PRODUCTION OF EXTRACELLULAR LIPASE FROM DIFFERENT FUNGUS AS CATALYST FOR THE ENZYMATIC SYNTHESIS OF BIODIESEL

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ABSTRACT

The lipase production activity of six different fungal strains belonging to genera Aspergillus niger, A. oryzae, A. awamori, Trichoderma sp., T. reesei and Penicillium chryzogenum was studied in a submerged fermentation batch. The Morphology and cultural characteristics of the producing strains were investigated microscopically to evaluate the physiological state and purity of the fungal cultures during the fermentation process. The cultivation was carried out in a nutritive modified Reese and Mandels medium containing olive oil as a sole carbon source in a rotary shaker for 120 h at 29±1°C.. P. chryzogenum and A. niger have shown the highest lipase activity among the studied fungal species: 3.5 and 4.3 U/ml, respectively. The maximum activity fo lipase corresponded to the end of exponential and the beginning of stationary phase of strain development. The optimum pH and temperature for enzymatic activity on P.chrysogenum and A. niger lipases were pH = 8.0, 40°C and 45°C respectively. The selected fungi are a good prospective for the development of industrial biotechnology in the process of biodiesel production.

Keywords: Extracellular lipase, microorganisms, fermentation, lipase activity, biodiesel.

1. INTRODUCTION

Lipases are glycerol ester hydrolases (EC 3.1.1.3), which hydrolyze the ester bond of the glyceride in the oil-water interface. During hydrolysis, lipases are attached to the glyceride acyl group to form an acyl-lipase complex, which then transfers its acyl group to the OH groups of water. However, in non-aqueous conditions, these hydrolytic enzymes can transfer acyl groups to carboxylic acid nucleophiles other than water [1]. The common action way of lipases as biocatalyst is:

$$\text{Fat (or) Oil} + \text{Water} \xrightarrow{\text{lipase}} \text{Fatty acids} + \text{Glycerol}$$

Lipases are produced by many microorganisms such as bacteria, fungi and yeasts. Of all these, fungi are recognized as the most powerful producers of lipases [2, 3]. They are also produced in the pancreas of mammals, including pigs and humans. It was also reported its existence in higher plants such as castor bean (*Ricinus communis*) and rapeseed (*Brassica napus*) [4]. Microbial lipase production is important from the economical and industrial standpoint. The industrial sector prefers lipases of microbial origin because of its availability in large quantities since they could have high yields. The importance of the use of lipases in industry is due to the following factors: (1) they act on a wide range of pH and temperature, (2) have high specificity, (3) do not require co-factors, and (4) they can catalyze a wide range of reactions [5]. The interest in microbial lipase production has increased in the last decades, due to its potential application in different industrial sectors such as food, waste water treatment, cosmetics, oil chemicals, pharmaceuticals, detergents [6] and the fuels sector [7]. Due to the wide variety of applications, there has been a renewed interest in developing sources of lipases. The present study reports the organism with the highest yield of lipase, with the necessary characteristics such as optimum pH, optimum temperature for use as biocatalyst in the synthesis of biodiesel. To this end, we analyzed the production of lipase from 6 different molds (*Aspergillus niger, Aspergillus oryzae, Aspergillus Awamory, Trichoderma sp, Trichoderma reesei and Penicillium chrysogenum*), taking into account that the extracellular lipases can be produced on a large scale under standard

laboratory conditions. A search from available information was made in order to find the most suitable substrate and the recommended values of the process parameters to be applied.

2. MATERIALS AND METHODS

2.1 Microorganisms and maintenance of culture

The cultures used in this study were Aspergillus niger, Aspergillus oryzae, Aspergillus Awamory, Trichoderma sp, Trichoderma reesei and Penicillium chrysogenum, and were donated by the University of Food Technologies in Plovdiv, Bulgaria [8]. Slants of Potato Dextrose Agar (PDA) were used for the maintenance of fungal cultures.

2.2 Taxonomic identification of fungi

The observed characteristics of the different crops for identification were the colony diameter, color, exudates and colony texture. The microscopic features for identification were the conidial head, conidiophores, color and shape of the vesicle length, conidia size, shape and texture. At the end the morphological features standard species were compared [9].

2.3 Culture media composition.

Lipase production was tested using submerged fermentation (SmF) on a Mineral Growth Medium (MGM). The MGM composition in g/l was: NaH_2PO_4 12, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.3 and $CaCl_2$ 0.1. Ammonium sulfate-urea from 0.75 to 0.34% and olive oil 2%, were used as nitrogen and carbon sources respectively. For Aspergillus and Penicillium species the initial pH was adjusted to 6 [10], [11] and for Trichoderma species the initial pH was adjusted to 5 [12].

2.4 Lipase production in SmF

The culture was grown in Erlenmeyer flasks containing mineral medium. The flasks were incubated at 28-30°C in a flask-shaker at 120 cycles/min for 4 days. The study samples were withdrawn regularly for every 24 h. Cells were separated and culture filtrate was used for the study of enzyme activity [13]. Figure 1 represents the sequence of this study.

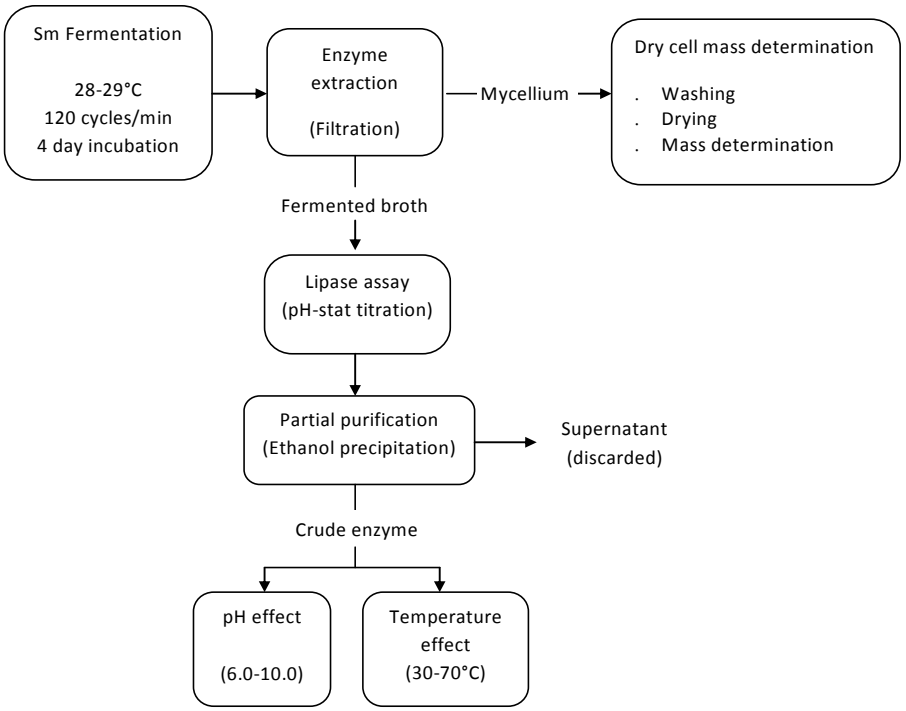

Figure 1. Flow diagram of experimental sequence.

2.5 Specific growth rate

To determine the specific growth rate (μ), natural log of biomass (Ln X) was plotted against time (t). The slope of the line at any moment gives the specific growth rate at that moment.

2.6 Lipase assay

The enzyme assay was performed with the cell free supernatant of fermented broth as the crude enzyme source. It was determined by the method of "pH-stat titration" in an emulsion made of gum acacia and olive oil as substrate [14].

2.9 Partial purification of lipase

Enzyme extract was treated with ethanol for enzyme precipitation. After that, the precipitate was dissolved in minimum amount of NaCl (0.5%) solution and collected in a sterile container and stored at 4°C for further analysis [15].

2.10 Effect of pH and temperature on enzyme activity

To determine the effect of temperature on lipase activity, the reaction was carried out at different temperatures ranging from 30 to 70°C [10]. Lipase activity was estimated following pH values within the range 6.0-10.0 to determine the optimal pH. To determine pH activity profile for the enzyme, assay was carried out maintaining the pH value by addition of standard alkali for 10 min at room temperature [16].

3. RESULTS AND DISCUSSION

3.1 Preliminary studies

The identification of microorganisms in the present study was important to confirm the integrity of cultures according to tests carried out during their isolation in the Department of Biochemistry and Microbiology of the University of Plovdiv. Figure 2 shows the macroscopic descriptions of the six micro-organisms. The microscopic observations proved the taxonomic integrity of these funguses.

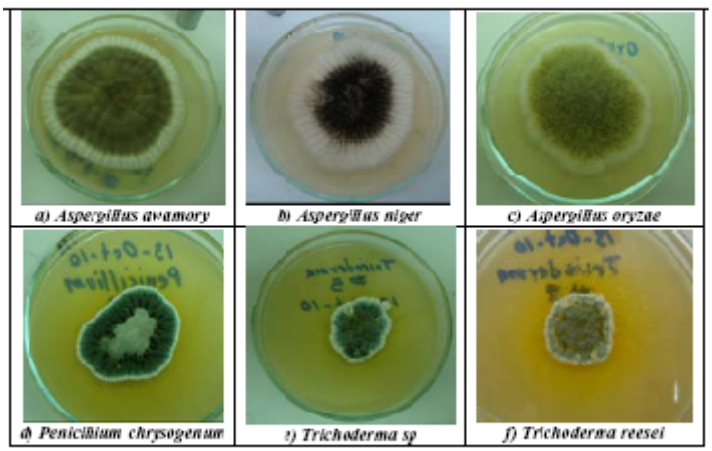

Figure 2. Macroscopic morphology of species under study: a*) A. awamory*, b) *A. niger*, c) *A.oryzae,* d) *P. chrysogenum* e) *Trichoderma sp* and f) *T. reesei*

3.2 Study of the production of lipase in SmF

The production of lipase by the tested microorganisms showed appreciable differences in their lipolytic activity. The mean lipolytic activity reached by fungi was about 7.6 and 4.8 times higher than by the other microorganisms. The results are displayed in Figures 3 and 4.

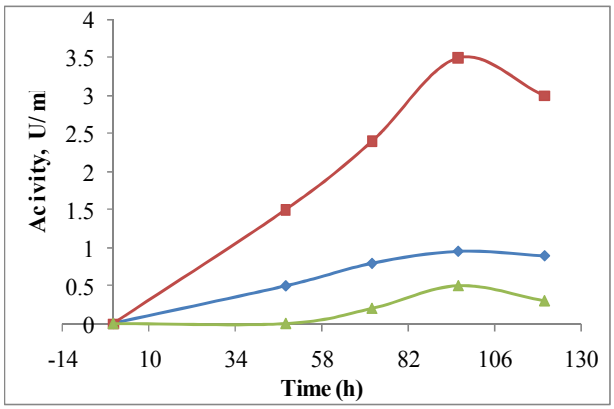

Figure. 3 Activity profiles of the fungus at different time. *A. awamory* (—◆—) *A. niger* (—■—), *A. oryzae* (—▲—).

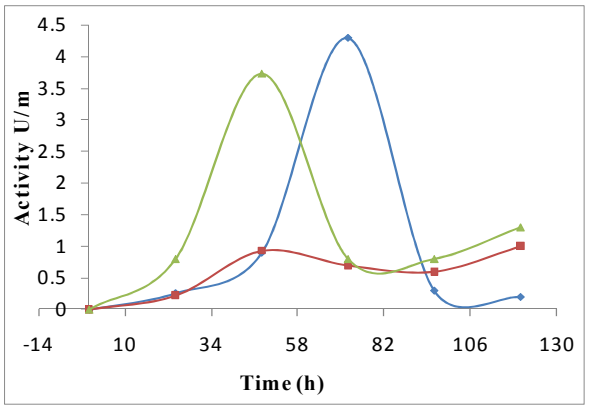

Figure. 4 Activity profiles of the fungus at different time *P. chrysogenum* (—◆—) *Trichoderma reesei* (—■—) *Trichoderma sp.* (—▲—).

In the case of Aspergillus species, lipase production started after 48 hours incubation (1.5 U/ml for *A .niger*), this means, after reaching the logarithmic growth phase. This suggests that the production of lipase by Aspergillus species was partly associated with the microorganism's growth. Subsequently, it was observed that the activity increased slowly until day 3. By day 4, the activity reached its maximum value (3.5 U/ml for *A. niger*). Finally, by day 5 the production rate had diminished, which could be due to proteolytic degradation of the enzyme system [13]. Lipase production by Trichoderma sp began around the first day of fermentation with an activation of 0.8 U/ml. Later there was a rapid increase in activity around the second day with a value of 3.8 U/ml, which is the highest recorded activity. Around day 3 the activity decreased markedly to a value 0.9 IU / ml. The species of Trichoderma reesei has similar behavior but with lower values of lipolytic activity. In contrast, the lipase production of Penicillium chrysogenum species started around day 2 with an activity of 0.9 U/ml, to increase rapidly to 4.3 U/ml around day 3 where it reaches its peak. On day 4 the activity of Penicillium dropped to 0.3 U/ml.

3.3 Effect of pH and temperature on *A.niger* and *P.chrysogenum* lipase activity

The optimum lipase activity of the enzyme extract was at pH 8.0 on both microorganisms. Lipase from *A.niger* was optimally active in a temperature range of 40 to 50 °C (Fig. 5), decreasing drastically at temperature above 60°C. Overall, lipases from *A.niger* strains have been reported to be active between 40 and 55°C [17]. While lipase from *P.chrysogenum* exhibits its optimal activity at 40°C (Fig.6). A similar result was also reported earlier [18].

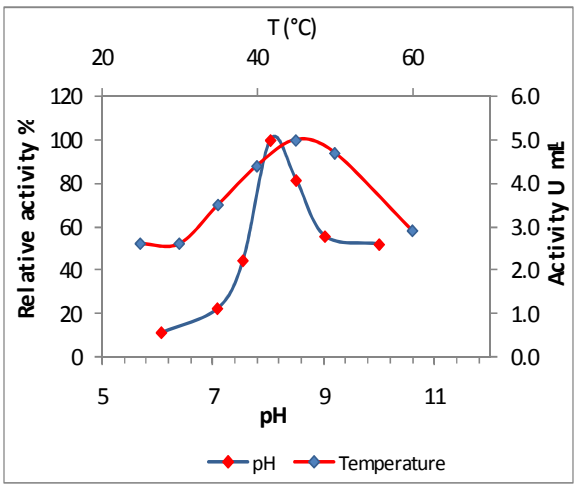

Figure 8. Effect of pH and temperature on *A.niger* lipase activity

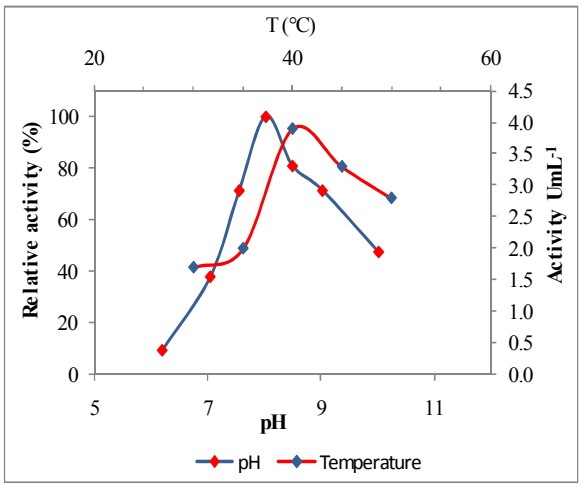

Figure 9. Effect of pH and temperature on *P.chrysogenum* lipase activity

4. CONCLUSION

According to the results obtained from SmF, the six strains showed lipases activities clearly. The two best strains which possess the highest activities were chosen on the basis of lipase activity assay for enzyme isolation. The optimum pH and temperature were determined for future observations of hydrolysis and transesterification reaction for the biodiesel production.

Acknowledgements

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