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RESEARCH ARTICLE

ISOLATION, PRELIMINARY CHARACTERIZATION AND ANTI-MICROBIAL ACTIVITY STUDY OF LIPASE PRODUCING BACTERIA FROM MEDICINAL WASTE

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ABSTRACT

Lipases (triacylglycerol hydrolases) are enzymes capable of hydrolysing lipids into fatty acids and glycerol. There are mainly three major categories of lipases- substrate-specific, regioselective and enantioselective. Microbial lipases are in huge demand due to its application in various industrial sectors. The present study aims to isolate lipase producing bacteria from biomedical waste of a pharmaceutical company located in West Bengal, India. Further, morphological and biochemical characterisation of the isolated bacterial sample was carried out. After serial dilution and spread plate method, the prominent clear zone observed in TBA agar (Tributyrin agar) media confirmed the isolation of lipase producing bacterial strain. The bacteria were biochemically characterised by different tests according to Bergey's manual. Further optimisation of growth parameters (i.e. media formulation, time, pH and temperature) and antimicrobial activities of isolated lipase producing bacteria have also been studied. The optimum parameters for bacterial growth were analysed by using spectroscopic methods at 600nm. The isolated bacteria exhibited its maximum growth at a temperature of 37°C and an optimum pH 7.0 in production media (PM) using 1% (w/v) olive oil (contain fatty acid act as a carbon and energy source for bacteria) after 48 hours of incubation.

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INTRODUCTION

Lipases are produced by plants, animals and microorganisms including bacteria and fungi. Among them microbial lipase is most important because- (1) easy production and isolation (grow in low time and low-priced media), (2) produced in large scale (3) effortlessness in genetic manipulation due to its high resistance over seasonal variations. The demand of microbial lipase is increasing day by day. The uses of microbial lipase markets are projected to reach USD 590.2 Million by 2023, growing at a CAGR of 6.8% from 2018 (Prem Chandra *et al*.2020). Lipases have huge application in different industrial sectors. From hydrolysis of milk fat, modification of the fatty acid chain lengths and to boost the flavour of cheese lipases are widely used in the dairy industry (Balcao *et al*. 1998, Ray *et al*. 2012). Lipases help to remove excess fat in meat manufacturing and fish industry so as to produce lean meat. It is also used to enhance the flavour for the fermentation of meat products (Xiao *et al*,2017) and to expand the superiority of fermented sausages. The emulsifying properties of egg lipids is improved by lipases resulting in better performance and addition rate of lesser egg yolk in managed food recipes such as in dressings and mayonnaise like products (Mirzanajaf-Zanjani *et al*.2019; Motta-Romero *et al*. 2017). Lipases are used for bioremediation to decontaminate samples from oil spills, oil-wet soils, industrial wastes and waste water tinged with lipids (Lailaja *et al*. 2007). They are widely useful in industrial waste water treatment such as managing food waste, dairy waste, grease from wool, and waste from oil mills employing the anaerobic processes (Agobo *et al*. 2017; Porwal *et al*. 2017). The chemical constituents of detergents cause ecological contamination and are hazardous for the fauna and flora. Lipases are used as a substitute of these unsafe constituents to protect the ecological niche (Bardach *et al*. 1967; Pettersson *et al*. 2000). Due to increase in environmental pollution resulting climate changes, production of greenhouse gases and decreasing amount of fossil fuels have encouraged improvement of biofuel/biodiesel technology from sustainable resources (Klek *et al*. 2016). Research work has reported a thermostable lipase obtained from *Acinetobacter baylyi* catalyses bioenergy, pharmaceutical manufacturing and trans-esterified palm oil to FAMEs (Norjannah *et al*. 2016, Rachmadona *et al*. 2020). Lipase isolated from *Candida antarctica* (CalB) are commonly used as catalyst for the manufacturing of personal care products, active pharmacological and food constituents due to their regio-, chemo- and enantioselectivity (Idris *et al*. 2012). To resolve the racemic mixtures and to synthesize the chiral building blocks lipases can be used for pharmaceuticals, agrochemicals and pesticide production (Lo Giudice *et al*. 2006). In the medical sector lipases are significant drug targets or marker enzymes. Their presence or increasing levels can indicate certain infection or disease and can be used as diagnostic tools (Wenk *et al*. 2005).The present study aims in the isolation, morphological and biochemical characterisation and anti-microbial study of lipase producing bacteria from medicinal waste.

MATERIALS AND METHOD

Sample collection: The sample was collected from medicinal waste of a pharmaceutical industry (ASG Biochem Private Ltd.) located in West Bengal, India in a sterile container.

Isolation of lipase producing bacteria: 1ml sample was added to 9ml sterilized doubled distilled water to make 10^{-1} dilution. Thereafter, different dilutions were made (up to 10^{-8}) from 10^{-1} dilution. 0.1 ml sample was then taken from different dilutions, spread on nutrient agar plates and incubated for 24-48 hours at 37^0C. After incubation several microbial colonies were seen on nutrient agar plate and then the number of colonies were counted by using CFU formula. From a total of 12 colonies obtained each colony was streaked on nutrient agar plate individually and repeated the steps several times for pure colony isolation of the bacteria. Among them one white colony of bacteria showed positive result (a clear zone after 48 hours of incubation at 37^0C) on TBA (Tributyrin agar) media confirming it as a lipase producing bacterial colony. Further, morphological and bacterial characterisation was performed with lipase producing bacterial strain.

Bacterial identification: At first Gram staining was done to identify the Gram character of the bacteria. The pure bacterial colony was inoculated in broth media (nutrient broth, trypticase soya broth) and trypticase soya agar. Different biochemical tests as mentioned in Bergey's manual for bacterial strain identification were performed.

Growth in different selective media: The selective media are those which permit the growth of some specific group or type of microorganisms while preventing or inhibiting the growth of others thus facilitating bacterial isolation. The bacteria were streak in different selective media: MacConkey agar, TSI agar, TCBS agar, mannitol salt agar, EMB agar.

Extracellular enzymatic activities of microorganisms: Different extracellular enzymatic tests which include Protease, Lipid hydrolysis, Gelatine hydrolysis were done to identify the extracellular enzymes produced by the bacteria.

Biochemical characterisation: Different biochemical tests which include Hydrogen sulphide production test, Indole test, Methyl red test, Voges-Proskauer test, Citric Acid Utilization test, Urease test, Catalase test, Oxidase test were also done.

Carbohydrate Fermentation Test: This test is helpful in the identification of bacteria which can ferment carbohydrate or those cannot ferment the carbohydrate. This test is based on the principle of acid or gas production. The type of media used for this test is phenol red carbohydrate broth (carbohydrates- glucose, sucrose, lactose, mannose etc).

Antibiotic sensitivity test: The word "Antibiotics" came from antibiosis or against life, anti- means fighting, opposing, or killing, and bios is the Greek word for "life,". Antibiotic helps to kill the bacteria or reduce the bacterial growth. In laboratory we mainly use Kirby-Bauer method to measure the antibiotic sensitivity or resistivity. In this test we placed different antibiotics discs on Muller-Hinton agar plates and after 24 hours incubation the zone of inhibition were measured.

Medium formulation of studied bacterial sample: For determination of maximum growth of studied bacteria, they were first grown in three different media- Nutrient broth (Peptone: 5 gm, Beef extract: 3 gm, NaCl: 5gm, Distilled water: 1000 ml), Production media (Peptone: 5 gm, Yeast extract:10 gm, NaCl: 5 gm, Olive oil: 1%, Distilled water: 1000 ml), Standard media (Peptone: 5 gm, Yeast extract: 5 gm, Glucose: 5 gm, NaCl: 0.25 gm, MgSO4,7H2O: 0.5 gm, Olive oil: 5%, Distilled water: 1000 ml). In this regard, 1% (w/v) inoculum was added to each of the media and incubated at 37^0C for different length of time (24, 48,72 hours). The bacteria reported maximum growth in production media at 48 hours as determined by using spectroscopic methods at 600nm.Each experiment was performed thrice.

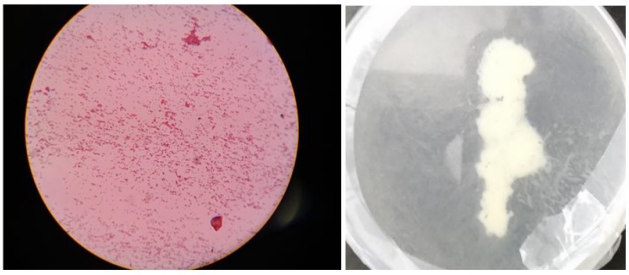
Optimum growth parameters determination of studied bacterial sample: For determination of the optimum parameters (pH, temperature), the bacteria were grown separately in production media at different pH (4, 5, 6, 7, 8 ,9) and different temperature (5^0C, 15^0C, 37^0C, 45^0C). The bacterial growth was measured by UV-Vis spectrophotometer by recording the absorbance at 600nm. All the analysis were performed thrice.

Bacterial growth cure: The bacterial growth was observed and growth curve was prepared by inoculating 1%(w/v) culture in 100 ml PM media where pH 7 was taken as optimum pH and 37^0C was taken as optimum temperature. The absorbance was recorded in different time interval at 600nm and performed in triplicate form.

RESULTS

The lipase producing bacteria was isolated from medicinal waste. Initially heterotrophic microorganisms were isolated on nutrient agar plate by spread plate technique. From a total of 12 different bacterial colonies, lipase producing bacteria were isolated by screening the promising clear zone on TBA agar plate at 48 hours. Further experimental studies were carried out using the particular lipase producing bacterial colony. All the experiments were carried out thrice and average value was reported.

Morphological and biochemical characterization result of studied bacteria



1(a) 1(b)

Fig1 a. Gram staining of isolated bacteria and (b) show the promising clear zone in TBA agar plate which confirmed the isolated bacteria was lipase positive

Morphology of bacteria	
Appearance	White, Glossy, smooth
Gram character	Gram negative
Pigmentation	Non pigmented
Shape	Rod shaped
Size	Small
Atmospheric oxygen requirement	Aerobic
Growth in different selective media	
MacConkey agar	+ve
TSI (Triple sugar iron) agar	+ve
TCBS agar	-ve
Mannitol salt agar	-ve
EMB Agar	-ve
Extracellular enzymatic activities of microorganisms	
Protease test	-ve
Lipid hydrolysis	+ve
Gelatine hydrolysis	-ve
Different biochemical tests of microorganisms	
Hydrogen sulphide production	-ve
Indole	-ve
Methyl red	+ve
Voges-Proskauer test	+ve
Citric Acid Utilization Test	-ve
Urease	-ve
Catalase	-ve
Oxidase	-ve
Carbohydrate fermentation tests	
Glucose	+ ve, gas production
Sucrose	+ ve, gas production
Inositol	+ ve, no gas production
Lactose	-ve
Mannitol	-ve

Antibiotics sensitivity analysis

Antibiotics	Zone of inhibition(mm)
Ampicillin (10 mcg)	No clear zone (Resistance)
Cefuroxime (30 mcg)	No clear zone (Resistance)
Penicillium (10 units)	No clear zone (Resistance)
Piperacillin (100/10 mcg)	37
Gentamycin (10 mcg)	26
Amikacin (30 mcg)	32
Azithromycin (15 mcg)	15
Chloramphenicol (30 mcg)	28
Ofloxacin (2 mcg)	29
Co-trimoxazole (25 mcg)	13
Ciprofloxacin (5 mcg)	30

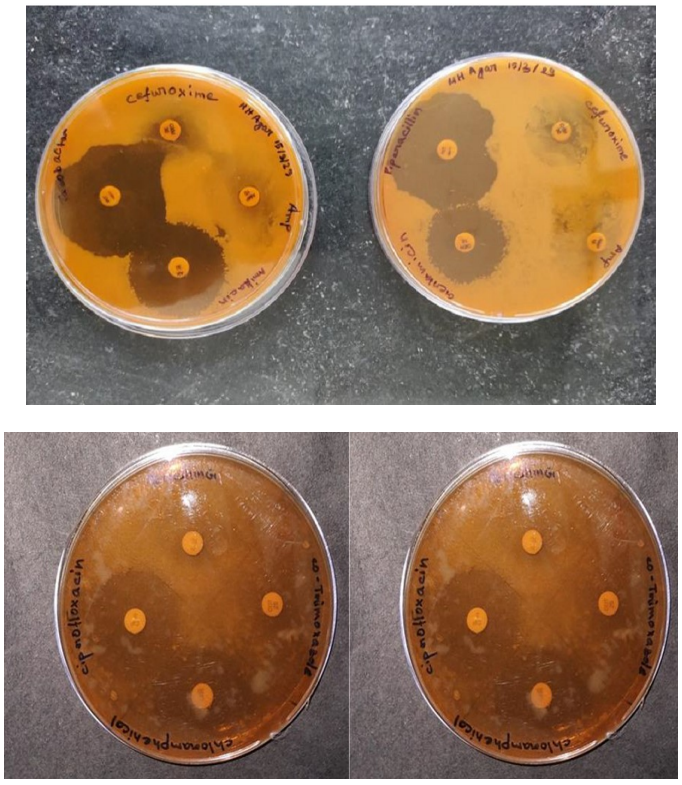


Fig. 2. Antibiotic sensitivity test result

Medium formulation for optimum growth

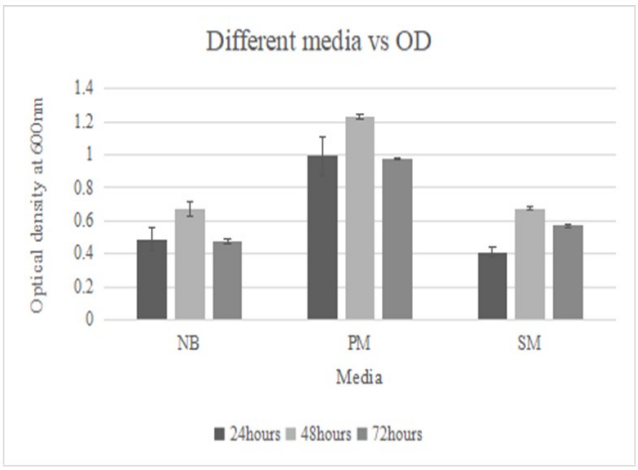


Fig. 3. Growth of bacteria in different growth medium – Nutrient broth (NB), Production media (PM), Standard media (SM). Experiments were performed in triplicate and the result represented the average of three experiments

Optimum pH of the bacterial growth

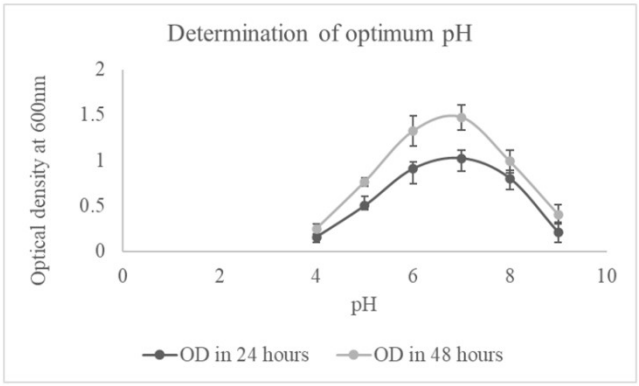


Fig 4. Determination of pH for the optimum growth of the bacteria. Experiments were performed in triplicate and the result represented the average of three experiments

Optimum temperature of the bacterial growth

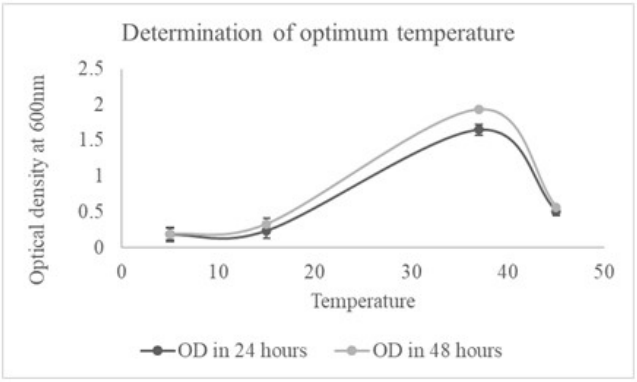


Fig: Determination of temperature for the optimum growth of the bacteria. The result represented average of three experiments.

Bacterial growth curve

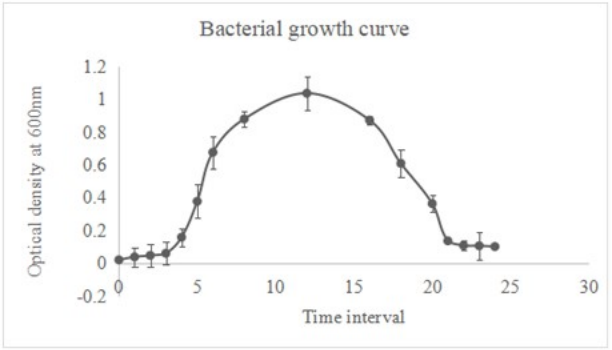


Fig.4. Bacterial growth curve. At different time intervals, microbial growth was determined and the experiment was performed in triplicate, the result represented average value.

Antimicrobial activity test of some medicinal plants and spices against studied bacteria: An antibacterial agent is that kills bacteria (bactericidal) or stops their growth (bacteriostatic agent). For this experiment plant parts and spices were collected from West Bengal, India. The plants parts and spices were cleaned, washed with distilled water and ethanol, dried and prepared the extract aseptically. The experiment was done by using cup plate method and incubated at 37^0C for 24 hours and measured the clear zone of inhibition.

Medicinal plants	Zone of inhibition (mm)
Neem (*Azadirachta indica)*	13
Aloe vera (Aloe barbadensis miller)	11
Basil (*Ocimum tenuiflorum*)	10
Malabar nut (*Adhatoda vasica*)	10
Species	**Zone of inhibition (mm)**
Cinnamon *(Cinnamomum verum)*	18
Clove *(Syzygium aromaticum)*	15
Cardamom *(Elettaria cardamomum)*	19
Black cumin *(Nigella sativa)*	10
Turmeric *(Curcuma longa)*	10
Ginger *(Ada aurantiaca)*	11

CONCLUSION

The present work report that the studied bacteria produce extracellular lipase which show a clear zone on TBA agar media. By using dichotomous keys and Bergey's Manual of Systematic Bacteriology we can predict the Genus Identification of Unknown Bacterial Cultures. It occurs from the morphological and biochemical characterisation the isolated bacteria producing extracellular lipase may belong to *Enterobacteriaceae, Pseudomonas or Haemophilus*. The bacteria showed optimum growth in production media containing 1% (w/v) olive oil after 48hrs at pH-7 and 37^0C. The antibiotic sensitivity test confirms the antibiotic Piperacillin (100/10 mcg) showed highest zone of inhibition(37mm) whereas, Ampicillin, Cefuroxime and Penicillium showed no clear zone on Muller-Hinton agar plate. Among the reported medicinal plants and spices, Neem (*Azadirachta indica)* and Cardamom *(Elettaria cardamomum)* showed more effectiveness than others against the studied bacteria.

CONFLICT OF INTEREST: The author declares there is no conflict of interest.

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