Journal of Molecular Catalysis B: Enzymatic 64 (2010) 155–161

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

An organic solvents tolerant and thermotolerant lipase from *Pseudomonas fluorescens* P21

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ARTICLE INFO

Article history:
Available online 24 September 2009

Keywords:
Organic solvents tolerance
Extracellular lipase
Pseudomonas fluorescens

ABSTRACT

In this study, 38 organic solvents tolerant bacteria have been isolated from 21 different soil and water environments and the bacterium called P21 has been isolated among them due to its ability of surviving in 30% toluene, which is highly toxic to microorganisms and its capability of high lipase production (3.5 U/l). The lipase production medium has been optimized and the medium containing olive oil as an inducer and peptone as a nitrogen source has been selected as the best ingredients for the highest lipase production conditions (15 U/l). After this optimization, initial lipase activity has increased to 638.3%.

In our study, to identify the physical and molecular properties of the lipase, the supernatant has been obtained at the highest production value in early stationer phase of the P21's growth curve and the supernatant has concentrated by ultrafiltration and ammonium sulphate precipitation. SDS-PAGE and zymogram analysis were exhibited two lipases with a molecular weight 15 and 38 kDa. Partially purified lipase is optimum active at pH 7.0 and 30 and 60 °C. The lipase activity is stable in the presence of organic solvents such as cyclohexane, styrene, hexane and heptane. The enzyme protects its 85.3% of its activity after incubating for one and half an hour at 70 °C.

P21 strain has identified as *Pseudomonas fluorescens* by biochemical and molecular techniques and has recorded as *P. fluorescens* P21 in National Center for Biotechnology Information (www.ncbi.nih.gov) with an accession number, FJ605510.

1. Introduction

The lipases from living organisms are used as catalysts for polymer synthesis in vitro. Fundamental studies on polymerizations revealed some remarkable capabilities of lipases for polymerization chemistry. For instance, lipases catalyze transesterification reactions between high-molecular-weight chains in melt conditions. The polymerization and transesterification studies generally demand the presence of organic solvents and high temperature [1].

Organic solvents tolerant (OST) lipases are required in biotechnological applications, especially in the production of biopolymeric materials, biodiesel and in the synthesis of fine chemicals [2]. Recently, lipases from pseudomonas are the most widely used in biotechnological application because of their potential in organic synthesis for high valuable chemicals and there are some of *Pseudomonas* sp. lipases commercially available [3–6].

Purified enzymes are expensive for large-scale industrial production. As mentioned above, in various industries organic solvents are used for reactions. Because of this, biocatalyst which does not lose its activity is very important for "green chemistry". If an organism is capable of producing enzyme under required conditions, it may not be necessary to purify it [1,7].

In this study, in order to discover new sources for OST-lipases, extremophile bacteria which can grow in a medium containing organic solvents and which can secrete lipolytic enzyme are isolated. One biotechnologically potential lipase that is active at 60 °C and highly stable in unpolar organic solvents was discovered from OST *Pseudomonas fluorescens* P21.

2. Material and methods

2.1. Chemicals and mediums

The solvents and olive oil were purchased from Sigma. The DNA polymerase and the primers were purchased from Roche. All the other chemicals were of the highest reagent grade and commercially available. The mediums used in this study are—enrichment culture medium [7]: 10.0% olive oil, 0.5% $(NH_4)_2SO_4$, 0.35% K_2HPO_4, 0.1% KH_2PO_4, 0.25% NaCl, and 0.05% $MgSO_4{\cdot}7H_2O$; nutrient medium (NM) [6]: 0.5% glucose, 0.5% peptone, 0.3% yeast extract, 0.25% NaCl and 0.05% $MgSO_4{\cdot}7H_2O$, pH 7.5. If the solid medium is necessary, 1.5% of agar was added into broth mediums.

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doi:10.1016/j.molcatb.2009.09.009

30 ml of olive oil was emulsified with 250 μl of Tween 80 and 50 ml of distilled water and the mixture was homogenized by blender, autoclaved separately and then added into mediums [8]. In Rhodamine B agar method, 20 mg of Rhodamine B is suspended in 20 ml of water and was added into lipoidal emulsion.

2.2. Isolation of solvent-tolerant lipase-producing microorganisms

Organic solvent-tolerant microorganisms were isolated according to Ogino et al. [7]. Briefly, 1 g of soil obtained from petroleum contaminated soils and water from Aegaean Sea was added into 9 ml of enrichment culture medium and was covered by 3 ml of cyclohexane. The test tubes were plugged with rubber stoppers to prevent evaporation of the organic solvent. The culture was incubated at 30 °C with shaking for 1 week. To isolate the organic solvent-tolerant bacteria, the enrichment medium was spread on a nutrient medium plate. The plate was overlaid with 7 ml of cyclohexane and incubated at 30 °C for 24 h. Growing colonies were purified by streaking single colonies on nutrient medium plate in the absence of cyclohexane several times. 5 μl of the nutrient medium in which organic solvent-tolerant strains grew were spotted on tributyrine (1%, v/v) agar plates. After incubation at 30 °C for 24 h the strains which have formed large clear zone around the colonies were selected as potential lipase-producing bacteria. In order to determine the true lipase-producing bacteria, potential lipase-producing bacteria were spotted on Rhodamine B (0.02%, w/v) agar plates. After 24 h of incubation at 30 °C colonies which showed orange fluorescence under UV irradiation were recorded as lipase-producing organic solvent-tolerant bacteria [8]. Quantitative lipase activity of the lipase-producing organic solvent-tolerant bacteria has done by p-nitrophenyl palmitate (pNPP) assay [9].

The strains were stored on Nutrient Medium (NM) agar slants at 4 °C and sub-cultured in every 4 weeks.

2.3. pNPP assay

pNPP assay was modified from Winkler and Stuckmann [9] and Gupta et al. [10] pNPP (30 mg) dissolved in 10 ml isopropanol and 90 ml Tris buffer (pH 7.4) in conjunction with Triton X-100 (200 mg) was used as substrate solution. 0.1 ml of the enzyme was added into 2 ml of the substrate solution and the reaction performed at the desired temperature and incubation time. Following the enzyme reaction, the amount of p-nitro phenol (pNP) released was estimated spectrophotometrically at 405 nm.

One unit of lipase activity was defined as the amount of enzyme liberating 1 μmol pNP in 1 min under standard assay conditions.

2.4. Organic solvents tolerance of isolated strains

Solvent tolerance was determined plate overlay solvent tolerance as described by Ogino et al. [7]. Briefly, 5 μl of an overnight culture (10^6 to 10^7 cfu/ml) was spotted on an NM plate. Plates were left open during approximately 20 min (until the drops were dry), and after that 30% of aliphatic (1-pentanol, 1-octanol, 1-decanol, n-hexane, n-heptane, n-octane, n-decane, dodecane) and aromatic organic solvents (benzene, toluene, p-xylene, styrene, benzene, ethylbenzene, cyclohexane) were poured onto the plates. The plates were put into screwed jars in order to minimize evaporation. After incubation at 30 °C for 24 h the colonies were examined.

2.5. Optimization of the media

2.5.1. Effect of lipids

To test the effect of different lipids on the lipase production, glucose in the NM was substituted with 10.0% (w/v) olive oil, soybean oil, almond oil, corn oil, sunflower seed oil, hazelnut oil and lard oil. The cultivation was performed with rotary shaking (200 rpm) at 30 °C. In this study, peptone was eliminated from the NM.

2.5.2. Effect of nitrogen sources

On the basis of the results regarding the effects of lipids, 10% of olive oil was added to the media and glucose was removed from the NM. In order to determine the effects of different nitrogen sources on lipase production, the peptone and yeast extract in the nutrient medium was replaced with organic nitrogen sources (peptone, tryptone, and casein) and inorganic nitrogen sources (ammonium hydrogen phosphate, ammonium sulphate, and sodium nitrate), each at a concentration of 0.3% (w/v).

2.5.3. Effect of organic solvent

The yeast extract and peptone in the modified NM was replaced with peptone (0.3%), renamed as lipase producing medium (LPM), and used in further study. In order to elucidate the effects of organic solvents on lipase production, 100 ml of LPM in 500 ml Erlenmeyer was inoculated with 10^6 to 10^7 cfu/ml of the P21 strain and overlaid with 30% of cyclohexane. 1 ml of sample was taken every 3 h for 24 h for lipase activity and growth curve (OD660). LPM medium without cyclohexane was used as control.

2.6. Partial purification of P21 lipase

2.6.1. Preparation of the lipase crude extract

After P21 strain was cultured at 30 °C at 200 rpm for 12 h, the supernatant was prepared by removing the cells in the culture by centrifugation at 10,000 rpm for 10 min. The oily part of the supernatant was then eliminated by a burette. The supernatant was filtered from 0.2 μm filter paper and the filtrate was used as the crude lipase in further studies.

2.6.2. Partial purification of lipase

In order to decide the most efficient method to concentrate the crude lipase, ammonium sulphate fractionation, acetone, trichloroacetic acid (TCA), chloroform/methanol precipitations [11] and ultrafiltration method were examined. 1600 ml of lipase crude extract was concentrated by ultrafiltration with a 10 kDa cut off membrane and precipitated by ammonium sulphate fractionation (25%, 85%). All the precipitates were resuspended in a minimal amount of buffer (0.01 M Tris–HCl, pH 7.4) and dialyzed against the same buffer by using successive large volumes of buffer. The process was continued till the last traces of ammonium sulphate were removed. All of the process was performed at +4 °C.

2.6.3. Determination of protein content

The protein content was determined according to the Bradford method [12] using the Bio-Rad assay reagent and bovine serum albumin (BSA) as the standard.

2.7. SDS-PAGE and zymogram

Protein samples were separated on 12% (w/v) SDS-PAGE gels under non-reducing conditions according to Laemli [13]. Following electrophoresis, SDS was removed from the gels by sequential washes in deionized water, 1% (v/v) Triton X-100 in 50 mM phosphate buffer and 0.2 M phosphate buffer, for 30 min each [14,15]. Activity staining (zymogram) was carried out according to Yadav et al. [14] with a modification; Victoria blue dye in the original method was replaced with 0.02% (w/v) Rhodamine B. The gel and overlay were incubated at 30 °C for 24 h and bands exhibiting orange fluorescence as lipase activity were defined under UV irradiation at 350 nm.

2.8. Effect of temperature on P21 lipase activity and stability

In order to determine the effect of temperature on activity, the lipase was incubated at various temperatures within the range 30–80 °C for 20 min. Residual activity was calculated by taking the activity at 30 °C as 100%. In a second set of experiments, the temperature stability was determined by preincubating the enzyme for 180 min at temperatures ranging between 0 and 70 °C and then measuring the remaining activity at 30 °C after adding the substrate. Residual activity was calculated by taking the activity at 30 °C as 100%.

2.9. Effect of pH on P21 lipase activity and stability

The optimal pH for enzyme activity was measured by incubating the enzyme substrate mixture at pH values ranging from 4.0 to 11.0 for 20 min at 30 °C. Residual activity at various pH was calculated by taking the activity at pH 7.0 at 30 °C as 100%. In another set of experiments, the pH stability was determined by incubating the enzyme in the optimum pH buffer for 180 min at 30 and 60 °C. Residual activity was expressed by taking the activity at pH 7.0 as 100%. The different buffers used were acetate (pH 4.0 and 5.0), phosphate (pH 6.0), Tris (pH 7.0–8.0), and glycine–NaOH (pH 9.0–11.0).

2.10. Organic solvent stability of the P21 lipase

The stability of the lipase in various organic solvents was determined by adding organic solvents into the enzyme solution at a 3:1 ratio. The mixture was incubated at 150 rpm at 30 °C. 1 ml of solution was extracted after 2 h [16]. Residual activity was expressed by taking the activity without any organic solvent as 100%.

2.11. Biochemical and molecular identification of P21

The VITEK2, an automated identification system, Gram negative (GN) test card (bioMérieux) was used for biochemical identification of P21 according to manufacturer's instructions.

P21 DNA was isolated from exponentially growing cells by NucleoSpin® Tissue kit according to manufacturer's instructions. Genomic DNA was utilized as a template for PCR amplification of 16S rDNA with Gm3F and Gm4R primers. Genomic DNA was amplified in a thermocycler (GeneAmp PCR system 2400, PerkinElmer, USA). The primers used were as follows: forward (GM3F) 5′-AGA GTT TGA TCM TGG C-3′ and reverse: (GM4R) 5′-TAC CTT GTT ACG ACT T-3′. The amplification conditions consisted of a cycle of predenature (95 °C, 5 min), 30 cycles of 95 °C (30 s), 46 °C (30 s), and 72 °C (2 min), plus one additional cycle with a final elongation (72 °C, 7 min), and then was maintained at 4 °C. The amplified products were evaluated via agarose gel electrophoresis. The PCR product, with a size of 1600 bp, was purified and ligated into the PGEM-T cloning vector (Promega) in accordance with the manufacturer's instructions, and was then transformed into *E. coli DH5α*. After selecting the blue colonies [17], the plasmid was then extracted and sequenced. A DNA homology search was conducted using the Genbank database (http://www.ncbi.nih.gov).

3. Results and discussion

3.1. Isolation solvent-tolerant lipase producer microorganisms

16 soil samples from petrochemistry industry, 1 waste sample from dye industry and 3 water samples from oil contaminated parts of harbor were investigated to obtain organic solvent-tolerant lipase producer microorganisms. 38 solvent-tolerant strains were isolated and 20 of them were lipase positive in tributyrine agar plates. Since both esterases and lipases can hydrolyze tributyrine [18], we corrected the "true lipases" by Rhodamine B plate method. By this technique, 12 strains, having bright pink zones, were selected as true lipase-producing bacteria (Fig. 1).

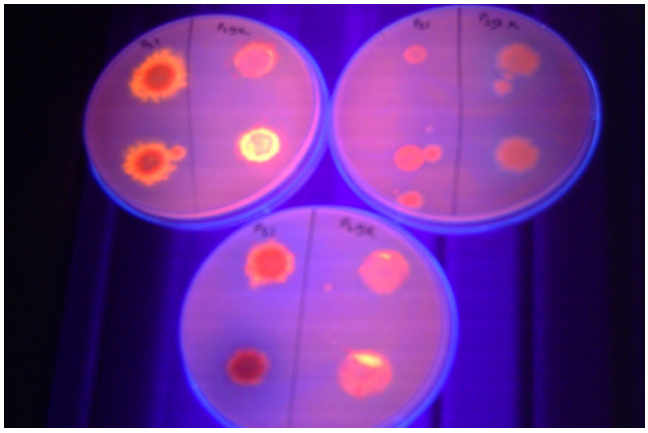

Fig. 1. Rhodamine B assays in different media. In LPM medium the bright pink zones can be seen in the first plate (P21, up left). In NM medium (bottom) P21 can grow but did not show lipolytic activity. In enrichment medium [6] the growth is very low and there is no lipolytic activity. P29K strain is another OST strain but does not show bright zones.

Quantitative lipase activities of the lipase-producing organic solvent-tolerant bacteria were shown in Table 1. As shown in Table 1, lipase activities of all strains are very close to each other.

3.2. Organic solvents tolerance of isolated strains

Organic solvent tolerance of isolated strains was taken account of the selection of the best organic solvents tolerant lipase-producing microorganism. As shown in Table 2, P21 was the most organic solvents tolerant lipase-producing microorganism with index log P_{ow} 2.69 and used in further study.

The effects of organic solvents on isolated microorganisms were investigated by plate spreading methods (Table 2). P21 can survive in all organic solvents except benzene and hexane. These results were compatible with plate spreading and broth inoculating methods except hexane. Although there was no colony in hexane-covered plate, the absorbance of P21 in NM broth with hexane was splendid (data not shown). The effects of hexane on P21 cell structure are due to the direct contact with organic solvents.

Considering the data from the last two experiments, we selected P21 as the organic solvents tolerant lipase-producing microorganism and used for further study.

Table 1
Lipase activities of the isolated organic solvent-tolerant strains. The activity was measured by pNPP method, at 30 °C.

No.	Activity (U/l)								
	1 h	3 h	6 h	9 h	12 h	15 h	18 h	21 h	24 h
P1	–	–	–	0.38	0.61	1.38	**2.56**	0.19	0.35
P3	–	–	–	0.19	0.32	0.96	**3.10**	1.66	0.54
P3k	–	–	–	–	0.64	0.83	**2.18**	1.15	1.09
P4	–	–	–	0.29	1.28	2.88	**3.20**	2.30	1.52
P6	–	–	–	1.28	1.02	1.02	**2.88**	0.90	0.10
P10	–	–	0.90	1.73	**3.20**	1.66	0.54	0.21	–
P13	–	–	0.70	1.47	2.11	2.02	**3.49**	3.36	2.18
P14	–	–	–	0.03	0.20	0.96	1.44	0.96	–
P16	–	–	–	0.82	1.18	0.80	**3.39**	–	–
P20	–	–	–	0.09	0.58	0.51	2.18	0.74	–
P21	–	–	0.50	1.98	3.33	**3.51**	3.36	1.52	1.50
P29	–	–	–	–	0.64	2.02	**3.36**	2.30	0.77

The bold numbers are the highest activities of the enzymes belonging to the strains.

Table 2
Organic solvents tolerance of isolated strains.

Solvents	Name	log P_{ow}	No.										
			P1	P3	P3K	P4	P6	P10	P13	P14	P16	P20	P21	P29K
Alcohols	1-Pentanol	1.4	+	+	+	–	+	+	+	+	+	+	+	+
	1-Octanol	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	1-Decanol	4.57	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Aliphatics	Cyclohexane	3.4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	n-Hexane	3.9	–	–	–	–	–	–	–	–	–	–	–	–
	n-Octane	4.9	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	n-Decane	6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Dodecane	6.8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Aromatics	Benzene	2.13	–	–	–	–	–	–	–	–	–	–	–	–
	Toluene	2.69	–	–	–	–	–	–	–	–	–	–	+	–
	Xylene	3.15	++	+	++	–	+	++	+	++	+	++	++	+
	Styrene	3.2	++	–	+	–	++	++	++	++	++	++	++	–
	Ethylbenzene	3.2	+++	+++	+++	++	+++	+++	++	+++	+++	++	+++	++

(–) no colony; (+) >5 colonies; (++) 5–15 colonies; (+++) >15 colonies.

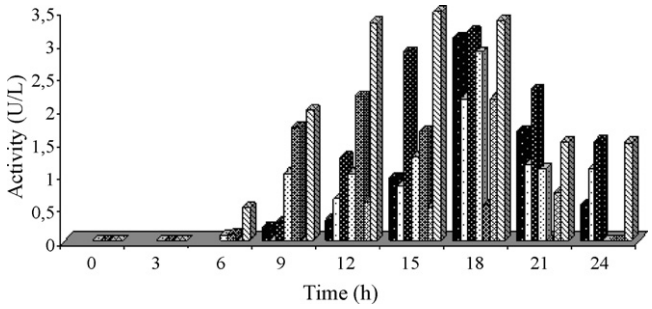

Fig. 2. Effects of lipids on lipase production. (■) sunflower seed oil; (□) lard oil; (▩) hazelnut oil; (▨) corn oil; (▩) almond oil; (▨) soybean oil; (▧) olive oil.

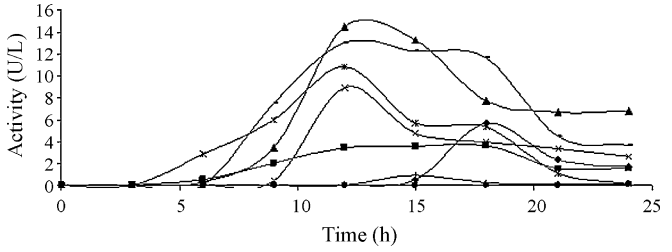

Fig. 4. Effects of nitrogen source on lipase production. 10% olive oil was added for inducing lipase (♦) peptone + yeast extract; (▲) peptone; (*) casein; (▌) ammonium hydrogen phosphate; (●) yeast extract; (×) tryptone; (■) ammonium sulphate; (▬) sodium nitrate.

3.3. Optimization of the media

3.3.1. Effect of lipids

The NM for lipase had glucose as carbon source. When glucose was replaced by various lipids, olive oil was the effective lipid for lipase production (3.5 U/l) (Fig. 2). When glucose in the NM was replaced with olive oil, the lipase yield was increased by 48.9% between 12 and 18 h (Fig. 3).

According to Fig. 3, it is obvious that olive oil acts as an inducer for lipases. It increases the activity almost two times at 12 h.

3.3.2. Effect of nitrogen sources

Yeast extract was the nitrogen source in the NM for the lipase. Yeast extract was replaced by various organic and inorganic nitrogen sources. Among the organic nitrogen sources tested, peptone exhibited a prominent effect on the yield of lipase in 12 h (15 U/l), while inorganic nitrogen source, sodium nitrate was as effective as peptone (13 U/l) (Fig. 4). As a conclusion after optimizations of the media, initial lipase activity was increased (638.3%).

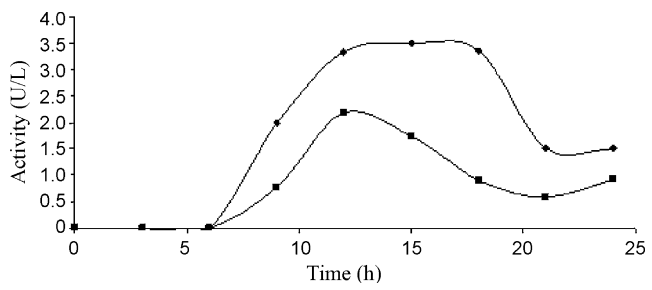

Fig. 3. Inducing effect of olive oil on lipase production. (■) Nutrient medium; (♦) nutrient medium containing olive oil.

3.3.3. Effect of organic solvent

On the basis of the hypothesis that, extracellular enzymes secreted by organic solvents tolerant microorganisms were stable in the presence of organic solvents, we have P21 produced lipase in the presence of cyclohexane. The growth in the presence and absence of cyclohexane was monitored spectrophotometrically at OD_{660} (Fig. 5).

The effect of cyclohexane on microbial growth in LPM broth was shown in Fig. 5. It is obvious that cyclohexane has an effect on doubling time. While the doubling time of the P21 in the presence of the cyclohexane is 141.3 min, it is 47.3 min in the absence of cyclohexane. Lipase production and cyclohexane resistance of P21 was cell-growth dependent (quorum sensing) and it reached the highest activity in the late logarithmic phase of the growth curve (Fig. 5).

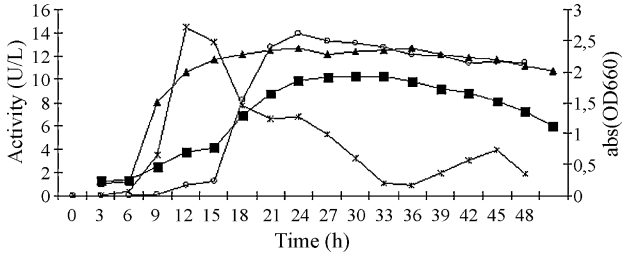

Fig. 5. Growth curve and lipase activity profile of P21 in medium, without cyclohexane and with cyclohexane (■) growth curve and (●) lipase activity of P21 in LPM supplemented with cyclohexane. (▲) and (*) growth of P21 and activity of P21 lipase in LPM used as control, respectively.

Table 3
Comparison of concentration methods of extracellular lipase.

	Activity (U/l)	Total protein (mg)	Specific activity (U/mg)	Efficiency of the method (%)
Lipase crude extract[a]	15.86	143.00	0.006	–
Acetone precipitation	0.40	5.96	0.003	93.0
TCA precipitation	0.16	1.46	0.006	80.4
Chloroform–methanol precipitation	–	–	–	–
$(NH_4)_2SO_4$ precipitation (%85)	5.20	30.8	0.008	97.1
Ultrafiltration	8.80	27.4	0.016	98.9

[a] Comparison has done for 50 ml of lipase crude extract.

Table 4
The summary of purification of lipase.

	Total activity (U)	Total protein (mg)	Specific activity ($\times 10^3$ U/mg)	Purification (fold)	Recovery (%)
Lipase crude extract	25.2	4578.3	5.5	1	100
Ultrafiltration	23.6	685.2	34.4	6.2	93.6
$(NH_4)_2SO_4$ precipitation	17.0	354	48.0	8.7	67.5

Furthermore, lipase in the presence of cyclohexane is more stable than the absence. Although the efficiency of lipase was similar in the mediums including and not including cyclohexane (±15.0 U/l), it was secreted earlier in the absence of cyclohexane (12 h).

3.4. Partial purification of P21 lipase

The methods used in precipitating or concentrating the lipase are the basic methods generalized for the primary purification step for extracellular enzymes. In order to decide the most efficient method to concentrate the lipase in crude extract, ammonium sulphate fractionation, acetone, trichloroacetic acid (TCA) and chloroform/methanol precipitations and ultrafiltration method were examined. The efficient method was decided with the formula given below, according to the recovered activity in the precipitates/retentate and supernatants/permeate for each method, independently.

$$\text{Efficiency of the method} = \frac{\text{Activity}_{\text{precipitates/retentate}}}{\text{Activity}_{\text{precipitates/retentate}} + \text{Activity}_{\text{supernatant/permeate}}}$$

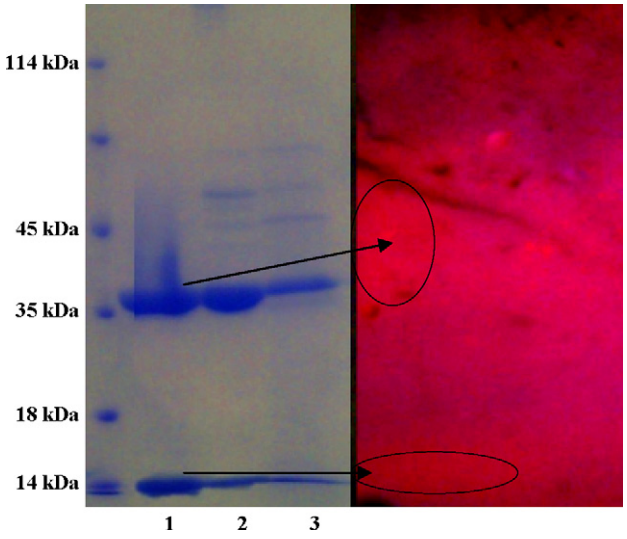

Fig. 6. SDS-PAGE and zymogram analysis of P21 lipase. Protein content of samples was determined by Bradford [10]. 1 mg of protein and peptide standards (Bio-Rad) were run on a 12% gel. Two major bands with molecular mass of 38 and 15 kDa were observed on SDS-PAGE by Coomassie brilliant blue staining (left). Both of them were hydrolyzed olive oil after zymography (right). Lane 1: $(NH_4)_2SO_4$ precipitate; lane 2: ultrafiltrate; lane 3: lipase crude extract.

It is obvious with Table 3, ultrafiltration and $(NH_4)_2SO_4$ precipitation are the most efficient methods (98.9% and 97.1%, respectively) for lipase and we used them consequently in high-scale purification.

1600 ml of lipase crude extract was partially purified and the summary of a typical purification of lipase is shown in Table 4. Lipase was purified to 8.7-fold purification with 67.5% yield.

Fig. 6 shows the SDS-PAGE and zymogram obtained with the above procedure after performing non-reducing PAGE on purification steps of lipase.

As seen in Fig. 6, the lipase activity occurring in the ammonium sulphate precipitate appeared as sharp, clear bands in SDS-PAGE led to a broadening of the orange-colored area in zymography under UV irradiation. Owing to the two bands in SDS-PAGE provoke activity in zymogram, we concluded that P21 secretes two different lipase into the medium with molecular mass of 38 and 15 kDa.

3.5. Thermal activity and stability of P21 lipase

As seen from Fig. 7a, the optimal activity for lipase was found to be in the temperature range 20–60 °C. As seen in Fig. 7b, the enzyme was stable for 150 min with the activity of 93.8% at 60 °C.

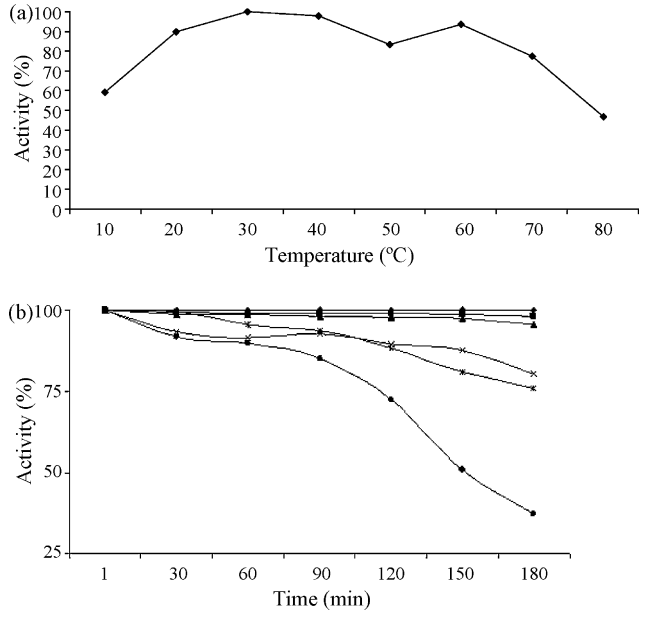

Fig. 7. Effect of temperature on activity (a) and stability (b) of purified lipase from P21. The enzyme activity (*A*) was measured using 25 mM Tris–HCl buffer (pH 7.4). (♦) 0 °C; (■) 30 °C; (▲) 40 °C; (×) 50 °C; (*) 60 °C; (●): 70 °C.

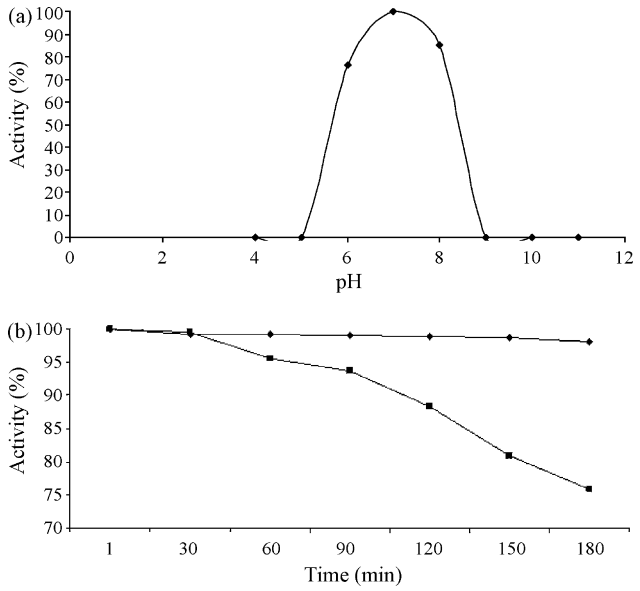

Fig. 8. Effect of pH on activity (a) and stability (b) of purified lipase from P21. The enzyme activity (a) was measured using acetate (pH 4.0 and 5.0), phosphate (pH 6.0), Tris (pH 7.0 to 8.0), and glycine–NaOH (pH 9.0–11.0) at 30 °C for 30 min. The pH stability (b) was measured in 25 mM Tris–HCl buffer (pH 7.0) at 30 °C (♦) and 60 °C (■) for 180 min.

At 70 °C for 120 min, it retained about 72% of the original activity. The optimum temperature of the lipase and its thermostability was higher as compared to other OST-lipases reported [19,20].

3.6. Optimal pH and pH stability

The optimal pH for lipase activity was found to be at 7.0 in Tris buffer (Fig. 8a). Below pH 5.0 and above pH 9.0, there was a gradual loss in activity (Fig. 8a). The pH stability of the lipase at pH 7.0 for 180 min exhibited that it was stable more than 180 min at 30 °C and is stable for 90 min at 60 °C. Its relative activity was more than 75% of the initial activity obtained at 60 °C (Fig. 8b).

3.7. Solvent stability of P21 lipase

The lipase produced by P21 is stable not only at high temperature, but also in unpolar solvents such as benzene, styrene, toluene, hexane, and heptane (Fig. 9). It is stable 2 h in heptane (91.4%), hexane (94.1%) and styrene (83.5%). Lipase of P21 also protected 66.7% of its activity in toluene for 2 h. This is considerably higher (~3-fold) than organic solvents tolerant LST-03 lipase [20].

Table 5
Identification of P21 by biochemical tests.

Biochemical test	Abbr.	Reaction
Ala-Phe-Pro-arylamidase	APPA	–
Adonitol	ADO	–
L-Pyrrolidonyl-aryl amidase	PyrA	–
L-Arabinose	IARL	–
D-Sellobiose	dCEL	–
B-galactosidase	BGAL	–
H_2S production	H2S	–
B-N-acetyl glucosaminidase	BNAG	–
Glutamyl aryl amidase pNA	AGLTp	–
D-Glucose	Dglu	+
Gamma-glutamyl transferase	GGT	+
Fermentation/glucose	OFF	–
B-glucosidase	BGLU	–
D-Maltose	DMAL	–
D-Mannitol	DMAN	(+)
D-Mannose	DMNE	+
B-xylosidase	BXYL	–
B-alanine arylamidase pNA	BAlap	+
L-Proline arylamidase	ProA	+
Lipase	LIP	–
Palatinose	PLE	–
Tyrosine arylamidase	TyrA	+
Urease	URE	–
D-Sorbitol	DSOR	–
Saccharose/sucrose	SAC	–
D-Tagatose	DTAG	–
D-Trehalose	DTRE	–
Citrate (sodium)	CIT	+
Malonate	MNT	–
5-Keto-D-gluconate	5KG	–
L-Lactate alcalinization	ILATk	+
Alpha-glucosidase	AGLU	–
Succinate alcalinization	SUCT	+
B-N-acetyl galactosaminidase	NAGA	–
Alpha-galactosidase	AGAL	–
Phosphatase	PHOS	–
Glycine arylamidase	GlyA	+
Ornitine decarboxylase	ODC	–
Lysine decarboxylase	LDC	–
L-Histidine asimilasyon	IHISa	+
Cormarate	CMT	+
B-glucoronidase	BGUR	–
O/129 resistance (comp. Vibrio.)	O129R	+
Glu-Gly-Arg-arylamidase	GGAA	–
L-Malate assimilation	IMLTa	+
Ellman	ELLM	–
L-Lactate assimilation	ILATa	+

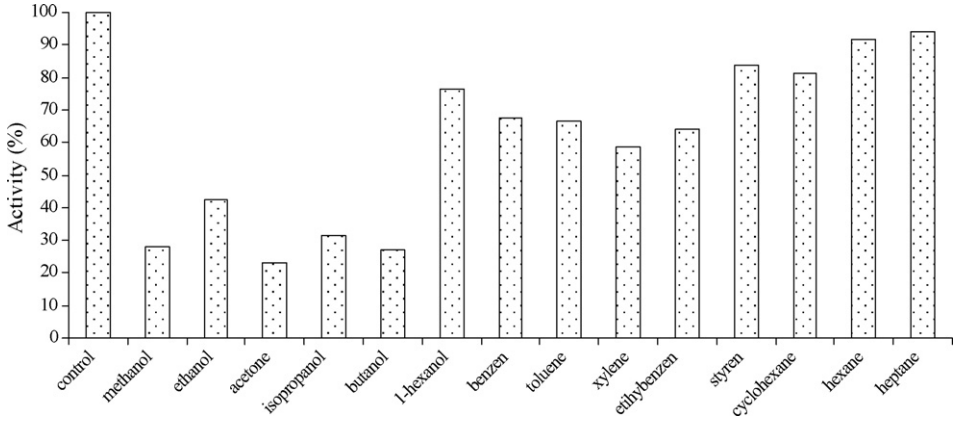

Fig. 9. Solvent stability of P21 lipase. Lipase activity was assayed by measuring the residual activity after incubating the enzyme in the presence of various organic solvents for 2 h.

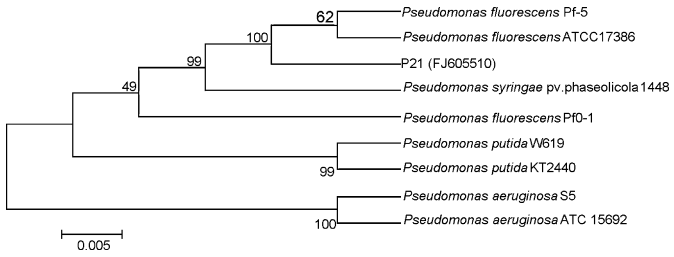

Fig. 10. Phylogenetic tree of P21.

However, the enzyme is unstable in polar organic solvents such as acetone, methanol, which is often used as a solvent for hydrocarbons.

3.8. Biochemical and molecular identification of P21

The results of the biochemical tests by VITEK2 Gram negative (GN) test card are shown in Table 5.

According to these results, P21 is *Pseudomonas aeruginosa*, *Pseudomonas putida* or *P. fluorescens*. In order to be sure, molecular identification of P21 has done. The sequences obtained from16s rDNA were compared with the sequences of bacteria from NCBI database by nucleotide BLAST program. Sequences were analyzed using fast minimum evolution method in the same database. P21 strain has identified as *P. fluorescens* by molecular techniques and has recorded as *P. fluorescens* P21 in NCBI database with an accession number, FJ605510.

Phylogenic tree was constructed by MEGA4 program. P21 and the relationship of closely related microorganisms were shown in Fig. 10.

4. Conclusions

The P21 lipase is obtained from an organic solvent-tolerant bacterium. This bacterium can secrete this lipase under stress conditions that includes presence of solvent and deficiency of easy to use carbon source. The stability in unpolar solvents and temperature makes us to think it is a useful biocatalyst in modifying lipids by means of its transesterification reaction.

Acknowledgements

This work was supported by a grant from The Scientific and Technological Research Council of Turkey (TUBITAK) and Gaziosmanpasa University Projects. We thank Dr. Juan Luis Ramos and Dr. Alejandro Acosta for their kind help.

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Glossary

Quorum sensing: A type of decision-making process used by decentralized groups to coordinate behavior. Many species of bacteria use quorum sensing to coordinate their gene expression according to the local density of their population.

Doubling time: The period of time required for a quantity to double in size or value. The doubling time is a characteristic unit (a natural unit of scale) for the exponential growth equation.