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Isolation of lipase producing *Bacillus* sp. from olive mill wastewater and improving its enzyme activity

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Abstract

The bacteria that could grow on media containing olive mill wastewater (OMW) were isolated and their lipase production capacities were investigated. The strain possessing the highest lipase activity among 17 strains grown on tributyrin agar medium was identified as *Bacillus* sp. The effect of initial pH on the lipase activity was investigated in tributyrin medium and pH 6 was found to be the optimal. The liquid medium composition was improved by replacing tributyrin with various carbon sources. Among the media containing different compositions of triolein, trimyristin, trilaurin, tricaprin, tricaprylin, tributyrin, triacetin, Tween 80, OMW, glucose, and whey; the medium contained 20% whey +1% triolein was found to give the highest lipase activity. Cultivation of *Bacillus* sp. in the optimal medium at pH 6 and 30 °C for 64 h resulted in the extracellular and intracellular lipase activities of 15 and 168 U/ml, respectively.

Keywords: *Bacillus* sp.; Isolation; Lipase; Olive mill wastewater

1. Introduction

Attempts to isolate microorganisms that produce lipase gain always attention since this enzyme is used in numerous biotechnological processes such as detergents, textile, and dairy industries; oil processing; production of surfactants; synthesis of chiral pharmaceuticals. Since each industrial application may require specific properties of the enzyme, there is an interest in finding new lipases that could create novel applications [1–3].

Many microorganisms such as bacteria, yeast and fungi are known to secret lipases during growth on insoluble organic substrates. Industrial wastes constitute a significance source for isolation of new organisms. Olive mill wastewater (OMW) is produced by olive oil plants and the phenolic contents and organic load of OMW create a serious environmental problem in the Mediterranean countries. Many research activities have been carried out to limit its discharge and to reduce its toxic content by using different chemical and biological treatment procedures [4,5]. Many fungal and bacterial strains were tested for aerobic biodegradation and detoxification of OMW [6–9]. OMW with its lipid and sugar contents in addition to tannins, polyphenols, polyalcohols and pectins [10] could be a good candidate to be used as a growth medium for microorganisms able to produce lipase.

Some studies report that some fungal strains obtained from different culture collections were able to grow and to produce significant amounts of lipase in OMW-based media. D'Annibale et al. [11] reported the growth of several fungal strains on OMW for extracellular lipase activity. Lanciotti et al. [12] used *Yarrowia lipolytica* strains for the treatment of OMW and for the production of lipase in OMW medium. However, the studies on the isolation of microorganisms from OMW and utilization of OMW as a growth medium for the production of lipase are very limited. Moreover, there is no report on the isolation of bacterial strains from OMW to be used for lipolytic activity. In the present work, we demonstrated, for the first time, that *Bacillus* sp. isolated from OMW exhibited a high lipase activity.

The literature reports that several *Bacillus* species isolated from several diverse environments produce lipase enzyme. In these studies, mostly the medium compositions that stimulate

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the production of lipase have been investigated. Kim et al. [16] isolated a lipase producing *Bacillus pumilus* strain from soil, cloned the lipase gene, expresses in *E. coli* and characterized its protein sequence as well as biochemical properties. Higher specifity of the lipase was found toward short-chain triglycerides than medium and long-chain ones. Ghanem et al. [17] reported the isolation of *Bacillus alcalophilus* as an alkalophilic thermostable lipase producer and investigated the influences of cations, pH and temperature on the activity of the enzyme. The authors found that the maximum lipase activity was at 60 °C and pH 10.6. Lindsay et al. [18] isolated *Bacillus* species from alkaline wash solutions used in dairy factories and investigated their physiology over a wide pH range. Castro-Ochoa et al. [19] isolated the strain of *Bacillus thermoleovorans* from hot spring and investigated the effects of pH, temperature, detergents and substrate specifity of the purified lipase. The strain showed the highest lipase activity toward *p*-nitrophenyl caprate (C_{10}) among *p*-nitrophenyl ester of C_2–C_{18}. Chen et al. [20] isolated *Bacillus* strains from milk powder production lines and investigated the activities of the strains toward different chain length substrates. They found that the strains showed higher activity toward short-chain length substrates than the others.

The purpose of the present study is to isolate a novel bacterial strain that is capable of producing lipase from OMW of the olive production plants located in Muğla-Milas (Turkey) and to increase its lipase production capacity by using OMW and whey that is a protein-reach industrial by-product in addition to pure fatty acids. The results of this work support the research for potential utilization of OMW as a source of new types of lipolytic bacteria.

2. Materials and methods

2.1. Isolation of lipolytic bacteria

Lipase producing microbial cultures were isolated from OMW originated in olive oil production plants located in Muğla-Milas (Turkey) and enriched by periodic subculturing of samples in Nutrient Broth (NB) media containing 20% (v/v) and 40% (v/v) OMW in successive. The composition of NB medium is (per liter) 5.0 g peptone and 3.0 g beef extract. The pH of the medium was adjusted to 7 with 0.1 M NaOH. The isolation process was performed by serial dilution of samples on tributyrin agar and egg yolk agar [13] plates according to standard techniques [14]. The composition of the tributyrin agar medium is (per liter) 5.0 g peptone, 3.0 g yeast extract, 10 ml tributyrin, and 15 g agar. Culture plates were incubated at 30 ± 1 °C (Memmert BE600) and periodically examined for 5 days. Colonies showing clear zones around them were picked out, purified on tributyrin agar plates and transferred to agar slants. The pure cultures developing on tributyrin agar medium were kept at +4 °C and transferred into fresh medium every 3 months. Isolated and purified high lipase producing bacterial cultures were identified according to cell morphology, gram staining and spore production properties [15].

2.2. Screening of lipolytic activity

Screening of isolated bacteria for their lipolytic activity was carried out in liquid tributyrin medium at pH 7. The cultures transferred into 100 ml of medium were incubated in a rotary shaker (New Brunswick Scientific Innova 4230) at 30 ± 1 °C and 100 rpm for 64 h. Samples taken from the incubation medium were analyzed for their extracellular and intracellular lipase activities.

2.3. Assay of lipase activity

Cells were separated from the incubation or cultivation medium by centrifugation (Hettich Rotina 35 R) at 14.000 rpm for 5 min at +4 °C and the supernatant was used as the source of extracellular enzyme. The harvested cells were washed with distilled water twice in microcentrifuge tubes, kept on ice and then sonicated (Sonics Vibra Cell) with a power input to 60 W and a frequency of 20 kHz for 5 min. The cell debris was removed by centrifugation at 14.000 rpm for 5 min at +4 °C and the clear supernatant was used to determine the intracellular lipase activity.

Lipase activity was determined using *p*-nitrophenol palmitate (*p*NPP) as substrate. The substrate solution was prepared by adding the solution A (30 mg of pNPP in 10 ml of isopropanol) into the solution B (0.1 g of gum arabic and 0.4 ml Triton X-100 in 90 ml of 50 mM Tris–HCl buffer, pH 8) with stirring until all was dissolved. The mixture of 9 ml of substrate solution and 1 ml of suitably diluted enzyme solution was incubated at 30 ± 1 °C for 15 min and absorbance was measured for 15 min at $\lambda = 410$ nm in a spectrophotometer (Shimadzu UV 2001). One unit of activity (*U*) was expressed as μmol of *p*-nitrophenol released per minute under the assay conditions.

2.4. Selection of lipolytic bacteria

Among numerous numbers of isolated bacteria, 17 strains grown on tributyrin and egg yolk agars showed better lipolytic activity than the others and subjected to identification tests. Some morphological and physiological characteristics and lipolytic activities of the strains are tabulated in Table 1. Strain 14, which was identified as *Bacillus* sp., was selected for the production of lipase enzyme in further experiments since it showed the highest intracellular lipolytic activity.

3. Results and discussion

3.1. Effect of pH on lipolytic activity

The effect of initial pH on the intracellular lipase activity of *Bacillus* sp. was investigated for pH 6–9 values throughout 45 h of cultivations carried out in tributyrin liquid medium at 30 °C and 100 rpm. The pH of the medium was adjusted to the desired value with 0.1 M HCl or 0.1 M NaOH. In the experiments, 1 ml of culture, which was formerly activated at the same pH for the lipase activity, was inoculated in 100 ml cultivation medium. The results given in Table 2 showed that higher lipase activity

Table 1
Morphological and physiological characteristics and lipolytic activities of isolated strains (pH:7, *T*: 30 °C; agitation rate: 100 rpm)

Strain no:	Morphology	Gram	Spore	Egg yolk agar[a]	Tributyrin agar[a]	Intracellular lipase activity (U/ml)		
						16 h	40 h	64 h
14	Rod	+	+	−	+	8.5 ± 0.2	3.3 ± 0.4	56.2 ± 0.1
12	Rod	+	+	−	+	9.6 ± 0.3	26.1 ± 0.1	41.8 ± 0.2
11	Coccus	+	−	+	+	5.4 ± 0.2	12.3 ± 0.1	18.3 ± 0.1
5	Rod	+	+	+	+	5.1 ± 0.1	16.8 ± 0.2	15.6 ± 0.3
13	Rod	+	+	−	+	9.3 ± 0.2	9.3 ± 0.3	14.4 ± 0.1
7	Rod	+	+	−	+	6.0 ± 0.1	9.9 ± 0.4	13.8 ± 0.2
8	Rod	−	−	−	+	9.9 ± 0.3	11.7 ± 0.2	13.5 ± 0.1
4	Rod	+	+	+	+	1.0 ± 0.4	11.4 ± 0.2	12.9 ± 0.2
2	Rod	+	+	+	−	1.3 ± 0.2	1.5 ± 0.3	5.7 ± 0.2
16	Rod	+	+	−	+	11.5 ± 0.5	4.8 ± 0.3	5.4 ± 0.3
1	Rod	−	−	−	+	[b]	[b]	[b]
3	Coccus	+	−	−	−	[b]	[b]	[b]
6	Rod	+	−	−	−	[b]	[b]	[b]
9	Rod	+	−	−	−	[b]	[b]	[b]
10	Rod	+	+	+	−	[b]	[b]	[b]
15	Rod	+	+	−	−	[b]	[b]	[b]
17	Rod	+	−	−	+	[b]	[b]	[b]

[a] The formation of clear zones around the colonies (+).
[b] No lipase production.

Table 2
Effect of initial pH on the lipolytic activity of *Bacillus* sp. (*T*: 30 °C; agitation rate: 100 rpm)

pH	Intracellular lipase activity (U/ml)		
	t = 16 h	*t* = 40 h	*t* = 45 h
6	7.2 ± 0.4	30.0 ± 0.2	23.6 ± 0.2
7	8.5 ± 0.2	3.3 ± 0.4	2.7 ± 0.3
8	16.1 ± 0.3	9.3 ± 0.2	7.8 ± 0.1
9	18.3 ± 0.2	18.0 ± 0.2	12.6 ± 0.1

were obtained at pH 6 and 9 than the other pH values where pH 6 led to higher and more stable enzyme activity. Although bacteria prefer pH around 7 for best lipase production, maximum activity at higher pH values were also reported in the literature [2].

3.2. Effect of medium composition on lipolytic activity

The effect of medium composition on the lipase activity of *Bacillus* sp. was investigated at 30 °C and 100 rpm throughout 64 h of cultivations. In the experiments, 1 ml of culture, which was formerly activated in the same medium for lipase activity, was inoculated in 100 ml of cultivation medium. All media included (per liter) 5.0 g peptone and 3.0 g yeast extract as the basal components. Each experiment was carried out in triplicates.

Tables 3 and 4 show the extracellular and intracellular lipolytic activities of *Bacillus* sp. cultivated in the media of different compositions at pH 6 and 9, respectively. At pH 6, intracellular lipase activities were found to be higher than extracellular activities and showed a maximum about 40 h cultivation regard-

Table 3
Effect of medium composition on the extracellular and intracellular lipase activities of *Bacillus* sp. (pH:6; *T*: 30 °C; agitation rate: 100 rpm)

Medium	Lipase activity (U/ml)					
	t = 16 h		*t* = 40 h		*t* = 64 h	
	Int.	Ext.	Int.	Ext.	Int.	Ext.
Triolein ($C_{18:1}$) (1%, v/v)	37.3 ± 0.5	9.6 ± 0.3	138.4 ± 0.7	32.2 ± 0.2	25.2 ± 0.4	14.1 ± 0.3
Trimyristin (C_{14}) (0.05%, v/v)	30.0 ± 0.2	4.2 ± 0.2	50.8 ± 0.3	50.8 ± 0.4	6.0 ± 0.2	3.9 ± 0.4
Trilaurin (C_{12}) (0.05%, v/v)	25.8 ± 0.2	3.9 ± 0.4	48.1 ± 0.2	4.8 ± 0.2	6.0 ± 0.5	3.0 ± 0.4
Tricaprin (C_{10}) (0.05%, v/v)	25.2 ± 0.3	4.8 ± 0.2	15.3 ± 0.1	6.0 ± 0.2	25.5 ± 0.6	1.5 ± 0.3
Tricaprylin (C_8) (1%, v/v)	22.5 ± 0.3	3.3 ± 0.2	22.5 ± 0.4	3.9 ± 0.3	14.1 ± 0.1	9.3 ± 0.3
Tributyrin (C_4) (0.5%, v/v)	7.2 ± 0.4	5.7 ± 0.3	30.0 ± 0.2	9.9 ± 0.2	7.8 ± 0.1	6.6 ± 0.3
Triacetin (C_2) (1%, v/v)	13.5 ± 0.2	3.0 ± 0.1	24.9 ± 0.5	3.0 ± 0.2	13.5 ± 0.4	6.0 ± 0.4
OMW (1%, v/v)	21.0 ± 0.3	6.9 ± 0.2	45.7 ± 0.7	6.6 ± 0.4	15.6 ± 0.3	13.5 ± 0.2
Tween 80 (1%, v/v)	27.3 ± 0.3	4.8 ± 0.1	18.0 ± 0.2	3.3 ± 0.3	49.9 ± 0.7	23.1 ± 0.2
Glucose (0.5%, v/v)	1.5 ± 0.2	–	–	–	–	–
Glucose (0.5%, v/v) and tributyrin (1%, v/v)	5.7 ± 0.4	–	–	–	–	–
Whey (20%, v/v)	3.9 ± 0.3	–	32.5 ± 0.2	–	24.3 ± 0.1	–
Whey (20%, v/v) and tributyrin (1%, v/v)	–	–	7.8 ± 0.4	–	8.1 ± 0.3	–

No lipase production.

Table 4
Effect of medium composition on the intracellular lipase activity of *Bacillus* sp. (pH: 9; *T*: 30 °C; agitation rate: 100 rpm)

Medium	Intracelluar lipase activity (U/ml)		
	$t = 16$ h	$t = 40$ h	$t = 64$ h
Triolein ($C_{18:1}$) (1%, v/v)	–	39.8 ± 0.4	40.9 ± 0.6
Trimyristin (C_{14}) (0.05%, v/v)	1.0 ± 0.2	3.3 ± 0.4	3.8 ± 0.2
Trilaurin (C_{12}) (0.05%, v/v)	0.6 ± 0.2	2.8 ± 0.2	5.0 ± 0.1
Tricaprin (C_{10}) (0.05%, v/v)	3.6 ± 0.1	6.9 ± 0.3	–
Tricaprylin (C_8) (1%, v/v)	6.8 ± 0.3	17.1 ± 0.5	8.0 ± 0.2
Tributyrin (C_4) (0.5%, v/v)	18.3 ± 0.2	18.0 ± 0.2	7.8 ± 0.3
Triacetin (C_2) (1%, v/v)	8.9 ± 0.2	9.9 ± 0.3	15.5 ± 0.1
Tween 80 (1%, v/v)	2.5 ± 0.4	8.9 ± 0.2	10.4 ± 0.2
OMW (1%, v/v)	8.7 ± 0.3	12.2 ± 0.1	13.8 ± 0.3

No lipase production.

less of the medium composition. Triglycerides of higher chain lengths provided higher enzyme activities. The highest intracellular and extracellular activities were achieved as 138.4 U/ml and 32.2 U/ml, respectively, in the medium including 1% (v/v) triolein. 1% (v/v) OMW and 1% (v/v) Tween 80 increased the lipase activity of the isolated *Bacillus* sp. The presence of glucose in the cultivation medium, however, depressed the production of lipase. Almost no enzyme activity was observed after 16 h in the media including glucose. The presence of whey in the cultivation medium increased the intracellular lipase activity and no extracellular enzyme activity was obtained in this protein-reach industrial by-product.

At pH 9, no extracellular lipase activity was obtained in all of the media tested and the highest intracellular lipase activity was observed in the presence of 1% (v/v) triolein as 40.9 U/ml after 64 h of cultivation. At pH 9, low chain length triglycerides, i.e., tributyrin and triacetin, provided higher enzyme activity compared to triglycerides with the carbon number of 8–14. No lipase activity was observed in the presence of glucose, tributyrin + glucose, whey, tributyrin + whey at pH 9.

The results of the present study showed that *Bacillus* sp. isolated from OMW exhibited its lipase activity in different extents depending on the composition and pH of the medium. This might be addressed to the production of different lipase isoenzymes varying with medium conditions.

The additional effect of whey over triglycerides on the lipase producing capacity of *Bacillus* sp. was investigated in another series of experiments. Fig. 1 shows the variations in intracellular and extracellular lipase activities as well as dry cell weight with cultivation time in the media composed of 20% (v/v) whey + 1% (v/v) triolein and 20% (v/v) whey + 1% (v/v) OMW in addition to 5.0 g peptone and 3.0 g yeast extract per liter at 30 °C and pH 6. The highest intracellular lipase activity was achieved in the presence of whey + triolein medium as 168.2 U/ml. The confident effect of whey in the medium containing OMW was also observed; however, the intracellular enzyme activities obtained in the presence of whey + OMW was lower compared to whey + triolein. It is seen that intracellular lipase activity was higher than extracellular activity and lipase production occurred simultaneously to the cell growth and continued after the stationary phase was reached.

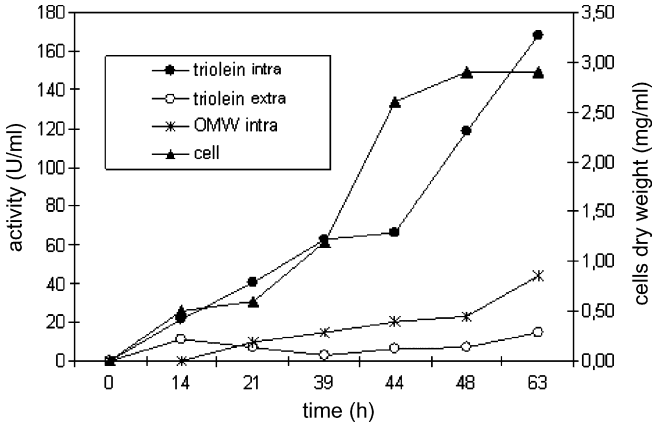

Fig. 1. Time course of lipase production by *Bacillus* sp. in the medium containing whey (20%), triolein or OMW (1%), peptone (5 g/l), yeast extract (3 g/l) ($T = 30$ °C, pH 6).

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