3. Materials and Methods

3.1. Sample collection

Soil samples were collected from Marakkanam salt pan (Latitude 12º14‘12N and Longitude 72º56‘28E), Tamil Nadu, India using sterile polythene bags randomly at a depth of 10 cm from the soil surface (Fig. 7). Samples were collected in the months of January 2014 and May 2014 (Fig. 8) and brought immediately to the laboratory for processing. On arrival to the laboratory, the samples were stored at 4˚C for short – term storage or at -20˚C for long periods.

3.2. Isolation and enumeration of halophilic bacteria

Halophilic bacteria were isolated using selective halophilic agar medium. The samples were serially diluted, an aliquot of 0.1 mL of each dilution from 10^{-3} to 10^{-6} was taken and spread on the surface of the sterile halophilic agar medium and incubated at 37˚C for 10 days. After the incubation period, pigmented colonies were selected and purified for further investigation (Rodriguez-Valera *et al*., 1982).

3.2.1. Composition of halophilic agar

The composition of the halophilic agar medium (HiMedia) was given in Table 2. The halophilic broth was prepared without adding agar.

Table 2: Composition of halophilic agar medium

Ingredients	**g / L**
Casein acid hydrolysate	10.000
Yeast extract	10.000
Proteose peptone	5.000
Trisodium citrate	3.000
Potassium chloride	2.000
Magnesium sulphate	25.000
Sodium chloride	250.000
Agar	20.000
Distilled water	1000mL
Final pH : 7.2±0.2	

Fig. 7: Map showing study area Marakkanam taluk, Villupuram district, Tamilnadu, India

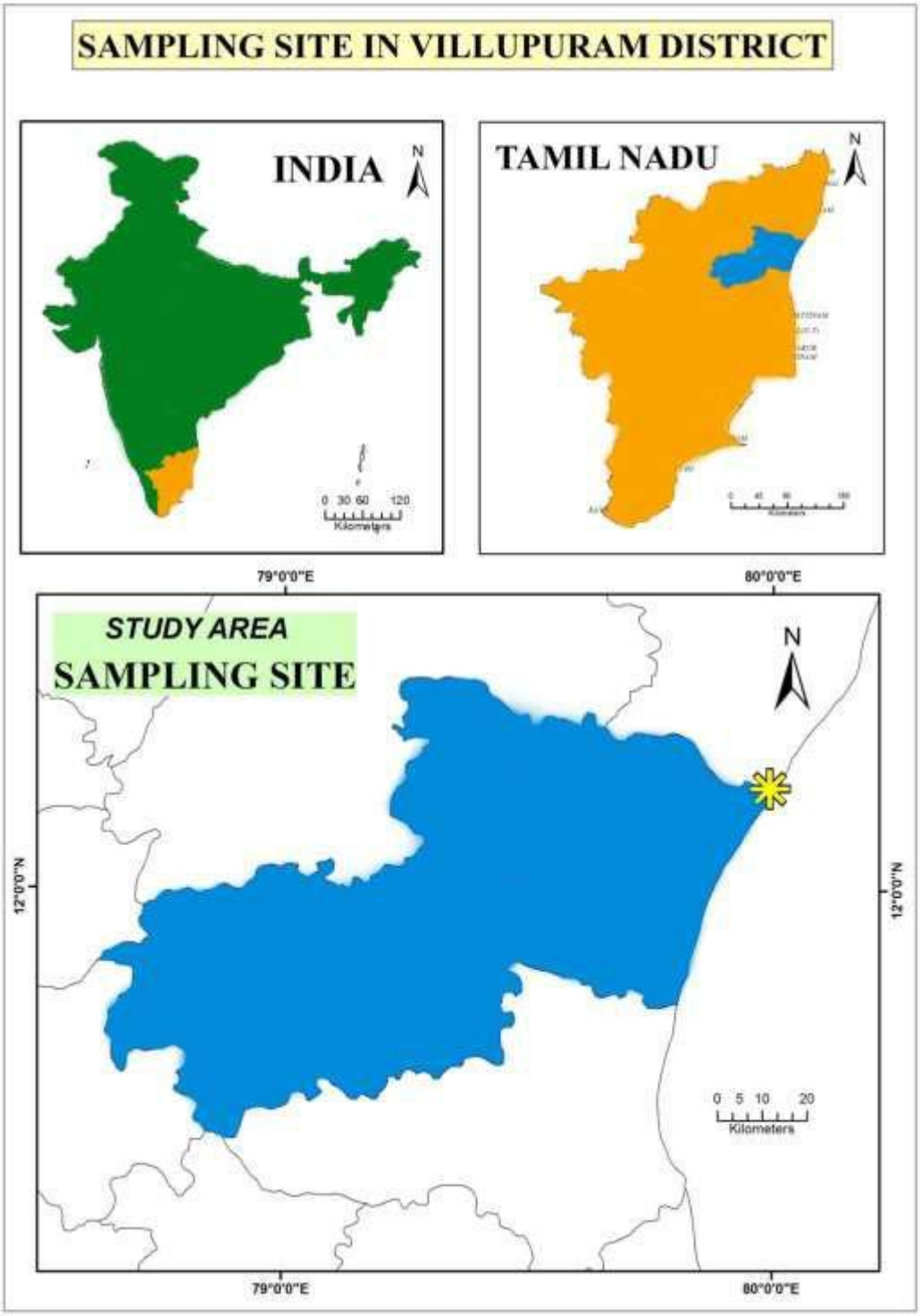

Fig. 8: Collection of soil samples at the sampling site

Note : (1)View of salt pan in Marakkanam on January 2014, (2) and (3) Collection of soil samples, (4) Concentrated brine with visible salt crystals, (5) and (6) View of salt pan on May 2014, (7) and (8) Collection of samples in May 2014, (9) Bright orange coloured brine solution.

3.3. Morphological and biochemical characterization of halophilic bacteria

The halophilic bacteria were subjected to various morphological and biochemical characterization. Biochemical tests were performed for selected halophilic bacterial isolates and 24 hours old bacterial cultures were used for all the biochemical tests.

3.3.1. Morphological characterization

3.3.1.1 Gram staining

Gram staining was performed for all the halophilic isolates obtained from Marakkanam salt pan, Tamil Nadu, India. Gram stain is a differential stain used to identify Gram positive and Gram negative bacteria. The pigment producing halophilic bacterial isolates were smeared and heat fixed on clean glass slides. The smears were flooded with methyl violet for 1 minute. After washing with distilled water, the smears were flooded with Gram's iodine for 1 minute. Then the smears were decolourised using ethanol for 30 seconds. Finally, the smears were flooded with safranine for 1 minute which acts as a counter stain. The slides were thoroughly and examined under oil immersion in a compound microscope. The morphology of the different pigment producing halophilic bacterial isolates was recorded (Vos *et al*., 2011).

3.3.2. Biochemical characterization

For biochemical tests, the halophilic bacteria isolates were grown in flasks and incubated at 37˚C. The optimal ionic content (per liter: $MgCl_2.6H_2O$ - 13 g, $MgSO_4.7H_2O$ - 20g, $CaCl_2$. $2H_2O$ - 1g, KCl - 4g, $NaHCO_3$ - 0.2 g, NaBr – 0.5g, NaCl - 250 g) was used in all the biochemical test media. The biochemical tests were performed by following the protocol of Colwell *et al*., (1979) and the isolates were identified based on Bergey's manual of systematic bacteriology (Krieg and Holt, 1984).

3.3.2.1. Indole test

Tryptophan is an important amino acid which can undergo oxidation by the enzyme tryptophanase produced by the bacteria and converted into various metabolic products such as indole, pyruvic acid and ammonia. The presence of indole is detected by adding Kovac‘s reagent which produces dark cherry red colour. Absence of cherry red colour indicates that tryptophan was not hydrolysed and demonstrates indole negative. The pigmented halophilic strains were inoculated into peptone water and kept for incubation at 37˚C for 24 hrs.

3.3.2.2. Methyl red test

All enteric bacteria produce acids at a pH6 in MR-VP broth which indicates methyl red positive by adding a drop of methyl red indicator. Absence of red coloration demonstrates negative. All the halophilic isolates were inoculated into MR-VP broth and kept for incubation at 37˚C for 24 hrs.

3.3.2.3. Voges Proskauer test

This test determines the presence of non-acidic or neutral end products such as acetyl methyl carbinol by oxidation of glucose. Baritt reagents such as alpha – napthol and potassium hydroxide solutions are used as a reagent used for this test. Presence of dark red colour indicates positive. The halophilic isolates were inoculated in MR – VP medium and incubated at 37˚C for 24 hrs.

3.3.2.4. Citrate utilization test

The test demonstrates which bacteria use citrate as a soul source of carbon for energy. This activity depends on the presence of an enzyme citrate permease produced by the microorganism which facilitates the transport of citrate into the cell. The selected halophilic isolates were inoculated in the citrate medium and incubated at 37˚C for 24 hrs. After incubation citrate positive isolates were identified by presence of growth on the surface of slants along with blue coloration. Citrate negative shows no growth without color change.

3.3.2.5. Carbohydrate fermentation

Fermentation of carbohydrates by the isolates under anaerobic condition was performed by using specific carbohydrate and a pH indicator (phenol red), which is red at neutral pH (7) and turns yellow at or below a pH of 6.8 due to organic acid production. All the halophilic isolates were inoculated into tubes containing 1 gm of specific carbohydrates (glucose, sucrose, arabinose, maltose, trehalose, cellobiose, and starch) and incubated at 37˚C for 24 hrs.

3.3.2.6. Catalase test

This test demonstrates the presence of an enzyme catalase in the organism. The enzyme catalyzes the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water. The presence of this enzyme in a halophilic bacterial isolate is apparent when small inoculum is introduced into hydrogen peroxide (3%) which shows air bubbles. All the halophilic isolates were subjected to catalase test under appropriate conditions.

3.3.2.7. Oxidase test

Oxidase test is used to detect the presence of cytochrome oxidase enzyme by specific bacteria. It was done in a disc impregnated with a reagent such as N, N, N‘, N‘ – tetramethyl-p-phenylenediamine (TMPD) which is a redox indicator. The oxidase positive organisms produce indophenol as end product. All the halophilic isolates were subjected to oxidase test by inoculating them into the oxidase disc. A positive oxidase test will results in a color change from violet to purple within 10-30 seconds. A negative oxidase test remains light-pink or absence of coloration.

3.4. Extraction of pigments from halophilic bacterial isolates

100 mL of halophilic broth was sterilized at 121˚C under 15lbs for 20 minutes separately inoculated with selected pigment producing halophilic bacteria and incubated at 37°C in an orbital shaker under 150 rpm. The pigments were extracted using acetone (intracellular) and ethyl acetate (extracellular) besides the

type of pigment produced by the isolates. A 24 hrs old culture broth incubated at 37°C under 150 rpm, was mixed with corresponding solvents and vortexed vigorously. The mixture was then centrifuged at 10,000 rpm, 0°C for 10 minutes. The resulting supernatant was collected and filtered through a 0.2 µm Whatman filter paper. The filtrate was concentrated using a rotary evaporator followed by extraction using methanol. The solvent extract was reconcentrated using rotary evaporator until minimal volume was obtained. This minimal volume of solvent extract was then transferred into a glass petri dish prior to drying in a vacuum drying oven. Dried pigment was dissolved in methanol for 2-3 times and used for further studies (Asker and Ohta, 1999).

3.5. Antioxidant activity by DPPH (1, 1-diphenyl-2-picrilhydrazyl) assay

The antioxidant activities of pigment extracts for all the selected twenty five isolates were estimated using quercetin as standard. The samples were prepared in methanol at a concentration of 500µg/mL and determined which is based on the principle of scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was added individually to the methanolic pigment extract of the halophilic isolates and stirred well. Then, the mixture was kept in dark for 30 minutes and the absorbance was measured at 517 nm against a blank. The scavenging effect was plotted against the time and the percentage of DPPH radical scavenging ability. All determinations were performed in triplicates. The percentage inhibition of the DPPH radical by the samples was calculated using the formula:

$$\%\ \text{of inhibition} = \frac{\text{Abs(C)} - \text{Abs(S)}}{\text{Abs(C)}} \times 100$$

Where Abs(C) is the absorbance of the control and Abs(S) is the absorbances of the sample i.e. pigment extract (Blois, 1958).

3.6. Cytotoxicity of the selected pigment producing halophilic bacteria

One of the most potential biological activities of halophilic isolates is their cytotoxic effect against various cancer cells. The selected five halophilic bacterial cultures which have shown highest antioxidant activity were justifiably chosen for cytotoxicity assay.

3.6.1. Preparation of HeLa cell line suspension and seeding of cells

A subculture of HeLa cell line in Dulbecco‘s Modified Eagle‘s Medium (DMEM) was trypsinized separately, after discarding the culture medium. 25 mL of DMEM with 10% FCS (Fetal Calf Serum) was added to the disaggregated cells in the flask. The cells were suspended in the medium by passing gently through the pipette and homogenized. One mL of the homogenized cell suspension was added to each well of a 24 well culture plate along with different concentration of samples – pigment extracts of *Chromohalobacter* sp. S2, *Chromohalobacter* sp. S6, *Oceanobacillus* sp. S12, *Bacillus* sp. S15, *Brevundimonas* sp. S17 (0 to 500 μg/mL). After 48 hours incubation, the cells were observed under microscope. With 80% confluence of cells, cytotoxic assay was carried out.

3.6.2. Cytotoxicity assay

After 48 hours incubation, the wells were added with MTT ((3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide) and left for 3 hours in room temperature. In all the wells, 100µL SDS in DMSO was added to dissolve the formazan crystals and the absorbances were read in Lark LIPR-9608 micro plate reader at 540 nm (Twentyman and Luscombe, 1987).

3.7. Molecular characterization of pigment producing halophilic bacteria

3.7.1. Genomic DNA extraction

The five selected pigment producing isolates were subjected to molecular characterization. The genomic DNA was extracted from selected five halophilic bacteria (*Chromohalobacter* sp. S2, *Chromohalobacter* sp. S6, *Oceanobacillus* sp. S12, *Bacillus* sp. S15 and *Brevundimonas* sp. S17) using the commercially

available DNA extraction kit (QIAGEN DNA). For DNA isolation, all the selected halophilic bacteria were grown in LB culture medium prepared at a salt concentration of 1M NaCl and pH7. 1mL of 24 hours grown bacterial cell suspension was transferred to 1.5 mL microcentrifuge tube and the cells were harvested by centrifugation in a centrifuge at 6000 rpm for 2 minutes at room temperature. The genomic DNA was extracted using QIAGEN DNA isolation kit. The extracted DNA was separated by means of 0.8% agarose gel electrophoresis and was stored at - 20˚C (Nowlan *et al*., 2006).

3.7.2. Polymerase Chain Reaction (PCR) amplification of 16S rRNA gene

In order to amplify 16S rRNA gene, polymerase chain reaction (PCR) was carried out using the primers 27f and 1522r. The sequences of the oligonucleotide primers used for 16S rRNA gene amplification were listed in Table 3. The components in the 40 µL of reaction mixture were given in the following Table 4.

Table 3: Sequences of 16S primer

Primer	Sequence 5'—3'
27f	AGAGTTTGATCCTGGCTCAG
1522r	AAGGAGGTGATCCACCCA

Table 4: Details of reaction mixture

Sl.No.	Reaction mixture	Volume
1	Ultra purified water	16µL
2	Primer Taq premix (Taq polymerase, dNTPs,2x buffer with Mg^{+})	20µL
3	Forward primer	1µL
4	Template DNA	2µL
5	Reverse primer	1µL

Amplification was carried out in a Thermocycler (icycler BioRad Laboratories, Inc., USA). The optimized PCR conditions were: Initial denaturation at 94˚C for 3 minutes, 30 cycles of cyclic denaturation at 94˚C for 1minute, cyclic annealing at 50˚C for 1 minute, cyclic extension at 72˚C for 1 minute and final extension at 72˚C for 5 minutes. After the reaction was completed, 10µL of amplified DNA was separated on 1.2% agarose, stained with ethidium bromide and recorded using gel documentation (Photonyx, Slite 140). A ready to use standard molecular marker supplied with DNAzyme^{TM} II DNA polymerase kit (Finzymes, Espoo, Finland) was included in the gel as marker DNA (Nowlan *et al*., 2006).

3.7.3. DNA Sequencing and phylogenetic analysis

3.7.3.1. DNA sequencing of 16S rRNA gene fragment

Finally the amplified PCR products were purified and subjected to DNA sequencing. The 16S rRNA purified PCR product (100 ng concentration) was subjected for sequencing using ABI DNA 3730 XL sequencer (Applied Biosystem Inc) at Macrogen, Korea. Sequencing of the 16S rRNA gene of the halophilic bacterial isolates was done from both the directions.

3.7.3.2. Computational analysis (BLAST) and identification of bacterial species

BLAST is a web based program that is able to align the search sequence to thousands of different sequences in a database and show the list of top matches. The obtained sequence was subjected to BLAST search and the bacterial species were determined. The percentages of sequence matching were also analyzed and the sequences was submitted to NCBI-Gen Bank using the available NCBI database (http://www.ncbi.nih.gov/BLAST/.) and obtained accession numbers (Sundaramanickam *et al*., 2015).

3.7.3.3. Phylogenetic analysis

The 16S rRNA sequences reported in the study was multiple-aligned using the CLUSTAL W, Version 1.7 (Thompson *et al*., 1994). The alignment was

corrected manually and converted to a distance matrix. The distance matrix was converted to a phylogenetic tree using the Neighbour - joining (NJ) algorithm (Saitou and Nei, 1987) of MEGA 5.05 with multiple substitutions corrected and positions with gaps excluded (Tamura *et al*., 2011).

3.7.3.4. Secondary structure prediction

The obtained nucleotide sequence was subjected to secondary structure prediction using web based program RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The query sequence of the respective halophilic bacteria was uploaded in the alignment box and it was submitted to RNAfold analyzer for secondary structure determination (Sloma and Mathews, 2015).

3.7.3.5. Restriction site analysis

The *in – silico* restriction site analysis of 16S rRNA in the selected pigmented producing halophilic bacterial species was analyzed by NEBCUTTER program version tools available online http://tools.neb.com/NEBcutter2/ index.pnp (Vincze *et al*., 2003).

3.8. Selection of potential isolate and growth analysis

The potential pigment producing halophilic bacterial isolate was selected besides their bright pigmentation, antioxidant activity and cytotoxicity. Then, the growth curve for the potential isolate was studied by optical density measurement of the culture broth at 600nm at regular intervals of 4 hours.

3.9. Optimization of culture condition for pigment production in *Bacillus licheniformis* S15

The effects of physical parameters such as pH, temperature; medium components (various carbon source and metal ions) was studied on pigment production *Bacillus licheniformis* S15. The parameters affecting pigment

production were investigated and validated. The variables were tested for increased pigment production with different carbon, pH, temperature and metal ions.

Among these variables different carbon, pH and temperature have shown more influence in the pigment production, hence it was taken further RSM based optimization.

3.10. RSM based optimization for pigment production in *Bacillus licheniformis* S15

RSM was used to optimize the medium constituents for pigment production. Several experimental designs have been considered to study such model and the Box Behnken Design (Box *et al*., 1978) followed by Aslan and Cebeci, (2007) was used. For the present study, three fractional factorial designs have been employed to fit the second order polynomial model, which indicated that 17 experiments were required for this procedure. The three significant independent variables X_1, X_2, and X_3 the mathematical relationship of the response Y on these variables can be approximated by quadratic (second degree) polynomial equation.

$$\mathbf{Y= b_o+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2+b_{13}X_1X_3 +b_{23}X_2X_3}$$

The _Design-Expert (File Version10.0.5.0) software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots and wireframe. This RSM was applied to the experimental data obtained using the statistical software (Khuri and Cornell, 1987).

3.11. Extraction and purification of pigment

The pigment from *Bacillus licheniformis* S15 was extracted using ethyl acetate and it was subjected to silica gel column chromatography (60×120mm) with n-hexane and ethyl acetate (2:1; v/v) as solvent. The eluted fraction was concentrated in rotary evaporator and used for further characterizations.

3.12. Characterization and structural elucidation of purified pigment

3.12.1. UV-Visible spectrophotometric analysis

The purified pigment extract was dissolved in ethyl acetate and then analyzed by scanning in UV-Visible spectrophotometer (Shimadzu, Japan) to detect the absorption maxima. The scanning range was 400-600nm (Khanafari *et al*., 2010).

3.12.2. Thin Layer Chromatography

The purified pigment extract of *Bacillus licheniformis* S15 was analysed using Thin Layer Chromatography (TLC) by coating the glass slide with silica gel 60. The extract was spotted on TLC sheet and separated using solvent system viz., petroleum ether: cyclohexane: ethyl acetate: acetone: methanol in different proportions. The developed plates were allowed to dry at room temperature and were identified by comparing with standard Rf values (Khanafari *et al*., 2010).

3.12.3. FT-IR analysis

FT-IR analysis of pigment extract was carried out as follows; A pinch of sample and 50mg KBr (spectroscopic grade) were thoroughly ground in mortar and the mixture was put on the pallet holder and pressure was applied through the hydraulic machine to make a thin film**.** FT-IR spectra were obtained on a FT = IR 8300 Shidmadzu spectrometer. The analysis conditions used were 16 scans at a resolution of 4cm measured between 400 and 4000 cm (Lóránd *et al*., 2002).

3.12.4. Mass spectrum

The purified pigment extract (0.5 g) was dissolved in DMSO prior to analysis using Electron spray ionization – Mass spectrometry (ESI-MS) (Lutnaes *et al*., 2002a).

3.12.5. NMR

The purified pigment was analysed for ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), HMBC NMR, HSQC NMR, COSY and DEPT 135 NMR using Bruker Avance 400 NMR spectrometer. Chemical shifts were reported relative to tetramethylsilane (TMS) with $CDCl_3$ as solvents (Lutnaes *et al*., 2002a).

3.13. Bioactivity of purified pigment

3.13.1. *In-vitro* anti-diabetic activity of purified pigment from *Bacillus licheniformis* S15

3.13.1.1. Inhibition of α -amylase enzyme by purified pigment

Starch solution (0.1% W/V) was obtained by stirring 0.1 g of potato starch in 100mL of 16 mM of sodium acetate buffer. The enzyme solution was prepared by mixing 27.5 mg of alpha-amylase in 100 mL of distilled water. The calorimetric reagent is prepared by mixing sodium potassium tartarate solution and 3, 5 di nitro salicylic acid solution. Both control and varying concentration of pigment extract (0-100µg/mL) from *Bacillus licheniformis* S15 were added with starch solution and left to react with alpha – amylase solution under alkaline conditions at 25˚C. The generation of maltose was quantified by the reduction of 3,5 diitro salicylic acid to 3-amino -5- nitro salicylic acids. The reaction was measured immediately after 3 minutes and OD value was taken at 540 nm (Malik and Singh, 1980). The percentage of inhibition was calculated by:

$$\% \text{ of inhibition} = \frac{\text{Abs(C)} - \text{Abs(S)}}{\text{Abs(C)}} \times 100$$

Where Abs(C) is the absorbance of the control and Abs(S) is the absorbance of the sample (Gopal *et al*., 2017).

3.13.1.2. Inhibition of α – glucosidase by purified pigment

The inhibitory activity of α - glucosidase was determined by incubating a solution of starch substrate 1mL (2% sucrose) with 0.2 M Tris buffer (pH 8) and

various concentration of purified pigment extract (0-100µg/mL) from *Bacillus licheniformis* S15 for 5 minutes at 37˚C.

The reaction was initiated by adding 1 mL of alpha- glucosidase enzyme (1U/mL) to it followed by incubation for 40 minutes at 35˚C.Then the reaction was terminated by the addition of the 2 mL of 6N HCL. Then the intensity of the colour was measured at 540 nm (Malik and Singh, 1980). The percentage of inhibition was calculated by:

$$\% \text{ of inhibition} = \frac{\text{Abs(C)} - \text{Abs(S)}}{\text{Abs(C)}} \times 100$$

where, Abs(C) is the absorbance of the control and Abs(S) is the absorbance of the sample (Gopal *et al*., 2017).

3.13.2. *In-vitro* anti inflammatory activity of purified pigment

3.13.2.1. Membrane stabilization activity

Fresh whole human blood (10mL) was obtained from clinical lab and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline. The reaction mixture consists of 1 mL of varying concentration of pigment extract (0-100µg/mL) and 1 mL of 10% RBCs suspension, saline was added to the control test tube. Aspirin was used as a standard. The reaction mixture was incubated in water bath at 56^0C for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. Percent membrane stabilization activity was calculated by the formula:

$$\text{Percentage stabilization} = \frac{\text{Abs(C)} - \text{Abs(S)}}{\text{Abs(C)}} \times 100$$

where, Abs(C) is the absorbance of the control and Abs(S) is the absorbance of the sample (Vidya *et al*., 2015).

3.13.2.2. Nitric oxide scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH was measured by Griess reaction (Marcocci *et al*., 1994). The reaction mixture (3mL) consisted of sodium nitroprusside (10mM) in phosphate buffer saline and the purified pigment (10, 25, 50 and 100µg/mL) was incubated at 25˚C for 150minutes. After incubation, 1.5mL of the reaction mixture was removed and 1.5mL of the Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% Napthylethyline diamine hydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Ascorbic acid was used as standard. Percent inhibition of nitric oxide scavenging was calculated using the following formula:

$$\text{Percentage inhibition} = \frac{\text{Abs(C)} - \text{Abs(S)}}{\text{Abs(C)}} \times 100$$

where, Abs(C) is the absorbance of the control and Abs(S) is the absorbance of the sample.

3.13.3. Antimicrobial activity of purified pigment extract from *Bacillus licheniformis* S15

3.13.3.1. Test-pathogenic microorganisms

Gram positive bacteria, *Staphylococcus aureus* (MTCC 96), yeast pathogen *Candida albicans* (MTCC 227) and mycelial fungus *Aspergillus fumicatus* (MTCC 2584) were used for *in- vitro* antimicrobial activity. These selected pathogenic strains were obtained from Microbial Type Culture Collection, IMTECH, Chandigarh.

3.13.3.2. Antibacterial activity of purified pigment

The antibacterial activity was determined by well diffusion method (Holder and Boyce 1994). About 25 mL of sterile Mueller Hinton agar was poured into a sterile Petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which 18 h grown (OD adjusted to 0.6) 100 µL of above said pathogenic

bacteria were transferred onto plate and made culture lawn by using sterile L-rod spreader. After five minutes, a sterile cork borer was used to make well on the agar. The test samples were dissolved in sterile saline and loaded in to wells with various concentrations such as 25, 50, 75 and 100 μg/mL. The solvent saline loaded well served as negative control and Azithromycin (30µg/mL) added well served as positive control. The plates were incubated at 37°C in for 24 h. After incubation, the antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).

3.13.3.3. Antifungal activity of purified pigment

The antifungal activity was determined by well diffusion methods (Rios and Recio, 2005). About 25 mL of potato dextrose agar was poured into a sterile petri plate (Himedia, Mumbai, India). The plates were allowed to solidify, after which two days grown fungal disc (5mm) of mycelial fungal pathogens were placed separately on to the mid of the agar plate and wells were made for mycelial fungi. The yeast pathogenic fungus was grown for 18 h and 100 µL of the culture was swabbed on to the potato dextrose agar (Himedia,Mumbai, India). The test sample was dissolved in ethyl acetate and loaded in to wells with various concentrations such as 25, 50, 75 and 100 μg/mL. The clotrimazole added well served as positive control. In case of mycelia fungi, the plates were incubated for 72 hours where as for yeast the plates were incubated for 24 hours. The antifungal activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (Himedia, Mumbai, India).