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# "ISOLATION AND IDENTIFICATION OF CAROTENOID PRODUCING HALOPHILIC BACTERIA AND ARCHAEA FROM SOLAR SALTERNS OF MULUND, MUMBAI"

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***Abstract:*** Halophilic microorganisms have capacity to tolerate and live under high salt concentrations by generating a balance inside and outside of the cell to avoid osmotic stress. These traits of halophilic microorganism generate a great interest to the scientific community due to their potential applications in the biotechnological industries. Three efficient carotenoid producing Halophiles SC-14, SC-15 and SC-16 were isolated and characterized from Solar saltern of Mulund, Mumbai, Maharashtra. Isolated halobacteria was identified as *Gordonia terrae*, *Rothia Kristinae* and *Niallia alba*. These salt tolerant organisms were characterized using morphological, biochemical assays. All the three isolates showed remarkable growth at pH 7 and temperature 30°C. However, variation was recorded in resistance of isolates to salt concentration. Remarkable carotenoid production by SC-14 was recorded at pH 7, temperature 30°C and NaCl concentration of 5 %. In our study antioxidant activity was exhibited by extracted pigments. In addition to their utilization as colorants many carotenoids have proved beneficial in prevention and treatment of certain cancers such as prostate cancer and in prevention of heart diseases by quenching free radicles.

**Keywords:** Carotenoids, Solar saltern, Phylogeny, antioxidant activity, Halophiles.

## I. INTRODUCTION

Hypersaline environments like salt lakes and solar salterns are extreme ecosystems in which the change in salinity is the dictating factor which determines microbial diversity at any given point of time .[1] Prokaryotic diversity in any ecosystem is an important factor to be considered because of its role in nutrient turnover, element recycling and as a potential hub for recovery of microorganisms for industrially important metabolic products. [2] Salterns originating microorganisms bear biotechnological potential for the production of hydrolytic enzymes, exopolysaccharide, carotenoid pigments etc. Carotenoids are naturally occurring yellow to orange red pigments synthesized as hydrocarbons (carotene; eg. lycopene, $\alpha$-carotene and $\beta$-carotene) or their oxygenated derivatives (xanthophylls, eg. lutein, $\alpha$-cryptoxanthin and $\beta$-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin) by plants, bacteria, algae and fungi. It is the ability of carotenoids to confer color therefore, commercial interest has been developed in these molecules predominantly as natural colorants. In addition to their utilization as colorants many carotenoids have proved beneficial in prevention and treatment of certain cancers such as prostate cancer and in prevention of heart diseases by quenching free radicles. [3] Besides this compatible solute produced in response to an osmotic stress by halophiles are also equally important. In this halophilic organisms mainly accumulate organic compounds like sugars, polyols, amino acids and their derivatives. These nonionic,highly water soluble compounds help to maintain osmotic equilibrium and stabilizes cell proteins in presence of high salt concentration and therefore called as compatible solutes. These solutes are commonly used as enzyme and or cell protectants [4,5],Halophilic bacteria and archaea have also been used to study haloviruses. Potential importance of halophiles in different industrial areas such as the leather industry [6], food preservation [7] food colorant production is also evident. [8] Besides food coloring properties the anti- carcinogenic, anti-inflammatory, radical scavenging properties of carotenoid are attracting researchers to develop new therapeutic products. Thus, the current study focuses on isolating halophilic bacteria from different Indian salt pans, screening and characterizing them using morphological, biochemical approaches, and identifying potential producers of carotenoid pigments. We have been made to study the diversity of cultivable halophilic microorganisms from interconnected multipond solar saltern of Mulund (E.) Mumbai (19°10'12''N, 72°57'18''E). These ponds are situated along western coast in Arabian Sea. Very less reports have showed community structure of these ponds till the date. Our investigation therefore aimed at characterization of prokaryotic biodiversity of these ponds.

## II. MATERIALS AND METHODS

### 2.1 Sample collection and isolation of pigment producing bacteria from solar saltern

The Sample was collected from marine solar slatterns of Mulund (E) ($19^0$10'12''N, $72^0$7'18''E) Mumbai from different locations around the area. They were mixed to get an adequate representation of the local micro-flora. The samples were collected in PET bottles. Different media were used for cultivation of microorganisms such as Nutrient agar, Luria Bertani agar with variable concentration of NaCl. All the samples were mixed in equal proportion and used for isolation of halophiles. Aliquots of 100µl of composite hypersaline samples were plated on solid media. Plates were observed after 14 days of incubation and number of colonies appeared and colony characteristics were recorded. Among the different media used for cultivation the medium that showed highest diversity andsupported faster growth was selected in further investigation. Using the selected medium minimum salt requirement for growth was determined by varying NaCl concentration in the range of 0 to 30 %. Different types of 3 colonies were observed and colony characters were recorded.

### 2.2 Purification and Selection of pigment producing bacterial isolates

The isolated colonies were purified by successive streaking on NB agar medium containing NaCl. Three potential isolates were selected based on the difference in the color of colonies. For this, isolates were streaked on NB agar medium and incubated at 37°C for 72 h and observed for the pigment production. The isolates that showed bright pigmentation were selected. Purified isolates were preserved as 30% glycerol stock at -80°C.

### 2.3 Identification of selected isolates

Selected colonies were subcultured on nutrient agar plates with 20% salt and incubated at 35°C. Microscopic and macroscopic features of grownisolates were recorded. Amongst many pigmented colonies seven intense pink colored fast growing colonies were selected for further analysis. Gram's staining was performed by Dussalt's modified method. [9] Catalase and oxidase activities, indole production, citrate utilization, lipase $H_2S$ productions, hydrolysis of gelatin, casein, starch, cellulose and urea were observed, Methyl red, Vogues–Proskauer tests and carbohydrate fermentation tests were performed using standard procedures. [10,11] Appropriate positive and negative controls were used in all these tests. Antibiotic susceptibility was tested by disc diffusion method using disc containing Bacitracin (10 units/disc), Polymyxin (300µg/disc), Ciprofloxacin(5µg/disc), Gentamycin (10 µg/disc) and Tetracycline (30 µg/disc) (Hi media Mumbai). [12]

### 2.4 Effect of pH, temperature and incubation time on growth of selected pigment producer

To check effect of the optimum temperature for growth and pigment production, isolates were grown in NB medium. Equal number of cells of the three isolates were inoculated in fresh NB medium and incubated at different temperatures of 30ºC, 37ºC and 40ºC for 18 h and observed for growth. To determine optimal pH for growth and pigment production, isolates were inoculated in NB media of varying pH (6, 7 and 8). pH of the media was set at 6 by using 0.1N HCl and 8 by 0.1N NaOH. Equal number of cells (10 µl culture representing 1 O.D at 620 nm) were inoculated in NB medium of different pH and incubated at 37°C for 18 h in an incubator shaker at 180 rpm and observed for growth by measuring absorbance at 620 nm. To determine optimal Salt Concentration for growth and pigment production, isolates were inoculated in NB media of varying salt Concentration (5%, 10% and 20%). Equal number of cells (10 µl culture representing 1 O.D at 620 nm) were inoculated in NB medium of different salt Concentration and incubated at 37°C for 18 h in an incubator shaker at 180 rpm and observed for growth by measuring absorbance at 620 nm. pH,temperature and salt concentration showing highest growth was selected and used for further analysis.

### 2.5 Production and Extraction of Microbial Pigments

Bacterial isolates were grown in NB medium as well as inNB agar medium. For large scale production of pigments, equal number of cells were inoculated in 1000 ml freshly prepared NB medium and incubated in an incubator shakerat temperature 37ºC at 180 rpm for 72 hours and visually observed for pigment production. After 72 h, bacterial culture was centrifuged at 7000 rpm for 30 min. After centrifugation, supernatant was discarded, and bacterial cellpellets were processed for extraction of pigments. To the cell pellets, 1ml mixture of acetone and methanol (3:1) was added and vortexed until the cell pellets turned colorless. The cell debris was then discarded, while the supernatant was transferred to a glass Petri plate and dried overnight in an incubator at 37°C. Dried pigment was scrapped out and dissolved in methanol. [13]

### 2.6 Assessment of Antioxidant activity using DPPH assay

The radical scavenging potential of pigments was studied through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Varying concentrations of pigments ranging from 100 µg/mL to 1000 µg/mL were prepared from stock. Total reaction volume was maintained at 4 mL, comprising of sample, methanol as diluent and 2 mL of DPPH was added to each tube. The reaction mixture was incubated in dark for approximately 30-45 min. Followed by the incubation period, the absorbance was measured at 515 nm using UV Visible spectrophotometer. [14,15]

Percent DPPH radical scavenging activity was determined using the formula. [14,15]

$$\frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100$$

## III.RESULTS

### 3.1 Isolation and screening of carotenoid producing halophilicbacteria

The traditional seasonal salt pan of Mulund, Mumbai, was sampled. Water samples were pale yellow in color and viscous (Figure-1(a)).

(**a**) (**b**)

**Figure 1**:(**a**) Crystallizer pond of saltern; (**b**) Sample collection at Solar saltern at Mulund, Mumbai, India.

Total 3 halophilic organisms were isolated and designated as SC-14, SC-15 and SC-16. Amongst the 3 isolates,2 were pigmented and intense coloured colonies.Colonies of all selected isolates were circular, orange to yellow in colour, small in size having raised elevation and entire margin. Out of all isolates two developed orange colonies while one isolate has developed yellow color colony. All isolates were Gram negative and circular in shape. All isolates showed luxuriant growth at 5% salt concentration at 30°C temperature.

SC-14 has used glucose, sucrose, maltose, fructose and trehalose as a carbon source and SC-15 has used all sugars as a carbon source except sorbitol. Whereas, SC-16 has used only glucose fructose and trehalose as energy source. However, none of the isolates has used sorbitol as carbon source. Enzyme profile of isolates showed that out of 3, SC-15 and SC-16 have secreted extracellular amylase. All the isolates showed positive oxidase test.

All the three isolates showed remarkable growth at pH 7, temperature 30°C and salt conc at 5 %. All the 3 isolates were tested for antibiotic susceptibility isolates. Both SC-15 and SC-16 were sensitive to polymyxin B, ciprofloxacin, Tetracyclin and resistant to gentamycin. Pattern of sugar utilization, enzyme profile and antibiotic sensitivity of isolates is given in Table 1.

Pattern of sugar utilization, enzyme profile and antibiotic sensitivity of isolates is given in Table 1. Identification of isolate was carried by comparing results with standard strain characteristics given in Bergey's Manual of Systematic Bacteriology. [16] SC-14, SC-15 and SC-16 were identified as *Gordonia terrae*, *Rothia Kristinae* and *Niallia alba* respectively.

**Table 1: Biochemical characteristics of isolated Carotenoid producers**

| **Characteristics** | **SC-14** | **SC-15** | **SC-16** |
|---|---|---|---|
| Morphology | Circular | Circular | Circular |
| Gram nature | - | - | - |
| Cell size (µm) | 2mm | 2mm | 2mm |
| Motility | Motile | Motile | Motile |
| Margin | Entire | Entire | Entire |
| Colony pigmentation | Orange | Yellow | Orange |
| Opacity | Opaque | Opaque | Opaque |
| NaCl Range for growth (%) | 5-20 | 5-20 | 5-20 |
| Temp. optimum °C | 30 | 30 | 30 |
| $P_H$ Optimum | + | + | - |
| **Utilization of** | | | |
| Glucose | + | + | - |
| Sucrose | + | + | + |
| Arabinaose | - | + | - |
| Lactose | - | + | - |
| Maltose | + | + | - |

| | | | |
|---|---|---|---|
| Fructose | + | + | + |
| Mannitol | - | + | - |
| Xylose | - | + | - |
| Sorbitol | - | - | - |
| Trehalose | + | + | + |
| Galactose | - | + | - |
| Indole production | - | - | - |
| **Enzyme Profile** | | | |
| Catalase | + | + | + |
| Amylase | - | + | + |
| Gelatinase | - | - | - |
| Urease | - | - | - |
| Protease | - | - | - |
| Cellulase | - | - | - |
| Oxidase | + | + | + |
| **Antibiotics suspetibility** | | | |
| Bacitracin (10 unit/disc) | ND | ND | ND |
| Polymyxin (300mcg/disc) | ND | S | ND |
| Ciprofloxacin (5mcg/disc) | ND | S | ND |
| Gentamycin (10mcg/disc) | ND | R | R |
| Tetracyclin (30mcg/disc) | ND | S | ND |
| Identified as | *Gordonia Terrae* | *Rothia kristinae* | *Niallia alba* |

ND= Not detected,
S= Sensitive R=Resistant

*Gordonia terrae* (SC-14) showing remarkable pigment production and fast growth was selected and used for pigment production (Figure-2(a)).

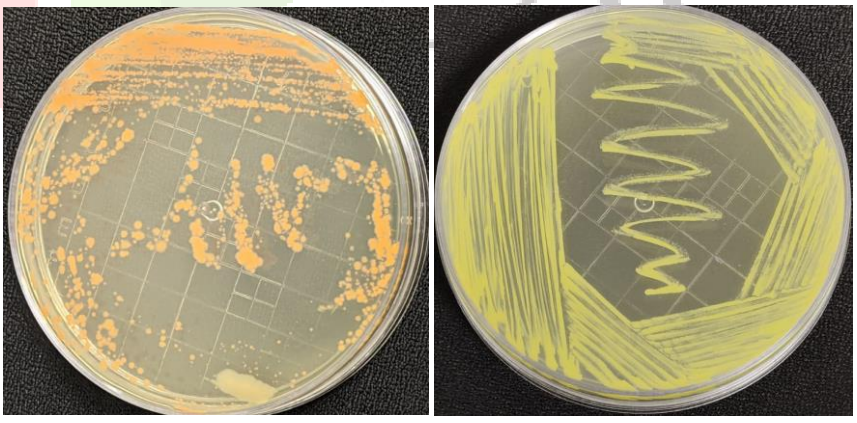

**Figure 2: Culture of (a) *Gordonia terrae* (b) *Rothia kristinae***

Remarkable carotenoid production by SC-14 was recorded at pH 7, temperature 30°C and NaCl concentration of 5% (Figures 3 (a)).

Initiation of carotenoid production was observed after 4 days of incubation and maximum carotenoid production was recorded after 7 days of incubation (Figure 3-5). The antioxidant activity of crude carotenoid extracted was tested and recorded activity was equivalent to antioxidant activity of standard lutine and astaxanthin. [17]

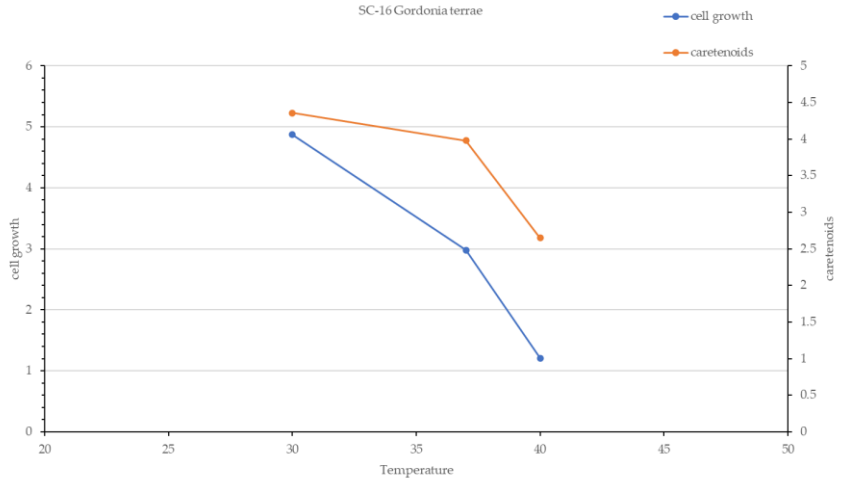


**Figure 3: Effect of temperature on growth and carotenoid production**

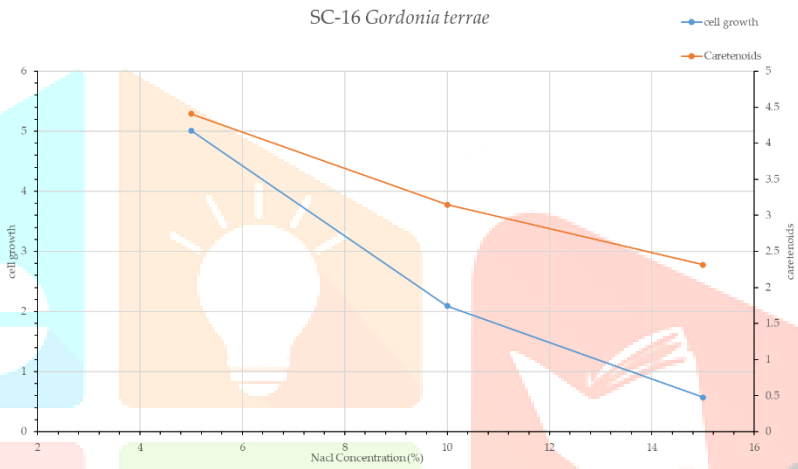


**Figure 4: Effect of NaCl on growth and carotenoid production**

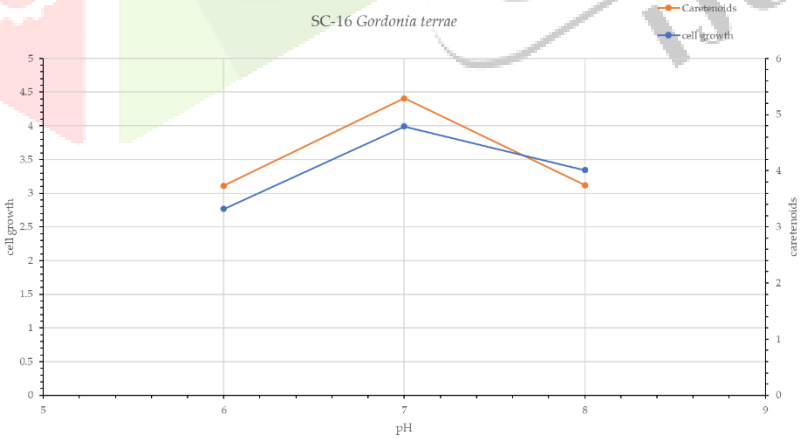


**Figure 5: Effect of pH on growth and carotenoid production**

## IV.DISCUSSION

Halophiles which inhabit almost in hypersaline environments like saline ponds, showed great potential toward production of various carotenoids including Dunaliella, producer of beta-carotene, Salinibacter ruber, rich in salinixanthin. [18] There exist many factors which influence carotenoid production by microbial cell factory and the most important and highly affecting ones especially in halophiles is salinity of culture condition since, adapting to salinity requires and also leads to altering in metabolic pathways. Hence the possibility of carotenoid over-production could be handled resourcefully. [19] In the present study we have isolated and identified Halophiles from the interconnected multipond solar saltern of Mulund (E.)Mumbai (19°10'12''N, 72°57'18''E). From identified isolates, remarkable carotenoid production by SC-16 was recorded at ph 7, temperature 30°C and nacl concentration of 5%. Similarly, previous study by Aparna et al., [20] showed carotenoid producing Halophiles from Solar saltern of Mulund, Mumbai, Maharashtra. Extensive studies on various hypersaline environments in different geographical locations have been conducted over the last few decades, as they are known to play a key role in the diversity that has permitted the isolation and taxonomic characterization of many halophilic species. [21,22] Many other studies have been studied from different parts of Maharashtra such as Unkeshwar hot spring, Bordi Region, and coastal region, Maharashtra. [23,24,25] Very few studies [20] have been done on isolation and identification of Halophiles from Solar saltern of Mulund, Mumbai, Maharashtra. Therefore, our study supports to add the microbial diversity of the solar saltern of Mulund. In present study, antioxidant activity was exhibited by extracted pigments. In addition to their utilization as colorants these carotenoids have proved beneficial in prevention and treatment of certain cancers such as prostate cancer and in prevention of heart diseases by quenching free radicles. Our research can be cutting edge ideas toward both preclinical studies and screening for anticancer activity of natural products.

## V. CONCLUSION

We have highlighted the presence of carotenoid producing *Gordonia Terrae.* in solar saltern of Mulund, Mumbai, India. The carotenoid production, extraction and characterization was also carried out. The results obtained revealed that the isolate *Gordonia Terrae* can be used as source of carotenoid.

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## References

1. Rodriguez-Valera, F., Ventosa, A., Juez, G., Imhoff, J.F. "Variation of environmental features and microbial populations with salt concentrations in a multi-pond saltern". *Microb Ecol* 11, (1985), 107–11.
2. Lorenz, P., Eck, J. "Metagenomics and industrial applications". *Nat Rev Microbiol* 3, (2005), 510–516.
3. Laura, P., Sabine, S., Khaneja, R., Bramley, P.M., Cutting, S.M., Michael, K., Sisse,l N., Synnove, L.J. "Bacterial carotenoids XXXI* C50- Carotenoid 5, ** Carotenoids of *Halobacteriumsalinarium* especially Bacterioruberin". *Acta Chemica Scandinavica*, 24, (1970), 2169-2182.
4. Margesin, R., Schinner, F. "Potential of halotolerant and halophilic microorganisms for biotechnology". *Extremophiles* 5, (2001), 73–83.
5. Oren, A. "Industrial and environmental applications of halophilic microorganism". *Environmental Technology* 8-9, (2010), 825-834.
6. Birbir, M., Ilgaz, A. " Isolation and identification of bacteria adversely affecting hide and leather quality". *Journal of the Society of Leather Technology Chemists* 80,(1996), 147–153.
7. Rodriguez-Valera, F. "In Halophilic Bacteria'. *Boca Raton: CRC Press* (1988), vol. I, 3–30.
8. Galinski, E. A. "Osmoadaptation in bacteria". *Advances in Microbial Physiology* 37, (1995), 273–328.
9. Dussault, H.P. "An improved technique for staining red halophilic bacteria". *J Bacteriol* 70,(1955), 484-485.
10. Aneja, K.R. "Experiments in Microbiology, Plant pathology and biotechnology", Fourth edition, New International Publisher, New Delhi, 2007.
11. Dave, S.R., Desai, H.B. "Microbial diversity at marine salternnear Bhavnagar, Gujarat, India". *Curr Sci*, **90**, (2006), 497-500.
12. Elevi, R., Assa, P., Birbir, M., Ogan, A., Oren, A. "Characterization of extremely halophilic Archaea isolated from the Ayvalik Saltern, Turkey". *World J Microbiol Biotechnol* 20, (2004), 719-725.
13. Asker, D., Ohta, Y. Production of canthaxanthin by *Haloferax alexandrines* under non-aseptic conditions and a simple, rapid method for its extraction, *Appl Microbiol Biotechnol* 2002, **58**, 743-750.
14. Koleva, I.I., Van Beek, T.A., Linssen, J.P.H., Groot, A., Evstatieva, L.N. "Screening of plant extracts for antioxidant activity: A comparative study on three testing methods". *Phytochem Anal* 13,2002, 8-17.
15. Om, P., Gondwal, M., Pant, A.K. "Essential oils composition and antioxidant activity of water extract from seeds and fruit pulp of *Skimmia anquetilia", Indian J Nat Prod Resour* **2**(4), (2011), 435-441.
16. Bergey, D.H., Krieg, N.R., Holt, J.G. "Bergey's manual of systematic bacteriology", Williams & Wilkins, Baltimore, 1984.
17. Yachai, M. "Carotenoid production by Halophilic Archaea and its application (Thesis)", Prince of Songkla University, 2009.
18. de Lourdes Moreno, M., Sanchez-Porro, C., Garcia, M.T., Mellado, E. "Carotenoids' production from halophilic bacteria". *Methods Mol Biol* 892,(2012), 207-17.
19. Margesin, R., Schinner, F. "Potential of halotolerant and halophilic microorganisms for biotechnology". *Extremophiles* 5, (2001),73-83.

20. Sardar, A.G., Pathak, A.P. "Isolation and characterization of carotenoid producing Haloarchaea from solar saltern of Mulund, Mumbai, India". *Indian Journal of Natural Products and Resources* 3(4), (2012), 483-488.
21. Das, D., Kalra, I., Mani, K., Salgaonkar, B.B., Braganca, J.M. "Characterization of extremely halophilic archaeal isolates from Indian salt pans and their screening for production of hydrolytic enzymes". *Environ. Sustain* 2, (2019), 227–239. doi:10.1007/s42398-019-00077-x.
22. Ventosa, A. "Taxonomy of moderately halophilic heterotrophic eubacteria. Halophil. Bacteria". 1, (1988), 71–84. doi:10.1007/978-1-4615-1869-3_13.
23. Anupama,P.P., Mukundraj, G.R. "Production and Characterization of Thermostable Amylase from *Bacillus korlensis* Isolated from Unkeshwar Hot Spring, Nanded". *International Journal of Biochemistry and Biomolecules* 5(2), (2019), 8–11.
24. Anupama, P.P., Vikas J., Supriya M., Bhoomi, D. "Assessment and determination of halophilic bacterial diversity and antimicrobial potential from mangrove ecosystems of bordi region, Maharashtra". *Journal of Advanced Scientific Research* (2021),147.
25. Girdhari,S., Pathak, A. "Thermophilic microbes present in coastal region of Maharashtra". *Bioinfolet* 19 (2), (2022), 124.