PH.D. THESIS

Analysis of extracellular lipase enzymes from zygomycetes fungi: enzyme production, characterization of synthetic and hydrolytic reactions

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INTRODUCTION

Lipase enzymes (EC 3.1.1.3) hydrolyze triacylglycerols, which are the major constituents of fats and oils. Besides, most lipases are able to catalyze the synthesis and translocation of ester linkages, mainly under low water content or non-aqueous conditions. In addition to their biological importance, lipases have important role in different biotechnological and industrial processes due to their diverse catalytic properties and substrate specificity. Their activities are utilized in the food-, pharmaceutical-, leather-, and detergent industries, as well as in the production of fine chemicals and biodiesel. Most of the current commercial enzymes are derived from microbial sources produced by bacteria or filamentous fungi. The main advantage of enzyme production by microbes is that relatively large amounts of enzyme can be produced economically. In addition, lipases derived from diverse microorganisms have different biochemical characteristics, namely substrate specificity, temperature and pH optimum and stability, etc.

Mucoromycota fungal group is one of the most important representatives of filamentous fungi. Besides of their ecological significance, medical, industrial, biotechnological and agricultural important species can be found between them. Among zygomycetes, many strains are known as good extracellular enzyme producers, however, a limited information available about their lipase production, and the biochemical characteristics of the produced hydrolytic and synthetic activities as well. However, identification and characterization of novel microbial lipases with promising hydrolytic and/or synthetic properties have special importance for industrial and biotechnological process development purposes.

Use of agro- and food industrial by-products such as crop and oilseed residues is a low-cost and environmental friendly biotechnological technique for production of lipase enzymes with industrial interest.

OBJECTIVES

The objective of our study was to identify extracellular lipase sources and enzymes from zygomycetes fungi, which can be used as a basis for further basic and applied researches. In addition, our aim was the examination of enzyme production by lipase producer isolates on various fermentation conditions applying agricultural and food industrial residues as substrates. Our goals included the isolation of enzymes and biochemical characterization of lipase-catalysed hydrolytic and synthetic reactions.

For this purpose, the following specific objectives have been formulated:

1. Screening of extracellular lipase production of zygomycetes strains belonging to the genera *Mucor, Rhizomucor, Rhizopus, Gilbertella*, *Dissophora*, *Gamsiella*, *Mortierella* and *Umbelopsis* using tributyrin contained agar plates.

2. Investigation of lipase production of selected strains under various culture conditions (e.g. effect of lipid inductors and substrates with high lipid content on the enzyme production).

3. Analysis of transesterification and esterification reactions catalysed by selected crude enzyme extracts. Characterization of the synthetic activities under various reaction conditions.

4. Purification and identification of lipase enzymes from extracts with high synthetic and hydrolytic activities.

5. Biochemical characterization of the hydrolytic activity of the purified lipases (e.g. determination of pH and temperature optimum and stability, kinetic parameters, substrate specificity, and analysis of potential inhibitors).

METHODS

Analysis of extracellular enzyme production:

- Tributyrin plate assay
- Inductive minimal medium
- Submerged fermentation (SmF) using mineral growth medium supplemented with wheat bran
- Solid state fermentation on plant derived substrates moisturised with water (SSF1) or mineral salt solution and olive oil (SSF2)

Determination of lipase activity:

- Assay of hydrolytic activity using *p*-nitrophenyl palmitate (*p*NPP) substrate
- Analysis of the transesterification using *p*NPP substrate and gas chromatography (GC) technique
- Determination of the esterification activity using gas chromatography (GC) technique

Determination of the total protein content:

- Bradford method
- Application of Qubit™ Fluorometer and Quant-iT Protein Assay Kit (Life Technologies)

Methods used for enzyme purification:

- Ammonium sulfate precipitation
- Size-exclusion chromatography
- Anion exchange chromatography
- Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
- Native Polyacrylamide Gel Electrophoresis (Native-PAGE)
- Protein staining with silver nitrate
- Zymogram analysis using fluorogenic and chromogenic substrates

Biochemical characterization of hydrolytic activities:

- Reaction mixtures to analyse the factors (e.g. temperature, pH, etc.) affecting the enzyme activity
- Determination of kinetic parameters by Lineweaver-Burk plot
- Regioselectivity studies using thin-layer chromatography (TLC)

RESULTS

1. Several Mucoromycota strains with high extracellular lipolytic activity have been identified. (Takó et al., 2012; Kotogán et al., 2014)

A total of 204 zygomycetes strains belonging to the genera *Gilbertella*, *Rhizomucor*, *Rhizopus*, *Mucor*, *Dissophora*, *Gamsiella*, *Mortierella* and *Umbelopsis* were screened for their tributyrin hydrolyzing capacity. According to the lipolytic halo and colony diameters, 21 promising lipase producers have been identified. From these, *Mo. alpina*, *Mo. echinosphaera*, *M. corticolus*, *R. miehei*, *Rh. oryzae*, *Rh. stolonifer*, *U. autotrophica*, *U. isabellina*, *U. ramanniana* var. *angulispora* and *U. versiformis* isolates were selected for further investigations.

2. Lipase production of lipolytic strains has been studied under various inductive conditions using submerged and solid-state fermentation. (Kotogán et al., 2014)

A range of different oils and oil-based materials were tested for their ability to induce the lipase production of the selected strains. In general, Tween 80 and olive oil proved to be good inducers for lipase production since most of the investigated fungi displayed high enzyme activity when the media was supplemented with these oils. Other lipid materials such as soybean, sesame- and cottonseed oils also enhanced the enzyme production of some isolates.

Wheat bran is well documented as a good inducer for lipolytic activity of various filamentous fungi. Therefore, lipase production of the selected strains was also tested in wheat bran-based submerged (SmF) and solid-state fermentation (SSF) systems. Wheat bran-based SmF resulted in higher volumetric activities for the *Mo. echinosphaera*, *Rh. stolonifer*, *U. autotrophica*, *U. ramanniana* var. *angulispora* and *U. versiformis* isolates than those obtained under minimal conditions. For each isolate, maximum lipase activities were observed at different phases of the fermentation. Two fermentation media were compared to evaluate the enzyme production on wheat bran-based SSF: a simple medium containing only distilled water to moisturize the wheat bran (SSF1) and a medium supplemented with mineral salt solution and 1.5% olive oil (SSF2). During the fermentation on SSF2, enzyme production of *R. miehei*, *Rh. oryzae* NRRL 1526, *Rh. stolonifer* and *U. versiformis* isolates improved considerably showing at least four times higher enzyme activities than on SSF1. When mineral salt solution and olive oil were used as supplements, the *R. miehei*, *M. corticolus*, *U. autotrophica*, *U. ramanniana* var. *angulispora* and *U. versiformis* also proved to be promising lipase producers on wheat bran, expressing specific activities of 4415, 1733.4, 416.3, 355.7 and 287.1 U/g of dry substrate,

respectively. Besides wheat bran, other plant residues with high lipid content were also tested, wherein the highest lipase yield could be detected on poppy seed and pumpkin seed grists.

3. Transesterification and esterification activities of crude zygomycetes lipases have been studied and characterized. (Kotogán et al., 2014; Kotogán et al., 2016)

In these experiments, crude lipase extracts obtained after wheat bran-based SSF of selected isolates were used. Transesterification activity was determined after 30 min incubation using *p*NP-palmitate (*p*NPP) as acyl donor and ethanol as acyl acceptor. The crude enzymes from *R. miehei*, *Rh. stolonifer*, *Mo. echinosphaera*, *Rh. oryzae* NRRL 1526 and NRRL 1472 and *M. corticolus* isolates showed the highest activities (1.7 – 4.8 U/mg).

To characterize the alcoholysis reactions, various alkanes and cycloalkanes were employed as reaction medium and their effect on the efficiency of transformation were examined. Generally, *n*-heptane was the most effective as reaction medium. Effect of temperature (20 – 50 °C) on transesterification reactions was investigated. The conversion rates increased linearly during the 6 h incubation at 40 °C, except for lipases of *Rh. oryzae* NRRL 1472, *U. autotrophica* and *U. versiformis*, which reached the maximal *p*NP conversion at the fourth hour. The *Rh. stolonifer*, *Rh. oryzae* NRRL 1526, *M. corticolus*, *Mo. echinosphaera* and *Mo. alpina* crude lipases showed faster initial conversion at 50 °C than 40 °C, which can be explained by the catalysis-stimulating effect of high temperature. Time course of the transesterification was also studied where reaction rate increased smoothly within the first 48 h, and a steady conversion was obtained after 96 h. The highest conversions, 90.5 and 88.5%, were achieved by *R. miehei* and *Mo. echinosphaera* enzymes. Transesterification of *p*NPP were also investigated using various alcohols as acyl acceptor molecules. The transesterification reaction takes place in the presence of all tested alcohols, and the highest *p*NPP conversion yields were achieved with ethanol. The effect of initial ethanol concentration on conversion was monitored by varying ethanol in the range of 0.85 – 5.1 M (5 – 30%, v/v). The conversion grew rapidly as the ethanol concentration increased up to 3.4 M by *R. miehei*, *Rh oryzae* NRRL 1526 lipases and up to 4.2 M by *Rh. stolonifer* and *M. corticolus* enzymes. When various acyl donor molecules were tested, higher *p*NP conversion yields were generally achieved for medium-chain aryl esters with C8 and C12-long fatty acids.

Esterification reactions between palmitic acid and ethanol were also investigated with some selected crude enzymes using gas chromatography detection of the obtained ethyl palmitate product. Concentrations of 9.77, 9.98 and 10.54 mg/L ethyl palmitate were obtained after 48-hour incubation for *Mo. echinosphaera* and *R. miehei* lipases, and after 24-hour for *Rh.*

stolonifer enzyme, respectively. Esterification reactions were much slower and resulted in less specific activities than transesterification. Fatty acid preference of the lipases during esterification was also investigated, where enzymes showed affinity for C16 – C18 fatty acids.

4. Lipase enzymes from crude extracts with high synthetic and hydrolytic activities have been purified. (Takó et al., 2017)

Based on our previous experiments, purification of *R. miehei* NRRL 5282, *Rh. oryzae* NRRL 1526, *Rh. stolonifer* SZMC 13609, *M. corticolus* SZMC 12031 and *Mo. echinosphaera* CBS 575.75 extracellular lipases were performed. To purify the enzymes, ammonium sulfate precipitation, size-exclusion and ion exchange separations were combined. Molecular weight of the purified enzymes was estimated by SDS-PAGE; which was approximately 55, 35, 28, 20 and 30 kDa for *R. miehei*, *Rh. oryzae*, *Rh. stolonifer*, *M. corticolus* and *Mo. echinosphaera* lipases, respectively. Zymogram analysis of the purified enzymes showed active lipases stained with 4-methylumbelliferyl nonanoate and *α*-naphthyl acetate. Each purified enzyme catalyzed the transesterification between ethanol and *p*NPP.

5. Hydrolytic activity of the purified lipases has been characterized under various reaction conditions. (Takó et al., 2017)

Biochemical characterization of the purified enzymes was performed considering several factors that can affect the hydrolytic activity. The temperature optimum for maximal lipolytic activity was 20 – 30 °C for *Mo. echinosphaera*, 30 °C for *M. corticolus* and *Rh. oryzae,* 40 °C for *R. miehei* and 50 °C for *Rh. stolonifer* enzymes. The *R. miehei* and *Rh. stolonifer* lipases can be considered as thermotolerant because they were stable up to 50 °C. Remarkably residual activities could be detected for the *Mo. echinosphaera* lipase at 20 °C, and for the *Rh. oryzae*, *Rh. stolonifer* and *M. corticolus* enzymes at 5 °C.

The *R. miehei* and *M. corticolus* enzymes had a pH optimum at slightly alkaline pH values from 6.8 to 7.4 and from 7.0 to 7.4, whereas that of the *Rh. oryzae* and *Rh. stolonifer* lipases was found to be in acidic pH range from 5.0 to 5.4 and from 4.6 to 5.0, respectively. The *R. miehei* and *M. corticolus* enzymes were stable from pH 7.0 to 8.0 and 6.2 to 7.4, respectively. The *Rhizopus* enzymes retained most of their initial activity at lower pH ranges: the *Rh. oryzae* enzyme was stable between pH 5.4 and 6.8, while the *Rh. stolonifer* between pH 4.2 and 5.4. The purified *Mo. echinosphaera* lipase had the pH optimum between pH 6.6 and 7.0; the enzyme proved to be active and stable between pH 4.6 to 8.0 and pH 3.4 to 8.0.

The *R. miehei* lipase had highest specificity for *p*NP esters with C6 – C12 acids, while the *Rhizopus* enzymes showed preference for C8 – C12 aryl substrates. In contrast, the *Mo. echinosphaera* and *M. corticolus* lipases exhibited wider substrate specificity, since they could effectively hydrolyze the *p*NP ester substrates with C3 – C10 and C4 – C12 long fatty acids, respectively. Triolein hydrolysis studies showed 1,3-regioselectivity for each purified lipase. Kinetic parameters of the purified lipases were also determined using different concentrations of *p*NPP substrate.

The effect of various metal ions and reagents on *p*NPP hydrolysis was also investigated. Significant inactivation was observed with Hg^{2+}, NBS and SDS. However, the Na^{+} and K^{+} in 5 mM concentration enhanced the activity of most enzymes by 5 – 37%, which may be due to its enzyme conformation stabilizing effect. In particular, the *Rh. stolonifer* and *Mo. echinosphaera* lipases showed high stability in most of the metal salts investigated.

Methanol, ethanol, propanol and isopropanol in low concentrations (5 – 10%, v/v) had no considerable effect on the *p*NPP hydrolysis catalyzed by the *R. miehei* and *Rh. oryzae* lipases. The *Mo. echinosphaera* lipase was stable in the presence of 5 – 15% (v/v) methanol, ethanol and isopropanol, while the *Rh. stolonifer* and *M. corticolus* lipases in concentrations up to 20% (v/v). Butanol and hexanol, and for certain enzymes, the isopentanol inhibited the *p*NPP hydrolysis. Increased *p*NF yield could be observed in some reaction mixtures as compared with the control, which may be attributed to the *p*NPP transesterification occurred as a result of the reduced water activity. All enzymes tested proved to be stable at high concentrations of *n*-hexane, cyclohexane, *n*-heptane and isooctane.

SUMMARY

1. 21 Mucoromycota strains with high extracellular lipolytic activity have been identified.
2. Lipase production of lipolytic strains has been studied under various inductive conditions using submerged and solid-state fermentation. Enhanced enzyme production was identified applying certain lipid materials and culture conditions.
3. Transesterification and esterification activities of crude zygomycetes lipases have been studied and characterized. To characterize the alcoholysis reactions various reaction conditions, acyl acceptors and donors with various chain lengths were employed. As far as we know, this is the first study on the catalyzation of synthetic reactions in organic media with extracellular *Mortierella* and *Umbelopsis* lipases.
4. Purification and isolation of *R. miehei* NRRL 5282, *Rh. oryzae* NRRL 1526, *Rh. stolonifer* SZMC 13609, *M. corticolus* SZMC 12031 and *Mo. echinosphaera* CBS 575.75 extracellular lipases were performed produced on wheat bran based fermentation systems. Our study is the first in which the isolation and characterization of extracellular lipase enzymes from *Rh. stolonifer* and *Mortierella* isolates have been described.
5. Hydrolytic activity of the purified lipases has been characterized under various reaction conditions. As a results, some thermotolerant as well as broad pH tolerant enzymes were identified. Each enzymes had 1,3-regioselectivity, and efficiently hydrolyzed *p*NP-esters with medium-chain fatty acids. The isolated lipases retained most of their initial activities in the presence of some metal ions, reagents and organic solvents. Our results suggest that the investigated lipases possess properties that can be valuable for future basic studies and biotechnological applications, particularly for the organic synthesis processes.

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