Lipase Activity Assay: Improvements to the Classical *p*-Nitrophenol Palmitate Spectrophotometrical Method

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Abstract

Gupta's lipase activity assay is widely used for checking lipase activity of isolates from different sources (olive oil mill wastewaters, sludge from waste water treatment plants, dairy industry effluents, oil contaminated soils, etc) when seeking for biotechnological applications for lipolytic strains in biofuels production, cosmetics, food and even pharmaceutical industry. However, this assay shows several limitations when the study of lipase activity requires the use of different pH, organic solvents or temperature conditions for one single strain or the comparison of the activity levels of several strains. In the present study, several improvements have been realized so the lipase assay can be applied to the simultaneous study of lipase activity of several bacterial strains under different conditions of pH, temperature and presence of organic solvents. Studied strains' lipases showed different performances at different pH, solvents and temperature conditions, and the most interesting conditions for using the isolates for methanolysis and ethanolysis of fatty acids were determined. Particularly, *Microbacterium* sp. S18 and *Proteus sp.* S53 showed a better activity level at pH 5 in presence of methanol, while their performance was quite similar both in ethanol and methanol at pH 8. Besides, feasibility of studied lipases for their application in different biotechnological fields could be sugested from the activity levels in presence of most used organic solvents.

Gupta's lipase activity assay limitations

Lipase assay developed by Gupta and colaborators is based in an spectrophotometrical method to determine the lipase activity of a bacterial culture throw the quantification of the *p*-nitrophenol released by lipase enzymes from the substrate *p*-nitrophenol palmitate (*p*NPP) (Gupta*, et al.*, 2002). In the normal assay, 1 ml of cell-free medium obtained by centrifugation of an overnight culture is mixed with 9 ml of substrate solution. This reaction mix is incubated at 30ºC for 30 minutes. The reaction is finished by incubation at 100ºC for 4 minutes before the spectrophotometrical determination.

Improvements to the protocol

Nevertheless, this lipase assay requires relatively long volumes of reagents and cultures, and the prolongated incubation time for each sample creates a bottleneck that reduces the applicability of the method to the study of multiple samples or multiple parameters. Besides, the assay has a low replicability because of *p*-NPP absorbance changes; as well as Triton X100, *p*-NPP and arabic gum emulsification problems when pH values are far from standard pH 8. Briefly, the changes made to the original protocol have been:

1. The buffer system includes citrate buffer to adjust pH 5-6, Sorensens' phosphate buffer to adjust pH 7-8, glycine-NaOH buffer to pH 9-10.4. Thus, pH of the reaction mix can be modified according to the needs of the study and the performance of the lipases can be analysed under pH conditions far from the standard and it is possible to make a more realistic prediction of the behaviour of the system where the enzyme will be deployed.
2. The emulsification problems have been fixed by directly disolving Triton X100 in isopropanol (200 µl/ml), resuspending the *p*-NPP directly with the obtained emulsifying solution (6.6 mg of *p*-NPP per ml of detergent solution) and suplementing the buffer with arabic gum (11 mg of arabic gum in 20 ml of buffer) prior to mixing it with the *p*-NPP-isopropanol-Triton solution. After incubating at the desired temperature for 45 minutes, absorbance at 410nm is readed in a microtiter reader.
3. The proportions of the different components of the reaction mix have been modified to improve the replicability and adapt the assay to 96 wells plates. Thus, 1 ml of TritonX100-isopropanol-*p*-NPP is added to 20 ml of arabic gum supplemented buffer. 50 µl of this mix are placed at each well. Finally, 100 µl of the supernatant from the strain under study are added to each well.
4. In order to study the effect of organic solvents in the activity of lipases present in the studied strains' supernatants, previously described lipase assay was modified as follows: Several strains supernatants' lipase activity was assayed in presence of organic solvents polyethileneglycol (PEG), glycerol, dimethyl sulfoxide (DMSO), methanol, ethanol, acetone, benzene and n-hexane. 400 µl of each supernatant were pippeted in glass chromatography vials containing 800µl of pH 5 adjusted citrate buffer or pH 8 adjusted Sorensens' buffer suplemented with 11.25% and 67.5% of each organic solvent. Vials were thoroughly agitated by vortex and, inmediately, 100 µl of the mix were transferred to the wells of a microtiter plate already containing 50 µl of lipase assay solution. Thus, final organic solvent concentration of each well was 5% or 30%. Microtiter plates were incubated at 30ºC for 45 minutes prior to the reading of absorbance at 410 nm.

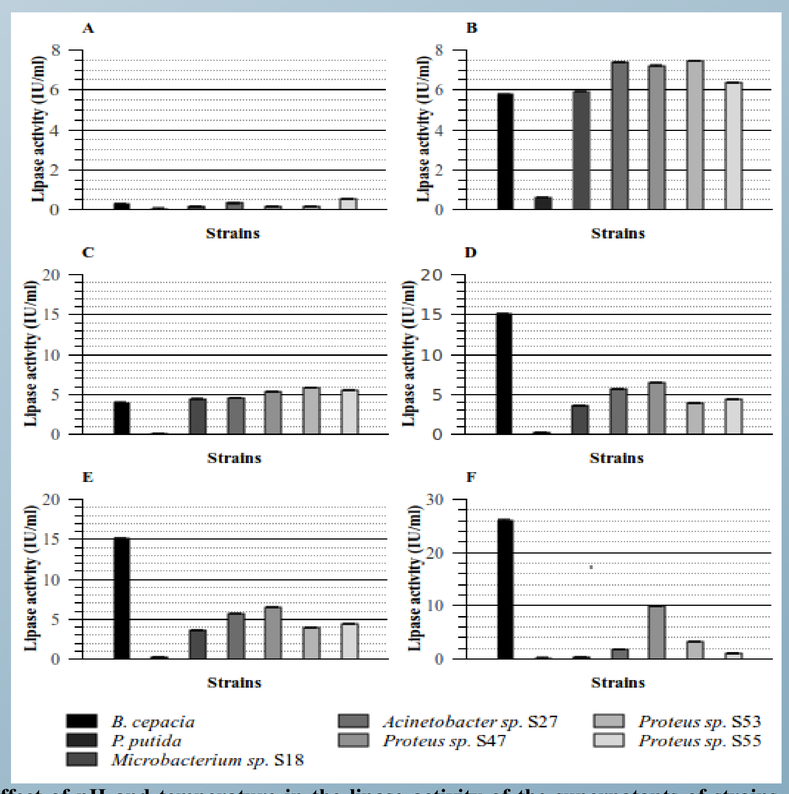

Figure 1. Effect of pH and temperature in the lipase activity of the supernatants of strains. (A) and (B) assays at 15ºC, under pH 5 and pH 8 respectively; (C) and (D) assays at 20ºC under pH 5 and pH 8 respectively; (E) and (F) assays at 30ºC under pH 5 and pH 8 respectively. *B. cepacia* and *P. putida* are included as positive and negative lipase activity controls, respectively.

Results

As illustrated in Figure 1, effect of temperature and pH on lipase activity of the different isolates can be monitored by incubation of the microplates at different temperatures after preparing them with the appropriate buffer. The effect of pH, temperature and the combination of both parameters can be studied attending to one of the strains, but also the differences within several strains under one particular temperature and pH combination can eassily be observed. Particularly, it can be observed that the strains' lipases tested showed a higher lipase activity at pH 5 when temperature of incubation was 20ºC and 30ºC, over the positive control level, whereas they showed lower lipase activities at pH 5 when temperature of incubation was set at 15ºC.

Similarly, as illustrated in Figure 2, effect of pH and organic solvent over the lipase activity of the different strains. For influence of particular conditions over a particular strain's lipase activity, it is better to look for its corresponding bar only. As an example, *Microbacterium sp.* S18 and *Proteus* sp. S53 lipases showed a better activity level in presence of methanol at pH 5, but its performance at pH 8 was quite similar with both ethanol or methanol. Thus, these strains' supernatants could be suitable for the methanolysis of fatty acids under acidic conditions as well as for the ethanolysis under alkaline conditions, so the two mentioned strains could be used for the production of biodiesel from fatty sources of variable pH and fatty acids quality. Besides, it is worth mentioning that trends of all the strains for determined solvents of interest can be observed when looking to the charts contained in figure 2 as a whole. Thus, as can be observed comparing pH5 and pH8 charts, all studied strains' lipases developed a higher activity in presence of acetone, benzene or n-hexane both at high (30%) and low (5%) concentration when reaction mix was adjusted at pH 5, while at pH 8 the higher lipase activity levels appeared in presence of low concentration of benzene (5%). These findings can be very interesting for fine chemicals synthesis from fatty precursors for both the cosmetics and pharmaceutical industry.

Conclusions

Proposed modifications to the classical *p*-NPP spectrophotometrical method for the determination of lipase activity of supernatants from lipolytic strains bring the opportunity to process more samples under different conditions, saving experimentation time and reducing the proportion of reagents needed for every sample. Besides, with the addition of organic solvents to the reaction mix, it is possible to determine the susceptibility or resistance of the lipase of interest to different organic solvents and have a more realistic approach to "real world" systems like processes of production of biodiesel or fine chemicals for food industry, cosmetics or pharmaceutical products.

References

*[1] Gupta N, Rathi P & Gupta R (2002) Simplified para-nitrophenyl palmitate assay for lipases and esterases. Analytical Biochemistry **311**: 98-99.*

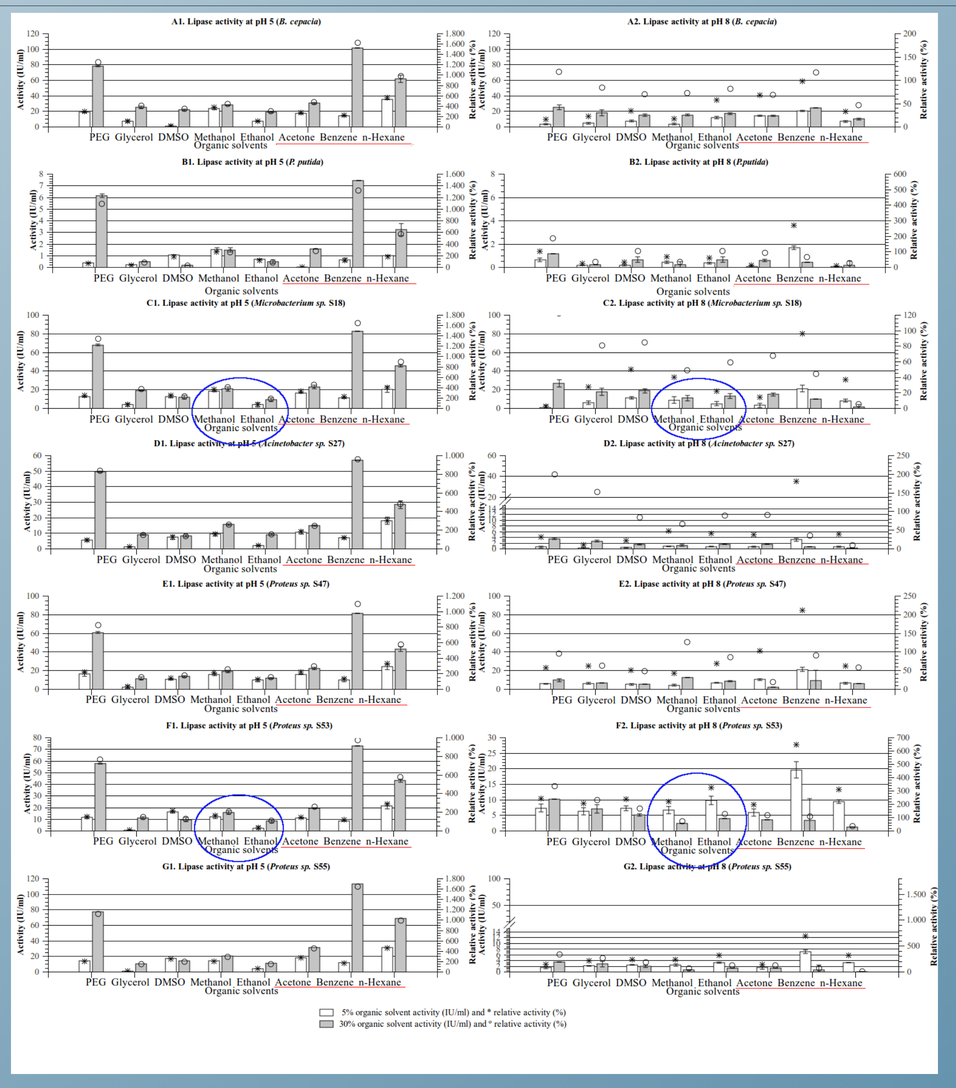

Figure 2. Organic solvents and pH effect over lipase activity of studied supernatants. Left side panels represent lipase activity for each strain under acidic (pH 5) conditions in presence of different solvents. Right side panels represent activities under alkaline conditions (pH 8) for the same strains and solvents. At each chart, left axis represents lipase activity whereas right axis represents relative activity (taking 100% as the lipase activity without addition of organic solvents). *B. cepacia* and *P. putida* are included as positive and negative lipase activity controls, respectively.