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The Effects of Heavy Metals and Molasses on Enzyme Activity of *Candida* Yeast

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ABSTRACT

Lipases are mainly applied in the food, abluent and medicine industries. Through the high production costs of lipase enzymes for industrial applications, cheap and eco-friendly enzyme production has gained great importance in recent years. Yeasts can produce lipase enzyme and grow in acidic media. In the present study, the act of Cu^{2+}, Ni^{2+} and molasses concentrations on the enzyme activity of *Candida* yeasts were investigated in a batch system. The maximum enzyme activities of microorganisms were determined at pH:4. Lipase enzyme activity was investigated changing metal ion and molasses sucrose concentrations by 25-250 mg/L and 1-20 g/L respectively. When molasses sucrose concentration was increased, the enzyme activity of all yeasts increased to 10 g/L, and the lipase enzyme activity decreased at the higher molasses concentrations. Enzyme activity of yeasts decreased with increasing both metal ion concentrations at constant molasses sucrose concentrations. Ni^{2+} cations were more inhibited to enzyme activity of all yeasts than Cu^{2+}. Among the yeasts, *Candida membranefeciens* (936.96 U/L) showed the highest enzyme activity in media containing a constant molasses concentration of 10 g/L.

Keywords: *Candida*, Lipase, Cu^{2+}, Ni^{2}, Enzyme activity.

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Introduction

Enzymes are known as biocatalysts with protein structure specific to a particular substrate naturally occur and can be synthesized artificially. The most common enzymes in nature are Amylase, cellulase, invertase, lactase, and lipase. Among these enzymes, lipases, which possess industrial and physiological importance are obtained from several herbs, bacteria, and yeasts. Hydrolysis of triglycerides to glycerol and free fatty acids through an oil-water interface is catalyzed by Lipases which are a member of hydrolase. Furthermore, they catalyze the hydrolysis and synthesis of other esters and play important role in transesterification [1,2]. Lipases exhibit a highly stable structure at high temperatures, pH, and organic solvents. Their enzymatic specificity regarding chemoselectivity, substrate selectivity, functional group selectivity, regional selectivity, stereoselectivity make it popular for the industry such as organic chemistry, detergent, milk, cosmetics, paper, and medicine [3,4]. Extracellular lipase is generally produced from various microorganisms and used in commercial applications [5]. Microbial lipases have some advantages such as high conversion efficiency of the substrate in comparison to other lipases that are produced from animal and plant cells [6]. One of the microorganisms that can produce lipase enzyme is yeasts. Among yeasts, *Candida* species can bind to metal ions in the environment as well as produce extracellular lipase with low nutritional requirements and high biomass production. Owing to active transport mechanisms, it transfers absorbed elements and accumulates them into cells as metal form [7]. The biomass of *Candida* yeast can be a source of mineral content by accumulating various substances that exceed the essential nutrients of the cells [8]. *Candida* yeasts are aerobic microorganisms and rapidly increase their biomass during aeration [9]. Optimum production conditions usually occur at 25-30 ^{o}C and pH 4.0-6.0 [2]. The enzyme activity of *Candida* yeasts is determined using appropriate carbon and nitrogen-based substrates in the nutrient medium and the presence of activating agents. Lipase activity may also change several parameters such as pH, temperature, substrate concentration, and inoculation level [10]. Lipid carbon sources, nitrogen sources, and micronutrients are required to obtain highly efficient lipase [11]. Furthermore, the synthesis and activation of lipase are affected by compounds that act as nitrogen source. Several nitrogen sources such as ammonium and sodium salts, yeast extracts, urea, peptone, etc. provide higher lipases activity for many microorganisms [12, 13]. Metal ions can stimulate or inhibit microbial enzyme production according to the genetic structure of the microorganism. Especially Ca^{2+}, effects structural and functional properties of enzymes, and plays a vital role in the production of some lipases with calcium-dependent metabolism. While Ca^{2+} ions increase enzyme activity, $Zn^{2+}$and ferric (Fe^{2+}, Fe^{3+}) ions strongly inhibit. Metal ions such as Co^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Al^{3+} and Na^{+} have slightly inhibitory effects on microbial lipase production [14,15].

For yeasts and fungi, acidic pH increases lipase production, and these microorganisms can grow in an acidic medium. A good lipase production efficiency was determined using *Rhodotorula glutinis* HL25 at pH:6 [16]. It was reported that lipase production and activity of *Penicillium sp.* section Gracilenta increased at pH:4 [17]. Temperature plays an important role in microbial lipase production. Low temperatures can decrease lipase enzyme production whereas high temperatures may cause enzyme degradation [18]. Temperature affects the enzyme activity of microorganisms according to microorganism type. Incubation time affects the lipase activity produced by the yeast due to optimum conditions for yeast growth and lipase production [19]. According to most studies, *Candida* yeasts are the most efficient lipase producer. *Candida utilis* [20], *Candida* [21], *Candida tropicalis* [22], (*Candida* albicans [23], *Candida* rugosa [24], *Candida cylindracea* [25], *Candida lipolytica* [26], *Candida antarctica* [27] can be given as examples.

Lipases are largely used in wastewater treatment under both aerobic and anaerobic conditions and are effective in water treatment for food and textile industries. For this reason, enzymatic techniques in water treatment have gained more attention because of specific properties such as enantiomeric, regional selectivity and substrate specificity [28]. Due to the *Candida* biomass can bioaccumulate metal ions, produce lipase enzyme and growth in metal contained media, uptake of Cu^{2+} and Ni^{2+} ions were investigated with changing molasses sucrose and Cu^{2+} and Ni^{2+} ion concentrations in our previous study [29]. We observed that biomass concentrations and specific growth rates increased when initial molasses sucrose concentration were increased in a medium containing metal and metal-free media. Specific growth rates of the yeasts were inhibited with Cu^{2+} and Ni^{2+} and the inhibition effect of Ni^{2+} ions was higher than Cu^{2+}. In this study, we aimed to investigate the enzyme activity of candida species in different fermentation media. Enzyme activities of yeasts were determined by changing growth media contents such as molasses and heavy metal concentrations in a batch reactor.

Material and Method

Candida membranafiens (*C. membranafiens*), *Candida utilis* (*C. utilis*), *Candida tropicalis* (*C. tropicalis*) and *Candida lipolytica* (*C. lipolytica*) were provided by the Department of Biology in Ankara University (Turkey). $(NH_4)_2SO_4$ and K_2PO_4 were bought from Sigma-Aldrich Company. Sugar factory which is in main Ankara (Turkey) donated molasses sucrose. Molasses consisting of 47-48% sugar was applied as the one carbon source of the yeasts for microbial growth [29].

Microorganism Growth Media

Growth media of *Candida* yeasts were prepared with 1 g/L $(NH_4)_2SO_4$ and K_2PO_4. Molasses sucrose concentration was varied from 1 to 10 g/L. Fermentation media was sterilized in an autoclave operated at 121°C at 0.99 bar for 15 minutes. Subcultures of yeasts were growth in 4 days by agitating at 150 rpm. 1 g $Cu(NO_3)_2.3H_2O$ and $Ni(NO_3)_2.6H_2O$ dissolved in water by one by and 1 g/L stock solutions of were prepared for Cu^{2+} and Ni^{2+} solutions. All experiments were conducted at pH:4 and 25 °C. Details of the adaptation experiments of the yeasts were discussed in our previous study. Microorganism concentration, residual Cu^{2+} and Ni^{2+} concentrations were determined at 360, 460 nm and 340 nm, respectively [29].

Analytical Procedure

P-nitro-phenylpalmitate (pNPP) method was used to the prediction of lipase activity of *Candida* yeasts. Experimental studies were compared in terms of lipase activity (U/L). In this method, the amount of enzyme needs to hydrolyze 1 µmol of pNP per minute describes as the one enzyme unit (U) of lipase activity. For the determination of enzyme activity spectrophotometrically, solutions A solution B and p-nitro-phenylpalmitate (pNPP) were used. For solution A: pNPP was dissolved in propan-2-ol and solution B: gum arabic and Triton X-100 was dissolved in distilled water. Solutions pH were adjusted to 8. Solution A was added to solution B by dropwise and it was used as the substrate solution. Tris buffer was used to adjust of pH as 8.5 [30]. This mixture was incubated for 30 min. at 150 rpm and 37°C. Enzyme activity of *Candida* yeasts was determined at 420 nm using a UV-vis spectrophotometer.

Results and Discussion

In our previous study we investigated microorganism growth by changing metal ions and molasses sucrose concentrations at pH: 4 and 25°C. The results indicated that biomass concentration was related to the metal concentrations in the fermentation medium and the physiological properties of the yeasts. pH was varied from 2 to 5 for the determination of pH effect on specific growth rate and maximum microorganism concentrations of *Candida* yeasts in a medium containing 10 g/L molasses sucrose. Maximum specific growth rate and microorganism concentrations were obtained at pH: 4. The highest specific growth rate and microorganism concentration were obtained as 0.308 h^{-1} and 3.111 g/L respectively using *C. lipolytica* in metal free medium. Molasses sucrose concentrations were varied from 1.0 to 20.0 g/L for investigation of growth rates of yeasts at pH: 4 and 25°C in metal-free media. Saturation kinetics were used to determination of the relevance of specific growth rate-substrate concentration. Detail results were presented in our previous study [29].

Lipase Enzyme Activity of Yeasts at Different Ph Values

Enzyme reaction rate changes with different hydrogen ion concentrations. Microbial growth and enzyme activity also changes the pH value of culture media. Most of the studies showed that the enzyme activity of yeasts is significantly sensitive to pH changes

during microbial growth and enzyme reaction slows down at pH values just next to the optimum pH value. In this case, the enzyme may denature and becomes inactive. Buffer solutions are used in enzyme studies to work at a constant optimum pH [31]. Lipase enzyme activities of yeasts at different pH values were investigated at pH: 2-5 and 10 g/L constant molasses sucrose concentration. From Figure 1, it was seen that lipolytic activity of yeasts increased with increasing pH up to 4.0. The highest value of enzyme activity was found as 934.52 U/L using *C. membranafiens*. Among the yeasts *C.utilis* showed the lowest lipase activity at pH:4 as 470.92 U/L (Figure 1). Keklikçioğlu Çakmak and Açikel (2015) reported that the lipase activity of *Candida utilis* was 788.5 U/L in aqueous media containing soybean oil at pH: 4 and 25°C [32].

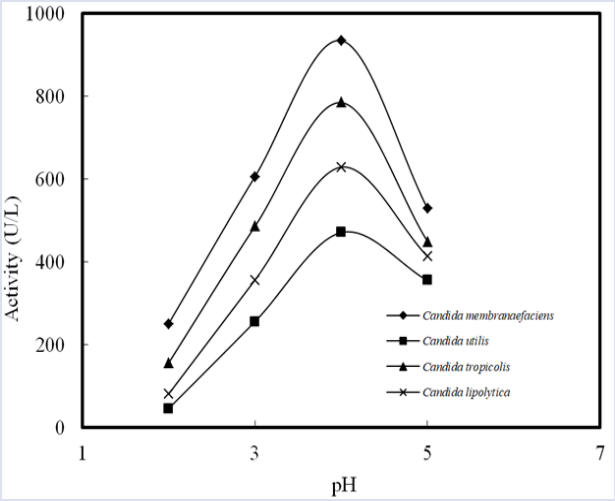

Figure 1. Lipase enzyme activity of yeasts at different pH values.

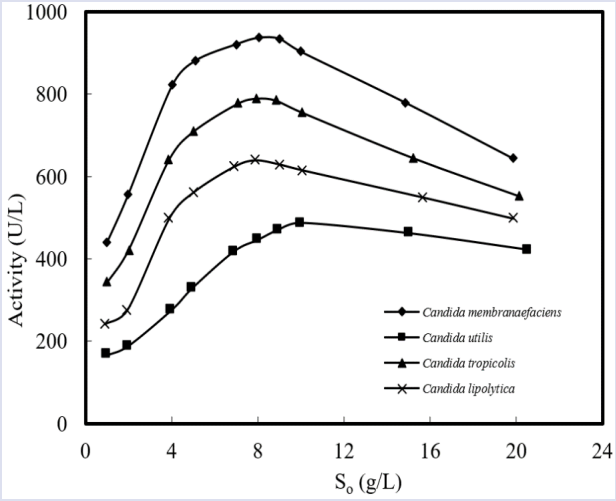

Figure 2. Lipase enzyme activity of yeasts at different molasses sucrose concentrations

Lipase Enzyme Activity of Yeasts at Different Molasses Sucrose Concentrations

Our results showed that initial molasses concentration influenced the enzyme activity of the yeasts. To investigate that, molasses sucrose concentrations were varied from 1.0 to 20.0 g/L, at pH 4.0 and at 25 ◦C. Enzyme activity of all yeasts increased with an increase in initial molasses sucrose concentration up to 10 g/L (Figure 2). It was attributed that higher molasses concentrations reduced the ethanol yield and efficiency of microorganisms. It also indicates catabolite or sugar inhibition that reduces enzyme activity in the fermentation medium. At high sugar concentrations, microorganisms produce ethanol rather than biomass under aerobic conditions. In this case, oxidative enzymes were less produced, and the cells shows fermentative metabolism [33]. Molasses contains trace elements such as K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^+, Cl^-, SO_4^-, PO_4^-, NO_3^- that did not show any significant influence on the lipid content in microorganisms [30]. Among the yeasts, *C. membranifaciens* showed the maximum activity at pH 4.0. It was determined as 903.41 U/L. Lipase activity of *C. membranifaciens* decreased from 903.41 U/L to 778.848 U/L with an increase in the initial molasses sucrose concentration from 10 to 15 g/L.

Açikel et al. (2011) used glucose and molasses sucrose as carbon sources for the determination of the lipase activity of *R. delemar*. They observed that the maximum lipase enzyme activity was obtained with 5 g/L of molasses sucrose [30]. Mihajlovski et al. (2016) examined the β-Amylase enzyme activity of *P. chitinolyticus* CKS1. Maximum β-amylase activity were stated as 2.237 U/ml under optimal conditions (inoculum concentration: 10%, incubation time: 83.07 h) [34]. Galvão de Morais et al. (2016) investigated lipase enzyme activity of *C. rugosa* at pH: 3.5 and 27 °C. They used 200 g/L of soybean molasses as a carbon nutrient. After 12 h, lipolytic activity was measured as 12.3 U/mL [35].

Lipase Enzyme Activity of Yeasts at Different Cu^{2+} and Ni^{2+} Ion Concentrations

The combined effect of single Cu^{2+} and Ni^{2+} ion and molasses concentrations on the lipase activities of *Candida* yeasts was studied. Initial Cu^{2+} and Ni^{2+} ion concentrations and molasses concentrations were varied from 25 to 250 mg/L and from 1 to 20 g/L respectively. Lipase activities of yeasts at different molasses and metal ion concentrations were presented in Table 1 and Table 2. The results showed lipase enzyme activity of yeasts increased with an increase in initial molasses sucrose concentration. But Cu^{2+} and Ni^{2+} ions showed adverse impact on the lipase production of *Candida* yeasts and lipase activity significantly decreased with an increase in Cu^{2+} and Ni^{2+} ion concentrations. It can be attributed that decreasing enzyme activity in metal-containing media may be related to the growth of microorganisms and was produced less enzyme as the microorganism growth decreases. This decrease might be due to a change in solubility of Cu^{2+} and Ni^{2+} ions, the catalytic properties of the enzyme and the properties of the ionized fatty acids at interfaces [14]. Lipase activities were determined as 701.04 U/L, 362.95 U/L, 578.52 U/L, 464.69 U/L for *C.*

membranaefaciens, *C. utilis*, *C. tropicalis*, *C. lipolytica*, respectively, at medium contained 10 g/L constant molasses and 200 mg Cu^{2+}/L (Figure 3 and Table 1). Under the same conditions, lipase activity decreased for all yeasts in a medium containing Ni^{2+} ions when compared to Cu^{2+} ions. For example, lipase activity was determined in a medium containing 200 mg Ni^{2+}/L and 10 g/L molasses as 634.19 U/L, 326.95 U/L, 525.65 U/L, 419.21 U/L for *C. membranaefaciens*, *C. utilis*, *C. tropicalis, C. lipolytica*, respectively (Figure 4 and Table 2).

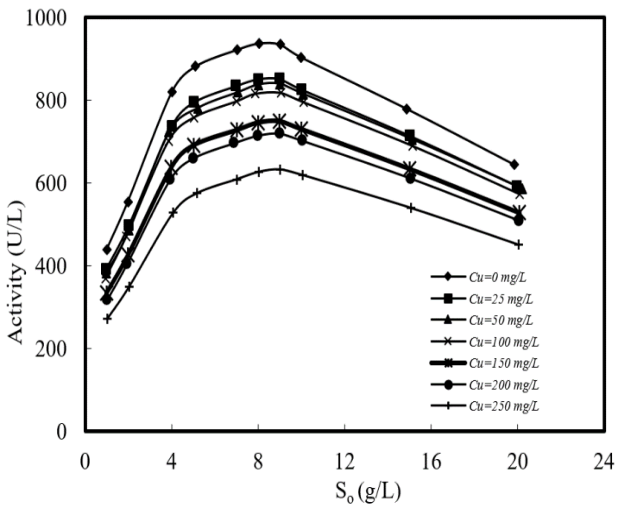

Figure 3. Lipase enzyme activity of *C. membrananaficiens* at different Cu^{2+} and molasses concentrations.

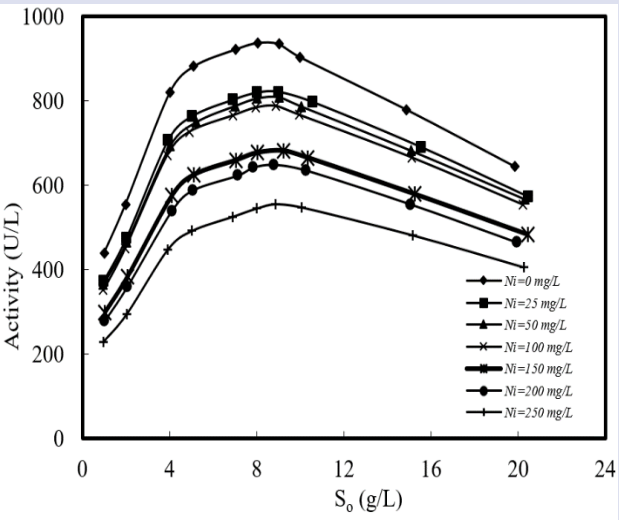

Figure 4. Lipase enzyme activity of *C. membrananaficiens* at different Ni^{2+} and molasses concentrations.

Lipase activities were determined as 903.4 U/L, 486.5 U/L, 755.2 U/L, 614.7 U/L respectively for *C.membranaefaciens*, *C. utilis*, *C. tropicalis*, *C. lipolytica* at 10 g/L molasses concentration in metal-free media (Table 3). For example, lipase activity decreased from 903.4 to 795.0 U/L with an increase in Cu^{2+} ion concentration 0.0 from to 100 mg/L at 10 g/L of constant molasses concentration using *C. membranaefaciens*. It was found that the inhibition effect of Ni^{2+} ions on lipase activity of all yeasts was higher than Cu^{2+} ions. Lipase activity decreased from 903.4 to 397.90 U/L with an increase in the initial Ni^{2+} concentration from 0 to 100 mg/L for *C. membranaefaciens* (Table 3). Lipase determination using living biomass, the chemical nature of metal ions plays an important role. Apart from essential trace metals for metabolic activities of microorganisms, higher concentrations of heavy metals might be deadly toxic and can be inhibited to enzyme activity [29, 36].

According to our results, molasses sucrose was efficient for lipase production by *Candida* species. In previous studies, it can be concluded that *Candida* species produced lipase and showed different enzyme activities in various fermentation mediums. Grbavcic et al. (2007) investigated the lipase activity of *Candida utilis* and they obtained the highest lipolytic activity of 284 U/dm^3 in a medium containing oleic acid and hydrolyzed casein as carbon and nitrogen sources, respectively, and supplemented with Tween 80 [37]. Andrade Silva et al. (2015) evaluated the use of cheese whey for lipase production by *Candida lipolytica*. The highest lipase activity was found as 118 U/mL at pH 5.0 -5.3 and 28 °C in the presence of cheese whey [26]. Rehman et al. (2014) investigated lipase activity of various yeast cultures including *Candida lipolytica* NRRL-Y-1095, *Candida utilis* NRRL-Y-900, *Candida tropicalis* NRRL-Y-1552 in a fermentation medium containing agro-industrial by-products. The maximum enzyme activity was determined as (1.12±0.09 U) with *C. utilis* NRRL-Y-900 when soybean meal was used in growth media [38].

Table 1. Lipase activities of yeasts at different initial molasses sucrose and Cu^{2+} ion concentrations

		U/L			
Yeast	**C_{oCu} (mg/L) S_o (g/L)**	**25**	**100**	**200**	**250**
C. memb.	2	496.29	470.81	404.35	348.95
	5	795.06	758.04	659.32	574.70
	10	825.71	795.00	701.04	617.93
	15	714.20	689.28	609.83	538.96
	20	592.08	572.78	508.42	450.50
C. uti.	2	162.32	154.06	132.07	113.66
	5	287.23	273.40	236.50	204.88
	10	430.09	413.55	362.95	318.19
	15	409.04	394.29	347.25	305.28
	20	374.91	362.28	320.15	282.24
C. tro.	2	373.23	353.85	304.56	263.28
	5	632.31	602.53	523.14	455.09
	10	682.74	657.07	578.52	509.04
	15	584.63	564.00	498.26	439.60
	20	502.70	486.13	430.89	381.17
C. lip.	2	242.18	229.45	197.34	170.22
	5	495.37	471.78	408.88	354.96
	10	549.52	528.63	464.69	408.15
	15	492.47	474.90	418.90	368.94
	20	447.75	432.82	383.07	338.30

Table 2. Lipase activities of yeasts at different initial molasses sucrose and Ni^{2+} ion concentration

Yeast	C_{oNi} (mg/L) S_o (g/L)	U/L 25	100	200	250
C. memb.	2	474.69	449.21	358.37	295.23
	5	763.33	726.31	587.04	491.84
	10	796.80	766.10	634.19	547.46
	15	690.05	665.13	553.76	481.32
	20	572.78	553.47	463.37	405.45
C. uti.	2	155.37	146.73	115.92	187.88
	5	275.37	261.54	209.49	329.40
	10	414.52	397.98	326.95	280.24
	15	394.75	379.99	314.05	461.16
	20	362.28	349.64	290.66	421.25
C. tro.	2	356.80	337.00	275.50	220.73
	5	606.79	577.01	473.52	388.46
	10	658.57	632.89	525.65	450.13
	15	564.65	544.02	453.14	391.90
	20	486.13	469.55	392.22	342.50
C. lip.	2	231.66	218.93	173.54	141.99
	5	475.15	451.56	362.82	302.16
	10	529.85	508.96	419.21	360.20
	15	475.45	457.88	379.37	328.31
	20	432.82	417.90	348.25	303.47

In recent years, green synthesis methods have gained importance in enzyme production. It is known as an environmentally friendly method that agricultural wastes are reused as a carbon source. Production of lipases using waste substrates such as molasses reduces the cost of obtaining valuable metabolites. *Candida* type yeasts are crucial, particularly for the food industry However, their specific properties enable *Candida* yeasts to be used in biotechnological processes such as lipase production and to be used in many commercial and industrial areas [39]. So, interest in the bioconversion of molasses sucrose into a value-added product such as lipase has increased. Molasses offers a wide range of potential uses in the manufacture of industrial enzymes like lipase and value-added bioproducts, according to recent studies and available data.

Lipase enzyme production using microorganism culture in a liquid medium is often preferred because of better control of growth and environmental conditions. This approach makes it possible to conduct essential optimization studies for maximum enzyme production. This study comparatively presents the inhibitory effect of heavy metals on the lipase production of *Candida* strains. Investigation of the survival of *Candida* type yeasts in a stressful environment has enabled the optimization of sustainable lipase production with wastes such as molasses.

Table 3. Decrease in lipase enzyme activities of yeasts in media contained 100 and 250 mg/L single Cu^{2+} and Ni^{2+} ions

Metal Ion (mg/L)		*C. membranefeciens*		*C. utilis*		*C. tropicalis*		*C. lipolytica*	
C_{oCu}	C_{oNi}	Activity U/L	Decrease %	Activity U/L	Decrease %	Activity U/L	Decrease %	Activity U/L	Decrease %
0.0	0.0	903.41	0.0	486.54	0.0	755.25	0.0	614.68	0.0
100.0	0.0	795.00	12.0	413.55	15.0	657.07	13.0	528.63	14.0
250.0	0.0	617.93	31.6	318.19	34.6	509.04	32.6	408.15	33.6
0.0	100.0	766.10	15.2	397.98	18.2	632.89	16.2	508.96	17.2
0.0	250.0	547.46	39.4	280.24	42.4	450.13	40.4	360.20	41.4

Conclusion

In this study, lipase enzyme activities of *Candida* species were investigated at pH:4 and 25 °C. Lipase activity of the *Candida* yeasts increased with an increase in initial molasses sucrose concentration to 10 g/L. In the experiments performed at a constant sucrose concentration of 10 g/L, maximum enzyme activity was obtained at pH 4.0 and *Candida membranaefaciens* showed the highest activity (934.52 U/L). It was found that the enzyme activities of all yeasts increased with the increasing initial sucrose concentration up to 10 g/L but it decreased above this value. We have found that molasses was a suitable carbon source for fermentation medium of *Candida* species. A significant decrease in enzyme activities of yeasts was observed when concentrations of Cu^{2+} and Ni^{2+} were increased. Enzyme activities of yeasts were inhibited with Cu^{2+} and Ni^{2+} ions and the contribution of Cu^{2+} ions in inhibition was lower than Ni^{2+}. The highest lipase activity was obtained using *Candida membranifaciens* in fermentation media not contained Cu^{2+} and Ni^{2+} ions singly.

Eco-friendly applications of lipase enzymes which were produced by *Candida* cells offer wastewater treatments that contain both lipid and heavy metal contaminations. Our studies showed that metal resistant *Candida* cells highly had lipase activity and they bioaccumulated Cu^{2+} and Ni^{2+} ions, simultaneously. We found that both microbial growth and lipase production depended upon initial pH, molasses and Cu^{2+} and Ni^{2+} ion concentrations of the growth medium. Compatible relevance was seen between lipase activity and biomass concentration. In our previous study, *Candida utilis* was sensitive to high concentrations of Cu^{2+} and Ni^{2+} with an

extension in lag phase duration, correlated with a decrease in lipase production. Although, we obtained the highest specific growth rate and microorganism concentration using *Candida lipolytica* in metal media, it did not show the highest lipase activity. So, there was an inverse relation between metal bioaccumulation and lipase activity. This difference may be due to the adaptation of yeasts to growth medium containing Cu^{2+} and Ni^{2+} ions, chemical properties of metals and differences in microorganism nature.

Our studies showed that *Candida* species can be innovative in lipase production and bioaccumulation of heavy metals from wastewaters. The study can also help other researchers in choosing an effective *Candida* strain for lipase production and metal bioaccumulation.

Conflicts of interest

There are no conflicts of interest in this work.

Acknowledge

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