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Lipase production by three thermophilic fungi

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Abstract:

Production of lipases by three thermophilic fungi, *Thermomyces lanuginosus*, *Talaromyces luteus* and *Rhizomucor pusillus* was investigated, influence of substratum, pH, temperature, carbon and nitrogen sources on lipase production was also studied. All the three fungi under study produced good amount of lipase constitutively. Lipase production was maximum at 45ºC and it was thermostable.

Keywords: Thermophilic fungi, lipase, influencing factors, *Thermomyces lanuginosus*, *Talaromyces luteus* and *Rhizomucor pusillus*

Introduction:

Lipases (triacylglycerol lipases E.C. 3.11.3) are enzymes which have been classically employed to carry on hydrolysis of triglycerides with concomitant production of free fatty acids.

Triacylglycerol ⟷ Diacylglyceroal+freefatty acids ⟷ Monoacylglycerol

+Free fatty acids ⟷ Glycerol + free fatty acids.

These enzymes also display catalytic activity towards a large variety of alcohols and acids in ester synthetic reactions under limited water activity. Synthesis of esters mediated by lipase has been carried out by numerous researchers to synthesize a variety of compounds which are important in day to day life. Lipases stand out amongst the most important biocatalysts carrying out novel reactions in both aqueous and non-aqueous media. This is primarily due to their ability to utilize a wide spectrum of substrates of high stability towards extremes of temperature, pH, organic solvents and chemo-regio- and enantio selectivity. Among lipases of plant, animal and microbial

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origin, it is the microbial lipase that finds wide applications. This is because microbes can be easily grown, more catalytic activity on wide variety of hydrolytic and synthetic reactions.

Brunke and Hube [1] and Rajan et al. [2] have excellently reviewed fungal lipases. Though production of lipases by mesophilic fungi was studied by several workers including Gasper et al. [3], Lima et al. [4]. Only limited reports are available on lipases of thermophilic fungi [5, 6, 7]. Hence, in the present investigations production of lipase by three thermophilic fungi was studied and discussed.

Material and Methods:

Isolation and identification of thermophilic fungi: An extensive and intensive survey of different substrates such as composting pits, bird nests, herbivore animals and soils were surveyed for the presence of thermophilic fungi. Different samples were collected aseptically in a sterile polythene bags and analysed for the presence of thermophilic fungi within 24 hrs. The thermophilic fungi were isolated by paired petri plate method as suggested by Cooney and Emerson [8].

Screening of thermophilic fungi on lipase production: The thermophilic fungi were primarily screened for lipase production on lipase screening medium containing rhodamine B agar medium at pH 7.0 [9]. The agar medium plates thus prepared were inoculated with 2 days old fungal strains and incubated at 45± 2 ˚C for 48 h. Lipase production was detected by irradiating the plates with UV light (350 nm). Fungal colonies that were considered positive for production of lipase showed orange fluorescent halo (Fig.1).

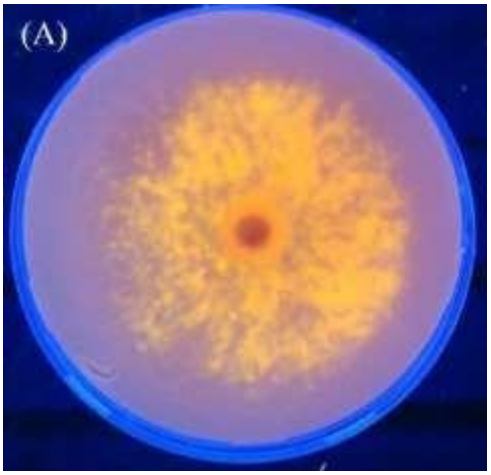

Fig 1. Fluorescent halo on rhodamine B agar medium

Assay of Lipase: Production of lipase was assessed in six different synthetic media, Yeast extract starch medium (A), Yeast extract starch medium + 0.1ml Triacetin (B), Medium (A) + 0.1ml Olive oil (C),Yeast extract Glucose medium (D), Medium (D) + 0.1ml Triacetin (E) and

Medium (D) + 0.1ml Olive oil (F). Urs et al. [10] method was adapted to assay lipases with some modifications. The reaction mixture consisting of 2 ml of triacetin substratum, 5 ml of citrate buffer (pH 8.0), 2 ml of enzyme and 0.5 ml of toluene was incubated at 45±2ºC for 3 hours for 8 days as it was found to be optimum for both for growth and lipase production. The reaction was terminated by adding 10 ml of absolute alcohol. The amount of acid produced was titrated with 0,05 N NaOH to a perceptible pink color using 1 ml of 1 % phenolphthalein solution as an indicator. Simultaneously, the blank was run with 2 ml of distilled water in place of enzyme. The activity was calculated from the difference between the control and experimental value. Lipase activity is expressed in International Units (IU) (0.1ml, 0.05 N NaOH required quantity was taken as 1 unit of enzyme activity).

Effect of pH and temperature on lipase production in YES medium by three thermophilic fungi was studied by making suitable conditions. Effect of carbon and nitrogen sources was also studied incorporating required amount of carbon or nitrogen sources so as to provide equal amount of carbon and nitrogen respectively in YES medium.

Results and Discussion:

Total of twenty eight fungal species belonging to 16 thermophilic and 12 thermotolerants were screened for the lipase activity and the results are presented in Table 1.

Table 1: Production of lipase by different thermophilic fungi

Name of the Fungi	Lipase*	Name of the Fungi	Lipase*
Absidia corymbifera	--	*H. stellata*	+
Acremonium thermophilum	--	*Rhizomucor miehei*	+
Aspergillus fumigatus	--	*R. pusillus*	+
A.nidulans	--	*Rhizopus arrhizus*	+
A. terreus	--	*R. microsporus*	+
A. flavus	+	*R. rhizopodiformis*	+
A. niger	--	*Malbranchea pulchella*	+
Chaetomium thermophile. V caprophile	--	*Myriococcum albomyces*	--
C. thermophile. V dissitum	--	*Pencillium duponti*	--
Chrysosporium fergusii	--	*P. purpurogenum*	--
Emericella nidulans	--	*Talaromyces luteus*	+
Humicola grisea	+	*Thermoscus aurantiacus*	--
H. fuscoatra	--	*Thermomyces lanuginosus*	+
H. insolens	--	*Torula thermophila*	--

* Orange fluorescence indicator positive for lipase production.

A. flavus, Humicola grisea, H. stellata, R. miehei, R. pusillus, Rhizopus arrhizus, R. microsporus, R. rhizopodiformis, M. pulchella, T.luteus and *T lanuginosus* were showed orange fluorescent halo on lipase screening medium, suggesting their potential to secrete lipase, while remaining fungi failed to produce lipase (Table 1).

The strains of *T. lanuginosus, T. luteus* and *R. pusillus* showed evidence of a maximum fluorescent halo. Therefore, these three strains were selected for lipase production under different cultural conditions.

Table 2 reveals that all the three thermophilic fungi under investigation could secrete lipase on different synthetic media assessed. However, the degree of production varied both with the medium and the fungus. *T. luteus* produced good amount of lipase.

When *T.lanuginosus* and *R.pusillus* preferred medium B, *T. luteus* opted for medium A for the production of lipase. All three fungi under investigation produce almost the same amount of lipase, irrespective of the presence or absence of lipid suggesting their constitutive nature.

Glucose medium (D) was inferior in the induction of lipase. Addition of olive oil to the medium D marginally increased the lipase production. Similarly, Aulakh and Prakash [11] reported the maximum production of lipase in medium containing cotton seed oil by *Aspergillus* spp. All the three thermophilic fungi preferred medium A over the medium D for the vegetative growth. Vegetative growth increased marginally with the addition of lipid. Efficiency of medium D did not improve significantly in the presence of lipid source. Lipase production also reached maximum by eighth day of incubation period in all the media tried. Similarly, Medium D was responsible for increased lipase production by *T.lanuginosus*. The pH drift in all the media growing all three fungi drifted towards alkaline side.

Table 2 : Production of Lipase during 8 days incubation period by three thermophilic fungi on different synthetic media

	T. lanuginosus			*T. luteus*			*R. pusillus*		
Medium	Dry wt (µg/ml)	pH	Lipase (in units)	Dry wt (µg/ml)	pH	Lipase (in units)	Dry wt (µg/ml)	pH	Lipase (in units)
Yeast extract Starch medium [A]	212.2	5.9	9.0	185.2	6.7	16.0	265.3	5.5	10.0
Medium A + 0.1 ml Triacetin [B]	221.8	5.2	10.2	210.4	6.9	18.0	268.4	5.2	12.2
Yeast extract Glucose Medium[C]	198.2	6.4	6.0	176.0	7.5	11.0	245.4	5.1	6.0
Yeast extract Glucose Medium 0.1ml Triacetin [D]	210.0	5.7	4.0	167.9	6.8	14.0	173.7	5.1	8.6
Medium A + 0.1 ml Olive oil [E]	156.8	5.2	8.0	202.5	5.5	14.2	245.2	4.8	9.0
Medium B+ 0.1 ml Olive oil [F]	182.1	5.9	6.4	155.8	6.4	11.0	231.7	4.4	9.0

Influence of pH was significant on the growth and lipase production by all the present fungi (table 3). pH 7.0 was optimum for lipase production by *T.luteus* and the lipase activity decreased with the increase of acidity or alkalinity. Similarly, *Geotrichum* spp produced maximum lipase at pH 7.0 [12]. Though *R.pusillus* could secrete lipase at the pH range studied, but its activity varied significantly with the pH of the medium. pH 6.0 was optimum as it supported maximum activity at this pH and the enzyme production decreased significantly both with the increase of acidity or alkalinity. Lipase activity was very low at pH 9.0. *T.lanuginosus* preferred an alkaline pH for the production of lipase, which was maximum at pH 8.0, while lipase production was minimum at pH 4.0 and pH 5.0. Similarly, *Fusarium solani* was reported to produce maximum lipase at pH 8.6 [13]. *T.lanuginosus* preferred pH 6.0 for the mycelium growth and only marginal growth was observed at pH 9.0. Decreased mycelia growth of *T.lanuginosus* was perceptible at pH 4.0. pH remained acidic in acidic media, while in media with initial alkaline pH it shifted to acidic side and the final pH was near neutral. No positive correlation could be observed between the vegetative growth and lipase production.

Table 3: Effect of pH on Lipase production during 8 days incubation period by three thermophilic fungi

Initial pH	*T.lanuginosus*			*T.luteus*			*R.pusillus*		
	Dry wt (µg/ml)	pH	Lipase (in units)	Dry wt (µg/ml)	pH	Lipase (in units)	Dry wt (µg/ml)	pH	Lipase (in units)
4.0	198.7	4.3	6.0	--	--	--	235.5	3.6	11.0
5.0	210.8	4.0	4.0	165.3	4.6	14.0	245.3	3.4	1.0
6.0	243.7	5.8	9.0	172.3	4.8	16.0	266.2	5.5	10.0
7.0	233.0	7.2	12.0	210.2	4.5	15.0	245.2	6.8	3.0
8.0	210.8	7.6	12.0	172.3	5.3	14.0	210.2	7.5	3.0
9.0	202.3	7.4	6.0	--	--	--	186.2	7.4	1.0

Results on influence of temperature on the production of lipase by three thermophilic fungi are précised in table 4.

Table 4 reveals that 45˚C was optimum for secretion of lipase by *T.luteus* and *R.pusillus*, while *T.lanuginosus* showed increasing trend of lipase production up to 50˚C and the production of enzyme decreased significantly at temperature 55˚C. *T.luteus* prefers a temperature of 45˚C for maximum lipase secretion. *R.pusillus* in general produced lipase in considerable amount at 45˚C and the enzyme production was marginal at 35˚C and 40˚C. Similarly, Abbas et al. [14] reported that the temperature has significant influence on lipase production by *Mucor* spp.

Table 4: Effect of temperature on Lipase production during 8 days incubation period by three thermophilic fungi

Temperature (ºC)	*T.lanuginosus*			*T.luteus*			*R.pusillus*		
	Dry wt (µg/ml)	pH	Lipase (in units)	Dry wt (µg/ml)	pH	Lipase (in units)	Dry wt (µg/ml)	pH	Lipase (in units)
35	153.8	5.9	6.0	166.3	4.8	5.8	222.2	5.5	3.0
40	186.3	5.8	5.0	172.3	4.6	11.0	245.3	5.7	4.0
45	243.7	6.0	12.0	178.2	4.8	16.0	266.2	5.5	10.0
50	210.2	6.7	12.0	164.2	4.6	11.0	245.2	6.8	7.8
55	198.2	7.6	6.0	172.3	5.3	13.0	210.2	7.5	1.0

Effect of carbon source on the production of lipase by three thermophilic fungi was studied and the results are précised in table 5.

Table 5 reveals that glucose supported maximum production of lipase, while succinic acid was responsible for least amount of lipase production by *T.lanuginosus*. Citric acid was responsible for total inhibition of lipase. Lactose was poor substratum for secretion of lipase by *T.lanuginosus. T.luteus* opted sucrose followed by D-fructose and maltose for production of lipase, while lipase production was least during its growth in D-mannose, L-sorbose and D-ribose and glycerol. Rest of the carbon sources supported the lipase production intermediately. *R.pusillus* produced moderate amount of lipase in medium containing citric acid, succinic acid, lactose, D-glucose, L-sorbose, D-ribose and D-xylose. Mannitol, maltose, glycerol and D-ribose and D-fructose induced good amount of lipase in *R.pusillus*. D-fructose supported good vegetative growth, while other carbon sources were inferior in supporting vegetative growth.

T.luteus preferred starch, sucrose, maltose, D-fructose, D-glucose, D-mannose and lactose for its vegetative growth, while other carbon sources supported comparatively low growth. The final pH in medium containing succinic acid growing *T. luteus* and *R. pusillus* was strongly acidic, while in medium containing the rest of the carbon sources was mild acidic.

Table 5: Influence of different carbon sources on Lipase production during 8 days incubation period by three thermophilic fungi

Carbon source	*T.lanuginosus*			*T.luteus*			*R.pusillus*		
	Dry wt (in µg)	pH	Lipase (in units)	Dry wt (in µg)	pH	Lipase (in units)	Dry wt (in µg)	pH	Lipase (in units)
D-glucose	225.7	5.6	12.0	182.3	4.8	12.0	245.3	5.5	9.0
D-fructose	228.3	5.5	9.0	214.2	5.2	14.0	254.3	5.7	6.0
D-galactose	221.8	6.1	8.0	198.3	5.1	9.0	222.3	5.9	6.0
D-mannose	225.3	5.7	6.8	210.3	4.7	6.0	247.8	5.7	8.0
L-sorbose	191.3	5.8	9.4	221.3	4.8	4.8	225.5	5.2	6.0
D-ribose	178.2	5.2	5.8	198.2	5.3	6.2	232.8	5.5	4.8
D-xylose	218.6	5.9	6.4	158.2	5.4	6.0	224.6	5.6	6.0
Sucrose	221.9	5.3	7.8	223.2	4.7	16.0	238.6	5.8	8.0
Maltose	210.6	5.3	8.0	227.3	4.8	14.0	243.6	5.8	7.0
Lactose	229.3	5.4	4.4	226.3	4.7	9.0	243.8	5.8	6.0
Citric acid	--	--	--	--	--	--	188.7	3.4	3.0
Succinic acid	135.0	3.7	4.0	176.2	4.5	3.8	191.6	3.5	3.0
Mannitol	178.9	5.2	9.2	210.2	4.7	9.8	255.6	5.7	14.0
Glycerol	112.3	5.7	8.8	197.3	4.6	6.2	254.2	5.7	6.9
Starch	197.2	5.6	7.0	223.4	4.7	14.0	247.9	5.7	8.0

Lipase production was maximum in medium containing L-asparagine, glutamine and L-glutamic acid by *T.lanuginosus*, while it was least in medium containing ammonium nitrate, L-methionine and L-histidine (table 6). Final pH was near neutral while, in medium containing L-histidine it was strongly acidic.

T.luteus opted L-glutamic acid, glycine and ammonium chloride for production of lipase, while ammonium sulphate, L-histidine, L-methionine, L-lycine were poor substrates. Similarly, Mhetras et al. [15] reported that *Rhizopus*, *Mucor* and *Geotrichum* preferred glycine and glutamic acid for maximum production of lipase. Rest of the nitrogen sources were intermediate in the induction of lipase. L-asparagine and L-aspartic acid were next preferred substrates for production of lipase.

When *T.lanuginosus* preferred L-asparagine over the aspartic acid for lipase production and vegetative growth, *T.luteus* and *R.pusillus* failed to distinguish between acid form / amine form of asparagine, as they secreted same amount of lipase. Similarly *T.luteus* preferred acid form over the amine form of glutamic acid for the vegetative growth and enzyme production.

The other two fungi *T.lanuginosus* and *R.pusillus* failed to distinguish between the two forms (acid and amide) for the enzyme production and vegetative growth. The different behavior of these fungi towards acid form and amide of these two amino acids is not in explainable as in some organisms amide is more active, while in others it is acid which is more active . However different behavior of present fungi with asparagine and glutamine needs more detailed investigations.

Table 6: Influence of different Nitrogen sources on Lipase production
8 days incubation period by three thermophilic fungi

Nitrogen source	*T.lanuginosus*			*T.luteus*			*R. pusillus*		
	Dry wt (in µg)	pH	Lipase (in units)	Dry wt (in µg)	pH	Lipase (in units)	Dry wt (in µg)	pH	Lipase (in units)
Ammonium Chloride	146.2	6.3	5.8	143.2	4.8	14.0	232.8	5.5	4.4
Ammonium nitrate	156.2	4.8	4.4	155.4	5.2	5.8	246.8	5.7	5.0
Ammonium Sulphate	162.3	5.2	8.0	166.3	5.1	3.6	211.7	5.9	6.0
L-arginine	225.3	5.7	7.2	174.8	4.7	8.2	265.8	5.7	12.0
L-asparagine	210.2	5.8	12.0	189.6	4.8	8.2	255.7	5.3	6.0
L-aspartic acid	187.2	5.2	7.0	210.5	5.3	7.2	262.8	5.5	8.2
L-glutamine	218.6	5.3	9.2	188.5	5.4	8.2	247.8	5.6	9.2
L-glutamic acid	221.9	3.2	9.0	206.3	4.7	12.0	238.6	5.8	7.0
L-Glycine	210.6	5.3	8.0	212.3	4.8	11.0	243.6	5.8	6.0
L-methionine	210.0	5.4	4.4	154.2	4.7	4.4	210.8	5.8	4.0
L-histidine	154.2	3.7	6.4	176.2	4.5	3.8	211.7	3.5	4.8
L-lysine	178.9	5.2	9.2	188.2	5.3	4.2	234.8	5.7	4.8
L-tryptophan	213.8	5.7	8.2	186.2	4.6	5.8	244.8	5.7	9.2
L-tyrosine	186.2	5.4	6.4	187.2	4.7	6.0	235.7	5.7	5.5
Yeast extract	197.2	6.2	8.0	210.8	4.7	14.0	247.9	5.7	8.0

Conclusion: All the present fungi were positive for lipase production. However, the degree of production varied with the fungus and environment under which the fungus grew. In view of the advantages of fermentative production of lipases by thermophilic fungi, more in-depth studies are needed.

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