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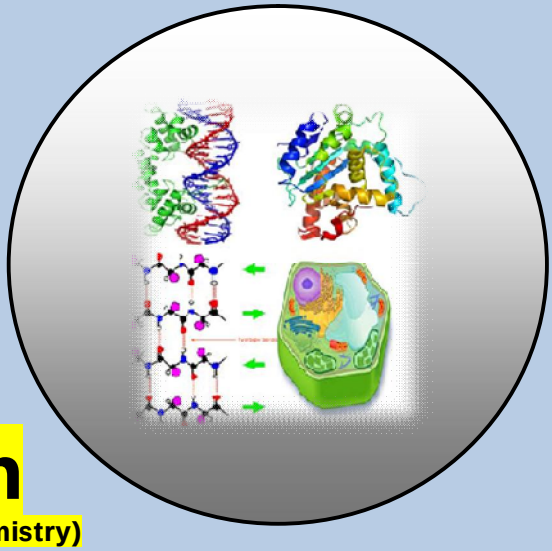
ISSN 0970-4973 (Print)
ISSN 2319-3077 (Online/Electronic)

Index Copernicus International Value
IC Value of Journal 4.21 (Poland, Europe) (2012)
Global Impact factor of Journal: 0.587 (2012)
Scientific Journals Impact Factor: 2.597

J. Biol. Chem. Research
Volume 31 (2) 2014 Pages No. 841-853



Journal of Biological and Chemical Research
(An International Journal of Life Sciences and Chemistry)

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 31, No. 2: 841-853 (2014)
An International Journal of Life Sciences and Chemistry

ISSN 0970-4973 Print
ISSN 2319-3077 Online/Electronic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RESEARCH PAPER

Received: 28/05/2014 Revised: 15/06/2014 Accepted: 20/06/2014

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ABSTRACT

The present paper deals with production of extra cellular alkaline lipase from Bacillus sp and Lactobacillus sp isolated from slaughter house soil sample. Some of the fermentation condition was checked for the production of lipase enzyme from the isolated organisms, to improve the enzyme production. The optimum pH of Bacillus sp grows on pH 9 and Lactobacillus sp pH 10 given the best result. The optimum incubation time 48 hr and temperature 40°C for both Bacillus sp and Lactobacillus sp given the high yield of lipase enzyme. The enzyme produced by submerged fermentation was studied using different agriculture waste such as coconut oil cake, groundnut oil cake and sesame oil cake. The maximum enzyme activity was achieved from coconut oil cake with supplement nutrient solution basal mineral medium with olive oil was given high level of enzyme production on Bacillus sp. The extracted lipase enzyme was partially purified. Purification was performed by ammonium sulfate precipitation and DEAE-Cellulose column using ion exchange chromatography. The molecular mass purified lipase was estimated to be approximately 45 KDa by SDS-PAGE.

Key words: Bacillus sp, Lactobacillus sp, Submerged fermentation and Alkaline lipase.

INTRODUCTION

Lipase acylglycerol hydrolases,n 3.1.1.3 are enzyme, the function of which is to catalyze the hydrolysis of triaclglycerols to fatty acids, mono, di, triacylalycerols and glycerol. Lipases are ubiquitous enzyme Brockerhoft and Jensen 1974. Lipases show optimum enzymatic activity at the oil-water interface contray to other esterases, which have optimum activity in homogeneous media with soluble substrates Maragoni AG 1994.

Lipases are found throughout all kingdom of life, which are prokaryotes including bacteria and archaea and eukaryotes including plant, animal and fungi. Microbial lipases are more useful than enzymes derived from plant and animal since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically and capable of rapid growth on inexpensive media Ghosh *et al*., 1996; Mala V and Dahot 1968; Marit *et al*., 1997 .

In plant mostly lipase are present in the form of food reserve tissues of growing seedling or especially in those which contains large amount of triacylcerols. The triacylcerols are converted to soluble sugars by the action of lipase which is then transported to the growing tissues to supply structural carbon and energy to provide support for the growth of young plants Mala V and Dahot 1968; Kapranchi kov *et al*., 2004; Dahot *et al*., 1987 .

The majority of lipases used for biotechnological purpose have been isolates from bacteria, fungi, Actinomyces and yeast. The bacterial species are preferred sources of lipases because they produce extracellular and intracellular enzyme Saxena *et al*., 1999 . Examples for lipase enzyme producing bacteria are *Bacillus megaterium* Ruiz *et al*.,2002 , *Pseudomonas aeroginosa* Shabtai Y and Daya-Mishne N.,1992 , *Psychrobacter* Zhang *et al*.,2007 , *Pseudomonas fluorescens* Duong *et al*.,1994 , *Pseudomonas aeroginosa* Wohlferth and Winkler UK.,1988 , *Acinetobacter calcoaceticus*, *Alcalingenes sp* Brune and Gotz 1992 , *Bacillus alcalophilus* Ghanem e*t al.,* 2000 , *Pyrococcus furiosus* Chandrayan *et al*., 2008 , *Rhodotorula pilimanae lipase I* Muderhwa *et al*., 1986 , *Salinivibrio sp,* strain SA-2 Mohammad *et al.,* 2008 , *Staphylococcus epidermidis* Joseph *et al*., 2006 , *Thermobifida fusca* Chen *et al*.,2008 , *Thermophilic Bacillus sp* J33 Nawani and Kaur 2004 .

The fungal species also produced lipase enzyme the chief producers of commercial lipases are *Aspergillus. niger, Candida cylindracea, Humicola lanuginosa, Mucor miechei*, *Rhizopus arrhizus, Rhizopus delemar, Rhizopus japonicus, Rhizopus niveus, Rhizuspus oryzae* Sharma *et al*.,2001 *Fusarium solani*, *Penicillium nitrocducesns* Ghosh *et al*.,1996 . Among mucorate the lipolytic enzymes of the moulds *Mucor hiemalis, Mucor michei, Mucor lipolyticus, Mucor pusillus* is well known as a producer of thermostable extracellular lipase Sztajer *et al*., 1988 .

Yeast strain produced by extracellular lipase enzyme. *Sporobolomyceso salmonicolor, Candida rugosa* Lazar *et al*., 1992 . Actinomyces are also producing extracellular and intracellular lipase enzyme Sztajer H *et al*., 1998. *Rhizophora mucronata* Fariha Hasan *et al*., 2006 , *Streptomyces griseus* Chowdary and Hamsaveni., 2001 .

Approximately 1000 tons of lipase is sold every year in the world. Lipase is generally added to the detergent primarily in association with proteases and celluloses Pandey *et al*., 1999 .

Potential industrial application of lipases comprises food additives Modification of aromas , fine chemicals ester synthesis , detergents hydrolysis of fats , waste water treatment decomposition and removal of leaginous substances , leather treatment fat removal from animal skin and pharmaceutical and the medical area Sharma *et al*., 2001; Pandey *et al*., 1999 . Lipases are widely used in the processing of fats and oil detergents and detergents and processing, the synthesis of fine chemical, pharmaceuticals, paper manufacture and production of cosmetic and pharmaceuticals. Lipase can be used to accelerate the degradation of fatty waste and polyurethane Takamoto T *et al*., 2001 . In paper industry, lipase enzyme used for removal of pitch from the paper making. Nippon Paper Industries, Japan, have developed a pitch control method that uses the *Candida rugosa* fungal Jaeger *et al*., 1999. The main processes in leather industry are the removal of subcutaneous fat and dehairing. Conventional methods including organic solvents and surfactants to remove fat from animal skins can be harmful to environment due to the production of dangerous final products like volatile organic compound VOC emissions Hasan *et al*. 2006. In present the study refers to the optimization conditions for the production of lipase enzyme, from *Bacillus spp* and *Lactobacillus spp* using various parameters such as pH, temperature, incubation time and production of lipase enzyme by submerged fermentation using agro waste.

MATERIAL AND METHODS

Screening and isolation of lipase producing organism on agar plates

The soil samples collected from Slaughter house area Rasipuram, TamilNadu, India. The samples were serially diluted and plated on nutrient agar plate and incubated at 37°C for 24 hours. The colonies appeared on nutrient agar medium. Selected colonies inoculate with Tributyrin agar medium incubated at 37°C for 24 hours. After incubation the plates were observed clear zone around the colonies Saxena Rk *et al*., 2000.

pH Tolerance of the selected isolated culture

Tributyrin agar plates were prepared with different pH rang from as 6,7,8,9 and 10. The plates were incubated at 37°C for 24 hours. After incubation of the plates were observed appearance of clear zone hydrolysis.

Identification of selected strain

The isolated organism was subjected to staining, motility and standard biochemical tests. The organism used in the present study has been indentified based on the test performed as species of *Bacillus sp* and *Lactobacillus sp*.

Production media

The production media used for basal mineral medium was prepared according to the procedure reported by Manresa E *et al*., 2000 , the composition of basal mineral medium used in this study was g/100 ml : $NH4_2SO_4$:0.5 $NaNO_3$:0.05;K_2HPO_4:0.1;KH_2PO_4:0.05;KCL:0.1;$MgSO_4$ $7H_2O$:0.03;$CaCO_3$:0.05; Yeast extract:1g The medium was supplemented with 0.05 ml of a trace elements solution with the following composition g/l :H_3BO_3:0.26; $CuSO_4$ $5H_2O$:0.5; $MnSO_4$ H_2O:0.5; $MoNa_2O_4$ $2H_2O$:0.06, $ZnSO_4$ $7H_2O$:0.7.

Then to this medium 2% olive oil was added. The medium was prepared inoculated 2 ml of overnight culture and incubated in mechanical shaker at 37ºC for 24 hours. After incubation the extracellular enzyme produced in the culture medium was extracted by centrifugation. The crude enzyme preparation thus obtained was assayed for lipase activity. (**Lipase Enzyme assays Kumar S *et al*., 2005.)**

The lipase activity of extracted crude enzyme from both submerged fermentation and solid state fermentation was determined separately using olive oil as a substrate.1ml Tris Hcl buffer pH 7.7 1 ml of distilled water pinch of bile salt for emulsification then add 0.1ml of the enzyme then incubated at 55ºC for 30 minutes. The reaction was terminated by adding 3ml of ethanol 100% followed by Titration method against 50mM NaoH to determine the amount of fatty acid liberated. One unit of enzyme activity was defined as one micro equivalent of fatty acid released from triglyceride in one hour at pH 7.5 and 55ºC.

Effect on the incubation time on enzyme production by using submerged fermentation

About 250 ml of conical flask to prepared 100 ml basal mineral broth added 2ml of olive oil adjusted the alkaline pH in *Bacillus sp* and *Lactobacillus sp*. The medium was sterilized to autoclave in 121°C for 15 minutes, after sterilization broth is cooled in 40°C the inoculated one loop full of selected colonies in two sets of conical flask. The flask was incubated at 37°C for 12, 24, 36, 48, 60 and 72 hours after incubation period the result was observed.

Effect on the temperature on enzyme production by using submerged fermentation

The clean conical flask were taken from 100 ml mineral broth and 2 ml olive oil the adjusted the Alkaline pH in *Bacillus sp* and *Lactobacillus sp*. Medium of the flask were sterilized and cooled the 40°C, after cooling the inoculated selected colonies. In each two sets conical flask. The flask were incubated in different temperature at 25°C,30°C,37°C,40°C and 45°C for 48 hours. After incubation period the result was observed.

Effect on the pH on enzyme production by using submerged fermentation

The clean conical flask was prepared from adjusted the pH 6,7,8,9 and 10 by *Bacillus sp*. as well as *Lactobacillus sp*. The medium was sterilized in autoclave 121°C for 15 minutes after sterilization cool the medium inoculated the specific selected bacteria one sets *Bacillus sp* and another set of *Lactobacillus sp.*

Production of lipase enzyme by using different agro waste by submerged fermentation

The substrate such as Coconut oil cake, Groundnut oil cake and Sesame oil cake was selected for this work. From each substrate; 10g was taken in 250 ml conical flask. Then the substrate was moistened with distilled water, nutrient solution basal mineral medium and nutrient solution basal mineral medium with olive oil Sankareswaran M *et al*., 2014 Table no: 1. The medium was prepared inoculated with overnight culture and incubated mechanical shaker 100 rpm at 40°C ± 1°C for 48 hours. After incubation the crude enzyme was extracted and analyzed for enzyme activity and then further purification lipase enzyme.

Partial purification of the lipase enzyme

Ammonium sulphate precipitation method was used for this work. 70% ice cold ammonium sulphate added to the cell free extract and incubated at overnight after the incubation the precipitation was found. The precipitation was centrifuged at 6000 rpm for 10 min the pellet was suspended in 0.1 M Tris HCl buffer. The enzyme was dialyzed using cellulose acetate membrane against Tris HCl buffer Sharma R *et al*., 2001.

Purification of lipase enzyme

The partially purified enzyme was subjected to DEAE-cellulose column using ion exchange chromatography to obtain pure and homogenous enzyme. The purified was subjected to determine molecular weight by SDS-PAGE.

RESULTS AND DISCUSSION

Lipases are widely used in the processing of fats and oil detergents and detergents and processing, the synthesis of fine chemical, pharmaceuticals, paper manufacture and production of cosmetic and pharmaceuticals. Lipase can be used to accelerate the degradation of fatty waste and polyurethane Takamoto T *et al*., 2001 .

The organism isolated from the soil sample used in the study has been identified as species of *Bacillus sp* and *Lactobacillus sp* based on the staining motility and biochemical reactions. Table No: 2 and 3.

Table 1. Combination of submerged fermentation system.

S.NO	Substrate	Moistening solution
1.	Coconut oil cake	Substrate with Distilled water. Substrate with nutrient solution basal mineral medium . Substrate with nutrient solution basal mineral medium with olive oil.
2.	Ground nut oil cake	Substrate with Distilled water. Substrate with nutrient solution basal mineral medium . Substrate with nutrient solution basal mineral medium with olive oil.
3.	Sesame oil cake	Substrate with Distilled water. Substrate with nutrient solution basal mineral medium . Substrate with nutrient solution basal mineral medium with olive oil.

The selected strain *Bacillus sp* and *Lactobacillus sp* was grown on Tributyrine agar medium. The pH range 8 to 11 almost all the pH value growth of bacteria in lipase production. The lipase production indicates the zone formation of the Tributyrine agar plate.

The bacterial, species of *Bacillus sp* and *Lactobacillus sp* in particular are capable of growing in alkaline pH and hence they are capable of secreting higher enzymes which are active at alkaline pH values Anderson *et al*., 1979.

Table 2. Biochemical Results for *Bacillus species.*

Biochemical Test	**Results**
Methyl Red test	Negative
Voges Proskar test	Negative
Citrate utilization test	Positive
Urease test	Positive
Triple Sugar Iron agar test	K/A
Oxidase test	Positive
Catalase test	Negative
Carbohydrate test: Glucose Lactose Sucrose Manitol Maltose	 AG A Negative Negative Negative
Motility test	Motile

K/A- Alkaline Slant Acid butt, AG-Acid Gas, A-Acid

Table 3. Biochemical Results for *Lactobacillus species.*

Biochemical Test	**Results**
Methyl Red test	Negative
Voges Proskar test	Negative
Citrate utilization test	Negative
Urease test	Negative
Triple Sugar Iron agar test	K/K
Oxidase test	Negative
Catalase test	Negative
Carbohydrate test: Glucose Lactose Sucrose Manitol Maltose	 A A A A A
Motility test	Motile

K/K- Alkaline Slant Alkaline butt, AG-Acid Gas, A-Acid

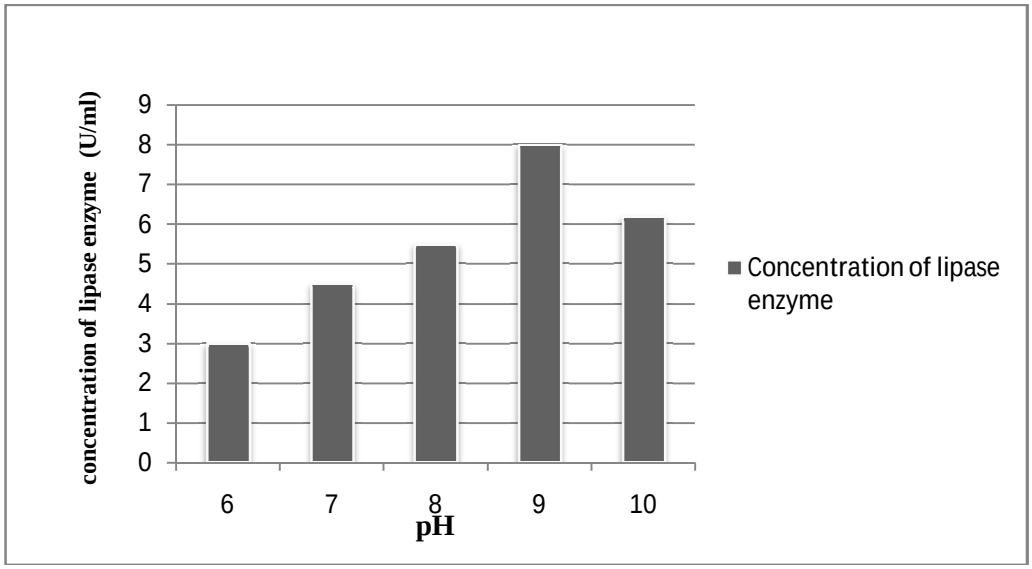

Fig 1. Effect of pH on enyme production by *Bacillus sp*

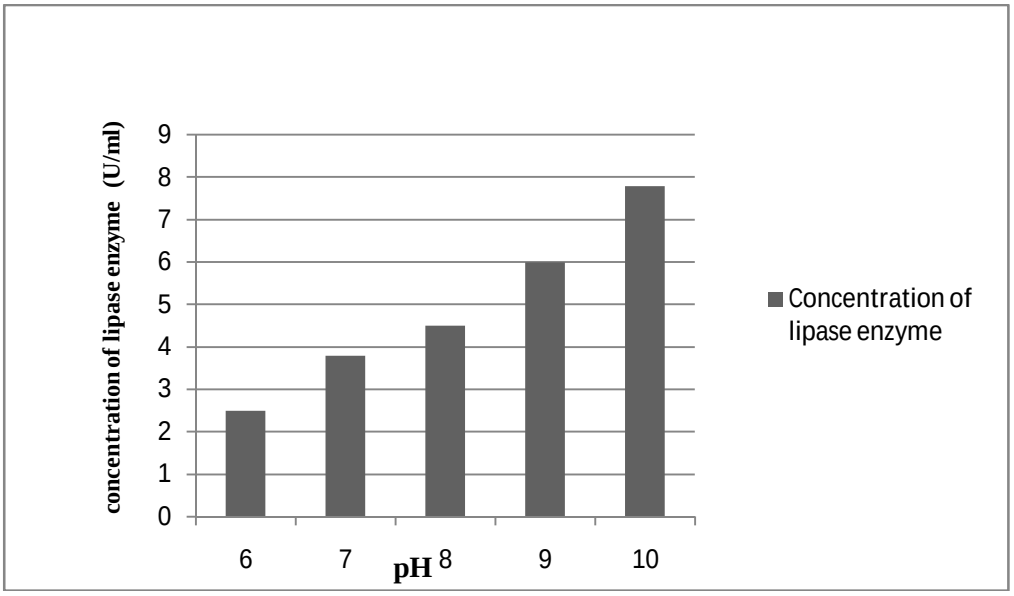

Fig 2. Effect of pH on enyme production by *Lactobacillus sp.*

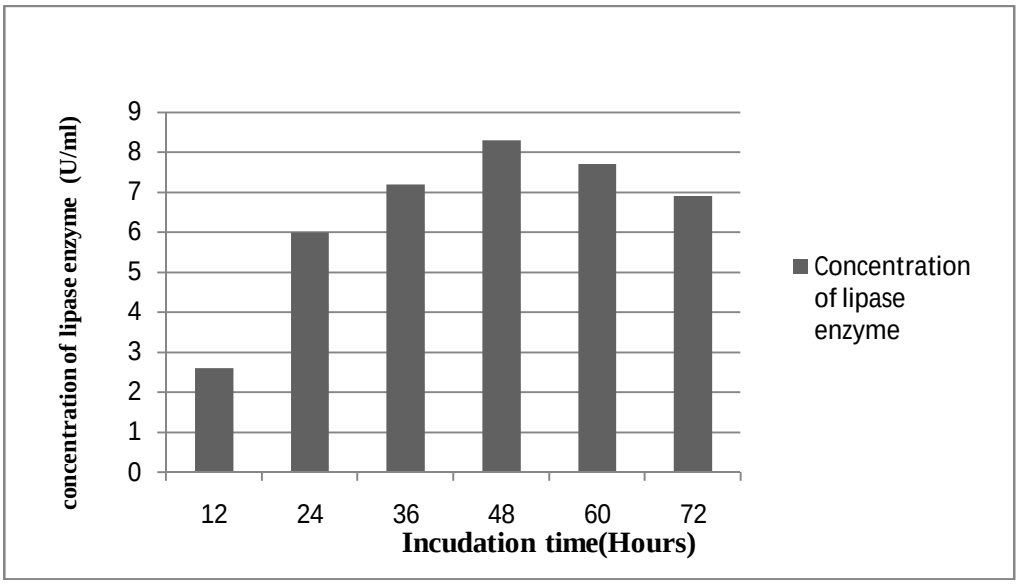

Fig 3. Effect of Incubation time on enyme production by *Bacillus sp.*

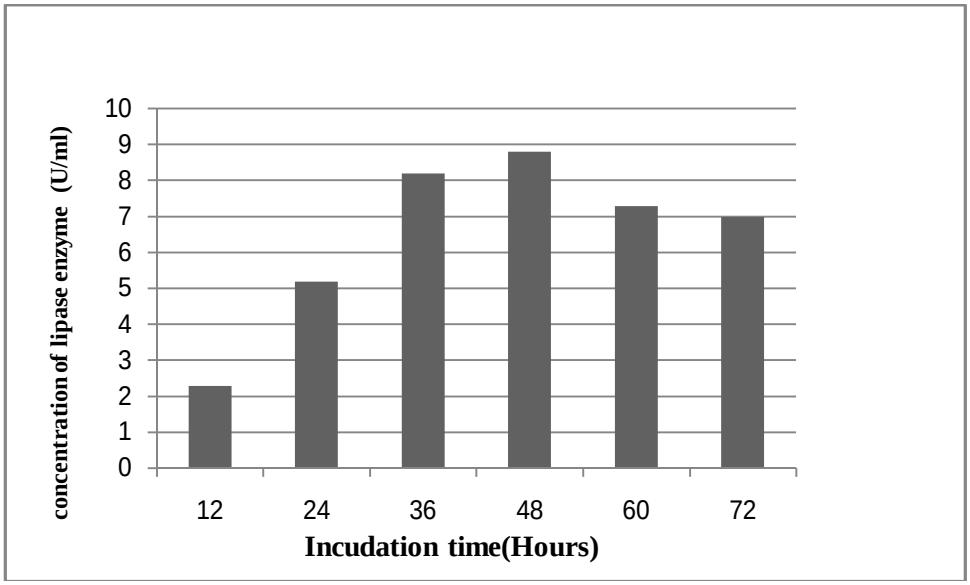

Fig 4. Effect of Incubation time on enyme production by *Lactobacillus sp.*

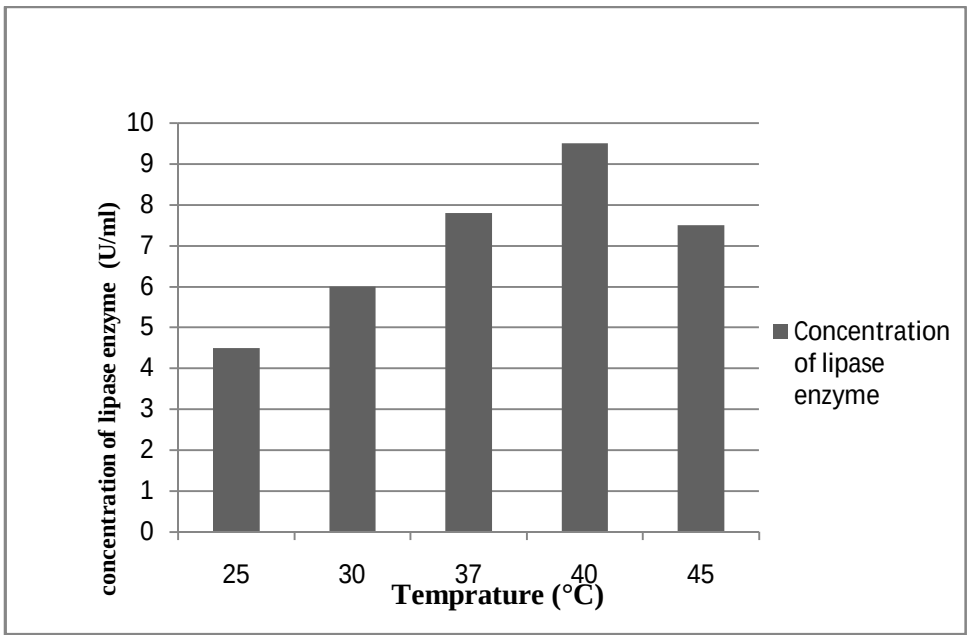

Fig 5. Effect of Temprature on enyme production by *Bacillus sp.*

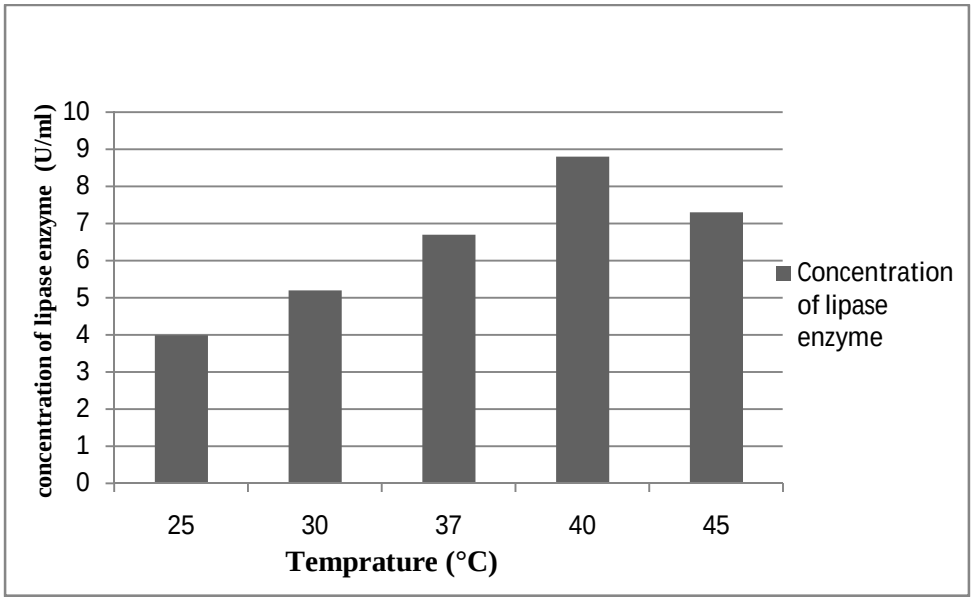

Fig 6. Effect of Temprature on enyme production by *Lactobacillus sp.*

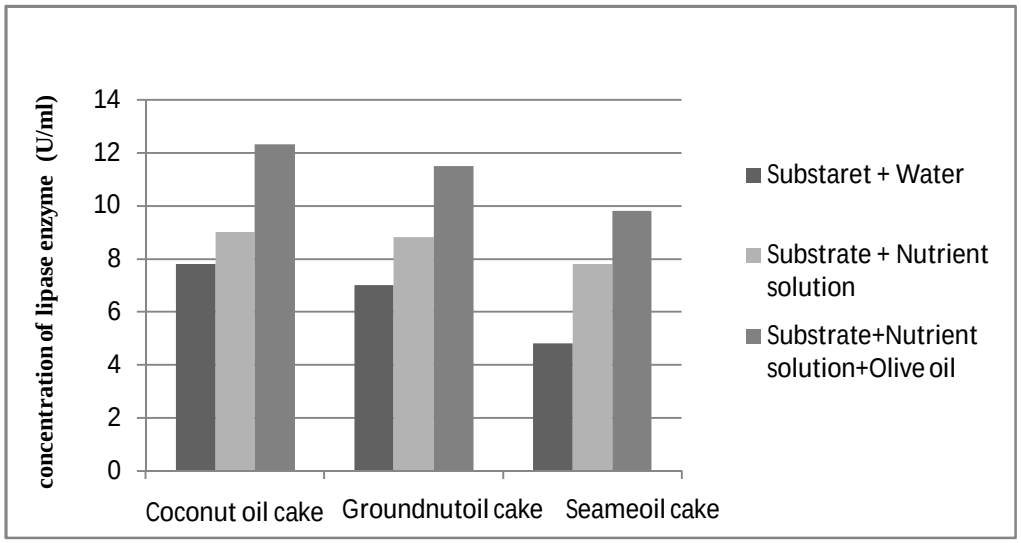

Fig 7. Enzyme production on Submerged Fermentation by *Bacillus sp.*

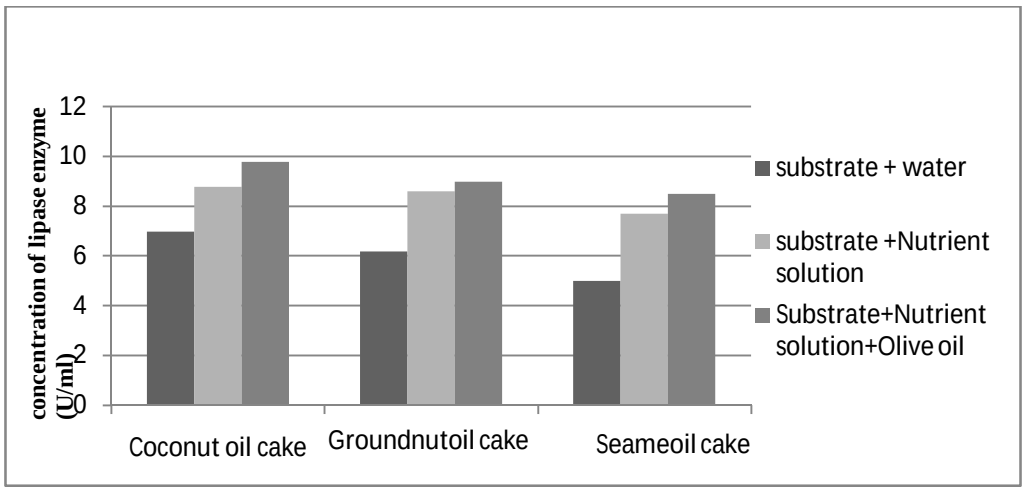

Fig 8. Enzyme production on Submerged Fermentation by *Lactobacillus sp.*

The *Bacillus sp* and *Lactobacillus sp* isolates grown on alkaline pH condition. *Bacillus sp* grown pH 9 production rate 8.0 U/ml and *Lactobacillus sp* pH 10 production rate 6.0 U/ml. Figure: 1 and 2. When the time course study was taken for the maximum enzyme production, maximum yield was found to be at 48 hours of incubation for both *Bacillus sp* and *Lactobacillus sp***.** Production of lipase enzyme both *Bacillus sp* 8.3 U/ml and *Lactobacillus sp* 8.8 U/ml . Figure: 3 and 4.

The optimum temperature for enzyme production with selected isolate of *Bacillus sp* and *Lactobacillus sp* was found to be 37°C**.**When the optimum temperature of enzyme production found at 40°C. Lipase enzyme was produced in both of *Bacillus sp* 9.5 U/ml and *Lactobacillus sp* 8.8 U/ml under 40°C. Figure: 5 and 6.

From these result, it has been observed that, coconut oil cake as substrate supplanted with nutrient solution basal mineral medium with olive oil broth has a higher yield of enzyme getting from *Bacillus sp* 12.3 U/ml and *Lactobacillus sp* 9.8 U/ml Figure: 7 and 8.
The cured lipase enzyme was dialyzed and purified by DEAE cellulose ion exchange column chromatography. The molecular weight of the purified lipase enzyme was confirmed as 45 kDa through SDS PAGE.

CONCLUSION

In conclusion, the selected isolates of *Bacillus sp* and *Lactobacillus sp* are a good candidate for production of alkaline lipase. The optimum pH 9 for *Bacillus sp* and pH 10 for *Lactobacillus sp,* the incubation time for both *Bacillus sp* and *Lactobacillus sp* 48 hours and optimum temperature for both *Bacillus sp* and *Lactobacillus sp* 40°C given high yield of lipase enzyme. Coconut oil cake as substrate supplement with olive oil could serve as an effective substrate for submerged fermentation, the high level lipase enzyme produced by *Bacillus species*, with submerged fermentation the enzyme production rate is 12.3 U/ml The organism is capable of producing a highly alkaline stable enzyme which is a demand of many industries.

ACKNOWLEDGEMENTS

The authors are thankful to our Institution of Muthayammal College of Arts and Science for providing the necessary material for this study.

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