



ISSN NO. 2320-5407

Journal homepage: <http://www.journalijar.com>  
Journal DOI: [10.21474/IJAR01](https://doi.org/10.21474/IJAR01)

INTERNATIONAL JOURNAL  
OF ADVANCED RESEARCH

## RESEARCH ARTICLE

### ISOLATION OF OLEAGINOUS ENDOPHYTES FROM NATURAL SOURCES.

Rakshita Govind, Santosh Kumar Jha, \*Hare Ram Singh.

Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India.

#### Manuscript Info

##### Manuscript History:

Received: 14 April 2016  
Final Accepted: 19 May 2016  
Published Online: June 2016

##### Key words:

Microbial lipid, polyunsaturated fatty acids, oleaginous, petroleum.

##### \*Corresponding Author

Hare Ram Singh.

#### Abstract

Petroleum is a natural source of energy being largely consumed by world's population. But consumption of petro products is not only exhausting availability of crude oils but also increasing greenhouse gases and creating expensive as well as challenging waste recycling issues. Microbial lipid could be an alternative to petroleum, containing a high fraction of polyunsaturated fatty acids which could serve as the potential source of significant quantities of transportation fuels. In the present study, an attempt has been made to isolate oleaginous endophytes, the microorganisms prevailing inside the plant part that is supposed to contain lipid in abundance within them. Further characterization of the isolates has been done which would give an ideal oleaginous microorganism, useful in the production of microbial lipid, an alternative source of petroleum.

Copy Right, IJAR, 2016,. All rights reserved.

#### Introduction:-

Energy is a critical input for socio-economics development, which is growing very rapidly day by day. The development can be directly related to energy consumption, the standard of living and demand for conventional sources of energy. Even though only approx. 16% of natural or renewable energy is used worldwide (Voloshin et al., 2015), it still plays a dominant role in current socio-economics development. Among these conventional energies, petroleum is the largest fuel used up by world's population (Brennan et al., 2008). Petro products are present everywhere in the society. Global demand for petroleum is predicted to increase by up to 40% by 2025 (Subramaniam et al., 2010). But since conventional energy has some limitations like lack of abundance, non-renewable characteristics, polluting and creating expensive and challenging waste recycling issues, these conventional sources of energy are predicted to last for only next few decades. The limiting factors of conventional sources of energy have compelled the researchers to find alternative sources of energy for maintaining and developing the socio-economics scenarios.

Biodiesel fuel is comparable or equivalent to petroleum diesel, only differing in the manner of origination, i.e. deriving from biological sources. It is produced from vegetable oils (Bunyaki et al., 2006), plant oils or animal fats by transesterification with low molecular weight alcohols (Lang et al., 2001). Soybean is a major source of biodiesel but other sources must include rapeseed, canola, palm, coconut seed, sunflower, and peanut. It can also be made from recycled cooking greases. These are esters of fatty acid (Subramaniam et al., 2010). It was first demonstrated by Rudolph Diesel in 1898 at World Exhibition in Paris with peanut oil as fuel (International Energy Report, 2008). Biodiesels can be used in its pure form but generally, it is blended with standard diesel fuel. The blends are indicated with the abbreviation Bxx, where xx represents the percentage of biodiesel in the mixture. It is safe to use and can be used with slight or no modifications. It has many advantages like it reduces CO<sub>2</sub> emission by 78%, lowers the carcinogenic properties of diesel fuel by 94%, degrades four times faster than conventional diesel fuels, decreases the dependence on imported oil, contributes to engine's ease of movement, decreases the clogging problem as it acts as solvent which loosens the deposits inside engine causing clogging problems, is safe to use, is nontoxic, has higher flashpoint than conventional diesel, since it burns at higher temperature, there are fewer chances to combust accidentally, also movement and transportation become easier.

Traditionally, it is made from vegetable oil, animal fats and waste cooking oils (Liu et al., 2008). But the production of biodiesel from plants is bit problematic because of a long time and a large amount of land required for plant cultivation from which these biodiesels can be made. Using of plant-based feedstock encounters a problem that their use for biodiesel production would turn away agricultural lands from their original purpose that is food production and compete with edible oils, thus leading to food insecurity and rise in food prices. This further led the researchers to find alternative sources of biodiesel which are cheap, time saving and easy to handle. (6, 7)

Some microbes can accumulate lipid in the cell more than 20% of their dry weight in the form of triacylglycerols. These microbes are called as oleaginous microorganisms (Certik 1999). These microbes can use various agro-industrial residues like molasses (Zhu et al., 2008), methanol (Rupcic et al., 1996), orange peel (Gema et al., 2002), monosodium glutamate (Xue et al., 2006), sewage sludge (Angerbauer, 2008), starch (Papnikolaou et al., 2007) and pectin (Papnikolaou et al., 2007).

Endophytes are the microorganisms that are supposed to prevail in the plant parts without visibly harming the plant (Fisher and Pertini, 1987). These endophytes have sometimes been found to produce chemical compounds which are similar to those of the host chemicals (aly et al., 2010).

This led to the development of Biofuel production from microorganisms which are major approach towards energy independence, reducing greenhouse gas emission, revitalizing rural communities, enhancing sustainable economic development (Chunjie et al., 2011)

The present study has been focused on the isolation of those endophytes which are oleaginous from the sources which have a higher percentage of lipid content.

## **Materials and methods:-**

**Sample Collection and Isolation of Endophytes:-** Leave samples of *Anacardi Occidentale* (Cashew), *Brassica* (Mustard), *Cymbopogon* (Citronella) were collected from BIT Medicinal Garden in a sterile plastic bag and brought to the laboratory. Processing of the sample was started within 2-3 hours of arrival. Rest of the sample was kept at 4°C for further use.

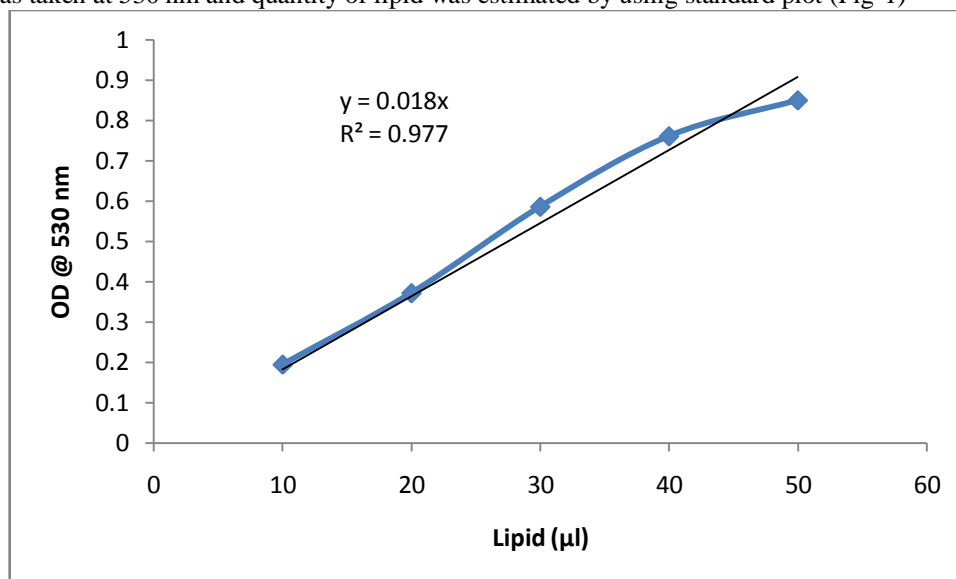
The isolation of endophytes was carried out using spread plate technique for which surface sterilization was done by cutting the sample into small pieces and washed with tap water, distilled and finally rinsed with Bavistine. Only one sample was handled at a time. Then samples were transferred to Laminar Air Flow and washed with chemicals 70% ethanol, 0.01%  $\text{HgCl}_2$  and 4%  $\text{NaOCl}$  for 30 seconds, 5 minutes and 2-3 minutes respectively in different sterile beakers (Ahmad et al., 2012). After every chemical wash, the samples were subsequently washed with sterile distilled water in separate sterile beakers. Once surface sterilization was done, the sides of the leaves were cut with sterile scalpel and forceps in a petriplate. Then these leaf pieces were picked up with forceps and placed on the Terrific Agar plates for 10 minutes. The plates were marked as control. Thereafter, leaf pieces placed on control plates were transferred to a mortar with the addition of 3 to 4 ml of sterile distilled water and crushed finely with the help of pestle. 200  $\mu\text{l}$  of this aqueous solution was poured on Terrific Agar plate and spread uniformly (Costa et al., 2012). This process was repeated for each of the samples. Control as well as test plates, both were incubated at 37°C for 24 to 74 hours. The selected colonies were streaked on slants for further use.

**Cell Lysis:-** For extracting the lipid, present within the cells, the cells were harvested by growing in nutrient broth medium and centrifuged. The pellets that were obtained were weighed and dissolved in chloroform. Thereafter, cells were lysed by the ultra-sonication method and subjected to centrifugation (Shilpa et al., 2012).

**Biochemical Characterization:-** The obtained endophytic colonies were characterized for cultural characteristic (on the basis of color, margin, texture, elevation and opacity of the colony), morphological characteristics (Grams staining was done and slides were observed under light microscope under 10X, 40X and 100X magnification so as to characterize on the basis of Grams reaction) and different biochemical tests were performed (Catalase Test, Methyl Red Test, Voges-Proskauer Test, Citrate Utilization Test, Hydrogen Sulphide Production Test, Nitrate Production Test, Starch Hydrolysis Test, Sugar Fermentation Test).

**Lipid Estimation:-** The lipid estimation was carried out using phosphovallin method. For this, 20  $\mu\text{l}$  of the samples were taken in different clean, dry test tubes. Then, 0.2 ml of  $\text{H}_2\text{SO}_4$  was added to each test tube. Tubes were mixed

well on vortex for 10 minutes. Then samples were cooled in water at room temperature. Thereafter, 10 ml of phosphovallinin was added to each tube and incubated for 10 minutes in water bath at 60° C. Finally, Optical density of samples was taken at 530 nm and quantity of lipid was estimated by using standard plot (Fig-1)



**Fig-1:-** Standard plot for estimation of quantity of lipid.

### Result and discussion:-

A total of 4 colonies were obtained on cashew test plate, 2 on Citronella plate, and 35 on Mustard test plate. Out of them, 1 colony was selected from both Cashew and Citronella and 2 from Mustard plates for further studies and characterization. The isolates were named as Cashew, Citronella, Col A, Col B. isolates.

The cultural characteristics of the obtained endophytes were studied and classified on the basis of color, texture, margin, shape, elevation, opacity. The observation has been tabulated in Table-1. The Gram's Reaction and the morphological characteristics of bacterial isolates were studied by preparing slides and observing under a light microscope at 10X, 20X, and 100X magnification (Table-2).

On the basis of biochemical tests (Table-3), the isolated endophytes were identified using ABIS Online Software. The isolate obtained from cashew isolate was identified as *Solibacillus silvestris* (88% identified by ABIS Online). The one isolated from citronella leaves *Paenibacillus curdlanolyticus* (88% identified by ABIS Online). From the mustard plant, two colonies were isolated. One was identified as *Bacillus megaterium* (68% identified by ABIS Online) and another colony was identified as *Paenibacillus jamilae* (73% identified by ABIS Online).

The lipid content in isolated endophytes was estimated in terms of by mass percentage composition. Among all the four endophytes, Cashew Isolates has highest lipid content of 30.58% (w/w) and Citronella Isolates having lowest lipid content of 0.039% (w/w). Col A and Col B having lipid content of 1.574% (w/w) and 12.87% (w/w) respectively. Since the lipid content of Cashew Isolates having higher than the 20% so it may be considered as an oleaginous endophyte.

**Table 1:-** Cultural characteristics of the isolated endophytes.

S. No.	Characteristics	Cashew Isolates	Citronella Isolates	Col A	Col B
1	Color	White	Creamy Whitish	Creamy Whitish	Cream
2	Texture	Smooth	Smooth	Smooth	Glossy and granular
3	Margin	Slight Undulate	Entire	Entire	Slimy
4	Shape	Circular	Circular	Circular	Circular
5	Elevation	Flat	Flat	Raised	Flat
6	Opacity	Opaque	Opaque	Opaque	Translucent

**Table 2:-** Gram's Reaction and morphological characteristics of the isolated endophytes.

Sample	Grams Reaction	Shape	Arrangement
Cashew Isolates	-ve	Cocci	In chain
Citronella Isolates	+ve	Cocci	Single
Col A	-ve	Rod	In chain
Col B	-ve	Cocci	In chain

**Table 3:-** Biochemical characteristics of isolated endophytes

S.No.	Biochemical Tests	Cashew Isolates	Citronella Isolates	Mustard Col. A	Mustard Col. B
1.	Starch Hydrolysis	-ve	+ve	+ve	+ve
2.	Nitrate Test	+ve	+ve	+ve	-ve
3.	Methyl Red Test	+ve	+ve	+ve	+ve
4.	Voges Proskeur Test	-ve	-ve	-ve	-ve
5.	H <sub>2</sub> S production Test	-ve	-ve	-ve	-ve
6.	Citrate Utilization Test	-ve	-ve	+ve	-ve
7.	Caesin Hydrolysis Test	-ve	+ve	+ve	+ve
8.	Catalase Test	-ve	-ve	-ve	-ve
9.	Lactose Fermentation Test	+ve	-ve	-ve	-ve

**Table 4:-** Lipid content in the isolated endophytes.

Sample	Lipid Content (w/w)
Cashew Isolates	30.58%
Citronella Isolates	0.039%
Col A	1.574%
Col B	12.87%

**Conclusion:-**

Oleaginous endophytes may be a promising alternative for the production of microbial lipid. In present study the endophytes isolated from the Cashew plants having higher lipid content of 30.58% so, it may be claimed as an oleaginous. The 16S rDNA based identification may further confirm the nature of this oleaginous endophytes.

**Acknowledgement:-**

Authors highly acknowledge the support from the Centre of Excellence (COE), TEQIP, Phase II (Ref NO. NPIU/TEQIP II/FIN/31/158; dated 16th April, 2013). The authors also gratefully acknowledge the department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi for providing facilities.

**References:-**

1. Ahmed, M., Hussain, M., Dhar, M.K. and Kaul, S. (2012): Isolation of microbial endophytes from some ethnomedicinal plants of Jammu and Kashmir. *Journal Nature Production and Plant Resource*. 2 :215-220
2. Aly, A.H., Debbab, A., Kjer, J. and Proksch, P. (2010): Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Divers*. 41(1): 1-16
3. Angerbauer, C., Siebenhofer, M., Mittelbach, M. and Guebitz, G.M. (2008): Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. *Bioresour. Technol*. 99:3051-3056.
4. Bunyakiat, K., Makmee, S., Sawangkeaw, R. and Ngamprasertsith S. (2006): Continuous production of biodiesel via transesterification from vegetable oil supercritical methanol. *Energy Fuels* 20:812-817
5. Certik, M. (1999): Effect of nitrogen sources on the activities of lipogenic enzymes in oleaginous fungus *Cunninghamella echinulata*. *Appl. Microbiol* 45:289-293.
6. Costa, L.E.O., Queiroz, M.V., Borges A.C., Moraes, C.A. and Araújo E.F. (2012): Isolation and characterization of endophytic bacteria from the leaves of the common bean (*Phaseolus vulgaris*). *Brazilian Journal of Microbiology*: 1562-1575
7. Fisher, P.J. and Petrini O. (1987): Location of fungal endophytes in tissues of *Suaeda fruticosa*: a preliminary study. *Trans. Br. Mycol. Soc.* 89:246-249.

8. Gema, H., Kavadia, A., Dimou, D., Tsagou, V., Komatis, M. and Aggelis, G. (2002): Production of c-linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel. *Appl. Microbiol., Biotechnol.* 58:303-307.
9. International Energy Outlook (2005)
10. Lang, X., Dalai, A.K., Bakshi, N.N., Reaney, M.J. and Hertz P.B. (2001): Preparation and characterization of biodiesels from various bio-oils. *Bioresources Technol.* 80: 53-62
11. Liu, Y.P., Zheng, P., Sun, Z.H., Ni, Y., Dong, J.J. and Zhu, L.L. (2008): Economical succinic acid production from cane molasses by *Actinobacillus succinogenes*. *Bioresour. Technol.* 99:1736-1742
12. Papanikolaou, S., Galiotou-Panayotou, M., Fakas, S., Komaitis, M. and Aggelis, G. (2007): Lipid production by oleaginous *Mucorales* cultivated on renewable carbon sources. *Eur. J. Lipid Sci. Technol.* 109:1060-1070.
13. Rupcic, J., Blagovic, B. and Maric, V. (1996): Cell lipids of *Candida lipolytica* grown on methanol. *J Chromatogr. A* 755: 75-80
14. Shilpa S, Jayakar, Rekha, Singhal, S. (2012): Development of efficient cell disruption method for release of lipoic acid from *Saccharomyces cerevisiae*. *Global Journal of Biotechnology & Biochemistry* 7(4): 90-99.
15. Subramaniam, R., Dufreche, S., Zappi, M. and Bajpai, R. (2010): Microbial lipids from renewable resources: production and characterization. *Journal Ind Microbiology Biotechnology* 37:1271–1287
16. Voloshin, R.A., Kreslavski, V.D., Zharmukhamedov, S.K., Bedbenov, V.S., Ramakrishna, S. and Allakhverdiev, S.I. (2015): Photoelectrochemical cells based on photosynthetic systems: a review. *Biofuel Research Journal* 6: 227-235.
17. Xue, F., Zhang, X., Luo, H., Tan, T. (2006): A new method for preparing raw material for biodiesel production. *Process Biochem.* 41(7):1699-1702.
18. Zhu, L., Zong, M. and Wu, H. (2008): Efficient lipid production with *Trichosporon* fermentans and its uses for biodiesel preparation. *Bioresour. Technol.* 99(16): 7881-7885.