Research Paper

Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (*Brassica napus*)

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Abstract

Mustard oil cake (*Brassica napus*), the residue obtained after extraction of mustard oil from mustard oil seeds, was investigated for the production of lipase under solid state fermentation (SSF) using the marine yeast *Yarrowia lipolytica* NCIM 3589. Process parameters such as incubation time, biomass concentration, initial moisture content, carbon source concentration and nitrogen source concentration of the medium were optimized. Screening of ten nitrogen and five carbon sources has been accomplished with the help of Plackett-Burman design. The highest lipase activity of 57.89 units per gram of dry fermented substrate (U/gds) was observed with the substrate of mustard oil cake in four days of fermentation.

Key words: lipase, mustard oil cake, 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) (Jaeger *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 fer-

mentation 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), *Jatropha curcas* seed cake (Mahanta *et al.*, 2008), niger seed oil cake (Imandi *et al.*, 2010a), and palm kernel cake (Imandi *et al.*, 2010b) have been used as the substrates for lipase production.

India is one of the leading producers of mustard rape seed along with Canada, USA, and some of the European countries. The production in India is about 5.7 million metric tons every year (FAO, 1994). Mustard seeds contain 30-35% oil and 34-39% protein (Singhal, 1986). Mustard oil cake (MOC) is prepared from the seeds of *Brassica cumpestris* (mustard). It is a valuable by-product left after the extraction of oil and is known as mustard oil cake. After extracting the oil from the seed, about 60% of residue is left as a cake which is available to the livestock industry. The composition of mustard cake varies with the variety, growing conditions and processing methods. These cakes contain 1-12% ether extract depending upon the method of oil extraction. The protein has a good balance of essential amino acids and relatively high methionine content (Prasad, 1978). However, low palatability of mustard cake is said to be the main problem for its utilization in ruminant diets. This problem is attributed to its glucosinolate content which yields hot and pungent metabolites upon hydrolysis due to the action of endogenous enzymes (Bell, 1984). Ruminants appear to be less susceptible to the toxic effects of glucosinolates compared with pigs and poultry. This is probably the result of glucosinolates being relatively unhydrolysed in the rumen.

Even though a large number of agricultural / food residues have been worked out as suitable substrates for SSF (Raimbault, 1998), there is no report on SSF of mustard oil cake so far to the best of our knowledge. Hence an attempt is made in this paper to utilize the MOC 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, initial 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

Mustard oil cake (*Brassica napus*), 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).

Microorganism and growth conditions

*Yarrowia lipolytic*a NCIM 3589, obtained from National Chemical Laboratory, Pune, India, was used throughout the study. 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. Sub-culturing was carried out once in 2 weeks and the culture was stored at 4 °C.

Inoculum and media 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). 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): KH_2PO_4: 0.1; $MgSO_4.7H_2O$: 0.05; $CaCl_2$: 0.01; NaCl: 0.01; H_3BO_3: 0.00005; $CuSO_4.5H_2O$: 0.000004; KI: 0.00001; $FeCl_3.4H_2O$: 0.00002; $ZnSO_4.7H_2O$: 0.00004; $MnSO_4.H_2O$: 0.00004 (Imandi, 2008). Out of various compounds tried, glucose and urea were ultimately selected as carbon and nitrogen sources respectively as per the Plackett-Burman design (Plackett and Burman, 1946). The contents were thoroughly mixed and autoclaved at 121 °C (15 psi) for 20 min.

Solid state fermentation and extraction of lipase

The sterilized substrate along with media as shown in the above section 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 and samples were withdrawn after 4 days of incubation. The crude enzyme from the fermented material was recovered by simple extraction method. For this, the fermented substrate 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 number 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 purpose of this optimization step was to identify which ingredients of the medium had a significant effect on lipase activity. The Plackett-Burman 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:

$$E_{(Xi)} = \frac{\sum Y_{(+)i} - \sum Y_{(-)i}}{\frac{L}{2}} \qquad (1)$$

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 extra-cellular lipase by the microorganisms (Shirazi *et al.*, 1998). The amount of lipase activity was observed daily during a period of five days. The maximum lipase activity was observed on fourth day as shown in Figure 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.

Effect of biomass concentration

Different concentration of biomass were tried to study their effect on lipase activity (Figure 2) so as to find an optimum biomass concentration in the fermentation process. A low biomass concentration may give insufficient biomass causing reduced product formation, whereas a higher biomass concentration may produce too much biomass leading to the poor product formation (Mudgetti, 1986). In our study, the maximum lipase activity (43.48 U/gds) was obtained with 2 g/L biomass concentration.

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 maximum lipase activity was obtained at 50% v/w initial moisture content as presented in Figure 3. 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 experimental runs as per the Plackett-Burman de-
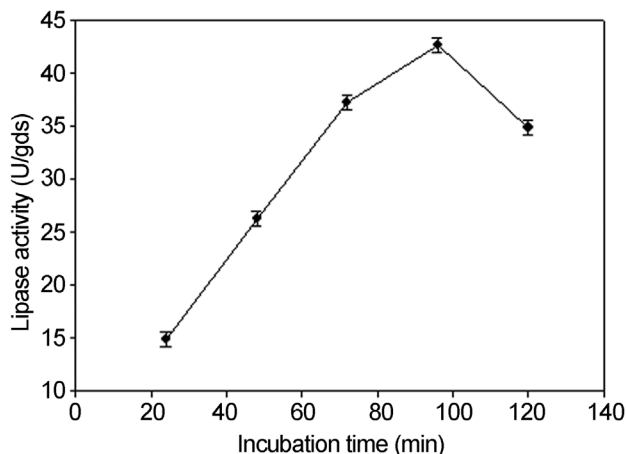

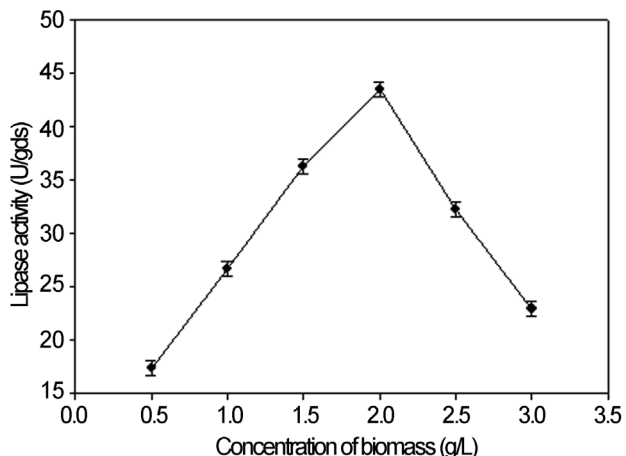**Figure 1** - Effect of incubation time on lipase activity.

Figure 2 - Effect of biomass concentration on lipase activity.

Figure 3 - Effect of initial moisture content on lipase activity.

Figure 4 - Pareto graph showing effect of various nitrogen sources on lipase production based on the observation of Plackett-Burman design.

sign and concentration of nitrogen sources were listed in Table 1. The experimental plan and corresponding lipase production were also shown in Table 1. The pareto graph (Figure 4) 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 four variables viz., urea, yeast extract, peptone, and malt extract indicates that they are significant. From the statistical analysis, it was also found that lipase production was affected by the above four nitrogen sources as evident from their F-values and p-values that were shown in Table 2. In addition, the coefficient of determination (R^2) of the model was found to be 0.9996 which explains the 99.96% variability of the data. Urea had the confidence level of 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. Further studies were conducted by taking urea at different concentrations. Figure 5 shows that 1.5 (% w/w) of urea yielded maximum lipase activity. However, at higher levels the production was reduced due to the inhibitory effects of urea.

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 3. The pareto graph (Figure 6) was used to show the effect of all the carbon sources on lipase production. A p-value less than 0.05 for

Table 1 - Plackett-Burman experimental design matrix for screening of various nitrogen sources for lipase production.

Run no.	Levels	Variables (% w/w)											Lipase activity (U/gds)
		Soyabean meal	Yeast extract	Peptone	Casein	Malt extract	Urea	$NH_4H_2PO_4$	$(NH_4)_2SO_4$	NH_4Cl	NH_4NO_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		+	-	+	-	-	-	+	+	+	-	+	45.91
2		+	+	-	+	-	-	-	+	+	+	-	29.92
3		-	+	+	-	+	-	-	-	+	+	+	36.12
4		+	-	+	+	-	+	-	-	-	+	+	51.46
5		+	+	-	+	+	-	+	-	-	-	+	29.23
6		+	+	+	-	+	+	-	+	-	-	-	45.04
7		-	+	+	+	-	+	+	-	+	-	-	49.27
8		-	-	+	+	+	-	+	+	-	+	-	39.23
9		-	-	-	+	+	+	-	+	+	-	+	45.34
10		+	-	-	-	+	+	+	-	+	+	-	48.82
11		-	+	-	-	-	+	+	+	-	+	+	43.82
12		-	-	-	-	-	-	-	-	-	-	-	43.14

the three variables viz., glucose, sucrose, and fructose indicates that they are significant. From the statistical analysis,
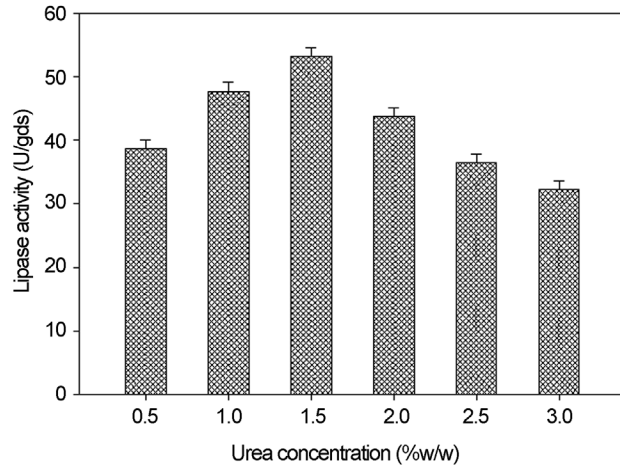

Figure 5 - Effect of urea concentration on lipase activity.
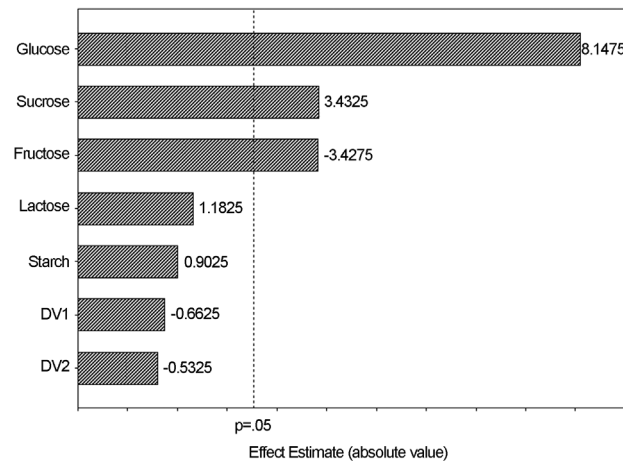

Figure 6 - Pareto graph showing effect of various carbon sources on lipase production based on the observation of Plackett-Burman design.

it was also found that lipase production was affected by the above three carbon sources as evident from their F-values and p-values which were shown in Table 4. In addition, the coefficient of determination (R^2) of the model was 0.9996 which explains the 99.96% variability of the data. Glucose had a confidence level of above 95% in comparison to other variables and thus considered to be highly significant for lipase production. Two dummy variables (DV1 & DV2) were employed to evaluate the standard errors of the experiment. Figure 7 presents the results of different concentrations of glucose on lipase activity.

Conclusions

The mustard oil cake demonstrated 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 MOC with

Table 2 - Effects for various nitrogen sources on lipase production from the results of Plackett-Burman design.

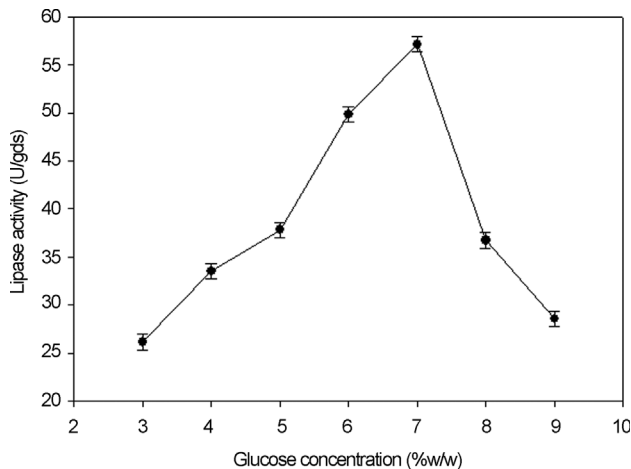| Variables	Effect (E)	t-value	p-value
Soyabean meal	-1.09000	-4.3187	0.144856
Yeastextract	-6.75000	-26.7445	0.023793[a]
Peptone	4.46000	17.6712	0.035988[a]
Casein	-3.06667	-12.1506	0.052276
Malt extract	-3.29000	-13.0355	0.048742[a]
Urea	10.03333	39.7535	0.016011[a]
$NH_4H_2PO_4$	0.87667	3.4735	0.178454
$(NH_4)_2SO_4$	-1.46333	-5.7979	0.108731
NH_4Cl	0.57667	2.2848	0.262638
NH_4NO_3	-1.42667	-5.6527	0.111470
DV	-0.59000	-2.3377	0.257335

Standard error = 0.252389.
[a]Significant at $p \leq 0.05$.

Table 3 - Plackett-Burman experimental design matrix for screening of different carbon sources for lipase production.

Run no.	Levels	Variables (%w/w)							Lipase activity (U/gds)
		Glucose	Sucrose	Fructose	Starch	Lactose	DV1	DV2	
	+	9	9	9	9	9	-	-	
	-	1	1	1	1	1	-	-	
1		-	-	-	+	+	+	-	43.76
2		+	-	-	-	-	+	+	49.29
3		-	+	-	-	+	-	+	46.42
4		+	+	-	+	-	-	-	54.82
5		-	-	+	+	-	-	+	39.28
6		+	-	+	-	+	-	-	48.24
7		-	+	+	-	-	+	-	41.68
8		+	+	+	+	+	+	+	51.38

DV: Dummy Variables.

Figure 7 - Effect of glucose concentration on lipase activity.

Table 4 - Effects for various carbon sources on lipase production from the results of Plackett-Burman design.

Variables	Effect (*E*)	t-value	p-value
Glucose	8.14750	45.4744	0.013997[a]
Sucrose	3.43250	19.1581	0.033200[a]
Fructose	-3.42750	-19.1302	0.033248[a]
Starch	0.90250	5.0372	0.124761
Lactose	1.18250	6.6000	0.095729
DV1	-0.66250	-3.6977	0.168145
DV2	-0.53250	-2.9721	0.206624

Standard error = 0.179167.
[a] Significant at $p \leq 0.05$.

urea 1.5% w/w, glucose 7% w/w, and 50% v/w moisture content yielded the maximum lipase activity (57.89 U/gds) in four days. The high lipase activity achieved in conjunction with the abundantly available mustard 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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