CHAPTER 3

ISOLATION, IDENTIFICATION AND OPTIMIZATION STUDIES OF HALOTOLERANT BACTERIA

3.1 INTRODUCTION

Optimization studies of growth factors for the halotolerant bacterial strain isolated from marine sea water and identified using biochemical and 16s RNA are discussed in this chapter.

The isolated strain from the sea water is optimized for the following physical growth parameters:

- Media composition
- Salinity
- Temperature
- Inoculum dosage
- pH
- Nitrogen source

3.2 MATERIALS AND METHODS

The chemicals used to formulate Mineral Salt Medium (MSM) and Basal Salt Medium (BSM), used in this study were of analytical grade and procured from M/s Hi-Media Biochemicals, India.
3.2.1 **Bacteria Isolation - Serial Dilution**

The serial dilution technique was adopted to isolate the microbial strain from the sea water. The 9 test tubes containing 9 ml of 0.85% saline water were prepared as per serial dilution technique. 1 ml of the saline water was added to the first test tube and mixed well using vortex mixer. 1 ml of saline water from this test tube was transferred to next test tube and so on. Various dilutions were plated on pre-sterilized Petri dishes containing nutrient agar (Koller and Jelinek 1976). Plates were incubated at 37°C for 48 h. Well-grown colonies were isolated and streaked over pre-sterilized agar slants separately. Further the slants were incubated at 37°C for 24 h and then labeled and stored in refrigerator for future use.

3.2.2 **Identification of the Strain**

The isolated strain was identified by both biochemical tests and 16s rRNA sequencing method and its morphological characteristics by Gram staining technique. Gram staining was a four-part procedure, used certain dyes to make a bacterial cell stand out against its background. A loop full of sample was taken and smeared on the slide. After heat fixing was over, the slide was flooded with crystal violet (primary stain) for 60 sec, and the specimen was washed. In the next step specimen was flooded with iodine solution (the Mordant) for 60 sec. Then, the specimen was rinsed with water. Decolorizer (ethanol) was added to the specimen to check for either gram (+) or Gram (-). Final step involved the addition of counter stain, Saffranin. It was allowed to stand for a minute. Later the specimen was rinsed with water; red colour appearance confirmed the presence of gram-positive species. Morphologically regular circular colonies were termed as cocci. The isolated halotolerant bacterial strain was identified as *S. lentus*, by 16s rRNA method. Appearance of white pigmentation in nutrient agar media is typical of *S. lentus*, and is shown in Figure 3.1.
3.2.3 Pre-Culture

Pre-culture was done in order to conduct growth experiments. Nutrient agar was used as a preservation media for the strain and nutrient broth was used for preparing inoculum. Nutrient broth was considered to be a standard medium for the growth of any unknown bacterial species. Test tubes were sterilized and nutrient broth (5 ml) was added. They were plugged tightly with non-absorbent cotton and sterilized in an autoclave at 121°C and 15 psi pressure. A loop full of grown colonies was removed with the sterilized wire loop. The mouth of the slant was again flamed before plugging to avoid external contamination. The entire procedure was carried out in the laminar hood. The inoculum was stabbed into the medium. The loop was pulled out of the tube. The neck of the tube was flamed and the cotton stopper was replaced. The scraper was flamed before setting it down on the table. The tube was labeled and incubated at 37°C for 24 h in a mechanical shaker for the oxygen supply required for the growth. The concentration of inoculum needed for any parameter study was obtained from this after 24 h. The appearance of turbidity in the test tube indicated the growth of the bacteria. The growth studies were carried out with this culture.

3.2.4 Rationale Behind the Selection of S. Lentus – A Halotolerant Bacterial Strain

The rationale behind the selection of a halobacterial strain was, it’s possible future requirement for the treatment of industrial effluents with high salinity and mixed industrial waste waters, especially effluents from leather manufacturing industry. Moreover, halo bacterial strains are reported (Asad et al 2006) to be efficient in degrading toxic xenobiotic compounds formed as a result of degradation of azo dyes.
3.3 EXPERIMENTAL

3.3.1 Effect of Growth Media

The growth was examined to study culture characteristics. Suspension for inoculum was obtained by growing the isolated organism S. lentus, on nutrient broth. A loop full of the strain was transferred aseptically from an agar slant to a test tube containing 5 ml of sterilized nutrient broth. It was incubated at 37°C for 24 h. This was served as inoculum for this experiment. The growth of microbial strain was studied in two different media. The media used were mineral salt medium contained the following chemicals (g/L) $K_2HPO_4$, 1.73; $KH_2PO_4$, 0.68; $MgSO_4\cdot7H_2O$, 0.1; NaCl, 4; $FeSO_4\cdot7H_2O$, 0.03; $NH_4NO_3$, 1.0; $CaCl_2\cdot2H_2O$, 0.02; glucose, 5.0. The composition of the basal mineral salt medium used in this study was as follow (g/L): $NaNO_3$, 4.0, NaCl 1.0, KCl 1.0, $CaCl_2\cdot2H_2O$ 0.1, $KH_2PO_4$ 3.0, $Na_2HPO_4\cdot12H_2O$ 3.0, $MgSO_4$ 0.2, $FeSO_4\cdot7H_2O$ 0.001; 2 ml trace element stock solution composed of (g/L): FeCl$_3\cdot6H_2O$ 0.08, ZnSO$_4\cdot7H_2O$ 0.75, CoCl$_2\cdot6H_2O$ 0.08, CuSO$_4\cdot5H_2O$ 0.075, MnSO$_4\cdotH_2O$ 0.75, H$_3$BO$_3$ 0.15, $Na_2MoO_4\cdot2H_2O$ 0.05. The initial pH was adjusted to 7. Two sets each for separate media were prepared. Each set contained 2 conical flasks (100ml) of 25 ml of media. The conical flasks were properly labeled and sterilized. The prepared suspension S. lentus, was inoculated in 25 ml pre-sterilized media and incubated at 37°C for 48 h. Samples were withdrawn aseptically in test tubes for every 8 h and examined for growth using spectrophotometer at 600 nm up to 48 h. The results are given in Figure 3.2.

3.3.2 Effect of NaCl Concentration on Bacterial Growth

Studies on the effect of NaCl, on bacterial growth were considered as an important module in this work. Isolated culture S. lentus, were inoculated in to sterilized MSM medium with different NaCl concentration
viz, 0.2%, 0.4%, 0.6%, and 0.8% (w/v), the inoculated samples were placed on incubated shaker at 37°C. Samples were collected every 8 h up to 48 h. Collected samples were examined for absorbance values at 600 nm using spectrophotometer. The results are given in Figure 3.3.

3.3.3 Effect of Temperature

The effect of temperature for S. lentus growth was studied, temperature was varied from 25, 30, 37 and 40 °C, culture was inoculated into sterilized MSM medium and samples were collected every 8 h, using spectrophotometer growth were monitored at 600 nm. The results are given in Figure 3.4.

3.3.4 Effect of Inoculum Concentration

Optimum inoculum dosage for an effective growth of the identified strain under optimized growth conditions was obtained by following the respective growth profiles at varying inoculum dosages. Inoculum dosage range was fixed between 2 – 8 % (v/v), Samples were inoculated in MSM media and incubated at 37°C for 48 h. Samples were withdrawn at regular time intervals and analyzed for absorbance at 600 nm. Corresponding growth profiles resulted were given in Figure 3.5.

3.3.5 Effect of pH

pH range was varied from 5-9, and the growth of identified halotolerant strain was carried out with the previously optimized MSM media, Well-grown fresh colonies (absorbance ≈1) of the halotolerant strain were added (4 % v/v) to MSM media and temperature was maintained at 37°C for 48 h. A minimum salinity of 0.4 % NaCl (w/v) was maintained in the MSM media (by adding externally NaCl under sterile condition) to ensure
halotolerance behavior. Samples were withdrawn at regular time intervals and analyzed for absorbance at 600 nm and growth curve was obtained as shown in Figure 3.6.

3.3.6 Effect of Nitrogen Source

For effective growth of identified strain on NB media, at optimized growth conditions, both organic (casein and urea) and inorganic (ammonium sulphate and ammonium nitrate) substances were used as co-substrates. All the co-substrates were added at a constant amount (0.1 % w/v) in MSM media and incubated at 37°C for 48 h. Samples were withdrawn at regular time intervals and analyzed for absorbance at 600 nm. Corresponding growth profiles resulted were given in Figure 3.7.

3.4 RESULTS AND DISCUSSION

3.4.1 Isolation of Halotolerant Strain

The incubated agar plates were analyzed for colony formation. Major colonies (cocci) were found to exist predominant. From the gram staining results, the isolated strains were found to be gram-positive respectively. The isolated halotolerant bacterial strain was identified as S. lentus, by 16s rRNA method and the nucleotide sequence of S. lentus has been submitted to GenBank of National Centre for Biotechnology Information (NCBI) of USA and has accession number JN673760. The results were given in Appendix 1.

3.4.2 Characteristics of S. lentus

S. lentus are gram-positive cocci that utilize glucose oxidatively.
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Figure 3.1  **White pigmentation of identified halotolerant S. lentus, in Nutrient agar media**

It has several features that distinguish it from other species of Staphylococcus:

- It can grow at 37°C
- Produces a white pigment
- Oxidase positive
- Coagulase negative
- Novobiocin resistant

### 3.4.3 Media Optimization

The isolated strains were grown in different media under similar physical environment and growth was ascertained by periodically measuring the absorbance of culture. The growth curve was plotted as in Figure 3.2.
Figure 3.2 Comparative plot of growth profile for S. lentus colonies grown in MSM (●) and BSM (■) media at pH 7, 37°C and 4 % Inoculum concentration

The microbial growth in different media established the requirement of nutrient for their cultivation. It was observed that microbial growth was found minimum in BSM compared to growth in MSM, during the same period. These observations could be taken as an indication that nitrogen was rate limiting. Hence it was decided that MSM would be used as the medium to conduct further experiments on Biological Reaction Calorimeter (Bio RTCal).

3.4.4 Effect of Salinity

The effect of salinity on growth for S. lentus was checked and the results are shown in Figures 3.3. The growth rate of isolated strain S. lentus exhibited optimum growth at concentration of 0.4 % NaCl by wt in mineral salt medium and show halotolerance up to 0.4 % NaCl by wt.
3.4.5 Effect of Temperature

The effect of temperature on growth for S. lentus was monitored individually and under various temperature conditions the results are shown in Figure 3.4. The growth was predominant in 37°C compared to the other temperature parameters.

Figure 3.3 Effect of salinity on growth of S. lentus in MSM at pH 7.0, 37°C and 4% Inoculum concentration. (Salinity %: 0.2 (■), 0.4 (●), 0.6 (▲), and 0.8 (▼)).

Figure 3.4 Effect of temperature on growth of S. lentus in MSM at pH 7.0, and 4% Inoculum concentration (Temperature, °C: 25 (■), 30 (●), 37 (▲), 40 (▼))
3.4.6 Effect of Inoculum Concentration

Figure 3.5 illustrates the effect of inoculum dosage on the growth of S. lentus. 4 % (v/v) inoculum concentration was found to be optimum for effective growth of S. lentus. Increasing the dosage levels at small increments above 4 % did not show any marked increase in cell growth. Excess inoculum dosage for a given composition of growth media would tend to inhibit the growth due to severe competition between the individual cells on assimilation of nutrients present in the growth media.

![Figure 3.5 Effect of inoculum concentration on the growth of S. lentus in MSM at pH 7, 37°C and 0.4 % NaCl (w/v). (Inoculum %: 2% (■), 4% (○), 6 % (▲), 8 % (▼).)](image)

3.4.7 Effect of pH

Figure 3.6 represents the comparative growth profiles resulted for growth of S. lentus under optimized growth conditions for varying pH levels. This comparative plot depicted that an optimum growth was observed on a neutral pH of 7.0. S. lentus was a well known neutrophile organism and therefore was the most suited one for the treatment of tannery dye effluents which is also found to have a neutral pH. Hence no pre-treatment was needed to correct the pH for biodegradation experiments.
3.4.8 Effect of Nitrogen Source

Apart from carbon co-substrates, effect of nitrogen substrates was also needed to be evaluated, as the dye effluents stream was composed of both nitrogen and carbon sources. Azo dye effluents waste stream was found composed of both organic and inorganic nitrogen’s. In Common Effluent Treatment Plants (CETP), urea was applied as a nitrogen co-substrate and therefore its effect on growth was also compared. Inorganic nitrogen sources like ammonium chloride and ammonium nitrate were also used to study their effect. Results (Figure 3.7) indicate that ammonium nitrate was observed as preferential co-substrate for maximum growth of S. lentus. This showed that the identified strain had ability to degrade the azo dyes.
Figure 3.7  Effect of nitrogen source on the growth of S. lentus in MSM at 4 % inoculum concentration 37°C, pH 7 and 0.4 % (NaCl w/v) salinity. (Nitrogen source: casein (■), Ammonium nitrate (●), Ammonium chloride (▲), urea (▼)).

3.5  CONCLUSIONS

- Gram-positive cocci bacterial strains were isolated from sea water. Strain was identified by both biochemical and 16s RNA sequencing method as S. lentus.

- Growth experiments performed showed an optimal growth at 0.4 % salinity (NaCl by wt) and exhibited a halotolerance up to 0.8 % level.

- The effect of various physical factors viz., Media, temperature, pH, inoculum conc., C-source and N-source on growth of S. lentus was done at shaker level.

- Optimal values of growth factors for enhanced growth of S. lentus were obtained from their respective growth profiles and were found as Mineral Salt Medium, 37°C, pH 7.0, 0.4 % NaCl (w/v) and 4 % inoculum (v/v), and ammonium nitrate 0.1% (w/v).