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**ORIGINAL PAPER**

# Optimization of nutrient medium components for lipase biosynthesis by *Aspergillus carbonarius* NRRL 369 and studying the dynamics of submerged cultivation in a laboratory bioreactor

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## Abstract

*Lipase production during submerged cultivation of Aspergillus carbonarius NRRL369 was studied. In order to increase lipase activity an optimal composite design was applied. Optimal concentrations of the nutrient medium components were found to be (g/l): rapeseed oil 20.0, Tween 80 20.0, meat extract 5.6, MgSO4 1.0, and KH2PO4 4.0. The result was confirmed by five experiments with average lipase activity 1.9 U/ml which was close to the predicted one 2.09 U/ml. In order of scaling up the process, dynamics of some parameters of the cultivation process in a laboratory bioreactor was studied. Lag phase was up to 25 h, exponential phase was between 25 and 40 h, and stationary phase was after 40 h. Lipase biosynthesis started at the end of the lag phase, and a significant lipase activity was observed at the end of the exponential phase and the beginning of the stationary phase.*

**Keywords**: lipase, submerged cultivation, *Aspergillus carbonarius*, bioreactor, optimal composite design

## 1. Introduction

Lipases (glycerol ester hydrolases) catalyze the hydrolysis of acylglycerols to fatty acids, diacylglycerols, monoacylglycerols and glycerol. Lipases have the ability not only to hydrolyze ester bonds, to transesterify triglycerides, and to allow the resolution of racemic mixtures but also to synthesize ester bonds in non-aqueous media [1, 2, 3]. They also have characteristic properties like substrate specificity, stereospecificity and the ability to catalyze heterogeneous reactions at the interface of water soluble and water insoluble systems. They differ from the other esterases in that their substrates are insoluble in water and they have maximum activity when adsorbed to the oil–water interface [4, 5, 6].

Microbial lipases have considerable industrial potential as catalysts for hydrolysis, synthesis and transesterification of triacyglycerols due to advantages such as high levels of production and diversity of stereospecific properties [7]. Researches are focused on lipase production, purification, structural characterization, clarification of action mechanism,

kinetics of lipase-catalyzed reactions, sequencing and cloning of lipase genes [8, 9, 10]. Increasing lipase production during the biosynthesis process is also an important step in industrial application of the enzyme.

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition. Lipases are inducible enzymes and the major factor for the expression of enzyme activity was reported to be the carbon source. These enzymes are generally produced in the presence of a lipid component such as different vegetable oils [11, 12, 13]. Lipase production by fungi strains is also significantly influenced by the type and concentration of nitrogen source in the nutrient medium. The use of complex organic nitrogen sources such as peptone and yeast extract is widely practiced in submerged cultivation for microbial lipase production [11, 14, 15]. The salt components of the nutrient medium are another factor effecting lipase production.

Widely used method to enhance lipase activity during the cultivation process is searching a variation of medium components and interactions among them during the cultivation process [4].

The conventional technique for determination these optima is by varying one parameter while keeping the others at constant levels. The major disadvantage of this single variable optimization is that it does not include the interaction effects among the variables. It is also time consuming and requires a number of experiments to determine optimum levels. In order to overcome these limitations, optimization can be done by statistical experimental design such as response surface methodology [4, 7, 16, 17].

A significant role for optimization of lipase production has the dynamics of process parameters. It allows investigation of enzyme biosynthesis and strain growth pattern, determination of the optimal process parameters and scaling up the process for larger bioreactor volumes.

The aim of the paper is to determine the optimal concentrations of meat extract, $MgSO_4$, and $KH_2PO_4$ for maximum biosynthesis of lipase by *Aspergillus carbonarius* NRRL369 and to study the dynamics of submerged cultivation in a laboratory bioreactor.

## 2. Materials and Methods

### 2.1. Microorganism maintenance and storage

The studied *Aspergillus carbonarius* NRRL369 strain from ARS Culture Collection was grown in the following medium (g/l): glucose 20.0, yeast extract 1.0; agar-agar 15.0. pH was adjusted to 7.0. The strain was cultivated at 27°C for 7 days and stored at 4°C.

### 2.2. Vegetative inoculum preparation

Spore suspension of 2.5 ml ($3\times10^7$ spores/ml) was added to 100 ml sterilized (at 121°C for 30 min) medium with pH 7.0 with the following composition (g/l): malt extract 10.0, yeast extract 4.0, glucose 4.0, $K_2HPO_4$ 1.0; $NaNO_3$ 2.5. The strain was cultivated on a rotary shaker (180 rpm) at 27°C for 48 h.

### 2.3. Submerged cultivation in shake flasks

Submerged cultivation was carried out in 500 ml flasks containing 100 ml medium with composition corresponding to the aim of the study. pH of the medium was adjusted to 7.0, then the medium was sterilized at 121°C for 30 min. 5.0 ml vegetative inoculum was used for inoculating each flask and cultivation was carried out at 27°C for 64 h at a rotary shaker (180 rpm). Biomass was removed by filtration and the culture liquid was tested for lipase activity.

### 2.4. Response surface methodology

Optimal composite design $2^3$ was used for optimization of the medium composition and for investigation of the interaction effect between meat extract, $MgSO_4$ and $KH_2PO_4$. For this study nutrient medium with the following composition was used (g/l): rapeseed oil 20.0, Tween 80 20.0, meat extract, $MgSO_4$, $KH_2PO_4$. Each parameter was studied at three different levels (Table 1).

**Table 1**. Values of independent variables at different levels of the optimal composite design

| Independent variables | Levels | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| $X_1$ Meat extract (g/l) | 1.0 | 4.0 | 7.0 |
| $X_2$ $MgSO_4$ (g/l) | 0.2 | 0.6 | 1.0 |
| $X_3$ $KH_2PO_4$, (g/l) | 2.0 | 4.0 | 6.0 |

A matrix of 14 experiments with three factors was generated. Lipase activity was taken as the dependent variable or response ($Y$). According to the obtained results a regression analysis was accomplished and a quadratic regression model was expressed as follows:

$$\hat{Y} = b_0 + \sum_{i=1}^{m} b_i.x_i + \sum_{i=1,\, j=i+1}^{m} b_{ij}.x_i.x_j + \sum_{i=1}^{m} b_{ii}.x_i^2 \qquad (1)$$

Where $\hat{Y}$ is the response variable, $b_0$, $b_j$, $b_{ij}$, $b_{ii}$ are the regression coefficients of the model, and $x_i$ and $x_j$ are coded levels of the independent variables.

The cultivation process was performed according to the procedure described above.

### 2.5. Submerged cultivation in a laboratory bioreactor

The experiments were performed in a laboratory bioreactor Biosat B plus with total volume 2.0 l on nutrient medium containing (g/l) rapeseed oil 20.0, Tween 80 20.0, meat extract 5.6, $MgSO_4$ 1.0, and $KH_2PO_4$ 4.0. The bioreactor was inoculated with 10 % (v/v) vegetative inoculum and cultivation was performed at 28°C, aeration 1.0 l/(l.min) and 450 rpm mixing. Dynamics of dry biomass, redox potential, $pO_2$, pH, lipase activity, and concentrations of soluble protein and lipids during submerged cultivation was investigated.

### 2.6. Lipase activity assay

Lipase activity was determined by spectrophotometric method using *p*-nitrophenyl palmitate as substrate buffered with phosphate buffer with pH 6.0 [16]. Reaction mixture with 2.4 ml of freshly prepared substrate (30 mg/100 ml) and 0.1 ml enzyme solution was incubated for 30 min at 35°C and the reaction was stopped by adding 1 ml 10% NaOH. After centrifugation absorbance was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released one μmol of p-nitrophenol per minute under the assay conditions.

### 2.7. Analytical assays

Biomass concentration was determined by drying at 110°C to a constant weight [18]. Concentration of soluble protein was determined by Lowry method [19], and lipid content – by Soxhlet assay [20].

## 3. Results and discussion

### 3.1. Optimization of nutrient medium composition

In a previous study *Aspergillus carbonarius* NRRL 369 was found to be a promising producer of lipase in a medium containing rapeseed oil, Tween 80 and meat extract. The optimal concentration of rapeseed oil and Tween 80 were determined to be 20.0 g/l [11]. In order to determine the optimal values of other critical medium components (meat extract,

$MgSO_4$ and $KH_2PO_4$) optimal composite design $2^3$ was performed in shake flasks cultivation (Table 2).

Table 2. Optimal composite design $2^3$

| № | Codded values | | | Lipase activity, U/ml | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $Y^a$ | $\hat{Y}$ |
| 1 | +1 | +1 | +1 | 1.71 | 1.54 |
| 2 | -1 | +1 | +1 | 0.22 | 0.32 |
| 3 | +1 | -1 | +1 | 0.46 | 0.27 |
| 4 | -1 | -1 | +1 | 0.17 | 0.33 |
| 5 | +1 | +1 | -1 | 1.44 | 1.57 |
| 6 | -1 | +1 | -1 | 0.45 | 0.35 |
| 7 | +1 | -1 | -1 | 1.38 | 1.57 |
| 8 | -1 | -1 | -1 | 1.75 | 1.63 |
| 9 | +1 | 0 | 0 | 1.02 | 1.06 |
| 10 | -1 | 0 | 0 | 0.52 | 0.48 |
| 11 | 0 | +1 | 0 | 1.52 | 1.92 |
| 12 | 0 | -1 | 0 | 2.32 | 1.92 |
| 13 | 0 | 0 | +1 | 0.48 | 0.59 |
| 14 | 0 | 0 | -1 | 1.36 | 1.25 |

$X_1$ – meat extract; $X_2$ – $MgSO_4$; $X_3$ – $KH_2PO_4$; $Y$ – experimental value; $\hat{Y}$ – predicted value

[a] results are a mean value of three replications

As a result of the optimal composite design the following mathematical model was developed:

$$\hat{Y} = 1.332+0.290.X_1-0.333.X_3+0.320.X_1.X_2+0.319.X_2.X_3-0.561.X_1^2+0.588.X_2^2-0.412.X_3^2 \quad (2)$$

The model had values for *Significance F* = 0.008 ($P$<0.01), which showed that it was adequate at $\alpha$=0.01. The value of correlation coefficient was relatively high $R^2$=0.91. The model was analyzed and maximum lipase activity was predicted to be $\hat{Y}$ = 2.09 U/ml. This value was achieved at codded values of the independent variables $X_1$ = 0.54, $X_2$ = 1.00 and $X_3$ = - 0.02, which corresponded to concentrations of meat extract 5.6 g/l, $MgSO_4$ 1.0 g/l and $KH_2PO_4$ 4.0 g/l.

On Figure 1 the interaction effect of the studied variables on lipase biosynthesis is presented.

In the range of 0.45-0.65 g/l $MgSO_4$ and 3.0-7.0 g/l meat extract, lipase activity remained constant at about 1.2 U/ml, and the highest value was observed at 1.0 g/l $MgSO_4$ and concentration of meat extract between 5.0 to 6.0 g/l (Figure 1a). Maximum lipase activity was achieved at concentrations of $MgSO_4$ 1.0 g/l and $KH_2PO_4$ 4.0 g/l (Figure 1b), and a clear optimum was seen at 5.6 g/l meat extract and 4.0 g/l $KH_2PO_4$ (Figure 1c). It can be concluded that lipase biosynthesis was highly influenced by the organic nitrogen source, phosphorous compounds, and salt components in the nutrient medium. Similar are the considerations for other mold strains producing lipase [16, 21, 22].

As a result of the optimization procedure a new nutrient medium for lipase biosynthesis was composed (g/l): rapeseed oil 20.0, Tween 80 20.0, meat extract 5.6, $MgSO_4$ 1.0, and $KH_2PO_4$ 4.0. A series of five experiments with the new nutrient medium was performed.

Average lipase activity of 1.90 U/ml was achieved with standard deviation $\sigma$ = 0.16 which was very close to the predicted value (2.09 U/ml).

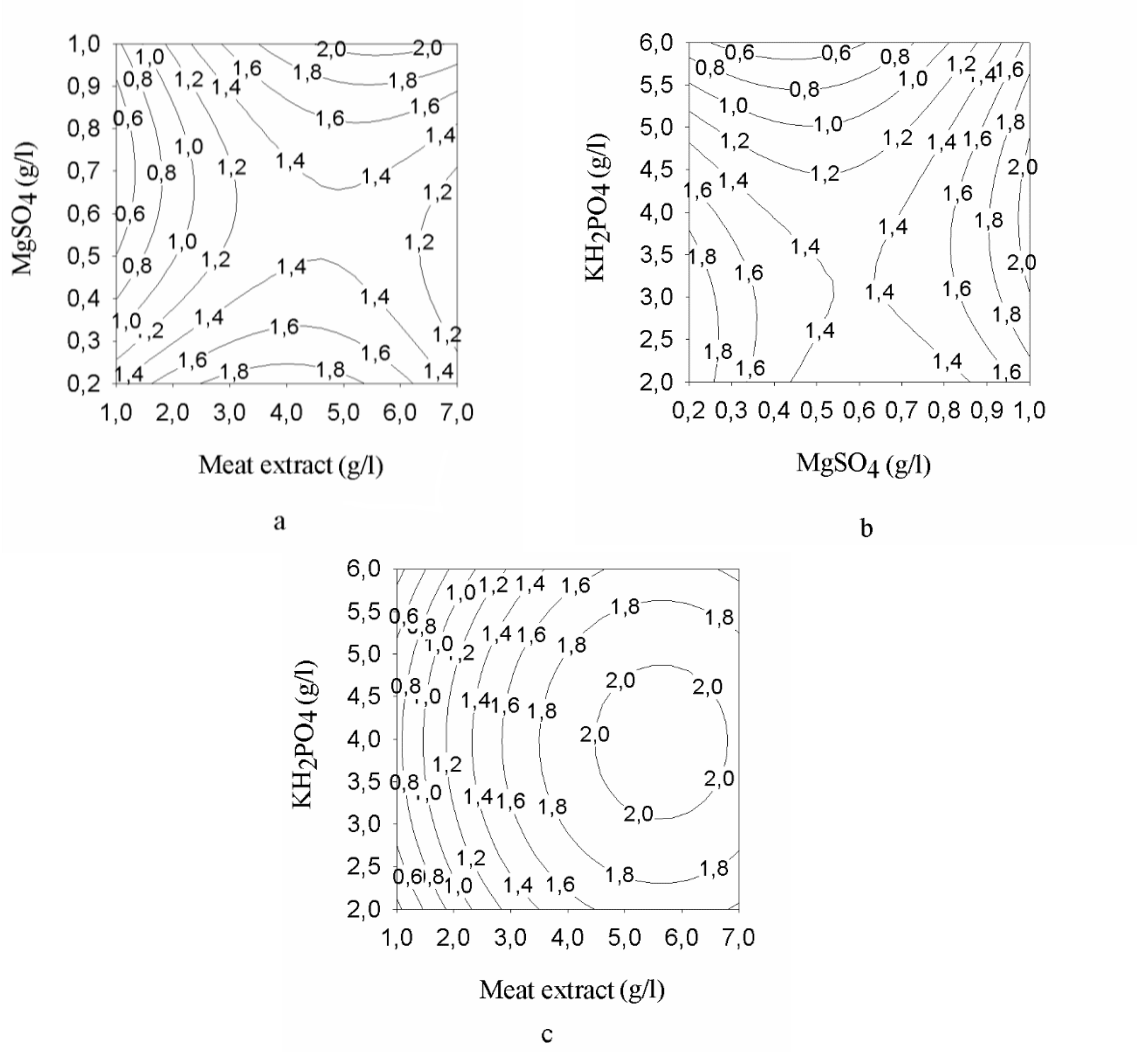


Figure 1. Response surface methodology: a. meat extract and $MgSO_4$, b. $MgSO_4$ and $KH_2PO_4$, c. meat extract and $KH_2PO_4$

### 3.2. Submerged cultivation in a laboratory bioreactor

Studying the dynamics of process parameters has a crucial role in optimization of lipase production. In order to scale up the process for lipase biosynthesis, a submerged cultivation in a laboratory bioreactor with the optimized medium was performed. On Figure 2 dynamics of biomass concentration, redox potential and dissolved oxygen is presented.

Dynamics of biomass concentration showed that lag phase of strain growth was up to about 25 h, the exponential growth phase was from 25 to 40 h, and the stationary phase was after 40 h. Dynamics of redox potential is an important criteria, which can be used for defining the growth phase of the strain and in scaling up the process for larger volumes of bioreactors. The redox potential decreased from the beginning of the cultivation to the end of the lag phase, and with the start of exponential growth phase at 25 h the value increased.

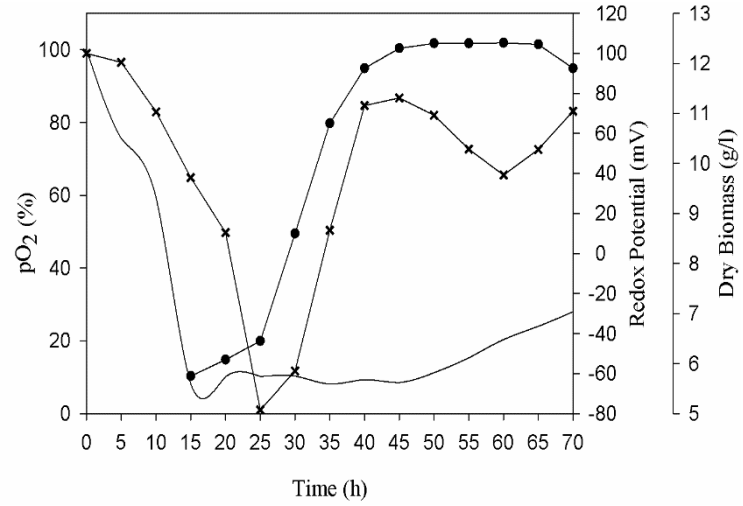


Figure 2. Dynamics of biomass concentration (●), redox potential (x) and dissolved oxygen (—)

Figure 3 shows the dynamics of concentrations of two main nutrient substrates – soluble proteins and lipids.

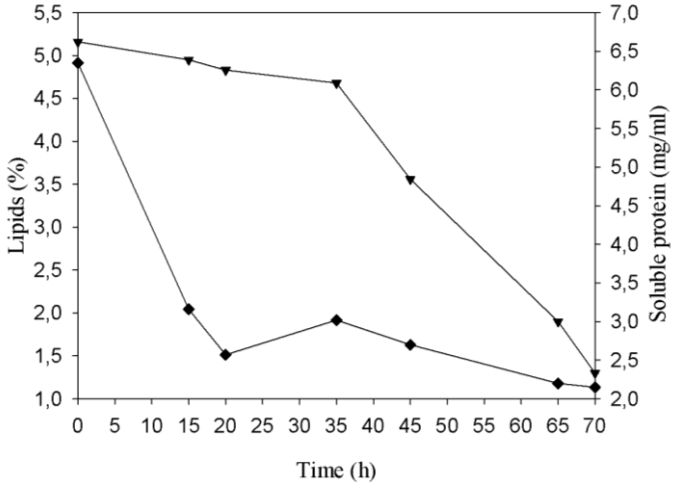


Figure 3. Dynamics of lipid (▼) and soluble protein (♦) concentration

During lag phase and exponential phase, protein concentration in the culture medium decreased from 6.35 mg/ml to 2.5 mg/ml at 25 h. In the same time interval, lipids concentration was not significantly changed. It could be assumed that during this period of growth the proteins from the nutrient medium were predominantly consumed.

During the first 30 hours of the process, which corresponded to the end of lag phase and beginning of exponential phase, pH of the medium remained almost unchanged (Figure 4). After 30 h of cultivation a sharp decrease of pH was observed, which was accompanied by biosynthesis of lipase. The most significant increase in lipase activity was observed from 35 to 65 h, which corresponded to the end of the exponential phase and the beginning of the stationary phase.

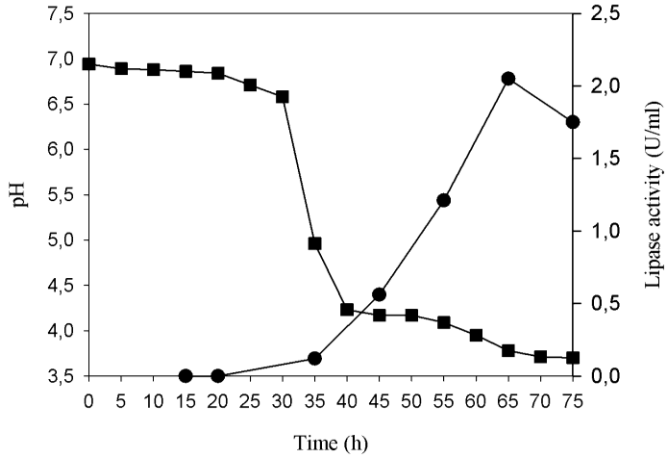


Figure 4. Dynamics of pH (■) and lipase activity (●)

Biosynthesis of lipase after 35 h was also connected with a sharp reduction in lipid substrate concentration in the medium. Maximal lipase production was observed between 45 and 65 h of the cultivation process. The highest lipase activity achieved in bioreactor cultivation was 2.05 U/ml at 65 h, which is comparable with the value obtained in shake flasks cultivation.

## 4. Conclusion

A new nutrient medium for lipase biosynthesis by *Aspergillus carbonarius* NRRL 369 in submerged cultivation was composed. It contained (g/l): rapeseed oil 20.0, Tween 80 20.0, meat extract 5.6, $MgSO_4$ 1.0, and $KH_2PO_4$ 4.0. In order of scaling up the process, dynamics of some parameters of the cultivation process in a laboratory bioreactor was studied. Lag phase was up to 25 h, exponential phase was between 25 and 40 h, and stationary phase was after 40 h. There was a clear correlation between the dynamics of the redox potential and the growth phases of the strain, which may be used for process scaling up. Biosynthesis of lipase depended largely on the growth phase of the strain. Lipase biosynthesis started at the end of the lag phase but a significant lipase activity was observed between 35-65 h, which corresponded to the end of the exponential phase and the beginning of the stationary phase.

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