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A study on the probabilities of the production of biodiesel from naturally isolated bacterial sources

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Abstract

Technically, biodiesel is vegetable oil methyl ester. It is formed by removing triglyceride molecule from vegetable oil in the form of glycerin (soap). Once the glycerin is removed from the oil, the remaining molecules are, to a diesel engine, similar to petroleum diesel fuel. There are some notable differences. The biodiesel molecules are very simple hydrocarbon chains, containing no sulfur, ring molecules or aromatics associated with fossil fuels. For about 30 years the thirst to find independence from these fossil fuels has driven man kind to those alternatives which not only give assurity in the quality of the fuel but also its renewability. These efforts have given the origin of biodiesel from plants and algal origin. Due to various industrial and commercial problems in these productions a new alternative source for biodiesel has taken the lime light. Bacterial based biodiesel production has taken new shape in this decade; especially the industrial importance of bacteria fermentations has assured that this source can bring big positive shift in the biodiesel market in the coming years. In this project main strategy is laid around isolating and analyzing various selected bacterial strains for their abilities to produce Fatty acid based biomolecules and also concentrates on the optimization of the nutrient medium to maximize lipid output for biodiesel production.

Key-Words: Acid value, Biodiesel, Esterification, *Corynebacterium rubrum,* Lipid extraction

Introduction

India occupies second place in population and 7th place in area in the World. Due to large population and the need of transportation made India top 5th country in the World in consumption of petroleum products. Yearly consumption of diesel in India is approximately 40 million tones, which constitutes about 40% of the total petro-products consumption (1). As these carbon sources are limited and the consumption of petro-products is increasing day by day there is a need of alternative resources, which includes solar energy, thermal energy, hydro energy and bio-energy (2). Among these alternative resources bio-energy is the most important and easily useful source for energy production. Production of diesel using plant sources is good alternative resource through which we can meet the demand for petroleum products (3, 4). Biodiesel is an alternative to diesel which is made from renewable resources such as vegetable oils (or) animal fats (5).

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As India is agriculture country most of its income comes from agriculture sector through the rural area the population mainly depends on the primary income source both men and women are involved in this sector of agriculture farming (6) . Biodisel from Bacteria is alternative source of income with agriculture sector. This can be done by any individual of any age, sex, qualification with proper guideness, investment and some space along with its primary source of income (7). Also it can be done on small and large scale (8, 9, 10). The oil derived from Bacteria will be future alternative for Biodiesel and Biopetrol (11). Corynebacterium is Gram-positive, rod-shaped bacteria which are widely distributed in nature and are mostly innocuous. Charecteristics of Corybacterium rubrum is catalase positive, nonspore-forming, nonmotile bacteria that are straight or slightly curved. Metachromatic granules usually represent stored phosphate regions. Their size ranges from 2-6 micrometers in length and 0.5 micrometers in diameter. Nonpathogenic species of Corynebacterium are useful in industrial applications like production of amino

acids, nucleotides, and other nutritional factors, bioconversion of steroids, degradation of hydrocarbons; cheese aging and production of enzymes (12, 13, 14, 15).

Material and Methods

Two types of bacterial strains were used for estimation of lipid content in this study. One was a commercially available strain with high lipid content- *Corynebacterium rubrum* [NCIM 2253] ATCC – 14898, ordered from NCIM, Pune, which was grown on a glycerol Asparagine agar (NCIM catalogue) for 10days to get a good growth and biomass, and the other type was naturally isolated filamentous bacteria. Three such natural strains were required for the study for comparative lipid analysis and were isolated as follows.

Sample area

The sample collection site was chosen to be a wastewater treatment plant as it's an ideal place for collection of filamentous bacteria as these bacteria prefer relaxed flowing water, are slow growers and fastidious, can grow when diverse nutrient conditions are available at such plants.

Sample collection

The mixed liquor samples were collected from the Sewage Treatment Plant, Amberpet, in sterile disposable sample collection bottles and were taken to the lab under non-contaminating conditions. The samples were inoculated in the decided media within 6hrs of sampling and incubated for further growth.

Fig. 1: Mixed Liquor in disposable sample bottle

Isolation of Culture

Isolation

Four different media were used for the inoculation and isolation of filamentous bacteria from the sludge sample R2A, I medium, Tryptone Glucose Yeast extract agar (TGY) and Casitone Glycerol Yeast autolysate broth base (CGY) (121). These media are specific for the growth of certain filamentous bacteria only, so a growth in them provisionally confirms the presence of the respective bacteria.

1 mL of sample was inoculated in 100 mL of each media in 250ml Erlenmeyer flasks and these conical flasks were incubated at room temperature, as filamentous bacteria grow well between 20- 28^{0}C. These flasks were half filled, so as to maintain minimal aerobic conditions for growth. Filamentous bacteria take 7 – 15 days for ideal growth. On the 10th day, sample from the each media was streaked on the agar of same composition on the respective media to check if growth has occurred.

Screening

The best media for alterations with reducing agents for enhanced lipid output was chosen on the basis of bacterial growth seen on the streaked plate. Mixed growths was obtained after initial streaking, but verious serial dilutions and sub-culturing thrice, isolated colonies were obtained in R2A media plates and hence that media was chosen for further media alteration experiments.

Fig. Error! No text of specified style in document.**-1: Corynebacteriumrubrum**

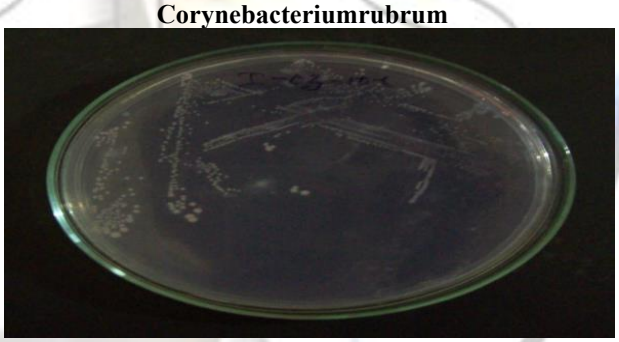

Fig. Error! No text of specified style in document.**-2: Natural strain grown on I medium**

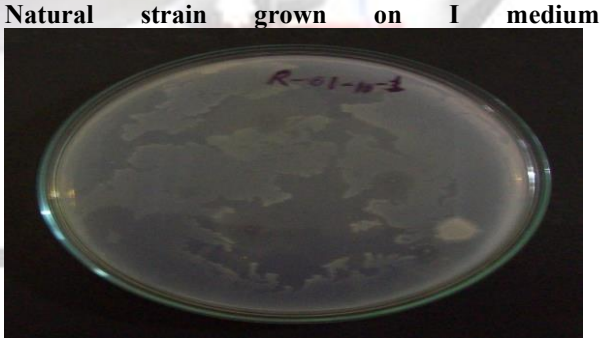

Fig. Error! No text of specified style in document.**-3: Natural strain grown on R2A medium**

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After the isolation and screening, the bacterial strains were grown in the presence of three different reducing agents under four different concentrations and their growth was assessed for lipid production. Reducing agents were used, as filamentous bacteria grow well in limiting oxygen conditions. The reducing agents were added after autoclaving as they get oxidized if added before. After the final experimental media was incubated for ample amount of time to allow growth of bacteria, it was then subjected to lipid extraction.

Lipid Extraction

Centrifuge the nutrient broth having grown culture (8 eppendorfs with 1ml sample in each) at 4500rpm for 4min. Concentrate the cell pellet by discarding the supernatant and suspending it new rounds of nutrient broth. Repeat this 4-5 times. Cell pellet is then suspended in 0.1% sodium Chloride and mixed well (add 1ml to each eppendorf). Centrifuge at 8000rpm for 8min. Discard the supernatant and transfer all the cell pellets in 1 or 2 eppendorfs. Store the pellets in -20 degree Celsius overnight. To the frozen biomass (if it is 100mg), add 114ml of solvent in a serial fashion with 15secs of shaking after each addition- Chloroform: Methanol: Water in the ratio of 1: 2: 0.8 respectively. Cover the beaker with aluminum foil (to prevent evaporation of solvents) and allow it to stand for 18hrs in a refrigerator. Phase separation of the biomass-solvent mixtures in the separatory funnels was achieved by adding chloroform and water to obtain a final chloroform/methanol/water ratio of 1/1/0.9 (v/v/v). A known portion of each total lipid extract recovered from the lower chloroform phase was used for further analysis.

Estimation of Acid Value

Acid value (a.k.a. Acid number) is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of chemical substance. The acid number is a measure of the amount of carboxylic acid groups in a chemical compound such as a fatty acid. In a typical procedure, a known amount of sample dissolved in organic solvent is titrated with a solution of potassium hydroxide with known concentration and with phenolphthalein as a color indicator.

The acid number is used to quantify the amount of acid present, for example in a sample of biodiesel. It is the quantity of base, expressed in milligrams of potassium hydroxide that is required to neutralize the acidic constituents in 1 g of sample.

Calculation Formula

$$\text{Acid value} = \frac{A \times N \times 56.1}{W}$$

Where: A – ml of 0.1N KOH consumed for sample.
N – Normality of KOH (0.1N)
W – Weight in gms of the sample (0.2gm)

The Lie Test

Lie test is performed to determine the amount of the base catalyst to be added to the transesterification reaction.

Results and Discussion

This study was undertaken to isolate naturally occurring bacteria having a substantial amount of lipid content. Filamentous bacteria were chosen because they have the ability to breakdown and ingest larger lipid molecules, this could be accounted for their higher lipid content. Characterization of these bacteria wasn't possible was many of them are gram-variable and sometimes show fake filaments; also they stick to each other as flocks.

As mentioned in the review, filamentous bacteria thrive well under either low oxygen conditions or low food conditions or both; hence a nutritionally minimal media- R2A was used. But as a part of the study it was also decided to maintain low oxygen conditions, hence a novel approach was used by addition of three reducing agents (individually) - used for anaerobic media preparations- under varying concentrations to maintain the distance between low oxygen and anaerobic conditions.

Commercial Strain	C
Natural strain- 1	N1
Natural strain- 2	N2
Natural strain- 3	N3

For a comparative analysis, a commercially available strain – *Corynebacteriumrubrum* (C) with high lipid content was also purchased from National Collection of Industrial Microorganisms (NCIM). Though this strain was never used for biodiesel application, still its unusually high lipid content made it favorable for this study.

Lipid extraction was performed with modified Bligh and Dyer protocol for bacterial cells. Most microorganisms rarely produce enough lipids to account for more than 10% of their dry weight. But, the lipid content of the industrial,

Corynebacteriumrubrum, was around 28% of original bacterial weight.

Reducing agent- Sodium Sulphide

Strain N1

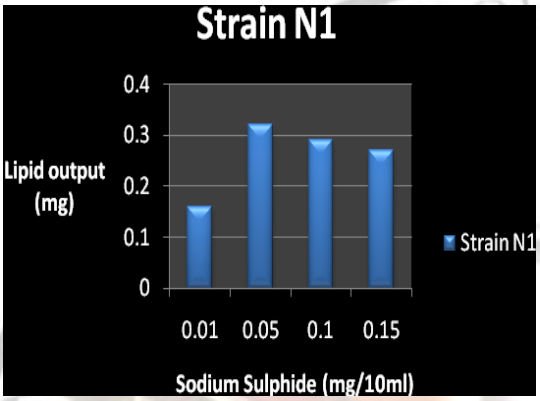

Fig. Error! No text of specified style in document.**-4: Effect of different concentrations of Sodium sulphide on Strain N1**

With Sodium sulphide as the reducing agent, Strain N1 showed maximum lipid content at concentration 0.05mg/10ml.

Strain N2

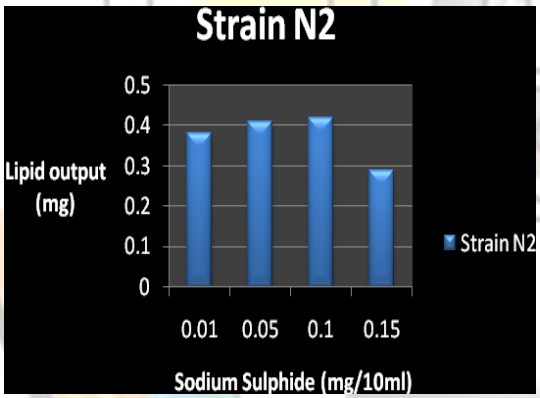

Fig. Error! No text of specified style in document.**-5: Effect of different concentrations of Sodium sulphide on Strain N2**

With Sodium sulphide as the reducing agent, Strain N2 showed maximum lipid content at concentration 0.1mg/10ml.

Strain N3

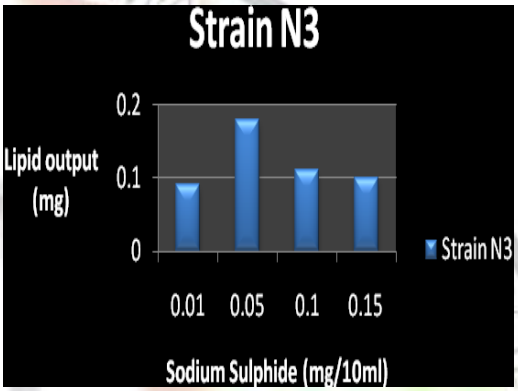

Fig. Error! No text of specified style in document.**-6: Effect of different concentrations of Sodium sulphide on Strain N3**

With Sodium sulphide as the reducing agent, Strain N3 showed maximum lipid content at concentration 0.05mg/10ml.

Lipid extractions were performed on 14 day old batches. For the reducing agent Sodium sulphide, Strain N1 showed better lipid output at 0.05mg/10ml concentration. Strain N2 showed better lipid output at 0.1mg/10ml concentration. Strain N3 showed better lipid output at 0.05mg/10ml concentration. Comparing the lipid output, it is seen that Strain N2 showed better lipid output with reducing agent Sodium Sulphide.

Reducing agent- Ferrous sulphide

Strain N1

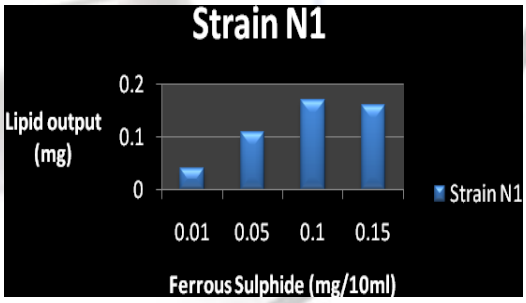

Fig. Error! No text of specified style in document.**-7: Effect of different concentrations of Ferroussulphide on Strain N1**

With Ferrous sulphide as the reducing agent, Strain N1 showed maximum lipid content at concentration 0.1mg/10ml.

Strain N2

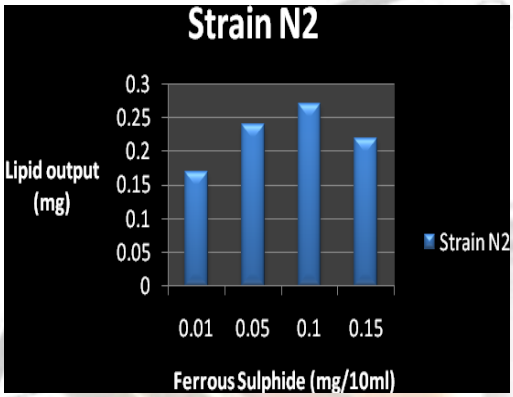

Fig. Error! No text of specified style in document.**-8: Effect of different concentrations of Ferroussulphide on Strain N2**

With Ferrous sulphide as the reducing agent, Strain N2 showed maximum lipid content at concentration 0.1mg/10ml.

Strain N3

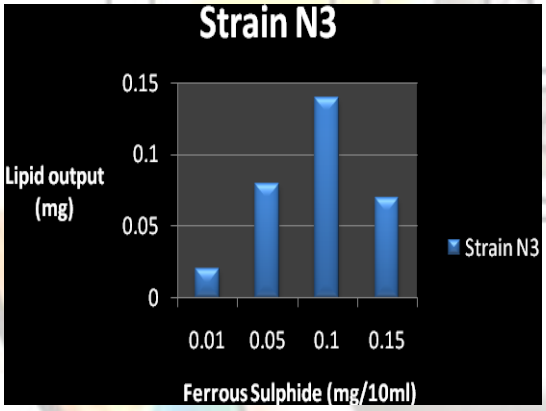

Fig. Error! No text of specified style in document.**-9: Effect of different concentrations of Ferroussulphide on Strain N3**

With Ferrous sulphide as the reducing agent, Strain N3 showed maximum lipid content at concentration 0.1mg/10ml.

For the reducing agent Ferroussulphide, Strain N1 showed better lipid output at 0.1mg/10ml concentration. Strain N2 showed better lipid output at 0.1mg/10ml concentration. Strain N3 showed better lipid output at 0.1mg/10ml concentration. Comparing the lipid output, it is seen that Strain N2 showed better lipid output with reducing agent Ferrous Sulphide.

Reducing agent- Titanium III citrate

Strain N1

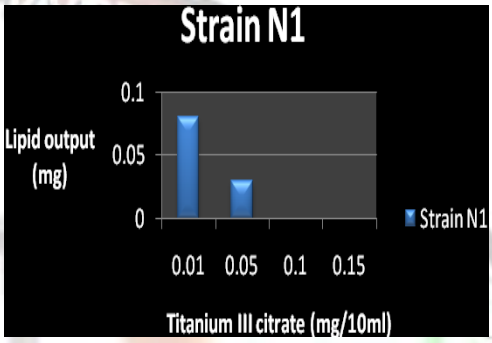

Fig. Error! No text of specified style in document.**-10: Effect of different concentrations of Titanium III citrate on Strain N1**

With Titanium III citrate as the reducing agent, Strain N1 showed maximum lipid content at concentration 0.01mg/10ml.

Strain N2

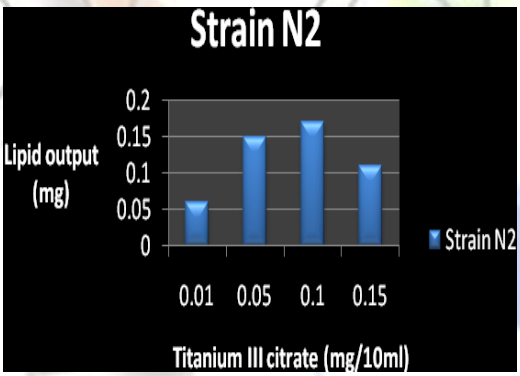

Fig. Error! No text of specified style in document.**-11: Effect of different concentrations of Titanium III citrate on Strain N2**

With Titanium III citrate as the reducing agent, Strain N2 showed maximum lipid content at concentration 0.1mg/10ml.

With Titanium III citrate as the reducing agent, Strain N3 showed maximum lipid content at concentration 0.1mg/10ml and 0.15mg/10ml.

For the reducing agent Titanium III citrate, Strain N1 showed better lipid output at 0.01mg/10ml concentration, while no readings were shown for the concentrations 0.1mg/10ml and 0.15mg/10ml. Strain N2 showed better lipid output at 0.1mg/10ml

concentration. Strain N3 showed better lipid output at 0.1mg/10ml and 0.15mg/10ml concentration. Comparing the lipid output, it is seen that Strain N2 showed better lipid output with reducing agent Titanium III citrate.

Strain N3

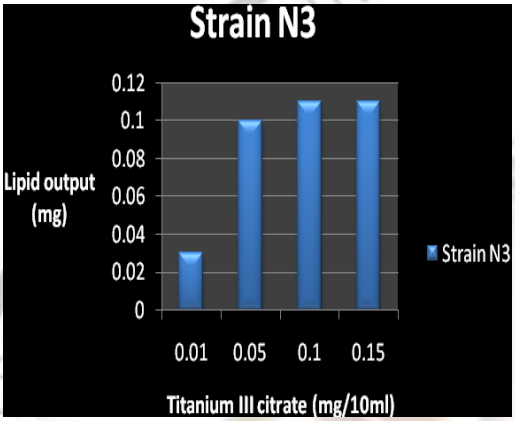

Fig. Error! No text of specified style in document.**-12: Effect of different concentrations of Titanium III citrate on Strain N3**

Addition of Reducing agents to media- ***Comprehensive Graphs***

Lipid extractions were performed after 14 days of incubation under varying concentrations of 3 different reducing agents, and following values were obtained.

Sodium Sulphide

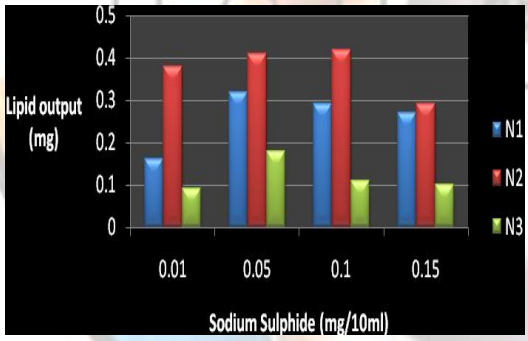

Fig. Error! No text of specified style in document.**-13: Comprehensive graph showing effect of Sodium sulphide on Strain N1, N2, N3**

Strain N1 showed better lipid output at 0.05mg/10ml concentration. Strain N2 showed better lipid output at 0.1mg/10ml concentration. Strain N3 showed better lipid output at 0.05mg/10ml concentration. Comparing the lipid output, it is seen that Strain N2 showed better lipid output with reducing agent- Sodium Sulphide.

However, taking all the aspects of the type of reducing agent, the concentration used and the amount of lipid extracted, it was observed that natural strain N1 showed maximum lipid output when the reducing agent used was Sodium sulphide at concentration (0.05mg/10ml), strain N2 showed maximum output when reducing agent was Sodium sulphide at concentration (0.1mg/10ml), whereas strain N3 showed maximum output when the reducing agent was Sodium sulphide at concentration (0.05mg/10ml). This showed that Sodium sulphide as a reducing agent can be used for media alterations to increase the lipid output in filamentous bacteria. Comparing the above data, strain N2 showed highest values for lipid extraction. Hence it should be taken in consideration for further characterization and experimental analysis.

Ferrous Sulphide

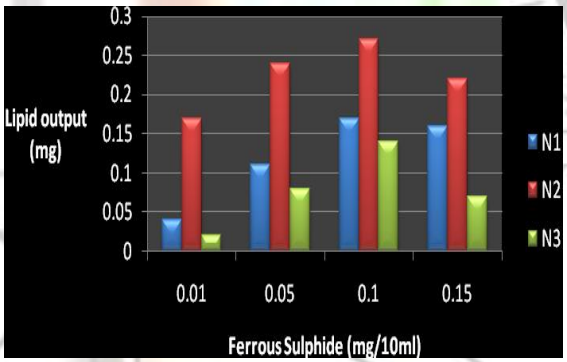

Fig. Error! No text of specified style in document.**-14: Comprehensive graph showing effect of Ferroussulphide on Strain N1, N2, N3**

Strain N1 showed better lipid output at 0.1mg/10ml concentration. Strain N2 showed better lipid output at 0.1mg/10ml concentration. Strain N3 showed better lipid output at 0.1mg/10ml concentration. Comparing the lipid output, it is seen that Strain N2 showed better lipid output with reducing agent- Ferrous Sulphide.

Titanium III citrate

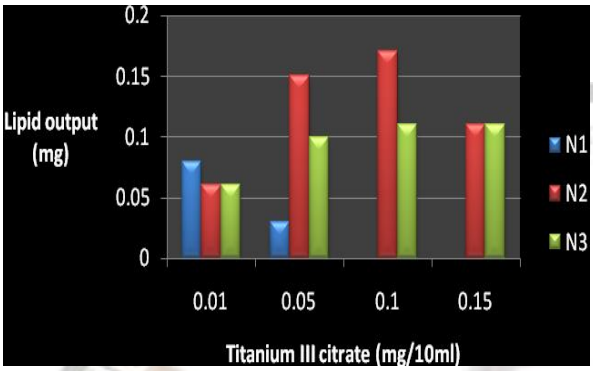

Fig. Error! No text of specified style in document.**-15: Comprehensive graph showing effect of Titanium III citrate on Strain N1, N2, N3**

Strain N1 showed better lipid output at 0.01mg/10ml concentration, while no readings were shown for the concentrations 0.1mg/10ml and 0.15mg/10ml. Strain N2 showed better lipid output at 0.1mg/10ml concentration. Strain N3 showed better lipid output at 0.1mg/10ml and 0.15mg/10ml concentration. Comparing the lipid output, it is seen that Strain N2 showed better lipid output with reducing agent-Titanium III citrate.

Acid Number

The Total Acid Number (TAN) is the amount of potassium hydroxide in milligrams that is needed to neutralize the acids in one gram of oil. High TAN [Total Acid Number] will cause: The formation of gums and lacquers on metal surfaces. Associated with increased viscosity of pumping losses. corrosion, particularly if water is present.

Strain N1

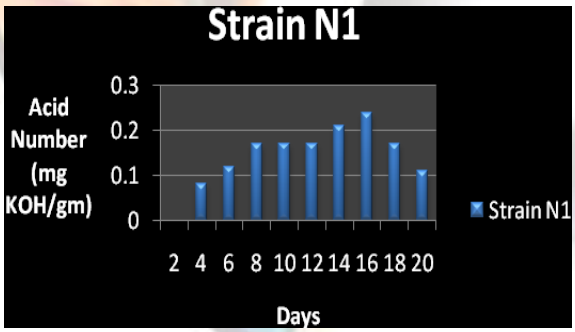

Fig. Error! No text of specified style in document.**-16: Acid number values for Strain N1**

The acid number was highest on day 16 and lowest on day 4. Day 2 showed no value.

Strain N2

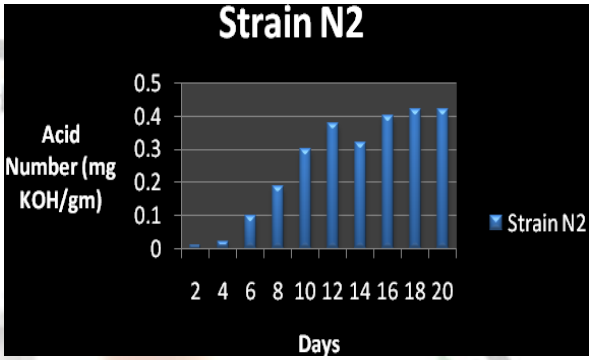

Fig. Error! No text of specified style in document.**-17: Acid number values for Strain N2**

The acid number was highest on day 18 and lowest on day 2.

Strain N3

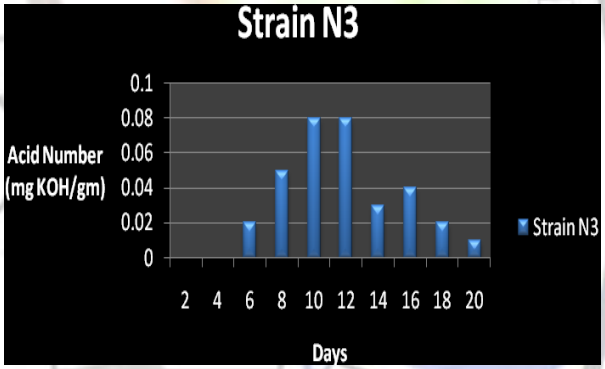

Fig. Error! No text of specified style in document.**-18: Acid number values for Strain N3**

The acid number was highest on day 10 and day12. No values were observed for day 2 and day 4.

Commercial Strain (*Corynebacteriumrubrum* [NCIM 2253] ATCC – 14898)

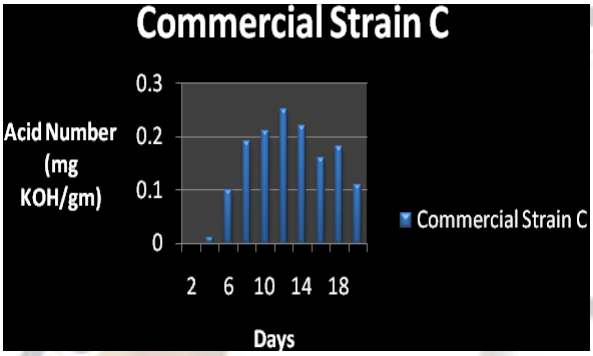

Fig. Error! No text of specified style in document.**-19: Acid number values for Commercial strain**

The acid number was highest on day 12 and lowest on day 4. Day 2 showed no value.

Comparison Graph

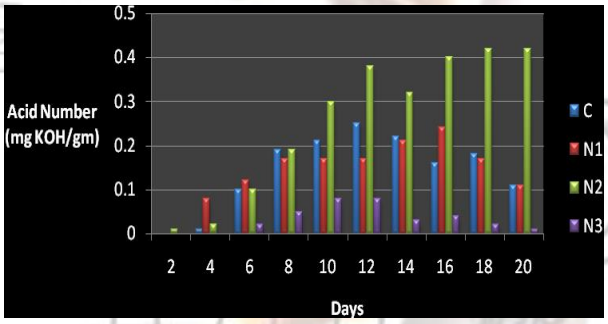

Fig. Error! No text of specified style in document.**-20: Comprehensive graph of Acid number values for Strain N1,N2,N3 and Commercial strain**

The above graph gives an idea of the Total Acid Number of all the 3 naturally isolated strains as well as the 1 commercial strain taken during different days of incubation. Acid number tests were performed on day 2, day 4 and so on till day 20 to determine the amount of potassium hydroxide consumed by certain constant amount of lipid. For strain N1, the acid number increased initially, and then followed a downward trend. It was highest on day 16. Day 2 showed no value. For strain N2, the acid number kept on increasing from day 2. It was highest on day 18 and day 20 and lowest on day 2. For strain N3, the acid number increased initially and then decreased gradually, it was highest on day 10 and day 12. No values were observed for day 2 and day 4. Even for the industrial strain, the acid number increased initially, and then followed a downward trend; it was highest on day 12. Day 2 showed no value.

Lie test values for Extracted Lipids

Lipid used	Lie Test Values [Batch I]*	Lie Test Values [Batch II]*	Lie Test Values [Batch III]*
N2M1			
[S1]	0.4	0.3	0.1
	0.3	0.3	0.3
	0.3	0.4	0.2
	Average 0.3	Average 0.3	Average 0.17
N1M1			
[S2]	0.3	0.2	0.2
	0.2	0.2	0.3
	0.2	0.2	0.4
	Average 0.23	Average 0.2	Average 0.2
N3M1			
[S3]	0.2	0.3	0.2
	0.1	0.2	0.1
	0.1	0.3	0.2
	Average 0.13	Average 0.26	Average 0.16

Batches I, II, III are duplicate batches with same media compositions and Strain type.

N2M1 – Natural Strain 2 when cultivated in Media Composition 1 showed maximum growth than other Medias.

N1M1 – Natural Strain 1 when cultivated in Media Composition 1 showed average growth in Media 1.

N3M1 – Natural Strain 3 when cultivated in Media Composition 1 showed below average growth in Media 1.

*Note: Extractions are done after 14 days

After lipid extractions, lie test was performed to determine the amount of the base catalyst (NaOH / KOH) to be added to the transesterification reaction. For lie test, the batches were incubated for 14 days. Though lie test values are used for transesterification reaction, due to time constrain, we were not able to perform the final step oftransesterification.

Conclusion

Lipid content of the three naturally isolated filamentous bacteria was performed and it was found that strain N2 produced a substantial amount of lipids which makes the strain a very potent strain for biodiesel research. This study also paves a way for further research into isolating more filamentous bacteria which have a significant amount of lipids. Further with the help of biochemical assays we can characterize the bacteria and then apply the knowledge of biochemistry and systems biology to study the lipid cycle and determine the pathways and genes associated with lipid production. With the help of bioinformatics tools, we can find the related genes involved in lipid production

in more related bacterial species and further extend the project. Also using molecular biology techniques, we can isolate the gene involved in lipid production; amplify it, so as to increase the lipid output.

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