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HEMOLYTIC, PROTEOLYTIC AND PHOSPHOLIPOLYTIC ACTIVITY OF EXOTOXIN PRODUCED BY *BACILLUS SPP*. ISOLATED FROM MANGROVE RHIZOSPHERE OF RATNAGIRI

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ABSTRACT

The mangrove rhizosphere environments are unique due to the hypersline and alkaline environment they offer. Also, mangrove rhizosphere has enormous diversity of all aerobic as well as facultative anaerobic bacteria. The isolates of the exotoxin producing *Bacillus spp* were obtained randomly from mangrove rhizosphere of Ratnagiri district on the west coast of India. The research in this regards was carried out with three unknown strains isolated from the mangrove rhizospheres. The biochemical analysis of these three stains was performed so as to identify them up to genus level. The organisms were grown on skimmed milk agar and egg yolk agar. The strains showed all three proteolytic, hemolytic and phospolytic activity. The Strains also showed fibrinolytic activity on blood clots. The isolates were found to be potent exotoxin producers. The nature and characters of these exotoxins has much similarity with known strains. The presence of these exotoxins might be able to indicate their survival in highly alkaline and hypersaline conditions.

Keywords: Rhizosphere, Mangroves Exotoxin, Proteolytic activity, hemolytic activity, Lipolytic activity

INTRODUCTION

The aim of this study was to isolate members of the *Bacillus* species from roots of Mangroves that produces exotoxins from Ratnagiri costal area. These are found ubiquitously and are one of the genera with largest 16S diversity and environmental diversity. One clade formed by *Bacillus anthracis*, *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus megaterium*, and *Bacillus thuringiensis* and under current classification standards should be a single species (within 97% 16S identity), but due to production of secondary metabolites in terms of toxins they are considered as separate species. Many *Bacillus* species are able to secrete large quantities of enzymes. (Johnson *et al.*, 1984). *Bacillus amyloliquefaciens* is a species of *Bacillus* that is the source of a natural antibiotic protein barnase (a ribonuclease), alpha amylase used in starch hydrolysis, the protease subtilisin used with detergents, and the BamH1 restriction enzyme used in DNA research. A portion of the *Bacillus thuringiensis* genome was incorporated into corn (and cotton) crops. The term "toxin" means the toxic material or product of plants, animals, microorganisms (bacteria, viruses, fungi, rickettsiae or protozoa), or infectious substances, or a recombinant or synthesized molecule, whatever their origin and method of production (Kenneth, 2008), (Kramer, *et al.*,

1984). It simply means it is a biologically produced poison. Toxins are poisonous products of organisms; unlike biological agents, they are inanimate and not capable of reproducing themselves. Toxins produced by microorganisms are important virulence determinants responsible for the pathogenicity of the pathogen and/or invasion of the host immune response (Kenneth, 2008).

Bacterial toxins are by-products produced by pathogenic microbes that have taken up residence in the body. (Cecilie, *et al.*, 2005) total of 333 Bacillus spp. isolated from foods, water, and food plants were examined for the production of possible enterotoxins and emetic toxins using a cytotoxicity assay on Vero cells, the boar spermatozoa motility assay, and a liquid chromatography-mass spectrometry method. Bacterium can enter a host by various means, such as consuming contaminated food or water. The type of bacterial toxins released depends on the species of invading bacteria. (Beattie, *et al*.1999) Toxigenesis, or the ability to produce toxins, is an underlying mechanism by which many bacterial pathogens produce disease (Kenneth, 2008). There are two main types of bacterial toxins, lipopolysaccharides which are cell associated and are often referred as endotoxins, and the other type is the exotoxins, which may be proteins, lipoproteins etc (Kenneth, 2008). In the nature there are number of Bacillus species are available that produces exotoxins out of them *Bacillus thuringiensi*s, *Bacillus cerus* and *Bacillus polymyxa* are potent one. *Bacillus thuringiensis* also occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well as on the dark surface of plants. Food born infections are also possible with certain *Bacullis spp*. (Lake, *et al.*, 2000) There are however many crystal-producing *Bacillus* strains that do not have insecticidal properties. It has been observed that most of the studies involving important toxins, have been to study the clinical manifestations and to some extent to determining the chemical nature. In this study, it has been attempted to isolate, and characterize the biochemical properties of these toxins *in vitro* obtained from Mangrove rhizosphere of Ratnagiri coastal area.

MATERIALS AND METHODS

This research was carried out from 10 January 2011 to 22 March2011.

Microorganism and growth medium: The organisms used in this study were isolated from roots of mangroves in the Ratnagiri coastal area. Isolation was done by using four quadrant striking method and respective biochemical's were done so as to determine genus level. The isolated three cultures were named as MRC1, MRC2 and MRC3 (Mangrove Rhizospher Culture) The organisms could be easily cultivated on nutrient agar and mineral sea agar medium. The compositions of these 2 media are as in Table 1 and 2: The organisms were maintained on nutrient agar medium but all other studies were carried out in the mineral sea agar medium (broth). Therefore, it was decided to see the growth pattern in the mineral sea agar medium. This was done by growing the organisms in liquid medium and recording the absorbance values at 530 nm.

1.1 Determination of Caseinase and Lecithinase activity: Toxin production: The 24 h old isolated culture (MRC1, MRC2 and MRC3) from Mangrove rhizosphere were grown on nutrient agar and separately inoculated in sterile 50 ml mineral agar base liquid medium which were kept for incubation at 37ºC on a rotary shaker with a speed of 200 r.p.m. for 6, 12, 18, 24 and 48 hours. (Five conical flasks for single isolate).

1.2 Acetone precipitation: After completion of each incubation period the medium was centrifuged at 2000 rpm for 20 min and cell free

medium containing the crude exotoxin was precipitated by using cold acetone for 24 h. Equal amount of acetone as that of the broth was used for the precipitation. After precipitation the mixture was centrifuged at 5000 rpm for 20 min at 2ºC. The residue was dissolved in 5 ml of 25 mM phosphate buffer at pH 7.0. It was concentrated against crystals of sucrose and kept in the refrigerator at 5ºC. Such a concentrate was then used for study of Caseinase (protease) and phospholipolytic (i.e. Lecithinase) activity.

1.3 Caseinase (Proteolytic) and Phospholipolytic (Lecithinase) activity: In each plate of milk agar (the composition of which is as shown in Table 3. Agar cup method was used for the determination of these activities. In each of these cups the 0.1 ml of the above precipitate was added and they were incubated for 24 and 48 h at 37ºC respectively. Zone of hydrolysis of casein on milk agar plate was measured. This was repeated 3 times to get a standard deviation less than 10. To check the Lecithinase activity the procedure was the same except that in place of milk agar, egg yolk emulsion (the composition of which is as in Table 4) was used. The zones were measured by the soap test using CuSO4 solution.

1.4 Determination of Hemolytic activity of Exotoxin obtained from three different isolates of Mangrove rhizosphere: This method was used for the calculation of hemolytic activity (i.e. Hb content) of exotoxin produced by three isolates of and was according to the recommendations of the International Committee for Standardization in Hematology (ICSH).

1.5 Procedure for testing Hemolytic activity of exotoxin of MRC1, MRC2 and MRC3 cultures: Separation of blood cells and plasma: Blood with anticoagulant was diluted with sterile saline in 1:10 proportion and 1 ml amount of this diluted blood was centrifuged at 1000rpm for 20 min at 2ºC. The sediment was washed with sterile saline (prevent hemolysis) and the final sediment was used to check hemolytic activity. The acetone precipitate suspended in 25 mM phosphate buffer was added with 5mg of washed blood cells. The mixture was incubated in water bath for 15 min at 37ºC and centrifuged at 5000rpm for 10 min. The Heme contents in supernatant were checked as per the method of Dacie and Lewis. (Dacie, J.V. *et al.*, 1968)

Determination of protein content of MRC1, MRC2 and MRC3 exotoxin: Protein content in exotoxin produced by MRC1, MRC2 and MRC3 cultures were estimated by Lowry method (Plummer, 1971).

Electrophoresis: The purity of exotoxin from MRC1, MRC2 and MRC3 were checked by SDS-PAGE, by the method of Laemmli *et al*. (1970). The bands were visualized by silver staining technique. The molecular mass of exotoxins of MRC1, MRC2 and MRC3 were determined on a calibrated scale with standard marker enzyme (Phosphorylase b 98 kDa, Bovine Serum Albumin 66 kDa, Oval albumin 43 kDa, Carbonic Anhydrase 29 kDa, Soya bean Trypsin Inhibitor 20 kDa).

RESULTS AND DISCUSSION

The strain MRC1, MRC2 and MRC3 had a lag period of 1 hour and followed by an exponential phase of 3 hours. (Fig No. 1, 2 and 3) This implies that food contaminated with this organism would contain the toxin within a short period of 1 hour. (EFSA, 2004) (Fig No. 4) Caseinase (Proteolytic) and Lecithinase (Phospholipase) activity of purified (dialysed) exotoxin of strain MRC1 after 12, 18, 24 and 48 hours respectively. (Fig No. 5) Caseinase and Phospholipolytic activity of purified (dialysed) toxin of strain MRC*2* after 12, 18, 24 and 48 hours respectively. (Fig No. 6) Caseinase and Phospholipolytic activity of purified (dialysed)

toxin of strain MRC*3* after 12, 18, 24 and 48 hours respectively. Figure No. 4 shown that at 18 hours there is highest Caseinase and Lecithinase activity for strain MRC1 than other periods and later decreases slightly. Figure No 5 also shown that at 18 hours there is highest Caseinase and Lecithinase activity for strain MRC2 than other periods and later decreases slightly. Figure No 6 shown that at 12 and 24 hours there is highest Caseinase and Lecithinase activity for strain MRC3 than other periods. Figure No. 7, 8 and 9 shown that Hemolytic activities of strain MRC1, MRC2 and MRC3. In this case, at 18 hours there is highest Hemolytic activity for all strains. Figure No.10 shows the Caseinase activity of stain MRC1, MRC2 and MRC3 on Milk agar in terms of zone diameter, but at 18 hours there is maximum zone diameter is observed for all strains. (Table No.2) Figure No.11 shows the Lecithinase activity of stain MRC1, MRC2 and MRC3 on Egg yolk Agar in terms of zone diameter but, again at 18 hours there is maximum zone diameter is observed for all strains. (Table No.3) In SDS-PAGE three separate bands are observed for three strains of MRC1, MRC2 and MRC3. (Figure No. 12)

Table No.1- In vitro Lecithinase activity (Zone diameter in mm) of strain MRC1, MRC2 and MRC3 at different time intervals.

Strains	**12 hr**	**18 hr**	**24 hr**	**48 hr**
MRC1	13mm	25mm	21mm	20mm
MRC2	15mm	20mm	15mm	17mm
MRC3	18mm	15mm	18mm	16mm

Table No.2- In vitro Caseinase activity (Zone diameter in mm) of strain MRC1, MRC2 and MRC3 at different time intervals.

Strains	**12 hr**	**18 hr**	**24 hr**	**48 hr**
MRC1	14mm	22mm	20mm	18mm
MRC2	13mm	22mm	16mm	16mm
MRC3	20mm	18mm	20mm	14mm

It is very evident that the Mangrove Rhizosphere possesses potent exotoxin producing microorganisms. The bioavailability of these microorganisms would be able to indicate their survival in highly alkaline conditions. The contamination of food items with these organisms will definitely cause food intoxication.

CONCLUSION

The exotoxins produced by three stains i.e., MRC1, MRC2 and MRC3 isolated from Mangrove Rhizosphere from Ratnagiri coastal area shows Caseinase, Lecithinase and hemolytic activity at 18 hours of its incubation in Mineral medium. These strains were highly proteolytic in nature. From the present study it is concluded that, Mangrove Rhizosphere has

heavily laden with tremendous diversity of various microorganisms. The purity of these exotoxins were detected by performing SDS-PAGE and it shows the potent proteins responsible for the Caseinase, Lecithinase and hemolytic activity. Further identification of these strains MRC1, MRC2 and MRC3 would be possible with 16s-rRNA sequencing so as to reveal species of these three strains.

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Figure No.1 Growth curve of Strain MRC1

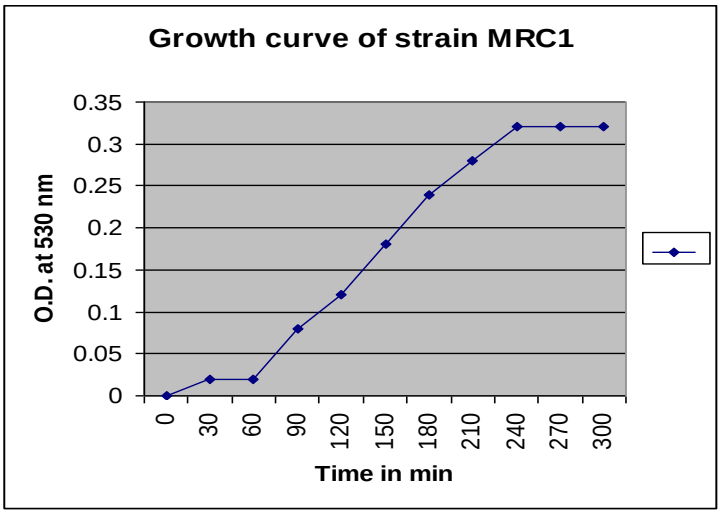


Figure No.2 Growth curve of Strain MRC2

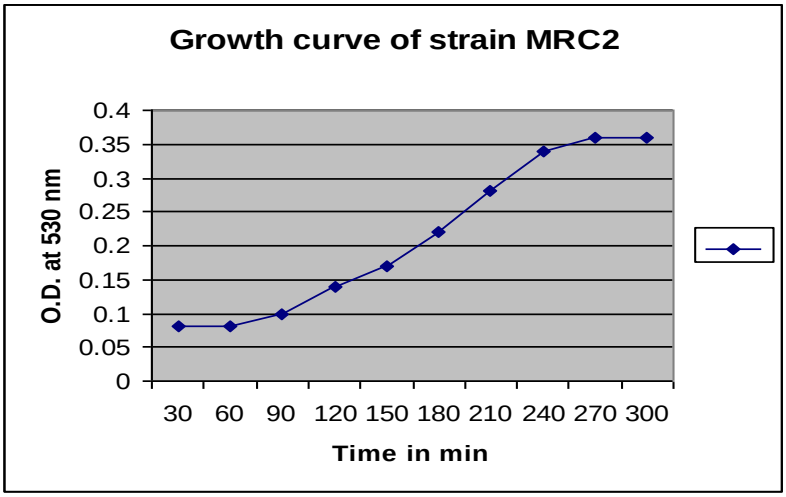


Figure No.3 Growth curve of Strain MRC3

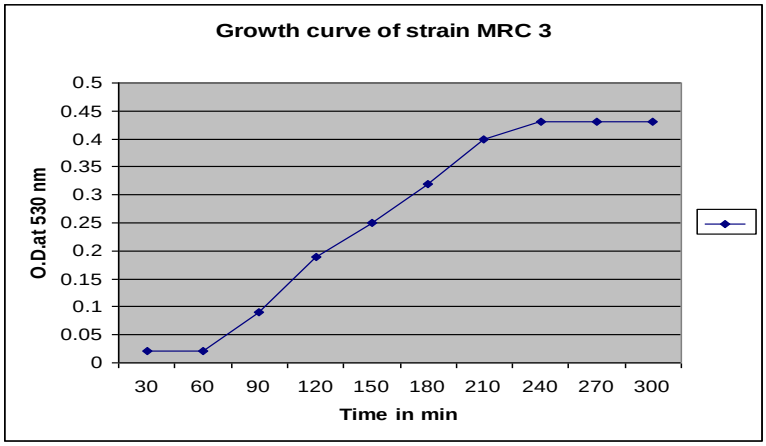


Figure No.4 Lecithinase and Caseinase activity of strain MRC1

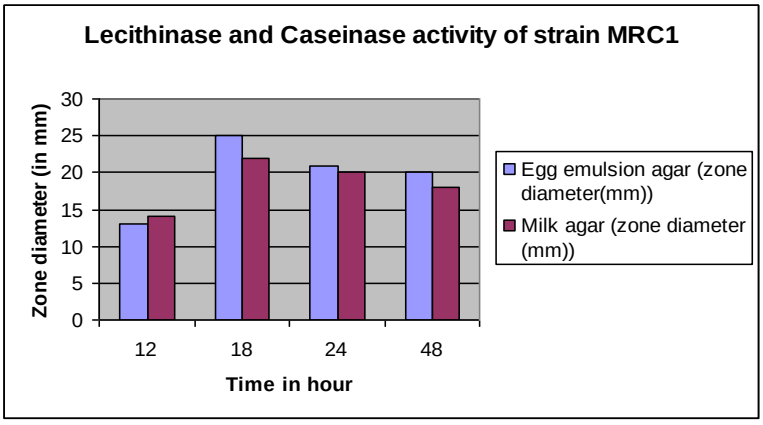


Figure No.5 Lecithinase and Caseinase activity of strain MRC2

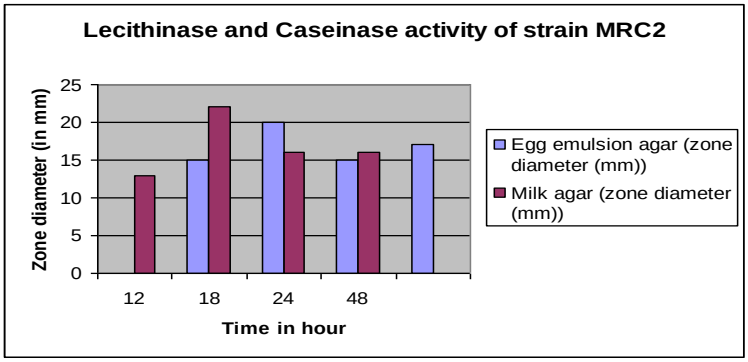


Figure No.6 Lecithinase and Caseinase activity of strain MRC3

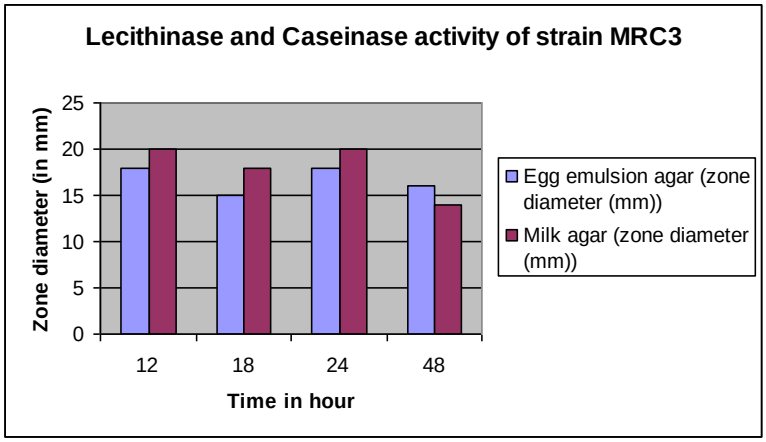


Figure No.7 Hemolytic activity of strain MRC1

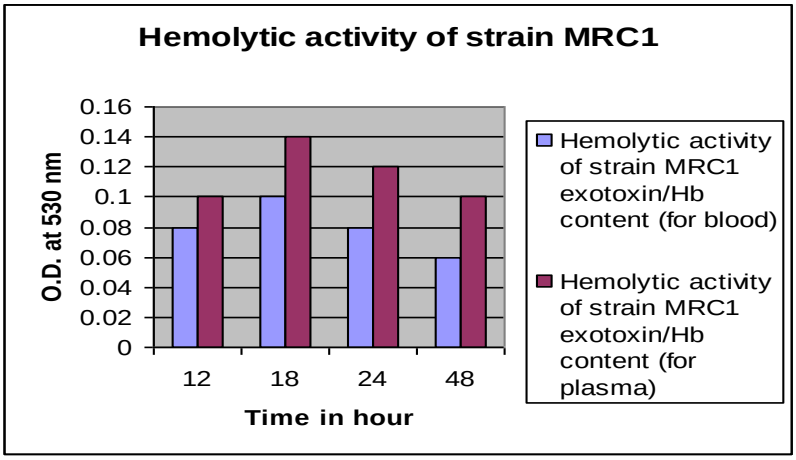


Figure No.8 Hemolytic activity of strain MRC2

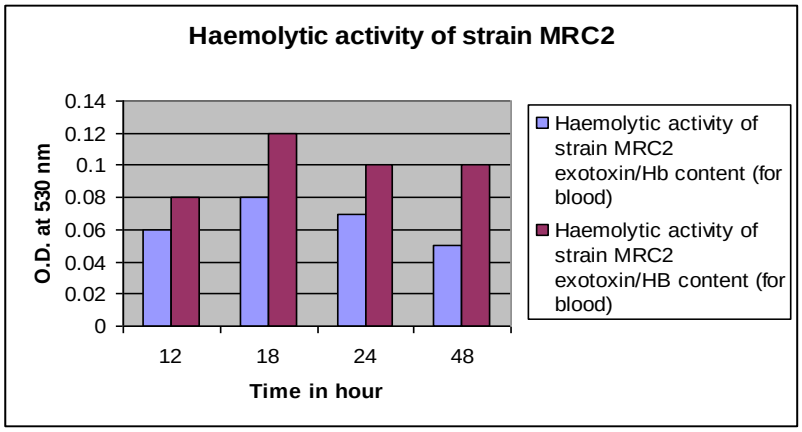


Figure No.9 Hemolytic activity of strain MRC3

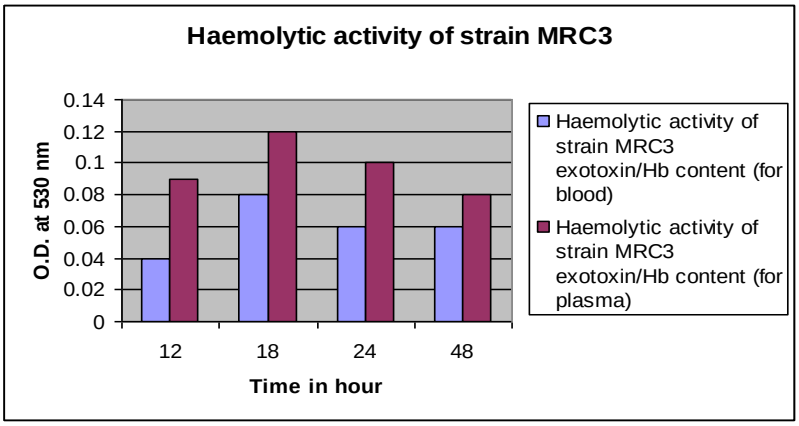
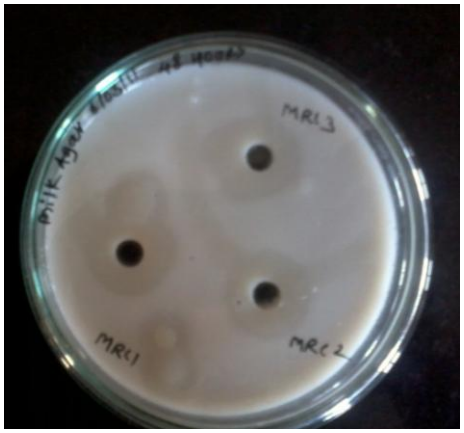
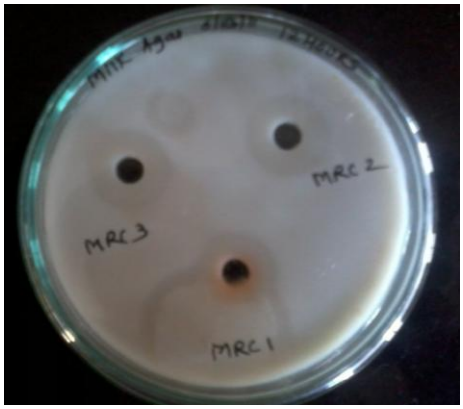


Figure No.10 Caseinase activity of stain MRC1, MRC2 and MRC3 on Milk agar.

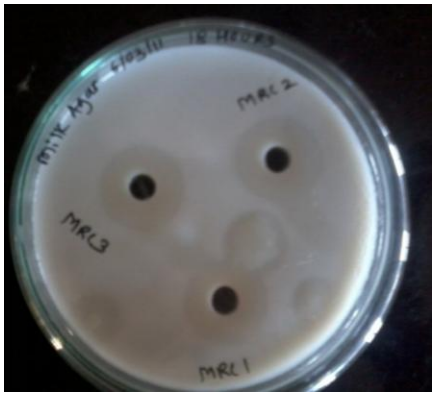
Milk Agar 12 Hours



Milk Agar 24 Hours

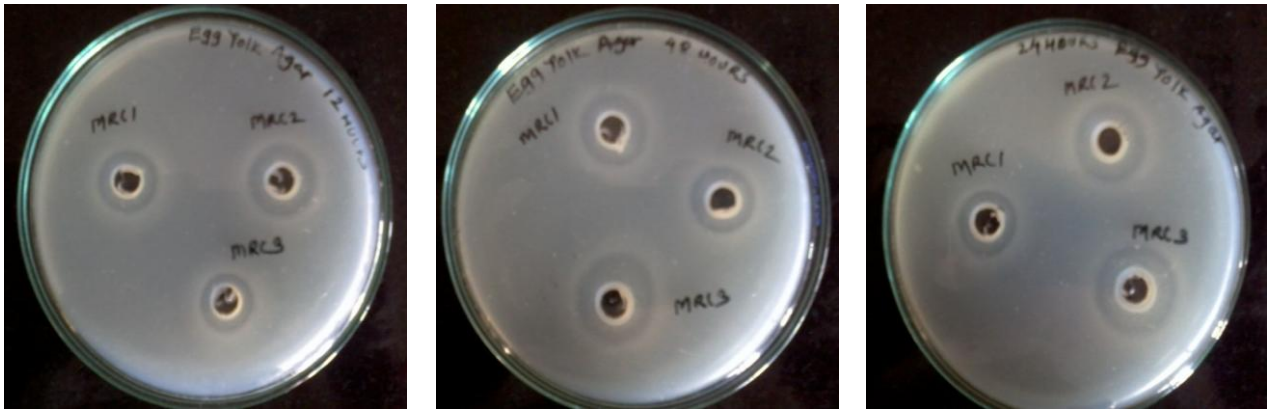


Milk Agar 48 Hours



Milk Agar 18Hours

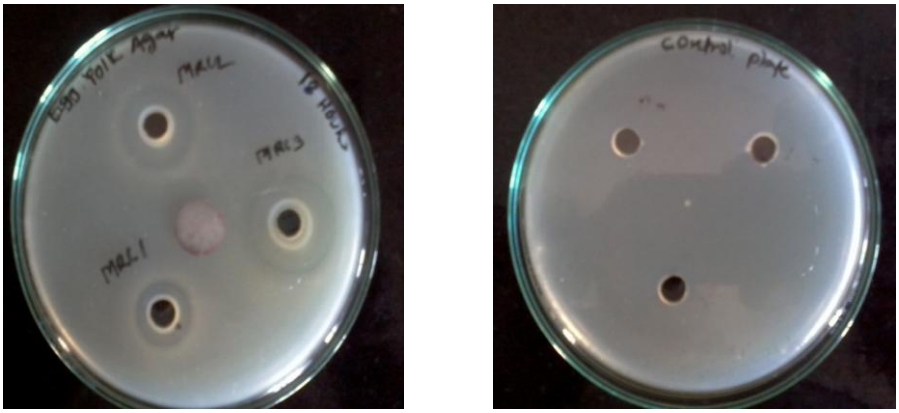
Figure No.11 Lecithinase activity of stain MRC1, MRC2 and MRC3 on Egg yolk Agar.



Egg Yolk agar 12 hours

Egg Yolk agar 48 hours

Egg Yolk agar 24 hours



Egg Yolk agar 18 hours

Control plate

Figure No.12 Electrophoresis of Exotoxin of strain MRC1, MRC2, and MRC3