Extracellular lipase of *Aspergillus niger* NRRL3; production, partial purification and properties

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Abstract Four strains of *Aspergillus niger* were screened for lipase production. Each was cultivated on four different media differing in their contents of mineral components and sources of carbon and nitrogen. *Aspergillus niger* NRRL3 produced maximal activity (325U/ml) when grown in 3% peptone, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.2% K_2HPO_4 and 1% olive oil:glucose (0.5:0.5). *A. niger* NRRL3 lipase was partially purified by ammonium sulphate precipitation. The majority of lipase activity (48%) was located in fraction IV precipitated at 50–60% of saturation with a 18-fold enzyme purification. The optimal pH of the partial purified lipase preparation for the hydrolysis of emulsified olive oil was 7.2 and the optimum temperature was 60°C. At 70°C, the enzyme retained more than 90% of its activity. Enzyme activity was inhibited by Hg^{2+} and K^+ , whereas Ca^{2+} and Mn^{2+} greatly stimulated its activity. Additionally, the formed lipase was stored for one month without any loss in the activity.

Keywords Lipase · *Aspergillus niger* · Culture conditions

Introduction

Lipases constitute the most important group of biocatalyst for biotechnological applications. Novel biotechnological applications have been successfully established using lipase for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agro-chemicals and flavor compounds [1–6]. The advantages of enzymatic hydrolysis over a chemical process are less energy requirement and higher quality of obtained product.

Lipase biosynthesis occurs in animals, plants and microorganisms. Microbial lipase are much more diverse in their enzymatic properties and substrate specifications, which gives it applicable values [7, 8]. Among the microbial lipase, molds are widely recognized as the best lipase sources and several references involving different industrial applications have been reported previously [9, 10]. *Aspergillus niger* is among the well-known lipase producers mainly used in the dairy industry [11, 12]. Studies on fermentation conditions for the production of extracellular lipase from *A. niger* showed variation among different strains of the fungus [12]. Different kinds and concentrations of carbon and nitrogen sources have a significant effect on lipase yield [13–15]. Despite the presence of a large number of already discovered and characterized lipolytic enzymes, the search for new lipase producers parameters remains important due to the wide range of practical industrial applications, which requires the availability of lipolytic enzymes displaying unique and specifically desired properties.

This work was undertaken to investigate the optimization of extracellular lipase production from a strain of *A. niger* in submerged culture and determine certain characteristics of partially purified enzyme.

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Materials and methods

Microorganisms and culture conditions

Strains of *A. niger* Van Tieghem, *A. niger* NRRL 599 and *A. niger* NRRL3 were provided by the culture collection of Northern Regional Research Laboratory (NRRL), USA. *A. niger* strains A10 and A20 were provided by the Center of Culture Collection, National Research Center (NRC), Egypt. The slants of *A. niger* were incubated in potato-dextrose-agar medium (PDA) at 30°C for 7 days. A spore suspension of about 10^5 – 10^6 spores/ml was prepared in sterile distilled water containing about 0.01% (v/v) Tween 80. This solution was used as a source of inoculum.

Lipase production

Test strains of *A. niger* were cultivated in media differing in the contents of mineral components and sources of carbon and nitrogen as follow:

Medium 1 (pH 6.0) contained 3% peptone, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl , 0.2% $\text{K}_2\text{H}_2\text{PO}_4$ and 1% olive oil: glucose (0.5:0.5) [16].

Medium 2 (pH 7.0) contained 2% soluble starch, 5% corn steep liquor, 0.2% K_2HPO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% CaCO_3 , 0.5% soybean oil (emulsion using gum arabic) [17].

Medium 3 (pH 7.0) contained 0.7% Na_2HPO_4 , 0.1% NH_4Cl , 0.002% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05% NaCl , 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3% KH_2PO_4 , and 5.0% decoction of defatted soybean flour [18].

Medium 4 (pH 4.0) contained 2% glucose, 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.2% KH_2PO_4 , 0.05% KCl , 0.056% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [19].

The microorganisms were grown in 250 ml flasks containing 50 ml of a culture media and incubated at 30°C and 200 rpm. On completion of cultivation, the biomass was harvested by filtration through a dried, weighted filter paper and washed more than one time with distilled H_2O , then dried in an oven at 70°C until complete dryness and then reweighed. The weight of the biomass was calculated in grams. The content of extracellular lipase was estimated according to the lipase activity in the supernatant.

Lipase activity

Lipase activity was determined according to the method described by Parry *et al* [20] using an emulsion of 10% olive oil in 10% gum Arabic. The emulsion was produced by treating the mixture of olive oil and gum Arabic solution in a top drive homogenizer for 10 min. The reaction mixture

contained 3 ml of substrate 2.5 ml of deionized water, 1 ml of 0.2 M Tris-HCL buffer (pH 7.5) and 1.0 ml lipase sample tested. The reaction was carried out at 37°C for 2 h in a shaking water bath, the reaction mixture was then supplemented with 10 ml ethanol. The amount of oleic was determined by titrating the hydrolysis products with 0.05N NaOH using thymolphthalein indicator. The control sample was prepared and treated similarly using boiled enzyme samples. The lipase activity values were calculated as the average of three parallel determinations displaying a variation coefficient lower than 5%. The amount of enzyme catalyzing the formation of one microequivalent (micromole) of oleic acid in 2 h at 37°C and pH 7.5 was taken as one lipase activity unit.

$$1 \text{ U/ml} = \frac{\text{Formation of } \mu\text{mol free fatty acids}}{1 \text{ ml enzyme solution}}$$

The protein determination was carried out by the method of Lowry *et al* [21].

Lipase activity and stability

Lipase activity was determined as mentioned before at 37°C and pH 7.5 [20].

The optimum pH and the optimum temperature for lipase activity was determined by carrying out the enzymatic assay at various pH and temperature.

Lipase stability was illustrated by preincubation of lipase at different pH with respect to pH stability or different temperature with respect to temperature stability. The remaining activity was measured at 37°C and pH 7.5 [13, 22, 23].

Partial purification of lipase from *Aspergillus niger* NRRL3

For characterization of the lipase, a partially purified preparation was obtained by filtration of the broth (to obtain the crude extract) followed by ammonium sulphate fractionation of the filtrate. Solid ammonium sulphate was added to the crude extract suspension (fraction I) at 30% saturation and allowed to stand overnight. The precipitate (fraction II) was obtained by centrifugation (14,000 g, 20 min at 4°C) while the resulting supernatant was further treated with solid ammonium sulphate at 50 % saturation. The precipitate (fraction III) was collected by centrifugation (14,000 g, 20 min at 4°C) and the supernatant was further treated with ammonium sulphate at 60% saturation. The precipitate (fraction IV) was obtained with centrifugation (14,000 g, 20 min at 4°C) was further treated with ammonium sulphate at 70% saturation. The precipitate (fraction V) was collected by centrifugation (14,000 g, 20 min at 4°C) and

the supernatant was finally treated with ammonium sulphate at 80% saturation. The precipitate (fraction VI) was obtained by centrifugation (14,000 g, 20 min at 4°C). All precipitates (II–VI) were resuspended in a minimal amount of distilled H₂O and dialyzed against distilled water using successive large volume of H₂O.

Results and discussion

The data in Table 1 illustrates that all strains of *A. niger* exhibited lipolytic activity, however, these strains differed

considerably in their potentiality for lipase biosynthesis and activity levels.

The nutrient media composition strongly affected the biosynthesis of the enzyme under study. Cultivation in medium 1, which contained glucose and olive oil as carbon sources, showed maximal lipolytic activities in *A. niger* A20, *A. niger* NRRL 599 and *A. niger* NRRL3 compared to the other three test media. Cultivation in medium 4, which contained only glucose as the carbon source, caused lipase inhibition in all the tested strains. Data in Table 1 shows that the most suitable media for lipase production were not the best one for growth; medium 3 represents the most suitable

Table 1 Production of extracellular lipase (LA) by strains of *Aspergillus niger* in different nutrient medium and yield of viable cell biomass

Medium	<i>A. niger</i> A20		<i>A. niger</i> NRRL599		<i>A. niger</i> NRRL3		<i>A. niger</i> A10	
	LA	DW	LA	DW	LA	DW	LA	DW
No. 1	190	0.77	320	0.73	325	0.877	250	0.991
No. 2	175	0.8	65	0.716	260	1.11	290	1.4
No. 3	125	2.8	25	1.95	310	1.58	275	2.3
No. 4	50	0.67	85	0.58	15	0.45	0.0	0.681

Incubation time: 4 days, LA in µ/ml, DW in g

Fig. 1. Effect of carbon (olive oil + glucose) and nitrogen source (peptone) concentration on lipase production by *Aspergillus niger* NRRL3

one for growth in all organisms tested but was not the best medium for lipase production. These results are in agreement with other cited results [24–26]. On the other hand, low lipase production accompanied by lowest biomass was obtained on medium 4 (pH 4). Otherwise, Ibrahim *et al* [27] and Betro *et al* [28] reported that the optimum pH for lipase production was dependent on the fungal species.

On the basis of the obtained results it was possible to substantially increase lipase production from *A. niger* species using medium 1, in which high lipase activity levels were produced by approximately all *A. niger* strains selected for the study. *A. niger* NRRL 599 and *A. niger* NRRL3 are equivalent in lipase activity. The production and characterization of lipase from *A. niger* NRRL599 has been reported [23]. To the best of our knowledge lipase production from *A. niger* NRRL3 has not been reported. This justifies its selection for subsequent work. The effect of initial pH on lipase production in olive oil medium was tested. *A. niger* NRRL3 was grown in medium 1 with a initial pH ranging from 3.5 to 8.0 (data not showed). The best initial pH for lipase production in medium 1 was 5.5–6.5 and as the pH was increased the lipase production decrease. These results agree with those obtained by Shafei [23], Seitz [29] and Papaparaskevas *et al* [30] who claimed that 6.0–6.5 is the optimum pH for *A. niger* and *A. oryzae*. The optimum medium found above was further improved as far as the concentration of the involved sources and components were concerned. The basic concentration levels were 3%, 2% and 1% and 1%, 2% and 3% (w/v) for nitrogen and carbon sources, respectively. Results in Figure 1 show that the optimum enzyme activity (640 U/ml) was obtained at a peptone concentration of 3% and a glucose and olive oil concentration of 2%. Mates and Sudakevitz recorded that growth and lipase production were considerably stimulated by increasing the peptone concentration [31]. They reported that on increasing the Bacto peptone concentration from 1.5 to 2%, 61% increasing in lipase activity formed by *Staphylococcus aureus* after 48 h incubation.

Enzyme activity however decreased with further increase in the concentration of the carbon source (3%). It might be due to the increase in fatty acid concentration, which

accumulated through hydrolysis of substrate (olive oil) suppressing lipase synthesis [32, 33]. The optimum lipase production was observed after 4 days in all treatments used, after which a sharp decrease in the lipase activity was demonstrated. Pau and Ibrahim [34] have shown that the optimum inoculum quantity (5×10^5 spore g^{-1} substrate) of *A. flavus* VSM A10 gives the optimum lipase activity after 4 days.

Partial purification of *Aspergillus niger* NRRL3 lipase

The profile of ammonium sulphate purification (Table 2) indicates that there is no enzyme activity in fraction II while the specific enzyme activity increased 6-fold in fraction III which precipitated at 30–50% saturation. The enzyme specific fraction activity increased to 18-fold with fraction IV which precipitated at 50–60% saturation. The results show that the majority of lipase activity (48%) was in fraction IV. This fraction was considered to be the partially purified enzyme and was used in subsequent studies.

Characterization of lipase from *Aspergillus niger* NRRL3

Effect of pH and temperature on activity and stability

As shown in Figure 2 when lipase activity was measured at a pH range from 5.7 to 9.0 using phosphate and Tris buffer, the enzyme was quite active over a pH 6.0–7.5, the optimum being at pH 7.2 (with both buffer used). These results are similar to other reported lipases [23, 26, 35–37] when the optimum pH for lipases activity ranged between 6.5 and 8.5.

To evaluate the stability of the enzyme with respect to pH, the lipase was preincubated in buffers between pH 6 and 9 for 60 min. The enzyme was found to be unstable, even at pH 7.2 where the activity decreased by 12% than that of original (without preincubation). Preincubation of the enzyme for 60 min at pH 8.0 resulted in decrease in its activity by more than 50%. Dimitris *et al* [13] reported that *A. niger* lipases rapidly denature at alkaline pH and lose ap-

Table 2 Profile for the partial purification of lipase from *Aspergillus niger* NRRL3

Fraction	Total protein (mg)	SEA (U/mg)	Total activity (U)	Recovery (%)	Purification fold
Culture supernatant I	18000	2.8	52000	100	1
II (0–30%)	723	0.0	0.0	-	-
III (30–50%)	310	18.68	5791	11.13	6.67
IV (50–60%)	494	50.65	25025	48.12	18.08
V (60–70%)	721	21.91	15801	30.38	7.82
VI (70–80%)	330	17.54	5791	11.13	6.26

SEA: Specific enzyme activity

Fig. 2 Influence of pH on lipolytic activity. Enzyme activity measured at pH 7.5 and olive emulsion as a substrate =100% of activity.

Fig. 3 Influence of temperature on lipolytic activity. Enzyme activity at 37°C and olive oil emulsion as substrate = 100% activity.

proximately 50% of the initial activity after 55 min.

The effect of temperature on enzyme activity and stability was investigated. Figure 3 illustrates that preincubation of lipase (without substrate) at over 60°C for 60 min caused a drastic loss of activity. When the enzyme was incubated with the substrate, the optimal temperature for reaction was about 60°C. In this case, incubation of the enzyme with a substrate at 60°C for 2 h (time of reaction) did not lead to any drastic loss of activity. A similar phenomenon of substrate stabilization of an enzyme at high temperatures, was observed in *Chromobacterium viscosum* lipase [38] and in Staphylococcal lipase [39]. In addition, Sugihara *et al* [38] reported rapid loss of lipase activity above 30°C in the absence of olive oil. On the other hand, the addition of olive oil or trimethylolpropane diallyl ether greatly stabilized the enzyme.

A characteristic feature of lipase is their adsorption to substrate/water interface, followed by activation [40, 41].

The interfacial binding of lipase was further demonstrated by several researchers [42, 43]. In their model of lipase action, Verger and de Hass [44] assumed that an active enzyme conformer was formed during or after the binding of the lipase to the surface of the insoluble substrate. No active enzyme was thought to occur in solution. Recent X-ray structure analyses of native and inhibitor-complexed *Rhizomucor miehi* lipase have revealed that a short peptide segment covering the active site in the absence of the substrate move in complexed enzyme so that the active site is exposed to the solvent [45, 46]. Concomitantly the hydrophobicity of the surface area around the active site increases, which should contribute to the strong binding of the enzyme to the interface. Thus, conformation changes during the adsorption are strongly suggested. We speculated that in *A. niger* NRRL3 lipase enzyme/substrate interaction conformation changes, or both caused the heat stabilization [22].

Effect of metal ions

The effect of various metal ions including Na⁺, K⁺, Zn²⁺, Ca²⁺, Hg²⁺, Mn²⁺, Mg²⁺ and Fe³⁺ (as chlorides except zinc as ZnSO₄·7H₂O) were examined by assaying the activity after incubation for 2 h at 37°C and pH 7.5, with 1 mM or 10 mM of each element as a final concentration. The results are represented as relative activities. The relative activity calculated on the basis of partially purified enzyme (before addition of any metal ions).

K⁺ and Hg²⁺ showed inhibitory effect (22% and 18%, respectively) at 1 mM, all the remaining ions used did not affect the enzyme activity at 1 mM. On increasing of the concentrations to 10 mM, the enzyme activity was slightly inhibited by Zn²⁺ and Fe³⁺; 75% and 38% of the enzyme activities were inhibited by addition of 10 mM of Hg²⁺ and K⁺, respectively (Table 3). Inhibition of lipases of

Table 3 Effect of metal ions on the lipase activity from *Aspergillus niger* NRRL3

Metal ions	Relative activity (%)	
	1 mM	10 mM
Na ⁺	100	120
K ⁺	78	62
Zn ²⁺	100	98
Ca ²⁺	100	170
Hg ²⁺	81	25
Mn ²⁺	100	151
Mg ²⁺	100	108
Fe ³⁺	100	95

Relative activity was calculated considering the activities of the partially purified enzyme without addition of any metal ions as control.

Bacillus and *Pseudomonas cepacia* by Hg^{2+} has been reported [40, 41]. Also Petrovic *et al* reported that K^+ at a concentration of 50 mM inhibited lipase activity. It is considered that metal ions generally from complexes with ionized fatty acids and change their solubility and behavior at the interface. However, the inhibition probably involves the catalytic site directly. Also the inhibitory effect might be attributed to binding of the thiol group of the enzyme or sulfhydryl groups, which may be present in the active center of the enzyme [42]. All remaining ions stimulated the enzyme activity, the greatest stimulation was achieved using Ca^{2+} and Mn^{2+} , 170% and 151%, respectively. Shippe reported that CaCl_2 accelerated the enzyme activity of *A. niger* and *Penicillium roqueforti* [43]. Also, calcium salts have been shown to accelerate the activity of pancreatic lipases as well as that of other several microbial lipases [47, 48]. Our results were in agreement with that reported by O'Leary and Weld, Shah and Wilson, Troller and Bozeman and Tyski *et al* who confirmed that Ca^{2+} stimulated lipolytic activity to some extent [49–52]. Lipase from *Acinetobacter* sp. was enhanced 1.5 fold in the presence of Ca^{2+} . Lee *et al* and Lelie *et al* reported that Hg^{2+} has an inhibitory effect whereas Ca^{2+} increases *Bacillus thermoleovorans* lipase activity [52, 53].

It was generally thought that the primary role of Ca^{2+} was to remove fatty acids formed as a result of hydrolysis as insoluble calcium soaps, and thus change the interfacial substrate–water relationship to favorable condition for the enzyme action [42].

Storage stability

Lipase preparations did not lose any activity when stored for 30 days at temperatures ranging from -20 to 4°C . Stephan *et al* had studied the effect of time and temperature on lipase activity. They found that lipase preparation did not lose any activity when stored for 35 days at temperatures ranging from 4 to 37°C . Lipases from *Penicillium roqueforti* appeared to be fairly stable at low temperature and could be held frozen for long periods without loss of activity [54].

Conclusion

In conclusion, the characterized thermolabile *A. niger* NRRL3 lipase would be useful for food processing including flavor generation of dairy products.

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