



STRAIN IMPROVEMENT AND EFFECT OF NATURAL INDUCER ON LIPASE PRODUCTION BY *RHIZOMUCOR PUSILLUS*

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ABSTRACT:

Lipases are widely used enzyme, known for its application in various industries. The fungal strains are preferred for lipase production since their enzymes are secreted extracellular and the extraction technique is simple. Thermophilic fungal enzymes are more important because of their stability at high temperature. During present investigation a thermophilic fungi *Rhizomucor pusillus* was isolated from decomposing leaves collected from forest of Nagpur district. Strain improvement of *R. pusillus* was carried out the increase Lipase production by using chemical mutagen EMS. Mutant strains derived were evaluated for yield of lipases production. Lipase activity of mutants was assayed by evaluation of quantity of fatty acid released in unit time during enzyme reaction and its measured by the quantity of NaOH required to maintain pH neutral. Present work also includes the effect of natural inducer such as wheat bran and olive oil on lipase production. It was concluded that the wheat bran is good inducer for lipase production as compare to olive oil during the process of submerged fermentation.

Keywords: Strain improvement lipase activity, inducer, fungi, *Rhizomucor pusillus*

INTRODUCTION:

Lipases are widely used enzyme, known for its applications in various industries. The fungal strains are preferred for lipase production since their enzymes are secreted extracellular and the extraction technique is simple. Thermophilic fungal enzymes are more important because of their stability at high temperature. Present investigation on a thermophilic fungus *Rhizomucor pusillus* (Lindt) Schipper was isolated from decomposing leaves collected from forest of Nagpur district. Mutant was developed earlier in same laboratory by using EMS. Quantitative estimation of lipase was carried out to understand effect of natural inducer during fermentation on lipase production.

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extraction technique is simple. Thermophilic fungal enzymes are more important because of their stability at high temperature. Present investigation on a thermophilic fungus *Rhizomucor pusillus* (Lindt) Schipper was isolated from decomposing leaves collected from forest of Nagpur district. Mutant was developed earlier in same laboratory by using EMS. Quantitative estimation of lipase was carried out to understand effect of natural inducer during fermentation on lipase production.

Rhizomucor pusillus – It is a thermophilic fungus that lives in hot environments such as decomposing leaf litter. Its growth optimum at 45° C and a maximum temperature 50° C or above and a minimum of 20 °C or above (Cooney Emerson, 1964, Maheshwari *et al.*, 2000). *Rhizomucor pusillus* structure shows rhizoids and branched sporangiophores. It is grey mycelium fungi grows naturally on dead and decaying

materials, most commonly found on composting and leaf litter during thermophilic phase of decomposition.

Classification: Kingdom-Emycota, Division-Zygomycota, Class-Zygomycets, Order-Mucorales, Family -Mucoraceae, Genus-*Rhizomucor*, Species-*pusillus*

Lipase:

- The hydrolysis of fats (lipids) catalyzes through an enzyme known as lipase.
- Lipase plays an important part in the processing, transport and digestion of dietary lipids (e.g. oils, fats, Triglycerides).
- In an oil-water interfaces, hydrolysis of free fatty acids and glycerol from triglycerides are done through hydrolysed under which lipases (triacylglycerol acylhydrolases: EC 3.1.1.3) is one of the category.
- Also, lipases catalyze the hydrolysis and transesterification of other esters as well as the synthesis of esters and exhibit enantio selective properties.
- Lipases becomes famous in most of the common used product such as detergent, food, cosmetic, pharmaceutical industries and organic synthesis due to its ability of chemical transformation (biotransformation).
- Recently many various plan of action in the chemical and pharmaceutical industries have use lipase in the synthesis of unblended drugs and agrochemicals (M. De M.D Maia *et al.*, 1999).

MICROBIAL SOURCES OF LIPASES:-

- Lipases are omnipresent in nature and are manufactured by various microorganisms, animals, and plants. The most widely extensively used class of enzymes in organic chemistry and biotechnological applications are lipases have microbial origin. A review of

the most recent (from 2004 to the present) potential microorganisms for lipase production in submerged fermentation.

FUNGI:-

- Most commercially important lipase-producing fungi are recognized as belonging to the genera *Geotrichum* sp., *Mucor* sp., *Rhizomucor* sp., *Aspergillus* sp., *Fusarium* sp., *Humicola* sp., etc. (Cihangir and Sarikava, 2004).
- Due to intense industrial application, new enzymes with specific characteristics in various branches of industrial activity is huge, therefore identification of novel enzymes with desired characteristics in relation to the substrate selectivity, thermo stability, pH and temperature optimum and optimization of its production resulting directly in reduced cost.

APPLICATION OF LIPASE:-

Lipases are generally apply in the processing of detergents and degreasing formulations, oils and fats, the synthesis of fine chemicals and pharmaceuticals, manufacturing of paper, processing of food, and cosmetic production (Rubin and Dennis,1997). The degradation of fatty waste (Masse *etal*, 2001) mostly accelerated by the use of Lipase. Fungi and bacteria derived the industrial microbial lipases. In 2002, the industrial market value for lipases was reported as 1.5 billion US dollars (Alain Houde *etal*.2004. Industrial applications of microbial lipases (Vulfson, 1994).

Present work:- Focus on factors relevant for the enhancement of enzymatic hydrolysis of triacylglycerols compounds using lipase extracted from thermophilic fungus *Rhyzomucor pusillus* and to study effect of mutagen on production of cellulase by evaluating cellulase activity of mutants generated.

Lipase [triacylglycerol acylhydrolase, EC 3.1.1.3] is omnipresent enzymes of substantial physiological significance and industrial potential. It catalyse the hydrolysis of triacylglycerols to glycerine and free fatty acids.

1. Isolation of thermophilic fungus *Rhizomucor pusillus* from samples of decomposing leaf litter collected from forest of Nagpur district.
2. Strain improvement of *Rhizomucor pusillus* by chemical mutagenesis for increased Lipase production by using chemical mutagen Ethyl Methane Sulphonate (EMS).
3. Mutants clones to be obtained by the mutagenesis program stabilize by culturing for minimum six generation.
4. Mutants derived by EMS treatment will be evaluated for yield of lipases. Lipase activity of Mutants was assayed for evaluation by the quantity of fatty acid released in unit time is measured by the quantity of NaOH required to maintain pH constant (Jayraman, J. (1981).

MATERIAL & METHODS:

Isolation of thermophilic fungi:-

Thermophilic fungi were isolated from samples of decomposing leaf litter collected from forest of Nagpur district.

Isolation techniques were used:- By (Waksman *et al* 1931; Waksman *et al.* 1939). The hyphal isolates were sub-cultured onto yeast starch (YpSs) agar (Cooney *et al.*1964) until pure cultures were obtained.

2. Procedure lipase assay:- By Jayraman, J. (1981):-

2ml of olive oil, neutralize to pH 7.0, and stir well with 100mg bile salt, i.e. sodium taurocholate

and 25ml of water. Addition of 2gm gum tragacanth has tens emulsification. Set the beaker on top of magnetic stirrer (cum hot plate) and stirrer the content slowly. Make 0.1 N NaOH and 50 mM phosphate buffer (pH 7.0). Lipase activity was determine by titrimetric method given by method (Jayraman, J. 1981); using Olive oil (Hi-media) as substrate emulsion. Crude enzyme was extracted from YpSs broth having six days old pure culture of *Rhizomucor pusillus*. Take centrifuge tubes and added the 1ml phosphate buffer, 2ml double distilled water and 3ml substrate containing homogenized 2ml olive oil, 2g gum tragacanth, 25ml double distilled water (pH maintain 7.0 stir the contents slowly). Note the room temperature; Dip the electrodes of pH meter in the reaction mixture. Note the pH and adjust to 7.0. Take centrifuge tube add 3ml substrate, 1ml phosphate buffer, 2ml double distilled water and add 1ml Crude enzyme. Instantly record the pH and set the timer on. Let it be pH at zero, (pH maintain to 7.0). Dip the electrodes of ph meter in the reaction mixture. Note the pH and adjust it to 7.0. After 30 min again dip the electrode to take the pH reading and added drops of 0.1N NaOH to bring pH to the initial value.

Note:- The volume of alkali consumed.

3. Effect of additives growth:- In *R. pusillus* fungus are have to show the effect of additive growth for that use additive wheat bran in different percentage (2% & 4%). We take the two set of petriplate pore the YpSs media then add with 2% and 4% additive. After that inoculate the fungi and day by day observed and measure the growth of colony.

RESULTS & DISCUSSION:

Isolation of thermophilic fungus *Rhizomucor pusillus* from samples of decomposing leaf litter collected from forest of Nagpur district.

Genetic improvement of *Rhizomucor pusillus* through mutation:- At 10 mg/ml EMS

concentration able to generate mutants with about survivability percentage near or less than 1 % and it is the requirement to generate mutants in fungi. Effective duration of exposure to EMS solution for fungus which is able to produce successful mutants was finding of 90 minutes and 120 minutes. Isolation of mutant after treatment of EMS. After the treatment of mutagen EMS, mutants pure cultured up to minimum six generations through spores for stabilization. The production of lipase and estimation of enzyme was carried out quantitatively by evaluating the enzymes complex.

Effect lipase production using wheat bran and olive oil as inducer by submerged

fermentation:- Enzyme extracted for lipase estimation from submerged fermentation using various inducer to enhance lipase activity where incubated at 45 °C for 5days. Control was taken without inducer. Pure culture of fungus was inoculated during submerged fermentation. Crude enzyme extract kept at 4°C till the procedure for assay started for lipase assay, (Jayraman, J .1981). When three ml enzyme use during the lipase activity. shows that the amount of NaOH required to neutralized substrate compared with control (without inducer), it shows 37% rise in enzyme activity with inducer as olive oil and with wheat bran 40% more enzyme activity, (Fig.No.2,Table No3). Wheat bran showed 3% more activity than olive oil as inducer.

B. Effect on enzyme production at different interval of time such as 5 days and 10 days:-

During enzyme assay when one ml enzyme used from the set of five days of incubation where wheat bran was inducer, shows NaOH required to neutralize fatty acid with inducer 14% equal to control no substantial increase in enzyme activity. After ten days of incubation lipase

activity shown, 60% increased as compared to control (Table No: 4, Fig. No.3)

Olive oil use as inducer:- When Olive oil use as inducer, and five days of incubation enzyme activity was shown 6.15% increase as compared to control. When one ml enzyme used during enzyme assay. When results of wheat bran and olive oil as inducer to enhance lipase activity, compared with control olive oil shown about 6% rise in enzyme production during five days of incubation.

Effect of wheat bran as additive on growth of R. pusillus:-

Growth of *R. pusillus* on solid nutrient medium with wheat bran as additive when compared with normal YpSs Emersion Agar medium. On second day as compared to control 0.20% growth rise found in two percent (2%) wheat bran as additive in nutrient medium. When four percent (4%) wheat bran was used as additive in nutrient medium rise was 0.22% this result indicated present wheat bran in nutrient medium favours fungal growth in a substantial way.(Table no.5, Fig.no.4, Plate no.2/3).

CONCLUSION

Experiment was Carry out with the aim to developed fungi with better capacity to produce lipase and industrially important enzyme through mutagenesis of *R. pusillus*. Success fully mutants of *R. pusillus* develop by Chemical mutagen EMS. Thermophilic fungus selected for enzyme production because such enzymes are thermo-stable which is an essential requirement for industrial utilization Jayraman, J.(1981) method is cost effective and less time consuming technique to evaluate lipase activity.

In the present work following techniques were standardized. Isolation of thermophilic fungi, Chemical mutagenesis, stabilization of mutant fungal strains, extraction and estimation of lipase enzyme activity. Effect of inducer such as olive oil

and wheat bran was observed during submerged fermentation under investigation to enhance lipase production from selected mutant. For selected mutant strain of *Rhizomucor pusillus*, showed Wheat bran as more effective inducer for lipase production during submerged fermentation when compared with olive oil used as inducer. Results showed consistency that rise in growth of *R. pusillus* on solid nutrient medium with wheat bran as additive when compared with normal YpSs Emersion Agar medium. During the investigation it has been observed that crude Enzyme concentration is an important factor during enzyme activity analysis. Low concentration of crude enzyme do not shows sufficient activity on substrate, purification of enzyme is essential for analysis of Lipase activity. All above parameters must be further analyzed in detail to optimize the lipase production.

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REFERENCES:

- Alain Houde, Ali Kademi, Danielle Leblanc. Lipase and their industrial application, Appl Biochem Biotechnol, 2004; 118(1):155-170.
- Brockman, H.L. and Borgstorm, B. (1984) Lipases, Elsevier, Amsterdam. pp. 34.
- Cihangir and Sarikaya E. 2004. Investigation of lipase production by a new isolate of *Aspergillus* sp. World J Microbiol Biotechnol. 20(2):193-197.
- Cooney, D. G. Emerson, R. 1964. Thermophilic fungi. Publ. San Francisco. 1-188.
- Jayraman, J. (1981) Jaeger K.E., B.W. Dijkstra, M.T. Reetz, Bacterial Biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications, Annu. Rev. Microbiol. 53 (2000) 315-351.
- Jayraman, J. (1981), in Laboratory manual in Biochemistry Wiley Eastern Limited New Delhi P. 133.
- Maheshwari R., Bharadwaj, G. Bhat, M. K. 2000. Thermophilic fungi: their physiology and enzymes. Microbiol. Mol. Biol. Rev. 64 (3): pp.461-488.
- M. De M.D Maia, Morais, M.M.C.D., Morais, M.A.D., Melo, E.H.M. and Filho, J.L.D.L. 1999. Production of extracellular lipase by the phytopathogenic fungus *Fusarium solani* FS1. Rev. Microbiol., 30(4):304-309.
- Masse L, Kennedy KJ, Chou SP. The effect of an enzymatic pretreatment on the hydrolysis and size reduction of fat particles in slaughter house waste water. J Chem Technol Biotechnol 2001; 76:629-35.
- Rubin and Dennis, 1997. editors. Lipase: Part A. Biotechnology Methods in enzymology. vol. 284. New York: Academic Press, pp.1-408.
- Vulfson, E.N. 1994. Industrial application of lipase. In: Woolley P, PETERSEN SB, editors. Lipases their structure, biochemistry and application. Cambridge, UK: Cambridge university press, 271-288.
- Waksman et al 1931; Waksman et al 1939, where two acyl moieties are exchanged between two acylglycerols (Balcao et al 1996). 643 R. Sharma et al/ Biotechnology advance (2001) 627-662.

Industry	Action	Product & its application
a) Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavouring agents in milk, cheese, and butter
b) Bakery foods	Flavour development	Shelf-life elongation
c) Pharmaceuticals	Trans esterification; hydrolysis	Speciality lipids, digestive acids
d) Libation	Improved aroma	Beverages
e) Cleaner	Hydrolysis of fats	Expulsion of oil stains from fabrics
f) Food dressing	Quality development	Mayonnaise, dressings, and whippings
g) Meat and fish	Flavour development	Meat and fish products, fat removal
h) Health food	Trans etherification	Health food
i) Meat and fish	Flavour development	Meat and fish products, fat removal
j) Fats and oils	Trans esterification; hydrolysis	Cocoa butter, fatty acids, glycerol
k) Cosmetics	Synthesis	Emulsifiers, moisturizers
l) Leather	Hydrolysis	Leather products
m) Paper	Hydrolysis	Paper with improved quality
n) Cleaning	Hydrolysis	Removal of fats

EMS treatment	No of Colonies survive after 90min. of EMS treatment	No of Colonies survive after 120 min. of EMS treatment	Control Colonies count
10mg/ml	40 colonies	11 colonies	79,2000
Percent survivability	0.005%	0.0013%	100%

	WHEAT BRAN			OLIVE OIL			Mutant without additive	
	NO	NaOH required (ml)	Mean (ml)	NO	NaOH required (ml)	Mean	NO	Mean
5ML ENZYME USE AS A REACTANT	1	2.3	1.95 (± 0.308)	1	1.7	1.88 (± 0.14)	1	1.66 (± 0.05)
	2	2.0		2	1.9			
	3	1.55		3	2.06			
3ML ENZYME USE AS A REACTANT	1	1.7	1.35 (± 0.49)	1	0.6	0.8 (± 0.28)	1	Mean
	2	1.0		2	1			1.25 (± 0.07)

NOTE:- Values in the parenthesis are Standard deviation

Table No. 5- Effect of wheat bran as inducer on growth of *R. pusillus*.

	CONTROL WITHOUT ADDITIVE				M90-D1 FUNGI WITH WHEAT BRAN 2%				M90-D1 FUNGI WITH WHEAT BRAN 4%			
	DAY 1		DAY 2		DAY 1		DAY2		DAY1		DAY2	
	Colony Diameter	Mean	Colony Diameter	Mean	Colony Diameter	Mean	Colony Diameter	Mean	Colony Diameter	Mean	Colony Diameter	Mean
1	6.6mm	<u>7.3</u> mm	17.8m m	<u>17.8</u> 6m	10.1m m	<u>9.77</u> mm	20.9m m	<u>20.73</u> mm	9.8mm	<u>10.3</u> mm	23.7m m	<u>22.8</u> mm
2	7.5mm	(± 0.62)	18.1m m	(± 0.20)	10.1m m	(± 0.57)	20.7m m	(± 0.15)	11.3m m	(± 0.81)	22.6m m	(± 0.66)
3	7.8mm		17.7m m		9.1mm		20.6m m		10mm		22.1m m	
Difference		0.07 3%		0.17%		0.09%		0.20%		0.10%		0.22%

Note:-Values in the parenthesis are Standard Deviations

Table No. 4:- Estimation of Lipase activity of Mutant=M90 D1 Of *Rhizomucor pusillus* (after 5days and 10 days comparatively) treatment

	NO	WHEAT BRAN		Mutant without additive	
		NaOH required (ml)	Mean	NaOH required (ml)	Mean
5 DAYS	1	0.26	0.21 (±0.061)	0.2	0.26 (±0.04)
	2	0.13		0.3	
	3	0.26		0.3	
10 DAYS	1	1.00	0.89 (±0.100)	0.1	0.2 (±0.094)
	2	0.76		0.3	
	3	0.93		0.3	

NOTE:- Values in the parenthesis are Standard deviation

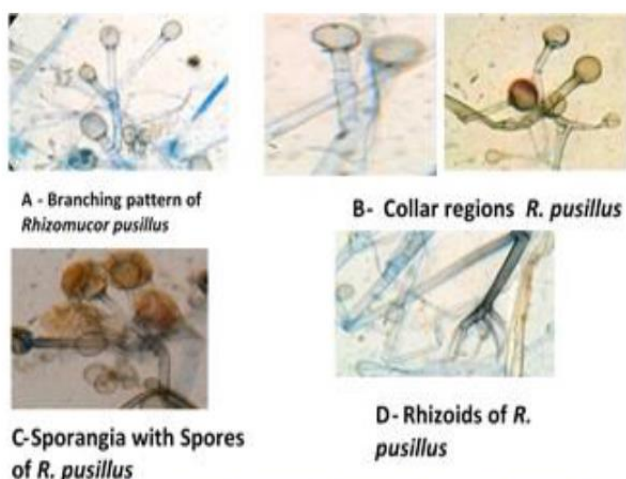


Figure 1: *Rhizomucor pusillus* morphology and colony features

