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Properties of an extracellular lipase from a traditional yoghurt yeast

[Geleneksel yoğurt mayasından elde edilen ekstraselüler lipazının özellikleri]

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ABSTRACT

Purpose: To produce an organic solvent tolerant extracellular lipase from a traditional yoghurt *Saccharomyces cerevisiae* isolate and to characterize this enzyme activity.

Material and Methods: A traditional yoghurt sample collected from a farm (Sivas-Turkey) was diluted and mixed with de Man, Rogosa and Sharp Medium (MRS) and poured into Petri dishes. One hundred colonies were selected and transferred onto MRS agar plates including Tween 80 as a lipase substrate. Only four colonies with lipase activity were incubated in MRS broth for 3 days at 37 ^{o}C and the extracellular proteins were precipitated with ethanol (95%v/v) from the supernatant. The precipitate with the highest lipase activity was selected for further analysis.

Results: The optimum pH and temperature were 7.0 and 37 ^{o}C, respectively. While (1 mM) Mn^{2+} caused a slight increase (~8%) Hg^{2+} or Zn^{2+} caused a substantial activity loss (~70%). Organic solvents (5%chloroform or n-butanol, v/v) increased activity ~2.5 fold. Five percent SDS or Triton X-100 increased activity by 8%and 14%, respectively. The enzyme could hydrolyse hydroxyethyl methacrylate (HEMA) and 2,3-epoxy propyl methacrylate (GMA),retained ~83%of its activity after 1 hour at 37 ^{o}C, lost no activity when stored at pH 7.0 and at 4 degree C for 60 days.

Conclusion: The properties of the extracellular yeast lipase was investigated using a number of physiological parameters. The results obtained indicated that it was an organic solvent- and detergent tolerant enzyme having the optimum activity at mild reaction conditions.

Key Words: Detergent tolerance, lipase activity, organic solvent tolerance, traditional yoghurt, yeast lipase.

Conflict of interest: The authors do not have a conflict of interest.

ÖZET

Amaç: Geleneksel yoğurt *Saccharomyces cerevisiae* izolatından organik çözücü tolerant ekstraselüler lipazının üretimi ve enzimatik özelliklerinin araştırılması.

Gereç ve Yöntemler: Divriği (Sivas-Türkiye) yöresinden elde edilen çiftlik yapımı yoğurt örneği de Man, Rogosa and Sharp (MRS) agarında seyreltilerek (%10) petri kaplarına döküldü. Yaklaşık yüz koloni secilerek Tween 80 içeren MRS agar plakalara ekildi.. Lipaz aktivitesi gösteren dört koloni MRS besiyerinde 3 gün 37 ^{o}C'de çoğaltıldı ve ekstraselüler proteinler süpernatandan etanol ile çöktürülerek alındı. En yüksek lipase aktivitesi gösteren protein ile enzim aktivite özelliklerinin araştırılmasına devam edildi.

Bulgular: Eksraselüler enzimin optimum pH ve sıcaklığı sırasıyla 7.0 ve 37 ^{o}C'dir. Mn^{+2} iyonu enzmi aktivitesini artırırken (%8), Zn^{+2} and Hg^{+2} iyonları aktiviteyi azaltmaktadır (~%70). %5 kloroform ve n-butanol varlığında enzim aktivitesinde ~2.5 kat artış gözlendi. Enzim aynı zamanda SDS (%108 relativ aktivite) ve Triton X-100 (%114 relativ aktivite) gibi deterjanlarda kararlılığını korudu. Enzim, hidroksietil metakrilat (HEMA) ve 2,3-epoksi propil metakrilatı (GMA) da substrat olarak kullandı. Enzim bir saat süreyle, 37 ^{o}C'de bekletildiğinde, aktivitesini %83'ün üzerinde koruduğu gözlendi. Enzim pH 7.0 fosfat tamponunda 60 gün boyunca aktivitesini kaybetmeden buzdolabında saklanabildi.

Sonuç: Eksrtraselüler maya lipazının özellikleri farklı fizyolojik parametreler kullanılarak incelendi. Elde edilen bulgulara göre bu enzim ılımlı tepkime koşullarında çalışan organik çözücü ve deterjan tolerant enzimdir.

Anahtar Kelimeler: Deterjan tolerant, geleneksel yoğurt, lipaz aktivitesi, organik solvent tolerans, maya lipazı.

Çıkar çatışması: Yazarlarların çıkar çatışması bulunmamaktadır.

Introduction

Lipases (EC.3.1.1.3) hydrolyze triglycerides into free fatty acids and glycerol moieties in aqueous environments. They are water soluble but catalyze lipophilic substrates at a water-lipid interface. [1,2]. They also catalyse acidolysis, alcoholysis, aminolysis and esterification [3].

Lipases are produced by organisms ranging from microbes to animals. Microbial lipases are produced by bacteria and fungi and secreted extracellularly. Yeasts are a traditional source because of their relatively simpler growth conditions [4-6]. *S. cerevisiae* is a versatile organism in making fermentation products such as dairy products, bread, beer and wine, and can also be used as a probiotic. [7, 8].

Microbial lipases have been used in detergent [9,10] and fat and oil industries [11], in production of flavouring agents [12], in biomedical and pharmaceutical industries [13-16] and as biosensors [17].

Enzymatic modification or synthesis of organic molecules is often performed by lipases in mono-, bi- or polyphasic or non-aqueous environments. [18-21]. The presence of lipases, both extra- and intracellular, in *S. cerevisiae* have been demonstrated by others [22, 23]. Hovewer in these studies the properties of the enzymes have not been characterized. We have used a crude fungal lipase secreted by a strain of *S. cerevisiae* isolated from an traditional yoghurt source, to determine its several substrates, pH and temperature optima, and the effect of several metals, and water miscible and immiscible organic solvents.

Materials and Methods

A traditional yoghurt sample obtained from a farm, produced for sale, was collected in a sterile glass container, at 4 °C, from the district of Divrigi, Sivas-Turkey. All chemicals used in MRS agar and culture media, were obtained from Merck AG (Darmstadt, Germany), and for activity studies from Sigma (St. Louis, MO, USA). Buffer and sample solutions were prefiltered through a 0.2 µm membrane (Whatman, Dassel, Germany). Water was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit.

Isolation and screening of lipase producing Yeast

Ten milliliters of yoghurt were diluted asceptically in 90 mL sterile peptone solution. Dilutions, up to 10^{-10}, were then prepared, and 1 mL aliquots of 10^{-5}-10^{-7} dilutions were mixed with 9 mL MRS agar and poured on plates that contained 1%peptone, 1%meat extract, 0.5 %yeast extract, 2%glucose, 0.2%K_2HPO_4, 0.5%sodium acetate, 0.2%triammonium citrate, 0.02%$MgSO_4.7H_2O$, 0.005%$MnSO_4.4H_2O$, (pH 6.3). Colonies were grown at 37ºC for 3 days in Petri dishes. One hundred 100 colonies that differed in colour and size, were obtained and transferred onto MRS agar plates supplemented with 1%Tween 80. The only four of the colonies surrounded by clear zones displayed fluffy and smooth morphology (with the naked eye), cream to light brown in colour, very similar to those of *S. cerevisiae* [24] were selected.

Extracellular lipase production

Each of the four colonies was inoculated into 250 mL MRS medium containing 1%Tween 80 and incubated at 37 °C overnight. Each culture was centrifuged for 15 min at 5,000 xg at 4°C, the supernatant was mixed with two volumes of 95%ethanol and the proteins were precipitated at -20 °C overnight. The precipitates were then air-dried and stored at -20 °C. Protein concentration was estimated from its absorbance at 280 nm in a UV/vis spectrophotometer (Optima, Tokyo, Japan) and calculated from a bovine serum albumin calibration curve.

Lipase activity

Lipase activity was measured using a modified version of the method described in [25]. Briefly, 250 mg olive oil were emulsified in 250 mg gum arabic (just these two chemical) in an ultrasonic water bath (Bandelin Sonorex Digitec, Germany). The emulsified substrate was then transferred into a test tube containing 5mL phosphate buffer (pH 7.0, 50 mM), and kept in a shaking water bath for 15 min at 37 °C. Then, 100 µl protein solution pH 7.0 phosphate buffer (1.0 mg/mL), was added and incubation continued for a further 15 min and the reaction terminated by adding 10 mL ethanol (95%). The fatty acids produced was determined by titration with 0.05 N NaOH and phenolphthalein as indicator. As a control, a sample without enzyme was treated in the same way. One unit of lipase activity was taken as one micromole of free fatty acid per minute per mg of protein. The protein precipitate yielding the highest lipase activity was used for the determining of lipase properties.

Effect of pH on lipase activity

Lipase activity was determined at pH from 3.0 to 10.0, at 37 °C, using as buffers: 50 mM acetate (pH 3.0-5.0), 50 mM phosphate (pH 6.0-8.0), and 50 mM carbonate buffer (pH 9.0-10).

Effect of temperature on lipase activity

Lipase activity was measured in 10 degree intervals at from 5 to 85 °C in 50 mM phosphate buffer (pH 7.0) and recorded as a percentage the highest value at each temperature [26].

Activity on different substrates

Enzyme activity was determined using olive oil, tributyrin, sunflower oil, Tween 20, Tween 40 or Tween 80 as substrates, after incubation at 37 °C for 5, 10, 15, 25, 45, and 60 min.

Kinetic studies

For the kinetic studies, activity of 100 µl protein solution

in pH 7.0 phosphate buffer (1.0 mg/mL) on 5 mL emulsion of olive oil concentration (from 10 to 100 mg/mL) was determined.

Effect of organic solvents detergents and metals

Activity was measured in presence of methanol, ethanol, acetone, toluene, benzene, ethyl acetate, 2,3-epoxypropyl methacrylate, hydroxymethyl methacrylate, n-butanol, chloroform, sodium dodecyl sulfate (SDS) or Triton X-100, each at 5%final concentration (v/v).

Activity was also measured in presence of of NaCl, KCl, $ZnSO_4$, $CaCl_2$, $MgCl_2$, $FeCl_3$, $HgCl_2$, or $MnCl_2$ (1mM final concentration) at 37 °C and in aqueous media.

Shelf-life

Precipitate dissolved in 50 mM phosphate buffer (pH 7.0) was stored at 4 °C and its activity was measured after 5, 15, 30, 45 or 60 days.

Thermal stability

100 µl protein solution (1.0 mg/mL) was prepared in 50 mM pH 7.0 phosphate buffer and incubated for 1 h at 37, 45, 55, 65 or 75 °C. Aliquots were taken at 10 min intervals and the activity determined.

Results and Discussion

Effect of pH and Temperature

The optimum pH and temperature for lipase activity were 7.0 (in phosphate buffer) and 37.0 °C respectively (Fig.1 and 2). Phosphate buffer was chosen since it stabilizes free fatty acid molecules [27] and most yeast lipases such as *Candida rugosa* function best under mild pH conditions [28]. The activity was totally inactivated by acetate buffer, pH 3-4 (Fig. 1). The specific activity was 120 U/mg with olive oil as substrate, in phosphate buffer at pH 7.0 and at 37 °C (The other three precipates showed activity as 60 U/mg, 65 U/mg, and 80 U/mg under the same experimental conditions).

Effect of Metal Salts

Contrary to the findings of others [29, 30], only Mn^{++} increased the lipase activity, by ~8%(Fig.3). Mg^{++} decreased activity by ~50 %(Fig.3). *Bacillus* sp. RN2 was also decreased activity in the presence of 1 mM Mg^{++} [31]. This could be due interaction of Mg^{++} with free fatty acids and formation of insoluble salts. Na^+ and K^+ each caused a ~20%decrease (Fig. 3). The highest inhibitory effect ~70%, Hg^{++} and Zn^{++} each inhibited by ~70%. These dramatic decreases may result from changes in the native structure of the lipase possibly by destruction of its SH-bonds. [35].

Effect of Organic Solvents

The effect of several organic solvents is summarized in Fig. 4. The highest activity (Fig. 4) was found with the n-butanol or chloroform (~250%), and toluene (~150%) while ethanol (~50%), acetone (~100%), and benzene (~86%) produced much increased activity by ~50%,! 100%and ~86%, respectively.. It has been hypothesized that, solvent-tolerant lipolytic enzyme secreted by organic solvent-tolerant Pseudomonas aeruginosa LST-03 are stable in presence of organic solvents [33]. The maximum relative activity, ~260%was with 2-3 epoxypropyl methacrylates (GMA). Hydroxymethyl methacrylate (HEMA) also increased activity ~170%

The detergents, SDS and Triton X -100 increased activity, ~8%, and ~14%, respectively. Yeast lipases from *Kurtzmanomyces* sp. I-11 active in presence of benzene, diethylether, and detergents such as SDS, have been reported [34].

Substrate hydrolysis

While we used olive oil for the measurement of specific activity the enzyme's highest activity was on Tween 80. The long chain length makes this molecule more hydrophobic thus increasing the surface area for the catalytic activity. Similar findings have been reported for Fusarium *oxysporum* f. sp. *vasinfectum* lipase [35].

Sunflower oil and Tween 40 had the same relative activity (85%), as did olive oil and tributyrin and Tween 20 (72%). These results confirm the effect of fatty acid chain length on the activity [21].

Incubation Period

With olive oil the reaction rate reached a maximum at 15 min.

Kinetic studies

The enzyme did not follow Michaelis-Menten kinetics. This could result from the substrates being water-insoluble and requiring the enzyme to function at the interface of the biphasic reaction system. This also demonstrates that the enzyme was a lipase and not an esterase [1, 36].

Shelf-life

No loss of activity was noted over 60 days at pH 7.0 and 4°C (Fig.5). Similar results have also been reported for the shelf life of thermophilic Bacillus sp. DH4 lipase [37]. Sequential freezing and thawing of the enzyme two times per days during 20 days did not cause detectable deterioration in activity (data not shown).

Thermal stability

Activity was inversely propotional to the temperature i.e., 83%was retained at 37°C, and 10%at 75 °C (Fig.6). Thus the enzyme was not thermostable and similar findings have also be found for *Aspergillus niger, Serratia marcescens* and *Bacillus cereus* lipases [30, 38, 39].

We conclude that our crude extracellular yeast lipase has the optimum activity at mild reaction conditions and has the potential to be useful in organic solvents and detergents

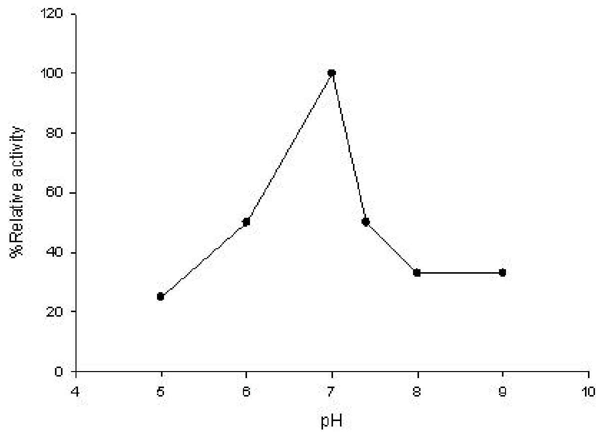

Figure 1. Effect of pH on lipase activity in 50 mM acetate, pH 3.0-5.0; 50 mM phosphate, pH 6.0-8.0; and 50 mM carbonate buffer, pH 9.0-10.0.

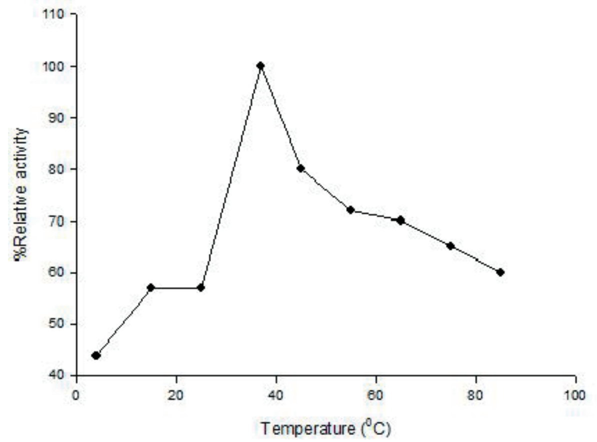

Figure 2. Effect of temperature on lipase activity at 5-85 °C, with 10 °C increments,for 30 min in 50 mM phosphate buffer, pH 7.0

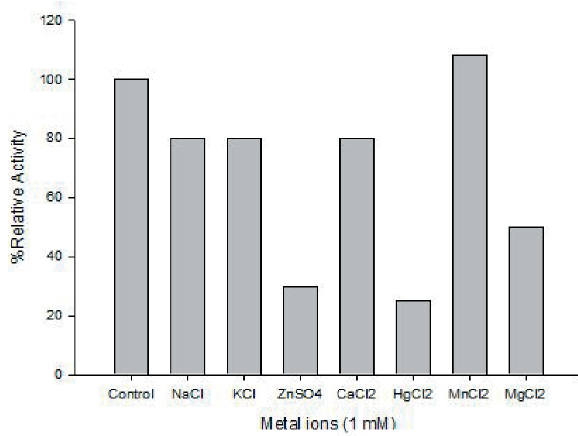

Figure 3. Effect of metals (1 mM) on lipase activity.

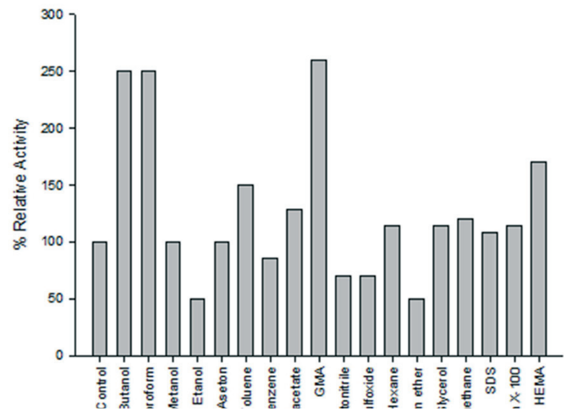

Figure 4. Effect of organic solvents (5%v/v) and detergents (5%v/v) on lipase activity. (GMA: 2,3-epoxypropyl methacrylate, HEMA: hydroxymethyl methacrylate).

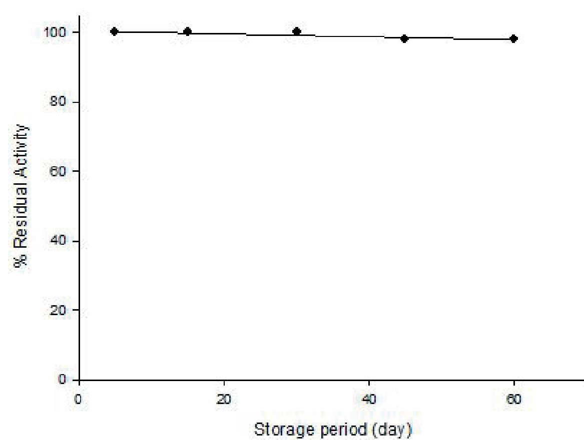

Figure 5. Storage stability of lipase at 4°C.

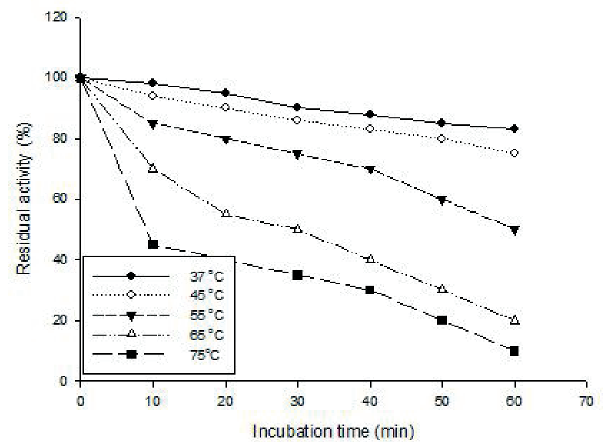

Figure 6. Thermal stability of lipase (at 37, 45, 55, 65, and 75 °C).

Table 1. Substrate specifity of lipase (reaction conditions: 250 mg substrate, 250 mg gum arabic, in 50 mM phosphate buffer, pH 7.0, at 37 °C, for 30 min)

Substrate (250 mg)	%Relative Activity
Olive oil	72
Sunflower oil	85
Tributyrine	72
Tween 80	100
Tween 40	85
Tween 20	72

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Conflict of interest: The authors do not have a conflict of interest.

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