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Production of Bioplastic (PHA) from Emulsified Cotton Seed Oil Medium by *Ralstonia Spp*.

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Abstract: Polyhydroxyalkanoates (PHAs) are polyoxoesters produced by a wide range of bacteria when they find themselves in an environment with an available carbon source but limited in additional nutrient's required for growth. Cotton seed oil is important agricultural product; it primarily consists of triacylglycerols in which three fatty acids are attached to glycerol backbone. Growth experiments with cotton seed oil are difficult to conduct in quantitative manner due to heterogeneity of the two phase medium. To overcome this obstacle a new culture method was developed with a newly designed medium. This medium was emulsified using the plant gum or resins as the emulsifying agent. Ralstonia spp was grown on the emulsified medium and PHA production was measured over time by cell dry weight method. The medium used in this study was minimal salt medium lacking nitrogen source designed to stimulate PHA accumulation by *Ralstonia spp*, and it contains fructose, cotton seed oil and water clarified solution of plant gum or resins in different concentration and trace elements and antibiotic was added. Additionally an extraction method was developed to monitor oil consumption. The cells accumulated high levels of PHB content i.e 78 - 80. % of cell dry weight was reached after 72 h. This method may prove to be useful for production of PHA from cotton seed oil and may also be useful for studying byproduct.

Key words: Cotton seed oil, Emulsifying agent, plant gum, Cell dry weight (CDW), Polyhydroxyalkanoates (PHAs), *Ralstonia spp*, Emulsified cotton seed oil medium.

Introduction:-

The world is facing the problem of plastic, which are non-biodegradable. In search of biodegradable plastic, it has been found that there are some microorganisms and plants which are producing biodegradable polymers, which had been used to produce biodegradable plastics from these polymers. Plastic pollution is "the accumulation in the environment of man-made plastic products

to the point where they create problems for wildlife and their habitats as well as for human populations." Plastic pollution is found "from Mount Everest to the bottom of the sea."

The genus *Ralstonia* is thus a most unusual genus, unifying species that are opportunistic human pathogens able to survive in oligotrophic environments with economically important plant pathogens and organisms that are of considerable biotechnological interest because of their potential for biodegradation a large list of chloroaromatic compounds and chemically related pollutants.[1] *Ralstonia eutropha* has a natural tendency that under stressed condition stop growing and put all its energy into making complex carbon compounds. [2]. *Ralstonia spp* is well known for its wide application in biopolymer production from palm oil, fruit waste and from grass. It is easily bioengineered by inserting some new genes and knocking out some genes. PHAs are renewable by nature; PHA would have been produced from renewable resources like plant oils [3, 4] sugars [5, 6, 7, 8] and carbon dioxide [9, 10, 11, 12].

Polyhydroxyalkanoates (PHAs) are polyoxoesters produced by a wide range of bacteria when they find themselves in an environment with an available carbon source but limited in additional nutrient(s) required for growth. Cotton seed oil is important agricultural product; it primarily consists of triacylglycerols in which three fatty acids are attached to glycerol backbone. Growth experiments with cotton seed oil are difficult to conduct in quantitative manner due to heterogeneity of the two phase medium. To overcome this obstacle a new culture method was developed with a newly designed medium.

The entitled study shows a great hope and a little way to solve the problem of using cotton seed oil directly in the medium and also solves the problem of pollution by plastic.

Materials and Methods:-

- Isolation and identification of *Ralstonia spp* from soil: Dextrose free tryptic soy broth (TSB) rich medium was used to maintain the culture. Isolation followed by Phenotypic and Genotypic Analysis of *Ralstonia spp*. was carried out.
- Shake Flask Experiment: 50-ml minimal media was used in 250-mL conical flasks. *Ralstonia spp* was grown aerobically at 30 °C and 200 rpm. O.D was taken at 600nm after 12 hrs interval.
- PHB production by Ralstonia Spp. in minimal medium with fructose: Minimal Medium with Sodium phosphate and K2SO4 lacking NH4Cl was prepared with fructose as a carbon source and trace elements were added. O.D was taken at 600nm after 12 hrs interval.
- PHB production by Ralstonia Spp. in Minimal Medium with cotton seed oil: Minimal Medium with Sodium phosphate and K2SO4, lacking NH4Cl was prepared with cotton seed oil as a carbon source and trace element was added. O.D was taken at 600nm after 12 hrs interval.
- PHB production by Ralstonia Spp. in emulsified cotton seed oil medium: This medium was emulsified using the plant gum or resins as the emulsifying agent. Plant gum or resins constitutes glycoprotein hence did not influence the growth of the

Ralstonia spp. To prepare the medium, a 10X solution of plant resin was prepared in water. Resins dissolve slowly at room temperature, so the solution was stirred fast for rapid dissolution. Resin solution was then centrifuged (10,500×g) to separate out insoluble particles [13]. Water, clarified Resin solution, and cotton seed oil were combined, along with the sodium phosphate and K2SO4 needed for the minimal medium. The medium used in this study was designed to stimulate PHA accumulation by *Ralstonia spp* contains Na_2HPO_4, K_2SO_4, $MgSO_4$, $CaCl_2$, water clarified solution of the plant gum or resins in different concentration and cotton seed oil, trace elements and antibiotic was added. O.D was taken at 600nm after 12 hrs interval.

- PHB and Dry cell weight estimation: PHB sample was extracted from *Ralstonia spp* biomass by the method described by Hahn et al. (1993) and the concentration was determined from the biomass by method described by Law and Slepecky (1960). For Dry Cell Weight determination, known volume of bacterial culture was centrifuged (8000 rpm, 15 min) and pellet was then lyophilized followed by determination of the dry weight of the lyophilized cell powder.

Result and Discussion:-

The effect of Emulsified cotton seed oil supplementation in the growth phase: Considering the low solubility of cotton seed oil at 30 °C, Which has no influence on the initial absorbance, it can be observed in Fig. 1 that an emulsified cotton seed oil from initial phase growth starts where as only in cotton seed oil increase in cotton seed oil concentration leads to an increase of the specific growth rate, which is confirmed by the slopes obtained at the beginning of the process for the different cotton seed oil and emulsified cotton seed oil concentrations tested. Akiyama *et al.* [*14*]

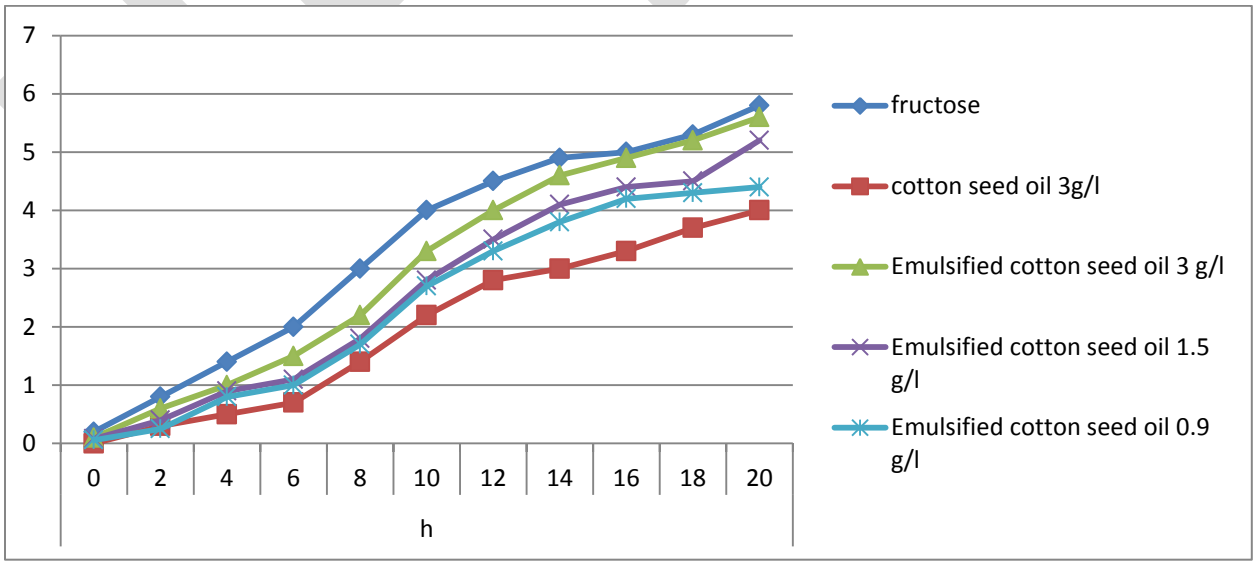

Fig: 1 **Fig 1.** Cell growth of *Ralstonia eutropha* (*A*/*A*0) with time for fructose, cotton seed oil and different concentrations of emulsified cotton seed oil used as supplement at 30 °C

The results obtained in this work allowed reaching a biomass concentration of 6.48 g/L for 6 h of cultivation, with fructose and emulsified cotton seed oil being used as substrate (30g/L) and emulsified cotton seed oil (3.0 g/L) as nutritional supplement. From Fig.1 we can also notice two exponential phases. The value of the first exponential phase increased as the Emulsified cotton seed oil concentration increased. Considering the first exponential phase and the biomass produced in the medium containing 3.0 g/L of emulsified cotton seed oil in 6 hours of cultivation, the cell yield was equivalent to 1.46 g/g, if just fructose was considered as substrate. This result shows clearly that the microorganism used another substrate. According to Rolls [15].

In order to conduct quantitative, reproducible experiments with fructose, cotton seed oil and Emulsified cotton seed oil as the carbon source, we developed an emulsified oil culture method for R. eutropha. Two-phase bacterial cultures have previously been investigated as a method for increasing the rate of biotransformation of compounds with low water solubility [16]. The PHB initially present in these cells was accumulated during pre culture.

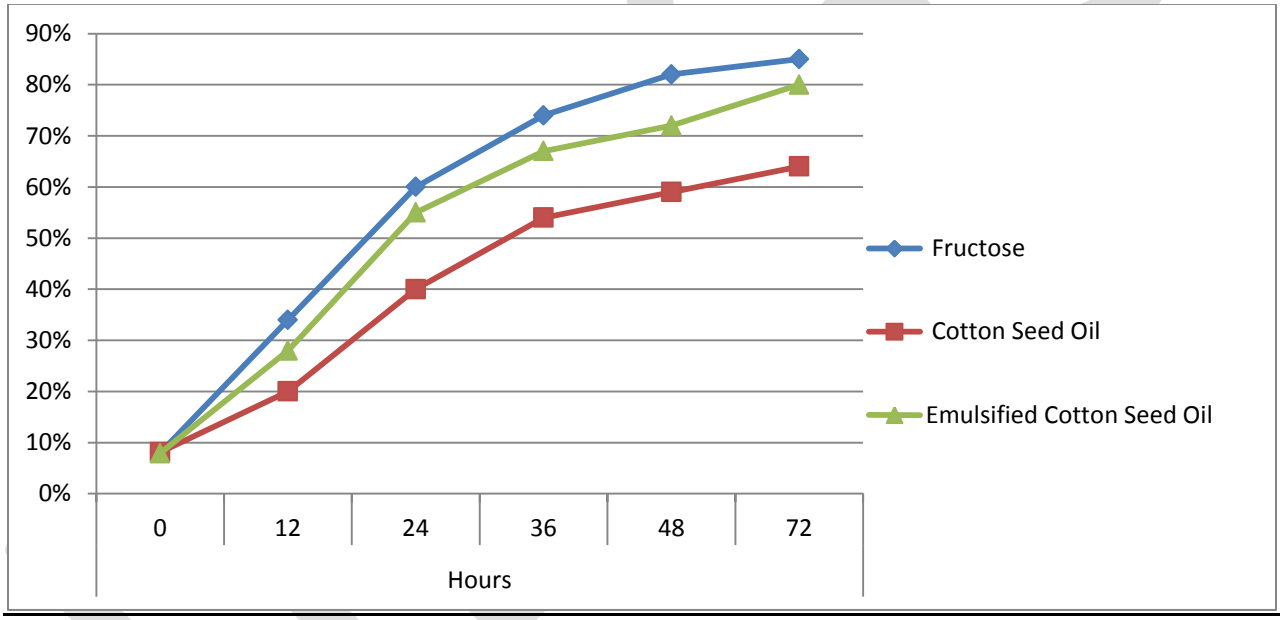

Fig No 2: Ralstonia was grown in Fructose Minimal Medium , Cotton Seed Oil Minimal Medium, and Emulsified Cotton Seed Oil Medium . PHB content as a CDW.

Our group is most interested in cotton seed oil, a major agricultural product in Vidarbha region of Maharashtra where farmer suicides due to poverty where cotton is a major crop. In this study the cells using fructose accumulated high levels of PHB content i.e 80 - 86. % of cell dry weight, the cells using emulsified cotton seed oil accumulated 78-80% PHB content of cell dry weight and cells using only cotton seed oil accumulated 60-64% PHB content of cell dry weight was reached after 72 h. Fig shows that cell finds difficulty to consume only cotton seed oil as a carbon source stored as PHA, where as the emulsified cotton seed oil is easily

consumed and stored as PHA when compared with fructose.

Conclusion:-

Emulsified Cotton seed oil has been projected to be more efficient carbon sources for industrial PHA production than sugars. This method may prove to be useful for production of PHA from cotton seed oil other vegetative oils and may also be useful for studying byproduct. The emulsified oil medium can be used in both shake flask and fermentors. There is one minor issue when using emulsified cotton seed oil. The emulsifying agent some parts gets precipitated at the time of autoclaving this will lead to slight decreased in PHB production but this issue neither had a major impact on growth of Ralstonia and PHB production.

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