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OPTIMIZATION OF PHYSIOLOGICAL PARAMETERS TO INCREASE LIPASE PRODUCTION IN THE FERMENTATION OF MUTATED STRAIN *ASPERGILLUS* SPTL-12(3)

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ABSTRACT

Physiological fermentation parameters like different salts, buffers and pH were studied at shake flask level for Aspergillus sp. TL12 (3). Better lipase production observed in media containing salts $MgSO_4$ 7 H_2O (20mM), $CaCl_2$ (20mM) and Na_2HPO_4 (20mM). Best lipase yield identified in 50 mM glycine Hcl buffer at pH 6. The size of the inoculum found 1 % spore suspension (~10^8 cells/ml) is an ideal for better lipase production. Physical parameters of fermentation were studied. Temperature at 26 °C with 150 RPM shaking was found better. The duration of fermentation was found 72 hours where maximum production of lipase was observed. By optimizing some of the physiological parameters, lipase production has been increased to 1450 U/ml which is much better results at shake flask level as compared to several published results.

KEY WORDS

Aspergillus TL-12(3), Lipase, Physiological parameters, Optimization, Shake flask fermentation

INTRODUCTION

Lipases [Triacylglycerol ester hydrolases (EC 3.1.1.3)] are enzymes which are having industrial importance. Microbial lipases are commercially important because of their unique properties and their bulk extracellular production, compared to lipases from other natural sources. Lipases are among the most widely used biocatalysts in the field of organic chemistry (1). Lipase production is dependent upon a number of factors including carbon, nitrogen, lipid sources, pH, temperature, aeration and innoculum size (2,3) Microbial lipases are produced mostly by submerged culture (4). Fungi are preferable lipase sources because fungal enzymes are usually produce extracellularly, facilitating easy extraction from fermentation media (5,6). Submerged fermentation found higher lipase production than static fermentation (7). Stimulation and inhibitory effects of temperature, pH and cationic salts were studied by S E Petrovic et al (8). Initial pH of the broth media and agitation was studied for *Fusarium oxysporium* by Hala Mohand Rifaat et al 2010 (9). Oxygen transfer plays an important role on production of lipase (10). Some of the lipases are able to catalyze reactions like estrification, transestrification and enantioselective hydrolyses (11). The selectivity of lipases has been exploited in industry in the synthesis of chiral compounds as well as in the resolution of racemic mixture (12). Hydrolytic enzymes like esterase and lipases are currently applied to cleave ester or amide bonds and also to synthesize when used in low or zero water concentration in organic solvents (13).

In this work, we report the optimum concentration of salts, buffers, hydrogen ions, aeration, and temperature and fermentation duration to produce increased production of lipase from *Aspergillus* mutated isolate TL-12(3).

MATERIALS AND METHODS

Culture conditions: Fungal strain *Aspergillus* TL-12(3) was obtained from our past work where the soil isolate TL-12 (personal collection) was mutated for higher production of lipase (14). This strain was grown on PDA slants. Slants were prepared with 2% Potato starch, 2%dextrose, and 1% agar with 1% tributyrin. The culture was maintained by periodic sub culturing and kept at room temperature (25 ^{0}C).

Inoculum preparation: Five days old PDA culture slant washed with 10 ml normal sterile saline (0.8 w/v) thus obtained spore suspension used for inoculation.

Separation of broth: Growth media filtered and centrifugation at 7000 rpm supernatant was taken for lipase assay and protein estimation.

Media composition: The medium for lipase production was selected from our past work (15) where optimized the carbon nitrogen and lipid sources to enhance lipase production for *Aspergillus* TL12 (3). Broth media components were 1 % soluble starch, 1% sucrose, 3% CSL, 1% casein and 1% olive oil.

Study of salts: Media were prepared with different salts $MgSO_4$, KH_2PO_4, NaCl, $CaCl_2$, $CaCO_3$, KCl, EDTA, $NaHCO_3$ and Na_2HPO_4 at 10 and 20 mM concentrations and pH was adjusted to 6.0.

Study of buffers: Media prepared with above composition and highest lipase producing mineral salts mixture consisted of $MgSO_4$ 7 H_2O (20mM), CaCl2(20mM) and Na_2HPO_4 (20mM) with different buffers which are 100mM Carbonate buffer pH 9.6, 100mM citrate phosphate buffer pH 6.0, 100mM Phosphates buffer pH 7.4, 50 mM Tris HCl pH 9.2, 50 mM Borate buffer pH 8.0, 10 mM Acetate buffer pH 5.5, 25 mM CHAPS pH 6.0, and 50 mM glycine HCl pH 6.0 were selected and prepared the media in these buffers. Media prepared in glycine HCl buffer has given the highest lipase activity was selected for further study.

Optimization of initial pH of fermentation: Media prepared in, best performed glycine Hcl (pH 6.0) buffer with all optimized components. The pH of the buffer was adjusted with 1N HCl/IN NaOH in the range of 4 to 9. 10ml of each formulated media added to 100 ml volumetric flask and autoclaved.

Optimization of inoculum: Different inoculums volumes tried to increase the lipase production.0.5% to 5%.

Optimization of temperature, duration and aeration of fermentation: Different fermentation temperatures, duration (up to 120 hours) aeration (100,150.200 rpm) was studied. Each optimized condition was introduced in next experiment. Media prepared with optimized ingredients and fermentation done at optimized parameters. Broth divided into equal parts and sterilized, cooled and inoculated. The flasks were fermented with different rpm like 100, 150, and 200.

Assay method: Lipase assay for fermentation broth was conducted by *Tietz.N.W et al* method (16) where release of free fatty acids from olive oil was measured. One unit of lipase activity was defined as the amount of enzyme liberating 1µmol of fatty acid per min.

Estimation of protein concentration: The protein content of the fermented broth was determined according to the Lowery's method (17)

RESULTS AND DISCUSSION

Results of this study were tabulated in **Table: 1**. among salts which are introduced in the medium, $MgSO_4$ 7 H_2O (20mM) has shown 600 U/ml with an increase of 144 %. Same salt at 10 mM concentration yielded 130 U/ml with an increase of 130%. Another salt KH_2 PO_4 edition in the media has exhibited 475 and 525 U/ml with 114 and 126% relative activity at 10 and 20 mM concentrations respectively. $CaCl_2$ (20mM) stimulated the lipase activity 154 % by yielding 641.6 U/ml and same salt at 10 mM stimulated the activity by 136% with an activity of 566.6 U/ml. Na_2HPO_4 (20mM) enhanced the activity 164% with an activity of 683.3U/ml. But NaCl, $CaCO_3$, KCl and EDTA were shown inhibition in lipase production when added to media at both the concentrations which were studied. $CaCO_3$, Na_2CO_3 and $NaHCo_3$ shown inhibition at 20 mM concentration and there is no much effect at 10 mM concentration. Mineral salt solutions consisted of $MgSO_4$ 7 H_2O (20mM), CaCl2 (20mM) and Na_2HPO_4 (20mM) were used as salt additives for media preparation in our further study.

Lipase production can increase by using different buffers (18).When media prepared in zwitwar ion buffer Glycine HCl (50 mM) highest lipase production 966 U/ml with 113.71% relative activity was found. Tris HCl yielded activity 883.3 U/ml with 103.91% relative activity. Media prepared in this buffer will be used in our future experiments. Though these buffers were improved considerable lipase production, but will help to maintain constant pH during the fermentation. Carbonate, Citrate, borate, acetate and chaps shown inhibition in lipase activity. When media prepared in these buffers lipase production reduced to, Carbonate (32%), citrate (24%), borate (42.15%), acetate (45%) and CHAPS (44.11%) (**Table 2**)

Initial pH of the culture broth is one of the most critical environmental parameters influences mycelial growth and lipase production (9, 19). Glycine HCl (50 mM) buffer when tested at different pH has different lipase activities were produced. (**Table 3**) In acidic pH Glycine HCl (pH 6.0) has sown highest lipase production (985 U/ml) with 78.9 U/mg specific activity.

Inoculum size was of 1% spore suspension has slightly better than 0.5%. But there is no much change has been observed in lipase production up to 5% of the spore suspension. (**Table 4**).

Table 1. Effect of different salts (10 and 20 mM concentrations) on lipase production of *Aspergillus* sp.TL-12(3)

S.No	Name of the salt		Activity (U/ml)	Specific activity (U/mg)	Biomass (mg/ 10ml)	Relative activity (%)
1	Control (without salt)		416.6	29.2	240	100
2	Na_2HPO_4	10mM	633.3	97.4	218	152
		20mM	683.3	127.2	210	164
3	$CaCl_2$	10mM	566.6	54.0	170	136
		20mM	641.6	93.4	190	154
4	$MgSO_4$	10mM	541.6	90.3	110	130
		20mM	600	80.0	125	144
5	$NaHCO_3$	10mM	416.6	60.6	230	100
		20mM	491.6	57.0	221	118
6	KH_2PO_4	10mM	475	88.5	200	114
		20mM	525	79.3	150	126
7	Na_2CO_3	10mM	375	34.0	190	90
		20mM	266.6	45.4	200	63.9
8	EDTA	10mM	308.3	32.5	30	74
		20mM	325	34.2	29	78
9	NaCl	10mM	300	39.3	115	72
		20mM	300	28.6	121	72
10	$CaCO_3$	10mM	216.6	36.1	131	51.9
		20mM	291.6	44.0	70	69.9
11	KCl	10mM	250	29.4	48	60
		20mM	233.3	25.2	61	56

Table 2: Effect of different buffers on lipase production of *Aspergillus* sp.TL-12(3)

Name of the buffer	pH of the broth	Activity (U/ml)	Specific activity (U/mg)	Biomass (mg/10ml)	Relative activity (%)
Control	6.0	850	60.1	88	100
Carbonate	9.6	275	41.5	134	32.00
Citrate	6.0	208.3	26.5	116	24.50
Phosphate	7.4	891.6	68.0	118	104.89
Tris Hcl	9.2	883.3	99.38	131	103.91
Borate	8.0	358.3	40.37	117	42.15
Acetate	6.0	383.3	30.97	29	45.00
CHAPS	6.0	375	32.25	153	44.11
Glycine Hcl	6.0	966.6	78.9	147	113.71

Table 3. Effect pH on lipase production of *Aspergillus* sp.TL-12(3)

Media pH	Activity (U/ml)	Specific activity (U/mg)	Biomass (mg /10 ml)	Relative activity (%)
4.0	316.6	53.88	157	32.14
5.0	925	73.33	178	93.9
6.0	985	89.6	168	100
7.0	733.3	54.31	120	74.4
8.0	591.6	43.82	171	60.06
9.0	700	82.35	180	71.06
10.0	550	55.72	181	55.83

Table 4. Effect inoculum size on lipase production of *Aspergillus* sp.TL-12(3)

% of spore suspension	ActivityU/ml	Specific activity(U/mg)	Biomass (mg/10ml)	Relative activity (%)
0.5	920.0	82.59	66	91.8
1.0	1008.0	60.90	83	100
2.0	883.3	62.25	81	82.3
3.0	833.3	69.44	63	89.4
4.0	883.3	60.00	109	96.5
5.0	841.6	64.16	54	90.6

Results of the studies on effect of temperature conditions during fermentation of the *Aspergillus* strain in the current investigations are presented in (**Table 5**). Lipase production found 14.84 % at 22 °C

and 25.59 % at 24 °C. It has been rapidly increased to maximum at 26°C. Fermentation at 26 °C has yielded 1245 U/ml with 102 U/mg specific activities. The biomass also showed highest at 26°C 168mg/10 ml broth. Then started decreasing and it reached to 78.49 % at 28 °C. Fermentation at 22 °C has shown lowest activity and biomass.

Duration of fermentation is an essential parameter. Production of lipase has been observed from 24 hours to 120 hours (**Table 6**) and it maintained the lipase activity up to 120 hours with slight decrease in activity by 79.87 %. At 72 hours 1283.3 U/ml highest lipase activity was observed. Lipase yields were optimum at 72 hours, this is correlating with Vishnupriya B et al 2010 (20) where they were reported maximum activity of 117.88 U/ml lipase activity in broth at 72 hours. Cihangir and Sarikaya [21] indicated that four days of incubation of *Aspergillus* sp. was optimum for its lipase activity, while biomass production increased after three days, where as in our case biomass and activity was found maximum at 3rd day of fermentation.

Effect of rpm on lipase fermentation plays major role on the yields (9, 22). Agitation has an influence on the production of metabolites during shake flask fermentation. Low shaking speeds are known to reduce the lipase activity by 60% (Alford *et al.,* 1965). However, Irina *et al.,* (2001) reported 100 rpm is better than higher rpm for fermentation aspects of *Geotrichum candidum*. **Fig. 1** presents the results of agitation speed experiments conducted on *Aspergillus* strain in the present investigations. The lipase enzyme activity as mentioned above reduced by 37.4% at lower agitation speed of 100rpm compared to the agitation speed of 150 rpm. At 150 rpm with all other optimized parameters, maximum activity of 1450U/ml along with 135.1U/mg specific activity and 168mg/10 ml biomass was obtained after 72 hours of fermentation duration. Reduction in lipase yield was observed at 200 rpm (1280U/ml). Medium rpm (150) has produced good lipase yields may be due to higher oxygen availability. When rpm is 200, because of vigorous shaking, the mycelial damage could have caused reduction in the lipase yield.

Table 5. Fermentation temperature to obtained highest lipase production *Aspergillus* sp TL-12(3)

Temperature (°C)	Activity (U/ml)	Specific activity (U/mg)	Biomass (mgs/10ml)	Relative activity (%)
22	184.8	19.45	16	14.84
24	318.6	34.44	134	25.59
26	1245	138.88	168	100
28	976.6	102.8	143	78.44
37	766.6	63.22	153	61.57

Table 6. Fermentation duration to find out harvesting time to obtained highest lipase production *Aspergillus* sp TL-12(3)

Duration (hours)	Activity (U/ml)	Specific Activity (U/mg)	Biomass dry wt (mg/10ml)	Relative activity (%)
24	193.3	23.79	19	15.06
48	1041.6	119.25	155	81.16
72	1283.3	135.05	180	100
96	1065	78.19	175	82.9
120	1025	71.81	174	79.87

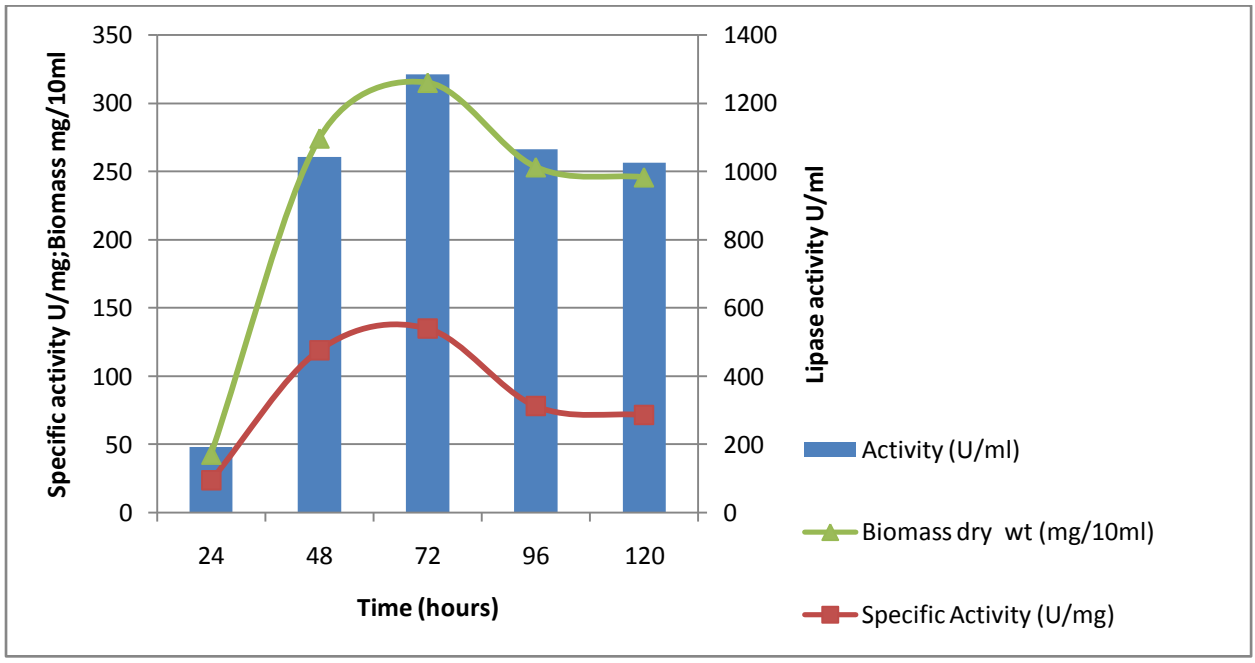

Fig1. Effect of aeration on enzyme activity by fermentation of *Aspergillus* sp. TL-12(3) at different rpm

CONCLUSIONS

The study of *Aspergillus* sp.TL-12(3) lipase production found better with additives of magnesium sulphate, phosphate salts and calcium chloride in lipase fermentation. Glycine HCl (pH 6.0) buffer found best among all other buffers tried. Aeration at 150 rpm found sufficient for the better lipase production. Fermentation temperature 26°C found an effective physical parameter for good growth and best lipase production. Duration of fermentation was found 72 hours where growth and lipase activity found maximum. In this study by optimizing some of the physiological parameters, we could able to increase the lapse activity to 1450 U/ml. By this study salts, buffers, pH, innoculum size, duration, temperature and rpm have significant contribution on improved production of lipase. The appreciable amount of lipase has been produced in shake flask level causing special interest and has much scope for further improvement in larger scale fermentation. Further research will be on purification characterization and application. Such research work is going on which can be published in future.

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