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Identification and characterization of psychrotrophic strain of *Planococcus maritimus* for glucosylated C_{30} carotenoid production

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Cold-adapted bacteria produce various pigments as their adaptive strategy. Here, we studied an aerobic, gram-positive motile coccoid bacterial strain KK21, isolated from the Siachen, Himalayas and characterized the major pigments present in it. The KK21 strain is capable of growth in a broad range of temperature (–4 to 37°C), pH (6.0-13.0) and salinity (0.5-8%). Phylogenetic analysis based on 16S rRNA gene sequence showed that it belongs to the genus *Planococcus* and closely related to *P. maritimus*. Total pigment extraction was best found in polar methanol. Alanine among amino acids, NaCl among inorganic salts and evening primrose amongst oils best supported the pigment production at 10°C after 6 days in BHI at neutral pH of 7.0. Maximum pigment production was recorded at 3% of NaCl concentration. In photoprotection activity, radiation exposed plates showed increased colony forming units and more than 3.5 times of total carotenoid production after 15 min exposure compared to the control group. Detailed characterization of orange pigment was done systematically by TLC, HPLC and MALDI-TOF. Chromatographic and MALDI-TOF data have revealed the exclusive presence of glycosylated C_{30}-carotenoid in *P. maritimus* KK21 with m/z of 655.871.

Keywords: Carotenoid pigment, Cold-adapted bacteria, Halotolerance, MALDI-TOF, Photoprotection activity, Psychrophile, Siachen Himalayas

Over half of the earth's surface viz. polar and high alpine regions, deep oceans, marine sediments, mountains, glaciers, cold deserts, permafrost soils, etc., is under extreme cold condition. Cold is a physical stress that drastically modifies all physical and chemical parameters of a living cell, thereby influences solute diffusion rates, enzyme kinetics, membrane fluidity and conformation, flexibility, topology, and interactions of macromolecules, such as DNA, RNA and proteins. However, these cold environments are teeming with life. Bacterial species adapted in extremely cold temperatures exhibit various adaptation mechanisms to regulate their metabolic functions. Several physiological and metabolic changes are included in adaptations that assist in growth in a number of ways. Survival is facilitated by synthesis of cryoprotectants, cold acclimation proteins, cold shock proteins, RNA degradosome, antifreeze proteins and ice nucleators[1-3]. Adaptations include changes in the outer cell membrane to changes in the central nucleoid of the cell; adaptations that have been required to increase membrane fluidity include an increased content of large lipid head group, proteins and non-polar carotenoid pigments[4]. However, these adaptive strategies did not seem to be widespread and studies showed more compact lipid head groups[5] and decreased non-polar carotenoid pigment synthesis in some psychrophiles[6].

Carotenoids are a group of extremely hydrophobic molecules with little or no solubility in water, which usually do not increase with increase in temperature[7]. They have an important role in photoprotection and photosynthesis in eukaryotes and mesophilic prokaryotes[8,9]. Temperature played a significant part in pigmentation strategies as reported in *Arthrobacter agili*[6] and *Micrococcus roseus*[10], in which pigment production was maximum at low temperature. Extracted pigments from cold-adapted bacteria showed multiple absorption peaks between 400-550 nm, characteristic of carotenoid pigments[11,12]. Many species of *Planococcus* have been isolated from diverse environments such as high Arctic permafrost, marine solar saltern, pigeon faeces, sea water, soil and cyanobacterial mats[13,14].

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In this study, we made an attempt to search for an alternative source of pigments and optimization of conditions for production of carotenoids for biotechnological applications. We describe *Planococcus Maritimus* KK21 isolated from the soil sample collected from Siachen, India. Apart from identifying the strain with a combination of phenotypic properties and phylogeny based on 16S rDNA sequence, we confirmed the presence of carotenoids by thin layer chromatography, purifyied by reverse phase HPLC C_{18} column and characterized the major pigment by MALDI-TOF.

Materials and Methods

Sample collection and isolation of strain

Soil samples were collected at random sites around Central post Siachen Himalayas, (35.421226°N 77.109540°E) in the month of September. The soil temperature varied from – 6 to + 6°C. The surface layer of 0·5 cm was cleared with a sterile spatula and the underlying soil was collected and brought to the laboratory in an ice box maintained at 4°C .

The bacterial strain was isolated from composite soil sample by dilution plate method on Antarctic Bacteria Medium (ABM, containing 0·5% peptone, 0·1% yeast extract, 1·5% agar)[15] at 15°C after 7 days of incubation.

Optimization of growth conditions

The optimum temperature and pH for growth of the isolate was determined and the culture was grown under the optimum conditions to determine the generation time. Salt tolerance was tested by supplementing the plates with appropriate concentrations of NaCl (0·5-8%). Cultures in the log phase of growth were observed under the phase contrast microscope (Leica, Germany) for cell shape and size. All tests were performed by incubating the cultures at 20°C. Growth under anaerobic conditions was determined after incubation at 20°C in anaerobically prepared Tryptone Peptone Glucose Yeast Extract serum vials flushed with nitrogen.

Enzymatic and biochemical activities

The amylolytic, proteolytic, and lipolytic activities of the isolate were determined by employing the method of Singh *et al.*[16]. Alkaline phosphatase, β-galactosidase[17] and cellulase activities[18] were qualitatively determined. Gelatin liquification capability of the isolates was checked on nutrient gelatin tube. Production of indole, utilization of citrate, reduction of nitrate to nitrite, and hydrolysis of Tween 80 and esculin were measured following standard procedures and the results of the biochemical tests were analyzed by comparing with Bergey's Manual of Systematic Bacteriology.

Utilization of amino acids as carbon source

The ability of the culture to utilize an amino acid as carbon source was checked by taking carbon compound in minimal medium without citrate (1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 1.5% agar). Sucrose at a concentration of 20 mM was added as carbohydrate source and different amino acids (21 in number) at 10 mM were added in medium, as described by Mechichi *et al.*[19]. Tests were scored positive when growth of strain occurred as compared with strains grown in minimal medium without carbon source.

Antibiotic sensitivity test

Sensitivity of the culture to various antibiotics was tested by using antibiotic discs purchased from Hi-media Pvt. Ltd., India. Ninety mm diameter sterile Petri dishes with ABM agar medium were used for surface spreading freshly grown cultures. Plates were incubated aerobically at 20°C for 48 h and zone of growth inhibition was analyzed by zone reader (Himedia, India).

Phylogenetic analysis

DNA was isolated by using the Gram-positive protocol of the DNeasy Blood and Tissue kit (Qiagen) and the 16S rRNA gene was PCR amplified by 16S rRNA bacterial universal primers fP1 (5'-GAGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGG CTACCTTGTTACGACTT-3'). The DNA after PCR amplification was purified using purification kit (MinElute™ Gel Extraction Kit, Qiagen). Purified PCR products were lyophilized and sent for sequencing to Chromous, Bangalore (India). BLAST search was done for phylogenetic analysis on http://blast.ncbi.nlm.nih.gov/Blast.cgi. A sequence similarity search was performed using BLAST N. Sequences of closely related taxa were retrieved and aligned with CLUSTAL X program[20]; the alignment was corrected manually. Representative sequences in the BLAST result showing >97% sequence identity and originating from low temperature habitats were also included in the alignment file in addition to the related reference strains, all the sequences were downloaded from nucleotide sequence libraries: Genbank, DDBJ, EMBL, (www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed with the help

of online phylogenetic tree constructing website (www.phylogeny.fr.).

Quantification and optimization of total carotenoids

The extraction of pigments from cell pellet was done in different polar and non-polar organic solvents. Basal medium was used as minimal growth medium and cell pellet to extract pigments with organic solvents[6,21-23]. A pre treatment step of cell disruption to extract total pigments from bacterial pellet was followed in which 10-15 mg lyophilized bacterial cell pellet was taken in 15 mL falcon tubes and ice cold HPLC grade methanol was added to it. It was then sonicated for 10 min at 3 s interval (0.5) pulses. Extraction of total pigments collected from cell lysate pellets of bacteria was done by repeated extractions, every time leaving in methanol for 15 min at room temperature of 26°C[24]. The extracts were collected in brown glass vials and total extracted pigments were filtered through 0.22 µm nylon syringe filters (Millipore, USA). Total volume of pigmented methanolic extract was used to quantify total carotenoid/ pigment content in bacteria according to the method of Rodriguez- Amaya & Kimura[25].

$$\text{Total carotenoid content in bacteria } (\mu g\ g^{-1}) = \frac{A_{Total} \times \text{volume (ml)} \times 10^4}{A^{1\%}_{cm} \times \text{sample wt. (g)}}$$

where, A_{Total}= Absorbance at 474 nm; Volume= Total volume of extract (ml); and $A^{1\%}_{cm}$= Absorption coefficient of carotenoids mixture in methanol.

To study the effect of cultivation temperatures on pigments/carotenoid production, the experiment was carried out at 5, 10, 15, 20, 25, 30, 37 and 42°C in ABM broth inoculated with culture, keeping all other conditions at their standard level. Similarly, in order to obtain the optimum incubation period for pigment production, ABM broth with inoculated culture was incubated for different time durations (24, 48, 72, 96, 120, 144 and 160 h) and then quantified for pigment. To determine the effect of pH of ABM on pigment production, experiments were performed with media of different pH (4, 5, 6, 7, 8, 9, 10, 11, 12) adjusted with 0.1M NaOH and 0.1N HCl. The optimum temperature, time and pH achieved as above were fixed for subsequent optimization experiments. The optimization for maximum production of pigments/ carotenoid was done for other variable parameters viz. different amino acids (alanine, asparagine, glutamic acid, glycine, leucine, proline, cystine, tryptophan, tyrosine and valine at a concentration of 20 mM), NaCl (0.5-10% conc.) inorganic salts ($FeCl_3$, NaH_2PO_4, $MnCl_2$, Na_2CO_3, KH_2PO_4, $(NH)_2SO_4$, NaCl, NH_4Cl and KCl) at a concentration of 20 mM), essential oils (0.2%) (Tea tree oil, Walnut oil, Lavender oil, Rosemary oil, Jojoba oil, Neroli oil, Sarson oil, Evening Primrose oil, Palm Rosa oil, Neem oil and Sweet Almond oil) and different culture media [Antarctic Bacteria Medium (ABM), Tryptic Soy Broth (TSB), Brain Heart Infusion Broth (BHI) and Nutrient Broth (NB)].

Carotenoid purification and identification

Partial purification of total extracted pigments was carried out on TLC plates in reference with beta-carotene as standard; 1:3, hexane:methanol was used as developing solvent in chamber [26]. Purification of extracted pigments was done using C_{18} reverse phase column (4.6×250 mm) HPLC system. 10 µL of 0.22 µm nylon syringe filtered (Millipore, USA) pigment extract was injected into the system and run was programmed for gradient mobile phase of A 9:1 methanol:DCM; B 1:1 methanol:dichloromethane; C 3:1 methanol:DCM; and D 3:1 DCM: methanol at a flow rate of 1 mL/min for 20 min run. Purified pigment peak fraction was collected and subjected to Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS) to obtain m/z ratio.

Photoprotection activity

To check the potential role of pigmentation as photoprotectant in bacteria, 6 mL of culture broth (in late log phase) was centrifuged at 8000 rpm to collect the cells. Serial dilution was made in normal saline and plated on nutrient agar plates, control set was not exposed to UV light while experimental set was exposed to 6W UV-radiations using UV lamp (Spectroline, USA) of 280-300 nm for 15, 20 and 30 min. Plates were stored in dark to avoid photoreactivation. The bacterial colonies were enumerated with respect to control set at the end of incubation of 3, 6, 8 days at 15°C and pigment produced was quantified by the equation provided by Rodriguiz-Amaya & Kimura[25].

Results

Growth conditions

The psychrotrophic isolate, *P. maritimus* KK21 was found to be well adapted to cold temperatures as it could grow between –4 to 37°C, optimum temperature being 15°C. The pH range tolerated was between 6.0-13.0 but the best growth took place from

7.0 to 9.0. Halotolerance spanned between 0.5 to 8% NaCl, however the best tolerance was up to 5% NaCl which showed that high salinity is not essential for its growth. This isolate grew luxuriously in aerobic conditions but could survive in anaerobic conditions also.

Identification and biochemical characterization

Cells of the selected strain were Gram-positive, aerobic, cocci, and occurred singly or in pairs. Electron microscopic picture of bacterial cells is given in Fig. 1. Colonies grown on trypticase soy agar were orange-coloured and about 1–2 mm in diameter, circular, smooth and convex.

KK21 strain was found to be positive for citrate utilization, casein hydrolysis, nitrate reduction and oxidase but negative for starch hydrolysis, urease production, hydrogen sulfide production, cellulase, esculin, β-galactosidase, lipase production, Tween 80 hydrolysis, gelatin liquification, Indole, methyl red and Voges–Proskauer reaction. Acid was produced from adonitol, cellobiose, D-arabitol, D-arabinose, D-galactose, D-maltose, D-mannose, D-melizitose, D-xylose, dextrose, dulcitol, fructose, inulin, inositol, L-arabinose, L-rhamnose, L-sorbose, lactose, melibiose, mannibiose, raffinose, salicin, sorbitol, sucrose and terahalose but not from glucose.

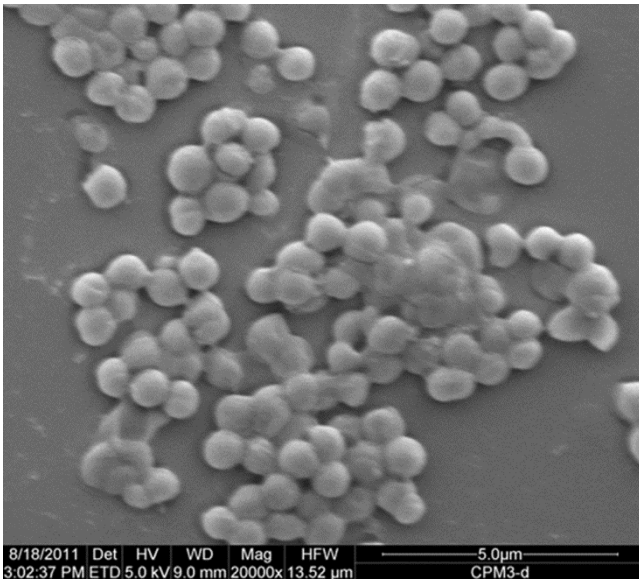

Fig. 1 — Scanning Electron micrographs of *Planococcus maritimus* KK21at magnification of 20000X.

The isolate utilized alanine, arginine, cystine, glycine, histidine, iso-leucine, leucine, lycine, methionine, proline, serine, threonine, tyrosine and valine as energy sources but was unable to utilize asparagine, glycine, 4-hydroxy proline, glutamic acid, glutamine, phenylalanine and tryptophan for the same. Biochemical test results are summarized in Table 1.

It showed sensitivity to azlocillin (75 µg), cefixime (5 µg),), lomefloxacin (10 µg), mezlocillin (75 µg), moxalactam (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), neomycin (30 µg), oxacillin (5 µg), oxytetracyclin (30 µg), piperacillin (100 µg), streptomycin (10 µg), sulphadiazine (300 µg), sulphafurazole (300 µg) and tobramycin (10 µg), but was resistant to aztreonam (30 µg), azlocillin (75 µg), carbencillin (100 µg) cefpodoxime (10 µg), cephradine (25 µg), kanamycin (30 µg), metronidazole (5 µg), nitrofurazone (100 µg), novobiocin (30 µg) and roxithromycin (30 µg).

Sequence analysis data from Genbank revealed that the isolate unambiguously belonged to the genus *Planococcus* with the close fit (99% similarity) to *P. maritimus* strain AMP-10. Thus, the isolate was proposed as *P. maritimus* and 1395 bp long partial sequence of 16S rRNA gene was submitted to

Table 1 — Summary of biochemical characteristics of *Planococcus maritimus*.

Name of test	Results
Citrate utilization; Casein hydrolysis; Nitrate reduction and Oxidase	Positive
Starch hydrolysis; Urease production; Hydrogen sulfide production; Cellulase; Esculin; β-galactosidase; Lipase production; Tween 80 hydrolysis; Gelatin liquification; Indole; Methyl red; and Voges–Proskauer reaction	Negative
Acid production by sugar utilization	Positive by following sugars: Inulin, D-arabitol, D-arabinose, adonitol, salicin, D-galactose, D-mannose, L-rhamnose, D-melizitose, L-sorbose, D-xylose, raffinose, cellobiose, terahalose, sucrose, L-arabinose,D-maltose, mannibiose, dulcitol, melibiose, fructose,inositol, sorbitol, lactose and dextrose
Carbon source utilized	Positive by following carbon sources: Proline, serine, threonine, arginine, alanine, tyrosine, cystine, valine, methionine,leucine, iso leucine, glycine, histidine and lysine

GenBank nucleotide sequence database with JN 638058 as its accession number. Phylogenetic relatedness of the isolate is shown in Fig. 2.

Carotenoid pigment production and purification

Extraction of pigments from cell pellet of strain KK21 was not found in octane, dichloroethane, petroleum ether, ethyl acetate, toluene, dietyl ether, ethanol, acetonitrile, DMSO, pyridinine, acetone, n-hexane, acetic acid, xylene and water. However, methanol gave positive results. Yellow orange pigments found in bacterial cells were carotenoids.

Parameters contributing in maximum pigment production in bacteria were checked; details given in graphical representation in Fig. 3 (A-H). Out of different amino acids tested, alanine produced maximum pigment (1.146 µg g^{-1}), followed by asparagine (1.140 µg g^{-1}) and least in glycine and glutamic acid (1.08 µg g^{-1}). $MnCl_2$ was found to be the best (1.2 µg g^{-1}) among inorganic salts, whereas NaH_2PO_4 the least (1.081 µg g^{-1}). Out of 4 media tested, TSB and BHI resulted in best production of carotenoid. The pigment production started at 24 h but steep increase was observed after 48 h, which remained constant up to 144 h and then declined. As regarding the effect of temperature, the strain KK21 could produce the pigment in wide range of temperatures i.e. from 5-42°C. However, 10°C was found to be most conducive. Among 11 essential oils tested, Evening primrose oil produced maximum carotenoid (1.149 µg g^{-1}), while the least was found in rosemary and sweet almond oils (1.066 µg g^{-1}). The isolate showed maximum carotenoid at neutral pH of 7 and minimum at basic pH of 12, while it was unable to show cellular growth at pH of 13. Halotolerance for the isolate was also checked; it showed optimum growth at 5% NaCl (carotenoid concentration of 1.098 µg g^{-1}), while maximum carotenoid production was observed at 3% NaCl concentration (1.186 µg g^{-1}). It can be inferred that none of the parameters resulted to be a limiting factor in pigment production.

Experimental set of radiation exposed nutrient agar plates showed exceptional increase in colony forming unit (CFU) of 365×10^6 at 15 min and decreased colony forming unit (CFU) of 298×10^2 at 20 min and

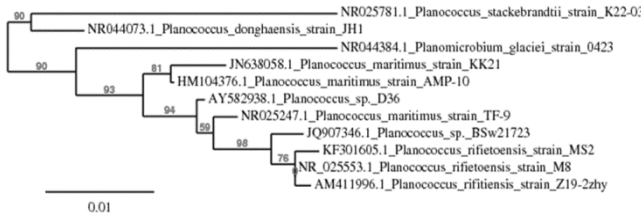

Fig. 2 — Neighbour-joining tree based on 16S rDNA sequences showing the position of *Planococcus maritimus* KK21. Bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points.

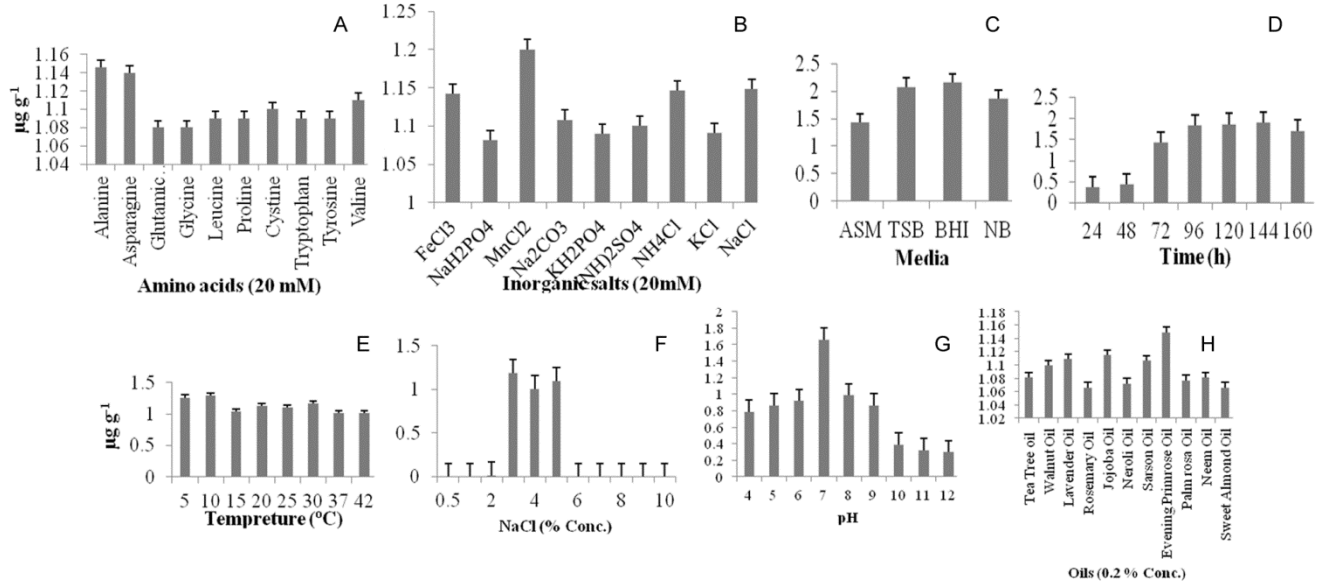

Fig. 3 — Factors contributing in optimization of pigment production (A) amino acids; (B) inorganic salts; (C) Brain Heart Infusion broth (2.16 µg g^{-1}) among four culture media tested; (D) After time period of 144 h (1.901 µg g^{-1}) of culture; (E) At a temperature of 10°C (1.297 µg g^{-1}); (F) in 3% NaCl concentration (1.186 µg g^{-1}); (G) Neutral pH of 7 appears to be best suited for maximum pigment production; and (H) Among 11 oils tested evening primrose oil (1.149 µg g^{-1}) gave maximum synthesis of pigments.

218×10^2 at 30 min, respectively compared to control set (without UV exposed plates) with CFU of 276×10^2. Total carotenoid content at the end of experiment came out to be 130 µg g^{-1} for control set, and 482, 158 and 156 µg g^{-1} for 15, 20 and 30 min exposed plates, respectively. It can be inferred that pigment production is a strategy that helps to survive the stressful conditions where bacteria reside in environment, as the radiation exposed plates showed increase in total pigment content.

Pigment extract from the isolate gave five pigment fractions on TLC plate with Rf of 0.97, 0.93, 0.91, 0.81 and 0.30. The HPLC chromatogram of extract from *P. maritimus* selected for the study showed two major peaks and several minor ones as can be ascertained from Fig. 4A. Both major peaks observed could be tentatively identified as belonging to carotenoid family on the basis of retention time (Peak 1 same as standard beta carotene) and absorption spectra at 458 nm and 437 nm, respectively. Purified peak fraction (peak 1) when subjected to MALDI-TOF, the $M+H^+$ peak was observed at m/z 655.871 as depicted in Fig. 4B. Carotenoids generally exhibit $M+H^+$ at m/z 537 approx.

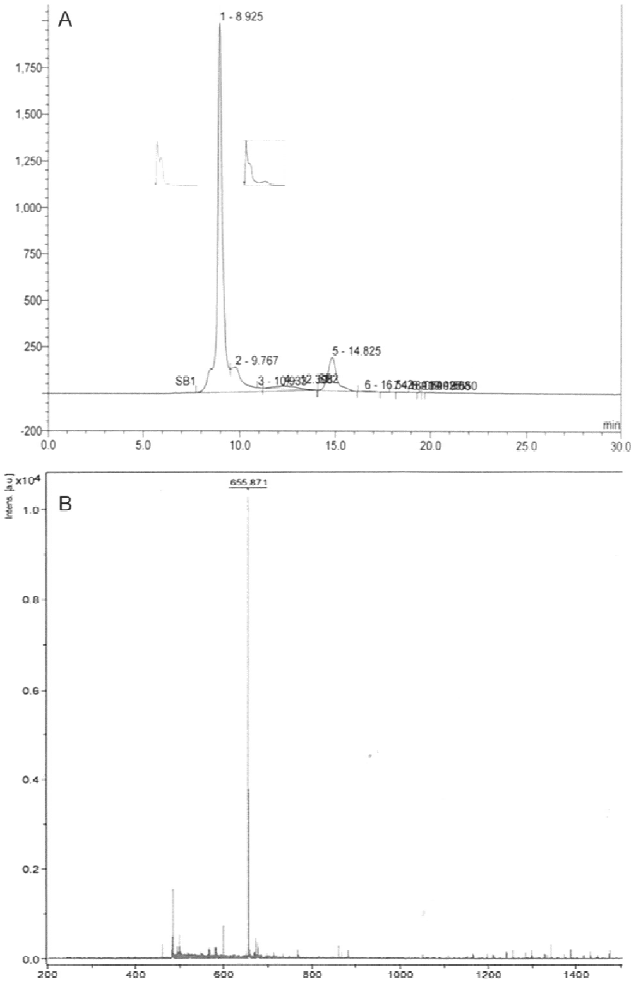

Fig. 4 — (A) HPLC chromatogram of pigment extract from *Planococcus maritimus* KK21 showing two major peaks and several minor peaks; and (B) MALDI-TOF showing $(M+H)^+$ at m/z 655.871

Discussion

Planococcus maritimus KK21 studied here could grow between wide range of temperature and pH. In earlier studies, carotenoid of *P. maritimus* KK21 was detected through FTIR and Raman spectroscopy techniques and found to have antimicrobial activity against four pathogenic bacterial strains viz. *Vibrio cholerae*, *Shigella dysenteriae*, *Bacillus cereus* and *Staphylococcus aureus*[27,28]. Most of the *Planococcus* species isolated so far seems to be well adapted to the extreme of temperatures or salt environment in which they occur. *P. halocry*ophils and *P. stackbrandii* were found to be best adapted to harsh conditions of Arctic permafrost and salty conditions as studied by Mayilraj *et al.*[13] and *P. antarcticus* and *P. psychrophilus* from Antarctica by Reddy *et al.*[14].

Yellow orange pigments found in bacterial cells were carotenoids. With very few exceptions, carotenoids are lipophilic in nature and as they are insoluble in water they need to be extracted in organic solvents. Factors affecting carotenoid production in *P. maritimus* were studied by Chaudhari & Jobanputra[29] in which they found best carotenoid production with sucrose, yeast extract, NH_4Cl, KH_2PO_4 and $MgSO_4.7H_2O$, moderate production with $(NH_4)_2SO_4$ and NaH_2PO_4, while least with peptone and $FeSO_4.7H_2O$ in the culture medium. Shatila and coworkers studied factors affecting pigment production in mesophilic strain of *Exiguobacterium aurantiacum* which showed maximum pigment production after 3 days of incubation[30]. Carotenoids have been lately used from industries to human health[31,32].

The radiation exposed nutrient agar plates exceptionally showed higher CFU after 15 min exposure but CFU decreased after 20 and 30 min exposure, and also increased total pigment content was observed after 15, 20 and 30 min exposure as compared to control during the experiments which proves the carotenoid production as one of the strategies of bacterial cells growing in extreme conditions to tolerate the stress conditions. Many organisms have developed mechanisms to compensate

damaging effects of UV radiations and possessed mainly nucleotide excision repair and photoreactivation. Low CFU in *Planococcus* plates irradiated with UV clearly demonstrates a dose dependent sensitivity to radiation and can be due to long exposure to radiations resulted in either damage of DNA or cell membrane. Increased total pigment content by survived cells in 30 min exposed plates in comparison to control signifies role of pigments (carotenoids) in photoprotection and protecting bacterial cell walls against damage. As carotenoids are known to act as an important photoprotectant in the UV rays that inflict damage on DNA and cell membrane[33,34]. Similar to our results, Vaz *et al.*[35] studied the role of pigmentation in photoprotection of yeast cells and concluded it as a survival strategy to cope up the stress. Likewise, high frequency of pigments in heterotrophic isolates from Antarctica and in recovered isolates from ice cores, glaciers, or marine surface waters suggested that pigmentation played a role in adaptation to cold environments[36-39]. Jaggandham *et al.*[40] however reported the role of carotenoids in modulation of membrane fluidity in bacteria growing under low temperature conditions.

Presence of glucosylated C_{30} carotenoids is typically found in a limited number of Gram positive bacteria i.e, *Methylobacterium rhodinum*[41], *Streptococcus faecium*[42], *Heliobacteria*[43] and *Staphylococcus aureus*[44]. Triterpenoids have been identified in the G(+)ve bacteria such as *Planococcus* by Shindo and his team[45] and β-apocarotenoids instead of 4,4'-diapocarotenoids in *Halobacillus* by Osawa and coworkers[46]. The spots with different migration rates are as follows: yellow (Rf 0.91, same as beta carotene standard), pink (Rf 0.80), light yellow (Rf 0.62) and pinkish orange (Rf 0.30). The yellow pigment gave absorption maxima at 458 nm and designated as carotenoid (C_{458}). TLC system of pigment extract from *Bacillus indicus* revealed yellow and orange *Bacillus* pigments with Rf values of 0.95 and 0.91, respectively[26]. KK21 strain showed $M+H^+$ peak at m/z 655.871, which was the same as methyl glucosyl-3,4-dehydro-apo-8'-lycopenoate in *P. maritimus* which gave m/z 625.37 due to attachment of sugar in the carotenoid[45].

Conclusion

The strain KK21 was validated as *Planococcus maritimus* and it was characterized for maximum pigment production. Detailed characterization of orange pigment by TLC, HPLC and MALDI-TOF revealed the exclusive presence of glycosylated C_{30}-carotenoid with m/z of 655.871.

Conflict of interest

Authors declare no conflict of interests or violation of Animal rights during the study.

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