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Isolation Characterization and Screening of fungal Lipase from oil contaminated Soil: An approach of best from Waste

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Abstract*— *Present scenario demands a more sustainable, ecofriendly and economic measures globally to deal with the growing problems of environmental issues. The main goal of this work is to opt for such ideas and technologies which involve cleaner and greener procedures for utilizing waste materials for deriving value added products. The soil pertaining to the areas of oil mills contains densely population of various microbes', especially fungal origin. These microbes are rich in lipase content (due to oil source). Thus in this we isolated fungal colonies from this oil rich soil, cultured in laboratory, fermented them under various conditions to extract fungal enzyme i.e. lipase and then used it for further applications. Lipases are highly versatile and industrially important enzymes. Deriving the lipases from waste soil is the main attraction of this work and is a venture strategizing the "best from waste" approach.

Keywords*— *fungal Lipase, oil contaminated Soil, waste materials.

I. INTRODUCTION

Since past few decades, there is a global drive to promote more selective and efficient green technology. Major advances in understanding of protein structure and function have enhanced availability of biocatalytic applications. Scientific breakthrough in enzyme directed biocatalytic transformation has become an important tool for rational designing of sustainable ecofriendly industrial process development [1]. Engineering of enzyme properties such as stability, activity, selectivity and substrate specificity tailor the rate of biocatalytic process. The use of hydrolases including lipase offers a remarkable tool for highly selective biocatalysis.

Lipase enzymes are triacylglycerol acyl hydrolases that catalyze the hydrolysis of glycerides and other fatty acid esters to glycerol and fatty acid under aqueous conditionLipases of microbial origin, mainly bacterial and fungal, have already established their vast potential regarding their usage in numerous biotechnological applications [2]. Fungal lipases are preferable over bacterial lipases because of their easier extraction and purification processes. Lipase-producing fungi have been found in various habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food [3], compost heaps, coal tips, and hot springs [5]. Lipolytic fungal species compete efficiently with other forms of life for survival by some important control mechanisms. Lipid sources seem to be generally essential for obtaining a high lipase producing fungi. Use of agro- and food industrial waste products such as soil near the oil processing industry might be a rich source for production of lipase with industrial interest [6]. The global market demands for industrial lipases application scope for development of new industrial process including leather.

1.1 LIPASES IN LEATHER INDUSTRY

Lipases have many applications and benefits in the pre tanning operations of leather processing. Lipases have the ability to bring about hydrolytic and synthetic reactions in both aqueous and non-aqueous media, hence have multifold applications in other post tanning operation as well. The most traditional application of lipases has been found in the degreasing operation which might be extended to a variety of other operation such as fat liquoring. Conventional fat liquoring entails a series of drawbacks. Uneven diffusion of fat molecules inside the hide and skin is responsible for uneven dyeing and finishing, waxy patches which demerits the product

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quality. Thus one strategy to overcome this difficulty is by utilizing fungal lipase for hydrolysis of fat liquor.

II. OBJECTIVE

The following specific objectives have been formulated:

- Isolation of fungal colonies from leather samples
- Screening of extracellular lipase producing fungi using tributyrin contained agar plates.
- Investigation of lipase production of selected strains under various culture conditions.
- Analysis of hydrolytic reactions catalyzed by selected extracts.
- Characterization of the hydrolytic activity of the crude enzyme on fat liquor
- Protein estimation using Ninhydrin assay
- Enzyme activity optimization
- Specific growth rate calculation of all the three isolates under various parameters of optimization

III. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Media

PDA medium was used for storage of fungal cultures or fresh seeding for preparation of liquid cultures. Composition of potato dextrose agar (PDA) medium. The potatoes (200 g) were first washed and cut into small pieces, then boiled in 1000 ml distilled water for 1 hour and filtered to get the potato infusion. Potato dextrose broth was prepared without the addition of agar keeping all other compositions same.

TBA medium was used for selective isolation of lipophilic fungi. Tributyrin was replaced by mustard oil in general composition of TBA medium whereas Tributyrin broth was prepared without the addition of agar. All other compositions were same.

3.1.2. Other chemicals

Lactophenol cotton blue was used for fungal staining. Tween 80, $CaCl_2$, KH_2PO_4, K_2HPO_4, HCl, NaOH, Ninhydrin, BSA were also required.

3.2. COLLECTION OF SOIL SAMPLE

For the present study samples were collected from soil and oil contaminated soil from Diamond Harbour, District-South 24 PGS, West Bengal, India. The soil samples were taken in appropriately labeled pre-sterilized plastics with the help of sterile spatula from the depth of 0.5 to 1.0 cm surface and sub surface.

3.3. ISOLATION AND SCREENING OF LIPASE PRODUCING FUNGAL STRAINS

3.3.1. Screening of isolates

1 g of soil sample were dissolved in 100 ml of sterile PDB in a 250 ml of conical flask and agitated in an orbital shaker at 120 rpm for 30 minutes at 37°C. PDB acts as selective media for fungi. The media composition is given in appendices . The sample (aqueous slurry) was serially diluted up to 10^{-15} dilution using sterile distilled water. 0.1 ml of each dilution was spread on PDA plates by spread plate technique to obtain isolated colonies after 72 h at 37°C of incubation. After the incubation, sixteen colonies with distinct morphology were easily isolated as they formed well dispersed surface colonies, particularly at higher dilutions. The pure cultures of the isolated strains were preserved in PDA medium under refrigerated conditions (4°C) and coded as C1 to C16.

3.4.1. Optimization of media parameters for profound enzyme activity

Fungal growth and lipase enzyme accumulation were studied in the present investigation in flask batch cultures under different growth conditions. These conditions include incubation time, incubation temperature, pH, different carbon and lipid sources, and surfactant. The aim of these experiments is the optimization of lipase enzyme production by the strain under investigation. Lipase production is influenced by these physiologically important growth parameters i.e. temperature, pH, lipid, carbon source, presence of surfactants and time [12]. Each experiment was repeated three times. 25 µL of mother culture were added into the 200 ml tributyrin broth from 1 to 3 hours at 20, 37, and 45°C. The lipase production was then evaluated over a wide range of pH 4, pH 7 and pH 10. Further the changes of lipase production in response to the following carbon source (1% w/v) were evaluated: glucose, sucrose and starch, mustard oil, castor oil, sunflower oil and anionic detergent.

3.4.1.1. Influence of specific media

The growth pattern of three isolates was characterised in PDB and TBB media. Tributyrin was replaced by mustard oil because it is easily available and its hydrolysis can be followed by measuring the increase in biomass.

3.4.1.2. Effect of temperature on fungal growth

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Temperature is one of the most important parameters regulating the activity of microorganisms in natural environments. Generally, there is an optimal temperature for the enzymatic activity produced by different microorganisms which is responsible for the biosynthesis or degradation of compounds. This optimal temperature may be similar or different from the optimal temperature of the microbial growth. Colony C1, C2 and C3 were cultured at temperature ranging from 20, 37 and 45°C to select the optimum temperature for maximum enzyme production by keeping the remaining parameters constant. To determine the effect of incubation temperature on mycelia growth which in turn influences enzyme production, Colony C1, C2 and C3 were cultured at under various temperatures ranging from 20, 37 and 45°C to select the optimum temperature for maximum enzyme production by keeping the remaining parameters constant at 120 rpm.

3.4.1.3. Effect of pH on fungal growth

Hydrogen ion concentration (pH) of the medium is considered one of the most important factors. It has a great influence on the growth of microorganisms as well as their physiological activity. The effect of initial culture pH on biomass yield was studied at different pH values in order to reach the maximum lipase enzyme. The present experiment aimed to investigate the influence of pH on lipase enzyme production by fungal isolates. Colony C1, C2 and C3 were cultured at varying pH from pH 4, pH 7 and pH 10 to select the optimum pH for maximum enzyme production by keeping the remaining parameters constant.

3.4.1.4. Effect of carbon source as lipase inducers

To investigate the effect of carbon sources on biomass yields by the local isolate of C1, C2 and C3, the fungi were cultured in tributyrin broth supplemented with different carbon source (1% w/v) like glucose, sucrose and starch at 30°C under shaking (120 rpm). Carbon source influences the biomass yield which in turn affects enzyme production. Remaining physiological parameters and media composition were unaltered.

3.4.1.5. Effect of substrate on biomass yield

Substrate specificity affects the mycelial growth and enzyme production. To evaluate the effect of various substrates on lipase activity, 1% mustard oil, sunflower oil and castor oil was added to the culture media, separately. Remaining culture conditions were maintained constant.

3.4.1.6. Effect of Tween 80 on biomass yield

The effect of the addition of surfactant on mycelial growth was evaluated by the addition of surfactant to the production medium. Colony C1, C2 and C3 were cultured in presence and absence of Tween 80 to select the optimum culture media for maximum enzyme production by keeping the remaining parameters constant.

IV. RESULT AND DISCUSSION

The only organisms isolated were fungi as bacteria did not grow on the isolation medium. This may be due to differential growth conditions, especially composition. Due to the oil rich environments of the substrates, special attention was given to screening of lipolytic enzymes. The enzyme activity was associated with growth of the cell and favorable environmental conditions.

4.1. MEASUREMENT OF GROWTH CURVE

The recorded optical density with an interval of 10 min is representative of increased cell biomass with different time interval. Graphs were plotted with time versus optical density. Growth and multiplication of microorganisms on any substrates is often considered as the first step toward its bioconversion. Activity of lipase is directed by the biomass yield. This biomass yield is controlled by a variety of factors such as type of substrate, pH, temperature, and position of esters fatty acids, stereo specificity and a combination of all. Therefore, in the work reported here, it was vital to institute an experimental design to test the effects of all the factors on the growth pattern of sample could be distinguished based on OD changes. The first phase was lag phase where no change in OD was observed. Rapid increase in OD indicates log phase. The next phase was the stationary phase, where there was no changes in OD. Last phase was death phase where negative slopes of the growth curve were observed.

4.1.1. Optimization of culture condition

4.1.1.1. Influence of specific media

4.1.1.2. Optimization of culture condition for temperature

A detailed characterization of temperature-dependent growth of three isolates demonstrates inter-isolate variation in growth performance (Fig.11). It demonstrates that environmental conditions, specifically temperature, exert a strong influence on growth performance of three fungal isolates. C1, C2 and C3 were cultured at temperatures 20, 37 and 45°C to optimize maximum growth for maximum enzyme production.

Differences in estimated growth curves among isolates were observed. It was observed that at 37 and 45°C, C3 showed maximum growth rate. But at 20°C, C1 showed growth rate to be maximum.C2 exhibited average growth rates under all the three temperature .

4.1.1.3. Optimization of culture condition for pH

pH is one of the most important factors affecting the fungal growth and development and their relationships have been investigated. At acidic pH C2 showed maximum growth whereas for C1 it was neutral and for C3, it was basic pH.

4.1.1.4. Optimization of culture condition for carbon source

Lipase production is frequently limited due to a negative effect exerted by the carbon source. This regulatory mechanism, termed carbon catabolite regulation (CCR), is widely distributed among microbial systems and functions primarily to assure an organized and sequential utilization of carbon sources, when more than one is present in the environment. Under this condition, the cell catabolizes the best carbon source which most rapidly supplies carbon and energy for growth present in the medium. Simultaneously, the synthesis of lipase utilizing other substrates is repressed until the primary substrate is exhausted.

4.1.1.5. Optimization of culture condition for inducer

The compound, the inducer (oil), is one of the major factors for biomass yields. It is able to “turn on” production in cells in such a way that the enzymes are produced only when needed. It has shown that presence of lipid (especially natural oils) stimulate lipase production . Colony C1, C2 and C3 showed maximum growth when the medium was supplemented with castor oil than mustard and sunflower oil .

4.1.1.6. Optimization of culture condition for surfactant

Tweens are nonionic surfactant which is widely employed as dispersing agent in the preparation of conidial suspensions of hydrophobic fungi. Emulsification leads to higher enzymatic activities in both cultures, though there are variations in enzyme levels depending on the presence or absence of Tween 80 and of different oils in growth media.

4.1.1.7. Optimization of culture condition for nitrogen source

As nitrogen sources, yeast extract, beef extract and meat extract were used and varied to find out the optimum source of nitrogen for all the three isolates. It was observed that for meat extract when used, maximum growth rate was observed compared to other two sources.C2 showed highest growth rate meat extract, little lesser in beef and lowest in yeast extract. In Beef and yeast extracts, C2 and C3 showed maximum growth respectively. C1 showed moderate growth in all the three sources.

V. CONCLUSION

In present investigation, an effort has been made to study the lipase activity of three test fungi and their application in fat liquoring during leather processing. These observations provided interesting perspectives, demonstrating that fungi isolated from oil-rich environments represent a source of several enzymes potentially exploitable for biotechnological purposes. The use of fungal lipases for catalyzing esterification reaction became considerable interest because lipase mediated hydrolysis is an energy saving process. The great advantages of fungal lipases are that they are easily amenable to extraction due to their extracellular nature, which will significantly reduce the cost. Microbial lipases have gained special industrial attention due to their ability to remain active under extremes of temperature, pH and organic solvents, and chemo-, regio and enantioselectivity.Lipase is frequently used to catalyze the hydrolysis of wide non-natural substrates in order to obtain enantio- and region selective substrates. Among those enzymes, lipase is predominantly used in several applications. Lipases have found application in the soaking, dehairing, bating, and degreasing operation in leather making. The great advantage of fungal lipases is that they are easily amenable to extraction due to their extracellular nature, which will significantly reduce the cost and makes these lipases more attractive than those bacteria.

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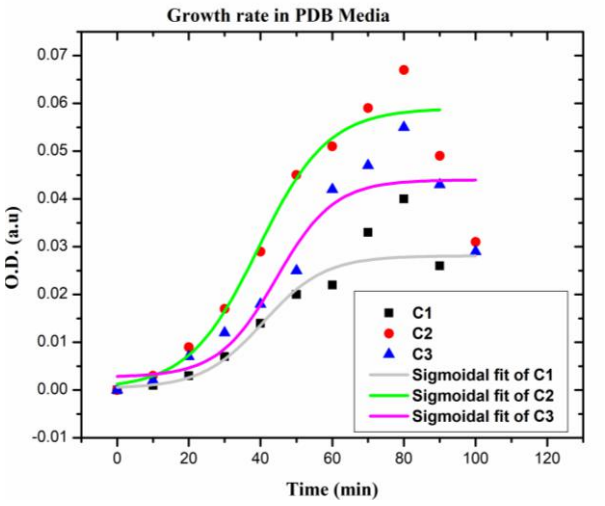
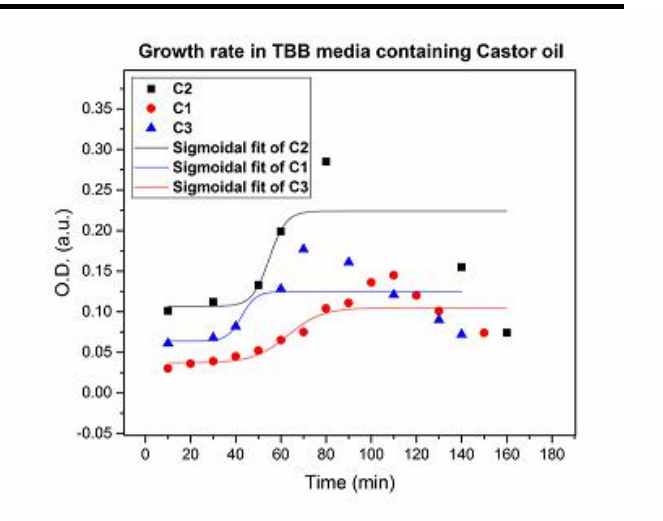
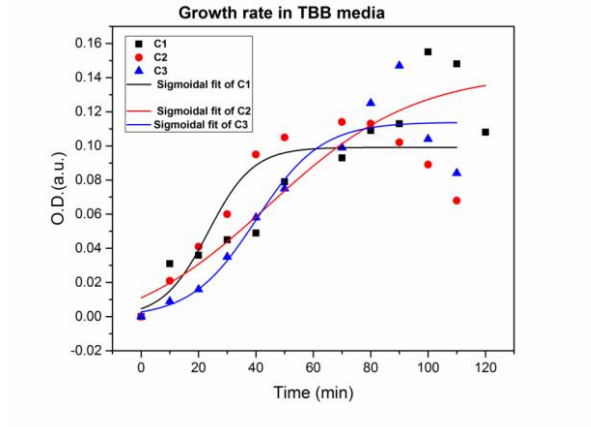
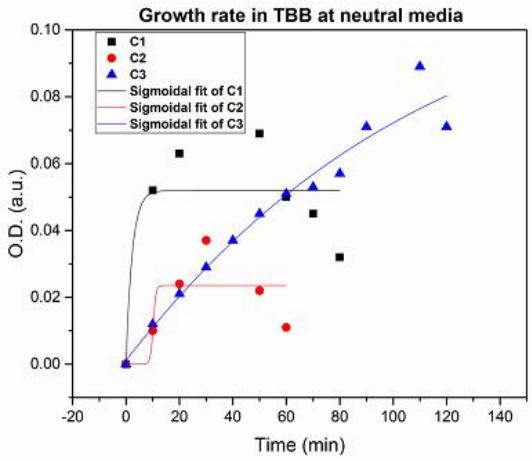
Growth rate curves:









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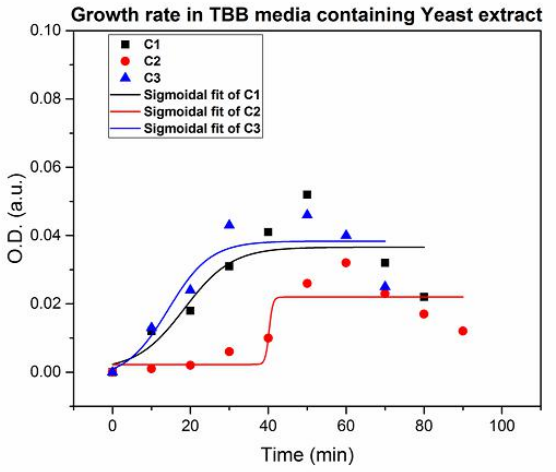

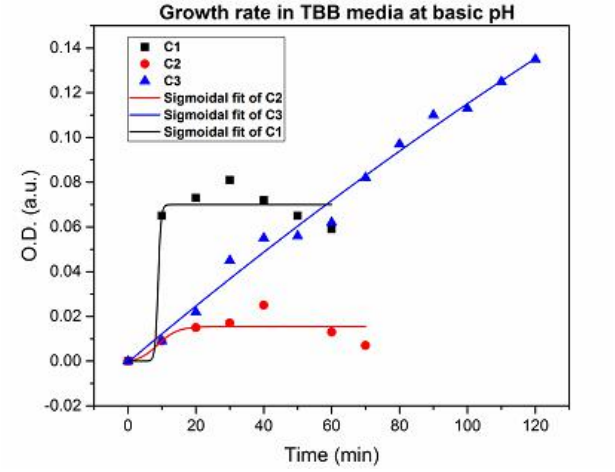

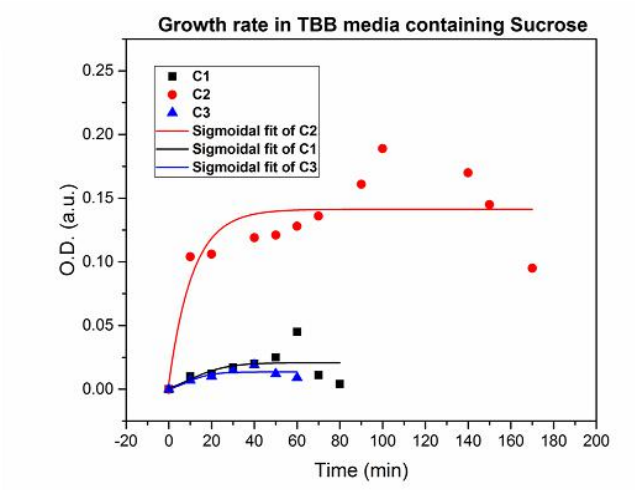

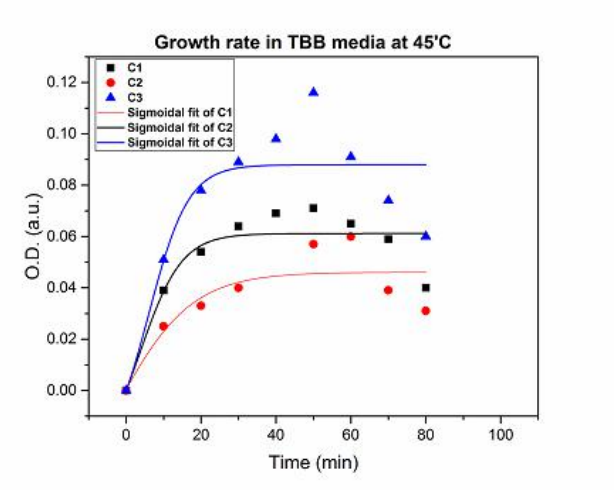

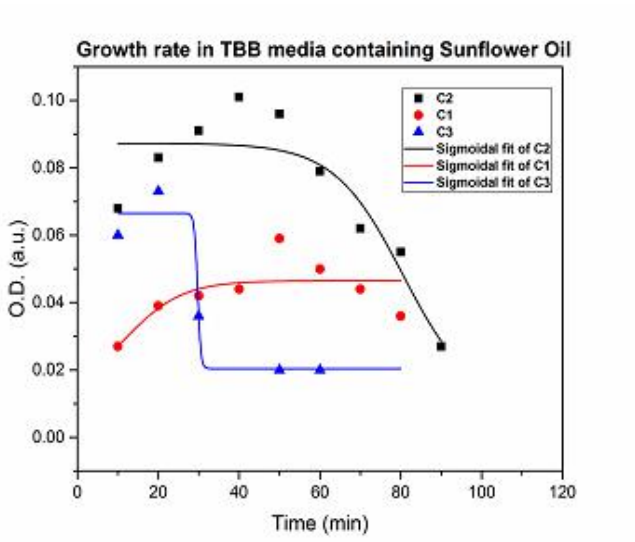

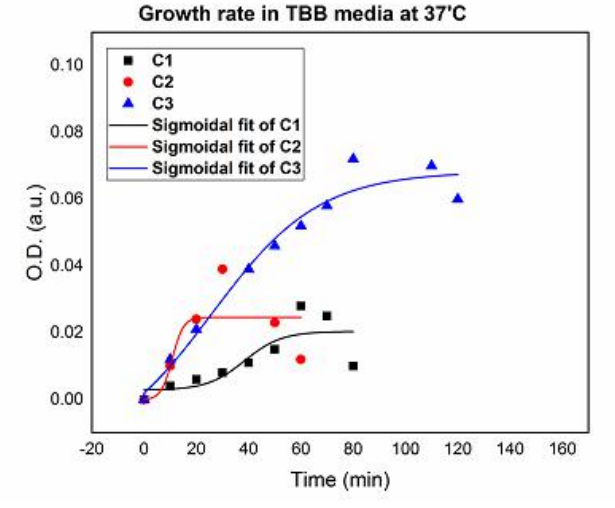

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