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Lipolytic Potentialities of Microbes from Oil Mill Effluent

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ABSTRACT

Lipases are the enzymes capable of catalyzing the hydrolysis and synthesis of esters formed from glycerol and long chain fatty acids. The production of lipase from *Bacillus subtilis* and *Aspergillus flavus.* After the isolation, lipase producing colonies were screened using tributyrin agar plates, the lipase producing cultures were obtained and maintained as stock cultures. The effect of different pH, temperature, incubation time and substrate concentration on the production of lipase was determined by the organisms on the three different substrates such as olive oil, coconut oil and sunflower oil. *Bacillus subtilis* produced the maximum amount of lipase in all physical parameters such as pH, temperature, incubation time and substrate concentration when compared to *Aspergillus flavus*.

Key words: Lipase, Tributyrin agar medium, *Bacillus subtilis, Aspergillus flavus.*

INTRODUCTION

Enzymes are biocatalyst which accelerates biological reactions. The sources of these enzymes are microorganisms, animal and higher plants. Animal enzymes used currently are lipase, trypsin, rennet etc. Microbial enzymes have two advantages over the plant and animal enzymes. They are economical and can be produced on large scale with in limited space and time. The amount produced depends on size of fermentor, types of microbial strain and growth condition. It can be easily extracted and purified[1]. Lipases are an important group of biotechnologically relevant enzymes because of their catalytic activity in both aqueous and non aqueous media.Lipase can capable of degrading lipid molecules. Lipase is secreted in the digestive tract that catalyses the breakdown of fats into individual fatty acids that can be absorbed into the blood stream. Lipases comprise a group of enzyme which catalyses the hydrolysis of triacylglycerols[2,3].

Lipase producing microorganisms have been found in diverse habitats such as industrial wastes,vegetable oil processing factories , dairies , oil seeds and decaying foods[4] compost heaps, coal tips and hot springs[5]. Lipase producing microorganism include bacteria, fungi, yeast, and actinomycetes. Lipase production increased when the medium was supplemented with inorganic nitrogen source (ammonium nitrate)[6]. Similarly the addition of ammonium sulphate and peptone to the medium enhanced lipase production by the type and concentration of carbon and nitrogen sources, culture pH. Lipase belongs to the class of serine hydrolases and therefore do not require any cofactor. The natural substrate of lipases are triacylglycerols having very low solubility in water. Under natural condition, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phases in which the enzyme is dissolved. Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction , growth

temperature and the dissolved oxygen concentration[7]. Vegetable oil processing plants are normally the big contributors of oily wastewater. Vegetable oil industries such as sunflower, olive and palm discharge large volumes of oily wastewaters. These wastewaters contain a very high volume of oil-in-water emulsions as their basic contaminant.

In order to achieve commercially important viable yields from microorganisms, optimization of physicochemical parameters is important. In the present study, isolation, screening and effect of physical parameters has been evaluated for the production of lipase by *Bacillus subtilis* and *Aspergillus flavus.*

MATERIAL AND METHODS

Isolation and screening of lipolytic organisms

The soil samples were collected from oil-mill areas of the coconut oil extracting industry at Mannargudi, Tiruvarur District, Tamil Nadu, South India were subjected to serial dilution, allowed to grow the colonies on the isolation agar medium and then subjected to staining and other biochemical procedures for identifying the bacteria by using Bergey's manual of systematic bacteriology [8]and fungi was identified wet mount technique[9]

The isolates were screened for lipolytic activity and lipolytic potential (R/r) using tributyrin agar medium. The strains were spread on tributyrin agar [10]and incubated for 24 hours at 30^{0}C . Then the bacterial colonies which formed clear zone around on the plates.

Inoculum preparation

Olive oil, coconut oil and sunflower oil were used as substrates. In order to prepare the inoculum, a loopful of cells from a freshly grown slant was transferred into a 250ml conical flask containing 50ml of minimal media containing KH_2PO_4 -3.0g, Na_2HPO_4 -6.0g, NaCl -5.0g, NH_4Cl_2 -0.2g, $MgSo_4$ -0.1g, dissolved in 1 litre of distilled water and incubated at 30^{o}C in a shaking incubator at 180rpm for 24hrs[11]

Lipase production

Lipase production was carried out using 3 different substrates like olive oil, coconut oil and sunflower oil under submerged fermentation. Reese's medium for fungi,containing,KH_2PO_4- 2g, $(NH_4)_2 So_4$ –1.4 g, $MgSo_4$ –0.3 g, $CaCl_2$ –0.3 g, Urea – 0.3 g, Trace element solution – 1ml, yeast extract – 0.05% ,dissolved in 1 litre distilled water. Conidial suspension at a concentration of 1x10^6 served as inoculum.

Optimization of Bioprocess variables on Lipase production

Various parameters like incubation time, pH, temperature and media components were altered to obtain theb maximum production of lipase. The lipase production was carried out by shake flask fermentation dublicates at appropriate conditions with 3% fresh inoculums. The flasks were incubated under shaking conditions at 150 rpm in a shaker incubator. After fermentation, enzyme activity was determined**.**

Effect of substrate concentration

The production medium supplemented with different substrates like olive oil, coconut oil and sunflower oil was estimated for lipase activity. 5,10 and 15 % of each substrates were added to the Erlenmeyer flasks separately. Then the Reese's broth and minimal broth was added to it and sterilized. After cooling , inoculated with *A.flavus* and *B.subtilis* culture broth. The flasks were incubated at 37ºC for 24hrs.

Effect of pH

The effect of pH on the production of lipase was carried out by using *Aspergillus flavus* and *Bacillus subtilis*.10ml of each substrates were added to separate Erlenmeyer flasks. After the addition of modified Reese's broth for fungal and minimal broth for bacteria, before sterilization pH of the fermentation medium was checked and adjusted to various pH units such as 4,5 and 6 using H_2So_4 and NaOH.After sterilization, the substrates were inoculated with *A.flavus* and *B.subtilis* separately and were incubated.

Effect of Temperature

10ml of each substrates were added to the Erlenmeyer flasks separately. Then the modified Reese's broth and minimal broth was added to it and sterilized. After cooling, inoculated with *A. flavus* and *B. subtilis* culture broth. The flasks were

incubated at 37^{0}C, 45^{0}C and 50^{0}C for 48hrs and 24hrs for *A.flavus* and *B.subtilis* respectively.

Effect of incubation time

The production of lipase was carried out by using submerged fermentation at time duration of 12,24 and 48 hrs for bacteria and 24,48 and 72 hrs for fungi.

Assay of lipase activity

Lipase activity was determined[12] according to the method using an emulsion of 10% olive oil in 10% gum arabic. The emulsion was produced by heating the mixture of olive oil and gum arabic solution in a top divine homogenizer for 10 minutes. The reaction mixture contained 3ml of 0.2M tris Hcl buffer and 1.0 ml crude enzyme sample was added. The reaction was carried out at 37^{0}C for 2 hours in a shaking water bath and the reaction mixture was supplemented with 10 ml ethanol. The amount of oleic acid was determined by titrating the hydrolysis products with 0.05N NaOH using phenolpthalin indicator. After 6 days of incubation, the flasks were retrieved from shaker, culture broth was transferred to sterile centrifuge tubes and centrifuged at 8000 rpm for 10 minutes. The crude filtrate containing enzyme was assayed for the activity and stored at 4^{0}C.

Statistical Analysis

The results obtained in the present investigation were subjected to statistical analysis like mean(X) and Standard deviation (SD).

RESULTS AND DISCUSSION

In this present study, the lipase production of *Bacillus subtilis* and *Aspergillus flavus* were compared using three different substrates such as olive oil, sunflower oil and coconut oil. The optimization of media conditions were also carried out.

Isolation and screening of lipase producing microorganisms

The isolated bacteria was identified as a gram negative, motile, rod shaped organism using Gram's staining and hanging drop technique. The bacterial isolates showed positive forVoges Proskauer, citrate utilization, catalase test and showed negative for Indole , Methyl red and urease test. According to the biochemical tests, the isolated organism was identified as *Bacillus subtilis*.Under microscopic observation, septate, branched, mycelium with yellowish-green colored conidia was observed. Their conidiospore inflates to form a vesicle, which is usually green colored. Thus the isolated fungus was identified as *Aspergillus flavus*.The lipase producing organisms *Bacillus subtilis* and *Aspergillus flavus* were identified with the help of the zone formation in the tributyrin agar medium. The zone is formed due to the organisms, which cleaves lipid molecules present in the tributyrin agar medium.

Table 1: Effect of pH on Lipase Production

S. No	Lipase activity of Organisms (IU/ml)	Substrates	pH		
			4	5	6
1.	*B.subtilis*	Olive Oil	2.86±0.06	2.53±0.29	3±0.23
		Coconut Oil	2.26±0.06	1.76±0.23	2.6±0.29
		Sunflower Oil	1.56±0.23	1.46±0.06	1.86±0.58
2.	*A.flavus*	Olive Oil	2.26±0.06	2.36±0.05	2.16±0.06
		Coconut Oil	1.73±0.11	1.63±0.08	1.43±0.11
		Sunflower Oil	1.4±0.23	1.2±0.17	1.1±0.16

Values are expressed as Mean ± Standard Deviation

Table 2: Effect of Temperature on Lipase Production

S. No	Lipase activity of Organisms (IU/ml)	Substrates	Temperature (°C)		
			25	37	45
1.	*B.subtilis*	Olive Oil	1.93±0.11	3.3±0.25	2.4±0.34
		Coconut Oil	1.5±0.46	2.46±0.06	1.8±0.87
		Sunflower Oil	1.2±0.10	1.5±0.64	1.2±0.57
2.	*A.flavus*	Olive Oil	3.2±0.46	1.96±0.40	1.93±0.11
		Coconut Oil	2.2±0.51	1.13±0.28	1.2±0.58
		Sunflower Oil	1.4±0.23	1.06±0.25	1.03±0.29

Values are expressed as Mean ± Standard Deviation

Table 3: Effect of Incubation Period on Lipase Production

S. No.	Lipase activity of Organisms (IU/ml)	Substrates	Incubation Period (hrs)			Lipase Productivity (IU/ml)
			12	24	48	
1.	*B.subtilis*	Olive Oil	0.56±0.26	2.53±0.48	1.63±0.46	
		Coconut Oil	0.29±0.17	2.3±0.29	1.33±0.28	1.76±0.07
		Sunflower Oil	0.09±0.03	2.06±0.40	1.16±0.23	
			24	**48**	**72**	
2.	*A.flavus*	Olive Oil	0.66±0.23	1.73±0.16	2.76±0.06	
		Coconut Oil	0.68±0.58	1.6±0.24	2.33±0.28	1.54±0.05
		Sunflower Oil	0.36±0.23	1.26±0.23	2.2±0.17	

Values are expressed as Mean ± Standard Deviation

Table 4: Effect of substrate concentration on lipase production

S. No	Lipase activity of Organisms (IU/ml)	Substrates	Temperature (°C)		
			25	37	45
1.	*B.subtilis*	Olive Oil	0.46±0.31	1.4±0.34	2.16±0.51
		Coconut Oil	0.3±0.10	1.73±0.11	2.1±0.06
		Sunflower Oil	0.2±0.12	0.68±0.55	1.93±0.61
2.	A.flavus	Olive Oil	0.6±0.34	0.96±0.40	0.23±0.46
		Coconut Oil	0.46±0.05	0.93±0.45	1.83±0.31
		Sunflower Oil	0.23±0.11	0.6±0.57	1.7±0.51

Values are expressed as Mean ± Standard Deviation

Assay of lipase production

The lipase producing bacterial strains were isolated from coconut oil mill soil and identified as *Bacillus* species. Among the different substrate tested, olive oil was found to be suitable for enhancing the lipase production by the screened *Bacillus* strains and the maximum lipase activity (0.0039 ug/ml) was recorded. The lipase activity of *Bacillus* species was maximum at pH 7 during the 24hrs of culture periods. Influence of the medium temperature indicated that the lipase production by the isolated strains was higher at 37^0 C [13] . The lipase productivity was estimated in the isolated two strains such as *Bacillus subtilis* and *Aspergillus flavus*. The results revealed that the maximum lipase productivity were noted in *B.subtilis* (1.76± 0.07) and *A.flavus* (1.54±0.05).

Optimization of lipase productivity

The lipase productivity of *B.subtilis* and *A. flavus* were analysed at various parameters such as pH, temperature, substrate concentration and incubation time.

Effect of substrate concentration

Lipase production was carried out by using different substrates such as olive oil, corn oil, gingelly oil, sunflower oil and cocconut oil.The maximum lipase activitys of olive oil concentration 0.5% for P. restricum (13 IU/ml)[14] . The lipase production of *B.subtilis* and *A.flavus* was determined under varying substrate concentration such as 5,10, and 15%.The maximum enzyme production was observed in 15% for *B.subtilis* (2.16± 0.51) and *A.flavus* (1.83± 0.31). The minimum amount of enzyme production was observed in 5% substrate concentration for *B.subtilis* (0.2± 0.12) and *A.flavus* (0.23± 0.11).

Effect of pH

The pH stability of the lipase was determined by the activity retained at different pH from 4.0 to 10.0 after 30 minutes of incubation. The pH stability curve showed that the lipase was stable at pH 6.0 to 8.0. The stability of data showed a decline in lipase activity below 6 and above 8[15].The lipase productivity were optimized using different pH ranges medium from 4,5 and 6. Maximum enzyme productivity was recorded at pH range 5 in both organism. At the same time, highest value was noted in *B.subtilis* (2.53±0.29) than *A.flavus* (2.36±0.05). Lowest lipase productivity were noted in low pH range 4 for *A.flavus* (1.4± 0.23).

Effect of temperature

The optimum temperature for lipase activity was carried out at different temperature from 25 to 60^0C. Lipase activity was found 100% at the temperature 50^0C.The activity of lipase decreased drastically at temperature above 60^0C. Lipase from *Aspergillus niger* strains have been reported to be active between 40 to 55^0C[16]. The lipase productivity were optimized using different temperature range from 25^0C , 37ºC and 45ºC. Maximum enzyme productivity were recorded at temperature range 37ºC in both organism. At the same time,the highest value was noted in *B.subtilis* (3.3±0.25) than *A.flavus* (1.96±0.11). Lowest lipase productivity were noted in low temperature range 25ºC for *B.subtilis* (1.2± 0.10).

Effect of incubation time

Lipolytic activity was detectable early in the incubation period. The maximum lipase activity was obtained after 5 days of incubation, while maximum cell mass was detected at 6 days after which the culture reached the stationary phase . The maximum lipase activity from *R.oligosporus* was obtained after 3 days[17]. Different hours of incubation time was maintained for lipase production by *B.subtilis* (ie) 12,24,48 hrs and *A.flavus* (i.e) 24,48,72 hrs. The maximum amount of enzyme production was observed in 48 hrs for *B.subtilis* (1.63±0.46) and 72 hrs for *A.flavus* (1.73±0.16). The minimum amount of lipase production was obtained in 12hrs for *B.subtilis* (2.53±0.48) and 24 hrs for *A.flavus* (0.68±0.58).

CONCLUSION

The lipase produced by *B.subtilis* was partially purified and characterized for optimum pH and temperature. *B.subtilis* can be recognized for lipase production for industrial application and much more research to optimize the fermentative process in order higher lipase production through this strain.

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