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Isolation and characterizations of halotolerant bacteria and identification by FAME analysis

KEYWORDS: FAME analysis, Salt tolerance, Antibiotic sensitivity, Decolourization.

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ABSTRACT *Micrococcus species constitute a diverse group of bacteria widely distributed in soil and the aquatic environment. In this study, micrococcus species isolated from the coastal environment of Gujarat (India) were identified, by morphological test, physiological biochemical methods and fatty acid methyl ester (FAME) analysis, as Kocuria rosea. Growth curve of this microorganism was measured (studied) and the generation time of Kocuria rosea was 3.86 hours. The isolated culture showed salt tolerance upto 20%.It was sensitive to antibiotics like Penicillin, Streptomycin and Tetracyclin, showed decolorization of Malachite green (50mg/l) under shaking conditions within 6 hours. However, decolorization was not observed at static conditions. The decolourization was confirmed by UV –VIS spectrophotometer.*

Introduction

Halotolerant organisms are known to tolerate extreme conditions of salt although they require normal salt concentration as has been shown by (Oren, 2002). In India, Gujarat contains 1600 km wide coastal areas including salt producing areas of Bhavnagar near Gulf of Khambhat. These areas are full of biological diversity as described previously (Dave and Desai, 2006). Micrococcus species show salt tolerance. The Gram–positive, aerobic, cocci-shaped, spherical bacteria of the Genus *Micrococcus* are the most widely represented organisms in the soil and fresh water frequently on the skin of man and other animals this was noticed by (Kamekura and Onishi, 1974). There is no record on the isolation of strains belonging to the genus *Kocuria* from the marine environment, although many members exhibit tolerance to high salt concentrations was observed by (Stackebrandt, Koch,Gvozdiak and Schumann, 1995) and (Kovács et. al,1999). A major class of synthetic dyes is widely used in textile, paper, printing, colour photography, rubber products, pharmaceuticals, food coloring, cosmetic and many other industries. Some of the dyes and dye degradation products are reported to be carcinogenic and mutagenic in nature, it was observed by (Novotny et. al, 2006).Thus, the effluents must be treated before releasing into the natural environment. Contamination of surface water with dyes released from Textile and Dye industries represents a serious problem. These dyes are recalcitrant to the microbial degradation and affect the usual biological treatment of the industrial effluents this was noticed by (Swamy and Ramsay, 1999). Malachite green (MG) is a tri-phenyl methane dye which has been used extensively for dyeing silk, wool, jute, leather, ceramic and cotton purposed by (Parshetti et. al, 2006). It is highly soluble in water. It is highly toxic to mammalian cells. Reduction of MG to leucomalachite green has been reported but leucomalachite green is also not environmentally friendly as per results of (Mitrowska and Posyniak, 2004). However, biodegradation of MG into non-toxic compounds and into simpler metabolic intermediates have been reported.

Present study includes isolation of halotolerant bacteria from soil and its identification. The diverse physiology of *Micrococcus* spp. requires elaborate biochemical tests for their identification. Advance in chromatographic analysis of whole call fatty acid methyl ester (FAME) profiles have made this technique sufficiently sensitive and reliable for identification of *Micrococcus* at species level. The isolated organism was studied for its Growth curve, Salt tolerance, Antibiotic sensitivity and Triphenylmethane (malachite green) dye decolorization.

Materials and Methods

Chemicals

Chemically treated media is used.

Sample collection

A soil sample along with sea water was collected in sterile plastic box with the help of spatula from a salt pan located near Bhavnagar (21°70'927"N, 72°22'4207"E, Gujarat).Temperature of the sample at the time of collection was 29°C and pH of the sample was 7.8. Sample was then transported to laboratory. Sample was filtered to remove soil particles.

Growth and Isolation of Bacterial cultures

0.5 ml of soil sample was diluted serially with double distilled sterilized water.100µl of 6^{th} dilution was placed on sterilized soybean casein digest agar contained in petridishes and incubated for 24 hours at 30 °C. The culture medium composition was Pancreatic digest of casein 1.7g, Peptic digest of soya bean meal 0.3g, Sodium chloride 0.5g, Dipotassium hydrogen phosphate 0.25g, Dextrose 0.25g, Agar 3g in 100 ml double distilled water with pH 7.3. The morphologically distinct types of colonies appeared in the petridishes were selected and purified on same sterilized (medium) agar plates.The purified bacterial strain was identified by biochemical tests and FAME analysis.

Characterization and Identification

The isolate was observed under the microscope, the colony morphology was noted with respect to color, shape, size, nature of colony and pigmentation. Gram reaction of the organism was studied. Isolates were subjected to a series of biochemical tests, which included Carbohydrates fermentation test, Methyl red test, Voges – proskauer test, Citrate utilization test, Indol production test, Urea hydrolysis test, and Nitrate reduction test, degradation of starch, casein, urea, gelatin, and catalase test.

Fatty acid methyl ester (FAME) analysis

Gas chromatographic analysis of whole cell fatty acid methyl ester (FAME) was performed for further identification and grouping of isolates. The culture was obtained from soil sample and pure cultured onto Trypticase soya broth agar (TSBA) media at 28°C for 24 hours. The fatty acids were extracted and methylated to form fatty acid methyl esters (FAME). These FAME's were analyzed using Gas Chromatography (Agilent 6850 Series II) with the help of MIDI Sherlock software for FAME. Aerobic library was used Fatty acid methyl ester extraction was performed using standard procedures. The fatty acid profiles generated were compared against an inbuilt Sherlock TSBA Library version 6.0B (s/n 160277) (MIDI

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Inc., DE, and USA). As described previously (Sasser et. al, 1990).

Growth curve of bacteria
100µl of pure bacterial culture was inoculated in 100ml of sterilized nutrient medium which contained peptone 1g, Meat extract 0.3g, sodium chloride 0.5g in 100 ml double distilled water. 3 to 4 ml of culture was withdrawn at different intervals (0, 1, 2, 3…27hours). Optical density of each aliquot was measured at 600nm.Generation time of bacteria was calculated

Salt tolerance test
Sterilized seven 250ml flasks with 100 ml soybean casein digest broth was prepared which contained different salt concentration (0%,3%,5%,10%,15%,20%).1 flask marked as control. Under aseptic condition each flask inoculated with loop full culture except control. Put all flasks on shaker at 120rpm at 37 °C for 22 hours. After 22 hour pH and O.D of culture flask was measured (Data not shown).

Antibiotic sensitivity test
The inhibition of *K.rosea* strains by various antibiotics was tested by standard agar wall diffusion technique. 0.2ml of young test culture was inoculated in sterilized top agar which contained 3% SCB and 0.5% agar and poured over the base agar which contained 3%scb and 2% agar (Hi-Media, Mumbai). Wells was filled with different quantity of antibiotics; the following antibiotics were added in wells Streptomycin (375mg/ml), penicillin (85.3mg/ml), tetracycline (250mg/ml).

Dye decolorization test by microorganism
Dyes and chemicals
Malachite green, crystal violet, methyl violet (triphenylmethane dyes) were used for dye decolorization experiments and were obtained from local market.

Dye decolorization experiments
All decolorization experiments were performed in triplicates. A 100µl of microbial culture was inoculated in 250ml Erlenmeyer flask containing 100ml nutrient broth. After 15 hours of incubation, all dyes were added at concentration of 50 mg/l and 3 ml of the culture media was withdrawn at different time intervals (e.g. 1, 2, and 3…7 hours). Aliquots were centrifuged at 6000 rpm for 15 minutes to separate the bacterial cell mass, clear supernatant was used to measure the decolorization at the absorbance maxima of the respective dyes. This study observed or examined, (1) Decolourization of Malachite green in static and shaking conditions. (2) Decolourization of different dyes (e.g. Malachite green, Crystal violate and Methyl violate). (3) Effects of different growth phases, concentrations and growth medium were observed in this experiment.

The percentage decolorization was calculated as follows:
Initial absorbance - Observed absorbance
Initial absorbance
Result and Discussion
Isolation of bacteria from soil of coastal area of Gujarat

Kocuria rosea can be well isolated by SCB agar plates at 28-38 °C at 24 hours from soil sample which collected from Bhavnagar, Gujarat. Similar species of *Kocuria* was identified by the genus *Kocuria* was proposed by (Stackebrandt et al.1995).Members of the genus have been isolated from different sources such as air, fermented sea food, mammalian skin, soil, the rhizoplane, freshwater or seawater, marine sediment and desert soil (Kloos et al. 1974; Stackebrandt et al. 1995 & Kovacs et al. 1999)

Identification of bacteria
Table 1: Colony characterization of bacteria was recorded and tabulated. Moreover, results of biochemical tests are also illustrated.

Morphological characteristics	Results
Size	Small
Elevation	Flat
Margin	Entire
Texture	Smooth
Pigment	Creamy /Yellow
Gram's reaction	Gram positive
Motility	Non motile
Configuration	Round
Biochemical tests	Results
Carbohydrate Fermentation	+ve
Methyl red	+ve
Voges-Proskauer	+ve
Citrate test	-ve
Indole production	-ve
Urea hydrolysis	-ve
Nitrate reduction	-ve
Starch hydrolysis	-ve
Casein hydrolysis	-ve
Gelatin hydrolysis	-ve
Catalase	+ve

Fame analysis
Gram staining of isolated bacteria was cocci shaped and violet in color so it was gram positive bacteria. Identification of the isolates was done by biochemical test and by fame analysis and the isolates were *Kocuria rosea*. Li et al, (2006) found pink or red pigments of *K.rosea*. On the other hand study of Kim et al, 2004 shows that gelatin and starch hydrolysis is positive. The MIDI Sherlock microbial identification system using RTSBA 6 method identified the organism to be *Kocuria rosea* with 0.516 SI (similarity index)

Growth curve of *kocuria rosea*
When bacteria are inoculated into a fresh nutrient medium, the resulting culture exhibits a characteristic growth curve of four distant phases. During the lag phase (1 to 7 hours) the cells prepared for synthesis of DNA and enzyme need for cell division. This is followed by the log phase (7 to 15 hours) where the culture reaches its maximum rate of growth for specific condition. This condition give rise to the stationary phase (16 to 20 hours) in which growth rate equals to death rate. Eventually there will be a decline in cell number – the death phase. (Above 21hours).Similar study was not found.

Salt tolerance test of *kocuria rosea*
Organism showed growth in 0-20% of salt concentration. It tolerates such a high salt concentration though the optimum growth condition of organism is between 0 to 3% salt concentrations. Biomass was decreased when salt was increased in media. Li et al. (2006) and Kim et al. (2004) addressed that *K. rosea* could grow up to 7% salt but growth is not possible in 10% salt.

Antibiotic sensitivity test of *kocuria rosea*
It was uniformly inhibited by streptomycin, penicillin and tetracycline. Antibiotic sensitivity of *Kocuria rosea* was measured in terms of zone of inhibition in (mm) (Data

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not shown). *K.rosea* is more sensitive against tetracycline drug because it gives maximum diameter of zone (Data not shown). Savini et al. (2010) found that *K.rosea* is susceptible against Netilmycin, Vancomycin, Ampicillin, Cefoperazone, and Meropenem. Altuntas et al. (2004) said that K.rosea can be treated with Vancomycin.

Dye decolorization test.

Decolorization at static and shaking condition

Kocuria rosea showed 100% decolorization of MG (50mg/l) within 6 hours at (120 rpm, RT) and 88% decolorization at static condition. However, Parshetti et al. (2006) found that MG decolourization was not seen in static condition (Data not shown).

Effect of different MG concentrations on the rate of decolorization

Maximum rate of MG decolorization was achieved at 25mg/l concentration. The rate of decolorization was decreased with increasing concentration of dye 100% decolorization of MG was observed at 25mg/l concentraton within 6 h respectively (Data not shown).

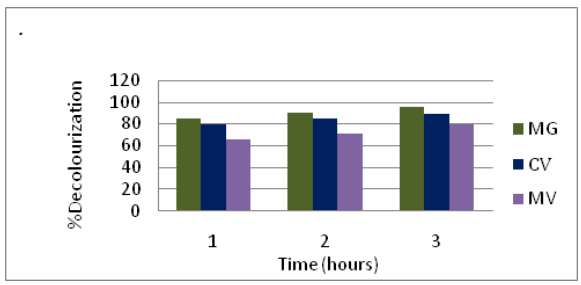
Decolourization of MG at different growth phases of *Kocuria rosea*

At lag phase decolourization was not observed.95% decolourization was observed in log phase of cell cycle in shaking conditions. Decline phase gave 88.9% decolorization of MG in static conditions

Decolourization of different dyes by Kocuria rosea

Kocuria rosea showed decolorization of triphenyl methane dyes(Malachite green, crystal violet, methyl violet 50mg/l). Data recorded in Figure (2).Among all three dyes maximum decolourization of MG was observed which was around 95%.. Thorat et al. (2010) showed 92% decolourization of bacterial cultures isolated from soil, collected from nearby area of dye industry.

Figure 2:% decolourization of three dyes (MG.CV.MV) by 15hours old culture of *K.rosea*.



Effect of different growth medium on the rate of decolourization

The effect of different medium was evaluated by adding 50mg/l MG in various growths medium**(**3%salt**)**Nutrient broth, soybean casein digest broth (3%), and Nutrient broth).90% of MG was observed in nutrient medium with 3% salt in shaking condition (120rpm at RT) (Data not shown).

Conclusion

Our study demonstrates isolation and identification, by GC-FAME analysis of halotolerant bacteria, *Kocuria rosea*. The identified bacterium was found to be salt tolerant, antibiotic sensitive and triphenylemethane (malachite green) dye decolourizer. Hence these bacteria can be used for the treatment of dye contaminated waste water.

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