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Isolation and Screening of Biosurfactant Producing Bacteria from Environmental Soil Samples of Rajkot Region

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ABSTRACT

Biosurfactants are valued microbial amphiphilic molecules with effective reducing surface tension & emulsification activity. Form the petrol & crude contaminated environmental soil sample, isolation and screening of Biosurfactant producing bacteria is done. Emulsification index, haemolytic activity and CTAB agar plate methods were used to screen the capability of isolates for producing Biosurfactant. Studies were also carried out using diesel, petrol, kerosene and engine oil as hydrocarbon source. Results of the present study suggested that these isolates have potential to use in oil degradation studies. The future aspects of this study will be to proceed further the biochemical and molecular characterization of potent isolates, to investigate and define the purification and characterization strategy of biosurfactant and to establish an optimized medium based on agro- industrial waste for scale up operations.

Keywords *Emulsifiers, MEOR, Bioremediation, surfactants, hydrocarbon degrading bacteria*

Surfactants, a short form for "surface-active-agents", are basically chemical compounds which lower the surface tension of a liquid, the interfacial tension between two liquids, or that between a liquid and a solid. These surfactants are produced by a variety of microorganisms such as yeasts, bacteria and filamentous fungi and thus are called biosurfactant. Biosurfactants have different properties such as they act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants. These are usually organic compounds that are amphiphillic in nature, which contains both the hydrophobic and hydrophilic component. The hydrophobic (non-polar) part of the biosurfactant is insoluble in water and may have a long-chain of fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The hydrophilic (polar) end can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Franzetti A, *et al*., 2008). A biosurfactant can have one of the following structures: glycolipids, mycolic acid, polysaccharide–lipid composite, lipoprotein/ lipopeptide, phospholipid, or the microbial cell surface itself. Biosurfactants can be characterized in two classes i.e. low molecular weight in which lower surface and interfacial tensions and high molecular weight molecules and the one bind tightly to surfaces. Micro-organisms producing biosurfactants help to amplify the bioavailability of hydrocarbons by increasing the contact between pollutant and the micro-organisms in the presence of the biosurfactant which helps in the enhancement bioremediation of hydrocarbon contamination. (Cooper, D. G. and Paddlock, 1983; Singh, V., 2012)

The general formation of biosurfactants is well known to be hampered by a lack of availability of economic and versatile products. At present there is only a very restricted offer of commercially available biosurfactants e.g., surfactin from *bacillus subtilis*, sophorolipids from *Candida bombicola,* and rhamnolipids from *Pseudomonas aeruginosa* (Banat I. M. *et al.* 2010). A variety of new biosurfactants respectively producing strains are the key issue in overcoming the economic hindrance of the production of biosurfactants. Therefore, increased efforts in the discovery of new biosurfactant producing microbes essentially be made by applying a wide range of different screening methods, which is the attention of this review. The principle aim in screening for new biosurfactants is finding new structures with strong interfacial activity, low critical micelle concentration(cmc), high emulsion capacity, good solubility and activity in a broad pH-range.

Table 1. Types of Biosurfactants (Satpute, *et al.*,2010)

Biosurfactant	Producing organisms	C-source	Charge
Surfactin	*Bacillus subtilus*	Glucose	Non-ionic
Cellobioselipids	*Ustilago sp.*	Vegetable oils	Anionic
Corynomycolates	*Arthrobacter sp.*	Different sugars	Non-ionic
Mannosylerythriollipids	*Candida sp.*	Glucose , soyabean oil	Non-ionic
Rhamanoselipids	*Pseudomonas sp.*	n-alkanes ,glycerol	Anionic
Sophoroselipids	*Torulopsis sp.*	Glucose , vegetable oil	Non-ionic/anionic
Lipopeptides	*Bacillus licheniformis*	glucose	Non-ionic

Therefore, the second goal in screening is the discovery of good production strains with high yields. Biosurfactants may be involved in pathogenesis due to their surface activity; however, for safety and controlling reasons, production strains should be non-pathogenic. In the above mentioned example of rhamnolipids this is not the case as *Pseudomonas aeruginosa*, the most common producing bacteria, is a pathogen. A variety of methods for the screening of biosurfactant producing microbes has been advanced and successfully applied. Since 1970th there have been various trials in this field. These screenings have mostly been restricted to a controllable number of samples. In current years automation and miniaturization have led to the development of high throughput methods for screening of biosurfactant producing strains. A wide application of such methods could eventually lead to the desired upsurge of new commercially interesting strains. An efficient screening strategy is the key to success in isolating new and desired microbes or their variants, because a large number of strains needs to be characterized. A complete approach for screening of new biosurfactants or production strains consists of three steps: sampling, isolation of strains and analysis of strains. (Dhail, 2012; Walter, *et al.,*2010; Satpute, *et al.,* 2010)

Types of biosurfactantmolecules

Several types of Biosurfactant(Table 1.) have been isolated and characterized including lipopeptides, glycolipids, polysaccharides-protein complexes, phospholipids, fatty acids and neutral lipids depending on carbohydrate moiety, glycolipids can further sub-divided into: sophorolipids(derived from sophorose a disaccharides),rhamanose lipid(derived from rhamanose)(Burger MM, *et al*., 1963), trehaloselipid(derived from trehalose) and surfactin produce from bacillus species. (Satpute, *et al.,* 2010; Asselineau, C.; Asselineau, J., 1978)

Microbial cell and biosurfactant

Biosynthetic and regulatory pathways are best known in *Pseudomonasfluorescens* producer of rhamanolipid and *Bacillus subtilus* producing surfactin. The main functions of bio-surfactant in microbial cells are emulsification of water insoluble substrates such as hydrocarbons and facilitate its passage into the cell to stimulate the growth. Similarly adhesion and desorption of cell is vital for the survival of microbes in unfavourable conditions like toxin accumulation, limited nutrients availability most biosurfactant are exolipids although in some cases they are cell bound the excretion can be detected in certain phases of growth cycle may be in late log phase or stationary phase as this is a secondary metabolite mainly produce in stress conditions. (Banat, *et al.,* 1995; Banat I. M. *et al.* 2010; Gautam, K. K. and Tyagi, V. K., 2005).

MATERIALS AND METHODS

Sample collection

The study was carried out between February 2013 and September 2013, on the environmental soil samples, which were collected from hydrocarbon contaminated regions such as nearby area of petrol pump and other crude oil contaminated regions near kalawad road, Rajkot, Gujarat. (Dhail, 2012; Satpute, *et al.,* 2010; Banat, *et al.,* 1995)

Isolation of microorganisms *(*Dhail, 2012; Anandraj, *et al., 2010;*Satpute, *et al.,* 2010; ABU-Ruwaida A. S., *et al*., 1991)

Growth conditions in solid and liquid enrichment

The composition of the Bushnell and hass

medium (BH) used in this study was the following(g/l) magnesium sulphate(0.2) ,calcium chloride(0.02), Monopotassium sulphate(1.0), dipotassium sulphate(1.0),ammonium nitrate (1.0),ferric chloride(0.05),pH-7.Nutrient-broth was also used in this having composition (g/l)Peptone (0.005),yeast extract(0.002),sodium chloride(0.002),Meat extract (0.005) pH-7. 2% to 3% agar powder was used if required. Hydrocarbon enrichment in solid media was done by vapor enrichment through filter paper dipped in respective hydrocarbons (petrol, diesel, and kerosene and engine oil).liquid media was given such enrichment through adding 2% respective hydrocarbons.

Liquid enrichment

Adding 1gm of soil from both the samples to 50ml of Nutrient-Broth and Bushnell&hass- Broth along with respective hydrocarbons

and flasks were kept for incubation at 170-180 rpm, 280c-300c temperature for 7 days using a shaker. After incubation of broths sub culturing was done twice in 15 ml of broths under the same hydrocarbon enrichment.

Solid enrichment

From the last sub culturing through spreading technique, cultures were spreaded on Nutrient- agar and Bushnell&hass- agar.i.e; selective medium for hydrocarbon degradaton.Above plates were given enrichment with four chosen hydrocarbon sources. After incubation plates were enumerated and morphologically different isolated colonies were selected for further experimentation.

Effect of glucose (Cooper, D. G. and Cavalero, D. A., 2003)

Before tests were performed the BH broths were differentiated as broth with glucose and broth without glucose, by adding 2% glucose as an additive in BH-broth, so as to see the changes in growth and secondary product formation.i.e; biosurfactant. Nutrient-broths were not used in further experimentation instead Bushnell&hass – Broth were used as it is selective media for hydrocarbon degraders(Guerra, *et al*., 1984).

Screening methods for detection of biosurfactant (Satpute, *et al.,* 2010; Cirigliano, M. and Carman, G., 1984; Das P, *et al*., 2008)

Haemolytic activity

It is a qualitative screening test for detection of BS producers. Solid media such as BH agar, nutrient agar (NA), supplemented with 5% fresh whole blood are used and composition of media are as followed (gm/100ml) peptone (138g),agar (2%),BH (3.27g), whole blood (5%).Isolates are either streaked, wells are prepared or simply a drop of BSandincubated at required temperature for 48 h and as a control anionic detergent SDS is used for comparisons of results. Visually yellowish-green colored area is seen as positive result of haemolytic activity may be an indication of red blood cell lysis due to cell membrane rupture caused by the presence of surface active molecules. Blood agar is a complex medium may contain other simple carbon sources other than hydrocarbons hence; it is very difficult to test the BS productivity of a culture at different culture conditions directly on the agar. Haemolytic activity however has been considered acriterionfor the detection of BS activity unreliable. As Shownin Fig.1

CTAB MethyleneBlue agar plate method

This technique was specially developed for detection of anionic surfactants produced by biosurfactant producing microbes, glycolipids such as rhamnolipids by *Pseudomonassp.* composition of CTAB agar consists of(gm/100ml) BH agar(3.27g) medium supplementedwith cetyltrimethylammonium bromide- CTAB (0.2g), methylene blue (0.0002%; 500µl), peptone (0.138g), glycerol(2.5ml),agar(3%). Anionic BS forms insoluble ion pair with the cationic CTAB-MB and formation of dark blue halo due to precipitation reaction between cationic and anionic compound around the culture is considered as positive for BS production. It is an excellent technique that has been usedgenerally for detection of glycolipids BS and as a positive control SDS anionic detergent.As shown in Fig.2.

Cell surface hydrophobicity assay

There is a straight association between cell surface hydrophobicity and BS production. The cells are collected by centrifugation (12,000g/30 min/4°C) and washed twice with 50mM phosphate buffer (K_2HPO_4 and KH_2PO_4, pH 7.0) through centrifugation and resuspended using the same buffer in such a way that absorption (A^{600}) is of 0.5. Now that pretreated 0.5 OD Cell suspensions (3 mL) are added to respective hydrocarbons (0.5 mL) and vortexed for 3 min and allowed to settle for 10 min for the hydrocarbon phase to separate completely with aqueous phase. The aqueous phase

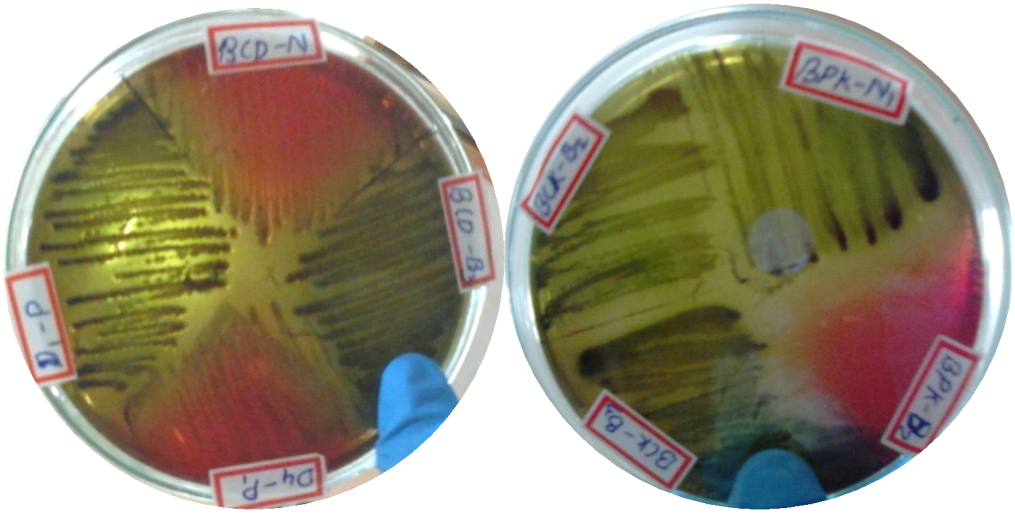


Fig. 1. Haemolytic assay

is removed and transferred to a 1mL cuvette to measure A^{600}. The decrease in A^{600} of the aqueous phase is taken as a measure of the cell surface hydrophobicity (H %), which is calculated as follows:

$$H\% = \frac{A^{\circ} - A}{A^{\circ} \times 100}$$

Where A^0 and A were A^{600} before and after mixing with hydrocarbon, respectively.

Depending upon the hydrocarbon utilization by microorganisms may have high or low surface hydrophobicity. Generally, those microbes which can take hydrocarbon by direct uptake mode, shows high surface hydrophobicity. Cell bound BS production is also associated with hydrocarbon uptake The microbes show low surface hydrophobicity when BS are released extracellularly, where hydrocarbon uptake is mediated through the BS, OD is less than 0.5 if the BS released is cell bounded and more if it is produced extracellularly.

Emulsification Capacity (E_{24})

Emulsification activity is measured by calculating E_{24},

$$E_{24} = \frac{\textit{Total Height of Emulsion}}{\textit{Total Height of Aqueous layer}} \times 100$$

Here respective hydrocarbons and biosurfactants are used in (1:2) taking 1ml hydrocarbon source and 2 ml biosurfactant. Vortexing is done for 2-3 minutes and kept for 24 hr for separation ofaqueous and emulsified layer, E_{24} is calculated by measurement of emulsion height and is divided by height of aqueous layer. The results of these are compared with SDS(Sodium dodecyl sulphate)anionic detergent as a positive control.(Satpute S. K. *et al*.,;Perfumo A, *et al*., 2009; Phetrong K, *et al*., 2008).

RESULTSHaemolytic activity

All of the above isolated were tested for haemolytic activity with and without 2% glucose. Among 39 isolates, 33 isolates were showing haemolytic activity with 2% glucose and 16isolates were showing haemolytic activity without 2% glucose.*Positive result for non glucose isolates*:BCP-B1,D4-P2,BPD-N,BPK-N2,BCK-N1,D1-P,BPE-B1,BCD-B1,BCD-B2,BCD-B3,BPE-B2,BCE-B1,BCE-B1,BCK-B2,BPE-N,BCK-B3.*Positive result for glucose isolates:*BCP-B1,BCP-B2,D4-P2,BPP-N7,BPD-N,BPD-B,BPK-N2,BCK-N1,BPK-B2,BCK-B1,K3-P,K2-P,BCE-N,D4-P3,D1-P,BPE-B1,BCD-B1,D4-P1,BCD-B2,BCD-B3,BPE-B2,BCE-B1,BCE-B2,BCK-B2,BCK-B3,D2-P,P1-P2,K1-P,D3-D3,BPE-N,D3-D2,BCP-N1.

CTAB Agar activity

All of the above isolated were tested for CTAB agar activity with and without 2% glucose. Among 39 isolates, 16isolates were showing CTAB agar activity with 2% glucose and 7 isolates were showing CTAB agar activity without 2% glucose.

Emulsification activity

All of the above isolated were tested for Emulsification activity with and without 2%

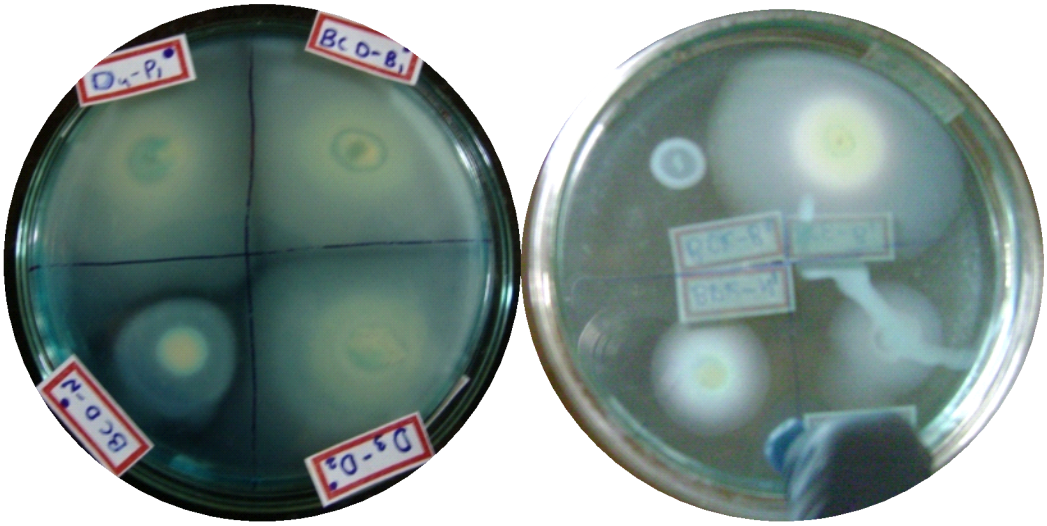
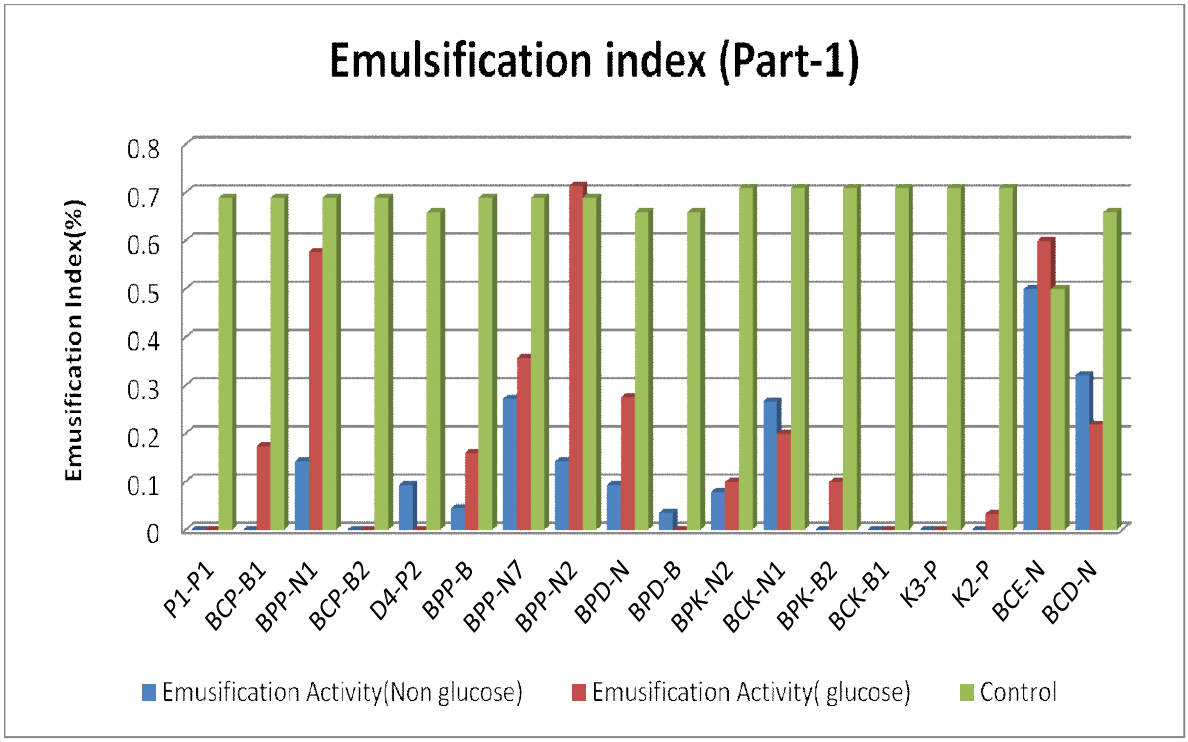


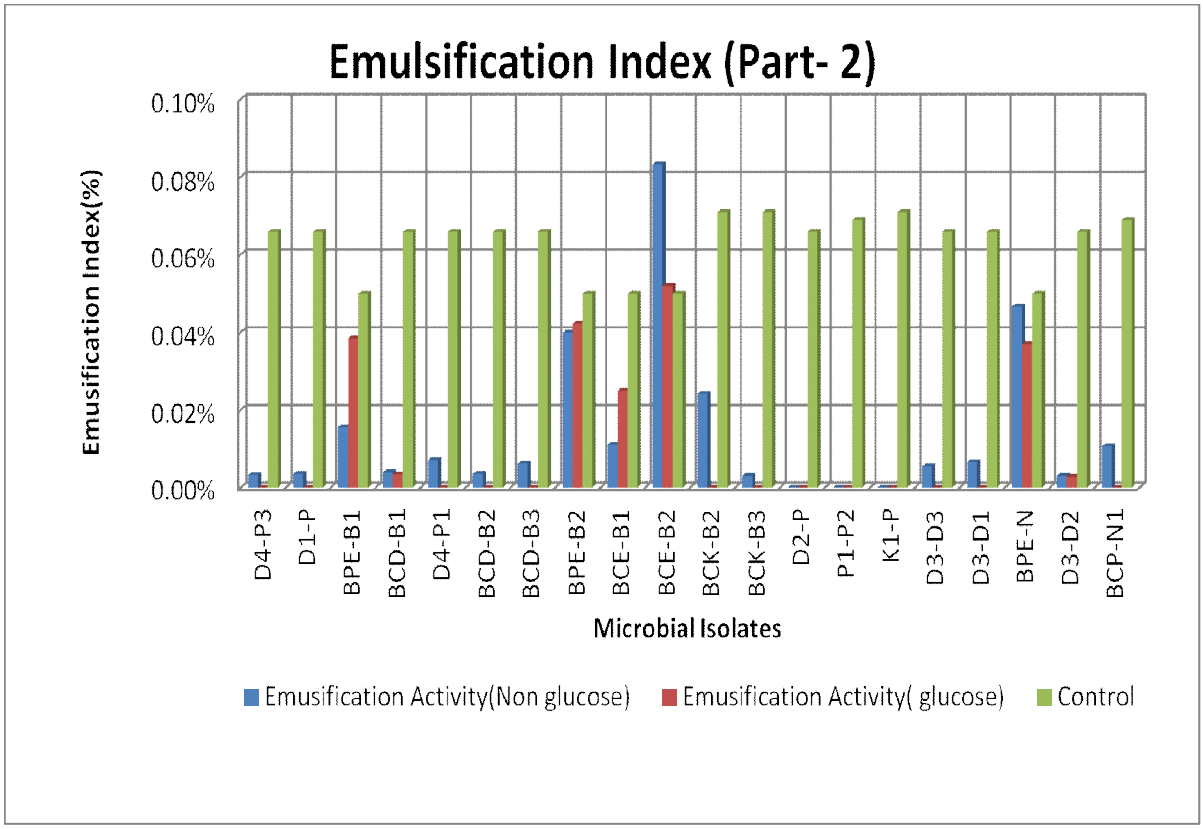
Fig. 2. CTAB-Agar assay

Fig. 3. Emulsification assay

glucose. Among 39 isolates, 29 isolates were showing Emulsification activity with 2% glucose and 19 isolates were showingEmulsificationactivity without 2% glucose.

Biosurfactant represents a group of compounds with versatile applications in biomedical sciences, bioremediation, microbial enhanced oil recovery and many more remedial applications. Bacterial isolates capable of producing biosurfactant were screened over various screening media and effect of glucose over biosurfactant production was analysed. Control sets of isolates in different screening methods show no significant biosurfactant activity, while isolates grown under hydrocarbon enriched screening media shown significant biosurfactant activity. Majority of isolates shown varied responses with relevance to glucose as component of their enrichment medium. Majority of isolates shown considerable haemolytic activity, proportion of which was higher in presence of glucose. Emulsification activity was higher in absence of glucose, where only isolate BPP- N2 has shown slightly higher emulasification activity in presence of glucose. All the isolates have shown greater CTAB blue halo zone size, where only the isolate BCD-N and D3- D2 has shown considerable zone size in presence of glucose also.





Future prospects

Future targets includes investigation of the potential isolates for the diverse type of biosurfactant and their yields along with the development of a cheap agro- industrial waste based medium for fermentation and scale up.

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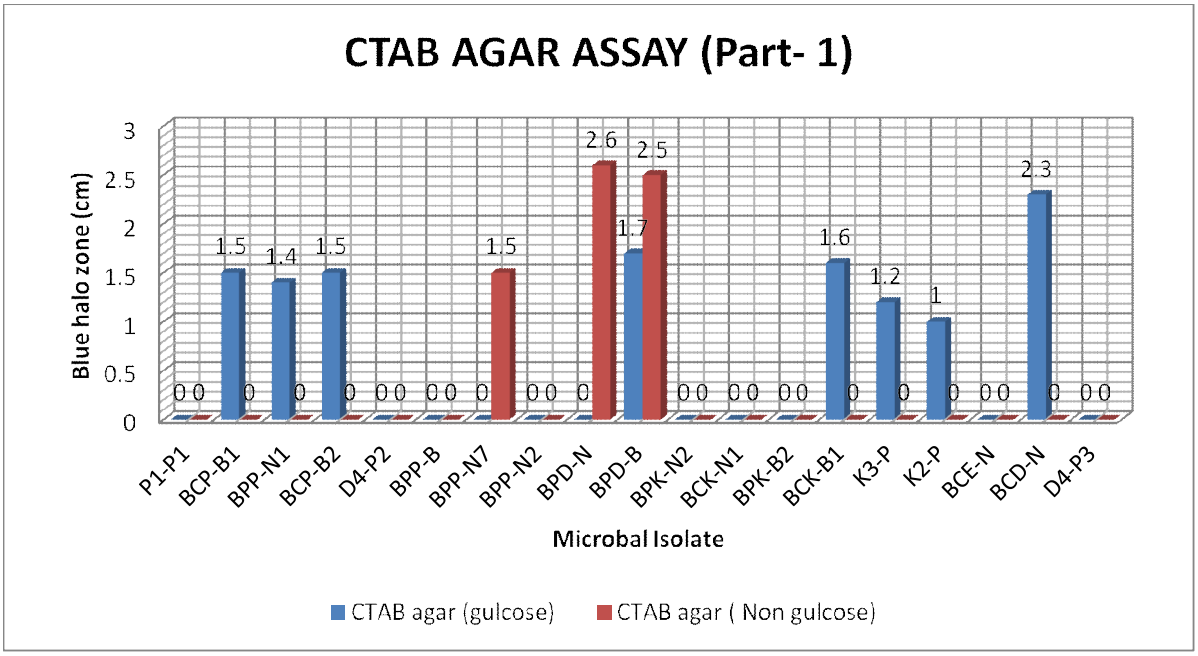

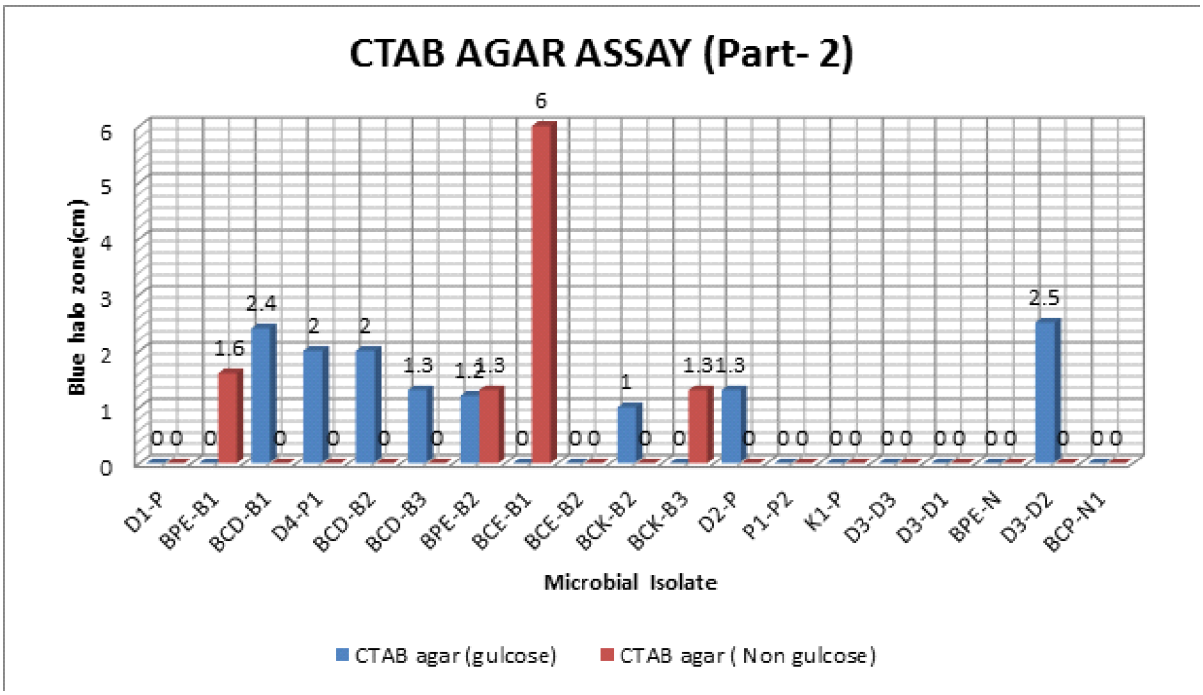

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