Isolation, Screening and Characterization of Lipase by *Enterobacter sakazaki* from farm soil

[1]Ramkumar Smita, [2]Mahida Ankita [3*]Intwala Siddhi M.
[1]P.G. student, [2]P.G. student , [3]Teaching Assistant,
[1] Department of Biosciences
[1]Veer Narmad South Gujarat University, Surat – 395007, Gujarat, India.

Abstract: The production of industrial enzymes, including fungi and bacteria has always been the industrial choice due to its large application and feasibility. Microbial lipases are used many industries like food flavouring industry, detergent and as ester and amino acid derivatives, biocatalytic resolution of pharmaceuticals, biosensors in agro chemicals, cosmetics, bioremediation etc. The present study describes the attempt of obtaining potential lipase producing bacteria from soil. 25 isolates were obtained by primary screening and from that 16 isolates were able to produce lipase enzyme. Based on quantity of the lipase produced by all isolates, A31 characterized as *Enterobacter sakazaki* which could be used as potent microbial source of lipase enzyme. The optimum pH for the maximum lipase production was 9 and optimum temperature was 40º C. Glucose incorporated as carbon source, yeast extract as nitrogen source and Tributyrene as substrate led highest lipase production. The presence of Cd^{+2} enhanced the lipase production. In the partial purification of lipase by ammonium sulphate precipitation, 30 % pellet gave 84 % yield purification and 70 % pellet gave 55 % yield purification.

Key words: Lipase, ammonium sulphate precipitation, pellet, tributyrene

I. INTRODUCTION

Lipases are found widely in nature, but microbial lipases are commercially significant because of low production cost. Lipases are enzymes that act on acylglycerols and catalyze the hydrolysis of fatty acids and glycerols. Lipases have the ability to hydrolyze long chain insoluble triglycerides into triglycerides, mono glycerides, glycerols and fatty acids (Heravi *et al* 2008) Lipases (E.C .3.1.1.3) are water – soluble enzymes, which are widely distributed in plants, animal's microbes. Lipase act on a variety of substrates such as natural oils, synthetic triglycerides and different carboxylic esters (Ferrato *et al* 1997). They remain enzymatically active in organic solvent, various physical and environmental conditions (Sharma *et al* 2011)
They are ubiquitous enzymes of considerable physiological significance as well as industrial potential. Microbial lipases appear to be one of the versatile class of enzymes with a number of potential biotechnological applications. With the rapid development of enzyme technology, lipases find promising future in organic chemical processing, biosurfactant synthesis, detergent industry, dairy industry, paper manufacturing industry, nutrition, cosmetics, pharmaceutical and agrochemical industries (Pradeep *et al* 2012). Development of lipase technologies for the synthesis of novel compounds of industrial importance will probably also result in their expansion into new areas.

The purpose of the present study is to isolate lipase producing microorganisms from various soil samples and increase the lipase activity by characterizing the medium.

II. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

For the present study, soil sample was collected from four different regions around Surat city. The samples were collected from Sayan farm soil (A1), from dumping site of Sayan (A2), from farm soil of Olpad region of Surat (A3) and from Bapalal garden of VNSGU (A4).

2.2 Isolation of lipolytic Bacteria

For the isolation of lipolytic bacteria, 1 gm of soil sample was dissolved in 9 ml of sterile Distill water. Then the samples were serially diluted from 10-1 to 10-10 and 0.1 ml of each aliquot and were spreaded on Nutrient agar plate. The plates were incubated at 30° C for 24 hours, after 24 hours observe the colony. Then isolated colonies were incubated in production medium (Patel P and Desai B, 2018).

2.3 Screening of Lipolytic Bacteria

2.3.1 Primary screening of lipolytic Bacteria

Primary screening of lipolytic bacteria was done by streaking a culture on a Sierra agar plate (peptone - 1 %, NaCl - 0.5 %, $CaCl_2.H_2O$ - 0.01 %, Tween 80 - 1 %, Agar - 3 %, pH (7.4 ± 0.2), Distill Water - 100 ml). Incubate all plate at 30°C in incubator for 24 hours. Observe the hollow zone on Sierra agar plate and measure the zone (Sierra, 1957).

2.3.2 Secondary screening of lipolytic Bacteria

Secondary screening of lipolytic bacteria was done by cup borer method. The production medium containing different cultures were respectively inoculated in a well of Sierra agar plate. The plates were incubated and the zones were observed around the well. The hallow zone around the well which contain bacterial culture identified as lipolytic colony or bacteria. (Sierra, 1957)

2.4 Lipase assay

The higher lipase producing bacterial colonies from secondary screening was selected and quantifies the lipase production. Lipase was determined titrimetrically on the basis of olive oil hydrolysis by modified method suggested by (Sagar, 2013). Approximately 10 ml of sample from incubated lipase production medium was taken and centrifuged it at 2500 rpm for 15 min. Different aliquots of supernatant was taken as a sample. Then the final volume was made 5ml which consists 75ml of 2% polyvinyl alcohol + 25 ml of olive oil. It was mixed thoroughly by vortexing. Then 4ml of sodium phosphate buffer (pH - 7) was taken in 100 ml of conical flask and incubated it at 32° C for 30 min on rotatory shaker at 150 rpm. The reaction was terminated by addition of 5 ml of acetone and then titrated against 1 M NaOH using phenolphthalein as indicator.

2.5 Characterization for lipase enzyme

2.5.1 Effect of pH on the activity of lipase

The lipolytic activity of the enzyme was determined by Olive oil assay in a pH range of 3,5,7,9 using different buffers at 1M concentration. After that, the assay was determined on the basis of olive oil hydrolysis by modified method suggested by (Sagar, 2013). Approximately 10 ml of sample from incubated lipase production medium was taken and centrifuged it at 2500 rpm for 15 min. Different aliquots of supernatant was taken as a sample. Then the final volume was made 5ml which consists 75ml of 2% polyvinyl alcohol + 25 ml of olive oil. It was mixed thoroughly by vortexing. Then 4ml of sodium phosphate buffer (pH - 7) was taken in 100 ml of conical flask and incubated it at 32° C for 30 min on rotatory shaker at 150 rpm. The reaction was terminated by addition of 5 ml of acetone and then titrated against 1 M NaOH using phenolphthalein as indicator (Kurhekar *et al* 2017).

2.5.2 Effect of temperature on the activity of lipase

Effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures in the range of 30,40,50,60 at pH 7 using phosphate buffer (1M). After that, the assay was performed as described for olive oil assay (Bora *et al* 2012).

2.5.3 Effect of Carbon source

Effect of different carbon source on lipase activity was determined by Olive oil assay using glucose, sucrose, lactose (0.5 %) as a same concentration. After that, the assay was performed as described for olive oil assay (Sumarsih *et al* 2018).

2.5.4. Effect of Nitrogen source

Effect of different nitrogen source on lipase activity was determined by Olive oil assay using different nitrogen source such as peptone (1%), beef extract (1%). After that, the assay was performed as described Olive oil assay (Chen *et al* 1992).

2.5.5 Effect of metal ions

The effect of heavy metal ions on the activity of enzyme was verified by adding of 0.5 % Cu^{+2} ($CuSo_4$), Fe^{+3} (FeCl3), Cd^{+3} (CdCl2) (Balakrishnan *et al* 2015).

2.5.6 Effect of Various Substrates

The effect of Substrate on the activity of lipase was verified by the addition of 1 % olive oil, tween 80, oleic acid, tributyrin (Komathi *et al* 2017).

2.6 Partial purification of Lipase (Kalapatapu *et al* 2015)

Partial purification of lipase was done by ammonium sulphate precipitation.

The production medium was inoculated by 24 hours old culture and incubated it on shaker at 30°C for 24 hours. After incubation, crude lipase was collected by centrifuging at 6000 rpm for 15 min. Crude lipases were collected and their lipolytic activity and protein concentration were determined. 50 ml of the crude sample lipase enzyme were first precipitated by 30 % saturation with solid ammonium sulfate according to

the ammonium sulfate concentration. The precipitated proteins were separated by centrifugation for 15 min at 8000 rpm. The resulted pellets were dissolved in minimum volume of buffer. From the 30% pellet and 80 % pellet determined enzyme activity protein concentration.

2.7 Characterization of Isolates

Gram Staining and the Biochemical characteristics (Indol, Methyl Red, Voes- Proskauer, Gelatin liquification , Citrate utilization, Urease, Lipid Hydrolysis, Triple Sugar iron test and sugar fermentation tests (Sucrose, maltose, lactose, xylose, and mannitol) were also carried out according to the standard methods described in Bergey's Manual of Determination Bacteriology (Holt,1993).

2.8 Identification of the Isolate Using Software (ABIS online)

ABIS Online – Advanced Bacterial Identification Software is a laboratory tool for bacterial identification based on morphological and biochemical characters, cultural characteristics, growth conditions, ecology and pathogenicity data.The given below is the link of the software.www.tgw1916.net/bacteria.logare.html

III.RESULTS AND DISCUSSION

3.1 Sample Collection

Soil samples were collected from different regions around surat city, from Sayan farm soil (A1), dumping site from Sayan (A2), farm soil of Olpad (A3), and from Bapalal garden of VNSGU (A4) respectively in the sterile container. The upper portion of the soil was removed and the soil was collected from the lower portion. The samples were preserved at 4-8 ° C till the bacterial screening and isolation. (Biswas *et al* 2016). Lipase producing bacteria were isolated from different soil sample. Total 25 isolates were isolated from the soil sample by using serial dilution technique on nutrient agar plates (Shaini and Jayasree, 2016). From (A1), 7 isolates were obtained, 10 isolates from (A2), 5 isolates from (A3) and 3 isolates from (A4) were obtained respectively. Secondary screening was performed on Serria Agar plates by cup borer technique (Sierra *et al* 1957). Upon screening 16 isolates showed clear zones of Tween - 80 hydrolysis. (Thomson *et al* 1999).

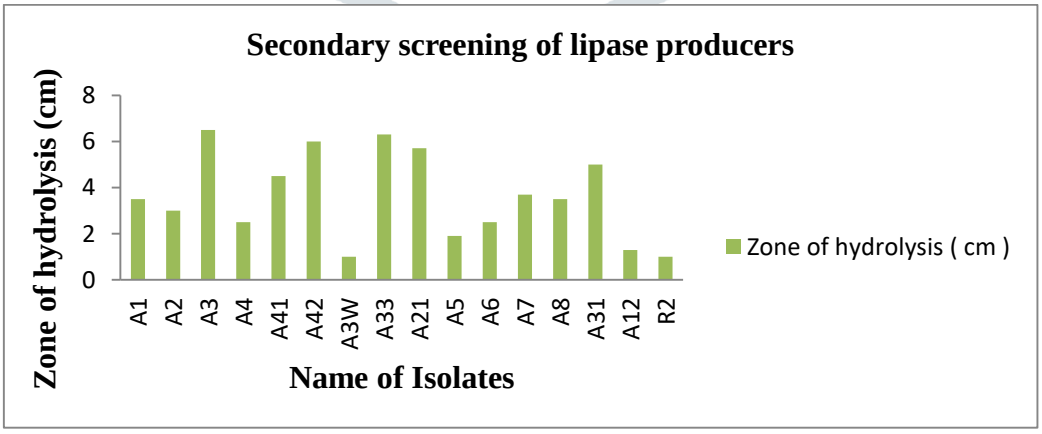

Fig - 1 Secondary Screening of Lipase producers

The precipitation of calcium salt takes place due to hydrolysis thus white opaque zones around the colonies were observed around the colonies (Gupta *et al* 2003). Among 16 isolates the highest zone of hydrolysis was found in isolate A3 - 6.5 cm, A33 - 6.3 cm, A42 - 6 cm, A21 - 5.7 cm and A31 - 5 cm

respectively. Based on literature review BSL2 gave 0.5 cm and ICK2 gave 0.68 cm respectively on Tributyrin agar (Musa *et al* 2018). Gram Reaction of the strains was done with the reference to Bergey's Manual of Determinative Bacteriology.

3.2 Assay of Lipase activity

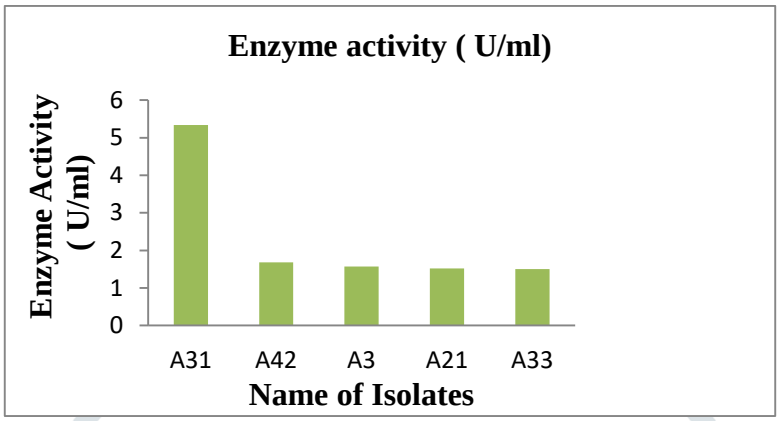

Fig - 2 Lipase activity of different isolates

The results indicated that the isolate A31 exhibited maximum lipase activity (5.338 U/ml) followed by other isolates. Hence, further studies were focused on the isolate A31. As reported from studies on other bacterial microbial lipase, the enzyme activity was found 0.569 U/ml (Musa *et al* 2018). The enzyme activity of *Staphylococcus spp* was reported 4.27 U/ml (Patel *et al* 2018) also *Pseudomnas aeruginosa KM110* was reported 0.46U/ml (Qamsari *et al* 2011).

3.3 Characterization of Lipase producing organism:

3.3.1 Effect of pH on Lipase activity

The effect of pH on lipase activity was studied by olive oil assay. Assay was carried out at different pH range from 3, 5, 7 and 9.

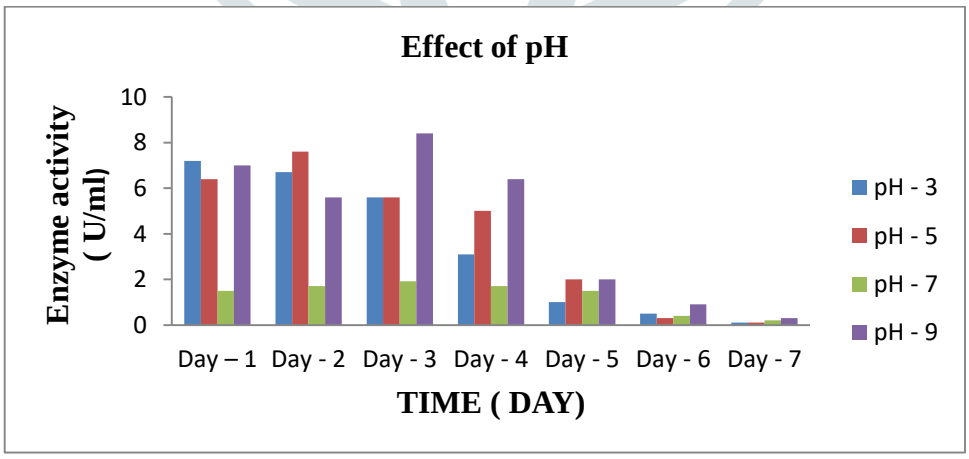

Fig - 4 Effect of pH on Lipase activity

A result of effect of pH on the enzyme activity is summarized in figure - 4. A31 isolates gave maximum activity at pH 9. Previous study shows that the isolate C1 and C2 gave maximum activity at pH 7.5

(Desai *et al* 2016) also *Pseudomonas putida SBS037* was reported to exhibit maximum enzyme activity at pH 7 (Singh *et al* 2013).

3.3.2 Effect of temperature on Lipase activity

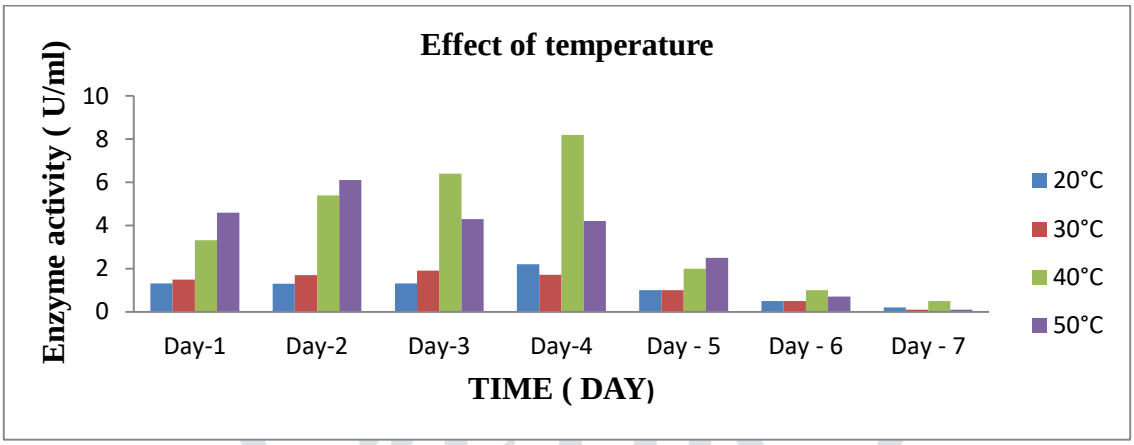

Fig - 5 Effect of temperature on Lipase activity

The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperature in the range of 20°C, 30° C, 40°C and 50°C. The results are shown in Fig-6. It was revealed that the optimum lipase activity of Bacillus strain was 37°C (Mohan *et al* 2008) while the A31 isolate gave more activity at 40°C. According to (Gilbret *et al* 2009) *Pseudomonas spp* showed maximum enzyme activity at 30°C. Also it was recorded that *Pseudomonas strain KE38* showed optimum growth at 25°C, but its lipase had maximum lipase activity at 45°C.

3.3.3 Effect of carbon source on lipase activity

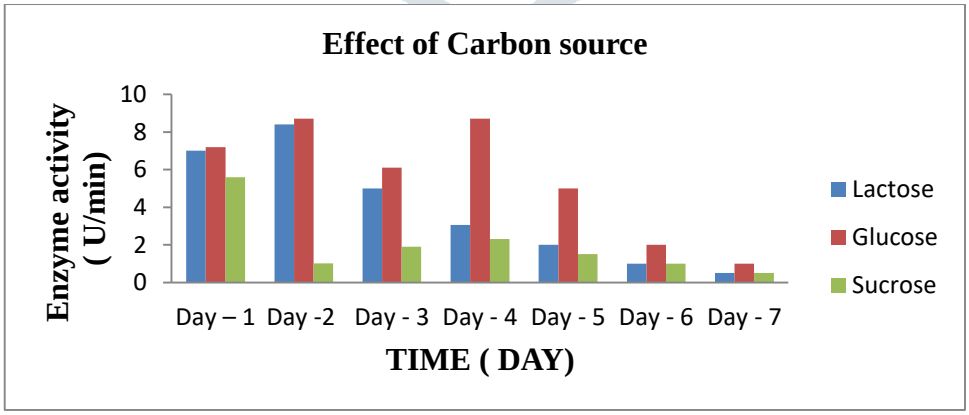

Fig - 6 Effect of carbon source on Lipase activity

Effect of different carbon source on lipase activity was studied by using different carbon sources, lactose (0.5 %), glucose (0.5 %), sucrose (0.5 %). From the result shown in figure - 6, it is clear that isolate A31 showed maximum enzyme activity with glucose as carbon supplement. It is recorded that *Pseudomonas putida SBS037* showed maximum enzyme activity with 1 % Dextrose (Singh *et al* 2013) and the maximum enzyme production in *Geobacillus stearothermophilus* was obtained with 1 % Glucose (Alashkar *et al* 2017).

3.3.4 Effect of nitrogen source on lipase activity

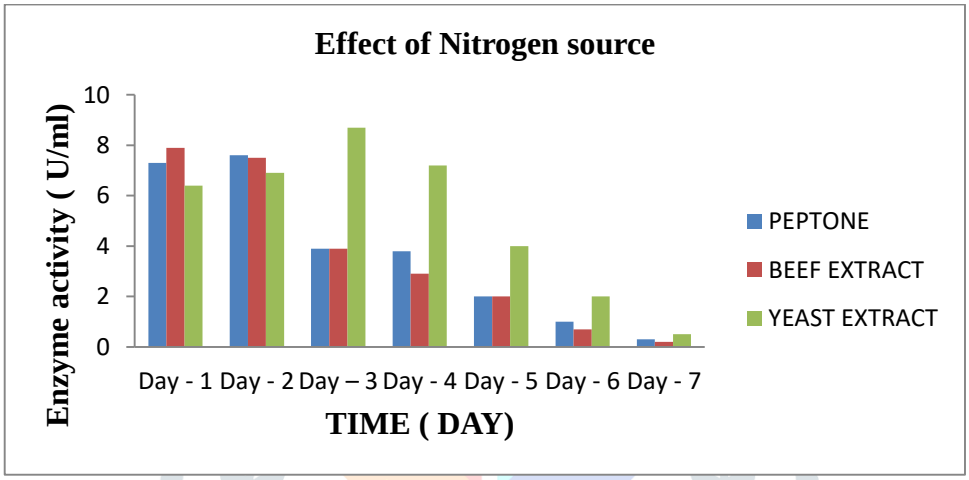

Fig - 7 Effect of nitrogen source on Lipase activity

Effect of nitrogen source on lipase activity was studied by olive oil assay. Assay was carried out by using different nitrogen source: peptone, beef extract, yeast extract. Effect of nitrogen source on lipase activity is shown in figure - 7. A31 isolate gave optimum activity in yeast extract medium. Based on literature review it is found that algae *Gelidium pusillum* showed maximum enzyme activity with Castor oil as nitrogen source (Sharmila *et al* 2014) also (Dong *et al* 1999) reported that inorganic nitrogen sources such as ammonium chloride and ammonium dihydrogen phosphate is effective in some bacterial species.

3.3.4 Effect of metal ions on lipase activity

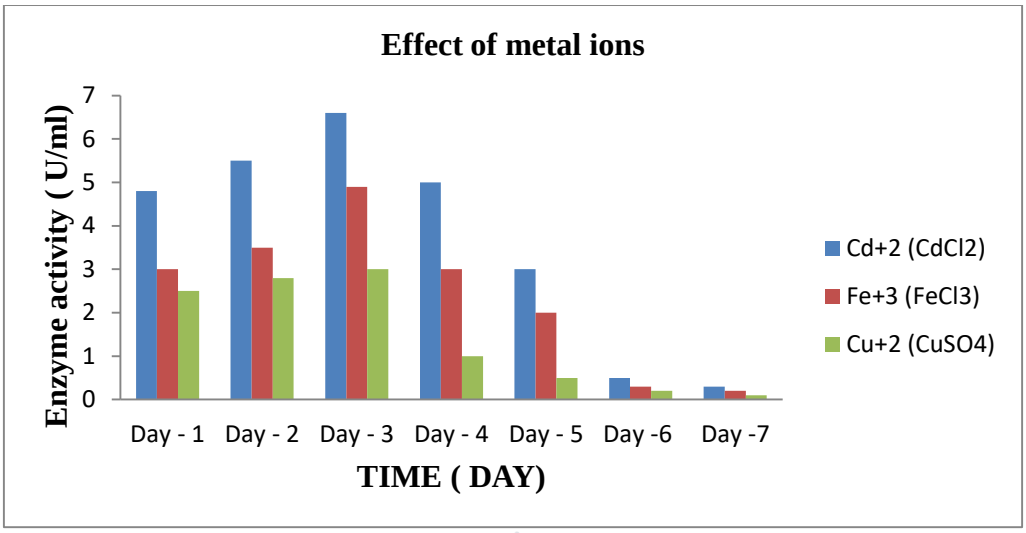

Fig - 8 Effect of metal ions on Lipase activity

Effect of various metal ions was carried out by olive oil assay by using different metal ions in medium. Results are shown in figure - 8. Cd^{+2} ($CdCl_2$) gave maximum activity than other metal ions. It is reported that *Pseudomonas xinjiangensis CFS14* exhibited maximum enzyme activity in the presence of $MgCl^{+2}$ metal ion (Lomthaisong *et al* 2012) and Ca^{+2} was also reported as the enhancers for bacterial lipases (Kulkarni and Gadre, 2002). It is noted that the presence of NaCl increased the lipase production in *Enterobacter gergoviae* and *Aeromonas veronii* (Kamladevi *et al* 2014).

3.3.5 Effect of substrate on lipase activity

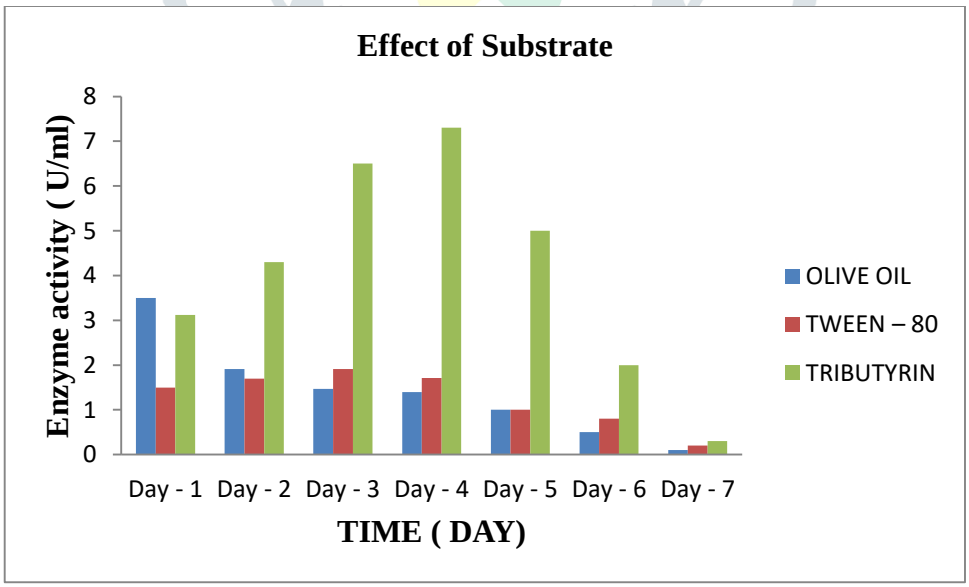

Fig - 9 Effect of substrates on Lipase activity

Effect of different substrate on lipase activity was carried out by olive oil assay using various substrates. Effect of substrate on lipase activity is shown in figure - 9 and Tributyrin gave maximum lipase activity

among all substrates. It was reported that *Pesudomonas gessardi* exhibited maximum enzyme activity in the presence of Protease Peptone (Veerapagu *et al* 2013) also among the various fatty acids oleic acid was found to be the best substrate for lipase production (Obradors *et al* 1993). It is also noted that wheat bran enhanced that lipase production in *Trichoderma harzianum* (Montero *et al* 2013).

3.3.6 Measurement of enzyme activity using characterized medium

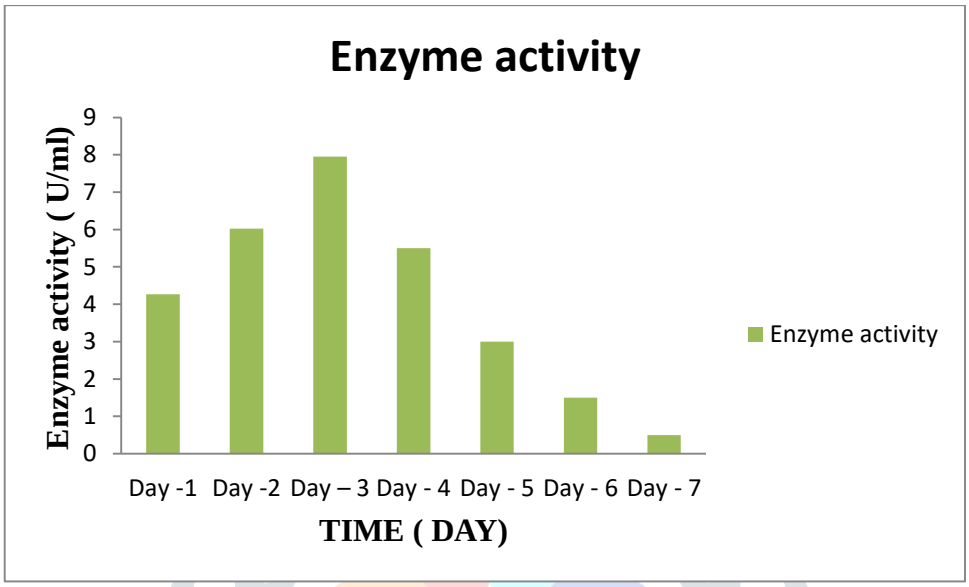

Fig - 10 Enzyme activity using characterized medium

By using the characterized medium which contain glucose as carbon source, yeast extract as a nitrogen source, Cd^{+2} as a metal ion, tributyrin as a substrate led highest lipase activity at 40°C and pH 9 as compared with other sources.

3.3.7 Partial purification of lipase enzyme by ammonium sulphate precipitation

Fraction	Volume	Enzyme activity (U/ml)	Total activity (U/min)	Total Protein (mg/ml)	Specific activity (U/ml)	Fold Purification (U/ml)	% yield Purification
Crude	40	0.02395	0.958	1.856	0.2507	0.0	100 %
30 % pellet	38	0.021365	0.81187	1.121	0.724	4.484	84 %
80 % pellet	35	0.0331	1.1585	0.056	0.299	0.056	55 %

Table - 1 Results of Partial purification of lipase enzyme by ammonium sulphate precipitation.

It was reported that the lipase enzyme produced from *Bacillus spp* purified with 60-80 % ammonium sulphate precipitation achieved 2.8 fold increase in the relative activity of the lipase in supernatant (Jaganathan and Jaiganesh, 2018). It was stated that the lipase enzyme was produced from *Pseudomonas* ADT3 partially purified approximately 2.9 fold with an overall yield of 64.4 % (Chatterjee *et al* 2014). The purification procedure resulted in 2.1 fold of lipase with a final yield of 20.8 % in case of *Micobacterium spp* (Tripathi *et al* 2014).

3.3.8 Results of Biochemical test

The results of biochemical tests are shown in table - 2.

No	Biochemical test	Result
1	Gram reaction	Gram –ve rod
2	MR test	- ve
3	VP test	-ve
4	Gelatin liquefaction test	-ve
5	Indole test	-ve
6	Citrate utilization test	+ve
7	Urease test	-ve
8	Lipid hydrolysis test	+ve
9	Triple sugar iron test	+ve
10	Sugar fermentation	
	Sucrose	Acid & gas formation
	Xylose	Acid & gas formation
	Mannitol	Acid & gas formation
	Fructose	Acid & gas formation
	Lactose	-ve

- *+ve = Poisitive *-ve = Poisitive

Bacterial identification was carried out using traditional method by morphological and biochemical characterisation of isolate using Bergy's Manual. Rather than the traditional method we also identified the bacteria using computational tool (ABIS).

It is a tool for Bacterial identification based on morphological, biochemical and cultural, growth conditions, ecology and pathogenicity data.

III. CONCLUSION

From the above study it is concluded that the isolated organism *Enterobacter sakazaki* is able to produce lipase enzyme. Further studies are recommended to enhance the enzyme production using biotechnology approach and statistical analysis.

IV. ACKNOWLEDGEMENT

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V. REFERENCES

- Angkawidijaja C. and Kanata S. (2006). Bacterial lipase secreted by the type 1 secretion system. Cellular and Molecular Life Sciences, 63, 2804-2817.
- Aria T., Yusa S., Kiremura K. (1997). Cloning and sequencing of the cDNA encoding lipase from *Trichosporonfermentans* WL-C12. Federation of European Microbiological Societies, 152, 183-188.
- Arpigny J. L., K. E. (1999). Bacterial lipolytic enzymes: classification and properties. Biochemical Journal, 343, 177-183.
- Brockerhoff H., Jensen R.G. (1974). Cholestrol esterase in lipolytic enzymes. New york: Academic Press. , 177-192.
- Cai-hong W., Run-fang G., Hong-wei Y., Ying-min J. (2008). Cloning and sequence analysis of a novel cold-adapted lipase gene from strain LIP35. Journal of Agricultural sciences, 7(10) , 1216-1221.
- Cihangir N, Sarikaya E. (2002). Investigation of lipase production by a new isolated strain of *Aspergillus sp*. World Journal of Microbiology & Biotechnology, 20 ,193-197.

- Dharmsthiti , Ammaranond S. (1997). Purification and characterization of lipase from a raw-milk yeast. Journal of Biotechnology and Applied Biochemistry, 26, 111-116.
- Gupta N, Shai V, Gupta R. (2007). Alkaline lipase from a novel strain *Burkholderia multivorans*: statistical medium optimization and production in a bioreactor. Journal of Processing Biochemistry. 42 ,518-526.
- Kulkarni N, Gadre RV. (2002). Production and properties of alkaline, thermophilic lipase from *Pseudomonas fluorenscens* NS2W. Journal of Industrial Microbiology and Biotechnology; 28:344-8.
- Masomian M, Rahman R, Salleh AB, Basri M (2013). A New thermostable and organic solvent-tolerant lipase from Aneurinibacillus thermoaerophilus strain HZ. Journal of Biochemistry, 48:169–75.
- Mobarak-Qamsari E, Kasra-Kermanshahi R, Moosavi-nejad Z. (2011). Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110. Iran Journal of Microbiology; 3:92-8.
- Patcha boonmahome and Wiyada mongkolthanaruk (2013). Lipase producing bacterium and its enzyme characterization. Journal of Life sciences and technologies, 1(4): 196- 200.
- Prasanna R.M, Mahendran V.S, Balakrishnan V.(2013). Isolation and identification of lipase producing organisms from diverse soil samples of kolli hills. International Journal of Current Microbiology and Applied Science; 1:205-10.
- Rashid, N., Shimada Y, Ezaki S, Atomi H and Imanaka T (2001). Low temperature lipase from psychrotrophic *Pseudomonas species* strain KB700A. Journal of Applied Environmental Microbiology. 67, 4064.
- Saeed H. M., Zaghlolu T.I., Khalil A.I. and Abdelbaeth M.T. (2005). Purification and characterization of two extra cellular lipases from *Pseudomonas aeruginosa* PS-X. Journal of microbiology. 54, 233.
- Sharma R., Christi Y and Banerjee U.C (2001). Production, purification, characterization and applications of lipases. Journal of Biotechnology 19: 627-662.
- Singh S., Joshi M. (2013). Screening and chactrization of lipase enzyme isolated from oily soil. International Journal of Biochemistry and Biotechnology Science, 2(2), 1-7.

- Sirisha E. , Rajasekhar N. and Lakshmi Narasu M. (2010). Isolation and optimization of lipase producing bacteria from oil contaminated soils. Advances in Biological Research, 4(5), 249-252.
- Stoyanova, V., Stanka G, Ivan I , Mariana M and Sonya T (2012). Lipolytic activity of genus *Pseudomonas*. Journal of Bioscience and Biotechnology. 163-168.
- Stuer, W., Jaeger K.E. and Winkler U.K. (1986). Purification of extracellular lipase from *Pseudomonas aeruginosa*. Journal of Bacteriology. 168, 1070.
- Tembhukar U R., Dama Z.B., Attarda N.P. and Zope P.S. (2012). Production and characterization of extracellular lipases of S*taphylococcus sp*. isolated from oil contaminated soil. Trends in Biotechnology Research 1: 36-41.
- Thakur S. (2012). Lipase its sources, properties and application: a review. International Journal of Scientific and Engineering Research, 3(7), 1-29.
- Upton C. and Buckley J. T. (1995). Protein sequence motifs: a new family of lipolytic enzymes. Trends in Biochemical Science, 20(5), 178-179.
- Veerapagg M, Sankara Narayanan A, Jeya K.R., Alagendran S (2014). Isolation and identification of a noval lipase producing bacteria from oil spilled soil. International Journal of Innovative Research in Science Engineering and Technology. 3:18122-28.
- Wang Y, Srivastava K.C, Shen G.J, Wang H.Y (1995). Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus strain*, A30-1(ATCC 53841). Journal of fermentation Bioengineering. 79: 433-8.
- Yasuo S, Masahito H, Koichi H. (2002). Use of tween 20 as a substrate for assay of lipase activity in soils. Journal of Soil Science Plant and Nutrition; 48(5):729-34.
- Zheng Chaocheng (2018). Growth characteristics and enzyme production optimization of lipase producing strain. Journal of Earth and Environmental Science 108-042087.