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Comparative studies of proteomes from extremophiles: elucidation of trends of thermal adaptation

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Abstract. *The systematic comprehensive comparative analysis of proteomes of extremophiles such as psychrophiles and thermophiles in reference to mesophiles was performed in the present study. The analysis reveals that psychrophilic proteins are rich in polar uncharged and small/tiny residues that facilitate their structural flexibiliy/heat lability, whereas thermophilic proteins favour charged, hydrophobic and aromatic residues associated with their thermostable nature. Interestingly, Gly, in spite of being a small/tiny residue, is preferred by thermophiles, while Leu, Cys, Met, His, Asp and Trp, though being hydrophobic, charged and aromatic residues, respectively are favoured by psychrophiles. Secondary structural comparisons demonstrate that the proportion of random coil is privileged in psychrophiles providing protein flexibility, whereas alpha helices and beta sheets are favoured by thermophiles conferring compactness to the proteins. The amino acid substitution pattern and correspondence analysis support the preference of particular amino acids as an adaptation of the proteins to the particular growth temperature.*

Key words: Psychrophilic; Thermophilic; Hydrophobic; Aromatic; Adaptation; Secondary structure.

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1 Introduction

Microorganisms that can thrive in extreme environmental conditions are known as extremophiles. Based on the optimal temperature of growth, the extremophiles can be classified as psychrophiles, mesophiles and thermophiles. Psychrophiles are capable to grow and reproduce at low-temperature environment, near or below freezing point of water like deep sea, glaciers, mountain regions etc, whereas thermophiles can thrive in hot temperature, near or above boiling point of water like hot springs, deep sea hydrothermal vents etc [1]. The microorganisms that grow best in moderate temperature, typically between 15^{o}C and 40^{o}C are called mesophiles. The ability to grow and reproduce at extreme thermal environment is a major challenge for the extremophiles. The hazards encountered by the extremophiles due to extreme hot and cold environments are the altered enzyme activity [2, 3], protein heat/cold denaturation and impaired protein folding [3, 4], solvent viscosity and high oxygen concentration of cold water [5, 6, 7] etc. To deal with these they require a vast array of adaptations at cellular as well as molecular levels. Thermolabile and thermostable nature of psychrophilic and thermophilic proteins, respectively [2] have extensive applications in different industries like biotechnology, food, detergent, petroleum, paper bleaching, baking, brewing etc [8, 9, 10, 11, 12].

Realizing the importance of psychrophilic and thermophilic proteins, many researchers concentrated on identifying factors contributing to cold-/heat-adaptation. Several studies have been performed to compare sequence and structural parameters between psychrophilic/thermophilic and mesophilic proteins [7, 13, 14, 15, 16, 17, 18, 19, 20]. The disparities among the results obtained from these previous studies have necessitated reviewing the work further. With the advent of the complete genome sequences of several extremophiles, the current manuscript presents a systematic comprehensive comparative analysis of proteomes of psychrophiles, mesophilies and thermophilies at primary and secondary structural levels to explain the trends of thermal adaptation. Also, the amino acid replacements among orthologs of psychrophiles, mesophilies and thermophilies are examined to understand which residues are instrumental in different thermal adaptations. In addition, by using correspondence analysis, the major trends of variation of proteome composition of these three groups of microbes are elucidated. In this context, the study considers complete genome sequences of five psychrophiles, nine mesophiles and six thermophiles from various phyla of bacteria/archaea with comparable GC-content to minimize the phylogenetic influence and the effect of mutational bias on amino acid usage, respectively.

2 Materials and methods

2.1 Retrieval of protein coding sequences

All protein coding sequences of the chromosomes of twenty microorganisms under study (Table 1) were retrieved from NCBI ftp site (ftp://ftp.ncbi.nlm.nih.gov/genomes/). Microorganisms considered for the present study are with optimal growth temperature ranging from -12^{0}C to 103^{0}C shown in Table 1. Moreover, the microorganisms were sampled with the criteria of single adaptation i.e. thermal adaptation, comparable GC-content (36.5-44.7) and were from various phylogeny to minimize the effect of other adaptations, GC-compositional bias on amino acid usage comparison and phylogenetic influence, respectively.

2.2 Parameters used for proteome analysis

Amino acid residues were classified into seven property groups as follows: small/tiny amino acid group includes Ala, Cys, Asp, Gly, Asn, Pro, Ser, Thr and Val; aromatic - Phe, Trp and Tyr; polar uncharged - Asn, Gln, Ser and Thr; charged - Asp, Glu, His, Arg and Lys; basic - His, Lys and Arg; acidic - Asp and Glu, Hydrophobic - Cys, Phe, Leu, Ile, Met, Val, Trp [18]. The frequencies of these seven property groups and each individual amino acids were calculated and compared among three categories of microorganisms under study. The significance of differences of each amino acid/ property group, if any, between psychrophiles, mesophiles and thermophiles was assessed by 2x2 contingency tables. The columns were for any two categories of microorganisms among psychrophiles, mesophiles and thermophiles and rows were for the counts of a particular amino acid residue/property group and the total amino acid counts for all 20 residues in the respective categories of microbes.

2.3 Amino acid replacement

Three sets of orthologous sequences between (i) mesophiles and psychrophiles, (ii) mesophiles and thermophiles and (iii) thermophiles and psychrophiles were retrieved using the program BlastP with cutoff value of E =1.0x10^{-10}. Sequences having more than 60%

Table 1: List of microorganisms under study

	Organism	OGT (^{O}C)	Genome size (Mb)	GC-content	Lineage
Psychrophiles	*Colwellia psychrerythraea* 34H (Cp)	8	5.37	38.0	Gamma-proteobacteria
	Psychrobacter arcticus 273-4 (Pa273)	-10	2.65	42.8	Gamma-proteobacteria
	Psychrobacter cryohalolentis K5 (PcK5)	-10	3.1	42.2	Gamma-proteobacteria
	Pseudoalteromonas haloplanktis TAC125 (PhTAC)	15	3.85	40.1	Gamma-proteobacteria
	Psychromonas ingrahamii 37(Pi37)	-12	4.6	40.1	Gamma-proteobacteria
Mesophiles	*Vibrio fischeri* ES114 (Vf)	37	4.28	38.4	Gamma-proteobacteria
	Coxiella burnetii Dugway 5J108-111(Cb)	37	2.25	42.1	Gamma-proteobacteria
	Bacillus subtilis (Bsub)	35	4.2	43.5	Firmicute
	Listeria monocygenes EGD (Lm)	37	2.94	38.0	Firmicute
	Listeria innocua Clip11262 (Li)	37	3.09	37.4	Firmicute
	Listeria welshimeri serovar 6b str. SLCC5334 (Lw)	37	2.8	36.5	Firmicute
	Bacteroides fragilis NCTC 9343 (Bf)	37	5.24	43.1	Bacteroidetes/Chlorobi
	Bacteroides vulgatus ATCC 8482B (Bv)	37	5.2	42.2	Bacteroidetes/Chlorobi
	Microcystis aeruginosa NIES-843) (Ma)	37	5.8	41.6	Cyanobacteria
Thermophiles	*Aquifex aeolicus VF5* (Aa)	96	1.59	43.3	Aquificaceae
	Clostridium thermocellum ATCC 27405 (Ct)	60	3.8	39.0	Firmicute
	Thermoanaerobacter tengcongensis MB4 (Tten)	75	2.69	37.6	Firmicute
	Pyrococcus abyssi GE5 (PaGE5)	103	1.77	44.7	Euryarchaeota
	Pyrococcus furiosus DSM 3638 (PfDSM)	100	1.9	40.8	Euryarchaeota
	Pyrococcus horikoshii OT3 (PhOT3)	98	1.7	41.9	Euryarchaeota

( ) Short form of each organism is written in parenthesis

similarity and less than 20% difference in length with the query were considered as orthologs. Then the amino acid sequences of each set of orthologs were aligned using the ClustalW program and the amino acid replacements were obtained in the form of a 20x20 matrix, using a program developed in C++ [21]. While studying amino acid replacements, the direction of conversion for a pair of residues (say, i to j) from mesophilic to psychrophilic, mesophilic to thermophilic and thermophilic to psychrophilic proteins was considered as 'forward direction' whereas *vice versa* is considered as 'reverse direction'. In unbiased condition, the ratio of conversion of a pair of residues in forward and reverse direction is expected to be 1:1. The violation of this ratio results in the gain or loss of a residue. Gain indicates that the number of replacements in forward direction is greater than that of the reverse direction. The significance of the gain was assessed by 2x2 contingency tables having 1 degree of freedom. For each pair of replacements, the first and second rows of the contingency table represented the number of replacements from one particular residue (i) to another (j) of the pair and the total count of the remaining replacements (say, k) from the residue i (where $k \neq j$), respectively.

2.4 Correspondence analysis

Correspondence analysis on amino acid usage was carried out using the CODONW 1.4.2 program. CODONW (developed by John Peden and available at ftp://molbiol.ox.ac.uk/win95.codonW.zip) is a widely used program to identify the major factors influencing the variation in codon and amino acid usage in an organism. Correspondence analysis creates a series of orthogonal axes to identify trends that explain the data variation, with each subsequent axis explaining a decreasing amount of the variation.

2.5 Secondary structure prediction

The secondary structural elements such as alpha helix, beta sheet and random coil of orthologous sequences from psychrophiles, mesophiles and thermophiles were computed using consensus secondary structure prediction program (utilizing default settings) available at http://umber.embnet.org/dbbrow-ser/bioactivity/NPS2.html.

Table 2: Comparisons of amino acid usage in psychrophiles, mesophilies and thermophiles

Amino acid	Average amino acid (%)		
	Psychrophile	Mesophile	Thermophile
Ala (A)	8.7*	7.46	6.43
Cys (C)	0.98*	0.96	0.78
Asp (D)	5.64*	5.29	4.88
Glu (E)	5.76	6.89	8.5*
Phe (P)	4.13	4.40	4.45*
Gly (G)	6.53	6.73	6.95*
His (H)	2.18*	1.94	1.48
Ile (I)	7.02	7.29	8.51*
Lys (K)	5.71	6.6	8.31*
Leu (L)	10.36*	9.85	9.73
Met (M)	2.48**	2.51*	2.29
Asn (N)	4.84*	4.68	4.16
Pro (P)	3.73	3.84	3.9*
Gln (Q)	4.61*	3.85	2.09
Arg (R)	4.06	4.43	4.9*
Ser (S)	6.78*	6.12	5.34
Thr (T)	5.6**	5.64#	4.57
Val (V)	6.61	6.58	7.67*
Trp (W)	1.13**	1.18*	1.01
Tyr (Y)	3.11	3.72	4.06*
Charged residues	23.35	25.15	28.07*
Basic residues	11.95	12.97	14.69*
Acidic residues	11.4	12.18	13.38*
Hydrophobic residues	32.71	32.82	34.44*
Aromatic residues	8.37	9.35	9.52*
Polar uncharged residues	21.87*	20.29	16.16
Small/Tiny residues	49.45*	47.3	44.68

*amino acid residues having higher frequencies in the respective group in comparison to other two at $p \leq 0.001$.
**amino acid residues having higher frequencies in psychrophiles in comparison to thermophiles at $p \leq 0.001$.
#non-significant difference.

3 Results and Discussion

3.1 Comparison of Primary Structure

The amino acid usage patterns of psychrophiles, mesophiles and thermophiles are determined to find out the effect of growth temperature on amino acid preferences of these three groups of microorganisms. The study indicates that the thermophiles prefer charged residues like Glu, Lys and Arg, hydrophobic residues like Ile, Val, Pro and aromatic residues like Phe and Tyr more significantly than mesophiles and psychrophiles (Table 2). On the other hand, the psychrophiles use polar uncharged residues like Ser, Asn, Gln, small/tiny residues like Ala, Cys, Asp more significantly than mesophiles and thermophiles (Table 2). In mesophiles, the frequencies of amino acids are moderate with respect to psychrophiles and thermophiles (Table 2). The preferences of charged, hydrophobic and aromatic amino acids in thermophiles are in accordance with the thermostable nature of heat-adapted proteins [14, 22]. The thermostability is conferred by different intermolecular interactions like electrostatic, hydrophobic and cation-pi interactions provided by charged, hydrophobic and aromatic residues, respectively [23]. In addition, the charged residues at the surface of the heat-adapted proteins contribute to their thermostability [24, 25]. In psychrophiles, the elevated usage of polar uncharged, small/tiny residues is related to the structural flexibiliy/heat lability of the cold adapted proteins [20]. In course of analysis of the preference of amino acids in psychrophiles, mesophiles and thermophiles, it is noticed that some amino acids are remarkably atypical in terms of their distribution in these three groups of microorganisms (Table 2). For instance, (i) Gly, although being a small destabilizing amino acid, is

preferred more by thermophiles than psychrophiles. The preference of Gly in thermophiles is probably due to its ability to provide stability to the heat-adapted proteins by making cross-strand pairing with aromatic residues, particularly with Phe and Tyr [26]. The stabilization of the thermophilic proteins is also facilitated by an electrostatic interaction between the amide group of Gly and the negatively charged ring of the overlying aromatic side chain [26]. (ii) Leu, being a hydrophobic residue, occurs more frequently in psychrophiles than in thermophiles. Although there is evidence of higher rate of Leu incorporation in bacterial proteins at freezing temperature [27], the role of Leu in molecular adaptation of proteins in cold environment is not clearly understood. (iii) Asp, being a charged residue, is preferred by psychrophiles than thermophiles. The apparent favouring of Asp in psychrophiles is consistent with the fact that this residue helps to decrease the unfolding transition temperature of proteins, effectively making them less heat-stable [17]. (iv) Cys, Met, Trp and His, in spite of their respective hydrophobic, aromatic and charged properties are preferred by psychrophiles than thermophiles. The plausible explanation of overrepresentation of these residues in psychrophiles may be that these residues have antioxidant properties which terminate high amount of free radical formation from the higher concentration of oxygen trapped in cold water and thus rescue the cells from damage [23, 28, 29, 30]. Apart from this, the unusual distribution of amino acids like Leu, Cys, Met, His in psychrophiles and Gly in thermophiles may be explained by the concept of chronology of evolution of amino acids [31]. As referred by the models proposed by three groups of investigators, Jordon et al., Trifonov, Miller [32, 33, 34], it is assumed that Gly was evolved and recruited to the genetic code early during the period when earth is dominated by thermophilic environment, while Leu, His, Cys, Met were late recruited to genetic code, evolved subsequently during the period of gradual cooling of earth to mesophilic and psychrophilic environments. Therefore, apart from growth temperature, the chronology of evolution of amino acids might sculpt the preference of amino acids in those extremophiles.

3.2 Amino Acid Substitution Pattern

Three sets of orthologous proteins (mesophiles & psychrophiles, mesophiles & thermophiles and thermophiles & psychrophiles) are compared to obtain all possible amino acid replacements (i.e $(20 \times 19)/2 = 190$ possible pairs of replacements) between the orthologous sequences in the direction of mesophiles to psychrophiles, mesophiles to thermophiles and thermophiles to psychrophiles. Table 3 shows the top 15 amino acid replacement pairs according to the most biased gain in the direction from mesophiles to psychrophiles, mesophiles to thermophiles, thermophiles to psychrophiles. The analysis of substitution patterns in the three sets of orthologous proteins depicts that (i) charged residues like Glu and Lys in mesophiles and thermophiles are replaced by polar uncharged residues like Gln, Ser, Thr, Asn, small/tiny residue like Ala and also the residues, Asp, Leu and Arg in psychrophiles and *vice versa.* (ii) Leu is gained in psychrophiles over charged (Lys), hydrophobic (Ile, Val) and aromatic (Phe) residues in mesophiles and thermophiles and *vice versa.* (iii) Similarly, polar uncharged residue Ser is gained in psychrophiles over another polar uncharged residue Thr in mesophiles and thermophiles and *vice versa.* (iv) Lys is acquired in thermophiles in cost of Arg in mesophiles and psychrophliles. It is to be mentioned here that although Lys and Arg both are preferred charged residues in thermophiles, Lys is more abundant than Arg (Table 2). The gain of Lys over Arg in thermophiles brings noticeable stabilization of the proteins in high temperature environment [35]. Thus, the amino acid substitution pattern confirms the preceding finding of amino acid preferences in psychrophiles, mesophiles and thermophiles.

3.3 Correspondence Analysis

To determine the trend of variation of amino acid usage of the proteomes of five psychrophiles, nine mesophiles and six thermophiles under study, Correspondence Analysis (COA) on Relative Amino Acid Usage (RAAU) was performed. The first four axes generated by COA on amino acid usages explain 46.51% of the total variations. Among them the first and second axes contribute 15.83%and 11.23% variations, respectively. The organisms under study thriving at different growth temperatures are clustered separately according to their thermal adaptation on the plane defined by first and second axes (Fig.1). To find out the factors playing important role in causing variation of proteome composition, axes are plotted against different parameters. Table 4 represents the parameters showing the strongest correlations with two principle axes generated from COA on RAAU. The charged residues, mainly Glu and Lys exhibit strong positive correlation whereas polar uncharged residues, mainly Gln and Ser and small/tiny residues, mainly Ala display strong negative correlation with both Axis1 and Axis2 (Table 4, Fig. 2, Fig. 4). Histidine is also strongly negatively correlated with both the axes (Table 4, Fig.2 and Fig. 4). Therefore, this finding suggests that residues related to ionic interaction and surface charge of thermostable heat-adapted proteins, structural flexibility/thermolability and resistance to high oxygen concentration of cold water of the cold-adapted proteins are the major factors that creates variation in amino acid composition of the proteomes obtained from psychrophiles, mesophiles and thermophiles. Interestingly, the plotting of

Table 3: Comparison of amino acid substitution pattern from mesophiles to psychrophiles, mesophiles to thermophiles, thermophiles to psychrophiles

Mesophile to Psychrophile				Mesophile to Thermophile				Thermophile to Psychrophile			
AA[S*]	F[**]	R[***]	gain	AA[S*]	F[**]	R[***]	gain	AA[S*]	F[**]	R[***]	gain
I-L	4717	3714	1003	A-E	1723	677	1046	I-L	4992	3095	1897
E-Q	1908	922	986	Q-K	1617	663	954	K-Q	1886	502	1384
E-A	1716	1034	682	L-I	4639	3733	906	E-Q	1953	634	1319
K-Q	1569	938	631	A-K	1499	624	875	E-A	1888	578	1310
E-S	1408	808	600	D-E	3039	2172	867	E-D	2766	1557	1209
E-D	2566	1977	589	T-K	1433	609	824	K-A	1673	531	1142
V-L	3064	2558	506	E-K	3103	2373	730	E-S	1521	652	869
K-A	1424	934	490	T-E	1271	640	631	K-S	1491	667	824
T-S	2034	1546	488	Q-E	1488	938	550	K-T	1303	495	808
K-S	1235	755	480	T-V	1384	835	549	E-T	1167	477	690
E-N	1161	783	378	T-I	1069	585	484	V-L	2884	2213	671
K-R	1872	1501	371	D-K	1288	808	480	K-R	2205	1565	640
K-L	1039	670	369	S-K	1179	735	444	K-D	1287	727	560
F-L	2031	1690	341	R-K	1232	800	432	T-S	1682	1134	548

*amino acid substitution, **forward substitution, ***reverse substitution

Table 4: Significant correlations of Axis1 and Axis2 of COA on RAAU with different parameters

Parameters	Axis1	Axis2
Alanine (A)	-0.94	-0.86
Glutamic acid (E)	0.95	0.96
Histidine (H)	-0.85	-0.82
Lysine (K)	0.95	0.91
Glutamine (Q)	-0.93	-0.98
Serine (S)	-0.90	-0.93
Charged residues	0.95	0.96
Basic residues	0.91	0.92
Acidic residues	0.90	0.87
Polar uncharged residues	-0.88	-0.96
Small/tiny residues	-0.88	-0.90

All data are significant at $p<0.0001$

amino acids in the plane of two main axes in Fig. 3 illustrates the amino acid preferences in psychrophiles and thermophiles and thereby, strengthens the assumption that the factors like molecular adaptation of proteins to respective growth temperatures and chronology of amino acid evolution together determine proteome composition of these two groups of microorganisms.

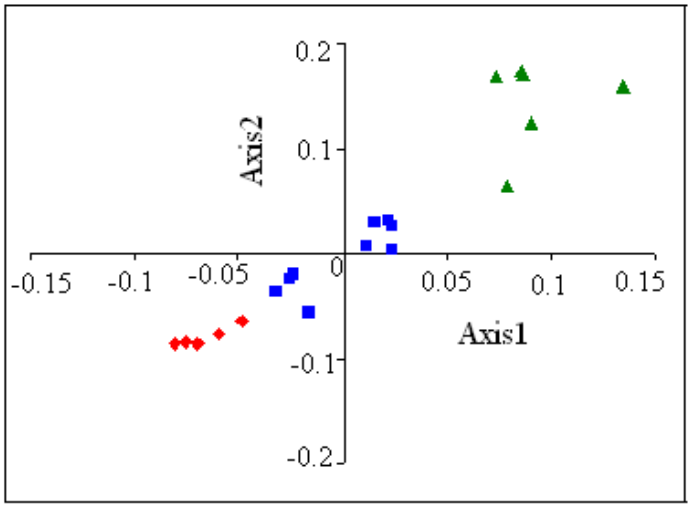

Fig. 1 Position of proteomes of psychrophiles, mesophiles and thermophiles along Axis1 and Axis2 generated by COA on RAAU. Red diamond, blue square and green triangle represent psychrophiles, mesophiles and thermophiles, respectively.

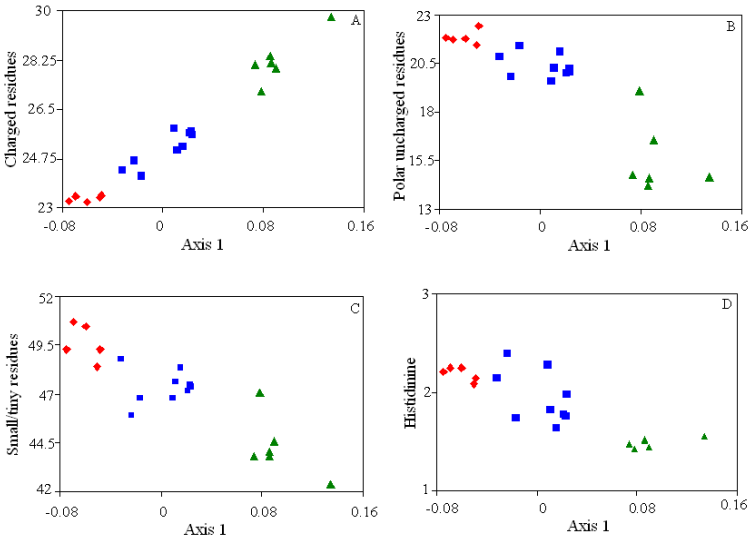

Fig. 2 Position of proteomes of psychrophiles, mesophiles and thermophiles along Axis1 generated by COA on RAAU has been plotted against charged residues (A), polar uncharged residues (B), small/tiny residues (C) and Histidine (D), respectively.

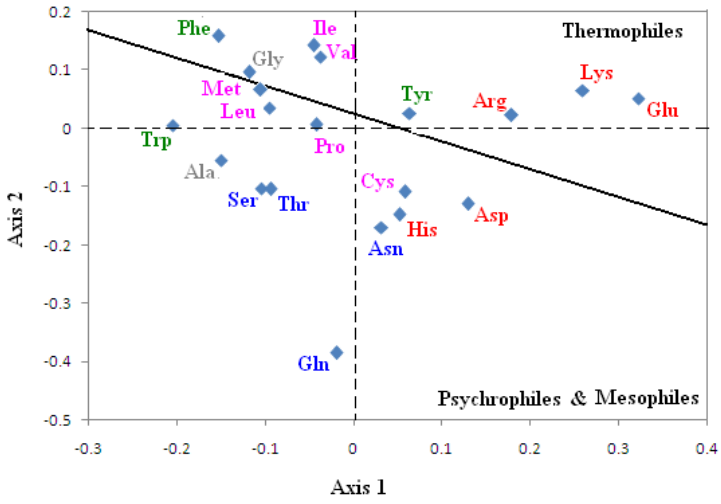

Fig. 3 The position of amino acids along Axis1 and Axis2 generated by COA on RAAU. Red, blue, pink, green and grey colours indicate charged, polar uncharged, hydrophobic, aromatic, small/tiny residues, respectively.

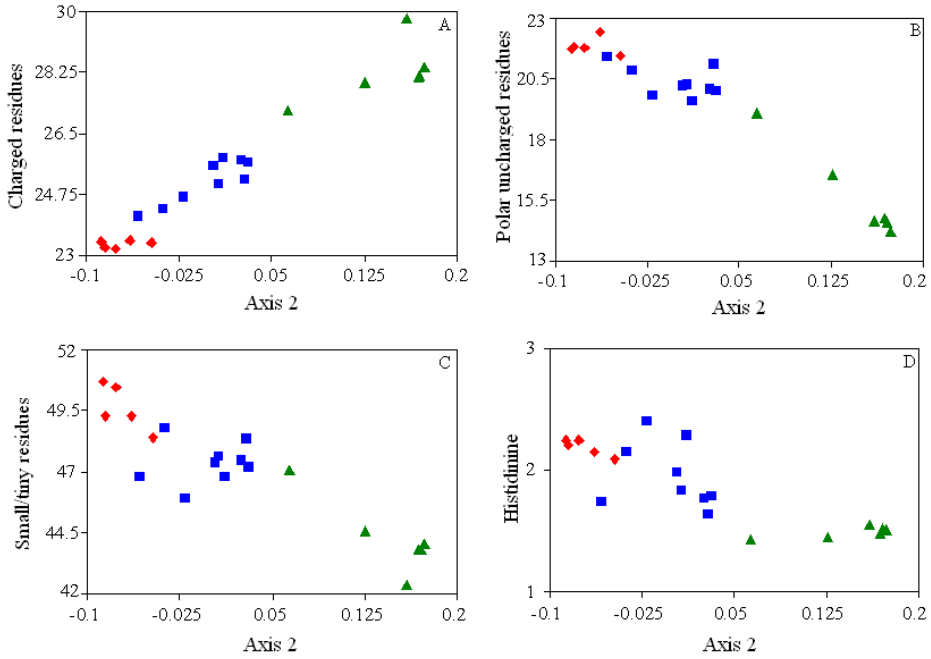

Fig. 4 Position of proteomes of psychrophiles, mesophiles and thermophiles along Axis2 generated by COA on RAAU has been plotted against charged residues (A), polar uncharged residues (B), small/tiny residues (C) and Histidine (D), respectively.

3.4 Comparison of secondary structure

To determine the effect of growth temperature on the secondary structural elements of the proteins, the secondary structures of the orthologous proteins of psychrophiles, mesophiles and thermophiles were compared. The result shows that the proportion of random coil is higher in psychrophiles (40%) than in mesophiles (39%) and thermophiles (36%), whereas alpha helices and beta sheets are higher in proportion in thermophiles (43% & 15%) than in mesophiles (40% & 14%) and psychrophiles (40% & 13%). This is in accordance with the fact that the higher percentage of random coil renders higher protein flexibility in psychrophiles, whereas higher proportion of alpha helix and beta sheet explains the relatively high protein compactness of thermophiles.

4 Conclusions

The present study systematically analyses the proteomes of five psychrophiles, nine mesophiles and six thermophiles at primary and secondary structural levels in order to elucidate the trends of thermal adaptation. The study reveals that charged (Lys, Arg, Glu), hydrophobic (Ile, Val, Pro, Gly) and aromatic (Phe, Tyr) residues are abundant in thermophilic proteins, a prerequisite to confer thermostability to the protein tertiary structure in order to retain functional optimality in extreme hot environment. Again, polar uncharged (Ser, Asn, Gln) and small/tiny residues (Ala, Cys, Asp) are frequent in psychrophilic proteins to provide flexibility to the protein tertiary structure for overcoming freezing effect of extreme cold environment. The interesting observation in this study is that some of the residues with counter physico-chemical properties meant to confer thermal resistance in extreme hot and cold environments are abundant in pychrophiles and thermophiles. For example, small/tiny residue Gly is favored by thermophiles, hydrophobic residues Leu, Cys, Met, charged residue His and aromatic residue Trp are favoured by psychrophiles. The overrepresentation of these residues, even though partly explained by their physico-chemical role in the respective thermal environment, is influenced by chronology of amino acid evolution to a greater extent. As well, the analysis of secondary structural elements depicts that random coil is proportionally higher in psychrophiles, while alpha helix and beta sheet are higher in thermophiles conferring flexible conformation and thermostability to tertiary structure of cold and heat-adapted proteins, respectively. Thus, the knowledge gained from this present study might be helpful to engineer proteins with optimal functionality in either cold or hot temperatures.

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