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Optimization of production conditions of lipase from *B. licheniformis* MTCC-10498

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Abstract

The initial broth containing 0.3 (%; w/v) yeast extract, 0.1% sodium nitrate (w/v) etc. were calibrated to a final pH of 7.5 to determine cumulative effect of all the selected components on lipase production by B. licheniformis MTCC 10498. The MB broth was autoclaved at 1.1 bar for 20 min. at 121°C. This broth was inoculated with 10% (v/v) of 36 h old seed culture and incubated under shaking at 55ºC for 72 h. The inoculated MB broth was harvested at 72 h by centrifugation (10, 000 X g for 20 min. at 4ºC; Sigma 3K30, Germany). The supernatant was filtered through Whatman filter paper no. 1. This enzyme preparation was termed as crude lipase. The lipase produced by B. Licheniformis MTCC-10498 in various batches was recorded. The pH was adjusted to 8.0 ± 0.2 and the final volume was made to 1000 ml with sterile distilled water. This crude lipase was subjected to lipase assay and finally 2.0 U/ml activity were recorded.

Keywords: *B. licheniformis, optimization, yeast extract, fatty acids, spectrophotometer.*

Introduction

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology bio-industry and have been used in *ex situ* lipid metabolism and ex situ multifaceted industrial applications[1,2]. A number of lipases have been studied and the majority of them originate from microorganisms such as *Achromobacter, Alkaligenes, Arthrobacter, Bacillus, Burkholdria, Chromobacterium* and *Pseudomonas*[3]. Thus the finding of new producers of lipases and the optimization of process/production strategy for enzyme are important aspects of current research impetus.

Material and Methods

Chemicals: $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, $FeSO_4.7H_2O$, $(NH_4)_2SO_4$ Celite-545 (S. D. Fine–Chem. Ltd., Hyderabad, India); yeast extract and gum acacia (Hi-Media Laboratory, Ltd., Mumbai, India); sucrose, KCl, KI, KNO_3, isopropanol, ammonium persulphate, 2- mercaptoethanol, HCl, bromophenol blue and molecular sieves (3Å X 1.5 mm; Merck-Ltd., Mumbai, India); p-nitrophenyl formate (p-NPF), p-nitrophenyl acetate (p-NPA), p-nitrophenyl benzoate (p-NPB) p-nitrophenyl caprylate (p-NPC), p-nitrophenyl laurate(p-NPL), p-nitrophenyl palmitate (p-NPP) from Alpha-aesar, Heysham, England. n-Hexane, Silica gel (0.040-0.063 mm, 230-400 mesh) acetic acid and triton–X100, tween-20, 40 and 80 (Qualigens Chemicals, Mumbai, India); phenyl methylsulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), sodioum lauryl sarcosine (SLS), acrylamide, bisacryl amide (N,N-methylenebisacrylamide) glycerol, glycine and Tris (2-hyroxymethyl-2-methyl-1, 3-propanediol) (Sigma Chemicals Co., USA). All chemicals were of analytical grade and were used as received.

Microorganism: For the present study, water samples were collected from hot-spring of Tattapani (Mandi District, Himachal Pradesh, India) in sterile containers. For the isolation of lipolytic microorganisms, 100 µl of water samples were plated on triolien and tributyrene agar plates. The formation of clear zone around the colony on the plate was considered as lipolytic microorganisms. In total, 101 isolates were found. Microorganisms which formed large clear zone around the colony were assayed for lipase activity[4]. The microorganism which shows highest lipase activity was designated as BTS-20. The microbial culture was maintained by repeated sub-culturing at 55°C on a mineral based (MB) medium, supplemented with 0.5% (w/v) sucrose and 1.0% (v/v) of cottonseed oil as a sole carbon source (pH 7.5). Further, identification and biochemical characterization was done by Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh. It was identified as *Bacillus licheniformis* MTCC 10498. Glycerol stocks were prepared (25%; v/v) and stored at -20 °C till further use.

Assay and Unit of lipase activity: Lipase assay were performed by a colorimetric method[4]. The absorbance of *p*-nitrophenol released were measured at 410_{nm} (Lab India, UV/ visible spectrophotometer, India). The enzyme activity was defined as µmole (s) of *p*-nitrophenol released per min by one ml of free enzyme or per g of immobilized enzyme (weight of matrix included) under standard assay conditions. Specific activity was expressed as µmole (s) of the *p*-nitrophenol released per min per mg of protein.

Preparation of inoculum of *Bacillus licheniformis* MTCC-10498: A loopful of *Bacillus licheniformis* MTCC-10498 culture taken from the MB-agar slant was asceptically transferred into 50 ml (250 ml Erlenmeyer's flask) of MB broth supplemented with sucrose (0.5%, w/v). The seeded broth was incubated at 55°C in a shaking incubator at 150 rpm up to 36 h. This inoculum was used to prepare glycerol stocks or to inoculate the production broth in the experiment performed.

Standardization of parameters for lipase production: Different parameters for production conditions *viz.* inoculum size, production temperature, pH, carbon, nitrogen sources, oil, surfactant, salt concentration, buffer system etc. were optimized as given below:

Optimization of inoculum size: Inoculum size was optimized by inoculating the production broth by using 36 h seed culture of varying size 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, and 12% (v/v). The sterile broth was inoculated with 10% v/v (5.2×10^6 cells/ml; A_{660}=2.0) of seed culture (36 h old). Lipase activity as well as growth of batch culture was estimated. The broths taken in 250 ml flasks (pH 7.5, 50 ml each flask) were incubated at 55°C for 36 h under shaking at 150 rpm. Thereafter, the harvested cell free broth (centrifuged at 10,000 X g for 10 minute at 4°C) was assayed for lipase activity.

Effect of incubation temperature and pH on lipase production: Most favorable production temperature was studied by incubating the production broth at different temperature of 45, 50, 55, 60 and 65°C and lipase activity was assayed in the cell-free broth after 72 h. For the production of pH, the production broth of varying pH *viz.* 7.0-10.0 were inoculated with seed culture and incubated at 150 rpm in a shaking incubator. Lipase activity was assayed by colorimetric method after 72 h incubation.

Effect of agitation rate and volume of lipase production: To optimize agitation rate for the enzyme production, the broth (50 ml in 250 ml Erlenmeyer's flask) was inoculated with 10% (v/v) inoculum at different rpm *viz.* 60, 90, 120, 150, and 180 rpm. The broth was harvested after 72 h and was assayed for lipase activity by colorimetric method. Most favorable production volume was studied by incubating the production broth at different volume *viz.* (25, 50, 100 and 150 ml). The broth was harvested 72 h and was assayed for lipase activity by a colorimetric method.

Effect of carbon and nitrogen sources on lipase production: Various carbon sources *viz.* (Mustard oil, Cotton seed oil, and Olive oil and Soyabean oil) were supplemented in the production broth. Each of the oil was emulsified with gum acacia (0.5%; w/v). Lipase activity was determined in cell-free broth after 72 h. The production broths having different concentrations (1.0, 2.0, 3.0 and 4.0%; v/v) of cotton seed oil were prepared and inoculated with the seed culture of bacterial isolate. After an incubation period of 72 h, the cell-free broth was assayed for lipase activity. Each of the various nitrogen sources (yeast extract, beef extract, urea, peptone and tryptone) were individually added to production broth at concentration of 0.5 (%;w/v). The lipase activity was assayed after 72 h incubation in cell-free broth. A 36 h old seed culture (10%; v/v) was inoculated in sterile broth containing anyone of the selected inorganic nitrogen sources that included ammonium nitrate, ammonium chloride, urea, sodium nitrate, and ammonium sulphate (0.3%; w/v) along with yeast extract (1.0%; w/v) and cotton seed oil (1%, v/v) in each of the flask. The flasks were incubated at 55± 2 ºC under continuous shaking (120 rpm). At periodic intervals 4 ml of culture broth was aseptically withdrawn to assay protein and lipase activity. To check the effect of different concentration of yeast extract on lipase production, the production broth was prepared with concentration (0.1, 0.3, 0.5 and 0.7%; w/v) of yeast extract in the production broth and the cell-free broth harvested after 72 h incubation at 55°C to determine the lipase activity.

Effect of surfactant on lipase production: The effect of each of the selected surfactant (viz., Sodium dodecyl sulphate, sodium lauryl sarcosine, tween-20, tween-40, tween-60, tween-80 and triton X-100 was separately evaluated by incorporating (0.5%; v/v) the surfactant to the production broth which was inoculated with 10% (v/v) of 36 h old inoculum.

Effect of carbohydrate on lipase production: To evaluate the effect of various carbohydrates (cellobiose, lactose, fructose, ribose, mannose, xylose, dextrose and sucrose) each of the carbohydrates (1%; w/v) was added into the production broth. The amount of lipase produced after 72 h was assayed in each case.

Effect of fatty acid on lipase production: The effect of various fatty acids as inducers (5 mM) each of the palmitic acid, triolein, tristearin, stearic acid, myristic acid, oleic acid and lauric acid) for lipase produced by *B. licheniformis* MTCC-10498 was studied by including separately each of the selected compound in the broth before sterilization. The broth dispensed in the flasks was seeded with 10% (v/v) of the 36 h old inoculum and flasks ware incubated under optimized temperature and pH. The amount of lipase produced in each case was determined at 72 h post inoculation.

Cumulative effect of optimized components on lipase production by *B. licheniformis* -MTCC 10498: The optimized broth containing 0.5 (%; w/v) yeast extract, 0.3% sodium nitrate and tween 80 (0.5%, w/v) was calibrated to a final pH of 7.5 to determine cumulative effect of all the selected components on lipase production by *B. licheniformis* MTCC 10498. The MB broth was autoclaved at 1.1 bar for 20 min. at 121°C. This broth was inoculated with 10% (v/v) of 36 h old seed culture and incubated under shaking at 55 ºC for 72 h. The inoculated MB broth was harvested at 72 h by centrifugation (10, 000 X g for 20 min. at 4ºC; Sigma 3K30, Germany). The supernatant was filtered through Whatman filter paper no. 1. This enzyme

preparation was termed as crude lipase. The lipase produced by *B. licheniformis* MTCC-10498 in various batches was recorded. The pH was adjusted to 7.5 ± 0.2 and the final volume was made to 1000 ml with sterile distilled water.

Growth and lipase profile of *Bacillus licheniformis* MTCC-10498: The seed culture (36 h old) at 10% (v/v) concentration was inoculated in the production broth (50 ml final volume taken in 250 ml Erlenmeyer flask). This flask was incubated at 55ºC in an incubator shaker (120 rpm; Genei shaker, Bangalore). At 4 h intervals, 2 ml of inoculated broth was aseptically sampled up to 120 h post inoculation. A_{660} value of each of the sample was recorded to determine the growth of the bacterial strain and the same was plotted against time. At the same time intervals, 2 ml of culture broth was separately withdrawn aseptically and cell-free broth (centrifugation at 10, 000 rpm for 10 minutes at 4ºC) was assayed at A_{410} to determine lipase activity with respect to the time post inoculation.

Lipase production under optimized conditions: The extracellular lipase production by *B. licheniformis* MTCC-10498 in the optimized medium broth was studied by inoculating the sterile broth with 10% (v/v) of 36 h old inoculum. The seeded broth was incubated at 55 ± 1 ºC for 72 h under continuous shaking (120 rpm). The lipase production in different batches (n = 5) were recorded. The culture broth was further processed to obtain purified lipase. The lipase activity was assayed using p-nitrophenyl palmitate (p-NPP), a chromomeric substrate. Lipase activity of crude lipase, purified or matrix bound lipase was assayed employing a modified colorimetric method (Winkler and Stuckmann, 1979). The stock solution (20 mM) of p-NPP was prepared in HPLC grade isopropanol. The reaction mixture contained 80 µl of p-NPP stock-solution, 20 µl of lipase and tris buffer (0.05 M, pH 8.5) to make final volume 3 ml. The reaction mixture was incubated at 55 °C for 10 min in a water bath (Banglore Genei Pvt. Ltd., Banglore). Further lipase reaction was stopped by chilling at -20°C for 5 min. Control containing heat inactivated (5 min in boiling water bath) enzyme was also incubated with each assay. The absorbance (A_{410}) of heat inactivated lipase was subtracted from the absorbance of the corresponding test sample. The absorbance A_{410} of the p-nitrophenol released was measured and expressed in millimoles (mM). The unknown concentration of p-nitrophenol released was determined from a refrence curve of p-nitrophenol (2-20 µg/ml in 0.05 M Tris buffer pH 8.0) (Appendix 1). Each of the assays was performed in triplicate unless otherwise stated and mean values ± S.D. were presented. Stock solutions of various p-nitrophenyl esters p-NPF, p-NPA, p-NPC and p-NPL were also prepared for use in the some of the experiments. Following two methods were used for the estimation of protein in the sample:

Spectroscopic method: Absorbance of protein sample was measured at 280 $_{nm}$ against the experimental buffer as blank using a spectrophotometer during column chromatography experiments[5] to determine protein concentration in the fractions.

Lowry's method: A standard quantitative assay for determining the protein content in a solution was used[6].

Results and Discussion

The bacterium strain used to produce an extracellular lipase in the present studies was characterized as an aerobic thermophillic, rod shaped of the genus *Bacillus* (figure 1 and figure 2) the strain was maintained by repeated sub-culturing on MB medium containing 1% (v/v) cotton seed oil. Culture stocks of *B. licheniformis* MTCC 10498 were prepared in glycerol (25% v/v; culture) and stored at -20 °C till further use.

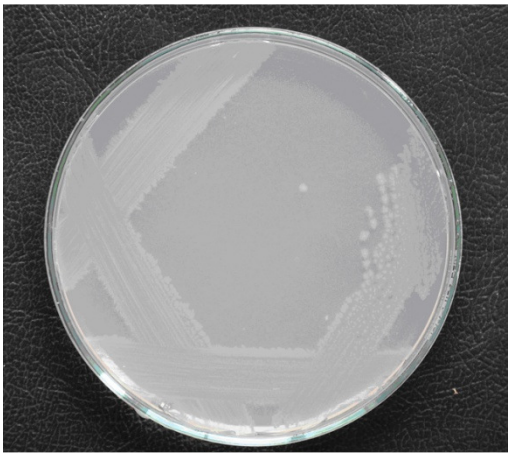

Figure-1
Showing single colony of *B. licheniformis* MTCC-10498 on MB agar plate

Optimization of production temperature: The *B. licheniformis* MTCC-10498 in the MB broth produced and accumulated maximum activity at 55 °C, reaching a highest enzyme activity of 0.327 ± 0.05 U/ml after 48 h post inoculation. A lower or higher cultivation temperature caused a decline in lipase production (table 1). In one liter of MB, $NaNo_3$ 1.0 g , yeast extract 3.0 g, sucrose 1.0 g, cotton seed oil 1% (v/v) was used.

Table-1
Effect of production temperature

Temperature (°C)	Lipase activity (U/ml)	Relative activity (%)
45	0.255 ± 0.07	78
50	0.301 ± 0.04	92
55	0.327 ± 0.05	100
60	0.228 ± 0.01	69.7
65	0.209 ± 0.01	63.9

Effect of pH on lipase production: Lipase production has been shown to be markedly dependent on pH in different species of microorganisms. The microorganism was grown in 50 ml of production medium with a pH range of 7.0-10.0 at 30˚C under shaking conditions for 36 hours. Maximum lipase production

was observed at pH 7.5 (~0.4 U/ml). The enzyme production decreased with increase in pH after 7.0 as shown in table 2.

Table-2
Effect of pH on lipase production

pH	Lipase Activity (U/ml)	Relative activity (%) (%)
7.0	0.349 ± 0.05	89.7
7.5	0.389 ± 0.02	100
8.0	0.356 ± 0.03	91.5
8.5	0.350 ± 0.02	89.9
9.0	0.327 ± 0.05	84.0
9.5	0.221 ± 0.02	57.5
10.0	0.109 ± 0.03	28.0

Effect of agitation rate on lipase production: The agitation rate was studied by varying rpm (60-180). A maximum lipase activity of 0.402 U/ml was recorded at rpm 120. Lipase activity decreased on either side by increasing or decreasing rpm (table 3).

Table-3
Effect of agitation rate on lipase production

Agitation rate (rpm)	Lipase activity (U/ml)	Relative activity (%)
60	0.150 ± 0.05	37
90	0.309 ± 0.01	78
120	0.402 ± 0.04	103
150	0.394 ± 0.01	98
180	0.389 ± 0.02	96

Optimization of inoculum concentration: When a 5% (v/v) inoculum of 36 h seed culture was employed in the production broth, 0.481 ± 0.05 U/ml of enzyme was produced at 55 ± 0.01 °C. After 5% inoculum concentration there is slight increase in lipase activity, however it is not economic (table 4). Inoculum contained nitrogen ($NaNO_3$ 1 g/L and yeast extract 3 g/L as well as the carbon sources (sucrose 5 g/L and cotton seed oil 1%; v/v).

Table-4
Optimization of inoculum concentration

Inoculum size (%; v/v)	Lipase activity (U/ml)
4	0.402 ± 0.04
5	0.481 ± 0.05
6	0.488 ± 0.04
7	0.512 ± 0.05
8	0.523 ± 0.01
9	0.535 ± 0.02
10	0.541 ± 0.01
11	0.535 ± 0.01
12	0.519 ± 0.01

Optimization of chemical parameters for lipase production:
Optimization of lipidic carbon sources: Amongst various carbon sources used in this study, the presence of cotton seed oil produced a relatively higher (0.882 U/ml) lipase activity in comparison to control (sucrose used as a sole carbon source), while addition of olive oil gave minimum activity (table 5). All potable oils were initially emulsified with 0.1% gum acacia. Control consisted of sucrose instead of oil in lipase production broth.

Table-5
Optimization of lipidic carbon sources

Lipidic carbon source (%; v/v)	Oil concentration vs. Lipase activity (U/ml)				
	Nil	0.5%	1.0%	1.5%	2.0%
Cotton seed oil (Ginni)	0.101 ± 0.01	0.672 ± 0.01	0.882 ± 0.05	0.702 ± 0.01	0.512 ± 0.01
Mustard oil	0.101 ± 0.01	0.517 ± 0.01	0.602 ± 0.02	0.585 ± 0.02	0.412 ± 0.01
Mineral oil	0.101 ± 0.01	0.312 ± 0.02	0.350 ± 0.03	0.313 ± 0.04	0.262 ± 0.02
Olive oil	0.101 ± 0.01	0.261 ± 0.01	0.252 ± 0.02	0.212 ± 0.01	0.212 ± 0.01

Effect of nitrogen sources on lipase production: Diverse nitrogen sources (both organic and inorganic) were tested individually by supplementing each of the selected N- sources used at 1.0% (w/v) in the production broth, yeast extract produced highest amount (0.982 U/ml) of lipase after 72 h of incubation at 55 ± 1°C. However, the control broth (broth without NaNO3 and yeast extract resulted in much lower activity (0.12 U/ml; table 6)). The broth containing 0.5% (w/v) YE was further supplemented with 1% additional YE as stated concentrations.

Table-6
Effect of organic N- sources on lipase production

N-sources (0.5%)	Lipase activity (U/ml)	Relative activity (%)
Beef extract	0.101 ± 0.02	10.2
Casein	0.112 ± 0.02	11.4
Peptone	0.092 ± 0.01	9.3
Tryptone	0.075 ± 0.02	7.6
Yeast extract	0.982 ± 0.02	100
Control (none)	0.120 ± 0.02	12.2

Table-7
Effect of 1% additional yeast extract on lipase production

Yeast extract (%, w/v)	Additional YE (1%,/v)	Lipase activity (U/ml)	Relative activity (%)
0.1	1.1	1.112 ± 0.02	113.2
0.3	1.3	1.123 ± 0.01	114.3
0.5	1.5	1.157 ± 0.01	117.8
0.7	1.7	1.178 ± 0.02	119.9
0.9	1.9	1.198 ± 0.01	121.9
1.0	2.0	1.208 ± 0.03	123.0
2.0	3.0	1.103 ± 0.01	112
3.0	4.0	1.056 ± 0.02	107.5
Control	0.5	0.982 ± 0.05	100

In a separate experiment when the amount of yeast extract was gradually increased above 0.5%, w/v by supplementation of additional yeast extract to the broth, it was noticed that additional presence of yeast extract promoted the production of lipase produced by *B. licheniformis* MTCC-10498 (table 7). Yeast extract at a final concentration of 3.0% (w/v) resulted in 1.103 U/ml lipase in the production broth. Since there is minor increase in promoting the yield of lipase by additional yeast extract, it is economical to use 0.5% (w/v) in subsequent experiments.

Effect of supplementation of inorganic nitrogen sources on lipase production: Among various inorganic N-sources that were additionally supplemented in the product broth (containing yeast extract 0.5%, w/v), additional supplementation of the $NaNO_3$ enhanced the lipase production to 1.062 U/ml (table 8). Optimal activity of combined nitrogen source was considered 100%.

Table-8
Effect of supplementation of inorganic nitrogen sources

Additional inorganic source (0.3%; w/v)	Lipase activity (U/ml)	Relative activity (%)
$NaNO_3$	1.062 ± 0.05	100
NH_4NO_3	0.355 ± 0.01	33.4
$(NH_4)_2SO_4$	0.542 ± 0.02	51.0
NH_4Cl	0.612 ± 0.03	57.6
Urea	0.512 ± 0.01	48.2
None (only YE)	0.672 ± 0.02	63.2

Effect of salts in lipase production: Addition of K_2HPO_4, KCl, $MgSO_4$ and $FeSO_4$ in the concentration of 0.1%, 0.05%, 0.05%, and 0.01% (w/v) enhanced the lipase activity up to 1.108, 1.128, 1.144 and 1.168 U/ml respectively. When any of the salts was not added lipase activity declined (table 9).

Table-9
Effect of addition of salts in lipase production

Salt (w/v, %)	Lipase Activity (U/ml)	Lipase activity without salt
K_2HPO_4 (0.1)	1.108 ± 0.05	0.608
KCl (0.05)	1.128 ± 0.05	0.577
$MgSO_4$ (0.05)	1.144 ± 0.02	0.712
$FeSO_4$ (0.01)	1.168 ± 0.05	0.633

Effect of carbohydrates as inducers on lipase production: To evaluate the effect of carbohydrates as inducer in lipase production in the broth, various carbohydrates were individually tested at a concentration of 1.0%. As evident all carbohydrates used in experiment induced the lipase activity (table 10). The control contains emulsified cottonseed oil (1.0%; v/v) and all optimized component till this step.

Table-10
Effect of carbohydrates as inducer

Carbohydrate (1.0%; w/v)	Lipase activity (U/ml)	Relative activity (%)
Cellobiose	1.364 ± 0.01	116.7
Lactose	1.216 ± 0.02	104.1
Fructose	1.106 ± 0.09	94.6
Ribose	1.196 ± 0.05	102.3
Mannose	1.182 ± 0.01	101.1
Xylose	1.219 ± 0.01	104.3
Dextrose	1.369 ± 0.01	117.2
Sucrose	1.412 ± 0.04	120
Nil	1.168 ± 0.05	100

Effect of surfactants on lipase production: Effect of supplementation of each of surfactants (0.05%; v/v) was studied using broth lacking both yeast extract as well as $NaNO_3$ (table 11). Most of the surfactant resulted in a decline in the amount of the lipase that varied from 0.1 to 0.3 U/ml in comparison to the control. However, the presence of tween-80 resulted in 1.808 U/ml lipase activities in the presence of 1.0% sucrose and 1.414 U/ml in the absence of sucrose (1.0%). Increasing or decreasing the concentration of tween-80 resulted in decline in activity. Lipase production broth was supplemented with sucrose (1%, w/v).

Table-11
Effect of surfactant on lipase production

Surfactant (0.5%; v/v)	Lipase activity (U/ml)	Relative activity (%)
SDS	0.712 ± 0.09	60.9
Sodium lauryl sarcosine (SLS)	0.636 ± 0.01	54.4
CTAB	0.767 ± 0.02	65.6
Tween 20	1.008 ± 0.01	86.3
Tween 60	0.810 ± 0.02	69.3
Tween 80	1.808 ± 0.02	154.7
Triton –X100	1.126 ± 0.01	96.4
Control (None)	1.168 ± 0.05	100

Effect of fatty acid (s) on lipase production: The inductive effect of presence of the fatty acids on lipase production by *B. licheniformis* MTCC-10498 was studied. Lipase activity is enhanced by each of the fatty acid when used in the production broth. Highest increase (189.5%) in the lipase activity was recorded when tri-olein was used (table 12). Control lacking in cotton seed oil.

Table-12
Effect of fatty acid (s) on lipase production

Fatty acid (5mM)	Lipase activity (U/ml)	Relative activity (%)
Palmitic acid	1.918 ± 0.01	164.2
Myristic acid	1.451 ± 0.01	124.2
Oleic acid	1.656 ± 0.03	141.7
Stearic acid	1.628 ± 0.04	139.3
Lauric acid	1.817 ± 0.02	155.5
Triolein	2.004 ± 0.01	171.5
Tristearin	2.001 ± 0.01	171.3
Tripalmitin	2.001 ± 0.02	171.3
Control	1.168 ± 0.02	100

Cumulative effect of optimized medium constituents on the production of lipase by *B. licheniformis* MTCC-10498: Optimized medium containing 1.0% (w/v) yeast extract, 0.3% (w/v) $NaNO_3$, 5mM trioein, 1% (w/v) sucrose, 0.5% tween -80 was adjusted to under optimized pH 8.0 and optimized temperature 55 ± 1ºC (table 13). * Cotton seed oil emulsified with 0.1% gum acacia was added to the sterile production broth. **Sucrose (1.0%, w/v), tween-80 (0.5% v/v) and triolein were added in the final concentration of 5mM in optimized broth.

Table-13
Comparison of initial MB and Optimized MB for lipase production

Constituents	Initial MB (g/L)	Optimized Broth (g/L)
Cotton seed oil*	----	10 ml
Yeast extract	3.0	5.0
NaNO3	1.0	3.0
K_2HPO_4	0.01	1.0
KCl	0.25	0.5
$MgSO_4$	0.25	0.5
$FeSO_4.7H_2O$	0.001	0.001
Additives**	Nil	---

Table-14
Batch wise yield of lipase

Batch	Volume (ml)	Lipase activity (U/ml)	Specific activity U/mg
1.	500	2.001 ± 0.02	1.01
2.	500	1.999 ± 0.04	1.02
3.	500	2.001 ± 0.02	1.03
4.	500	2.002 ± 0.02	1.00
5.	500	2.006 ± 0.01	1.00
6.	500	1.998 ± 0.02	1.00
Average	500	2.001 ± 0.02	1.01

Growth and lipase profile of *B. licheniformis* MTCC-10498: The production broth when inoculated with 10% (v/v) of 36 h old seed culture produced optimal amount of lipase activity (2.0 U/ml), specific activity (1.01 U/mg) and protein (1.88mg/ ml) at 72 h post inoculation (figure 2) under optimized conditions. The culture broth rendered cell free by centrifugation at 10,000 x g at 4°C for 10 min was filtered through Whatman filter paper no. 1 and assayed for lipase activity using p-NPP.

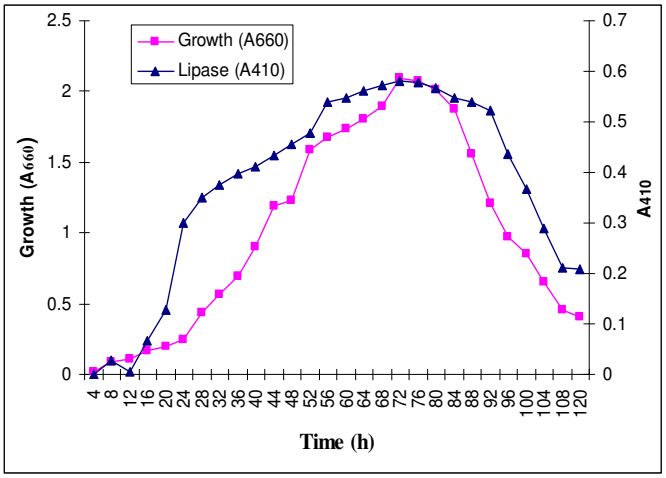

Figure-2
Growth and lipase profile of *B. licheniformis* MTCC-10498 using 5% inoculum.

In the present study, a thermotolerant bacterial isolate *Bacillus licheniformis* MTCC-10498 that produce a novel lipase was exploited to obtain the this enzyme in its purified form. To enhance the lipase production in broth by B. *licheniformis* MTCC-10498 effect of inducers fatty acids and carbohydrates etc. are studied. It was obvious that different microorganism possess different modes of carbohydrate metabolism. Production is significantly influenced by other carbon sources, such as sugars, alcohols, polysaccharides, whey, casamino acids and other complex sources[7].

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, such as peptone and yeast extract, which have been used as nitrogen source for lipase production by various *Bacillus* spp. *viz*., *B. alkalophilus*, *B. coagulans*, *B. cereus*, and *B. licheniformis* strain H1[8-12]. *R. glutinis* seems to require organic nitrogen sources (e.g., yeast extract and tryptone), an inorganic nitrogen source such as ammonium phosphate appears to favor lipase production[14]. Production of the extracellular and cell bound enzymes were reported to depend on the carbon and nitrogen composition of the medium[14,15]. Inorganic nitrogen sources such as sodium nitrate have also been reported to be effective in some microbes[16].

Ca^{2+} stimulated[17] the lipase activity and most of the metal ions (Mg^{2+}, Fe^{2+} Cu^{2+} and $Mn2^{+}$) had strong inhibition on lipase

production from *Burkholderia* sp. Ions of calcium, magnesium and sodium were reported to greatly enhance the lipase production from different type of microorganisms. For instance, lipase production by *B. multivorans* was enhanced[18] in the presence of Ca^{2+}; improvement of lipase production was done by *P. fluorescens* NS2W in the medium containing[19] Mg^{2+} and Ca^{2+}; stimulation of lipase production using *Burkholderia* sp. GXU56 by addition of Mg^{2+} was observed[20]; and, addition of 1.5% NaCl to the medium induced high level of lipase production[21] by *P. fluorescens* NS2W.

Lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range[22] (pH 3-12). Bacterial lipases generally have temperature optima in the range[23] 30-60°C. A highly thermotolerant lipase has been reported from *B. stearothermophilus*, with a half-life[24] of 15-25 min at 100°C. In another similar study with metal ions (1 mM) and chelating agents, *P. pseudoalcaligenes* F-111 lipase activity was 60% inhibited by Fe^{3+} but not by Ca^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+} , metal chelators (EDTA, o-phenanthrolin) did not significantly affect the alkaline lipase activity[25].

Non-specific reversible inhibitors are compounds that do not act directly at the active site but inhibit lipase activity by changing the conformation of lipase or interfacial properties. Surfactants, bile salts[26] and proteins belong to this group of inhibitors. However, surfactants and bile salts activate the enzyme in some cases. Previously, a moderately thermophillic bacteria *B. coagulans* MTCC 6375 exhibited an extracellular lipase activity[27].

Conclusion

Bacillus licheniformis MTCC-10498 lipase showed optimal activity by manipulating various physical and kinetic parameters (i.e., temperature, pH, agitation rate, carbon, nitrogen, inoculum size, salt concentration etc.).

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