Yeast as a Viable and Prolonged Feedstock for Biodiesel Production

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Abstract-Demand of alternative fuels is increasing day by day due to the present crisis of petroleum based fuels. Biodiesel is one of the most demanding alternative fuel, which is produced either by animal or plant based feedstock. Both types of feedstocks are facing problem of constant availability in sufficient quantity for prolonged time period. This problem could be solved, if a source having higher lipid content is found in sufficient quantity. One probable solution is to use microorganisms; especially oleaginous species, because of their higher lipid content and almost similar composition as plant/animal lipid. For this experiment, yeast was selected because of several reasons like easy availability, rapid growth rate, higher lipid accumulation capacity, capable to grow on a variety of media etc. In this experiment, yeast was adapted to accumulate maximum quantity of lipids by providing metabolic stress condition, which was later converted into biodiesel by acid transesterification. Elimination of lipid extraction step has made the process much faster and easier as compared to traditional methods. Presence of fatty acid methyl esters were confirmed by high performance thin layer chromatography (HPTLC) and gas chromatography (GC). Fatty acids composition was determined by gas chromatography (GC) by comparing with standards.

Keywords-economic production of biodiesel, yeast biomass, single step transesterification method, prolonged feedstock.

1. Introduction

Biodiesel, a non-petroleum based diesel fuel could be defined as alkyl esters of long chain fatty acids. In the principle process of biodiesel production which is also known as transesterification, lipids (mainly triglycerides, TG) are converted into fatty acid methyl/ethyl esters (FAME/FAEE) in the presence of suitable catalyst and alcohols. These catalysts may be either chemical or enzymatic.[1-5] Major feedstocks used for biodiesel production are based on either animal or plant sources. Common plant feedstocks involve rapeseed oil, soybean oil and jatropha oil, while chicken fat and fish oil are mostly used animal based feedstocks.[2], [5-8] Problem with these kinds of sources are their limited availability which becomes a major limiting factor in biodiesel production. Now a days, scientists have also focused on algae(micro and macro) and genetically modified microorganisms as an alternative feedstock due to limited availability of other sources.[9-11] It is assumed that, as this biodiesel is produced from biological feedstocks, it is renewable, eco-friendly and biodegradable. Reference have also shown that this kind of biodiesel contains less sulfur as well as CO and it also lacks polyaromatic hydrocarbons as present in petroleum diesel [2].

As mentioned before, major obstacles with plant and animal based sources are their higher cost and lesser availability. Cost could be reduced by using comparatively cheaper feedstocks like waste oils and greases but again availability is a major problem.[5], [12] To solve this problem, scientists have focused on the oleaginous microorganisms, as it is possible to produce sufficient biomass by using fermentors and ultimately production of higher quantity of lipids in the form of stored lipids within the cell.[13] Other advantages also include smaller area for production as compared to the plants, easy and rapid oil extraction methods and ability to grow on a wide variety of media. Yeast, fungi and certain microalgae are examples for

such microorganisms.[13], [14] Major lipid components found in yeast and fungi are triglycerides having C16 and C18 long chain fatty acids, which are very similar to the vegetable oil.[15-17] Under normal growth conditions, microorganisms do not accumulate higher quantity of lipid, but their ability could be enhanced by providing metabolic stress conditions.[16-18] One of the most common and widely used methods is to provide limited supply of nitrogen with excess carbon source. Under nitrogen limiting condition, replication of yeast is inhibited after certain growth cycles and excess sugar is accumulated in the form of triglycerides within cell as reserved energy source.[16], [19]

In the present study, yeast was isolated and adapted for accumulation of lipids by providing metabolic stress conditions. Yeast biomass was directly used for acid transesterification without prior lipid extraction, which is an essential step for both animal and plant based sources. Presence of fatty acid methyl esters was determined by HPTLC and GC.

2. Materials and Methods

2.1. Reagents and Chemical

All the reagents were of AR grade. Methanol and n-hexane were purchased from Merck Ltd., India. Phosphomolybdic acid and standard FAME were purchased from Sigma Aldrich, USA. All the other chemicals were purchased from Himedia Laboratories Pvt. Ltd, India.

2.2. Sample Collection

Marine water samples were collected from "The Bay of Khambhat", Gujarat, India in autoclaved bottles and were transferred to the laboratory under cold condition. Samples were immediately preceded for experiment in the laboratory.

2.3. Isolation and Identification of Yeast

The samples were serially diluted and spreaded on GYE agar (Glucose - 2.0%, Yeast Extract - 1.0%, peptone - 1.0%, pH-6.0). Plates were incubated at 25˚C for 48 hrs. Selected colonies were further spreaded on GYE plates to obtain pure culture. Differential media viz. Molybdate Agar and Bismuth Sulphite Glucose Glycine Yeast (BiGGY) Agar were used for primary identification of yeast.(Bump and Kunj (1968), Rale and Vakil (1884) and Atlas and Parks (1993)) For confirmation, identification of microorganism with 18s rRNA sequencing was done.

2.4. Adaptation for Lipid Accumulation

From previous studies, it was found that yeast are able to accumulate lipids in the form of triglycerides(storage lipid) if they are grown in media containing less nitrogen and higher carbon.[16], [17], [20] So a specialized media was designed in the laboratory for the experiment in which optimum concentration of glucose, inorganic nitrogen and organic nitrogen was determined, which favors maximum lipid accumulation. Effect of incubation time on lipid accumulation was also determined. Fermentation was initiated by inoculating 10% of 24hrs old culture grown in GYE broth to the specialized media.

2.5. Extraction of Lipid

Lipid was extracted only for comparative analysis in HPTLC and to calculate percentage conversion of total lipids into biodiesel. After 5 days of incubation on a rotary shaker at 200 rpm at 25˚C, produced biomass was collected by centrifugation at 8500 rpm for 20mins. The pellet was washed twice with distilled water and used for lipid extraction. Blight and dyer method with some modifications was used for lipid extraction[21]. In the process, cell suspension was prepared by suspending known quantity of biomass in known volume of sterile distilled water. To this, mixture of chloroform: methanol (1:2) (3.75mL/mL of suspension) was added and vortex atleast for 15mins. Then 1.5mL of chloroform/mL of suspension was added and mixed for 2 minutes. In the next step, 1.5mL of distilled water/mL was added and mixed. After centrifugation at 8000 rpm for 10mins, lower solvent phase was collected and dried at room temperature. The residue was suspended in known volume of chloroform: methanol (2:1) and stored at 4 -8 ˚C.

2.6. Direct Transesterification

Among the various methods of transesterification, acid transesterification was chosen for production of FAMEs. Acid transesterification was selected because studies of Demirbas (2008) and Fallon et al. (2007) have shown that, it is possible to convert variety of lipids into their respective esters by acid transesterification without prior extraction. In this modified process, dried yeast biomass was mixed with 20 volume of methanol containing concentrated sulfuric acid to a final concentration of 0.02 mol/L. Reaction was carried out in a tightly closed glass bottle at 70˚ C with constant stirring for 24 hrs. At the end of reaction, suspension was cooled and filtered. To the filtrate, hexane was added to recover the FAMEs. Upper hexane layer containing FAMEs was collected and used for various analyses. Similar procedure was used to produce FAMEs from yeast grown under normal condition to compare FAMEs composition produced by yeast grown under nitrogen limiting media.

2.7. HPTLC Analysis

HPTLC was carried out by using CAMAG V instrument (Germany). Silica Gel G 254 plates were used as stationary phase, on which a known quantity of previously extracted yeast lipids and esterified product were applied. The plate was developed in hexane:diethyl ether(9:1). Plate was stained with phosphomolybdic acid (5% in ethanol) and heated at 105˚C until the spots were visualized. Stained plates were scanned using CAMAG V scanner at 534 nm.

2.8. Gas Chromatography Analysis

GC analysis of methyl esters of fatty acids produced by yeast biomass grown under normal as well as metabolic stress conditions were carried out in Shimadzu GC -2014, (Restek 1 capillary column 15 meters length (0.25micron id), temp. range 150-280oC, FID Detector, detection temp. 300˚C, injection volume-1 µL with spilt ratio 1:10).

3. Results and Discussion

3.1. Colony Identification

After 48hrs of incubation, smooth medium olive colonies with olive bottom appeared on Molybdate Agar and on BIGGY's agar media, black to brown colonies with no pigment diffusion were observed, both of these were indication of presence of *Candida species.*[23], [24] Pigment formation of colony is a function of reduction of particular constituent present in the media. Reduction of such compounds leads to formation of certain colour complexes which reveal the identity of certain microorganisms. Hence, it could be used for primitive identification of microbes. Molybdate and bismuth are examples of such compounds. 18s rRNA sequence have confirmed the yeast as *Candida albicans.*

3.2. Media Standardization and Lipid Accumulation

As a result of media standardization, it was found that media containing glucose - 70.0 gm/lit, ammonium sulfate - 0.1 gm/lit, magnesium sulfate - 1.5 gm/lit, potassium dihydrogen phosphate - 0.4 gm/lit, yeast extract - 0.70 gm/lit, (pH - 6.0) has favored maximum lipid accumulation.(Figure 1-3) This is because under nitrogen limiting condition yeast are not able to produce certain enzymes, reproductive enzymes are among them [17]. This unable yeast to reproduce and it tends to absorb glucose from the surrounding media, which is converted in fat and stored in the cell as reserve energy sources [19], [20]. When cultures were grown in media containing higher concentration of inorganic and organic nitrogen source, a rapid decrease in lipid accumulation was seen.(Figure. 2 and. 3) No doubt biomass obtained at higher concentration was higher but lipid content was lower. Reason behind this is availability of sufficient quantity of nitrogen for production of reproductive enzymes which leads to increase in the biomass rather than accumulation of lipids [16], [20]. In term of stress level, lower concentration of nitrogen salt creates higher metabolic stress condition in the microbes which triggers accumulation of lipids in cell much faster than the higher nitrogen concentration where the stress level is comparatively low.

Not only media constituents but also, incubation time has shown significant effect on lipid accumulation. It was found that, in the initial phase there was very low rate of accumulation but after 72hrs rate increased significantly and reached maximum at 120hrs. After 120hrs, lipid content was reduced gradually.(Figure. 4) In the initial phase of growth, sufficient quantity of nitrogen molecules were present required for cell division, so cells tends to divide rather than accumulation of lipids but after certain cycles microbes starts facing stress condition which has triggered lipid accumulation [16], [20]. At very late phase of growth cycle, lipid quantity of lipid decreases because cells have started using stored lipids to fulfill their energy requirement to survive.

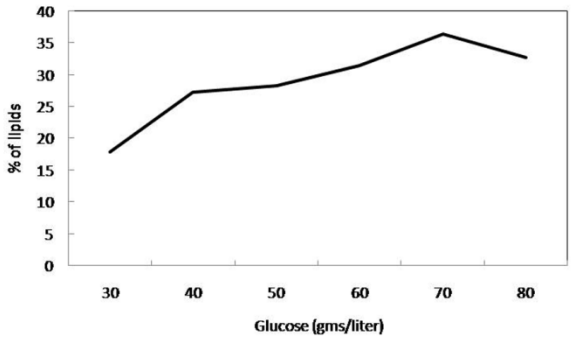

Fig. 1. Effect of glucose concentration on lipid accumulation (Optimum concentration of glucose was 70.0 gm/liter. Above this concentration microbes were not able to accumulate lipids in higher quantity.)

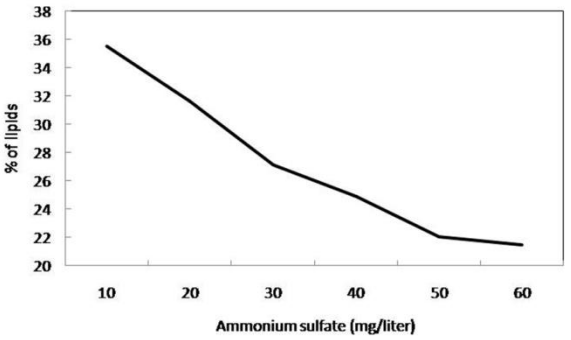

Fig. 2. Effect of ammonium sulfate concentration on lipid accumulation

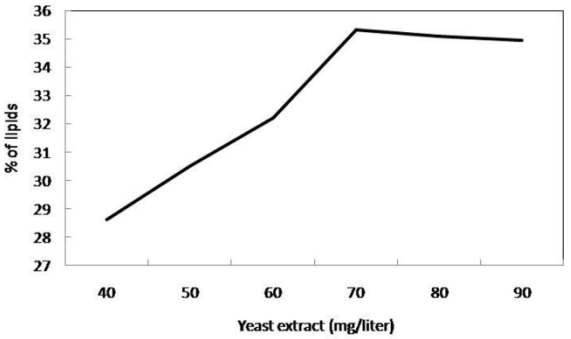

Fig. 3. Effect of yeast extracts concentration on lipid accumulation (Figure 2 and 3 : Gradual decrease in the lipid accumulation capacity was noticed as the concentrations of nitrogen sources both organic and inorganic were increased. Optimum concentration for ammonium sulfate was only 10mg/liter and for yeast extract it is 70mg/liter.)

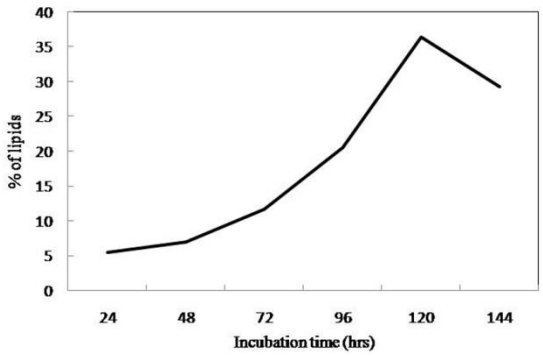

Fig. 4. Effect of incubation time on lipid accumulation (In the initial phase of incubation rate of accumulation was very low, which was increased significantly after 72hrs and reached maximum at120 hrs. After 120hrs of incubation gradual decrease in lipid accumulation was found.)

In this experiment, yeast was successfully adapted to accumulated lipids up to 37% of its dry weight, which was very higher than normal which is around 5% only.[25], [26] This quantity is also similar to Jatropha which has on an average 35% of total lipids. Because of higher growth rate of yeast; it is also possible to produce sufficient quantities of biomass using fermentors within shorter period of time to resolve the problem of availability.

3.3. HPTLC Analysis

Many previous studies have suggested that lipids could be easily separated on the basis of their chemical nature.[27], [28] Keeping polarity in mind, only non-polar solvent system was used for separation of non-polar compounds i.e. FAMEs, triglycerides, diglycerides and monoglycerides. FAMEs being highly non-polar run fastest with Rf value 0.91 followed by triglyceride, diglycerides and monoglycerides (Figure. 5).

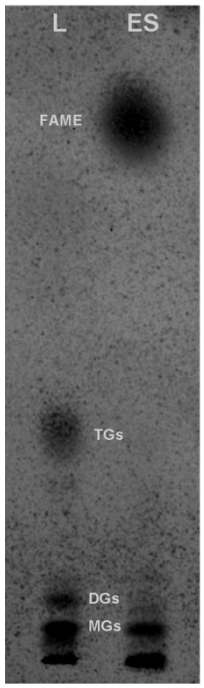

Fig. 5. HPTLC analysis of yeast lipid and yeast biodiesel (TGs: triglycerides, DGs: diacylglycerides, MGs: Monoacylglycerides, FAMES: fatty acid methyl esters, L: Yeast lipid, Es: esterified product)(Figure indicates that, all the TGs were converted into FAMEs resulted into absence of spot of TGs in ES lane. Another important observation was lower intensity of DGs and MGs in ES lane as compared to L lane, which indicates a few quantity of DGs and MGs were also been converted in to FAMEs.)

Similar kind of results were obtained by Yunoki et al(2004) while working on fatty acid esters production from potato pulp by lactic acid producing fungus. Freeman and West(1966) have noted that addition of smaller quantity of acetic acid favors separation of polar compounds and lowers the rate of migration of non-polar compounds. It was found that almost 90% of total lipids (w/w) were converted into FAMEs. Another important observation was not only the triglycerides but also some quantity of diglycerides and monoglycerides were converted into fatty acid methyl esters during the reaction, resulted into reduction of band intensity. (Figure. 4) This suggests that diglycerides and monoglycerides could also be used for production of biodiesel. But again, it is very difficult to get enough quantity of diglycerides and monoglycerides for production of biodiesel.

3.4. GC Analysis

Non-polar capillary column gives separation of compounds on the basis of their boiling point and degree of unsaturation.[29], [30] Here, unsaturated fatty acids methyl esters elute faster than the respective saturated fatty acids methyl esters. When product was compared with standard, it was found that methyl esters of fatty acids produced from yeast grown under normal condition gave only four prominent peaks of C16:0, C16:1, C 18:0 and C18:1, while product produced by yeast grown in metabolic stress condition have given peaks of C16:0, C16:1, C18:0, C18:1, C18:2, C20:0 and C22:0 fatty acid methyl esters along with many other peaks of structural lipids.(Figure. 6) This variation in lipid composition was because of lower concentration or absence of certain lipids during normal growth cycle which were not easily detectable with gas chromatography. But when the cells were forced to accumulate lipids, the concentration of these lipids had increased upto detectable concentration. The composition of biodiesel produced by yeast has very similar composition as compared to the biodiesel produced from either plant or animal based feedstocks, where C16 and C18 fatty acid chains are prominent [2], [6]. Many other peaks were also observed apart from these peaks which may be of some structural fatty acids as whole cell was used for transesterification. Results also suggested that longer stationary phase favors the production of saturated fatty acids as compared to unsaturated fatty acids in presence of excess carbon and sufficient aeration. Brown and Rose (1969) have noted that temperature and dissolved oxygen has great effect on fatty acid composition of cells. According to them extremely lower temperature (< 20ºC) favors accumulation of unsaturated fatty acid while temperature above that favors saturated fatty acids. Not only these, they have also shown that limited concentration of nitrogen leads to accumulation of mainly C16 and C18 fatty acids within cell. [31]

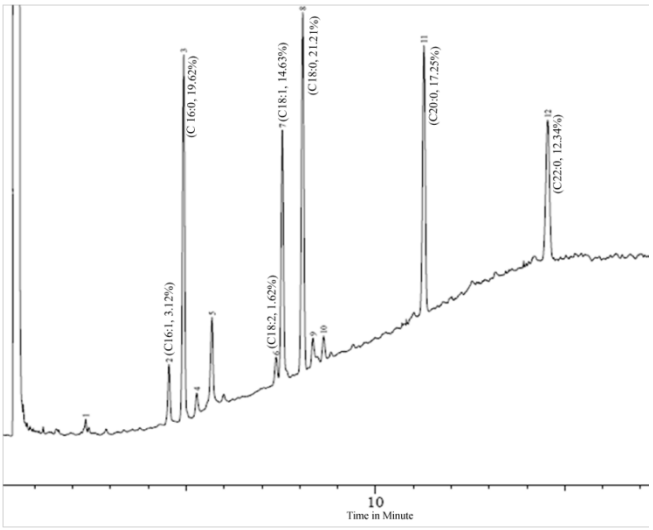

Fig. 6. GC Analysis (GC analysis indicates presence of many fatty acid methyl esters with different concentrations. Chain length and percentage concentration are mentioned in the figure along with degree of unsaturation. Rests of the peaks were may be of structural lipids as whole biomass was used for transesterification.)

From all the results obtained in this study it was found that yeast could be one of the most preferable and viable sources for biodiesel production. Rapid growth rate of yeast is able the problem of feedstock availability. Direct transesterification and single step purification of biomass makes the process faster and easier. Similar composition of fatty acid esters makes it more compatible to the biodiesel produced from others sources.

4. Abbreviations

TG : triglycerides, DG : diglycerides, MG : monoglycerides, FAME : fatty acid methyl ester, HPTLC : high performance thin layer chromatography, GC : gas chromatography,

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