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Astaxanthin from psychrotrophic *Sphingomonas faeni* exhibits antagonism against food-spoilage bacteria at low temperatures

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ABSTRACT

Food production and processing industry holds a perpetual relationship with microorganisms and their by-products. In the present study, we aimed to identify beneficial cold-adapted bacteria devoid of any food spoilage properties and study their antagonism against common food-borne pathogens at low temperature conditions. Ten isolates were obtained on selective isolation at 5 °C, which were spread across genera *Pseudomonas*, *Sphingomonas*, *Psychrobacter*, *Leuconostoc*, *Rhodococcus*, and *Arthrobacter*. Methanol extracts of strains were found to contain several bioactive metabolites. Among the studied isolates, methanol extracts of *S. faeni* ISY and *Rhodococcus fascians* CS4 were found to show antagonism against growth of *Escherichia coli*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Listeria monocytogenes* and *Vibrio fischeri* at refrigeration temperatures. Characterization of the abundant yellow pigment in methanol extracts of *S. faeni* ISY through UV–Vis spectrophotometry, high performance liquid chromatography (HPLC) and mass spectrometry (LC-MS) revealed the presence of astaxanthin, which, owing to its presence in very large amounts and evidenced to be responsible for antagonistic activity of the solvent extract.

Introduction

Microbial life has played a substantial role in the field of food production and processing since historic times. Several of them including representatives from bacteria, fungi and yeasts play a beneficial role, but at the same time, many microorganisms also remain to be notorious spoilage agents which often cause large scale human ailments and monetary losses in food industry. Despite the effectiveness of low-temperature to restrict mesophilic food spoiling bacteria, cold-adapted bacteria which can survive and proliferate even under low temperatures remain to pose a continuing threat (Remenant et al., 2015; Russell, 2002). Moreover, physical treatments and control of temperatures require human and mechanical inputs, which escalate costs. According to the Center for Disease Control and Prevention (CDC), over 22 cases of food based *Staphylococcus* sp. poisoning, 6 cases of *Salmonella* sp. poisoning and shiga toxin-producing *E. coli* from frozen food products are reported every year (Rounds et al., 2013). *Bacillus cereus* has been reported to produce emetic toxin at 12 and 15 °C leading to food spoilage (Finlay et al., 2000). Regardless of their mesophilic nature, several strains of *E. coli*, *S. typhi*, *B. cereus*, *S. aureus* and *L. monocytogenes* also have been reported to be associated with the refrigerated foods (Ayres, 2007; Nufer et al., 2007). These records suggest that there exists a vast requisite for techniques and products that can effectively control food-spoilage and pathogenic bacteria at low temperatures.

Presently, there is a growing interest in the exploration of eco-friendly biological products which are also cost-effective, such as metabolites from extremophiles which serve as a natural source of products for industrial applications (O'Brien et al., 2004). Organisms that inhabit extreme environments have often been reported to synthesize several novel bioactive metabolites most of which serve as anti-tumor or antibacterial agents (O'Brien et al., 2004; Barros et al., 2013). Though metabolite synthesis by bacteria is primarily to aid their nutrient accumulation by successfully eradicating the competing microbes, such metabolites produced by extremophiles are often also found to have potential applications. Among such extremophilic bacteria, cold-adapted bacteria have not been extensively investigated for their antagonistic properties compared to other extremophiles (Sánchez et al., 2009). Therefore, through the present study, we aimed to isolate non-spoilage beneficial cold-adapted bacteria that naturally occur in the vicinity of

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food products and study their potential to control common food borne and human pathogens.

Materials and methods

Sample collection and isolation of bacteria

Isolation of psychrotolerant bacteria was carried out by sampling anthropogenic cold environments such as refrigerated food products and cold storage facilities available in the vicinity of Vellore, India. Samples were collected as fresh or processed food products and swabs. The temperature during transportation until isolation of bacteria was maintained at 4 ± 1 °C. Serial dilution and plating of samples in triplicates on pre-cooled sterile standard plate count (SPC) agar (5 g/L of HiVeg hydrolysate, 2.5 g/L of yeast extract, 1 g/L of dextrose, 9 g/L of agar) followed by incubation at 7 °C for 10 days was carried out to selectively isolate psychrotolerant bacteria.

Screening and characterization of bacteria

Distinctive colonies were selected based on difference in morphology and sub-cultured on SPC agar to obtain axenic cultures. Cultures were then stored at −80 °C in 80% glycerol. Phylogenetic characterization of the isolates was carried out using 16S rRNA gene sequences amplified using primers 5′-GAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions 8–27) and 5′-ACGGCTACCTTGTTACGACTT-3′ (*E. coli* positions 1494–1513). The amplified products were purified and sequencing was carried out at Macrogen (Seoul, South Korea). The 16S rRNA gene sequence of the isolates was aligned using ClustalW and Neighbor-joining phylogenetic trees constructed using MEGA 5.0. A bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1000 re-assembling (Mageswari et al., 2012).

Enzyme activities and extraction of bioactive compound

Purified isolates were subjected to screening for production of hydrolytic enzymes amylase, cellulase, pectinase and DNase using plate assay methods (Rohban et al., 2009). For extraction of bioactive metabolites, sequential extraction using organic solvents hexane, chloroform and methanol was carried out to separate bioactive components from the cell pellets. Isolates were grown in nutrient broth until stationary phase and cells were harvested by centrifugation at 10,000 rpm for 15 min at 4 °C. The obtained pellets were washed thrice using 1× PBS (Phosphate Buffered Saline) and suspended in 25 mL of respective organic solvent overnight in an orbital shaker. The suspension was then centrifuged and the supernatants were collected. The pellets were again treated with the extraction solvent and the procedure repeated. The combined supernatants were evaporated under vacuum and dissolved in dimethyl sulfoxide (DMSO) for further experiments (Faijes et al., 2007).

Bioactive compound analysis

The bioactive components present in the respective solvent extracts were assayed using standard protocols (Firdouse and Alam, 2011). The extracts were tested for presence of alkaloids, flavanoids, terpenoids, saponins, anthocyanins, glycosides, amino acids and anthroquinones.

Antibacterial activity of the extract

The extracts re-dissolved in DMSO were used to test their antagonistic property against pathogenic bacteria. Freshly grown cultures of the common food borne and human pathogens *E. coli* (NCIM 2809), *S. typhi* (MTCC 733), *P. mirabilis* (NCIM 2388), *E. aerogenes* (NCIM 5139), *V. fischeri* (MTCC 3438), *L. monocytogenes* (MTCC 1143), *B. cereus* (NCIM 2458), *Streptococcus faecalis* (NCIM 2405), *S. pyogenes* (NCIM 2608) were harvested and their population adjusted to 5×10^5 CFU/mL using sterile LB media. The pathogens were then uniformly spread over Muller Hinton agar plates using sterile cotton swabs. Wells were cut in the media and 50 μL aliquots of the extracts were added to the wells. Sterile DMSO served as control. The plates were incubated at 7 °C for 1 week and zones of clearance were measured from the edge of the wells (Wietz et al., 2012).

Characterization of pigment in methanol extracts of S. faeni *ISY*

UV–vis spectra of the pigment present in methanol extract of strain *S. faeni* ISY was measured to find λ_{max} by scanning at a range of 300–700 nm using GeneQuant 1300 UV spectrophotometer (GE Healthcare). Identification of the pigment using HPLC was performed on reverse phase silica C_{18} column using Shimadzu LC 20A system equipped with double pump (LC-20AT) and UV–vis detector (SPD-20A), isocratic solvent acetone/methanol/tetrahydrofuran (70:28:2) at a flow rate of 1.0 mL and detected at 467 nm. For LC-MS analysis the pigment in the methanol extract of strain ISY was purified by preparative TLC using pre-coated Silica gel plates (Merck, Bombay, India) and acetone: methanol (7:3, vol/vol) as solvent. LC-MS analyses were carried out using an ACQUITY UPLC™ system (Waters, Milford, MA, USA). Detection of the pigment was carried out using an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). Mass Lynx™ software version 4.1 was used for data acquisition and processing. UPLC chromatographic separations were performed on a reversed-phase C_{18} column and a gradient system with the mobile phase consisting of solvent A: water:formic acid (99.99:0.1, v/v) and solvent B: acetonitrile:formic acid (99.99:0.1, v/v). The column was thermostated at 32 °C and the sample temperature was set at 25 °C. Before use, all solutions were filtered through 0.2 μm nylon membrane (Rivera et al., 2011).

Results and discussion

Isolation and phylogeny of cold-adapted bacteria

Screening of bacteria from artificially cold environments and food samples led to the isolation of ten bacterial strains which were distributed as five Gram negative rods (ISD, C1, CR2, ISO, ISY), two Gram positive rods (CS1, CS4) and three Gram positive cocci (ISW, IMW2, CR1) (Table 1). All of the isolated strains exhibited positive growth in a psychrotolerant range of temperature conditions ranging from 0 to 30 °C above which no growth was observed in all the isolates. The 16S rRNA gene sequences were submitted to Genbank and their accession numbers along with the strain identification are as follows, HQ911364(CR2), HQ911365(ISD), HQ911366(C1), HQ911367(ISO), HQ911368(IMW2), JF766372(ISW), JF766376(CS1), JN990379(CR1), JN990378(ISY), JN990377(CS4) (Fig. 1).

Phylogenetic analysis of the 16S rRNA gene revealed that phylum *Proteobacteria* contributed to the highest number of strains with six representatives followed by phyla *Actinobacteria* and *Firmicutes* which were represented by two strains respectively. Strain ISW showed close genetic phylogeny with *Psychrobacter alimentarius*, a psychrophilic bacterium isolated from squid jeotgal, a traditional Korean fermented seafood (Yoon et al., 2005). Strains ISO and ISY showed close similarity with an orange pigmented psychrotolerant bacterium *S. faeni*, isolated from air and dust (Busse et al., 2003). CS1 showed 99% similarity with *Arthrobacter rhombi*,

Table 1
Morphological and phylogenetic characteristics of psychrotrophic isolates.

Isolate	Environment	Colony morphology	Gram staining	Closest neighbor
ISY	Cold storage	Yellow color, gummy, medium	G− Rod	*Sphingomonas faeni*
IMW2	Cold storage	White, pinheaded, soft	G+ Cocci	*Leuconostoc mesenteroides*
ISO	Cold storage	Orange color, smooth, small	G− Rod	*Sphingomonas faeni*
ISD	Cold storage	Pale white, irregular, mucoid	G− Rod	*Pseudomonas simiae*
C1	Refrigerated carrot from supermarket	White, gummy, smooth	G− Rod	*Pseudomonas marginalis*
CS1	Cold storage swab	Pale yellow color, small, mucoid	G+ Rod	*Arthrobacter rhombi*
CS4	Cold storage swab	Pale orange color, small, smooth	G+ Rod	*Rhodococcus fascians*
ISW	Cold storage	Whitish, Medium, smooth	G+ Cocci	*Psychrobacter alimentarius*
CR1	Refrigerated coriander from supermarket	White, pinheaded, smooth	G+ Cocci	*Leuconostoc mesenteroides*
CR2	Cold storage	White, tiny, smooth	G− Rod	*Pseudomonas azotoformans*

isolated from Greenland halibut (Osorio et al., 1999). BLAST and EZtaxon analysis revealed that the isolates IMW2, CR1, CS4 and TU2 showed 99% similarity with *L. mesenteroides, R. fascians* and *R. erythropolis*. Genera such as *Arthrobacter*, *Pseudomonas*, *Psychrobacter*, *Rhodococcus* and *Sphingomonas* are well represented by several cold-adapted species and it was observed that a majority of the identified species here and their nearest neighbors had their origins in cold environments such as alpine soil, streams and the Polar Regions.

Bioactive compound analysis

Metabolite extracts from the isolates were analyzed for the presence of several bioactive compounds. Primarily, among the used organic solvents, we observed that methanol was found to be the most efficient which was indicated by the presence of an array of metabolites including flavonoids, terpenoids, alkaloids, glycosides, anthroquinonens, anthocyanins, saponins and amino acids (Table 2). Secondly, we observed that methanol was equally efficient in extraction of metabolites from the bacteria regardless of their Gram-negative or positive membrane and phylogeny. Presence of terpenoids, alkaloids and amino acids were observed in hexane extracts whereas chroloform extracts indicated presence of terpenoids, alkaloids, anthroquinones and saponins. However, both these organic solvents were not efficient in extracting these metabolites from all the studied strains (Table 2). Methanol has also been reported to be efficient for extraction of metabolites from bacteria in previous reports (Maharjan and Ferenci, 2003; Winder et al., 2008). Moreover, to the best of our knowledge, bioactive compounds analysis has not been extensively studied in the genera of isolates reported here in the present study.

Antibacterial activity of the methanolic extract

Metabolites in methanol extracts of all the isolates were evaluated for antagonism against pathogenic bacteria at refrigeration temperatures. Comparing the results among the studied isolates, extracts from strains *S. faeni* (ISY) and *R. fascians* (CS4) showed antagonism against several Gram-positive and Gram-negative pathogens (Table 3). Methanol extracts of *S. faeni* ISY were found to control *E. coli*, *P. mirabilis*, *E. aerogenes*, *L. monocytogenes* and *V. fischeri*. Similarly, methanol extract of *R. fascians* CS4 was found to show antagonism against *E. coli*, *P. mirabilis* and *L. monocytogenes* (Fig. 2). Several representatives of genus *Sphingomonas* have been reported to produce anticyanobacterial compounds such as argimicins and antimicrobial compounds (Romanenko et al., 2007).

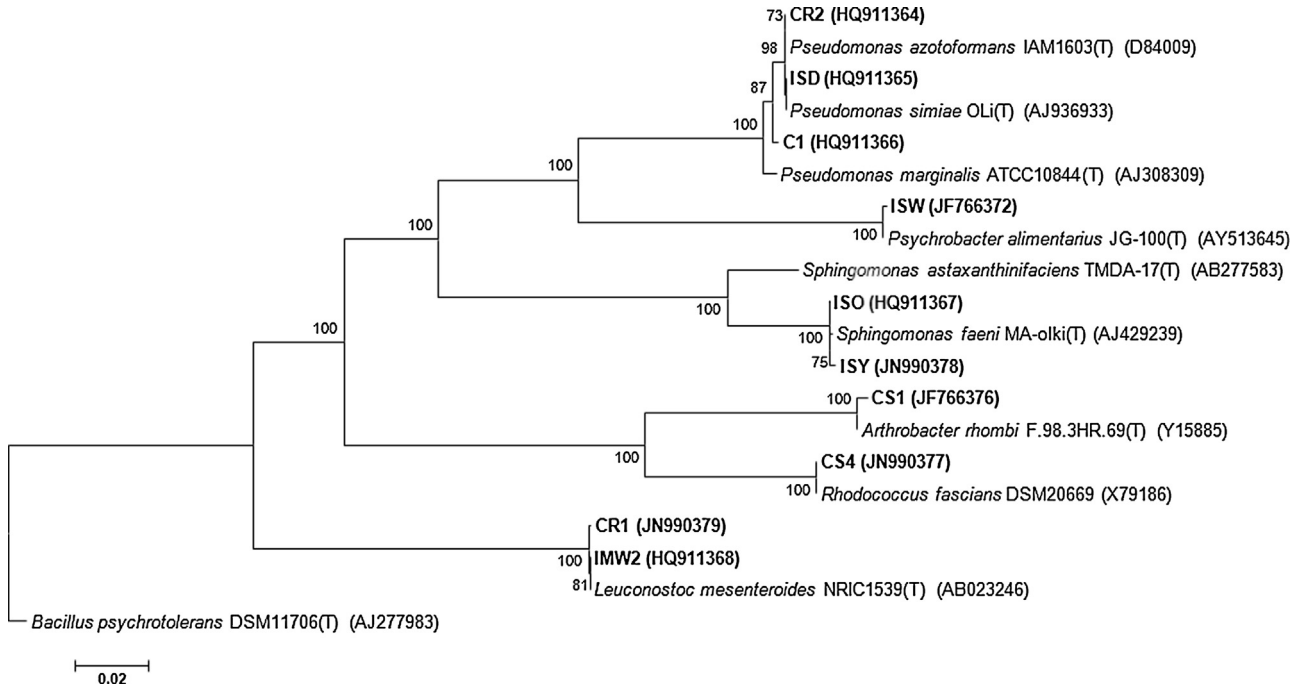

Fig. 1. Neighbor-joining tree showing the relative positions psychrotolerant bacterial isolates based on 16S rDNA sequencing. *Bacillus psychrotolerans* DSM11706(T) was used as an outgroup reference. GenBank accession numbers of individual member strains are given in parentheses. Scale bar corresponds to 0.02 units of the number of base substitutions per site.

Table 2
Bioactive chemical analysis of methanol, hexane and chloroform extracts.

Test strains	Flavanoids			Terpenoids			Alkaloids			Glycosides			Anthraquinones			Antho-cyanins			Saponins			Amino acids		
Extracts	M[a]	H[b]	C[c]	M	H	C	M	H	C	M	H	C	M	H	C	M	H	C	M	H	C	M	H	C
ISY	+	–	–	+	+	+	+	–	–	–	–	–	+	–	–	+	–	–	–	–	–	+	–	–
IMW2	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–
ISO	+	–	–	+	+	+	+	–	+	–	–	–	+	–	–	+	–	–	–	–	–	+	–	–
ISD	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–
C1	–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–	–	–	+	–	–	+	+	–
CS1	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
CS4	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ISW	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CR1	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
CR2	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	+	+	–	–

[a] Methanol.
[b] Hexane.
[c] Chloroform.

Table 3
Antibacterial activity of methanolic extract against pathogenic bacteria.

Test pathogens	ISY	IMW2	ISO	ISD	C1	CS1	CS4	ISW	CR1	CR2	P.C (T)[a]
Zone of inhibition (mm)											
Escherichia coli (NCIM 2809)	18 ± 0.43	–	5 ± 0.09	–	–	–	11 ± 0.43	–	–	–	30 ± 0.02
Salmonella typhi (MTCC 733)	–	–	–	–	2 ± 0.45	–	–	–	3 ± 0.40	2 ± 0.59	29 ± 0.03
Proteus mirabilis (NCIM 2388)	23 ± 0.47	–	10 ± 0.42	–	–	–	18 ± 0.71	–	–	2 ± 0.70	30 ± 0.02
Bacillus cereus (NCIM 2458)	–	–	–	–	–	–	–	–	–	–	–
Enterobacter aerogenes(NCIM 5139)	12 ± 0.71	–	–	–	–	–	–	–	–	–	27 ± 0.02
Streptococcus faecalis(NCIM 2405)	–	–	–	–	–	–	–	–	–	–	23 ± 0.04
Streptococcus pyogenes(NCIM 2608)	–	–	–	–	–	–	–	–	–	–	22 ± 0.05
Listeria monocytogens (MTCC 1143)	19 ± 0.72	–	–	–	–	–	14 ± 0.75	–	–	17 ± 0.41	–
Vibrio fischeri (MTCC 111)	22 ± 0.72	–	–	–	–	–	–	–	–	–	32 ± 0.03

[a] P.C (T), positive control (Tetracycline 30 μg). The mean value and standard error expressed were replicates of three individual experiments.

Sphingomonas sp. isolated from a marine mollusc exhibited antimicrobial activity against several pathogens including *S. aureus*, *B. subtilis*, *E. faecium*, *Citricoccus* sp. and *Candida albicans* (Romanenko et al., 2008). Studies have also reported on antimicrobial nature of metabolites from members of *Rhodococcus.* Bacterium *R. erythropolis* was found to produce three types (Group I, Group II and Group III) of antibiotic compounds which were antagonistic against taxonomically different groups of bacteria (Kitagawa and Tamura, 2008). Antibiotic compound lariatin extracted from *Rhodococcus* sp. K01B0171 that exhibited antibiotic activity against several *Mycobacterium* species (Iwatsuki et al., 2006). Apart from this, trehalosolipids (a type of glycolipid) produced by *Rhodococcus* spp. also displayed antibacterial activity (Franzetti et al., 2010).

In our study, *S. faeni* (ISO, ISY) and *R. fascians* (CS4) did not produce any hydrolytic enzyme responsible for food spoilage (data not shown). Further, they were found to effectively control growth of pathogenic bacteria under storage temperatures. Therefore these two strains can be taken as putative agents for biocontrol during food storage or processing in an ecofriendly approach provided that additional supporting data can be obtained from further research. During our studies we observed that methanol extracts of *S. faeni* ISY were strongly colored and contained profuse amounts of

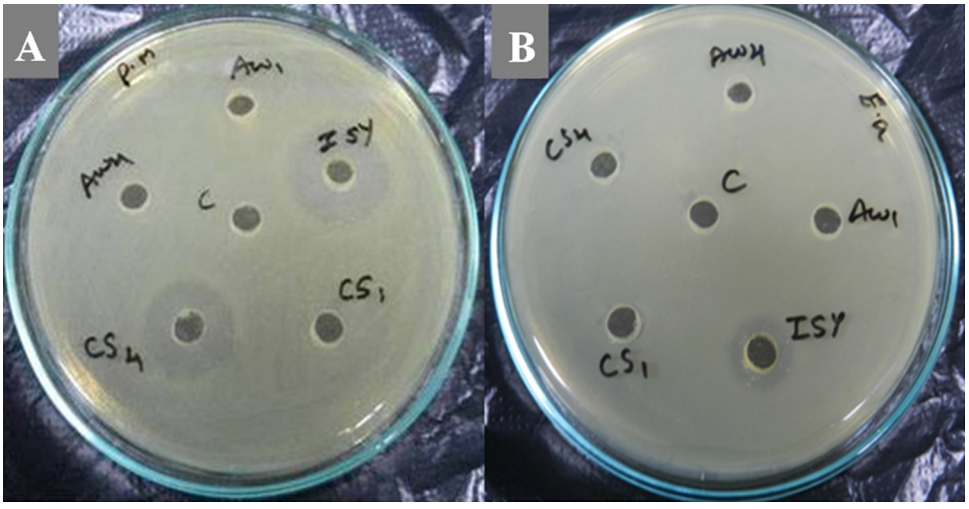

Fig. 2. Antibacterial activity of methanolic extracts of the psychrotrophic strains against *Proteus mirabilis* (A) and *Enterobacter aerogens* (B) at 7 °C. Zone of clearance indicates the growth inhibition of pathogens.

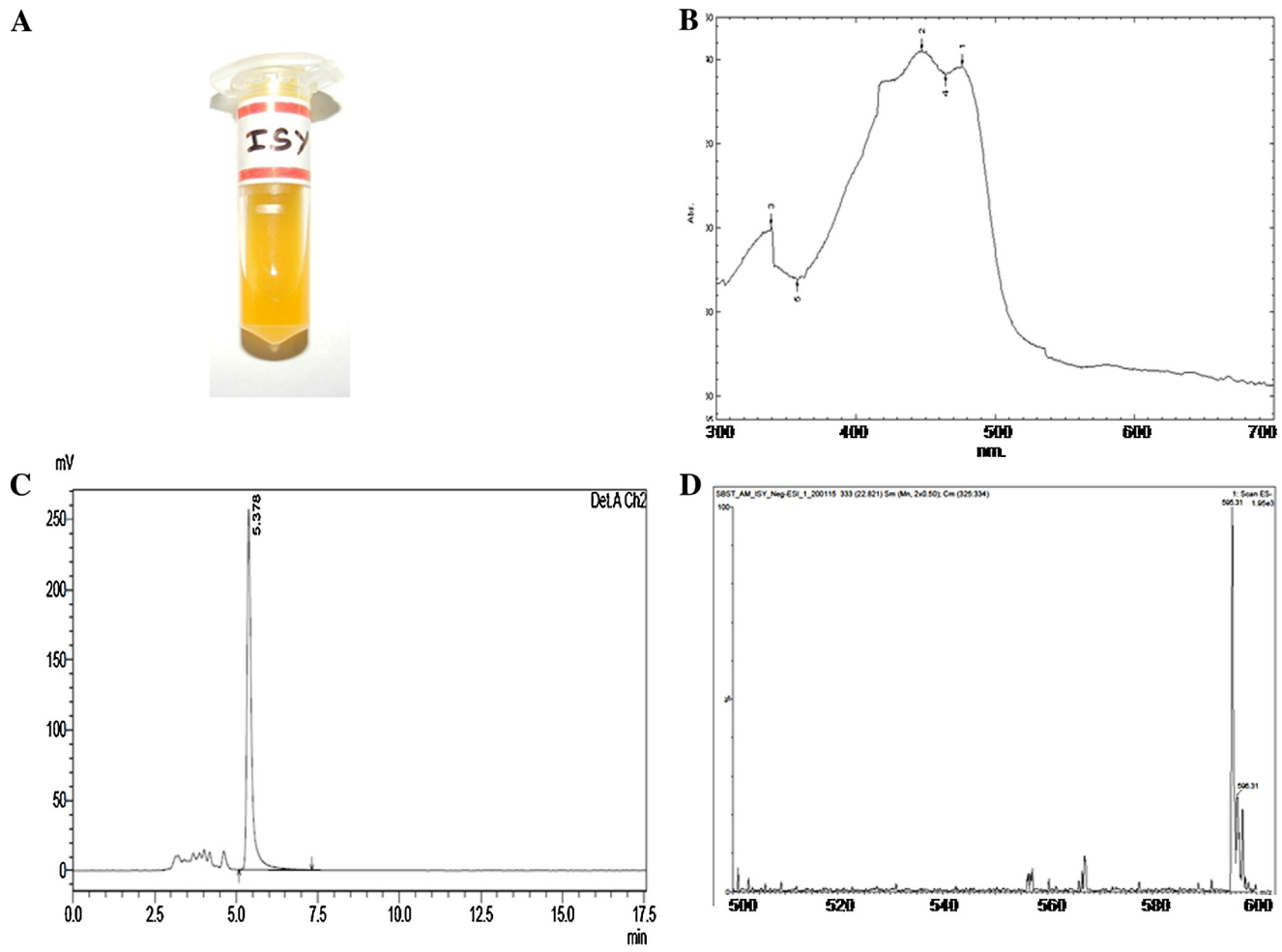

Fig. 3. (A) Yellow colored pigment extracted from *S. faeni* (ISY); (B) UV–vis spectrum of methanolic extract ranging from 300 to 700 nm; (C) HPLC chromatogram of methanolic extract of *S. faeni* (ISY); (D) LC-MS revealed the presence of carotenoids in *S. faeni* (ISY) methanolic extract representing with their molecular mass (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

pigment. Pigmentation is ubiquitous to several extremophilic bacteria and may result in response to an abiotic environmental stress condition (Chattopadhyay, 2006). In extremophilic bacteria pigments such as carotenoids and flexirubins prevent the cells from damage and stabilizing the membrane (Dieser et al., 2010). Moreover, such pigments are also found to possess antagonistic properties and natural pigments have a wide demand as antimicrobial agent in food preservation compared to synthetic pigments because of their low toxicity and decomposing nature (Nakamura et al., 2003). Carotenoids like zeaxanthin from rose hips, lutein from golden delicious apple, astaxanthin from *Haematococcus pluvialis* were found to be potent antimicrobial agents (Kirti et al., 2014; Horváth et al., 2012).

Analysis of the methanolic extract by UV – vis spectra, HPLC and LC-MS

Our strain *S. faeni* (ISY) produced yellow colored pigment (Fig. 3A). The pigment in methanolic extract of *S. faeni* (ISY) was determined using λ_{max} value (450 and 480 nm) by spectrophotometric analysis (Fig. 3B). Carotenoid type pigments have absorption at 450–490 nm. Prior studies from *Sphingomonas* sp. have also reported production of a yellow pigment which was identified nostoxanthin (Silva et al., 2004). An Antarctic psychrotolerant bacterium *Sphingobacterium antarticus* has been reported for the production of carotenoids (Jagannadham et al., 2000). Other reported carotenoids from *Sphingomonas* sp. are astaxanthin and β-carotene (Silva et al., 2004; Asker et al., 2007).

HPLC analysis indicated that the methanolic extract pigment of *S. faeni* (ISY) has retention time similar to astaxanthin (Fig. 3C). Thus, we identified that the strain ISY produced carotenoid pigment which was putatively identified as astaxanthin. Production of astaxanthin has been reported as a distinct characteristic of bacterium *S. astaxanthinifaciens* (Asker et al., 2007). LC-MS analysis for exact identification of the carotenoid revealed that the *S. faeni* (ISY) extract were found to contain abundant quantities of astaxanthin. The protonated molecules with *m*/*z* 595.31 in the methanolic extract of *S. faeni* ISY was identified as [astaxanthin–(M-H)]$^-$ (Fig. 3D). The purified fraction was collected and examined against *P. mirabilis* for the antagonistic activity and a zone of inhibition was clearly observed around the well (Fig. 4).

Genus *Paracoccus* is a prominent bacterial genera reported to synthesize astaxanthin including *P. marcusii* (Harker et al., 1998), *P. carotinifaciens* (Tsubokura et al., 1999), *P. haeundaensis* (Lee et al., 2004), *Paracoccus sp.* MBIC 01143 (Chougle and Singhal, 2012). Among members of genus *Sphingomonas*, Few reports only available on synthesis of astaxanthin and its derivative (astaxanthin dideoxyglycoside) by *S. astaxanthinifaciens* and *Sphingomonas sp.* PB304 (Asker et al., 2007; Kim et al., 2014). Astaxanthin from *Haematococcus pluvialis* was reported to exhibit antagonistic

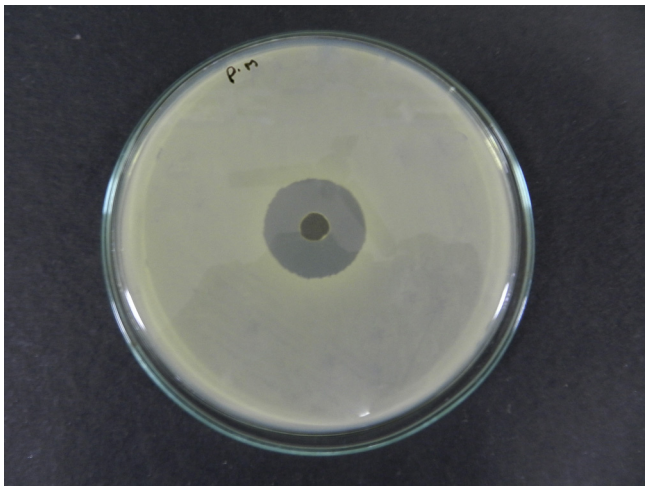

Fig. 4. Antagonistic activity of purified fraction of astaxanthin from methanolic extract of *S. faeni* (ISY) against *Proteus mirabilis* at 7 °C. Zone of clearance indicates the growth inhibition of pathogen.

activity against *Helicobacter pylori* (Horváth et al., 2012). Astaxanthin isolated from marine yeast exhibited antibacterial activity against *B. subtilis*, *S. typhi*, *S. aureus* and *P. aeroginosa* (Ushakumari and Ramanujan, 2013).

Conclusions

In conclusion, among the metabolite extracts from the ten isolates, *S. faeni* ISY methanolic extract exhibited maximium antibacterial activity against *E. coli*, *P. mirabilis*, *V. fischeri*, *E. aerogenes* and *L. monocytogenes.* Moreover, strain *S. faeni* ISY was not found to synthesize hydrolytic enzymes responsible for food spoilage. Characterization *of S. faeni* ISY methanolic extract revealed the presence of carotenoid pigment in the extract. Further studies using HPLC and LC-MS to establish the nature of the compound responsible for the antibacterial activities revealed that the carotenoid in *S. faeni* ISY extract was astaxanthin. To the best of our knowledge, this is the extensive report of antibacterial activity detected in *S. faeni*. From the results we understand that strain *S. faeni* ISY or its derived astaxanthin pigment has the potential to be used as an eco-friendly method to control food-spoilage as well as pathogenic bacteria. Further research can lead to development of strategies and risk-assessment to employ the strain *S. faeni* ISY for control of pathogenic bacteria during low temperature conditions.

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