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Growth and Production Kinetics, Extraction, and Characterization of Microbial Biosurfactants

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Abstract:
Biosurfactants (BSs) are amphipathic, surface-active molecules produced by microorganisms and can reduce the surface tension and interfacial tension. The present study emphasizes the Growth, production and characterization of the biosurfactant produced by *Pseudomonas aeruginosa, Micrococcus luteus and Serratia marcescens*. These three isolates study for growth Kinetics and production kinetics. From growth kinetics and production kinetics found that Maximum biomass as well as biosurfactant production by *Pseudomonas aeruginosa* was at 28 hours, *Micrococcus luteus* was at 24 hours and *Serratia marcescens* was at 120 hours. HPLC analysis of biosurfactant shows major peak and minor peak with different retention time on the basis of sample spend a different amount of time on the column according to its chemical composition. This study shows that the biosurfactant produced from *Pseudomonas aeruginosa, Micrococcus luteus and Serratia marcescens* was identified as glycolipid.

Keywords: Biosurfactant, Growth Kinetics, Production Kinetics, Glycolipid

Introduction

Diverse microorganisms produce a number of surface-active agents primarily in order to adapt and grow on a variety of substrates among other natural functions (Banat 2010). These biosurfactants are produced under various growth and environmental conditions reported to be mainly involved in increasing the solubility and availability of various water immiscible substrates (Chrzanowski et.al 2012). Synthetic surfactants are chemically amphipathic molecules that reduce the surfacetension at oil and water interface, thereby increasing the solubility of water immiscible substances. Due to their diverse and extensive application, there is growing concern over their environmental impacts and difficulty in being degraded easily in the environment. This necessitated the discovery of alternative whichis equally efficient but environment friendly. Recently much attention has been given towards biosurfactant over chemically synthesized surfactants due to their ecological acceptance, low toxicity, biodegraded effectiveness at extreme temperature or pH values and widespread applicability (Mnif and Ghrini et.al). these biosurfactant have been used as alternatives for synthetic surfactants during last decade and have many industrial and environmental application such as enhanced oil recovery, crude oil drilling, Lubrication, bioremediation of Pollutants, detergency and solubilization. They have application also in food processing, cosmetics and healthcare industries.

Biosurfactant also showed important biological activities including antimicrobial, insecticidal, immune modulative and anti-tumoral activities. It has also been observed that microbial biosurfactant are advantageous overplant-based surfactants because of the scale upcapacity, rapid production, and multi-functional properties. Biosurfactant are amphiphilic biological compound produced extracellularlyor as a part of cell membrane by a variety of yeast, bacteria and filamentous fungi by usingsubstrates such as sugar, oil and wastes.

These molecules comprise complex structure which are grouped either low molecular weight compounds such as glycolipid, lipopeptide or high molecular weight compounds such as polymeric biosurfactant. The major classes of biosurfactant includes glycolipids, lipopeptide, lipoproteins, phospholipid and fatty acids and polymeric surfactants. Studied have indicated that microbial biosurfactants have the same properties as synthetic surfactants in the term of detergency, emulsification, wetting, the formation of foam, the dispersion and solubilization of hydrophobic substances, and the modification of surfaces. Biosurfactants also have advantages over synthetic surfactants, such as compatibility with the skin, low toxicity and irritability, and greater biodegradability. Moreover, the literature indicates that biosurfactants are promising for use in cosmetics and personal hygiene products due to their anti-adherence, antifungal, antiviral and antibacterial activities against a number of pathogens.

In this study there are three isolates has beentaken which was earlier isolated for biosurfactant production i.e *Pseudomonas aeruginosa, Micrococcus luteus, and Serratia marcescens.* These three-isolate characterized by HPLC (High performance liquid chromatography). This study shows growth kinetics, production kinetics and characterization of biosurfactant produced by organisms. Determination of production curve is necessary to estimate to the biosurfactant production related to the biomass of microorganism. Characterization ofbiosurfactant is important to know the structure of compound. These characterization of biosurfactant carried out by HPLC analysis. Different biosurfactant contains different chemical group which can be used in various applications in many industries.

Materials and methods

Producing organism

The producing microorganisms, *Pseudomonas aeruginosa, Micrococcus luteus and Serratia marcescens* was earlier isolated from engine oil contaminated soil from Navi Mumbai area and collected from Department of Microbiology, Institute ofscience. The stock culture of the microorganism was retrieved and sub-cultured in Nutrient Agar by the quadrant-streak plate technique at 37°C for 24 hours. Fresh culture was stored at 4°C for further process.

Growth kinetics

To study growth kinetics all three Cultures were grown in a100 ml of mineral salt medium with 2% engine oil as carbon source. *Pseudomonas aeruginosa* and *Micrococcus luteus* incubated for 2 days at 37°C at 135 rpm in shaker condition and *Serratia marcescens* incubated for 7 days at 37°C at 135 rpm in shaker condition on the basis of pilot studies. Absorbanceof *Pseudomonas aeruginosa* and *Micrococcus luteus* has been measured after every 4 hours till 48 hours and absorbance of *Serratia marcescens* has been measured after every 24 hours till 216 hours, using colorimeter at 620 nm. Graph of Absorbance verses Time plotted indicated growth curve of all organisms (Swetha Sunkar et al.,2019).

Production Kinetics

For biosurfactant production three 500 ml flasks has been taken for all three microorganisms in which 300 ml of mineral salt medium was added. The flasks were sterilized and 2% sterile engine oil was added in a medium as a sole carbon source. In each flask 2% fresh bacterial growth of each microorganism was added and incubate under shaking condition at 37°C at 135 rpm for 2 days and 7 days accordingly. To determine biosurfactant production, the cultures is centrifuge at 4°c 10,000 rpm for 15 minutes. Then Production of biosurfactant was investigate in cell free supernatant using anthrone assay. The anthrone assay was used to quantify the amount of glycolipid present in the culture supernatant. Rhamnose and Glucose was used as standard at concentration range 10-100 µg/ml as described earlier (Swetha Sunkar et al.,2019; Khademolhosseini R et al., 2019).

Extraction of biosurfactant compound by Acid Precipitation

This extraction technique is used in combination with acid precipitation for more efficient yields. Removed cells by centrifuge at 12,000g for 15 min. Acidify by addition of concentrated HCl to pH 2.0. The precipitate formed was allowed to settle at 4°C for overnight and was collected by centrifugation at 12,000 rpm for 20 min. Precipitate was dissolved in sodium bicarbonate solution (0.1M) then extracted with equal volume of chloroform- methanol mixture (2:1 v/v). The organic phase from the separating funnel was collected in a glass petri dish and dried (T. J. P. Smyth et al.,2014).

HPLC Analysis

Small Amount of sample of glycolipid was air dry and was Dissolved in 1 ml water. Centrifuge to remove particulate material. Using gradient HPLC with UV detection at 244 nm, connect HPLC column. mobile phase A (Acetonitrile) and B (water) Gradient conditions should be set as follows; 50% A and 50% B for 3 min, then to 100% mobile phase A over 19 min and held for 5 min, followed a change to 50% A over 3 min and held for 10 min. Set flow rate at 1.0 ml/min with an injection volume of 50µl (T. J. P. Smyth et al.,2014).

Results and Discussion

From growth kinetics and production kinetics found that Maximum biomass as well as biosurfactant production by ***Pseudomonas aeruginosa*** was at 28 hours (Figure 1). The observation that both biomass and biosurfactant production peaked at 28 hours indicates that this time point is critical for maximizing biosurfactant yield. This could be an optimal harvest time in large-scale production, ensuring maximum recovery of biosurfactant before the cells begin to decline in viability. The simultaneous peak suggests that the cells likely began to experience nutrient limitations (e.g., carbon or nitrogen) at 28 hours. This limitation may have triggered biosurfactant production as a survival strategy, allowing the cells to access otherwise inaccessible substrates or form protective biofilms. The balance between cell growth and biosurfactant production is essential for industrial processes. By identifying the optimal time for maximum production, you can fine-tune the fermentation process to enhance biosurfactant yield while maintaining efficient growth conditions.

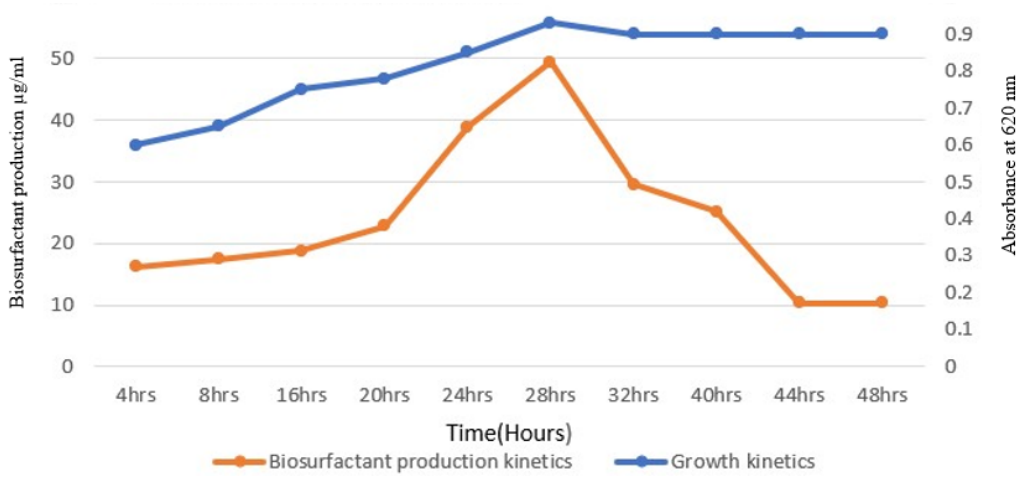

Figure 1: This table shows Growth kinetics and biosurfactant production by Pseudomonas aeruginosa

From growth kinetics and production kinetics found that Maximum biomass as well as biosurfactant production by ***Serratia marcescens*** was at 120 hours (Figure 2). The fact that maximum biosurfactant production occurs at 120 hours suggests that this time point is crucial for maximizing yield. For industrial-scale biosurfactant production, harvesting the culture at or around 120 hours would ensure that the highest levels of biosurfactants are obtained before the culture enters decline phase. The peak in biosurfactant production at 120 hours is likely a response to metabolic stress caused by nutrient limitations. *Serratia marcescens* may upregulate biosurfactant synthesis as a survival strategy to access additional nutrients or to establish a more favourable microenvironment, such as through biofilm formation. The timing of biosurfactant production is influenced by nutrient availability. Optimizing the medium composition or implementing fed-batch or continuous feeding strategies could potentially extend the production period or increase the overall yield by prolonging the stationary phase.

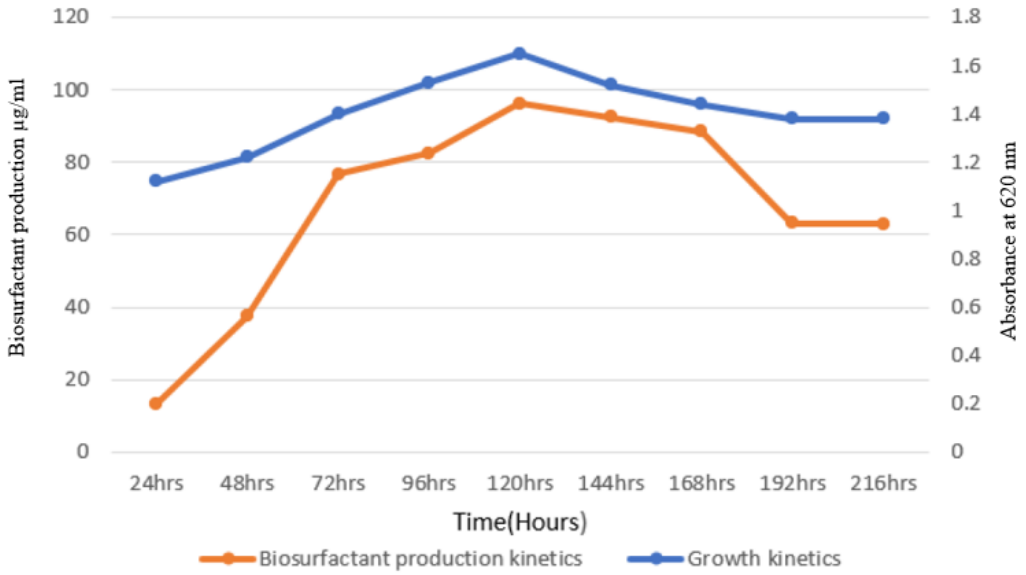

Figure 2: This table shows Growth kineticsand biosurfactant production by *Serratia marcescens*

From growth kinetics and production kinetics found that Maximum biomass as well as biosurfactant production by ***Micrococcus luteus*** was at 24 hours (Figure 3). Since both biomass and biosurfactant production peaked at 24 hours, this time point is critical for maximizing biosurfactant yield. For industrial processes, harvesting at or near 24 hours would maximize both cell mass and biosurfactant recovery. The peak in biosurfactant production at 24 hours is likely a response to nutrient limitations in the medium. When cells experience stress from depleted nutrients, they redirect their metabolism toward biosurfactant production as a means of accessing new energy sources and enhancing their survivability in hostile conditions. To further enhance biosurfactant production, future research could explore nutrient optimization or control over fermentation conditions. Adjusting the carbon and nitrogen ratios or employing fed-batch strategies could extend the period of biosurfactant production or increase yield.

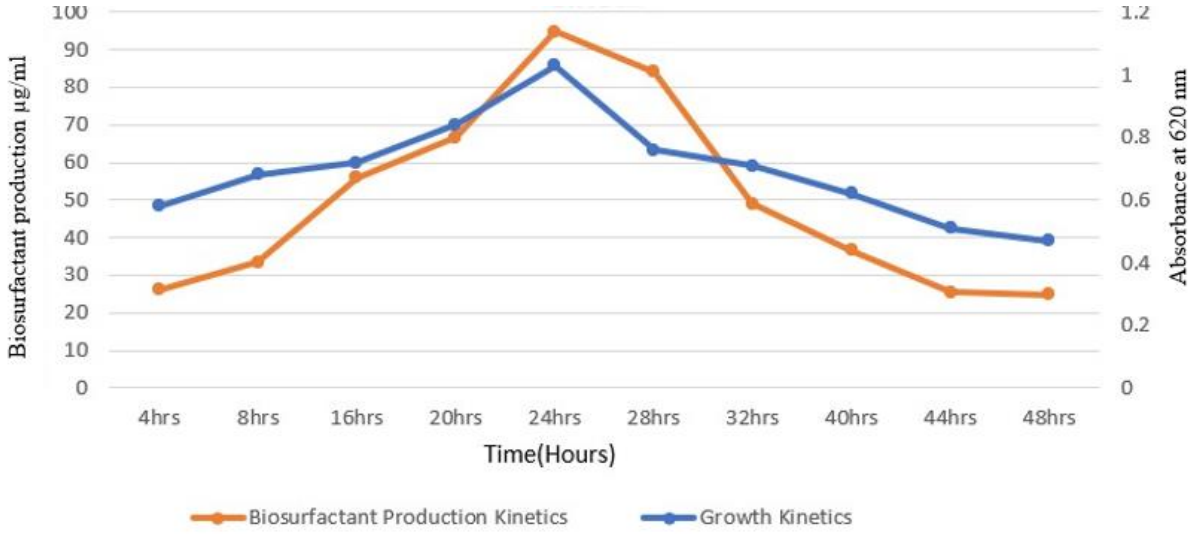

Figure 3: This table shows Growth kinetics and biosurfactant production by *Micrococcus Luteus*

High performance liquid chromatography analysis

HPLC Chromatogram of *Pseudomonas aeruginosa*

HPLC analysis of biosurfactant shows major peak at 4.961 and minor peak at 2.474, 5.232 and 6.072 (Figure 4). This can be shows that presence of Glycolipid type of biosurfactant. The major peak at RT 4.961 suggests the primary glycolipid component in the sample. Since glycolipids consist of a hydrophilic sugar group and a hydrophobic lipid tail, this peak is likely associated with the most dominant glycolipid compound in your sample. The moderate retention time indicates a balanced amphiphilic structure.

Minor Peak at 2.474 Minutes is early eluting peak is likely due to a more polar (hydrophilic) component, such as a sugar moiety without a lipid tail or with a very short lipid chain. In biosurfactant mixtures, this might represent unreacted sugar molecules or highly polar glycolipid forms. Other minor Peak at 5.232 Minutes could correspond to a variant of the primary glycolipid, possibly a di-glycolipid (e.g., di-rhamnolipid), where two sugar units are attached to the lipid tail, making it slightly more hydrophobic than the mono-glycolipid, hence the longer retention time. Also, minor Peak at 6.072 Minutes with the longest retention time likely represents the most hydrophobic component in

the sample. This could be a lipid-rich glycolipid or an isomer with a longer fatty acid chain or more complex lipid structure. The later elution suggests a strong interaction with the hydrophobic stationary phase of the column.

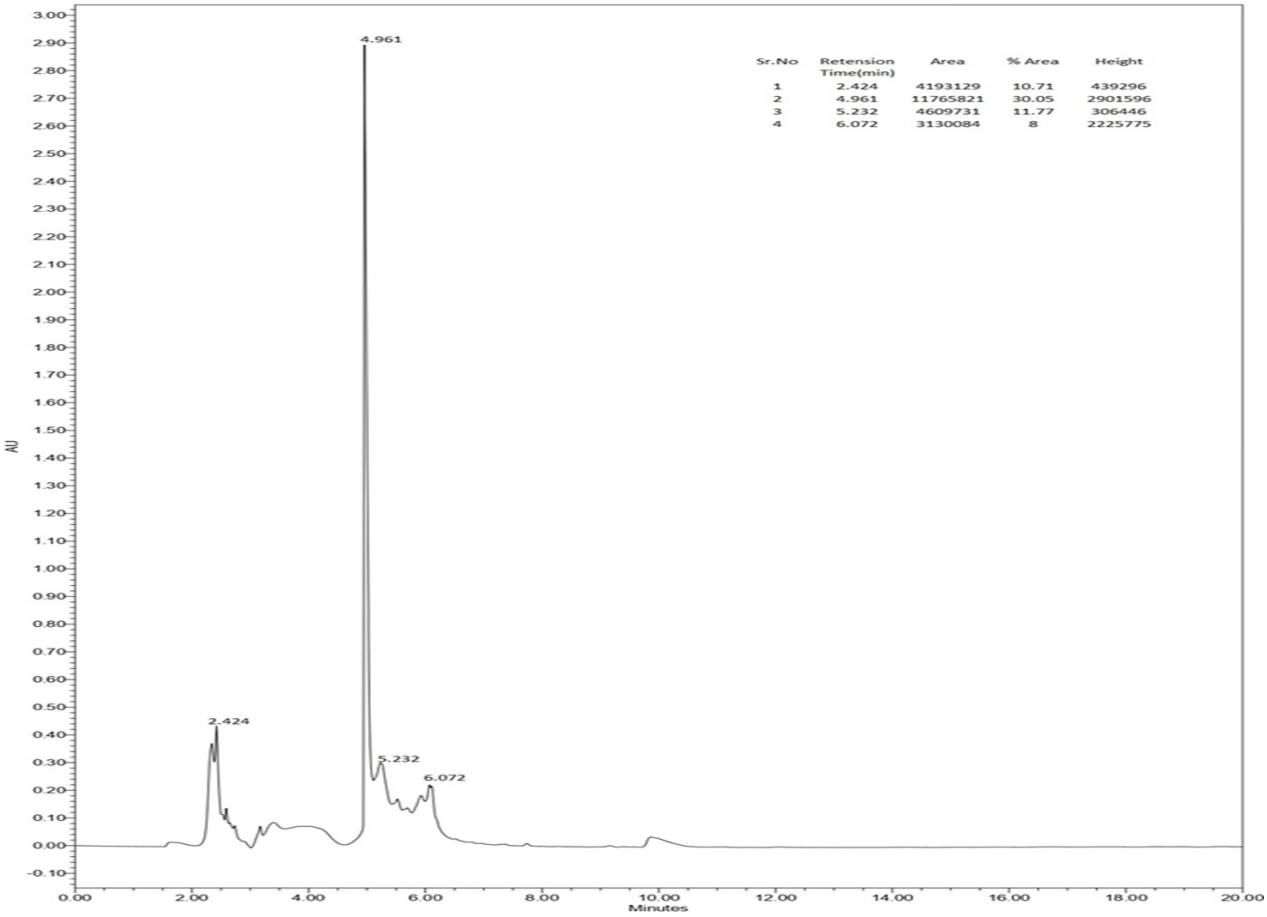

Figure 4: HPLC Chromatogram of *Pseudomonas aeruginosa*

HPLC Chromatogram of *Serratia marcescens*

HPLC analysis of biosurfactant shows major peak at 2.366 and minor peak at 2.902 (Figure 5). This can be shows that presence of glycolipid type of biosurfactant. The major peak at RT 2.366 suggests the presence of the main glycolipid component. Since glycolipids consist of a sugar moiety attached to a lipid chain, this peak likely corresponds to a more hydrophilic portion of the glycolipid structure (likely the sugar moiety), which elutes earlier due to weaker interactions with the hydrophobic stationary phase of the column.

The minor peak at RT 2.902 suggests the presence of a secondary or less abundant component in the biosurfactant sample. This might indicate another glycolipid variant or isomer. Since it elutes slightly later, it is likely more hydrophobic (possibly due to a longer lipid chain or additional lipid groups).

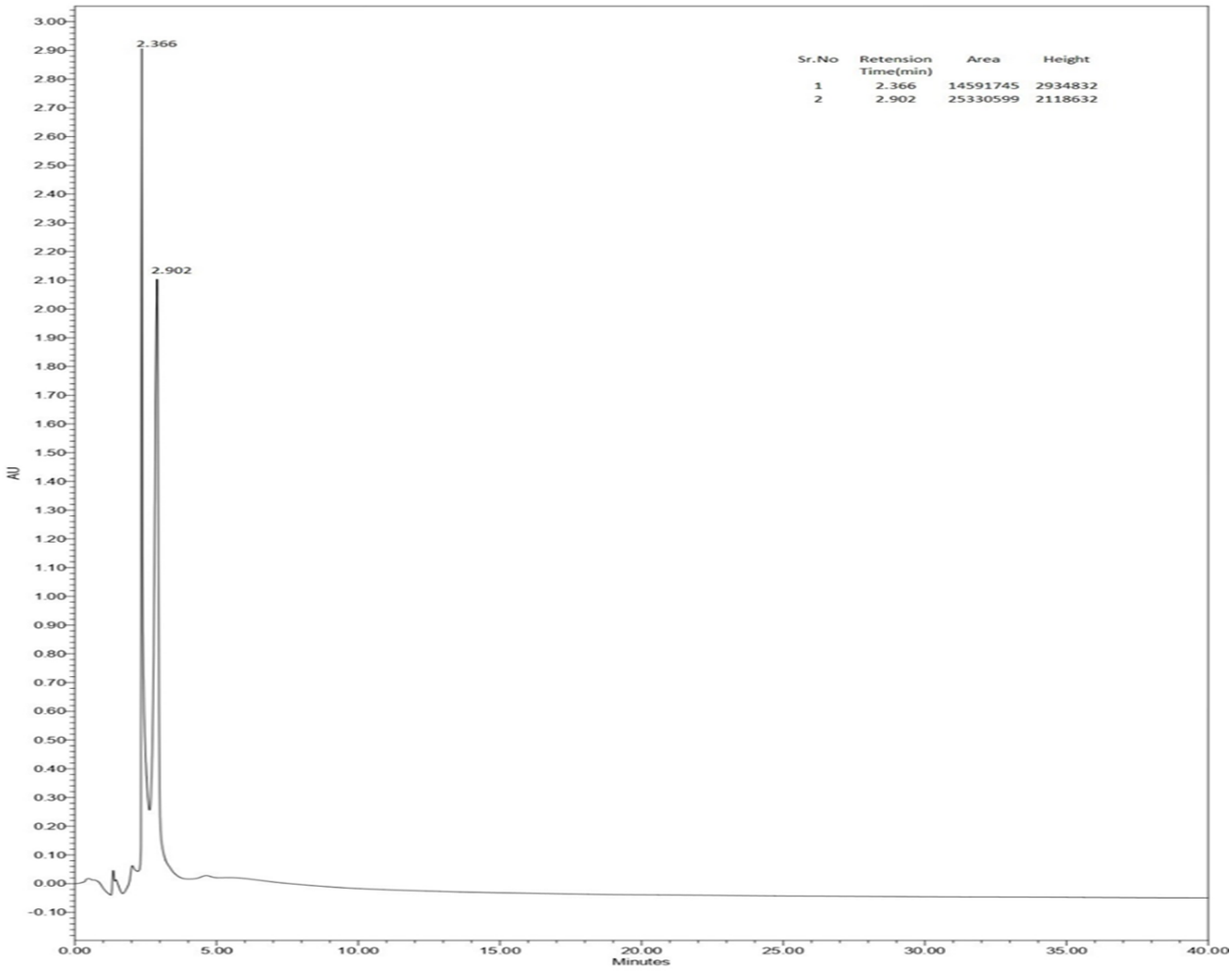

Figure 5: HPLC Chromatogram of *Serratia marcescens*

HPLC Chromatogram of *Micrococcus luteus*

HPLC analysis of biosurfactant shows major peak at 6.462 and 2.521 and minor peak at 6.049 and 6.246 (Figure 6). This can be showsthat presence of Rhamnolipid biosurfactant. Major Peak at 6.462 likely corresponds to the hydrophobic lipid moiety of the glycolipid. The relatively high retention time indicates that this component has stronger interactions with the non-polar column, suggesting a more lipophilic structure and Peak at 2.521 which appears earlier in the chromatogram, represents a more polar compound. In the case of glycolipids, this peak may correspond to the sugar (glycoside) portion of the biosurfactant, which interacts less with the hydrophobic column and elutes sooner.

Where, Minor Peaks presents at 6.049 and 6.246. These minor peaks, appearing close to the major peak at 6.462, might indicate the presence of closely related glycolipid congeners or isomers. Glycolipid biosurfactants are often produced as mixtures, and the slight variations in retention time could reflect structural differences (e.g., variations in fatty acid chain length or the number of sugar units).

Suggest further analysis using techniques such as MS or NMR to confirm the molecular structure of the all identified glycolipids and to identify the specific glycolipid types present in the sample.

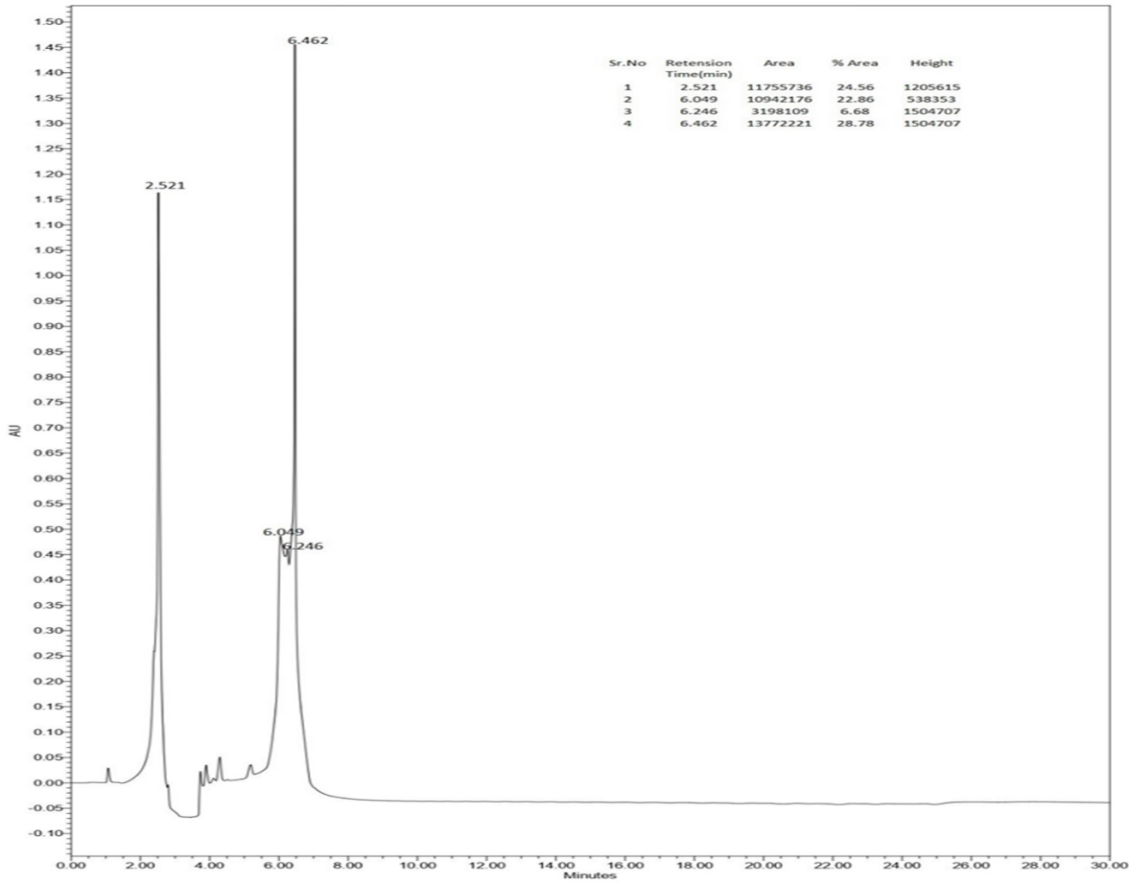

Figure 6: HPLC Chromatogram of *Micrococcus Luteus*

Conclusion

Growth kinetics and Production curve of microorganisms such as *Pseudomonas aeruginosa*, *Micrococcus luteus* and *Serratia marcescens* was observed using carbohydrate estimation by Anthrone reagent. From that we found that Maximum biomass as well as biosurfactant production by *Pseudomonas aeruginosa* was at 28 hours, for *Micrococcus luteus* at 24 hours and *Serratia marcescens* at 120 hours. After that, extraction of biosurfactant was done using Chloroform: Methanol method. The extract was brownish in colour. Analysis of biosurfactant produced by microorganisms was done using HPLC technique. This technology helps to determine the chemical structure of molecule. HPLC reveals that *Pseudomonas aeruginosa, Serratia marcescens* and *Micrococcus luteus* can be produces glycolipid type of biosurfactant. Consider performing further analysis using techniques like Mass Spectrometry (MS) or Nuclear Magnetic Resonance (NMR) to confirm the molecular structure of the biosurfactant. These methods can provide detailed insights into the composition and structural features, helping to verify the identity and purity of the biosurfactant produced.

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