International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.8, No.3, pp 988-996, 2015

Optimization of Conditions for the Formation of Lipase Enzyme from *Aspergillus* Brown Colored Fungi Isolated from Sun Flower Oil Contaminated Soil

V.Narasimhan[1]*, B.Valentin Bhimba[2]

[1]Research Scholar, Sathyabama University / Department of Biotechnology, Shri Andal Alagar College of Engineering, Mamandur– 603 111, India

[2]Department of Biotechnology, Sathyabama University, Chennai - 600119, India

Abstract: Lipase enzymes catalyze the conversion of vegetable oils into fatty acids/esters that are probable substitute for diesel fuel. The activity but depends on the strain, conditions of formation, usage, etc. An *Aspergillus*, brown colored stain having high lipolytic activity, with NCBI accession number KP715156 was isolated from a Sun flower oil contaminated soil. Most important, major and minor medium components and culture conditions that strongly influence the release of highly active lipase enzyme were identified by Plackett - Burman design. Glucose, ammonium nitrate, ferrous sulphate and calcium chloride at a basic pH exhibited strong influence on the formation of active lipase enzyme. The optimization of the major factors were carried out, individually. The optimum conditions of formation were determined to be glucose: olive oil ratio of 1:2, ammonium nitrate: yeast ratio of 1:4, 6 g/L of ferrous sulphate and 4 g/L of calcium chloride at 30-35°C and pH 7 to 7.5.
Keywords: Screening; Medium components; culture conditions; Plackett-Burman design; Optimization.

Introduction:

Lipase enzymes belong to the general classification (E.C. 3.1.1.3). Lipases primarily catalyze the hydrolysis of tri-acyl glycerols but are also versatile biological catalysts (1, 2) capable of catalyzing the synthesis of esters through trans-esterification, thio-esterification and aminolysis (3). This ability is being applied for the conversion of vegetable oils into lower alcohol esters referred as Bio-Diesel- a renewal fuel alternative to the fast depleting petroleum stocks (4). Cost effective production of a highly active and selective lipase enzyme through, isolation or development of micro organism and optimization of culture conditions is crucial for the commercialization of the biological process. Micro organisms produce either internal or external lipase enzyme to utilize the fatty material as their food. Fungi have been considered as better source for extra cellular lipase production (5-8). But the formation and activity of the extra cellular lipase enzymes, varies depending on the organism, strain, medium, culture conditions of carbon & nitrogen source, temperature, pH etc (9-10). So, the general classical strategy of medium selection may not be good enough to find the ideal conditions suitable for the organism in question, for bio-diesel production. Each organism / strain has to be treated on their own merit to optimize their suitable medium components, conditions etc. But optimization of the many variables will require a large number of experiments that may sometimes be impractical. Plackett and Burman have proposed a method of selection of the most important among many parameters that strongly influence any process (11). This report details the identification of factors strongly important for the lipase formation using Plackett and Burman method and the optimized conditions of the factors, for the highly active Aspergillus-brown colored fungi with accession number KP715156 isolated from the sun flower oil contaminated soil.

Materials and Methods:

Microorganism: A pure culture of *Aspergillus* brown colored strain fungi, exhibiting relatively high lipase activity than other four fungal strains on tween-80, Rhodamine-B and Phenol Red agar plates was isolated from an oil contaminated soil of a sunflower oil processing unit, in Tamil Nadu, India. The fungi was identified by PCR amplified ITS nucleotide sequence analysis, as having close resemblance to *Aspergillus* terreus *isolate PKU F22 18S ribosomal RNA gene* and given accession number KP715156. (12).

All the chemicals used for the medium and analysis are of analar grade purchased from Sigma, Hi Media laboratories.

Plackett-Burman design:

Processes may involve many variables. The variables individually or collectively may influence the process responses differently. Optimization of all these variables may be very difficult and may not be necessary also. Statisticians Robin L. Plackett and J.P. Burman proposed a design of identifying the most important variables that has strong influence on the responses with minimum number of experiments. The design involves a total experiments of Y (Y= X+1= multiples of four) for X variables. Variables not having so pronounced effects are also permitted to be included and considered as dummy variables. In a simplistic design, each variable can be considered at two- high and low concentration levels.

Fermentation process depends on many variables in terms of growth medium -major components of carbon, nitrogen, minor nutrients of sodium, potassium, phosphrous, iron, zinc, and culture conditions of pH, temperature etc. The medium may also contain additional inputs as inducers, inhibitors.

Plackett - Burman design of sixteen experiments with fifteen variables was devised. Of them ten were taken up for screening and five were taken at constant concentration ie dummy variables in all the experiments. The ten medium components taken up for screening are: Glucose and olive oil as carbon source, ammonium nitrate and ammonium chloride as inorganic nitrogen source, ferrous sulphate, calcium chloride and zinc sulphate to supply the minor nutrients of iron, calcium and zinc. Literature reported variables affecting the formation or release of extra cellular lipase like calcium (13), sodium do-decyl alkyl sulphonate (SDS) were also included. The reaction conditions of pH and temperature were also considered for screening. A two level design at high or low levels of each of these components were followed. The two- high and low levels of the tested variables are given in the table 1. The concentrations and conditions of the test variables of the sixteen experiments of the Plackett – Burman design are shown in table 2.

The following medium components, yeast (4 g/L), magnesium sulphate (2 g/L), di-hydrogen sodium phosphate (3 g/L), potassium di-hydrogen phosphate (3 g/L) and tween-80 (2 g/L) were considered as dummy variables and are of same concentrations in all screening experiments of Plackett-Burman design.

All the medium components were taken in 100 ml of distilled water and sterilized in a 250 ml conical flask. The medium was inoculated with *Aspergillus* stain at 6% (v/v) and kept on an incubator shaker for 48 hours. The responses in terms of bio-mass production, lipase protein concentration and lipase activity were considered to identify the most important factors.

Biomass weight:

The fermented liquor was filtered on a WhatmanNo.1filter paper. The filter mass on the filter paper was air dried and weighed for bio-mass estimation.

Table 1. Two factor levels of components of Plackett-Burman design

Sl.No	Variable	Low level	High level
1	Glucose	0.5 g/100ml	3 g/100ml
2	Olive Oil	0.5 g/100ml	3 g/100ml
3	Ammonium Nitrate	0.5 g/100ml	2 g/100ml
4	Ammonium Chloride	0.5 g/100ml	2 g/100ml
5	Ferrous Sulphae	0.1 g/100ml	0.5 g/100ml
6	Zinc Sulphate	0.1 g/100ml	0.5 g/100ml
7	Calcium Chloride	0.1 g/100ml	0.5 g/100ml

8	SDS	0.04 g/100ml	0.2 g/100ml
9	pH	6	9
10	Temperature	30°C	37°C

Table 2. Components levels in the Plackett-Burman design experiments

Sl. No	Test Variables	Experiment Number															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Glucose (g/100 ml)	3	3	3	0.5	0.5	3	0.5	0.5	3	3	3	0.5	0.5	3	0.5	0.5
2	Olive oil (g/100 ml)	0.5	3	3	3	0.5	0.5	3	0.5	0.5	3	3	3	0.5	0.5	3	0.5
3	Ammonium Nitrate (g/100 ml)	0.5	0.5	2	2	2	0.5	0.5	2	0.5	0.5	2	2	2	0.5	0.5	2
4	Ammonium Chloride (g/100 ml)	2	0.5	0.5	2	2	2	0.5	0.5	2	0.5	0.5	2	2	2	0.5	0.5
5	Ferrous Sulphate (g/100 ml)	0.1	0.5	0.1	0.1	0.5	0.5	0.5	0.1	0.1	0.5	0.1	0.1	0.5	0.5	0.5	0.1
6	Zinc Sulphate (g/100 ml)	0.1	0.1	0.5	0.1	0.1	0.5	0.5	0.5	0.1	0.1	0.5	0.1	0.1	0.5	0.5	0.5
7	Calcium Chloride (g/100 ml)	0.5	0.1	0.1	0.5	0.1	0.1	0.5	0.5	0.5	0.1	0.1	0.5	0.1	0.1	0.5	0.5
8	SDS (g/100 ml)	0.2	0.2	.04	.04	0.2	.04	.04	0.2	0.2	0.2	.04	.04	0.2	.04	.04	0.2
9	pH	9	9	9	6	6	9	6	6	9	9	9	6	6	9	6	6
10	Temperature (°C)	30	37	37	37	30	30	37	30	30	37	37	37	30	30	37	30

Protein estimation:

The protein content of the filtered solution was estimated by Bradford method and measuring the absorbance at 595nm.

Titrimetric assay:

Lipolytic activity was measured according to Watanabe *et al*.,(14). A reaction mixture containing, 2.5 ml of 0.1M Tris.HCl buffer pH 8.0, 2.5 ml of 25% (v/v) olive oil in 2% (v/v) polyvinyl alcohol solution and 1 ml of enzyme filtrate, was incubated at 30°C on reciprocal shaker at 45 cycles/min. After 10 min, 10 ml of acetone/ethanol (1:1 v/v) solution was added to stop reaction. The free fatty acid released is estimated by titration with 0.05 N, NaOH using thymolphthalein as an indicator. Culture filtrate, boiled for 10 min was used as control. One unit of lipase was defined as amount of enzyme required to release 1 mole of fatty acids per minute under the specific conditions.

Optimization Studies:

The most important components of carbon sources, nitrogen sources, ferrous sulphate, calcium chloride and pH, identified by the Plackett–Burman design of experiments were optimized individually.

The concentrations of medium components, magnesium sulphate (2 g/L), di-hydrogen sodium phosphate (3 g/L), potassium di-hydrogen phosphate (3 g/L) and tween-80 (2 g/L) are same for all the optimization experiments. As SDS and zinc sulphate were not showing much influence they were dropped as medium components in optimization studies. For the optimization of each screened variable, the other screened variables were taken at their optimized or maximum levels. All the components concerned were taken in 100 ml of distilled water and sterilized in a 250 ml conical flask. The medium was inoculated with *Aspergillus* stain at 6% (v/v) and kept inside an incubator shaker at 100 rpm for 48 hours.

Effect of carbon sources on lipase production:

Glucose is an important factor for the lipase formation (15). Vegetable oil or fatty ester are said to be essential for the initialization of the fungi to produce extra cellular lipase enzymes (16). Though only glucose showed greater influence on the lipase activity, the optimization of the glucose was so considered in relation to olive oil ratio. Different glucose to olive oil ratios was used. Other strongly influencing components, ammonium nitrate (20 g/L), ferrous sulphate (5 g/L) and calcium chloride (5 g/L) and pH (9) at 37°C were taken at their high values in the experiments.

Effect of nitrogen sources on lipase production:

Ammonium nitrate was utilized better as nitrogen source than ammonium chloride. Another nitrogen source, namely yeast was also present at constant concentration as a dummy variable. The optimization of the inorganic against organic nitrogen source (ammonium nitrate: yeast) was done by changing the relative proportion of them. Carbon source for all other optimization experiments of nitrogen, iron, calcium, pH and temperature consisted of glucose: olive oil ratio of 1:2 (10:20 g/L).

Effect of ferrous sulphate on lipase production:

Optimization of ferrous sulphate was carried out with medium concentrations of glucose: olive oil ratio (10:20 g/L) and ammonium nitrate: yeast ratio (2:8 g/L) and by varying the ferrous sulphate concentrations from 2 g/L to 10 g/L.

Effect of calcium chloride sources on lipase production:

Calcium chloride concentration optimization was done by regularly increasing the level to 10 g/L at the optimized concentrations of carbon, nitrogen and ferrous sulphate.

Effect of pH on lipase production:

The fermentation medium in different flasks at the optimized concentrations of carbon, nitrogen, iron and calcium were adjusted to different pH values (with citrate buffer for pH between 3 & 6, phosphate buffer for pH between 7 & 8 and borate buffer for pH=9) before inoculation and then incubated at 37°C.

Effect of temperature on lipase production:

The fermentation medium in 250 ml conical flasks adjusted to pH 7.5 was incubated at different temperatures with continuous shaking at 100 rpm for 48 hours.

Results and Discussion:

Bio mass yield, Bredford protein estimation and the lipase titrametric assay responses, from the important factors identification experiments are summarized in table 3. All the three responses exhibit variations among experiments, indicating influences by variables. (Fig.1). Of them, the lipase activity was taken for screening of the most important components for optimization studies. Sum of the lipolytic activity values of experiments corresponding to the high value and low value of each of these test variables were calculated and given in table 4. The difference between the summation of high and low levels for each variable and from that the average of the square of the difference were calculated. Large values of the average indicate a strong influence of the test variable on the lipolytic activity of the extra cellular enzyme.

Table 3. Experimental responses of the Plackett- Burman design

Response	Experiment Number															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Bio Mass (mg/ml)	-	0.2	0.2	-	-	0.22	-	-	0.18	0.3	-	-	-	0.4	-	-
Absorbance	0.2	1.5	0.6	0.2	0.2	1.6	0.2	0.2	1.3	1.8	0.3	0.2	0.2	2.4	0.2	0.2
Lipolytic activity (U/ml)	0.2	2.33	0.92	0.2	0.2	1.92	0.2	0.2	1.7	2.3	1.0	0.2	0.2	4.33	0.2	0.2

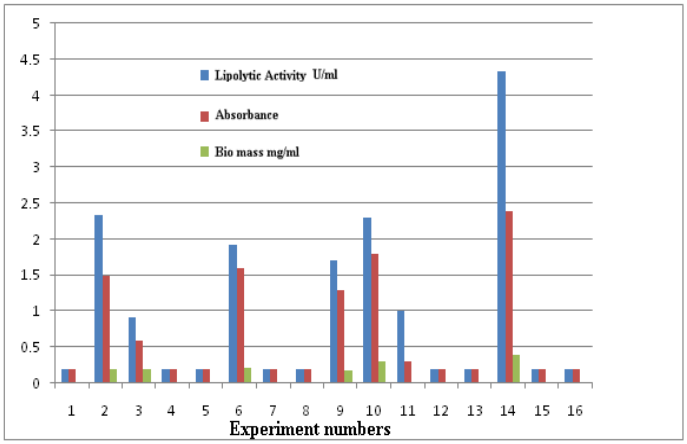

FIG 1. Plackett-Burman design experimental responses

Table 4. Screening the most important components of the medium.

Calculation	Lipolytic activity responses of									
	Glucose	**Olive oil**	**Ammonium Nitrate.**	**Ammonium Chloride**	**Ferrous Sulphate**	**Zinc Sulphate**	**Calcium Chloride**	**Sodium Do decyl Sulphate**	**pH**	**Temperature**
∑H	14.96	7.55	3.12	8.95	11.38	8.97	3.1	7.35	14.67	7.45
∑L	1.6	8.57	13.17	7.55	4.62	7.33	13.75	5.72	1.6	8.77
Diff	+13.36	-1.02	-10.05	+1.4	+6.76	+1.64	-10.65	+1.63	-13.07	-1.32
$\sum^2/8$	**22.31**	1.03	**12.6**	0.25	**5.76**	1.51	**14.16**	0.33	**21.37**	0.22

Glucose, ammonium nitrate, ferrous sulphate, calcium chloride and basic pH exhibit larger values and hence are identified as the most important components/ conditions that exert strong influence on the activity of the lipase formed.

Optimizations of these important variables were carried out individually. Glucose and olive oil relative concentrations were optimized for the carbon source for the fungi. The bio mass yield, protein concentration and the lipolytic activity of the lipase enzyme, observed of the experiments are given in table 5. Fig 2 shows the variation of the responses. A glucose –olive oil ratio of 1:2 exhibited maximum formation of bio mass, protein and lipase activity.

Table 5. Optimization of Carbon source

Experiment No	Glucose (g/100 ml)	Olive oil (g/100 ml)	Biomass (mg/ml)	Absorbance	Lipolytic activity (U/ml)
1	3.0	0.0	.51	1.9	3.50
2	2.5	0.5	.3	.5	1.25
3	2.0	1.0	.3	.6	1.25
4	1.5	1.5	.54	2.2	3.58
5	**1.0**	**2.0**	**.72**	**2,9**	**4.80**
6	0.5	2.5	.65	2.6	4.33
7	0.0	3.0	.54	2.1	3.58

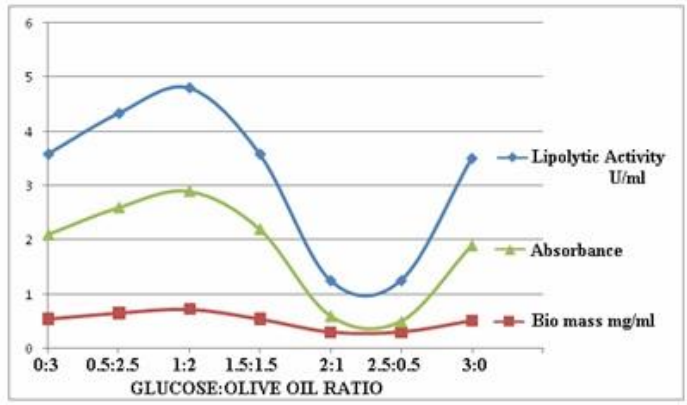

FIG 2. Optimization of Carbon Source

Table 6. Optimization of Nitrogen source

Experiment No	Ammonium nitrate (g/100ml)	Yeast (g/ml)	Biomass (mg/ml)	Absorbance	Lipolytic activity (U/ml)
1	1.0	0.0	0.10	-	0.17
2	0.8	0.2	0.30	0.8	0.50
3	0.6	0.4	0.30	1.2	0.83
4	0.4	0.6	0.53	2.0	2.50
5	**0.2**	**0.8**	**0.74**	**3.1**	**5.41**
6	**0.0**	**1.0**	**0.71**	**3.1**	**5.41**

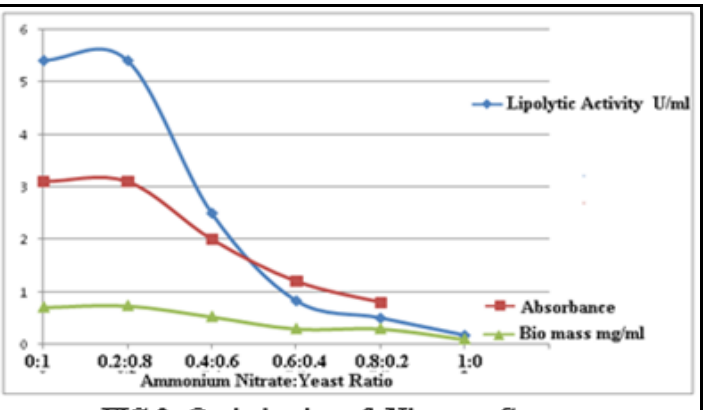

FIG 3. Optimization of Nitrogen Source

Ammonium nitrate was the preferred inorganic nitrogen source by the fungi. Concentration of this ammonium nitrate against the organic yeast source was estimated with differing proportions of them. Table 6 provides the observations of these experiments and the yield pattern are visualized in Fig 3. Experiments with ammonium nitrate-yeast ratio of 1:4 and 100% yeast without ammonium nitrate gave high responses of lipase formation and activity.

Table 7 Optimization of minor nutrient – Iron

Experiment No	Ferrous sulphate (g/100ml)	Biomass (mg/ml)	Absorbance	Lipolytic activity (U/ml)
1	0.2	0.2	0.4	1.3
2	0.4	0.3	0.5	1.6
3	**0.6**	**0.6**	**1.4**	**2.1**
4	0.8	0.5	1.5	1.8
5	1.0	0.5	1.6	1.8

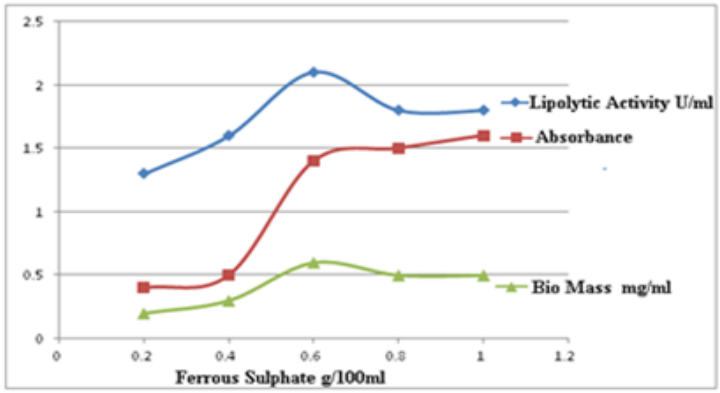

FIG 4. Optimization of Iron

Results of ferrous sulphate concentration variation studies on the lipase enzyme are in table 7 and Fig 4. 6 g/L of ferrous sulphate concentration resulted in high bio mass and lipase activity.

Optimization of calcium chloride experiments showed maximum activity at 4 g/L calcium chloride concentration.(Table 8 and Fig 5)

Table 8 Optimization of minor nutrient – Calcium

Experiment No	Calcium Chloride (g/100 ml)	Biomass (mg/ml)	Absorbance	Lipolytic activity (U/ml)
1	0.2	0.4	1.4	2.1
2	**0.4**	**0.8**	**1.8**	**2.9**
3	0.6	0.6	1.2	1.9
4	0.8	0.2	0.8	0.8
5	1.0	0.2	0.7	0.8

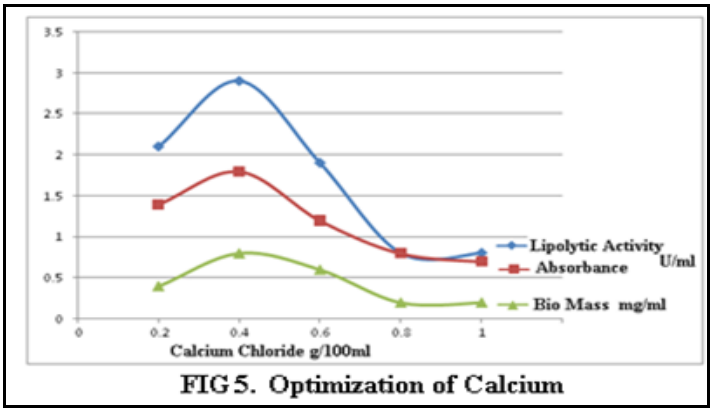

FIG 5. Optimization of Calcium

Placket-Burman design for identification was carried out at one acidic and basic pH conditions. pH of the medium was buffered at pH values from 5 to 9 to select the better operating conditions. Almost neutral pH (7.5) was observed to be good enough for the fungi to grow and produce maximum lipase activity as observed in Table 9 and Fig 6.

Table 9. Optimization of culture conditions- pH

Experiment No	pH	Biomass (mg/ml)	Absorbance	Lipolytic activity (U/ml)
1	5.0	-	0.2	-
2	5.5	-	0.2	-
3	6.0	-	0.2	-
4	6.5	0.3	0.5	0.4
5	7.0	0.5	1.6	1.8
6	**7.5**	**0.9**	**2.1**	**2.8**
7	8.0	0.9	1.8	2.5
8	9.0	0.8	1.7	2.3

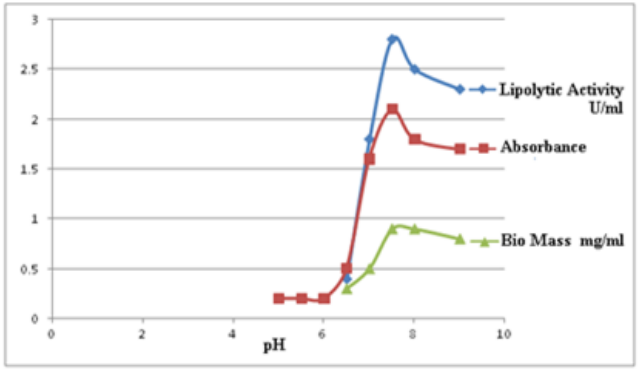

FIG 6. Optimization of pH

Fermentations were conducted at increasing temperatures from 25°C up to 55°C. Maximum lipase activity was observed at temperatures 30°C and 35°C (Table 10 and Fig 7).

Table 10. Optimization of culture conditions - Temperature

Experiment No	Temperature (˚C)	Biomass (mg/ml)	Absorbance	Lipolytic activity (U/ml)
1	25	0.6	1.8	1.9
2	**30**	**0.8**	**2.3**	**2.6**
3	**35**	**0.8**	**2.2**	**2.4**
4	40	0.7	1.6	1.8
5	45	0.6	1.6	1.8
6	50	0.4	0.9	0.8
7	55	0.2	0.7	0.6

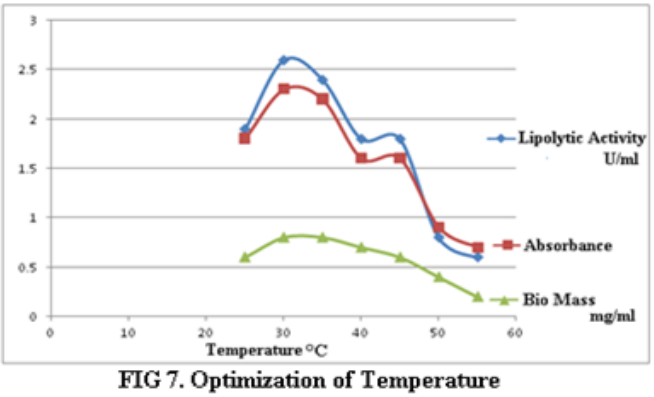

FIG 7. Optimization of Temperature

Medium components and conditions suitable for maximizing the production of an *Aspergillus terreus* stain *having NCBI* accession number KP715156 was determined using Plakett-Burman design. Glucose, ammonium nitrate, iron, calcium, pH and temperature were identified as important components strongly influencing the growth and release of the active lipase enzyme.

A carbon : olive oil ratio of 1:2, ammonium nitrate: yeast ratio of 1:4, 6 g/L of ferrous sulphate as a source for iron, 4 g/L of calcium chloride (for calcium) in a pH range of 7.5 and temperature around 30°C were estimated to be the optimum conditions of fermentation, for the production of lipase enzyme from the *Aspergillus* strain isolated from the sun flower oil contaminated soil.

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