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A study on the probabilities of the production of biodiesel fromnaturally isolated bacterial sources

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Abstract

Biodiesel is a methyl ester derived from vegetable oil. Glycerin (soap) is created by isolating triglyceride molecules from vegetable oil. Once the glycerin is removed, the molecules in the oil are functionally equivalent to petroleum diesel fuel in a diesel engine. There are a few key distinctions. There is no sulfur or ring molecules or aromatics in biodiesel, and the hydrocarbon chains are quite simple. For the last 30 years, mankind's need for freedom from these fossil fuels has propelled it toward alternatives that guarantee both fuel quality and sustainability. Thanks to these efforts, biodiesel may now be produced from algae and plants. A new alternative biodiesel source has recently come to the forefront as a solution to the many industrial and commercial issues plaguing these processes. The industrial significance of bacterial fermentations, in particular, has promised that this source may deliver a significant positive change in the biodiesel market in the next years, giving rise to a new era of biodiesel production based on bacteria. The focus of this research is on optimizing the nutritional medium to optimize lipid output for biodiesel generation, and it centers on isolating and studying several chosen bacterial strains for their capacities to create Fatty Acid based biomolecules.

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

Introduction

India has the world's second-largest population and the seventh-largest landmass. India is the fifth most oil-hungry nation because of its huge population and growing transportation needs. Diesel accounts for over 40% of India's annual petro-products consumption (1), with annual usage hovering around 40 million tons. Solar energy, thermal energy, hydro energy, and bio-energy (2) are all viable alternatives to using fossil fuels, which are becoming more depleted as their demand rises. Bio-energy stands out as the most significant and practically applicable alternative energy source. Diesel made from plants is a promising renewable resource that might help us fulfill the growing demand for petroleum goods (3, 4). Biodiesel is a substitute to diesel which is manufactured from renewable resources such as vegetable oils (or) animal fats (5).

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The majority of India's rural population relies on agriculture as their primary source of income, and many people in urban areas also work in the agricultural industry (6). Bacterial biodiesel offers an alternate revenue stream for the farming industry. Anyone, regardless of age, gender, or educational background, may achieve this if given the right guidance, investment, and space to do so, in addition to its principal source of revenue (7). Also it may be done on little and big scale (8, 9, 10). Bacterial oil is a promising replacement for petroleum-based biofuels like biodiesel and biopetrol (11). Gram-positive, rod-shaped Corynebacterium bacteria are common in the environment and seldom cause illness. Corybacterium rubrum is characterized by the fact that it is catalase positive, does not produce spores, and does not move about on its own. Phosphate is often contained in metachromatic granules. They may be anywhere from 2 and 6 micrometers long and 0.5 micrometers wide. Corynebacterium species that are not harmful to humans have several commercial uses, including the synthesis of amino acids.

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 othertype was naturally isolated filamentous bacteria. Three such natural strains were required for the study forcomparative 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.

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.

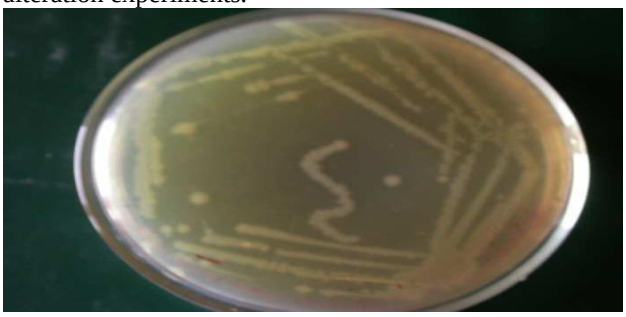

Fig. Error! No text of specified style in document.**-1: Corynebacteriumrubrum**

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

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 Pardon the pun! Document has no content formatted as indicated.-3: R2A medium-grown natural strain

After the bacterial strains were isolated and screened, they were cultured in the presence of three distinct reducing agents at four distinct concentrations, and their lipid synthesis was then evaluated over the course of their growth. Filamentous bacteria thrive in low-oxygen environments, which was exploited by the use of reducing chemicals. Since adding reducing agents before to autoclaving would have resulted in their oxidation, they were introduced afterwards. After the final experimental mixture had been incubated for a sufficient period of time to permit bacterial growth, lipid extraction was performed.

Separation of Lipids

Culture cultured in nutrient broth should be centrifuged at 4500 rpm for 4 minutes. Discard the supernatant and suspend the cell pellet in fresh nutrient broth to concentrate the cells. Do this four or five times. After adding 1ml to each eppendorf, suspend the cell pellet in 0.1% sodium Chloride and mix well. Spin for 8 minutes @ 8,000 rpm. The supernatant should be discarded, and the cell pellets should be placed in 1 or 2 eppendorf tubes. Put the pellets in a freezer overnight at -20 degrees. For every 100 milligrams of frozen biomass, 114 milliliters of solvent should be added in a serial method, with 15 seconds of shaking between additions. The solvents should be chloroform, methanol, and water in that order. Wrap the beaker in aluminum foil (to keep the solvents from evaporating) and chill it for 18 hours. Adding chloroform and water to a final chloroform/methanol/water ratio of 1/1/0.9 (v/v/v) allowed for phase separation of the biomass-solvent mixtures in the separatory funnels. Each complete lipid extract was taken in a measured amount from the chloroform-distillate phase.

Calculating the Acid Content

One gram of a material has an acid value (also known as an acid number), which is the amount of potassium hydroxide (KOH) in milligrams needed to neutralize it. The acid number of a chemical substance, such a fatty acid, indicates how many carboxylic acid groups it has. Standard practice calls for titrating a sample dissolved in an organic solvent with a solution of potassium hydroxide of known concentration and a

color indicator, such as phenolphthalein.

A sample of biodiesel, for instance, might have its acidity measured using the acid number. To neutralize the acidic components in 1 g of sample, this value represents the amount of base, in milligrams of potassium hydroxide, needed. Calculation Formula

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 thrivewell 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, thelipid content of the industrial, *Corynebacteriumrubrum*, was around 28% of originalbacterial weight.

Reducing agent- Sodium SulphideStrain N1

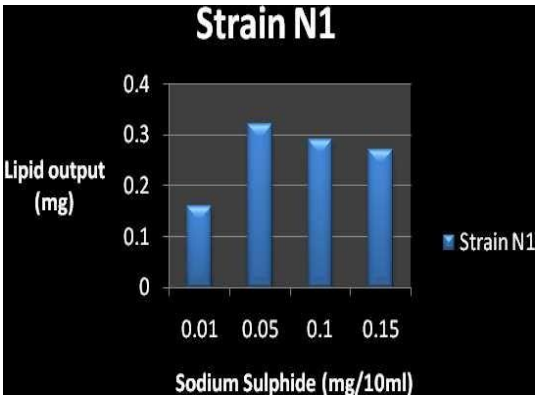

Fig. Error! No text of specified style in document.**-4: Effect of different concentrations of Sodium sulphide on Strain N1**

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

Strain N2

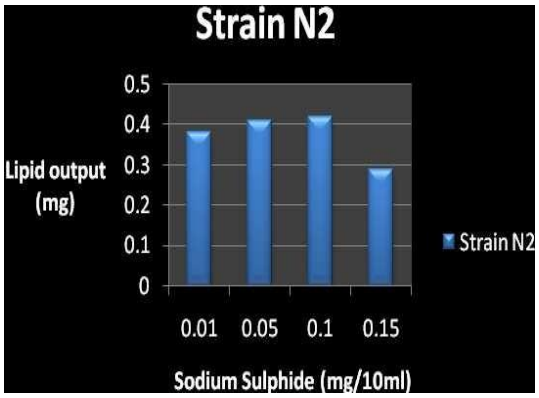

Fig. Error! No text of specified style in document.**-5: Effect of different concentrations of Sodium sulphide on Strain N2**

With Sodium sulphide as the reducing agent, Strain N2 showed maximum lipid content at concentration 0.1mg/10ml. Strain N3

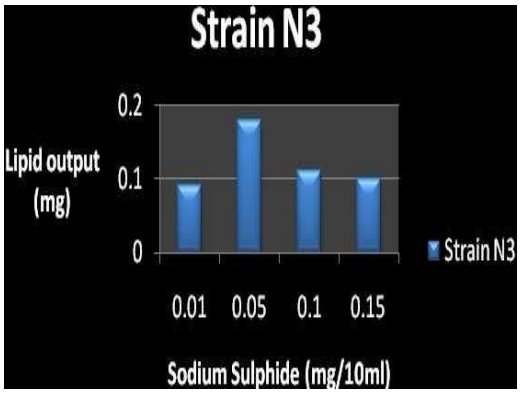

Fig. Error! No text of specified style in document.**-6: Effect of different concentrations of Sodium sulphide on Strain N3**

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 sulphideStrain N1

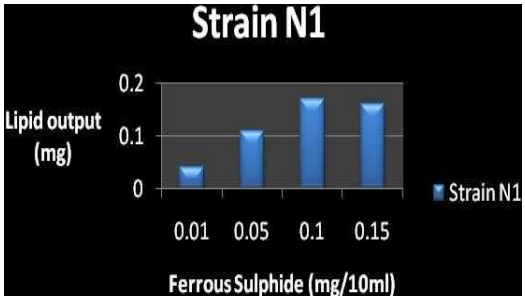

Fig. Error! No text of specified style in document.**-7: Effect of different concentrations of Ferroussulphide on Strain N1**

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

Strain N2

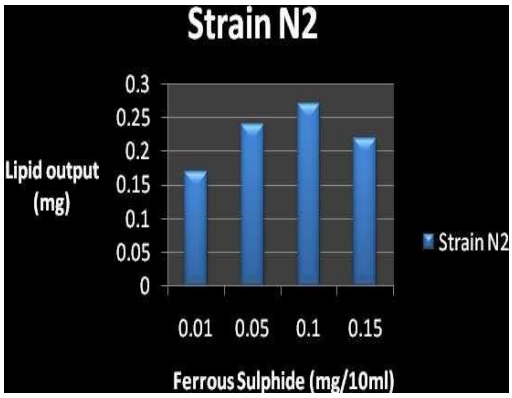

Fig. Error! No text of specified style in document.**-8: Effect of different concentrations of Ferroussulphide on Strain N2**

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

Strain N3

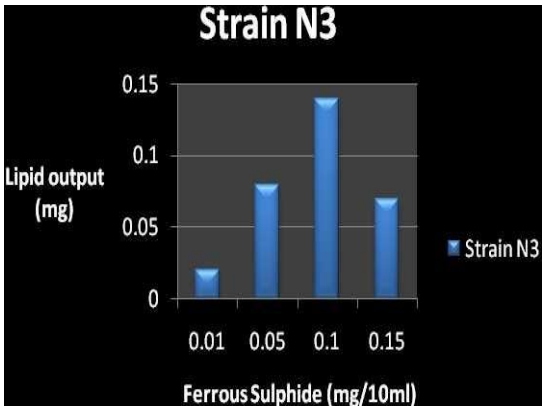

Fig. Error! No text of specified style in document.**-9: Effect of different concentrations of Ferroussulphide on Strain N3**

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 citrateStrain N1

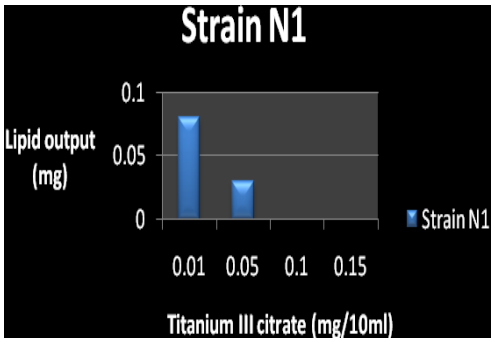

Fig. Error! No text of specified style in document.**-10: Effect of different concentrations of Titanium III citrate on Strain N1**

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

Strain N2

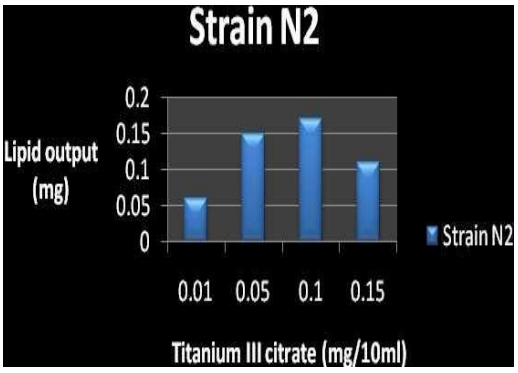

Fig. Error! No text of specified style in document.**-11: Effect of different concentrations of Titanium III citrate on Strain N2**

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

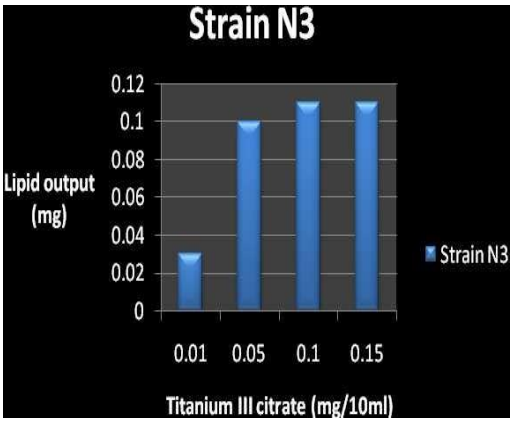

Fig. Error! No text of specified style in document.**-12: Effect of different concentrations of Titanium III citrate on Strain N3**

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

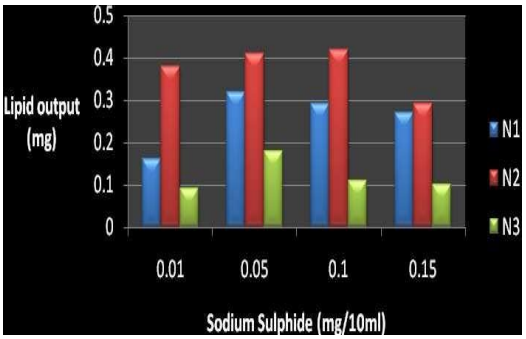

Fig. Error! No text of specified style in document.**-13: Comprehensive graph showing effect of Sodium sulphide on Strain N1, N2, N3**

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 showedmaximum 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 infilamentous 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

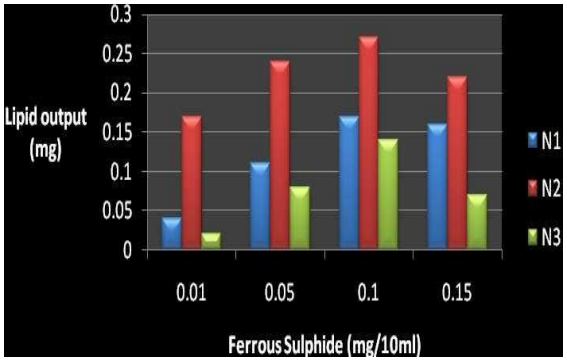

Fig. Error! No text of specified style in document.**-14: Comprehensive graph showing effect of Ferroussulphide on Strain N1, N2, N3**

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

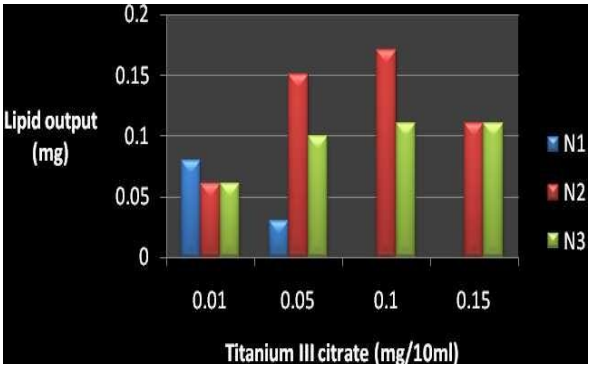

Fig. Error! No text of specified style in document.**-15: Comprehensive graph showing effect of Titanium III citrate on Strain N1, N2, N3**

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

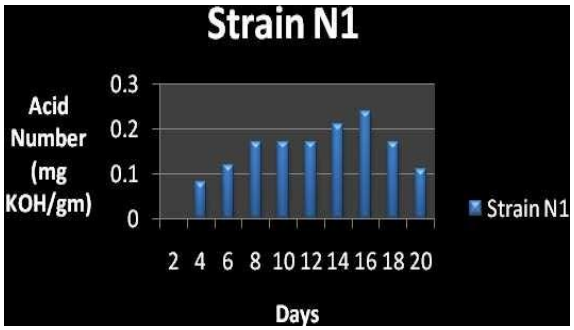

Fig. Error! No text of specified style in document.**-16: Acid number values for Strain N1**

The acid number was highest on day 16 and lowest on day 4. Day 2 showed no value. **Commercial Strain (*Corynebacteriumrubrum* [NCIM 2253] ATCC – 14898)**

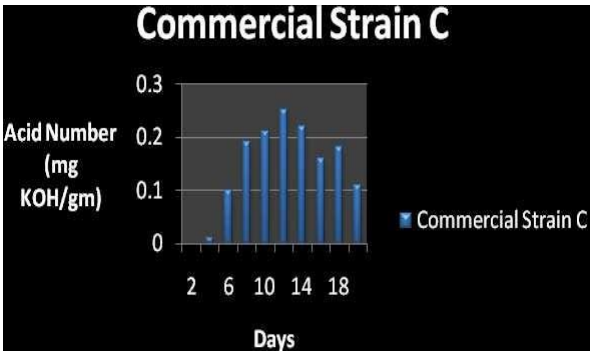

Fig. Error! No text of specified style in document.**-19: Acid number values for Commercial strain**

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

Comparison Graph

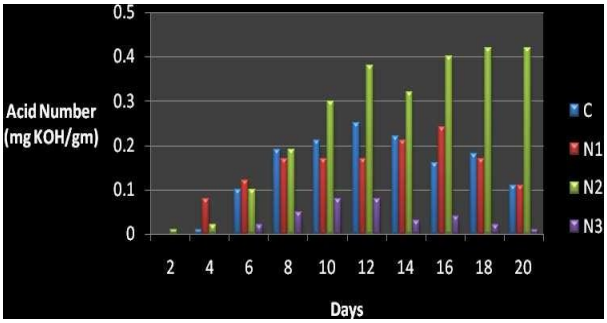

Fig. Error! No text of specified style in document.**-20: Comprehensive graph of Acid number values for Strain N1,N2,N3 and Commercial strain**

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 day2, 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 onincreasing 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 Media1.

*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

Strain N2 generated a large quantity of lipids, making it a particularly powerful strain for biodiesel research, when compared to the other two naturally isolated filamentous bacteria tested for lipid content. More research into isolating filamentous bacteria rich in lipids is made possible by this findings as well. Biochemical tests allow us to learn more about the bacteria, and then we can use what we know about biochemistry and systems biology to investigate the lipid cycle and identify the pathways and genes involved in lipid synthesis. The relevant genes involved in lipid formation may be identified with the use of bioinformatics techniques.

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