Int.J.Curr.Microbiol.App.Sci (2025) *14*(02): 103-115

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 14 Number 2 (2025)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2025.1402.009

Proteomic Analysis of Thermophilic Starter *Streptococcus thermophilus* NCDC74 on Low Temperature

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Keywords

CSP, Cryoprotection, Mowse score, Proteomic, Peptide mass fingerprinting

Article Info

Received:
18 December 2024
Accepted:
26 January 2025
Available Online:
10 February 2025

A B S T R A C T

Low temperature adaptation of a thermophilic starter *Streptococcus thermophilus* NCDC74 (St74) (routinely used for production of *'Dahi'* (yoghurt) in Indian Dairy Industries) was analysed by physiology and proteomic approach. Cold shock at 20°C for 2, 4 and 6h to St74 demonstrated a factorial increase in freeze-thaw survival of 80%, 800% and 250% respectively, compared to the control (42°C). Proteomic analysis of cold-induced cells at 20°C for 6h revealed 23 spots, which were differentially expressed. Of the differentially expressed proteins, 14 and 9 were of low (< 10 kDa) and high molecular weight (> 10 kDa) respectively. Peptide mass finger printing (PMF) analysis revealed that spot1 (MW ~7.2 kDa) matched with cold shock protein A (cspA) fragment of *Lactobacillus casei* (Mowsescore: 164) and spot3 (MW ~7.2 kDa) matched with csp fragment of *Leuconostoc mesenteroides*, thus suggesting their strong role in cryoprotection. General stress proteins induced during cold shock include lp_0704 (MW 8 kDa) from *L. plantarum*, dnaA from *L. monocytogenes* and acetolactate synthases of *B. cereus*. Two repressed spots (5 and 6) include chorismate synthase of *S. epidermidis*, t1pA from *C. tetani* (spot5), and ABC transporter from *C. perfringens* respectively (spot6). Cold inducible proteins identified in high MW include glyceraldehyde-3-phosphate dehydrogenase, parvulin-like peptidyl-prolyl isomerase, (PrsA), PTS (phosphotransferase) system and cIp protease, cIpB, UTP-glucose-1-phosphate uridylyltransferase among the repressed spots. Results revealed increased freeze thaw survival capacity after adaptation to low temperature as well as identification of several proteins that include csps, general stress proteins and repressed proteins in St74.

Introduction

India is a land of traditional fermented foods of both dairy (like dahi, chaach, dahi chenna, kanji, etc.) and non-dairy origin, and lactic acid bacteria (LAB) are the predominant microbes in these products. Their GRAS (Cross *et al.,* 2002; Puertollano *et al.,* 2008) status allows them to be widely used in fermentation. Thermophilic lactic acid bacteria (TLAB) *Streptococcus thermophilus* is routinely used in *'dahi'* (a summer fermented product

of India) making. During commercial production process these are subjected to various stress conditions induced by lyophilisation, concentration, extreme (heat, cold) temperatures, salt and sugar treatments, rising lactic acid concentration with concomitant pH shift, and nutrient deprivation (Somkuti & Steinberg, 1999) that affect organoleptic quality of the final product. Therefore, stress response studies of TLAB have aroused interest among researchers.

Low temperature adaptation of *S. thermophilus* that might help overcome major post processing complexity in dairy industry, such as, acidification and freezing is particularly significant for strain improvement studies. Such adaptation of an industrially important strain *S. thermophilus* NCDC74 has been investigated by proteomic analysis in the present study.

Materials and Methods

Collection and Maintenance of Bacterial Strain

Streptococcus thermophilus NCDC74 (St74) procured from National Collection of Dairy Culture (NCDC), National Dairy Research Institute (NDRI), Karnal was grown at 42 °C in M17 broth (HiMedia Pvt. Ltd, India) containing 1% (w/v) lactose (LM17), and in chalk litmus milk. While the stock culture was maintained at -80 °C in glycerol, the working sets were prepared by inoculating the culture in the broth of both the media at 42 °C, and maintained in a refrigerator through fortnight sub-culturing.

Growth Estimation

St74 was grown in LM17 broth at 42 °C, until mid-exponential phase (OD_{650} ~ 0.5). This was used to inoculate 200 ml of fresh media (LM17) in duplicate at 1% (v/v). The inoculated broth was incubated at 42 (control), 25, 20 and 15 °C for 36h.

The growth was monitored spectrophotometrically at 3 h intervals by measuring the OD at 650 nm (CARY1, USA), and confirmed through the simultaneous cell counts by standard plate count method (42 °C; 48h). The DMFit curve fitting programme designed by Baranyi (IFR, Norwich, UK) was used for the log counts data fit applying Baranyi function (Baranyi & Roberts, 1994). The growth rate (μ_{max}/h) was ascertained from the resultant programme.

Low Temperature Stress Response of ST74

Low temperature adaptation

St74 was grown in five separate flasks containing 60 mL of LM17 broth each, at 42 °C until mid log phase (OD_{650} = 0.524), centrifuged (3000 rpm, 10 min) at 4 °C, and pelleted. Four sets of these pellets were resuspended in the same volume of precooled LM17 medium (at 25, 20, 15 and 10 °C). The fifth (control) set was incubated at 42 °C, and all of them were incubated for 36h. In a duplicate set, the cultures were added with 100 µg chloramphenicol mL^{-1} in order to arrest new protein formation. Both sets (with and without chloramphenicol) were carried out in duplicates for the reproducibility of the results and the mean data are presented. The OD and cell counts were estimated at every 3 h interval.

Freeze-thaw survival

Freeze-thaw survival study was performed as per Wouters *et al.,* (1999). Five mL mid-log phase (OD_{650} = 0.524) culture from each flask, *i.e.*, control (at 42 °C) and cold-shocked cells (at 10, 15 and 20 °C, all for 2, 4 and 6 h) maintained in LM17 broth was centrifuged at 9000 rpm for 5 min, and resuspended in 5 mL of fresh LM17 medium. All suspensions were freeze-thawed (frozen at – 20 °C, 24 h; thawed at 30 °C, 4 min cycle; the cycle repeated for four times). Cell counts were determined just before and after each consecutive freeze-thaw cycle for both chloramphenicol-treated and chloramphenicol-untreated cells, through colony forming units (CFUs) after 48 h incubation at 42 °C on LM17 agar plates. All experiments were in duplicate and the mean data are presented. The per cent survival and tolerance factor was calculated as per the formulae:

Survival (%) = (posttreatment CFUs/pretreatment CFU) X 100

Tolerance factor = % survival of adapted cells/%survival of control cells.

Proteomic Analysis

2D-Gel Electrophoresis

Protein was extracted from 200 mL of both control (42 °C) and treated (20 °C; 6 h) cultures in triplicate. Samples were washed twice in 50 mM phosphate buffer

(pH 7.0), resuspended in 4-5 mL sonication buffer which constituted 150 mM Tris HCl, pH 7.0; 1 mM EDTA, 1 mM mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (pH 7.0) and incubated overnight at 4 °C. The pellets were thawed to room temperature and sonicated (Branson Sonic Power Company, Danbury, Connecticut) on ice for 4-5 min (30 sec burst followed by one min cooling on ice in cycle) followed by centrifugation (3000 rpm; 15 min).

The supernatants were lyophilised and the protein contents were estimated (Bradford, 1976). Sample clean up were performed to remove salts, nucleic acids etc. by 2D-clean up kit (Amersham biosciences).

High resolution 2D PAGE were followed as per standard procedure (O'Farrell, 1975) with a BioRad 2D-IEF system (Bio-Rad India Pvt. Ltd).

Immobilised pH gradient (IPG) strips (17 cm, pH gradient range 4-7; Biorad) were rehydrated in rehydration buffer. 200 µG of the samples were mixed with 100 µL of rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2 % biolytes (3-10), traces of bromophenol blue) and loaded at the acidic end of the IPG strips. Overnight rehydration was done at 20 °C. A final voltage of 50,000 VH was set for isoelectric focusing (IEF) carried out with a PROTEAN IEFCELL (BIORAD) in a three step process. The second dimension SDS PAGE was carried out as per standard procedure in 13 % gel (Laemelli, 1970). Calibration of molecular weight markers and isoelectric points were performed by using comigrating standards from Amersham Biosciences. The gels were silver-stained as per Blum *et al.,* (1987). Careful visual analyses of scanned gel pictures were done to differentiate spots in control and treated cells.

Peptide mass finger printing (PMF) analysis

Selected pieces from 2D gel were cut carefully, rehydrated, trypsin-digested and analysed for PMF (Leverrier *et al.,* 2003) using 2D-nano LC-MS (LC-MSD Trap XCT, Agilent 1100 series). Mascot peptide mass fingerprinting search program from Matrix science (http://www.matrixscience.com) were used for data analyses. At least three matching peptides were taken into consideration for a match to be considered significant ($p<0.05$). Nucleotide accession numbers for the sequences were reported as listed in Tables 3 and 4.

Results and Discussion

Growth of St74

The correlation coefficient between the OD and the CFU were in the range of 0.6689 (control), 0.8778 (25 °C) and 0.7265 (20 °C). The exponential phase for the control cells were achieved in 9 h (OD_{650} = 0.524), whereas the cells grown at 25 and 20 °C achieved the same at 12h (OD_{650} = 0.573) and 24 h (OD_{650} = 0.506). At 15 and 10 °C, a significant decrease in the growth rate was observed and no growth was discernible until 36 h as compared to others (*i.e.*, 0.5 h^{-1} at 42 °C, 0.2 h^{-1} at 25 °C and 0.008 h^{-1} at 20 °C).

Thus, the temperatures for the optimal growth and subminimal growth for St74 were 42 °C and 20 °C, respectively. The calculated minimum temperature for growth of this strain has been reported to be 18 °C (Ratkowsky *et al.,* 1982; Wijtzes *et al.,* 1995).

Low temperature adaptation of St74

The adaptation capacity of St74 was within 3-4 h when shifted from 42 to 25 °C and that of 5-6 h when shifted from 42 to 20 °C (Fig. 1), confirming the low-temperature adaptation efficiency of the strain. At 15 °C, it attained an exponential growth in 15 h (OD_{650} = 0.575) but was not able to survive after 20 h. A cold shock from 42 to 10 °C arrested its growth beyond 10 h (Fig. 1).

Freeze-thaw survival capacity of St74

At 42 °C, about 0.01 % cells survived four successive freeze-thaw cycles. Adapted cells (20 °C) demonstrated an 80, 800 and 250 fold increased survival with 2, 4 and 6 h treatments, respectively against the control (Fig. 2), whereas exposure of cells to 10 °C had only a inappreciable increment (8, 11 and 2 fold with 2, 4 and 6 h treatments, respectively) in freeze-thaw survival (Fig. 3).

Inhibition of adaptive response was observed in chloramphenicol treated cells indicating that the protein synthesis during the adaptation process was essential. Conversely, chloramphenicol-treated cells exposed to 20 °C for 4 and 6 h did not result in complete inhibition of the adaptive response where a nine fold rise was discernible (Fig. 2).

Identifying cold shock proteins in St74 by 2D-Proteomics

Changes in protein synthesis in St74 during cold adaptation

Gene expression modification during stress adaptation expressed as protein in both control and cold-treated cells of St74 was compared by 2D PAGE (Fig. 4, Plates 1 & 2). More than 150 spots were expressed with the illustrated separation and staining conditions. Important cold induced proteins were observed for exposed cells at 20 °C for 6 h. Out of 23 spots identified in control and 6 h gels, 14 spots were differentially expressed as low molecular weight (LMW) proteins (< 10 kDa) whereas 9 spots were of high molecular (HMW) region (> 10 kDa) (Tables 1 and 2). A total of 15 spots (Nine in HMW and six in LMW) were identified by peptide mass finger printing (PMF) analysis using 2D-nano LC-MS.

Proteins of low and high molecular weight region

Since visual analyses of the images were performed, it was difficult to interpret the fold induction or fold repression. However, a total of five new spots (spot numbers 1, 8, 9, 11 and 13), six induced spots (spot numbers 2, 3, 7, 10, 12 and 14) and three repressed spots (spot numbers 4, 5 and 6) could be interpreted (Tables 1 and 3; Fig. 4; Plates 1 and 2). All these spots were expressed in LMW region. Spots 1 (New), 2, 3, 7 (induced), and 5 and 6 (repressed) were identified through PMF analysis (Table 3).

These proteins seemed to be playing significant role in cold-adaptation. Spot 1 in 6 h treatment (red box in Fig. 4) appeared as a novel protein (MW ~7.2 kDa). PMF analysis revealed that spot 1 matched with the cold shock protein A (csp A) fragment of *Lactobacillus casei* with the highest probability based Mowse score of 164 ($P<0.05$) (Table 3). Two other important peptide fragments of this protein matched with Holo (acyl-carrier-protein) synthases (EC 2.7.8.7) from *Mycoplasma mycoides* (Mowse score 54) and zinc transport transcriptional repressor from *S. thermophilus* CNRZ1066 (Mowse score 44) respectively.

Spots 2, 3 and 7 were acknowledged among upregulated (induced) proteins. The significant hits of all these spots are presented in Table 3. Spot 3 matched with one major CSP fragment of *Listeria grayii* (Mowse score 48). Several proteins showing significant hits among the two repressed spots (5 and 6) are given in Table 3 that includes probable ABC transporter from *Clostridium perfringens*, and methyl accepting chemotaxis protein t1pA of *Clostridium tetani*, among others.

Nine spots (A, V, D, Q, K, M, P, I, E; 4 new, 2 upregulated, 3 downregulated in the same sequence) differentiated in HMW region were characterised by PMF analysis (Table 4; Fig. 4). Among new important proteins, spot A matched with glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12) of *Lactobacillus delbrueckii* (Mowse score 44). Spot V matched with riboflavin biosynthesis protein and several hypothetical proteins. Spot D matched with large subunit of phage terminase *Bacillus licheniformis* (Mowse score 47). Out of the two identified upregulated (induced) spots (K and M), spot K matched against Cu/Zn-containing superoxide dismutase of *Bacillus cereus*, PTS system of lactose-specific enzyme IIA [imported] - *Clostridium acetobutylicum* amino acid amidohydrolase [imported] - *Lactococcus lactis* subsp. lactis (strain IL1403), PTS system of lactose-specific enzyme IIA [imported] - *Clostridium acetobutylicum* and several hypothetical proteins. Several important proteins identified by analysing spot M are, L-serine dehydratase α-subunit of *Streptococcus thermophilus* CNRZ1066, abi829 protein of *Lactococcus lactis* plasmid including several QSR proteins like two component multidrug ABC transporter ATP-binding protein of *Bacillus clausii* (strain KSM-K16) (Table 4). Among the downregulated (repressed) spots, P matched with ATP-dependent Clp protease and E matched with UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) of *Lactobacillus plantarum*. *Pyruvate kinase* from *L.casei* matched (Mowse Score, 106) with spot I.

St74 as a TLAB recorded growth optima of 42 °C and growth minima of just lower than 20 °C, with the best and uniform growth at 25 °C. The figures reported by Wouters *et al.,* (1999) for *S. thermophilus* CNRZ302 were 42 °C and just below 15 °C, respectively. The difference in the growth minima might be attributed to the different origin of the strain, as the environmental factors significantly affect the growth and often species and strain specificity (Tripathy, 2006; Jan *et al.,* 2000). St74 showed a growth inhibition when exposed to 10 °C from 42 °C, but was able to adapt within 5-6 h when exposed to 20 °C. The increased freeze-thaw survival of the (20 °C) adapted cells was in line with the observation of Wouter *et al.,* (1999) who reported a 100 fold increased post-freezing survival in *L. lactis* MG1363

grown at 30 °C with a treatment of 10 °C for 4 h. The adaptation is blocked by chloramphenicol suggesting the role of protein synthesis during adaptation. However, the adaptation process could not be completely blocked by chloramphenicol when the cells were exposed to 20 °C for 4 and 6 h. This phenomenon could be attributed to the incomplete block of protein synthesis and constitutive production of non-cold-induced 7 kDa CSPs as reported by Lee *et al.,* (1994) and Wouters *et al.,* (1998) observed in both *E. coli* and *L. lactis*.

Proteomics study suggested the role of various proteins during cold adaptation of St74. Spot 1 expressed in 6 h gel was found to be a new spot and matched with cold shock protein A (CSP A) fragment (Mol. Wt ~7.2 kDa; pI ~4.6) of *Lactobacillus casei* with the highest probability based Mowse score of 164 ($P<0.05$). This spot also showed 100 % sequence coverage with other bacterial CSPs including that of *Lactobacillus*.

Among others, 7 kDa CSPs were identified in *S. thermophilus* CNRZ 302 by Wouters *et al.,* (1999), whereas Perrin *et al.,* (1999) reported the presence of a new CSP of 21.5 kDa in *S. thermophilus* PB18 along with 7.5 kDa protein. The 7 kDa CSPs also corresponded to a family of *csp* genes of *L. lactis* MG1363 (Wouters *et al.,* 1998). Another important hit of this spot matched with the zinc transport transcriptional repressor from *S. thermophilus* CNRZ1066 (Mowse score 44).

As zinc is an essential constituent of many proteins**,** and is poisonous to the cell in large concentrations, its transport is regulated by *AdcR* in *Streptococcus* group. The *adcR* gene is also present in *S. thermophilus* CNRZ 1066 (Bolotin *et al.,* 2004). But the role of this protein in cold shock response in *S. thermophilus* and other microbes is yet to be ascertained. However, in *Arabidopsis thaliana*, cold-inducible zinc finger-containing glycine-rich RNA-binding protein has been found to be responsible for the freeze-tolerance (Kim *et al.,* 2005).

Among the three induced spots (2, 3 and 7) of LMW range, spot 3 matched with the major CSPs of *Listeria grayi* (Mowse score 48, Mol. wt 4.98, pI 4.21) and of *Leuconostoc mesenteroides* (Mol. wt 7.2; pI 4.72). This suggested the presence of CSP family of proteins in St74. Spot 2 matched with the chromosomal replication initiation protein DnaA [imported] of *Listeria monocytogenes* (Mowse score 47) and hypothetical protein of *Enterococcus faecalis* (Mowse score 48) (Table 3). Atlung & Hansen (1999) conferred that DnaA is a CSP in *E. coli*. Induction of DnaA protein in this study also confirmed the role of this protein in cold shock in *S. thermophilus*. The significant hits of spot 7 matched with several proteins including small subunit of acetolactate synthase III of *Bacillus cereus* (strain ATCC 10987) (Table 3).

Acetolactate synthases are found in plants, fungi and bacteria that synthesise the branched-chain amino acids by their own. Weinberg *et al.,* (2005) reported the expression of acetolactate synthases in the *hyperthermophilic archaeon*, *Pyrococcus furiosus* upon exposure to low temperature. The correlation of this enzyme with low temperature is not reported through proteomics approach in any other *Lactobacillus* and *Streptococcus* groups yet.

The significant hits of spot 5 (repressed spot) include AE007317 NID of *Streptococcus pneumoniae* R6 (a putatively uncharacterised protein), HsdS.- *Mycoplasma gallisepticum* type I restriction modification system specificity subunit domain protein, AE016747 NID (chorismate synthase**)** of *Staphylococcus epidermidis* ATCC 12228, and that of spot 6 include methyl-accepting chemotaxis protein tlpA of *Clostridium tetani*, hypothetical protein UU482 [imported] of *Ureoplasma urealyticum*, probable ABC transporter from *Clostridium perfringens*, AE001437 NID (ATPase component of ABC transporter) of *Clostridium acetobutylicum* ATCC 824. Beckering *et al.,* (2002) demonstrated the repression of several proteins including chorismate synthase and ABC transporter (ATP-binding protein) during cold-shock in *B. subtilis* by transcriptomic approach.

Presently, the roles of other repressed proteins identified in this study have not been ascertained. This is the first proteomic analysis report identifying such proteins repressed during cold shock in *S. thermophilus*. In HMW region, spots A and V were identified as new protein spots. Significant hits of spot A as presented in Table 4 related to GAPDH, PTS system, and PrsA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a rate-limiting enzyme of the glycolytic pathway under starvation conditions in microbes, expressed during cold shock in *Bacillus subtilis* (Graumann *et al.,* 1996), and thus correlates with the finding in this study. The glycolytic activity of *Lactococcus lactis* subjected to low temperature was regulated by CcpA-HPr (Ser-P) control circuit, and GAPDH was not involved (Graumann *et al.,* 1996).

Table.1 Low (<10 kDa) and high (>10 kDa) molecular weight protein spots (ref. Fig. 4)

Spot No. (6h)	Low Molecular Weight	High Molecular Weight
New Spots[#]		
1	Present	
8	Present	
9	Present	
11	Present	
13	Present	
A		**Prominent**
V		**Prominent**
D		**Prominent**
Q		**Prominent**
Induced spots[$]		
2	Prominent	
3	Prominent	
7	Prominent	
10	Prominent	
12	Prominent	
14	Prominent	
K		**Prominent**
M		**Prominent**
Repressed spots		
4 (Prominent in control)	Light	
5 (Present and prominent in control)	Absent	
6 (Dark in control)	Light	
P (Prominent in control)		**Absent**
I (Prominent in control)		**Absent**
E (Prominent in control)		**Light**

[#]: Absent in Control; [$]: Light in Control

Figure.1 *S. thermophilus* NCDC 74 subjected to cold shock (arrow) at different temperatures (°C). Growth assessment was performed in terms of OD_{650} for the control cells (grown at 42 °C) followed by a cold shock from 42 °C to 25 °C, 20 °C, 15 °C and 10 °C.

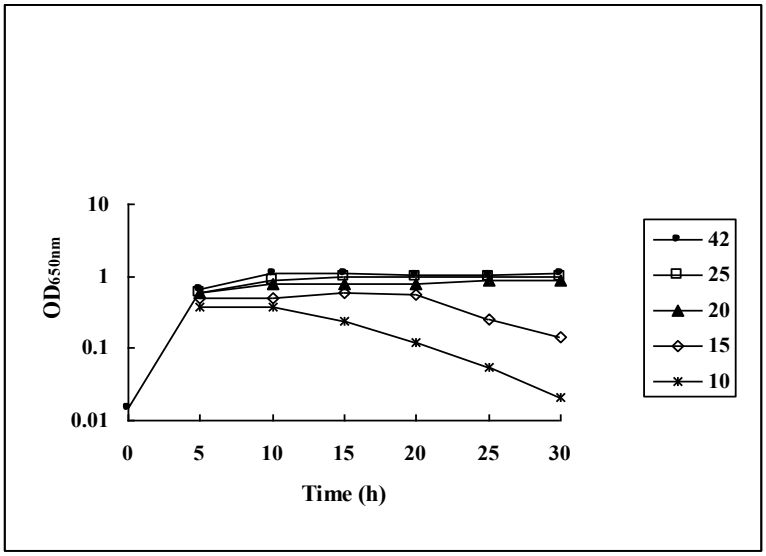


Table.2 Peptide mass fingerprinting (PMF) analysis of low molecular weight (<10 kDa) proteins of cold-stressed *S. thermophilus* NCDC 74 (in control and 6 h gel)

Spot no.	Homologous protein	Microorganism	NCBInr accession no*	Queries matched	Mowse score†	pI	Mol. mass (kDa)
New							
1	Cold shock protein A	*Lactobacillus casei*	Q70LR3_LACCA	6	164	4.6	**7.2**
	Holo-[acyl-carrier-protein] synthase	*Mycoplasma mycoides (subsp. mycoides SC*	Q6MT35_MYCMS	1	54	-	**-**
	Zinc transport transcriptional repressor	*Streptococcus thermophilus* CNRZ1066	Q5M1P4_STRTR	1	44	-	**-**
Induced							
2	Hypothetical protein	*Enterococcus faecalis (Streptococcus faecalis).*	Q830L7_ENTFA	1	48	11.98	**4.70**
	Chromosomal replication initiation protein dnaA	*Listeria monocytogenes*	AB 1432	2	47	5.73	**51.3**
3	Major cold shock protein (fragment)	*Listeria grayi*	Q48555_LISGR	1	48	4.21	**4.98**
	Cold shock protein	*Leuconostoc mesenteroides*	Q66UK1_LEUME	1	30	4.72	**7.2**
7	Acetolactate synthase III, small subunit	*Bacillus cereus*	Q73A48_BACC1	1	47	7.95	**8.52**
Repressed							
5	Putative uncharacterized protein	*Streptococcus pneumoniae R6*	Q8CYI8	5	61	26.5	**5.71**
5	Type I restriction-modification system specificity (S) subunit domain protein HsdS	Mycoplasma gallisepticum	Q7NAH4_MYCGA	2	46	23.4	**9.45**
6	Chorismate synthase	*Enterococcus faecalis* (*Streptococcus faecalis*)	Q9ANY8_ENTFA	2	40	42.4	**7.00**
6	Methyl-accepting chemotaxis protein tlpA	*Clostridium tetani*	Q895R0_CLOTE	2	47	63.5	**5.04**
	Probable ABC transporter	***Clostridium perfringens***	**Q8XK97_CLOPE**	**8**	**45**	**59.3**	**5.03**

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits

*The NCBInr library was searched by using the Mascot programme (http://www.matrixscience.com); †Probability-based Mowse score – -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology ($p<0.05$)

Table.3 Peptide mass fingerprinting (PMF) analysis of high molecular weight (>10 kDa) proteins of cold-stressed *S. thermophilus* NCDC 74 (in control and 6 h gel)

Spot no.	Homologous protein	Microorganism	NCBInr accession no*	Queries matched	Mowse score†	pI	Molecular mass (kDa)
New							
A	Glyceraldehyde-3-phosphate dehydrogenase	*Lactobacillus delbrueckii*	Q6F1H0_MESFL	2	44	5.51	**36.5**
	Parvulin-like peptidyl-prolyl isomerase, *B. subtilis* ortholog	*Clostridium acetobutylicum*	D97295	2	43	9.08	**37.6**
	PTS system, beta-glucoside-specific, IIB component	*Listeria monocytogenes*	Q71W88_LISMF	1	42	5.4	**109.4**
V	Cell surface protein	*Bacillus cereus*	Q816D8_BACCR	2	48	5.27	**37.6**
	Riboflavin biosynthesis protein	*Staphylococcus aureus*	Q6G9U1_STAAS	1	41	5.69	**36.69**
Induced							
K	Cu/Zn-containing superoxide dismutase	*Bacillus cereus*	Q6RT16_BACCE	1	49	4.9	**30**
M	L-serine dehydratase alpha subunit.-	*Streptococcus thermophilus* CNRZ1066	Q5LZ51_STRTR	6	59	4.7	**29.8**
	Two-component sensor histidine kinase BH0397 [imported] -	*Bacillus halodurans*	E83699	6	42	6.03	**59.13**
Q	Predicted Permease	*Clostridium acetobutylicum*	AAK78123	1	41	9.23	**45.7**
Repressed							
P	ATP-dependent C1p protease, ATP-binding subunit C1pB	*B .thuringiensis*	Q6HM12_BACHK	3	50	5.35	**97.6**
I	Pyruvate kinase	*Lactobacillus casei*	Q6XBK1_LACCA	2	106	5.20	**62.7**
E	**UTP—glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)**	***Lactobacillus plantarum***	**D7V9Z9_LACPL**	**5**	**140**	**6.04**	**34.40**

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits

*The NCBInr library was searched by using the Mascot programme (http://www.matrixscience.com); †Probability-based Mowse score – -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 42 indicate identity or extensive homology ($p<0.05$)

Figure.2 Low temperature adaptation of *S. thermophilus* NCDC 74 expressed in terms of freeze thaw survival capacity. *S. thermophilus* NCDC 74 was cold shocked from 42 °C to 20 °C. Percent survival is expressed as the percentage of surviving cells in relation to the amount before freezing (100%). Cm: Chloramphenicol

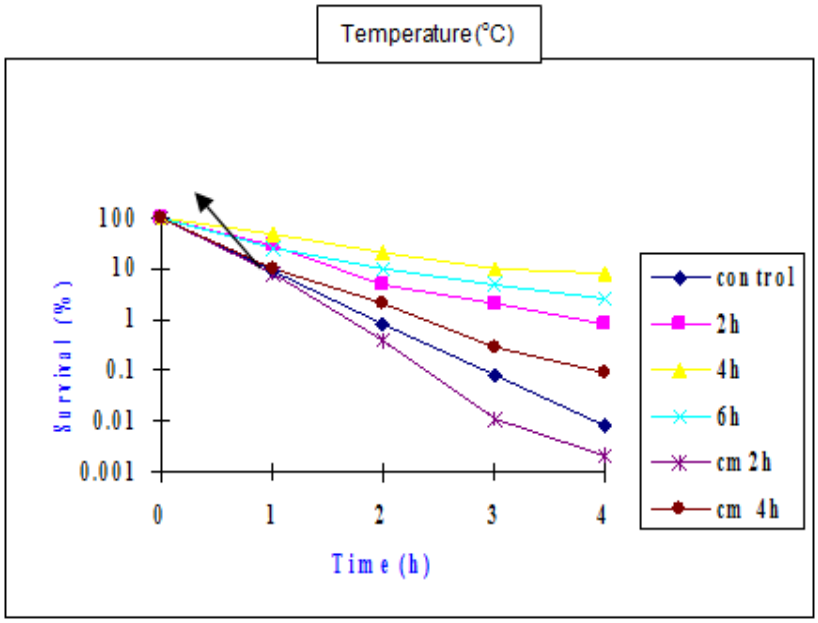


Figure.3 Low temperature adaptation of *S. thermophilus* NCDC 74 cold shocked from 42 to 10ºC. Freeze thaw survival capacity was expressed as the percentage of surviving cells in comparison to the amount prior to freezing (100%). Cm: Chloramphenicol

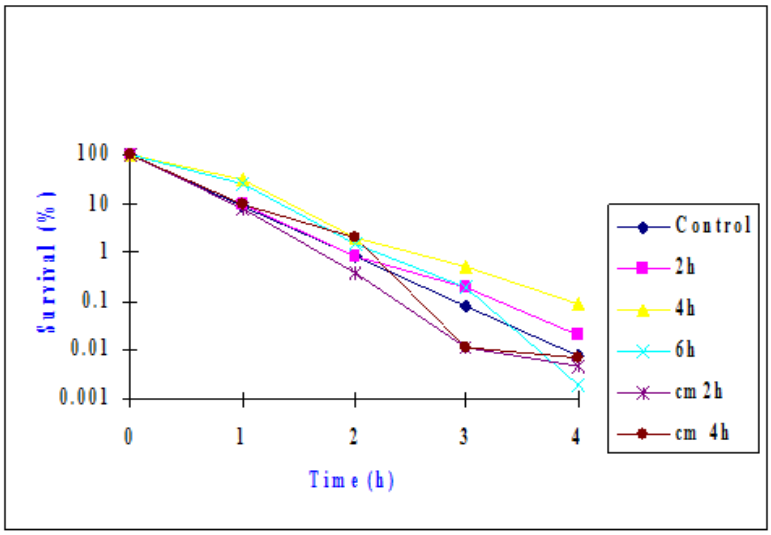
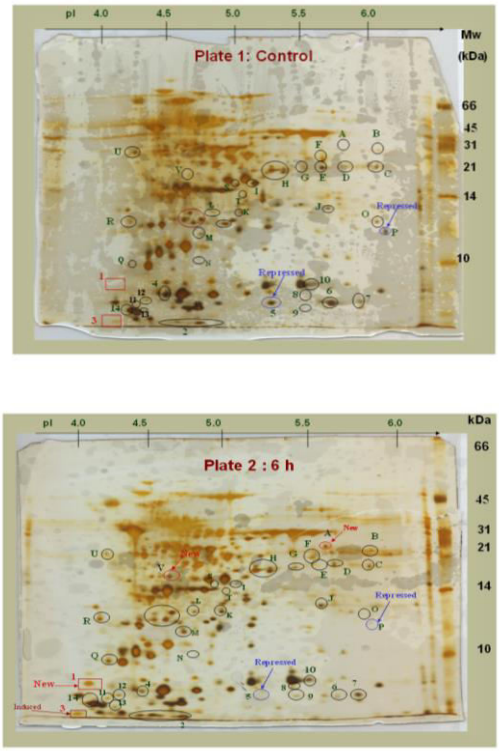


Figure.4 Plates 1 and 2: Proteomic analysis of whole cell (cell-free) extracts of *S. thermophilus* NCDC 74. Gels are representative of three repeated experiments. Plate1: 2D gel image of *S. thermophilus* NCDC 74 grown at 42ºC. Plate2: 2D gel image of *S .thermophilus* NCDC 74 exposed to cold shock from 42 °C to 20 °C for 6h. Proteins of low molecular weight are numbered as 1, 2 etc. whereas proteins of high molecular weight are represented by Capital letters *e.g.*, A, B etc. Spots matched with cold shock proteins are boxed.



Non-sugar-specific proteins enzyme I and HPr combined with the sugar-specific enzyme II are responsible for catalysis of PTS system in lactic acid bacteria (Luesink *et al.,* 1999). Transportation of glucose and lactose in lactic acid bacteria is performed by phosphotransferase (PTS) system. The IIB component of PTS system induced in *S. thermophilus* in this study also indicates the involvement of HPr and other proteins of PTS system in glycolysis. Besides this, several proteins of unknown functions have also been identified (data not presented here).

Another significant hit of spot A protein PrsA also was a cold inducible protein (CIP) recognised in this study. The PrsA protein helped in post-translocational folding of exported proteins in *Bacillus subtilis* and is dependable with the homology of a PrsA segment of parvulin-type peptidyl-prolyl *cis*/*trans* isomerases (PPIase) (Vitikainen *et al.,* 2004). The PPiB protein in *B. subtilis* belonging to the family of cyclophilins that catalyse the isomerisation of peptidyl prolyl bonds is cold-shock induced. Thus, PPiB is important during low temperature adaptation of *B. subtilis*, and this could be the case for other bacterial cyclophilins (Herrler *et al.,* 1994). The trigger factor in *E. coli*, which possesses prolyl isomerase activity, is a cold inducible protein (CIP) (Jones & Innouye, 1994), which leads to the assumption that isomerase activity is important at low temperatures (Grauman *et al.,* 1996). Peptidyl-prolyl *cis*/*trans* isomerases were also induced in cold-adapted archaeon *Methanococcoides burtonii* (Goodchild *et al.,* 2004). Thus, the induction of PTS system in St74 when exposed to low temperature is not surprising. Spot V matched with riboflavin biosynthesis and several hypothetical proteins. Riboflavin synthesis protein induction was also observed in cold-adapted archaeon *Methanococcoides burtonii* (Goodchild *et al.,* 2004).

Of the spots P and E of HMW region, spot P matched with the ATP-dependent C1p protease, ATP-binding subunit C1pB. The Clp ATPases can function both as molecular chaperones and as regulator components of the proteolytic complex (Gottesman *et al.,* 1997; Wawrzynow *et al.,* 1996; Wickner & Maurizi, 1999). Most of the clp genes are heat-stress induced as revealed

in *B. subtilis* and *E. coli* (Yura *et al.,* 1996; Kruger & Hecker, 1998). The c1p genes related to class III group of heat shock gene in B. subtilis are negatively regulated by the CtsR repressor, and are regulated by the σ^{32} factor in *E. coli*. In the present study, the repression of this protein in 6 h suggested the down-regulation of both *C1pP* and *C1pB* genes of St74 may be involved in protein synthesis during cold stress. Porankiewicz *et al.,* (1997) demonstrated that the induction of *C1pP1* and *C1pB* in cyanobacteria occurred during cold shock. They suggested that, *C1pB* might renature and solubilise the aggregated proteins at low temperatures at which the translation was repressed. Spot E, another identified repressed protein, matched with UTP-glucose-1-phosphate uridylyltransferase which catalyses the conversion of D-glucose-1-phosphate and UTP into UDP-D-glucose. This enzyme is induced during acid tolerance response in *L. lactis* (Verneuil *et al.,* 2005). Down-regulation of this protein suggested that the organism may not cope with the production of this protein during a cold shock.

St74 has the capacity to adapt to low temperature (within 5-6 h at 20 °C). The freeze-thaw survival increased in adapted cells compared to the control. Proteome analysis identified two cold shock proteins (spots 1 and 3) of 7.2 kDa molecular weight region. Other cold shock proteins identified were zinc transport transcriptional repressor (AdcR) (Mowse score 44) and holo acyl carrier protein synthase (Mowse score 54). Several cold inducible proteins identified include glyceraldehyde-3-phosphate dehydrogenase, PTS system, riboflavin biosynthesis biosynthesis protein, PrsA, dnaA, acetolactate synthase III, superoxide dismutase, l-serine dehydratase, and two component sensor histidine kinase. The identified repressed proteins include chorismate synthase, Type I restriction-modification system specificity subunit domain protein HsdS, methyl-accepting chemotaxis protein tlpA, ATP-dependent C1p protease, ATP-binding subunit C1pB, UTP—glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) among others. All these proteins were identified when the culture was exposed to 20 °C for 6 h. For the first time, some of the above proteins were identified using proteomic approach in St74. The observed post-freezing increased survival would definitely help in preserving this commercial strain. However, future studies regarding the exact mechanisms of these proteins in relation to low temperature adaptation in *S. thermophilus* require an integrated Omics approach.

Acknowledgements

The authors acknowledge the Director, NDRI for providing the necessary facilities and funding for this work. The facilities utilised at the School of Biotechnology for completion of the work is also acknowledged.

Author Contributions

Prangya P. Tripathy: Investigation, formal analysis, writing—original draft. Ashok Kumar Mohanty: Validation, methodology, writing—reviewing. P. K. Aggarwal:—Formal analysis, writing—review and editing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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How to cite this article:

Prangya P. Tripathy, Ashok Kumar Mohanty and Aggarwal, P. K. 2025. Proteomic Analysis of Thermophilic Starter *Streptococcus thermophilus* NCDC74 on Low Temperature. *Int.J.Curr.Microbiol.App.Sci.* 14(02): 103-115.
doi: https://doi.org/10.20546/ijcmas.2025.1402.009