Effect of Na^+ and K^+ on Lipase Catalyzed Hydrolysis of Ester

A

Thesis Submitted

In partial fulfillments of requirements for the

Degree of

Master of Science

in

Chemistry

Submitted By

Parul Sharma

(Reg. no. 30702013)

Under the supervision of

Dr.Amjad Ali

Lecturer

School of Chemistry and Biochemistry

Thapar University

Patiala ,147004

June, 2009

- 2 -

Acknowledgements

I thank almighty for his mercy and blessings upon me.

I take this opportunity to thank my guide Dr. Amjad Ali for his guidance and support in doing this thesis. He has instilled in me the knowledge and motivation to learn more about the subject.

I am grateful to Prof. Sushcel Mittal for approving this thesis to me.

I am thankful to all the Ph.D. scholars especially Dinesh Sir, Nirankar Sir, Vishal Sir, and Jasminder Sir for their timely help and support.

The laboratory staff especially Chander Sir, Praveen Sir were very forthcoming and helpful in every possible way and I am highly obliged to them.

I thank all my friends who constantly motivated me and supported me throughout the thesis

I thank my parents and my brothers Ankit and Naman who are building blocks of my life.

Place: Patiala

Date: 24.6.09

Parul Sharma

Certificate

This is to certify that the thesis entitled "Effect of Na^+ and K^+ on lipase catalyzed hydrolysis of ester", being submitted by Ms Parul Sharma in partial fulfillment of the requirement for the award of degree of Master of Science (Chemistry) in the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafide work carried out under the supervision of Dr. Amjad Ali and that no part of this thesis has been submitted for the award of any other degree.

Dr.Amjad Ali,
Lecturer,
Thapar University.

Susheel Mittal 24/6/09
Head,
(Dr. Susheel Mittal)
School of Chemistry and Biochemistry,
Thapar University.

RK Sharma 30/6/09
Dean, Academic Affairs,
(Dr. R.K. Sharma)
Thapar University.

A

Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled "Effect of Na^+ and K^+ on lipase catalyzed hydrolysis of ester", in partial fulfillment of the requirements for the award of the degree of Masters in Chemistry, School of Chemistry and Biochemistry (SCB), Thapar University, Patiala, is my own work during the period of Jan 2009 to May 2009, under the supervision of Dr. Amjad Ali Lecturer, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Place: Patiala

Date: 24.6.09

Parul Sharma

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

Dr.Amjad Ali

Thesis Supervisior,

Lecturer (SCB),

Thapar University

Dr. Susheel Mittal

Head, (SCB)

Thapar University.

List of Contents

Introduction:-

To satisfy the global requirement of the industrial process we should have enzymes showing optimal activities at maximum degree of extremity of pH, temperature and salt concentration. Thermopile microorganisms are found to be potential and good alternative source of thermo stable enzymes. The extremophilic, especially thermopile bacteria can be isolated from the natural high temperature environments distributed throughout the world and found in association of tectonically active sites. Lipases (EC 3.1.1.3) are comprised a group of enzymes which catalyze the hydrolysis of triacylglycerols. In the recent years, the interest on lipase has grown significantly. The development of technologies using lipases for the synthesis of novel compounds will result in their expansion into new areas and increase in number of industrial applications. Lipases are extremely versatile enzymes, showing many interesting properties of industrial applications. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional methods.

Enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetic industries. In the above scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. However, with the realization of the biocatalytic potential of microbial lipases in both aqueous & non aqueous media in the last one and a half decades, industrial fronts have shifted towards utilizing this enzyme for a variety of reactions of immense importance.

It is in the last decade that lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis. The enantioselective and regioselective nature of lipases have been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substituents, bio fuels, and for synthesis of personal care products and flavour enhancers. Thus, lipases are today the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists. Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases and therefore do not require any cofactor. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural

conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved (Figure 1). Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The occurrence of the lipase reaction at an interface between the substrate and the aqueous phase causes difficulties in the assay and kinetic analysis of the reaction. The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyze them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids.

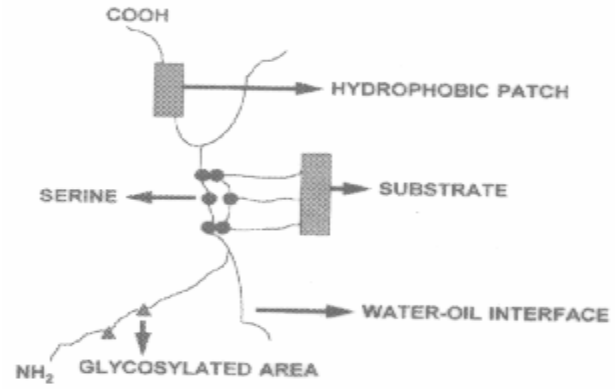


Figure -1 Diagrammatic representation of lipase molecule showing its main features.

Today, lipases stand amongst the most important biocatalysts carrying out novel reactions in both aqueous and non aqueous media. Advantage while using lipase, this is primarily due to their ability to utilize a wide variety of substrates, high stability towards extreme condition of temperature, pH and organic solvents, and chemo-, regio- and enantioselectivity. More recently, the determination of their three-dimensional structure has thrown light into their unique structure–function relationship. Among lipases of plant, both animal and microbial origin, it is the microbial lipases that find immense application because microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions. Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts.

Lipase:-

A lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester bonds in water–insoluble, lipid substrates. Lipases thus comprise a subclass of the esterase.

Sources of lipase:-

Lipase enzymes found in plants and animals plays a vital role in lipolytic metabolism. Pancreatic lipase of porcine origin is one of the earliest recognized and is still the best known lipase. Lipase is also produced (both extracellular and intracellular) by many bacteria, algae, fungi.

Structure:-

Though lipase differ widely in the number of amino acids in their primary sequence, yet the common feature of all lipase is that the active site is build of three amino acids serine, aspartate or glutamate and histidine (catalytic triad).The three dimensional structure of all lipase follow a common motif the α/β fold of hydrolayse (figure 3), where lipase is consisting of eight most parallel β sheets surrounded on both sides by α helices.

The triad and Oxonian-stabilizing residue are thought to compose the active centres of lipase. A helical (lid or flap)which block the active centre is responsible for the important characterization of lipase interfacial –activation which let them to distinguish from other hydrolayse such as serine proteases & esterases.Based on x-ray structure of lipase before and after binding to the substrate, researchers believe that lipase are activated before they take part in biochemical transformation reactions. A freely dissolved lipase in the absence of an aqueous/lipids interface resides in its inactive state and a part of the enzyme molecule cover the active (figure 2) site. However when the enzyme contacts the interface of biphasic water-oil system a short alpha helix (the lid) is folded back leading to activation of lipase enzyme**.**

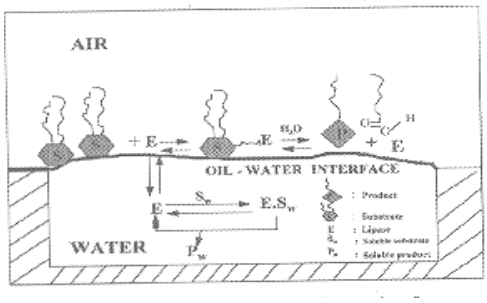


Figure :2 Showing aqueous -lipids interface.

In other words, when no substrate present, the lid closed and the enzyme is inactive, where as in the presence of substrate, water –oil interface exists and the lid opened than the lipase is active(figure 4) :below show Pseudomonas lipase open configuration lipase molecule obtained from source pseudomonas cepacia.It is obtained from X-ray diffraction pattern at resolution 2.1Å. Its enzyme commission number is 3.1.1.3.

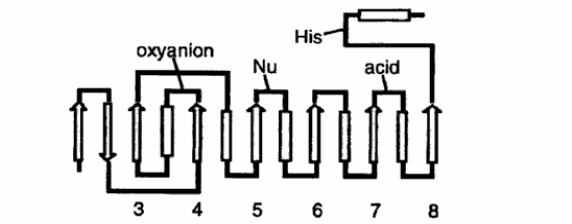


Figure: 3 Systematic representation of α /β fold of hydrolyses.

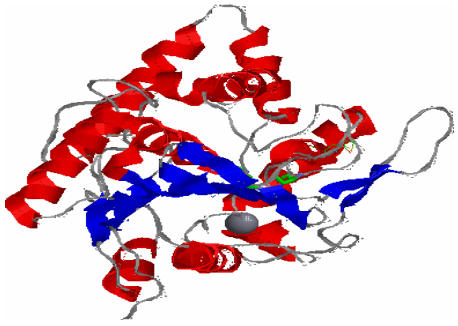


Figure: 4 Showing *Pseudomonas* lipase open configuration.

METHOD:	X-ray diffraction
RESOLUTION:	2.1 Angstroms
SOURCE:	*Pseudomonas cepacia*
EC:	3.1.1.3
CHAIN:	Null
MOLECULE:	Lipase

Lipase enzyme as shown in fig 5 contains a catalytic triad that usually consists of a serine, a histidine and aspartic acid and most of lipase doesn't have any metal ion attached with the active site. Serine amino acid is embedded in the consensus sequence G-X-S-X-G (X= any amino acids) at the active site of the lipase enzyme.

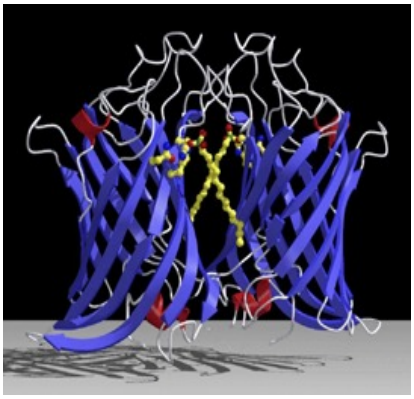


Figure: 5 3-D structure of lipase of *Pseudomonas aeruginosa*.

Applications of lipase:-

Lipases in polymer synthesis.

Lipases in synthesis of ingredients for personal care products.

Lipases in synthesis of triglycerides.

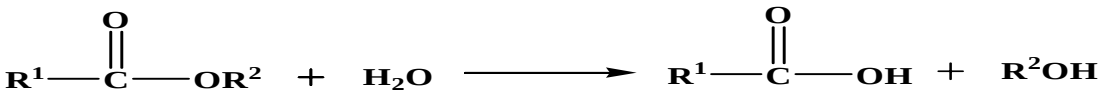
Lipases in synthesis of surfactants.

Lipases in detergents.

Lipases in dairy industry.

Hydrolysis:-

The hydrolysis of the lipids forms a heterogeneous reaction system made up of two liquid phases. The aqueous phase consists of water and glycerol, the homogeneous lipid phase consists of fatty acids and glycerides. The hydrolysis of the lipids takes place in the lipid phase in several stages via diglycerides and monoglycerides. Reaction accelerates in presence of acid catalyst and requires high temperature and high pressure in absence of catalyst.

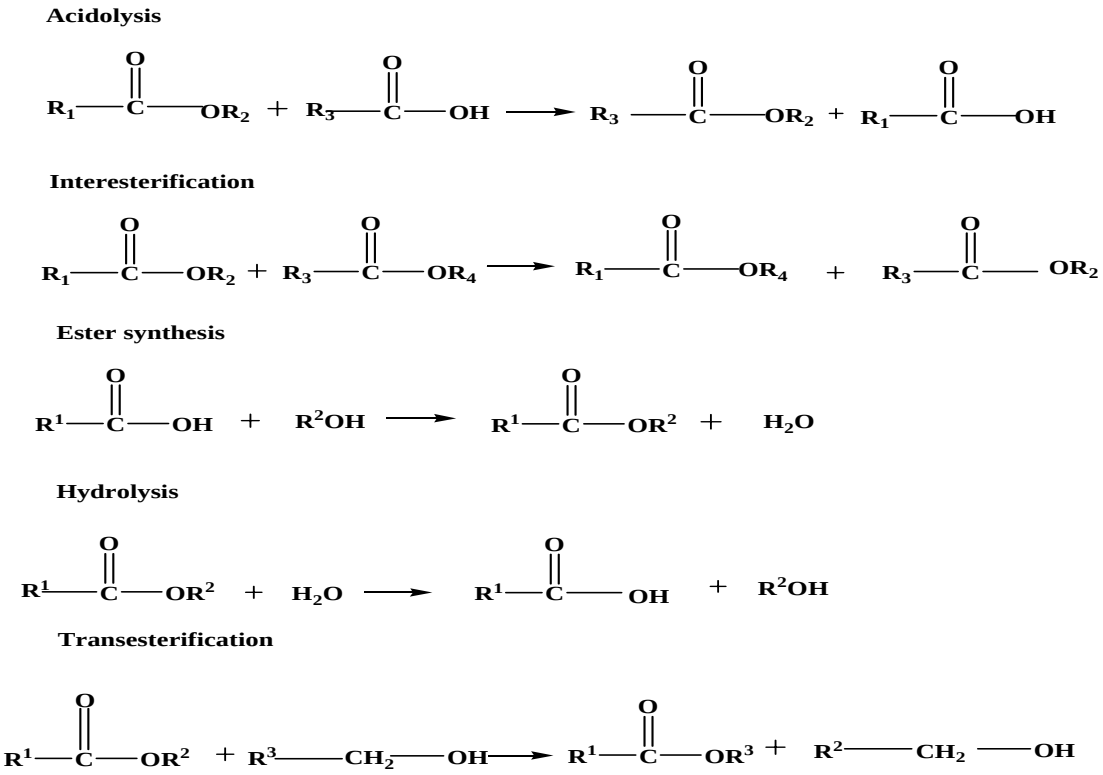


Scheme 1: General equation of hydrolysis reaction.

Esterification:-

Ester formation occurs through a condensation reaction between a carboxylic acid (fatty acid) and alcohol[1]. This reaction being slow and requires acid catalyst like strong acids viz. sulphuric acid, phosphoric acid, organic sulfonic acids and hydrochloric acid.

Reaction of Lipase:-



Scheme 2: Showing types of reaction catalyzed by lipase.

Catalysts:-

Transesterification reactions require catalysts to increase the rate of reaction. Catalyst used in these reaction could be homogeneous or heterogeneous (acid catalyzed, alkali catalyzed, enzyme catalyzed) and generally alkali-catalyzed transesterification is much faster than acid-catalyzed and enzyme catalyzed reactions[1]. However if feedstock used has high free fatty acid and moisture contents, alkali catalyzed reaction leads to the soap formation and in such cases acid-catalyzed transesterification is suitable[2-3]. The acids used as a catalyst could be sulphuric acid, phosphoric acid, hydrochloric acid or organic sulfonic acid and alkalis include sodium hydroxide, sodium methoxide, potassium hydroxide, potassium methoxide, sodium amide, sodium hydride, potassium amide and potassium hydride.

Biocatalysts as lipase are also one of the major catalysts which are explored for their biotechnological applications. Lipase (acylglycerol hydrolyses (HAG) E.C.3.1.13) are a class

of enzymes which catalyzes the hydrolysis of long chain triglycerides to fatty acids, mono-, di- and triacylglycerols and glycerol[4]. Lipases show optimum activity at the oil-water interface thus can be used to produce biodiesel in the presence of water[5]. Lipases have a wide application in industries ranging from food, pharmaceutical, detergents, oleo-chemicals, cosmetics, agriculture, leather, tea and current focus is on biodiesel production industry[6].

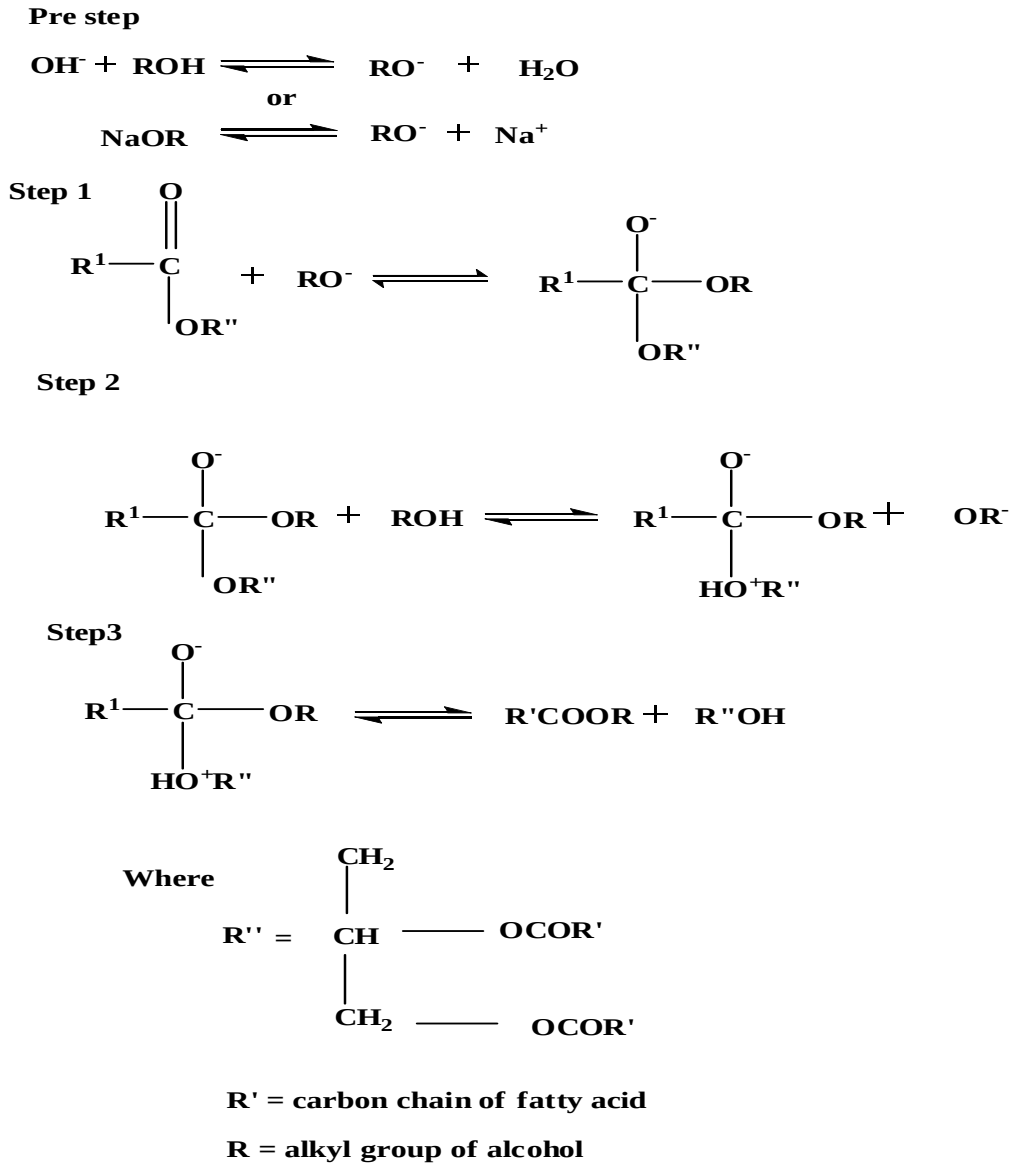
Alkali catalyst

It was observed that transesterification was faster when catalyzed by alkali. The alkaline catalyst reported in literature includes, sodium alkoxides, sodium or potassium hydroxides as well as sodium or potassium carbonates. Sodium methoxide was found to be more effective than sodium hydroxide[7-8] because of the assumption that a small amount of water was produced upon mixing NaOH with MeOH as shown in scheme 3, opposite results were reported.

$$NaOH + MeOH \rightarrow MeONa + H_2O$$

Scheme 3: Reaction between NaOH and MeOH

The alkali catalyzed transesterification of triglycerides is fast and high conversions have been achieved easily at temperatures ranging from 40 to 65 ^{o}C. In addition, alkaline catalysts are less corrosive than acidic compounds. The amount of catalyst that should be added to the reaction varies from 0.5% to 1% w/w[9], but some authors prefer any values between 0.005% and 0.35% w/w[10]. The mechanism of alkali catalyzed transesterification is shown in scheme 4. Sodium hydroxide is widely used as catalyst in large-scale production of biodiesel due to its relatively low cost, though it requires feedstock free from FFAs and moisture contents. The homogenous alkali catalyst has several other drawbacks viz., it produces alkali salt contaminated glycerin and biodiesel which require further treatment for purification[12].



Scheme 4: Mechanism of base catalyzed reaction.

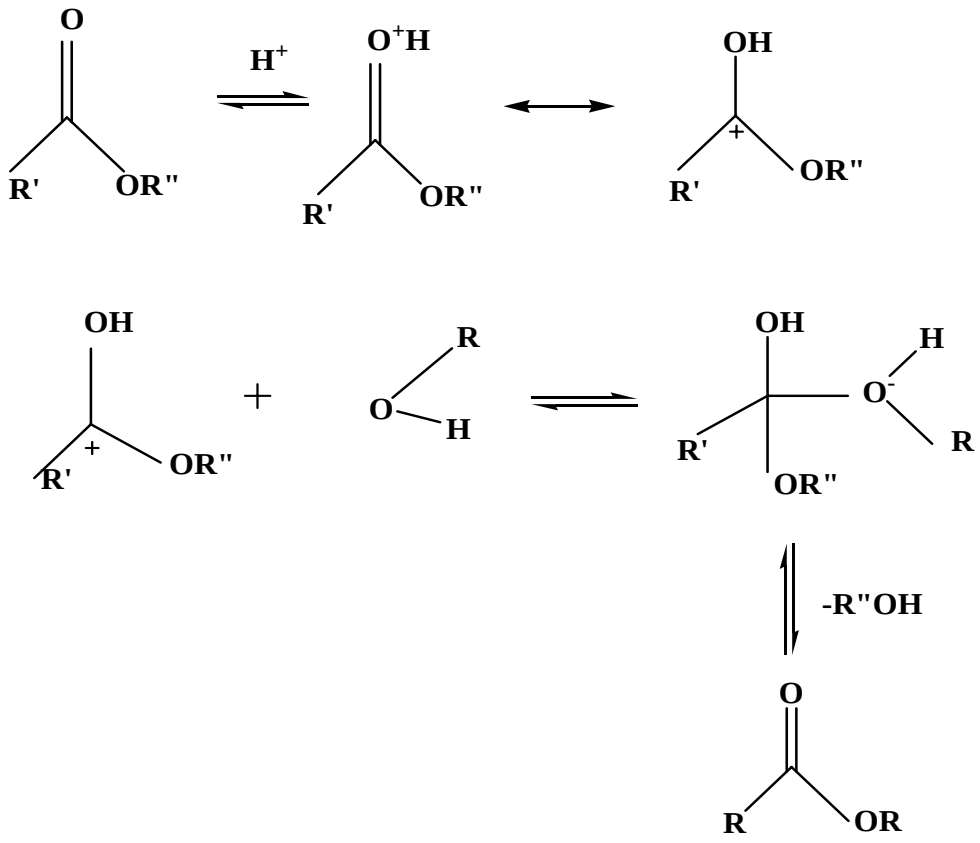
In order to overcome the problem associated with alkali homogeneous catalyst, use of heterogeneous alkali catalyst has been reported in literature[12]. These catalysts simplify the post-treatment of the products (separation and purification), do not produce soaps and could be reused [13].

A large number of heterogeneous alkali catalysts have been reported in the literature, including zeolites, clays, guanidines heterogenized on organic polymers, ion-exchange resins

and metal oxides. The heterogeneous catalysts were used at 5 mol% in contrast to the 3 mol% used with the homogeneous catalyst. Na/NaOH/g-Al_2O_3 heterogeneous alkaline catalysts were applied for the transesterification of soybean oil with methanol using hexane as co-solvent[12]. Monteiro and Cruz studied the transesterification of soybean oil using mixed oxides viz., ZrO_2- SiO_2, KOH/ZrO_2-SiO_2, Co_2O_3-SiO_2, Mo_2O_5-SiO_2, Na_2O-SiO_2, La_2O_3 (10%)-MCM-41, MgO (10%)-MCM-41, BaO (10%)- MCM-41, CaO and MgO as catalysts. However, the heterogeneous catalysts are expensive and their preparation is complicated too, which may require high thermal conditions, which adds to its cost and minimizes their use on industrial scale for the production of biodiesel. Heterogeneous catalysts are moisture and acid sensitive which further refrains them to be a potent industrial catalyst[14].

Acid catalyst

Acid catalysts were used when feedstock has relatively high concentration of FFAs and moisture, as in such cases base catalyzed reaction leads to the formation of soap. Commonly used catalyst involves Brownsted acids viz., sulphuric, hydrochloric and organic-sulphuric acids and among this sulphuric acid is most commonly used acid[15], however, few reports in literature reported the use of sulfonic acid. The mechanism of acid catalyzed transesterification of vegetable oil (for a monoglyceride) is shown in scheme 5.



R' = carbon chain of fatty acid

R = alkyl group of alcohol

Scheme 5: Mechanism of acid catalyzed transesterification

Acid catalyst

Acid catalysts give high yield in esters comparative to the alkali catalyzed or enzyme catalyzed transesterification, but the reaction is very slow, requires temperature above 100 °C. The 99% conversion of vegetable oil in to biodiesel in the presence of 1 mol% sulphuric acid and with a molar ratio of alcohol/oil 30:1 at 65 °C after 50 hrs of reaction period was

reported[3].Acid catalysts are corrosive in nature and this problem can be overcome by using heterogeneous solid acid catalysts. Like heterogeneous alkali catalysts, acids can also be impregnated to solid support viz., hydrous zirconia, alumina, silica and activated carbon. 12–Tungstophosphoricacid on hydrous zirconia as support has been reported in literature to produce biodiesel from low quality canola oil containing 20 % wt free fatty acid [16].

Effect of metal ion on lipase extracted from different species

Proteases from the genus Bacillus have attracted considerable attention due to commercial application of its hydrolytic activity in detergent, food, leather, textile, and pharmaceutical Industries. In addition, proteases have also been employed for synthetic reaction in non-aqueous environment such as in the presence of organic solvent. Though the presence of organic solvent favours the enzymatic synthesis of peptide bonds, it has a limitation of solubility and stability of protease. Therefore, methods have been developed for stabilizing enzymes in the presence of organic solvents. Microorganisms tolerant to organic solvents and also producing enzymes tolerant to these solvents have been isolated[17].The solvent-tolerant microorganisms can provide opportunities for whole-cell biotransformation in the presence of organic solvents, and the enzymes they produce are likely to exhibit natural resistance to organic solvents as well. Organic solvent-tolerant bacterial strains can also be employed for degradation of hydrophobic organic solvents present as pollutants. So far, most of the work on the production of enzymes and the mechanism for organic solvent tolerance has been conducted on Pseudomonas sp. On the contrary, Bacillus sp., which is traditionally used for commercial production of protease, has not been employed extensively. The effect of metal ions on the hydrolytic activity of protease was studied by adding different divalent ions to the buffer before measuring the activity of the enzyme. The inclusion of Ca^{2+} resulted in an activation of 70% protease activity, while Mn^{2+} and Mg^{2+}at the same concentration had lowered the activity by 20% and 15%, respectively. On the other hand, Zn^{2+} and Cu^{2+} had an inhibitory effect leading to a decrease in activity by 30% and 70%, respectively.

An extra-cellular lipase produced by *Bacillus licheniformis* MTCC 6824. The crude lipase extract had an activity of 41.7 LU/ml of culture medium when the bacterium was cultured for 48 h at 37 °C and pH 8.0 with nutrient broth supplemented with sardine oil as carbon source. The pure enzyme is a monomeric protein and has an apparent

molecular mass of 74.8 kDa. The lipase had a V_{max} and K_m of 0.64 mM/mg/min and 29 mM, respectively, with 4-Nitrophenylpalmitate as a substrate, as calculated from the Line weaver–Burk plot[18].The lipase exhibited optimum activity at 45 °C and pH 8.0, respectively. The enzyme had half-lives ($T_{1/2}$) of 82 min at 45 °C, and 48 min at 55 °C. The catalytic activity was enhanced by Ca^{2+} (18%) and Mg^{2+} (12%) at 30 mM. The lipase was inhibited by Co^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} even at low concentration (10 mM).

Lipase (EC.3.1.1.3) from *Candida* sp. 99-125 was separated into four isoforms (isoform A, isoform B, isoform C, and isoform D) by two steps of ion- exchange chromatography. The isoforms had a little distinct optimum temperature in the range of 20–35 °C, and the same optimum pH (8.0)[19]. They remained to be active in methanol, ethanol and ethylene glycol at the concentration of 10% and 20% (v/v) and acetone at the concentration of 10% (v/v), and sensitive to EDTA. The metal ion Ca^{2+} and Mg^{2+} had mild effect on lipase activity.

Purification and characterization of an intracellular lipase produced by *Rhizopus chinenesis* cultured in solid-state fermentation was investigated. Through four successive purification steps, the enzyme was purified to homogeneity with an apparent molecular mass of 36 kDa. The lipase was active for pH between 7.0 and 9.0 and temperatures[20] 20-45 °C. Lipase activity was slightly increased in the presence of Ca^{2+} and Mg^{2+}, but strongly inhibited by Hg^{2+}.

Objective:-

To study the effect of metal ion (NaCl and KCl) on lipase catalyzed hydrolysis reaction and their comparison with pure enzyme.

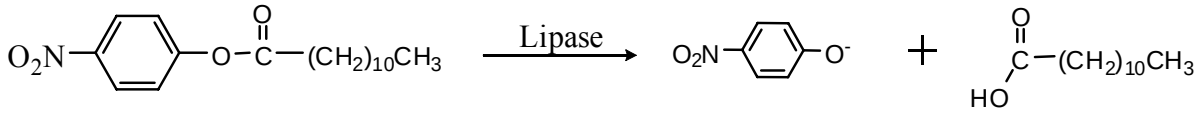
Material and methods:-

P-Nitrophenyllaurate (98%, GC) and lipase enzyme (activity 187 U/gram) of *Aspergillus Niger* were obtained from Sigma Aldrich (USA); NaCl (99.5 %), KCl (99.5%) Sodiumphosphatemonobasic and sodiumphosphatedibasic (98%) were obtained from LOBA-Chemie.All the chemicals and solvents were used as received without any purification or recrystallization.Absorption spectra to determine the kinetic parameters were recorded on double beam UV-Visible spectrophotometer (PERKIN ELMER LAMDA 35) and data were saved on a computer attached with the instrument using PERKIN ELMER UV WINLAB software for further analysis. Insoluble material from the lipase solution was removed by using centrifuge (Superspin R-V/F_M Plasto Crafts) at 5,000 rpm. Software Origin Pro 7.5 and Microsoft excel 2007 was used to draw the curves and was fitted using the protocols available with the software.

Experimental work:-

Preparation of ***p***-nitrophenyllaurate solution

The stock solution of the substrate, *p*-nitrophenyllaurate (*p*-NPL), of concentration 0.1 M was prepared in pure ethanol and stored in refrigerator. This solution was used for further dilutions in order to make the final solution of appropriate concentration.



P-nitrophenyllaurate (colourless) *p*-nitrophenolate (yellow)

Scheme 6: Hydrolysis of *p*-nitrophenyllaurate to *p*-nitrophenolate.

p-nitrophenyllaurate has been taken as model compound for triglyceride ester as it has the ester bond and upon hydrolysis yielded *p*-nitrophenol as shown in scheme 6, which is chromogenic (λ_{max} = 405nm) and could be followed easily by visible spectroscopy.

Preparation of phosphate buffer solution

Solutions of 0.2 M sodiumphosphatemonobasic and 0.2 M sodiumphophatedibasic were prepared in distilled water and mixed to prepare 0.1 M buffer of pH 8. To prepare 100 ml of phosphate buffer of pH 8, 47.35 ml of 0.2M sodiumphosphatedibasic and 2.65 ml of 0.2 M sodiumphosphatemonobasic were added and the volume was made upto 100 ml. The pH of the buffer was measured by pH instrument and adjusted, if required, by adding HCl or NaOH of 0.1M.

Preparation of enzyme solution

The solution of lipase enzyme of *Aspergillus Niger* was prepared of concentration 4mg/ml by stirring 80 mg of enzyme in 20 ml distilled water for 30 mins at room temperature. Solution, so prepared, was centrifuged for 5 min at 5000 rpm and clear supernatant lipase solution were collected and stored in refrigerator for further use.

Preparation of diluted metal salts

The solutions of 10 mM concentration of inorganic salts NaCl, KCl were prepared by dissolving the appropriate amount in distilled water. All the solutions were stored in refrigerator for further use.

Hydrolysis of *p*-NPL by pure lipase enzyme

The reaction mixture was prepared in glass cuvette by mixing 0.5ml phosphate buffer of 0.1 M concentration (pH 8) with 1.2ml *p*-nitrophenyllaurate of varying the concentrations from $1X10^{-4}$M, $5X10^{-3}$Mand $5X10^{-4}$M, 0.5 ml ethanol and then 0.7 ml enzyme of 4mg/ml concentration was added to it. Absorption spectra of this reaction mixture were recorded in the range of 350 to 450 nm against a blank having 1 ml buffer, 0.7 ml enzyme and 1 ml ethanol. Product, *p*-nitrophenol, formed in this reaction shows maximum absorbance (λ_{max}) at 405 nm and a plot between absorbance vs. time shows the progress of reaction with the passage of time. Concentration of *p*-nitrophenol formed during the reaction was calculated from the absorbance (λ_{max} = 405 nm; ε = 14,080 $M^{-1}cm^{-1}$) and plotted against time to calculate the initial rate of reaction.

Result and discussion:-

Progress of Enzyme-Substrate reaction

The progress of the hydrolysis of *p*-nitrophenyllaurate were monitored by recording the absorption spectra in the wavelength range 350 to 450 nm.Formation of *p*-nitrophenolate was observed by the increase in the absorbance at 405 nm as shown in figure 6 to 7.

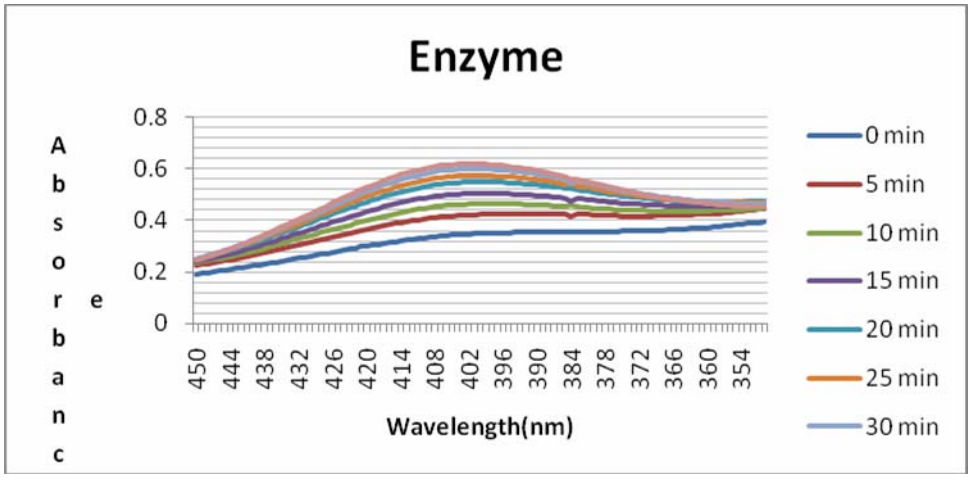


Figure:-6 Progress of lipase catalyzed hydrolysis reaction of p-nitrophenyllaurate.

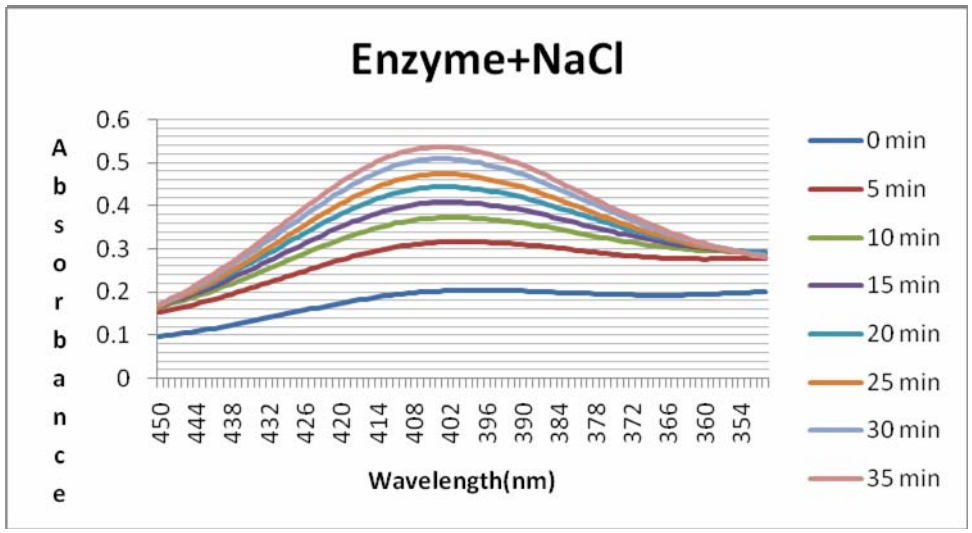


Figure:-7 Progress of lipase catalyzed hydrolysis reaction of *p*-nitrophenyllaurate in the pressence of NaCl.

Absorbance Vs Time graph

A plot between maximum absorbance and time interval is plotted at λmax is equal to 405 nm. From graph between maximum absorbance and time at different intervals shows that absorbance increase with respect to time in each case as shown in figure 8 to 9.

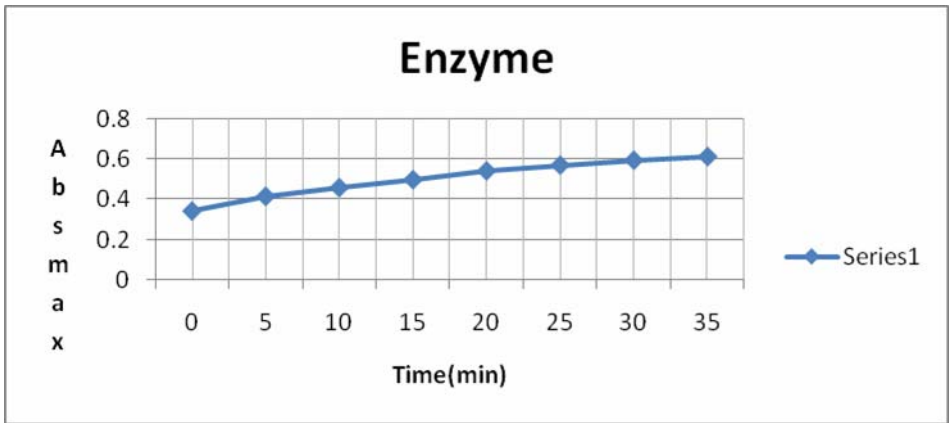


Figure:-8 Graph showing increase in absorbance of non-treated lipase.

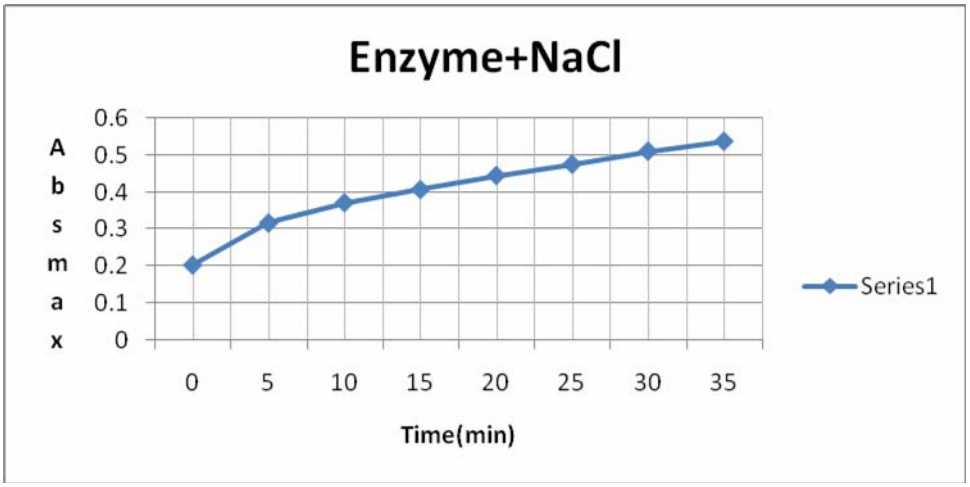


Figure:-9 Graph showing increase in absorbance when lipase treated with NaCl.

Conversion of absorbance into concentration of p-nitrophenol:-

The rate of formation of product (*p*-Nitrophenol) was monitored by change in absorbance, which was further converted into concentration following the conventional Beer- Lamberts

law for absorbance. The formula used for converting absorbance to concentration of product formed

$$A = \varepsilon c l$$

Where A = absorbance at λ_{max}

ε = molar extinction of coefficient of *p*-nitrophenol taken as 14080 M^{-1} cm^{-1}.

l = path length of light traveled is 1 cm i.e. width of glass cuvette.

Concentration and time graph shows (figure10 to 11) that as time interval increases the conversion of p nitrophenyllaurate to *p* nitrophenol also increases. From concentration time graph slope the initial rate for the formation of *p*- nitophenol has been calculated and this initial rate has been used while plotting the Line weaver-Burk plot.

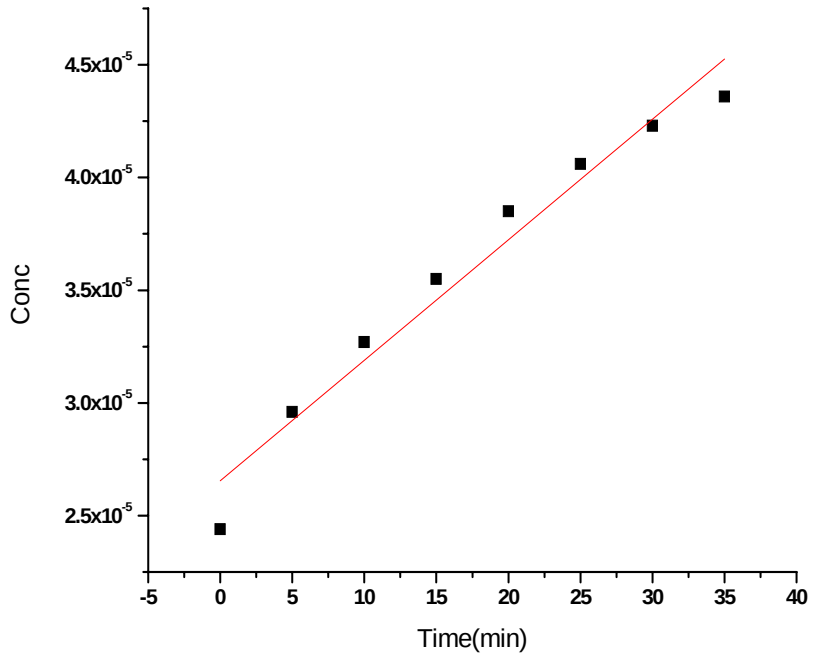


Figure:-10 Increase in concentration of product of non-treated lipase.

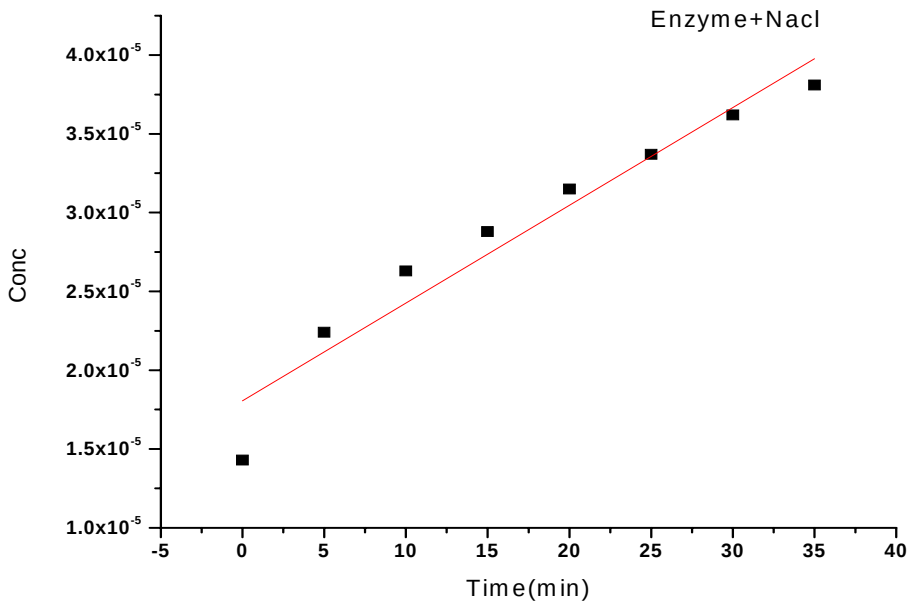


Figure:-11 Increase in concentration of product when lipase treated with metal ion.

For first order reaction:-

First order rate equation:

$$k=1/t \ln (a/a-x)$$

For calculating first order reaction formula used:-

$$k=1/t (\ln A_0-A\infty/A_t-A\infty)$$

Where A_0 =absorbance at time initial time (i.e. 0).

$A\infty$= absorbance at maximum time.

A_t= absorbance at different time interval.

In almost all the graph a straight line is obtained which shows that substrate which is p - nitrophenyllaurate follows first order kinetics as shown in figure 12 to 13.

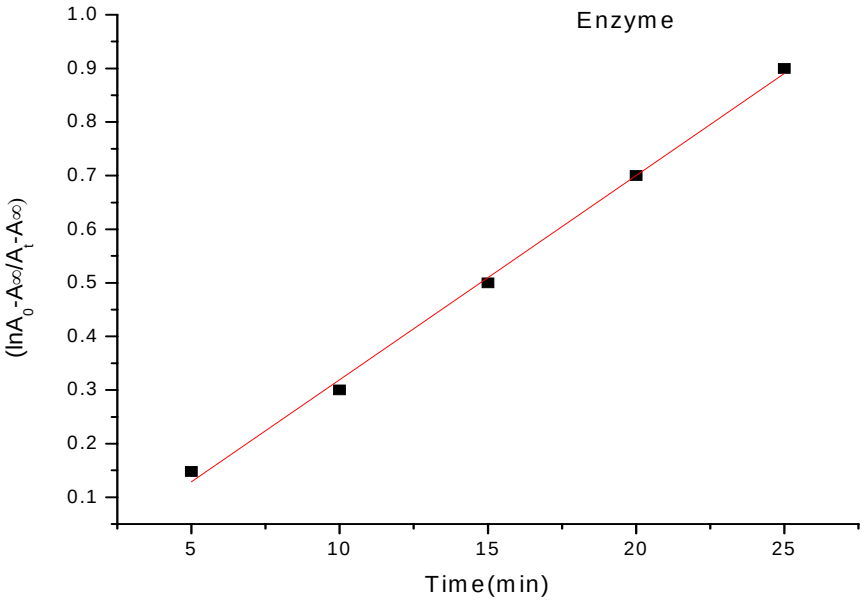


Figure:-12 First order rate curve for non treated lipase.

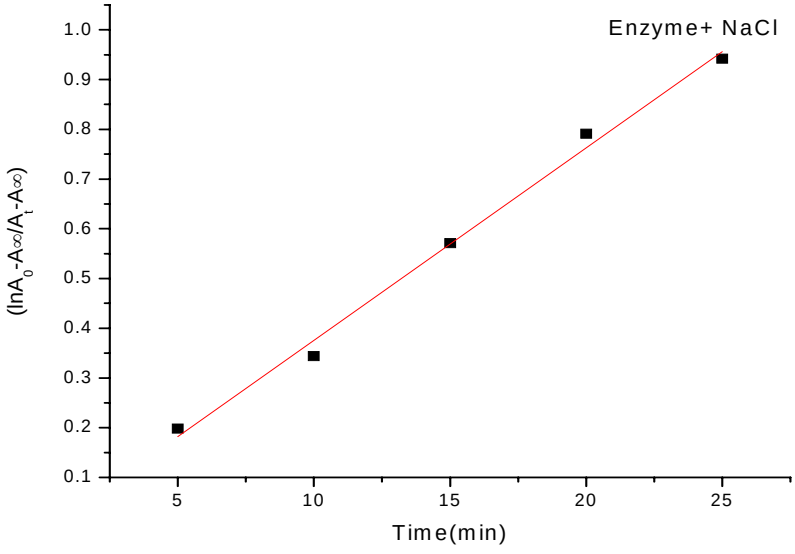


Figure:-13 First order rate curve for lipase treated with NaCl.

Line weaver-Burk plot:-

The Line weaver-Burk plot is plotted between inverse of initial rate and inverse of substrate concentration. From this we are able to calculate value of Vmax from intercept of Y-axis and value of Km from intercept of X-axis. The K_M and V_{max} were calculated from Line weaver-Burk plot by extrapolating the line till negative X-axis and the points where this line cuts on X and Y axis were represented as $-1/K_M$ and $1/V_{max}$ respectively Kcat is calculated from Vmax / Enzyme conc. in M.

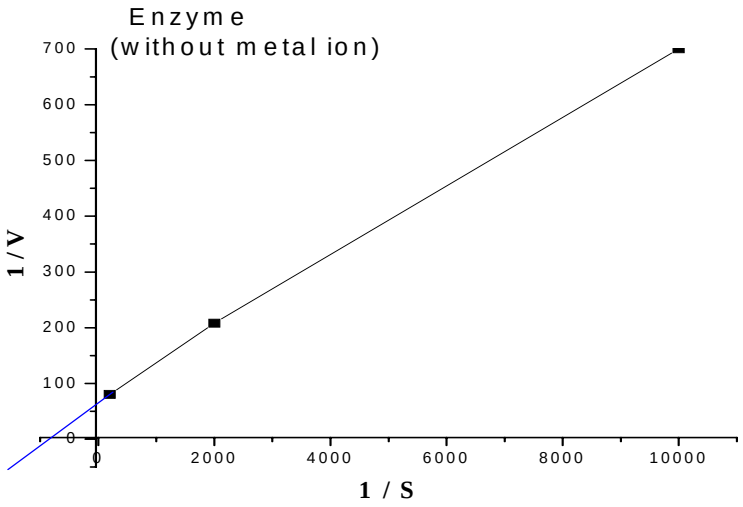


Figure : 14 Line weaver-Burk plot of lipase enzyme for the *p*-NPL hydrolysis

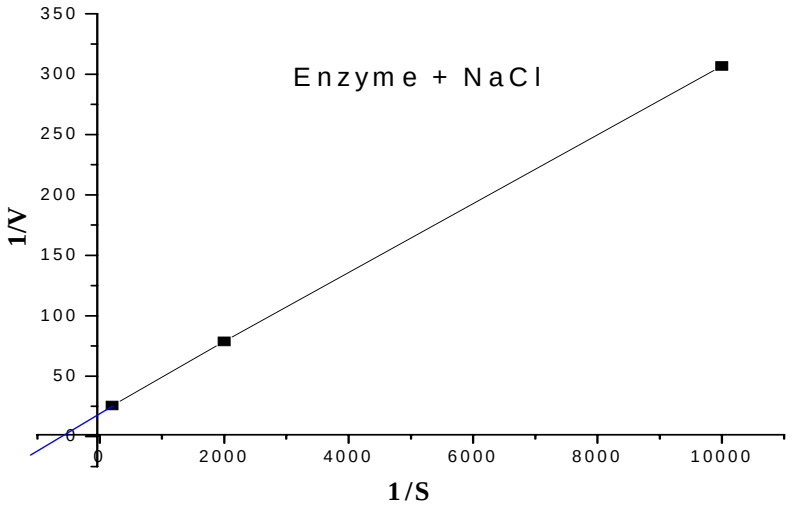


Figure:15 Line weaver-Burk plot of lipase enzyme for the *p*-NPL hydrolysis in presence of NaCl.

Table 2: Comparison of the of Km and Vmax between pure enzyme and in the presence of Na^+ and K^+.

Metal ion	Km (M)	Vmax (M/min)	Kcat (min^{-1})
Nil	0.002	0.0598	0.679
KCl	0.00030	0.0167	0.189
NaCl	0.0011	0.0156	0.1772

From Line weaver-Burk plot we are able to obtained value of Km and Vmax. As we compare the value of Km and Vmax of enzyme (without metal ion), KCl and NaCl the value of Km and Vmax decreases with respect to enzyme .This shows that NaCl and KCl shows

uncompetitive effect so in presence of metal ion the activity of enzyme-substrate reaction decrease .The enzyme posses particular kind of active site where substrate bind and get converted to product. Any distortion or modification of active site led to loss of catalytic activity of enzymes. So it may say that with binding of NaCl or KCl there may be some modification in the active site of enzyme due to which inhibition of reaction takes place, however detailed studies are require to understand the actual case of the decrease of the rate of reaction in the presence of Na^+ and K^+.

Conclusion:-

The hydrolysis of *p*-nitrophenyllaurate to *p*-nitrophenol was catalyzed by lipase enzyme which cleaves the ester bond present in the substrate. When we plot a graph between absorbance and time, with increase in time increase in absorbance is observed in all cases as we move from conc. $5X10^{-4}$ to $5X10^{-3}$M. Similar trend is found when plot between conc. and time is considered. As, we plot (lnA_0-$A\infty$/A_t-$A\infty$) and time a straight line is obtained which shows that substrate i.e. *p*-nitrophenyllaurate shows first order kinetic.

The functional/ catalytic activity of an enzyme is attributed to its structure and conformational properties and the tertiary structure of an enzyme results into a defined spatial arrangement. The enzyme possesses a characteristic active site where the substrate binds to the enzyme and gets converted into product and any modification or distortion to the active site may result (in the present case possibly due to enzyme-metal ion interactions) into the loss of catalytic activity of the enzyme.

The metal ions, being small in size, can probably bind to the lipase enzyme changing its conformational properties and distorting the active site thus inhibiting its activity. So it could be proposed that binding of NaCl or KCl causing some changes in the active site of enzyme due to which inhibition of reaction takes place, however detailed studies are require to understand the actual case of the decrease of the rate of reaction in the presence of Na^+ and K^+.

References:-

1. H.N. Basu and M.E. Norris, *US Patent 5525126*, 1996.

2. F.J. Spurles and D. Price, *US Patent 2*, 1950, 366-494.

3. B.Freedman, E.H.Pryde and T.L.Mounts, *Journal of American oil chemist society*, 1984, **61(10)**, 1638-43.

4. A.Marek and W. Bednarski, *Journal of Biotechnology Letters*, 1996, **18 (10)**, 1155-1160.

5. A.G.Marangoni, *Journal of Biochemistry Biophysics Resource Communission* 1994, **200**, 1321-1328.

6. R.Gulati, J.Isar, V. Kumar, A.K. Prasad, V.S.Parmar and R.K.Saxena, *Journal of Pure Applied Chemistry.* 2005, **77(1)**, 251-262.

7. B.Freedman, R.Butterfield and E.Pryde, *Journal of American oil chemist society*, 1997, **74 (11)**, 1457-63.

8. L.Hartman, *Journal of American oil chemist society*, 1956, **33**, 129-132.

9. B.K.Barnwal and M.P.Sharma, *Renewable Sustained Energy Review*, 2005, **9(4)**, 363–78.

10. F Ma, and M.A.Hanna, *Journal of Bioresource Technology*, 1999, **70**, 1-15.

11. H.Fukuda, A.Kondo and H.Noda,*Journal of Bioscience Bioengineering*, 2001, **92(5)**, 405–416.

12. H.J.Kim, B.S, Kang, M.J.Kim, Y.M.Park, D.K.Kim, J.S.Lee and K.Y.Lee, *Journal of Cataysis. Today*, 2004, **93**, 315.

13. G. Vicente, M.Martínez and J.Aracil, *Journal of Bioresouce Technology*, 2004, **92**, 297.

14. W.Xie, H.Peng and L.Cheng, *Journal of molecular catalysis*, 2005, **246**, 24-32.

15. B.Freedman, R.Butterfield and E.Pryde, *Journal of American oil chemist society*, 1997, **74 (11)**, 1457-63.

16. M.G.Kulkarni, R.Gopinath, L.C.Meher and A.K.Dalai, *Journal of Green chemistry*, 2004, **8**, 1056-1062.

17. R.Sareen and P. Mehra, *Journal of Applied Microbial Biotechnology*, 2008, **79**,399-405.

18. K.Chakraborty and R.PaulRaj, *Journal of Food Chemistry*, 2008, **109**,727-736.

19 F. Dayan, Y. Mingrui, T. Tanwei and Z. Xiz, *Journal of Molecular Catalysis*, 2009, **56**, 115-121.

20. S.Y.Sun, X.Yan and W. Dong, *Journal of Chemical Technology and Biotechnology,* 2008, **84**, 435-441