Developing Lipase catalyzed quantitative way to detect the amount of monoglycerides in Biodiesel

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1. Introduction

The three primary driving forces behind biofuels are the worlds increasing requirement of petroleum with diminishing supply of fossil fuels, global warming and the intention to reduce the dependence on fuel imports. Biodiesel is a mixture of alkyl esters of higher fatty acids, which arises within transesterification reaction of vegetable oils. Most biofuels are produced by straightforward production process are readily biodegradable and non-toxic, have low emission profiles and can be used at it is or blended with conventional fuels. Still biodiesel has many problems faced during storage conditions that will allow highest levels of stability. Glycerol is created during the biodiesel production process as a co-product. After separation of glycerol phase, an impermissible amount of free glycerol and total glycerol remains in biodiesel. Free glycerol refers to the amount of glycerol that was left in the final biodiesel product while selling in market with maximum permissible concentration 0.02%wt. Total glycerol refers to impurities present after purification with maximum permissible amount of 0.24%wt set by the ASTM D 6751 specification.

Since glycerol is mostly insoluble in biodiesel, all the glycerol was removed by either settling or centrifugation. Free glycerol may be available either as suspended droplets or in a very small amount dissolved in biodiesel. The water washing process helps to remove most of the glycerol from biodiesel. Fuel with high free glycerol than specified by ASTM D6751 will have problem with glycerol settling in storage tanks, creating a mixture that can plug fuel filters and cause combustion problems in the engine. Therefore it is necessary to determine the amount of free glycerol in biodiesel (Hájek et al., 2006).

There are several methods of free and total glycerol determination in laboratory. Gas chromatography (GC) and High Performance Liquid Chromatography (HPLC) are frequently used to check the level of free and total glycerin in B100 biodiesel following a ASTM method (Mahajan et al., 2006) however, GC and HPLC analyses of biodiesel consume valuable personnel and instrumentation time (Hájek et al., 2006) These methods are very specific, may not suite for routine analysis, and process monitoring. To have a quick field testing device, enzyme based catalytic assays or biosensors are developed. Completed enzymatic systems

eliminate the use of caustic reagents, extraction solvents, high temperature baths and adsorption mixture (Lapenaite et al., 2006). Small, laboratory instruments were focused on specific fuel quality characteristic. While, various multifunctional instruments use infrared technology indicating free and total glycerol, water content, free fatty acids and cloud point. However, like field test kits, these instruments do not use the ASTM testing protocols. A typical biosensor would save the time and money to determine the measure of total glycerin (Templeton et al., 2009) Accurate level determination in a few minute span may save lot of time for industries instead of sending outside laboratory testing which cost $80 -$150 per test (Gerpen, 2007). GC and HPLC determination procedures are considered less suitable for glycerol analysis particularly the complexity and investigated matrices makes it more expensive and require a long time for sample and standard preparation (Lapenaite et al., 2006). Since level of total glycerol depend on the quality of biodiesel, a correct, easier and cheaper method will be required by industries to decide on further purification process.

2. Research Explanation

Objective

1. To design an experimental test with lipase catalyzed reaction for measuring amount of impurities (especially monoglycerides) content in biodiesel in cost effective way.
2. To evaluate the time and temperature needed for different concentration of lipase at various biodiesel pH measurement and perform the reaction needed.

Importance to Monoglycerides

An important criterion for a good quality biodiesel is the completion of transesterification reaction (Biodiesel Reaction). At a certain amount of alcohol combine the catalyst with the feedstock vegetable oil or fat and mix it well at a temperature of 55 °C the result is a mixture of glycerol and Biodiesel (ASTM D 6584).

Oil + Alcohol + Catalyst --> Biodiesel + Glycerol

The total glycerol content produced are in the forms of mono-, di-, and triglycerides. During the transesterification, the amount of monoglycerides formed are larger than the other two (Rapaka, 2012). The monoglycerides formed as the result of different feed stocks have inherently different properties like raised cloud point which may give negative effect during winter conditions and storage stability (Worldwide Fuel Charter Committee, 2009). They can also lead to increased injector deposits (Component, 1912). Therefore, in addition to overall limit for total glycerol 0.24%, EN14214 specifies the maximum amount of individual to be 0.80% monoglycerides on the mass basis.

Detection using lipase catalyzed analysis

Lipase is important enzyme catalyst that catalyzes transesterification reaction to produce methyl esters (Templeton et al., 2009). Potentiometric biosensor makes use of ion-selective electrodes to transduce the biological reaction into an electrical signal. But, instead of immobilizing the lipase enzyme as a membrane surrounding the probe of a pH- meter (Vadgama, 1986) direct introduction of lipase would save time to create membrane and easy process for the reaction to complete and also noted that degradation of membrane occurred after each time the sample was tested (Ju and Kandimalla, 2008).

$$glycerides + H20 \xrightarrow{lipase} glycerol + fatty\ acids + H^+$$

The enzyme reaction with the sample reacted with glycerol will release hydrogen ions and can be measured by a pH probe. The measurement with change in pH read as mV signal in the meter. With optimum pH for lipase of 7.5-8.0 (Soares et al., 1999), the production of fatty acids by means of transesterification reaction in the specific reaction conditions may result in pH changes due to dissociation of fatty acids. The change in pH is proportional to the concentration of triglyceride in solution (Lapenaite et al., 2006). Sometimes the pH of a solution can change with temperature, on the dissociation of weak acids and bases, and the dissociation of water itself. In fact, any solution with a pH 7 or above will have some degree of temperature dependence. How much this will affect the measurement will depend upon the composition of the solution and how high the nominal process temperature is above 25°C. This behavior is frequently the explanation

for discrepancies between laboratory and pH measurements (Emerson Process Management, 2010). The experimental procedure was altered so that the enzyme lipase was added directly to the biodiesel sample and the pH probe was used to measure the release of hydrogen ions.

3. Methodology

Amount of monoglycerides (Impurities)

To eliminate variability, different amount of monoglyceride (Impurities) was added for each level. Considering 0.80 max %m/m as the recommended allowable levels by ASTM D 6584, the total levels needs to be introduced into biodiesel is calculated with glycerol grade levels as 17mg, 27mg & 48mg for the maximum of 50 ml biodiesel sample.

Amount of Lipase (Catalyst)

The amount of lipase was calculated for each levels of Monoglycerides with the respective lipase units and molecular weight (358.55g/mol) with mass above 60kDa (Öztürk, 2001). Since lipase is directly not soluble in oil, it is dissolved into the soap solution and introduced into the sample. the calculated levels would be 5.2mg, 8.2mg & 14.7mg comparative with levels of monoglycerides.

Temperature constancy

The stability of enzyme during the reaction was maintained by the temperature. Chances of lipase to act different is possible with specific temperature. The exact value of physical property can be minimized by comparing approximation process. The thermal stability can be verified by the pH difference and the time taken to reach the minimum. in case of high temperatures range the lipase becomes inactivate (Miranda et al., 2011). For instance, the values of 25 ° to 35 ° C was taken as phases. However, variation in lipase does not change. This kind of thermal stability was conducted after inspecting the better concentration points.

Experimental setup

The experiment was carried out using variable like temperatures, amount of lipase concentration, and amount of impurities (monoglycerides) based on the maximum allowable for bound glycerol levels. Calibrated lab quest pH sensor introduced into freshly prepared purified one batch biodiesel on a hotplate with varying temperatures of 25 °C & 30 °C used to take readings continuously. Lipase was calculated with

respective monoglyceride levels according to molar mass used as impurities to add biodiesel. Readings were noted with time and pH at random intervals. The total experiment with different combinations were listed in below table.

Table 1:Experimental Dependent Variables

Temperature	Monoglycerides	Lipase
25 ° C	0mg	0mg
	17mg	5.2mg
30 ° C	27mg	8.2mg
	48mg	14.7mg

The biodiesel used for this experiment were analyzed for the Gas Chromatography earlier. According to the certificate of analysis from Gorge Analytical, the total monoglycerides in the biodiesel were found to be 0.550% mass of the total glycerol 0.159% mass.

4. Results and Discussion

The change in pH noted immediately after the lipase is added. For the pH to reach minimum or stable it took approximately 10-15 min. To keep the observed value consistent the probe was left over in the sample and the projected value after reaching minimum pH to a stable, eventually started to increase over time. The reason might be either the amount of lipase was not enough for the reaction to further process or, there are still the impurities present in it to purify them (Califano et al., 2014) . Within all combinations of 25 °C a ratio for each monoglyceride molecule, 2 times of lipase enzyme is needed to react in the transesterification reaction as shown in figure 1. The combinations show better results of reaching pH 7 in only 2 times the lipase amount needed. Keeping pH 7 to reach as target, only 3 of the observations barely reached ~7.25 (figure 3) which proves that impurities at these combinations will need more lipase in the reaction. Within all combinations of 35°C, temperatures variable doesn't really have effects because

chances of overreaction occur in sample reaction. Though we have an observation which reached almost pH of 7 it is only because of increasing temperature but the reaction was not stable enough in these levels.

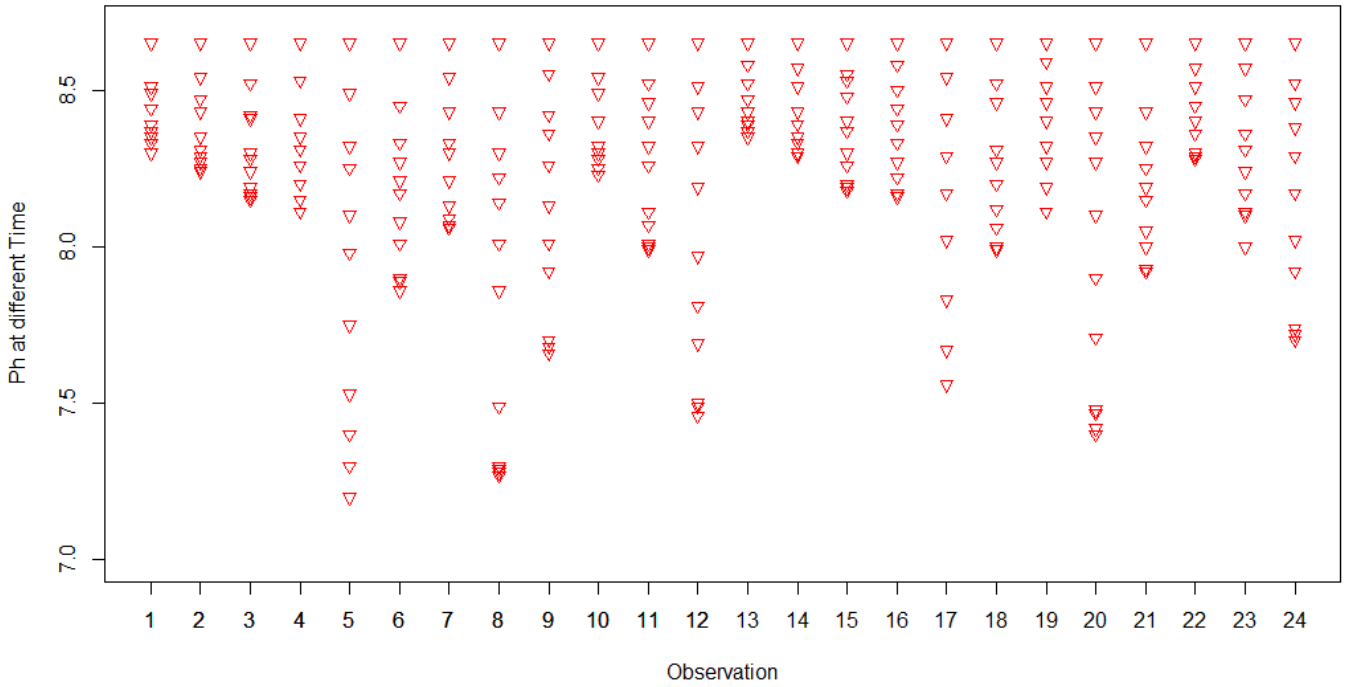

Figure 1: All 24 Observations, the maximum pH reached

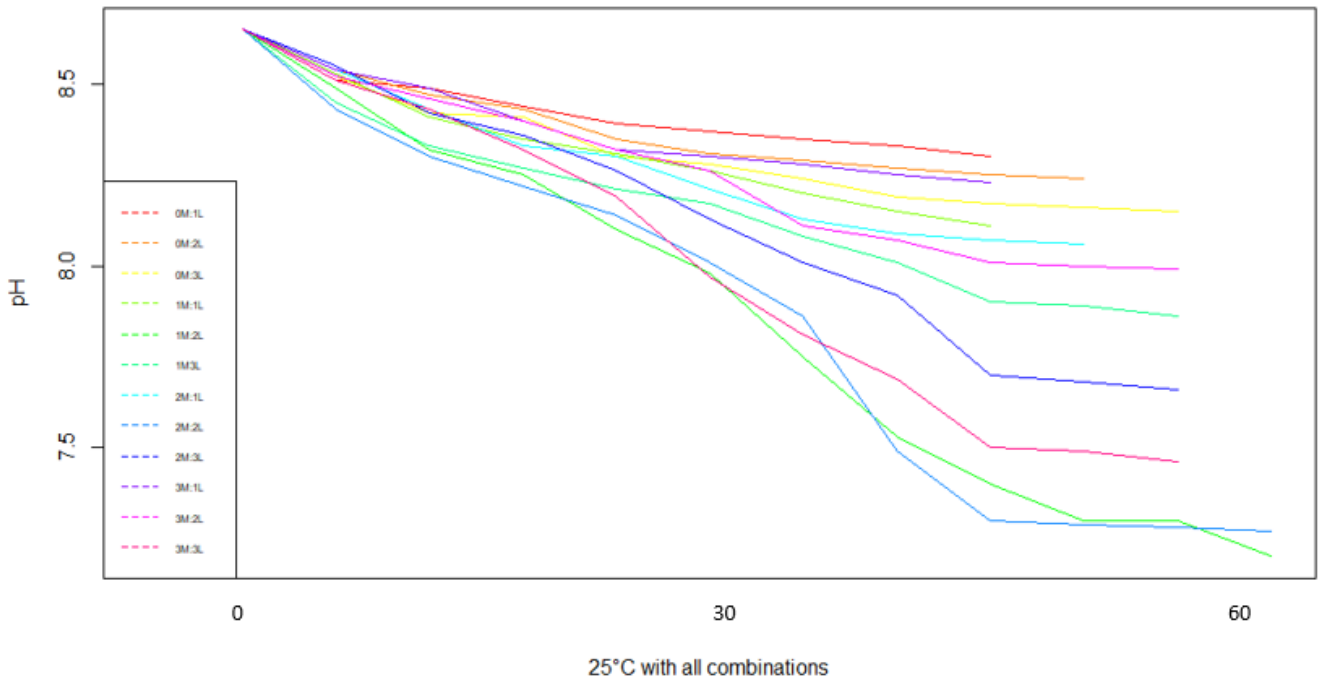

Figure 2: Time vs pH (All combinations of 25 ° C) – (M-Monoglycerides : L-Lipase Ratio) in each observations

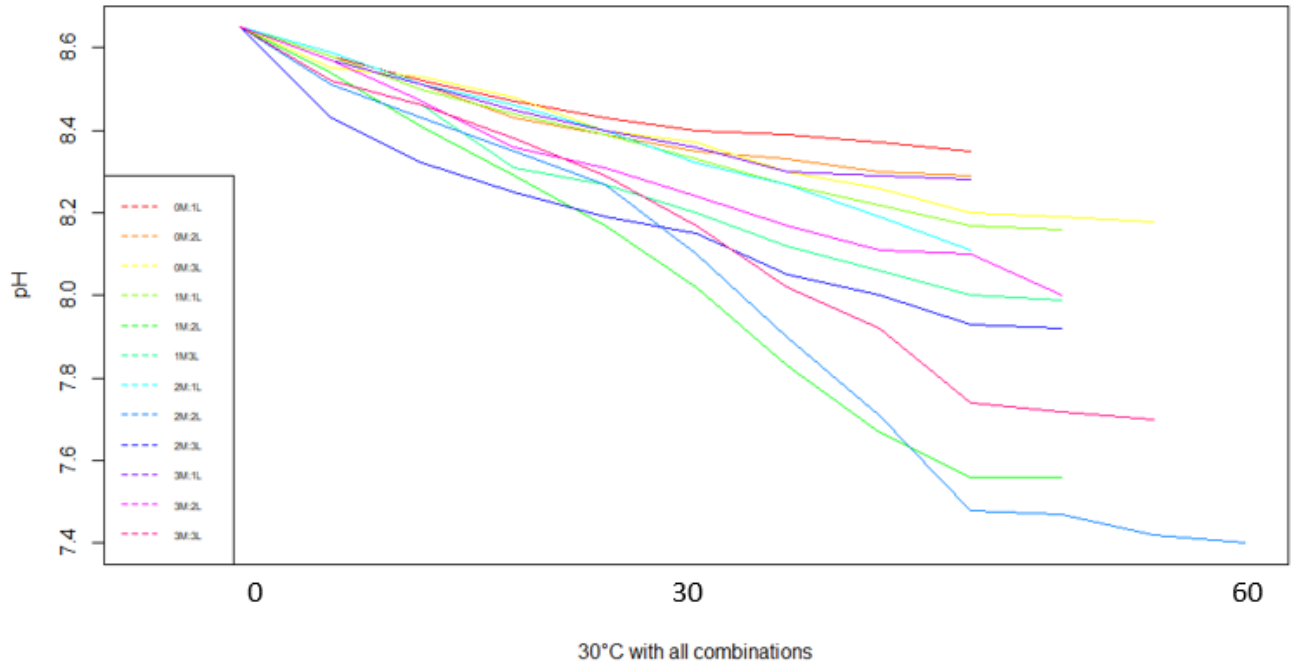

Figure3: Time vs pH (All combinations of 30 ° C) – (M-Monoglycerides : L-Lipase) Ratio in each observations

Regression analysis was performed through R programming. At any possible combinations, the monoglycerides content were compared with the lipase and the temperature variability. Multiple linear regression statistical techniques used to describe the function of one or more predictor variables which can help to understand and predict the behavior of complex systems. Most of the comparative results with variables, the estimates and the t-value provide a negative number for lipase concentration and were not significant with the temperature. In regression results, if the correlation coefficient is negative, it provides statistical evidence of the negative relationship between the variables. The increase in the first variable (lipase) will cause the decrease in the decrement in the second. When we have two variables of temperatures 25°C and 30°C and comparative pH variable with time from 2-60min & lipase concentrations with selective monoglycerides levels it is understandable that from the regression estimates the temperature variable doesn't have any effect on the reaction sample. However, pH levels are positive which shows that change in pH has always been effective with any number of lipase levels in sample reaction. When we have a regression estimates for the three-way interaction including two temperature difference, the monoglycerides and the lipase shows a significant difference. But, at any levels temperature variable would not make any

suitable environment for change in pH as of with monoglycerides and lipase do. When the amount of lipase is increased three times more than that of the monoglycerides content, the variables becomes significant. But if the lipase value was decreased the negative factor becomes visible with the temperature factor always being in negative. This proves that with all the Beta values =0 for the null hypothesis becomes true and the alternative hypothesis becomes false. The data is a real-time experience collection measurements so even if the R squared value is small it doesn't matter. Though all the p-value is very high the interactions between the variables (temperature, monoglycerides and the lipase) were not there which is good. This helps the individual variables to make good effect in the experiment.

5. Conclusion

With all this in concerns, finding the amount of monoglycerides using a free-lipase technique had always been a difficult take to experiment them. At any point of sample analysis, the temperature of 25 ° C, lipase of 8mg for 17 mg of monoglycerides made a significant difference in the biodiesel to reach the minimum ph. More than the procedure figured out for the technique, the analysis for each variable has always been some basic criteria to understand the sample and verify the technique. Analyzing the Total glycerol (Mono, Di & Tri – glycerol) would be the best thing for any sample overall rather than testing for a composition. But if the free lipase techniques help us to figure out even the Total glycerol that would be little help for the industries. The procedure for the free lipase techniques has been improved in a better way which helped to understand that temperature, and lipase concentration should be set a benchmark for any experiment. However, getting separate results for each composition has been a difficult path to understand and implement in this experiment. Detailed procedure in this project will surely help to stat with the free lipase techniques but understanding each sample with various lipase would be a better option to work in future.

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Appendix

Codes

```
#linear regression
#-------------------------
results=lm(Mono~Lipase)
#multiple regression with all combinations
#-----------------------------------------------------
results=lm(ph~(temp+mono+lipase+temp*mono+temp*lipase+lipase*mono),data25)
#Linear regression with all dependent variable
#-----------------------------------------------
results=lm(ph~(temp+mono+lipase),data25)
#plots
#---------
#Time series with all observations plots
#-----------------------------------------------
#Read data
Data1=read.csv("timeseries.csv", 1)
Data2=timeseries[,-1] #delect 1st column
#define every column
x=25C Min Lipase:35C Max Lipase #observation
y1=Data2[,1]
y2=Data2[,2]
y3=Data2[,3]
y4=Data2[,4]
y5=Data2[,5]
y6=Data2[,6]
y7=Data2[,7]
y8=Data2[,8]
y9=Data2[,9]
y10=Data2[,10]
y11=Data2[,11]
y12=Data2[,12]
#Main plot function
matplot(x,
cbind(y1,y2,y3,y4,y5,y6,y7,y8,y9,y10,y11,y12),
pch=6, xlim=c(1,24),ylim=c(7,8.7),col = "red",xlab = "Observation",ylab = "Ph at different Time")
axis(1,1:24)
#Time Vs pH graph for both temperatures
#-----------------------------------------------------
Install.package(zoo)
zoo.basket2<-as.zoo(basket1)
basket1<-
cbind(comb13,comb14,comb15,comb16,comb17,comb18,comb19,comb20,comb21,comb22,comb23,comb24)
xrange<-range(0:12)
tsRainbow<-rainbow(ncol(zoo.basket2))
plot(x=zoo.basket2,col=tsRainbow,screens=1,xlim=c(0,12),xlab="30°C with all combinations",ylab="pH")
legend(x="bottomleft",legend=c("0M:1L","0M:2L","0M:3L","1M:1L","1M:2L","1M3L","2M:1L","2M:2L","2M:3L","3M:1L","3M:2L","3M:3L"),lty=2,col=tsRainbow)
xtick<-seq(from=0,to=,by=)
axis(1,at=xtick,labels=TRUE)
levels(Time) = c(0,2,5,7,10,15,20,25,35,40,50,60)
#-------------
#ggplot
p1<-ggplot(example,aes(x=observation, y=ph))
  + theme(legend.position =
"top",axis.text=element_text(size=6))
p1+geom_bar()
d=ggplot(example, aes(x=observation, y=ph))
d+geom_line()
d+geom_point(aes(color=cut))
#--------------
qplot(lipase, ph, data=data, geom=c("boxplot", "jitter"))
boxplot(ph~lipase,mono)
boxoplot(phbtos)
boxplot(ph~mono,main="phbtosn")
boxplot(ph~lipase,main="phbtosn")
#-----------------------------
#nonlinear
curve.fit=nls(rate~b,data=,
              start=list(b1=b1.0,b2=b2.0))
#---------------------------------
```