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Proceeding Paper

Investigating Culture Media for Obtaining Lipolytic Biocatalysts Based on *Rhizopus oryzae* Fungi †

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Abstract: *Rhizopus oryzae* is widely distributed in nature and can be isolated from different substrates such as decomposing vegetables, fruits and various soils. It is generally classified as GRAS filamentous fungi and commonly used in the production of oriental traditional food such as tempeh or peka. This microorganism has a great industrial potential due to the capability to synthesize enzymes (glucoamylases, cellulases and lipases) and organic acids (lactic acid, fumaric acid). The most studied enzymes of the fungi are lipases (ROL). Therefore, the aim of the study was the selection of growth medium content and initial pH rate, which would provide high lipase synthesis yield in 5 days shaken cultures. Two fractions of lipases were investigated in order to obtain lipase biocatalysts: extracellular enzymes present in supernatant and cell-bound lipases in biomass. There were used nutrient-rich media: YPG (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), YPO (10 g/L yeast extract, 20 g/L peptone, 20 g/L olive oil), YMG (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L glucose), YMO (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L olive oil) and mineral media: SMG (10 g/L peptone, 14 g/L KH_2PO_4, 2.4 g/L K_2HPO_4, 0.4 g/L $MgSO_4$, 20 g/L glucose) and SMO (10 g/L peptone, 14 g/L KH_2PO_4, 2.4 g/L K_2HPO_4, 0.4 g/L $MgSO_4$, 20 g/L olive oil). Fungi biomass and supernatant were separated and used to measure lipase activity by a spectrophotometric method based on the hydrolysis of *p*-nitrophenyl laurate. The results showed that the highest lipase activity after 5 days of cultivation was reached in YPO medium for biomass (from 7- to 60-fold higher results depending on compared variant of culture media) and YMG for supernatant (from 3- to 6.5-fold higher results depending on used variant of culture media). The addition of citric acid resulted in two times increase of the activity of produced lipases after 5 days of cultivation.

Keywords: *Rhizopus oryzae*; lipases; culture medium

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1. Introduction

Rhizopus oryzae is widely distributed in nature and mostly isolated from alcoholic beverages or oriental foods in China, Japan and Indonesia [1]. This microorganism is well known as a primary or secondary colonizer, by fast invasion of digestible substrates, because of its rapid growth. *R. oryzae* can use different plant compounds and polysaccharides as an energy and carbon source. It is generally classified as GRAS filamentous fungi and broadly employed in industry due to capability to synthesize a great variety of prod-

ucts like enzymes (proteases, cellulases, lipases) and organic acids (lactic and fumaric acids) [2]. The most studied enzymes of this fungi are lipases, because they can carry out esterification and transesterification in organic media. They are classified as enzymes that hydrolyze fats and oils with subsequent release of free fatty acids, diacyloglycerols, monoacylglycerols and glycerol. *R. oryzae* lipases (ROL) activity has a strong *sn*-1,3 regiospecificity which makes its activity attractive for several industrial processes such as fat and oil modification for structured lipids production [3,4]. Therefore, the aim of the study was the selection of growth medium content and initial pH rate, which would provide high lipase synthesis yield in shaken cultures of *R. oryzae*.

2. Materials and Methods

2.1. Materials

Microorganism *Rhizopus oryzae* DSM 2199 was purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany). Chemicals and medium ingredients were purchased from Sigma-Aldrich (Burlington, MA, USA) and Avantor Performance Materials (Gliwice, Poland).

2.2. Methods

2.2.1. Culture Media and Fungi Cultivation

Culture media used in the research are presented in Table 1. Inoculation was conducted by adding 1 mL of *R. oryzae* spores suspension to 200 mL sterile medium and then cultured on a rotary shaker (150 rpm) for 3 and 5 days in 30 °C. To investigate the influence of medium acidification the medium with 1 g/L NH_4NO_3, 1 g/L $(NH_4)_2SO_4$, 4 g/L K_2HPO_4, 2 g/L KH_2PO_4, 1 g/L NaCl, 10 g/L% olive oil and 1 g/L yeast extract was used. The medium was acidified with 10% citric acid. At the end of experiment biomass was separated from supernatant by filtration.

Table 1. Culture media composition.

Culture Media	Composition
YPG	10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose
YPO	10 g/L yeast extract, 20 g/L peptone, 20 g/L olive oil
YMG	3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L glucose
YMO	(3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L olive oil
SMG	10 g/L peptone, 14 g/L KH_2PO_4, 2.4 g/L K_2HPO_4, 0.4 g/L $MgSO_4$, 20 g/L glucose
SMO	10 g/L peptone, 14 g/L KH_2PO_4, 2.4 g/L K_2HPO_4, 0.4 g/L $MgSO_4$, 20 g/L olive oil

2.2.2. Hydrolytic Activity Measurements

Hydrolytic activity was evaluated by a spectrophotometric method of measuring the progress of the p-nitrophenyl laurate hydrolysis described by Kapturowska et al. [5]. Hydrolytic activity was measured in both fractions, i.e., extracellular enzymes present in supernatant and cell-bound lipases in biomass. The reaction was carried out in Erlenmeyer flask. Firstly, the 0.3 mmol of p-nitrophenyl laurate was dissolved in 2 mL of heptane. Secondly, 1 g of biomass was dissolved in 15 mL of distilled water. In reaction 15 mL of supernatant was also used. Substrate and biocatalyst were stirred for 30 min at 37 °C. The absorbance was measured at 410 nm in UV/Vis spectrophotometer. The unit of lipase enzymatic activity was 1U, i.e., the amount of enzyme that released 1 μmol of p-nitrophenol per minute under the assay conditions.

2.2.3. Statistical Analysis

Statistical analysis was performed using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). The Shapiro–Wilk test was used for the normality of data distribu-

tion, while the Brown–Forsythe test was used to assess the equality of variances. The results were analyzed using a one-way analysis of variance (ANOVA) and Tukey's post hoc test. The significance level was $\alpha = 0.05$.

3. Results

Two fractions of *R. oryzae* lipases were investigated in order to obtain biocatalysts: extracellular enzymes present in supernatant and cell-bound lipases in mold biomass. Different culture media were examined which contained glucose or olive oil as carbon source. As can be seen in Figure 1 the highest lipolytic activity for cell-bound lipases was reached in 72 h culture in YPO medium (7.466 U/g), which was plentiful in nutrients and included olive oil abundant in oleic acid. In the rest of culture media the lipolytic activity was significantly lower. For extracellular lipases the maximum lipase activity was obtained in YMG medium (0.571 U/mL). (Figure 2). The lipolytic activity was slightly higher than for other used media which contained glucose as carbon source (YPG, SMG) and considerably higher for media YPO, YMO, SMO.

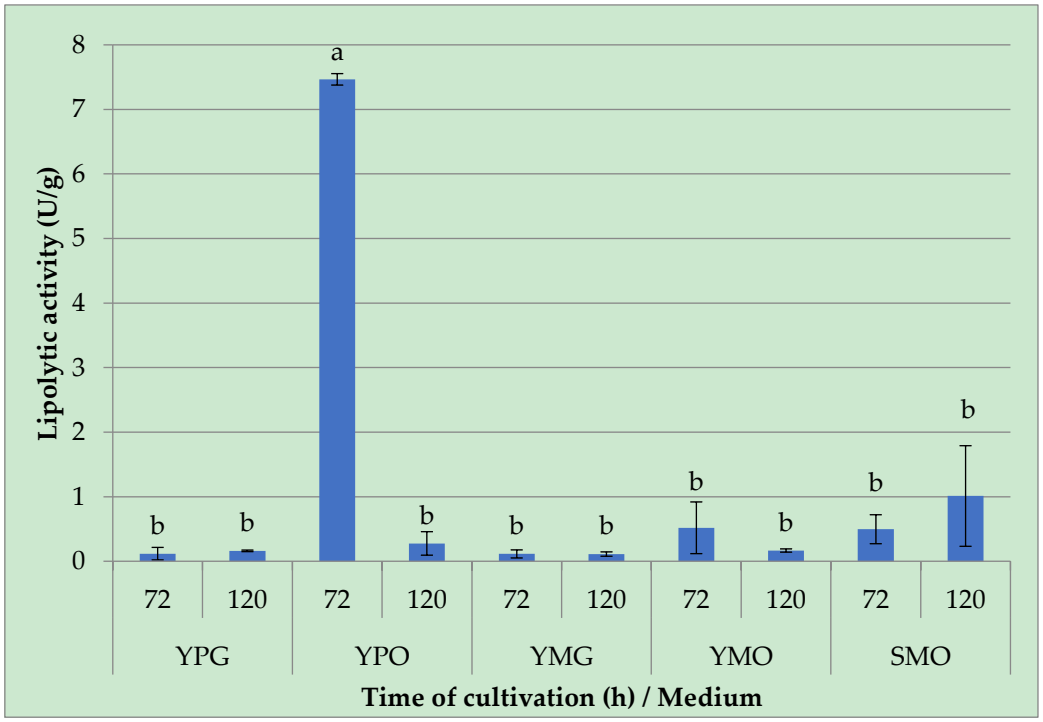

Figure 1. Lipolytic activity of cell-bound lipases in *R. oryzae* biomass in 72 h and 120 h shaken culture. The values with the same letter did not differ significantly ($\alpha = 0.05$).

Similar culture conditions were used with strain of *R. oryzae* WPG (ROL_W). Salah et. al. [6] observed that the maximal production of extracellular lipase was reached at pH 6, 30 °C and 72 h of growth in medium with glucose as a carbon source. The presence of triacylglycerols such as lipids in olive oil has not yet been examined for lipase synthesis by *R. oryzae* cells.

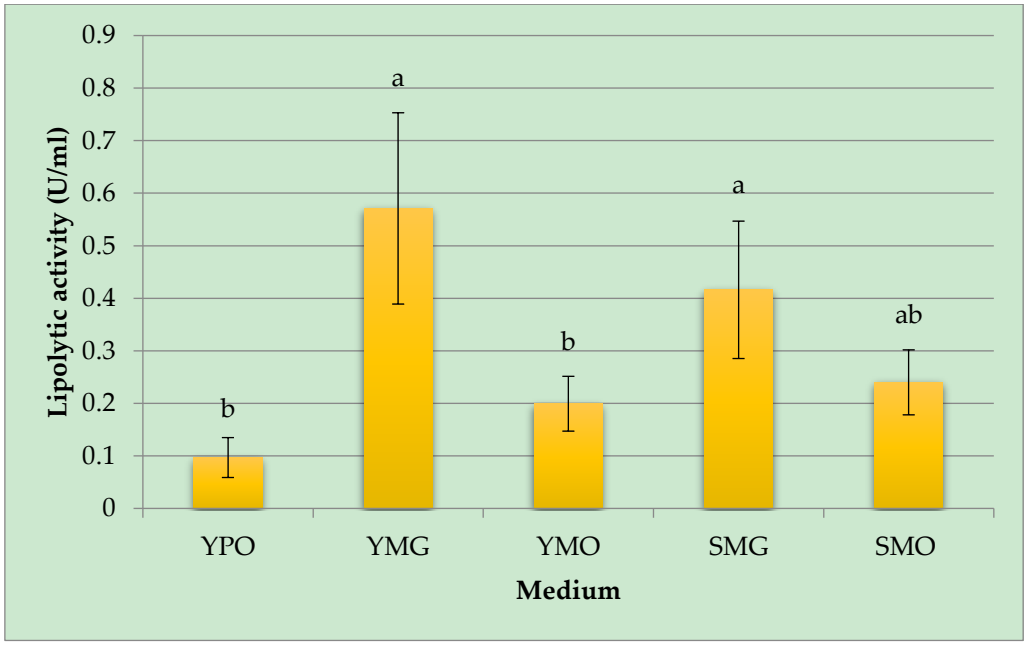

Figure 2. Lipolytic activity of *R. oryzae* extracellular enzymes in 72 h shaken culture. The values with the same letter did not differ significantly (α = 0.05).

The second step of research investigated the effect of citric acid addition to the medium on the production of lipases. In this experiments different types of medium were used and the results were presented in Table 2. The addition of citric acid to medium slightly increased the lipolytic activity in 120 h of culture growth but the difference was not relevant. According to another studies it was shown that the lipase activity of *R. oryzae* is active at high pH but decrease in the presence of acetone, ethers, alkanes and chloroalkanes [1,3]. As can be seen the lipolytic activity of lipases in supernatant was significantly lower comparing with previous experiments and did not obtain satisfactory levels.

Table 2. Lipolytic activity of extracellular enzymes produced by *R. oryzae*. The values with the same letter did not differ significantly (α = 0.05).

Lipolytic Activity (U/mL)			
Medium with addition of citric acid		Medium without addition of citric acid	
Time cultivation:		Time cultivation:	
72 h	120 h	72 h	120 h
0.018 ± 0.017 a	**0.034 ± 0.010 A**	**0.008 ± 0.002 a**	**0.015 ± 0.004 B**

4. Conclusions

In this study it was proven that obtaining two fractions of lipases: extracellular enzymes present in supernatant and cell-bound lipases in biomass from *Rhizopus oryzae* is possible. More investigations for selecting conditions of microorganism growth are needed because in the research only one medium which consisted olive oil (YPO) brought satisfying effects for receiving high results for lipolytic activity. The secretion and lipolytic enzymes activity to culture media did not significantly change in pH rate 3 comparing with pH rate 7.

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