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Proteomics of *E.coli Nissle 1917* in Responce to *Cocos nucifera* sap and Wine

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Proteomics of *E.coli Nissle 1917* in responce to *Cocos nucifera* sap and Wine

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ABSTRACT

In the present study, we described the protein profile experimentally by 2D-PAGE and MALDI analysis to understand the stress mechanisms of cocoti sap and wine on *E.coli* Nissle 1917. We isolated one newly expressed protein from cocoti wine treated gel which is not present in both control and cocoti sap treated sample i.e. P21 prophage-derived head-stabilizing proteinVG03_ECOL6 (3n1) also called as Head protein gp3. This protein mainly activities related to the viral life cycle. It helps to attach the viral gene into host. The growth rate was delayed in cocoti wine treated *E.coli* Nissle *1917* when compared to control and cocoti sap treated samples. Stress mechanism induce many proteins they are involved in metabolic process, hydrolase activity, lyase activity, quinone binding, phosphotransferase system, carbohydrate metabolism, DNA binding, DNA repair, transferase activity, oxidoreductase, purine metabolism, transcription anti-termination, transcription regulation and other related activities.

We proved that the predicted protein structure quality, resolution, density and error plot values by QMEAN analysis. Based on these results, only two differentially expressed proteins under sap stress showed that the significant results, which were N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1, PTPB1_ECOLI and DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57. In case of wine stress, the differentially expressed proteins were Transcription anti-termination protein RFAH- ECO57 NusA and PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase showed significant results. ProtParam analysis indicating that the multiple physico-chemical characters of differentially expressed proteins were differed and compared. The phylogenetic tree represents the relationship in-between the differentially expressed proteins, were showed siblings (related) as well as monophytic clade.

Reviewer

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PREFACE

Probiotics are viable microorganisms that are increasingly used for treatment of a variety of diseases. Probiotics are used instead of antibiotics. Antibiotics works against both pathogenic and non-pathogenic organisms. Pathogenic organisms can develop resistance against antibiotics. The bacterial stress response enables bacteria to survive adverse and fluctuating conditions in their immediate surroundings. Various bacterial mechanisms recognise different environmental changes and mount an appropriate response. A bacterial cell can react simultaneously to a wide variety of stresses and the various stress response systems interact with each other by a complex of global regulatory networks.

Alcoholism is the major problem in rural areas, people prefer palm wine to drink because it is low cost and easily available in rural areas. Due to lack of sanitation conditions it is easily contaminated by the microorganisms because it is a natural media for microorganisms while the people suffer when consumed over fermented palm wine, sometimes it may leads to death.

The present theme of the research is concentrated on proteomics of probiotics and its resistance against cocoti sap and wine. By the utilisation of “omics” technology viz, homology modelling, Ramachandran’s plot analysis, the ProtParam and phylogenetic analysis of the differentially expressed proteins were compared under cocoti sap and wine stress.

This concept provides the rationale for selective therapeutic manipulation of the abnormal microbiota by probiotics for the intestinal diseases. *Escherichia coli* Nissle 1917 have demonstrated the capacity of probiotics to reduce intestinal inflammation.

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(K. Chandrasekhar)

ABBREVIATIONS

S.No	Short cut	Expansion
1	2-DE	2-D electrophoresis
2	3-D	3-Dimentional
3	HPA	3-hydroxy picolinic acid
4	AQUA	Absolute Quantification of Proteins
5	ATP	Adenosine Tri Phosphate
6	APS	Ammonium persulfate
7	ANOVA	Analysis of Variance
8	BLAST	Basic Local Alignment Search Tool
9	BCA	Bicinchoninic acid
10	CO_2	Carbon dioxide
11	DNA	Deoxy ribonucleic acid
12	DTT	Dithiothreitol
13	ESI	Electro Spray Ionization
14	ESI-Q-IT-MS	Electrospray Ionization Quadrupole Ion Trap- Mass Spectrometry
15	*E. coli*	*Eschericia coli*
16	EcN	*Eschericia coli* Nissle 1917
17	FAS	Fetal Alcohol Syndrome
18	FT-ICR	Fourier Transform Ion Cyclotron Resonance
19	FOS	*Fructooligosaccharides*
20	GI	Gastro Intestinal Tract
21	GRAS	Generally Regarded as Safe
22	GRAVY	Grand Average Hydropathicity
23	HK	Histidine Kinases
24	IR	Infrared radiation
25	IEF	Iso Electric Focusing
26	pI	Iso Electric Point
27	ICAT	Isotope Coded Affinity Tags
28	LAB	Lactic acid Bacteria
29	LC-MS/MS	Liquid chromatography Mass/ Mass-spectrometry
30	MS	Mass Spectrometry
31	MALDI-TOF	Matrix Assisted Laser Desorption/Ionization- Time of Flight
32	MIC	Minimum Inhibitory Concentration
33	MOWSE	Molecular Weight Search
34	NCBI	National Center for biotechnological Information
35	NTU	Nephelo Turbidity Units
36	NMR	Nuclear Magnetic Resonance
37	NAM	Nutrient Agar Medium
38	O.D	Optical Density
39	PMF	Peptide Mass Fingerprinting
40	PA	Picolinic acid
41	PAGE	Poly Acrylamide Gel Electrophoresis
42	PSD	Post-Source Decay
43	PTM	Post-Translational Modifications
44	PDB	Protein Data Bank
45	QMEAN	Quality Model Energy Analysis
46	RNA	Ribonucleic acid

47	RMSD	Root Mean Square Deviation
48	SAGE	Serial Analysis of Gene Expression
49	SA	Sinapic acid
50	SDS	Sodium Dodecyl Sulphate
51	SD	Standard Deviation
52	S.V	Standard Variance
53	SPSS	Statistical Package for the Social Sciences
54	SELDI-TOF-MS	Surface Enhanced Laser Desorption/Ionization- Time of Flight-Mass Spectrometry
55	TMS	Tandem Mass Spectrometry
56	TEMED	Tetra Methyl Ethylene Diamine
57	UV light	Ultra Violet light
58	VICAT	Visible Isotope Coded Affinity Tags
59	WHO	World Health Organisation
60	CHCA	α- Cyano-4- hydroxyl cinnamic acid

1. INTRODUCTION

Microbial cultures have been used for thousands of years in food and alcoholic fermentations, and in the past century have undergone scientific scrutiny for their ability to prevent and cure a variety of diseases. Elie Metchnikoff in 1907 first introduced the probiotics concept. Probiotics are defined as the living microorganisms administered in a sufficient number to survive in the intestinal ecosystem. Probiotic bacteria are friendly to the host and protect the host against infection by secreting antimicrobial substance (Klaenhammer *et al*., 1999). Probiotics are a heterogeneous group of non-pathologic bacteria that are functionally defined by their ability to allay inflammation when introduced into the inflamed intestine. During the periods of stress, illness or antibiotic treatment, the gut flora is often changed in favour of harmful bacteria that may cause overgrowth of the harmful bacteria and its subsequent invasion of the system lead to inflammatory, immunological, neurological and endocrinological problems. Induction of the growth of beneficial bacteria is one of the possible solutions to normalize the health conditions. This could be achieved by the supplementation of viable bacterial cells into the host. Probiotics can help to build up the beneficial bacterial flora in the intestine and completely exclude the pathogenic bacteria. Many beneficial bacteria produce Vitamin K - a very important blood coagulation factor. The efficacy of a probiotic effect often depends on the mechanism by which they exert their activity. When a person takes antibiotics, both the harmful bacteria and the beneficial bacteria are killed. So, Probiotics are gaining more and more interest as alternatives for antibiotics or anti-inflammatory drugs.

Probiotic bacteria:

Probiotics involving a number of different bacterial species and strains, mainly lactic acid bacteria (LAB). Lactic acid Bacteria considered as “Generally Regarded as safe” (GRAS) and there were no reports of any harmful effects from the consumption of these bacteria (Gilliland, 1990). Probiotics are the beneficial microorganisms administered in a sufficient number to survive in the intestinal ecosystem. They must have a positive effect on the host (Gismondo *et al*., 1999). The term 'probiotic' was first used by Lilly and Stillwell in 1965 to describe the 'substances secreted by one microorganism that stimulate the growth of another. Now-a-days probiotics available commercially as supplements like tablets, capsules and powders (Weese *et al*., 2002). Most commercially available probiotic products sold for use in companion animals contain *Lactobacillus spp.* or *Bifidobacterium spp* (Weese *et al.,* 2011). Certain species of enterococci are also commonly used. Probiotic dosing varies depending on the product and specific indication. No consensus exists about the minimum number of microorganisms that must be ingested to obtain a beneficial effect (Farnworth *et al*., 2008). In the digestive system, probiotics improve food digestion directly by

helping the body to assimilate nutrients, through contributing to the metabolism of bile acids and accelerating their elimination, as well as by producing digestive enzymes.

Prebiotics:

Non-digestible substances that provide a beneficial physiological effect for the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria are known as prebiotics. Most prebiotics are used as food ingredients in biscuits, cereals, chocolate, spreads, and dairy products. Lactulose is a synthetic disaccharide used as a drug for the treatment of constipation and hepatic encephalopathy. The prebiotic oligo-fructose is found naturally in many foods, such as wheat, onions, bananas, honey, garlic, and leeks. Oligofructose can also be isolated from chicory root or synthesized enzymatically from sucrose.

Normal intestinal micro flora:

The human intestinal tract is a very complex internal environment. It filled with various species of bacteria and yeasts that should assist digestion kill harmful or pathogenic infections and even help to produce many vitamins and other chemical substances needed for our health and long life. The name given to these organisms that live in our intestines is called intestinal flora. The GI tract is a biologically diverse and complicated system which contains around 10^{14} bacterial cells and up to 1000 species. The role of normal intestinal flora plays in various physiologic processes of man. Conversion of bilirubin to urobilinogens ($C_{33}H_{44}N_4O_6$) takes place in the intestine through the activity of the intestinal flora. The intestinal micro flora may prevent infection by interfering with pathogens. The flora includes low populations of potentially pathogenic organisms such as *Clostridium difficile*. Antibiotics that upset the balance of the normal flora can favour both infections by exogenous pathogens and overgrowth by endogenous pathogens. Gut flora consists of a complex of microorganism species that live in the digestive tracts of animals and is the largest reservoir of human flora. Intestinal micro flora is important for the immune system, the development of normal intestinal morphology and maintenance of a continued and immunologically balanced inflammatory response. The GI tract always releases hormones to help regulate the digestive process. These hormones, including gastrin, secretin, cholecystokinin and grehlin, are mediated through intracrine or autocrine mechanisms, indicating that the cells releasing these hormones are conserved structures throughout evolution (Nelson *et al.*,2005). The gastrointestinal tract is also a prominent part of the immune system (Richard Coico *et al*., 2003). The immune system must work hard to prevent pathogens from entering into blood and lymph. Intestinal bacteria serve to prevent the overgrowth of potentially harmful bacteria in the gut. A ratio of 80-

85% beneficial to 15-20% potentially harmful bacteria generally is considered normal within the intestines.

Surface micro flora of human body:

Human body surface tissues, i.e. skin and mucous membranes are constantly in contact with environmental organisms and become readily colonized by various microbial species. The mixture of organisms regularly found at any anatomical site is referred to as the normal flora, except by researchers in the field who prefer the term "indigenous micro biota". The normal flora of humans consists of a few eukaryotic fungi and protists, but bacteria are the most numerous and obvious microbial components of the normal flora.

Table- 1: Bacteria commonly found on the surfaces of the human body.

Bacterium	Skin	Conjunctiva	Nose	Pharynx	Mouth	Lower GI	Ant. urethra	Vagina
Staphylococcus epidermidis (1)	++	+	++	++	++	+	++	++
*Staphylococcus aureus** (2)	+	+/-	+	+	+	++	+/-	+
Streptococcus mitis				+	++	+/-	+	+
Streptococcus salivarius				++	++			
*Streptococcus mutans** (3)				+	++			
*Enterococcus faecalis** (4)				+/-	+	++	+	+
*Streptococcus pneumoniae** (5)		+/-	+/-	+	+			+/-
*Streptococcus pyogenes** (6)	+/-	+/-		+	+	+/-		+/-
Neisseria sp. (7)		+	+	++	+		+	+
*Neisseria meningitidis** (8)			+	++	+			+
Enterobacteriaceae(Escherichia coli)* (9)		+/-	+/-	+/-	+	++	+	+
Proteus sp.		+/-	+	+	+	+	+	+
*Pseudomonas aeruginosa** (10)				+/-	+/-	+	+/-	
*Haemophilusinfluenzae** (11)		+/-	+	+	+			
*Bacteroides sp.**						++	+	+/-
Bifidobacterium bifidum (12)						++		

Lactobacillus sp. (13)				+	++	++		++
*Clostridium sp.** (14)					+/-	++		
Clostridium tetani (15)						+/-		
Corynebacteria (16)	++	+	++	+	+	+	+	+
Mycobacteria	+		+/-	+/-		+	+	
Actinomycetes				+	+			
Spirochetes				+	++	++		
Mycoplasmas				+	+	+	+/-	+

Note: ++ = nearly 100 % ; + = common (about 25 %); +/- = rare (less than 5%) ; * = potential pathogen.

Role of probiotics on human health:

Probiotics have formed a vital part of Mediterranean and Middle Eastern diets for thousands of years, in the form of fermented milk and vegetable products such as yogurt and pickles (Mateos *et al., 2005;* Pataky *et al*., 2009).The new science of research on probiotics is called *Pharmabiotics,* uses probiotic organisms as natural pharmaceutical agents in the treatment and prevention of disease along with promoting longevity. Pharmabiotics provides an almost limitless source of biologically active materials which can influence human health. Probiotic bacteria are involved with our lives and health that they are considered as a part of the human organism (Hill *et al.,* 2010*;* Rauch *et al.,* 2011*;* Shanahan *et al.,* 2011). With advancing technology, scientists are now able to select specific strains of organisms to accomplish precise tasks (O'Hara *et al., 2007*). The probiotic microorganisms consist mostly of the strains of the genera *Lactobacillus* and *Bifidobacterium*, but strains of *Bacillus, Pediococcus*, yeast and some *E. coli* species are also been found as probiotics. Together they play an important role in the protection of the organism against harmful microorganisms and also strengthen the host's immune system. The probiotic bacteria attach to the intestinal wall where they increase the number of beneficial bacteria regulating and maintaining balance between the beneficial and harmful bacteria. Probiotics increase the immune function in our body over all 83% of immunity is located in intestine, promote digestive system, enhancing the absorption of food and nutrients. Supporting vitamin production and destroying of toxins and carcinogens.

Figure -1: List of various diseases that are possibly treated by probiotics.

***Eschericia coli* as a probiotic:**

E. coli is the best known bacterium that regularly associates itself with humans, being an invariable component of the human intestinal tract. Even though *E.coli* is the most studied of all bacteria, and we know the exact location and sequence of 4,288 genes on its chromosome, we do not fully understand its ecological relationship with humans. The beneficial effects of *E. coli* strain Nissle 1917 (EcN) on various gastrointestinal disorders were initially discovered during the World War. EcN has evolved into one of the best characterized probiotics, and its therapeutic efficacy and safety have convincingly been proven (Kruis *et al*., 1997; Rembacken *et al*., 1999; Kruis *et al*., 2004). In 1917, before Sir Alexander Fleming's discovery of penicillin, the German professor Alfred Nissle isolated a non-pathogenic strain of *Escherichia coli* from the faeces of a First World War soldier who did not develop enterocolitis during a severe outbreak of shigellosis. Disorders of the intestinal tract were frequently treated with viable non-pathogenic bacteria to change or replace the intestinal micro biota. The *Escherichia coli* strain Nissle 1917 is one of the few examples of a non-LAB probiotic. *Escherichia coli* strains play a pivotal role within the unique intestinal micro ecological system, which consists of an enormous variety and quantity of different microorganisms. These microorganisms can be found as physiological constituents of the intestinal micro flora both in healthy individuals and under pathological conditions in the course of various gastrointestinal

diseases. Several lines of evidence suggest that *E. coli* is involved in the pathogenesis of IBD (Hart *et al*., 2002).

History of Cocoti palm sap and wine:

Cocos nucifera namely Coconut palms are one of the oldest flowering trees in the world. For centuries throughout the tropics, cocoti palm wine is the collective name for alcoholic bevarages produced by the spontaneous fermentation of the sap of cocoti palm trees. Palm wine is also called as kallu, palm toddy, or simply toddy , is an alcoholic beverage created from the sap of various species of palm tree such as the Palmyra, date palms and coconut palms (Rundel *et al.,* 2002). This drink is common in various parts of Asia and Africa and goes by various names, such as emu and oguro in Nigeria, nsamba in Democratic Republic of the Congo, nsafufuo in Ghana, *kallu* in South India etc. Palm wine is an important socio-economic, nutrition and healthy item of many Nigerians especially the low-income rural dwellers. The sap can be fermented and distilled for alcohol (*ogogoro*) (Ezeronye *et al.,* 1998).

Cocoti sap:

The traditional practice of "Tapping" coconut trees for their prized "Sap" is a time- honoured art form. The nutrient- rich sap that exudes from the blossoms before they mature into coconuts is used to make unique and nutritious food products. Cocoti sap is collected by tapping method. Tapping methods vary with the type of palms locality of the coconut palm is tapped by pulverising or crushing the flowers and the sap that issues from the injury is collected (Swing *et al.,* 1977). The sap is extracted and collected by a tapper. Typically the sap is collected from the cut flower of the palm tree. A container is fastened to the flower stump to collect the sap. The white liquid that initially collects tends to be very sweet and non-alcoholic before it is fermented. The taste of sap varies with the length of time the sap has been fermenting, and with the length of time a particular incision has yielded sap. The fresh sap from a newly tapped tree collected through a funnel is very sweet and becomes progressively dry or sour as it ferments. Sap which has fermented for about 24 hours has a sour taste and has premium value for palm wine drinkers.

Cocoti sap is tapped from immature inflorescence is a clear, sugary solution. It ferments rapidly upon exudation from the tree. As a result to obtain unfermented samples for analysis is often difficult. Many scientists have reported the chemical composition of the cocoti palm sap (Bassir, 1962; Chinasara, 1968; Faparusi *et al.,* 1972*;* Okafor, 1978).

Table- 2: Chemical composition of Palm Sap.

Chemical	Bassir , 1962	Chinasara, 1968	Faparusi *et al.*, 1972	Okafor, 1978
Sucrose (%)	4.29 (+/-) 1.4	13	3.00	11.2
Glucose	3.31(+/-) 0.9	-------	1.20	0.95
Fructose	-------	-------	1.20	1.0
Raffinose	-------	-------	0.80	-------
Protein	-------	0.36	-------	-------
Ammonia	0.38-0.015	-------	-------	-------
Vit C (mg/100 ml)	-------	10	-------	18.6
Vit B_{12}(Pg/ml)	-------	-------	-------	160.00

The freshly gathered cocoti sap is oyster white in colour, has a nearly neutral pH, and is readily inherently sweet tasting by nature. Whereas, the sap from a maple tree (as well as the juice from an agave cactus) has very little readily available sweetness, and requires long heating times in order to produce the sweet syrup you purchase in the bottle. The nutrient- rich coconut sap comes right out of the tree naturally abundant in 17 Amino Acids (the building blocks of protein), broad-spectrum B- Vitamins, Vitamin C as well as Fructo oligosaccharides (FOS) a prebiotic that promotes digestive health.

Table- 3: Vitamin content of freshly- gathered cocoti sap.

Vitamin	Fresh Coconut sap value (mg/dl)
Thaimine (B-1)	77.00
Riboflavin(B-2)	12.20
Niacin (B-3)	40.60
Pantothenic acid (B-5)	5.20
Pyridoxine (B-6)	38.40
Biotin (B-7)	0.17
Folic acid (B-9)	0.24
Cyanocobalamin (B-12)	Trace
Choline	9.00
Inositol	127.70
Vitamin C	23.4

Table - 4: Mineral content of cocoti sap.

Minerals	Macronutrient mg/l ppm in Dry matter
Nitrogen (N)	2,020.0
Phosphorous (P)	790.0
Potassium (K)	10,300.0
Calcium (Ca)	60.0
Magnesium (Mg)	290.0
Sodium (Na)	450.0
Chlorine (Cl)	4,700.0
Sulphur (S)	260.0
Boron (B)	6.3
Zinc (Zn)	21.2
Manganese (Mn)	1.3
Copper (Co)	2.3
Iron (Fe)	21.9

Cocoti wine:

Palm wine generally refers to a group of alcoholic beverages obtained by fermentation from the saps of palm trees (Agu *et al*., 1999). Palm wine is an alcoholic beverage obtained from the fermentation of the sugary sap of various palm samples by the presence of various microorganisms especially the bacteria and yeast. During fermentation, the sugars in the palm sap are metabolized to alcohol and organic acids with the results that the sap loses its sweetness (Okafor, 1977). After fermentation, oyster colour change in white and sweet taste change into sour. The natural fermented palm wine contains 5 - 6 % v/v ethanol (Nwokeke, 2001). The major chemical constituents of palm wine are sugar, protein, water-soluble vitamin of the B-group, titratable organic acids, alcohol and water (Eschie, 1978; Ojimelukwe, 2000). Palm wine and its distillate are important solvent in herbal medicinal administration; pregnant women consume it fresh for the sweetness and nutrition while nursing mothers drink it warm to enhance breast milk production. The microorganisms on palm wine fermentation produce lactic acid and CO_2 that make the palm wine anaerobic and leaven the

product. It has been established that consumption of alcohol during pregnancy results in profound developmental and behavioral effects on the fetus and offspring, called Fetal Alcohol Syndrome (FAS) (Desroches *et al.,* 1987; Breese *et al.,* 1993). Alcoholism among women has increased during the last few decades. The excess intake of alcohol for a long time period causes fatty liver (Ramakrishnan *et al.,* 1976) and accumulation of fat in the heart and kidneys (Ramakrishnan *et al.,* 1973) and in brain (Ramakrishnan *et al.,* 1983). Previous studies have shown that maternal consumption of alcohol/Toddy reduced the body weight of both dams and fetuses and altered carbohydrate metabolism (Lal *et al.,* 1997). Fermented palm wine exposure could cause prenatal osteo-inhibitory effects on bones (Eluwa *et al.,* 2010).Consumption of ethanol during pregnancy causes reduction in the weight of the fetus and hepatic glycogen content (Desroches *et al*., 1987; Breese *et al.,* 1993). Palm wine caused significant decrease in testosterone levels. Similar report was given by Das *et al*., (2009) in rats treated with Aeglemermelos extract.

Table – 5: Chemical constituents in palm wines (6% sugar).

Component (%)	Palm wine	Extended	Formulated
Sugar	6	6	6
Alcohol	1.4	1.7	1.6
Protein	0.245	0.231	0.201
Titratable acidity	0.276	0.192	0.24
pH	3.80	3.85	4.2

Health effects:

According to recent studies by the World Health Organisation (WHO), alcohol consumption is a leading contributor to chronic disease and recognized as a strong risk factor affecting health in developed countries such as the United States and Canada (Rehm *et al.,* 2006). The WHO global burden of disease project estimated that in developed countries alcohol was responsible for 9.2% burden of disease, behind tobacco (12.2%) and high blood pressure (10.9%).There are many kinds of palm wines especially in Africa, with various names. Palm wine contains many components such as a heavy suspension of fungi and bacteria, fermentation agents which give the palm wine a milky white flocculent appearance (Morah,1995), different kind of volatile constituents (Uzochukwu *et al.,* 1994), chemical basis for aroma (Lasekan *et al.,* 2007) mineral elements which may change from one production to another and must of all alcohol. Toddy caused reduction in weight gain and weight of fetuses and also altered carbohydrate metabolism (Lal *et al.,* 1997).

Palm wine caused non-significant changes in body weight of rats after treatment for 30 days, this suggests that palm wine was not toxic to the animals as well as non-androgenic in nature, since androgens are known to possess anabolic activities. Similar report was given by (Gonzales *et al.*, 2006) in rats. Palm wine caused significant decrease in sperm motility (Verma *et al.*, 2002).Treatment of rats with palm wine caused mild interstitial congestion and oedema. Similar results were reported by (Manna *et al.,* 2005) as well as (Mohammed *et al.,*2007) in rats treated with deltamethrin and sumithion. These could be due to (i) increased hydrostatic pressure (ii) reduced oncotic pressure (iii) lymphatic obstruction or (iv) Sodium retention (Kumar *et al*., 1999).

Genomics and proteomics:

The genome is defined as the complete set of genes inside a cell. Genomics is "the study of function and interactions of all the genes in the genome, including their interactions with environmental factors (Collins *et al.,* 2006*).*Completion of human genome sequencing in 2006 started a new era of science. Since that time genomes of many organisms have been deciphered. The word "PROTEOMICS" might come from the word "GENOMICS". By the study of genome full DNA sequence of several biological species including human has been determined. Under these circumstances, the focus of life science is moving from genome to proteins, which are biologically synthesized from genome. Aim of proteomics is to study the structure and function of all proteins of a biological species to reconstruct the total biological function of the life is called "PROTEOMICS". Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" - specifically, the study of their small-molecule metabolite profiles. Metabolomics may provide information on an additional level of regulation called, metabolic regulation (Rossell *et al*., 2006). Metabolomics is the valuable technique for the all-encompassing profiling (Von Roepenack-Lahaye *et al*., 2004; Krishnan *et al*., 2005; Moco *et al*., 2006).

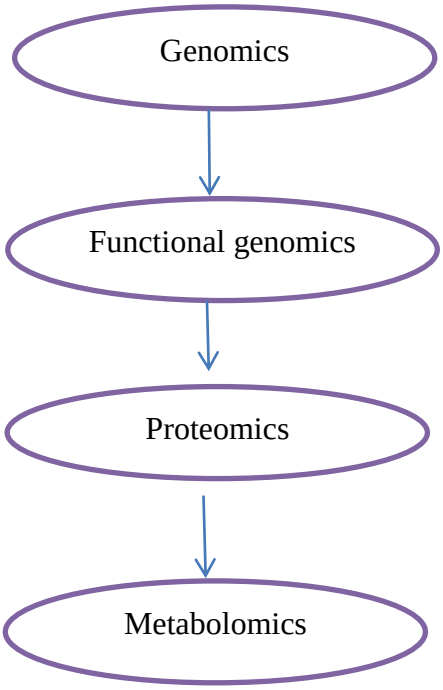


Figure- 2: Diagrammatic representation of genomics to proteomics.

What is the need of proteomics:

The study of genomics we are not getting accurate results in all cases. It is impossible to make clear and (more) comprehensible mechanisms of disease, aging and effects of the environment only by studying the genome. Only through the study of proteins protein modifications can be characterized and the targets of drugs are to be identified.

Proteomics:

The terms “proteomics” and “proteome” were coined by Marc Wilkins and colleagues in the early 1990s and mirror the terms “genomics” and “genome,” which describe the entire collection of genes in an organism. Proteomics is a tool for studying the proteome, i.e., the set of proteins expressed under a defined physiological condition in an organism or cell or tissue. Proteomics is the large scale of study of proteins, particularly their function and structure. Proteins are the vital parts of the living organisms (Naven, 2002; Twyman, 2004). Organisms respond at the molecular level due to adverse conditions such as chemicals and metal stress different origins by the rapid and acceleration in the synthesis of class of proteins called as stress proteins. All proteins are encoded by the genome of the organism. While the genome is a relatively invariable reserve of potential functions, the proteome reveals the active functions in the organism. The proteome changes continuously and a large amount of information on the functional responses of the organism can be obtained by studying the proteome under different physiological conditions.

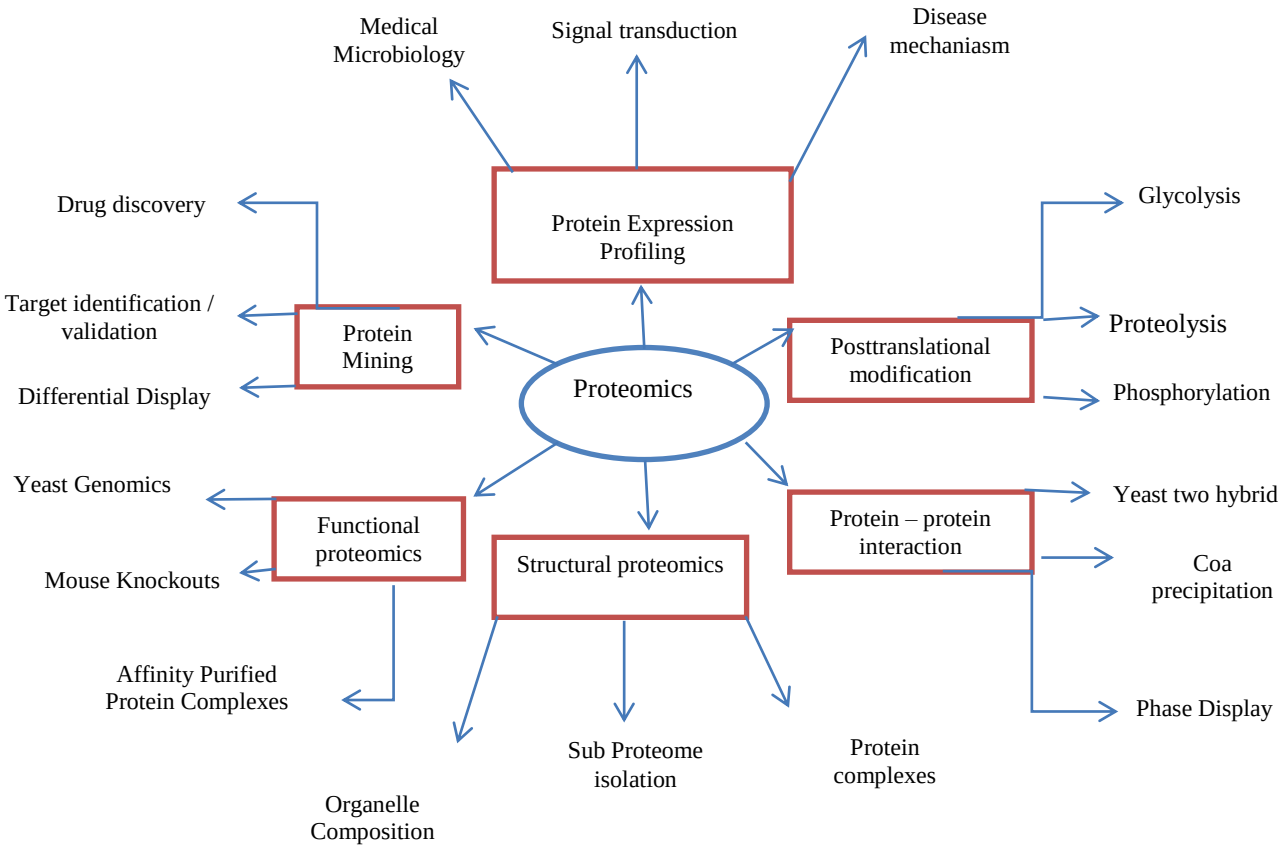


Figure-3: Applications of proteomics in different fields.

Types of proteomics:

The term "proteomics" was first coined in 1995 and was defined as the large-scale characterization of the entire protein complement of a cell line, tissue, or organism (Wikins *et al.*, 1995; Wasinger *et al.*, 1995; Anderson *et al.*, 1996). Protein expression profiles change several times during the cell cycle and are heavily affected by a number of intracellular and extracellular stimuli (temperature, stress, apoptotic signals, etc.) (Godovac- Zimmermann *et al.*, 2001). Proteomics are mainly classified into three types i.e. Functional Proteomics, Structural Proteomics and Differential proteomics.

Functional proteomics:

Functional proteomics is the large-scale study of proteins at the functional activity level, such as expression and modification. Currently proteome investigations are focused on mainly two major areas i.e. expression and functional proteomics. Expression proteomics to indicate the down regulation and up regulation of protein levels, in functional proteomics to characterise protein activates, multi protein complexes, and signaling pathways (Pandey *et al*., 2000; Hinsby *et al.*, 2003).

Structural proteomics:

The major challenges in structural proteomics include the determination and prediction of atomic resolution 3-D structures of proteins on a genome-wide scale for better understanding their structure-function relationships (Smith, 2000). Recent advances in the fields of X-ray crystallography and NMR spectroscopy (Montelione *et al*., 2000; Abola *et al*., 2002) have allowed structural biologists to gloss the structures and biological functions of proteins by determining their atomic coordinates.

Differential proteomics:

The aim of differential proteomics is to obtain information about all proteins in a sample. It provides difference between healthy and treated samples. (Bantscheff *et al*., 2007; Nikolov *et al.*, 2012). Several approaches can be used and these typically involve electrophoresis and chromatography combined with mass spectrometry.

Protein expression studies:

In recent years, the analysis of mRNA expression by various methods has become increasingly popular. These methods include serial analysis of gene expression (SAGE) (Velculescu, 1995) and DNA microarray technology (Shalon, 1996). However, the analysis of mRNA is not a direct reflection of the protein content in the cell. Consequently, many studies have now shown a poor correlation between mRNA and protein expression levels (Abbott**,** 1999; Ideker, 2001).

Tools Used in Proteomics field:

In Proteomics field, combinations of Analytical techniques are used to analyse the protein samples. The first step in all proteomic studies is the separation of a mixture of proteins. This can be carried out using 2-D gel electrophoresis technique in which proteins are separated based on their individual Molecular weight and charges. The spots obtained in 2-D electrophoresis are separated for subjected to mass spectrometric analysis of each protein present in the mixture. Then the proteins were denatured, reduced, alkylated and digested with trypsin. Tryptic peptides were analysed by using MALDI-TOF.

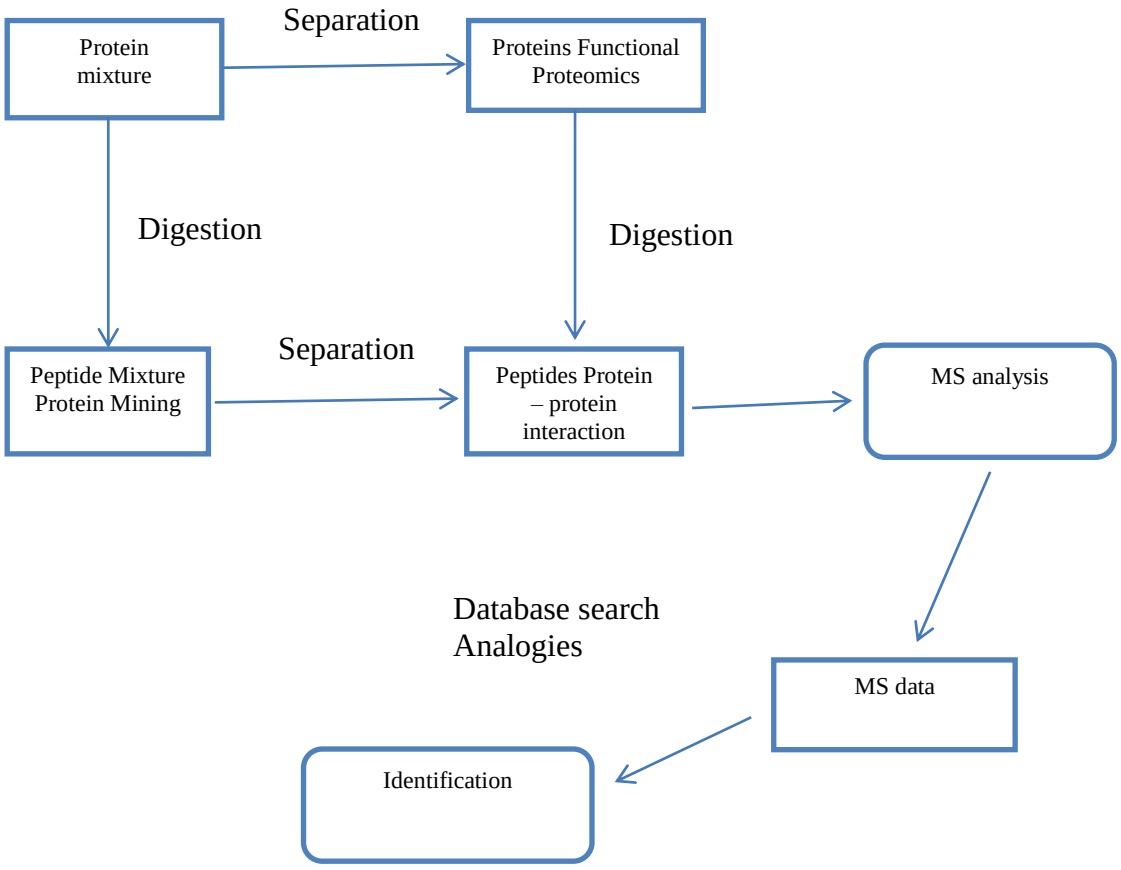


Figure- 4: General flow schemes for proteomic analysis.

MALDI-TOF:

"Matrix Assisted Laser Desorption/Ionization- Time of Flight." (MALDI-TOF) is first introduced by Hillenkamp and Karas in 1988 (Kara *et al*., 1988) has become a wide spread analytical tool for peptides, proteins and other bio-molecules. MALDI is an advanced stage from early stage research towards proteomics. The principle behind MALDI-TOF-MS revolves around the rapid- volatilization of a sample embedded in a UV-absorbing matrix followed by time of flight mass spectrum analysis. Several chemical and physical pathways have been suggested including gas-phase photo ionization, ion-molecule reactions, disproportionate, excited- state proton transfer, energy pooling thermal ionization, and of preformed ions.

Sample for MALDI is uniformly mixed in a large quantity of matrix. Different types of Matrix's are available; the matrix absorbs the U. V. light (nitrogen laser light, wavelength 337nm) and converts it to heat energy. Heat rapidly and is vaporized, together with the sample. Charged ions of various sizes are generated on the sample slide. The charged ions move faster through the drift space until they reach the detector. Based on the time it takes for the ion to drift down the flight tube to the detector. Lighter ions have higher velocities than heavier ions and reach the detector first.

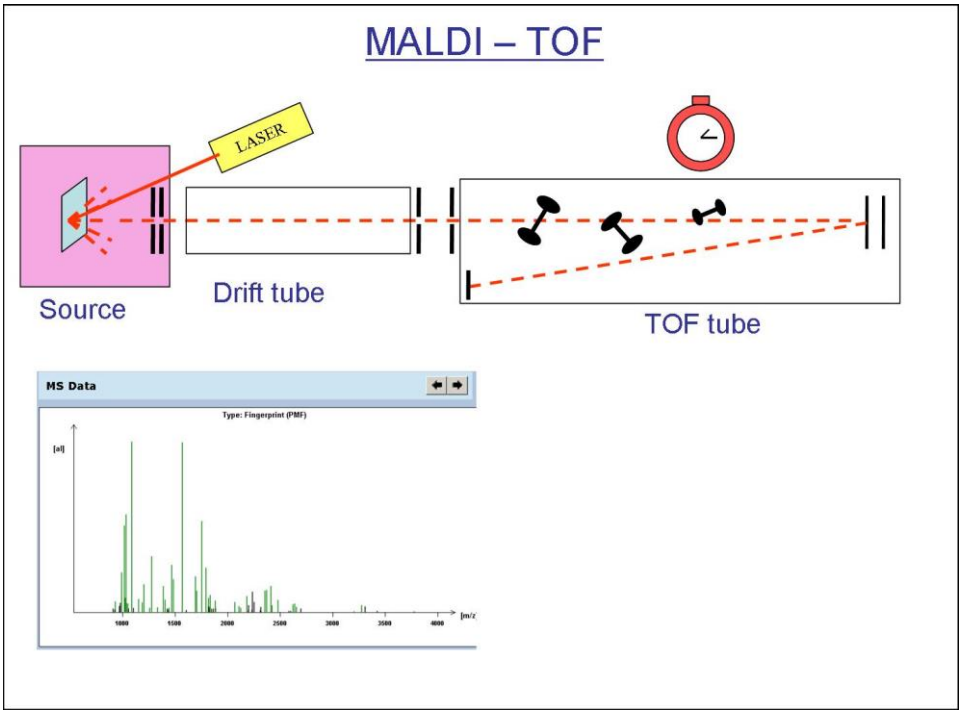


Figure-5: MALDI-TOF image.

Matrix:

Matrix is the supporting material in sample analysis, the choice of the matrix is crucial for success in MALDI experiments. Matrix consists of crystallized molecules, matrix act as a first absorber of the UV laser radiation and breaks down, expanding into the gas phase. A good matrix obey the following characters i.e. they are a fairly low molecular weight, but are large enough not to evaporate during sample preparation or while standing in the spectrometer. They are often acidic, therefore act as a proton source to encourage ionization of the analyte. Basic matrices have also been reported. They have a strong optical absorption in either the UV or IR range so they rapidly and efficiently absorb the laser irradiation. This efficiency is commonly associated with chemical structures incorporating several conjugated double bonds, as seen in the structure of cinnamic acid.

Table - 6: Matrix compounds for MALDI-TOF analysis.

S.No	Name of the compound	Another name	Solvent	Wave length(nm)	Application
1	3,5 dimethyl-4-hydroxyl cinnamic acid	Sinapic acid, Sinapinic acid; SA	Acetonitrile, Water, acetone, chloroform	337,355, 266	Peptides, proteins, lipids
2	4-hydroxy-3-methoxy cinnamic acid.	Ferulic acid	Acetonitrile, water, propanol	337,355, 266	Proteins
3	α- Cyano-4-hydroxyl cinnamic acid	CHCA	Acetonitrile, water, ethanol, acetone.	337,355	Peptides, lipids and nucleotides
4	Picolinic acid	PA	Ethanol	266	Oligonucleotides
5	3-hydroxy picolinic acid	HPA	Ethanol	337,355	Oligonucleotides
6	2,5-Dihydroxy benzoic acid.DHB,	Gentistic acid	Acetonitrile, water, methanol, acetone, chloroform	337,355, 266	Peptides, nucleotides, oligonucleotides, oligosaccharides.

MS/MS analysis:

Mass spectrometry is an analytical technique that produces spectra of masses of the atoms or molecules comprising a sample of material. Mass spectrometry is an important newly emerging method for the characterization of proteins. In MS analysis a sample which may be solid, liquid or gas is ionized. The ions are separated according to their mass-to-charge ratio (Sparkman *et al*., 2000). Two types of methods are used to ionization of whole protein are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Total protein mass analysis is preliminary conducted using either time-of-flight (TOF) MS or Fourier transform ion cyclotron resonance (FT-ICR). Protein sample enzymatically digested into smaller peptides using trypsin. These peptides introduced into mass spectrometer and identified by peptide mass fingerprinting (PMF), tandem mass spectrometry (TMS). MALDI-TOF is often the preferred instrument because it allows a high sample throughput and several proteins can be analyzed in a single experiment, if complemented by MS/MS analysis.

For each MS/MS spectrum, software is used to determine which peptide sequence in a database of protein or nucleic acid sequences gives the best match.

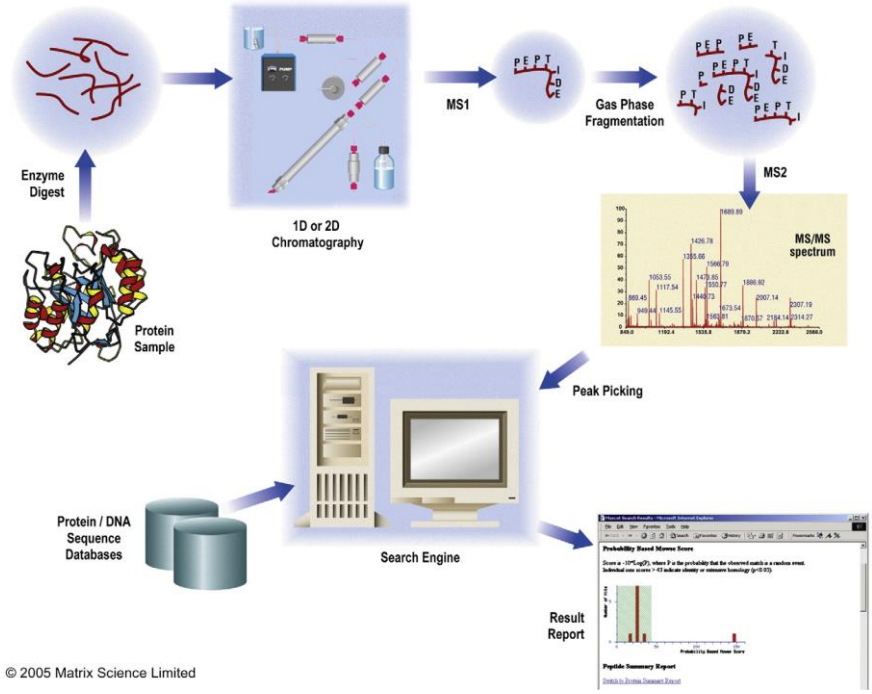


Figure-6: Workflow for protein identification and characterization using MS/MS data. (Collected from website@2005 Matrix Science Limited)

Advanced methods in Proteomics:

Different types of techniques are available presently for protein identification
ICAT- Isotope Coded Affinity Tags
VICAT- Visible Isotope Coded Affinity Tags
AQUA- Absolute quantification of Proteins.
ESI-Q-IT-MS- Electrospray Ionization Quadrupole Ion Trap- Mass Spec
SELDI-TOF-MS- Surface Enhanced Laser Desorption/Ionization- Mass Spec.

Vaporization of proteins:

In MALDI-TOF analysis, the analyte is first co-crystallized with matrix compound, usually an ultraviolet (UV) absorbing weak organic acid, after which laser radiation of this analyte- matrix mixture results in the vaporization of the matrix which carries the analyte with it. The matrix therefore plays a key role by strongly absorbing the laser light energy and causing indirectly, the analyte to vaporize. The matrix also serves as a proton donor and receptor, acting to ionize the analyte in both positive and negative ionization modes, respectively.

Protein identification:

Protein identification is a major process in proteomics field. Two major techniques are used to identification of proteins i.e. MALDI-TOF and LC-MS/MS based on protein fingerprinting, peptide sequencing. In MALDI-TOF, sample is digested with trypsin mixed with matrix then allowed to examine under MS spectrum, it generates the massed of all peptides. Protein identification and analysis software performs a central role in the investigation of proteins from two-dimensional (2-D) gels and mass spectrometry. For protein analysis, information in protein database can be used to predict certain properties about a protein which can be useful for its empirical investigation.

Two main MALDI-MS based identification methods are used to describe the protein sample i.e. i) Peptide mass fingerprinting ii) Post-source decay (PSD) analysis.

Peptide mass fingerprinting:

Peptide mass fingerprinting is also known as peptide mass mapping/ protein fingerprinting it is an analytical technique for protein identification that was developed by several groups independently (Pappin *et al.*, 1993; Henzel *et al*., 1993). In this method, sample first sample cleaved into smaller peptides, masses can be accurately measured with a mass spectrometer such as MALDI-TOF. These masses are then compared to either a database containing known protein sequences or even the genome. This is analysed by using computer programs/ software tools that translate the known genome of the organism into proteins, then theoretically cut the proteins into peptides, and measure the accurate masses of the peptides from each protein. Then compare to the masses of the peptides of the unknown protein to the theoretical peptide masses of each protein encoded in the genome. High-mass accuracy is required for this method to be of use. Sometimes no matches are found or the level of certainty is too low. The results are statistically analyzed to find the best match.

Post- source decay:

Post-source decay (PSD) is a process specific to the ion source utilizing matrix-assisted laser desorption/ ionisation and operating in vacuum. In the post-source decay, parent ions fragment in a process of laser-induced fragmentation. Time interval suitable for observation of the post-source decay in the reflection starts after the precursors leave the ion source and ends prior to the moment when the precursors enter the ion mirror (Kaufmann *et al.*, 1994). The kinetic energy of fragment of mass m in the post-source decay significantly differs from that of parent ions of mass M is proportional to m/M. So, the distribution of kinetic energies for the PSD ions is extremely large. Not surprisingly, it cannot be compensated in “classic” single or double- stage reflections. To achieve acceptable mass resolution for PSD ions which masses typically distributed over broad mass range, these ions are accelerated to energies substantially (Kurnosenko *et al.*, 2010) exceeding the initial energy of precursor ions.

Post-translational modifications (PTM):

Proteins are created by ribosomes translating m-RNA into polypeptide chains. These polypeptide chains undergo PTM, (such as folding, cutting and other processes), before becoming the mature protein product. A protein is a chain of amino acids. During protein synthesis, 20 different amino acids can be incorporated to become a protein. *Escherichia coli* is a host

commonly used for expression of proteins in research, diagnostic, therapeutic, and industrial applications (Baneyx, 1999). Non-enzymatic post translational modifications could be the result of unavoidable interference between bacterial metabolic pathways and the abundant heterologously expressed protein. Such modifications result in heterogeneity of the expressed protein (Mark *et al.*, 2014). In the case of therapeutic proteins, efforts have to be made to correct such metabolic interference to help ensure the highest level of protein quality. Non-enzymatic glycation is one type of post translational modification with important implications. This type of reaction has been extensively evaluated in higher eukaryotes (Lindsay *et al.*, 1997) and in prokaryotes (Casey *et al.*, 1995; Geoghegan *et al.*, 1999; Yan *et al.*, 1999; Kim *et al.*, 2001; Mironova *et al.*, 2003).

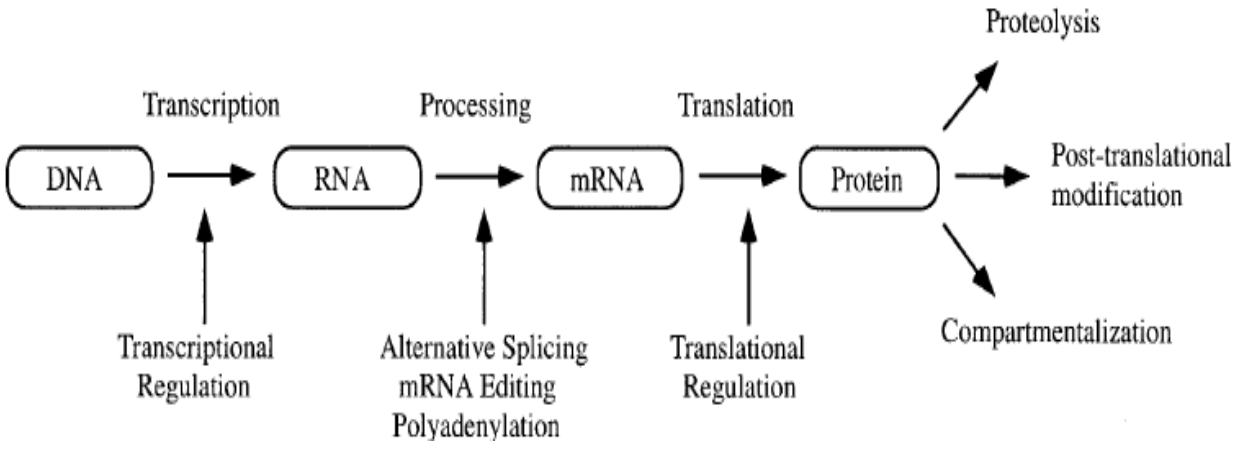


Figure-7: Schematic presentation of post-translational modification.

Homology modelling:

Homology modeling is also known as comparative modeling of protein, the technique used for the identification of one or more known protein structures likely to resemble the structure of the query sequence to residues in the template sequence (Kaczanowski *et al.*, 2010). Homology model is dependent on the quality of the sequence alignment and template structure (Marti-Renom *et al*., 2010*)*. The sequence alignment and template structure are used to produce a structural model of the target. Homology modeling can produce high-quality structural models when the target and template are closely related which has inspired the formation of a structural genomics consortium dedicated to the production of representative experimental structures for all classes of protein folds (Williamson, 2000). Homology modeling process mainly divide into four important steps i.e. template selection, target alignment, model construction and model assessment (Marti-Renom *et al*.,

2010*)*. The first two steps are performed by identifying the template production of sequence alignment. The remaining two steps involve the process to improve the quality of the final model. Protein threading also known as fold recognition can also use as a search technique for identifying templates to be used traditional homology modeling methods (Peng Jian *et al.,* 2011). Homology models can also be used to identify the difference between related proteins that have not all been solved structurally (Sanchez *et al*., 1998).

Protein Threading:

Protein threading also known as fold recognition is a method of protein modeling. It is used to model those proteins which have the same fold as proteins of known structures but do not have homologous proteins with known structure. It differs from the homology modeling. Protein threading approaching can be divided into four groups' i.e. i) No variable length gaps allowed. ii) No pair wise interactions considered in scoring function. iii) No optimal solution guarantee. iv) Exponential run-time.

Peptide mass finger printing:

Peptide mass finger printing is also known as Peptide mass mapping (or) Protein finger printing (Aebersold *et al*., 2001). It is an analytical technique for protein identification of proteins following separation by 2-D gel electrophoresis, SDS-PAGE (Sodium Dodecyl Sulphate) or liquid chromatography (Wang *et al.,* 2003). The advantage of this method is that masses of the peptides have to be known. 2-D gel electrophoresis is the most preferred method for protein separation prior to peptide mass finger printing. After the cleavage of proteins, digest with enzymes commonly used enzyme is trypsin. Trypsin is the favoured enzyme for peptide finger printing; it is relatively cheap, highly effective and generates peptides with average size amino acids, ideally suited for analysis by MS. The masses of the sample compared with known protein sequence or the genome (Shevchenko *et al*., 1996). The mass of these peptide fragments is then calculated and compared to the peak list of measured peptide masses.

Role of Bio-informatics tools in proteomics:

Bioinformatics is the branch of science which uses the applications of information technology and computer science into the field of molecular biology. Paulien Hogeweg invented the term Bioinformatics in 1979 to study the processes of information technology into biological systems. Bioinformatics is a rapidly developing interdisciplinary science, new and improved versions of the software and data banks are released very frequently. Mostly used methods in proteomic field such as sequence analysis and comparative proteomics may provide valuable aid and provide insights to different areas of research. Mainly used software tools are MASCOT, SWISS-PROT, FUGUE, PYROL2, PDB Viewer, Rampage validation tool.

Basic Local Alignment Search Tool (BLAST) is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or nucleotides of DNA sequences. FASTA is a DNA and protein sequence alignment software package first described by David J. Lipman and William R. Pearson in 1985. Both of these methods follow heuristic method that almost always works to find related sequences in a database search. FASTA is a short common pattern in query and database sequences and joins these into alignment. BLAST is similar to FASTA, but gains a further increase in speed by searching only for rarer, more significant patterns in DNA and protein sequences. Currently available FASTA package include special translated search algorithms that correctly handle frame-shift errors when comparing nucleotide to protein sequence data. BLAST can be used for several purposes such as identifying species, locating domains of the sequence, and DNA mapping. CLUSTAL-W is a tool used for multiple alignments of the sequences. MASCOT is a software search engine that uses mass spectrometry data to identify proteins from peptide sequence database (Koenig *et al.,* 2008). RasMol is a computer program written for molecular graphics visualization intended and used primarily for the depiction and exploration of biological macromolecule structures, such as those found in the Protein Data Bank. It was originally developed by Roger Savle in the early 90s (Roger Sayle *et al.,* 1995). Phyre2 (Protein Homology/AnalogY recognition Engine) is a web based service for protein structure prediction (Kelley *et al.,* 2009). FUGUE is a program used for recognising distant homologues by sequence- structure comparison. FUGUE scans a database of structural profiles, calculates the sequence-structure compatibility scores and produces a list of potential homologues and alignments (Shi *et al.,* 2001). PDB (Protein Data Bank) is a repository for the 3-D structural data of proteins and nucleic acids. The data obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists from around the world. PDB play a key role in structural biology (Berman, 2008). Uniprot is a comprehensive, high quality database of protein sequence and

functional information, many entries being derived from genome sequencing projects. Rampage server is used for to check the protein structure validation. Ramachandran's plot is a way to visualize dihedral angles φ against ψ of amino acid residues in protein structure. It shows the possible conformations of φ and ψ angles for a polypeptide. Few search engines for MS/MS data analysis are given in the following table-7.

Table-7: Search engines for uninterrupted MS/MS data.

InsPect	http://proteomics.ucsd.edu/LiveSearch/
Mascot	http://www.matrixscience.com/search_form_select.Html
MS-Tag (Protein Prospector)	http://prospector.ucsf.edu/prospector/cgibin/msform.cgi?form = ms tag standard
OMSSA	http://pubchem.ncbi.nlm.nih.gov/omssa/index.htm
PepProbe	http://bart.scripps.edu/public/search/pep_probe/search.jsp
Phenyx	http://phenyx.vital-it.ch/pwi/login/login.jsp
Popitam	http://www.expasy.org/tools/popitam/
RAId_DbS	http://www.ncbi.nlm.nih.gov/CBBResearch/qmbp/RAId_DbS/index.html
Sonar	http://hs2.proteome.ca/prowl/knexus.html
X!Tandem (The GPM)	http://thegpm.org/TANDEM/index.html

Mascot is a software search engine that uses mass spectrometry data to identify proteins from peptide sequence databases. Mascot is widely used by research facilities around the world. Mascot uses a probabilistic scoring algorithm for protein identification that was adapted from the MOWSE algorithm (Koenig *et al*., 2008). The search parameters are represented in the following table-8.

Table -8: Mascot parameters.

Parameter	**Value**
Database	Swissprot
Taxonomy	*E.coli*
Maximum missed cleavage	1
Fixed modifications	Carbamidomethyl (C)
Variable modifications	oxidation (M)
Enzyme	Trypsin
Peptide tolerance	200-1200 ppm
Peptide charge	+1
MS/MS tolerance	0.2-2 Da
Data format	Mascot generic

Protein structural studies:

Protein is a polymer of Amino acids. By conversion of polypeptide chain under 40 residues is often identified as a peptide, rather than a protein. Protein – protein bond interactions such as hydrogen bonding, ionic interactions, Van der Waals forces and hydrophobic interactions. By using specific techniques to determine their structure the techniques are X-ray crystallography, NMR spectroscopy were used. Proteins are classified based on their arrangement.

Primary structure:

Primary structure refers to amino acid linear sequence of the polypeptide chain. Poly peptides are unbranched polymers. Proteins can become cross-linked, most commonly by disulphide bonds, and the primary structure also requires specifying the cross linking atoms. Two ends of the polypeptide chain are referred to as the carboxy terminus and the amino terminus based on the nature of the free group on each extremity.

Secondary structure:

Secondary structure refers to highly regular local sub-structures. The most common secondary structures are alpha helices and beta sheets. A rough estimation of a polymer is 40% α-helix and 20% β- sheets can often be estimated spectroscopically.

Tertiary structure:

Tertiary structure refers to 3-D structure of a single, double, or triple bonded protein molecule. Tertiary structure will have a single polypeptide chain "backbone" with one or more protein secondary structures, protein domains. α- helix and β- sheets are folded into a component globular structure.

Quaternary structure:

Quaternary structure is the three-dimensional structure of a multi-subunit protein. Structures of the quaternary protein were determined by a variety of experimental techniques that require a sample of protein in a variety of experimental conditions. A Variety of bonding interaction including hydrogen bonding interactions, salt bridges, and disulphide bonds hold the various chains into a particular geometry. There are two major categories of proteins with quaternary structure-fibrous and globular.

Root Mean Square Deviation:

The Root mean square deviation (RMSD) is a frequently used measure of the differences between values predicted by a model (or) an estimator and the values actually observed. In the study of globular protein conformations one customarily measures the similarity in 3-D structure by the RMSD of the Cα atomic coordinate after optimal rigid body superposition. The applications of RMSD are in Bio-informatics the RMSD is the measure of the average distance between the atoms of superimposed proteins. In protein nuclear magnetic resonance spectroscopy, the RMSD is used as a measure to estimate the quality of the obtained bundle of structures. In structure based drug design, the RMSD is a measure of the difference between a crystal conformation of the ligand conformation and a docking prediction.

Phylogenetic analysis:

Phylogenetic tree is a branching tree showing the relationships among various biological species. The similarity of biological functions and molecular mechanisms in living organisms strongly suggests that species from a common ancestor. Molecular phylogenetics uses the structure and function of molecules and how they change over time infer these evolutionary relationships (Liddell *et al.,* 1968).

Based on evolutionary theory, all organisms on earth have descended from a common ancestor, which means that any set of species, extant or extinct is related. This relationship is called a phylogeny, and is represented by phylogenetic trees (Linder *et al.,* 2005).

The confidence statements made about such trees will be main focus. Biologists have also begun to adapt Bayeesian methods based on Markow chain Moute Carlo computations using parametric evolutionary models (Li *et al*., 2000).

Protparam analysis:

The tool ProtParam is allows the computation of various physical and chemical properties that can be deducted from a protein sequence (http://web.expasy.org/protparam/). The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average hydropathicity (GRAVY) (John *et al*., 2005). Extinction coefficient indicates that how much light a protein absorbs at a certain wavelength. It is useful to have an estimation of this coefficient for a protein which a spectrophotometer when purifying it (Gill *et al.,* 1982). The half-life is a prediction of the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. The instability index provides an estimate of the stability of your protein. Instability index is smaller than 40 is predicted as stable, greater than 40 the protein may be unstable. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine and leucine). It may be regarded as a positive factor for the increase of thermostability of globular proteins. GRAVY value of a predicted protein calculated as the sum of hydropathy values of all amino acids, divided by the number of residues in the sequence (kyte *et al.,* 1982).

2. OBJECTIVES

In combination with 2-D electrophoresis, MALDI-TOF MS/MS is a one of the technique for protein identification. In the present study, by using applied proteomic techniques analyse the altered patterns of protein expression that occur in probiotic *E.coli* Nissle *1917* with response to cocoti sap and wine. Differentially expressed proteins identified by using MALDI-TOF MS/MS are performed.

1. Collect the *E.coli* Nissle 1917 from Ardeypharm GmbH, Herdecke, Germany and cultivate in Nutrient agar medium (NAM).
2. Analyse the growth profile of *E.coli* Nissle 1917 in Nutrient agar medium in presence or absence of cocoti sap and wine sample and find the minimum inhibitory concentration.
3. Isolation of protein from cocoti sap and wine treated probiotic *E.coli* Nissle 1917 by using sonication and centrifugation techniques.
4. Purification of protein sample by using Bio-Rad purification kit and quantify the protein sample with the help of BCA kit method.
5. Evaluation of differentially expressed proteins in response to cocoti sap and wine, the proteins can separate individually by 2D-PAGE and allowed for MALDI-TOF-MS/MS analysis.
6. Scan the vaporized proteins of *E.coli* Nissle *1917* by MALDI-TOF MS/MS. The results are analysed by using MASCOT data base search.
7. Protein structure prediction of differentially expressed proteins is formulated under cocoti sap and wine stress by using homology modelling.
8. Validation of the predicted proteins by QMEAN analysis.
9. Phylogenetic relationship of the expressed proteins under cocoti sap and wine stress.
10. Complete analysis of expressed protein physico- chemical characters by using Protparam server.

3. REVIEW OF LITERATURE

Status of Probiotics:

Eschericia coli Nissle 1917 played a major role in probiotic field after *Lactobacillus, E.coli* Nissle 1917 isolated in 1917 at the time of second world war based on its potential to protect from presumably infectious gastroenteritis, intestinal disorders, urinary tract diseases initial therapeutic success was noted and then it is used to chronic inflammatory conditions (Schultz, 2008; Krishnamoorthy *et al*., 2012). Isolated two lactic acid bacteria from 20 fermented samples of plant fermented beverages (PFB) were analyzed for probiotic properties. Acid tolerant (pH 3), thirty six *Lactobacillus* sp. and thirty *Streptococcus* sp. showed good resistance (2%) in bile salt even after exposure for 48 h. The organisms showed high specific growth rate and inhibitory action against potent food borne pathogenic bacteria. According to Resta-lenert *et al.,* (2003) probiotic *Streptococcus thermophilus* and *Lactobacillus acidophilus* can prevent invasion of entero invasive *E.coli* enhance intestinal epithelial barrier function by amplifying phosphorylation of occluding and ZO-1(zonula occludens-1) in vitro. *E.coli* Nissle has evolved into one of the best characterized probiotics, and its therapeutic efficiency and safety have convincingly been proven (Kruis *et al.,* 2004; Westendorf *et al.,* 2005; Henker *et al.,* 2007). The work on these lines is promising, even though their mechanism of action is still under investigation. A potential mechanism by which probiotics may exhibit their beneficial activities is modulation of epithelial barrier function (Dotan *et al.,* 2005).

Probiotics and Gut Microflora:

Gut microflora controls several aspects of bodily function including certain type of cancer (khan *et al*., 2012). Normally large number of *Lactobacilli* is observed in the intestine, but they rapidly decline after infancy (Balamurugan *et al.,* 2008). Majority of the organisms are strictly anaerobic. They comprise the main part of the human normal gut microflora and appear in the stool a few days after birth and subsequently raise the number (Matto *et al.,* 2004). Living microorganisms that enter the gastrointestinal tract (GIT) in an active state and exert a positive influence on the host tissues are called probiotics (Bohm *et al*., *2006*). The potential therapeutic role of probiotics in the prevention or treatment of GIT diseases is earmarked (Mach., 2006). At present *E.coli* Nissle is contained in a probiotic drug called Mutaflor. In recent years, there has been considerable progress in understanding the mechanisms of probiotic action and in the future this should help to select suitable bacterial strains which could beneficially affect mucosal barrier function, immune responses, and suppression of inflammation (Meijerink *et al.,* 2013). The

knowledge of the effects of simultaneous administration of drugs and probiotics on drug pharmaco kinetics is still very limited. The complexity of mechanisms by which the fate of orally administered drugs could be affected by probiotics is recently reported (Stojancevic *et al.,* 2013). Role of both human and commensal microbiota components in drug efficacy and toxicity was recently documented and pointed out (Haise *et al.,* 2013). Some scientists say that great quote "Life without gut bacteria would be extremely unpleasant, if not possible" (Gibson *et al.,* 1999).

The study showed that the application of proteomic tools provided an overview of the proteins present in *E.coli* Nissle under *Cocos nucifera* sap and wine stress conditions. It confirmed that proteins belonging to the vascular system are involved in various biological functions like stress and defence reactions, redox reactions, signal and the transport of the substances and sugar metabolism.

When the cell is under stress arising from oxidation, heat, infection, toxic contamination or any other stressful condition, proteins may unfold and expose residues in their structure that under normal conditions are hidden and shielded from chemical reactions. As a consequence of stress, these residues can easily interact and form aggregates which may harm or even kill the cell (Krebs *et al*., 2003). Under such conditions all cells produce stress proteins to protect the cell from damage.

Probiotics may stimulate immunity, regulate immune signaling pathways, and produce anti-pathogenic factors. Probiotics may produce secreted factors that stimulate or suppress cytokines and cell-mediated immunity. These factors may also interfere with key immune signaling pathways such as the NF-kB and MAP kinase cascades. Probiotics may produce that factors inhibit pathogens and other commensal bacteria, effectively enabling these microbes to compete effectively for nutrients in complex communities. Microbes that produce antipathogenic factors may be regulated by master regulatory genes in particular classes of bacteria. Probiotics mainly present in gastrointestinal tract, plays an important role as an interface between the host and the environment. It is colonized by about 10 trillion microbes of many different species (O'Hara *et al.,* 2006). Intestinal epithelial cells have the capacity to distinguish pathogenic from non-pathogenic bacteria on the basis of their invasiveness and the presence of flagella, although the extract mechanisms that allow them to do this have not been elucidated fully (Borchers *et al.,* 2009). The importance of the intestinal microflora composition in physiological process in the GIT is becoming more evident and has led to new possibilities for prevention and therapy of diseases (Dominguez-Bello *et al*., 2008; Sekirov *et al*., 2010; Kau *et al*., 2011). There is a growing interest in probiotics as a safe way of changing the intestinal bacterial flora. It is possible to increase the proportion of Lactic acid bacteria

(LAB) and *Bacillus sp*. In the gastrointestinal microflora by consumption of probiotics or by oral administration of specific non-digestible substrates, such as oligofructose, termed as prebiotics (Parracho *et al.*, 2007).

***E. coli* Nissle 1917- A model organism:**

E.coli is the most commonly used bacteria *E.coli* Nissle 1917 is one of the oldest, most well-characterised probiotic agents and has shown promising results in treatment of various intestinal diseases (Sonnenborn *et al*., 2009). At the time of World War II it is isolated from solider feces. Completed genomic studies on *E.coli* Nissle 1917 including sequencing, DNA-DNA hybridisation, t-RNA screening (Grozdanov *et al*., 2003) and even low-coverage genomic shotgun sequencing (Sun *et al*., 2005). Until now, however, the whole genome sequence has been inaccessible. Serotyping of *E. coli* Nissle 1917 has identified the presence of a K5 antigen, which is known to be composed of N-acetyl heparosan (a precursor to the anticoagulant pharmaceutical heparin), a group 2 capsular polysaccharide (CPS) consisting of a repeating [¡4) -D-glucuronic acid (GlcA) (1 ¡ 4) N-acetyl--D-glucosamine (GlcNAc) (1¡] n disaccharide unit. Under certain growth conditions, *E. coli* Nissle 1917 produces significantly more CPS than *E. coli* K5, making the organism attractive as a production strain for bioengineered heparin.

Important aspects when select as a probiotic strain:

The significance of human origin has been debated recently, but currently successful strains are indicated to be of human origin. It can also argue that a probiotic strain can function better in a similar environment like human gut to where it was originally isolated from; safety aspects include the following specifications.

1) Strains for human use are preferable of human origin.
2) They are isolated from healthy human GI Tract
3) They have a history of association with diseases such as infective endocarditis or GI disorders
4) They have history of being non-pathogenic.
5) They do not deconjugate bile salts
6) They do not carry transmissible antibiotic resistance genes.

The *E. coli* is a common bacterium present in our gut, it is a gram-negative rod shaped bacteria, about 1.1–1.5 μm x 2.0 – 6.0 μm in size. It grows under aerobic and anaerobic conditions (facultative anaerobic), because it possesses two different redox systems (menaquinone and ubiquinone) which enable it to derive energy from catabolic metabolism under both aerobic and anaerobic conditions. Under optimal growing conditions, the rate of cell division of the *E. coli* bacteria is very fast, the number of bacterial cells can double every 20 minutes. However, the circumstances that are ideal for this population dynamics are not achieved in the bacteria are normal environment. Midtvedt, in 1998 reported that doubling of cells in the caecum of the rat after about 100 minutes, while in the human gut it may take 30 hours. Various strains of *E. coli* have been classified serologically on the basis of their surface antigens O, K and H. O antigens represent the heat-stable constituents of the lipo polysaccharide complex (LPS) of the outer cell membrane, K antigens represent polysaccharides of the capsule and H antigens represent whip or flagellar antigens.

E.coli M-17 is a novel probiotic drug with beneficial effects on the GI tract. EC-M17 is believed to be a direct descendant of the M17 strain first identified by the Russian bacteriologist L. G. Peretz in 1933 (Fitzpatrick *et al.*, 2008). This strain used extensively in humans as a therapy for GI diseases such as colitis, inflammatory bowel disease and infections. Anti-colitis action of EC-M17 is mediated by modulation of immune processes attributed to an inhibitory effect on NF-kB signaling.

Cocoti sap and wine:

Isolation of microorganisms from palm wine *Saccharomyces cerevisiae* dominated in yeast, *Lactobacillus plantarum, Leuconostoc meseteroides* were the dominated organisms. Acetic acid bacteria were isolated after third day when levels of alcohol had become substantial. The pH, lactic and acetic acid concentrations during the tapping were among 3.5 -4.0 %, 0.1-0.3% and 0.2-0.4% respectively, while the alcohol contents of samples collected within the day were between 3.24% to 4.75% and palm wine held for 24 h, over 7.0% and in palm oil wine alcohol content is 1.4% and 2.82%.

Limited consumption of Date sap was found to improve the treatment of haemoglobin deficient anaemic patients and to supplement vitamin-B 12 levels in the Vitamin deficient patients (Debmalya *et al.,* 2008). The organisms *Saccharomyces cerevisiae, Debaryomyceshansenii, geotrichumlactis and Zygosaccharomycesrouxii*are isolated from freshly tapped palm wine (Boboye

et al., 2008). Kadere *et al.,* (2008) has isolated *Acetobacter* and *Gluconobacter* in coconut toddy. Palm sap is a rich medium capable of supporting the growth of various types of micro-organisms as high number of aerobic mesophiles, lactic acid bacteria, yeasts and acetic acid bacteria were found in palm wine (Amoa-Awua *et al*., 2007). It has been reported that fermented palm wine exposure could cause prenatal osteo-inhibitory effects on bones (Eluwa *et al*., 2010).

Palm wine caused changes in body weight of rats after treatment for 30 days this suggests that palm wine was not toxic as well as non-androgenic in nature, since androgens are known to possess anabolic activities. Several reports suggests that palm wine shows negative effect on rat reproductive system (Verma *et al*., 2002; Gonzales *et al.,* 2006; Das *et al.,* 2009). The reason could be due to increased hydrostatic pressure, reduced oncotic pressure, lymphatic obstruction and sodium retention (Kumar *et al*., 1999). In rats Palm wine (10 mL kg-1 bw) caused significant decrease in testosterone level (0.12±0.02 ng mL-1; relative to control 1.38±0.52 ng mL-1). Palm wine (10 mL kg-1 bw) caused significant decrease in sperm counts, motility and viability but no significant change in morphology (Oyedeji *et al*., 2012).

Palm wine causes hypoglycemia, was seen in treated rats when compare to ethanol palm wine causes more effect on gestation. According to Lal *et al*., (1997) key enzymes of citric acid cycle and gluconeogenesis were inhibited on administration of both alcohol and toddy. It shows effect on carbohydrate metabolism. Palm wine increased activity of glycolytic enzymes.

In previous studies, composition of sugars analysis of fresh cocoti sap contains sucrose was the major sugar component in sugars content varied from 9.40g/100ml to 12.24g/100ml. It contains 0.36- 1.5% of proteins and minerals when compared to the date palm wine revealed that sugars are the major components (92-95% dry matter basis) with the dominance of sucrose. It contains also 2.7-5% of proteins and 2.3-2.6% of minerals (Ben Thabet *et al*., 2009). The nutrients – rich in coconut sap comes right out of the tree naturally abundant in 17 Amino acids, broad- spectrum B-Vitamins, Vitamin C as well as FOS. The protein quantification assays revealed that the proteins content was about 0.2g/ml which was comparable with that of the coconut palm exudates 0.1g/ml but was lower than the proteins concentrations of cucumber (60g/l) and of pumpkin (35g/l) phloem sap (Walz *et al.,* 2002; Nakamura *et al*., 2004).

Physico - chemical stress on *E.coli:*

E.coli in response to physical (heat) and chemical (benzyl alcohol) stress elucidate the common and differing elements of the stress response originating in cellular membranes caused by external stress signals of a different nature (high temperature and membrane fluidising agent), by observing overlapping changes at the membrane level. It is expected that signals generated within the membranes might cause HSR and acquisition of cellular thermotolerence in a similar manner independently from the nature of the membrane perturber. The present study addressed the validity of the membrane sensor hypothesis in *E.coli*, which was chosen as our model organism due to its different cellular simplicity and because it is biochemically and genetically well characterised. A reporter system was also developed to study the transcription of heat shock genes, including heterologous promoter sequences of cyanobacterial heat shock genes recognised in an *E.coli* host (Georgopoulos *et al.,* 1993). In proteome analysis total 93 proteins are identified that are phosphorylated in E.coli upon heat shock. These are include chaperones, signaling molecules, ion-channels, proteins involved in transcription and translation process, in amino acid biosynthesis, oxidoreduction, energy metabolism, cell motility and cell membrane structure. Changes in stress signaling pathways are achieved mostly through the activation of protein- tyrosine kinases (Kim *et al.,* 2002).

Cellular cross-protection by stress:

Cellular cross-protection occurs when the stress response induced by one specific type of stress, gives cells increased resistance to other types of stress (Mary, 2003; Vattanaviboon, 2003). An example of this kind of protection is demonstrated with stress-induced thermo-tolerance, where *Escherichia coli* cells given a non-lethal heatshock (42 °C) down-regulated normal protein production and begin production of HSPs, and so are later able to survive what would otherwise be a lethal heat shock (46 °C). This is due to the up-regulation of stress proteins at many levels (e.g. mRNA synthesis and stability, translational efficiency) that can protect cells from other stress. Cross-protection is not universal, and it can also occur in specific ways. For example, heat shock may protect against hydrogen peroxide.

Booth (2002) proposed that “Stress is any change in the genome, proteome or environment that imposes either reduced growth or survival potential”. The cellular response depends on the severity of the stress. Under slight stress, growth continues at the same rate and cells fully adapt to

the new conditions. Under severe stress the growth rate is reduced but cells adapt and tolerate the conditions while under extreme stress, growth cease and cells switch to a survival mode (Storz *et al*., 2000). The key aspects to surviving environmental stress are the cell's ability to maintain the integrity of the cell membrane, the integrity of DNA and the ability to properly fold proteins (Booth, 2002). An understanding of the physiological, biochemical and molecular mechanisms involved in response of *E.coli* to environmental stresses is essential for assessing, predicting, and minimizing the health risks and can offer insight into designing effective methods to control their growth.

When the bacteria expose to high temperature into less time period it produce heat shock response proteins these proteins are unfolded and damaged proteins, such as exposure to harmful chemicals (antibiotics, solvents) or overproduction of endogenous and recombinant proteins. In *E.coli*, heat shock response consists of the induction of more than 20 different heat shock proteins (HSPs), the majority of proteases that degrade misfolded and abnormal proteins. Bacterial cells exposed to one type of stress it can also condition them against other, seemingly unrelated, stresses, when bacteria are challenged with high osmolality (Fletcher *et al.*, 2001).

Proteomics of stress responses of potentially probiotic bacteria:

Proteomics is an excellent approach for studying changes in bacterial metabolism and, e.g., stress responses during the progression of growth. The proteome of the potential probiotic *L.plantarum* WCFS1 was mapped at mid- and late-exponential and early- and late-stationary phases, and growth phase-dependent differences were detected in the abundances of 154 protein spots (Cohen *et al*., 2006). In a study of *L. plantarum* REB1, isolated from fermented feed, and the potential probiotic *L. plantarum* MLBPL1, isolated from white cabbage, both the growth phase (lag, early exponential, late exponential, and early stationary phases) dependent and strain-dependent differences in the proteomes were compared (Koistinen *et al.*, 2007). Proteome maps of *L. casei* Zhang cells grown until the exponential and stationary phases were also compared. Forty-seven protein spots showed growth phase-dependent production, and the major up-regulated proteins in the stationary phase were stress proteins and proteins involved in carbohydrate and energy metabolism, and they were suggested to be involved in the stress response mechanisms of *L. casei* (Wu *et al.*, 2010).

Current Status of the *E.coli* Proteome:

E.coli proteome study can be classified into two main types i.e., Proteomics for biology and Proteomics for biotechnology. An enormous number of *E.coli* proteome studies have focused on improving our biological knowledge proteins and findings members of regulations and stimulations under particular conditions (VanBogelen, 2003) and these studies are referred to as proteomics for biology. Other groups have studied the *E.coli* proteome under various genetic and environmental perturbations in an effort to develop strategies for improving cellular properties and enhancing the production of bio-products based on comparative proteome profiles and these studies are referred to as proteomics for biotechnology.

Proteomic studies of gut microflora explain the molecular mechanisms, expression patterns of proteins and enzymes in response to dietary components and therapy provide a rationale for the development of new active ingredients. For instance, a nano-high-performance liquid chromatography/mass spectrometry (nano-HPLC/MS) system was established to separate proteins of *E. coli* in a two-dimensional manner by combining strong cation exchange (SCX) and reverse phase (RP) chromatography. Peptides were eluted online to an ion trap MS instrument and further analyzed by tandem MS fragmentation for identification using the Swissprot database. Differentially expressed proteins on glucose and lactose were identified. Similarly, lactic acid bacteria that are widely used in the agro-food industry have been characterized by proteomic techniques as reviewed in Champomier-Verges *et al.,* (2002). More recently, the proteome of *Bifidobacterium* has received considerable attention. Adaptation to Low-pH tolerance response and tolerance of bile stress are among the main limiting factors to ensure survival of *Bifidobacterium* in the intestinal environment of humans. Comparing protein patterns of strains grown with or without bile showed 34 different proteins whose expression was regulated (Sanchez *et al.,* 2005; Sanchez *et al.,* 2007).

Protein identification methods:

New methods in protein identification have led to the application of mass spectrometry to the identification of proteins by Peptide Mass Fingerprinting (PMF). MS analysis is an effective tool for identification, characterization and quantification of proteins that are integral components of the processes essential for life. Mass spectrometry relies on the formation of gas- phase ions that can be isolated electrically based on their mass-to- charge ratio. Mass spectrometry (MS) has been

widely used in forensic science in the identification of compounds, particularly illicit drugs. MS is a technique that allows the detection of compounds by separating ions by their unique mass (mass-to-charge ratios) using a mass spectrometer. The method relies on the fact that every compound has a unique fragmentation pattern (mass spectrum). The sample is ionized; the sample ions are separated based on their differing masses and relative abundance.

Matrix Assisted Laser Desorption/Ionisation (MALDI) is a soft ionization technique used in spectrometry, allowing to analysis the biomolecules like DNA, protein, peptides. Biomolecules and synthetic polymers have low volatility and are thermally unstable, which has limited the use of MS as a means of characterization. These problems have been minimized through the development of MALDI-TOF MS, which allows for the mass determination of biomolecules by ionization and vaporization without degradation, a Laser beam used to ionize the sample (Wu *et al.,* 1994). Currently high-through put proteomic tools based on high resolution mass spectrometers and shotgun strategies provide the opportunity to study the physiology of bacterium at high-resolution (Armengaud., 2010; Armengaud., 2013).Recently, efforts have focused on developing new high-throughput techniques for studying microbial cells and complex communities. Among them, proteomics is increasingly being used (Aires *et al.,* 2011). Several studies reported that bile salts can cause stress on probiotic organisms present in gastrointestinal tract, the stress effects are protein misfolding and denaturation, DNA damage, the formation of secondary structure in RNS and intracellular acidification (Begley *et al*., 2005; Lebeer *et al.,* 2008).The most of the proteomic studies on pH responses have been performed under specific aerobic and/or anaerobic conditions, allowing identification of new classes of acid- and base-dependent regulators and dissection of the relationship between pH and oxygen levels (Wu *et al*., 2009).

Enterohemorrhagic and enteropathogenic *E. coli* (EHEC and EPEC) strains are human pathogens that are responsible for food-borne epidemics in many countries. Proteomics offers a powerful platform for analyses of the disease process and of bacterium-host interactions at the protein level. Pathogenesis and identifying markers for laboratory diagnoses of these pathogens (Li *et al*., 2004). Proteomics has been extensively used in understanding and treatment of molecular diseases in humans. The worldwide emergence of antibiotic-resistant bacteria poses a serious threat to human health. For the first time report, potential role of a multi-drug efflux pump system in laboratory *E. coli* strain resistance to piperacillin/ tazobactam, and wild type E. coli ATCC25922 (Santos *et al.,* 2010). For any infection, bacteria must first adapt to the host environment and grow.

When *E.coli* under hyperosmolarity stress results in rapid loss of water (plasmolysis), loss of turgor, and shrinkage of the cell (Weber *et al*., 2005). Within the minutes, respiration ceases, both the intracellular ATP concentration and the cytoplasmic pH increases. Many studies reported the cellular membrane of *E.coli* is a vital factor that allows for cells acclimate to external stresses and is also one of the components highly affected by organic solvents like alcohol (Isaac *et al.,* 2005). Most of the researchers have proposed that the plasma membrane is the most affected target of organic solvents and plays a significant role in adapting to stress, alcohols are sensitive toxins to *E.coli* as tolerances of n-butanol and ethanol are only 0.5-1% and 4-5% respectively.

Proteomics is an excellent approach for studying changes in bacterial metabolism and response. The proteome of the potential probiotic *L. plantarum* WCFS1 was mapped at mid- and late-exponential and early- and late-stationary phases, and growth phase-dependent differences were detected in the abundances of 154 protein spots (Cohen *et al*., 2006). In a study of *L. plantarum* REB1, isolated from fermented feed, and the potential probiotic *L. plantarum* MLBPL1, isolated from white cabbage, both the growth phases dependent and strain-dependent differences in the proteomes were compared (Koistinen *et al*., 2007). Proteome maps of *L. casei* Zhang cells grown until the exponential and stationary phases were also compared. 47 protein spots showed growth phase-dependent production and the major up-regulated proteins in the stationary phase were stress proteins and proteins involved in carbohydrate and energy metabolism and they were suggested to be involved in the stress response mechanisms of *L. casei* (Wu *et al*., 2009).

MATERIALS AND METHODS

Culture collection:

The probiotic bacterium, *Escherichia coli* Nissle 1917 was obtained from the culture collection of Ardeypharm GmbH, Herdecke, Germany (www.bionity.con.en). The bacterial culture of *E. coli* Nissle 1917 were grown in 50 ml nutrient broth supplemented with 100 mg/L Ampicillin at 37^0C, 100 rpm for overnight. Then 50 µl of overnight cultures were transferred into 50 ml broth and grown until OD reached 0.5 OD (McFarland standard). Cells were adjusted to equal OD = 0.05 in nutrient broth supplemented with 100 mg/L Ampicillin.

Table- 9: Composition of Nutrient broth

Ingredients	Amount (Gms/L)
Peptone	5g/L
Sodium chloride	5g/L
Beef extract	1.5g/L
Yeast extract	1.5g/L
Final pH (at 25 ^{0}c)	7.4 ± 0.2

Sample collection:

Collection of palm sap and wine:

Fresh palm sap samples were collected (Wilson, 1996) from coconut palm trees in a sterilized reagent bottles from Goud's (palm tappers) in Tirupati rural, A.P, INDIA. Sample were transported immediately to the laboratory for analysis, some of the sap separated to allowing for fermentation making palm wine (fermented palm sap is known as palm wine) at room temperature 25-28^0c. These samples were filtered by using vacuum pump. The samples were undergoing for analysis of Physico-chemical factors.

Physical factors: Colour, Odour, Taste, pH and Turbidity.

Chemical factors: Acidity and Alcohol.

Colour:

Four test tubes were taken. One tube was filled with cocoti wine and another filled with cocoti sap. Remaining tubes filled with standard solutions like potassium chloroplatinate and cobaltous chloride and observed the colour parallel to the eye with white background (Yohannes *et al*., 2004).

Odour:

Take one liter wide mouth bottle and cleaned it with hydrochloric acid pour it out and find the smell it. Then rinsed with distilled water two times washed out and find the smell it. The sample rinsed and observed the odour (Nordin *et al.,* 2004).

Taste:

After tasting salt and sugar solutions and distilled water, finally recorded our samples taste for the result (Stephen *et al.,* 2013)

pH:

pH was analysed (Maloney *et al.,* 2011) by using pH analyser (MBI pH METER MODEL 3-D)

Turbidity:

Turbidity indicates the growth of microorganisms in the samples. Distilled water is taken into a cuvette and inserted it into the holder and calibrate by using zero knob to set zero. By using standard solutions to set the calorimeter finally and introduced the samples and analyse the turbidity of sap and wine (Baton Rouge, 2007).

Acidity:

5 ml of wine/sap sample was taken into clean conical flask and homogenise by gentle shaking. To this, added 2 drops of phenolphthalein indicator and it was titrated against 0.1N NaOH. A clean burette was taken and filled with NaOH and initial volume was recorded by drop wise release of NaOH burette to wine/ sap sample at complete neutralisation. Turning of a pink colour development was recorded. Likewise, three concurrent values were recorded by taking same wine/ sap sample. By taking standard neutralisation of 0.1N NaOH (100 ml) can neutralise 9 gms of lactic acid. The amounts of lactic acid present in the given wine/ sap was evaluated. To calculate acidity,

we can use the following formula. The concentration of acetic acid in the media was determined by titration following the protocol described by Accolas *et al.*, (1977).

$$\% \text{ total acidity} = \frac{\text{ml of alkali x Normality of alkali x 9}}{\text{Weight of sampling}}$$

Alcohol:

Different concentrations of standard alcohol samples were prepared like 0.1, 0.3, 0.5, 0.7, and 0.9 in test tubes. These test tubes were made up to 1 ml with distilled water, again these test tubes filled with1% 1 ml of $K_2Cr_2O_7$ and 5 ml of concentrated H_2SO_4. Blank also prepared along with the samples. After incubation period recorded the readings. A standard curve was plotted by taking O.D values on Y-axis and concentration of alcohol on X- axis (Frank Rogerson *et al*., 2006).

Purification of the samples:

Vacuum pump filtration:

Vacuum pump filtration mainly used to separate microorganisms from fluids / broth/ liquid samples (Jensen William, 2006). Filter holder connect with funnel by using iron clamp Nitrocellulose membrane filters are used filter poured size 0.02µ. Cocoti sap and wine solution poured into funnel passes through filter and filtrate can be collected into filtered flask connected with vacuum pump.

Determination of MIC (Minimum Inhibitory Concentration):

MIC of Two sample solutions i.e. Cocoti sap and wine was determined by using Macro Dilution method (Andrews, 2001). The MIC was determined in 250 ml conical flasks containing 150 ml Nutrient broth medium was prepared. 2 sets of sterile test tubes total 14 were taken. Then the medium was poured in test tube of 10 ml volume. Then equal volume of bacterial (*E.coli* Nissle 1917) culture (OD 0.5 McFarland standard) 10 µl was added. Then, the samples of sap and wine were added in test tubes respectively, with increased concentrations. The sap concentrations were taken as 60, 80, 100, 120, 140, 160,180µl, in case of wine concentrations 60, 80, 100, 120, 140, 160µl were taken, maintained control and blank, and the tubes were allowed to incubation. The initial readings were recorded before incubation. Record the readings every one hour incubation period, the O.D values were taken at different time intervals at 670nm. Minimum 5-6 readings were recorded.

The minimum inhibitory concentration i.e. the lowest concentration of metal required to cause inhibition of bacterial growth. Which concentration shows approximately 50% growth inhibition was selected as stress concentration for the subsequent proteomic analysis.

Protein Extraction:

The proteomes of three different samples were compared. The first sample served as the control, corresponding to the *E.coli* Nissle *1917* population grown in the absence of sap and wine, and the second and third samples were *E.coli* Nissle *1917* populations adapting to the sap and wine samples. The turbidity of the culture which gradually increases indicates bacterial growth. Transfer the grown culture into fresh tube sterile conditions. Centrifuged the tube 12,000 rpm for 10 min, maintaining the temperature at 4^0C, transfer the pellet to a micro centrifuge tube and washed this pellet thoroughly with phosphate buffer to remove the unwanted debris.

The re-suspended pellet was sonicated on ice is enable the bacterial cells to break. So that the contents were released sonication involves the use of high energy sound waves that capable of breaking outer membrane of cell. All cells contains including protein of interest leak out of the disrupted membrane carryout the sonication procedure for 30 sec with the pulse of 1 sec at 40% amplitude, once it was completed, centrifuged the contents and collected supernatant. The supernatant was treated as protein sample.

Proteins were extracted by the method of Trizol protein extraction method (Chomczynski *et al.*, 1987). Trizol reagent consisting of guanidine thiocyanide, phenol and chloroform to the supernatant obtained after sonication, mixed the contents thoroughly by vortex mixture. Added chloroform to this solution, mixed the content and place the tube on ice for few minutes, centrifuged the tube at 2000 rpm for 5 minutes. Three distinct layers were obtained at this stage the top most is the aqueous layer containing RNA, inter phase is the protein and the bottom layer contains DNA. Discarded the transparent top layer having the RNA, then added absolute alcohol to the remaining layers and mixed the solution as well centrifuged the contents at 2000 rpm for 5 minutes. The DNA forms a white precipitate at the bottom of the tube, while the protein remains clear in the supernatant. Supernatant was collected in a fresh tube, and then added chilled acetone to this tube and mixed well by vortex mixture. The solution was stored at -20 ^{0}C at least one hour before

centrifugation. Protein pellet was collected, discarded the supernatant and dried the protein pellet at room temperature. Reconstitutes the dried pellet with rehydration buffer and stored overnight at -20 ^{0}C before carry out protein quantification.

Protein quantification:

Protein quantification was done by three methods i.e. Lowry's method and Bradford method and BCA kit method when compare to three methods BCA kit method gives better comprehensive results than the other methods.

Lowry's method:

In Lowry's method (Jakob *et al.,* 2002) first prepared Bovine serum albumin (BSA) working standards in test tubes upto 1 ml by using distilled water. To this solution, 4.5 ml of reagent - 1(Na_2CO_3, NaOH, Na-K Tartrate, and $CuSO_4$.5 H_2O) was added and allowed to incubation for 10 minutes. After incubation 0.5ml of reagent-II (Folin-Phenol) was added and allowed to incubate for 30 minutes. Blank was also maintained and prepared unknown solutions as mentioned above. The absorbance was measured at 660nm and standard graph was plotted. The amounts of proteins presents in the unknown sample were estimated using standard graph. O.D values on Y- axis and concentrations of known samples on X- axis were taken and calculate the unknown concentrations.

Bradford method:

Bradford method is a colorimetric analysis method used to measure the concentration of protein (Bradford, 1976). Standard protein samples were prepared with BSA ranges from 0.1-0.8 mg/ml, to this test tube added 5 ml of Bradford reagent (Coomassie-Brilliant blue G250+ phosphoric acid + glycerol) allowed to incubate for 10-30 minutes and read each of the standard and unknown samples at 595nm.

Bicinchoninic method:

Protein content was estimated by BCA (Bicinchoninic acid) method (Olsen *et al.,* 2007) to quantify the protein concentration. Before 2-D PAGE analysis, it was required to determine the

concentration of proteins in the samples, so that the same amounts of protein can be applied onto the gels for better comparison with control sample. Protein concentrations in the samples were determined based on the method of BCA (Bicinchoninic acid). Protein samples and unknown samples were prepared, mixed the BCA reagents A and B (50:1(v/v)). Then 25 µl of each standard and unknown sample were pipetted and transferred to microtiter plate. 200 µl of BCA working reagent was added to each well and allowed for incubation at 37 ^{0}C for 30 min, BCA protein assay Kit method utilizes a copper (Cu^{2+}) salt which can be reduced to the cuprous state by proteins. The generated Cu^{2+} ion forms an intensely coloured complex with the bicinchoninic acid reagent with a very strong absorbance band at 562nm by using Elisa reader. The intensity of the blue complex is proportional to the amount of protein in the sample. The BCA Protein assay is suitable for measuring protein concentration in the range of 0.5-30 µg protein (0.01-0.6 mg/ml).

2-D clean-up method:

Protein samples were collected which contains some salts and detergents. So, it requires clean-up process by using 2-D clean-up kit from GE Healthcare. 300 µl of precipitant was added to the extracted protein sample (www.gelifesciences.com). The concentration of the protein 1-100 µl sample containing 1-100 µg protein per sample. Vortex the mixture and incubate on ice for 15 minutes again added 300µl of co-precipitant to this protein solution and mixed well with the help of vortex mixture. Centrifuged the tube at 12,000 rpm for 5 minutes and remove the supernatant. Washed the pellet by using de-ionised water to this pellet and 25 µl of de-ionised water was added vortex the pellet for 5-10 sec and discarded the water. 1ml of chilled wash buffer and 5 µl wash additive were added to this pellet vortex the tube 20-30sec every 10 minutes, repeated this step at least 3-5 times. Chill the wash buffer at -20^0 C for at least one hour before starting the experiment. Again centrifuged the tube and discard the supernatant, pellet allowed to air dry. Resuspend the pellet in rehydration or sample solution of choice. Now the sample is ready to load on gel.

2-D gel electrophoresis:

Two-dimensional gel electrophoresis (2-D electrophoresis) is a powerful and widely used method (http://www.bio-rad.com) for the analysis of complex protein mixtures extracted from cells, tissues or other biological samples. This technique separates proteins in two steps, based on charge/ isoelectric point (pI) and molecular weight (MW).

Isoelectric Focusing:

Isoelectric focusing was carried out on 18 cm immobilize strips which provided a linear gradient from pH 4 to 7 (Bio-Rad) by a PROTEAN IEF Cell (Bio-Rad). For isoelectric focusing (IEF), the first dimension of 2-DPAGE, we used immobilized pH gradient (IPG) strips at both pH 4 to 7, linear gradient and carried out the experiment by using a protean IEF Cell (Bio-Rad). In the first step of IEF, we applied our protein samples and the rehydration procedure was carried out in IEF focusing tray. After rehydration, we carried out focusing. We used length focusing tray suitable for our IPG strips which was cleaned before use. First of all, we placed paper wicks above the electrodes and soaked them with 8µl ultra-pure water.

Then we diluted our protein samples to 500µg with rehydration buffer and 2 % Ampholytes were mixed with the sample. We loaded both control and stress *E.coli* Nissle 1917 sap and wine treated samples. Then we placed 320µl of our protein sample into a certain point in IEF focusing tray and avoided bubble formation. Then IPG strips were taken out of -20 ºC and allowed to thaw for 5 minutes at room temperature. Afterwards the protective layer above the IPG strips was removed and the strips were positioned in the IEF focusing tray gel side down and positioned with the positive end of the strip to the positive electrode and the negative end to the negative electrode.

To minimize evaporation and urea crystallization, the strip was then covered with 2 ml of mineral oil. Finally, the focusing tray was covered and allowed to stand for one hour at room temperature. After one hour, the IEF tray was placed into Protean IEF Cell then IPG strips were actively rehydrated with 50V application per strip for 12 hours. After rehydration, we carried out 6-stepped focusing which was as follows: phase 1, linear gradient up to 250 Volts in 15 minutes; phase 2, linear gradient up to 500 Volts in 30 minutes; phase 3, rapid gradient up to 1000 Volts in 1 hour; phase 4, linear gradient up to 5000 Volts in 3 hours; phase 5, linear gradient up to 10,000 Volts in 3 hours; and phase 6, linear gradient up to 10,000 Volts in 12 hours. Working temperature was set to 20ºC in IEF. Because above 20ºC temperature urea might be crystallized and below 20ºC temperature which cause carbamylation. So, active temperature (20º C) is necessary. It took approximately 20h for focusing to finish. After the IEF run was complete, IEF focusing tray and IPG strips were removed and strips were placed at -80ºC or immediately equilibrated with equilibrium buffers for the second dimension of the experiment.

Equilibration of Strips:

After IEF, the strips containing the focused proteins were equilibrated. This procedure is applied for formation of SDS-protein complexes, reducing the disulphide bonds and to alkylate the sulfhydryl groups. After we applied this procedure, proteins had completely unfolded structure and carried only negative charges. We used two equilibration buffers both of them containing SDS, Tris-Hcl pH 8.8, glycerol, and urea. Equilibration buffer-I contained DTT and equilibrium buffer- II contains Iodoacetamide instead of DTT. DTT is a reducing agent required for cleavage of disulphide bonds between cysteine residues. Iodoacetamide is an alkylation agent used for preventing disulphide bond formation by alkylating free sulfhydryl groups in cysteine residues.

Preparation of equilibrium buffer I:

6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20 % glycerol and 2 % DTT.

1.81 g of urea, 1.25 ml of Tris-HCl pH 8.8, 0.1 g of SDS, 1 ml of glycerol, and 0.1 g of DTT. The final volume was adjusted to 5 ml with water for one strip.

Preparation of equilibrium buffer II:

6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20% glycerol and 2.5 % Iodoacetamide.
1.81 g of urea, 1.25 ml of Tris-HCl pH 8.8, 0.1 g of SDS, 1 ml of glycerol, and 0.125 g of iodoacetamide (Sigma). The final volume was adjusted to 5 ml with water for one strip.

Equilibration buffers were prepared fresh just before use and focused IPG strips were placed into equilibration trays gel side up. Afterwards strips were treated with equilibration buffer I for 15 minutes with gentle shaking. After 15 minutes, strips were taken to a new dry tray and treated with equilibrium buffer II for 15 minutes with gentle shaking.

Table- 10: 2-D electrophoresis rehydration buffer components.

Components	Function	Final concentration	Comments
Urea	Denatures and solubilizes proteins	8 M urea or 9 M urea in some cases • For urea/ thiourea solution, use 5–8 M urea	• Urea solutions are prepared fresh or stored frozen at –20°C. • Deionize urea solutions on a mixed-bed ion exchange resin using manufacturer's recommendations. • Thiourea is used to increase solubility of some proteins (Rabilloud, 1998)
Detergent	Solubilizes the proteins and helps to maintain the proteins in solution during rehydration and IEF	Total detergent concentration range is 0.5–4%	• Use non-ionic or zwitter ionic detergents such as CHAPS, NP-40, CHAPSO, and sulfobetaines (SB3-10) (Chevallet *et al.*, 1998). • Ionic detergents such as SDS are not recommended.
Reducing agent	Cleaves the disulphide bonds in the proteins	DTT or DTE at 20 mM to 100 mM	• Use DTT or DTE (dithioerythritol). • β-mercaptoethanol is not recommended for reduction (Righetti *et al.*, 1982).
Ampholytes	Helps in solubilizing proteins and aids in maintaining the pH gradient 0.2–2%	Note: Higher ampholyte concentration requires longer focusing times.	• Use the appropriate carrier ampholytes based on the pH range of the IPG strip. • May use ampholytes pH 3–10 for all pH ranges of IPG strips.

Table- 11: Types of Rehydration:

Passive rehydration	Active rehydration
IEF parameters 20^0 C, 50 µA per strip 2:00 h at 20^0C	S1 Step-n-hold - 50 V 10:00 hrs S2 Step-n-hold - 500V for 1:00 h S3 Gradient - 1000V during 1:00 h S4 Gradient - 8000V during 3:00 hrs S5 Step-n-hold - 8000V for 56000 V hrs

The entire IEF takes 24h to finish. At the end of an IEF run strips can be stores at -80^{0}C for later use. It is convenient to start it in the next day so that it is ready for running the second dimension during the next day.

SDS-PAGE:

SDS-PAGE experiments were performed in Bio-Rad PROTEAN II XL electrophoresis cell. Dimensions of the glass plates were 16 x 20 cm for inner plate and18.3 x 20 cm for outer plate. Prior to analysis, the glass plates were assembled according to the instructions in the manufacturer's manual (http://www.lifetechnologies.com).

Pouring SDS-Polyacrylamide Gels:

As it is stated above, SDS-polyacrylamide gel contains only the resolving gel but not the stacking gel. The resolving gel for SDS-PAGE should be prepared the day before SDS-PAGE analysis, and kept at 4°C overnight. The composition of acrylamide for the resolving gel was chosen as 12% so that components of the gel could be prepared according to this value. Preparation of the resolving gel components were described below.

Preparation of Acrylamide mixture:

Acrylamide 30.0 g and Bis-acrylamide 0.8 g were dissolved in distilled water and made up to 100 ml. pH of the mixture was adjusted to 7.0 and stored at 4^{0}C.

Preparation of 1.5M Tris-HCl Buffer pH 8.8:

18.5 g of Tris base dissolved in 80ml of distilled water, pH adjusted to 8.8 with 1 n HCL made up to 100ml with water , stored at 4^{0}C.

Preparation of 0.5M Tris-HCl Buffer pH 6.8:

6.0g of Tris base was dissolved in 60 ml of distilled water pH adjusted to 6.8 with in HCL made up to 100 ml with water , stored at 4^{0}C.

Preparation of 10% SDS: 1 g of SDS (AppliChem) was dissolved in10 ml of distilled water.

Preparation of 10% ammonium persulfate:

1 g of ammonium persulfate (Sigma) was dissolved in 10 ml of distilled water. 2-D polyacrylamide gels were prepared for samples of 15°C and 30°C in a comparative manner. Since the volume of each gel amount was approximately 35 ml, necessary volume of each solution component was adjusted to prepare 110 ml of 12%SDS-polyacrylamide gel.

Table -12: 12 % separating gel components for three gels.

Separating gel components	Volume (110 ml) 12%.
Distilled water	37.4
1.5 M Tris-HCL, pH 8.8	27.5
Acrylamide/Bis acrylamide solution	44
10% ammonium persulfate	2.2ml
TEMED	100µl
SDS	0.275µl

After the preparation of all solvents they were mixed in a beaker in the above order, gel has polymerised, decant the overlay, prepare the staking monomer, add TEMED and then ammonium persulfate (APS) because to avoid degasing and pour into the gel apparatus. Insert the comb and allow polymerizing completely before running.

The final mixture was then swirled rapidly and poured into the gap between the glass plates without delay. After the completion of polymerization (~30 minutes), upper side of the gels were covered with distilled water and they were kept at 4°C overnight.

Running the Gel:

After period of equilibration of IPG strip, a 100 ml-graduated cylinder was filled with 1X Tris/glycine/SDS buffer and any bubbles on the surface of the buffer was removed using Pasteur pipette. Then, equilibrated IPG strips were dipped briefly into the graduated cylinder respectively to be rinsed in the buffer. After that, each strip was laid gel side up on to the longer (back) glass plate,

and connected with the gel without any air bubble at the interface. The glass plates were then held vertically by placing them in the gel box, and one ml of preheated overlay agarose solution was pipetted into the IPG well of each gel. After allowing the overlay agarose solution to solidify for 5 minutes, the reservoir of the gel box and the gap between the gels were filled with 1X Tris/glycine/SDS running buffer. Water circulation for controlled cooling of electrophoresis was enabled and electrophoresis was started according to the conditions. The migration of the Bromophenol Blue which is present in the overlay agarose solution was used to monitor the progress of the electrophoresis. When it reached the bottom of the gel, electrophoresis was stopped. The given Table (13) illustrates the running conditions of SDS-PAGE.

Table- 13: 2-D PAGE running conditions.

Program	**Power conditions**	**Approximate running time**
constant current	16 mA	1-hour
constant voltage	180V	8-7 hours

Preparation of 1-X Tris/glycine/SDS running buffer:

15.0 g of Tris-base and 72 g of glycine were dissolved in 900 ml of distilled water. To this mixture, 50 ml of 10% (w/v) SDS was added on and the final volume was adjusted to 1000 ml with distilled water. 200 ml of this solution was taken and diluted to 1000 ml with distilled water before use.

Table -14: 5 X Running Buffer, pH 8.3

Components	**volume**
Tris Base	50g
Glycine	72g
SDS	5g
Distilled water	1000ml

Protein gel staining techniques:

After 2-D PAGE, the separated proteins have to be visualized, either by “universal” or by "specific" staining methods. Since the concentrations of individual proteins in a single cell differ between six or seven orders of magnitude, ranging from several millions of copies/cell for some highly abundant proteins (*e.g.*, glycolytic enzymes) to a few copies/cell for very low abundant proteins, these enormous variations in protein concentrations are a major challenge for almost all currently available protein detection methods. The most important properties of protein visualization methods are high sensitivity (low detection limit), high linear dynamic range (for quantitative accuracy), reproducibility, and compatibility with post electrophoretic protein identification procedures, such as mass spectrometry. Unfortunately, currently no staining method for 2-D gels meets all requirements for proteome analysis.

Here we use Colloidal coomassie staining technique, after electrophoresis was finished; gels were removed from glass plates and transferred to a large tray. Gels were rinsed with milli Q Distilled water. For Colloidal coomassie blue detection, gels were fixed for 1 h in 20% ethanol 7% acetic acid then wash gel in water 3 times for 10 minutes each. Remove the wash solution and cover the gel with Colloidal coomassie stain. Stain the gel with continuous gentle agitation for at least 3 hrs for maximal sensitivity. Specific staining can be seen in 30–90 minutes. For convenience, gels may be left in the stain solution overnight (16–18 hrs) without over staining. Rinse the gel in 10% methanol (ethanol), 7% acetic acid for 30–60 minutes. This rinse step decreases background fluorescence, wash gel in water before imaging.

Image analysis by using Image master 2-D platinum 6.0:

Image analysis is the most important step in proteomics is to identify the differentially expressed proteins between control and stress samples run on a series of 2-D gels i.e., the protein spots that have been down-regulated, up-regulated or decreased or increased the spot size. Once these gel features have been found, the proteins of interest can be identified using MS (Model voyager De-STR, applied Bio-systems, Foster, CA, USA). This goal is usually accomplished with the help of computerized image analysis systems. The colloidal coomassie blue stained gels were scanned and photographed by gel scanner (Typhon variable mode imager) with a 488 nm laser. The 2-D gel image analysis was performed with image master 2-D platinum 6.0 software and each gel was analyzed for spot detection. The gel image showing the higher number of spots and the best

protein spot sets were created, pattern was chosen as a reference template, and spots in a standard gel were then matched across all gels. Spot quantity values were normalized in each gel dividing the raw quantity of each spot by the total quantity of all the spots included in the standard gel. In order to analyse gel similarities or experimental variations such as disparities in stain intensity or sample loading, one can produce Scatter plots for groups. Scatter plots give an idea of the relationship between the spot values from two gels by searching for the linear dependence between the spot values of one gel in comparison to another gel. Spot sizes, Mean, Standard deviation, Coefficient of Variation in each group were determined. Transferred the data square root to $Sin^{-1}\sqrt{p}$. After performing $Sin^{-1}\sqrt{p}$ transformation independent samples t- test was conducted in order to compare the two groups and identify sets of proteins that showed a statistically significant difference with a confidence level of 0.05 and a minimum two fold of variation. The spots in these sets were excised from gels using Spot Cutter for further analysis of spots and explain the steps involved in using Image master 2D platinum 6.0.

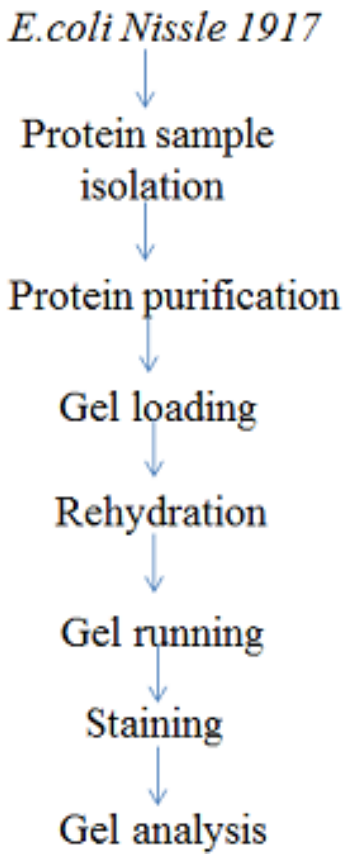


Figure-8: Flow chart of 2-D analysis work flow.

Statistical analysis of the gel regulation:

Independent t-test (Fisher Box *et al.*, 1987) was performed to analyse the significant difference between up and down regulation values. The t-test can be used even if sample sizes are very small, as long as the variables within each group are normally distributed and the variation of scores within the two groups is equal. With the t-test, the test statistic used to generate p-values has a student's distribution with n-1 degrees of freedom

Table -15: Up-regulation values of proteins under exposure of sap and wine treatment.

Spot no	Sap up-regulation	Wine up-regulation	Average Up-regulation	p-value	Square root(p)	Variable
427	1.4392	1.0146	1.2269	0.1021	0.3195	18.6327
488	1.3880	1.6292	1.5086	0.1255	0.3542	20.7444
478	1.0343	1.4729	1.2536	0.1043	0.3229	18.8384
415	1.3391	1.4478	1.3934	0.1160	0.3405	19.9073
472	1.0400	1.3275	1.1841	0.0985	0.3138	18.2884
324	0.9836	1.1779	1.0807	0.0899	0.2998	17.4456
348	1.0285	1.1774	1.1029	0.0918	0.3029	17.6319
507	0.6155	1.0965	0.8560	0.0712	0.2668	15.4739
468	1.1728	1.0039	1.0883	0.0906	0.3009	17.5117
345	1.6316	1.0024	1.3170	0.1096	0.3310	19.3295
			Total = 12.0115			

Table- 16: Down- regulation values of proteins under exposure of sap and wine treatment.

Spot no	Sap down-regulation	Wine down -regulation	Average Down-Regulation	p-value	Square root(p)	Variable
595	2.1437	1.1589	1.6513	0.20607	0.45395	26.9974
656	1.9544	1.4716	1.7130	0.21377	0.46236	27.5395
457	1.7074	1.4133	1.5603	0.19472	0.44127	26.1849
276	1.3755	0.9965	1.1860	0.14801	0.38472	22.6264
376	1.4453	2.3600	1.9026	0.23743	0.48727	29.1613
			Total =8.0132			

The Up-regulation and Down-regulation values from the data were calculated, and get the p-values and Variables. Transferred the data square root to $Sin^{-1}\sqrt{p}$. After performing $Sin^{-1}\sqrt{p}$ transformation independent samples, t- test was conducted.

Protein identification:

Mass spectrometry is an important emerging method (Model voyager De-STR, applied Bio-systems, Foster, CA, USA) for the characterization of proteins from excised 2-D gel spots as these methods are very sensitive, it requires small amount of sample and have the capacity for high sample throughput protein analysis. Recent advances in mass spectrometry also allow the investigation of post translational modifications including phosphorylation and glycosylation. Matrix-assisted laser/desorption ionization mass spectrometry (MALDI-MS) is the most commonly used technique to perform Peptide mass fingerprinting (PMF). It is also known as Peptide mass mapping.

Peptide mass fingerprinting by MALDI-TOF and sequencing by tandem mass spectrometry have evolved into the major methods for identification of proteins following separation by two-dimensional gel electrophoresis. This technique, which is user friendly and quite fast. The standard approach to identify proteins includes separation of proteins by gel electrophoresis. In gel electrophoresis special caution must be taken to avoid contamination of the protein samples with keratin. To avoid contamination gels should be sealed as soon as possible after staining. Subsequently, the proteins are cleaved with sequence specific end- proteases or Proteolytic enzymes such as trypsin, chymotrypsin, mainly trypsin is used for protein digestion to produce peptides with molecular masses in the optimal range for MS analysis. For protein identification, the experimentally obtained finger print masses are compared with the theoretical peptide masses of proteins stored in databases by means of mass search programs.

Peptide mass fingerprinting MALDI-TOF-MS:

Spots were picked from colloidal coomassie blue stained gels placed into tubes, add 200 µl of 25 mM NH_4HCO_3 / 50% acetonitrile and vortex these mixture for 10 min discard the supernatant. Repeat this step 3- 4 times until the gel pieces are colorless and discard the supernatant. Spots were treated with 100% acetonitrile for dehydration allowed to stand for few minutes until the gel pieces shrink and turn white. Remove the acetonitrile and the spots allowed for drying by using Speed Vac process. 25µg trypsin solution was added to the dried spots. Allow the gel pieces to re-hydrate with trypsin at 4^0C for 60 minutes and remove the supernatant was discarded to minimize auto-digested for 10 min. 25mM NH_4HCO_3 was added it will prevent the gel from drying, incubate at 37^0C overnight (12-16 hrs).

On the sample slides 0.25µl α-cyano-4-hydroxycinnamic acid in acetonitrile (1mg/ml) were pipetted and air dried (seed-layer). Then 0.5 µl digested sample was added to the slide and mixed with 0.5 µl matrix solution (15 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile + 0.5% tri-fluoroacetic acid) and air dried for 10minutes.

Mass spectrometry was performed by using the (Model Voyager-DE STR, Applied Bio-systems, Foster, CA, USA). The spectra measured for unknown peptides were compared against the mass peaks derived from calibration of internal standards. Spectra were collected over the mass

range of 800–3500 Da and integrated with the MASCOT 2.2 search engine (Matrix Science, http://www.matrixscience.com) was used for spot proteins identification by querying the trypsin digested peptide fragment data using the reference database NCBInr with *E. coli*. Known keratin masses and trypsin auto digest products were excluded from the searches. Partial modifications of proteins by carbaminomethylation of cysteine and oxidization of methionines were taken into account. Protein homology was assigned, if at least four peptide masses with a sequences coverage 15% or higher were matching within a maximum of 100-ppm error spread across the data set. The number of missed cleavage to be significant difference ($p< 0.05$).

Table - 17: Recommended Search parameters for search MALDI-MS and MS/MS data.

Search parameter	**Value**
Database	NCBInr
Taxonomy	*E.coli*
Enzyme	Trypsin
Allowed up to	1 missed cleavage
Fixed modifications	None
Variable modifications	Carbamidomethyl (cysteine) Oxidation (Methionine)
Protein mass	None
ICAT	None
Data format	Mascot generic

Generation of 3-D- structure models –Homology modeling:

In this study, the 3-D structures of the stress expressed proteins are modeled generated by using phyre-2 programme. The primary structure for the stress expressed proteins can be obtained from SWISS-PROT database (http://www.expasy.org/). The modeling step can be carried out by searching the stress expressed protein sequences against the databases of well-defined template sequences derived from protein Data Bank entry (http://www.rcsb.org/pdb/).

The modeling step can be carried out by searching the metal stress expressed protein sequences against the databases of well-defined template sequences derived from Protein Data Bank entry (http://www.rcsb.org/pdb/).

1. Homology derived restraints on the 3-D geometrical information including the distances and dihedral angels in the unknown query sequence, obtained from its alignment with the template structures (Mac Kerell *et al*., 1998).
2. Stereo chemical restraints such as bond length and bond angle preferences, obtained from the CHARMM22 molecular mechanics force field (Sali *et al.*, 1994).
3. Statistical preferences for dihedral angles and non-bonded inter atomic distances, obtained from a representative set of known protein structures (Marti-Renom *et al.*, 2001).

The SWISS PROT searches the templates used for model building, which is representative of multiple structure alignments that can be obtained from CLUSTAL W. (Larkin *et al.,* 2007). Sequence profiles are defined as the sequence position- specific scoring matrix. This scoring matrix is designed for both the differently expressed proteins under stress proteins (target) sequences and the template by searching in contrast with the Swiss-Prot/ TrEMBL database of sequences. The BUILD PROFILE module of phyre-2 executes this sequence profile construction. The BUILD PROFILE command has many options. Unrecognized residues are ignored. In this study, the structural homolog search is set to use the BLOSUM62. Because, the parameters for the gap penalties are set to the appropriate values for the BLOSUM62 matrix. A match is reported if its falls below the threshold set. Lower E value thresholds are more stringent and report fewer matches. Many hits were displayed on the basis of the sequence identity and E value between the protein sequences.

Multiple sequence alignment:

Multiple alignments of protein sequences (MPS) are important tools in studying sequences. (Chenna *et al.*, 2003). CLUSTAL X, W is a general purpose multiple sequence alignment programs for proteins. It produces biologically meaningful sequence alignment of divergent sequences. It calculate the best match for the selected sequences, it brings out both evolutionary and structural similarity among the proteins encoded by each sequence in the alignment. Evolutionary relationships can be seen via viewing Cladograms or Phylograms. Sequences can be aligned across their entire length or only in certain regions. This is true for pair wise alignment and multiple alignments. Global alignments need to use gaps while local alignments can avoid them, aligning regions between gaps.

Target-template alignment:

The alignments between the stress expressed proteins and templates are executed by SWISS MODELL. Target sequence, template structure matches are determined by aligning the target sequence profile against the template profiles, by using CLUSTALW and MUSCLE programmes (Schwede *et al.,* 2003). In order to analyze the close relation between the target and template protein sequences we carry out the comparative modeling procedure. Comparative modeling requires the information on target template alignment. Now the matching parts of the template structure and unknown sequence were realigned by the use of Kalign-SBC, it is fast and accurate multiple sequence alignment tool.

This command executes a global dynamic programming method for comparison between the target-template sequences and also relies on the observation that evolution tends to place residue insertions and deletions in the regions that are solvent exposed, curved, outside secondary structure segments, and between two Cα-β positions close in space (Melo *et al.,* 2002). Gaps are included between the target-template alignments, in order to get maximum correspondence between the protein sequences. Gaps in these regions of high correspondence are favoured by the variable gap penalty function that is executed from the template structure alone.

In principle, the error between the target-template alignments is greatly minimized almost by one-third relative to the present day sequence alignment methods. Models are built for each of the sequence-structure matches using SWISS MODEL. Nevertheless, there is clearly a need for even more accurate sequence-structure alignments and for using multiple template structures, so that more accurate models are obtained. The resulting models are then evaluated by a composite model quality criterion that depends on the compactness of a model, the sequence identity of the sequence-structure match and statistical energy Z-scores.

Homology Modeling Using the Swiss-Model Server:

In this section, we are discussing about the generation of the three dimensional structure for the unknown metal stress expressed protein sequence (target) with templates as its suitable structural homolog. SWISS MODELL is the software tool used for 3-D model building (Arnold *et al.,* 2009). Successful model building requires at least one experimentally determined 3-D structure that shows significant amino acid sequence similarity with target sequence. Building a homology model contains four main step i.e. identification of structural template, alignment of target of sequence and template structure, model building, and model quality evaluation. These steps can be

repeated until a satisfying modeling result is achieved. Each of the four steps requires specialized software and access to up-to-date protein sequence and structure databases.

Protein sequence and structure database necessary for modeling are accessible from the workspace and are updated in regular intervals. Software tools for template selection, model building, and structure quality evaluation can be involved from within the workspace. The output of this module displays many restraints parameters between the target template alignments including the distances, main chain dihedral angles, side chain dihedral angles, disulphide dihedral angle, NMR distant restraints and non−bonded restraints between these two proteins (Mac Kerell *et al.,* 1998). These relationships are expressed as conditional probability density functions (pdf's) and can be used directly as spatial restraints.

Homology modeling (Guex *et al.,* 2009) is used to build three-dimensional models for a protein target based upon a single or multiple templates with known structure. The accuracy of homology modeling is dependent on the fact that evolutionarily related protein sequences often have similar three-dimensional structures. The homology modeling procedures is a multi-step process that can be summarized in the following steps i.e. Sequence alignment of the target and template, target backbone generation, target loop modeling, target side-chain modeling and target model refinement.

Structure validation:

Validation refers to the procedure for assessing the quality of deposited atomic models (structure validation) and for assessing how well these models fit the experimental data. Validation parameter includes the covalent bond distances and angles, stereo chemical validation, atom nomenclature are taken care. Moreover all the distances between the atoms including the water oxygen atoms and all polar atoms (oxygen and nitrogen) of the macromolecules, ligands and solvent is calculated (http://psvs-1_4-dev.nesg.org/).The results are displayed along with the PROCHECK server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum) and Ramachandran ‘s plot (Rampage Ramachandran’s plot server).

Ramachandran’s plot displays the phi and psi backbone conformational angles for each residue in a protein. The phi angle is the angle of right-handed rotation around N-Ca bond and the psi angle is the angle of right-handed rotation around Ca-C bond. Phi and psi angles are also used in the classification of some secondary structure elements such as alpha helix and beta turns. In a Ramachandran’s plot, the core or allowed regions indicates the preferred areas for psi/phi angle pairs for all residues in a protein. If the determination of protein structure is reliable, most pairs will be in the favoured regions of the plot, some pairs will be in the allowed region, and only a few will appear in ‘disallowed’ regions.

Phylogenetic analysis:

The phylogenetic tree (phylogeny) (http://www.phylogeny.fr/) or evolutionary tree is a branching diagram or tree showing the inferred evolutionary relationships between the studied data. The data must be comprised of homologous types. Computational phylogenetics is the application of computational algorithms, methods and programs to phylogenetic analyses. The goal is to assemble a phylogenetic tree representing a hypothesis about the evolutionary ancestry of a set of genes, species, or other taxa. The studied data is allowed to phylogenetic analysis by using Bio-Edit tool version 7.0.9, it is used for several modes of alignment, Automated ClustalW alignment, Plasmid drawing and annotation.

Analysis of physico-chemical parameters of a sequence:

Protparam is one among the protein analysis tool available on the ExPasy server. (http:// www.expasy.org/tools/protparam.html). It is used for calculating various physiochemical parameters of a provided protein. The protein sequence is the only input provided to calculate such parameters. The protein can be either is specified as a UniProtKB/Swiss-Prot accession number or ID or as sequences of amino acids. The various parameters computed by Protparam are molecular weight, amino acid composition, extinction coefficient, estimated half-life, theoretical pI, and Grand average of hydropathicity (GRAVY), aliphatic index and instability index.

4. RESULTS AND DISCUSSION

E.coli Nissle 1917 is a Gram –ve rod shaped probiotic bacteria. It is present in human gut. Protein samples of *E.coli* Nissle *1917* exposed to *Cocos nucifera* sap and wine treatment was analyzed on 2-D PAGE and differentially expressed proteins were identified by using Image master 2-D platinum 6.0. MALDI-TOF MS/MS and Mascot search were also performed to determine the differentially expressed protein structure and characterization. Proteomics can produce more accurate results and comprehensive information than what genomics studies can provide because protein expressions are regulated not only at transcriptional level but also at translational levels resulting in more details about expressed proteins and their interactions than genome based prediction.

The reason for selecting *E.coli* Nissle 1917, it is easy to handle, short-term replication period, and cultivated in normal lab conditions. Probiotic *E.coli* Nissle 1917 has been reported in various gastrointestinal disorders ranging from childhood diarrhoea to inflammatory bowel diseases. The strain is a Non LAB and non-pathogenic member of the Gram – negative bacteria species *E.coli.* Even though *E. coli* is the most studied of all bacteria, and we know the exact location and sequence of 4,288 genes on its chromosome, we do not fully understand its ecological relationship with humans and this strain have been well characterized (Grozdanov *et al.,* 2004).

Cocoti wine is the fermented sap of palm trees. It has been reported fermented palm wine exposure could cause prenatal osteo-inhibitory effects on bones (Eluwa *et al*., 2010). Palm wine (10ml kg^{-1}/body weight) caused significant decrease in sperm count, motility and viability but no significant change in morphology (Oyedeji *et al.*, 2012). Palm wine per orally caused 21.8% decrease in gastric acid secretion. But 4% ethanol caused significant increase, while 12% sucrose caused significant decrease in gastric acid secretion in rats. The inhibitory effect of palm wine may be of clinical significance in the management of peptic ulcer prone subjects in this region (Lbu *et al.,* 1986). The effects of fermented Cocoti bevarages are similar to those of the other high alcohol content liquors and drinking too much can result in the symptoms of alcohol poisoning (Ratsch *et al*., 1998).

In this study we analysed three different types of protein samples isolated from *E.coli* Nissle 1917, i.e. one is control sample remaining two were treated samples causing the stress conditions with reference to the cocoti sap and cocoti wine. We analysed all the well-known physico - chemical properties of cocoti sap and wine.

Physical factors: Colour, Odour, Taste, pH and Turbidity.

Chemical factors: Acidity and Alcohol.

Colour of the sample solution (cocoti sap and wine) was measured by using 1.245g of potassium chloroplatinate and 1 g of cobaltous chloride dissolved in distilled water and makes it to 1000ml. The colour of cocoti sap is in white and cocoti wine is in milky white because in fermentation process, microorganisms evolved CO_2 gas with white bubbles. In case of odour, cocoti sap evolves sweet smell but wine gives sour smell with foam which indicates formation of carbon dioxide. Taste of the cocoti sap is better than the wine taste. Wine was sour/ tangy due to the acidic level. When fermentation, time increases taste of wine also differed. Sap is in sweet taste when it undergoes fermentation taste converts into sour. The pH falls sharply with in the 3 hours in both sap

and wine. The fall is from near neutral of pH 6.8 to pH 4.5. In case of wine, the pH rate decreases very sharply within 3 hours the pH converts from 4.2 to 3.1. After 12 hours, the pH stabilizes around 2.5 in wine.

Table – 18: pH difference in between cocoti sap and wine.

S.No	Time intervals in minutes	pH meter readings	
		Sap	Wine
1	30	6.8	4.2
2	60	6.3	4.1
3	90	6.1	3.8
4	120	5.7	3.5
5	150	5.1	3.3
6	180	4.5	3.1

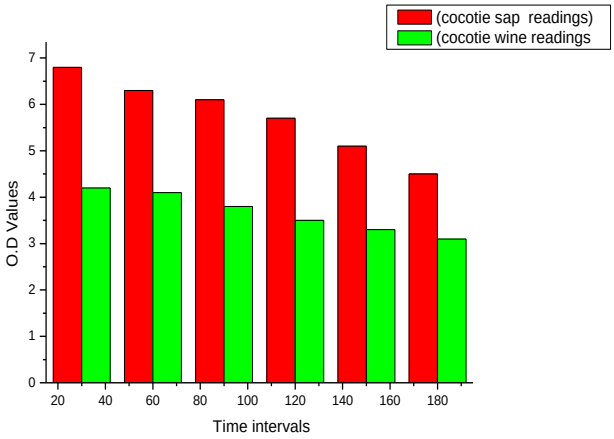


Figure-9: pH difference in-between cocoti sap and wine.

The turbidity of cocoti sap was slightly transparent solution, but in the case of wine it is different. Turbidity indicating the growth rate of microorganisms (or) fermentation rate was enhanced due to higher fermentation processing. Old cell debris is settled down on bottom of the container. Cells within a culture scattering the light make it harder to see cloudy. It should be above 2 NTU (Nephelo Turbidity Units). For analysis of total acidity, titration method was employed in seven samples. The average total amount of acidity in cocoti sap and wine was 2.59g/100ml and 4.75g/100ml respectively. There is a sharp rise in titratable acidity of cocoti sap after 4 hours. This is probably due to the *Acetobacter* species. Conversion of alcohol to acetic acid may have occurred. The causation of active fermentation creates aerobic conditions which favours this organism.

Table-19: **Total acidity levels of cocoti sap and wine.**

S.No	Volume of the sample (ml)	Volume of the NaOH rundown (ml)	
		Sap	wine
1	5	3.0	4.7
2	5	2.7	4.1
3	5	2.4	4.7
4	5	2.1	4.4
5	5	2.2	4.6
6	5	2.4	4.5
7	5	2.1	4.4

Cocoti sap and wine consists of so many microorganisms. They enhance the rate of alcohol fermentation. Microorganisms convert the sap sugars into alcohol and amino acids. Alcohol percentage majorly depends on temperature and pH. Cocoti sap concentration is 70µl/ml and wine alcohol content is 120µl/ml. Fermentation is a metabolic process that converts sugar to acids, gases and alcohol with the help of microorganisms like yeast and bacteria. The following equation represents the fermentation process in a single step.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Table-20: Total alcohol content in cocoti sap and wine.

S.No	Sample volume in ml	D.Water in ml	Concentration µl/ ml	$K_2Cr_2O_7$ solution in ml	H_2SO_4 solution in ml	O.D values
1	0.1	0.9	100	1	5	0.10
2	0.2	0.8	200	1	5	0.21
3	0.3	0.7	300	1	5	0.22
4	0.4	0.6	400	1	5	0.28
5	0.5	0.5	500	1	5	0.31
6	Blank	0.0	000	1	5	0.00
7	cocoti sap(0.5)	0.5	----	1	5	0.11
8	cocoti wine(0.5)	0.5	----	1	5	0.16

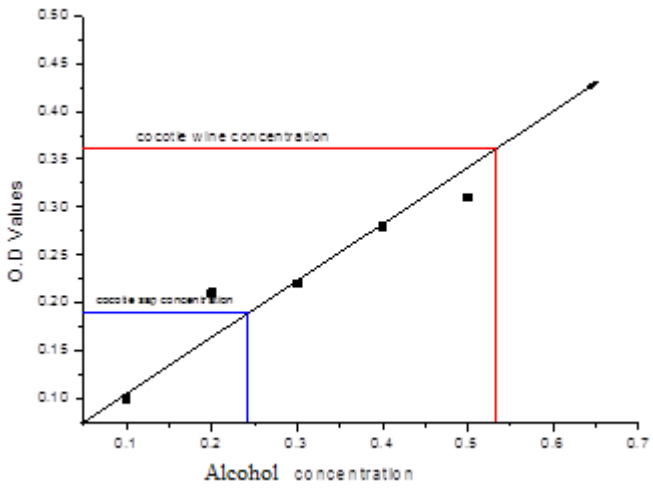


Figure-10: Alcohol concentration in cocoti sap and wine.

Minimum Inhibitory concentration Determination:

High concentration of cocoti wine and also sap causes damage to our intestine because sample contains some amount of micro elements. High concentration of wine and sap can affect the metabolic activities of *E.coli.* The term Minimum Inhibitory Concentration (MIC) i.e. the lowest concentration of sample required to cause inhibition of bacterial growth, which concentration shows approximately 50% growth inhibition. It was selected as stress concentration for the subsequent proteomic analysis. 180 µl of the cocoti sap sample inhibit 50% growth of the *E.coli* Nissle *1917.* In case of the cocoti wine sample inhibition range of concentration is 120 µl. At this particular concentrations *E.coli* can't shows more resistance compared to the lower concentrations (John *et al*., 2005).

Table: 21: By dilution methods, the O.D values represented the cocoti sap influence on *E.coli.*

Time intervals

concentrations	1	2	3	4	5
S1	0.06	0.05	0.09	0.08	0.07
S2	0.09	0.10	0.14	0.12	0.10
S3	0.14	0.11	0.19	0.16	0.16
S4	0.11	0.10	0.15	0.14	0.14
S5	0.09	0.09	0.14	0.12	0.11
S6	0.08	0.08	0.12	0.08	0.08

Table-22: Two-way ANOVA for sap influence on *E.coli*.

Source of Variation	df	Sum of Squares	Mean sum of square	F- calculated value	p-value
Time intervals	4	7.055	1.764	21.2123**	0.000
concentrations	5	14.764	0.082		
Error	20	1.640			
Total	29	23.459			

Table -23: By dilution methods, the O.D values represented the cocoti wine influence on *E.coli*.

Time intervals

concentrations	1	2	3	4	5
W1	0.03	0.02	0.06	0.06	0.06
W2	0.03	0.04	0.05	0.04	0.04
W3	0.02	0.04	0.08	0.03	0.03
W4	0.03	0.02	0.05	0.03	0.03
W5	0.03	0.03	0.05	0.03	0.03
W6	0.01	0.02	0.05	0.04	0.04

Table-24: Two way ANOVA for wine influence on *E.coli*

Source of variation	df	Sum of Squares	Mean sum of Square	F-calculated value	p-value
Concentrations	5	5.857	1.171	1.463^{NS}	0.24^{NS}
Time intervals	4	25.274	6.319	7.889**	0.001^{**}
Error	20	16.019	0..801		
Total	29	47.15			

Note: NS: Not Significant; **: Highly Significant

ANOVA was carried out by taking concentrations as rows and time intervals as columns the results of two way ANOVA represented in the table-23.

P- value for concentrations in greater than 0.05 whereas for time intervals is less than 0.05, we concluded that there is no significant difference occur between concentrations and there is high significance difference between time intervals.

Growth curves:

The present results indicating that the high concentration of cocoti sap and wine shows pathogenic effect on probiotic *E.coli* growth. Growth curves also evidenced to the said statement. The control samples shows higher O.D values than cocoti sap and wine treated samples. The results concluded that fermented cocoti wine shows higher pathogenic effect on probiotic *E.coli* when compared to the sap. Both are harmful on gastro intestinal probiotic organisms and it may leads to the disorders of the human intestine. Growth curves were analysed and presented in the following table-25 and in figure-11.

Table- 25: Growth curves of *E.coli* under cocoti sap and wine treatment.

Time intervals (min)	O.D values		
	Control	Palm sap treated	Palm wine treated
30	0.03	0.02	0.02
60	0.05	0.05	0.02
90	0.09	0.09	0.06
120	0.16	0.12	0.11
150	0.21	0.15	0.12
180	0.26	0.17	0.12
210	0.28	0.17	0.12
240	0.28	0.17	0.12

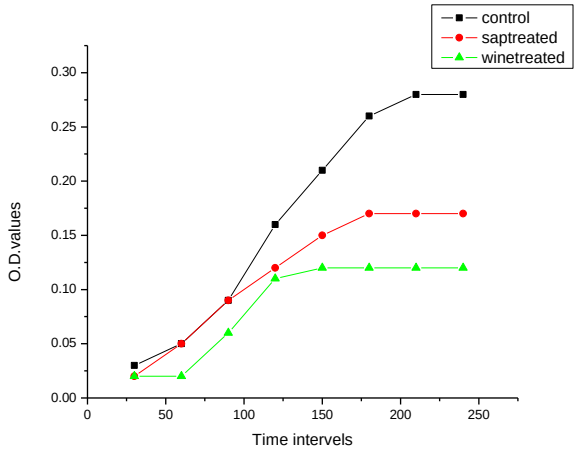


Figure-11: Growth curves of control, sap and wine treated *E.coli.*

Proteins isolation and purification:

There are several methods to isolate proteins from the cell or tissue i.e. sonication, freezing, homogenization, thawing by high pressure, filtration or permeabilization by organic solvents. We used sonication process for isolation of proteins from *E.coli* cells. Re-suspended pellet was sonicated on ice is enable the bacterial cells to break. So that, the contents were released sonication involves the use of high energy sound waves that capable of breaking outer membrane of cell. All cells contains including protein of interest leak out of the disrupted membrane carry out the sonication procedure for 30 sec with the pulse of 1 sec at 40% amplitude, once it was completed, centrifuged the contents and collected the supernatant. Protein samples were purified because 2-D electrophoresis is very sensitive to salts and detergents.

Proteins quantification:

Protein quantification is a major step in 2-D electrophoresis because it is based on the quantification rate sample should be uploaded into the IPG strips.

Quantification:

Total proteins of the control, sap and wine treated *E.coli* through the Lowry's method were analysed and presented in the following table-26 and in figure-12.

Analysis of proteins by Lowry's method:

Table-26: Total protein concentration in control, cocoti sap and wine treated *E.coli* by Lowry's method.

Concentrations	O.D values
0.2	0.1349
0.4	0.2901
0.6	0.4261
0.8	0.6830
1.0	0.9019
Normal	0.5155
Sap	0.6065
Wine	0.4641

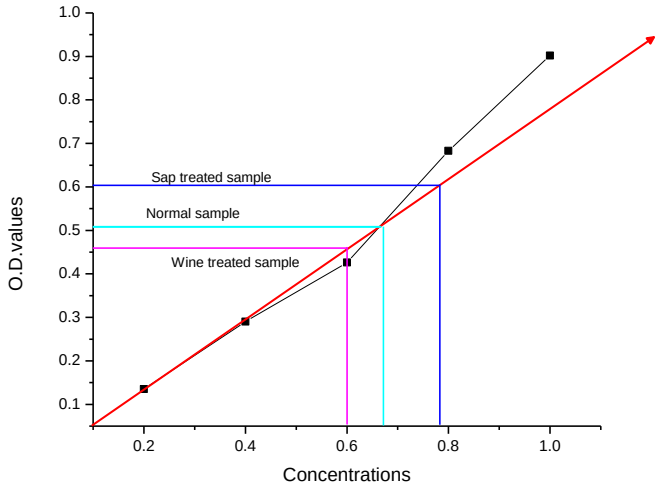


Figure-12: Total protein concentration in control, cocoti sap and wine treated *E.coli* by Lowry's method.

Bradford Method:

Total proteins of the control, sap, and wine treated *E.coli* through the Bradford method were analysed and presented in the following table-27 and in figure-13.

Analysis of proteins by Bradford – method:

Table-27: Total protein concentrations control, cocoti sap and wine treated *E.coli* by Bradford's method.

Concentrations	O.D values
0.2	0.1349
0.4	0.2901
0.6	0.4261
0.8	0.6830
1.0	0.9019
Normal	0.5855
Sap	0.6765
Wine	0.5041

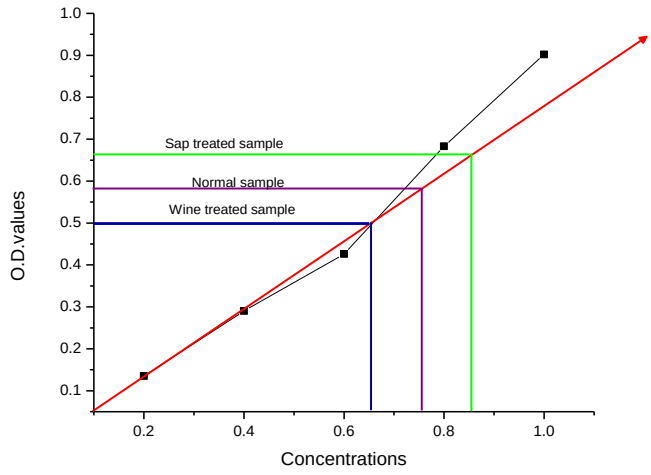


Figure-13: Total protein concentrations control, cocoti sap and wine treated *E.coli* by Bradford's method.

Analysis of proteins by BCA kit method:

Total proteins of the control, sap, and wine treated *E.coli* through the BCA method were analysed and presented in the following table 28 and in figure-14.

Analysis of proteins by BCA kit method:

Table-28: Total proteins concentration in control, cocoti sap and wine treated *E.coli* by BCA kit analysis.

S.No	Standard	control	Sap treated	Wine treated
1	0.3345	0.156	0.1556	0.1346
2	0.3641	0.142	0.1631	0.1275
3	0.3885	0.1422	0.1642	0.1239
Average	0.3623	0.1467	0.1609	0.1286
Mean ± SD	0.022	0.0065	0.0038	0.0044

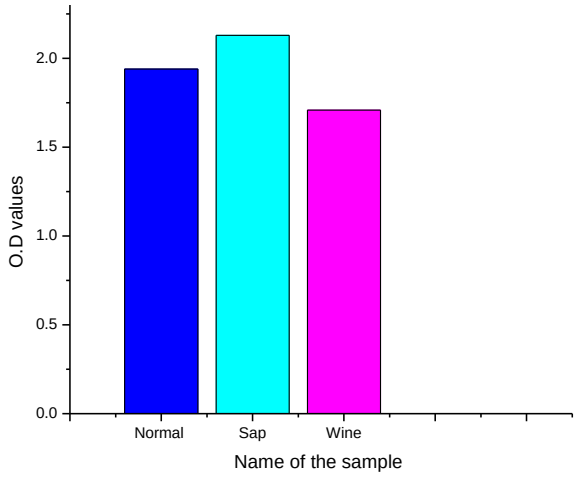


Figure -14: Histographic representation of protein concentrations.

Table-29: Total protein concentration in control, sap and wine treated samples.

Methods	control sample µg/ µl	Sap treated sample µg/ µl	Wine treated sample µg/ µl
Lowry's method	0.68	0.79	0.60
Bradford method	0.76	0.86	0.65
BCA kit method	1.94	2.13	1.709

Statistical analysis:

Statistical analysis was made using the statistical package for the social sciences (SPSS) 15.0 software. Data was analysed by one-way analysis of variance (ANOVA). In all statistical analyses, $p<0.05$ was taken as the level of significance.

Table-30: Analysis of Variance (ANOVA) of the protein concentrations.

S.V	DF	SS	MSS	Fc	Ft
Rows	2	2.85	1.42		F_{tr} at 5% significant
Errors	6	0.14	0.02	61.73	variance – 3.11
Total	8	2.99			

ANOVA indicating $F_{cr} > F_{tr.}$ H_o (Hypothesis) is rejected so all the method effects are not same.

Results showing that the protein concentrations in the sap and wine by using three different methods are variable. Out of the three methods, the analysis of protein concentrations BCA kit method was more appropriate and accurate method when compared to Lowry's and Bradford methods. The protein concentrations in control and wine treated samples are less when compared to sap treated sample, because sap has a positive influence on the growth of microorganisms. The protein concentration in the wine treated sample is decreased than the control indicating the protein synthesis has been arrested. Due to cocoti wine treatment, the protein concentration is decreased when compared to the normal sample and sap treated samples indicating wine is toxic to the probiotic *E.coli* Nissle 1917. When bacterial cell growth increases automatically protein content is increased, quantification of protein is an important for proteomics, especially before proceeding IEF (Iso Electro Focusing) BCA reagent is approximately 10 times more sensitive. BCA assay is easier and faster method than the Lowry's and Bradford's method. It is a preliminary study and also evidenced the further research work; 1D-PAGE, 2D-PAGE, MS analysis, protein expression, and clinical diagnosis of altered protein levels in body fluids, indicative of a variety of toxicants/diseases.

Differentially expressed proteins in sap and wine treated *E.coli* identified by 2-D gel electrophoresis:

The effect of cocoti sap on *E.coli* Nissle 1917 is caused changes in protein synthesis. We analyzed the proteomic response to sap, to identify differentially expressed proteins, which could be important for resistance to this sample. *E.coli* Nissle 1917 cells were treated with 180µl sap. Isolated sap treated and untreated protein samples from *E.coli* Nissle 1917 and then sample can be subjected to comparative 2-D gel electrophoresis. Variations were analysed by using Image master 2D platinum software based on the spot size /spot variation. When compared to the normal, spot size increases in treated sample, the type of spot named as up-regulated spot, the spot size decreases named as down-regulation. We noticed 7 differentially expressed proteins induced by sap treatment. Over all these 5 proteins showed good structure prediction remaining of these proteins concentration is low for structure prediction. Search results shows significant difference, which considered as ($P< 0.05$). *E.coli* cells under stress compared to control cells, the stressed cells induce many proteins.

We analysed control, cocoti sap and wine treated protein samples by using 2-D gel electrophoresis. In gel images, it shows the variations in all the three samples i.e. control, cocoti sap and cocoti wine treated samples. Based on the protein regulation, we were noticed 15 differentially expressed spots in cocoti sap and wine treated sample when compared to control sample. Differentially expressed proteins of *E.coli* Nissle 1917 under cocoti sap and wine stress 2-D gel electrophoresis were represented in the following figures -15, 16 &17.

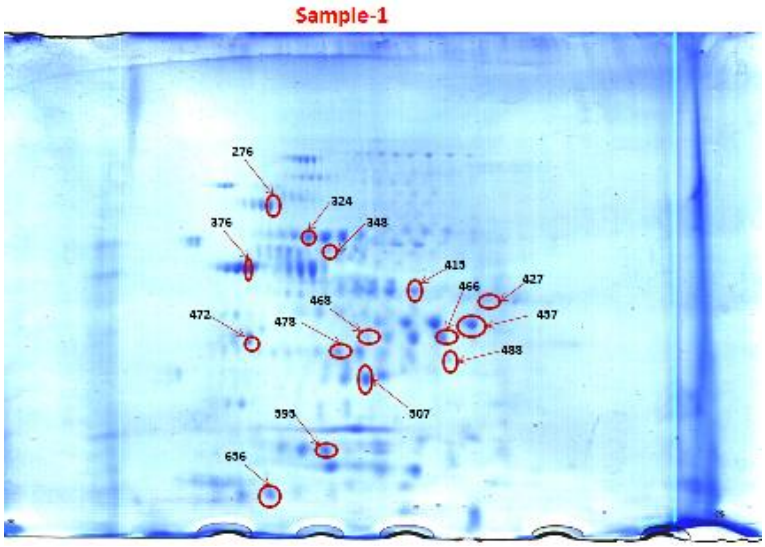


Figure- 15: Representative 2-D gel electrophoresis image of control *E.coli* Nissle 1917, covering pI range of 4 to 7. The locations of the spots are marked on the gel.

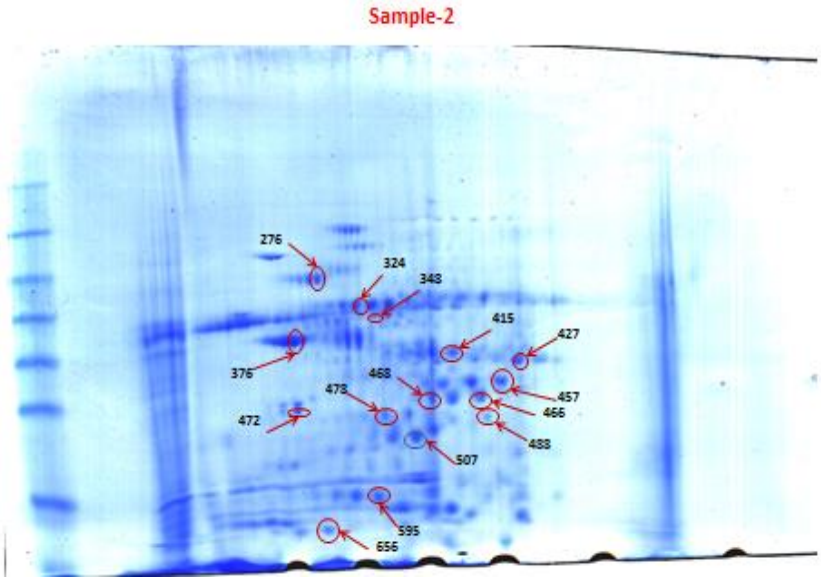


Figure-16: Representative 2-D gel electrophoresis image of cocoti sap treated *E.coli* Nissle 1917, covering pI range of 4 to 7. The locations of the spots are marked on the gel.

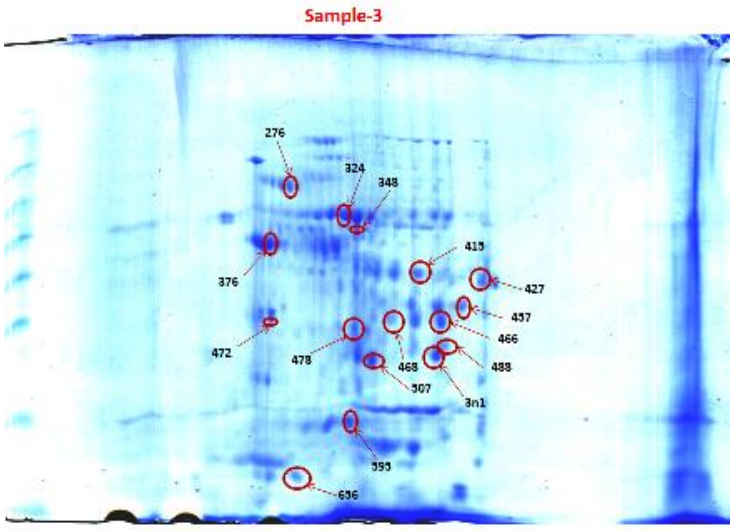
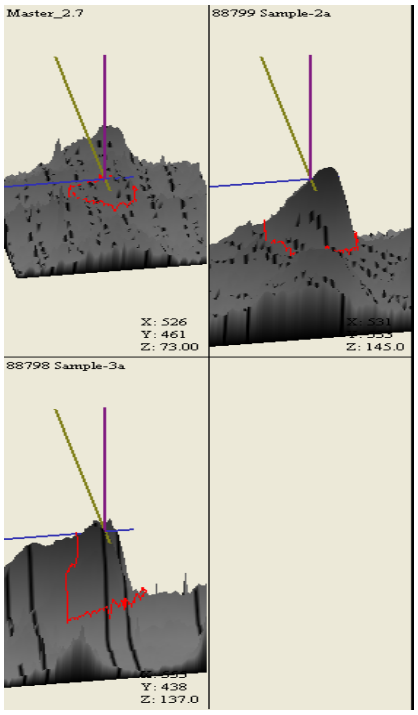


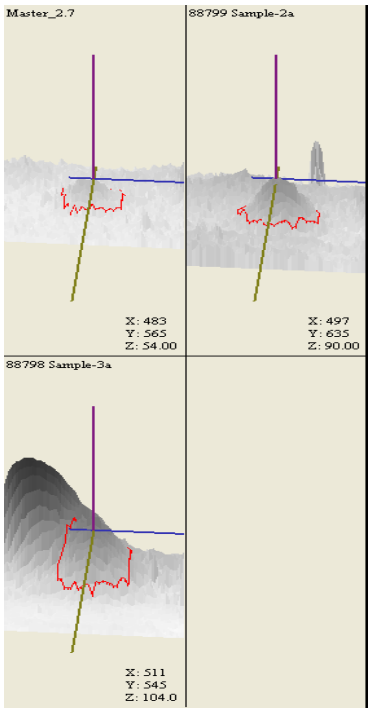
Figure-17: Representative 2-D gel electrophoresis image of cocoti wine treated *E.coli* Nissle 1917 covering pI range of 4 to 7. The locations of the spots are marked on the gel.

Differentially expressed *E.coli* Nissle 1917 proteins regulation with response to cocoti sap and wine:

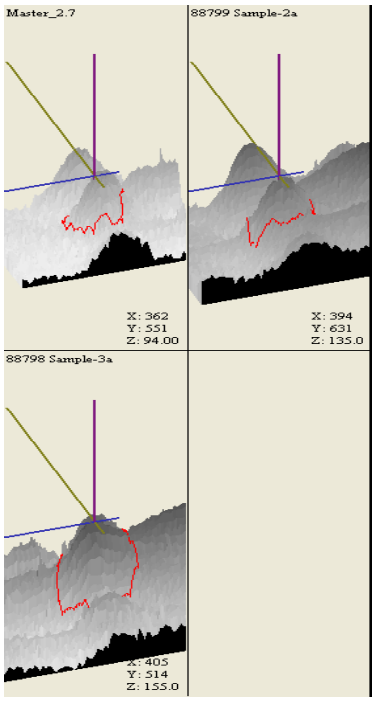
Analysis of differentially expressed proteins is one of the major challenges in proteomics. Identification of expressed proteins, whose encoding genes are differentially expressed. Its significance in biology and medicine is evident. Like in order to understand how diseases affect organisms, one can differentially compare the expression in healthy and diseased cells (Curreli *et al*., 2001). To investigate the response of the bacteria, *E.coli* Nissle 1917 was treated with cocoti sap and wine at a sub-inhibitory concentration. Cocoti sap and wine was first determined to have an MIC against *E.coli* Nissle 1917. Total bacterial proteins were extracted and separated by 2-D electrophoresis. Proteins on the gels were stained with coomassie blue dye and compared with those prepared from control and treated samples. Images were analysed by using 2-D platinum 6.0 software. We noticed over all 800 proteins in our gel samples, in this 370 protein spots were visualised clearly, in that considers 15 spots were isolated based on the regulation. Ten spots shows up regulation and remaining shows down regulation. One newly expressed protein was isolated from cocoti wine treated gel when compared to the control and also cocoti sap treated gels.



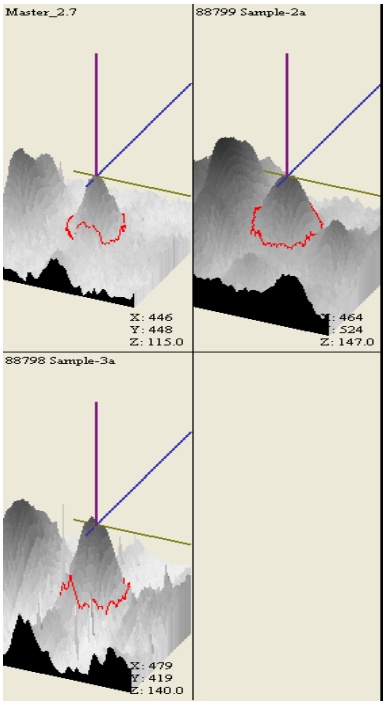
3-D view for protein spot 427



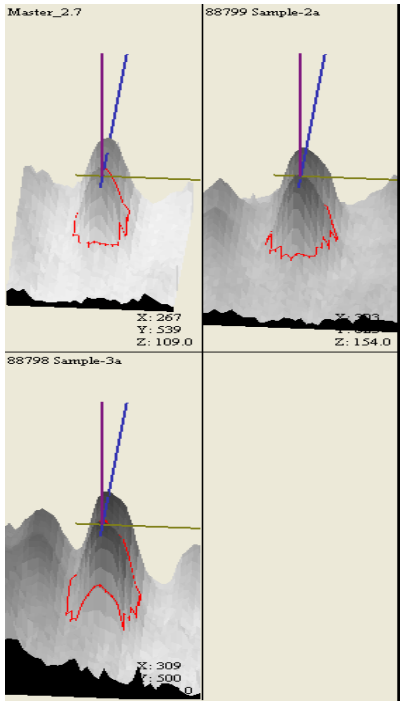
3-D view for protein spot 488



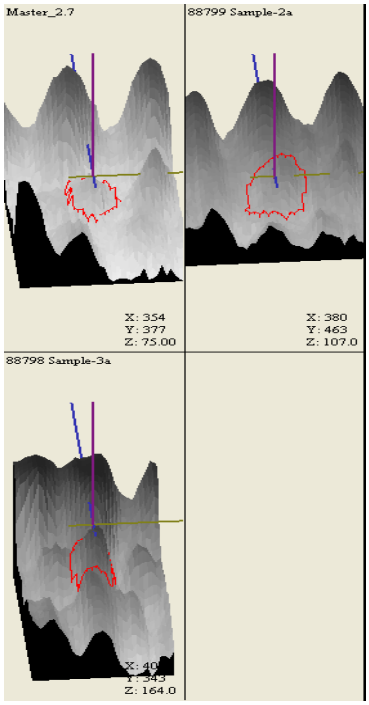
3-D view for protein spot 478



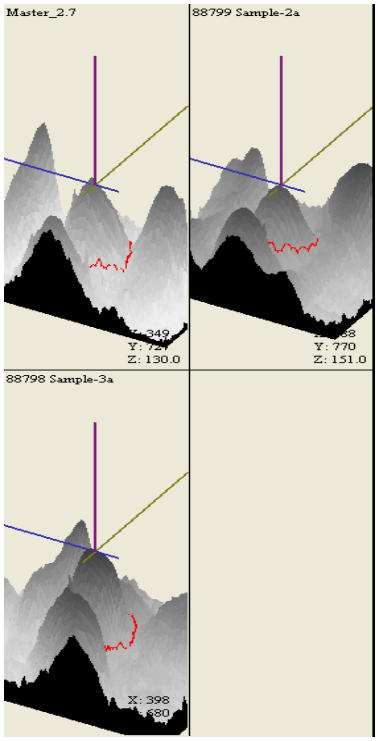
3-D view for protein spot 415



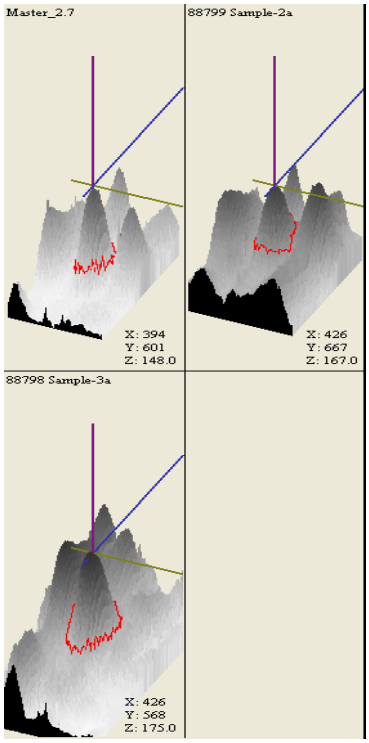
3-D view for protein spot 472



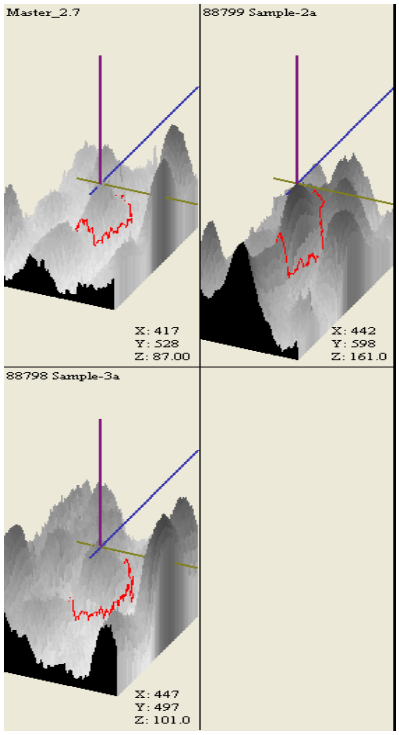
3-D view for protein spot 324



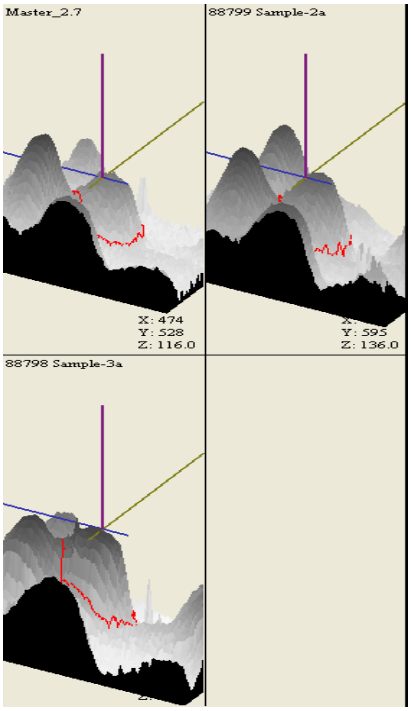
3-D view for protein spot 348



3-D view for protein spot 507

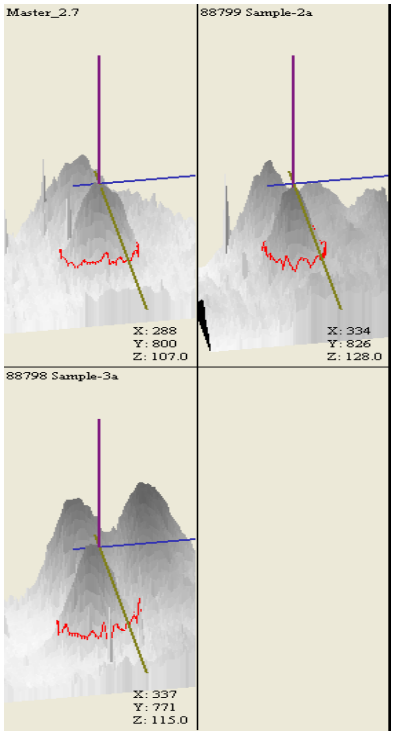


3-D view for protein spot 468

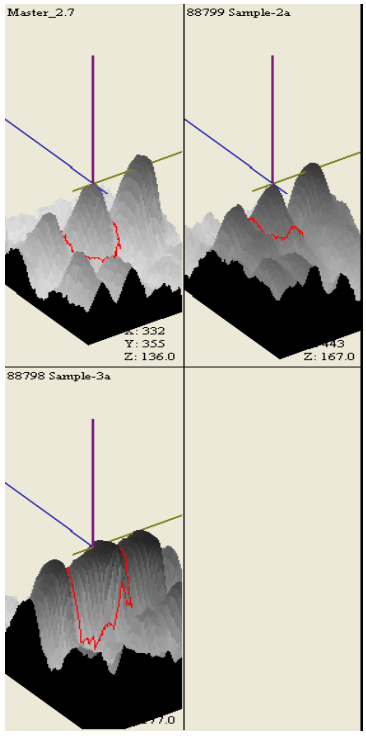


3-D view for protein spot 466

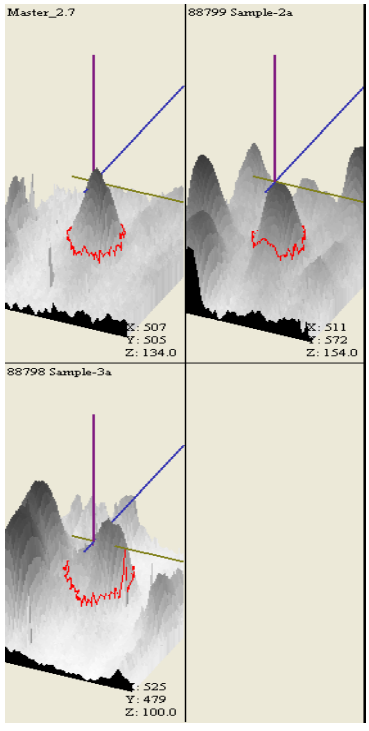
Figure- 18: Up- regulation of protein expression 3-D images



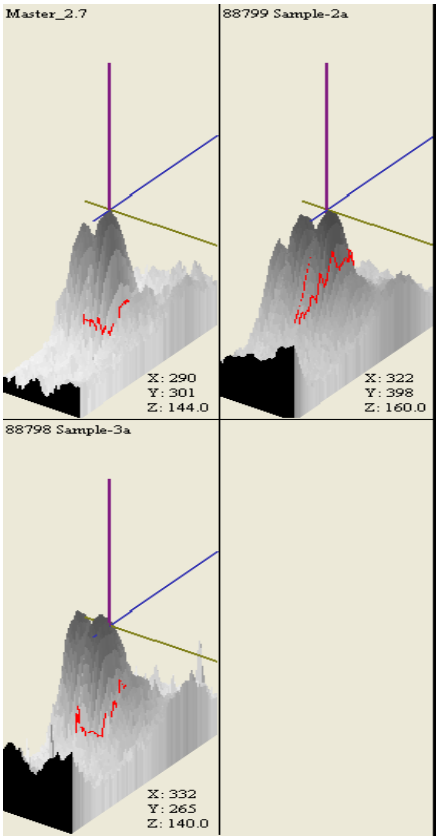
3-D view for protein spot 595



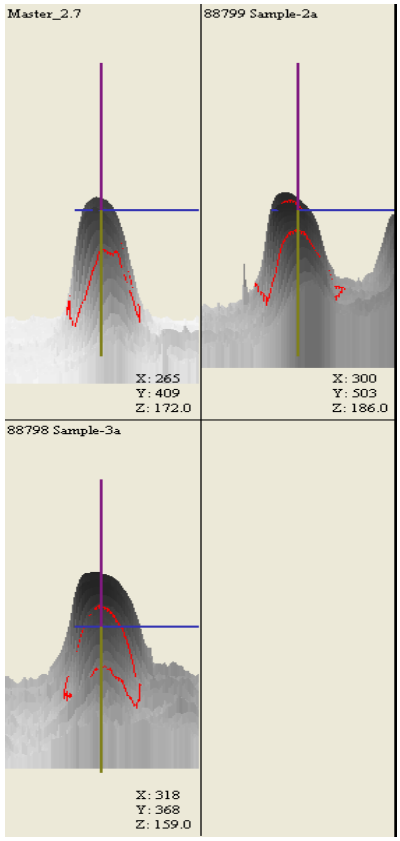
3-D view for protein spot 656



3-D view for protein spot 457



3-D view for protein spot 276



3-D view for protein spot 376

Figure- 19: Down regulation of protein 3-D images

In cocoti sap treatment, differentially expressed proteins belong to different functional groups. The protein spot identified in gel with the number of 595, belongs to the class contains UPF0401 protein ECP_3010, which is involved in purine metabolism. The protein spot identified in gel with the number of 472, belongs to the class contains Transcriptional regulatory protein BaeR, BAER_ ECOL6, which is involved in transcription regulation. The protein spot identified in gel with the number of 457, belongs to the class contain Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli), which is involved in electron carrier activity, The protein spot identified in gel with the number of 427, belongs to the class contains formate hydrogenase, subunit E [*Eschericia coli* F11], which is involved in Quinone, NAD, Nickel binding activity. The protein spot identified in gel with the number of 488, belongs to the class contain DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57, which is involved in regulation of the metabolic activities. The protein spot identified in gel with the number of 468, belongs to the class contain N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI, which is involved in phosphotransferase system. The protein spot identified in gel with the number of 348, belongs to the class contain t-RNA-Specific adenosine deaminase monomer, which is involved in the deamination reactions.

Table- 31: List of newly expressed proteins of *Eschericia coli* Nissle 1917 identified by MS/MS analysis under cocoti sap stress.

S.No	Spot	Differentially expressed proteins	Molecular weight	Calculated pI value	Protein Score	Number of Amino acids	Sequence coverage (%)
1	595	UPF0401 protein ECP Y3010_ECOL5	8744.9	6.54	50	77	76
2	472	Transcriptional regulatory protein BAER_ECOL6	140620.3	5.54	32	240	15
3	457	Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli)	48369.6	5.4	29	450	9
4	427	Formatehydrogenlyase subunit HYCE_ECOLI	64980.3	6.15	47	569	8
5	488	DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57	9378.6	5.46	26	82	26
6	468	N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI	17622.2	6.28	58	158	19
7	348	t-RNA-Specific adenosine deaminase monomer	26109.1	8.5	33	167	21

1). UPF0401 protein ECP Y3010_ECOL5:

It is a phase protein, Uniprot id of the protein S1GQX9 and the taxonomic identifier number is -1182698 mostly the protein involves molecular functions like hydrolase activity acting on ester bonds and metabolic process. Esterase does exist different their substrate specifically, their protein structure and functions phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric acid monoesters into a phosphate ions and a molecule with a free hydroxyl group. This action is directly opposite to that of phosphorylases and kinases which attach phosphate groups to their substrates by using energetic molecule like ATP. Protein phosphorylation plays a crucial role in biological functions and controls nearly every cellular process, including metabolism, gene transcription and translation, cell-cycle propagation, cytoskeletal rearrangement,

protein-protein interactions, protein stability, cell movement and apoptosis. This process dependent on the highly regulated and opposing actions of PKs and PPs, through changes in the phosphorylation of key proteins. Histone phosphorylation, along with methylation, ubiquitination, sumoylation and acetylation, also regulates access to DNA through chromatin reorganisation. UPF0401 protein ECP Y3010_ECOL5 is a down regulated protein with response of cocoti sap treatment. We noticed the protein spot in pH range around 7 and the molecular mass close to 8744.9 Da. The percentage of sequence coverage, calculated pI and protein score were presented in table-31.

2). Transcriptional regulatory protein BAER_ECOL6:

Transcriptional regulatory protein involved in transcription regulation, DNA binding, phosphorelay response regulator activity. Taxonomic identification number of this protein is 1181761 and the Uniprot id L4RK56, total sequence length of this protein 240 AA, molecular weight of this protein 140.6kda. The percentage of sequence coverage, calculated pI and protein score were presented in table-31.

The protein responds to a phosphorelay sensor to initiate a change in cell state or activity. This is a type of intracellular signal transduction, this process first elucidated by studies of the action of hormones such as epinephrine, which signals the breakdown of glycogen in anticipation of muscular activity. The activity of the response regulator is regulated by transfer of a phosphate from a histidine residue in the sensor, to an aspirate residue in the response regulator. Many but not all response regulators act as transcriptional regulators to elicit a response. These protein consist a membrane bound histidine kinase that senses a specific environmental stimulus and a corresponding response that mediates the cellular response, mostly through differential expression of a target genes (Mascher *et al*., 2006). Signal transduction occurs through the transfer of phosphoryl groups from adenosine triphosphate (ATP) to a specific histidine residue in the histidine kinases (HK). This is an auto-phosphorylation reaction. It transfers the phosphoryl group to an internal receiver domain, rather than to separate RR protein (Hoch *et al*., 2001; Varughese, 2002). The phosphoryl group is then shuttled to histidine phosphotransferase (HPT) and subsequently to a terminal response regulator which can evoke the desired response. Some histidine kinases (HK) are bi-functional, catalysing both the phosphorelation and dephosphorelation of their cognate response regulators (RRs). In case of Arabidopsis thaliana it consists three cytokinin receptors (Arabidopsis Histidine Kinases [AHK2, AHK3,AHK4]) are hybrid His Kinases that contain a fused receiver domain in

addition to an input (a cytokinin binding CHASE domain) and a His kinase domain (Inoue *et al.*, 2001; Suzuki *et al*., 2001; Yamada *et al*., 2001). Regulatory protein involved in gene expression. Receptor binds to a DNA binding site which is sometimes located near the promoter, sites of DNA sequence where regulatory proteins bind are called enhancer sequences. Regulatory proteins are often needed to be bound to a regulatory binding site to switch a gene on (activator) or to shut off a gene (repressor).

3). PMBA-Eco57– protein pmbA 0s *E.coli*:

Molecular weight of this protein 48.3kDa, Uniprot id L3P9L0 and taxonomic identifier number is 1182674. Peptidase pmba influence protease activity. Proteases involved in Microcin B17 maturation and in sensitivity to the DNA gyrase inhibitor LetD. Chromosome TldD/ TldE act as a protease (Allali *et al*., 2002) that is involved in sensitivity to DNA gyrase inhibitor LetD. CcdB (encoded by the F plasmid) by effecting degradation of the F-plasmid encoded LetD inhibitor, LetA.CcdA (Allali *et al.,* 2002). TldD and TldDE are also involved in proteolytic processing of the antibiotic Microcin B 17 (encoded by the pMccB17 plasmid) prior to its export from the cell. TldE and GroE have been proposed to antagonize ZfiA- mediated inhibition to the binding of the F-plasmid-encoded LetD DNA gyrase inhibitor to the DNA gyrase A subunit. Calculated pI of protein sample, protein score and percentage of sequence coverage were presented in table-31.

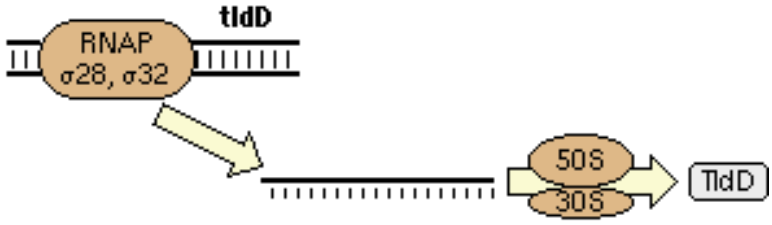


Figure-20: Gene tldD regulation summary

4). Formate hydrogenlyase subunit HYCE_ECOLI:

Formate hydrogenlyase is a membrane bound complex from *Eschericia coli*. It oxidizes formic acid (HCOOH or HCO_2H) to carbon dioxide (CO_2) and molecular hydrogen (H) (Magalon *et al*., 2000). Under anaerobic growth conditions and fermentation of sugars (glucose), it exists in two forms. One form by constituted by formate dehydrogenase H and hydrogenase 3, and the other one is formate dehydrogenase and hydrogenase 4, the presence of small protein subunits, carriers of

electrons, is also probable. Other proteins may also be involved in formation of the enzyme complex, which requires the presence of metal (nickel_ cobalt). This protein has homology with one of the subunits of NADH: ubiquinone Oxidoreductase of the respiratory chain and also involves Oxidoreductase activities (Sawers, 1994). Subunit composition of formate hydrogenlyase complex is [(FdhF)((HycD)(HycC)(HycF)(HycG)(HycB)(HycE)]. The percentage of sequence coverage, calculated pI and protein score were presented in table-31. Taxonomic identifier number of the protein is 340197and the Uniprot id of the protein is B3HQ53.

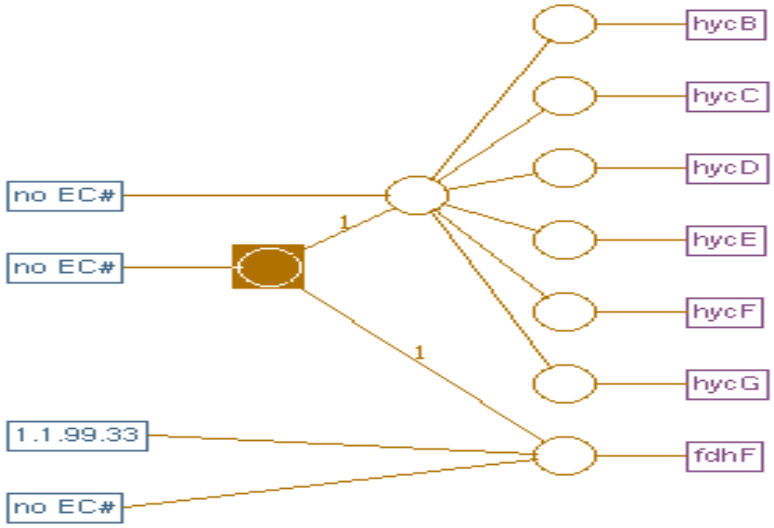


Figure-21: Composition of formate hydrogenlyase complex.

5). DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57:

Dinl (DNA – damage inducible protein l) like protein family is a family of short proteins (Ramirez *et al.,* 2000). *Escherichia coli* Dinl, a LexA-regulated SOS gene product, shut off the initiation of the SOS response when over expressed in-vivo. The SOS response, a set of cellular phenomena exhibited by eubacteria, is initiated by various causes that include DNA damage – induced replication arrest, and is positively regulated by the co-protease activity of RecA. Genetic studies indicates that Dinl physically interact with RecA to inhibit its co-protease activity (Yoshimasu *et al.,* 2003). The other Din1 like proteins are presented in the table -32. Taxonomic identifier number of the protein is 868147 and the Uniprot id of the protein is H4NQH2. The percentage of sequence coverage, calculated pI and protein score were presented in the table -31.

Table -32: DNA damage- inducible protein Dinl- like family

Accession	Protein name	Species	Family	Amino acids
P0A1G0	Protein ImpC	*Salmonella* typhimurium	DNA damage-inducible protein DinI-like	82
P0A1G1	Protein ImpC	*Escherichia coli*	DNA damage-inducible protein DinI-like	82
P0A1G2	Protein ImpC	*Shigella* flexneri	DNA damage-inducible protein DinI-like	82
P0A1G3	Virulence protein MsgA	*Salmonella* typhimurium (strain LT2 / SGSC1412 / ATCC 700720)	Salmonella typhimurium (strain LT2 / SGSC1412 / ATCC 700720)	79
A1JN43	DNA-damage-inducible protein I	*Yersinia* enterocolitica serotype O:8 / biotype 1B (strain NCTC 13174 / 8081)	DNA damage-inducible protein DinI-like	81
P0A1G4	Virulence protein MsgA	*Salmonella* typhi	DNA damage-inducible protein DinI-like	79
P0ABR1	DNA-damage-inducible protein I	*Escherichia coli* (strain K12)	DNA damage-inducible protein DinI-like	81
P0ABR2	DNA-damage-inducible protein I	*Escherichia coli* O6:H1 (strain CFT073 / ATCC 700928 / UPEC)	DNA damage-inducible protein DinI-like	81
P0ABR3	DNA-damage-inducible protein I	*Escherichia coli* O157:H7	DNA damage-inducible protein DinI-like	81
P0ABR4	DNA-damage-inducible protein I	*Shigella* flexneri	DNA damage-inducible protein DinI-like	81
P21320	DinI-like protein in retron EC67	*Escherichia coli*	DNA damage-inducible protein DinI-like	77
P41063	SOS operon TUM protein	Enterobacteria phage 186	DNA damage-inducible protein DinI-like	146
P58217	DinI-like protein Z2083/ECs2153	*Escherichia* coli O157:H7	DNA damage-inducible protein DinI-like	80

6). N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1:

The protein N- acetylgalactosamine specific phosphotransferase enzyme IIB component 1 involved in protein-N (PI)-phosphohistidine- sugar phosphotransferase activity. The phosphoenolpyruvate-dependent sugar phosphotransferase system (Sugar PTS), a major

carbohydrate active- transport system, catalyzes the phosphorylation of incoming sugar substrates concomitantly with their translocation across the cell membrane. This system is involved in N-acetylgalactosamine transport (Ray *et al.,* 2004). AgaVWEF (N-acetyl-galactosamine PTS aga-operon (comprising agaVWEF), the cryptic galNAc PTS permease, belongs to the functional superfamily of the PEP-dependent, sugar transporting PTS. If all of its components were present, AgaVWEF would take up exogenous GalNAc, releasing the phosphate ester into the cell cytoplasm in preparation for metabolism (Brinkkotter *et al*., 2000). The percentage of sequence coverage, calculated pI and protein score were presented in table-31. Taxonomic identifier number of the protein is 1116033 and the Uniprot id of the protein is M8THA9.

7). t-RNA-Specific adenosine deaminase monomer:

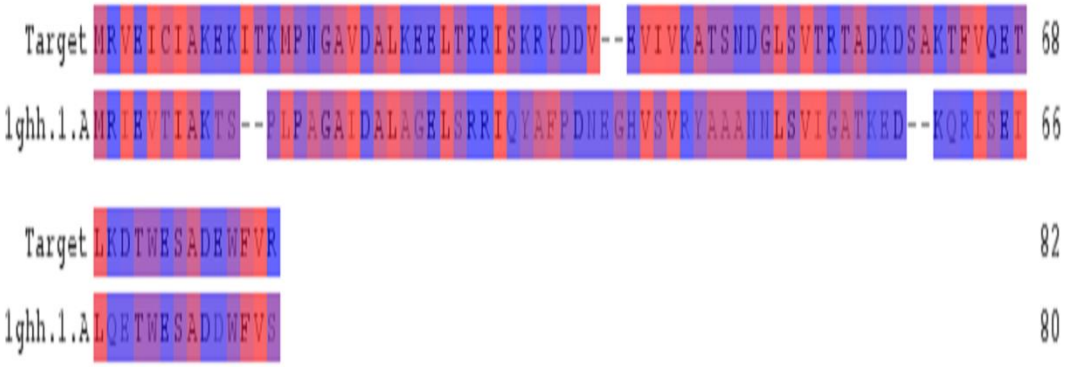
Taxonomic identifier of the protein sample is 868141and Uniprot code H4L196, length of the sequence 167AA, molecular weight of this protein sample is 26kDa. TadA is a tRNA- specific adenosine deaminase that belongs to the family of adenosine at position 34 of tRNA ^{Arg2} resulting in an inosine at this position, which is the wobble base of the anticodon. Substrate requirements have been evaluated, the anticodon stem and loop are found to be sufficient for inosine formation (Wolf *et al.,* 2002). TadA can form a homodimer in vitro, but it is unknown whether it functions as a homodimer in vivo (Wolf *et al.,* 2002). A crystal structure of TadA has been solved at 2.0 Å resolutions (Kim *et al.,* 2006). Adenosine deaminase (ADA) is considered one of the key enzymes of purine metabolism (Glader *et al.,* 1983). The high degree of amino acid sequence conservation suggests the crucial nature of ADA in the purine salvage pathway (Cristalli *et al.,* 2001). The calculated pI, percentage of sequence coverage and protein score were presented in the table -31.

Catalysis of the reaction: adenosine + H2O = inosine + NH3, in a tRNA molecule.

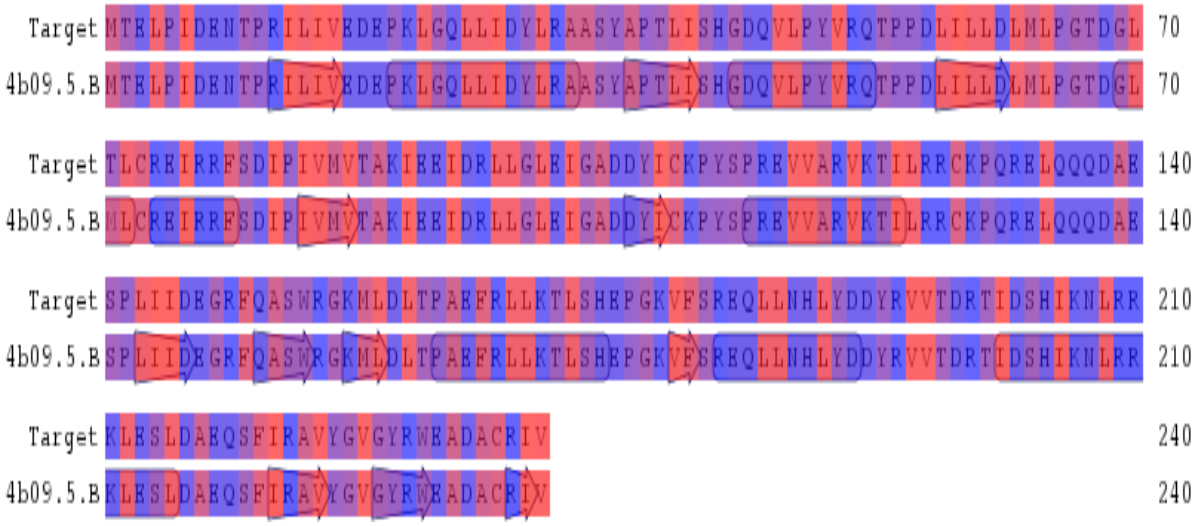
Sequence alignment of differentially expressed proteins under sap stress:

The target sequence was searched with BLAST search against Protein Data Bank, which one has a high level of sequence identity with target protein selected as a template protein. Templates were determined by superimposition of the two structures and multiple sequence alignment was performed with CLUSTAL W (Larkin *et al.,* 2007) program to identify the set of conserved residues alignment are represented in the following figures - 22 a to 22 g.

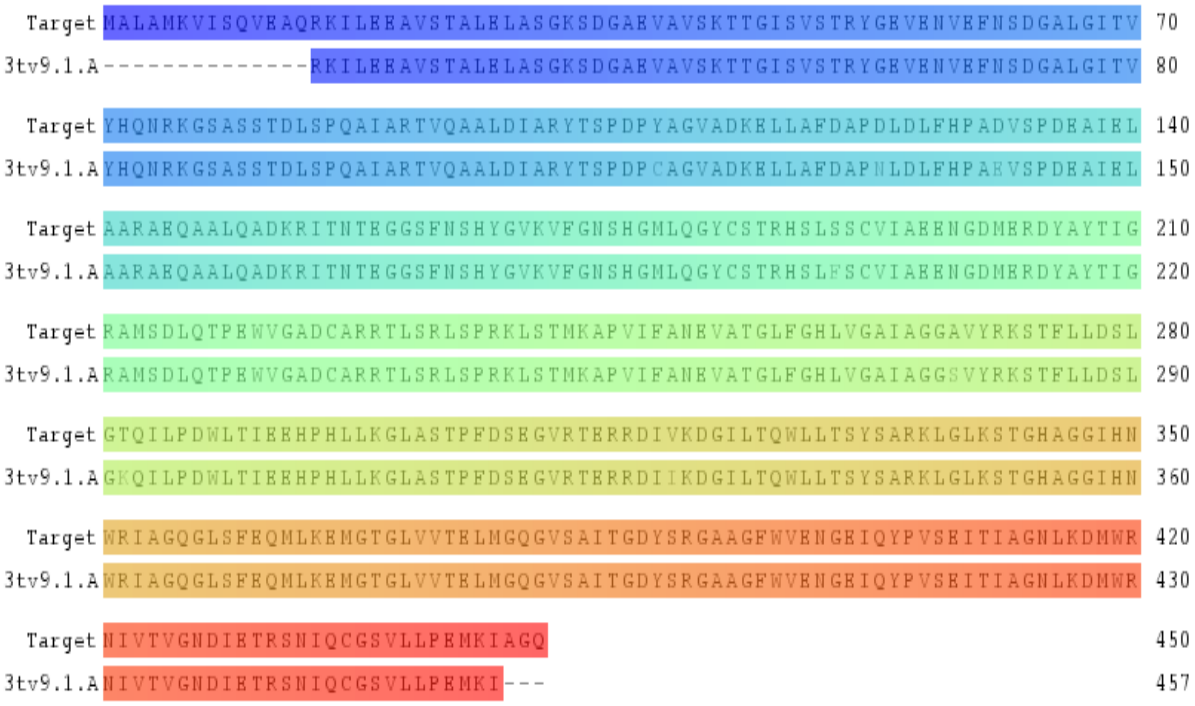
2HJJ protein was the selected template for UPF0401 target protein, **4B09** protein was the selected template for transcriptional regulatory protein BAER_ECOL6 target protein, **3TV9** was the selected template for Protein PmbA-Eco 57 target protein, **2FUG** was the selected template for protein Formatehydrogenlyase subunit HYCE_ECOLI target protein, **1GHH** protein was the selected template for Dinl-like protein Z3305/ECs 2939 target protein, **3EYE** was the selected template for N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1 target protein and **1Z3A** was the selected template for t-RNA specific adenosine deaminase monomer target protein. Amino acid sequences of these proteins were represented in the following figures -22 a to 22 g.



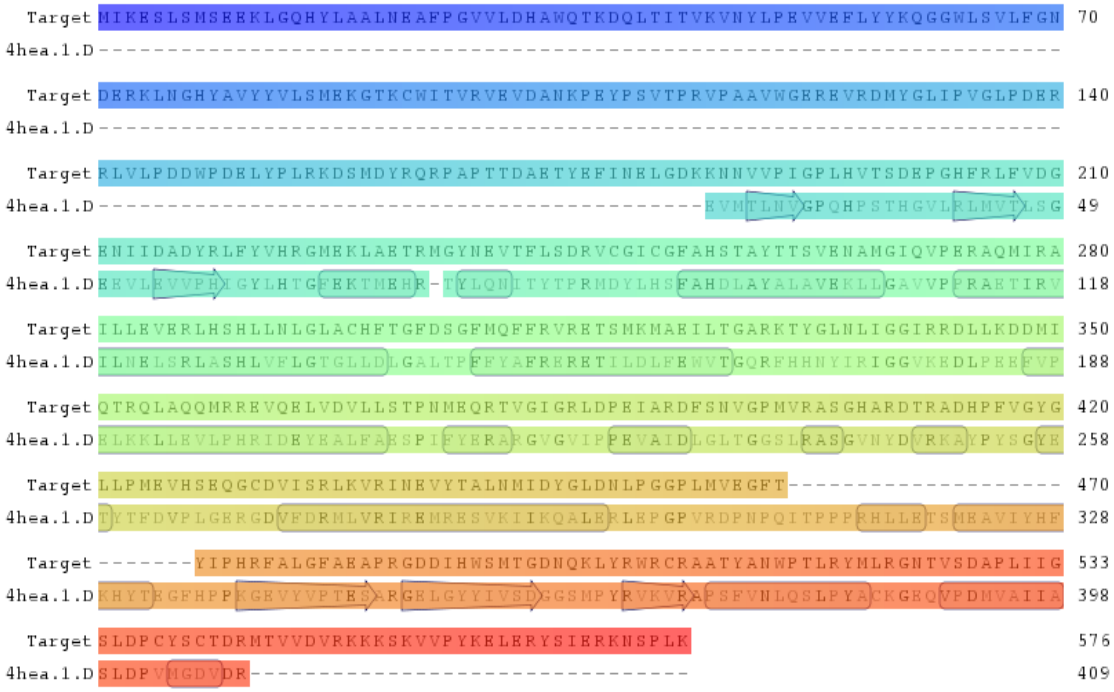
a) UPF0401 protein ECP Y3010_ECOL5 target protein aligned with 2HJJ template**.**



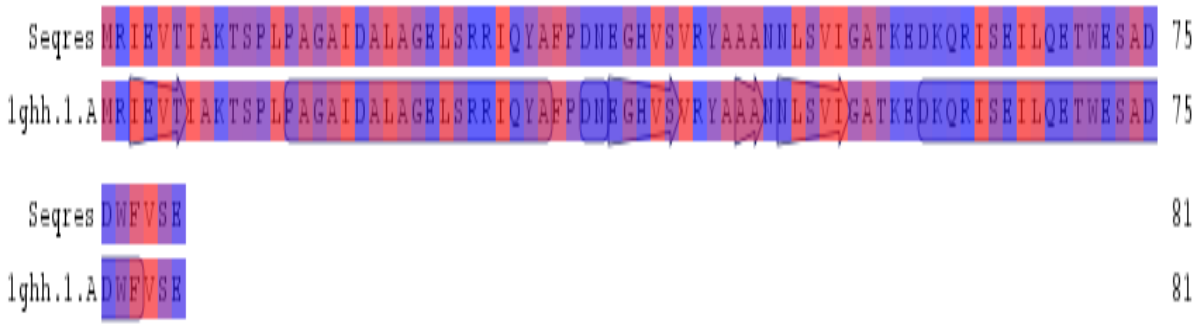
b) Transcriptional regulatory protein BAER_ECOL6 target protein aligned with 4B09 template.



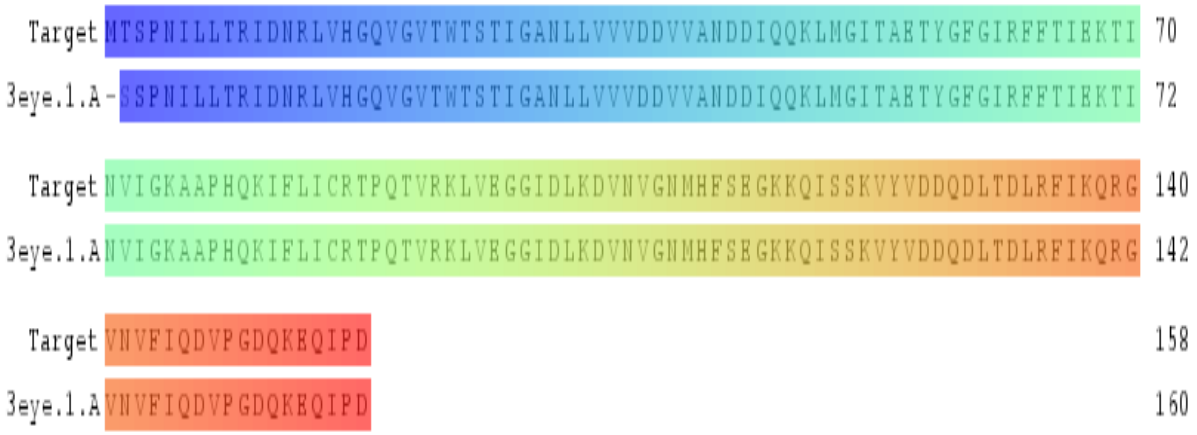
c) Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli) target protein aligned with 3TV9 template.



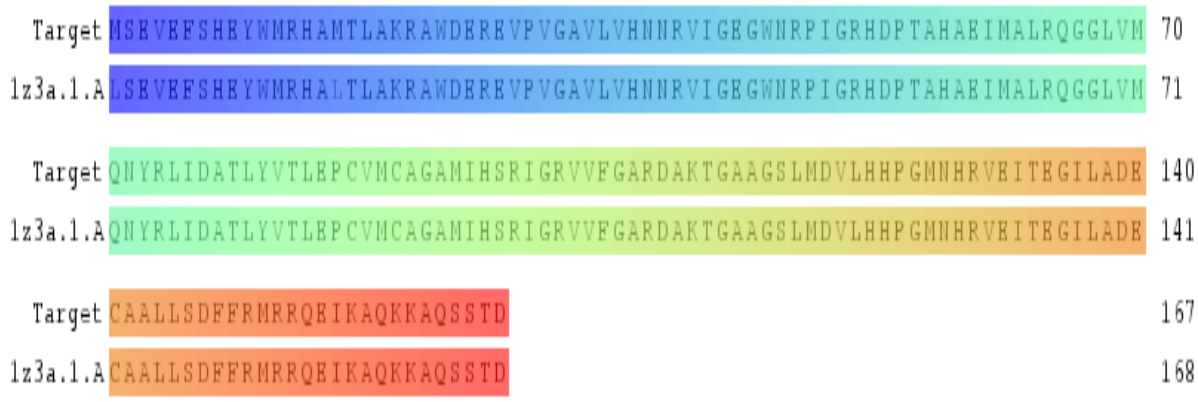
d) Formatehydrogenlyase subunit HYCE_ECOLI target protein aligned with 2FUG template protein



e) DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 target protein aligned with 1GHH template protein.



f) N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI target protein aligned with 3EYE template protein.



g) t-RNA-Specific adenosine deaminase monomer target protein aligned with 1Z3A template protein.

Figure- 22: Alignment of the amino acid sequences of differentially expressed proteins. The results were generated with CLUSTAL W multiple sequence alignment tool.

Homology modelling of differentially expressed proteins under sap stress:

The sequences were analysed with the help of Phyre-2 (protein Homology/analogy Recognition Engine V 2.0) server for obtaining pdb file. The final 3-D structure obtained with the help of RasMol version 2.6 software programme.

2HJJ selected as a reference structure for modeling of UPF0401 protein. Overall 76% sequence identity of the protein, it is sufficient for creating an acceptable model prediction, in Swiss pdb we get 11pdb templates out of this select a least energy. The structure has 45-H-bonds, 2 helices, number of strands-4 and 6- turns were showed in the following figure -23-a.

4B09 is selected as reference structure for modelling Transcriptional regulatory protein BAER_ECOL6. The protein sequence identity is 15%, it is sufficient for creating an acceptable model prediction, in Swiss pdb we get 50 templates, out of this we select best match one. The structure has 113-H-bonds, 7-Helices, 11 strands and 17 turns were showed in the following figure-23-b.

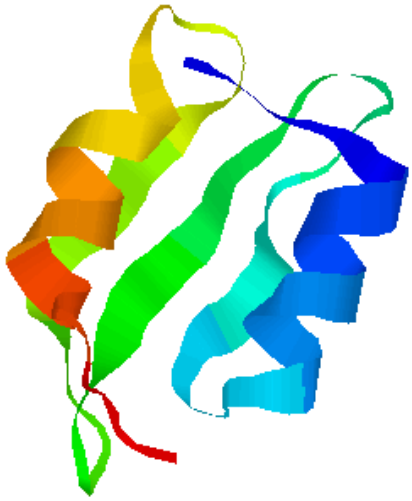
3TV9 template selected as reference structure for modelling protein pmbA (PMBA-Eco57-protein pmba 0s E.coli). Percentage of sequence identity is 9%, in Swiss pdb we get 18 templates out of this we select best matched one for prediction. The structure has 311-h-bonds, 11 helices, 32 strands and 42 turns were showed in the following figure-23-c.

2FUG selected as a reference structure for modelling Formatehydrogenlyase subunit HYCE_ECOLI protein. Sequence identity is 8% and in Swiss pdb we get 11pdb templates out of this we select a least energy for prediction. The structure has 226-H-bonds, 14 helices, 9 strands and 33 turns were showed in the following figure -23-d.

1GHH template protein selected as a reference structure for modelling Dinl-like protein Z3305/ECs in prophase CP-933VDINI1_ECO57. Sequence identity of this protein is 76% and we get 11pdb templates from Swiss pdb. 3-D structure of this protein consists 55-H-bonds, 5 helices, 4-strands and 8 turns showed in the following figure -23-e.

3EYE template protein selected as a reference structure for modelling N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1 PTPB1_ECOLI. Sequence identities matched with target is 19% and we get 28 pdb templates from Swiss pdb. 3-D structure of this protein consists of 113-H-bonds, 8-helices, 12 strands and 12 turns showed in the following figure -23-f.

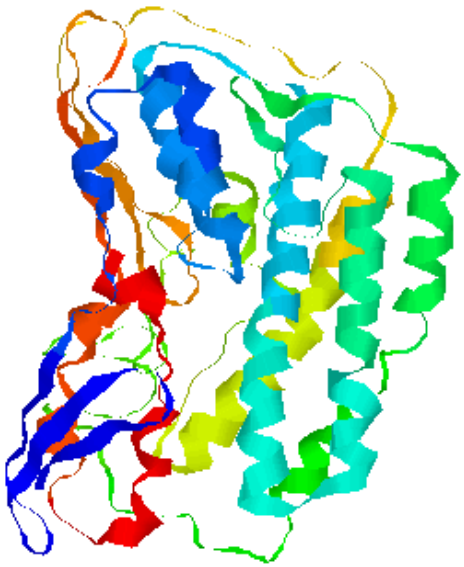
1Z3A is selected as reference structure for modelling t-RNA-Specific adenosine deaminase monomer. Sequence identities matched with 21% and we get 79 pdb templates from Swiss pdb. 3-D structure of this protein consists of 115-H-bonds, 5- helices, 10 strands and 17 turns showed in the following figure -23-g.



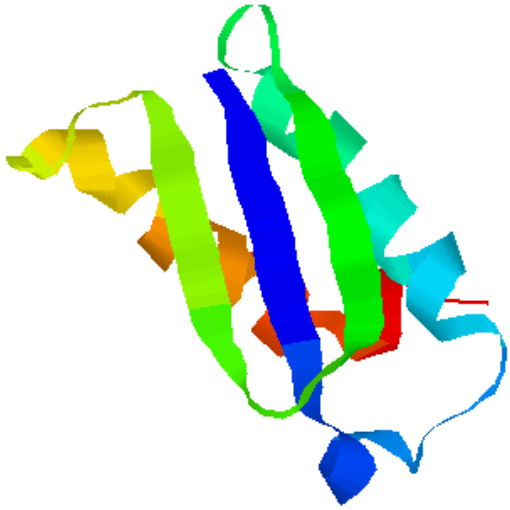
a) 3-D structure of UPF0401 protein**:**

b) 3-D Structure of Transcriptional regulatory protein BAER_ECOL6**:**

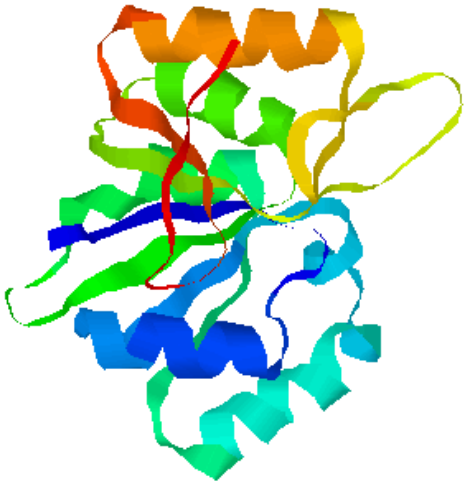
c) 3-D structure of protein PmbA (PMBA-Eco57-protein pmba 0s E.coli).



d) 3-D structure of Formatehydrogenlyase subunit HYCE_ECOLI protein.



e) 3-D structure of Dinl-like protein Z3305/ECs in prophase CP-933VDINI1_ECO57.



f) 3-D structure of N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1 PTPB1_ECOLI.

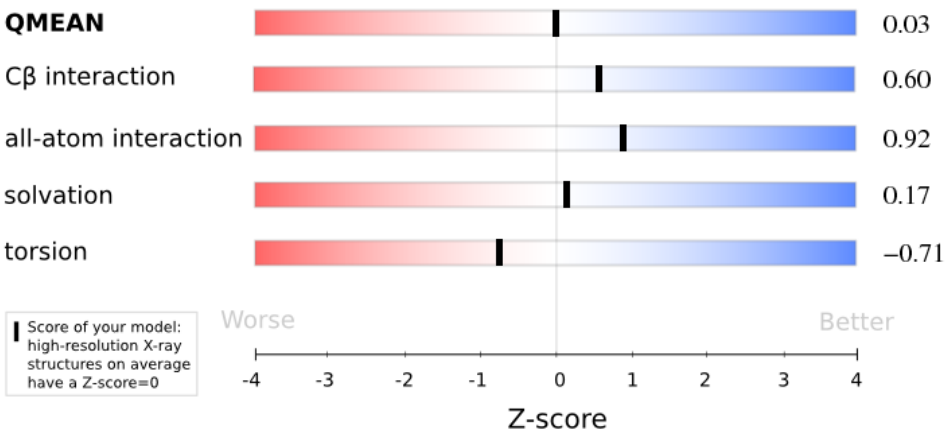
g) 3-D structure of t-RNA-Specific adenosine deaminase monomer

Figure-23: 3-D structures of differentially expressed proteins under sap stress:

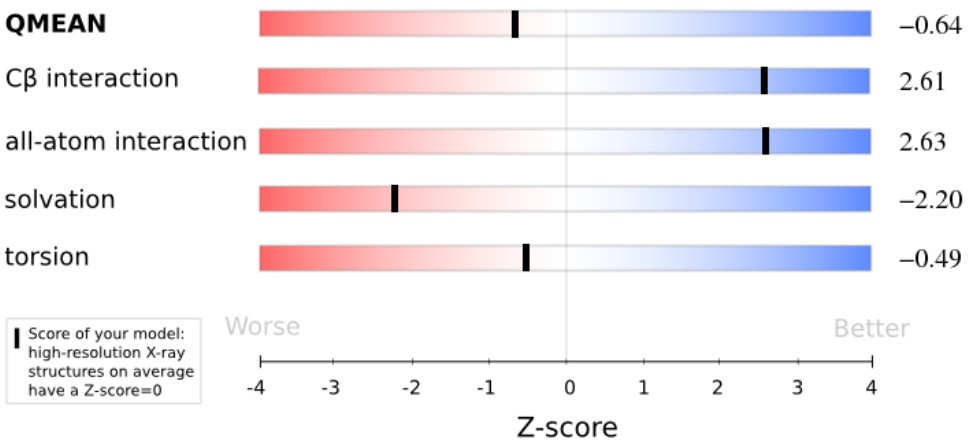
QMEAN analysis for the quality resolution structure:

The QMEAN scoring function estimates the global quality of the models on the basis of a linear combination of six structural descriptions, four of them are statistical potentials of mean force. The local geometry is analysed by a torsion angle potential over three consecutive amino acids. The distance –dependent interaction potentials based on Cβ atoms and all toms, respectively are used to assess long-range interactions. A solvation potential describes the burial status of the residues. The analysis of these Z-scores of the individual terms can help identifying the geometrical features responsible for an observed large negative QMEAN Z-score. Models of low quality are expected to have strongly negative Z-scores for QMEAN but also for most of the contributing terms. Large negative values correspond to red regions in the colour gradient. Good structures are expected to have all sliders in the light red to blue region. The quality of resolution structure of differentially expressed proteins under sap stress were represented in the following figures-24 a to 24 g. In UPF0401 protein quality resolution structure except torsion angle value remaining all values are positive this indicates it is a good structure. In Transcriptional regulatory protein BAER_ECOL6 protein Cβ, all- atom interactions shows positive values correspond blue regions, solvation values shows negative value remaining two shows light red region indicates that the protein structure is low quality prediction structure. In case of Protein PmbA except solvation values remaining all the values were negative it indicates the structure was in poor quality prediction. The protein Formatehydrogenlyase subunit HYCE_ECOLI quality of resolution structure refers all the values were negative it indicates the structure is in poor quality prediction. In case of DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 protein all angle shows positive values except torsion angle, this value also shows light red colour indicates the

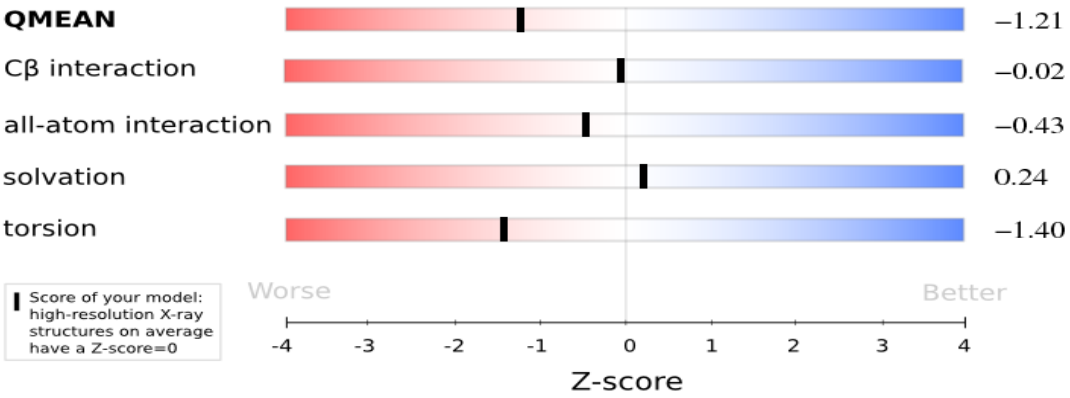
structure is better quality compare to Protein PmbA, Formatehydrogenlyase subunit HYCE_ECOLI. In case of N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI Cβ values are in neutral position and the remaining values are positive, these values indicate that the predicted structure is good. The protein t-RNA-Specific adenosine deaminase monomer all values were negative except all-atom interaction value, this indicates poor quality structure.



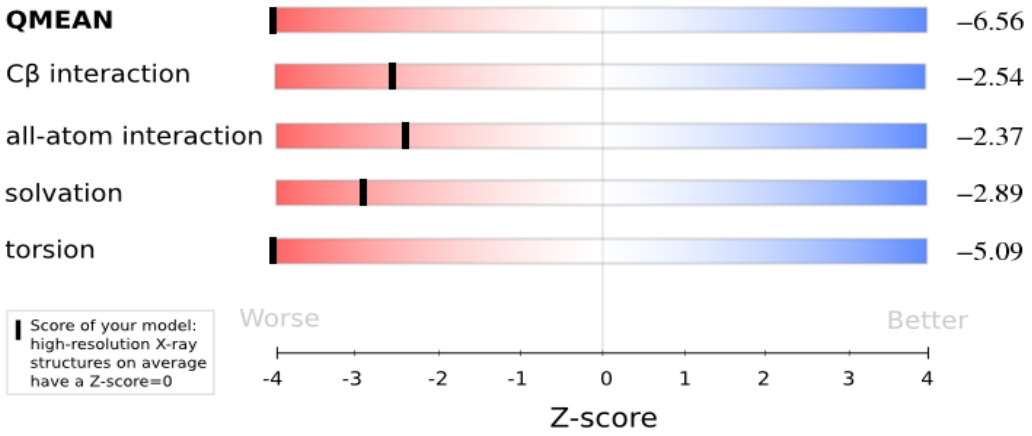
a) Quality of resolution structure of UPF0401 protein:



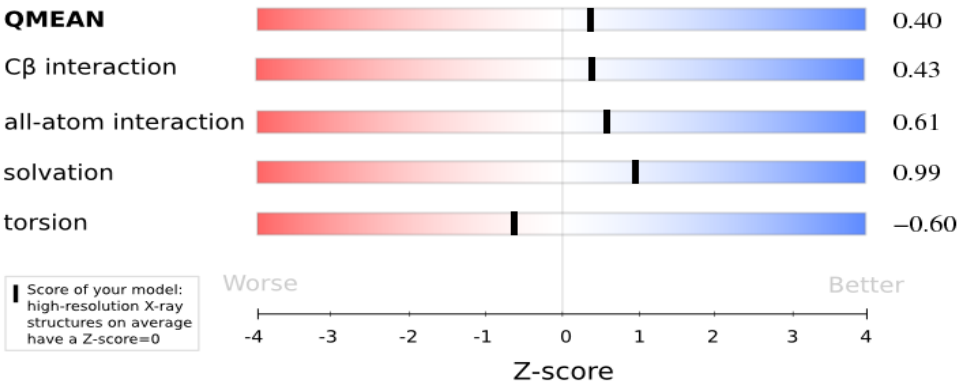
b) Quality of resolution structure of Transcriptional regulatory protein BAER_ECOL6



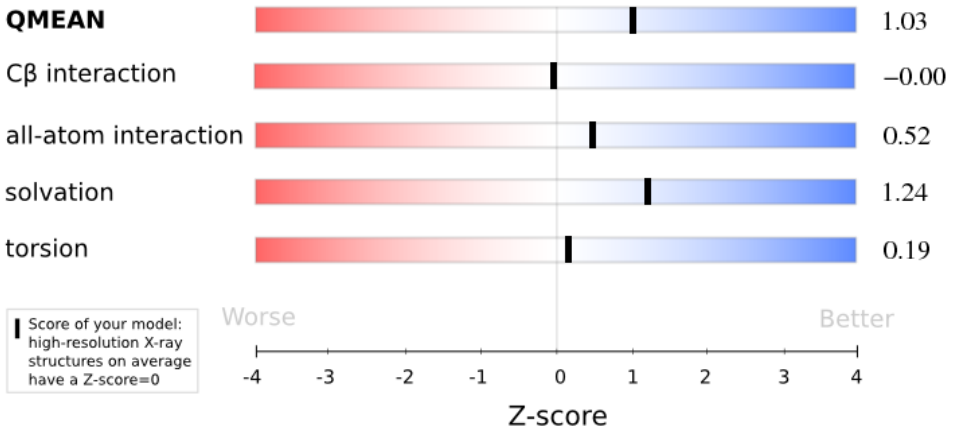
c) Quality of resolution structure of Protein PmbA



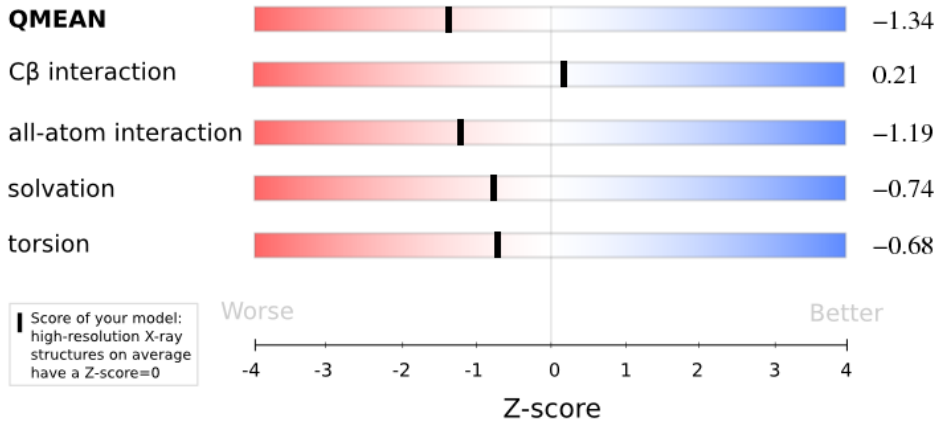
d) Quality of resolution structure of Formatehydrogenlyase subunit HYCE_ECOLI



e) Quality of resolution structure of DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57.



f) Quality of resolution structure of N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI**.**



g) Quality of resolution structure of t-RNA-Specific adenosine deaminase monomer.

Figure -24: Quality of resolution structure of differentially expressed proteins under sap stress.

QMEAN scoring:

The QMEAN score is a composite score consisting of a linear combination of 6 terms. The pseudo-energies of the contributing terms are given below together with their Z-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography (Benkert *et al*., 2008). The Z-score of a protein is defined as the energy separation between the native fold and the average of an ensemble of misfolds in the units of the standard deviation of the ensemble. The Z-score is often used as a way of testing the knowledge-based potentials for their ability to recognize the native fold from other alternatives. QMEAN scoring

function of differentially expressed proteins under sap stress were presented in the following tables -33 a to 33 g.

Table -33: QMEAN scoring function of differentially expressed proteins under sap stress.

a) QMEAN scoring function of UPF0401 protein ECP Y3010_ECOL5

protein model name	595_ pdb
C_beta interaction energy	-35.74 (Z-score: 0.60)
All-atom pairwise energy	-2469.22 (Z-score: 0.92)
Solvation energy	4.01 (Z-score: 0.17)
Torsion angle energy	-11.05 (Z-score: -0.71)
Secondary structure agreement	82.8% (Z-score: -0.12)
Secondary structure agreement	75.0% (Z-score: 0.21)
Total QMEAN-score	0.722 (Z-score: 0.04)

b) QMEAN scoring function of Transcriptional regulatory protein BAER_ECOL6

protein model name	472_ pdb
C_beta interaction energy	-133.86 (Z-score: 2.61)
All-atom pairwise energy	-8293.81 (Z-score: 2.63)
Solvation energy	4.55 (Z-score: -2.20)
Torsion angle energy	51.76 (Z-score: -0.49)
Secondary structure agreement	84.2% (Z-score: 0.23)
Secondary structure agreement	66.5% (Z-score: -2.38)
Total QMEAN-score	0.617 (Z-score: -1.64)

c) QMEAN scoring function of Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli)

protein model name	457_ pdb
C_beta interaction energy	-135.64 (Z-score: -0.02)
All-atom pairwise energy	-9082.02 (Z-score: -0.43)
Solvation energy	-44.31 (Z-score: 0.24)
Torsion angle energy	-88.40 (Z-score: -1.40)
Secondary structure agreement	83.0% (Z-score: 0.63)
Secondary structure agreement	77.1% (Z-score: -0.72)
Total QMEAN-score	0.674 (Z-score: -1.10)

d) QMEAN scoring function of Formatehydrogenlyase subunit HYCE_ECOLI

protein model name	427_ pdb
C_beta interaction energy	-5.96 (Z-score: -2.54)
All-atom pairwise energy	-4276.52 (Z-score: -2.37)
Solvation energy	-5.49 (Z-score: -2.89)
Torsion angle energy	10.06 (Z-score: -5.09)
Secondary structure agreement	71.6% (Z-score: -1.94)
Secondary structure agreement	72.5% (Z-score: -1.66)
Total QMEAN-score	0.471 (Z-score: -3.51)

e) QMEAN scoring function of DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO5

protein model name	488_ pdb
C_beta interaction energy	-55.53 (Z-score: 0.43)
All-atom pairwise energy	-2931.58 (Z-score: 0.61)
Solvation energy	-12.29 (Z-score: 0.99)
Torsion angle energy	-16.40 (Z-score: -0.60)
Secondary structure agreement	84.0% (Z-score: -0.09)
Secondary structure agreement	85.2% (Z-score: 1.42)
Total QMEAN-score	0.851 (Z-score: 0.84)

f) QMEAN scoring function of N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI

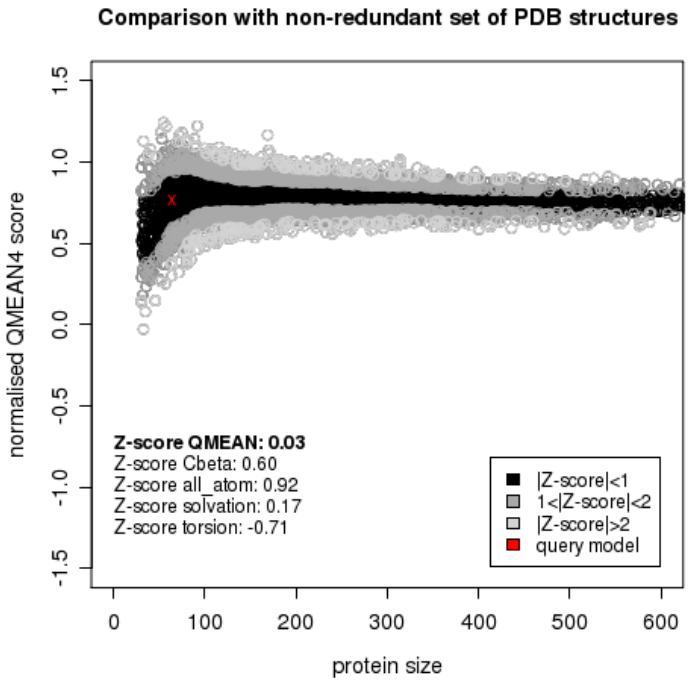
protein model name	468_ pdb
C_beta interaction energy	-92.32 (Z-score: -0.00)
All-atom pairwise energy	-5987.88 (Z-score: 0.52)
Solvation energy	-22.80 (Z-score: 1.24)
Torsion angle energy	-44.62 (Z-score: 0.19)
Secondary structure agreement	88.2% (Z-score: 0.71)
Secondary structure agreement	81.7% (Z-score: 0.73)
Total QMEAN-score	0.822 (Z-score: 0.76)

g) QMEAN scoring function of t-RNA-Specific adenosine deaminase monomer

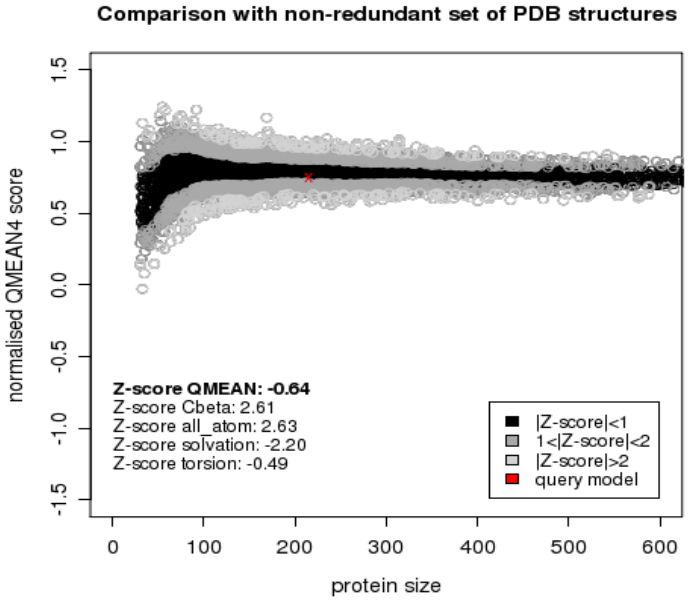
protein model name	348_ pdb
C_beta interaction energy	-99.98 (Z-score: 0.21)
All-atom pairwise energy	-3468.40 (Z-score: -1.19)
Solvation energy	-9.82 (Z-score: -0.74)
Torsion angle energy	-34.22 (Z-score: -0.68)
Secondary structure agreement	87.2% (Z-score: 0.57)
Secondary structure agreement	80.8% (Z-score: 0.51)
Total QMEAN-score	0.778 (Z-score: 0.26)

QMEAN analysis for estimating the quality of differentially expressed proteins structure under sap stress:

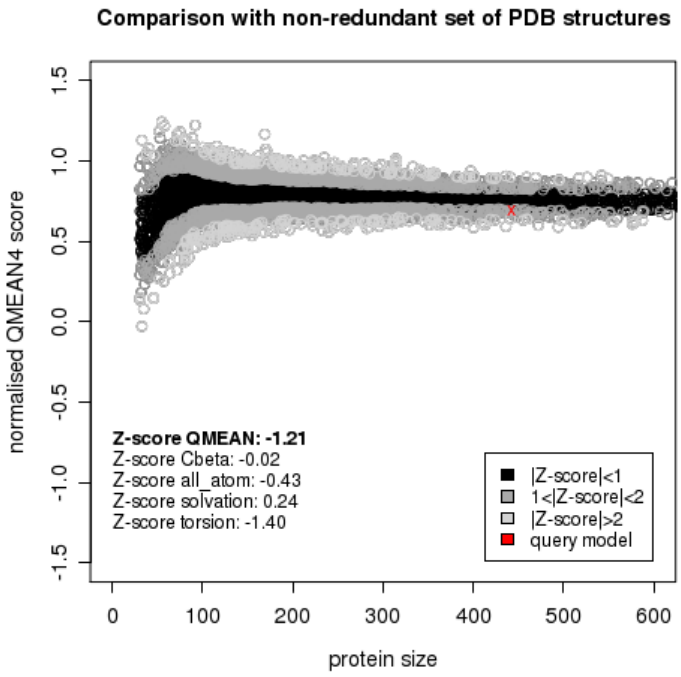
The areas built by the coloured in different shades of grey in the plot on the left hand side represent the QMEAN scores of the reference structures from the PDB. The model QMEAN score is compared to the scores obtain for experimental structures of similar size and Z- score is calculated. Z-score value of differentially expressed protein values under sap stress were presented in the following tables 25 a to 25 g. UPF0401 protein Z-score is 0.03, Transcriptional regulatory protein BAER_ECOL6-Z-score is -0.64, Protein PmbA (PMBA-Eco57– protein pmba 0s *E.coli*)-Z-score is -1.21, Formatehydrogenlyase subunit HYCE_ECOLI -6.56, DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 Z-score 0.40, N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI Z- score of this protein 1.03, t-RNA-Specific adenosine deaminase monomer protein Z-score is -1.34. Standard Q-MEAN score indicates that models with the normalized QMEAN score range is 0.3-0.4 should be considered as bad, between 0.4 - 0.5 considered as medium and above 0.5 the structure is good, based on these standard values the protein quality of DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 is medium in range, N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI Z- protein quality range is good.



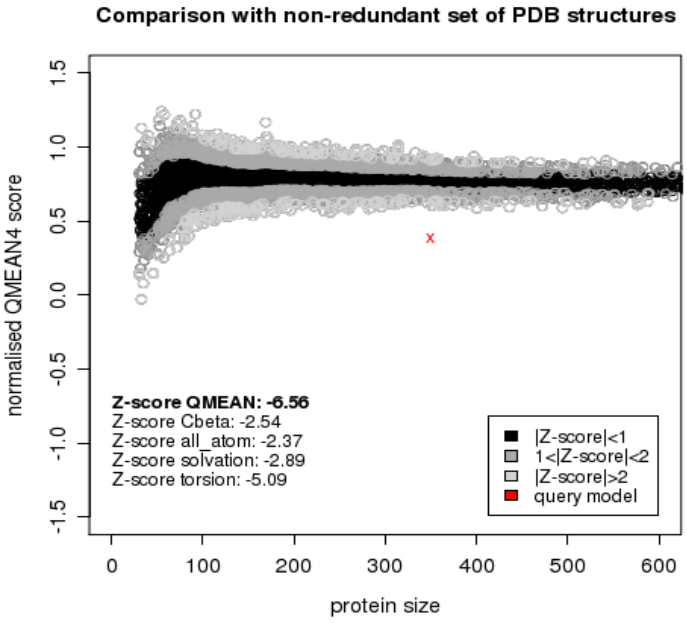
a) Quality estimation of UPF0401 protein ECP Y3010_ECOL5.



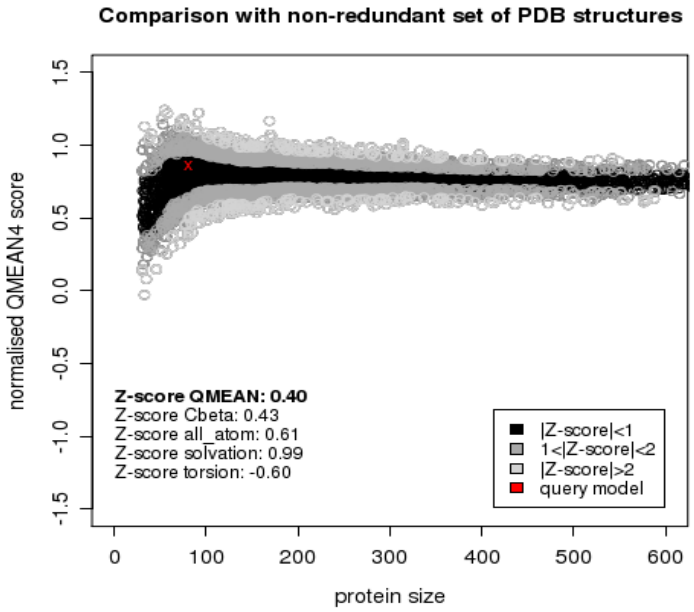
b) Quality estimation of Transcriptional regulatory protein BAER_ECOL6.



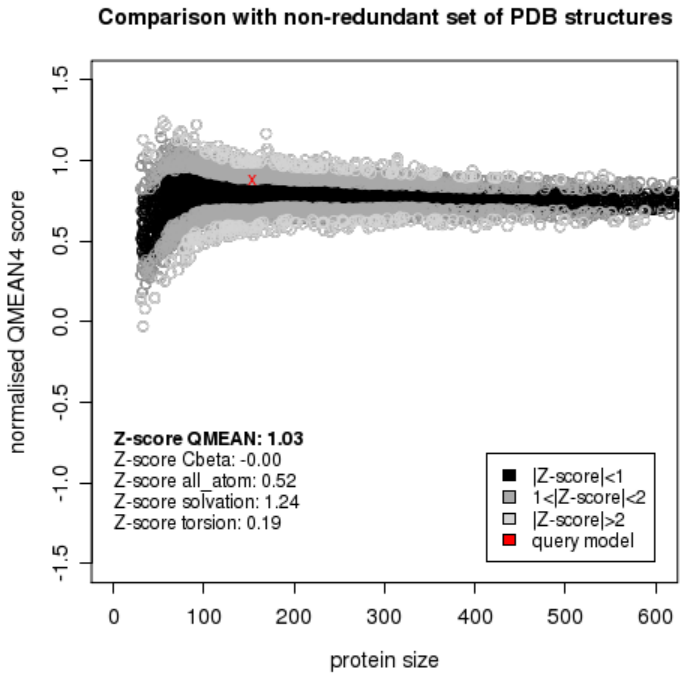
c) Quality estimation of Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli)



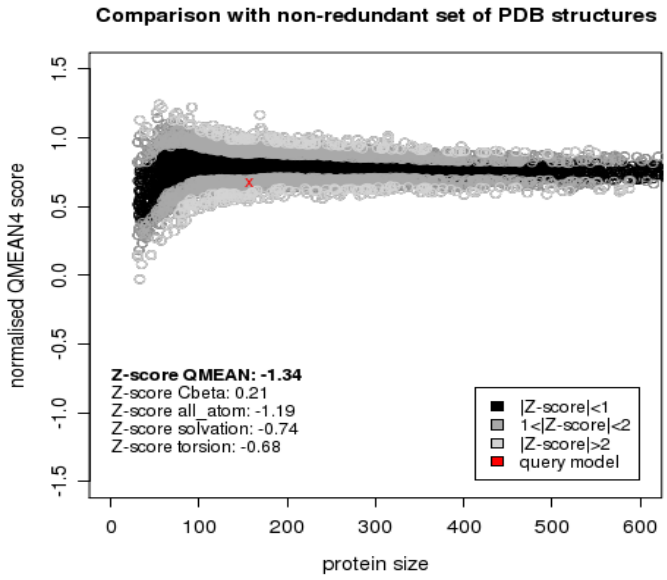
d) Quality estimation of Formatehydrogenlyase subunit HYCE_ECOLI



e) Quality estimation of DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57



f) Quality estimation of N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI

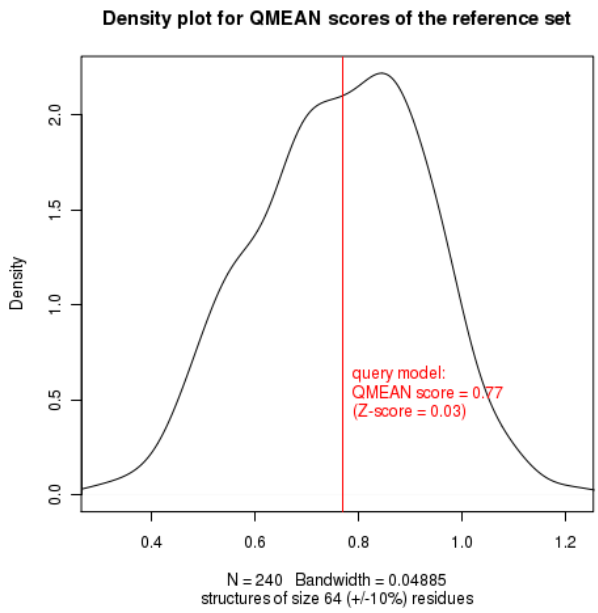


g) Quality estimation of t-RNA-Specific adenosine deaminase monomer

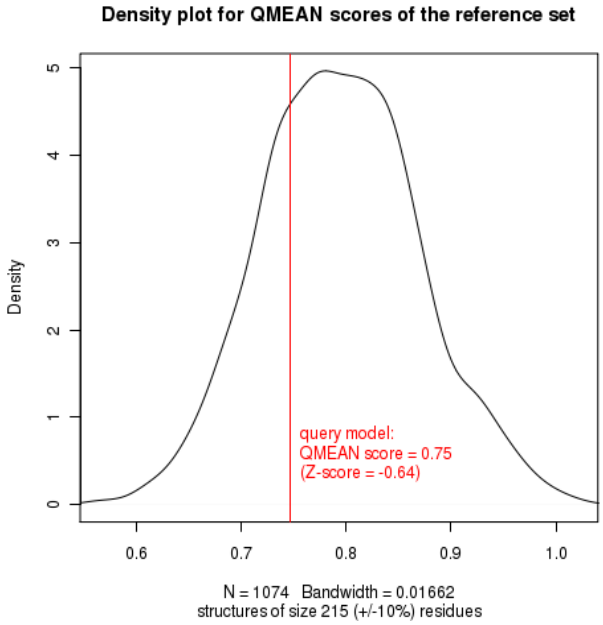
Figure - 25: QMEAN analysis for estimating the quality of protein structure.

Density plot analysis for QMEAN score of the reference set:

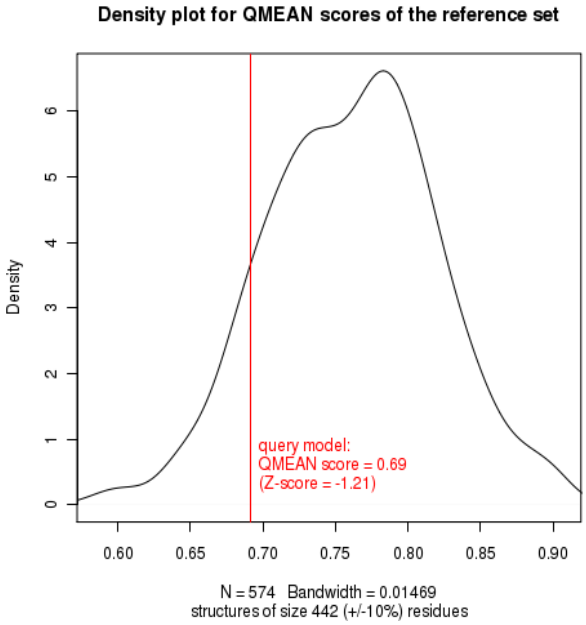
Density plot visualizing the QMEAN Z- score distribution of theoretical protein structure models. The plot explains experimental reference of the structure indicates black line and the quality of the protein indicates red in colour. The plot in the middle shows the density plot (based on QMEAN score) of all reference models used in the Z-score calculation. The plot basically is a projection of the first plot for given protein size. By drawing density plot we taken QMEAN score on X-axis and density values on Y-axis, the plot represents QMEAN values of the plot, compare to the all sap treated expressed proteins N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI and DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 shows highest scores and it supports the values of Z- score. Density plot analysis for QMEAN score of differentially expressed proteins under sap stress were represented in the following figure -26 a to 26g.



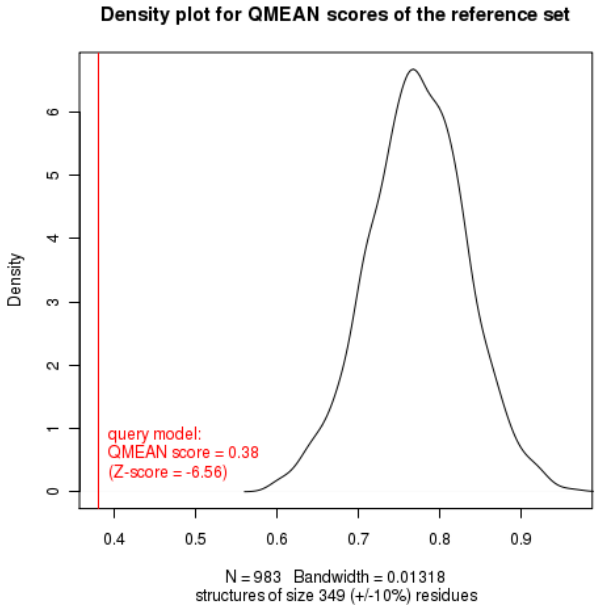
a) Density plot for UPF0401 protein ECP Y3010_ECOL5



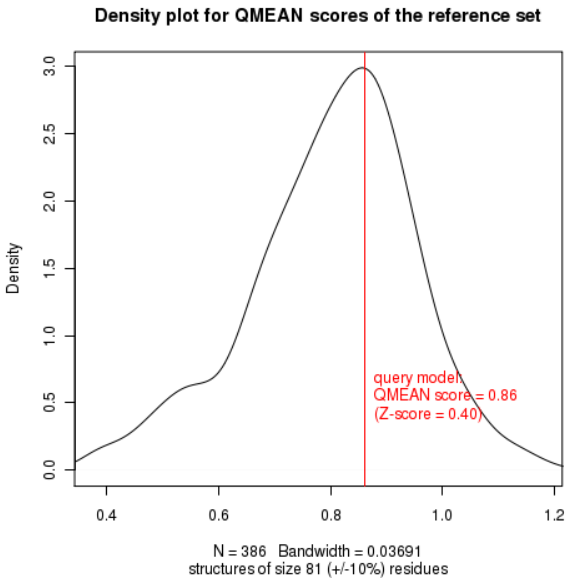
b) Density plot for Transcriptional regulatory protein BAER_ECOL6



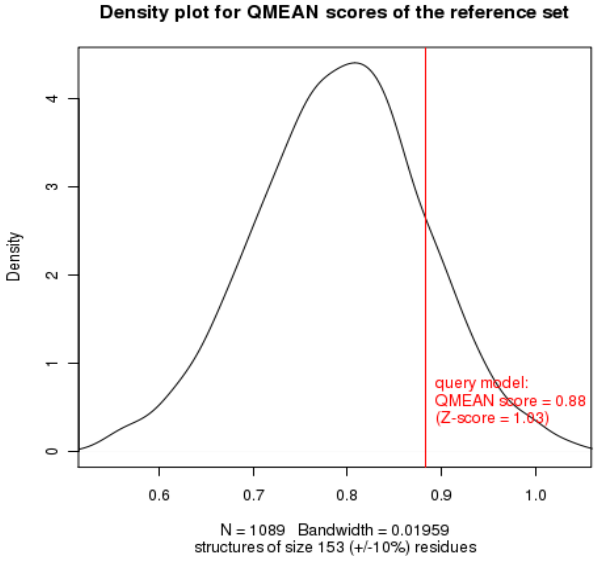
c) Density plot for Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli)



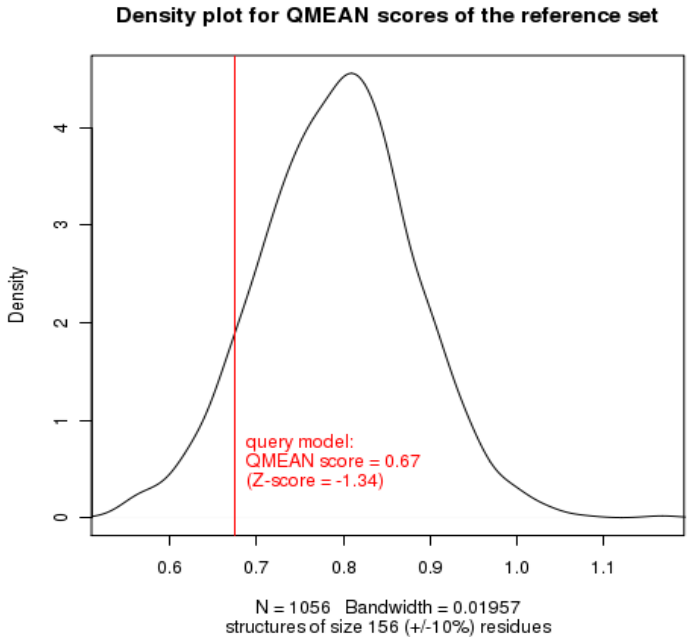
d) Density plot for Formatehydrogenlyase subunit HYCE_ECOLI



e) Density plot for DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57



f) Density plot for N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI

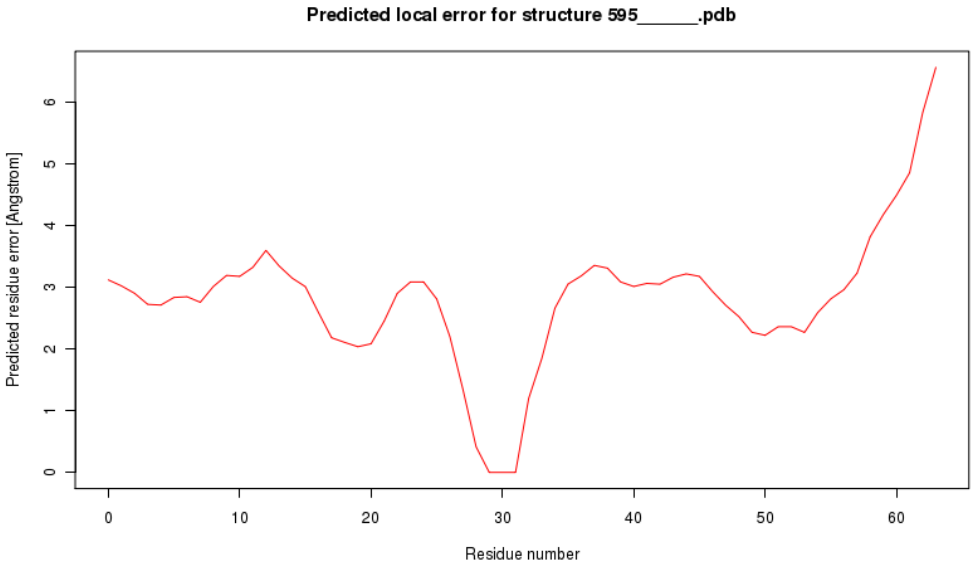


g) Density plot for t-RNA-Specific adenosine deaminase monomer

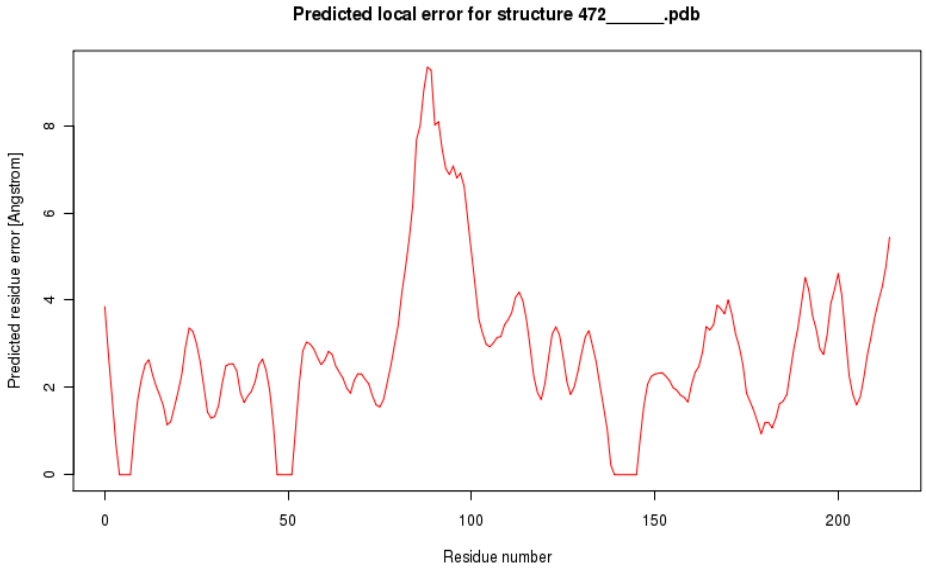
Figure -26: Density plot for QMEAN scores of the reference set

Predicted local error for the structure pdb:

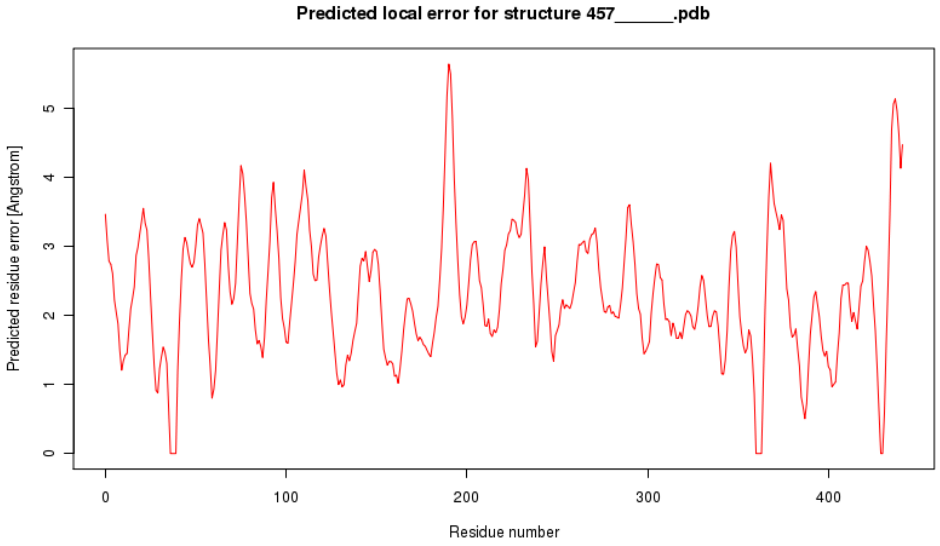
The error can rise in prediction where an insertion or deletion mutation or a gap in a solved structure result in a region of target sequence for which there is no corresponding template. Residue error plot used model energy profile with estimated residue errors along the sequence. Error values were represented. Predicted local error plot, Residue number was taken on X-axis and predicted residue error was taken on Y-axis. Lower resolution (2.5-3 A^0) indicates the structure is good based on this DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 and N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI showed less error values in structure prediction. Error plot values of differentially expressed proteins under sap stress were represented in the following figures -27 a to 27 g.



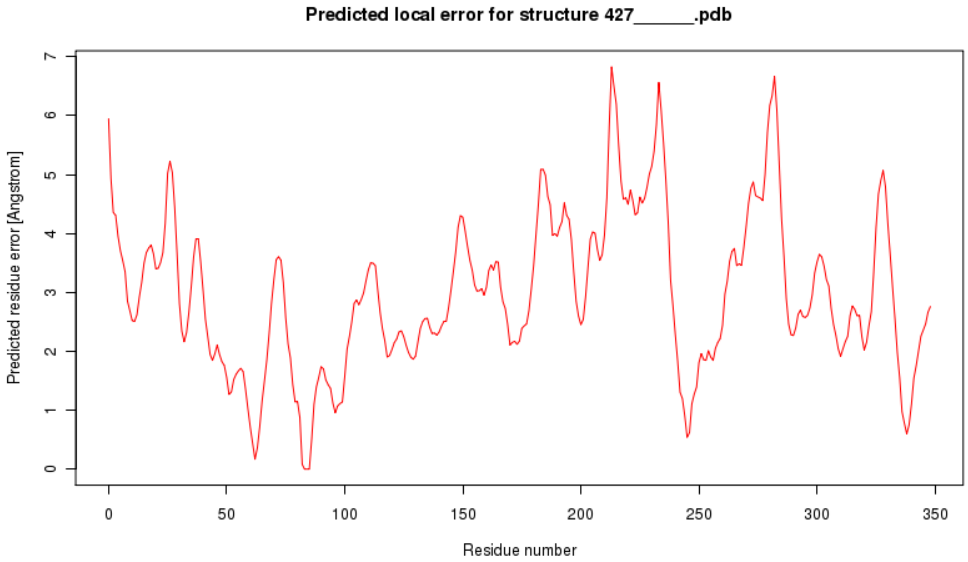
a) Predicted local error for UPF0401 protein ECP Y3010_ECOL5



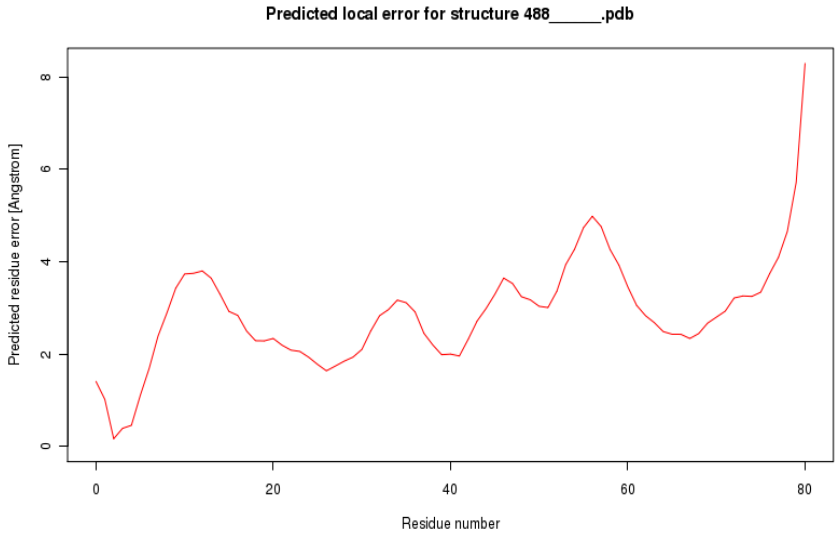
b) Predicted local error for Transcriptional regulatory protein BAER_ECOL6



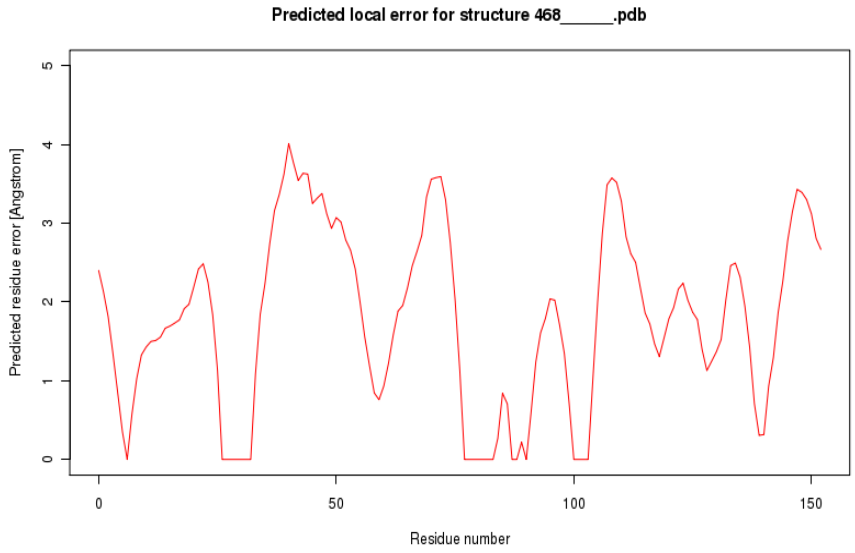
c) Predicted local error for Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli)



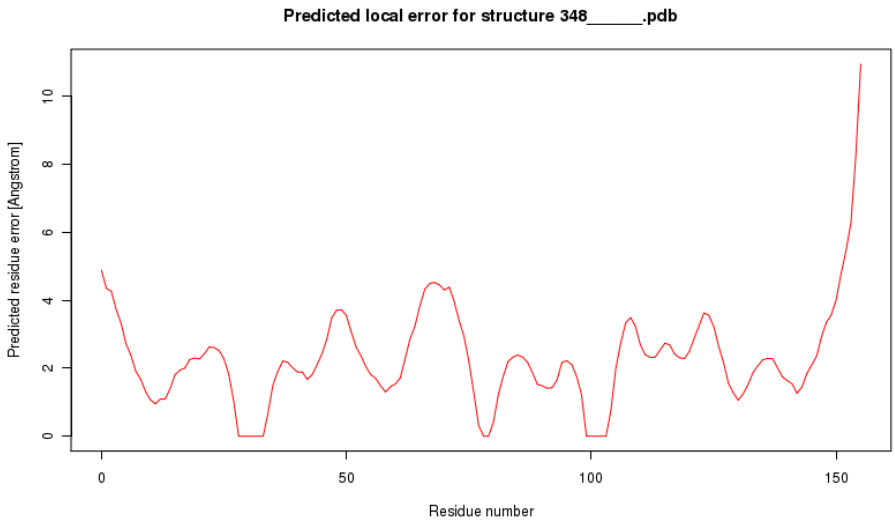
d) Predicted local error for Formatehydrogenlyase subunit HYCE_ECOLI



e) Predicted local error for DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57



f) Predicted local error for N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI



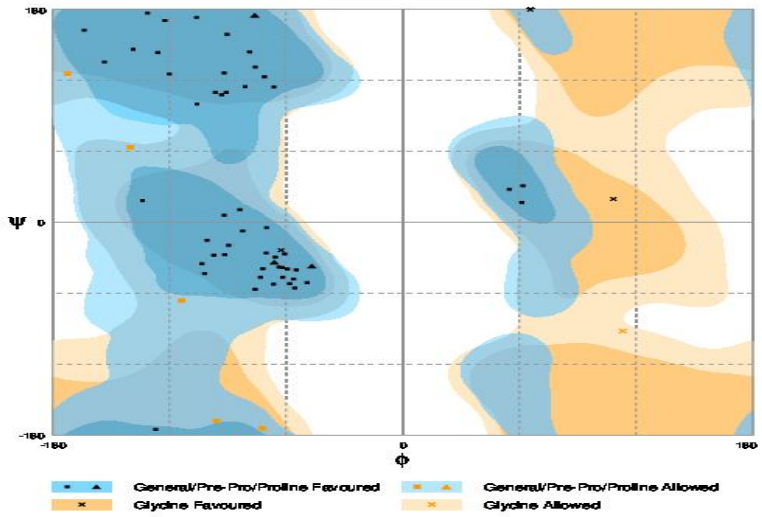
g) Predicted local error for t-RNA-Specific adenosine deaminase monomer

Figure- 27: Predicted local error for the structure of differentially expressed proteins pdb under sap stress

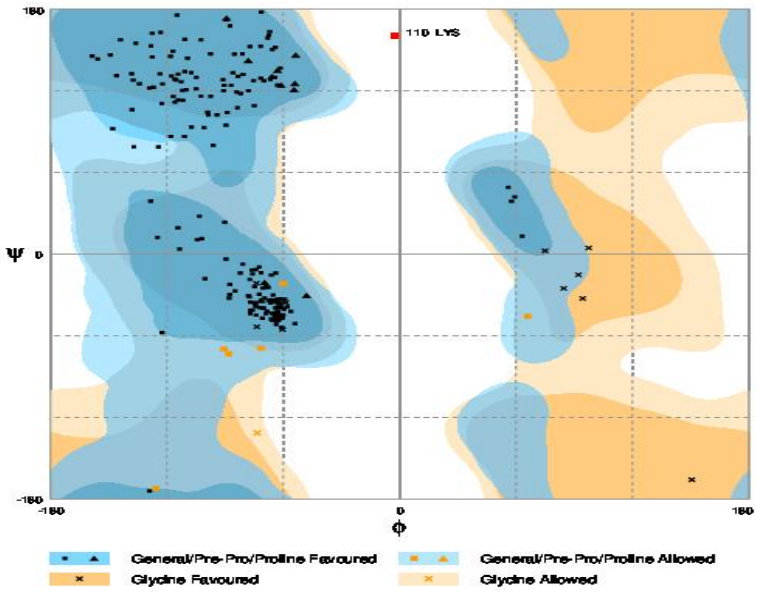
Validation of protein samples:

Validation of the protein model was done with RAMPAGE server (Lovell *e al.*, 2000). After the refinement process, validation of the model was carried out using Ramachandran plot. The ψ and φ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized. Validation of protein samples of differentially expressed proteins under sap stress were represented in the following figures -28 a to 28 g.

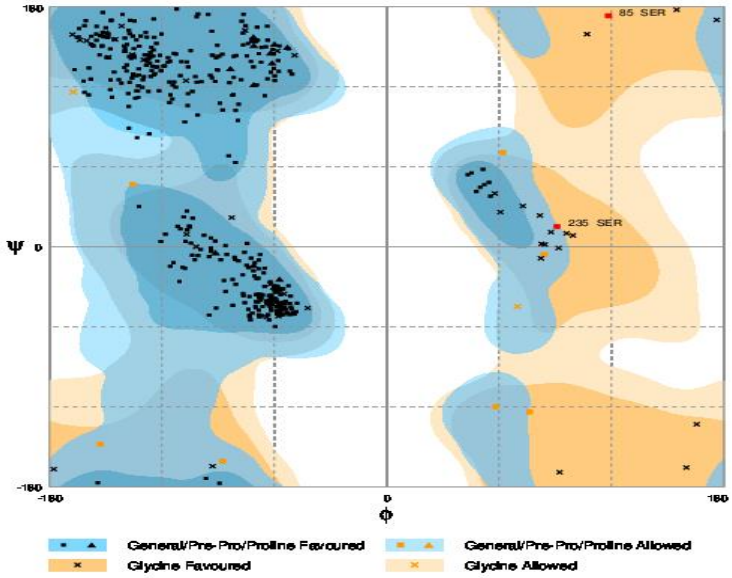
Ramachandran's plot of UPF0401 protein shows that 94.8% (146) favoured and allowed regions, 5.2% (8) of the residues was in disallowed regions. Ramachandran's plot of Transcriptional regulatory protein BAER_ECOL6 shows Lysine and Aspargine in disallowed region. This plot shows 96.2% (203) favoured region, 3.3% (7) allowed region and the remaining 0.5 % (1) outlier region. In case of Protein PmbA (PMBA-Eco57– protein pmba 0s *E.coli*), the plot shows 97.5% (429) favoured region, 2.0% (3) allowed region and the remaining 0.5 % (2) outlier region. The protein Formatehydrogenlyase subunit HYCE_ECOLI showed that the ψ and φ distributions of the Ramachandran's plots of glycine, proline regions were summarized. 76.9% (267) of residues in favoured region, 15% (52) of residues in allowed regions and 8.1% (28) of the residues in outlier regions. In case of DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 the ψ and φ distributions of the Ramachandran's plots of non-glycine, non-proline residues were summarized. 94.9% (75) of favoured region (non-glycine and non-proline), 2.5% (2) in allowed regions (Glycine and Proline) and 2.5% (2) in disallowed regions (Glycine and Proline residues). In case of N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI the ψ and φ distributions of the Ramachandran plots of non-glycine, non-proline residues were summarized. 98% (148) of favoured regions (non-glycine, non-proline), 2% (3) of allowed regions (glycine, proline) and there was no residues in disallowed regions. The protein t-RNA-Specific adenosine deaminase monomer the ψ and φ distributions of the Ramachandran plots of non-glycine, non-proline residues were summarized. 94.8% (146) of favoured regions (non-glycine, non-proline), 5.2% (8) of allowed regions (glycine, proline) and there was no residues in disallowed regions.



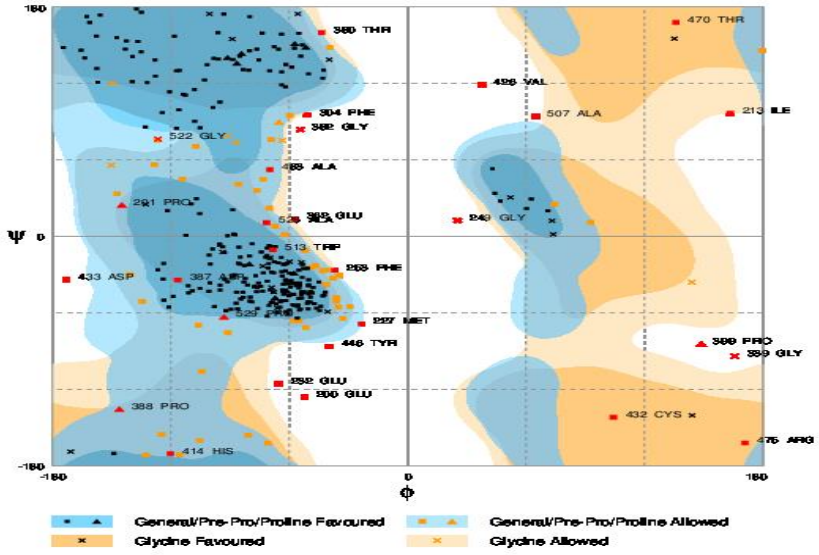
a) : UPF0401 protein validation by Ramachandran's plot analysis.



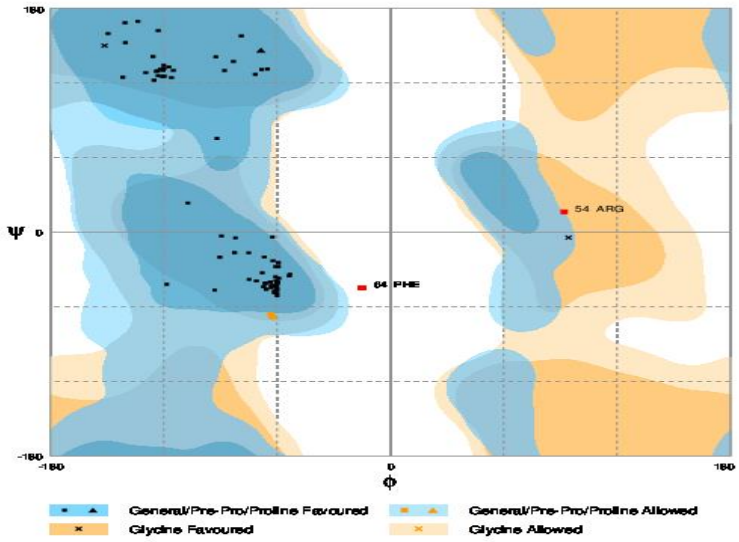
b) Transcriptional regulatory protein validation by Ramachandran's plot analysis



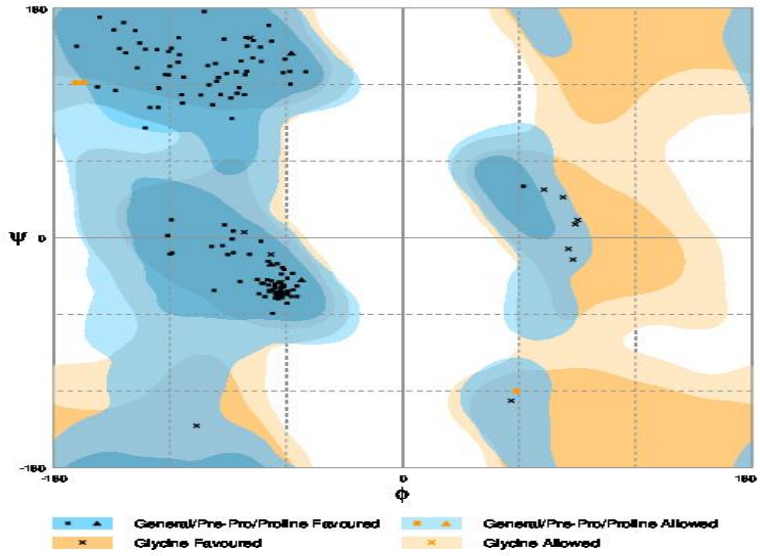
c) Protein PmbA validation by Ramachandran's plot analysis



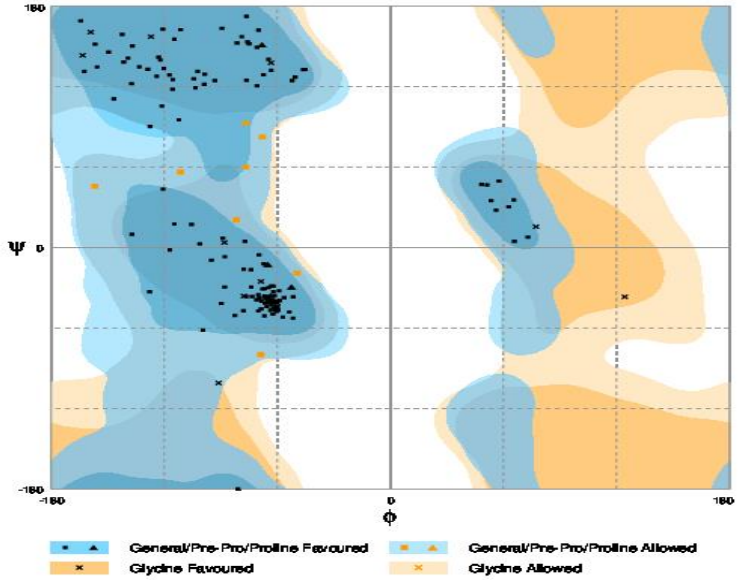
d) Formatehydrogenlyase subunit HYCE_ECOLI protein validation Ramachandran's plot analysis.



e) DinI-like protein validation by Ramachandran's plot analysis



f) N-acetylgalactosamine-specific phosphotransferase enzyme IIB component1 Protein validation by Ramachandran's plot analysis.



g) t-rna specific adenosine deaminase TADA-ECO57

Fig-28: Validation of protein sample by Ramachandran's plot analysis.

Global quality validation of proteins:

The analysis provides both global and site-specific measures of protein structure quality. Global quality measures are reported as Z scores, based on calibration with a set of high-resolution X-ray crystal structures. PSVS is particularly useful in assessing protein structures determined by NMR methods, but is also valuable for assessing X-ray crystal structures or homology models. RMS Deviation is a good measure of accuracy.

Tab -34: Validation scores of differentially expressed proteins under sap treatment.

Quality score of the model, Close contacts and deviations from ideal geometry were represented in the following table- 34 (1 to 7.1).

1) UPF0401 protein ECP Y3010_ECOL5 protein global quality scores.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.33	0.45	-0.66	-0.28	63.49
Z-score[1]	-2.09	-0.83	-2.28	-1.66	-9.37

1.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	1.9Å
RMS deviation for bond lengths	0.36 Å

2) Transcriptional regulatory protein BAER_ECOL6 global quality scores.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.30	0.46	-0.22	-0.10	29.63
Z-score[1]	-2.57	-0.79	-0.55	-0.59	-3.56

2.1) Close contacts and deviations from ideal geometry

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	3.1 Å
RMS deviation for bond lengths	0.50 Å

3) Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli) global quality scores

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.46	0.56	-0.29	-0.04	83.74
Z-score[1]	0.00	-0.37	-2.83	-0.24	-12.84

3.1) Close contacts and deviations from ideal geometry

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	3.4 Å
RMS deviation for bond lengths	0.049 Å

4) Formatehydrogenlyase subunit HYCE_ECOLI global quality scores

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.33	0.06	-1.04	-0.56	158.79
Z-score[1]	-2.09	-2.44	-3.78	-3.31	-25.72

4.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	4.0 Å
RMS deviation for bond lengths	0.060 Å

5) DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57 global quality scores.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.45	0.80	-0.07	0.27	56.97
Z-score[1]	-0.16	-0.62	-0.59	1.60	-8.25

5.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	5.0 Å
RMS deviation for bond lengths	0.068 Å

6) N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI Global quality scores

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.48	0.69	0.03	0.10	3.70
Z-score[1]	0.32	0.17	0.43	0.59	0.89

6.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	1.6 Å
RMS deviation for bond lengths	0.014 Å

7) t-RNA-Specific adenosine deaminase monomer global quality scores

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.44	0.53	-0.22	-0.12	20.15
Z-score[1]	0.32	0.50	-0.55	-0.71	-1.93

7.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	1.7 Å
RMS deviation for bond lengths	0.015 Å

Table -35: List of newly expressed proteins of *Eschericia coli* Nissle *1917* identified by Mass Spectrometry by using peptide mass fingerprinting (PMF) analysis under cocoti wine stress.

S.No	Spot	Differentially Expressed protein name	Molecular weight	Calculated pI value	Protein Score	Number of Amino acids	Sequence coverage (%)
8	3n1	P21 prophage-derived head-stabilizing proteinVG03_ECOL6	7620.8	10.70	37	68	41
9	507	PUR7-eco24-phosphoribosylamidazole-succinocarboxamide synthase	26955	5.05	32	237	16
10	378	Probable adenosine monophosphate-protein transferase FIC_ECOLI	22960	5.16	29	200	23

11	276	UPF0033 protein YEDF_	8638.9	4.83	38	77	53
12	324	Transcription anti-termination protein RFAH- ECO57 NusA	18340.2	8.55	35	162	17
13	415	30S ribosomal protein RS11_ECO24	18903.7	9.71	34	177	25
14	478	Small toxic polypeptide LDRA_ECOLI	4013.8	10.83	25	35	94
15	466	Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57	16949.7	6.79	32	159	25

Under the influence of wine stress, *E.coli* Nissle 1917 shows eight differentially expressed proteins. The protein spot identified in the gel with the number of **3n1**, belongs to the class contains P21 prophage-derived head-stabilizing proteinVG03_ECOL6, which is involved in viral life cycle activities. The protein spot identified in the gel with the number of **507**, belongs to the class contains PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase, which is involved in catalytic activities. The protein spot identified in the gel with the number of **378**, belongs to the class contains Probable adenosine monophosphate-protein transferase FIC_ECOLI, which is involved in regulation mechanisms. The protein spot identified in the gel with the number of **276,** belongs to the class contains UPF0033 protein YEDF, which is involved in cellular response to DNA damage stimulus. The protein spot identified in the gel with the number of **324**, belongs to the class contains Transcription anti-termination protein RFAH- ECO57 NusA, which is involved in the anti-termination and termination process. The protein spot identified in the gel with the number of **415**, belongs to the class contains 30S ribosomal protein RS11_ECO24, which is involved in protein binding. The protein spot identified in the gel with the number of **478**, belongs to the class contains small toxic polypeptide LDRA_ECOLI, which involved in the cell signaling process. The protein spot identified in the gel with the number of **466**, belongs to the class contains Xanthine dehydrogenase iron sulphur binding subunit XDHC-Eco57, which is involved in the electron transport mechanism. Differentially expressed proteins under wine stress were presented in the following table -35.

P21 prophage-derived head-stabilizing proteinVG03_ECOL6 :

Uniprot id of the protein L4E621 and the taxonomic identifier number is -1182672 mostly the protein activities related to the viral life cycle, Head protein gp3 is the alternative name of this protein, sequence length 68 AA. It helps to attach the viral gene into host and involved set of processes include attachment and entry of the virus particle and translation of viral mRNA by host ribosomes. Molecular weight of this protein 7.6 kDa, calculated pI and sequence coverage identity and total protein score were presented in table-35.

PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase:

The protein involved in purine metabolism, protein Uniprot id is L3F5K4 and the taxonomic id is 1181759. Alternate name of this protein SAICAR synthase (Nelson *et al.,* 2005). It is an intermediate in the formation of purines. The conversion of ATP, L-aspartate, and 5-aminoimidazole-4-carboxyribonucleotide to 5- aminoimidazole-4-(N-succinylcarboxamide) ribonucleotide, ADP and phosphate by phosphoribosylaminoimidazole-succinocarboxamide synthase, represents the eighth step of the denovo purine nucleotide biosynthesis. Molecular weight of this protein 26.9 kDa, calculated pI and sequence coverage identity and total protein score were mentioned in table - 35.

ATP + 5-amino-1-(5-phospho-D-ribosyl) imidazole-4-carboxylate + L-aspartate ⇌ ADP + phosphate + (S)-2-[5-amino-1-(5-phospho-D-ribosyl) imidazole-4- carboxamido] succinate.

Probable adenosine monophosphate-protein transferase FIC_ECOLI:

The protein involved in transferase activity, taxonomic identifier number 550685 and the Uniprot id H1E4X9. The protein also known as cell filamentation protein fic. It is a component in base excision repair pathway, it replace the mismatched and fill in the gap with the correct base, by using the template strand as the reference (Yuan Liu *et al.,* 2007).

Catalytic activity: ATP + [protein] = diphosphate + [protein]-AMP

Molecular weight of this protein 22.9kDa, calculated pI and sequence coverage identity and total protein score were presented in table- 35.

UPF0033 protein YEDF:

Molecular weight of this protein 8.6 kDa, calculated pI and sequence coverage identity and total protein score were presented in table-35. It belongs to UPF0033 family proteins. It involves in cellular response to DNA damage stimulus means, change the activity of a cell like movement, expression, secretion and enzyme production as a result of indicating damage to its DNA from environmental errors during metabolism (Hayashi *et al*., 2006).

Transcription anti-termination protein RFAH- ECO57 NusA:

Molecular weight of the protein is 18.3 kDa, calculated pI and sequence coverage identity and total protein score were mentioned in table-35. Uniprot id of the protein is POAFF7/6 and taxonomic id 199310. It is an essential component of the RNA polymerase elongation complex, is involved in transcriptional elongation, termination, anti-termination, cold shock and stress-induced mutagenesis the protein involved in a variety of cellular and viral termination and antitermination processes, such as Rho dependent transcriptional termination, and phage lambda N-mediated transcriptional antitermination (Tomar *et al*., 2013). Also important for coordinating the cellular responses to DNA damage by coupling the processes of nucleotide excision repair and translesion synthesis to transcription.

30S ribosomal protein RS11_ECO24:

Molecular weight of this 18.9kDa, Uniprot id of the protein is T9ELL3 and taxonomic identification number 1281225. This protein binds to the 23s rRNA, and its secondary structure. It is located near the subunit interface in the base of the L7/L12, and near the t-RNA binding site of the peptidyl transferase center (Rasko *et al.*, 2008). Calculated pI and sequence coverage identity and total protein score were mentioned in table -35.

Small toxic polypeptide LDRA_ECOLI:

Uniprot id of this protein in L4K737 and taxonomic identifier is 83333. Molecular weight of this protein is 4013.8 Daltons. Calculated pI and sequence coverage identity and total protein score were presented in table-35. It belongs to toxic peptide ldr family total 3 proteins in this family i.e. LdrB,LdrD. LdrA/LdrC. Toxic peptide whose overexpression causes rapid cell killing and nucleoid

condensation of the host cell. A type l toxin antitoxin (TA) system where expression of the proteinaceous toxin is controlled by an antisense sRNA, in this case RdlA or RdlC (Kawano *et al*., 2002). Only a few of these TA systems have been mechanistically characterised, the mechanisms used to control expression of the toxin gene are not necessarily the same.

Xanthine dehydrogenase iron sulphur binding subunit - XDHC-Eco57:

Molecular weight of this protein is 16949.7 Daltons, Uniprot code of this protein is K3TSG9 and taxonomic identifier is 1005482. The protein involved in Xanthine dehydrogenase activity, electron carrier activity, iron- sulphur cluster binding, metal ion binding activity. Iron –sulphur are found metalloproteins, such as ferredoxins, coenzyme Q-cytochrome c reductase and nitrogenase. Iron- sulphur clusters are best known for their role in the Oxidation-reductions of mitochondrial electron transport. Xanthine dehydrogenase can be converted to Xanthine oxidase by reversible sulfhydryl oxidation or by irreversible proteolytic modification. Xanthine dehydrogenase cause xanthinuria, may contribute to adult respiratory syndrome, and may potentiate influenza infection through an oxygen metabolite-dependent mechanism (Ichida *et al.,* 1993). Calculated pI and sequence coverage identity and total protein score were presented in table -35.

Template alignment of differentially expressed proteins under wine stress:

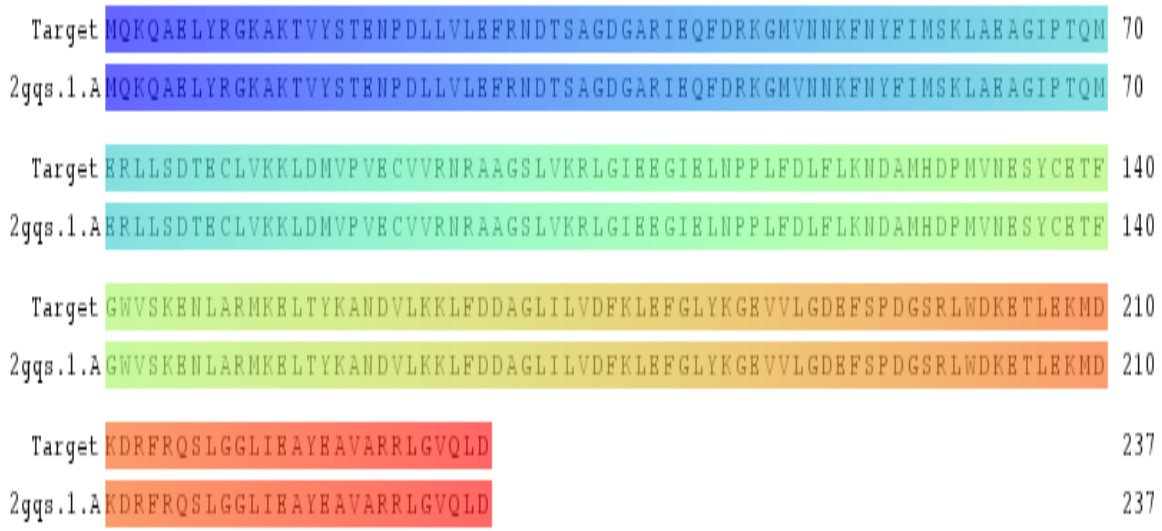
Homology models were obtained from wine stress proteins based on MASCOT search data sequence, on wine treatment eight proteins were isolated from 2-D gel and overall all proteins gave good prediction structure. Differentially expressed proteins were presented in the above table -35.

Eight homology model proteins were obtained differentially expressed proteins under wine stress, based on X- ray structures of templates. The target sequence was searched with BLAST against Protein Data Bank, which one has a high level of sequence identity with target protein selected as a template protein. Templates were determined by superimposition of the two structures and multiple sequence alignment was performed with CLUSTAL W (Larkin *et al.,* 2007) program to identify the set of conserved residues of alignment were represented in the following figures 29a to 29h.

1HYW protein was selected as a template for P21 prophage-derived head-stabilizing proteinVG03_ECOL6 target protein, **2Z02** protein was selected as a template for PUR7-eco24-phosphoribosylamidazole-succinocarboxamide synthase target protein, **3ZC7** was selected template

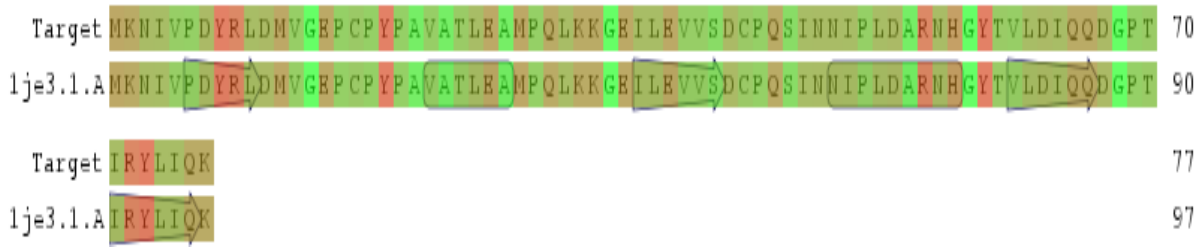
for Probable adenosine monophosphate-protein transferase FIC_ECOLI target protein, **1JE3** was selected template for protein UPF0033 protein YEDF_ target protein, **4MTN** protein was selected template for Transcription anti-termination protein RFAH- ECO57 NusA target protein, **3J5E** was selected template for 30S ribosomal protein RS11_ECO24 target protein. **C4B1** was the selected template for Small toxic polypeptide LDRA_ECOLItarget protein and **1ZXI** was selected template for Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57. Amino acid sequences of these proteins were represented in the following figure 29-a to 29h.

a) Alignment of the amino acid sequences of P21 prophage-derived head-stabilizing protein VG03 ECOL6 protein with **1HYW**.

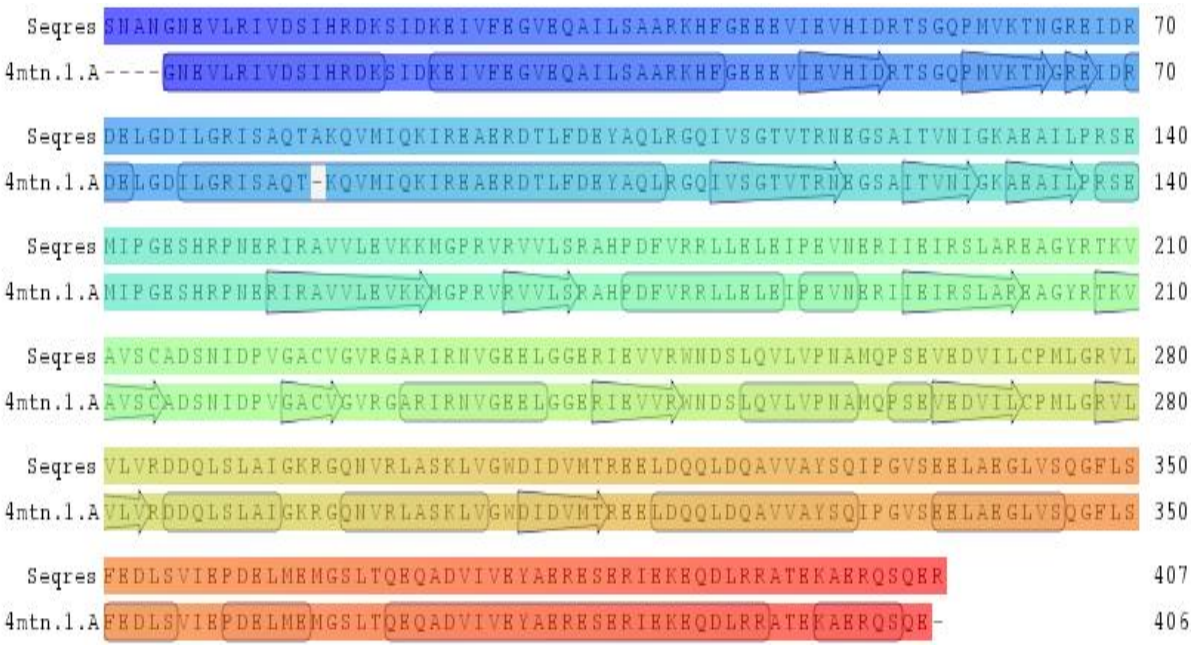


b) Alignment of the amino acid sequences of PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase protein with **2Z02**.

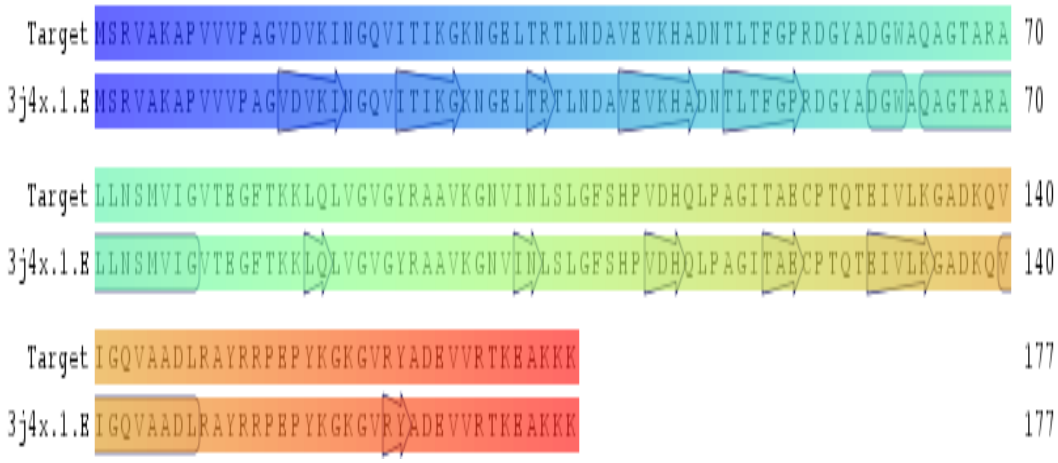
c) Alignment of the amino acid sequences of Probable adenosine monophosphate-protein transferase FIC_ECOLI protein with **3ZC7.**



d) Alignment of the amino acid sequences of UPF0033 protein YEDF_ ECO57 with **1JE3**



e) Alignment of the amino acid sequences of Transcription anti-termination protein RFAH- ECO57 NusA with **4mtn**.



f) Alignment of the amino acid sequences 30S ribosomal protein RS11_ECO24 with **3J5E**

g) Alignment of the amino acid sequences of Small toxic polypeptide LDRA_ECOLI protein with **C4B1**.

h) Alignment of the amino acid sequences of Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein with **1ZXi**

Fig-29: Alignment of the amino acid sequences of differentially expressed proteins under wine stress. The results were generated with CLUSTAL W multiple sequence alignment tool.

Homology modelling of differentially expressed proteins under wine stress:

The sequences were analysed with the help of Phyre-2 (protein Homology/analogy Recognition Engine V 2.0) server for obtaining pdb file. The final 3-D structure obtained with the help of RasMol version 2.6 software programme.

1HYW selected as a reference structure for modeling of P21 prophage-derived head-stabilizing proteinVG03_ECOL6 protein. Overall 41% sequence identity of the protein, it is sufficient for creating an acceptable model prediction, in Swiss pdb we get 16 pdb templates out of this select a least energy. The structure has 41-H-bonds, 2 helices, No.of strands-2 and 4- turns were represented in the following figure -30a.

2Z02 is selected as reference structure for modelling PUR7-eco24-phosphoribosylamidazole-succinocarboxamide synthase. The protein sequence identity is 16%, it is sufficient for creating an acceptable model prediction, in Swiss pdb we get 29 templates, out of this we select best match one. The structure has 172-H-bonds, 9-Helices, 22 strands and 21 turns were represented in the following figure-30-b.

3ZC7 template selected as reference structure for modelling Probable adenosine monophosphate-protein transferase FIC_ECOLI. Percentage of sequence identity is 23%, in Swiss pdb we get 28 templates out of this we select best matched one for prediction. The structure has 129-H-bonds, 9 helices, 6 strands and 17 turns were represented in the following figure 30-c.

1JE3 selected as a reference structure for modelling UPF0033 protein YEDF_ protein. Sequence identity is 53% and in Swiss pdb we get 83 pdb templates out of this we select a least energy for prediction. The structure has 48-H-bonds, 2 helices, 6 strands and 7 turns were represented in the following figure -30-d.

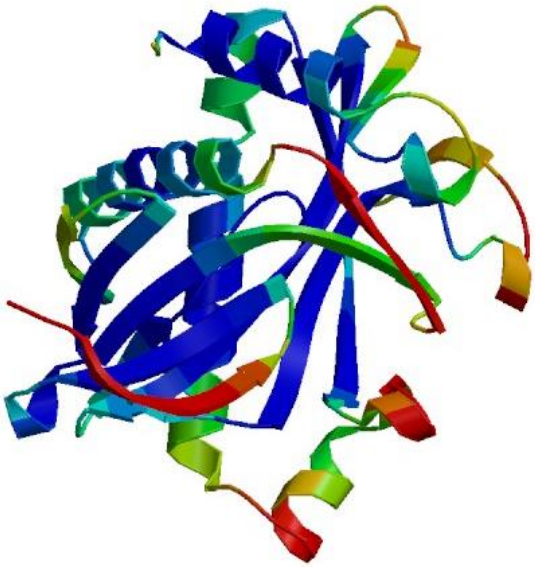
4MTN template protein selected as a reference structure for modelling Transcription anti-termination protein RFAH- ECO57 NusA. Sequence identity of this protein is 17% and gets 50 pdb templates from Swiss pdb. 3-D structure of this protein consists of 305-H-bonds, 19 helices, 18-strands and 29 turns were showed in the following figure-30-e.

3J5E template protein selected as a reference structure for modelling 30S ribosomal protein RS11_ECO24. Sequence identities matched with target is 76% and we get 8 pdb templates from Swiss pdb. 3-D structure of this protein consists of 89-H-bonds, 3-helices, 14 strands and 20 turns represented in the following figure-30f.

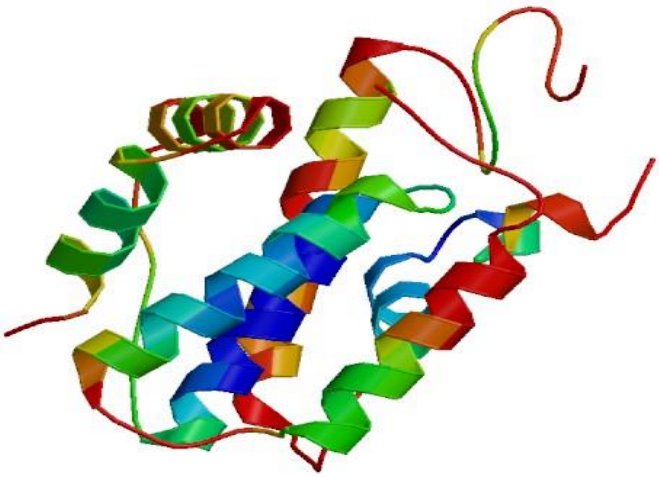
C4B1 is selected as reference structure for modelling Small toxic polypeptide LDRA_ECOLI. Sequence identities matched with 94% and we get 11 pdb templates from Swiss pdb. 3-D structure of this protein consists of 23-H-bonds, 3- helices, and 1 turns represented in the following figure-30g.

1ZXI template protein selected as a reference structure for modelling Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57. Sequence identities matched with target is 25% and we get 89 pdb templates from Swiss pdb. 3-D structure of this protein consists of 98-H-bonds, 10-helices, 7 strands and 21s turns represented in the following figure-30-h.

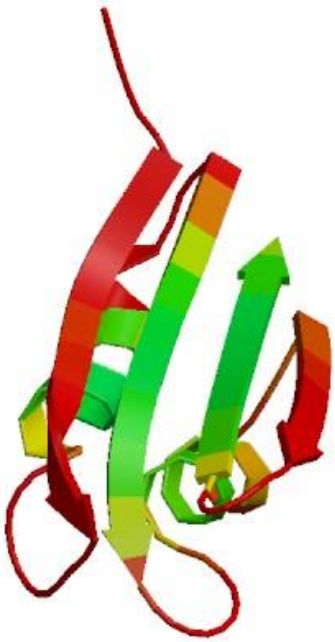
a) 3-D structure of P21 prophage-derived head-stabilizing protein VG03 ECOL6



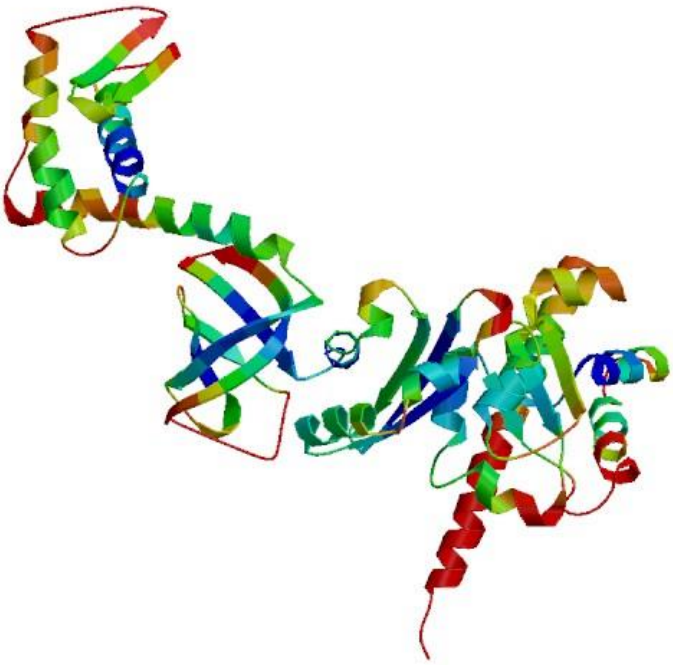
b) 3-D structure of PUR7-eco24- phosphoribosylamidazole- succinocarboxamide synthase Protein



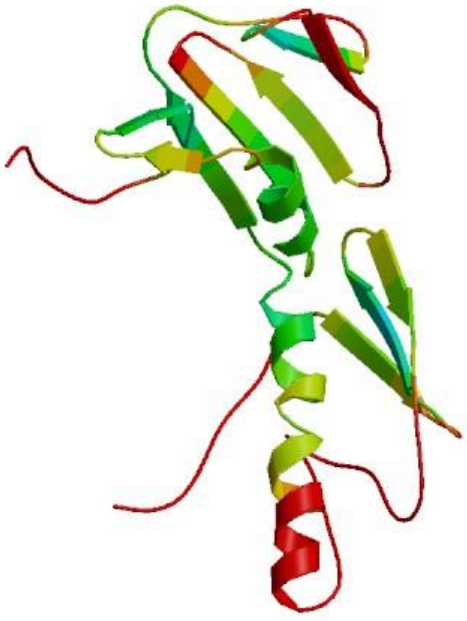
c) 3-D structure of Probable adenosine monophosphate-protein transferase FIC_ECOLI



d) 3-D structure of UPF0033 protein YEDF_ECO57 protein

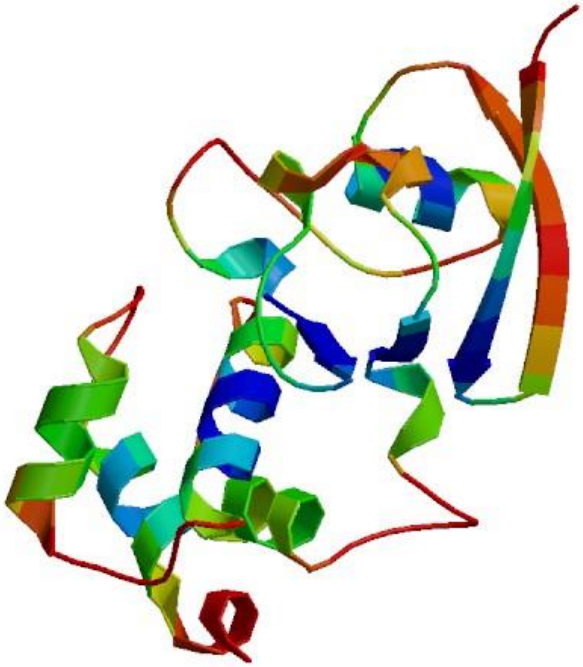


e) 3-D structure of Transcription anti-termination protein RFAH- ECO57 NusA



f) 3-D structure of 30S ribosomal protein RS11_ECO24

g) 3-D structure of Small toxic polypeptide LDRA_ECOLI

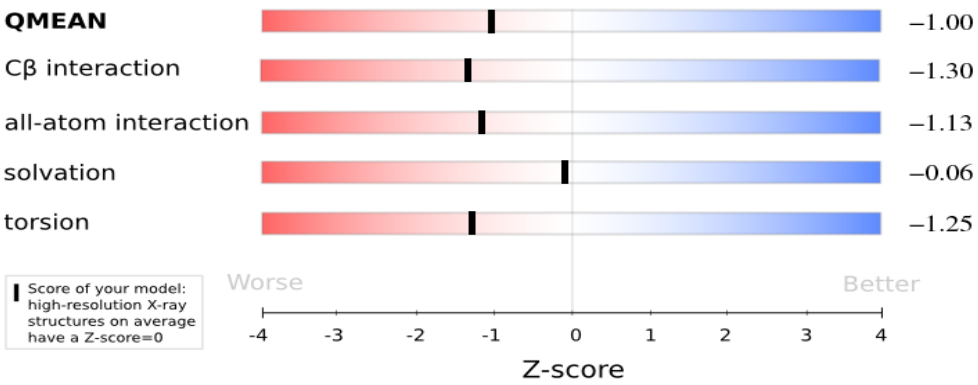


h) 3-D structure of Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein

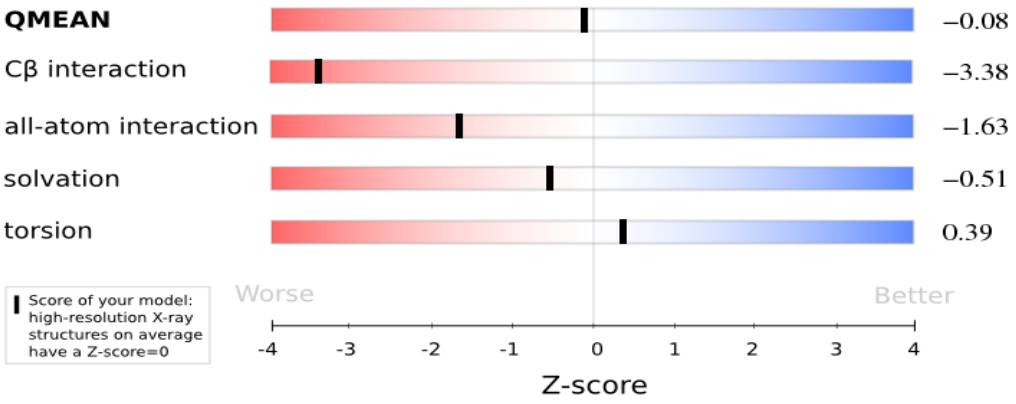
Figure- 30: Homology modelling of differentially expressed proteins under wine stress.

QMEAN analysis for the quality resolution structure:

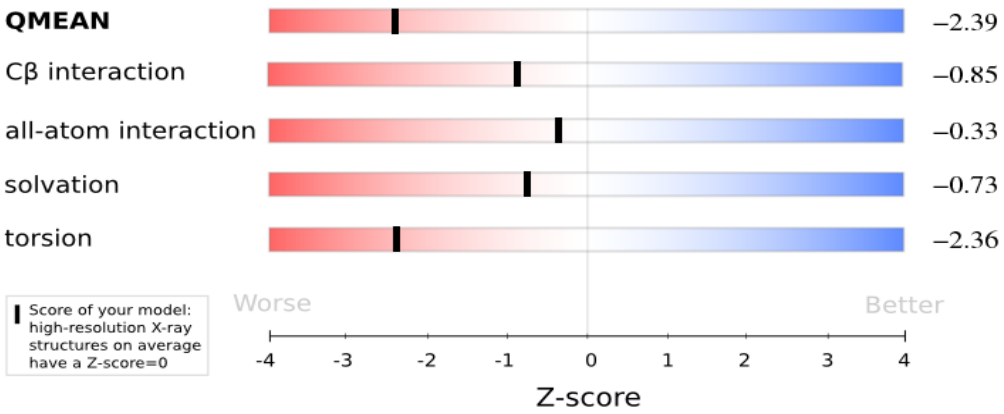
The QMEAN scoring function estimates the global quality of the models on the basis of a linear combination of six structural descriptions, four of them are statistical potentials of mean force. The local geometry is analysed by a torsion angle potential over three consecutive amino acids. The distance –dependent interaction potentials based on Cβ atoms and all toms, respectively are used to assess long-range interactions. A solvation potential describes the burial status of the residues. The analysis of these Z-scores of the individual terms can help identifying the geometrical features responsible for an observed large negative QMEAN Z-score. Models of low quality are expected to have strongly negative Z-scores for QMEAN but also for most of the contributing terms. Large negative values correspond to red regions in the colour gradient. Good structures are expected to have all sliders in the light red to blue region. The qualities of resolution of differentially expressed proteins under sap stress were represented in the following figures-31a to 31h. In P21 prophage-derived head-stabilizing proteinVG03_ECOL6 protein quality resolution structure all the values are negative indicates the structure is poor quality structure. In PUR7-eco24-phosphoribosylamidazole-succinocarboxamide synthase only torsion angle value is positive remaining all values were negative, the protein structure is low quality prediction structure. In case of Probable adenosine monophosphate-protein transferase FIC_ECOLI protein shows all values were negative it indicates the structure was in poor quality prediction. The protein UPF0033 protein YEDF_ quality of resolution structure refers all the values were negative it indicates the structure is low quality prediction. In case of Transcription anti-termination protein RFAH- ECO57 NusA protein all angle shows positive values except torsion angle and QMEAN values shows light red colour indicates the structure is better quality compare to UPF0033 protein YEDF. In case of 30S ribosomal protein RS11_ECO24 except solvation remaining all are negative values indicate predicted structure is low quality. All values of the protein Small toxic polypeptide LDRA_ECOLI were negative of this protein indicating poor quality structure. In case of Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein also showed negative values indicates the quality of structure is poor.



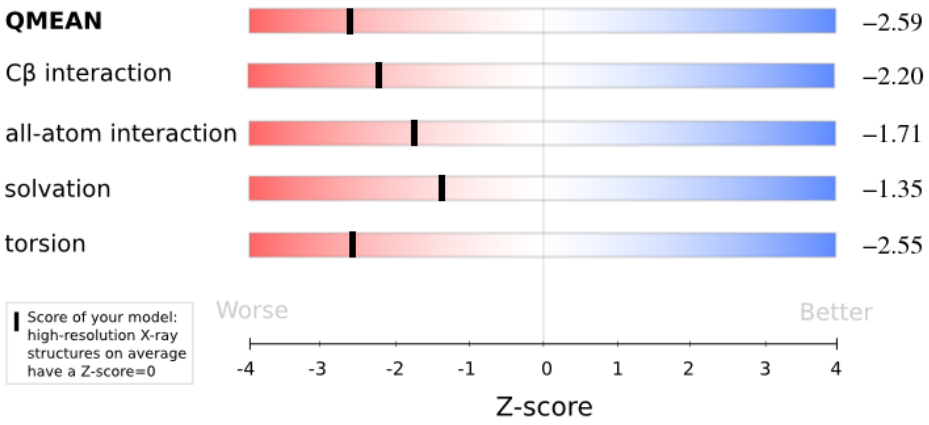
a) Quality of resolution structure of P21 prophage-derived head-stabilizing proteinVG03_ECOL6 protein:



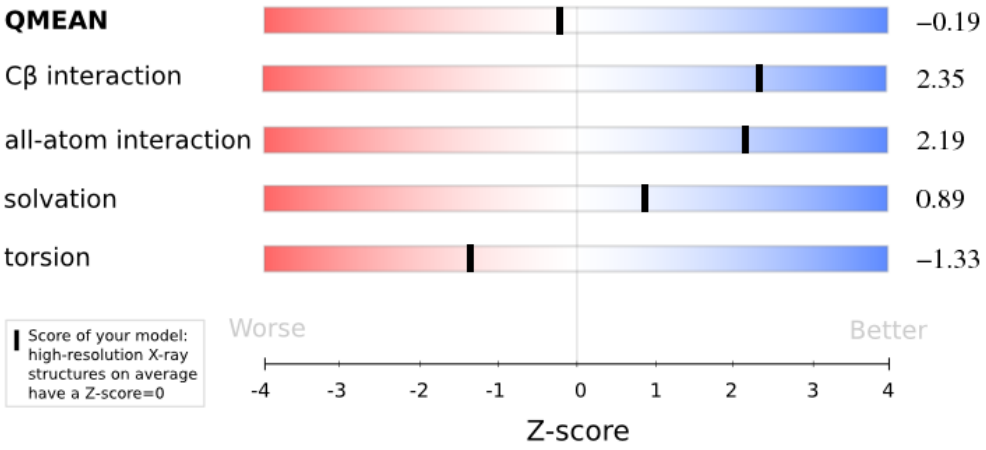
b) Quality of resolution structure of PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase.



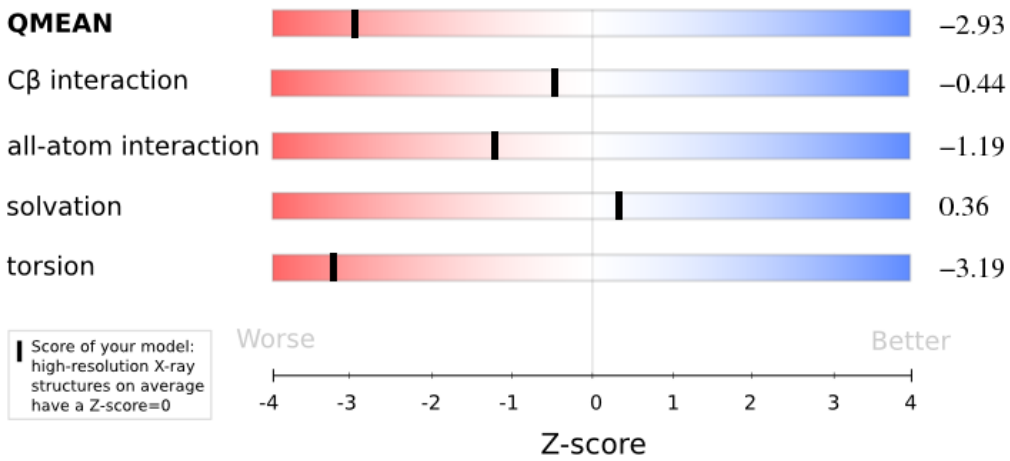
c) Quality of resolution structure of Probable adenosine monophosphate-protein transferase FIC_ECOLI.



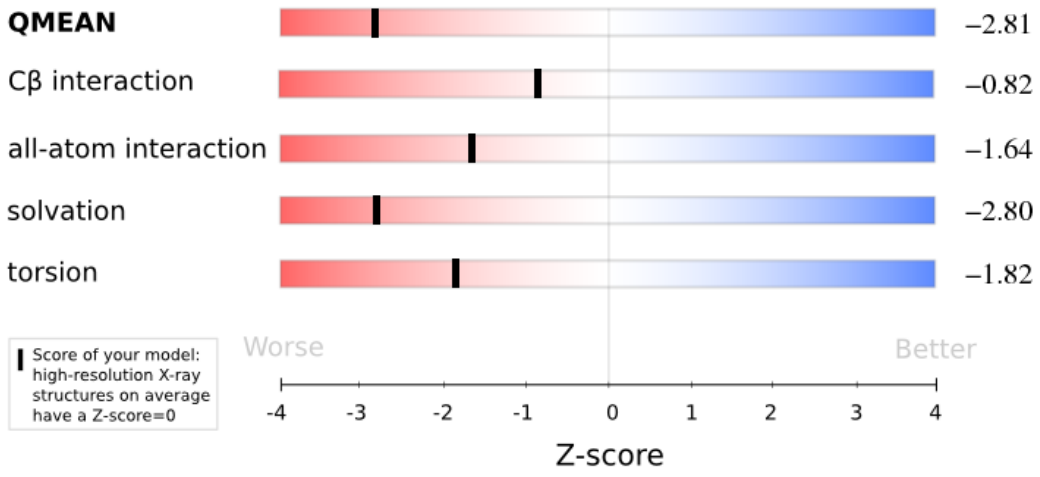
d) Quality of resolution structure of UPF0033 protein YEDF



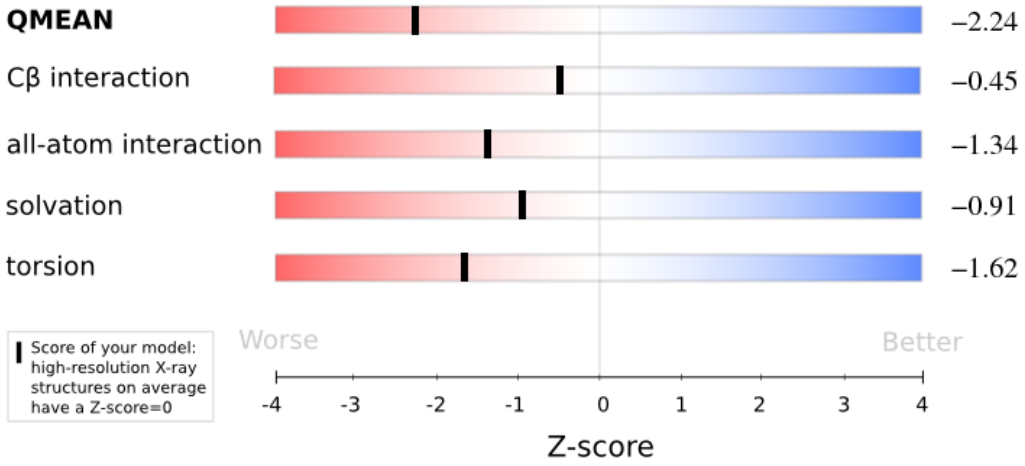
e) Quality of resolution structure of Transcription anti-termination protein RFAH- ECO57 NusA



f) Quality of resolution structure of 30S ribosomal protein RS11_ECO24



g) Quality of resolution structure of Small toxic polypeptide LDRA_ECOLI



h) Quality of resolution structure of Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57

Fig -31: Quality of resolution structure of differentially expressed proteins under wine stress.

QMEAN score:

The QMEAN score is a composite score consisting of a linear combination of 6 terms. The pseudo-energies of the contributing terms are given below together with their Z-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography (Benkert *et al*., 2008). The Z-score of a protein is defined as the energy separation between the native fold and the average of an ensemble of misfolds in the units of the standard

deviation of the ensemble. The Z-score is often used as a way of testing the knowledge-based potentials for their ability to recognize the native fold from other alternatives. QMEAN score values of differentially expressed proteins under sap stress were represented in the following tables -36a to 36h.

Table -36: QMEAN scoring function of differentially expressed proteins under wine stress

a) QMEAN scoring function of P21 prophage-derived head-stabilizing proteinVG03_ECOL6

protein model name	3n1_ pdb
C_beta interaction energy	-1.75 (Z-score: -1.30)
All-atom pairwise energy	-711.19 (Z-score: -1.13)
Solvation energy	-1.42 (Z-score: -0.06)
Torsion angle energy	-5.22 (Z-score: -1.25)
Secondary structure agreement	87.9% (Z-score: 0.32)
Secondary structure agreement	79.3% (Z-score: 0.80)
Total QMEAN-score	0.716 (Z-score: 0.05)

b) QMEAN scoring function of PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase

protein model name	507_ pdb
C_beta interaction energy	7.56 (Z-score: -3.38)
All-atom pairwise energy	-4574.16 (Z-score: -1.63)
Solvation energy	-20.29 (Z-score: -0.51)
Torsion angle energy	-73.51 (Z-score: 0.39)
Secondary structure agreement	75.5% (Z-score: -1.16)
Secondary structure agreement	77.2% (Z-score: -0.49)
Total QMEAN-score	0.698 (Z-score: -0.77)

c) QMEAN scoring function of Probable adenosine monophosphate-protein transferase FIC_ECOLI

protein model name	378_ pdb
C_beta interaction energy	-71.46 (Z-score: -0.85)
All-atom pairwise energy	-5391.09 (Z-score: -0.33)
Solvation energy	-13.35 (Z-score: -0.73)
Torsion angle energy	-18.15 (Z-score: -2.36)
Secondary structure agreement	86.6% (Z-score: 0.50)
Secondary structure agreement	80.6% (Z-score: 0.29)
Total QMEAN-score	0.722 (Z-score: -0.46)

d) QMEAN scoring function of UPF0033 protein YEDF

protein model name	276_ pdb
C_beta interaction energy	-0.49 (Z-score: -2.20)
All-atom pairwise energy	-841.95 (Z-score: -1.71)
Solvation energy	3.29 (Z-score: -1.35)
Torsion angle energy	0.77 (Z-score: -2.55)
Secondary structure agreement	77.9% (Z-score: -0.73)
Secondary structure agreement	66.2% (Z-score: -1.07)
Total QMEAN-score	0.445 (Z-score: -2.07)

e) QMEAN scoring function of Transcription anti-termination protein RFAH- ECO57 NusA

protein model name	324_ pdb
C_beta interaction energy	-201.50 (Z-score: 2.35)
All-atom pairwise energy	-11754.71 (Z-score: 2.19)
Solvation energy	-49.07 (Z-score: 0.89)
Torsion angle energy	-80.99 (Z-score: -1.33)
Secondary structure agreement	82.7% (Z-score: 0.41)
Secondary structure agreement	75.5% (Z-score: -1.03)
Total QMEAN-score	0.664 (Z-score: -1.21)

f) QMEAN scoring function of 30S ribosomal protein RS11_ECO24

protein model name	415_ pdb
C_beta interaction energy	-55.24 (Z-score: -0.44)
All-atom pairwise energy	--2614.96 (Z-score: -1.19)
Solvation energy	20.11 (Z-score: 0.36)
Torsion angle energy	-5.12 (Z-score: -3.19)
Secondary structure agreement	75.4% (Z-score: -1.09)
Secondary structure agreement	73.7% (Z-score: -0.87)
Total QMEAN-score	0.515 (Z-score: -2.55)

g) QMEAN scoring function of Small toxic polypeptide LDRA_ECOLI

protein model name	478_ pdb
C_beta interaction energy	-0.08 (Z-score: -0.82)
All-atom pairwise energy	22.19 (Z-score: -1.64)
Solvation energy	9.56 (Z-score: -2.80)
Torsion angle energy	0.31 (Z-score: -1.82)
Secondary structure agreement	60.7% (Z-score: -1.92)
Secondary structure agreement	32.1% (Z-score: -3.40)
Total QMEAN-score	-0.069 (Z-score: -3.75)

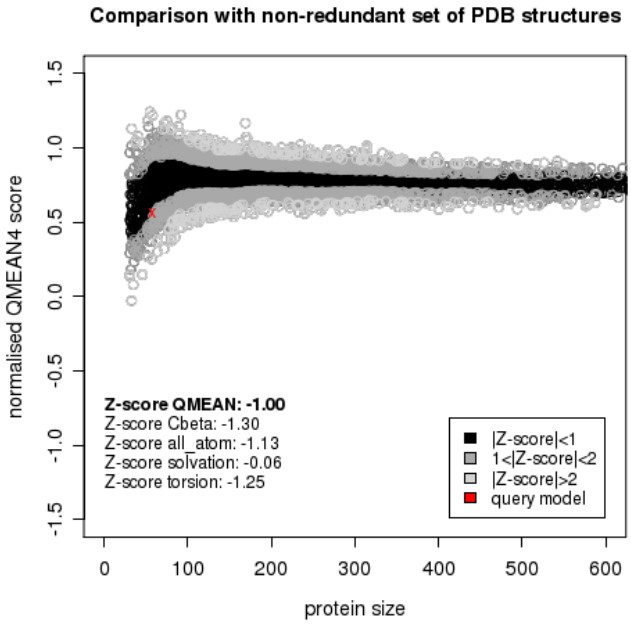
h) QMEAN scoring function of Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57

protein model name	466_ pdb
C_beta interaction energy	-69.56 (Z-score: -0.45)
All-atom pairwise energy	-2838.56 (Z-score: -1.34)
Solvation energy	-8.68 (Z-score: -0.91)
Torsion angle energy	-21.90 (Z-score: -1.62)
Secondary structure agreement	87.2% (Z-score: 0.57)
Secondary structure agreement	75.6% (Z-score: -0.40)
Total QMEAN-score	0.663 (Z-score: -0.95)

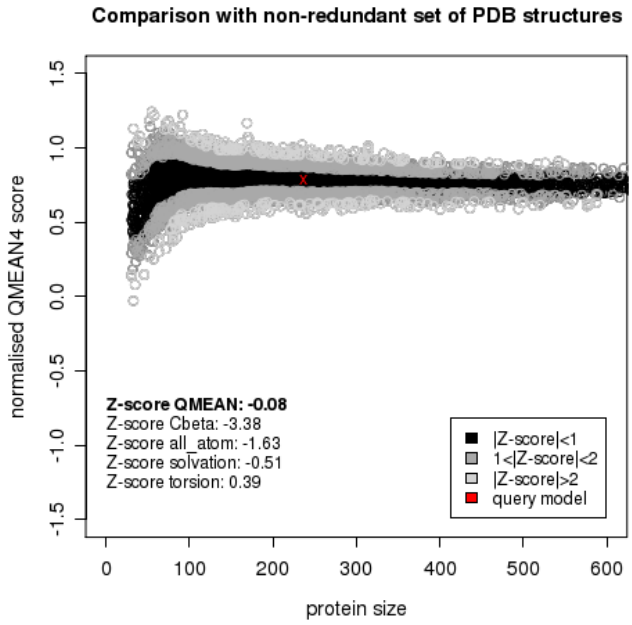
QMEAN analysis for estimating the quality of protein structure:

The areas built by the coloured in different shades of grey in the plot on the left hand side represent the QMEAN scores of the reference structures from the PDB. The model QMEAN score is compared to the scores obtain for experimental structures of similar size and Z- score is calculated. P21 prophage-derived head-stabilizing proteinVG03_ECOL6 Z-score is -1.00, PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase -Z-score is -0.08, Probable adenosine monophosphate-protein transferase FIC_ECOLI Z-score is -2.39, UPF0033 protein YEDF Z-score -2.59, Transcription anti-termination protein RFAH- ECO57 NusA Z- score of this

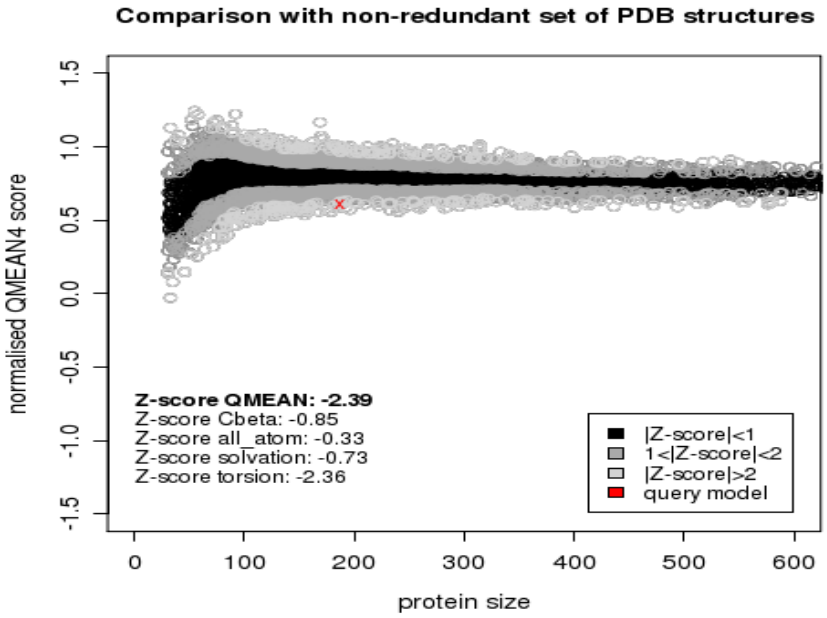
protein -0.19, 30S ribosomal protein RS11_ECO24 Z-score is -2.93. Small toxic polypeptide LDRA_ECOLI Z-score -2.81, Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 Z-score -2.24. Standard Q-MEAN score indicates that models with the normalized QMEAN score range is 0.3-0.4 should be considered as bad, between 0.4 - 0.5 considered as medium and above 0.5 the structure is good, based on these standard values the differentially expressed proteins under wine stress the quality is poor because all were showed negative Z- scores. QMEAN quality of the differentially expressed proteins under wine stress were represented in the following figures (32 a to h).



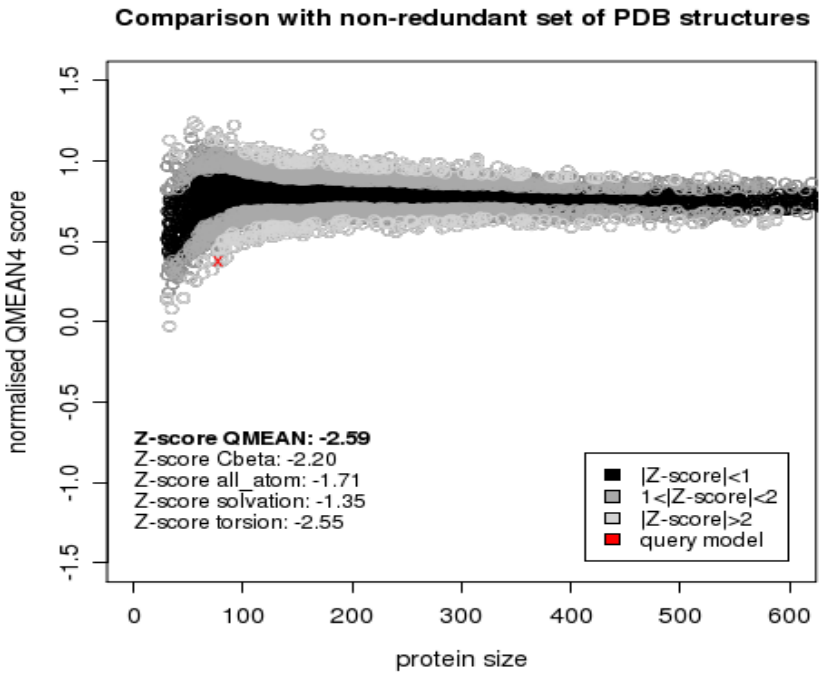
a) Quality estimation of P21 prophage-derived head-stabilizing proteinVG03_ECOL6



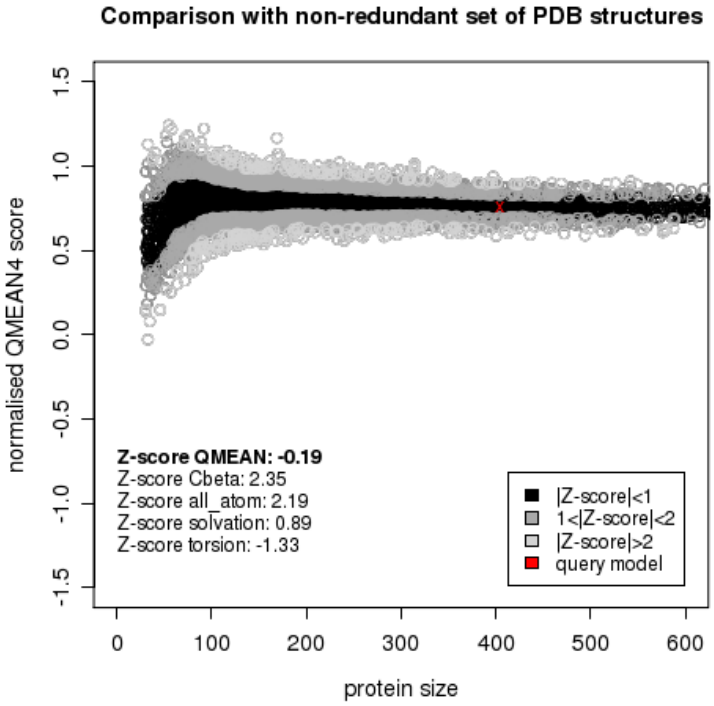
b) Quality estimation of PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase.



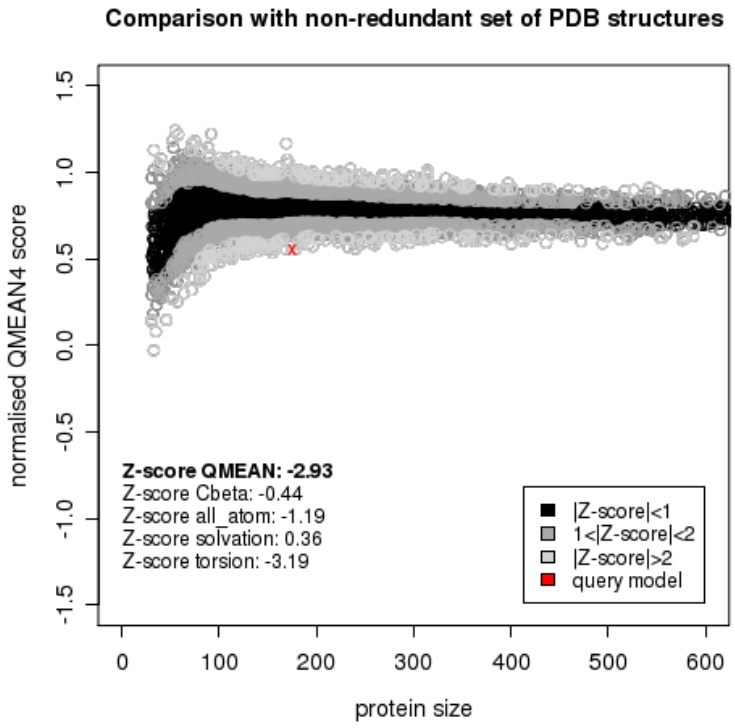
c) Quality estimation of Probable adenosine monophosphate-protein transferase FIC_ECOLI



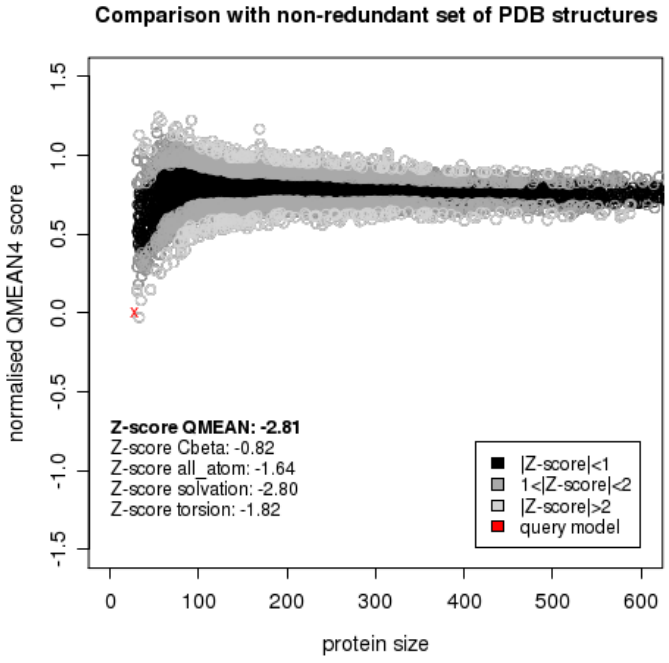
d) Quality estimation of UPF0033 protein YEDF



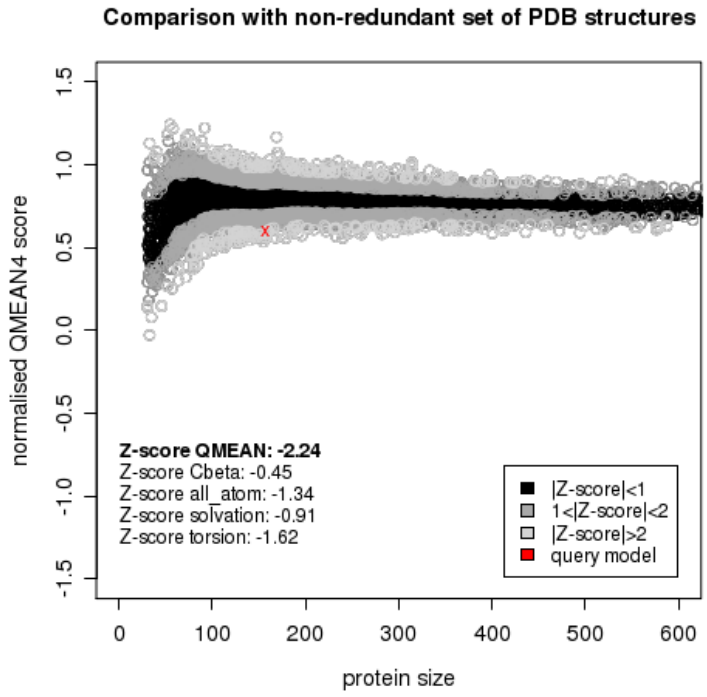
e) Quality estimation of Transcription anti-termination protein RFAH- ECO57 NusA



f) Quality estimation of 30S ribosomal protein RS11_ECO24



g) Quality estimation of Small toxic polypeptide LDRA_ECOLI

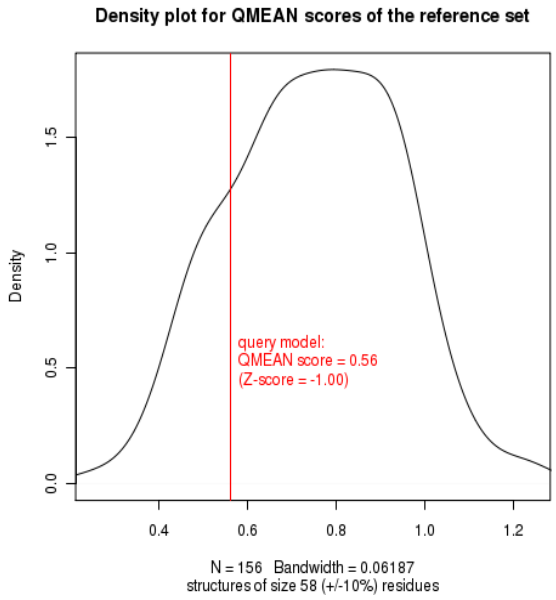


h) Quality estimation of Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57

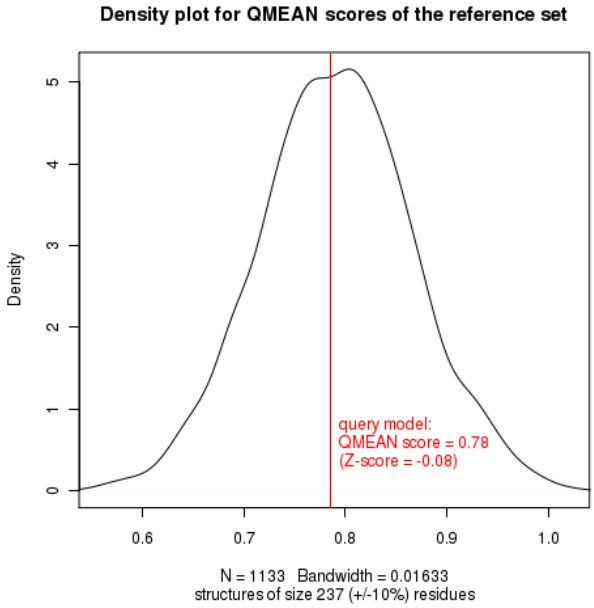
Figure -32: QMEAN analysis for estimating the quality of differentially expressed proteins under wine stress.

Density plot analysis for QMEAN score of the reference set:

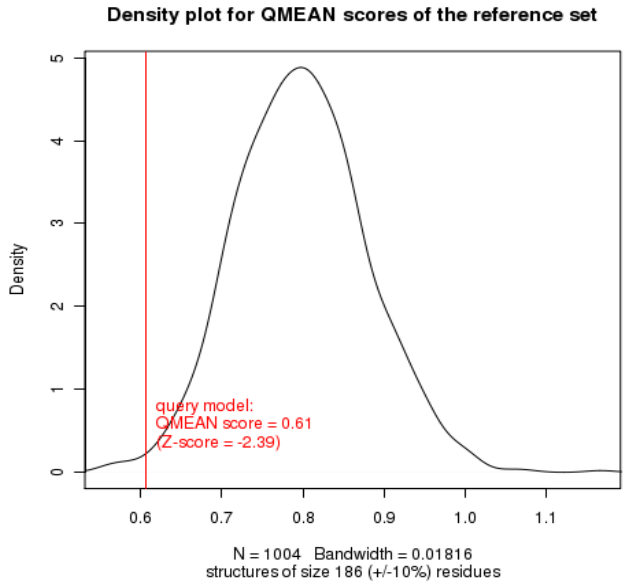
Density plot visualizing the QMEAN Z- score distribution of theoretical protein structure models. The plot explains experimental reference of the structure indicates black line and the quality of the protein indicates red in colour. The plot in the middle shows the density plot (based on QMEAN score) of all reference models used in the Z-score calculation. The plot basically is a projection of the first plot for given protein size. By drawing density plot we taken QMEAN score on x-axis and density values on y-axis, the plot represents QMEAN values of the plot, compare to the all wine treated expressed proteins, Transcription anti-termination protein RFAH- ECO57 NusA and PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase showed better results compared to the remaining expressed proteins. It explains the quality of the structure is medium. Density plot analysis for QMEAN score of differentially expressed *E.coli* Nissle 1917 proteins under cocoti wine stress were represented in the following figures-33a to 33h.



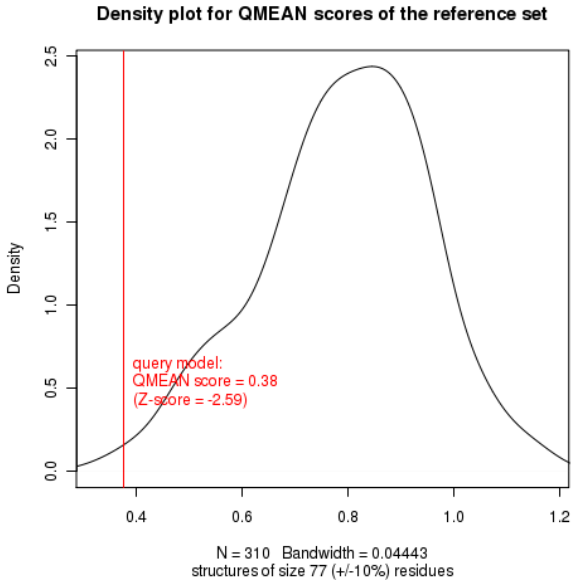
a) Density plot for P21 prophage-derived head-stabilizing proteinVG03_ECOL6.



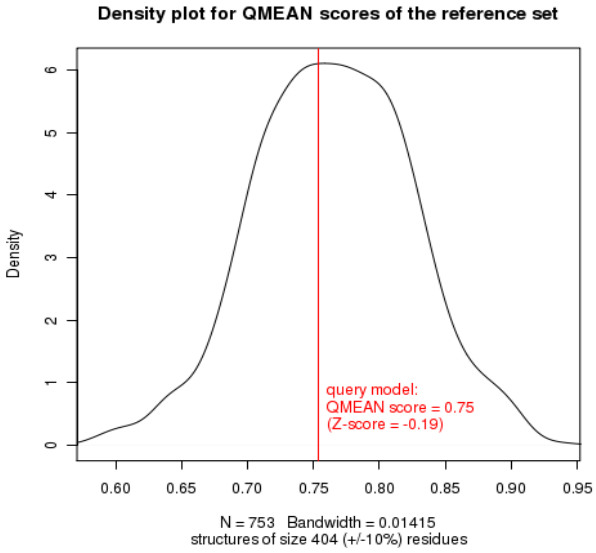
b) Density plot for PUR7-eco24-phosphoribosylamidazole-succinocarboxamide synthase



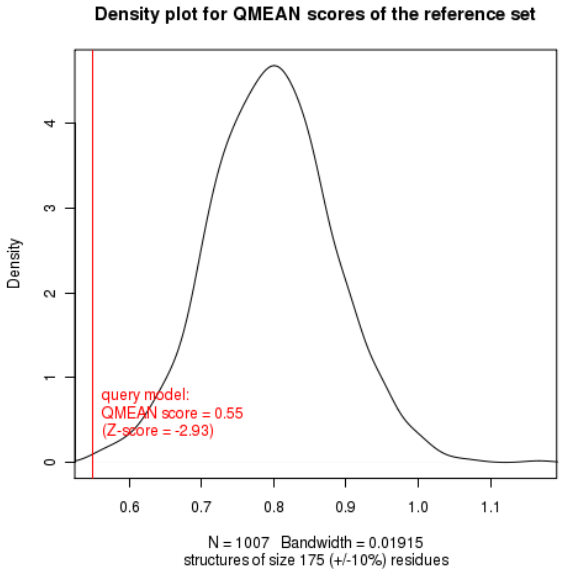
c) Density plot for Probable adenosine monophosphate-protein transferase FIC_ECOLI



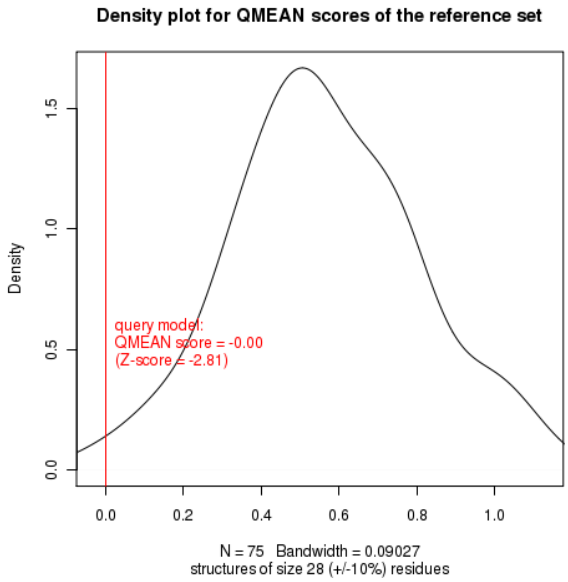
d) Density plot for UPF0033 protein YEDF



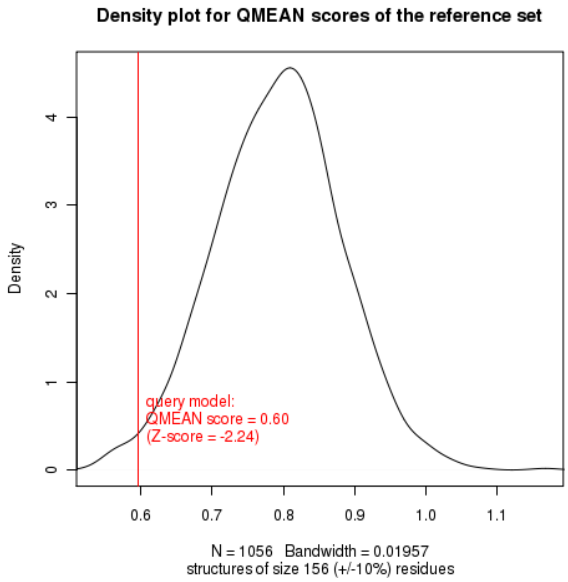
e) Density plot for Transcription anti-termination protein RFAH- ECO57 NusA



f) Density plot for 30S ribosomal protein RS11- ECO24



g) Density plot for Small toxic polypeptide LDRA_ECOLI

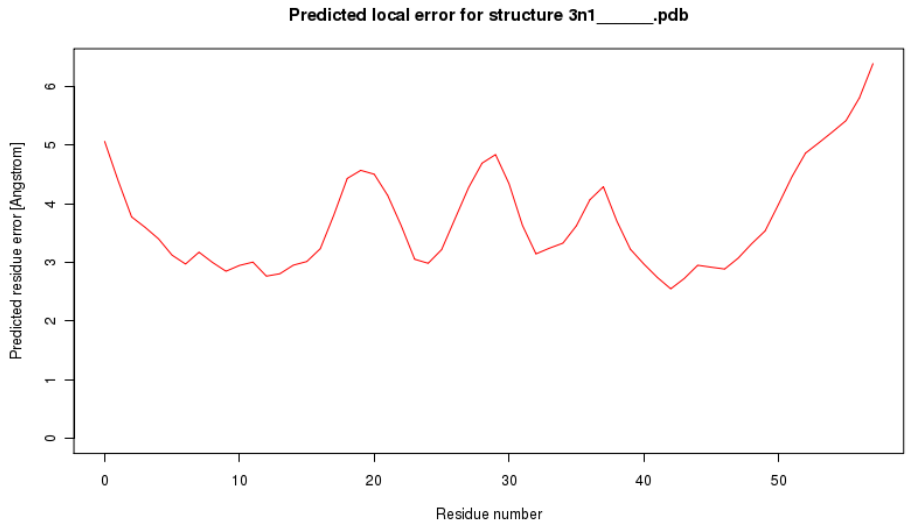


h) Density plot for Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57

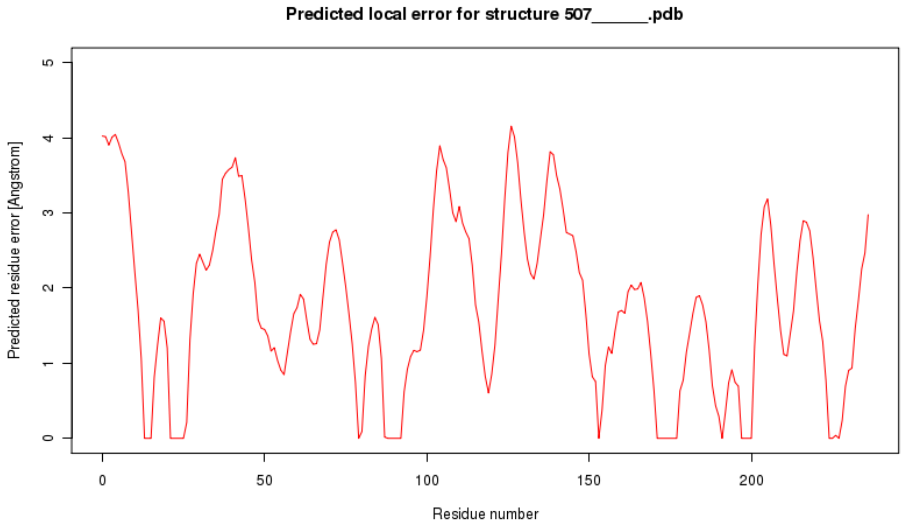
Figure -33: Density plot for QMEAN scores of differentially expressed proteins under wine tress.

Predicted local error for the structure pdb:

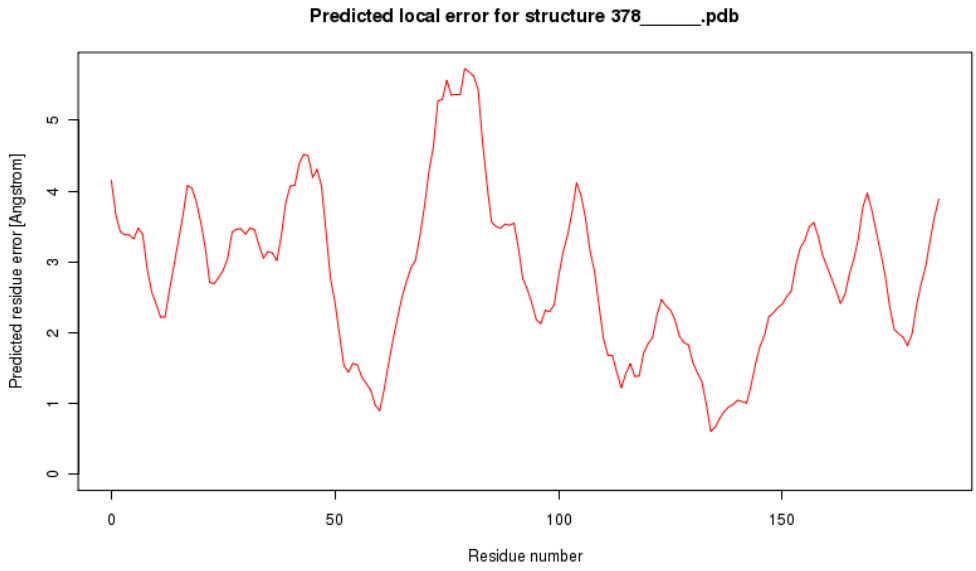
The error can rise in prediction where an insertion or deletion mutation or a gap in a solved structure result in a region of target sequence for which there is no corresponding template. Residue error plot used model energy profile with estimated residue errors along the sequence. Error values were represented. Predicted local error plot, Residue number was taken on X-axis and predicted residue error was taken on Y-axis. Lower resolution (2.5-3 A^0) indicates the structure is good, based on this Transcription anti-termination protein RFAH- ECO57 NusA showed less error values, in structure prediction when compare to remaining protein error plots. Error plot values of differentially expressed proteins of *E.coli* Nissle 1917 under wine stress were represented in the following figures -34a to 34h.



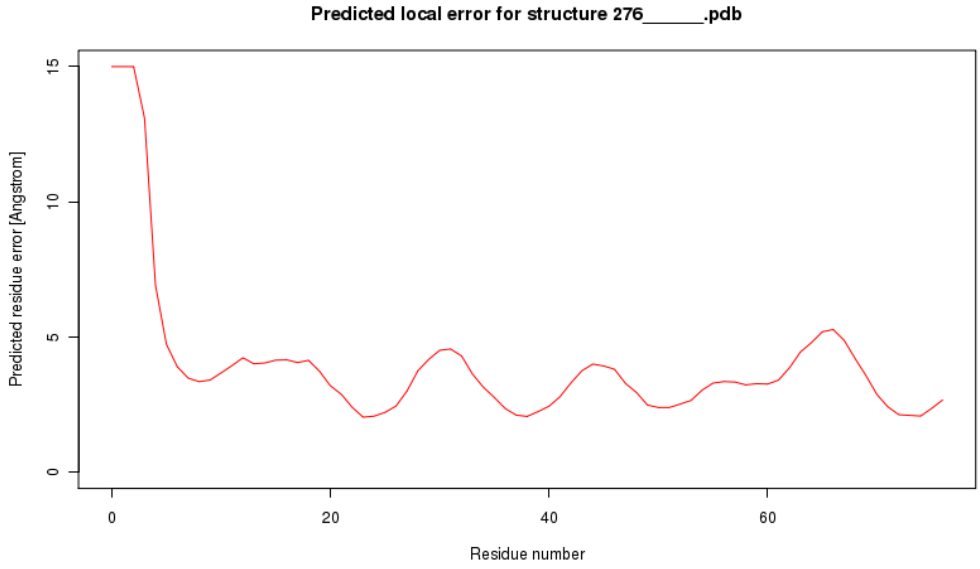
a) Predicted local error for P21 prophage-derived head-stabilizing proteinVG03_ECOL6



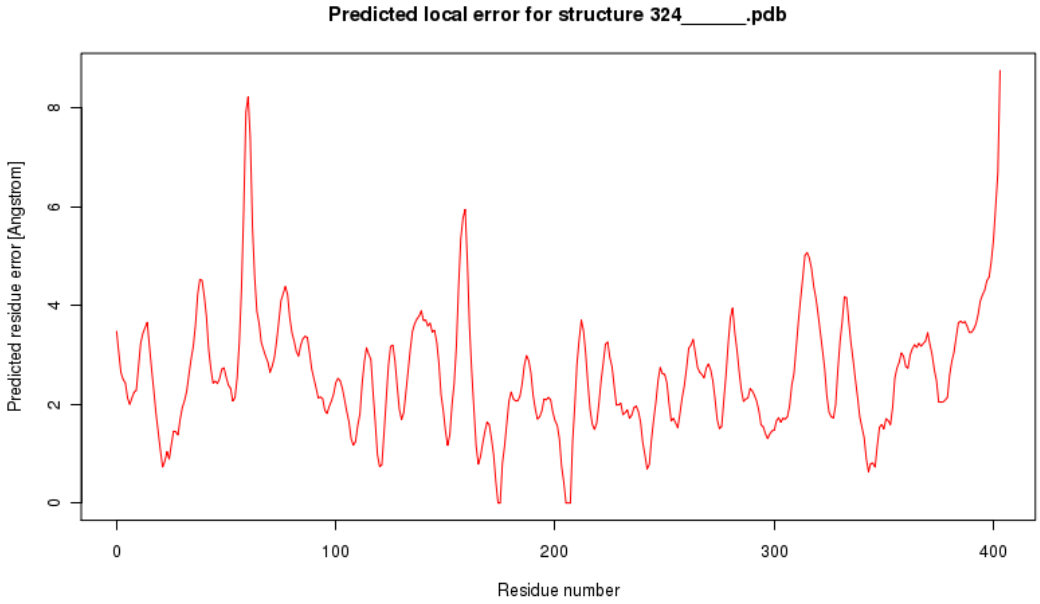
b) Predicted local error for PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase



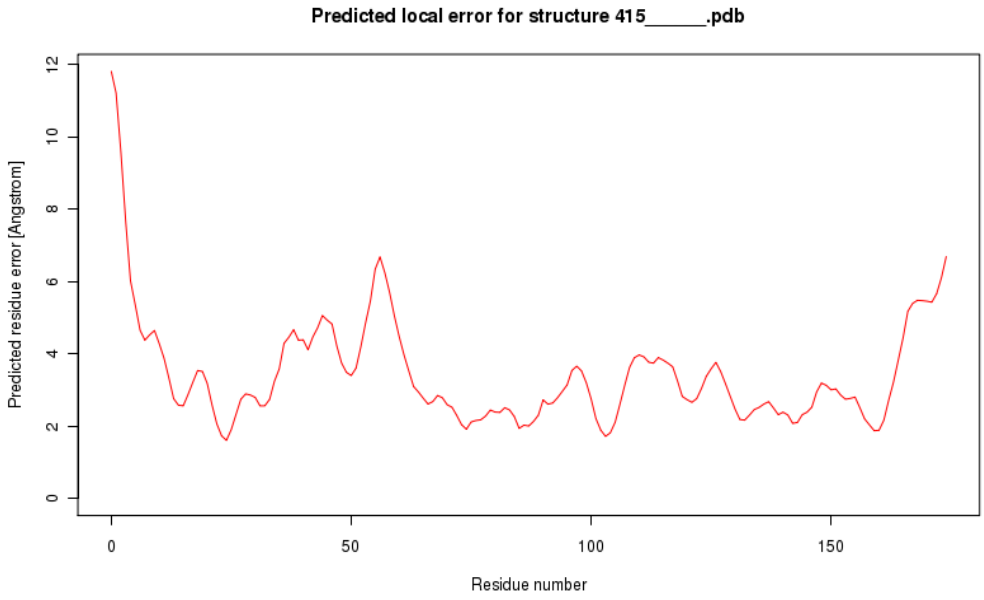
c) Predicted local error for Probable adenosine monophosphate-protein transferase FIC_ECOLI



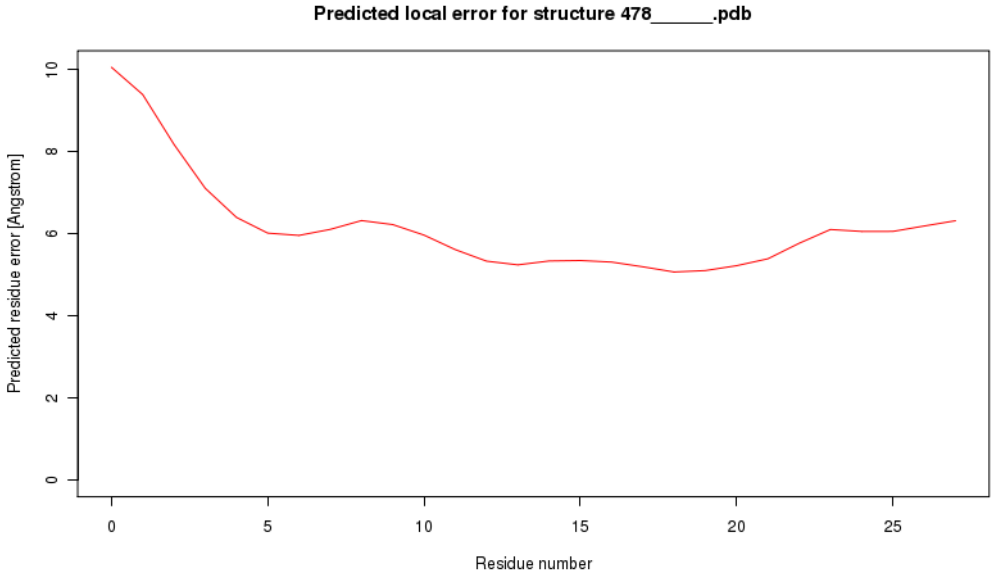
d) Predicted local error for UPF0033 protein YEDF



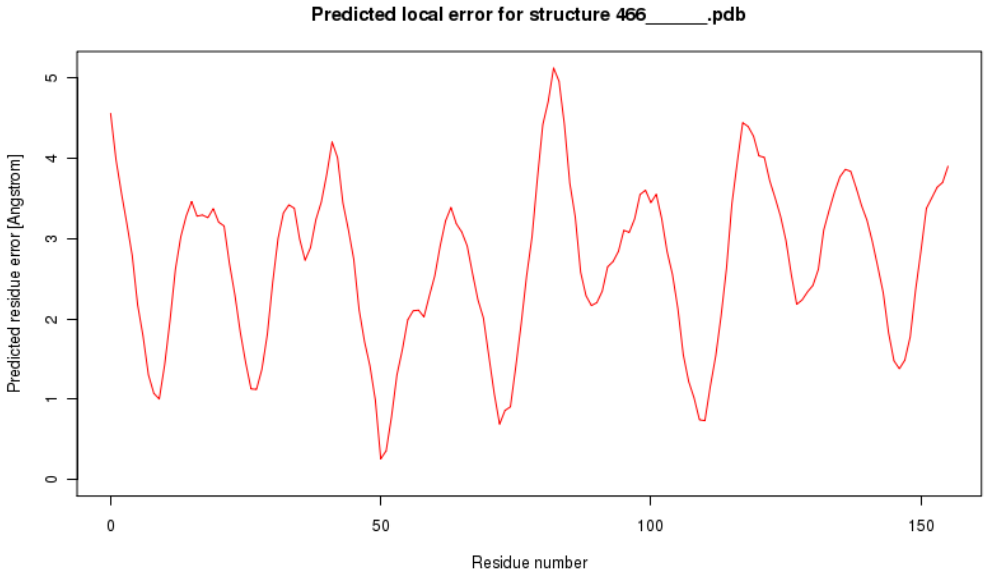
e) Predicted local error for Transcription anti-termination protein RFAH- ECO57 NusA



f) Predicted local error for 30S ribosomal protein RS11_ECO24



g) Predicted local error for Small toxic polypeptide LDRA_ECOLI



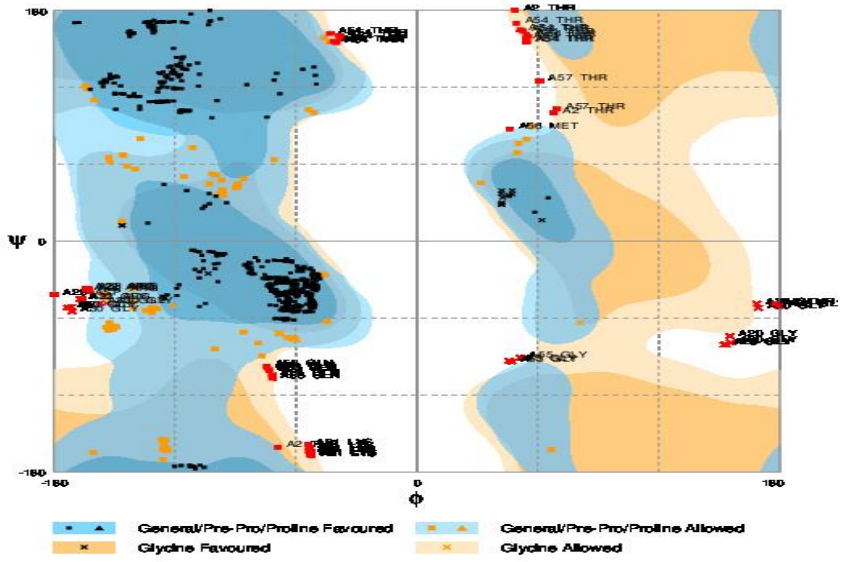
h) Predicted local error for Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57

Figure- 34: Predicted local error for the structure of differentially expressed proteins Pdb under wine stress.

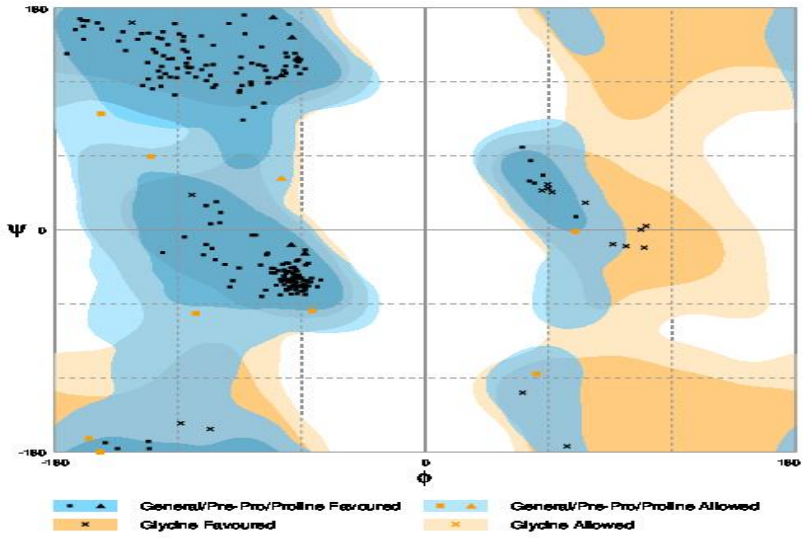
Validation of protein samples:

Validation of the protein model was done with RAMPAGE server (Lovell *et al.,* 2000). After the refinement process, validation of the model was carried out using Ramachandran's plot. The ψ and φ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized. Validations of differentially expressed proteins under wine stress were represented in the following figures- 35a to 35h.

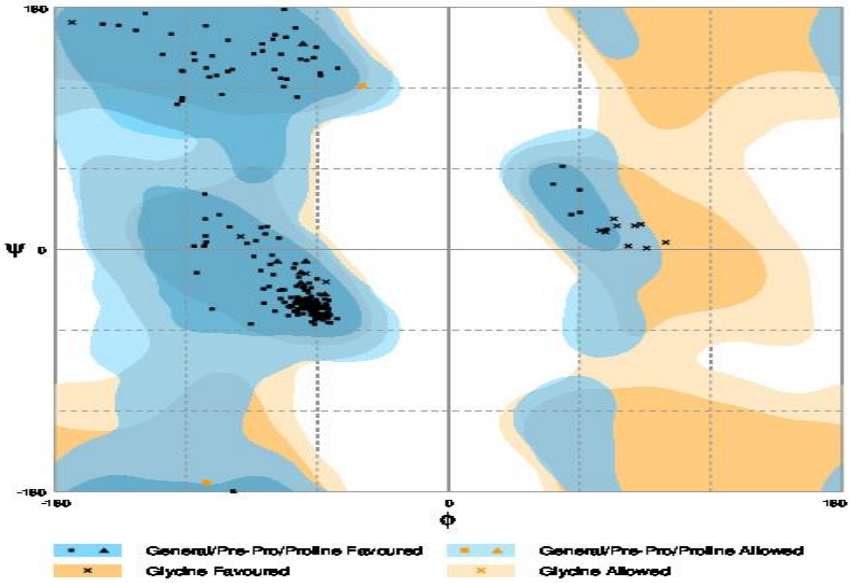
Validation of protein P21 prophage-derived head-stabilizing proteinVG03_ECOL6 shows 78.6% (4) of favoured regions (non-glycine, non-proline), 16% (9) of allowed regions (glycine, proline) and 5.4% (3) (glycine, proline residues) in disallowed regions. In PUR7-eco24-phosphoribosylamidazole-succinocarboxamide synthase shows 96.2% (226) of favoured regions (non-glycine, non-proline), 3.8% (9) of allowed regions (glycine, proline) and there is no residues in disallowed regions. In case of Probable adenosine monophosphate-protein transferase FIC_ECOLI the plot shows 94.5% (172) of favoured regions (non-glycine, non-proline), 4.9% (9) of allowed regions (glycine, proline) and 0.5% (1) (glycine, proline residues) in disallowed regions. The protein UPF0033 protein YEDF showed the ψ and φ distributions of the Ramachandran's plots of glycine, proline regions were summarized. 70.3% (52) of favoured regions (non-glycine, non-proline), 24.3% (18) of allowed regions (glycine, proline) and 5.4 %(4) (glycine, proline residues) in disallowed regions. In case of Transcription anti-termination protein RFAH- ECO57 NusA protein the ψ and φ distributions of the Ramachandran's plots of non-glycine, non-proline residues were summarized. 94.5% (380) of favoured regions (non-glycine, non-proline), 4.2% (17) of allowed regions (glycine, proline) and 1.2 %(5) (glycine, proline residues) in disallowed regions. In 30S ribosomal protein RS11_ECO24 the ψ and φ distributions of the Ramachandran's plot of non-glycine, non-proline residues were summarized. 85.0% (147) of favoured regions (non-glycine, non-proline), 9.8% (17) of allowed regions (glycine, proline) and 5.2% (9) (glycine, proline residues) in disallowed regions. The protein Small toxic polypeptide LDRA_ECOLI the ψ and φ distributions of the Ramachandran's plots of non-glycine, non-proline residues were summarized. 92.3% (24) of favoured regions (non-glycine, non-proline), 3.8% (1) of allowed regions (glycine, proline) and 3.8% (1) (glycine, proline residues) in disallowed regions. In Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein showed 94.8% (146) of favoured regions (non-glycine, non-proline), 3.9% (6) of allowed regions (glycine, proline) and 1.3%(2) (glycine, proline residues) in disallowed regions.



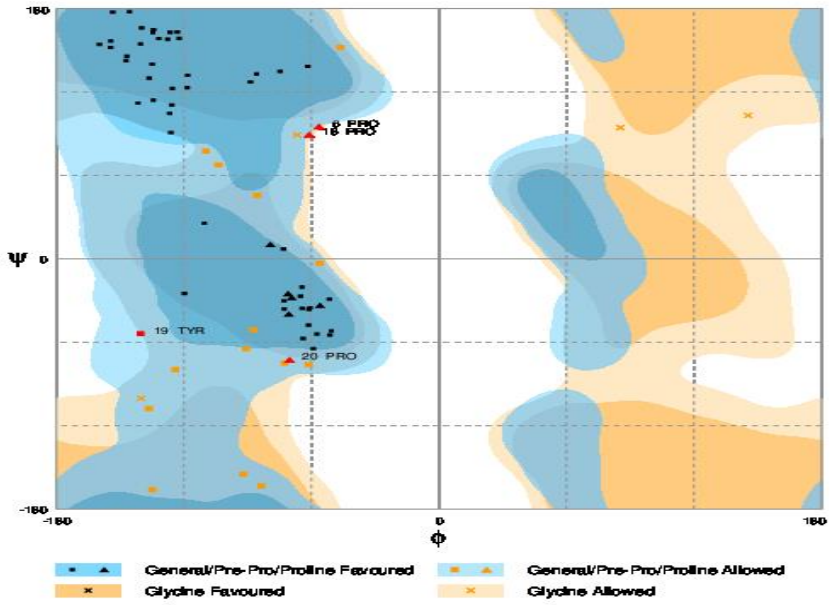
a) P21 prophage-derived head-stabilizing proteinVG03_ECOL6 validation by Ramachandran's plot analysis.



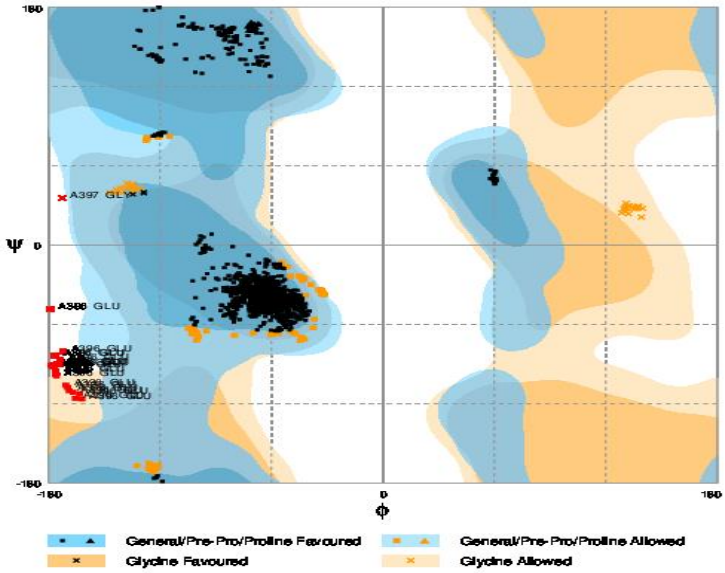
b) PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase protein validation by Ramachandran's plot analysis.



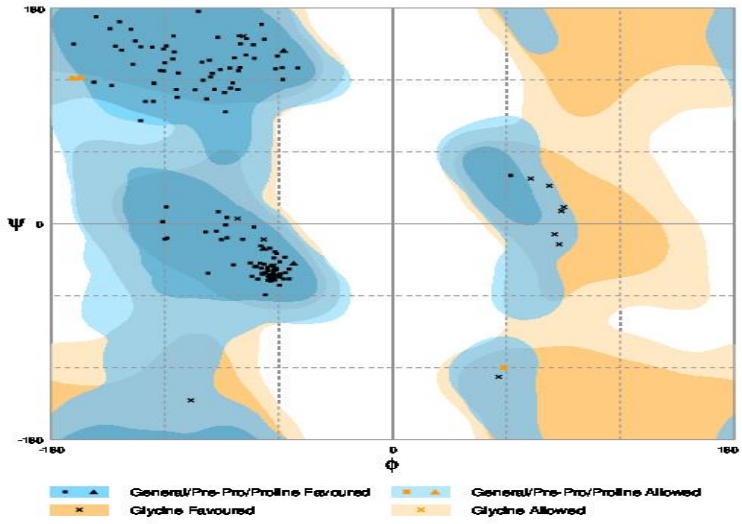
c) Probable adenosine monophosphate-protein transferase FIC_ECOLI protein validation by Ramachandran's plot analysis.



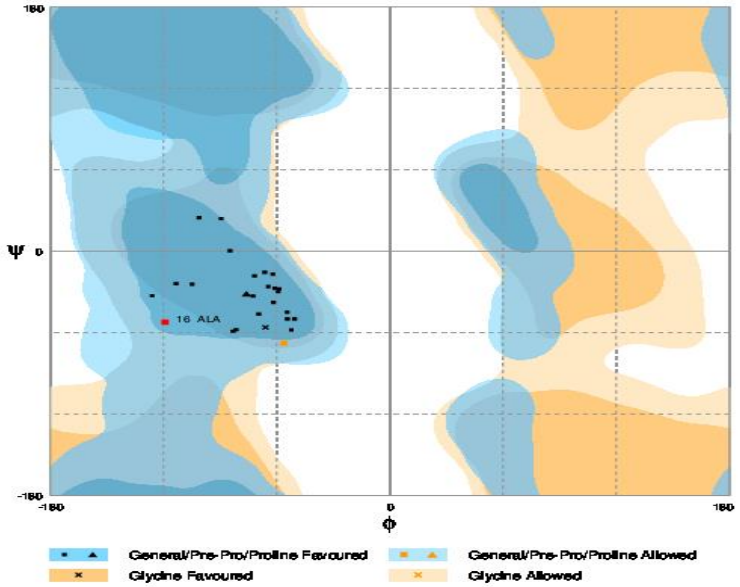
d) UPF0033 protein YEDF protein validation by Ramachandran's plot analysis.



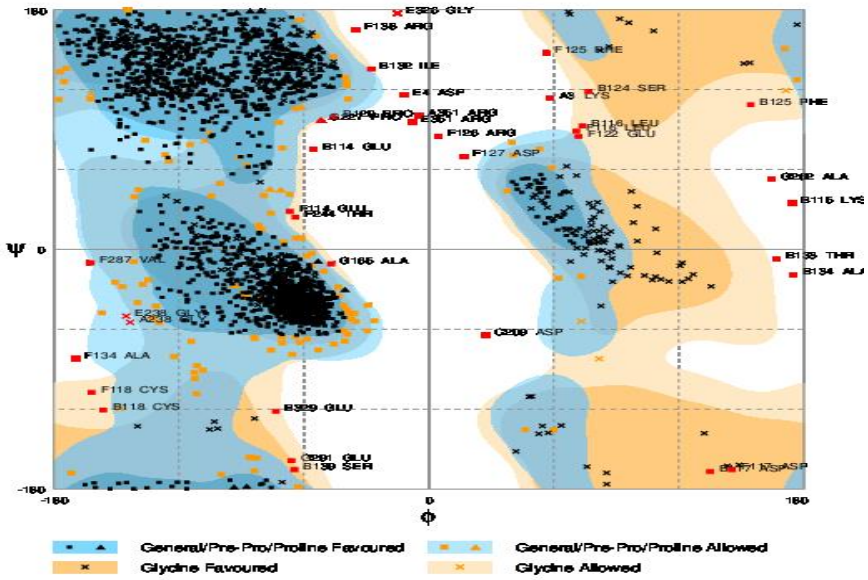
e) Transcription anti-termination protein RFAH- ECO57 NusA protein validation by Ramachandran's plot analysis.



f) 30S ribosomal protein RS11_ECO24 protein validation by Ramachandran's plot analysis.



g) Small toxic polypeptide LDRA_ECOLI protein validation by Ramachandran's plot analysis.



h) Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein validation by Ramachandran's plot analysis.

Figure-35: Validation of protein sample by Ramachandran's plot analysis.

Global quality validation of proteins:

The analysis provides both global and site-specific measures of protein structure quality. Global quality measures are reported as Z scores, based on calibration with a set of high-resolution X-ray crystal structures. PSVS is particularly useful in assessing protein structures determined by NMR methods, but is also valuable for assessing X-ray crystal structures or homology models. RMS Deviation is a good measure of accuracy.

Table -37: Validation scores of differentially expressed proteins under wine stress

Quality score of the model, Close contacts and deviations from ideal geometry were presented in the following tables -37 (1- 8.1).

1) P21 prophage-derived head-stabilizing proteinVG03_ECOL6 global quality scores.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.28	0.57	-0.81	-0.69	271.24
Z-score[1]	-2.89	-0.33	-2.87	-4.08	-45.02

1.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	2.7 Å
RMS deviation for bond lengths	0.10 Å

2) PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase global quality scores

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.42	0.47	-0.15	-0.02	11.33
Z-score[1]	-0.64	-0.74	-0.28	-0.12	-0.42

2.1) Close contacts and deviations from ideal geometry

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	1.3 Å
RMS deviation for bond lengths	0.06 Å

3) Probable adenosine monophosphate-protein transferase FIC_ECOLI global quality scores.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.40	0.69	0.00	0.18	65.71
Z-score[1]	-0.96	0.17	0.31	1.06	-9.75

3.1) Close contacts and deviations from ideal geometry

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	3.5 Å
RMS deviation for bond lengths	0.056 Å

4) UPF0033 protein YEDF_ global quality score.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.07	0.27	-1.26	-1.44	59.31
Z-score[1]	-6.26	1.57	-4.64	-8.52	-8.65

4.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	0.5 Å
RMS deviation for bond lengths	0.003 Å

5) Transcription anti-termination protein RFAH- ECO57 NusA global quality score.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.36	0.70	-0.01	0.23	61.82
Z-score[1]	-1.61	0.21	0.28	1.36	-9.08

5.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	4.3 Å
RMS deviation for bond lengths	0.062 Å

6) 30S ribosomal protein RS11_ECO24 global quality score.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.41	0.44	-1.15	-0.65	66.37
Z-score[1]	-0.80	-0.87	-4.21	-3.84	-9.86

6.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	2.5 Å
RMS deviation for bond lengths	0.031 Å

7) Small toxic polypeptide LDRA_ECOLI global quality score.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	-0.26	-1.18	-0.51	-0.21	55.91
Z-score[1]	-11.56	-7.57	-1.69	-1.24	-8.07

7.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	2.3 Å
RMS deviation for bond lengths	0.11 Å

8). Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 global quality score.

Program	verify 3D	ProsaII (-ve)	Procheck (phi-psi)[3]	Procheck (all)[3]	MolProbity Clashscore
-Raw score	0.39	0.47	-0.12	0.12	84.52
Z-score[1]	-1.12	-0.74	-0.16	0.71	-12.98

8.1) Close contacts and deviations from ideal geometry.

Number of close contacts (within 2.2 Å)	0
RMS deviation for bond angles	4.1 Å
RMS deviation for bond lengths	0.067 Å

Phylogenetic analysis of cocoti sap and wine expressed probiotic *E.coli* Nissle 1917 proteins:

Phylogeny is the evolutionary history of a particular group of organisms or their genes. Phylogeny can be represented in a phylogenetic tree which graphically represents the lines of descent among organisms or their genes. Phylogenetic analysis of differentially expressed *E.coli* Nissle 1917 proteins were represented in the following figure-36.

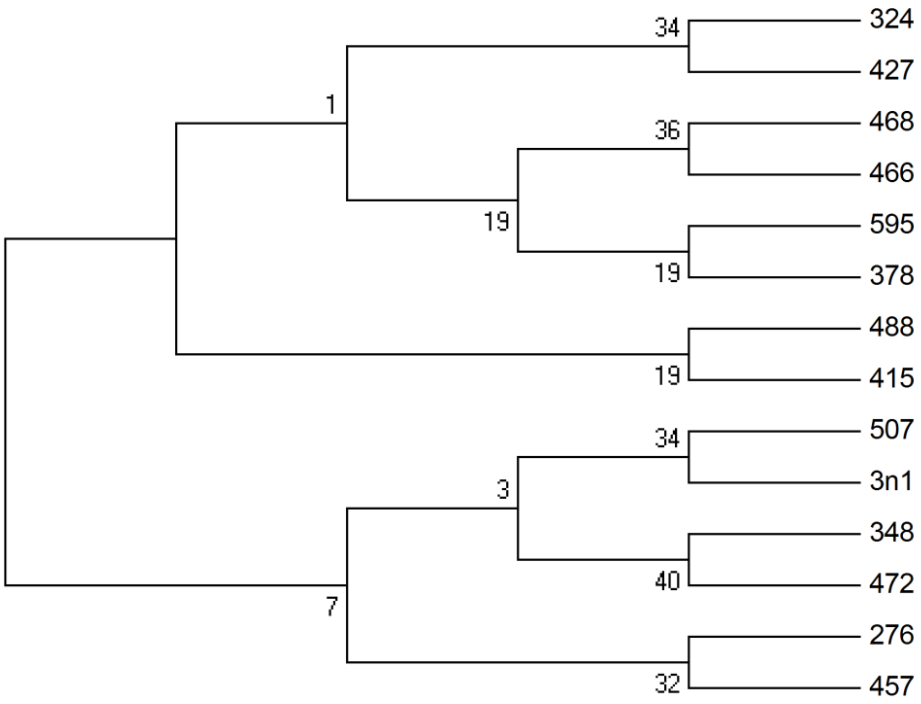


Figure -36: Phylogenetic analysis of *E.coli* stress expressed proteins with response to cocoti sap and wine.

The method of bootstrapping is the multinomial non-parametric bootstrap as applied in the binomial setting. The tree were represented in the following figure- 36, showed bootstrap values at the inner modes, for example, 34 means the protein spot 324 and protein spot 427 were showed siblings (related) in 34% of the boot strap replications, the protein spots 468, 466 and 595, 378 were grouped together in what is called a monophytic clade, in 19% bootstrap relationship replications (Aldous, 2001). Protein spot 348, 472 were showed sibling in 40% of bootstrap replications.

ProtParam analysis:

Extinction coefficients indicate how much light absorbs a protein at a certain wavelength, the half-life is a prediction of the time it takes for half of the amount of protein in a cell to disappear after its synthesis in the cell. ProtParam relies on the "N-end rule", which relates the half-life of a protein to the identity of its N-terminal residue, The instability index provides an estimate of the stability of the protein in a test tube, the aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It may be regarded as a positive factor for the increase of thermostability of globular proteins. The GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. ProtParam form analysis of differentially expressed *E.coli* Nissle 1917 proteins under cocoti sap and wine treated stress were presented in the following tables -38a to 38g and 39a to 39h.

Table-38: ProtParam form analysis of differentially expressed *E.coli* Nissle 1917 proteins under cocoti sap treated stress:

a). t-RNA-Specific adenosine deaminase monomer.

Formula of the protein	$C_{814}H_{1298}N_{248}O_{235}S_1$
Total No.of atoms	2608
Extinction coefficient	2109
Molecular weight	18735.5 Daltons
Estimated half-life	>10 hours
Instability index	39.77
Aliphatic index	82.99
GRAVY	-0.231

The instability index (II) is computed to be 39.77 this classifies the protein as stable.

b) Formatehydrogenlyase subunit HYCE_ECOLI.

Formula of the protein	$C_{2894}H_{4522}N_{804}O_{842}S_{29}$
Total No.of atoms	9091
Extinction coefficient	84730
Molecular weight	64980.3 Daltons
Estimated half-life	>10 hours
Instability index	29.76
Aliphatic index	84.41
GRAVY	-0.35

The instability index (II) is computed to be 29.76 this classifies the protein is stable.

c). Protein PmbA (PMBA-Eco57– protein pmba 0s E.coli).

Formula of the protein	$C_{2116}H_{3377}N_{595}O_{669}S_{13}$
Total No.of atoms	6773
Extinction coefficient	49640
Molecular weight	48369.6 Daltons
Estimated half-life	>10 hours
Instability index	32.98
Aliphatic index	87.84
GRAVY	-0.16

The instability index (II) is computed to be 32.98 this classifies the protein as stable

d). N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1. PTPB1_ECOLI.

Formula of the protein	$C_{785}H_{1270}N_{216}O_{235}S$
Total No.of atoms	2510
Extinction coefficient	8480
Molecular weight	17622.2 Daltons
Estimated half-life	>10 hours
Instability index	22.51
Aliphatic index	102.85
GRAVY	-0.126

The instability index (II) is computed to be 22.51 this classifies the protein as stable

e). Transcriptional regulatory protein BAER_ECOL6.

Formula of the protein	$C_{1233}H_{1992}N_{342}O_{362}S_8$
Total No.of atoms	3937
Extinction coefficient	24660
Molecular weight	27655.9
Estimated half-life	>10 hours
Instability index	47.80
Aliphatic index	106.83
GRAVY	-0.281

The instability index (II) is computed to be 47.80 this classifies the protein as unstable.

f). DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57.

Formula of the protein	$C_{408}H_{668}N_{114}O_{132}S$
Total No.of atoms	1325
Extinction coefficient	12490
Molecular weight	9378.6 Daltons
Estimated half-life	>10 hours
Instability index	27.26
Aliphatic index	79.63
GRAVY	-0.584

The instability index (II) is computed to be 27.26. This classifies the protein as stable.

g). UPF0401 protein ECP Y3010_ECOL5.

Formula of the protein	$C_{387}H_{605}N_{113}O_{113}S$
Total No.of atoms	1221
Extinction coefficient	14105
Molecular weight	8744.9 Daltons
Estimated half-life	>10 hours
Instability index	63.64
Aliphatic index	84.94
GRAVY	-0.408

The instability index (II) is computed to be 63.64.This classifies the protein as unstable

Table - 39: ProtParam form analysis of cocoti wine treated Expressed proteins:

a) P21 prophage-derived head-stabilizing proteinVG03_ECOL6

Formula of the protein	$C_{324}H_{564}N_{108}O_{99}S$
Total No.of atoms	1097
Extinction coefficient	1490
Molecular weight	7620.8
Estimated half-life	>10 hours
Instability index	56.26
Aliphatic index	108.97
GRAVY	-0.291

The protein does not contain any Trp residues. This could result in more than 10% error in the computed extinction coefficient.

The instability index (II) is computed to be 56.26 so this indicates the protein is unstable.

b). UPF0033 protein YEDF.

Formula of the protein	$C_{382}H_{615}N_{101}O_{116}S$
Total No.of atoms	1219
Extinction coefficient	6085
Molecular weight	8638.9 Daltons
Estimated half-life	>10 hours
Instability index	47.99
Aliphatic index	98.70
GRAVY	-0.27

This protein does not contain any Trp residues. This could result in more than 10% error in the computed extinction coefficient.

The instability index (II) is computed to be 47.99. This classifies the protein as unstable

c). Transcription anti-termination protein RFAH- ECO57 NusA.

Formula of the protein	$C_{833}H_{1313}N_{223}O_{233}S$
Total No.of atoms	2607
Extinction coefficient	13075
Molecular weight	18340.2
Estimated half-life	>10 hours
Instability index	35.21
Aliphatic index	93.27
GRAVY	-0.141

The instability index (II) is computed to be 35.21 the protein is stable.

d). Probable adenosine monophosphate-protein transferase FIC_ECOLI.

Formula of the protein	$C_{1028}H_{1578}N_{282}O_{301}S$
Total No.of atoms	3197
Extinction coefficient	31525
Molecular weight	22960 Daltons
Estimated half-life	>10 hours
Instability index	43.13
Aliphatic index	86.90
GRAVY	-0.360

The instability index (II) is computed to be 43.13 this classifies the protein as unstable.

e). 30S ribosomal protein RS11_ECO24.

Formula of the protein	$C_{837}H_{1374}N_{244}O_{247}S_3$
Total No.of atoms	2705
Extinction coefficient	12950
Molecular weight	18903.7 Daltons
Estimated half-life	>10 hours
Instability index	19.48
Aliphatic index	91.41
GRAVY	-0.227

The instability index (II) is computed to be 19.48 this classifies the protein as stable.

f). Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57.

Formula of the protein	$C_{730}H_{1202}N_{204}O_{225}S_{16}$
Total No.of atoms	2377
Extinction coefficient	10595
Molecular weight	16949.7 Daltons
Estimated half-life	>10 hours
Instability index	42.33
Aliphatic index	88.99
GRAVY	0.072

The instability index (II) is computed to be 42.33 this classifies the protein as unstable.

g). Small toxic polypeptide LDRA_ECOLI.

Formula of the protein	$C_{190}H_{291}N_{49}O_{43}S$
Total No.of atoms	575
Extinction coefficient	16500
Molecular weight	4013.8 Daltons
Estimated half-life	>10 hours
Instability index	23.18
Aliphatic index	117.43
GRAVY	0.760

The instability index (II) is computed to be 23.18 This classifies the protein as stable.

h). PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase.

Formula of the protein	$C_{1200}H_{1908}N_{320}O_{362}S_1$
Total No.of atoms	3802
Extinction coefficient	21555
Molecular weight	26995 Daltons
Estimated half-life	>10 hours
Instability index	37.62
Aliphatic index	88.02
GRAVY	-0.370

The instability index (II) is computed to be 37.62 this classifies the protein as stable

6. SUMMARY

E.coli Nissle 1917 played a major role in probiotic field after *Lactobacillus*. *E.coli* Nissle 1917 is one of the oldest, most well-characterised probiotic agents and has shown promising results in treatment of various intestinal diseases. *E.coli* can survive extreme acid stress conditions. Proteomic studies of gut microflora explain the molecular mechanisms, expression patterns of proteins and enzymes in response to dietary components and therapy provide a rationale for the development of new active ingredients.

The study showed that the application of proteomic tools provided an overview of the proteins present in *E.coli* Nissle under *Cocos nucifera* sap and wine stress conditions. It confirmed that proteins are involved in various biological functions under stress and defence reactions. The aim of the study is to understand the structure prediction and functions of the differentially expressed stress proteins of *Eschericia coli* under *Cocos nucifera* sap and wine stress.

E.coli Nissle 1917 collected from culture collection center Germany, maintained on Nutrient broth 50 µl of overnight cultures were transferred into 50 ml broth and grown until OD reached 0.5 OD (McFarland standard). Cells were adjusted to equal OD = 0.05 in nutrient broth supplemented with 100 mg/L ampicillin. Test samples of cocoti sap and cocoti wine were collected from rural area of Tirupati and it was filtered with vacuum pump filtration by using 0.02µ Nitrocellulose membrane filters, then analysed the physico- chemical characters of the sample. Various concentrations of cocoti sap and wine were added into the broth, then allow for incubation at room temperature for 5 hours. Measured the growth curves of experimental organism for every half-an-hour by using spectrophotometer (Techcomp) at 620nm. The minimum inhibitory concentration (MIC) of cocoti sap and wine was determined by Macro-dilution method. Growth was monitored using spectrophotometer in every half an hour at 670nm. Which concentration shows approximately 50% growth inhibition was selected as stress concentration for the subsequent proteomic analysis.

Proteins were extracted from bacterial cells by the method of Trizol protein extraction method, centrifugation and sonication techniques were used for cell lysis. Quantify the protein sample by Bicinchoninic acid (BCA) method. 2-D gel electrophoresis was carried out to study differentially expressed proteins under cocoti sap and cocoti wine stress conditions. pH 4 to 7 (Bio-Rad) range IPG strips were used for Isoelectric focusing. 500 µg of rehydration buffer and 2% Ampholytes were mixed with the purified protein sample. Then 320 µl of sample were loaded in strips and allowed for rehydration. After rehydration, we carried out 6-stepped focusing which was as follows: phase 1, linear gradient up to 250 Volts in 15 minutes; phase 2, linear gradient up to 500 Volts in 30 minutes; phase 3, rapid gradient up to 1000 Volts in 1 hour; phase 4, linear gradient up to 5000 Volts in 3 hours; phase 5, linear gradient up to 10,000 Volts in 3 hours; and phase 6, linear gradient up to 10,000 Volts in 12 hours. Working temperature was set to 20ºC in IEF. After Isoelectric focusing, proteins were separated in the second dimension by using 12% SDS-PAGE gel and 80V was maintained through while running the second dimension. After second dimension, gels were stained with coomassie blue stain. We noticed over all 800 proteins in our gel, 370 spots were visualized clearly. Gels were digitized using gel scanner (Typhon Variable Mode imager), and allowed for gel analysis by using Image master 2-D platinum 6.0 software. It quantify the protein spots and showed the variation between control and treated gel samples, the spot size indicates, up and down-regulation of the protein. The expressed protein spots were separated by using spot cutter and these spots can be analysed by MS- for protein identification. The protein spots were dehydrated with acetonitrile and dried at 40^0C, the samples were digested with Proteolytic enzyme (trypsin) cleaved protein sample mixed with matrix (3, 5 dimethyl-4- hydroxyl cinnamic acid) compound, prepared a sample slide consists digested protein sample and matrix compound air dried for ten minutes. Mass spectrometry was performed and the spectra measured for unknown peptides were compared against the mass peaks derived from calibration of internal standards. Spectra were collected over the mass range of 800-3500Da. Generated spectra values were converted into Mascot Generic Format (MGF), MGF is a simple human readable format for MS/MS data. MASCOT search engine was used for spot identification by querying for fixed modifications of protein by Carbamidomethyl of cysteine and oxidation of Methionine, database NCBInr, taxonomy *E.coli,* and enzyme trypsin were selected for reference, peptide tolerance, peptide charge, mass tolerance were changed based on protein. Number of missed cleavage sites was allowed up to one. Searching for high percentage sequence coverage, number of related sequences to find a related protein.

Swiss Model (http://swissmodel.expasy.org/) alignment mode were selected for template alignment, 3-D model was generated by using phyre-2 software (http://www.sbg.bio.ic.ac.uk/) used to obtained Protein Data Bank(PDB) file, RasMol software (http://rasmol.org/) were used to analyse the 3-D structure of protein sample. The final structure was analyzed by Ramachandran's plot drawn by using Rampage Ramachandran's server (http://mordred.bioc.cam.ac.uk/). Further analysis of pdb structure by using pdb validation tool, Z-score and error value of structure were analysed by QMEAN server (http://swissmodel.expasy.org), finally the protein physico- chemical characters, like molecular weight, molecular formula, half-life of the protein, extinction coefficient, grand average of hydropathicity (GRAVY), total number of atoms and instability index were determined by using ProtParam server.

7. CONCLUSION

In the present study, we described the protein profile experimentally by 2D-PAGE and MALDI analysis to understand the stress mechanisms of cocoti sap and wine on *E.coli* Nissle 1917. We isolated one newly expressed protein from cocoti wine treated gel which is not present in both control and cocoti sap treated sample i.e. P21 prophage-derived head-stabilizing proteinVG03_ECOL6 (3n1) also called as Head protein gp3. This protein mainly activities related to the viral life cycle. It helps to attach the viral gene into host. The growth rate was delayed in cocoti wine treated *E.coli* Nissle *1917* when compared to control and cocoti sap treated samples. Stress mechanism induce many proteins they are involved in metabolic process, hydrolase activity, lyase activity, quinone binding, phosphotransferase system, carbohydrate metabolism, DNA binding, DNA repair, transferase activity, oxidoreductase, purine metabolism, transcription anti-termination, transcription regulation and other related activities.

We proved that the predicted protein structure quality, resolution, density and error plot values by QMEAN analysis. Based on these results, only two differentially expressed proteins under sap stress showed that the significant results, which were N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1, PTPB1_ECOLI and DinI-like protein Z3305/ECs2939 in prophage CP-933VDINI1_ECO57. In case of wine stress, the differentially expressed proteins were Transcription anti-termination protein RFAH- ECO57 NusA and PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase showed significant results. ProtParam analysis indicating that the multiple physico-chemical characters of differentially expressed proteins were differed and compared. The phylogenetic tree represents the relationship in-between the differentially expressed proteins, were showed siblings (related) as well as monophytic clade.

Finally we concluded that *E.coli* Nissle 1917 exhibited low resistance to cocoti sap. Three differentially expressed proteins showed under cocoti wine stress negative effect on human health. P21 prophage-derived head-stabilizing proteinVG03_ECOL6 protein helps to attach the viral gene into host, Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein chance to cause xanthinuria (respiratory syndrome), may contribute to adult respiratory syndrome, and may potentiate influenza infection through an oxygen metabolite-dependent mechanism. Small toxic polypeptide LDRA_ECOLi protein under wine stress is influencing on cell-signaling.

In my post-doctoral research, these three differentially expressed proteins 1) P21 prophage-derived head-stabilizing proteinVG03_ECOL6 protein, 2) Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 and 3). Small toxic polypeptide LDRA_ECOLi are to be undergoing for drug designing which will be useful for bio-pharmaceutical industries to prepare the drugs against the toxicity of wine treatment.

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34	Density plot for QMEAN scores of differentially expressed protein under wine stress
	a). Density plot for P21 prophage-derived head-stabilizing proteinVG03_ECOL6
	b). Density plot for PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase
	c). Density plot for Probable adenosine monophosphate-protein transferase FIC_ECOLI
	d).Density plot for UPF0033 protein YEDF
	e). Density plot for Transcription anti-termination protein RFAH- ECO57 NusA
	f). Density plot for 30S ribosomal protein RS11_ECO24
	g). Density plot for Small toxic polypeptide LDRA_ECOLI
	h). Density plot for Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57
35	Predicted local error for the structure of differentially expressed wine stressed proteins Pdb under wine stress
	a). Predicted local error for P21 prophage-derived head-stabilizing proteinVG03_ECOL6
	b). Predicted local error for PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase
	c). Predicted local error for Probable adenosine monophosphate-protein transferase FIC_ECOLI
	d). Predicted local error for UPF0033 protein YEDF
	e). Predicted local error for Transcription anti-termination protein RFAH-ECO57 NusA
	f). Predicted local error for 30S ribosomal protein RS11_ECO24
	g).Predicted local error for Small toxic polypeptide LDRA_ECOLI

	h). Predicted local error for Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57
36	Validation of protein sample by Ramachandran's plot analysis
	a). P21 prophage-derived head-stabilizing proteinVG03_ECOL6 validation by Ramachandran's plot analysis
	b). PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase protein validation by Ramachandran's plot analysis
	c). Probable adenosine monophosphate-protein transferase FIC_ECOLI protein validation by Ramachandran's plot analysis.
	d). UPF0033 protein YEDF protein validation by Ramachandran's plot analysis
	e). Transcription anti-termination protein RFAH- ECO57 NusA protein validation by Ramachandran's plot analysis.
	f). 30S ribosomal protein RS11_ECO24 protein validation by Ramachandran analysis
	g). Small toxic polypeptide LDRA_ECOLI protein validation by Ramachandran's plot analysis.
	h). Xanthine dehydrogenase iron sulphur binding subunit XDHC_Eco57 protein validation by Ramachandran's plot analysis.
37	Phylogenetic analysis of *E.coli* stress expressed proteins with response to cocoti sap and wine

Annexure-I (Supplementary Data)

1. Sequences of differentially expressed *E.coli* Nissle 1917 proteins under cocoti sap stress.

a) t-rna specific adenosine deaminase TADA-ECO57 (spot no- 348)

MSEVEFSHEYWMRHAMTLAKRAWDEREVPVGAVLVHNNRVIGEGWNRPIGRHD
PTAHAEIMALRQGGLVMQNYRLIDATLYVTLEPCVMCAGAMIHSRIGRVVFGARD
AKTGAAGSLMDVLHHPGMNHRVEITEGILADECAALLSDFFRMRRQEIKAQKKAQ
SSTD

b) Formate hydrogenase subunit 5 HYCE- E.coli (spot no- 427)

MIKESLSMSEEKLGQHYLAALNEAFPGVVLDHAWQTKDQLTITVKVNYLPEVVEF
LYYKQGGWLSVLFGNDERKLNGHYAVYYVLSMEKGTKCWITVRVEVDANKPEY
PSVTPRVPAAVWGEREVRDMYGLIPVGLPDERRLVLPDDWPDELYPLRKDSMDYR
QRPAPTTDAETYEFINELGDKKNNVVPIGPLHVTSDEPGHFRLFVDGENIIDADYRL
FYVHRGMEKLAETRMGYNEVTFLSDRVCGICGFAHSTAYTTSVENAMGIQVPERA
QMIRAILLEVERLHSHLLNLGLACHFTGFDSGFMQFFRVRETSMKMAEILTGARKT
YGLNLIGGIRRDLLKDDMIQTRQLAQQMRREVQELVDVLLSTPNMEQRTVGIGRL
DPEIARDFSNVGPMVRASGHARDTRADHPFVGYGLLPMEVHSEQGCDVISRLKVRI
NEVYTALNMIDYGLDNLPGGPLMVEGFTYIPHRFALGFAEAPRGDDIHWSMTGDN
QKLYRWRCRAATYANWPTLRYMLRGNTVSDAPLIIGSLDPCYSCTDRMTVVDVR
KKKSKVVPYKELERYSIERKNSPLK

c) PMBA-Eco 57 – protein pmba 0s E.coli 0157:H7 (spot no-457)

YHQNRKGSASSTDLSPQAIARTVQAALDIARYTSPDPYAGVADKELLAFDAPDLDL
FHPADVSPDEAIELAARAEQAALQADKRITNTEGGSFNSHYGVKVFGNSHGMLQG
YCSTRHSLSSCVIAEENGDMERDYAYTIGRAMSDLQTPEWVGADCARRTLSRLSPR
KLSTMKAPVIFANEVATGLFGHLVGAIAGGAVYRKSTFLLDSLGTQILPDWLTIEEH
PHLLKGLASTPFDSEGVRTERRDIVKDGILTQWLLTSYSARKLGLKSTGHAGGIHN
WRIAGQGLSFEQMLKEMGTGLVVTELMGQGVSAITGDYSRGAAGFWVENGEIQY
PVSEITIAGNLKDMWRNIVTVGNDIETRSNIQCGSVLLPEMKIAGQ

d) N acetyl galactosamine – specific phosphor transferase IIB component 1 (spot no-468)

MTSPNILLTRIDNRLVHGQVGVTWTSTIGANLLVVVDDVVANDDIQQKLMGITAET
YGFGIRFFTIEKTINVIGKAAPHQKIFLICRTPQTVRKLVEGGIDLKDVNVGNMHFSE
GKKQISSKVYVDDQDLTDLRFIKQRGVNVFIQDVPGDQKEQIPD

e) BAER-Ecol6- Transcriptional regulatory protein os-E.coli (spot no- 472)

MTELPIDENTPRILIVEDEPKLGQLLIDYLRAASYAPTLISHGDQVLPYVRQTPPDLIL
LDLMLPGTDGLTLCREIRRFSDIPIVMVTAKIEEIDRLLGLEIGADDYICKPYSPREVV
ARVKTILRRCKPQRELQQQDAESPLIIDEGRFQASWRGKMLDLTPAEFRLLKTLSHE

PGKVFSREQLLNHLYDDYRVVTDRTIDSHIKNLRRKLESLDAEQSFIRAVYGVGYR
WEADACRIV

f) DINI-E.co 57- Dini like protein Z3305/Ecs 2939 in prophage (spot no-488)

MRVEICIAKEKITKMPNGAVDALKEELTRRISKRYDDVEVIVKATSNDGLSVTRTA
DKDSAKTFVQETLKDTWESADEWFVR

g) Y3010-E.coli5- UPFO401 protein ECP-3010 (spot no- 595)

MPGCTSRLLPEGPFSRNQALAVTTAYLNVLIEDDQGTHFRLVIRNAEGQLRWRCW
NFEPDAGKQLNPYLASEGILRQ

2. Sequences of differentially expressed *E.coli* Nissle 1917 proteins under cocoti wine stress.

a) VGO3-ECol6- p21 prophase- derived head stabilising protein.(spot no- 3n1)

MVTVAELQALRQARLDLLTGKRVVSVQKDGRRIEYTAASLDELNRAINDAESVLG
TTRCRRRPLGVRL

b) YEDF-ECO57- UPF0033 protein Yedf (spot no- 276)

MKNIVPDYRLDMVGEPCPYPAVATLEAMPQLKKGEILEVVSDCPQSINNIPLDARN
HGYTVLDIQQDGPTIRYLIQK

c) Transcription anti-termination protein NsuA RFAH- ECO57 (spot no- 324)

MNKEILAVVEAVSNEKALPREKIFEALESALATATKKKYEQEIDVRVQIDRKSGDF
DTFRRWLVVDEVTQPTKEITLEAARYEDESLNLGDYVEDQIESVTFDRITTQTAKQ
VIVQKVREAERAMVVDQFREHEGEIITGVVKKVNRDNISLDLGNNAEAVILREDML
PRENFRPGDRVRGVLYSVRPEARGAQLFVTRSKPEMLIELFR
IEVPEIGEEVIEIKAAARDPGSRAKIAVKTNDKRIDPVGACVGMRGARVQAVSTELG
GERIDIVLWDDNPAQFVINAMAPADVASIVVDEDKHTMDIAVEAGNLAQAIGRNG
QNVRLASQLSGWELNVMTVDDLQAKHQAEAHAAIDTFTKYLDIDEDFATVLVEEG
FSTLEELAYVPMKELLEIEGLDEPTVEALRERAKNALATIAQAQEESLGDNKPADD
LLNLEGVDRDLAFKLAARGVCTLEDLAEQGIDDLADIEGLTDEKAGALIMAARNIC
WFGDEA

d) FIC-E.coli – Full probable adenosine monophosphate –protein transferase fic (spot no-378)

MSDKFGEGRDPYLYPGLDIMRNRLNIRQQQRLEQAAYEMTALRAATIELGPLVRG
LPHLRTIHRQLYQDIFDWAGQLREVDIYQGDTPFCHFAYIEKEGNALMQDLEEEGY
LVGLEKAKFVERLAHYYCEINVLHPFRVGSGLAQRIFFEQLAIHAGYQLSWQGIEK
EAWNQANQSGAMGDLTALQMIFSKVVSEAGESE

e) RL6-Eco 24- 30s ribosomal protein L6(spot no- 415)

MSRVAKAPVVVPAGVDVKINGQVITIKGKNGELTRTLNDAVEVKHADNTLTFGPR
DGYADGWAQAGTARALLNSMVIGVTEGFTKKLQLVGVGYRAAVKGNVINLSLGF
SHPVDHQLPAGITAECPTQTEIVLKGADKQVIGQVAADLRAYRRPEPYKGKGVRY
ADEVVRTKEAKKK

f) XDHC-Eco57- Xanthine dehydrogenase iron sulphur binding subunit (spot no- 466)

MNHSETITIECTINGMPFQLHAVPGTPLSELLREQGLLSVKQGCCVGECGACTVLVD
GTAIDSCLYLAAWAEGKEIRTLEGEAKGGKLSHVQQAYAKSGAVQCGFCTPGLIM
ATTAMLAKPREKPLTITEIRRGLAGNLCRCTGYQMIVNTVLDCEKTK

g) LDRA-E.coli- Small toxic polypeptide Ldra/LdRc (spot no- 478)

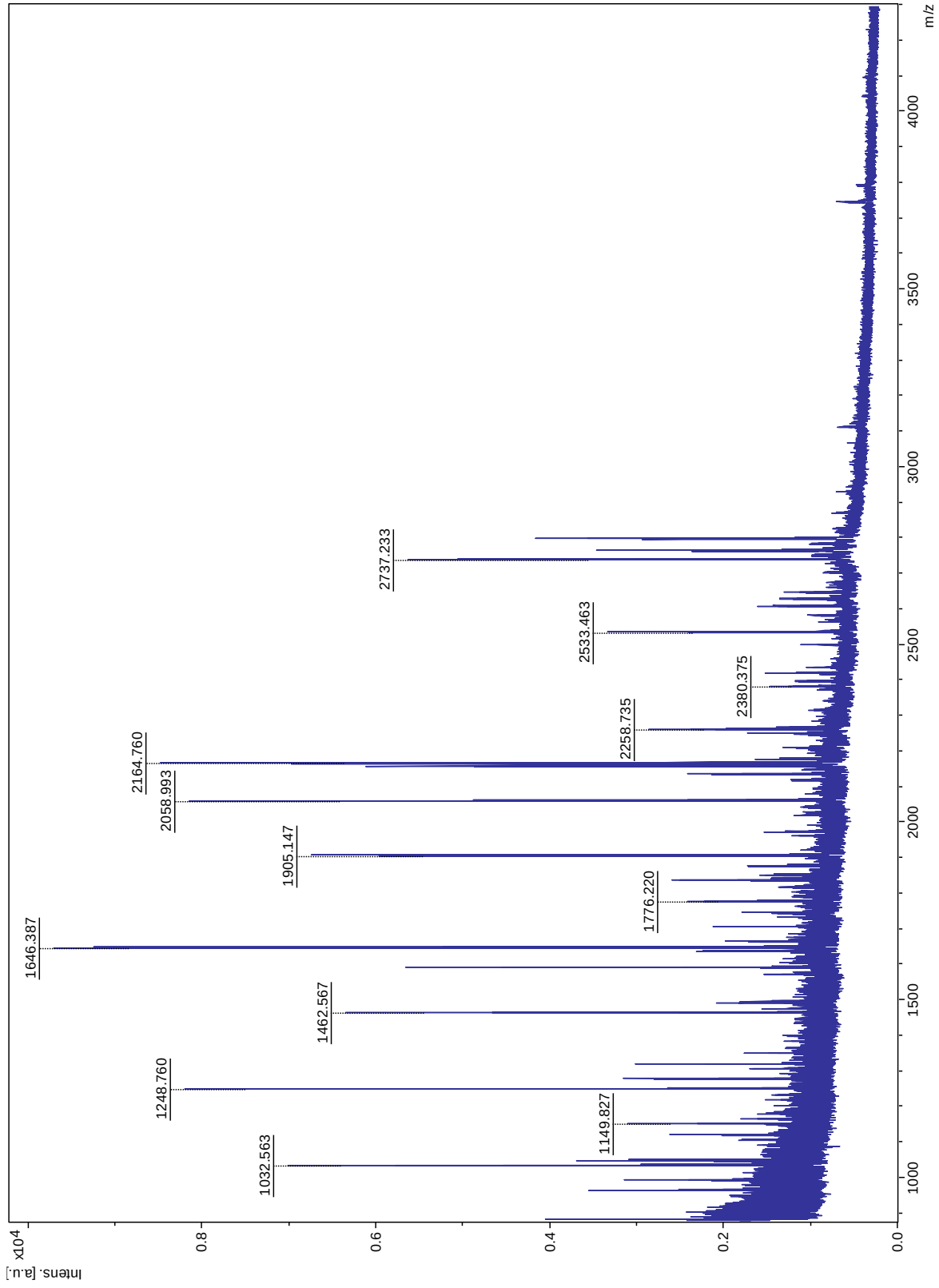
MTLAQFAMIFWHDLAAPILAGIITAAIVSWWRNRK

h) PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase (spot no- 507)

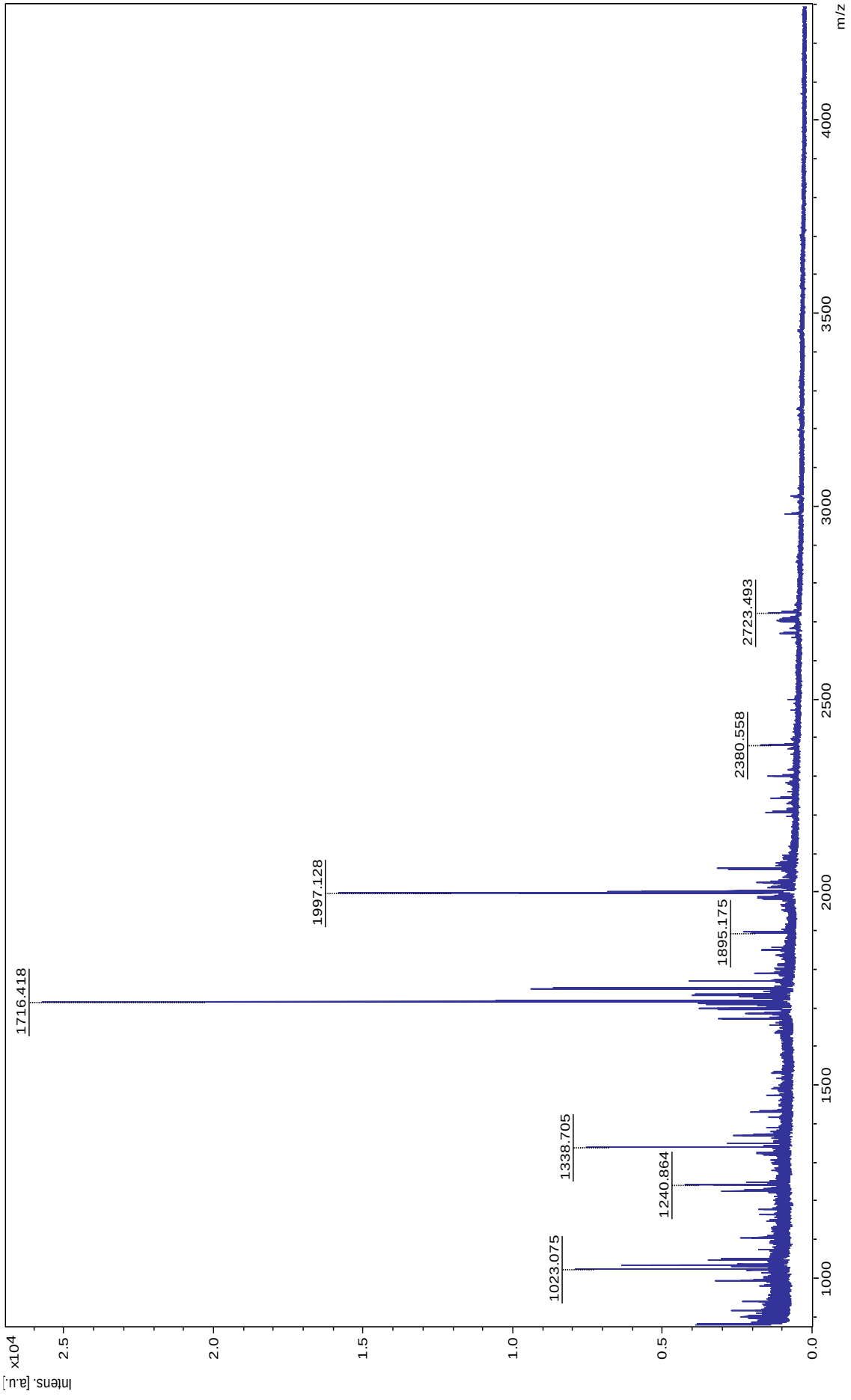
MQKQAELYRGKAKTVYSTENPDLLVLEFRNDTSAGDGARIEQFDRKGMVNNKFN
YFIMSKLAEAGIPTQMERLLSDTECLVKKLDMVPVECVVRNRAAGSLVKRLGIEEG
IELNPPLFDLFLKNDAMHDPMVNESYCETFGWVSKENLARMKELTYKANDVLKKL
FDDAGLILVDFKLEFGLYKGEVVLGDEFSPDGSRLWDKETLEKMDKDRFRQSLGG
LIEAYEAVARRLGVQLD

1) Mass-Spectra images of differentially expressed *E.coli* Nissle 1917 proteins under sap stress

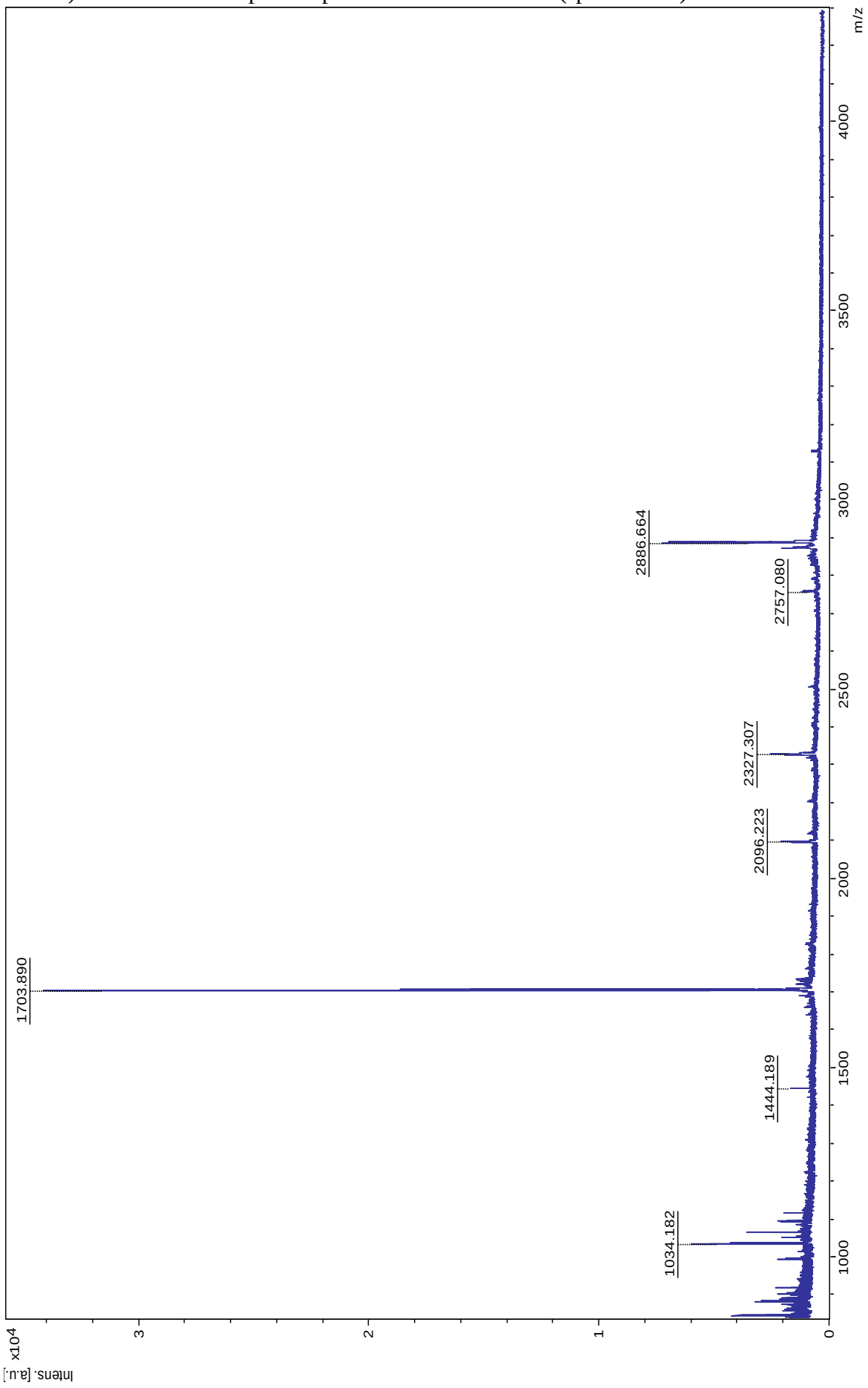
A) t-rna specific adenosine deaminase TADA-ECO57 (spot no- 348)



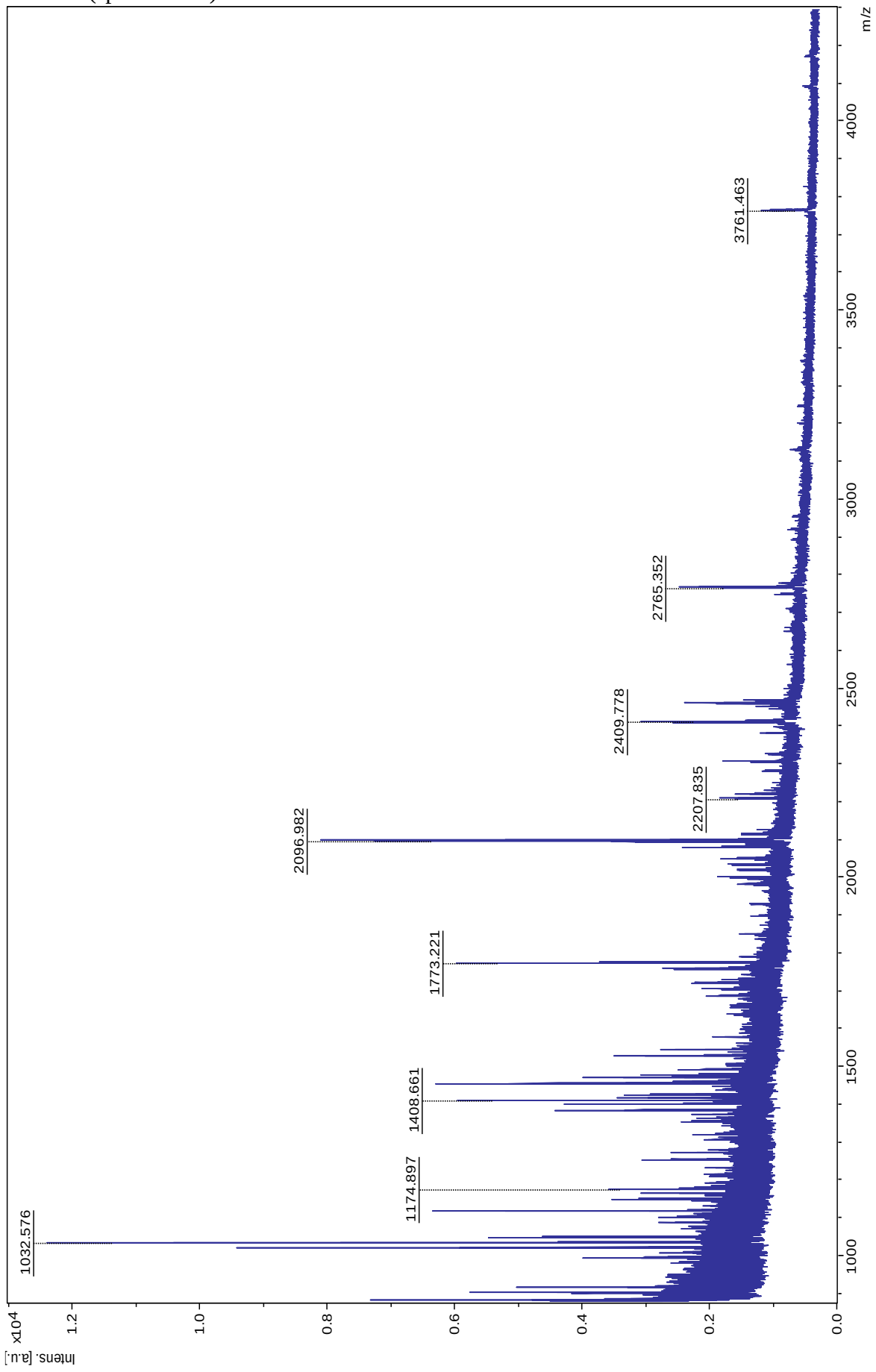
B) Formate hydrogenase subunit 5 HYCE- E.coli (spot no- 427)



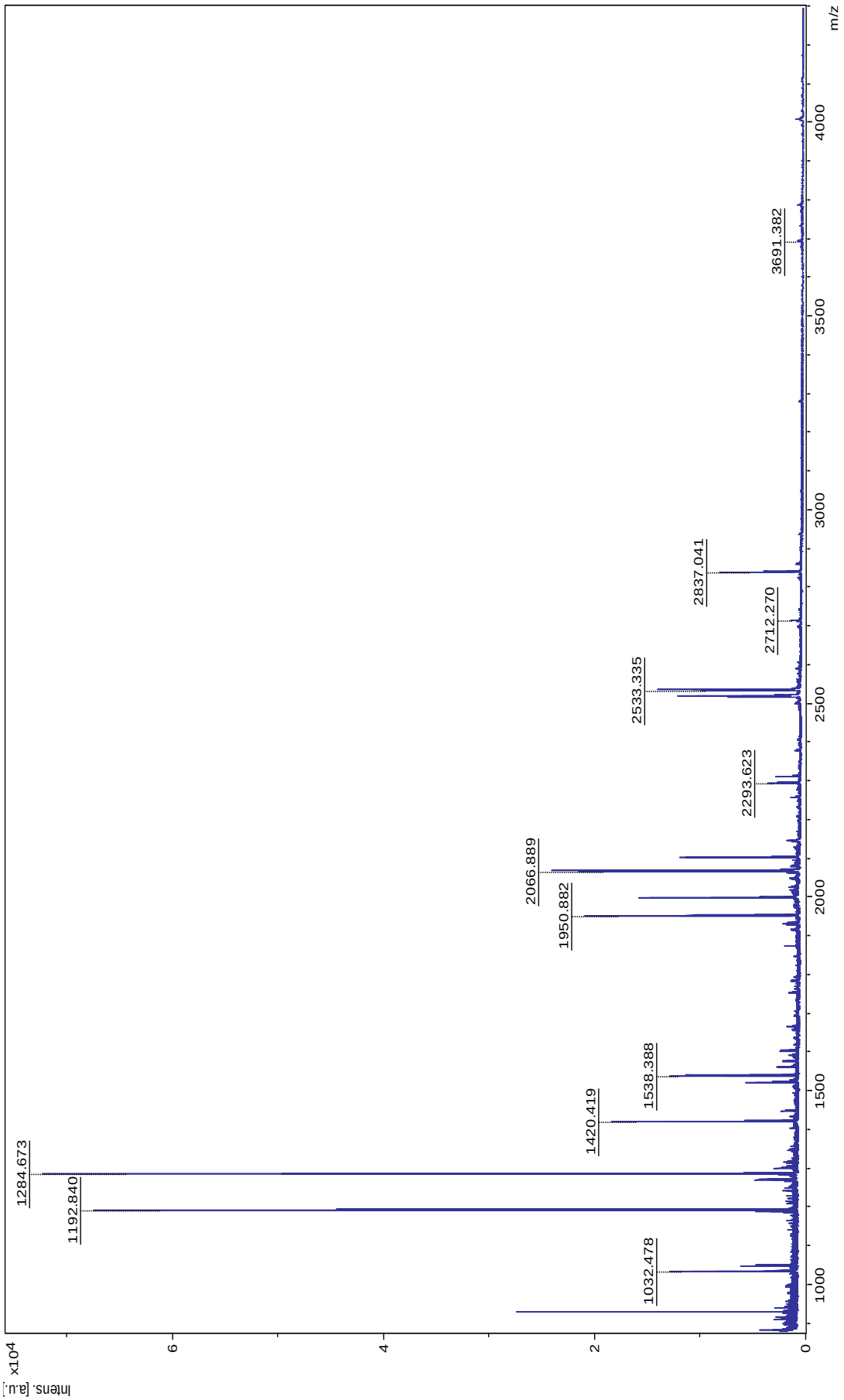
C) PMBA-Eco 57 – protein pmba 0s E.coli 0157:H7 (spot no-457)



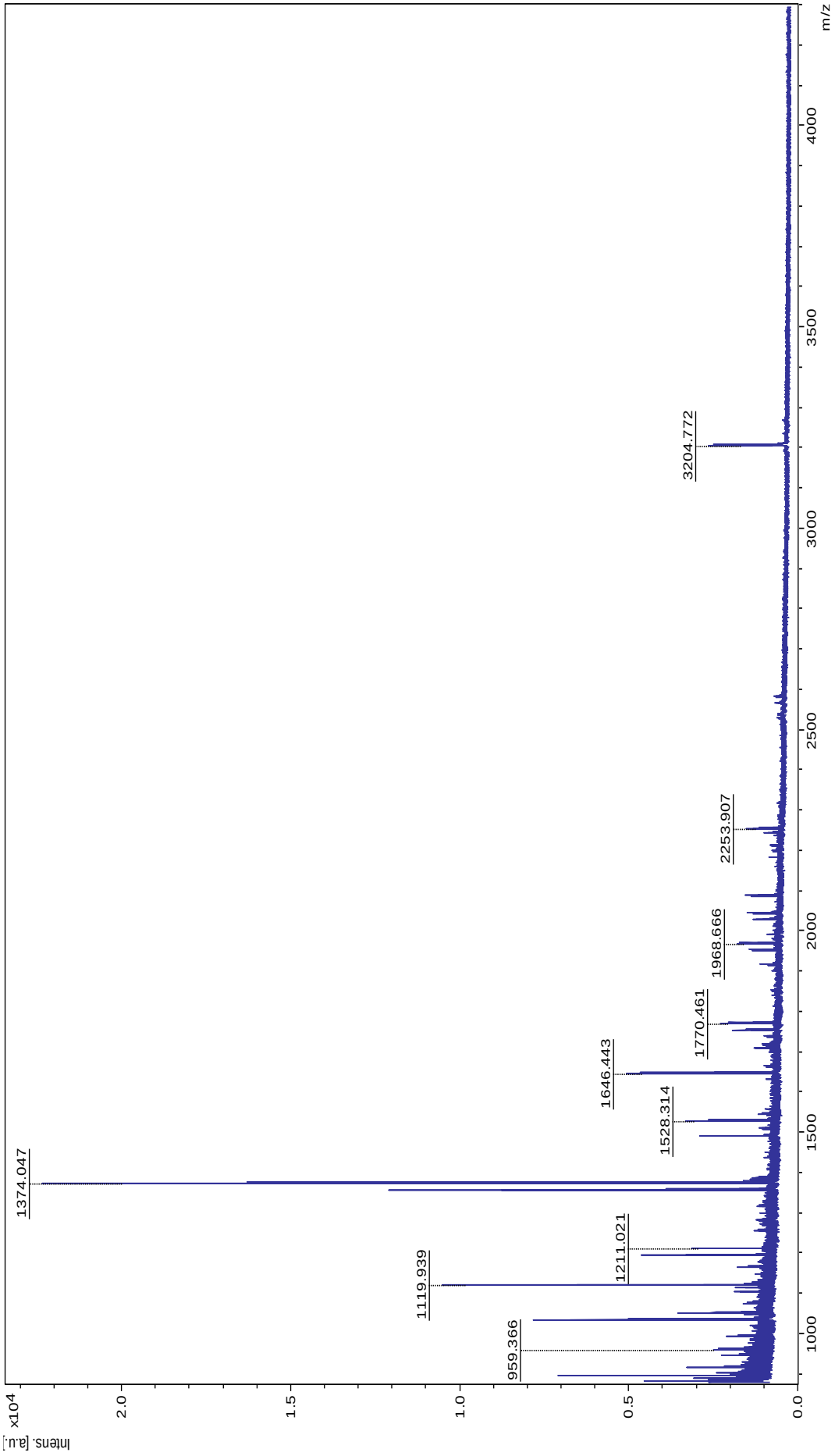
D) N acetyl galactosamine – specific phosphor transferase IIB component 1 (spot no-468)



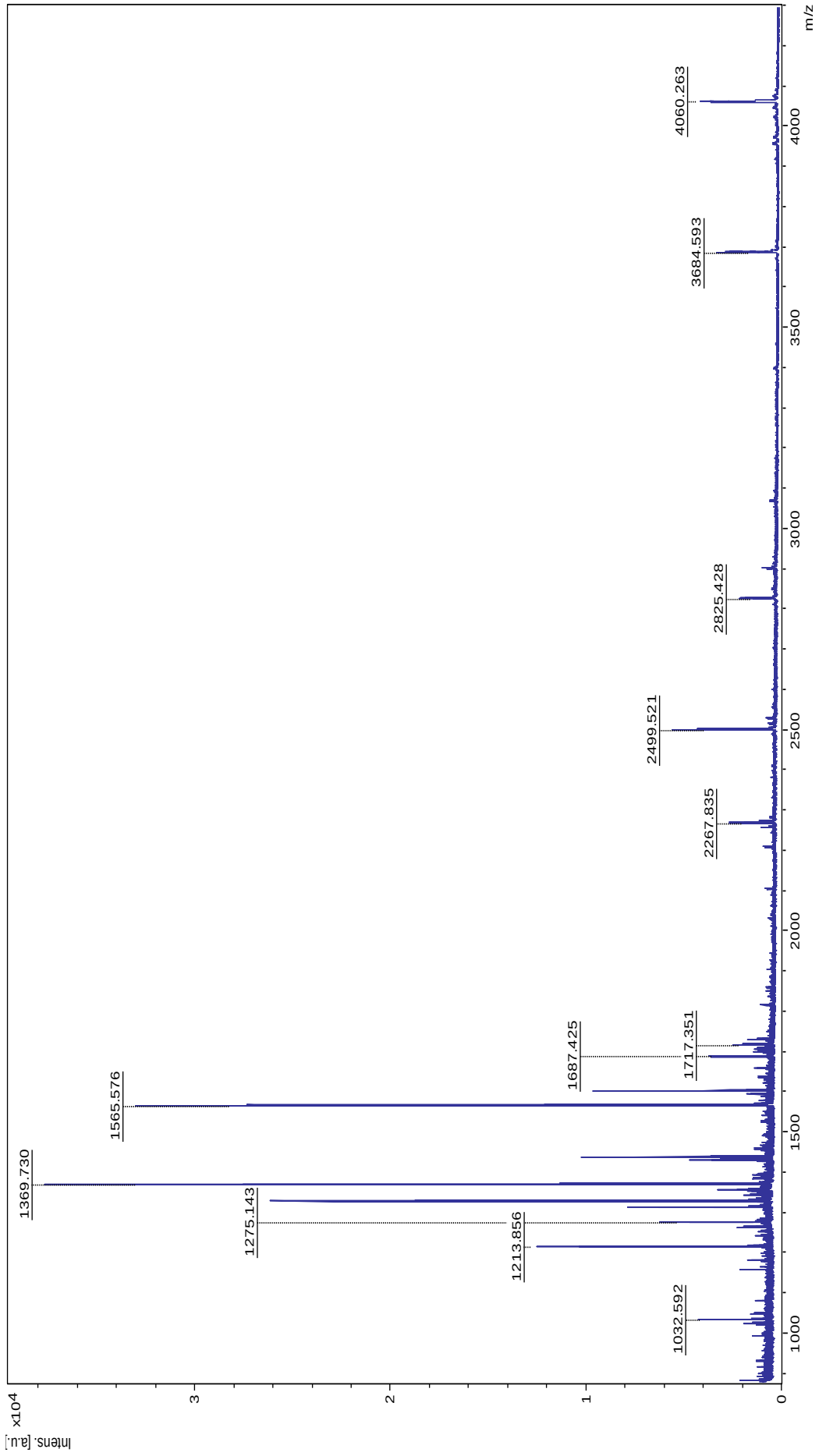
E) BAER-Ecol6- Transcriptional regulatory protein os-E.coli (472)



F) DINI-E.co 57- Dini like protein Z3305/Ecs 2939 in prophage (spot no-488).

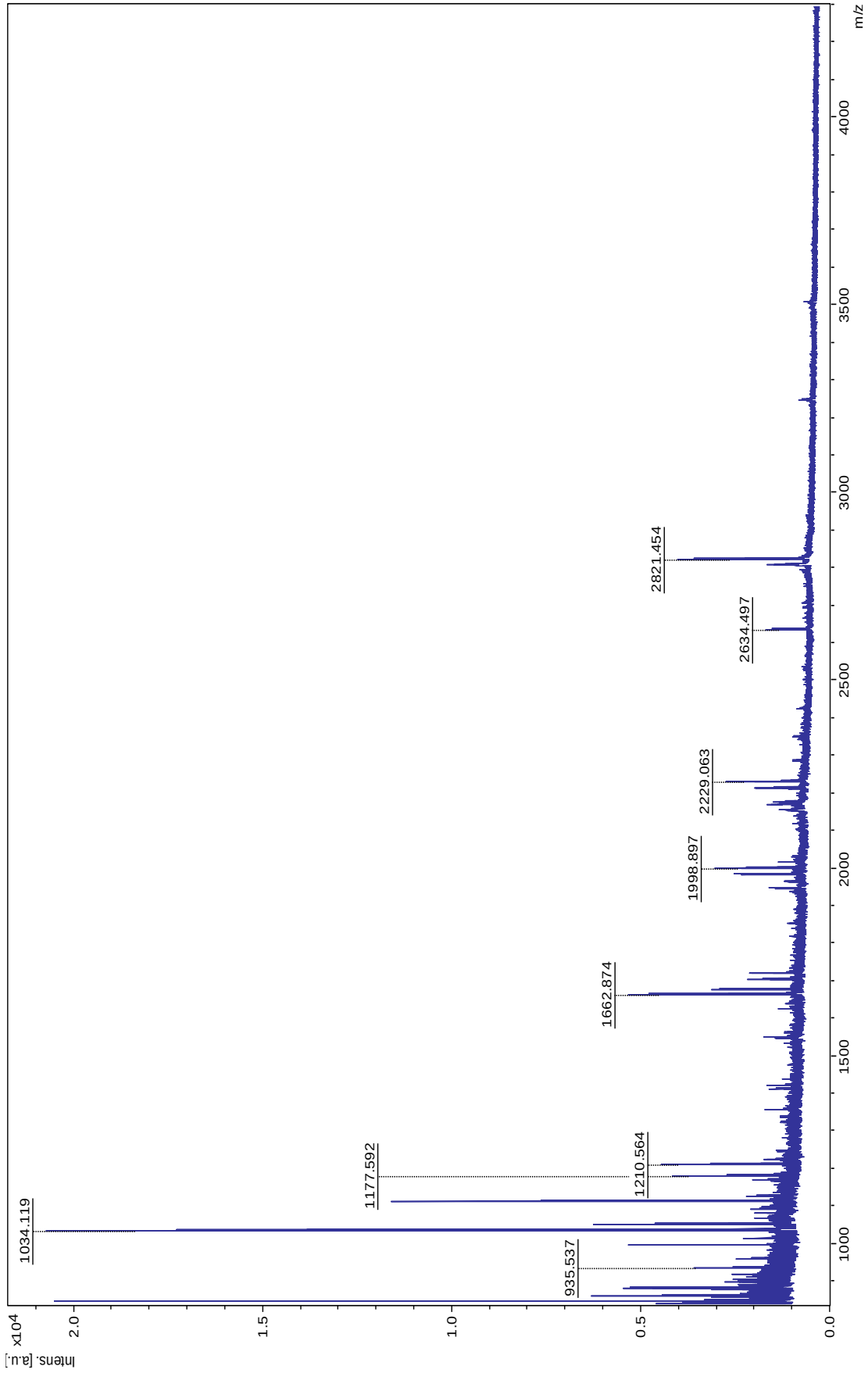


G) Y3010-E.coli5- UPFO401 protein ECP-3010 (spot no- 595)

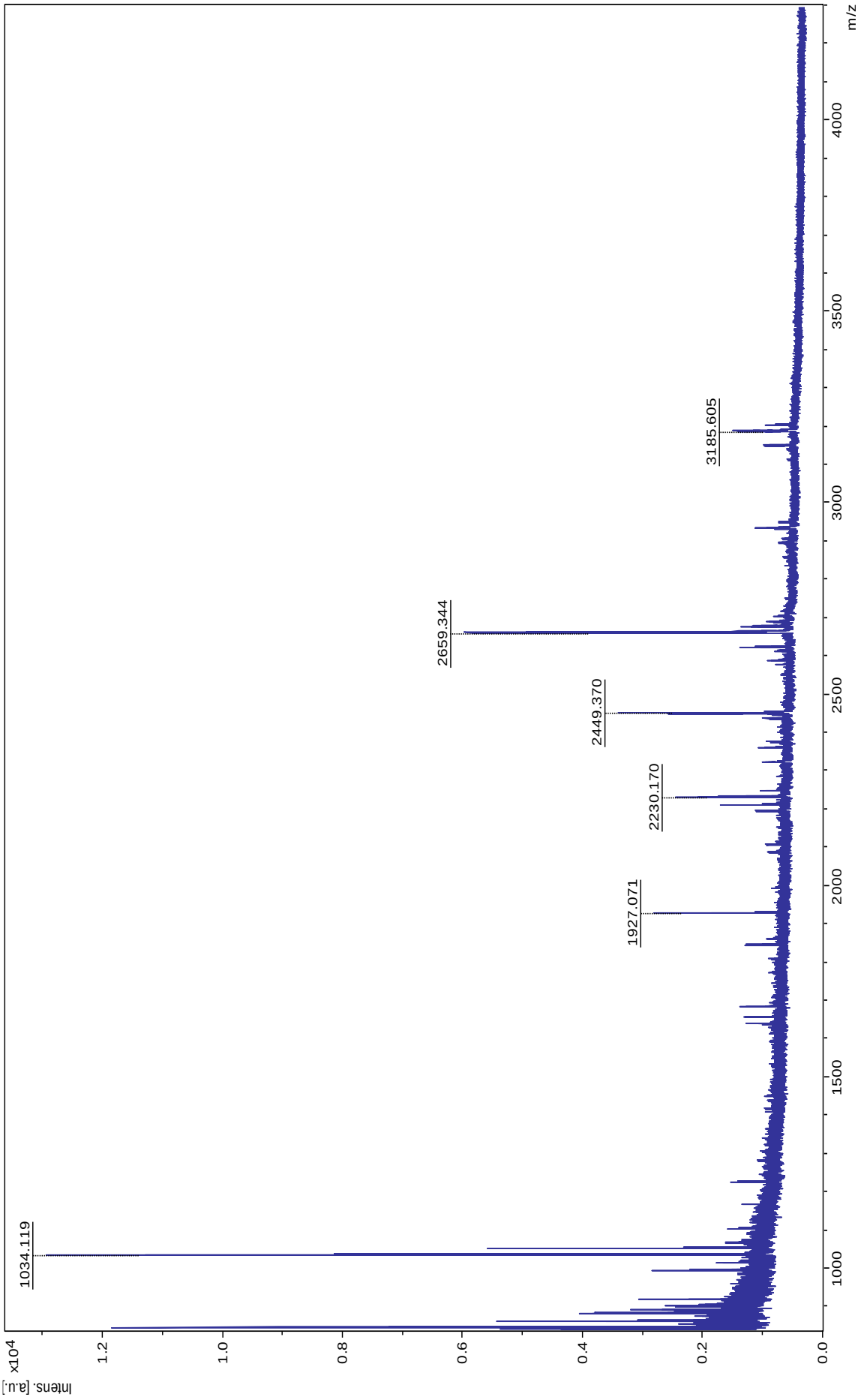


2) Mass-Spectra images of differentially expressed *E.coli* Nissle 1917 proteins under wine stress

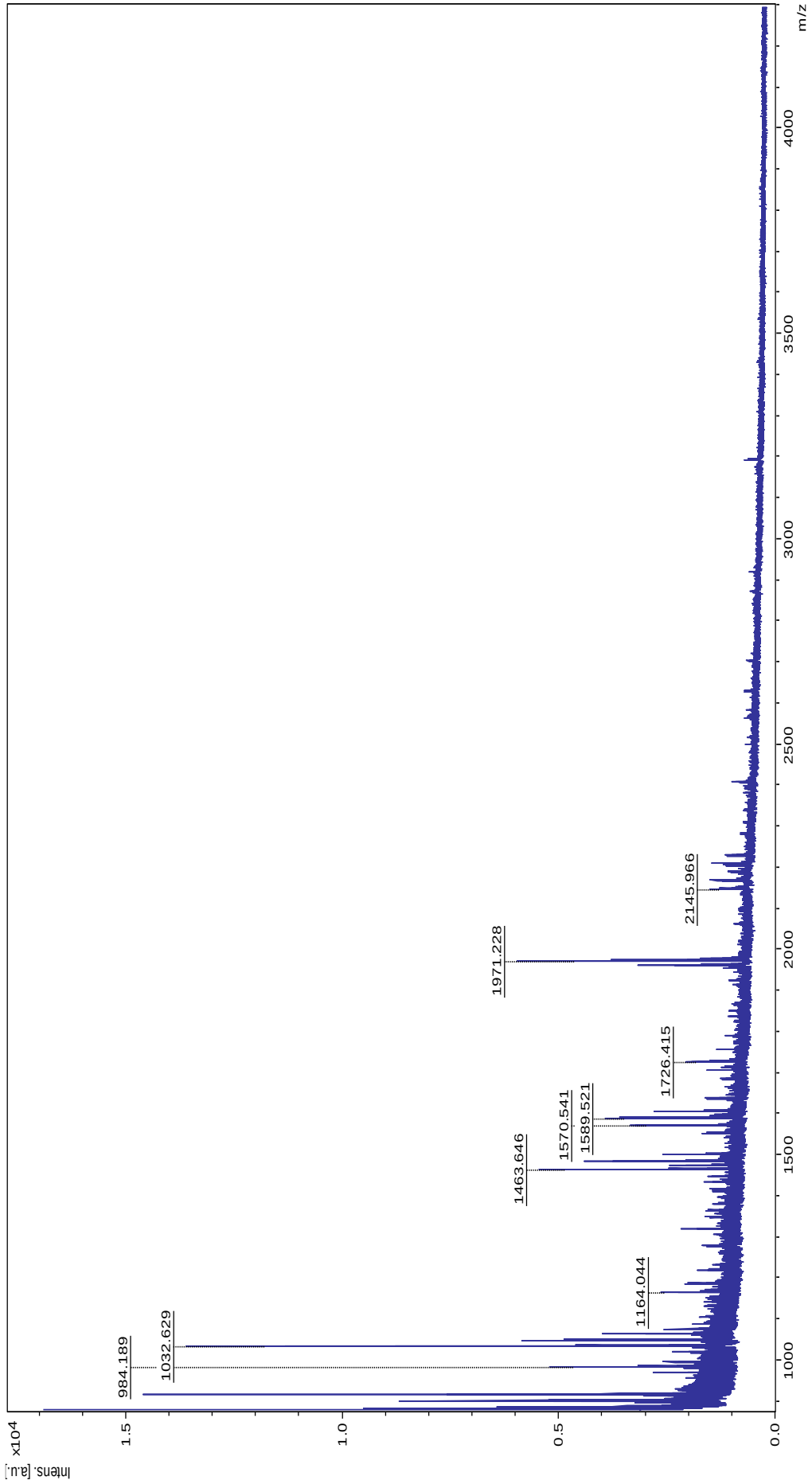
A) VGO3-ECol6- p21 prophase- derived head stabilising protein.(spot no- 3n1)



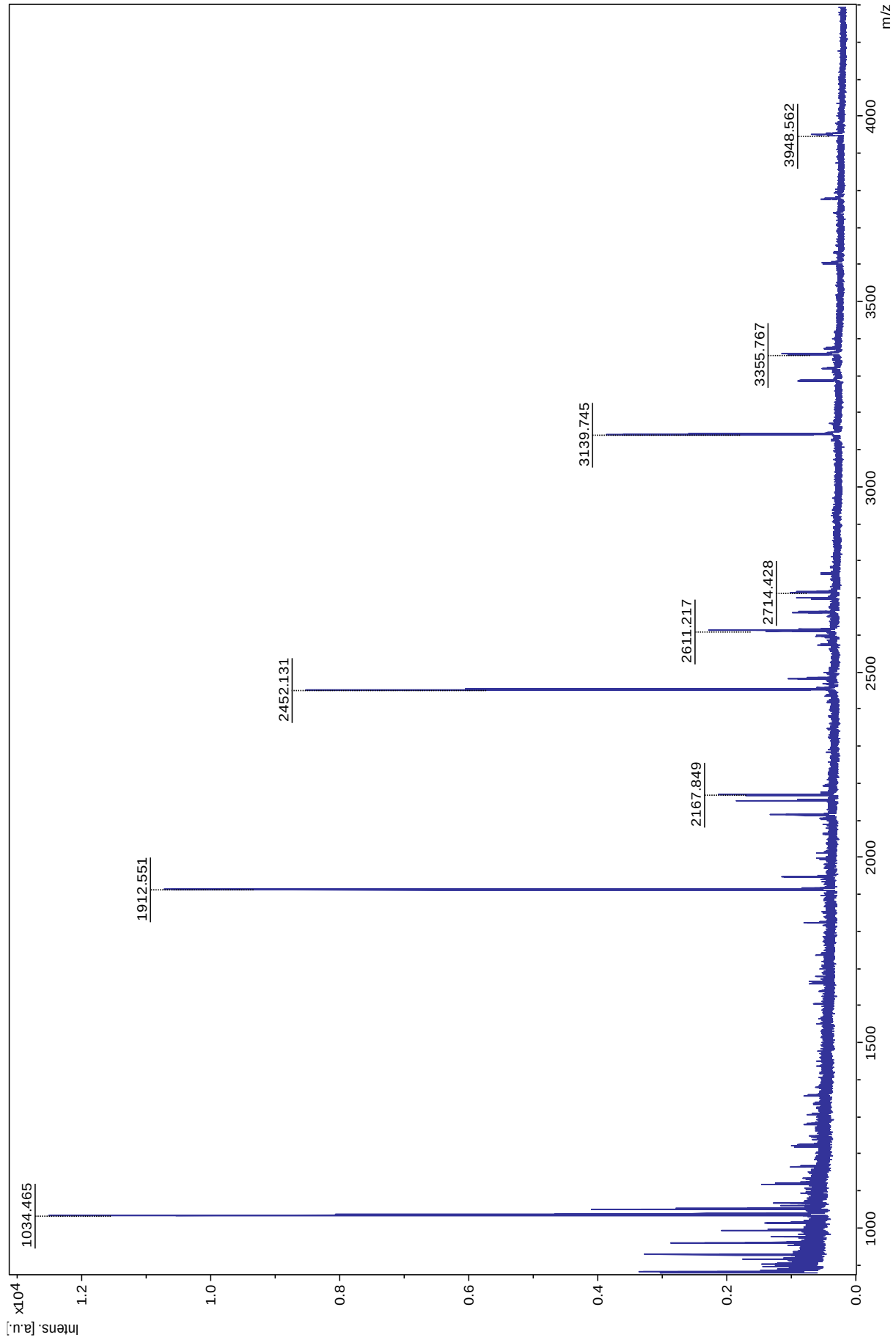
B) YEDF-ECO57- UPF0033 protein Yedf (spot no- 276)



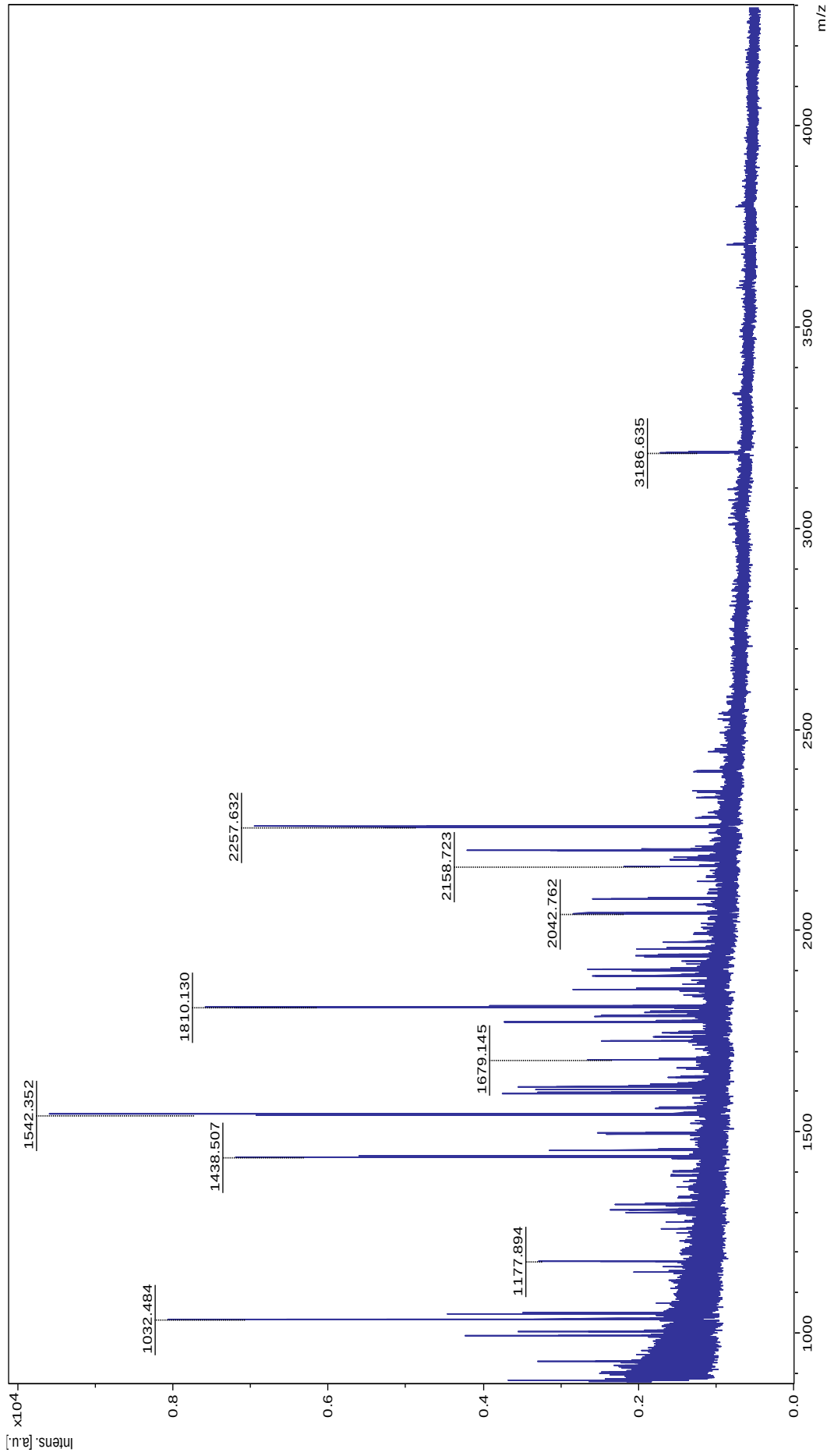
C) Transcription anti-termination protein NsuA RFAH- ECO57 (spot no- 324)



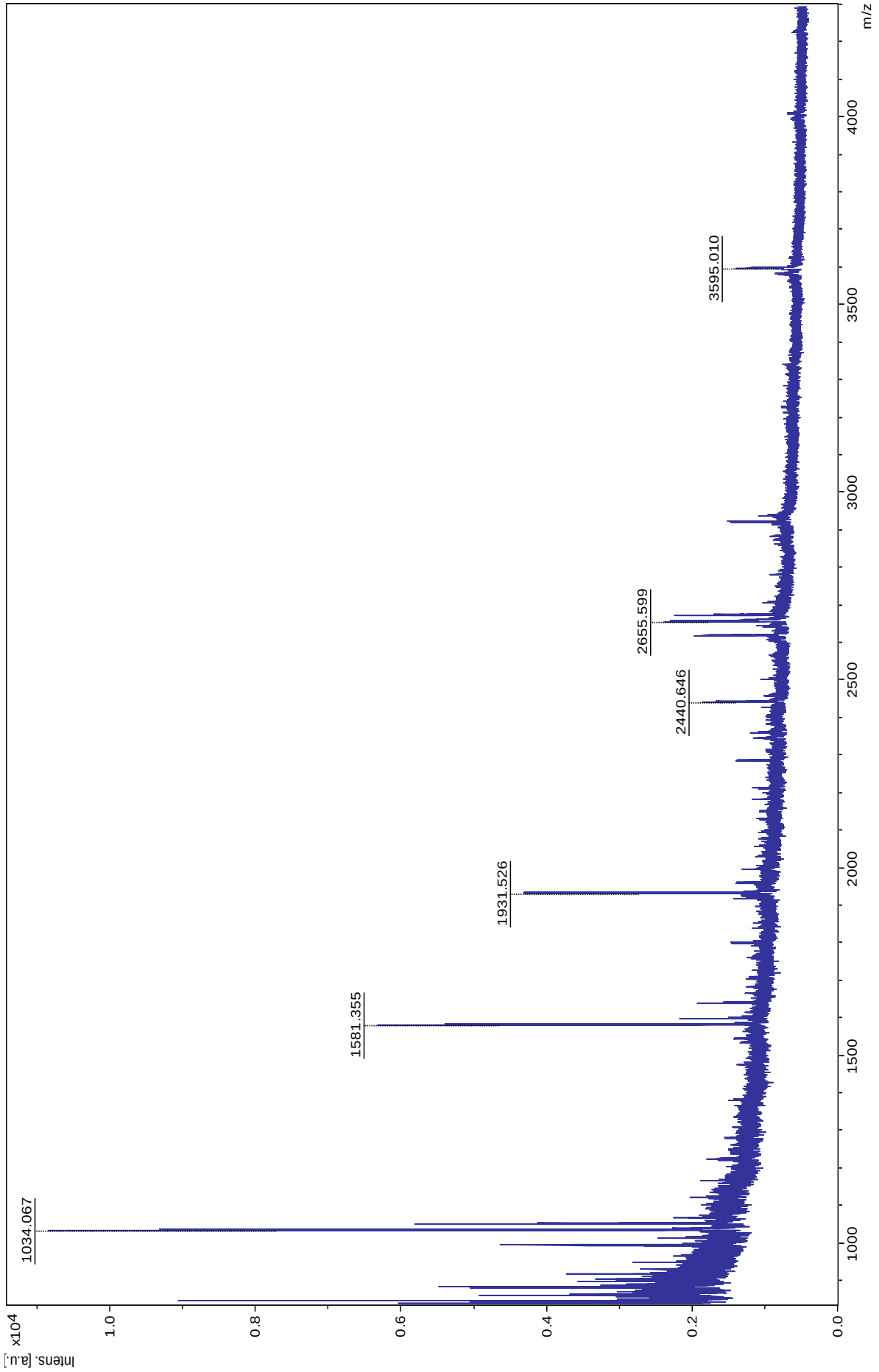
D) FIC-E.coli – Full probable adenosine monophosphate –protein transferase fic (spot no-378)



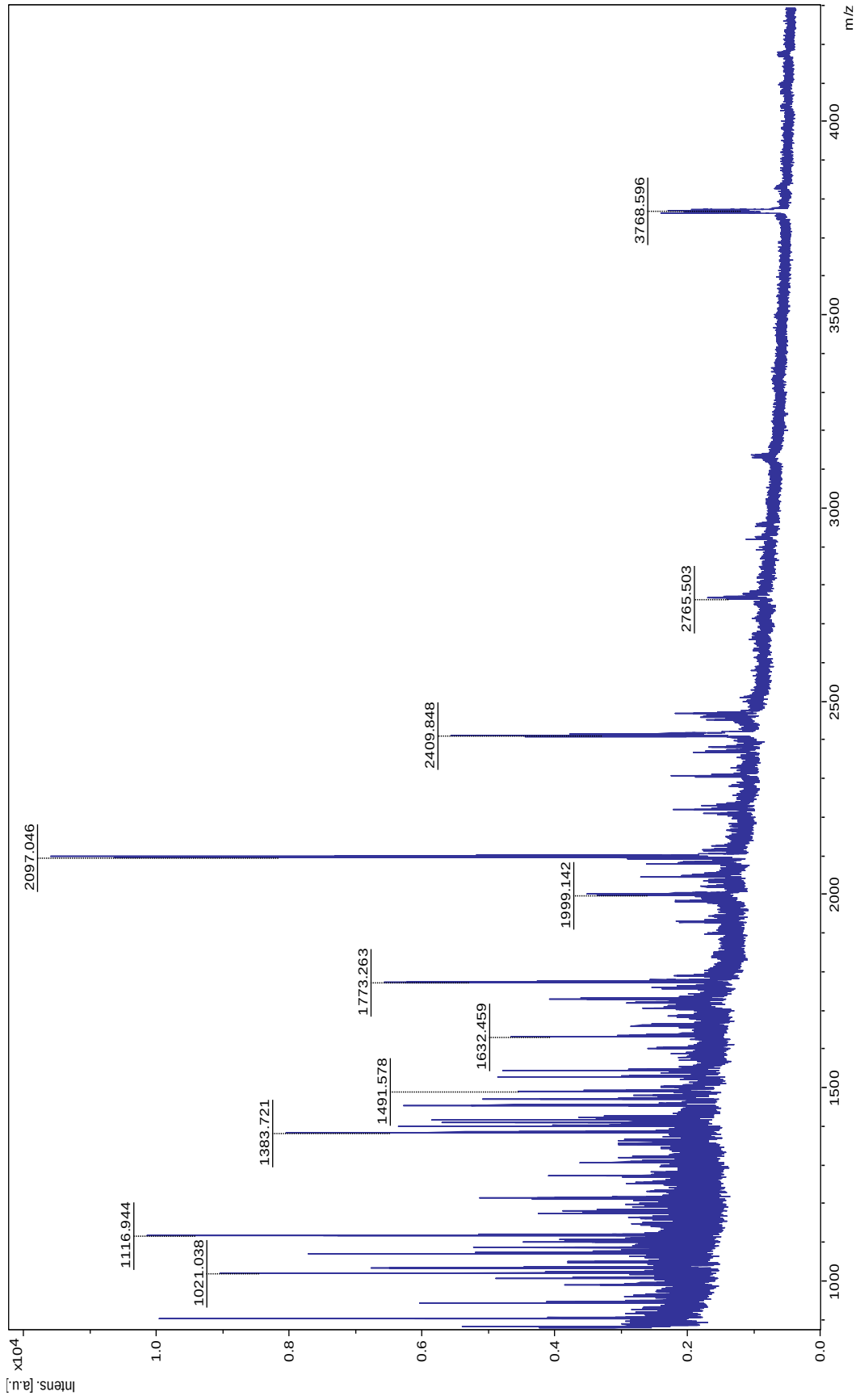
E) RL6-Eco 24- 30s ribosomal protein L6 (spot no- 415).



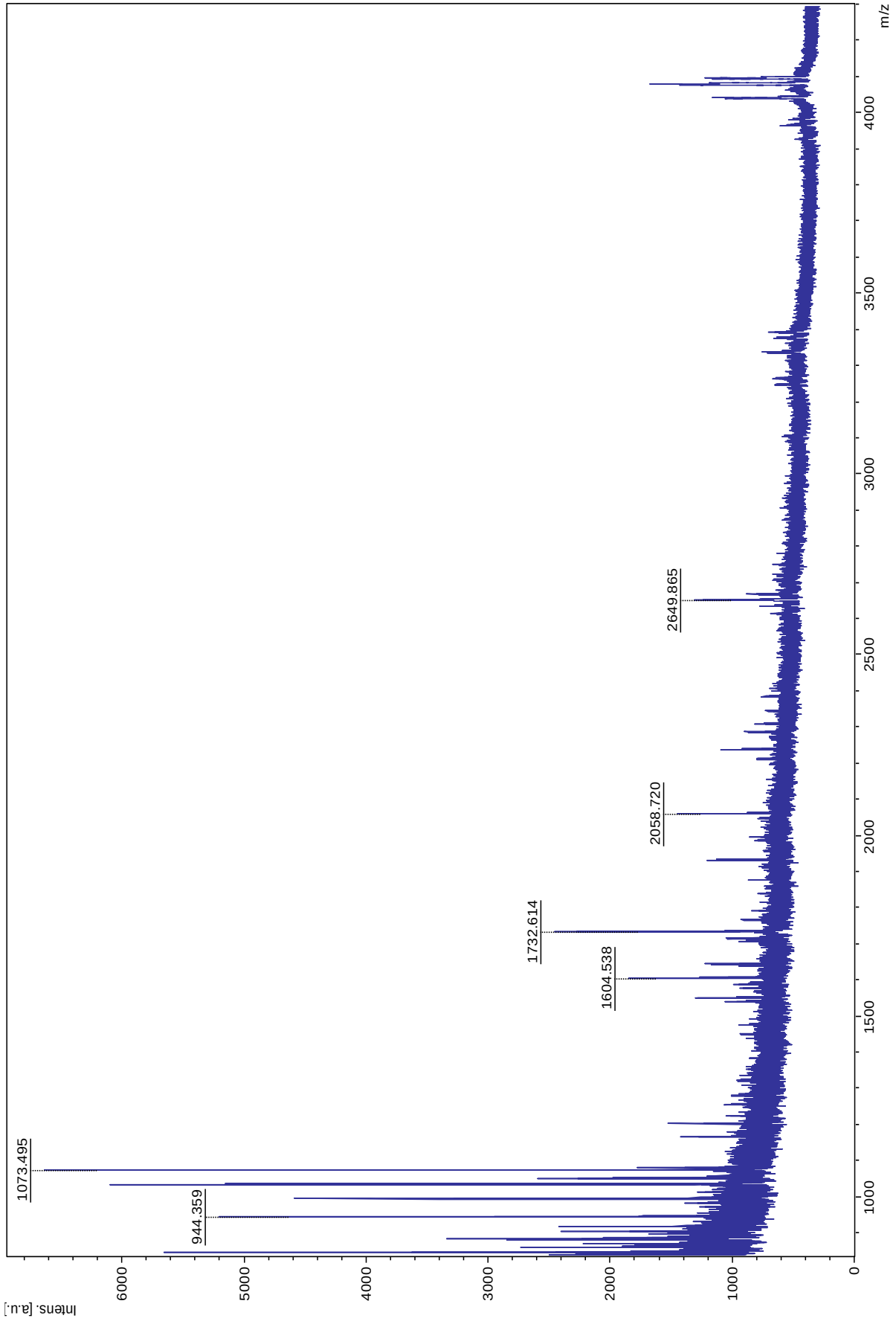
F) XDHC-Eco57- Xanthine dehydrogenase iron sulphur binding subunit (spot no- 466)



G) LDRA-E.coli- Small toxic polypeptide Ldra/LdRc (spot no- 478)



H) PUR7-eco24- phosphoribosylamidazole-succinocarboxamide synthase (spot no-507)



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