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# Rapid Determination of the Synthetic Activity of Lipases/Esterases via Transesterification and Esterification Zymography

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## ABSTRACT

*A new simple and extremely versatile zymography method based on transesterification and esterification reactions was developed in this work. The method consists in building a transesterification or esterification reaction medium according to the goal of the research. Since commercial enzymes and crude extracts commonly consist in pools of proteins, this methodology provides a means to determine which protein are responsible for the enzymatic activity desired. The protocol may be potentially used as a high-throughput screening of lipases or esterases that catalyzes the synthesis reaction for biodiesel and biolubricants production.*

Keywords: transesterification reactions, zymography, biodiesel, lipase and biolubricants.

## INTRODUCTION

Zymography is a method that consists in the use of a polyacrylamide gel electrophoresis (PAGE) to determine enzymes activity that has been largely use not only for proteolytic enzymes reactions [Paech et al., 1993, García-Tejedor et al., 2015] but also for lipases and esterases detection in both hydrolytic [Johri et al., 2001], and synthetic reactions [Kwon et al., 2011]. This method is based on staining the protein band responsible for the desired activity usually by precipitation of a reaction product. The use of this technique for the detection of transesterification activity has not been reported until the present date. Thus, the aim of this work is to describe one new, quick, and simple method that allows the detection of enzymes able to catalyze transesterification reactions, such as the biodiesel and biolubricants production [Aguieiras et al. 2014, Da Silva et al., 2015]. The methodology could be useful in high-throughput screening of lipases/esterases, permitting the identification of the enzyme/s that is/are the responsible/s for the detected activity, as this should be coupled to a protein band in the PAGE experiment, allowing even possible future identification, extraction and purification of specific lipases/esterases.

## MATERIALS AND METHODS

The pre-stained molecular weight SeeBlue® Plus 2 was obtained from Novex by Life Technologies (PMW). Lipase AY Amano 30 (AY) was purchased from Amano Enzyme Inc. (Japan). Lipozyme TL 100L (TL) was provided by Novozymes S/A (Denmark). Lipomod 34 MDP™ (MDP) was from Biocatalysts Inc. (USA) and Lipase from *Pseudomonas* sp. type

XIII (XIII) was purchased from Sigma Aldrich. The enzyme preparation consists of a dry fermented solid (DFS) of *Rhizomucor miehei* fungus, previously reported by Aguieiras et al. (2014) and the crude enzyme extract was obtained as described by Gombert et al. (1999). The oleic acid was purchased from VETEC, Sigma Aldrich (Brazil), the trimethylolpropane (TMP) from Sigma Aldrich (Brazil) and the methyl esters from castor oil (castor biodiesel esters) was provided by PETROBRAS (Rio de Janeiro, Brazil). All enzymes were diluted to an appropriated concentration according to their specific activity in 5 mM phosphate buffer, pH 7.0 to be used at zymography assays.

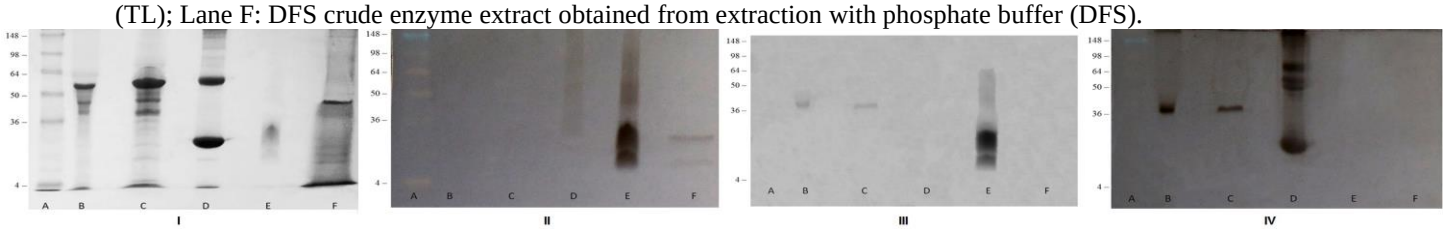
Enzyme samples were loaded, without boiling, in 12 % running and 4 % stacking PAGE gels. Sample protein has been diluted in Laemmli's sample-loading buffer [Laemmli, 1970] excluding β-mercaptoethanol and containing bromophenol blue and SDS. The electrophoretic running gel was performed at 180 V at room temperature. The gel was stained with Coomassie Brilliant Blue to identify proteins bands. For the zymography same SDS-PAGE procedures were performed. After the electrophoretic run, gels were soaked with 1 % Triton X-100 in 5 mM phosphate buffer pH 7.0, for 20 min. Subsequently, they were washed with distilled water and re-equilibrated in 5 mM phosphate buffer pH 7.0 for 10 min. Finally, the gels were submerged in three reaction media which were prepared according to the final activity desired. For biodiesel production, the medium was composed of ethanol and oleic acid in a molar ratio of 1:1. For biolubricant reaction, the medium consisted of oleic acid and TMP, in 3:1 molar ratio. The third reaction castor biodiesel esters and TMP in a molar ratio of 4.5:1 were used [Da Silva et al., 2015]. For all reaction medium, the substrates were blended together and the gels subjected to each reaction. They were left stirring in a shaker in these reaction mixtures at a minimum rotation (approximately 50 rpm) at 45°C for 24h when TMP is used for biolubricant reaction and 3h in the case of ethanol for biodiesel reaction, however, the time can vary according to the enzyme activity. The gels were washed and conditioned with acetic acid 1% until image acquisition.

## RESULTS AND DISCUSSION

Commercial enzymes and crude extract consist of a complex mixture of proteins (Fig.1: Gel I). Lipases and esterases with synthetic activities were detected in the transesterification and esterification zymograms (Fig.1: Gels II, III, IV) by the *in situ* white precipitates that were obtained when the reaction occurred, corresponding to fatty acid esters formed by the enzyme-catalyzed reaction between fatty acids/ esters and alcohols/ polyols. These white precipitates (bands) are shown in black due to a digital color inversion. Thus, it was possible to identify the enzymes that are responsible for the synthetic activity of commercial preparations and DFS extract upon the target substrates (Fig.1: Gels II, III, IV). The precipitates were water-insoluble, well-shaped and assuredly adhered, and could not be removed by washing in buffers or solvents such as hexane, chloroform, ethanol or Folsch reagent, even under sonication. Moreover, the removal was not possible by friction, showing the material was not adsorbed onto to the mesh, but was synthesized through the polyacrylamide network. In addition, stains or any other similar deposits were not observed throughout gel, discarding the possibility that the substrates would spontaneously precipitate. It assures the suitability of the method for such detection.

Fig 1: Gel I: SDS-PAGE of all commercial enzymes and crude extract; Gel II: Esterification zymogram of biodiesel production from the reaction of oleic acid:ethanol, molar ratio 1:1. Gel III: Esterification zymogram of biolubricant production from the reaction of oleic acid: TMP, molar ratio 3:1; Gel IV: Transesterification

zymogram of biolubricant production from the reaction of castor biodiesel esters: TMP, molar ratio 4.5:1.Gels I, II, III and IV: Lane A: Pre-stained molecular protein standard (MPS); Lane B: Lipase AY Amano 30 (AY); Lane C: Lipomod 34 MDP (MDP); Lane D: Lipase from *Pseudomonas* sp. Type XIII (XIII); Lane E: Lipozyme TL (TL); Lane F: DFS crude enzyme extract obtained from extraction with phosphate buffer (DFS).




The commercial lipase XIII showed two major protein bands, about 64 kDa and 20 kDa, and other minor bands (Fig. 1: Gel I: Lane D). These two major bands showed activity in the transesterification zymogram between castor biodiesel esters and TMP so as other two bands with high activity (Fig. 1: Gel IV: Lane D). It indicates that these minority proteins could be interesting for one specific biotechnological process. The DFS crude extract consists in a *pool* of proteins in which could be present lipases or esterases [Aguieiras et al., 2014]. The crude enzyme extract contains diffuse bands distributed along the gel lane (Fig.1: Gel I: Lane F). Two bands (<36 kDa) are responsible for the reaction of oleic acid and ethanol molar ratio 1:1 (Fig. 1: Gel II: Lane F). The commercial enzyme Lipozyme TL 100 L presented a diffuse band around 30 kDa (Fig. 1: Gel I: Lane E) that resulted in a significant activity, as well as other minor bands displayed some activity (Fig. 1: Gels II and III). Amano 30 and Lipomod$^{TM}$ 34 MDP are described as lipases derived from the yeast *Candida rugosa*. This yeast produces several isoforms of lipases around 60 kDa [Trbojević et al., 2013], corresponding to the main band of the preparation as the band of major intensity (Fig.1: Gel I: Lanes B and C). However, there are other minor bands showed in the same figure but only one of these bands (about 40 kDa) was responsible for the activity showed in Fig. 1: Gels III and IV.

Da Silva et al. (2015) developed a biolubricant production from transesterification between castor biodiesel esters and TMP catalyzed by lipase 34P (whose was discontinued and substituted by 34 MDP$^{TM}$ at Biocatalysts). Based on these results, we developed a zymogram transesterification to investigate the possibility of determine the specific bands of protein which are able to catalyze this biolubricant reaction. Zymography was performed applying the transesterification medium previously developed by them. The results were shown in Fig. 1: Gel IV. The enzymes AY (Lane B), 34 MDP (Lane C) and XIII (Lane D) presented bands with white precipitate, indicating a high potential of these enzymes to produce trimethylolpropane esters from this substrate. The production of superior alcohol esters with lubricating properties via enzymatic processes has been increasingly studied, as well as the biodiesel production [Akerman et al. 2011, Da Silva et al. 2015, Malhotra et al., 2015]. In this context, we developed a procedure to detect enzymatic biolubricants production by zymography using oleic acid and TMP as substrates[Akerman et al. 2011, Da Silva et al. 2015, Cavalcante et al., 2014], because of their worldwide access and large use in literature for the biolubricants production (Fig. 1: Gel III). Besides AY, 34MDP and TL presented positive activity in transesterification between oleic acid and TMP; the first two enzymes did not show synthetic activity over oleic acid and ethanol (Fig. 1: Gel II). In addition, the same two enzymes presented transesterification activity when the substrate was the castor biodiesel esters (Fig. 1: Gel IV). The enzyme XIII, who formed products in the castor biodiesel esters reaction (Fig. 1: Gel IV), gave a negative result with oleic acid as substrate (Fig.1: Gels II,

III). In parallel, the TL commercial preparation showed activity for the oleic acid, but did not show activity when the substrate was castor biodiesel esters (Fig. 1: Gel IV). The diversity of results for the reactions highlights the need to apply a specific test for each enzyme activity, based on reactions and applications of interest.

Thus, is possible to indicate which protein bands are more active, and also, whose protein band will have more affinity for a specific substrate. It has great importance since commercial enzymes commonly consists of a pool of proteins, whose their activities are measured as hydrolytic activity upon classical and robust substrates in the literature, such as olive oil and p-nitrophenyl laurate.

## CONCLUSIONS

The methodology proposed in this work is a new simple and extremely versatile zymography method based on transesterification and esterification reactions. This methodology could is being useful to determine, in a pool of several proteins, the ones responsible for the synthetic activity required since, in many times, they are not the main proteins in commercial or non-commercial enzyme preparations. Thus, this protocol may be potentially used as a high-throughput screening of enzymes – lipases and esterases – that catalyzes the reaction for biodiesel or biolubricant production, presenting a new alternative process optimization, allowing trying the best method according to the biotechnology application.

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