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Isolation and Biochemical Analysis of Bacteria associated with Dried Stigma of Saffron (*Crocus sativus*)

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ABSTRACT

The stigma and pistil of saffron are used to extract chemical compounds such as crocin, picrocrocin, crocetin and safranal; and volatile oils for flavor respectively. The work is to isolate microbiome associated with stigma of saffron. 15 morphotypes obtained from Kashmir and 32 from Kishtwar. Their morphological characterization showed that the smooth textured colonies more in Kishtwar and rough textured colonies more in Kashmir. The IC_{50} values of radical scavenging revealed that antioxidant activity of Kishtwar is more as lower IC_{50} value corresponds to a higher antioxidant activity of sample. The IC_{50} values of ferrous chelation revealed that antioxidant activity of Kishtwar is more as lower IC_{50} value corresponds to a higher antioxidant activity of sample. Hence, Kishtwar has more antioxidant activity. The flavonoid and phenolic content of Kashmir is higher than that of Kishtwar. Of the biochemical assays conducted, only tannins and Glycosides are present in stigma of saffron. Further work can be done to unveil the bacterial property to produce the pigments that act as favouring agent that can be used in bio-industry.

Introduction

Various studies on the plants have revealed that plants genome contributes to the structure and function of the plant microbiome and also the interations between host (plant) and the microorganisms. According to these studies the microbiome can be considered as an extension of the host genome. Microbiomes associated with above-ground (phyllosphere), below-ground (rhizosphere) (Oldroyd *et al.,* 2011; Kent *et al.,* 2002) and internal (endosphere) tissues of the same plant and those occupying the same niche of different plants have been studied (Gilbert *et al.,* 2010; Tumbaugh *et al.,* 2007) and it was seen to be

very different, particularly when the microbiome is viewed at fine taxonomic levels such as genus, species and strain.

Specific metabolic capabilities are required to use host-derived carbon sources and tolerate host defenses; abiotic conditions, such as temperature, moisture and pH. Thus, it becomes necessary to study microbial association to unravel the role of microbes in plant growth promotion. *Piriformospora indica*, an endophytic fungus colonizes the roots of a variety of plant species and promotes their growth The stigmas are covered by glandular trichoms cuticle wrinkles and scale wax (Negbi *et al.,* 1999).

It contains many chemical compounds that are antioxidants such as alpha-crocin, zea-xanthin, lycopene, carotenes etc (Wani *et al.,* 2012). Flower pistil contains volatile oils which gives pleasant flavor such as safranal, phenethenol, cincole, pinene, borneol, geraniol, limonene, para-cymene, linaloan, terpinen-4-ol etc. Kim-Kwon and co-workers, 2012 reported that bacteria 5B6 (*Bacillus* sp.) has the potential to colonize in the phyllosphere and act as a biocontrol agent. The number of shoot sprouts significantly decreased beyond the untreated controls. The quantity of crocin, picrocrocin, crocetin and safranal compounds extracted from stigma of saffron was high in the plants which were soil drenched with *Bacillus* spore solution 14 weeks after sowing date.

Plant growth-promoting rhizobacteria, *Pseudomonas* and *Bacillus* species, have been applied to many agricultural crops to enhance, plant biomass, and/or disease control (Chen *et al.,* 2007). The treatment conditions that yielded the best growth produced only modest improvements in a few saffron constituents.

Study of bacterial diversity associated with stigma of saffron from Kashmir and Kishtwar using Metagenomic approach. Isolation of bacterial diversity associated with stigma of saffron from Kashmir and Kishtwar using cultivation based approach. To compare the antioxidant properties, phenolic content, flavonoid contents and chemical analysis of bacteria isolated from stigma of saffron from Kashmir and Kishtwar.

Materials and Methods

The microbial diversity was analyzed from the stigma samples collected from Kishtwar and Kashmir using the culture dependent and culture independent approaches. The saffron is well known to be grown in Jammu and Kashmir (longitude: 34° 5′ 24″ N; latitude: 74° 47′ 24″ and altitude 1585 mts. above sea level). The stigma of Kashmir and Kishtwar were dried in J&K Agricultural department, Jammu. The dried stigma of Kashmir and Kishtwar were collected from J&K Agricultural department, Jammu.

Microbial diversity analysis using cultivation independent approach

Three methods were used i.e. Pang's method (2011); Wetcher's method (2013); and Zhao's method (2011). The three procedures are as follows:

Pang's *et al.,* 2011

Modified chemical lysis method was carried out for Metagenomic DNA extraction by Yeates *et al.,* (1998). 20 g stigma was suspended in 50 ml DNA extraction buffer [100 mMTris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 1.5 M NaCl] and 1 ml of Lysozyme (10 mg/ml) was added and the sample was incubated at 37˚C for 1 h. Then in sample, 2 ml SDS (20%, w/v) and 15 µl of proteinase K (20 mg/ml) were added and it was further incubated at 65˚C with 2 h. The mixture was then centrifuged at 6,000 rpm for 10 min to remove stigma residues. Supernatant was then transferred into a clean

tube, and then precipitated by using half-volume of PEG (30%, w/v) / NaCl (1.6 M) and incubated at room temperature for another 2 h. Then DNA was pelleted and resuspended in 20 ml of TE (10 mMTris-HCl, 1 mM sodium EDTA, pH 8.0). Further purification of this metagenomic DNA extract was done by using equal volume of P:C:I (phenol/ chloroform/ isoamyl-alcohol (25:24:1)). Subsequently the metagenomic DNA was precipitated with 0.1 volume of NaCl (5 M) and 2.5 volume of absolute ethanol. The DNA pellet was then recovered by centrifugation at 13,000 rpm for 30 min. DNA pellet was washed by 70% (v/v) ethanol, air-dried and dissolved in 1 ml TE.

Zhao's method; 2011

For sodium dodecyl sulfate (SDS)-based DNA extraction CTAB was used in the buffer. Stigma samples of 5 g were mixed with 13.5 ml of DNA extraction buffer (100 mMTris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and in centrifuge tubes 100 ml of proteinase K (10 mg/ml) was added by shaking at 225 rpm for 30 min at 37˚C. After the shaking treatment, 1.5 ml of 20%SDS was added, with gentle end-over-end inversions every 15 to 20 min., the samples were incubated in a 65˚C water bath for 2 h. The supernatants were collected after centrifugation at 6,000 3 *g* for 10 min at room temperature and transferred into 50 ml centrifuge tubes. By adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10s and incubating at 65˚C for 10 min, centrifuged as before, the DNA was extracted two more times. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of iso-propanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000gfor 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 ml.

Wetcher's *et al.,* 2013

In 2 ml microcentrifuge tube containing one milliliter of sterile PPB (pH 7.2) was added to 500 mg of stigma. After vortexing for 1 min at high speed, the mixture was centrifuged at 325g for 30 s in a microcentrifuge. The supernatant was then transferred to a 1.5 ml microcentrifuge tube and 400 ml of polyvinyl-polypyrrolidone slurry (100 mg/ml PVPP in PPB at pH 7.2) was added using a large-bore pipette tip, and the mixture was vortexed at high speed for 30s. To this, 2 ml of 3M $CaCl_2$ was added, and the microcentrifuge tube was vortexed for 30s and centrifuged as stated above. The supernatant was then transferred carefully to a clean 1.5-ml microcentrifuge tube, to which 20 ml of a lysozyme solution (25 mg/ml) and 10 ml of a proteinase K solution (20 mg/ml) were added. The tube was inverted several times to mix the contents and placed in a 37˚C water bath for 10 min and then placed in a 55˚C water bath for 10 min. Thirty micro liters of 20% (w/v) SDS was added to the tube and the contents were mixed by inverting several times, and the tube was placed in an 80˚C water bath for 10min. Immediately after removal from the water bath, 400 ml of the PVPP slurry was added to the contents of the tube which was mixed by gentle inversion, placed on ice for 5 min, and then centrifuged at 16,000g for 10 min. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube followed by the addition of 0.7 volumes of 100% iso-propanol, the tube was inverted several times and centrifuged at 16,000g for 5 min. After centrifugation, the supernatant was discarded and the remaining pellet was washed in 500 ml of 70% ethanol,

centrifuged for 5 min at 16,000g, and air dried for 5 min. The resulting pellet was resuspended in 25 ml of TE.

Microbial diversity analysis using cultivation dependent approach

Eight different media i.e. Nutrient Agar (NA), (LB), (R-2-A), Water agar (WA), Czapek Dox Agar (CD), Soyabean Casein Digest Agar (SCD), King's B medium Base Agar, Minimal Agar(MA) were used. The 0.4gm of stigma was mixed in 18ml of Milli-Q. This solution was incubated at 37°C for 1 day. The quantity of Milli-Q was kept more than the required quantity as the stigma soaked 3-4 ml of Milli-Q. There is no osmotic shock to the bacteria because the saffron provides the conditions to inhibit the osmotic shock.

These dilutions were spread on the plates of nine media prepared and autoclaved previously and incubated at 37°C for bacterial isolates. The saffron stigma was crushed in previously autoclaved pestle mortar so that both the endophytes and exophytes can be isolated separately.

To check the gram positive and gram negative bacteria

The pure colonies were isolated and the Gram's staining was done to check for gram positive and gram negative bacteria. The shape of bacteria was also analyzed.

Antioxidant, Flavonoid, Phenolic and Biochemical assay of the bacterial isolates

Antioxidant activity

Isolated bacterial cultures from Kashmir and Kishtwar were aggregated separately and their extract was prepared. This extract was taken at different concentrations (20µl, 40µl, 60µl, 80µl and 100µl) of Kashmir and Kishtwar.

DPPH radical scavenging assay

To the extract methanol was added and the volume was raised to 1000µl. To this 1ml DPPH (prepared in methanol) add 2ml acetate buffer were added and the mixture was incubate for 30 min. in dark. The OD was taken at 517nm. Measurement of radical inhibition properties of C. sativus was carried out according to the method described by Braca *et al.,* (1971) with some modifications. Ascorbic acid was used as positive control and % inhibition was determined according to the following equation

$$\%\text{Inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \text{x}100$$

Three experimental replicates were taken for the assay. The IC_{50} values were calculated as the concentration of extracts causing 50% inhibition of DPPH radical, a lower IC_{50} value corresponds to a higher antioxidant activity of sample.

Ferrous chelation

To the extract methanol was added and the volume was raised to 3ml. To this 60 µl of $FeCl_2$ and 120µl of Ferrozine were added and OD was taken at 562nm. Measurement of radical scavenging properties of C. sativus was carried out according to the method described by Carter *et al.,* (1971) with some modifications. Gallic acid was used as positive control and % scavenging was determined according to the following equation:

$$\%\text{Scavenging} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \text{x}100$$

Three experimental replicates were taken for the assay. The IC_{50} values were calculated as the concentration of extracts causing 50%

scavenging of ferrous ions, a higher IC_{50} value corresponds to a higher antioxidant activity of sample.

Phenolic content

To the extract distilled water was added to raise the volume to 1ml. To this 1ml FC reagent and 300µl of NA_2CO_3 was added and OD was taken at 765nm.

Flavonoid content

To the extract distilled water was added to raise the volume to 3 ml. To this 300µl $NaNO_2$ was added and incubated for 5 min. To this 300µl $AlCl_3$ was added and incubated at 6 min. Then 2ml NaOH was added to 2.4 ml distilled water and OD was taken at 510nm.

Biochemical assay

Test for Tannins

To 20µl sample 980 µl methyl-hydroxide was added. To this 1 ml of 5% ferrous chloride ($FeCl_3$) was added. Formation of bluish or greenish black precipitates shows that the presence of Tannins.

Test for Saponins

To 20µl sample 980 µl methyl-hydroxide was added. To this 2.5 ml of distilled water was added and shook vigorously. Then some drops of refined oil were added. Formation of foam shows presence of Saponins.

Test for Coumerins

To 20µl sample 980 µl methyl-hydroxide was added. To this 1 ml of 10% sodium-hydroxide (NaOH) and 1 ml chloroform was added. Yellow colour of solution shows the presence of Coumerins.

Test for Terpenoids

To 20µl sample 980 µl methyl-hydroxide was added. To this 1 ml chloroform and 1.5 ml of concentrated sulphuric acid (H_2SO_4) was added. The reddish brown colouration at the interface shows the presence of Terpenoids.

Test for Quinones

To 20µl sample 980 µl methyl-hydroxide was added. To this 1 ml of diluted NaOH was added. Blue- green or red colour shows the presence of Quinone.

Test for Glycosides (Keller-Killani test)

To 20µl sample 980 µl methyl-hydroxide was added. To this 2 ml GAA and one drop of $FeCl_3$ and 1 ml of concentrated H_2SO_4 was added.

Brown ring at the interface indicates the presence of de-oxy sugar characteristics of Glycosides. A violet ring may appear below brown ring. White or greenish ring may form uniformly throughout the acetic acid layer.

Results and Discussions

Microbial diversity analysis using cultivation independent approach

The DNA obtained was by all the three methods i.e. Pang's; Zhao's and Wetcher's method but the DNA obtained was not in the purified form and need standardization of new protocol especially for stigmal isolations. 0.8% agarose gel was prepared for analysis of isolated DNA samples: a) Control DNA shown in well no 4 and 5; b) Well numbers 1 and 6 show metagenomic DNA isolated by Pang's method from stigma of Kashmir and Kishtwar respectively; c) Well numbers 2 and 7 show metagenomic DNA isolated by Zhou's method from stigma of Kashmir and Kishtwar

respectively; d) Well numbers 3 and 8 show metagenomic DNA isolated by Wetcher's method from stigma of Kashmir and Kishtwar respectively.

Microbial diversity analysis using cultivation dependent approach

The various morphotypes were isolated shown in Table I.

Antioxidant activity

DPPH scavenging property

The graph shows that microbial extract of Kishtwar has more DPPH scavenging activity than that of microbial extracts of Kashmir. Hence, Kishtwar has more antioxidant activity.

Ferrous chelation antioxidant property

The graph shows that ferrous chelation property is more in case of microbial extracts from Kashmir than that of the microbial extracts from Kishtwar. Hence, Kishtwar has more antioxidant activity.

Antioxidant DPPH percentage scavenging and Ferrous percentage chelation properties of the extracts of the bacterial isolates

The metagenomic DNA has been extracted from soil, rhizosphere, corms and other underground parts of the plant but no Metagenomic DNA isolation has been done from the upper parts of the flower like stigma, leaves, etc. The protocol used for the isolation of DNA from stigma does not produce purified DNA and had protein and other contaminants and so could not be sequenced and needs optimization. The bacteria isolated from the stigma were all grams positive rods or cocci and no grams negative bacteria were isolated. The morphotypes obtained from Kashmir are 15 and from Kishtwar are 23. The smooth textured colonies were more in Kishtwar than Kashmir and rough textured colonies were more in Kashmir than in Kishtwar. Yellow coloured colonies are more in Kishtwar whereas white coloured colonies are more in Kashmir. Pale-coloured colonies are same in both the cases. Kashmir has one orange coloured colony.

Table.1 Morphotypes obtained using different media

Name of media	Kishtwar	Kashmir
R-2-A Agar media	5	7
Water Agar media	1	-
Soyabean Casein Digest media	4	1
NA Agar media	6	4
LB Agar media	6	3
Czapek Dox media	1	-
King's B Agar media	-	-
Minimal Agar media	-	3

The morphotypes obtained a) from Kashmir are 15 and b) from Kishtwar are 23.

Table.2 Bacterial isolates from Kishtwar

Name of organism	Plate	Shape	Texture	Colour	Gram's Stain	Shape
SN1	LB	Flower like	Rough	White	Positive	Cocci
SN2	LB	Mat forming	Smooth	Pale yellow	Positive	Rod
SN3	LB	Round	Smooth	Yellow	Positive	Rod
SN4	LB	Round	Smooth	Yellow	Positive	Cocci
SN5	NA	Concentric ring like	Smooth	Yellow	Positive	Cocci
SN6	R-2-A	Concentric ring like	Smooth	White	Positive	Cocci
SN7	SCD	Round	Shiny, smooth	Yellow	Positive	Cocci
SN8	R-2-A	Round	Smooth	White	Positive	Cocci
SN9	SCD	Mat forming	Smooth	Yellow	Positive	Cocci
SN10	CD	Irregular	Slimy	Yellow	Positive	Cocci
SN11	SCD	Irregular	Slimy	Yellow	Positive	Cocci
SN12	NA	Round	Smooth	Yellow	Positive	Cocci
SN13	NA	Round	Smooth	Yellow	Positive	Cocci
SN 14	LB	Flower like	Smooth	White	Positive	Cocci
SN 15	WA	Round	Smooth	Pale yellow	Positive	Cocci
SN 16	LB	Round	Smooth	Yellow	Positive	Cocci
SN 17	R-2-A	Concentric ring like	Smooth	Yellow	Positive	Cocci
SN 18	R-2-A	Flower shaped	Smooth	White	Positive	Cocci
SN 19	R-2-A	Round	Smooth	White	Positive	Cocci
SN 20	NA	Round	Smooth	Yellow	Positive	Cocci
SN 21	NA	Round	Smooth	Yellow	Positive	Cocci
SN 22	SCD	Mat forming	Smooth	Yellow	Positive	Rod
SN 23	NA	Round	Smooth	Yellow	Positive	Cocci

Table.3 Bacterial isolates from Kishtwar

Name of organism	Plate	Shape	Texture	Colour	Gram's Stain	Shape
SN a	NA	Round	Smooth	Yellow	Positive	Cocci
SN b	NA	Mat forming	Smooth	White	Positive	Cocci
SN c	NA	Mat with thread like structures	Feathery to touch	Yellow	Positive	Cocci
SN d	R-2-A	Mat forming	Smooth	White	Positive	Cocci
SN e	R-2-A	Round	Smooth	White	Positive	Cocci
SN f	R-2-A	Round	Smooth	Pale Yellow	Positive	Cocci
SN g	SCD	Round	Smooth	Pale Yellow	Positive	Cocci
SN h	R-2-A	Mat with thread like structures	Feathery	White	Positive	Cocci
SN i	R-2-A	Mat forming	Smooth	White	Positive	Cocci
SN j	R-2-A	Concentric ring like	Smooth	White	Positive	Cocci
SN k	R-2-A	Round	Smooth	White	Positive	Cocci
SN l	MM	Round	Smooth	White	Positive	Cocci
SN m	MM	Round	Smooth	Orange	Positive	Cocci
SN n	MM	Round	Smooth	White	Positive	Cocci
SN o	NA	Round	Flower like	White	Positive	Cocci
SN p	LB	Round	Smooth	Yellow	Positive	Rod
SN q	LB	Mat forming	Smooth	White	Positive	Cocci
SN r	LB	Mat with thread like structures	Feathery to touch	Yellow	Positive	Cocci

Table.4 Comparison of the Antioxidant activities: DPPH radical scavenging and Ferrous chelation activity of the bacterial isolates from Kashmir and Kishtwar

Concentration of samples	DPPH radical scavenging (OD at 517nm)		Ferrous chelation (OD at 562nm)	
	Kashmir	**Kishtwar**	**Kashmir**	**Kishtwar**
20 µl	0.158 Å	0.312 Å	1.123 Å	1.024 Å
40 µl	0.18 Å	0.335 Å	1.146 Å	0.99 Å
60 µl	0.212 Å	0.359 Å	1.019 Å	0.987 Å
80µl	0.236 Å	0.372 Å	0.99 Å	0.963 Å
100 µl	0.273 Å	0.391 Å	1.93 Å	0.941 Å

Table.5 Percentage scavenging and percentage chelation

Volume of extract	% Scavenging		% Chelation	
	Kishtwar	Kashmir	Kishtwar	Kashmir
20 µl	70.6	85.13	5	- 4.3
40 µl	68.5	83	8	- 6.5
60 µl	66.2	80	9	5.5
80 µl	65	77.84	10	7.99
100 µl	63.2	74.32	12.71	19.6

Table.6 Phenolic content

Concentration of samples	Phenolic content (OD at 765nm)	
	Kashmir	Kishtwar
20 µl	0.20 Å	0.13 Å
40 µl	0.29 Å	0.14 Å
60 µl	0.39 Å	0.20 Å
80µl	0.53 Å	0.29 Å
100 µl	0.61 Å	0.34 Å

Table.7 Flavonoid content

Concentration of samples	Flavonoid content (OD at 510nm)	
	Kashmir	Kishtwar
20 µl	0.15 Å	0.14 Å
40 µl	0.16 Å	0.15 Å
60 µl	0.25 Å	0.17 Å
80µl	0.30 Å	0.29 Å
100 µl	0.42 Å	0.32 Å

Table.8 Biochemical assay

Assay	Kashmir	Kishtwar
Tannins	+	++
Coumerins	-	-
Terpenoids	-	-
Quinone	-	-
Glycosides	+	+

Fig.1 Gel picture showing metagenomic DNA isolated from stigma of Kashmir and Kishtwar.

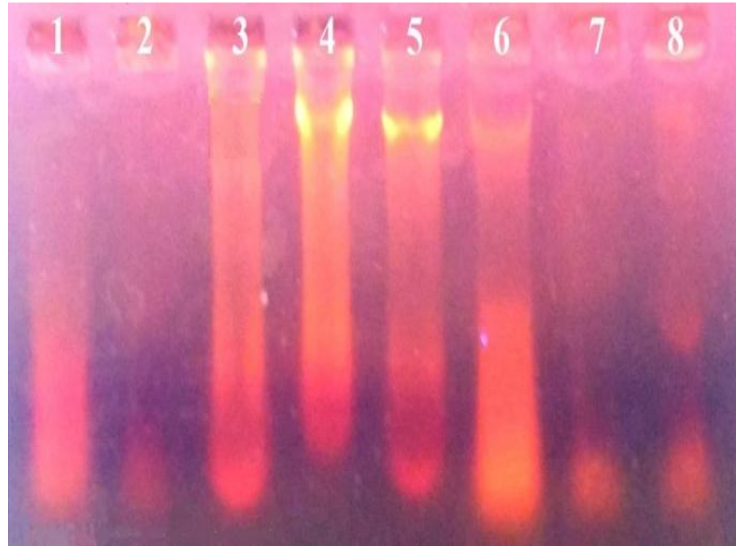


Fig.2 Various morphotypes from a) Kashmir and b) Kishtwar

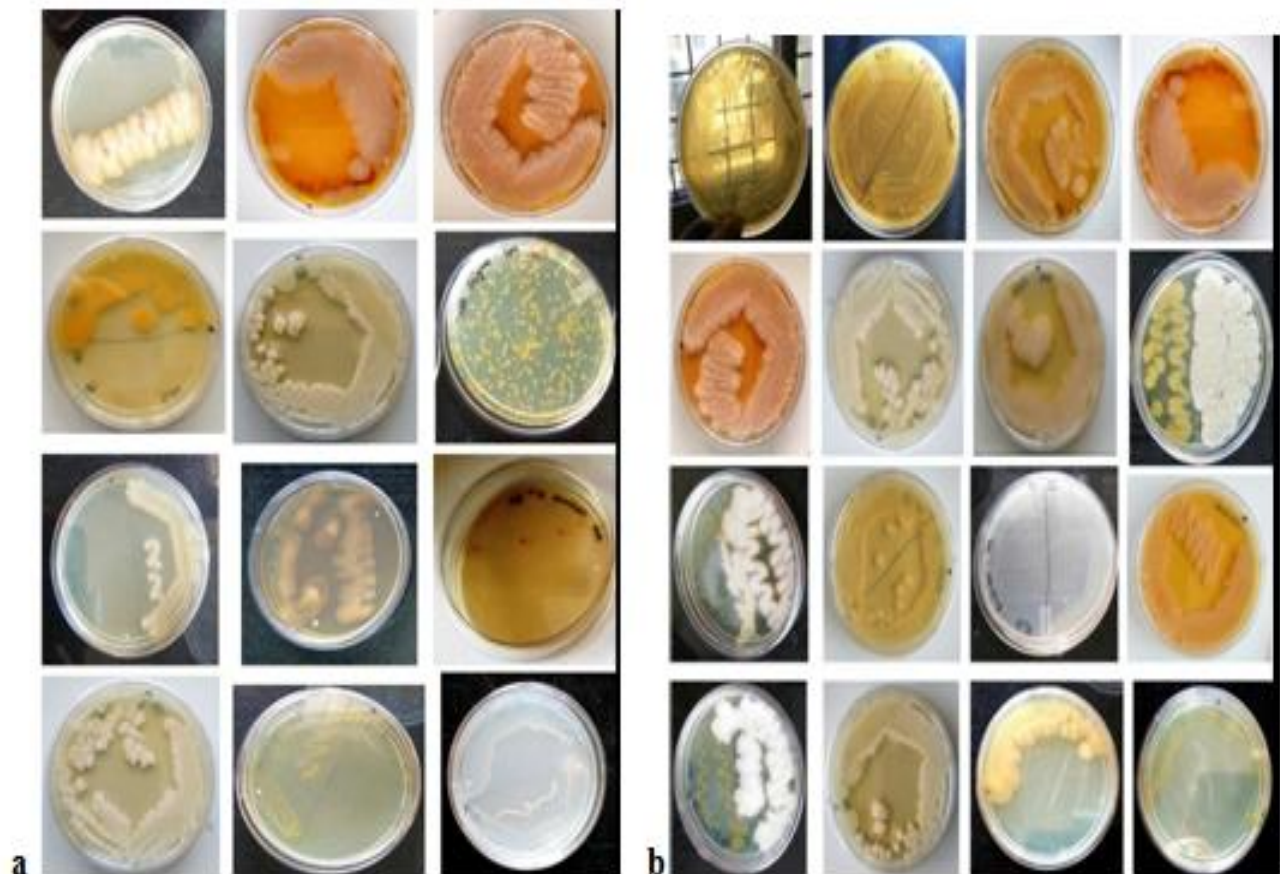


Fig.3 Graphs showing the differences in texture in Saffron from Kashmir and Kishtwar. Smooth textured colonies are more in Kishtwar whereas Rough textured colonies are more in case of Kashmir

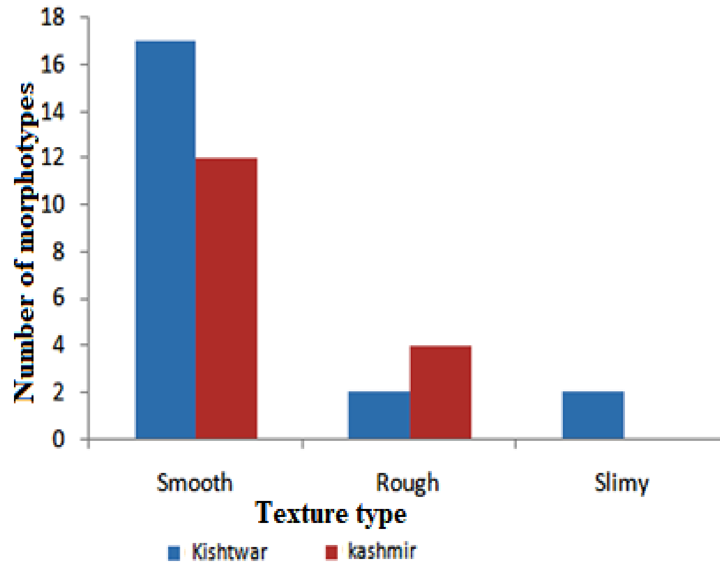


Fig.4 Graphs showing the differences in gram's staining in Saffron from Kashmir and Kishtwar. Theisolates from the stigma were all grams positive rods or cocci and no grams negative bacteria were isolated.

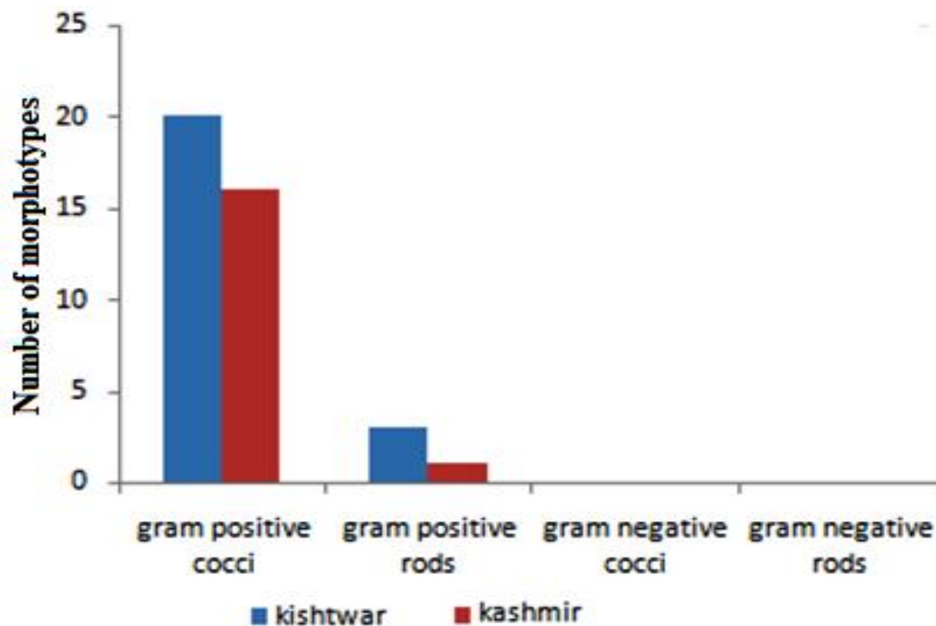


Fig.5 Antioxidant properties of extracts from Saffron.

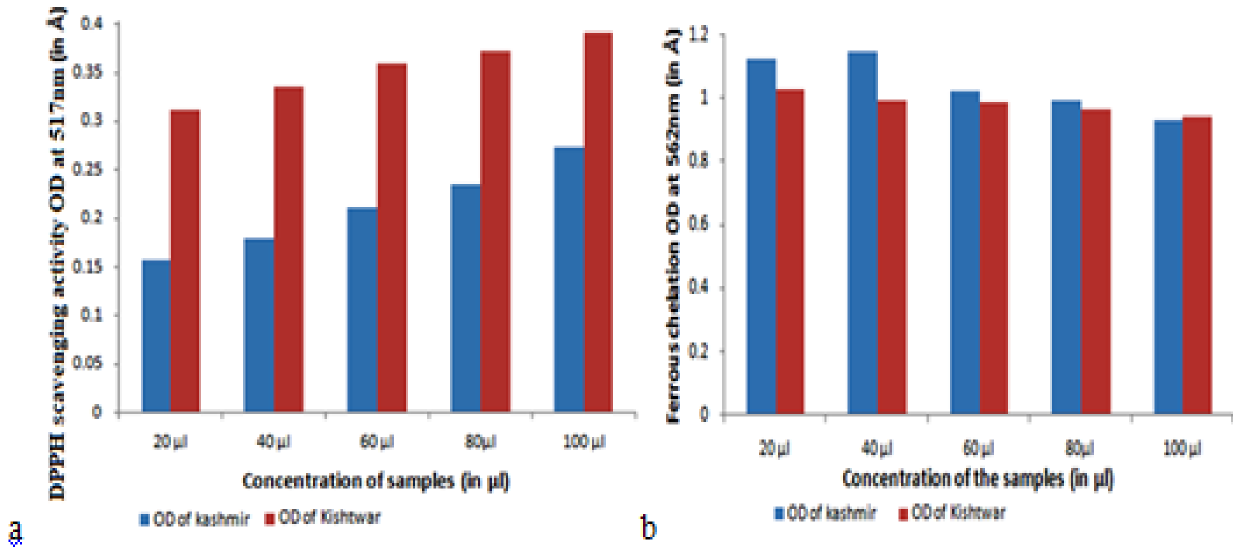
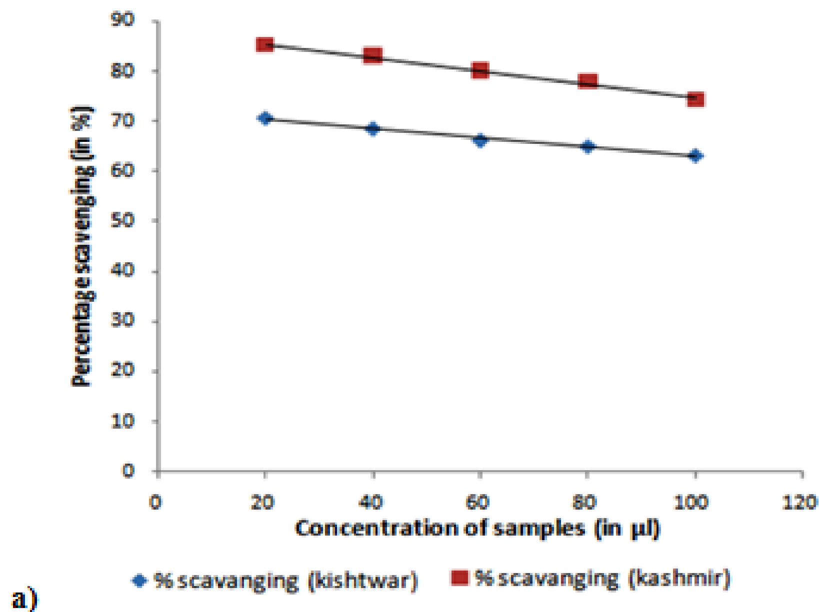


Fig.6 Antioxidant free radical scavenging and Ferrous chelation properties of the extracts of the bacterial isolates: The above graphs show that the a) Percentage scavenging activity of extract from Kashmir is less than that of Kishtwar. Hence, Kishtwar has more antioxidant property (scavenging decreases with increase in the concentration, the antioxidant property is increasing). b) Percentage chelation is more in extracts from Kashmir than that of Kishtwar. Hence, Kishtwar has more antioxidant property (chelation increases with increase in concentration, antioxidant property increases).



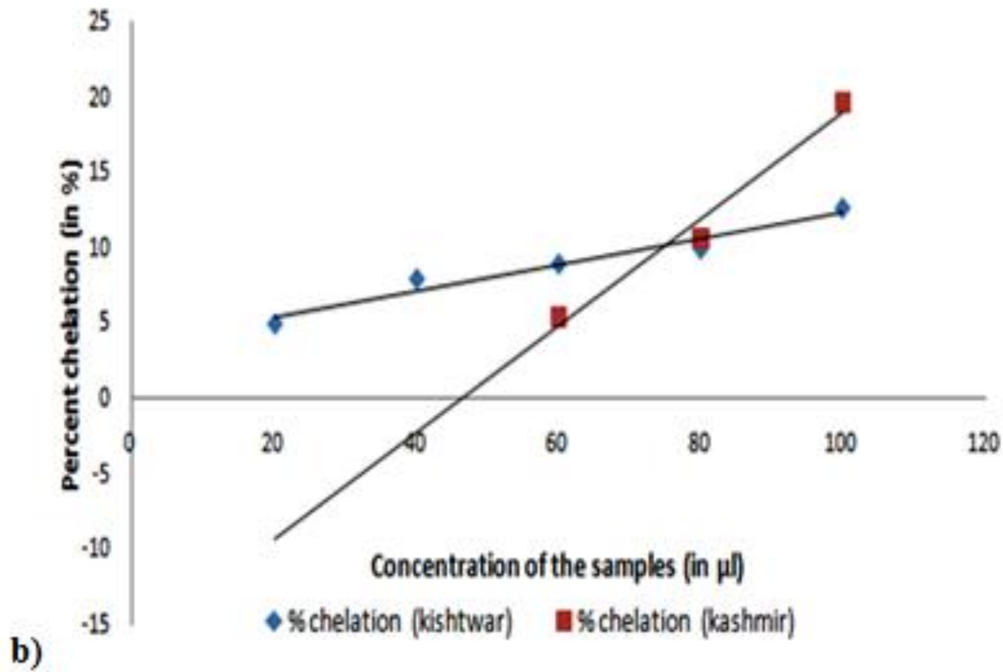


Fig.7 Phenolic content: Phenolic content of the extracts of Kishtwar is more than that of the phenolic content of the extracts of Kashmir.

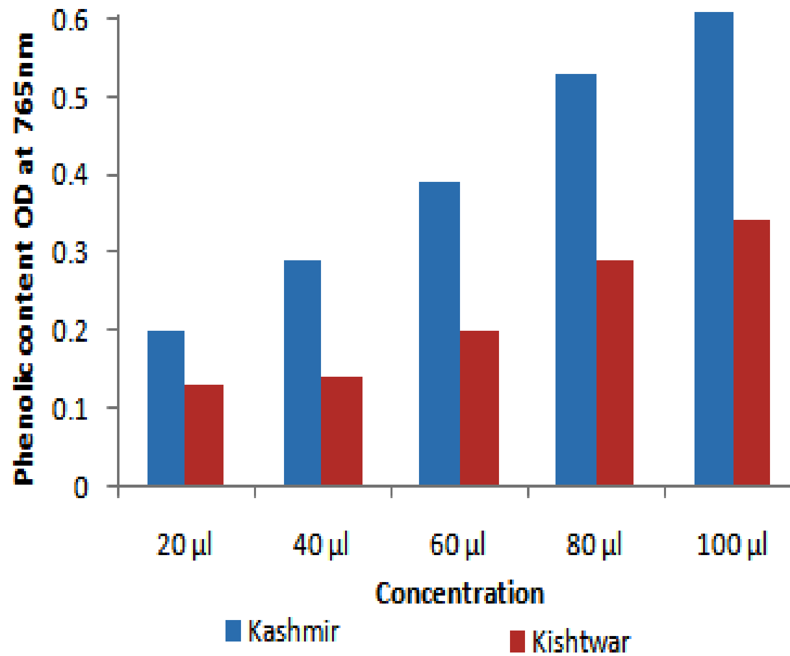


Fig.8 Flavonoid content: Flavonoid content of the extracts of Kishtwar is more than that of the Flavonoid content of the extracts of Kashmir.

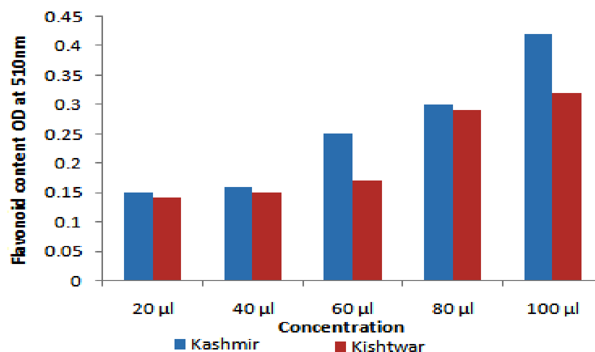
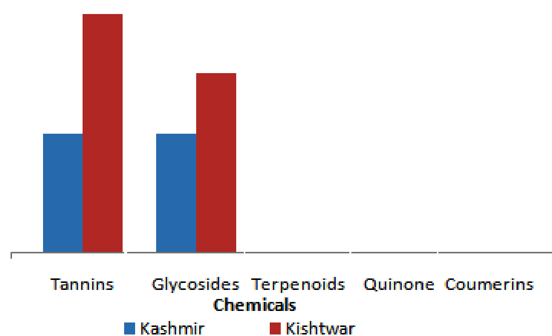


Fig.9 Biochemical assay. It shows that tannins and glycosides are present in the extracts of microbes from the stigma of Kashmir and Kishtwar.



The IC_{50} values of radical scavenging revealed that antioxidant activity of Kishtwar is more as lower IC_{50} value corresponds to a higher antioxidant activity of sample. The IC_{50} values of ferrous chelation revealed that antioxidant activity of Kishtwar is more as lower IC_{50} value corresponds to a higher antioxidant activity of sample. Hence, Kishtwar has more antioxidant activity. The flavonoid and phenolic content of Kashmir is higher than that of Kishtwar. Of the biochemical assays conducted, only tannins and Glycosides are present in stigma of saffron.

The Metagenomic DNA isolated methods used are used for soil samples. Hence, the Metagenomic DNA was not isolated in the purified form and hence could not be sequences. This needs further study for standardizing a protocol for Metagenomic DNA from stigma samples. Further work can be done to unveil the bacterial property to produce the pigments that act as favouring agent that can be used in bio-industry.

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