Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

Biochemical Characterization of Extracellular Lipase from an Improved Strain of *Penicillium Citrinum* KU613360.

LAKKAKULA BHAGYA LAKSHMI[1], AMRUTHA V AUDIPUDI*
[1] Freshman Engineering Department, Lakireddy Bali Reddy College of Engineering, Mylavaram, Krishna District, Andhra Pradesh
*Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur District, Andhra Pradesh

*Corresponding author Email ID: *audipudiamrita@gmail.com*

Abstract:

In the present study lipase purified from an improved strain of ***Penicillium citrinum*** **KU613360** *was subjected to biochemical characterization. The lipase showed maximum activity at optimum pH of 6.5 andtemperature 40°C respectively.It wasobserved that lipase showed specificity for long chain fatty acids (Triolein (C18;1) and Trilinoclenin (C18; 3)) than short chain fatty acids.The metal ion stability studies indicate that the lipase activity was maximum with* Mn^{2+}, Ca^{2+}, Mg^{2+} *(1mM and 10mM)and the lipase* activity *is inhibited by Hg and Zn.The kinetic parameters of improved strain of****P.citrinum*** were *also evaluated and the recorded value of* K_m *was found to be 19.33±0.010mM and* V_{max} *was 83.33±0.06 (µmole* $min^{-1}mg^{-1}$*) respectively. Thus, from our results it was concluded that the improved strain has considerable capability and potentiality for the industrial applications.*

Key words: Lipase, ***Penicillium citrinum,***mutated strains, medium, Optimization.

Introduction:

Various microscopic classes of organisms of prokaryotic and eukaryotic origin (bacteria, yeast,moulds and few protozoan's)were identified to exudes lipase enzyme for digestion of lipid compounds (Luciane *et al.,* 2015; Sarma*et al.,* 2016).Among themfilamentous fungi are identified as good sources of extracellular lipase for mass production and are believed to be having many industrial applications.Many species belonging to *Mucor, Rhizopus, Aspergillus, Geotrichum* and *Pencillium* are broadly indentified as excellent sources for lipase production. (Nema *et al*., 2019; Contesini *et al.,* 2010).The copious industrial purposes of lipases have stirred along termsignificance in isolation of new forms of lipases from novel fungal sources and robust efforts have been shown on the modular engineering of enzyme with preciseactivities or capable of showing improved performance for industrial applications (Mehta *et al.,* 2017).Not only at the industrial application has the lipases gained its own

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

implications in bioremediation technology in varied environment (Balaji*et al.*, 2014; Kargir and Rao, 2011). Industrial and domestic waste harbour fungal species of greater potential in degrading fats and oils. Besides waste disposal, bioconversions by fungal activities results in the production of vast number of useful substances. Thus, unused and vast quantity of waste can be transformed into a useful resource for further use(Gopinath, 2013). Keeping all the above factors in mind and wide prospective of lipase enzyme, an attempt was made to analyse the biochemical parameters ofpurified lipase isolated from a mutant strain of ***Penicillium citrinum*KU613360.**

Materials and Methods:

Microorganism and Lipase production: The experimental fungal strain used in the present work was isolated from vegetable oil contaminated soil samples collected from the oil spill mills near Guntur District, Andhra Pradesh, India. The qualitative screening for hydrolytic activity of the lipids was done on Olive oil-Rhodamine B Agar and tributryin Agar medium. The micro-morphology of the isolated fungal strain was studied by viewing lactophenol cotton blue wet mount preparation. Based on the data, the isolate was assigned to the genus *Penicillium*. Confirmation of the assigned toxon was carried out using 18S r RNA gene sequence analysis.

The Sequencing analysis was carried out by Macrogen, South Korea. The sequence obtained was initially analyzed at National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (Blast) and phylogenetic tree was constructed to identify the isolate. Based on the similarity studies, the sequence of the isolate showed 97.4% similarity to *Pencillium citrinum.* Then, the sequence of the identified species was deposited in Genbank with accession number KU613360. To enhance the lipase activity, mutational analysis was carried for the isolated strain ***Penicillium citrinum*KU613360**. The strain improved by the combination of UV and Et. Br treatment (Data not shown here) was selected used in these further experiments.

Lipase assay and estimation: Lipase activity was assayed quantitatively by titrimetric method using olive oil as substrate and by measuring the liberated fatty acid as described by Burket *et al.,* (2004) with slight modifications. The reaction mixture (5ml) containing 1 ml olive oil (emulsified with 7% W/V gum Arabic in 50 mM phosphate buffer pH 6.5) as substrate. The reaction was initiated by adding 1ml of appropriately diluted enzyme solution to the reaction mixture and incubated 15 min at temperature 37 ± 2°C. The reaction was

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

stopped by adding of 15 ml of acetone:ethanol (1:1 v/v). The released free fatty acid was titrated with 0.05 N NaOH to a thymolphttalein end point. Control was made with similar conditions without the inoculation of enzyme source. One IU of lipase was defined as the enzyme that liberates 1 µmol of fatty acid per min at 37°C ±2, pH 6.5.

Biochemical characterization and enzyme kinetics

The mutated strain which showed improved enzyme production (data not given) was grown on optimized medium containing Peptone (0.7gm), fructose (0.5 gm) and inducers [Lin seed oil (5.0%w/v)], and substrate like NPP (p-nitrophenyl Palmitate (C16:0), in presence of 2mM Mn^{2+} metal ion, incubated at a temperature of 40°C and pH of 6.5 for 96 hrs. Then, the purified enzyme, after salt precipitation, dialysis, Ion exchange chromatography (DEAE-Sepharose) and Gel chromatography (Sephacryl S-100 column), wasthen subjected to biochemical characterization and study of enzyme kinetics by applying the following steps.

a) Effect of pH and pH stability: The effect of free enzyme, isolated from mutated ***P. citrinum***strainwas by subjected to pH, in the range of 4.0-10.0 at 55°C in the improved medium by using olive oil as substrate. A relative activity was calculated as the ratio of the activity of free enzyme measured at different pH to the activity of enzyme at the standard conditions (pH 8.0, 55°C). Free Enzyme stability was also determined in the improved medium by exposing free lipase to pHs in the range of 4.0-11.0 for 1 hr at 4°C, by using four different buffers: 50mM Glycine-HCl (pH3.0-4.0), 50mM sodium acetate (pH 5.0-6.0), 50mM Phosphate buffer (pH7.0), 50mM Tris-HCl (pH8.0-10.0). Residual activity was calculated as the ratio of the activity of lipase enzyme measured after incubation to the activity of the enzyme under standard condition.

b) Effect of Temperature and Temperature stability: To indentify the effect of temperature lipase and its stability, the experiment was carried out in the range of 30-90°C at standard pH 8.0 using olive oil as substrate in improved medium. A relative activity was calculated as the ratio of the activity of free enzyme measured at different temperatures to the activity of enzyme at the standard conditions (pH 8.0, 55°C). Free Enzyme thermo-stability was also determined in the improved medium by exposing free lipase to temperature (40-90°C) for 1 hr. The enzyme was then cooled to room temperature and activity was measured under standard conditions. Residual activity was calculated as the ratio of the activity of lipase enzyme measured after incubation to the activity of the enzyme under standard condition.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

The first order thermal deactivation constant (k_d) and half-life ($t_{1/2}$) for free enzyme was calculated by using the equations (i) and (ii)

$$\ln A = \ln A_0 - k_d t \text{----------(i)}$$

$$t_{1/2} = \ln 0.5 / -k_d \text{----------(ii)}$$

In addition, the free enzyme of activation (ΔG_d) for the deactivation process was calculated from equation **(iii)**

$$\Delta G_d = -RT \ln [k_d h / k_B T] \text{……(iii)}$$

Where k_d is the deactivation constant (h^{-1}). k_B Boltzmann's constant (1.38 x 10^{-23} jK^{-1}), h planck's constant (1.84 x 10^{-37}), R the gas constant (8.314 j mol^{-1} K^{-1}) and T the temperature (K)

c) Effect of Metal ions and its Concentrations:

Different metal ions like Ba, Ca, Co, Cu, Fe, Hg, Mg, Mn, Ni, and Zn were tested with the concentration of 1mM and 10 mM on the activity of the mutated strain of ***P. citrinum***by keeping all the other optimum conditions stable.

d) Kinetic analysis of free enzyme:

The Michaelis –Menten (MM) equation was used to correlate the dependence of enzyme activity on substrate concentration for free lipase enzyme. The kinetic parameters of V_{max} and K_m was derived from MM model and estimated from the experimental data by using non-linear regression function in Graph Pad PRISM software.

$$V = V_{max} S / K_m + S$$

Where, V_{max} is the highest value of lipase activity (IU mg^{-1}) and K_m half – saturation constant (mM) determined from substrate concentration (S) that gives a ½ V_{max} of free lipase isolated enzyme(Dheeman *et al.*, 2011).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

Results and Discussion:

Enzyme purification:

The enzyme produced after optimization of the mutant strain on the culture conditions are subjected to purification methods and molecular mass was estimated by SDS PAGE analysis and was found to be 35kDa (data not shown here)and was studied for biochemical characterization and enzyme kinetics study.

Biochemical characterization of lipase:

i) Effect of pH and pH stability:

The optimum pH of purified lipase from mutant strain ***P. citrinum***was assayed at pH 4-10 by both standard p-NPP spectrophotometric and tritrimetric assay. The pH 6.5 was found to be the optimum pH at which the lipolytic activity was maximum followed by a gradual decrease in the enzyme activity with the further increase in the pH (Fig 1).The enzyme showed good stability retaining more than 80% of its activity in the pH range 6-8.5 after 24hrs (Fig 1). This may be due to the alterations in the protein active domains and ionization in the protein molecule and thus reducing the availability of active sites for substrate binding. The observations are in agreement with work done on other fungal lipases by Dheeman *et al.*,(2011) and Tako *et al.*,(2017). Jayaprakash and Ebenezer,(2012) andPera *et al.*,(2006) also reported similar observations regarding the pH stability of fungal lipases from *Aspergillus japonicus*and*A. niger*, respectively.

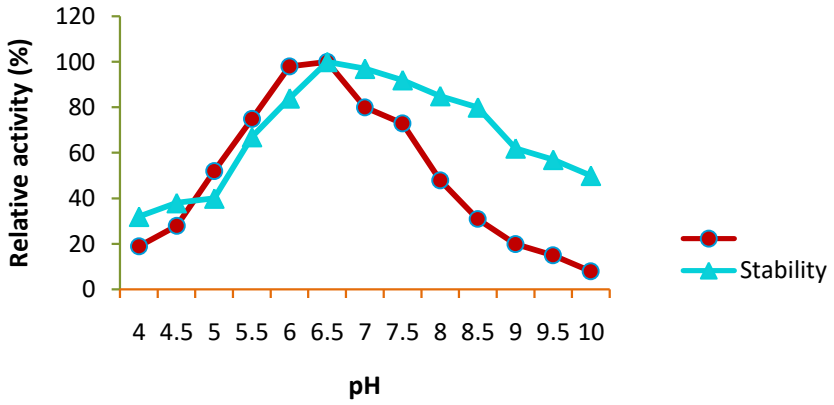

Fig 1 : Optimum pH and pH stabilityfor Lipase activity

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

Effect of Temperature and Temperature stability:

In order to determine the optimum temperature of the enzyme, the isolated lipase enzyme was incubated at different temperatures ranging from 20°C to 60°C. The temperature optimum was observed to be 40°C and retained 100% of initial activity after 1hr incubation in the temperature range 40-50°C, above 50°C to 60°C, upon incubation the enzyme activity declined. **(Fig2a**).The observation and recorded values are in correlation with the study and results obtained from other *Penicillium*species (Ferrer *et al.*, 2000; Dheeman etal., 2011).Similar reports are also published with various other fungal strains like *A. japonicus* (Jayaprakash and Ebenezer,2012), *T.harzianum* (Ulker *et al.*, 2011) and *A.niger* (Pera *et al.*, 2006; Sarkar and Laha,2013). To confirm the tolerance of free lipase towards high temperatures, investigation was carried out on the thermal stability of free enzyme. The free enzyme lipase was incubated at various temperatures (40-90°C) for 1hr and the residual activity was measured. Free lipase enzyme retained 100% of initial activity after 1 hr incubation in the temperature range 40-50°C. Above 60°C-70°C, upon incubation for 1 hr, the free lipase enzyme was completely inactivated. At 50°C the lipase displayed a significant thermal stability with residual activity of 35% (Fig 2b).Thus, the high activity and stability of the purified lipase from mutant strain of ***P. citrinum*KU613360** can be used as a potential biocatalyst in processes operating at high temperatures.

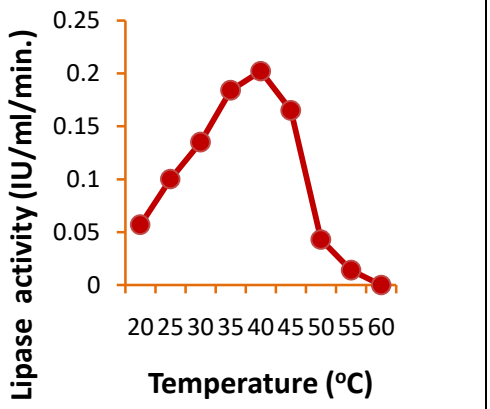

Fig 2a : Thermal stability of the Lipase activity

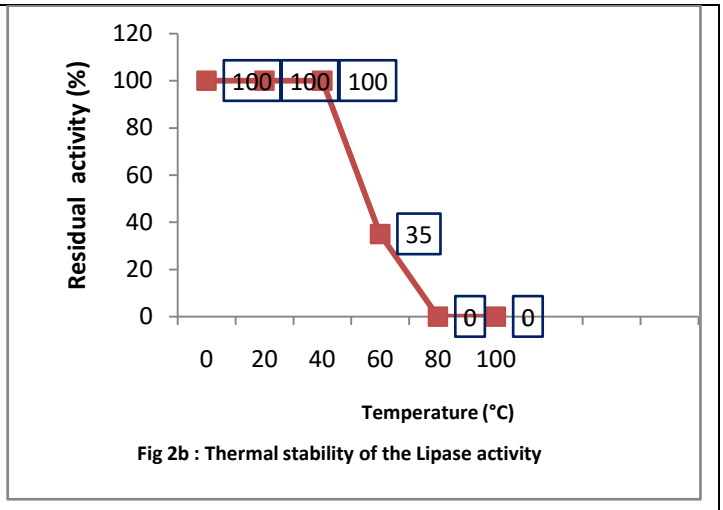

Fig 2b : Thermal stability of the Lipase activity

Time-dependent thermal inactivation of free enzyme:

To investigate the effect of free enzyme stability the purified enzyme was incubated with different time intervals at 40°C and their residual activity was determined **(Fig 3).** The lipase

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

after 60 min, the enzyme in solution lost its activity. The enzyme deactivation constant (*Kd*) was calculated from the slopes of the best fit curves obtained by exponential regression when residual activity was plotted against pre incubation time. The deactivation of free lipase enzyme obeyed first order exponential deactivation kinetics .reaction. The 1.22 *Kd* (h^{-1}) and t ½ (hr) was calculated from the graph **(Fig 3; Table 1)**as 0.55 .

Table 1: Time-dependent thermal inactivation of free enzyme:

Enzyme form	*Kd* (h^{-1})	t(1/2)	ΔG_d (KJ mol-1)
Free Lipase	1.06	0.55	106.15

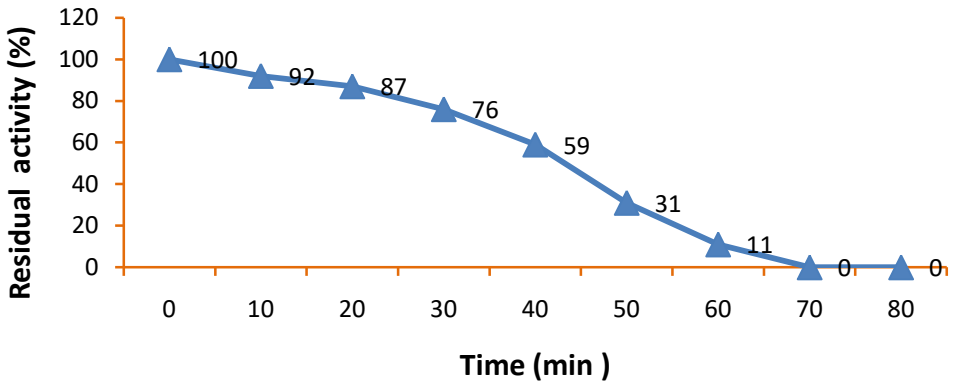

Fig 3: Time dependent thermal stability of Lipase activity

Effect of Substrate specificity on lipase activity:

Various types of substrates specificities among lipases will contribute for differences in the geometry and size of their active domains (Aloulou *et al.,* 2006).Many of the lipases reported from *penicillium* strains prefer short chain fatty acids triglycerides or their methyl esters (Bancerz *et al.,* 2005; Song *et al.,* 2008). However the lipase obtained from the mutated stain of ***P. citrinum*KU613360** showed specificity for long chain fatty acids triglycerides, which suggests the potentiality of the enzyme in bioremideation applications and food industries (Rodrigues et al,2010; Dheeman *et al.,* 2011)**(Table 2)**. Similar results are also reportedby Jayaprakash and Ebenezer, (2012)on*A.japonicus* strain.

Table 2: Effect of substrates (Triacylglycerol and fatty acids methyl esters) on lipase activity.

Substrates	Concentrations	Relative activity (% ±SD)

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

	(mM)	
Triacyl glycerols		
Triacetin (C2:0)	**20**	**32.53±2.17**
Tributryin (C4:0)		**49.51±2.24**
Tricaprylin (C8:0)		**56.78±2.84**
Triolein (C18;1)		**131.91±4.01**
Trilinolein (C18:2)		**90.39±2.07**
Trilinoclenin (C18:3)		**92.98±3.08**
Fatty acids methyl esters		
Caproic acidmethyl esters (C6:0)	**20**	**4.92±1.03**
Lauric acid methyl esters (C20:0)		**5.25±1.18**
Stearic acid methyl esters (C18:0)		**9.92±1.01**
Oleic acid methyl esters (C18;1, *Cis*-9)		**12.25±2.30**

Effect of metal ions on lipase activity:

The effect of metals ions was tested at 1 and 10mM concentration on thepurified enzyme protein at its optimum pH of 6.5 in 20mM citrate-phosphate buffer **(Fig 4**). Lipase activity was enhanced or stabilised in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}. This type of enhancement in the lipase production and stabilisation was also reported in other fungal *Penicillium*species (Bancerz *et al.,* 2005; Dheeman *et al.,* 2011). Hg^{2+} and Zn^{2+} metal ions showed the inhibitory effect on the lipase activity as it was previously reported from the *P. aurantiogriseum* and *P. simplicissimum* (Lima *et al.,* 2004 and Sztajer *et al.,* 1989). This evidence explains the presences of cysteine amino acid residues in the lipase (Sugihara *et al.,* 1996). With 10mM concentration of metal ions (Ca, Mg and Mn) the lipase activity was stabilised and enhanced**(Fig 4)**. The results are also in agreement with the other fungal stains of *Rhizopus oryzae* and *Rhizomucor miehei*as reported by Miklos Tako *et al.*,(2017). Other investigations by Yu *et al.*,(2009), Katiyar and Ali, (2013), Costa-Silva *et al.*,(2014) and Colla *et al.*,(2015) on fungal lipase supports our study.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

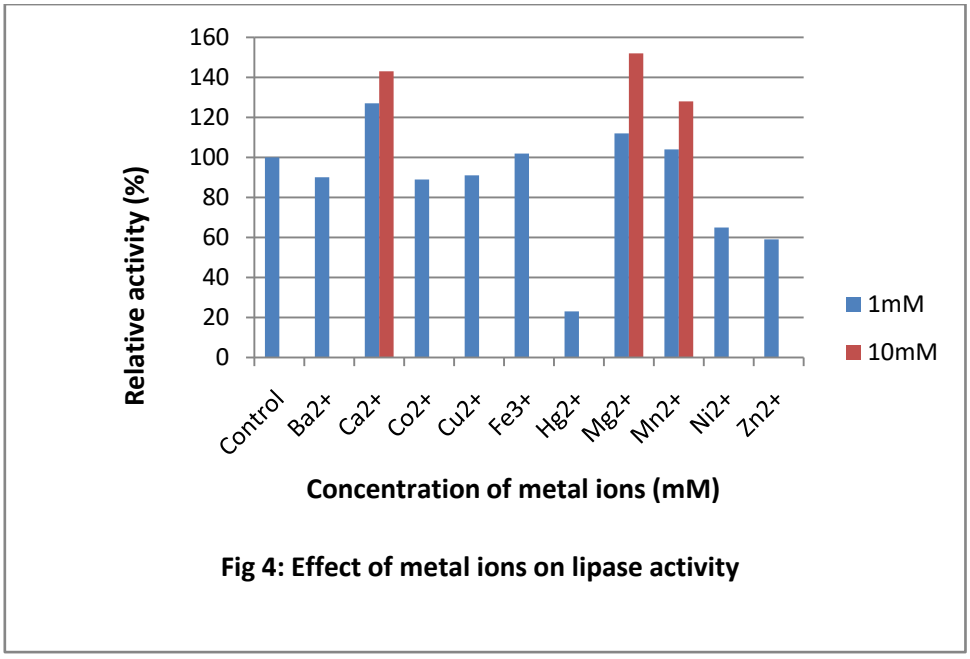

Fig 4: Effect of metal ions on lipase activity

Determination of Kinetic constants of free lipase enzyme isolated from mutated strain of *P. citrinum* :

The kinetic analysis of purified lipase from the mutated strain of ***P. citrinum*KU613360**, performed using p-NPP at 40 °C produced a linear Lineweaver-Burk plot corroborating the Michaelis-Menten behaviour of the enzyme **(Fig 5a,b)**, The Vmax of 83.33 ±0.06 µmol $min^{-1}mg^{-1}$, Km of 19.33±-.010 mM. **(Table 3)**

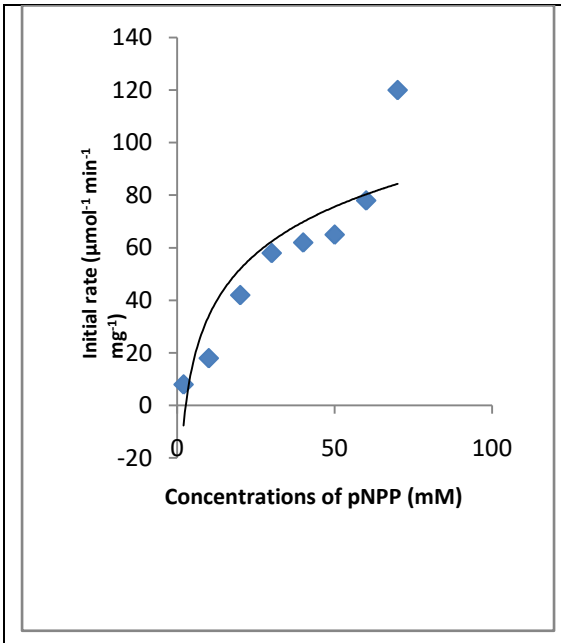

Fig 5a: The kinetic behaviour of Mutant lipase.

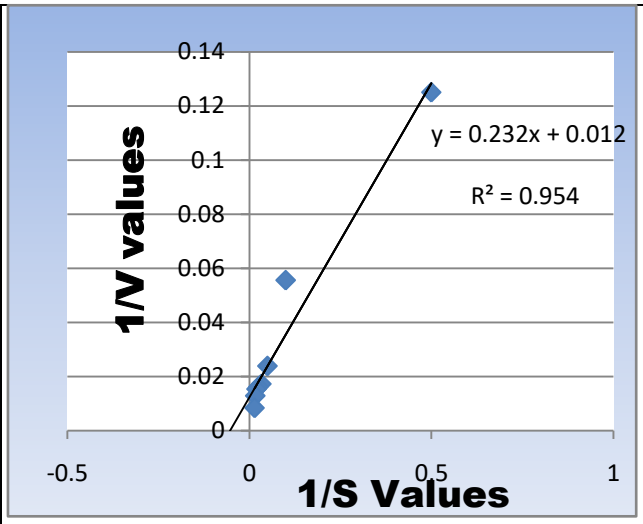

Fig 5b: **The kinetic behaviour of Mutant lipase standard graph.**

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

Table 3: Kinetic parameters of free lipase isolated from mutated strain of *P. citrinum*.

Enzyme -form	Km (mM)	Vmax ($IUmg^{-1}$)	Vmax/Km
Free lipase	19.33±-.010	**83.33**±0.06	**4.31**

The Vmax/Km value is expressed as the catalytic efficiency (Hu *et al.*, 2006; Kermasha and Bisakowski, 1998).

Conclusions:

An extracellular lipase purified from mutated strain of ***Penicillium citrinum*KU613360** showed maximum activity at 40° C with optimum pH was found to be 6.5 and metal ion Mg^{2+} has 100% relative activity providing stability to the lipase. We havesuccessfully developed a promising mutant strain, byemploying a simpler technique of chemicalmutagenesis, which can be exploited at bioreactor levelfor industrial production of lipase.

Acknowledgments

Authors are thankful to Head, FreshmanEngineering Department, Lakireddy Bali ReddyCollege of Engineering, Mylavaram, and Head,Department of Botany and Microbiology (UGC-SAP& DST – FIST *(SR/FST/LS1-627/2015)* funded), Acharya NagarjunaUniversity, for providing thefacilities.

References:

1) Colla, L. M., Ficanha, A. M., Rizzardi, J., Bertolin, T. E., Reinehr, C. O., & Costa, J. A. V. (2015). Production and characterization of lipases by two new isolates of Aspergillus through solid-state and submerged fermentation. *BioMed Research International*, *2015* Article ID 725959, 9

2)Sharma, A. K., Sharma, V., & Saxena, J. (2016). Characterization of Lipase from Wild (LPF-5) and Mutant (HN1) Strain of Aspergillus niger. *Int. J. Curr. Microbiol. App. Sci*, *5*(11), 681-690.

3) Nema, A., Patnala, S. H., Mandari, V., Kota, S., & Devarai, S. K. (2019). Production and optimization of lipase using Aspergillus niger MTCC 872 by solid-state fermentation. *Bulletin of the National Research Centre*, *43*(1), 82.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

4)Contesini, F. J., Lopes, D. B., Macedo, G. A., da Graça Nascimento, M., & de Oliveira Carvalho, P. (2010). Aspergillus sp. lipase: potential biocatalyst for industrial use. *Journal of Molecular Catalysis B: Enzymatic*, *67*(3-4), 163-171.

5) Mehta, A., Bodh, U., & Gupta, R. (2017). Fungal lipases: a review. *Journal of Biotech Research*, *8*.: 58-77

6)Balaji, V., Arulazhagan, P., & Ebenezer, P. (2014). Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. *Journal of Environmental Biology*, *35*(3), 521-9.

7) Karigar, C. S., & Rao, S. S. (2011). Role of microbial enzymes in the bioremediation of pollutants: a review. *Enzyme research*, *2011*.1-11.

8) Gopinath, S. C., Anbu, P., Lakshmipriya, T., & Hilda, A. (2013). Strategies to characterize fungal lipases for applications in medicine and dairy industry. *BioMed research international*, *2013*.

9) Iftikhar, T., Niaz, M., Abbas, S. Q., Zia, M. A., Ashraf, I., Lee, K. J., & Haq, I. U. (2010). Mutation induced enhanced biosynthesis of lipases by Rhizopus oligosporus var. microsporus. *Pak. J. Bot*, *42*(2), 1235-1249.

10) Burkert, J. F. M., Maugeri, F., & Rodrigues, M. I. (2004). Optimization of extracellular lipase production by Geotrichum sp. using factorial design. *Bioresource technology*, *91*(1), 77-84.

11) Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J biol Chem*, *193*(1), 265-275.

12) Dheeman, D. S., Antony-Babu, S., Frías, J. M., & Henehan, G. T. (2011). Purification and characterization of an extracellular lipase from a novel strain Penicillium sp. DS-39 (DSM 23773). *Journal of Molecular Catalysis B: Enzymatic*, *72*(3-4), 256-262.

13) Takó, M., Kotogán, A., Papp, T., Kadaikunnan, S., Alharbi, N. S., & Vágvölgyi, C. (2017). Purification and properties of extracellular lipases with transesterification activity and 1, 3-regioselectivity from Rhizomucor miehei and Rhizopus oryzae. *Journal of microbiology and biotechnology*, *27*(2), 277-288.

14)Jayaprakash, A., & Ebenezer, P. (2012). Purification and characterization of Aspergillus japonicus lipase from a pig fat production medium. *Journal of Academia and Industrial Research*, *1*(1), 1-7.

15) Pera, L. M., Romero, C. M., Baigori, M. D., & Castro, G. R. (2006). Catalytic properties of lipase extracts from Aspergillus niger. *Food Technology & Biotechnology*, *44*(2).247-252

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 7758 - 7770
Received 25 April 2021; Accepted 08 May 2021.

16) Ferrer, M., Plou, F. J., Nuero, O. M., Reyes, F., & Ballesteros, A. (2000). Purification and properties of a lipase from Penicillium chrysogenum isolated from industrial wastes. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, *75*(7), 569-576.

17)Ülker, S., Özel, A., Çolak, A., & Karaoğlu, Ş. A. (2011). Isolation, production, and characterization of an extracellular lipase from Trichoderma harzianum isolated from soil. *Turkish Journal of Biology*, *35*(5), 543-550.

18)Sarkar, D., & Laha, S. (2013). Optimization of extracellular lipase enzyme production from Aspergillus niger by submerged and solid-state Fermentation process. *Int. J. Pharma Bio Sci.*, 4(4): 978-985.

19) Aloulou, A., Rodriguez, J. A., Fernandez, S., van Oosterhout, D., Puccinelli, D., & Carrière, F. (2006). Exploring the specific features of interfacial enzymology based on lipase studies. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1761*(9), 995-1013.

20)Bancerz, R., Ginalska, G., Fiedurek, J., & Gromada, A. (2005). Cultivation conditions and properties of extracellular crude lipase from the psychrotrophic fungus Penicillium chrysogenum 9′. *Journal of Industrial Microbiology and Biotechnology*, *32*(6), 253-260.

21)Song, X., Qi, X., Hao, B., & Qu, Y. (2008). Studies of substrate specificities of lipases from different sources. *European journal of lipid science and technology*, *110*(12), 1095-1101.

22) Rodriguez, J. A., Mateos, J. C., Nungaray, J., González, V., Bhagnagar, T., Roussos, S., ... & Baratti, J. (2006). Improving lipase production by nutrient source modification using Rhizopus homothallicus cultured in solid state fermentation. *Process Biochemistry*, *41*(11), 2264-2269.

23)Lima, V. M. G., Krieger, N., Mitchell, D. A., & Fontana, J. D. (2004). Activity and stability of a crude lipase from Penicillium aurantiogriseum in aqueous media and organic solvents. *Biochemical Engineering Journal*, *18*(1), 65-71.

24) Sztajer, H., & Maliszewska, I. (1989). The effect of culture conditions on lipolytic productivity of Penicillium citrinum. *Biotechnology letters*, *11*(12), 895-898.

25) Sugihara, A., Shimada, Y., Takada, N., Nagao, T., & Tominaga, Y. (1996). Penicillium abeanum lipase: Purification, characterization, and its use for docosahexaenoic acid enrichment of tuna oil. *Journal of fermentation and bioengineering*, *82*(5), 498-501.

26) Yu X, Xu Y, Wang R. 2010b. Enhancement of activity of *Rhizopus chinensis* lipase by directed evolution. Journal of Biotechnology 150(1):344.

27)Katiyar, M., & Ali, A. (2013). Effect of metal ions on the hydrolytic and trans esterification activities of Candida rugosa lipase. *Journal of Oleo Science*, *62*(11), 919-924.

28) Costa-Silva, T. A., Souza, C. R. F., Oliveira, W. P., & Said, S. (2014). Characterization and spray drying of lipase produced by the endophytic fungus Cercospora kikuchii. *Brazilian Journal of Chemical Engineering*, *31*(4), 849-858.

29) Hu, J., Li, S., & Liu, B. (2006). Properties of immobilized pepsin on modified PMMA microspheres. *Biotechnology Journal: Healthcare Nutrition Technology*, *1*(1), 75-79

30) Kermasha, S., & Bisakowski, B. (1998). The effect of immobilization on the hydrolytic/interesterification activity of lipase from Rhizopus niveus. *Journal of the American Oil Chemists' Society*, *75*(12), 1791-1799.