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Comparative Metabolic Profiling of Halotolerant Bacterial Strains and Identification of Novel-Species Specific Metabolites

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VIT University, Tamil Nadu, India



VIT[®]
UNIVERSITY
(Estd. u/s 3 of UGC Act 1956)

VELLORE ■ CHENNAI

www.vit.ac.in

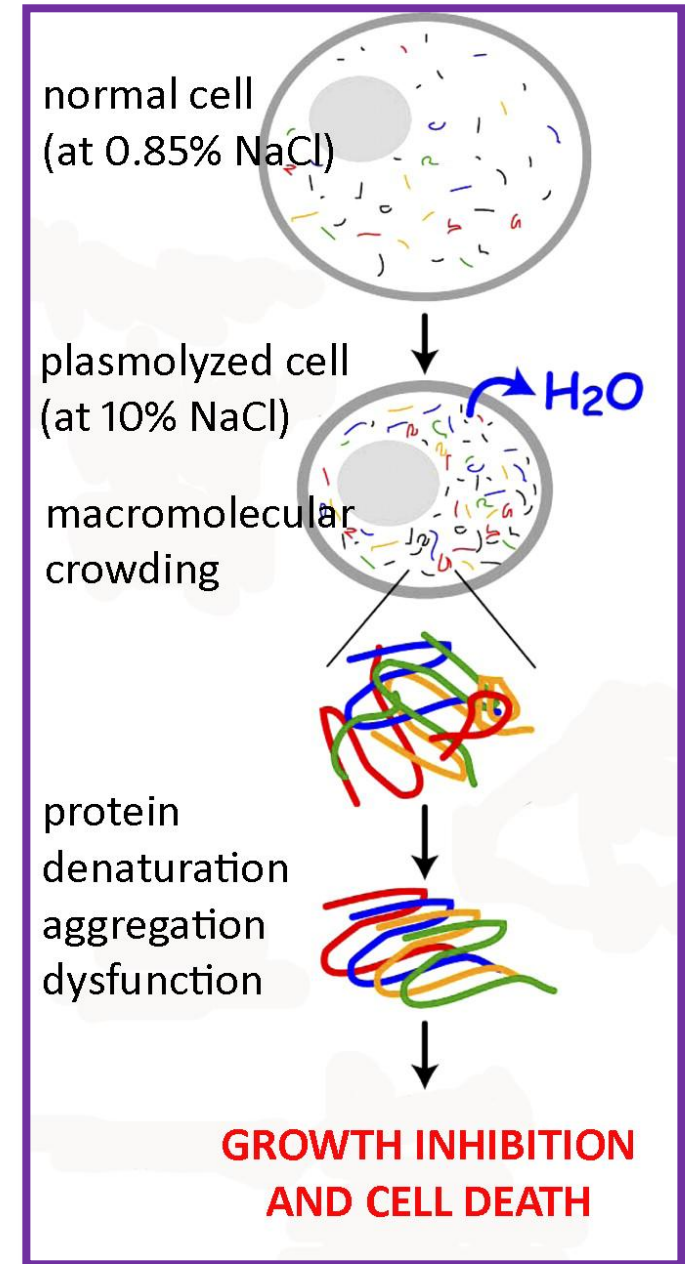
4th International Conference and Exhibition on
Metabolomics & Systems Biology
Philadelphia, USA

The Research Focus

Saline environments - marine water, salt lakes, brines, salterns, saline soils, salted foods, etc.

Halophilic and halotolerant bacteria have developed two types of strategies (Galinski and Truper, 1994)

1. Salt-in strategy - K^+ , Cl^-
2. Organic solute-in strategy - polar neutral/zwitterionic organic compounds



The 'Bacterial' Challenge

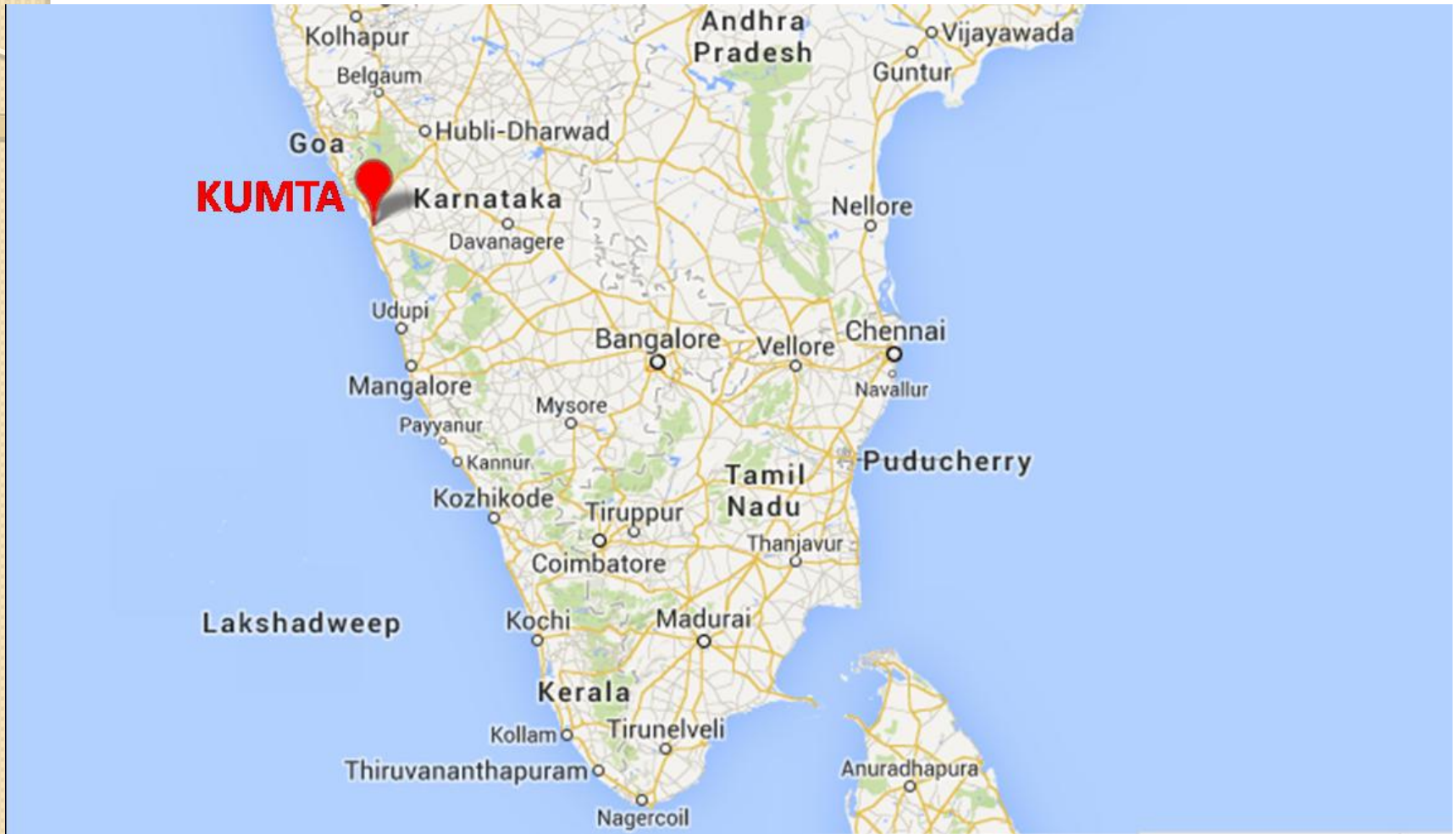
Protein stability – one of the most **challenging problems in halotolerant** bacteria during osmotic stress due to

- reduced water activity
- macromolecular crowding

Compatible solutes help in protecting the protein stability and function under such stress conditions – survival of these organisms

Maintaining protein stability during **various biotechnological processes** – protein expression, purification, formulations, storage, harsh conditions employed in various processes – food and chemical processing, detergents - a challenging and daunting task

The Starting Point

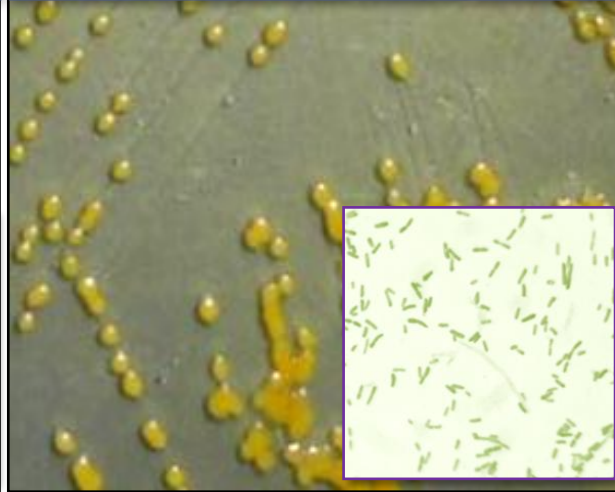


The Isolates

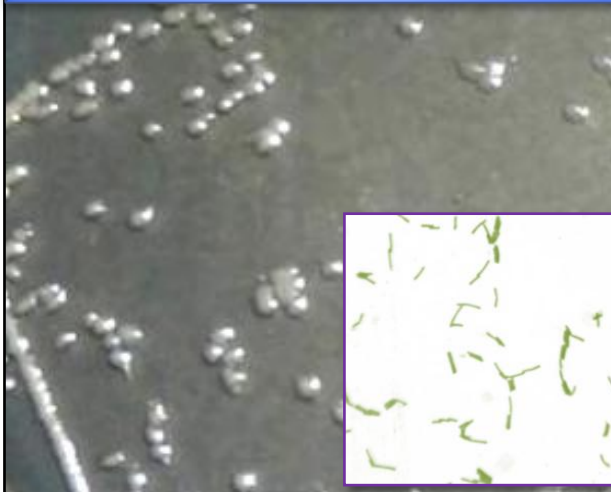
Halomonas hydrothermalis
VITP09 (FJ743438)



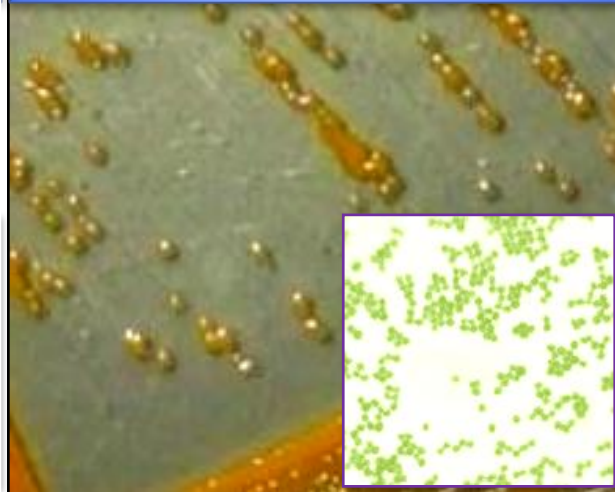
Bacillus aquimaris
VITP04 (FJ687490)



Virgibacillus dokdonensis
VITP14 (HQ929427)

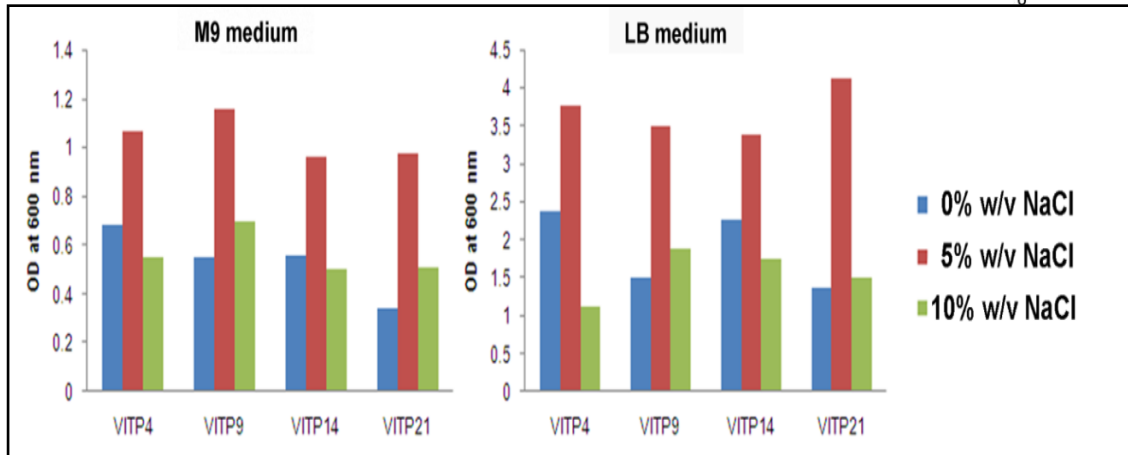
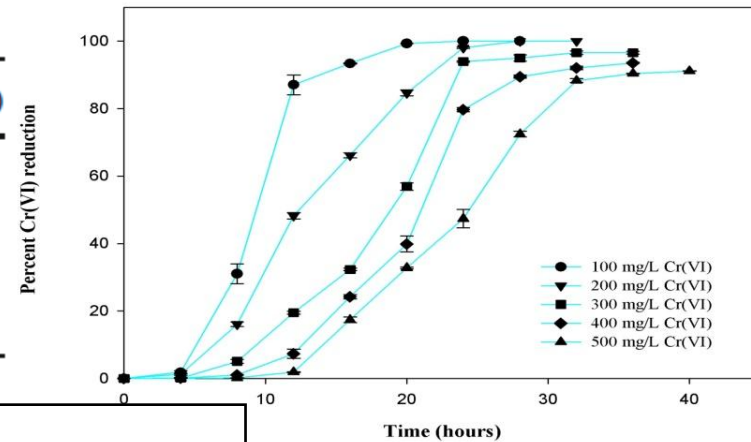


Planococcus maritimus
VITP21 (HQ829429)



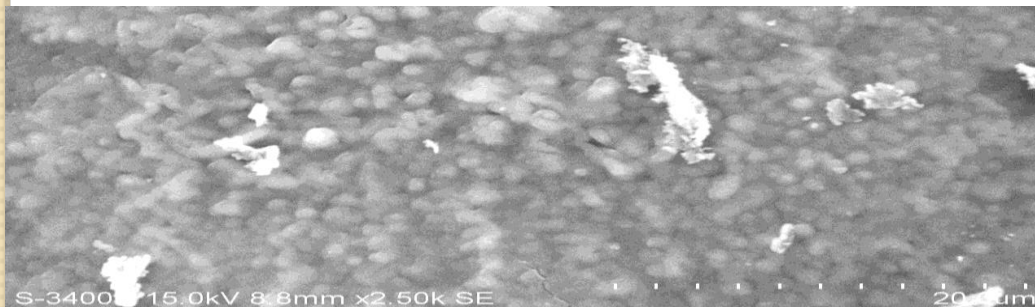
Products & Adaptability

Substrates (1 mg ml ⁻¹)	Relative activity (%)
Casein	100.36±1.87
Egg albumin	64.56±1.81
Gelatin	93.10±1.75
BSA	68.40±1.48

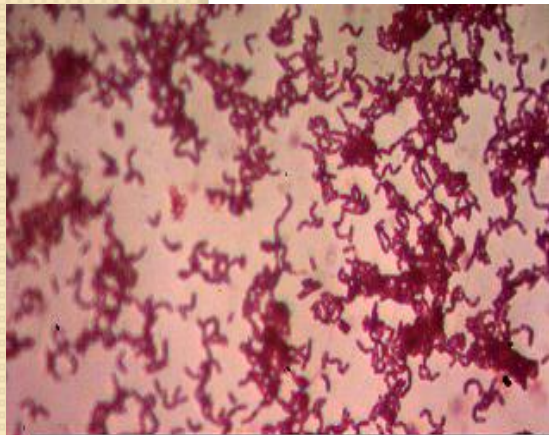
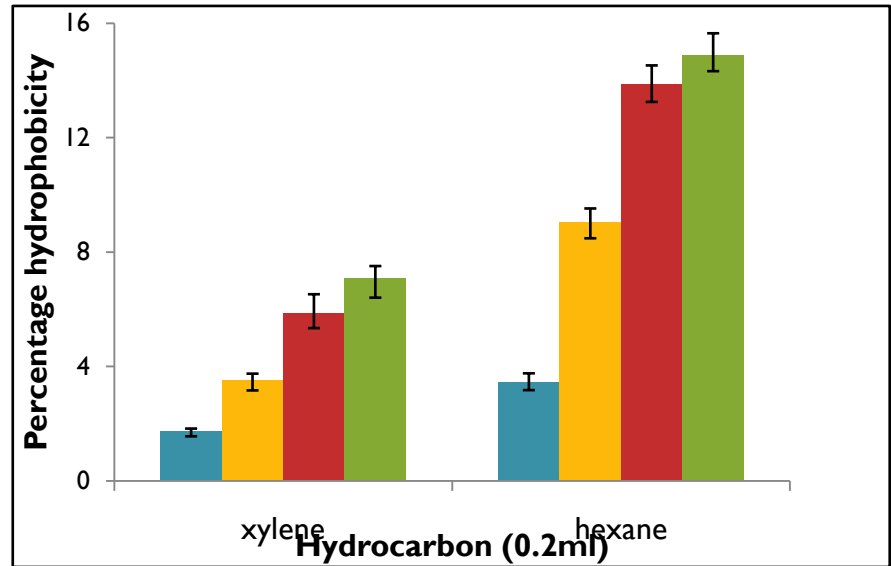


Protease
Amylase
Keratinase
Cellulase
Xylanase

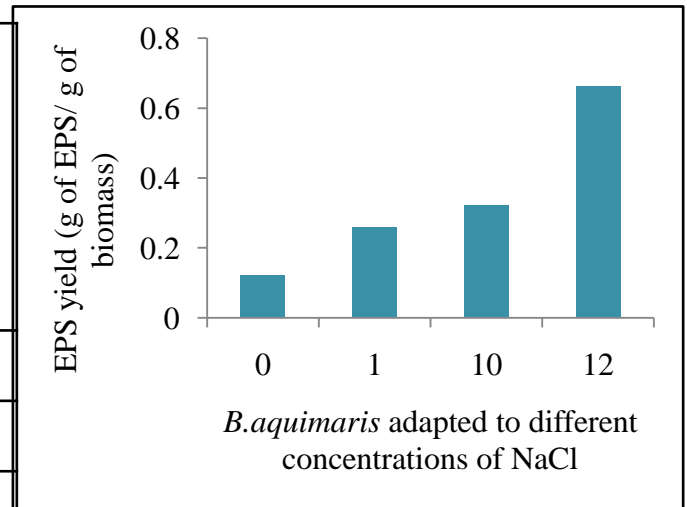
Reduction of heavy
metal ions



Natural & Induced Adaptability



Amount of NaCl (%)	Amount of NaCl (M)	Number of passages required to reach 1 OD
1	0.25	4
10	2.5	8
12	3	15

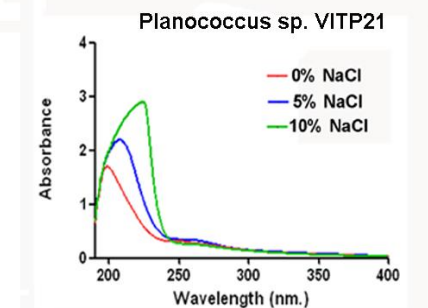
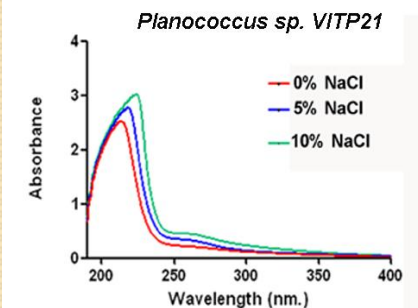
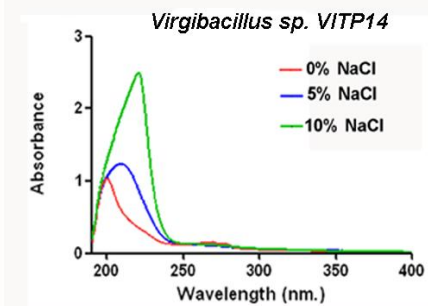
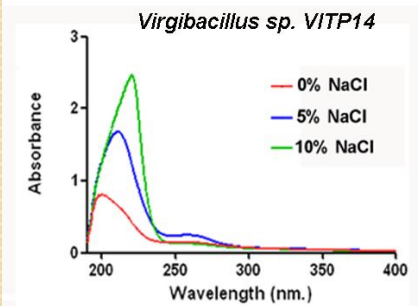
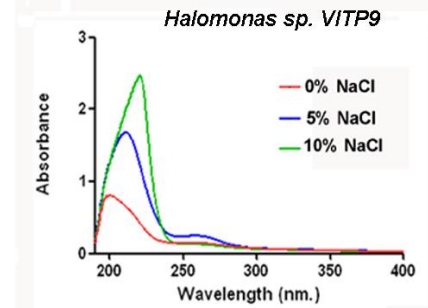
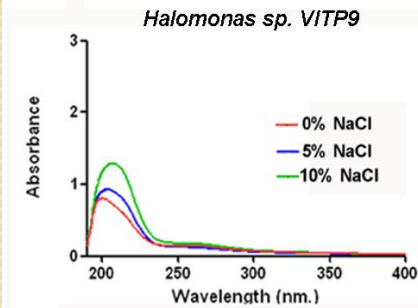
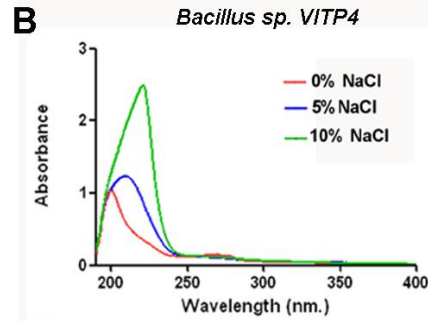
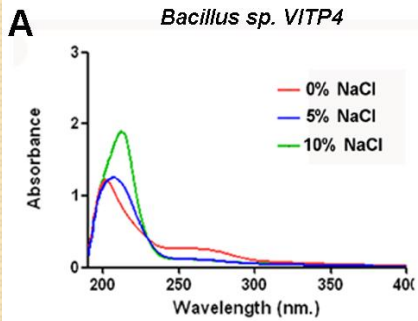


With shift in temperature and pH optimum and also increased stability under saline conditions

M9

LB

SALT STRESS INDUCED BIOCHEMICAL CHANGES

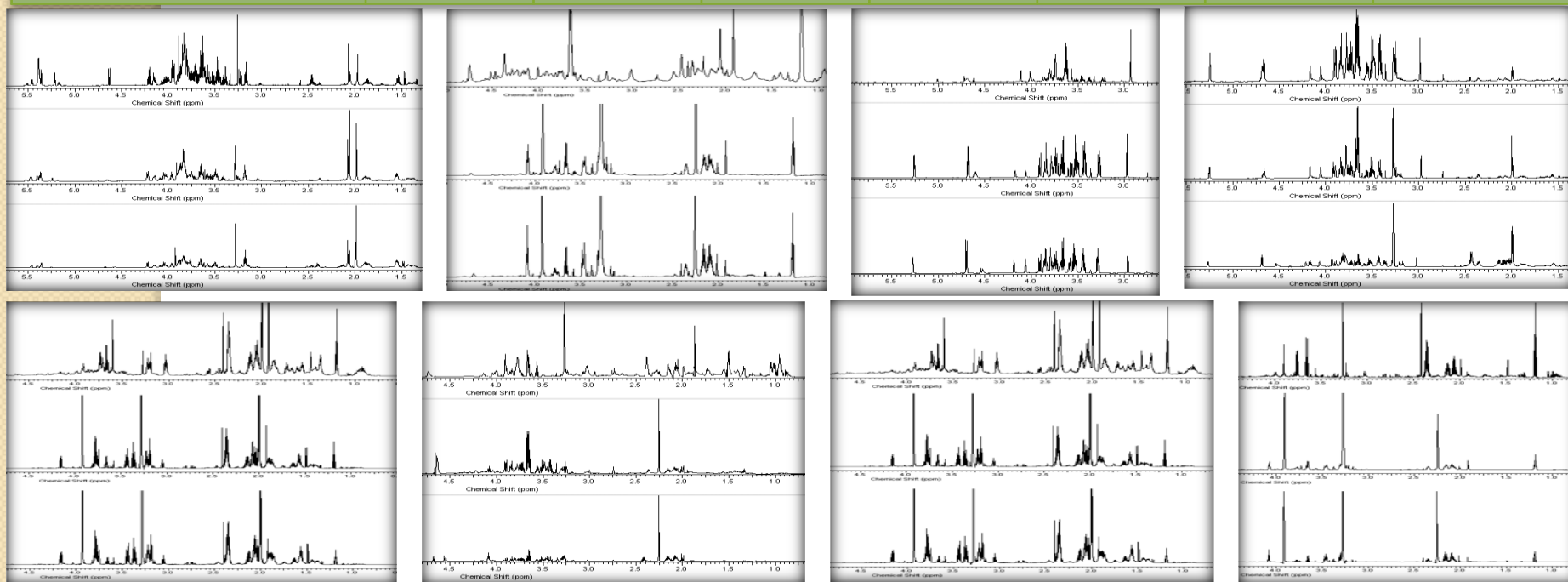


- Changes in the intracellular organic solute pools analyzed – UV spectra of the extracts (200 – 400 nm) - varied with respect to the salt concentration, growth media and the type of species.

- Could be assumed that the composition of the intracellular organic solutes **changes with increasing salt concentrations, type of growth media and bacterial species.**

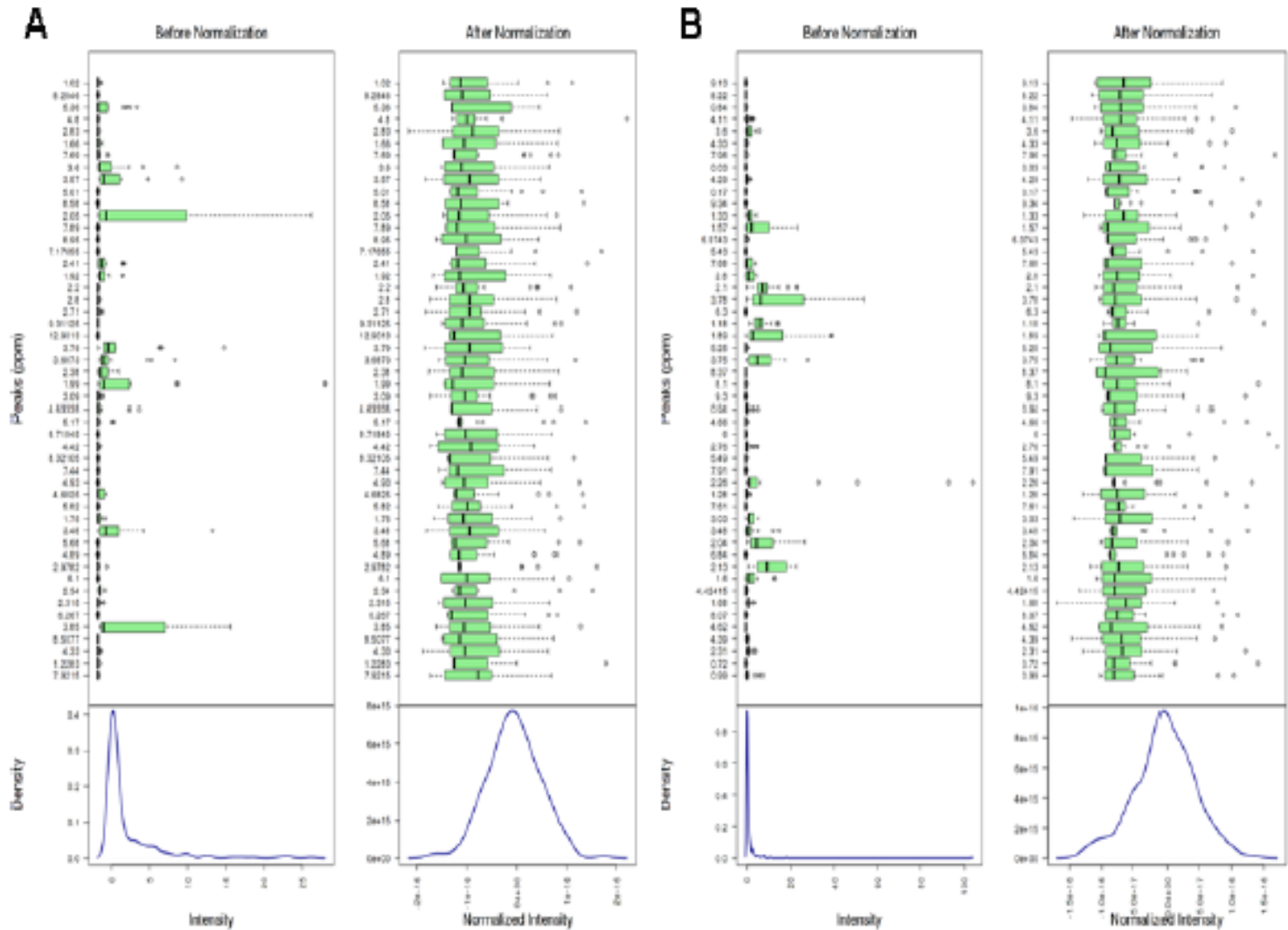
SPECTRAL COMPLEXITY

	0% NaCl		5% NaCl		10% NaCl		Total
Growth Medium	M9	LB	M9	LB	M9	LB	
No of cultures (4 bacterial species)	4	4	4	4	4	4	24
No of NMR samples (Biological replicates)	8	8	8	8	8	8	48

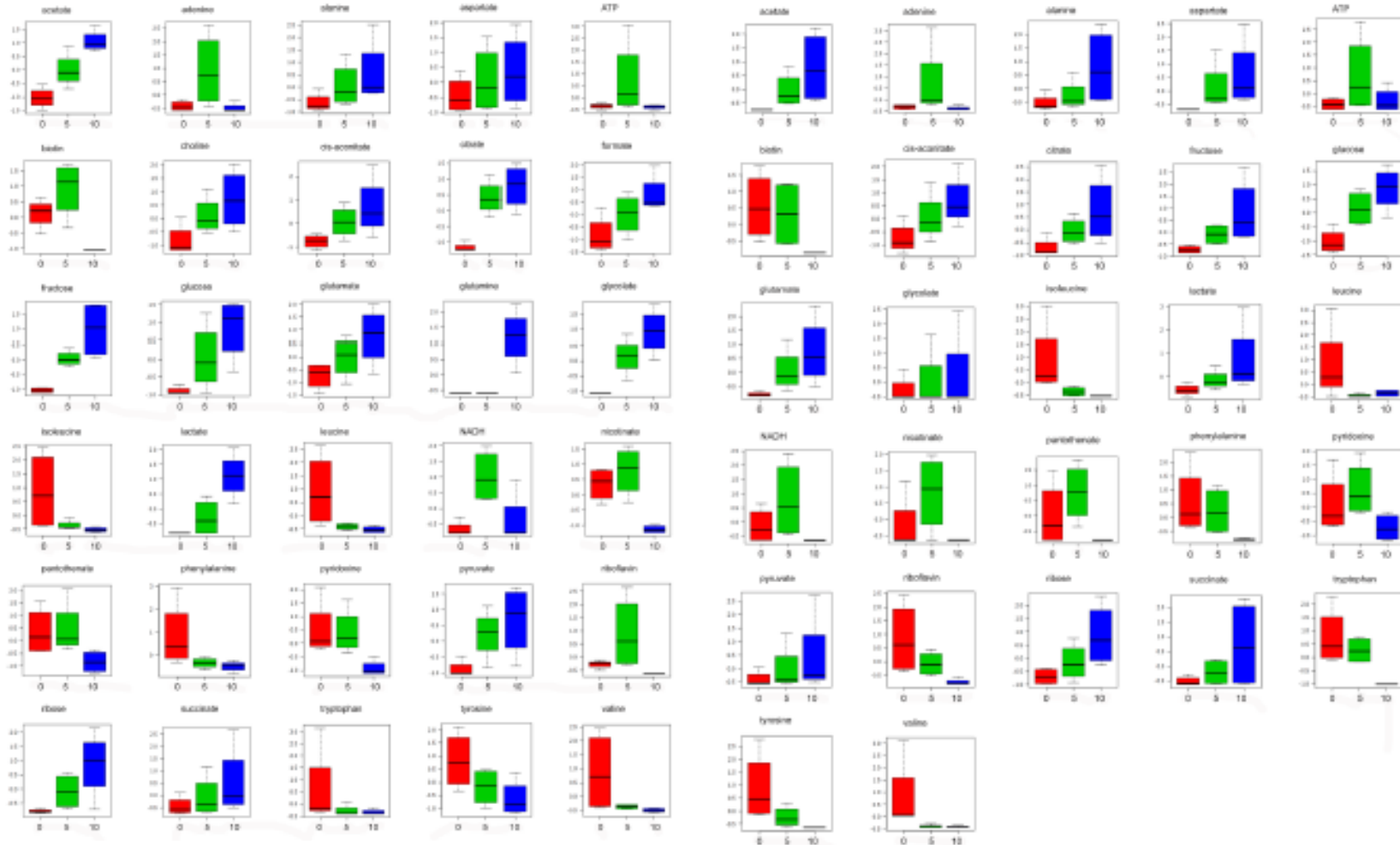


METABOANALYST 2.0 was used to analyse the data

The Data Normalisation



Concentration of Selected Metabolites



Pathway Analysis

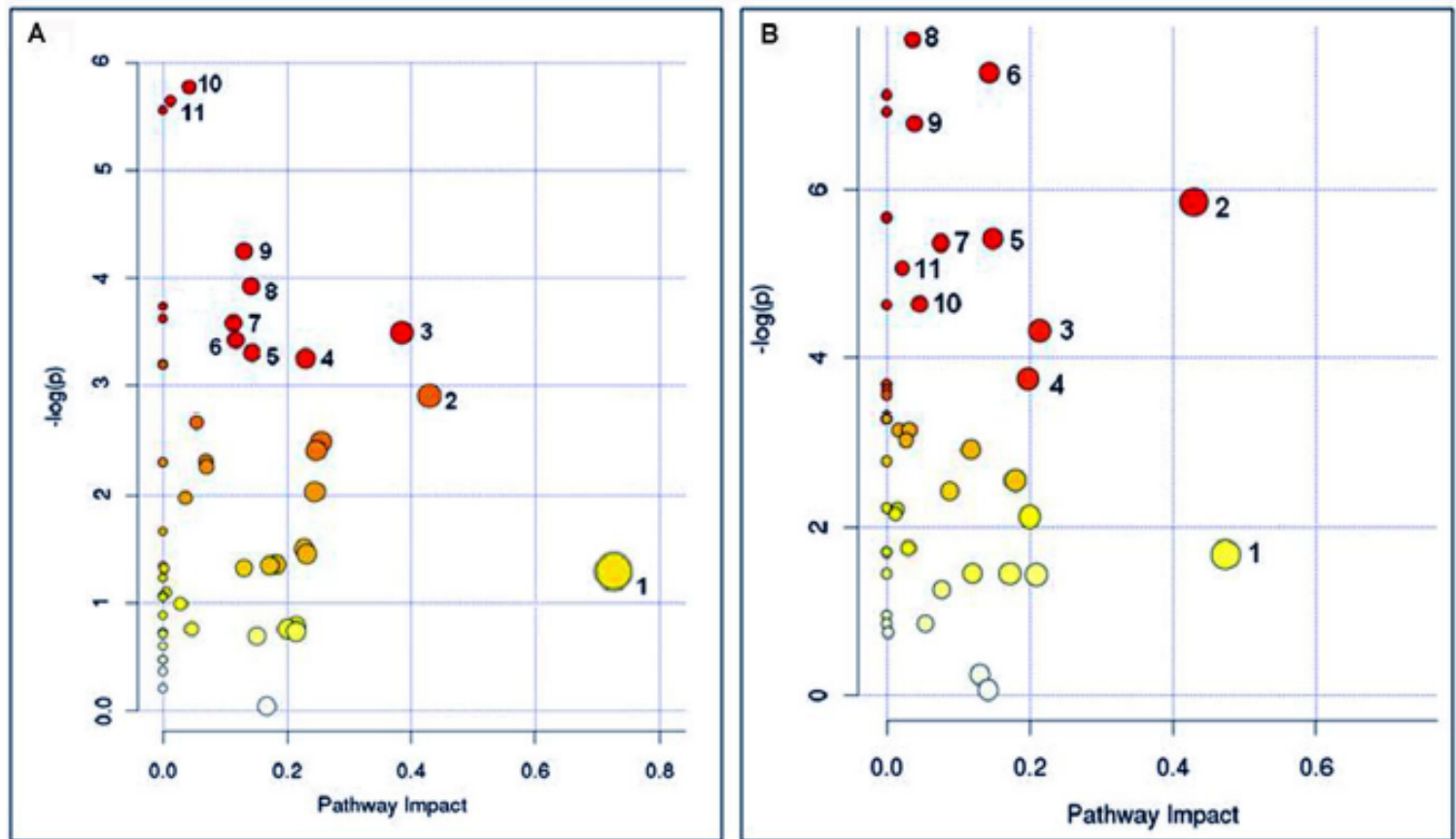
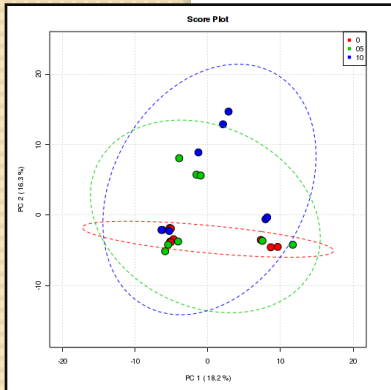


Figure 4.8 Summary of the pathway analysis for the salt-stress response of the cells grown in minimal medium (A) and complex medium (B). All the matched pathways are represented as circles. The color intensity and the radius of the circle indicate the p value and pathway impact value respectively. Key : 1. Alanine, aspartate and glutamate metabolism, 2. Pyruvate metabolism, 3. Tricarboxylic acid metabolism, 4. Glyoxylate metabolism, 5. Glycolysis metabolism, 6. Pentose phosphate pathway, 7. Valine, leucine and isoleucine metabolism, 8. Phenylalanine, tyrosine and tryptophan metabolism, 9. Pantothenate metabolism, 10. Biotin metabolism, 11. Glycerophospholipid, metabolism

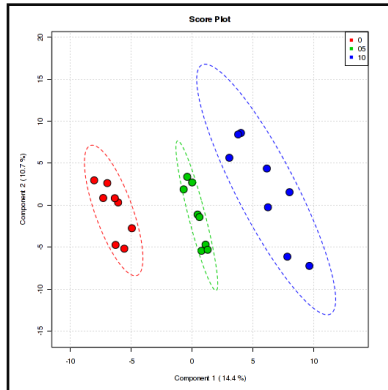
CHEMOMETRIC ANALYSIS

Cells grown in M9 medium supplemented with 0% (red), 5% (green) and 10% (blue) NaCl

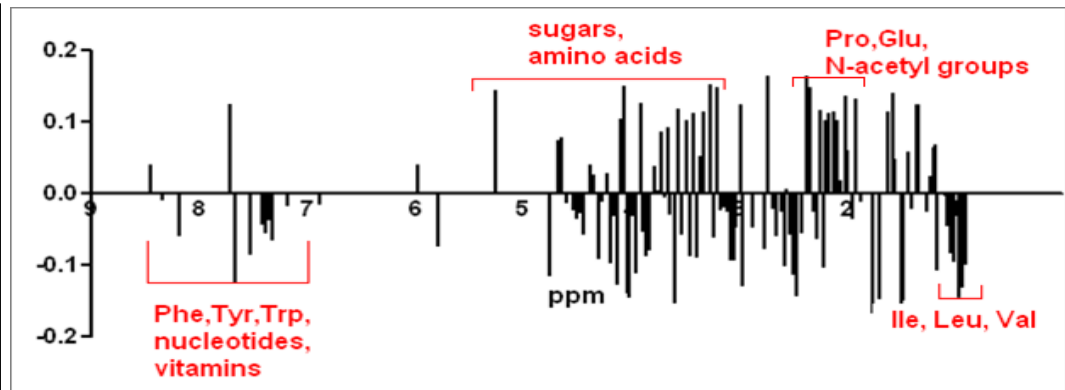
PCA score plot



PLS-DA score plot



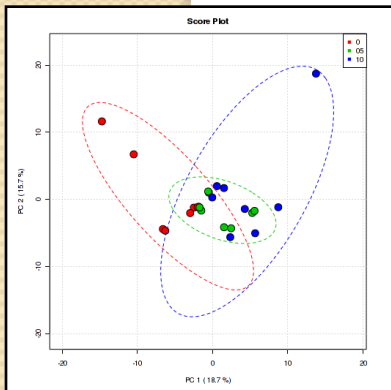
PLS-DA loadings



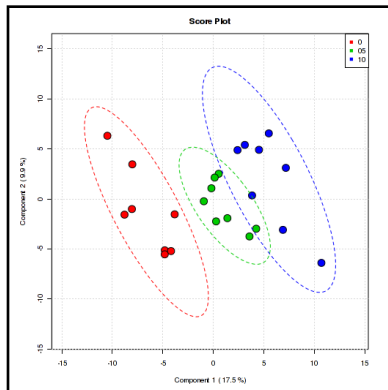
$p < 0.05$, $R^2 = 0.93$ and $Q^2 = 0.79$

Cells grown in LB medium supplemented with 0% (red), 5% (green) and 10% (blue) NaCl

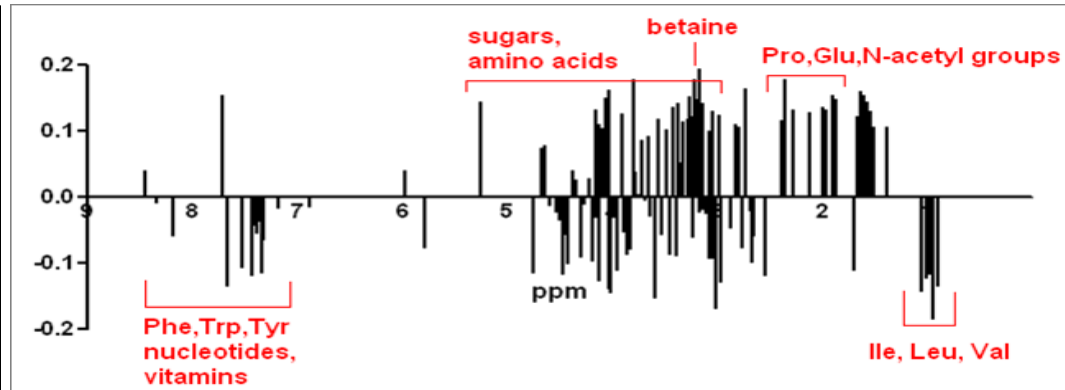
PCA score plot



PLS-DA score plot



PLS-DA loadings



$p < 0.05$, $R^2 = 0.82$ and $Q^2 = 0.65$

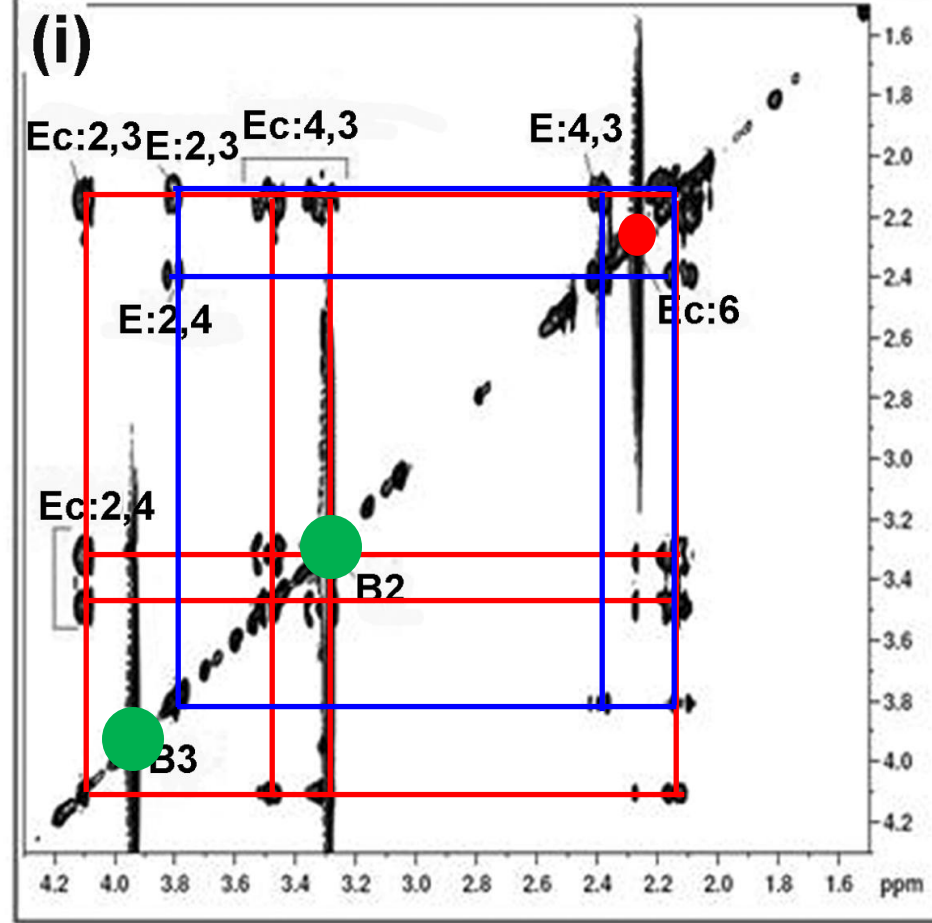
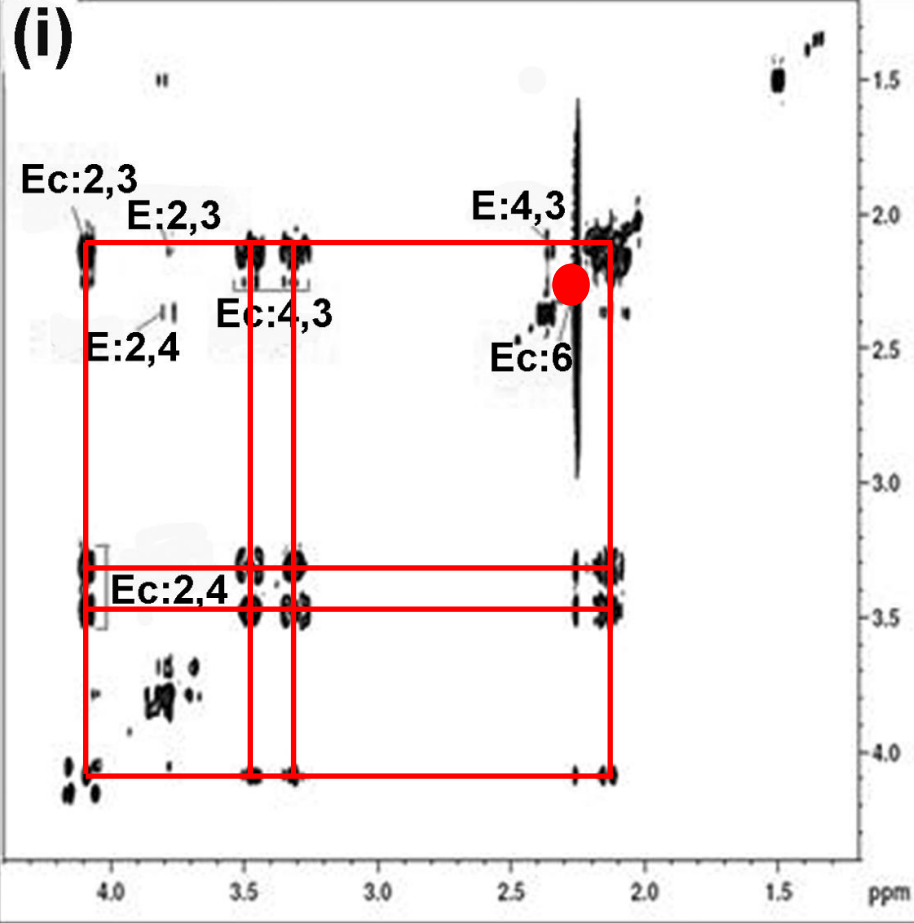
STRESS RESPONSIVE METABOLITES

Putative metabolites		Related pathway
Up-regulated		
Amino acids	Aspartate	Alanine, aspartate and glutamate metabolism
	Alanine	Alanine, aspartate and glutamate metabolism
	Glutamine	Alanine, aspartate and glutamate metabolism
Sugars	Glucose	Glycolysis metabolism, Pentose phosphate pathway
	Fructose	Glycolysis metabolism
	Ribose	Pentose phosphate pathway
Organic acids	Pyruvate	Pyruvate metabolism, Tricarboxylic acid metabolism, Glycolysis metabolism, Glyoxylate metabolism
	Citrate	Tricarboxylic acid metabolism, Glyoxylate metabolism
	Succinate	Tricarboxylic acid metabolism, Glyoxylate metabolism
	Cis-aconitate	Tricarboxylic acid metabolism, Glyoxylate metabolism
	Formate	Pyruvate metabolism, Glyoxylate metabolism
	Acetate	Pyruvate metabolism, Glycolysis metabolism
	Lactate	Pyruvate metabolism
	Glycolate	Glyoxylate metabolism
Down-regulated		
Amino acids	Phenyl alanine	Phenylalanine, tyrosine and tryptophan metabolism
	Tyrosine	Phenylalanine, tyrosine and tryptophan metabolism
	Tryptophan	Phenylalanine, tyrosine and tryptophan metabolism
	Valine	Valine, leucine and isoleucine metabolism
	Isoleucine	Valine, leucine and isoleucine metabolism
	Leucine	Valine, leucine and isoleucine metabolism
Vitamins	Pantothenate	Pantothenate metabolism
	Biotin	Biotin metabolism

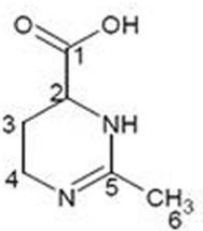
TOCSY SPECTRUM OF *Halomonas sp.* VITP9

M9

LB

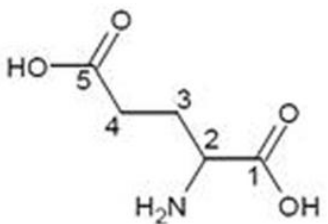


Ectoine (Ec)

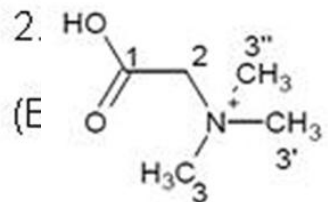


2.0 (C3), 2.2 (C4), 3.2 (C4), 3.4 (C5)
(Ceylan et al.)

L-glutamate (E)



Glycine betaine (B)

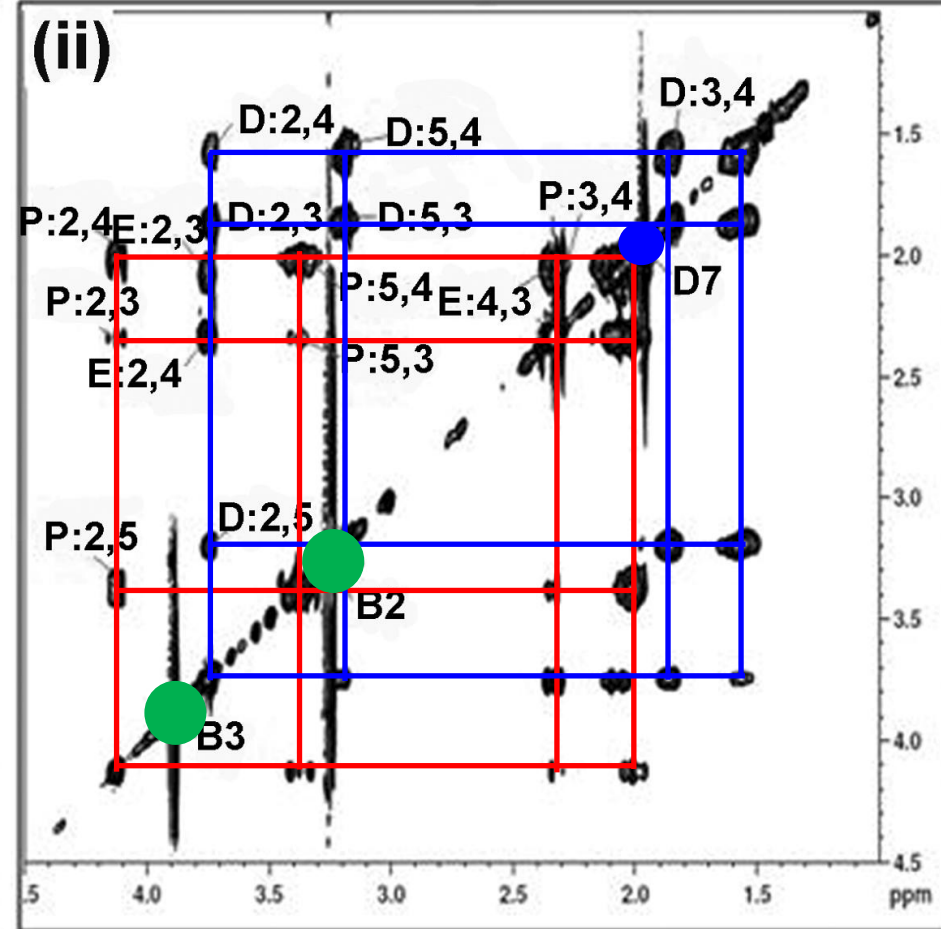
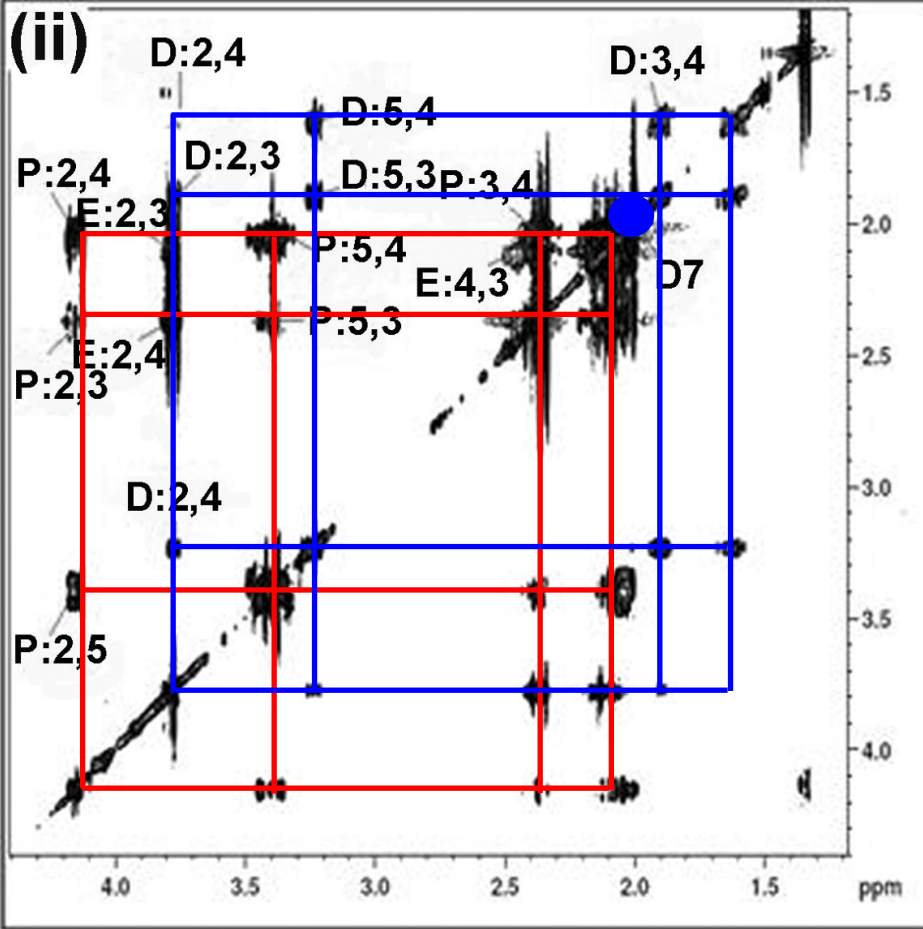


3.2 (C2), 3.8 (C3)
(BMRB database)

TOCSY SPECTRUM OF *Bacillus sp.* VITP4

M9

LB

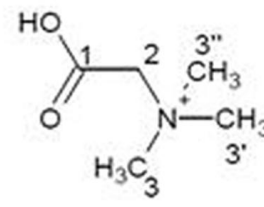
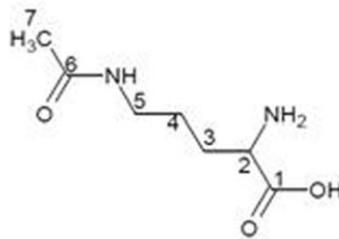
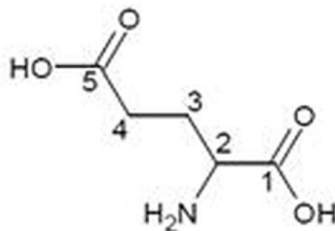
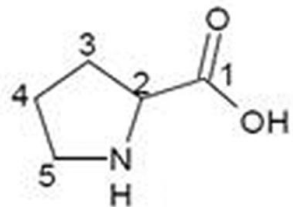


L-Proline (P)

L-glutamate (E)

N δ -acetyl ornithine (D)

Glycine betaine (B)



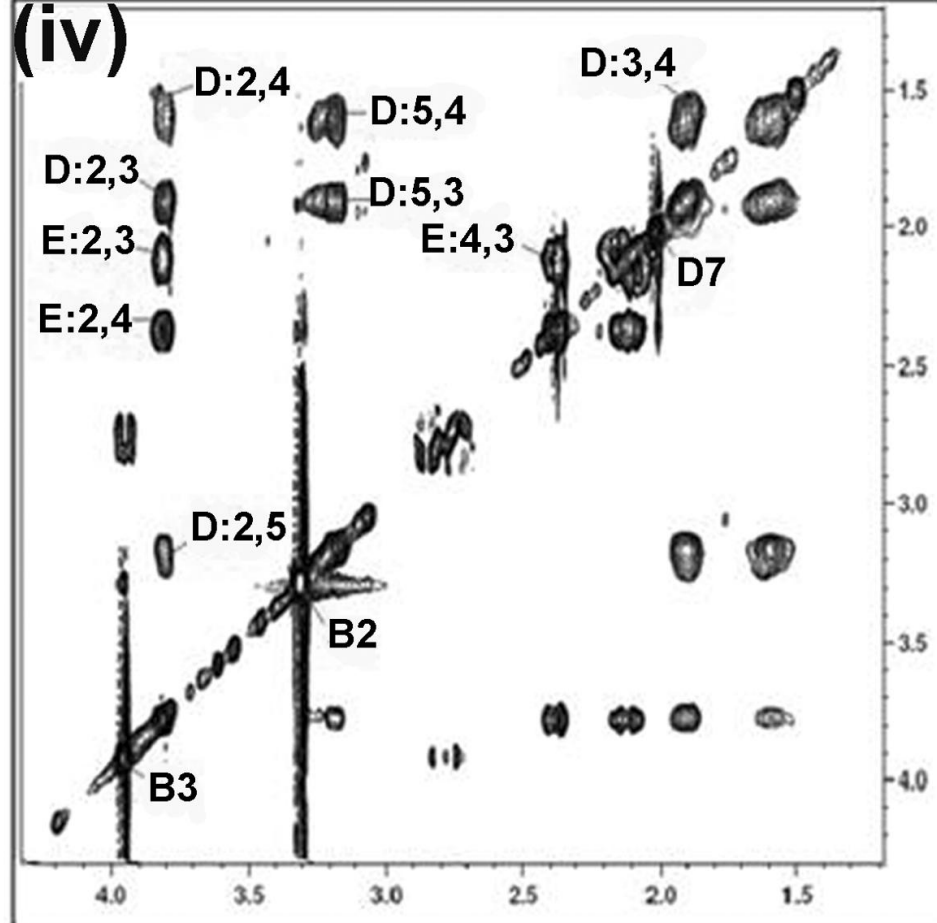
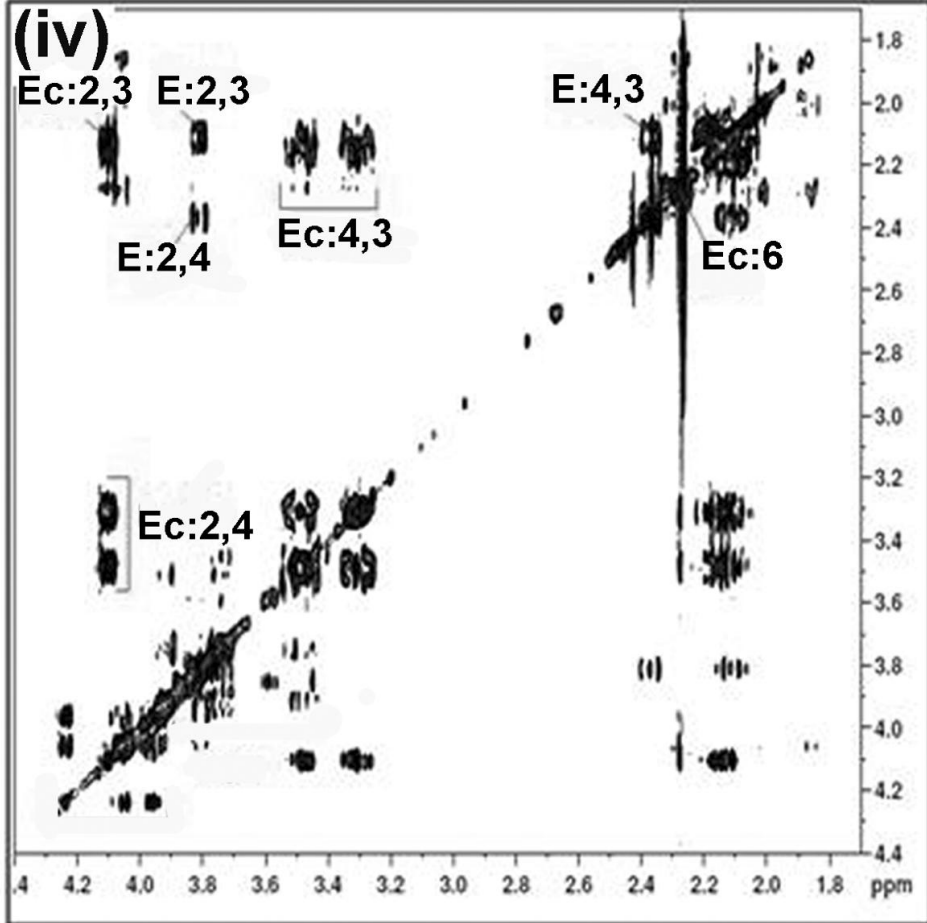
3.2 (C2), 3.8 (C3)

(BMRB database)

TOCSY SPECTRUM OF *Virgibacillus* sp. VITP14

M9

LB

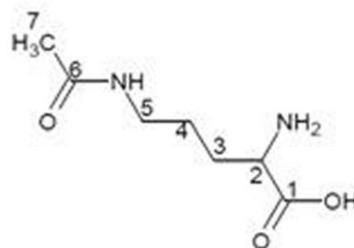
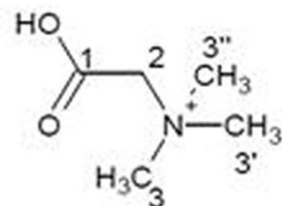
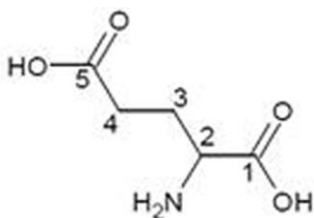
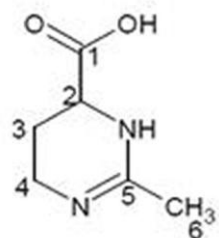


Ectoine (Ec)

L-glutamate (E)

Glycine betaine (B)

N δ -acetyl ornithine (D)



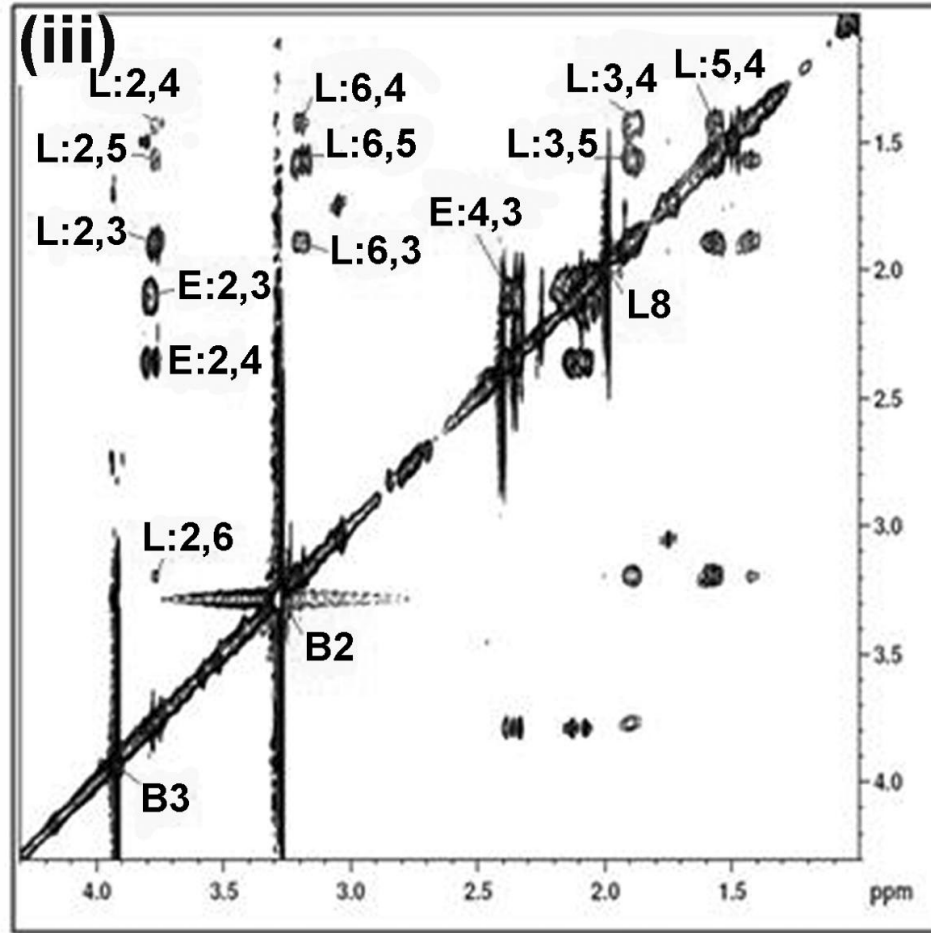
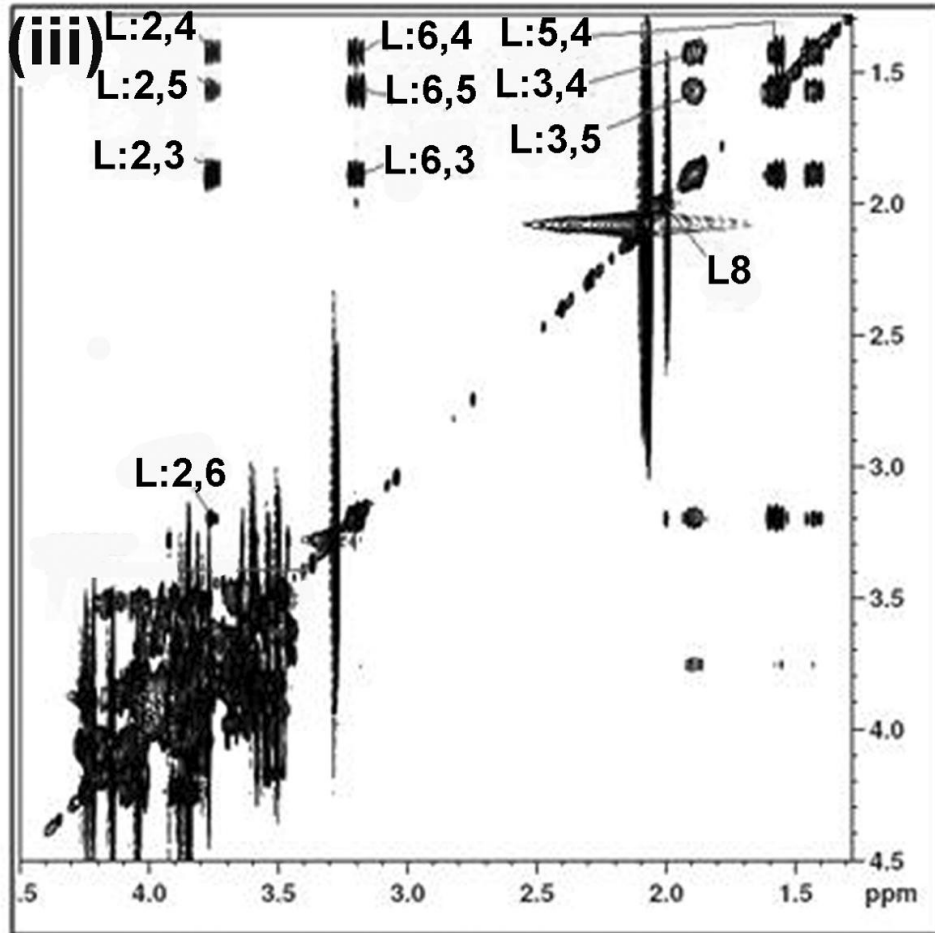
1.5 (C4), 1.7 (C3),
1.85 (C7), 3.1 (C5), 3.6 (C2)

(Adio et al 2011)

TOCSY SPECTRUM OF *Planococcus sp.* VITP21

M9

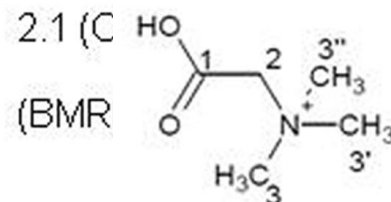
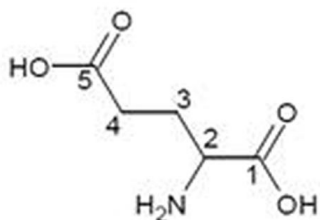
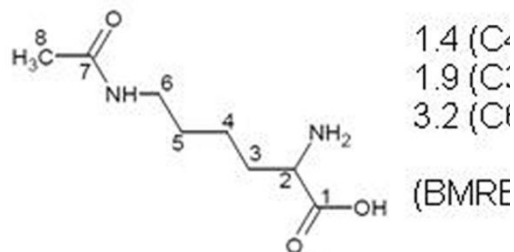
LB



Nε-acetyl L-lysine (L)

L-glutamate (E)

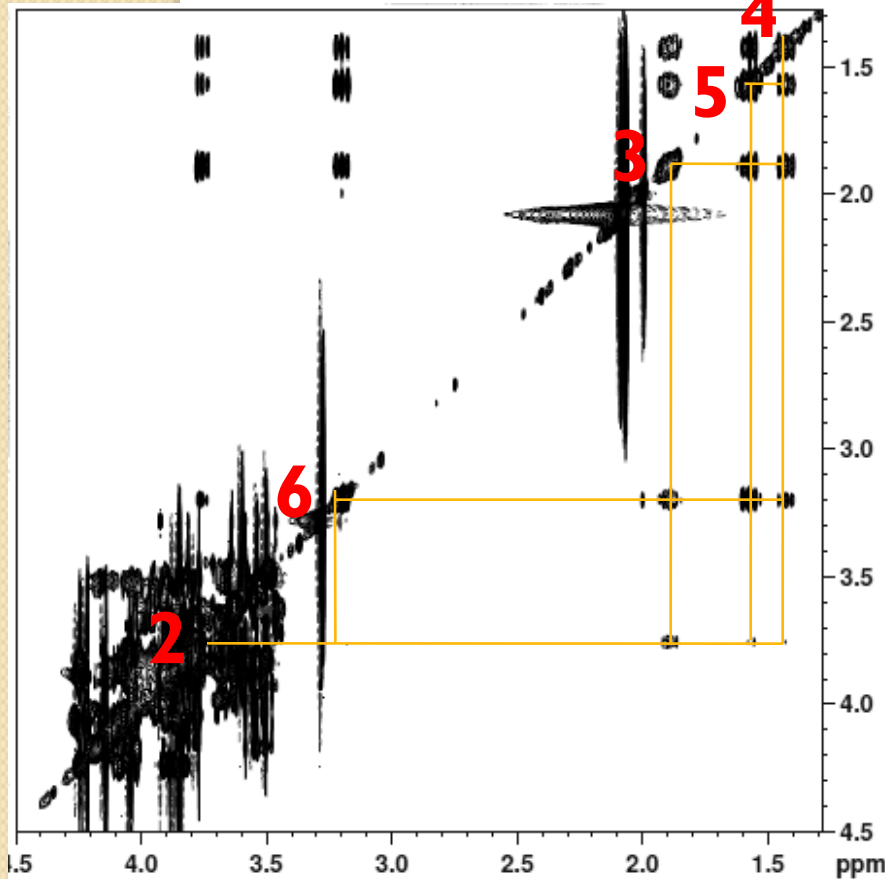
Glycine betaine (B)



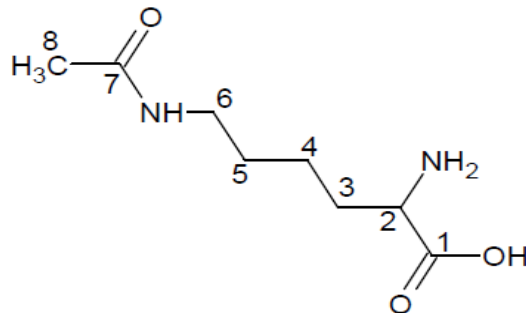
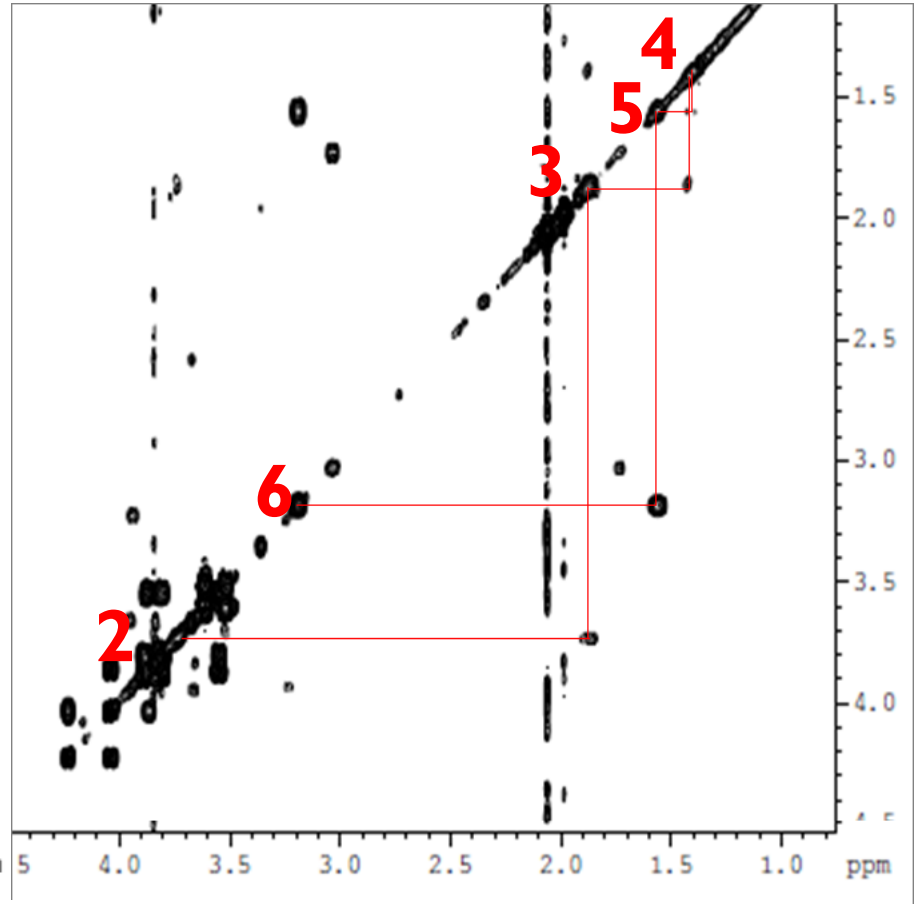
3.2 (C₂), 3.8 (C₃)
(BMRB database)

N ϵ -acetyl lysine

TOCSY



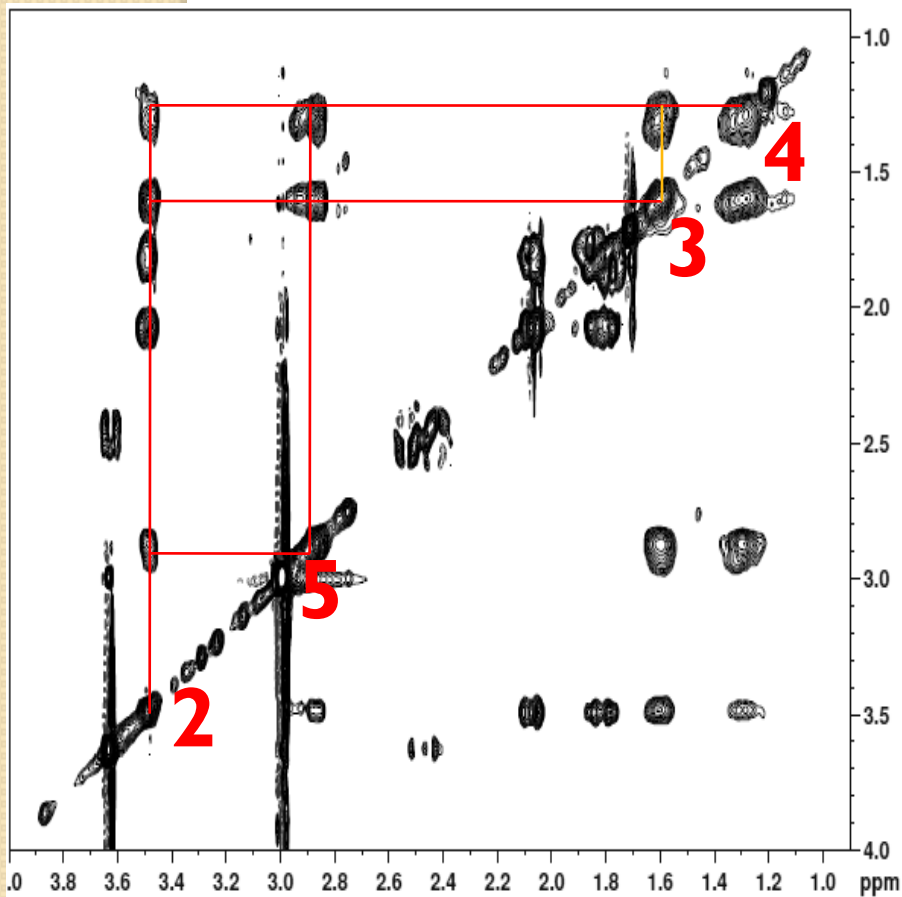
COSY



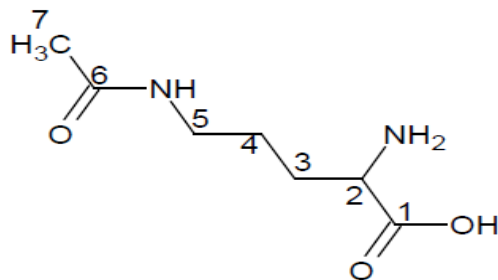
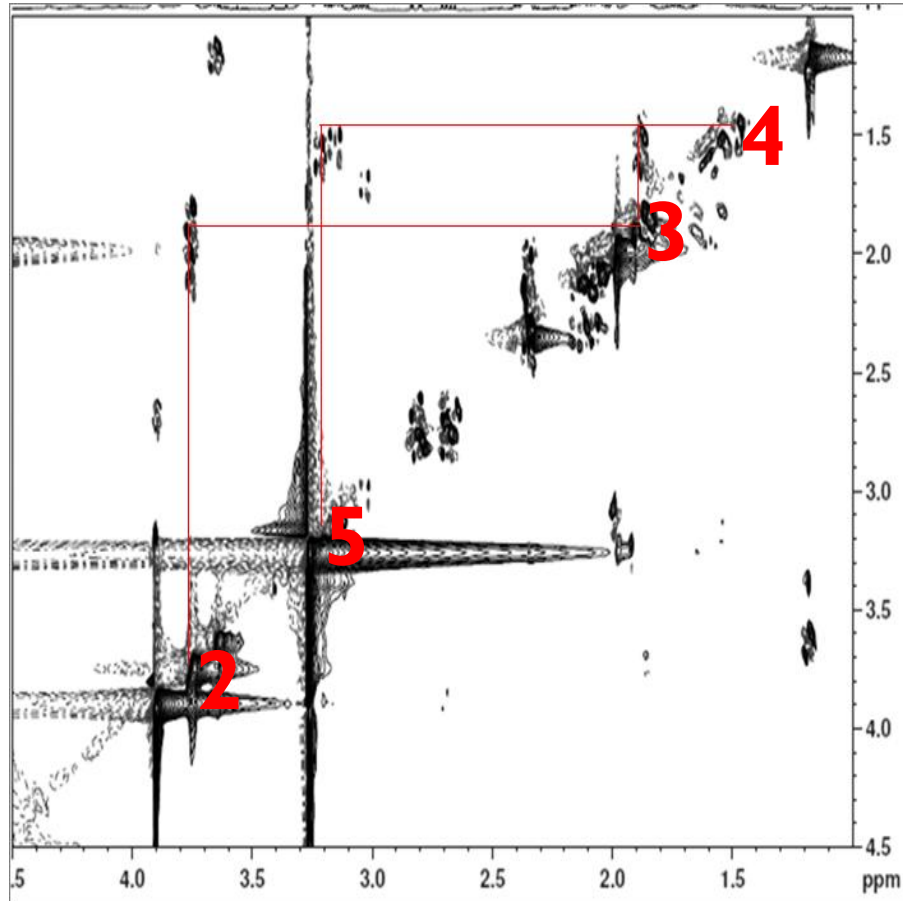
N ϵ -acetyl lysine

N δ -acetyl ornithine

TOCSY



COSY

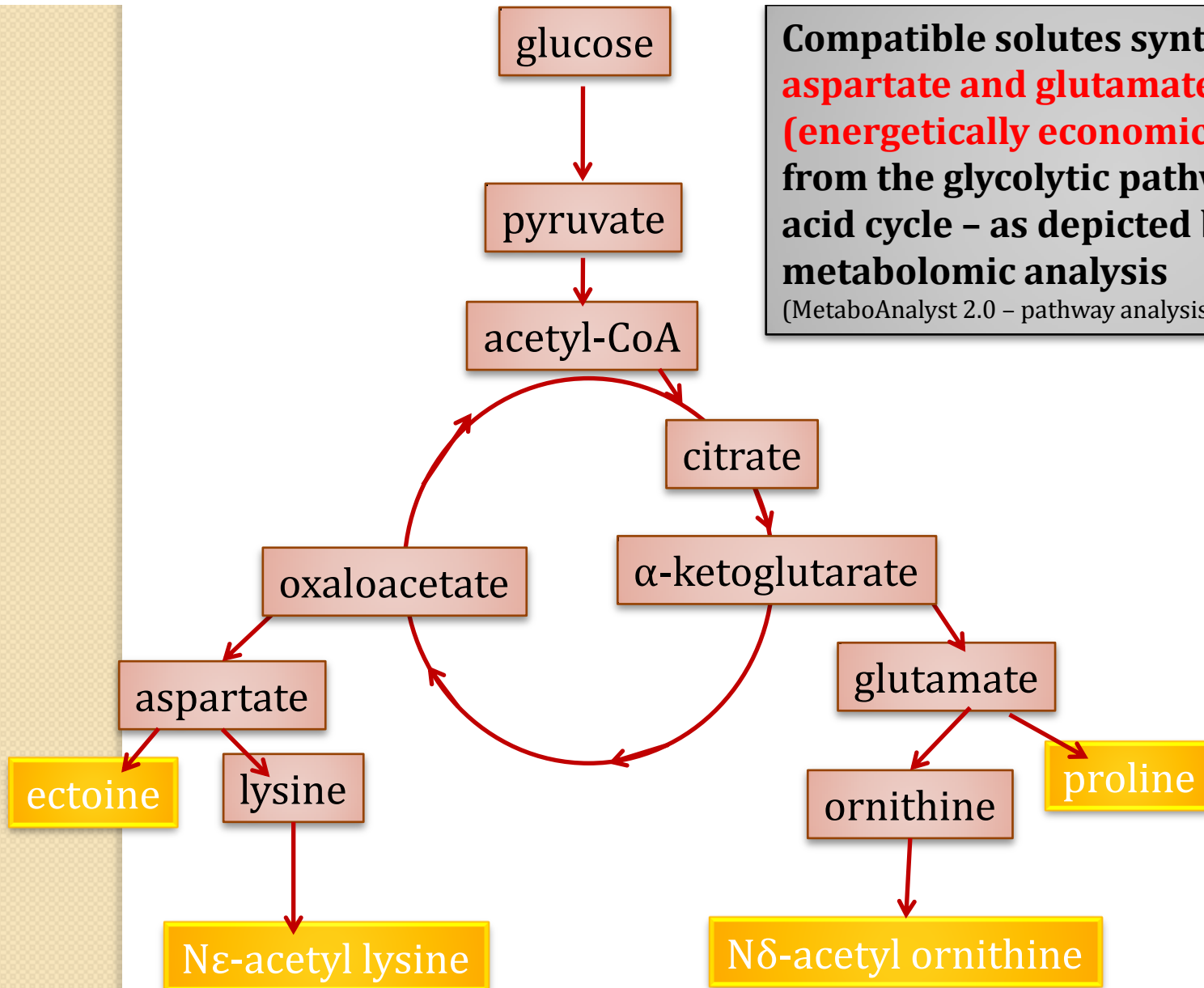


N δ -acetyl ornithine

DISTRIBUTION OF OSMOLYTES IDENTIFIED WITHIN THE STRAINS

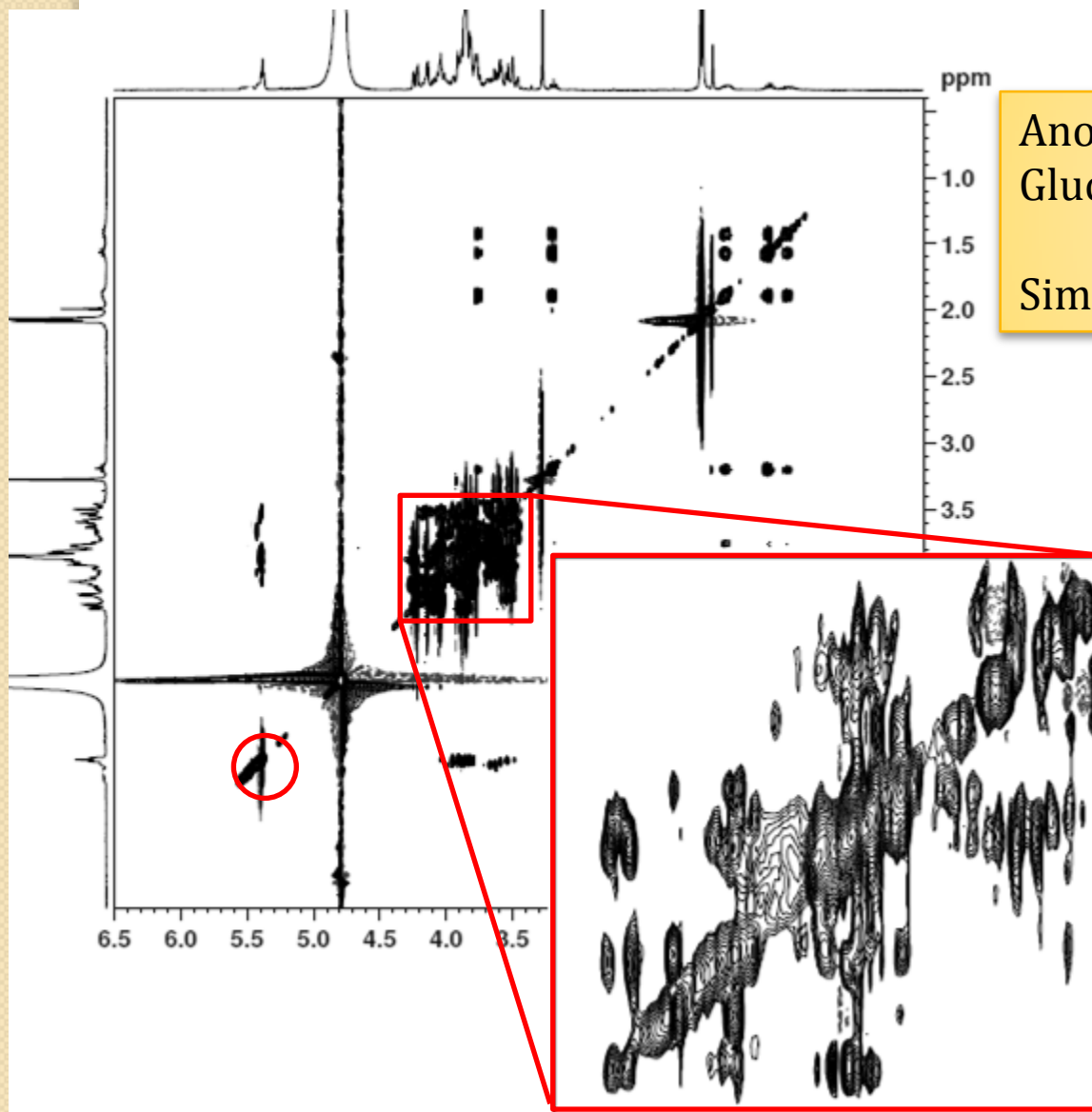
Bacterial strain	Minimal medium	At 0% w/v NaCl (mM)	At 5% w/v NaCl (mM)	At 10% w/v NaCl (mM)	Complex medium	At 0% w/v NaCl (mM)	At 5% w/v NaCl (mM)	At 10% w/v NaCl (mM)
<i>Halomonas sp. VITP9</i>					Glycine betaine	0	100.36±1.	137.52±3.
	Ectoine	0	41.56±2.1	96.95±2.7	Ectoine	0	31.56±1.5	83.50±2.8
	Glutamate	0.88±0.2	26.51±2.1	54.62±6.2	Glutamate	1.95±0.1	8.60±0.9	14.90±0.5
	Aspartate	0	0.13±0.0	0.21±0.0	Aspartate	0.24±0.0	0.39±0.0	0.48±0.1
<i>Bacillus sp. VITP4</i>					Glycine betaine	0.33±0.3	65.57±2.2	83.75±1.7
	Proline	1.34±0.4	45.61±6.6	112.07±4.1	Proline	0	36.18±2.5	60.80±1.
	Nδ-acetyl ornithine	0.40±0.0	4.16±1.1	24.70±4.6	Nδ-acetyl ornithine	0.62±0.1	28.20±1.7	36.45±0.7
	Ornithine	0.13±0.0	0.23±0.0	0.31±0.0	Ornithine	0	3.23±0.0	5.32±0.0
	Glutamate	1.34±0.0	66.01±1.4	98.59±1.9	Glutamate	18.64±2.3	36.62±2.3	44.71±0.4
	Aspartate	0	0.19±0.0	0.42±0.1	Aspartate	0	0.89±0.1	3.65±0.1
<i>Virgibacillus sp. VITP14</i>	Ectoine	0	32.95±7.1	69.44±7.8	Glycine betaine	2.32±0.5	62.91±4.3	112.44±0.
					Nδ-acetyl ornithine	2.25±0.3	6.25±1.5	12.79±2.5
					Ornithine	0.02±0.0	3.45±0.0	7.18±0.2
	Glutamate	7.35±2.0	26.85±1.3	64.65±4.7	Glutamate	11.62±0.5	21.37±1.9	37.42±2.2
	Aspartate	0.04±0.0	0.10±0.0	0.145±0.0	Aspartate	4.11±0.6	8.38±0.8	10.89±1.0
<i>Planococcus sp. VITP21</i>					Glycine betaine	2.07±0.8	78.80±2.5	113.32±0.
					Proline	0	4.36±0.9	36.05±1.4
	Nε-acetyl lysine	3.45±0.1	45.12±1.5	107.49±2.1	Nε-acetyl lysine	3.05±0.2	11.60±1.9	31.29±1.0
	Lysine	0.53±0.0	4.46±0.1	8.53±1.0	Lysine	3.04±0.1	5.34±0.5	10.32±0.5
	Glutamate	1.30±0.1	13.40±0.5	27.10±2.5	Glutamate	9.62±2.2	21.37±1.9	36.92±1.5
	Aspartate	0.76±0.5	1.38±0.4	2.86±0.2	Aspartate	5.91±0.0	12.81±0.0	15.94±0.1

METABOLIC PATHWAY ANALYSIS – SYNTHESIS OF NITROGENOUS OSMOLYTES



Compatible solutes synthesized from **aspartate and glutamate family (energetically economical)**, directed from the glycolytic pathway and citric acid cycle – as depicted by the **metabolomic analysis**
(MetaboAnalyst 2.0 – pathway analysis)

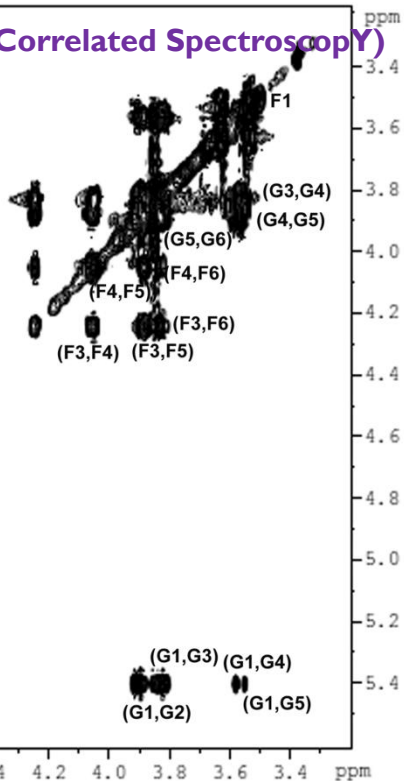
UNKNOWN SUGAR-OSMOLYTE FROM *Planococcus* sp. VITP21



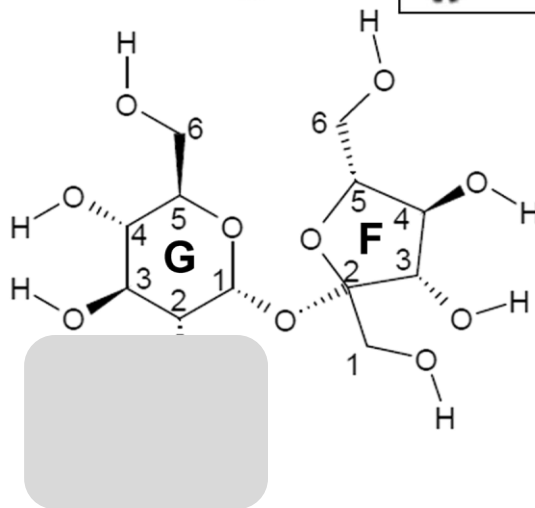
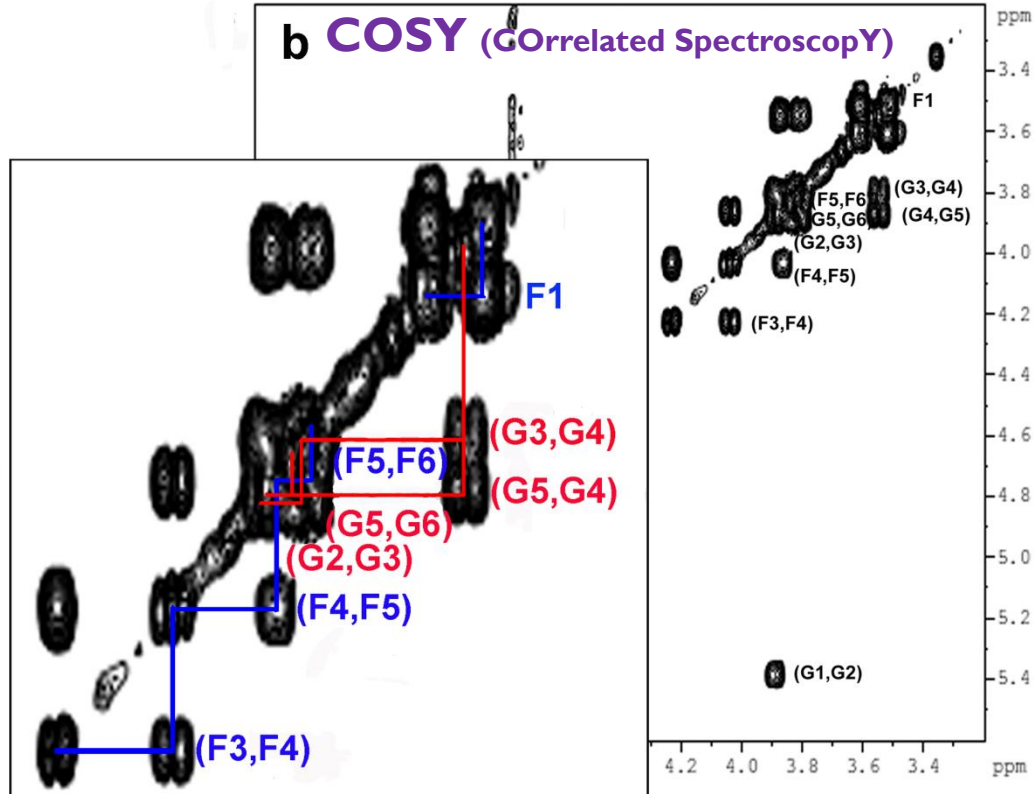
Anomeric signal \approx 5.3 ppm
Glucose and Fructose moieties
Similarity with sucrose

HOMONUCLEAR 2D NMR

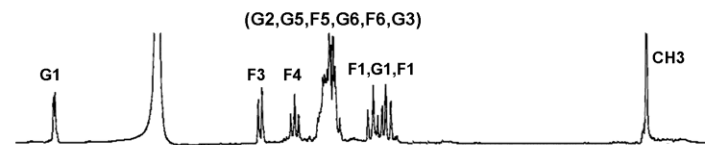
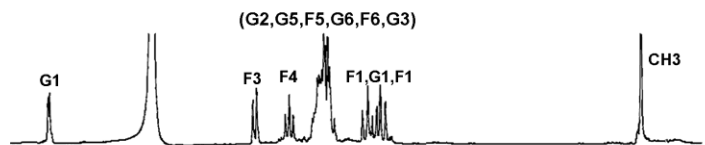
a TOCSY (Total Correlated Spectroscopy)



b COSY (Correlated Spectroscopy)

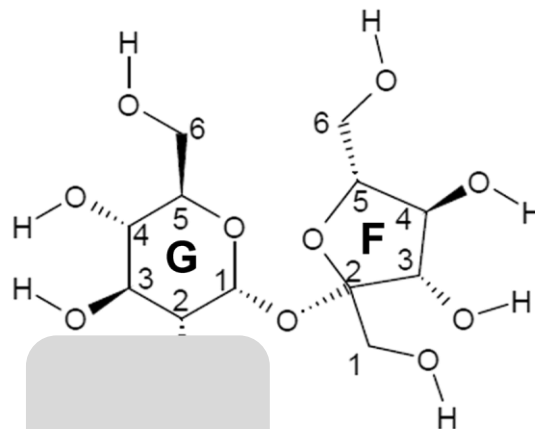
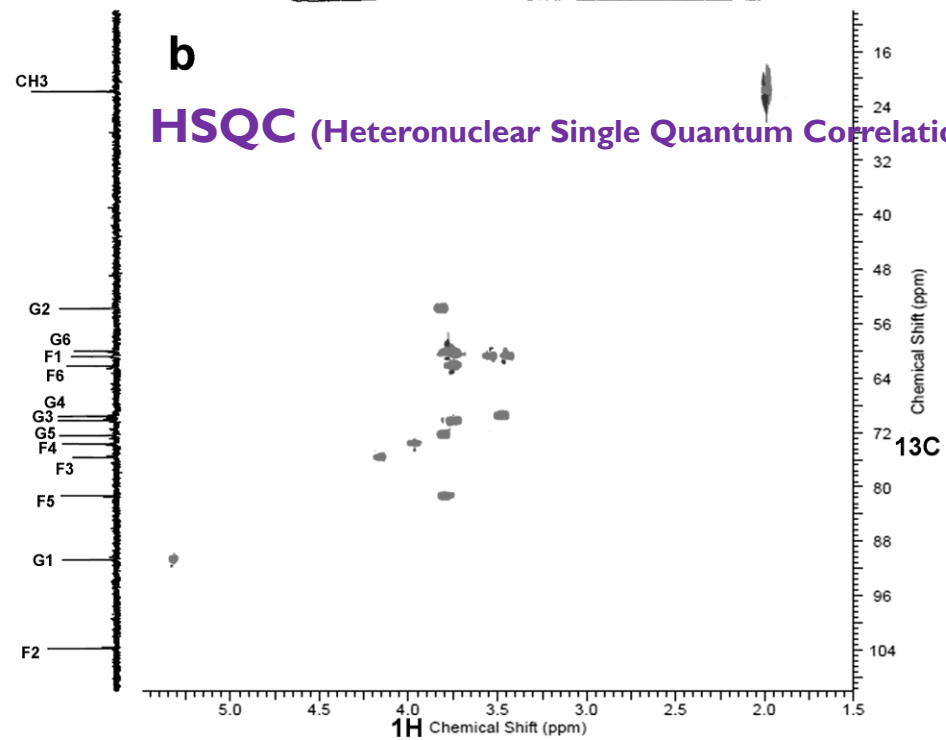
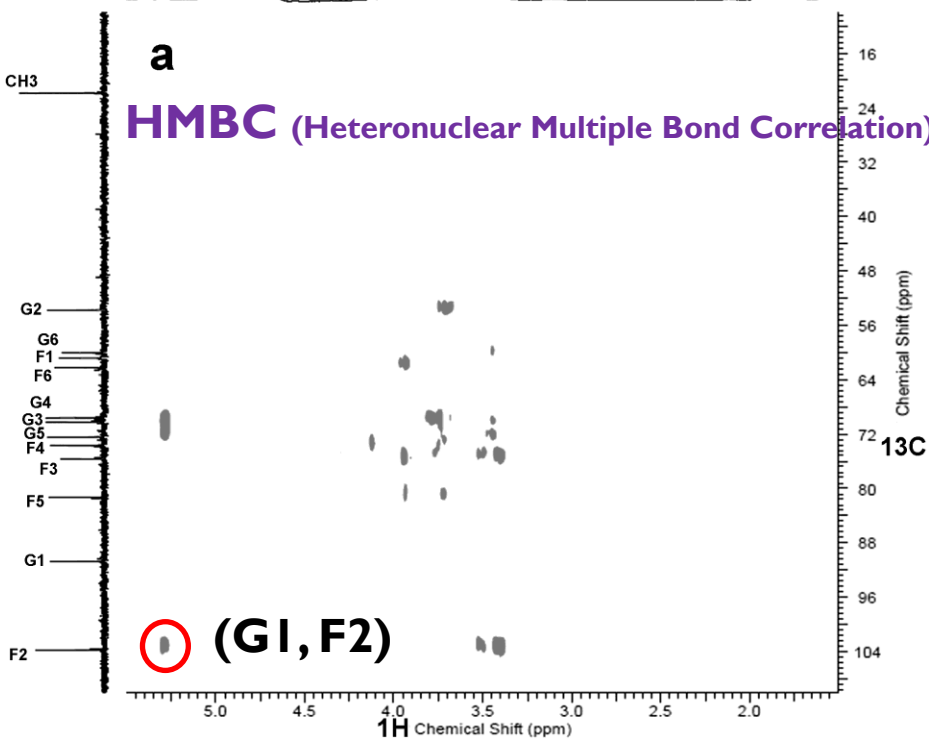


HETERONUCLEAR 2D NMR



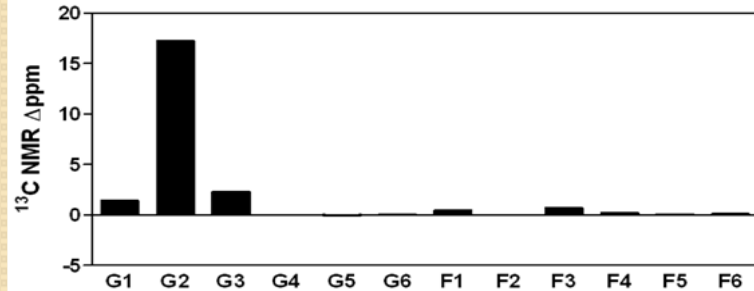
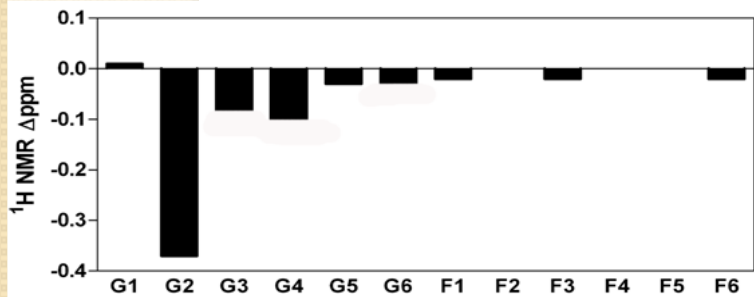
a
HMBC (Heteronuclear Multiple Bond Correlation)

b
HSQC (Heteronuclear Single Quantum Correlation)

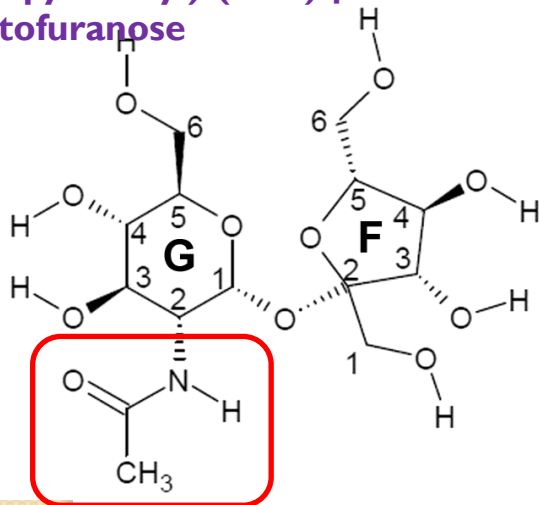


STRUCTURE ELUCIDATION OF THE UNKNOWN SUGAR-OSMOLYTE

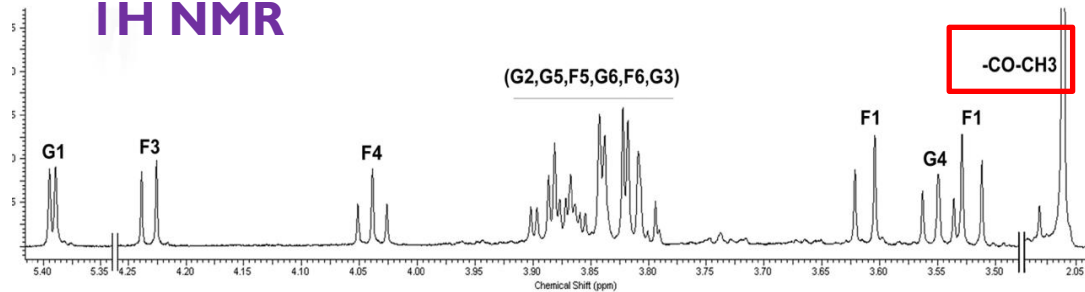
Differences in ppm values between sucrose and unknown sugar-osmolyte



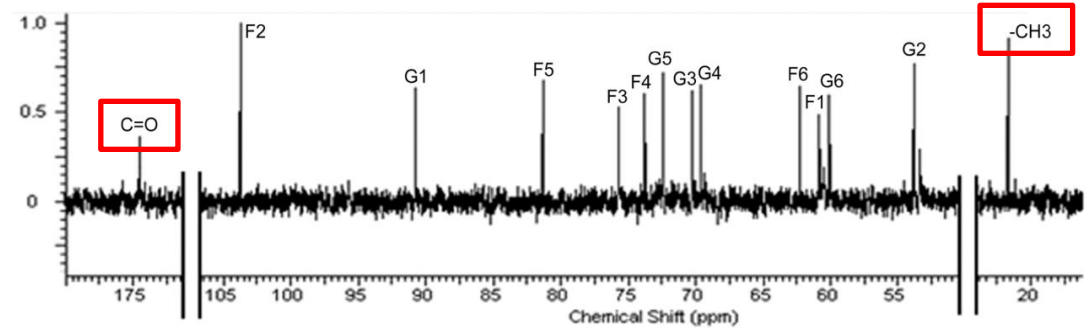
(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 \rightarrow 2)- β -D-fructofuranose



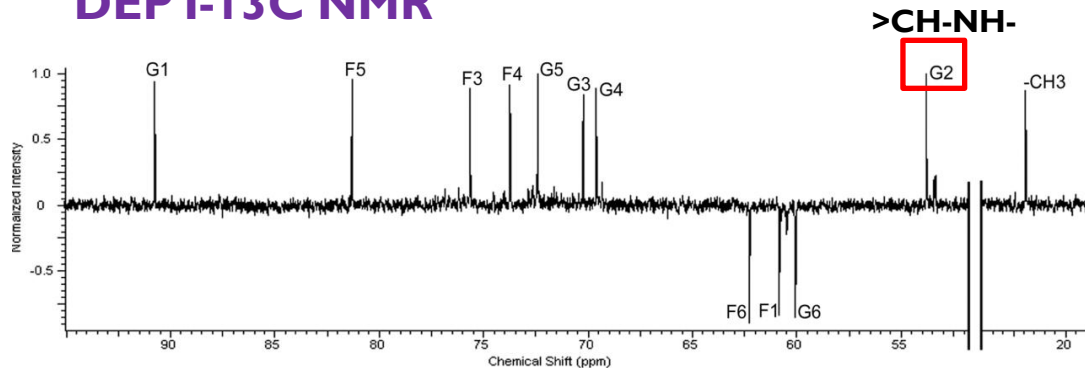
$^1\text{H NMR}$



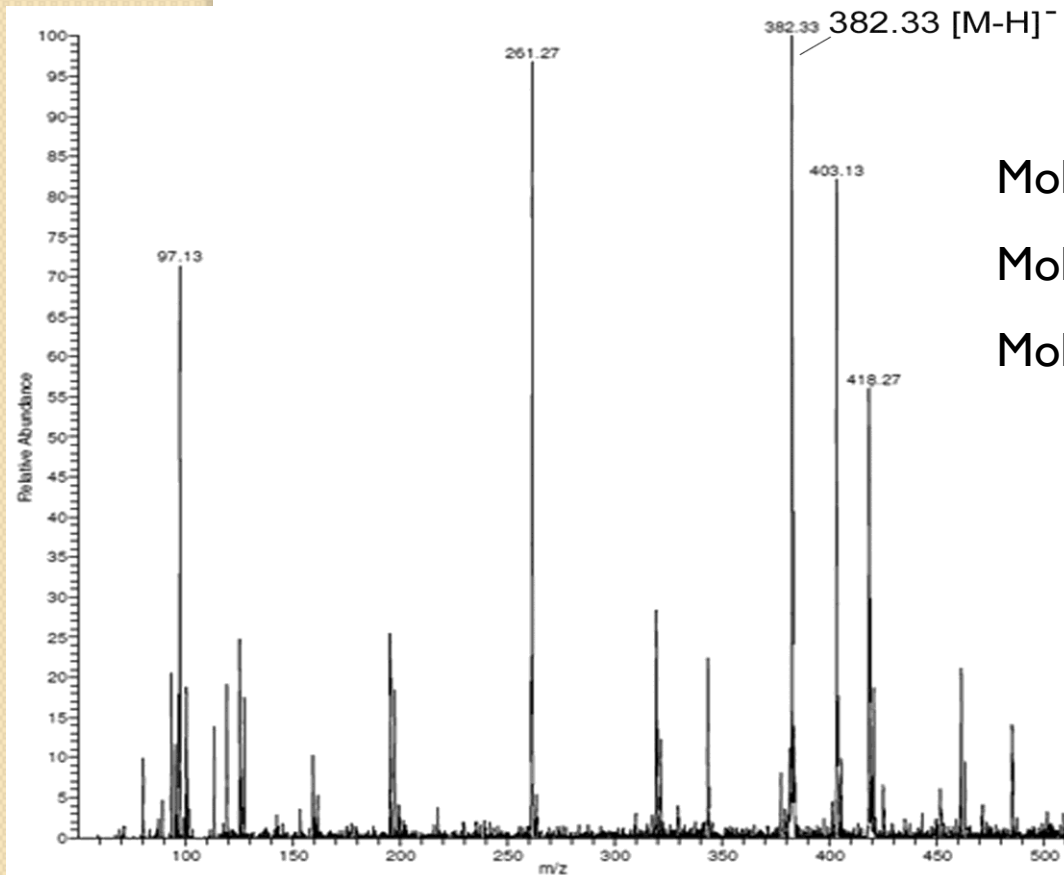
$^{13}\text{C NMR}$



DEPT- $^{13}\text{C NMR}$



SUPPORTING INFORMATION – ESI-MS

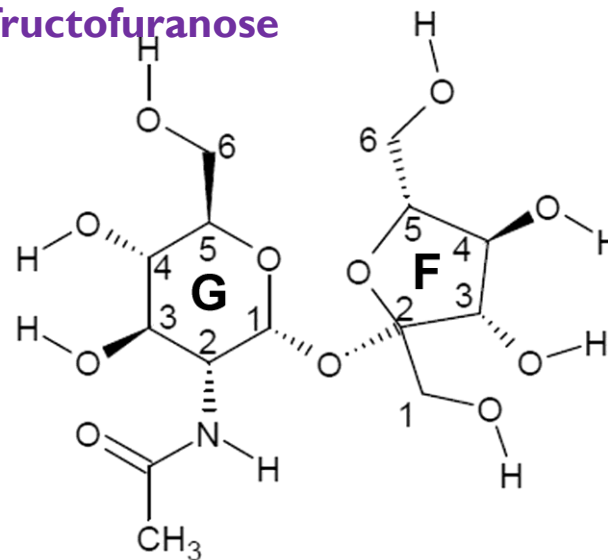


Molecular formula : **C₁₄H₂₅O₁₁N₁**

Molecular weight : **383.35**

Molecular ion [M-H]⁻ : **382.34 (theoretical)**
: **382.33 (experimental)**

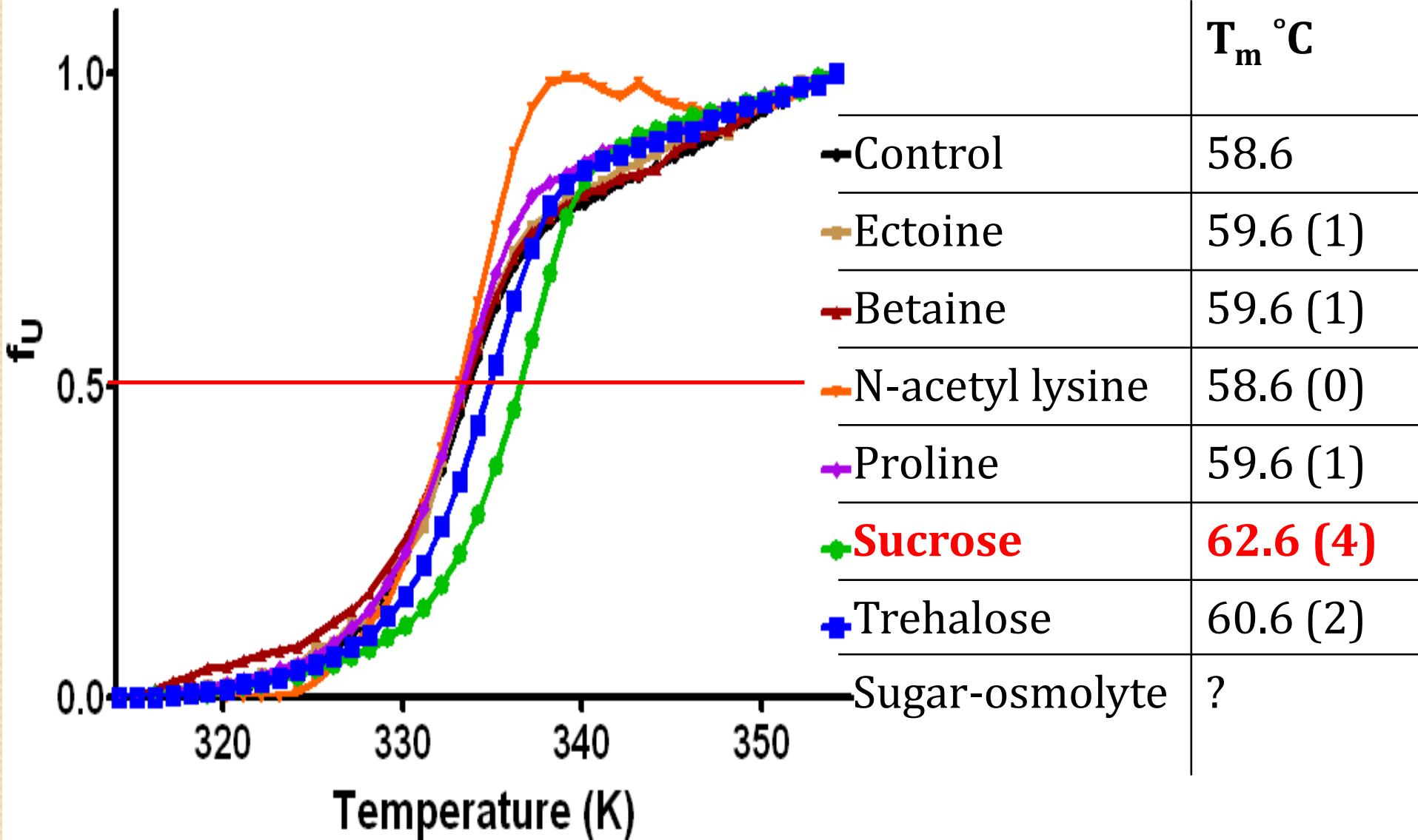
(2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1- \rightarrow 2)- β -D-fructofuranose



This sugar-osmolyte has not been previously reported for its natural synthesis.

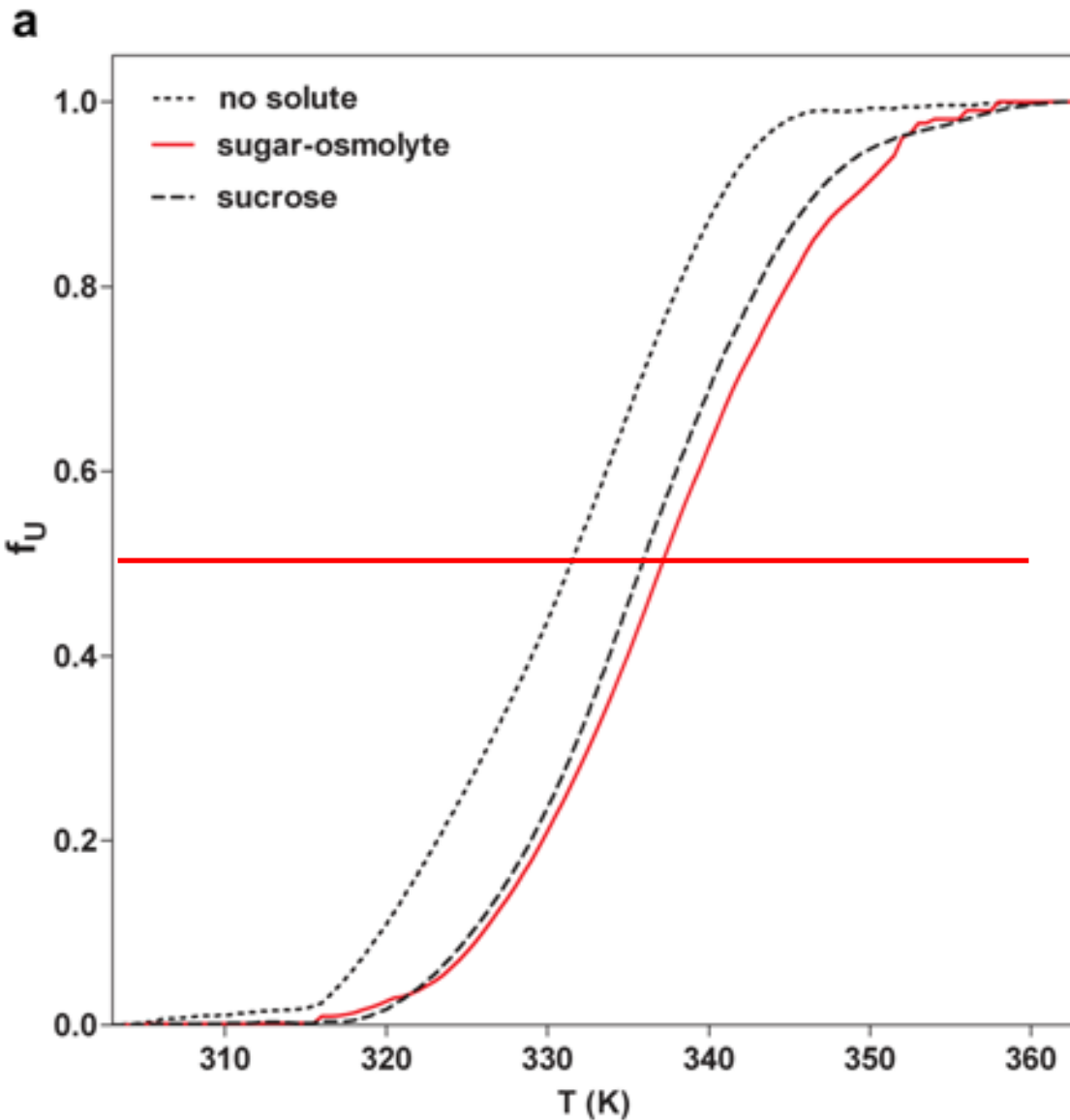
EFFECT OF THE SOLUTES ON PROTEIN STRUCTURAL STABILITY – FLUORESCENCE

At pH 7



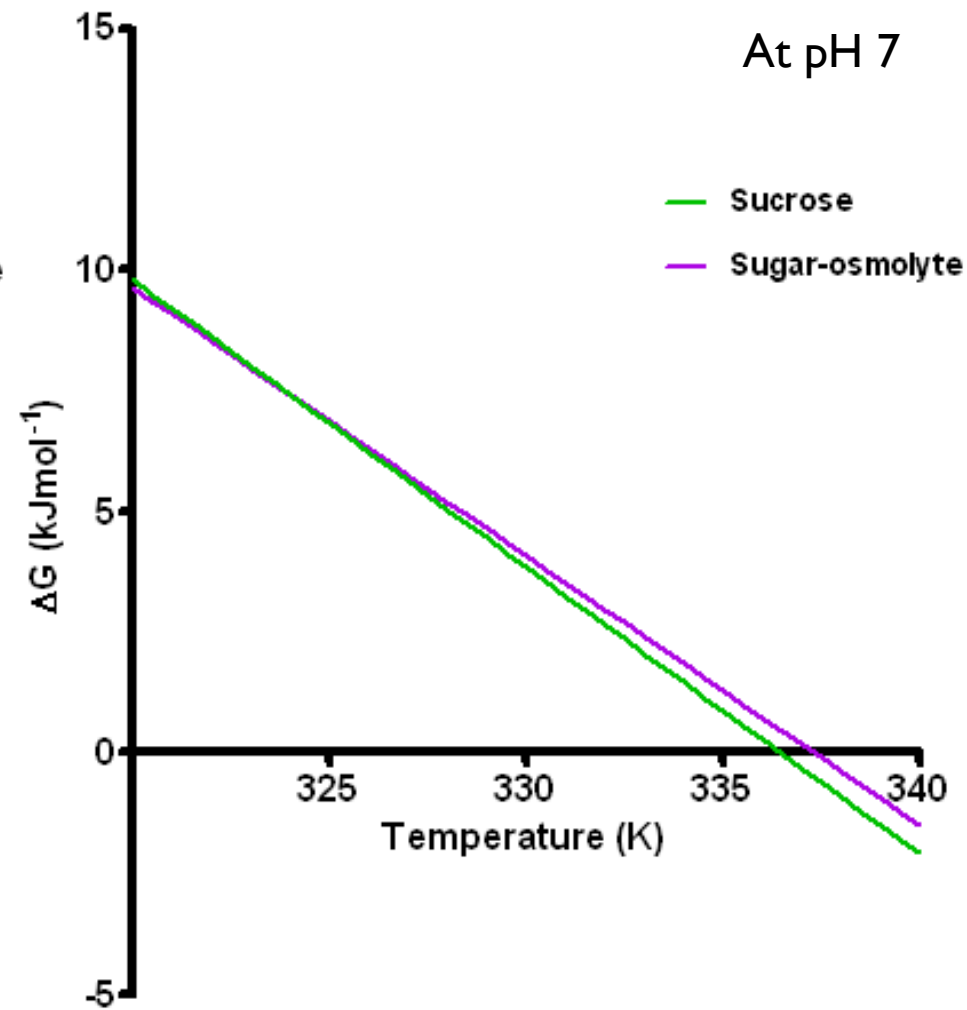
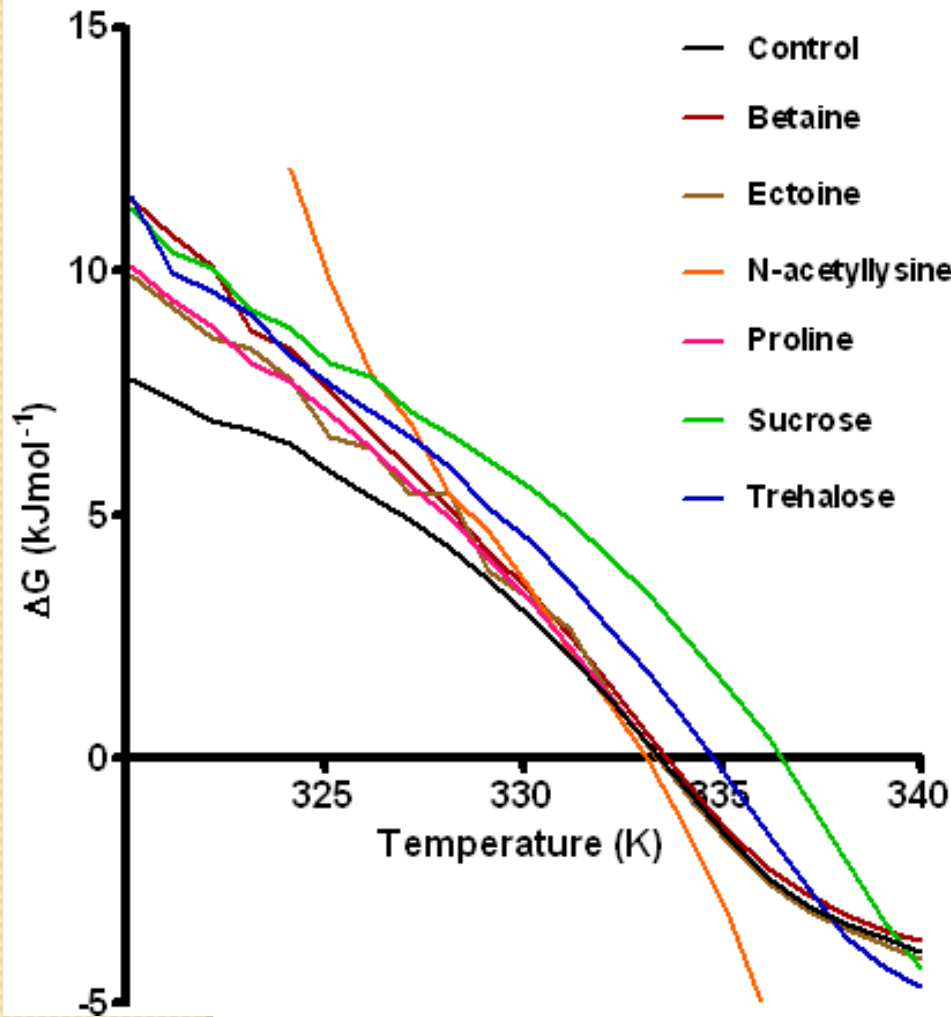
EFFECT OF THE SUGAR-OSMOLYTE ON PROTEIN STRUCTURAL STABILITY – CIRCULAR DICHROISM

At pH 7



	T_m °C
Control	57.8
Sucrose	61.4 (3.6)
Sugar-osmolyte	62.5 (4.7)

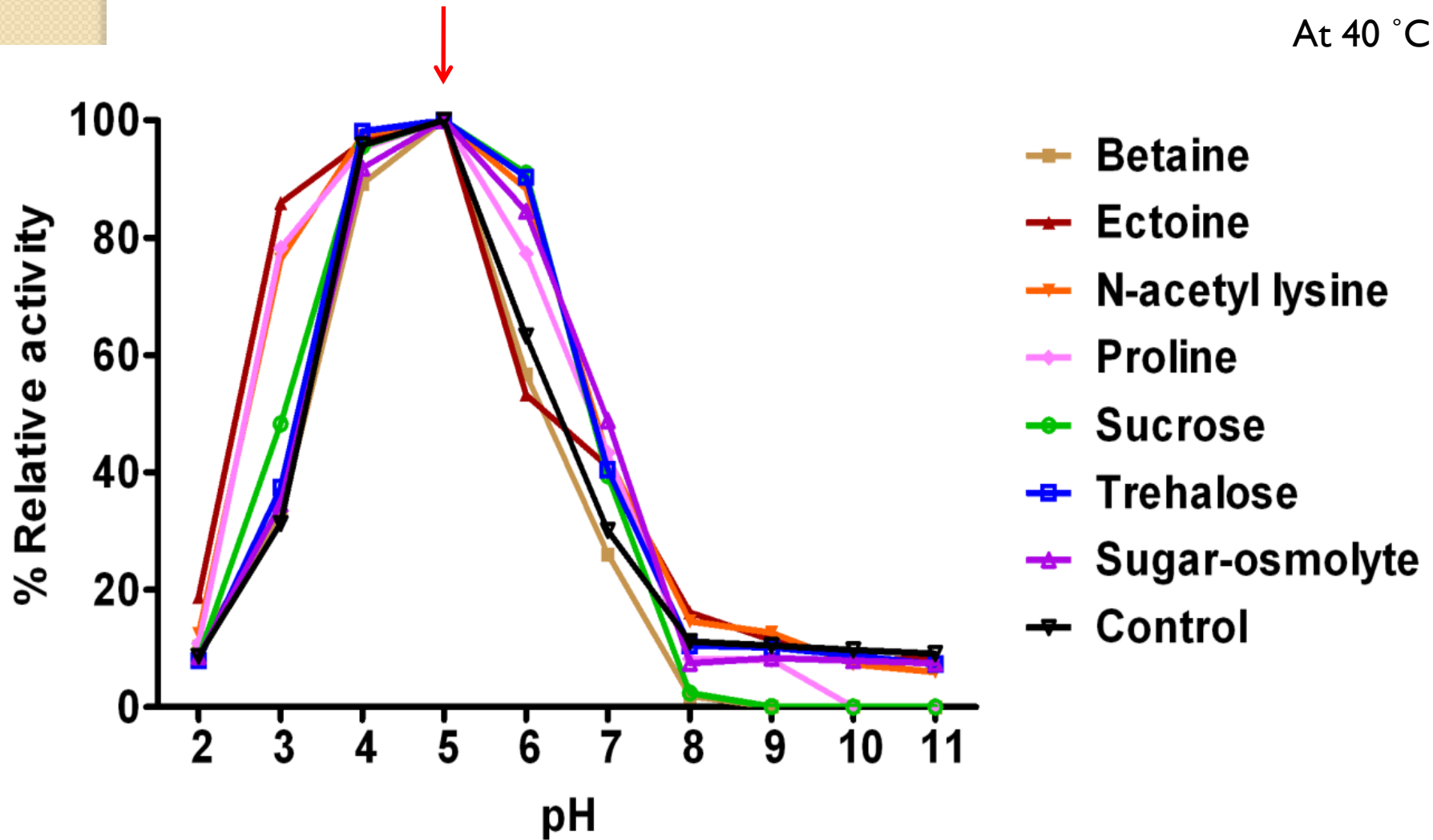
EFFECT OF THE SOLUTES ON GIBBS FREE ENERGY OF UNFOLDING



Osmolytes modulated protein function by manipulating their stability - increased the T_m and ΔG_U

EFFECT OF SOLUTES ON pH PROFILE

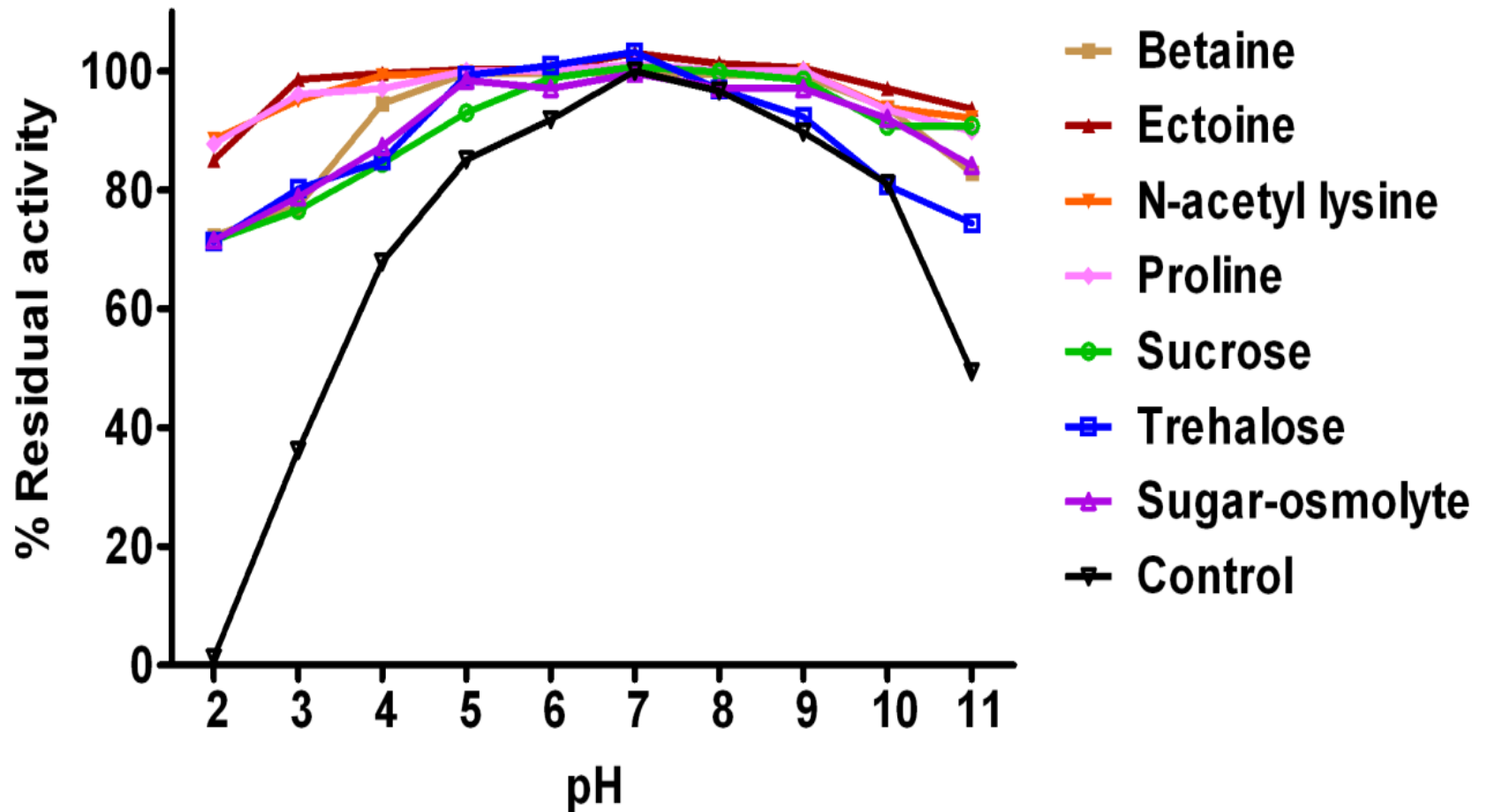
At 40 °C



Optimum activity at pH 5

EFFECT OF SOLUTES ON pH STABILITY

At 40 °C



Solutes increased stability at all pH ranges

STRUCTURAL STABILITY AT pH 5

	T_m °C	T_{agg} °C
Control	50.5	42.1
Ectoine	52.5 (2)	49.7 (7.6)
Betaine	52.5 (2)	45.4 (3.3)
N-acetyl lysine	51.5 (1)	44.9 (2.8)
Proline	51.5 (1)	44.1 (2)
Sucrose	59.6 (9)	50.8 (8.7)
Trehalose	53.5 (2.9)	45.7 (3.6)
Sugar-osmolyte	60.9 (10.4)	46.2 (4.1)

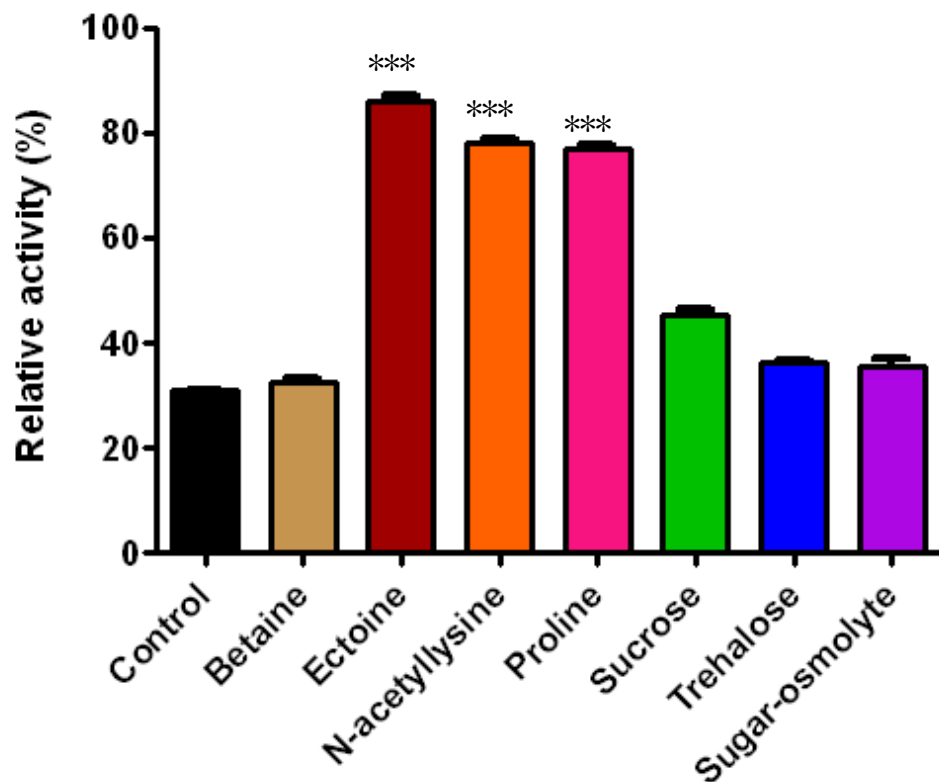
Improved the structural stability - shifting the N \leftrightarrow D equilibrium towards left

by delaying unfolding - increase in T_m

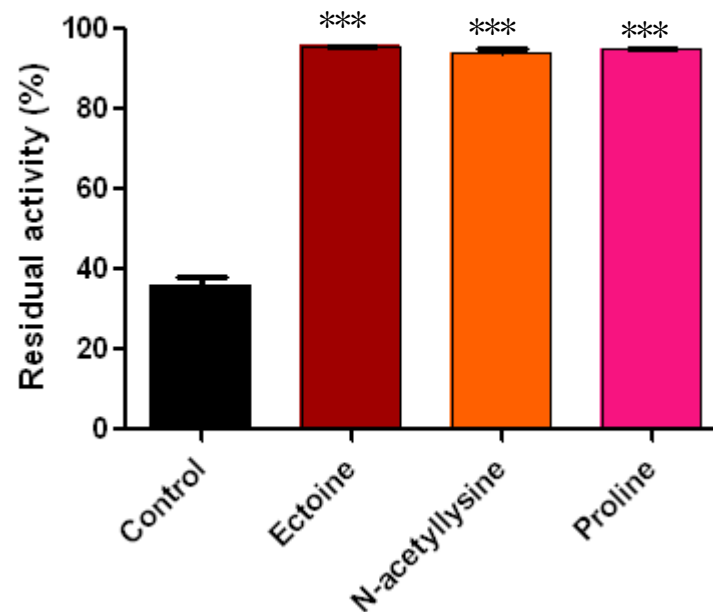
by inhibiting aggregation - increase in T_{agg}

IMPROVED ACTIVITY AND STABILITY AT ACIDIC pH 3

Catalytic activity at pH 3



Catalytic stability at pH 3

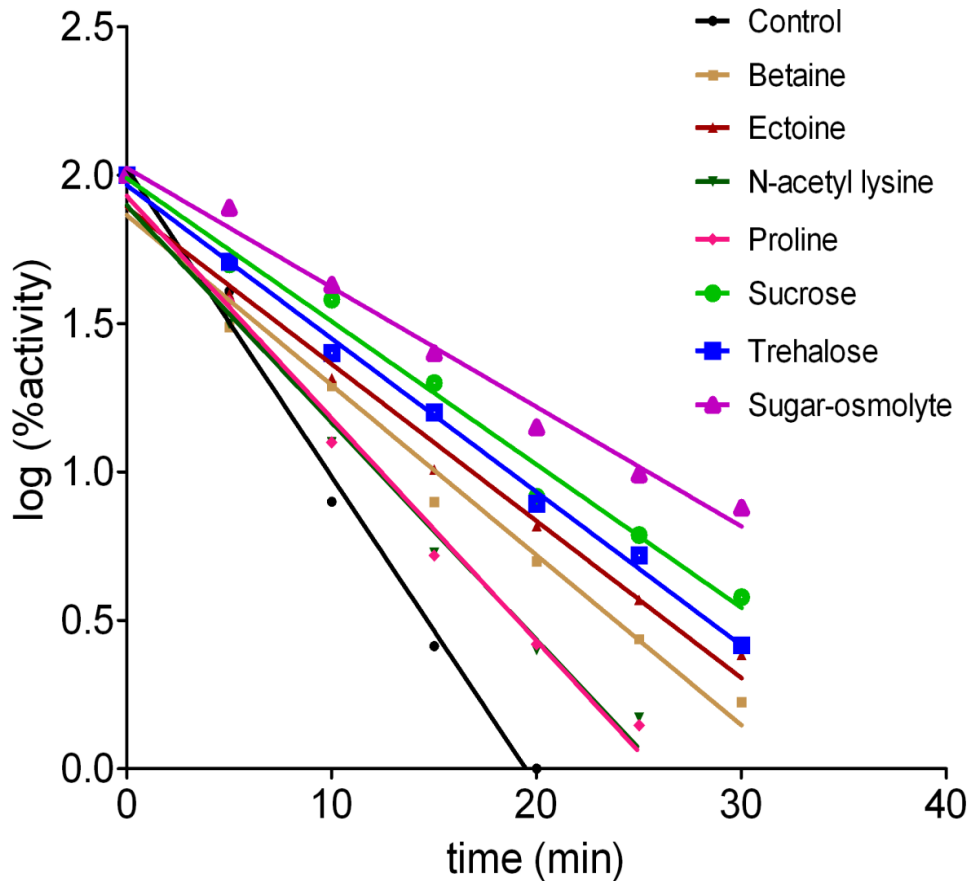


Structural stability at pH 3

	T _m °C
Control	30.5
Ectoine	40.5 (10)
N-acetyl lysine	41.5 (11)
Proline	34.5 (4)

Catalytic activity, catalytic stability and structural stability are improved by solutes –N-acetyl lysine

OPERATIONAL STABILITY – INACTIVATION KINETICS AT 50°C AND pH 5



	t1/2 (min)
Control	6.67
Ectoine	13.11
Betaine	12.10
N-acetyl lysine	9.47
Proline	9.25
Sucrose	14.37
Trehalose	13.41
Sugar-osmolyte	17.22

- **Proof-of-concept for osmolyte induced protein stability under denaturing stress conditions**
- **Such comparative studies result in the identification of suitable solutes for improving the activity and stability of the protein of interest**

SALIENT FEATURES OF THE STUDY

1. Synthesis / accumulation of species specific and growth media dependent compatible solutes to achieve halotolerance
2. Changes in the levels of metabolites of the aspartate and glutamate metabolic pathway to restore homeostasis
3. Two strains (*Planococcus maritimus* VITP21 and *Bacillus aquimaris* VITP4) capable of synthesizing rarely occurring N-acetylated diamino acids, N ϵ -acetyl lysine and N δ -acetyl ornithine were reported.
4. A novel sugar osmolyte, (2-acetamido-2-deoxy- α -D-glucopyranosyl)-(1 \rightarrow 2)- β -D-fructofuranose, synthesized de-novo by *Planococcus maritimus* VITP21 was discovered.
5. These compatible solutes could enhance the stability of proteins

Acknowledgement

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IISc, Bangalore

SIF, VIT

VIT University

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