

Optimization of Process Parameters for the Production of Lipase in Solid State Fermentation by *Yarrowia Lipolytica* from Niger Seed Oil Cake (*Guizotia Abyssinica*)

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Abstract

The production of extra cellular lipase in Solid State Fermentation (SSF) using *Yarrowia lipolytica* NCIM 3589 with Niger seed oil cake (*Guizotia abyssinica*) has been made. Different parameters such as incubation time, inoculum level, initial moisture content, carbon level and nitrogen level of the medium were optimized. Screening of various process variables has been accomplished with the help of Plackett–Burman design. The maximum lipase activity of 26.42 units per gram of dry fermented substrate (U /gds) was observed with the substrate of Niger seed oil cake in four days of fermentation. STATISTICA 6.0 was used for implementing Plackett–Burman design.

Keywords: Lipase; Niger seed oil cake; Optimization; Plackett–Burman design; *Yarrowia lipolytica*

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes. They hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. In recent years, there has been an increasing interest in the study of lipases mainly due to their potential applications as medicines (digestive enzymes), food additives (flavour modifying enzymes), clinical reagents (glyceride-hydrolysing enzymes) and cleaners (detergent additives) (Sharma et al., 2001). Additionally, a promising application field for lipases is in the biodegradation of plastics such as polyhydroxyalkanoates (PHA) and polycaprolactone (PCL) (Jager et al., 1995; Mochizuki et al., 1995). Lipases would be economically manufactured in solid state fermentation.

Solid state fermentation (SSF) is defined as the fermentation of solids in the absence of free water; however, the substrate must possess enough moisture to support the growth and metabolism of microorganisms. Recently, several reports have been published indicating the application of this culture in upgrading the food and industrial wastes and in the production of fine chemicals and enzymes. The utilization of by-products and wastes from food and industrial sources has several advantages over submerged fermentation such as superior productivity, simple techniques, reduced energy requirements, low wastewater output, improved product recovery and the reduction in production costs (Ashok, 2003). In SSF, any type of substrate, including industrial wastes, could be used to enhance the production of enzymes because of their richness in fatty acids, triacylglycerols and /or sugars. The use of cheap raw materials would diminish the operating costs of the process. Moreover,

total capital investment for lipase production has been reported to be significantly lower in solid state fermentation than in submerged fermentation (Castilho et al., 2000). Most studies on lipolytic enzymes production with bacteria, fungi and yeasts have been performed in submerged fermentation; however, there are only few reports on lipase synthesis in solid state fermentation. In recent years, considerable research has been carried out using agricultural wastes, which are renewable and abundantly available to produce value-added products. For example babassu oil cake (Gombert et al., 1999), olive cake and sugar cane bagasse (Cordova et al., 1998), gingelly oil cake (Kamini et al., 1998), wheat bran (Mahadik et al., 2002), rice bran (Rao et al., 1993) and *Jatropha curcas* seed cake (Mahanta et al., 2008) have been used as the substrates for lipase production.

Niger seed oil cake (*Guizotia abyssinica*) is cultivated in tropical countries and is quite expensive as it is imported usually from Ethiopia and India. Niger seed oil has been identified as a potential biodiesel crop because of the presence of 50–60% the oil called biocrude, which can be converted into biodiesel by chemical or lipase mediated esterification (Gubitz et al., 1999). The niger oil cake is either used as an animal feed or disposed to the soil as the waste materials. Since they are rich in fatty acids, triacylglycerols and / or sugars and other nutrients, they can serve as the potential substrates for lipase production using SSF. Hence an attempt is made in this paper to utilize the niger seed oil cake as a substrate for the production of lipase by solid state fermentation. It was under taken to optimize the key process variables, including incubation time, inoculum level, ini-

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Received January 22, 2010; **Accepted** February 27, 2010; **Published** February 27, 2010

Citation: Imandi SB, Karanam SK, Garapati HR (2010) Optimization of Process Parameters for the Production of Lipase in Solid State Fermentation by *Yarrowia lipolytica* from Niger Seed oil Cake (*Guizotia Abyssinica*). J Microbial Biochem Technol 2: 028-033. doi:10.4172/1948-5948.1000019

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Citation: Imandi SB, Karanam SK, Garapati HR (2010) Optimization of Process Parameters for the Production of Lipase in Solid State Fermentation by *Yarrowia lipolytica* from Niger Seed oil Cake (*Guizotia abyssinica*). *J Microbial Biochem Technol* 2: 028-033. doi:10.4172/1948-5948.1000019

tial moisture content, carbon level, and nitrogen level of the medium for the production of lipase using this oil cake under SSF.

Materials and Methods

Substrate

Niger seed oil cake (*Guizotia abyssinica*), procured from a local oil extracting unit of Vizianagaram, India was used as the substrate. It was dried at 60°C for 72 h to reduce the moisture content to around 5 %, and ground to the desired size (2 mm).

Chemical analysis of niger oil cake

Niger press cake is very dark in color. It contains 24–34% protein, 4–14% oil, 8–24% crude fiber, 20–28% carbohydrates and 8–12% ash (Nasirullah et al., 1982; Seegeler, 1983). The cake has more or less a balanced essential amino acid composition. It is, however, deficient in lysine and threonine (Mehansho et al., 1973). The cake contains about 5% N, 2% P₂O₅ and 1.5% K₂O (Weiss, 1983).

Microorganism

Yarrowia lipolytica NCIM 3589, obtained from National Chemical Laboratory, Pune, India, was used throughout the study.

Growth conditions

The culture was maintained on MGYP slants having the composition (%): malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5 and agar agar 2.0. The pH of the medium was adjusted to 6.4–6.8 and culture was incubated at 30°C for 48 h. Subculturing was carried out once in 2 weeks and the culture was stored at 4°C.

Inoculum preparation

The *Yarrowia* strain was cultivated in a medium containing peptone 5 g, yeast extract 3 g and sodium chloride 3 g per liter of distilled water. The cells were cultivated in this medium at 30°C on a shaker at 200 rpm for 24 h (Imandi et al., 2008).

Media preparation

Ten grams of substrate was weighed into a 250 ml Erlenmeyer flask and to this a supplemental salt solution was added to the desired moisture level. The composition of the salt solution was as follows (% w/w): KH₂PO₄: 0.1; MgSO₄.7H₂O: 0.05; CaCl₂: 0.01; NaCl: 0.01; H₃BO₃: 0.00005; CuSO₄.5H₂O: 0.000004; KI: 0.00001; FeCl₃.4H₂O: 0.00002; ZnSO₄.7H₂O: 0.00004; MnSO₄.H₂O: 0.00004 (Imandi, 2009). Glucose and urea were taken as carbon and nitrogen sources respectively as per the Plackett–Burman design. The contents were thoroughly mixed and autoclaved at 121°C (15 psi) for 20 min.

Solid state fermentation

The sterilized substrate including media as shown in the above Section 2.6 was inoculated with 2 ml of inoculum. The contents were mixed thoroughly and incubated in a slanting position at 30°C. All the experiments were carried out in duplicate and samples were withdrawn after 4 days of incubation.

Extraction of lipase

The crude enzyme from the fermented material was recovered by simple extraction method. For this, the fermented sub-

strate was mixed thoroughly with 100 ml of distilled water and the contents were agitated for 1 h at the room temperature in a rotary shaker at 150 rpm. At the end of extraction, the liquid was filtered off through Whatman No.1 filter paper and the resulting clear filtrate was used for lipase assay.

Lipase assay

Lipase activity was assayed by the colorimetric method of Winkler and Stuckmann, (1979) by measuring the micromoles of 4–nitrophenol released from 4–nitrophenyl palmitate. One unit of lipase activity was defined as the enzyme amount that releases 1 μmol of *p*–nitrophenol per minute under assay conditions. Enzyme activity was expressed as units/gram of the initial dry substrate (U/gds).

Experimental design and optimization

Plackett–burman design

The Plackett–Burman (Plackett and Burman, 1946) statistical experimental design is a versatile method for screening the important variables. The total number of experiments to be carried out is $K + 1$, where K is the number of variables. Each variable is represented at two levels, high and low denoted by (+) and (–) respectively. The statistical software package STATISTICA 6.0 (Stat-Ease Inc., Tulsa, OK, USA) was used for analyzing the experimental data.

The effect of each variable on lipase activity was calculated by using the following equation:

$$E_{(xi)} = \frac{\sum Y_{(+i)} - \sum Y_{(-i)}}{L / 2} \quad (1)$$

where $E_{(xi)}$ is the effect of levels of the tested variables, $Y_{(+i)}$ and $Y_{(-i)}$ are the lipase activity from the experimental runs in which the variables being tested are added to the medium at their maximum and minimum levels respectively and L is the number of experiments carried out. When the value of concentration effect ($E_{(xi)}$) of the tested variable is positive, the influence of the variable is greater at the high concentration, and when it is negative, the influence of the variable is greater at the low concentration.

Results and Discussion

Effect of incubation time

The incubation time is an important factor for the production of extracellular lipase by the microorganisms (Shirazi et al., 1998). In this study with *Yarrowia lipolytica* NCIM 3589, lipase activity increased from 24 h onwards and reached maximum production (10.18 U/gds) after 96 h of incubation (Table 1). At longer incubation periods, the lipase activity decreased which might be due to the depletion of nutrients, accumulation of toxic end products, and the change in pH of the medium, or loss of moisture. Various investigators have reported different

Incubation time (hrs)	Lipase activity (U/gds)
24	0.94
48	2.73
72	5.62
96	10.21
120	5.47

Table 1: Effect of incubation time on lipase activity.

incubation periods for optimal lipase production. Maximum lipase activity was achieved after 48 h of incubation by Ul-Haq et al. (2002) with *Rhizopus oryzae*. Cordova et al. (1998) reported the maximum lipase activity with *R.pullis* after 24 h of incubation using the mixture of olive oil cake and sugar cane bagasse as the substrate. In another study, the maximum lipase activity by *Aspergillus niger* occurred after 5 days of incubation (Mahadik et al., 2002). Benjamin and Pandey, (1997) obtained maximum production of lipase by *Candida rugosa* after 3 days of incubation.

Effect of inoculum level

Different levels of the inoculum were tried to study their effect on lipase activity (Figure 1) so as to find an optimum inoculum level in the fermentation process. A lower inoculum may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass leading to the poor product formation (Mudgetti, 1986). In our study, the maximum lipase activity (10.21 U/gds) was obtained with 2 ml (20 % v/w) inoculum level. Some researchers have used different levels of inoculum for lipase production employing different microorganisms. Maximum lipase production by *Rhizopus oligosporus* was achieved by Ul-Haq et al. (2002) with 1 ml of inoculum. An inoculum concentration of 1.07×10^8 spores/ 10 g of substrate was found to be optimal for lipase production by

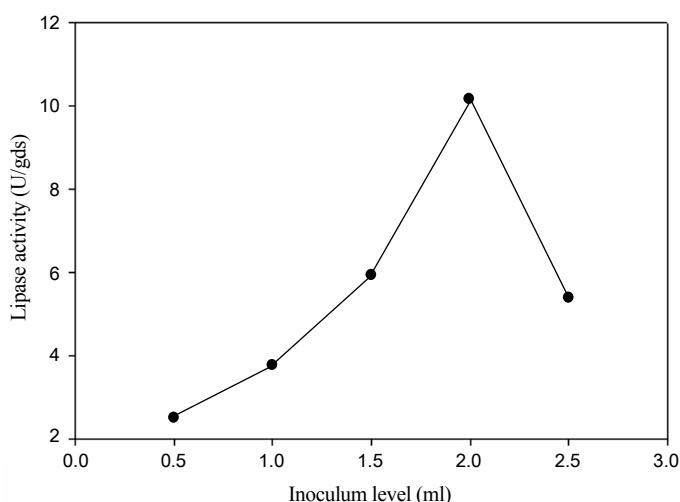


Figure 1: Effect of inoculum level on lipase activity.

Aspergillus niger (Kamini et al., 1998). Diaz et al. (2006) used an inoculum concentration of 3×10^7 spores/g of dry substrate for maximum lipase production with *R. homothallicus*.

Effect of initial moisture content

Moisture content of substrate plays a vital role for the microbial growth and for effecting biochemical activities in SSF (Babu and Rao, 2007). The influence of initial moisture content on lipase production by *Yarrowia lipolytica* was presented in Figure 2. Initial moisture content of 60 %v/w had shown maximum lipase production. Lipase production was decreased at higher moisture content probably because of decrease in porosity and hence decrease in gaseous exchange leading to suboptimal growth and less enzyme production as indicated by Silman et al., 1979 and at lower moisture content probably low moisture content causes reduction in the solubility of nutrients of the substrate, lowers the degree of swelling, and creates higher water tension as suggested by Perez-Guerra et al., 2003. Mahanta et al. (2008) reported initial moisture content at 50 % of substrate as ideal for lipase production using *Jatropha curcas* seed cake as the substrate.

Identification of important nitrogen source using Plackett-Burman design

A total of ten nitrogen sources were screened through twelve

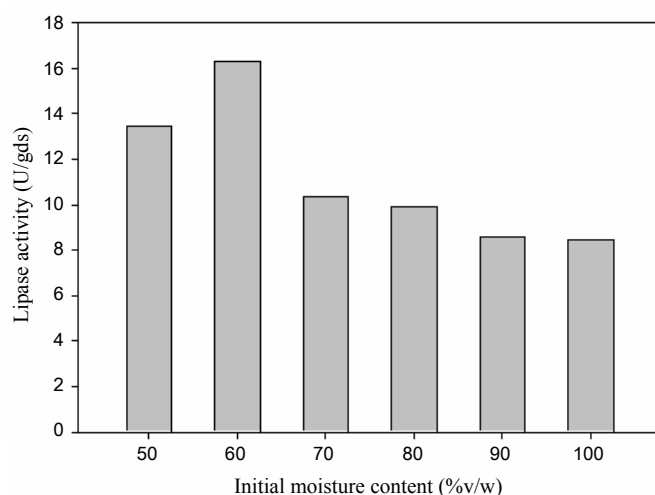


Figure 2: Effect of initial moisture content on lipase activity.

Run no.	Levels	Variables (% w/w)											Lipase activity (U/gds)
		Soyabean meal	Yeast extract	Peptone	Casein	Malt extract	Urea	NH ₄ H ₂ PO ₄	(NH ₄) ₂ SO ₄	NH ₄ Cl	NH ₄ NO ₃	DV	
	+	5.0	5.0	5.0	5.0	5.0	3.0	3.0	3.0	3.0	3.0	-	
	-	1.0	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.5	-	
1		+	-	+	-	-	-	+	+	+	-	+	16.56
2		+	+	-	+	-	-	-	+	+	+	-	12.67
3		-	+	+	-	+	-	-	-	+	+	+	15.75
4		+	-	+	+	-	+	-	-	-	+	+	19.98
5		+	+	-	+	+	-	+	-	-	-	+	12.35
6		+	+	+	-	+	+	-	+	-	-	-	17.28
7		-	+	+	+	-	+	+	-	+	-	-	18.99
8		-	-	+	+	+	-	+	+	-	+	-	14.26
9		-	-	-	+	+	+	-	+	+	-	+	16.97
10		+	-	-	-	+	+	+	-	+	+	-	17.48
11		-	+	-	-	-	+	+	+	-	+	+	16.14
12		-	-	-	-	-	-	-	-	-	-	-	16.94

DV: Dummy Variable

Table 2: Plackett-Burman experimental design matrix for screening of various nitrogen sources for lipase production.

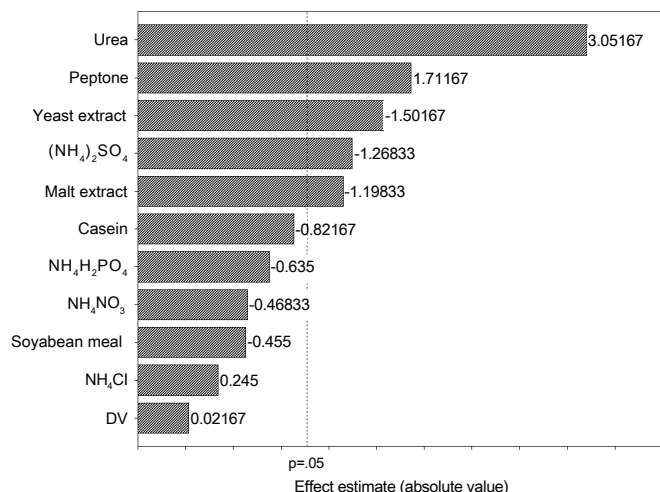


Figure 3: Pareto graph showing effect of various nitrogen sources on lipase production based on the observation of Plackett–Burman design.

Variables	Effect (E)	t-value	p-value
Soyabean meal	-0.45500	-6.2695	0.100694
Yeast extract	-1.50167	-20.6917	0.030743 ^a
Peptone	1.71167	23.5854	0.026976 ^a
Casein	-0.82167	-11.3219	0.056084
Malt extract	-1.19833	-16.5120	0.038508 ^a
Urea	3.05167	42.0495	0.015137 ^a
NH ₄ H ₂ PO ₄	-0.63500	-8.7498	0.072444
(NH ₄) ₂ SO ₄	-1.26833	-17.4766	0.036387 ^a
NH ₄ Cl	0.24500	3.3759	0.183336
NH ₄ NO ₃	-0.46833	-6.4532	0.097873
DV	0.02167	0.2985	0.815301

Standard error = 0.072573

^aSignificant at $p \leq 0.05$

Table 3: Effects for lipase production from the results of Plackett–Burman design.

experimental runs. The experimental plan and corresponding lipase production were shown in Table 2. The Pareto graph (Figure 3) was used to show the effect of all nitrogen sources (both organic and inorganic) on lipase production. A p -value of less than 0.05 for the five variables viz., urea, peptone, yeast extract, (NH₄)₂SO₄, and malt extract indicates that they are significant. From the statistical analysis, it was also found that lipase production was affected by the above five nitrogen sources as evident from their F -values and p -values as shown in Table 3. In addition, the coefficient of determination (R^2) of the model was

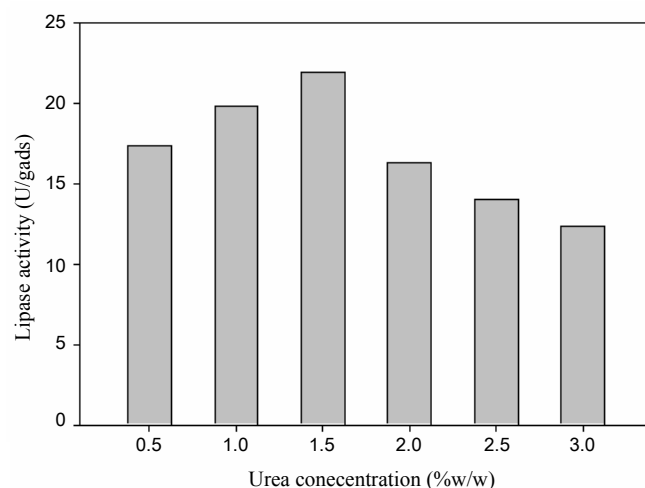


Figure 4: Effect of urea concentration on lipase activity.

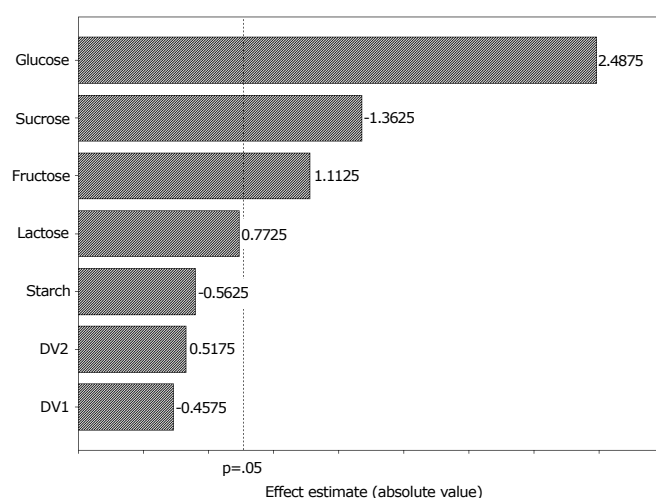


Figure 5: Pareto graph showing effect of various carbon sources on lipase production based on the observation of Plackett–Burman design.

found to be 0.9998 which explains the 99.98% variability of the data. Urea had the confidence level more than 95% in comparison to the other variables and thus considered to be highly significant for lipase production. Here one dummy variable (DV) was employed to evaluate the standard errors of the experiment. Urea was found to be good nitrogen source for lipase production with *C. rugosa* (Banjamin and Pandey, 1997). The effect of different concentrations of urea on lipase production was studied, and the results are presented in Figure 4.

Run no.	Levels	Variables (%w/w)							Lipase Activity (U/gds)
		Glucose	Lactose	Fructose	Sucrose	Starch	DV1	DV2	
	+	9	9	9	9	9	-	-	
	-	1	1	1	1	1	-	-	
1		-	-	-	+	+	+	-	16.86
2		+	-	-	-	-	+	+	21.79
3		-	+	-	-	+	-	+	19.97
4		+	+	-	+	-	-	-	21.14
5		-	-	+	+	-	-	+	19.51
6		+	-	+	-	+	-	-	22.28
7		-	+	+	-	-	+	-	20.67
8		+	+	+	+	+	+	+	21.75

DV: Dummy Variables

Table 4: Plackett–Burman experimental design matrix for screening of different carbon sources for lipase production.

Identification of important carbon source using Plackett–Burman design

Five carbon sources were screened by eight experimental runs. The experimental plan and corresponding lipase production were shown in Table 4. The pareto graph (Figure 5) was used to show the effect of all the carbon sources on lipase production. A p -value less than 0.05 for the three variables viz., glucose, sucrose, and fructose indicates that they are significant. From the analysis, it was also found that lipase production was affected by the above three carbon sources as evident from their F -values and p -values as shown in Table 5. In addition, the coefficient of determination (R^2) of the model was 0.9998 which explains the 99.98% variability of the data. Glucose had a confidence level of above 95% in comparison to other variables and thus was considered to be highly significant for lipase production. Two dummy variables (DV1 & DV2) were employed to evaluate the standard errors of the experiment. The effect of different concentrations of glucose on lipase production was studied and the results are presented in Figure 6.

Conclusions

Deoiled Niger seed cake was assessed for its suitability as substrate for lipase production by solid state fermentation (SSF). The seed cake supported good microbial growth and enzyme production as evident by its chemical composition. Marine yeast *Yarrowia lipolytica* NCIM 3589 was used for the fermentation. The presence of Niger seed oil cake with moisture content of 60% yielded of maximum lipase activity (26.42 U/ gds) in four days. The high lipase activity achieved in conjunction with the

Variables	Effect (E)	t -value	p -value
Glucose	2.48750	39.800	0.015992 ^a
Lactose	0.77250	12.360	0.051395
Fructose	1.11250	17.800	0.035728 ^a
Sucrose	-1.36250	-21.800	0.029182 ^a
Starch	-0.56250	-9.000	0.070447
DV1	-0.45750	-7.320	0.086435
DV2	0.51750	8.280	0.076516

Standard error = 0.0625

^aSignificant at $p \leq 0.05$.

Table 5: Effects for lipase production from the results of Plackett–Burman design.

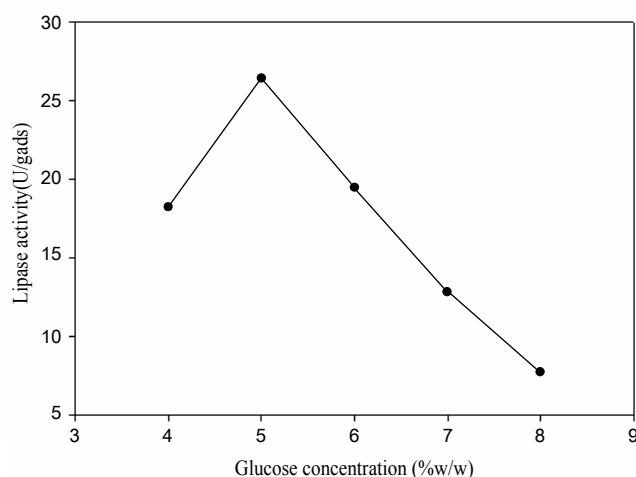


Figure 6: Effect of glucose concentration on lipase activity

abundantly available Niger seed oil cake in the state of Andhra Pradesh, India, paved a way for the industrial exploitation of this substrate under solid state fermentation using the indigenous *Yarrowia lipolytica* NCIM 3589 as a suitable micro organism.

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