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Isolation, Screening and optimization of lipase producing Staphylococcus spp. from oil mill soil

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Abstract

Lipases involve an unmistakable spot as biocatalysts and have an expansive range of biotechnological applications. The security of these proteins in natural solvents has driven them into outskirts regions of natural combination prompting the planning of novel medications, surfactants, bio responsive mixes, and oleochemicals.. In the present study, bacterial cultures were isolated from various sources of which 8 were found to be positive for lipase production. All these 8 isolates were screened by their zone of hydrolysis observed on the Tributyrin agar plate. The strain showing the maximum zone of hydrolysis on the tributyrin agar plate was identified by morphological and biochemical characters as *Staphylococcus* spp. The 5% inoculums size for production, groundnut oil cake as substrates, greatest lipase creation was noticed at 48 hours of growth, pH 8 was notice to be an ideal pH, maximum growth at 37°C temperature, Fermentation in shake flask improved the lipase yield, moisture found of 80% was ideal for growth. Results presented in this study indicate that the *Staphylococcus spp*. isolated from our present study may be abused as a potential producer for industrial production of lipase.

Keywords: Lipase production, TBA (tributyrin agar), *Staphylococcus spp*., optimization of media components.

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Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are hydrolytic enzymes that catalyze the hydrolysis of triacylglycerols into monoacylglycerols, diacylglycerols, free fatty acids, and glycerols (1). Also under low water conditions lipases catalyze ester synthesis and transesterification.

lipases are used industrially for a long time and have a high potential for various applications, their use is presently limited because of the high cost and lack of lipases with features required for certain applications (2). On account of the colossal variety in applications, the accessibility of lipases with explicit attributes is as yet a restricting element. In this manner to look for new lipases with various qualities and improve lipase creation keep on being significant examination themes. (1).

Thus, lipases are most important enzymes of selecting for organic chemists, pharmaceuticals, biophysicists, bio-chemical and practice engineers, biotechnologists, microbiologists, and biochemists. Looking with the wide situation of lipase application commercialization of lipase creation is a main area of interest. Microbes are the tools for commercial production. Commercially valuable lipases are generally obtain from microorganisms to produce a broad range of extracellular lipases. Extracellular lipases are produced from microorganisms like as bacteria, mould, yeast, etc. Besides that, the plants and animals can also produce lipases. (3). Bacterial lipases have been very much considered contrasted with plant and fungi lipases. Bacterial lipases are glycol-proteins, but a few extracellular bacterial lipases are lipoproteins.. The majority of bacterial lipases reported so far is constitutive and are distracted in their substrate specificity, and only some bacterial lipases are thermostable. (4).

Structure Of Lipase The 3-D structure of lipase in fungus *(Rhizomucor miehei)* and human pancreas were determined in 1990. These enzymes have molecular weight ranges from 19 to 61 KD. All enzymes exhibit a characteristic folding pattern -sheet consisting of up to eight various β-strands (β1-β8) connected by up to six α-helicals (A-F).

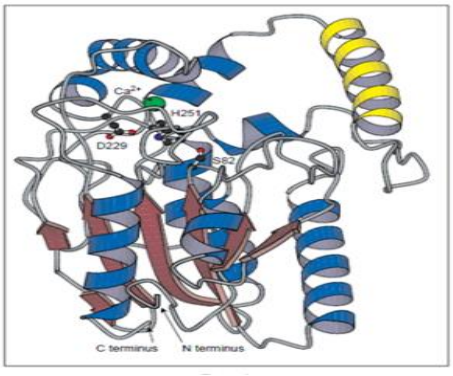

Figure-1:- Structure of lipase. (β strands are represented as arrows and a-helices as coil; the yellow helix could form a 'lid' over the active site)

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Lipases are activated only when adsorbing to an oil-water interface. Interfacial activation of lipases occurs at the lipid-water interface, a event that can be traced to the exceptional structural character of this division of enzymes. (5).

In nature, the lipases available from from different sources have considerable disparity in their reaction specificities: this property is generally referred to as enzyme specificity. Thus, as of the fatty acid part, a few lipases have an resemblance for short-chain fatty acids (acetic, butyric, capric, caproic, caprylic, etc.), a few have a first choice for unsaturated fatty acids (oleic, linoleic, linolenic, etc.), while lots of others are nonspecific and randomly crack the fatty acids into the triglycerides. (6).

Materials and methods

Sample Collection, Isolation, and Screening:

For the present study, soil samples were collected from various sources i.e. Anand oil mill, garage, petrol pump, and hotel. Soil samples were gathered at 4 to 5 cm depth with the help of the sterile spatula. The sample was put in sterile plastic bags and transferred to the laboratory for isolation of lipase producing organisms under laboratory condition.

Lipolytic microorganisms were isolated from collected soil samples. For this, 1gm of the sample was mixed in 10 ml of sterile D/W and kept for some time. Then it was serially diluted (10^{-1} to 10^{-5}). Then diluted samples were placed on modified nutrient agar.

Microbial lipases have a great potential for commercial applications due to their stability, selectivity, and broad substrate specificity. Following protease and lipases are measured to be the third-largest group based on the entire sales quantity. (7). The marketable utilize of lipase is a thousand rupees business that comprises a large range of different applications. The majority of enzymes in present business use is of microbial source and is created in straight aerobic submerged fermentation which allows them greater organizes of the situation of growth than solid-state fermentation. The the majority dynamic species belong to the genera *Geotricum spp., Penicillium spp., Aspergillus spp. and Rhizomucor spp*. There are also a certain number of lipases produced by yeast, most of them belonging to the *Candida* genus. (8).

The dominant organisms were isolated and individually streaked on TBA plates and the formation of halo zones around the colony on tributyrin agar was considered as the positive colony. After incubation of 24 hours, a total of 8 colonies viewing clear zones were picked and their diameter was measured.

The 8 isolates were subjected to rapid screening by using the Rhodamine B method (9). The culture was spotted in the centre of the plate and incubated for 48 hrs. Lipase production was monitored by irradiating plates with UV light under a UV transilluminator.

The morphological characteristics of isolated 8 positive organisms were studied. The organism showing the highest zone of clearance on tributyrin agar was chosen for further study. The selected organism was

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examined for various morphological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology

Identification of Selected Organism

The selected isolate was identified by cultural and morphological characteristics as well as by performing biochemical tests such as sugar fermentation test, catalase, Voges-Proskauer test, methyl red test, gelatinase test, indole test, Simmon citrate utilization test, starch hydrolysis test, and triple sugar iron test.

Inoculums Preparation

To prepare the inoculums, a loopful of cells from a freshly grown slant of organisms was transferred into the 150 ml of the conical flask containing 50 ml of minimal media (without agar). The media was autoclaved before use. After inoculation, the flask was put on the shaker at 37^0C and 150 rpm.

Media Preparation

10 gms of desired oil cake was suspended into the 90 ml of the minimal media into 250 ml flasks. The media was autoclave at 121^0C and 15lbs pressure for 20 minutes. It was cooled before using.

Lipase assay

the raw enzyme obtained and perform Lipase assay and find enzyme activity. The activity of lipase was determined as described in the literature (Winkler and Stuckman, 1979) with the following modification, 10ml of isopropanol containing 30mg of p-nitrophenyl acetate was mixed with 90 ml of 0.05M of phosphate buffer (pH 8.0), containing 207.0 mg of sodium deoxycholate and 100mg of gum acacia. According to this method, 2.4 ml of freshly prepared p-nitrophenyl acetate substrate solution was mixed with 0.1ml of crude enzyme. After 15min of incubation at 37°C, optical density was measured at

410nm against a control. One unit of lipase activity is defined as the quantity of enzyme releasing 1 µmole of p-nitrophenol per minute under assay condition.

Effect of Type of Substrate on Lipase Production

The above production media (90ml + 10 gm substrate) with the different substrates were prepared in 250ml flasks. The substrates used were groundnut oil cake, coconut oil cake, castor oil cake, and sesame oilcake. The media were autoclaved at 15lbs and 121^0C for 20 minutes. . Flasks were incubated on the shaker for 150 rpm and 37^0C temperature. The samples were collected every 12 hours and lipase assay was performed.

Effect of Incubation Time on Lipase Production

The optimum time was measured by incubating flasks, which have groundnut oil cake as a substrate in the fermentation medium, on the shaker for 150 rpm and 37^0C temperature. The samples were withdrawn at 12 hours, 24 hours, 36 hours, 48 hours, 60 hours, and 72 hours and lipase assay was carried out at regular intervals.

Effect of pH on Lipase Production

Optimization of pH was carried out by incubating groundnut oil cake containing fermentation medium at different pH of 5, 6, 7, 8, 9 & 10 in orbital shaker incubator at 150 rpm. The samples were withdrawn at regular time and lipase assay was performed.

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Effect of Temperature on Lipase Production

Optimization of temperature was carried out by incubating groundnut oil cake containing fermentation medium at different temperatures of 28°C, 37 °C & 45°C in an orbital shaker incubator at 150 rpm. The samples were collected at regular intervals and lipase assay was performed.

Effect of Moisture Content on Lipase Production

For the optimization of moisture content, media were prepared with moisture concentration of 50, 60, 70, 80, 90 & 100% with ground-nut oil cake as substrate. The samples were withdrawn at standard intervals and lipase assay was performed.

Effect of Shaking and Static Condition on Lipase Production

For comparison of shaking and static condition on lipase production, one flask by groundnut oil cake containing fermentation medium was incubated on a shaker at 150rpm at37°C and another flask was incubated in the static condition in the incubator. The samples were withdrawn at regular time and lipase assay was performed.

Results and Discussion

Isolation, Screening and Characterization of Lipase Producing Microorganism

Eight bacterial strains (LP01 to LP08) were observing to be full-grown on TBA (tributyrin agar) and produced a clear zone surrounding the colony. The diameters of the zones were measured for all 8 bacterial strains.

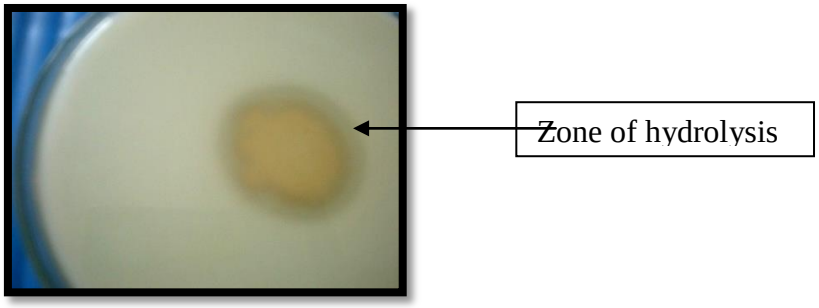

Figure- 2:- Zone of hydrolysis of LPO2 strain TBA (tributyrin agar) plate

Out of these 8 isolates, LP02 had shown the maximum zone of clearance. So LP02 was used for further study. LP02 was Gram-positive cocci and nonmotile. Based on colony characteristics, morphological characteristics, and biochemical tests, the selected isolate was identified as *Staphylococcus sp.*

BIOCHEMICAL TEST OF LPO2 STRAIN (*Staphylococcus sp.*):-

***Table:- 1* Biochemical Test of LP02 Strain (*Staphylococcus sp.*)**

Sr no.	Biochemical media	Test	Observation of results
1.	1% peptone broth	Indole production	Negative
2.	GPB	Methyl red	Positive

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3.	GPB	VP test	Negative
4.	Simmons citrate agar slant	Citrate utilization	Negative
5.	Motility agar	Motility	Positive
6.	N-gelatine	Gelatinase activity	Positive
7.	Sugar broth Glucose Lactose Maltose Sucrose Mannitol	Sugar fermentation.	 Only acid production Negative Only acid production Only acid production Only acid production
8.	N-agar	Catalase	Positive
9.	N-broth	coagulase	Positive
10.	Starch agar plate	Starch hydrolysis	Positive
11.	Casein agar plate	Casein hydrolysis	Positive

RESULT OF TSI AGAR SLANT:-

Butt	Slant	Gas	H_2S
Yellow	Yellow	Negative	Negative

Effect of Inoculums Size on Lipase Production

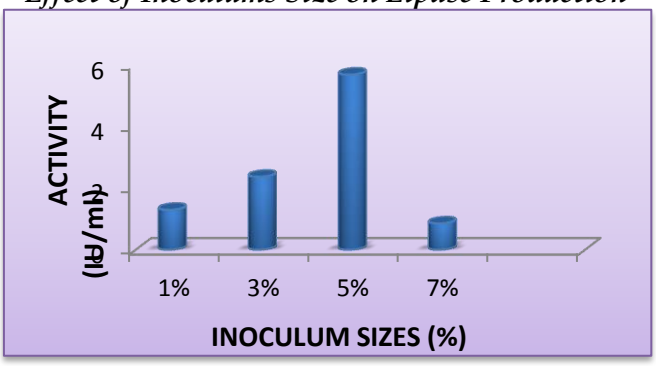

Figure- 3:- ***Effect of inoculums size on lipase production***

The inoculums size influences the lipase production very efficiently. At the different concentrations i.e. 1%, 3%, 5% and 7% inoculums sizes, the lipase production was obtained. At the 5% inoculums, the maximum activity of lipase was found. The maximum activity was (5.67 IU/ml).

The enzyme activity decreases with the increasing the size of the inoculum might be due to dumping of cells which could have reduced availability of substrate and reduced oxygen uptake rate and also enzyme release.

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Effect of Type of Substrate on Lipase Production

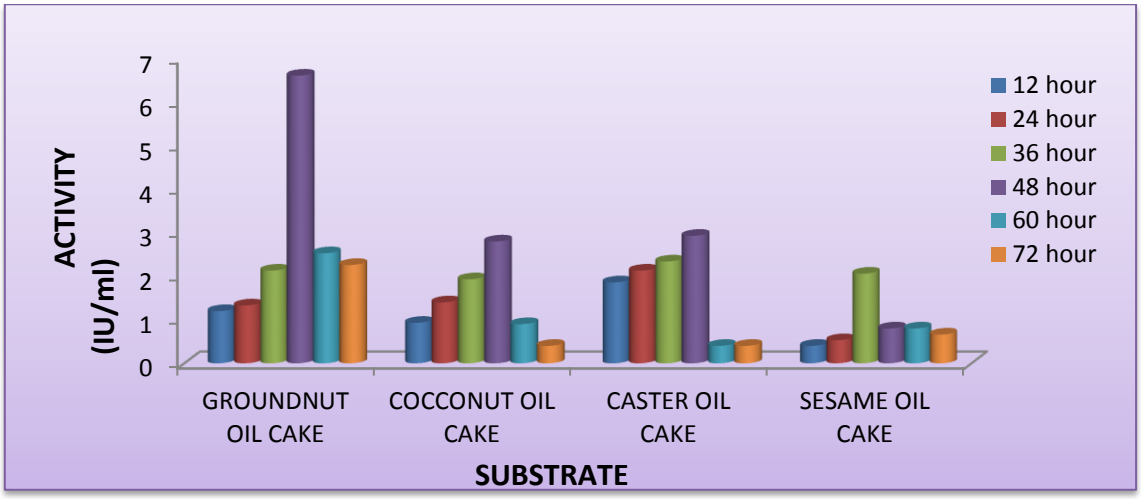

Figure- 4:- Effect of type of substrate on lipase production

From all the substrate, the highest lipase activity was found with groundnut oil cake as substrate. The highest lipase activity was 6.60 IU/ml.

Different substrate occupied surface area according to their sizes. Due to its simple penetration, the microbial mass of the bacterial culture showed a high growth rate with groundnut oil cake as a substrate due to which more lipase production was observed. The less lipase production at a higher level was due to the low mass transfer rate and difficulty in penetration of the organism.

Singh *et al*. (2010) found groundnut oil cake is the best among all substrates used in the case of *B. Subtilis* OCR-4 lipase production (10). In contradiction, Chaturvedi *et al*. (2010) reported that *B. Subtilis* showed higher lipase activity than grown in medium added with coconut oil cake as substrate (11). Mohan *et al*. (2008) reported that high levels of lipase activity were obtained in *Bacillus* strains when olive oil was used as the substrate (12).

The lipase production was increased gradually from 12 hours (1.2 IU/ml) to 48 hours (6.60 IU/ml) and after that, the production decreased at 60 hours (2.53 IU/ml). The production was observed to be optimum in the formulated medium at 48 hours.

Pogaku *et al*. (2010) reported that the *Staphylococcus sp*.Lp 12 showed maximum lipase activity at 48 hours of incubation (13). Sarkar *et al*.(1998) have found that Pseudomonas strain isolated from soil gave a maximum yield of lipase after 72 hours of incubation.

Lipases are produced by bacterial growth and with optimum production at the late exponential growth phase (Gupta *et al*.,2004). Therefore depending on environmental situation and the features of the microorganism itself, the best incubation

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time is depends on the duration of log-phase where restriction of growth elements occur.

Effect of Incubation Time on Lipase Production

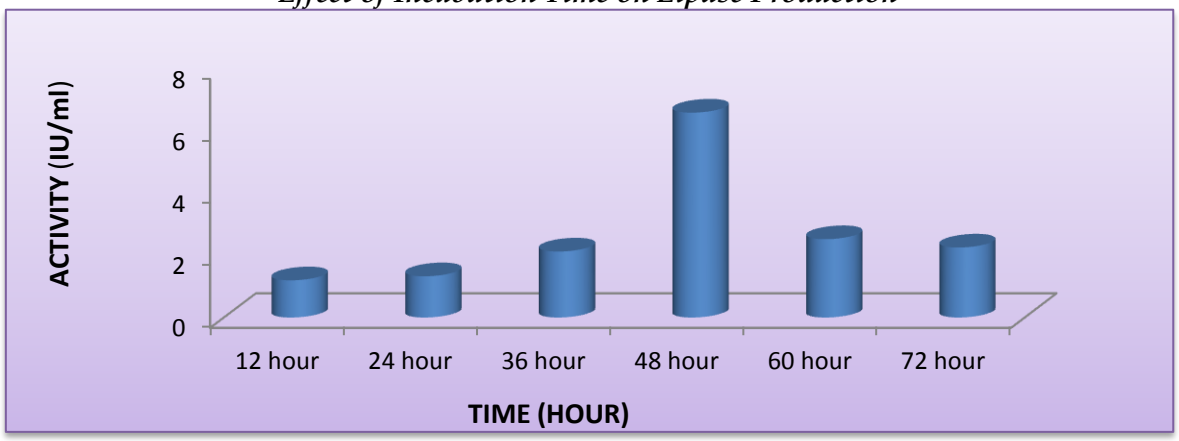

Figure- 5:- Effect of incubation time on lipase production

A quick decrease in lipase production after 48 hours of cultivation might be due to proteolytic degradation of enzyme system. The decrease of lipase productivity at the later on fermentation stage might be explained by pH in-activation, proteolysis, or both and the increase of protease activity was the result of the liberate of intracellular protease.

Effect of pH on Lipase Production

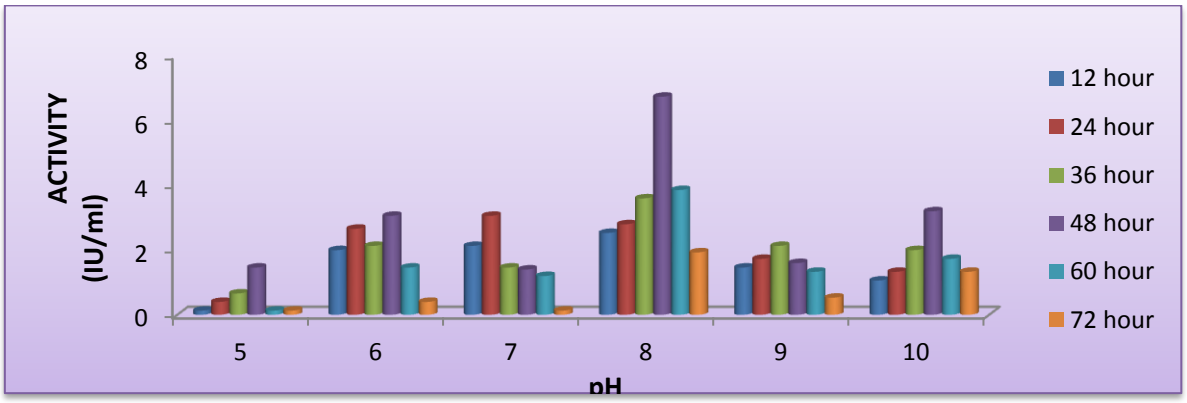

Figure- 6:- Effect of pH on lipase production

The enzyme activity improved with an primary increase in pH and optimum was found at pH 8. Additional increase in pH further than optimum caused a fast decrease in the enzyme activity. These results could be expected, considering that this microorganism was isolated from soil and natural habitats where the pH is neutral or alkaline.

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Pogaku *et al*. (2010) revealed that the lipase production by *staphylococcus sp* Lp 12 was maximum at pH 8 (13). Gao (2004) reported that *Serratia marcescens* preferred slightly acidic pH for lipase production (14). Marcin *et al*. (1993) reported that P.aeroginosa MB preferred neutral pH. Khoramnia *et al*. observe maximum lipase activity at pH 8 with *Staphylococcus xylosus* (15).

Effect of Temperature on Lipase enzyme Production

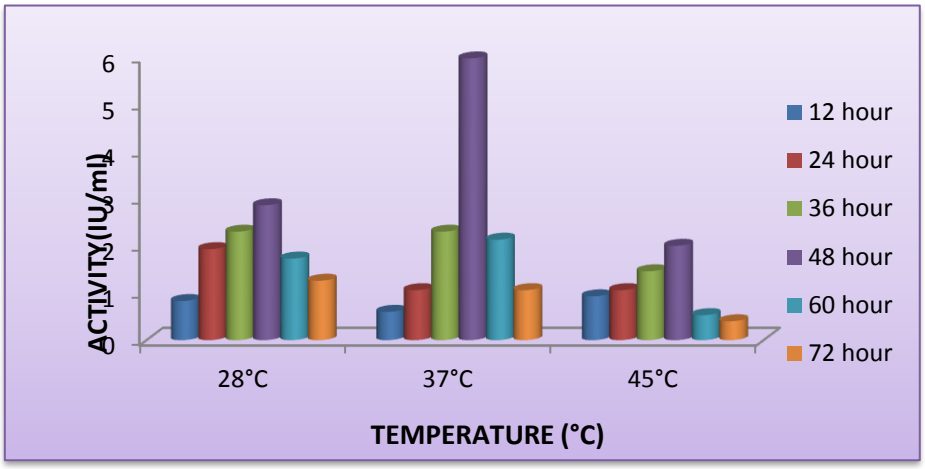

Figure- 7:- Effect of temperature on lipase enzyme production

In this study, the temperature effect on the activity of lipase production reveals that the optimum temperature was 37°C (5.98 IU/ml).

Mohan et al., (2008) reported that the lipase production by the isolated strains was higher (0.0021 µg/ml/min) at 37°C with compared to 27°C and 47°C temp (12). *Walavalkar and Bapat (2001)* have reported that the activity of lipase from *Staphylococcus spp*. was maximum at 37°C (16).

The thermal stability of lipase activity is related to its configuration and subsequently the melting point. The compact 3-D structure of the enzyme is thermodynamically more stable and catalytically more active. Above a certain temperature, the enzyme loses the compact 3-D structure required for the enzyme activity i.e. enzyme is denatured. Incubation of many enzymes at temperatures above about 50°C leads to fairly rapid loss of catalytic activity, although many enzymes can withstand exposure to high temperature.

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Effect of Moisture Content on Lipase Production

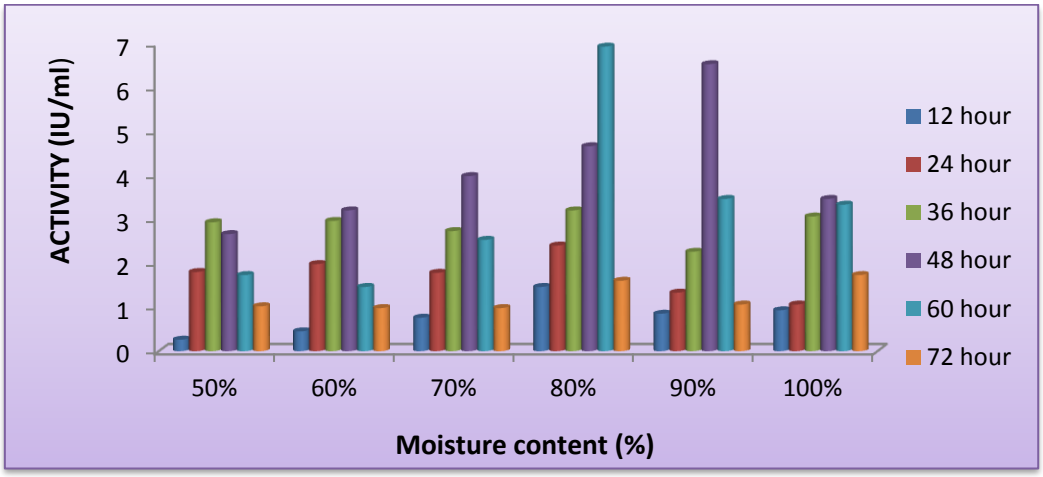

Figure- 8:- Effect of moisture content on lipase production

The original moisture content of 50% lipase yield was 2.66 IU/ml which considerably increase with the increase in moisture content. The maximum yield was at 80% (6.93 IU/ml). Variation in the first moisture content of substrate observed that the enzyme production was associated to the accessibility of moisture content.

Chaturvedi *et al*. reported highest lipase activity at 70% moisture content. Singh *et al*. also reported the highest lipase activity at 70% moisture content (11).

Effect of Shaking and Static Condition for Lipase Production

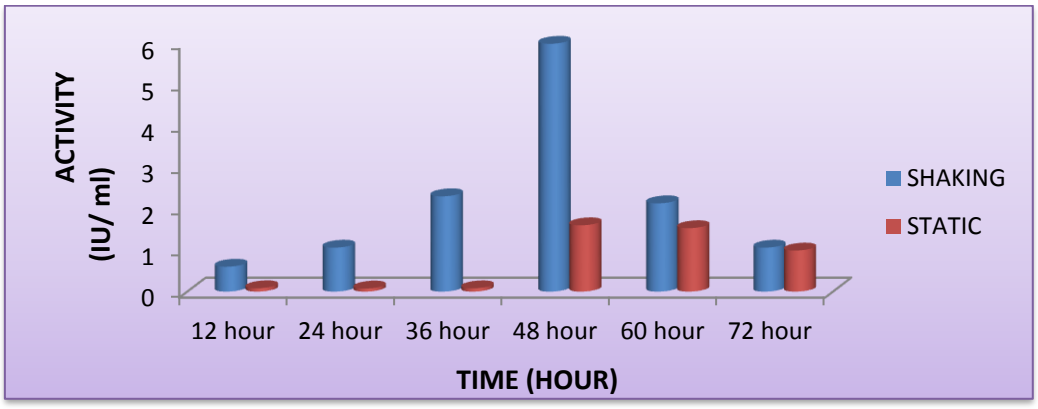

Figure- 9:- Effect of shaking and static condition for lipase production

As the result of the comparison between static and shaking conditions, the highest lipase activity was found in shaking conditions which was 5.98 IU/ml.

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This is because of the oxygen limitation. In static conditions, the microorganisms cannot yield oxygen at the lower side. So cannot grow properly. Therefore lipase activity decreases. Whereas in shaking condition the organisms get sufficient oxygen and can be grown properly. So lipase activity becomes higher.

Conclusion

In the present study, bacteria were isolated from different sources of which 8 were observing to be positive for lipase production. All these 8 isolates were screened by their zone of hydrolysis observed on the Tributyrin agar plate. The strain showing the maximum zone of hydrolysis on the tributyrin agar plate was recognized through morphological and biochemical characteristics as *Staphylococcus* spp. The 5% inoculums size was observe to be ideal for the lipase production by *Staphylococcus* spp. and highest lipase activity was 5.67 IU/ml. Among the different substrates experienced, groundnut oil cake was observed to be suitable for enhancing the lipase production by the isolated and screened *Staphylococcus* spp. and the highest lipase activity was 6.60 IU/ml. The highest lipase production was found at 48 hours of growth. The pH 8 was observed to be an ideal pH for optimum production of lipase by *Staphylococcus* spp. and highest lipase activity was 6.73 IU/ml. The influence of medium temperature indicated that the lipase production by *Staphylococcus* spp. was higher (5.98 IU/ml) at 37°C temperature when compared with those at 28°C and 45°C. Fermentation in shake flask improved the lipase yield with an activity of 6.93 IU/ml with moisture found of 80% using oil cake of groundnut as a substrate. Results presented in this study indicate that the *Staphylococcus* spp. isolated from our present study may be used as a potential producer for industrial production of lipase.

Conflict of Interest: Authors declares no conflict of interest

Authors Contributions: Each and every author had contributed to the manuscript.

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