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Research Article

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Quantification of bacterial biomass by DNA analysis

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ABSTRACT

Quantification of microbial biomass is very important for proper understanding of the microbial process in environment. There are many different methods available to estimate the bacterial biomass but they all suffer from some limitations. Due to the recent development in molecular methods, it is possible to quantify the bacterial biomass by analyzing the genomic DNA. The aim of this research work was to estimate the bacterial biomass by quantification of DNA extracted from halophilic bacterial isolates during the growth. For this purpose, two bacterial strains JSO1 and JSO2 isolated from halophylic environment were used. The strains isolated were sequenced and identified as Micrococcus lylae (JSO1) and Micrococcus luteus (JSO2). After monitoring the growth, the cultured samples were used for extraction of DNA and were analyzed by UV spectrophotometer. JSO1 showed 13µg/ml growth compared to JSO2 which had 7.2µg/ml of DNA. JSO2 showed better purity of the DNA ranging from 1.72 to 1.84 than JSO1 which had 1.69 to 2.15. JSO1 showed higher DNA growth than JSO2 but purity was seen higher in JSO2 than JSO1. Relationship between turbidity and DNA concentration was significant, but the level of significance was low suggesting DNA quantification may be useful to quantify the bacterial biomass.

Keywords: Genomic DNA, halophile, microbial biomass, phylogenetic tree, turbidity.

INTRODUCTION

Halophiles are extremophile organisms that can thrive in environments with very high concentrations of salt. The name comes from the Greek for "salt-loving". They consist of archaea, bacteria and eukarya. According to the metabolic activity of halophiles, they include oxygenic and anoxygenic phototrophs, aerobic heterotrophs, denitrifiers, fermenters, sulfate reducers, and methanogens [1]. These extremophiles can be found anywhere with a concentration of salt five times greater than the salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds. The presence of osmoprotectants such as polyols, betaines [2], ectoines [2,3], beta-carotene [2] etc. in their cytoplasm help them to tolerate the salt environment. These organic compounds make these organisms very important for exploitation in industries, pharmaceutical, textile etc [1].

In order to study the microorganisms, estimation of bacterial biomass is very much essential [4]. The quantification of biomass is needed for scaling up in commercial biotechnology. Different methods used for the quantification of microbial cells are ATP [5], muramic acid, lipopolysaccharide, organic carbon [6], etc. but the limitation of these processes are that they are either commonly present every living organism or in specific group of organism. Another technique used is direct counting but it does not show the exact population present in the environment and overlapping of cells cannot give the exact estimation of number in a sample. Other methods used are

bioluminescence, photometric methods including fluorescence, flow cytometry [7] which also undergo some or the other limitations.

In growth studies, the increase in turbidity of the medium is considered proportional to the increase in optical density of the medium that may directly relate to the increase in the bacterial biomass i.e. cell number of bacteria. One limitation of this method is both the dead and living cell will contribute to the turbidity thereby allowing false estimation of biomass in the culture broth. DNA quantification may be a good method for measuring and quantifying of bacterial biomass in an environmental sample. The living biomass can only have an intact cell and used for extraction of DNA. So quantification of biomass using genomic DNA analysis is a reliable and sensitive technique.

The aim of this research work was to estimate the bacterial biomass by quantification of DNA extracted from bacterial strain during the growth. The purpose of this experiment was also to investigate the relationship between DNA concentration and turbidity measurement during the growth.

EXPERIMENTAL SECTION

Sample Collection

Halophilic soil samples were collected from Ottapalam, Kerala, India. The samples collected in sterile polythene bags were transported to Molecular and Microbiology Research Laboratory and stored at 4°C until further analysis.

Media Preparation

To isolate microorganisms from the soil sample, nutrient agar medium was used. The composition of liquid medium was as follows (gram per 1000 ml of distilled water) peptone (10); meat extract (10); agar (15) and pH 7.2 ± 0.2 and autoclaved at 121^0C for 15 min at 15 lbs pressure. Isolation of halophilic organisms medium was incorporated with 3 % of sodium chloride.

For DNA extraction, Luria bertani broth was used. The composition of medium was as follows (gram per 100 ml of distilled water) tryptone (1.0); yeast extract (0.5); without agar and pH was maintained at 7.2 ± 0.2. Isolation of halophilic organisms medium was incorporated with 3 % of sodium chloride.

Isolation of Microorganisms

1 g soil sample were serially diluted up to 10^{-5} and 10^{-6} and plated by spread plate method on nutrient agar plates and incubated under aerobic conditions at 37°C for 24 hours. Isolation was done in duplicate and plates without inoculation were maintained as negative control. The colonies that were grown on plates were randomly selected for further study. The isolated microorganisms were preserved in agar slants at 4 ^{0}C.

Study of Growth Kinetics of Bacterial Strains

LB medium in side arm flasks was inoculated with culture and incubated at 30 ^{0}C in orbital shaker at 120 rpm for 5 days (120 hrs). Turbidity was measured as an indicator of bacterial growth. Measurement was taken at 600 nm of the samples at 3 hours interval using calorimeter (Systronics 9130, Ahmedabad, India) for 5 days. After measuring the growth at regular intervals of 3 hours, 1 ml of broth was transferred to a sterile eppendorf tube. The eppendorf tubes were centrifuged at 10,000 rpm for 10 min and then the supernatant was discarded. The pellet was used for DNA extraction.

Procedure for DNA Extraction

1 ml CTAB buffer was added to the pellet in each of the eppendorf tubes. 7 µl beta-mercaptoethanol and 1 µl proteinase K were added and kept in the water-bath for 45 minutes at 65^{o}C. Centrifugation was done at 10,000 rpm for 10 minutes at 25^{o}C. The supernatant was transferred to fresh vial and 200 µl of phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added to it and mixed well. Centrifugation was done and this step was repeated. The supernatant was transferred to fresh vial and 300 µl of chloroform:isoamylalcohol (25:1) was added and centrifuged. The supernatant was collected and 60 µl of sodium acetate was added along with 120 µl isopropanol to precipitate DNA. Centrifugation was done at 10,000 rpm for 10 minutes at 25^{o}C and the supernatant was discarded. 200 µl of 70% ethanol was added to the pellet and again centrifuged for 5 minutes at 4^{o}C. The DNA pellet was air dried and dissolved in 100 µl in TE buffer. Electrophoresis was carried out to check the banding pattern of DNA of the samples. 15 µl of this sample was loaded in a well and agarose gel electrophoresis was run at 50 volts [8].

Quantification of Bacterial DNA

Quantitative analysis of DNA of the samples was done using UV-Vis spectrophotometer (Elico BL-222, Chennai, India). 5 µl of the samples was diluted in 3000 µl of distilled water (dilution factor equal to 600). Absorbance was measured at 260 nm and 280 nm [9].

Quantity and quality of DNA can be calculated using the following formula

Quantity of DNA = (A_{260} x 50 x dilution factor) / 1000 - (a)

Quality of DNA = (A_{260} / A_{280}) - (b)

Identification of the Strain

The bacterial strain JSO1 and JSO2 were identified using 16S rRNA sequencing. The PCR analysis was performed as follows: universal 16S rRNA primer 518F and 800R were used for amplification of 16S rRNA. The 16S rRNA gene sequences obtained from the isolate JSO1 and JSO2 were compared with other bacterial sequences by using NCBI BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pair wise identities. Multiple alignments of these sequences were carried out by Clustal W2 (www.ebi.ac.uk/clustalw). Phylogenetic trees were constructed in Jalview software using neighbor joining with distance calculation. The 16S rRNA sequence of JSO1 and JSO2 were deposited in NCBI with an accession number KC351486 and KC351487 respectively. Bacterial sequencing was done by Macrogen, Inc., Seoul, Korea.

RESULTS AND DISCUSSION

Based on the sequence analysis of the bacterial isolates by BLASTN for JSO1 and JSO2 were identified as *Micrococcus lylae* and *Micrococcus luteus* respectively. The phylogenetic tree of the results was shown in Fig. 1 and the neighboring distance between *Micrococcus lylae* (JSO1) and *Micrococcus luteus* (JSO2) was found to be 0.29 and 0.51 respectively.

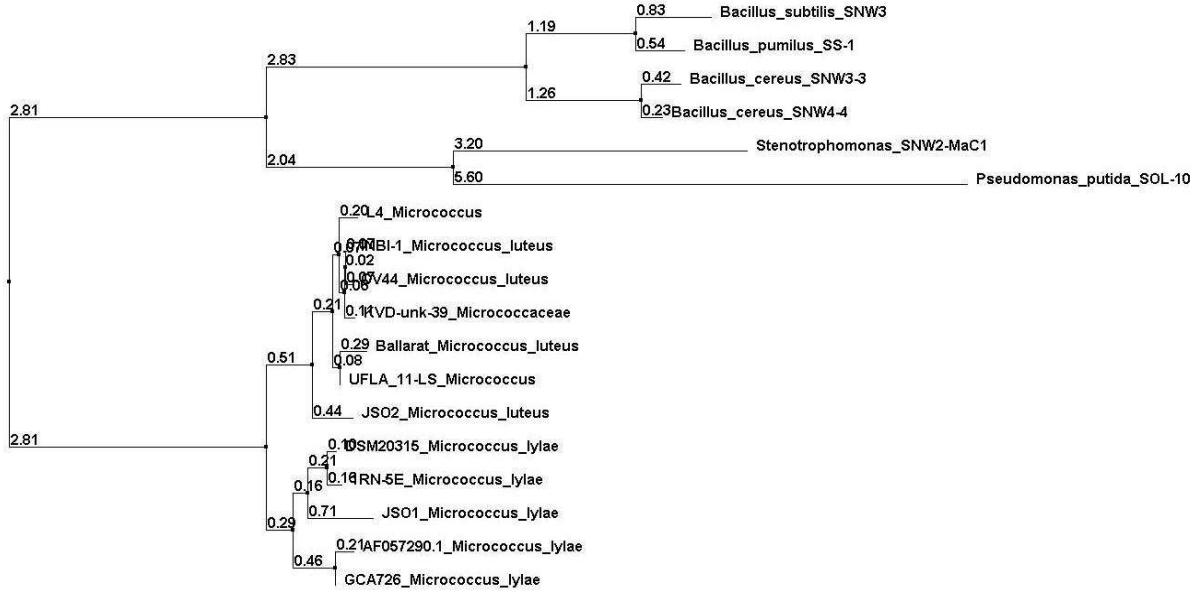

Fig. 1: Phylogenetic tree representing *Micrococcus lylae* (JSO1) and *Micrococcus luteus* (JSO2) with neighbor joining distance

Results were expressed as mean ± standard deviation. Figure 2 clearly showed that in the initial period, both the strains *M. lylae* (JSO1) and *M. luteus* (JSO2) showed significant growth till 12 hour. A gradual increase in the growth of bacteria was observed till 54 hours and then it was found to be stagnant till 108th hour for *M. luteus* (JSO2) and up to 81st hr in *M. lylae* (JSO1). Increase in quantity of DNA with increase in growth time for both *M.lylae* (JSO1) and *M. luteus* (JSO2) was observed. Except on the 1st day *M. luteus* (JSO2) showed lowere levels of DNA than *M.lylae* (JSO1) during the growth but on the last day of estimation, *M. lylae* (JSO1) showed maximum value of DNA which was about two times of the DNA in *M. luteus* (JSO2). *M. lylae* (JSO1) showed a value of 13µg/ml whereas *M. luteus* (JSO2) showed 7.2µg/ml of DNA on the last day of the experiment (Fig.3). In fig. 3, the extracted DNA of *M. lylae* (JSO1) and *M. luteus* (JSO2) were compared.

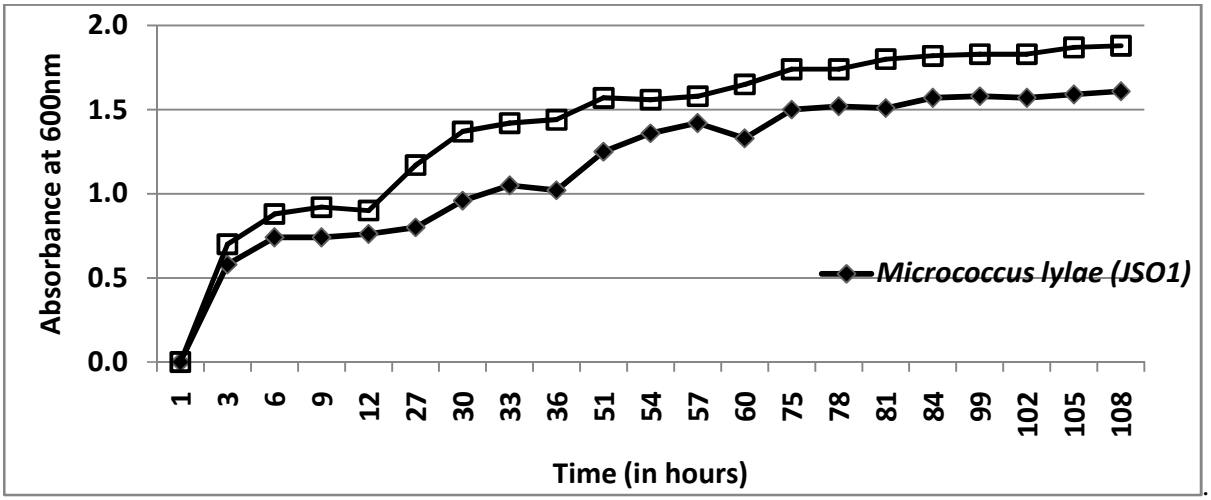

Fig. 2: Growth of *Micrococcus lylae* (JSO1) and *Micrococcus luteus* (JSO2)

The quality of DNA relates to the purity of DNA. The purity of DNA is generally calculated by A260/ A280 in which the quality of DNA can be one of these three situations:
(i) 1.75 to 1.9 = DNA is good
(ii) less than 1.75 = protein contamination and
(iii) more than 1.9 = RNA contamination

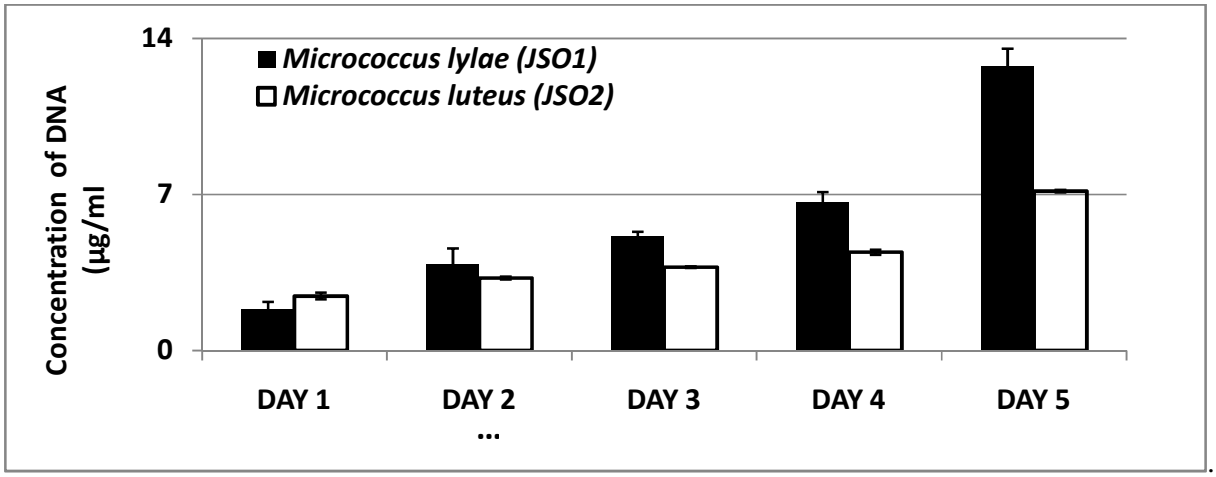

Fig 3: Comparison of the extracted DNA of strain *Micrococcus lylae* (JSO1) and *Micrococcus luteus* (JSO2) during the growth

The quality of DNA in *M. lylae* (JSO1) and *M. luteus* (JSO2) ranged from 1.69 to 2.15 and 1.72 to 1.84 respectively during the growth. The DNA obtained from *M. luteus* (JSO2) showed better purity of DNA than *M. lylae* (JSO1).

DNA concentration and turbidity values obtained during growth were correlated (fig.4). The R^2 values of samples obtained indicated the correlation but the level of significant was not very high. Since the growth was measured as turbidity, the values of turbidity may not reflect the growth of bacteria. That means the two variables of x and y axes are dependent on each other and directly proportional to each other. This figure also graph showed approximately similar values of R^2 for bacteria *M. lylae* (JSO1) and *M. luteus* (JSO2). Since DNA was an indicator of live cells, they may provide a better estimation of growth than the turbidity.

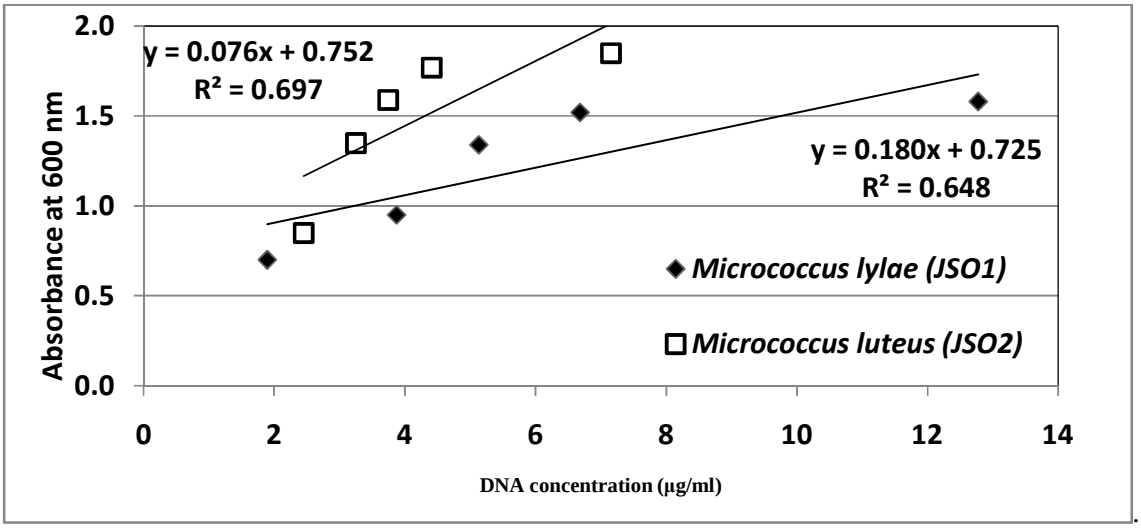

Fig 4: Relationship between growth of bacterial isolates turbidity and the extracted DNA concentrations of *Micrococcus lylae* (JSO1) and *Micrococcus luteus* (JSO2)

CONCLUSION

In this study, it is clear that biomass of the bacterial growth in the culture medium can be quantified by the concentration of DNA in the sample. Growth studies by turbidity measurement at an absorbance of 600nm are generally used for biomass estimation of microorganisms in a sample [10]. But much difference is seen in theoretical and practical ways of biomass analysis by this turbidity method. After reaching the stationary phase, there is seen no decline in the curve, the metabolites produced by the cells and dead biomass present will show turbidity giving a false interpretation of living microorganisms in the culture medium.

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