The Libyan Conference on Chemistry and Its Applications (LCCA 2021)
(15 – 16 December, 2021)

Lipase and Protease Production in Submerged and Solid State Fermentation by Candida rugosa using Olive Mills Wastes

Omar A. S. Moftah[1] and Zakia M. Moftah[2]
[1]Department of Chemistry Faculty of Sciences- Alasabaa, University of Gharyan, Libya.

[3]Department of General Sciences, Faculty of Agriculture, University of Ziantan, Libya.

ARTICLEINFO

Article history:
Received 15 April 2021
Accepted 30 April 2021
Available online 11 April 2022

Keywords:
olive oil cake, olive mill waste water, lipase, protease, fermentation, *C. rugosa*

Corresponding author :
Omarali702@yahoo.co.uk

ABSTRACT

To study the ability of using olive oil industry wastes by yeast strain *Candida. rugosa* to grow in solid state fermentation for lipase and protease production. Lipase production in submerged fermentation of olive mill waste water. The enzymes production was ranged from 4.2 to 9.8 IU/g of lipase on the second day of fermentation period and from 21 to 64.5 IU/g of protease on the third day of fermentation period. The supplementation of the olive oil cake with yeast extract as a nitrogen source significantly increased the lipase (157%) and protease (49 %) production. The chemical composition of olive oil cake estimated before and after fermentation process, results shown that the nutritional value was improved to be use as animal feed. Submerged fermentation of olive mill waste water shown that the yeast *C. rugosa* could growth on undiluted sample, and to produce up to 220.1 U/ dm^{-3} of lipase in 1st day, lipase production was found to be promoted by the addition of tween 80. The highest lipolytic activity of 1667.5 IU dm^{-3} was achieved after 6 days of submerged cultivation in supplemented olive mill wastewater. The results indicated that olive mill waste water and olive oil cake seemed to provide necessary nutrients and physical support for the yeast to grow and produce enzymes. The fermentation of olive mills wastes could be a good technique to save our environment and produce a valuable thing by using harmful wastes.

Introduction

Current agricultural and industrial practices have led to the generation of large amounts of various low-value or negative cost crude wastes, which are difficult to treat and valorize. The production of agro-industrial waste pollutants has become a major problem for many industries. The olive oil industry generates large amounts of olive mill wastes (OMWs) as by-products that are harmful to the environment (Mafakher etal 2010). About 214 kg olive oil, 496 kg crude olive cake, 40 kg of leaves and 1633 kg of olive mill wastes water (OMWW) are produced from 1 tonne of fresh olives (Salehmin etal 2014). However, OMWs have simple and complex carbohydrates that represent a possible carbon resource for fermentation processes. In addition, OMWs generally contain variable quantities of residual oil; the amount of are mainly depends on the extraction process. Therefore, OMWs could be used as the substrate for the synthesis of biotechnological high-value metabolites that their utilization in this manner may help solve pollution problems (D'Annibale etal 2006). Olive oil cake contains a rather high amount of nutritionally valuable substances such as sugars, protein and lipids, but generally it has low crude protein content, high crude fiber and high unsaturated fatty acids contents (Rigo etal 2010). Oil cakes are traditionally used as feed ingredients for farm animals. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new areas have opened for their utilization as raw materials for the production of value-added fine products like enzymes, antibiotics, mushrooms, antioxidants etc. Agro-industrial residues are generally considered as convenient substrates for biotechnological processes (Pandey etal 2000). Research efforts have been directed mostly toward extracellular lipase and/or protease production by fungus such as *Rhizhopus* sp., *Aspergillus* sp., *Penicillium* sp. on different oil cakes (Cordova etal 1998, , Sandhya etal 2005). While the potential of yeast as a strain to grow on solid substrates seems clear, few researchers have investigated the synthesis of lipases by yeasts using oil cakes and this mode of culture. Benjamin and Pandey (1997) cultivated *Candida rugosa* on coconut oil cake for lipase production using SSF and SmF systems showing that enzyme yields were higher in the former (Benjamin& Pandey 1998). Lipases and proteases are the most versatile biocatalysts due to their wide range of applications. Their applications are found in the detergent, food, leather, textile, oil, fat, cosmetic, paper and pharmaceutical industries (Sharma etal 2001). Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world account for approximately 40% of the total worldwide enzyme sale (Gupta etal 2002). The industrial applications of proteases go back to 1914 as detergent additives (Massucco 1980), food industries, leather (Andrade etal 2002), meat processing (Gibb and Stroh 1987l), cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Aleksieva and Peeva 2000). They also have medical and pharmaceutical applications (Benslimane etal 1995). lipases in general, and lipase from *Candida rugosa* in particular have been extensively investigated as tools of biological transformations catalyzed by enzymes in synthetic organic chemistry. In this way, lipase has been employed in the hydrolysis and synthesis of many organic

compounds (Valero etal 1991). *Candida rugosa* is a well-known lipase producer microorganism. (Benjamin& Pandey 1997). *Candida rugosa* lipase, has been widely used to catalyze hydrolysis, esterification, alcoholysis, acidolysis and trans esterification reactions yielding free fatty acids, diacylglycerol, monoacylglycerol, glycerol and/or specific ester compounds which have many biotechnological applications (Akoh & Shaw 2004).

To the best of our knowledge, so far, no attempts have been made to use the olive oil cake as a substrate for the production of lipase and protease in SSF by this yeast. The present study is aimed to valorize olive oil processing wastes (OMW and OOC) as substrate mediums for cultivation of the yeast *Candida rugosa* in order to facilitate the production of lipases and protease.

Experimental

Organism

The microorganism used in this study, *Candida rugosa*, was maintained on malt agar slants at 4 ºC. One day old culture grown in malt broth was used as the inoculum.

Characterization of the substrates

OMW samples were collected from various traditional olive oil mills in Alasaba- Libya, and used as fermentation medium for the submerged yeast cultivation. The substrate samples were characterized before fermentation for total solids, chemical oxygen demand (COD), pH, crude protein, phenols, reducing sugars and total lipids. The results were mentioned in the previous work (Moftah et al. 2013). Olive mill wastewater (OMW) characteristics make it a suitable resource to be used as a microbial culture media to produce value-added compounds, such as enzymes.

Samples of OOC were also obtained from various traditional olive oil mills in Alasaba- Libya, and used as a natural substrate for the solid-state fermentation (SSF). The composition of olive oil cake was described in the previous paper (Moftah et al. 2012). Olive oil cake has a relatively high protein and fat content, suggesting that the cake could be suitable substrate for SSF. The mean moisture of the cake was 51% w/w. They were packaged in vacuum-sealed packages and stored at 4 ºC until used.

Submerged fermentation using olive mill wastewater as a substrate

The submerged fermentation was performed by distributing 100 cm^3 of undiluted samples of the OMW to the Erlenmeyer flasks and sterilizing them at 121 ºC (at 1.2 bar pressure) for half an hour prior to inoculation with 1 % (v/v) of the yeast culture in malt broth. Fermentation was carried out in thermostat shaker at 30 ºC at 150 rpm. Samples were withdrawn at 24-hours intervals and tested for lipase activity. Composition of the OMW was optimized by the addition of ammonium sulfate (0.6 % w/v), yeast extract (0.1 % w/v), maltose (0.5 % w/v), olive oil (0.3 % w/v) and peptone I (0.1 % w/v).

Solid state fermentation on olive oil cake

Substrate was dried and sieved to provide the particle size between 0.2- and 0.5-mm. Experiments were carried out in 150 cm^3 Erlenmeyer flask with 5 g of well ground dry substrate supplemented with 0.15 g of yeast extract. Then, 1 cm^3 of distilled water was added and the contents of the flask were mixed and autoclaved at 121 ºC for 20 min. Unless otherwise mentioned, SSF was carried out by inoculating olive oil cake (initial moisture content adjusted to 50 %) with 500 µL of inoculum followed by incubation at 30 ºC. The water added with the inoculums was also considered in moisture correction. Optimization studies were performed by varying the moisture content of the substrate and amount of inoculum. The effect of addition of various carbon and nitrogen supplementation was also studied for optimal lipase production.[21] Simple and complex carbon source (maltose, oleic acid and starch) were used at 1 % to investigate their effect on lipase production.

Extraction of the enzymes

The crude enzyme was extracted by mixing a known quantity of fermented substrate with sterilized distilled water (1:5, w/w) incubation in an orbital shaker (Ika KS 4000-Werke, GmbH & Co.KG) at 30 ºC and 180 rpm for 30 min. A part of the liquid phase was used for determination of yeast cell growth, while the rest was centrifugated at 12,000 rpm for 10 min. The supernatant was used for determination of lipase activity.

Lipase activity assay

Lipase activity was determined by hydrolysis of the *p*-NPP (*p*-nitrophenyl palmitate) substrate by lipases according to the method described previously.[24] The amount of liberated *p*-nitrophenol was measured spectrophotometrically during the first 3 minutes of reaction. One unit of enzyme activity (IU) is defined as the amount of enzyme that formed 1 µmol of *p*-nitrophenol per minute (ε=1500 $dm^3\ mol^{-1}\ cm^{-1}$) under the assay conditions.

Protease activity assay

The proteolytic activity was measured using azocasein as a substrate. The method is based on the reaction of the enzyme sample with a 2% azocasein solution at 37 °C and pH 9.0. One unit of proteolytic activity was defined as the quantity of enzyme that produced a unitary difference in absorbance between the reaction blank and the sample under the assay conditions.

Results and discussion

Lipase and protease production by *Candida rugosa* in solid state fermentation on olive oil cake as a substrate*:*

The incubation time of *Candida rugosa* on olive oil cake as a substrate to produce lipase and protease is given in Fig. 1. The lipase production was ranged from 4.2 to 9.8 IU/g of substrate and the activity was found to be higher on the second day of fermentation and decreased thereafter. Our results are in agreement with a (Salihu etal 2012) while using ground nut oil cake as a substrate. But it's much lower than that achieved by *Penicillium restrictum* using soybean meals supplemented with urea (Gombert etal 1999), *Aspergillus versicolor CJS-98* using jatropha seed cake (Veerabhadrappa etal 2014) and *Pseudomonas aeruginosa PseA* (Mahanta etal 2008). Their protease production was ranged from 21 to 64.5 IU/g of substrate and the maximum of activity was on the third day; which is similar or near to our results by *Candida utilis* (Moftah et al. 2013).

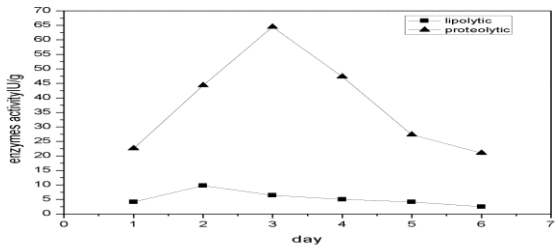

Fig. 1: The incubation time of *Candida rugosa* on Libyan olive oil cake. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

Effect of initial moisture content of substrate

The moisture content is a critical factor in solid-state fermentation. The optimum initial moisture content for lipase and protease production was determined by adjusting the initial moisture content of the fermentation substrate to varying levels of 50 - 75%. A moisture level of 55% (v/w) was found to be optimum for lipase production 12.7 U/g and protease production 58.7 IU/g. Fig. 2 explain that the lipase and protease production were highest at 55 % initial moisture content of substrate.

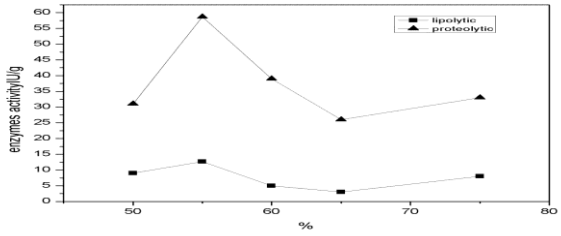

Fig. 2: Effect of different initial substrate moisture on lipase and protease production. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

Effect of inoculums size on enzymes production

The effect of inoculum size on lipase and protease production was studied by conducting the fermentation with different inoculum levels. Various inoculum size (0.5, 1.0, 1.5, 2.0, and 3 ml) were tried to study their effect on enzymes production. The higher production of lipase 9.2 U/g and of protease 45.3U/g was obtained at 0.5 cm^3. High inoculum levels are inhibitory in nature. (Asgher etal 2006).

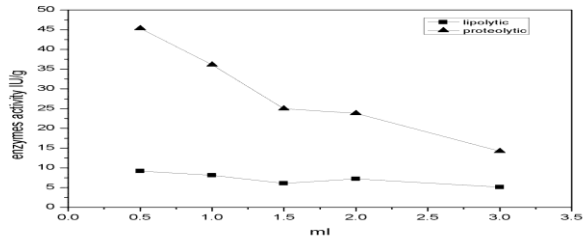

Fig. 3: Inoculum size effects on lipase and protease production. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

Effect of carbon supplements on enzymes production

Influence of various carbon supplements on enzymes production was studied by adding various supplements namely maltose, oleic acid and starch 1% (w/w) to fermentation medium. Of the three different carbon supplements used as enrichment, maltose was good carbon supplement which gave maximum lipase activity 19.9 U/g and protease production 39.9 U/g. It was reported that the presence of 2% maltose resulted in the highest lipase yield using Penicillium notatum (Pandey 1992). Mahanta (Mahanta etal 2008) reported that the presence of maltose in the growth media enhanced the lipase production by *Pseudomonas aeruginosa* and *Candida rugosa* respectively. (Rao et.al 1993) reported that by *Candida rugosa*. And our previous study (Moftah et al. 2013).

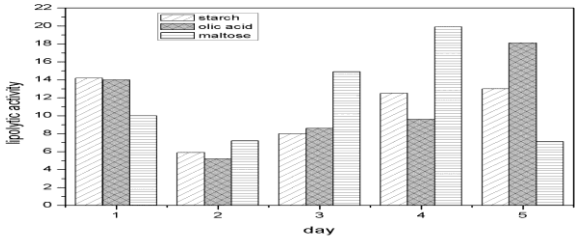

Fig. 4: Effect of carbon supplementations on lipase production. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

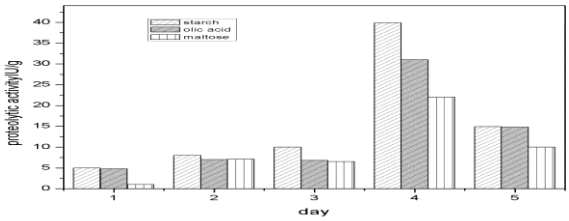

Fig. 5: Effect of carbon supplementations on protease production. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

Effect of nitrogen supplements on enzymes production

Influence of nitrogen supplements on enzyme production was studied by adding various supplements namely yeast extract, peptone and ammonium nitrate to fermentation media. Of the three different nitrogen supplements used as enrichment, it was observed yeast extract a good nitrogen supplement which gave maximum lipase production 25.2 U/g and protease
production 96.3 U/g. this is in agreement with our previous studies by *Yarrowia lipolytica* (Moftah et al. 2013)and *Candida utilis* (Moftah et al. 2012).

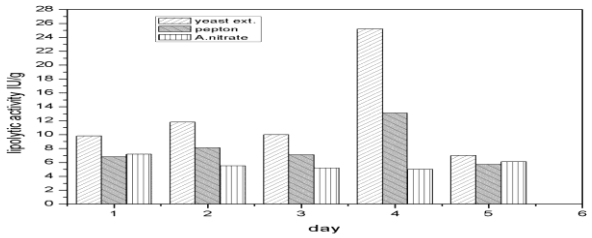

Fig.6: Effect of nitrogen supplementations on lipase production. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

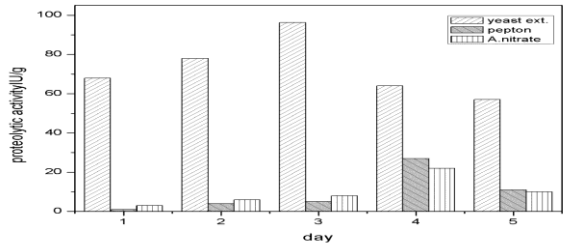

Fig. 7: Effect of nitrogen supplementations on protease production. Fermentation was performed on 5 g of fermented olive cake (0.5-mL inoculum) at 30 °C with shaking

Lipase production by *Candida rugosa* in submerged fermentation using olive oil mill waste water:

An initial experiment in conical flasks with liquid OMW-based media showed that the yeast *Candida rugoza* could growth on undiluted OMW, and to produce up to 220.1 U/L of lipase in 1st day, figure 8. lipase production was found to be promoted by the addition of tween 80 figure 9, but the highest production was with adding ammonium sulfate (0.6 % w/v), yeast extract (0.1 % w/v), maltose (0.5 % w/v), olive oil (0.3 % w/v) and peptone I (0.1 % w/v). figure 10 shows that *Candida rugosa* gave the highest lipase production (1667.5 U/L) in supplemented medium on the sixth day of fermentation period leading to the more than 7-folf higher compared to the production value in un-supplemented medium as shown in figure 11. The highest result is higher than that obtained by (Grbavćic´ et al 2011). And that mentioned by (Salehmin et al 2014).

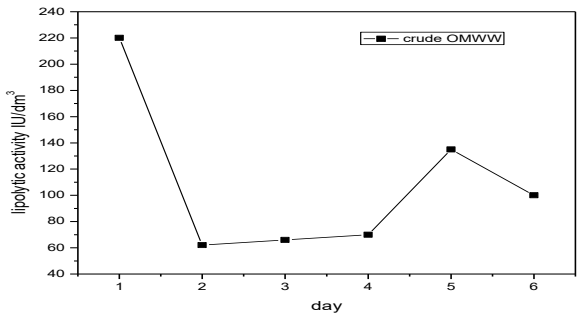

Fig.8: lipase production by *Candida rugosa* in un-supplemented OMW . Fermentation was performed on 100 cm^3 of crude olive mill waste water (1-cm^3 inoculum) at 30 °C with shaking

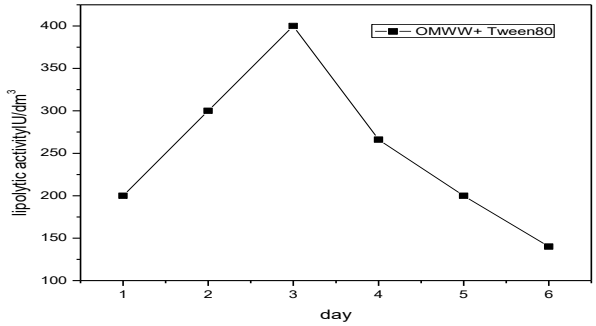

Fig.9: lipase production by *Candida rugosa* in un-supplemented OMW with tween 80. Fermentation was performed on 100 cm^3 of crude olive mill waste water (1-mL inoculum) at 30 °C with shaking

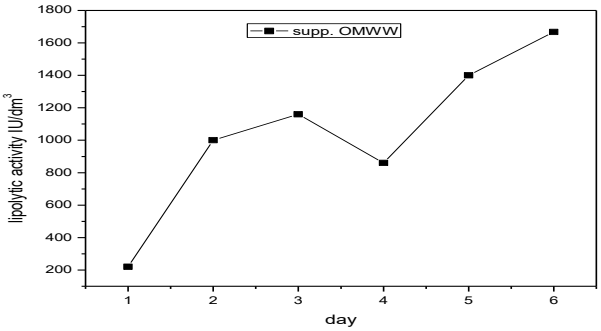

Fig. 10: lipase production by *C. rugosa* in supplemented OMW with adding ammonium sulfate (0.6 % w/v), yeast extract (0.1 % w/v), maltose (0.5 % w/v), olive oil (0.3 % w/v) and peptone I (0.1 % w/v).

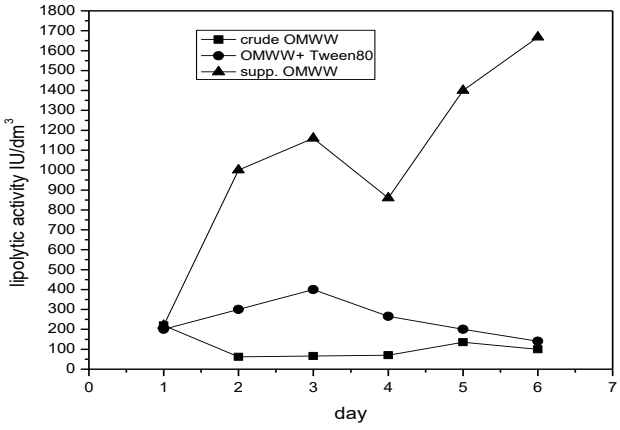

Fig. 11: Improvement in lipase production by optimization of growth medium

This study shows that fermentation techniques could be suitable to prevent effects of olive mills wastes on environment. *Candida rugosa* strain can be successfully utilized for olive oil processing wastes treatment and valorization. OMW and OOC seemed to provide necessary nutrients and physical support for the yeast to grow and

produce enzymes but lipase production can be further optimized by media supplementation and/or change in physical settings of the experiment. The amount of lipase and protease in initial study is promising and it could be interesting to try for the production of other industrial enzymes from different microbes.

References

1- Mafakher L, Mirbagheri M, Darvishi F, Nahvi I, Esfahani H Z and Emtiazi G. (2010). Isolation of lipase and citric acid producing yeasts from agro-industrial wastewater. *New Biotechnol*. 27(4) 337-340

2- Salehmin M. N. I., Annuar M.S. M and Chisti Y. (2014). High cell density fed-batch fermentation for the production of a microbial lipase. Biochem. Engineering J. 85: 8–14.

3- D'Annibale A, Sermanni G G, Federici F and Petruccioli M. (2006). Olive mill wastewaters: a promising substrate for microbial lipase production. *Bioresource Technol.*. 97: 1828 – 1833

4- Rigo E, Ninow J L, Di Luccio M , Oliveira J V, Polloni A E, Remonatto D Arbter F, Vardanega R, Oliveira D and Treichel H. (2010). Lipase production by solid fermentation of soybean meal with different supplements. LWT- *Food Sci. Technol*.43 (2010) 1132e1137.

5- Pandey A, Soccol, C. R. and Mitchell, D. (2000). New developments in solid state fermentation: I-bioprocesses and products, *Process Biochem*. 35: 1153- 1169.

6- Cordova, J., Nemmaoui, M., Alaaoui, M.I., Morin, A., Roussos, S., Raimbult, M., Benjilali, B. (1998). Lipase production by solid state fermentation of olive cake and sugar cane bagasse. *J. Mol. Catal. B: Enzym*. 5, 75-78.

7- Sandhya, C., Sumantha, A., Szakacs, G., Pandey, A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. Process Biochem. 40, 2689-2694.

8- Benjamin, S., & Pandey, A. (1998). Candida rugosa lipases: Molecular biology and versatility in biotechnology. Yeast, 14, 1069–1087.

9- Sharma, R., Chisti, Y., Banerjee, U.C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnol. Adv.* 19, 627-662.

10- Gupta, R., Beeg QK, Loranz P., (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol*., 59(1):15-32.

11- Massucco, A. E., (1980). Production of alkaline protease from *Bacillus subtilis* NRRL 3441. *Rev. Argent. Microbiol*., 12(2), 52-58. C.F.C.A., 94(21), 172849R (1981).

12- Gomaa, M. A.; Abou-Zied, M. M.; Moustfa, M. M. and El-Habashy, M. (1987). Factors effecting protease enzymes production by some *Aspergillus* and *Bacillus* strains. *Ann. Meet A. M. Soc. Microbiol*. 87 (0)272.

13- Andrade, V. S.; Sarubbo, L. A.; Fukushima, k.; Miyaji, M.; Nishimura, K. and de Campos-Takaki, G. M., (2002). Production of extracellualr proteases by *Mucor circinelloides* using D-glucose as carbon sources/substrate. *Braz. J. Microbiol.* 33: 106-110.

14- Gibb, G. D. and Strohl, W. R. (1987). Physiological regulation of protease activity in *Streptomyces peucetius. Can. J. Microbiol*. 34: 187-190.

15- Aleksieva, P. and Peeva, L., (2000). Investigation of acid protinase biosynthesis by the fungus *Humicola Lutea* 120-5 in an airlift bioreactor. *Enzyme Microb. Technol*., 26: 402-405.

16- Benslimane, C.Lebrihi, A., Lounes A., Lefebvre, G., Germain, P. (1995) Influnce of dextrins on the assimilation of yeast extract amino acids in culture of *Streptomyces ambofaciens* producer of spiramycin. *Enzyme. Microb. Technol*., 17: 1003-1013.

17-Valero F, Del Rio J. L, Poch M and Sola C. (1991). Fermentation behavior of lipase production by Candida rugosa growing on different mixture of glucose and olive oil . *J. ferment. Bioengineering*. Vo. 72, no.5, 399 – 401.

18-Benjamin S and Pandey A. (1997). Enhancement of lipase production during repeated batch culture using immobilized *Candida rugosa. Process Biochemistry*, Vol. 32, No. 5, pp. 437-440.

19- Akoh, C. C., Lee, G. C., & Shaw, J. F. (2004). Protein engineering and application of Candida rugosa lipase isoforms. *Lipids*, 39, 513–526.

20- Moftah O. A. S, Grbavcic S. Z, Moftah W. A. S, Lukovic N D,. Prodanovic O. L, Jakovetic S. M, and Knezevic-Jugovic Z D. (2013). Lipase production by *Yarrowia lipolytica* using olive oil processing wastes as substrates. *J. Serb. Chem. Soc.* vol. 78, no. 6, pp. 781–794, 2013.

21- Moftah O. A. S, Grbavčić S, Žuža M., Luković N., Bezbradica D., Knežević-Jugović Z. (2012). Adding value to the oil cake as a waste from oil processing industry: production of lipase and protease by *Candida utilis* in solid state fermentation. *Appl. Biochem. Biotechnol*. 166 (2012) 348- 364.

22- Salihu A., Alam. M. Abdulkarim M. I and Salleh H. M. (2012). lipase production: An insight in the utilization of renewable agricultural residues. *Resour. Conserve. recycl*.58: 36 – 44.

23- Gombert A K, Pinto A L., Castilho L R. And Freire D M.G. (1999). Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. *Process Biochemistry* 35 : 85–90.

24- Veerabhadrappa M. B., Shivakumar S B, and Devappa S. (2014). Solid-state fermentation of Jatropha seed cake for optimization of lipase, protease and detoxification of anti-nutrients in Jatropha seed cake using *Aspergillus versicolor* CJS-98. *J. Bioscience . Bioengineer*. Vo. 117 No. 2, 208-214.

25- Mahanta, N., Gupta, A., & Khare, S. K. (2008). Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using Jatropha curcas seed cakeas substrate. *Bioresource Technol.* 99, 1729–1735

26- Asgher M., Asad M.J. and Legge R.L .(2006). Enhanced lignin peroxidase synthesis by *Phanerichaete chrysosporium* in solid state

bioprocessing of a lignocellulosic substrate, *World J. Microbiol. Biotechnol*., 22, 449–453

27- Pandey A. Production of glucoamylase in solid state fermentation. In: Malik VS, Sridhar P, editors. Industrial Microbiology. New Delhi: IBH & Oxford Publishing Co, 1992:525–37.

28- Rao PV, Jayaraman K, Lakshmanan CM. (1993). Production of lipase by *Candida rugosa* in solid state fermentation. 1: Determination of significant process variables. *Process Biochem* . 28:385–9.

29- Grbavćic´ S, Bezbradica D , Izrael-Zivkovic´ L , Avramovic´ N , Milosavic´ N , Karadzic´ I and Knezevic´-Jugovic Z. (2011). Production of lipase and protease from an indigenous *Pseudomonas aeruginosa* strain and their evaluation as detergent additives: Compatibility study with detergent ingredients and washing performance. *Bioresource Technol.* 102 : 11226–11233.

30- Salehmin M.N.I. , Annuar M.S.M. and Y. Chisti. (2014). High cell density fed-batch fermentation for the production of a microbial lipase. *Biochem. Engineering J*. 85: 8–14.