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LIPASE PRODUCTION FROM *Aspergillus niger* BY SOLID STATE FERMENTATION AND ITS APPLICATIONS

SUMATHY R[1*] AND VIJAYALAKSHMI.M[2]

[1]Dwaraka Doss Goverdhan Doss Vaishnav College, Gokulbagh. No. 833, E.V.R. Periyar Highroad, Arumbakkam, Chennai.
[2]Department of Biotechnology, Dr. M.G.R. Educational and Research Institute University, Maduravoyal,Chennai.

ABSTRACT

This study reviews the application of Solid State Fermentation (SSF) to the production of lipase enzyme. Lipase production has been investigated by SSF of agro-industrial wastes like coconut oil cake, wheat rava, wheat bran, gingelly oil cake etc., by *Aspergillus niger* MTCC 2594. Highest enzyme production was obtained by combination of wheat bran, coconut oil cake, wheat rava (5:2:1), with moisture content of 1:1.5, 250µl of spore suspension at 30°C. Media supplementation with nitrogen source as commercial casein (0.5%) and Palm oil increased enzyme production, to 559U/g DS (Dry substrate) and with a productivity rate of 7.76 Units per hour. Initial studies on enzymatic hydrolysis of different vegetable oils by lipase produced by SSF showed that palm oil was hydrolyzed efficiently to 74.49% after 72 hours of reaction time and with 50U of lipase.

KEYWORDS: Lipase, *Aspergillus niger,* Solid state fermentation (SSF)

SUMATHY R
Dwaraka Doss Goverdhan Doss Vaishnav College, Gokulbagh.
No. 833, E.V.R. Periyar Highroad, Arumbakkam, Chennai.

INTRODUCTION

Lipases (triacylglycerols acylhydrolases) (E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. They catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface [1] and do not hydrolyze dissolved substrates in the bulk fluid. Lipases are subclass of esterases and it can split the esters of glycerine and fatty acids like triolein and tripalmitin.Lipases are serine hydrolases and display less activity in aqueous solutions containing soluble substrates. The interest in lipase has grown over the last few years due to their excellent catalytic properties [2] and their diverse industrial applications, for example,additives in detergents, the elaboration of dietetic foods for use in the food industry, obtaining bioactive molecules in the pharmaceutical industry and pure optical compounds in chemical synthesis processes [3,] as well as modifications of fats and lipids by hydrolysis and esterification reactions [4.] Lipases occur widely in bacteria, yeasts and fungi [5–7]. In recent literature reported that about 90% of lipases are obtained from microbial sources but among fungi, *Aspergillus niger* is considered to be the best extracellular enzyme producers. The term "Solid State Fermentation"(SSF) refers to the growth of microorganisms on solid materials in the absence of free liquid.SSF has many advantages over submerged fermentation (SmF), including an economical use of space that is required for fermentation, simplification of the fermentation media,superior yields and no requirement for complex machinery[8]. Although the vast majority of the literature on SSF refers to fungal systems, there are actually very few reports on lipase production in SSF by *A. niger* [9,10.] The objective of this study was the production of lipase by *A. niger* by SSF and characterization of the enzyme in regards to its stability in relation to temperature, pH and the optimization of the temperature and pH conditions for obtaining higher lipase activity.

MATERIALS AND METHODS

Microorganisms and maintenance of culture

Aspergillus niger was maintained by subculturing on to Czapek Dox Agar slants, It was grown at 30 ºC for 5 days and then stored at 4 ºC.

Substrates

Coconut oil cake (COC), Gingelly oil cake (GOC), Wheat bran (WB), Wheat rava (WR) were used as substrates.

Fermentation

10g of different substrates were taken in a series of 250mL Erlen-Meyer flasks and moistened with 15mL of water to an initial moisture content of 70% w/v and sterilized at 15 lbs/in^2 for 20min.

Preparation of spore suspension and inoculation

To the slants, 2mL of sterile distilled water was added and spores were disturbed by gentle scraping off to obtain a dense spore suspension. 250µL of spore suspension was added to each flask and mixed well using a sterilized spatula.

Incubation

The contents of the flasks after mixing were incubated at 30^{o}C under static condition for 5 days.

Extraction of the enzyme

At the end of fermentation period, 1g of moldy substrate was homogenized with 10mL of 0.1M phosphate buffer (pH 7.0) using mortar and pestle. Then it was filtered with a muslin cloth and the filtrate was centrifuged at 10,000rpm for 20min. The supernatant was used as enzyme source.

Titrimetric assay

Titrimetric method measures the rate of neutralization of sodium hydroxide by the release of fatty acids from the triglycerides as a function of time and hence the quantification of free fatty acids released is an estimate of lipase activity. Ota and Yamada, 1962[11] have

reported a titrimetric method using olive oil as a substrate and polyvinyl alcohol as an emulsifier.

Lipase assay

5 mL of emulsion and 4 mL of phosphate buffer (pH 7.0) was added to 100mL Erlen-Meyer flasks and mixed well. The flasks were preincubated at 37^{o}C for 10min.After preincubation, 1mL of the enzyme was added and incubated at 37^{o}C for 20min.20 mL of acetone was added to stop the reaction. Adding 1mL of the enzyme after addition of acetone served as control. The release of fatty acid was estimated by titration against 0.05 N NaOH using phenolphthalein as indicator. Appearance of permanent pale pink color was the end point. One unit of lipase activity is defined as one micromole of free fatty acid liberated per mL of enzyme per minute under standard assay conditions and is expressed as U/mL/min.

$$\text{Lipase activity (Units/ml/min)} = \frac{\text{Volume of NaOH consumed x Normality of NaOH x 1000}}{\text{Time of incubation (20 min) x Volume of enzyme}}$$

Volume of NaOH consumed = Volume of NaOH Consumed by (Test - Control).

Protein estimation

Estimation of protein content in the sample is used to find the specific activity of the enzyme. Here amount of protein present in crude enzyme was done by Lowry method (Lowry *et al.,* 1951).

Optimization studies

Consecutive optimization studies were carried out by varying the initial moisture content, inoculum concentration ($0.54x10^7$ – $4.3x10^7$ CFU/g substrate), inducers (5%v/w). The effect of environmental variables such as pH and temperature was determined by altering the initial pH of the medium from 6.0 - 8.0 and varying the incubation temperature (25^0C, 30^0C, 37^0C). Using the optimal parameters, the time coarse studies on lipase production were carried out.

Effect of various substrates on lipase production by A. niger

Different substrates like coconut oil cake, gingelly oil cake, wheat bran, wheat rava, rice bran, cotton oil cake etc., were used. 10g of each substrate was weighed into 250mL conical flask, 15mL of distilled water was added for moisture and autoclaved. After cooling to room temperature, 250µl of spore suspension was inoculated into each flask and mixed well. After incubation lipase enzyme assay was performed at 24. 48, 72, 96, 120 hours and the activity was calculated.

Effect of moisture content on lipase production by A. niger

Effect of moisture content on the growth and production of lipase enzyme were observed by mixing the different volumes of distilled water with the substrates (WB, WR, COC), autoclaved, inoculated and incubated at room temperature. The enzyme activity was assayed and calculated.

Effect of pH

The trisubstrate medium was incubated at different pH like 6, 7 and 8. Activity of lipase enzyme produced was assayed at 48, 72, 96, 120 hours, per gram of wet and dried substrate were calculated.

Effect of Temperature

The trisubstrate medium was incubated at different temperatures like 25^{o}C, 30^{o}C, 37^{o}C. Activity of *lipase* enzyme produced were assayed at 48, 72, 96, 120 hours, per gram of wet and dried substrate were calculated.

Effect of oil inducers

0.5% of different vegetable oils and fish oils were added to the trisubstrate medium and activity of lipase enzyme produced were assayed at 48, 72, 96, 120 hours and recorded.

Effect of nitrogen sources

0.1% of different nitrogen sources like cornsteep liquor, urea, soybean meal, commercial casein, commercial ammonium

sulphate, were added to the trisubstrate medium. Activity of lipase enzyme produced were assayed at 48, 72, 96, 120 hours and recorded.

APPLICATION STUDIES OF LIPASE

Effect of solvents in hydrolysis

The reaction mixture consisted of 1.25 mL of hexane, heptane, DMSO, Benzene + 13.75mL of 0.1M Phosphate buffer pH 7.0 + 1g of palm oil + 5mL of enzyme solution (50U) was taken in a 100mL volumetric flask. The flasks were incubated at 37^{O}C, 150rpm for 72 h. The reaction was stopped by adding 10mL of acetone, few drops of phenolphthalein was added and titrated against 0.1N alcoholic KOH. The end point is the appearance of pink color. The acid value and hydrolysis ratio was calculated.

RESULTS AND DISCUSSION

LIPASE PRODUCTION BY SOLID STATE FERMENTATION

In the present study, *Aspergillus niger* showed the maximum lipase activity was obtained after 96 hrs of incubation with the combination of substrates wheat bran, coconut oil cake and wheat rava (467 U/g). The amount of protein produced by *Aspergillus* niger 20 mg/ml

OPTIMIZATION STUDIES

Effect of various substrates on lipase production by A. niger by SSF

The effect of different substrates such as gingelly oil cake (GOC), coconut oil cake (COC), wheat bran (WB), wheat rava (WR) on lipase production is shown in table. Among the different substrates, COC and WR (fine) were found to be the best and the lipase activities were 413 U/g dry substrate and 323 U/g wet substrate at 72h respectively. (Table 5.1, Fig 5.1) The universal suitability of WB may be attributed to the presence of sufficient nutrients and is able to remain loose even in moist conditions, thereby providing a large surface area**.** Oil cakes are rich in fibre and have high concentration of non-starch polysaccharides (NSP). Oil cakes such as palm kernel oil cake, sesame oil cake and coconut oil cake contain 14-20% of crude protein. However, GOC contains 40-50% of crude protein. Fat content of the oil cake is dependent on the oil extraction method. They generally have <2-3% fat**.** Coconut oil cake serves as a good substrate, which not only provides nutrients but also good surface area for proper growth and aeration [12]. The higher enzyme yields in the mixed substrate (Table 5.2, Fig 5.2) indicate that the trace elements in the different substrates in the mixed state enabled the organism to yield more by favorably influencing its biochemical pathways for lipase production. Further optimization studies were carried out using this substrate by altering the different parameters one at a time, the moisture content, oil inducers, pH, temperature, nitrogen supplements for maximal lipase production.

Table 5.1
Effect of different substrates on lipase production

S. No	Substrates	Lipase activity (U/L/min)							
		48 hours		72 hours		96 hours		120 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	Wheatbran	50	102	70	182	70	214	100	193
2.	Gingelly oil cake	65	184	80	279	123	288	110	211
3.	Coconut oil cake	69	292	85	283	**131**	**413**	95	248
4.	Wheat rava (Coarse)	58	176	75	205	80	283	75	217
5.	Wheat rava (Fine)	60	180	79	244	100	323	85	229

DS – Dry Substrate; WS – Wet Substrate

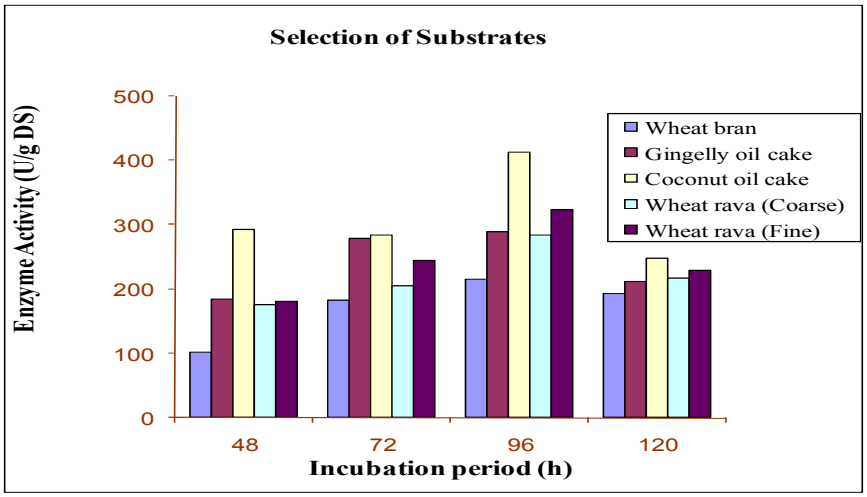
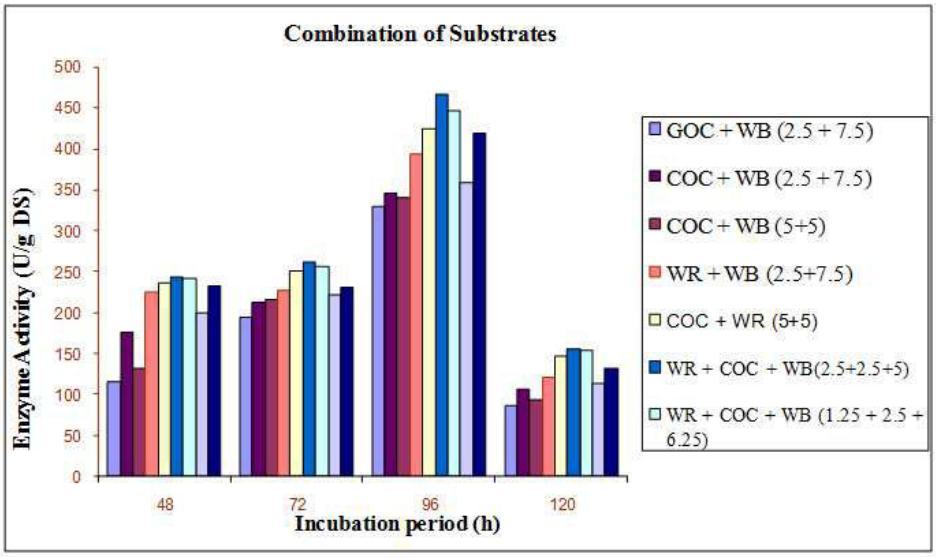


Figure. 5.1
Selection of Substrates

Table 5.2
Effect of combination of substrates on lipase production by A. niger by solid state fermentation

S. No	Substrates	Lipase activity (U/mL/min)							
		48 hours		72 hours		96 hours		120 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	GOC + WB (2.5 + 7.5)	41	116	55	194	102	329	75	86
2.	COC + WB (2.5 + 7.5)	50	176	62	213	112	347	90	107
3.	COC + WB (5 + 5)	43	132	58	216	111	341	80	93
4.	WR + WB (2.5 + 7.5)	63	225	70	227	115	393	103	120
5.	COC + WR (5 + 5)	70	236	79	250	120	424	108	146
6.	WR + COC + WB (2.5 + 2.5 + 5)	75	244	82	262	**132**	**467**	115	156
7.	WR + COC + WB (1.25 + 2.5 + 6.25)	72	241	80	256	127	446	113	154
8.	GOC + COC + WB (5 + 2.5 + 5)	58	200	66	221	114	359	96	113
9.	WR + COC + WB (6.25 + 2.5 + 1.25)	66	233	75	230	118	419	105	132

DS – Dry Substrate; WS – Wet Substrate;WR - Wheat Rava; WB - Wheat Bran; COC - Coconut Oil Cake; GOC - Gingelly Oil Cake



Effect of moisture content on lipase production by A. niger by SSF

An initial moisture content of 1:1.5 was found to be optimal and the activity was 429 U/g dry substrate at 96h. An increase or decrease in moisture content significantly affects the lipase production. The optimal moisture content for lipase production using this substrate is 1:1.5 (substrate w/v). Moisture of the substrate and humidity, aeration and agitation in solid state fermentation are other important factors which govern SSF. Moisture level has great impact on the physical properties of the solid substrate. Water is very present in very limited amount in the solid state fermentation system and thus optimum moisture content is important as it determines the productivity of a solid state fermentation process. Moisture content in solid state fermentation can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and liberation through fungal metabolism and also due to environmental factors. The moisture content in the substrate can also depends on the types of microorganisms and the substrate used in the solid state fermentation. At the same time, the amount of moisture content also varies depending upon the water binding characteristics of the substrate. Moisture level lower than the optimum level does not allow a good diffusion of solutes and gas and the cell metabolism slows down. Moisture level higher than optimum decrease the porosity of the solid substrate, prevents oxygen penetration, promotes development of stickiness, increases the chances of contamination [13]

Table 5.3

Effect of moisture content on lipase production by A. niger by solid state fermentation

S. No	Moisture content	Lipase activity (U/mL/min)							
		48 hours		72 hours		96 hours		120 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	1 : 0.5	51	132	58	157	108	176	82	104
2.	1 : 1.0	57	192	63	200	116	416	96	113
3.	1 : 1.5	74	240	82	271	**130**	**429**	113	154
4.	1 : 2.0	62	214	78	235	118	423	106	122

Note: DS – Dry Substrate; WS – Wet Substrate

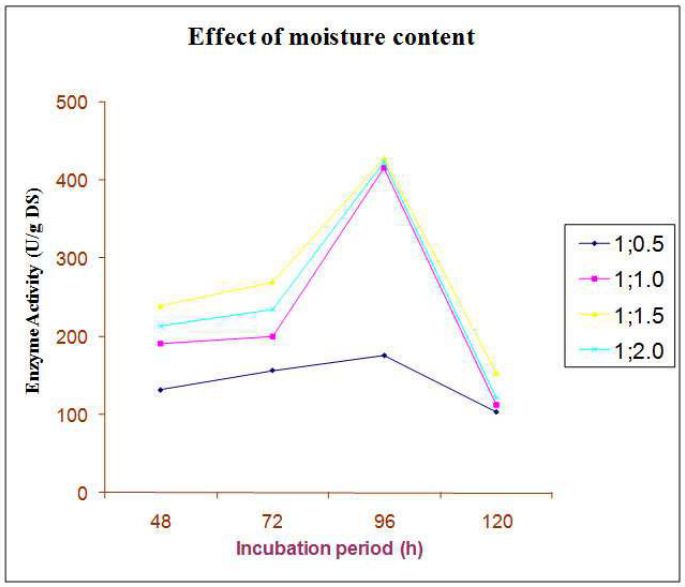


Figure 5.3
Effect of Moisture Content on Lipase Production

Effect of pH on lipase production by A. niger by SSF

The effect of pH on lipase production was studied. Maximal lipase production with an activity of 467 U/g dry substrate was obtained with the control system. The determination of a suitable pH of the fermentation medium is critical for the success of solid state fermentation system [13]

Table 5.4
Effect of pH on lipase production by A. niger by solid state fermentation

S. No	Ph	Lipase activity (U/mL/min)					
		48 hours		72 hours		96 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	Distilled water	72	209	86	254	**136**	**467**
2.	6	33	105	46	157	50	196
3.	7	68	186	79	236	104	385
4.	8	55	158	62	215	82	298

Note: DS – Dry Substrate; WS – Wet Substrate

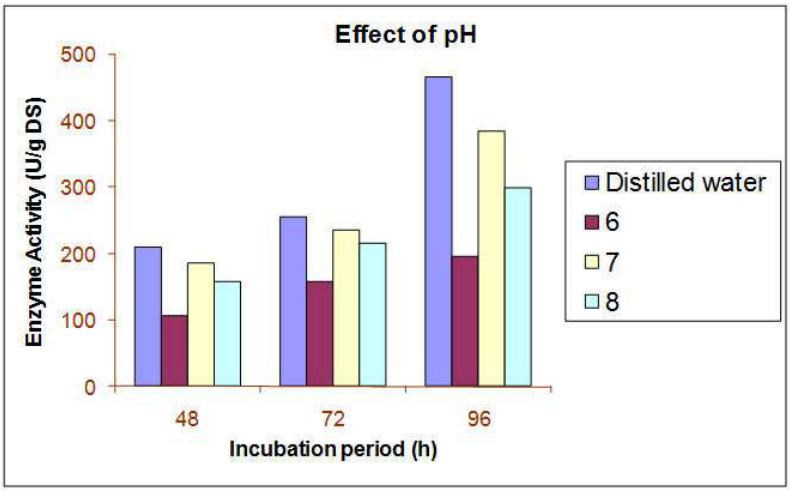


Figure. 5.4
Effect of pH on lipase production SSF

Effect of temperature on lipase production by A. niger by SSF

The effect of fermentation temperature (25°C, 30°C, 37°C) on lipase production was studied. Optimal lipase activity of 461 U/g dry substrate was obtained at 30°C at 96h of growth, while the enzyme activity at 28°C and at 37°C was found to be lower than that obtained at 30°C at 96h.The maintenance of constant temperature during the entire period of fermentation is essential in solid state fermentation system because of the large amount of metabolic heat generation and mass transfer effects. Temperature plays an important role in solid state fermentation as it significantly affects the germination of spores. However, as the spore germinates, the optimum temperature for mycelial propagation may change[14]. In the present study, 30°C proved to be the best temperature for the enzyme synthesis.

Table 5.6
Effect of temperature on lipase production by A. niger by SSF

S. No	Temperature (°C)	Lipase activity (U/mL/min)					
		48 hours		72 hours		96 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	25	66	227	80	237	132	447
2.	30	73	241	84	256	**136**	**461**
3.	37	52	211	78	201	106	433

Note: DS – Dry Substrate; WS – Wet Substrate

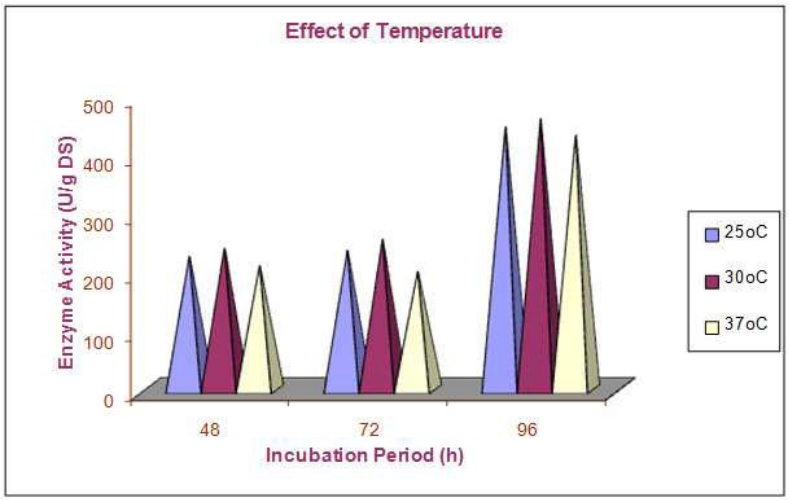


Figure. 5.6
Effect of temperature on lipase production

Effect of oil inducers on lipase production by A. niger by SSF

Supplementation of the solid substrate medium with gingelly oil, coconut oil, sunflower oil, palm oil and olive oil at a concentration of 0.5% v/w was observed. Palm oil induced about 10% increase in lipase production when compared with that of control. (Table 5.7, Fig 5.7) Olive oil in combination with nitrogen sources increased the lipase production but the presence of carbon sources in the olive oil significantly decreased both the lipase activity and biomass content. This result may be due to the limited availability of these carbon sources to the fungus in the media supplemented with oil [15]

Table 5.7
Effect of oil inducers on lipase production by A. niger by SSF

S. No	Oil inducers	Lipase activity (U/mL/min)							
		48 hours		72 hours		96 hours		120 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	Control	72	231	82	237	136	462	114	260
2.	Gingelly oil	16	43	106	448	111	319	96	276
3.	Sunflower oil	14	36	152	476	104	283	89	264
4.	Palm oil	22	58	**170**	**541**	164	510	144	491
5.	Coconut oil	86	116	136	442	121	297	122	316
6.	Olive oil	24	62	58	215	76	259	83	290

Note: DS – Dry Substrate; WS – Wet Substrate

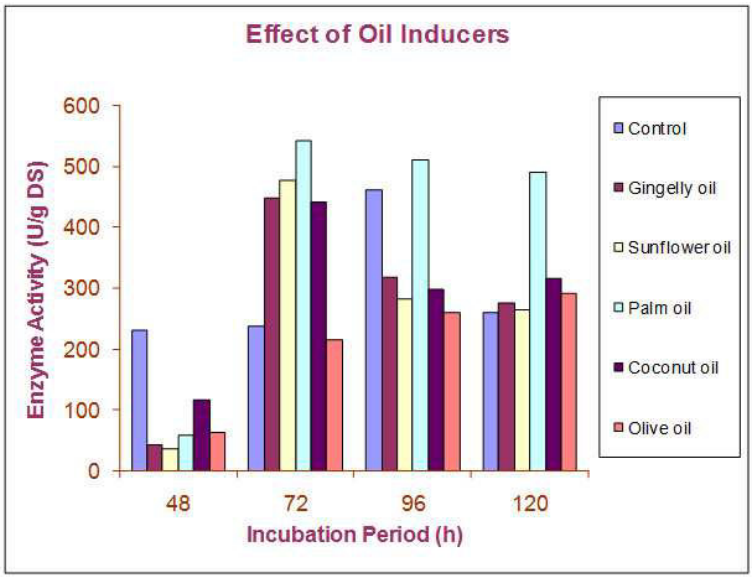


Figure. 5.7
Effect of oil inducers on lipase production by SSF

Effect of nitrogen inducers on lipase production by A. niger by SSF

Trisubstrate medium supplemented with different nitrogen sources (0.1%) like Cornsteep liquor, soybean meal, Commercial ammonium sulphate, Commercial casein, Urea. Medium induced with 0.1% commercial casein has shown 559 U/gDS, (Table 5.8), where as Rodriguez *et al.*, 2006 has reported a similar observation using casein with *Penicillium restrictum* in SSF. On the other hand Lima et al., found that lipase production in *P. aurantiofriseum* was stimulated using ammonium sulphate.

Table 5.8
Effect of nitrogen inducers on lipase production by A. niger in SSF

S. No	Inducers	Lipase activity (U/mL/min)							
		48 hours		72 hours		96 hours		120 hours	
		U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS	U/g WS	U/g DS
1.	Corn steep liquor	55	168	80	254	125	411	25	86
2.	Soybean meal	20	61	85	250	40	133	20	88
3.	Commercial casein	40	123	**175**	**559**	160	481	90	242
4.	Urea	40	121	125	317	55	186	20	80
5.	Commercial Ammonium sulphate	70	236	70	247	75	269	35	123
6.	Control	75	278	85	292	135	457	100	314

Note: DS – Dry Substrate; WS – Wet Substrate

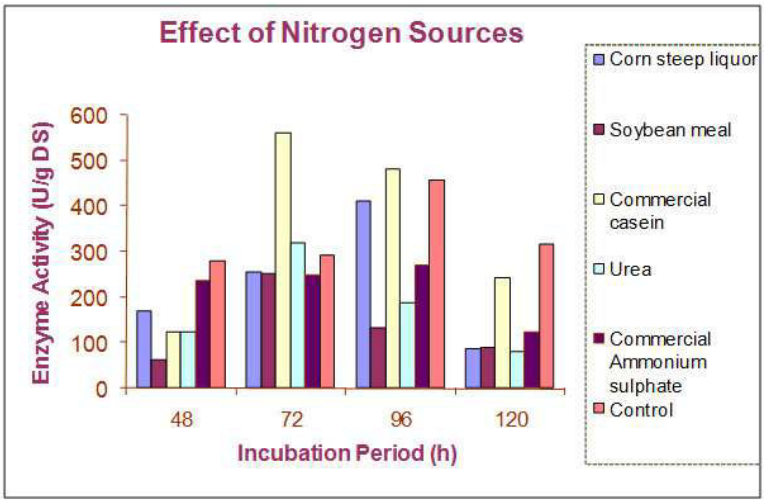


Figure. 5.8
Effect of nitrogen inducers on lipase production by A. niger by SSF

APPLICATION OF ENZYME

Effect of Solvents

The effect of different additives has been investigated in order to achieve maximum hydrolytic rates by *Aspergillus niger* lipase. However, the addition of different solvents such as Hexane, Heptane, DMSO and Benzene are not observed to be efficient for the hydrolytic reaction. This observation is quite economical aspect of enzyme hydrolysis and imparts simplicity in hydrolytic operations in an industrial scale.

Table: 5.11
Effect of Solvents in Palm oil Hydrolysis

S. No	Solvents	Hydrolysis percentage at 72h
1.	Phosphate buffer	**74.49**
2.	Hexane	46.79
3.	Heptane	37.55
4.	DMSO	70.04
5.	Benzene	12.89

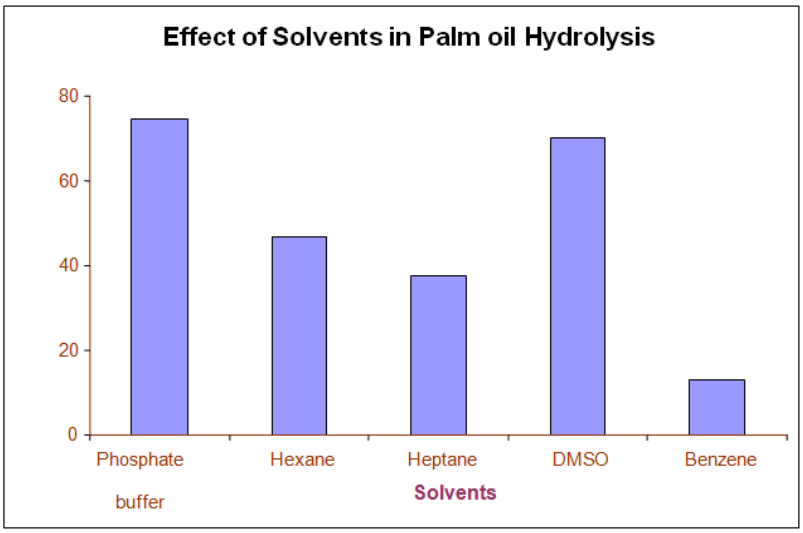


Figure. 5.10
Effect of Solvents

CONCLUSION

The yield achieved under optimum growth conditions is higher than those reported earlier by various fungi under submerged conditions. The substrates used are easily available in abundance as oil-mill wastes in all seasons throughout the year. By using cheaper substrates, we have tried to lower down the input cost for enzyme production, thereby making it possible to be used at a large scale in industries. Enzymatic hydrolysis of palm oil by using the lipase produced by SSF was studied. The effect of different solvents was studied and 0.1M phosphate buffer at pH 7 showed the best result. Further optimization studies like, effect of solvent concentration, enzyme concentration, additives are to be performed.

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