Production of Lipase by Newly Isolated *Rhodotorula mucilaginosa* by Using Molasses, Important Environmental Pollutant

Hammamchı H.[1,*] Cihangir N.[2]

[1] Hacettepe University, Department of Biology, 06800 Beytepe, ANKARA TURKEY.

[2] Hacettepe University, Department of Biology, 06800 Beytepe, ANKARA TURKEY.

*corresponding author:

e-mail: hi_haman@yahoo.com

Abstract This present work describes the production and biochemical characterization of lipase by *Rhodotorula mucilaginosa* in a culture supplemented with molasses. This study reveals the utilization of renewable resource for their cost effective production and influence on the process under various conditions. In the production of lipase, culture conditions and media components are investigated as important parameters. After optimizing the incubation periods, effects of molasses concentrations, medium pH and incubation temperature. Olive oil with various concentrations as a different carbon sources were incorporated into the production medium and lipase activity were determined. The activity and specific activity of lipase immobilized on alginate. Maximum lipase activity was obtained during 6th day of fermentation at 150 rpm, pH 5, and 30°C temperature. When the concentration of the molasses in medium supplemented with olive oil *Rhodotorula mucilaginosa* showed the highest lipase activity in the medium with 1.0% molasses. Besides These results, important environmental pollutant molasses; can use as a cheap carbon source for biotechnological applications.

Keywords: lipase, Production, Yeasts, Molasses.

1. Introduction

Lipase is ubiquitous enzymes that catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification, and aminolysis It is also one of the most adaptable classes of industrial enzymes with catalyzing capability in aqueous and organic solvents [Treichel H, de Oliveira D, Mazutti MA, Di Luccio M, Oliveira JV. 2010]. In the production of cosmetics they can function as both active ingredients in a cosmetic formulation and biocatalysts in the synthesis of specific cosmetic chemicals [M.B. Ansorge-Schumacher, O. Thum, 2013]. Although lipases are produced widely in nature by various animals, plants, and microorganisms, only lipases of microbial origin, mainly bacterial and fungal, are commercially significant due to their stability, selectivity and broad substrate specificity as well as their easy extraction and potential for an unlimited supply. In addition diversity in catalytic activity, high yield, and cheaper cost of production and use of renewable resources. Moreover, not only are microbial lipases stable in organic solvents but they also do not require cofactors, in addition to encompassing broad substrate specificity [R. Gupta, N. Gupta, P. Rathi, Appl. 2004]. *Rhodotorula sp. such as Rhodotorula glutinis and Rhodotorula mucilaginosa* is one of the lipase producing microorganisms, which has been widely studied and has a potential for industrial production since it offers advantages over others in terms of high growth rate and the use of low-cost substrates. It can grow in several inexpensive agricultural raw materials such as sugar cane juice, peat extract, whey, grape must, beet molasses, hydrolyzed mung bean waste flour, soybean and corn flour extracts and sugar cane molasses [Potumarthi R, Subhakar C, Vanajakshi J, & Jetty A, 2008]. Several strategies have been used to model the fermentation process and to optimize the process parameters using conventional and statistical experimental designs. Several factors have been noted to affect the extracellular lipase production by fungi varies such as pH, temperature, kind of carbon and nitrogen sources, aeration, cultivation conditions, inorganic salts and the composition of the growth medium [N. Cihangir, and E. Sarikaya. 2004]. Therefore, the improvement in productivity of lipases can be achieved through manipulation of nutritional as well as physical parameters. This can serve as an inference for commercial success of any industrial production process. The agro-industrial residues products i.e. sugarcane molasses have been proved to be nutritionally enriched and thus can be used for production of many industrial products such as lipase enzyme by microorganisms. Molasses are the residue left after the crystallization of sucrose. It is the most economical source of carbohydrate for ethanol and citric acid fermentation. It contains reduced polymeric sugars that can further react to form fermentable sugar during enzymatic hydrolysis.

2. Material & Method

2.1. Isolation and identification of isolates for lipase activity:

Microorganism *was* isolated from the village which is located near Eskisehir city. Previously prepared PDA media (Potato dextrose agar) were kept open for 10

minutes in the chicken coop after that petries were incubated for 5 days at 30°C. Growth was observed after incubation. Microorganism strain used in this study was sented to Bioeksen Medical and Biotechnology Research Center for species identification. As a result of 18S rRNA analysis carried out, was determined that the microorganism is *Rhodotorula mucilaginosa.*

2.2. **Enzyme assay with tributyrin as a substrate:** The enzyme assay was performed with tributyrin as a substrate. The isolate were inoculated in medium that contains : %1 Agar, % 0,5 pepton %3 Yeast extract. Medium was sterilised by autoclaving at 121º C for 15 min. They were cooled to 60º C and then combined. After inoculation of the isotale, the plates were incubated for 7 day at 30^{0}C. Clear zones were taken as the indication of lipase activity.

2.3. **Molasses as Sole Production Medium for lipase activity:** To study the efficiency of renewable substrate from agricultural – waste product molasses as a sole medium sources on lipase activity, different concentrations of molasses varying from %1 to %5 were used for the production of lipase. The pH of the medium was adjusted to 5.0. The medium was poured in each Erlenmeyer flasks. The medium was sterilized at 121^0 C for 15 minutes. The Erlenmeyer flasks were incubated at 30^0 C on a rotary shaker at 150 rpm for 1-10 days. At regular time intervals, cell growth and lipase activity were determined. Culture samples were collected after 5 days the yeast growth was determined by the Spectrophotometric measuring absorbance at 450 nm after that the *Rhodotorula mucilaginosa* biomass was separated through Whatman No: 1 filter paper. The clarified supernatant was used as the source of enzyme. Lipase activity was quantified by free fatty acid titration. Lipolytic activity was calculated following equaiton: 50 x Expended KOH / 30 = U/ml followed by incubation at 30 ^{0}C for 6 days. Lipase activity was determined after 6 th days of incubation at 30 ^{0}C under shaking conditions.

2.4. **Effect of Incubation Periods on enzyme activity and stability:** Activity assays were done at 30^{0}C on a rotary shaker at 150 rpm for 1-10 days. Stability assays were done by incubating lipase for 30-90 min prior to the activity assay. Assays were carried out in triplicate.

2.5. **Effect of pH on enzyme activity:** Effect of pH on enzyme activity was determined by measuring the enzyme activity at different pH values ranging from 3 to 11 values at pH: 5.0, 30ºC and 150 rpm throughout 6 days of cultivations.

2.6. **Effect of temperature on enzyme activity and stability:** The optimal temperature for lipase activity was determined within the range of 10-40°C. The thermostability was determined by measuring the residual activity under standard assay conditions following the pre-incubation of the enzyme solution at 10 - 90°C for 30 min. Assays were carried out in triplicate.

2.7. **Effect of olive oil on enzyme activity and stability:** The enzyme production was estimated by using olive oil in addition to determined molasses medium. Olive oil with various concentrations from %1 to %5 as a different carbon sources were incorporated into the molasses production medium, by incubation at 30 ^{0}C for 6 days and lipase activity was determined. In order to investigate the effect of substrate concentration in the reaction medium which is the enzyme substrate mixture on lipase activity, %0 to %3 to of the olive oil used as the substrate was incubated with the enzyme source at 30 ° C and then the activity in the media was determined and plotted. Assays were carried out in triplicate.

3. Results

3.1. Isolation and identification of *Rhodotorula mucilaginosa:*

Microorganism was isolated from the village which is located near Eskisehir city, was selected to investigate lipase activity. As a result of 18S rRNA analysis carried out, was determined that the microorganism is *Rhodotorula mucilaginosa.*

3.2. Enzyme assay with tributyrin as a substrate:

Rhodotorula mucilaginosa showed clear lip olytic zone on tributyrin agar medium at 30°C.

3.3. Molasses as Sole Production Medium for lipase activity:

The effect of different concentrations of molasses varying from %1 to %5 on the enzyme activity was determined at pH: 5.0 and *Rhodotorula mucilaginosa* showed the lipase activity (6.6 U/ ml) at 1% molasses. Molasses is the by product obtained from sugar factory. The cost of molasses is lover than other sugars, so this material was chosen as a suitable carbon sugar sources for lipase production from *Rhodotorula mucilaginosa*, as shown in Fig. 1.

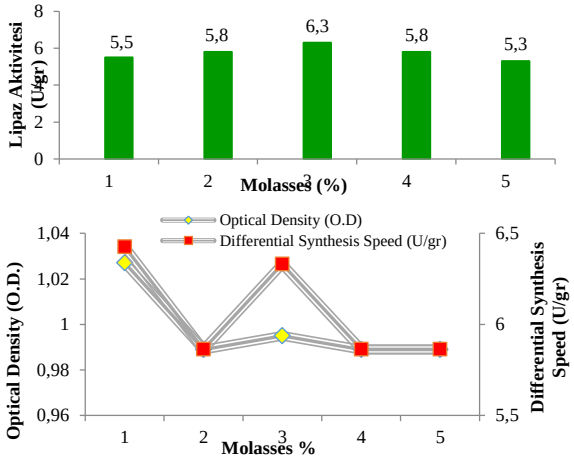

Fig.1. Effect of Various Molasses Consentrations as Sole Production Medium for lipase activity.

3.4. Effect of Incubation Periods on enzyme activity and stability:

Maximum production of lipolytic enzyme by *Rhodotorula mucilaginosa* was recorded at 6th day, further there was gradual decrease in growth of the organism and enzyme production. The growth rate in 7th day was very low. 8.16 U/ml maximum enzymatic activity was observed in 6th day incubation period. The organism did not show enzyme production after 6th day as shown in Fig. 2. When the reaction was kept at 30^{0}C for 50 min, the lipase retained almost 9.25 U/ml activity. However, the optimal activity was lost after 50 min, as shown in Fig. 3.

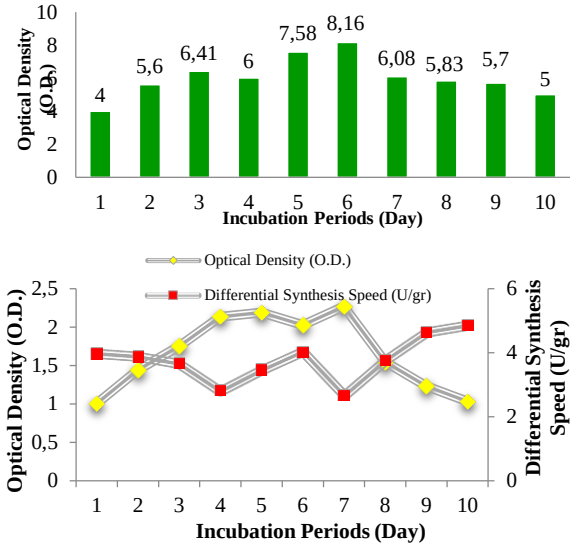

Fig. 2. Effect of Incubation Periods on enzyme activity.

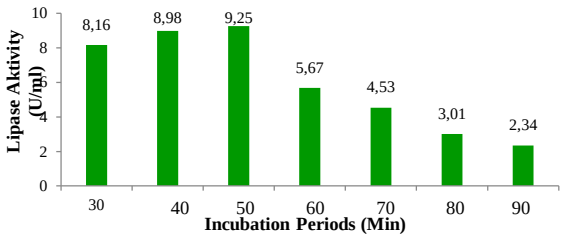

Fig.3. Effect of Incubation Periods on enzyme stability.

3.6. **Effect of pH on enzyme activity:**

The effect of the pH on the lipolytic enzyme activity of *Rhodotorula mucilaginosa* was examined at various pH ranging from 3 to 9, as shown in Fig. . The optimum pH and maximum enzymatic activity was recorded at 6th day pH: 5.0, 8.08 U/ml. At pH 4.0-5.0 showed maximum enzyme unit 8 and 8.08 U/ml respectively. lipase exhibited stable activity at pH levels from 4.0 to 11.0 with the maximum activity at pH 7, as shown in Fig. 4.

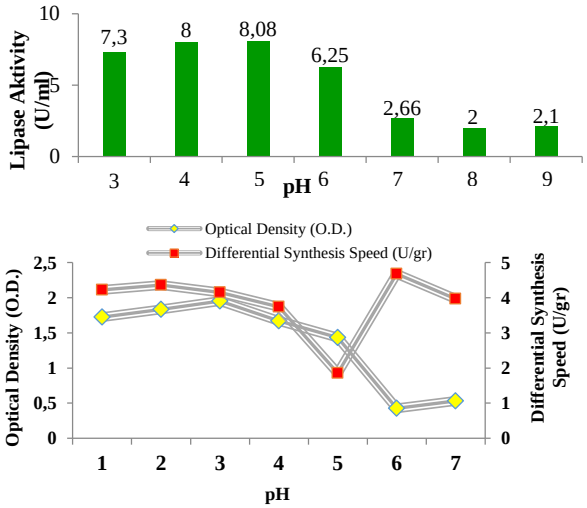

Fig.4. Effect of pH on enzyme activity.

3.7. **Effect of temperature on enzyme activity and stability:**

The effect of temperature on the activity of enzyme was determined at various temperature ranging from 10°C to 40°C at pH: 5.0 and *Rhodotorula mucilaginosa* showed good enzyme activity between 20°C to 30°C. The optimum enzyme production was at 30°C and reduction was observed in enzyme activity above or below 40°C, as shown in Fig. 5. When the reaction was kept at 60^{0}C for 30 min, the lipase retained almost 9 U/ml activity, whereas only 25% of the maximal activity was lost after incubation at 30-50^{0}C for 30 min. However, the activity was substantially reduced at temperatures higher than 70^{0}C, as shown in Fig. 6.

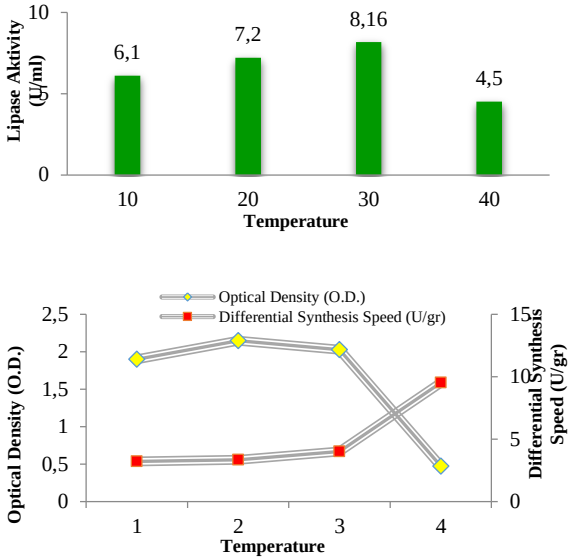

Fig.5. Effect of temperature on enzyme activity.

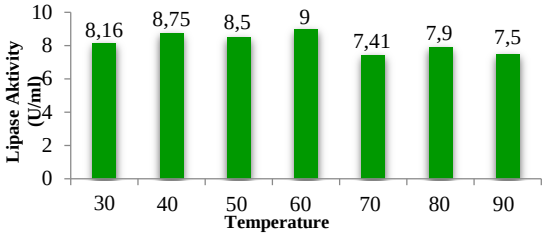

Fig.6. Effect of temperature on enzyme stability:

3.5. **Effect of olive oil on enzyme activity and stability:**

When diefferent concentration of molasses medium (%1 to %5) supplemented with %1 olive oil *Rhodotorula mucilaginosa* showed the highest lipase activity (10.3 U/ml), as shown in Fig. 7. However, when adding molsses higher than %3 the lipase activity decreased. In contrast, the total cell was increased from 1.267 to 1.289. The substrate concentration was determined as 1% olive oil, as shown in Fig. 8.

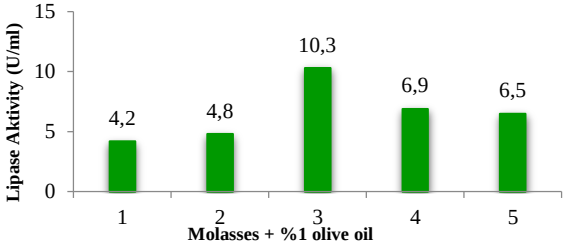

Fig.7. Effect of olive oil on enzyme activity

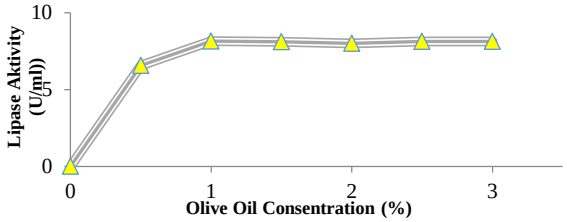

Fig.8. Effect of olive oil on enzyme stability.

3.8. Determination of Lipase Production and Activity in Determined Optimum Conditions:

Maximum lipase activity 11.65 U/ ml was obtained during 6th day of fermentation at 150 rpm, pH 5, 30°C temperature when the concentration of the molasses in medium supplemented with 1% olive oil *Rhodotorula mucilaginosa* showed the highest lipase activity in the medium with 1.0% molasses.

4. Discussion

The results of qualitative screening of lipolytic activity revealed that cultivating yeast in the acidified (pH 5.5) tributirate medium showed lipolytic activity with a clear zon on it. Tributirate medium with a different pH has been reported in several studies to lipolytic activity revealed [Paskevicius A. 2001]**.** In the present study, microorganism growth and enzyme production was determined during 1-10 days. The maximum amount of lipase activity was obtained on the 6th day. Most published experimental data have shown that the researcher was determined the effect of incubation time/day on microorganism growth and enzyme production [Pallavi Pogaku, A. Suresh, P. Srinivas and S. Ram Reddy. (2010), Dharmendra K. Parihar, 2012]. Similar observations were found in *Aspergillus terreus*, was showed maximum lipolytic enzyme activity at 5th day [R. Sumathi and R. S. Meerabai, 2012]. The relative activity was measured under assay conditions. In the previously published literature almost all studies in this domain have employed various pH and temperatures. Other research showed that *Aspergillus terreus* was showed maximum lipase activity at pH 7 [R. Sumathi and R. S. Meerabai, 2012]. However, pH 5 was suitable for *Aspergillus niger* and *Rhizopus japonicus* and pH 8 was found to be suitable for high lipase activity for *Aspergillus fumigatus* [Jitender, Sharma, Rajesh Kumar and Avatar Singh. 2004]. Similar observations were found in Cryptococcus sp. At pH 5.0 [Kamini, n. r,. Fujii, T., Kurosu, T., and Lefuji, H., 2000]. Though lipases have been reported form many sources, temperature dependence on lipase production by *Rhodotorula mucilaginosa* when tested proved that 30°C was suitable. Similar observations were found in and *Aspergillus terreus* [R. Sumathi and R. S. Meerabai, 2012], whereas *Humicola lanuginosa* exhibited high temperature optima between 40-45°C [Omar, IC., M. Hiyashi and S. Nagai. (1987] and Fusarium solani FSI showed the highest lipase activity below 35 °C but above this temperature activity losses were observed [M.M.D. Maia a,b, A. Heasley b , M.M. Camargo de Morais b , E.H.M. Melo d , M.A. Morais Jr. b,c, W.M. Ledingham b, J.L. Lima Filho. 2001]. *A. niger ATCC MYA-135* and *Rhizopus oryzae* were showed maximum lipolitic activity at 30.3°C [M. N. Hosseinpour, G. D. Najafpour, H. Younesi, M. Khorrami, Z. Vaseghi, 2012]. Most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase production [Mobarak-Qamsari E, Kasra-Kermanshahi R, Moosavi-nejad Z. 2011]. When the production medium was supplemented with trace elements using corn oil, olive oil and sesame oil, the lipase specific activity was increased [M.M.D. Maia a,b, A. Heasley b , M.M. Camargo de Morais b , E.H.M. Melo d , M.A. Morais Jr. b,c, W.M. Ledingham b , J.L. Lima Filho. 2001]. Similar findings was observed in *Aspergillus terreus* [R. Sumathi and R. S. Meerabai, 2012] and Dextrose was the best carbon source for maximum lipase production in *Aspergillus niger* [Elliaiah, P., T. Prabhakar, B. Ramakrishna, A.T. Taleb, and K. Adinarayana. 2002]. The best results in the production of lipase from *Candida rugosa*, were obtained with the use of olive oil as the carbon source [Fadiloglu S, Erkmen O. 2002] and in another work with *Candida* sp. sesame oil was promoted optimal lipolytic activity [Tan T., Zhang M., Wang B., Ying C., Deng L. 2003]. Other study reported that the *Sporidiobolus pararoseus* showed maxmimum lipase activity (26.9 U/mL) when %1 of olive oil was used as carbon source in fermentation medium. Molasses as a raw material at different concentration was taken for the study to estimate the yield for lipase. The lipase activity (12.91 U/mL.) was maximum at 1.5% concentration of molasses [Sasmita Sabat, V. Krishna Murthy, Pavithra M, PalaMayur and Anitha Chandavar. 2012] *Rhodotorula mucilaginosa*-MTCC 8737 also resulted good production with molasses, Increase in molasses concentration resulted in decreased lipase production by increase in viscosity of the fermentation broth [Potumarthi R, Subhakar C, Vanajakshi J, & Jetty A, 2008]. Other research showed that the highest dry cell biomass (1.67 g/L) and rhamnolipid (1.45 g/L) yields were observed, on 2% total sugars-based molasses [Z.A. Raza, M.S. Khan, Z.M. Khalid. 2007]. However, produced lipase from P. verrucosum by SSF using Soybean meal, sugar cane molasses and Soybean meal was the best substrate [Kempka *et al*. 2008]. Other research investigated the the cultivation of the fungus in the medium containing babassu cake and sugar cane molasses led to a lipase activity of 26.4 U/g by SSF in fixed-bed bioreactor [Cavalcanti *et al*. 2005]. The cultivation medium was babassu cake, supplemented with 6.25% (w/w) sugar cane molasses [Gutarra, M. L. E. 2003]**.** A similar trend was observed in *Mucor geophillus* , when it was grown on 5% molasses mineral medium evaluated maximum production of lipase (44.56 U/ml) [Naqvi, S. H., Khan, M.Y., Rafiq, M. & Dahot, M. U. 2012].

CONCLUSION

Lypolytic enzyme are most useful enzymes known and having great significance. The main purpose of this studies was investigation and characterization of lipase enzymes which was isolated by *Rhodotorula mucilaginosa* gave positive results to lipase activity in different lipase assay protocols. According to the results, The enzyme exhibited maximum activity in pH 4.0 and 5.0, but the optimum pH was investigated as pH: 5.0 and activity was low at acidic pH. Additionally the optimum grow temperature was determined as 30°C for lipase activity. The results obtained in this study show that as a carbone source: olive oil and maltose were the most suitable substrate for maximum lipase production by *Rhodotorula mucilaginosa* maltose and as a nitrogen source Peptone had shown highest lipolytic enzyme activity.

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