LIPASE PRODUCTION THROUGH SOLID-STATE FERMENTATION USING AGRO-INDUSTRIAL RESIDUES AS SUBSTRATES AND NEWLY ISOLATED FUNGAL STRAINS

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ABSTRACT
Extracellular lipase production by Penicillium chrysogenum, Trichoderma harzianum and Aspergillus flavus was carried out through solid state fermentation using agro-industrial residues as substrates. For all three strains, the growth temperature was 29±1 °C, and 65 % w (g/gds) moisture content. The effect of three factors on lipase production rate was investigated: initial pH (6.0 and 7.0), time of fermentation (72 h, 96 h and 120 h), and type of mixed substrate (wheat bran-olive oil, and wheat bran-castor oil cake). The process was optimized applying a mixed level factorial design. Fermentation time and pH were found to have positive effects on lipase production and secretion rates. However, the time effect was larger than initial pH. Type of substrate demonstrated minor effective importance than the other two factors, and Aspergillus flavus showed the larger lipase production among the three strains. Results indicated that the three fungal strains were able to grow and produce lipase in both culture mediums. The maximum lipase activity achieved was 121.35 U/gds by Aspergillus flavus, which was five and nine times the lipase produced by Trichoderma harzianum and P. chrysogenum respectively, at the same conditions. An initial neutral pH and 96 h of fermentation time were the optimum conditions for lipase production by Aspergillus flavus.


Keywords: lipase production, Penicillium chrysogenum, Trichoderma harzianum, Aspergillus flavus, agro-industrial residues, solid state fermentation.

Introduction
Lipases, also known as triacylglycerol ester hydrolases (E.C. 3.1.1.3), belong to the class of serine hydrolases that contain the consensus sequence G-X1-S-X2-G as a catalytic moiety, where G is glycine, S is serine, X1 is histidine, and X2 is glutamic acid or aspartic acid. They are versatile biocatalysts which find various applications. They catalyze not only hydrolysis reactions in aqueous solutions (25), but also synthesis processes such as esterification and transesterification in non-conventional media (13, 15, 25).

Since lipases are enzymes of industrial interest, their production should be combined with cost reduction, which can be achieved through the use of low-cost culture media (residues) from the agro-industry (10). In some applications of these enzymes, e.g. in wastewater treatment and biodiesel production, the cost of the biocatalyst is a limiting factor in the viability of the industrial process (3, 23,31).

A way to obtain low-cost lipases is the process termed Solid-State Fermentation (SSF) (3, 17, 28). The essence of SSF is in the use of a solid culture medium as a nutrient source and support for microorganism growth. Several studies have been carried out to obtain lipases by SSF using different agro-industrial residues, such as wheat bran (18), gingelly oil cake (14), rice husks (21), castor bean waste (10), and mixed substrates (5, 20). Various residues from biodiesel production, arising from the oil extraction of soybean, castor bean, and jatropha curcas seeds, among others (24), have been exploited, too. Godoy et al. (10) used castor bean waste – a residue from castor bean seeds, with no utility due to its toxicity and alkalinity, as a solid culture medium in SSF, eliminating its toxicity and reducing its allergenicity.

Enzyme production costs could also be reduced by obtaining enzymes with high activity, which can be achieved through optimization of the production conditions. Experimental design techniques are widely used as tools in the optimization of enzyme production processes (1, 7, 8, 16). Such techniques allow determining the optimum conditions for obtaining high activity levels and for testing the possible effects from the interaction between the studied variables.

This work is focused on lipase production through solid-state fermentation, taking advantage of the potential of three newly isolated filamentous fungi: Penicillium chrysogenum, Trichoderma harzianum and Aspergillus flavus, and using agro-industrial residues of low cost: wheat bran–olive oil (WBO) and wheat bran–castor oil cake (WBC) as solid matrices. The objective of the study was to identify the effect of initial pH, time of fermentation, and type of mixed substrate...
on lipase production rate, and define the optimum experimental conditions for lipase production.

**Materials and Methods**

**Microorganisms and propagation**

*Penicillium chrysogenum* and *Aspergillus flavus* strains were obtained from the Department of Biochemistry and Microbiology, University of Plovdiv (Bulgaria), and were described in a previous work (9). *Trichoderma harzianum* strain was obtained from the Department of Phytopathology, UABC (4). All strains were selected as promising lipase producers in SSF. Pure cultures of fungal strains were obtained by subculturing onto a Potato Dextrose Agar (PDA) medium and maintained at 4 °C. The spore suspension for inoculation was prepared by adding 4 mL of sterile distilled water to 10-day-old culture plates; and the spores were dislodged using a sterile spatula under aseptic conditions and counted in a cell-counting chamber.

**Solid-state fermentation**

Two different substrates were tested for lipase production: wheat bran with 2 % olive oil as lipase inducer (WBO), and a mixture of wheat bran and 30 % castor oil cake (WBC). The final oil content in both substrates was 9.0 %. The substrate WBC contained 3.6 % w castor oil with 84 % ricinoleic acid composition (24). These materials were purchased from local markets. Castor bean seeds were treated with oil expeller laboratory equipment in order to obtain the oil cake. This residue was ground in a mortar and separated in a sieve shaker. The final waste particle size was 0.71 mm in diameter. Both substrates were supplemented with mineral growth medium (MGM) containing: NaH\(_2\)PO\(_4\) 1.8 % w, g/gds; K\(_2\)HPO\(_4\) 0.3 % w, g/gds; Mg\(_{2}\)SO\(_4\).7H\(_2\)O 0.045 % w, g/gds; and CaCl\(_2\) 0.0375 % w, g/gds. A combination of urea-ammonium sulfate at 0.75 % to 0.34 % w and glucose at 2 % w, were used as nitrogen and carbon sources, respectively. The moisture of the culture medium was fixed at 64 % w/w. The initial pH was adjusted to the set up value. The fermentations were carried out in a lab scale. The glass flasks containing 5 g of the dry substrate and 9.0 mL of MGM at fixed pH were autoclaved. After cooling, the flasks were inoculated with 10\(^6\) spores/gds and incubated at 29 °C to 30 °C. The flasks were rotated twice a day for aeration. Fermentation samples (whole flasks) were removed at fixed intervals and assayed for pH, protein content and enzyme activity. Cultivations were performed in two replicate culture flasks, and the analysis was carried out in triplicate.

**Preliminary analysis of wheat bran and castor bean oil cake**

To determine the moisture content, 5 g of the substrate was dried at 100 °C for 8 h, cooled and weighed until a constant weight value was reached. The content of lipids and total nitrogen was estimated with standard methods (6, 12).

**Effect of initial pH (6 and 7), time of fermentation (72 h, 96 h, and 120 h), and type of mixed substrate (WBO and WBC), on the production of extracellular lipase by *Penicillium chrysogenum*, *Trichoderma harzianum* and *Aspergillus flavus*. Response: lipase activity, U/gds.**

<table>
<thead>
<tr>
<th>Time</th>
<th><em>Penicillium chrysogenum</em></th>
<th><em>Trichoderma harzianum</em></th>
<th><em>Aspergillus flavus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6</td>
<td>pH 7</td>
<td>pH 6</td>
</tr>
<tr>
<td>WBO</td>
<td>72 h</td>
<td>14.77</td>
<td>13.38</td>
</tr>
<tr>
<td>WBC</td>
<td>96 h</td>
<td>17.22</td>
<td>12.67</td>
</tr>
<tr>
<td></td>
<td>120 h</td>
<td>18.39</td>
<td>14.97</td>
</tr>
</tbody>
</table>

**Analysis of variance for lipase activity**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td>2</td>
<td>77567.3</td>
<td>38783.7</td>
<td>2262.03</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>4937.7</td>
<td>2468.9</td>
<td>143.99</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>Substrate</td>
<td>1</td>
<td>16.3</td>
<td>16.3</td>
<td>9.5</td>
<td>0.335</td>
<td>Non-significant</td>
</tr>
<tr>
<td>Initial pH</td>
<td>1</td>
<td>388.7</td>
<td>388.7</td>
<td>22.67</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>Fungi*Time</td>
<td>4</td>
<td>3811.8</td>
<td>952.9</td>
<td>55.58</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>Fungi*Substrate</td>
<td>2</td>
<td>1424.3</td>
<td>712.2</td>
<td>41.54</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>Fungi*Initial pH</td>
<td>2</td>
<td>207.6</td>
<td>103.8</td>
<td>6.05</td>
<td>0.004</td>
<td>Significant</td>
</tr>
<tr>
<td>Time*Substrate</td>
<td>2</td>
<td>837.9</td>
<td>418.9</td>
<td>24.43</td>
<td>0.000</td>
<td>Significant</td>
</tr>
<tr>
<td>Time*Initial pH</td>
<td>2</td>
<td>196.0</td>
<td>98.0</td>
<td>5.72</td>
<td>0.006</td>
<td>Significant</td>
</tr>
<tr>
<td>Substrate*Initial pH</td>
<td>1</td>
<td>6.7</td>
<td>6.7</td>
<td>0.39</td>
<td>0.534</td>
<td>Non-significant</td>
</tr>
</tbody>
</table>
Enzyme extraction
After fermentation, a 1 % NaCl solution (pH 7.0, 10 mL/g) was added to each flask containing the fermented solids. Enzyme extraction was carried out by magnetic stirring (200 rpm) at room temperature for 30 min. Then, solid–liquid separation was performed by pressing and vacuum filtration through a double-layered muslin cloth. In order to remove cells and spores, the culture was filtered through filter paper (Whatman No 2) and then the filtrate was vacuum-filtered again through membrane filters with 0.45 µm porosity. The clear supernatant obtained was used as extracellular enzyme (18).

Enzyme activity and protein determination
Lipase activity was determined by the spectrophotometric method (22) with slight modifications for rapid and routine measurement, using p-nitrophenyl butyrate (p-NPB) as a substrate. The enzyme activity was measured by the addition of 0.10 mL of the crude enzyme to a solution of 3.8 mL of 100 mmol/L phosphate buffer (pH 8.02), 0.1 % v/v of Triton X-100 and 0.10 mL of 5 mmol/L p-NPB. The hydrolysis reaction was carried out at 30 °C and measured over time up to 10 min at 410 nm. One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per minute, under the assay conditions. The protein content of the crude enzyme extract was determined by the Bradford method using bovine serum albumin as a standard (2), and was expressed as milligram of total proteins per milliliter of enzyme extract.

Factorial design of experiments
A mixed level full factorial design was used for the analysis of the experimental data obtained by varying the factors affecting lipase production. All experiments were performed in duplicate. Main points were highlighted through appropriate graphs. Data analysis was performed with Minitab statistical software 15.0.

Results and Discussion
As mentioned above, extracellular lipase production by Penicillium chrysogenum, Trichoderma harzianum and Aspergillus flavus was carried out through SSF with the locally available WBO and WBC as solid matrices. SSF with agriculture and food residues is well suited for enzyme production (30). The effect of three factors on the lipase production rate was investigated, namely: type of mixed substrate, initial pH (6.0 and 7.0), and time of fermentation (72 h, 96 h and 120 h).

Olive oil is the commonly used substrate in lipase production by filamentous fungi. It serves not only as an inducer for lipase generation, but also as a carbon source for microorganisms growth (27). In this study, as shown in Table 1, the addition of olive oil and castor oil cake to wheat bran as a substrate considerably influenced enzyme production. It was found that both mixed substrates: WBO and WBC are suitable for obtaining of lipase. However WBO, being more easily available compared to WBC, seems to be the better choice for extracellular lipase production by Penicillium chrysogenum and Trichoderma harzianum. In contrast, Aspergillus flavus fermentation indicated that WBC provides an optimum response: ~ 121 U/gds at pH 7 and 96 h of fermentation time. It may be suggested that this effect was caused by the fatty acids released from the metabolism of the oil present, and the better lipase production appears to be correlated with the content of fatty acids (32).

It is noteworthy that, to the best of our knowledge, lipase production by Trichoderma harzianum through SSF with castor oil cake as a substrate has not been previously described. However, other agro-industrial wastes commonly exploited such as babassu cake, gingelly oil cake, jatropha seed cake, and castor bean residues have been reported to produce lipase in SSF. The stated values are: 26.4 U/gds (11), 630 U/gds (18), 384 U/gds (20), 976 U/gds (19), and 44.8 U/gds (10) from Penicillium simplicissimum, Aspergillus niger NCIM 1207, Aspergillus niger MTCC 2594, Pseudomonas aeruginosa, and P. simplicissimum, respectively. Some of them present lower activity compared to the one reported here by using T. harzianum. Thus, this strain could be of importance for highly active lipase production.

The pH value of the growth medium is a factor of crucial importance and could drastically influence the microbial growth and enzyme production during SSF. As known, each microorganism possesses a unique optimum pH, as well as a pH range for its growth and activity. Filamentous fungi are considered to be able to thrive over a broad range of pH under solid state culture, since the solid substrate has a better buffering capacity (26). However, the data obtained in this study demonstrated that lipase production by T. harzianum and by A. flavus was affected by the pH change in the investigated pH range (Table 1), and the neutral pH was shown to be most favorable for lipase production. Initial pH change from 6 to 7 did not show a significant effect on lipase production by P. chrysogenum.

Another factor strongly affecting enzyme production is the fermentation time. Time increase from 72 h to 120 h led in general to a significant augmentation of lipase activity by all the microorganisms. Apparently, maximum lipase activity is achieved at 120 h of fermentation (Table 1). Beyond these incubation periods, a decrease in the enzyme activity may occur, which might be related to the depletion of the nutrients or denaturation of the enzyme caused by the interaction with other components present in the medium or due to pH change (29). For instance, A. flavus showed a decline in the rate of lipase production by 1.2 times after 96 h.

The comparison of the obtained data presented in Table 1 demonstrates that the best lipase producer strain is Aspergillus flavus, followed by Trichoderma harzianum and Penicillium chrysogenum at the conditions of the experiments.

The significance of the various effects and of their interaction on lipase production was evaluated by analysis of variance (ANOVA). The obtained data are listed in Table 2.

As the data in Table 2 demonstrate, eight effects have p-values less than 0.05, thus they do affect lipase production.

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To assist the analysis, a Pareto diagram was constructed, illustrating the magnitude of the effects (Fig. 1). The effect of the most important factors was found to decrease as follows: Fungi > Fermentation time > Fungi*Fermentation time > Fungi*Substrate > Time*Substrate > pH > Substrate.

**Fig. 1.** Pareto diagram illustrating the magnitude of the effects (%) affecting lipase production

Optimum conditions for lipase production by *Penicillium chrysogenum*, *Trichoderma harzianum* and *Aspergillus flavus* identified by applying the mixed level factorial design were found to be: pH 7 and fermentation time of 96 h. The optimal substrate for *Penicillium chrysogenum* and *Trichoderma harzianum* is WBO. However, maximum lipase activity was achieved with agro-industrial residues such as wheat bran–castor oil cake and *Aspergillus flavus* as lipase producer in the above-mentioned optimal conditions.

**Conclusions**

In this study we investigated the effect of the experimental conditions on extracellular lipase production by *Penicillium chrysogenum*, *Trichoderma harzianum*, and *Aspergillus flavus* through solid-state fermentation, using agro-industrial residues as substrates. The major factors influencing enzyme production were identified as: fermentation time, followed by the interactions between fungi and fermentation time, and fungi and type of mixed substrate. The pH change had a minor effect. The investigation showed that SSF is a suitable technique for lipase production, employing *Aspergillus flavus*. Further, the short fermentation time (96 h) and the use of agro-industrial residues such as castor oil cake make SSF a promising process of commercial importance.

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