Discussion

Catalysts for Transesterification

The transesterification process is catalyzed by alkalis, acids or enzymes. However, the use of alkali catalysts is 100% in commercial sector. The most common alkaline catalysts are sodium hydroxide (NaOH) and potassium hydroxide (KOH) (Schuchardt *et al.,* 1998; Marchetti *et al.,* 2008; Robles *et al.,* 2009). Other alkaline catalysts include carbonates, methoxide, sodium ethoxide, sodium prop oxide and sodium butoxide (Fukuda *et al.,* 2001). These chemicals proved to be the most economic because of higher conversion rate of esters under a low temperature and pressure environment and short reaction time (Leung *et al.,* 2010). The main drawback of the technology is the sensitivity of alkaline catalysts with respects to feedstock purity. The presence of free fatty acids and water in the feedstock has a significant impact on the transesterification process besides the multi step purification of end products. Alkaline transesterification requires treatment for the waste water that is produced from the process (Ghaly et al., 2010)

The amount of waste produced is approximately 0.2 ton per ton biodiesel produced. The need for extensive downstream processing makes alkaline transesterification expensive and not environmentally friendly (Fjerbaek *et al.,* 2009).

The second commercially used catalysts are acid-catalysts. The most commonly employed acids are: Sulfuric acid, hydrochloric acid and sulfonic adic. Despite the fact that yield is very high and no soap formations, the corrosive nature of acid, very slow reaction rate and higher temperature conditions limit the use of the technology for esterrification reactions (Freedman *et al.,* 1984; Nacovsky *et al.,* 2007).

The present study revealed that for Base catalyst KOH was suitable than NaOH and acid-base catalyzation using KOH, though proved to be good, yield of biodiesel was better in base method (Table 29a, 29b, figure 4a, 4b, 5a, 5b).

The acid and alkali transesterification processes are energy intensive and require extensive downstream processing (Xu and Wu, 2003). Post treatments are required after the completion of transesterification reaction as the end products are a mixture of esters, glycerol, mono-and diacylglycerols, pigments, unreacted alcohol, catalyst and tri, di and monoglycerides. These post treatment include a multi-step purification of end products which include: (a) separation of glycerol by gravitational settling or centrifugation, (b) neutralization of the catalyst, (c) deodorization and (d) removal of pigments (Antczak *et al.,* 2009; Banerjee and Chakraborty, 2009).

Enzymatic transesterification is, therefore, an attractive method for biodiesel production over chemical methods because of the reduced feedstock limitations, downstream processing and environmental impact (Jegannathan *et al.,* 2008). The use of enzyme catalysts eliminates these problems associated with acid and alkali catalysts as well as presents other production benefits.

Unlike the alkaline catalysts, enzymes do not form soaps so there is no restriction on free fatty acid content (Harding *et al.,* 2007; Fjerbaek *et al.,* 2009). Unlike the acid catalysts, enzymes are not severely inhibited by water, so there is little concern about water production (Dizge and Keskinler, 2008). Since the enzymes are capable of completely converting free fatty acids to FAAEs, low cost feedstocks such as waste oils and lard can be used (Fukuda *et al.*, 2001). The enzymes are most often immobilized

when used which simplifies the separation of products, produces a high quality glycerol and allows for the reuse of the catalyst (Akoh *et al.,* 2007; Robles *et al.,* 2009).

Lipases as biocatalysts:

Lipases (triacylglycerol acyhydrolases, EC 3.1.1.3) constitute a diverse and ubiquitous family of enzymes which are produced by animals, plants and microorganisms. The animal lipase most commonly used is the pancreatic lipase. Plant lipases include papaya lates, oat seed lipase and castor seed lipase (Akoh *et al.,* 2007). Microbes have been found to produce high yields of lipases compare to the animal and plants. Because their bulk production is easier, commercialization of microbial lipases and their involvement in enzymatic biodiesel production are more common than animal and plant ones (Hasan *et al.,* 2006; Akoh *et al.,* 2007; Antczak *et al.,* 2009). Lipases from microorganisms (bacterial and fungal) are the most used as biocatalysts in biotechnological applications and organic chemistry.

The physical and biochemical properties vary among lipases. As such, each industrial application requires lipases with specific properties. Therefore, there is always interest in new lipases that could be used in new applications (Aires-Barros *et al.,* 1994; Abramic *et.al.,* 1999). It is the stability of lipase that allows them to catalyze the unnatural reaction of transesterification (Jegannathan *et al.,* 2008).

The limitations of using lipases in biodiesel production include: (a) significant cost, (b) the risk that glycerol inhibits the lipase by covering it, due to its accumulation in the reaction mixture; (c) initial activity may be lost because of volume of the oil

molecule (Marchetti *et al.,* 2008; Robles *et al.,* 2009). However, more research is needed in order to be able to use modified lipase on a large scale.

Microbial lipases:

Microbial lipases come from a variety of sources. Gupta *et al.* (2004) referenced 38 distinct bacterial sources from which common lipase are derived. Of these microorganisms, *Candida rugosa, Pseudomonas cepacia, Pseudomonas fluorescens, Rhizomucor miehei* have produced the most effective lipases for transesterification (Vasudevam and Briggs, 2008).

The lipases produced by organisms can be used in various application sectors in different form: extracellular or intracellular (immobilized and regiospecific). Extracellular lipase refers to the use of the enzyme that has been previously extracted from the producing organism and purified. Intracellular lipase refers to the use of the enzyme while it is still contained in the producing organism (Robles *et al.,* 2009). Both extracellular and intracellular lipase can be immobilized on a solid support (Jegannathan *et al.,* 2008). Lipases can also be regiospecific which means they only act on specific bonds of the triglyceride molecule (Robles *et al.,* 2009).

In the present investigation an attempt has therefore been made to identify lipase producing fungi and their possible applications for biodiesel production from *Jatropha* and *Pongamia* oil.

Out of the twenty fungi identified as lipase producers only six species have been selected as source of intracellular and extracellular enzymes for transesterification so far. In the present study all the six fungi such as *Aspergillus terreus, Mucor racemosus,*

Penicillium candidum, Rhizopus oryzae and *Trichoderma viride* were grown in selective and suitably modified media using either *Jatropha* oil or *Pongamia* oil. The culture filtrates of five fungi excluding *Aspergillus terreus* were used as crude enzyme source namely extracellular enzyme. This enzyme was allowed to catalyze *Jatropha*/*Pongamia* oil for conversion into biodiesel.

The process parameters such as suitability of solvent, amount of enzyme needed were standardized and found that for maximum conversion of *Jatropha* oil, 40 ml of methanol and 50 ml culture filtrates per 100 ml of oil was necessary, for *Pongamia* oil, 50 ml of methanol and 50 ml of culture filtrates per 100 ml of oil was found to be suitable for conversion into biodiesel (Table 30a-e, 31a-e and Table 40a-e, 41a-e).

The use of methanol as solvent has been standardized initially during Base catalyzation (Figure 4a, 4b, and 6a, 6b) and Acid-Base catalyzation (5a, 5b and 7a, 7b).

Extracellular / whole cell enzyme

The biggest issue with enzymatic biodiesel is the cost of enzymes. Thus, eliminating the costly step (the purification needed for extracellular lipases) has led to the use of whole cells as biocatalysts. Direct use of compact cells afford intracellular production of lipase or fungal cells immobilized within porous biomass support particles as a whole biocatalyst represents an attractive process for bulk production of biodiesel and polyesters (Iftikhar *et al.*, 2008). Only a handful of microorganisms have been used as whole cell biocatalysts: *Candida antarctica, Rhizopus chinensis, Rhizopus oryzae* and *Saccharomyces cerevisiae*,with the latter being the least popular option (Fukuda *et al.*, 2008; 2009; Robles *et al.*, 2009).

In the present study the whole cell/intracellular lipases were studied for their catalyctic property by using vacuum dried mycelium. This eliminates the purification procedures. Matsumoto *et al*., (2001) and Shiraga *et al*., (2005) have added the air dried and oven dried organisms directly to the reaction without immobilization. Effect of using various amounts of whole cell biocatalyst on transesterification was also tested by Guang and Birma (2010). Whole cell biocatalyst grown and reacted at room temperature can achieve biodiesel yield of about 90% and can convert almost 100% triglycerides into either biodiesel or FFA. However, this required a reaction time of at least 96 hours. Whole sale biocatalyst derived from *Rhizopus oryzae* (ATCC 10260) was able to produce biodiesel with the yield about 75% of virgin oil 80% for waste vegetable oil and 55% for brown grease with the 72 hour traces reaction using methanol is a solvent free water containing system at room temperature.

In the present investigation parameters such as concentration of solvent (oil : solvent ratio) suitability of buffer, the amount of catalyst needed were studied. The results have shown that for *Jatropha* oil conversion for 100 ml of oil, 40 ml of methanol, 40mg of whole cell enzyme in 40 ml of Phosphate buffer was suitable for *Aspergillus terreus, Penicillium candidum, Rhizopus oryzae* and *Trichoderma viride.* Similarly, *Mucor racemosus* showed a maximum yield in Phosphate buffer (Table 32, 33 & 34). For *Pongamia* oil 50 ml of methanol, 50 ml and 50 mg of whole cell enzyme in 50ml of Tris HCL buffer was found suitable for all organisms such as *Aspergillus terreus, Mucor racemosus, Penicillium candidum, Rhizopus oryzae* and *Trichoderma viride* (Table 42, 43 & 44) for yield up to 80 – 85%.

A molar ratio of alcohol to oil is needed for the transesterification reaction to proceed at a reasonable rate. Generally, the greater the molar ratio of alcohol to oil the faster the reaction rate, as long as the alcohol is soluble in the reaction mixture (Antczak *et al.,* 2009). When a portion of the alcohol remains insoluble (in excess) it forms droplets which coat the enzyme causing it deactivation. Many authors stressed that the alcohol employed in transesterification must be completely dissolved (especially methanol) implying that there is an optimum alcohol to oil molar ratio which allows for the fastest reaction rate (Jeong and Park, 2008).

As a guideline, if the alcohol has less than three carbons it is likely to inhibit the lipase enzyme since its solubility is less than three carbons it is likely to inhibit the lipase enzyme since its solubility is less than the stoichiometric ratio. Methanol and ethanol typically are soluble at 1/2 and 1/3 of their stoichiometric amounts respectively. Alcohols with greater than three carbons typically do not cause any inhibition since they often dissolve in the feedstock in their stoichiometric ratios (Shimade *et al.,* 2002; Robles *et al.,* 2009). In an organic solvent reaction, an excess amount of alcohol is needed in order to achieve a satisfactory reaction rate and a FAAE yield. Typically, in a solvent system, methanol to oil molar ratios should be in the range.

Partially purified / purified enzyme

In our study when partially purified enzymes were used the amount of alcohol needed was 100 ml of oil, 40 ml methanol and 40 ml of partially purified enzymes for *Jatropha* oil. For *Pongamia* oil, per 100 ml of oil, 40 ml of methanol and 40 ml of

partially purified enzyme was found to be suitable for all five organisms (Table 35 & 45).

When purified and lyophilized enzyme was used for *Jatropha* oil/*Pongamia* oil 40 ml of methanol and 25 ml purified enzyme was found suitable. Only *Rhizopus oryzae* enzyme was tested for this purpose (Table 38 & 48).

Oil to alcohol ratio

As per the classical reaction of the transesterification a 1:3 molar ratio of alcohol to triglycerides is needed. In practice, the ratio needs to be higher to drive the equilibrium to a maximum ester yield. Conversion of *Pongamia* and *Jatropha* oil required 1:4 ratio of oil to alcohol to yield the maximum ethyl esters in the present study. ratio of oil to alcohol gave only 20% conversion where as 1:4 gave maximum conversion up to 94% In case of higher ratios like 1:6 conversion rate dropped and only up to 30% ethyl esters were formed due to inactivation of lipase by excess of ethanol present in the reaction. Shah *et.al*., in 2004 reported the same ratio of alcohol to oil for enzymatic esterification of *Jatropha* oil by *Chromobacterium viscosum*. For maximum conversion of oleic acid in to ethyl oleate by purified lipase from same organism required same ratio of oil to ethanol was reported earlier in previous studies.

Temperature and incubation period

All the transesterification procedure were carried out at 35 – 40^0C because the lipase activity of the fungi studied in the present investigation was higher only in this temperature (Table 25 & 26). It was observed by Jayshree and Asmita (2015) that

reaction which was carried out at 30^0C gave maximum fatty acid ethyl esters from both *Jatropha* and *Pongamia* oil. Sudden drop was observed after 35^0C and only 30% ethyl esters were produced. It was suggested by them as due to lesser amount of lipase production from *Rhizopus* strain JK1 cells when temperature was increased beyond 30^0C.

Chen & Lin in, 2010 have reported growth associated transesterification of soybean oil using *R.oryzae* whole cells with only 72.6% yield which was maximum after 24h. Adachi et.al., 2013 studied methanolysis of palm oil, one of the recombinant strain studied showed methyl ester content of nearly 100% but which is after completion of 96h at elevated temperatures. Whereas, we report 80-85% transesterification of *Jatropha* and *Pongamia* oil with the use of five days old cultures of five fungi under investigation.

Suitability of solvent

Ethanol gave the maximum conversion in case of both the oils as compared with the methanol.When ethanol was used as acyl acceptor 95% of ethyl esters were formed from *Pongamia.* According to the studies by Marchetti., *et.al.* 2007, the toxic nature of methanol and its availability issue, it is important to have a potent acyl acceptor candidate to achieve maximum conversion of triglycerides to fatty acid alkyl esters. Lipase from the *Rhizpous* strain JK-1 showed high stability in presence of ethanol as compared to methanol.

These results are contray to the reports of Hama, *et. al.,* in 2006 and Tamalampudi, *et. al.,* in 2008 where methanol was better acyl acceptor for maximum

production of esters. Also in recent reports of Adachi, *et. al.,* 2013, recombinant *Aspergillus oryzae* was more stable in methanol when compared with ethanol and when methanol was used as acyl acceptor maximum conversion of palm oil was achieved. Our results also confirm the suitability of methanol for lipase catalysis.

Comparison of commercial and immobilized enzyme

When immobilize enzyme of *Rhizopus oryzae* was tested for biodiesel yield from *Jatropha/Pongamia* oil the results were not appreciable, since the matrix and enzyme together made the solution extremely viscous. However, Tamalampudi et al. (2008 **)** have compared *R.oryzae* for immobilized lipase with one of the most common biocatalyst for biodiesel production, Novozym 435. They showed that *R.oryzae* lipase is more efficient than Novozym 435, regardless of alcohol type. Our results suggest that lyophilized enzyme of *Rhizopus oryzae* yields biodiesel on par with commercial enzyme (Table 36, 46, 38, 48) when both the oils were used.

Table 55

Determination of enzyme activity in the sources of catalyst used for transesterification of *Jatropha* oil

Enzyme name	Microorganism	Enzyme activity (U/mL)	Amount of enzyme used for transesterification	Total Units	Biodiesel yield in %
Extracellular enzyme	*Mucor racemosus*	4.81	50	240.5	68
	Rhizopus oryzae	5.12	50	256	75
	Aspergillus terreus	4.13	50	206.5	68
	Penicillium candidum	4.96	50	248	70
	Trichoderma viride	4.81	50	240.5	70
Partially purified enzyme	*Mucor racemosus*	4.86	40	194.4	80
	Rhizopus oryzae	4.94	40	197.6	86
	Aspergillus terreus	4.74	40	189.6	80
	Penicillium candidum	4.41	40	176.4	81
	Trichoderma viride	4.92	40	196.8	80
Intracellular enzyme	*Mucor racemosus*	4.83	40	193.2	70
	Rhizopus oryzae	4.97	40	198.8	75
	Aspergillus terreus	4.89	40	195.6	65
	Penicillium candidum	4.54	40	181.6	71
	Trichoderma viride	4.74	40	189.6	72
Purified enzyme	*Rhizopus oryzae*	4.99	25	124.75	94
Commercial enzyme		5.87	25	146.75	96

From the table it is understood that 200 – 250 units of enzyme is needed for conversion of *Jatropha/Pongamia* oil into 80 – 85% of biodiesel, irrespective of the source of enzyme viz, extracellular, intracellular and partially purified enzyme. However when purified / commercial enzymes were used nearly 125-130 units alone were needed.

Table 56

Determination of enzyme activity in the sources of catalyst used for transesterification of *Pongamia* oil

Enzyme name	Microorganism	Enzyme activity (U/mL)	Amount of enzyme used for transesterification	Total Units	Biodiesel yield in %
Extracellular enzyme	*Mucor racemosus*	4.21	50	210.5	60
	Rhizopus oryzae	5.02	50	251	75
	Aspergillus terreus	4.18	50	209	68
	Penicillium candidum	4.09	50	204.5	63
	Trichoderma viride	4.18	50	209	58
Partially purified enzyme	*Mucor racemosus*	4.95	40	198	83
	Rhizopus oryzae	4.99	40	199.6	89
	Aspergillus terreus	4.91	40	196.4	80
	Penicillium candidum	4.33	40	173.2	80
	Trichoderma viride	4.83	40	193.2	80
Intracellular enzyme	*Mucor racemosus*	4.02	50	201	80
	Rhizopus oryzae	4.92	50	246	88
	Aspergillus terreus	4.63	50	231.5	78
	Penicillium candidum	3.95	50	197.5	76
	Trichoderma viride	4.00	50	200	76
Purified enzyme	*Rhizopus oryzae*	5.06	25	126.5	94
Commercial enzyme		5.03	25	125.75	96

Characteristics of biodiesel

Flash and fire points of both the biodiesels were higher than the international standard, other characteristics viz., viscosity, Iodine value and saponification value were almost on par with standards but higher than petroleum diesel. Cloud and Pour points were significantly lesser than diesel and calorific value was less than diesel but ash value was equal to diesel. (Table 49, 50)

Engine Performance

Engine efficiency and emissions study showed the results almost on par with petroleum diesel in terms of Brake Thermal Efficiency (BTE), Total Fuel Consumption (TFC) and Specific Fuel Consumption (SFC) upon increase in load (Figure 9 and 10).

GC-MS profile

GC-MS profile of both the oil showed the presence of methyl ester which contribute to the biodiesel characteristics (Figure 10, 11 and Table 53, 54).

BLAST studies

The best lipase producer namely Rhizopus oryzae which also showed higher biodiesel yield with its different lipases such as extracellular, whole cell, Partially purified, Purified and Lyophilized was subjected to authentication using 18srRNA sequence by BLAST studies and the species showed 98% similarity with several strains of Rhizopus oryzae available in the NCB database (Figure 12).

Cost effective ratio

Realistic cost estimates and improvement in process, economics are the key factors in the commercial success of any technology. Therefore, an attempt was made to reduce down the cost of enzyme production by three fungi, which produces very high level of lipase under submerged fermentation in very cost effective media. Using the lipase enzyme, biodiesel production was carried out and the cost economics ratio when calculated suggested that the cost of biodiesel thus produced could be 40 % higher than

the cost of conventional diesel. If the glycerol and methanol available at the bottom layer were used for other purpose the cost could be reduced to another 15%.

To overcome these drawbacks, which may limit the availability of biodiesel fuel, enzymatic processes using both Extra cellular and Intracellular lipases have recently been developed. The latter process is much simpler since recovery of unreacted methanol find waste water treatments are unnecessary. In addition, only a simple concentration is required to recover glycerol. Since the cost of lipase production is the main hurdle to commercialization of the lipase-catalyzed process, several attempts have been made to develop cost-effective systems.

To avoid serious degradation of lipase activity in the presence of a high concentration of methanol, a novel operation with stepwise addition of methanol has been developed. The use of intracellular lipase as a whole cell bio-catalyst is also an effective way to lower the lipase production cost, since complex purification is not necessary.

Another useful approach to reducing the production cost is to use solvent-tolerant lipases. Several microorganisms that produce solvent-tolerant lipases, either 1,3-specific from *Fusarium sp.* (Shimada *et al,* 1993) or non-specific from *Pseudomonas* and *Bacillus* sp. (Sugihara *et al.,* 1992; Iizumi *et al.,* 1990; Sugjhara *et al,* 1991; Ogino *et al,* 1999), have been reported. These lipases are stable in most water - immiscible solvents, but their stability generally decreases somewhat in water-miscible solvents such as methanol and ethanol. However, activity of the lipase from *Fusarium heterosporum* was found to slightly increase in the presence of a low concentration of methanol.

Further enhancement of lipase production may be achieved by genetic engineering. High levels of expression of lipases from several microorganisms (Bertolini *et al*., 1995; Nagao *et al*., 1996; Takahashi *et al*., 1998) have been successfully achieved using *Saccharomyces cerevisiae* as the host. Among these the lipase cDNA from *F. heterosporum* increased lipase productivity 3-fold over that of the original strain.

In the light of these findings, by the use of whole cell bio catalytic process with stepwise addition of methanol, significant reduction in the cost the production of biodiesel could be expected. Such novel system would be promising at industrial scale for enzymatic production of biodiesel.

Scope for future work

Modeling and scale up studies

The experimental data obtained in the present experiments should be modeled as protocols for enzymatic transesterification in scaling up the process for industrial applications. This would determine the actual ability of this enzyme on a commercial scale.