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Isolation, Identification of Aspergillus niger and Optimization of Extracellular Lipase Production

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ABSTRACT

Seven different fungal strains were isolated from various sites on the campus of Stella Maris College (Autonomous), Chennai and identified as Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Pencillium citrinum, Mucor racemosun, Cladosporium cladosporoides, Rhizopus stolonifer apart from some sterile hyphae. The fungal isolates were tested for lipase production. It was found that Aspergillus niger showed better zone for lipase enzyme. 15g of Groundnut was found to be ideal for extra cellular lipase production by A. niger (509 U/g). Glucose as a carbon source (602 U/g) and Ammonium nitrate was used as the nitrogen source (579 U/g) showed the maximum enzyme production. Olive was an excellent substance (579 U/g) and production of the enzyme enhanced between temperatures of 40ºC (534 U/g) - 45ºC (579 U/g). The optimum pH was 6.5(681 U/g). The maximum protein content was found to be at 4 ºC (114 µg) and pH 6.5 (122 µg). Maximum zone of hydrolysis (4mm) was observed at 15g as substrate concentration while it was 5mm when dextrose was used as the carbon source. When Potassium Nitrate was used as the nitrogen source the zone of hydrolysis was 4mm. A maximum zone of was observed for olive oil and gingely oils. The maximum zone of hydrolysis was 3mm at 28 and 37 ºC and 5mm at pH 6.5. SDS PAGE analysis of the crude lipase enzyme showed the presence of a prominent protein band of 40KDa apart from some other proteins of lower molecular weight.

Key words*: Fungi, Atmospheric air, Lipase, Groundnut, olive oil, Aspergillus niger*

Introduction

Lipases (triacylglycerol acylhydrolase EC3.1.1.3) are hydrolytic enzymes that catalyze the hydrolysis of fats and oils to free fatty acids and glycerol at the oil water interface [1]. Lipases belong to the class of serine hydrolases that do not require a cofactor [2]. Lipases are widely used in hydrolysis, esterification, transesterification, alcoholysis [3].They are the most widely used enzymes in organic synthesis [4]. Microorganism such as bacteria, yeast, molds, animals and plants produce lipases for digestion of lipid material [5] but microbial lipases are of significance due to the specificity of reaction and steriospecificity [6]. Lipase producers have been found in diverse habitats such as soil, oil contaminated soil, oil processing factories, dairy, industrial waste [4]. Filamentous fungi belonging to the genera of Aspergillus spp, Pencillium spp, Trichoderma spp, *Geotrichum candidum*, Rhizomucor spp, Rhizopus spp, *Candida rugosa*, *Cunninghamella verticillate*, *Humicola lanuginose*, *Lipomyces starkeyi* are described as potent lipase producers [6, 2]. Commercial lipases are from microbial source with fungi being able to produce the enzyme in more intense and diversified way [7]. Fungal lipases are extracellular enzymes that can be recovers from the fermentation broth with much ease [8]. Lipases can be produced by submerged fermentation (SmF) or by solid state fermentation (SSF) process and fungi are better adapted to grow on SSF [9] due to their ability to grow with minimum amount of water [10]. Lipases have been topic of active research for their varied application in the file of medicine, detergent, dairy, food, leather, pulp and paper, cosmetics, biodiesel and waste water management [11, 12]. *Aspergillus niger* has been considered to be promising for the production of lipases with industrial application as they can be grown on SSF and the amount of extra cellular enzyme produced is also in large amounts [13, 14]. The current study was undertaken to evaluate the various variables that influence lipase production by *Aspergillus niger*.

1. Materials and methods

1.1. Collection of Sample and Isolation of Fungi

Sabouraud dextrose agar (Hi Media) was used as an artificial media to support the growth of fungi isolate from open environment. Samples were collected from different places in Stella Maris College such as canteen area, Main gate, C-C block, Pond area and M-0-1. The plates were exposed to atmospheric air for 15 minutes at different places in the campus. The exposed plates were incubated at 37C for 3-4 days.

1.2. Partial Identification of Fungal Isolates

Partial identification of fungal isolates was done by visual observation in Petri plate cultures and morphological studies in slide culture. For visual observation, the isolates are grown in Potato Dextrose agar (PDA) were used. The mode of mycelial growth and colour of each isolate was examined daily. For the micro-morphological studies, a piece of PDA block containing the fungi was detached by a sharp sterile needle. The shape size, arrangement and development of conidiophore, phialides, conidiospheres and phialospores were studied using Lacto phenol cotton blue. The identified fungi were corroborated by Dr. S. Bhuvaneswari, Assistant Professor, Department of Botany, Bharathi Women's college, Chennai.

1.3. Screening Tests for Lipase Activity

The isolated pure culture was screened for the production of extracellular lipase using screening medium such as Tween 20 lipolytic media [15], Tween 80 agar plates [16] , Tributyrin agar [17] plates, Rhodamine B agar [18] and Chromogenic agar plate [19]. Briefly, single spore of the pure culture was placed in center of the plate and the Petri plates were incubated at 37ºC for 24hours.

1.4. Enzyme Production by Solid State Fermentation

Conidial suspension was used as inoculum and the number of spores was counted in a Neubauer hemocytometer and the concentration of the spores was adjusted to $1x10^9$ with sterile distilled water. Ground nut was soaked overnight and left for sprouting. It was coarse grind and used as substrate. Tween 20 lipolytic liquid media was used as the production media (peptone-10g, sodium chloride-5g, calcium chloride-0.1g, Tween 20-10ml, distilled water-1000ml, pH 6.0). The medium was autoclaved and 10ml of pre sterilized Tween 20 was added to it. 10g of coarse grind groundnut was weighed in a conical flask and 24ml of pre sterilizes Tween 20 liquid media was added. 5ml of the spore suspension was inoculated and incubated in static condition at room temperature for 5days. Duplicates were maintained at the same condition.

1.5. Enzyme Extraction

The enzyme was extracted at the end of fermentation period by adding 10ml of phosphate buffer saline (pH 7.0) to the flask and kept in an incubator shaker for 2 hours at 200rpm. The culture was filtered through Whatman filter paperNo.1 and the filtrate was centrifuged at 10,000rpm at 4ºC for 20 minutes. The clear supernatant was used as enzyme source and for other experimental analysis.

1.6. Titrimetric Assay Method

The enzyme was assayed by titrimetric method using olive oil. A unit lipase is defined as the amount of enzyme which releases one micromole of fatty acid per minute under specified assay conditions. One milliliter of the culture supernatant was added 5ml of the reaction mixture (Olive oil-10ml, Gum Acaia-10g, calcium chloride-0.6g, phosphate buffer (pH 7.0)-100ml). The liberated fatty acid was titrated against 0.1N NaOH using phenolphthalein as indicator. The end point was indicated by appearance of pink colour. The enzyme unit was calculated as follows [20].

Lipase activity = Amount of NaOH x Normality of NaOH x 100/ Fresh weight of the biomass

1.7. Optimization of Lipase Enzyme Production by Different Inducers

Different parameters were employed to check the enzyme productivity viz., substrate concentration, carbon, nitrogen, lipid source (oils) temperature, pH were maintained in duplicates [20].

2.7.1. Effect of Substrate Concentration

The substrate concentration was varies as 10g, 15g, 20g, 25g and 30g was incubated with spore suspension at room temperature for 5 days.

2.7.2. Effect of Carbon Source

Glucose, fructose, lactose, sucrose and dextrose were added (1% concentration) individually to the medium

and their effect on extra cellular lipase production was estimated.

2.7.3. Effect of Nitrogen Source

Nitrogen source such as sodium nitrate, ammonium nitrate, ammonium sulphate, potassium chloride and beef extract was given separately in the medium in 1% concentration and its effect on enzyme production was studied.

2.7.4. Effect of Oil Substance

Five oil sources viz., sunflower oil, gingely oil, coconut oil, corn oil and olive oil were added in 1% concentration to the substrate and incubated for 5 days. The effect of these oil substances on enzyme production was studied.

2.7.5. Effect of Temperature

The medium was incubated with spore suspension incubated at different temperature such as 4ºC, 27ºC, 37ºC and 40ºC for 5 days.

2.7.6. Effect of pH

The pH of the medium was adjusted to 4.0, 5.0, 5.5, 6.0 and 6.5 and its effect on enzyme production was studied after an incubation period of 5 days.

1.8. Protein Estimation

The amount of protein present was estimated by the standard method of [21].

1.9. Enzyme Assay

The activity of the enzyme extracted was assayed using Tween- 20 medium by cut wells (3mm) using gel punch. To this wells, the enzyme was added and plates were incubated at room temperature and observed for the development of opaque zone.

2.10. Partial Purification of the Enzyme

2.10.1. Ammonium Precipitation Method

Solid ammonium sulphate was added to the culture filtrate with stirring to bring the saturation to 30% and allowed to stand for 4hrs at 4°C and the precipitate was removed by centrifugation. Additional ammonium sulphate was added to the supernatant to bring saturation to 60% the precipitate were collected, dissolved in distilled water and the solution was dialyzed against water for 48hrs in a dialysis bag [22]. The dialyzed enzymatic fraction were subjected to protein analysis by [21] method and lipase activity.

2.10.2. SDS-PAGE of the Partially Purified Enzyme

The partially purified enzyme was visualized on a 10% SDS-PAGE following the standard protocol of [23] Laemmli, 1970.

2. Results and Discussion

In the current study seven different fungal strains were isolated and identified as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Pencillium citrinum*, *Mucor racemosun*, *Cladosporium cladosporoides*, *Rhizopus stolonifer* apart from some sterile hyphae (Table 1). The fungal isolates were tested for lipase production. It was found that *Aspergillus niger* showed better zone for lipase enzyme and was selected for further study. [24] have reported *Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium*, *Trichoderma* and *Curvularia* from soil samples in which *A. niger* was dominant similar to the present study.

2.1. Screening Tests for Lipase Activity

The screening of lipolytic activity was carried out using tributyrin agar medium which showed a zone of hydrolysis of 3mm, similar to the tech of [25] have reported the use of tributyrin clearing zone techniques in the production of lipase by certain soil fungi. Tween 20 agar plates were used as an indication of extra cellular lipase activity and similar work was reported earlier by [26]. A pH based detection method using phenol red was used for the detection of lipase in crude enzymes as was done by [19]. In this study *A. niger* showed higher lipolytic activity in the presence of surfactant Tween 20 which showed a zone of hydrolysis ranging from 0.5mm to 5mm. Hence Tween 20 appeared to be the best inducer. [27] suggested a double effect of Tween 20 and Tween 80 that can serve as inducers as their chemical nature is similar to that of some natural substrate as well as the surfactants stimulate the enzyme release. [28] found that surfactant Tween 20 could be helpful for lipase production.

2.2. Optimization of Lipase Enzyme Production

In the present study a substrate concentration (sprouted ground nut) of 15g was found to be ideal for extra cellular lipase production by *A. niger* (509 U/g). Least amount of lipase was produced at a concentration of 30g (322 U/g) (Table 2). Sprouted groundnut used as substrate for lipase production was reported by [29]. [20] have reported that soyabean meal of 10g showed maximum lipase production in *Malbranchea pulchella* var. *sulfurea*. Glucose as a carbon source showed the maximum enzyme production (602 U/g) followed by Fructose (549 U/g) (Table 2). Maximum growth was obtained with glucose in *Fusarium oysporum* [30] and *Aspergillus niger* [31]. The amount of enzyme produced when Ammonium nitrate was used as the nitrogen source was 579 U/g followed by ammonium sulphate (517 U/g) (Table 2). [28] found that *Candida rugosa* produces optimum lipase when ammonium nitrate was used as a nitrogen source. Olive was an excellent substance (579 U/g) for the production of lipase enzyme followed by gingely oil (Table 2). [32] have reported olive oil as the best inducer for lipase production (6 U/ml). [33] reported that the production of lipase by *Trichoderma viride* in a complex medium containing olive oil was high at a temp of 30ºC. In the present study maximum enzyme production was observed at room temperatures of 28ºC (686 U/g) (Table 2). [9] stated that high activity of lipase from Pencillium sp. was recorded at 35 to 60ºC. [34] reported the maximum lipase production by Rhizopus sp at 20ºC. A pH 6.5 was found to be conducive for the production of the enzyme (681 U/g) (Table 2). This result was in concordance with a report of [28] Song et al., 2001 who reported pH 6.5 to be ideal for lipase production. [4] has reported optimum production of lipase in *Aspergillus saprophyticus* at 35ºC and pH 7.

2.3. Protein Estimation

The amount of protein was estimated as 84 µg for 15g of ground nut as substrate concentration. When dextrose was used as the carbon source the protein was 190 µg and it was 134 µg when Ammonium nitrate was used as the nitrogen source. When olive oil and gingely oils were used as the oil substances individually, the protein content was 180 µg. The maximum protein was found to be at 4 ºC (114 µg) and pH 6.5 (122 µg) (Table 2). There was an increase in the lipase production and protein content at a substrate concentration of 15g. Similar results have been reported by [4].

2.4. Enzyme Assay

Maximum zone of hydrolysis (4mm) was observed at 15g as substrate concentration while it was 5mm when dextrose was used as the carbon source. When Potassium Nitrate was used as the nitrogen source the zone of hydrolysis was 4mm. A maximum zone of was observed for olive oil and gingely oils. The maximum zone of hydrolysis was 3mm at 28 and 37 ºC and 5mm at pH 6.5 (Table 2).

2.5. SDS-PAGE of the Partially Purified Enzyme

SDS PAGE analysis of the crude lipase enzyme showed the presence of a prominent protein band of 40KDa apart from some other proteins of lower molecular weight. Partially purified samples too showed the presence of 40KDa protein. [32] have also reported a 40KDa protein band from lipase enzyme of *Aspergillus niger*.

In the presence study preliminary work was carried for partial purification of lipase enzymes, further study need to be carried out for purification and characterization of lipase in *A. niger.*

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Table 1 Enumeration of Fungal Isolates Collected from Various Sites

Fungal Species	Canteen			Main Gate			C.C Block			Pond			M-0-1		
Plates	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Isolates	No of isolates			No of isolates			No of isolates			No of isolates			No of isolates		
Aspergillus niger	3	4	3	3	2	3	1	3	2	1	2	0	1	1	3
Aspergillus flavus	1	1	0	1	2	1	3	2	3	2	0	1	1	1	1
Aspergillus fumigatus	1	1	1	3	0	1	2	1	2	3	2	2	1	1	2
Pencillium citrinum	3	1	1	0	1	1	1	2	1	1	1	1	0	1	1
Mucor racemosun	1	1	1	1	2	3	2	1	3	0	1	1	1	2	2
Cladosporium cladosporoides	1	1	2	3	2	1	1	2	1	1	1	1	1	0	1

Inducers		Lipase Activity U/g	Protein Content µg	Zone of Hydrolysis (mm)
Substrate Concentration	10g	502	34	2
	15g	512*	84*	4*
	20g	438	32	2
	25g	509	50	2
	30g	322	28	1
Carbon Source	Glucose	602*	125	3
	Fructose	549	171	3
	Lactose	186	130	2.5
	Sucrose	522	110	2
	Dextrose	356	190*	5*
Nitrogen Source	Ammonium nitrate	579*	134	1
	Potassium Nitrate	275	102	4*
	Sodium Nitrate	325	101	2
	Beef extract	312	96	2
	Ammonium Sulphate	517	126*	3
Oil Substance	Olive oil	579*	180*	4*
	Sunflower oil	412	171	3
	Gingely oil	543	180	4*
	Corn oil	513	102	1
	Coconut oil	506	94	2
Temperature	4ºC	421	86	0.5
	28 ºC (Room Temperature)	686*	114*	3*
	37 ºC	481	104	3*
	40 ºC	534	40	1
pH	4.0	405	130*	3
	5.0	582	102	4
	5.5	464	134	4
	6.0	509	120	5*
	6.5	681*	122	5*

Rhizopus stolonifer	1	1	0	0	1	1	0	0	0	2	0	0	3	1	0	
Sterile hyphae	0	0	0	5	1	1	0	0	0	2	0	0	3	1	0	

Table 2 Effect of Different Inducers on Lipase Production by *A. niger*