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Isolation, Screening and Optimization of Lipase Producing Microorganisms from Industrial Waste

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Abstract
Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. The many applications of lipases include specialty organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses. In view of the increasing understanding of lipases and their many applications in high-value syntheses and as bulk enzymes, these enzymes are having an increasing impact on bioprocessing. In this study, microbes isolated from industrial waste are screened for the lipase production.

Keywords
Industrial waste, Microbes, Enzymes, Lipases.

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INTRODUCTION
Many attempts have been made to isolate lipase producing microorganisms since this enzyme is used in numerous biotechnological processes including food, leather, cosmetic, detergents and pharmaceutical industries and industrial wastes management (1). Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long- chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional methods (2). Many microorganisms such as bacteria, yeast and fungi are known to secret lipases. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc (3). The most important stage in a biological process is optimization to improve and increase the efficiency of the process without increasing the cost. The purpose of the present study is to isolate new organism from industrial waste and to characterize them for use in industrial application.

According to Enzyme Nomenclature given by International Union of Biochemistry and Molecular Biology (IUBMB), Lipolytic enzymes are grouped into 2 main categories, those which can hydrolyze triglycerides at the water/oil boundary are termed lipases or, more systematically, triacylglycerol

hydrolases, and those which attack phospholipids are termed Phospholipases (4).

The aim of this study was to isolate and select lipase producing microbes from effluent waste from industries. A total of 3 samples were collected from waste dump sites of dairy industry, fish market, oil press (chekku). 20 isolates of bacteria were isolated using Nutrient Agar medium and 16 isolates of fungi were isolated using Czapek dox agar medium.

MATERIALS AND METHODS

Collection of samples

Each sample was collected in dry, sterile polypropylene containers and transported immediately to lab. These containers were maintained at 4°C to ensure the minimal biological activity. The entire area of sample was swabbed using the sterile cotton swab immersed in normal saline, the swab was then used for culture.

Isolation of bacteria

The bacterial species were isolated from the collected samples with the help of conventional serial dilution technique [5]. In this technique sample suspension was prepared by adding soil mixed with waste (1g) was added to 10 ml of sterile water (the stock) and shaken vigorously for at least 1 minute. The dilute was then sedimented for a short period. Sterile dilution blanks were marked sequentially starting from stock and10^{-1} to 10^{-4}. One ml from the stock was transferred to the 10^{-1} dilution blank using a fresh sterile pipette. One ml from the 10^{-1} dilution was transferred to the 10^{-2} tube for each succeeding step then from the 10^{-2} to the 10^{-3}, then from the 10^{-3} to the 10^{-4}. From each dilution tube 0.1 ml of dilution fluid was transferred into Nutrient Agar culture media and incubated at 37 °C for 24 hours.

Screening of bacteria

Tributyrin Plate Assay

The bacterial isolates were screened for lipolytic activity on tributyrin agar plates. A loopful of isolate was streaked into the tributyrin medium and incubated at 37°C for 24 hours. After incubation the isolates were observed for lipolysis i.e. zone of hydrolysis around the colony. Lipase production is indicated by the formation of clear zone around the colonies grown on tributyrin- containing agar plates [6, 7]

Optimization conditions for lipase producing bacteria

a) Optimum pH

Weigh 2.4g of LB Broth and dissolve in 100ml of distilled water. Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inculcated loop full of Bacterial Culture and mixed with 10ml of LB Broth. Incubate the cells at 37°C overnight with shaking. Prepare 100ml of LB broth, aliquot 10ml of broth in to 30ml Boiling tube and Label each flask indicating different pH from 2-10. Adjust the pH of each flask as mentioned in the label (use 0.1N HCl and 0.1N NaOH provided in the flask). Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inoculate all the flasks with 50µl overnight culture of given organism and keep it in shaker at room temperature for 24 hours. aliquot small volume of culture in a cuvette and note down the OD value at 600nm. Plot of graph X axis against OD value in y –axis and find the optimum pH of the grown culture.

b) Optimum temperature

Weigh 2.4g of LB Broth and dissolve in 100ml of distilled water. Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inoculated loop full of Bacterial Culture and mixed with 10ml of LB Broth. Incubate the cells at 37°C overnight with shaking. Prepare 100ml of LB Broth, aliquot 10ml of broth in to 30 ml Boiling tube. Label the flask indicating the temperature. (10 °C, 25°C, 30°C, 37°C, 45°C, 60°C). Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inoculate all the flasks with 50µl overnight culture of given organism and incubate at various temperature for 24 hrs (10 °C, 25°C, 30°C, 37°C, 45°C, 60°C). Duplicate or triplicate flasks are maintained for each temperature. After incubation, the optical density of each culture is measured at 600nm, using LB broth as blank. Plot of graph X axis against OD value in y –axis and find the optimum temperature of the grown culture

c) Optimum day

Weigh 2.4g of LB Broth and dissolve in 100ml of distilled water. Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inculcated loop full of Bacterial Culture and mixed with 10ml of LB Broth. Incubate the cells at 37°C overnight with shaking. Aliquot small volume of culture in a cuvette and note down the OD value at 600nm for 1-7 days. Plot of graph X axis against OD value in y –axis and find the optimum day of the grown culture.

Identification of lipase producing bacterial strain

The isolate which shows maximal lipase activity TBA plate assay is considered as positive colonies for lipase enzyme production [8].

Isolation of fungi

Serial dilution techniques were used for the isolation of fungi. In this technique sample suspension was prepared by adding soil mixed with waste (1g) was added to 10 ml of sterile water (the stock) and shaken vigorously for at least 1 minute. The dilute was then sedimented for a short period. Sterile dilution blanks were marked sequentially starting

from stock and10^{-1} to 10^{-4}. One ml from the stock was transferred to the 10^{-1} dilution blank using a fresh sterile pipette. One ml from the 10^{-1} dilution was transferred to the 10^{-2} tube for each succeeding step then from the 10^{-2} to the 10^{-3}, then from the 10^{-3} to the 10^{-4}. From each dilution tube 0.1 ml of dilution fluid was transferred into Czapek dox Agar culture media and incubated at room temperature for 48 hours.

Screening of fungi

Optimization conditions for lipase producing fungi

a) Optimum pH

Weigh 3.9g of Potato Dextrose Broth and dissolve in 100ml of distilled water. Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inculcated loop full of fungal Culture and mixed with 10ml of Potato Dextrose Broth. Incubate the cells at 25°C overnight with shaking. Prepare 100ml of Potato Dextrose broth, aliquot 10ml of broth in to 30ml Boiling tube and Label each flask indicating different pH from 2-10. Adjust the pH of each flask as mentioned in the label (use 0.1N HCl and 0.1N NaOH provided in the flask). Sterilize the broth by autoclaving at 121lbs for 20 minutes. Inoculate all the flasks with 50µl overnight culture of given organism and keep it in shaker at room temperature for 24 hours. Aliquate small volume of culture in a cuvette and note down the OD value at 600nm. Plot of graph X axis against OD value in y –axis and find the optimum pH of the grown culture.

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c) Optimum day

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Identification of fungal strain

Dissolve 0.5ml olive oil, 0.073g Calcium chloride, 0.65g nutrient broth, 0.75g agar in 35ml of distilled water and adjust pH 7.3 and add 0.005g rhodamine B dye mix well and make up to 50 ml distilled water. Pour to 20 ml rhodamine B olive oil media in petriplate. Then in the solidified plate cut three wells first one 20µl bacteria sample and second one 30 µl bacteria sample and third one 50µl bacteria sample were loaded and incubated at 20°C for to the 24 hrs [10].

Identification by sequencing method

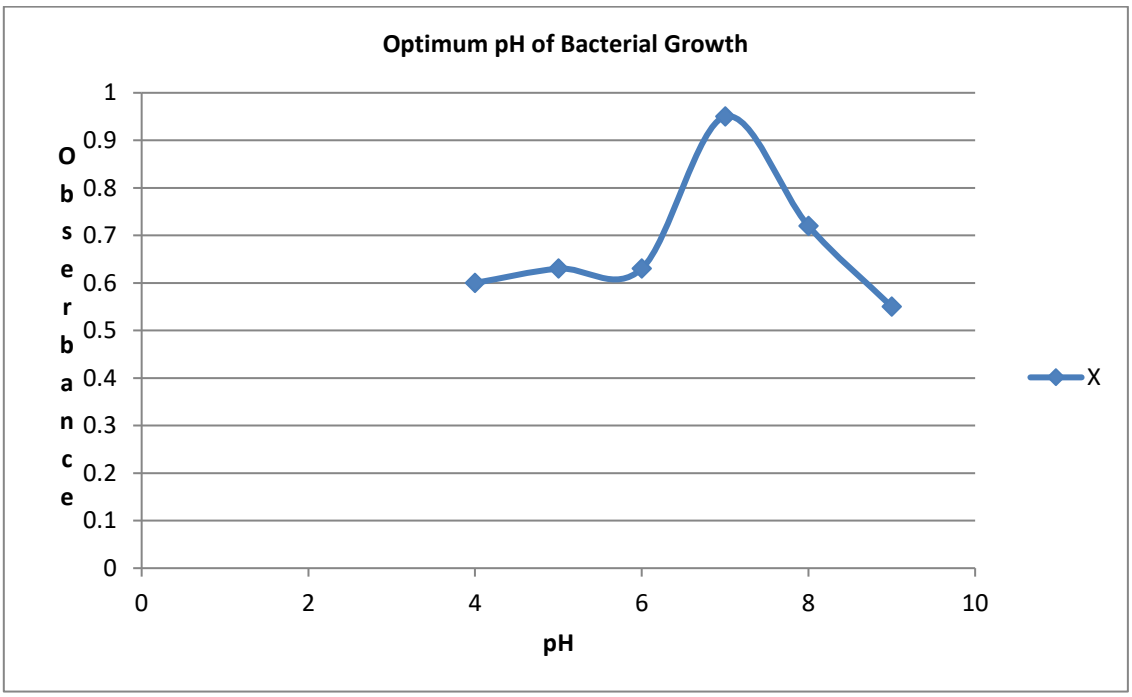
The isolate which shows maximal lipase activity in TBA plate assay and on ROA plate assay are considered as positive colonies for lipase enzyme production. The isolate which produces maximum lipase production in the lipase screening liquid medium was selected as a novel lipase producing bacteria and fungi and was identified by phenotypic characterization based on morphological, biochemical and physiological characters [13]. Genomic DNA was isolated by CTAB method. A PCR was performed in the Thermal cycler (Eppendorf, Germany) using the genomic DNA. The identity of the sequence obtained was established by comparing with the gene sequences in the database using BLAST software [14].

RESULTS AND DISCUSSION

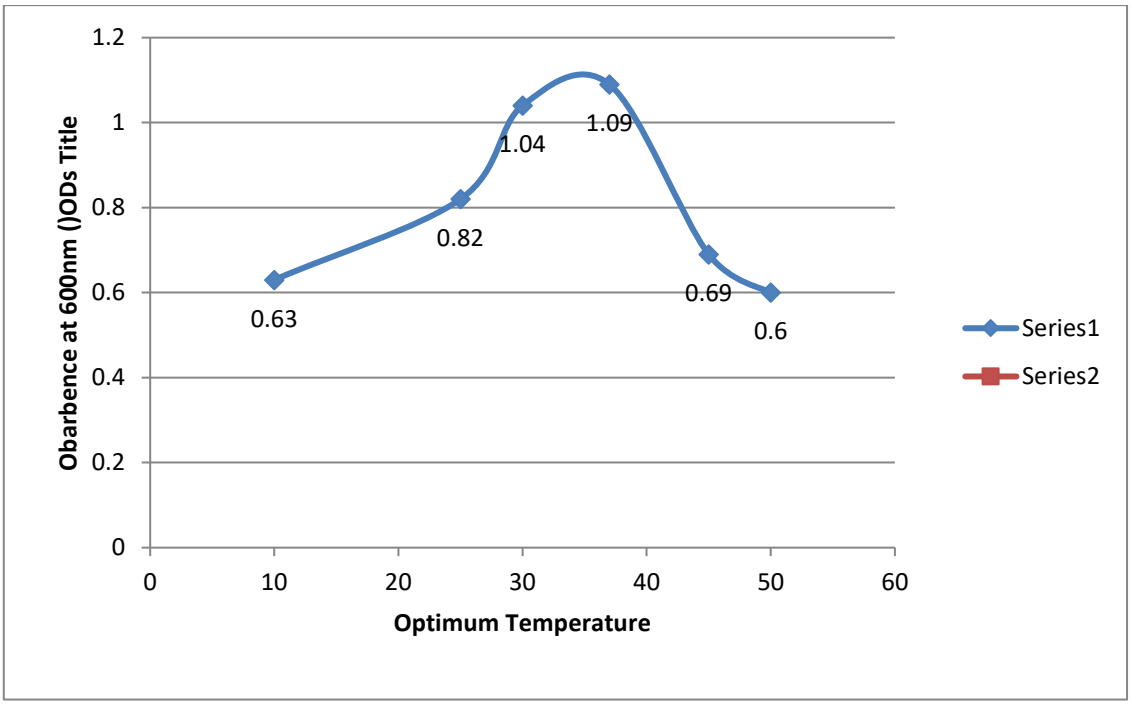
Lipases are currently used in different industrial products and processes and new areas of applications are constantly being added, which include the production of single cell protein, cosmetics, pulping, lubricants etc. [9]. Hence in the present study an attempt has been made to isolate lipase degrading microorganism from the Dairy and oil industrial waste of Coimbatore district and to investigate the ability of these microorganisms to produce lipases. All the samples were aseptically subjected to serial dilutions and plated on Nutrient Agar (NA) and Czapek dox agar by spread plate method.

Optimization results of bacteria

Lipases produced by potential isolates were also analyzed for its optimum activity at different pH. Lipases were found to give better activity at pH 6-8.

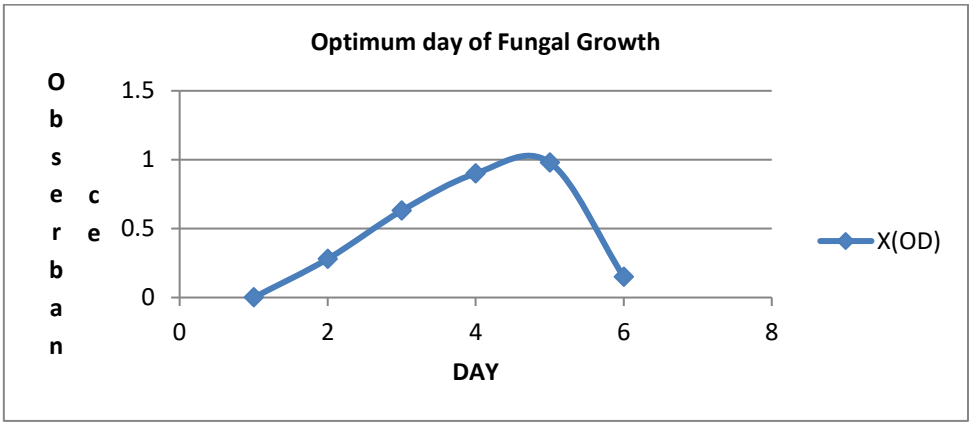


Lipases produced by potential isolates were also analyzed for its optimum activity at different temperatures. Lipases were found to give better activity at 37^{0}C temperature.

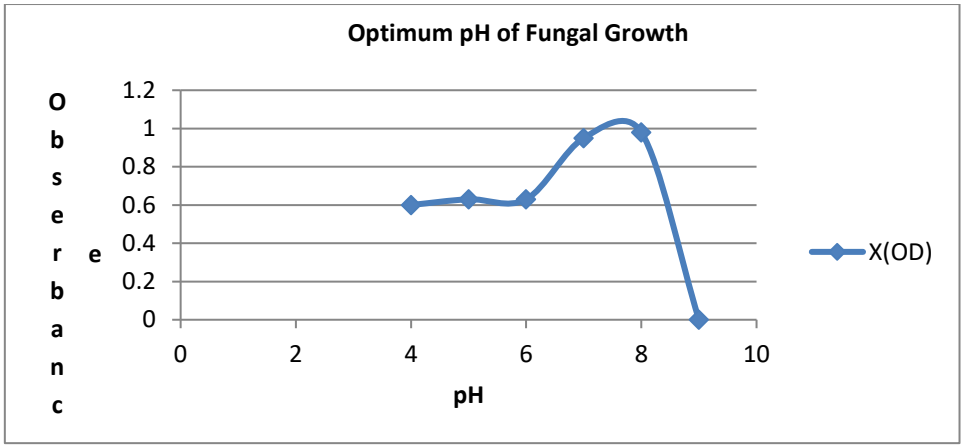


Optimization results of fungi

Lipases produced by potential isolates were also analyzed for its optimum activity at different day. Lipases were found to give better activity at day 6.



Lipases produced by potential isolates were also analyzed for its optimum activity at different pH. Lipases were found to give better activity at pH 7-8.



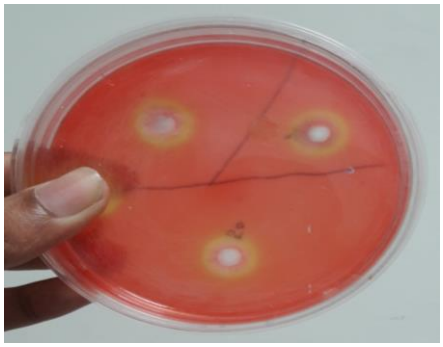
Screening of lipase producing bacteria

Screening for bacterial lipase producer using TBA plate assay was evident by the formation of clear zone around the streaking. Three isolate exhibited high lipolytic activity, five isolate showed moderate, one isolate showed low lipolytic activity and four isolates no lipolytic activity on TBA plate assay.

Screening of lipase producing fungi

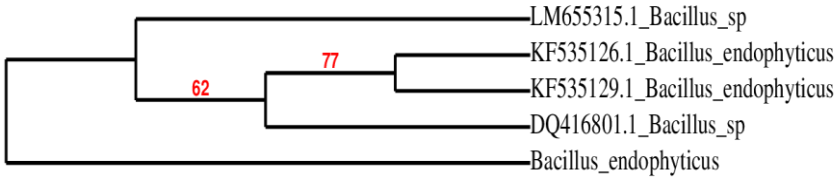
Screening for true fungal lipase producer using rhodamine B olive oil plate assay was evident by the formation of orange fluorescent halos around fungal colonies visible upon UV irradiation. Seven isolate exhibited high lipolytic activity (>40mm), six isolate showed moderate (25 to below 40 mm), three isolates showed low lipolytic activity (< 25 mm) on

rhodamine B olive oil plate assay. Since both esterase's and lipases can hydrolyze tributyrin, rhodamine B-olive oil-agar plate assay was performed in further screening in order to select efficient lipase producing bacteria [11]. Similar results were reported that 38 solvent-tolerant strains were isolated from different environment and 20 of them were lipase positive in tributyrin agar plates [12] and among which only 12 strains showed lipase activity in rhodamine B plate method.



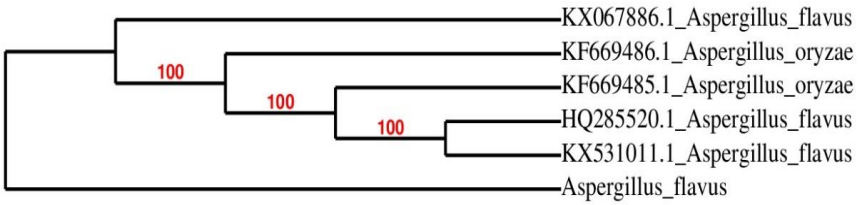
Molecular Identification and phylogenetic analysis of selected isolates

Molecular Identification Molecular techniques utilizing amplification of target DNA provide alternate methods for diagnosis and identification. To identify the experimental strain exactly according to 16S rRNA sequence analysis as well as taxonomical studies, genomic DNA of the strain was used as template to amplify partial 16S rRNA. Finally, the obtained partial 16S rRNA sequence of this strain was analyzed with BLAST. It was found to have 90-99% identity with different strains of Bacillus. Among them, it showed high similarity (99%) with Bacillus endophyticus. Therefore, it could be concluded that the isolated strain was Bacillus endophyticus. A phylogenetic tree was also constructed based on the homology of known 16S rRNA sequences.



Phylogenetic tree showing relationship of a novel lipase producing Bacillus endophyticus with other Bacillus sp.

To identify the isolated fungal strain according to 18S rRNA sequence analysis as well as taxonomical studies, genomic DNA of the strain was used as template to amplify partial 18S rRNA. Finally, the obtained partial 18S rRNA sequence of this strain was analyzed with BLAST. It was found to have 90-99% identity with different strains of Aspergillus. Among them, it showed high similarity (99%) with Aspergillus flavus. Therefore, it could be concluded that the isolated strain was Aspergillus flavus. A phylogenetic tree was also constructed based on the homology of known 18S rRNA sequences.



Phylogenetic tree showing relationship of a novel lipase producing Aspergillus flavus with other Aspergillus sp.

CONCLUSION

The samples were collected from dairy and oil industry waste around Coimbatore area. The microbes were isolated and identified by referring previous work done on the similar area. The isolates that gives maximum lipase production were screened and sequencing was done to identify their species.

FURTHER WORK

Further the work can be concentrated in the areas such as purification of lipase enzyme through salt precipitation, TLC. Even further down streaming process will be helpful to purify the crude lipase enzyme to attain its 100% purity. This enzyme is highly essential and will be applied in wide spectrum like in human diet, detergent industries, tanning industries, etc.

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