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Industrial Important Micro-Fungal Lipase Enzyme Production and Optimization from Western Ghats of Sathuragiri Hills of Litter Soil Micro-Fungal Isolates

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Abstract: Litter Soil samples were collected from twelve different locations of Sathuragiri Hills (Western Ghats, Tamilnadu). Micro-fungal species were encounter in potato dextrose agar medium and five microfungal species were identified for lipase production by primary screening. The after Screening micro-fungal lipase assay showed maximum lipase record with *Cladosporium cladosporioides* (354 U/ml), followed by *Penicillium citrinum* (311U/ml), *Fusarium oxysporum* (264 U/ml) and *Curvularia lunata* (238U/ml) by different optimization process such different pH, carbon, nitrogen sources and different substrates. The extracellular protein were recorded in different production medium of *C. cladoporioides* (378 U/ml), *P. citrinum* (321U/ml), *Fusarium oxys*porum (315 U/ml).The highest lipase activity was recorded in ground nut oil cake which act as good substrate followed by lactose, yeast extract, pH-6.0 for good carbon and nitrogen source.

Key words: Micro-fungal lipase · Optimization · Industrial enzyme · Litter soil

INTRODUCTION

Microbial diversity from various habitats such as soil, river water, hypersaline lakes and insects offers vast opportunities for exploration, as these habitats are the source of useful biomolecules which are a great relevance to the biotechnology industry and include enzymes, fatty acids, pigments, antibiotics [1]. Leaf litter is a dead plant material like leaves that provides food and shelter to a variety of invertebrates that break down the leaves, which feeds the soil and other wildlife. In plant litter decomposition in forest ecosystem, fungi play a central role through nutrient cycling and humus formation in soil because they colonize the lignocelluloses matrix in litter that other organisms are unable to decompose. Micro-fungal species possible to convert forest waste residues into liquid biofuels, Cellulases, hemicellulases and pectinases. Therefore they have a wide range of potential applications in food biotechnology. Exploration of fungi for the production of industrially important cellulase, amylase, protease, is gaining momentum. Enzymes are produced by various micro-organisms including bacteria, fungi and yeast are considered as important products obtained for human needs through microbial sources. The advantage of using micro-organisms for the production of enzymes is economical and microbes are easy to manipulate to obtain enzymes with desired characteristics.

Lipase (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyses hydrolysis of long chain acylglycerol at an oil water interface. These are enzymes belonging to the group of hydrolases that present as main biological function to catalyze the hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol [2]. Lipases are also used in various reactions due to their high specificity, prevention of product and substrate deterioration and decreased energy consumption. The advantage of the enzymatic hydrolysis over the chemical process is less energy requirements and higher quality of the obtained products [3].

Microbial lipases are also more stable than their corresponding plant and animal enzymes and their production is more convenient, safer and can be obtained in bulk at low cost [4]. Enzymes however are biodegradable and since they usually are dosed at 0.1 to 1.0 % of substrate. Numerous species of fungi, yeast and bacteria produce lipases. Different microorganisms have

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been used for the production of lipases such as *Rhizopus* species, *Rhizomucor mehei, Aspergillus niger, Penicillium* species, *Bacillus subtilis*. Gopinath [5] reported about 34 wild fungal species associated with edible oil mill wastes which were isolated by the serial dilution technique. This study also confirmed that the isolated fungi present on a wide range of substrates in the ambient environment and these results could also provide basic data for further investigations on fungal extracellular enzymes. *Rhizopus* species is among the most well known lipase producers and its enzyme is suitable for use in many industrial applications [6].

Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration [7]. Fungal lipases are typically produced in submerged fermentation processes, with the use of complex culture media containing sources of organic compounds such as yeast extract, soymeal, corn steep liquor and egg yolk [8]. The yield of extracellular lipase increased 2-5 times by the addition of Arabic gum gum arabic, possibly due to the cell surface-bound lipase [9]. Present study focuses on optimization of industrial important micro-fungal strains to lipase production by different pH, substrate, carbone and nitrogen sources used to enhance lipase production.

MATERIALS AND METHODS

Sample Collection: Soil and litter samples were collected from Sathuragiri in12 different locations (Western Ghats, Tamil nadu). Using a sterilized spatula, 50g of soil sample was collected from each location using sterile zip cover. The samples from the 12 different sites were conveyed to the Microbiology Laboratory within the same day, where they were analyzed.

Fungal Isolation from Soil Samples: One gram soil was mixed thoroughly in 99ml of sterile water in a conical flask and shaken thoroughly. From this initial suspension, serial dilutions were prepared .One ml of the required dilution (1/1000) was pipette into five replicate plates containing potato dextrose agar medium with tetracycline and amoxicillin antibiotic. The plates were incubated at room temperature 36°C in glass chambers under aseptic conditions for 4 days and then examined for fungal growth.

Fungal Isolation from Leaf Litter Sample: The litter sample contains three layers; they differentiated as L-upper most layer consisting of recently fallen senescent leaves lying loosely on the surface of the soil. F1 layer –immediately below the L layer with dark brown leaves having high moisture content, usually compacted. F2 layer-lower most layer below F1 layer and the soil surface in an advanced stage of decomposition. The semi-decomposed leaf litter soil was collected in the region of F2 layer.

The leaves were washed thoroughly in water and segments of 1 cm^2 were cut from the midrib portion of each leaf and surface sterilized by immersing in 70% ethanol for 1 min, followed by 4% sodium hypochlorite (v/v) for 2 min and finally washed in sterile water for 1min. One gram of surface sterilized leaves were mixed thoroughly in 100 ml of sterile water in a glass tube and shaken thoroughly. From this initial suspension, serial dilutions were prepared .One ml of the required dilution (1/1000) was pipette into five replicate plates containing potato dextrose agar medium with antibiotic. The plates were incubated at room temperature in glass chambers under aseptic conditions for 4 days and then examined for fungal growth. All fungal colonies developed were recorded.

Identification of the Isolated Fungal Strain: The isolated fungus was identified based on morphological and microscopic features. A drop of lacto-phenol cotton blue stain was placed on a clean glass slide to which fungal mycelia and spores of the test strain were added. The stain was gently mixed with fungal structures and a cover slip was placed over the preparation. Slides prepared were sealed with DPX mountant. The prepared slide was examined microscopically at 40x under compound microscope. Identification of the fungal species was done using "The Genera of Hyphomycetes from soil" by Gorege [10] Compendium of soil fungi by Domsch [11].

Qualitative Screening for Lipase Production: The fungal isolates were qualitatively screened for lipase production by cultivating on enzyme screening medium containing 1% Tributyrin. All the plates were incubated at 37°C for 5 days. Zone of clearance around the microbial growth indicated the production of lipase. On the basis of the area of clearance, five fungal potential isolates were selected for further studies on lipase production [12].

Submerged Culture Fermentation: Submerge culture fermentation mediated enzyme production were evaluated by adding the mineral salt medium and the substrate of the enzyme Olive oil 1% (Tween 20 as a surfactant) for lipase, fermentation was carried out at 37°C under 120 rpm in shaker. After incubation OD measured by 410 nm.

Assay of Lipase Activity: Lipase activity was determined with p-NPP (para Nitrophenyl palmitate) method. In this method p-NPP was hydrolysed by lipase to give p-NP (para Nitrophenol) which gave yellow color absorbance of which was measured spectrophotometrically at 410 nm. The substrate for this reaction composed of solution A and solution B. Solution A contained 40 mg of p-NPP dissolved in 12 ml isopropanol. SolutionB contained 0.1 g of gum arabic and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water. The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring to obtain an emulsion that remains stable for 2 h. The assay mixture contained 1 ml of the substrate, 0.5 ml of buffer (Potassium phosphate buffer, pH 7, 0.1 M), 0.1 ml of enzyme (the filtrate) and volume was made up to 4 ml with distilled water. This was incubated at 50°C for 30 min. The enzyme activity was stopped by adding 0.2 ml of isopropanol. The absorbance was measured at 410 nm against enzyme free blank. The standard graph was prepared by using p-NP (20-100 µM) and absorbance taken at 410 nm [12].

Estimation of Protein by Lowry's Method: One ml of partially purified protein solution was taken in a test tubes and 2 ml of alkaline copper sulphate reagent (analytical reagent) was added, mixed and incubate at room temperature for 10 mins. Then 0.2 ml of reagent Folin Ciocalteaus solution (reagent solutions) was added to each tube and incubated for 30 min. Zero the UV - spectrometer with blank and take the optical density (measure the absorbance) at 660nm.The absorbance plotted against protein concentration to get a standard calibration curve. By using the absorbance of unknown sample, the concentration of the unknown sample was determined using Bovine serum albumin (BSA) standard curve [13].

RESULTS

Micro-Fungal Isolates and Primary Screening of Lipase: The five potential strains were isolated from Sathuragiri hills soil litter samples by serial dilution technique (Fig. 1). The micro-fungal species were obtained by pure culture technique (Fig. 2). In primary screening of mico-fungal species gives form halo zone formation on the Tribytrin agar medium in plate assay method and further isolates of fungal species such as *Penicillium citrinum,* (Fig. 3) *Fusarium oxysporum,* (Fig-4) *curuvularia lunata,* (Fig. 5) *Alternaria alternata* and *Cladosporium cladosporioides* submerged fermentation of lipase production.

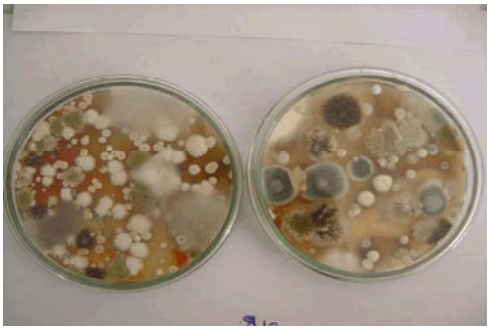

Fig. 1: Micro-fungal isolates in serial dilution technique

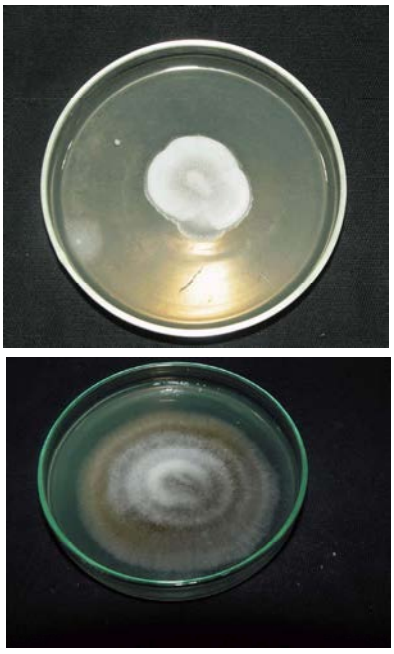

Fig. 2: Micro-fungal isolates in pure culture form

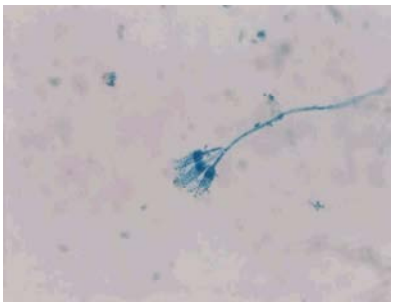

Fig. 3: *Penicillium citrinum*

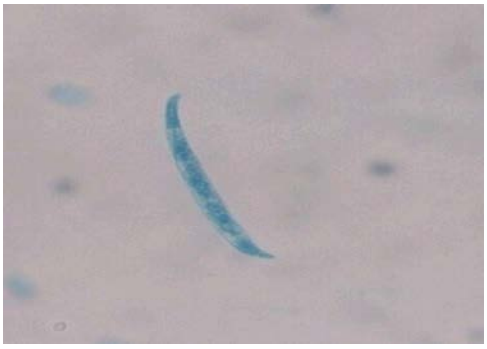

Fig. 4: *Fusarium oxysporum*

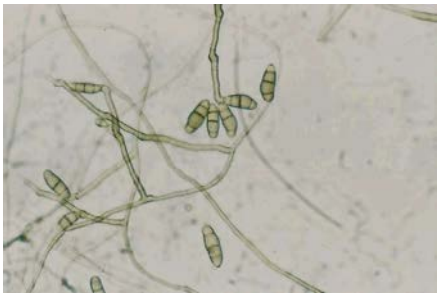

Fig. 5: *Curuvularia lunata*

Extracellular Lipase Production: Quantitative screening of extracellular fungal lipase was done by inoculating the qualitatively screened fungal species *Penicillium citrinum, Cladosporium cladosporioides, Fusarium oxysporum, curuvularia lunata, Alternaria alternata* on the submerged culture fermentation medium containing 1% Olive oil. Results in (Table 1) indicated that the highest lipase production was observed with *Cladosporium cladosporioides* (84 U/ml), followed by, *P.citrinum* (79 U/ml) and *Fusarium oxysporum* (78 U/ml), *curuvularia lunata* (72 U/ml) and *Alternaria alternata* (62 U/ml). The extracellular protein were recorded maximum in production media for *Cladosporium cladosporioides* (99 U/ml), *P. citrinum*, (94 U/ml), *F.oxys*porum (92 U/ml), *A.alternata* (91 U/ml), *C.lunata* (92 U/ml).

Effect of Carbon Source: Effect of different carbon sources such as lactose, maltose, sucrose, fructose, glucose, were evaluated for the production of extracellular lipase by using quantitatively screened fungal species. Of all the carbon sources, lactose showed maximum lipase production with *P. citrinum* (326 U/ml), followed by *F. oxysporum* (294 U/ml), *A. alternata* (244 U/ml), *C. cladosporioides* (131 U/ml), *C. lunata* (129 U/ml) further obtained results were shown (Table 2).

Effect of Nitrogen Source: Effect of different nitrogen sources such as, Ammonium nitrate, Ammonium sulphate, Peptone, Yeast extract, Sodium nitrate were evaluated for the production of extracellular lipase by using quantitatively screened fungal species. Of all the nitrogen sources yeast extract showed maximumin lipase production with *Cladosporium cladosporioides* (354 U/ml), followed by *C.lunata* (268 U/ml), *F. oxysporum* (161 U/ml), *P.citrinum* (156 U/ml), *A.alternata* (146 U/ml), further obtained results were shown (Table 2).

Effect of pH: Effect of various pH such as, pH 5.0, 5.5, 6.0, 6.5, 7.0 were evaluated for the production of extracellular lipase by using quantitatively screened fungal species. Maximum in lipase production was observed at pH 6.0 with *F.oxysporum* (99 U/ml), followed by *F. solani* (91 U/ml), *P. citrinum* (88 U/ml), *C. lunata* (87 U/ml) and *A. alternata* (87 U/ml) further obtained results were shown in (Table 2).

Effect of Different Substrate: Effect of different substrate such as, Wheat Bran, Rice Bran, Coconut oil cake, Groundnut oil cake, Sesame oil cake were evaluated for the production of extra cellular lipase by using quantitatively screened fungal species. Of all the substrate, Groundnut oil cake showed maximum lipase production with *F. oxysporum* (117 U/ml), followed by *A. alternata* (116 U/ml), *P. citrinum* (112 U/ml), *C. lunata* (111 U/ml) and *F. solani* (99 U/ml), further obtained results were shown in (Table 2).

Optimized Media Composition for Lipase Production: Optimum fermentation media composition for lipase production was designed based on result obtained in the optimization of lipase production. High level lipase production was evaluated by adding Ground nut oil cake as a substrate, lactose as a carbon source, yeast extract as a nitrogen source, pH 6.0, incubation time 5 day and mineral salt medium was added as a nutrient supplement (Table 3). Maximum increase in lipase production was observed in *Clodosporium cladosporioides* (346 U/ml), followed by *P. citrinum* (311 U/ml), *F. oxysporum* (264 U/ml), *C. lunata* (238 U/ml) and *A. alternata* (256 U/ml), The extracellular protein were recorded maximum in production media for C*ladosporium cladoporioides* (378U/ml), *P. citrinum* (321U/ml), *F. oxysporum* (315 U/ml), *C. lunata* (298 U/ml) (Table 4).

Table 1: Quantitative screening of lipase production on submerged fermentation

		Lipase assay (U/ml)			Protein estimation (U/ml)		
S.No	Fungal species	3rd	5th	7th	3rd	5th	7th
1	*P. citrinum*	45	79*	56	68	94*	59
2	*F.oxys*porum	57	78*	57	69	92*	55
3	*A.alternata*	47	62*	49	72	91*	61
4	*C. cladosporioides*	61	84*	52	79	99*	63
5	*C.lunata*	55	72*	51	75	92*	64

*Maximum level of lipase production

Optimization of micro-fungal lipase production

Table 2: Quantitative assay of lipase production on 5th day of incubation

Optimization of Lipase Production (U/ml)					
Quantitatively screened fungal species	*P. citrinum*	*F.oxys*porum	*A.alternata*	*C. cladosporioides*	*C.lunata*
Effect of Ph					
pH – 5. 0	69	86	74	71	69
pH – 5.5	84	98	84	89	86
pH – 6. 0	88*	99*	87*	91*	87*
pH – 6. 5	79	78	62	84	72
pH – 7. 0	67	57	62	59	55
Effect of carbon source					
Fructose	120	165	110	105	95
Glucose	105	113	120	84	79
Lactose	326*	294*	244*	131*	129*
Maltose	99	121	119	121	124
Sucrose	176	229	137	143	131
Effect of nitrogen source					
Ammonium nitrate	134	145	135	109	114
Ammonium sulphate	149	139	129	129	122
Peptone	124	128	143	165	112
Yeast extract	156*	161*	146*	354*	268*
Sodium nitrate	123	138	167	145	142
Effect of different substrate (Agro waste)					
Coconut oil cake	102	85	92	72	78
Groundnut oil cake	112*	117*	116*	99*	111*
Sesame oil cake	89	92	93	98	102
Wheat Bran	87	75	82	79	91
Rice Bran	78	87	86	79	81

* Maximum level of lipase production

Table 3: Optimum fermentation technology for lipase production

Media components	Media composition
Substrate	Groundnut oil cake
Carbon source	Lactose
Nitrogen source	Yeast extract
pH	6.0
Incubation time	5 days
Nutrient supplement	Mineral salt medium

Table 4: Lipase production on optimized fermentation medium

		Lipase assay (U/ml)			Proteinestimation (U/ml)		
S.No	Fungal species	3rd	5th	7th	3rd	5th	7th
1	*P. citrinum*	194	311*	131	219	321*	187
2	*F.oxys*porum	187	264*	123	218	315*	165
3	*A.alternata*	179	256*	145	223	311*	189
4	*C. cladoporioides*	167	346*	135	216	378*	198
5	*C.lunata*	187	238*	165	216	298*	168

* Maximum level of lipase production

DISCUSSION

The substrate concentration, pH range changes and micro-fungal selection was alter the fermentation products [14]. In present study also highest lipase production was obtained by alteration of micro-fungal substrate, pH, carbon, nitrogen sources and micro-fungal strain. Micro-fungal Lipase play role in oily hydrocarbons biodegradation and environmental polluted soil oil degradation. Lipase activity has been used as biochemical and biological parameter for testing hydrocarbon degradation and very useful in industrial sector [15]. Different microorganisms have been used for the production of lipases such as *Rhizopus* species, *Rhizomucor mehei, Aspergillus niger, Penicillium* species*, Bacillus subtilus*. Gopinath [5]. In present study lipase production was encounter in *Aspergillus*, *penicillum* micro-fungal genera.

Various workers have reported the less amount of lipase production. Puthli and Benjamin [16, 17] reported separately the low amount of lipase 1.84 U after 66 hours and 12.55 U after 72 hours secreted by *Candida rugosa*. Mahadik [18] also produced less amounts of lipase yield 4.0 U lipase and 13 U by *Aspergillus niger* and *Penicillium restrictum* in submerged fermentation condition. While Pinheiro [19] studied on lipase production from *Penicillium verrucosum* and obtained maximal enzyme yield 3.22 U 96 hours and 2.63 U at 72 hours in conventional and industrial fermentation medium respectively. Furthermore, *Mucor griseocyanus* produced 0.113 U in optimized fermentation condition [20]. In iftikhar [21] reported that bacterial bacillus species produced 32 U of lipase after 72 hours incubation. In present study five potential micro-fungal species that form halo zone formation on the Tribytrin agar medium were selected for lipase production on the submerged fermentation. The highest level of lipase production was observed in *Cladosporium cladosporioides* (354 U/ml), followed by *P. citrinum* (326 U/ml), *F. oxysporum* (294 U/ml), *C. lunata* (268 U/ml) and *A. alternata* (244 U/ml), for optimization of fermentation was carried out under the condition with different carbon source, nitrogen source, different pH, optimum fermentation media composition for lipase production was designed based on result obtained in the optimization of lipase production. High level lipase production was evaluated by adding Ground nut oil cake as a substrate, lactose as a carbon source, yeast extract as a nitrogen source, pH 6.0, incubation time 5 day, and mineral salt medium was added as a nutrient supplement. Maximum increase in lipase production was observed in *Clodosporium cladosporioides* (346 U/ml), followed by *P. citrinum* (311 U/ml), *F. oxysporum* (264 U/ml), *C. lunata* (238U/ml) and *A.alternata* (256 U/ml).

CONCLUSION

In relation to the different tested fungal strains, *Clodosporium cladosporioides* was the best producer for extracellular lipase and its synthesis is induced by the presence of a lipid source. The present study focus on industrial important micro-fungal lipase which enhanced by cheapest ground nut oil cake as a substrate, lactose as a carbon source, yeast extract as a nitrogen source, pH 6.0, incubation time 5 day and mineral salt medium was added as a nutrient supplement and in this optimization very help for industrial scale up of lipase production.

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