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SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF THE OLEAGINOUS ACTINOBACTERIUM *STREPTOMYCES FRADIAE* FOR LIPID PRODUCTION

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ABSTRACT
Producing lipids for biofuel conversion requires saccharification of simple sugars by oleaginous microorganisms during fermentation. This work investigates the biomass of oleaginous actinobacteria required for lipid production. Waste Rice Water (WRW) was used as a substrate for microbial biomass production. Substituting WRW during fermentation influenced biomass growth in the oleaginous actinobacterium *Streptomyces fradiae*. The efficiency of biomass production in delivering the highest lipid content during the exponential growth phase was evaluated. The total lipid content was evaluated using the calorimetric sulfo-phosphovannilin assay. WRW at 25% dilution proved to be an alternative substrate for the ISP2 (International *Streptomyces* Project 2) growth medium, which showed a maximum yield of 60.3g/L of biomass and total lipid content of 38.6% (w/w). Enzymatic degradation of starch has resulted in simple sugar formation, and fermentation of simple sugars further initiated biomass and lipid accumulation. The relatively higher carbon and nitrogen content of WRW increases lipid accumulation in the oleaginous actinobacteria *Streptomyces fradiae*. This indicates that starch from WRW was completely degraded by the oleaginous actinobacteria *Streptomyces fradiae* and shows a notable impact on lipid production.

KEYWORDS: Microbial biomass, Lipid production, Fermentation and Waste Rice Water.

INTRODUCTION

Biofuels have attracted global attention for their efficient carbon dioxide emissions. Fossil energy fuels are continuously decreasing due to climate change and various other factors, leading to global warming. This raises energy demand and increases the necessity for alternative fuels **(1).** Biofuel feedstock can be derived from vegetable oils, animal fats, and microbial oils. The fatty acids in microbial lipids containing triglycerides (TAG) must be esterified into long-chain mono-alkyl esters through the transesterification process into fatty acid methyl esters (FAME) before blending with commercial diesel fuel **(2).** The renewability of the feedstock is a beneficial property of biofuel. Microbial lipids alone have renewable potential without any limitations in fuel availability. The special feature of microbial fuels enables quick blending with diesel oil at any desired proportion without compromising the fuel quality. Thus, eliminates the need for engine modifications in vehicles to substitute microbial fuels **(3).**

Oleaginous microorganisms, specifically *Acinetobacter, Mycobacterium*, and *Streptomyces* belonging to the genus *Actinobacteria*, accumulate lipids in the form of TAG at high levels **(4).** Lipid biosynthesis in *Actinobacteria* occurs intracellularly, initiated by a cell wall composed of a simple peptidoglycan mesh surrounded by the cytoplasmic membrane **(5).** Oleaginous *Actinobacteria* consume carbon sources in the form of simple sugars, such as glucose, starch, lactose, sucrose, and xylose, and accumulate intracellular lipids **(6)**. The biofuel quality depends on the lipid fatty acid profile **(7).** Microbial lipids are more beneficial than vegetable oil or animal fats, with minimal requirements for cultivating microorganisms. Microbial biomass cultivation has huge advantages, including faster biomass growth in limited land space, lower maintenance, easy scalability, and no requirement for seasonal and climatic changes **(8).** Triglycerides with polyunsaturated fatty acids (PUFA) in microbial lipids exhibit a higher degree of unsaturation and share similar chemical structures with vegetable oils **(9).** Microbial fuel is considered the most beneficial fuel as it is non-toxic, biodegradable, and eco-friendly **(10).** To enhance lipid accumulation, a limited amount of starch is required during microbial fermentation **(11).** Starch is one of the affordable and widely used components for growing oleaginous microorganisms because it provides a carbon source that readily supports lipid biosynthesis **(12).**

2. METHODOLOGY

2.1 Oleaginous *Streptomyces fradiae* isolation

Microbial lipids were produced by the potential actinobacterium *Streptomyces fradiae* isolated from coastal soil sediments of the Indian east coast, Mamallapuram, Tamil Nadu. Among 53 isolated Actinobacterium strains, we selected fatty acid producers based on our previously published method **(13).** Calorimetric analysis using 0.1%

w/v 2, 3, 5 Triphenyl Tetrazolium Chloride (TTC) dye, mixed with 10 mL of each culture. After 60 minutes of incubation at 25°C, the culture turns red, indicating the presence of fatty acids **(14).** Molecular identification of the lipid-producing actinobacterium was performed using the 16S rRNA sequencing method **(15).** The biochemical and physiological characteristics were analyzed by the standard protocol of the International *Streptomyces* Project (ISP) **(16).** WRW contains starch that solidifies after sterilization and arrests actinobacterial filament growth. Hence, WRW was diluted with water 25%, 50%, and 75% dilutions. The 25% dilution was suitable for the growth of actinobacteria. Further experiments were carried out using 25% WRW with a two-series experimental setup for standard results. The shake-flask fermentation method was opted for biomass production.

2.3 Effect of pH on reducing sugar

The starch in WRW was degraded by *Streptomyces fradiae* during Simultaneous Saccharification and Fermentation (SSF), was investigated based on pH and reducing sugar concentration. Microbial cultures were prepared in 2000 mL Erlenmeyer flasks containing 1000 mL of basal medium supplemented with 25%WRW substrate as the carbon source. The initial pH of the culture medium was adjusted to 6.5 using 1 N HCl or NaOH and sterilized. Fermentation was carried out by inoculating *S. fradiae* (10% v/v inoculum) into the WRW and ISP2 culture medium. Both flasks were incubated at 30 °C with shaking at 160 rpm for 5 days. Reducing sugar concentration was quantified at 24 h intervals using the dinitrosalicylic acid (DNS) method **(17)**.

2.3 Biomass harvest and lipid analysis

The biomass was harvested from the substrate on the exponential growth phase at the 5th day of fermentation, by centrifugation at 6000rpm for 10 minutes. Microbial oil was extracted from the biomass using the Bligh and Dyer method **(18).** The total lipid content from the extracted microbial oil was calculated based on the phospho-vanillin assay. The homogenized biomass was heated directly with concentrated sulfuric acid followed by the addition of vanillin and phosphoric acid. This reaction immediately formed a pink-colored product. This colour change was due to the oxidation of unsaturated lipids that further condensed to ketones. Hence, the ketones got condensed with vanillin in the presence of an acid catalyst. Finally, the highly unsaturated lipids were absorbed using a visible light UV spectrophotometer **(19).**

3. RESULTS AND DISCUSSION

3.1 Oleaginous *Actinobacteria Streptomyces fradiae* in biofuel application

Actinobacteria were selected as a potential candidate for lipid production due to the lower possibility of contamination, compared to other microorganisms involved in biofuel feedstock production **(20).** It also reaches its exponential growth phase within 72 hours of fermentation in the low-cost substrate WRW by utilizing starch as a carbon source. Natural flocculation of biomass after fermentation made the biomass harvest simple for scaling biofuel applications. Isolation and screening of lipid producing *Actinobacteria* were identified as *Streptomyces fradiae* based on phylogenetic analysis using the 16s rRNA sequencing technique. 16s rDNA of the potential strain JJ1 contains 416bp (NCBI GenBank Acc No. MK733985). Molecular identification revealed 99% homology with *S.fradiae* KP698740 in BLAST. Morphology of *Streptomyces fradiae* showed sporulated mycelium producing yellow pigments and possessed an earthy odor with filamentous rods in 0.2-5µm size. **(Fig.1)** shows the proliferated biomass image of Scanning Electron Microscope (SEM) before and after fermentation. In the present study, microbial lipids were produced from the isolated oleaginous *Actinobacteria Streptomyces fradiae*. WRW substrate reduced the cultivation cost and promoted lipid accumulation efficiently to produce biofuel. Shake flask fermentation induced natural flocculation of biomass weighed 60.3g/L with oil content 38.6% (w/w).

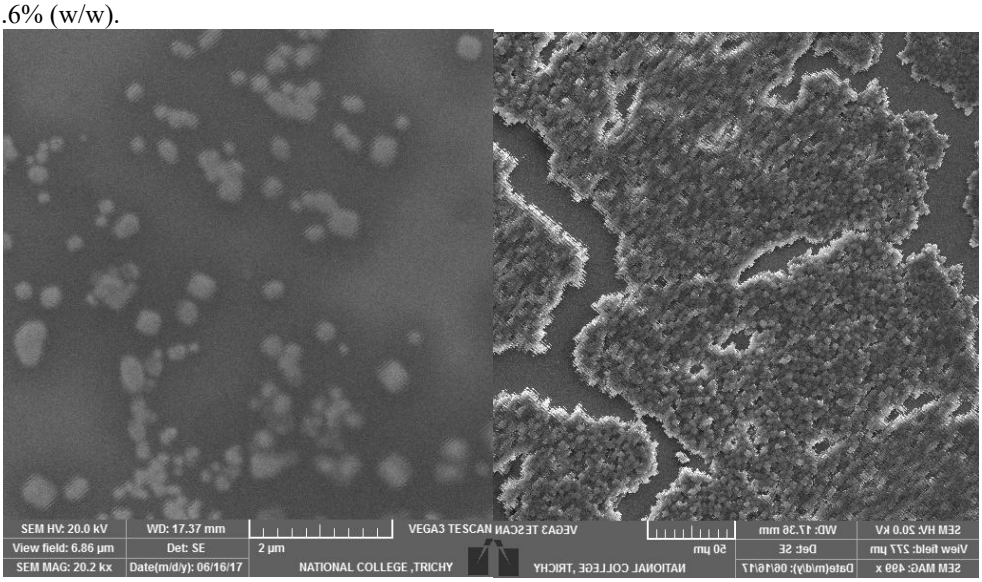

Fig.1 SEM image of biomass before and after fermentation

3.2 Efficacy of lipid extraction

Natural flocculation enables the separation of biomass from the WRW substrate. The biomass pellets were harvested, followed by cell lysis to induce the release of microbial lipids by agitation using ultrasonication, as stated by the Bligh & Dyer method **(Fig.2)**. The maximum amount of lipid was extracted from 60.3g/ L of biomass, releasing 38.6% (W/W) of total lipid content estimated using the phospho-vanillin assay**.** The total fatty acid compounds comprised 85.4 wt %. This satisfied the basic requirement for lipid conversion to biofuel **(21)**. These findings demonstrate that the natural flocculation efficacy of *Streptomyces fradiae* along with ultrasonication-assisted extraction is a strong strategy for maximizing lipid yield from oleaginous actinobacteria, thereby strengthening their potential as sustainable biofuel feedstock.

Fig.2 Lipid extracted from *Streptomyces fradiae*

3.3 Effect of simultaneous Saccharification and Fermentation on biomass

The SSF process has improved the microbial biomass yield of *Streptomyces fradiae* through the dual activation of enzymatic saccharification with microbial metabolism. Rapid conversion of sugars by substrate utilization has enhanced lipid productivity. The results imply that RWW medium yields are consistently higher, reaching ~60.3 g/L by Day 5, compared to ~59.5 g/L in ISP2. Both media showed a linear increase in biomass yield, indicating active microbial growth throughout the incubation period. Scanning e**(Fig.3)**. Previous studies also stated that SSF improves substrate conversion efficiency by reducing sugar inhibition by channelling carbon flux directly into microbial growth and lipid biosynthesis **(22)**. The observed increase in biomass yield under SSF can be attributed to the continuous release of fermentable sugars from the substrate starch available in WRW, which also sustained microbial activity and reduced lag phases. Similar findings were reported in oleaginous actinobacteria, algae and yeast systems, where SSF not only improved lipid yield but also enabled affordable scaling by integrating Saccharification and fermentation into a single step **(23)**. Thus, the present study confirms that SSF offers a robust strategy for enhancing microbial biomass and lipid productivity in *S. fradiae*, aligning with broader biotechnological applications of consolidated bioprocessing.

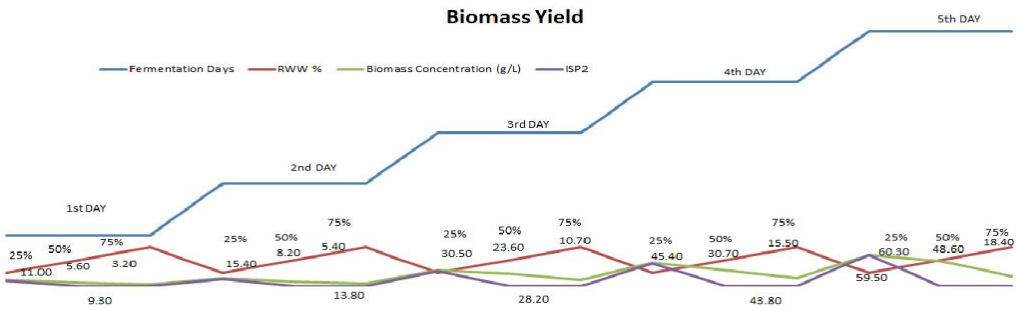

Fig.3 Effect of incubation time on biomass yield in WRW and ISP2 medium

CONCLUSION

Based on the above results, this study validates a compelling point that lipids produced by *Streptomyces fradiae* in WRW proved to be the cost-effective alternate substrate with enhanced biomass and lipid productivity. The sustainability of the biofuel can be achieved by utilizing these renewable microorganisms having oleaginous

property. These findings highlight the efficacy of WRW as fermentation medium fulfilling the fundamental requirements for lipid biosynthesis. The integration of enzymes with reducing sugars improves biomass yield and lipid accumulation. Future Research will focus on scaling up SSF systems, optimizing substrate specificity and to explore genetic engineering strategies to further enhance lipid biosynthesis pathways that aids in futuristic bioenergy applications.

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