Production of extracellular lipase by *Bacillus megaterium* AKG-1 in submerged fermentation

Anurag Sekhon1, Neetu Dahiya2, Ram P Tewari3 and Gurinder S Hoondal3*

1Molecular Genetics and Microbiology, The University of Texas at Austin 1, University Station A5000
Austin TX 78712-0162, USA
2Department of Biotechnology and 3Department of Microbiology, Panjab University, Chandigarh-160 014, India

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*Bacillus megaterium* AKG-1, a soil isolate, has been found to produce thermostable lipase during submerged fermentation (SF). Mannitol at a concentration of 0.2% (w/v) was the best carbon source for lipase production (848 units/mL). Among oils, soyabean oil gave highest lipase yield (1160 units/mL), followed by coconut oil (912 units/mL). Lipase yield was maximum (1000 units/mL) with wheat bran (1%) as sole carbon and nitrogen source, followed by neem seed cake (810 units/mL) and cotton seed cake (790 units/mL).

**Keywords**: *B. megaterium* AKG-1, submerged fermentation, optimization, carbon source

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**Introduction**

Lipases are ubiquitous enzymes produced by all biological systems, viz. animals, plants and microorganisms. In eukaryotes, lipases are involved in various stages of lipid metabolism and are found in energy reservoir tissues. Microorganisms produce a wide spectrum of lipases that differ in their enzymatic characteristics, such as substrate specificity, pH, temperature activity and stability profile. Microbial lipases are most suitable for industrial applications because of their ease of production, relatively inexpensively by fermentation, wide variety and stability in organic solvents, which enables their use in organic synthesis—an emerging field in biotechnology1. With the rapid development of enzyme technology, many new potential biotechnological applications for lipases have been identified in the areas of detergent industry, food industry, paper manufacturing industry, biosurfactant synthesis, organic synthesis of cosmetics and pharmaceuticals. There are several reports on isolation of lipase producing microorganisms and effect of nutritional factors on their growth and lipase production2-15.

The present study reports the optimization of nutritional parameters for production of a thermostable lipase from *Bacillus megaterium* AKG-1 under submerged fermentation conditions.

**Materials and Methods**

**Growth Kinetics and Lipase Production under Shaking and Stationary Conditions**

Nutrient broth, pH 7.0 (100 mL in 250 mL flask) was inoculated with 1.0% of 16 h old seed culture of *Bacillus megaterium* AKG-1 and incubated at 37°C under shaking (150 rpm) and stationary conditions. Thereafter, microbial growth and lipase production were monitored.

**Effect of pH**

Effect of initial pH of medium on lipase production was studied by inoculating buffered nutrient broth (pH 4.0-11.0) with 1.0% of 16 h old seed culture of *Bacillus megaterium* AKG-1 and incubating at 37°C under shaking (150 rpm) conditions. Cell growth and lipase production were determined after 24 h.

**Effect of Carbon Sources**

Flasks containing minimal medium (Na₂HPO₄, 0.7; KH₂PO₄, 0.3; NH₄Cl, 0.1; and NaCl, 0.5%, pH 7.5) supplemented with 0.2% (w/v) sugars (glucose, sucrose, lactose and mannitol) were inoculated with 1%, 16 h old seed culture. Inoculated flasks were kept on rotary shaker (150 rpm) at 37°C. In another experiment, cell density and lipase activity were determined after 24 h of inoculation of medium with different mannitol concentrations (0.1-2.0%, w/v).
Effect of Nitrogen Sources
Nitrogen sources (peptone, yeast extract, tryptone, soyameal, biopeptone, corn steep liquor, ammonium chloride, ammonium nitrate, sodium nitrate, and urea) were incorporated at 0.2% concentration in minimal medium containing 0.2% mannitol. Cell density and lipase activity were determined after 24 h of incubation at 30°C under shaking conditions.

Effect of Oils, Tween-80 and Bile Salts
Oil (1.0%, v/v), Tween-80 (0.1-1.0%, v/v) and bile salts (0.2%, v/v) were added as additional carbon source in optimized production medium (pH 7.5). Lipase activity was determined after 24 and 48 h of incubation at 37°C under shaking conditions.

Effect of Seed Cakes and Wheat Bran
Seed cakes and wheat bran were used at 1.0% (w/v) concentration in minimal medium without any other carbon and nitrogen source. The medium was inoculated with 1.0% (v/v) 16 h old seed culture. Lipase activity was measured after 24 and 48 h of incubation at 37°C.

Enzyme Assay
Lipase activity was measured spectrophotometrically using p-nitrophenyl laurate as substrate and measuring formation of p-nitrophenol (PNP) at 410 nm. The assay mixture contained 800 µL of 50 mM Tris-maleate buffer (pH 7.0), 100 µL substrate solution (0.4% p-nitrophenyl laurate in ethanol), 100 µL enzyme (appropriately diluted). The mixture was incubated at 55°C for 5 min. Absorbance was read at 410 nm. One unit of enzyme activity was defined as the amount of enzyme, which releases one nmole of PNP per minute per mL under standard assay conditions.

Results and Discussion
Growth Kinetics and Lipase Production under Shaking and Stationary Conditions
Under shaking conditions, the maximum cell growth of B. megaterium AKG-1 was obtained after 20 h (cell density 600 nm, 1.700) of incubation, while the maximum lipase yield (350 unit/mL) was observed after 27 h of incubation (Fig. 1). Thereafter both microbial growth as well as lipase yield showed a sharp decline. On the other hand, cell biomass reached slowly to its highest value after 27 h of incubation under stationary conditions, but the increase was equivalent to that observed under shaking conditions. However, the maximum lipase level (230 units/ml) was observed in 34 h old culture, which was comparatively lower vis-a-vis culture grown under shaking conditions (data not shown). Thus, the culture grown under shaking conditions was found to be the best and used in subsequent studies.

Effect of pH
B. megaterium AKG-1 could grow in wide pH range of 4.0-11.0 (Fig. 2). However, the best lipase yield was observed in pH range 6.5-8.0, the optimal being pH 7.5 (325 units/mL, cell density 600 nm, 1.5). The optimal production of lipase by Bacillus strain J33 and B. thermocatenulatus DSM 730 was also reported at pH 8.0 and 7.4, respectively.

Effect of Sugars as Carbon Sources
Mannitol at a concentration of 0.2% (w/v) was found to be the best carbon source among sugars for
lipase production by *B. megaterium* AKG-1 and gave an enzyme yield 848 units/mL (Table 1). Although higher concentrations [0.4-2.0% (w/v)] of mannitol resulted in increased cell biomass, the lipase activity was reduced (data not shown). Glucose, galactose, xylose and sucrose [0.2% (w/v)] also gave similar cell biomass but comparatively lower enzyme activity (520-776 units/mL). The present finding confirms the earlier reports of Mahadik *et al*18, where repressive effect of glucose on lipase production was demonstrated. However, xylose, glucose and mannitol were suggested as good carbon sources for lipase production by *Rhizopus oligosporus*19. Ginalska *et al*13 reported stimulatory effect of sucrose on lipase production by *Geotrichum* sp. strain R59.

**Effect of Nitrogen Sources**

Among inorganic nitrogen sources, the best growth and enzyme activity were observed in the presence of NH₄NO₃ (cell density 600 nm, 0.635; enzyme activity, 110 units/mL; Table 2). However, no detectable enzyme activity was observed in the presence of NH₄Cl and urea. On the other hand, peptone was found to be the best nitrogen source for lipase production (810 units/mL) among the organic nitrogen sources, followed by corn steep liquor (784 units/mL) and yeast extract (760 units/mL; Table 2). Earlier reports also indicated that peptone is the preferred nitrogen source for lipase production20-22. However, tryptone was found to be the best nitrogen source for lipase production by *Yarrowia lipolytica*12. Burkert *et al*15 demonstrated stimulatory effects of ammonium nitrate and corn steep liquor on lipase production by *Geotrichum* sp.

**Effect of Oils, Tween-80 and Bile Salts**

Addition of oils to growth medium resulted in enhanced lipase production by *B. megaterium* AKG-1 (Fig. 3). Soyabean oil gave the highest enzyme yield (1160 units/mL), followed by coconut oil (912 units/mL). In case of tributyrin and glycerol, the growth was slow and the maximum enzyme activity was achieved after 48 h as compared to 24 h with other oils. Several workers have reported the enhancing effect of oils on lipase production. Corn oil was reported to be the best lipid source for *Aspergillus terreus* and *Bacillus* A 30-123,24. Soyabean oil stimulated the lipase production by *Acremonium strictum*, *A. oryzae* and *Geotrichum* sp.15,21,25. Stimulatory effects of olive oil on lipase production were reported in *Y. lipolytica*12, *Bacillus* sp.9 and *Aspergillus* sp.26. However, Gupta *et al*14 reported inducible effect of glycerol on lipase production by *Bacillus* sp.

Tween-80, incorporated at various concentrations into the production medium increased both the growth and enzyme production. The optimal production occurred with 0.25% (v/v) Tween-80 (cell density 600nm, 1.6; enzyme activity, 920 units/mL) (Fig. 4). Increase in Tween-80 concentration although did not
affect the growth much, but caused decrease in lipase yield. Espinosa et al\textsuperscript{27} reported a positive effect on lipase yield by \textit{R. delemar} when the production medium was supplemented with Tween-80 (0.02-2.0%). Incorporation of Tween-80 (1.0%, v/v) in culture medium enhanced the lipase production by \textit{A. strictum}\textsuperscript{25}. Nahas et al\textsuperscript{19} used 0.7% Tween-80 to increase lipase production by \textit{R. oligosporus}. Stimulatory effect of Tween-80 was also reported on lipase production by \textit{Bacillus} sp.\textsuperscript{9}.

Lipase production was recorded more in the presence of cholic acid (21%), lithocholic acid (15%) and sodium deoxycholate (4%), and less (8%) in the presence of sodium taurocholate as compared to control (Fig. 5). Sodium deoxycholate was reported to increase lipase production by \textit{Lactobacillus delbrueckii} subsp. \textit{bulgaricus}\textsuperscript{28}.

**Effect of Seed Cakes and Wheat Bran**

All seed cakes, used in the present study, enhanced the lipase production (Fig. 6). The maximum lipase yield were observed with cotton and neem seed cakes, i.e. 780 and 800 units/mL, respectively, which were comparable to those obtained with peptone and mannitol supplemented medium (710 units/mL). Thus, seed cakes, peptone and mannitol could be best sources for lipase production by \textit{B. megaterium} AKG-1.

Wheat bran was found to be the best individual source that gave the lipase yield of 1000 units/mL, whereas mustard seed cake gave the minimum yield (580 units/mL). Earlier reports showed that the optimized production medium for \textit{Bacillus} sp. THL027\textsuperscript{29} contained 1.0% rice bran. However, wheat bran was used as nitrogen source for lipase production by \textit{L. delbrueckii} subsp. \textit{bulgaricus}\textsuperscript{28}.

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**References**

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