 5720; Gp, Gamma Proteobacterium NOR51-B; Cl, *Congregibacter litoralis* KT71; He, *Halomonas elongata* DSM 2581; Ep, *Erwinia pyrifoliae* Ep1/96; Ta, *Tolumonas auensis* DSM 9187; B, *Burkholderiales* bacterium.

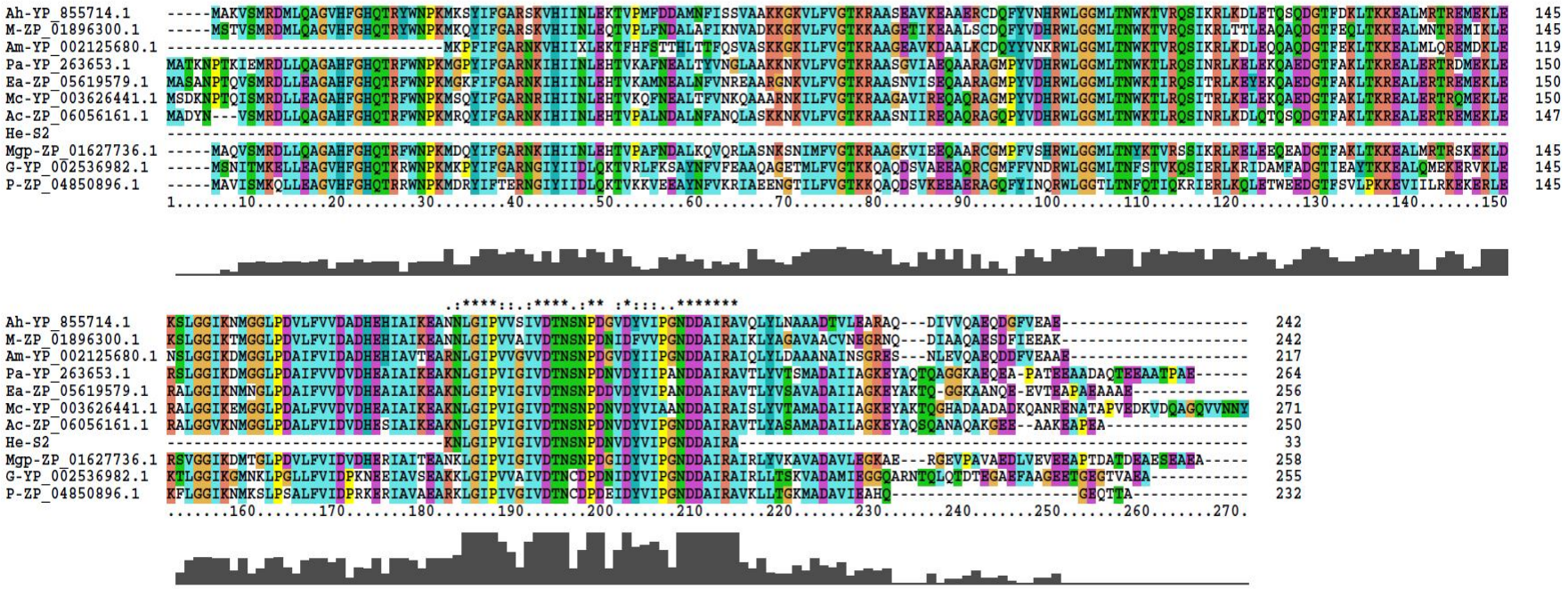


Fig: 35Aii. Multiple sequence alignment for peptide (m/z 3281.0785) of ribosomal protein S2. Ah, *Aeromonas hydrophila* ATCC 7966; M, *Moritella* sp. PE36; Am, *Alteromonas macleodii*; Pa, *Psychrobacter arcticus* 273-4; Ea, *Enhydrobacter aerosaccus* SK60; Ac, *Acinetobacter calcoaceticus* RUH2202; He, *Halomonas eurihalina* DSM 5720; Mgp, Marine Gamma Proteobacterium; G, *Geobacter* sp. FRC-32; P, *Paenibacillus* sp. str. D14.

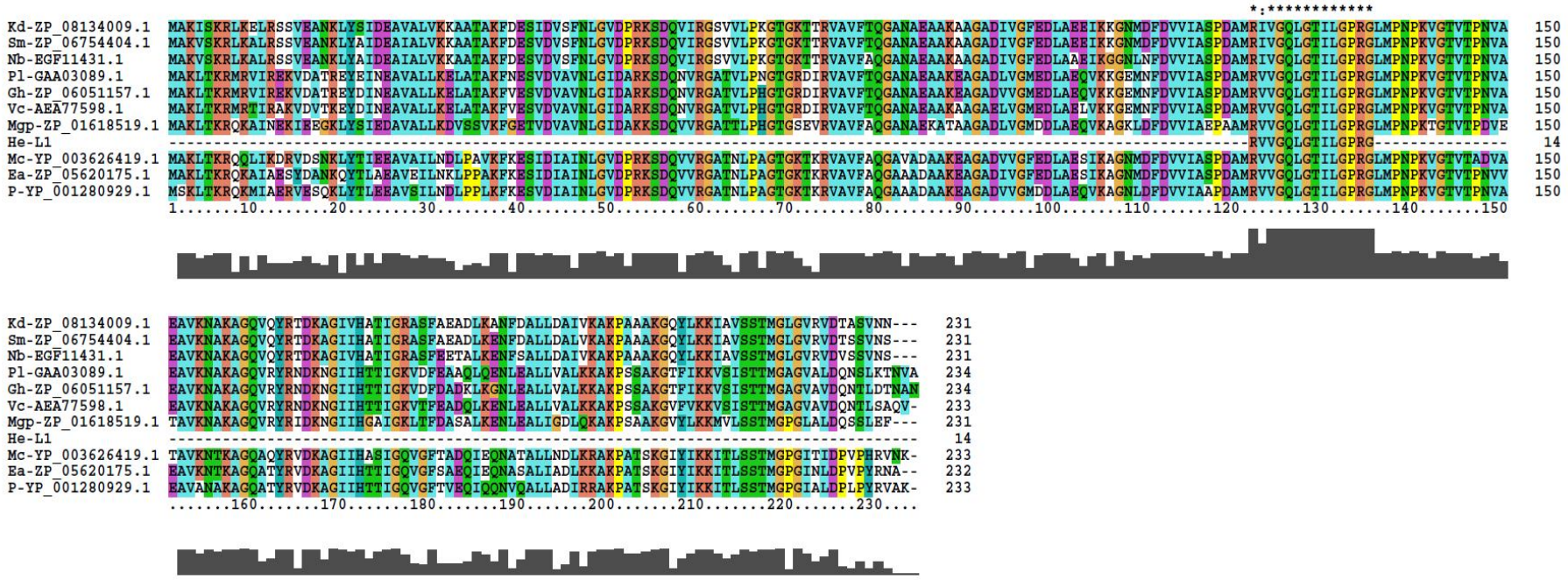


Fig. 35Bi. Multiple sequence alignment for peptide (m/z 1209.71) of ribosomal protein L1a. Kd, *Kingella denitrificans* ATCC 33394; Sm, *Simonsiella muelleri* ATCC 29453; Nb, *Neisseria bacilliformis* ATCC BAA-1200; Pl, *Photobacterium leiognathi*; Gh, *Grimontia hollisae* CIP 101886; Vc, *Vibrio cholerae* LMA3894-4; Mgp, Marine Gamma Proteobacterium HTCC 2143; He, *Halomonas eurihalina* DSM 5720; Mc, *Moraxella catarrhalis* RH4; Ea, *Enhydrobacter aerosaccus* SK60; P, *Psychrobacter* sp. PRwf-1.

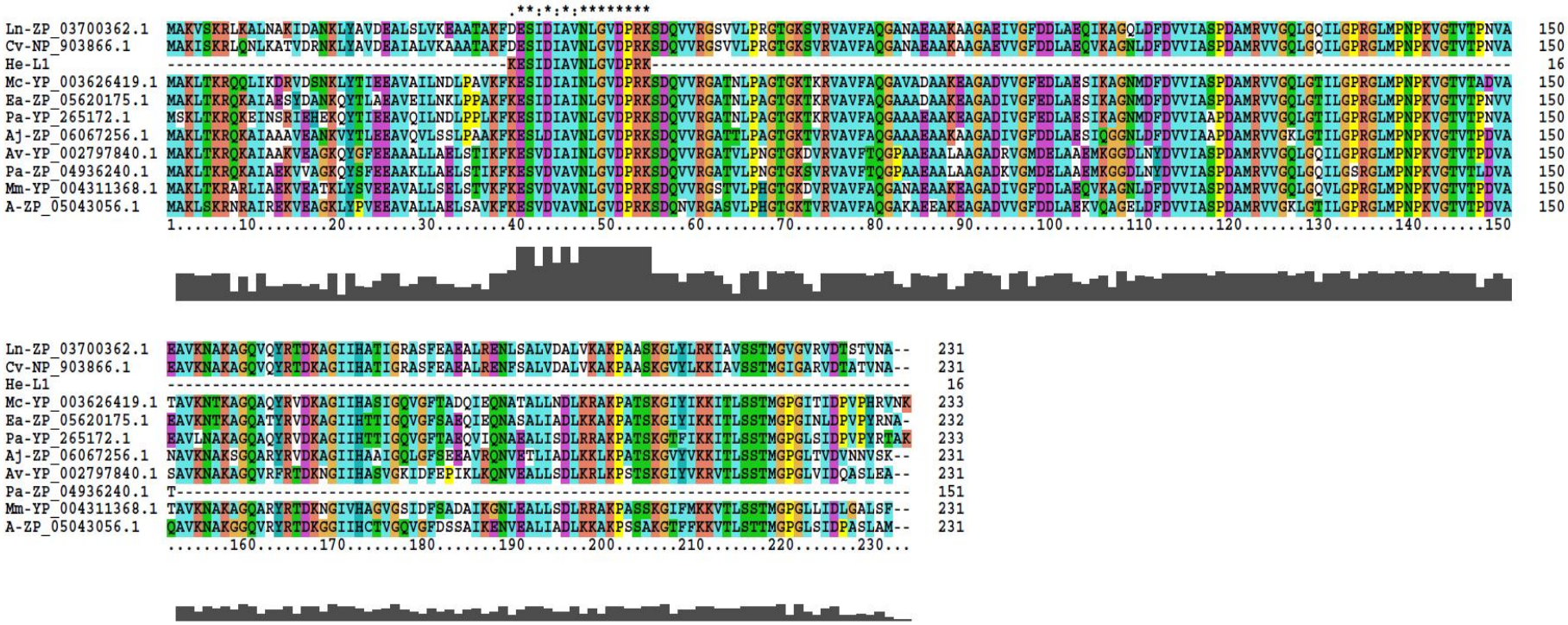


Fig: 35Bii. Multiple sequence alignment for peptide (m/z 1497.76) of ribosomal protein L1a. Ln, *Lutiella nitroferrum* 2002; *Chromobacterium violaceum* ATCC 12472; He, *Halomonas eurihalina* DSM 5720; Mc, *Moraxella catarrhalis* RH4; Ea, *Enhydrobacter aerosaccus* SK60; Pa, *Psychrobacter arcticus* 273-4; Aj, *Acinetobacter junii* SH205; Av, *Azotobacter vinelandii* DJ; Pa, *Pseudomonas aeruginosa* 2192; Mm, *Marinomonas mediterranea* MMB-1; A, *Alcanivorax* sp. DG881.

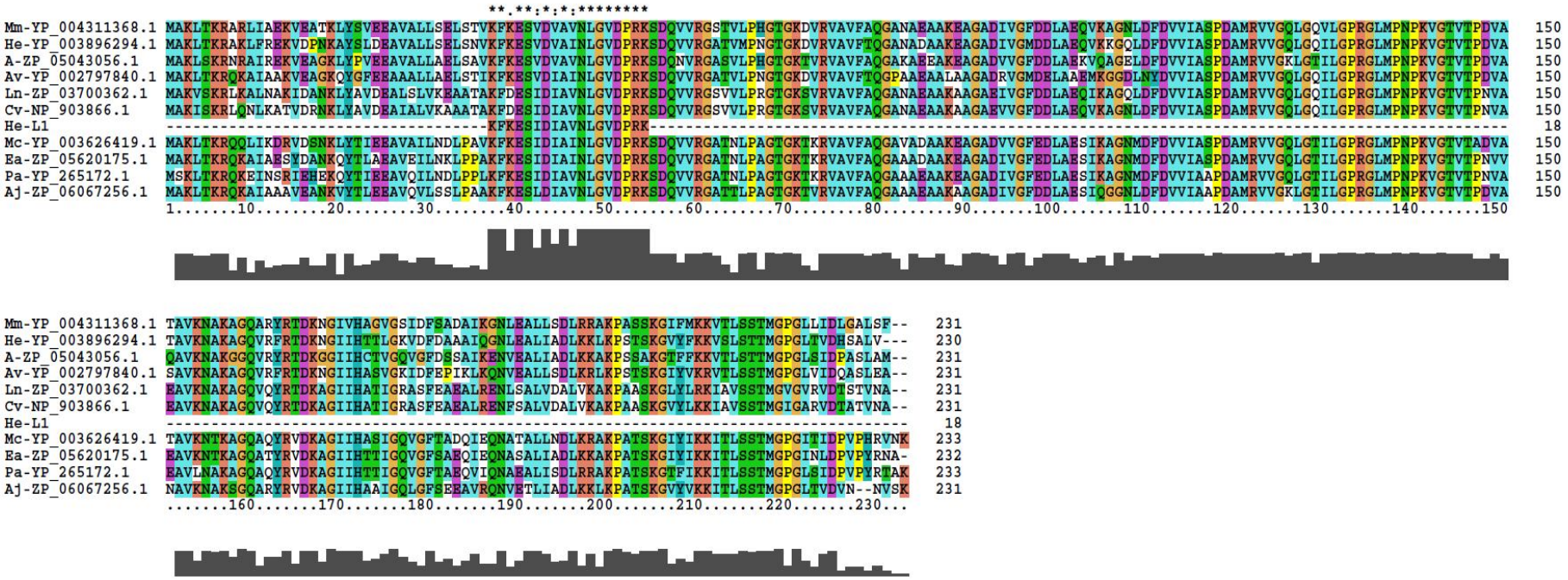


Fig: 35Biii. Multiple sequence alignment for peptide (m/z 1772.92) of ribosomal protein L1a. Mm, *Marinomonas mediterranea* MMB-1; He, *Halomonas elongata* DSM 2581; A, *Alcanivorax* sp. DG881; Av, *Azotobacter vinelandii* DJ; Ln, *Lutiella nitroferrum* 2002; Cv, *Chromobacterium violaceum* ATCC 12472; He, *Halomonas eurihalina* DSM 5720; Mc, *Moraxella catarrhalis* RH4; Ea, *Enhydrobacter aerosaccus* SK60; Pa, *Psychrobacter arcticus* 273-4. Aj, *Acinetobacter junii* SH205.

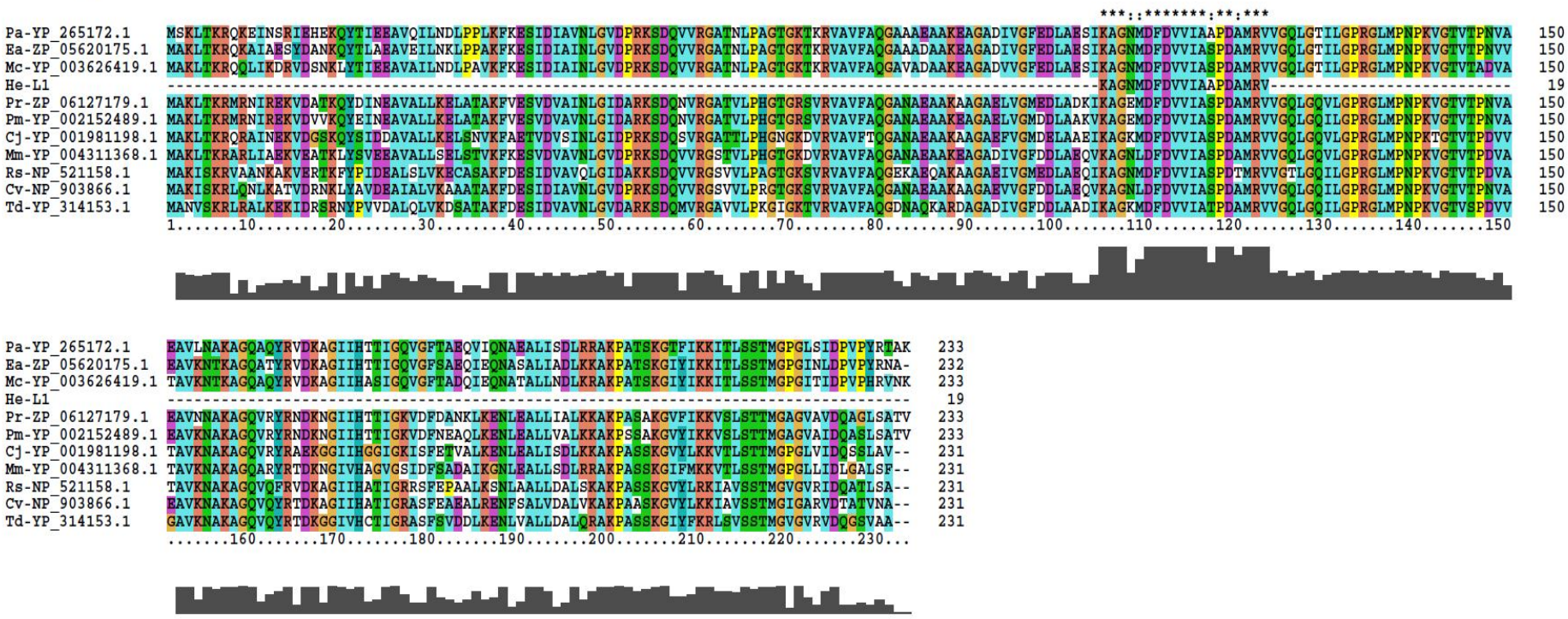


Fig: 35Biv. Multiple sequence alignment for peptide (m/z 1824.79) of ribosomal protein L1a. Pa, *Psychrobacter arcticus* 273-4; Ea, *Enhydrobacter aerosaccus* SK60; Mc, *Moraxella catarrhalis* RH4; He, *Halomonas eurihalina* DSM 5720; Pr, *Providencia rettgeri* DSM 1131; Pm, *Proteus mirabilis* HI4320; Cj, *Cellvibrio japonicus* Ueda107; Mm, *Marinomonas mediterranea* MMB-1; Rs, *Ralstonia solanacearum* GMI1000; Cv, *Chromobacterium violaceum* ATCC 12472; Td, *Thiobacillus denitrificans* ATCC 25259;

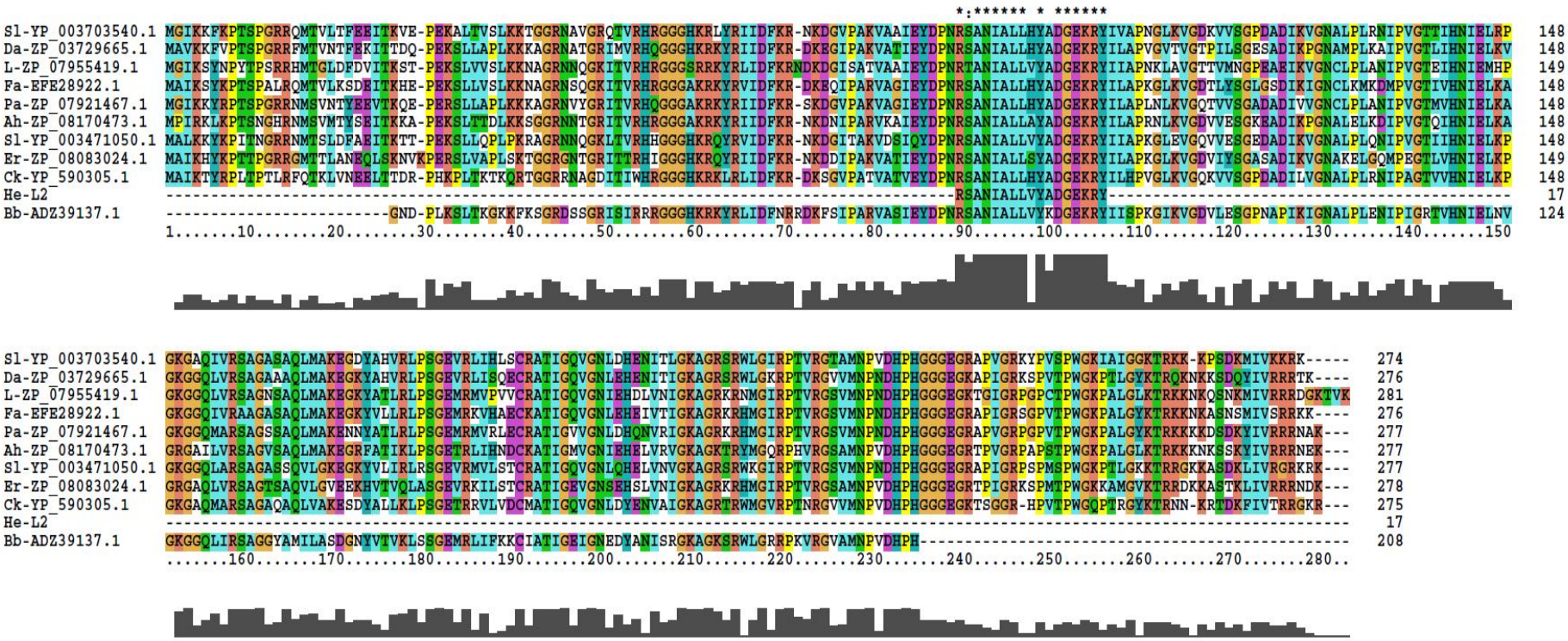


Fig: 35Ci. Multiple sequence alignment for peptide (m/z 1619.75) of ribosomal protein L2. Sl, *Syntrophothermus lipocalidus* DSM 12680; Da, *Dethiobacter alkaliphilus* AHT 1; L, *Lachnospiraceae* bacterium; Pa, *Pseudoramibacter alactolyticus* ATCC 23263; Ah, *Anaerococcus hydrogenalis* ACS-025-V-Sch4; Sl, *Staphylococcus lugdunensis* HKU09-01; Er, *Erysipelothrix rhusiopathiae* ATCC 19414; Ck, Candidatus *Koribacter versatilis* Ellin345; He, *Halomonas eurihalina* DSM 5720; Bb, *Borrelia burgdorferi*.

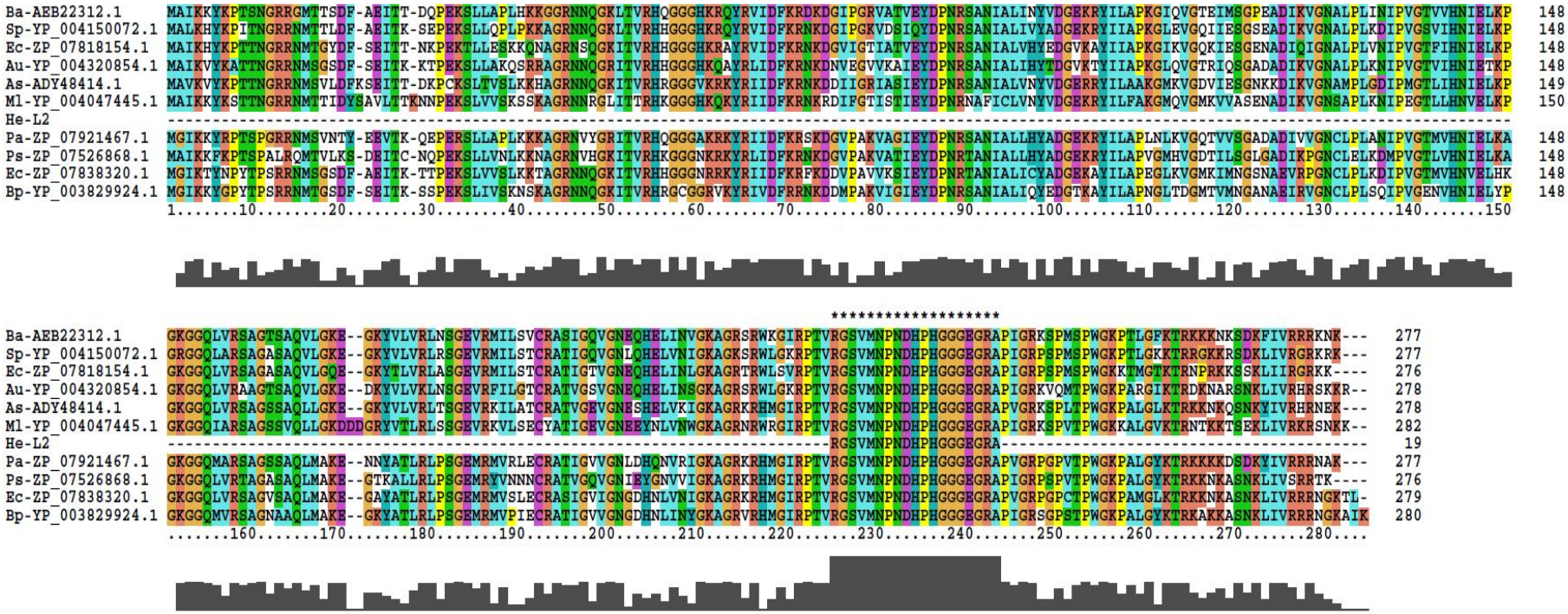


Fig: 35Cii. Multiple sequence alignment for peptide (m/z 1733.60) of ribosomal protein L2. Ba, *Bacillus amyloliquefaciens* TA208; Sp, *Staphylococcus pseudintermedius* HKU10-03; Ec, *Eremococcus coleocola* ACS-139-V-Col8; Au, *Aerococcus urinae* ACS-120-V-Col10a; Ml, *Mycoplasma leachii* PG50; He, *Halomonas eurihalina* DSM 5720; Pa, *Pseudoramibacter alactolyticus* ATCC 23263; Ps, *Peptostreptococcus stomatis* DSM 17678; Ec, *Eubacterium cellulosolvens* 6; Bp, *Butyrivibrio proteoclasticus* B316.

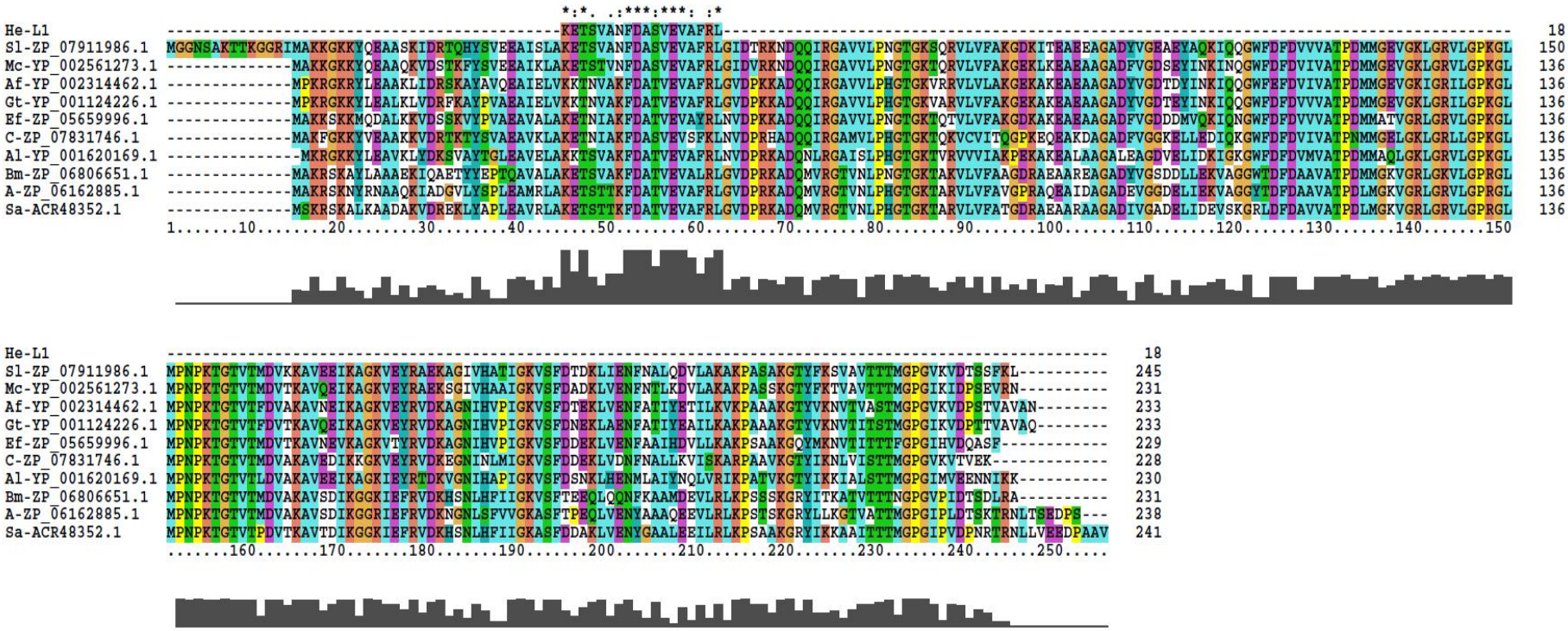


Fig: 35D. Multiple sequence alignment for peptide (m/z 1741.84) of ribosomal protein L1b. He, *Halomonas eurihalina* DSM 5720; Sl, *Staphylococcus lugdunensis* M23590; Mc, *Macrococcus caseolyticus* JCSC5402; Af, *Anoxybacillus flavithermus* WK1; Gt, *Geobacillus thermodenitrificans* NG80-2; Ef, *Enterococcus faecium* 1,230,933; C, *Clostridium* sp. HGF2; Al, *Acholeplasma laidlawii* PG-8A; Bm, *Brevibacterium mcbrellneri* ATCC 49030; A, *Actinomyces* sp. oral taxon 848 str. F0332; Sa, *Streptomyces actuosus*;

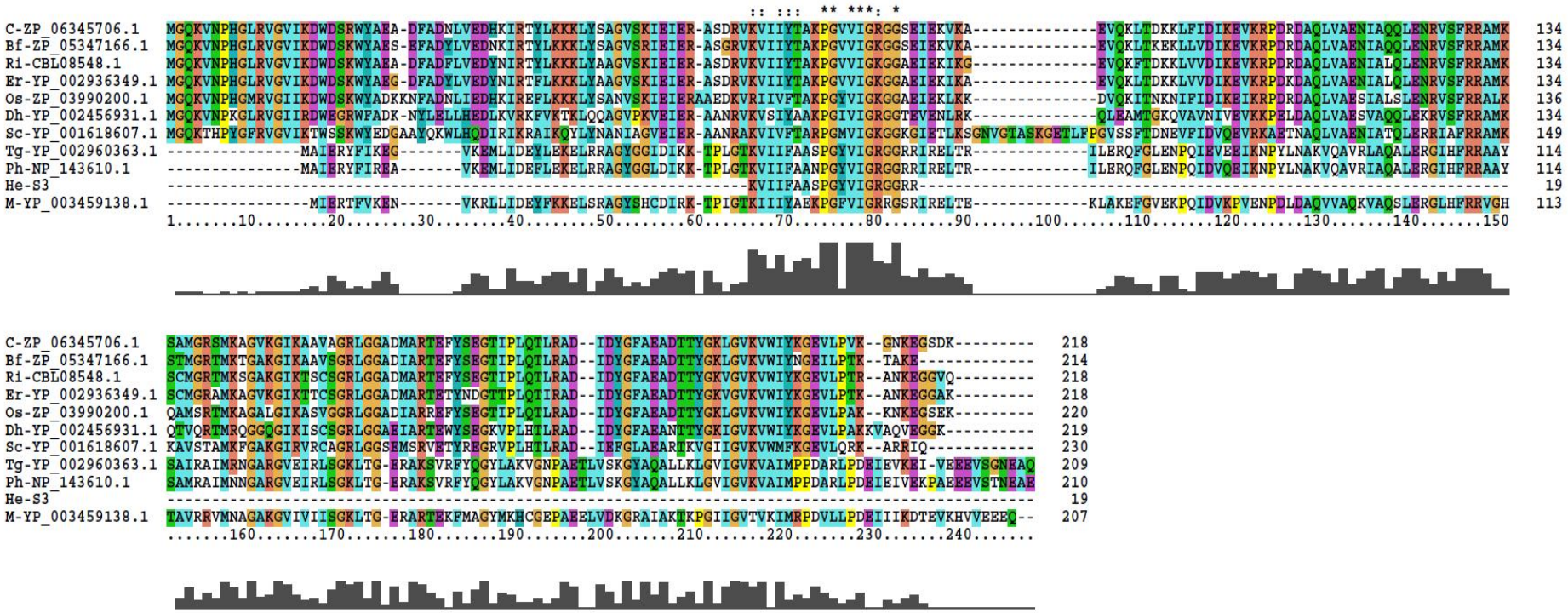


Fig: 35E. Multiple sequence alignment for peptide (m/z 1733.13) of ribosomal protein S3. C, *Clostridium* sp. M62/1; Bf, *Bryantella formatexigens* DSM 14469; Ri, *Roseburia intestinalis* M50/1; Er, *Eubacterium rectale* ATCC 33656; Os, *Oribacterium sinus* F0268; Dh, *Desulfitobacterium hafniense* DCB-2; Sc, *Sorangium cellulosum* 'So ce 56'; Tg, *Thermococcus gammatolerans* EJ3; Ph, *Pyrococcus horikoshii* OT3; He, *Halomonas eurihalina* DSM 5720; M, *Methanocaldococcus* sp. FS406-22.

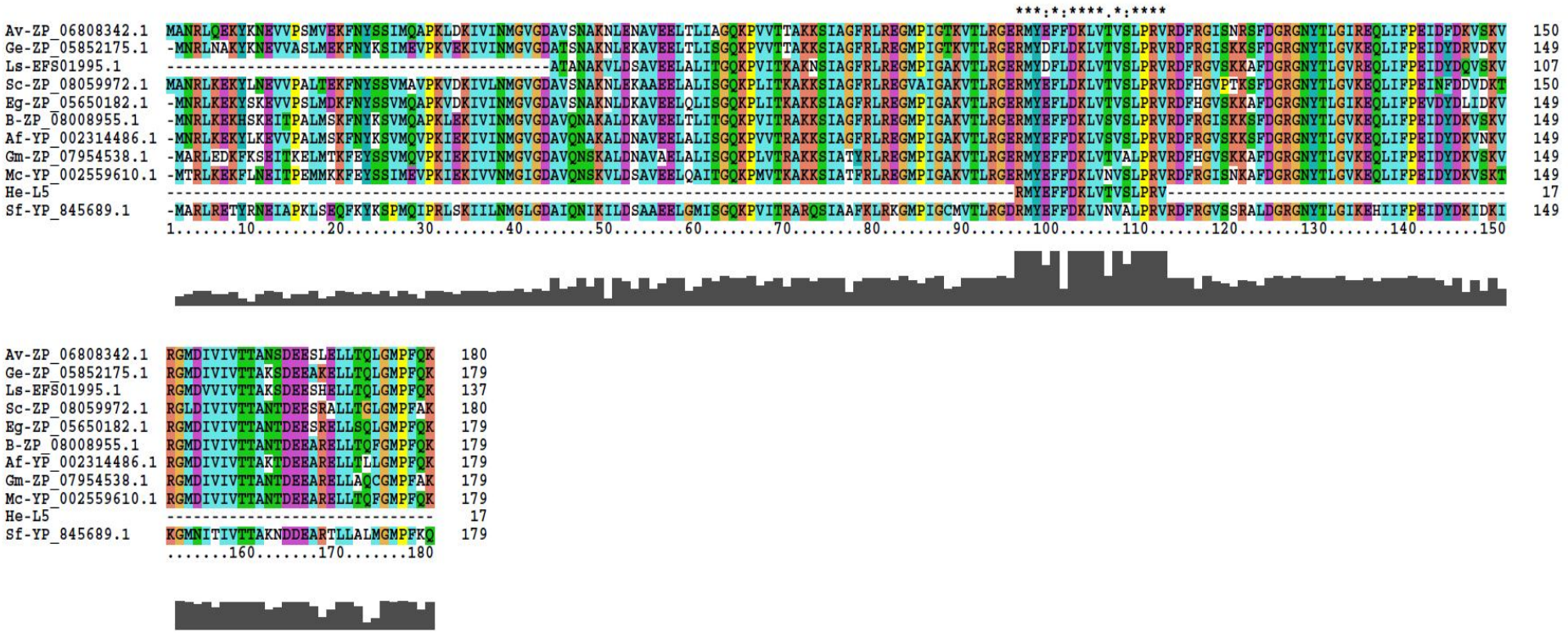


Fig: 35F. Multiple sequence alignment for peptide (m/z 1861.16) of ribosomal protein L5. Av, *Aerococcus viridans* ATCC 11563; Ge, *Granulicatella elegans* ATCC 700633; Ls, *Listeria seeligeri* FSL S4-171; Sc, *Streptococcus cristatus* ATCC 51100; Eg, *Enterococcus gallinarum* EG2; B, *Bacillus* sp. 2_A_57_CT2; Af, *Anoxybacillus flavithermus* WK1; Gm, *Gemella moribillum* M424; Mc, *Micrococcus caseolyticus* JCSC5402; He, *Halomonas eurihalina* DSM 5720; Sf, *Syntrophobacter fumaroxidans* MPOB;

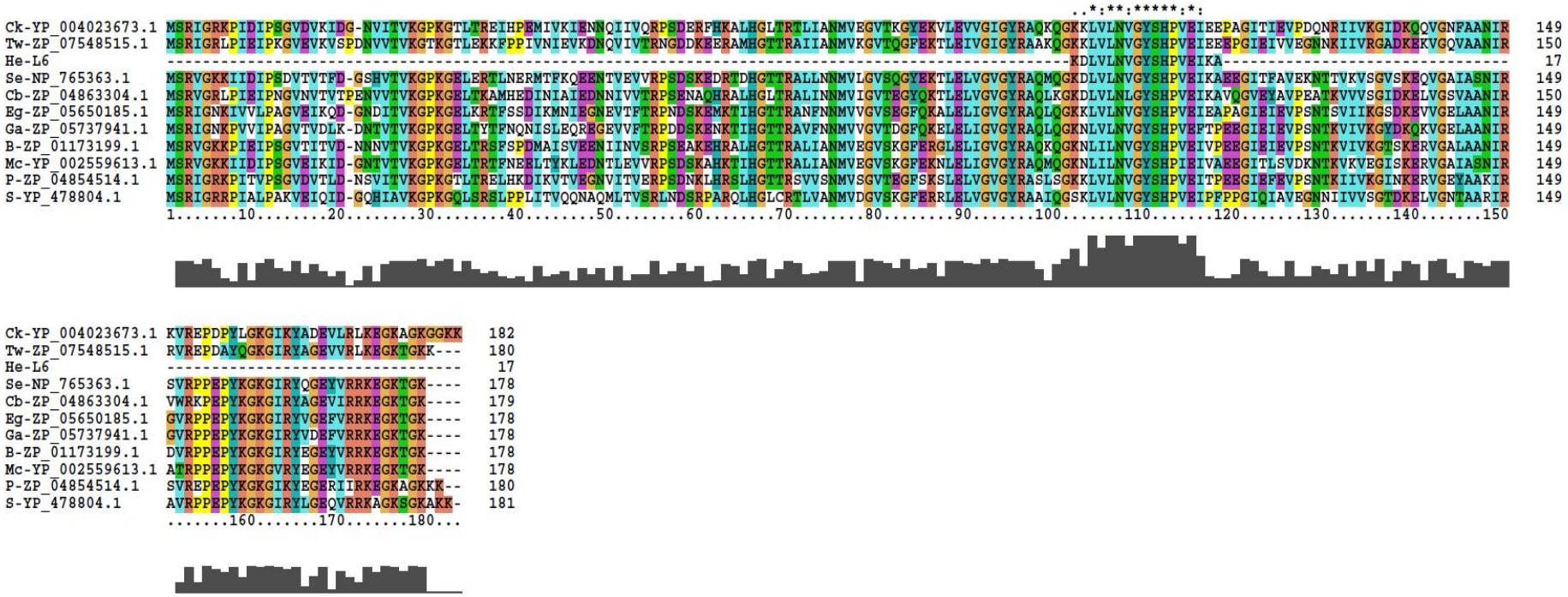


Fig: 35G. Multiple sequence alignment for peptide (m/z 1682.89) of ribosomal protein L6. Ck, *Caldicellulosiruptor kronotskyensis* 2002; Tw, *Thermoanaerobacter wiegelii* Rt8.B1; He, *Halomonas eurihalina* DSM 5720; Se, *Staphylococcus epidermidis* ATCC 12228; Cb, *Clostridium botulinum* D str. 1873; Eg, *Enterococcus gallinarum* EG2; Ga, *Granulicatella adiacens* ATCC 49175; B, *Bacillus* sp. NRRL B-14911; Mc, *Macrococcus caseolyticus* JCSC5402; P, *Paenibacillus* sp. oral taxon 786 str. D14; S, *Synechococcus* sp. JA-2-3B'a(2-13);

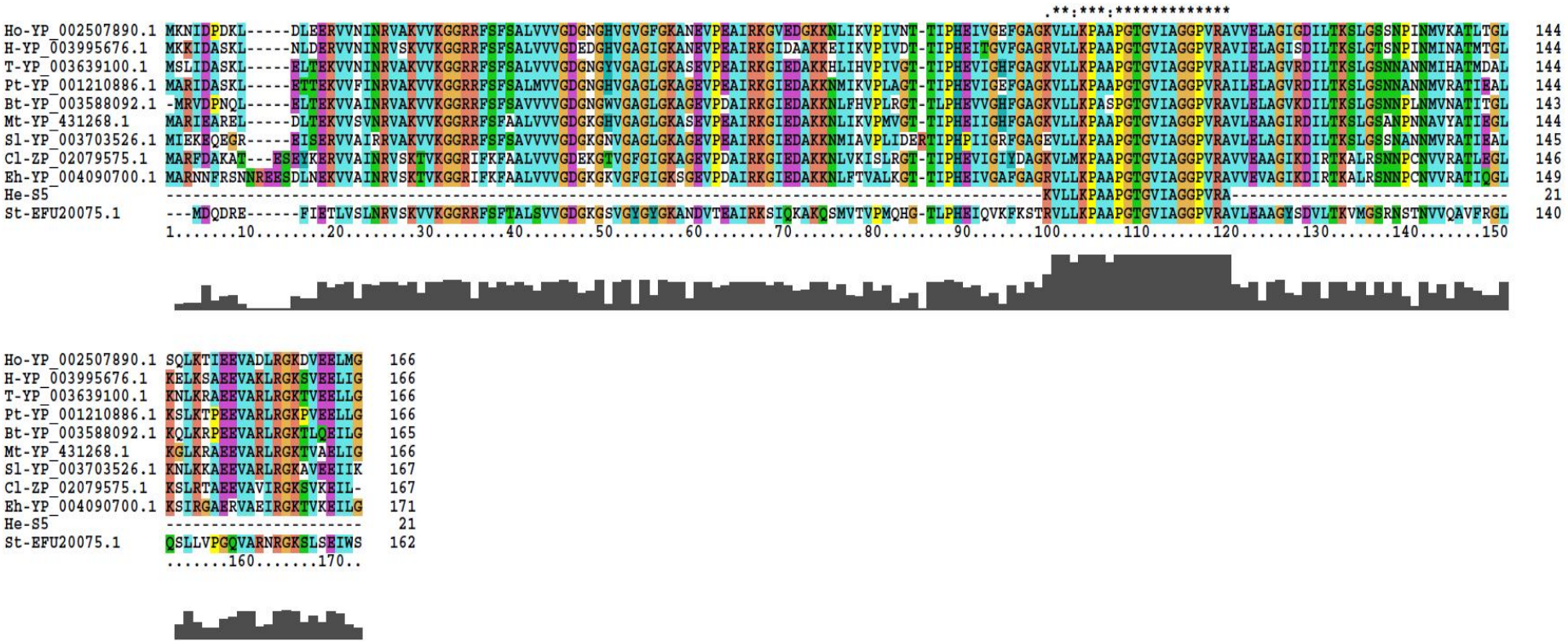


Fig: 35H. Multiple sequence alignment for peptide (m/z 1773.06) of ribosomal protein S5. Ho, *Halothermothrix orenii* H 168; H, *Halanaerobium* sp.; T, *Thermincola* sp. JR; Pt, *Pelotomaculum thermopropionicum* SI; Bt, *Bacillus tusciae* DSM 2912; Mt, *Moorella thermoacetica* ATCC 39073; Sl, *Syntrophothermus lipocalidus* DSM 12680; Cl, *Clostridium leptum* DSM 753; Eh, *Ethanoligenens harbinense* YUAN-3; He, *Halomonas eurihalina* DSM 5720; St, *Spirochaeta thermophila* DSM 6578.

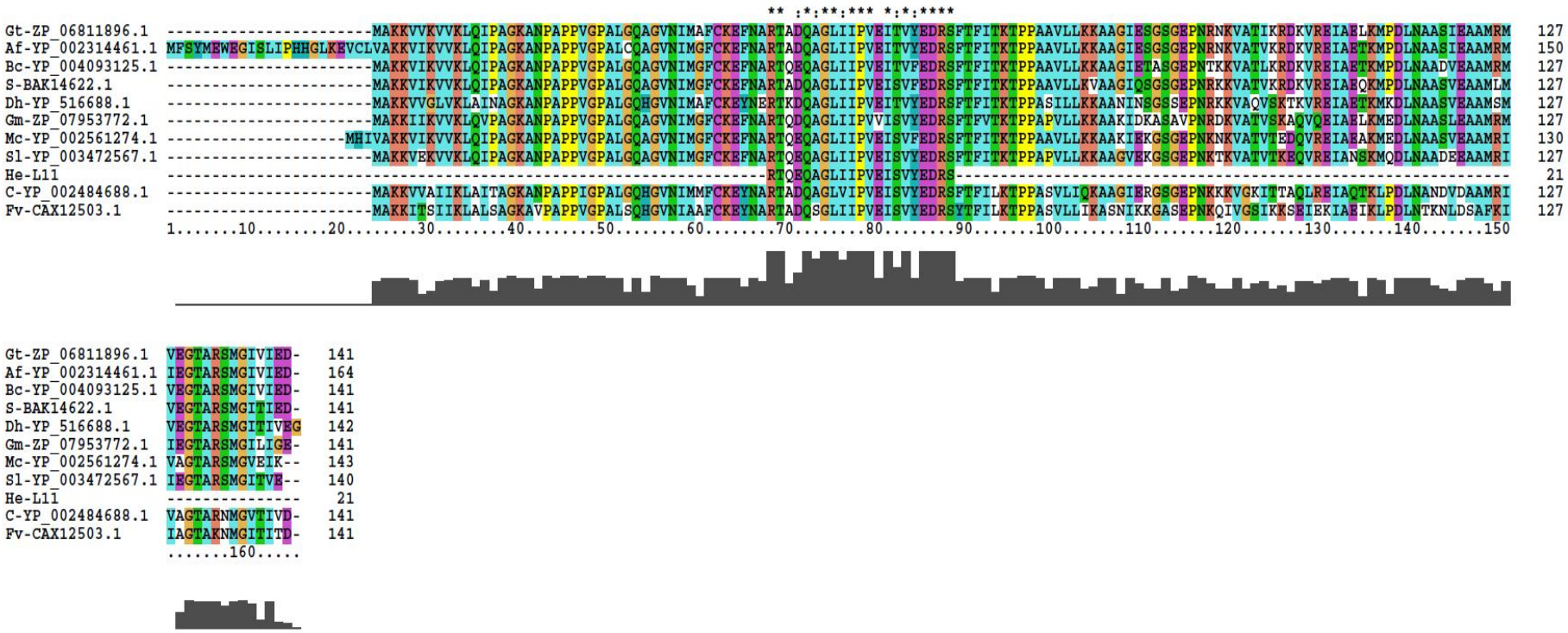


Fig: 35I. Multiple sequence alignment for peptide (m/z 2160.17) of ribosomal protein L11. Gt, *Geobacillus thermoglucosidasius* C56-YS93; Af, *Anoxybacillus flavithermus* WK1; Bc, *Bacillus cellulosilyticus* DSM 2522; S, *Solibacillus silvestris* StLB046; Dh, *Desulfitobacterium hafniense* Y51; Gm, *Gemella moribillum* M424; Mc, *Micrococcus caseolyticus* JCSC5402; Sl, *Staphylococcus lugdunensis* HKU09-01; He, *Halomonas eurihalina* DSM 5720; C, *Cyanothece* sp. PCC 7425; Fv, *Fucus vesiculosus*.

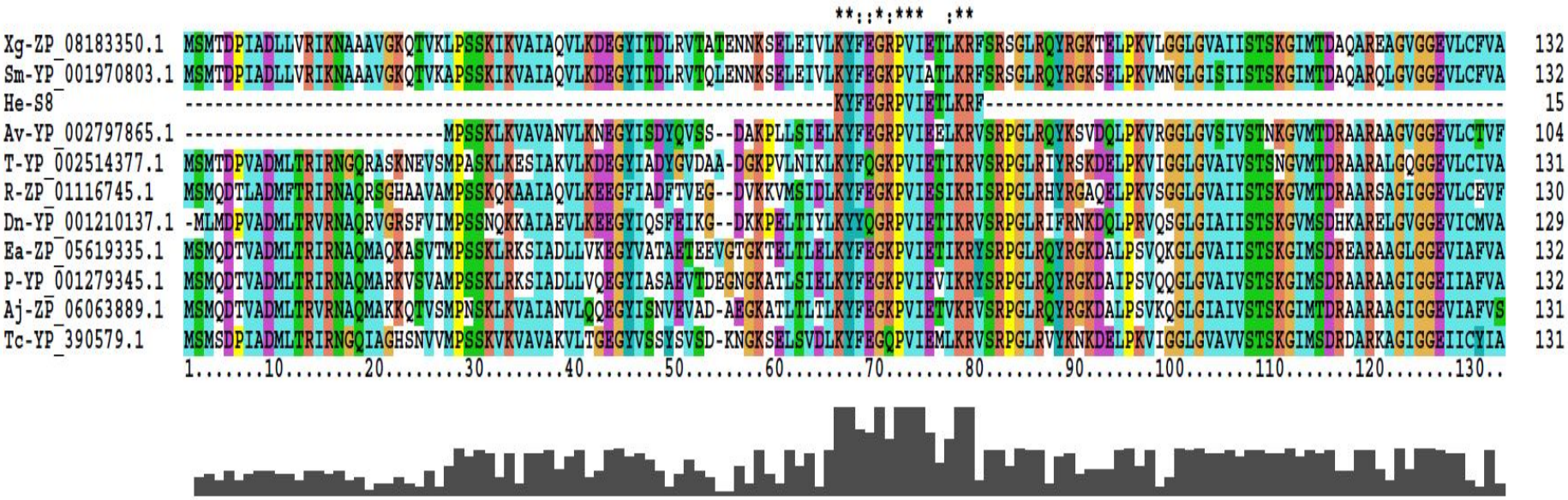


Fig: 35J. Multiple sequence alignment for peptide (m/z 1608.12) of ribosomal protein S8a. Xg, *Xanthomonas gardneri* ATCC 19865; Sm, *Stenotrophomonas maltophilia* K279a; He, *Halomonas eurihalina* DSM 5720; Av, *Azotobacter vinelandii* DJ; T, *Thioalkalivibrio* sp. HL-EbGR7; R, *Reinekea* sp. MED297; Dn, *Dichelobacter nodosus* VCS1703A; Ea, *Enhydrobacter aerosaccus* SK60; P, *Psychrobacter* sp. PRwf-1; Aj, *Acinetobacter johnsonii* SH046; Tc, *Thiomicrospira crunogena* XCL-2.

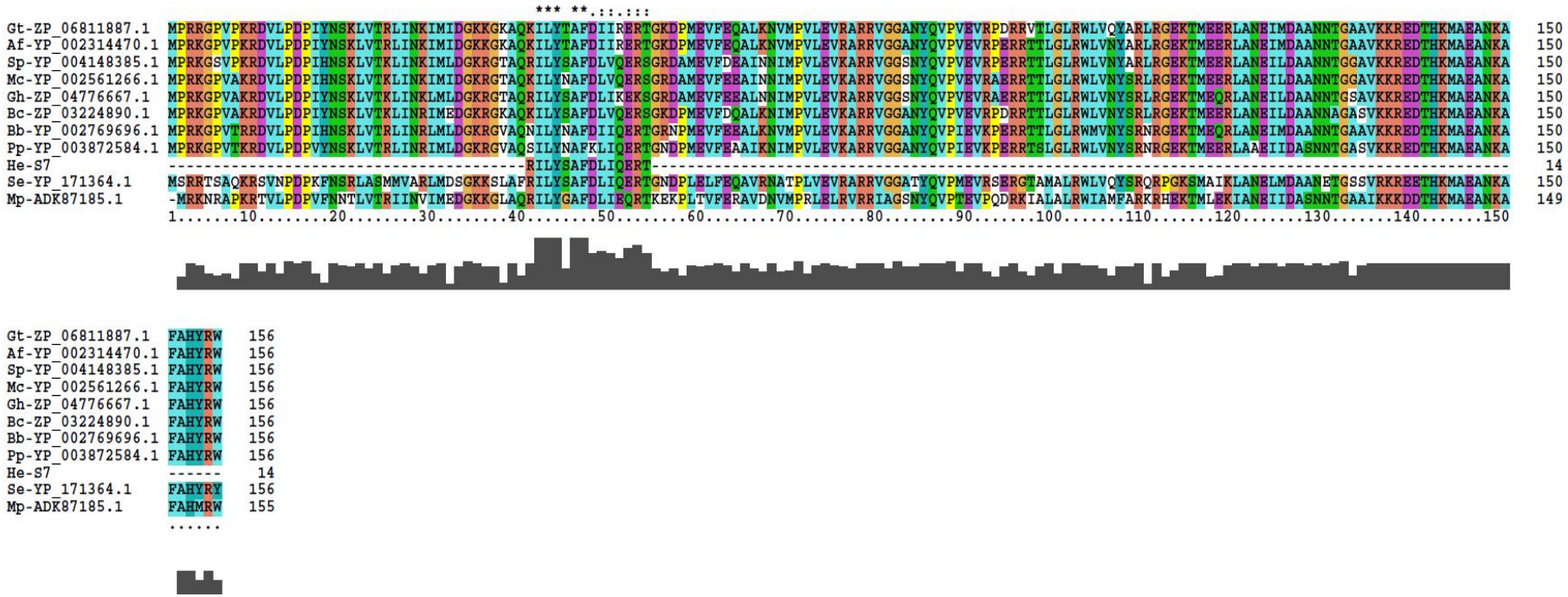


Fig: 35Ki. Multiple sequence alignment for peptide (m/z 1468.00) of ribosomal protein S7a. Gt, *Geobacillus thermoglucosidasius* C56-YS93; Af, *Anoxybacillus flavithermus* WK1; Sp, *Staphylococcus pseudintermedius* HKU10-03; Mc, *Macrococcus caseolyticus* JCSC5402; Gh, *Gemella haemolysans* ATCC 10379; Bc, *Bacillus coahuilensis* m4-4; Bb, *Brevibacillus brevis* NBRC 100599; Pp, *Paenibacillus polymyxa* E681; He, *Halomonas eurihalina* DSM 5720; Se, *Synechococcus elongatus* PCC 6301; Mp, *Mycoplasma pneumoniae* FH.

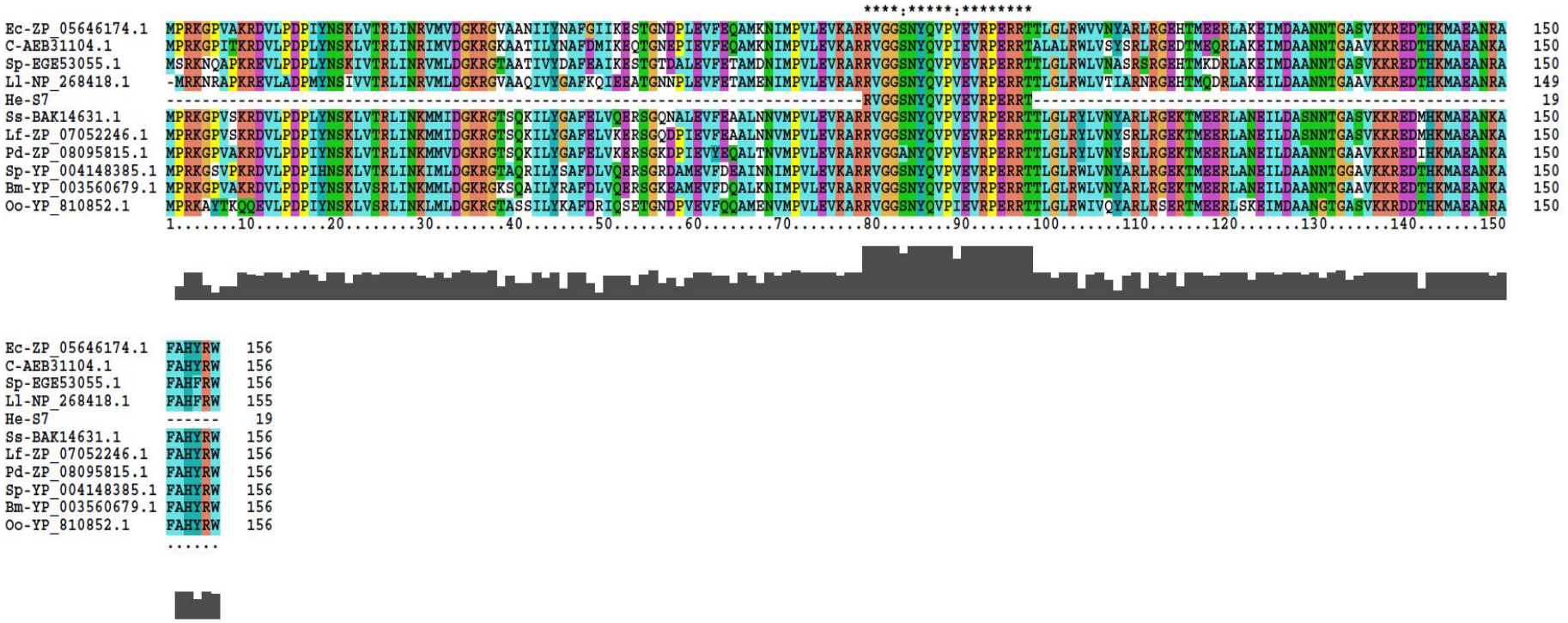


Fig: 35Kii. Multiple sequence alignment for peptide (m/z 1942.36) of ribosomal protein S7a. Ec, *Enterococcus casseliflavus* EC30; C, *Carnobacterium* sp. 17-4; Sp, *Streptococcus parauberis* NCFD 2020; *Ll, Lactococcus lactis*; He, *Halomonas eurihalina* DSM 5720; Lf, *Lysinibacillus fusiformis* ZC1; Pd, *Planococcus donghaensis* MPA1U2; Sp, *Staphylococcus pseudintermedius* HKU10-03; Bm, *Bacillus megaterium* QM B1551; Oo, *Oenococcus oeni* PSU-1.

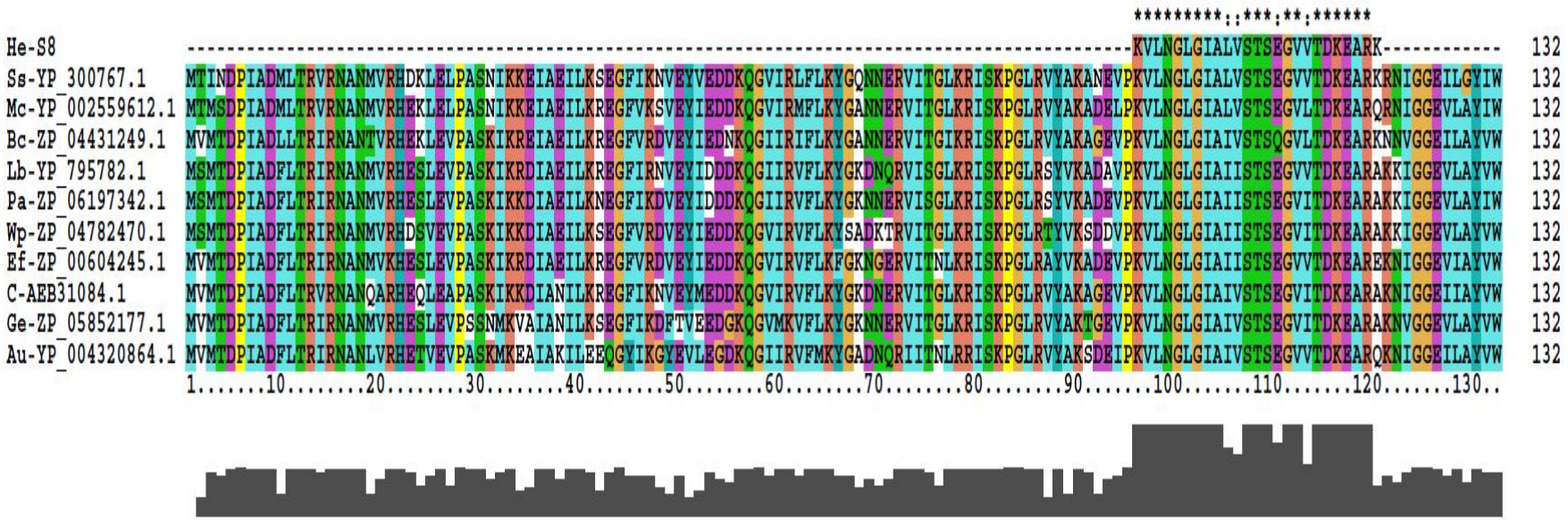


Fig: 35Li. Multiple sequence alignment for peptide (m/z 2328.88) of ribosomal protein S8b. He, *Halomonas eurihalina* DSM 5720; Ss, *Staphylococcus saprophyticus* ATCC 15305; Mc, *Macrococcus caseolyticus* JCSC5402; Bc, *Bacillus coagulans* 36D1; Lb, *Lactobacillus brevis* ATCC 367; Pa, *Pediococcus acidilactici* 7_4; Wp, *Weissella paramesenteroides* ATCC 33313; Ef, *Enterococcus faecium* DO; C, *Carnobacterium* sp. 17-4; Ge, *Granulicatella elegans* ATCC 700633; Au, *Aerococcus urinae* ACS-120-V-Col10a.

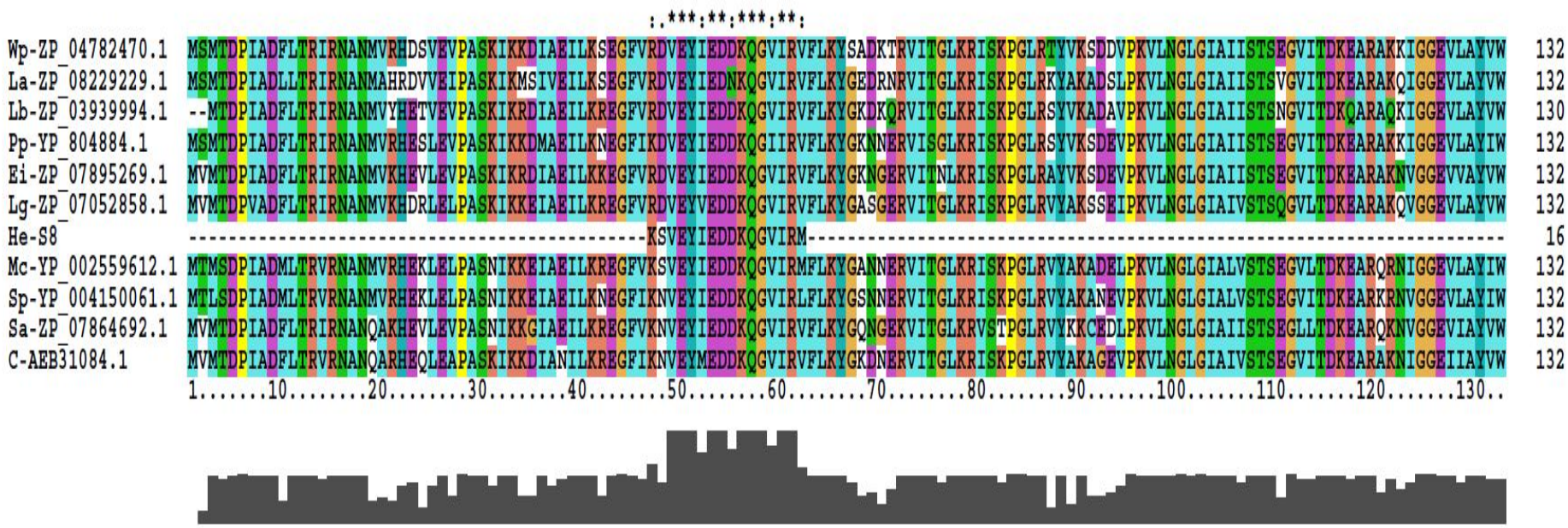


Fig: 35Lii. Multiple sequence alignment for peptide (m/z 1651.22) of ribosomal protein S8b. Wp, *Weissella paramesenteroides* ATCC 33313; La, *Leuconostoc argentinum* KCTC 3773; Lb, *Lactobacillus brevis* ATCC 27305; Pp, *Pediococcus pentosaceus* ATCC 25745; Ei, *Enterococcus italicus* DSM 15952; Lg, *Listeria grayi* DSM 20601; He, *Halomonas eurihalina* DSM 5720; Mc, *Macrococcus caseolyticus* JCSC5402; Sp, *Staphylococcus pseudintermedius* HKU10-03; Sa, *Streptococcus anginosus* F0211; C, *Carnobacterium* sp. 17-4.

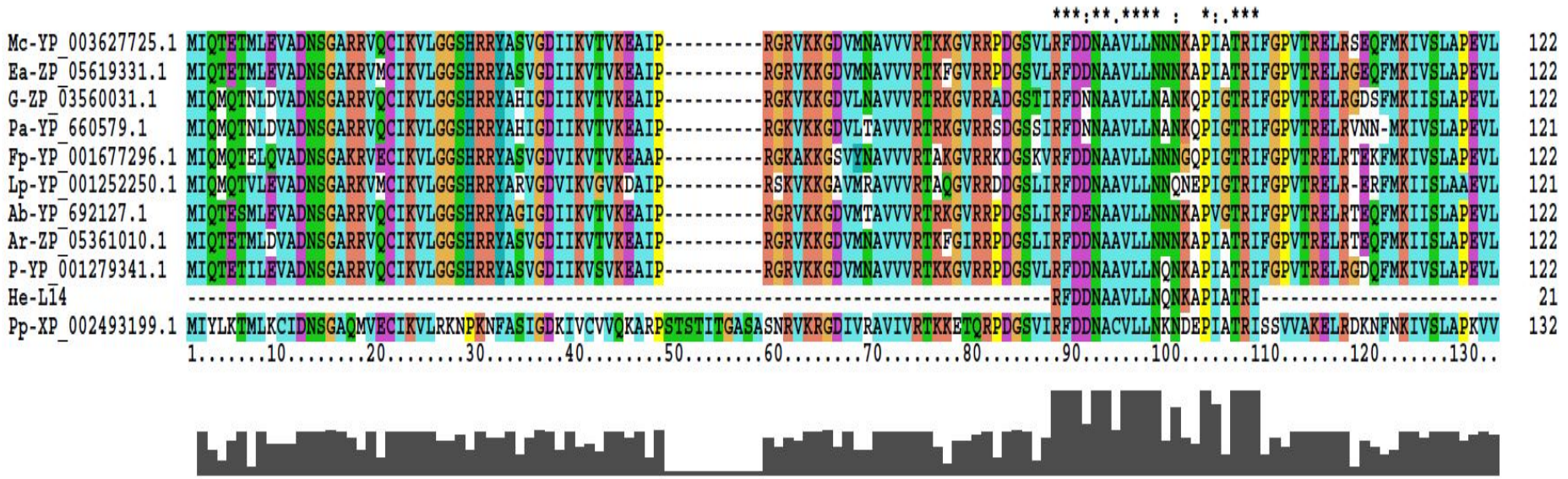


Fig: 35M. Multiple sequence alignment for peptide (m/z 2071.72) of ribosomal protein L14. Mc, *Moraxella catarrhalis* RH4; Ea, *Enhydrobacter aerosaccus* SK60; G, *Glaciecola* sp. HTCC2999; Pa, *Pseudoalteromonas atlantica* T6c; Fp, *Francisella philomiragia* ATCC 25017; Lp, *Legionella pneumophila* str. Corby; Ab, *Alcanivorax borkumensis* SK2; Ar, *Acinetobacter radioresistens* SK82; P, *Psychrobacter* sp. PRwf-1; He, *Halomonas eurihalina* DSM 5720. Pp, *Pichia pastoris* GS115.

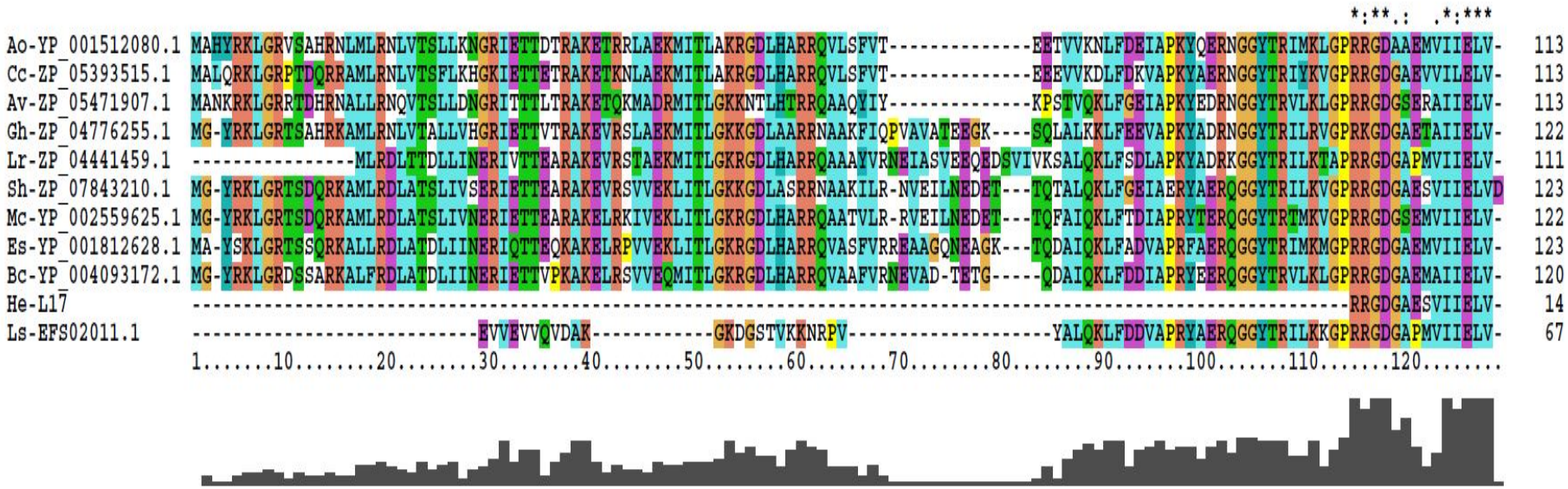


Fig: 35N. Multiple sequence alignment for peptide (m/z 1358.10) of ribosomal protein L17. Ao, *Alkaliphilus oremlandii*; Cc, *Clostridium carboxidivorans* P7; Av, *Anaerococcus vaginalis* ATCC 51170; Gh, *Gemella haemolysans* ATCC 10379; Lr, LMS2-1; *Lactobacillus rhamnosus*; Sh, *Staphylococcus hominis* C80; Mc, *Macrococcus caseolyticus* JCSC5402; Es, *Exiguobacterium sibiricum* 255-15; Bc, *Bacillus cellulosilyticus* DSM 2522; He, *Halomonas eurihalina* DSM 5720; Ls, *Listeria seeligeri* FSL S4-171.

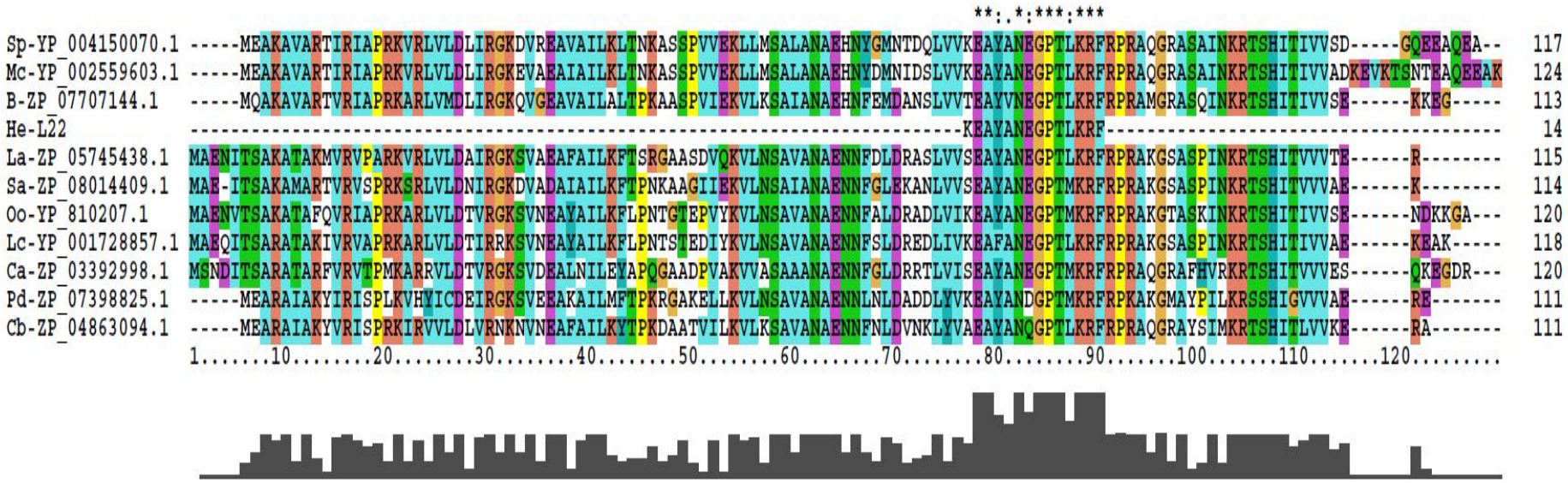


Fig: 35O. Multiple sequence alignment for peptide (m/z 1349.08) of ribosomal protein L22. Sp, *Staphylococcus pseudintermedius* HKU10-03; Mc, *Macrococcus caseolyticus* JCSC5402; B, *Bacillus* sp. m3-13; He, *Halomonas eurihalina* DSM 5720; La, *Lactobacillus antri* DSM 16041; Sa, *Streptococcus anginosus* 1_2_62CV; Oo, *Oenococcus oeni* PSU-1; Lc, *Leuconostoc citreum* KM20; Ca, *Corynebacterium amycolatum* SK46; Pd, *Peptoniphilus duerdenii* ATCC BAA-1640; Cb, *Clostridium botulinum* D str. 1873.

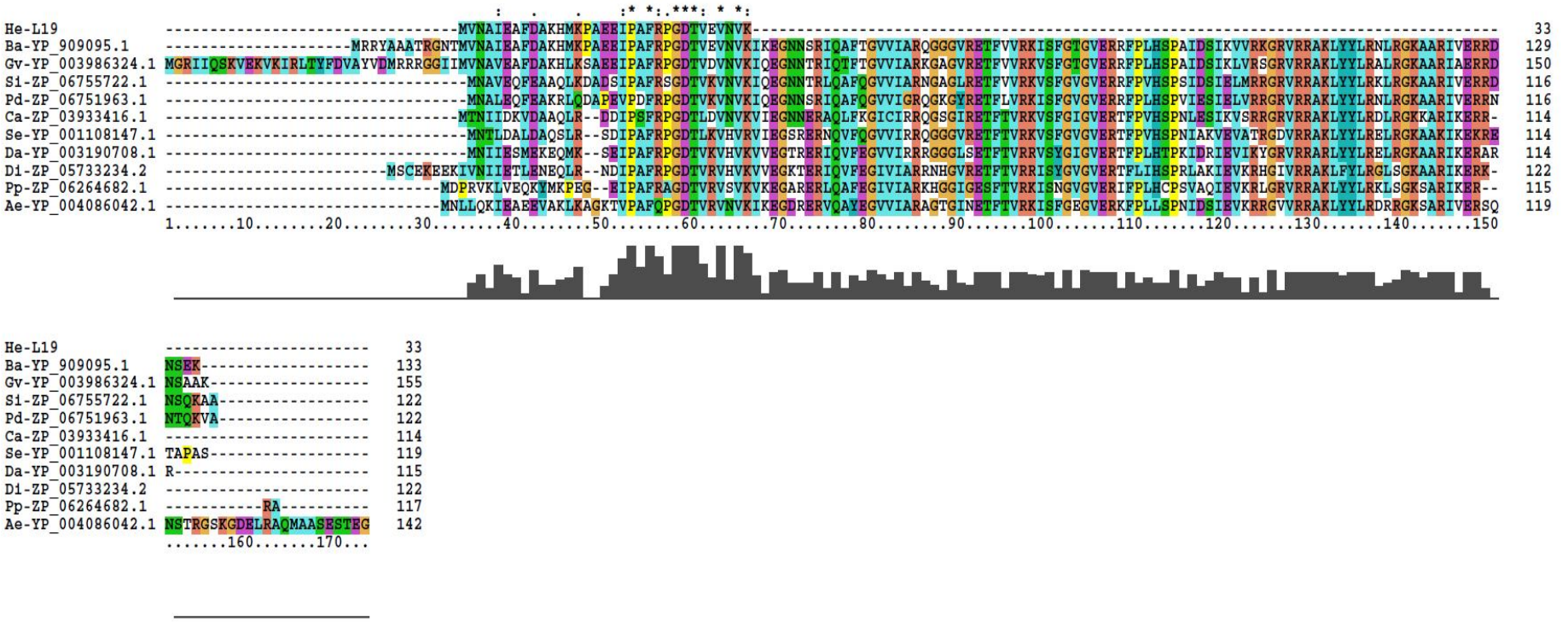


Fig: 35P. Multiple sequence alignment for amino acid sequence of ribosomal protein L19. He, *Halomonas eurihalina* DSM 5720; Ba, *Bifidobacterium adolescentis* ATCC 15703; Gv, *Gardnerella vaginalis* ATCC 14019; Si, *Scardovia inopinata* F0304; Pd, *Parascardovia denticolens* F0305; Ca, *Corynebacterium accolens* ATCC 49725; Se, *Saccharopolyspora erythraea* NRRL 2338; Da, *Desulfotomaculum acetoxidans* DSM 771; Di, *Dialister invisus* DSM 15470; Pp, *Pyramidobacter piscolens* W5455; Ae, *Asticcacaulis excentricus* CB 48.

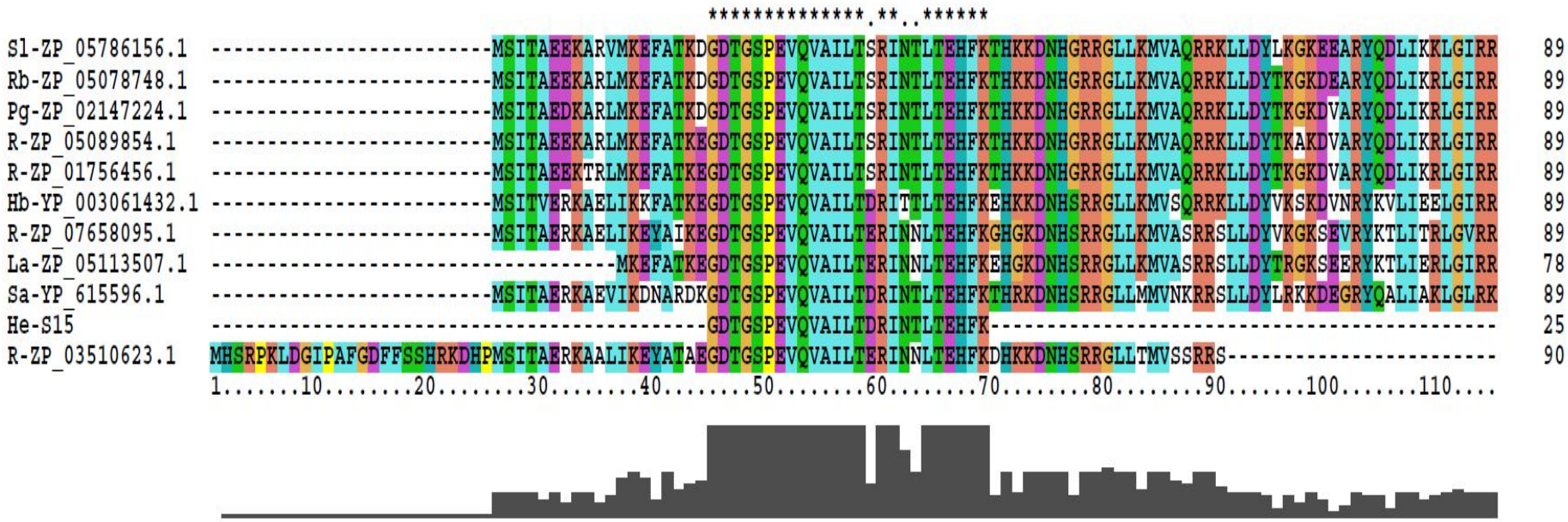


Fig: 35Q. Multiple sequence alignment for amino acid sequence of ribosomal protein S15. Sl, *Silicibacter lacuscaerulensis* ITI-1157; Rb, *Rhodobacterales bacterium* Y4I; Pg, *Phaeobacter gallaeciensis* BS107; R, *Ruegeria* sp. R11; R, *Roseobacter* sp. SK209-2-6; Hb, *Hirschia baltica* ATCC 49814; R, *Roseibium* sp. TrichSKD4; La, *Labrenzia alexandrii* DFL-11; Sa, *Sphingopyxis alaskensis* RB2256; He, *Halomonas eurihalina* DSM 5720; R, *Rhizobium etli* 8C-3.

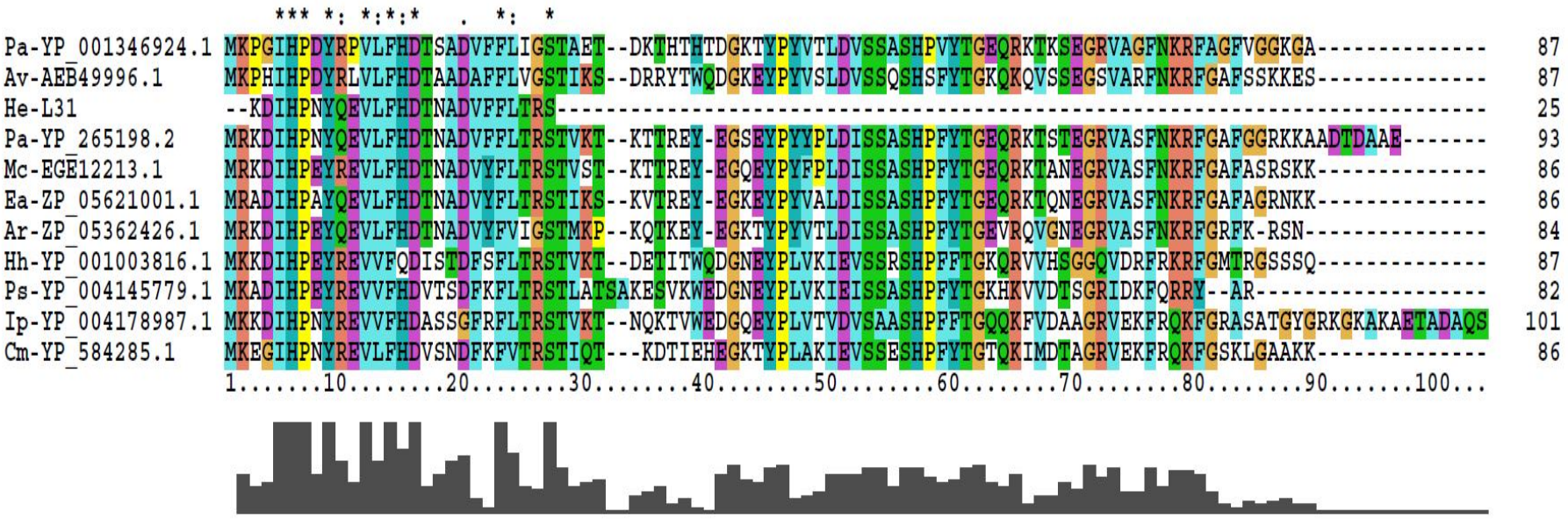


Fig: 35R. Multiple sequence alignment for peptide (m/z 2792.36) of ribosomal protein L31. Pa, *Pseudomonas aeruginosa PA7*; Av, *Aeromonas veronii* B565; He, *Halomonas eurihalina* DSM 5720; Pa, *Psychrobacter arcticus* 273-4; Mc, *Moraxella catarrhalis* 7169; Ea, *Enhydrobacter aerosaccus* SK60; Ar, *Acinetobacter radioresistens* SK82; Hh, *Halorhodospira halophila* SL1; Ps, *Pseudoxanthomonas suwonensis* 11-1; Ip, *Isosphaera pallida* ATCC 43644; Cm, *Cupriavidus metallidurans* CH34.

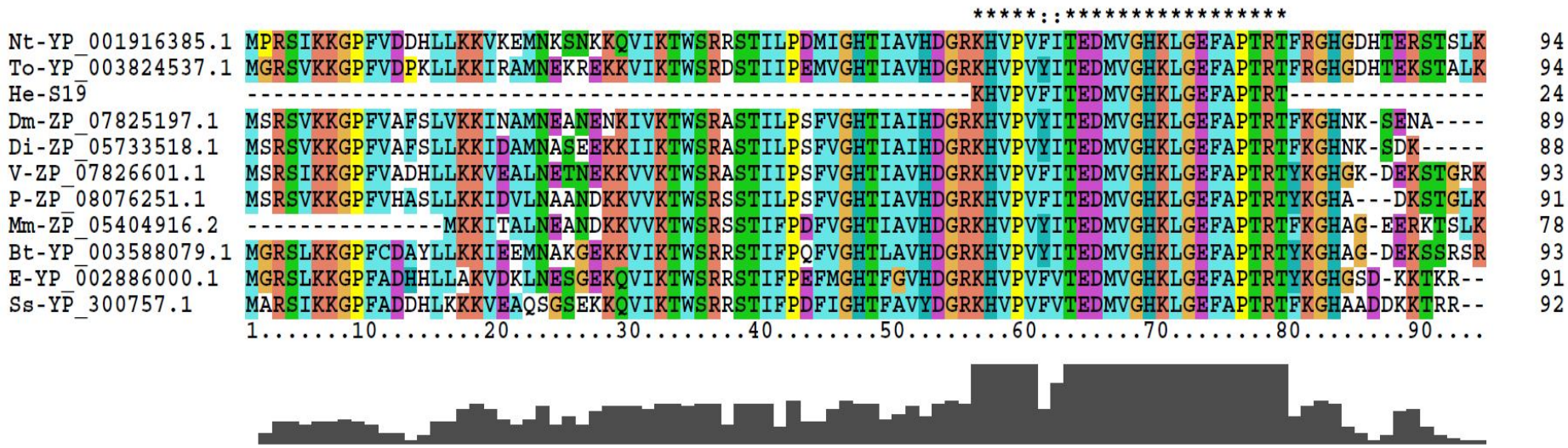


Fig: 35S. Multiple sequence alignment for peptide (m/z 2496.16) of ribosomal protein S19. Nt, *Natranaerobius thermophilus* JW/NM-WN-LF; To, *Thermosediminibacter oceani* DSM 16646; He, *Halomonas eurihalina* DSM 5720; Dm, *Dialister microaerophilus* UPII 345-E; V, *Veillonella* sp. oral taxon 158 str. F0412; P, *Phascolarctobacterium* sp. YIT 12067; Mm, *Mitsuokella multacida* DSM 20544; Bt, *Bacillus tusciae* DSM 2912; E, *Exiguobacterium* sp. AT1b; Ss, *Staphylococcus saprophyticus* ATCC 15305;

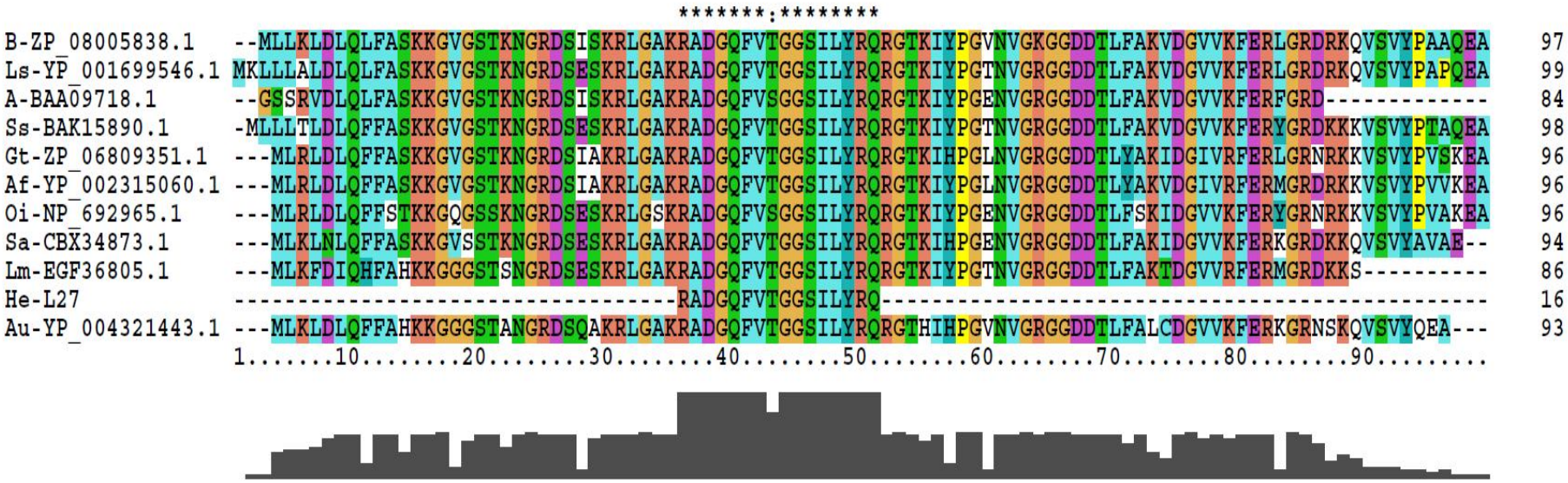


Fig: 35Ti. Multiple sequence alignment for peptide (m/z 1484.13) of ribosomal protein L27a. B, *Bacillus* sp. 2_A_57_CT2; Ls, *Lysinibacillus sphaericus* C3-41; A, *Arthrobacter* sp. TE1826; Ss, *Solibacillus silvestris* StLB046; Gt, *Geobacillus thermoglucosidasius* C56-YS93; Af, *Anoxybacillus flavithermus* WK1; Oi, *Oceanobacillus iheyensis* HTE831; Sa, *Staphylococcus aureus* ECT-R 2; Lm, *Listeria monocytogenes* 1816; He, *Halomonas eurihalina* DSM 5720; Au, *Aerococcus urinae* ACS-120-V-Col10a.

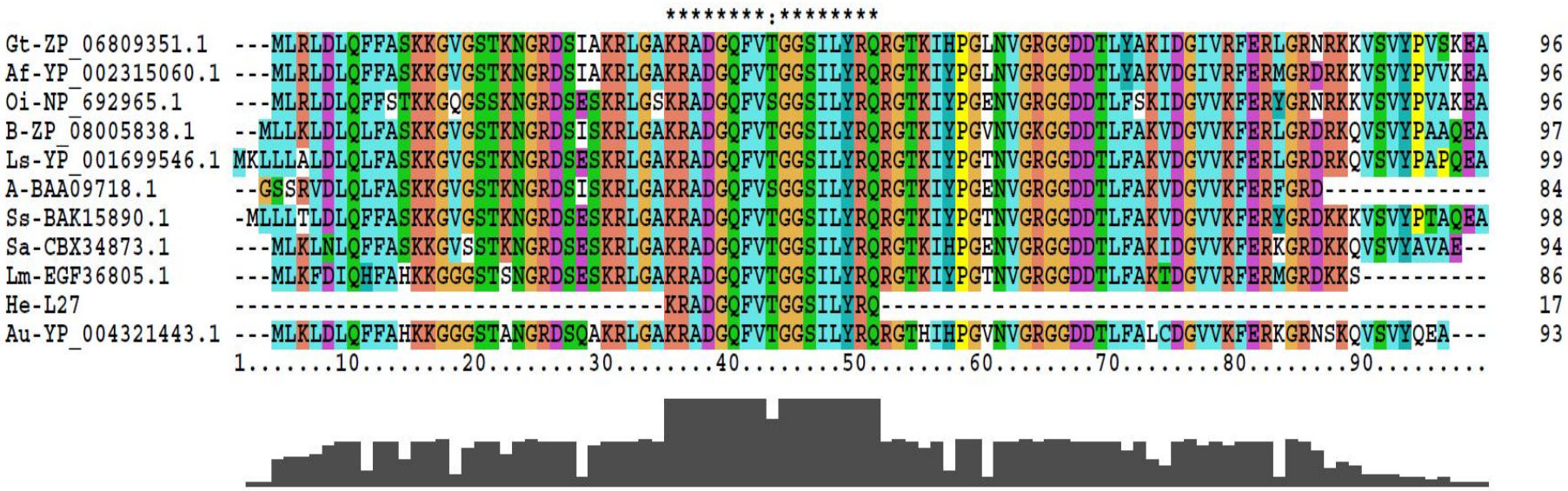


Fig: 35Tii. Multiple sequence alignment for peptide (m/z 1640.32) of ribosomal protein L27a. Gt, *Geobacillus thermoglucosidasius* C56-YS93; Af, *Anoxybacillus flavithermus* WK1; Oi, *Oceanobacillus iheyensis* HTE831; B, *Bacillus* sp. 2_A_57_CT2; Ls, *Lysinibacillus sphaericus* C3-41; A, *Arthrobacter* sp. TE1826; Ss, *Solibacillus silvestris* StLB046; Sa, *Staphylococcus aureus* ECT-R 2; Lm, *Listeria monocytogenes* 1816; He, *Halomonas eurihalina* DSM 5720; Au, *Aerococcus urinae* ACS-120-V-Col10a.

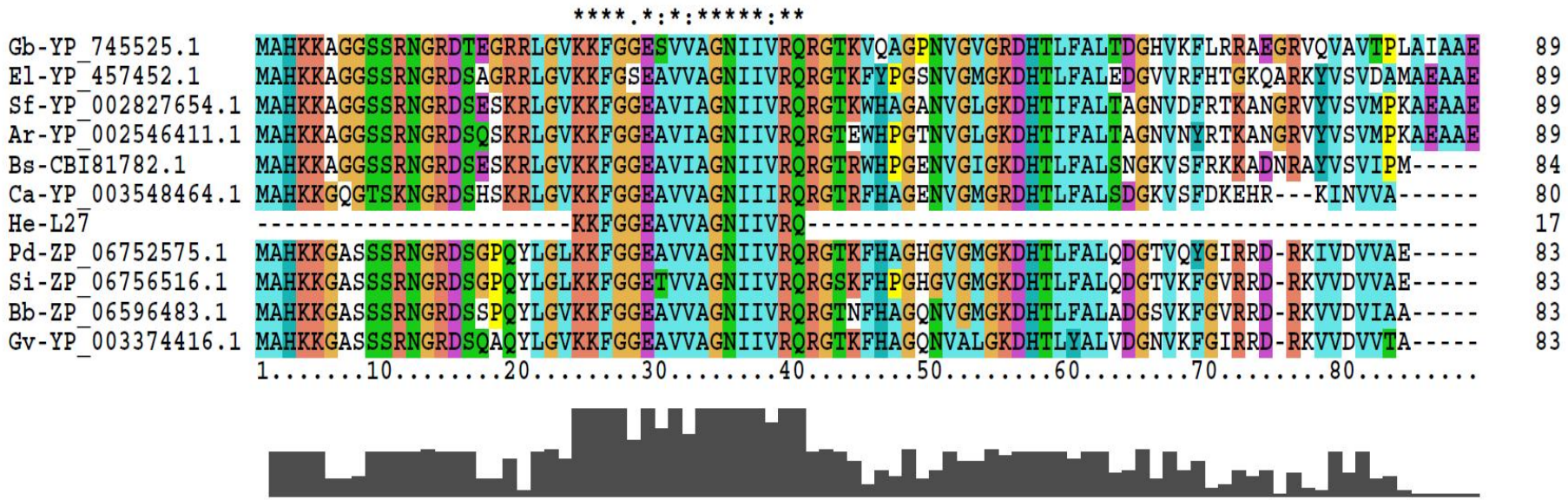


Fig: 35U. Multiple sequence alignment for peptide (m/z 1529.93) of ribosomal protein L27b. Gb, *Granulibacter bethesdensis* CGDNIH1; El, *Erythrobacter litoralis* HTCC2594; Sf, *Sinorhizobium fredii* NGR234; Ar, *Agrobacterium radiobacter* K84; Bs, *Bartonella schoenbuchensis* R1; Ca, *Coraliomargarita akajimensis* DSM 45221; He, *Halomonas eurihalina* DSM 5720; Pd, *Parascardovia denticolens* F0305; Si, *Scardovia inopinata* F0304; Bb, *Bifidobacterium breve* DSM 20213; Gv, *Gardnerella vaginalis* 409-05.

Purification of ribosomal protein S1:

The position in two-dimensional gels in acidic high molecular weight region (top right corner) where ribosomal protein S1 from gram negative bacteria (like *E. coli*) migrates contains several closely spaced protein spots. From this region we could not pick any protein spot for MALDI-TOF MS analysis. We tried to purify ribosomal protein S1 homologue (if any) by exploiting its strong poly (U) binding property using affinity chromatography on poly (U)-sepharose column. Poly (U)-sepharose chromatography of whole ribosomes under high salt conditions results in elution of ribosomes free of S1 with S1 strongly bound to the column which can be eluted by urea buffer. Poly (U)-sepharose chromatography of ribosomes was carried out as described under methods section. Poly (U)-sepharose chromatography of ribosomes with 1 M NH_4Cl buffer resulted in the specific removal of S1 from the ribosomes as in *E. coli*. Elution of the column with 7 M urea and 1 M NH_4Cl buffer resulted in the elution of protein S1. Elution profile of *H. eurihalina* ribosomes on poly (U)-sepharose was given in Fig: 36A. The profile showed a single sharp peak. Electrophoretic analysis of the ribosomes eluted with 1 M NH_4Cl buffer showed that S1 was removed from ribosomes and that of peak fractions showed S1 was specifically eluted with 1 M NH_4Cl and 7 M urea (Fig: 36B). Molecular weight of the protein was determined by SDS-PAGE as 61 kDa, same as that of *E. coli* as shown in Fig: 36B.

MALDI analysis and identification of S1 protein:

S1 protein was subjected to MALDI- TOF MS analysis to obtain peptide mass fingerprint. The data obtained with peptide Mass fingerprint (M 2 values) were used to search databases (Swiss-Prot, NCBI Nr) using software MASCOT search software (Matrix Science USA). The protein was identified as homolog of bacterial of 30 S ribosomal protein S1. The results of the peptide mass fingerprints data base search with the Mascot search were presented in Fig: 36C. The results indicated greater homology of S1 protein to those from

genera *Vibrio* spp., *Aeromonas* spp., *Psychrobacter* spp. and *Marinomonas* sp. The observed molecular weight is about 61 kDa in all organisms. The peptide sequence was searched against non-redundant proteins sequences database using BLAST search. Putative conserved domains corresponding to S1 protein encoded by *rpsA* gene were detected in all the hits. Multiple sequence alignment of the data obtained was given in Fig: 36D. The peptide length was of 16 amino acids and is found to be homologous to 132–148 amino acids region, which is just upstream of the repeat of one of the nucleic acid binding domains of S1. These results show that the S1 peptide detected in *H. eurihalina* is highly conserved among other eubacteria.

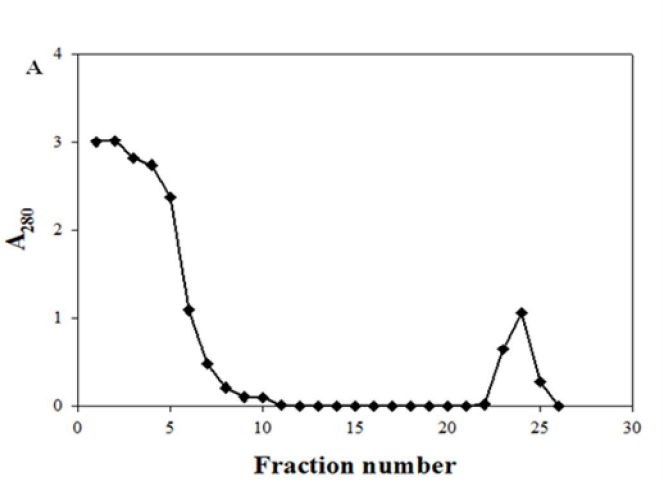


Fig: 36A. Elution profile of *H. eurihalina* ribosomes on poly (U)-sepharose column. Fraction number 1-20 were eluted with 1 M ammonium chloride buffer, fraction number 20-26 were eluted with 1 M ammonium chloride and 7 M urea buffer.

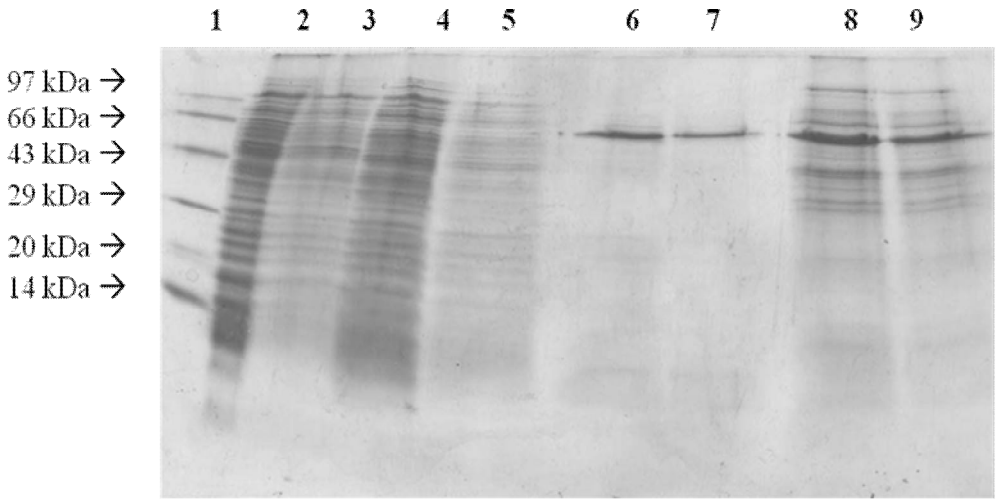
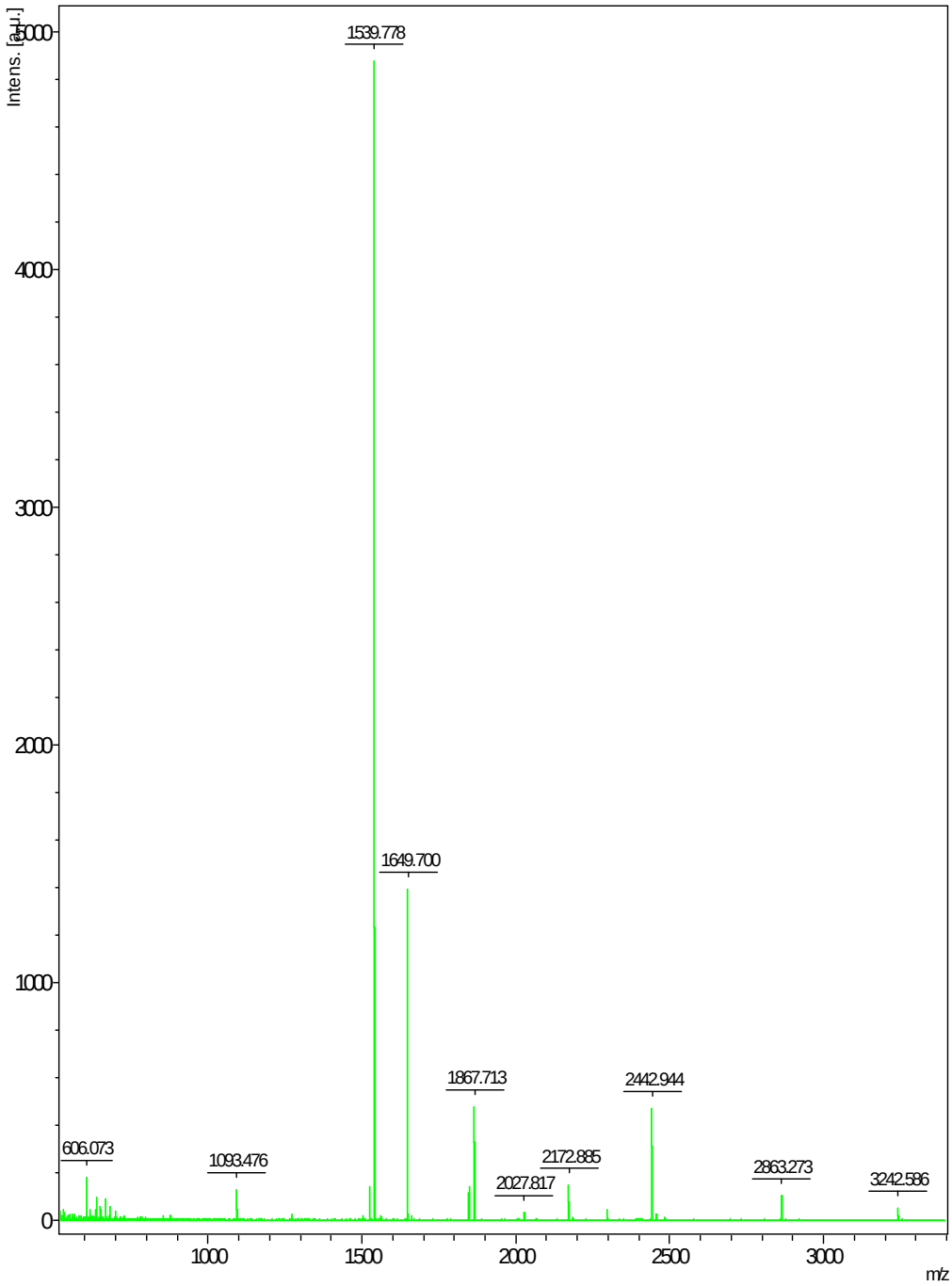


Fig: 37B. SDS-PAGE analysis of poly (U)-sepharose column fractions. Lane 1: Molecular weight marker, lane 2: *H. eurihalina* ribosomes, lane 3: break through, lane 4-5: fraction numbers 3 and 5, lane 6-7: fraction numbers 24 and 25 and lane 8-9: *E. coli* S1.

Fig: 36C. Ingel-digestion - Peptide mass fingerprint:

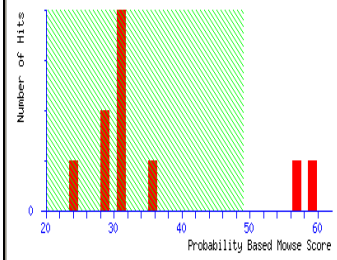


Mascot Search Results

User : madhurarekha
Email : madhurarekha@rediffmail.com
Search title :
MS data file : DATA.TXT
Database : NCBInr 20081113 (7308559 sequences; 2531675135 residues)
Taxonomy : Bacteria (Eubacteria) (3675643 sequences)
Timestamp : 17 Nov 2008 at 09:09:08 GMT
Protein hits : gi|15641917 30S ribosomal protein S1 [Vibrio cholerae O1 biovar eltor str. N16961]
gi|90416549 30S ribosomal protein S1 [marine gamma proteobacterium HTCC2207]
gi|27904178 30S ribosomal protein S1 [Buchnera aphidicola str. Bp (Baizongia pistaciae)]
gi|145220609 hypothetical protein Mflv_0003 [Mycobacterium gilvum PYR-GCK]
gi|83949745 hypothetical protein ISM_01585 [Roseovarius nubinhibens ISM]
gi|108760891 non-ribosomal peptide synthase [Myxococcus xanthus DK 1622]
gi|167834984 sensor histidine kinase [Burkholderia thailandensis MSMB43]
gi|159039332 hypothetical protein Sare_3801 [Salinispora arenicola CNS-205]
gi|148555365 FeoA family protein [Sphingomonas wittichii RW1]
gi|42524701 hypothetical protein Bd3333 [Bdellovibrio bacteriovorus HD100]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 49 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



gi|15641917 **Mass:** 61129 **Score:** 90 **Queries matched:** 1

30 S ribosomal protein S1 [Vibrio cholera O1 biovar eltor str. N16961]

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Peptide
1539.7000	1538.6927	1538.8933	-0.2006	0	90	3.7e-06	1	R.AFLPGSLVDVRPIR.D

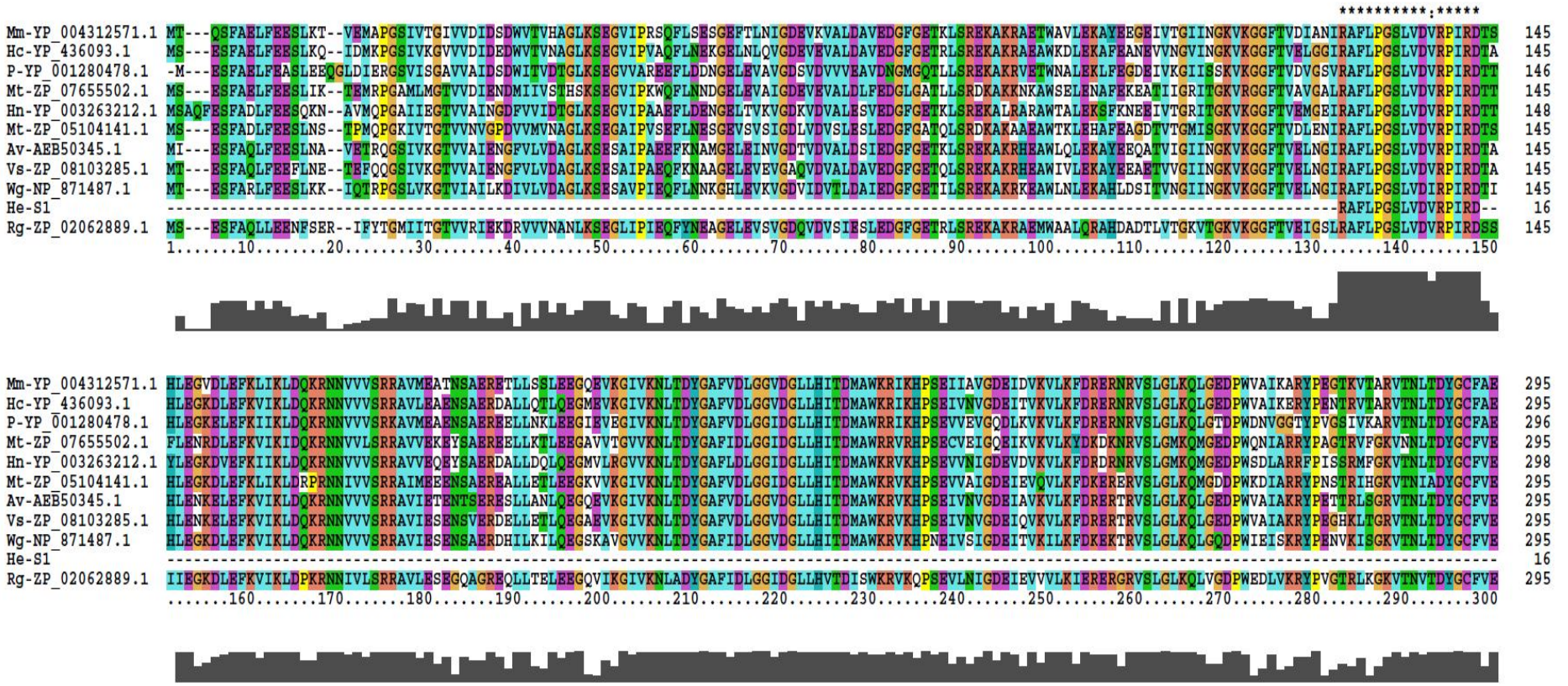
Proteins matching the same set of peptides:

gi|27366257 **Mass:** 60979 **Score:** 90 **Queries matched:** 1

30 S ribosomal protein S1 [Vibrio vulnificus CMCP6]

gi|28898804 **Mass:** 61019 **Score:** 90 **Queries matched:** 1

30 S ribosomal protein S1 [Vibrio parahaemolyticus RIMD 2210633]


Mm-YP_004312571.1
Hc-YP_436093.1
P-YP_001280478.1
Mt-ZP_07655502.1
Hn-YP_003263212.1
Mt-ZP_05104141.1
Av-AEB50345.1
Vs-ZP_08103285.1
Wg-NP_871487.1
He-S1
Rg-ZP_02062889.1

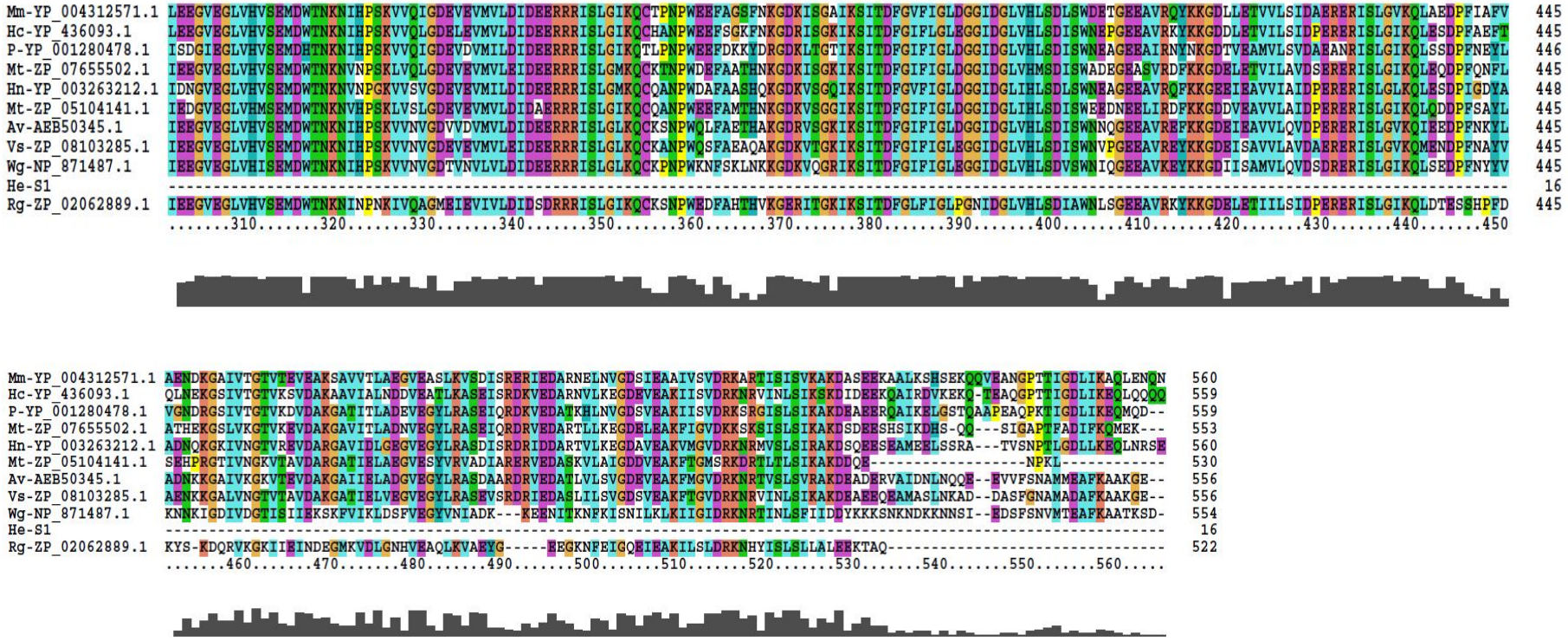


Fig: 36D. Multiple sequence alignment for peptide (m/z 1539.70) of ribosomal protein S1. Mm, *Marinomonas mediterranea* MMB1; Hc, *Hahella chejuensis* KCTC 2396; P, *Psychrobacter* sp. PRwf-1; Mt, *Methylobacter tundripaludum* SV96; Hn, *Halothiobacillus neapolitanus* c2; Mt, *Methylophaga thiooxidans* DMS010; Av, *Aeromonas veronii* B565; Vs, *Vibrio sinaloensis* DSM 21326; Wg, *Wigglesworthia glossinidia*; He, *Halomonas eurihalina* DSM 5720; Rg, *Rickettsiella grylli*;

Cold shock proteins in *H. eurihalina*

Introduction:

During our studies on S-100 to isolate S1, we observed the presence of two proteins exhibiting strong poly (U) binding capacity. Both these proteins were identified as cold shock DNA binding proteins.

Adaptation to environmental stress is essential to the survival of microorganisms and microorganisms produce different kinds of proteins when they are subjected to stress. Membrane fluidity decreases upon the temperature downshift (cold shock) and organisms overcome this by increasing the synthesis of membrane bound-desaturase as in case of *E. coli*, *Bacillus* and *Synechococcus*. Cold shock also causes stabilization of the secondary structures in RNA and DNA and reduced efficiency of translation, transcription and DNA replication. These deleterious effects are overcome by induction of cold shock proteins (CSPs) (Phadtare *et al*, 1999). Many cold shock proteins were identified to date in both prokaryotes and eukaryotes. In *E. coli* CspA, CspB and CspG were identified as major cold shock proteins (Etchegaray, 1999). In *Bacillus subtilis*, CspB, CspC and CspD were identified and they were found to be homologous to *E. coli* CspA (Ermolenko, 2002).

CSPs are not usually found in eukaryotes, but exist as a nucleic acid-binding domain within multidomain proteins, called CSD (Cold Shock Domain) proteins (Sommerville, 2006). Homologues of CSPs were identified in eukaryotes and Cla h 8 from mould *Cladosporium herbarum* was the first one to be reported. Cla h 8 shows 76% amino acid identity with *E. coli* CspA, although it displays nucleic acid binding properties more similar to those of the eukaryotic CSD, and Cla h 8 has been suggested to represent an evolutionary link between bacterial Csps and eukaryal CSDs (Falsone *et al*, 2002) .

Cold shock proteins in *E. coli* are of two types: class I proteins are expressed at extremely low levels at 37 ºC and are dramatically induced to very high levels after exposure to a downshift in temperature. Class II proteins are present at 37 ºC also and are not dramatically induced after cold shock (<10-fold) (Phadtare *et al*, 1999).

CspA, CspB, CspG, CsdA, RbfA, NusA and PNP are class I proteins (Thieringer *et al*, 1998). CspA, CspB and CspG have been proposed to function as RNA/DNA chaperones. CsdA is a ribosome associated protein with RNA unwinding activity, RbfA is a ribosome binding factor, and NusA is involved in termination and antitermination of transcription and PNP is a ribonuclease. The recombination factor RecA, the initiation factor IF-2, the nucleoid-associated DNA-binding protein H-NS, and the subunit of topoisomerase DNA gyrase GyrA are class II proteins (Thieringer *et al*, 1998). Trigger factor (TF) is induced upon cold shock in *E. coli* and enhances viability at low temperatures (Kandror and Goldberg, 1997).

E. coli encodes nine *csp* genes (*cspA* to *cspI*), of which *cspA*, *cspB*, *cspG* and *cspI* are induced by cold shock (Etchegaray *et al*, 1996; Goldstein *et al*, 1990; Lee *et al*, 1994; Nakashima *et al*, 1996; Wang *et al*, 1999). *cspC* and *cspE* are constitutively expressed and *cspD* is induced by nutrient deprivation. *cspF* and *cspH* has not been associated with any particular growth condition or phenotype (Bae *et al*, 1999; Yamanaka and Inouye, 1997; Yamanaka *et al*, 1994) .

Poly (U)-cellulose chromatography of S-100:

S1 associates loosely with ribosomes and is usually lost during centrifugation and is found in S-100, in the case of *E. coli*. Poly (U)-cellulose chromatography of S-100 was performed as described in methods to purify S1 from S-100, as in the case of *E. coli*. Elution profile of S-100 on poly (U)-cellulose column was given in Fig: 37A. S-100 obtained from both logarithmic and stationary phase cells resulted in the same elution profile. Native-PAGE analysis of peak fractions from logarithmic and stationary phase S-100 eluted on poly (U)-cellulose column was shown in Fig: 37B.

Results showed that even though elution profile was similar, logarithmic phase fractions contained two proteins (low molecular weight) and stationary phase fractions contained one protein with very strong affinity to poly (U). Native-PAGE results showed that both these proteins are acidic; the protein observed in both the growth phases was more acidic in comparison to the one which was observed only in logarithmic phase.

It can also be observed that only negligible amount of S1 is present in S-100 fraction, in contrary to *E. coli*. So, it can be concluded that the association of S1 is strong in the case of *H. eurihalina* ribosomes, unlike *E. coli* ribosomes.

SDS-PAGE and Two-dimensional gel analysis:

SDS-PAGE analysis of logarithmic and stationary phase S-100 and poly (U)-cellulose peak fractions was shown in Fig: 38A & B. Results showed that, both the proteins were of low molecular weight. Molecular weight of the protein observed in both the growth phases was around 12 kDa and that of the protein observed only in logarithmic phase was around 8 kDa.

Acidic two-dimensional gel electrophoresis of the proteins was carried out as described in methods. The two-dimensional gel electrophoresis of the partially purified proteins obtained from logarithmic and stationary phases was given in 39A & B. These results showed that the protein observed in both the growth phases is more acidic in comparison to the protein observed only in logarithmic phase. These results support the data obtained by Native-PAGE analysis.

Identification of the proteins by MALDI-TOF MS analyses:

Both the proteins were identified as cold shock DNA binding proteins by MALDI-TOF MS analysis. The peptide mass fingerprints and their respective identities obtained for 12 kDa and 8 kDa proteins were given in Fig: 40A & 41A respectively. 12 kDa protein was found to be homologous to cold shock proteins belonging to the family CspA.

The peptide sequences thus obtained were used to search similar proteins sequences using BLAST search. A S1-like cold shock domain (CSD) was detected for 12 kDa cold shock protein peptide sequence. It is a member of the oligonucleotide/oligosaccharide binding fold (OBD) and is found in archaea, eukaryotes and eubacteria.

Multiple sequence alignment of the proteins sequences, generated with BLAST search was carried out, along with 12 kDa and 8 kDa cold shock protein peptide sequences using ClustalX2 were given in Fig: 40B & 41B respectively.

The results showed that the peptide from 12 kDa protein is of 28 amino acid length and it is found to be homologous with C-terminal sequence (41-68 a.a.) of other cold shock proteins. Peptide from 8 kDa protein is of 19 amino acid length and it is also found to be homologous to sequence near C-terminal region (42-60 a.a.) of other cold shock proteins.

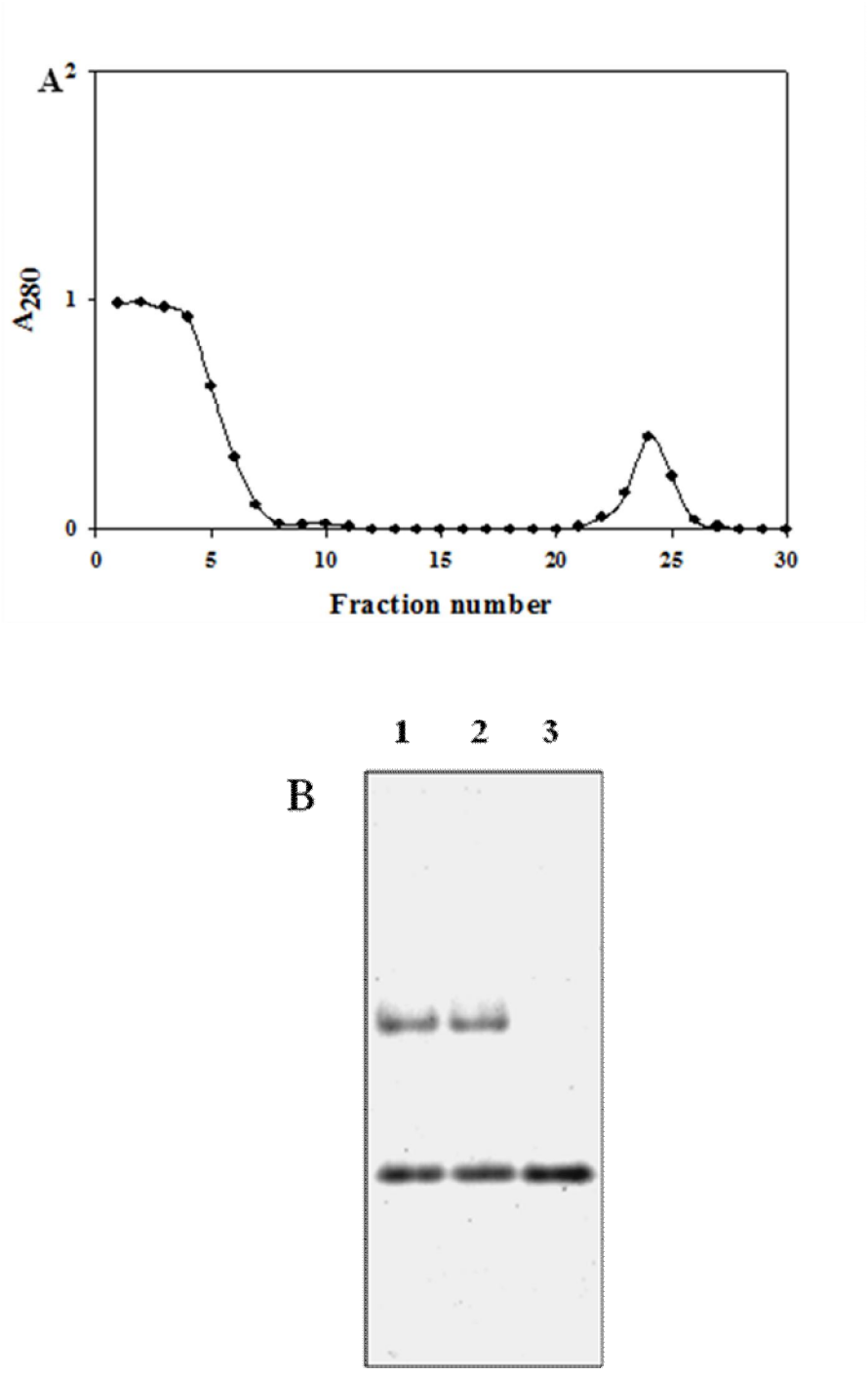


Fig: 37. Purification of strong poly (U) binding proteins from S-100. A. Elution profile. Fractions 1-20 eluted with 1 M ammonium chloride buffer. Fractions 21-30 eluted with 1M ammonium chloride and 8M urea buffer. B. Native-PAGE analysis. Lane 1-2: Fractions 24 and 25 of log phase elution, lane 3: Fraction 24 of stationary phase S-100 elution.

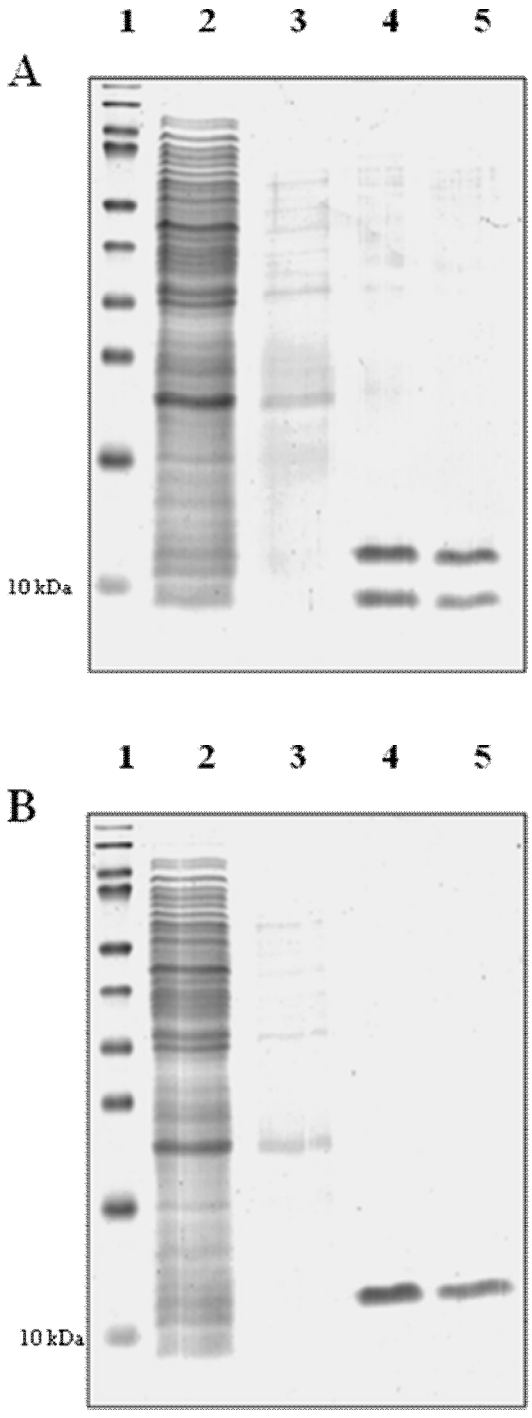


Fig: 38. SDS-PAGE analysis: A. Fractions from log phase S-100. B. Fractions from stationary phase S-100. Lane 1: Molecular weight marker, lane 2: logarithmic/stationary phase S-100, lane 3: Fraction number 3 and lanes 4-5: Fractions 24 and 25.

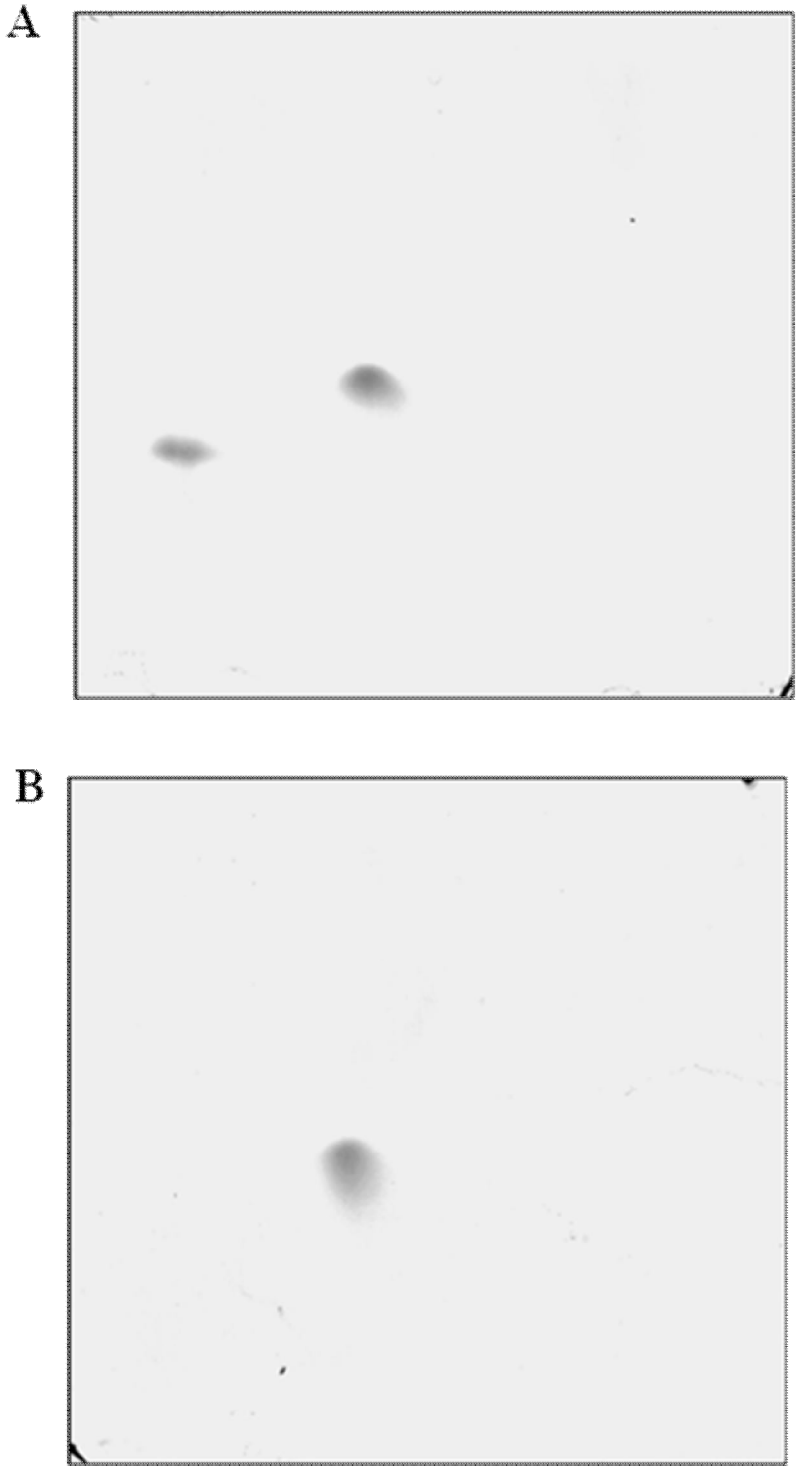
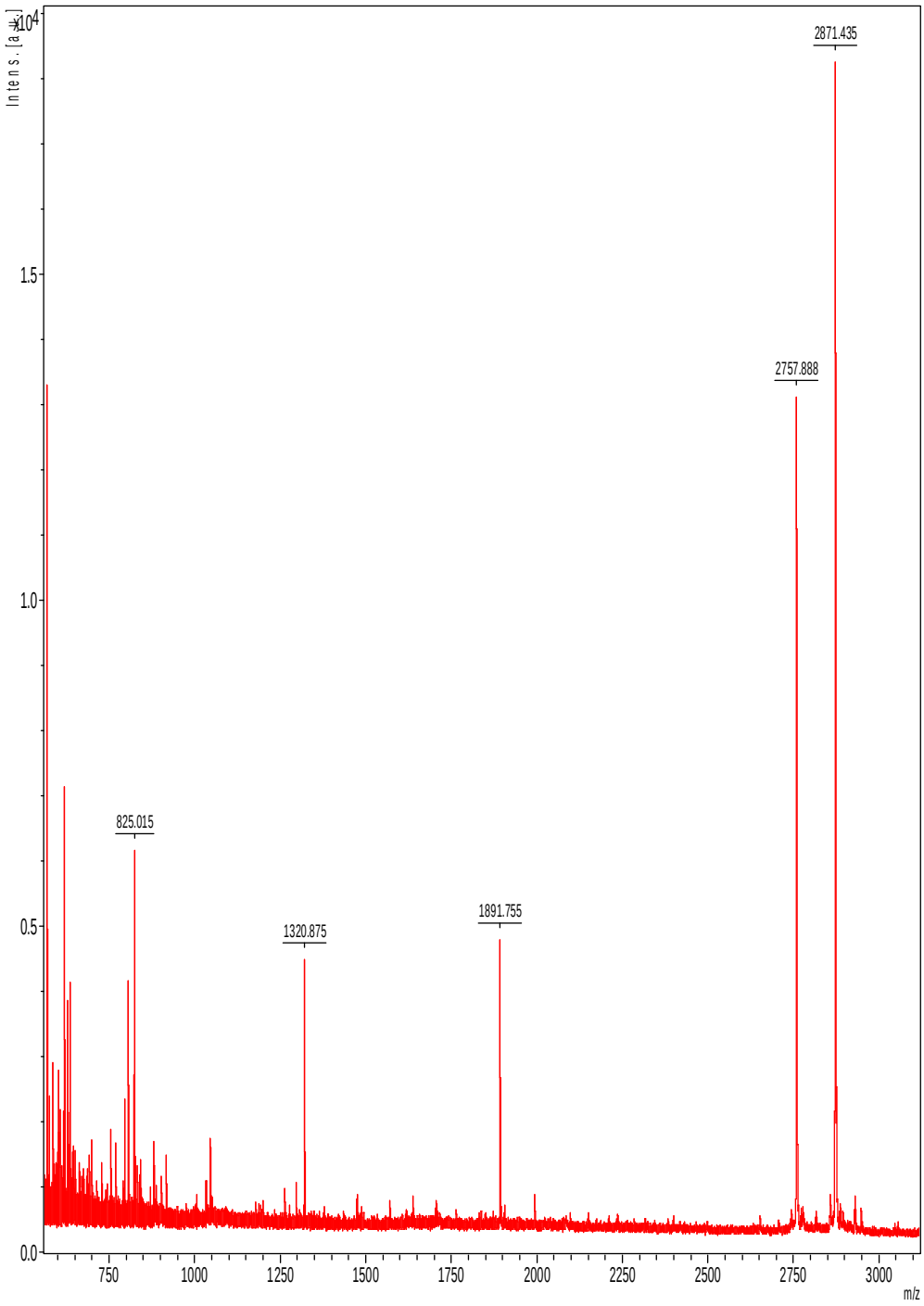
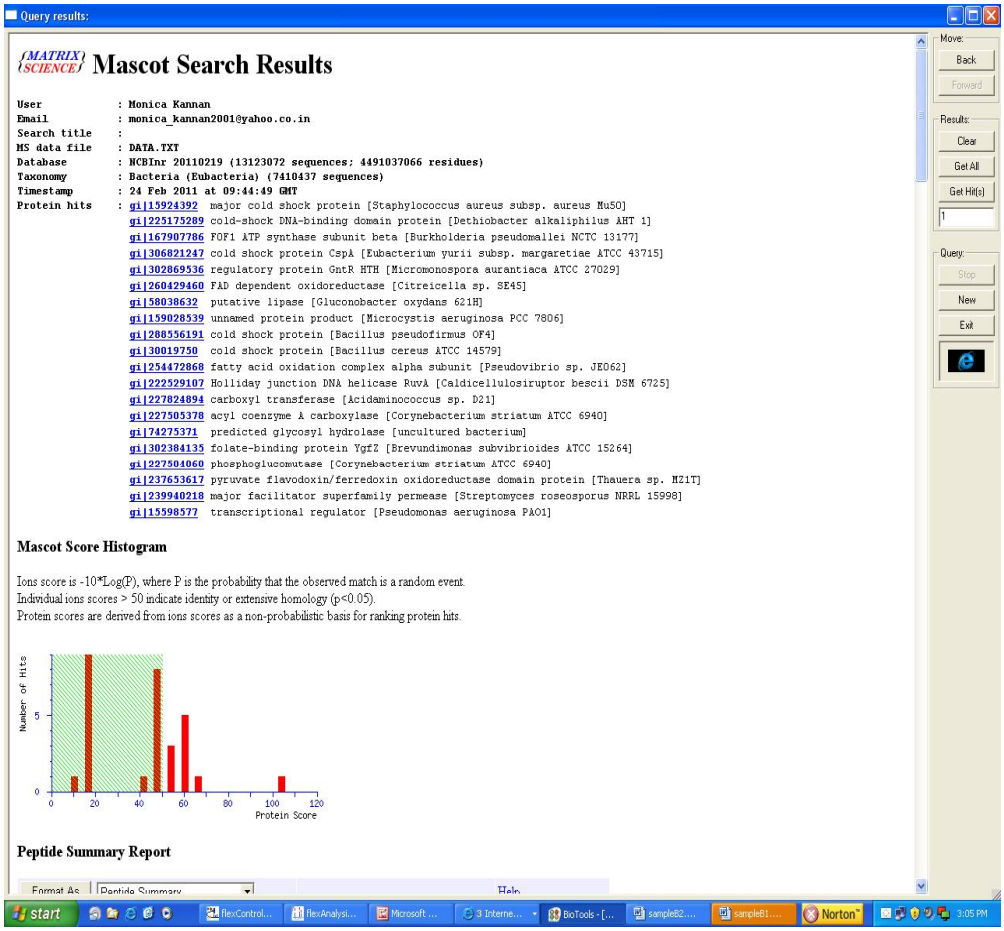


Fig: 39. Two-dimensional gel electrophoresis of poly (U)-binding proteins from S-100. A. Poly (U)-binding proteins from logarithmic phase S-100. B. Poly (U)-binding proteins from stationary phase S-100.

Fig: 40A. Ingel digestion: Peptide mass fingerprint





gi|15924392 **Mass:** 7317 **Score:** 104 **Matches:** 1(1) **Sequences:** 1(1)

major cold shock protein [Staphylococcus aureus subsp. aureus Mu50]

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2757.8882	2756.8809	2756.3617	188	1	104	2.3e-07	1	U	K.SLEEGQAVEFEVVEGDRGPQAANVVK.L

Proteins matching the same set of peptides:

gi|20302392 **Mass:** 7283 **Score:** 104 **Matches:** 1(1) **Sequences:** 1(1)

cold shock protein [Staphylococcus aureus]

gi|224476532 Mass: 7331 Score: 104 Matches: 1(1) Sequences: 1(1)

putative cold shock protein [Staphylococcus carnosus subsp. carnosus TM300]

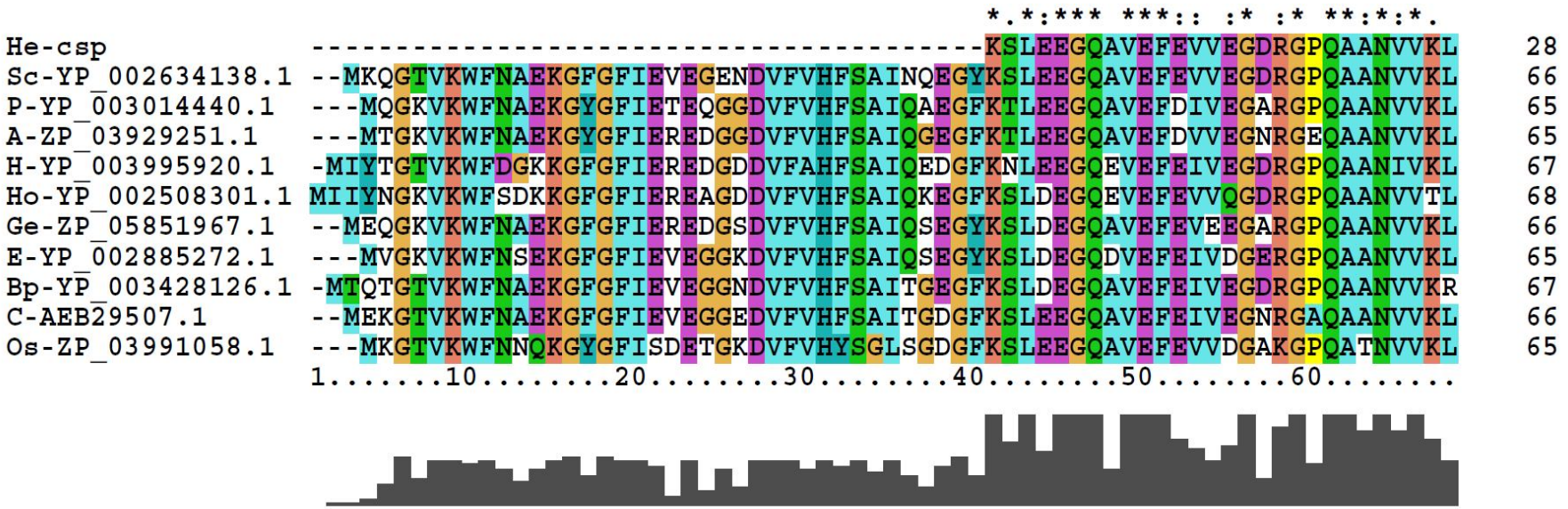
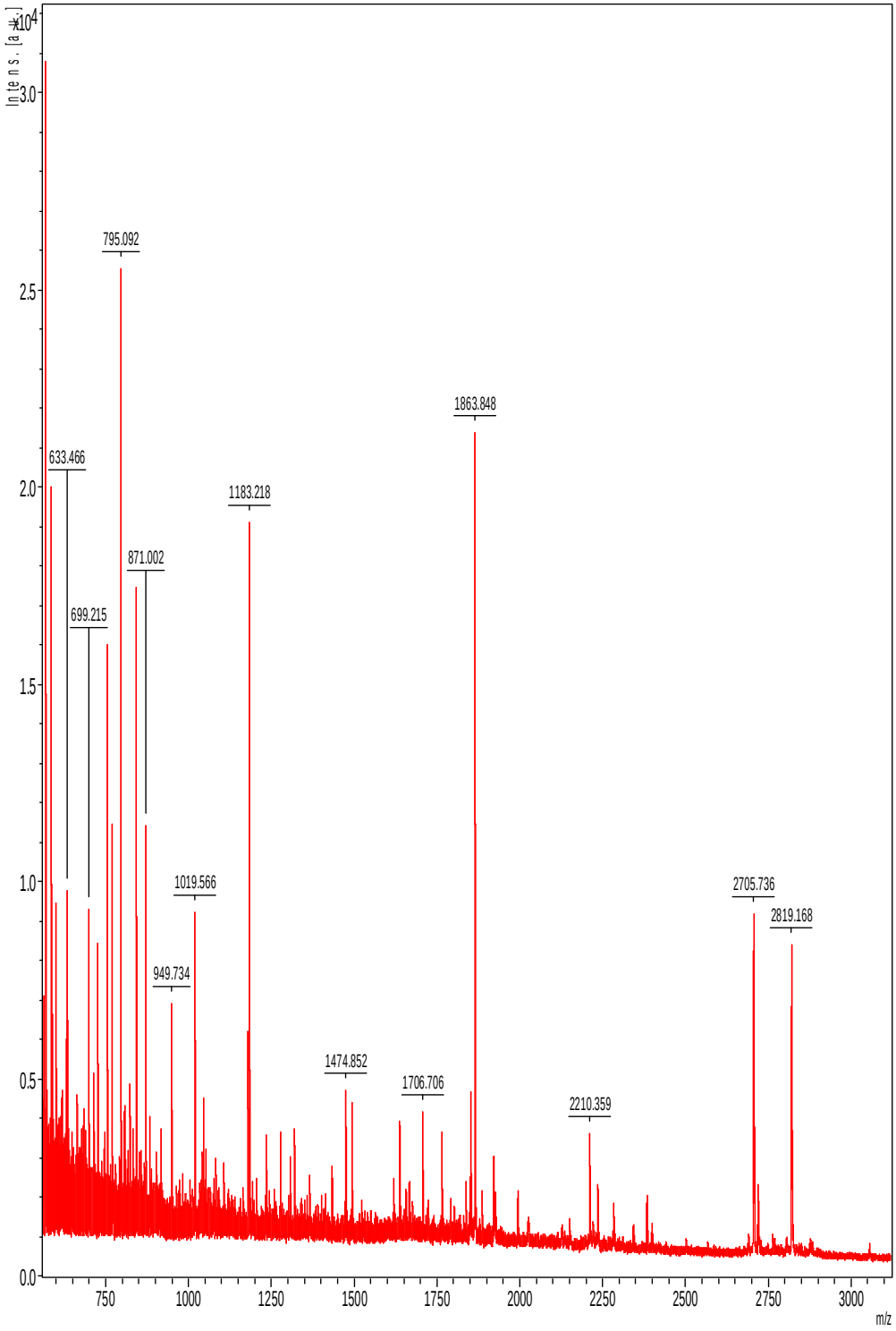
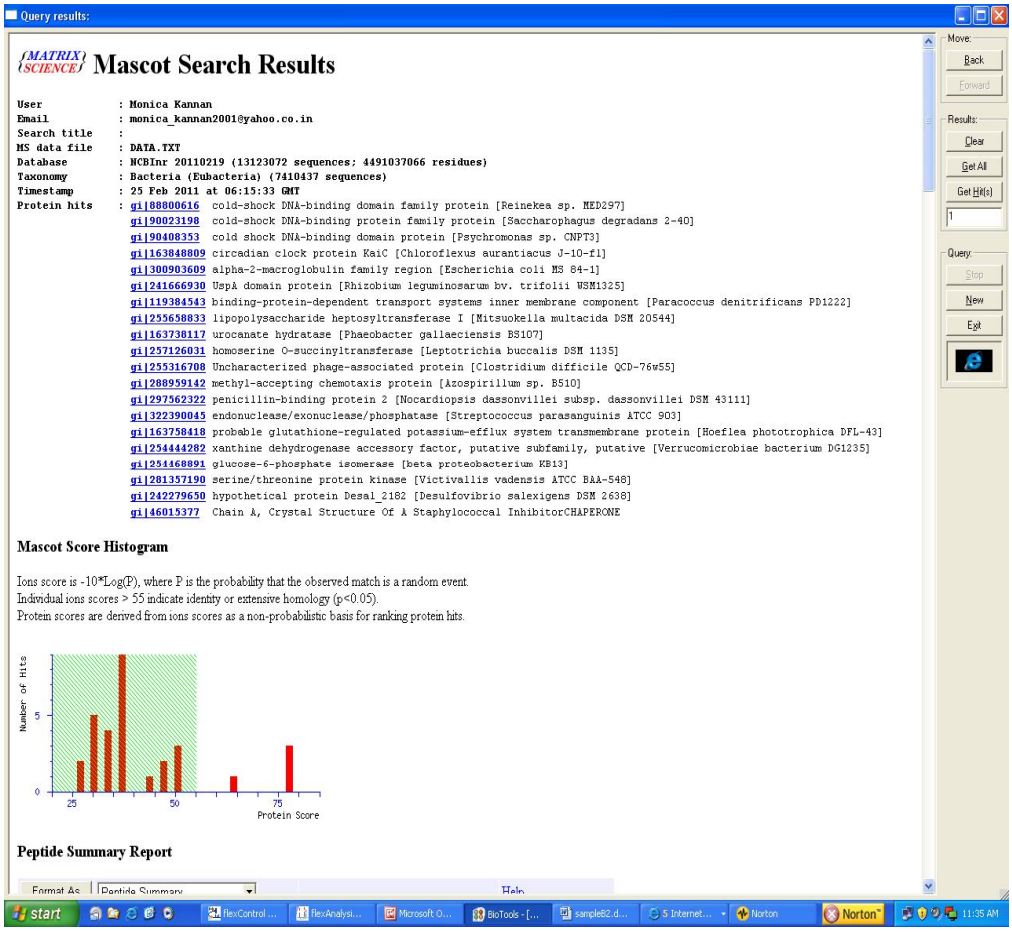


Fig: 40B . Multiple sequence alignment for peptide (m/z 2757.8882) of 12 kDa cold shock protein. He, *Halomonas eurihalina* DSM 5720; Sc, *Staphylococcus carnosus* TM300; P, *Paenibacillus* sp. JDR-2; A, *Acidaminococcus* sp. D21; H, *Halanaerobium* sp.; Ho, *Halothermothrix orenii* H 168; Ge, *Granulicatella elegans* ATCC 700633; E, *Exiguobacterium* sp. AT1b; Bp, *Bacillus pseudofirmus* OF4; C,*Carnobacterium* sp. 17-4; Os, *Oribacterium sinus* F0268.

Fig: 41A. Ingel digestion-Peptide mass fingerprint:





gi|88800616 **Mass:** 7275 **Score:** 78 **Matches:** 1(1) **Sequences:** 1(1)

cold-shock DNA-binding domain family protein [Reinekea sp. MED297]

Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1863.8481	1862.8408	1862.9738	-71.38	1	78	0.00031	1	U	K.TLAEGQKVEFTVTQGQK.G

Proteins matching the same set of peptides:

gi|90408760 **Mass:** 7359 **Score:** 78 **Matches:** 1(1) **Sequences:** 1(1)

Cold shock protein [Psychromonas sp. CNPT3]

gi|109897864 Mass: 7404 Score: 78 Matches: 1(1) Sequences: 1(1)

cold-shock DNA-binding domain-containing protein [Pseudoalteromonas atlantica T6c]

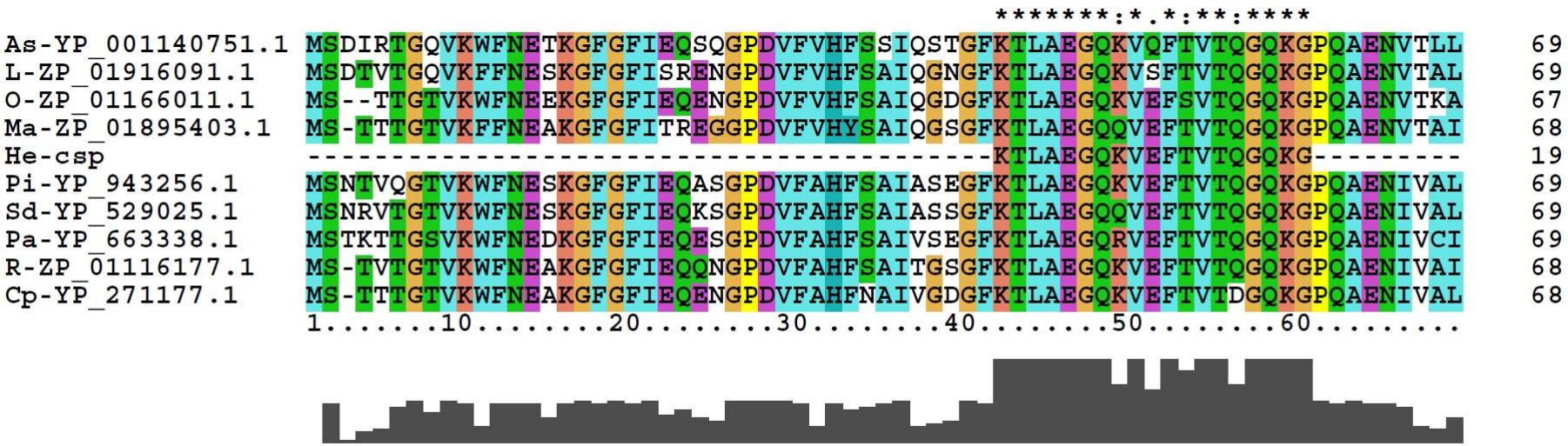


Fig: 41B. Multiple sequence alignment for peptide (m/z 1863.8481) of 8 kDa cold shock protein. As, *Aeromonas salmonicida*; L, *Limnobacter* sp. MED105; O, *Oceanospirillum* sp. MED92; Ma, *Marinobacter algicola* DG893; He, *Halomonas eurihalina* DSM 5720; Pi, *Psychromonas ingrahamii* 37; Sd, *Saccharophagus degradans* 2-40; Pa, *Pseudoalteromonas atlantica* T6c; Cp, *Colwellia psychrerythraea* 34H; R, *Reinekea* sp. MED297;

DISCUSSION

In the present study, work was carried out on the characterization of moderately halophilic ribosomes, partial purification and identification of poly (U) binding proteins: S1 from ribosomes and proteins from post ribosomal supernatant of *Halomonas eurihalina* DSM 5720.

H. eurihalina ribosomes were chosen to study the structural organization of moderately halophilic ribosomes as *H. eurihalina*, exhibits strong euryhaline nature, imparting on the cells the ability to grow in a wide range of salt concentrations. Thus, allowing us to study the variations in ribosomes, if any, in different salt concentrations.

The number of proteins in *H. eurihalina* ribosomes is more compared to mesophilic *E. coli* ribosomes. Two-dimensional electrophoretic pattern of moderately halophilic ribosomes indicates similarity to *E. coli* ribosomes, in terms of nature. Unlike extremely halophilic archaea, which contain more number of acidic ribosomal proteins, moderately halophilic bacteria contain more number of basic proteins like other eubacteria (Falkenberg, 1976).

Comparison of *H. eurihalina* logarithmic phase ribosomal two-dimensional pattern with stationary phase ribosomal pattern revealed the variation in the protein profile. Concentration of protein was less for a number of ribosomal proteins from stationary phase ribosomes. Growth cycle coupled variation in ribosomal protein profile was reported in *E. coli* by Wada *et al* in 2000 (Wada *et al* 2000).

Agarose gel electrophoretic pattern of *H. eurihalina* rRNA shows that the rRNA species are similar to *E. coli* rRNA, in terms of migration. Halophilic rRNA from logarithmic and stationary phase did not show any variation.

Growth curves of *H. eurihalina* in varying salt (NaCl) concentrations shows that the growth rate of the bacteria is not affected up to 10% NaCl concentration. The growth rate is slowed down in media containing 15% NaCl and a prolonged lag phase is observed in media containing 20% NaCl. These results show that the bacteria can withstand high concentrations of salt up to 20%. Once the lag period was over cells were able to grow at the same rate as the cells growing in regular NaCl concentration.

Comparison of two-dimensional electrophoretic pattern of ribosomal proteins isolated from bacteria grown under extremely halophilic conditions (20% NaCl containing media) with those from bacteria grown under moderate halophilic conditions showed that the ribosomal protein profile is similar (basic nature) under both the conditions. These results suggest that there is no variation in the charge contributed by post translational modification.

Sucrose density gradient studies of halophilic ribosomes on 5-20% gradients indicate that *H. eurihalina* logarithmic phase ribosomes are compactly organized in comparison *E. coli* ribosomes, where as stationary phase ribosomes showed density similar to *E. coli* ribosomes. This may be explained by relating to the rRNA and protein profiles of halophilic ribosomes. rRNA of the both species showed similar electrophoretic mobility, whereas halophilic ribosomes showed more number of proteins compared to *E. coli* ribosomes. Thus resulting in compact organization of *H. eurihalina* ribosomes. In stationary phase ribosomes some of the proteins were found to be missing and thus less compact, compared to logarithmic phase ribosomes.

Studies on 10-30% sucrose density gradients revealed that halophilic ribosomes continued to be in associated form even under low magnesium ion concentration such as 0.2 mM. Studies were also carried out in presence of different salts to dissociate subunits. The results show a significant peak shift in presence of salts such as potassium glutamate or sodium chloride, indicating the stripping off of some ribosomal proteins from ribosomes as revealed by

SDS-PAGE analysis. Even, in the presence of high salt (500 mM) and low magnesium ion concentration (0.2 mM), ribosomes continued to maintain their undissociated form, indicating the absence of magnesium dependent dissociation of subunits in *H. eurihalina* ribosomes. These results also indicate that the ribosomal subunits are tightly coupled. Conditions which favour dissociation of ribosomes from other species (*E. coli*, eukaryotes) resulted in unfolding of the ribosomal particles.

Further, we carried out sucrose density gradient studies on S-30 of *E. coli* and *H. eurihalina* for the presence of native ribosomal subunits. To our surprise, the results showed the absence of native ribosomal subunits in *H. eurihalina* logarithmic and stationary S-30, hinting at the probable absence of subunit dissociation or the requirement of a strong ribosome dissociation factor activity (IF-3 like) for the formation of 30 S and 50 S subunits in *H. eurihalina*.

Hydrophobic interaction chromatography, which includes treatment of ribosomes with high salt (1.5 M) and low magnesium (0.2 mM) concentration, was also tried. Krillov (1978) demonstrated the isolation of subunits from *E. coli* by hydrophobic interaction chromatography. *H. eurihalina* logarithmic and stationary phase ribosomes remained in their undissociated monomer form even with hydrophobic interaction chromatography supporting the results obtained by sucrose density gradient studies and gel exclusion chromatography.

We have analyzed the effect of magnesium ion concentration on the subunit dissociation of *H. eurihalina* ribosomes by light scattering studies. The amount of light scattered by ribosomal subunits will be less than the amount of light scattered by associated ribosomes. In presence of low magnesium ion concentration viz., 1 mM also there is no decrease in the amount of light scattered by ribosomes of *H. eurihalina*. Under the same conditions *E. coli* ribosomes showed dissociation as indicated by decreased light scattering with lower magnesium ion concentration. These studies show that magnesium ion

concentration dependent dissociation of ribosomes is absent in *H. eurihalina*. These results support the results obtained by sucrose density gradient studies and hydrophobic interaction chromatographic studies that the ribosomes from *H. eurihalina* are composed of tightly coupled 30 S & 50 S subunits.

Structural organization of moderately halophilic ribosomes was studied based on the changes in the conformation and stability of ribosomes up on treatment with different salts and reagents as a function of magnesium ion concentration in the presence of pancreatic RNase A. These studies might provide valuable information regarding interactions or mechanisms involved in the stabilization of protein-nucleic acid complexes at high salt concentrations. Pancreatic RNase A was used as the enzymatic activity is unaffected, under the conditions used (varying salt concentrations).

The conformation of ribosomes depends on many factors such as primary and secondary structures of the ribosomal RNA, its interaction with ribosomal proteins, free electrostatic energy of different rRNA regions and finally the topologarithmicy of ribosomal particles.

The changes in conformation of ribosomes were detected by the increase or decrease in the susceptibility of treated ribosomes to RNase A or by variation in the amount of RNase A required to reach maximum level of degradation and their resistance to unfolding treatments. The results were analyzed by comparing with that of similarly treated mesophilic *E. coli* ribosomes.

Magnesium ions play a major role in the stability of ribosomes. Magnesium interacts with the nucleic acids in two distinct ways i.e. diffuse binding and site binding (Misra and Draper, 1998). In diffuse binding, fully hydrated divalent ions interact with the polynucleotide via nonspecific long-range electrostatic interactions. In site bound magnesium is found within a deep electronegative pocket formed by inter helical cleft where the sugar-

phosphate backbone is buried within the RNA (Conn *et al* 2002). Diffusely bound magnesium ions strongly promote RNA folding by preferentially interacting with regions of high electronegative electrostatic potential created by tertiary structure formation. Binding of magnesium to sites with very high electrostatic potential can also favour the tertiary structure folding (Misra and Draper, 2002).

RNase A susceptibility assays were done at different magnesium concentrations as large difference in RNA stability could be used to distinguish the change in the conformation of ribosomes.

The susceptibility of ribosomes at lower magnesium concentrations (0.1 mM) was more compared to that at high magnesium concentration (1 mM) at room temperature. Lowering of magnesium concentration results in unfolding of the ribosome structure (Gesteland, 1966), which makes the ribosome more susceptible to RNase A due to exposure of single stranded rRNA regions.

RNase A sensitivity assays at different magnesium concentrations indicates that the rate of degradation of *H. eurihalina* ribosomes in the presence of RNase A was significantly less compared to that of *E. coli* ribosomes. Degradation of both logarithmic and stationary phase ribosomes of *H. eurihalina* in $TNM_{0.1}$Me was less and required many fold concentration of RNase A to reach the same amount of degradation compared to *E. coli* ribosomes.

H. eurihalina ribosomes are less susceptible to RNase A compared to their mesophilic counterparts. The less susceptibility of *H. eurihalina* ribosomes indicate that they may be stabilized by interactions other than those formed by interaction with magnesium. The rate of degradation of *H. eurihalina* ribosomes is less, due to compact structural organization of ribosomes. These results show that halophilic ribosomes maintain their

structural integrity with variety of non covalent interactions with strong hydrophobic component.

The amount of enzyme required by the *H. eurihalina* logarithmic phase ribosomes to obtain 20% degradation was 26 times more compared to that required by the *E. coli* ribosomes to obtain 30% degradation. The amount of enzyme required by *H. eurihalina* stationary phase ribosomes to obtain 25% degradation was 10 times more compared to that required by the *E. coli* ribosomes to obtain 30% degradation.

In the case of *E. coli*, degradation does not reach an absolute saturation state and residual rate of break down is observed due to the degradation induced unfolding, which makes the ribosomes more susceptible to RNase A. Whereas, in the case of *H. eurihalina*, degradation has reached an absolute saturation state indicating that degradation induced unfolding is absent in the case of both logarithmic and stationary phase ribosomes. These observations indicate that *H. eurihalina* ribosomes are very compact and the RNA available for RNase A digestion is provided only by low magnesium ion concentration induced unfolding.

In comparison with 30% degradation of *E. coli* ribosomes, even after addition of 26 fold and 10 fold more RNase A the maximum amount of degradation reached by *H. eurihalina* logarithmic and stationary phase ribosomes is only 20% and 25% respectively, indicating the inherent resistance of *H. eurihalina* ribosomes to RNase A treatment. It also shows that the *H. eurihalina* logarithmic phase ribosomes are considerably more resistant to RNase A treatment compared to stationary phase ribosomes indicating significantly strong and compact structural organization of ribosomes isolated from exponential phase cells and that the stationary phase ribosomes are more unfolded. These results are in conformity with sucrose density gradient sedimentation of stationary phase ribosomes. This difference may also be

contributed by the absence of primary rRNA binding protein (L2) in stationary phase ribosomes.

Low sensitivity of *H. eurihalina* ribosomes to RNase A could be due to more extensive interactions that exist between the functional groups of ribosomal proteins and rRNA domains than that of the mesophilic ribosomes, which lead to the formation of more compact structural organization. Presence of more number of ribosomal proteins or high protein to RNA ratio in halophilic ribosomes may also help in stronger protein-rRNA interactions.

Studies on the effect of different salts and their concentrations on RNase A sensitivity of *H. eurihalina* ribosomes shows the protective effect of different salts at 250 mM concentration with an exception of sodium chloride, which does not exhibit any protective effect at any concentration. These studies also show that at 250 mM concentration, potassium chloride is exhibiting highest stabilizing activity closely followed by ammonium chloride. Potassium glutamate also exhibited some extent of protection against RNase A activity. Sodium chloride, the salt which is vital for the growth of the bacteria is the only salt among the group of salts tested, which does not exhibit any protective activity.

RNase A sensitivity assays with polyamine treated ribosomes indicated the higher stability of halophilic ribosomes in the presence of spermine or spermidine compared to mesophilic ribosomes.

RNase A sensitivity assays showed that *H. eurihalina* ribosomes treated with EtBr are less sensitive to RNase A compared to their mesophilic counterparts. Both logarithmic and stationary phase ribosomes showed similar resistance to EtBr. This may be due to fewer distortions produced by the EtBr in case of *H. eurihalina* ribosomes compared to mesophilic ribosomes. *H. eurihalina* rRNA in ribosomes may not be easily available for EtBr intercalation, which may be due to the formation of an internal compact and

poorly hydrated core created mainly by the hydrophobic protein-protein interactions.

Resistance of halophilic ribosomes to unfolding reagents like urea was studied. Urea treatment unfolds the ribosome resulting in removal of some of the ribosomal proteins. This unfolding can be detected by the increase in the absorbance of the solution (Langer *et al* 1975). Urea was found to influence the H-bonding (Petermann *et al* 1972).

A large increase in the absorption at 260 nm is observed up on addition of urea. Broadening of the spectrum in all regions was observed from 220 nm to 300 nm with increasing concentrations of urea in case of *E. coli* ribosomes suggesting urea induced unfolding of the ribosome. In case of *H. eurihalina* logarithmic phase ribosomes the increase was very less where as stationary phase ribosomes showed pattern similar to *E. coli* ribosomes. These results show that the logarithmic phase ribosomes are organized in such a way that makes them resistant to unfolding treatments i.e., with urea. On the other hand stationary phase ribosomes are unfolded by urea. This could be because of the already partially unfolded nature of stationary phase ribosomes.

Greater resistance showed by the halophilic ribosomes to urea also indicates that the component rRNA and protein molecules are held together by strong cooperative interactions. Probably hydrophobic bonds in protein-protein contact surfaces could play an essential role in conferring the high degree of stability to the halophilic ribosomes. Thus, results of sedimentation profile, RNase A sensitivity studies, ethidium bromide intercalation, urea unfolding suggest that *H. eurihalina* logarithmic phase ribosomes are more compact than stationary phase ribosomes and *E. coli* ribosomes.

65 spots were observed consistently in logarithmic phase gels, indicating the possibility of presence of more number of ribosomal proteins in *H. eurihalina*. The presence of more number of proteins may be one of the

reasons for the stability of the halophilic ribosomes. These proteins might be organized in such a way, which makes the rRNA unavailable to degradation by RNase A and thus making them more resistant to RNase A treatment. In stationary phase ribosomes, some of the proteins were found to be missing and many of them showed decrease in the concentration. Increased RNase A susceptibility of stationary phase ribosomes in comparison to logarithmic phase ribosomes also supports this view. We also found that some of the proteins (eg: L1, L27, S7, S8 and S15) existed in two isoforms or truncated forms and showed homology to the same protein.

The decrease in several of the ribosomal proteins in stationary phase can be explained by either low concentration or their binding to ribosomes could be affected by missing of some of the primary rRNA binding proteins (eg: L2, L17, S7, S15 etc.).

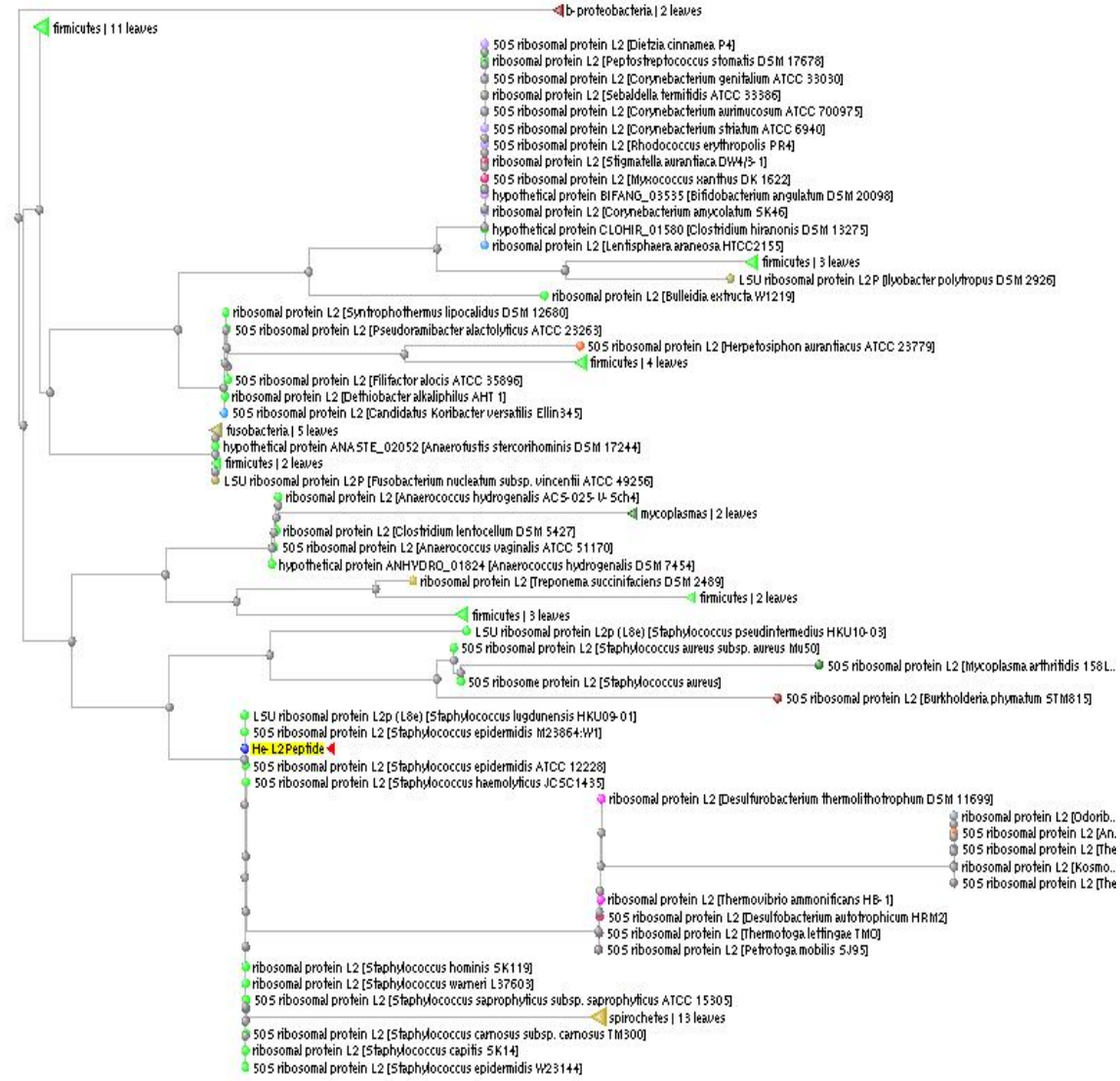
MALDI-TOF MS analyses of the ribosomal proteins showed that majority of the identified *H. eurihalina* ribosomal proteins are homologous to eubacteria. None of them were found to be homologous to ribosomal proteins of extremely halophilic archaea. Only one protein (S3) was found to be homologous to S3 from *Euryarchaeota*. S3 forms complex with S10 and S14 and binds to the lower part of 30 S subunit. S3 is one of the ribosomal protein which is highly conserved in eubacteria, archaea and eukaryotes.

Multiple sequence alignments of the identified peptides with homologous ribosomal proteins from bacteria encompassing several genera, phyla and kingdoms showed that these peptides exhibited considerable homology to these proteins. Homology was found to both gram positive and gram negative eubacterial ribosomal proteins, although the results showed that several of the ribosomal proteins showed greater homology to ribosomal proteins of gram positive species (phyla *Firmicutes*). Interestingly, *H. eurihalina* is a gram negative bacterium classified under *Proteobcateria*, a class in which all of the bacteria are gram negative. As already mentioned

ribosomal protein S3 showed greater sequence homology to *Euryarchaeota* than to eubacteria. The present work is useful in studying phylogenetic relatedness of *Halomonas* with in prokaryotic kingdom and also across the kingdoms (prokaryotes, archaea and eukaryotes).

Phylogenetic analyses of L2 and S3 showed that *H. eurihalina* are more distantly related to *Proteobacteria* and closer to *Firmicutes* and *Rubrobacteria*. Phylogenetic trees of relationship for L2 peptide sequences were given in Fig: 42 & 43. However the peptide query (m/z 1619.75) shares significant homology to RNA binding domain in L2 that is conserved in eubacteria, archaea and eukaryotes (Nakagawa *et al* 1999). Phylogenetic tree of L2 peptide m/z 1733.60 showed that the peptide is highly conserved among different domains of life. Phylogenetic tree of S3 was given in Fig: 44 and the analysis showed that S3 is phylogenetically related to members of *Euryarchaeota*, *Crenorarchaeota* followed by members of *Firmicutes* and *Ascomycetes*.

It is worth mentioning about the study by Vishwanath *et al* (2004) who have analysed ribosomal protein sequences from bacteria, archaea and eukaryotes by bioinformatics approach. Their study shows that three types of ribosomal protein sequences blocks which are universal blocks (conserved in both phylodomains prokaryotes and eukaryotes), bacterial blocks (unalignable with any archaeal counterparts) and archaeal blocks (unalignable with bacterial counterparts). Their study points to a complex evolutionary nature of prokaryotes. Furthermore, Martini *et al* (2007) have used ribosomal protein gene based phylogeny for finer differentiation and classification of *Phytoplasmas*. Thus, 16 S rDNA based phylogeny can be refined by using ribosomal protein based phylogenetic studies.



Blast names color map
β-proteobacteria
firmicutes
high GC Gram+
fusobacteria
δ-proteobacteria
bacteria
GNS bacteria
mycoplasmas
spirochetes
unknown
aquificales
CFB group bacteria
thermotogales

Fig: 42. Phylogenetic tree of the relationship among L2 protein (peptide m/z 1619.75) from *H. eurihalina* and other organisms.

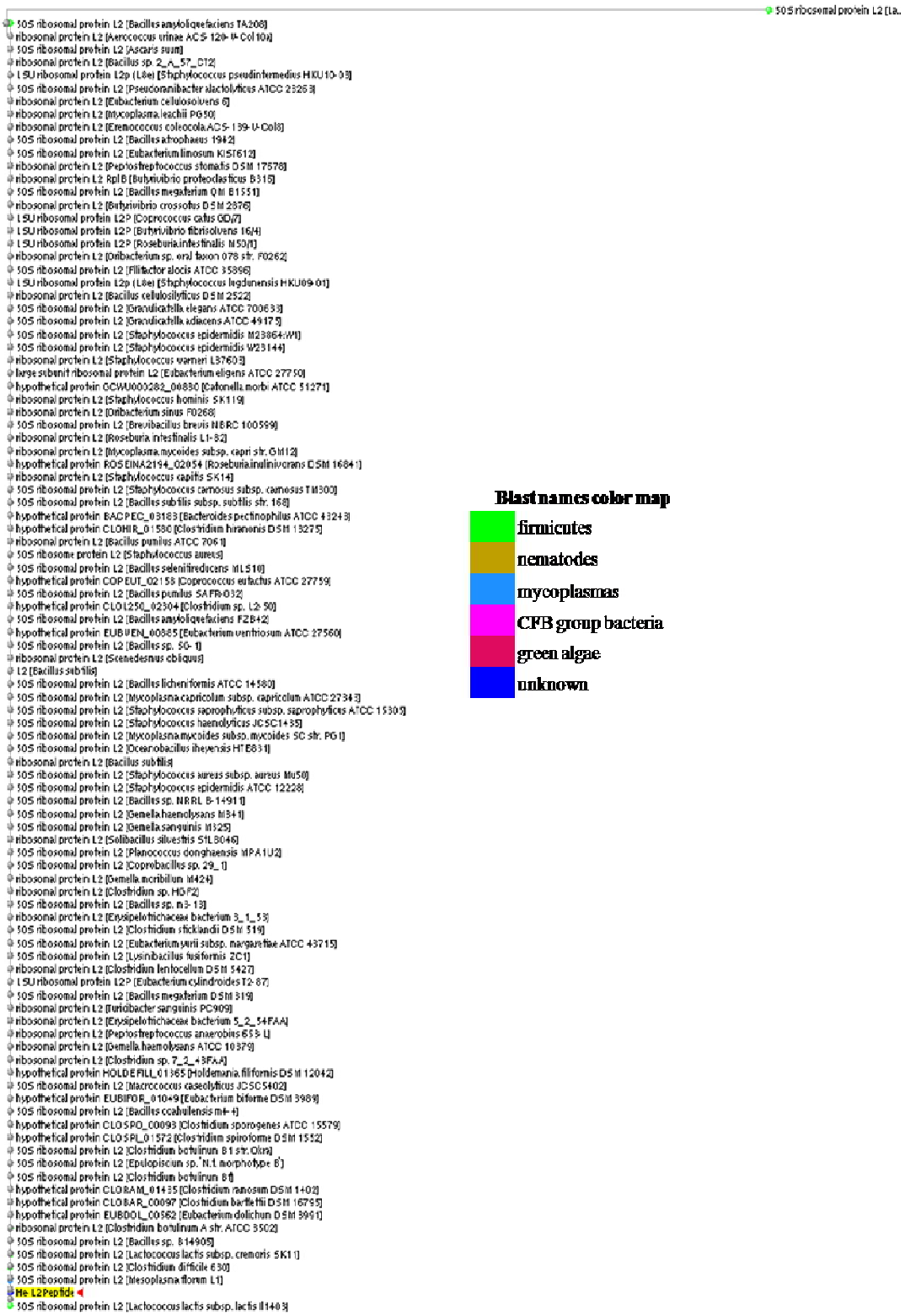
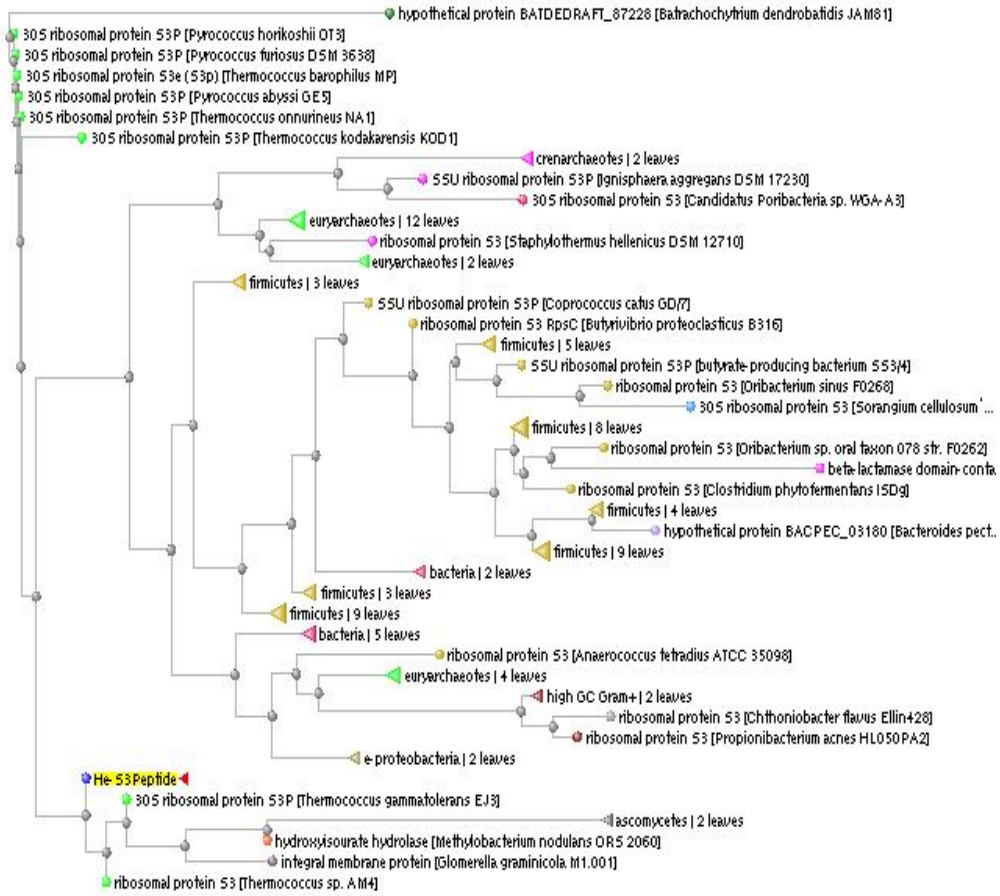


Fig: 43. Phylogenetice tree of the relationship among L2 protein (peptide m/z 1733.60) from *H. eurihalina* and other organisms.



Blast names color map
chytrids
euryarchaeotes
crenarchaeotes
bacteria
firmicutes
δ-proteobacteria
CFB group bacteria
high GC Gram+
verrucomicrobia
ε-proteobacteria
unknown
ascomycetes
α-proteobacteria

Fig: 44. Phylogenetic tree of relationship among S3 protein (peptide m/z 1733.13) from *H. eurihalina* and other organisms.

S1 protein was purified and identified from *H. eurihalina* and its molecular mass was found to be 61 kDa, similar to *E. coli* S1. In case of *E. coli*, S1 is loosely associated with ribosomes and is lost during ribosome preparation and purification. Thus majority of S1 is found in S-100 extract instead of ribosomes in *E. coli*. We tried to find whether in *H. eurihalina*, S1 is present in S-100. We observed that only negligible amount of S1 was found in S-100 in case of both logarithmic phase and stationary phase S-100 of *H. eurihalina*. These results showed that S1 association was not loose with *H. eurihalina* (logarithmic and stationary phases) ribosomes, as in the case of *E. coli*. During these studies we observed the presence of two low molecular weight (12 kDa, 8 kDa), strong poly (U) binding proteins in *H. eurihalina* S-100. These proteins were found to be cold shock proteins and 8 kDa protein was found to be missing in stationary phase.

Cold shock proteins are stress induced in mesophilic bacteria (*E. coli*, *B. subtilis*). However, presence of these proteins in abundance may indicate that these proteins may have a function in the organism growing under extreme salt concentrations. Presence of both the proteins in logarithmic phase S-100 may be of more advantage than the presence of single protein in stationary phase S-100.

SUMMARY

Thesis includes Introduction, Materials and methods, Results (Chapter 1, 2, 3 and 4), Discussion, Summary and References.

Introduction

This chapter deals with the introduction about halophiles, distribution of halophiles among universal phylogenetic tree based on rRNA sequence homologies, a brief account on *H. eurihalina* general properties, adaptation to halophilic environments and biomolecule profile of moderate halophiles. A detailed account on ribosomes, their structure, their halophilic counterparts along with their comparison to archaeal and eukaryotic ribosomes, detailed account of S1 protein, including its purification, physical properties, structure and its role were also given. Objectives and scope of the present investigation were also mentioned.

Materials and methods

This chapter includes materials and a detailed description of the methodologies used to carry out the present work. The methodologies described include growth of organism under moderate and extremely halophilic conditions, isolation of ribosomes, extraction of ribosomal proteins and rRNA, gel electrophoresis, two dimensional gel electrophoresis, sucrose density gradient studies, hydrophobic interaction chromatography, spectrophotometric measurement of the degradation of ribosomes in the presence of RNase A under variety of conditions, light scattering studies, MALDI-TOF MS identification of ribosomal proteins, computational analyses and poly (U) affinity chromatography of ribosomes and S-100.

Results

Chapter: 1. Growth phase and growth media dependent variation of ribosomes

Growth phase dependent variation of ribosomes

Growth phase dependent variation of ribosomes was studied based on the growth curve determination, isolation of ribosomes from logarithmic and stationary phase cultures, extraction of ribosomal proteins and rRNA, their analysis by SDS-PAGE and agarose gel electrophoresis. Logarithmic and stationary phase ribosomal proteins were analysed and compared in terms of two-dimensional gel electrophoresis. The two dimensional gel electrophoretic mobility pattern of moderately halophilic ribosomal proteins was also compared with two dimensional gel electrophoretic mobility of their non halophilic counterpart, *E. coli*.

Growth media dependent variation of ribosomes

Growth media dependent variation of ribosomes was studied based on the results obtained by determination of growth curves in the presence of varying concentrations of salt (NaCl) and isolation of ribosomes from bacteria grown under moderately halophilic and extremely halophilic conditions, and their analysis and comparison in terms of SDS-PAGE and two-dimensional gel electrophoresis.

Chapter: 2. Strucutral studies on ribosomes from logarithmic and stationary phase *H. eurihalina*

Structural studies of halophilic ribosomes were carried out using different methods of ribosomal subunit separation and studying the stability of ribosomes treated with intercalating agents, different salts, polyamines in

presence of RNase A and the stability of halophilic ribosomes was compared with that of mesophilic ribosomes. The structure of ribosomes was studied by their resistance to unfolding treatment i.e., with urea. Results were analyzed by comparing with that of the similarly treated mesophilic *E. coli* ribosomes. Light scattering studies were also performed to study the effect of magnesium ion concentration on ribosomes.

Magnesium ion dependent subunit dissociation

Absence of magnesium ion dependent ribosomal subunit dissociation was determined by sucrose density gradient centrifugation studies of ribosomes using different salts in the presence of low magnesium concentration, hydrophobic interaction chromatography and light scattering studies. Sucrose density gradient studies of S-30 were also performed. These studies showed the absence of magnesium dependent dissociation of ribosomal subunits and also the absence of native ribosomal subunits in *H. eurihalina*. These results establish that at low magnesium concentration also the ribosomes are maintaining their monomeric undissociated form.

RNase A degradation studies with treated and untreated ribosomes

Stabilities of mesophilic (*E. coli*) and halophilic (*H. eurihalina*) ribosomes treated with reagents like polyamines, different salts and intercalating agents were studied using RNase A as a probe.

The rate of degradation and percentage of degradation of *Halomonas eurihalina* ribosomes was less compared to *E. coli* ribosomes at different magnesium ion concentrations. However treatment of ribosomes with different salts showed that at optimum salt concentration, some salts exhibit protective effect on halophilic ribosomes against RNase A treatment. The results indicate the importance of salt concentration in stabilizing the halophilic ribosomal structure.

Ribosome unfolding studies with urea

Ribosome unfolding studies with urea treated ribosomes showed more unfolding in case of *E. coli* ribosomes than both logarithmic and stationary phase ribosomes of *H. eurihalina*. The unfolding of *H. eurihalina* logarithmic phase ribosomes was less compared to stationary phase ribosomes. These results indicate that *H. eurihalina* ribosomes are more resistant to urea treatment and the rRNA-protein interactions are stabilized by strong hydrophobic interactions in halophilic ribosomes compared to mesophilic ribosomes.

Chapter: 3. Proteomic studies of ribosomal proteins from *H. eurihalina*

Proteomic studies of ribosomal proteins were carried out to identify their identity. Numbering of ribosomal proteins was given based on their electrophoretic mobilities. 65 proteins were identified in logarithmic phase gels. Some proteins were found to be missing in stationary phase gels (eg: L2, L19 and S12); where as some proteins showed decrease and some other proteins were found to be increased in stationary phase gels in comparison to logarithmic phase gels. Homologues of 26 bacterial ribosomal proteins were identified in *H. eurihalina* ribosomal proteins. Some proteins like L1, L27, S7, S8 and S15 were found in two isoforms or truncated forms and both of them showed homology to same protein. Even though good spectra were obtained, some of the proteins do not show homology to any protein indicating at the possible presence of proteins unique to *H. eurihalina*.

Many of the identified *H. eurihalina* ribosomal proteins were found to be homologous to ribosomal proteins from *Firmicutes*, *Proteobacteria* and *Actinobacteria*. One protein each was found to be homologous to ribosomal proteins from *Euryarchaeota* (S3) and *Cyanobacteria* (S7). These observations showed that ribosomal proteins of moderate halophile are homologous to ribosomal proteins of bacteria distributed among different phyla and kingdoms.

These results also show that none of the ribosomal proteins were homologous to ribosomal proteins from extremely halophilic archaea.

Multiple sequence alignments were carried out for the peptide sequence identified by MS/MS analyses and significant homology was observed for these peptides with ribosomal proteins of other bacteria.

Chapter: 4. Partial purification and identification of S1 from ribosomes and low molecular weight, strong poly (U)-binding proteins from S-100 of *H. eurihalina*

Purification and identification of S1 protein from ribosomes

Poly (U)-sepharose column chromatography was used to specifically isolate poly (U)-binding proteins from ribosomes. The purified protein was identified as S1 by MALDI-TOF MS. The results showed that *H. eurihalina* ribosomes contain S1 and its molecular weight was estimated to be 61 kDa, similar to its mesophilic counterpart, *E. coli*. The results also show that S1 associates tightly with *H. eurihalina* ribosomes as, S1 was found only in negligible amounts in S-100.

Purification and identification of poly (U)-binding proteins from S-100

Poly (U)-cellulose chromatography was used to isolate poly (U)-binding proteins from S-100. The results showed that *H. eurihalina* S-100 contains two low molecular weight, very strong poly (U) binding proteins. The molecular weight of the proteins was estimated to be around 12 kDa and 8 kDa. Both proteins were observed in logarithmic phase S-100, whereas 8 kDa protein was absent in stationary phase S-100. Both the proteins were identified to be homologous to cold shock proteins.

Discussion

Two dimensional gel electrophoretic studies of *H. eurihalina* ribosomes showed that the ribosomal proteins of moderate halophiles are basic in nature, unlike their extremely halophilic archaeal counterparts. Two- dimensional gel electrophoresis studies also revealed that ribosomal proteins isolated from moderately halophilic bacteria grown under extremely halophilic conditions were similar to ribosomal proteins isolated from moderately halophilic bacteria grown under moderately halophilic conditions.

Sucrose density gradient studies showed that logarithmic phase ribosomes of *H. eurihalina* are denser compared to *E. coli* ribosomes. In spite of using different methods to separate ribosomal subunits, none of them resulted in separation of subunits, indicating the compact nature of *H. eurihalina* ribosomes. The studies also indicated the absence of native ribosomal subunits in *H. eurihalina*.

RNase A degradation studies with ribosomes at different magnesium concentrations indicated that *Halomonas* ribosomes are stabilized by strong RNA-protein interactions compared to mesophilic ribosomes and also that logarithmic phase ribosomes showed more resistance to RNase A than stationary phase ribosomes. In presence of optimum salt concentration of salts like potassium chloride, ammonium chloride moderately halophilic ribosomes showed more resistance to RNase A activity.

Many of the ribosomal proteins were identified by MALDI-TOF MS and majority of them showed extensive homology to ribosomal proteins of other bacteria (both gram positive and gram negative). S3 showed homology to *Euryarchaeota* members, but none of the ribosomal proteins identified, showed homology to ribosomal proteins of extreme halophiles. L2 and S12 were identified to be missing in stationary phase ribosomes, along with many ribosomal proteins showing growth cycle coupled variation. Multiple sequence

alignments carried out with ribosomal proteins of other organisms showed extensive homology and highly conserved nature of the identified peptides.

Poly (U)-binding protein, partially purified from ribosomes was identified as S1. Two poly (U)-binding proteins were partially purified from S-100 and both of them were identified as cold shock proteins. Among them, 8 kDa protein showed growth cycle coupled variation. Multiple sequence alignments showed that the identified peptides were highly conserved among other organisms.

Summary

It includes the summary of each chapter.

References

This chapter includes list of references cited in different chapters in an alphabetical orders.

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